Standard Operating Procedure **SOP040**

Title: Manual Culture of Primary Human Mesenchymal Progenitor Cells

Location: CBE Laboratory Unit

1. PURPOSE

To establish a Standard Operating Procedure for the routine manual isolation, growth, cryopreservation and sub-culture of adherent human mesenchymal progenitor cells.

2. SCOPE

To describe an experimental protocol for the manual sub culture of human mesenchymal progenitor cells, including aseptic technique, pipetting, swabbing, handling flasks, cleaning and Quality Control.

3. RESPONSIBILITES

Good laboratory practice should be observed for handling potentially infectious material.

All work involving the handling of potential pathogens should be performed in a Class II biological safety cabinet appropriate for the organism involved.

Keep work surfaces free of clutter. Clean working areas with a suitable disinfectant (1:50 chemgene and 70% IMS) between operations and allow 15 minutes between handling different cell types.

Use personal protective equipment (PPE) - Wear gloves and a laboratory coat throughout procedure.

Maintain separate bottles of media and separate pipette tips for each cell type cultivated

Do not allow stored media to go out of date

Handle limited numbers of cell vessels at one time to reduce the risk of contamination and spread of bacteria or mycoplasma.

Check that equipment (incubators, centrifuges, BSC, micropipettes etc) is cleaned, serviced and calibrated as appropriate.

All disposable contaminated items and spent media must be disinfected and autoclaved in accordance with SOP 003.

Any contaminated sharps must be disposed of in a sharps bin.

Correctly label reagents including flasks, medium and ampoules.

When work is finished, uncontaminated and autoclaved waste should be disposed of through the waste management system.

4. EQUIPMENT AND MATERIALS

4.1. Reagents:

i. **Dulbecco's Modified Eagles' Medium (DMEM)** low glucose, sterile-filtered, cell culture tested (Cambrex, 12-707F, 500ml)

ii. **Glutamax™**, Invitrogen,

iii. **Non-essential amino acid solution (NEAA)**

iv. **Hanks' Balanced Salt Solution (HBSS)** sterile, cell culture tested (Cambrex, 10-543F)

v. **Human bone marrow** – 25 ml Cambrex

vi. **Histopaque-1077 Hybri-Max**, defined density liquid, sterile (Accuspin™ tubes, Sigma, A7054)

vii. **Foetal Bovine Serum** (**FBS**) – hMSC qualified, EU standard, south American origin (Brazil), screened for viruses (IBR, PI3 and BVD) and the absence of mycoplasma, 0.2μm filtered (Invitrogen, 12662-029, reserved batch)

viii. **0.25% Trypsin-EDTA** solution, 2.5 g porcine trypsin and 0.2 g EDTA•4Na per liter of Hanks' Balanced Salt Solution with phenol red, sterile-filtered, cell culture tested (Sigma, T4049)

ix. **0.4% Trypan Blue** solution, liquid, sterile-filtered, cell culture tested (Sigma-Aldrich, T8154)

x. **DMSO freezing medium**, Sigma

Note: Antibiotics/Antimyotics could possibly be used, but are not in general use in the CBE. If to be used use at 1%.

4.2. Equipment:

i. Class II Biological Safety Cabinet

ii. Microscope

iii. Centrifuge
iv. Centrifuge tubes: Corning plug seal cap, sterile, polypropylene, non-pyrogenic, RCF:9400xg, max 50mL (Fisher, CFT-643-021J)
v. Medium aspiration pump
vi. CO_2 incubator (37˚C, 5% CO_2)
vii. Disposable Pipettes (sterile) and pipettes aids
viii. Disposable plastic tubes (~15ml)(sterile)
ix. Haemocytometer or Nucleocounter NC3000
x. T175 Flasks: Nunc EasY Flask, tissue culture, angled neck, polystyrene radiation sterilised, filter cap, 175cm^2 growth area (Fisher, TKT-130-210T)
xi. Water bath (37˚C)
xii. Planer controlled rate freezer
xiii. Cryostorage tank

5. PROCEDURE

All manipulations with open culture vessels or media must be performed in a biological safety cabinet using sterile techniques. Refer to SOP 009.

5.1 Preparation

All solutions, glassware, culture flasks etc should be sterile and all procedures carried out under aseptic conditions and in the sterile environment of the Class II BSC.

5.1.1 Prepare working solutions:

- DMEM supplemented with 10% Foetal Bovine Serum (FBS), 1% NEAA and 2mM Glutamax (1% stock).

NB: Supplemented media should be kept at 4°C and stored for no longer than 1 week.

- If cryopreserving cells defrost Sigma DMSO freezing medium and keep on ice until ready to use

5.1.2 Inspect Disposables

- Inspect all disposables (e.g. culture vessels, pipettes) for cracks or damage.

5.1.3. Open the BSC and clean working area with 1:50 Chemgene and spray everything in using 1:50 Chemgene.

5.1.4. Assess Culture Vessel(s) for Contamination before any process: Examine all culture(s) using an inverted microscope to confirm the absence of bacterial or fungal contamination and the absence of signs of distress (floating cells, excessive debris). Cultures contaminated or exhibiting signs of distress should be discarded.

5.2 Isolation and seeding of hMSCs

5.2.1. Transfer the bone marrow (BM) aspirate (25ml) into a 50 ml falcon tube. Add an equal volume of DMEM-LG-10FB. Mix thoroughly with a pipette (six 10 ml strokes).

5.2.2 Take an Accuspin tube and spin it at 800 g for 30 seconds to ensure all the Histopaque is beneath the divide. At room temperature (lower may cause clumping), add half the BM media mixture to the top of each of two Accuspin tubes.

5.2.3 Centrifuge at 800g on a swing-arm rotor for exactly 20 minutes with the centrifuge brake off (SOP 015).

5.2.4 Using a pipette, discard the upper transparent layer (plasma) to within 0.5 cm of the opaque interface layer (mononuclear cells).

5.2.5 Transfer the opaque layer to a new 50 ml falcon tube, add 10 ml of DMEM-LG-10FB, mix with a pipette, and centrifuge at 300 g for 10 minutes at 4ºC.

5.2.6 Discard the supernatant, re-suspend in 30 ml DMEM-LG-10FB, count and assess viability using 1 ml cell suspension in the Cedex cell counter (SOP).

5.2.7 Adjust the cell suspension volume and seed at 20000 viable cells per cm^2 in a T175 flasks (3 500 000 mononuclear cells per flask in 40 ml). Incubate at 37 ºC/5% CO_2 in a humidified incubator. Label each cell culture vessel with cell type, lot number, passage number, date, media and lot number, and operators initial.

5.2.8 On the 2^{nd} and 5^{th} day after seeding add 25 ml DMEM-LG-10FB to the flask.

5.2.9. On the 8^{th} day after seeding change the culture media by aspirating and discarding the old media and replacing with 40 ml DMEM-LG-10FB.

- Record the size of colonies, number of colonies, and rate of growth of colonies in each flask. Keep a photographic record of cell growth. Keep cells out of the incubator for a set time for monitoring (4 minutes per day).
- Record media Osmolality, Ph.

5.3 Subculture of hMSCs

5.3.1 On the 12th day after seeding aspirate the cell culture media and then add 8 ml room temperature trypsin-EDTA solution and incubate at 37ºC for 10 minutes.

5.3.2 Add 16 ml DMEM-LG-10FB and use the mixture to rinse any remaining weakly attached cells off the surface with a pipette (ignore more adherent trypsin resistant cells as these are less likely to be hMSCs). Gently Pipette cells without foaming to ensure a single cell suspension.

5.3.3 Centrifuge suspension from all the culture dishes at 300g for 5 minutes, remove and discard the supernatant, and re-suspend cell pellet in 20 ml DMEM-LG-10FB. Count the cells using 1 ml cell suspension in the Cedex cell counter (SOP).

5.3.4 Seed cells at 2500 cells/cm^2 (437500 per T175 flask) in 40 ml media and label each cell culture vessel with cell type, lot number, passage number, date, media and lot number, and operators initial. Replace media on the 4^{th} day after passage.

5.3.5 On the 7^{th} day after the first passage repeat the procedure from step 5.3.1 – 5.3.4. The cells should be evenly spread compared with the original colonies and only require 5 minutes exposure to trypsin for further sub-cultivation.

5.3.6 On the 7^{th} day after the second passage repeat the procedure from step 5.3.1 – 5.3.4. The cells should be evenly spread compared with the original colonies and only require 5 minutes exposure to trypsin for further sub-cultivation.

5.3.7 On the 7^{th} day after the third passage repeat the procedure from step 5.3.1 – 5.3.4. The cells should be evenly spread compared with the original colonies and only require 5 minutes exposure to trypsin for further sub-cultivation.

- Record any deviation in length of incubation with trypsin (try to keep standard). Record all cell counts. Keep a photographic record of cell growth. Keep cells out of the incubator for a limited time for monitoring (less than 5 minutes).

5.4 Cryopreservation of hMSCs

5.4.1 Label cryovials with a lot number and record cell type, lot number, passage number, date, media and lot number, and operator in a lab book along with references to lab book information relating to the pre-culture of the cells.

5.4.3 Centrifuge MNC suspension from step 5.2.6 at 300 g for 10 minutes. Discard supernatant. Resuspend cells at 100 x 10^6 cells/ml in DMEM-LG-10FB.

5.4.4 Add 50 µl of this cell suspension to each cryo vial.

5.4.5 Add 1 ml of freezing medium to each cryovial. Load the cryovials into a vial holder from the cryobank.

5.4.6 Rapidly transfer the cryovials to the controlled rate freezer (no more than 5 minutes after cells first exposed to freezing medium) and reduce the temperature at 1ºC per minute to -80 ºC (SOP 031). Once this temperature is reached transfer to the liquid nitrogen freezer.

5.5 Thawing of cryopreserved hMSCs

5.5.1 Defrost the required number of cryovials each containing 1 million cells by transferring to a 37ºC water bath. Remove from the water bath whilst still partially frozen.

5.5.2 Transfer the vial to the BSC (during in which the sample should become completely liquid) and gently transfer the sample into a 50 ml falcon containing 35 ml of pre-warmed DMEM-LG-10FB (minimise exposure to DMSO).

5.5.3 Centrifuge at 250 g for 5 minutes, discard supernatant, and re-suspend gently in 4 ml DMEM-LG-10FB.

5.5.4 Combine 100 µl cell suspension with 100 µl trypan blue and count viable cells using a Neubauer haemocytometer (SOP 034). Or use the Nucleocounter NC3000 with cassettes (See SOP 121)

5.5.4 Seed 20000 viable cells per cm^2 in a T175 flasks (3 500 000 mononuclear cells per flask in 40 ml) and proceed as from step 5.2.7

- Record time required in water bath (standardise), cell viability and cell attachment. Keep a photographic record of cell culture progress.

5.6 Output specification

P1 – Yield 3.85 – 4.55 million cells (10%-30%) increase.

P2 and subsequent passages – Yield 2.625 – 4.375 million cells (600%-1000%) increase

5.7 Clean Up Cell Culture Waste

- Discard all unused spent media and unused cells according to SOP 003.
- Discard all plasticware in a biohazard bag and discard sharps in a biohazard sharps

container.

- Reusable items must be soaked with 1% Virkon solution for a minimum of 1hr prior to washing and autoclaving.
- All apparatus (e.g. BSC, incubator) used in the Cell Culture should be cleaned regularly. Refer to equipment SOPs.

5.8 Quality Control - Signs of Instability or Deterioration in Cell Cultures

- Screen for the presence of Mycoplasma (refer to SOP 036) and other organisms (by macro or microscopic examination).

- Cell cultures should be routinely examined for changes in pH (optimal pH range for cell culture is 7.0 - 7.4). Phenol red is commonly used as an indicator. It is purple at pH 7.8, reddish-pink at pH 7.6, red at pH 7.4, becoming orange at pH 7.0, yellow at pH 6.5, and lemon yellow below pH 6.5. If phenol red is not present in the media, pH readings may be obtained by using pH paper or a pH meter.

- Cell cultures should be routinely examined for changes in characteristic cell morphology. Observe for excessive rounding of attachment dependent cells (cells will round during cell division), sloughing (shedding of cells off the culture vessel), increased cytoplasmic vacuolization, increased granularity around the nucleus, crenated (serrated) outer membrane edge, and retracted (shrunken) cells.

- Changes in cell growth may be attributed to cell damage during splitting (poor technique), aging of cells, changes in media or media components, contamination, and incubator temperature and/or gas flow changes.

6. DOCUMENTATION

6.1 Record changes to the protocol in a laboratory notebook

6.2 Record starting vial or culture vessel information in laboratory notebook.

6.3 Record and retain all suppliers data on primary cells supplied in laboratory notebook.

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SOP Version History

Version Reviewed	Date Revised/ Reviewed	Revision Summary	New Version Number
01	**01/12/2015**	Annual Review- Put into lean SOP template.	**02**
02	**26/06/18**	**Addition of 1:20 and 1:50 Chemgene for use in cleaning.** **Removed Antibiotic/antimyotics – as not used in the CBE for general use.**	**03**