PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

3D *In Vitro* HepG2 Spheroid Model

This is a SOP recommended for external use by PATROLS

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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WP4 Partners

Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	30/01/2019	Initial Document	Samantha Llewellyn
1.1	14/03/2019	Amended SOP to include comments from WP4	Samantha Llewellyn
1.2	12/04/2019	Final SOP uploaded to PATROLS Server	Samantha Llewellyn
1.3	12/06/2020	Update to Section 5.1, 5.8 & 7.	Samantha Llewellyn
1.4	08/04/2021	Update to SOP Template	Samantha Llewellyn

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1 Introduction:

DOMAIN: Advanced 3D *In Vitro* Model Systems for Hazard Assessment

Due to the rapid development and implementation of a diverse array of engineered nanomaterials (ENM), it is inevitable that humans will be exposed to ENM on a regular basis. There are currently many international activities in place to assess the potential toxicity of these materials over acute, long-term, and repeated low-dose exposure scenarios. Hepatic toxicology is key when considering ENM exposure, as it widely known that the liver is a major site of ENM accumulation post exposure (Gieser and Kreyling, 2010; Modrzynska, J., 2018). Based upon the accepted understanding that 2D hepatocyte models do not accurately mimic the complexities of intricate multi-cellular interactions and metabolic activity observed *in vivo*, a greater focus into developing robust and physiologically relevant *in vitro* 3D liver models for *in vivo* substitute technologies has been established (Breslin and O'Driscol, 2013; Shah *et al.,* 2018). Current 3D *in vitro* hepatic models consisting of mono-cultures (hepatocytes only) or co-cultures (hepatocytes with non-paranchymal cells) range from microtissues or spheroids in ultra-low adhesion plates, hanging-drop, embedding in matrices and/or scaffolds and microfluidic cell culture platforms; all of which are deemed effective *in vitro* models for hepatic toxicity assessment. However, the majority of these model systems are high maintenance, require specialized equipment and are expensive. Furthermore, these models are often static (*i.e*. non-dividing cell models) which limits genotoxicity assessment as it deems them unsuitable for use with the cytokinesis-block micronucleus assay. In line with the 3Rs principles to replace, reduce and refine animal experimentation, this SOP has been established to develop an advanced 3D *in vitro* hepatic model capable of reliably evaluating hazard endpoints following acute, longer term and repeated ENM exposures in a routine and easily accessible manner.

1.1 Scope and limits of the protocol

This SOP was established with the intention to be used for developing advanced 3D hepatic cultures *in vitro* which can provide a more physiologically relevant assessment of the hazards associated with ENM exposures over both an acute or longer term, repeated dose regime. This model system enables multiple toxicological endpoints to be analysed (*e.g.* liver function tests, pro-inflammatory markers, viability and genotoxicity).

Limitations of the Protocol:

1. Longevity of the Culture System

   The 3D HepG2 spheroid model system is only viable for 14 days in culture (to date) as continued proliferation results in the formation of a necrotic core in the centre of the spheroid. As a result, longer term or repeated exposures of up to 7-10 days can be conducted on this 3D model system as the viability of cells within the spheroid during this period remain above 80%.

1.2 Validation State of protocol

Level of advancement towards standardization	**Level reached** (please mark only one with "X")
Stage 1: Internal laboratory method under development	
Stage 2: Validated internal laboratory method	
Stage 3: Interlaboratory tested method	
Stage 4: Method validated by Round Robin testing	X
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	N
Has the method been submitted for standardisation (to OECD, CEN, ISO,…) in its own right or as part of another standardisation project?	N
Is the method included in an existing standard (or ongoing standardisation work)	N
If yes, specify	[standard reference number, eg. EN 17199-4]

2 Terms and Definitions:

Agglomerate

Collection of weakly or medium strongly bound *particles* where the resulting external surface area is similar to the sum of the surface areas of the individual components.

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.2]

Aggregate

Particle comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components.

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent or ionic bonds, or those resulting from sintering or complex physical entanglement, or otherwise combined former primary particles.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.3, modified — Note 1 adapted.]

Nanomaterial

Material with any external dimension in the *nanoscale* or having internal structure or surface structure in the nanoscale.

Note 1 to entry: This generic term is inclusive of *nano-object* and *nanostructured material*.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.4]

Engineered nanomaterial

Nanomaterial designed for specific purpose or function

[SOURCE: ISO/TS 80004-1: 2016, definition 2.8]

Spheroid

A spherical, heterogeneous cluster of proliferating, quiescent, and necrotic cells in culture that retain three-dimensional architecture and tissue-specific functions

[SOURCE: Spheroid Culture in Cancer Research (Bjerkvig, 1992)]

Substance

Single chemical element or compound, or a complex structure of compounds.

[SOURCE: ISO 10993-9:2009, definition 3.6]

Teratogen

A chemical agent that can disturb or cause a malformation in the development of an embryo or fetus. Teratogens may cause a birth defect in the child or cause termination of the pregnancy.

[SOURCE: OSHA SOP 3.21, definitions 3.21.1]

3 Abbreviations:

ATCC – American Type Culture Collection

DMEM – Dubecco's Modified Eagle Medium

DMSO - Dimethyl sulfoxide

EDTA – Ethylenediaminetetraacetic Acid

ENM – Engineered Nanomaterial

FBS – Foetal Bovine Serum

IVIVE – *In Vitro In Vivo* Extrapolation

PBS – Phosphate Buffered Saline

Pen/Strep – Penicillin/Streptomyocin

PPE – Personal Protective Equipment

4 Principle of the Method:

To establish a physiologically relevant 3D hepatocyte cell line based *in vitro* model system for both acute orlong-term, repeated ENM hazard assessment.

The protocol can be broken down into 5 key stages:

1. Culturing Cryopreserved HepG2 Cells
2. Sub-culturing HepG2 Cells
3. HepG2 Spheroid Preparation
4. HepG2 Spheroid Transfer from Hanging Drop to Agarose Suspension
5. HepG2 Spheroid Harvesting

5 Description of the Method:

5.1 Biological setting & test system used:

This SOP should be carried out under sterile conditions and in a Class 2 Laminar Tissue Culture Hood.

- The advanced *in vitro* 3D model utilised is a Human Caucasian Hepatocellular Carcinoma derived epithelial cell line, HepG2 (ATCC®-HB-8065™, American Type Culture Collection) spheroid model, established and described in PATROLS Deliverable 4.1 SOP '3D *In Vitro* HepG2 Spheroid Model'. Cryopreserved HepG2 cells were frozen down in 10% DMSO and 90% FBS.

- For further information regarding the selected cell line, please see https://www.atcc.org/products/all/HB-8065.aspx

5.2 Chemicals and reagents used:

Agarose (CAS# 9012-36-6) (A9539-50G, Sigma Aldrich®, UK)

0.05% Trypsin-EDTA (5300-054, GIBCO®, Paisley, UK),

FBS (10270-106, GIBCO®, Paisley, UK)

DMEM with 4.5g/L D-Glucose, L-Glutamine (41965-039, GIBCO®, Paisley, UK).

DMEM, phenol-red free with 4.5g/L D-Glucose, L-Glutamine with Hepes (21063-029, GIBCO®, Paisley, UK).

Erythrosin B (CAS# 15905-32-5) (200964, Sigma Aldrich®, UK)

PBS pH 7.4 1X, $MgCl_2$ and $CaCl_2$ Free (14190-094, GIBCO®, Paisley, UK),

Pen/Strep (15140-122, Penicillin/Strepmyocin 100X or 10,000U/ml, GIBCO®, Paisley, UK).

Trypan Blue Solution (CAS# 72-57-1) (T8154-100mL, Sigma Aldrich®, UK)

Virkon Disinfectant (Rely+OnTM Virkon®, DuPont, UK)

5.3 Apparatus and equipment used:

Equiverlant equipment purchased from alternative suppliers can be used if desired, although the products with specific manufacturer details listed below are recommended for this SOP.

- Liquid Nitrogen
- Cell Freezing Aid
- Cryogenic Sample Box
- Laminar Class II Tissue Culture Hood

- 37˚C and 5% CO_2 ISO Class 5 Hepa Filter Incubator
- Water Bath (37˚C)
- Autoclave Tape
- 50mL or 100mL Glass Bottles
- Centrifuge
- T-25 (690175, Greiner Bio-One, UK) and T-75 (660175, Greiner Bio-One, UK) Tissue Culture Flask
- Pipette Boy
- 5mL, 10mL and 25mL Pipettes
- P1000 and P200 micropipettes
- P300 and P50 multi-channel pipettes
- Non-Filtered, Sterile 200µl and 1000µl Pipette tips
- 50mL Conical Falcon Tubes
- 50mL Skirted Falcon Tubes
- 9.4cm Square, Petri Dish (688161, Greiner Bio-One, UK)
- Nunc™ MicroWell™ 96-Well Microplates (167008, ThermoFisher Scientific, Denmark)
- Haemocytometer and coverslip
- 0.2mL and 1.0mL Eppendorf Tubes
- 1.0mL Syringe
- 0.45µm Filter Unit
- Microwave
- Light Microscope

5.4 Reporting of protected elements:

This SOP does not have any associated patent restrictions, specific licenses, material transfer agreements or commercial purchase requirements required to perform the protocol described.

5.5 Health and safety precautions:

Standard health and safety precautions associated with working within a laboratory environment and performing mammalian cell culture, as described by the European Agency for Safety and Health at Work (https://osha.europa.eu/en/safety-and-health-legislation/european-guidelines), should be adopted when conducting this SOP. In addition, all health and safety precautions outlined in the MSDS data sheets associated with the specific chemicals required must also be followed.

SOP Specific Health and Safety Precautions:

- **Heating of Agarose for Coating 96 Well Plates**

  Coating the 96-well plates with agarose involves heating the agarose gel at 900 Watt for 30 seconds in a microwave. This procedure results in piping hot, liquid agarose which if spilt onto the skin may burn and cause injury. Furthermore, care must be taken when handling the glass bottle containing the liquid agarose as this too can be very hot.

  To reduce the risk of injury, appropriate PPE (gloves, labcoat, closed footwear) must be worn at all times. Prepare a small volume (*e.g.* 10mL) of agarose gel in an closed, glass bottle and be careful not to overheat (*i.e.* stop heating if agarose begins to boil). Take care handling melted agarose and leave in microwave for 20-30 seconds following heating before removing the glass bottle from the microwave. For spills and burns wash area with running water, and then seek medical advice.

- **Trypan Blue Exclusion Assay for Cell Viability**

  Trypan Blue solution is a known teratogen, so it is advised that any pregnant individuals take extra caution when handling the chemical and should follow additional health and safety guidelines stated by the Occupational Safety and Health Administration (OSHA) in SOP 3.21 Reproductive Toxins, Mutagens, Teratogens and Embryotoxins – Procedures for Safe Handling and Storage. However, alternative cell viability dyes (*e.g.* Eryothrosyn B) could be used as a direct replacement instead if preferred.

5.6 Applicability:

Whilst this SOP is solely for the set-up of an *in vitro* model, this HepG2/KC co-culture liver spheroid model is applicable for use with both chemicals and engineered nanomaterials. This has been demonstrated with Aflatoxin B1 (A6636, Sigma Aldrich), Benzo[a]pyrene (B1760, Sigma Aldrich), Titanium Dioxide (NM-105, JRC), Zinc Oxide (NM-111, JRC), Silver (576832, Sigma Aldrich), Barium Sulfate (NM-220, JRC) and Cerium Dioxide (NM-212, JRC).

5.7 Reagent preparation:

5.7.1 Cell Culture Medium

In preparation for the SOP to be performed, it is advised that the cell culture medium is prepared and pre-warmed at 37˚C for 30 minutes prior to use. Cell culture medium can be prepared sterile as follows:

- Into a 500mL of 1X DMEM with 4.5g/L D-Glucose and L-Glutamine (41965-039, GIBCO®, Paisley, UK), add 50mL of heat inactivated FBS and 5mL Penicillin/Streptomycin antibiotic.

Order of addition does not need to be specific, but medium needs to be mixed by inverting the medium bottle after each addition. Once prepared, cell culture medium (with supplements) can be stored at 4˚C for a maximum of 3 months.

5.7.2 1.5% Agarose Gel

Agarose gels should be prepared and autoclaved prior to the day of plate coating (*i.e.* day 3 post seeding). To prepare a 1.5% agarose gel, weigh 0.15g of agarose into a clean, 50mL or 100mL glass bottle then add 10mL of phenol-red free DMEM medium (21063-029, GIBCO®, Paisley, UK). Securely place the lid on the al the glass bottle and seal with autoclave tape prior to 1 hour sterilization in the autoclave at 230˚C. Sterilized agarose can then be stored at room temperature for up to a month. The agarose medium is solid at room temperature and will need to be microwaved at maximum (900 W) Watt for 30 seconds before coating the plates. Agarose gel can be reheated a maximum of 3 times.

5.8 Procedure:

The following steps are conducted under sterile conditions in a Class 2 Laminar tissue culture cabinet to reduce potential for contamination.

5.8.1 Culturing Cryopreserved HepG2 Cells

Human hepatocellular carcinoma cell line HepG2, obtained from ATCC, was cultured in 1X DMEM with 4.5g/L D-Glucose and L-Glutamine (41965-039, GIBCO®, Paisley, UK) supplemented with 10% FBS and 1% Penicillin/Streptomycin antibiotic.

i. Pre-warm DMEM cell culture medium (including the supplements) in a 37°C water bath for 30 minutes.
ii. Remove one vial of HepG2 cells from liquid nitrogen and thaw in a 37°C water bath for 2-3 minutes, whilst gently swirling the vial to allow for uniform thawing of the cell suspension. Take care not to submerge the vial above the O-ring in order to reduce the potential for contamination.
iii. Once thawed, remove the vial from the water bath and spray generously with 70% Ethanol to decontaminate the outer surface of the vial before placing under a sterile, Class II Laminar Tissue Culture hood.
iv. Carefully pipette the contents of the cryovial of HepG2 cells into a falcon tube containing 9ml of pre-warmed DMEM cell culture medium (with supplements).
v. Using a 10mL strippette, transfer 10mL of the cell suspension into a T-25 flask and incubate the culture for 3 days (from seeding) at 5% CO_2 and 37°C until ~80% confluency is reached before undergoing sub-culture into a larger T-75 flask.

5.8.2 Sub-Culturing HepG2 Cells

Cells were sub-cultured under sterile conditions by trypsinisation with 0.05% trypsin/EDTA solution pre-warmed in a 37°C water bath for 30 minutes. At no point should the cells be allowed to dry out:

i. Cells form an adherent monolayer, from which media can be removed by tipping into Virkon disinfectant.

ii. The monolayer is then immediately washed to remove all traces of existing media by rinsing the flask twice with 3mL of sterile room temperature 1X PBS solution. PBS is also discarded into Virkon.

iii. Once PBS wash is removed, add 5ml of pre-warmed 0.05% trypsin-EDTA solution, ensuring to cover the entire surface of the cells and incubate cells for 6-8 minutes at 37°C and 5% CO_2.

iv. Following this, gently tap the flask to dislodge the cells from the bottom of the flask and then add 5 ml of DMEM cell culture medium (with supplements) to neturalise the trypsin enzyme.

v. Transfer the cell suspension into a 50ml centrifuge tube and pipette the cell suspension up and down thoroughly to ensure that cells are completely disassociated.

vi. Centrifuge the diluted cell suspension at 230 g for 5 minutes.

vii. Discard the supernatant into Virkon and re-suspend cell pellet in 25ml of DMEM cell culture medium (with supplements).

viii. Transfer cell suspension into a T-75 flask and incubate at 37°C and 5% CO_2 for a further 3 days before undergoing spheroid preparation. Once the HepG2s have had time to acclimatise and reach ~80% confluency, the cells can be counted and seeded ready for spheroid preparation.

5.8.3 HepG2 Spheroid Preparation

i. Repeat sub-culture steps stated above, except following centrifugation, re-suspend the cell-pellet in 1mL of DMEM culture medium pre-warmed in a 37˚C water bath. Pipette cell suspension up and down thoroughly.

ii. Count and check cell viability using the Trypan Blue Exclusion Assay (see section 5.5 for health and safety precaution and alternative assay dyes); 1:1 ratio of cell suspension to pre-filtered 0.4% trypan blue solution. Prior to cell counting, using a 1mL syringe take 1mL of trypan blue solution and filter with a 0.45µm filter unit into a sterile, 1mL Eppendorf tube.

iii. Transfer 10µl of filtered, trypan blue solution into a 0.2mL Eppendorf tube and add 10µl cell suspension. Remaining filtered trypan blue

solution can be stored at room temperature up to 3 months for future use.

iv. Spray the Haemocytometer thoroughly with 70% ethanol and wipe dry with a sterile paper towel before securing the coverslip on top using breath vapour. Sliding the coverslip across the breath moistened surface induces cohesive forces by generating newton rings.

v. Gently pipette the trypan blue cell suspension up and down using a P-1000 pipette (to reduce sheer stress) before adding 10µl to the haemocytomer. Ensure the solution is dispersed by capillary action underneath the cover slip and covers the entire grid without any bubbles.

vi. Under the microscope, count the live (unstained) and dead (stained blue) cells found in the four large corner squares (as shown in Fig.1).

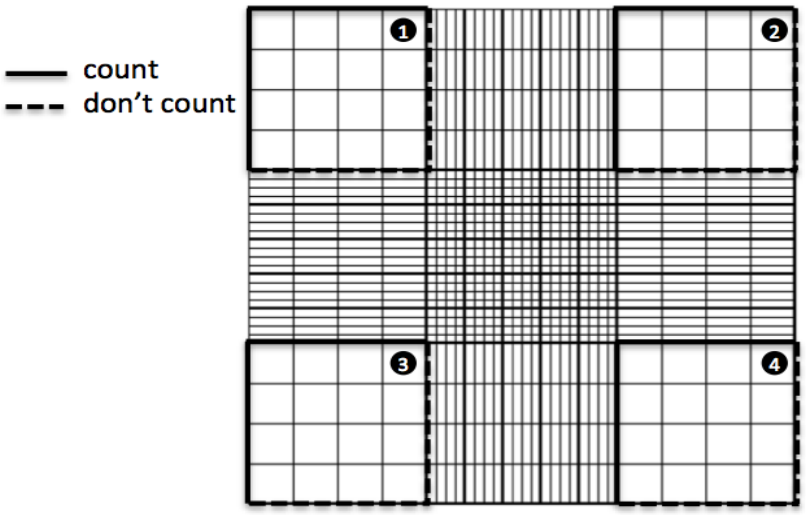

Figure 1: Counting Cells using a Haemocytometer (www.hemocytometer.org, 2018)

vii. Using the following calculation, calculate the average number of live, viable cells (unstained) present in the sample:

Total Number of Cells/mL = Live Cell Count x $\frac{dilution}{\#of\ squares\ counted}$ **x 10,000**

Where:

Dilution *refers to how many times the stock solution was diluted in trypan blue (2X in this case).*

# of squares counted *refers to the four large corner squares of the haemocytometer counted*

viii. Based on the viable HepG2 cell count and using the following formula:

ix. $C_1V_1=C_2V_2$

Where:

C_1 = the concentration of viable cells you currently have

V_1 = the volume of cell suspension you currently have

C_2 = the concentration of cell suspension you want

V_2 = the volume of cell suspension you want

x. Prepare a 10mL stock solution of HepG2 cell suspension with DMEM cell culture medium set at a concentration of $2.0x10^5$ cells/mL in order to get 4000 HepG2 cells per 20µl hanging drop.Mix the cell suspension thoroughly by gently pipetting up and down using a P-1000 to ensure all cells are fully suspended within the media.

xi. To the wells of a 96-well cell culture plate add 100µl of sterile, room temperature PBS to prevent the hanging drops from drying out during incubation.

xii. Take the lid of the 96-well cell culture plate, invert it and carefully pipette 20µl drops of the cell suspension into the centre of each well groove of the lid, as shown in Figure 2. This step can be done using a multi-channel pipette but it is advised that only 2 - 4 drops are performed at once as multiple seeding can affect the accuracy and placement of the drops. It is essential that the drops are centred within the grooves of the wells laid out on the lid otherwise they will not hang in the centre of the wells when the lid of the plate is turned over, and

are at risk of falling off into the plate altogether. Gently flip the lid of the 96-well plate, so the drops are now hanging and carefully place on top of the 96-well plate.

Note: It is advised when seeding that the pipette is angled as close to vertical (i.e. ≤45°) as possible in relation to the lid of the plate. This ensures compact droplets are plated, which will aid the formation of spherically shaped spheroids. In addition, it is worth noting that excessive disturbance to the incubator can result in misshapen sheroids.

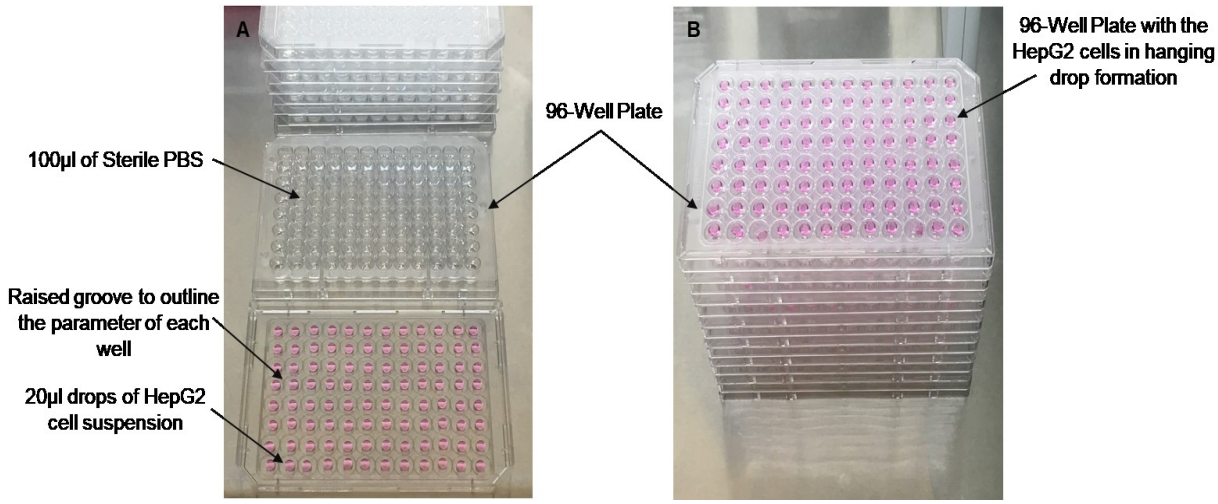

Figure 2: Displays (A) the HepG2 cells seeded in 20µl drops onto the lid of a 96-well plate whilst (B) shows the HepG2 cells post-seeding in the hanging drop model to allow for spheroid formation.

xiii. Place the whole 96 well plate with lid gently into an incubator at 37°C and 5% CO_2 for 3 days prior to spheroid transfer onto agarose.

Note: Extra care must be taken not only when transporting the plates to/from the incubators, but when opening and closing the incubator in general as the excessive movement can cause the plates to shift and the spheroids to either fall or form incorrectly.

5.8.4 HepG2 Spheroid Transfer from Hanging Drop to Agarose Suspension

Day 3 post seeding into hanging drops, the spheroids are transferred into the wells of the same 96-well plate all of which are coated with a fine layer of 1.5% agarose gel as instructed below. The agarose coating prevents the HepG2 spheroids from adhering to the base of wells and forming a cellular monolayer, thus retaining their 3D spheroid structure.

i. On Day 3 post seeding, remove the 96-well plate containing the HepG2 hanging drop spheroids out of the incubator and carefully flip the lid so the spheroids are no longer hanging.

ii. Using a multichannel pipette, remove and discard the 100µL of PBS previously added to the base of the 96-well plate. Allow the plates to air-dry for 2-3 minutes whilst heating the agarose in preparation for coating.

iii. Using the 1.5% agarose gels previously prepared, heat the glass bottle containing the agarose gel for 30 seconds in a microwave at the maximum watt (*i.e*. 900W). To coat two 96-well plates, one bottle of pre-preapred 1.5% agarose gel should be sufficient.

iv. Once melted, gently swirl the agarose by rotating the glass bottle to remove any bubbles before adding 50µl of agarose into the base of each well.

Note: When adding the agarose, ensure not to angle the plate >45° as the agarose sets quickly and will not form a flat, level layer. An uneven agarose layer can disrupt spheroid growth. It is important to work efficiently at this stage to prevent the agarose from solidifying before the plate is completely coated.

v. Allow the plate to stand for 2 minutes at room temperature before adding 100µl of pre-warmed DMEM cell culture medium (with supplements) on top of the solid agarose layer in each well.

vi. Flip the lid of the 96-well plate and place back on top of the 96-well plate so the spheroids are now hanging once again.

vii. Centrifuge the plate for 3 minutes at 200g in order to transfer the spheroids from the hanging drop formation into the individual wells of the 96-well plate.

viii. Following the transfer, the HepG2 spheroids should now be suspended in the cell culture medium and allowed to settle for 24 hours in the incubator at 37°C and 5% CO_2.

ix. HepG2 spheroids of this size can then be exposed to either chemical or ENM treatments on Day 4 post seeding (*i.e*. 24 hours after transfer to agarose coated plates).

In order to maintain cell viability over extended culture periods, it is advised that the cell culture medium is refreshed every 3 days. To do this, gently aspirate 50µl of the cell culture medium from the surface of the well and replace with a fresh 50µl of DMEM cell culture medium. Take care not to remove or disturb the spheroid tissue when perfoming a medium change.

5.8.5 HepG2 Spheroid Harvesting

Following either chemical or ENM exposure treatments, both cell culture medium or spheroid tissue can be harvested for multiple endpoint analysis. Depending on the endpoint analysis, spheroids can either be harvested individually (*e.g.* for image analysis or cytotoxicity cell counts) or pooled together (*e.g*. for the cytokinesis block micronucleus assay or ELISAs for inflammatory cytokines).

To harvest the spheroids:

i. Remove the 96-well plate from the incubator.

ii. Using a P-200 pipette, aspirate the 100µl of cell culture medium including the spheroid tissue from each well and collect in a sterile, 15mL falcon tube. Take care to avoid contact with the agarose.

iii. Once collected, centrifuge the spheroid suspension at 230g for 5 minutes.

iv. Remove the supernatent and store at -80˚C for further endpoint analysis (*e.g.* liver function tests) at a later date.

v. Re-suspend the pellet of spheroids in 1mL of sterile, room temperature PBS (1X).

vi. Once washed, centrifuge the spheroid suspension again at 230g for 3 minutes.

vii. Discard the supernatant, re-suspend in 500µl of 0.05% trypsin-EDTA solution and incubate for 6-8 minutes at 37°C and 5% CO_2.

viii. Following incubation, gently pipette the trypsinized cells up and down to fully disassociate and re-suspend the HepG2 cells prior to neutralising with 1mL of DMEM cell culture medium.

ix. Centrifuge the diluted cell suspension at 230 g for 5 minutes.

x. Discard the supernatant into Virkon and re-suspend cell pellet in 1mL of DMEM cell culture medium or PBS (1X) depending on the endpoint to be analysed.

5.9 Quality control & acceptance criteria:

To ensure the quality of the HepG2 cultures, pay careful attention to any morphological changes (*e.g.* cells become more rounded in shape) or excessive clumping in 2D monolayer cultures, which should be avoided prior to seeding into 3D spheroids. Figure 3 displays a phase contrast image displaying the natural morphology of HepG2 2D culture and can be used as a point of reference to ensure the cells are still viable before seeding in spheroids.

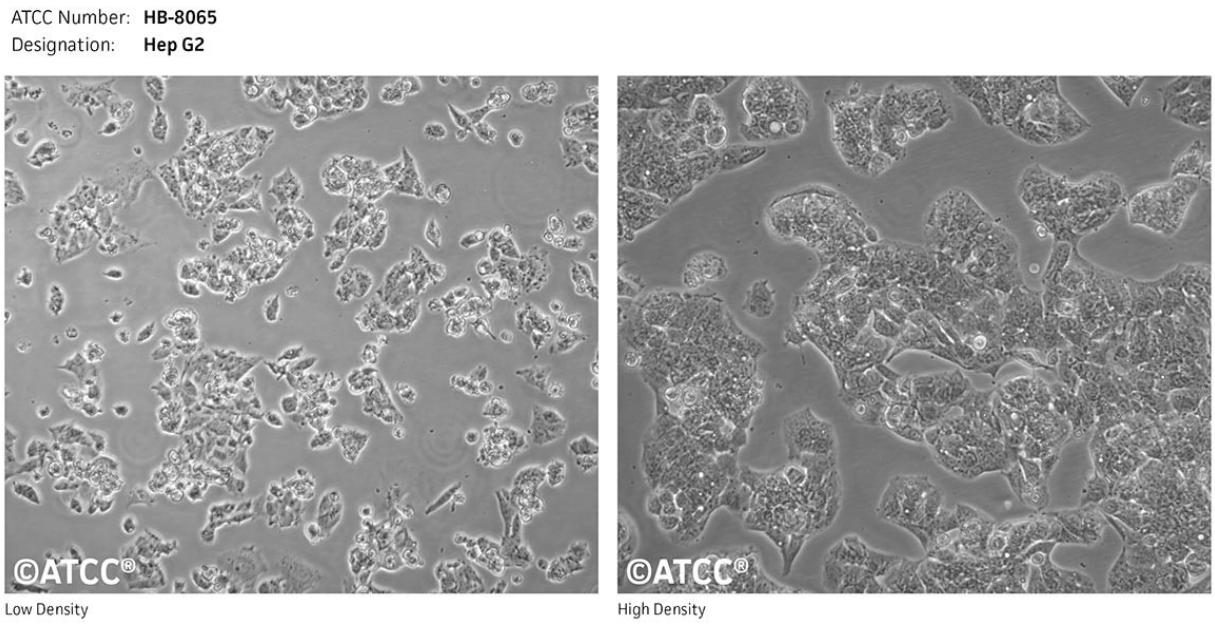

Figure 3: Light microscopy image displaying the natural morphology of the HepG2 (ATCC HB-8065) cell line provided by American Type Culture Collection.

Date Accessed: 12/06/2020

https://www.lgcstandards-atcc.org/products/all/HB-8065.aspx#characteristics

Following seeding HepG2 cells into spheroids, it is important to check the spheroids have formed properly prior to agarose transfer or chemical/ENM treatment. HepG2 spheroids produced using the hanging drop method usually take **3 days** post seeding to form compact, spherical shaped spheroids as shown in Figure 4 (A - B). HepG2 spheroids which have formed correctly and are acceptable to be used for *in vitro* toxicological assessment must have a compact, spherical shaped structure with a smooth surface and no visual projections. Figure 4 provides examples of good quality (D-F) and a poor quality (G-I) spheroids. The latter of which should be discarded.

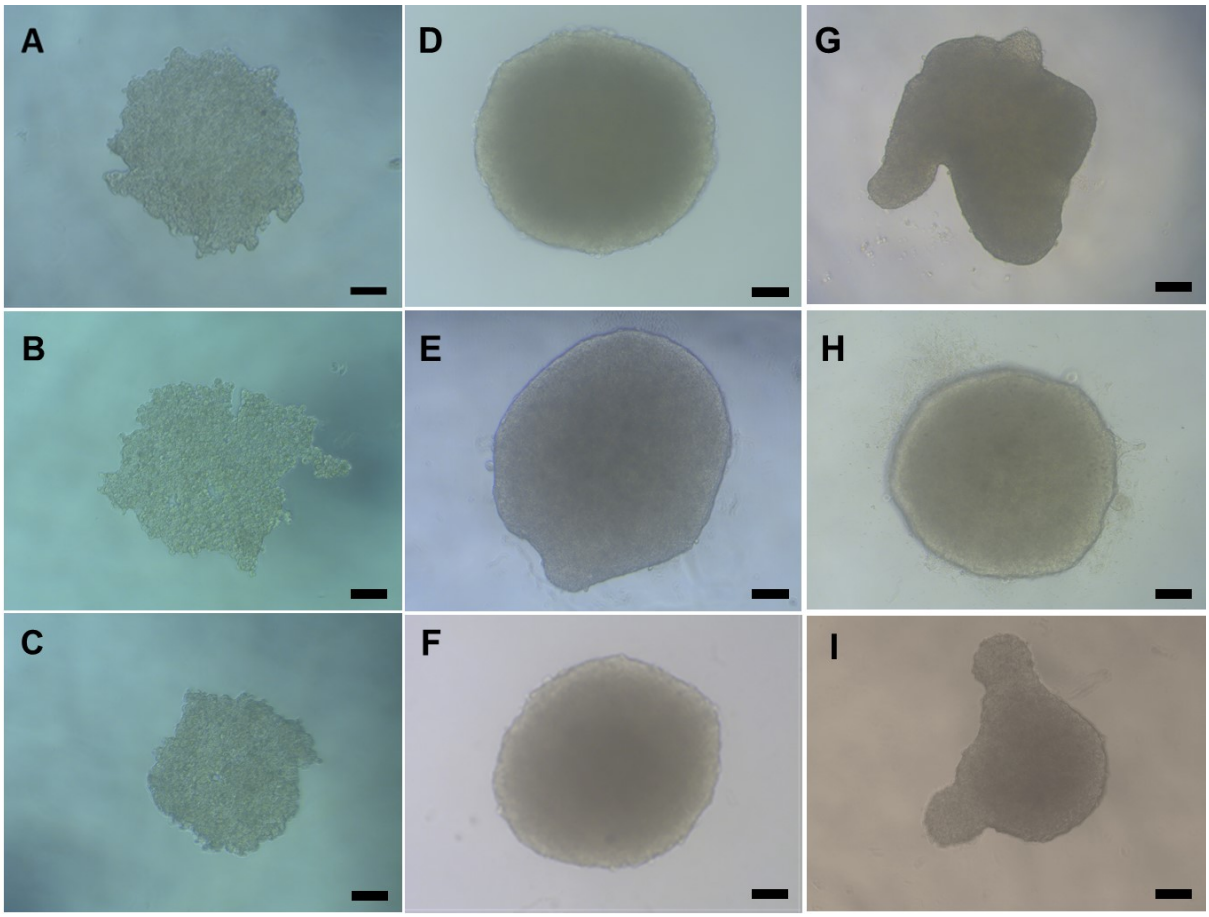

Figure 4: Light microscopy images displaying the natural morphology of the HepG2 spheroids formed *via* the hanging drop method on Day 2 (A-C) and Day 4 (D-I) post seeding. (D-F) are examples of good quality HepG2 spheroids whilst (G-I) shows poorly formed spheroids. All images were taken on a X20 objective using a Axiovert 40C, Zeiss Microscope. Scale bar represents 20µm.

(Samantha Llewellyn, 2018)

To further confirm HepG2 spheroid viability, a basic colorimetric BCG Albumin Assay (MAK124, Sigma Aldrich, UK) can be performed to assay their liver-like functionality. Based on a 4PL standard curve-fit, characterisation data indicates Albumin levels for HepG2 spheroids seeded at 4000 cells/spheroid should be above 50.0ng/μL.

6 Data Analysis and Reporting of Data:

Not applicable for this SOP.

7 Publications:

Llewellyn, S. V., Conway, G. E., Shah, U. K., Evans, S. J., Jenkins, G. J. S., Clift, M. J. D., Doak, S. H. Advanced 3D Liver Models for In vitro Genotoxicity Testing Following Long-Term Nanomaterial Exposure. *J. Vis. Exp.* (160), e61141, doi:10.3791/61141 (2020).

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8 References:

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