SOP: **Generation and Propagation of Cultured Human Amniotic Fluid Mesenchymal Stem/Stromal Cells (AF-MSCs)**
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Cell Information

Cultured human Amniotic Fluid Mesenchymal Stem/Stromal Cells (AF-MSCs) are isolated from second trimester amniotic fluid samples, collected during scheduled amniocentesis for prenatal diagnosis between the 15th and 18th weeks of gestation at the Alexandra Hospital, University of Athens, Medical School, Athens, Greece.

Second trimester AF samples are obtained following written informed consent, approved by the Ethical Committee of the Alexandra Hospital and the Bioethics Committee of the School of Medicine of the University of Athens, Athens, Greece

Amniocentesis was performed under aseptic conditions. Using a 22-gauge needle and under ultrasonographic control, 10-15 ml of AF was aspirated for each sample according to the Alexandra Hospital regulations. The procedure-related spontaneous abortion rate ranges from 0.06 to 0.5%.

Materials List

1. Dulbecco's Modified Eagle Medium (DMEM) (1X), liquid (high glucose), with L-glutamine (Life Technologies, Cat# 41966-029)
2. Fetal Bovine Serum (Life Technologies, Cat# 10500-064)
3. Penicillin/Streptomycin, liquid (Life Technologies, Cat# 15140-122)
4. Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid (Lonza, Cat# LONZ17-516Q)
5. 75cm^2 and 25cm^2 Tissue Culture Flasks and 6 well tissue culture plates
6. Conical Polypropylene Centrifuge Tubes (15mL and 50mL)
7. Graduated Serological Pipets (1, 5, 10, 25, 50mL)
8. Trypsin-EDTA (10X), (Life Technologies, Cat# 15400-054)
9. Accutase Enzyme Cell Cell Dissociation Reagent (Life Technologies, Cat# A11105-01)
10. DMSO, (Sigma-Aldrich, Cat# D2650)
11. Freezing Medium (90% FBS, 10% DMSO)
12. Freezing cryotubes (Corning, Cat# 430489)
13. Beckman Coulter Centrifuge
14. -80 Freezer (Thermo Scientific)
15. Mr. Frosty™ Freezing Container (Thermo Scientific)
16. Neubauer counting chamber
17. Trypan blue (BIOCHROM, Cat#L6323)
18. Microscope
19. Marker pen

Growth Medium for AF-MSCs

400mL DMEM high Glucose Medium
100mL FBS
5mL Penicillin/Streptomycin

Freezing Medium for AF-MSCs Cells

9mL FBS
1mL DMSO

Procedure

A. Starting Cell Culture and select colonies of AF-MSCs

1) Following collection, the excess volume from each AF sample used for prenatal diagniosis (5-10mL), which is normally discarded, is centrifuged at 1,300 rpm for 10 min.
2) Resuspended the pellet in DMEM supplemented with 20% (vol/vol) FBS.
3) Plate 1 x 10^5 AF cells/well into six- well plates.
4) To culture, place the plate in a 37°C, 5% CO2 humidified incubator for approximately 18-20 days, when the first colonies appeared.
5) Change medium was changed every 5 days.
6) When CFUs are formed, label the colonies on the plate with marker and mechanically select the spindle-shaped (SS) AF-MSCs colonies, using a 200µL tip, and sub-culture them separately to higher passages.
7) Freeze at early passages.

B. Thawing Frozen AF-MSCs and Starting Cell Culture

1) When ready to start cell culture, quickly thaw 1 vial in a small container of room temperature water or a in a 37^{o}C waterbath.
2) After about 10-15 seconds, dispense contents of vial into a 15mL conical and centrifuge tube containing 8mL complete culture medium.
3) Pellet cells at 1200 rpm x g for 10 minutes.
4) Re-suspend cell pellet in 5mL complete culture medium and dispense into one 25cm^2 tissue culture flask (1.5 x 10^5 AF-MSCs/25cm^2 tissue culture flask)
5) To culture, place the flask in a 37°C, 5% CO_2 humidified incubator.

C. Sub-culture

1) Propagate cells for 3-4 days, changing medium every 1-2 days.
2) Aspirate medium.
3) Wash cells with 1X D-PBS.
4) Add 1 mL of Trypsin to 25cm^2 tissue culture flask (2mL for 75cm^2 tissue culture flask) and let sit at room temperature for 5-10 minutes, or until cells detach.
5) Immediately remove cells, rinse tissue culture plate with equal amount of growth medium to collect residual cells, and pellet at 1200rpm x g for 5 minutes.
6) Gently re-suspend cell pellet in growth medium.
7) Perform 1:2 to 1:4 split every 3-4 days.

D. Harvest

1) At time of harvest, rinse plates with PBS.
2) Add 3mL Accutase and incubate for 10-15 minutes at 37°C.
3) Remove cells to 15mL conical centrifuge tube and rinse dish with culture medium to collect residual cells.
4) Pellet cells at 300 x g for 5 minutes.
5) Wash pellet in PBS
6) Count number of cells and proceed to SOP for cultured cells: crypreservation of adherent mammalian tissue culture cells, nuclei isolation, and DNaseI treatment.

Key publications:

1. Roubelakis MG, Pappa KI, Bitsika V, Zagoura D, Vlahou A, Papadaki HA, Antsaklis A, Anagnou NP. Molecular and proteomic characterization of human mesenchymal stem cells derived from amniotic fluid: comparison to bone marrow mesenchymal stem cells. Stem Cells Dev. 2007 Dec;16(6):931-52.
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2. Roubelakis MG, Bitsika V, Zagoura D, Trohatou O, Pappa KI, Makridakis M, Antsaklis A, Vlahou A, Anagnou NP.
   In vitro and in vivo properties of distinct populations of amniotic fluid mesenchymal progenitor cells. J Cell Mol Med. 2011 Sep;15(9):1896-913. doi: 10.1111/j.1582-4934.2010.01180.x.
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3. Zagoura DS, Roubelakis MG, Bitsika V, Trohatou O, Pappa KI, Kapelouzou A, Antsaklis A, Anagnou NP. Therapeutic potential of a distinct population of human amniotic fluid mesenchymal stem cells and their secreted molecules in mice with acute hepatic failure. Gut. 2012 Jun;61(6):894-906. doi: 10.1136/gutjnl-2011-300908. Epub 2011 Oct 13.
   http://www.ncbi.nlm.nih.gov/pubmed/21997562

4. Zagoura DS, Trohatou O, Bitsika V, Makridakis M, Pappa KI, Vlahou A, Roubelakis MG, Anagnou NP. AF-MSCs fate can be regulated by culture conditions.Cell Death Dis. 2013 Apr 4;4:e571. doi: 10.1038/cddis.2013.93.
   http://www.ncbi.nlm.nih.gov/pubmed/23559005

5. Roubelakis MG, Trohatou O, Roubelakis A, Mili E, Kalaitzopoulos I, Papazoglou G, Pappa KI, Anagnou NP.Platelet-rich plasma (PRP) promotes fetal mesenchymal stem/stromal cell migration and wound healing process. Stem Cell Rev. 2014 Jun;10(3):417-28. doi: 10.1007/s12015-013-9494-8.
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6. Roubelakis MG, Tsaknakis G, Pappa KI, Anagnou NP, Watt SM.
   Spindle shaped human mesenchymal stem/stromal cells from amniotic fluid promote neovascularization. PLoS One. 2013;8(1):e54747. doi: 10.1371/journal.pone.0054747. Epub 2013 Jan 24.
   http://www.ncbi.nlm.nih.gov/pubmed/23359810