Annex I. List of viability testing methods (non-inclusive) of cell cultures

1. Structural cell damage (non-invasive)	
Evaluation of overall cell shape, cytoplasmic structure, flatness and outline properties on a good phase contrast light microscope	Screening assay that covers many forms of damage with high sensitivity, if observer is experienced. May be automated and rendered quantitative to some extent by high content imaging. • **Advantages:** high throughput (if automated), non-invasive, repeatable on same well over time. • **Disadvantages:** No clear prediction model (only qualitative data, no exact cell death definition), no standalone approach; requires extensive experience of operator.
LDH-release test	Cells with intact membrane retain their content of LDH enzyme; LDH is released when cell membranes rupture (non-viable cells), and the enzyme can then be measured in the supernatant. To give fully quantitative data, the assay requires normalisation to the total LDH content of the positive control well(s). It can to some extent be repeated for the same culture at different time points. • **Advantages:** Measurement of a definite/unambiguous cell death endpoint; can be combined with cell function assays. Allows cells to be used for other purposes, if only supernatant is sampled. • **Disadvantages:** Information only for cell populations. Requires normalisation to the total LDH content of a culture well (extra wells for cytotoxicity positive control treated). Frequently high background LDH levels are observed (e.g., from serum components; signal/noise ratio can be bad in some culture media or with some cell types). Problems with long-term assays involving medium changes. Not a very sensitive measure of cytotoxicity
2. Structural cell damage (invasive)	
Membrane penetration using dyes to detect 'cytotoxicity' (e.g., naphthalene black, trypan blue, propidium iodide, ethidium bromide, EH-1)	Dyes are selected so that they stain non-viable cells, but do not enter cells with an intact cell membrane. Some of the dyes stain the entire cell (e.g., trypan blue), others stain the nucleus/DNA (e.g., propidium iodide). Dyes that only stain dead cells usually need a combination with a method that stains/identifies all cells (such as phase contrast for trypan blue, or a nuclear counterstain (H-33342, acridine orange, SYTO-13) for fluorescent dyes. • **Advantages:** Rapid and usually easy to interpret. Gives information

	on the single cell level. High throughput and absolute quantification are possible (high content imaging). • **Disadvantages:** May overestimate viability since apoptotic cells continue to have intact membranes and may appear viable. Some dyes (e.g., trypan blue, H-33342) are cytotoxic, so that the evaluation has to be performed rapidly. Trypan blue and ethidium bromide, are toxic/CMR classified chemicals and the use should be restricted.
Retention of dyes within intact cells to detect 'viability' (e.g., fluorescein diacetate or calcein-AM)	After dye exposure, viable cells fluoresce when observed under UV light. The lipid-soluble dyes are transformed by cellular enzymes (esterases) into lipid-insoluble fluorescent compounds that cannot escape from cells with intact membranes. Thus, cells can be observed under a microscope (single cell analysis) or with a fluorescence plate reader (population analysis). The dyes are often used in combination with a cytotoxicity stain (e.g., propidium iodide). • **Advantages:** Rapid and usually easy to interpret. Gives information on the single cell level (including morphological information on the cell shape). High throughput and absolute quantification are possible (high content imaging, fluorescent plate reader or FACS). • **Disadvantages:** Some cells leak the dyes; some cells actively export the dyes through P-gp activity. Many fluorescent dyes are prone to photo-bleaching, and some may be sensitive to their local environment (pH etc.).
Evaluation of programmed cell death/apoptosis markers	As programmed cell death is a universal biological process based on defined cellular biochemical pathways and organelle changes, the activation of cell-death-associated pathways is often used as surrogate marker for cell death. An example for such a pathway is the activation of caspases (detectable in populations by enzymatic analysis or in single cells by staining) or the activation of endonucleases (detectable on population level as DNA-fragmentation). Moreover, a typical type of chromatin condensation (detectable by DNA stains) and the display of phosphatidylserine on the outside of the plasma membrane (detectable by Annexin staining) is highly correlated with apoptotic death. • **Advantages:** Adds mechanistic information to cytotoxicity data. Several endpoints are easy to quantify and useful for high through-put measurements. • **Disadvantages:** Not all types of cell death may be detected by a given endpoint. Needs to be combined with a general cytotoxicity test. Some endpoints are prone to artefacts (Annexin staining) and some staining techniques (TUNEL, caspase-3) lead to an un-intentional selection of subpopulations. Caspase activity measurement does not easily yield a prediction model for the extent of cell death.

3. Cell growth	
Cell counting	For some cell populations, continued growth is a defining feature, and thus impaired growth needs to be considered as a reduction of viability. Notably, impaired growth/proliferation is not necessarily correlated with cell death; it is thus rather a functional viability endpoint than a cytotoxicity measure. A special case for growth is the increase in cell size without proliferation. This feature is e.g., seen for the extension of neurites by neurons. The gold standard analytical endpoint for the growth/proliferation endpoint is counting (or morphometry). There are many ways of counting cells, either as single particles (e.g., by FACS or HCI) or by assessing a biochemical parameter correlated to cell number (e.g., DNA content). • **Advantages:** Growth can be a sensitive parameter of cell well-being. • **Disadvantages:** Growth is a functional endpoint, not necessarily linked to cytotoxicity; artefacts for growth endpoints may arise from inhomogeneous growth of subpopulations: moreover, growth may hide ongoing cell death, and thus needs careful control in combination with cytotoxicity assays.
BrdU or EdU incorporation	Measures new DNA synthesis based on incorporation of the easily detectable nucleoside analogs BrdU (or EdU) into DNA. BrdU can be detected e.g., by fluorescent-labelled antibodies in permeabilised cells. Alternatively, radiolabelled thymidine can be used. • **Advantages**: Measurement on single cell level. Easy to quantify and use at high throughput. • **Disadvantages**: BrdU/EdU can be cytotoxic; no information available on how often one given cell has divided. High cost and effort compared to counting.
Staining of cellular components that are proportional to overall cell mass (proteins by e.g., sulforhodamine B or crystal violet; DNA by Hoechst H-33342)	These assays evaluate a surrogate measure of overall cell mass and assume that it correlates with total cell number. In non-proliferating cells, or with continuous ongoing proliferation, the endpoints are also frequently used as indicators of cytotoxicity, as dead cells often detach from plates and reduce the overall cell mass. • **Advantages**: Simple and cheap; lots of historical data. • **Disadvantages:** Mostly not a single cell measure but only population level. Protein staining is only a surrogate endpoint of real cell number. For DNA quantification with Hoechst 33342: fluorescent probe penetration, bleaching, and cytotoxicity are issues to be considered. Crystal violet is a toxic/CMR classified chemicals and the use should be restricted.

4. Cellular metabolism	
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, or similar tetrazolium dye reduction assays (e.g., WST-8)	Biochemical activity (mostly mitochondrial metabolism; production of reducing equivalents like NAD(P)H) in viable cells causes reduction of the tetrazolium dye. The resultant formazan is extracted and measured spectrophotometrically. The rate of formation of formazan corresponds to the function of essential cellular processes like respiration. • **Advantages:** High throughput, easy, robust, low cost. Used in several ISO standards and OECD test guidelines. High sensitivity. Can be used for tissue constructs. • **Disadvantages:** Measures amount of viable cells (only indirect measure of cell death), and needs control for contribution of proliferation. Cells with reduced mitochondrial function may appear non-viable. Inhibition of cell metabolism by the test item causes low values in the assay which is not necessarily related to cell viability. Some test items interfere with the assay e.g., by reducing the dye why interference testing is recommended. Measurement usually not on single cell level. Some cell cultures need long time to reduce sufficient amount of dye (no sharp time point for viability definition). Assessment of kinetic of the reduction may be necessary to ensure proper selection of incubation time with a tetrazolium dye (to avoid reaching plateau of OD).
Resazurin reduction assay (sometimes called Alamar blue)	Similar to tetrazolium reduction assays. Fluorescent/absorbent resorufin is formed from resazurin through mitochondrial metabolism of viable cells. • **Advantages:** Many tests can be performed rapidly in multi-well dishes. Cells can be tested repeatedly (non-invasive measurement). High sensitivity. • **Disadvantages:** Cells with reduced mitochondrial function may appear non-viable. Some test items interfere with the assay (e.g., superoxide also reduces the dye) why interference testing is recommended. Measurement only on population level. Some cell cultures need a long time to reduce sufficient resazurin (no sharp time point for viability definition).
Mitochondrial depolarisation assays (based on fluorescent indicator dyes)	Many organelle functions are used as endpoints of general cell health. Most frequently, mitochondrial function is assessed (see MTT, resazurin). One mitochondrial test on the single cell level is the measurement of mitochondrial membrane potential by addition of potential sensing fluorescent dyes like JC-1, TMRE, MitoTracker, etc. Quantification is by HCI or FACS • **Advantages**: Fast, inexpensive, high throughput; single cell information.

	• **Disadvantages**: As for MTT (measures cell function, not cytotoxicity). Artefacts by test items that affect mitochondria specifically. Artefacts by test items that affect plasma membrane potential. Artefacts due to bleaching, quenching and unquenching, and due to shape changes and clustering of mitochondria.
Neutral Red Uptake (NRU) (ISO 10993)	A cell organelle function assay assessing lysosomal function. Active cells accumulate the red dye in lysosomes and the dye incorporation is measured by spectrophotometric analysis. • **Advantages:** Low cost. Used in several ISO standards and OECD test guidelines. Historic data base. • **Disadvantages:** Normalisation required for quantitative measurement, e.g., with protein content or number of cells. Usually only gives information at the population level. Not suited for tissue constructs and certain cell lines. Not suitable for test items that affect lysosome function.
ATP assays	Measurement of the total ATP content in a cell population. Dying cells fail to produce ATP, have an increased ATP consumption, and may lose ATP through perforations of the plasma membrane. For the test, cell lysates are prepared, and the ATP content is assesses by a luminometric assay. • **Advantages:** Fast, high throughput • **Disadvantages:** No single cell data, expensive, requires a luminometer, as MTT: measurement of viable cell mass, not a direct measure of cytotoxicity. Artefacts as for other mitochondrial tests.