PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Guidance Document for macrophage differentiation from THP-1 cells

This is a a) SOP used by members of PATROLS only or (b) SOP recommended for external use by PATROLS

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Version	Approval Date	Description of the change	Author(s) of change
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1 Introduction:

The THP-1 cell line was purchased from the American Typce Culture Collection (ATCC). THP-1 cells are monocytes originally harvested from a one-year old male with acute monocytic leukaemia. Cells are received in cryovials, cultured in suspension, and can be differentiated into macrophages (dTHP-1) using phorbol 12-myristate-13-acetate (PMA).

1.1 Scope and limits of the protocol

THP-1 cells are differentiated over 4 days, 2 days with PMA and 2 days in recovery medium. This must be considered when using this protocol.

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	x
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.]

Nanoscale

Length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE : ISO/TS 80004-1: 2016, definition 2.1]

Nanotechnology

Application of scientific knowledge to manipulate and control matter predominantly in the *nanoscale* to make use of size- and structure-dependent properties and phenomena distinct from those associated with individual atoms or molecules, or extrapolation from larger sizes of the same material.

Note 1 to entry: Manipulation and control includes material synthesis.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.3]

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]

Nano-object

Discrete piece of material with one, two or three external dimensions in the *nanoscale*.

Note 1 to entry: The second and third external dimensions are orthogonal to the first dimension and to each other.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.5]

Nanostructure

Composition of inter-related constituent parts in which one or more of those parts is a *nanoscale* region.

Note 1 to entry: A region is defined by a boundary representing a discontinuity in properties.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.6]

Nanostructured material

Material having internal *nanostructure* or surface nanostructure.

Note 1 to entry: This definition does not exclude the possibility for a *nano-object* to have internal structure or surface structure. If external dimension(s) are in the *nanoscale*, the term nano-object is recommended.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.7]

Engineered nanomaterial

Nanomaterial designed for specific purpose or function

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

Manufactured nanomaterial

Nanomaterial intentionally produced to have selected properties or composition.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.9]

Incidental nanomaterial

Nanomaterial generated as an unintentional by-product of a process.

Note 1 to entry: The process includes manufacturing, bio-technological or other processes.

Note 2 to entry: See “ultrafine particle” in ISO/TR 27628:2007, 2.21

Particle

Minute piece of matter with defined physical boundaries.

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general particle definition applies to *nano-objects*.

[SOURCE: ISO 26824:2013, 1.1]

Substance

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

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

3 Abbreviations:

ALI – Air-liquid interface

ATCC – American Tissue and Cell Collection

CCM – Cell Culture Medium

DMSO - Dimethyl sulfoxide

FBS - Fetal bovine serum

PBS – Phosphate Buffered Saline

RPMI - Roswell Park Memorial Institute-1640 Medium

4 Principle of the Method:

This method aims to standardise the aseptic culture and cryopreservation of THP-1 cells in culture flasks. THP-1 cells have a population doubling time of 26 hrs in optimal cell culture settings medium with serum.

This protocol will be broken into key stages:

1. Culturing cryopreserved cells
2. Sub-culturing cells
3. Freezing cells

5 Description of the Method:

5.1 Biological setting & test system used:

This SOP should be carried out under laboratory based conditions, with all work performed under sterile conditions and at a minimum of Biological Safety Level 1 conditions.

Approximately twice yearly, a new batch of cells is purchased from ATCC. This new batch is cultured, aliquoted in approximately 10 vials, and each aliquot is cultured and aliquoted again in approximately 10 vials. These latter vials are used for the experiments, till a maximum of 20 passages. No authentication is done.

5.2 Chemicals and reagents used:

- DMSO (276855, Sigma Aldrich®, UK)
- FBS (10270-106, GIBCO®, Paisley, UK)
- PBS pH 7.4 1X, $MgCl_2$ and $CaCl_2$ Free (14190-094, GIBCO®, Paisley, UK),
- Penicillin/streptomycin (10,000 U/mL) – Gibco (ThermoFischer)
- PMA (phorbol 12-myristate 13-acetate; CAS Number 16561-29-8) - Sigma P8139-1MG or P8139-5MG
- RPMI 1640 (11879020, GIBCO®, Paisley, UK)
- THP-1 cells – ATCC TIB 202 (or THP-1null cells - InvivoGen)
- Trypan Blue Solution (CAS# 72-57-1) (T8154-100mL, Sigma Aldrich®, UK)
- Virkon (Rely+OnTM Virkon®, DuPont, UK)

5.3 Apparatus and equipment used:

All tissue culture equipment was sourced from Greiner Bi-One, UK unless stated otherwise.

- 37˚C and 5% CO_2 ISO Class 5 Hepa Filter Incubator
- 0.2 mL and 1.0 mL Eppendorf Tubes

- 5 mL, 10 mL and 25 mL sterile pipettes
- 50 mL Centrifuge Tubes
- 50 mL Skirted Falcon Tubes
- Autoclave Tape
- Cell Freezing Aid
- Cell culture companion plate (353502, Corning)
- Cell culture insert (353091, Corning)
- Centrifuge
- Haemocytometer
- Laminar Class II Tissue Culture Hood
- Light Microscope
- Liquid Nitrogen
- P1000 and P200 micropipettes
- Pipette Controller
- Non-Filtered, Sterile 200µl and 1000µl Pipette tips
- T-25 (690175) and T-75 (660175) Tissue Culture Flask
- Water Bath (37˚C)

5.4 Reporting of protected elements:

To the best of our knowledge, 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:

Prior to any use of this SOP a full risk assessment should be completed, considering all potential risks associated with chemicals equipment and use, in compliance with

national regulation. Training of personnel should be completed before any person is working with the SOP.

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.

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 assays (*e.g.* Eryothrosyn B) could be used instead if preferred.

5.6 Reagent preparation:

5.6.1 Cell Culture Medium (CCM)

RPMI supplemented with

- 1% Penicillin-Streptomycin (corresponds to 100 Units/mL Penicillin and 100 µg/mL Streptomycin)
- 1% L-glutamine (corresponds to 2 mM L-Glutamine)
- 10% FBS (heat inactivated, please follow the ATCC protocol for heat inactivation (https://www.atcc.org/~/media/PDFs/Culture%20Guides/AnimCellCulture_Guide.ashx, page 19))

Culture medium and supplements do not require pre-heating before supplementation but should be stored at between 4-6°C after preparation. Can be kept for 3 months at this temperature.

Antibiotics are added to the medium to reduce bacterial contamination.

In a 500 mL medium bottle add:

- 5.5 mL Penicillin-Streptomycin
- 5.5 mL L-glutamine
- 50 mL FBS

Order of addition does not need to be specific, but medium needs to be mixed after each addition.

5.6.2 Freezing Medium

Complete cell culture medium (as prepared in section 3.5.1) is combined with 5% (v/v) DMSO to make complete freezing medium, at the time of freezing.

5.7 Procedure:

5.7.1 Thawing Cells

1. Warm all required reagents to 37°C in a water bath (~20-30 min).
2. Place 9 mL of complete cell culture medium into a 15 mL centrifuge tube
3. Collect frozen vial from liquid nitrogen storage
4. Defrost vial in water bath at 37°C
5. Pipette slowly (drop by drop) the contents of the vial into a centrifuge tube with 9 mL of prewarmed cell culture medium and centrifuge at 1200 rpm for 5 min.
6. Pellet the cells at 1200 rpm for 5 mins

From here on, all steps need to be completed under aseptic conditions.

7. Discard the supernatant into waste (this is laboratory specific so follow laboratory specific protocols) and re-suspend (via pipetting up and down) the cell pellet in 1 mL of prewarmed cell culture medium.
8. Resuspend cell pellet in 5 mL complete culture medium
9. Transfer the re-suspended cells into the pre-prepared cell culture medium in the T25 flask (from step 2) and incubate the culture at 37°C and 5% CO_2.

Allow a few days to grow in a T25 flask before transferring to a larger T75 flask to expand cell cultures.

10. Change the cell culture medium every 2-3 days. Cells are spun into a pellet by pouring contents of flask into a 15 ml centrifuge tube and centrifuge at 1200rpm for 5mins. The pellet is then resuspended in the fresh medium pipetted up and down to ensure mixing and then pipetted into the flask and the flask replaced back into the incubator.

5.7.2 Sub-Culture

- Cells should be passaged at least 2 times after thawing, before use in experiments or before freezing, and they should be passaged no more than 60 times in total (minimal published references show data beyond this point, and cell doubling time may decrease beyond this point).

Protocol

1. Visually check the cell culture medium colour and cells under the light microscope (fig.1)

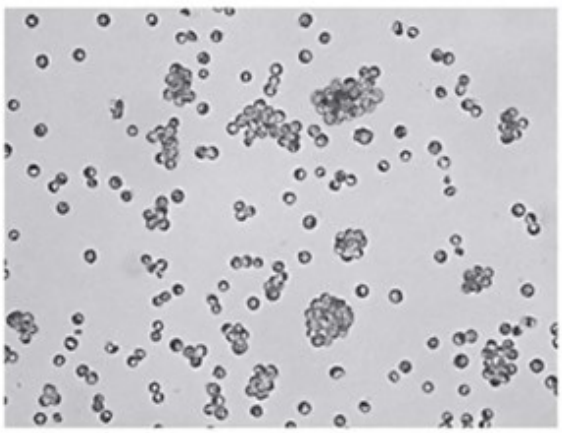

Figure 1 – THP-1 cells in culture

From here on, all steps need to be completed under aseptic conditions.

2. Transfer the cell suspension from the flask to a 50 ml centrifuge tube.
3. Centrifuge at 1200 rpm for 5 min (24°C; the setting of “accelerate” should be 8 (on a scale of 0-10); the setting of “brake” should also be 8 (on a scale from 0-10).

4. Add 10 µL sterile-filtered trypan blue to a clean microcentrifuge tube and set aside.
5. After centrifugation, pour off the supernatant, use a pipette to remove any excess supernatant.
6. Add 1 mL complete cell culture medium to the pellet and re-suspend using a pipette. Transfer 10 µL of cell suspension to the 10 µl trypan blue prepared in step 4 and mix well.
7. Attach a cover slip to a haemocytometer using breath vapour, then pipette 10 µL of cell/trypan blue solution into a counting chamber.
8. Count the cells in the 4 large squares (Nr. 1-4) (fig. 2).

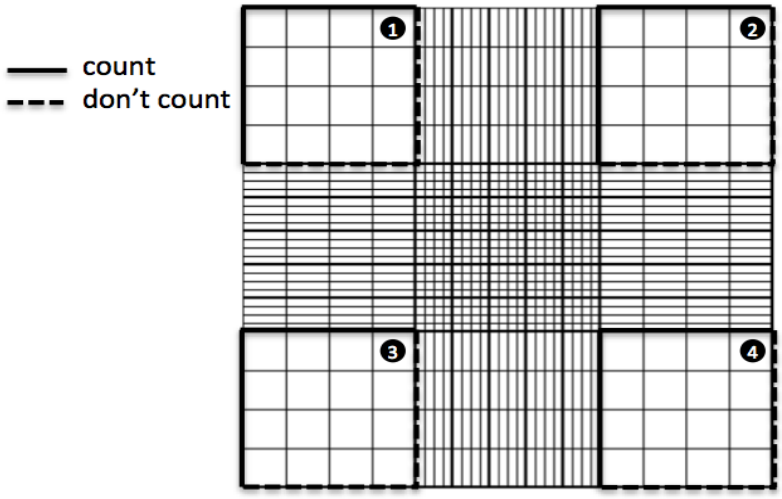

Figure 2 - Diagram of a haemocytometer

9. Using the following calculation, count the number of living cells (not labelled with trypan blue) that are in your sample.

$$\text{Total cells/mL} = \text{counted cells} \times \frac{dilution}{\#of\ squares\ counted} \times 10'000$$

The term 'dilution' refers to how many times the stock solution was diluted in trypan blue (in this case 2x) and the '# of squares counted' refers to the number of larger haemocytometer sections on which cells were counted (i.e. the outside 4 big squares). Using the following calculation, count the percentage of viable cells:

$$\frac{Live\ cell\ count\ (white\ cells)}{Total\ cell\ count\ (blue+white\ cells)} = \%\ \text{viable cells}$$

A worked example of cell counting can be found in the appendix. Using the live cell number calculated above, determine the dilution required to get the correct cell number of between $1.5x10^5$ and $7.5x10^5$ cells per mL. The seeding density depends on number of flasks required and when the flasks are required. For example, a higher seeding density is used if confluent flasks are required sooner.

$$C_1V_1=C_2V_2$$

Where:

C_1=the concentration you currently have

V_1 = the volume you have of your current concentration

C_2 =the concentration you want

V_2 =the volume you want

10. For continuing cell culture, prepare a cell suspension concentration of $2x10^5$ cells/mL. The total volume per T75 flask is 15 mL.
11. Add the required volume of complete cell culture medium (calculated in step 9) into a new flask and then add the cell suspension. Mix well by pipetting up and down.
12. Label the flask with cell type, operator's name, date, and passage number.

Note : THP-1 cells should not be passaged more than 20 times. Therefore, it is critical to prepare an adequate number of frozen stocks at early passages.

5.7.3 Freezing Cells

Notes

- Aliquots of early-passage cells should be frozen. Expand the cells so that at least 10 vials can be frozen.

- Record the total number of passages (at the time of sub-culture) and other data when freezing the cells. A log of the historical passage number is also important.
- Store the cryovials in different liquid nitrogen tanks if available to create a back-up of cells in case a liquid nitrogen tank fails

Protocol:

- Cryopreservation follows the same steps as that of the sub-culture protocol (section 3.8.2 until step 7).

1. Count cells to determine current cell density (as outlined in section 3.6.2, step 8).
2. Dilute cells to $1x10^6$ cells/mL in prewarmed cell culture medium.
3. Slowly add 5% (v/v) DMSO.
4. Pipette 1mL of the diluted cells into the freezing medium into a cryovial.
5. Place the cryovial into a suitable cryopreservation chamber and leave overnight at -80°C before removing and putting into liquid nitrogen for long-term storage.

5.7.4 Differentiation of THP-1 cells to macrophage like cells (dTHP-1):

- In the flow cabinet, prepare the PMA stock solution by adding pure ethanol to the tube containing the PMA (instead of ethanol, DMSO may be used), until a final concentration of 100 µg/ml. Dissolve by inverting the tube several times and visually check for complete dissolution. It is strongly advised to aliquot the PMA solution to prevent multiple freezing/thawing. Store at -20°C. *Alternatively, resuspend 1 mg PMA in 1.6 ml DMSO to obtain a concentration of 1mM; this solution is further diluted 100 times to obtain a stock solution of 10 µM.*

- Thaw the PMA stock solution at room temperature. When thawed, invert the tube several times. In the flow cabinet, take the 5x10^5 cells/ml THP-1 cell culture (see above) and add by pipetting the appropriate volume of PMA stock solution such that a 1000-fold dilution of PMA is obtained (100 ng/ml). For instance, if the THP-1 cell culture has a volume of 5 ml, add 5 µl PMA stock solution. *Alternatively, add 20 µl of the 10 µM stock solution per 10 ml cell suspension to obtain a final concentration of 20 nM.*

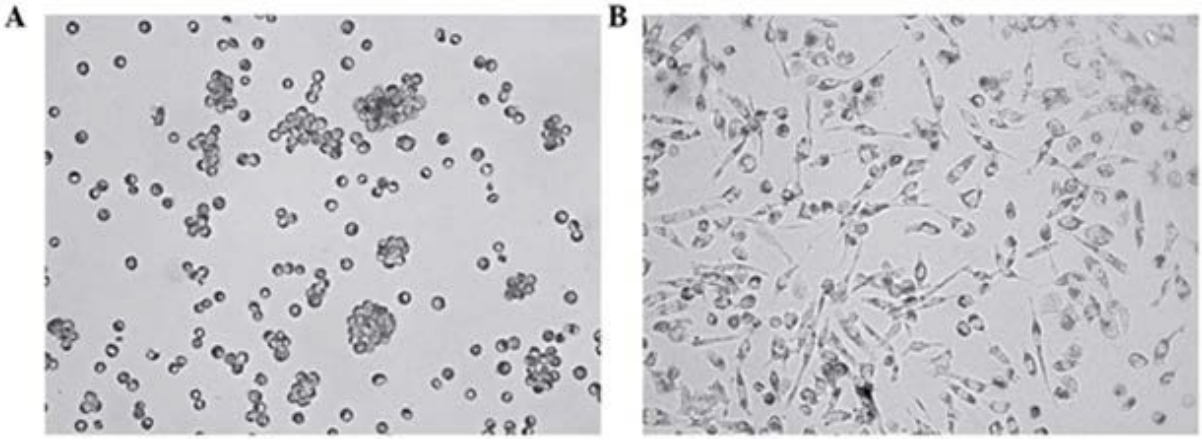

Figure 3. 3A shows THP-1 cells; 3B shows PMA-stimulated THP-1 cells.

- Add 100 µl of the cell suspension to each well of a 96-well flat bottom well plate (2x104 cells/well). Preferably, use a multichannel pipette. *Alternatively, cells can be differentiated in T-25 flasks (10^5 cells/ml) or by adding 1 ml of the cell suspension to each well of a 12-well plate (105 cells/well).*
- Leave the cells for 48 hours to differentate before changing the media to fresh CCM and leaving for an additional 48 hours for recovery before using them (see Figure 3).

5.8 Quality control & acceptance criteria:

Not applicable for this current SOP.

6 Data Analysis and Reporting of Data:

Not applicable for this current SOP.

7 Publications:

Not applicable for this current SOP.

8 References

https://www.hemocytometer.org/hemocytometer-protocol/