Project: EUNCL

# Hep G2 Hepatocarcinoma Cytotoxicity Assay

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# Table of Content

# 1 Introduction

This protocol describes the cytotoxicity testing of nanoparticle formulations in human hepatocarcinoma cells (Hep G2), as part of the in vitro EU-NCL preclinical characterization cascade. The protocol utilizes two methods for estimation of cytotoxicity, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) release [1-2].

# 2 Principle of the Method

1. MTT Assay

   MTT is a yellow, water-soluble tetrazolium dye that is reduced by live cells to a water-insoluble, purple formazan. The amount of formazan can be determined by solubilizing it in DMSO and measuring it spectrophotometrically. Comparisons between the spectra of treated and untreated cells can give a relative estimation of cytotoxicity [3].

2. LDH Assay

   LDH is a cytoplasmic enzyme that is released into the cytoplasm upon cell lysis. The LDH assay, therefore, is a measure of membrane integrity. The basis of the LDH assay: a) LDH oxidizes lactate to pyruvate, b) pyruvate reacts with the tetrazolium salt INT to form formazan, and c) the water-soluble formazan dye is detected spectrophotometrically [4, 5].

# 3 Applicability and Limitations (Scope)

The SOP describes in vitro methods for evaluation of cytotoxicity of nanoparticle formulations using the MTT (metabolic activity) and LDH-assays (membrane activity) for Hep G2 cells. For both assays, potential interferences of the nano formulations on the assay readout should be taken into account during design of experiments and interpretation of data. Existing available literature should therefore be reviewed during design of experiments in order to evaluate the applicability of the assays.
The assays provide basic information of the cytotoxicity of the nanoparticle formulations. The assays does not provide information of sub lethal cellular effects or detailed mechanistic information of the toxic effect experienced by the cells.

# 4 Related Documents

**Table 1:**

| **Document ID** | **Document Title** |
|---|---|
| EUNCL-GTA-001 | *LLC-PK1 Kidney Cytotoxicity Assay* |

# 5 Equipment and Reagents

## 5.1 Equipment and Culture ware

1. Costar 96 well flat bottom cell culture plates (Nunc, 3598)
2. Greiner 96 well flat bottom polystyrene plates (Greiner, 655163)
3. Half area 96 well plate (Costar, 3695)
4. Falcon® 40 µm Cell strainer (Falcon, 352340)
5. Plate reader (Safire – Tecan or equivalent)
6. Orbital plate shaker
7. Incubator, 37˚C with 5% $CO_2$ and 95% humidity

## 5.2 Reagents

1. Hep G2 (human hepatocarcinoma)(LGC Standards, HB-8065, LOT 62591368)
2. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma, M5655)
3. Dimethyl sulfoxide (Sigma, D5879)
4. Dimethyl sulfoxide Hybri-Max ™ (Sigma, D2650), for cryopreservation of cells
5. Glycine (Sigma, G7126 or G7403)
6. Sodium chloride (Sigma, S7653)
7. 10% Triton-X-100 (Sigma, 93443)
8. Digitonin (Sigma, D141-500MG)
9. RPMI 1640 (GIBCO, 31870-025)
10. Fetal bovine serum (Sigma, F7524, LOT 025M3302)
11. L-Glutamine (Sigma, G7513)
12. Penicillin-Streptomycin (Gibco, 15140122)
13. Dissociation reagent e.g Trypsin/EDTA or TrypLE ( *e.g* Gibco, 12605-010)
14. Biovision LDH-cytotoxicity assay kit (Biovision, K311-400)
15. PBS (Difco, BR0014)

## 5.3 Reagent Preparation

Cell culture medium

The complete medium for Hep G2 is prepared by adding 2 mM L-Glutamine, 10% FBS and 100 U/mL Penicillin-Streptomycin to RPMI 1640 medium. The medium is stable for up to 4 weeks at 4˚C.

Positive Controls

Prepare all positive controls fresh for each round of assay, same control can be used for all reading point (0, 4, 24 and 48 hr)

1. 0.1% Triton-X-100:

   Prepare a 20X stock (2%) of Triton-X-100 by adding 2 mL of Triton-X-100 to 8 mL of RPMI 1640 Cell Culture Media (with 10% FBS). Sterilize by filtering through a 0.2 μm filter.
2. Digitonin stock solution is made by solubilizing digitonin in DMSO to a final concentration at 20 mg/mL. Mix until a clear and homogeneous solution is obtained. The stock solution can be aliquoted and frozen at -20˚C. Prepare a 2X digitonin solution for internal plate control by diluting digitonin stock (20 mg/mL) in RPMI 1640 Cell Culture Media to a final concentration of 60 μg/mL.

MTT assay

1. MTT solution:

   Prepare 5 mg/mL MTT in PBS. Sterilize by filtering using a 0.2 μm filter. The solution can be stored for up to one month at 4˚C in the dark, or freeze at -20 ˚C.
2. Glycine Buffer:

   Prepare 0.1 M glycine (MW 75.07) with 0.1 M NaCl (MW 58.44) in MQ water, pH 10.5.

   Sterilize by filtering using a 0.2 μm filter. The solution can be stored at room temperature for up to 4 weeks.

LDH assay

1. Reconstitute catalyst in 1 mL $dH_2O$ for 10 min with occasional vortexing. The solution is stable for 2 weeks at 4˚C.
2. Reaction mixture (for one 96-well plate): Add 125 μL of reconstituted catalyst solution to 5.63 mL of dye solution. Once thawed, the kit components are stable for 2 weeks stored at 4˚C. Reconstituted catalyst solution should be added to the dye solution immediately before use.

# 6 Procedure

## 6.1 Flow chart

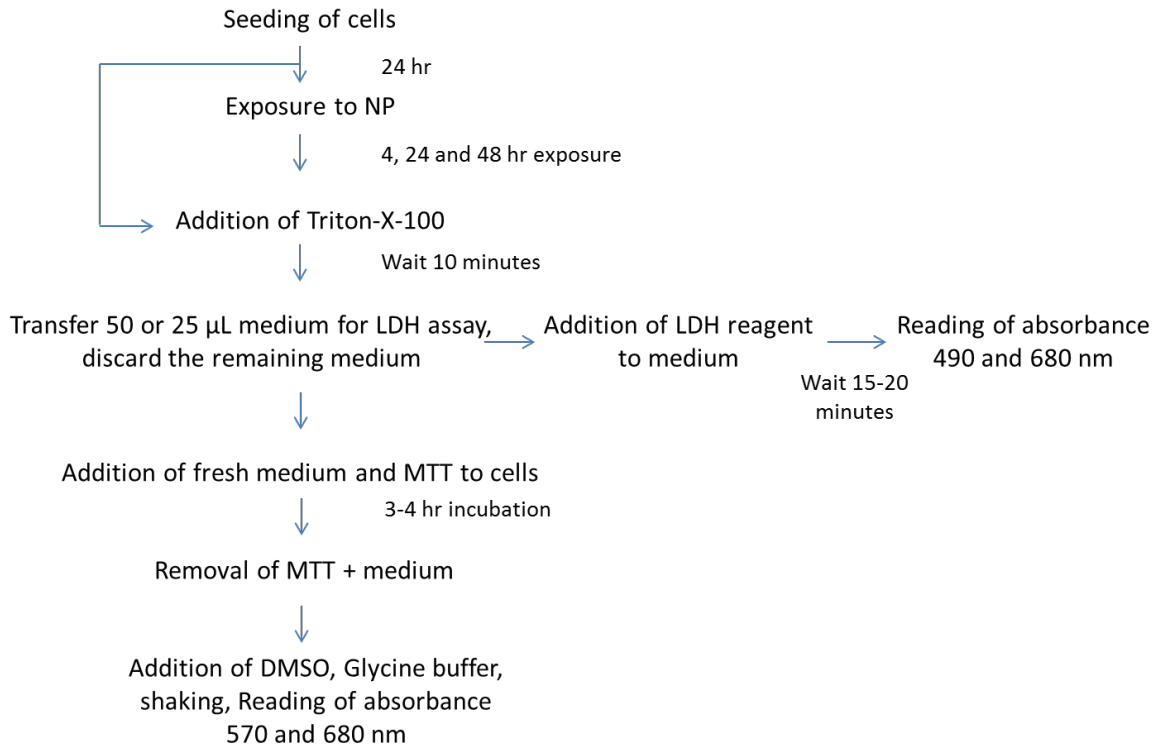


Figure 1: Brief outline of the workflow.

## 6.2 Cell handling

The Hep G2 cell line used by EU-NCL is obtained from LGC Standards, see Annex for detailed information about thawing and propagation of a set of stock ampoules. 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 amount of cells are achieved (see Subcultivation). On the day of experiment, perform cell seeding as described in 6.4, continue with addition of samples the following day as described in 6.5.

**Subcultivation**

Hep G2 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) Dulbecco's phosphate-buffered saline without calcium and magnesium.
3. Add 2.0-3.0 mL dissociation reagent (e.g Trypsin/EDTA or TrypLE®), incubate at 37˚C for 5 minutes, and gently knock culture flasks to detach most of the cells.
4. Resuspend cells in medium containing FBS to stop trypsination.
5. Transfer and dilute in new culture vessels.
6. Incubate the culture at 37˚C in a humidified atmosphere with 5% $CO_2$ in a suitable incubator.

## 6.3 Cell seeding

Cell preparation (or as recommended by supplier, see also 6.3.)

1. Harvest cells from prepared flasks, the cells should be cultivated for minimum 3 passages before use for experiment. **Note**: Limit to 20 passages from vial from cell bank at LGC Standards (Figure 3).
2. Hep G2 cells detach as small clusters of cells during trypsination. Large clusters of cells can contribute to uneven cell distribution in well plate resulting in higher assay coefficient of variations. A more homogeneous cell suspension can be achieved by filtering the cell suspension through a cell strainer (*e.g.* Falcon® 40 µm Cell Strainer, 352340).
3. Count cell number using a coulter counter or hemocytometer.
4. Dilute cells to a density of 5 x $10^5$ cells/mL in RPMI1640 (10% FBS) cell culture media.
5. Plate 100 µL cells/well as per plate format (Annex) for four 96-well plates (time zero, 4, 24, and 48 hr sample exposure). See plate design.
6. Incubate plates for 24 hr at 5% $CO_2$, 37˚C and 95% humidity. Cells are grown to approximately 80% confluence (Figure 2).

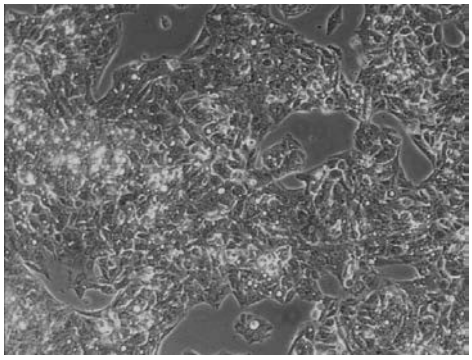

**Figure 2. Hep G2 Cell Culture.** Image was taken with a phase contrast microscope at 225X magnification. Hep G2 cells are approximately 80% confluent at this stage.

## 6.4 Assay procedure

The cells are prepared in 96-well plates as described in section 6.4. See also plate design in Annex. The format indicates no cells in rows A and H as they serve as particle blanks to be subtracted from cell treatment wells. Each plate accommodates two samples (Rows A–D and E-H). Each nanoparticle is tested at nine dilutions. Column 11 receives the Triton X-100 at the end of the relevant time point and column 12 receives the positive control Digitonin.

**Time Zero Plate (MTT Assay)**

1. Remove time zero plate from the incubator and add 100 µL fresh culture media to all wells. Then add 10 µL of 20X Triton-X-100 to the positive control wells in column 11 (see plate format in Annex) for a final concentration of 0.1% Triton-X-100 (see Section 5.3). Let the plate set for 10 minutes at room temperature.
2. Remove 50 µL of media from each well and transfer it to another plate (Greiner, 655163), maintaining plate format. Transfer 25 µL if half area plates are used for LDH assay (Costar, 3695). Use this plate immediately for the LDH assay (see Section below).
3. Remove remaining media from original plate and discard.
4. Add 200 µL fresh media to all wells.
5. Add 50 µL MTT (see Section 5.3) to all wells.
6. Cover with aluminum foil and incubate at 37˚C for 3-4 hrs.
7. Aspirate and discard media.
8. Add 200 µL DMSO to all wells to solubilize the MTT formazan crystals.
9. Add 25 µL glycine buffer (see Section 5.3) to all wells. Cover with aluminum foil and place on shaker to mix for 10 minutes in room temperature.

10. Read absorbance at 570 nm on plate reader using a reference wavelength of 680 nm.

**Test Samples and Positive Control Addition**

All samples are run in triplicates with 9 dilutions each (see Plate Map).

1. The highest concentration of nanoparticle tested should be at the limit of solubility or determined by the potency of the test material.
2. Dilute the test compound in media, making a total of nine 1:4 dilutions at 2X of the desired final concentration. Different dilution series (i.e. 1:2, 1:10) can be done depending on test material potency and availability.
3. Add 100 µL of each sample dilution and positive control to 4, 24 and 48 hr exposure plates as per the plate format (Annex), and place in 37˚C incubator with 5% $CO_2$ and 95% humidity for indicated time. Note that Triton X-100 control is added later at the end of the exposure time, so that these wells receive only 100µL media at this point (See step 1 in MTT assay). Alternatively, sample and positive control can be made at desired final concentration. Media is aspirated from the wells on the test plates, and sample and positive control are added at 200 µL per well as per the plate format (Annex).

**Test Plates, 4, 24 and 48 hr Exposures (MTT Assay)**

1. At the end of each exposure time point, remove plate from incubator and add 10 µL of 20X Triton-X-100 to positive control wells (see plate format in Annex) for a final concentration of 0.1% Triton-X-100 (see Section 5.3). Let the plate set for 10 minutes at room temperature.
2. Remove 50 µL of media from each well and transfer it to another plate (Greiner, 655163), maintaining plate format. Transfer 25 µL if half area plates are used for LDH assay (Costar, 3695). Use this plate immediately for the LDH assay (see Section below).
3. Remove remaining media from original plate and discard.
4. Add 200 µL fresh media to all wells.
5. Add 50 µL MTT to all wells.
6. Cover with aluminum foil and incubate for 37˚C for 3-4 hr.
7. Aspirate and discard media.
8. Add 200 µL of DMSO to each well.
9. Add 25 µL of glycine buffer to each well. Cover with aluminum foil and place on shaker to mix for 10 minutes in room temperature.

10. Read absorbance at 570 nm on plate reader using a reference wavelength of 680 nm.

**Test plates, 0, 4, 24 and 48 hr Exposures (LDH Assay)**

*(Adapted from Biovision LDH Cytotoxicity Assay Kit, K311-400)*

1. Add 50 µL of the Reaction Mixture (see Section 5.3) to each well of transfer plate. Alternatively add 25 µL of the Reaction Mixture if half area plates are used. Shake plate on an orbital shaker briefly to mix samples.
2. Incubate at room temperature for up to 20 minutes in the dark.
3. Read the plate on a plate reader at 490 nm using a reference wavelength of 680 nm.

## 6.5 Calculations

All samples, positive, negative, and media controls are run in triplicate (e.g., rows B-D or E-G). Each well will be subtracted from its respective cell-free blank (e.g., B2-A2 or G3-H3) in the following calculations. The average of these three values should be used in the equations below for the positive and negative controls (e.g., [(B10-A10) + (C10-A10) + (D10-A10)]/3 = mean media control absorbance for sample 1, or [(B11-A11) + (C11-A11) + (D11-A11)]/3 = mean Triton-X-100 positive control absorbance for sample 1. Sample 2 uses the blank row H for subtractions.).

1. MTT Assay

$$\%\ \text{Cell Viability} = \frac{\text{sample absorbance} - \text{cell free sample blank}}{\text{mean media control absorbance}} \times 100$$

2. LDH Assay

$$\%\ \text{Total LDH Leakage} = \frac{(\text{sample absorbance} - \text{cell free sample blank}) - \text{mean media control absorbance}}{\text{mean TritonX positive control absorbance} - \text{mean media control absorbance}} \times 100$$

3. Mean, SD and %CV should also be calculated for each positive control, negative control and unknown sample.

# 7 Quality Control, Quality Assurance, Acceptance Criteria

**Acceptance criteria**

1. A set of assay wells exposed to a dilution series (9 dilutions, 3 parallel wells per concentration) of the positive control digitonin for 24 hours is included in each assay batch, starting at 140 µg/ml. The EC50 value of the digitonin positive control is used for monitoring of assay performance. Plot values for each assay batch.

Assays batches in which the EC50 value of the digitonin positive control are outside of 3 standard deviations should be discarded.

2. The viability in wells with the plate internal digitionin control should be less than 50 %.
3. The replicate coefficient of variations of the positive controls and of the samples should be within 50%.
4. The assay is acceptable if criteria 1 and 3 are met. Otherwise, the assay should be repeated until acceptance criteria are met.
5. If the acceptance criteria are met, determine the highest concentration of the nanoparticulate material that does not interfere with the assay system indicated in rows A and H.
6. The concentration–response curves for the 48 hr MTT and LDH data should be classified as having complete (two observed asymptotes) or incomplete (second asymptote not obtained) curves, single point activity (activity at the highest concentration only), or no activity. For all complete 48 hr concentration–response curves, a nonlinear fit of the sigmoidal Hill equation should be performed, and an estimate of potency ($EC_{50}$ - Calculated using Graphpad Prism), efficacy ($E_{max}$), minimum response ($E_0$), and Hill slope ($\gamma$) from the Hill equation (below) fit should be reported. Any excluded points (excluded by outlier analysis) should also be reported.

$$E = E_0 + [ (E_{max} – E_0)C\gamma / EC\gamma_{50} \cdot C\gamma]$$

# 8 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.

# 9 Abbreviations

| | |
|---|---|
| CV | coefficient of variation |
| DMSO | dimethyl sulfoxide |
| FBS | fetal bovine serum |
| Hep G2 | Human hepatocarcinoma cells |
| INT | 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride |

| | |
|---|---|
| LDH | lactate dehydrogenase |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MW | molecular weight |
| NaCl | Sodium Chloride |
| PBS | phosphate buffered saline |
| SD | standard deviation |

# 10 References


1. ISO 10993-5, Biological evaluation of medical devices: Part 5, Tests for *in vitro* cytotoxicity.
2. F1903 – 98, Standard Practice for Testing for Biological Responses to Particles in vitro.
3. Alley, et al. (1988) Cancer Res. 48:589-601.
4. Decker, T. & Lohmann-Matthes, M.L. (1988) J. Immunol Methods 15:61-69.
5. Korzeniewski, C. & Callewaert, D.M. (1983) J. Immunol Methods 64:313-320.
6. Freshney, R. I. (2010). Culture of animal cells: a manual of basic technique and specialized applications. (pp. 87-88, 193) Hoboken, N.J., Wiley-Blackwell
7. NCL Method GTA-2, Version 1.2, November 2015
   LGC Standards

# 11 Annex

## 11.1 Cell handling and storage

### 11.1.1 Cell origin and provenance

Hep G2 Human hepatocarcinoma cells are used as functional models in the hepatocarcinoma cytotoxicity assay and must be well characterized and validated. Validated cell lines are obtained from LGC Standards, upon arrival these should be propagated and cryopreserved at a low passage number. In order to minimize variations between EU-NCL labs due to cell cultivation, cell lines used in the EU-NCL-GTA02 assay should not be propagated above 20 passages from original vials from LGC Standards (See figure 3) [6, 7].

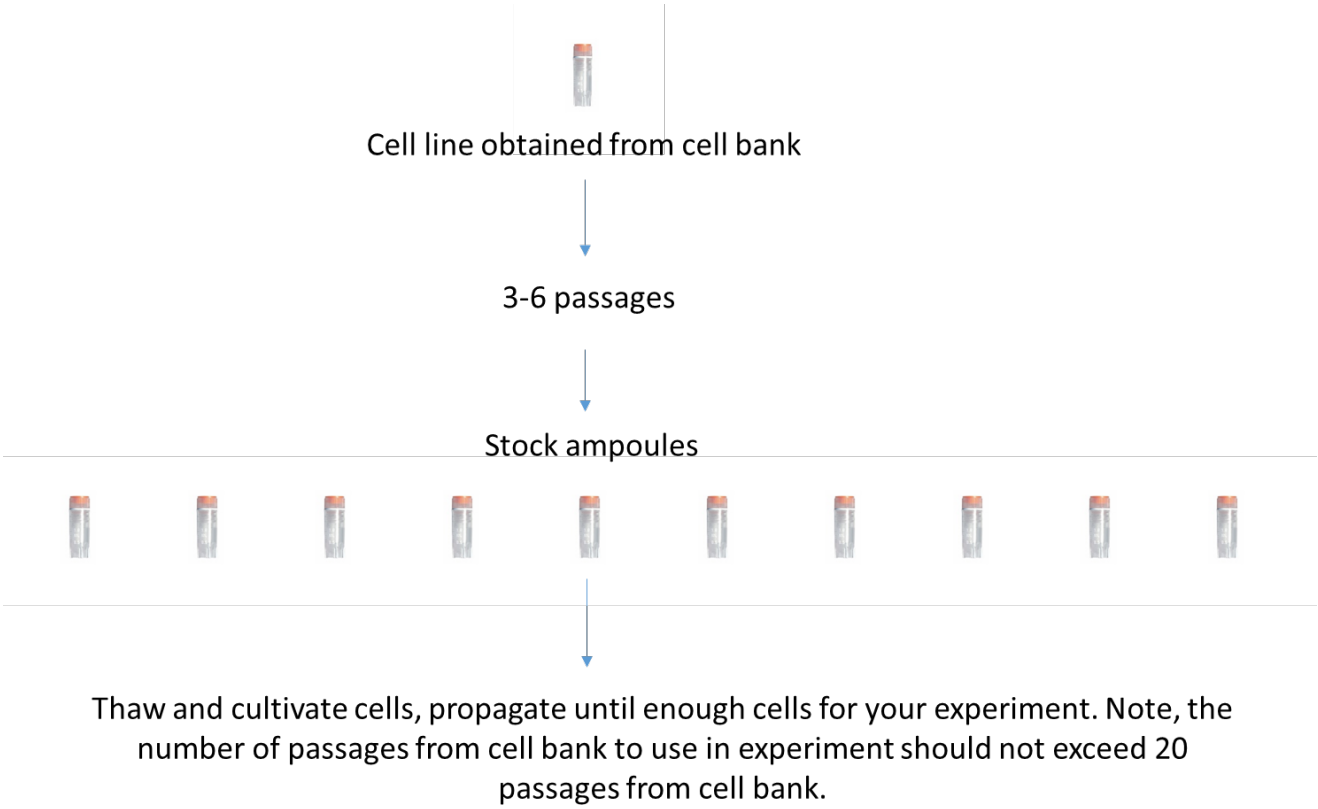


Figure 3: Propagation of stock ampoules from LGC Standards.

### 11.1.2 Thawing Procedure

Thawing of frozen ampoule was done according to instructions from cell bank [8].

1. Thaw the vial by gentle agitation in a 37˚C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid, approximately 1-2 minutes.

2. Remove the vial from the water bath as soon as the content is thawed, and decontaminate by spraying with 70% ethanol. Place the vial into biosafety cabinet and work under strict aseptic conditions.
3. Transfer the vial content to a sterile 15 mL conical centrifuge tube containing 9.0 mL preheated complete culture medium, and spin at 140 x G for 5 minutes. Discard supernatant.
4. Resuspend the cell pellet gently in 2-3 mL of the complete cell medium (this small volume makes it easier to achieve a homogeneous suspension). Gently dilute the cell suspension to the recommended final volume in culture vessel.
5. Incubate the culture at 37˚C in a humidified atmosphere with 5% $CO_2$ in a suitable incubator.

### 11.1.3 Propagation of stock ampoules

Stock ampoules were propagated by thawing vial from cell bank according to thawing procedure (11.2.2). For Hep G2 the vial from cell bank the vial from cell bank was thawed, old medium containing freeze agent was removed by diluting in fresh media before spinning down the cells. Cell pellet was resuspended in fresh media and transferred to a T25 culture vessel. The cell line became 70-80% confluent after three days, and was passaged three times before freezing of stock ampoules.

P1: Thaw to 10 mL in T25 (3 days)

P2: Split and seed to cell density between $6x10^4$ – $1x10^5$ cells/$cm^2$ (3-5 days)

P3: Split and seed to cell density between $6x10^4$ – $1x10^5$ cells/$cm^2$ (3-5 days)

P4: Split and seed to cell density between $6x10^4$ – $1x10^5$ cells/$cm^2$ (3-5 days), freeze stock ampoules when cells become 70-80% confluent.

**Freezing Procedure**

Always start by preparing all reagents, vials and equipment needed for freezing cells. After the cells are out of the incubator, minimal time should be used for such preparation steps.

1. Harvest cells according to the description for subculturing. After dissociation of cells, dilute them in fresh culture medium.
2. Measure cell density and viability by taking out an aliquot of the cell suspension. Calculate the necessary volume of freezing media (See Freezing Media underneath).
3. Transfer cell suspension to centrifuge tubes, and spin down cells at 140 x G for 5-7 minutes. Avoid very dense cell suspensions as these might be difficult to spin down.
4. Remove all medium.
5. Gently resuspend cells to wanted concentration in freezing medium. Freeze Hep G2 cells at 2 x $10^6$ – 5 x $10^6$ cells/mL in cryogenic vials, aliquot 1 mL in each vial. **Note:** For Hep G2 it can be difficult to achieve a homogeneous dispersion, resuspending cells in two steps can improve the viability through cryopreservation. Resuspend cells to double concentration in cell media (e.g if cells should be resuspended in 10 mL of freezing media, then resuspend them in 5 mL culture medium with FBS). Work with cell suspension until satisfactory homogeneous suspension is obtained. Gently add the 2xfreezing medium (meaning with 2xconcentration of DMSO or similar cryoprotectant), pipette to mix, and transfer 1 mL suspension to each premarked cryovial.

6. Transfer cryovials to freezing containers ( CoolCell®), and place the container in -80˚C freezer, leave for at least four hours before transferring to storage in liquid nitrogen.

**Freezing Media**

To prepare Hep G2 freezing media use complete supplemented medium and add sterile DMSO to a final volume of 5% (v/v). To make prepare 2xfreezing medium, use complete supplemented medium and add sterile DMSO to a final volume of 10% (v/v).

### 11.1.4 Plate map with positioning of samples and controls in assay plates

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Media TS1, D9 | Media TS1, D8 | Media TS1, D7 | Media TS1, D6 | Media TS1, D5 | Media TS1, D4 | Media TS1, D3 | Media TS1, D2 | Media TS1, D1 | Media | Media Triton-X 0.1% | Media DIGI 30 µg/mL |
| B | Cells TS1, D9 | Cells TS1, D8 | Cells TS1, D7 | Cells TS1, D6 | Cells TS1, D5 | Cells TS1, D4 | Cells TS1, D3 | Cells TS1, D2 | Cells TS1, D1 | Cells Media | Cells Triton-X 0.1% | Cells DIGI 30 µg/mL |
| C | Cells TS1, D9 | Cells TS1, D8 | Cells TS1, D7 | Cells TS1, D6 | Cells TS1, D5 | Cells TS1, D4 | Cells TS1, D3 | Cells TS1, D2 | Cells TS1, D1 | Cells Media | Cells Triton-X 0.1% | Cells DIGI 30 µg/mL |
| D | Cells TS1, D9 | Cells TS1, D8 | Cells TS1, D7 | Cells TS1, D6 | Cells TS1, D5 | Cells TS1, D4 | Cells TS1, D3 | Cells TS1, D2 | Cells TS1, D1 | Cells Media | Cells Triton-X 0.1% | Cells DIGI 30 µg/mL |
| E | Cells TS2, D9 | Cells TS2, D8 | Cells TS2, D7 | Cells TS2, D6 | Cells TS2, D5 | Cells TS2, D4 | Cells TS2, D3 | Cells TS2, D2 | Cells TS2, D1 | Cells Media | Cells Triton-X 0.1% | Cells DIGI 30 µg/mL |
| F | Cells TS2, D9 | Cells TS2, D8 | Cells TS2, D7 | Cells TS2, D6 | Cells TS2, D5 | Cells TS2, D4 | Cells TS2, D3 | Cells TS2, D2 | Cells TS2, D1 | Cells Media | Cells Triton-X 0.1% | Cells DIGI 30 µg/mL |
| G | Cells TS2, D9 | Cells TS2, D8 | Cells TS2, D7 | Cells TS2, D6 | Cells TS2, D5 | Cells TS2, D4 | Cells TS2, D3 | Cells TS2, D2 | Cells TS2, D1 | Cells Media | Cells Triton-X 0.1% | Cells DIGI 30 µg/mL |
| H | Media TS2, D9 | Media TS2, D8 | Media TS2, D7 | Media TS2, D6 | Media TS2, D5 | Media TS2, D4 | Media TS2, D3 | Media TS2, D2 | Media TS2, D1 | Media | Media Triton 0.1% | Media DIGI 30 µg/mL |

Abbreviations:
TSX: Test sample number X
DX: Dilution number X
DIGI: Digitonin plate internal control sample