Materials/Equipment:

1. Centrifuge
2. Refrigerator
3. -80°C freezer
4. Tank with liquid nitrogen (LN2)
5. Cell counting chamber with cover
6. Biosafety Cabinet Class IIA (BSC)
7. Mr. Frosty™
8. LN2 gloves
9. Lab coat
10. Goggles
11. Face mask / protector
12. Sterile petri dish
13. Tweezers
14. Scalpel

Solutions/Medium:

1. Dulbecco's Modified Eagle Medium Low Glucose (Sigma cat #D5546)
2. Fetal Bovine Serum (Gibco cat #10270106)
3. L-Glutamine (Sigma Cat #G7513)
4. Dulbecco's PBS solution without ca/mg (Lonza cat #17-516F)
5. Antibiotic/antimycotic (AB/AM) (Sigma cat #A5955)
6. CASO agar (Fluka cat #22095)
7. Trypsin 0.05% (Gibco cat #25300-062)
8. 0.36% and 0.22% Trypan Blue solution (Sigma cat #T8154)
9. Collagen type I, Calf Skin (Sigma Cat #C8919)
10. Dimethyl Sulfoxide (MP Biomedicals Cat #196055)

Consumables:

1. Nitrile gloves
2. Surgery mask
3. 70 % ethanol
4. 70 % ethanol spray
5. Small and big autoclavable bags
6. Sterile 15 and 50 mL centrifuge tube
7. Sterile 2 ml, 5 ml, 10 ml and 25 ml serological pipet
8. Sterile 1.5 ml microtubes

9. Micropipette and tips
10. Disposable petri dish
11. T75 flasks

Work sheet:

Name of officer : _____
Date of surgery : _____

Researcher:

A. Umbilical Cord Transport Procedure

☐ Umbilical cord with 10-20 cm in length will be obtained from mother post-sectio caesarean

☐ Put the tissue into 250 ml schott duran bottle containing _____ ml (target: 200 ml) PBS completed with 2% antibiotic/antimycotic (Ab/Am) until the tissue is covered, contain in coolbox filled with ice packs, and transported to the lab. **Note: The collected umbilical cord should be processed as soon as possible (less than 4 hours).**

Researcher :

B. Sterility test in BSC class IIA:

☐ Pipette _____ ml (target: 0.3-0.5 ml) of transport solution onto a CASO-agar plate, let it diffuse into agar, seal with parafilm, and incubate at 32 °C (upside-down).

☐ Put the tissue using sterile forceps onto the agar surface of a 2nd CASO-agar plate (weigh and roll it over), seal with parafilm and incubate at 32 °C (upside-down).

Researcher:

C. Isolation of Mesenchymal Stem Cells

☐ Transfer the umbilical cord into a new petri dish

☐ Soak the umbilical cord with 10 ml PBS + 1% Ab/Am and incubate in room temperature for 5-10 minutes, cut into 1 cm length, while cleaning the umbilical cord from the remaining blood, change the PBS solution until the tissue is totally white

☐ Move the tissue into a new petri dish and cut into 1 mm^2 size using scalpel, occasionally add few drops of PBS solution to prevent the tissue from drying out

☐ Put the cut tissues on __ collagen-coated petri dishes (target: 8-10 petri dishes)with some spacing in between (a distance of ±1 cm)

☐ Dry the small pieces of tissue until the pieces are attached onto the surface of the petri dish inside the BSC for ±10 minutes.

☐ Add __ ml (target: 5 ml) of complete DMEM (**see appendix**) + 20% FBS

☐ Incubate at 37 °C, 5% CO_2

☐ Observe the cell migration and change medium every 3-5 days until day 20.

☐ Observe the cell migration on the 20th day, detach and dispose umbilical cord pieces from the petri dish, then add new medium __ ml (target: 5 ml)

☐ Continue incubating the cells at 37 °C, 5% CO_2, change the medium every 3-5 days, until cell confluence is reached

Researcher:

D.Trypsinizing and Harvesting MSCs

☐ After cell reached confluence, discard all the supernatant, rinse the cells with 5 ml of PBS

☐ Trypsinize the cells with 2 mL trypsin 0.05% per petri dish. Incubate at 37 °C, 5% CO_2 for 5-10 minutes (or until cells are detached from the petri dish)

☐ Add the complete DMEM +20 % FBS 2 mL into each petri dish to stop the trypsin activity

☐ Collect all the cell suspension into a centrifuge tube.

☐ Centrifuge at 1500 rpm for 5 minutes

☐ Discard the supernatant, and resuspend with __ mL (target: 5 mL) of complete DMEM + 20% FBS

☐ Count the cells using trypan blue exclusion method

☐ **Isolated cells (IC) counting**

Counter I : ☐☐

Total volume of cell suspension : _____ ml
Dilution factor : _____
Viable cells : _____/4 squares
MSCs per ml : _____
Total MSCs : _____

Counter II : ☐☐

Total volume of cell suspension : _____ ml
Dilution factor : _____
Viable cells : _____/4 squares
MSCs per ml : _____
Total MSCs : _____

Resume
MSCs/ml : _____
Total MSCs : _____
Viability : _____ %

☐ Seed the cells in a T75 flask to culture the cells (P1) with the addition of ___ ml (target: 15 ml) Complete DMEM + 20% FBS

☐ Keep incubating the cells at 37 °C, 5% CO_2

☐ Change the medium every 3-5 days until the cell reached confluence, then trypsinize the cells to obtain P2 and so on

☐ Some of the cells (depends on the necessity) on Passage 3 (P3) will be cryopreserved.

Researcher:

E. Freezing the Cells

☐ Detach cells using trypsin and collect the cell suspension (**refer to step 1-4 from section D**)

☐ Centrifuge at 1500 rpm for 5 minutes

☐ Discard supernatant after centrifugation and re-suspend the cells pellet in _____ µl (target: maximum 900 µl/cryotube) of cold complete DMEM + 20% FBS (**see appendix**) with cell density of ±2 million cells/cryotube for _____ ea cryotubes.

☐ Transfer cells suspension into sterile cryotubes

☐ Add _____ ml (target: same volume with complete DMEM +20% FBS) freshly prepared cold freezing medium and mix it once with pipette, immediately close and transfer to Mr. Frosty™

☐ Keep the cryotube inside Mr. Frosty™ and put Mr. Frosty™ inside -80 °C freezer to achieve a cooling rate of 1 °C per minute, overnight. **Note:** check the amount of propanol inside Mr. Frosty™ container and make sure the volume is right.

☐ Transfer the cryotubes for storage in the liquid nitrogen (-196 °C)

☐ At -196 °C, the frozen cells can be preserved as long as needed

**** If the liquid nitrogen is not available yet, alternatively the frozen cells can be preserved at -80°C freezer for half a year.**

Researcher:

F. Conditioned Medium Production

☐ Cells on P5 or P6 will be cultured in complete DMEM + 20% FBS

☐ Incubate cells at 37 °C, 5% CO_2, until cell reached confluence

☐ Dispose the medium, then add complete DMEM to starve the cells (minimum nutrition)

☐ Incubate at 37 °C, 5% CO_2, for 72 hours

☐ Collect the medium into a centrifuge tube (50 ml)

☐ Centrifuge at 1500 rpm for 5 minute

☐ Collect the conditioned medium (CM) into a new centrifuge tube (50 ml) for further use or;

☐ Store the CM at -80 °C freezer for up to 3 months

APPENDIX :

Medium Preparation

Complete DMEM preparation

☐ Supplement 0.584 g/L L-Glutamine (Sigma Cat # G7513) to Dulbecco's Modified Eagle Medium Low Glucose (Sigma cat #D5546)

☐ Mix completely

Freezing medium preparation:

☐ In a 15 or 50 mL centrifuge tube, mix FBS, completed DMEM, and DMSO with 2:7:1 ratio.

☐ Sterilize using 0.22 µm filter

REPUBLIK INDONESIA
KEMENTERIAN HUKUM DAN HAK ASASI MANUSIA

SURAT PENCATATAN

CIPTAAN

Dalam rangka pelindungan ciptaan di bidang ilmu pengetahuan, seni dan sastra berdasarkan Undang-Undang Nomor 28 Tahun 2014 tentang Hak Cipta, dengan ini menerangkan:

Nomor dan tanggal permohonan	:	EC00201973964, 2 Oktober 2019
Pencipta		
Nama	:	**Dr. dr. Siufui Hendrawan, M.Biomed**
Alamat	:	Jl. Letjen S. Parman No.1, Universitas Tarumanagara, Fakultas Kedokteran, Jakarta Barat, Dki Jakarta, 11440
Kewarganegaraan	:	Indonesia
Pemegang Hak Cipta		
Nama	:	**Dr. dr. Siufui Hendrawan, M.Biomed**
Alamat	:	Jl. Letjen S. Parman No.1, Universitas Tarumanagara, Fakultas Kedokteran, Jakarta Barat, Dki Jakarta, 11440
Kewarganegaraan	:	Indonesia
Jenis Ciptaan	:	**Buku Panduan/Petunjuk**
Judul Ciptaan	:	**Conditioned Medium Umbilical Cord Mesenchymal Stem Cell Preparation**
Tanggal dan tempat diumumkan untuk pertama kali di wilayah Indonesia atau di luar wilayah Indonesia	:	30 September 2019, di DKI Jakarta
Jangka waktu pelindungan	:	Berlaku selama hidup Pencipta dan terus berlangsung selama 70 (tujuh puluh) tahun setelah Pencipta meninggal dunia, terhitung mulai tanggal 1 Januari tahun berikutnya.
Nomor pencatatan	:	000156983

adalah benar berdasarkan keterangan yang diberikan oleh Pemohon.
Surat Pencatatan Hak Cipta atau produk Hak terkait ini sesuai dengan Pasal 72 Undang-Undang Nomor 28 Tahun 2014 tentang Hak Cipta.

a.n. MENTERI HUKUM DAN HAK ASASI MANUSIA
DIREKTUR JENDERAL KEKAYAAN INTELEKTUAL

Dr. Freddy Harris, S.H., LL.M., ACCS.
NIP. 196611181994031001