PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Nanomaterial Pre-treatment with Simulant Fluids to Mimic Oral and Inhalation Exposures for Hazard Assessment using 3D Liver Models *In Vitro*

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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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	12/03/2020	Initial Document	Samantha Llewellyn
1.1	10/04/2020	Amended SOP to include comments from WP4	Samantha Llewellyn
1.2	14/04/2020	Final SOP uploaded to PATROLS Server	Samantha Llewellyn
1.3	08/04/2021	Update to SOP Template	Samantha Llewellyn

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1 Introduction:

DOMAIN: Advanced 3D *In Vitro* Model Systems and Nanomaterial Exposure Regimes for Hazard Assessment

There are currently many international activities in place to assess the potential toxicity of engineered nanomaterials (ENMs) over acute, long term, and repeated low-dose exposure scenarios. Hepatic toxicology is key when considering ENM exposure, as the liver is understood to be a major site of secondary ENM accumulation post exposure (Gieser and Kreyling, 2010; Modrzynska, J., 2018). However, it is highly unlikely that the liver would be exposed to pristine ENM due to their systemic translocation through the body and interaction with a variety of biological membranes and fluids (*e.g*. changeable pH environments, proteins and microbes). As a result, the original 'pristine' ENMs are often transformed in a manner that may result in changes to surface chemistry, morphology and dissolution. Additionally, the protein corona that forms on the surface of the ENM may become modified according to the biological compartment the material is located in, which will subsequently affect how these materials interact with biological systems, also influencing their toxicological profile. This SOP therefore describes a protocol for the simulated transformation of ENMs to be applied prior to the use of *in vitro* 3D liver cell model-based hazard evaluation experiments. As depicted in Figure 1, the protocol involves the pre-treatment of ENM in gastro-intestinal tract (GIT) simulant fluid or lung simulant fluid, representing oral and inhalation exposures respectively. Following this initial step, the ENM are transferred to human blood plasma to simulate their translocation into the circulatory system. The aim of pre-treating ENM in these simulant fluids before exposing them to cell cultures is therefore to enhance the physiological relevance of ENM exposure regimes upon *in vitro* 3D liver systems.

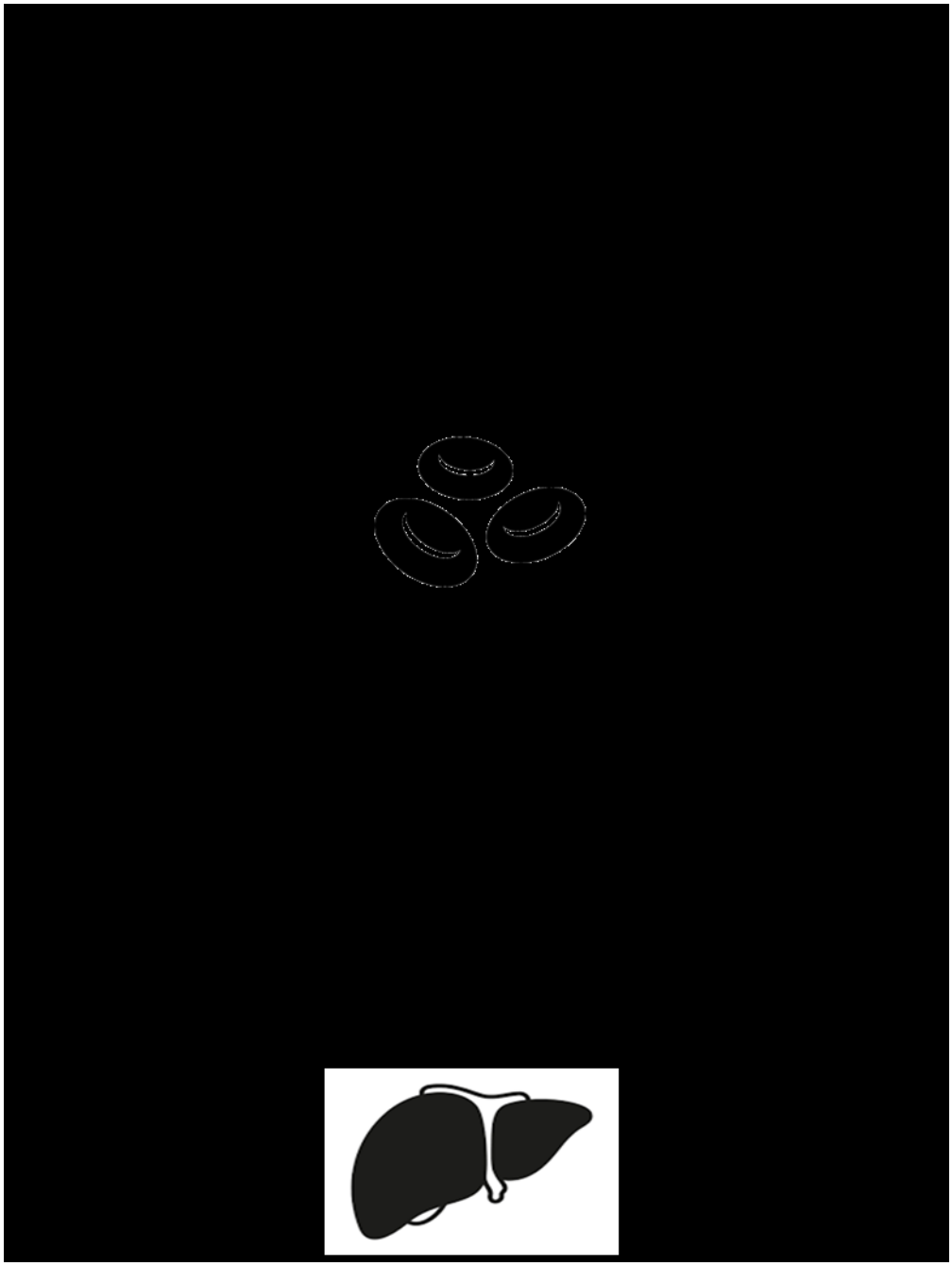

Figure 1: Schematic representation to illustrate the sequential incubations required for simulating the translocation of ENMs following both oral and inhalation exposure routes to the liver.

(Samantha Llewellyn, 2020)

1.1 Scope and limits of the protocol

This SOP was established with the intention to further enhance the physiological relevance of ENM exposure and hazard assessment protocols for secondary organ systems (*i.e.* organs that wouldn't naturally be exposed to neat, pristine ENMs), specifically, the liver. The aim of this SOP is to provide a way in which ENM exposure to 3D liver models can be adapted to account for the systemic translocation and potential modification of the ENMs prior to deposition in the liver, relevant to both acute and long-term, repeated dosing regimes. This model system enables multiple toxicological endpoints to be analysed (*e.g.* liver function tests, pro-inflammatory markers, viability, genotoxicity etc).

Limitations of the Protocol:

1. Lack of Microbiome Influence

   This SOP does not include the addition of microflora components or metabolites, which may also have an impact in the processing of ENMs following translocation through the body after oral exposure and before they reach secondary organs such as the liver.

2. Lung Simulant Fluid Selection

   The particular lung simulant fluid selected can be complex to develop and characterise. As a result, collaboration with Prof. Ben Forbes and his research group at Kings College London, UK would be required.

3. Ethical Approval and Vaccinations for Human Blood Plasma Handling

   Ethical approval would be required from the local governing authority or ethics committee, as well as up to date and relevant vaccinations for the principle researcher to allow for handling and use of human blood plasma in this SOP.

1.2 Validation of the 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	X
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]

3 Abbreviations:

BSA – Bovine Serum Albumin

DMEM – Dulbecco's Modified Eagle Medium

DMSO - Dimethyl sulfoxide

ECACC - European Collection of Authenticated Cell Cultures

EDTA – Ethylenediaminetetraacetic Acid

ENM – Engineered Nanomaterial

FBS – Foetal Bovine Serum

GIT – Gastro-Intestinal Tract

IVIVE – *In Vitro In Vivo* Extrapolation

PBS – Phosphate Buffered Saline

Pen/Strep – Penicillin/Streptomycin

PPE – Personal Protective Equipment

4 Principle of the Method:

To enhance the physiological relevance of ENM exposure regimes for secondary organ systems (*e.g.* liver) *in vitro*, this SOP looks to provide a way to mimic the transformation of ENMs as they translocate through the body following oral and inhalation exposure via the use of sequential incubations in relevant simulant fluids.

The protocol can be broken down into 4 key stages:

1. ENM Preparation
2. ENM Gastric / Intestinal Digestion Simulant Pre-treatment
3. ENM Lung Simulant Pre-treatment
4. ENM Human Blood Plasma Simulant Pre-treatment
5. ENM Liver Model Exposure

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 where possible. However, certain stages in the initial ENM pre-treatment require motion and temperature sensitive conditions and thus will have to be conducted outside of a sterile, 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'.
- 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:

Engineered Nanomaterials

FBS (10270-106, GIBCO®, Paisley, UK)

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

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

Distilled H_2O

Sodium Chloride (NaCl)

Hydrogen Chloride (HCl)

Sodium Carbonate (Na_2CO_3)

Sodium Bicarbonate ($NaHCO_3$)

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

Simulated Lung Fluid (Prof. Ben Forbes. Kings College London, UK)

5.3 Apparatus and equipment used:

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

- Weighing Balance with Weighsafe system
- 20mL Glass Scintillation Vials (Scint-Burk glass pp-lock & aluminium foil, WHEA986581, Wheaton Industries Inc., USA)
- Steel Spatula
- Laminar Class II Tissue Culture Hood
- 37˚C and 5% CO_2 ISO Class 5 Hepa Filter Incubator
- Orbital Shaker
- Water Bath (37˚C)
- 1L Glass Bottles
- Magnetic Stirrer
- pH Meter
- Autoclave
- Autoclave Tape
- Centrifuge
- Pipette Boy
- 5mL and 10mL 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

- Nunc™ MicroWell™ 96-Well Microplates (167008, ThermoFisher Scientific, Denmark)
- 0.2mL and 1.0mL Eppendorf Tubes
- 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:

5.5.1 Preparation of the Gastric and Intestinal Fluids

Caution must be taken when preparing the gastric and intestinal simulant fluids as they are a strong acidic and alkaline solution respectively, which can cause irritation if in contact with the eyes, skin or inhaled directly in close proximity.

5.5.2 Preparation and Handling of the Human Blood Plasma

IMPORTANT: Before having any contact with human blood plasma, the researcher carrying out the experiment as described in this SOP must ensure they have current and relevant vaccinations (*i.e*. Hepatitis B). Furthermore, it is essential they adhere to the health and safety legislation set out by the local governing body (*e.g.* in the UK, all institutions must abide by the Human Tissue Act) when working with human tissues and blood products.

5.6 Applicability:

This SOP is designed for use with powder based ENMs, but the protocol can be adapted accordingly. ENMs, specifically Titanium Dioxide (NM-105, JRC) and Silver (576832, Sigma Aldrich), have been applied to demonstrate this SOP. This SOP was designed to be utilized with *in vitro* liver models, but can be adapted to complement other organ systems defined as secondary sites of ENM deposition.

5.7 Reagent preparation:

As described in the PATROLS Deliverable 4.1 IUF Digestion Protocol, the following GIT digestion simulant fluids are prepared as follows:

5.7.1 Gastric Solution (34mM NaCl/HCl) – pH 2.7

Weigh out 1.98g of NaCl powder and add into a 1L glass bottle containing 1L of ddH_20. Once added, gently mix the solution and leave on a magnetic stirrer until all the NaCl has fully dissolved. Test the pH of the solution using a pH meter and adjust to pH 2.7 accordingly using HCl (1 N) acid.

5.7.2 Intestinal Solution (50mM Carbonate/Bicarbonate buffer) – pH 9.5

Into a 1L glass bottle containing 1L of ddH_2O, add 0.84g of Na_2CO_3, 3.58g of $NaHCO_3$ and 2.00g of NaCl. Once added, give a gentle swirl to mix the solution and leave on a magnetic stirrer until all the powder has fully dissolved before testing the pH of the solution to be pH 9.5.

Sterilize both the gastric and intestinal digestion simulant fluids using an autoclave at 230˚C for 1 hour.

5.7.3 Lung Simulant Fluid (SLF) – pH 7.7

The simulated lung fluid (SLF) selected consists of key components found in healthy human respiratory tract lining fluid (RTLF), including major soluble proteins (Albumin, IgG and Transferrin), abundant lipids (DPPG, DPPC and Cholesterol) and antioxidants (Ascorbate, Glutathione and Urate). Important to note, that this SLF does not contain any surfactant proteins, although does cover many of the other important components of (human) lung fluid. SLF is

prepared by Prof. Ben Forbes and his research group in Kings College London, as described in Hassoun et al., 2018.

A

Simulated Lung Fluid (SLF)	
Ingredient	Concentration in 10mL SLF Aliquots
DPPC	4.8 mg/mL
DPPG	0.5 mg/mL
Cholesterol	0.1 mg/mL
Albumin	8.8 mg/mL
IgG	2.6 mg/mL
Transferrin	1.5 mg/mL
Ascorbate	122.14 µM
Urate	95.47 µM
Glutathione	161.61 µM
Gentamicin	10 µL
HBSS	775 µl

B

Simulated Lung Fluid (SLF)	
Physiochemical Property	Reconstituted freeze-dry SLF
pH	7.7 ± 0.1
Conductivity [mS/m]	14.6 ± 0.2
Viscosity [Pa.s x10^{-3}]	1.111 ± 0.015
Surface Tension [mN/m]	55.6 ± 0.7

Table 1: **Composition and Physiochemical Properties of the Simulated Lung Fluid**. Highlights the (A) composition and (B) physiochemical properties of the simulated lung fluid (Hassoun *et al*., 2018)

Aliquots of stabilized, freeze-dry SLF can be stored at room temperature for up to 3 months and then reconstituted in 10mL of de-ionised water on day of ENM exposure.

5.7.4 Human Blood Plasma

Collect human blood from healthy volunteers into 10mL anti-coagulant K2-EDTA coated vacutainer tubes (367525, BD Biosciences, USA). Whole blood samples must be processed within 24 hours of donation, kept at room temperature and out of direct sunlight prior to plasma harvest.

Plasma Preparation:

- Take at least 30mL of whole human blood and add 3mL of Histopaque®-1077 (10771, Sigma, UK), pre-warmed to room temperature. For 30mL of blood, *ca.* a 10mL yield of plasma should be possible.
- Centrifuge the whole human blood collected at 500g for 10 minutes, at room temperature with no brake or acceleration applied.
- Harvest the plasma supernatant and aliquot up to 10mL of plasma into 50mL conical falcon tubes to avoid repeated freeze-thawing of plasma samples as it can negatively affect the quality of the specimen (Rai *et al.*, 2005). Store at -80°C until ready for use.

5.8 Procedure:

Please take careful note of the ENM concentration after each pre-treatment step described within this section as the test material will become increasingly diluted through each step of the simulation protocol. This may result in the sample being too dilute for the final ENM exposure range required.

5.8.1 ENM Preparation

1. Set up the weighing balance, ensuring it is calibrated and the HEPA filter is on. Prior to weighing any ENMs, it is important the correct PPE is worn as outlined in the OSHA guidelines described in Section 5.5.
2. Into a 20mL glass scintillation flask, weigh out exactly 4.0mg of ENM using a steel spatula. Ensure the lid of the scintillation flask is secured before removing from the weighing balance.

5.8.2 ENM Gastric / Intestinal Digestion Simulant Pre-treatment

1. Under a sterile laminar tissue culture hood, add 1000µL of gastric solution to the 4mg of ENMs to generate a 4mg/mL solution. As a control, add 1000µL of gastric solution to an empty scintillation vial without ENMs.
2. Incubate the ENM gastric solution for 30 minutes at 37°C on an orbital shaker set to 120rpm.

3. Following incubation, add 0.12 times intestinal solution to the volume of gastric solution (*i.e.* 0.12 x 1000µL vol. of gastric solution = 120µL intestinal solution) to generate 1.12mL GIT digestion solution with an ENM concentration of 3.57mg/mL.
4. Incubate the GIT digestion solutions on an orbital shaker at 120rpm for 30 minutes at 37°C.

5.8.3 ENM Lung Simulant Pre-treatment

1. Reconstitute the freeze-dry lyophilised SLF powder in 10mL of de-ionized water and gently pipette up and down to mix thoroughly.

Note: Reconstituted SLF can be stored at 4°C for up to 14 days.

2. Into a 15mL falcon tube, dilute the dispersed ENM stock solution with reconstituted SLF to result in a 4mg/mL solution.
3. Incubate the ENM suspension on an orbital plate shaker at 60 rpm for 1 hour at 37°C and 5% CO_2.

5.8.4 ENM Human Blood Plasma Simulant Pre-treatment

1. Whilst the ENM GIT digestion or lung simulant pre-treatment are incubating, thaw an aliquot of human blood plasma in a 37°C water bath.
2. Once fully thawed, centrifuge the plasma at 4°C for 2 minutes at 3000g.
3. Harvest supernatant into a fresh 15mL falcon tube.
4. Transfer plasma to either:
    a. The **ENM digestion solution (3.57mg/mL)** at a ratio of 11 parts blood plasma: 9 parts ENM solution (*i.e.* 1120µL ENM digestion solution + 1370µL plasma = 55% blood plasma). This yields a **1.61mg/mL concentration**.
    b. The **ENM lung simulant solution (4.00mg/mL)** at a ratio of 11 parts blood plasma: 9 parts ENM solution (*i.e.* 1120µL ENM lung simulant + 1370µL plasma = 55% blood plasma). This yields a **1.90mg/mL concentration**.
5. Follow this with a 60 minute incubation on shaker at 120rpm at 4°C.

5.8.5 ENM Liver Model Exposure

1. Once the final incubation has elapsed, the pre-treated ENMs are now ready to be further diluted in media to the desired final concentrations in preparation for exposure to the cell cultures. Ensure to include the appropriate negative controls, all of which should not contain any ENMS. For example, the following controls may be appropriate for the oral simulant exposure regime:
    - Gastric Solution Only
    - Intestine Solution Only
    - Gastric + Intestine Solution
    - Human Blood Plasma Only
    - Gastric, Intestine + Human Blood Plasma
2. To expose the *in vitro* 3D liver spheroid model to ENMs, carefully aspirate 50µl of cell culture medium from the surface of the wells in the 96-well plate. Be mindful not to disturb or remove the spheroid from the well during this process.
3. Following this, add 50µL of cell culture medium containing the final desired concentration of pre-treated ENMs and leave for the required exposure period in a 37°C and 5% CO_2 incubator.

*Note: If following this SOP in conjunction with the PATROLS SOP '3D In Vitro HepG2 Spheroid Model', it is important to note that any final concentrations will need to be double concentrated as they become **diluted two fold** when added to the model. This is due to replacing only 50µL out of the 100µL total volume of media in the well, in order to minimise any disturbance to the spheroid.*

Worked Example:

Final desired concentration: 10µg/mL from a stock of 1.61mg/mL in the ENM digestion solution.

Dilution factor in 3D liver spheroid model: **2**

Total volume required: 5mL for 1 plate based on the following calculation -

$$\mathbf{C_1 \times V_1 = C_2 \times V_2}$$

Where:

- C_1 – Starting Stock Concentration – **1.61mg/mL**
- V_1 – Volume of Starting Stock Solution – **V_1**
- C_2 – Final Working Concentration – **10.0µg/ml (0.01mg/mL)**
- V_2 – Volume of Final Working Solution – **5mL**

$$1.61\text{mg/mL} \times V_1 = 0.01\text{mg/mL} \times 5\text{mL}$$
$$V_1 = (0.01 \times 5) / 1.61$$
$$V_1 = 0.031056\text{mL}$$
$$V_1 = 31.06\mu\text{L}$$

(31.06µL x 2 = **62.11µL**) of 1.61mg/mL pre-treated ENMs to give 10µg/mL in 5mL total volume.

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 100µL volume of cell culture medium per well and replace with a fresh 50µL of DMEM cell culture medium. Take care not to remove or disturb the spheroid tissue or sedimented ENM when performing a medium change.

5.8.6 HepG2 Spheroid Harvesting

Following ENM exposure treatments, both cell culture medium and 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 cytokinesis block micronucleus assay).

To harvest the spheroids:

1. Remove the 96-well plate from the incubator
2. Using a P-200 pipette, aspirate the 100µL of cell culture medium including the spheroid tissue from each well and collect them altogether in a sterile, 15mL falcon tube. Take care to avoid contact with the agarose.

3. Once collected, centrifuge the spheroid suspension at 230g for 5 minutes.
4. Transfer the supernatant to 1.5mL Eppendorf tubes and store at -80˚C for further endpoint analysis (*e.g.* liver function tests) at a later date.
5. Re-suspend the pellet of spheroids in 1mL of sterile, room temperature PBS (1X).
6. Once washed, centrifuge the spheroid suspension again at 230g for 3 minutes.
7. 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.
8. 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.
9. Centrifuge the diluted cell suspension at 230 g for 5 minutes.
10. 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:

5.9.1 HepG2 Spheroid Model *(if selected for use alongside this protocol)*

To ensure the quality of the HepG2 cultures is maintained from seeding through to ENM exposure and harvest, post-seeding it is important to check the spheroids have formed properly prior to 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 - C). 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.

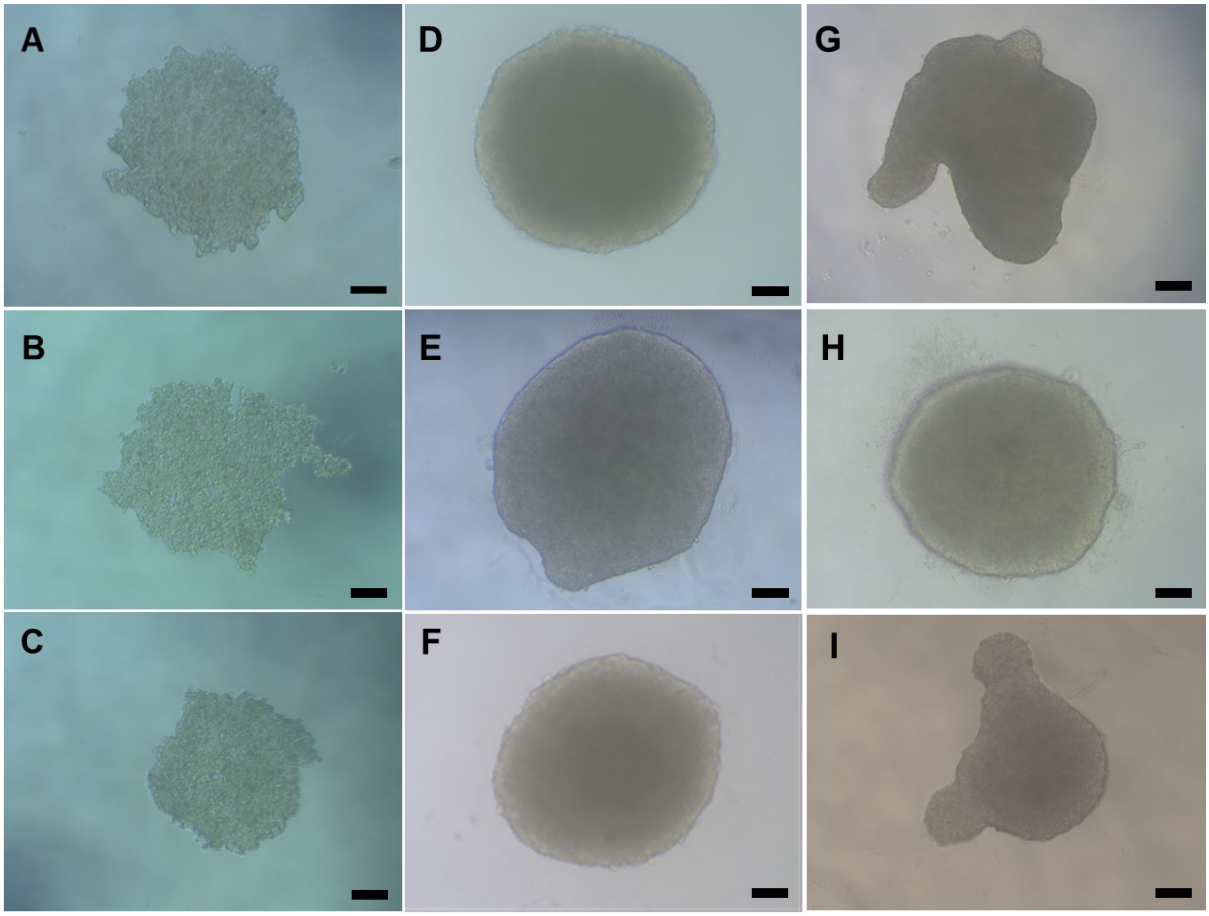

Figure 2: Light microscopy images displaying the natural morphology of the HepG2 spheroids formed *via* the hanging drop method on Day 2 (A-C) and Day 4 (D-I) post seeding. (D-F) are examples of good quality HepG2 spheroids whilst (G-I) shows poorly formed spheroids. All images were taken on a X20 objective 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. Albumin levels for HepG2 spheroids seeded at 4000 cells/spheroid should be above 50.0ng/μL.

5.9.2 ENM Simulant Fluid Suspensions

It is vital to check and adjust the pH of the simulant fluids if necessary before use to ensure they are an accurate representation of the biological fluids they are intended to mimic (*i.e.* gastric solution should be at an acidic pH of 2.7 whilst the intestinal solution should be of an alkaline nature, pH 9.5).

Throughout the ENM pre-treatment process, it is important to ensure the ENMs are as well dispersed within the simulant fluids as possible to ensure accurate dosimetry throughout the sequential incubations and subsequent ENM liver exposure. Figure 3 illustrates the difference in appearance between a well dispersed (A) and poorly dispersed (B) ENM solution.

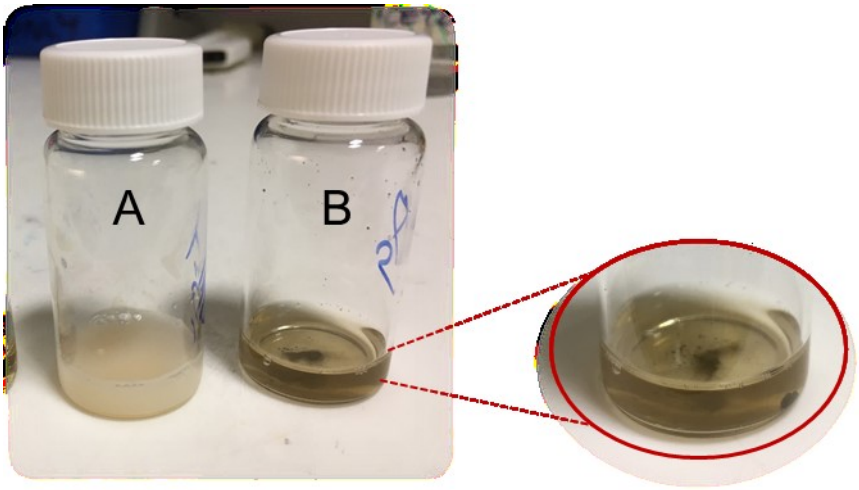

Figure 3: Variation in ENM dispersion quality displayed following pre-treatment with oral exposure simulants resulting in (A) a well dispersed TiO_2 (NM110) ENM solution and (B) a poorly dispersed Ag (Sigma) ENM solution.

6 Data Analysis and Reporting of Data:

Not applicable for this SOP.

7 Publications:

Llewellyn S. V., Kämpfer A., Keller J.G., Vilsmeier K., Büttner V., Ag Seleci D., Schins R.P.F., Doak S.H., and Wohlleben W. Simulating Nanomaterial Transformation in Cascaded Biological Compartments to Enhance the Physiological Relevance of *In Vitro* Dosing Regimes: Optional or Required? *Small* (2021), e2004630. doi/10.1002/smll.202004630.

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