# MPS-003
# NPC CELL CULTURE FOR MPS

October 19, 2020
University of Pittsburgh Drug Discovery Institute

AUTHOR:

Richard DeBiasio — Date: 10/17/24

APPROVED:

Gregory M. LaRocca — Date: 10/19/24

Jacqueline Brown — Date: 10/19/24

| Revision History | | | |
|---|---|---|---|
| ***Version*** | ***By*** | ***Date*** | ***Revisions*** |
| TCTC 2 | Richard DeBiasio | 18-Mar-12 | Update for TCTC |
| 1.0 | Richard DeBiasio | 20-Oct-19 | Reformat for MPS |

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## A. PURPOSE AND APPLICABILITY

This protocol details the procedure used to maintain cells and provide cell suspensions for the MPS models used in the Liver microphysiology project. Cells are maintained in T75 flasks and when needed for experimental use in the model, the cells grown on flasks are used to generate cell suspensions that are then seeded into the SQL-SAL devices.

The cells used in the MPS models are intended to perform the function of the related cells in the human liver. LSECs serve as endothelial cells, THP-1 cells serve as Kupffer cells, and LX-2 cells serve as stellate cells.

## B. SUMMARY OF METHOD

Cells are maintained in vitro prior to use on the day of MPS model assembly. Cells are purchased, cryopreserved, passaged and defrosted as detailed in this SOP.

## C. DEFINITIONS

| | |
|---|---|
| SOP | Standard Operating Procedure |
| NPC | Liver Non-Parenchymal Cells |
| MPS | Microphysiology System |
| FBS | Fetal Bovine Serum |
| LSEC | Liver Derived Sinusoidal Endothelial Cells |
| DDI | University of Pittsburgh Drug Discovery Institute |
| HMM | Hepatocyte Maintenance Medium |
| IF | Immunofluorescence |
| PMA | phorbol 12-myristate 13-acetate |

## D. QUALIFICATIONS

1. Personnel must be trained on this protocol before performing these cell culture techniques.
2. Laboratory personnel will be trained on this SOP and essential instrumentation by qualified, experienced personnel.
3. Successful completion of training will be indicated by initialing and dating of the training record by the Trainer and Trainee.

## E. HEALTH AND SAFETY WARNINGS

- Cells, chemicals, media, reagents, and laboratory materials are handled according to the manufacturer's instructions or information stated in the Safety Data Sheets (SDS).
- Cells of human origin must be handled using universal precautions.
- All chemicals must be handled in compliance with the University of Pittsburgh's Chemical Hygiene Plan Safety Manual.
- All biological material must be handled in compliance with the University of Pittsburgh's Biosafety Guidelines.

## F. CAUTIONS

- Avoid microbial contamination by using aseptic technique

## G. INTERFERENCES

- Possible microbial contamination of cell or reagents

## H. EQUIPMENT AND SUPPLIES

### H1. CELLS

- LSEC
  - Samsara, catalog #HLLEC p1
  - Selected by lot (LINK)
  - SEM imaged to check fenestrations (LINK)
- THP-1 Cells [monocytes]
  - ATCC, catalog #TIB 202
- LX-2 Cells [stellate]
  - Millipore, catalog #SCC064

### H2. CELL-LINE MEDIA

- For LSEC
  - EBM-2
  - Prepare complete medium per manufacturer's recommendation
- For THP-1
  - RPMI
  - 10% FBS
  - 1% Pen/Strep
  - 2mM L-Glutamine
- For LX-2
  - DMEM
  - 2% FBS
  - 1% Pen/Strep
- Freezing Medium for LSEC
  - 90% FBS
  - 10% DMSO
- Freezing Medium for THP-1 and LX-2
  - 95% Complete Medium
  - 5% DMSO

### H3. SUPPLIES AND REGENTS

- EBM-2
  - Lonza, catalog #CC-3156 [or #CC-4176 single aliquots]
- RPMI

- Hyclone, catalog #SH30096.01
- DMEM
  - Life Technologies, catalog #11965-092
- FBS
  - Corning, catalog #35-010-CV
- L-Glutamine
- Pen/Strep
  - Hyclone, catalog #SV30010
- T75 Vented Tissue Culture Flask
- T150 Vented Tissue Culture Flask
- T150 Collagen I Coated Flasks
  - Corning, catalog #354486
- TrypLE Express
  - Life Technologies, catalog #12605-010
- Trypsin-EDTA
  - Hyclone, catalog #SV 30031.01
- Trypan blue
  - Sigma, catalog #T8154
- DMSO
  - Alfa Aesar, catalog #42780
- PMA
  - Calbiochem, catalog #524400
  - 400µg/mL stock stored at -80°C
    - 1mg PMA into 2.5mL DMSO
- TNF-α
  - Thermo, catalog #PHC3015
- Mouse anti-ICAM-1 Antibody
  - Invitrogen, catalog #MA5407
- Alexafluor 488 goat anti-mouse
  - Thermo, catalog #A11001

### H4. EQUIPMENT

- Nalgene Cell Freezer
  - Thermo, catalog #5100-0001

# I. PROCEDURAL STEPS

## I1. MAINTAINING CELLS IN CULTURE

### 1. General

1. For maintenance purposes cells are generally split on Mondays and Fridays.
2. Cultures should be split when they are approximately 70-80% confluent.
3. This split schedule may change to accommodate experimental requirements for cells.
4. THP-s and LX-2 cells are maintained in culture for no more than 20 passages.
5. To initiate a culture (note: see LSEC protocol for specifics):
    a. partially submerge frozen vial in a 37°C waterbath until only a small quantity of ice is visible in the vial. This should take less than two minutes.
    b. Avoid contaminating the vial seal with water.
    c. Rinse the vial with ethanol to decontaminate the surface before opening it.
    d. Transfer the contents of the vial to a 75 $cm^2$ flask containing 10 ml of medium.
    e. Incubate the flask.
    f. After 24 hours change the medium, then re-incubate until use.

### 2. Cell Counting with Hemacytometer

1. Dilute cells 1:2 with trypan blue (e.g., 50 µL cell suspension plus 50 µL trypan blue) in order to determine viability.
2. Some cells may be diluted more than 1:2 in order to obtain an optimal concentration for counting, but trypan blue should always be diluted 1:2
3. Count viable and non-viable cells separately in order to calculate viability
    a. Use the viable count to calculate cell number
    b. Cells with viability less than 80% are not to be used

### 3. LSEC Passage

These cells have a limited number of doublings in vitro due to loss of phenotypic characteristics of endothelial cells, and for this reason are not generally sub-cultured. These cells are instead defrosted on the day of use in the MPS devices.

1. LSECs are chosen by lot based on the following criteria:
    a. SEM imaging to check the fenestrations within the first week of culture—see *Electron Microscopy*
    b. ICAM-1 IF labeling post 25 ng/ml TNF-alpha for 16 hours—see *ICAM-1 Staining*
2. After thawing vial, transfer contents to a 15mL centrifuge tube containing 5 ml of pre-warmed medium. Rinse the vial 2X with 4 mL medium, for a total of 10 mL.
3. Centrifuge cells at 200g for 5 minutes.
4. Re-suspend the pellet in 10 ml of pre-warmed medium.
5. Dispense 1 mL of cell suspension into each of ten T150 collagen 1 coated flasks containing 20 mL of cell medium.
6. Incubate at 37°C, 5% $CO_2$.
7. Replace medium every 2-3 days as needed.
8. When flasks reach confluency, they are cryopreserved.

a. Detach cells from the flask using TrypLE Express. Do five flasks at a time.
    i. Wash cell surface 2x with 4 mL pre-warmed PBS per flask.
    ii. Incubate in 4 mL TrypLE per flask for 7 minutes at 37C.
    iii. Stop the TrypLE by addition of 4 mL medium per flask. Transfer to a 50 mL centrifuge tube.
    iv. Serially rinse the flasks with 5 mL of medium. Transfer to the centrifuge tube for a total of 45 mL cells suspension. 10 flasks will equal two tubes or 45 mL.
b. Perform a viable cell count, take the average of the two tubes.
c. Centrifuge the cells at 200g for 5 minutes.
d. Aspirate the supernatant.
e. Resuspend the combined pellets at 1 x $10^6$ cells/ml in pre-warmed cryopreservation medium.
f. Aliquot 1 ml per vial into labeled freezer vials
g. Transfer the vials to the Nalgene Cell Freezer then freeze overnight at -80°C.
h. Relocate the vials to LN2 for long term storage.

**4. THP-1 Passage**

1. Since THP-1 cells grow in suspension, splitting involves dilution of cells into fresh medium at a split ratio of 1:5 or 1:10 depending upon experimental needs.
2. Pipet up and down a few times to break up any clumps of cells that may have formed.
3. It is recommended that these cells be maintained at a density of less than 1 x $10^6$ per mL.

**5. LX-2 Passage**

1. LX-2 cells are generally grown to 70-80% confluency prior to routine passage.
2. Aspirate media from the flask using a Pasteur pipette.
3. Rinse cells with 5 ml TrypLE using 5 mL pipette.
4. Aspirate wash using a Pasteur pipette.
5. Transfer 1 ml TrypLE per flask using a 5 mL pipette.
6. Allow cells to detach at 37°C. Observe at frequent intervals, usually 2-5 minutes is sufficient to release cells.
7. Add 9 ml fresh medium.
8. Pipette up and down few times to break down the cells into a single-cell suspension.
9. Transfer the cells into 1-2 T75 flasks depending upon experimental needs with a split ratio of 1:3 or 1:5.
10. Add sufficient medium into each flask to equal 10 ml total.
11. Place flasks into the incubator.

## I2. CRYOPRESERVATION

**1. LSEC**

LSEC are not cryopreserved by DDI

**2. THP-1**

1. Defrost cells into a T75 flask and passage after 4-5 days (1 x $10^6$ cells/ml) with a split ratio of 1:3 into 3 T75 flasks at 10 ml per flask.
2. After 3-5 days passage into 4-6 T75 flasks at 10 ml per flask for a split ratio of 1:2.
3. After 3-5 days count cells and resuspend in Freezing medium (maintenance medium plus 5% DMSO) at a concentration of 2 x $10^6$ cells/ml.
4. Add 1 ml of cell suspension to a labeled vial, freeze at -80°C in a Nalgene cell freezer for one day, then transfer to LN2 for long term storage.

**3. LX-2**

1. Defrost cells into a T75 flask and passage after 4-5 days (70-80 % confluent) with a split ratio of 1:4 into 2 T150 flasks.
2. Trypsinize the cells using TrypLE and passage into 6 T150 flasks for a split ratio of 1:3 using all cells.
3. When the cells are approximately 80% confluent trypsinize the flasks using TrypLE, count cells and resuspend in Freezing medium (maintenance medium plus 5% DMSO) at a concentration of 1-2 x $10^6$ cells/ml.
4. Add 1 ml of cell suspension to a labeled vial, freeze at -80°C for one day in a Nalgene cell freezer then transfer to LN2 for long term storage.

## I3. Preparation for Experimental Use

**1. LSEC**

1. LSECS are defrosted on the day of use in the MPS model.
2. Cells are defrosted, resuspended in HMM used in the MPS model and a viable count is performed.
3. Resuspend the cells at the appropriate number for the MPS model.

**2. THP-1**

1. Two days before intended use, transfer 5 million cells into each of two T75 flasks.
2. Add PMA to the flasks at a final concentration of 200 ng/ml.
3. Incubate the treated flasks for 48 h during which time the cells will become adherent.
4. Aspirate the medium and non-adherent cells and remove the now adherent THP-1 cells using TrypLE as above, but incubate for 15 minutes, remove detached cells with a pipette then scrape any residual cells from the flask using a cell scraper.
5. Re-suspend cells from both flasks in a total of 2 ml RPMI medium and perform a viable cell count.
6. Transfer the appropriate number of cells into a centrifuge tube as per MPS protocol.

**3. LX-2**

1. A flask(s) of cells is chosen for use that has a cell density of approximately 70-80%.
2. Trypsinize per normal procedure, and perform a viable cell count.
3. Transfer the appropriate number of cells into a centrifuge tube as per MPS protocol.

## J. DATA AND RECORDS MANAGEMENT

Cell culture maintenance and usage records are maintained in laboratory notebooks associated with the experiments being done.

## K. QUALITY ASSURANCE AND QUALITY CONTROL

- Mycoplasma testing is done at the time of cryopreservation for THP-1 and LX-2 cells using the ATCC mycoplasma testing kit.
- Cell culture contamination is avoided by using acceptable sterile technique and visual inspection for media cloudiness along with appropriate cell morphology.
- Cell culture passage records are maintained in laboratory notebooks and in the MPS database as appropriate.

## L. REFERENCES

1. Minta, JO. et al. In Vitro Induction of Cytologic and Functional Differentiation of the Immature Human Monocytelike Cell Line U-937 With Phorbol Myristate Acetate. AJP. 1985, 119: 111-126.
2. Xu, L et al. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. GUT 2005, 54:142-151.
3. Li, X et al. A glass based, continuously zonated and vascularized human liver acinus microphysiological system (vLamps) designed for experimental modeling of diseases and ADME/TOX. Lab Chip 2018 18(17) 2614-31
4. LSEC: Lifenet.Health NPC-AD-LEC-P1
5. THP-1 : https://www.atcc.org/Products/All/TIB-202.aspx#culturemethod
6. LX-2: https://www.sigmaaldrich.com/catalog/product/mm/scc064?lang=en&region=US

## M. APPENDIX

### M1. ELECTRON MICROSCOPY

The protocol for the scanning electron microscopy (SEM) was provided by the Center for Biologic Imaging (CBI, University of Pittsburgh).

Briefly, the samples were fixed with 2.5% Glutaraldehyde for 1 hr,followed with 1% $OsO_4$ treatment for 1 hr. Samples were dehydrated using a graded series of ethanol solutions and then air-dried overnight. After removing the PET membranes from the intermediate layer and mounting them on specimen stubs, the samples were sputter-coated with 3.5 nm platinum (Cressington Scientific, Watford, UK), then imaged on a JSM-6330F electron microscope (JEOL, Peabody, MA).

See figure below for checklist details of protocol.

**SEM RUN UP CHECKLIST**

***All tissues are to be handled with gloves in the hood.***

Sample No.: _____

| Procedure: | Time: |
|---|---|
| 1. 2.5% Glutaraldehyde ___ | 1 hour* |
| 2. PBS ___ ___ ___ | 15 min each |
| 3. 1% $OsO_4$ ___ | 1 hour * |
| 4. PBS ___ ___ ___ | 15 min each |
| 5. 30% EtOH ___ | 15 min |
| 6. 50% EtOH ___ | 15 min |
| 7. 70% EtOH ___ | 15 min |
| 8. 90% EtOH ___ | 15 min |
| 9. 100% EtOH ___ ___ ___ | 15 min each |
| 10. HMDS (if not critical point drying) | 15min |
| 11. Air-drying | overnight |

*In normal processing never leave glut or $OsO_4$ more than 1 hour.

## M2. ICAM-1 STAINING

Cells are plated in a ???? and grown for X days. Then, they are treated with TNFα for 16 hours. Following treatment, cells are fixed and labeled. Positive labeling for ICAM-1 is acceptable.

1. Wash cells with PBS
2. Fix using 2% paraformaldehyde in PBS for 30 min at 4°C
3. Wash 3X with PBS
4. Wash 1X for 15 min with 150 mM glycine in PBS
5. Was 1X with PBS
6. Permeabilize for 1h with 0.1 % Triton X-100 in PBS at RT
7. Rinse 3X 10 minutes with PBS
8. Block for 1 hour at RT with PBS X 1% BSA
9. Incubate overnight at 4 deg C with Primary diluted 1:100 in PBS + 0.5% BSA
10. Wash 3X 15 min each with PBS
11. Incubate with Secondary 1:300 for 1 hour at RT in dark
12. Rinse 3X 10 min each with PBS
13. Incubate devices for 15 min with 100 µl PBS + 4 µg/ml Hoechst
14. Rinse 1X with PBS
15. Image cells.