PATROLS
Advanced Tools for NanoSafety Testing

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 760813

PATROLS Standard Operating Procedures (SOP)

3D *In Vitro* HepG2, Kupffer Cell Co-Culture 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).

Authored by:

S.V. Llewellyn, Swansea University

Reviewed by:

Dr. G. E. Conway, Dr. M.J.D. Clift, Prof. G.J.S. Jenkins and Prof. S.H. Doak, Swansea University

Dr. A Kermanizadeh, University of Bangor

Prof. V Stone, Heriot-Watt University

Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	09/03/2020	Initial Document	Samantha Llewellyn
1.1	22/04/2020	Amended SOP to include comments from WP4	Samantha Llewellyn
1.2	27/04/2020	Final SOP uploaded to PATROLS Server	Samantha Llewellyn
1.3	01/10/2020	Updated to inc. new experimental data	Samantha Llewellyn
1.4	08/04/2021	Update to SOP template	Samantha Llewellyn

Table of Contents

1 Introduction:

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

In an effort to replace, reduce and refine the reliance on animal experimentation, there is great attention towards advancing current 3D *in vitro* model systems that offer features with more physiological relevance and enhanced predictivity of toxicological output. Hepatic toxicology is key when considering both chemical and engineered nanomaterial (ENM) exposure, as the liver is vital in metabolic homeostasis and detoxification as well as being a major site of ENM accumulation post exposure. An important adaptation to hepatic models, is the addition of liver specific macrophages known as Kupffer Cells (KCs). These cells are located in the liver lining of the sinusoids and are a major part of the liver innate immunity. They are heavily involved in the mediation of inflammatory responses, immune-mediated hepatotoxicity, liver injury and regeneration (Blizer *et al*., 2006; Dixon *et al*., 2013). KCs account for 20% of the total hepatic cell population, resulting in a 1 to 4 ratio of KCs to hepatocytes and are known to phagocytose pathogens, recruit neturophils and release both (pro-)inflammatory (*e.g*. IL-1β, IL-6 and TNF-α) cytokines and (pro-)fibrotic markers (*e.g.* TGF-β) (Dixon *et al*., 2013; Kolios *et al*., 2006; Rose *et al*., 2016). The release of these factors influence and regulate the phenotypes of neighbouring hepatocytes and other non-parenchymal cells (*e.g*. stellate cells and endothelial cells), such that they can trigger signalling pathways that regulate cell proliferation, viability and cell death as well as functional cell changes (*i.e*. hepatocyte drug-metabolising enzyme activities) (Dixon *et al*., 2013; Li *et al.*, 2020; Nguygen-Lefebvre *et al.,* 2015). Subsequently, KCs can mediate chronic inflammation and liver fibrosis as a result of oxidative stress induced by cytokine release. Not only do these resident macrophages play an important anti-inflammatory role, but their presence within an advanced *in vitro* model allows for secondary genotoxicity to be assessed.

There are a wide range of 3D *in vitro* liver models available on the market, all with benefits and limitations extensively reviewed by Lauske *et al*., 2019. These models are often static (*i.e*. non-dividing cell models) which limits genotoxicity assessment as it deems them unsuitable for use with the gold-standard cytokinesis-block

micronucleus assay (OECD TG487). Furthermore, there is a distinct lack of 3D *in vitro* liver models with the capability to emulate secondary genotoxicity; denoted as the main genotoxic mechanism *in vivo* (Evans *et al*., 2017; Lauske *et al*., 2019). Secondary genotoxicity arises *in vivo* as a result of chronic inflammation caused by the recruitment and activation of immune cells (*e.g.* macrophages, neutrophils), resulting in the continual release of inflammatory mediators that subsequently induce DNA damage in the surrounding epithelial tissues. The standard *in vitro* 2D monoculture test systems for genotoxicity evaluation are only capable of detecting primary genotoxicity, thereby overlooking a key DNA damage mechanism that occurs *in vivo*, particularly following exposure to ENM. Previous work within the PATROLS project provided a 3D hepatocyte model able to support ENM hazard assessment endpoints, including the evaluation of primary genotoxicity *in vitro.* Therefore, this SOP has been established to develop an advanced 3D *in vitro* hepatic coculture model capable of reliably evaluating hazard endpoints and enabling the assessment of secondary genotoxicity, 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 co-culture model system enables multiple toxicological endpoints to be analysed (*e.g.* liver function tests, pro-inflammatory markers, viability and genotoxicity) whilst providing the added tissue complexity of multiple cell types.

Limitations of the Protocol:

1. Longevity of the Culture System

   The 3D HepG2, Kupffer cell 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, according to the Trypan Blue Assay, remain above 80%.

2. Primary Human Kuppfer Cells Limited Supply

   Primary Human KCs are expensive, cannot be sub-cultured and with limited stocks can introduce donor to donor variation; reducing the reproducibility and cost effective nature of the hepatic spheroid model.

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	X
Stage 2: Validated internal laboratory method	
Stage 3: Interlaboratory tested method	
Stage 4: Method validated by Round Robin testing	
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

ECACC - European Collection of Authenticated Cell Cultures

EDTA – Ethylenediaminetetraacetic Acid

ELISA – Enzyme-Linked ImmunoSorbent Assay

ENM – Engineered Nanomaterial

FBS – Foetal Bovine Serum

IVIVE – *In Vitro In Vivo* Extrapolation

KC – Kupffer Cell

LPS - Lipopolysaccharide

OCT Medium – Optimal Cutting Temperature Medium

PBS – Phosphate Buffered Saline

Pen/Strep – Penicillin/Streptomyocin

PPE – Personal Protective Equipment

4 Principle of the Method:

The purpose of this SOP is to further advance the existing 3D hepatocyte cell line (HepG2) based spheroid model, enhance the physiological relevance of the *in vitro* model system for both acute or longer term (7–10 day), repeated ENM hazard assessment, and to provide a novel test system that is able to support the evaluation of secondary genotoxic mechanisms. To do this, a coculture with both hepatocyte cells and primary human liver macrophages (*i.e*. kuppfer cells) will be established.

The protocol can be broken down into 5 key stages:

1. HepG2/KC Cell Suspension Preparation
2. HepG2/KC 3D Co-culture Spheroid Formation
3. 3D Co-culture Spheroid Transfer from Hanging Drop to Agarose Suspension
4. 3D Co-culture Spheroid Harvesting
5. HepG2/KC 3D Spheroid Integration Analysis

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 co-culture model utilised is an adaptation of the 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'.

- The co-culture model includes the addition of primary human macrophage cells; human hepatic kupffer cells (HP-KC-F, ZenBio Inc.) in a 1:4 ratio with HepG2 hepatocytes.
- For further information regarding the selected cell lines, please see https://www.atcc.org/products/all/HB-8065.aspx for HepG2 and https://www.zen-bio.com/products/cells/hepatocytes.php for Human Kupffer cells.

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)

VECTASHIELD® PLUS Antifade Mounting Medium with DAPI (H-2000-10, Vector Laboratories)

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)

Human Hepatic Kupffer Cell Plating Medium (KC-1, ZenBio, UK)

Human Hepatic Kupffer Cell Maintenance Medium (KC-2, ZenBio, UK)

Mouse Monoclonal Antibody [KP1] to CD68 (ab222914, Alexafluor® 488, Abcam, UK)

OCT Medium (12542716, Cryomatrix, FisherScientific, 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:

Equivalent 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
- 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
- Pasteur Pipette
- 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
- Glass Microscope Slides
- µ-Slide Angiogenesis Chambered Microcope Slide (81501, Ibidi®)
- 0.2mL, 0.5mL and 1.0mL Eppendorf Tubes
- Cryogenic Sample Box

- 1.0mL Syringe
- 0.45µm Filter Unit
- Microwave
- Light Microscope
- Confocal 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 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 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 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), Titanium Dioxide (NM-105, JRC) and Silver (576832, Sigma Aldrich).

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. Additionally, it is advised that a second bottle of medium is prepared in parallel which must be kept cold (*i.e.* at 4˚C) in order to re-suspend the kupffer cells when fresh out of liquid nitrogen.

Cell co-culture medium is a 75:25 split of the HepG2 medium to Human KC specific medium, which should be prepared sterile as follows:

- HepG2 Medium - 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.

- Human Hepatic KC Medium – Consists of two types of culture medium already pre-prepared by the manufacturer, with the components of each medium as described below in Table 1. Further information can be found here: https://www.zenbio.com/pdf/ZBM0099.00%20Human%20Hepatic%20Kupffer%20Cell%20Manual.pdf

Table 1: Composition of the pre-prepared human hepatic KC plating and maintenance medium required from the manufacturer for culturing the HepG2/KC co-culture effectively.

Human Hepatic KC Medium (A) Plating Medium (250ml) (Cat#. KC-1, ZenBio, UK)	Human Hepatic KC Medium (B) Maintenance Medium (250ml) (Cat#. KC-2, ZenBio, UK)
• DMEM +high glucose (4.5g/L) • Insulin - *human recombinant* • Transferrin, human halo • Selenium • L-alanyl-L-glutamine • Glycine • Non-essential Amino Acids ; L-Alanine, L-Asparagine, L-Aspartic, L-Glutamic Acid, L-Proline & L-Serine • HEPES • Fetal Bovine Serum (FBS) • Penicillin/Streptomycin • Amphotericin B	• RPMI 1640 • L-alanyl-L-glutamine • HEPES • Fetal Bovine Serum (FBS) • Penicillin • Streptomycin • Amphotericin B
Storage and Expiration Date	Storage and Expiration Date
Medium is provided ready to use and	Media can be stored and will remain

prepared fresh prior to shipment. Store at 4°C for up to 30 days post shipment.	stable either at 4°C until the expiration date on the bottle label or at -20°C for 6 months. Medium is stable for 30 days post thawing.

- HepG2/KC Co-culture Medium (75:25) – In a 50ml falcon tube, add 37.5ml of HepG2 medium and 12.5ml of Hepatic KC Medium A (Table 1); this plating medium will be required for seeding the co-culture spheroids. For the plating medium composition, make two 50ml tubes in parallel, one of which can be kept at 4°C for re-suspending the KCs when fresh out of liquid nitrogen.
- In another 50ml falcon tube, prepare the maintenance medium consisting of 37.5ml of HepG2 meduim then 12.5ml of Hepatic KC Medium B (Table 1). This medium will be required for the remaining duration of the culture.

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

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, and then add 10mL of phenol-red free DMEM medium (21063-029, GIBCO®, Paisley, UK). Securely place the lid on 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 ECACC, 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

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

iv. 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.

v. 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.

vi. 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.

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

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

ix. 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/KC Cell Suspension Preparation

i. Following HepG2 sub-culture steps as stated in **Section 5.7.2**, after centrifugation of trypsinised HepG2 cells, re-suspend the cell pellet in 1mL of DMEM culture medium pre-warmed in a 37˚C water bath. Gently pipette cell suspension up and down thoroughly.

ii. In parallel to performing HepG2 sub-culture, safely remove a vial of cryopreserved human KCs from liquid nitrogen and hold in a 37˚C water bath until completely thawed. Immediatley remove the vial from the water bath, wipe dry and rinse the vial with 70% Ethanol before placing it in the sterile cell culture hood.

iii. Using a P-1000 pipette, gently transfer the human KCs from the vial to a 15mL conical tube containing 9mL of cold (4˚C) HepG2/KC Co-culture Medium A (*i.e.* 75%:25% HepG2 to KC plating culture medium, as described above in Section 5.6.1). Place the tube on ice to prevent the KCs from attaching to the plastic ware prematurely.

Note: At a physiological temperature of 37˚C, KCs are very "sticky" and will quickly adhere to the surface of the plastic ware, thus it is essential the culture medium remains cold (4˚C) until you're ready to seed the spheroids.

iv. Centrifuge the 15mL conical tube containing the human KCs for 5 mins at 4˚C and 500g.

v. Aspirate the supernatant and re-suspend the cell pellet in 1mL of cold HepG2/KC Co-culture Medium A in preparation for viability counting.

vi. Count and check cell viability of both the HepG2 and human KCs using the Trypan Blue Exclusion Assay (see Section 5.5 for health and safety precaution and alternative assay dyes). It is important to note, the viability of the recovered KCs is quite variable and can be anywhere between 70 – 100%. 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.

vii. To apply a 1:1 ratio of cell suspension to pre-filtered 0.4% trypan blue solution; transfer 10µl of filtered, trypan blue solution into a 0.2mL Eppendorf tube and add 10µl cell suspension. The remaining filtered trypan blue solution can be stored at room temperature up to 3 months for future use.

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

ix. 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.

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

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

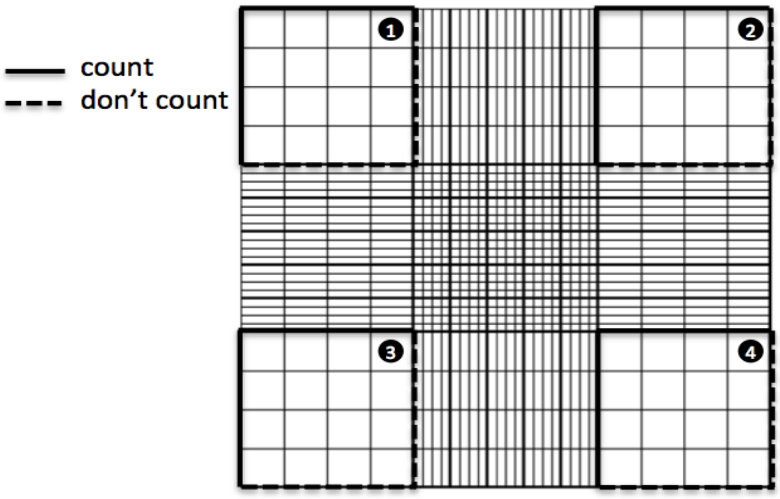

Figure 1: Counting Cells using a Haemocytometer (www.hemocytometer.org, 2018)

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

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

xii. Based on the total number of cells per mL and using the formula below determine the individual volumes required of each cell stock solution in order to maintain a 4:1 ratio of HepG2s to KCs in the final co-culture cell suspension:

$$C_1V_1=C_2V_2$$

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

In order to seed **1 full 96-well cell culture plate**, **2.0mL of coculture cell suspension** would be required.

- HepG2 Cells - A concentration of **$1.6x10^5$ cells/mL** suspensed in HepG2/KC Co-culture Medium A in order to have **3200 HepG2 cells** per 20µl hanging drop.
- Human KCs – A concentration of **$4.0x10^4$ cells/mL** suspensed in HepG2/KC Co-culture Medium A in order to have **800 KCs** per 20µl hanging drop.

Worked Example:

Number of 96-well plates to seed: **6**

Total Volume (V_2) of coculture suspension required: **6 x 2.0mL = 12mL**

HepG2s

Total Cell Count per mL: 2,372,000

($1.6 \times 10^5 \times 12mL$) / 2,372,000 = **0.81 mL of HepG2s**

KCs

Total Cell Count per mL: 475,400

($4.0 \times 10^4 \times 12mL$) / 475,400 = **1.01 mL of KCs**

HepG2/KC Coculture Stock Solution

Total Vol. Required = 12 mL

12mL – 0.81mL – 1.01mL =

10.18mL of HepG2/KC Co-culture Medium A required.

xiii. Based on the worked example above, add 10.18mL of pre-warmed HepG2/KC Co-culture Medium A into a 50mL conical tube before adding in the HepG2 cells (0.81mL) and KCs (1.01mL) to bring the coculture cell suspension to the final volume of 12mL.

Thus, add the individual volumes of each cell suspension into the remaining volume of HepG2/KC Co-culture Medium A to bring the coculture cell suspension to the final volume required to seed the desired number of 96-well plates. Mix the cell suspensions thoroughly by gently pipetting up and down using a P-1000 to ensure all cells are fully suspended within the media.

5.8.4 HepG2/KC 3D Co-culture Spheroid Formation

i. 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.

ii. Take the lid of the 96-well cell culture plate, invert it and carefully pipette 20µl drops of the mixed co-culture cell suspension prepared earlier into the centre of each well groove of the lid, as illustrated in Figure 2. This step can be done using a multi-channel pipette but it is advised that only 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. 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.

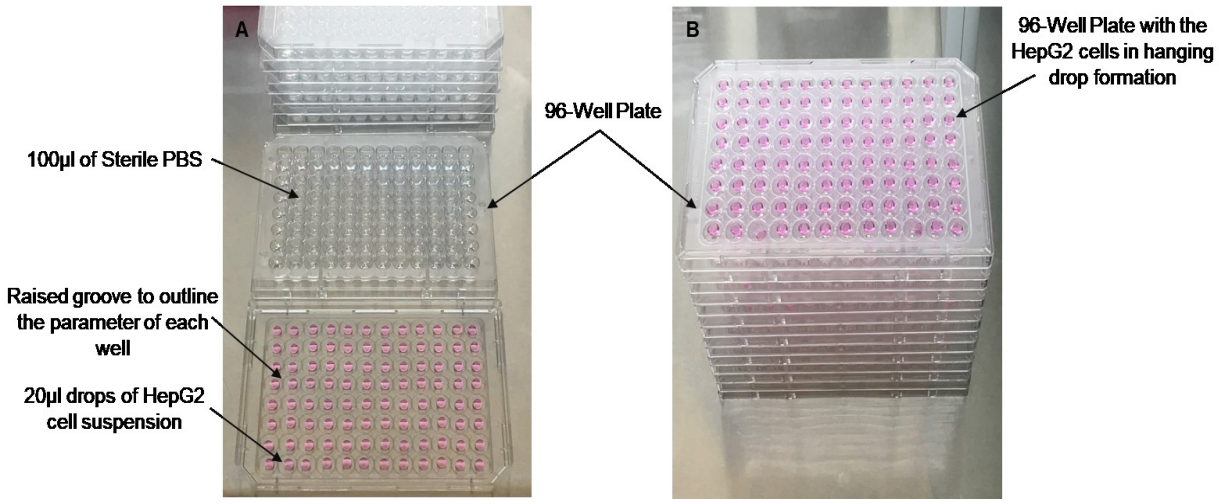

Figure 2: Displays (A) the HepG2 cells seeded in 20µl drops onto the lid of a 96-well plate whilst (B) shows the HepG2 cells post-seeding in the hanging drop model to allow for spheroid formation.

iii. 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 during general use as the excessive movement can cause the plates to shift and the spheroids to either fall or form incorrectly.

5.8.5 3D Co-culture 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/KC 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/KC hanging drop spheroids from 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 HepG2/KC Co-culture Medium B (i.e. 75%:25% HepG2 to KC maintenance culture medium, as described above in Section 5.6.1) 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/KC 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/KC 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 HepG2/KC Co-culture Medium B. Take care not to remove or disturb the spheroid tissue when perfoming a medium change.

5.8.6 3D Co-culture 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 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 conical 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 coculture 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/KC cells prior to neutralising with 1mL of HepG2/KC Co-culture Medium B.

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 HepG2/KC Co-culture Medium B or PBS (1X) depending on the endpoint to be analysed.

5.8.7 3D HepG2/KC 3D Spheroid Cell Integration Analysis

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

II. Using a P-200 pipette, aspirate the 50µl of cell culture medium including the spheroid tissue from each well and collect in a sterile, µ-Slide chambered microscope slide. Take care to avoid contact with the agarose.

Note: When adding the spheroids to the chamber wells of the µ-Slide microscope slide and during any subsequent washes, try to keep the spheroid away from the edges and as close to the centre of the well as possible as this will help to ensure a clear image of the spheroid is taken when using the confocal microscope.

III. Once collected, remove as much of the 50µl of cell culture medium from the chamber well as possible and replace with 50µl of room temperature 1X PBS.

IV. Perform three PBS washes by removing as much of the 50µl of 1X PBS as possible and replacing with a fresh 50µl of 1X PBS.

V. On the third PBS wash, remove 25µl of 1X PBS and add 25µl of 8% Paraformaldehyde (PFA) at room temperature for 30 mintutes.

VI. Perform a further three PBS washes by removing as much of the 50µl of 1X PBS as possible and replacing with a 50µl of fresh, room temperature 1X PBS.

VII. On the third PBS wash, remove 50µl of 1X PBS and add 50µl of 0.1% Triton-X in 1X PBS for 5 minutes at room temperature.

VIII. Remove 50µl of 0.1% Triton-X in 1X PBS and block overnight at 4°C in 50µl of preprepared, sterile-filtered 3% BSA.

IX. Following overnight incubation in 3% BSA block, wash the spheroids three times with 1X PBS by removing as much of the 50µl of 3% BSA block as possible and replacing with a 50µl of fresh, room temperature 1X PBS.

X. On the third PBS wash, remove 50µl of 1X PBS and add 50µl of 1:50 CD68 positive fluorescent anti-body in preprepared, sterile-filtered 3% BSA. Incubate overnight (minimum of 12 hours), in the dark at 4°C.

Note: The Alexafluor CD68 positive fluorescent antibody identifies the Kupffer cells within the co-culture.

XI. Following overnight incubation with Alexafluor CD68 positive fluorescent antibody, wash the spheroids three times with 1X PBS by removing as much of the 50µl of CD68 antibody and replacing with a 50µl of fresh, room temperature 1X PBS.

XII. On the third PBS wash, remove 50µl of 1X PBS and add 50µl of 1:1000 VECTASHIELD® DAPI in 3% BSA. Incubate overnight (minimum of 12 hours), in the dark at 4°C.

XIII. Perform a final set of three PBS washes by removing as much of the 50µl of VECTASHIELD® DAPI and replacing with a 50µl of fresh, room temperature 1X PBS.

XIV. On the final PBS wash, remove all 50 of 1X PBS and allow the spheroid to air dry, in the dark, for 15 mins before imaging the spheroids using a confocal microscope.

5.9 Quality control & acceptance criteria:

To ensure the quality of the HepG2 cultures, pay careful attention to any morphological changes (*e.g.* cells becoming 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 demonstrating the natural morphology of HepG2 2D cultures and can be used as a point of reference to ensure the cells are still viable before seeding in spheroids

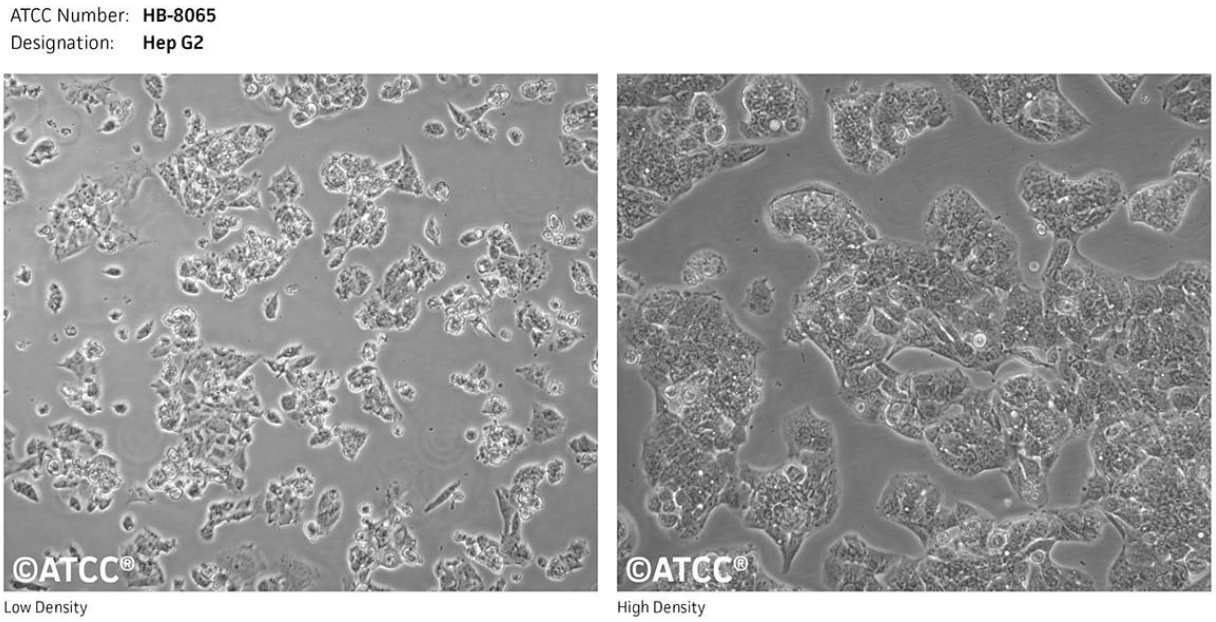

Figure 3: Light microscopy image displaying the natural morphology of the HepG2 (ATCC HB-8065) cell line provided by American Type Culture Collection.

Date Accessed: 12/06/2020

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

Following seeding of HepG2/KC cells into spheroids, it is important to check the spheroids have formed properly prior to agarose transfer or chemical/ENM treatment. HepG2/KC 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 - C). HepG2/KC spheroids which have formed correctly and are acceptable for use in *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 (A-C) and a poor quality (D-F) spheroids. The latter of which should be discarded.

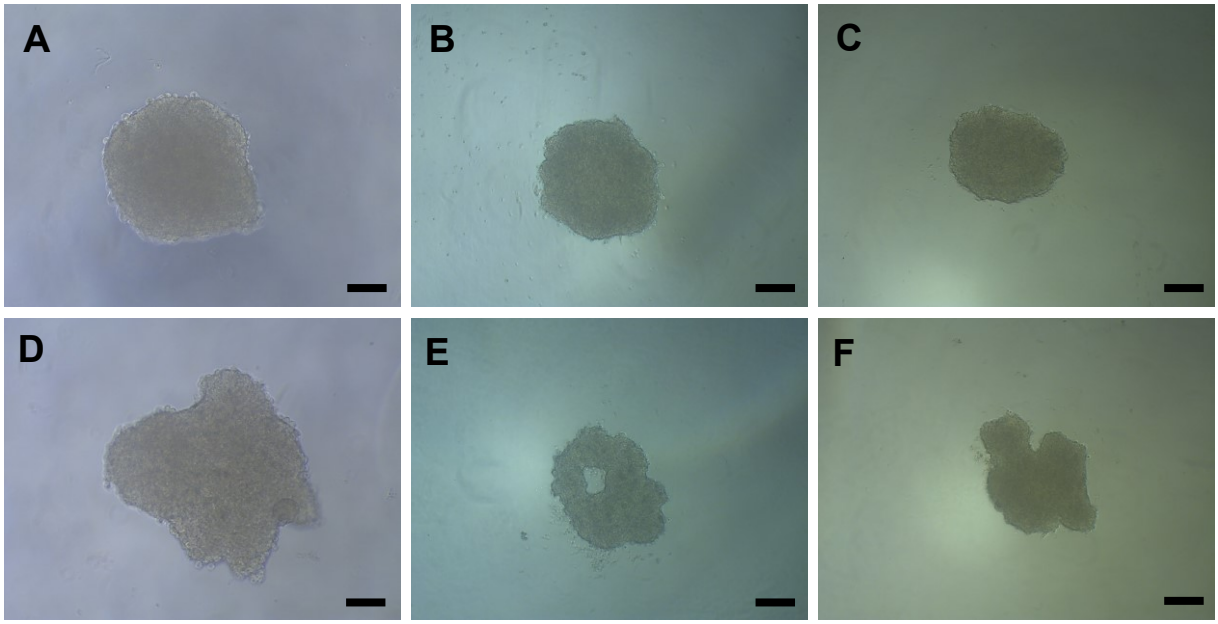

Figure 4: Light microscopy images displaying the natural morphology of the HepG2/KC spheroids formed *via* the hanging drop method on Day 4 (A-F) post seeding. (A-C) are examples of good quality HepG2 spheroids whilst (D-F) shows poorly formed spheroids. All images were taken on a X20 objective using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

(Samantha Llewellyn, 2020)

Further to this, it is important to confirm the KCs are fully integrated within the HepG2 spheroid and are equally distributed throughout the spheroid. To verify this, CD68 and DAPI fluorescently labelled images of the spheroids can be taken as described above in Section 5.7.7, to highlight the proportion and distribution of hepatic macrophages to epithelial cells. Figure 5 demonstrates an effective liver co-culture

spheroid with full KC integration (CD68 positive cells in green). It is not feasible to assess the integration of every spheroid, but it is advised that at least one spheroid per plate is evaluated.

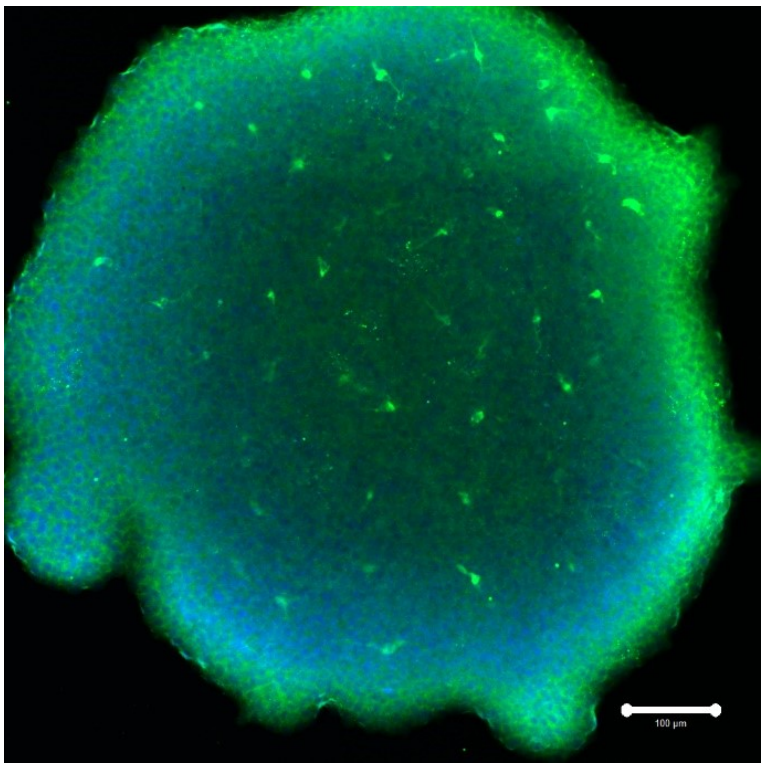

Figure 5: Confocal microscopy image of a HepG2/KC co-culture spheroid on Day 1, *i.e*. 24 hours after agarose transfer and 4 days after seeding. Green fluorescence represents CD68 (ab222914, Abcam, UK) positive staining for the KCs, whilst the blue fluorescence signifies the DAPI nuclear staining. Scale bar represents 100µM.

(Samantha Llewellyn, 2020)

To further confirm HepG2/KC spheroid viability, a basic colorimetric BCG Albumin Assay (MAK124, Sigma Aldrich, UK) and Urea Assay (MAK , Sigma Aldrich, UK) can be performed following manufacturers instructions to assay their liver-like functionality. Based on a 4PL standard curve-fit for Albumin and linear standard curve-fit for Urea, characterisation data indicates Albumin and Urea levels for HepG2/KC spheroids seeded at 4000 cells/spheroid should be above 50.0ng/µL and 0.25ng/µL respectively.

Furthermore, with the addition of macrophages, it is important to assess the capability of the model to respond effectively to a known positive inflammatory inducer (*e.g.* TNF-α protein or LPS). To assess this a solid phase sandwich ELISA for IL-8 (DY208, IL-8/CXCL8 DuoSet ELISA, R&D Systems, UK) can be performed using the pooled cell culture supernatents. Baseline characterisation indicates that on Day 1 of spheroid culture, initial IL-8 levels without the presence of an inflammatory inducing agent should be below 100pg/mL. Following acute (24 hour) activation with a positive control for (pro-)inflammatory response, in this case 0.25µg/mL of TNF-α protein (NBP2-35076-50µg, Bio-techne, UK), IL-8 release should be between 300pg/mL and 600pg/mL for a significant positive response.

6 Data Analysis and Reporting of Data:

Not applicable for this SOP.

7 Publications:

Not applicable for this SOP.

8 References:

Bilzer, M., Roggel, F. and Gerbes, A., 2006. Role of Kupffer cells in host defense and liver disease. *Liver International*, 26(10), pp.1175-1186.

Dixon LJ, Barnes M, Tang H, Pritchard MT, Nagy LE. 2013. Kupffer cells in the liver. *Compr Physiol,* 3, pp785-797.

Evans, S., Clift, M., Singh, N., de Oliveira Mallia, J., Burgum, M., Wills, J., Wilkinson, T., Jenkins, G. and Doak, S., 2016. Critical review of the current and future challenges associated with advancedin vitrosystems towards the study of nanoparticle (secondary) genotoxicity. *Mutagenesis*, 32(1), pp.233-241.

Kolios, G., 2006. Role of Kupffer cells in the pathogenesis of liver disease. *World Journal of Gastroenterology*, 12(46), p.7413.

Lauschke, V., Shafagh, R., Hendriks, D. and Ingelman-Sundberg, M., 2019. 3D Primary Hepatocyte Culture Systems for Analyses of Liver Diseases, Drug Metabolism, and Toxicity: Emerging Culture Paradigms and Applications. *Biotechnology Journal*, 14(7), p.1800347.

Li, F., Cao, L., Parikh, S. and Zuo, R., 2020. Three-Dimensional Spheroids With Primary Human Liver Cells and Differential Roles of Kupffer Cells in Drug-Induced Liver Injury. *Journal of Pharmaceutical Sciences*,.

Nguyen-Lefebvre AT, Horuzsko A. 2015. Kupffer cell metabolism and function. *J Enzymol Metab*, 1, pp101.

Osha.europa.eu. (2019). European guidelines - Safety and health at work - EU-OSHA. [online] Available at: https://osha.europa.eu/en/safety-and-health-legislation/european-guidelines [Accessed 27 Jan. 2019].

Rose KA, Holman NS, Green AM, Andersen ME, LeCluyse EL. 2016. Co-culture of hepatocytes and Kupffer cells as an in vitro model of inflammation and drug induced hepatotoxicity. *J Pharm Sci,* 105, pp950-964.