Project:

High Content Screening Assay for Cell Viability

Assessment of cellular responses to nanomaterials by means of HCS multiparametric assays: cell viability using live/dead fluorescent stains

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Contents

1 Introduction

The acute cytotoxicity of nanomaterials (or test substances) can be assessed following various exposure routes and exposure times using the High Content Screening (HCS) platform. One such technique is by monitoring the number of live and dead cells using fluorescent dyes. Following exposure to the materials, the cellular viability of the treated cells can be determined using dyes that are specific for live and dead cells based on the integrity of their plasma membranes, and intracellular esterase activity. Consideration should be taken to ensure that experimental design, cellular models, methods of exposure, and analytical metrics are in line with OECD [1, 2] and European Food Safety Authority (EFSA) guidelines [3].

As such, this protocol describes the approach for testing the cell viability of human cell lines to nanoparticles (NPs) using the HCS platform by automated imaging and determination of both live and dead cells based on cell counts and fluorescent intensities.

2 Principle of the Method

Procedures followed are in accordance with REACH on the hazard assessment of chemical entities [4-6] and aspects of the ISO 10993-5 guidelines for in vitro cytotoxicity assessment on the use of liquid samples [7].

Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). Ethidium homodimer (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. Cell nuclei can be visualized using Hoechst 33342. This enables the determination of total cell counts, as well as the calculation of cell nuclear area, which can be markers of a cytotoxic insult [8].

The determination of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events that do not affect these cell properties may not be accurately assessed using this method. Background fluorescence levels are inherently low with this assay technique because the dyes are virtually non-fluorescent before interacting with cells.

The determination of the cell viability using the HCS assays detailed in this SOP relate to the thus refers to the measurement of cell counts using an automated fluorescent microscope, imaging sufficient fields per well to obtain sufficient numbers of cells to be analysed to provide robust data. In the case of this SOP, cells are images using the Cytell Cell Imaging System, and analysis is conducted using the InCell Investigator 1.6 software package (all GE Healthcare, United States). Other imaging and analysis instruments and software packages can also be used [9].

3 Applicability and Limitations (Scope)

This SOP describes the *in vitro* methods for the evaluation of the cytotoxicity of nanoparticles using the HCS platform to measure the total cell count, and the number of live and dead cells using specific fluorescent dyes. The protocol is based on the assessment of the A431 epidermoid carcinoma cell line, a cell model used to access skin exposure to the nanomaterial, using the Molecular Probes ™ LIVE/DEAD ® Viability/Cytotoxicity Kit *for mammalian cells* (Invitrogen). Other cell lines can be used. If the HCS platform used does not have the capabilities to detect a specific dye, for example far-red capabilities to detect EthD-1, this can be excluded, with the number of dead cells being calculated as the total cell count less cell count for cells stained green (calcein). The assay, and this SOP, does not provide detailed mechanistic information of the toxic effect experienced by the cell.

Interference of the NP with the assay readouts should be considered, particularly if the NP under examination exhibits fluorescent properties (quantum dots for example), during experimental planning and interpretation of the results. Existing available literature should therefore be reviewed during design of experiments in order to evaluate the applicability of the assays.

Test material dilutions are carried out in the presence of serum. This may cause aggregation of the test material, and so stability studies in such media should be carried out. Further details on experimental design and characterisation protocols can be found in scientific literature or by contacting EUNCL core expert teams. Methodology for the characterisation of test materials in complex media (such as cell culture medium) are available in EUNCL-PCC-21, EUNCL-PCC-22, EUNCL-PCC-23, and EUNCL-PCC-35. Where appropriate serum-free medium should be considered for use, as variations and inconsistencies in serum quality can lead to unquantifiable uncertainties in experimental procedures and results [1].

Finally, the protocols for image analysis are specific to the InCell Investigator software. A comprehensive review of other HCS capable equipment can be found in the literature [9].

4 Equipment and Reagents

4.1 Equipment

- Fluorescence microscope – GE Healthcare Cytell Imaging system (or equivalent)
- Image analysis software – GE Healthcare InCell Investigator 1.6
- 96 well flat bottom polystyrene cell culture plate (Nunc, 167008)
- Incubator, 37˚C with 5% CO2 and 95% humidity

4.2 Reagents

- LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells and Hoechst 33342 (Molecular Probes ™, Invitrogen, L3224)
    - Absorption/Emission maxima for dyes used:
        - Hoechst Dye = 350/461 nm
        - Calcein = 494/517 nm
        - Ethidium homodimer-1 in presence of DNA = 528/617 nm
- Positive control compound – valinomycin (Sigma, V3639-5ML)

- A431 (epidermoid carcinoma cell line) (ATCC CRL-1555)
- DMEM (Sigma, D5796)
- Fetal bovine serum (Sigma, F9665-500ML)
- Penicillin-streptomycin (Sigma, P4458-100ML)
- Dimethyl sulfoxide (Sigma, D5879)
- 37 % formaldehyde (Sigma, 252549-25ML)
- Dissociation reagent – TrypLE (or equivalent) (BioSciences, 0040090DG)
- PBS (Fischer, 092810305)

4.3 Reagent Preparation

4.3.1 **Cell culture medium**

The complete medium for A431 is prepared by adding 10% FBS and 1% Penicillin-Streptomycin to DMEM medium. The medium is stable for up to 4 weeks at 4˚C.

4.3.2 Positive control

Prior to nanomaterials exposure, it would be advisable to identify the assay response to the specific cell line adopted for the experimental investigation. Thus, a titration dose range finding has to be carried out on valinomycin where to identify the specific EC50 for the cell line in question. Typical dose range goes from 50 to 200 µM in at least 4 concentration doses. This will require a separate plate where to carry out the dose finding. Once this is identified

Prepare all positive controls fresh for each assay. For the specific case here reported for A431 cell line, dilute valinomycin to 120 µM in cell culture medium. Stock solution used in this SOP is at 1 mg/mL concentration, equating to 899 µM. The concentration of valinomycin should be titrated to find the EC50 and EC90 concentration for each cell line.

4.3.3 Determine the optimal dye concentrations

Best results are obtained by adjusting the dye concentrations to achieve distinct labelling of live cells with calcein AM and of dead cells with EthD-1. The optimal concentrations are likely to vary depending on the cell type. In general, it is best to use the lowest dye concentration that gives sufficient signal. The following method can be used to determine optimal dye concentrations:

1. Remove the LIVE/DEAD ® assay reagent from the freezer and allow them to warm to room temperature
2. Prepare some samples of live cells as well as of dead cells in wells of the 96 well plates. Kill the cells using any preferred or validated method (e.g. 70% methanol for 30 mins)
3. Using samples of dead cells, select and EthD-1 concentration that stains the dead cells nuclei bright red without staining the cytoplasm (titrate the concentration from 0.1 to 10 µM EthD-1)
4. Using samples of dead cells, select a calcein AM concentration that does not give significant fluorescence in the dead cell cytoplasm (titrate from 0.1 to 10 µM calcein AM)
5. Using samples of live cells, check that calcein AM concentration selected in step 4.3.3.4 generates sufficient fluorescence signal in the live cells (titrate to a higher concentration if signal is too low).

4.3.4 Preparation of Calcein AM/EthD-1 solution

This protocol makes 10 mL of a 2 µM calcein AM and 4 µM EthD-1 solution. Cells with higher esterase activity require less calcein AM, with similar EthD-1 concentrations.

1. Remove the LIVE/DEAD ® assay reagent from the freezer and allow them to warm to room temperature
2. Add 20 uL of the supplied 2 mM EthD-1 stock solution (Component B) to 10 mL of sterile, tissue culture grade PBS, vortexing to ensure thorough mixing
3. Transfer 5 uL of the supplied 4 mM calcein AM stock solution (Component A0 to the 10 mL EthD-1 solution. Vortex thoroughly to mix
4. Note that aqueous solutions of calcein AM are susceptible to hydrolysis and so solutions should be used within one day

4.3.5 Hoechst 33342 staining/fixation solution

Add 2.5 ml of 37 % formaldehyde and 12.3 µL of Hoechst Dye to 22.5 ml of PBS and cover with foil to keep out the light. Incubate at 37 °C.

5 Procedure

5.1 General remarks

This procedure is based on guidelines set out in ISO 10993:5 for tests for *in vitro* cytotoxicity [7]. Time points and concentrations tested should be used that appropriate for the required outcome

5.2 Flow chart

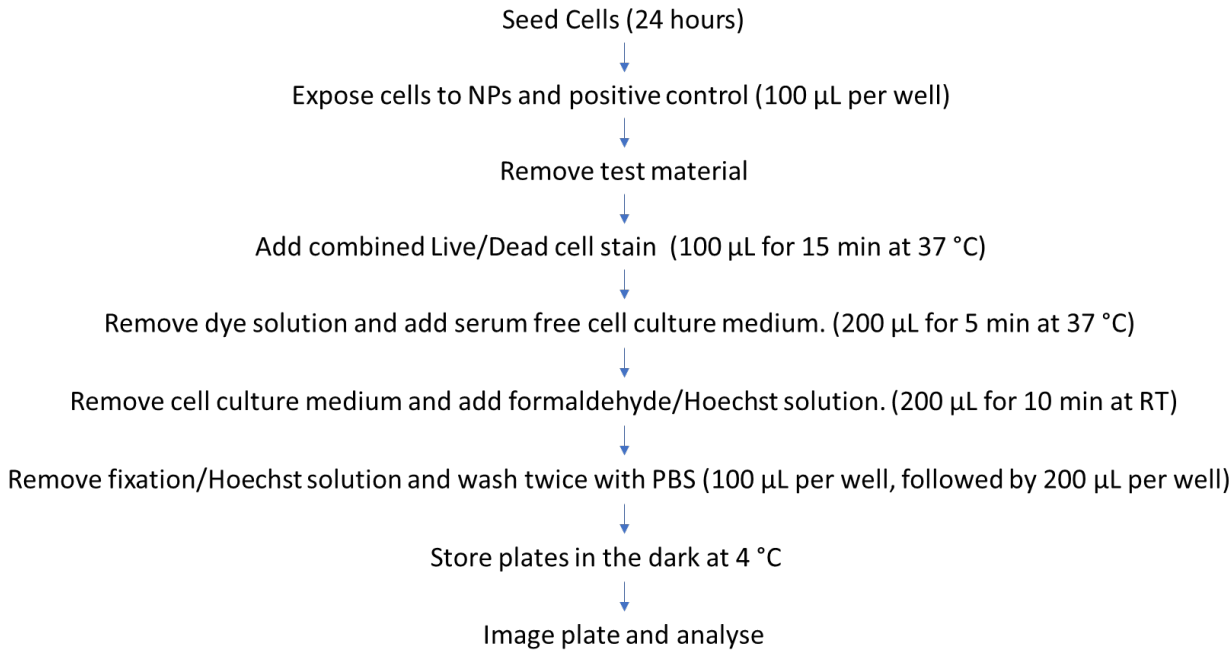

Figure 1: Brief outline of experimental workflow for HCS assay

5.3 Cell handling

The A431 cell line used in this study were obtained from the ATCC. Information relating to thawing, propagation, and creation of stocks is similar to that as described in EUNCL-GTA-001 and EUNCL-GTA-002. These stock ampoules serve as starting point for all experiments. After thawing of stock ampoules, cells are subcultured for 3 passages or more until enough cells are achieved (see Subcultivation). On the day of experiment, perform cell seeding as described in 5.4, continue with addition of samples the following day as described in 5.5.2.

5.3.1 Subcultivation

A431 cells are kept in a sub confluent state by routinely passaging twice or three times a week to seeding densities between 6 x 10^4 – 1 x 10^5 cells /cm^2.

Given volumes are for 75 cm^2 flask – proportionally reduce or increase amount of dissociation medium for culture vessel of other size.

1. Remove and discard culture medium.
2. Wash the cell layer twice by gently rinsing it with 10-15 mL preheated (37˚C) PBS.
3. Add 2.0-3.0 mL dissociation reagent (e.g TrypLE®), incubate at 37˚C for 5 minutes, and gently knock culture flasks to detach most of the cells.
4. Resuspend cells in complete cell culture medium to stop trypsination.
5. Transfer and dilute in new culture vessels.
6. Incubate the culture at 37˚C in a humidified atmosphere with 5% CO2 in a suitable incubator.

5.4 Cell Seeding

1. Harvest cells from prepared flasks, the cells should be cultivated for minimum 3 passages before use for experiment.
2. Count cell number using a coulter counter or haemocytometer.
3. Dilute cells to a density of 1 x 10^5 cells/mL in cell culture media.
4. Plate 200 µL cells/well per plate per time point (4, 24 hr, etc).
5. Incubate plates for 24 hr at 5% CO2, 37˚C and 95% humidity.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Test Material Conc 6			Negative control (both stains)			Test Material Conc 6 (NCS)			Cell BGC	
C		Test Material Conc 5			Negative control- Calcein AM			Test Material Conc 5 (NCS)			Cell BGC	
D		Test Material Conc 4			Negative control EthD-1			Test Material Conc 4 (NCS)			Cell BGC	
E		Test Material Conc 3			Positive control- Calcein AM			Test Material Conc 3 (NCS)			Spare	
F		Test Material Conc 2			Postive Control EthD-1			Test Material Conc 2 (NCS)			Spare	
G		Test Material Conc 1			Positive control (both stains)			Test Material Conc 1 (NCS)			Spare	
H												

Figure 2: Representative plate layout for Live/Dead assay. NCS: No cell control, BGC: background control (no stains). Spare wells are included for completeness, and can be used for internal quality control, such as exposure to valinomycin EC50 value. Outer wells are filled with PBS to minimise possibility of edge effects.

5.5 Assay procedure

5.5.1 Procedural Notes

1. Do not allow plate wells to become dry at any time during the protocol.
2. Perform all steps at room temperature unless otherwise indicated.
3. The protocol is optimized for A431 cells seeded in 96-well plates. Using conditions other than those indicated may necessitate optimization.
4. The DMSO concentration exposed to the cells at less than < 0.1%.
5. For best results, use low velocity fluid dispensing (≤ 4 ml/minute) with an automatic pipetting device.
6. For best results, aspirate with a weak vacuum. Toxic compounds may weaken cell adherence to the substrate, thus aspiration with moderate and strong vacuums will cause significant cell loss. To minimize cell loss, add and remove solutions at the edge of the well while tilting the plate without ever touching the bottom of the plate.
7. Please refer to the guide and instructions provided with the software and the HCS instrument for optimal implementation of the assay.

5.5.2 Procedure for 4, 24 hr, etc. exposure

1. Dilute test compounds to appropriate concentration in complete culture medium. Add 100 µL of the test compound or the positive control compound (120 µM valinomycin) to desired wells.
2. Incubate plates at 37°C in 5% CO_2 for 24 hours.
3. Remove test compound and add 100 µL of LIVE/DEAD staining solution per well.
4. Control wells should also be treated as shown below, and are used for calculations in subsequent section:
    a. Positive control cells treated with EthD-1 only (Dead cell intensity max)
    b. Positive control cells treated with calcein AM only (Dead cell intensity min)
    c. Negative control cells treated with EthD-1 only (Live cell intensity min)
    d. Negative control cells treated with calcein AM only (Live cell intensity max)
5. Incubate plates at 37°C in 5% CO_2 for 15 minutes.
6. Gently aspirate media and staining solution from each well and add 200 µL of warmed (37°C) Fixation/Hoechst solution.
7. Incubate plates for 10 minutes at room temperature.
8. Gently aspirate Fixation/Hoechst Solution and add 100 µL of PBS to each well.
9. Gently aspirate the wash and fill wells with 200 µL PBS.
10. Seal plate and evaluate on the Cytell, imaging 12 fields per well at random locations.
11. Analyse images using InCell Investigator
12. Store sealed plates in the dark at 4°C.
13. Experiment should be completed in triplicate

5.6 Qualitative image acquisition protocol using Cytell Imaging system

Note: The following procedures are specific to the InCell Investigator software (GE Healthcare). Other image analysis programs are also available, and as such methods of analysis may differ

The 96 well plate is placed into the stage of the HCS system and an automated imaging protocol created. The software allows for the selection of wells to be imaged, and the number of fields to be imaged per well. 10x magnification is used to obtain the optimal resolution while ensuring the number of cells imaged is maximized.

The focus settings and exposure times are selected automatically by the system. These can then be fine-tuned manually using the integrated controls. The user is required to check the focus and exposure times for each fluorescent channel separately to ensure optimal settings are used. It is good practice to verify that the exposure settings are optimizing for both the untreated and positive control wells to allow for appropriate image analysis following completion of the imaging. Acceptance criteria for image suitability are available from the various instrument manufacturers, with a comprehensive discussion of methods for detection out-of-focus and over saturated images being available within the scientific literature [10].

It should also be noted that experimental data generated, including images, must remain in compliance with FDA 21 CFR Part 11 regulations.

5.7 Quantitative image analysis using InCell Investigator software

Note: The following procedures are specific to the InCell Investigator software (GE Healthcare). Other image analysis programs are also available, and as such methods of analysis may differ.

Analysis of the images requires the classification of the of each wavelength used (each dye) as a specific designation. This enables the segmentation of the image based on the colour channels

- Hoechst (blue channel, Wave 1: nucleus) represents the nuclei and is used as the basis of the image segmentation, marking the location of each cell and enabling the quantification of cell counts. The change in nuclear area can also give information relating to the viability of the cell
- Calcein (green channel, Wave 1: nucleus): designation of this image channel as nucleus (as above) allows the software to count the number of cells stained with calcein, i.e. live cells
- EthD-1 (red channel, Wave 1: nucleus): designation of this image channel as nucleus allows the software to count the number of cells stained with EthD-1, i.e. dead cells.

Nuclei are segmented using top-hat segmentation, enabling the accurate selection of closely positioned nuclei. Minimum nuclear size was determined through selection of the smallest nuclei using the arrow tool, which estimates the size based on local background intensity, and returns the measured object's area in square microns.

To generate the required live/dead cell counts, each of the above need to analysed separately, yielding first: the total cell counts, secondly: the number of cells stained with calcein, and finally: the number of cells stained with EthD-1. Running the analyse in batch mode allows these 3 analyse protocols to be run in succession automatically, thereby increasing the throughput.

5.7.1 Method based on Fluorescent Intensity

Determination of the percentage of live and dead cells can also be done by measuring the fluorescent intensity of the calcein and EthD-1 stains, as demonstrated below:

- Hoechst (blue channel, Wave 1: nucleus) represents the nuclei and is used as the basis of the image segmentation, marking the location of each cell and enabling the quantification of cell counts. The change in nuclear area can also give information relating to the viability of the cell
- Calcein (green channel, Wave 2: cell) is designated as a "cell mask". Detection, and colocalisation of this dye within the cytoplasm is used to distinguish viable cells
- EthD-1 (red channel, Wave 3: organelle) is classified as an "organelle". The fluorescent intensity within the nucleus is used to distinguish dead cells.

Here, these results yield the fluorescent intensity of the live (calcein) and dead (EthD-1) containing cells.

The Z'-factor of the assay, as extensively reported for high content screening investigation, can also be calculated, where Z' =1 is an ideal assay, Z'=0 is suitable for binary yes/no responses, and Z' < 0 in unacceptable. The Z-factor is calculated as shown below:

$$Z'factor = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

Where μ_p and σ_p are the mean and standard deviation values of the positive control (or treated samples) and μ_n and σ_n are those of the negative control [10]. A Z' Factor > 0.5 is an determined as being an "excellent assay for screening" [11].

It should also be noted that experimental data generated, including images, must remain in compliance with FDA 21 CFR Part 11 regulations.

5.8 Calculations

All samples, positive, negative, and NP controls are run in triplicate, with each well will be subtracted from its respective cell-free blank to account for any fluorescence that may be due to the test material. The average of these three values should be used in the equations below. In the below equations, as specified by the assay developer, only the mean cell intensity refers to the fluorescent intensity of calcein within the cytoplasm, and organelle intensity refers to the fluorescent intensity of the EthD-1 within the nucleus.

$$\%\ Live\ Cells = \frac{Mean\ sample\ cell\ intensity\ fluoresence - (\text{Live cell intensity})_{min}}{(\text{Live cell intensity})_{max} - (\text{Live cell intensity})_{min}} \times 100$$

$$\%\ Dead\ Cells = \frac{Mean\ sample\ organelle\ intensity\ fluoresence - (\text{Dead cell intensity})_{min}}{(\text{Dead cell intensity})_{max} - (\text{Dead cell intensity})_{min}} \times 100$$

$$\%\ Nuclear\ Area = \frac{Mean\ sample\ nucelar\ area}{Mean\ nuclear\ area\ of\ untreated\ Control} \times 100$$

- Mean, SD and %CV should be calculated for each set of samples tested

- Results should be plotted and presented as mean ± standard error of the mean for repeated experiments.
- The sample EC50 value can be calculated by monitoring the increase in the % Dead cells (or EthD-1 fluorescent intensity) as the test material concentration increases. It is recommended to generate a nonlinear fit (based on at least three independent measurement for each datapoint) calculating the EC50 as the concentration of the test material that induces and increase in the fluorescence of EthD-1 halfway between the baseline and maximum using statistical software (e.g. GraphPad Prism).

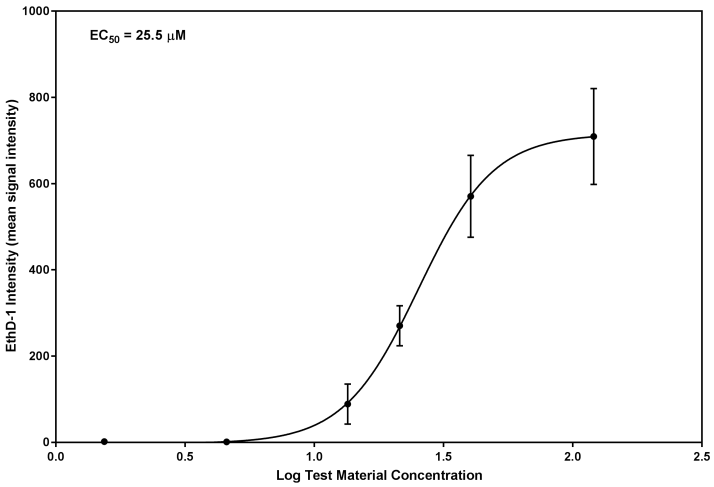

Figure 3: Representative dose-response curve and EC50 generated for cells treated with varying concentration of a test material. Cell viability percentage versus the concentration of test material can also be plotted.

6 Quality Control, Quality Assurance, Acceptance Criteria

- Assay wells exposed to a series of dilutions of valinomycin should be used as internal quality controls. The EC50 value can be used in monitoring the assay performance.
    - If the value lies outside the linear response of the EC50 curve then the assay has to be repeated.
- The nuclear area, and cell viability of the internal valinomycin positive control should be less than 50%
    - If not, the assay has to be repeated since either the cell did not respond as from cell technical specification or the assay did not work (see point above).
- Assays in which the internal controls are outside of these ranges should be discarded.
    - If the internal control estimation is out of the specification then the validity for the testing of the nanomaterials are in question. As above, the entire, assay, including positive controls, should be repeated.
- A 30 % reduction in cell viability is deemed to be a marker of cytotoxicity [7, 12].
    - The cell supplier technical specification should indicate or else please check literature for different cell viability cut offs.
- The sum of the % live cells and % dead cells must not be greater than 100 %.
    - If this sum is greater than 100 then possible explanations has to be found:

- Exceeding cell fragmentation is present in the well, please revise your cell seeding density or check for exceeding nanomaterial aggregation (not quantified during physico-chemical characterisation). For the latter, nanoparticle aggregates could be counted as cells or organelles depending on the size of aggregates and sensitivity of the analytical thresholds.

7 Health and Safety Warnings, Cautions and Waste Treatment

Please refer to available H.S.E information for any nano formulations evaluated in the assays. Note that some of the listed reagents are hazardous and must be handled with precaution. Please refer to safety data sheets for each reagent, wear protective equipment, and dispose waste according to local regulations.

8 Abbreviations

NP: Nanoparticle

RT: Room temperature

PBS: Phosphate buffered saline

SD: standard deviation

% CV: Percentage coefficient of variation

FBS: fetal bovine serum

EC: Effective concentration

9 Related Documents

Table 1:

Document ID	Document Title	URL
EUNCL-GTA-001	LLC-PK1 Kidney Cytotoxicity Assay	http://www.euncl.eu/about-us/assay-cascade/PDFs/Toxicology/EUNCL_GTA_001.pdf?m=1468937871&
EUNCL-GTA-002	HepG2 hepatocarcinoma cytotoxicity	http://www.euncl.eu/about-us/assay-cascade/PDFs/Toxicology/EUNCL_GTA_002.pdf?m=1468937871&
EUNCL-PCC-21	Measuring NP aggregation propensities with DLS	http://www.euncl.eu/about-us/assay-cascade/PDFs/PCC/EUNCL-PCC-021.pdf?m=1468937870&
EUNCL-PCC-22	Size, Size Distribution	http://www.euncl.eu/about-us/assay-cascade/PDFs/PCC/EUNCL-PCC-022.pdf?m=1468937868&

EUNCL-PCC-23	Particle Tracking Analysis	http://www.euncl.eu/about-us/assay-cascade/PDFs/PCC/EUNCL_PCC_023.pdf?m=1526712237&
EUNCL-PCC-35	Drug release in complex media	

10 References

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