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Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery

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Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In-Vitro Testing Battery

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
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Foreword

Developmental neurotoxic chemicals are a diverse set of substances that have the potential to interfere with the normal development of the nervous system, which, if perturbed without compensation, may lead to adverse effects on nervous system structures and/or functions. Current regulatory approaches to determine the potential developmental neurotoxicity of chemicals in humans are based on in vivo test protocols that require prenatal and postnatal exposure of pregnant rats followed by assessment of offspring for physical and neurodevelopmental landmarks, clinical observations, and behavioural and neuropathological endpoints (e.g., OECD Test Guideline (TG) 426 'Developmental Neurotoxicity Study', OECD 2007). Use of the in vivo developmental neurotoxicity test guideline has been limited due to its resource intensive nature in terms of cost, time and number of animals used, as well as a lack of chemical alerts that trigger it. In recognition of the challenges for traditional methods of toxicity testing (NRC, 2007; Kavlock et al., 2018) there has been a paradigm shift, moving away from chemical safety decisions based on in vivo animal tests using apical endpoints and towards the application of in vitro testing and within integrated approaches to testing and assessment IATA (OECD 2016). This shift in approach relies on identifying and assessing the disruption of molecular events and cellular pathways associated with adverse outcomes in humans using in vitro models and higher throughput technologies. Thus, new approach methodologies (NAMs) including in vitro (omics, cell-based, tissue-based etc.) assays, in silico models, and other computational approaches, are being developed to provide information on chemical hazard and risk for humans while limiting the use of animals. International efforts have proposed NAMs for developmental neurotoxicity testing resulting in the Developmental Neurotoxicity In Vitro Battery (DNT IVB); an EFSA/OECD workshop was organised in 2016 to discuss the possible uses of an in vitro battery of assays (Fritsche et al., 2017; OECD 2017a). The assays in this battery are designed to detect changes in a number of important neurodevelopmental processes at the cellular level that are regulated by multiple signalling pathways at the molecular level. Chemical perturbations in these processes may result in adverse outcomes at the organ and individual level. However, it is acknowledged that this battery does not contain all components that are important for certain mode(s) of action that can lead to adverse neurodevelopmental outcomes (e.g., endocrine and immune system perturbations).

The purpose of the present document is to provide initial recommendations on the evaluation of data developed with the DNT IVB (e.g., hit vs non-hit, uncertainties, biological coverage). The major aims are to describe the assays that comprise the battery in terms of neurodevelopment, provide criteria that allows evaluation of the relevance of the data to developmental neurotoxicity, and to assist in the determination of the degree of certainty in any positive or negative findings to better inform use of DNT in vitro data in regulatory hazard determinations. Although a list of regulatory needs identified by international working groups is provided, the GD is not intended to guide the use of results in human hazard and risk assessments which will be driven by regulatory needs.

- Assay relevance: Assays were designed to detect changes in cellular processes that underly normal nervous system development.
- Assay Inclusion: The assays included in the current iteration of the DNT IVB are those that: 1) were deemed ready for use in screening and prioritisation (Fritsche et al. 2017; Bal-Price et al. 2018; Sachana et al. 2019); 2) have been tested using a common set of chemicals (see Appendix E); 3) data have been analysed using the USEPA's ToxCast Pipeline (TCPL)

(ToxCastDB v3.5, https://www.epa.gov/chemical-research/exploring-toxcast-data); and 4) have detailed methodological descriptions in the ToxTemp format (Krebs et al. 2019) in Appendix B.

- Chemicals: There have been a total of 476 compounds tested in one or more DNT IVB assays, and 81 compounds tested in all the assays of the DNT IVB. Chemicals tested included: 1) assay-specific chemicals used in assay development; 2) a list of possible positive and negative developmental neurotoxicants; and 3) chemicals important to funding agencies.
- Negative findings: At this time negative results from the DNT IVB should not be interpreted as a lack of DNT potential. This is due to the uncertainties associated with the in vitro methods used, as well as the lack of coverage for some critical neurodevelopmental processes.
- Adversity: Currently interpretations of adversity is outside the scope of the document. As with many in vitro test methods, to date there is no analysis correlating specific levels of changes in vitro with in vivo neurodevelopmental outcomes.
- Validation: A classic validation of the DNT IVB (e.g., inter-laboratory testing of all assays using defined lists of positive and negative compounds) according to the OECD Guidance Document (GD) 34 on the 'Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment' has not been conducted. This uncertainty is clearly stated in this DNT IVB GD and should be considered by regulatory authorities for use in a fit-for-purpose manner driven by their decision needs. Information on the current level of validation of the individual assays can be found in the ToxTemp files in Appendix B.
- This battery was developed based on current knowledge; it should be considered an evergreen document with improvements and revisions occurring as new assay methods and more chemical test data become available to improve the predictive capacity of the test battery, and as the reliability of each individual test method is improved through further validation work.

The original draft of this document was developed by Kevin M. Crofton and William R. Mundy and published in 2021 (Crofton and Mundy, 2021). A second version was developed after review and revisions by the co-Chairs of the Expert Group, Andrea Terron (EFSA), Timothy Shafer (US EPA), with input from Expert Group members Susanne Hougaard Bennekou, Stefan Masjosthusmann, Ellen Fritsche, Anna Price, Kate Willet, Francis Bailey, Rex FitzGerald, the Secretariat, and the external experts Jack Fowle and Pamela Lein. The current version is the outcome of two commenting rounds by the members of the OECD Expert Group on the DNT IVB and two commenting rounds by the Working Party of the National Coordinators of the Test Guidelines Programme (WNT).

The Working Party of the National Coordinators of the Test Guidelines Programme approved these Initial Recommendations at its 35th meeting in April 2023. This document is published under the responsibility of the Chemicals and Biotechnology Committee.

Abbreviations Table	
AChE	Acetylcholinesterase
AO	Adverse Outcome
AOP	Adverse Outcome Pathway
ATP	Adenosine Triphosphate
B	Background
BMC	Benchmark Concentration
BMR	Benchmark Response
CV	Coefficient of Variation
CYPs	Cytochromes
DNT	Developmental Neurotoxicity
DNT IVB	Developmental Neurotoxicity In Vitro Battery
EC	Effective Concentration
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
ESCs	Embryonic Stem Cells
EU	European Union
GD	Guidance Document
HTS	High-Throughput Screening
IATA	Integrated Approaches to Testing and Assessment
ITS	Integrated Testing Strategy
iPSC	Induced Pluripotent Stem Cells
IUF	Leibniz Research Institute for Environmental Medicine
IVB	In Vitro Battery
IVIVE	In Vitro to In Vivo Extrapolation

KE	Key Event
KER	Key Event Relationship
LDH	Lactate Dehydrogenase
MEA	Multi-Electrode Assay
MeHg	Methylmercury
MIE	Molecular Initiating Event
NAMs	New Approach Methodologies
NNF	Neuronal Network Formation
NPCs	Neural Progenitor Cells
NSCs	Neural Stem Cells
NT	Neurotoxicity
NTP	National Toxicology Program
OECD	Organisation for Economic Cooperation and Development
S	Signal
S/B	Signal-to-background ratio
S/N	Signal-to-noise ratio
SD	Standard Deviation
SOP	Standard Operating Procedure
TG	Test Guideline
ToxPi	Toxicological Prioritisation Index
(Q)SAR	(Quantitative) Structure Activity Relationships
UKON	University of Konstanz
USEPA	US Environmental Protection Agency
WoE	Weight of Evidence

Table of contents

1 Introduction

Background and Goal

1. Developmental neurotoxicants are a diverse set of substances that have the potential to interfere with the normal development of the nervous system, which, if perturbed without compensation, may lead to irreversible adverse effects on nervous system structures and/or functions (Mileson and Ferenc 2001; USEPA 1998). Development of the nervous system involves a complex interplay between multiple processes that occur both prenatally and postnatally and are developmental stage and location dependent. This has led to the general observation that the developing nervous system is particularly vulnerable to environmental chemicals (Grandjean and Landrigan 2006; 2014). Due to the vulnerability and potentially high societal costs of adverse impacts on neurodevelopment, the potential health effects following exposure to environmental chemicals led to development of in vivo testing batteries starting in the 1980's, and development of testing guidelines by the USEPA in 1986, with refinement by OECD in 2007.

2. Current USEPA and OECD DNT Test Guidelines require assessing the impact of prenatal and postnatal exposure on the development of physical and developmental landmarks, clinical observations, behavioural and neuropathological endpoints (OECD 2007). However, there has been limited use of these Test Guidelines, with a total of approximately 165 chemicals assessed to date using either USEPA/OECD DNT or TG443 extended one-generation test guidelines (Crofton 2020; Makris et al., 2009; OECD 2008). This is the result of a number of factors, including: the limited regulatory requirement for DNT testing as compared to some other Test Guidelines, testing is both time (e.g., 1-2 years) and resource-consuming, limited triggered testing by chemical alerts, the need to reduce animal use and uncertainties in DNT guideline data about variability, sensitivity and coverage of critical neurodevelopmental functions (Paparella et al., 2020; Tohyama 2016; Tsuji and Crofton 2012). This limited testing, coupled with an increasing need to assess the potential hazards of hundreds of pesticides and thousands of industrial chemicals, has resulted in calls for the development and use of New Approach Methodologies (NAMs) that reduce the use of animals (Kavlock et al., 2018) and are efficient and predictive for DNT testing (Bal-Price et al., 2015a; Barbosa et al., 2015; Crofton et al., 2011; Fritsche et al., 2017; Lein et al., 2005).

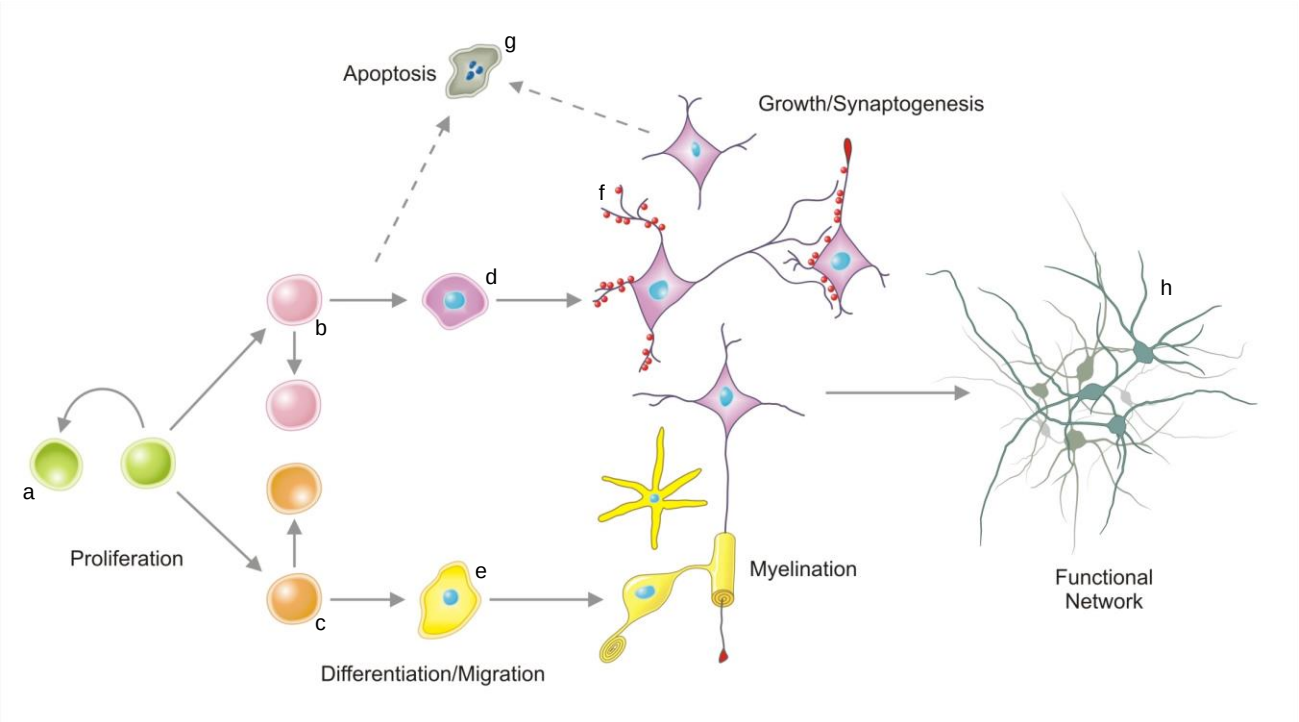
3. The output from the 2016 OECD/EFSA DNT workshop was a consensus that an existing in vitro testing battery (IVB) could immediately be used for screening and prioritisation (OECD 2017a), and that further work was needed to gain international acceptance for hazard identification and characterisation. This formed the basis of several global projects. These include a long-running project by the USEPA to develop and validate in vitro DNT methods (USEPA 2020a), a project led by EFSA to generate more data from the DNT IVB (Masjosthusmann et al., 2020), one led by the Danish EPA to test high priority pesticides, and another by the US National Toxicology Program (Behl et al., 2019) to develop a rapid and cost-effective screening strategy to prioritise replacements for classes of chemicals (e.g., flame retardants). Furthermore, in response to the outcome and the recommendations of this workshop, in 2017 the OECD convened an international Expert Group to develop a framework for evaluation of in vitro DNT testing and use of DNT IVB data in Integrated Approaches to Testing and Assessment (IATA) based on case studies using DNT IVB data.

4. The overall purpose of the present document is to provide initial recommendations on the evaluation of data developed with the DNT IVB. The major aims are to describe the assays that comprise the battery in terms of neurodevelopment, provide criteria that allows evaluation of the relevance of the data to developmental neurotoxicity, and to assist in the determination of the degree of certainty in any positive or negative in vitro bioactivity to better inform use of DNT in vitro data in regulatory hazard determinations. This document is not intended to guide the use of results in human hazard and risk decisions. Specific criteria for such use will likely be available in specific regulations or developed by regulatory authorities who will determine acceptability based on their needs.

The Developmental Neurotoxicity In Vitro Battery (DNT IVB)

5. The DNT IVB developed for the EFSA funded research project was based on the OECD/EFSA review of available in vitro DNT assay (Fritsche et al., 2017; OECD 2017a). This review proposed a set of assays that are not based on molecular targets, as no comprehensive list of targets is currently known, but instead on a number of fundamental neurodevelopmental processes (Figure 1.1, see Section Developmental Neurotoxicity In Vitro Battery (description of assays) for details). This battery of assays has been reviewed by expert panels (Bal-Price et al., 2018; Fritsche et al., 2017; Sachana et al., 2019), with the consensus opinion that they are ready for use in the regulatory arena.

Figure 1.1. Fundamental neurodevelopmental processes necessary for proper nervous system development. In vivo studies have shown that several biological processes at the cellular level are essential for nervous system development. Neural stem cells (NSC, green, a) proliferate and differentiate into multiple types of neural progenitor cells (NPCs) including neuronal progenitors (light purple, b) and glial progenitors (orange, c). These proliferate, migrate, and differentiate into neurons (purple, d) and glia (yellow, e). As cells mature, they extend neurites and form synapses (red, f). Surplus cells undergo apoptosis (grey, g). When these events happen in a coordinated fashion, cell-cell interactions result in a functional neuronal network (olive, h). (modified from Aschner et al., 2017, original by William Mundy and John Havel).



6. The use of the assays in the DNT IVB that measure changes in neurodevelopmental processes is based on the assumption that changes in these processes will reflect the integration of chemical disruptions in multiple up-stream molecular events (Lein et al., 2007; Lein et al., 2005; Radio and Mundy 2008). This battery contrasts with many other in vitro batteries that cover a single cellular pathway (e.g., oestrogen receptor, Judson et al., 2015), in that it seeks to predict the impact of xenobiotic exposures on the development of a tissue or organ (i.e., brain) via multiple cellular pathways. To date about 476 chemicals have been tested in up to 17 assays in the DNT IVB (see Appendix E), using animal- and human-based cell cultures that can measure changes in proliferation, differentiation, apoptosis, migration, neurite formation, synaptogenesis, and neural network formation (Harrill et al., 2018; Masjosthusmann et al., 2020; Shafer et al., 2019). Each assay includes assessment of cell viability and/or cytotoxicity. This document was developed based on current knowledge; it should be considered an evergreen document with improvements and revisions occurring as new assay methods (e.g., Culbreth et al., 2012; Loser et al., 2021) and more chemical test data become available to improve predictive capacity of the whole test battery, and as the reliability of each individual test method is improved through further validation work. A summary of ongoing work to improve the DNT IVB can be found on the OECD DNT IVB Expert Group webpage (https://www.oecd.org/chemicalsafety/testing/developmental-neurotoxicity.htm).

Target Uses

7. It is important to note that currently there is not sufficient evidence that the DNT IVB can replace the use of OECD TG426 and the DNT Cohorts 2A and 2B in OECD TG443 for derivation of all hazard-based decisions. Instead, targeted DNT IVB testing should be guided by a problem formulation approach based on regulatory needs and acceptability using the IATA framework (OECD 2016; Sachana and Leinala 2017; Sakuratani et al., 2018) (see Section on Integrated Approaches to Testing and Assessment (IATA) for DNT). Below is a list of needs for some regulatory frameworks identified by international working groups (Bal-Price et al., 2018; Fritsche et al., 2017; Sachana et al., 2019).

- Follow-up testing for positives identified by quantitative structure activity relationships ((Q)SAR), read-across and other predictive computational models of developmental neurotoxicity.
    - Screening for prioritisation
        - Screening of large numbers of chemicals that lack or have limited data on DNT (e.g., Tox21/ToxCast, Health Canada prioritisation scheme).
        - Screening of small numbers of structure/class specific chemicals (e.g., Behl et al., 2019).
- Single chemical hazard assessments
    - When no in vivo DNT data exists, in some regulatory frameworks data from the DNT IVB assays may be used to determine if, and what, follow-up testing (e.g., orthogonal assays, alternative species (e.g., zebrafish), or test guideline study) could be conducted.
    - If existing in vivo DNT data is equivocal, data from DNT IVB testing could be used to inform the Weight of Evidence (WoE)-based IATA assessment for DNT.
    - When DNT in vivo data exists and is negative, but concern exists from new findings or novel in vitro assays (i.e., not currently covered in the DNT IVB), the DNT IVB may be used to inform the WoE.
    - When data from an MIE[1] based assay or alternative species assay (e.g., zebrafish) data exist, a regulatory choice could be to run the DNT IVB to inform the WoE-based assessment.

[1] More information on the AOP Framework can be found in Section 2 and Appendix C.

Target Chemicals

8. The target chemicals for possible use in the DNT IVB are industrial chemicals, pesticides, and environmental contaminants, including metabolites and environmental degradates. There are known limitations of chemical testing capability in in vitro assays related to the physiochemical characteristics (e.g., volatility, high reactivity, hazard level, and limited solubility in assay appropriate media) of the test compounds (Richard et al., 2020; Richard et al., 2016). These types of chemical limitations for the individual assays in the DNT IVB should be described in assay documentation as per OECD GD211 Guidance document for describing non-guideline in vitro test methods (OECD 2017b) and/or the ToxTemp format (Krebs et al., 2019). Descriptions for assays in the DNT IVB, using the ToxTemp format, can be found in Appendix B.

9. Currently, a total of 81 compounds have been tested in all 17 assays in the DNT IVB[2]. A total of 97 compounds have been tested in 14 or more assays, and 331 compounds have been tested in at least four assays. A total of approximately 476 compounds have been tested in one or more of the assays in the DNT IVB. Appendix E provides a list of which assays have been conducted for each chemical.

Aims and Context

10. This document provides initial recommendations on how to evaluate data from the DNT IVB (e.g., hit vs non-hit, uncertainties, biological coverage). It is not intended to guide the use of results in human hazard and risk assessments. Specific criteria for such use will likely be available in specific regulations or developed by regulatory authorities who will determine acceptability based on their needs. Nor is it intended to provide guidance for all in vitro assays purported to detect DNT (e.g., available in published literature). The structure of these initial recommendations should be expanded in the future to encompass improvements to the current assays in the DNT IVB, updated validation information, and/or new and novel assays that complement or expand the DNT IVB as it currently exists.

11. The assays in the DNT IVB were chosen based on international discussions on the ability of existing in vitro DNT assays to measure neurodevelopmental processes and the readiness of the assays, at a screening level, to test large numbers of chemicals (Bal-Price et al. 2018; Fritsche et al. 2017; Sachana et al. 2019). The specific assays included in the current iteration of the DNT IVB are those that: 1) were deemed ready for use in screening and prioritisation (Bal-Price et al. 2018; Fritsche et al. 2017; Sachana et al. 2019); 2) have been tested using a common set of chemicals (see Appendix E); 3) data have been analysed using the USEPA's ToxCast Pipeline (TCPL), a common normalisation and dose-response modelling approach (ToxCastDB v3.5, https://www.epa.gov/chemical-research/exploring-toxcast-data); and 4) have detailed descriptions in the ToxTemp format in Appendix B.

12. These initial recommendations were developed based on the testing of a set of chemicals that is limited compared to the overall chemical universe (Richard et al., 2020), and a set of assays that do not include all critical processes within the developing nervous system (see Section Developmental Neurotoxicity In Vitro Battery (description of assays). Thus, there are uncertainties in the predictive accuracy of the DNT IVB that cannot currently be quantified. This uncertainty will only be reduced with the testing of additional chemical libraries that encompass a greater portion of chemical space (Richard et al., 2020) and by integrating additional assays in the DNT IVB (see Section Uncertainties (summary)).

[2] As of March 2023

This uncertainty needs to be considered when using data from the DNT IVB within any regulatory assessment (see Section Usage in hazard assessments).

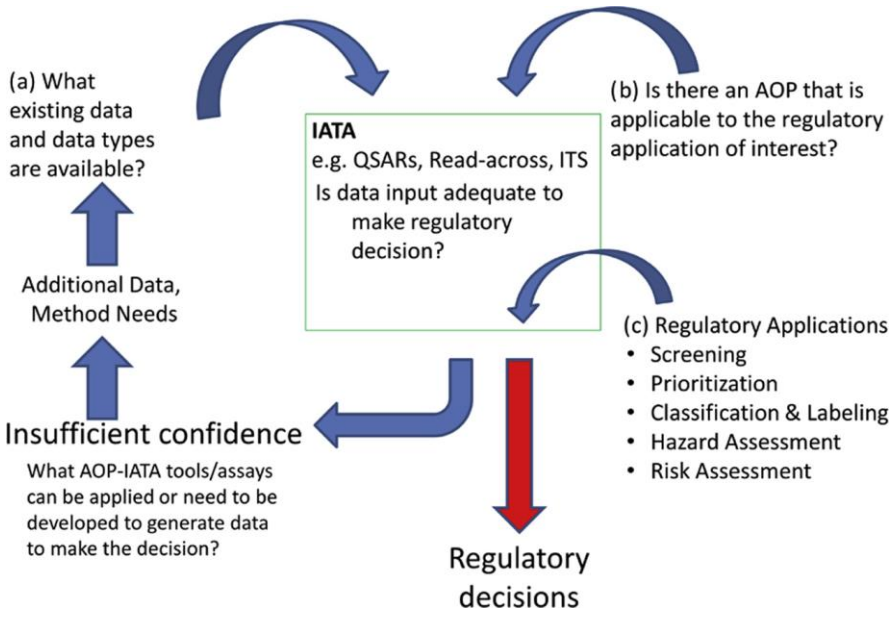
13. The remainder of this document contains a background on DNT assays, a brief description of the assays in the battery, elements for evaluation and use in WoE considerations for both individual assays and the battery, and a brief review of available case studies. This document is intended to briefly review the science that supports the DNT IVB and to provide initial guidance on how to evaluate data from the DNT IVB.

2 Context and Description of the DNT IVB

Integrated Approaches to Testing and Assessment (IATA) for DNT

14. IATA is a framework developed by OECD that allows for the integration of all available hazard and possibly exposure data, including in silico, in chemico, in vitro and in vivo, for use in chemical regulatory assessments (OECD 2016). IATAs are "pragmatic, science-based approaches for chemical evaluations in the context of hazard or risk assessments that rely on an integrated analysis of existing information, with optional use of the adverse outcome pathway (AOP) framework, coupled with the generation of new information if necessary. IATAs follow an iterative approach to answer a defined question in a specific regulatory context, taking into account the acceptable level of uncertainty associated with the decision context." (OECD 2016; Sachana and Leinala 2017; Sakuratani et al., 2018).

Figure 2.1. A conceptual framework for an Adverse Outcome Pathway (AOP)-informed Integrated Approach to Testing and Assessment (IATA) to support regulatory assessments. (ITS = Integrated Testing Strategy), QSAR = Quantitative Structure Activity Relationships) (from Tollefsen et al., 2014).



15. Problem formulation is the first step in IATA development and is critical to the use of the DNT IVB. This involves an understanding of the scope and needs of the risk assessment objective, data requirements, and the level of acceptable uncertainty associated with the decision being made (Tollefsen et al., 2014).

16. Regulatory needs in an IATA can range from screening of large chemical classes for prioritisation of follow-up testing, to regulatory acceptance of the use of new chemicals (see also Section Target Chemicals). An IATA is based on an evaluation of all existing evidence and may include use of AOPs or other frameworks. Figure 2.1 illustrates these approaches, which can be iterative if necessary. If the uncertainties in the IATA are acceptable within the context of a given regulatory need, the IATA may be used for that regulatory purpose. If the uncertainties in the IATA are not acceptable due to insufficient confidence of the totality of available information, additional data that can address the uncertainties should be identified and input into the IATA. One basic approach of IATAs is to apply the AOP framework to integrate and organise the various data sources. AOPs allow the mapping of data to molecular initiating events (MIEs) and key events (KEs) at the molecular, cellular, organ levels, that lead to adverse outcomes (AOs) at organismal and population levels (Ankley et al., 2010; OECD 2016). A diagram that illustrates potential components of a DNT AOP is shown in Figure 2.2.

17. Within AOP-based IATA it is important to clearly articulate the uncertainties in the use of the AOP framework for DNT. First, only a limited number of putative and reviewed AOPs exist for DNT (Bal-Price et al., 2015b; Spinu et al., 2019) (see Appendix C). Second, most of the assays used in the DNT IVB do not measure MIEs, but instead downstream KEs. Third, the basic assumption underlying the DNT IVB is that if a chemical disrupts neurodevelopmental processes in vitro, then it has the potential to do the same in vivo (Lein et al., 2007; Lein et al., 2005; Radio and Mundy 2008). Understanding these intrinsic uncertainties is critical to inform regulatory authorities in the use of data derived from the DNT IVB. As more data are generated using the DNT IVB, and with further AOP development and improved understanding of mechanisms underlying developmental neurotoxicity (see Section 4 Integration of Evidence), the uncertainties in the use of DNT IVB will be reduced.

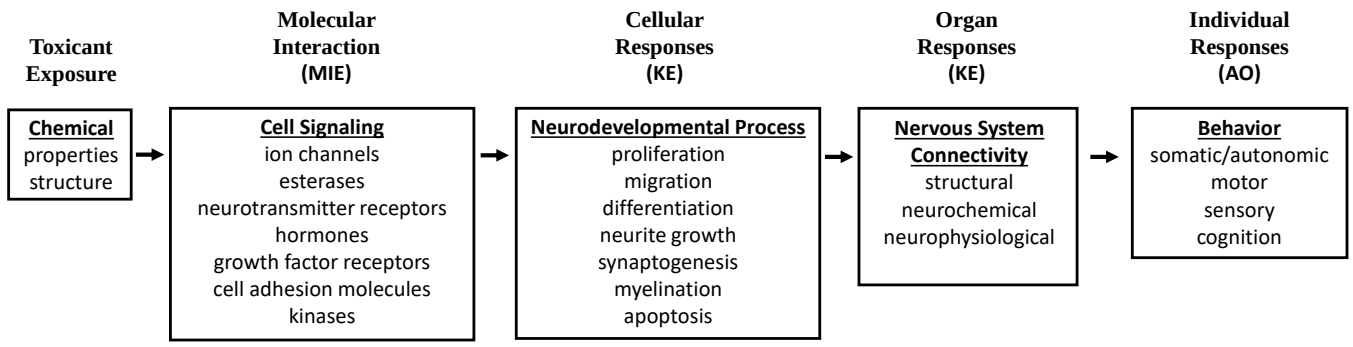


Figure 2.2. This diagram illustrating some potential components of an Adverse Outcome Pathway (AOP) for developmental neurotoxicity. This AOP framework provides examples that show the relationships between molecular initiating events (MIEs), and key events (KEs) at the cellular and organ levels which may lead to an adverse outcome (AO) for the individual.

18. An overview of DNT AOPs and the relationship to KEs measured in the DNT IVB assays is found in Appendix C.

Developmental Neurotoxicity In Vitro Battery (DNT IVB)

19. In vivo development of the nervous system proceeds through a series of coordinated biological processes that are essential for the formation of normal brain structure and function. While the timing, duration, and spatial location of these processes differ between species, the fundamental processes are remarkably conserved across invertebrate, non-mammalian vertebrate and mammalian organisms (Sanes et al., 2011). These fundamental neurodevelopmental processes include progenitor cell proliferation, differentiation into neuronal and glial cells, migration, apoptosis, axonal and dendritic outgrowth, myelination, synapse formation and formation of functional neural networks (Figure 1.1). In vivo, perturbation of one or more of these neurodevelopmental processes during nervous system development may, if not compensated, result in adverse effects on brain structure and/or function (Rice and Barone 2000; Rodier 1994). Thus, use of the DNT IVB is based on the hypothesis that chemicals affecting one or more of these neurodevelopmental processes in vitro have the potential to do so in vivo (Lein et al., 2007; Lein et al., 2005; Radio and Mundy 2008). While this hypothesis is supported by a limited number of examples demonstrating a correlation of chemical effects on neurodevelopmental processes in vitro and in vivo (Section Plausibility), this does not, in any way, imply that in vitro changes in these processes, de facto, predict an adverse outcome in vivo. Some aspects of these neurodevelopmental processes can be examined as discrete, measurable events in vitro that provide biologically relevant information about chemical perturbations at the cellular level. In terms of the AOP framework, the assays which comprise the DNT IVB can be considered as cellular/organ level KEs in an AOP for DNT (Figure 2.2). An advantage of measuring cellular-level neurobiological processes (e.g., migration) in cell-based assays (i.e., assays in which cell cultures are exposed to the test chemical) is that the intact cell integrates potential chemical actions at multiple upstream targets (MIEs) and cellular pathways (KEs) (Lein et al., 2007; Lein et al., 2005; Radio and Mundy 2008). For use in hazard identification, characterisation, or chemical screening, cell-based assays cast a wide net (Cooper et al., 2017), in that they can detect chemical effects in the absence of prior knowledge of MIEs. In addition, use of the AOP framework for aims to identify the potential links between MIEs, KEs and AOs and to provide mechanistic data supporting the relevance of the data derived from the DNT IVB. Concurrent measurement of cell viability and/or cytotoxicity in cell-based assays allows for the evaluation of data in terms of a selective effect of a chemical on a neurodevelopmental process (i.e., changes occurring in the absence of an overall effect on cell health or viability), further supporting the relevance of in vitro data to perturbation of neurodevelopment.

20. Due to the many known and unknown modes of action for chemical effects on nervous system development, any individual in vitro assay is unlikely to cover all potential targets of developmental neurotoxicants. Rather, a battery of assays that partly cover some key neurodevelopmental processes has been proposed that should increase the ability to detect developmental neurotoxicants. Numerous test methods (assays) have been developed for assessment of chemical effects on aspects of neurodevelopmental processes in vitro (e.g., Fritsche et al., 2017; Schmidt et al., 2017). These assays use a variety of neural cell types derived from human and animal sources (test systems) and measure molecular, morphological, and electrophysiological endpoints. In most cases, methods that are amenable to medium- or high-throughput testing are used. Details on published methods for measuring the neurodevelopmental processes at the cellular level and their application for use in regulatory DNT testing have been reviewed (OECD 2017a). The conclusion of that review was that for many of the neurodevelopmental processes, in vitro assays are available that permit quantitative assessment of relevant endpoints (i.e., measured variable) (Bal-Price et al., 2018). Due to advances in stem cell biology that have made human cells more accessible, test systems using human-derived neural cells are becoming more common; such test systems are preferred since they should minimise interspecies extrapolation issues. However, human neural cells can be difficult to culture, and the developmental timeline of such cells may be much longer than rodent-derived neural cells (Barry et al., 2017; Nimtz et al., 2020; Odawara et al., 2016). For some processes (e.g., neural network function), assays

incorporating human-derived neurons and glia are being developed (Nimtz et al., 2019), but currently only assays using rodent-derived cell cultures were deemed ready for screening. An international expert group on DNT completed an analysis that scored and ranked the available in vitro assays for their readiness for use in chemical screening/prioritisation or risk assessment (Bal-Price et al., 2018). Readiness criteria included assay descriptions (e.g., purpose, relevance, cell type, reference chemicals,

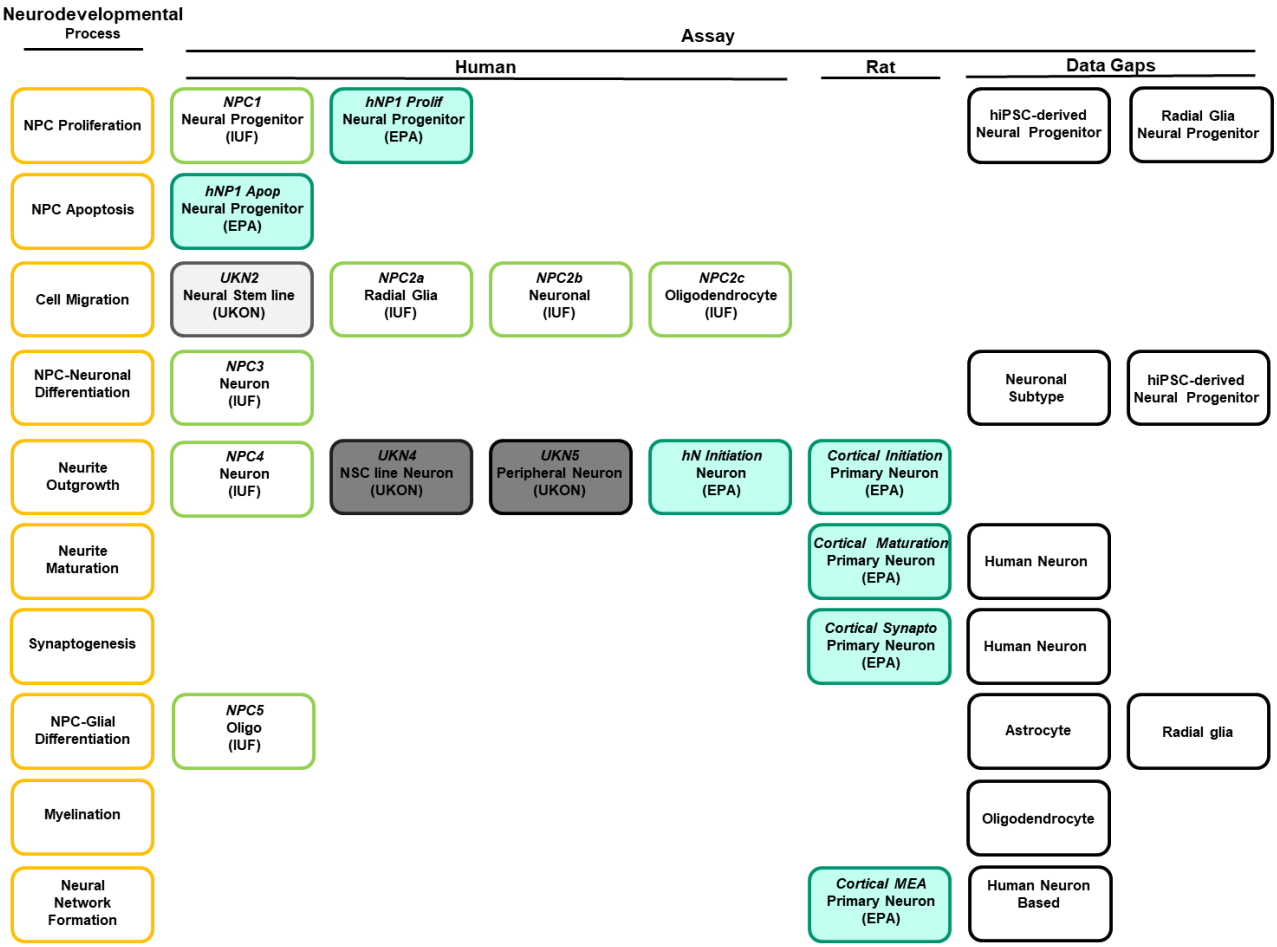


Figure 2.3. Assays in the current DNT IVB and assays identified as high priority for development (modified and adapted from Masjosthusmann et al., 2020). Assays are grouped according to the neurodevelopmental process evaluated (rows) and test system used (columns). Each assay box lists the abbreviated assay name, cell type and assay development laboratory. Assays which need further development, and not included in the current DNT IVB, are identified as data gaps. Each assay is represented as a box which lists the test method name (italics), the test system (cell type used), and the home institution of the developer (IUF =Leibniz Research Institute for Environmental medicine – green ; UKON =University of Konstanz - gray; EPA= US Environmental Protection Agency – blue). Other abbreviations can be found in the Abbreviations List.

exposure, technical limitations), performance (e.g., repeatability, variability, sensitivity/specificity), data evaluation (e.g., dynamic range, curve fitting, benchmark response) and application (e.g., chemicals, relevant pathways/AOPs, prediction models). Additional information used to evaluate assay readiness included results from assays that have already screened tens to hundreds of chemicals. Based on the recent reviews of DNT in vitro assays (Bal-Price et al., 2018; Fritsche et al., 2017), a battery of assays (DNT IVB) has been assembled that covers a majority of the key neurodevelopmental processes

(Masjosthusmann et al., 2020). Figure 2.3 illustrates the assays in the current battery (in total 17 assays) organised by key neurodevelopmental processes (rows). The figure also indicates the species origin of the test system used and lists neurodevelopmental processes where assays should be a high priority for development. In many cases, multiple test methods are available to assess a single neurodevelopmental process. These assays are currently applied in the battery in a complementary manner (as opposed to orthogonal assays) including some that model the similar neurodevelopmental processes using distinct test systems (see Textbox 1).

Textbox 1.

Complementary Assays: Assays that measure similar endpoints and are conducted in conjunction rather than as follow-ups to primary screening. These assays may use similar or different technologies or test species and may evaluate different levels of biological complexity. Such assays can provide additional certainty when they are all positive or negative. However, different findings between the assays are not necessarily indicative of a false positive in that said differences could be due to differences in assay methods such as cell type, species, level of biology evaluated, or assay technology.
Orthogonal Assays: An assay performed following the primary assay to differentiate between compounds that generate false positives from those substances that are genuinely active against the target. Such assays use different reporter or assay technologies in an effort to confirm that activity of the compound is directed toward the biological target of interest, or if negative suggest that the original substance activity was most likely assay format-dependent and not specific to the biology of interest. In some cases, orthogonal assays can be run at the same time (adapted from Thorne et al., 2010).

21. However, due to differences between test systems (e.g., neural stem cell versus primary neural cell cultures) and test methods (e.g., developmental timing, assay duration, endpoints, exposures), different outcomes from assays for the same neurodevelopmental process are possible. Three assays in the DNT IVB that measure migration are a case in point. The UKN2 assay utilises neural crest cells, a type of neural stem cell that migrates towards tissues outside the nervous system during early embryogenesis and can differentiate into both neural and non-neural cells (Mayor and Theveneau 2013). In contrast, the NPC2a assay uses radial glia cells that normally migrate as a prerequisite for cortex formation (Borrell and Gotz 2014), and the NPC2c assay uses oligodendrocytes, a more mature cell that arises and migrates later in development (Barateiro and Fernandes 2014). The biology that regulates migration is different in these test systems (Minoux and Rijli 2010; Ortega et al., 2012; Sild and Ruthazer 2011), providing different targets and pathways for testing chemical interactions. Another example is the neuronal network formation assay (NNF). The cortical cell-based Multi-Electrode Assay (MEA) (Shafer et al., 2019), and human NNF assay being developed (Nimtz et al., 2020), use the same basic cell types (neurons and astrocytes), but from different species(rat primary neocortex versus human induced pluripotent stem cell- and primary astrocyte-derived). Test results from these assays may be different due to differences in developmental timing (Saavedra et al., 2021) as well as differences between neural cells derived from different brain regions and in the pathways and proteins expressed in rats versus humans (Robbins et al., 2010). Thus, to facilitate evaluation of the results in terms of the stated test purpose it is important that both the test system and test method be clearly described and annotated by the assay developer. This information is documented in an organised format developed for cell-based assays (see below) and is found in Appendix B. Table 2.1 provides a general description of the types of cell cultures used as test systems in the current DNT IVB. Each of these different types of neural cultures can be derived from rodent or human tissue.

Table 2.1. General descriptions of neural cell cultures used as test systems in the current DNT IVB	
Type of Cell Cultures	***Description***
Neural Stem Cells (NSC)	Cells that can self-renew and proliferate indefinitely. They produce progeny cells (e.g., neural progenitor cells such as radial glia) that ultimately give rise to a majority of cells in the nervous system. They can be derived from embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC).
Neural Progenitor Cells (NPC)	Derived as progeny of neural stem cells or directly from foetal brain tissue, they have a more limited proliferative ability. They can terminally differentiate into neurons (including many subtypes) and glia (astrocytes and oligodendrocytes).
Neurospheres	3D culture of neural stem cells and neural progenitor cells that grow in small clusters and can terminally differentiate into a mixed culture of neurons and glia.
Primary Cells	Immature neurons and glia derived directly from brain tissue.

22. Test methods for the DNT IVB are designed to model aspects of one or more neurodevelopmental processes in vitro. The primary purpose of each method is to evaluate whether test chemicals can alter these processes as a function of development in vitro. A general description of each neurodevelopmental process modelled in the DNT IVB along with potential consequences of chemical disruption and endpoints measured is listed in Table 2.2. Note that these are neurodevelopmental processes that may not necessarily reflect developmental timing, location, or neuronal subtype aspects.

Table 2.2. General descriptions of neurodevelopmental processes in the DNT IVB	
Process	***Description***
Proliferation	Division of neural stem cells and neural progenitor cells resulting in an increase in cell number. Changes in proliferation can result in an incorrect cell number (increase or decrease) and altered brain growth. It is measured directly by assessing the number of cells undergoing DNA replication or inferred by measuring a change in cell number over time.
Apoptosis	Programmed death of cells resulting in a decrease in cell number. Changes in apoptosis can result in an increase or decrease in cell number and altered brain growth. It is measured by assessment of cell nucleus morphology or detection of biochemical markers specific to the apoptotic pathway.
Migration	Movement of neural progenitor, glial or neuronal cells from their point of origin to a final position. Changes in migration can result in cells in the wrong position and abnormal brain structure. It is measured by assessing the number of cells moving into a defined area, or the distance moved by individual cells.
Neuronal Differentiation	Process in which a neural progenitor cell changes to a specific type of neuron. Changes in differentiation can result in altered cell numbers for specific populations of neurons, changing brain structure and function. It is measured by assessment of the number of cells expressing markers specific for neurons and neuronal subtypes.
Neurite Growth	Outgrowth of morphological processes relatively early in neuronal differentiation. Neurites eventually develop into dendrites or axons. Changes in neurite growth can alter the number and length of axons and dendrites, changing brain structure and connectivity between neurons. It is measured by counting the number of cells elaborating processes or morphological assessment of neurite length.
Neurite Maturation & Synaptogenesis	Maturation of neurites into the specialized processes of dendrites and axons which then form synapses responsible for communication between neurons. Changes in neuronal maturation and synaptogenesis alter neuronal connectivity, changing network formation and brain function. They are measured by morphological assessment of axon and dendrite length and counting of the number of synapses.
Glial Differentiation & Maturation	Process in which a neural progenitor cell changes to a specific type of glia (radial glia that support formation of cortical architecture, astrocytes that support neuronal function, and oligodendrocytes that myelinate axons). Changes in differentiation can result in altered numbers of glia and reduced

	myelination, changing brain structure and function. Differentiation and maturation are measured by assessment of the number of cells expressing markers specific for glial subtypes.
Network Formation	Process in which neurons and glia grow and make functional contacts with each other, exemplified by spontaneous generation and propagation of electrical action potentials within a network. Changes in network formation and function can result in altered neural connectivity and altered brain function. It is measured by electrophysiological assessment of coordinated electrical activity of neurons and glia grown on electrode arrays.
Viability/Cytotoxicity	Test methods for each neurodevelopmental process should also include a concurrent measure of cell viability (or its converse, cytotoxicity) as a baseline for comparison of potential non-specific effects of chemical exposure. Cell viability can be assessed by counting cells with normal morphology (based on cell body and nucleus size), delineation of live/dead cells based on uptake or exclusion of vital dyes, or biochemical assessment of active cell metabolism. Cytotoxicity is typically assessed by measuring parameters associated with loss of cell membrane integrity, including leakage of intracellular proteins and enzymes, or exposure of DNA.

23. In vitro DNT test methods have been reviewed (Bal-Price et al., 2018; OECD 2017a), and those that constitute the current DNT IVB are presented in Table 2.3, which is organised by the neurodevelopmental process being modelled in vitro for chemical testing (i.e., the test purpose). For each test method, a brief description is provided of the test system, exposure scheme, and endpoints measured. To facilitate the evaluation of data from cell-based test methods and potential use in regulatory settings, the OECD has formulated guidance for a more detailed documentation for non-guideline and new approach methods (OECD 2017b). This guidance outlines the standard information that should be provided by developers to assess the quality of data produced and the potential utility in regulatory applications. A more recent and expanded template called ToxTemp, expanding upon OECD GD211, was developed to guide the user in the details required, specifically for the description of cell-based test systems (Krebs et al., 2019). For the DNT IVB, assay descriptions based on these formats are provided in Appendix B.

Table 2.3. Assays Currently in the DNT In Vitro Battery

Test Method (Assay)	Test System (Cell culture)	Assay Duration/ Chem exposure	DNT Endpoint	Viability/Cytotoxicity Endpoint
Proliferation				
NPC1	human NPC grown as proliferating 3D neurospheres	72 h / 72 h	neurosphere area, BrdU incorporation in dividing cells	Resazurin reduction /LDH release
hNP1 Prolif	human NPC	24 h / 24 h	BrdU incorporation in dividing cells	ATP level
Apoptosis				
hNP1 Apop	human NPC	24 h / 24 h	apoptosis pathway (Caspase) activation	ATP level
Migration				
UKN2	human NSC-derived neural crest cells	72 h / 24 h	number of cells moving into defined area	Calcein-AM vital dye
NPC2a	human NPC grown as differentiated 3D neurospheres	72 h / 72 h 120 h / 120 h	mean distance of radial glia (nuclei negative for neuronal and oligodendrocyte markers) from edge of sphere	Resazurin reduction/LDH release
NPC2b	human NPC grown as differentiated 3D neurospheres	120 h / 120 h	mean distance of tubulin-positive neurons from edge of sphere	Resazurin reduction/LDH release
NPC2c	human NPC grown as differentiated 3D neurospheres	120 h / 120 h	mean distance of O4-positive oligodendrocytes from edge of sphere	Resazurin reduction/LDH release
Neuronal Differentiation				
NPC3	human NPC grown as differentiated 3D neurospheres	120 h / 120 h	number of tubulin-positive neurons	Resazurin reduction/LDH release
Neurite outgrowth				
NPC4	human NPC grown as differentiated 3D neurospheres	120 h / 120 h	neurite length & area	Resazurin reduction /LDH release
UKN4	human NSC-line (v-myc transformed)	72 h / 24 h	neurite area	Calcein-AM vital dye
UKN5	human iPSC-derived peripheral (sensory) neurons	24 h / 24 h	neurite area	Calcein-AM vital dye
hN initiation	human iPSC-derived neurons	48 h / 48 h	neurite length	cell morphology
Cortical initiation	rat primary neocortex	48 h / 48 h	neurite length	cell morphology
Neurite Maturation and Synaptogenesis				
Cortical maturation	rat primary neocortex	288 h / 120 h	dendrite length	cell morphology

Cortical synapses	rat primary neocortex	288 h / 120 h	synapse number	cell morphology
Glial Differentiation				
NPC5	human NPC grown as differentiated 3D neurospheres	120 h / 120 h	number of O4-positive oligodendrocytes	Resazurin reduction /LDH release
Neural Network Formation				
Cortical MEA	rat primary neocortex	288 h / 288 h	action potential spike and burst parameters related to network connectivity	Resazurin reduction/total LDH

Developmental Neurotoxicity (DNT) versus Neurotoxicity (NT) In Vitro

24. Testing for DNT is needed due to the fact that the developing brain may be more vulnerable to chemical toxicity than the adult brain. A major reason for increased vulnerability is the dynamic nature of development itself. During this period there is a sequential and coordinated expression of molecular signalling pathways underlying neurodevelopmental processes and subsequent changes in nervous system structure and connectivity (Semple et al., 2013). Chemical disruption during critical time windows can lead to both reversible and permanent effects (Rice and Barone 2000; Rodier 1994).

25. In vitro assays that measure the impact of chemicals on the nervous system can be divided into two broad categories: 1) assays for DNT using test systems that aim to model neurodevelopmental processes; and 2) assays for neurotoxicity (NT) that use test systems that aim to model the steady state observed in the adult brain and may not model developmental processes. Assays in the DNT IVB are included in the first category and are designed to assess the impact of exposures on aspects of neurodevelopmental processes. In vitro test systems for developmental neurotoxicity are, by definition, in a dynamic state, undergoing changes in cellular status over time. For example, in proliferation assays the number of neural progenitor cells increases over time (Moors et al., 2009), while for neurite outgrowth assays neuron cell number is relatively constant but the length of neurite processes is increasing (Harrill et al., 2010). This contrasts with the second category, NT assays, which use in vitro test systems that are designed to assess neurotoxicity in cell cultures using mature-type cells that have reached a relatively stable equilibrium. These mature neural cell cultures do not necessarily exhibit the complex and dynamic changes in cell status associated with active neurodevelopment.

Developmental Exposure In Vitro

26. Proper evaluation of data from DNT in vitro assays requires a good understanding of the exposure regimen used. While there are several factors to consider, the most important is the timing and duration of exposure relative to the timing of dependent variable measurement. A rapid and acute chemical exposure applied to the in vitro test system over a relatively short time (e.g., seconds to minutes) prior to assessment of the endpoint is not a long enough exposure since it does not encompass a period that includes a significant change in the measured endpoint related to a neurodevelopmental process. However, it may provide information on direct effects of a chemical on cell function.

27. Developmental exposures require time periods between chemical application and endpoint assessment that coincide with the dynamic changes in cell status associated with neurodevelopmental processes. Since exposures may be different for each test method and test system, they must be documented with reference to the dynamic change in cell status as described above. The actual

exposure scheme can range from a single application without media change at the start of the test to multiple applications (with or without media changes) at regular periods throughout the test. For the DNT IVB, the test duration can range from 24 hours to weeks. For example, proliferation is a process that can occur relatively rapidly in neural progenitor cells. In the DNT IVB, the hNP1 proliferation assay uses a single chemical application at the start of the 24-hr exposure period, during which the cells are undergoing cell division. In contrast, neural network formation is a more prolonged process that includes axon and dendrite outgrowth and synapse formation occurring over days to weeks. In the rat Cortical MEA assay, multiple applications of the chemical are made along with media changes at regular intervals throughout a 12-day test period (Brown et al., 2016). For this assay, the delay of 48 hr between chemical application and endpoint assessment minimises the chance of detecting rapid and acute effects of chemicals on neural network activity that would not necessarily reflect a change in neurodevelopment. Acute exposures can, however, provide information on MIEs by examining the direct effects of a chemical on cell signalling pathways that regulate neural cell electrophysiology. For example, MEA assays using mature cortical cell cultures have been used to study the effects of chemicals on action potentials following a single acute exposure (i.e., chemical addition to the cells minutes prior to recording) (Strickland et al., 2018; Valdivia et al., 2014).

28. The suitability of the chemical exposure scheme should be determined with reference to the developmental status of the test system at the beginning and end of the test for each individual test method. Information on the exposure scheme (including a graphical timeline indicating addition of chemical(s) and medium, assessment of endpoints, etc.) in the context of the overall cell culture scheme is provided in Section 5 of ToxTemp (Krebs et al., 2019). Further documentation of the test method should demonstrate that the exposure scheme is appropriate for examining chemical effects on a particular neurodevelopmental process based on the use of positive control compounds (Crofton et al., 2011).

3 Elements for Establishing WoE

Background

29. Any WoE assessment, not only for DNT IVB data, may follow OECD GD311 (OECD 2019a) or equivalent internal guidance of regulatory authorities. WoE guidance provides a set of principles and elements that promotes a consistent and clear approach to ensure transparency and avoidance of unreasonable bias in the WoE decision processes. The overall aim of data evaluation is to determine data quality, based on relevance and reliability.

30. Relevance of the test methods and data should be determined by an a priori problem formulation as a critical first component in the use of data from the DNT IVB in regulatory assessments. A "fit for purpose" or "context appropriate" approach (Andersen et al., 2019; Cote et al., 2016; Griesinger et al., 2016; Judson et al., 2013) can be used to determine whether the uncertainties in the data (or dataset) are deemed acceptable for the regulatory need (see also: Parish et al. 2020; Patterson et al. 2021; van der Zalm et al. 2022; Carmichael et al. 2022). IATAs encompass this concept (see Section Integrated Approaches to Testing and Assessment (IATA) for DNT and Figure 2.1) with two possible outcomes after consideration of all empirical information against the regulatory need; either the available information is adequate and supports a decision, or the uncertainty due to data gaps is too large and a decision cannot be made.

31. WoE will require assessments of both data for the individual assays (see Section Criteria for individual assay evaluation below) as well as the entire data set from all assays (see Section Evaluation of the DNT IVB for chemical testing below). Ideally, all assays used in the DNT IVB and the battery itself should have been validated for predicting adverse in vivo developmental neurological outcomes according to OECD principles (see Section Uncertainties (summary)). However, the lack of a formal validation, including inter-laboratory transferability, (i.e., OECD GD34) should not impede the use of data from the DNT IVB in a fit-for purpose manner for some regulatory assessments (Parish et al., 2020; Patterson et al., 2021; van der Zalm et al., 2022). The important issue is that the uncertainties in the assays and available data are transparently communicated to regulatory authorities, who will decide if such uncertainty is acceptable given their decision needs (Weinberg 1972). To date, data from the DNT IVB was used in a WoE-based case study for some organophosphate pesticides (USEPA 2020b), in IATAs for deltamethrin (OECD 2022a) and flufenacet (OECD 2022b), and in IATAs for the neonicotinoids, acetamiprid (OECD 2022c) and imidacloprid (OECD 2022d). See the complete list of examples in Appendix D.

Generic In Vitro WoE Issues

32. All in vitro data, not just data from the DNT IVB, must meet criteria for use in regulatory assessments. These criteria include adherence to guidance for reporting test systems and methods, data analyses, and transparency, and may vary based on the regulatory context.

33. First among these criteria is whether the in vitro data to be used was generated using OECD Test Guidelines or national equivalents. If not, documentation of the test methods should, at a minimum, adhere to OECD GD286 Guidance Document on Good In Vitro Method Practices (GIVIMP) (OECD 2018) and GD211 (OECD 2017b). GD286 provides guidance for both development and implementation of in vitro test methods using good scientific, technical, and quality practices. The goal of GD286 is to enable test developers to establish robust in vitro methods and those utilising the in vitro method to apply the assay following the established good practice, thereby reducing uncertainties in predictions from in vitro data and assisting in the acceptance of such data by regulatory authorities. All present and future DNT IVB assays should be compliant with this guidance.

34. The rapid development of in vitro technologies has led to the recognition that in vitro test methods without applicable Test Guidelines may be useful within various regulatory frameworks (EFSA 2021). OECD GD211 (OECD 2017b) outlines the type of information that should be provided for each assay, by the assay developers. The test system and methods should be described in enough detail to allow assessment of data quality and its potential use in regulatory applications. It is important to note that GD211 acknowledges that due to rapid technological developments this guidance "may thus need to be revised in the near future". Indeed, a revision has already been developed (i.e., ToxTemp) that has improved on details that should be provided for NAMs (Krebs et al., 2019).

35. The assays included in the DNT IVB proposed in this initial recommendations were developed consistent with the principles of OECD GD286, and all assays have descriptions that include the information required in the newly expanded ToxTemp format in Appendix B (Masjosthusmann et al., 2020).

WoE Issues Specific to DNT Battery

36. There are limitations of the DNT IVB that must be considered in any WoE assessment. Some are similar to limitations of other in vitro test methods. These include lack of, or unknown metabolic competence of the assays, limitations of testing methods for volatiles or DMSO insoluble chemicals, limited exposure durations, and potency estimates based on nominal media concentrations (Thomas et al., 2018; Tice et al., 2013). Examples of limitations specific to the DNT IVB are listed in Table 3.1 (see also Section Uncertainties (summary)).

Table 3.1. Examples of WoE limitations in evaluation of DNT IVB

- The lack of assays for several cellular processes and systemic processes known to be critical for normal neurological development (see Sections Developmental Neurotoxicity In Vitro Battery (description of assays) and Evaluation of the DNT IVB for chemical testing).
- Need for development of additional AOPs to increase mapping of the KEs covered in the DNT IVB.
- A relatively limited number of tested chemicals as compared to current accepted batteries (e.g., ER activation).
- Uncertainty in the overall specificity and sensitivity of the DNT IVB due to limited testing of DNT reference chemicals and comparison of results to a curated in vivo developmental neurotoxicity study database.
- A need for a consensus-based and regulatory driven tiered testing strategy to be used in IATAs.

Data Availability

37. A key component of any WoE is transparency in all decisions. For high-throughput screening (HTS) data an especially critical component is the availability of all data on the activity or inactivity of a compound in an assay. Examples of good practice in data availability and analyses include the Tox21 and ToxCast datasets available from NCATS (https://tripod.nih.gov/tox21/assays/), NIEHS Division of Translational Toxicology (DTT) CEBS database (https://cebs.niehs.nih.gov/cebs/), and US EPA ToxCast programs (https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data), in which the publicly available data is adequate to foster transparency and independent replication of analyses.

38. To aid in transparency and increasing confidence in data usage, it is critical that there is public access to both all the underlying data as well as the analysis algorithm code. Results from some previous analyses are available as processed data (Frank et al., 2017; Harrill et al., 2018; Masjosthusmann et al., 2020; Shafer et al., 2019). Processed data has been through normalisation, curve fitting and hit calls, unprocessed data has not been through these processes. Some additional assays from the NIEHS DTT project have hit calls, but only summary data are available (Developmental NeuroToxicity Data Integration and Visualization Enabling Resource, DNT-DIVER https://doi.org/10.22427/NTP-DATA-002-00062-0001-0000-1). The recent release of ToxCastDB v3.5 provides public access to both unprocessed and processed data, the underlying analysis code, and results for all 17 assays analysed using the ToxCast data analysis pipeline. This includes hit information, curve fit comparisons, and a number of flags or warnings that the processing pipeline has identified as possible false positive or false negative findings.

Criteria for Individual Assay Evaluation

39. Evaluation and appropriate use of the data derived from NAMs such as the individual in vitro assays that comprise the DNT IVB should be based on demonstrating that they have biological and toxicological relevance (e.g., scientific rationale for use of the test system, linking endpoints measured to an adverse outcome) and acceptable technical qualification (e.g., repeatability, reliability, predictive capacity) (Judson et al., 2013). As described in Section 2, the battery of assays in the DNT IVB are based on an understanding of the underlying neurobiological processes required for normal brain development, with each assay modelling aspects of a potential KE at the cellular level in the AOP framework for DNT (Figure 2.2). Evaluation of the utility and performance of any specific assay will therefore be influenced by how well the in vitro test system can recapitulate aspects of a particular neurodevelopmental process. Supporting information for the evaluation and use of data from individual assays is listed in Table 3.2 (see Krebs et al. 2019 for details). While the assays in the DNT IVB were developed in different laboratories using multiple technologies, common criteria for evaluating the state of readiness (i.e., fit-for-purpose use) were applied across all assays in the DNT IVB. The type and amount of information describing individual assays, including the availability, and testing of reference chemicals, will vary.

40. It is important to note that these initial recommendations do not provide extensive details and analyses of the assays in the DNT IVB. Instead, it highlights those factors deemed most important for assessing how and when the data may be used in a fit-for-purpose regulatory context, including screening and prioritisation of chemicals or targeted testing for single chemical hazard assessment (see also Section Target Chemicals). A detailed description of the information and data used for evaluation of the individual DNT IVB assays is documented in Appendix B. These descriptions use the ToxTemp format (Krebs et al., 2019) which explicitly provides the acceptance criteria for test elements needed for evaluation of assay performance. A summary of the information described in the ToxTemp is shown in Table 3.3. Further sources of data documenting assay relevance and performance can be found in

publications from the assay developers as documented in assay descriptions in Appendix B, and several reviews evaluating the readiness of DNT in vitro assays for regulatory use (Bal-Price et al., 2018; Fritsche et al., 2017; Masjosthusmann et al., 2020). The following text provides examples and recommendations for evaluating test elements that are critical for determining assay performance and informing data interpretation.

41. *Application of the test system to assess the key neurodevelopmental processes*. Because the assays are designed to recapitulate basic processes of neurodevelopment, the ability of the test system to reproducibly undergo quantifiable developmental changes over time using a multi-well plate format is a prerequisite for assay performance. All assays in the DNT IVB are known to use test systems that assess neurodevelopmental processes (Fritsche et al., 2017). DNT cell culture test systems should be characterised and their ability to accurately quantify developmental changes in an endpoint related to the KE demonstrated (Crofton et al., 2011). Additional endpoints to concurrently assess aspects of cell health and viability should be included. This information is typically provided in peer-reviewed publications by the assay developers and can include data showing control of the culture conditions that influence the dynamic range of the endpoint. For example, published data that evaluate the KE of neurite outgrowth are available for LUHMES cells (UKN4 assay; (Stiegler et al., 2011), and human neuronal cells (hN initiation assay; (Harrill et al., 2011a). These publications characterised cell growth

Table 3.2 Critical elements to be considered when assessing data from individual assays in the DNT IVB

Critical Element	***Evaluation Criteria***
The test system assesses change in one or more aspects of neurodevelopmental processes	Characterisation of neurodevelopmental process in vitro including demonstration that endpoints accurately quantify developmental change in a key event over time. Evaluation of the ability of measured endpoints to quantify chemical-induced changes in the key event, as well as concurrent measurement of viability/cytotoxicity.
Assay quality and repeatability	Estimates of criteria such as signal to noise ratio, linear range, and baseline variation of the measured endpoint(s). Within laboratory repeatability of the assay is demonstrated in medium- to high-throughput testing format using multi-well plates.
Chemical screening with training set	Use of training set (endpoint-specific positive controls and negative controls) to demonstrate ability of assay to test multiple chemicals in concentration-response mode and provide initial indication of predictive ability.
Data analysis and identification of a reference point	Methods for data normalisation and curve fitting for concentration-response analysis is documented. Derivation and justification of a reference point identifying critical effect levels based on concentration-response data.
Descriptions of chemical activity	Description of procedure used to classify chemicals as active or inactive in the assay. Determination of selective effect on neurodevelopmental process versus general effect on cytotoxicity. Supported by correct identification of positive and negative controls.

in 96-well plate format, quantified neurite outgrowth over time using automated measurement of neurite number, length, and/or area as endpoints, and simultaneously assessed cell viability. Both studies used endpoint-specific controls (see Textbox 2) to demonstrate the dynamic range of the test system and characterise concentration-related changes in neurite outgrowth.

42. *Assay quality and repeatability in medium- to high-throughput testing mode*. Characterisation of the utility of in vitro assays for chemical testing should include estimates of the signal (S, endpoint measurement under maximal conditions), the background (B, endpoint at baseline or solvent control conditions) and the variation between wells in a multi-well plate format. The signal-to-background ratio (S/B = mean signal / mean background) and the signal-to-noise ratio (S/N = mean signal – mean background / standard deviation of background) can be determined to provide an indication of the dynamic range of the assay. While there is no consensus on an acceptable value, larger numbers generally indicate better dynamic range. For example, in the UKN4 assay, 30,000 cells/well and a time period of 2 h and 24 h after replating were used to evaluate neurite outgrowth, which resulted in a S/N ratio greater than 50 (Stiegler et al., 2011). However, S/B and S/N ratios do not adequately take into consideration assay variability between wells. Another measure, the z' factor, has been developed that provides a measure of assay quality that takes account of both the signal window and assay variability (Zhang et al., 1999). The higher the z' value the more discriminating the assay, with a z' value of > 0.5 generally considered acceptable for complex cell-based assays like those in the DNT IVB. In the UKN4 neurite outgrowth assay example, the z' score was 0.6 (Stiegler et al., 2011). Further discussion of these criteria can be found in OECD 286 (OECD 2018).

Textbox 2.

Endpoint-Specific Controls are chemicals that are known to alter the endpoint of concern in a particular test system, also termed "endpoint-selective controls" or "mechanistic tool compounds". Within a known concentration range, they selectively alter the key neurodevelopmental event (e.g., cell proliferation, neurite outgrowth) without affecting general test system characteristics including cell viability.

Table 3.3. Summary of information in the ToxTemp documentation format with reference to assays in the DNT IVB (Krebs et al. 2019)

Section	Information
1. Overview	Descriptive title and brief abstract describing test method and endpoints used to assess key neurodevelopmental process(es) related to DNT.
2. General information	Test method name, version, related databases, depositor, and contact person.
3. Description of general features of the test system source	Supplier and source of cells. Definition and characterisation of cells, including description of type of neural cell culture (e.g., Neural Progenitor Cells, Primary Cells), species, and format (e.g., 2-D, neurosphere). Procedures for characterisation and maintenance of cells (including acceptance criteria), and differentiation of cells towards the final test system.
4. Definition of test system as used in the test method	Description of the cell culture protocol in terms of the neurodevelopmental process tested. What subpopulation of neural cells are present during the assay (e.g., subpopulations of neurons, glia)? How is the cell culture manipulated (addition of mitotic inhibitors, growth factors, etc.) during the assay? Is there endogenous metabolic capacity in the test system (e.g., CYPs)? Is there any transporter activity? How closely does the test system reflect the *in vivo* neural tissue being modelled?
5. Test method exposure scheme and endpoints	Description of the exposure scheme: how and when are cells exposed to the test compound (including a graphical timeline) in terms of the overall culture protocol (when cells are plated, medium changes, endpoint measurement)? Definition of specific endpoints measured in relationship to the neurodevelopmental process tested. Is cytotoxicity assessed concurrently? Are any reference endpoints included (e.g., cell number, protein content) to be used for normalisation? What endpoint-specific controls (positive, negative, unspecific) are used? What rules are applied to determine that the assay is performing as expected and that the results are acceptable?
6. Handling details of the test method	Description and documentation of the execution of the assay, with reference to a Standard Operating Procedure (SOP). Provides details for preparation and addition of test compounds, including definition of the concentration range. Precautions and uncertainties that can affect with the assay should be noted (e.g., compounds that are volatile or auto fluorescent, known sources of variation, need for special supplies or equipment).
7. Data management	Description of raw data format, definition and handling of outliers, and processing of raw data to summary data.
8. Prediction model and toxicological application	Which neurodevelopmental processes are modelled in the test method? What toxicological endpoints (e.g., neurite length, cell number, etc.) are used in the prediction model? How does the model classify the results in terms of toxicity (e.g., hit, no hit, borderline) and selectivity (e.g., neurotoxicity vs cytotoxicity)? What is the performance of the test method (e.g., sensitivity and specificity, z-score, etc.) and what compounds were used to make that determination? What is the application domain of the test method and how does it fit into the DNT IVB?
9. Publication/ validation status	Provide a list of key publications describing the development and use of the test method. Is the test method linked to an AOP or is there information on mechanistic validation? Is it part of a formal pre-validation or validation study?
10. Test method transferability	What experience/training is required? Has the test method been transferred to another laboratory?
11. Safety, ethics, and specific requirements	Are there any specific hazards or safety requirements? Is any aspect of the test method licensed or protected by intellectual property rights?

43. Variation between plates and over time (i.e., between experimental runs) is also used to evaluate repeatability. This information can be obtained by assessing endpoint response under baseline (solvent control) and treated (endpoint-specific positive control) conditions in multiple plates across independent cultures. For example, repeatability of neurite outgrowth was compared in the hN assay

and the rat primary cortical initiation assay across multiple plates and cultures (Harrill et al., 2011b). The results showed that an endpoint-specific positive control (lithium chloride) consistently decreased neurite outgrowth in both assays, with a coefficient of variation (CV) of 27% in human neurons and 6% in rat primary cortical neurons. Similar data for other assays in the DNT IVB can be found in the EFSA DNT publication (Masjosthusmann et al., 2020) and Appendix B. This information is important for understanding the difference in sensitivity between assays. These results illustrate that the assays in the DNT IVB differ in their variability, and thus will not be able to detect the same effect size. This will be an important consideration in the calculation of a reference point and formulation of the data analysis algorithm (see below), especially when comparing data across the battery of assays.

44. *Use of a training set*. To further demonstrate the biological relevance and utility of an assay, as well as its practical ability to rapidly and efficiently screen moderate numbers of chemicals, a larger set of chemicals, called a training set (Crofton et al., 2011) should be used (see Textbox3).

Textbox 3.

A **Training Set** is a list of chemicals including those that are known to reliably elicit a response to the specific neurodevelopmental endpoint of interest (positives), and those that do not elicit a response (negatives). Evidence for an effect, or lack thereof, should come from in vitro data using multiple test systems.

45. It is important to note that for the assays in the DNT IVB, there is not a standard training set for each of the neurodevelopmental processes. Rather, training sets have been selected and annotated by the individual assay developers, and often include several endpoint-specific controls. Examples of training sets for in vitro assays of the neurodevelopmental process of neurite outgrowth are described for the hN, UKN4 and hNPC assays (Harrill et al., 2011b; Krug et al., 2013; Masjosthusmann et al., 2020). It is important to note that training set chemicals are specific to the neurodevelopmental endpoint being measured, and different training sets will be necessary for the different assays in the DNT IVB. In the above examples, the training sets for assays of neurite outgrowth included chemicals that selectively affected signalling pathways involved in neurite extension, as well as environmental toxicants that have multiple or unknown modes of action that include known effects on neurite outgrowth (Harrill et al., 2011b; Krug et al., 2013). Similarly, chemicals affecting signalling underlying hNPC migration, neuronal or oligodendrocyte differentiation were used as a training set for those endpoints (Masjosthusmann et al., 2020). In comparison, a training set for an assay for proliferation included chemicals that selectively altered DNA replication and cell division, as well as some of the same non-selective environmental toxicants that affect neurite outgrowth that also affect proliferation (Mundy et al., 2010). The use of a training set which includes chemicals that have a positive effect, or no effect, informs the demonstration of the ability of an assay to test multiple chemicals at the same time in concentration-response mode (i.e., throughput), and can provide an initial indication of its predictive ability. All assays in the DNT IVB were developed using training sets selected by the assay developer.

46. *Data analysis and identification of a reference point*. The methods used to analyse data from the DNT IVB assays require two major steps. The first is a statistical data analysis algorithm and the second is the choice of a reference point (see Textbox 4).

Textbox 4.

The **Reference Point** is a point on the concentration-response curve corresponding to an estimate of potency (e.g., EC_{50}) or a threshold defining a critical level of response (e.g., 20% change from control).

47. Data analyses typically start with plate corrections. Assays in the DNT IVB normally include solvent controls and a wide concentration range for a chemical within a multi-well plate. This plate map is then repeated across multiple plates, and for large numbers of chemicals testing can occur over multiple cultures and experimental runs. The initial step may include pre-processing of raw plate data, such as subtracting out plate blanks (Nimtz et al., 2019). This step may vary depending upon the technology used to measure the endpoint (e.g., fluorescence plate reader, automated imaging, etc.). The next step is normalisation of data to control wells within a plate to correct for plate-to-plate variability. In high-throughput screening assays there are several methods for normalisation based on calculating percent of control. These include dividing raw values by the average of the within plate solvent control, or, under the assumption that most compounds are inactive, including wells containing low chemical concentrations in the average of the solvent controls (Malo et al., 2006). Normalised concentration-response data are then pooled across all experiments and subjected to curve fitting using multiple models (including linear and non-linear models), and the best fit curve selected and used for determination of a reference point. The normalisation and curve fitting methods have been clearly stated for each assay in the DNT IVB and are found in the assay description in Appendix B. Automated data analysis pipelines have been developed for high-throughput screening assays (e.g., Filer et al., 2017; Hsieh et al., 2015), and models are also available to estimate the uncertainties in estimated potency and efficacy endpoints (Watt and Judson 2018). Moving forward, data analysis should be performed in a consistent and transparent manner for all assays in the DNT IVB. Confidence in any results is vastly improved when all aspects of the statistical analyses are publicly available and allow replication of findings.

48. A consensus choice of reference points (effect concentrations) for chemical effects observed using in vitro assays in the DNT IVB has not yet been formalised (Hardy et al. 2017). Based on standard quantitative analysis of chemical-receptor interactions, many in vitro pharmacologic and toxicologic studies focus on determining the concentration giving rise to a 50% response (i.e., EC50) (Goodman 1996). While using this approach can be valuable in ranking the potency of chemicals and prioritising them for further testing (e.g., Paul Friedman et al., 2016), it does not necessarily consider biological variation or relevance of the degree of in vitro change to possible in vivo adverse outcomes. For this purpose, the Benchmark Concentration (BMC) approach has been recommended by the EFSA Scientific Committee (Hardy et al., 2017). This approach fits a concentration-response curve to all data points and generates the BMC as the concentration that is associated with a predefined level of response, that is, the Benchmark Response (BMR). Ideally the in vitro BMR would be chosen based on the understanding of the relationship between the endpoint measured (neurodevelopmental process) and the adverse outcome in vivo (DNT). Because there are currently few AOPs documented quantitative relationships between DNT IVB endpoints and in vivo neurodevelopmental outcomes, the selection of an in vitro BMR for a particular assay is based on expert judgement and scientific consensus of stakeholders. For individual assays in the DNT IVB this choice was made by the assay developer after considering the variation in a particular assay and limiting the number of false positives. Ongoing work is aimed at development of a consolidated data analysis pipeline that uses the same process for choices of in vitro BMRs across assays, to begin to address this issue (Paul Friedman, personal communication). This effort will allow estimation of the false positive and false negative rates of the entire battery. Preventing false negatives in DNT IVB screening efforts is critical and is needed to decrease the uncertainty in hazard assessments.

49. In the absence of a biological basis for selecting the in vitro BMR, its value can be defined statistically as an effect size that is higher than the biological (control) variability of the measured endpoint (Davis et al., 2011). It is important to note that this statistical approach can result in different in vitro BMRs for each endpoint, since the variability will change with the different test systems and methods used in each assay. On a practical basis the in vitro BMR is determined by quantifying the

variability across all control wells in all plates used within a data set or experiment. For screening studies, this can include hundreds of plates. Again, there is not a formalised approach for either calculating the assay variability or setting the in vitro BMR using this calculated value, and the decision is left to the assay operator. One method used in evaluating variability of high-throughput screening data in the USEPA ToxCast program is determining the baseline median absolute deviation (BMAD), a robust statistic of control variability that is resilient to outliers (Leys et al., 2013). This approach yielded coefficients of variation in control values ranging from 2-22% across some in vitro DNT assays (including the endpoint of cytotoxicity) developed at the USEPA (Harrill et al., 2018). Another method normalises the lowest compound concentration (assuming the lowest concentration has no effect) to the solvent controls within a plate and calculates the standard deviation (SD) between the means of the lowest concentration over all plates in an experiment. This resulted in deviations ranging from 1.5-27% across the assays developed at the IUF - Leibniz Research Institute for Environmental Medicine (Masjosthusmann et al., 2020). Once the variation has been determined, a modifying factor is used to account for the distribution of values in the control well population. Thus, the in vitro BMR may be set for example, at a level above 2xSD (accounting for 95% of the variation in control wells) or 3xSD (accounting for 99% of the control wells).

50. Currently, in vitro BMRs have been set for some assays based on both expert judgement and statistical considerations as determined by the assay developer. For example, for cell migration (UKN2 assay), in vitro BMRs of 25% for migration and 10% for cytotoxicity were used based in part on expert judgement of in vitro biological significance (i.e., what is a meaningful extent of reduction), in part on statistical considerations (all positive controls showed a reduction in migration of > 25%), and in part on graphical comparisons of effective concentrations for positive control chemicals and “unspecific” controls (chemicals that show general cytotoxicity in cell cultures) (Nyffeler et al., 2017b; Zimmer et al., 2012).

51. *Data Analysis and Statistical Model*. Data analysis for the in vitro DNT assay results requires a statistical model which is an algorithm or set of rules for determining whether a chemical has altered the key neurodevelopmental process assessed in the assay. These decisions should include: 1) an evaluation as to whether a change in an endpoint has reached or surpassed the level set as the in vitro BMR; and 2) a determination as to whether the effect is selective for the neurodevelopmental process examined or is a result of a general effect on cell health and viability (i.e., distinguish DNT-specific hits from nonspecific hits). The former is based on the statistical analyses of the data to determine whether a threshold for a hit has been met or exceeded. To determine selectivity, the chemical concentration affecting the neurodevelopmental endpoint (e.g., migration distance, neurite length, etc.) is compared to a concurrent measurement of the concentration affecting cell health (e.g., a measure of cytotoxicity or viability).

52. The statistical algorithm would then categorise a chemical as either inactive, active and selective, or active but non-selective (Textbox 5). In some cases, a fourth category defined as borderline has been used (Delp et al., 2018; Masjosthusmann et al., 2020). Borderline classifications may be defined by an overlap of the confidence intervals for the in vitro BMC of the neurodevelopmental process and in vitro BMC for cytotoxicity/viability (Masjosthusmann et al., 2020). Examples for chemical concentration-response curves illustrating each category have been reported (Delp et al., 2018; Harrill et al., 2018) and are illustrated in Figure 22 from Masjosthusmann et al. (Masjosthusmann et al., 2020).

53. From the description provided above it is apparent that the decision as to whether a chemical has a selective effect on neurodevelopment (i.e., is a DNT-specific hit) will depend upon the choice of the level of response set for the in vitro BMRs for the neurodevelopmental endpoint and a non-specific endpoint such as cytotoxicity/viability, and the procedure used to define the degree of separation required between the resulting in vitro BMCs. To date, the procedure for determining the degree of

separation is not standardised but has been set by the assay developer. For the DNT IVB assays, a common approach has been used whereby testing of each assay includes both endpoint-specific controls (positive control chemicals that alter the endpoint of interest) and unspecific controls (chemicals that show general cytotoxicity in cell cultures). The degree of separation of the in vitro BMCs for the neurodevelopmental endpoint and cytotoxicity is compared for each group of controls, with the expectation is that it will be larger for the endpoint-specific controls and smaller for the unspecific controls. Another approach is to use cytotoxicity data from multiple assays to determine the "cytotoxicity burst" (c.f., Escher et al., 2020; Judson et al., 2016). All these approaches assume that the presence of cytotoxicity is not just a confound, but instead a cause of any changes in the assay endpoint. The degree of separation between the concentrations that change the DNT assay, and the cytotoxicity assay will impact the rate of false positive and false negatives, and the criteria can also be adjusted to balance or limit false negatives, should this be a priority for regulatory use.

54. Statistical determination of the degree of separation most often examines the ratio of the in vitro BMC for cytotoxicity to the in vitro BMC for the neurodevelopmental endpoint for each unspecific control chemical (e.g., EC50 cytotoxicity/EC50 neurodevelopmental endpoint). The ratios are averaged and the variation (SD or 95% confidence interval) calculated. The degree of separation is then chosen as a value that accounts for the upper bound of the variation of the ratios. For example, in the rat cortical MEA Neural Network Formation assay, the mean of these ratios was 1.9 (i.e., network formation endpoints were on average affected by unspecific controls at slightly lower concentrations than cytotoxicity). The upper 95% confidence interval of the ratio was 2.8, so a ratio of 3 was chosen as a threshold for chemicals to be considered as having selective effects (Frank et al., 2017; Shafer et al., 2019). Similar calculations were done for neurite outgrowth in the UKN4 assay, resulting in an average ratio of 1.4 with a SD of 0.8 (Krug et al., 2013). In this case, variation was accounted for using 3xSD, and a ratio of 4 was used as the threshold (Delp et al., 2018; Krug et al., 2013). To verify the appropriateness of the threshold, ratios from the endpoint-specific controls and unspecific controls should be examined to see if they are classified correctly as selective or nonselective, respectively.

55. A second approach is to set individual fixed in vitro BMR levels for the neurodevelopmental and cytotoxicity/viability endpoints which consider differences in the underlying biological process and/or the baseline variability. Different in vitro BMR levels can be compared, and the most appropriate chosen based on a ratio that correctly classifies the endpoint-specific and unspecific control chemicals. This approach has been used for the UKN2 cell migration assay (Nyffeler et al., 2017b). An in vitro BMR of 10% for cytotoxicity/viability was used based on biological plausibility that changes in viability below this value are not meaningful. In contrast, an in vitro BMR of 25% for migration was used based on experimental findings that unspecific control chemicals may cause up to a 25% change of migration, but not beyond. Using the EC10 cytotoxicity/EC25 migration ratio, all unspecific control chemicals had a ratio ≤ 1.11, whereas endpoint-specific control chemicals reached a ratio of > 1.3. Thus, the EC10 cytotoxicity/EC25 migration ratio of 1.3 was used as the threshold to classify selective chemicals (Nyffeler et al., 2017a).

56. As described above, the outcome of the data analyses that interpret chemical data from individual assays in the DNT IVB is dependent upon variables that are inherent to the test method (complexity of the test system, measurement technology used, biological variability, etc.), but also upon

Textbox 5. Potential Chemical Categories Based on Assay Results:

Inactive – An in vitro BMR not reached for either the neurodevelopmental process or cytotoxicity/viability.
Active and selective – An in vitro BMC for the neurodevelopmental process separated from the BMC for cytotoxicity/viability.
Active but non-selective – An in vitro BMC for the neurodevelopmental endpoint is not separated from the BMC for cytotoxicity/viability.
Borderline – chemicals for which the separation between the in vitro BMC for the neurodevelopmental endpoint and the in vitro BMC for cytotoxicity/viability is not clear.

decisions by the assay developer/operator. These decisions include expert judgement on the level of change in an endpoint considered as biologically relevant in vitro (i.e., relative to assay positive controls in the training set), what constitutes a DNT-specific effect, and the statistical evaluation of variability. Currently, most statistical algorithm set parameters that are relatively conservative to maximise both sensitivity (the correct prediction of a positive compound) and specificity (the correct prediction of a negative compound) (e.g., Filer et al., 2017; Hsieh et al., 2015). The parameters used by assay developers for assays in the DNT IVB were chosen to limit the number of false positives in the individual assays. An important advantage of public access to the data and analysis algorithms is that it allows reanalyses that can incorporate adjustment of these parameters to focus on balancing or limiting false negatives, should this be a priority for regulatory use. However, screening of chemicals for potential developmental neurotoxicity involves multiple (unknown) targets and complex biological processes. In this case, the analysis parameters can be altered to be fit-for-purpose. When screening for prioritisation the specificity could be altered to allow for a higher number of false positives, so that a smaller number of potential neurotoxicants will be missed. For example, in the UKN2 cell migration assay the in vitro BMR level for the migration endpoint could be reduced from 25% to 20% (leading to more chemicals classified as active) and the selectivity threshold could be reduced from 1.3 to 1.2 (leading to more chemicals classified as selective). This approach may be desirable when testing large numbers of chemicals for which there is little or no toxicology data. It is necessary to evaluate each assay to determine suitable analysis parameters to ensure that the sensitivity and specificity of the assay are appropriate for the use to which the DNT IVB is put.

Evaluation of the DNT IVB for chemical testing

57. *Predictive performance*. To date there has been limited use of the current DNT IVB in chemical testing. This is mainly the result of research to date that has focused on assay development and refinement efforts. In addition, chemical testing will be facilitated by the assembly of a set of annotated DNT reference chemicals (see Textbox 6).

Textbox 6.

DNT Reference Chemicals are chemicals with evidence for in vivo developmental neurotoxicity identified in studies of humans or animals (positives), as well as chemicals with evidence that they do not result in developmental neurotoxicity in vivo (negatives).

58. The importance of a set of reference chemicals cannot be overstated because it allows estimations of performance, in terms of sensitivity and specificity, of both individual assays and the assay battery. Currently, there is no consensus set of reference chemicals for use in development and validation of DNT in vitro assays. Ideally, a large set of structurally diverse chemicals should be identified (Richard et al., 2020; Richard et al., 2016). Testing this set of DNT reference chemicals across all the assays would allow for the evaluation of the predictive performance of the entire battery. Several groups have evaluated the available evidence for in vivo developmental neurotoxicants to be included as reference chemicals. Short lists of so called gold standard chemicals that are generally acknowledged to be developmental neurotoxicants in humans have been proposed (Grandjean and Landrigan 2006; 2014; Rees et al., 1990). Mundy et al. (Mundy et al., 2015) evaluated approximately 500 peer-reviewed publications and USEPA DNT guideline studies and found evidence for in vivo developmental neurotoxicity in mammals and/or humans for approximately 100 chemicals. Importantly, this list included only chemicals where empirical evidence of DNT was reported from two or more different laboratories. Similarly, a workshop consensus (Aschner et al., 2017) identified approximately 30 chemicals as being in vivo developmental neurotoxicants. Individual laboratories employing assays

in the DNT IVB have, to date, used lists of proposed positive compounds derived from the above publications that include empirical findings of in vivo DNT. Only recently has there been a review publication for negative reference compounds, and it lists only eight chemicals that have empirical findings of no DNT in vivo (Martin et al., 2022). Thus, in the work examining DNT IVB assays, these same laboratories used more extensive lists of proposed negative reference chemicals, chosen primarily based on the authors' expert opinions and not necessarily on empirical negative in vivo data (see in publications listed below for details). Performance estimates of the DNT IVB assays, indicated by the ability to correctly detect a positive (sensitivity) and reject a negative (specificity) based on positive and negative reference chemicals chosen by the authors are summarised below. A summary of all tested reference chemicals is in Appendix A.

59. A set of 75 proposed DNT reference chemicals was assessed in the cortical MEA network formation assay (see Figure 2.3) developed at the USEPA (Frank et al., 2017; Shafer et al., 2019). The chemical set included 61 proposed positives and 14 proposed negatives. Based on a selective hit (i.e., a hit that is DNT endpoint-specific as compared to cytotoxic) in at least one of the seventeen network parameters assessed, the sensitivity was 61% and the specificity was 86%. If the data is considered in terms of any active hit (selective or not selective) in at least one network parameter, the sensitivity was 75% and the specificity was 86%.

60. A set of chemicals consisting of 53 proposed positives and 14 proposed negatives were tested in a suite of DNT IVB assays (see USEPA assays in Figure 2.3) including hNP1 proliferation, hNP1 apoptosis, hN neurite initiation, cortical neurite initiation, and cortical maturation and synaptogenesis (Harrill et al., 2018). Based on selective effects, the combined assays had a sensitivity of 68% and specificity of 93%. When all active hits are considered (regardless of selectivity) the sensitivity was 87% and the specificity was 71%. In both cases, the combined results of all five assays had a better sensitivity and specificity than any individual assay (Harrill et al., 2018).

61. Output from the European Food Safety Authority (EFSA) and Danish EPA projects involved testing at the University of Konstanz (UKON) and the IUF in Düsseldorf using ten DNT IVB assays (see Fig 2.3). This work tested a library of about 100 compounds, assembled from multiple sources, including 29 proposed DNT positives and 17 proposed DNT negatives (for details see Masjosthusmann et al., 2020). The assays tested endpoints including, proliferation, migration, differentiation, neurite outgrowth, and oligodendrocyte differentiation. For selective effects, the combined assays had a sensitivity of 83% and a specificity of 88%. Based on all active hits (regardless of selectivity) from the combined assays, the sensitivity was 83% and the specificity was 82%. Further analysis showed that performance was optimal when results from all assays were combined (Masjosthusmann et al., 2020).

62. These data indicate that use of all 17 assays currently in the DNT IVB can detect a majority of the in vivo developmental neurotoxicants in the current reference chemical set, with a relatively small number of false positives (Harrill et al., 2018; Masjosthusmann et al., 2020; Shafer et al., 2019). The data support the previous international consensus that the battery could be used in the current form for chemical screening (Fritsche et al., 2017; OECD 2017a). As discussed below, a more complete understanding of the predictive performance of the DNT IVB will be possible when a standardised DNT reference chemical set is tested using the entire suite of assays (Table 2.3). The data have been analysed using the USEPA's ToxCast Pipeline (TCPL) (ToxCastDB v3.5). Such analyses will be critical to estimate performance of the entire DNT IVB, and importantly, allow interlaboratory comparisons for assays that measure similar processes.

63. Based on selective hits, the predictive ability across all assays in the DNT IVB exhibit a sensitivity range from 61 to 83%. This is comparable to many other in vitro screening assays. For context, performance estimates of the DNT IVB, can be compared to in vitro assays used for chemical screening for other outcomes such as carcinogenicity, hepatotoxicity, and endocrine disruption. The ability of a battery of commonly used in vitro genotoxicity tests (Ames assay, mouse lymphoma assay,

and the in vitro micronucleus or chromosomal aberrations assay) was evaluated for its ability to predict rodent carcinogens and non-carcinogens, based on a database of over 700 reference chemicals (Kirkland et al., 2005). Using an optimal combination of three assays the sensitivity was 93%, but the specificity was only 29%. Vorrink et al. (Vorrink et al., 2018) evaluated the use of human hepatic spheroid cultures to predict hepatotoxicity of 123 reference compounds with positive or negative clinical evidence for drug-induced liver injury. The results showed a sensitivity of 69% and a specificity of 100%. Judson et al. (2015) used a battery of assays to screen for oestrogen receptor active chemicals with a set of 18 in vitro assays covering multiple KEs. Computational model classifications were derived for 1812 substances, and model performance was compared to data for specific reference chemicals (Judson et al., 2015), and depending on the combination of tests used, the prediction model achieved a sensitivity of 97% and specificity of 89%, as compared to guideline in vitro tests and the uterotrophic assay.

64. Two factors contribute to the uncertainty in assessing the performance of the battery. The first is the lack of a comprehensive analysis to assess performance of the entire data set. The second is the extent of coverage in the DNT IVB for all critical neurodevelopmental processes (Figure 2.3). Several gaps in coverage of neurodevelopment processes and cell types have been acknowledged, including assays for neuroectodermal formation, peripheral nervous system specific processes, astrocyte differentiation and maturation, the blood-brain and placental barriers, microglia regulation of neuronal growth and connectivity, neuronal subtype specification, and axon myelination (see Section 2 Context and Description of the DNT IVB). It is expected that addition of assays that account for these aspects of neurodevelopmental processes will increase the predictive ability of the battery.

65. Several factors, also common to most in vitro assays, contribute to the uncertainty in predicting in vivo developmental neurotoxicity. Brief descriptions of the most important of these are listed below. For a more complete review see (Barbosa et al., 2015; Fritsche et al., 2015; Harry et al., 1998; Harry and Tiffany-Castiglioni 2005; Lein et al., 2005; Smirnova et al., 2014)

- Incomplete coverage of complex interactions within the brain during development. Factors that regulate normal brain development in vivo including cell-to-cell communication within and between brain regions, neurotransmitter and growth factor signalling (Cameron et al., 1998; Cowan and Petri 2018; Ojeda and Avila 2019) are not fully accounted for in the current neural cell culture models. Multicellular test systems (including neurospheres and rat primary cortical cultures) contain limited aspects of cell-to-cell interactions like auto- or paracrine signalling (Kartvelishvily et al., 2006; Masjosthusmann et al., 2018; Ogunshola et al., 2002), thus there is a need for additional assays to cover these complex interactions.
    - Use of human- and animal-derived neural cell cultures. Some assays in the DNT IVB use animal derived cells rather than human cells, and while the fundamental processes are similar, the potential for species-specific effects is unknown. Also, the current DNT IVB does not fully account for sex or human genetic diversity that may influence susceptibility to chemical-induced developmental neurotoxicity (i.e., gene x environment interaction). These factors may result in lower sensitivity and specificity.
- Uncertainties in extrapolation of significant effects found in in vitro models of neurodevelopmental processes to adverse outcomes in vivo. This is hampered by a lack of empirical data to correlate specific levels of changes in the assays to alterations in the corresponding process in vivo leading to neurodevelopmental outcomes.
    - ADME (absorption, distribution, metabolism, and excretion). Like most other in vitro assays/batteries, the ADME processes that determine chemical exposure in vivo are mostly absent in the current DNT IVB. Many in vitro test systems, including the DNT IVB, have minimal or unknown metabolic capacities compared to liver (DeGroot et al., 2018; Ferguson and Tyndale 2011; Hedlund et al., 2001). In addition, developmental changes in in situ metabolism including

the influence of glia are not completely understood. Thus, the DNT IVB may not accurately predict the potential DNT of chemicals that are activated or detoxified. Importantly, there are issues specific to nervous system exposure during development that are not currently included in the DNT IVB, e.g., test methods and kinetics models for chemical transport across the placental (Gingrich et al., 2021; Wong et al., 2020) and blood brain barriers (Ball et al., 2013; Barbosa et al., 2015; Delsing et al., 2020).

- Systemic impacts of hormones and immune signalling on brain development. Circulating steroid and thyroid hormones are known to impact a wide variety of neurodevelopmental processes including sexual dimorphism (Adhya et al., 2018; Bernal 2000). Chemicals that disrupt maternal thyroid hormone levels in vivo can result in developmental neurotoxicity (Miller et al., 2009; Zoeller and Rovet 2004). Several in vitro screening assays have been employed to test the ToxCast and/or Tox21 chemical libraries for a number of known MIEs linked to disruption of thyroid homeostasis and consequent downstream impacts on the developing nervous system (Hornung et al., 2018; Olker et al., 2019; Paul-Friedman et al., 2019; Paul Friedman et al., 2016; Wang et al., 2018). It should be noted that some assays in the DNT battery may be modified to study hormonal mechanisms (e.g.,(Klose et al., 2021)), but they do not currently meet the four criteria for inclusion in the battery. In addition, immune regulators (microglia), the gut/brain axis, and the placental/brain axis also regulate brain development (Cowan and Petri 2018). While endocrine activity and immune signalling are outside the domain of the DNT IVB, incorporation of such data streams will expand coverage of MIEs and KEs for known DNT AOPs, and thus reduce the probability of false negatives. This is an example of how results from the DNT IVB should be assessed in the context of all other available relevant data.

66. The next step to a better understanding of the predictive performance of the entire DNT IVB requires thorough analysis of all data from the 17 assays. The recent release of ToxCastDB v.3.5 has allowed this process to begin. The combined in vitro data can then be compared to any existing in vivo findings, including compounds in a consensus DNT reference chemical list (see paragraph 75 and Appendix A). At this time there are no predictive in silico computational DNT models (Crofton et al., 2022). Development of computational models that use data from multiple assays of the DNT IVB for prediction could be feasible, similar to the model describing oestrogen receptor activation (Judson et al., 2015), where data from in vitro assays covering multiple KEs of the oestrogen receptor activation pathway were used to construct a computational model predicting endocrine disruption. This approach requires the ability to link measured MIEs, KEs and adverse outcomes, but current AOPs describing developmental neurotoxicity are incomplete. However, information on important signalling pathways modulating neurodevelopment is available for some assays in the DNT IVB (Masjosthusmann et al., 2020). These data, obtained by using selective pathway inhibitors, begin to describe the biological applicability domain of the DNT IVB, and provide the basis for further AOP development. Until these types of analyses are completed, it is recommended that all available assays in the DNT IVB should be used within a fit-for-purpose approach.

67. *Evaluation of results across the battery*. Data from chemical testing using all 17 assays in the DNT IVB summarised above (Frank et al., 2017; Harrill et al., 2018; Masjosthusmann et al., 2020; Shafer et al., 2019) show that most chemicals that are hits are active in more than one assay. This is not surprising, and likely the result of two reasons. First, the key neurodevelopmental events modelled in the in vitro battery are controlled by signalling pathways that are not specific for individual processes, but rather contribute to multiple processes (Masjosthusmann et al., 2020). Thus, chemical actions at a single target (MIE) can affect multiple downstream KEs and neurodevelopmental endpoints. Second, some known developmental neurotoxicants are pleotropic, i.e. may act at multiple MIEs (e.g., Klocke and Lein 2020; Prince et al., 2019). As a result, chemical testing has shown a spectrum of chemical effects across the in vitro battery.

68. Evaluation of the potency and selectivity of chemicals across all assays in the battery may be useful in some regulatory frameworks for chemical prioritisation and may provide information on chemical hazard, as well as in the context of cumulative risk assessment. Evaluation of results across the DNT IVB will be facilitated when a consensus approach using a common set of methods and parameters to analyse data is determined. This would include both the concentration-response analysis and determination of a reference point and selectivity (i.e., a DNT-specific hit). Currently the parameters summarising chemical response data from the individual assays (in vitro BMRs and BMCs, selectivity, etc.) have been derived using a variety of different approaches (see Section Criteria for individual assay evaluation). It is suggested that a consensus should be sought for the definition and statistical determination of the values used as the BMR level and the degree of separation between the DNT-specific endpoint and cytotoxicity (i.e., selectivity). There is currently an ongoing effort to harmonize this process (Shafer 2021).

69. As potency is a critical determinant of hazard, chemicals may be ranked and prioritised based on comparison of relative potencies across all assays in the battery. There are a number of ways this ranking could be done. For example, prioritising chemicals that are highly potent in a single assay, or prioritising chemicals that are potent in multiple assays. Further development of a consensus-based statistical algorithm is needed for ranking and prioritising results from the DNT IVB. Regardless, several procedures and tools have been applied to the data currently available for the DNT IVB in order to provide both an overall ranking of chemicals and a graphical visualisation of chemical groups or clusters with similar properties.

- Selective versus non-selective effects. The use of selectivity to filter in vitro data will exclude chemicals that are designated as active as the result of a non-specific effect on cell health. Current DNT IVB data described above suggest that accounting for cytotoxicity improves the correct rejection of chemicals that are DNT negatives (i.e. specificity) but may decrease identification of DNT positives (i.e., sensitivity) (e.g., Harrill et al., 2018). Some well-known DNT chemicals [(e.g., methylmercury (MeHg)] are very potent in vitro, and the degree of separation between the DNT endpoint-specific in vitro BMCs and cytotoxic BMCs can be small; an appropriate cut-off for selective effects can be difficult to define. As more data are collected on chemical effects, it will be possible to compare results from the battery both with and without using selectivity as a filter. In addition, potency estimates for both DNT endpoint-specific effects and cytotoxicity can be compared to values obtained for in vitro assays examining other (non-DNT) effects (see Section Chemical potency in DNT IVB assays versus other in vitro endpoints).
- MSE (Most Sensitive Endpoint). As an initial approach, a chemical can be ranked by potency using the lowest in vitro BMC identified after testing in the DNT IVB. The assay with the lowest in vitro BMC is designated as the MSE, and in addition to use for ranking, it may be considered as a relevant endpoint for further targeted testing. There may be other assays, however, with in vitro BMCs in the range of the MSE that should also be considered. Ranking chemicals by the MSE is illustrated in Masjosthusmann et al. (2020). A caveat on the use of the MSE is that it compares assays that use different benchmark response levels (e.g., BMR of 10% for radial glia migration (NPC2a) versus an in vitro BMR of 30% for oligodendrocyte differentiation (NPC5)). In theory, this could bias the MSE to assays with lower benchmark responses.
- Hierarchical cluster analysis. Chemical effects based on both the number of endpoints affected and the potency for each endpoint can be analysed using hierarchical clustering. The result is a heatmap that provides a grouping of chemicals with similar effect profiles (e.g., Harrill et al., 2018; Masjosthusmann et al., 2020). The data can be used to prioritise chemicals based on potency and number of endpoints affected and facilitate identification of chemicals with a common mode of action.
- ToxPi (Toxicological Prioritization Index). ToxPi is a data modelling tool that combines multiple sources of data and can provide prioritised orders of chemical toxicity based on endpoint

classes, as well as a visualisation of the weight of the contributing factors (Reif et al., 2010). The use of ToxPi to compare chemicals in a class and rank their potential for developmental neurotoxicity is illustrated in Masjosthusmann et al. (2020).

70. *Consistency of evidence within the DNT IVB*. Examination of chemical outcomes across the DNT IVB can also be considered in terms of consistency between multiple assays for the same neurodevelopmental endpoint (e.g., multiple assays for neurite outgrowth) or a known biological relationship between assays at increasing levels of complexity (e.g., the upstream KE of neurite outgrowth proceeding and necessary for the downstream KE of neuronal network formation). In the first case, quantitative analysis has shown that chemical effects in related assays (e.g., UKN4 and UKN5 assays for neurite outgrowth) can be highly correlated (Masjosthusmann et al. 2020). For an individual chemical, consistency of an outcome between different assays for the same endpoint increases the strength of evidence. In the second case, data showing that a chemical alters two KEs in a neurodevelopmental pathway (e.g., neurite outgrowth in the cortical maturation assay and neuronal network formation in the cortical MEA assay) increases the biological plausibility of the effect.

Chemical potency in DNT IVB assays versus assays for other endpoints

71. An important consideration in the evaluation of findings from the DNT IVB is the comparison of chemical potency in neurodevelopmental assays to potency in other in vitro toxicity assays. This allows a differentiation between neural specific effects and disruption of non-specific cellular processes such as cell stress. This can be considered in two ways.

72. The first approach is comparing the potency of a given chemical that is considered a DNT-specific hit to data from other cell types, i.e., non-neural cell types. To do this the potency of DNT-specific chemicals from the DNT IVB could be compared to their potencies calculated in the “cytotoxicity burst” region from all other non-DNT in vitro assays (Escher et al., 2020; Judson et al., 2016). This burst region is the concentration range showing in vitro activity across multiple assay endpoints that is associated with non-specific cellular processes. Examples include activation of cell stress pathways, disruption of proteins or membranes, or broad low-affinity non-covalent interactions (Judson et al., 2016). This approach, while not conducted yet for DNT IVB results, can inform the specificity of the DNT effect relative to non-specific effects across a wide biological spectrum.

73. The second approach is to compare the potency of a DNT-specific hit to the potency in specific hits from other non-DNT assays (e.g., assays for hepatic nuclear receptors, hormone transactivation, proliferation, apoptosis) (Thomas et al., 2013). This has been done to a limited degree for some assays in the DNT IVB (Delp et al., 2018; Masjosthusmann et al., 2020; Nyffeler et al., 2017a; Shafer et al., 2019). These comparisons have demonstrated that for some chemicals the in vitro potency estimates for the DNT IVB assays were below those found for all Tox21 assays (Delp et al., 2018; Ryan et al., 2016), demonstrating the importance of DNT in vitro assays as part of any overall assessment. Failure to include DNT in vitro assays would, for some chemicals, underestimate in vitro potency.

4 Integration of Evidence

Background

74. Use of evidence from the DNT IVB may be guided by several factors, including: 1) the consistency of in vitro data derived from complementary assays within the battery itself; 2) biological plausibility based on existing AOPs for adverse developmental neurological outcomes and any available in vivo data; 3) incorporation of available in vitro to in vivo extrapolation (IVIVE) exposure modelling that extrapolates in vitro exposures to in vivo; and 4) weighting of known uncertainties of the IVB and any existing in vivo data against the regulatory needs. These factors are all integrated with the IATA framework.

Predictive Power

75. While subject to limitations the predictive power of the battery is currently similar to other in vitro (See Section Evaluation of the DNT IVB for chemical testing); estimates of sensitivity range from 61 to 87%, and specificity from 71 to 93% for different groups of DNT assays (Harrill et al., 2018; Masjosthusmann et al., 2020; Shafer et al., 2019). These numbers will evolve over time as the science evolves, and 1) a more comprehensive consensus set of reference chemicals (positive and negative) is defined and tested, 2) new assays are developed that fill known biological gaps in the current in vitro models of neurodevelopmental processes, and 3) there is the development of consensus-based data pipelines and publicly available databases.

Plausibility

76. Any use of results from the DNT IVB must be integrated with all other available information to determine the potential plausibility of xenobiotics to cause alterations in the developing nervous system. Data integration should be conducted using WoE methods such as OECD GD311 (OECD 2019a; 2019b) or equivalent internal guidance of regulatory authorities. This may include data from in silico or read-across predictions, data from in vitro assays or alternative species, data from in vivo animal studies, and human data from clinical or epidemiological studies. Evaluation of findings for single chemicals, or structurally similar chemicals, should be made in the context of known neurobiology pathways that underlie neurodevelopment. Note that for some chemicals or chemical classes (e.g., new or untested chemicals) data on DNT may be non-existent or extremely limited. Below are several examples of the integration of DNT IVB findings with other information that support plausibility.

77. The scientific basis of the DNT IVB is the use of assays for KEs at the cellular level that are plausibly related to the modes of action of developmental neurotoxicants in vivo. At a basic level, plausibility is supported by data demonstrating the correlation of effects of chemicals on neurodevelopmental processes in vitro and in vivo. A number of known developmental neurotoxicants alter KEs in vitro and have the analogous effect after in vivo exposures, with subsequent changes in

brain structure and function (Guo et al., 2013; Jones et al., 1996; Miller 1986; Tingling et al., 2013; Yang et al., 2009; Yu et al., 2010). As a detailed example, MeHg alters the rate of radial glial migration in the NPC2 assay and neural crest cell migration in the UKN2 assay (Nyffeler et al., 2017a), as well as neuronal differentiation and maturation in the NPC3, NPC4, UKN4 and UKN5 assays (Masjosthusmann et al. 2020). And it is known that in vivo developmental exposure to MeHg disturbs neurodevelopment by altering cortical migration processes and results in adverse neuronal differentiation and cortical organisation (e.g., Choi 1986; Guo et al., 2013). Consistency with other in vitro data for the same key neurodevelopmental process adds to the plausibility. For example, MeHg has also been shown to alter migration in other complementary in vitro assays (Kunimoto and Suzuki 1997; Sass et al., 2001), as well as in trophoblast cells (Tucker and Nowak 2018). Thus, consistency of a change in a KE in the DNT IVB assay to an upstream mechanism for the same chemical increases plausibility.

78. There are many signalling pathways that are known to initiate and regulate the KEs controlling neurodevelopment, and chemical or genetic perturbation of these pathways can result in adverse outcomes in brain structure and function. Demonstration of a chemical effect on both a key neurodevelopmental process (measured in the DNT IVB) and on a signalling pathway underlying that process provides evidence for a plausible mode of action. For example, distinct from its role as an esterase to hydrolyse acetylcholine in the adult brain, acetylcholinesterase (AChE) acts as a morphogen during early brain development and can regulate neurite outgrowth (Bigbee et al., 1999). Cholinesterase inhibitors that bind to the morphogenic site on AChE, including chlorpyrifos and its active metabolite chlorpyrifos oxon, can inhibit neurite outgrowth in vitro (Masjosthusmann et al., 2020), in some cases at concentrations that do not inhibit the esterase activity (Das and Barone 1999; Howard et al., 2005). Additional work showed that chlorpyrifos inhibited axon growth in vivo in developing zebrafish (Yang et al., 2011). There is also limited data suggesting chlorpyrifos can alter neurite outgrowth in vivo in mammals (Qiao et al., 2003). This data supports the plausibility of DNT IVB data for inhibition of neurite outgrowth for chlorpyrifos, and although incomplete, provides a plausible AOP leading from AChE binding to altered brain growth (USEPA 2012).

79. Plausibility is also enhanced by mapping changes in the KEs measured in the DNT IVB to existing AOPs. Appendix C maps DNT IVB assay endpoints to KEs in currently available DNT AOPs. For example, NNF is a KE in two proposed AOPs, and NNF is a KE in the current DNT IVB. Recently, EFSA developed an IATA for the integration of all available in vitro data, including data from the DNT IVB, in a developmental neurotoxicity hazard characterization for deltamethrin, a pyrethroid insecticide (OECD 2022a) (see Appendix D). This case study utilised data streams that included: data from the DNT IVB, results of a systematic review of both human and animal studies in the peer reviewed literature, and a DNT OECD TG426 study. This was followed by development of an AOP that links changes in voltage gated sodium channels, through downstream impacts on NNF, to alterations in neurodevelopment expressed as adverse impacts on neurobehaviour. A Bayesian network analysis was used to determine the probability of occurrence of downstream KEs and identified the largest uncertainty data gap as being the lack of empirical support for the biological understanding of the key event relationship (KER) between changes in neuronal network functioning and the adverse behavioural outcome (OECD 2022a). This is an example of how DNT IVB data can inform a hazard characterisation for a pesticidal chemical.

Incorporation of IVIVE

80. As with all in vitro data, the use of data from the DNT IVB in vitro assays to inform human hazard assessment requires the extrapolation from the concentration used in the in vitro assay to identify relevant in vivo exposure levels. Thus, it is important to understand that dose, per se, in many in vitro assays is estimated as the nominal media concentration. The relationship between the media concentration and cellular or target concentration is influenced by several variables, including, but not

limited to binding to media serum proteins and/or the plastics of cell plates, active vs passive uptake into cells or multi-cellular tissues, cell density, and the degree of any metabolism and parent compound stability in the culture (Paini et al., 2019). New models are available to predict partitioning to in vitro compartments, i.e. cells, serum constituents in exposure media, microtiter plate plastic, headspace and extracellular matrices (Kramer et al., 2015; Proenca et al., 2021). Use of media concentrations as a surrogate for cellular concentrations has, especially for lipophilic compounds, been shown to underpredict cell concentrations by orders of magnitude (Croom et al., 2015; Mundy et al., 2004; Schreiber et al., 2010; Shafer and Hughes 2010). Important to note is that actual analytical measurement of media and cellular concentrations in in vitro DNT studies, including the DNT IVB, are very rarely done when screening chemicals. Single chemical assessments should follow existing GLP for practices for homogeneity, concentration, and stability of the test item. Computational models are available for extrapolation from in vitro concentrations to an external in vivo exposure level. The level of uncertainty that may be tolerated varies depending on the context of use. For instance, the current models can be used as part of the weight of evidence analysis to inform relative sensitivity of endpoints such as AChE inhibition versus morphometric changes in an vivo assay. These models involve the use of empirical data for a limited number of in vitro ADME parameters (e.g., binding to serum proteins, disappearance rates of parent chemicals in hepatic cultures) to predict a human oral equivalent dose, a dose which would result in steady-state in vivo blood concentrations equivalent to the in vitro concentration that alters a response by 50% (e.g., Wetmore et al., 2012). Data have been developed to predict oral equivalents for thousands of chemicals (e.g., (Breen et al., 2021; Sipes et al., 2017; Wetmore et al., 2012). To date, this has been done for some DNT IVB assays (Algharably et al., 2021; Dobreniecki et al., 2022; Shafer 2019). It is important to point out that some extrapolated human equivalent doses have extremely high uncertainty (e.g., (Wambaugh et al., 2019; Wetmore et al., 2012). Data gaps in IVIVE for developmental toxicity, including DNT, include a need to incorporate placental transfer, and in addition estimating brain concentrations and the foetal and postnatal development of the blood brain barrier. Oral equivalents calculated from in vitro assays can be combined with human exposure estimates for large numbers of chemicals to develop risk-based prioritisations (Wambaugh et al., 2019). IVIVE can also be used for single chemical assessments. This approach was recently used in a follow up publication to an IATA case study for deltamethrin (OECD 2022a; Maass et al., 2023). While it is acknowledged that progress has been made on development of IVIVE methods for extrapolating results from in vitro assays to an in vivo equivalent dose that can be used in a quantitative risk assessment, there is lack of an internationally accepted guidance on IVIVE procedures and models.

Uncertainties

81. The development of the nervous system, arguably the most complicated organ in the body, involves integration of intracellular, intercellular, interregional, and systemic interactions that occur in development-stage and regional specific manners. Due to the lack of knowledge of all possible molecular targets that, if disrupted, will alter nervous system development, the DNT IVB was designed to measure changes in some of the critical cellular processes downstream from potential molecular targets. Thus, the DNT IVB is different than other in vitro batteries that assess one intracellular pathway (e.g., intracellular oestrogen signalling pathway). Therefore, uncertainties in the evaluation of data from the DNT IVB (see previous sections) will be different compared to other batteries of in vitro assays. In addition to known uncertainties common to all in vitro assays (e.g., metabolism, untested chemical domains), the DNT IVB has additional uncertainties. These include the lack of assays for some critical developmental KEs (e.g., myelination), incomplete coverage of cell-to-cell interactions even in multicellular test systems (e.g., MEA, neurospheres), unknown neuronal subtypes, maturation stage, and complementary assays for only some processes (e.g., migration). The dearth of a consensus set of animal or human positive and negative reference chemicals adds uncertainty to the predictive nature of the DNT IVB and should be a high priority effort in the future (see Appendix A). These uncertainties

are described in previous sections. Details of ongoing work to improve the DNT IVB and address some of these uncertainties, including inter-laboratory transferability, can be found on the OECD DNT IVB Expert Group webpage (https://www.oecd.org/chemicalsafety/testing/developmental-neurotoxicity.htm). Importantly, these uncertainties related to the DNT IVB must be judged in light of the problem formulation.

Usage in Hazard Assessments

82. It is important to note that this document does not provide guidance on follow-up testing or tiered testing that would inform use of in vitro data in hazard assessments. Such testing could include orthogonal assays (i.e., not part of the current DNT IVB) to confirm positives or negatives, or further chemical characterisation using alternative species (e.g., C. elegans, zebrafish), mechanistic testing in rodent, or use of the DNT TG426 or OECD TG443.

83. Development of a tiered decision framework for DNT, which has been previously advocated (Bal-Price et al., 2018; Bushnell et al., 2010; Coecke et al., 2007; Crofton et al., 2011; Fritsche 2017; Lein et al., 2007), has only recently begun. One framework has been proposed (Masjosthusmann et al., 2020) that includes multiple tiers starting with toxicokinetics, then the DNT IVB, targeted follow-up in vitro testing, and targeted in vivo testing in rats only when necessary. This approach advocates using a WoE at each tier to determine if sufficient information is available within the context of a given regulatory need, prior to progression to the next tier. A draft tiered testing framework, which was developed to facilitate international discussion, is currently under review by the OECD.

84. Considering the currently limited use of data from the DNT IVB, it is recommended that regulatory jurisdictions create frameworks that are fit-for-purpose, taking into account scientific uncertainties and practical limitations of existing test methods, to incorporate the DNT IVB into their regulatory process that is reflective of their needs. This should include critical comparisons of the uncertainties and limitations of both the in vitro and in vivo test methods (e.g., (Ly Pham et al., 2020; NAFTA 2016; Paparella et al., 2020). Critical appraisals of in vivo based approaches may serve as an objective benchmark to be met or overcome with any new approach considered (NASEM 2022).

85. Despite the uncertainties summarised above, data from the DNT IVB assays have been used to inform several hazard and risk decisions. For example, data from the NNF assay is being used, along with data from many other in vitro assays, to prioritise large number of perfluorinated chemicals for further testing (USEPA 2019). Prioritisation for flame retardant alternatives is being made based on the combination of data from both in vitro DNT assays and zebrafish (Behl et al., 2015; OECD 2022e). Data from multiple DNT IVB assays has been proposed for use in a WoE for organophosphates (USEPA 2020b). Data from the entire DNT IVB were recently used by EFSA to develop IATA case studies for deltamethrin and flufenacet (OECD 2022a,b). This effort resulted in an AOP-informed DNT risk assessment using all available hazard-related information (e.g., in vitro, toxicokinetics, epidemiology, in vivo animal data) (see also Appendix D). A recent publication summarised a WoE analysis that integrated in vivo data on DL-glufosinate and data from some DNT IVB assays for both DL- and L-glufosinate. Using the DNT IVB data in a WoE approach, a waiver for a new in vivo DNT guideline study testing the enriched isomers (L-glufosinate ammonium, and L-glufosinate acid) was supported (Dobreniecki et al., 2022). These examples illustrate how data from the DNT IVB can be applied to a wide variety of use contexts.

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List of Appendices and Filenames

Appendix	Title	Filenames
A	Proposed Positive and Negative Control Compounds for Use in Performance Evaluations of DNT IVB Assays	Appendix A
	• Lists of Positive and Proposed Negative Control Compounds for Use in Performance Evaluations of DNT IVB Assays	Appendix A.1A
	• Lists of Positive and Proposed Negative Control Compounds for Use in Performance Evaluations of DNT IVB Assays with References and Rationale	Appendix A.1B
B	Assay Names	Appendix B
	• NPC1	Appendix B.1
	• NPC2a	Appendix B.2
	• NPC2b	Appendix B.2
	• NPC2c	Appendix B.2
	• NPC3	Appendix B.2
	• NPC4	Appendix B.2
	• NPC5	Appendix B.2
	• UKN2	Appendix B.3
	• UKN4	Appendix B.4
	• UKN5	Appendix B.5
	• Cortical Initiation	Appendix B.6
	• Cortical Maturation	Appendix B.7
	• Cortical Synapto	Appendix B.7

	• Cortical MEA	Appendix B.8
	• hN initiation	Appendix B.9
	• hNP1 Apop	Appendix B.10
	• hNP1 Prolif	Appendix B.10
C	Adverse Outcome Pathway as an Underlying Framework for Developing In Vitro DNT Testing Strategies	Appendix C
D	List of DNT IATAs	Appendix D
E	List of all chemicals tested in the DNT IVB assays	Appendix E

Appendix A. Proposed Positive and Negative Control Compounds for Use in Performance Evaluations of DNT IVB Assays

The purpose of this Appendix is to provide a list of proposed DNT Reference chemicals. The importance of a list of Refence chemicals cannot be understated as it provides the data necessary to calculate estimates of sensitivity and specificity for both individual assays and the entire DNT IVB. This proposed list was generated to foster international discussion that would lead to a consensus list of chemicals to be used in the development and performance evaluations of assays in the Developmental Neurotoxicity In Vitro Battery (DNT IVB). This includes chemicals deemed to be developmental neurotoxicants in humans and/or chemicals with evidence of developmental neurotoxicity in mammalian animal models. The majority of chemicals in this list were deemed to have evidence of DNT from animal models and were collated from publications that contained expert opinions as well as publications that conducted extensive literature reviews. The minority of chemicals listed as positive in humans were based on mostly on expert opinions.

Appendix A.1A provides a list of proposed positive and negative control chemicals used in studies of performance of the DNT IVB. Table 1 lists chemicals used as positive reference chemicals, and Table 2 lists chemicals used as negative reference chemicals.

Appendix A.1B provides the references used to generate the proposed positive and negative chemicals. The list of positive compounds was compiled from workshop reviews, reviews of published data, and the US EPA's Neurotoxicity Risk Assessment Guideline (see references for details). The table also provides the rationale for the negative reference chemicals, which in some cases differed from lab to lab and may not have been based on known negative in vivo DNT testing. For example, some chemicals may be listed negative in vitro due to lack of metabolism to an active metabolite. Some compounds were deemed to be negative based on a review of literature data (Martin et al., 2022).

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Lists of Positive and Proposed Negative Control Compounds for Use in Performance Evaluations of DNT IVB Assays

Appendix A.1A, Table 1. Proposed positive DNT reference chemicals used in studies of performance of the DNT IVB. Details and references describing selection of in vivo positive DNT compounds can be found in Appendix A.1B. An 'X" indicates whether or not the chemical was tested in any of the four published reports, and the lack of an X for a chemical indicates that it has not been tested in any of the four publications.

Compound	Harrill et al. (2018)	Frank et al. (2017) & Shafer et al. (2019)	Masjusthusmann et al. (2020)
Acrylamide	x	x	x
Allethrin		x	
Aluminum			
Aminonicotinamide. 6-	x	x	
Amphetamine	x		
Arsenic	x	x	
Aspartame			
Azacytidine			
Benomyl			
Benzene			
Bisphenol A	x	x	
Bis(tri-n-butyltin)oxide	x	x	
Bromodeoxyuridine			
Butylated hydroxyanisole			
Cadmium	x	x	x
Caffeine	x	x	
Carbamazepine	x	x	
Carbon monoxide			
Chlordecone			
Chlordiazepoxide	x	x	
Chlorine dioxide			
Chlorpromazine	x	x	x
Chlorpyrifos	x	x	x
Cocaine	x	x	
Colcemid			
Colchicine	x	x	
Cyclophosphamide	x	x	

Cypermethrin		x	
Cytosine arabinoside	x	x	
DDT, p,p'-			
Deltamethrin	x	x	x
Dexamethasone	x	x	x
Diazepam	x	x	
Diazinon			
Dieldrin	x	x	
Di-(2-ethylhexyl) phthalate		x	
Diethylstilbestrol	x	x	
Dioxin			
Diphenylhydantoin	x	x	x
Domoic acid		x	x
Epidermal Growth Factor			
Ethanol			
Ethylene thiourea			
Fluoride	x		
Fluorouracil, 5-	x	x	
Fluoxetine	x	x	
Haloperidol	x	x	x
Halothane			
Heptachlor	x	x	
Heroin			
Hexachlorobenzene			
Hexachlorophene	x	x	x
Hydroxyurea	x	x	
Iminodipropionitrile, 3,3-		x	
Ketamine	x	x	x
Lead	x	x	x
Lidocaine			
Lindane		x	
Lysergic acid diethylamide			
Maneb	x	x	x
Manganese	x	x	x
3,4-Methylenedioxymethamphetamine			
Methadone			
Methanol			
Methimazole		x	
Methotrexate	x	x	
Methylazoxymethanol			x
Methylmercury	x	x	x
Methyl parathion			

Monosodium glutamate			
Methyl-4-phenyl-1,2,3,6-tetrahydropyridine			
Naloxone	x	x	
Naltrexone			
Nicotine	x	x	x
Ozone			
Paraquat	x	x	x
Parathion		x	
Penicillamine			
Perchlorate			
Perfluoroalkyls			x
Permethrin	x	x	
Phenobarbital	x	x	
Phenylacetate			
Polybrominated diphenyl ethers	x	x	x
Polychlorinated biphenyls			
Propranolol			
Propylthiouracil, 6-	x	x	
Retinoic acid	x	x	x
Tebuconazole	x	x	x
Tellurium			
Terbutaline	x	x	x
Tetrachloroethylene			
Tetrahydrocannabinol			
Thalidomide	x	x	
Toluene			
Triamcinolone			
Tri-n-butyltin			x
Trichlorfon			x
Trichloroethylene			
Triethyl lead			
Triethyltin	x	x	x
Trimethyltin	x	x	
Valproic acid	x	x	x

Appendix A.1A, Table 2. Summary table of proposed negative DNT reference chemicals used in studies of performance of the DNT IVB.

Compound	Harrill et al. (2018)	Frank et al. (2017) & Shafer et al. (2019)	Masjusthusmann et al. (2020)
Acetaminophen	x	x	x
Acetylsalicylic acid		x	x
Amoxicillin	x	x	x
Ascorbic acid		x	
Buspirone			x
Captopril	x		x
Chloramben	x		
Chlorpheniramine			x
Cotinine	x		
Diethylene glycol	x		x
Doxylamine succinate			x
Erythromycin		x	
Famotidine			x
Fluconazole	x		
Folic acid		x	
Glycerol		x	x
Glyphosate	x	x	
Ibuprofen			x
Isoniazid	x		
Loperamide	x		
Mannitol, D-		x	x
Metformin			x
Metoprolol			x
Penicillin VK			x
Phenol	x		
Propylene glycol		x	
Saccharin	x	x	x
Sodium benzoate	x	x	x
Sorbitol, D	x	x	x
Tetracycline		x	
Warfarin			x

Lists of Positive and Proposed Negative Control Compounds for Use in Performance Evaluations of DNT IVB Assays with References and Rationale

Appendix A.1B, Table 1. Summary Table of DNT Reference Chemicals Based on In Vivo Data and Rationale for use in In Vitro Studies *

Compound	Positive or Negative	Rees et al (1990)	EPA Neurotox RA Guideline (1998)	Grandjean papers (2006, 2014)	Mundy et al List 1 (2015)	Aschner et al (2017)	Martin Negatives (2022)**	Harrill et al (2018)	Frank et al. (2017) & Shafer et al (2019)	Masjusthusman et al (2020)***	Masjusthusmann et al Rationale for Use as Negative (from Aschner et al., 2017)	Frank et al Rationale for Use as Negative	Shafer et al Rationale for Use as Negative	Harrill et al 2018 Rationale for Use as Negative
Acrylamide	Positive				x			x	x	x				
Allethrin	Positive				x				x					
Aluminum	Positive				x h									
6-aminonicotinamide	Positive				x			x	x					
Amphetamine	Positive				x			x						

Arsenic	Positive			h	x h	x		x	x					
Aspartame	Positive				x									
Azacytidine	Positive		x		x									
Benomyl	Positive				x									
Benzene	Positive				x									
Bisphenol A	Positive				x			x	x					
Bis(tri-n-butyltin)oxide	Positive				x			x	x					
Bromodeoxyuridine	Positive				x									
Butylated hydroxyanisole	Positive				x									
Cadmium	Positive		x		x h	x		x	x	x				
Caffeine	Positive				x			x	x					
Carbamazepine	Positive				x h			x	x					
Carbon monoxide	Positive				x									
Chlordecone	Positive		x		x									
Chlordiazepoxide	Positive				x h			x	x					
Chlorine dioxide	Positive				x									
Chlorpromazine	Positive				x	x		x	x	x				

Chlorpyrifos	Positive			h	x h	x		x	x	x				
Cocaine	Positive	x h			x	x h		x	x					
Colcemid	Positive				x									
Colchicine	Positive				x			x	x					
Cyclophosphamide	Positive				x			x	x					
Cypermethrin	Positive				x				x					
Cytosine arabinoside	Positive				x			x	x					
DDT, p,p'-	Positive		x	h										
Deltamethrin	Positive				x			x	x	x				
Dexamethasone	Positive				x h	x		x	x	x				
Diazepam	Positive				x			x	x					
Diazinon	Positive				x									
Dieldrin	Positive				x			x	x					
Di-(2-ethylhexyl) phthalate	Positive				x				x					
Diethylstilbestrol	Positive				x			x	x					
Dioxin	Positive				x									
Diphenylhydantoin	Positive	x h			x h	x		x	x	x				
Domoic acid	Positive				x	x			x	x				

Epidermal Growth Factor	Positive				x									
Ethanol	Positive	x h	x h	h	x h	x h								
Ethylenethiourea	Positive				x									
Fluoride	Positive			h	x h			x						
5-Fluorouracil	Positive				x			x	x					
Fluoxetine	Positive				x			x	x					
Haloperidol	Positive				x	x		x	x	x				
Halothane	Positive				x									
Heptachlor	Positive				x			x	x					
Heroin	Positive	x h			x	x h								
Hexachlorobenzene	Positive				x									
Hexachlorophene	Positive				x h	x h		x	x	x				
Hydroxyurea	Positive				x			x	x					
Iminodipropanenitrile, 3,3-	Positive				x	(negative)			x		Neurotoxicant requiring metabolic activation. Low toxicity if test			

											system lacks activating enzymes			
Ketamine	Positive				x	x		x	x	x				
Lead	Positive	x h	x	h	x h	x h		x	x	x				
Lidocaine	Positive				x									
Lindane	Positive					x			x					
Lysergic acid diethylamide	Positive				x									
Maneb	Positive				x	x		x	x	x				
Manganese	Positive			h	x h	x		x	x	x				
3,4-Methylenedioxymethamphetamine	Positive					x								
Methadone	Positive	x h												
Methanol	Positive		x		x	x								
Methimazole	Positive				x				x					
Methotrexate	Positive				x			x	x					
Methylazoxymethanol	Positive				x	x				x				
Methylmercury	Positive	x h	x h	h	x h	x h		x	x	x				

Methyl parathion	Positive				x									
Monosodium glutumate	Positive				x									
MPTP	Positive				x	x								
Naloxone	Positive				x			x	x					
Naltrexone	Positive				x									
Nicotine	Positive				x	x		x	x	x				
Ozone	Positive				x									
Paraquat	Positive				x	x		x	x	x				
Parathion	Positive				x				x					
Penicillamine	Positive				x									
Perchlorate	Positive			h	x									
Perfluoroalkyls (PFOA, PFOS)	Positive					x				x				
Permethrin	Positive				x			x	x					
Phenobarbital	Positive				x h			x	x					
Phenylacetate	Positive				x									
Polybrominated diphenyls				h	x h	x		x	x	x				
Polychlorinated	Positive	x h	x h	h	x h	x h								

biphenyls														
Propranolol	Positive				x									
Propylthiouracil	Positive				x			x	x					
Retinoic acid	Positive				x h	x		x	x	x				
Tebuconazole	Positive				x			x	x	x				
Tellurium	Positive				x									
Terbutaline	Positive				x h	x		x	x	x				
Tetrachloroethylene	Positive			h										
Tetrahydrocannabinol	Positive				x h									
Thalidomide	Positive				x			x	x					
Toluene	Positive			h	x	x								
Triamcinolone	Positive				x									
Tri-n-butyltin	Positive				x					x				
Trichlorfon	Positive				x					x				
Trichloroethylene	Positive				x									
Triethyl lead	Positive				x									
Triethyltin	Positive				x	x		x	x	x				
Trimethylti	Positive				x			x	x					

n														
Valproic acid	Positive				x h	x		x	x	x				
X Irradiation	Positive	x h												
Acetaminophen	Negative							x	x	x	Negative in most systems up to mM levels, but has been discussed as *in vivo* DNT toxicant	Assay negative control	Shafer-not tested	Two criteria: 1) absence of effects in any of the USEPA ToxCast in vitro bioactivity assays (https://comptox.epa.gov/dashboard), or 2) lack evidence of developmental neurotoxicity in a review of the published literature (Pub Chem).
Acetylsalicylic acid	Negative								x	x	Unknown	not tested	Commonly used substances and/or drugs that are used during pregnancy without established adverse	

													neurodevelopmental outcomes.	
Ampicillin	Negative													
Amitryptiline	Negative										Drugs that are acceptable during pregnancy			
Amoxicillin	Negative							x	x	x	Drugs that are acceptable during pregnancy	GRAS	Shafer - not tested	GRAS and/or FDA Pregnancy Risk Category #2.
Anthracence	Negative										Polycyclic aromatic hydrocarbon; may act via Ah receptor, but has no target in many human DNT/NT test systems			

Ascorbic acid	Negative						x		x		No evidence from previous work of neurite outgrowth inhibition	not tested	Commonly used substances and/or drugs that are used during pregnancy without established adverse neurodevelopmental outcomes.	
Atropine	Negative										Drugs with low likelihood to affect DNT test systems due to their well characterized side effects and mode of action (may have direct effects on neural networks)			
Bismuth	Negative													

Buspirone	Negative									x	Unknown			
Captopril	Negative							x		x	Drugs with extracellular targets			Two criteria: 1) absence of effects in any of the USEPA ToxCast in vitro bioactivity assays (https://comptox.epa.gov/dashboard), or 2) lack evidence of developmental neurotoxicity in a review of the published literature (Pub Chem).
Chlorambe n	Negative							x						Two criteria: 1) absence of effects in any of the USEPA ToxCast in vitro bioactivity assays(https://comptox.epa.gov/dashboard), or 2) lack evidence of developmental neurotoxicity in a review of the published

														literature (Pub Chem).
Chlorpheniramine	Negative									x	Unknown			
Cotinine	Negative							x						Two criteria: 1) absence of effects in any of the USEPA ToxCast in vitro bioactivity assays (https://comptox.epa.gov/dashboard), or 2) lack evidence of developmental neurotoxicity in a review of the published literature (Pub Chem).
Dabigatran	Negative										Drugs with extracellular targets			

Deferoxamine mesylate	Negative										Iron chelator, tolerated at mM levels			
Deprenyl	Negative										Antidepressant/parkinsonian drug, inhibitor of monoamine oxidase-B (1 mM range)			
Diethylene glycol	Negative							x		x	No pronounced bioactivity, sometimes not entering cells, tolerated to mM level; belongs to "trivial" controls (low usefulness for specificity			Two criteria: 1) absence of effects in any of the USEPA ToxCast in vitro bioactivity assays (https://comptox.epa.gov/dashboard), or 2) lack evidence of developmental neurotoxicity in a review of the published literature (Pub Chem).

											calculations) with solvents			
Dimethylformamide	Negative										Generally low toxicity up to mM range			
Dimethylsulfoxide (DMSO)	Negative										Generally low toxicity up to mM range			
Dinitrofuran	Negative						x				Neonicotinoid pesticide without DNT effects in many systems (may however affect neuronal network assays)			

diphenhydramine	Negative										Drugs that are acceptable during pregnancy			
Doxylamine succinate	Negative									x	Unknown			
Erythromycin	Negative								x			not tested	Commonly used substances and/or drugs that are used during pregnancy without established adverse neurodevelopmental outcomes.	
Famotidine	Negative									x	Unknown			
Fipronil	Negative										Pesticide tested clearly negative for DNT; may be cytotoxic at > 10 µM; may have indirect			

											effects through cramp induction (zebrafish)			
Fluconazole	Negative							x						Two criteria: 1) absence of effects in any of the USEPA ToxCast in vitro bioactivity assays (https://comptox.epa.gov/dashboard), or 2) lack evidence of developmental neurotoxicity in a review of the published literature (Pub Chem).
Folic acid	Negative								x			not tested	Commonly used substances and/or drugs that are used during pregnancy without established adverse	

													neurodevelopmental outcomes.	
Furosemide	Negative										Drugs with low likelihood to affect DNT test systems due to their well characterized side effects and mode of action (may have direct effects on neural networks)			
Galactosamine hydrochloride	Negative													

Glucosamine	Negative										No pronounced bioactivity, sometimes not entering cells, tolerated to mM level; belongs to "trivial" controls (low usefulness for specificity calculations) with solvents			
Glycerol	Negative						x		x	x	No pronounced bioactivity, sometimes not entering cells, tolerated to mM level;	not tested	Commonly used substances and/or drugs that are used during pregnancy without established adverse neurodevelo	

											belongs to "trivial" controls (low usefulness for specificity calculations) with solvents		pmental outcomes.	
Glyphosate	Negative							x	x		Pesticide tested negative for DNT; low cytotoxicity	listed as UNKNOWN in Frank	Not tested nor listed as negative in Shafer	Two criteria: 1) absence of effects in any of the USEPA ToxCast in vitro bioactivity assays (https://comptox.epa.gov/dashboard), or 2) lack evidence of developmental neurotoxicity in a review of the published literature (Pub Chem).
Ibuprofen	Negative						x			x	Drugs that are acceptable during pregnancy			

Isoniazid	Negative							x						Two criteria: 1) absence of effects in any of the USEPA ToxCast in vitro bioactivity assays (https://comptox.epa.gov/dashboard), or 2) lack evidence of developmental neurotoxicity in a review of the published literature (Pub Chem).
Lactose	Negative										No pronounced bioactivity, sometimes not entering cells, tolerated to mM level; belongs to "trivial" controls (low			

											usefulness for specificity calculations) with solvents			
Levetiracetam	Negative										Drugs with low likelihood to affect DNT test systems due to their well characterized side effects and mode of action (may have direct effects on neural networks)			

Loperamide	Negative							x						Two criteria: 1) absence of effects in any of the USEPA ToxCast in vitro bioactivity assays (https://comptox.epa.gov/dashboard), or 2) lack evidence of developmental neurotoxicity in a review of the published literature (Pub Chem).
Mannitol, D-	Negative						x		x	x	No pronounced bioactivity, sometimes not entering cells, tolerated to mM level; belongs to "trivial" controls (low	not tested	Commonly used substances and/or drugs that are used during pregnancy without established adverse neurodevelopmental outcomes.	

											usefulness for specificity calculations) with solvents			
Metformin	Negative									x	Unknown			
Metoclopramide	Negative										Drugs that are acceptable during pregnancy			
Metoprolol	Negative									x	Drugs that are acceptable during pregnancy			
Mifepristone	Negative													
Naloxone	Negative										Drugs with low likelihood to affect DNT test systems due to their well characterized side effects and mode of action			

											(may have direct effects on neural networks)			
Omeprazole	Negative						x				Drugs with primary target only in stomach/liver; low likelihood to have DNT effects			
Penicillin VK	Negative									x	Unknown			
Phenol	Negative							x						Two criteria: 1) absence of effects in any of the USEPA ToxCast in vitro bioactivity assays (https://comptox.epa.gov/dashboard), or 2) lack evidence of developmental neurotoxicity in a review of the

														published literature (Pub Chem).
Pomalidomide	Negative										Thalidomide analog, no DNT up to 200 µM			
Propylene glycol	Negative								x			GRAS	not tested nor listed as negative in Shafer	
Propylthiouracil	Negative										Hormone modifiers little relevant to *in vitro* DNT test system targets			
RU38486	Negative										Hormone modifiers little relevant to in vitro DNT test system			

											targets			
Saccharin	Negative						x	x	x	x	Artificial sweetener, very low toxicity	listed as UNKNOWN in Frank	not tested nor listed as negative in Shafer	GRAS and/or FDA Pregnancy Risk Category #2.
Selegiline hydrochloride	Negative						x							
Seroquel	Negative										Drugs with low likelihood to affect DNT test systems due to their well characterized side effects and mode of action (may have direct effects on neural networks)			
Sodium benzoate	Negative							x	x	x	Unknown	GRAS	not tested nor listed as	GRAS and/or FDA Pregnancy

													negative in Shafer	Risk Category #2.
Sorbitol, D (glucitol, D)	Negative							x	x	x	No pronounced bioactivity, sometimes not entering cells, tolerated to mM level; belongs to "trivial" controls (low usefulness for specificity calculations) with solvents	GRAS	Commonly used substances and/or drugs that are used during pregnancy without established adverse neurodevelopmental outcomes	GRAS and/or FDA Pregnancy Risk Category #2.
Statins	Negative										Drugs with low likelihood to affect DNT test systems due to their well characteri			

											zed side effects and mode of action (may have direct effects on neural networks)			
Sulfisoxazole	Negative													
Sumatriptan	Negative										Drugs that are acceptable during pregnancy			
Testosterone	Negative										Hormone modifiers little relevant to *in vitro* DNT test system targets			
Tetracycline	Negative								x			not tested	Commonly used substances and/or drugs that are used during pregnancy without	

													established adverse neurodevelopmental outcomes.	
Tiotropium	Negative										Drugs with low likelihood to affect DNT test systems due to their well characterized side effects and mode of action (may have direct effects on neural networks)			
Trolox	Negative										Water-soluble vitamin E analog; caspase inhibitor (usable at 100 µM)			

Ursodeoxycholic acid	Negative										Drugs with low likelihood to affect DNT test systems due to their well characterized side effects and mode of action (may have direct effects on neural networks)			
Verapamil	Negative										Drugs with low likelihood to affect DNT test systems due to their well characterized side effects and mode of action (may have			

											direct effects on neural networks)			
Warfarin	Negative									x	Drugs with primary target only in stomach/liver; low likelihood to have DNT effects			
zVAD-fmk	Negative										Water-soluble vitamin E analog; caspase inhibitor (usable at 100 µM)			
Notes:														
*** Based on animal (x) and/or human (h) data**														
**** Listed as "favourable" (see Martin et al., 2022 for definition)**														
***** Unknowns are listed as the undefined term "ML Prioritization" in Masjusthusmann et al (2021)**														

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Appendix B. Assay Descriptions

Detailed description of the information and data used for evaluation of the individual DNT IVB assays is documented in Appendix B. These descriptions use the ToxTemp format (Krebs et al., 2019) which explicitly provides the acceptance criteria for test elements needed for evaluation of assay performance.

Appendix B.1

Author: Stefan Masjosthusmann, Ellen Fritsche, Katharina Koch, Kristina Bartmann
Date: 10.03.2023
Version: 20230310_v2

Overview

Descriptive full-text title

Assessment of human neural progenitor cell proliferation (NPC1)

Abstract

The human developing central nervous system may be more vulnerable to adverse effects of chemical agents than the adult brain. At present, due to the knowledge gap concerning hazard identification for human neurodevelopmental toxicity (DNT), there is an urgent need for testing and subsequent regulation of chemicals for their potential to interfere with the developing nervous system. Primary human neural progenitor cells (hNPCs) cultivated as three-dimensional floating spheres are able to represent several key processes of brain development. In the neural progenitor cell proliferation assay (NPC1), hNPCs are plated in 96 well plates as 3-dimensional spheres and exposed to test compounds. Thereby the process of NPC proliferation can be studied. This DNT-specific endpoint is studied in combination with general cell viability and cytotoxicity. Cortical human NPC proliferation is a critical process during brain development that, if disturbed, may lead to alterations in brain development and cause cognitive dysfunction. Currently, cortical NPC proliferation is one of the many processes, which are assessed in the OECD TG426 by neuropathological evaluation of certain brain regions as well as neurobehavioral tests. According to the readiness criteria as published by Bal-Price et al. (2018), the neural progenitor cell proliferation assay obtained the readiness score A.

Assay summary:

toxicological target	developing brain
test system	primary human neural progenitor cells (hNPCs) from human cortex (Gestation week (GW) 16-19)
readout(s)	sphere size, DNA synthesis as chemiluminescence measurement, viability and cytotoxicity as fluorescence intensity
biological process(es)	fetal NPC proliferation viability, cytotoxicity
(human) adverse outcome(s)	cognitive dysfunction

hazard(s)	adverse effect on cell proliferation
endpoint of current regulatory studies	not directly
validation/evaluation	readiness analysis: readiness score A, according to Bal-Price et al. (2018)

General information

Name of test method

Neural progenitor cell proliferation assay (NPC1)
by sphere size (NPC1a)
by BrdU incorporation (NPC1b)

Version number and date of deposition

20230310_v2

Summary of introduced changes in comparison to previous version(s)

changes according to comments

Assigned data base name

NPC1a_DNT_hNPC_prol_72h_20200702v1.2
NPC1b_DNT_hNPC_prol_72h_20200702v1.2

ToxCast invitroDB name:
IUF_NPC1b_proliferation_BrdU_72hr
IUF_NPC1a_proliferation_Area_72hr

Name and acronym of the test depositor

IUF – Leibniz Research Institute for Environmental Medicine

Name and email of contact person

Ellen Fritsche: ellen.fritsche@iuf-duesseldorf.de

Name of further persons involved

Stefan Masjosthusmann: stefan.masjosthusmann@iuf-duesseldorf.de
Kristina Bartmann kristina.bartmann@iuf-duesseldorf.de
Katharina Koch katharina.koch@iuf-duesseldorf.de

Reference to additional files of relevance

none

Description of general features of the test system source

Supply of source cells

Commercial supplier, Lonza, Verviers, Belgium

Overview of cell source component(s)

Primary human neural progenitor cells (hNPCs) are provided as cryopreserved 3D neurospheres from Lonza, Verviers, Belgium. The material originates from the human brain cortex of different gestational ages (GW16-19). Sex is either specified or determined before the cells are used.

Characterization and definition of source cells

$1x10^6$ hNPCs per vial are obtained from Lonza (#PT-2599) and expanded. Lonza provides the cells with a viability of at least 20%. FACS analysis confirmed that proliferating neurospheres express the cell type-specific CNS neural stem cell and progenitor cell markers nestin, SRY-box 2 (SOX2), and Ki67 (Koch et al., 2022). Moreover, proliferating hNPCs react to growth factor stimuli (epidermal growth factor (EGF) and recombinant human fibroblast growth factor (FGF)) with increased proliferation, while simultaneous pharmacological inhibition of the EGF receptor (EGFR; PD153035) impaired the proliferation increase. Upon transfer of hNPC neurospheres on poly D-lysine/laminin matrix and cultivation in the absence of growth factors (EGF and FGF), the hNPCs differentiate into effector cells expressing markers of neurons (β-III-tubulin(TUBB3), astrocytes (Glial Fibrillary Acidic Protein (GFAP)), radial glia cells (nestin) and oligodendrocytes (O4) (Baumann et al., 2015; Schmuck et al., 2017; Koch et al., 2022).

Acceptance criteria for source cell population

The following acceptability criteria have been tested at the supplier (Lonza) and are prerequisites for the shipment to customers:

- tested positive for TUBB3 and GFAP after differentiation
- tested free of HIV, HBV and HC
- tested negative both in sterility test and for mycoplasma contamination
- cell count of $1.2x10^6$ cells/mL
- viability of at least 20%
- Adherence of <=50%

The proliferative capacity of Lonza hNPCs was reported previously (Moors et al., 2009; Baumann et al., 2015; Klose et al., 2021a).

Variability and troubleshooting of source cells

The sphere size at day 0 of cell thawing can be different depending on the donor.
In the 3- to 4-week expansion period different donors can show differences in their proliferative capacity (spheres need longer, 3 instead of 4 weeks, to reach the acceptable minimum size of 0.2 – 0.5 µm). After the first mechanical dissociation, there are no observable or measurable inter-individual differences.

Critical consumables
The proliferation medium does not contain serum or serum replacement.
The use of EGF and recombinant human fibroblast growth factor (FGF) is critical for sphere growth. FGF contains 1% bovine serum albumin and is thus prone to batch effects.

Critical handling
The thawing medium contains DMSO in a concentration that affects cell health which is why thawed cells should quickly be diluted in proliferation medium (30 mL of media for one vial of cells).

It is recommended to add FGF to the proliferation medium directly before thawing.

At the end of week two of the expansion period (see below), the spheres should be transferred to petri dishes coated with poly-(2-hydroxyethyl methacrylate) (poly-Hema) to prevent cell attachment.

Attached cells that are not differentiated can be gently detached using a 1000 µL pipet. To avoid repeated attachment, all cells should be transferred to a new poly-Hema coated petri dish.

Medium containing FGF should not be stored longer than 1 week at 4°C.

During the first two weeks, the medium should be removed using a 1000 µL pipet to keep the accidental removal of small spheres to a minimum. In addition, removed medium should be kept in a new petri dish under culture conditions until the next feeding day, to transfer accidentally removed spheres back to the culture.

The neurospheres should be well distributed in the petri dish to prevent aggregation. This is especially important after mechanical dissociations.

It is important to avoid frequent re-opening of the incubators, to ensure constant CO_2 and temperature levels. Furthermore, the smallest vibrations can lead to aggregations of neurospheres.

The number of passages after thawing influences the proliferation capability of neurospheres. Neurospheres should not be used for the NPC1 assay after passage 6.

Differentiation towards the final test system

Cells are frozen in liquid nitrogen and have to be cultivated in proliferation medium at 37°C and 5% CO_2 after thawing. The medium contains Dulbecco's modified Eagle medium and Hams F12 (2:1) supplemented with 2% B27, 20 ng/mL EGF, 20 ng/mL recombinant human FGF, 100 U/mL penicillin and 100 µg/mL streptomycin. The thawing is performed by repeated addition and removal of proliferation medium to the vial until all cells are transferred to a tissue culture flask containing proliferation medium. The cells are carefully resuspended and distributed to 10 cm petri dishes filled with fresh, prewarmed proliferation medium. The cells are fed by replacing half the medium with new medium every two to three days (Monday, Wednesday, and Friday). At each feeding day, the culture is checked for impurities (e.g. fibers or other debris). Impurities and the removed media are transferred to a new petri dish (waste dish). If spheres are mistakenly sorted out during feeding, they can be rescued and placed back in the original culture dish. After 3-4 weeks, neurospheres reach the acceptable size of 0.2 – 0.5 mm for passaging by mechanical dissociation. Therefore, neurospheres are mechanically dissociated into pieces of 0.15 - 0.25 mm edge length (depending on the desired sphere size after passaging) using a tissue chopper, which then round-off again to uniform sized neurospheres within 1 day in proliferation medium. By using this method, neurospheres are expanded every week. Starting at week 2, poly-Hema coated dishes are used for the cultivation procedure.

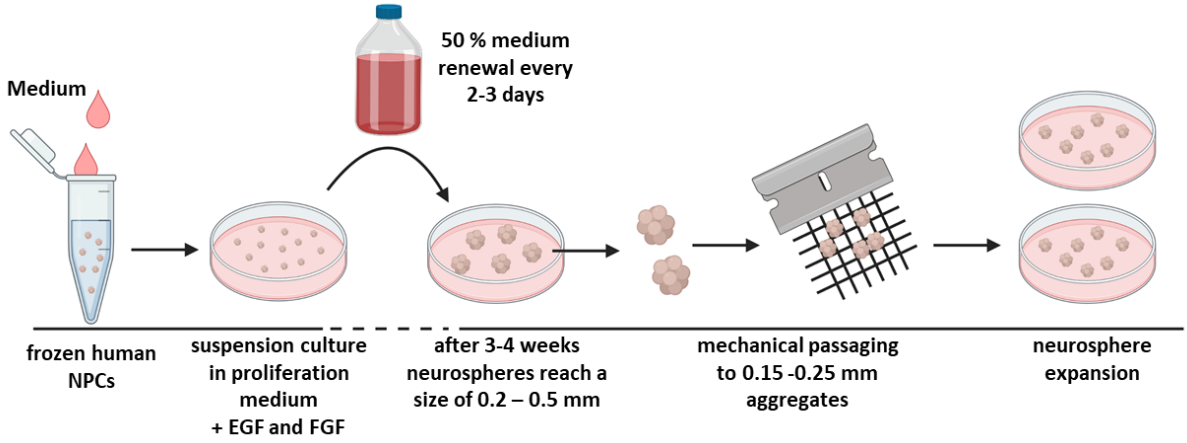

Figure 1 differentiation towards the final test system. hNPC are thawed by repeated addition and removal of proliferation media. The resuspended cells are distributed to cell culture dishes and cultivated in proliferation media containing EGF and FGF for three to four weeks with 50% media exchange every two to three days. When the spheres reach a size of 0.2-0.5 mm they are expanded by mechanical passaging every 7 days.

Reference/link to maintenance culture protocol

See SOP in DB-ALM format (Appendix I in Masjosthusmann et al., 2020). Detailed protocols are also available as publications (Baumann et al., 2014; Nimtz et al., 2019) or upon request (ellen.fritsche@iuf-duesseldorf.de).

Definition of the test system as used in the method

Principles of the culture protocol

After the cell expansion period, the cells are cultured for up to four weeks in which they are passaged every week as described in 0. Between one to three days after passaging, depending on the size chosen for passaging, spheres at a size of 0.3 mm are used in the assay.

For the assessment of neural progenitor cell proliferation, the spheres are plated in poly-Hema coated 96-well U-bottom plates filled with proliferation medium containing growth factors (EGF and FGF). One 0.25 - 0.35 mm big sphere is plated in the middle of each well. Within 3 days NPCs proliferate and grow in size. Cultivation during the test method is performed at 37°C and 5% CO_2 at a pH of 7.2-7.6. As a positive control, spheres are cultivated in absence of growth factors (EGF and FGF), which dramatically reduces proliferation.

Acceptance criteria for assessing the test system at its start

To be used in the test method, neurospheres have to display a perfectly round shape with no disintegrated borders. One neurosphere with 300 µm in diameter contains around 2.6 x 10^3 cells.

Additionally, the basic neurospheres culture is checked for mycoplasma contamination every three months and controlled for fungal and bacterial contamination by visual inspection at each feeding and plating day.

Acceptance criteria for the test system at the end of compound exposure

The proliferative capacity of hNPCs is assessed by cultivating them in either medium supplemented with the human growth factors EGF and FGF basic (as described in 4.1) or deprived of them (positive control). Over the 3 days in culture, hNPCs approximately increase their size on average by 33% (Koch et al., 2022).
For this process the following acceptance criteria are defined for the solvent control containing the solvent of the highest test compound concentration (SC; mean of at least three replicates):

Proliferation by area (slope of sphere area)	1000-3000 pixels/day
Proliferation by BrdU (BrdU raw values):	raw values of treatment conditions must not be lower than the positive control
Proliferation by BrdU (BrdU raw values):	raw values of the SC must be significantly higher than the positive control

Variability of the test system and troubleshooting

Sources of variation:
Selection of spheres: Depending on the researcher and the availability of spheres, the size of selected spheres can differ in a range of 0.25 – 0.35 mm.

Primary hNPCs are a complex multicellular system with a self-organized sphere composition. Due to the complex multicellular and self-organizing nature, the test system is subject to some heterogenicity, which is represented as the biological variability of some of the measured endpoints.

The variability for the different endpoints is shown in 0 “Test Performance”.

Metabolic capacity of the test system

Primary hNPC under proliferating and differentiating conditions do not express CYP1A1 and CYP1B1 (Gassmann et al., 2010).

Other metabolic pathways are not characterized.

Omics characterization of the test system

Proliferating, three day differentiated, and five day differentiated hNPCs were analyzed for changes in their transcriptomic profile. Several key neurodevelopmental processes (migration, neuronal differentiation, glial differentiation) and genes regulating these processes (Bone morphogenetic protein (BMP), Notch and EGF signaling) were identified and characterized on a functional level (Masjosthusmann et al., 2018).

Transcriptomic effects of exposure to 8 flame retardants were analyzed in hNPCs differentiated for five days (Klose et al., 2021)

Features of the test system that reflect the *in vivo* tissue

hNPCs reflect the following in vivo tissue features:

NPC1 – fetal NPC proliferation (3D, primary cells) corresponding to in vivo growth during the fetal phase. Proliferating hNPCs progressively increase in sphere size by on average 33% within three days of

proliferation in the presence of EGF and FGF. Moreover, they express the cell type-specific CNS neural stem and progenitor cell markers nestin and SOX2 (Koch et al., 2022). EGFR signaling is indispensable for proper brain development in vivo and increasingly expressed over time (Romano and Bucci, 2020). In line with that, exposure of proliferating hNPCs to the EGFR inhibitor PD153035 impaired the proliferative capacity (Koch et al., 2022).

Commercial and intellectual property rights aspects of cells

For the source cells, Lonza holds donor consent and legal authorization that provides permission for all research use.

Reference/link to the culture protocol

See SOP in DB-ALM format (Appendix I in Masjosthusmann et al., 2020). Detailed protocols are also available as publications (Baumann et al., 2014; Nimtz et al., 2019) or upon request (ellen.fritsche@iuf-duesseldorf.de).

Test method exposure scheme and endpoints

Exposure scheme for toxicity testing

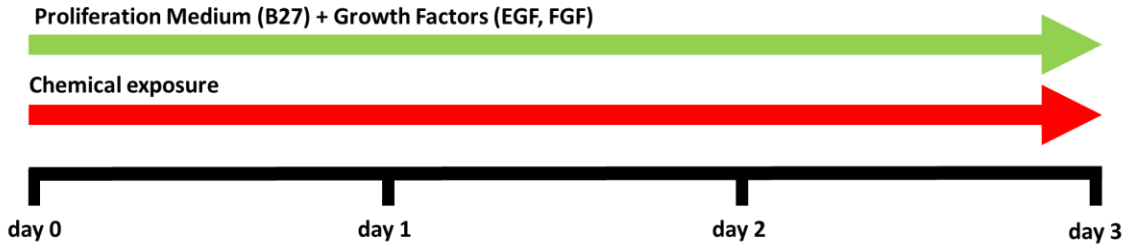

Figure 2: Exposure scheme. Neurospheres are plated on poly-Hema coated 96-well U-bottom plates containing proliferation medium and are exposed to increasing compound concentrations over a cultivation time of 72 hours.

0.3 mm big hNPCs are plated as described in 0. Cells are plated according to the plating scheme in Figure 4 in the already prepared test solutions. Exposure starts on the plating day (day 0) and is continued over three days, without chemical renewal, until the experiment is terminated (Figure 2).

Endpoint(s) of the test method

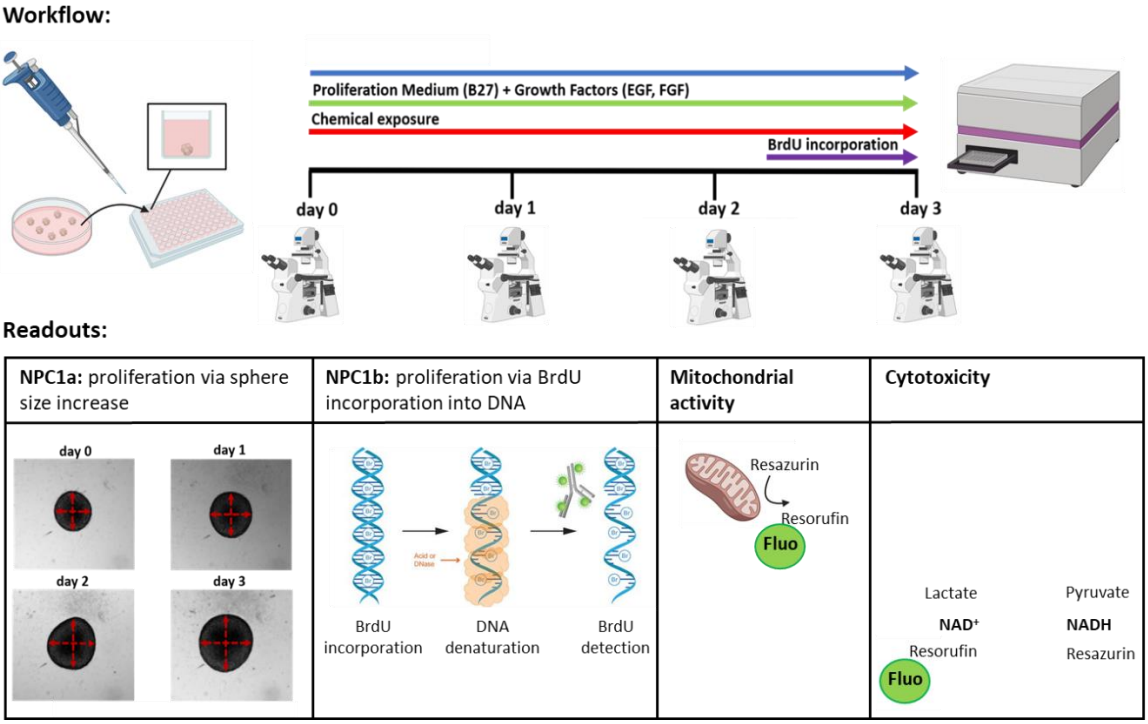

Figure 3: Endpoint assessment. Neurospheres are plated in 96-well U-bottom plates and exposed to increasing compound concentrations in proliferation medium over a cultivation period of 72 h. Sphere size is determined every day via brightfield images (2-dimensional assessment). The assay is terminated by the assessment of cell viability, cytotoxicity, and proliferation by BrdU.

Primary DNT specific endpoints of the test method are:

1 . proliferation by area (NPC1a)
2 . proliferation by BrdU (NPC1b)

Secondary endpoints are:

1 . cytotoxicity 72 h
2 . viability 72 h

All endpoints are generated from the same experimental run and from each well/sphere in the 96-well plate.

Overview of analytical method(s) to assess test endpoint(s)

Primary endpoints:

1. Proliferation by area (72h; NPC1a) is assessed as the slope of the increase in sphere size (amount of pixels in the bright-field image, sphere area) over 72 h measured by brightfield microscopy using high content imaging at 0 h, 24 h, 48 h, and 72 h.

2. Proliferation by BrdU (72h; NPC1b) is assessed as BrdU incorporation (as an indirect measure of DNA synthesis) over the last 16 h of compound exposure. It is measured as a luminescence signal (relative luminescence unit) in a multi-plate reader after 72 h.

Secondary endpoints:

1 . Cytotoxicity 72 h is assessed as membrane integrity by measuring the amount of LDH leaked from cells with damaged plasma membranes. LDH-dependent reduction of resazurin to resorufin is measured in the supernatant of each well as fluorescence of the reaction product resorufin (relative fluorescence unit) in a multi-plate reader after 72 h of compound exposure.

2 . Viability 72 h is assessed as mitochondrial activity by measuring the amount of resazurin reduced to fluorescent resorufin (relative fluorescence unit) in a multi-plate reader in the last two hours of the 72 h proliferation and compound exposure period.

Technical details (of e.g. endpoint measurements)

All technical details for the test method are available in the SOP in DB-ALM format (Appendix I in Masjosthusmann et al., 2020). Detailed protocols are also available as publications (Baumann et al., 2014; Nimtz et al., 2019) or upon request (ellen.fritsche@iuf-duesseldorf.de).

Endpoint-specific controls/mechanistic control compounds (MCC)

All endpoint-specific controls are run for each experiment (plate).

1. Controls for Primary endpoints:
   hNPC proliferation is diminished by the withdrawal of growth factors (EGF and FGF). Spheres are plated in medium not containing EGF and FGF. This positive control demonstrates the physiological functionality of the growth factor-dependent regulation of hNPC proliferation. Inhibition of the growth factor-dependent proliferation causes a reduction of proliferation to 0% of the solvent control (SC, see 5.7) for proliferation by area (NPC1a) and 0-40% of the SC for proliferation by BrdU (NPC1b).

2. Controls for Secondary endpoints:
   0.2 % Triton X-100 is used as a positive control for cell viability and cytotoxicity since it lyses the cell and therefore causes a maximal response for both endpoints. This positive control is run on each experimental plate.

Positive controls

The NPC1 hNPC proliferation assay correctly identified the following compounds that are known to cause DNT in humans or in vivo (Masjosthusmann et al., 2020; Blum et al., 2023):

Cadmium chloride
Dexamethasone
Hexachlorophene
Chlorpromazine hydrochloride
Methylazoxymethanol acetate
all-trans-Retinoic acid

Tributyltin chloride
Sodium valproate
all-trans-Retinoic acid

Negative and unspecific controls

The solvent control (SC) is used as a negative control that is run on each experimental plate. Each solvent has to be established for its use as a solvent control by comparing the effect of the SC to the effect of medium only. Established solvent controls show the same response as the medium control.

The SC is used to assess if the acceptability criteria for NPC1 proliferation are met and for normalization of the compound exposure and the positive control response.

Established SCs are:
DMSO: 0.3% v/v; 0.2% v/v; 0.1% v/v
DPBS: 2% v/v
ddH_2O: 2% v/v
MeOH: 0.1% v/v

Other negative control compounds that were identified as negative in this assay and are known to not affect neurodevelopmental endpoints *in vivo* include (Masjosthusmann et al., 2020):

Acetaminophen
Amoxicillin
Aspirin
Buspirone
Chlorpheniramine maleate
D-Glucitol
Diethylene glycol
D-Mannitol
Doxylamine succinate
Famotidine
Ibuprofen
Metformin
Metoprolol
Penicillin VK
Saccharin
Sodium benzoate
Warfarin

Features relevant for cytotoxicity testing

Cytotoxicity and cell viability are assessed for each sphere plated in the assay.

Acceptance criteria for the test method

General acceptance criteria:

1. At least three replicate values (technical replicates) need to be present for each condition (concentration) to be accepted for the data analysis.

2. At least five conditions and the solvent control need to be present for the experiment to be accepted in the data analysis for concentration response modeling.

Throughput estimate

The methods described here are set up in a 96-well plate format with automated image acquisition, analysis, and data evaluation. Pipetting steps such as compound dilutions, as well as the viability and cytotoxicity assays can be automated using a liquid handling system.

In the fully automated set up, 10 plates with 8 conditions (Figure 4) and 4 replicates per condition can be run in one week by two laboratory technicians. This results in the generation of 400 data points for each endpoint within one week (excluding all controls). The throughput is therefore estimated as medium.

Handling details of the test method

Preparation/addition of test compounds

The method is set up for 8 test conditions including 7 compound concentrations and one SC, including 2 different compounds on one 96-well plate. The test conditions are prepared in a serial dilution from the stock solution (Figure 4).

Stock solutions are prepared by diluting the compound in the solvent (e.g. DMSO) in a concentration that allows the preparation of the highest test concentration without exceeding the highest acceptable solvent concentration (see 0). For DMSO the highest acceptable solvent concentration is 0.3% which means that the stock concentration needs to be at least 1000x higher than the highest test concentration.
Stock solutions in non-sterile solvents (e.g. water or PBS) have to be sterile filtrated using a sterile syringe filter (diameter = 0.2 µm). Adsorption to the filter needs to be considered.

Stock solutions are aliquoted and stored at -20°C. A stock solution is not thawed more than three times.

For the preparation of the test condition, the stock solution is diluted to the highest test concentration (default 1:1000) in proliferation medium. All following dilutions are prepared by serial dilution of the highest concentration in proliferation medium with solvent (in the concentration of the highest test concentration). The default serial dilution is 1:3 which covers a concentration range from e.g. 20 µM to 27 nM (729-fold). Depending on the desired concentration range, the dilution can be adjusted to 1:2, 1:5, 1:10, or other.

The SC is prepared by adding the solvent to proliferation medium in the same concentration as the highest test concentration.

100 µL of the compound dilutions and the SC are added to each well of a 96 well plate (Figure 4). Alternatively, the serial dilution can be prepared directly in the 96 well plates.

One hNPC sphere is added to each well after the medium equilibrated for 15 to 30 min at 37°C and 5% CO_2.

To subtract the background fluorescence of the phenol red-containing medium, 4 wells with medium only (Background, without cells, Figure 4) are prepared for the viability and cytotoxicity assays.

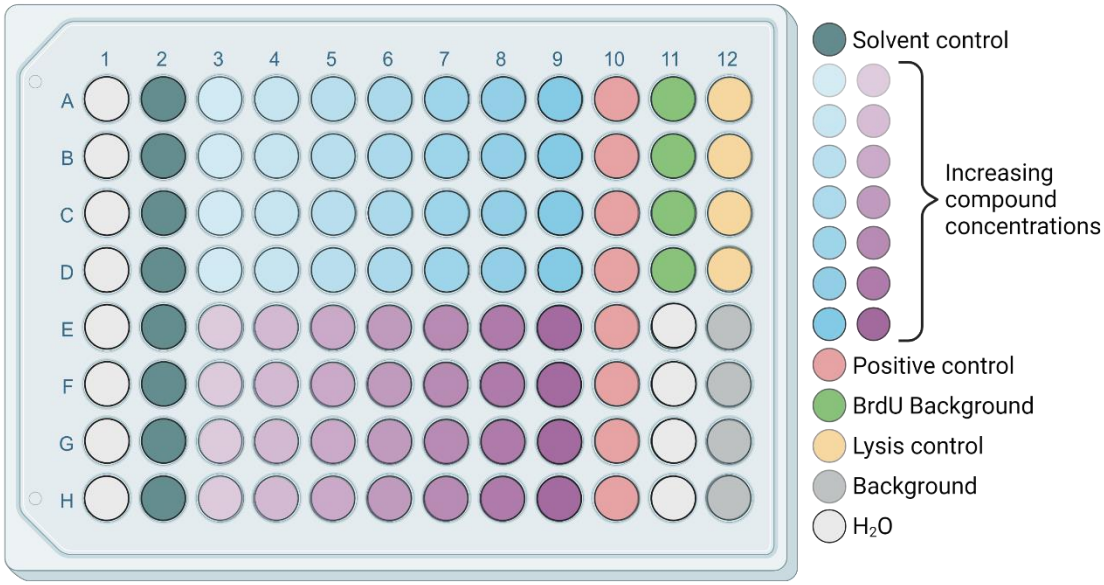

Figure 4 Plating Scheme. The type of solvent control depends on the solvent of the compound that was tested. 7 compound concentrations (for each compound) are plated in a serial dilution from lowest (left) to highest (right) concentration. The positive control for cell proliferation is proliferation medium without EGF and FGF. BrdU background is used for the proliferation assay by BrdU (spheres in SC medium). Background and lysis control are used for cell viability and cytotoxicity assays. 2 different compounds can be investigated on one plate (blue and purple).

Day-to-day documentation of test execution

Documentation for each experiment including meta data and the experimental data that is collected in the Automated Experimental Evaluation or AXES sheet.
Meta data such as plating date, experimenter, NPC individual, NPC passage, compound, compound concentrations and a plate map are reported in these sheets. Depending on the endpoint, the experimental data is collected during or at the end of the experiment. Each raw data point (including all outliers) is collected in the AXES sheet. All deviations from the standard procedure are documented in a comment section of the AXES sheet.

Practical phase of test compound exposure

The practical phase of the test compound exposure follows the description in the SOP in DB-ALM format (Appendix I in Masjosthusmann et al., 2020) or upon request (ellen.fritsche@iuf-duesseldorf.de).
Deviation(s) from the SOP are documented in the comment section of the AXES sheet.

Errors (e.g. pipetting in wrong well or wrong volume pipetted) are also documented in the comment section of the AXES sheets. Data points of the affected well are marked in the AXES sheet and excluded from the analysis.

Concentration settings

Starting concentrations and concentration ranges are defined based on the following factors:

- toxicological relevance of the compound (i.e. internal human exposures, effects at lowest concentrations)
- solubility of the compound
- highest useable solvent concentration

These factors are determined based on available information (databases/literature) or experimentally (e.g. solubility test).

Uncertainties and troubleshooting

Problematic compounds:

- Volatile compounds
- High lipophilicity (high K_{OW})
- Low solubility in established solvents
- Fluorescent compounds (possible interference with viability and cytotoxicity assays)

Critical handling steps:

- If different plate types are used, the test system and method need to be re-established.
- Outer wells have to be filled with H_2O because of edge effects.
- Automating the pipetting steps using a liquid handling system for coating, preparation of the plates, viability and cytotoxicity assays reduces the variability and the user bias.

Sources of variation:

- Pipetting steps: Each pipetting step is a source of variation. Especially in the viability and cytotoxicity assays where the volume pipetted determines the final readout.

Detailed protocol (SOP)

See SOP in DB-ALM format (Appendix I in Masjosthusmann et al., 2020) or upon request (ellen.fritsche@iuf-duesseldorf.de).

Special instrumentation

- Incubator for cell culture, e.g. CB170 (Binder)
- Tissue chopper, e.g. McIlwain tissue chopper (Campden Instruments)
- Multi-plate reader for fluorescence and luminescence measurements, e.g. Infinite M200 Pro reader (Tecan)
- Bright-field microscope, e.g. High Content Analysis (HCA) platform Cellomics ArrayScan(Thermo Fisher)
- Liquid handling system (necessary to achieve the throughput described above), e.g. MICROLAB STAR® M; Hamilton
- Hair dryer, e.g. PHD5767 (Bosch)

Possible variations

There are no established variations of the assay.

Cross-reference to related test methods

The hNP1 assay (CCTE_Mundy_HCI_hNP1_Pro assay) uses proliferating neural stemcells derived from a neuroepithelial cell lineage of WA09 human embryonic stem cells. The assay measures cell proliferation using BrdU labeling in combination with a immunocytochemical staining and high content imaging. In contrast to the NPC1 assay cells are plated as a 2D monolayer instead of the free-floating 3D spheroids used in the NPC1 assay.

Data management

Raw data processing, curve fitting and hit definition (prediction) are performed using CRStats (Concentration-Response Statistics), an R package for concentration-response analysis automation for in vitro test systems optimized for multi-well plate experiments based on drc (https://github.com/iuf-duesseldorf/fritsche-lab-CRStats; Keßel et al., (pre-print at BioRxiv; doi: 10.1101/2022.10.18.512648)).

Raw data format

The raw data format is different depending on the endpoints.

For all endpoints assessed in a multi-plate reader (viability, cytotoxicity, BrdU incorporation) the raw data formats are excel files containing values (one for each endpoint, timepoint and well) measured as relative fluorescence/luminescence units. These values are transferred from the original excel file into the AXES sheet. The original excel output file is saved for traceability of the data.

The sphere size is automatically measured in the Cellomics scan software (Version 6.6.0; Thermo Scientific) and copied into the AXES sheet. Original brightfield images are archived for 10 years.

Outliers

Mathematical procedures to define outliers are not applied. Data points from wells where technical problems are known or obvious are excluded from the analysis.

Possible technical problems:
- pipetting errors
- spillover from lysis
- problems in fixation of singularized cells

All wells with technical problems are marked in the AXES sheet.

Raw data processing to summary data

If not otherwise stated, all data processing steps are performed in an R based evaluation tool that was designed for data processing, curve fitting and point of departure evaluation of in vitro concentration response toxicity data.

Data processing describes all processing steps of raw data that are necessary to obtain the final response values including the normalization, curve fitting and benchmark concentration calculation.

Processing (or pre-processing) steps depend on the endpoint and are described below:

Proliferation by BrdU: subtraction of mean BrdU background from each raw response value.

$$Background\ corrected\ response\ [RLU] = raw\ response\ [RLU] - Background\ BrdU\ [RLU]$$

Proliferation by area: slope of the sphere size over 3 days of proliferation (d0, d1, d2, d3). The calculated slope is used as raw data input for the data base (DB) and is thus not calculated in the R based evaluation tool.

Viability: subtraction of mean background from each response value.

$$Background\ corrected\ response\ [RFU] = raw\ response\ [RFU] - Background\ [RFU]$$

Cytotoxicity: no pre-processing

Curve fitting

The data is normalized to the SC and re-normalized to the starting point of the curve.
For the normalization to the SC the median of each replicate data point is normalized to the median of the SC in the respective experiment.

For the cytotoxicity assays the following normalization is used instead of the normalization to the SC. Here again each response value is normalized using the median of the lysis control and the median of the solvent control.

$$\text{normalized response} = \frac{\text{lysis control} - \text{response}}{\text{lysis control} - \text{solvent control}}$$

The R package drc is used to calculate the optimal fit for each experiment. For calculations of curve fits and BMCs, the data from independent experiments is pooled (median of all replicate values for one concentration). Several non-linear models are run with the concentration response data of each endpoint and the Akaike´s information criteria is used to determine the best fit.

For re-normalization of the data, the response value of the curves starting point is determined and used to re-normalize all response values. Therefore, each mean response value is divided by the starting point of the curve and multiplied with 100. For the re-normalized response values the curve fitting is repeated to produce the final concentration response curve.

For deriving a reference point (RP) or point of departure (Pod) the Benchmark Concentration (BMC) approach as recommended by the EFSA Scientific Committee (Hardy et al., 2017) is applied. The BMC approach makes use of all data points that define the fitted concentration response curve. Thereby, the BMC is defined

as the concentration that is associated with a specific change in response, the Benchmark Response (BMR). The BMR is a value of effect size and should be defined as an effect size that is higher than the general variability of the measured endpoint. The BMR is therefore determined based on the variability of the respective endpoint.

BMR for NPC1:

proliferation by area	BMR30
proliferation by BrdU	BMR30
cytotoxicity 72 h	BMR10
viability 72 h	BMR30

Based on the BMR and the concentration response curve, the evaluation tool calculates the BMC, as well as upper and lower confidence limits (BMCU and BMCL respectively) based on the predict function in the R package drc. The predict function calculates the prediction bands around the concentration response curve based on the deviation between independent experiments and gives an estimation of the area that is expected to enclose 95% of future data points. The BMCL is thereby defined as the intersection of the lower band and the BMR while the BMCU is defined as the intersection of the upper band and the BMR. The confidence intervals are used to access the uncertainty of the BMC. If the BMCU is 1.5 times above the test range, the original BMCU is replaced by 1.5x the highest tested concentration.

Internal data storage

All raw data is stored on a server with a daily server back up for at least 10 years.

Metadata

All metadata is collected in the AXES sheet (see 0) together with all raw data.

The metadata gives information on:

The experiment:
- start and end date of the experiment
- experimenter

The cell source:
- human individual
- cells thawing date
- passage of cells
- date of cell passaging

The compound:
- compound identity
- stock concentration
- all dilution steps
- solvent and solvent concentration

The controls:
- control identity

- preparation of controls

Metadata file format

All metadata is collected in an Excel format.

Prediction model and toxicological application

Scientific principle, test purpose and relevance

Primary hNPCs are isolated from the fetal brain cortices and can be used to measure proliferation, a process of brain growth during the fetal phase of prenatal development.

The test system therefore measures adverse events in the young (fetal) developing brain.
Different types of NPC exist in the developing brain. Besides ventricular zone NPC, radial glia cells serve as cortical progenitor cells responsible for cortical expansion and folding. As whole cortices were used for cell preparation, this is not a specific NPC type but rather a mix of NPCs found in fetal human cortex during development.

The toxicological events that are modeled concern events that influence proliferation of NPCs found in human cortex during the fetal phase.

Prediction model

Two prediction models (PM) are applied for the NPC1 assay. One PM for a downregulation (PM downregulation) and one PM for an upregulation (PM upregulation) in cell proliferation.

PM downregulation
The PM uses hit definition based on comparison of the confidence intervals (CI) for the BMC of the DNT-specific endpoint (BMCs) and the unspecific endpoint (cytotoxicity/viability; BMCus).

Thereby the following four hit classifications apply:

“no hit”	The compound is not defined as a hit.
“specific hit”:	The compound is defined as hit and the CI’s do not overlap, meaning that the upper confidence limit of the specific endpoint (BMCUs) is lower than the lower confidence limit of the unspecific endpoint (BMCLus).
“borderline hit”:	The compound is defined as hit and the CI of the specific endpoint overlaps by less than, or equal to 10% with the CI of the unspecific endpoint.
“unspecific hit”	The compound is defined as hit and the CI of the specific endpoint overlaps by more than 10% with the CI of the unspecific endpoint.

The compound is classified as a hit, if the concentration response curve generates a BMC and if the CI is within the test range. In case the CI spans above the test range, the compound is only classified as a hit, if the highest test concentration is significantly different from the lowest test concentration. The adjusted significance ($p<0.05$) is thereby determined using a Tukey HSD test.

The decision process for the prediction model is described in the flow chart in Figure 5.

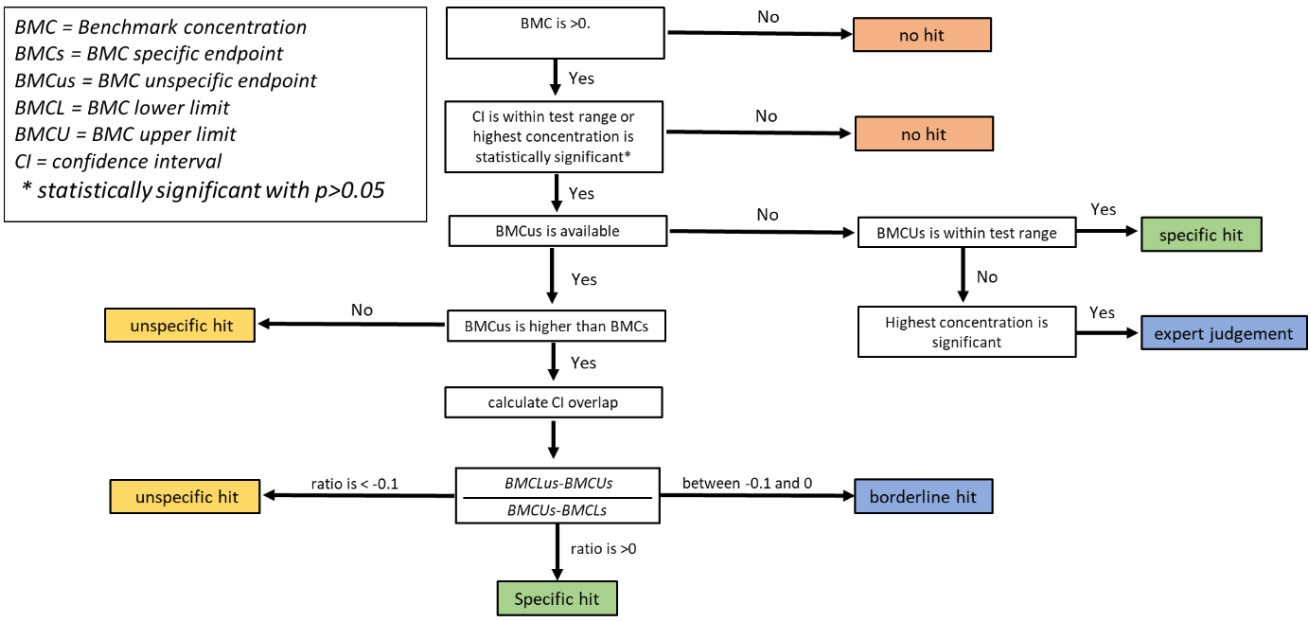

Figure 5: Decision tree for the PM for down regulation applied for the test method NPC1. Overview of the decisions leading to the classification of a compound in one of four categories: "no hit", "specific hit", "borderline hit" and "unspecific hit".

Specific consideration for the prediction model:

In case no confidence limits are available for the unspecific endpoint because the BMR is not reached, the BMCLus is assumed to be the highest tested concentration. If the CI of the specific endpoint additional spans above the test range for a compound identified as "hit" based on statistical significance, expert judgement is applied to define if the "hit" is specific or unspecific. Therefore, all datapoints of the unspecific and specific endpoint of the highest test concentration are compared. If these data points do not overlap, the compound is classified as "specific hit" otherwise as "unspecific hit".

In general, BMCs based on the same BMR are compared (e.g. BMC_{30us} vs BMC_{30s}). In case the BMC_{30us} is not available for an endpoint that allows the generation of a BMC_{10us}, the BMC_{10us} is used instead. If the classification of this comparison is "unspecific hit", the compound will be flagged as "check manually" as the BMC_{10us} is lower than the BMC_{30us} leading to a higher probability of a false classification. To avoid such false classifications, expert judgment is needed.

Compounds can also be flagged as "check manually", if the classification, based on the viability is different from the classification based on the cytotoxicity and if the confidence interval is very wide (BMCU/BMCL > 25), which means a high uncertainty for the BMC estimation. In both cases expert judgment is needed to decide on the classification.

The expert judgement is an individual decision process that accounts for effect size, curve progression, statistical significance and overall standard deviation. If the concentration response curves do not give enough information for decision by expert judgement, additional testing or testing in a different concentration range should be performed.

PM upregulation

In contrast to the PM for downregulation, the PM for upregulation is based on a hit definition without the comparison of confidence intervals (CI) between the specific and unspecific endpoint. The reason is, that specific and unspecific endpoints do not have the same relationship during an induction, compared to a reduction in the endpoint. A loss in general cell health will likely result in an effect on cell proliferation, while an induction in cell health (measured as mitochondrial activity) does not necessarily increase cell proliferation.

The following three hit classifications apply:

"no hit"	The compound is not defined as hit.
"specific hit":	The compound is defined as hit and the effect is no artifact due to loss in cell health.
"unspecific hit"	The compound is defined as hit in only the unspecific endpoints.

The compound is classified as hit, if the concentration response curve generates a BMC and if the CI is within the test range. In case the CI spans above the test range, the compound is only classified as hit, if the highest test concentration is significantly different from the lowest test concentration. The adjusted significance ($p<0.05$) is thereby determined using a Tukey HSD test.

The decision process for the prediction model is described in the flow chart in Figure 6.

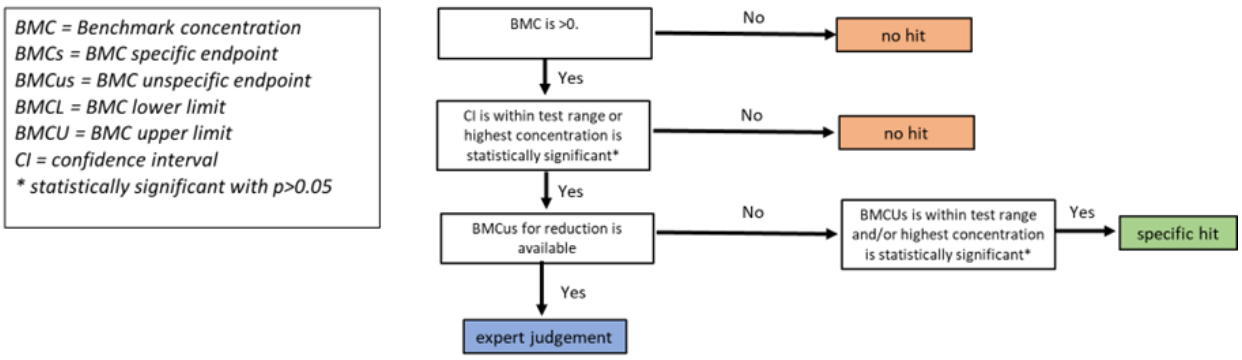

Figure 6: Decision tree for the PM for upregulation applied for the test method NPC1. Overview of the decisions leading to the classification of a compound in one of three categories: "no hit", "specific hit", and "unspecific hit".

In case a compound is classified as hit together with a reduction in an unspecific endpoint, it needs to be clarified if the induction is a specific effect or an artifact, due to a loss in cell viability or an increase in cytotoxicity. Therefore, expert judgement is applied, which accounts for effect size, curve progression, statistical significance, overall standard deviation but also morphological changes of the spheres. Expert judgement is additionally applied, if the confidence interval is very wide (BMCU/BMCL > 25) which means a

high uncertainty for the BMC estimation.

Prediction model setup

The prediction model is set up as a statistical model which uses the 95 % confidence intervals (assessed based on the prediction bands around the concentration response curve) to determine the uncertainty of a hit definition. In the case of high uncertainty in the confidence interval, (e.g. because the CI spans above the tested concentration range) the model additionally considers the statistical significance between the highest and the lowest test concentrations. Next to the hit definition, the uncertainty given by the 95 % CI is also considered in the specificity analyses (see 8.2 for a more detailed description).

The model has been tested on a set of 17 DNT negative- and 9 DNT positive compounds (see 5.6 and 5.7). All negative compounds were correctly classified as “no hit” and 3 positive compounds were correctly classified as “specific hit” for DNT.

Test Performance

The following parameters were assessed to quantify the assay variability:

Intra-experimental variation (SC) is the mean coefficient of variation (CV) ± SD of the CV of all replicates of the solvent control from one experiment across all (n>350) experiments.

Inter-experimental variation (raw) is the variability as CV between the raw SC across all independent experiments (n>360) before normalization.

Inter-experimental variation (low conc.) is the variability as CV across all independent experiments (n>390) after normalization based on the response of the lowest test concentration. It is assumed that the lowest test concentration does not affect any of the endpoints measured.

Inter-experimental variation (positive controls) is the variability of the positive control across all independent experiments (n>360) after normalization. For further detail, see Fig. 7.

Positive control:
proliferation media without growth factors (EGF, FGF)

Table 1 summarizes the assay performance in terms of variability of each endpoint in the assay. This variability spans different individuals where cells were derived from.

Table 1: Assay variability quantified as CV. Inter-experimental variation (positive controls) is only given for specific endpoints.

Endpoint	Intra-experimental variation (SC)	inter-experimental variation (raw)	Inter-experimental variation (low con.)	Inter-experimental variation (positive

				controls)
proliferation by area (NPC1a)	19.4 ±9.4 %	34.3%	21.4%	1.1%
proliferation by BrdU (NPC1b)	13.2 ±5.9 %	65.4%	28.1%	10.3%
cytotoxicity [72h]	1.3 ±1.6 %	37.7%	7.9%	
viability [72h]	5.8 ±3.5 %	11.6%	9.1%	

Sensitivity and specificity of the NPC1 assay are determined based on a set of 9 predicted human DNT positive compounds and 17 predicted human DNT negative compounds (Masjosthusmann et al., 2020; Blum et al., 2023).
Based on this compound set the following performance parameters are obtained for the NPC1 assay.

Specificity: 100 %
Sensitivity: 33%

Here, it is important to mention, that it is not expected that the NPC1 assay identifies all DNT positive compounds as not all of those compounds act via an effect on cell proliferation on the developing brain. For most compounds the exact MoA for their neurodevelopmental adversity is not precisely known, yet other mechanisms like neuron/glia differentiation, neurite outgrowth, synaptogenesis and neuronal network formation are amongst the known DNT MoAs. It is therefore recommended that this assay is run as one part of an in vitro DNT battery.

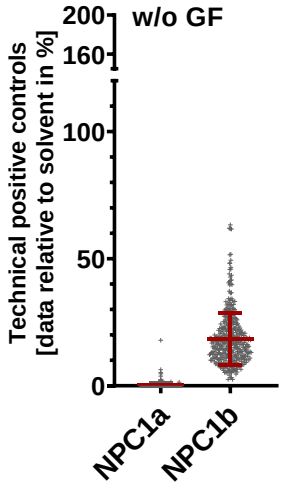

Figure 7: Inter-experimental variability of endpoints specific positive controls normalized to the solvent control in %. Proliferation medium without growth factors (w/o GF) serves as a positive control for NPC1a (proliferation by area) and NPC1b (proliferation by BrdU). In red, the mean ± standard deviation (SD) is depicted. NPC1a: 0.14% ± 1.1%; NPC1b: 18.5% ± 10.3%.

In vitro – in vivo extrapolation (IVIVE)

Parameters for in vitro – in vivo extrapolation are not yet determined.

Applicability of test method

Toxicological applicability domain
The following compound classes have been tested successfully:

- Industrial chemicals
- pesticides and biocides
- cosmetics ingredients
- pharmaceuticals

Compounds need to be soluble in a solvent at a solubility where the solvent does not produce effects by itself in the test system (see 0 for established solvents).

Compounds that are volatile or have a high lipophilicity have not been tested and might need more sophisticated exposure methods such as ‘passive dosing’.

Biological applicability domain
Neural progenitor cell proliferation is based on primary hNPC obtained from the fetal human cortex. As mentioned in 0 “Scientific principle” the method represents NPC proliferation during the fetal period.

Next to the endpoints represented by this test method, there are several other necessary neurodevelopmental endpoints which need to be studied using other test methods.

Neurodevelopmental processes not represented by this test method:
Neural Crest Cell (NCC) Migration
NPC apoptosis
Neuronal migration
Oligodendrocyte migration
Radial glia migration
Neuronal differentiation
Oligodendrocyte differentiation
Neuronal morphology
Synaptogenesis
Neuronal network formation
Neural Rosette Formation
hiPSC-derived NPC proliferation
hiPSC-NPC neuronal differentiation
Neuronal subtype differentiation
Astrocyte Differentiation and Maturation
Astrocyte Reactivity
Microglia reactivity
Myelination

For a complete assessment of developmental neurotoxicity, the test method needs to be part of a test battery.

The information on signaling pathways modulating the neurodevelopmental endpoints of the test method is summarized in Table 2. This describes the so far tested biological application domain of the assay.

Table 2: Signaling pathways studied in the test method. EGF(R): epidermal growth factor (receptor); CREB: cAMP-response element; COX-2: cyclooxygenase-2; PDGFR: platelet-derived growth factor; mTOR: mammalian target of rapamycin; RAR: retinoic acid receptor; GR: glucocorticoid receptor; PKC: protein kinase C; Bis-I: Bisindolylmaleimide I.

DNT Assay	Signaling Pathway	Model Compound	Reference
NPC1	EGFR	lack of EGF, EGFR antagonist	Masjosthusmann et al., 2018
	CREB	KG-501	unpublished
	COX-2	Celecoxib	
	PDGFR	CP-673451	
	mTOR	Everolimus, MHY1485	
	NOTCH	DAPT	
	ETC complex I	MPP+	Masjosthusmann et al., 2020
	RAR	all-*trans* retinoic acid	
	GR	Dexamethasone	
	RHO	Narciclasine	
	PKC	Bis-I	

Incorporation in test battery

To assess the hazard for developmental neurotoxicity it is recommended that this assay is used as one assay in a battery of assays (see 0 “Applicability of test methods”)

For the assessment of chemical action on the endpoints represented by this test method, the test method can be used as stand-alone test method.

The test method is currently used in the set-up of a DNT test battery.

Publication/validation status

Availability of key publications

Key Publications concerning the test method are:

Blum et al., 2023
Koch et al., 2022
Klose et al., 2021
Masjosthusmann et al., 2020
Nimtz et al., 2019
Masjosthusmann et al., 2018
Baumann et al., 2016a
Baumann et al., 2016b
Baumann et al., 2014
Fritsche et al., 2011
Moors et al., 2009

(Potential) linkage to AOPs

No AOP linkage.

Steps towards mechanistic validation

See:
0 Characterization and definition of source cells
0 Omics characterization of the test system
4.7 Features of the test system that reflect the in vivo tissue
0 Applicability of test method

Pre-validation or validation

To date, 123 unique compounds (as defined by unique DTXSIDs) have been tested successfully in this assay.

No formal OECD 34 validation study has been done (eg., ring trials with a standard set of known positive and negative controls).

Linkage to (e.g. OECD) guidelines/regulatory use

Test is not linked to regulatory guidelines.

Test method transferability

Operator training

For operators with a basic training in cell culture practices a four-week training period for handling of the test system and training in the assay is recommended. The operators should have basic understanding in image analysis and data evaluation with respect to concentration response fitting.

Transfer

The test method has been used by multiple operators over a period of 18 months. However, inter operator variability has not been determined.

Safety, ethics and specific requirements

Specific hazards; issues of waste disposal

No specific requirements.

Safety data sheet (SDS)

Reference to MSDS is given in the SOP in DB-ALM format (Appendix I in Masjosthusmann et al., 2020) or

upon request (ellen.fritsche@iuf-duesseldorf.de).

Specific facilities/licenses

No specific facilities are required.
No specific ethical approval is required.

Commercial aspects/intellectual property of material/procedures

There are no commercial aspects or intellectual properties to be considered.

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Appendix B.2

Author: Stefan Masjosthusmann, Ellen Fritsche; Katharina Koch, Kristina Bartmann
Date: 10.03.2023
Version: 20230310_v2

Overview

Descriptive full-text title

Assessment of human neural progenitor cell migration and differentiation (NPC2-5)

Abstract

The human developing central nervous system may be more vulnerable to the adverse effects of chemical agents than the adult brain. At present, due to the knowledge gap concerning hazard identification for human neurodevelopmental toxicity (DNT), there is an urgent need for testing and subsequent regulation of chemicals for their potential to interfere with the developing nervous system. Primary human neural progenitor cells (hNPCs) cultivated as three-dimensional floating spheres are able to represent several key processes during brain development. In the neural progenitor cell migration and differentiation assay (NPC2-5), hNPCs are plated on an extracellular matrix, and migrate and differentiate out of the sphere core. Thereby the processes radial glia migration, migration of neurons and oligodendrocytes as well as differentiation into neurons and oligodendrocytes and neurite outgrowth can be studied. Those DNT-specific endpoints are studied in combination with general cell viability and cytotoxicity. Cell migration and differentiation are critical processes during brain development that, if disturbed, lead to alterations in brain development and may cause cognitive dysfunction. Currently, cortical NPC migration- and differentiation-related processes are some of the many processes, which are assessed in the OECD TG426 by neuropathological evaluation of certain brain regions as well as neurobehavioral tests. According to the readiness criteria as published by Bal-Price et al. (2018), the neural progenitor cell migration and differentiation assay obtained readiness scores between A and B depending on the endpoint.

Assay summary:

toxicological target	developing brain
test system	primary human neural progenitor cells (hNPCs) from human cortex (Gestion week (GW)16-19)
readout(s)	migration distance, cell number (all cells) number of neurons/oligodendrocytes, neurite length, neurite area, fluorescence intensity
biological process(es)	radial glia/neuronal/oligodendrocyte migration, neuronal/oligodendrocyte differentiation, neuronal morphology, viability, cytotoxicity

(human) adverse outcome(s)	cognitive dysfunction
hazard(s)	adverse effect on cell migration and differentiation
endpoint of current regulatory studies	not directly
validation/evaluation	readiness analysis: readiness score A and B (depending on the endpoint), according to Bal-Price et al. (2018)

General information

Name of test method

Neural progenitor cell migration and differentiation assay (NPC2-5)
- radial glia migration (NPC2a)
- neuronal migration (NPC2b)
- oligodendrocyte migration (NPC2c)
- neuronal differentiation (NPC3)
- neuronal morphology (NPC4)
- oligodendrocyte differentiation (NPC5)

Version number and date of deposition

20230310_v2

Summary of introduced changes in comparison to previous version(s)

changes according to comments

Assigned data base name

NPC2a_DNT_hNPC_mig_72h_20200317v1.1
NPC2a_DNT_hNPC_mig_120h_20200317v1.1
NPC2b_DNT_hNPC_mig_120h_20200317v1.1
NPC2c_DNT_hNPC_mig_120h_20200317v1.1
NPC3_DNT_hNPC_diff_120h_20200317v1.1
NPC4_DNT_hNPC_diff_120h_20200317v1.1
NPC5_DNT_hNPC_diff_120h_20200317v1.1

ToxCast invitroDB name:
IUF_NPC2a_radial_glia_migration_72hr
IUF_NPC2a_ radial_glia_migration_120hr
IUF_NPC2b_neuronal_migration_120hr
IUF_NPC2c_oligodendrocyte_migration_120hr
IUF_NPC3_neuronal_differentaition_120hr
IUF_NPC4_neurite_length_120hr
IUF_NPC4_neurite_area_120hr
IUF_NPC5_oligodendrocyte_differentiation_120hr

Name and acronym of the test depositor

IUF – Leibniz Research Institute for Environmental Medicine

Name and email of contact person

Ellen Fritsche ellen.fritsche@iuf-duesseldorf.de

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Katharina Koch katharina.koch@iuf-duesseldorf.de

Reference to additional files of relevance

none

Description of general features of the test system source

Supply of source cells

Commercial supplier, Lonza, Verviers, Belgium

Overview of cell source component(s)

Primary human neural progenitor cells (hNPCs) are provided as cryopreserved 3D neurospheres from Lonza, Verviers, Belgium. The material originates from the human brain cortex of different gestational ages (GW16-19). Sex is either specified or determined before the cells are used.

Characterization and definition of source cells

$1x10^6$ hNPCs per vial are obtained from Lonza (#PT-2599) and expanded. Lonza provides the cells with a viability of at least 20%. FACS analysis confirmed that proliferating neurospheres express the cell type-specific CNS neural stem cell and progenitor cell markers nestin, SRY-box 2 (SOX2), and Ki67 (Koch et al., 2022). Moreover, proliferating hNPCs react to growth factor stimuli (epidermal growth factor (EGF) and recombinant human fibroblast growth factor (FGF)) with increased proliferation, while simultaneous pharmacological inhibition of the EGF receptor (PD153035) impaired the proliferation increase. Upon transfer of hNPC neurospheres on poly D-lysine/laminin matrix and cultivation in the absence of growth factors (EGF and FGF), the hNPCs differentiate into effector cells expressing markers of neurons (β-III-tubulin (TUBB3), astrocytes (Glial Fibrillary Acidic Protein (GFAP), radial glia cells (nestin) and oligodendrocytes (O4) (Baumann et al., 2015; Schmuck et al., 2017, Koch et al., 2022).

Acceptance criteria for source cell population

The following acceptability criteria have been tested at the supplier (Lonza) and are prerequisites for the

shipment to customers:

- tested positive for TUBB3 and GFAP after differentiation
- tested free of HIV, HBV and HC
- tested negative both in sterility test and for mycoplasma contamination
- cell count of $1.2x10^6$ cells/mL
- viability of at least 20%

The proliferative capacity of Lonza hNPCs was reported previously (Moors et al., 2009; Baumann et al., 2015; Klose et al., 2021a).

Variability and troubleshooting of source cells

The sphere size at day 0 of cell thawing can be different depending on the donor.
In the 3- to 4-week expansion period different donors can show differences in their proliferative capacity (spheres need longer, 3 instead of 4 weeks, to reach the acceptable minimum size of 0.2 – 0.5 µm). After the first mechanical dissociation, there are no observable or measurable inter-individual differences.

Critical consumables
The proliferation medium does not contain serum or serum replacement.
The use of epidermal growth factor (EGF) and recombinant human fibroblast growth factor (FGF) is critical for sphere growth. FGF contains 1% bovine serum albumin and is thus prone to batch effects.

Critical handling
The thawing medium contains DMSO in a concentration that affects cell health which is why thawed cells should quickly be diluted in proliferation medium (30 mL of media for one vial of cells).

It is recommended to add FGF into the proliferation medium directly before thawing.

At the end of week two of the expansion period (see below), the spheres should be transferred to petri dishes coated with poly-(2-hydroxyethyl methacrylate) (poly-Hema) to prevent cell attachment.

Attached cells that are not differentiated can be gently detached using a 1000 µL pipet. To avoid repeated attachment, all cells should be transferred to a new poly-Hema coated petri dish.

Medium containing FGF should not be stored longer than 1 week at 4°C.

During the first two weeks, the medium should be removed using a 1000 µL pipet to keep the accidental removal of small spheres to a minimum. In addition, removed medium should be kept in a new petri dish under culture conditions until the next feeding day, to transfer accidentally removed spheres back to the culture.

The neurospheres should be well distributed in the petri dish to prevent aggregation. This is especially important after mechanical dissociations.

It is important to avoid frequent re-opening of the incubators, to ensure constant CO_2 and temperature levels. Furthermore, the smallest vibrations can lead to aggregations of neurospheres.

Differentiation towards the final test system

Cells are frozen in liquid nitrogen and have to be cultivated in proliferation medium at 37°C and 5% CO_2 after

thawing. The medium contains Dulbecco's modified Eagle medium and Hams F12 (2:1) supplemented with 2% B27, 20 ng/mL EGF, 20 ng/mL recombinant human FGF, 1% penicillin and streptomycin. The thawing is performed by repeated addition and removal of proliferation medium to the vial until all cells are transferred to a tissue culture flask containing proliferation medium. The cells are carefully resuspended and distributed to 10 cm petri dishes filled with fresh, prewarmed proliferation medium. The cells are fed by replacing half the medium with new medium every two to three days (Monday, Wednesday, and Friday). At each feeding day, the culture is checked for impurities (e.g. fibers or other debris). Impurities and the removed media are transferred to a new petri dish (waste dish). If spheres are mistakenly sorted out during feeding, they can be rescued and placed back in the original culture dish. After 3-4 weeks neurospheres reach the acceptable size of 0.2 – 0.5 mm for passaging by mechanical dissociation. Therefore, neurospheres are mechanically dissociated into pieces of 0.15 - 0.25 mm edge length (depending on the desired sphere size after passaging) using a tissue chopper, which then round-off again to uniform sized neurospheres within 1 day in proliferation medium. By using this method, neurospheres are expanded every week. Starting at week three poly-Hema-coated dishes are used for the cultivation procedure.

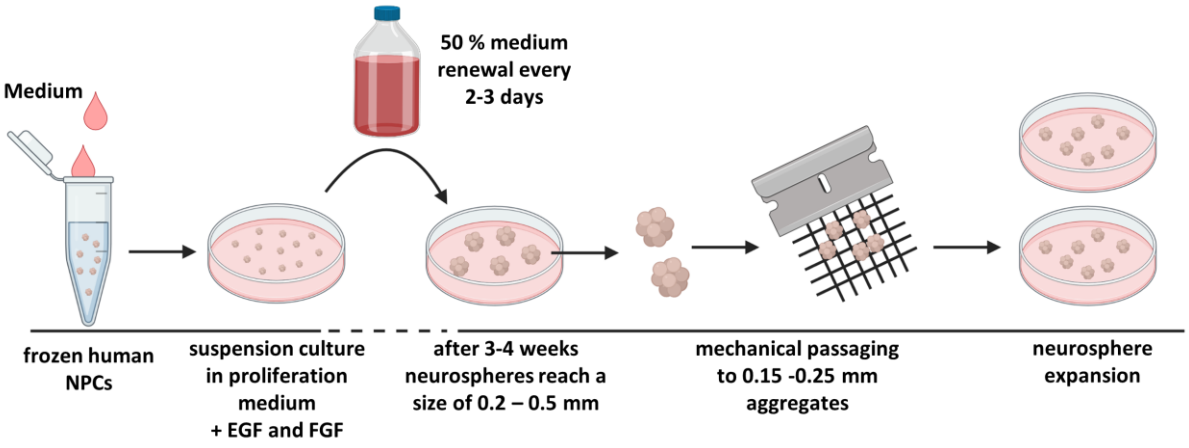

Figure 8 differentiation towards the final test system. hNPC are thawed by repeated addition and removal of proliferation media. The resuspended cells are distributed to cell culture dishes and cultivated in proliferation media containing EGF and FGF for three to four weeks with 50% media exchange every two to three days. When the spheres reach a size of 0.2-0.5 mm they are expanded by mechanical passaging every 7 days.

Reference/link to maintenance culture protocol

See SOP in DB-ALM format (Appendix J in Masjosthusmann et al. 2020). Detailed protocols are also available as publications (Baumann et al. 2014; Nimtz et al. 2019) or upon request (ellen.fritsche@iuf-duesseldorf.de).

Definition of the test system as used in the method

Principles of the culture protocol

After the cell expansion period, the cells are cultured for up to four weeks in which they are passaged every week as described in 0. Between one to three days after passaging spheres at a size of 0.3 mm are used in the assay.

For the assessment of neural progenitor cell migration and differentiation, the spheres are plated on poly-D-

lysine/laminin coated 96-well flat bottom plates in differentiation medium (N2) to initiate migration and differentiation. Therefore, one sphere of 0.3 mm diameter is plated in the middle of a well. The differentiation medium consists of DMEM and Ham's F12 at a ratio of 2 to 1 supplemented with 1% N2 and 1% penicillin and streptomycin. Within 5 days NPCs radially migrate out of the sphere core and differentiate into radial glia cells (nestin positive), neurons (β-III-tubulin positive), oligodendrocytes (O4 positive) and astrocytes (GFAP positive). Cultivation during the test method is performed at 37°C and 5% CO2 at a pH of 7.2-7.6.

Acceptance criteria for assessing the test system at its start

The spheres need to be rounded and have a size of 0.25 – 0.35 mm to be used in the test method.

Additionally, the basic neurospheres culture is checked for mycoplasma contamination every three months and controlled for fungal and bacterial contamination by visual inspection at each feeding and plating day.

Acceptance criteria for the test system at the end of compound exposure

As described in 0 the cells radially migrate out of the sphere core and differentiate into the effector cells. For this process the following acceptance criteria are defined for the solvent control (SC; median of at least three replicates):

Radial glia migration 72h:	700 – 1500 µm
Radial glia migration 120h:	700 – 1600 µm
Cell number:	2500 – 6000
Percentage of neurons	≥ 1.5 %
Percentage of Oligodendrocytes	≥ 1.5 %

Variability of the test system and troubleshooting

Sources of Variation:
Selection of spheres: Depending on the researcher and the availability of spheres, the size of selected spheres can differ.

Primary hNPCs are a complex multicellular system with a self-organized sphere composition as well as migration and differentiation. Due to the complex multicellular and self-organizing nature, the test system is subject to some heterogenicity which is represented as biological variability of some of the measured endpoints.

The variability for the different endpoints is shown in 8.4 "Test Performance".

Metabolic capacity of the test system

Primary hNPCs under proliferating and differentiating conditions do not express CYP1A1 and CYP1B1 (Gassmann et al., 2010).

Primary hNPCs during differentiation, have the capacity to up-regulate glutathione-dependent protective strategies upon reactive oxygen species (ROS) exposure (Masjosthusmann *et al*, 2019).

Gene expression levels of genes involved in the antioxidative defense (glutathione peroxidase 1 (GPX1), superoxide dismutase 1 (SOD1), catalase (CAT)) were comparable between the *in vitro* system and

developing human brains *in vivo* and show similar expression levels (Masjosthusmann et al., 2019).

Other metabolic pathways are not characterized.

Omics characterization of the test system

Proliferating, three day differentiated, and five day differentiated hNPCs were analyzed for changes in their transcriptomic profile. Several key neurodevelopmental processes (migration, neuronal differentiation, glial differentiation) and genes regulating these processes (Bone morphogenetic protein (BMP), Notch and EGF signaling) were identified and characterized on a functional level (Masjosthusmann et al., 2018).

Features of the test system that reflect the *in vivo* tissue

hNPCs reflect the following in vivo tissue features:

In differentiation culture:

NPC2a – radial glia cell migration → corresponding to radial glia cell migration during corticogenesis in vivo. The migrated hNPCs exhibit the characteristic elongated radial glia (RG)-like morphology and express the RG-markers nestin and GFAP as well as the proliferation marker Ki-67 (Koch et al., 2022). In accordance with in vivo studies, exposure to EGF (0.5-1 ng/mL) after neurosphere plating enhances hNPC migration compared to the solvent control (Koch et al., 2022). As a second human-relevant key regulator of migration, hNPCs respond to SRC-family kinase inhibition (PP2) with reduced migration (Moors et al., 2007; Koch et al., 2022). The migration speed of hNPCs in vitro is in the same range as migrating mouse granule cells in vivo (Baumann et al., 2016, Fahrion et al., 2012).

NPC2b – migration of young cortical neurons on radial glia scaffolds → corresponding to cortical neuronal radial migration.

NPC2c – oligodendrocyte migration → corresponding to oligodendrocyte migration during corticogenesis.

NPC3 – fetal neuronal differentiation into young neurons → corresponding to cortical neurogenesis in vivo.
Over the time course of differentiation, neurons expressing β-III-tubulin progressively appear in the migration zone representing approximately 20% of the mixed culture after 5 days. In line with observations in vivo, inhibition of the Notch signaling pathway during hNPC differentiation increases neuronal numbers compared to the solvent control (Koch et al., 2022). Moreover, narciclasine, an activator of Ras homolog family member A (RhoA), reduces neuronal differentiation of hNPCs cultured for 5 days in a concentration-dependent manner (Masjosthusmann et al., 2020; Blum et al., 2023). Likewise, RhoA inactivation stimulated axon regeneration and recovery of hindlimb function after spinal cord injury in mice (Dergham et al., 2002).

NPC4 – neurite length, neurite area of young primary fetal neurons → corresponding to axon/dendrite formation in vivo.
During the 5 days of hNPC differentiation, neurite maturation is characterized by an elongation of neurites and an increase in neurite area (Koch et al., 2022). Increase in RhoA activity caused morphological alterations in rat cortical neurons in vivo (Chen et al., 2018). In line with that, RhoA activator narciclasine reduced both neurite area and neurite length (Masjosthusmann et al., 2020).

NPC5 – oligodendrocyte formation from fetal hNPCs → corresponding to oligodendrogenesis during the fetal phase of brain development.
Differentiation of hNPCs over 5 days progressively generates cells expressing the oligodendrocyte-marker O4, which exhibit the typical oligodendrocyte morphology with multiple branched processes necessary to

ensheath neuronal axons (Moors et al., 2009; Koch et al., 2022). Differentiation of hNPCs in the presence of the Notch inhibitor DAPT concentration-dependently decreases the percentage of O4-positive cells compared to the solvent control (Koch et al., 2022). Moreover, oligodendrocyte differentiation is negatively influenced by bone morphogenic protein (BMP) 7 (Baumann et al., 2015) and BMP2 (Masjosthusmann et al., 2018), proteins of the transforming growth factor β family. These data demonstrate that two major developmental pathways, i.e. Notch and BMP, are functional in hNPCs.

Primary hNPCs are self-organized and produce auto- and paracrine cues like heparin-binding EGF-like growth factor (HB-EGF) and neuregulins that guide migration and differentiation. Moreover, they express extracellular matrix (ECM) proteins like laminin subunits, fibronectin and collagens (microarray data from Klose et al. 2021). Hence, effects concerning ECM-like adhesion defects can be assessed with this assay (Barenys et al. 2017). Various signaling cues that guide migration and differentiation processes of the neurosphere assay are summarized in Koch et al., 2022.

Commercial and intellectual property rights aspects of cells

For the source cells, Lonza holds donor consent and legal authorization that provides permission for all research use.

Reference/link to the culture protocol

See SOP in DB-ALM format (Appendix J in Masjosthusmann et al. 2020). Detailed protocols are also available as publications (Baumann et al. 2014; Nimtz et al. 2019) or upon request (ellen.fritsche@iuf-duesseldorf.de).

Test method exposure scheme and endpoints

Exposure scheme for toxicity testing

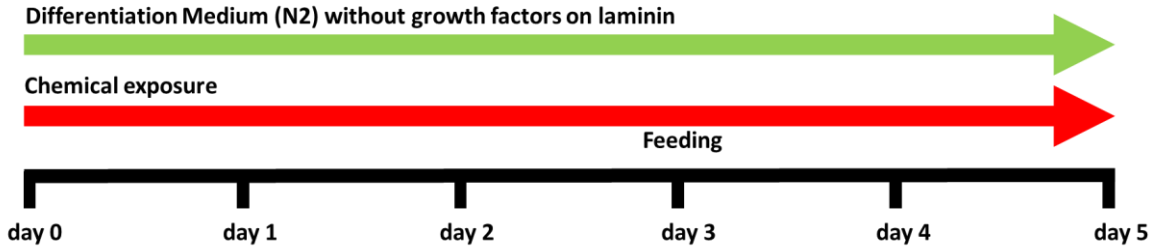

Figure 9 Exposure scheme. Spheres are plated in poly-D-lysine (PDL)/Laminin-coated 96 well Flat-bottom plates in differentiation medium and exposed to increasing compound concentrations over a cultivation time of 120 h. Half of the medium is replaced after 72h of cultivation.

hNPCs of 0.3 µm diameter are plated as described in 0. Cells are plated according to the plating scheme in Figure 4 in the already prepared test conditions. Exposure starts at day 0 of differentiation and is continued over five days of differentiation until the experiment is terminated. Cells are fed with fresh medium on day 3 of differentiation (Figure 2). Therefore, half of the test condition solution (e.g. solvent control or compound dilution) is replaced by freshly prepared test condition solution.

Endpoint(s) of the test method

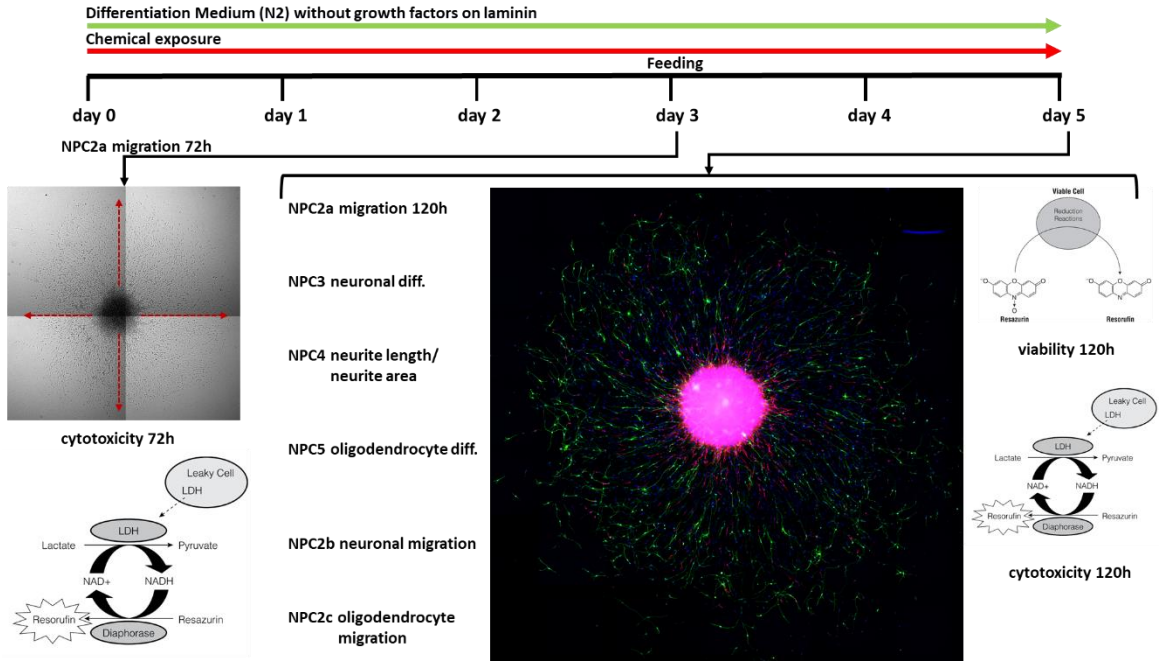

Figure 10 Endpoint assessment. Migration distance and cytotoxicity is determined after 72 h. The assay is terminated by the assessment of cell viability and cytotoxicity as well as cell fixation after 120 h. Immunocytochemistry is performed to assess Hoechst-positive nuclei, β-III-tubulin-positive neurons and O4-positive oligodendrocytes. On the immunocytochemical images, migration after 120 h, neuronal and oligodendrocyte number, neuronal morphology and neuron/oligodendrocyte specific migration is assessed

Primary DNT specific endpoints of the test method are:

3. radial glia migration 72 h (NPC2a)
4. radial glia migration 120h (NPC2a)
5. neuronal migration 120 h (NPC2b)
6. oligodendrocytes migration 120h (NPC2c)
7. neuronal differentiation 120 h (NPC3)
8. neurite length 120 h (NPC4)
9. neurite area 120 h (NPC4)
10. oligodendrocyte differentiation 120h (NPC5)

Secondary endpoints are:

3. cell number 120 h
   used for normalization of neuronal and oligodendrocyte differentiation
4. cytotoxicity 72 h
5. cytotoxicity 120 h
6. viability 120 h

All endpoints are generated from the same experimental run and from each well/sphere in the 96 well plate.

Overview of analytical method(s) to assess test endpoint(s)

Primary endpoints:

1. Radial glia migration 72 h is assessed as migration distance in µm from the edge of the sphere core to the edge of the migration area based on brightfield images of each well (Figure 3). Therefore, each plate is scanned after 72 h in culture in an automated high content imaging device under 5% CO_2 and 37°C. Images are exported and the sphere size in four directions is measured manually using ImageJ.

All other primary endpoints are assessed based on an ICC staining image for each sphere. Therefore, cells are fixated after 120 h in culture and an ICC staining with Hoechst for nuclei, β-III-tubulin for neurons and O4 for oligodendrocytes is performed. The plates are scanned using an automated high content imaging device and all nuclei and their positions are determined automatically based on their intensity and size. Images are imported to the Omnisphero software (https://omnisphero.com) to run the image analysis that measures the following endpoints.

2. Radial glia migration 120h is assessed as the migration distance in µm between the sphere core and the edge of the migration area based on ICC images of Hoechst-positive nuclei. By identification of each nuclei's position in relation to the sphere core, the migration distance can be calculated. Therefore, a density distribution mask is calculated. By scanning the images of the nuclei channel, the algorithm can determine relatively more or less dense image areas. By identifying the densest area in the image, the sphere core can be detected. For identification of the migration, it is assumed, that the nuclei density decreases with increasing distance to the sphere core. Once the density hits a pre-defined threshold, the outer boundaries are determined and the sphere itself can be mapped out in a polynomial bounding box. Derived from this box, the actual size and migration distance can be calculated for each well.

3. Neuronal migration 120 h is the mean distance of all neurons from the edge of the sphere core in relation to the radial glia migration and is determined based on the position of each neuron (see neuronal differentiation).

4. Oligodendrocyte migration 120 h is the mean distance of all oligodendrocytes from the edge of the sphere core in relation to the radial glia migration and is determined based on the position of each oligodendrocytes (see oligodendrocyte differentiation).

5. Neuronal differentiation is defined as the number of all β-III-tubulin-positive cells in percent of the cell number (Hoechst-positive cells) in the migration area after 120 h of differentiation. The identification of neurons is done automatically using a convolutional neural network (CNN). Training of the CNN was done based on manually annotated experiments (Förster et al., 2021).

6. Neurite length 120 h is the mean length in µm of all neurons (see neuronal differentiation) that are identified by the skeletonization algorithm in Omnisphero (https://omnisphero.com).

7. Neurite area 120 h is the mean area in pixels (without nuclei) of all neurons (see neuronal differentiation) that are identified by the skeletonization algorithm in Omnisphero (https://omnisphero.com).

8. Oligodendrocyte differentiation 120 h is defined as the number of all O4-positive cells in percent of the cell number (Hoechst-positive cells) within the migration area after 120 h of differentiation. The

identification of oligodendrocytes is done automatically using a convolutional neural network (CNN). Training of the CNN was done based on manually annotated experiments (Förster et al., 2021).

Secondary endpoints:

3. Cell number is the number of all Hoechst-positive nuclei detected within the area between the sphere core and the outer boundaries of the migration area (see radial glia migration 120 h).

4. Cytotoxicity 72/120 h is assessed as membrane integrity by measuring the amount of LDH leaked from cells with damaged plasma membranes. LDH-dependent reduction of resazurin to resorufin is measured in the supernatant of each well as fluorescence of the reaction product resorufin (relative fluorescence unit) in a multiplate reader after 72/120 h of differentiation and compound treatment.

5. Viability 120 h is assessed as mitochondrial activity by measuring the amount of resazurin reduced to fluorescent resorufin (relative fluorescence unit) in a multiplate reader in the last two hours of the 120 h differentiation and compound treatment period.

Technical details (of e.g. endpoint measurements)

All technical details for the test method are available in the SOP in DB-ALM format (Appendix J in Masjosthusmann et al. 2020). Detailed protocols are also available as publications (Baumann et al. 2014; Nimtz et al. 2019) or upon request (ellen.fritsche@iuf-duesseldorf.de).

Endpoint-specific controls/mechanistic control compounds (MCC)

Primary Endpoints:
All endpoint-specific controls are run on each experimental plate and for each human individual and passage number. Endpoint-specific control effects on NPC2a, NPC3 and NPC5 from n>40 experiments are shown in figure 4. The locations of the endpoint-specific controls on the experimental plate are shown in figure 5.

3. Radial glia migration 72 h: The endpoint-specific control for radial glia migration after 72 h is the SRC kinase inhibitor PP2. SRC family kinases represent one pathway that regulates radial glia migration of differentiating hNPCs (Moors et al., 2007). Inhibition of this pathway with PP2 (10 µM) causes a reduction of radial glia cell migration to values between 0 and 60 % of the solvent control.

4. Neuronal differentiation: The endpoint specific control for neuronal differentiation is EGF. EGF is a growth factor that stimulates radial glia proliferation and migration and inhibits neuronal differentiation (Ayuso-Sacido et al., 2010; Jenny Baumann et al., 2016). 20 ng/mL EGF reduces neuronal differentiation to values between 0 and 50% of the solvent control.

5. Oligodendrocyte differentiation: The endpoint specific control for oligodendrocyte differentiation is BMP7. BMP7 promotes the BMP signaling cascade which upregulates astroglia differentiation and maturation and inhibits oligodendrocyte formation (Jenny Baumann et al., 2016; Gross et al., 1996; Mabie et al., 1997). 100 ng/mL BMP7 reduces oligodendrocyte differentiation to values between 0 and 60 % of the SC.

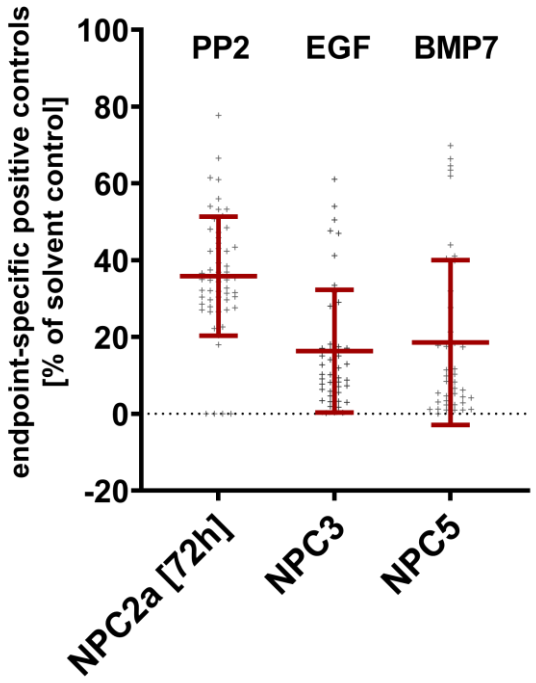

Figure 4: Effects of endpoint-specific positive controls. The SRC kinase inhibitor PP2 reduces radial glia migration (NPC2a), epidermal growth factor (EGF) reduces neuronal differentiation (NPC3) and bone morphogenic protein 7 (BMP7) reduces oligodendrocyte differentiation (NPC5).

Secondary Endpoints:
0.2 % Triton X-100 is used as a positive control for cell viability and cytotoxicity since it lyses the cell and therefore causes a maximal response for both endpoints. This positive control is run on each experimental plate (Figure 5).

Positive controls

The NPC2-5 hNPC migration and differentiation assay correctly identified the following compounds that are known to cause DNT in humans or in vivo (Masjosthusmann et al., 2020; Blum et al., 2023):

Methylmercury(II) chloride
Cadmium chloride
Hexachlorophene
2,2',4,4'-Tetrabromodiphenyl ether
Dexamethasone
Manganese(II) chloride
Chlorpromazine hydrochloride
Haloperidol
Paraquat dichloride hydrate
Trichlorfon

Deltamethrin
Sodium valproate
Tebuconazole
Tributyltin chloride
Potassium perfluorooctanesulfonate

Negative and unspecific controls

The solvent control (SC) is used as negative control that is run on each experimental plate. Each SC has to be established by comparing the effect of the SC to the effect of the media control. Established solvent controls show the same response as the media control.

The SC is used to assess if the acceptability criteria for radial glia migration, neuronal and oligodendrocyte differentiation are met and to normalize the compound treatment and the positive control response.

Established solvent controls are:
DMSO: 0.1 % v/v
DPBS: 2 % v/v
ddH2O: 2 % v/v
MeOH: 0.1% v/v

Other negative control compounds that were identified as negative in this assay and are known to not affect neurodevelopmental endpoints in vivo include (Masjosthusmann et al., 2020; Blum et al., 2023):

Acetaminophen
Amoxicillin
Aspirin
Buspirone
Chlorpheniramine maleate
D-Glucitol
Diethylene glycol
D-Mannitol
Doxylamine succinate
Famotidine
Ibuprofen
Metformin
Metoprolol
Penicillin VK
Saccharin
Sodium benzoate
Warfarin

Features relevant for cytotoxicity testing

Differentiating hNPCs are a multicellular system consisting of radial glia, neurons (1.5-16 %), oligodendrocytes (1.5-11%) and astrocytes within the migration area. The measurement of cytotoxicity and viability therefore always represents all cells within the migration area.
Because of the higher percentage of GFAP-positive radial glia and astrocytes, these two cell types are overrepresented in the assessment of cytotoxicity and viability.

The measure of cell viability assessed by the Alamar blue assay (mitochondrial reductase activity) strongly depends on the number of cells in the migration area. Therefore, a reduction in cell number either due to a reduced migration distance or a lower cell number in the migration area will lead to a reduction in the Alamar blue signal despite the cell viability is not necessarily affected (Figure 3 in Nimtz et al. 2019). Therefore, as soon as the migration distance or cell number in the migration area are reduced, the Alamar blue (viability) assay is not used as a measure for viability, but the cytotoxicity assay (based on LDH release) is taken as a reference for the specific DNT endpoints to determine if the effect is specific or not.

Acceptance criteria for the test method

General acceptance criteria (individual experiment):

3. At least three technical replicate values need to be present for each condition to be accepted for the data analysis.
4. At least five conditions need to be present for the experiment to be accepted in the data analysis for concentration response modeling.

Endpoint dependent acceptance criteria:

The acceptance criteria described below is the MEAN response of at least three replicates of the SC:

- radial glia migration 72 h: 700 – 1500 µm
- radial glia migration 120h: 700 – 1600 µm
- neuronal differentiation: ≥1.5 % neurons
- oligodendrocyte differentiation: ≥1.5 % oligodendrocytes
- cell number: 2500 – 6000

The acceptance criteria described below is the response for each replicate and is applied to all conditions:

1 . neuronal migration: ≥ 5 neurons
2 . oligodendrocyte migration ≥ 5 oligodendrocytes

Throughput estimate

The methods described here are set up in a 96-well plate format with automated image acquisition, analysis, and data evaluation. Pipetting steps such as coating of 96-well plates, compound dilutions, feeding, cell viability and cytotoxicity assays can be automated using a liquid handling system.

In the fully automated setup, 5 plates with 8 conditions (Figure 5) of 2 test compounds each and 4 replicates per condition can be run in one week by two technical assistants. This results in the generation of 320 data points for each endpoint within one week (excluding all controls). The throughput is therefore estimated as medium.

Handling details of the test method

Preparation/addition of test compounds

The method is set up for 8 test conditions including 7 compound concentrations and one SC. The test conditions are prepared in a serial dilution from the stock solution (Figure 5).

Stock solutions are prepared by diluting the compound in the solvent (e.g. DMSO) in a concentration that allows the preparation of the highest test concentration without exceeding the highest acceptable solvent concentration (see 0). For DMSO the highest acceptable solvent concentration is 0.1% which means that the stock concentration needs to be at least 1000x higher than the highest test concentration.
Stock solutions in non-sterile solvents (e.g. water or PBS) have to be sterile filtrated using a sterile syringe filter (diameter 0.2 µm). Adsorption to the filter needs to be considered.

Stock solutions are aliquoted and stored at -20°C. A stock solution is not thawed more than three times.

For the preparation of the test condition the stock solution is diluted to the highest test concentration (default 1:1000) in differentiation medium. All following dilutions are prepared by serial dilutions of the highest concentration in differentiation medium containing solvent (in the concentration of the highest test concentration). The default serial dilution is 1:3 which covers a concentration range from e.g. 20 µM to 27 nM (729-fold). Depending on the desired concentration range, the dilution can be changed to 1:2, 1:5, 1:10 or other.

The SC is prepared by adding the solvent to differentiation media in the same concentration as the highest test concentration.

100 µL of the compound dilutions and the SC are added to a 96-well plate (Figure 4).

The serial dilution can also be prepared directly in the 96 well plates.

hNPCs are added to each well after a 15 to 30 min equilibration period at 37°C and 5 % CO_2.

To subtract the background fluorescence of the phenol red-containing medium, 4 wells with medium only (Background, medium without cells, Figure 5) are prepared for the viability and cytotoxicity assays.

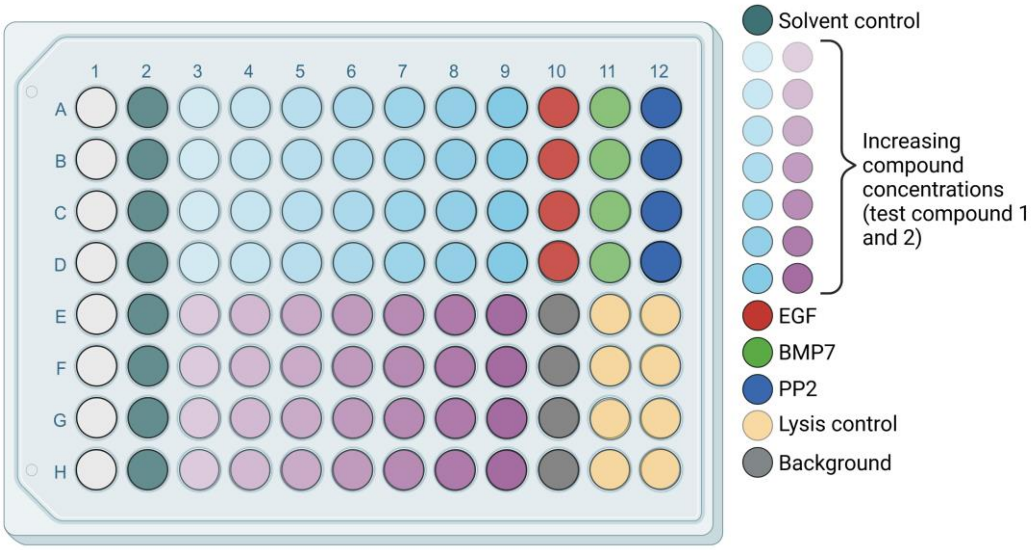

Figure 5 Plating Scheme. The solvent control depends on the solvent of the compound that is tested. 7 compound concentrations are plated in a serial dilution from lowest (left) to highest (right) concentration. Lysis control (4 replicates for each timepoint, 72h and 120h) and background control (4 replicates) are used for the viability and cytotoxicity assays. Endpoint-specific controls for radial glia migration (PP2), neuronal differentiation (EGF) and oligodendrocyte differentiation (BMP7) are performed on each experimental plate (4 replicates each).

Day-to-day documentation of test execution

Documentation for each experiment including meta data and the experimental data that is collected in the Automated Experimental Evaluation or AXES sheet.
Meta data such as plating date, experimenter, NPC individual, NPC passage, compound, compound concentrations and a plate map are reported in these sheets. Depending on the endpoint, the experimental data is collected during or at the end of the experiment. Each raw data point (including all outliers) is collected in the AXES sheet. All deviations from the standard procedure are documented in a comment section of the AXES sheet.

Practical phase of test compound exposure

The practical phase of the test compound exposure follows the description in the SOP in DB-ALM format (Appendix J in Masjosthusmann et al. 2020) or upon request (ellen.fritsche@iuf-duesseldorf.de). Deviation from the SOP are documented in the comment section of the AXES sheet.

Errors (e.g. pipetting in wrong well or wrong volume pipetted) are also documented in the comment section of the AXES sheets. Data points of the affected well are marked in the AXES sheet and excluded from the analysis.

Concentration settings

Starting concentrations and concentration ranges are defined based on the following factors:

- toxicological relevance of the compound (i.e. internal human exposures, effects at lowest concentrations)
- solubility of the compound
- highest useable solvent concentration

These factors are determined based on available information (databases/literature) or experimentally (e.g. solubility test).

Uncertainties and troubleshooting

Problematic compounds:

- Volatile compounds
- High lipophilicity (high K_{OW})
- Low solubility in established solvents
- Fluorescent compounds (possible interference with viability and cytotoxicity assay)

Critical handling steps:

- The poly-D-lysine/laminin coating as well as the plate format and plate type (F-bottom) are critical for cell migration and differentiation. Coated plates should be stored at 4°C for no longer than 7 days. If different plate types are used, the test system and test method need to be re-established.
- Outer wells have to be filled with H_2O because of edge effects.
- Automating the pipetting steps using a liquid handling system for coating, preparation of the plates, feeding of the plate, viability and cytotoxicity assays reduces the variability and the user bias.

Sources of variation:

- Pipetting steps: Each pipetting step is a source of variation. Especially in the viability and cytotoxicity assays where the volume pipetted determines the final readout.
- ICC: The staining consists of multiple washing steps. As pipetting errors add up in each washing step, there is variation in the dilution of blocking solution and antibodies.
- NPC differentiation: The biological variation between the differentiation of different NPC spheres cannot be controlled and can lead to variations in the endpoints neuronal and oligodendrocyte differentiation.

Known Pitfalls:

- Spill over from the lysis control wells to other wells can happen and needs to be controlled by the operator. It is important to completely empty the 3-day lysis control wells after they have been used and fill them with water, to avoid spill over during the last 2 days of the experiment.
- Multiple washing steps in the ICC staining lead to added pipetting errors and may cause differences in the volume in each well. Here the operator needs to control that the spheres are always covered by PBS. Differences between the wells can be corrected by the operator.

Detailed protocol (SOP)

See SOP in DB-ALM format (Appendix J in Masjosthusmann et al. 2020). Detailed protocols are also available as publications (Baumann et al. 2014; Nimtz et al. 2019) or upon request (ellen.fritsche@iuf-duesseldorf.de).

Special instrumentation

- Incubator for cell culture
- Tissue chopper
- Multiplate reader for fluorescence measurement
- High content imaging device for automated fluorescence microscopy
- Liquid handling system (necessary to achieve the throughput described above)

Possible variations

Other endpoints accessible with the test system (human NPCs):

- Measurement of BMP2 dependent astrocyte maturation (Masjosthusmann et al., 2018)
- Distribution of the neuronal density within the migration area (Schmuck et al., 2017)
- Oligodendrocyte maturation (Katharina Dach et al., 2017)
- Migration pattern (Barenys et al., 2016)

Other exposure schemes of NPC2-5 endpoints:

- 24 h NPC2a migration (Jenny Baumann et al., 2016)
- 72 h NPC3 neuronal differentiation (Jenny Baumann et al., 2016)

Cross-reference to related test methods

No related test methods

Data management

Raw data processing, curve fitting and hit definition (prediction) are performed using CRStats (Concentration-Response Statistics), an R package for concentration-response analysis automation for in vitro test systems optimized for multi-well plate experiments based on drc (https://github.com/iuf-duesseldorf/fritsche-lab-CRStats; Keßel et al. (pre-print at BioRxiv; doi: 10.1101/2022.10.18.512648)).

Raw data format

The raw data format is different depending on the endpoints.

For all endpoints assessed in a multiplate reader (viability and cytotoxicity), the raw data format are excel files containing values (one for each endpoint, timepoint and well) measured as relative fluorescence units. These values are transferred from the original excel file into the AXES sheet. The original excel output files are saved for traceability of the data.

The radial glia migration after 72h, which is measured manually in ImageJ, is directly copied into the AXES sheet as values in µm. Original brightfield images are archived for 10 years.

All other raw data is computed from the ICC images in the Omnisphero software and is exported and saved as one csv file. From there the values are again transferred to the AXES sheets. The following data is exported from Omnisphero:

- number of all cells in the migration area (cell number)
- number of all neurons in the migration area
- number of all oligodendrocytes in the migration area
- radial glia migration (µm)
- mean neuronal migration (µm)
- mean oligodendrocyte migration (µm)
- neurite length (µm or pixels)
- neurite area (pixel)

All original ICC images are archived for 10 years.

Outliers

Mathematical procedures to define outliers are not applied. Data points from wells where technical problems are known or obvious are excluded from the analysis.

Possible technical problems:

- pipetting errors
- spillover from lysis
- washed-off sphere without indication of cytotoxicity or reduction in cell viability
- wrong or no identification of migration area and sphere core
- problems in ICC staining
    - cells dried out
    - wrong illumination
    - blurry pictures

All wells with technical problems are marked in the AXES sheet.

Raw data processing to summary data

If not otherwise stated, all data processing steps are performed in an R based evaluation tool that was designed for data processing, curve fitting and point of departure evaluation of in vitro concentration response toxicity data.

Data processing describes all processing steps of raw data that are necessary to obtain the final response values including the normalization, curve fitting and benchmark concentration calculation.

Processing (or pre-processing) steps depend on the endpoint and are described below:

radial glia migration 72 h: The mean of four replicate measures of each sphere. The mean of four measures per well is used as raw data input and is not calculated in the R based evaluation tool.

radial glia migration 120 h: no pre-processing.

cell number 120 h: no pre-processing.

neuronal differentiation: the number of all neurons is divided by the number of all cells (in the migration area).

$$neuronal\ differentiation\ [\%] = \frac{\#\ neurons}{\#\ cells} * 100\ \%$$

neuronal migration 120 h: mean neuronal migration (in the migration area) divided by radial glia migration 120 h.

$$migration\ distance\ neurons\ [\%] = \frac{mean\ migration\ distance\ all\ neurons\ [\mu m]}{migration\ distance\ radial\ glia\ 120\ h\ [\mu m]} * 100\ \%$$

oligodendrocyte differentiation: number of all oligodendrocytes is divided by the number of all cells (in the migration area).

$$oligodendrocyte\ differentiation\ [\%] = \frac{\#\ oligodendrocytes}{\#\ cells} * 100\ \%$$

oligodendrocyte migration 120 h: mean oligodendrocyte migration (in the migration area) divided by the radial glia migration 120 h.

$$migration\ distance\ oligo.\ [\%] = \frac{mean\ migration\ distance\ all\ oligo.\ [\mu m]}{migration\ distance\ radial\ glia\ 120\ h\ [\mu m]} * 100\ \%$$

Viability: subtraction of mean background from each response value.

$$Background\ corrected\ response\ [RFU] = raw\ response\ [RFU] - Background\ [RFU]$$

where RFU is a relative fluorescent unit of the plate reader output

Cytotoxicity: no pre-processing

Neurite Area: no pre-processing

Neurite length: no pre-processing

Curve fitting

The data is normalized to the SC and re-normalized to the starting point of the curve.
For the normalization to the SC each replicate data point is normalized to the median of the SC in the respective experiment.

For the cytotoxicity assays the following normalization is used instead of the normalization to the SC. Here again each response value is normalized using the median of the lysis control and the median of the solvent control.

$$\text{normalized response} = \frac{\text{lysis control} - \text{response}}{\text{lysis control} - \text{solvent control}}$$

The R package drc is used to calculate the optimal curve-fit for each experiment (https://github.com/iuf-duesseldorf/fritsche-lab-CRStats; Keßel et al. (pre-print at BioRxiv; doi: 10.1101/2022.10.18.512648). For calculations of curve fits and BMCs, the data from independent experiments is pooled (median of all replicate values for one concentration). Several non-linear models are run with the concentration response data of each endpoint and the Akaike´s information criterion is used to determine the best fit.

For re-normalization of the data, the response value of the curves starting point is determined and used to re-normalize all response values. Therefore, each mean response value is divided by the starting point of the curve and multiplied by 100. For the re-normalized response values the curve fitting is repeated to produce the final concentration response curve.

For deriving a reference point (RP) or point of departure (Pod) the Benchmark Concentration (BMC) approach, as recommended by the EFSA Scientific Committee (Hardy et al., 2017), is applied. The BMC approach makes use of all data points that define the fitted concentration response curve. Thereby, the BMC is defined as the concentration that is associated with a specific change in response, the Benchmark Response (BMR). The BMR is a value of effect size and should be defined as an effect size that is higher than the general variability of the measured endpoint. The BMR is therefore determined based on the variability of the respective endpoint.

BMR for NPC2-5:

Primary DNT specific endpoints of the test method are:

radial glia migration 72 h (NPC2a)	BMR10
radial glia migration 120h (NPC2a)	BMR10
neuronal migration 120 h (NPC2b)	BMR30
oligodendrocyte migration 120h (NPC2c)	BMR30
neuronal differentiation 120 h (NPC3)	BMR30
neurite length 120 h (NPC4)	BMR30
neurite area 120 h (NPC4)	BMR30
oligodendrocyte differentiation 120h (NPC5)	BMR30

Secondary endpoints are:

cell number 120 h	BMR30
cytotoxicity 72 h	BMR10
cytotoxicity 120 h	BMR10
viability 120 h	BMR30

Based on the BMR and the concentration response curve, the evaluation tool calculates the BMC, as well as upper and lower confidence limits (BMCU and BMCL respectively) based on the predict function in the R package drc. The predict function calculates the prediction bands around the concentration response curve based on the deviation between independent experiments and gives an estimation of the area that is expected to enclose 95% of future data points. The BMCL is thereby defined as the intersection of the lower band and the BMR while the BMCU is defined as the intersection of the upper band and the BMR. The confidence intervals are used to access the uncertainty of the BMC. If the BMCU is 1.5 times above the test range, the original BMCU is replaced by 1.5x the highest tested concentration.

Internal data storage

All raw data is stored on a server with a daily server back up for at least 10 years.

Metadata

All metadata is collected in the AXES sheet (see 6.2) together with all raw data.

The metadata gives information on:
The experiment:
- start and end date of the experiment
- experimenter

The cell source:
- human individual
- cells thawing date
- passage of cells
- date of cell passaging

The compound:
- compound identity
- stock concentration
- all dilution steps
- solvent and solvent concentration

The controls:
- control identity
- preparation of controls

Metadata file format

All metadata is collected in an Excel format.

Prediction model and toxicological application

Scientific principle, test purpose and relevance

Primary hNPCs are isolated from the fetal brain and can be used to model neurodevelopmental processes like migration of radial glia, neurons and oligodendrocytes, neurite outgrowth and differentiation into neurons and oligodendrocytes within this test method.

The test system therefore measures adverse events in the young (fetal) developing brain.
Thereby, the biological processes that are modeled are:
1. radial glia cell migration (NPC2a)
2. migration of young cortical neurons (NPC2b)
3. oligodendrocyte migration (NPC2c)
4. fetal neuronal differentiation into young neurons (NPC3)
5. neurite outgrowth of young fetal neurons (NPC4)
6. oligodendrocyte formation of fetal NPC (NPC5)

The toxicological events that are modeled include events that impact the above-mentioned biological processes in any direction (increase or decrease). Thereby, neurodevelopmental processes represented by the NPC2-NPC5 assay are guided by a variety of pathways known to contribute to the respective processes

in vivo as assessed by hNPC exposure to in vivo relevant signaling pathway modulators (summarized in Table13, Masjosthusmann et al. 2020; Koch et al., 2022). All the endpoints can be assessed in a high content format within one experiment due to the multi-cellularity and high developmental dynamic of the neurospheres. In addition, the strength of the system is that it allows KE-based assessment of human neurodevelopment without the need to perform species extrapolation. Moreover, the self-organized mixed culture system contains cell-cell interactions e.g. via gap junction channels as cells die when cell-cell communication is blocked by gap junction blockers (unpublished observations). Hence, the system is superior to single-cell 2D systems, since due to its nature it mimics a multitude of neurodevelopmental processes. One limitation of the hNPC model is the restricted timing. These endpoints represent early processes of neurodevelopment during the fetal period. Neurons stay immature hence later processes like synaptogenesis cannot be studied. Moreover, correct positioning, like one can study in vivo with cortical layering, cannot be studied here, since the assay mimics cortical radial glia and neuronal migration but does not cortical layer formation.

The test method predicts the hazard of a chemical to induce developmental neurotoxicity by causing neurophysiological, functional and behavioral changes in the developing nervous system.

Prediction model

Two prediction models (PM) are applied for the NPC2-5 assay. One PM for a downregulation (PM downregulation) and one PM for an upregulation (PM upregulation) in cell differentiation and migration.

PM down regulation

The PM uses hit definition based on comparison of the confidence intervals (CI) for the BMC of the DNT-specific endpoint (BMCs) and the unspecific endpoint (cytotoxicity/viability; BMCus).

Thereby the following four hit classifications apply:

“no hit”	The compound is not defined as hit.
“specific hit”:	The compound is defined as hit and the CI’s do not overlap, meaning that the upper confidence limit of the specific endpoint (BMCUs) is lower than the lower confidence limit of the unspecific endpoint (BMCLus).
“borderline hit”:	The compound is defined as hit and the CI of the specific endpoint overlaps by less than or equal to 10% with the CI of the unspecific endpoint.
“unspecific hit”	The compound is defined as hit and the CI of the specific endpoint overlaps by more than 10% with the CI of the unspecific endpoints.

The compound is classified as a hit, if the concentration response curve generates a BMC and if the CI is within the test range. In case the CI spans above the test range, the compound is only classified as hit, if the highest test concentration is significantly different from the lowest test concentration. The adjusted significance ($p<0.05$) is thereby determined using a Tukey HSD test.

The decision process for the prediction model is described in the flow chart in **Figure 11**.

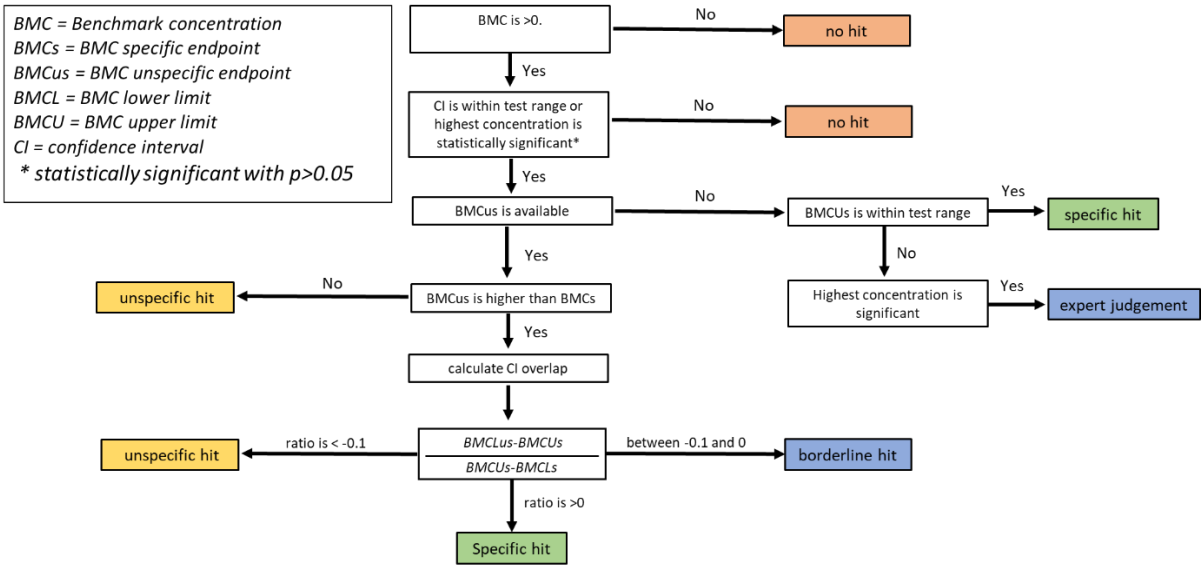

Figure 11: Decision tree for the PM for downregulation applied for test method NPC2-5. Overview of the decisions leading to the classification of a compound in one of four categories: "no hit", "specific hit", "borderline hit" and "unspecific hit".

Specific consideration for the prediction model:

In case no confidence limits are available for the unspecific endpoint because the BMR is not reached, the BMCLus is assumed to be the highest tested concentration. If the CI of the specific endpoint spans above the test range for a compound identified as "hit" based on statistical significance, expert judgement is applied to define, if the "hit" is specific or unspecific. Therefore, all datapoints of the unspecific and specific endpoint of the highest test concentration are compared. If these data points do not overlap, the compound is classified as "specific hit", otherwise as "unspecific hit".

In general, BMCs based on the same BMR are compared (e.g. BMC_{30us} vs BMC_{30s}). In case the BMC_{30us} is not available for an endpoint that allows the generation of a BMC_{10us}, the BMC_{10us} is used instead. If the classification of this comparison is "unspecific hit", the compound will be flagged as "check manually" as the BMC_{10us} is lower than the BMC_{30us} leading to a higher probability of a false classification. To avoid such false classification expert judgement is needed.

Compounds can also be flagged as "check manually", if the classification based on the viability is different from the classification based on the cytotoxicity and if the confidence interval is very wide (BMCU/BMCL > 25), which means a high uncertainty for the BMC estimation. In both cases expert judgement is needed to decide on the classification.

The expert judgement is an individual decision process, that accounts for effect size, curve progression, statistical significance and overall standard deviation. If the concentration response curves do not give enough information for decision by expert judgement, additional testing or testing in a different concentration range should be performed.
For compounds that reduce the cell number or radial glia migration the viability is not used as unspecific endpoint. The reason is that an effect in both endpoints indirectly affects cell viability. In this case only the cytotoxicity is used for the classification.

PM upregulation

In contrast to the PM for downregulation, the PM for upregulation is based on a hit definition without the

comparison of confidence intervals (CI) between the specific and unspecific endpoint. The reason is that specific and unspecific endpoint do not have the same relationship during an induction compared to a reduction in the endpoint. A loss in general cell health will likely result in an effect in e.g cell migration or differentiation, while an induction in cell health (measured as mitochondrial activity) does not necessarily increase these endpoints.

The following three hit classifications apply:

"no hit"	The compound is not defined as hit.
"specific hit":	The compound is defined as hit and the effect is not an artifact due to loss in cell health.
"unspecific hit"	The compound is defined as hit in only the unspecific endpoints.

The compound is classified as hit, if the concentration response curve generates a BMC and if the CI is within the test range. In case the CI spans above the test range, the compound is only classified as hit, if the highest test concentration is significantly different form the lowest test concentration. The adjusted significance ($p<0.05$) is thereby determined using a Tukey HSD test.

The decision process for the prediction model is described in the flow chart in figure 7.

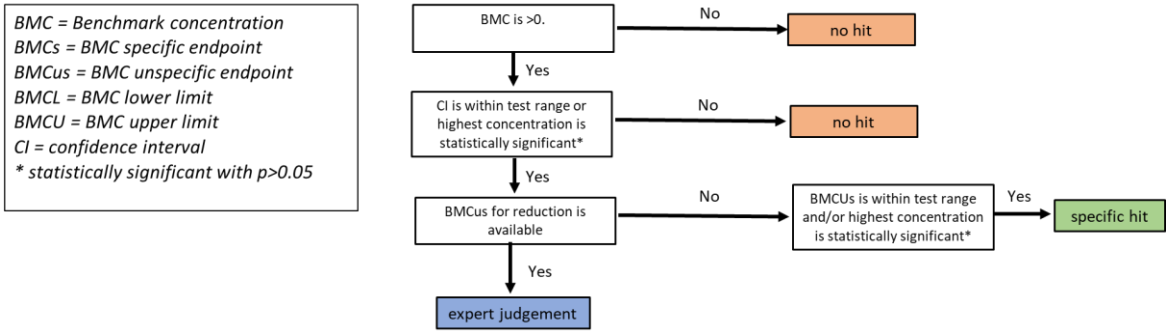

Figure 7: Decision tree for the PM for upregulation applied for test method NPC2-5. Overview of the decisions leading to the classification of a compound in one of three categories: "no hit", "specific hit", and "unspecific hit".

In case a compound is classified as hit together with a reduction in an unspecific endpoint, it needs to be clarified if the induction is a specific effect or an artifact due to a loss in cell viability or an increase in cytotoxicity. Therefore, expert judgement is applied which accounts for effect size, curve progression, statistical significance, overall standard deviation but also morphological changes of the spheres. Expert judgement is additionally applied if the confidence interval is very wide (BMCU/BMCL > 25) which means a high uncertainty for the BMC estimation.

Prediction model setup

The prediction model is set up as a statistical model which uses the 95 % confidence intervals (assessed based on the prediction bands around the concentration response curve) to determine the uncertainty of a

hit definition. In the case of high uncertainty in the confidence interval, (e.g. because the CI spans above the tested concentration range) the model additionally considers the statistical significance between the highest and the lowest test concentration. Next to the hit definition the uncertainty given by the 95 % CI is also considered in the specificity analyses (see 8.2 for a more detailed description).

The model has been tested on a set of 17 DNT negative- and 9 human DNT positive compounds (see 5.6 and 5.7). All negative compounds were correctly classified as “no hit” while 6 positive compounds were correctly classified as “specific hit”. Here it is important to mention, that it is not expected that the NPC2-5 assay identifies all DNT positive compounds as not all of those compounds act via the mode-of-action (MoA) represented by the NPC2-5 assay. For most compounds the exact MoA for their neurodevelopmental adversity is not precisely known, yet other mechanisms like synaptogenesis and neuronal network formation are amongst the known DNT MoAs.

Test Performance

Table 1 summarizes the assay performance in terms of reproducibility of the assay.

Intra-experimental variation (SC) is the mean coefficient of variation (CV) ±SD of the CV of all replicates of the solvent control from each experiment across n>400 experiments.

Inter-experimental variation (raw values) is the variability across the raw values of all independent experiments (n>400) before normalization in percent.

Inter-experimental variation (low conc.) is the variability across all independent experiments (n>400) after normalization based on the response of the lowest test concentration. It is assumed that the lowest test concentration does not affect any of the endpoints measured.

Inter-experimental variation (positive controls) is the variability of the respective positive controls across all independent experiments (n>40) after normalization.

Positive controls:
radial glia migration 72h (NPC2a) → PP2
neuronal differentiation (NPC3) → EGF
oligodendrocyte differentiation (NPC5) → BMP7

Table 3: Assay variability quantified as coefficient of variance (CV)

Endpoint	Intra-experimental variation (SC)	inter-experimental variation (raw values)	Inter-experimental variation (low con.)	Inter-experimental variation (positive controls)
radial glia mig. 72h (NPC2a)	5.1 ±1.9%	11.3 %	5.2 %	15.5 %
radial glia mig. 120h (NPC2a)	5.6 ±2.3%	9.6 %	6.1 %	
neuronal mig. (NPC2b)	10.7 ±4.3%	19.8 %	11.5 %	
oligodendrocyte mig. (NPC2c)	9.4 ±4.9%	13.1 %	10.9 %	
cell number (NPC2c)	12.4 ±4.8%	23.3 %	14.4 %	
neuronal diff. (NPC3)	23.0 ±8.9%	48.4 %	30.5 %	15.8 %

neurite length (NPC4)		25.9 %	23.31	
neurite area (NPC4)		28.6 %	23.1 %	
oligodendrocyte diff. (NPC5)	35.1 ±15.9%	54.3 %	34.2 %	22.2 %
cytotoxicity [72h]	1.4 ±1.8%	24.4 %	8.6 %	
cytotoxicity [120h]	1.5 ±1.8%	37.6 %	6.6 %	
viability [120h]	8.4 ±3.3%	15.7 %	9.2 %	

Sensitivity and specificity of the NPC2-5 assay are determined based on a set of 9 predicted human DNT positive compounds and 17 predicted human DNT negative compounds (Masjosthusmann et al., 2020; Blum et al., 2023).

Based on this compound set the following performance parameters are obtained for the NPC2-5 assay.

Specificity: 100 %
Sensitivity: 66.7%

Here it is important to mention, that it is not expected that the NPC2-5 assay identifies all DNT positive compounds as not all of those compounds act via the MoA represented by the NPC2-5 assay. For most compounds the exact MoA for their neurodevelopmental adversity is not precisely known, yet other mechanisms like synaptogenesis and neuronal network formation are amongst the known DNT MoAs. It is therefore recommended that this assay is run as one part of an in vitro DNT battery.

In vitro – in vivo extrapolation (IVIVE)

Parameters for in vitro – in vivo extrapolation are not yet determined.

Applicability of test method

Toxicological applicability domain
The following compound classes have been tested successfully:

- Industrial chemicals
- Pesticide/biocides
- cosmetics ingredients
- pharmaceuticals

Compounds need to be soluble in a solvent at a solubility where the solvent does not produce effects by itself in the test system (0 for established solvents).

Compounds that are volatile or have a high lipophilicity have not been tested and might need more sophisticated exposure methods such as ‘passive dosing’.

Biological applicability domain
The neural progenitor cell migration and differentiation assay (NPC2-5) is based on primary hNPCs obtained from the human fetal cortex. As mentioned in 0 “Scientific principle”, the method depicts migration of radial glia, neurons and oligodendrocytes, neurite outgrowth and differentiation into neurons and oligodendrocytes from fetal hNPCs.

Next to the endpoints represented by this test method there are several other necessary neurodevelopmental endpoints which need to be studied using other test methods.

Neurodevelopmental processes not represented by this test method:
- NPC proliferation (NPC1)
- Neural Crest Cell (NCC) Migration
- NPC apoptosis
- Neuronal morphology
- Synaptogenesis
- Neuronal network formation
- Neural Rosette Formation
- hiPSC-derived NPC proliferation
- hiPSC-NPC neuronal differentiation
- Neuronal subtype differentiation
- Astrocyte Differentiation and Maturation
- Astrocyte Reactivity
- Microglia reactivity
- Myelination

For a complete assessment of developmental neurotoxicity, the test method needs to be part of a test battery.

The information on signaling pathways modulating the neurodevelopmental endpoints of the test method are summarised in Table 2. This describes the so far tested biological applicability domain of the assay.

Table 4: Signaling pathways studied in the test method. Abbreviations: AKT, AKT serine/threonine kinase; BMP, bone morphogenic protein; COX-2, cyclooxygenase 2; CREB, CAMP responsive element binding protein; EGFR, epidermal growth factor receptor; ETC complex I, electron transport chain complex 1; GSK3, glycogen synthase kinase 3; LXR, liver X receptor; mTOR, mechanistic target of rapamycin kinase; NO-cGMP, nitric oxide cyclic guanosine monophosphate; PDGFR, platelet derived growth factor receptor; PKC, protein kinase C; PLC, phospholipase C; PPARβ/δ, peroxisome

proliferator activated receptor beta/delta; RHO, ras homolog family member; ROCK, Rho associated coiled-coil containing protein kinase ; RXR, retinoid X receptor; SRC, SRC proto-oncogene; THR, thyroid hormone receptor; WNT, wingless

DNT Assay	Signaling Pathway	Model Compound	Literature
NPC2a	SRC	PP2	Baumann et al., 2015; Masjosthusmann et al., 2018; Moors et al., 2007
	EGFR	AG1478, PD153035, EGF	
	PKC	PMA/Bis-I	
	NO-cGMP	7-NI, ODQ, Rp-8-Br-cGMP	Tegenge et al., 2011
	ROCK	Y27632	
	PLC	m-3M3FBS	unpublished
	CREB	db-cAMP	
	mTOR	MHY1485	
	GSK3	CHIR	
	WNT	CHIR	
	RHO	Narciclasine	Masjosthusmann et al., 2020
	ETC complex I	Rotenone	
	PKC	Bis-I	
NPC3	Notch	DAPT	Baumann et al., 2015; Masjosthusmann et al., 2018
	EGFR	EGF, PD153035	
	RHO	Narciclasine	Masjosthusmann et al., 2020
	ETC complex I	Rotenone	
	THR	Triiodothyronine	unpublished
	RXR	Bexarotene	
	PKC	Bis-I	
	GSK3	CHIR	
	WNT	CHIR	
NPC4	PKC	Bis-I	Masjosthusmann et al., 2020
	ETC complex I	Rotenone	
	RHO	Narciclasine	
	WNT	CHIR	unpublished
	GSK3	CHIR	
	AKT	LY294002	
NPC5	BMP	BMP2, BMP7	Dach et al., 2017; Masjosthusmann et al., 2018
	-	Ascorbic Acid	
	Notch	DAPT	
	EGFR	PD153035	
	WNT	CHIR, IWP2	unpublished
	COX-2	Celecoxib	
	mTOR	MHY1485, Everolimus	
	GSK3	CHIR	
	CREB	cAMP	
	AKT	LY294002	
	PLC	m-3M3FBS	
	PDGFR	CP-673451	
	PPARβ/δ	GW0742	
	LXR	GW3965	
	PKC	Bis-I	Masjosthusmann et al., 2020
	RHO	Narciclasine	

Incorporation in test battery

To assess the hazard for developmental neurotoxicity it is recommended that this assay is used as one assay in a battery of assays (see 0 “Applicability of test methods”)

For the assessment of chemical action on the endpoints represented by this test method, the test method can be used as stand-alone test method.

The test method is currently used in the setup of a DNT test battery.

Publication/validation status

Availability of key publications

Key Publications concerning the test method are:

Blum et al., 2023
Koch et al., 2022
Klose et al., 2021
Masjosthusmann et al., 2020
Nimtz et al., 2019
Masjosthusmann et al., 2019
Masjosthusmann et al., 2018
Dach et al., 2017
Schmuck et al., 2017
Baumann et al., 2016
Baumann et al., 2014
Fritsche et al., 2011
Moors et al., 2009

(Potential) linkage to AOPs

NPC2 is linked to the AOP “Disrupted laminin-beta1-integrin interaction leading to developmental neurotoxicity”. The AOP is part of the OECD Project with the ID1.83 and the Coaching number C3-#8.

NPC5 is linked to an AOP on the binding to voltage gated sodium channels, which leads to impaired behavioral function (Hernández-Jerez et al., 2021).

Steps towards mechanistic validation

See:
0 Characterization and definition of source cells
0 Omics characterization of the test system
4.7 Features of the test system that reflect the in vivo tissue
0 Applicability of test method

Pre-validation or validation

To date, 123 unique compounds (as defined by unique DTXSIDs) have been tested successfully in this assay.

No formal OECD 34 validation study has been done (e.g., ring trials with a standard set of known positive and negative controls).

Linkage to (e.g. OECD) guidelines/regulatory use

Test is not linked to regulatory guidelines.

Test method transferability

Operator training

For operators with a basic training in cell culture practices a four-week training period for handling of the test system and training in the assay is recommended. The operators should have basic understanding in image analysis and data evaluation with respect to concentration response fitting.

Transfer

The test method has been used by multiple operators over a period of 18 months. However, inter operator variability was not been determined.

The test method is currently part of project that involves the lab to lab transfer and testing of a set of 35 chemicals.

Safety, ethics and specific requirements

Specific hazards; issues of waste disposal

No specific requirements.

Safety data sheet (SDS)

Reference to MSDS is given in the SOP in DB-ALM format (Appendix J in Masjosthusmann et al. 2020) or upon request (ellen.fritsche@iuf-duesseldorf.de).

Specific facilities/licenses

No specific facilities are required.
No specific ethical approval is required.

Commercial aspects/intellectual property of material/procedures

There are no commercial aspects or intellectual properties to be considered.

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Appendix B.3

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Date: 17.03.2023
Version: 220428_v2

Overview

Descriptive full-text title

Assay to test impairment of migration of human neural crest cells (cMINC; UKN2) – V2.0

Abstract

This in vitro test method uses human neural crest cells (NCCs) generated from induced pluripotent stem cells (iPSC). It assesses disturbances of NCC migration during fetal development. The number of migrated NCCs, as well as cell viability are measured simultaneously using high content imaging. Thereby the processes of cell migration and cell death are measured in cells exposed to potential toxicants for 24 h. The data of this method are meant to predict developmental disorders and malformations e.g., neural tube defects or craniofacial malformations caused by compound exposure during fetal development. The method has a well-established prediction model, but it has not undergone formal validation and it has not been part of a ring trial. It has been used in the screening of several medium-sized compound libraries. According to the readiness criteria as published by Bal-Price et al. (2018) the neural crest cell migration assay obtained the readiness score A-.

General information

Name of test method

Circular migration inhibition of NCC (cMINC) assay, UKN2

Version number and date of deposition

This is Version 2.0 of the protocol “Assay to test impairment of migration of human neural crest cells (cMINC; UKN2) – V2.0). It was assembled and deposited in March 2023. A previous version was assembled in 2019 in the context of the EU-ToxRisk project (see publication Krebs et al., 2020)

Summary of introduced changes in comparison to previous version(s)

Changes compared to V1 refer mainly to the generation of the test system and the cell line used. Test procedures and parameters remain unchanged.

Assigned data base name

UKN2a_DART_NC_cMIGR_24h_02

Name and acronym of the test depositor

University of Konstanz (UKN), Germany

Name and email of contact person

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Reference to additional files of relevance

- An important reference is the DB-ALM Protocol n° 195. The original iPSC are in the meantime cultured feeder-free (see 3.2)
- Raw data file
- Data processing file

Description of general features of the test system source

Supply of source cells

The human induced pluripotent stem cell (hiPSC) line IMR90_clone_#4 has been bought from WiCell, Wisconsin in 2012 and a masterstock has been frozen. From the masterstock several working stocks have been prepared. The working stocks are regularly thawed and can be continously maintained due to self-renewal and pluripotency capabilities of the cells. The cells are maintained up to 10 passages before a new vial of the working stock is thawed.

Overview of cell source component(s)

Undifferentiated hiPSC cells (IMR90, WiCell) are maintained as monoculture on Laminin-521 coating in essential 8 (E8) medium. The cells grow in colonies, and are split weakly. The cells show self-renewal and pluripotency characteristics (regular testing). The cells can be differentiated into several different cell types.

Characterization and definition of source cells

- **ATCC number**: CCL-186
- **Origin**: Homo sapiens, human
- **Tissue**: lung
- **Cell type**: fibroblast

- **Gender**: female
- **Morphology**: fibroblast
- **Culture properties:** adherent
- **Disease:** no disease was diagnosed
- **Age:** 16 weeks gestation
- **Ethnicity:** Caucasian
- **Expression:** Cells express the pluripotency markers Oct4, nanog and Tra-1-60

Table 1. Short tandem repeat (STR) signature of cell line confirms 100% loci homology

	ATCC (CCL-186)	University of Konstanz; Dept. of In-Vitro-Toxikology and Biomedicin
Cell line	**IMR-90**	**IMR90**
Date		09.11.2018
D5	12	12
D5'	13	13
D13	11	11
D13'	13	13
D7	9	9
D7'	12	12
D16	10	10
D16'	13	13
vWA	16	16
vWA'	19	19
TH01	8	8
TH01'	9,3	9,3
TPOX	8	8
TPOX'	9	9
CSF1	11	11
CSF1'	13	13
Amel	X	X
Amel'	X	X

Acceptance criteria for source cell population

The cells have to be pathogen-free (regular testing for mycoplasma).
The iPSC maintenance is regularly checked for expression of pluripotency markers (Oct4, Nanog, Tra-1-60) by immunocytochemistry.

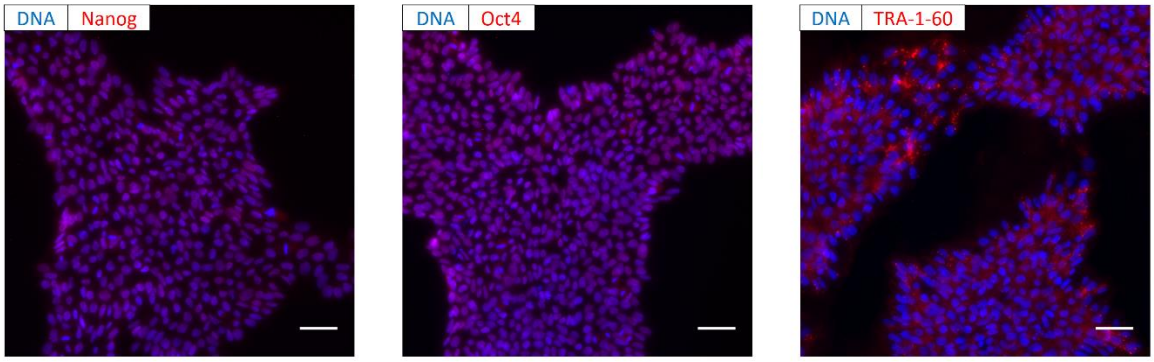


Figure 12: Immunostaining of relevant pluripotency markers in maintenance culture

Variability and troubleshooting of source cells

- hiPSC can be maintained up to 10 passages, high passage number might influence performance of cells
- Too little or too high cell density leads to detachment of cells or spontaneous differentiation
- If cells start to differentiate, cells should be discarded immediately
- Cells have to be maintained as colonies and not as single cells. Therefore splitting should be performed as fast as possible
- Batch effects of critical additives (e.g. holo-transferrin for iron supply of cells or TGF-β which maintains cell pluripotency) can lead to differentiation of cells at low passage number.

Differentiation towards the final test system

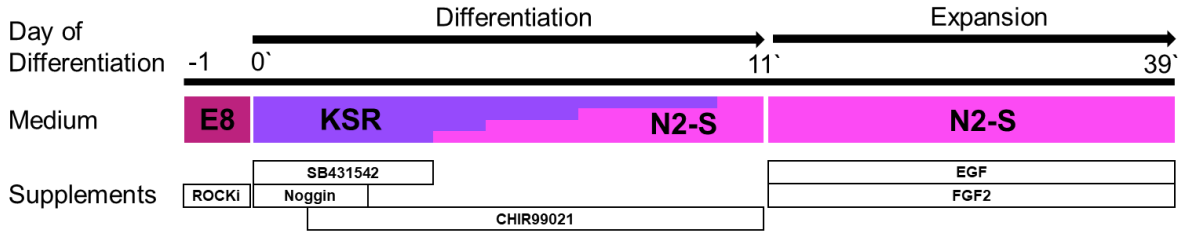


Figure 2: Differentiation scheme from hiPSCs to neural crest cells

NCCs were differentiated from hiPSCs following the modified protocol of Mica et al. (2013) (https://doi.org/10.1016/j.celrep.2013.03.025). Therefore, IMR90 iPSCs were plated on Matrigel coated 6-well plates at a density of 100'000 cells/cm^2 in E8 medium containing 10 µM ROCK (Rho-associated protein kinase)-inhibitor Y-27632. After one day cells reached a confluency of 80-100% and differentiation was initiated (day 0') by a medium change to KSR medium (Knock out DMEM (Dulbecco's Modified Eagle Medium), 15% knock out serum replacement, 1% GlutaMax, 1% MEM NEAA (minimum essential medium - non-essential amino acids) solution, 50 µM 2-mercaptoethanol) supplemented with 20 ng/ml Noggin and 10 µM SB431542. From day 2 on cells were treated with 3 µM CHIR99021. Noggin and SB431542 were withdrawn at day 3 and 4, respectively. Beginning at day 4, the KSR medium was gradually replaced with N2-S medium (DMEM/F12 (Dulbecco's Modified Eagle Medium/Ham's F-12), 1.55 mg/ml glucose, 1% GlutaMax, 0.1 mg/ml apotransferin, 25 µg/ml insulin, 20 nM progesterone, 100 µM putrescine, 30 nM selenium) in 25% increments. Cells were collected at day 11, resuspended in N2-S medium supplemented with 20 ng/ml EGF (epidermal growth factor) and 20 ng/ml FGF2 (fibroblast growth factor 2) and seeded as

droplets (10 µl) on poly-L-ornithine (PLO)/Laminin/Fibronectin coated 10 cm dishes. Cells were expanded by weekly splitting. From now on seeding as droplets was not necessary and medium was changed every second day. After 35-39 days, cells were cryopreserved at a concentration of $4*10^6$ cells/ml in 90% N2-S medium and 10% dimethyl sulfoxide (DMSO) (Merck Millipore) until further use.

Reference / link to maintenance culture protocol

DB-ALM Protocol n° 195

Definition of the test system as used in the method

Principles of the culture protocol

A highly homogeneous pre-differentiated population of neural crest cells is added to coated wells. In the middle of the well is a silicone stopper that prevents cells from settling in a circular area in the middle of the well. The cells are kept viable and alive by the presence of EGF and FGF in the medium, and they become adherent overnight. When the stopper is removed, the cells form a dense monolayer in the culture dish, with a sharply demarcated circular area in the middle that is free of cells. Due to their natural spontaneous migration behaviour, the cells move into the cell-free area. The cells are still in a proliferative state. Proliferation at the edge of the cell free area contributes to a small extent to “apparent migration behaviour”. This has been characterized and quantified in detail (Nyffeler et al. 2016). It is accounted for by counting cells in a narrower circle than the original circular area.

Acceptance criteria for assessing the test system at its start

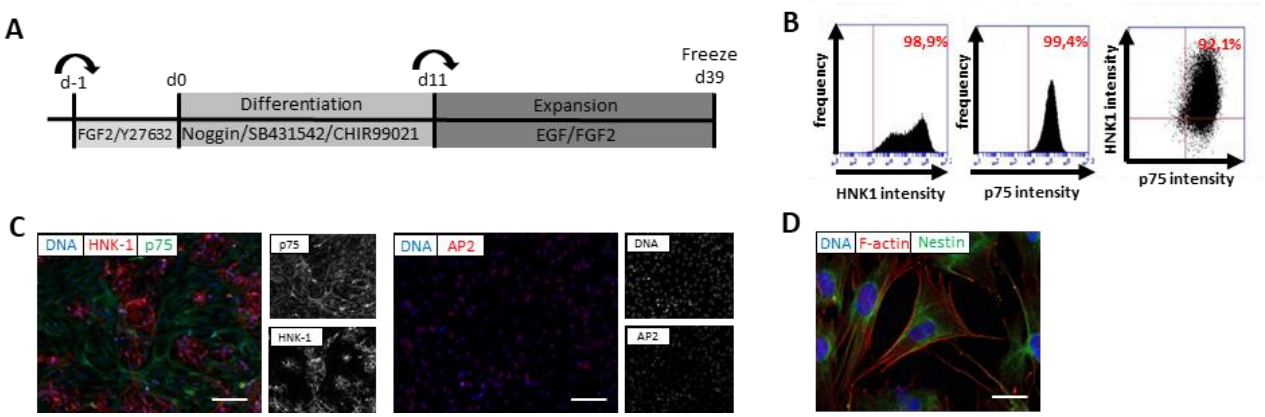


Figure 3: Characterization of the cellular system.

(A) Differentiation schema of NCCs from human induced pluripotent stem cells. (B) Expression of the NCC markers HNK-1 and p75 was monitored by FACS analysis. (C) After thawing the cells were immunofluorescently labelled for the typical NCC markers p75, HNK1 and AP2. Scale bar: 50 µm. (D) Typical mesenchymal like cell morphology of NCCs. Cells were double-stained for nestin and f-actin. Scale bar: 10 µm.

The derived NCCs are ≥ 90% positive for the NCC marker human natural killer-1 (HNK1) and the nerve low affinity nerve growth factor receptor p75. Additionally, the cells express the stem cell microfilament protein nestin and AP2. On the other hand, none of the cells show expression of the astrocyte marker GFAP, the

central nervous system precursor cell marker Pax6 and the neuronal marker βIII tubulin. Furthermore, the functional capability of the cells is checked by performing the cMINC assay with endpoint specific controls. The cells have to be pathogen-free to be used in further experiments (regular testing for mycoplasma after thawing). After thawing the viability should be > 90%.

Acceptance criteria for the test system at the end of compound exposure

After compound treatment, the negative controls should fulfil the following:

- cells should be migrated into the cell free area

- cell viability should be > 90%

Variability of the test system and troubleshooting

Causes of variability:

- High passage number of iPSC maintenance might influence NCC differentiation
- Too little cell density at the start of differentiation can cause problems (confluency should be 80-100%)
- For the freeze and thaw processes a fast handling is important to avoid cells to stay at 10% DMSO for too long. This can decrease number of viable cells after thawing.

Metabolic capacity of the test system

No specific information available.

Omics characterization of the test system

Transcriptomics data (unpublished) will become available from the originator lab (Leist) upon request.

Features of the test system that reflect the *in vivo* tissue

- The cells are able to migrate mostly as single cells; they show some evidence of collective migration
- Cells show invasive behaviour in a 3D environment (e.g. transwell assay)

Commercial and intellectual property rights aspects of cells

The cells are not protected by patents or any other licences.

Reference / link to the culture protocol

Brief description is in section 3 of this file.

Detailed maintenance is described in the DB-ALM SOP n° 195 available at:

http://cidportal.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/DBALM/VER2-0/online/DBALM_docs/195_P_cMINC.pdf

A lab-internal handling protocol is also available upon request to the Leist-lab.

Test method exposure scheme and endpoints

Exposure scheme for toxicity testing

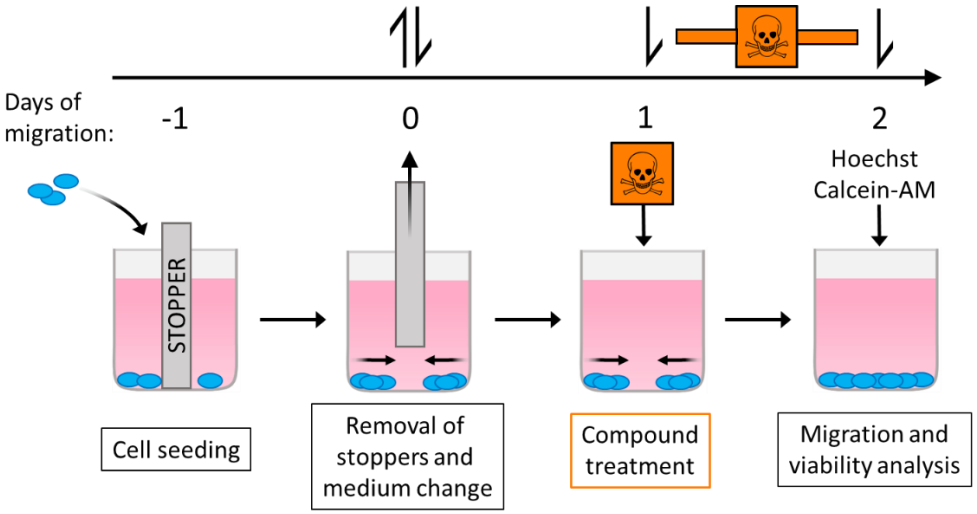


Figure 4: Exposure scheme and assay procedure.

Day -1: The wells are coated with PLO/laminin/fibronectin. Then, silicone stoppers are placed into the wells of a 96-well plate. Cells are seeded around the stoppers and allowed to attach.
Day 0: The silicone stoppers are removed and the medium is replaced with pre-warmed, fresh N2-S medium containing the cytokines EGF and FGF. Cells are allowed to migrate into the cell free area.
Day 1: 25 µl of the 5x concentrated toxicants are added to the wells. The cells plus toxicants are incubated for 24 h.
Day 2: Cells are stained with calcein-AM and Hoechst (H-33342) for 30 min before imaging with a high content imaging microscope. Quantification of migration and viability is done by high content imaging analysis.

Endpoint(s) of the test method

Specific Endpoint: Migration inhibition
Reference endpoint: Cell viability
Note: Migration induction is also possible to assess, but complex in its toxicological interpretation. Therefore only unidirectional migration is assessed as an endpoint if the method is used in a standardized testing procedure in the prediction model.

Overview of analytical method(s) to assess test endpoint(s)

Migration inhibition: NCCs are plated around silicone stoppers in a culture dish and are allowed to migrate into the cell free area upon removal of the stopper. The number of migrated cells into the cell free zone is quantified 24 h after toxicant treatment. Migration inhibition of NCCs after treatment with toxicants is measured relative to control conditions (solvent control cells). For the quantification the cells are stained with calcein-AM and H-33342 for 30 min at 37 °C. The center of the well (migration zone) is imaged in four tiles with a 5x objective. Afterwards the four images are stitched together to obtain one image. For migration quantification, a software tool has been developed (http://invitrotox.uni-konstanz.de/). With the help of this software the previously cell-free area can be estimated and the number of H-33342 and calcein double-

positive cells in the region of interest (ROI) can be counted. The diameter of the ROI was chosen so that 150 to 300 cells were in the ROI in untreated conditions. An Excel table containing the number of viable cells in the ROI of all wells of the plate is generated by the software.
Cell viability: Cell viability is measured after 48 h outside the migration zone in the same well. The cells are stained with calcein-AM and H-33342 and four fields outside the migration zone are imaged with a 10x objective. Viability is defined as the number of H-33342 and calcein double-positive cells, viable cells are determined by an automated algorithm. An excel file is generated with the number of viable cells in each well. Migration and Viability are normalized to untreated controls.

Technical details (of e.g. endpoint measurements)

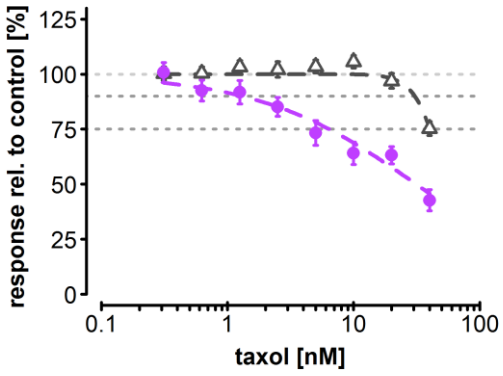
Quantification of migration
An automated microplate reading microscope (Array-ScanII HCS Reader, Cellomics, PA) equipped with a Hamamatsu ORCA-ER camera (resolution 1024 x 1024; run at 2 x 2 binning) was used for image acquisition. Four fields per well were imaged. Images were recorded in 2 channels using a 5x objective and excitation/emission wavelengths of 365 ± 50/535 ± 45 to detect H-33342 in channel 1 and 474 ± 40/535 ± 45 to detect calcein in channel 2. Pictures were exported from the microscope and for migration quantification, a software tool has been developed (http://invitrotox.uni-konstanz.de/). With the help of this software the previously cell-free area can be estimated and the number of H-33342 and calcein double-positive cells in the region of interest (ROI) can be counted. The diameter of the ROI was chosen so that 150 to 300 cells were in the ROI in untreated conditions. An Excel table containing the number of viable cells in the ROI of all wells of the plate is generated by the software.

Quantification of individual viable cells by imaging
For a quantitative assessment of viable cells, the same wells that were used to assess migration were analyzed using another image analysis algorithm of the ArrayScan VTI 700 Series software (v. 7.6.2.4) as described earlier in Stiegler et al. (2011) (doi: 10.1093/toxsci/kfr034) and Krug et al. (2013) (doi: 10.1007/s00204-013-1072-y). Nuclei were identified in channel 1 as objects according to their size, area, shape, and intensity. Nuclei of apoptotic cells with increased fluorescence were excluded. Viable nuclei were defined as H-33342 and calcein double-positive cells. An Excel table containing the number of viable cells is generated.

Endpoint-specific controls / mechanistic control compounds (MCC)

Example 1: Cell migration requires dynamic variability of the cytoskeleton, e.g. actin reorganisation. Cytochalasin D is a known inhibitor of actin polymerisation and therefore inhibits cell migration at non-cytotoxic concentrations.

Example 2: Migration depends on the dynamic instability of microtubules in the leading edge of a migrating cell. Taxol inhibits breakdown of microtubules and leads to migration inhibition at non-cytotoxic



concentrations.

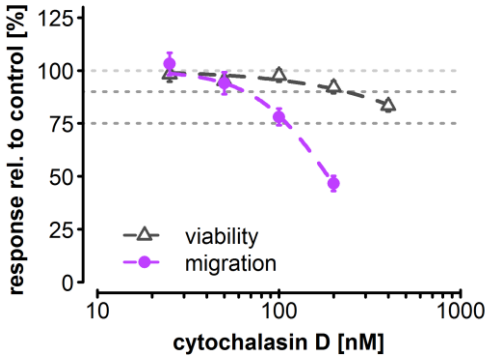


Figure 5: Examples of endpoint specific controls in the cMINC assay

Positive controls

Positive control: Cytochalasin D (200 nM), LiCl (10 mM), taxol (10 nM)

Negative and unspecific controls

Negative control: solvent (0.1% DMSO final concentration), paracetamol, ASS

Features relevant for cytotoxicity testing

Cells still proliferate after thawing and proliferation can interfere with the migration result. About 30% of the cells undergo mitosis during the assay.

Acceptance criteria for the test method

A test is discarded if the positive control did not inhibit migration, e.g. if the cell number in the migration zone is ≥ 75% of control.
If the negative control decreases migration ≥ 10%, the test is discarded.

Throughput estimate

Data point = one biological replicate (→ usually 4 technical replicates); each concentration/condition of a compound counts as a data point.
768 data points per month
2 compounds per plate, 6 different concentrations of each compound per plate (see figure 6) → 12 data points (1 plate)

16 plates can be done per week → correlates to 32 compounds → 192 data points per week
4 weeks per month → 768 data points per month.

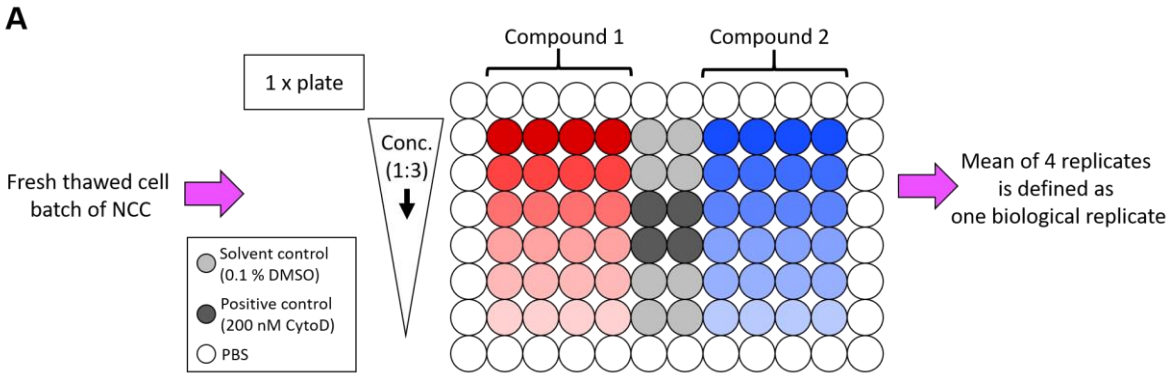


Figure 6: A typical 96-well plate layout of the cMINC

Handling details of the test method

Preparation / addition of test compounds

- Compounds are stored according to the manufacturer's instructions (e.g., 4°C, room temperature, -20°C).
- Preferable solvent is DMSO. The used DMSO is stored in a lightproof, air-tight bottle at room temperature.
- Final DMSO concentration on the cells is 0.1%
- After dissolving the compounds which are delivered in a solid/powder form, all compound solutions are aliquoted into volumes sufficient for one experiment (i.e., one biological replicate). In this way repeated freezing and thawing and therefore damaging the compound's stability and efficiency can be avoided.
- For conducting an experiment, a compound aliquot is thawed and diluted with 'DMEM/F12 Advanced' without supplements in a separate deepwell-plate.
- All compound dilutions in the master plate contain 0.5 % DMSO, so that a final concentration of 0.1 % DMSO is reached on the cells. The highest compound concentration is diluted with medium 1:200 without DMSO as 0.5 % is already reached with the DMSO the compound is solved in, the serial dilution is done with N2-S medium supplemented with 20 ng/ml EGF and FGF and 0.5 % DMSO.
- 25 µl of the 5x concentrated compound dilutions are added to the cells using a multichannel pipette.

Day-to-day documentation of test execution

Plate maps are defined prior to the experiment and documented in the lab book and files (Excel files) are stored on the work group server.
Concentrations and compound dilutions are calculated prior to the experiment.
Experimental procedures are noted manually in a paper lab book.

Practical phase of test compound exposure

The experimenter plans the experiment according to Cellomics microscope availability (has to be booked in advance).
Pipetting errors are marked directly on the plate maps and are documented in the lab book.
The paper lab book is taken to cell culture rooms and errors are documented in there right away.
The technical replicates were pipetted from left to right. The highest concentration is located at the top row.

Concentration settings

2 compounds per plate with 6 concentrations

As default a serial dilution 1:3 is used, i.e., a concentration range from e.g. 100 µM → 0.4 µM.
Serial dilutions of compounds are prepared in a separate deepwell-plate, from which 25 µl are transferred to the according plates with attached cells using a multichannel-multistepper pipette. N2-S medium supplemented with 20 ng/ml EGF and FGF is used for dilution.
Dilution steps can be adapted to be more narrow (e.g., 1:1.5)

Uncertainties and troubleshooting

- Compound solubility in stock and during dilution is too low (stock solved in 100% DMSO, final concentration of the solvent on the cells is 0.1% DMSO)
- Some compounds show autofluorescence and interfere with the detection of calcein-AM or H-33342.
- To prevent negative edge effects, only the inner 60 wells of a 96-well plate are used and the edge wells were filled with PBS.
- Focusing failure of Array Scan VTI HCS Reader (Cellomics, PA) can be a problem that produces outliers; as well as imaging only one channel.
- Highly trained/automated handling with multichannel and multistepper pipette is necessary to achieve little variance.
- Different cell batches vary in the cell number due to freezing conditions and the differing proliferation rates of the lots. The variation between the plates of one experiment is lower than 5% and the variation between different experiments is lower than 20%. The results were always normalized to untreated controls.
- Operators can get trained within 2-4 weeks. Cell seeding and medium change should be performed as fast as possible to keep cells as short as possible at room temperature. The more practice an operator has, the faster the critical steps can be performed.
- Substances are added when pipette tips are touching the wall of the wells right above the medium surface. When the substance solution is pipetted too high above the medium surface, the droplet may just stick to the wall of the well without flowing down into the medium.

Detailed protocol (SOP)

Protocol nº 195 in DB-ALM data base

http://cidportal.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/DBALM/VER2-0/online/DBALM_docs/195_P_cMINC.pdf

Updated SOP can be made available by laboratory upon request:
marcel.leist@uni-konstanz.de

Special instrumentation

- The method requires a Cellomics Array Scan VTI HCS high content reader that may not be present in the standard lab. (Alternative automated microscopes and software tools to perform migration assays exist and the method might be transferred in the future)
- Silicone stoppers (Platypus Technologies, Madison, WI, US).

Possible variations

a) further additional endpoints:
- EdU (5-Ethynyl-2′-deoxyuridine) staining can be performed within the assay to measure proliferation

b) other analytical endpoints:
cell viability by:
- fluorimetric measurement of resazurin conversion
- measurement of extracellular LDH *(lactate dehydrogenase)*
- measurement of luminescence indicating ATP (*adenosine triphosphate)* content

c) other exposure:
- The exposure time for toxicants can be increased up to 48 h. But longer exposure time increases the effect of toxicants on viability and cell proliferation.

d) experimental variation:
- AraC (cytosine arabinoside) addition to prevent proliferation effects on migration result. About 30% of the cells divide during the 24 h of the assay period. Therefore a cell proliferation inhibiting compound would reduce the cell number by 25% in the migration zone and therefore results in 80% viability and 75% migration measurement. But a reduction of migration by > 25% is unlikely to be explained by effects on proliferation (Nyffeler J. et al., 2016).
- other cells may be used (derived from different iPSC)

Cross-reference to related test methods

The scratch assay is another method to analyse cell migration. In comparison to the cMINC assay the throughput of the scratch assay is low (Zimmer B. et al., 2012).

Data management

Raw data format

Raw data is extracted by copy-paste in Excel files (example file available upon request).
Data from all technical replicates are collected in one file.

Outliers

1. Mathematical procedures to define outliers have not been defined. Data points that are far off (i.e. more than the known endpoint variability which would be 25% for migration) are discarded. Biological outliers do practically not exist, most far data points are the result of technical problems (focus not found, only one channel imaged, etc.)
2. All raw data (incl. outliers) are stored.
3. Technical outliers make up 1-0.1%.

Raw data processing to summary data

- Array Scan VTI HCS Reader (Cellomics, PA) takes images (optionally bitmap or tiff-format; 512 x 512 pixels, 8bit or 16bit)
- For migration quantification images from the Array Scan VTI HCS Reader are exported in 8-bit tiff format and loaded to the “Ringassay” software (http://invitrotox.uni-konstanz.de/) for calculating the number of migrated cells.
- Images are locally analyzed using the Array Scan software, algorithm quantifies cell count (viable cells)
- data are copy-pasted into an Excel sheet, further analysis is done with Excel + GraphPad Prism

Curve fitting

The data are analyzed with Excel and represented with GraphPad Prism.
For the concentration curve, a nonlinear regression fit is calculated. The fitting method is least squares. If a non-linear curve fit is not possible, a linear curve fit is performed. The curve deriving from the fit is a 4-parameter log function. To calculate the EC25 value, this log-function is solved for y=25% of the total scale, not for 25% of the min-max scale (see example below). Treated concentrations are analyzed for deviation from control. Sometimes it is analyzed whether the deviation of neurite growth is different from the deviation of viability via comparison of the means ± deviation using two-way ANOVA + Tukey-Kramer post hoc testing. Significance levels of data points compared to solvent control are determined via one-way ANOVA followed with non-parametric Dunnett's post test.

BMC values with their upper and lower confidence intervals (BMCU and BMCL) are calculated via the publically available online software:
http://invitrotox.uni-konstanz.de/BMCeasy/

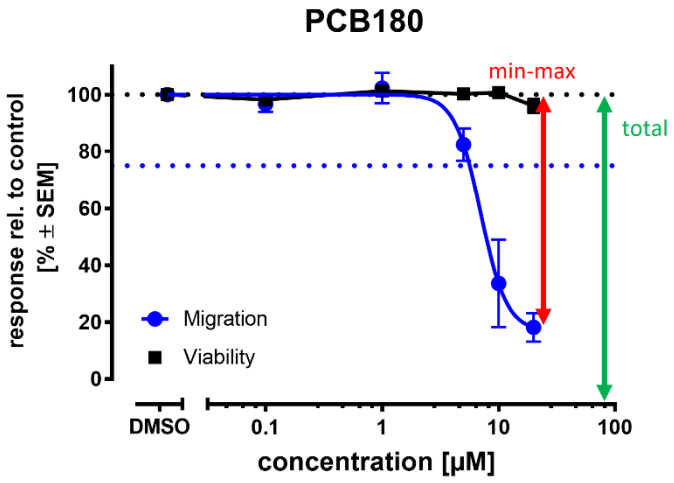


Figure 7: Exemplification of curve fit and relative effect responses

Internal data storage

The data are firstly stored on the microscope computer and then exported to other servers (lab group server and university server), which are back-upped regularly.

Metadata

The metadata are documented, stored and exported as text document (log)-files to the according scheme: (local PC)_descriptor(date and time)_XXX.log:
The following metadata are stored:

- cellinsight-pc_160429130003_AutomationControllerIni
- cellinsight-pc_160429130003_kineticprotocol
- cellinsight-pc_160429130003_protocol
- cellinsight-pc_160429130003_scan
- cellinsight-pc_160429130003_ScanIni
- cellinsight-pc_160429130003_spooling
- cellinsight-pc_160429130003.spooled

Metadata file format

Metadata files are available.

Prediction model and toxicological application

Scientific principle, test purpose and relevance

Migration of NCCs is an essential process during fetal development. Impaired NCC migration triggered genetically or by toxicants can lead to malformations and disorders e.g. Hirschsprung's disease or Treacher-Collins syndrome.
The cMINC assay models the effects of NCC migration under toxicant exposure.
The test captures endpoints like spina bifida or cleft palate also measured during developmental toxicity regulatory studies.

Prediction model

Three different models are used:

1. prediction model for screening:
hit = inhibition of NCC migration while viability is not changed:
Migration ≤ 80% of DMSO control
Viability ≥ 90% of DMSO control

2. prediction model for compound hazard evalaution:
hit confirmation testing: EC10 Viability (V) / EC 25 Migration (M) ≥ 1.3 → specific migration inhibitor of NCCs

3. prediction model for borderline compounds:
A ratio of BMC10 Viability (V) / BMC**L**25 Migration (M) ≥ 1.3 is considered a borderline hit. In some scenarios the viability does not reach the BMC10 Viability (V) necessary for the ratio calculations. In this case the highest tested concentration (HTC) was used. Schematic representation of the complete prediction model is shown in Fig. 8 below.

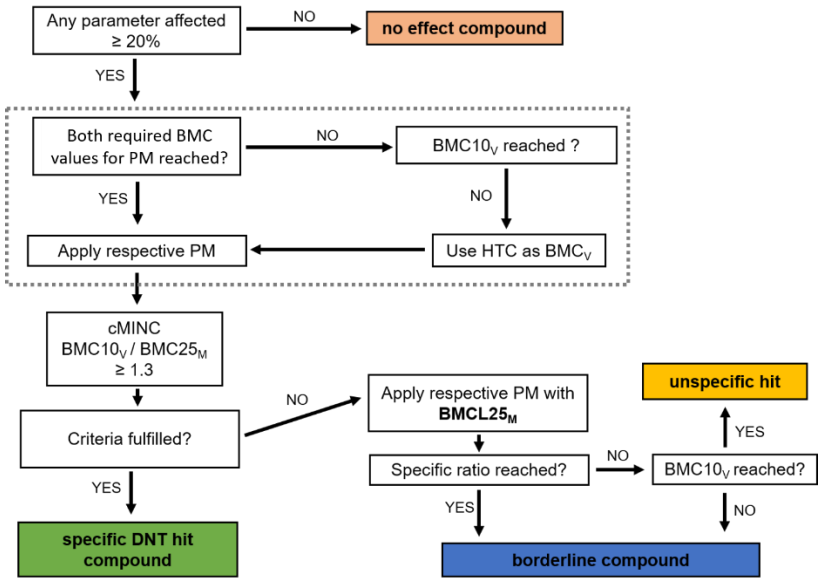


Figure 8: UKN2 Prediction model classification tree

A full overview with a schematic representation of the UKN4 prediction model can also be found in supplementary figure 3 of Blum et al., 2022.

Prediction model setup

a) The prediction model was established using the following compounds (Nyffeler et al. 2016):
- acrylamide
- arsenic trioxide (As_2O_3)
- cadmium chloride ($CdCl_2$)
- lithium chloride (LiCl)
- PCB180
- retinoic acid
  ➔ Positive controls

- cytochalsin D
- taxol
- colchicine
  ➔ Endpoint-specific controls

- silver nitrate ($AgNO_3$)
- cytarabine (AraC)
- aphidicolin
- L-homocysteine
- MG-132
- staurosporine
- triton X-100
  ➔ Unspecific toxicants

b) The prediction model has been applied to screen the 80 compound library of NTP (Nyffeler et al., 2017). The prediction model including the borderline classification has been applied to screen a 120 compound library (Blum et al., 2022; Masjosthusmann et al., 2020).

c) The process is documented in Nyffeler et al. 2016

d) Sensitivity and specificity require still definition of a good standard and have therefore not been done.

Test performance

Some background on the test performance is given in chapters 8.2/8.3 (prediction model).

Several performance parameters for the test were obtained in several separate evaluation rounds.

A first evaluation was done during the first publication of the model and its applications (Nyffeler et al. 2016). Here, a panel of well-selected positive and negative controls have been tested. Accordingly, the specificity was 100% and the sensitivity was > 90 %. In dedicated experiments, S/N ratios of > 20 and a z' of > 0.5 have been determined. The compound used as positive control cytochalasin D was run across 35 different assay plates. The migration percentage relative to the solvent control varied between 30 and 70% across all plates (Nyffeler et al. 2016, Supplementary S2).

Later, the test has been used in screening campaigns, and real-live performance data under broader screen conditions have been obtained. The different performance data need to be considered, when a compound is a hit in a screen, or whether it has been specifically evaluated in a hit follow-up or a mechanistic project.

A first screen application has been the NTP80 screen (80 compounds provided by the US NTP). Data are published in Nyffeler et al. 2017.

A second screen application has been the cross systems case study of the EU-ToxRisk project. The baseline variation is indicated in Krebs et al., 2020. Moreover, an overview is given for 19 compounds on the BMC/BMCL ratio as measure of readout certainty.

A third screen was performed in the context of the EFSA DNT test battery evaluation with 120 compounds (Masjosthusmann et al. 2020). From this screen the following performance indicators were obtained:

A: **Specificity of DNT IVB: 100%**

→ Also with cMINC as standalone assay in 17 `tool negatives´ tested (Masjosthusmann et al. 2020).

B: **Sensitivity of DNT IVB: 82.7%**

→ With cMINC combined in a full DNT battery and a selected set of 27 positive compounds with evidence for DNT (Masjosthusmann et al. 2020).

C: **Baseline variation (intra-experimental)**

Migration: 14.8 ± 4.3%

Cell viability: 6 ± 3%

D: **Baseline variation (inter-experimental)**

Migration: 15.6%

Cell viability: 9.7%

E: **Variation of a positive control run on each (inter-experimental)**

Migration: 32.2%

Definition of values C-E

C: **Baseline variation (intra-experimental)** is the mean coefficient of variation CV±SD of the CV of all replicates of the solvent control from each experiment across n>200 experiments.

D: **Baseline variation (inter-experimental)** is the variability across all independent experiments (n>200) after normalization based on the response of the lowest test concentration. It was assumed that the lowest test concentration does not affect any of the endpoints measured.

E: **Variation of a positive control run on each (inter-experimental)** is the variability of the positive control

across all independent experiments (n>40) after normalization. Example for a positive control that on average reduced the specific endpoint down to 40% (relative to solvent control) and a calculated variability of 50%: 0.5 x 40% = +/- 20% → The positive control with mean of 40% varies from 20% to 60%.

In vitro – in vivo extrapolation (IVIVE)

1. Estimated lipid content and albumin concentration in in vitro test media and human plasma:

Medium	Lipid content (ml/ml)	Albumin concentration (µM)
UKN2	2.8E-6	5.6
Human plasma	6000	600

2. A three-step (physiology-based) pharmacokinetic (PBPK) modelling strategy has been used to evaluate the clinical relevance of the in vitro concentrations, which impair NCC migration in the scratch assay. The in vivo plasma concentrations of the tested compounds were within the same range as the concentrations used in the scratch assay (Zimmer B. et al., 2014).

3. No special considerations known.

Applicability of test method

Pesticides, flame retardants, polychlorinated biphenyls (PCBs), drugs have been detected.
The exact applicability domain is not yet clear.
Volatile compounds and substances that are not water-soluble cannot be measured.

Incorporation in test battery

a) Strengths:

- Medium throughput (The term “medium” is derived from a comparison and in relation to a number of other known DNT NAMs)
- Automated microscopy

b) The comparable scratch assay is a low throughput assay. The orthogonal transwell-assay is also of lower throughput.
c) The assay has been considered as part of the ESNATS (Embryonic Stem cell-based Novel Alternative Testing Strategies) screen battery.
d) The test can to some extent be stand-alone (positive hits are meaningful). The ‘negative hits’ provide little information and require other tests in a developmental toxicity test battery

Publication / validation status

Availability of key publications

Establishment of a human cell-based in vitro battery to assess developmental neurotoxicity hazard of chemicals
Blum, J. et al. Chemosphere, 2022. PMID: 36328314

Neurodevelopmental toxicity assessment of flame retardants using a human DNT in vitro testing battery.
Klose, J. et al. Cell Biol Toxicol. 2021. PMID: 33969458

Establishment of an a priori protocol for the implementation and interpretation of an in-vitro testing battery for

the assessment of developmental neurotoxicity.
Masjosthusmann, S. et al. EFSA Supporting Publications. 2020; 17(10): 1938E.

The EU-ToxRisk method documentation, data processing and chemical testing pipeline for the regulatory use of new approach methods.
Krebs, A. et al. Arch. Toxicol., 2020. PMID: **32632539**

A structure-activity relationship linking non-planar PCBs to functional deficits of neural crest cells: new roles for connexins.
Nyffeler, J. et al. Arch. Toxicol., 2018. PMID: 29164306

Combination of multiple neural crest migration assays to identify environmental toxicants from a proof-of-concept chemical library.
Nyffeler, J. et al. Arch. Toxicol., 2017. PMID: 28477266

Design of a high-throughput human neural crest cell migration assay to indicate potential developmental toxicants.
Nyffeler, J. et al. ALTEX, 2016. PMID: 27463612

Identification of transcriptome signatures and biomarkers specific for potential developmental toxicants inhibiting human neural crest cell migration.
Pallocca, G. et al. Arch.Toxicol., 2016. PMID: 26705709

Profiling of drugs and environmental chemicals for functional impairment of neural crest migration in a novel stem cell-based test battery.
Zimmer, B. et al. Arch. Toxicol., 2014. PMID: 24691702

Evaluation of developmental toxicants and signaling pathways in a functional test based on the migration of human neural crest cells.
Zimmer, B. et al. Environ. Health Perspect., 2012. PMID: 22571897

(Potential) linkage to AOPs

Test method is not linked to AOP.

Steps towards mechanistic validation

a) Cells express typical NCC markers, are of human origin and are able to migrate
b) Cell migration requires dynamic variability of the cytoskeleton, e.g. actin reorganization and the dynamic instability of microtubules in the leading edge. If this is inhibited by cytochalasin D or taxol migration of NCCs is inhibited.
c) A formal mechanistic validation has not been performed.

Pre-validation or validation

To date, 141 unique compounds (as defined by unique DTXSIDs) have been tested successfully in this assay. No formal OECD 34 validation study has been done (e.g., ring trials with a standard set of known positive and negative controls).
In total, >200 different compounds were tested in the cMINC assay. The test method was developed using a compound training set (Nyffeler et al. 2016). It was used for an 80 compound screening library from the US National Toxicology Program (NTP) (Nyffeler et al. 2017). The test method was part of a DNT hazard assessment for 120 compounds in a DNT testing battery. The later compound set includes potential DNT

positive and DNT negative compounds (Blum et al., 2022).

Linkage to (e.g. OECD) guidelines / regulatory use

Test is not linked to regulatory guidelines.

Test method transferability

Operator training

Experiences are required in:
- cell culture
- multichannel/multistep pipetting
- handling of Array Scan VTI HCS Reader (Cellomics, PA) and its software
- Microsoft Excel
- Ringassay software
- GraphPad Prism

Operator is trained and guided by a highly experienced instructor. Approximately 4 weeks will be needed for a smooth assay performance.
Learning iPSC culture and cell differentiation takes several months.

Transfer

The assay hasn't been transferred or applied in other labs.

Safety, ethics and specific requirements

Specific hazards; issues of waste disposal

No specific requirements.

Safety data sheet (SDS)

SDS are available in the university of Konstanz DaMaRIS database (**Da**ngerous **Ma**terials **R**egistry **I**nformation **S**ystem).

Specific facilities / licenses

Work requires S1 cell culture laboratories (genetically modified cells).
No specific facilities are required.
No specific ethical approval is required.

Commercial aspects / intellectual property of material / procedures

To our best knowledge, no elements needed to conduct the experimental part of the test method are protected. Programs used to conduct the analysis of the data (Microsoft Excel and GraphPad Prism) need to

be purchased or obtained by license agreement, however data analysis and plotting can be done with other, freely available tools.

Appendix B.4

Author: Jonathan Blum, Marcel Leist
Date: 17.03.2023
Version: 220428_v2

Overview

Descriptive full-text title

Assay to test compound-derived impairment in neurite outgrowth in human dopaminergic neurons (NeuriTox; UKN4) – V2.0

Abstract

This *in vitro* test method is based on human neurons (LUHMES cells) at a stage of neurite growth. Is assesses (a) disturbances in the development of the nervous system/brain structures, and (b) direct damage to the adult nervous system, by exposure to toxicants. The neurite area (which serves as indirect measurement of neuronal interconnectivity) of stained differentiating neurons as well as cellular viability are measured simultaneously using high content imaging. The processes of neurite outgrowth and cell viability are assessed. The data of this method are meant to predict (a) developmental disorders in children caused by compound exposure during fetal development, and (b) damage to the developing nervous system, in particular to dopaminergic parts of the nervous system. The method has not undergone formal validation and has not been part of a ring trial. It predicts some aspects of neurotoxicity, but not all aspects covered by an in vivo neurotoxicity study (TG424). It has been used in the screening of medium-sized compound libraries, has undergone some mechanistic evaluation, and has been linked to AOP id3 in aopwiki.org (parkinsonian motor deficits). According to the readiness criteria as published by Bal-Price et al. (2018) the NeuriTox assay obtained the readiness score A.

General information

Name of test method

NeuriTox test, UKN4

Version number and date of deposition

This is Version 2.0 of the protocol “Assay to test compound-derived impairment in neurite outgrowth in human dopaminergic neurons (NeuriTox; UKN4) – V2.0)”. It was assembled and deposited in March 2023. A previous version was assembled in October 2019 in the context of the EU-ToxRisk project (see publication Krebs et al., 2020).

Summary of introduced changes in comparison to previous version(s)

Changes compared to V1 refer mainly to changed parameters in the prediction model.
Test procedures remain unchanged.

Assigned data base name

UKN4a_DART_LUH_neurite_24h

Name and acronym of the test depositor

University of Konstanz (UKN), Germany

Name and email of contact person

Prof. Dr. Marcel Leist
marcel.leist@uni-konstanz.de
Tel: +49-7531885037

Name of further persons involved

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jonathan.blum@uni-konstanz.de

Anna-Katharina Ückert (PhD student, experimenter)
anna-katharina.ueckert@uni-konstanz.de

Reference to additional files of relevance

- An important reference is the DB-ALM Protocol n° 200 (the new prediction model remains to be updated)
- Raw data file
- Data processing file
- Internal SOP

Description of general features of the test system source

Supply of source cells

The LUHMES cells have been brought from Lund (Sweden) to Konstanz in 2006 (Lotharius et al., 2005) and a masterstock has been frozen. The cells used for the test method are continuously generated by cell culture. Approximately every 4-5 years, a vial from the cell masterstock is thawed and extensively expanded. These cells are then frozen as working stock. A new cell batch is thawed every 4 weeks from this working stock; cells are cultured until passage 20-25.

Overview of cell source component(s)

LUHMES cell line
LUHMES cells originate from the ventral mesencephalon of an 8 week old human, female fetus. They exhibit the same characteristics as MESC2.10 cells.

LUHMES cells can be differentiated into morphologically and biochemically mature **dopamine-like neurons**

following exposure to tetracycline, GDNF (glial cell line-derived neurotrophic factor), and db-cAMP for 6 days.

They are usually cultured in a **2D monolayer**, but have also been shown to grow into 3D structures (Smirnova et al., 2015, Brull et al., 2020). It was shown that co-culture with mouse astrocytes leads to important cell-cell interactions in culture (Gutbier et al., 2018). Co-culture with hiPSC derived astrocytes (see also Spreng et al., 2022, doi: 10.3390/cells11172644) in 2D and 3D-like LUHMES is also possible (Brull et al., 2020).

Characterization and definition of source cells

- **ATCC number**: LUHMES ATCC® CRL-2927™; LUHMES cells used at the University of Konstanz differ from LUHMES cells deposited and distributed by ATCC. The assay described in here is based on UKN (University of Konstanz) LUHMES cells.
- **Origin**: mesencephalon of an 8 week old human fetus, subclone of the tetracycline-controlled, v-myc-overexpressing human mesencephalic-derived cell line MESC2.10. MESC2.10 has been conditionally immortalized with a LINX v-myc retroviral vector with a tet-off system.
- **Gender**: female
- **Morphology**: upon differentiation, cells form neuronal network, see figure below
- **Doubling time:** approx. 14 -20 h (depending on passage)
- **Phenotype:** Dopaminergic (DA)-phenotype; tyrosine hydroxylase (TH) expression depends on the presence of cAMP in the culture medium. Under differentiation conditions, cells extend neurites after 24h. They display extensive growth cones, but synapse formation is not clear. After 6 days of differentiation, cells express voltage-dependent ion channels and show **electrical activity**.
- **Expression:** Cells express α-synuclein (Parkinson's disease) (Schildknecht et al., 2013), all standard synaptic proteins and all proteins required for AD (Alzheimer's disease) pathology (Aβ-formation, Tau hyperphosphorylation) (Scholz et al., 2013; Scholz et al., 2018).

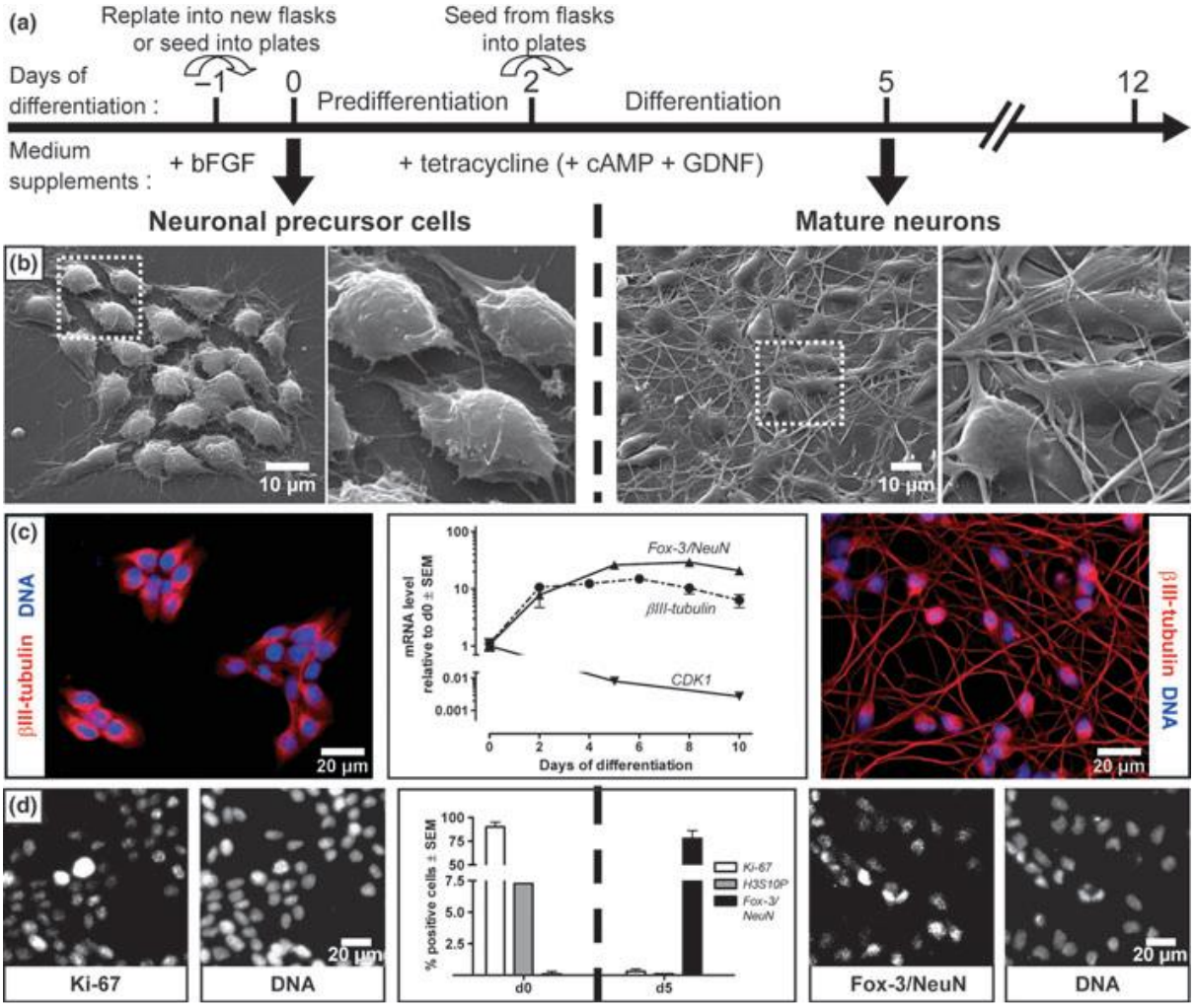


Fig. 1: Conversion of proliferating LUHMES cells into post-mitotic neurons. LUHMES were grown and differentiated either on glass cover slips or in multi-well plates. Cells were either fixed for microscopy or lysed for RNA extraction at different stages between day 0 and day 10 (d0–d10). (a) Schematic representation of the 2-step differentiation procedure, initiated by the absence of the cytokine basic fibroblast growth factor (bFGF) and addition of tetracycline. Unless mentioned otherwise, dibutyryl cAMP (cAMP) and glial cell derived neurotrophic factor (GDNF) were present throughout the differentiation. (b) Representative scanning electron microscopy (SEM) images of undifferentiated (d0) and differentiated (d5) LUHMES with marked squares shown at higher magnification. (c) LUHMES were immunostained on d0 and d5 for bIII-tubulin and nuclei were labeled by DNA staining with H-33341 dye. The mRNA expression levels of bIII-tubulin, Fox-3/NeuN and cyclin-dependent kinase 1 (CDK1) were determined after different days of maturation by RT-qPCR. (d) The proliferative status of d0 and d5 cells was quantified by immunostaining of Ki-67, H3S10P and Fox-3/NeuN. It is indicated as percentage of positive nuclei relative to all nuclei, as identified by DNA staining with H-33342. Quantitative data are expressed as means ± SEM from three independent differentiations. Figure from Scholz et al., 2011.

Table 1. Short Tandem Repeat signature: (lab internal data)

	ATCC website	UKN LUHMES	ATCC LUHMES

Amelogenin	X	X	X
CSF1PO	13,14	13,14	13,14
D5S818	11,13	11,13	11,13
D13S317	9,11	9,11	9,11
D7S820	11,13	11,13	11,13
D16S539	11,12	11,12	11,12
vWA	14,17	14,17	14,17
THO1	7,9.3	7,9.3	7,9.3
TPOX	8	8	8
Penta D		12,13	12,13
D8S1179		12,13	12,13
FGA		19,21	19,21
D3S1358		17,18	17,18
D21S11		30,31	30,31
D18S51		12	12
Penta E		11,13	11,13

Acceptance criteria for source cell population

The cells have to be pathogen-free to be used in further experiments (regular testing for mycoplasma after thawing). Cells of the working stock are checked for marker gene expression once the stock is created, but not every thawed cell batch is checked. There only visual inspection is done. The cells should be of oval morphology, and should not form extensive aggregates and clustering. Approx. 5-10% of cell death (dead cells floating in flask) is considered normal, increased cell death one passage after thawing justifies to discard of the cell batch. Culture medium should not be yellow. Proliferating cells from working stock batches should only be used up to passage 20-25.

Variability and troubleshooting of source cells

- Plastic coating is critical for even cell distribution; problems with coating often leads to cell clumping and aggregation
- Cells maintained and distributed by ATCC might behave differently than cells from the stock at University of Konstanz (Gutbier et al., 2018)
- Passage number of working stock cells might influence cellular behavior
- Problems with plastic ware obtained from other suppliers than indicated in the DB-ALM SOP n° 200 have occurred in the past
- Too little or too high cell density can cause problems (confluency should never exceed 40-85%)

Differentiation towards the final test system

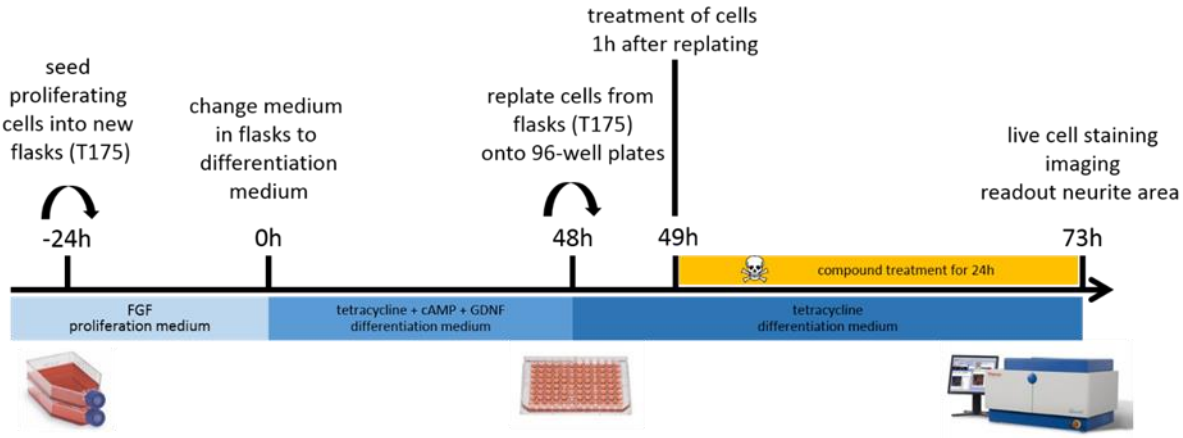


Fig. 2: Differentiation scheme and assay procedure including exposure scheme.

day -1: Proliferating LUHMES cells are seeded in proliferation medium
day 0: Medium is changed from proliferation medium to differentiation medium
day 2: Replating to 96-well plates, toxicant treatment
day 3: Readout

Differentiation is initiated by the exposure to tetracycline, GDNF (glial cell line-derived neurotrophic factor), and db-cAMP (tetracycline shuts down v-myc expression).

Coating of flasks and plates
principle:
plates and flasks are coated with a poly-L-orthinine (PLO) and fibronectin solution in ion-exchanged and purified MilliQ-H_2O overnight in the cell culture incubator. The next day the coated plastic ware is washed with MilliQ water once and dried under sterile conditions in the cell culture hood. The coated plastic can be stored at 4°C up to 4 weeks.

Differentiation
principle:
For differentiation, the following growth factors are added: GDNF (glial cell-derived neurotrophic factor), dibutyryl-cAMP (Cyclic adenosine monophosphate) and tetracycline.
If differentiation medium without cAMP and GDNF is required, the volume of those components is replaced with Advanced DMEM/F12 (Dulbecco's Modified Eagle Medium/Ham's F-12).

Reference / link to maintenance culture protocol

External document is available
Mainentance
principle:
The growth factor bFGF is added to the medium of proliferating cultures. Cells are passaged every 2-3 days (Monday – Wednesday – Friday). Minimum confluence for LUHMES cells should not fall below 40%. A confluence of 85% should not be exceeded.
For splitting, cells are counted every time and seeded in the according cell numbers:
For 2 days: 3 million per T75 flask
6 million per T175 flask
For 3 days: 1 million per T75 flask
2.7 million per T175 flask

Definition of the test system as used in the method

Principles of the culture protocol

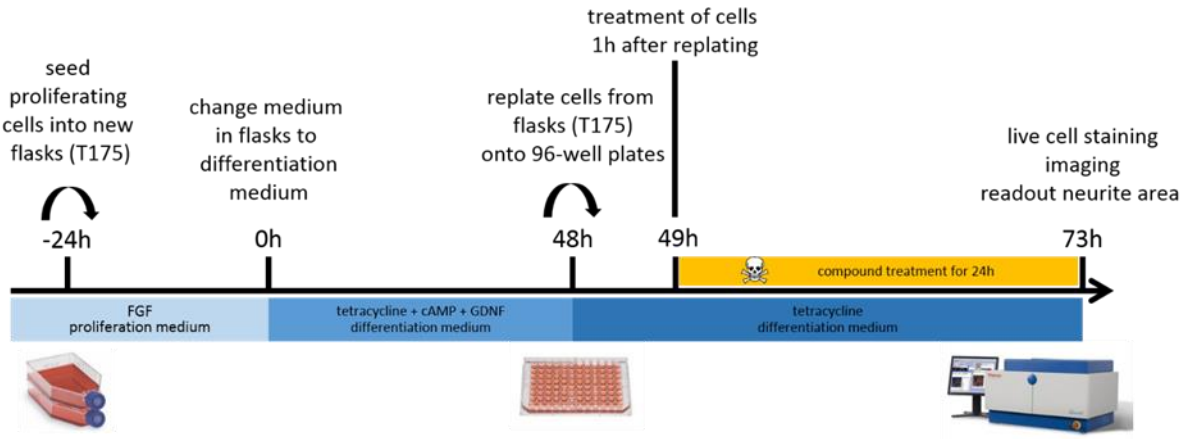


Fig. 3: Differentiation scheme and assay procedure including exposure scheme.

day -1: Proliferating LUHMES cells are seeded in proliferation medium
day 0: Medium is changed from proliferation medium to differentiation medium
day 2: Cell number reaches about 30-40 Mio per T175 flask.
Cells are trypsinized and replated onto 96-well plates (30'000 cells/well in 90 µl) in differentiation medium without cAMP and GDNF.
At about 30 min - 2 h after seeding, when cells have attached, the compounds are added (10 µl of each dilution; total volume 100 µl)
day 3: 23.5 h after toxicant treatment, cells are live-stained with H-33342 and calcein-AM and incubated for 30 min. After 24 h of treatment (including staining), the cells are imaged using a high-content microscope (Cellomics).

Acceptance criteria for assessing the test system at its start

- Cells should have neurites approx. as long as their cell body when they are trypsinized for re-plating
- They must not be contaminated
- They should have a confluency of about 60%
- The moment the cells are being treated, cells are not attached to the plate completely yet and still appear round when seen through a microscope

→ Criteria are not quantified and are mainly based on visual inspection

Acceptance criteria for the test system at the end of compound exposure

After compound treatment, the negative controls should fulfil the following:
- Neurites should be at least as long as cell bodies
- Medium should not be orange/yellow
- Appropriate cell confluency of 40-60%

→ Criteria are not quantified and are mainly based on visual inspection

Variability of the test system and troubleshooting

Causes of variability:
- Problems with coating (different PLO/fibronectin batches; problems with water quality):
  → If problems occur: Wash twice after coating
  Wash with PBS instead of MilliQ-H_2O
  Don't store plates and flasks in the fridge, use them immediately
- Different cell passages:
  → Cells have different morphology and behavior the older they get; thawing a new batch might be useful
- Lots of different plates/flasks:
  → Plastic might be different, if the manufacturer delivers from a different/new lot
- Differences between the vials of one cell "lot"
- Different lots of medium and supplements
- Cells too confluent
  → impaired metabolism
  → too slow differentiation (autocrine proliferation stimulation)
  → discard cells in that case

Cell lot = cells that have all been frozen at the same time. Usually 10-15 flasks (T175) are frozen in numerous vials.
Cell batch = vial with approx. 3 Mio cells frozen
Cell passage = cells of each vial thawn are passaged up to 17 times

Metabolic capacity of the test system

Dopamine transporter is expressed and used for MPP^+ (1-methyl-4-phenylpyridinium) transport.

Omics characterization of the test system

Microarray analysis has been used to compare differentiated LUHMES on day 3 (end of UKN4) and day 6 to undifferentiated LUHMES cells (Data source: https://kops.uni-konstanz.de/bitstream/handle/123456789/28842/Weng_288423.pdf;sequence=1).
Epigenetic modifiers have been extensively characterized (Weng et al., 2014).
Genes relevant to AD have been extensively characterized (Scholz et al., 2011).
Genes relevant for neuronal receptor composition have been extensively characterized (Loser et al., 2020; Loser et al., 2021). Genes triggered by mitochondrial toxicants have been identified (Delp et al., 2021).
There is a complete transcriptome data set on LUHMES differentiation from day 2 – day 10: It is deposited at the EBI data base under accession-ID S-TOXR1833 (public as of March 2022).

Features of the test system that reflect the *in vivo* tissue

- As neurons of the central nervous system they express the dopamine transporter DAT
- Cells grow axons and neurites in course of their differentiation
- They express various neuronal transporters and receptors (e.g. purinergic receptors, nicotinic acetylcholine receptors (Loser et al., 2020)).
- They are electrophysiologically active and excitable, produce action potentials (Loser et al., 2020)

Commercial and intellectual property rights aspects of cells

The cells are not protected by patents or any other licences.

Reference / link to the culture protocol

Brief description is in section 3 of this file.
The maintenance is described in the DB-ALM SOP °200 available at:
http://cidportal.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/DBALM/LATEST/online/DBALM_docs/200_P_UKN4.pdf
Another lab-internal SOP is also available upon request to the Leist-lab.

Test method exposure scheme and endpoints

Exposure scheme for toxicity testing

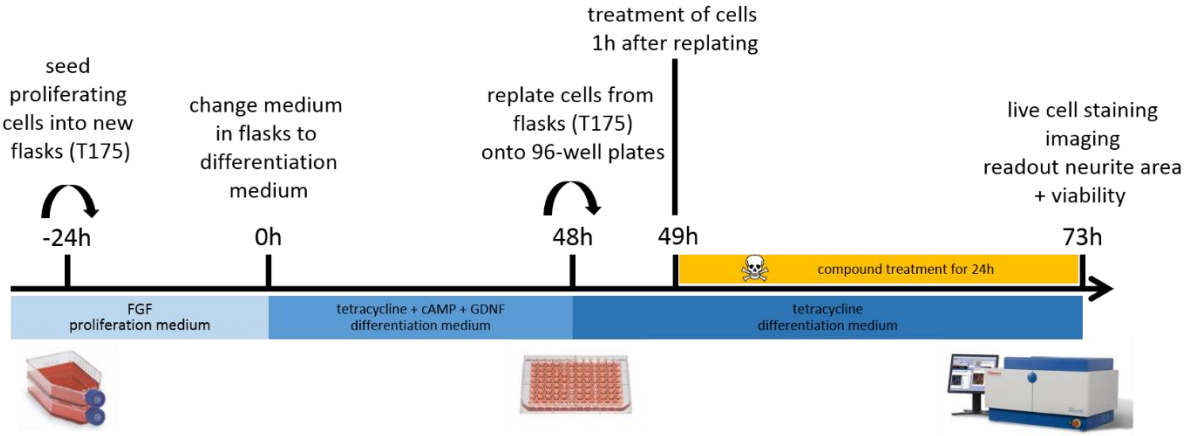


Fig. 4: Differentiation scheme and assay procedure including exposure scheme.

day -1: LUHMES cells are seeded in proliferation medium
day 0: Medium is changed from proliferation medium to differentiation medium
day 2: Cells number reaches about 30-40 Mio per T175 flask
Cells are trypsinized and replated onto 96-well plates (30'000 cells/well in 90 µl)
At about 30 min - 2 h after seeding when cells have attached, the compounds are added (10 µl of each dilution). **Toxicant exposure for 24 h from day 2 to day 3 of differentiation.**
day 3: 23.5 h after toxicant treatment, cells are stained with H-33342 and calcein-AM and incubated for 30 min. After 24 h the cells are imaged using a high-content microscope (Cellomics).

Endpoint(s) of the test method

Test endpoints: 1) neurite area (main endpoint)
2) cell number
3) % of viable cells (reference endpoint)

Overview of analytical method(s) to assess test endpoint(s)

Cells are stained with calcein-AM to mark viable cells. Co-staining with Hoechst H-33342 allows the identification of any cell.

Cells are stained for 30 min at 37°C and 5% CO_2 in the incubator.

The cell staining is imaged in a Cellomics Array Scan VTI HCS reader.
Hoechst H-33342 staining is imaged in channel 1 (UV-Hoechst); calcein staining is imaged in channel 2 (Green-FITC). Exposure times are set manually.

To measure the neurite area, the software acquires the Hoechst signal in channel 1 to identify the cells as objects (via identification of the nuclei), and the calcein-AM signal in channel 2 to measure neurite area. Double positive cells are counted as viable.

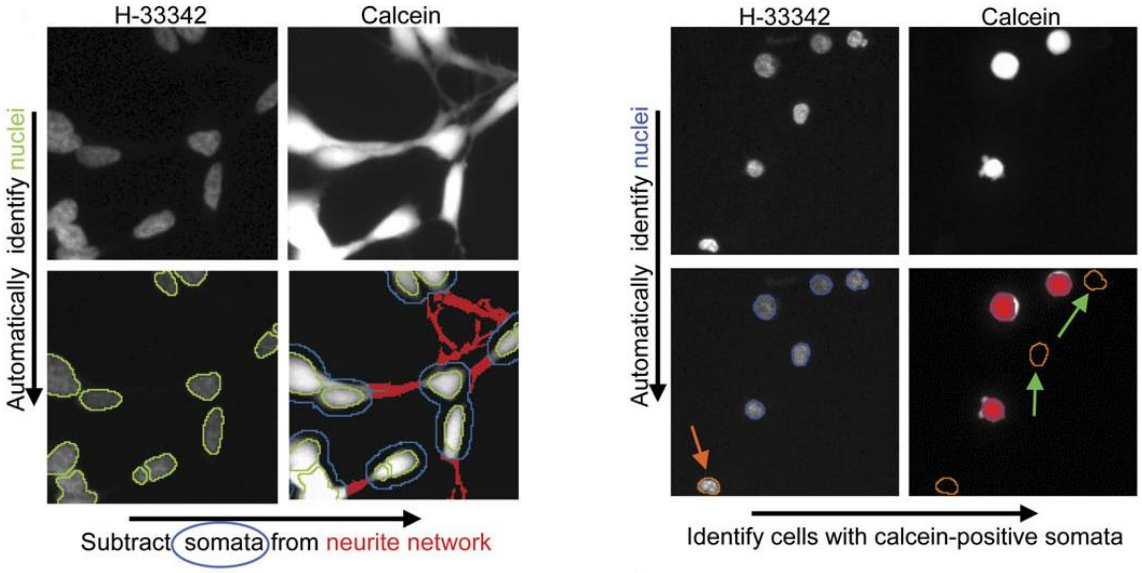


Fig. 5: Exemplification of object identification via automated algorithm in UKN4

Technical details (of e.g. endpoint measurements)

Quantification of neurite outgrowth
An automated microplate reading microscope (Array-ScanII HCS Reader, Cellomics, PA) equipped with a Hamamatsu ORCA-ER camera (resolution 1024 x 1024; run at 2 x 2 binning) was used for image acquisition. Ten fields per well were imaged. Images were recorded in 2 channels using a 20x objective and excitation/emission wavelengths of 365 ± 50/535 ± 45 to detect H-33342 in channel 1 and 474 ± 40/535 ± 45 to detect calcein in channel 2. In both channels, a fixed exposure time and an intensity histogram-derived threshold were used for object identification. Neurite pixels were identified using the following image analysis algorithm: nuclei were identified as objects in channel 1 according to their size, area, shape, and intensity which were predefined on untreated cells using a machine-based learning algorithm, and manual selection of nuclei to be classified as intact. The nuclear outlines were expanded by 3.2 µm in each direction, to define a virtual cell soma area (VCSA) based on the following procedure: The average width of the cytoplasm ring (distance nucleus - cell membrane) of LUHMES cells was experimentally determined to be 2.3 µm. Size irregularities were not always due to growing neurites, as determined by combined F-actin/tubulin beta-III staining. To avoid scoring of false positive neurite areas, the exclusion ring (VCSA) was made bigger than the average cell size. Then, we used two control compounds (U0126 and bisindolylmaleimid I) to vary the expanded outlines from 0.6 to 4 µm. We found 3.2 µm to be optimal both to detect neurite growth over time and to identify reduced neurite growth with high sensitivity. All calcein-positive pixels of the field (beyond a given intensity threshold) were defined as viable cellular structures (VCSs). The threshold was dynamically determined for each field after flat field and background correction and intensity normalization to 512 gray values and was set to 12% of the maximal brightness (channel 63 of 512). The VCS defines the sum of all

somata and neurites without their assignment to individual cells. In an automatic calculation, the VCSAs, defined in the H-33342 channel, were used as filter in the calcein channel and subtracted from the VCS. The remaining pixels (VCS - VCSA) in the calcein channel were defined as neurite area.

Quantification of individual viable cells by imaging

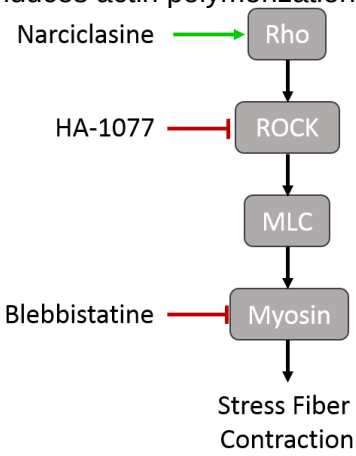
For a quantitative assessment of viable cells, the same images used to assess neurite area were analysed using another image analysis algorithm: nuclei were identified in channel 1 as objects according to their size, area, shape, and intensity. Nuclei of apoptotic cells with increased fluorescence were excluded. A VCSA was defined around each nucleus by expanding it by 0.3 µm into each direction. Calcein-AM staining, labelling live cells, was detected in channel 2. The algorithm quantified the calcein intensity in the VCSA areas. Cells having an average calcein signal intensity in the VCSAs below a predefined threshold were classified by the program as “not viable”. Valid nuclei with a positive calcein signal in their cognate VCSA were counted as viable cells. A positive calcein signal was based on measurements of the average intensity (normal cells: 1300 ± 115, threshold: < 50) and the total integrated intensity (normal cells: 186,000 ± 23,600, threshold < 1000) of cells.

Endpoint-specific controls / mechanistic control compounds (MCC)

Positive control for neurite growth inhibition:
Narciclasine: activates Rho

Positive control for neurite growth enhancement:
HA-1077: Rho-associated kinase inhibitor
Blebbistatine: inhibits myosin II

Rho/ROCK/LIM kinase/cofilin pathway:
induces actin polymerization, key regulator of the cytoskeleton and cell polarity



Positive controls

Positive control: narciclasine (50 nM final concentration)

Negative and unspecific controls

Negative control: solvent (0.1% DMSO final concentration), mannitol, paracetamol, aspirin, galloflavin.

Features relevant for cytotoxicity testing

Cells are highly sensitive to toxicants (Tong ZB 2016). Cell death is easily quantified, LDH release always shows very high baseline activity.

Acceptance criteria for the test method

Positive control narciclasine:
Neurite area ≤ 75% of DMSO control
Viability ≥ 90% of DMSO control (or not significantly changed)

Negative control DMSO:
Neurite area ≥ 35,000 pixels per well

Throughput estimate

Data point = one biological replicate (→ usually 3 technical replicates); each concentration/condition of a compound counts as data point
1200 data points per month
5 compounds per plate, 10 different concentrations of each compound per plate (see figure 6) → 50 data points (3 plates)
one plate correlates to one technical replicate → 3 plates for 3 technical replicates

6 plates can be done per day (correlates to 10 compounds → 100 data points)
→ 3 days of readout per week → 300 data points per week; 4 weeks per month → 1200 data points per month.

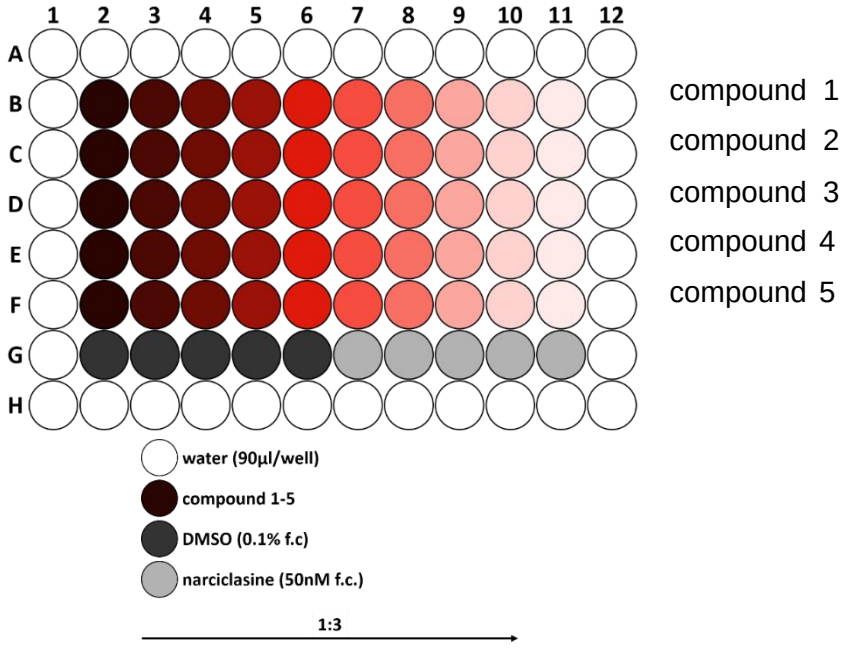


Fig. 6: Typical plate layout of the UKN4 test method

Replicate	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
N1	Direct Differentiation		Cell seeding and treatment	Live staining and Cellomics readout			
N2	Pre-Differentiation	Differentiation (change medium)		Cell seeding and treatment	Live staining and Cellomics readout		
N3					Pre-Differentiation	Differentiation (change medium)	
	Cell seeding and treatment	Live staining and Cellomics readout					

Fig. 7: Typical weekly work schedule of the UKN4 test method

Handling details of the test method

Preparation / addition of test compounds

- Compounds are stored according to the manufacturer's instructions (e.g. 4°C, room temperature, -20°C).
- Preferable solvent is DMSO. The used DMSO is stored in a lightproof, air-tight bottle at room temperature.
- Final DMSO concentration on the cells is 0.1%
- After dissolving the compounds, which are delivered in a solid/powder form, all compound solutions are aliquoted into volumes sufficient for one experiment (i.e. one biological replicate). In this way repeated freezing and thawing and therefore damaging the compound's stability and efficiency can be avoided.
- For conducting an experiment, a compound aliquot is thawn and diluted with 'DMEM/F12 Advanced' without supplement in a separate deepwell-plate.
- All compound dilutions in the deepwell plate contain 1% DMSO, so that a final concentration of 0.1% DMSO is reached on the cells. The highest compound concentration is diluted with medium 1:100 without DMSO as 1% is already reached with the DMSO the compound is solved in, the serial dilution is done with DMEM/F12 Advanced without supplement and 1% DMSO.
- The compound dilutions (10 µl each) are added to the cells using a multichannel-multistepper pipette, 6 filter tips at a time, dispense mode, speed for uptake is set to medium, speed for output is set to high. 40 µl are taken in and 10 µl are released on each plate.

Day-to-day documentation of test execution

Plate maps are defined prior to the experiment and documented in the lab book and files (Excel files) are stored on the work group server.
Concentrations and compound dilutions are calculated prior to the experiment.
Experimental procedures are noted manually in a paper lab book.

Practical phase of test compound exposure

The experimenter plans the experiment according to Cellomics microscope availability (has to be booked in advance) and availability of a sufficient number of cells.
Pipetting errors are marked directly on the plate maps and are documented in the lab book.
The paper lab book is taken to cell culture rooms and errors are documented in there right away.
The technical replicates were pipetted from left to right. Pipetting starts with the highest concentration at the left column.

Concentration settings

5 compounds per plate
As default a serial dilution 1:3 is used, i.e. a concentration range of 19683-fold is covered (e.g. from 20µM →

1nM).
Serial dilutions of compounds are prepared in a separate deepwell-plate, from which 10 µl are transferred to the according plates with attached cells using a multichannel-multistepper pipette. DMEM/F12 Advanced cell medium without supplements is used for dilution.
Dilution steps can be adapted to be more narrow (e.g. 1:1.5)

Uncertainties and troubleshooting

- Compound solubility in stock and during dilution is too low (stock solved in 100% DMSO, final concentration of the solvent on the cells is 0.1% DMSO)
- Some compounds show autofluorescence and interfere with the detection of calcein-AM or H-33342.
- To prevent negative edge effects, only the inner 60 wells of a 96-well plate are used and the edge wells were filled with PBS/MilliQ water.
- Focusing failure of Array Scan VTI HCS Reader (Cellomics, PA) can be a problem that produces outliers; as well as imaging only one channel.
- Highly trained/automated handling with multichannel and multistepper pipette is necessary to achieve little variance.
- Operators can get trained within 2-4 weeks. Cell seeding and medium change should be performed as fast as possible to keep cells as short as possible at room temperature. The more practice an operator has, the faster the critical steps can be performed.
- Substances are added when pipette tips are touching the wall of the wells right above the medium surface. When the substance solution is pipetted too high above the medium surface, the droplet may just stick to the wall of the well without flowing down into the medium.

Detailed protocol (SOP)

Protocol nº 200 in DB-ALM data base
http://cidportal.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/DBALM/LATEST/online/DBALM_docs/200_P_UKN4.pdf

Updated SOP can be made available by laboratory upon request:
marcel.leist@uni-konstanz.de

Special instrumentation

The method requires a Cellomics Array Scan VTI HCS high content reader that may not be present in the standard lab.
Alternative automated microscopes and software tools to perform neurite outgrowth assays exist and the method might be transferred in the future.

Possible variations

a) further additional endpoints:
- metabolic activity (resazurin reduction)
- glutathione levels
- staining of tubulin
- analysis of differentiation markers by qPCR or immunostaining

b) other analytical endpoints:
cell viability by:

- fluorimetric measurement of resazurin conversion
- measurement of extracellular LDH
- measurement of luminescence indicating ATP content

c) other exposure:
- compound can be washed out → acquisition on day 4
- longer exposure is possible
- later exposure is possible (from day 5 on) in order to measure more mature neurite networks
- the medium can be changed to contain galactose instead of glucose. This increases the sensititivity of the cells to inhibitors of mitochondrial respiration (Delp et al., 2019)

d) variants for recording of neurite growth:
- neurite growth by GFP-labelled cells (Schildknecht S 2013)

Cross-reference to related test methods

UKN3a assay to test compound-derived neurite integrity impairment in human mature dopaminergic neurons after long-term compound exposure - Protocol no 202 in DB-ALM
UKN3b assay to test compound-derived neurite integrity impairment in human mature dopaminergic neurons - Protocol no 196 in DB-ALM

Data management

Raw data format

Raw data is extracted by copy-paste in Excel files (example file available).
Data from all technical replicates are collected in one file.

Outliers

1. Mathematical procedures to define outliers have not been defined. Data points that are far off (i.e. more than the known endpoint variability which would be 25% for neurite outgrowth) are discarded. Biological outliers do practically not exist, most far data points are the result of technical problems (focus not found, only one channel imaged, etc.)
2. All raw data (incl. outliers) are stored.
3. Technical outliers make up 1-0.1%.

Raw data processing to summary data

- Array Scan VTI HCS Reader (Cellomics, PA) takes images (optionally bitmap or tiff-format; 512 x 512 pixels, 8bit or 16bit)
- Images are locally analyzed using the Array Scan software, algorithms quantify neurite area and cell count (nuclei)
- data are copy-pasted into an Excel sheet, further analysis is done with Excel + KNIME + GraphPad Prism and BMCeasy.

Curve fitting

The data are analyzed with Excel and represented with GraphPad Prism.

For the concentration curve, a nonlinear regression fit is calculated. The fitting method is least squares. If a non-linear curve fit is not possible, a linear curve fit is performed. The curve deriving from the fit is a 4-parameter log function. To calculate the EC50 value, this log-function is solved for y=50% of the total scale, not for 50% of the min-max scale (see example below). Treated concentrations are analyzed for deviation from control. Sometimes it is analyzed whether the deviation of neurite growth is different from the deviation of viability via comparison of the means ± deviation using two-way ANOVA + Tukey-Kramer post hoc testing. Significance levels of data points compared to solvent control are determined via one-way ANOVA followed with non-parametric Dunnett's post test.

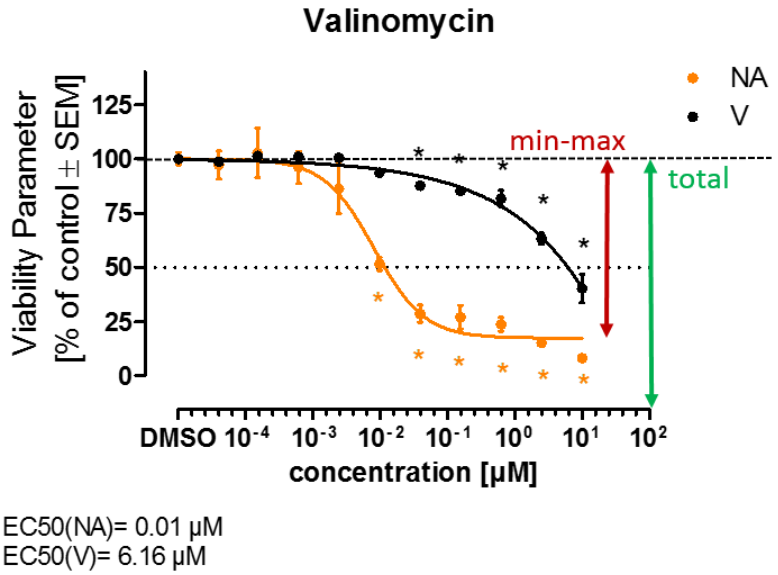


Fig. 8: Exemplification of curve fit and relative effect responses

NA = neurite area, V = viability

BMC values with their upper and lower confidence intervals (BMCU and BMCL) are calculated via the publically available online software:
http://invitrotox.uni-konstanz.de/BMCeasy/

Internal data storage

The data are firstly stored on the microscope computer and then exported to other servers (lab group server and university server), which are back-upped regularly.

Metadata

The metadata are documented, stored and exported as text document (log)-files to the according scheme: (local PC)_descriptor(date and time)_XXX.log:
The following metadata are stored:

- cellinsight-pc_160429130003_AutomationControllerIni
- cellinsight-pc_160429130003_kineticprotocol
- cellinsight-pc_160429130003_protocol
- cellinsight-pc_160429130003_scan

- cellinsight-pc_160429130003_ScanIni
- cellinsight-pc_160429130003_spooling
- cellinsight-pc_160429130003.spooled

Metadata file format

Metadata files are available.

Prediction model and toxicological application

Scientific principle, test purpose and relevance

LUHMES cells used in this test method represent cells of the central nervous system with a dopaminergic phenotype.
The UKN4 test method models neurite outgrowth as a biological process and assesses viability of the cells in parallel. The cells are used in an early developmental stage (day 2 of differentiation) and chemical exposure occurs during this development. Therefore, the UKN4 test method assesses hazards for developmental neurotoxicity. It can be integrated into adverse outcome pathways as an important key event to predict potential adverse outcomes in humans.

Prediction model

Three different models are used:

1. prediction model for screening:
hit = decrease/increase in neurite area while viability is not changed (compare to narciclasine positive control):
Neurite area ≤ 80% of DMSO control
(For early screening tiers 80% can be used as a more conservative filter than 75%)
Viability ≥ 90% of DMSO control

2. prediction model for compound hazard evaluation:
hit confirmation testing; BMC25 Viability (V) / BMC25 Neurite Area (NA) ≥ 4 → specifically neurotoxic
3. prediction model for borderline compounds:
A ratio of BMC25 Viability (V) / BMC**L**25 Neurite Area (NA) ≥ 4 is considered a borderline hit. In some scenarios the viability does not reach the BMC25 necessary for the ratio calculations. In this case the highest tested concentration (HTC) was used. Schematic representation of the complete prediction model is shown in Fig. 9 below.

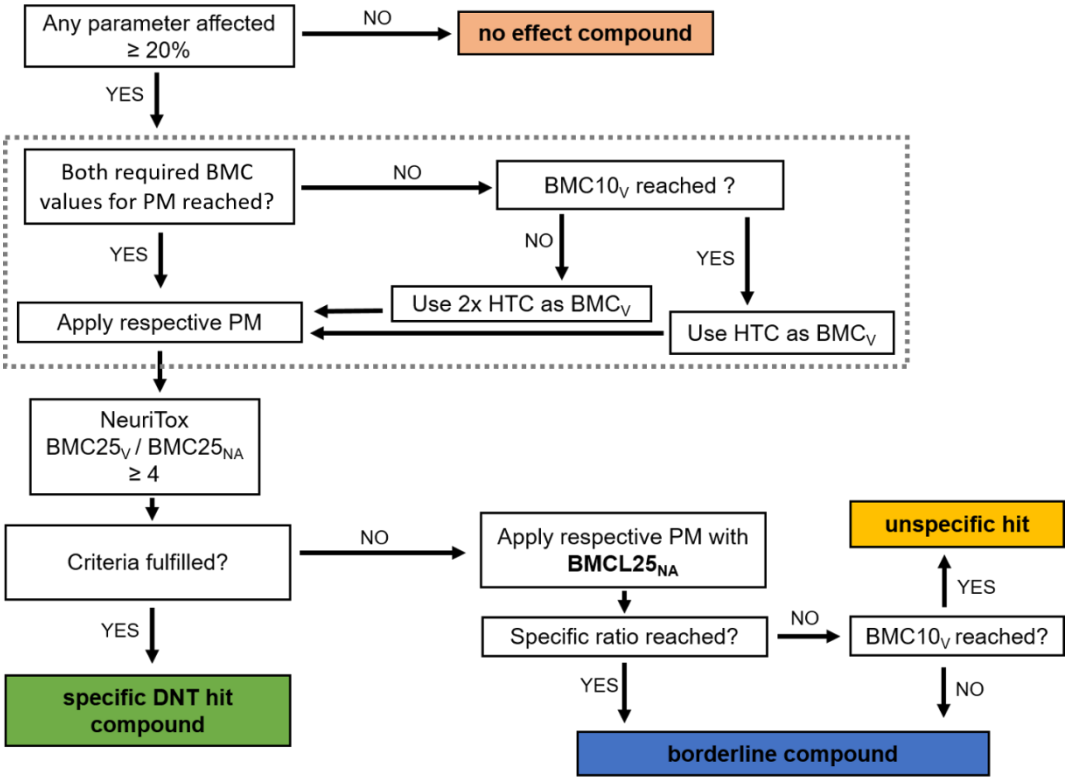


Fig. 9: UKN4 prediction model classification tree

A full overview with a schematic representation of the UKN4 prediction model can also be found in supplementary figure 3 of Blum et al., 2022.

Prediction model setup

a) The prediction model was established using the following compounds (Stiegler 2011; Krug 2013):
-colchicine
-vincristine
-nocodazole
➔ Positive controls
-etoposide
-buthionine sulfoximine (BSO)
➔ Unspecific toxicants affecting general viability
-cycloheximide
-paraquat
➔ Rules of assay interpretation, criteria to define a positive test result
- carbonyl cyanide 3-chlorophenylhydrazone (CCCP)
- 2,4-dinitrophenol (2,4-DNP)
- Sodium dodecyl sulfate (SDS)
-tween-20
- potassium chromate (K2CrO4)
- Hoechst (H-33352)
- tertiary butyl hydroperoxide (tBuOOH)

➔ Unspecific toxicants

b) The prediction model has been applied to screen 80 compound library of NTP (Delp et al., 2018). The prediction model including the borderline classification has been applied to screen a 120 compound library (Blum et al., 2022; Masjosthusmann et al., 2020).

c) The process is documented in Krug et al., 2013 and Stiegler et al., 2011

d) Sensitivity/ specificity have not been defined due to a lack of reference compounds. Below, a set of compounds that triggers specific inhibition of neurite growth is shown (light blue). Other compounds are cytotoxic without specific effects on neurites (orange).

The use of the updated prediction model
BMC25 Viability (V) / BMC25 Neurite Area (NA) ≥ 4
was validated by comparing classifications derived by the initial and the updated prediction model. The reason for updating the prediction model was that a decrease of 50% in neurite area and viability cannot always be achieved with our range of test concentrations. An effect of 25% is more often observed and came to similar results when applied (Delp et al., 2018).

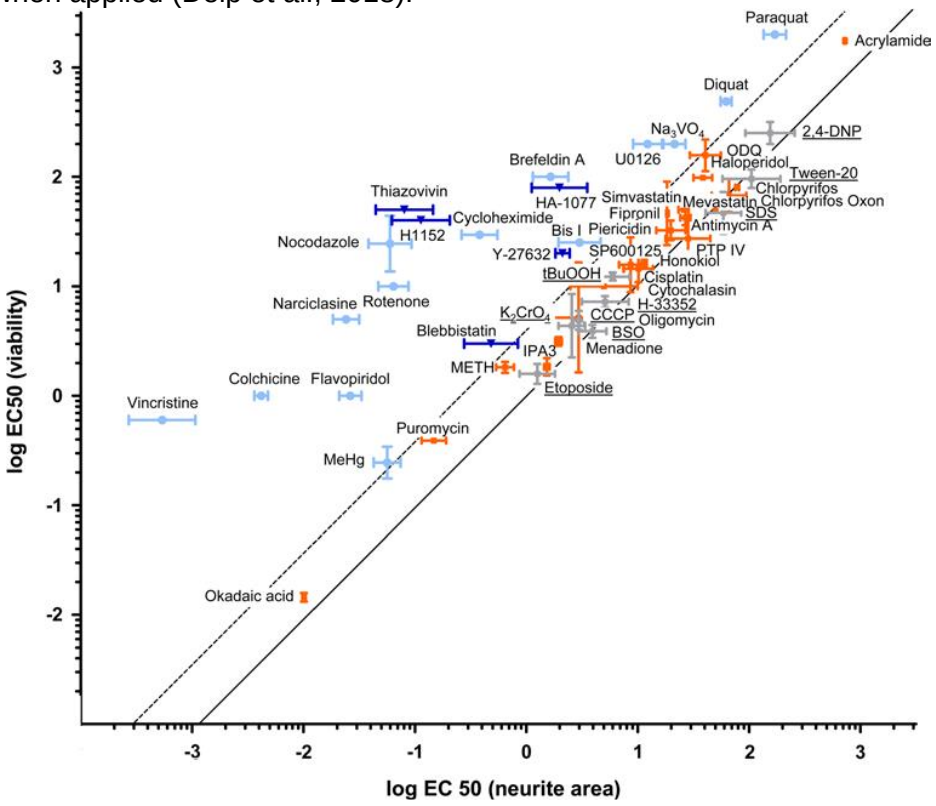


Fig. 10: Compound set tested to establish prediction model in UKN4 (from Krug et al., 2013)

Test performance

Some background on the test performance is given in chapters 8.2/8.3 (prediction model).

Several performance parameters for the test were obtained in several separate evaluation rounds.

A first evaluation was done during the first publication of the model and its applications (Stiegler et al. 2011,

Krueg et al. 2013). Here, a panel of well-selected positive and negative controls have been tested. Accordingly, the specificity was 100% and the sensitivity was > 90 %. In dedicated experiments, S/N ratios of > 20 and a z' of > 0.5 have been determined. The compound narciclasine (run on each plate as positive control) was tested across 36 different test plates and 12 independent assay runs. The neurite area relative to the solvent control varied between 40% and 75% across all plates with a viability value constantly >90% (Delp et al. 2018).

The test has been used in screening campaigns, and real-live performance data under broader screen conditions have been obtained. The different performance data need to be considered, when a compound is a hit in a screen, or whether it has been specifically evaluated in a hit follow-up or a mechanistic project.

A first screen application has been the NTP80 screen (80 compounds provided by the US NTP). Data are published Delp et al. 2018.

A second screen application has been the cross systems case study of the EU-ToxRisk project. The baseline variation is indicated in Krebs et al., 2020. Moreover, an overview is given for 19 compounds on the BMC/BMCL ratio as measure of readout certainty.

A third screen was performed in the context of the EFSA DNT test battery evaluation with 120 compounds (Blum et al., 2022; Masjosthusmann et al. 2020). From this screen the following performance indicators were obtained:

A: **Specificity of DNT IVB: 100%**

→ Also ith NeuriTox as standalone assay in 17 `tool negatives´ tested (Masjosthusmann et al. 2020).

B: **Sensitivity of DNT IVB: 82.7%**

→ With NeuriTox combined in a full DNT battery and a selected set of 27 positive compounds with evidence for DNT (Masjosthusmann et al. 2020).

C: **Baseline variation (intra-experimental)**

Neurite area: 11.7 ± 4.5%

Cell viability: 3.8 ± 3.5%

D: **Baseline variation (inter-experimental)**

Neurite area: 15.3%

Cell viability: 4.3%

E: **Variation of a positive control run on each (inter-experimental)**

Neurite area: 38.5%

Definition of values C-E

C: **Baseline variation (intra-experimental)** is the mean coefficient of variation CV±SD of the CV of all

replicates of the solvent control from each experiment across n>200 experiments.

D: **Baseline variation (inter-experimental)** is the variability across all independent experiments (n>200) after normalization based on the response of the lowest test concentration. It was assumed that the lowest test concentration does not affect any of the endpoints measured.

E: **Variation of a positive control run on each (inter-experimental)** is the variability of the positive control across all independent experiments (n>40) after normalization. Example for a positive control that on average reduced the specific endpoint down to 40% (relative to solvent control) and a calculated variability of 50%: 0.5 x 40% = +/- 20% → The positive control with mean of 40% varies from 20% to 60%.

In vitro – in vivo extrapolation (IVIVE)

1. Estimated lipid content and albumin concentration in in vitro test media and human plasma:

Medium	Lipid content (mg/l)	Albumin concentration (µM)
UKN4	2.9	5.8
Human plasma	6000	600

this information can be used to calculate from nominal to free concentrations of compounds tested (https://doi.org/10.1007/s00204-020-02802-6))

2. The test has not been used extensively for IVIVE. However, data from the cell model (but different exposure scheme) have been used for IVIVE modelling (Loser et al., 2021, b). The test has also been used in projects with potency estimates and dose estimates (Klose et al., 2021; van der Stel et al., 2021).
3. No special considerations known.

Applicability of test method

Test is sensitive to cytoskeletal toxicants, some signaling modifiers and flame retardants. Polycyclic aromatic hydrocarbons (PAH) and HDAC inhibitors have no effect.

Incorporation in test battery

a) Strengths:

- Medium to high throughput (The term “medium to high throughput” is derived from a comparison and in relation to a number of other known DNT NAMs)
- Automated microscopy

b) compared to UKN5 (which quantifies neurite outgrowth of peripheral neurons), UKN4 measures neurite outgrowth specifically of CNS dopaminergic neurons. This was shown by treatment with MPP^+, which is transported by the dopamine transporter (DAT) and had an effect in UKN4, but not UKN5 peripheral neurons, which lack the DAT transporter.
c) specific effects on central nervous system dopaminergic neurons. Implementation in a DNT battery was investigated (Blum et al., 2022; Masjosthusman et al., 2020).
d) Preferential use in first tier, no complementary assays required. Fast cell supply compared to other potential DNT battery assays (cells ready to treat after 2 days) + short assay time (24 h) + number of data points possible to generate in one run make this assay a potential “pre-screen/first tier”.

Publication / validation status

Availability of key publications

“Establishment of a human cell-based in vitro battery to assess developmental neurotoxicity hazard of chemicals.”
Blum, J. et al. Chemosphere, 2022. PMID: 36328314

“Progressive degeneration of human mesencephalic neuron-derived cells triggered by dopamine-dependent oxidative stress is dependent on the mixed-lineage kinase pathway.”
Lotharius J, Falsig J, van Beek J, Payne S, Dringen R, Brundin P, Leist M.
J Neurosci. 2005 Jul 6;25(27):6329-42. DOI: 10.1523/JNEUROSCI.1746-05.2005. PMID: 16000623

“Rapid, complete and large-scale generation of post-mitotic neurons from the human LUHMES cell line.”
Scholz D, Pöltl D, Genewsky A, Weng M, Waldmann T, Schildknecht S, Leist M.
J Neurochem. 2011 Dec;119(5):957-71. DOI: 10.1111/j.1471-4159.2011.07255.x. PMID: 21434924

“Assessment of chemical-induced impairment of human neurite outgrowth by multiparametric live cell imaging in high-density cultures.”
Stiegler NV, Krug AK, Matt F, Leist M.
Toxicol Sci. 2011 May;121(1):73-87. DOI: 10.1093/toxsci/kfr034. PMID: 21342877

"Control of Abeta release from human neurons by differentiation status and RET signaling."
Scholz, D., Y. Chernyshova and M. Leist
Neurobiol Aging. 2013 Jan;34(1):184-99. DOI: 10.1016/j.neurobiolaging.2012.03.012. PMID: 22534065

“Evaluation of a human neurite growth assay as specific screen for developmental neurotoxicants.”
Krug AK, Balmer NV, Matt F, Schönenberger F, Merhof D, Leist M.
Arch Toxicol. 2013 Dec;87(12):2215-31. DOI: 10.1007/s00204-013-1072-y. PMID: 23670202

“Generation of genetically-modified human differentiated cells for toxicological tests and the study of neurodegenerative diseases.”
Schildknecht S, Karreman C, Pöltl D, Efrémova L, Kullmann C, Gutbier S, Krug A, Scholz D, Gerding HR, Leist M.
ALTEX. 2013;30(4):427-44. DOI: 10.14573/altex.2013.4.427. PMID: 24173167

“A LUHMES 3D dopaminergic neuronal model for neurotoxicity testing allowing long-term exposure and cellular resilience analysis.”
Smirnova L, Harris G, Delp J, Valadares M, Pamies D, Hogberg HT, Waldmann T, Leist M, Hartung T.
Arch Toxicol. 2015 Dec 8. DOI: 10.1007/s00204-015-1637-z. PMID: 26647301

"Reduced Abeta secretion by human neurons under conditions of strongly increased BACE activity."
Scholz, D., Y. Chernyshova, A. K. Uckert and M. Leist
J Neurochem. 2018 Oct;147(2):256-274. DOI: 10.1111/jnc.14467. PMID: 29804308

“Major changes of cell function and toxicant sensitivity in cultured cells undergoing mild, quasi-natural genetic drift.”
Gutbier S, May P, Berthelot S, Krishna A, Trefzer T, Behbehani M, Efremova L, Delp J, Gstraunthaler G, Waldmann T, Leist M.
Arch Toxicol. 2018 Dec;92(12):3487-3503. DOI: 10.1007/s00204-018-2326-5. PMID: 30298209

“A high-throughput approach to identify specific neurotoxicants/ developmental toxicants in human neuronal cell function assays.”
Delp J, Gutbier S, Klima S, Hoelting L, Pinto-Gil K, Hsieh JH, Aichem M, Klein K, Schreiber F, Tice RR, Pastor M, Behl M, Leist M.
ALTEX. 2018;35(2):235-253. DOI: 10.14573/altex.1712182. Erratum in: ALTEX. 2019;36(3):505. PMID: 29423527

“Stage-specific metabolic features of differentiating neurons: Implications for toxicant sensitivity.”
Delp J, Gutbier S, Cerff M, Zasada C, Niedenführ S, Zhao L, Smirnova L, Hartung T, Borlinghaus H, Schreiber F, Bergemann J, Gätgens J, Beyss M, Azzouzi S, Waldmann T, Kempa S, Nöh K, Leist M.
Toxicol Appl Pharmacol. (b) 2018 Sep 1;354:64-80. DOI: 10.1016/j.taap.2017.12.013 Erratum in: Toxicol Appl Pharmacol. 2019 Jun 1;372:70. PMID: 29278688

"Major changes of cell function and toxicant sensitivity in cultured cells undergoing mild, quasi-natural genetic drift."
Gutbier, S., P. May, S. Berthelot, A. Krishna, T. Trefzer, M. Behbehani, L. Efremova, J. Delp, G. Gstraunthaler, T. Waldmann and M. Leist
Arch Toxicol. 2018 Dec;92(12):3487-3503. DOI: 10.1007/s00204-018-2326-5. PMID: 30298209

"Development of a neurotoxicity assay that is tuned to detect mitochondrial toxicants."
Delp, J., M. Funke, F. Rudolf, A. Cediel, S. H. Bennekou, W. van der Stel, G. Carta, P. Jennings, C. Toma, I. Gardner, B. van de Water, A. Forsby and M. Leist
Arch Toxicol. 2019 Jun;93(6):1585-1608. DOI: 10.1007/s00204-019-02473-y. PMID: 31190196

"Incorporation of stem cell-derived astrocytes into neuronal organoids to allow neuro-glial interactions in toxicological studies."
Brull, M., A. S. Spreng, S. Gutbier, D. Loser, A. Krebs, M. Reich, U. Kraushaar, M. Britschgi, C. Patsch and M. Leist
ALTEX. 2020 Mar; 37(3): 409-428. DOI:10.14573/altex.1911111. PMID: 32150624

"Establishment of an a priori protocol for the implementation and interpretation of an in-vitro testing battery for the assessment of developmental neurotoxicity."
Masjosthusmann, S., J. Blum, K. Bartmann, X. Dolde, A.-K. Holzer, L.-C. Stürzl, E. H. Keßel, N. Förster, A. Dönmez, J. Klose, M. Pahl, T. Waldmann, F. Bendt, J. Kisitu, I. Suciu, U. Hübenthal, A. Mosig, M. Leist and E. Fritsche
EFSA Supporting Publications. 2020; 17(10): 1938E.
https://doi.org/10.2903/sp.efsa.2020.EN-1938

"Human neuronal signaling and communication assays to assess functional neurotoxicity."
Loser, D., J. Schaefer, T. Danker, C. Moller, M. Brull, I. Suciu, A. K. Uckert, S. Klima, M. Leist and U. Kraushaar
Arch Toxicol. 2021 Jan;95(1):229-252. DOI: 10.1007/s00204-020-02956-3. PMID: 33269408

"Neurotoxicity and underlying cellular changes of 21 mitochondrial respiratory chain inhibitors."
Delp, J., A. Cediel-Ulloa, I. Suciu, P. Kranaster, B. M. van Vugt-Lussenburg, V. Munic Kos, W. van der Stel, G. Carta, S. H. Bennekou, P. Jennings, B. van de Water, A. Forsby and M. Leist
Arch Toxicol. 2021 Feb;95(2):591-615. DOI: 10.1007/s00204-020-02970-5.PMID: 33512557

"Neurodevelopmental toxicity assessment of flame retardants using a human DNT in vitro testing battery."
Klose, J., M. Pahl, K. Bartmann, F. Bendt, J. Blum, X. Dolde, N. Forster, A. K. Holzer, U. Hubenthal, H. E. Kessel, K. Koch, S. Masjosthusmann, S. Schneider, L. C. Sturzl, S. Woeste, A. Rossi, A. Covaci, M. Behl, M. Leist, J. Tigges and E. Fritsche

Cell Biol Toxicol. 2021 May 10. DOI: 10.1007/s10565-021-09603-2. PMID: 33969458

"Functional alterations by a subgroup of neonicotinoid pesticides in human dopaminergic neurons."
Loser, D., M. G. Hinojosa, J. Blum, J. Schaefer, M. Brüll, Y. Johansson, I. Suciu, K. Grillberger, T. Danker, C. Möller, I. Gardner, G. F. Ecker, S. H. Bennekou, A. Forsby, U. Kraushaar and M. Leist
Arch Toxicol. (b) 2021 Jun;95(6):2081-2107. DOI: 10.1007/s00204-021-03031-1. PMID: 33778899

"New approach methods (NAMs) supporting read-across: Two neurotoxicity AOP-based IATA case studies."
Van der Stel, W., G. Carta, J. Eakins, J. Delp, I. Suciu, A. Forsby, A. Cediel-Ulloa, K. Attoff, F. Troger, H. Kamp, I. Gardner, B. Zdrazil, M. J. Mone, G. F. Ecker, M. Pastor, J. C. Gomez-Tamayo, A. White, E. H. J. Danen, M. Leist, P. Walker, P. Jennings, S. Hougaard Bennekou and B. Van de Water
ALTEX. 2021;38(4):615-635. DOI: 10.14573/altex.2103051. PMID: 34114044

(Potential) linkage to AOPs

Test method could be potentially linked to the following AOPs in AOPwiki:

- AOP 48 : Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.
  → Organ effects: Neurodegeneration, decreased neuronal network function
  → Organism effects: Impairment of learning and memory
- AOP 13: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities.
  → Organ effects: Decreased neuronal network function
  → Organism effects: Impairment of learning and memory
- AOP 3: Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits.
  → Organ effects: degeneration of DA neurons of nigrostriatal pathway
- AOP 42: Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals
  → Adverse Outcome: Cognitive function decreased
- AOP 54: Inhibition of Na+/I- symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children.
  → Organ effects: decreased neuronal network function
  → Organism effects: learning and memory deficits
- AOP 8: Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals.
  → Adverse Outcome: Altered neurodevelopment
- AOP 134: Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals.
  → Adverse Outcome: Cognitive function decreased

Steps towards mechanistic validation

a) LUHMES are dopaminergic, express DAT, TH. Are of human origin, form network
b) Tubulin plays a major role in neurite outgrowth and if inhibited by colchicine/vincristine/nocodazole neurite outgrowth is reduced. If the Rho/Rock pathway is activated, neurite outgrowth is enhanced.
c) A formal mechanistic validation has not been performed. Reversibility and protection/counterregulation by mechanistic compounds have been shown (Stiegler 2011; Krug AK).
d) The test covers a fundamental neurodevelopmental process. In some contexts, this might be seen as a

key event of an AOP (Smirnova 2014, Bal-Price 2015 (ISTNET))

Pre-validation or validation

To date, 143 unique compounds (as defined by unique DTXSIDs) have been tested successfully in this assay. No formal OECD 34 validation study has been done (e.g., ring trials with a standard set of known positive and negative controls).
In total, >200 different compounds were tested in the NeuriTox assay. The test method was developed using a compound training set (Krug et al. 2013). It was used for an 80 compound screening library from the US National Toxicology Program (NTP) (Delp et al. 2018). The test method was part of a DNT hazard assessment for 120 compounds in a DNT testing battery. The later compound set includes potential DNT positive and DNT negative compounds (Masjosthusmann 2020).

Linkage to (e.g. OECD) guidelines / regulatory use

Test is not linked to regulatory guidelines.

Test method transferability

Operator training

Experiences are required in:
- LUHMES cell culture
- multichannel/multistep pipetting
- handling of Array Scan VTI HCS Reader (Cellomics, PA) and its software
- Microsoft Excel
- KNIME
- GraphPad Prism

Operator is trained and guided by a highly experienced instructor. Approximately 4 weeks will be needed for a smooth assay performance.

Transfer

Test system (UKN LUHMES cells) has been transferred and established to numerous other labs. The NeuriTox assay has been successfully transferred to one other lab for tool compound testing and data comparison.

Safety, ethics and specific requirements

Specific hazards; issues of waste disposal

No specific requirements.

Safety data sheet (SDS)

SDS are available in the university DaMaRIS database (**Da**ngerous **Ma**terials **R**egistry **I**nformation **S**ystem).

Specific facilities / licenses

Work requires S1 cell culture laboratories (genetically modified cells).
No specific facilities are required.
No specific ethical approval is required.

Commercial aspects / intellectual property of material / procedures

To our best knowledge, no elements needed to conduct the experimental part of the test method are protected. Programs used to conduct the analysis of the data (Microsoft Excel and GraphPad Prism) need to be purchased or obtained by license agreement, however data analysis and plotting can be done with other, freely available tools.

Appendix B.5

Author: Jonathan Blum, Marcel Leist
Date: 17.03.2023
Version: 220428_v2

Overview

Descriptive full-text title

Assay to test compound-derived impairment in neurite outgrowth in human iPSC-derived immature dorsal root ganglia (iDRG) neurons (PeriTox; UKN5) – V2.0

Abstract

This in vitro test method is based on human iPSC-derived immature dorsal root ganglia (iDRG) neurons at a stage of neurite growth. It assesses (a) disturbances in the development of the (peripheral) nervous system, and (b) direct damage to the peripheral nervous system, by exposure to toxicants. The neurite area (which serves as indirect measurement of neuronal interconnectivity) of stained differentiating neurons, as well as cellular viability are measured simultaneously using high content imaging. The processes of neurite outgrowth and cell death are measured. The data of this method are meant to predict (a) developmental disorders in children caused by compound exposure during fetal development, and (b) damage to the developed nervous system, in particular to the peripheral nervous system. The method has not undergone formal validation and has not been part of a ring trial. It predicts some aspects of neurotoxicity, but not all aspects covered by an in vivo neurotoxicity study (TG426). It has been used in the screening of medium-sized compound libraries, has undergone some mechanistic evaluation, and has been linked to AOP-279 (AOPwiki ID) / ETR09N (EU-ToxRisk AOP task ID) (Peripheral neuropathy caused by microtubule interacting drugs). According to the readiness criteria as published by Bal-Price et al. (2018) the PeriTox assay obtained the readiness score A-
.

General information

Name of test method

PeriTox test, UKN5

Version number and date of deposition

This is Version 2.0 of the protocol “Assay to test compound-derived impairment in neurite outgrowth in human iPSC-derived immature dorsal root ganglia (iDRG) neurons (PeriTox; UKN5) – V2.0)”. It was assembled and deposited in March 2023. A previous version was assembled in 2019 in the context of the EU-ToxRisk project (see publication Krebs et al., 2020).

Summary of introduced changes in comparison to previous version(s)

Changes compared to V1 refer mainly to the generation of the test system and the cell line used. Test procedures and parameters remain unchanged.

Assigned data base name

UKN5_DART_iDRG_24h_02

Name and acronym of the test depositor

University of Konstanz (UKN), Germany

Name and email of contact person

Prof. Dr. Marcel Leist
marcel.leist@uni-konstanz.de
Tel: +49-7531885037

Name of further persons involved

Anna-Katharina Holzer (PhD student, experimenter)
anna-katharina.holzer@uni-konstanz.de

Jonathan Blum (PhD student)
jonathan.blum@uni-konstanz.de

Reference to additional files of relevance

- An important reference is the DB-ALM Protocol n° 218 The original iPSC are in the meantime cultured feeder-free (see 3.2)
- Raw data file
- Data processing file

Description of general features of the test system source

Supply of source cells

The human induced pluripotent stem cell (hiPSC) line EPITHELIAL-1 has been bought from Sigma-Aldrich, Germany in 2018 and a masterstock has been frozen. From the masterstock several working stocks have been prepared. The working stocks are regularly thawed and can be continously maintained due to self-renewal and pluripotency capabilities of the cells. The cells are maintained up to 8 passages before a new vial of the working stock is thawed.

Overview of cell source component(s)

The human induced pluripotent stem cell line iPSC EPITHELIAL-1 (Cat# IPSC0028) is purchased from Sigma-Aldrich, Taufkirchen, Germany as a frozen suspension of single cells. iPSC EPITHELIAL-1 cells are produced via reprogramming of epithelial cells from a Caucasian female (24 years) using OSKM retrovirus. Pluripotency was certified by gene and protein expression of pluripotency markers.
The maintenance culture is cultured in colonies under feeder-free conditions on Laminin-521 coating in Essential 8 (E8) medium. The cells are split weekly.

Characterization and definition of source cells

- **Tissue**: epithelium
- **Gender**: female
- **Culture properties:** adherent
- **Disease:** no disease was diagnosed
- **Age:** 24 year old
- **Ethnicity:** Caucasian
- **Expression:** iPSC EPITHELIAL-1 express all expected pluripotency markers, such as OCT4, NANOG, SSEA4 and SOX1.
- **Short tandem repeat (STR) analysis:** confirms, at least 80% loci homology of observed

STR loci	Data-sheet (Sigma/ Merck)		wt (our data)	
TH01	9	9.3	9	9.3
D5S818	12	13	12	13
D13S137	8	12	8	12
D7S820	8	11	8	11
D16S539	11	12	11	12
SCF1P0	10	12	10	12
AMEL	X	X	X	X
vWA	16	16	16	16
TPOX	8	9	8	9
D3			15	16
D21			28	30
D18			13	16
PentaE			7	10
Penta D			8	11
D8			10	14
FGA			22	25
D19			13	13
D2			17	20

Figure 1: STR analysis of Sigma iPSC0028 (from Holzer et al., 2022a)

Acceptance criteria for source cell population

The cells have to be pathogen-free to be used in further experiments (regular testing for mycoplasma). The iPSC maintenance is regularly checked for expression of pluripotency markers (Oct4, Nanog, Tra-1-60) by immunocytochemistry.

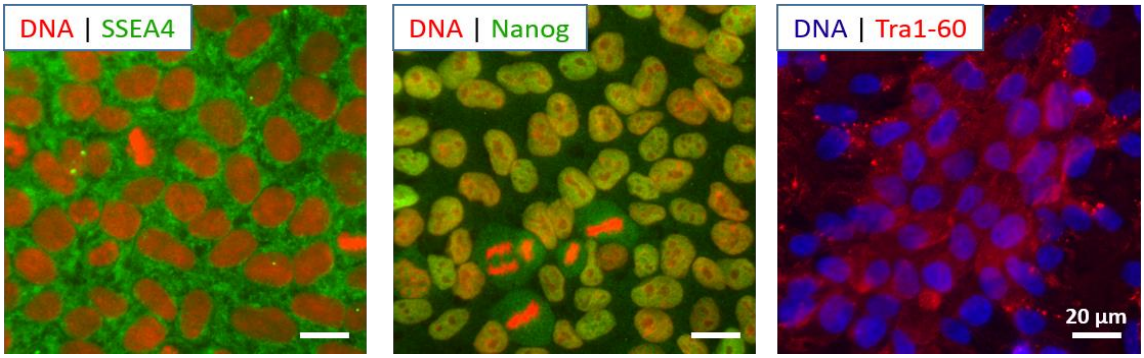


Figure 2: Pluripotency marker expression in iPSC checked via immunostaining

The cells should grow in colonies with sharp edges, no spontaneously differentiated cells should be visible. Stem cells are split every 5-7 days whenever they reach >80% confluency. The cells can be used for differentiation from passage 2 on until passage 8.

Variability and troubleshooting of source cells

- hiPSC can be maintained up to 8 passages, high passage number might influence performance of cells
- Too little or too high cell density leads to detachment of cells or spontaneous differentiation
- If cells start to differentiate, cells should be discarded immediately
- Cells have to be maintained as colonies and not as single cells. Therefore splitting should be performed as fast as possible, iPSC have to be detached and seeded as clumps. Avoid single cells.
- Plastic coating is critical for even cell distribution; problems with coating often leads to cell detachment, especially at the edges of culture dishes
- Batch effects of critical additives (e.g. holo-transferrin for iron supply of cells or TGF-β which maintains cell pluripotency) can lead to differentiation of cells at low passage number

Differentiation towards the final test system

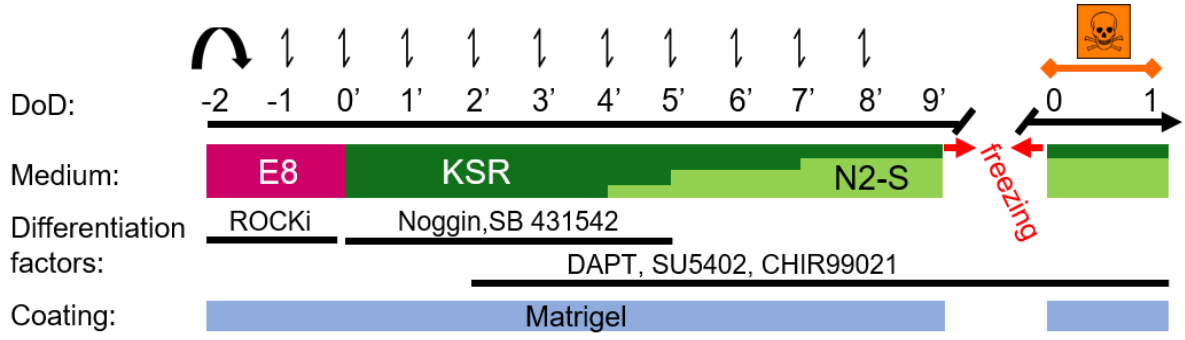


Figure 3: UKN5/PeriTox differentiation scheme

Culture is essentially as described in Hoelting et al. (2016) with minor changes detailed in Holzer et al. (2022a) and Holzer et al. (2022b).
Medium components:

25% KSR:
Knockout DMEM with
15 % knockout serum replacement,
2 mM Glutamax,
0.1 mM MEM non-essential amino acids and
50 µM beta-mercaptoethanol

75% N2:
DMEM/F12 medium
1 % Glutamax
1.55 mg/ml glucose
0.1 mg/ml apotransferrin
25 µg/ml insulin
100 µM putrescine
30 nM selenium
20 nM progesterone)

External SOP document is available and published in Holzer et al. (2022a).

Neural differentiation:

The human pluripotent stem cell line EPITHELIAL-1 is prepared for neural differentiation on day of differentiation minus 2 (DoD -2) by replating the pluripotent stem cells in a single cell suspension onto Matrigel coated plates in Essential 8 (E8) medium. This E8 is freshly supplemented with 10 ng/ml Rock inhibitor Y-27632.

On DoD0', neural differentiation is started by adding neural differentiation medium KSR and the combination of 4 small molecule pathway inhibitors. From DoD0'-5', Noggin (17.5 ng/ml) and SB-431642 (10 µM) are added and CHIR99021 (1.5 µM), SU5402 (5 µM) and DAPT (γ-Secretase inhibitor IX, 5 µM) are added on DoD2'-9'. From DoD4' onwards, the KSR medium is gradually replaced by N2-S medium.

On DoD9' the cells are cryopreserved in FCS (fetal calf serum)/10% DMSO.

After thawing, cells are cultured in 25% KSR and 75% N2-S supplemented with CHIR99021 (1.5 µM), SU5402 (5 µM) and DAPT (5 µM). Cells are seeded on 96-well-plates in a density of 100.000 cells / cm^2. One hour after seeding, cells have attached to the plate and compounds for the treatment can be added.

Coating of plates:

Frozen matrigel is resolved and diluted 1:40 in cold DMEM/F12 (Dulbecco's Modified Eagle Medium/Ham's F-12) medium. Plates are coated with diluted matrigel (6-well plate: 1 ml/well, 96-well plate: 50 µl/well) and incubated for 30 min at 37°C.

Reference / link to maintenance culture protocol

Maintenance
principle:
The iPSC line EPITHELIAL-1 is cultured in Essential 8 (E8) medium under feeder-free conditions on Laminin-521 coated plastic dishes. Cells are passaged every 5-7 days, or as soon as the cells reach >80% confluency.
For splitting, cells are detached as clumps using EDTA, diluted 1:35-50 in prewarmed medium (depending on culture confluency) and reseeded in E8 medium on Laminin-521 coated plastic dishes.

The cells are checked for basic stem cell morphological characteristics as cell growth in defined colonies, the

expression of marker genes and proteins like Oct-4 and Nanog and the absence of spontaneously differentiated cells.
As soon as differentiated cells are spotted in the stem cell culture, a new batch of cells is thawed.

Definition of the test system as used in the method

Principles of the culture protocol

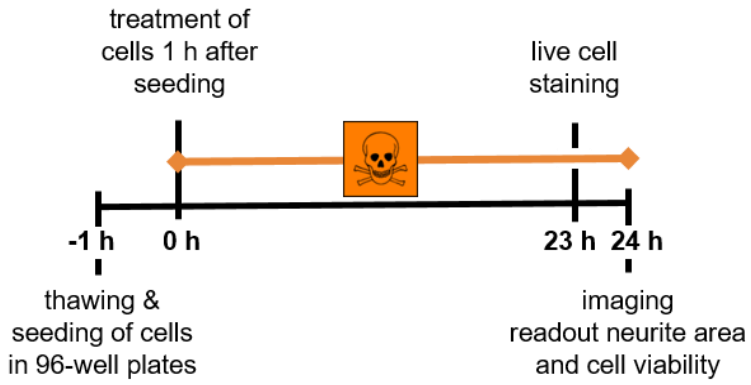


Figure 4: UKN5/PeriTox exposure scheme

The previously differentiated immature peripheral neurons are thawed and seeded on matrigel coated plates (1:40 diluted) in 75 µl medium composed of 75% N2-S medium and 25% KSR medium, supplemented with CHIR99021 (1.5 µM), SU5402 (5 µM) and DAPT (γ-Secretase inhibitor IX, 5 µM) at a density of 100.000 cells/cm^2.
One hour after seeding, treatment compounds are added to the cells in 25 µl of similar to culture medium in which cells were seeded.
23 h after toxicant application, cells are live-stained with H-33342 and calcein-AM and incubated for 60 min.
After 24 h of treatment (including staining), the cells are imaged using a high-content microscope (Cellomics VTI Array Scan).

Acceptance criteria for assessing the test system at its start

Cells should be attached to the plate (appear flattened at the edges) when the toxicant treatment is applied
→ as the cells are freshly thawed for the test run, there are no quantifiable criteria the culture can be checked for before toxicant treatment. However, neurite growth and appearance of the control cells are checked visually before live staining of the cells.

In general, cells are checked for the expression of the (sensory) neuronal markers Brn3A, Islet-1, peripherin and βIII tubulin (on DoD1, 4 and 7 after thawing).

Acceptance criteria for the test system at the end of compound exposure

After compound treatment, the negative controls should fulfil the following:
- control cells should have properly grown neurites, neurite area quantification (via Cellomics) has to be > 150,000 pixels in the control wells.

Variability of the test system and troubleshooting

Causes of variability:
- different differentiations:
  → contaminating, non-neuronal cells might be present in some differentiations for unknown reasons during the differentiation process.
- lots of different plates/flasks:
  → plastic might be different if the manufacturer delivers from a different/new lot
- differences between the vials of one cell "lot"
- different lots of medium and supplements

differentiation = cells that have all been differentiated from the one iPSC passage and frozen at the same time. Usually one to three 6-well plates are frozen in numerous vials with $8x10^6$ cells/vial.

Metabolic capacity of the test system

No specific information available.

Omics characterization of the test system

Transcriptomics data (unpublished) will become available from the originator lab (Leist) upon request.

Features of the test system that reflect the *in vivo* tissue

- As neurons of the peripheral nervous system they express peripherin, Brn3A, Islet-1
- They express various neuronal receptors and channels (e.g. purinergic receptors, TRP channels) and especially the tetrodotoxin-resistant voltage-gated sodium channel $Na_v1.8$ which is specifically expressed in dorsal root ganglia
- They are electrophysiologically active and excitable
- Cells do not exhibit the typical pseudo-unipolar morphology of dorsal root ganglion-neurons

Commercial and intellectual property rights aspects of cells

The cells are not protected by patents or any other licenses.

Reference / link to the culture protocol

Brief description in section 3 of this file.

The maintenance is described in the DB-ALM SOP available at:
http://cidportal.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/DBALM/LATEST/online/DBALM_docs/218_P_PeriTox.pdf

Or in SOP attached in Holzer et al., 2022a supplementary file 1.

A lab-internal handling protocol is also available upon request to the Leist-lab.

Test method exposure scheme and endpoints

Exposure scheme for toxicity testing

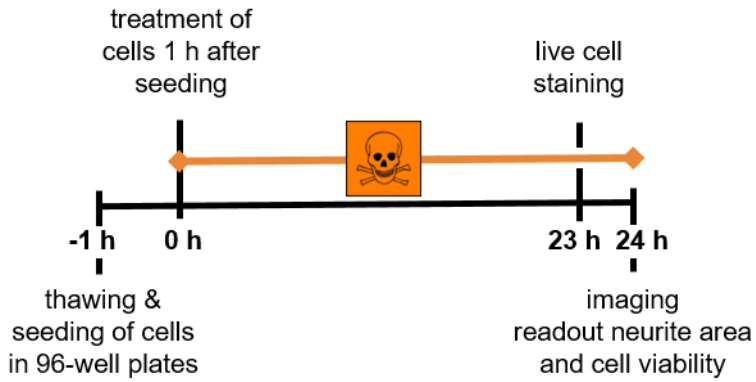


Figure 5: UKN5/PeriTox exposure scheme

The previously differentiated immature peripheral neurons are thawed and seeded on matrigel coated plates (1:40 diluted) in 75 µl medium composed of 75% N2-S medium and 25% KSR medium, supplemented with CHIR99021 (1.5 µM), SU5402 (5 µM) and DAPT (γ-Secretase inhibitor IX, 5 µM) at a density of 100.000 cells/cm^2.
One hour after seeding, treatment compounds are added to the cells in 25 µl of culture medium similar to culture medium in which cells were seeded.
23 h after toxicant application, cells are live-stained with H-33342 and calcein-AM and incubated for 60 min.
After 24 h of toxicant treatment (including staining), the cells are imaged using a high-content microscope (Cellomics VTI Array Scan).

Endpoint(s) of the test method

Test endpoints: 1) neurite area (specific endpoint)
2) cell number
3) % of viable cells (reference endpoint)

Overview of analytical method(s) to assess test endpoint(s)

Cells are stained with calcein-AM to mark viable cells. Co-staining with Hoechst H-33342 allows the identification of any cell.

Cells are stained for 60 min at 37°C and 5% CO2 in the incubator.

The cell staining is imaged in a Cellomics Array Scan VTI HCS reader.
Hoechst H-33342 staining is imaged in channel 1 (UV-Hoechst); calcein staining is imaged in channel 2 (Green-FITC). Exposure times are set manually.

To measure the neurite area, the software acquires the Hoechst signal in channel 1 to identify the cells as objects (via identification of the nuclei), and the calcein-AM signal in channel 2 to measure neurite area. Double positive cells are counted as viable.

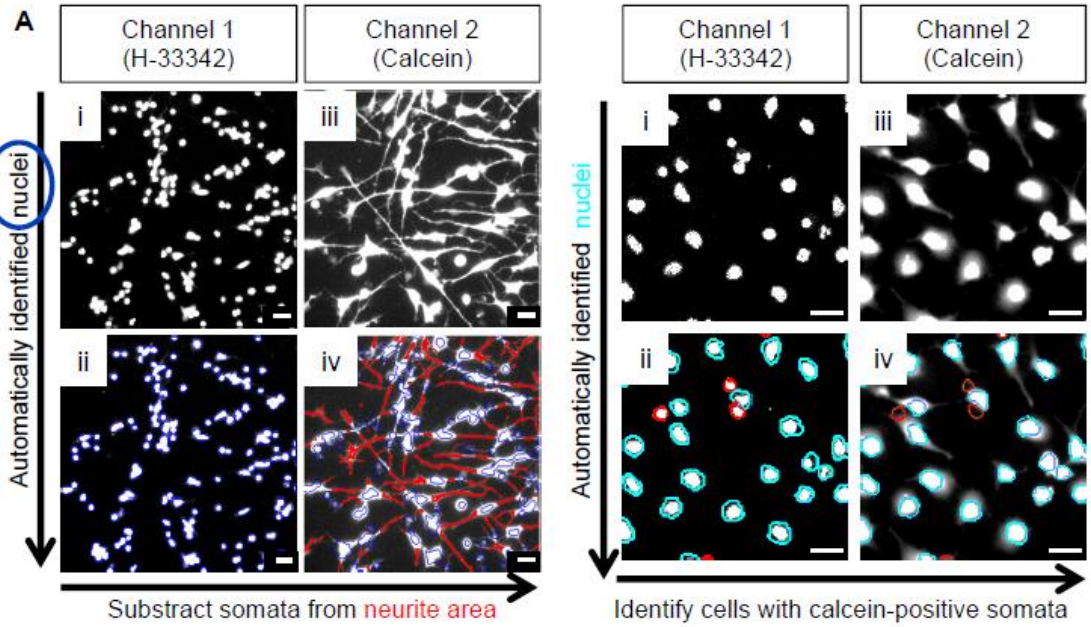


Figure 6: Exemplification of object and structure identification via automated algorithm in UKN5 assay analysis

Technical details (of e.g. endpoint measurements)

Quantification of neurite outgrowth

An automated microplate reading microscope (Array-ScanII HCS Reader, Cellomics, PA) equipped with a Hamamatsu ORCA-ER camera (resolution 1024 x 1024; run at 2 x 2 binning) was used for image acquisition. Ten fields per well were imaged. Images were recorded in 2 channels using a 20x objective and excitation/emission wavelengths of 365 ± 50/535 ± 45 to detect H-33342 in channel 1 and 474 ± 40/535 ± 45 to detect calcein in channel 2. In both channels, a fixed exposure time and an intensity histogram-derived threshold were used for object identification. Neurite pixels were identified using the following image analysis algorithm: nuclei were identified as objects in channel 1 according to their size, area, shape, and intensity which were predefined on untreated cells using a machine-based learning algorithm, and manual selection of nuclei to be classified as intact. The nuclear outlines were expanded by 3.2 µm in each direction, to define a virtual cell soma area (VCSA) based on the following procedure: All calcein-positive pixels of the field (beyond a given intensity threshold) were defined as viable cellular structures (VCSs). The threshold was dynamically determined for each field after flat field and background correction and intensity normalization to 512 gray values and was set to 12% of the maximal brightness (channel 63 of 512). The VCS defines the sum of all somata and neurites without their assignment to individual cells. In an automatic calculation, the VCSAs, defined in the H-33342 channel, were used as filter in the calcein channel and subtracted from the VCS. The remaining pixels (VCS - VCSA) in the calcein channel were defined as neurite area.

Quantification of individual viable cells by imaging

For a quantitative assessment of viable cells, the same images used to assess neurite area were analysed using another image analysis algorithm: nuclei were identified in channel 1 as objects according to their size, area, shape, and intensity. Nuclei of apoptotic cells with increased fluorescence were excluded. A VCSA was defined around each nucleus by expanding it by 0.3 µm into each direction. Calcein-AM staining, labelling live cells, was detected in channel 2. The algorithm quantified the calcein intensity in the VCSA areas. Cells having an average calcein signal intensity in the VCSAs below a predefined threshold were classified by the program as “not viable”. Valid nuclei with a positive calcein signal in their cognate VCSA were counted as

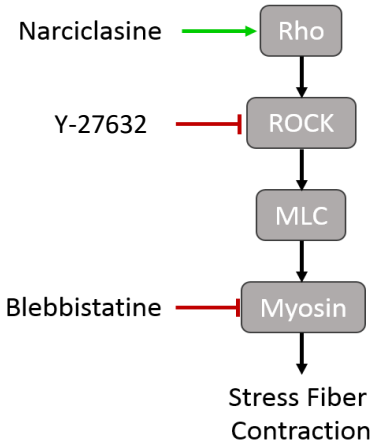
viable cells. A positive calcein signal was based on measurements of the average intensity (normal cells: 1300 ± 115, threshold: < 50) and the total integrated intensity (normal cells: 186,000 ± 23,600, threshold < 1000) of cells.

Endpoint-specific controls / mechanistic control compounds (MCC)

Endpoint-specific control for neurite growth inhibition:
Vincristine: microtubule toxicant
Colchicine: microtubule polymerization inhibitor
Cytochalasin D: actin polymerization inhibitor
Narciclasine: activates Rho

Endpoint-specific control for neurite growth enhancement:
Y-27632: ROCK inhibitor
Blebbistatine: inhibits myosin II

Rho/ROCK/LIM kinase/cofilin pathway:
induces actin polymerization, key regulator of the cytoskeleton and cell polarity



Positive controls

Positive control: narciclasine (50 nM final concentration)

Negative and unspecific controls

Solvent control: 0.1% DMSO final concentration (standard)
Up to 0.5% DMSO final concentration was tested in this test system and can be also used as solvent control
The concentration of the solvent control is aligned with the highest final DMSO concentration in wells treated with toxicants which is normally at 0.1% DMSO.

Further possible negative control compounds: e.g. mannitol, paracetamol

Features relevant for cytotoxicity testing

Cell death can easily be quantified.
Distinguishing slowed proliferation from cell death is not an issue for this test system as the cells are mainly

post-mitotic at the time point of toxicant exposure.

Acceptance criteria for the test method

Positive control narciclasine (50 nM):
Neurite area ≤ 75% of DMSO control
Viability ≥ 90% of DMSO control (or not significantly changed)

Negative control DMSO:
Neurite area ≥ 150,000 pixels per well

5.10 Throughput estimate

Data point = one biological replicate (→ usually 3 technical replicates); each concentration/condition of a compound counts as a data point
1440 data points per month
3 compounds per plate, 6 different concentrations of each compound per plate, 3 technical replicates per plate (see figure) → 18 data points (1 plate)

5 plates can be done per day (correlates to 15 compounds → 90 data points)
→ 4 days of readout per week → 360 data points per week; 4 weeks per month → 1440 data points per month.

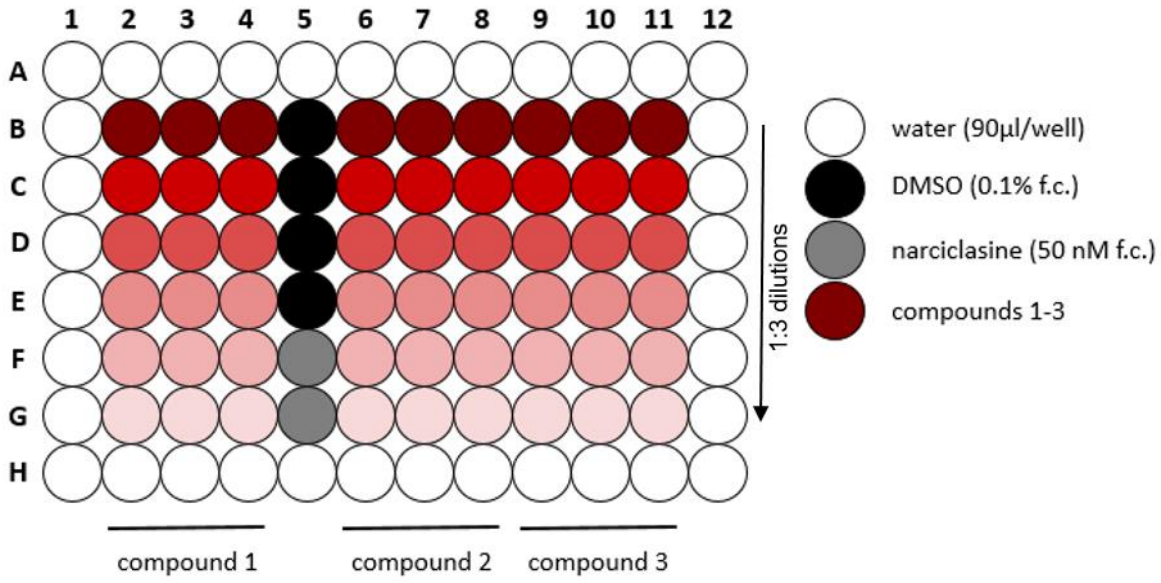


Figure 7: UKN5/PeriTox typical plate layout

Handling details of the test method

Preparation / addition of test compounds

- Compounds are stored according to the manufacturer's instructions (e.g. 4°C, room temperature, -20°C, -80°C).

- Preferable solvent is DMSO. The used DMSO is stored in a lightproof, air-tight bottle at room temperature.
- After dissolving the compounds which are delivered in a solid/powder form, all compound solutions are aliquoted into volumes sufficient for one experiment (i.e. one biological replicate). In this way repeated freezing and thawing and therefore damaging the compound's stability and efficiency can be avoided.
- final concentration of DMSO is 0.1%
- For conducting an experiment, a compound aliquot is thawed and diluted with culture medium (75% N2-S medium, 25% KSR medium, supplemented with CHIR99021 (1.5 µM), SU5402 (5 µM) and DAPT (5 µM)).
- All compound dilutions in the master plate contain 0.4% DMSO, so that a final concentration of 0.1% DMSO is reached on the cells. The highest compound concentration is diluted with medium 1:250 without DMSO as 0.4% is already reached with the DMSO the compound is solved in. The serial dilution is done with culture medium and 1% DMSO.
- The compound dilutions (25 µl each) are added to the cells using a multichannel pipette, 6 filter tips at a time. Pipetting has to be performed slowly.

Day-to-day documentation of test execution

Plate maps are defined prior to the experiment and documented in the lab book and files (Excel files) are stored on the work group server.
Concentrations and compound dilutions are calculated prior to the experiment.
Experimental procedures are noted manually in a paper lab book.

Practical phase of test compound exposure

The experimenter plans the experiment according to Cellomics microscope availability (has to be booked in advance) and availability of a sufficient number of cells.
Pipetting errors are marked directly on the plate maps and are documented in the lab book.
The paper lab book is taken to cell culture rooms and errors are documented in there right away.
The technical replicates were pipetted from left to right.

Concentration settings

3 compounds per plate
As default a serial dilution 1:3 is used, i.e. a concentration range of 1024-fold is covered (e.g. from 100 µM → 100 nM).
Serial dilutions of compounds are prepared in a separate deepwell-plate, from which 25 µl are transferred to the according plate with attached cells using a multichannel pipette.
Dilution steps can be adapted (e.g. 1:1.5, 1:4)

Uncertainties and troubleshooting*

- Compound solubility in stock and during dilution is too low (stock solved in 100% DMSO, final concentration of the solvent on the cells is 0.1% DMSO)
- Some compounds show autofluorescence and interfere with the detection of calcein-AM or H-33342.
- To prevent negative edge effects, only the inner 60 wells of a 96-well plate are used and the edge wells were filled with PBS or water.
- Focusing failure of Array Scan VTI HCS Reader (Cellomics, PA) can be a problem that produces outliers; as well as imaging only one channel.
- Highly trained/automated handling with multichannel and multistepper pipette is necessary to achieve little variance.

- Operators can get trained within 2-4 weeks. Cell seeding and medium change should be performed as fast as possible to keep cells as short as possible at room temperature. The more practice an operator has, the faster the critical steps can be performed.
- Substances are added when pipette tips are touching the wall of the wells right above the medium surface. When the substance solution is pipetted too high above the medium surface, the droplet may just stick to the wall of the well without flowing down into the medium.

Detailed protocol (SOP)

Protocol nº 218 in DB-ALM data base:

http://cidportal.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/DBALM/LATEST/online/DBALM_docs/218_P_PeriTox.pdf

Or see SOP attached in Holzer et al., 2022a supplementary file 1.

Updated SOP can be made available by laboratory upon request:
marcel.leist@uni-konstanz.de

Special instrumentation

The method requires a Cellomics Array Scan VTI HCS high content reader that may not be present in the standard lab.
Alternative automated microscopes and software tools to perform neurite outgrowth assays exist and the method might be transferred in the future.

Possible variations

a) further additional endpoints:
- metabolic activity (resazurin reduction)
- glutathione levels
- staining of tubulin
- analysis of differentiation markers by qPCR or immunostaining

b) other analytical endpoints:
cell viability by:
- fluorimetric measurement of resazurin conversion
- measurement of extracellular LDH
- measurement of luminescence indicating ATP content

c) other exposure:
- compound can be washed out → acquisition on day 2
- longer exposure is possible
- later exposure is possible (from day 3 on) in order to measure effects on more mature neurons

Cross-reference to related test methods

There is no related test.

Data management

Raw data format

Raw data is extracted by copy-paste in Excel files (example file available upon request).
Data from all technical replicates are collected in one file.

Outliers.

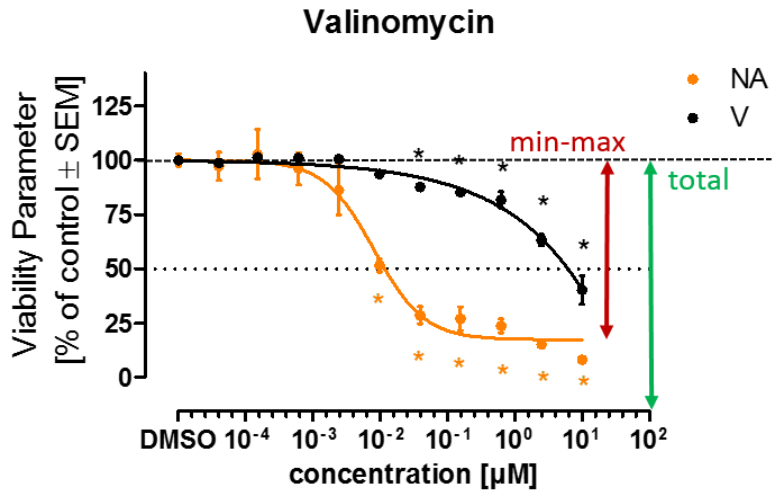
1. Mathematical procedures to define outliers have not been defined. Data points that are far off (i.e. more than the known endpoint variability which would be 25% for neurite outgrowth) are discarded. Biological outliers do practically not exist, most far data points are the result of technical problems (focus not found, only one channel imaged, etc.)
2. All raw data (incl. outliers) are stored.
3. Technical outliers make up 1-0.1%.

Raw data processing to summary data

- Array Scan VTI HCS Reader (Cellomics, PA) takes images (optionally bitmap or tiff-format; 512 x 512 pixels, 8bit or 16bit)
- Images are locally analyzed using the Array Scan software, algorithms to quantify neurite area, total cell count (nuclei) and viable cell count.
- data are copy-pasted into an Excel sheet, further analysis is done with Excel + GraphPad Prism

Curve fitting

The data are analyzed with Excel and represented with GraphPad Prism.
For the concentration curve, a nonlinear regression fit is calculated. The fitting method is least squares. If a non-linear curve fit is not possible, a linear curve fit is performed. The curve deriving from the fit is a 4-parameter log function. To calculate e.g. the EC50 value, this log-function is solved for y=50% of the total scale, not for 50% of the min-max scale (see example below). Treated concentrations are analyzed for deviation from control. Sometimes it is analyzed whether the deviation of neurite growth is different from the deviation of viability via comparison of the means ± deviation using two-way ANOVA + Tukey-Kramer post hoc testing. Significance levels of data points compared to solvent control are determined via one-way ANOVA followed with non-parametric Dunnett's post test.



EC50(NA)= 0.01 µM
EC50(V)= 6.16 µM

Figure 8: **Exemplification of curve fit and relative effect responses**

Note: Curve shown was generated in UKN4 assay and does not represent UKN5 data but the statistical analysis and curve fitting principle which is similar in both assays.
NA = neurite area, V = viability

BMC values with their upper and lower confidence intervals (BMCU and BMCL) are calculated via the publically available online software:
http://invitrotox.uni-konstanz.de/BMCeasy/

Internal data storage

The data are firstly stored on the microscope computer and then exported to other servers (lab group server and university server), which are backed-up regularly.

Metadata

The metadata are documented, stored and exported as text document (log)-files to the according scheme: (local PC)_descriptor(date and time)_XXX.log:
The following metadata are stored:

- cellinsight-pc_160429130003_AutomationControllerIni
- cellinsight-pc_160429130003_kineticprotocol
- cellinsight-pc_160429130003_protocol
- cellinsight-pc_160429130003_scan
- cellinsight-pc_160429130003_ScanIni
- cellinsight-pc_160429130003_spooling
- cellinsight-pc_160429130003.spooled

Metadata file format

Metadata files are available.

Prediction model and toxicological application

Scientific principle, test purpose and relevance

Immature peripheral neurons used in this test method represent the (developing) peripheral nervous system.

The test method therefore measures adverse effects on peripheral neurons that directly or subsequently affect neurite growth and integrity or the cell viability in general.

The test method not only predicts the hazard to induce developmental neurotoxicity but also to induce neurotoxicity in mature peripheral neurons, as these are highly dependent on an intact cytoskeleton due to their enormous length. Any adverse interference with the cytoskeleton in the state of developing neurons might therefore also present an adverse interference in mature peripheral neurons. Therefore, this test method can be related to adverse human outcomes like peripheral neuropathies.

Prediction model

Three different models are used:

1. prediction model for screening:
hit = decrease/increase in neurite area while viability is not changed (compare to narciclasine positive control:
Neurite area ≤ 75% of DMSO control
Viability ≥ 90% of DMSO control

2. prediction model for compound hazard evaluation:
hit confirmation testing; BMC25 Viability (V) / BMC25 Neurite Area (NA) ≥ 3 → specifically neurotoxic

3. prediction model for borderline compounds:
A ratio of BMC25 Viability (V) / BMC**L**25 Neurite Area (NA) ≥ 3 is considered a borderline hit. In some scenarios the viability does not reach the BMC25 necessary for the ratio calculations. In this case the highest tested concentration (HTC) was used. Schematic representation of the complete prediction model is shown in the scheme below.

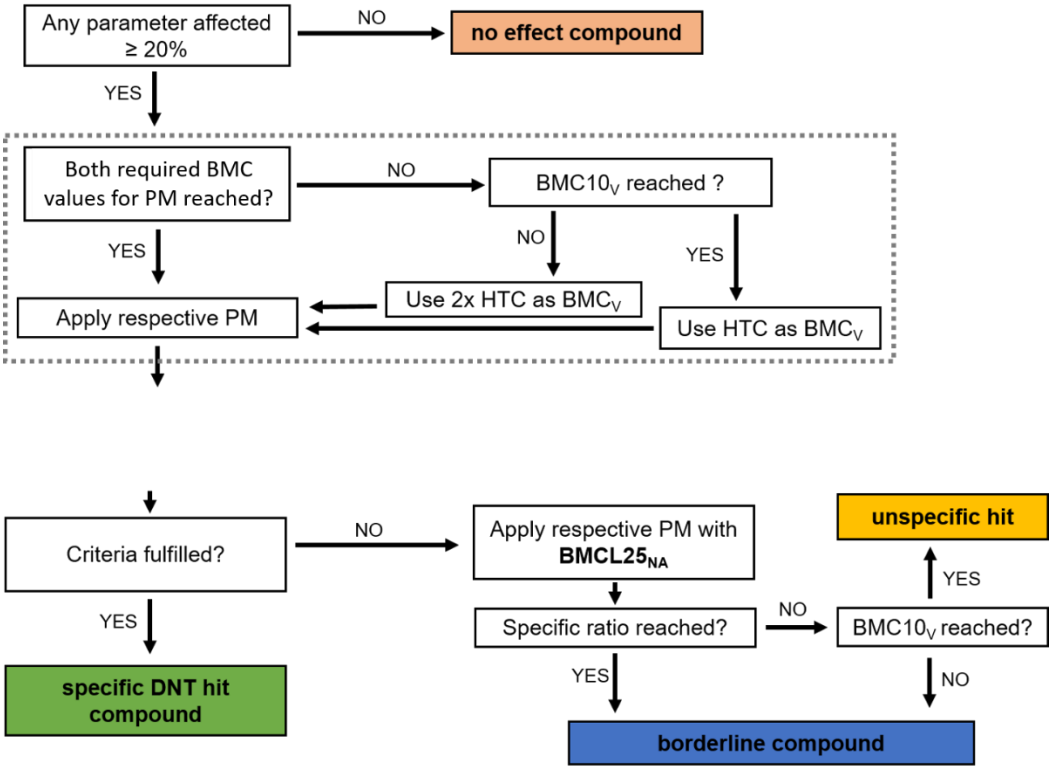


Figure 9: **Prediction model decision tree for UKN5 assay**

A full overview with a schematic representation of the UKN4 prediction model can also be found in supplementary figure 3 of Blum et al., 2022.

Prediction model setup

To design the initial prediction model for the PeriTox test, we took the following steps:
(a) use of the "ratio" of EC50 (viability)/EC50 (neurites) as the primary endpoint
(b) measurement of this value for "unspecific toxicants" (the uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP), sodium dodecyl sulfate (SDS), Octoxinol 9 (Triton-X100), and the topoisomerase inhibitor etoposide.
The average ratio was 1.376±0.39)
(c) definition of a "noise band" (4SD(standard deviations) from the average of the ratios of these compounds)
(d) definition of compounds with a ratio outside the noise band (EC50 ratio of >3) as "neurite specific."
The use of the updated prediction model
BMC25 Viability (V) / BMC25 Neurite Area (NA) ≥ 3
was validated by comparing classifications derived by the initial and the updated prediction model. The reason for updating the prediction model was that a decrease of 50% in neurite area and viability cannot always be achieved with our range of test concentrations but to reach an effect of 25% is more reasonable. The initial prediction model therefore often used EC50 values that were only based on a pure mathematical curve fit. However, the now used BMC25 is more related to the data that was practically obtained.
The prediction model has been applied to screen the 80 compound library of NTP (Delp et al., 2018). The prediction model including the borderline classification has been applied to screen a 120 compound library (Blum et al., 2022; Masjosthusmann et al., 2020).

Test performance

Some background on the test performance is given in chapters 8.2/8.3 (prediction model).

Several performance parameters for the test were obtained in several separate evaluation rounds.

A first evaluation was done during the first publication of the model and its applications (Hoelting et al. 2016). Here, a panel of well-selected positive and negative controls have been tested. Accordingly, the specificity was 100% and the sensitivity was > 90 %. In dedicated experiments, S/N ratios of > 20 and a z' of > 0.5 have been determined. Operator reproducibility was shown in Hoelting et al. 2016 (Supplementary 3) for the compound colchicine.

The test has been used in screening campaigns, and real-live performance data under broader screen conditions have been obtained. The different performance data need to be considered, when a compound is a hit in a screen, or whether it has been specifically evaluated in a hit follow-up or a mechanistic project.

A first screen application has been the NTP80 screen (80 compounds provided by the US NTP). Data are published Delp et al. 2018.

A second screen application has been the cross systems case study of the EU-ToxRisk project. The baseline variation is indicated in Krebs et al., 2020. Moreover, an overview is given for 19 compounds on the BMC/BMCL ratio as measure of readout certainty.

A third screen was performed in the context of the EFSA DNT test battery evaluation with 120 compounds (Blum et al., 2022; Masjosthusmann et al. 2020). From this screen the following performance indicators were obtained:

A: **Specificity of DNT IVB: 100%**

→ Also with PeriTox as standalone assay in 17 `tool negatives´ tested (Masjosthusmann et al. 2020).

B: **Sensitivity of DNT IVB: 82.7%**

→ With PeriTox combined in a full DNT battery and a selected set of 27 positive compounds with evidence for DNT (Masjosthusmann et al. 2020).

C: **Baseline variation (intra-experimental)**

Neurite area: 7.8 ± 4.3%

Cell viability: 5.5 ± 3.4%

D: **Baseline variation (inter-experimental)**

Neurite area: 21%

Cell viability: 15.7%

E: **Variation of a positive control run on each (inter-experimental)**

Neurite area: 17.5%

Definition of values C-E

C: **Baseline variation (intra-experimental)** is the mean Coefficient of variation CV±SD of the CV of all replicates of the solvent control from each experiment across n>200 experiments.

D: **Baseline variation (inter-experimental)** is the variability across all independent experiments (n>200) after normalization based on the response of the lowest test concentration. It was assumed that the lowest test concentration does not affect any of the endpoints measured.

E: **Variation of a positive control run on each (inter-experimental)** is the variability of the positive control across all independent experiments (n>40) after normalization. Example for a positive control that on average reduced the specific endpoint down to 40% (relative to solvent control) and a calculated variability of 50%: 0.5 x 40% = +/- 20% → The positive control with mean of 40% varies from 20% to 60%.

In vitro – in vivo extrapolation (IVIVE)

1. Lipid and Albumin content is not known. Medium used during toxicant treatment is as follows:

25% KSR:
Knockout DMEM with
15 % knockout serum replacement,
2 mM Glutamax,
0.1 mM MEM non-essential amino acids and
50 µM beta-mercaptoethanol

75% N2:
DMEM/F12 medium
1 % Glutamax
1.55 mg/ml glucose
0.1 mg/ml apotransferrin
25 µg/ml insulin
100 µM putrescine
30 nM selenium
20 nM progesterone)

2. The test has not been used for IVIVE or other use of potency information.
3. No special considerations known.

Applicability of test method

Test is sensitive to cytoskeletal toxicants, some signaling modifiers and flame retardants. Polycyclic aromatic hydrocarbons (PAH) and HDAC inhibitors have no effect.

Incorporation in test battery

a) Strengths:

- Medium to high throughput (The term “medium to high throughput” is derived from a comparison and in relation to a number of other known DNT NAMs)

- Automated microscopy

b) compared to UKN4 (which quantifies neurite outgrowth of central neurons), UKN5 measures neurite outgrowth specifically of peripheral neurons. This was shown by treatment with MPP+ which is transported by the dopamine transporter (DAT) and had effect in UKN4, but not UKN5 as peripheral neurons lack the DAT transporter. Furthermore, toxicants known to specifically induce peripheral neuropathies, like proteasome inhibitors (e.g. Bortezomib) or acrylamide were shown to have neurite specific effects in UKN5 but not in UKN4 (Hoelting et al. 2016)
c) specific effects peripheral neurons. The test method is currently used in the setup of a DNT test battery.
d) Preferential use in first tier, no complementary assays required for the assessment of chemical effects on the endpoints investigated by this test method. Fast cell supply compared to other potential DNT battery assays + short assay time + number of data points possible to generate in one run make this assay a potential "pre-screen/first tier".

Publication / validation status

Availability of key publications

Establishment of a human cell-based in vitro battery to assess developmental neurotoxicity hazard of chemicals
Blum, J. et al. Chemosphere, 2022. PMID: 36328314

Generation of nociceptor-enriched sensory neurons for the study of pain-related dysfunctions. Holzer, A-K. et al. Stem Cells Transl Med., 2022**a**; PMID: 35689659

Specific attenuation of purinergic signaling during bortezomib-induced peripheral neuropathy in vitro.
Holzer, A-K. et al. Int J Mol Sci., 2022**b**; PMID: 35409095

Stem cell-derived immature human dorsal root ganglia neurons to identify peripheral neurotoxicants.
Hoelting, L. et al. Stem Cells Transl Med, 2016. PMID : 26933043

A high-throughput approach to identify specific neurotoxicants / developmental toxicants in human neuronal cell function assays.

Delp, J. et al. Altex, 2018. PMID : 29423527

The EU-ToxRisk method documentation, data processing and chemical testing pipeline for the regulatory use of new approach methods.
Krebs, A. et al. Arch. Toxicol., 2020. PMID: 32632539

Establishment of an a priori protocol for the implementation and interpretation of an in-vitro testing battery for the assessment of developmental neurotoxicity.
Masjosthusmann, S. et al. EFSA Supporting Publications. 2020; 17(10): 1938E.

Neurodevelopmental toxicity assessment of flame retardants using a human DNT in vitro testing battery.
Klose, J. et al. Cell Biol Toxicol. 2021; PMID: 33969458

(Potential) linkage to AOPs

Test method could be potentially linked to the following AOPs in AOPwiki:

- AOP 249 : Microtubule interacting drugs lead to peripheral neuropathy
  → Adverse Outcome: Sensory axonal peripheral neuropathy

Steps towards mechanistic validation

a) Cells express typical sensory neuronal markers, are of human origin and form a network
b) Tubulin plays a major role in neurite outgrowth and if the dynamic instability of microtubules is inhibited by compounds like colchicine/vincristine/taxol neurite outgrowth is reduced. If the Rho/Rock pathway is activated, neurite outgrowth is enhanced.
c) A formal mechanistic validation has not been performed. Reversibility has been shown (Hoelting et al., 2016).
d) The test rather covers a fundamental neurodevelopmental process than a key event (Smirnova 2014, Bal-Price 2015 (ISTNET))

Pre-validation or validation

To date, 145 unique compounds (as defined by unique DTXSIDs) have been tested successfully in this assay. No formal OECD 34 validation study has been done (eg., ring trials with a standard set of known positive and negative controls).
In total, >200 different compounds were tested in the PeriTox assay. The test method was developed using a compound training set (Hoelting et al. 2016). It was used for an 80 compound screening library from the US National Toxicology Program (NTP) (Delp et al. 2018). The test method was part of a DNT hazard assessment for 120 compounds in a DNT testing battery. The latter compound set includes potential DNT positive and DNT negative compounds (Masjosthusmann et al. 2020).

Linkage to (e.g. OECD) guidelines / regulatory use

Test is not linked to regulatory guidelines.

Test method transferability

Operator training

Experiences are required in:
- cell culture
- multichannel/multistep pipetting
- handling of Array Scan VTI HCS Reader (Cellomics, PA) and its software
- Microsoft Excel
- GraphPad Prism

Operator is trained and guided by a highly experienced instructor. Approximately 4 weeks will be needed for a smooth assay performance.
Learning iPSC culture and cell differentiation takes several months.

Transfer

The assay hasn’t been transferred or applied in other labs.

Safety, ethics and specific requirements

Specific hazards; issues of waste disposal

No specific requirements.

Safety data sheet (SDS)

SDS are available in the university DaMaRIS database (**Da**ngerous **Ma**terials **R**egistry **I**nformation **S**ystem).

Specific facilities / licenses

Work requires S1 cell culture laboratories (genetically modified cells) due to retroviral reprogrammed hiPSC line used for the assay.
No specific facilities are required.
No specific ethical approval is required.

Commercial aspects / intellectual property of material / procedures

To our best knowledge, no elements needed to conduct the experimental part of the test method are protected. Programs used to conduct the analysis of the data (Microsoft Excel and GraphPad Prism) need to be purchased or obtained by license agreement, however data analysis and plotting can be done with other, freely available tools.

Appendix B.6

Author: Tim Shafer, Jackson Keever
Date: 10.03.2023
Version: 1

Overview

Descriptive full-text title

High-Content Imaging Assay to Screen for Changes in Neurite Outgrowth Due to Chemical Exposure in Neurons Derived from Rat Primary Cortical Cells

Abstract

The High Content Imaging Assay to Screen for Changes in Neurite Outgrowth was developed to screen large numbers of compounds for potential developmental neurotoxicity in vitro. During the development of the nervous system, many processes occur to give rise to a functional and healthy neural network. These important neurodevelopmental processes may be disrupted by potential toxicants, resulting in developmental neurotoxicity. Among these processes is neurite outgrowth – the physical outward growth of neurites (eventually axons and dendrites) of individual neurons that allows them to make connections with other neurons and ultimately gives rise to the physical network of cells. This assay utilizes a high-content imaging solution to describe neurite outgrowth in a rat primary cell culture, via the immunocytochemical labelling of cell bodies and neurites. An automated image analysis protocol is employed to systematically identify targeted structures based on preassigned criteria. Ultimately, changes in the number and length of young outgrowths is quantified and inhibition of more than 30% results in a hit call. The assay is performed on a 96-well plate, allowing for a medium-to-high throughput screening of chemicals. According to the readiness criteria as published by Bal-Price et al. (2018) the rat cortical neurite outgrowth assay obtained the readiness score A.

Assay summary:

toxicological target	→	developing brain
test system	→	Rat primary cortical cells from PND0 rat pups
readout(s)	→	number of cells per valid field, total neurite length per neuron, number of neurites per neuron, and number of neurite branch points per neuron.
biological process(es)	→	Neurite outgrowth, immunohistochemical staining, viability, cytotoxicity.
(human) adverse outcome(s)	→	CNS dysfunction

hazard(s)	→	adverse effect on neurite outgrowth
endpoint of current regulatory studies	→	no
validation/evaluation	→	readiness analysis

General information

Name of test method

Rat Cortical Neurite Outgrowth Assay (NOG)

Version number and date of deposition

20211215_v1.1

Summary of introduced changes in comparison to previous version(s)

original version

Assigned data base name

ToxCast invitro database assay identification: *CCTE_MUNDY_HCI_ Cortical_NOG*

Name and acronym of the test depositor

United States Environmental Protection Agency (EPA)

Name and email of contact person

Tim Shafer (Shafer.Tim@epa.gov)

Name of further persons involved

Kathleen Wallace (Wallace.Kathleen@epa.gov)
Theresa Freudenrich (Freudenrich.Theresa@epa.gov)
Seline Choo (Choo.Selina@epa.gov)
Jackson Keever (Keever.Jackson@epa.gov)

Reference to additional files of relevance

No additional supporting files submitted

Description of general features of the test system source

Supply of source cells

Cell cultures are isolated from the cortical neurons from Long-Evans rat pups on postnatal day 0. Pregnant dams are supplied by Charles River Laboratories; delivered to the US EPA facility on GD16 and held in animal colonies until they give birth.

Overview of cell source component(s)

Primary rat cortical cultures are prepared on site from the neocortex dissected from the CNS of newborn (PND0) Long-Evans rat pups using a standard protocol (Section 3.7). In a typical culture, cells are isolated from the combined cortices of 3-5 pups, seeded onto a Poly-L-Lysine coated 96-well plate at a density of 10,000 cells/well and are allowed 2 hours to attach. The cells are maintained in a humidified incubator at 37°C and 5% CO_2. Sex of pups is not determined, and cultures are presumed to consist of a mixture of male and female pups since multiple pups are used for each culture.

Characterization and definition of source cells

Primary cortical cultures consist of a mixture of glutamatergic and gabaergic neurons, as well as glial cells (oligodendrocytes and a few microglia) as characterised by immunocytochemistry and functional responses to pharmacological agents. (Mundy and Freudenrich, 2000; McConnell et al., 2011; Frank et al., 2017).

Acceptance criteria for source cell population

Before plating the cells, the following criteria must be fulfilled:

- The rat pups used for cortical culture must be less than one day old, preferably newborn.
- Minimum cell viability of 85% verified by trypan blue dye exclusion.
- Cell cultures examined under the microscope are free of microorganisms.
- Each time medium is prepared, a 1.0 mL sample is placed onto a sterility plate and incubated at 37°C. Sterility plates are checked daily for contamination and contaminated cell cultures should not be used. Media color changes may indicate contamination or improper CO_2 levels.

Variability and troubleshooting of source cells

Some variability is inherent in the system since new cultures are made from different animals for each preparation and hence assay. This would reflect normal biological variation. In addition, during the plating process, cells will randomly distribute and attach to the bottom of the 96 well plate and this will result in random differences in the networks formed, which may contribute to variability.

Serum-free media which reduces variability due to differences in the content of growth factors and other critical nutrients found in serum.

Each time medium is prepared, a 1.0 mL sample is placed into a plate and incubated at 37ºC to test for sterility. It is strongly recommended that media be made at least 2 days before use. If the media becomes purple or yellow, examine the plate for contamination. When the media is cloudy, this indicates contamination by some microorganism and the media should be discarded. If the media is bright pink to purple but no cloudiness is present, this may indicate a possible problem with the CO_2 level in the incubator. Examine other cultures in the incubator for color changes and measure the CO_2 level in the incubator with the Fyrite. The plate may also be examined under the microscope for microorganisms.

Critical consumable

The cultivation medium is supplemented with B27. The cultivation medium should be discarded 7 days after addition of the B27 supplement.

The cell culture procedure employs a cortical buffer for digestion, consisting of 137 mM NaCl, 5 mM KCl, 170 µM Na_2HPO_4, 205 µM KH_2PO_4, 5 mM glucose, 59 mM sucrose, and 100 U/mL penicillin/0.1 mg/mL

streptomycin (Gibco Cat# 1510-122). A cortical medium is used during cell attachment, consisting of DMEM with GlutaMax (Gibco Cat# 1056-010), 10% Horse Serum, heat inactivated (Gibco Cat. No. 26050-088), 10 mM HEPES, 100 U/mL penicillin/0.1 mg/mL streptomycin. The final media used in the culture, NB/B27 media, consists of 500ml Neurobasal-A Medium (1X, Gibco Cat# 10888), 10ml B-27 Supplement (50X, Gibco 17504-044), 5 ml GlutaMax (100X, Gibco 35050-061), and 5 ml Pen-Strep (Gibco Cat# 15140-122), pH adjusted to 7.4.

Differentiation towards the final test system

Once plated, primary cortical neurons rapidly extend neurites. Given the proper growth conditions, primary cortical neurons will develop extensive neurite networks containing both axons and dendrites as well as synaptic connections. A stereotypic pattern of neurite outgrowth is observed in primary cortical neurons prepared in dissociated culture. As cortical neurons mature, not only do they undergo periods of extensive neurite outgrowth, the neurites also differentiate into specialized neurite sub-populations: i.e. axons and dendrites. Axons are comparatively smaller caliber neurites whose rapid growth phase initiates prior to the growth and differentiation of dendrites. In primary cortical cultures, axons grow rapidly, branch extensively and form dense complex axon networks through the culture. Dendrites are comparatively larger caliber neurites whose growth phase initiates after the onset of rapid axonal growth. Maturing cortical dendrites can have proximal to distal tapered appearance and a branched appearance.
In addition to difference in the morphology of axons and dendrites, certain cytoskeletal and microtubule associated proteins can be used to distinguish axons from dendrites in maturing and mature cultures. The low molecular weight microtubule associated protein tau, as well as some forms of phosphorylated neurofilaments are selectively expressed in axons. In contrast, the high molecular weight microtubule associated protein MAP2 is selectively expressed in dendrites and neuronal cell bodies. The cytoskeletal protein β_{III}-tubulin is expressed in both axons and dendrites as well as in neuronal cell bodies. Previous studies in the literature have used the cytoplasmic localization of these proteins to distinguish axons from dendrites.

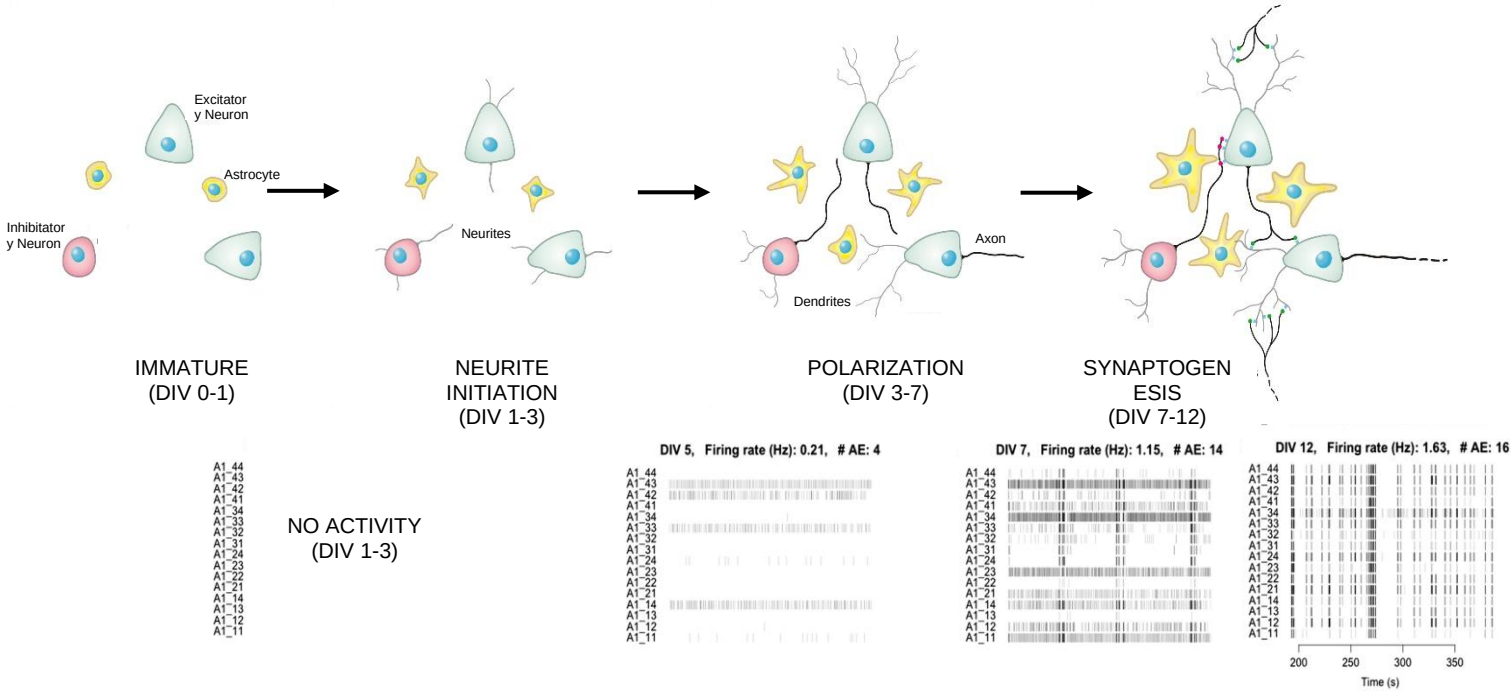


Figure 3.6.1. Schematic representation of the development of cortical cultures (upper panels) over days in vitro (DIV). This illustrates the major cell types in the culture and the morphological changes (neurite initiation, polarization and synaptogenesis. Lower panels: Raster plots illustrating the development of network activity in neuronal cultures over 12 DIV. Control (non-treated) wells from DIV 5, 7, 9, and 12 are shown. In each panel, time is on the x-axis and data from each electrode is plotted in rows on the y-axis. The naming convention is as follows: "A1" indicates that the data are from well A1 of the plate, while 11, 12, 13, etc indicate the row and column position, respectively, of the electrodes. A vertical "tick" mark indicates each event on that electrode that exceeds the spike threshold (>8x root mean square noise levels). Heavier shading indicates groups of events occurring closely in time (eg, bursts). The overall mean firing rate (MFR; in Hz) and number of Active Electrodes for each time point are shown above each panel. Neuronal activity, bursting and coordinated activity (eg, simultaneous bursts on multiple channels (eg, "network spikes") increase with time.

Reference/link to maintenance culture protocol

EPA Operating Protocol: NHEERL-H/ISTD/SBB/KAW/2017-01-r2 "Cortical cell culture" (Available upon request; Email: Shafer.tim@epa.gov)

Definition of the test system as used in the method

Principles of the culture protocol

The Neurite Outgrowth assay uses 96-well microtiter plates for the duration of this procedure.

This culture was plated at a seeding density of 10,000 cells/well on a 96-well plate, prepared as described in section 3.7. Cells were administered via a 90 µL media (DMEM + 10% serum) drop directly onto each of the wells. After a 2-hour attachment period, the plating media was removed and replaced with 90 uL of NeuroBasal media containing B27 and the cells returned to the incubator.

This assay utilizes high-content imaging analysis. Following exposure (see section 5), cells are fixed and

nuclei (Hoechst 33342) and neurites (βIII tubulin) are stained using immunocytochemical approaches. Plates are then placed in the Cellomics ArrayScan VTi HCS Reader is used for automated image acquisition and analysis of neurite outgrowth. Bioapplications automatically analyze the images and determine the various parameters reported in section 5.

The primary culture model consists of glutamatergic (excitatory) neurons, gabaergic (inhibitory) neuron, astrocytes and sparse microglia (Harrill et al., 2011; Frank et al., 2017).

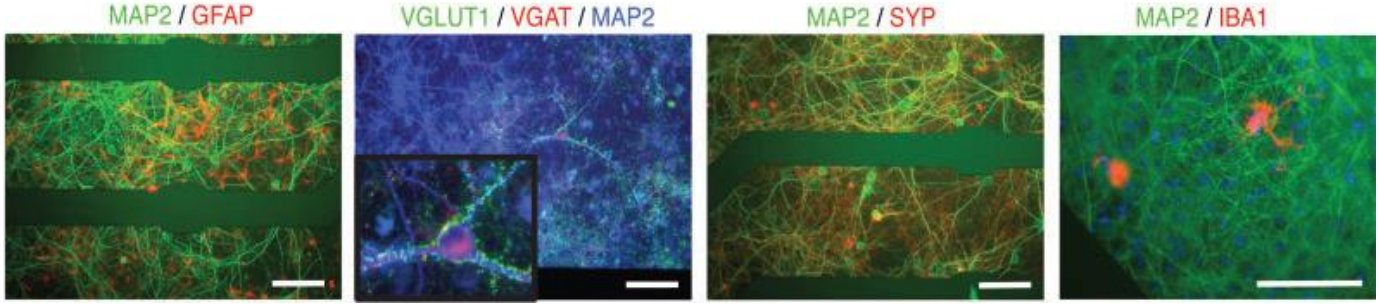


Figure 4.1.1 Representative images of DIV 12 cortical networks grown on 48-well MEA plates. A dense culture is maintained over the electrode array that contains microtubule-associated protein 2 (MAP2) staining of dendrites, Glial fibrillary acidic protein (GFAP)–positive astrocytes, punctate vesicular glutamate transporter 1 (VGLUT1), and vesicular GABA transporter (VGAT) staining of synaptic vesicles, punctate synaptophysin (SYP) staining of presynaptic vesicles, and a small percentage of Ionized calcium binding adapter molecule 1 (IBA1)–positive microglia. Scale bar=100 mm. From Frank et al., 2017.

Acceptance criteria for assessing the test system at its start

As noted above, viability of cells should be 85% or greater at the time of plating. Cells will be visually examined for attachment to the substrate, proper cell density, cell health, and any signs of contamination. Some of these parameters are subjective but are based on the experience and knowledge of the individual doing the assay.

Acceptance criteria for the test system at the end of compound exposure

For data generated with the ArrayScan to be acceptable for use, a cellular endpoint-specific chemical standard (typically rac-1) will be used as an internal control in the culture plate being used. For any particular endpoint, the chemical standard will be based on the scientist's expertise and understanding of the biology of the endpoint being measured and endpoint-specific data from the literature. The effect of the chemical standard must be within +/- 10% of the expected value (e.g. for an expected chemical result of a 50% change from control, the value should be between 40 to 60 %) to be accepted. If the effect of the chemical standard is outside of this range, the data from that particular culture plate will not be used.

Variability of the test system and troubleshooting

Sources of Variation:
Variability may be due to different numbers of male and female pups selected for each culture. In addition, the random distribution of excitatory and inhibitory cells in the culture, and their random location in each well may contribute to variability in the neurite outgrowth in each well.

Metabolic capacity of the test system

We have not extensively characterized the metabolic capacity of our primary cortical cultures. mRNA expression of various Cyp enzymes is low on DIV 1, however, by DIV 14, mRNA for Cyp 211c >> 4x1 > 2d4 > 1s1 > 1a1. Functional expression of these proteins has not been confirmed (Shafer et al., 2015).
Other metabolic pathways are not characterized.

Omics characterization of the test system

Transcriptomic characterization of the test system is currently underway.

Features of the test system that reflect the *in vivo* tissue

Cell model reflects the following in vivo tissue features:

- Presence of excitatory glutamatergic neurons
- Presence of inhibitory gabaergic neurons
- Presence of glial cells (astrocytes, microglia)
- A functional switch in $GABA_A$ receptor activation occurs between DIV 6 and 8, wherein prior to this, activation of the receptor is excitatory and drives an increase in intracellular calcium, whereas after this switch, activation of the receptor is inhibitory and results in an influx of chloride ions into the cells (Inglefield and Shafer 2000).
- Elaboration of neurites, with subsequent specialization into axons and dendrites (Harrill et al., 2013)
- Formation of synapses (Harrill et al., 2011)

Commercial and intellectual property rights aspects of cells

For primary cortical cells, N/A

The software for bioapplications used by the Cellomics ArrayScan VTi HCS Reader are proprietary.

The assay procedure as described above is nonproprietary. It contains some proprietary materials, listed below, which the protocol is optimized for. Utilizing materials from other providers may or may not necessitate changes to the procedure, including seeding density, culture media, media change schedule, days in vitro prior to testing, etc. For details relating to the development of this procedure, please refer to Harrill et al., 2011.

Reference/link to the culture protocol

See section 0.

Test method exposure scheme and endpoints

Exposure scheme for toxicity testing

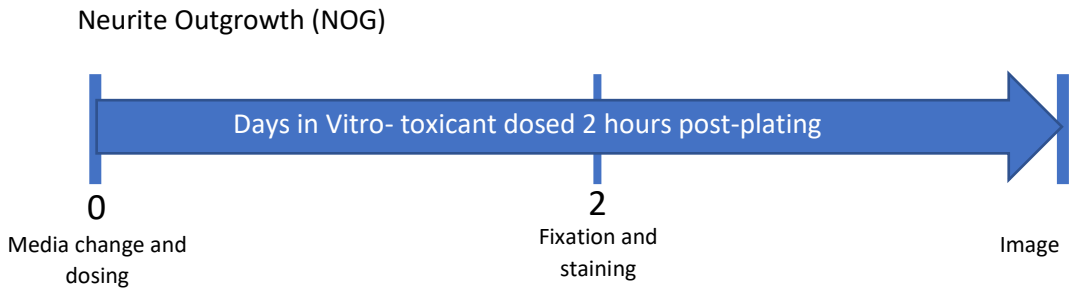


Figure 5.1.1. Exposure scheme. Cells are plated onto 96 well plates and allowed 2 hr for attachment to the substrate. Two hours post plating cells are exposed to chemicals with a full media change. After 48 hours post-treatment, Cells are fixed and stained using rabbit anti-βIII-tubulin primary antibody and Alexa Fluor-488 secondary antibody to label neuronal cell bodies and neurites. A Cellomics ArrayScan VTi HCS Reader is used for automated image acquisition and analysis of neurite outgrowth

Exposure starts 2 hours post-plating by adding 10 µl of the working solution to 90 µl of media in the wells for a 1/10 dilution. Forty-eight hours after chemical treatment cells are fixed by direct addition of 100 µl of warm (37°C) Dulbecco's phosphate buffered saline (DPBS) fixative solution containing 8% paraformaldehyde, 8% sucrose, and 0.1% of 3 mg/ml Hoechst 33342 into each well. Primary antibodies are prepared by dilution in Immunocytochemical Staining Buffer (ISB), 10X Dulbecco's PBS, 0.1% Saponin, 5% Bovine Serum Albumin, 0.5% NaN_3 (Sodium Azide) and βIII-tubulin (rabbit anti-βIII-tubulin, 1:800) followed by Alexa Fluor-488 secondary (1:500) to label neuronal cell bodies and neurites. A Cellomics ArrayScan VTi HCS Reader is used for automated image acquisition and analysis of neurite outgrowth.

Endpoint(s) of the test method

Table 5.2.1 Endpoints assayed

Neurodevelopmental Process	Assay Name (*Cell Type*)	ACID	AEID	Endpoint Name	Description
	MUNDY_HCI_Cortical_NOG (*1° rat cortical cultures*)	2699	2777	BPCount_loss	Morphology of α_{III}-tubulin labeled neurons as measured using automated microscopy. Measurements of neurite length (Neurite_Length), the number of neurites (NeuriteCount) and the number of neurite branch points (BPCount) per cell are calculated for each assay well. Decreases in any of these measures are associated with inhibition of neurite outgrowth. The number of neurons per well (NeuronCount) is also measured. Decreases in the number of neurons per well as compared to control is indicative of cytotoxicity.
		2698	2778	NeuriteCount_loss	
		2697	2779	Neurite_Length_loss	
		2696	2780	NeuronCount_loss	

[a] These endpoints are measured using a luminescent plate reader. All other endpoints for all other assays are measured using high-content imaging (HCI). [b] The rat cortical neurite outgrowth and rat cortical neurite maturation and synaptogenesis assays, respectively, are performed in the same *in vitro* cell model. However, the

timing and duration of chemical exposures differs across the respective assays in order to different phases of *in vitro* neuronal development. [c] MAP2 is a cytoskeletal protein that localizes specifically to dendrites: i.e., specialized neurites that receive incoming signals from other neurons in an integrated neuronal network. In primary rat cortical cultures, dendrites develop slower than axons and measurement of dendritic morphology is an indicator of neurite maturation.

All endpoints are generated from the same experimental run and from each well in the 96 well plate.

Overview of analytical method(s) to assess test endpoint(s)

The Cellomics Neuronal Profiling BioApplication utilizes the BR3 and BR4 polarity algorithms for this assay. The purpose of these algorithms is to selectively quantify axon and dendrite lengths in primary cortical cultures during development. Quantitation is based upon the differential labeling patterns observed using antibodies targeted against β_{III}-tubulin. The BR3 and BR4 algorithms are "paired protocols", meaning that images are captured and analyzed with one protocol (BR3) followed by an off-line or "disk-scan" with the second protocol (BR4). These algorithms are appropriate for use in primary cortical cultures grown for 1 to 7 days *in vitro* at densities ranging from 2000 to 10,000 cells / well. Well-level population averages were used as the statistical unit of measure. Complete concentration-response curves for chemical effects on neurite outgrowth and cell viability were generated within a 96-well plate using one well per concentration. Experiments were repeated in triplicate on separate plates. All data were normalized to the vehicle control wells within a plate.

Technical details (of e.g. endpoint measurements)

A Cellomics ArrayScan VTi HCS Reader (Thermo Fisher Scientific) is used for automated image acquisition and analysis of neurite outgrowth (Operating Procedure for High Content Imaging of Neurite Outgrowth: OP-NHEERL-H/ISTD/SBB/TMF/2018-008-r1. Images are acquired using a 20x Pan NeoFLUAR (NA = 0.4) objective with a solid state LED light source, and an XF100 two channel dichroic filter set with excitation at 365(50) and 475(40) and emission at 535(45). Images are analyzed using the Cellomics Neuronal Profiling BioApplication (version 4) to measure neurite morphology. Optimization of nuclear masking and selection, cell body masking and selection, and neurite tracing parameters is performed on untreated cultures at 48 h after initial plating. In each well, multiple unique fields-of-view are acquired until at least 300 neurons are counted. Four morphological features are quantified: 1) number of cells (neurons) per field, 2) total neurite length per neuron, 3) number of neurites per neuron, and 4) number of neurite branch points per neuron. Neurites are defined as processes > 10 µm in length.

Endpoint-specific controls/mechanistic control compounds (MCC)

This assay was developed using the following compounds that had previously been demonstrated to inhibit neurite outgrowth in vitro (see Harrill et al., 2011 for details):

Methylmercury chloride
t-retinoic acid
Bis-indolylmaleimide I (Bis-I)
Lead acetate
U0126
Dexamethasone

In the assay, each experimental plate is equipped with 8 wells of positive controls to verify proper assay

performance. Rac 1 inhibitor is used as a positive control for neurite outgrowth at 10 µM and 30 µM for 4 wells at both concentrations for each plate.

Positive Controls

This assay has been evaluated against 53 compounds that have evidence of DNT in vitro (Harrill et al., 2018). These 53 compounds were selected based on an evaluation of the literature by Mundy et al., 2015. See Harrill et al., 2018 for details on the compounds selected.

Negative and unspecific controls

The following compounds were used as negative/unspecific controls in the development of this assay. They previously had been shown not to alter neurite outgrowth in vitro (see Harrill et al., 2011 for details):

Saccharin sodium salt
Acetaminophen
Glyphosate
Dimethyl Phthalate
Amoxicillin
D-Sorbitol

The solvent control (SC) is used as negative control that is run on each experimental plate. Each SC must be established by comparing the effect of the SC to the effect of the media control. Established solvent controls show the same response as the media control. For each 96-well plate, 8 wells of in-plate solvent controls of 0.1% DMSO are run concurrently with each row of chemicals.

Features relevant for cytotoxicity testing

Primary cortical cultures are a multicellular system consisting of excitatory and inhibitory neurons, glia (oligodendrocytes, astrocytes) and a few microglia. The measurement of cytotoxicity and viability therefore always represents all cells within the culture.

When using the Cellomics Neuronal Profiling BioApplication, Neuron count per valid field is collected as a measure of cell viability by giving a measure of the quantification of live cells in a given area.

Acceptance criteria for the test method

As stated previously, an image analysis protocol is used to automatically identify targeted structures based on preassigned criteria. Changes in the number and length of young outgrowths is quantified; see section 8.2 for criteria for hit calls. Each individual plate is accessed for expected results based on previous experiments.

As with all cell-based experimentation, maintain proper sterile technique and good cell maintenance practices. In plating cells, an aliquot is to be counted and assessed for viability. If less than 85% of the cells are viable, the cells are not used. Wells in which erroneous volumes of treatment compound are added should be discarded. Each time media is prepared, a sterility test plate is prepared by placing at least one 1.0 mL sample of cell media into the plate.

Plates should be monitored for contamination throughout the experiment. Contamination may be indicated by yellow and/or cloudy media. Contaminated wells should be emptied of media and treated with a bleach solution. Any plate with contaminated wells should be monitored more frequently and carefully as contamination can often spread to multiple wells. Data from contaminated wells should not be analyzed.

Throughput estimate

The assay as described below is medium-to-high throughput. It uses primary cortical rat neurons, seeded on a 96-well plate. Each plate may contain 8 test compounds at up to 11 concentrations, in addition to vehicle controls, assay positive controls, and blanks. Cell cultures are exposed to chemicals for 48 hours prior to fixation and analysis. Each experiment should be replicated on three separate plates from the same culture preparation.

Handling details of the test method

Preparation/addition of test compounds

The experimental compounds were each prepared in stock solutions at 1000-fold concentrations of 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, and 30 mM in DMSO, ethanol, or double-distilled H2O (ddH2O) based on solubility. Training chemicals known to be especially potent or to have low solubility at high concentrations were not prepared at high concentrations. Chemicals that were known to be present in vivo at very high concentrations were tested at the appropriate order of magnitude.

Dosing solutions were prepared from each of these stocks by diluting 1/100 into Medium. In a 96 deep well plate, 5 µL of 1000X test chemical is diluted with 495 µL of Neuro Basal medium with B27. Cells were exposed to chemicals 2 hours after plating by diluting a volume of the 10x dosing solution 1/10 into the wells of the 96-well plates. This results in a final vehicle concentration (DMSO, ethanol or water, depending on solubility of the compound) of 0.1%. The cells were maintained in a humidified incubator at 37°C and 5% CO_2. A typical experimental plate layout is illustrated in Figure 6.1.

Stock solutions are aliquoted and stored at -20°C. Freeze/thaw cycles should be avoided with compound stock solutions, therefore it is best to prepare an aliquot of stock solution to be thawed and used once for

each treatment.

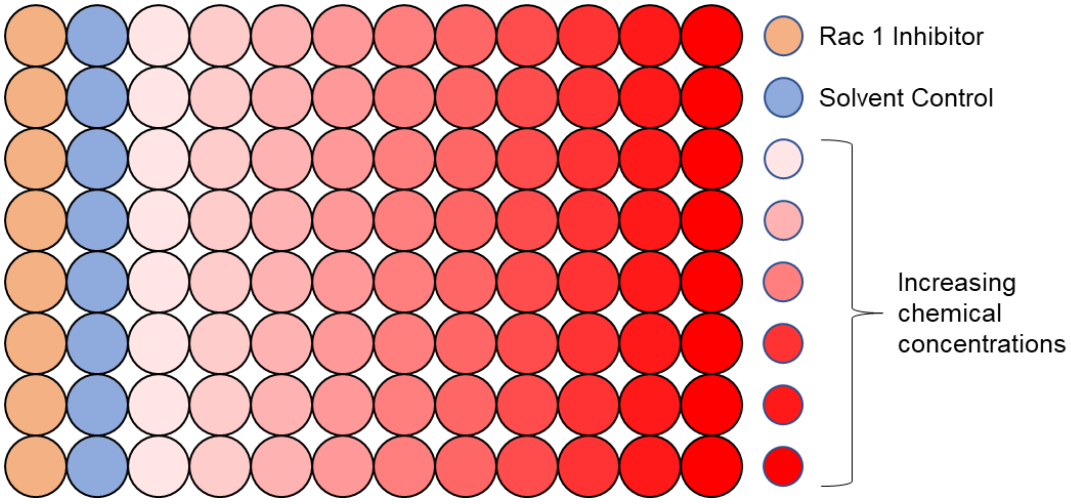


Figure 6.1. Plating Scheme. 10 compound concentrations are plated in a serial dilution from lowest (left) to highest (right) concentration. Positive control (Rac 1 inhibitor) and Solvent Control are plated at 8 replicates each. Rac 1 inhibitor is dosed at 4 wells of 30µM and 4 wells of 10 µM. Solvent control depends on the solvent of the compound that was tested.

Day-to-day documentation of test execution

A plate map for all three plates is created prior to plating cells. All data relevant to the rat cortical neurite outgrowth assay is stored on an online OneNote notebook.

Practical phase of test compound exposure

All aspects of the experiment are recorded in an online OneNote laboratory notebook. This includes any documentation of adherence to platemaps, potential errors, and any other variable that may impact the assay and interpretation of results. Projects are typically subjected to review by EPA Quality Assurance Managers.

Concentration settings

Ten compound concentrations are tested on each plate, with ½ log unit spacing between concentrations (e.g. 10, 3, 1 µM, etc). Standard upper concentrations tested are either 100 or 30 µM, depending on the solubility of the compounds as well as the highest concentration which can be provided in a stock solution from test set providers (e.g. EPA's ToxCast program).

Start concentrations and concentration ranges are defined based on the following factors:

- toxicological relevance of the compound (i.e. internal human exposures, effects at lowest concentrations)
- concentration of stock compound in solutions provided by suppliers of chemical sets (e.g. for EPA's ToxCast library chemicals are typically supplied at 20 mM in DMSO).
- the highest concentration of solvent (e.g. DMSO) that can be tolerated by the assay.
- solubility of the compound

Uncertainties and troubleshooting

Problematic compounds:

- volatile compounds
- high lipophilicity (high K_{OW})
- low solubility in established solvents
- Fluorescent compounds (possible interference with viability and cytotoxicity assay)

Critical handling steps:

- For compounds that may have some volatility, or to ensure against effects due to evaporation of media, plate sealers may be used.
- Networks are sensitive to disruption and this can impact activity, therefore, when manipulating plates at any step, care should be given to prevent jostling as much as possible. Media and other solution changes should be done gently so as not to wash cells off the bottom of the plate.

Sources of variation:

- Many of the steps performed in the Neurite Outgrowth assay are sensitive to pipetting errors. Care should be taken to pipette slowly and steadily to prevent disrupting attached cells.
- Immunocytochemical staining consists of multiple washing steps. Variation can be caused by various aspiration and pipetting steps that are needed to perform staining. Pipetting errors can also cause variation of dilution of antibodies and buffers. Additionally, a slow, consistent aspiration should be used to prevent aspiration of cells.

Known Pitfalls:

- Careful attention should be paid to the age of antibodies used for staining, as old antibodies will yield poor results. Do not use antibodies after their expiration date and store them according to the vendors instructions.

Caveats:

- No extracellular growth “cues” are provided in this assay.
- Different neuronal (sub)populations express different proteins (which may also vary between different time points) and thereby also only a limited number of potential intracellular targets for toxicants are present in these different neurite outgrowth tests (even if testing different neuron types between different tests).

Detailed protocol (SOP)

Dosing: OP-NHEERL-H/ISTD/SBB/TMF/2018-004r1; Chemical exposure of cells in cell culture plates. Immunostaining: OP-NHEERL/ISTD/SBB/TMF/2018-010-r2; Immunocytochemistry on cells in 96 well plates. High Content imaging OP-NHEERL/ISTD/SBB/TMF/2018-008r1; Operating procedure for high content imaging of neurite outgrowth (Available upon request; email: Shafer.tim@epa.gov).

Special instrumentation

- Incubator for cell culture
- Cellomics® ArrayScan® VTI HCS Reader (ThermoFisher Scientific)

Possible variations

Measurement of neurite outgrowth is a common way to assess the effects of chemicals on development of neuronal morphology, and a number of publications are available on this topic (Ryan et al., 2016; Li et al., 2021). Other approaches may be valid for assessment of chemical effects on neurite outgrowth but may or may not have been evaluated to the extent of the assay described herein.

Data management

Raw data format

Image files (*.C01 files) are saved to a network drive. These files can be reanalyzed by re-applying the bioapplication software. The data extracted are saved to a network drive as *.xls (excel) files, with 1 file containing all extracted feature values per experimental plate. The original excel output files are saved for traceability of the data.

Outliers

Mathematical procedures to define outliers are not applied. The tcpl curve fitting program (Filer et al., 2017) is robust with respect to minimizing the impact of outliers.

Data points from wells where technical problems are known or obvious are retained in the data file but are excluded from the analysis by marking them as “well quality 0”.

Some example technical problems:
- pipetting errors
- contamination

Raw data processing to summary data

Bioapplication software analyze the image files and extract the relevant features (neurite length, branch points, etc) and save these data as excel (*.xls) files. R scripts are used to scrape the data from the *.xls files. Data are transformed to the “long” data format, with 1 row for each well-feature pair.

Normalization, Curve fitting and BMC calculation

Data are analyzed using the ToxCast Pipeline (tcpl) approach as described by Filer et al., 2017. A summary of techniques applied is in table 7.

Table 7.4 Methods applied in tcpl for the rat cortical neurite outgrowth assay

ToxCast Data Pipeline Level	HCI assays: Methods Applied
mc0: pre-processed data input	Data are raw input
mc1: mapping to well and column indexes	Auto
mc2: transformation	No transformation
mc3: normalization	Baseline value (bval) was calculated as the median value for the vehicle control wells (DMSO) on a by-plate basis; No positive control value was used in normalization (pval=0); the response was calculated as percent of DMSO vehicle control.
mc4: BMAD	An approximation of noise around the baseline signal, the baseline median absolute deviation, was

calculation type for curve-fitting	calculated based on the vehicle control wells on each plate.
mc5: Hitcall and potency determination	The cutoff for a positive response was the greater of 30% or 3*BMAD.
mc6: caution flags on fitting	Flags for single point hit at maximum concentration (6), flags for single point hit not at the maximum concentration screened (7), inactives with multiple median responses above baseline (8), noisy curves relative to the assay (10), actives with borderline efficacy (11), inactives with borderline efficacy (12), low concentration gain-loss curve-fits (15), possibly overfitting (16), hitcalls with less than 50% efficacy (17), model fits with AC_{50} less than lowest concentration tested (18) were assigned to all; additionally cell viability assays were assigned "viability gain-loss fit" (19)

Internal data storage

Data collected from the Arrayscan VTI are saved as **.C01 files* on a laboratory network drive. This network drive resides on EPA servers which are backed up daily. As per US Government regulations, these files will be maintained for at least 20 years.

Metadata

Metadata is saved in *.xlsx files, with 1 file for each group of 3 plates prepared on the same date. R scripts are used to scrape the metadata from the files, merge the metadata with the experimental data for each well, and save the result in a *.RData file.

Metadata file format

The metadata file format is *.xlsx.

Prediction model and toxicological application

Scientific principle, test purpose and relevance

During the development of the nervous systems, many processes occur to give rise to a functional and healthy neural network and hence nervous system. These important neurodevelopmental processes may be disrupted by potential toxicants, resulting in developmental neurotoxicity. Among these processes is neurite outgrowth – the physical outward growth of neurites (eventually axons and dendrites) of individual neurons that allows them to make connections with other neurons and ultimately gives rise to the physical network of cells that connect the nervous system together. This assay utilizes a high-content imaging solution to describe neurite outgrowth in a rat primary cell culture, via the immunocytochemical labelling of cell bodies and neurites. An automated image analysis protocol is employed to systematically identify targeted structures based on preassigned criteria. Ultimately, changes in the number and length of young outgrowths is quantified and inhibition of more than 30% results in a hit call. The assay is performed on a 96-well plate, allowing for a medium-to-high throughput screening of chemicals.

Prediction model

The cutoff for a positive response in each assay endpoint is set as 3*BMAD or a 30% change from DMSO controls, and compounds with treatment levels reaching this cutoff are then subjected to curve fitting in tcpl, from which AC_{50} values are generated (see table 7.4). The PM is based on a comparison between the AC_{50} value for the NOG-specific endpoint and the AC_{50} value cytotoxicity/viability effect.

Thereby the following classifications apply:

"specific hit": a threefold difference between the AC_{50} value for NOG endpoints and the most potent cytotoxicity endpoint. Where no cytotoxicity endpoint had an AC_{50} value, then the highest concentration tested is used.

"non-specific hit": Less than a threefold difference exists between AC_{50} value for the NOG-specific endpoint and the most potent cytotoxicity AC_{50} value.

It should be noted that there are other valid approaches to determining specificity. For example, one could calculate the area under the curve of the specific endpoint that is below the AC_{50} value for cytotoxicity.

"inactive": the compound was not active in NOG and cytotoxicity endpoints.

Prediction Model Setup

Test Performance

Table 8.4.1 summarizes the assay performance in terms of variability of each endpoint in the assay.

ACID	Assay component name	Median	MAD	CV
2699	MUNDY_HCI_Cortical_NOG_BPCount	3.47	0.28	8.36
2698	MUNDY_HCI_Cortical_NOG_NeuriteCount	2.95	0.04	1.76
2697	MUNDY_HCI_Cortical_NOG_NeuriteLength	128.36	10.45	8.42
2696	MUNDY_HCI_Cortical_NOG_NeuronCount	26.29	1.81	7.6

ACID = Assay Component Identification

For performance assessment in this assay, the following compounds are used as assay positive controls as they have been previously demonstrated to disrupt neurite outgrowth in in vitro systems:

Bisindolylmaleimide I
Lithium Chloride

Table 8.4.2 summarizes the z prime (z'), strictly standardized median deviation (SSMD) and signal-to-noise (SN) for assay positive control compounds.

AENM	MED.RESP	MED.HITC	CHEM	CONC.UM	Z'	SSMD	SN
MUNDY_HCI_Cortical_NOG_NeuriteLength_loss	42.66	1	Bisindolylmaleimide I	3	0.31	4	4.76
MUNDY_HCI_Cortical_NOG_NeuronCount_loss	*18.52*	*0*	*Bisindolylmaleimide I*	*3*	*0*	*0*	*-0.1*
MUNDY_HCI_Cortical_NOG_BPCount_loss	48.39	1	Lithium chloride	3	0.06	3	9.07
MUNDY_HCI_Cortical_NOG_NeuriteCount_loss	24.66	0	Lithium chloride	3	0	3	3.58
MUNDY_HCI_Cortical_NOG_NeuriteLength_loss	42.66	1	Lithium chloride	3	0.3	5	6.98

Abbreviations: AENM=assay endpoint name; MED.RESP=median response; MED.HITC=median hitcall; CHEM=chemical; CONC=concentration of tested CHEM in micromolar; Z'=z prime score; SSMD=strictly standardized median deviation; SN=Signal to noise ratio.

In vitro-in vivo extrapolation

IVIVE of data from this assay has been conducted based on the activity (e.g. AC_{50}) values obtained from curve fitting. Because in vitro toxicokinetic information (e.g. lipid and protein content of cells, volume of cells) are not readily available, these extrapolations have been based on the nominal concentration of test article in the medium. Adjusted Equivalent Doses (AEDs) were estimated using the high-throughput toxicokinetic (HTTK) information and models available in the httk R package (v1.8; Pearce et al., 2017), which functionalizes an approach similar to the one previously used by Wetmore et al. (2012). For complete details, see EPA 2020.

Applicability of test method

Toxicological application domain

To date, 176 unique compounds (as defined by unique DTXSIDs) have been tested successfully in this assay. This includes the following compound classes:

Industrial chemicals
Pesticides and metabolites (e.g. oxons)
pharmaceuticals
metals and organometals

Biological application domain

Next to the endpoints represented by this test method there are several other necessary neurodevelopmental endpoints which need to be studied using other test methods.

Neurodevelopmental processes not represented by this test method:

- Neural Crest Cell (NCC) Migration
- NPC apoptosis
- Synaptogenesis
- Neural Rosette Formation
- hiPSC-derived NPC proliferation
- Network formation
- hiPSC-NPC neuronal differentiation Network formation
- Neuronal subtype differentiation
- Astrocyte Differentiation and Maturation
- Astrocyte Reactivity
- Microglia reactivity

For a complete assessment of developmental neurotoxicity, the test method needs to be part of a test battery.

Incorporation in test battery

To assess the hazard for developmental neurotoxicity it is recommended that this assay is used as one assay in a battery of assays (see 8.6 "Applicability of test methods")

For the assessment of chemical action on the endpoints represented by this test method, the test method can be used as a stand-alone test method.

The test method is currently used in the setup of a DNT test battery.

Publication/validation status

Availability of key publications

Key Publications concerning the test method are:

Druwe I, Freudenrich TM, Wallace K, Shafer TJ and Mundy WR. Comparison of human iPSC-derived neurons and rat primary cortical neurons as in vitro models of neurite outgrowth. Applied in vitro Toxicology, 2016. 2, 26-36.

Harrill JA, Freudenrich TM, Robinette BL, Mundy WR.Comparative sensitivity of human and rat neural cultures to chemical-induced inhibition of neurite outgrowth. Toxicol Appl Pharmacol. 2011 Nov 1;256(3):268-80. doi: 10.1016/j.taap.2011.02.013.

Harrill JA, Freudenrich T, Wallace K, Ball K, Shafer TJ, Mundy WR.Testing for developmental neurotoxicity using a battery of in vitro assays for key cellular events in neurodevelopment. Toxicol Appl Pharmacol. 2018 Sep 1;354:24-39. doi: 10.1016/j.taap.2018.04.001.

Potential linkage to AOPs

Key event #382, aberrant dendritic morphology is relevant to this assay, and is part of the AOP titled: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities.

Steps towards mechanistic validation

a) Information demonstrating how the test system is biologically relevant to humans in terms of cell types, signaling pathways, etc

Formation of neural networks during development is a process that is highly conserved across all mammalian species, including humans. In vitro, neural cultures derived from rodents and human IPSC models develop neural networks over a period of days in culture that include the extension of neurites that ultimately form axons and dendrites. This process occurs in every type of neuron to some extent, and the signaling pathways involved are highly conserved across species.

b) Interventions (pathway knockdown, specific inhibitors (i.e. mechanistic controls, which may be part of the training set) that show expected effects on the assay

This assay has been developed by using mechanistic control compounds known to disrupt neurite outgrowth in cortical neurons (Harrill et al., 2011; Druwe et al., 2016).

c) Formal mechanistic validation

There has been no formal validation of this assay. This test method was developed following the criteria established in Crofton et al., 2011, where a set of assay positive controls has been tested (Harrill et al., 2011), followed by a test set of compounds (Harrill et al., 2018).

d) Is there a correspondence to human (in vivo?) changes?

To date, no specific studies have been conducted with chemicals to demonstrate a correspondence to human in vivo changes.

Pre-validation or validation

No OECD 34 validation study has been done. The test method is part of a pre-validation study that test the DNT hazard assessment for 83 Compounds in a DNT test battery. The compound set includes potential DNT positive and DNT negative compounds.

Linkage to (e.g. OECD) guidelines/regulatory use

Test is not linked to regulatory guidelines.

Test method transferability

Operator training

For operators with a basic training in cell culture practices a four-week training period for handling of the test system and training in the assay is recommended. The operators should have basic understanding in neurobiology, toxicology, image analysis and data evaluation with respect to concentration response fitting.

Transfer

The test method has been used by multiple operators over a period of 5 years. However, inter-operator variability has not been formally determined. In addition, assessment of neurite outgrowth using high content imaging is a widely utilized metric in the published literature, indicating that transfer of this specific protocol would not be difficult.

Safety, ethics and specific requirements

Specific hazards; issues of waste disposal

The Neurite Outgrowth assay itself has no specific hazards. However, chemicals being tested in the NOG may pose both human health and environmental hazards. Therefore, appropriate personal protective equipment should be worn by operators, and appropriate waste disposal practices should be followed.

Safety data sheet (SDS)

SDS should be supplied by the manufacturer or supplier of the chemicals being tested and should be kept on file as appropriate for legal guidelines for the location of the facility where testing is occurring.

Specific facilities/licenses

No specific facilities are required.
Use of live rodents will require the approval of the appropriate institutional animal care committee. Note that many vendors supply frozen primary cortical cells which would avoid this issue. However, the performance of these cells has not been verified in this assay.

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Appendix B.7

Author: Joshua Harrill, Tim Shafer
Date: 10.03.2023
Version: 1.0

Overview

Descriptive full-text title

Synaptogenesis and neurite maturation assay in rat primary cortical neurons.

Abstract

Synaptogenesis is a critical process in nervous system development whereby neurons establish specialized contact sites which facilitate neurotransmission. Early life exposure to chemicals can result in persistent deficits in nervous system function at later life stages. These effects are often the result of abnormal development of synapses. The synaptogenesis and neurite maturation assay apply automated high content image analysis (HCA) technology to examining synapse formation in rodent primary mixed cortical cultures. During the first 15 days in vitro (DIV) cortical neurons develop a network of polarized neurites (i.e., axons and dendrites) and expression of the pre-synaptic protein synapsin increases over time. The localization of punctate synapsin protein in close apposition to dendrites also increases, indicating an increase in synapse formation. According to the readiness criteria as published by Bal-Price et al. (2018) the rat cortical maturation and synaptogenesis assay obtained the readiness score A.

Assay summary:

toxicological target	→	developing brain
test system	→	primary cortical cells from postnatal day 0 rat
readout(s)	→	Neurite count, neurite length, cell body spot count, NeuriteSpotCountPerNeuron, synapse count, NeuriteSpotCountPerNeuriteLength, neuron count
biological process(es)	→	synaptogenesis, neurite maturation viability, cytotoxicity
(human) adverse outcome(s)	→	cognitive dysfunction
hazard(s)	→	adverse effect on synapse formation
endpoint of current regulatory studies	→	no
validation/evaluation	→	readiness analysis

General information

Name of test method

Rat Cortical Synaptogenesis and Neurite Maturation Assay

Version number and date of deposition

20220103_v1.1

Summary of introduced changes in comparison to previous version(s)

“original version”

Assigned data base name

ToxCast invitro database assay identification: *MUNDY_HCI_Cortical_Synap&Neur_Matur*

Name and acronym of the test depositor

United States Environmental Protection Agency (EPA)

Name and email of contact person

Tim Shafer (shafer.tim@epa.gov)

Name of further persons involved

Joshua Harrill (harrill.joshua@epa.gov)

Reference to additional files of relevance

Number of supporting files:

1. Standard Operation Procedure (Section 3.7)

Description of general features of the test system source

Supply of source cells

Rat primary cortical neurons prepared from postnatal day (PND) 0 pups. Pregnant dams are supplied by Charles River Laboratories; delivered to the US Environmental Protection Agency (EPA) facility on gestational day (GD)16 and held in animal colonies until they give birth.

Overview of cell source component(s)

Primary rat cortical cultures are prepared on site from the neocortex dissected from the central nervous system (CNS) of newborn (PND0) Long-Evans rat pups using a standard protocol (Section 3.7). In a typical culture, cells are isolated from the combined cortices of 3-5 pups, seeded onto a Poly-L-Lysine coated 96-well plate at a density of 10,000 cells/well and are allowed 2 hours to attach. The cells are maintained in a

humidified incubator at 37°C and 5% CO_2. Sex of pups is not determined, and cultures are presumed to consist of a mixture of male and female pups since multiple pups are used for each culture.

Characterization and definition of source cells

Primary cortical cultures consist of a mixture of glutamatergic and gabaergic neurons, as well as glial cells (oligodendrocytes and a few microglia) as characterized by immunocytochemistry and functional responses to pharmacological agents (Freudenrich and Mundy, 2000; McConnell et al., 2011; Frank et al., 2017).

Acceptance criteria for source cell population

Before plating the cells, the following criteria must be fulfilled:

- The rat pups used for cortical culture must be less than one day old, preferably newborn.
- Minimum cell viability of 85% verified by trypan blue dye exclusion.
- Cell cultures examined under the microscope are free of microorganisms.

Each time medium is prepared, a 1.0 mL sample is placed onto a sterility plate and incubated at 37°C. Sterility plates are checked daily for contamination and contaminated cell cultures should not be used. Media color changes may indicate contamination or improper CO_2 levels

Variability and troubleshooting of source cells

Some variability is inherent in the system since new cultures are made from different animals for each preparation and hence assay. This would reflect normal biological variation. In addition, during the plating process, cells will randomly distribute and attach to the bottom of the 96-well plate and this will result in random differences in the synapses formed, which may contribute to variability.

Serum-free media which reduces variability due to differences in the content of growth factors and other critical nutrients found in serum.

Each time medium is prepared, a 1.0 mL sample is placed into a plate and incubated at 37ºC to test for sterility. It is strongly recommended that media be made at least 2 days before use. If the media becomes purple or yellow, examine the plate for contamination. When the media is cloudy, this indicates contamination by some microorganism and the media should be discarded. If the media is bright pink to purple but no cloudiness is present, this may indicate a possible problem with the CO2 level in the incubator. Examine other cultures in the incubator for color changes and measure the CO2 level in the incubator with the Fyrite. The plate may also be examined under the microscope for microorganisms.

Critical consumable

The cultivation medium is supplemented with B27. The cultivation medium should be discarded 7 days after addition of the B27 supplement.

The cell culture procedure employs a cortical buffer for digestion, consisting of 137 mM NaCl, 5 mM KCl, 170 µM Na_2HPO_4, 205 µM KH_2PO_4, 5 mM glucose, 59 mM sucrose, and 100 U/mL penicillin/0.1 mg/mL streptomycin (Gibco Cat# 15140-122). A cortical medium is used during cell attachment, consisting of DMEM with GlutaMax (Gibco Cat# 1056-010), 10% Horse Serum, heat inactivated (Gibco Cat. No. 26050-088), 10 mM HEPES, 100 U/mL penicillin/0.1 mg/mL streptomycin. The final media used in the culture, NB/B27 media, consists of 500ml Neurobasal-A Medium (1X, Gibco Cat# 10888), 10ml B-27 Supplement (50X, Gibco 17504-044), 5 ml GlutaMax (100X, Gibco 35050-061), and 5 ml Pen-Strep (Gibco Cat# 15140-122), pH adjusted to 7.4.

Differentiation towards the final test system

Once plated, primary cortical neurons rapidly extend neurites. Given the proper growth conditions, primary cortical neurons will develop extensive neurite networks containing both axons and dendrites as well as synaptic connections (Figure 3.6.1). A stereotypic pattern of neurite outgrowth is observed in primary cortical neurons prepared in dissociated culture. As cortical neurons mature, not only do they undergo periods of extensive neurite outgrowth, the neurites also differentiate into specialized neurite sub-populations: i.e. axons and dendrites. Axons are comparatively smaller caliber neurites whose rapid growth phase initiates prior to the growth and differentiation of dendrites. In primary cortical cultures, axons grow rapidly, branch extensively and form dense complex axon networks through the culture. Dendrites are comparatively larger caliber neurites whose growth phase initiates after the onset of rapid axonal growth. Maturing cortical dendrites can have proximal to distal tapered appearance and a branched appearance.

In addition to difference in the morphology of axon and dendrites, certain cytoskeletal and microtubule associated proteins can be used to distinguish axons from dendrites in maturing and mature cultures. The low molecular weight microtubule associated protein tau, as well as some forms of phosphorylated neurofilaments are selectively expressed in axons. In contrast, the high molecular weight microtubule associated protein MAP2 is selectively expressed in dendrites and neuronal cell bodies. The cytoskeletal protein βIII-tubulin is expressed in both axons and dendrites as well as in neuronal cell bodies. Previous studies in the literature have used the cytoplasmic localization of these proteins to distinguish axons from dendrites. Expression of both pre-synaptic proteins, such as synaptophysin, vGLUT1 (excitatory synaptic vesicle protein) and vGAT (inhibitory synaptic vesicle protein) are also observed in close proximity to MAP2 (to label dendrites) and the post-synaptic protein, PSD95 (Harrill et al., 2011; 2015), indicating the formation of anatomical synapses. Typically, the cultures show an ontogeny of activity that occurs rapidly in the first 12-14 days in vitro (DIV) and then becomes more stable in terms of the network activity thereafter (see Cotterill et al., 2016). Establishment of synchronous activity across the network indicates the formation of functional synapses.

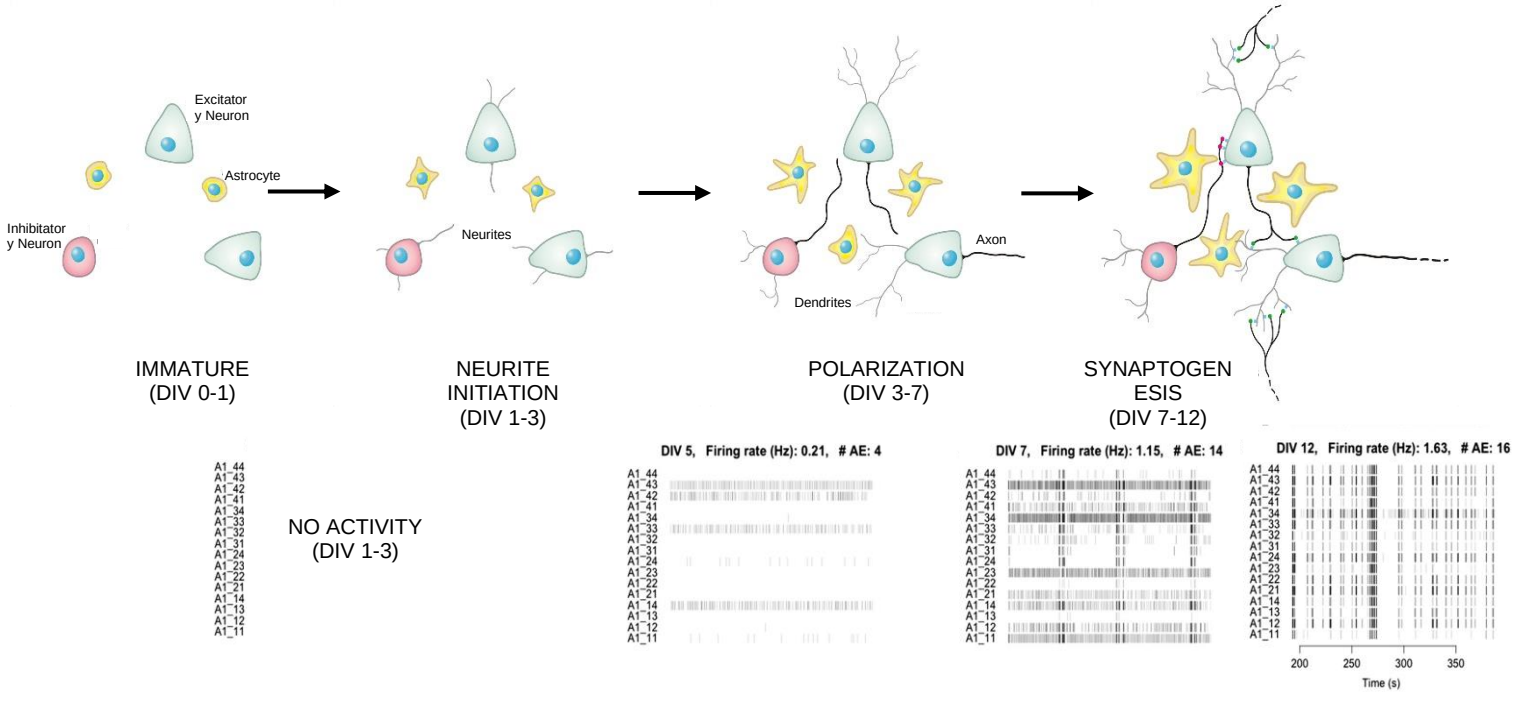

Figure 3.6.1. Schematic representation of the development of cortical cultures (upper panels) over days in vitro (DIV). This illustrates the major cell types in the culture and the morphological changes (neurite initiation, polarization and synaptogenesis). Lower panels: Raster plots illustrating the development of network activity in neuronal cultures over 12 DIV. Control (non-treated) wells from DIV 5, 7, 9, and 12 are shown. In each panel, time is on the x-axis and data from each electrode is plotted in rows on the y-axis. The naming convention is as follows: "A1" indicates that the data are from well

A1 of the plate, while 11, 12, 13, etc. indicate the row and column position, respectively, of the electrodes. A vertical "tick" mark indicates each event on that electrode that exceeds the spike threshold (>8x root mean square noise levels). Heavier shading indicates groups of events occurring closely in time (e.g., bursts). The overall mean firing rate (MFR; in Hz) and number of Active Electrodes for each time point are shown above each panel. Neuronal activity, bursting and coordinated activity (e.g., simultaneous bursts on multiple channels (e.g., "network spikes") increase with time.

Reference/link to maintenance culture protocol

EPA Operating Protocol NHEERL-H/ISTD/SBB/KAW/2017-01-r2 "Cortical cell culture" (Available upon request: Email: Shafer.tim@epa.gov)

Definition of the test system as used in the method

Principles of the culture protocol

The Cortical Maturation and Synaptogenesis assay uses 96-well microtiter plates for the duration of this procedure.

This culture was plated at a seeding density of 10,000 cells/well on a 96-well plate, prepared as described in Section 3.7. Cells were administered via a 90 µL media (DMEM + 10% serum) drop directly onto each of the wells. After a 2-hour attachment period, the plating media was removed and replaced with 90 uL of NeuroBasal media containing B27 and the cells returned to the incubator.

This assay utilizes high-content imaging analysis. Following exposure (see section 5), cells are fixed and nuclei (Hoechst 33342), synapses (synaptophysin) and dendrites (MAP2) are stained using immunocytochemical approaches. Plates are then placed in the Cellomics ArrayScan VTi HCS Reader which is used for automated image acquisition and analysis of synaptogenesis. Bioapplications automatically analyze the images and determine the various parameters reported in section 5.

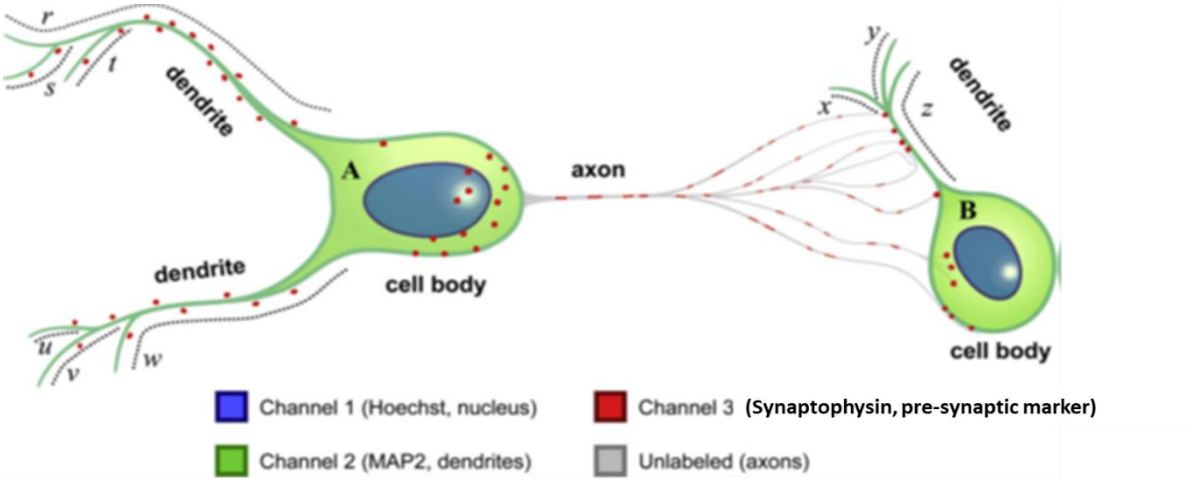

Figure 4.1.1 Morphological features of synaptogenesis. Schematic representation of primary cortical neurons and the morphological features associated with synaptogenesis. Cell bodies are selected based on optimized geometric and fluorescent intensity based parameters using the Bioapplications. Dendrites are separated from cell bodies based on a width gating criteria. Total dendrite length is calculated by adding together the length of non-overlapping line segments

(dotted lines r–z). Punctate synaptophysin labeling is only quantified if it is close apposition to a MAP2 positive cell body or dendrite.

Acceptance criteria for assessing the test system at its start

As noted above, viability of cells should be 85% or greater at the time of plating. Cells will be visually examined for attachment to the substrate, proper cell density, cell health, and any signs of contamination. Some of these parameters are subjective but are based on the experience and knowledge of the individual doing the assay

Acceptance criteria for the test system at the end of compound exposure

For data generated with the ArrayScan to be acceptable for use, a cellular endpoint-specific chemical standard (sodium orthovanadate) will be used as an internal control in the culture plate being used. For any particular endpoint, the chemical standard will be based on the scientist's expertise and understanding of the biology of the endpoint being measured and endpoint-specific data from the literature. The effect of the chemical standard must be within +/- 10% of the expected value (e.g. for an expected chemical result of a 50% change from control, the value should be between 40 to 60 %) to be accepted. If the effect of the chemical standard is outside of this range, the data from that particular culture plate will not be used.

Variability of the test system and troubleshooting

Sources of Variation:
Variability may be due to different numbers of male and female pups selected for each culture. In addition, the random distribution of excitatory and inhibitory cells in the culture, and their random location in each well may contribute to variability in the formation and neurites and synapses that arise in each well.

The variability for the different endpoints is shown in 0 "Test Performance".

Metabolic capacity of the test system

We have not extensively characterized the metabolic capacity of our primary cortical cultures. mRNA expression of various Cyp enzymes are low on DIV 1, however, by DIV 14, mRNA for Cyp 211c >> 4x1 > 2d4 > 1s1 > 1a1. Functional expression of these proteins has not been confirmed (Shafer et al., 2015).

Other metabolic pathways are not characterized. Omics characterization of the test system

Transcriptomic characterization of the test system is currently underway.

Features of the test system that reflect the *in vivo* tissue

Cell model reflects the following in vivo tissue features:

- Presence of excitatory glutamatergic neurons
- Presence of inhibitory gabaergic neurons
- Presence of Glia (astrocytes, microglia)
- A functional switch in $GABA_A$ receptor activation occurs between DIV 6 and 8, wherein prior to this, activation of the receptor is excitatory and drives an increase in intracellular calcium, whereas after this

switch, activation of the receptor is inhibitory and results in an influx of chloride ions into the cells.
- Elaboration of neurites, with subsequent specialization into axons and dendrites (Harrill et al., 2013)
- Formation of synapses (Harrill et al., 2011)

Commercial and intellectual property rights aspects of cells

For primary cortical cells, N/A

The assay procedure as described above is nonproprietary. It contains some proprietary materials, listed below, which the protocol is optimized for. Utilizing materials from other providers may or may not necessitate changes to the procedure, including seeding density, culture media, media change schedule, days in vitro prior to testing, etc. Reference/link to the culture protocol

Reference/link to the culture protocol.

See section 0.

Test method exposure scheme and endpoints

Exposure scheme for toxicity testing

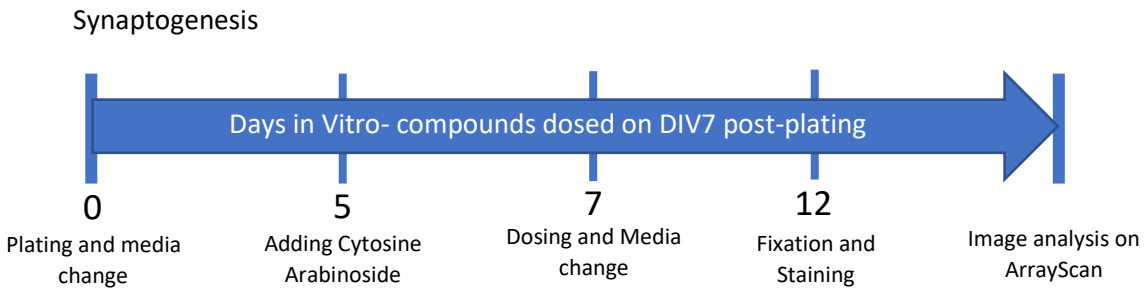

Figure 5.1.1. Exposure scheme for the synaptogenesis assay. Cells are plated onto 96 well plates and allowed 2 hr for attachment to the substrate. At DIV 5, Cytosine Arabinoside (AraC) is added to arrest glial cell growth. Chemicals are dosed and a media change is performed at DIV 7. Cells are fixed with 20% paraformaldehyde and Hoechst Dye. Cells are stained using Millipore Mouse anti-MAP2$_{MAB3418}$ (1:800) and Millipore Rabbit anti-Synaptophysin$_{sc-1750}$ (1:250) primary antibodies and Alexa Fluor-488 goat anti-mouse and AlexaFluor-546 goat anti-rabbit secondary antibodies to label neuronal cell nuclei, synapses and neurites. A Cellomics ArrayScan VTi HCS Reader is used for automated image acquisition and analysis of neurite outgrowth

The assay is conducted as summarized in the plating scheme in Figure 4.1.1. Cells are plated according to the protocol in section 3.7. Exposure to test compounds starts at day in vitro (DIV) 7 of differentiation and is continued through DIV 12 when the experiment is terminated. Cells are fed with fresh medium at day 3 of differentiation (Figure 2). Therefore, half of the test condition solution (e.g. solvent control or compound dilution) is replaced by freshly prepared test condition solution.

Endpoint(s) of the test method

Table 5.2.1 Endpoints assayed in the synaptogenesis assay

Neurodevelopmental Process	Assay Name (*Cell Type*)	ACID	AEID	Endpoint Name	Description
Neurite Maturation and Synaptogenesis [b]	MUNDY_HCI_ Cortical_Synap& Neur_Mature (*1° rat cortical cultures*)	2707	2781	BPCount_loss	Morphology of MAP2 [c] and synapsin labeled neurons as measured using automated microscopy. Measurements of neurite length (NeuriteLength), the number of neurites (NeuriteCount) and the number of neurite branch points (BPCount) per cell are calculated for each assay well. Decreases in any of these measures are associated with inhibition of neurite maturation. In addition, the number of pre-synaptic puncta in the cell body compartment (CellBodySpotCount) and the neurite compartment (NeuriteSpotCountPerNeuron) are counted in each assay well. The number of cell bodies and neurite-associated puncta are combined to calculate the total number of synapses (SynapseCount). The number of neurite-associated puncta are also quantified per unit length of neurite measured (NeuriteSpotCountPerNeuriteLength). Decreases in any of these features are associated with inhibition of synaptogenesis. The number of neurons per well (NeuronCount) is also measured. Decreases in the number
		2702	2782	NeuriteCount_loss	
		2706	2783	NeuriteLength_loss	
		2705	2784	CellBodySpotCount_loss	
		2704	2785	NeuriteSpotCountPerNeuron_loss	
		2703	2786	NeuriteSpotCountPerNeuriteLength_loss	
		2701	2787	SynapseCount_loss	
		2708	2788	NeuronCount_loss	

Neurodevelopmental Process	Assay Name (*Cell Type*)	ACID	AEID	Endpoint Name	Description
					of neurons per well as compared to control is indicative of cytotoxicity.
Endpoints are measured using high-content imaging (HCI). [b] The rat cortical neurite outgrowth and rat cortical neurite maturation and synaptogenesis assays, respectively, are performed in the same *in vitro* cell model. However, the timing and duration of chemical exposures differs across the respective assays in order to differentiate phases of *in vitro* neuronal development. [c] MAP2 is a cytoskeletal protein that localizes specifically to dendrites: i.e., specialized neurites that receive incoming signals from other neurons in an integrated neuronal network. In primary rat cortical cultures, dendrites develop slower than axons and measurement of dendritic morphology is an indicator of neurite maturation.					

All endpoints are generated from the same experimental run and from each well in the 96 well plate.

Overview of analytical method(s) to assess test endpoint(s)

Primary endpoints:

All primary and secondary endpoints are assessed based on an immunocytochemical staining (ICC) of images for each well. Five days after chemical treatment cells were fixed with warm (37°C) 4% paraformaldehyde containing 1.5 µg/ml Hoechst 33342 for 20 min followed by permeabilization and blocking steps. Cell bodies and dendrites were labeled using a rabbit primary antibody for microtubule associated protein 2 (MAP2) (Millipore Catalog AB5622, 1:800) and mouse antibody for synaptophysin (Santa Cruz catalog number SC-17750, 1:200) followed by AlexaFluor 488 goat anti-rabbit secondary antibody (Molecular Probes catalog number A11034, 1:500) and AlexaFluor 546 goat antimouse secondary antibody (Molecular Probes catalog number A11029, 1:500). Well-level population averages were used as the statistical unit of measure. Complete concentration-response curves for chemical effects on neurite outgrowth and cell viability were generated within a 96-well plate using one well per concentration. Experiments were repeated in triplicate on separate plates (technical replicates). All data were normalized to the vehicle control wells within a plate.

Secondary endpoint:

The number of cells per field was used as an indicator of cell viability at the time of fixation. Cellomics Neuronal Profiling BioApplication utilizes the BR3 and BR4 polarity algorithms for this assay. The purpose of these algorithms is to selectively quantify dendrite lengths in primary cortical cultures during development. Quantitation of synaptic puncta and dendrite lengths is based upon the differential labeling patterns observed using antibodies targeted against synaptophysin and MAP2 respectively. The BR3 and BR4 algorithms are “paired protocols”, meaning that images are captured and analyzed with one protocol (BR3) followed by an off-line or “disk-scan” with the second protocol (BR4). These algorithms are appropriate for use in primary cortical cultures grown for up to 12 days in vitro at densities ranging from 2000 to 10,000 cells / well. Well-level population averages were used as the statistical unit of measure. Complete concentration-response curves for chemical effects on dendritic outgrowth and synaptic puncta and cell viability were generated within a 96-well plate using one well per concentration. Experiments were repeated in triplicate on separate plates (technical replicates). All data were normalized to the vehicle control wells within a plate.

Technical details (of e.g. endpoint measurements)

A Cellomics ArrayScan VTi HCS Reader (Thermo Fisher Scientific) is used for automated image acquisition and analysis of cortical maturation and synaptogenesis (Operating Procedure for High Content Imaging of

Neurite Outgrowth: OP-NHEERL-H/ISTD/SBB/TMF/2018-008-r1). Images are acquired using a 20x Pan NeoFLUAR (NA = 0.4) objective with a solid state LED light source, and an XF100 three channel dichroic filter set with excitation at 365(50) and 475(40) and emission at 535(45). Images are analyzed using the Cellomics Neuronal Profiling BioApplication (version 4) to measure neurite morphology. Optimization of nuclear masking and selection, cell body masking and selection, and neurite tracing parameters is performed on untreated cultures at DIV12 after initial plating. In each well, multiple unique fields-of-view are acquired until at least 200 neurons are counted. Eight morphological features are quantified (see table 8.4.1) Neurites are defined as processes > 10 µm in length.

Endpoint-specific controls/mechanistic control compounds (MCC)

This assay was developed using the compounds in Table 5.5.1 that had previously been demonstrated to inhibit neurite maturation in vitro (see Harrill et al., 2011 for details):

Table 5.5.1 Chemicals used as endpoint specific controls (From Harrill et al., 2011. See primary source for all references in this table).

Test chemicals.

Molecular target/pathway	Compound	Source	Pharmacological action	Concentrations tested	References
Neuronal membrane polarization	KCl	Fisher	n/a	0.3, 1, 3, 10, 30 mM	Baker et al. (1991a,b, 1992), Moulder et al. (2003, 2006) and Sohya et al. (2007)
Protein tyrosine phosphatase activity	Na_3VO_4	Sigma–Aldrich	Inhibitor	0.1, 0.3, 1, 3, 10 µM	Dunah et al. (2005), Lee et al. (2010a), Lim et al. (2009)
Cholesterol synthesis	Mevastatin	Calbiochem	Inhibitor	0.3, 1, 3, 10, 30 µM	Fester et al. (2009), Mauch et al. (2001), and Suzuki et al. (2007)
NKCC1 Cl^- transporter activity	Bumetanide	Sigma–Aldrich	Inhibitor	0.1, 0.3, 1, 3, 10, 30 µM	Nakanishi et al. (2007) and Wang and Kriegstein (2008)
Estrogen receptor	Tamoxifen	Calbiochem	Antagonist	0.1, 0.3, 1, 3, 10, 30 µM	Fester et al. (2009), Hu et al. (2007), and Murphy and Segal (1996)
Protein kinase C activity	Bis1	Calbiochem	Inhibitor	0.1, 0.3, 1, 3, 10 µM	Hama et al. (2004), Kuroda et al. (1992), and Meier et al. (2003)
Adenosine transporter activity	Dipyridamole	Calbiochem	Inhibitor	1, 3, 10, 30, 60 µM	Mitchell et al. (1993)
A1 adenosine receptor activity phosphodiesterase activity ryanodine receptor mediated Ca^{+2} release	Caffeine[1]	Calbiochem	Antagonist inhibitor stimulator	0.1, 0.3, 1, 3, 10 mM	Yoshimura (2005)[a]

[a] Caffeine is a synaptic modulator which can affect multiple targets at the concentrations used in the present study.

In the assay, each experimental plate is equipped with 6 wells of positive controls to verify proper assay performance. Sodium Orthovanadate is used as a positive control for neurite maturation at 10 µM in3 wells for each plate.

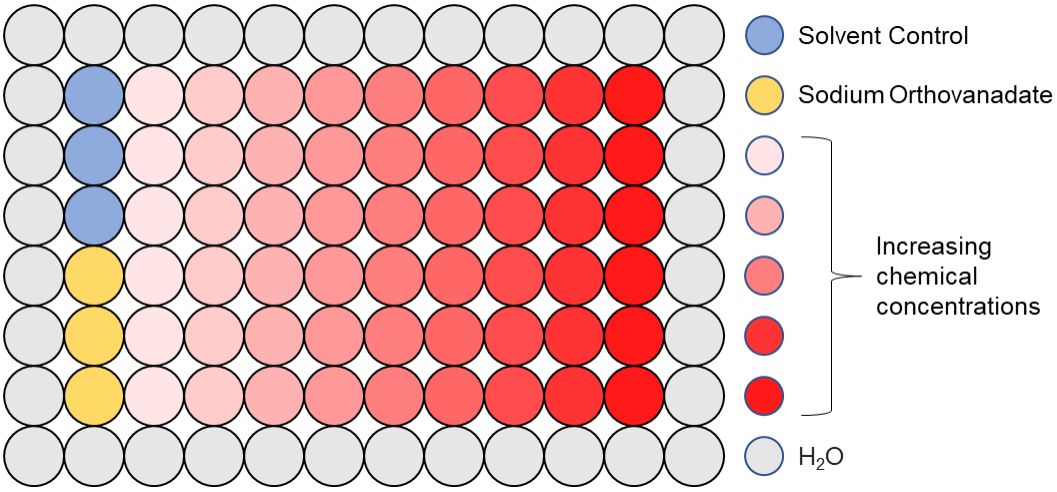

Figure 5.5.1. Plating Scheme. 9 compound concentrations are plated in a serial dilution from lowest (left) to highest (right) concentration. Positive control (Sodium Orthovanadate) and Solvent Control are plated at 3 replicates each. Sodium Orthovanadate is dosed at 3 wells of 10 µM. Solvent control depends on the solvent of the compound that was tested.

Secondary Endpoints:
Solvent controls (typically 0.1% DMSO) are run in triplicate wells on each plate, as indicated in Figure 5.5.1.

Positive Controls

This assay has been evaluated against 53 compounds that have evidence of DNT in vitro (Harrill et al., 2018). These 53 compounds were selected based on an evaluation of the literature by Mundy et al., 2015. See Harrill et al., 2018 for details on the compounds selected.

Negative and unspecific controls

The following compounds were used as negative/unspecific controls in the development of this assay. They previously had been shown not to alter neurite maturation in vitro (see Harrill et al., 2011 for details):

Saccharin sodium salt
Acetaminophen
Glyphosate
Dimethyl Phthalate
Amoxicillin
D-Sorbitol

The solvent control (SC) is used as negative control that is run on each experimental plate. Each SC must be established by comparing the effect of the SC to the effect of the media control. Established solvent controls show the same response as the media control. For each 96-well plate, 3 wells of in-plate solvent controls of 0.1% DMSO are run concurrently with each row of chemicals.

Features relevant for cytotoxicity testing

Primary cortical cultures are a multicellular system consisting of excitatory and inhibitory neurons, glia (oligodendrocytes, astrocytes) and a few microglia. The measurement of cytotoxicity and viability therefore always represents all cells within the culture.

When using the Cellomics Neuronal Profiling BioApplication, Neuron count per valid field is collected as a measure of cell viability by giving a measure of the quantification of live cells in a given area.

Acceptance criteria for the test method

As stated previously, an image analysis protocol is used to automatically identify targeted structures based on preassigned criteria. Changes in the number and length of neurites, synaptic puncta and synaptic puncta per unit length or per cell body are quantified (see table 8.4.1 for all endpoints); see section 8.2 for criteria for hit calls. Each individual plate is accessed for expected results based on previous experiment.

As with all cell-based experimentation, maintain proper sterile technique and good cell maintenance practices. In plating cells, an aliquot is to be counted and assessed for viability. If less than 85% of the cells are viable, the cells are not used. Wells in which erroneous volumes of treatment compound are added should be discarded. Each time media is prepared, a sterility test plate is prepared by placing at least one 1.0 mL sample of cell media into the plate.

3. Plates should be monitored for contamination throughout the experiment. Contamination may be indicated by yellow and/or cloudy media. Contaminated wells should be emptied of media and treated with a bleach solution. Any plate with contaminated wells should be monitored more frequently and carefully as contamination can often spread to multiple wells. Data from contaminated wells should not be analyzed

Throughput estimate

The assay as described below is medium-to-high throughput. It uses primary cortical rat neurons, seeded on a 96-well plate. Each plate may contain 6 test compounds at up to 9 concentrations, in addition to vehicle controls, assay positive controls, and blanks. Cell cultures are exposed to chemicals for 5 days prior to fixation and analysis. Each experiment should be replicated on three separate plates from the same culture preparation.

Handling details of the test method

Preparation/addition of test compounds

The experimental compounds were each prepared in stock solutions at 1000-fold concentrations of 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, and 30 mM in DMSO, ethanol, or double-distilled H2O (ddH2O) based on solubility. Training chemicals known to be especially potent or to have low solubility at high concentrations were not prepared at high concentrations. Chemicals that were known to be present in vivo at very high concentrations were tested at the appropriate order of magnitude.

Dosing solutions were prepared from each of these stocks by diluting 1/100 into Medium. In a 96 deep well plate, 5 µL of 1000X test chemical is diluted with 495 µL of Neuro Basal medium with B27. Cells were exposed to chemicals on DIV7 by diluting a volume of the 10x dosing solution 1/10 into the wells of the 96-well plates. This results in a final vehicle concentration (DMSO, ethanol or water, depending on solubility of the compound) of 0.1%. The cells were maintained in a humidified incubator at 37°C and 5% CO2. A typical experimental plate layout is illustrated in Figure 5.5.1.

Stock solutions are aliquoted and stored at -20°C. Freeze/thaw cycles should be avoided with compound

stock solutions, therefore it is best to prepare an aliquot of stock solution to be thawed and used once for each treatment.

Day-to-day documentation of test execution

A plate map for all three plates is created prior to plating cells. All data relevant to the rat cortical maturation and synaptogenesis assay is stored on an online OneNote notebook.

Practical phase of test compound exposure

All aspects of the experiment are recorded in an online OneNote laboratory notebook. This includes any documentation of adherence to platemaps, potential errors, and any other variable that may impact the assay and interpretation of results. Projects are typically subjected to review by EPA Quality Assurance Managers.

Concentration settings

Nine compound concentrations are tested on each plate, with ½ log unit spacing between concentrations (e.g. 10, 3, 1 uM, etc). Standard upper concentrations tested are either 100 or 30 uM, depending on the solubility of the compounds as well as the highest concentration which can be provided in a stock solution from test set providers (e.g. EPA's ToxCast program)

Start concentrations and concentration ranges are defined based on the following factors:

- toxicological relevance of the compound (i.e. internal human exposures, effects at lowest concentrations)
- concentration of stock compound in solutions provided by suppliers of chemical sets (e.g. for EPA's ToxCast library chemicals are typically supplied at 20 mM in DMSO).
- the highest concentration of solvent (e.g. DMSO) that can be tolerated by the assay.
- solubility of the compound (highest useable solvent concentration)

Uncertainties and troubleshooting

Problematic compounds:

- volatile compounds
- high lipophilicity (high K_{OW})
- low solubility in established solvents
- Fluorescent compounds (possible interference with viability and cytotoxicity assay)

Critical handling steps:

- For compounds that may have some volatility, or to ensure against effects due to evaporation of media, plate sealers may be used.
- Networks are sensitive to disruption and this can impact activity, therefore, when manipulating plates at any step, care should be given to prevent jostling as much as possible. Media and other solution changes should be done gently so as not to wash cells off the bottom of the plate.

Sources of variation:

- Many of the steps performed in the Neurite Maturation assay are sensitive to pipetting errors. Care should be taken to pipette slowly and steadily to prevent disrupting attached cells.
- Immunocytochemical staining consists of multiple washing steps. Variation can be caused by various aspiration and pipetting steps that are needed to perform staining. Pipetting errors can also cause

variation of dilution of antibodies and buffers. Additionally, a slow, consistent aspiration should be used to prevent aspiration of cells.

Known Pitfalls:

- Careful attention should be paid to the age of antibodies used for staining, as old antibodies will yield poor results. Do not use antibodies after their expiration date and store them according to the vendors instructions.

Detailed protocol (SOP)

Dosing: OP-NHEERL-H/ISTD/SBB/TMF/2018-004r1; Chemical exposure of cells in cell culture plates. Immunostaining: OP-NHEERL/ISTD/SBB/TMF/2018-010-r2; Immunocytochemistry on cells in 96 well plates. High Content imaging: High content imaging-synaptogenesis (SOP currently in process, number to be assigned) (Available upon request; email: Shafer.tim@epa.gov).

Special instrumentation

- Incubator for cell culture
- Cellomics® ArrayScan® VTI HCS Reader (ThermoFisher Scientific)

Possible variations

Synaptogenesis can be quantified in rat hippocampal cells by similar approaches (Harrill et al., 2015), and assays for synaptogenesis also are available for human inducible pluripotent stem (IPS) cell-derived neurons (Pistolatto et al., 2020). However, assays in hippocampal and human IPS neurons have not been evaluated as extensively as this protocol.

Data management

Raw data format

Image files (*.C01 files) are saved to a network drive. These files can be reanalyzed by re-applying the bioapplication software. The data extracted are saved to a network drive as *.xls (excel) files, with 1 file containing all extracted feature values per experimental plate. The original excel output files are saved for traceability of the data.

Outliers

Mathematical procedures to define outliers are not applied. The tcpl curve fitting program (Filer et al., 2017) is robust with respect to minimizing the impact of outliers.

Data points from wells where technical problems are known or obvious are retained in the data file but are excluded from the analysis by marking them as "well quality 0".

Some example technical problems:
pipetting errors
contamination

Raw data processing to summary data

Bioapplication software analyze the image files and extract the relevant features (neurite length, synaptic puncta, etc) and save these data as excel (*.xls) files. R scripts are used to scrape the data from the *.xls files. Data are transformed to the "long" data format, with 1 row for each well-feature pair.

Normalization, Curve fitting and BMC calculation

Data are analyzed using the ToxCast Pipeline (tcpl) approach as described by Filer et al., 2017. A summary of techniques applied is in table 7.4

Table 7.4.1. Methods applied in tcpl for the rat cortical maturation and synaptogenesis assay.

ToxCast Data Pipeline Level	HCI assays: Methods Applied
mc0: pre-processed data input	Data are raw input
mc1: mapping to well and column indexes	Auto
mc2: transformation	No transformation
mc3: normalization	Baseline value (bval) was calculated as the median value for the vehicle control wells (DMSO) on a by-plate basis; No positive control value was used in normalization (pval=0); the response was calculated as percent of DMSO vehicle control.
mc4: BMAD calculation type for curve-fitting	An approximation of noise around the baseline signal, the baseline median absolute deviation, was calculated based on the vehicle control wells on each plate.
mc5: Hitcall and potency determination	The cutoff for a positive response was the greater of 30% or 3*BMAD.
mc6: caution flags on fitting	Flags for single point hit at maximum concentration (6), flags for single point hit not at the maximum concentration screened (7), inactives with multiple median responses above baseline (8), noisy curves relative to the assay (10), actives with borderline efficacy (11), inactives with borderline efficacy (12), low concentration gain-loss curve-fits (15), possibly overfitting (16), hitcalls with less than 50% efficacy (17), model fits with AC_{50} less than lowest concentration tested (18) were assigned to all; additionally cell viability assays were assigned "viability gain-loss fit" (19)

Internal data storage

Data collected from the Arrayscan VTI are saved as **.C01 files* on a laboratory network drive. This network drive resides on EPA servers which are backed up daily. As per US Government regulations, these files will be maintained for at least 20 years.

Metadata

Metadata is saved in *.xlsx files, with 1 file for each group of 3 plates prepared on the same date. R scripts are used to scrape the metadata from the files, merge the metadata with the experimental data for each well, and save the result in a *.RData file.

Metadata file format

Metadata is saved in *.xlsx files.

Prediction model and toxicological application

Scientific principle, test purpose and relevance

During the development of the nervous system, many processes occur to give rise to a functional and healthy neural network and hence nervous system. These important neurodevelopmental processes may be disrupted by potential toxicants, resulting in developmental neurotoxicity. Among these processes is synaptogenesis, where individual cells form close connections that allow for communication by chemical neurotransmitter. These interconnections between groups of neurons give rise to networks of cells that connect the nervous system together. This assay utilizes a high-content imaging solution to describe synaptogenesis in a rat primary cell culture, via the immunocytochemical labelling of cell bodies, neurites and presynaptic structures. An automated image analysis protocol is employed to systematically identify targeted structures based on preassigned criteria. Ultimately, changes in the number of synaptic connections are quantified and inhibition of more than 30% results in a hit call. The assay is performed on a 96-well plate, allowing for a medium-to-high throughput screening of chemicals.

Prediction model

The cutoff for a positive response in each assay endpoint is set as 3*BMAD (Baseline median absolute deviation) or a 30% change from DMSO controls, and compounds with treatment levels reaching this cutoff are then subjected to curve fitting in tcpl, from which AC50 values are generated (see table 7.4). The PM is based on a comparison between the AC50 value for the maturation- and synaptogenesis -specific endpoint and the AC50 value cytotoxicity/viability effect. For this assay, the cell body spot count (Table 8.4.2) serves as the measure of cytotoxicity as this represents a loss of cell bodies (e.g. neurons).

Thereby the following classifications apply:

“specific hit”: a threefold difference between the AC50 value for NOG endpoints and the most potent cytotoxicity endpoint. Where no cytotoxicity endpoint had an AC50 value, then the highest concentration tested is used.

“non-specific hit”: Less than a threefold difference exists between AC50 value for the NOG-specific endpoint and the most potent cytotoxicity AC50 value.

It should be noted that there are other valid approaches to determining specificity. For example, one could calculate the area under the curve of the specific endpoint that is below the AC50 value for cytotoxicity.

“inactive”: the compound was active in NOG and cytotoxicity endpoints.

Prediction model setup

This assay was developed using a training set of chemicals (see Harrill et al., 2011), and then further evaluated with a test set of chemicals that had 33 putative positive and 13 putative negative DNT chemicals (see Harrill et al., 2018). See sections below for additional details.

All endpoints in this assay are fit in the down direction. For the viability endpoints, fitting in the up direction (increased viability) is not logical since viability of controls is typically quite high (>90%). The synaptogenesis parameters, can be fit in both the up and down direction. However, to date, the vast majority of compounds tested cause decreases in these parameters, which can be interpreted as decreased synapse formation, and for which we have assay positive controls. Biological meaning of changes in the up direction (increased synapse parameters) is difficult to interpret due to the lack of assay positive controls that alter parameters in

the up direction.

Test Performance

Table 1.1 summarizes the assay performance in terms of reproducibility of the assay, while Table 8.4.2 summarizes performance of the assay in terms of median z', median strictly standardized median deviation and median signal-to-noise (SN).

Table 8.4.1 Assay variability quantified as median, median absolute deviation (MAD) and coefficient of variance (CV) of control wells.

ACID	Assay component name	Median	MAD	CV
2707	MUNDY_HCI_Cortical_Synap&Neur_Matur_BPCount	10.33	0.78	7.39
2706	MUNDY_HCI_Cortical_Synap&Neur_Matur_NeuriteCount	4.88	0.17	3.66
2705	MUNDY_HCI_Cortical_Synap&Neur_Matur_NeuriteLength	396.04	28.48	9.41
2702	MUNDY_HCI_Cortical_Synap&Neur_Matur_CellBodySpotCount	14.14	1.69	13.91
2703	MUNDY_HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuron	64.17	12.26	16.24
2708	MUNDY_HCI_Cortical_Synap&Neur_Matur_SynapseCount	77.69	16.62	16.56
2704	MUNDY_HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuriteLength	0.18	0.01	8.24
2701	MUNDY_HCI_Cortical_Synap&Neur_Matur_NeuronCount	23.42	1.24	5.41

Table 8.4.2 Assay performance

aeid	AENM	CHEM	DTXSID	CONC.(uM)	Z'	SSMD	SN
2782	CellBodySpotCount_loss	Bisindolylmaleimide I	DTXSID50157932	3.02	0	-1	-1.41
2784	NeuriteLength_loss	Bisindolylmaleimide I	DTXSID50157932	3.02	0.3	5	4.91
2785	NeuriteSpotCountPerNeuriteLength_loss	Bisindolylmaleimide I	DTXSID50157932	3.02	0	0	-0.01
2786	NeuriteSpotCountPerNeuron_loss	Bisindolylmaleimide I	DTXSID50157932	3.02	0.29	5	6.31
2787	NeuronCount_loss	Bisindolylmaleimide I	DTXSID50157932	3.02	0	-2	-2.88
2788	SynapseCount_loss	Bisindolylmaleimide I	DTXSID50157932	3.02	0.34	5	5.99
2781	BPCount_loss	Sodium orthovanadate	DTXSID2037269	10	0	3	5.14
2782	CellBodySpotCount_loss	Sodium orthovanadate	DTXSID2037269	3.02	0	0	0.62
2783	NeuriteCount_loss	Sodium orthovanadate	DTXSID2037269	10	0	2	2.87
2784	NeuriteLength_loss	Sodium orthovanadate	DTXSID2037269	3.02	0	3	5.77
2785	NeuriteSpotCountPerNeuriteLength_loss	Sodium orthovanadate	DTXSID2037269	3.02	0	1	0.67
2786	NeuriteSpotCountPerNeuron_loss	Sodium orthovanadate	DTXSID2037269	3.02	0	2	1.95
2787	NeuronCount_loss	Sodium orthovanadate	DTXSID2037269	3.02	0	1	1.99
2788	SynapseCount_loss	Sodium orthovanadate	DTXSID2037269	3.02	0	1	1.28

Abbreviations: aeid=assay endpoint identification; AENM=assay endpoint name; CHEM=chemical; DTXSID= Distributed Substance-Searchable Toxicity database (DSSTox) substance identifier; CONC=concentration of tested CHEM in micromolar; Z'=z prime score; SSMD=strictly standardized median deviation; SN=Signal to noise ratio.

In vitro – in vivo extrapolation (IVIVE)

To date, IVIVE has been based on the nominal concentration of test compound in the media as information

on the in vitro toxicokinetics are not well established. The bioactivity of the in vitro DNT new approach methodologies (NAM) data was transformed into administered equivalent doses (AEDs) using high-throughput toxicokinetic (HTTK) data and models following the principles of reverse dosimetry (Bell et al., 2018; Sipes et al., 2017; Wambaugh et al., 2018; Wetmore et al., 2012). This methodological approach, was used to compute an AED in units of milligram per kilogram bodyweight per day (mg/kg/day) from the NAM-derived concentration at 50% maximal activity (log10-AC50). The IVIVE approach relies on several high-level assumptions: 1) a nominal in vitro assay concentration approximates an in vivo plasma concentration that would correspond to a similar effect; 2) in vivo plasma concentration can be approximated based on steady-state kinetics; and, 3) a toxicokinetic model can be constructed using estimates of species-specific physiology and Phase I and Phase II enzyme-driven hepatic clearance. The HTTK information was built into the "httk" R package (version 2.0.3) (Pearce, et al. 2017) which uses Monte Carlo simulation to incorporate population variability in the model.

Applicability of test method

Toxicological application domain

To date, 176 unique compounds (as defined by unique DTXSIDs) have been tested successfully in this assay. This includes the following compound classes:

Industrial chemicals
Pesticides and metabolites (e.g. oxons)
pharmaceuticals
metals and organometals

Biological application domain

Next to the endpoints represented by this test method there are several other necessary neurodevelopmental endpoints which need to be studied using other test methods.

Neurodevelopmental processes not represented by this test method:

- Neural Crest Cell (NCC) Migration
- NPC apoptosis
- Neural Rosette Formation
- hiPSC-derived NPC proliferation
- Network formation
- hiPSC-NPC neuronal differentiation Network formation
- Neuronal subtype differentiation
- Astrocyte Differentiation and Maturation
- Astrocyte Reactivity
- Microglia reactivity

For a complete assessment of developmental neurotoxicity, the test method needs to be part of a test battery.

Incorporation in test battery

To assess the hazard for developmental neurotoxicity it is recommended that this assay is used as one assay in a battery of assays (see 0 "Applicability of test methods")

For the assessment of chemical action on the endpoints represented by this test method, the test method can be used as a stand-alone test method.

The test method is currently used in the setup of a DNT test battery.

Publication/validation status

Availability of key publications

Key Publications concerning the test method are:

Harrill JA, Robinette BL, Mundy WR. Use of high content image analysis to detect chemical-induced changes in synaptogenesis in vitro. Toxicol In Vitro. 2011 Feb;25(1):368-87. doi: 10.1016/j.tiv.2010.10.011.

Harrill JA, Freudenrich T, Wallace K, Ball K, Shafer TJ, Mundy WR. Testing for developmental neurotoxicity using a battery of in vitro assays for key cellular events in neurodevelopment. Toxicol Appl Pharmacol. 2018 Sep 1;354:24-39.

Potential linkage to AOPs

This assay can be linked to Key Event #385 “Decreased Synaptogenesis” in the AOP Wiki database. This KE is found in two AOPs, both related to neurodevelopment:

AOP 13. Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities

AOP 54. Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment

Steps towards mechanistic validation

a) Information demonstrating how the test system is biologically relevant to humans in terms of cell types, signaling pathways, etc

Formation of synapses during development is a process that is highly conserved across all mammalian species, including humans. In vitro, neural cultures derived from rodents and human inducible pluripotent stem cell models develop neural networks over a period of days in culture that include the extension of neurites that ultimately form axons and dendrites and structural and functional synapses. This process occurs in every type of neuron to some extent, and the signaling pathways involved are highly conserved across species.

b) Interventions (pathway knockdown, specific inhibitors (i.e. mechanistic controls, which may be part of the training set) that show expected effects on the assay

This assay has been developed by using mechanistic control compounds known to disrupt neurite maturation in cortical neurons (Harrill et al., 2011; 2015).

c) Formal mechanistic validation

There has been no formal validation of this assay. This test method was developed following the criteria established in Crofton et al., 2011, where a set of assay positive controls has been tested (Harrill et al., 2011), followed by a test set of compounds (Harrill et al., 2018).

d) Is there a correspondence to human (in vivo?) changes?

To date, no specific studies have been conducted with chemicals to demonstrate a correspondence

to human in vivo changes.

Pre-validation or validation

No OECD 34 validation study has been done. The test method is part of a pre-validation study that test the DNT hazard assessment for 83 Compounds in a DNT test battery. The compound set includes potential DNT positive and DNT negative compounds.

Linkage to (e.g. OECD) guidelines/regulatory use

Test is not linked to regulatory guidelines.

Test method transferability

Operator training

For operators with a basic training in cell culture practices a four-week training period for handling of the test system and training in the assay is recommended. The operators should have basic understating in image analysis and data evaluation with respect to concentration response fitting.

Transfer

The test method has been used by multiple operators over a period of >5 years. However, inter-operator variability has not been formally determined.

Safety, ethics and specific requirements

Specific hazards; issues of waste disposal

The Neurite Maturation and Synaptogenesis assay itself has no specific hazards. However, chemicals being tested in the NOG may pose both human health and environmental hazards. Therefore, appropriate personal protective equipment should be worn by operators, and appropriate waste disposal practices should be followed.

Safety data sheet (SDS)

SDS should be supplied by the manufacturer or supplier of the chemicals being tested and should be kept on file as appropriate for legal guidelines for the location of the facility where testing is occurring

Specific facilities/licenses

No specific facilities are required.
Use of live rodents will require the approval of the appropriate institutional animal care committee. Note that many vendors supply frozen primary cortical cells which would avoid this issue. However, the performance of these cells has not been verified in this assay.

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Appendix B.8

Author: Tim Shafer
Date: 10.03.2023
Version: 1

Overview

Descriptive full-text title

Multiwell Microelectrode Array to Screen for Developmental Neurotoxicity in Rat Cortical Neurons via Assessing Changes in Neural Network Formation Parameters During Extended Chemical Exposure

Abstract

This Multiwell Microelectrode Array (mwMEA) Network Formation Assay was developed to screen large numbers of compounds for potential to disrupt the formation of interconnected networks of neurons ("neural networks") development in vitro. During the development of the nervous system, many processes occur to give rise to a functional and healthy neural network. These important neurodevelopmental processes may be disrupted by potential toxicants, resulting in developmental neurotoxicity. This assay is designed to monitor for disruption of the formation of functional neural network activity over a developmental period; during both in vivo and in vitro neurodevelopment, neurons elaborate processes and form synapses which connect them together, forming networks of neurons that communicate with one another. Assessment of neural network formation is achieved by treating primary cortical cultures on a 48-well mwMEA plate with chemicals of interest and monitoring changes in electrical activity over a twelve-day period during development of network activity. This assay provides an assessment of seventeen parameters that describe different aspects of network activity. This assay employs a multiplexed approach, which includes in-well assessments of cell viability and cell health. The use of a 48 well plate allows for a medium-throughput screening of chemicals. According to the readiness criteria as published by Bal-Price et al. (2018) the network formation assay obtained the readiness score A.

Assay summary:

toxicological target	→ developing brain
test system	→ Rat primary cortical cells from PND0 rat pups
readout(s)	→ multiple readouts related to general activity, bursting and connectivity of neurons
biological process(es)	→ network formation, neurite outgrowth, synaptogenesis, functional connections, cytotoxicity
(human) adverse outcome(s)	→ CNS dysfunction

hazard(s)	→	adverse effect on functional measures of network activity
endpoint of current regulatory studies	→	no
validation/evaluation	→	readiness analysis

General information

Name of test method

Rat Cortical Network Formation Assay (NFA)

Version number and date of deposition

20211215_v1.1

Summary of introduced changes in comparison to previous version(s)

“original version”

Assigned data base name

ToxCast Invitro database assay ID: *CCTE_Shafer_MEA_dev; CCTE_Shafer_MEA_LDH (cytotoxicity); CCTE_Shafer_MEA_AB (cytotoxicity)*

Name and acronym of the test depositor

United States Environmental Protection Agency (USEPA)

Name and email of contact person

Tim Shafer (shafer.tim@epa.gov)

Name of further persons involved

Seline Choo
Kathleen Wallace
Theresa Freudenrich

Reference to additional files of relevance

Number of supporting files:

2. Network Formation Assay Standard Operation Procedure (Section 3.7)

Description of general features of the test system source

Supply of source cells

Rat primary cortical neurons prepared from post natal day (PND) 0 pups obtained from pregnant Long Evans rats purchased from Charles River Laboratories delivered to the US EPA facility on gestational day (GD)16 and held in animal colonies until they give birth.

Overview of cell source component(s)

Primary rodent cortical cell cultures are prepared on site from the neocortex dissected from the central nervous system of newborn rats using a standardized protocol (Section 3.7). In a typical culture, cells are isolated from the combined cortices of 3-5 pups, plated onto multiwell microelectrode array plates and allowed 2 hrs to attach. The cells are maintained in a humidified incubator at 37°C and 5% CO_2. Sex of pups is not determined and cultures are presumed to consist of a mixture of male and female pups since multiple pups are used for each culture.

Characterization and definition of source cells

Primary cortical cultures consist of a mixture of glutamatergic and gabaergic neurons, as well as glial cells (oligodendrocytes and a few microglia) as charqacterized by immunocytochemistry and functional responses to pharmacological agents. (Mundy and Freudenrich, 2000; McConnell et al., 2011; Frank et al., 2017).

Acceptance criteria for source cell population

Before plating the cells, the following criteria must be fulfilled:

- The rat pups used for cortical culture must be less than one day old, preferably newborn.
- Minimum viability of 85% verified by trypan blue exclusion
- Cell cultures examined under the microscope are free of microorganisms.
- Each time medium is prepared, a 1.0 mL sample is placed onto a sterility plate and incubated at 37°C. Sterility plates are checked daily for contamination and contaminated cell cultures should not be used. Media color changes may indicate contamination or improper CO_2 levels.

Cultures are checked daily for signs of microbial contamination. Contaminated wells are cleaned out.

Variability and troubleshooting of source cells

Some variability is inherent in the system since new cultures are made from different animals for each preparation and hence assay. This would reflect normal biological variation. In addition, during the plating process, cells will randomly distribute and attach to the bottom of the MEA plate and this will result in random differences in the networks formed, which may contribute to variability.

Serum-free media which reduces variability due to differences in the content of growth factors and other critical nutrients found in serum.

Each time medium is prepared, a 1.0 mL sample is placed into a plate and incubated at 37ºC to test for sterility. It is strongly recommended that media be made at least 2 days before use. If the media becomes purple or yellow, examine the plate for contamination. When the media is cloudy, this indicates contamination by some microorganism and the media should be discarded. If the media is bright pink to purple but no cloudiness is present, this may indicate a possible problem with the CO2 level in the incubator. Examine other cultures in the incubator for color changes and measure the CO2 level in the incubator with the Fyrite. The plate may also be examined under the microscope for microorganisms.

Critical consumable
The cultivation medium is supplemented with B27. The cultivation medium should be discarded 7 days after

addition of the B27 supplement.

The cell culture procedure employs a cortical buffer for digestion, consisting of 137 mM NaCl, 5 mM KCl, 170 µM Na_2HPO_4, 205 µM KH_2PO_4, 5 mM glucose, 59 mM sucrose, and 100 U/mL penicillin/0.1 mg/mL streptomycin (Gibco Cat# 15140-122). A cortical medium is used during cell attachment, consisting of DMEM with GlutaMax (Gibco Cat# 1056-010), 10% Horse Serum, heat inactivated (Gibco Cat. No. 26050-088), 10 mM HEPES, 100 U/mL penicillin/0.1 mg/mL streptomycin. The final media used in the culture, NB/B27 media, consists of 500ml Neurobasal-A Medium (1X, Gibco Cat# 10888), 10ml B-27 Supplement (50X, Gibco 17504-044), 5 ml GlutaMax (100X, Gibco 35050-061), and 5 ml Pen-Strep (Gibco Cat# 15140-122), pH adjusted to 7.4.

Critical handling:

Network activity is sensitive to disturbances. Therefore, it is important to allow the plates to have time (minimum of 15 mins) to equilibrate activity following movement from the incubator to the Maestro recording device. In addition, temperature and CO_2 levels should be maintained as changes in these parameters can also impact network activity. The Maestro device does have environmental controls for these two parameters, but the operator must ensure that they are activated and that CO_2 supplies do not run out during an experiment.

Differentiation towards the final test system

Cortical cells are plated at a high density in the center of the well where the microelectrodes are located. At early days in vitro neurons in the culture spontaneously extend neurites (neurite initiation), which become axons and dendrites (polarization) after a few days. Finally, synaptic connections are made between days in vitro (DIV) 7 and 15. Neurons become electrically active over the same time-frame. Electrical activity begins as unorganized, random events first observed on DIV 3-5, and increases thereafter and becomes progressively more organized into bursts on individual electrodes and organized (synchronous) bursts across all of the electrodes in a single well (Figure 3.6.1). Typically, the cultures show an ontogeny of activity that occurs rapidly in the first 12-14 days in vitro (DIV) and then becomes more stable in terms of the network activity thereafter (Figure 3.6.2. see Cotterill et al., 2016).

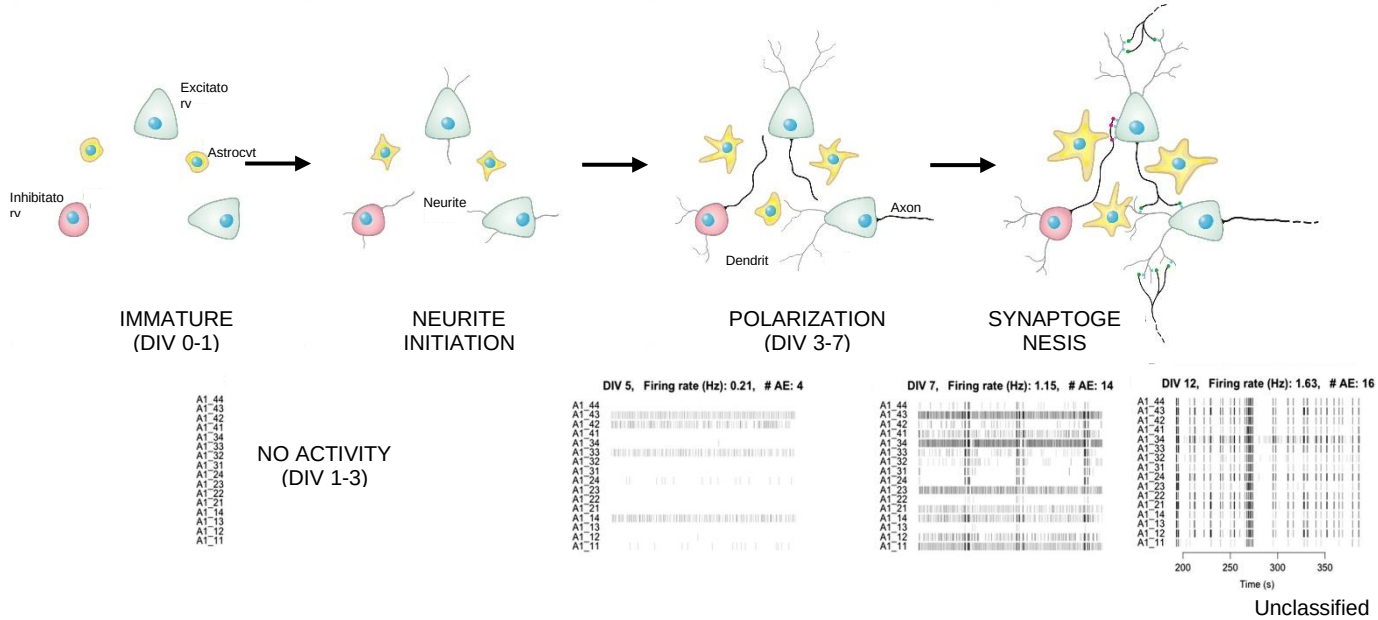

Figure 3.6.1. Schematic representation of the development of cortical cultures (upper panels) over days in vitro (DIV). This illustrates the major cell types in the culture and the morphological changes (neurite initiation, polarization and synaptogenesis). Lower panels: Raster plots illustrating the development of network activity in neuronal cultures over 12 DIV. Control (non-treated) wells from DIV 5, 7, 9, and 12 are shown. In each panel, time is on the x-axis and data from each electrode is plotted in rows on the y-axis. The naming convention is as follows: "A1" indicates that the data arefrom well A1 of the plate, while 11, 12, 13, etc indicate the row and column position, respectively, of the electrodes. A vertical "tick" mark indicates each event on that electrode that exceeds the spike threshold (>8x root mean square noise levels). Heavier shading indicates groups of events occurring closely in time (eg, bursts). The overall mean firing rate (MFR; in Hz) and number of Active Electrodes for each time point are shown above each panel. Neuronal activity, bursting and coordinated activity (eg, simultaneous bursts on multiple channels (eg, "network spikes") increase with time.

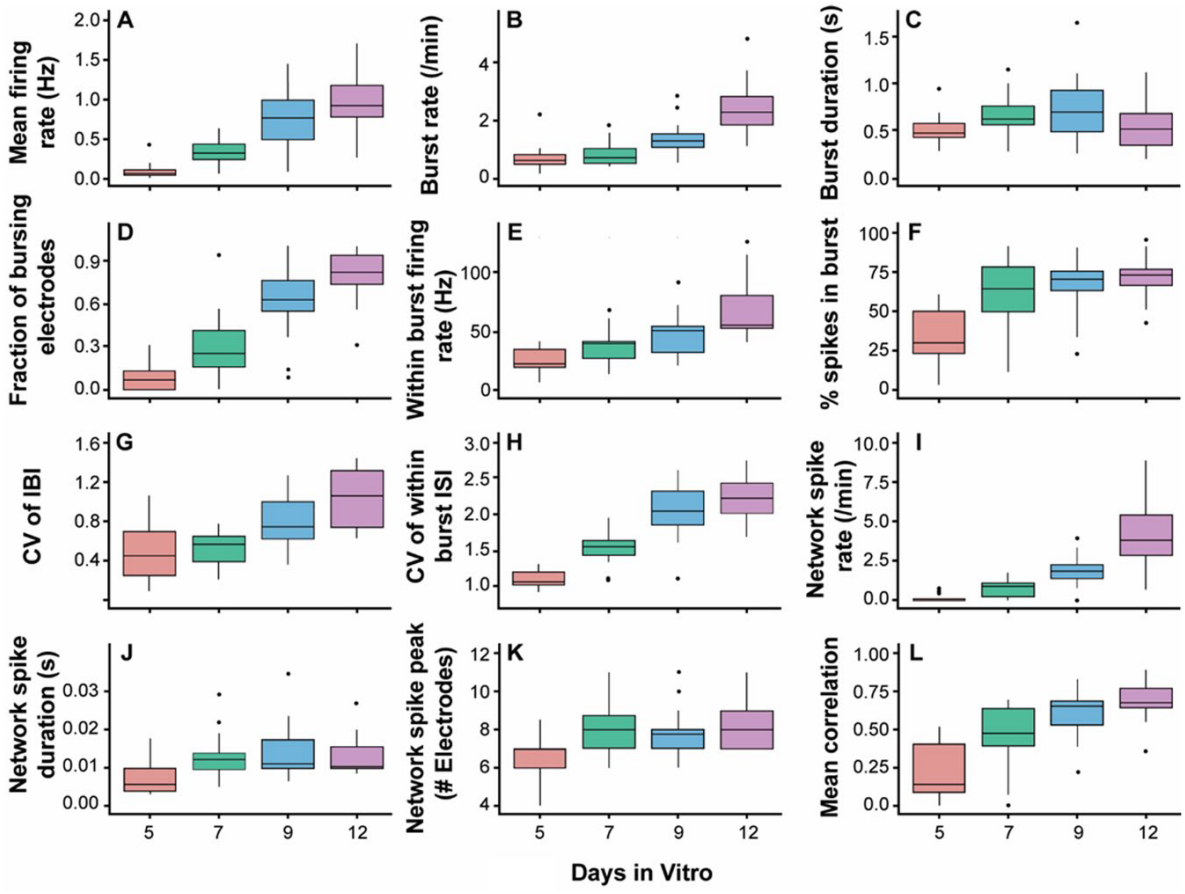

Figure 3.6.213 Ontogeny of network activity in primary cultures of cortical cells for the Network Formation Assay. Mean firing (A) and burst rates (B) increase with development. Box plots showing median and interquartile range are shown for

n = 16 plates. (C) Burst duration. (D) Fraction of bursting electrodes. (E) Within-burst firing rate. (F) percentage of spikes in bursts. (G) CV of interburst interval (IBI). (H) CV of within-burst interspike interval (ISI). (I) Network spike rate. (J) Network spike duration. (K) Network spike peak. (L) Mean pairwise correlation. CV = coefficient of variation. From Cotterill et al., 2016.

Reference/link to maintenance culture protocol

Primary cultures of rat neocortex are newly prepared for each experiment. (See: OP No. NHEERL-H/ISTD/SBB/KAW/2017-01-r2 “Cortical cell culture” (available upon request; email shafer.tim@epa.gov))

Definition of the test system as used in the method

Principles of the culture protocol

48-well microelectrode array plates (Axion Biosystems Inc., Atlanta GA) were utilized for the duration of this procedure. Each of the 48 wells on these plates contains an array of 16 microelectrodes that allow for extracellular recording of electrical activity in the cells plated on top of the electrodes. Measurement of the electrical activity is non-invasive, and recordings can be made from the same plate over multiple different days of the culture. In cortical neurons, this allows for the measurement of extracellular action potentials over the period during which networks form in the MEA plate. On the day prior to culture, each well was coated thoroughly with 150 µL of 0.05% polyethyleneimine (PEI, Sigma Cat# P3143) in 50 mM HEPES (Sigma Cat# H7523) at a pH of 8. The plate was incubated at 37°C for one hour. PEI was rinsed out with 500 µL of sterile water three times. Plates were stored at 4°C until the day of culture. Complete culture details are found in the are available upon request to shafer.tim@epa.gov OP No. NHEERL-H/ISTD/SBB/KAW/2017-01-r0).

This culture was plated at a seeding density of 1.5 x 10^5 cells/well on a 48-well MEA plate, prepared as described above. Cells were administered via a 25 µL media/laminin (20 ng/ml; Sigma Cat# L2020-1MG) drop directly onto the microelectrode array, as adding the cells with the laminin results in better attachment than pre-coating with laminin. After 2 hours, an additional 475 µL of cortical media was added and the cells returned to the incubator. As described in section 3.1, over the course of 12 DIV, networks of electrically active excitatory and inhibitory neurons form spontaneously.

The primary culture model consists of glutamatergic (excitatory) neurons, gabaergic (inhibitory) neuron, astrocytes and sparse microglia (Harrill et al., 2011; Frank et al., 2017).

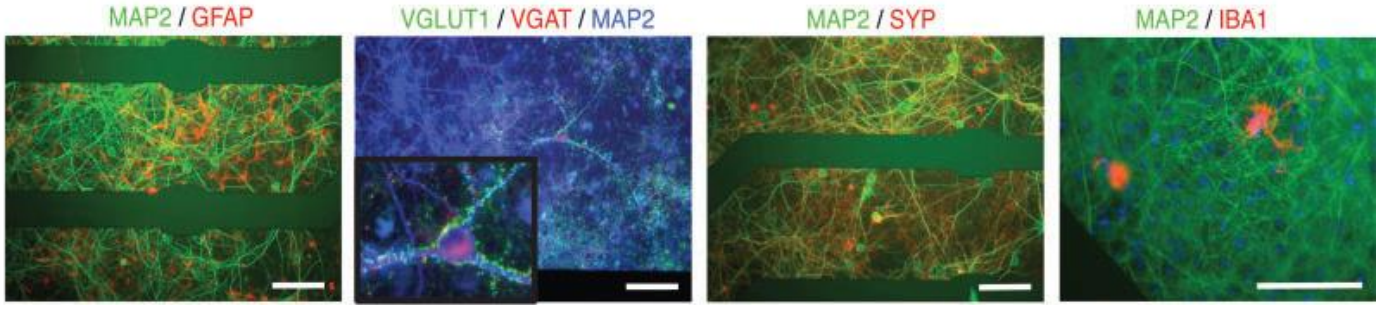

Figure 4.1.1 Representative images of DIV 12 cortical networks grown on 48-well MEA plates. A dense culture is maintained over the electrode array that contains microtubule-associated protein 2 (MAP2) staining of dendrites, Glial fibrillary acidic protein (GFAP)–positive astrocytes, punctate vesicular glutamate transporter 1 (VGLUT1), and vesicular GABA transporter (VGAT) staining of synaptic vesicles, punctate synaptophysin (SYP) staining of presynaptic vesicles, and a small

percentage of Ionized calcium binding adapter molecule 1 (IBA1)–positive microglia. Scale bar=100µm. From Frank et al., 2017.

Acceptance criteria for assessing the test system at its start

As noted above, viability of cells should be 85% or greater at the time of plating.

Acceptance criteria for the test system at the end of compound exposure

By DIV 12, control (untreated) or DMSO (or other solvent treated) wells should have developed robust network activity that shows visible bursts of activity and coordinated bursts of activity across multiple electrodes in the same well

Variability of the test system and troubleshooting

Sources of Variation:
Variability may be due to different numbers of male and female pups selected for each culture. In addition, the random distribution of excitatory and inhibitory cells in the culture, and their random location in each well may contribute to variability in the formation and activity levels of the spontaneous networks that arise in each well.

Metabolic capacity of the test system

We have not extensively characterized the metabolic capacity of our primary cortical cultures. mRNA expression of various Cyp enzymes are low on DIV 1, however, by DIV 14, mRNA for Cyp 211c >> 4x1 > 2d4 > 1s1 > 1a1. Functional expression of these proteins has not been confirmed (Shafer et al., 2015).

Other metabolic pathways are not characterized.

Omics characterization of the test system

Transcriptomic characterization of the test system is currently underway.

Features of the test system that reflect the *in vivo* tissue

Cell model reflects the following in vivo tissue features:

- Presence of excitatory glutamatergic neurons
- Presence of inhibitory gabaergic neurons
- Presence of glia cells (astrocytes, microglia)
- A functional switch in $GABA_A$ receptor activation occurs between DIV 6 and 8, wherein prior to this, activation of the receptor is excitatory and drives an increase in intracellular calcium, whereas after this switch, activation of the receptor is inhibitory and results in an influx of chloride ions into the cells (Inglefield and Shafer, 2000).
- Elaboration of neurites, with subsequent specialization into axons and dendrites (Harrill et al., 2013)
- Formation of synapses (Harrill et al., 2011)

Commercial and intellectual property rights aspects of cells

For primary cortical cells, N/A.

The assay procedure as described above is nonproprietary. It contains some proprietary materials, listed below, which the protocol is optimized for. Utilizing materials from other providers may or may not necessitate changes to the procedure, including seeding density, culture media, media change schedule, days in vitro prior to testing, etc. For details relating to the development of this procedure, please refer to Frank et al. (2017) and Brown et al. (2016).

Multi-well microelectrode array plates and assay readout detection technology and AxIS software are commercially available from Axion Biosystems Inc. (Atlanta Ga). MEA systems are also available from other manufacturers. Cell viability assays utilized are commercially available from Promega (Madison, WI). Cell viability assays are also available from other manufacturers.

Reference/link to the culture protocol

See section 0 for information on the culture protocol

Test method exposure scheme and endpoints

Exposure scheme for toxicity testing

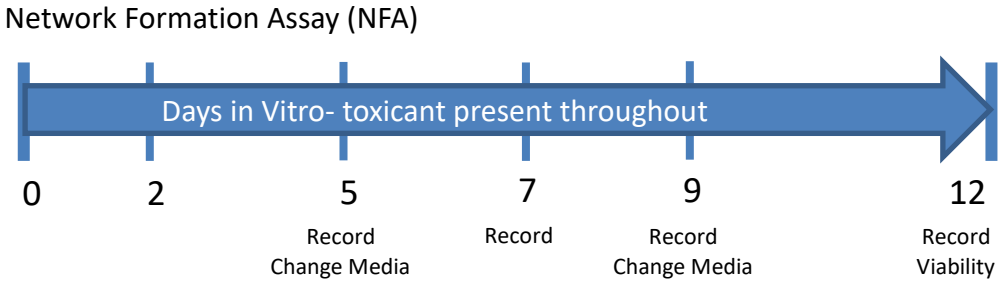

Figure 5.1.114 Exposure scheme. Cells are plated onto mwMEA plates and allowed 2 hr for attachment to the substrate. Starting on DIV 5, 15 min recordings of spontaneous network activity are made on DIV 5 and end on DIV 12.

Exposure starts at day 0 of plating and is continued over twelve days of network development until the experiment is terminated. Cells are fed with fresh medium on DIV 5 and 9, following the recordings on those days. (Figure 2). The entire volume of media in the well is exchanged and any vehicle or compound treatment is renewed. Complete details are found in OP-NHEERL/ISTD/SBB/TJS/2015-03-r0 (available on request to shafer.tim@epa.gov.

Endpoint(s) of the test method

Network activity and its development is complex and can be measured by many different parameters. Typically, 17 different parameters are measured in addition to two measures of cell viability.

Table 5.2 Endpoints in the NFA test.

Activity Type	Type	Parameter	Level	ACID	AEIDs	Description
General Activity	Emp	Mean Firing Rate, *CCTE_Shafer_MEA_dev_firing_rate_mean*	Electrode	2471	2494, 2495	The mean firing rate on each electrode was calculated, with the well level value equal to the mean across all active electrodes.
	Emp	Burst Rate, *CCTE_Shafer_MEA_dev_burst_rate*	Electrode	2472	2496, 2497	The number of bursts per minute. Max-interval method used with parameters: ISI to start =0.1s, ISI to end =0.25s, min IBI =0.8, min duration =0.05s, min no. spikes = 5.
	Emp	Number of Active Electrodes, *CCTE_Shafer_MEA_dev_active_electrodes_number*	Electrode	2473	2498, 2499	Number of electrodes firing at or above 5 spikes per minute.
	Emp	Number of Actively Bursting Electrodes, *CCTE_Shafer_MEA_dev_bursting_electrodes_number*	Electrode	2474	2500, 2501	Number of electrodes with burst rates of above 0.5 bursts per minute.
Bursting Activity	Der	Interspike Interval (ISI) within a burst, *CCTE_Shafer_MEA_dev_per_burst_interspike_interval*	Electrode	2475	2502, 2503	Time interval between spikes within a burst (ms).
	Emp	Percentage of Spikes in Burst, *CCTE_Shafer_MEA_dev_per_burst_spike_percent*	Electrode	2476	2504, 2505	The number of spikes within a burst over total spike count x 100.
	Der	Mean Burst Duration, *CCTE_Shafer_MEA_dev_burst_duration_mean*	Electrode	2477	2506, 2507	Mean duration of a burst (ms).
	Der	Mean interburst interval, *CCTE_Shafer_MEA_dev_interburst_interval_mean*	Electrode	2478	2508, 2509	Mean time interval between bursts (sec).
Network Connectivity	Emp	Number of Network Spikes, *CCTE_Shafer_MEA_dev_network_spike_number*	Well	2479	2510, 2511	Number of spikes in network spikes.
	Der	Network Spike Peak, *CCTE_Shafer_MEA_dev_network_spike_*	Well	2480	2512, 2513	The number of electrodes active at peak of network spike.

Activity Type	Type	Parameter	Level	ACID	AEIDs	Description
		peak				
	Der	Network Spike Duration, *CCTE_Shafer_MEA_dev_spike_duration_mean*	Well	2481	2514, 2515	The average duration (ms) of a network spike.
	Der	SD of Network Spike Duration, *CCTE_Shafer_MEA_dev_network_spike_duration_std*	Well	2482	2516, 2517	Standard deviation of network spike duration.
	Der	ISI in Network Spike, *CCTE_Shafer_MEA_dev_per_network_spike_interspike_interval_mean*	Well	2483	2518, 2519	Mean inter-spike interval for spikes in network spikes.
	Der	Mean number of Spikes in Network Spikes, *CCTE_Shafer_MEA_dev_per_network_spike_spike_number_mean*	Well	2484	2520, 2521	Number of spikes in network spike.
	Emp	% Spikes in Network Spike, *CCTE_Shafer_MEA_dev_per_network_spike_spike_ percent*	Well	2485	2522, 2523	Ratio of spikes in network spikes over total spikes x 100.
	Emp	Mean Correlation, *CCTE_Shafer_MEA_dev_correlation_coefficient_mean*	Well	2486	2524, 2525	The average of all pairwise correlation between all electrodes.
	Emp	Normalized Mutual Information, *CCTE_Shafer_MEA_dev_mutual_information_norm*	Well	2487	2526, 2527	Normalized mutual Information between all electrodes in the well.
Cell viability	Emp	LDH, *CCTE_Shafer_MEA_dev_LDH_dn*	Well	2488	2529	Cell viability was assessed using lactate dehydrogenase (LDH).
	Emp	AB, *NHEERL_MEA_AB_dn*	Well	2489	2530	Cell viability was assessed using CellTitre blue (AB).

ACID=assay component identification; AEID=assay endpoint identification; Emp=emperical; Der=derived.

All endpoints are generated from the same experimental run and from each well/network in the 48 well plate.

Overview of analytical method(s) to assess test endpoint(s)

Endpoints listed in table 5.2 are calculated from the recorded data on DIV 5-12 using custom R scripts. Those scripts are freely available on GitHub (https://github.com/dianaransomhall/meadq and https://github.com/sje30/sjemea)

Technical details (of e.g. endpoint measurements)

All technical details for the test method are available in the NFA OP (Section 3.7).

Endpoint-specific controls/mechanistic control compounds (MCC)

1- Bisindolylmaleimide 1 (Bis-1; PKC inhibitor): inhibits neurite outgrowth
2- Domoic Acid: inhibits neurite outgrowth and has been shown to inhibit ontogeny of cortical network activity (Hogberg et al., 2011)
3- Loperamide: mu opoid agonist. Inhibits network ontogeny at concentrations lower than cytotoxicity
4- Mevastatin: inhibits synaptogenesis in vitro (Harrill et al., 2011).
5- Sodium Orthovanidate: inhibits synaptogenesis in vitro (Harrill et al., 2011).

Due to the limited wells on the 48 well plate, MCC are not run on each plate but an assay positive control compound (e.g. Bisphenol A) and an Assay negative control compound (e.g. glyphosate) are typically included on plates at least once during testing of a set of compounds or at least every 12 weeks.

Positive controls

This assay has been evaluated against 63 compounds that have evidence of DNT in vitro (Shafer et al., 2019). These 63 compounds were selected based on an evaluation of the literature by Mundy et al., 2015. See Shafer et al., 2019 for details on the compounds selected.

Negative and unspecific controls

The solvent control (SC) is used as negative control that is run on each experimental plate. Each SC has to be established by comparing the effect of the SC to the effect of the media control. Established solvent controls show the same response as the media control.

Established SCs are:
DMSO: 0.1 % v/v
ddH2O: 0.1 % v/v
DMSO/Ethanol: 0.1 % v/v
Ethanol: 0.1 % v/v

Other negative control compounds that were identified as negative in this assay (Brown et al., 2016; Frank et al., 2017; Shafer et al., 2019) and are known to not affect neurodevelopmental endpoints in vivo are:

Acetaminophen
Amoxicillin
Aspirin
D-mannitol
Erythromycin
Glycerol
Glyphosate
Propylene glycol
Tetracycline

Sodium Benzoate
Saccharin

Features relevant for cytotoxicity testing

Primary cortical cultures are a multicellular system consisting of excitatory and inhibitory neurons, glia (oligodendrocytes, astrocytes) and a few microglia. The measurement of cytotoxicity and viability therefore represents all cells within the culture.

The measure of cell viability assessed by the CellTiter Blue assay (mitochondrial reductase activity) depends on the metabolic activity of cells present in the well. Reduced cell viability as measured by CellTiter Blue indicates either fewer cells present and/or a reduced metabolic capacity of the cells in the well.

Lactate dehydrogenase (LDH) is a large intracellular molecule that when present in the extracellular media indicates a damaged cell membrane. By washing the cells, and then lysing the remaining cells in the well, the LDH indicates the relative number of remaining viable cells in the well. Since LDH has a half-life in the media of approximately 9 hr, measuring media LDH accumulated over a longer period of time is confounded by degradation of the LDH.

In the NFA, only the cellular LDH is measured at the end of the 12 DIV exposure period. This then reflects the total amount of cells remaining in the well and in treated wells, the relative cytotoxicity over the exposure period can be determined by comparing the cellular LDH in the treated well to the cellular LDH in the control/solvent treated wells.

Acceptance criteria for the test method

If properly performed, spiking and bursting activity should develop between DIV 2 and 12, following a clear ontogeny, with random spiking activity developing first, followed by bursting and coordinated bursting across multiple electrodes. Significant amounts of activity are not observed until DIV 5. The average number of active electrodes of control wells should be between 12 and 16 by DIV 12. Viability in control wells should also be 90% or greater.

Signals are detected based on thresholds unique to each electrode – six times the standard deviation of the root mean square (rms) noise. Due to this, any individual electrode having an rms noise level of greater than 5 µV is grounded and no data are collected from that electrode. Once grounded, an electrode must remain grounded for all subsequent treatments. Care must be taken in the selection of electrodes to record or ground (not collect data from) during the experiment. If any given well on a plate has more than 50% of electrodes grounded, data from that well should be collected but assigned a “well quality 0” value (indicates not to use that data, see section 7). Typically, less than 4 electrodes on an entire plate (of 768 total electrodes) should need to be grounded. If more than 10 electrodes across an entire plate need grounding, this may indicate problems with the equipment or plates such as bad contacts between the plate and Maestro device. Rejection of data due to noise issues should be rare.

As with all cell-based experimentation, maintain proper sterile technique and good cell maintenance practices. In plating cells, an aliquot is to be counted and assessed for viability. If less than 85% of the cells are viable, the cells are not used. Wells in which erroneous volumes of treatment compound are added should be discarded. Each time media is prepared, a sterility test plate is prepared by placing at least one 1.0 mL sample of cell media into the plate.

Plates should be monitored for contamination throughout the experiment. Contamination may be indicated by yellow and/or cloudy media. Contaminated wells should be emptied of media and treated with a bleach solution. Any plate with contaminated wells should be monitored more frequently and carefully as

contamination can often spread to multiple wells. Data from contaminated wells should not be analyzed.

Throughput estimate

The methods described here are described for a 48 well plate format. Typically, 6 plates can be made in one culture, which allows testing 12 compounds in triplicate. If cultures are made every 14 days, 24 compounds per month can be screened in triplicate at multiple concentrations.

Handling details of the test method

Preparation/addition of test compounds

The method is set up to test 6 compounds at 7 concentrations plus a solvent control. Example dilution plate and final plate maps are provided in figures 6.1.1 and 6.1.2, respectively

The experimental compounds are each prepared in stock solutions at 1000-fold concentrations of 0.03, 0.1, 0.3, 1.0, 3.0, 10, and 30 mM in DMSO, ethanol, or double-distilled H_2O (ddH_2O) based on solubility. For each experiment, working solutions of the chemicals were prepared by adding 5 µL of chemical to 95 µL of media for a 1/20 dilution. Cells were treated by adding 10 µL of the working solution to 500 µL of media in the wells for a 1/50 dilution, resulting in a total 1/1000 dilution of chemical stock and 0.1% final concentration of DMSO, ethanol or ddH_2O vehicle.

Stock solutions are aliquoted and stored at -20°C. Freeze/thaw cycles should be avoided with compound stock solutions, therefore it is best to prepare an aliquot of stock solution to be thawed and used once for each treatment on DIV 0, 5 and 9.

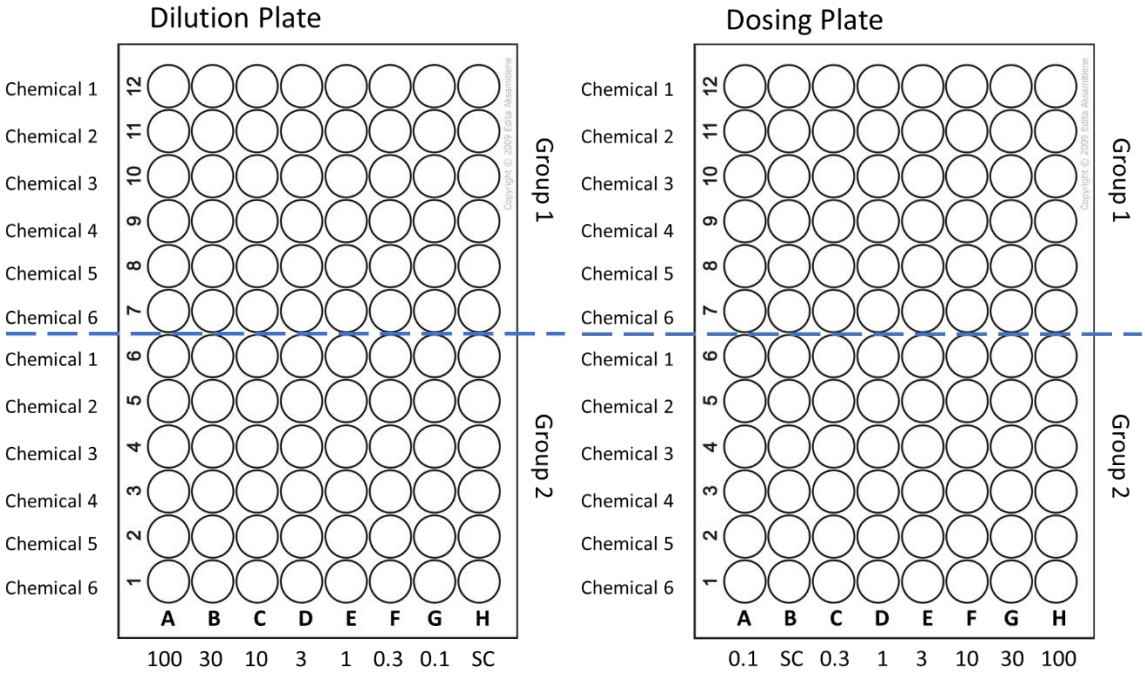

Figure 6.1.1. Dilution and Dosing Scheme. Neat compounds are aliquoted on the Column A on the dilution plate. Solvent control (SC) is added to the Column H and transferred to Column B, C, D, E, F, and G. Serial dilution is performed from left to right across the plate. On the dosing plate, the compounds are diluted 1:20 with NB Supplemented media. They are further diluted 1:50 into their corresponding treatment well on the MEA plate.

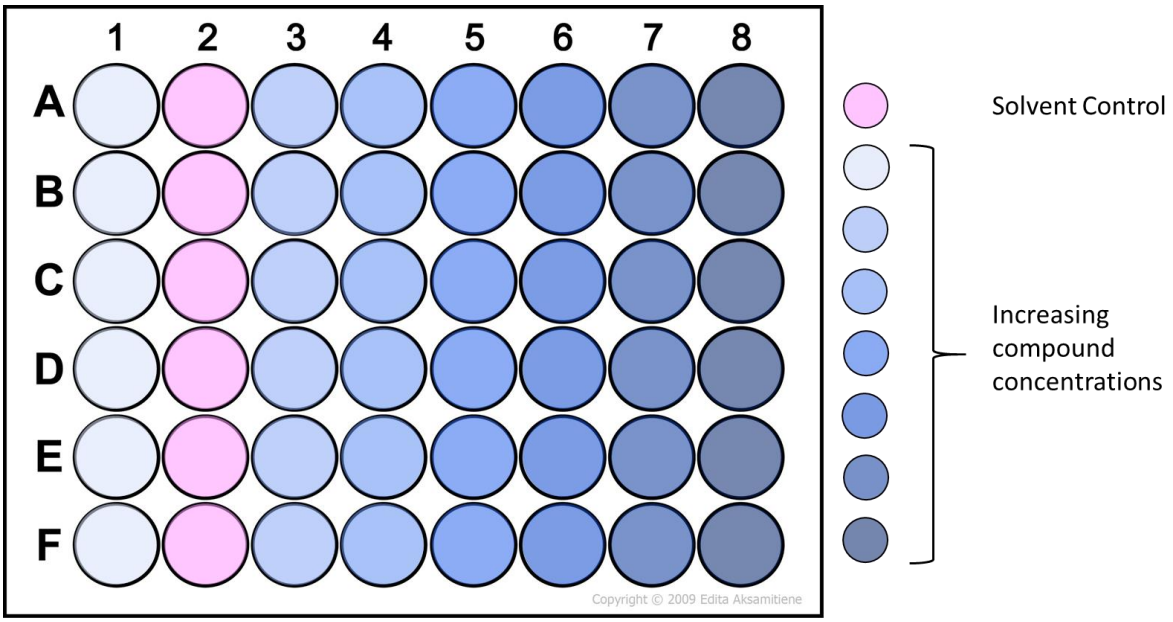

Figure 6.1.2. Final MEA platemap. Solvent controls are on the second column, and the compound treatment are increasing in concentration from left to right of the plate. There are three replicates of each concentration of each compound treatment on three different MEA plates. On each MEA plates, each compound are rotated in different rows, to minimize edge well effect.

Day-to-day documentation of test execution

Documentation for each experiment including meta data and the experimental data is collected in the Practical phase of test compound exposure.

The Practical phase of the test compound exposure follows the description in the NFA SOP (Section 3.7). Deviations from the SOP are documented in the laboratory notebook.

Errors (e.g. pipetting in wrong well or wrong volume pipetted) are also documented in the laboratory notebooks. This information is then captured in later analyses by setting the well quality to zero in tcpl (see analysis section below), which indicates that data from that well should not be used for analyses.

Practical phase of test compound exposure

All aspects of the experiment are recorded in an online OneNote laboratory notebook. This includes any documentation of adherence to platemaps, potential errors, and any other variable that may impact the assay and interpretation of results. Projects are typically subjected to review by EPA Quality Assurance Managers.

Concentration settings

Seven compound concentrations are tested on each plate, with ½ log unit spacing between concentrations (e.g. 0.3, 1, 3, 10 uM, etc). Standard upper concentrations tested are either 100 or 30 uM, depending on the solubility of the compounds as well as the highest concentration which can be provided in a stock solution from test set providers (e.g. EPA's ToxCast program).

Uncertainties and troubleshooting

Problematic compounds:

- volatile compounds
- high lipophilicity (high K_{OW})
- low solubility in established solvents
- Fluorescent compounds (possible interference with viability and cytotoxicity assay)

Critical handling steps:

- For compounds that may have some volatility, or to ensure against effects due to evaporation of media, plate sealers may be used.
- Networks are sensitive to disruption and this can impact activity, therefore, when moving plates from the incubator into the Maestro for recording, they should be given a period of 15-20 min to equilibrate prior to collecting data.

Sources of variation:

- Pipetting steps: Each pipetting step is a source of variation
- Source of cells for the culture may differ from culture to culture due to the use of mixed sex cultures, where rat pups are not sexed prior to use in cultures.

Known Pitfalls:

- Allow cells to equilibrate for 15 or 20 min when moving from the incubator to the Maestro device, as this can impact activity
- Check settings on Maestro AxIS software, as this can change depending on the user and type of experiment. Incorrect software settings for plate type, filters activity measures and other parameters will result in data that are not useful.

Detailed protocol (SOP)

See OP: r0 Data Acquisition and Analysis for Recording from Neuronal Cultures with the Axion MaestroPro Multielectrode Array (MEA) System. OP For cytotoxicity measurements (In process, number to be assigned), see CellTiter Blue® Cell Viability Assay: OP-NHEERL-H/ISTD/SBB/TJS/2015-006-r2; Cell titer blue cell viability assay and for LDH see OP: NHEERL/ISTD/SBB/TJS/2015-04-r0 Measuring LDH release from cortical cells in a multiwell microelectrode array. (All protocols are available upon request: email shafer.tim@epa.gov).

Special instrumentation

- Standard cell culture equipment.
- Multi-well microelectrode array plates
- Multi-well MEA amplifier (e.g. Axon Instruments Maestro System)
- Note: raw MEA data files can be quite large, so additional data storage capacity is recommended.

Possible Variations

Other endpoints:

- Additional endpoints can be extracted from the recordings of activity. However, many MEA endpoints have high correlation with each other, so extraction of additional endpoints may not provide any additional information and should be done with caution. Replacement of some endpoints with others that are deemed more robust would be recommended if data supports it.

Other exposure schemes:

- Because the MEA measurements are non-invasive, different exposure schemes and timepoints could be considered. In addition, some investigators (Hogberg et al., 2011) have employed pharmaceutical challenges as an additional measure of network responsiveness. However, any alterations in the protocol would require additional characterization to demonstrate that it is a sensitive and informative measure.

Cross-reference to related test methods

Data management

Raw data format

The raw data format for MEA recordings is in the form of *.raw files from the Axion AxIS software. *_spike_list.csv and *_spike_count.csv files can also be recorded during the experiment and/or generated by replaying the *.raw file.

For all endpoints assessed in a multiplate reader (viability and cytotoxicity) the raw data format are excel files containing values (one for each endpoint, timepoint and well) measured as relative fluorescence units. The original excel output files is saved for traceability of the data.

Outliers

Mathematical procedures to define outliers are not applied as the tcpl fitting approach is robust and designed to limit the impact of outliers (Filer et al., 2017). Data points from wells where technical problems are known or obvious are retained in the data file but are excluded from the analysis by marking them as “well quality 0”.

Some example technical problems:

- pipetting errors
- contamination
- noisy electrodes

Raw data processing to summary data

If not otherwise stated, all data processing steps are performed in an R based script that were designed for data processing and curve fitting.

Data processing describes all processing steps of raw data that are necessary to obtain the final response values including the normalization, curve fitting and benchmark concentration calculation.

Processing (or pre-processing) steps depend on the endpoint and are described below:

The collected data is analyzed by parsing the measured parameters (Table 5.2) describing network function via R statistics programing software. These parameters are obtained from the *_spike_list.csv files that are saved during recordings or can be generated by replaying the *.raw files. Eight parameters were measured that are considered empirical („emp“ in Table 5.2) measures of network activity, and eight additional parameters of network activity were derived from („der“ in Table 5.2) these empirical parameters. An additional parameter, normalized mutual information (MI) was computed for each recording, which is a measure of synchrony that scales with increasing complexity of a network.

Any instance of a derived data point being undefined (such as burst duration in a well without bursting) was set to a value of 0 for analysis. Some parameters are measured at the well level and some at the electrode level. Those measured at the electrode level are averaged across the array to give well level measurements (as in mean fire rate, etc.) across all parameters.

The data are graphed as parameter vs DIV for all 17 parameters. A trapezoidal area under the curve (AUC) calculation was performed for selected parameters of every concentration, given by the equation:

$$AUC_P = 0.5 * (DIV_1 - 2) * (P_1 - 0) + 0.5 * \sum_{k=2}^{n-1} (DIV_{k+1} - DIV_k) * (P_{k+1} - P_k)$$

where *k* is the time point, *n* is the number of time points, *P* is the parameter value at time point *k*, and DIV is the day in vitro at time point *k*. This AUC value reflects the overall maturity of the neural network after the 12 days of development and condenses concentration effects across time (DIV). Scripts for the data analysis are written in R programming language and are freely available on GitHub (https://github.com/dianaransomhall/meadq and https://github.com/sje30/sjemea).

Curve fitting

AUC data are then analyzed using the ToxCast Pipeline (tcpl) approach as described by Filer et al., 2017. A summary of techniques applied is in table 7.4

Table 7.4 Methods applied in tcpl for the NFA assay.

ToxCast Data Pipeline Level	MEA NFA: Methods Applied
mc0: pre-processed data input	Data are pre-processed to obtain AUC values by assay component
mc1: mapping to well and column indexes	Auto
mc2: transformation	No transformation
mc3: normalization	Baseline value (bval) was calculated as the median value for the vehicle control wells (DMSO) on a by-plate basis; No positive control value was used in normalization (pval=0); the response was calculated as percent of DMSO vehicle control. The response was multiplied by -1 for the “up” endpoints such that all endpoints are curve-fit in the positive direction.

mc4: BMAD calculation type for curve-fitting	An approximation of noise around the baseline signal, the baseline median absolute deviation, was calculated based on the vehicle control wells on each plate.
mc5: Hit-call and potency determination	The cutoff for a positive response in each assay endpoint was set as 3*BMAD.
mc6: caution flags on fitting	Flags for single point hit at maximum concentration (6), flags for single point hit not at the maximum concentration screened (7), inactives with multiple median responses above baseline (8), noisy curves relative to the assay (10), actives with borderline efficacy (11), inactives with borderline efficacy (12), low concentration gain-loss curve-fits (15), possibly overfitting (16), hit-calls with less than 50% efficacy (17), model fits with AC_{50} less than lowest concentration tested (18) were assigned to all; additionally cell viability assays were assigned "viability gain-loss fit" (19)

Internal data storage

Neural activity data collected through the Maestro Pro system are saved as raw files. Data analysis can be performed on the raw file using Axion software. These raw recordings are backed up in Drobo external drives. Due to their large size, raw files are maintained until data are published. Efforts are underway to be able to permanently store these files.
All spikelist and other files derived from the analysis is stored on EPA servers which are backed up daily. As per US Government regulations, these files will be maintained for at least 20 years.

Metadata

For the MEA experimental data, metadata is collected in csv files. R scripts are used to scrape the metadata from the files, merge the metadata with the experimental data for each well, and save the result in the Hierarchical Data Format version 5 file format. For the cytotoxicity and viability experimental data, metadata is collected in xlsx files. R scripts are used to scrape the metadata along with the experimental values and save the result in a csv file.

Metadata file format

Metadata file formats are csv, xlsx, and h5.

Prediction model and toxicological application

Scientific principle, test purpose and relevance

One of the major ways information is stored, encoded and shared in the nervous system is by electrical signals, and the primary signal is the rapid depolarization of the neuronal membrane known as the action potential. The rates and patterns of action potentials that are transmitted from one neuron to another neuron encode specific information and are vital to nervous system function. The development of this complex network of neurons in the nervous system is the result of integrated networks of neurons and glia. Both in vivo and in vitro, the development of bursting and coordinated electrical activity are intrinsic properties of neural networks. The development of these activities is essential to neural network function. By extension, these activities are crucial to the development of the nervous system as a whole. This assay seeks to screen for compounds that disrupt these properties as a rat cortical cell culture develops into a network. The biological processes and patterns of network activity are highly conserved across different species, including

rodents and humans. As such, compounds flagged by this assay may be considered to be potentially developmentally neurotoxic and considered for further analysis.

Prediction model

The cutoff for the benchmark response in each assay endpoint is set as 3*BMAD, and compounds with treatment levels reaching this cutoff are then subjected to curve fitting in tcpl, from which AC_{50} values are generated (see table 7.4). The PM is based on a comparison between the AC_{50} value for the NFA-specific endpoint and the AC_{50} for value cytotoxicity/viability effect.

Thereby the following classifications apply:

"specific hit": a threefold difference between the AC_{50} value for the NFA specific endpoint and the most potent cytotoxicity endpoint. Where no cytotoxicity endpoint had an AC_{50} value, then the highest concentration tested is used.

"non-specific hit": Less than a threefold difference exists between the AC_{50} value for the NFA-specific endpoint and the most potent cytotoxicity AC_{50} value.

It should be noted that there are other valid approaches to determining specificity. For example, one could calculate the area under the curve of the specific endpoint that is below the AC_{50} value for cytotoxicity.

"inactive": the compound was active in <3 NFA endpoints, including cytotoxicity.

Prediction model setup

This assay was developed using a training set of chemicals (see Brown et al., 2016), and then further evaluated with a test set of chemicals that had 63 putative positive and 13 putative negative DNT chemicals (see Frank et al., 2017; Shafer et al., 2019). See sections below for additional details.

All endpoints in this assay are fit in the down direction. For the viability endpoints, fitting in the up direction (increased viability) is not logical since viability of controls is typically quite high (>90%). The network formation parameters, can be fit in both the up and down direction. However, to date, the vast majority of compounds tested cause decreases in network development parameters, which can be interpreted as decreased network formation, and for which we have assay positive controls. Biological meaning of changes in the up direction (increased network parameters) is difficult to interpret due to the lack of assay positive controls that alter parameters in the up direction. The exception to this are parameters, which tend to increase as other parameters decrease with decreasing activity (e.g. interburst intervals increase as the burst rate decreases).

Test Performance

Table 1 summarizes the assay performance in terms of variability of each endpoint in the assay.

For this assay, the following compounds are used as assay positive controls as they have been previously demonstrated to disrupt network formation and/or synaptogenesis in in vitro systems (Hogberg et al., 2011; Harrill et al., 2011).

Loperamide
Bisindolylmaleimide I
L-Domoic acid
Mevastatin

Sodium orthovanadate

Table 8.4.1 Variability of NFA endpoints (MAD- median absolute deviation; CV- coefficient of variation)

ACID	Assay component name	Median	MAD	CV
2471	CCTE_Shafer_MEA_dev_firing_rate_mean	8.81	2.04	21.26
2472	CCTE_Shafer_MEA_dev_burst_rate	16.88	3.47	20.52
2473	CCTE_Shafer_MEA_dev_active_electrodes_number	88.5	7.04	8
2474	CCTE_Shafer_MEA_dev_bursting_electrodes_number	71.75	7.41	10.21
2475	CCTE_Shafer_MEA_dev_per_burst_interspike_interval	0.3	0.07	24.23
2476	CCTE_Shafer_MEA_dev_per_burst_spike_percent	414.86	41.35	10.4
2477	CCTE_Shafer_MEA_dev_burst_duration_mean	3.98	0.86	25.27
2478	CCTE_Shafer_MEA_dev_interburst_interval_mean	208.93	42.93	21.88
2479	CCTE_Shafer_MEA_dev_network_spike_number	272.5	51.52	23.04
2480	CCTE_Shafer_MEA_dev_network_spike_peak	73.33	5.81	8.08
2481	CCTE_Shafer_MEA_dev_spike_duration_mean	1.24	0.16	13.92
2482	CCTE_Shafer_MEA_dev_network_spike_duration_std	0.4	0.08	23.91
2483	CCTE_Shafer_MEA_dev_inter_network_spike_interval_mean	235.92	60.07	26.4
2484	CCTE_Shafer_MEA_dev_per_network_spike_spike_number_mean	285.63	45.06	16.99
2485	CCTE_Shafer_MEA_dev_per_network_spike_spike_percent	73.01	10.12	18.76
2486	CCTE_Shafer_MEA_dev_correlation_coefficient_mean	1.33	0.17	15.67
2487	CCTE_Shafer_MEA_dev_mutual_information_norm	0.04	0.01	21.38
2488	CCTE_Shafer_MEA_dev_LDH	0.98	0.07	8.06
2489	CCTE_Shafer_MEA_dev_AB	20318.9	1448.87	6.93

ACID=Assay Component Identification; MAD = Median absolute deviation; CV = coefficient of variation

Table 8.4.2 provides the performance of the assay as measured by median z-prime (median.Zprm), median strictly standardized median deviation (median.SSMD) and median signal-to-noise (median.SN) ratio

chemical	dsstox_substance_id	Median.Zprm	Median.ssmd	Median.SN
Loperamide	DTXSID00880006	0.1693	2.16	2.16305
Bisindolylmaleimide I	DTXSID50157932	0.1693	2.16	2.16305
L-Domoic acid	DTXSID20274180	0.1323	1.975	2.16305
Mevastatin	DTXSID4040684	0.1693	2.16	2.16305
Sodium orthovanadate	DTXSID2037269	-1.7455	0.145	0.3335

Sensitivity and specificity were determined in Shafer et al. (2019) as 78 and 84%, respectively based on the analyses of 63 predicted positive and 13 predicted negative chemicals.

In addition, reproducibility of results has been evaluated by repeated testing of compounds in the assay.

Table 8.4.3 summarizes reproducibility of results.

Chemical	Sample	Positive AEIDs	Minimum log10-	Mean log10-AC50	SD(log10-AC50 by AEID and	Reproducibility Score[a]

			AC50		Chemical), average	
2,2',4,4'-Tetrabromodiphenyl ether	EX000285	16	0.492	1.07	1.59	Strong
2,2',4,4'-Tetrabromodiphenyl ether	EX000461	7	-1.87	-1.32	1.59	
Loperamide HCl	EX000411	20	-0.68	0.0282	0.561	Strong
Loperamide HCl	MEA20201109A12	19	-2.52	-0.978	0.561	
Loperamide HCl	MEA20201109A13	19	-1.46	-0.443	0.561	
6-Propyl-2-thiouracil	EX000421	17	0.728	1.26	na	Weak
6-Propyl-2-thiouracil	TP0001649B07	0	na	na	na	
Acephate	EPAPLT0167A01	2	-0.0493	0.964	na	Equivocal
Acephate	TT0000177A04	0	na	na	na	
Acetaminophen	MEA20201109A6	0	na	na	na	Equivocal
Acetaminophen	MEA20201109A7	0	na	na	na	
Acetaminophen	MEA20201109A8	2	-1.15	0.00882	na	
Bisphenol A	EX000420	5	0.412	0.753	0.403	Strong
Bisphenol A	MEA20201109A9	14	0.8	1.12	0.403	
Captopril	EPAPLT0169G09	2	1.34	1.66	na	Strong
Captopril	EX000456	1	1.22	1.22	na	
Chlorpyrifos	EX000384	19	0.661	1.18	0.132	Strong
Chlorpyrifos	TT0000177E02	18	1.1	1.22	0.132	
Chlorpyrifos oxon	EX000378	16	-0.832	0.56	na	Weak
Chlorpyrifos oxon	TT0000177G02	0	na	na	na	
D-Glucitol	EPAPLT0169A05	0	na	na	na	Strong
D-Glucitol	EX000322	0	na	na	na	
D-Glucitol	EX000400	0	na	na	na	
Dexamethasone	EPAPLT0169F08	2	-0.427	0.0799	na	Equivocal
Dexamethasone	EX000395	0	na	na	na	
Di(2-ethylhexyl) phthalate	EX000324	1	-0.414	-0.414	1.05	Weak
Di(2-ethylhexyl) phthalate	EX000422	16	0.301	0.872	1.05	
Diazinon	EPAPLT0170D06	2	1.06	1.22	na	Weak
Diazinon	TT0000177H01	17	1.51	1.74	na	
Dimethoate	EPAPLT0167G06	15	1.07	1.27	0.295	Strong
Dimethoate	TT0000177H02	18	1.55	1.69	0.295	
Glyphosate	MEA20201109A10	0	na	na	na	Strong
Glyphosate	MEA20201109A11	0	na	na	na	
Hexachlorophene	EX000335	20	-0.713	0.0341	0.0907	Strong
Hexachlorophene	EX000392	19	-0.275	0.0973	0.0907	
Malathion	EPAPLT0167G08	18	-0.0959	0.797	0.253	Strong
Malathion	TT0000177D02	19	0.865	1.16	0.253	
Methamidophos	EPAPLT0167A08	5	-1	0.665	na	Weak
Methamidophos	TT0000177B02	0	na	na	na	

Permethrin	EX000346	19	0.756	0.944	0.255	Strong
Permethrin	EX000463	19	1.1	1.3	0.255	
Trichlorfon	EPAPLT0170D03	13	0.281	0.862	0.447	Strong
Trichlorfon	TT0000177F01	18	0.547	1.56	0.447	

[a]Reproducibility Score

Strong: replicates were consistently positive with >3 hits or consistently negative with 0 hits
Equivocal: 1 replicate was between 1 and ≤3 hits and 1 replicate was negative
Weak: 1 replicate was positive and 1 was replicate negative or equivocal
Sample=identification number of the sample for tracking in ToxCast

In vitro-In vivo Extrapolation (IVIVE)

IVIVE of data from this assay has been conducted based on the activity (e.g. EC_{50}, AC_{50}, tipping point) values obtained from curve fitting. Because in vitro toxicokinetic information (e.g. lipid and protein content of cells, volume of cells) are not readily available, these extrapolations have been based on the nominal concentration of test article in the medium. Adjusted Equivalent Doses (AEDs) were estimated using the high-throughput toxicokinetic (HTTK) information and models available in the httk R package (v1.8; Pearce et al., 2017), which functionalizes an approach similar to the one previously used by Wetmore et al. (2012). See Shafer et al., 2019 or EPA 2020 for complete details.

Applicability of test method

Toxicological application domain
To date, 451 unique compounds (as defined by unique DTXSIDs) have been tested successfully in this assay. Data for 395 of these chemicals are currently available on the EPA Comptox Dashboard (https://comptox.epa.gov/)This includes the following compound classes:

- Industrial chemicals (>150 chemicals tested)
- Pesticides and metabolites (e.g. oxons) (>75 chemicals tested)
- pharmaceuticals (>52)
- metals and organometals
- cosmetics ingredients

Compounds need to be soluble in a solvent at a solubility where the solvent does not produce effects by itself in the test system (see section 5.70for established solvents).

Nicotine produced only small effects in the assay, and additional characterization of nicotinic responses are needed to determine the applicability of this method for detecting nicotinic compound effects.

Compounds that are volatile would need specialized modifications to test as the assay is conducted at 37ºC, and these compounds would likely evaporate to some extent depending on the duration of the experiments and the vapor pressure of the compound. To date, volatile compounds have not been tested in this assay.

While mixtures can be tested in the assay, it has not been evaluated for its ability to distinguish additive from non-additive effects of mixtures to date. If proper experimental design is used and the chemical properties are compatible with the assay, it is anticipated that the NFA could be used for this purpose.

As MEAs are based on electrophysical signals, fluorescent and/or colored chemicals will not interfere with measurements of network activity, however, they may interfere with the cell viability measurements.

Biological application domain

The Network Formation assay is based on the spontaneous formation of neural networks by cells obtained from PND0 rat cortex.

Next to the endpoints represented by this test method there are several other necessary neurodevelopmental endpoints which need to be studied using other test methods.

Neurodevelopmental processes not represented by this test method:

- Neural Crest Cell (NCC) Migration
- NPC apoptosis
- Neuronal morphology
- Synaptogenesis
- Neural Rosette Formation
- hiPSC-derived NPC proliferation
- hiPSC-NPC neuronal differentiation
- Neuronal subtype differentiation
- Astrocyte Differentiation and Maturation
- Astrocyte Reactivity
- Microglia reactivity

For a complete assessment of developmental neurotoxicity, the test method needs to be part of a test battery.

Incorporation in test battery

The test method is currently used in OECD's the Developmental Neurotoxicity In Vitro Battery to assess the potential hazard for developmental neurotoxicity (see 0 "Applicability of test methods")

For the assessment of chemical action on the endpoints represented by this test method, the test method can be used as a stand-alone test method.

Publication/validation status

Availability of key publications

Key Publications concerning the test method are:

Johnstone AF, Gross GW, Weiss DG, Schroeder OH, Gramowski A, Shafer TJ.Microelectrode arrays: a physiologically based neurotoxicity testing platform for the 21st century. Neurotoxicology. 2010 Aug;31(4):331-50. doi: 10.1016/j.neuro.2010.04.001.
PMID: 20399226

Robinette BL, Harrill JA, Mundy WR, Shafer TJ. In vitro assessment of developmental neurotoxicity: use of microelectrode arrays to measure functional changes in neuronal network ontogeny. Front Neuroeng. 2011 Jan 20;4:1. doi: 10.3389/fneng.2011.00001.
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(Potential) Linkage to AOPs

Changes in neuronal network formation are found in Key Events #618 and 386. KE 618 is part of an AOP 48 titled “ionotropic glutamatergic receptors and cognition”. KE386 is included in 7 different AOPs, the most relevant to developmental neurotoxicity is one titled: Oxidative stress and Developmental impairment in learning and memory (AOP 17).

Steps towards mechanistic validation

a) Information demonstrating how the test system is biologically relevant to humans in terms of cell types, signaling pathways, etc.

Formation of neural networks during development is a process that is highly conserved across all mammalian species, including humans. In vitro, neural cultures derived from human inducible pluripotent stem cell (IPSC) models develop neural networks with patterns of spiking and bursting that are similar to those of rodent cells (Saavedra et al., 2021). However, the time period over which that activity develops is longer than rodent cultures.

b) Interventions (pathway knockdown, specific inhibitors (i.e., mechanistic controls, which may be part of the training set) that show expected effects on the assay

This assay has been developed by using mechanistic control compounds known to disrupt neurite outgrowth and synaptogenesis in cortical neurons (Robinette et al., 2011; Brown et al., 2016).

c) Formal mechanistic validation

There has been no formal mechanistic validation of this assay. This test method was developed following the criteria established in Crofton et al., 2011, where a set of assay positive controls has been tested (Brown et al., 2016), followed by a test set of compounds (Frank et al., 2017; Shafer et al., 2019).

d) Is there a correspondence to human (in vivo?) changes?

To date, no specific studies have been conducted with chemicals to demonstrate a correspondence to human in vivo changes. However, the basic electrical properties measured in MEA recordings provide the

biological substrate and mechanistic underpinnings for in vivo electroencephalogram recordings made clinically in humans and other mammals. Further, neural cultures grown on MEAs from patient derived IPSC cells from various different neurological diseases demonstrate disruptions in network activity (Gullo et al., 2014; Pelkonen et al., 2020; Que et al., 2021) and MEAs have been used to evaluate compounds causing convulsions s in vivo (Odawara et al., 2018).

Pre-validation or validation

To date, 451 unique compounds (as defined by unique DTXSIDs) have been tested successfully in this assay.

No formal OECD 34 validation study has been done (eg., ring trials with a standard set of known postive and negative controls).

The test method is part of a pre-validation study that test the DNT hazard assessment for 83 Compounds in a DNT test battery. The compound set includes potential DNT positive and DNT negative compounds.

Linkage to (e.g. OECD) guidelines/regulatory use

Test is not linked to regulatory guidelines.

Test method transferability

Operator training

For operators with a basic training in cell culture practices a minimum four-week training period for handling of the test system and training in the assay is recommended. This should be under the supervision of someone experienced in running the NFA. The operators should have basic understanding in neurobiology, toxicology and electrophysiology as well as data evaluation with respect to concentration response fitting.

Transfer

The test method has been used by multiple operators over a period of 5 years. However, inter operator variability has not been formally determined. Many of the compounds that were tested as multiple samples (Table 8.4.2) were tested by different operators with similar results. In addition, ring trials have been performed that demonstrate high intralaboratory replicability for MEA based approaches to assess responses of mature neural networks to acute exposures to neurotoxic/neuroactive compounds (Novellino et al., 2011; Vassallo et al., 2017).

Safety, ethics and specific requirements

Specific hazards; issues of waste disposal

The NFA itself has no specific hazards. However, chemicals being tested in the NFA may pose both human health and environmental hazards. Therefore, appropriate personal protective equipment should be worn by operators, and appropriate waste disposal practices should be followed.

Safety data sheet (SDS)

SDS should be supplied by the manufacturer or supplier of the chemicals being tested, and should be kept

on file as appropriate for legal guidelines for the location of the facility where testing is occurring.

Specific facilities/licenses

No specific facilities are required.
Use of live rodents will require the approval of the appropriate institutional animal care committee. Note that many vendors supply frozen primary cortical cells which would avoid this issue. However, the performance of these cells has not been verified in this assay.

Commercial aspects/intellectual property of materials/procedures

The Axion AxIS software is a commercial product and requires a license. Kits used for cytotoxicity assessment are supplied by commercial vendors and subject to their licensing. There are no other commercial/intellectual property issues.

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Appendix B.9

Author: Tim Shafer, Kathleen Wallace
Date: 10.03.2023
Version: 1.0

Overview

Descriptive full-text title

High-Content Imaging Assay to Screen for Changes in Neurite Outgrowth Due to Chemical Exposure in Neurons Derived from human Induced Pluripotent Stem Cells.

Abstract

High Content Imaging Assay to Screen for Changes in Neurite Outgrowth was developed to screen large numbers of compounds for potential developmental neurotoxicity in vitro. During the development of the nervous system, many processes occur to give rise to a functional and healthy neural network. These important neurodevelopmental processes may be disrupted by potential toxicants, resulting in developmental neurotoxicity. Among these processes is neurite outgrowth – the physical outward growth of neurites (eventually axons and dendrites) of individual neurons that allows them to make connections with other neurons and ultimately gives rise to the physical network of cells. This assay utilizes a high-content imaging solution to describe neurite outgrowth in an iPSC-based cell culture, via the immunocytochemical labelling of cell bodies and neurites. An automated image analysis protocol is employed to systematically identify targeted structures based on preassigned criteria. Ultimately, changes in the number and length of young outgrowths is quantified and inhibition of more than 30% results in a hit call. The assay is performed on a 96-well plate, allowing for a medium-to-high throughput screening of chemicals. According to the readiness criteria as published by Bal-Price et al. (2018) the human neurite outgrowth assay obtained the readiness score of A.

Assay summary:

toxicological target	→	developing brain
test system	→	induced pluripotent stem cells (iPSC) neurons
readout(s)	→	Neurite count, neurite length, neuron count
biological process(es)	→	neurite outgrowth, viability, cytotoxicity
(human) adverse outcome(s)	→	cognitive dysfunction
hazard(s)	→	adverse effect on neurite outgrowth
endpoint of current regulatory studies	→	no

validation/evaluation → readiness analysis

General information

Name of test method

Neurite outgrowth assay in human iPSC derived neurons

Version number and date of deposition

20220105_v1.0

Summary of introduced changes in comparison to previous version(s)

“original version”

Assigned data base name

ToxCast invitro database assay identification: *CCTE_Mundy_HCI_CDI_NOG*

Name and acronym of the test depositor

United States Environmental Protection Agency (US EPA)

Name and email of contact person

Tim Shafer (shafer.tim@epa.gov)

Name of further persons involved

Theresa Freudenrich
Kathleen Wallace

Reference to additional files of relevance

Number of supporting files:
1. Standard Operation Procedure (Section 3.7)

Description of general features of the test system source

Supply of source cells

Commercial supplier, FujiFilm Cellular Dynamics, Inc, Madison, Wisconsin. Note: an alternative source of cells are hN2 cells from Aruna. However, these cells are no longer commercially available.

Overview of cell source component(s)

Human glutamatergic-enriched cortical neurons derived from induced pluripotent stem (iPS) cells are

provided as cryopreserved cells, from FujiFilm Cellular Dynamics, Inc, Madison, Wisconsin. Material originates from human blood of a male aged 50 - 59 at sampling.

Characterization and definition of source cells

$6x10^6$ iCell GlutaNeurons are obtained from FujiFilm Cellular Dynamics (#R1061) and cultured to the SOP (section 3.7). FujiFilm Cellular Dynamics provides the cells with a viability of at least 70% by trypan blue exclusion.

After 7 days in vitro (DIV) the cells are nestin negative, MAP2, DCX and βIII-tubulin positive, and express the presynaptic protein synaptophysin.

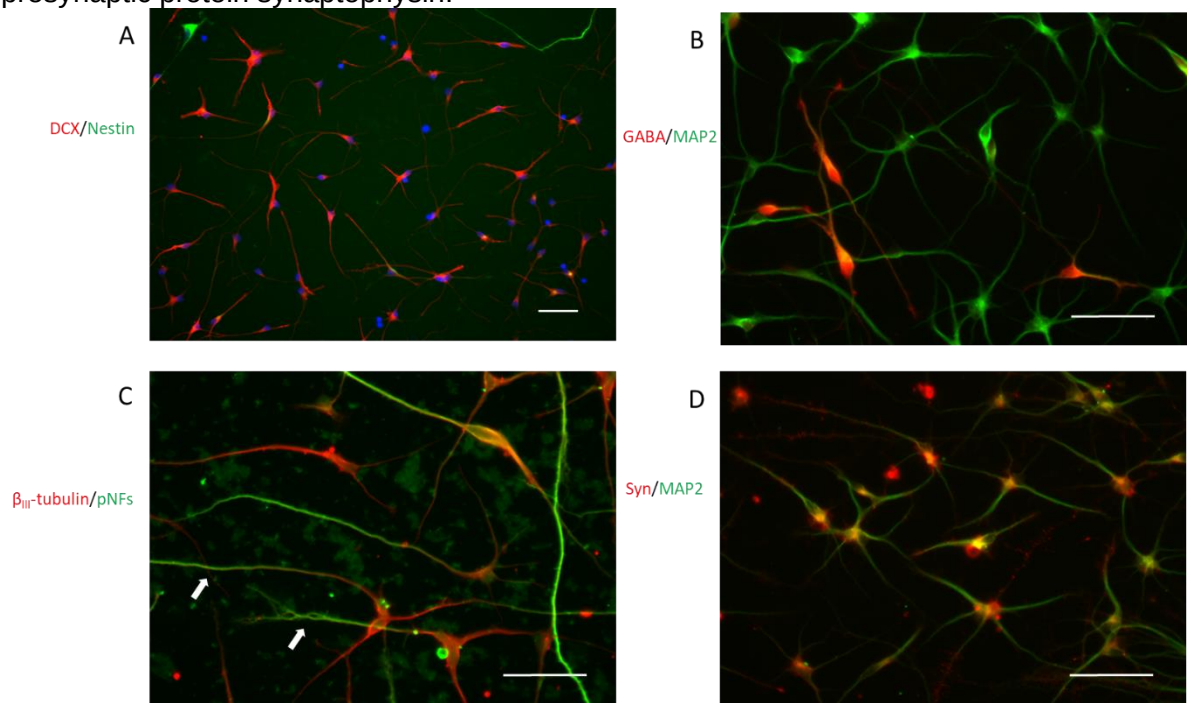

Figure 3.3.1. Characterization of iCell neurons. Cells were plated at 5,000 cells per well in a 96-well plate and fixed on DIV 7. The cells were then immunostained for neuronal markers, including (A) DCX (early neuron) and nestin (neuroprogenitor); (B) MAP2 (neuronal cell bodies and dendrites) and GABA (inhibitory neuron); (C) βIII-tubulin (neuronal cell bodies and neurites) and phosphorylated neurofilament (axons [arrows]); and (D) MAP2 (neuronal cell bodies and dendrites) and synaptophysin (presynaptic vesicle protein). In all images, scale bar = 50 µm. Figure from Druwe et al., 2016.

Acceptance criteria for source cell population

Before thawing of the cells the following criteria have to be fulfilled:

- tested positive for neuron specific betaIII tubulin (Tuj^+)/ $Nestin^-$ at 3 days post thaw
- identity confirmation (single nucelotide poylmorphism (SNP) genotyping)
- tested negative in sterility test and for mycoplasma
- cell count of ≥ 6 $x10^6$ cells/vial
- viability ≥ 70%
- cell type ≥ 70% Glutamatergic

These acceptability criteria are provided by FujiFilm Cellular Dynamics Inc.

Variability and troubleshooting of source cells

Thawing the cryopreserved cells and coating the cell culture surface generates the variability

Critical consumables
The BrainPhys Neuronal Medium, iCell Nervous System Supplement, iCell Neural Supplement B, and N-2 Supplement must be protected from light.

The maintenance medium is supplemented with laminin, iCell Nervous System Supplement, iCell Neural Supplement B and N-2 Supplement. The medium should be discarded 14 days after addition of the supplements.

Critical handling
Following the thawing procedure provided by the manufacturer is critical to obtain optimum viability.

Precise timing during thaw is critical to maximizing viable cell recovery.

Drop-wise addition of the complete BrainPhys medium to the cell suspension is critical to minimize osmotic shock and ensure maximum viability and attachment.

Avoid repeated pipetting of the thawed iCell GlutaNeurons cell suspension.

Differentiation towards the final test system

Not applicable.

Reference/link to maintenance culture protocol

OP: iCell GlutaNeurons OP-NHEERL-H/ISTD/SBB/KAW/2018-01-r2 (Available upon request; email: Shafer.tim@epa.gov)

Definition of the test system as used in the method

Principles of the culture protocol

Cells are cultured on clear 96 well culture plates sequentially coated with poly-L-ornithine and laminin (Sigma). The cells are thawed from frozen stock vials obtained from CDI (CDI Technologies catalog number C1033) that are kept in liquid nitrogen until use, resuspended in Brain Phys medium (StemCell technologies catalog number 05790) supplemented with iCell Neuronal Supplement B (CDI catalog number C0129), iCell nervous System Supplement (CDI catalog number M1031) N-2 supplement (Life Technologies catalog number 17502048), laminin (Sigma catalog number L2020) and Penicillin/streptomycin (Life Technologies catalog number 15140122). The cells are centrifuged at 400xg for 5 min at room temperature. Cells are plated in 96 well culture plates at a density of 5000 live cells in 100 µl of media per well and maintained in a humidified incubator at 37ºC and 5 % CO_2. Other cell culture products (DMEM, StemPro media) have not been evaluated for growth and maintenance of these cells).

Acceptance criteria for assessing the test system at its start

As noted previously, viability of cells should be 70% or greater at the time of plating.

Acceptance criteria for the test system at the end of compound exposure

Every experiment is run in triplicate. Data generated from a replicate that is more than 2x the mean of the other replicates will be evaluated for potential problems before being accepted. If rejected, rationale for rejection will be recorded in the experimental notebook.

Negative (solvent) control – Dimethyl sulfoxide (DMSO), CAS 67-68-5
Water, CAS 7732-18-5
Ethanol (EtOH), CAS 64-17-5
DMSO/EtOH, 1:1 mix

Positive control – Rac1 Inhibitor, CAS 1177865-17-6.

Variability of the test system and troubleshooting

Sources of Variation:

Sources of variation include in parameters due to plating on different days (day to day or vial to vial variation), as well as operator to operator variation.

These cells are all derived from the same source and should have highly consistent performance.

The variability for the different endpoints is shown in 0 “Test Performance”.

Metabolic capacity of the test system

Metabolic pathways have not characterized extensively to date.

Omics characterization of the test system

Transcriptomic characterization of the test system is currently underway.

Features of the test system that reflect the *in vivo* tissue

- Newly formed neurons undergo a series of extensive morphological changes as they mature including emergence of neurites, neurite outgrowth, neurite branching and establishment of cell–cell contacts (i.e. synaptogenesis).
- Presence of excitatory glutamatergic neurons
- Presence of inhibitory gabaergic neurons
- This test system does not contain glia

Commercial and intellectual property rights aspects of cells

For the source cells, CDI holds donor consent and legal authorization that provides permission for all research use.

Reference/link to the culture protocol

See 0.

Test method exposure scheme and endpoints

Exposure scheme for toxicity testing

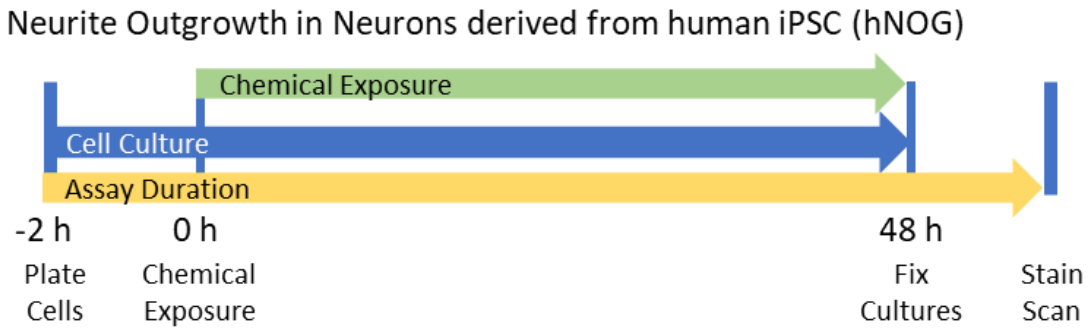

Figure 5.15.1 Exposure scheme. iCell GlutaNeurons are plated in Poly-L-Ornithine/Laminin coated 96 well Flat-bottom plates in complete BrainPhys media and exposed to increasing compound concentrations over a cultivation time of 48 h.

Endpoint(s) of the test method

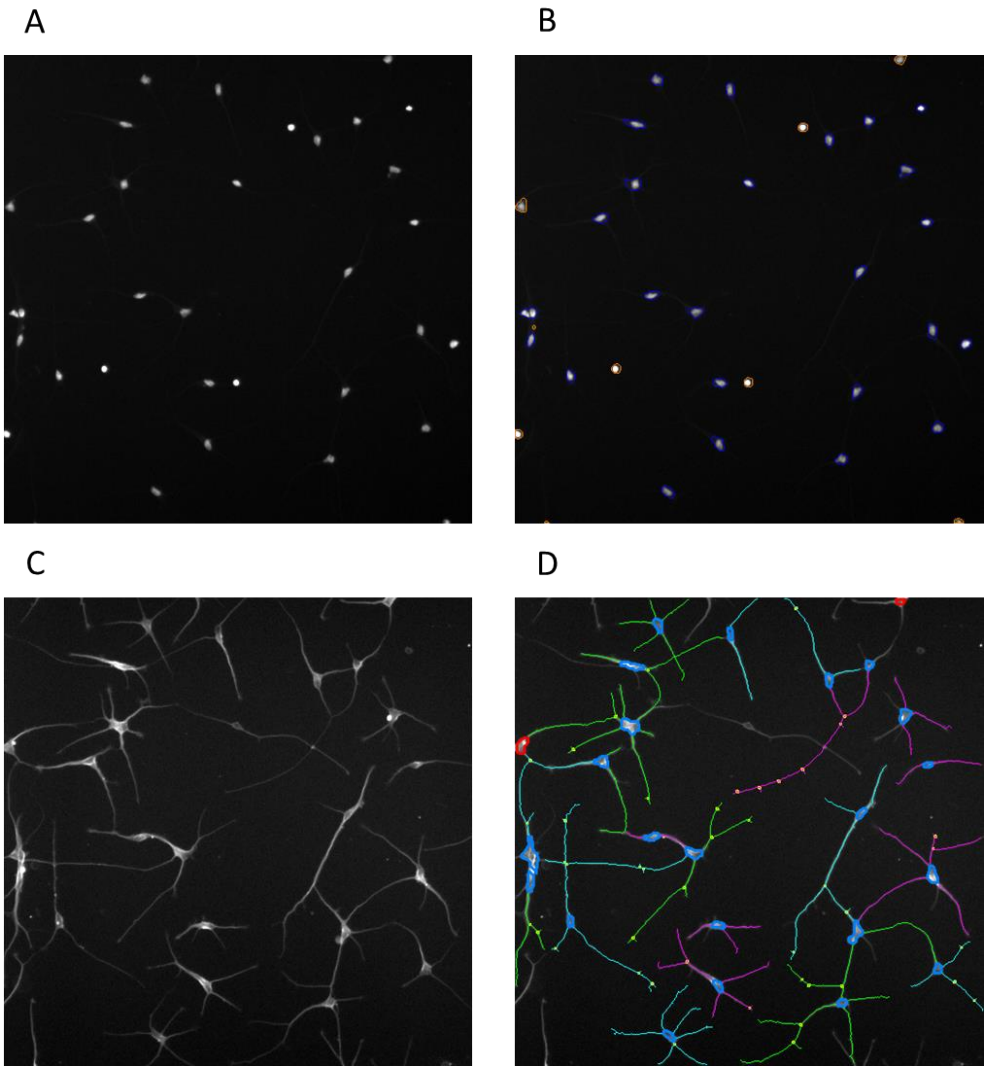

Figure 5.2.1 Automated image analysis of neurite outgrowth. Automated image analysis of neuronal morphology. (A) Channel 1: Hoechst-labeled nuclei. (B) Analysis of channel 1 showing accepted nuclei outlined in a dark blue mask and rejected nuclei outlined by an orange mask. (C) Channel 2: βIII-tubulin labeled neuronal cell bodies and neurites. (D) Analysis of channel 2 showing neuronal cell bodies associated with a valid nucleus outlined in light blue and neurites emerging from the cell bodies traced in light blue, green, and purple. Yellow dots represent branch points. Scale bar = 100 µm. From Druwe et al., 2016.

Table 5.16.1 Endpoint assessment: Morphology of βIII -tubulin labeled neurons as measured using automated microscopy.

Neurodevelopmental Process	ACID	AEID	Endpoint Name/TCPL Endpoint Name	Description
Neurite outgrowth (NOG) initiation	2695	2789	BranchPointTotalCountPerNeuronCh2 / BPCount_loss	Neuron Structure
	2694	2790	NeuriteTotalCountPerNeuronCh2 / NeuriteCount_loss	Neuron Structure
	2693	2791	NeuriteTotalLengthPerNeuronCh2 / Neurite_Length_loss	Neurite Length
	2696	2780	SelectedNeuronCountPerValidField / NeuronCount_loss	Cell Viability / Quality Control

Measurements of neurite length (NeuriteLength), the number of neurites (NeuriteCount) and the number of neurite branch points (BPCount) per cell are calculated for each assay well. Decreases in any of these measures are associated with inhibition of neurite outgrowth. The number of neurons per field (NeuronCount) is also measured. Decreases in the number of neurons per well as compared to control is indicative of cytotoxicity. ACID=assay component endpoint identification; AEID = assay endpoint identification; TCPL = toxcast pipeline.

Overview of analytical method(s) to assess test endpoint(s)

Primary endpoints:

All primary and secondary endpoints are assessed based on an immunocytochemical staining (ICC) of images for each well. Forty-eight hours after chemical treatment cells were fixed with warm (37°C) 4% paraformaldehyde containing 1.5 µg/ml Hoechst 33342 for 20 min followed by permeabilization and blocking steps. Cell bodies and neurites (axons and dendrites) were labeled using a rabbit primary antibody for βIII-tubulin (Biolegend 802001, 1:800) followed by AlexaFluor 546 goat anti-rabbit secondary antibody (Molecular Probes, 1:500). Well-level population averages were used as the statistical unit of measure. Complete concentration-response curves for chemical effects on neurite outgrowth and cell viability were generated within a 96-well plate using one well per concentration. Experiments were repeated in triplicate on separate plates. All data were normalized to the vehicle control wells within a plate.

Secondary endpoint:

The number of cells per field was used as an indicator of cell viability at the time of fixation.

Technical details (of e.g. endpoint measurements)

A Cellomics ArrayScan VTi HCS Reader (Thermo Fisher Scientific) is used for automated image acquisition and analysis of neurite outgrowth (Operating Procedure for High Content Imaging of Neurite Outgrowth: OP-NHEERL-H/ISTD/SBB/TMF/2018-008-r1) Images are acquired using a 20x Pan NeoFLUAR (NA = 0.4) objective with a solid state LED light source, and an XF100 two channel dichroic filter set with excitation at 365(50) and 475(40) and emission at 535(45). Images are analyzed using the Cellomics Neuronal Profiling BioApplication (version 4) to measure neurite morphology. Optimization of nuclear masking and selection, cell body masking and selection, and neurite tracing parameters is performed on untreated cultures at 48 h after initial plating. In each well, multiple unique fields-of-view are acquired until at least 300 neurons are counted. Four morphological features are quantified: 1) number of cells (neurons) per field, 2) total neurite

length per neuron, 3) number of neurites per neuron, and 4) number of neurite branch points per neuron. Neurites are defined as processes > 10 µm in length.

Endpoint-specific controls/mechanistic control compounds (MCC)

Rac 1 Inhibitor (Inhibitor of small GTPase Rac 1 protein), Rac1 and Cdc42, members of the Rho GTPase family, positively regulate neurite extension through reorganization of the actin cytoskeleton.

This assay was developed following the methods outlined in Crofton et al., 2011, using a training set of chemicals shown in Table 5.5.1.

Table 5.5.1. Training set of chemicals

Compound	*CAS*	*Vehicle*	*Source*	*Concentration (µM)*	*Reference*[a]
Bisindolylmaleamide-1	133052-90-1	DMSO	EMD/Millipore	0.01–10	Shi et al.[27]
Lead	6080-56-4	H_2O	Sigma	0.01–30	Audesirk et al.[28]
Lithium	7447-41-8	H2O	Sigma	10–10,000	Takahashi et al.[29]
Methylmercury	115-09-3	DMSO	Sigma	0.03–10	Krug et al.[30]
Rac1 inhibitor	477865-17-6	H2O	EMD/Millipore	1–50	Blakely et al.[31]
Sodium orthovanadate	13721-39-6	H2O	Sigma	0.01–30	Mandel and Banker[32]
trans-Retinoic acid	302-79-4	DMSO	Sigma	0.01–30	Radio et al.[33]
U0126	109511-58-2	DMSO	Promega	0.01–30	Krug et al.[30]
Etoposide	33419-42-0	DMSO	Sigma	0.01–10	Krug et al.[30,b]
Acetaminophen	103-90-2	DMSO	Sigma	0.03–30	NR
Glyphosate	1071-83-6	H_2O	Chem Service	0.03–30	NR

[a]Reference for effect of compound on neurite outgrowth *in vitro*.
[b]Nonspecific control.
NR, not reported in the literature; CAS, chemical abstracts service number.

Positive Controls

Each experimental plate is equipped with 8 wells of positive controls to verify proper assay performance. Rac 1 inhibitor is used as a positive control for neurite outgrowth at 10 µM and 30 µM for 4 wells at both concentrations for each plate.

Negative and unspecific controls

The solvent control (SC) is used as negative control that is run on each experimental plate. Each SC has to be established by comparing the effect of the SC to the effect of the media control. Established solvent controls show the same response as the media control.

Established solvent controls are:

Dimethyl sulfoxide (DMSO), CAS 67-68-5 - 0.1 % v/v
Water, CAS 7732-18-5 - 0.1 % v/v
Ethanol (EtOH), CAS 64-17-5 - 0.1 % v/v
DMSO/EtOH, 1:1 mix - 0.1 % v/v

Features relevant for cytotoxicity testing

CDI Igluta™ neurons are a multicellular system consisting of excitatory and inhibitory neurons. The measurement of cytotoxicity and viability therefore always represents all cells within the culture. It dies not

differentiate between excitatory and inhibitory neurons.

When using the Cellomics Neuronal Profiling BioApplication, Neuron count per valid field is collected as a measure of cell viability by giving a measure of the quantification of live cells in a given area.

Acceptance criteria for the test method

General acceptance criteria:

As stated previously, an image analysis protocol is used to automatically identify targeted structures based on preassigned criteria. Changes in the number and length of young outgrowths is quantified; see section 8.2 for criteria for hit calls. Each individual plate is accessed for expected results from controls based on previous experiments.

As with all cell-based experimentation, maintain proper sterile technique and good cell maintenance practices. In plating cells, an aliquot is to be counted and assessed for viability. If less than 85% of the cells are viable, the cells are not used. Wells in which erroneous volumes of treatment compound are added should be discarded. Each time media is prepared, a sterility test plate is prepared by placing at least one 1.0 mL sample of cell media into the plate.

Plates should be monitored for contamination throughout the experiment. Contamination may be indicated by yellow and/or cloudy media. Contaminated wells should be emptied of media and treated with a bleach solution. Any plate with contaminated wells should be monitored more frequently and carefully as contamination can often spread to multiple wells. Data from contaminated wells should not be analyzed.

Throughput estimate

The methods described here are set up in a 96 well plate format with automated image acquisition and analysis and data evaluation. Pipetting steps such as coating of 96 well plates and compound dilutions can be automated using a liquid handling system.The methods described here are described for a 96 well plate format. Typically, 9 plates can be made from one vial of cells, which allows testing 24 compounds in triplicate (technical replicates). If cultures are made every 14 days, 48 compounds per month can be screened in triplicate at multiple concentrations. The throughput is therefore estimated as medium.

Handling details of the test method

Preparation/addition of test compounds

The method is set up to test 8 compounds at 10 concentrations plus a solvent control. Example dilution plate and final plate maps are provided in figures 6.1.1 and 6.1.2, respectively

The experimental compounds are each prepared in stock solutions at 1000-fold concentrations of 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, 30 and 100 mM in DMSO, ethanol, DMSO/ethanol or double-distilled H_2O (ddH_2O) based on solubility. For each experiment, working solutions of the chemicals were prepared by adding 5 µL of chemical to 495 µL of media for a 1/100 dilution. Cells were treated by adding 11 µL of the working solution to 100 µL of media in the wells for a 1/10 dilution, resulting in a total 1/1000 dilution of chemical stock and 0.1% final concentration of solvent. Note: the addition of 11 ul to 100 ul is not an exact 1:100 dilution and will result in a 0.01% difference from nominal concentration that will have to be corrected for when determining actual final concentration.

Stock solutions are aliquoted and stored at -20°C. Freeze/thaw cycles should be avoided with compound stock solutions.

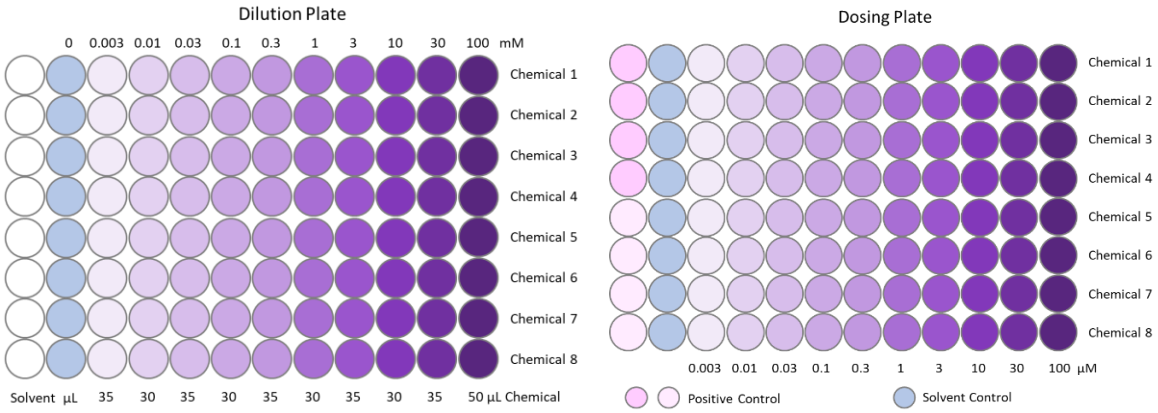

Figure 6.1.1 Dilution and Dosing Scheme. Stock solutions are aliquoted on Column 12 of the dilution plate. Solvent control (SC) is added to Column 2 and transferred to Columns 3, 4, 5, 6, 7, 8, 9, 10 and 11. Serial dilution is performed from right to left across the plate. On the dosing plate, the compounds are diluted 1:100 with Complete BrainPhys media. They are further diluted 1:10 into their corresponding well on the 96 well plate. Note: the concentrations indicated on the Dosing plate (along the bottom), indicate the final concentration that will be achieved following dilution in the test plate.

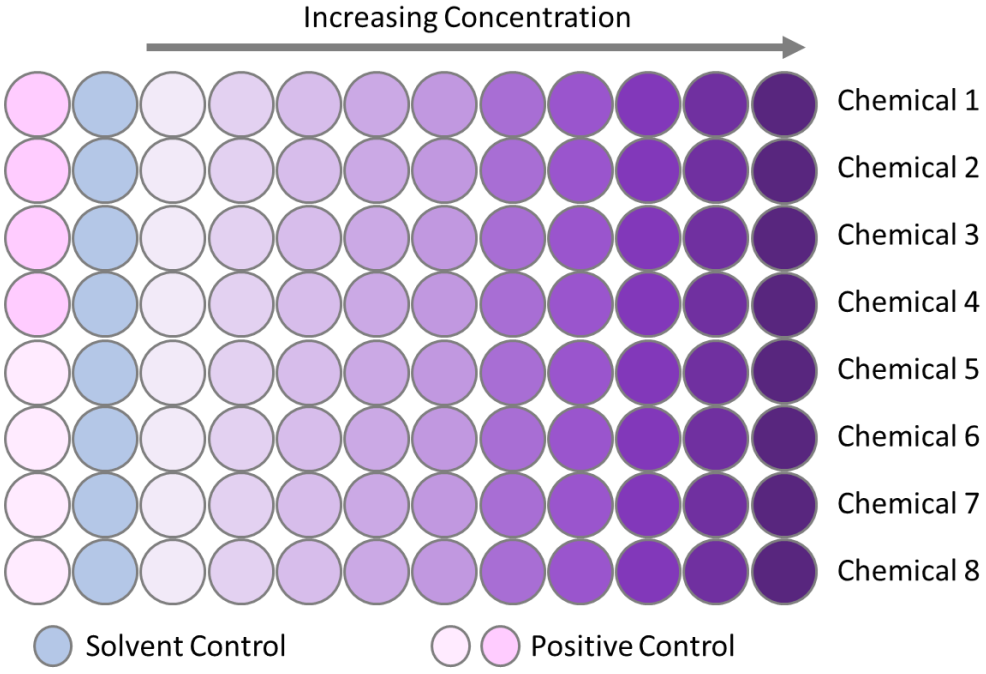

Figure 6.1.2. Final plate map: Positive controls are in the first column. Solvent controls are on the second column, and the compound treatment are increasing in concentration from left to right of the plate. There are three replicate plates tested, The compounds are rotated in different rows, to minimize edge well effect.

Day-to-day documentation of test execution

A plate map for all three plates is created prior to plating cells. All data relevant to the human neurite outgrowth assay is stored on an online OneNote notebook.

Practical phase of test compound exposure.

All aspects of the experiment are recorded in an online OneNote laboratory notebook. This includes any documentation of adherence to platemaps, potential errors, and any other variable that may impact the assay and interpretation of results. All projects are subjected to review by EPA Quality Assurance Managers.

Concentration settings

Ten compound concentrations are tested on each plate, with ½ log unit spacing between concentrations (e.g. 10, 3, 1 uM, etc). Standard upper concentrations tested are either 100 or 30 uM, depending on the solubility of the compounds as well as the highest concentration which can be provided in a stock solution from test set providers (e.g. EPA's ToxCast program).

Start concentrations and concentration ranges are defined based on the following factors:

- toxicological relevance of the compound (i.e. internal human exposures, effects at lowest concentrations)
- concentration of stock compound in solutions provided by suppliers of chemical sets (e.g. for EPA's ToxCast library chemicals are typically supplied at 20 mM in DMSO).
- the highest concentration of solvent (e.g. DMSO) that can be tolerated by the assay.
- solubility of the compound

Uncertainties and troubleshooting

Problematic compounds:

- volatile compounds
- high lipophilicity (high K_{OW})
- low solubility in established solvents

Critical handling steps:

- For compounds that may have some volatility, or to ensure against effects due to evaporation of media, plate sealers may be used.

Sources of variation:

- Many of the steps performed in the Neurite Outgrowth assay are sensitive to pipetting errors. Care should be taken to pipette slowly and steadily to prevent disrupting attached cells.
- Immunocytochemical staining consists of multiple washing steps. Variation can be caused by various aspiration and pipetting steps that are needed to perform staining. Pipetting errors can also cause

variation of dilution of antibodies and buffers. Additionally, a slow, consistent aspiration should be used to prevent aspiration of cells.

Known Pitfalls:

- Careful attention should be paid to the age of antibodies used for staining, as old antibodies will yield poor results. Do not use antibodies after their expiration date and store them according to the vendors instructions.

Caveats:

- No extracellular growth "cues" are provided in this assay.
- Different neuronal (sub)populations express different proteins (which may also vary between different time points) and thereby also only a limited number of potential intracellular targets for toxicants are present in these different neurite outgrowth tests (even if testing different neuron types between different tests).

Detailed protocol (SOP)

Two hours after plating, cells are exposed to chemicals by adding 11 µl of the working solution to 100 µl of media in the wells for a 1/10 dilution, resulting in a 0.1% final concentration of DMSO, and then returned to the incubator. Rac1 Inhibitor (10 and 30 µM) is used as a positive control.

Forty-eight hours after chemical treatment cells are fixed by direct addition of 110 µl of warm (37°C) Dulbecco's phosphate buffered saline (DPBS) fixative solution containing 8% paraformaldehyde, 8% sucrose, and 0.1% of 3 mg/ml Hoechst 33342 into each well. The amount of fixative solution added is equivalent to the amount of media in each well. After 20 minutes of incubation at room temperature the cells are rinsed twice with DPBS, then permeabilized for 30 min in permeabilization/blocking buffer (0.2% Triton X-100 and 2% BSA in PBS). Primary antibodies are prepared by dilution in Immunocytochemical Staining Buffer (ISB: 10X Dulbecco's PBS (Gibco 14080-500), 0.1% Saponin, 5% Bovine Serum Albumin,0.5% NaN3 (Sodium Azide)) with βIII-tubulin (rabbit anti-βIII-tubulin, Biolegend 802001, 1:800) followed by Alexa Fluor-488 secondary (1:500) to label neuronal cell bodies and neurites.

A Cellomics ArrayScan VTi HCS Reader (Thermo Fisher Scientific) is used for automated image acquisition and analysis of neurite outgrowth. Images are acquired using a 20x Pan NeoFLUAR (NA = 0.4) objective with a solid state LED light source, and an XF100 two channel dichroic filter set with excitation at 365(50) and 475(40) and emission at 535(45). Images are analyzed using the Cellomics Neuronal Profiling BioApplication (version 4) to measure neurite morphology. Optimization of nuclear masking and selection, cell body masking and selection, and neurite tracing parameters is performed on untreated cultures at 48 h after initial plating. In each well, multiple unique fields-of-view are acquired until at least 300 neurons are counted. Four morphological features are quantified: 1) number of cells (neurons) per field, 2) total neurite length per neuron, 3) number of neurites per neuron, and 4) number of neurite branch points per neuron. Neurites are defined as processes > 10 µm in length. The number of cells per field is used as an indicator of cell viability at the time of fixation. Well level population averages are used as the statistical unit of measure.

Dosing: OP-NHEERL-H/ISTD/SBB/TMF/2018-004r1; Chemical exposure of cells in cell culture plates. Immunostaining: OP-NHEERL/ISTD/SBB/TMF/2018-010-r2; Immunocytochemistry on cells in 96 well plates. High Content imaging OP-NHEERL/ISTD/SBB/TMF/2018-008r1; Operating procedure for high content imaging of neurite outgrowth (Available upon request; email: Shafer.tim@epa.gov).

Special instrumentation

- Incubator for cell culture

- Cellomics® ArrayScan® VTI HCS Reader (ThermoFisher Scientific)

Possible variations

Measurement of neurite outgrowth is a common way to assess the effects of chemicals on development of neuronal morphology, and a number of publications are available on this topic (Ryan et al., 2016; Li et al., 2021). Other approaches may be valid for assessment of chemical effects on neurite outgrowth but may or may not have been evaluated to the extent of the assay described herein.

Data management

Raw data format

Image files (*.C01 files) are saved to a network drive. These files can be reanalyzed by re-applying the bioapplication software. The data extracted are saved to a network drive as *.xls (excel) files, with 1 file containing all extracted feature values per experimental plate. The original excel output files are saved for traceability of the data.

Outliers

Mathematical procedures to define outliers are not applied. The tcpl curve fitting program (Filer et al., 2017) is robust with respect to minimizing the impact of outliers.
Data points from wells where technical problems are known or obvious are excluded from the analysis.

Possible technical problems:
- pipetting errors
- problems in ICC staining
    - cells dried out
    - wrong illumination
    - blurry pictures

All outliers are marked in the laboratory notebook.

Raw data processing to summary data

Bioapplication software analyze the image files and extract the relevant features (neurite length, branch points, etc) and save these data as excel (*.xls) files. R scripts are used to scrape the data from the *.xls files. Data are transformed to the “long” data format, with 1 row for each well-feature pair.

Normalization, Curve fitting and BMC calculation

Data are analyzed using the ToxCast Pipeline (tcpl) approach as described by Filer et al., 2017. A summary of techniques applied is in table 7.4

Table 7.4 Methods applied in tcpl for the human neurite outgrowth assay

ToxCast Data Pipeline Level	HCI assays: Methods Applied

mc0: pre-processed data input	Data are raw input
mc1: mapping to well and column indexes	Auto
mc2: transformation	No transformation
mc3: normalization	Baseline value (bval) was calculated as the median value for the vehicle control wells (DMSO) on a by-plate basis; No positive control value was used in normalization (pval=0); the response was calculated as percent of DMSO vehicle control.
mc4: BMAD calculation type for curve-fitting	An approximation of noise around the baseline signal, the baseline median absolute deviation, was calculated based on the vehicle control wells and the 2 lowest concentrations of the test wells on each plate.
mc5: Hitcall and potency determination	The cutoff for a positive response was the greater of 30% or 3*BMAD.
mc6: caution flags on fitting	Flags for single point hit at maximum concentration (6), flags for single point hit not at the maximum concentration screened (7), inactives with multiple median responses above baseline (8), noisy curves relative to the assay (10), actives with borderline efficacy (11), inactives with borderline efficacy (12), low concentration gain-loss curve-fits (15), possibly overfitting (16), hitcalls with less than 50% efficacy (17) were assigned to all; additionally cell viability assays were assigned "viability gain-loss fit" (19)

Internal data storage

Data collected from the Arrayscan VTI are saved **.C01 files* on a laboratory network drive. This network drive resides on EPA servers which are backed up daily. As per US Government regulations, these files will be maintained for at least 20 years.

Metadata

Metadata is saved in *.xlsx files, with 1 file for each group of 3 plates prepared on the same date. Typically, 9 plates are prepared from one vial of cells. Groups of 3 plates are used as technical replicates. This allows up to 24 chemicals to be tested from one vial of cells. R scripts are used to scrape the metadata from the files, merge the metadata with the experimental data for each well, and save the result in a *.RData file.

Metadata file format

The metadata file format is *.xlsx.

Prediction model and toxicological application

Scientific principle, test purpose and relevance

During the development of the nervous system, many processes occur to give rise to a functional and healthy neural network and hence nervous system. These important neurodevelopmental processes may be disrupted by potential toxicants, resulting in developmental neurotoxicity. Among these processes is neurite outgrowth – the physical outward growth of neurites (eventually axons and dendrites) of individual neurons that allows them to make connections with other neurons and ultimately gives rise to the physical network of cells that connect the nervous system together. This assay utilizes a high-content imaging solution to describe neurite outgrowth in a human iPSC-derived neuronal cell culture, via the immunocytochemical labelling of cell bodies and neurites. An automated image analysis protocol is employed to systematically identify targeted structures based on preassigned criteria. Ultimately, changes in the number and length of young outgrowths is quantified and inhibition of more than 30% results in a hit call. The assay is performed on a 96-well plate, allowing for a medium-to-high throughput screening of chemicals.

Prediction model

The cutoff for a positive response in each assay endpoint is set as 3*BMAD or a 30% change from DMSO controls, and compounds with treatment levels reaching this cutoff are then subjected to curve fitting in tcpl, from which AC_{50} values are generated (see table 7.4). The predicition model is based on a comparison between the AC_{50} value for the NOG-specific endpoint and the AC_{50} value for a cytotoxicity/viability effect.

Thereby the following classifications apply:

"specific hit": a threefold difference between the AC_{50} value for hNOG endpoints and the most potent cytotoxicity endpoint. Where no cytotoxicity endpoint had an AC_{50} value, then the highest concentration tested is used.

"non-specific hit": Less than a threefold difference exists between AC_{50} value for the hNOG-specific endpoint and the most potent cytotoxicity AC_{50} value.

It should be noted that there are other valid approaches to determining specificity. For example, one could calculate the area under the curve of the specific endpoint that is below the AC_{50} value for cytotoxicity.

"inactive": the compound was not active in hNOG and cytotoxicity endpoints.

Prediction Model Set-up

This assay was developed using a training set of chemicals (see Druwe et al., 2016), and then further evaluated with a test set of chemicals that had 53 putative positive and 13 putative negative DNT chemicals (see Harrill et al., 2018). See sections below for additional details.

All endpoints in this assay are fit in the down direction. For the viability endpoints, fitting in the up direction (increased viability) is not logical since viability of controls is typically quite high (>90%). The neurite outgrowth parameters, can be fit in both the up and down direction. However, to date, the vast majority of compounds tested cause decreases in neurite outgrowth parameters, for which we have assay positive controls. Biological meaning of changes in the up direction (increased neurite outgrowth parameters) is difficult to interpret due to the lack of assay positive controls that alter parameters in the up direction.

Test Performance

Table 8.4.1summarizes the assay performance in terms of reproducibility of the assay.

Assay component name	Median	MAD	CV
MUNDY_HCI_hN2_hNOG_BPCount	0.56	0.0741	13.5
MUNDY_HCI_hN2_hNOG_NeuriteCount	1.66	0.0593	3.63
MUNDY_HCI_hN2_hNOG_NeuriteLength	75.2	4.76	7.15
MUNDY_HCI_hN2_hNOG_NeuronCount	23.4	3.56	13.5

For this assay, the following compounds are used as assay positive controls as they have been previously demonstrated to inhibit neurite outgrowth in in vitro systems:

Loperamide
Bisindolylmaleimide I
Lithium Chloride

Table 8.4.2 summarizes the performance of the assay positive control Lithium Chloride.

AENM	COFF	MED.RESP	MED.HITC	CONC.UM	Z'	SSMD	SN
MUNDY_HCI_hN2_hNOG_BPCount_loss	37.7	79.34	1	10000	0	3	3.62
MUNDY_HCI_hN2_hNOG_NeuriteCount_loss	30	43.53	1	10000	0.38	8	10.45
MUNDY_HCI_hN2_hNOG_NeuriteLength_loss	30	62.56	1	10000	0.58	9	13.25
MUNDY_HCI_hN2_hNOG_NeuronCount_loss	*32.45*	*44.25*	*1*	*10000*	*0*	*2*	*3.63*

In vitro to in vivo extrapolation

IVIVE of data from this assay has been conducted based on the activity (e.g. EC_{50}, $AC_{50,}$ tipping point) values obtained from curve fitting. Because in vitro toxicokinetic information (e.g. lipid and protein content of cells, volume of cells) are not readily available, these extrapolations have been based on the nominal concentration of test article in the medium. Adjusted Equivalent Doses (AEDs) were estimated using the high-throughput toxicokinetic (HTTK) information and models available in the httk R package (v1.8; Pearce et al., 2017), which functionalizes an approach similar to the one previously used by Wetmore et al. (2012).

Applicability of test method

Toxicological application domain
To date, 213 unique compounds (as defined by unique DTXSIDs) have been tested successfully in this assay. The following compound classes have been tested successfully:

Industrial chemicals
cosmetics ingredients
pharmaceuticals

Biological application domain

Next to the endpoints represented by this test method there are several other necessary neurodevelopmental endpoints which need to be studied using other test methods.

Neurodevelopmental processes not represented by this test method:
- Neural Crest Cell (NCC) Migration
- NPC apoptosis
- Synaptogenesis
- Neural Rosette Formation
- hiPSC-derived NPC proliferation
- Network formation
- hiPSC-NPC neuronal differentiation Network formation

- Neuronal subtype differentiation
- Astrocyte Differentiation and Maturation
- Astrocyte Reactivity
- Microglia reactivity

For a complete assessment of developmental neurotoxicity, the test method needs to be part of a test battery.

Incorporation in test battery

To assess the hazard for developmental neurotoxicity it is recommended that this assay is used as one assay in a battery of assays (see 06 “Applicability of test methods”)

For the assessment of chemical action on the endpoints represented by this test method, the test method can be used as a stand-alone test method.

The test method is currently used in the setup of a DNT test battery.

Publication/validation status

Availability of key publications

Key Publications concerning the test method are:

Harrill JA, Freudenrich TM, Robinette BL, Mundy WR.Comparative sensitivity of human and rat neural cultures to chemical-induced inhibition of neurite outgrowth. Toxicol Appl Pharmacol. 2011 Nov 1;256(3):268-80. doi: 10.1016/j.taap.2011.02.013.

Druwe I, Freudenrich TM, Wallace K, Shafer TJ and Mundy WR. Comparison of human iPSC-derived neurons and rat primary cortical neurons as in vitro models of neurite outgrowth. Applied in vitro Toxicology, 2016. 2, 26-36.

Harrill JA, Freudenrich T, Wallace K, Ball K, Shafer TJ, Mundy WR. Testing for developmental neurotoxicity using a battery of in vitro assays for key cellular events in neurodevelopment. Toxicol Appl Pharmacol. 2018 Sep 1;354:24-39. doi: 10.1016/j.taap.2018.04.001.

Potential linkage to AOPs

Key event #382, aberrant dendritic morphology is relevant to this assay, and is part of AOP 13 titled: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities.

Steps towards mechanistic validation

a) Information demonstrating how the test system is biologically relevant to humans in terms of cell types, signaling pathways, etc

This is a human model, so no species extrapolation is necessary. Outgrowth of neurites is a process that relies on conserved signaling pathways that are implimented at different times in different parts of the nervous system, so alterations in this assay may indicate a hazard for impacts on morphology that differ in different brain regions depending on the timing of exposure.

b) Interventions (pathway knockdown, specific inhibitors (i.e. mechanistic controls, which may be part of the training set) that show expected effects on the assay

This assay has been developed by using mechanistic control compounds known to disrupt neurite outgrowth in cortical neurons (Harrill et al., 2011; Druwe et al., 2016).

c) Formal mechanistic validation

There has been no formal validation of this assay. This test method was developed following the criteria established in Crofton et al., 2011, where a set of assay positive controls has been tested (Harrill et al., 2011), followed by a test set of compounds (Harrill et al., 2018).

d) Is there a correspondence to human (in vivo?) changes?

To date, no specific studies have been conducted with chemicals to demonstrate a correspondence to human in vivo changes.

Pre-validation or validation

No OECD 34 validation study has been done. The test method is part of a pre-validation study that test the DNT hazard assessment for 83 Compounds in a DNT test battery. The compound set includes potential DNT positive and DNT negative compounds.

9.5. Linkage to (e.g. OECD) guidelines/regulatory use

Test is not linked to regulatory guidelines.

Test method transferability

Operator training

For operators with a basic training in cell culture practices a four-week training period for handling of the test system and training in the assay is recommended. The operators should have basic understanding in image analysis and data evaluation with respect to concentration response fitting.

Transfer

The test method has been used by multiple operators over a period of 5 years. However, inter-operator variability has not been formally determined. In addition, assessment of neurite outgrowth using high content imaging is a widely utilized metric in the published literature, indicating that transfer of this specific protocol would not be difficult.

Safety, ethics and specific requirements

Specific hazards; issues of waste disposal

The Neurite Outgrowth assay itself has no specific hazards. However, chemicals being tested in the hNOG may pose both human health and environmental hazards. Therefore, appropriate personal protective equipment should be worn by operators, and appropriate waste disposal practices should be followed.

Safety data sheet (SDS)

SDS should be supplied by the manufacturer or supplier of the chemicals being tested and should be kept on file as appropriate for legal guidelines for the location of the facility where testing is occurring.

Specific facilities/licenses

No specific facilities are required.
No specific ethical approval is required.

References

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Appendix B.10

Author: Theresa Freudenrich, Tim Shafer
Date: 10.03.2023
Version: 1

Overview

Descriptive full-text title

Cell Viability, Apoptosis and High-Content Imaging Assay to Screen for Changes in neuroprogenitor cell Proliferation Due To Chemical Exposure.

Abstract

The High Content Imaging Assay to Screen for Changes in Proliferation was developed to screen large numbers of compounds for potential developmental neurotoxicity in vitro. During the development of the nervous system, many processes occur to give rise to a functional and healthy neural network. These important neurodevelopmental processes may be disrupted by potential toxicants, resulting in developmental neurotoxicity. Among these processes is proliferation – neural circuit function can be drastically affected by variations in the number of cells that are produced during development. Defects in neural stem cell proliferation that result in the generation of incorrect cell numbers or defects in neural stem cell differentiation can cause microcephaly or megalencephaly (Homen,et.al. 2015). Apoptosis is a form of programmed cell death that also plays a critical role in development of the nervous system. In particular, cells that fail to become integrated into neural networks during neural development often undergo apoptosis. Chemical exposure can result in changes in apoptosis and such changes may, like changes in proliferation, alter the number of cells in the nervous system, resulting in developmental neurotoxicity. This assay utilizes a high-content imaging solution to determine the number of proliferating cells in a human neural progenitor cell line by the immunocytochemical labelling of the nucleus and the proliferating cell bodies. An automated image analysis protocol is employed to systematically identify targeted structures based on preassigned criteria. Ultimately, changes in the number of proliferating cells is quantified and reduction of more than 30% results in a hit call. The assay is performed on a 96-well plate, allowing for a medium-to-high throughput screening of chemicals. Cell viability and apoptosis measurements are performed simultaneously in 96 well plates using commercial assays to determine toxicity of chemicals and death by apoptosis. According to the readiness criteria as published by Bal-Price et al. (2018) this assay obtained the readiness score of A.

Assay summary:

toxicological target	→	developing brain
test system	→	human neural progenitor cells

readout(s)	→	cell number, proliferation, cell intensity
biological process(es)	→	Proliferation, apoptosis
(human) adverse outcome(s)	→	cognitive dysfunction
hazard(s)	→	adverse effect on cell proliferation and cell viability; indication of apoptosis
endpoint of current regulatory studies	→	no
validation/evaluation	→	readiness analysis

General information

Name of test method

Human Neural Progenitor Proliferation, Cytotoxicity and Apoptosis Assay

Version number and date of deposition

20220104_v1.0

Summary of introduced changes in comparison to previous version(s)

original version

Assigned data base name

ToxCast invitro database assay identification: *CCTE_Mundy_HCI_hNP1 (Apoptosis and Cytotoxicity); CCTE_Mundy_HCI_hNP1_Pro (Proliferation)*

Name and acronym of the test depositor

United States Environmental Protection Agency (USEPA)

Name and email of contact person

Tim Shafer (shafer.tim@epa.gov)

Name of further persons involved

Theresa Freudenrich (freudenrich.theresa@epa.gov)
Kathleen Wallace (wallace.kathleen@epa.gov)
Jackson Keever (keever.jackson@epa.gov)

Reference to additional files of relevance

Number of supporting files:
1. Standard Operation Procedure (Section 3.7)

Description of general features of the test system source

Supply of source cells

Human neural progenitor cells (hNP1) were obtained as cryopreserved cells from ArunA Biomedical, Athens Georgia. The cells were thawed and expanded to produce a stock which is used for all experiments and replenished as needed.

Overview of cell source component(s)

Human neural progenitor cells (hNPC) were obtained as a cryopreserved single cell suspension from ArunA Biomedical, Athens Georgia.

Characterization and definition of source cells

hNP1 human neural progenitor cells are fully differentiated, derived as adherent cells from human embryonic stem cell (ESC) WA09 line. The cells are shipped frozen in a vial with 1 x 10^6 cells. hNP1 Human Progenitors were characterised for expression of Nestin and Sox2, which are markers of progenitor status.

Acceptance criteria for source cell population

After thawing, a trypan blue exclusion viability count is done. Cells above 80% viability are acceptable. Cell populations below 80% will not be used and a new vial will be thawed and tested. To maintain stability, cells are grown for several passages then expanded and cryopreserved. This cryopreserved passage is used as a stock for cells of the same passage and is the beginning point for all testing assays.

Variability and troubleshooting of source cells

As with any cell line, the more subculturing performed, the more likely there will be genetic drift. This critical element is controlled by not expanding beyond a certain passage.

Critical consumables for the hNP1 cells are:

The usage of a poly-L-ornithine/laminin coating that is no older that four weeks from coating. Poly-L-Ornithine should be reconstituted, sterile filtered, aliquoted and aliquots should not be refrozen. Laminin should be thawed slowly at 2-8ºC, aliquoted and stored at -20ºC or -80ºC.

The usage of Knock-out™DMEM/F12 supplemented with StemPro® Neural Supplement, GlutaMax, bFGF and EGF. The Proliferation medium should be aliquoted for feedings and protected from light. The shelf life is four weeks at 4ºC.

The Knockout DMEM/F12, StemPro Neural Supplement and TrypLE Express must be protected from light.

Repeated freezings do not affect the strength of the StemPro Neural Supplement.

The Proliferation Medium should be warmed (room temperature to 37ºC) for all feedings.

Flasks should be at a density on the high side, 5 x10^5 for a T25 flask, 1-3x10^6 for a T75 flask.

All consumables are controlled for shelf-life following recommendations from the manufacturer.

Differentiation towards the final test system

- Establishment of Stock cell supply. The vial of cells obtained from ArunA are thawed in a 37ºC water bath with agitation. Thawed cells are transferred to a 15 mL conical tube and Proliferation Media (Knockout™ DMEM/F12 supplemented with 2% StemPro®, 20 ng/mL EGF, 20 ng/mL recombinant human FGF, 1% GlutaMax™) is quickly added dropwise. Cells are spun at 200xg for 5 minutes at room temperature. The pellet is resuspended in Proliferation media and a sample is taken to determine cell number and viability. Cells are seeded at 5 x 10^5 cells/mL in a T25 flask pre-coated with poly-ornithine and laminin. The flask is placed in a 37ºC/5% CO_2 humidified incubator. Cells are fed every other day during the work week until the flask is at 90% confluency. Cells are sub-cultured and expanded until a pre-determined passage is reached (P7). A large batch of cells is then frozen into cryotubes at 3 x 10^6cells/vial. This becomes the stock from which all experimental cells are drawn.
- Culture of cells for experiments. For an experiment, cells are removed from liquid nitrogen and after thawing are cultivated in proliferation medium at 37°C and 5 % CO_2. Cells are spun at 200xg for 5 min at room temperature. The pellet is resuspended in Proliferation media and a sample is taken to determine cell number and viability. Cells are seeded at 5 x 10^5 cells/mL in a T25 flask pre-coated with poly-L-ornithine and laminin. The flask is placed in a 37ºC/5% CO_2 humidified incubator. Cells culture media is changed every 2-3 days until the flask is at 90% confluency. Cells are sub-cultured and expanded for two more passages each with an increasing number of cells and flask size (T25 to T75) to get to the needed cell number.

Reference/link to maintenance culture protocol

Operating Protocol OP- I-BCTD-RADB-SOP-3311-1 Human neuroprogenitor cells (hNP1). (Available upon request: email: shafer.tim@epa.gov)

Definition of the test system as used in the method

Principles of the culture protocol

After the establishment of the stock (see section 3.6.1), one vial is removed from liquid nitrogen and thawed for each experiment. The cells are sub-cultured twice to allow for recovery and expansion before use. Before each subculturing the cells are observed under a microscope for attachment, confluency, and any sign of contamination. After each trypsinization, a trypan blue exclusion count is used to determine that cells are proliferating, and that viability remains above 80%. Cultivation during the expansion is performed at 37°C and 5% CO2 at a pH of 7.2-7.6.

Acceptance criteria for assessing the test system at its start

The cells are inspected visually for attachment to the plate surface. Before each subculturing the cells are observed under a microscope for attachment, confluency, and any sign of contamination. After each trypsinization, a trypan blue exclusion count is used to determine that cells are proliferating, and that viability remains above 80%. Cultivation during the expansion is performed at 37°C and 5% CO2 at a pH of 7.2-7.6.

Acceptance criteria for the test system at the end of compound exposure

The cells are inspected visually for attachment and contamination. Data from contaminated wells is noted and discarded.

Variability of the test system and troubleshooting

Variability is reduced by using the same passage for each experiment. Potential areas that may cause issues are the age of the coated surface, the age of the media and the health of the cells. These are avoided by only using freshly coated plates (made the day of plating), using medium that is not expired and allowing the cells to achieve confluency before using.

Metabolic capacity of the test system

Unknown

Omics characterization of the test system

Transcriptomic characterisation of the test system is currently underway.

Features of the test system that reflect the *in vivo* tissue

These neuroprogenitor cells express nestin and Sox2, which are markers of neural progenitor status and are expressed by neuroprogenitor cells in vivo.

Commercial and intellectual property rights aspects of cells

The cells were commercially available and trademarked by ArunA Biomedical. However, ArunA no longer sells them. Fisher Scientific appears to have cells from the original EnStem line but these were not the same as the hNP1. They also have other neural progenitors. While cells from these other providers are likely to perform similarly in this assay, their responses would need to be fully characterized.

The tests for cytotoxicity, CellTiter-Glo® 2.0 Assay (Promega G9242),
and apoptosis, Caspase-Glo® 3/7 Buffer (Promega G810A) and Caspase-Glo® 3/7 Substrate (Promega G811A), are patented.

Reference/link to the culture protocol

See section 3.7.

Test method exposure scheme and endpoints

Exposure scheme for toxicity testing

Cells are plated on pre-coated poly-L-ornithine/laminin clear bottom and opaque 96 well plates (day 0). Plates are placed in a 37ºC, 5% CO_2 incubator for 40 to 44 hours at which time plates are removed from the incubator and 10 µL of a freshly made chemical solution is added to each well. Twenty hours after the addition of the chemicals, the clear plates are removed from the incubator and 11 µL of a BrdU solution is added to each well. The plates are returned to the incubator for four hours. After four hours, the plates are fixed with a 4% paraformaldehyde solution. Twenty-four hours after chemical addition, opaque plates are removed from

incubator and cytotoxicity and apoptosis assays are performed.

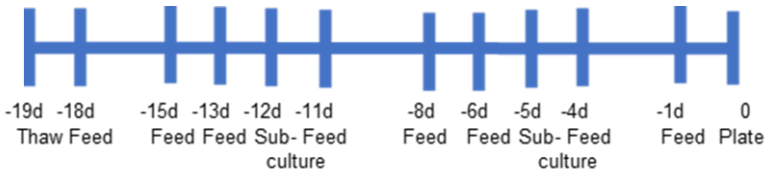

Figure 5.1.1 Expansion of Cells. HNP1 cells are thawed and expanded according to the scheme above. Here "Feed" means a media change.

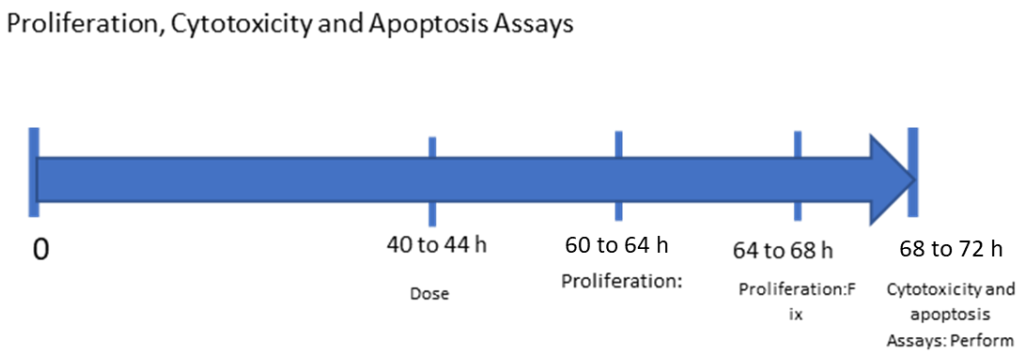

Figure 5.1.17 Exposure scheme. Cells are plated onto 96 well clear and opaque plates and allowed 40-44 hr for attachment to the substrate and recovery. Between 40 and 44 hours all plates are exposed to the chemical ("Dose"). Twenty-four hours after exposure, cells are either fixed or assayed.

Endpoint(s) of the test method

The primary DNT specific endpoints of this test method is BrdU positive cells. Secondary endpoints are apoptotic cell number and cytotoxicity. All endpoints are generated from the same experimental cells but on sister plates.

The normalization endpoint is an in-plate negative control.

Neurodevelopmental Process	Assay Name (*Cell Type*)	ACID	AEID	Endpoint Name	Description
Neural Progenitor Cell Proliferation	MUNDY_HCI_hNP1_Pro (*human hNP1 neuroprogenitors*)	2711	2795	MeanAvgInten_loss	Intensity of BrdU labeling in the nucleus of each cell, averaged across all cells in a well. A decrease as compared to control is indicative of decreased cell proliferation.
		2709	2796	ResponderAvgInten_loss	Percentage of cells with intensity of BrdU labeling $\geq$ 3X background. A decrease as compared to control is indicative of decreased cell proliferation

Neurodevelopmental Process	Assay Name (*Cell Type*)	ACID	AEID	Endpoint Name	Description
		2710	2797	ObjectCount_loss	The number of nuclei per well. A decrease as compared to control is indicative of cytotoxicity.
Neural Progenitor Apoptosis / Viability	MUNDY_HCI_hNP1 (*human hNP1 neuroprogenitors*)	2691	2793	Casp3_7_gain [a]	Intensity of luminescent signal produced by caspase 3/7 cleavage of a detection reagent. The signal produced is proportional to the number of apoptotic cells. An increase as compared to control is indicative of increased apoptosis.
		2700	2794	CellTiter_loss [a]	Intensity of luminescent signal produced by detection of cellular ATP. The signal produced is proportional to the number of viable cells. A decrease as compared to control is indicative of cytotoxicity.

ACID=assay component identification; AEID = assay endpoint identification

Overview of analytical method(s) to assess test endpoint(s)

The primary endpoint of BrdU positive cells is assessed based on an immunocytochemical staining (ICC) image of cells in each well. Cells are fixed 24 hours after chemical exposure then an ICC staining with Hoechst for nuclei and anti-BrdU for BrdU positive cells is performed. The plates are scanned using an automated high content imaging device and all nuclei and BrdU positive cells are identified automatically based on their intensity and size. Images are analyzed by the software to give the following endpoints:

1. % Responders_Avg IntensityCh2
   Several negative (solvent) control wells are chosen to determine exposure times for the plate. Each well is auto-focused then auto-exposed in both
   Channel 1 (Nuclei) and Channel 2 (BrdU positive). The average of several wells is taken to determine exposure times. The time for each channel is fixed. Using the fixed exposure times, an image set from a negative control well is obtained and the algorithm is run. The mean average intensity for channel 2 (Mean_Avg Inten Ch2) is collected and three more data points are obtained. These are averaged and the number obtained is placed in the Assay Parameter table in the AvgIntCh2_Level High line. Any cell with an intensity above this number is counted as a BrdU positive cell.

2. Selected Object Count per Valid Field
   The selected object count is any object meeting the pre-set parameters listed in the Assay Protocol. The Assay Protocol is developed over a period of time by using multiple plates under a number of circumstances to account for normal culture to
   culture or staining variability then testing plates to see if these parameters are accurate.

Cytotoxicity and apoptosis assays are performed in parallel.

Technical details (of e.g. endpoint measurements)

Apoptosis Assay: With the lid removed from the plate, read in the BMG FLUOstar OPTIMA Fluorescence/Luminescence Microplate Reader following the instructions in OP-NHEERL/ISTD/SBB/TMF/2013-007-r0 using the "Caspase Glo" protocol (set "Gain" at 3500). All data and calculations are recorded in an Excel spreadsheet and stored in a laboratory drive.

Cytotoxicity Assay With the lid removed from the plate, read in the BMG FLUOstar OPTIMA Fluorescence/Luminescence Microplate Reader following the instructions in OP-NHEERL/ISTD/SBB/TMF/2013-007-r1 using the "CellTiter-Glo protocol" (in the program set "Gain" at 3500). All data and calculations are recorded in an Excel spreadsheet and stored in a laboratory drive.

Data are stored on a dedicated server.

Endpoint-specific controls/mechanistic control compounds (MCC)

This assay was developed using the compounds in table 5.5.1 (apoptosis) and 5.5.2 (proliferation). See the original publications (Druwe et al., 2015 for apoptosis and Harrill et al., 2018 for proliferation) for citations in tables 5.5.1 and 5.5.2.

Table 5.5.1 Training set of chemicals for evaluation of apoptosis.

Training set chemicals for evaluation of apoptosis.

Compound	CAS	Vehicle	Source	Apoptosis in vitro[a]
Positive				
Arsenite	7784-46-5	H_2O	Fisher Scientific	Sidhu et al., 2006
Chlorpyrifos oxon	5598-15-2	DMSO	Chem Service	Culbreth et al., 2012
Dexamethasone	50-02-2	DMSO	Sigma	Yu et al., 2010
Ketamine	33795-24-3	DMSO	Sigma	Bai et al., 2013
Manganese	73913-06-1	DMSO	Sigma	Tamm et al., 2008
Methylmercury	115-09-3	DMSO	Sigma	Tamm et al., 2006
Staurosporine	62996-74-1	DMSO	EMD	Akhtar et al., 2006
Tributyltin	1461-22-9	DMSO	Sigma	Suzuki and Ishido, 2011
Trimethyltin	56-24-6	H_2O	ICN Biomedical	Yoneyama et al., 2009
Negative				
Acetaminophen	103-90-2	DMSO	Sigma	NR[b]
Amoxicillin	26787-78-0	DMSO	Sigma	NR
Glyphosate	1071-83-6	H_2O	Chem Service	NR

[a] Reference for effect of compound on apoptosis in neuroprogenitor cells in vitro.
[b] Not reported in literature.

Positive Controls

6. Proliferation
   Aphidicolin: Inhibits DNA replication, blocks cell cycle progression

7. Cytotoxicity and Apoptosis
   Staurosporine: Inhibits the proliferation, alters the cell cycle distribution, and induces apoptosis

This assay has been evaluated against 53 compounds that have evidence of DNT in vitro (Harrill et al., 2018). These 53 compounds were selected based on an evaluation of the literature by Mundy et al., 2015. See Harrill et al., 2018 for details on the compounds selected.

Negative and unspecific controls

The following compounds were used as negative/unspecific controls in the development of this assay. They previously had been shown not to alter apoptosis or proliferation in vitro:

Saccharin sodium salt
Acetaminophen
Glyphosate
Dimethyl Phthalate
Amoxicillin
D-Sorbitol

The solvent control (SC) is used as a negative control that is run on each experimental plate. Each SC has to be established by comparing the effect of the SC to the effect of the media control. Established solvent controls show the same response as the media control.

Established solvent controls are: DMSO, Water, Ethanol, Ethanol/DMSO

Features Relevant for Cytotoxicity Testing

The Caspase-Glo® 3/7 Assay is a homogeneous, luminescent assay that measures caspase-3 and -7 activities. These members of the cysteine aspartic acid-specific protease (caspase) family play key effector roles in apoptosis in mammalian cells. The assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. Addition of Caspase-Glo® 3/7 Reagent in an “add-mix-measure” format results in cell lysis, followed by caspase cleavage of the substrate and generation of a luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present.

The Cell Titer-Glo 2 Cell Viability assay is a method for determining the number of viable cells in culture based on the quantification levels of ATP present (indicative of metabolically active cells). It is an assay which results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The ATP reacts with beetle luciferin in the presence of recombinant firefly luciferase and results in a stable luminescent signal. The measure of cell viability assessed by the CellTiter Glo 2 assay depends on the metabolic activity of cells present in the well. Reduced cell viability indicates either fewer cells present and/or a reduced metabolic capacity of the cells in the well.

Acceptance Criteria for the Test Method

High Content Imaging: For data generated with the ArrayScan to be acceptable for use, a cellular endpoint-specific chemical standard will be used as an internal control in the culture plate being used. For any endpoint, the chemical standard will be based on the scientist’s expertise and understanding of the biology of the endpoint being measured and endpoint-specific data from the literature. The effect of the chemical standard must be within +/- 10% of the expected value (e.g., for an expected chemical result of a 50% change from control, the value should be between 40 to 60 %) to be accepted. If the effect of the chemical standard is outside of this range, the data from that culture plate will not be used.

Cytotoxicity and Apoptosis: A positive control (staurosporine) which is a chemical known to cause toxicity is included in eight wells on each plate. A negative control (eight wells) which is the solvent used to dilute the test chemicals is also included. Data from the control wells will be compared to historical controls for the same cell type. Data should fall within ± 10% of the historical controls.

Throughput Estimate

The methods described here are described for a 96 well plate format. Typically, 18 plates can be made in one culture (Six for Proliferation, six for apoptosis, six for cytotoxicity), which allows testing 16 compounds in triplicate technical replicates. With thawing and expansion plating can occur every 14 days, allowing 32 compounds in triplicate at multiple concentrations to be screened per month

Handling details of the test method

Preparation/addition of test compounds

The experimental compounds were each prepared in stock solutions at 1000-fold concentrations of 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, and 30 mM in DMSO, ethanol, DMSO/ethanol or double-deionized H2O (ddH2O) based on solubility. Training chemicals known to be especially potent or to have low solubility at high concentrations were not prepared at high concentrations. Chemicals that were known to be present in vivo at very high concentrations were tested at the appropriate order of magnitude.

Dosing solutions were prepared from each of these stocks by diluting 1/100 into Medium. In a 96 deep well plate, 5 µL of 1000X test chemical is diluted with 495 µL of Proliferation Media. Cells were exposed to chemicals 48 hours after plating by diluting a volume of the 10x dose concentration (in appropriate solvent, depending on solubility of the compound) of 0.1%. The cells were maintained in a humidified incubator at 37°C and 5% CO2.

Stock solutions are aliquoted and stored at -80°C. Freeze/thaw cycles should be avoided with compound stock solutions; therefore, it is best to prepare an aliquot of stock solution to be thawed and used once for

each treatment.

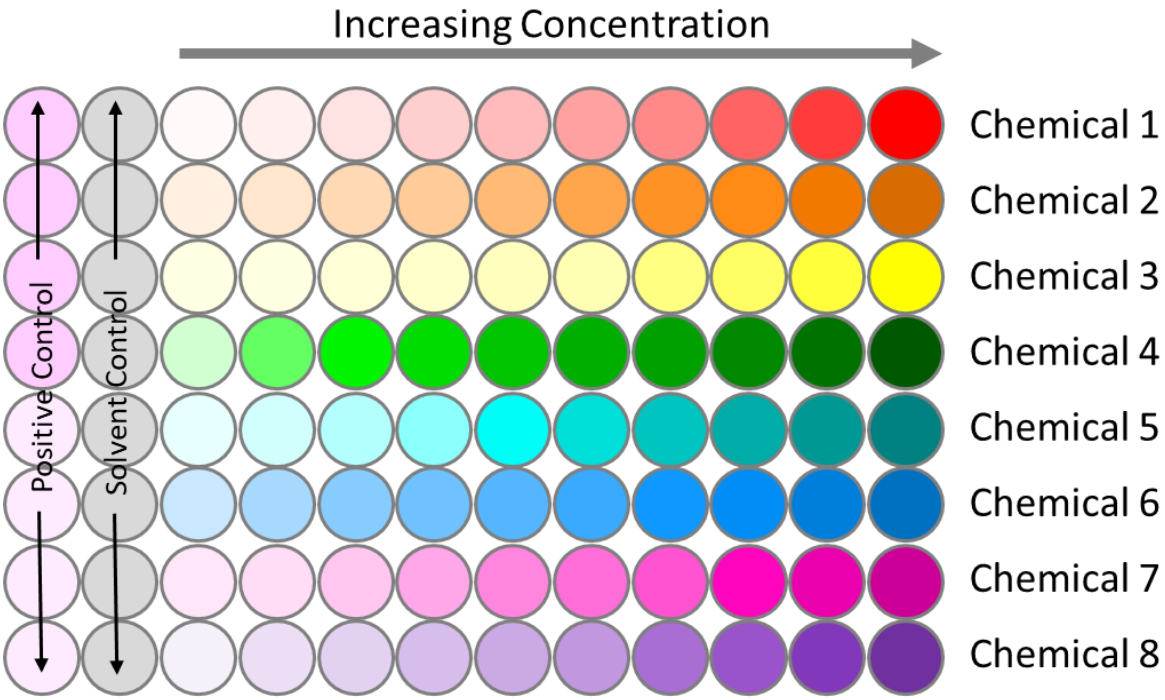

Figure 6.1. Plating Scheme. 10 compound concentrations are plated in a serial dilution from lowest (left) to highest (right) concentration. Positive control (Aphidicolin or Staurosporine) and Solvent Control are plated at 4 replicates each of one concentration. Solvent control depends on the solvent of the compound that was tested.

Day-to-day documentation of test execution

Plate maps contain all information for the set-up of a plate: chemicals to be tested, concentrations of those chemicals, and negative and positive controls. A plate map for all three plates is created prior to plating cells. Data Sheets have been created to record all information on the procedures. These sheets are scanned and placed into the OneNote online notebook. All data relevant to the assay is stored on an online OneNote notebook created for the specific project.

Practical phase of test compound exposure

Notes on any deviation or error are made on the data sheets. Errors are defined (coding based on QA requirements), initialed and dated. All aspects of the experiment are recorded (scanned) in an online OneNote laboratory notebook. This includes any documentation of adherence to plate maps, potential errors, and any other variable that may impact the assay and interpretation of results. Projects are typically subjected to review by EPA Quality Assurance Managers.

Concentration settings

Ten compound concentrations in serial dilutions are tested in each plate.

As default a serial dilution of 1:3 is used, i.e., a concentration range of 729-fold is covered (e.g., from 20 µM

to 27 nM). Depending on the need, other serial dilutions such as 1:2, 1:5, 1:10 can be used.

Start concentrations and concentration ranges are defined based on the following factors:

- toxicological relevance of the compound (i.e., internal human exposures, effects at lowest concentrations)
- solubility of the compound
- concentration of stock compound in solutions provided by suppliers of chemical sets (e.g., for EPA's ToxCast library chemicals are typically supplied at 20 mM in DMSO).
- the highest concentration of solvent (e.g., DMSO) that can be tolerated by the assay.
- solubility of the compound (highest useable solvent concentration)

Uncertainties and troubleshooting

Problematic compounds:
- volatile compounds
- high lipophilicity (high K_{OW})
- low solubility in established solvents
- Fluorescent compounds (possible interference with viability assay)

Critical handling steps:
- The poly-L-ornithine/laminin coating is critical for good cell attachment. Coated plates should be stored at 4°C for no longer than 7 days. Preferably freshly coated plates are best.

Sources of variation:
- Pipetting steps: Each pipetting step is a source of variation.

Known Pitfalls:
- Multiple washing steps in the ICC staining can lead to the wash off of cells. Slow and careful pipetting, the use of a manifold on low aspiration and the use of wide orifice tips can alleviate this issue.

Caveats:
- different subpopulations of neuroprogenitors express different proteins (that also vary between different developmental timepoints).

Detailed protocol (SOP)

OP-NHEERL-H/ISTD/SBB/KAW/2018-02-r1: The "Caspase-Glo" 3/7 assay (Available upon request; Email: Shafer.tim@epa.gov).
OP-NHEERL/ISTD/SBB/TMF/2013-007-r1: Operation of the BMG FLUOstar OPTIMA Fluorescence / Luminescence Microplate Reader using the "CellTiter-Glo protocol"
OP-NHEERL/ISTD/SBB/TJS/2017-001-r2; BrdU staining for proliferation in 96 well plates.
All OPs are available upon request; Email: Shafer.tim@epa.gov

Special instrumentation

- Biological Safety Cabinet
- Incubator for cell culture
- Multiplate reader for luminescence measurement
- Cellomics® ArrayScan® VTI HCS Reader (ThermoFisher Scientific)

Possible variations

Not Applicable

Cross-reference to related test methods

Not applicable

Data management

Raw Data Format

Image files (*.C01 files) are saved to a network drive. These files can be reanalyzed by re-applying the bio application software. The data extracted are saved to a network drive as *.xls (excel) files, with 1 file containing all extracted feature values per experimental plate. The original excel output files are saved for traceability of the data.

Outliers

Mathematical procedures to define outliers are not applied. The tcpl curve fitting program (Filer et al., 2017) is robust with respect to minimizing the impact of outliers.

Data points from wells where technical problems are known or obvious are retained in the data file but are excluded from the analysis by marking them as “well quality 0”.

Some example technical problems:
- pipetting errors
- contamination

Raw data processing to summary data

Bio application software analyze the image files and extract the relevant features (%responders, object count, etc) and save these data as excel (*.xls) files. R scripts are used to scrape the data from the *.xls files. Data are transformed to the “long” data format, with 1 row for each well-feature pair.

Normalization, Curve fitting and BMC calculation

Data are analyzed using the ToxCast Pipeline (tcpl) approach as described by Filer et al., 2017. A summary of techniques applied is in table 7.4.

Table 7.4 Methods applied in tcpl for the proliferation, apoptosis and viability assay

ToxCast Data Pipeline Level	HCI assays: Methods Applied
mc0: pre-processed data input	Data are raw input
mc1: mapping to well and column indexes	Auto
mc2: transformation	No transformation

mc3: normalization	Baseline value (bval) was calculated as the median value for the vehicle control wells (DMSO) on a by-plate basis; No positive control value was used in normalization (pval=0); the response was calculated as percent of DMSO vehicle control.
mc4: BMAD calculation type for curve-fitting	An approximation of noise around the baseline signal, the baseline median absolute deviation, was calculated based on the vehicle control wells on each plate.
mc5: Hitcall and potency determination	The cutoff for a positive response was the greater of 30% or 3*BMAD.
mc6: caution flags on fitting	Flags for single point hit at maximum concentration (6), flags for single point hit not at the maximum concentration screened (7), inactives with multiple median responses above baseline (8), noisy curves relative to the assay (10), actives with borderline efficacy (11), inactives with borderline efficacy (12), low concentration gain-loss curve-fits (15), possibly overfitting (16), hitcalls with less than 50% efficacy (17), model fits with AC_{50} less than lowest concentration tested (18) were assigned to all; additionally cell viability assays were assigned "viability gain-loss fit" (19)

Internal data storage

Data collected from the Arrayscan VTI are saved as **.C01 files* on a laboratory network drive. This network drive resides on EPA servers which are backed up daily. As per US Government regulations, these files will be maintained for at least 20 years.

Metadata

Metadata is saved in *.xlsx files, with 1 file for each group of 3 plates prepared on the same date. Data from each plate are considered technical replicates. R scripts are used to scrape the metadata from the files, merge the metadata with the experimental data for each well, and save the result in a *.RData file.

Metadata file format

The metadata file format is *.xlsx.

Prediction model and toxicological application

8.Scientific principle, test purpose and relevance

BrdU, or bromodeoxyuridine, is a synthetic nucleic acid that may be incorporated into DNA during replication in lieu of thymine. Cells undergoing DNA replication – S-phase of the cell cycle – incorporate this BrdU into their DNA, but cells in other phases of the cell cycle may not. Since only four hours are allotted for cells to incorporate BrdU into their DNA, not enough time is given for S-phase cells to begin mitosis and pass the BrdU-label to their progeny. Antibodies are selected to screen for this nucleoside to demonstrate which cells were actively dividing at the time of BrdU exposure (after 20hr of chemical exposure). The purpose of this test is to identify compounds that may interfere with the normal neurodevelopmental process of neurprogenitor cell proliferation. Chemicals that interfere with proliferation may result in too few, or too many cells in the nervous system. Both of these conditions have been associated with developmental neurotoxicity following chemical exposures.

Prediction model

The cutoff for a positive response in each assay endpoint is set as 3*BMAD or a 30% change from DMSO

controls, and compounds with treatment levels reaching this cutoff are then subjected to curve fitting in tcpl, from which AC_{50} values are generated (see table 7.4). The PM is based on a comparison between the AC_{50} value for the proliferation-, apoptosis-, and viability/cytotoxicity-specific endpoint and the AC50 value cytotoxicity/viability effect.

Thereby the following classifications apply:

"specific hit": a threefold difference between the AC_{50} value for apoptosis or proliferation endpoints and the most potent cytotoxicity endpoint. Where no cytotoxicity endpoint had an AC_{50} value, then the highest concentration tested is used.

"non-specific hit": Less than a threefold difference exists between AC_{50} value for the proliferation-, apoptosis-, and viability/cytotoxicity-specific endpoint and the most potent cytotoxicity AC_{50} value.

It should be noted that there are other valid approaches to determining specificity. For example, one could calculate the area under the curve of the specific endpoint that is below the AC_{50} value for cytotoxicity.

"inactive": the compound was not active in proliferation, apoptosis, and cytotoxicity endpoints.

Prediction Model Set-up

This assay was developed using a training set of chemicals and then further evaluated with a test set of chemicals that had 53 putative positive and 13 putative negative DNT chemicals (see Harrill et al., 2018). See sections below for additional details.

All endpoints in this assay are fit in the down direction. For the viability endpoints, fitting in the up direction (increased viability) is not logical since viability of controls is typically quite high (>90%). The proliferation parameters can be fit in both the up and down direction. However, to date, the vast majority of compounds tested cause decreases in proliferation parameters, including assay positive controls. Biological meaning of changes in the up direction (increased proliferation parameters) is difficult to interpret due to the lack of assay positive controls that alter parameters in the up direction.

Test performance

- Table 8.4.1 summarizes the assay performance in terms of variability of each endpoint in the assay.

Table 8.4.1

ACID	Assay component name	Median	MAD	CV
2711	MUNDY_HCI_hNP1_Pro_MeanAvgInten	87.75	11.49	12.72
2710	MUNDY_HCI_hNP1_Pro_ResponderAvgInten	33.7	4.16	11.9
2709	MUNDY_HCI_hNP1_Pro_ObjectCount	92.88	8.45	8.51
2691	MUNDY_HCI_hNP1_Casp3_7	65272	2228.35	3.8
2700	MUNDY_HCI_hNP1_CellTiter	714037	18172.2	1.96

ACID=assay component identification; MAD=median absolute deviation; CV=coefficient of variation

For the assessment of chemical action on the endpoints represented by this test method, the test method can be used as a stand-alone test method.

For performance assessment in this assay, the following compounds are used as assay positive controls as

they have been previously demonstrated to disrupt proliferation/apoptosis in in vitro systems:

Staurosporine for apoptosis/viability
Aphidicolin for proliferation

Table 8.4.2 summarizes the z prime (z'), strictly standardized median deviation (SSMD) and signal-to-noise (SN) for assay positive control compounds.

Table 8.4.2

Activity type	AEID	AENM	MED.RESP	MED.HITC	CHEM	CONC.UM	Z'	SSMD	SN
Apoptosis / viability	2793	MUNDY_HCI_hNP1_Casp3_7_gain	199.41	1	Staurosporine	1	0.8	19	54.59
	2794	*MUNDY_HCI_hNP1_CellTiter_loss*	*70.3*	*1*	*Staurosporine*	*10*	*0.75*	*18*	*19.69*
Proliferation	2795	MUNDY_HCI_hNP1_Pro_MeanAvgInten_loss	47.12	1	Aphidicolin	10	0	3	2.99
	2796	*MUNDY_HCI_hNP1_Pro_ObjectCount_loss*	*20.83*	*0*	*Aphidicolin*	*10*	*0*	*1*	*1.57*
	2797	MUNDY_HCI_hNP1_Pro_ResponderAvgInten_ loss	87.14	1	Aphidicolin	10	0.1	4	4.77

AEID=assay endpoint identification; AENM=assay endpoint name; MED RESP=median response; MED HITC=median hitcall; CHEM=chemical; CONC UM= concentration in micromolar

The test method is currently used in the setup of a DNT test battery.

In vitro – in vivo extrapolation (IVIVE)

IVIVE of data from this assay has been conducted based on the activity (e.g. EC_{50}, $AC_{50,}$ tipping point) values obtained from curve fitting. Because in vitro toxicokinetic information (e.g. lipid and protein content of cells, volume of cells) are not readily available, these extrapolations have been based on the nominal concentration of test article in the medium. Adjusted Equivalent Doses (AEDs) were estimated using the high-throughput toxicokinetic (HTTK) information and models available in the httk R package (v1.8; Pearce et al., 2017), which functionalizes an approach similar to the one previously used by Wetmore et al. (2012).

Applicability of test method

Toxicological application domain
The following compound classes have been tested successfully:

- Industrial chemicals
- cosmetics ingredients
- pharmaceuticals

Compounds need to be soluble in a solvent at a solubility where the solvent does not produce effects by itself

in the test systems (5.7 for established solvents).

Compounds that are volatile or have a high lipophilicity have not been tested and might need more sophisticated exposure methods such as 'passive dosing'.

Biological application domain
This assay is based on human embryonic stem cell derived neuroprogenitor cells . As mentioned in 8.1 "Scientific principle" The method represents proliferation, apoptosis and viability.

Next to the endpoints represented by this test method there are several other necessary neurodevelopmental endpoints which need to be studied using other test methods.

Neurodevelopmental processes not represented by this test method:
- Neural Crest Cell (NCC) Migration
- Neuronal morphology
- Synaptogenesis
- Neuronal network formation
- Neural Rosette Formation
- hiPSC-derived NPC proliferation
- hiPSC-NPC neuronal differentiation
- Neuronal subtype differentiation
- Astrocyte Differentiation and Maturation
- Astrocyte Reactivity
- Microglia reactivity

For a complete assessment of developmental neurotoxicity, the test method needs to be part of a test battery.

Incorporation into Test Battery

To assess the hazard for developmental neurotoxicity it is recommended that this assay is used as one assay in a battery of assays (see 8.6 "Applicability of test methods")

For the assessment of chemical action on the endpoints represented by this test method, the test method can be used as a stand-alone test method.

The test method is currently used in the setup of a DNT test battery.

Publication/validation status

Availability of key publications

Druwe et al., 2015. Sensitivity of neuroprogenitor cells to chemical-induced apoptosis using a multiplexed assay suitable for high-throughput screening. Toxicology 2015 Jul 3;333:14-24. doi: 10.1016/j.tox.2015.03.011.

(Potential) Linkage to AOPs

The AOPWiki lists numerous Key Events (KEs) that are relevant to cell proliferation. The most directly relevant are KEs 1821 (decreased cell proliferation) 870/1555 (increased cell proliferation). However, to date, none of the AOPs containing these KEs are relevant to DNT.

Steps Toward Mechanistic Validation

a) Information demonstrating how the test system is biologically relevant to humans in terms of cell types, signaling pathways, etc.

These are human cells, thus it is not necessary to extrapolate across species. Proliferation of neuroprogenitor cells during development relies on highly conserved across signaling pathways. The same signals in vivo that allow continued proliferation, or signal exiting the cell cycle and differentiation also cause continued proliferation or cell cycle exit in vitro.

b) Interventions (pathway knockdown, specific inhibitors (i.e., mechanistic controls, which may be part of the training set) that show expected effects on the assay

This assay has been developed by using mechanistic control compounds known to disrupt proliferation in neural progenitor cells.

c) Formal mechanistic validation

There has been no formal validation of this assay. This test method was developed following the criteria established in Crofton et al., 2011, where a set of assay positive controls has been tested followed by a test set of compounds (Harrill et al., 2018).

d) Is there a correspondence to human (in vivo?) changes?

To date, no specific studies have been conducted with chemicals to demonstrate a correspondence to human in vivo changes.

Pre-validation or Validation

No OECD 34 validation study has been done. The test method is part of a pre-validation study that test the DNT hazard assessment for 83 Compounds in a DNT test battery. The compound set includes potential DNT positive and DNT negative compounds.

Linkage to (e.g. OECD) guidelines/regulatory use

Test is not linked to regulatory guidelines.

Test method transferability

Operator training

For operators with a basic training in cell culture practices a four-week training period for handling of the test system and training in the assay is recommended. The operators should have basic understanding in image analysis and data evaluation with respect to concentration response fitting.

Transfer

The test method has been used by multiple operators over a period of greater than 5 years. However, inter operator variability has not been determined. Given the availability of multiple commercial approaches for measuring cell proliferation, transfer of this protocol to other laboratories would not be difficult.

Safety, ethics and specific requirements

Specific hazards; issues of waste disposal

This assay itself has no specific hazards. However, chemicals being tested in this assay may pose both human health and environmental hazards. Therefore, appropriate personal protective equipment should be worn by operators, and appropriate waste disposal practices should be followed.

Safety data sheet (SDS)

SDS should be supplied by the manufacturer or supplier of the chemicals being tested and should be kept on file as appropriate for legal guidelines for the location of the facility where testing is occurring.

Specific facilities/licenses

No specific facilities are required.
No specific ethical approval is required. No specific license is required.

Commercial aspects/intellectual property of material/procedures

As noted in section 4.8, the hNP1 cells were sold by ArunA Biomedical, but are no longer commercially available. However, ArunA is willing to enter into licensing agreements with contract laboratories that wish to use these cells. The kits mentioned for apoptosis and viability are also commerically available. However, the assay itself is not subject ot any other intellectual property issues, and could be conducted by anyone with access to the cells and other materials.

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Appendix C. Adverse Outcome Pathway as an Underlying Framework for Developing In Vitro DNT Testing Strategies

The existing AOPs for DNT outcomes should facilitate the application of mechanistic knowledge of toxicity pathways (i.e., physiological signaling pathways perturbed upon chemical exposure) into regulatory assessment since adverse outcomes (AOs) are of regulatory relevance. Intermediate key events (KEs) represent pathways of toxicity at different biological levels (cellular, tissue and organ) which are empirically observable and measurable (Ankley et al., 2010). Therefore, in vitro DNT assays can utilize the KEs identified in DNT AOPs to detect potential developmental neurotoxicants. A battery of such in vitro test methods that relies on mechanistic KEs derived from AOPs should increase scientific confidence in *in vitro* data, and decrease uncertainty in the regulatory acceptance of in vitro assays, supporting paradigm shift towards a mechanistically-driven hazard identification and characterization and possibly risk assessment.

Furthermore, empirical evidence for describing a key event relationship (KER) is based on relevant data found either from existing literature or studies specifically designed for the purpose of AOP development. In vitro assays anchored to KEs may support the overall weight of evidence and causal linkages (KERs) leading to the AO. This is an important component of an IATA used in a flexible and fit-for-purpose approach suitable for various regulatory needs.

Understanding the likelihood of effects (e.g., initiation of a toxicity pathway) occurring at lower, cellular levels of biological complexity through, for example in vitro testing or (Q)SAR, can also help to inform whether testing at higher levels of biological organisation (i.e., in vivo) is warranted.

Additionally, in vitro assays that allow an evaluation of the key neurodevelopmental processes specific for brain development such as cell proliferation, migration, differentiation, synaptogenesis, neuronal network formation and function etc., often overlap with KEs identified in DNT AOPs, strengthening the biological context for the applied in vitro assays. Indeed, it is strongly documented in the existing literature that if these key neurodevelopmental processes are sufficiently perturbed upon exposure to a chemical, this can lead consequentially to DNT effects.

However, an approach based on individual AOPs (assays anchored to KEs) presents the limitation of being able to identify only a small number of positive “hits” (developmental neurotoxicants) eliciting toxicity through the specific AOP(s). Therefore, it has been proposed to identify key events common to many pathways described in individual AOPs. Following this recommendation, i.e., building network(s) of the existing individual AOPs relevant to DNT and determining the common KEs within such network (s), will facilitate the selection of the most critical/robust in vitro assays suitable to identify a number of developmental neurotoxicants, targeting various signalling pathways and triggered by diverse MIEs. Table C.1 lists the assay names in the DNT in vitro battery (see Appendix B for more detailed information). Table C.2 lists the neurodevelopmental processes and other endpoints that are assessed in the DNT IVB assays. Table C.3 illustrates how the neurodevelopmental processes measured in the DNT IVB assays map to Key Events in existing developmental neurotoxicity AOPs. These include AOPs in the AOP-Wiki that are currently under development or WPHA/WNT Endorsed, as well as AOPs that have been published but are not in the AOP-Wiki.

Furthermore, incorporation of supplementary information derivedfrom DNT in vitro mechanistic studies and other alternative approaches (e.g., QSAR, read across) would increase weight of evidence when, if necessary, combined with DNT in vivo testing where results may often be equivocal or open to different interpretations and, if so, by what mechanisms. This can be achieved by using a battery of in vitro assays which permit evaluation of a range of key pathways that mediate DNT effects, perturb neurodevelopmental processes at different developmental exposure

windows and KEs identified in the existing AOPs relevant to DNT, using human-based models derived from induced pluripotent stem cells (hiPSCs), rather than rodent test systems to avoid interspecies differences.

In addition, it is important to be able to define threshold(s) for KEs (quantitative AOPs), allowing discrimination between changes observed in in vitro studies as adaptive processes normally found in biological systems in vivo from those that are predictive of adverse outcomes. Coupling the adverse or adaptive nature of the measured endpoints with absorption, distribution, metabolism, and excretion (ADME) data and exposure information derived from in vitro to in vivo extrapolation (IVIVE) will increase the level of confidence in the information derived from in vitro assays anchored to KEs, especially if based on human neuronal/glial cells derived from hiPSCs (mimicking human biology) and coupled with models of chemical kinetics and dynamics, being more predictive for an in vivo exposure scenario.

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Table C.1. Assay names and neurodevelopmental processes assessed in the DNT in vitro battery. Assay names are from Table 2.3 in the Initial Reccommendations. Note the ToxTemplate files in Appendix B contain detailed information for all the assays.

Assay Name	Process	Appendix Number
NPC1	Proliferation	Appendix B.1
NPC2a	Migration	Appendix B.2
NPC2b	Migration	Appendix B.2
NPC2c	Migration	Appendix B.2
NPC3	Neuronal Differentiation	Appendix B.2
NPC4	Neurite Outgrowth	Appendix B.2
NPC5	Glial Differentiation	Appendix B.2
UKN2	Migration	Appendix B.3
UKN4	Neurite Outgrowth	Appendix B.4
UKN5	Neurite Outgrowth	Appendix B.5
Cortical Initiation	Neurite Outgrowth	Appendix B.6
Cortical Maturation	Neurite Maturation	Appendix B.9
Cortical MEA	Neural Network Formation	Appendix B.7
Cortical Synapto	Synaptogenesis	Appendix B.9
hN initiation	Neurite Outgrowth	Appendix B.10
hNP1 Apop	NPC Apoptosis	Appendix B.6
hNP1 Prolif	NPC Proliferation	Appendix B.8

Table C.2. Neurodevelopmental processes and other endpoints in the DNT IVB assays.	
1	Proliferation
2	Neuronal differentiation
3	Neurite outgrowth (neurite length, neurite area)
4	Oligodendrocyte differentiation
5	Neural Crest Cell Migration
6	Neurite Outgrowth of Neural Crest Cells
7	Neuronal network formation and function
8	Synaptogenesis
9	Cell viability
10	Cytotoxicity

Table C.3. Processes and assays included in the DNT IVB mapped to Key Events in developmental neurotoxicity AOPs*.

AOPs *(AOP-Wiki)*	**Key Events (KEs) and corresponding in vitro assay**							
AOP 12 ***(AOP-Wiki, WPHA/WNT Endorsed)***	***Title: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development Leads to neurodegeneration with impairment in learning and memory in aging*** *(AOP-Wiki: https://aopwiki.org/aops/12)*							
KEs	**Binding of antagonist to the NMDA Receptor (MIE)**	Inhibition of NMDA Receptor	Decreased calcium influx	Reduced levels of BDNF	Cell injury/ death	Neuro-inflammation	Neurodege-neration	**Impairment of lerning and memory (AO)**
In vitro assays					Viability, Cytotoxicity (hNP1 Apop assay)		Viability, Cytotoxicity (hNP1 Apop Assay)	
AOP 13 ***(AOP-Wiki, WPHA/WNT Endorsed)***	***Title: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities*** *(AOP-Wiki: https://aopwiki.org/aops/13)*							

KEs	**Binding of antagonist, NMDA receptors (MIE)**	Inhibition of NMDA Receptor	Decreased calcium influx	Reduced levels of BDNF	Reduced Presynaptic Release of glutamate	Aberrant, Dendritic morphology	Cell injury & Death	Decreased Synaptogenesis	Decreased Neuronal network function	**Impairment of lerning and memory (AO)**
In vitro assays						Neurite length and neurite area (UKN4, Cortical Maturation assays)	Viabilty, Cytotoxicity (hNP1 Apop assay)	Synaptogenesis (Cortical Synapto assay)	Neuronal network formation and function (Cortical MEA assay)	
AOP (under development, EFSA Scientific Opinion 2021)	***Title: Binding of deltamethrin to Voltage Gated Sodium Channels (VGSCs) leads to the disruption action potential resulting in impairment of behavioral function (sensory motor reflex and learning)***									
KEs	**Binding to VGSC (MIE 1)** **Binding to Ryanodine receptors (MIE 2)**	Disruption of sodium channel gate kinetics hannel gate kinetics (KE1 For MIE1)	Disruption of intracellular Ca channel kinetics (KE1 for MIE2)	Disruption of action potential generation (KE 2)	Disruption of axon terminal depolarization changes in neurotrans-mitter release	Decreased oligodendro-cytes differentiation (KE5)	Increase of intracellular sodium in microglia cells. (KE6)	Altered neuronal network function (KE4)	**Impairment behavioural function (AO)**	

					(KE3)					
In vitro assays				Neuronal network formation and function (Cortical MEA assay)	Neuronal network formation and function (Cortical MEA assay)	Oligodedro-cytes differentiation (NPC5 assay)		Neuronal network formation and function (Cortical MEA assay)		
AOP 42 ***(AOP-Wiki, WPHA/WNT Endorsed)***	***Title: Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals*** *(AOP-Wiki: https://aopwiki.org/aops/42)*									
KEs	**Thyroperoxidase Inhibition (MIE)**	TH synthesis, Decreased	T4 in serum, Decreased	T4 in neuronal tissue, Decreased	Hippocampal gene expression, Altered	Hippocampal anatomy, Altered	Hippocampal Physiology, Altered	**Cognitive Function, Decreased (AO)**		
In Vitro assays							Synapto-genesis, Neuronal network formation and function			

							(Cortical MEA & Synapto assay)			
AOP ***(outlined in Hassan et al., 2017)***	***Thyroid hormone (TH) synthesis inhibition and development of a cortical brain malformation, a cortical heterotopia***									
KEs	**Thyroperoxidase inhibition (in dam and Fetus) (MIE)**	TH release in dam and fetus decreased	TH in serum of dam and fetus decreased	TH in fetus brain decreased	**Cortical heterotopia (AO)**					
In vitro assays										
AOP 54 ***(AOP-Wiki, WPHA/WNT Endorsed)***	***Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment*** *(AOP-Wiki: https://aopwiki.org/aops/54)*									
KEs	**Inhibition, Na+/I-symporter (NIS) (MIE)**	Thyroidal Iodide, Decreased	TH synthesis, Decreased	T4 in serum, Decreased	T4 in neuronal tissue, Decreased	BDNF, Reduced	GABAergic interneurons, Decreased	Synapto-genesis, Decreased	Neuronal network function, Decreased	**Impairment, Learning and memory (AO)**

In vitro assays								Synapto-genesis (Synapto assay)	Neuronal network formation and function (Cortical MEA assay)	
AOP 8 ***(AOP-Wiki, under development)***	***Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals***									
KEs	**Xenobiotic nuclear receptor activation (MIE)**	Increased Phase II catabolism / Increased hepatic transport	Decreased T4/T3 serum levels	Decreased tissue TH concentration	Altered neuro-development	**Neurological dysfunction (AO)**				
In vitro assays										
AOP 17 ***(AOP-Wiki, EAGMST Under Review)***	***Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory***									

KEs	**Binding to Thiol/seleno-proteins involved in protection against oxidative stress (MIE)**	Decreased protection against oxidative stress	GSH depletion	Oxidative stress	Glutamate dyshomeo-stasis	Cell injury/death	Neuroinfla-mmation	Tissue resident cell activation/ Increased Pro-inflamma-tory mediators	Decrease of neuronal network function	**Impairment, learning and memory (AO)**
In vitro assays						Viability Cytotoxicity (hNP1 Apop)			Neuronal network formation and function (Cortical MEA assay)	
AOP 134 ***(AOP-Wiki, under development)***	***Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals***									
KEs	**Inhibition, Na+/I-symporter (NIS) (MIE)**	Thyroid hormone synthesis, Decreased	Decrease of Thyroidal iodide	Thyroxine (T4) in serum, Decreased	Thyroxine (T4) in neuronal tissue, Decreased	Hippocampal gene expression, Altered	Hippocampal anatomy, Altered	Hippocampal Physiology, Altered	**Cognitive Function, Decreased (AO)**	

In vitro assays								Neuronal network formation and function (MEA assay)**		
AOP 152 ***(AOP-Wiki, under development)***	***Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity***									
KEs	**Binding, Transthyretin in serum (MIE)**	Desplace-ment, Serum thyroxine (T4) from transthyretin	Increased, Free serum thyroxine (T4)	Increased, Uptake of thyroxine into tissue	Increased, Clearance of thyroxine from serum	T4 in serum, Decreased	T4 in neuronal tissue, Decreased	Hippocampal gene expression, Altered	Hippocampal anatomy and physiology Altered	**Cognitive Function, Decreased (AO)**
In vitro assays									Neuronal network formation and function (MEA assay)**	

AOP 275 *(AOP-Wiki, under development)*	***Histone deacetylase inhibition leads to neural tube defects***									
KEs	**Histone deacetylase inhibition (MIE)**	Histone acetylation, increase	Altered, Gene Expression	Altered differentiation	**Neural tube defects (AO)**					
In vitro assays				Neurite Outgrowth of Neural Crest Cells Neurite length and neurite area (NPC4, UKN4 and hN initiation assay)						
AOP 300	***Thyroid Receptor (TR) Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals***									

(AOP-Wiki, under development)										
KEs	**TR Antagonism (MIE)**	Hippocampal gene expression, Altered	Hippocampal anatomy, Altered	Hippocampal Physiology, Altered	**Cognitive Function, Decreased (AO)**					
In vitro assays										
AOP *(US EPA report, 2017)*	***Inhibition of deiodinase results in decreased thyroxine (T4) to triiodothyronine (T3) conversion and subsequent adverse neurodevelopmental outcomes***									
KEs	**Deiodinase inhibition (MIE)**	Decreased T4 and/or T3 in target tissues	TR binding/trans-activation	Gene expression modifications	Neurodevelop-mental Alterations	**Neurological and cognitive impairments (AO)**				
In vitro assays										
AOP *(US EPA report, 2017)*	***Interference with thyroid receptor and subsequent adverse neurodevelopmental outcomes***									
KEs	**Thyroid receptor binding (MIE)**	Decreased T4 and/or T3 in target tissues	TR binding/trans-activation	Gene expression modifications	Neurodevelop-mental Alterations	**Neurological and cognitive impairments (AO)**				

In vitro assays										
AOP ***(US EPA report, 2017)***	***Interference with thyroid hormone transport results in decreased T4 in brain and subsequent adverse neurodevelopmental outcomes***									
KEs	**Thyroid hormone transport interference (MIE)**	Decreased T4 and/or T3 in target tissues	TR binding/trans-activation	Gene expression Modifica-tions	Neurodevelo-pmental Alterations	**Neurological and cognitive impairments (AO)**				
In vitro assays										
AOP III ***(Cristina Suñol*** *In Bal-Price et al., 2017)*	***Binding of antagonist to GABA A receptor results in hyperexcitability and convulsions***									
KEs	**Binding of antagonists to GABAA Receptor (MIE)**	Inhibition of $GABA_A$ Receptor	Reduced/ blocked CL^- influx	Reduced inhibitory signals	Disinhibition of Excitatory pathways	Increased excitatory activity in neuronal network	**Seizures/ Convulsions (AO)**			
In vitro assays						Neuronal network formation and function				

						(Cortical MEA assay)				
AOP IX *(Pamela J. Lein in Bal-Price et al., 2015)*	***The interaction of non-dioxin-like PCBs with ryanodine receptors (RyRs) causes their sensitization affecting neuronal connectivity that results in behavioral deficits***									
KEs	**Biding to RyRs (MIE)**	RyRs sensitization	Altered neuronal calcium oscillations	Activation of calcium dependent signalling	Altered dendritic arborization and synapto-genesis	Increased neuronal apoptosis	Altered neuronal networks and pathways	**Behavioral deficits (learning, memory, psychomotor, attention) (AO)**		
In vitro assays					Neurite length and neurite area, Synapto-genesis (UKN4, hN initiation & Cortical Synapto assay)	Viability, cytotoxicity	Neuronal network formation and function (Cortical MEA assay)			

AOP *(outlined in von Stackelberget al., 2015)*	***Exposure to Mixtures of Metals and Neurodevelopmental Outcomes***									
KEs	**Activationof MEK-ERK1/2 in Astrocytes (MIE)**	Calcium Signaling	ROS Generation and Oxidative Stress	Apoptosis in Astrocytes	Neurotrans-mitter Release	**Learning and Cognitive Deficits (AO)**				
In vitro assays				Viability, cytotoxicity						
AOP *(outlined in von Stackelberget al., 2015)*	***Exposure to Mixtures of Metals and Neurodevelopmental Outcomes***									
KEs	**Multiple potential events for individual metals (MIEs)**	Mitochon-drial dysfunction	Reactive oxygen species/oxidative stress	Apoptosis	Synapse impairment; neurodegene-ration	**Learning and Cognitive Deficits (AO)**				
In vitro assays				Viability,	Synapto-genesis					

				cyto-toxicity (hNP1 Apop assay)	(Cortical Synapto assay) Viability, cytotoxicity					
AOP *(outined in Barenys et al., 2020)*	***Interference of a compound with the serotonin transporter and enzyme synthesis during brain development leading to decreased motor functions in children***									
KEs	**Unknown SERT interaction and TPH inhibition? (MIE)**	Depletion of 5HT	Impaired trophic 5HT function during neurodeve-lopment	KE3 KE 3.1, KE 3.2, KE 3.n unknow n	Altered neuroanato mical structure	**Decreased motor function in children (AO)**				
In vitro assays										
AOP *(outined in Barenys et al., 2020)*	***Alteration of DA receptor signaling during development leading to a cortical imbalance of excitatory and inhibitory neurons in cortex causing decreased motor functions in children***									
KEs	**Unknown**	Alteration of	Altered tangential migration of	Cortical imbalanc e of	Unknown	**Decreased motor**				

	(MIE)	dopamine receptor signaling during development	GABAergic neurons	excitatory and inhibitory neurons		**function in children (AO)**				
In vitro assays				(Cortical MEA assay)						

* The regulatory acceptance of the AOPs listed above range from WPHA/WNT endorsed, under development to not included in AOP-Wiki but outlined in the cited papers.

** In the DNT IVB 'Cortical MEA assay' is based on primary rat cortex neurons but exactly the same assay can be performed using neurons from any other brain structures, including hippocampus.

Appendix D. List of DNT IATA and Publications that Used Data from DNT IVB Assays

Table D.1 – List of DNT IATA case studies that have used data from the DNT IVB and their current status.

Title and lead	Chemicals or Chemical Class	Current Status	Reference
EFSA: Case study for the integration of in vitro data in the developmental neurotoxicity hazard identification and characterisation using deltamethrin as a prototype chemical	Deltamethrin	Published in the OECD Series on Testing and Assessment in September 2022 Reviewed by the OECD IATA Case studies project team, and endorsed at the 6th meeting of the Working Party on Hazard Assessment in June 2022 Initial draft published in EFSA Journal	- No. 362 Case study for the integration of in vitro data in the developmental neurotoxicity hazard identification and characterisation using deltamethrin as a prototype chemical (Annex 1; Annex 2, Annex 3: Excel File; Annex 4: Excel File). ENV/CBC/MONO(2022)24 -EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), Hernández-Jerez A, Adriaanse P, Aldrich A, Berny P, Coja T, Duquesne S, Focks A, Marinovich M, Millet M, Pelkonen O, Pieper S, Tiktak A, Topping C, Widenfalk A, Wilks M, Wolterink G, Crofton K, Hougaard S, Paparella M, Tzoulaki I, 2021. Scientific Opinion on Development of Integrated Approaches to Testing and Assessment (IATA) case studies on developmental neurotoxicity (DNT) risk assessment. EFSA Journal 2021;19(5):6599, 67 pp. doi:10.2903/j.efsa.2021.6599
EFSA: Case study for the integration of in vitro data in the developmental neurotoxicity hazard identification and characterisation using flufenacet	Flufenacet	Published in the OECD Series on Testing and Assessment in September 2022 Reviewed by the OECD IATA Case studies project team, and endorsed at the 6th meeting of the Working Party on Hazard Assessment in June 2022 Initial draft published in EFSA Journal	-No. 363 Case study for the integration of in vitro data in the developmental neurotoxicity hazard identification and characterisation using flufenacet (Annex 1; Annex 2, Annex 3: Excel File). ENV/CBC/MONO(2022)25 -EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), Hernández-Jerez A, Adriaanse P, Aldrich A, Berny P, Coja T, Duquesne S, Focks A, Marinovich M, Millet M, Pelkonen O, Pieper S, Tiktak A, Topping C, Widenfalk A, Wilks M, Wolterink G, Crofton K, Hougaard S, Paparella M, Tzoulaki I, 2021. Scientific Opinion on Development of Integrated Approaches to Testing and Assessment (IATA) case studies on developmental neurotoxicity (DNT) risk assessment. EFSA Journal 2021;19(5):6599, 67 pp. doi:10.2903/j.efsa.2021.6599
US: Organophosporus flame retardants, a case study on the use of IATA for DNT to prioritize a class of compounds	Brominated flame retardants	Published in the OECD Series on Testing and Assessment in September 2022 Reviewed by the OECD IATA Case studies	- No. 364 Case study on the use of Integrated Approaches for Testing and Assessment for DNT to prioritize a class of

		project team, and endorsed at the 6th meeting of the Working Party on Hazard Assessment in June 2022	Organophosphorus flame retardants. ENV/CBC/MONO(2022)26
EuToxRisk: Case Study on the use of Integrated Approaches for Testing and Assessment for developmental neurotoxicity hazard characterisation of acetamiprid	Neonicotinoids	Published in the OECD Series on Testing and Assessment in September 2022 Reviewed by the OECD IATA Case studies project team, and endorsed at the 6th meeting of the Working Party on Hazard Assessment in June 2022	- No. 365 Case Study on the use of Integrated Approaches for Testing and Assessment for developmental neurotoxicity hazard characterisation of acetamiprid (Annex 1). ENV/CBC/MONO(2022)27
EuToxRisk: Case Study on the use of Integrated Approaches for Testing and Assessment for developmental neurotoxicity hazard characterisation of imidacloprid and the metabolite desnitro-imidacloprid	Neonicotinoids	Published in the OECD Series on Testing and Assessment in September 2022 Reviewed by the OECD IATA Case studies project team, and endorsed at the 6th meeting of the Working Party on Hazard Assessment in June 2022	- No. 366 Case Study on the use of Integrated Approaches for Testing and Assessment for developmental neurotoxicity hazard characterisation of imidacloprid and the metabolite desnitro-imidacloprid (Annex 1). ENV/CBC/MONO(2022)28
US: Case study in use of DNT IVB data in WoE for glufosinate herbicides	Glufosinate isomers	Published in a peer-reviewed journal	Dobreniecki S, Mendez E, Lowit A, Freudenrich TM, Wallace K, Carpenter A, Wetmore BA, Kreutz A, Korol-Bexell E, Friedman KP, Shafer TJ. Integration of toxicodynamic and toxicokinetic new approach methods into a weight-of-evidence analysis for pesticide developmental neurotoxicity assessment: A case-study with DL- and L-glufosinate. Regul Toxicol Pharmacol. 2022 Apr 9;131:105167. doi: 10.1016/j.yrtph.2022.105167. Epub ahead of print. PMID: 35413399.

Appendix E. List of All Chemicals Tested in the DNT IVB Assays

The following tables provide an overview of all compounds that have been tested in any of the 17 assays in the DNT IVB. The list was created in January 2022 based on output from ToxCast InVitroDB. Appendix Table E.1 is a list of assay names from Table 2.3 in the Guidance Document with information on the associated neurodevelopmental process tested and the appendix number for the ToxTemp file that describes the assay. Table E.2 provides the assay names from Table 2.3 and the names of the variable measurements in the ToxCast InVitro database. Appendix Table E.3 is a list of all compounds tested as of February 2022, including CAS_RN and DTXSID numbers. No attempt was made to combine compounds that were tested in different salt forms (e.g., trimethyltin chloride and hydroxide). The number one (1) in this table indicates that the compound was tested in an assay, and it does not provide any indication of bioactivity. Preliminary analysis of bioactivity from the DNT IVB assays is now available in ToxCastDB 3.5.

The following is a brief summary of the data in this table:

- A total of 476 compounds have been tested in one or more of the assays. It is important to note that there has been no attempt to merge compounds tested in different salt forms.
- A total of 3177 individual assays were run (i.e., chemicals x assays for which it was tested).
- 81 compounds were tested in all 17 assay
- 97 compounds were tested in 14 or more assays
- 331 compounds were tested in 4 or more assays.

Appendix Table E.1 List of assay names from Table 2.3 in the Guidance Document with associated neurodevelopmental process, and the appendix number for the ToxTemp file that describes the assay.

Assay Name in Table 2.3	Neurodevelopmental Process	Appendix Number
NPC1	NPC Proliferation	Appendix B.1
NPC2a	Cell Migration	Appendix B.2
NPC2b	Cell Migration	Appendix B.2
NPC2C	Cell Migration	Appendix B.2
NPC3	Neuronal Differentiation	Appendix B.2
NPC4	Neurite Outgrowth	Appendix B.2
NPC5	Glial Differentiation	Appendix B.2
UKN2	Cell Migration	Appendix B.3
UKN4	Neurite Outgrowth	Appendix B.4
UKN5	Neurite Outgrowth	Appendix B.5
Cortical Initiation	Neurite Outgrowth	Appendix B.6
Cortical Maturation	Neurite Maturation	Appendix B.7
Cortical Synapo	Synaptogenesis	Appendix B.7
Cortical MEA	Neural Network Formation	Appendix B.8
hN initiation	Neurite Outgrowth	Appendix B.9
hNP1 Apop	Apoptosis	Appendix B.10
hNP1 Prolif	Proliferation	Appendix B.10

Appendix Table E.2 List of assay names from Table 2.3 in the Guidance Document with ToxCast InVitro database variable names. See Appendix B files for assay details.

Assay Names in Table 2.3 of the Guidance document	Assay Variable names and Definitions in the ToxCast InVitro Database					
	asid assay source id	asnm assay source name	aid assay ID	anm assay ID	acid assay component id	acnm assay component name
UNK5	27	UKN	691	UKN5_HCS_SBAD2	2500	UKN5_HCS_SBAD2_neurite_outgrowth
	27	UKN	691	UKN5_HCS_SBAD2	2501	UKN5_HCS_SBAD2_cell_viability
UNK2	27	UKN	840	UKN2_HCS_IMR90	2650	UKN2_HCS_IMR90_neural_migration
	27	UKN	840	UKN2_HCS_IMR90	2651	UKN2_HCS_IMR90_cell_viability
UNK4	27	UKN	841	UKN4_HCS_LUHMES	2652	UKN4_HCS_LUHMES_neurite_outgrowth
	27	UKN	841	UKN4_HCS_LUHMES	2653	UKN4_HCS_LUHMES_cell_viability
NPC1	30	IUF	846	IUF_NPC1	2687	IUF_NPC1a_proliferation_BrdU_72hr
	30	IUF	846	IUF_NPC1	2688	IUF_NPC1b_proliferation_area_72hr
	30	IUF	846	IUF_NPC1	2689	IUF_NPC1_viability_72hr
	30	IUF	846	IUF_NPC1	2849	IUF_NPC1_cytotoxicity_72hr
NPC2A	30	IUF	858	IUF_NPC2-5	2763	IUF_NPC2a_radial_glia_migration_72hr
	30	IUF	858	IUF_NPC2-5	2764	IUF_NPC2a_ radial_glia_migration_120hr
NPC2B	30	IUF	858	IUF_NPC2-5	2765	IUF_NPC2b_neuronal_migration_120hr

NPC2C	30	IUF	858	IUF_NPC2-5	2766	IUF_NPC2c_oligodendrocyte_migration_120hr
NPC3	30	IUF	858	IUF_NPC2-5	2767	IUF_NPC3_neuronal_differentiation_120hr
NPC4	30	IUF	858	IUF_NPC2-5	2768	IUF_NPC4_neurite_length_120hr
	30	IUF	858	IUF_NPC2-5	2769	IUF_NPC4_neurite_area_120hr
NPC5	30	IUF	858	IUF_NPC2-5	2770	IUF_NPC5_oligodendrocyte_differentiation_120hr
	30	IUF	858	IUF_NPC2-5	2771	IUF_NPC2-5_cytotoxicity_72hr
	30	IUF	858	IUF_NPC2-5	2772	IUF_NPC2-5_cytotoxicity_120hr
	30	IUF	858	IUF_NPC2-5	2773	IUF_NPC2-5_cell_number_120hr
	30	IUF	858	IUF_NPC2-5	2774	IUF-NPC2-5_viability_120hr
hNP1 Apop	31	CCTE_MUNDY	848	CCTE_Mundy_HCI_hNP1_Casp3_7	2691	CCTE_Mundy_HCI_hNP1_Casp3_7
hNP1 Prolif	31	CCTE_MUNDY	851	CCTE_Mundy_HCI_hNP1_CellTiter	2700	CCTE_Mundy_HCI_hNP1_CellTiter
	31	CCTE_MUNDY	853	CCTE_Mundy_HCI_hNP1_Pro	2709	CCTE_Mundy_HCI_hNP1_Pro_ObjectCount
	31	CCTE_MUNDY	853	CCTE_Mundy_HCI_hNP1_Pro	2710	CCTE_Mundy_HCI_hNP1_Pro_ResponderAvgInten
	31	CCTE_MUNDY	853	CCTE_Mundy_HCI_hNP1_Pro	2711	CCTE_Mundy_HCI_hNP1_Pro_MeanAvgInten
Cortical initiation	31	CCTE_MUNDY	850	CCTE_Mundy_HCI_Cortical_NOG	2696	CCTE_Mundy_HCI_Cortical_NOG_NeuronCount
	31	CCTE_MUNDY	850	CCTE_Mundy_HCI_Cortical_NOG	2697	CCTE_Mundy_HCI_Cortical_NOG_NeuriteLength

	31	CCTE_MUNDY	850	CCTE_Mundy_HCI_Cortical_NOG	2698	CCTE_Mundy_HCI_Cortical_NOG_NeuriteCount
	31	CCTE_MUNDY	850	CCTE_Mundy_HCI_Cortical_NOG	2699	CCTE_Mundy_HCI_Cortical_NOG_BPCount
Cortical maturation	31	CCTE_MUNDY	852	CCTE_Mundy_HCI_Cortical_Synap&Neurite_Matur	2701	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuronCount
	31	CCTE_MUNDY	852	CCTE_Mundy_HCI_Cortical_Synap&Neurite_Matur	2705	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteLength
	31	CCTE_MUNDY	852	CCTE_Mundy_HCI_Cortical_Synap&Neurite_Matur	2706	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteCount
	31	CCTE_MUNDY	852	CCTE_Mundy_HCI_Cortical_Synap&Neurite_Matur	2707	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_BPCount
Cortical Synapto	31	CCTE_MUNDY	852	CCTE_Mundy_HCI_Cortical_Synap&Neurite_Matur	2708	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_SynapseCount
	31	CCTE_MUNDY	852	CCTE_Mundy_HCI_Cortical_Synap&Neurite_Matur	2702	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_CellBodySpotCount
	31	CCTE_MUNDY	852	CCTE_Mundy_HCI_Cortical_Synap&Neurite_Matur	2703	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuron
	31	CCTE_MUNDY	852	CCTE_Mundy_HCI_Cortical_Synap&Neurite_Matur	2704	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuriteLength
hN initiation	31	CCTE_MUNDY	867	CCTE_Mundy_HCI_CDI_NOG	2836	CCTE_Mundy_HCI_CDI_NOG_BPCount
	31	CCTE_MUNDY	867	CCTE_Mundy_HCI_CDI_NOG	2837	CCTE_Mundy_HCI_CDI_NOG_NeuriteCount
	31	CCTE_MUNDY	867	CCTE_Mundy_HCI_CDI_NOG	2838	CCTE_Mundy_HCI_CDI_NOG_NeuriteLength

	31	CCTE_MUNDY	867	CCTE_Mundy_HCl_CDI_NOG	2839	CCTE_Mundy_HCl_CDI_NOG_NeuronCount
	31	CCTE_MUNDY	849	CCTE_Mundy_HCl_hN2_NOG	2692	CCTE_Mundy_HCl_hN2_NOG_NeuronCount
	31	CCTE_MUNDY	849	CCTE_Mundy_HCl_hN2_NOG	2693	CCTE_Mundy_HCl_hN2_NOG_NeuriteLength
	31	CCTE_MUNDY	849	CCTE_Mundy_HCl_hN2_NOG	2694	CCTE_Mundy_HCl_hN2_NOG_NeuriteCount
	31	CCTE_MUNDY	849	CCTE_Mundy_HCl_hN2_NOG	2695	CCTE_Mundy_HCl_hN2_NOG_BPCount
Cortical MEA	20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2471	CCTE_Shafer_MEA_dev_firing_rate_mean
	20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2472	CCTE_Shafer_MEA_dev_burst_rate
	20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2473	CCTE_Shafer_MEA_dev_active_electrodes_number
	20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2474	CCTE_Shafer_MEA_dev_bursting_electrodes_number
	20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2475	CCTE_Shafer_MEA_dev_per_burst_interspike_interval
	20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2476	CCTE_Shafer_MEA_dev_per_burst_spike_percent
	20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2477	CCTE_Shafer_MEA_dev_burst_duration_mean
	20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2478	CCTE_Shafer_MEA_dev_interburst_interval_mean

20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2479	CCTE_Shafer_MEA_dev_network_spike_number
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2480	CCTE_Shafer_MEA_dev_network_spike_peak
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2481	CCTE_Shafer_MEA_dev_spike_duration_mean
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2482	CCTE_Shafer_MEA_dev_network_spike_duration_std
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2483	CCTE_Shafer_MEA_dev_inter_network_spike_interval_mean
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2484	CCTE_Shafer_MEA_dev_per_network_spike_spike_number_mean
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2485	CCTE_Shafer_MEA_dev_per_network_spike_spike_percent
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2486	CCTE_Shafer_MEA_dev_correlation_coefficient_mean
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2487	CCTE_Shafer_MEA_dev_mutual_information_norm
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2488	CCTE_Shafer_MEA_dev_LDH
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2489	CCTE_Shafer_MEA_dev_AB
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2819	CCTE_Shafer_MEA_dev_burst_rate_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2820	CCTE_Shafer_MEA_dev_interburst_interval_mean_DIV12

20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2821	CCTE_Shafer_MEA_dev_burst_duration_mean_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2822	CCTE_Shafer_MEA_dev_per_burst_interspike_interval_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2823	CCTE_Shafer_MEA_dev_firing_rate_mean_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2824	CCTE_Shafer_MEA_dev_mutual_information_norm_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2825	CCTE_Shafer_MEA_dev_bursting_electrodes_number_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2826	CCTE_Shafer_MEA_dev_active_electrodes_number_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2827	CCTE_Shafer_MEA_dev_spike_duration_mean_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2828	CCTE_Shafer_MEA_dev_network_spike_duration_std_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2829	CCTE_Shafer_MEA_dev_inter_network_spike_interval_mean_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2830	CCTE_Shafer_MEA_dev_per_network_spike_spike_number_mean_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2831	CCTE_Shafer_MEA_dev_network_spike_number_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2832	CCTE_Shafer_MEA_dev_network_spike_peak_DIV12
20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2833	CCTE_Shafer_MEA_dev_per_network_spike_spike_percent_DIV12

	20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2834	CCTE_Shafer_MEA_dev_per_burst_spike_percent_DIV12
	20	CCTE_SHAFER	573	CCTE_Shafer_MEA_dev	2835	CCTE_Shafer_MEA_dev_correlation_coefficient_mean_DIV12

Appendix Table E.3. List of all compounds tested as of February 2022, including CAS_RN and DTXSID numbers. The number one (1) in this table indicates that the compound was tested in an assay, and it does not provide any indication of bioactivity.

Chemical Name	CAS_RN	dsstox_substance_id	Cortical Initiation	Cortical Maturati	Cortical MEA	Cortical Synapto	hN initiatio	hNP1 Apop	hNP1 Prolif	NPC1	NPC2a	NPC2b	NPC2c	NPC3	NPC4	NPC5	UKN2	UKN4	UKN5
((2,2,3,3-Tetrafluoropropoxy)methyl)oxirane	19932-26-4	DTXSID70880230			1		1	1	1										
((Perfluorooctyl)ethyl)phosphonic acid	80220-63-9	DTXSID30627108			1		1	1	1										
(-)-Cocaine hydrochloride	53-21-4	DTXSID2048903															1	1	1
(-)-Nicotine	54-11-5	DTXSID1020930	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
(+/-)-3,4-Methylenedioxymethamphetamine hydrochloride	64057-70-1	DTXSID5048893															1	1	1
(Heptafluorobutanoyl)pivaloylmethane	17587-22-3	DTXSID3066215			1		1	1	1										
(Heptafluoropropyl)trimethylsilane	3834-42-2	DTXSID70400078			1		1	1	1										
(Methylcyclopentadienyl)tricarbonylmanganese	12108-13-3	DTXSID9027738			1														

(Perfluoro-5-methylhexyl)ethyl 2-methylprop-2-enoate	50836-66-3	DTXSID60379901			1		1	1	1										
(Perfluorobutyryl)-2-thenoylmethane	559-94-4	DTXSID7060332			1		1	1	1										
(S)-1-Anilino-4-methyl-2-methylthio-4-phenylimidazolin-5-one	161326-34-7	DTXSID2034590	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1-(Perfluorofluorooctyl)propane-2,3-diol	94159-84-9	DTXSID80881157			1		1	1	1										
1-(Perfluorohexyl)octane	133331-77-8	DTXSID20440585			1		1	1	1										
1,1,1,3,3-Pentafluorobutane	406-58-6	DTXSID5073901			1														
1,1,1,5,5,5-Hexafluoro-2,4-pentanedione	1522-22-1	DTXSID4061753			1		1	1	1										
1,1-Dimethylpiperidinium chloride	24307-26-4	DTXSID1024170	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1,2-Propylene glycol	57-55-6	DTXSID0021206			1														
11:1 Fluorotelomer alcohol	423-65-4	DTXSID80375107			1		1	1	1										
11-H-Perfluoroundecanoic acid	1765-48-6	DTXSID5061954			1		1	1	1										
17beta-Estradiol	50-28-2	DTXSID0020573			1														
1-Bromopentadecafluoroheptane	375-88-2	DTXSID9059919			1		1	1	1										
1-Ethyl-3-methylimidazolium diethylphosphate	848641-69-0	DTXSID9047889			1														

1H,1H,10H,10H-Perfluorodecane-1,10-diol	754-96-1	DTXSID50369896			1		1	1	1										
1H,1H,11H,11H-Perfluorotetraethylene glycol	330562-44-2	DTXSID00380798			1		1	1	1										
1H,1H,2H,2H-Perfluorohexyl iodide	2043-55-2	DTXSID1047578			1		1	1	1										
1H,1H,2H,2H-Perfluorooctyl iodide	2043-57-4	DTXSID2047565			1														
1H,1H,2H-Perfluoro-1-decene	21652-58-4	DTXSID7074616			1		1	1	1										
1H,1H,5H,5H-Perfluoro-1,5-pentanediol diacrylate	678-95-5	DTXSID5060986			1		1	1	1										
1H,1H,5H-Perfluoropentanol	355-80-6	DTXSID0059879			1		1	1	1										
1H,1H,6H,6H-Perfluorohexane-1,6-diol diacrylate	2264-01-9	DTXSID80379721			1		1	1	1										
1H,1H,7H-Dodecafluoro-1-heptanol	335-99-9	DTXSID9059832			1		1	1	1										
1H,1H,7H-Perfluoroheptyl 4-methylbenzenesulfonate	424-16-8	DTXSID30340244			1		1	1	1										
1H,1H,8H,8H-Perfluoro-3,6-dioxaoctane-1,8-diol	129301-42-4	DTXSID70381090			1		1	1	1										
1H,1H,8H,8H-Perfluorooctane-1,8-diol	90177-96-1	DTXSID30396867			1		1	1	1										
1H,1H,9H-Perfluorononyl acrylate	4180-26-1	DTXSID00194615			1		1	1	1										
1H,1H-Heptafluorobutyl epoxide	1765-92-0	DTXSID10379254			1		1	1	1										

1H,1H-Perfluoro-3,6,9-trioxadecan-1-ol	147492-57-7	DTXSID40380797			1		1	1	1									
1H,1H-Perfluorooctyl acrylate	307-98-2	DTXSID5059799			1		1	1	1									
1H,1H-Perfluoropentylamine	355-27-1	DTXSID60377826			1		1	1	1									
1H-Perfluoro-1,1-propanediol	422-63-9	DTXSID9059969			1		1	1	1									
1-Iodo-1H,1H,2H,2H-perfluoroheptane	1682-31-1	DTXSID9061881			1		1	1	1									
1-Iodo-1H,1H,2H,2H-perfluorononane	2043-52-9	DTXSID90880156			1			1	1									
1-Iodopentadecafluoroheptane	335-58-0	DTXSID5059828			1		1	1	1									
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine	28289-54-5	DTXSID8040933														1	1	1
1-Methyl-4-phenylpyridinium iodide	36913-39-0	DTXSID40880040			1					1	1	1	1	1	1	1	1	1
1-Methylbenzene	108-88-3	DTXSID7021360														1	1	1
1-Naphthalenol, 1-(N-methylcarbamate)	63-25-2	DTXSID9020247	1	1	1	1	1	1	1	1	1	1	1	1	1			
1-Naphthol	90-15-3	DTXSID6021793								1	1	1	1	1	1	1	1	1
1-Pentafluoroethylethanol	374-40-3	DTXSID70880134			1		1	1	1									
1-Propenylperfluoropropane	355-95-3	DTXSID70379270			1													

2-(m-Chlorophenoxy)propionic acid	101-10-0	DTXSID9034232			1														
2-(Perfluorobutyl)-1-ethanesulfonic acid	757124-72-4	DTXSID30891564			1		1	1	1										
2-(Perfluorobutyl)ethyl acrylate	52591-27-2	DTXSID1068772			1		1	1	1										
2-(Perfluorohexyl)ethanol	647-42-7	DTXSID5044572			1		1	1	1										
2-(Perfluorohexyl)ethyl methacrylate	2144-53-8	DTXSID3047558			1		1	1	1										
2-(Perfluorohexyl)ethylphosphonic acid	252237-40-4	DTXSID20179883			1		1	1	1										
2-(Perfluorooctyl)ethanthiol	34143-74-3	DTXSID20337446			1		1	1	1										
2-(Perfluorooctyl)ethyl acrylate	27905-45-9	DTXSID5067348			1		1	1	1										
2-(Trifluoromethoxy)ethyl trifluoromethanesulfonate	329710-76-1	DTXSID00442840			1		1	1	1										
2,2,2-Trifluoroethyl perfluorobutanesulfonate	79963-95-4	DTXSID60380390			1		1	1	1										
2,2,3,3,4,4,4-Heptafluorobutyl methacrylate	13695-31-3	DTXSID3065586			1		1	1	1										
2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-Pentadecafluorooctyl methacrylate	3934-23-4	DTXSID5063235			1		1	1	1										
2,2,3,3-Tetrafluoropropyl acrylate	7383-71-3	DTXSID10224331			1		1	1	1										
2,2',3,4,4',5,5'-Heptachlorobiphenyl	35065-29-3	DTXSID6038299															1	1	1

2,2',4,4',5,5'-Hexabromodiphenyl ether	68631-49-2	DTXSID4030047			1												1	1	1
2,2',4,4',5-Pentabromodiphenyl ether	60348-60-9	DTXSID9030048			1					1	1	1	1	1	1	1	1	1	1
2,2',4,4'-Tetrabromodiphenyl ether	5436-43-1	DTXSID3030056	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2,2-Bis(4-hydroxyphenyl)-1,1,1-trichloroethane	2971-36-0	DTXSID8022325			1														
2,2-Difluoroethyl triflate	74427-22-8	DTXSID30378880			1		1	1	1										
2-Amino-2H-perfluoropropane	1619-92-7	DTXSID70481246			1														
2-Aminohexafluoropropan-2-ol	31253-34-6	DTXSID80382093			1		1	1	1										
2-Ethylhexyl 2,3,4,5-tetrabromobenzoate	183658-27-7	DTXSID9052686			1														
2-Ethylhexyl diphenyl phosphate	1241-94-7	DTXSID1025300	1	1	1	1				1	1	1	1	1	1	1			
2H,2H,3H,3H-Perfluorooctanoic acid	914637-49-3	DTXSID20874028			1		1	1	1										
2-Methoxyethanol	109-86-4	DTXSID5024182			1														
2-Perfluorooctylsulfonyl-N-ethylaminoethyl alcohol	1691-99-2	DTXSID6027426			1		1	1	1										
2-Vinylperfluorobutane	239795-57-4	DTXSID50379359			1														
3-(Perfluoro-2-butyl)propane-1,2-diol	125070-38-4	DTXSID10382147			1		1	1	1										

3-(Perfluoro-3-methylbutyl)-1,2-propenoxide	54009-81-3	DTXSID00379884			1		1	1	1										
3-(Perfluoroheptyl)propanoic acid	812-70-4	DTXSID90382620			1		1	1	1										
3-(Perfluorohexyl)-1,2-epoxypropane	38565-52-5	DTXSID30880413			1		1	1	1										
3-(Perfluoroisopropyl)-2-propenoic acid	243139-64-2	DTXSID40380257			1		1	1	1										
3-(Perfluorooctyl)propanol	1651-41-8	DTXSID10379991			1		1	1	1										
3-(Perfluoropropyl)propanol	679-02-7	DTXSID60379269			1		1	1	1										
3,3,4,4,5,5,6,6,6-Nonafluorohexene	19430-93-4	DTXSID6047575			1														
3,3',5,5'-Tetrabromobisphenol A	79-94-7	DTXSID1026081			1					1	1	1	1	1	1	1			
3,3-Bis(trifluoromethyl)-2-propenoic acid	1763-28-6	DTXSID30170109			1		1	1	1										
3,3'-Iminobispropanenitrile	111-94-4	DTXSID2041464	1	1	1	1	1	1	1										
3:1 Fluorotelomer alcohol	375-01-9	DTXSID4059914			1		1	1	1										
3:3 Fluorotelomer carboxylic acid	356-02-5	DTXSID00379268			1		1	1	1										
3H,3H-Perfluoro-2,4-hexanedione	20825-07-4	DTXSID90174941			1		1	1	1										
3H-Perfluoro-2,2,4,4-tetrahydroxypentane	77953-71-0	DTXSID70379295			1		1	1	1										

3H-Perfluoro-4-hydroxy-3-penten-2-one	1694-30-0	DTXSID90275806			1														
3-Iodo-2-propynyl-N-butylcarbamate	55406-53-6	DTXSID0028038			1														
4,4'-Sulfonyldiphenol	80-09-1	DTXSID3022409			1														
4,5-Dihydro-2-mercaptoimidazole	96-45-7	DTXSID5020601	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4:2 Fluorotelomer alcohol	2043-47-2	DTXSID1062122			1		1	1	1										
4:4 Fluorotelomer alcohol	3792-02-7	DTXSID60377821			1		1	1	1										
4H-Cyclopenta(def)phenanthrene	203-64-5	DTXSID1024887			1														
4H-Perfluorobutanoic acid	679-12-9	DTXSID50892417			1		1	1	1										
5,5-Diphenylhydantoin	57-41-0	DTXSID8020541	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5-Azacytidine	320-67-2	DTXSID9020116	1	1	1	1	1	1	1								1	1	1
5-Chloro-2-methyl-3(2H)-isothiazolone	26172-55-4	DTXSID9034286			1														
5-Fluorouracil	51-21-8	DTXSID2020634	1	1	1	1	1	1	1										
5H-Octafluoropentanoyl fluoride	813-03-6	DTXSID70379730			1														
5H-Perfluoropentanal	2648-47-7	DTXSID20337466			1														

6:1 Fluorotelomer alcohol	375-82-6	DTXSID00190950			1		1	1	1										
6:2 Fluorotelomer phosphate monoester	57678-01-0	DTXSID90558000			1		1	1	1										
6:2 Fluorotelomer sulfonic acid	27619-97-2	DTXSID6067331			1		1	1	1										
6-aminopyridine-3-carboxamide	329-89-5	DTXSID5051446	1	1	1	1	1	1	1								1	1	1
6H-Perfluorohex-1-ene	1767-94-8	DTXSID10379850			1														
6-Methyl-2-thiouracil	56-04-2	DTXSID2020890			1														
6-Propyl-2-thiouracil	51-52-5	DTXSID5021209	1	1	1	1	1	1	1										
7:3 Fluorotelomer alcohol	25600-66-2	DTXSID50382621			1		1	1	1										
8:2 Fluorotelomer sulfonic acid	39108-34-4	DTXSID00192353			1		1	1	1										
9-Chloro-perfluorononanoic acid	865-79-2	DTXSID30382104			1		1	1	1										
Abamectin	71751-41-2	DTXSID8023892			1														
Acenaphthene	83-32-9	DTXSID3021774			1														
Acenaphthylene	208-96-8	DTXSID3023845			1														
Acephate	30560-19-1	DTXSID8023846	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Acetaminophen	103-90-2	DTXSID2020006	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
Acetamiprid	135410-20-7	DTXSID0034300	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Acibenzolar-S-methyl	135158-54-2	DTXSID1032519	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Acrylamide	79-06-1	DTXSID5020027	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
Aldicarb	116-06-3	DTXSID0039223	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
Aldrin	309-00-2	DTXSID8020040			1														
Allethrin	584-79-2	DTXSID8035180	1	1	1	1	1	1	1								1	1	1
all-trans-Retinoic acid	302-79-4	DTXSID7021239	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Allyl perfluoroisopropyl ether	15242-17-8	DTXSID10370988			1														
alpha-Cypermethrin	67375-30-8	DTXSID7041201	1	1	1	1	1	1	1								1	1	1
Ammonium perfluorooctanoate	3825-26-1	DTXSID8037708			1		1	1	1										
Amoxicillin	26787-78-0	DTXSID3037044	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ampicillin	69-53-4	DTXSID4022602	1	1	1	1	1	1	1								1	1	1
Anthracene	120-12-7	DTXSID0023878	1	1	1	1	1	1	1										

Arsenic oxide (As2O3)	1327-53-3	DTXSID0020103															1	1	1
Aspartame	22839-47-0	DTXSID0020107	1	1	1	1	1	1	1								1	1	1
Aspirin	50-78-2	DTXSID5020108	1	1	1	1	1	1	1	1	1	1	1	1	1	1			1
Atrazine	1912-24-9	DTXSID9020112			1														
Auramine hydrochloride	2465-27-2	DTXSID9020114			1														
Azinphos-methyl	86-50-0	DTXSID3020122	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Azoxystrobin	131860-33-8	DTXSID0032520			1														
Benomyl	17804-35-2	DTXSID5023900	1	1	1	1	1	1	1								1	1	1
Bensulide	741-58-2	DTXSID9032329	1	1	1	1	1	1	1										
Benz(a)anthracene	56-55-3	DTXSID5023902			1														
Benzo(b)fluoranthene	205-99-2	DTXSID0023907			1														
Benzo[a]pyrene	50-32-8	DTXSID2020139			1														
Benzo[e]pyrene	192-97-2	DTXSID3023764			1														
Benzo[g,h,i]perylene	191-24-2	DTXSID5023908			1														

Benzo[k]fluoranthene	207-08-9	DTXSID0023909			1														
Berberine chloride	633-65-8	DTXSID8024602			1														
beta-Cyfluthrin	1820573-27-0	DTXSID8032330	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
beta-Cypermethrin	1224510-29-5	DTXSID6052871								1	1	1	1	1	1	1	1	1	1
Bifenthrin	82657-04-3	DTXSID9020160			1														
Bis(1H,1H-perfluoropropyl)amine	883498-76-8	DTXSID50381992			1		1	1	1										
Bis(2-ethylhexyl) tetrabromophthalate	26040-51-7	DTXSID7027887			1														
Bis(tributyltin)oxide	56-35-9	DTXSID9020166	1	1	1	1	1	1	1										
Bisindolylmaleimide I	133052-90-1	DTXSID50157932			1					1	1	1	1	1	1	1			
Bisphenol A	80-05-7	DTXSID7020182	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
Bisphenol AF	1478-61-1	DTXSID7037717			1														
Bisphenol B	77-40-7	DTXSID4022442			1														
Boric acid (H3BO3)	10043-35-3	DTXSID1020194			1														
Boscalid	188425-85-6	DTXSID6034392	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Buspirone	36505-84-7	DTXSID2022707	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Busulfan	55-98-1	DTXSID3020910			1														
Butylated hydroxyanisole	25013-16-5	DTXSID7020215	1	1	1	1	1	1	1								1	1	1
Cadmium chloride	10108-64-2	DTXSID6020226	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Cadmium(II) chloride hydrate (2:5)	7790-78-5	DTXSID4040183	1	1	1	1	1	1	1										
Caffeine	58-08-2	DTXSID0020232	1	1	1	1	1	1	1								1	1	1
Captan	133-06-2	DTXSID9020243			1														
Captopril	62571-86-2	DTXSID1037197	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Carbamazepine	298-46-4	DTXSID4022731	1	1	1	1	1	1	1								1	1	1
Carbofuran	1563-66-2	DTXSID9020249			1														
Cariporide mesylate	159138-81-5	DTXSID3047344			1														
Chloramben	133-90-4	DTXSID2020262	1	1	1	1	1	1	1								1	1	1
Chlordane	57-74-9	DTXSID7020267			1														
Chlordiazepoxide hydrochloride	438-41-5	DTXSID40880060	1	1	1	1	1	1	1										

Chlorendic acid	115-28-6	DTXSID2020268			1														
Chlorethoxyfos	54593-83-8	DTXSID2032344	1	1	1	1		1	1										
Chlorpheniramine maleate	113-92-8	DTXSID4020321	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Chlorpromazine hydrochloride	69-09-0	DTXSID7024827	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Chlorpyrifos	2921-88-2	DTXSID4020458	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Chlorpyrifos oxon	5598-15-2	DTXSID1038666	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Chlorpyrifos-methyl	5598-13-0	DTXSID6032352	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Chrysene	218-01-9	DTXSID0022432			1														
Clodinafop-propargyl	105512-06-9	DTXSID6032354			1														
Clothianidin	210880-92-5	DTXSID2034465	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Clotrimazole	23593-75-1	DTXSID7029871			1														
Clove leaf oil	8000-34-8	DTXSID8044175			1														
Cocaine	50-36-2	DTXSID2038443	1	1	1	1	1	1	1										
Colchicine	64-86-8	DTXSID5024845	1	1	1	1	1	1	1										

Cotinine	486-56-6	DTXSID1047576	1	1	1	1	1	1	1								1	1	1
Coumaphos	56-72-4	DTXSID2020347	1	1	1	1	1	1	1										
CP-409092	194098-25-4	DTXSID2047276			1														
CP-457920	220860-50-4	DTXSID4047254			1														
Cyclophosphamide monohydrate	6055-19-2	DTXSID6024888	1	1	1	1	1	1	1								1	1	1
Cyfluthrin	68359-37-5	DTXSID5035957	1	1	1	1	1	1	1								1	1	1
Cymoxanil	57966-95-7	DTXSID6032358	1	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1
Cypermethrin	52315-07-8	DTXSID1023998	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Cytarabine	147-94-4	DTXSID3022877	1	1	1	1	1	1	1								1	1	1
Cytarabine hydrochloride	69-74-9	DTXSID5024891	1	1	1	1	1	1	1									1	1
DDT	50-29-3	DTXSID4020375			1														
Deltamethrin	52918-63-5	DTXSID8020381	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dexamethasone	50-02-2	DTXSID3020384	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dextroamphetamine sulfate	51-63-8	DTXSID2057865	1	1	1	1	1	1	1										

D-Glucitol	50-70-4	DTXSID5023588	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
Di(2-ethylhexyl) phthalate	117-81-7	DTXSID5020607	1	1	1	1	1	1	1										
Diacetylmorphine hydrochloride monohydrate	5893-91-4	DTXSID90735793															1	1	1
Diazepam	439-14-5	DTXSID4020406	1	1	1	1	1	1	1										
Diazinon	333-41-5	DTXSID9020407	1	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1
Diazoxon	962-58-3	DTXSID5037523	1	1	1	1		1	1										
Dibasic sodium arsenate (Na2HAsO4.7H2O)	10048-95-0	DTXSID3032048															1	1	1
Dibenz[a,c]anthracene	215-58-7	DTXSID9049245			1														
Dibenz[a,h]anthracene	53-70-3	DTXSID9020409			1														
Dichloromethyl((perfluorohexyl)ethyl)silane	73609-36-6	DTXSID00223797			1		1	1	1										
Dichlorvos	62-73-7	DTXSID5020449	1	1	1	1	1	1	1										
Dicrotophos	141-66-2	DTXSID9023914	1	1	1	1		1	1										
Dieldrin	60-57-1	DTXSID9020453	1	1	1	1	1	1	1										
Diethylene glycol	111-46-6	DTXSID8020462	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Diethylstilbestrol	56-53-1	DTXSID3020465	1	1	1	1	1	1	1										
Difluoromethyl 1H,1H-perfluoropropyl ether	56860-81-2	DTXSID0074059			1														
Difpas-pyrazole	151506-44-4	DTXSID6048175			1														
Dimethoate	60-51-5	DTXSID7020479	1	1	1	1	1	1	1	1	1	1	1	1	1	1		1	1
Dimethoxymethyl((perfluorohexyl)ethyl)silane	85857-17-6	DTXSID40235137			1			1	1										
Dinotefuran	165252-70-0	DTXSID7034549	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Diphenhydramine hydrochloride	147-24-0	DTXSID4020537			1														
Disulfoton	298-04-4	DTXSID0022018	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D-Mannitol	69-65-8	DTXSID1023235	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Doxylamine succinate	562-10-7	DTXSID7020552	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Emamectin benzoate	155569-91-8	DTXSID0034566	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Enadoline	124378-77-4	DTXSID4047258			1														
Endosulfan	115-29-7	DTXSID1020560	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Endosulfan I	959-98-8	DTXSID9037539								1	1	1	1	1	1	1	1	1	1

Endosulfan sulfate	1031-07-8	DTXSID3037541								1	1	1	1	1	1	1			1
Endrin	72-20-8	DTXSID6020561			1														
EPTC	759-94-4	DTXSID1024091			1														
Erythromycin	114-07-8	DTXSID4022991	1	1	1	1	1	1	1								1	1	1
Esfenvalerate	66230-04-4	DTXSID4032667			1														
Esketamine hydrochloride	33643-47-9	DTXSID601027734	1	1	1	1	1	1	1										
Ethanol, 2-butoxy-, hydrogen phosphate	14260-97-0	DTXSID3065740								1	1	1	1	1	1	1			
Ethoprop	13194-48-4	DTXSID4032611	1	1	1	1	1	1	1										
Ethyl pentafluoropropionyl acetate	663-35-4	DTXSID20880144			1		1	1	1										
Ethyl perfluorobutyl ether	163702-05-4	DTXSID0073118			1														
Etofenprox	80844-07-1	DTXSID9032610	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Eugenol	97-53-0	DTXSID9020617			1														
Famotidine	76824-35-6	DTXSID5023039	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Fenamiphos	22224-92-6	DTXSID3024102			1														

Fenitrothion	122-14-5	DTXSID4032613			1														
Fenpropathrin	39515-41-8	DTXSID0024002			1														
Fipronil	120068-37-3	DTXSID4034609	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Firemaster 550	860302-33-6	DTXSID70880073			1														
Flubendiamide	272451-65-7	DTXSID4047672	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Fluconazole	86386-73-4	DTXSID3020627	1	1	1	1	1	1	1										
Flufenacet	142459-58-3	DTXSID2032552	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Fluorene	86-73-7	DTXSID8024105			1														
Fluorotelomer alcohol 8:2	678-39-7	DTXSID7029904			1		1	1	1										
Fluoxastrobin	361377-29-9	DTXSID2034625			1														
Fluoxetine hydrochloride	56296-78-7	DTXSID7020635	1	1	1	1	1	1	1								1	1	1
Flurothyl	333-36-8	DTXSID5046516			1														
Flusilazole	85509-19-9	DTXSID3024235			1														
Folic acid	59-30-3	DTXSID0022519			1														

Fosthiazate	98886-44-3	DTXSID0034930	1	1	1	1	1	1	1										
Galactosamine hydrochloride	1772-03-8	DTXSID4031356	1	1	1	1	1	1	1								1	1	1
Glufosinate-ammonium	77182-82-2	DTXSID1024120			1					1	1	1	1	1	1	1	1	1	1
Glufosinate-P	35597-44-5	DTXSID401020544			1														
Glufosinate-P ammonium	73777-50-1	DTXSID101020545			1														
Glycerol	56-81-5	DTXSID9020663	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Glyphosate	1071-83-6	DTXSID1024122	1	1	1	1	1	1	1										
Haloperidol	52-86-8	DTXSID4034150	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Heptachlor	76-44-8	DTXSID3020679	1	1	1	1	1	1	1										
Heptachlor epoxide B	1024-57-3	DTXSID1024126	1	1	1	1	1	1	1										
Heptafluorobutyl iodide	374-98-1	DTXSID4059912			1		1	1	1										
Heptafluorobutyramide	662-50-0	DTXSID2060965			1		1	1	1										
Hexachlorophene	70-30-4	DTXSID6020690	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hexafluoroamylene glycol	376-90-9	DTXSID3059927			1		1	1	1										

Hexafluoroglutaryl chloride	678-77-3	DTXSID0060985			1		1	1	1										
Hydroxyurea	127-07-1	DTXSID6025438	1	1	1	1	1	1	1										
Ibuprofen	15687-27-1	DTXSID5020732	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Imidacloprid	138261-41-3	DTXSID5032442	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Indoxacarb	173584-44-6	DTXSID1032690	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Isodecyl diphenyl phosphate	29761-21-5	DTXSID3025465			1					1	1	1	1	1	1	1			
Isoniazid	54-85-3	DTXSID8020755	1	1	1	1	1	1	1										
Kepone	143-50-0	DTXSID1020770	1	1	1	1	1	1	1								1	1	1
Ketamine hydrochloride	1867-66-9	DTXSID4040137								1	1	1	1	1	1	1	1	1	1
Lactofen	77501-63-4	DTXSID7024160			1														
L-Ascorbic acid	50-81-7	DTXSID5020106	1	1	1	1	1	1	1								1	1	1
L-Domoic acid	14277-97-5	DTXSID20274180			1					1	1	1	1	1	1	1	1	1	1
Lead(II) acetate trihydrate	6080-56-4	DTXSID3031521	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Lindane	58-89-9	DTXSID2020686			1												1	1	1

Loperamide hydrochloride	34552-83-5	DTXSID00880006	1	1	1	1	1	1	1										
Lovastatin	75330-75-5	DTXSID5020784			1														
Malaoxon	1634-78-2	DTXSID9020790	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Malathion	121-75-5	DTXSID4020791	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Mancozeb	8018-01-7	DTXSID0034695	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Maneb	12427-38-2	DTXSID9020794	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Manganese dichloride	7773-01-5	DTXSID9040681	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Manganese(II) acetate	638-38-0	DTXSID5027279			1														
Manganese(II) sulfate monohydrate	10034-96-5	DTXSID4020795														1	1	1	
Mercuric chloride	7487-94-7	DTXSID5020811			1														
Metaflumizone	139968-49-3	DTXSID6040373	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Metformin	657-24-9	DTXSID2023270	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Methadone hydrochloride	1095-90-5	DTXSID2020501			1														
Methamidophos	10265-92-6	DTXSID6024177	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Methimazole	60-56-0	DTXSID4020820	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Methotrexate	59-05-2	DTXSID4020822	1	1	1	1	1	1	1								1	1	1
Methoxychlor	72-43-5	DTXSID9020827			1														
Methyl 2H,2H,3H,3H-perfluoroheptanoate	132424-36-3	DTXSID50441560			1														
Methyl 3H-perfluoroisopropyl ether	568550-25-4	DTXSID70537191			1		1	1	1										
Methyl parathion	298-00-0	DTXSID1020855	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Methyl perfluoro(3-(1-ethenyloxypropan-2-yloxy)propanoate)	63863-43-4	DTXSID8044969			1		1	1	1										
Methyl perfluorobutanoate	356-24-1	DTXSID4059881			1														
Methyl perfluoroethyl ketone	374-41-4	DTXSID90285748			1														
Methyl perfluorohexanoate	424-18-0	DTXSID20335700			1														
Methyl viologen dichloride hydrate	75365-73-0	DTXSID001044510															1	1	1
Methylazoxymethanol acetate	592-62-1	DTXSID1025568								1	1	1	1	1	1	1	1	1	1
Methylmercuric(II) chloride	115-09-3	DTXSID5020813	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Metoprolol	51384-51-1	DTXSID2023309	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Mevastatin	73573-88-3	DTXSID4040684			1														
Mifepristone	84371-65-3	DTXSID5023322	1	1	1	1	1	1	1								1	1	1
Mirex	2385-85-5	DTXSID7020895			1														
Molinate	2212-67-1	DTXSID6024206			1														
Morphine hydrochloride trihydrate	6055-06-7	DTXSID30904880															1	1	1
Naled	300-76-5	DTXSID1024209	1	1	1	1		1	1										
Naloxone hydrochloride dihydrate	51481-60-8	DTXSID90199452	1	1	1	1	1	1	1										
Naphthalene	91-20-3	DTXSID8020913			1														
Narciclasine	29477-83-6	DTXSID70183677								1	1	1	1	1	1	1	1	1	1
N-Ethylperfluorooctanesulfonamide	4151-50-2	DTXSID1032646			1		1	1	1										
N-Methyl-N-(2-hydroxyethyl)per fluorooctanesulfonamide	24448-09-7	DTXSID7027831			1		1	1	1										
N-Methyl-N-trimethyl silylheptafluorobutyramide	53296-64-3	DTXSID40379666			1		1	1	1										
N-Methylperfluoro octanesulfonamide	31506-32-8	DTXSID1067629			1		1	1	1										
Nonafluoropentanamide	13485-61-5	DTXSID60400587			1		1	1	1										

o,p'-DDT	789-02-6	DTXSID6022345			1														
Octafluoroadipamide	355-66-8	DTXSID80310730			1		1	1	1										
Octamethylcyclotetrasiloxane	556-67-2	DTXSID7027205	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Octylbicycloheptene dicarboximide	113-48-4	DTXSID6032562			1														
Omethoate	1113-02-6	DTXSID4037580	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Oxidopamine hydrochloride	28094-15-7	DTXSID0045838			1														
p,p'-DDD	72-54-8	DTXSID4020373			1														
p,p'-DDE	72-55-9	DTXSID9020374			1														
Paraquat dichloride	1910-42-5	DTXSID7024243	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
Parathion	56-38-2	DTXSID7021100	1	1	1	1	1	1	1										
Penicillin VK	132-98-9	DTXSID7021102	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Pentafluoropropanoic anhydride	356-42-3	DTXSID70870515			1														
Pentafluoropropionamide	354-76-7	DTXSID0059871			1		1	1	1										
Penthiopyrad	183675-82-3	DTXSID6058005								1	1	1	1	1	1	1	1	1	1

Perfluamine	338-83-0	DTXSID9059834			1		1	1	1										
Perfluoro-(2,5,8-trimethyl-3,6,9-trioxadodecanoic) acid	65294-16-8	DTXSID70276659			1		1	1	1										
Perfluoro(4-methoxybutanoic acid)	863090-89-5	DTXSID60500450			1		1	1	1										
Perfluoro-1-iodohexane	355-43-1	DTXSID7047566			1		1	1	1										
Perfluoro-1-octanesulfonyl chloride	423-60-9	DTXSID90315130			1		1	1	1										
Perfluoro-2,5-dimethyl-3,6-dioxanonanoic acid	13252-14-7	DTXSID00892442			1		1	1	1										
Perfluoro-2-methyl-3-oxahexanoic acid	13252-13-6	DTXSID70880215			1		1	1	1										
Perfluoro-3,6,9-trioxatridecanoic acid	330562-41-9	DTXSID50375114			1		1	1	1										
Perfluoro-3,6-dioxaheptanoic acid	151772-58-6	DTXSID30382063			1		1	1	1										
Perfluoro-3,6-dioxaoctane-1,8-dioic acid	55621-21-1	DTXSID20375106			1		1	1	1										
Perfluoro-3-methoxypropanoic acid	377-73-1	DTXSID70191136			1		1	1	1										
Perfluoro-4-isopropoxybutanoic acid	801212-59-9	DTXSID60663110			1		1	1	1										
Perfluorobutanedioic acid	377-38-8	DTXSID8059928			1			1	1										
Perfluorobutanesulfonic acid	375-73-5	DTXSID5030030			1		1	1	1										

Perfluorobutanesulfonyl fluoride	375-72-4	DTXSID20861913			1													
Perfluorobutanoic acid	375-22-4	DTXSID4059916			1		1	1	1									
Perfluorobutyraldehyde	375-02-0	DTXSID10190946			1													
Perfluorocyclohexanecarbonyl fluoride	6588-63-2	DTXSID80379781			1		1	1	1									
Perfluorodecanoic acid	335-76-2	DTXSID3031860			1		1	1	1									
Perfluoroglutaryl difluoride	678-78-4	DTXSID50218052			1													
Perfluoroheptanesulfonic acid	375-92-8	DTXSID8059920			1		1	1	1									
Perfluoroheptanoic acid	375-85-9	DTXSID1037303			1		1	1	1									
Perfluorohexanedioic acid	336-08-3	DTXSID4059833			1		1	1	1									
Perfluorohexanesulfonamide	41997-13-1	DTXSID50469320			1		1	1	1									
Perfluorohexanesulfonic acid	355-46-4	DTXSID7040150			1		1	1	1									
Perfluorohexanoic acid	307-24-4	DTXSID3031862			1		1	1	1									
Perfluoroisobutyl methyl ether	163702-08-7	DTXSID5042326			1													
Perfluorononanoic acid	375-95-1	DTXSID8031863			1		1	1	1									

Perfluorooct-1-ene	559-14-8	DTXSID40204489			1		1	1	1										
Perfluorooctanamide	423-54-1	DTXSID60195123			1		1	1	1										
Perfluorooctanamidine	307-31-3	DTXSID70381151			1		1	1	1										
Perfluorooctane	307-34-6	DTXSID0059794			1		1	1	1										
Perfluorooctanesulfonamide	754-91-6	DTXSID3038939			1		1	1	1										
Perfluorooctanesulfonamido ammonium iodide	1652-63-7	DTXSID8051419			1		1	1	1										
Perfluorooctanesulfonic acid	1763-23-1	DTXSID3031864			1		1	1	1										
Perfluorooctanesulfonyl fluoride	307-35-7	DTXSID5027140			1		1	1	1										
Perfluorooctanoic acid	335-67-1	DTXSID8031865			1		1	1	1	1	1	1	1	1	1	1	1	1	1
Perfluoropentanamide	355-81-7	DTXSID70366226			1		1	1	1										
Perfluoropentanedioic acid	376-73-8	DTXSID8059926			1		1	1	1										
Perfluoropentanoic acid	2706-90-3	DTXSID6062599			1		1	1	1										
Perfluoropropanoic acid	422-64-0	DTXSID8059970			1		1	1	1										
Perfluoropropyl trifluorovinyl ether	1623-05-8	DTXSID0061826			1														

Perfluorotetradecanoic acid	376-06-7	DTXSID3059921			1		1	1	1										
Perfluorotridecanoic acid	72629-94-8	DTXSID90868151			1		1	1	1										
Perfluoroundecanoic acid	2058-94-8	DTXSID8047553			1		1	1	1										
Permethrin	52645-53-1	DTXSID8022292	1	1	1	1	1	1	1										
PharmaGSID_47330	NOCAS_47330	DTXSID9047330			1														
Phenanthrene	85-01-8	DTXSID6024254			1														
Phenobarbital	50-06-6	DTXSID5021122			1														
Phenobarbital sodium	57-30-7	DTXSID0021123	1	1	1	1	1	1	1										
Phenol	108-95-2	DTXSID5021124	1	1	1	1	1	1	1								1	1	1
Phenylmercuric acetate	62-38-4	DTXSID7021150			1														
Phorate	298-02-2	DTXSID4032459	1	1	1	1		1	1										
Phosmet	732-11-6	DTXSID5024261	1	1	1	1	1	1	1										
Picoxystrobin	117428-22-5	DTXSID9047542			1														
Piperonyl butoxide	51-03-6	DTXSID1021166			1														

Pirimiphos-methyl	29232-93-7	DTXSID0024266	1	1	1	1	1	1	1										
Potassium perfluorobutanesulfonate	29420-49-3	DTXSID3037707			1		1	1	1										
Potassium perfluorohexanesulfonate	3871-99-6	DTXSID3037709			1		1	1	1										
Potassium perfluorooctanesulfonate	2795-39-3	DTXSID8037706	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Potassium perfluorooctanoate	2395-00-8	DTXSID00880026			1			1	1										
Prallethrin	23031-36-9	DTXSID0032572			1														
Pravastatin sodium	81131-70-6	DTXSID6047525			1														
Profenofos	41198-08-7	DTXSID3032464	1	1	1	1	1	1	1										
Pymetrozine	123312-89-0	DTXSID2032637	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Pyraclostrobin	175013-18-0	DTXSID7032638			1														
Pyrene	129-00-0	DTXSID3024289			1														
Reserpine	50-55-5	DTXSID7021237			1														
Resmethrin	10453-86-8	DTXSID7022253			1														
Rotenone	83-79-4	DTXSID6021248			1					1	1	1	1	1	1	1			

S-(+)-Methamphetamine hydrochloride	51-57-0	DTXSID8048864															1	1	1
Saccharin	81-07-2	DTXSID5021251								1	1	1	1	1	1	1			
S-Bioallethrin	28434-00-6	DTXSID2039336	1	1	1	1	1	1	1								1	1	1
Selegiline hydrochloride	14611-52-0	DTXSID9044584	1	1	1	1	1	1	1								1	1	1
Sevoflurane	28523-86-6	DTXSID8046614			1														
Simvastatin	79902-63-9	DTXSID0023581			1														
Sodium arsenite	7784-46-5	DTXSID5020104	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
Sodium benzoate	532-32-1	DTXSID1020140	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Sodium chlorite	7758-19-2	DTXSID8021272	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Sodium fluoride	7681-49-4	DTXSID2020630	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
Sodium L-glutamate hydrate	6106-04-3	DTXSID0047240	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Sodium orthovanadate	13721-39-6	DTXSID2037269			1														
Sodium perchlorate	7601-89-0	DTXSID1034185								1	1	1	1	1	1	1	1	1	1
Sodium perfluorooctanoate	335-95-5	DTXSID40880025			1		1	1	1										

Sodium saccharin hydrate	82385-42-0	DTXSID7021992	1	1	1	1	1	1	1										
Sodium valproate	1069-66-5	DTXSID5037072	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Spirodiclofen	148477-71-8	DTXSID6034928	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Spiroxamine	118134-30-8	DTXSID1034212			1														
Sulfisoxazole	127-69-5	DTXSID6021292	1	1	1	1	1	1	1								1	1	1
Tamoxifen	10540-29-1	DTXSID1034187			1														
tau-Fluvalinate	102851-06-9	DTXSID7037555	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Tebuconazole	107534-96-3	DTXSID9032113	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
Tebupirimfos	96182-53-5	DTXSID1032482	1	1	1	1	1	1	1										
Tefluthrin	79538-32-2	DTXSID5032577			1														
Tembotrione	335104-84-2	DTXSID5047037	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Terbufos	13071-79-9	DTXSID2022254	1	1	1	1	1	1	1										
Terbutaline hemisulfate	23031-32-5	DTXSID3045437	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
tert-Butylhydroquinone	1948-33-0	DTXSID6020220			1														

tert-Butylphenyl diphenyl phosphate	56803-37-3	DTXSID6024701	1	1	1	1				1	1	1	1	1	1	1			
Tetracycline	60-54-8	DTXSID7023645	1	1	1	1	1	1	1								1	1	1
Tetraethylthiuram disulfide	97-77-8	DTXSID1021322			1														
Tetramethrin	7696-12-0	DTXSID6032649			1														
Thalidomide	50-35-1	DTXSID9022524	1	1	1	1	1	1	1										
Thiacloprid	111988-49-9	DTXSID7034961	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Thiamethoxam	153719-23-4	DTXSID2034962	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Topramezone	210631-68-8	DTXSID0034722	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Tri-allate	2303-17-5	DTXSID5024344	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Triamcinolone	124-94-7	DTXSID1040742	1	1	1	1	1	1	1								1	1	1
Tribufos	78-48-8	DTXSID1024174	1	1	1	1		1	1										
Tributyltin chloride	1461-22-9	DTXSID3027403	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Tributyltin methacrylate	2155-70-6	DTXSID9035204	1	1	1	1	1	1	1								1	1	1
Trichlorfon	52-68-6	DTXSID0021389	1	1	1	1		1	1	1	1	1	1	1	1	1	1	1	1

Triclosan	3380-34-5	DTXSID5032498			1														
Triethoxy((perfluorohexyl)ethyl)silane	51851-37-7	DTXSID1074915			1		1	1	1										
Triethyltin bromide	2767-54-6	DTXSID9040712	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Trifloxystrobin	141517-21-7	DTXSID4032580			1														
Trimethyltin chloride	1066-45-1	DTXSID6042496															1	1	1
Trimethyltin hydroxide	56-24-6	DTXSID9032240	1	1	1	1	1	1	1										
Tri-o-cresyl phosphate	78-30-8	DTXSID6032192	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Triphenyl phosphate	115-86-6	DTXSID1021952			1					1	1	1	1	1	1	1			
Triphenyl phosphates isopropylated	68937-41-7	DTXSID4028880	1	1	1	1				1	1	1	1	1	1	1			
Triphenyl phosphite	101-02-0	DTXSID0026252			1														
Tris(1,3-dichloro-2-propyl) phosphate	13674-87-8	DTXSID9026261	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Tris(2-butoxyethyl) phosphate	78-51-3	DTXSID5021758								1	1	1	1	1	1	1			
Tris(2-chloroethyl) phosphate	115-96-8	DTXSID5021411			1					1	1	1	1	1	1	1			
Tris(2-chloroisopropyl)phosphate	13674-84-5	DTXSID5026259	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Tris(methylphenyl) phosphate	1330-78-5	DTXSID4021391	1	1	1	1				1	1	1	1	1	1	1			
tris(Trifluoroethoxy)methane	58244-27-2	DTXSID30395037			1		1	1	1										
Valinomycin	2001-95-8	DTXSID9041150			1														
Warfarin	81-81-2	DTXSID5023742	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Z-Tetrachlorvinphos	22248-79-9	DTXSID1032648	1	1	1	1	1	1	1										
λ-Cyhalothrin	91465-08-6	DTXSID7032559	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1