Stemgent® StemRNA™-SR Reprogramming Using Blood-Derived EPCs

Overview

This protocol describes procedures for reprogramming human blood-outgrowth endothelial progenitor cells (EPCs) using self-replicative RNA (srRNA) to generate induced pluripotent stem cells (iPSCs). The Stemgent StemRNA™-SR Reprogramming Kit supports the reprogramming of five wells in a standard 6-well plate format.

StemRNA-SR reprogramming experiments can be performed under both atmospheric conditions (21% O_2) and decreased oxygen levels (5% O_2). However, the reprogramming process is more efficient under low oxygen levels, often yielding 2 to 5 times as many iPSC colonies[1]. Stemgent recommends using a 5% O_2 hypoxic incubator for increased efficiency in reprogramming experiments.

This protocol specifically outlines the procedures for reprogramming one well of EPCs of a 6-well tissue culture plate. **Please scale appropriately for larger experiments.**

> **NOTE:** Please read and understand this entire protocol prior to beginning any experiments. To maintain sterility, all procedures (except as indicated) should be performed in a biological safety cabinet.

Caution

These procedures use EPCs derived from human blood. These cells are a potential source of infection with blood-borne pathogens. Prior to beginning the experiment, consult with your institution's biosafety group for specific guidelines on how to minimize your exposure. Appropriate personal protective equipment (lab coats, gloves, safety glasses, etc.) should be worn throughout these procedures.

Contents:

Stemgent® StemRNA™-SR Reprogramming Using Blood-Derived EPCs

Abbreviations

bFGF	Basic fibroblast growth factor (FGF-basic), human
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulfoxide
EGM	Endothelial growth medium
EPC	Human blood-outgrowth endothelial progenitor cell
FBS	Fetal bovine serum
hESC	Human embryonic stem cell
HSA	Human serum albumin
iPSC	Induced pluripotent stem cell
OKSiM	Oct4, Sox2, Klf4, IRES, c-Myc
PBS	Phosphate-buffered saline
RT-PCR	Reverse transcriptase polymerase chain reaction
srRNA	Self-replicative RNA

Stemgent® StemRNA™-SR Reprogramming Using Blood-Derived EPCs

Required Materials

PRODUCT DESCRIPTION	CAT. NO.	AMOUNT	STORAGE
StemRNA™-SR Reprogramming Kit **OKSiM-srRNA (5 µg, 100 µg/µL)** **microRNA Reprogramming Cocktail (20 µM)** **B18R Recombinant Protein, Carrier-free (0.5 mg/mL)**	Stemgent 00-0075 Stemgent 05-0038 Stemgent 05-0036 Stemgent 03-0017	 50 µL 35 µL 100 µL	-80 °C
NutriStem™ XF/FF Culture Medium	Stemgent 01-0005	500 mL	-20 °C
FGF-basic, Human Recombinant (bFGF)	Stemgent 03-0002	50 µg	-80 °C
StainAlive™ TRA-1-60 (Dylight™ 488), mouse anti-human	Stemgent 09-0068	100 µL	4 °C
Lipofectamine® MessengerMAX™ Transfection Reagent	Life Technologies LMRNA001	Per manufacturer's instructions	
DMEM, high glucose	Life Technologies 11965-092	Per manufacturer's instructions	
Puromycin (10 mg/mL)	Life Technologies A11138-02	Per manufacturer's instructions	
PBS	Life Technologies 10010-23	Per manufacturer's instructions	
Trypsin-EDTA (0.05%), phenol red	Life Technologies 25300054	Per manufacturer's instructions	
EGM™ Bullet Kit	Lonza CC-3162	Per manufacturer's instructions	
FBS, mESC-qualified	Hyclone SH30070.03E	Per manufacturer's instructions	
Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units 500 mL; Pore Size: 0.20 µm	Thermo Scientific 569-0020	Per manufacturer's instructions	
Matrigel® hESC-qualified Matrix	Corning 354277	Per manufacturer's instructions	
Human Serum Albumin (HSA)	-	-	
Standard Tissue Culture laboratory supplies and equipment			

Optional Reagents & Equipment

PRODUCT DESCRIPTION	CAT. NO.
RNAse Zap™	Ambion AM9780
Low oxygen incubator	-

Related Stemgent Protocols

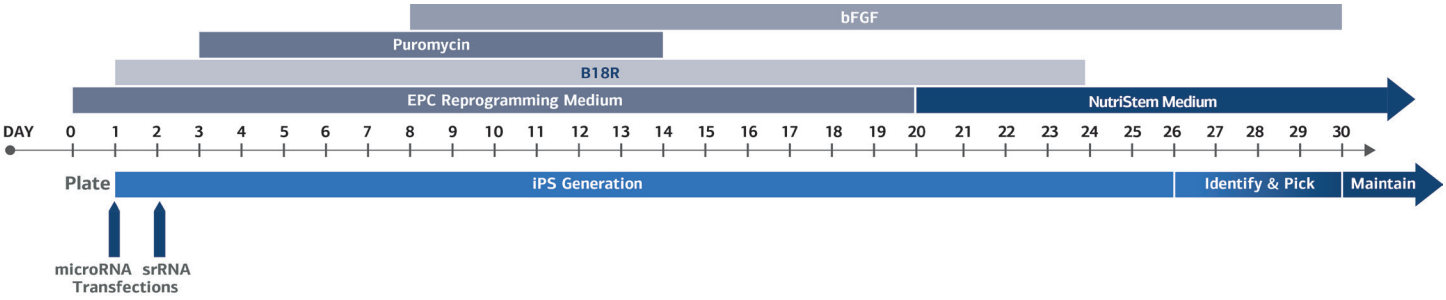
For derivation and culturing of EPCs:

"EPC Derivation from Whole Blood", available through Stemgent Technical Support.
E-mail: tech.support@stemgent.com

For live staining with Stemgent StainAlive TRA-1-60 antibody to verify pluripotency prior to picking colonies:

"Protocol: Immunocytochemistry of Live Cells", which can be found on the Stemgent website at: www.stemgent.com/support/protocols.

Experimental Timeline



Day 0: Plate target cells

Day 1: microRNA transfection and start B18R supplementation

Day 2: srRNA transfection

Day 3: Start puromycin selection

Day 8: Start bFGF supplementation

Day 14: End puromycin selection

Day 20: Switch to NutriStem medium

Day 24: End B18R supplementation

Reprogramming Protocol

Step 1: Material Preparation

1.1 Preparation of EPC Reprogramming Medium

1. Thaw FBS in refrigerator overnight.
2. Add 50 mL FBS and the EGM-2 SingleQuots (all except for the SingleQuot-provided heparin and FBS) to 500 mL EBM-2 basal medium, to obtain **EPC Reprogramming Medium**.

NOTE: Heparin is a highly charged protein that will interfere with the transfection reagent.

3. Filter **EPC Reprogramming Medium** using 0.2 µm filter unit.
4. Store **EPC Reprogramming Medium** at 4 °C for up to four weeks.

NOTE: 50 mL of the **EPC Reprogramming Medium** is sufficient to reprogram one well of a 6-well plate.

1.2 Human bFGF Solution (4 µg/mL stock solution)

1. Briefly centrifuge the lyophilized vial of bFGF.
2. Reconstitute the lyophilized bFGF in 500 µL 10 mM Tris pH 7.6.
3. Transfer the reconstituted bFGF to a 15 mL conical tube.
4. Add 12 mL of 0.1% HSA in PBS to yield a 4 µg/mL working solution.
5. Filter sterilize the working solution using a 0.2 µm pore size, low protein-binding filter.
6. Aliquot the working solution into single-use volumes in sterile, low protein-binding microcentrifuge tubes.
7. Store the bFGF aliquots at -20 °C for up to 6 months.

1.3 B18R Recombinant Protein (0.5 mg/mL stock solution)

1. Thaw vial of B18R recombinant protein on ice. Once thawed, keep the vial on ice at all times.
2. Briefly centrifuge the vial to collect the contents at the bottom of the tube.
3. Pipet 6.6 µL B18R solution directly to the bottom of 15 sterile, low protein-binding microcentrifuge tubes. This is sufficient volume for one well of a 6-well plate for 8 days.
4. Freeze and store the B18R aliquots at -80 °C until use.
5. After thawing, store one aliquot at 4 °C and use within 8 days.

1.4 Puromycin (1 mg/mL stock solution)

1. Thaw vial of puromycin on ice. Once thawed, keep the vial on ice at all times.
2. Dilute puromycin in PBS to 1 mg/mL working solution.
3. Store at 4 °C for up to four weeks.
4. Aliquot remaining puromycin into sterile microcentrifuge tubes and store at -20 °C.

Step 2: Preparation of Target Cells

2.1 Preparation of EPCs

Prepare EPCs as described in the Stemgent protocol "EPC Derivation from Whole Blood." Derivation and Preparation of EPCs from whole blood will take approximately 18-21 days before they are ready to be plated for the reprogramming experiment.

NOTE: Each reprogramming experiment is completed in one well of a 6-well plate of cells.

2.2 Plating of EPCs for Reprogramming Experiment – DAY 0

1. Following manufacturer's instruction, coat an appropriate number of wells of a 6-well plate with Corning Matrigel extracellular matrix at least 1 hour prior to seeding EPCs.
2. Remove the used culture medium from the T25 flask of exponentially growing EPCs to be harvested.
3. Add 5 mL of PBS to the culture surface of the flask to wash, then carefully aspirate the PBS.
4. Add 3 mL of 0.05% Trypsin/EDTA to the culture surface of the flask and incubate for 3 to 5 minutes at 37 °C and 5% CO_2.
5. Tap the flask to completely detach the cells from the culture surface.
6. Add 6 mL of **EPC Reprogramming Medium** to the flask to neutralize the Trypsin/EDTA.
7. With a 5 mL pipet, transfer the harvested cell suspension from the flask to a 15 mL conical tube. Pipette up and down gently to disrupt the cell aggregates.
8. Centrifuge the cells for 5 minutes at 250 x g.
9. Remove the supernatant and resuspend the pellet in 1 mL of **EPC Reprogramming Medium**.
10. Count the cells and calculate the live cell density.
11. To each well of the Corning Matrigel-coated plate, add 2 mL of the EPC suspension to a final cell density of 2.0 x 10^5 cells per well.
12. Incubate the cells overnight in an incubator set at 37 °C, 5% CO_2 and 21% O_2.

Step 3: Transfections and Media Changes

Optional: Wipe down all working surfaces (gloves, reagent bottles, biosafety cabinet surfaces, pipettors, etc.) with RNAse Zap.

3.1 microRNA (miRNA) Transfection – DAY 1

1. Warm **EPC Reprogramming Medium** in a 37 °C water bath.
2. Prepare **B18R-supplemented EPC Reprogramming Medium** according to the following:

EPC Reprogramming Medium:	**2000.0 µL**
B18R (0.5 mg/mL)	**0.8 µL**
Final Volume:	**2000.8 µL**
Final B18R Concentration	*200 ng/mL*

3. Remove the **EPC Reprogramming Medium** from each well of the EPC reprogramming plate and replace with 2 mL of **B18R-supplemented EPC Reprogramming Medium**.
4. Equilibrate MessengerMax and DMEM at room temperature for at least 15 minutes.
5. Thaw the microRNA Reprogramming Cocktail supplied in the StemRNA-SR kit at room temperature, then immediately place on ice.
6. Label two sterile, RNAse-free 1.5 mL microcentrifuge tubes “A” and “B”. Tube A will be used for DMEM plus microRNA Reprogramming Cocktail. Tube B will be used for DMEM plus MessengerMax.

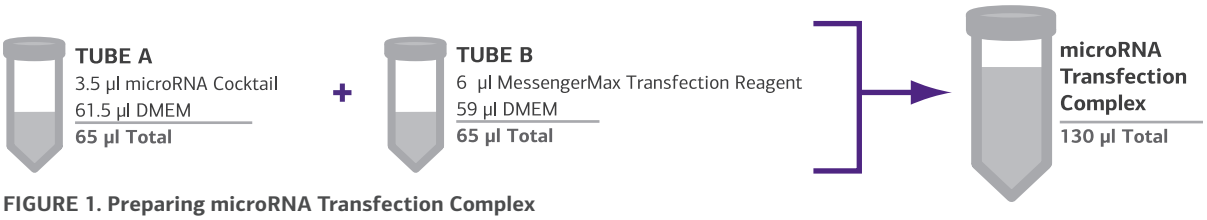


FIGURE 1. Preparing microRNA Transfection Complex

7. To Tube A add 61.5 µL DMEM, then add 3.5 µL microRNA Cocktail from the StemRNA-SR kit.
8. To Tube B add 59 µL DMEM , then add 6 µL MessengerMax transfection reagent.
9. Mix the contents of each tube by tapping the bottom of the tube with your finger.
10. Using a pipettor, transfer the entire contents of tube B to tube A drop-wise at the meniscus level to generate the **microRNA Transfection Complex**.
11. Mix the **microRNA Transfection Complex** by tapping the bottom of the tube, then incubate at room temperature for 15 minutes.
12. Add 130 µL of **microRNA Transfection Complex** to a single well of cells in the EPC reprogramming plate by tilting the plate and pipetting drop-wise into medium in the well. Mix by rocking in the X- and Y-directions.
13. Return the EPC reprogramming plate to a hypoxic incubator (5% O_2) and incubate overnight.

3.2 Self-Replicative RNA (srRNA) Transfection – DAY 2

Optional: Wipe down all working surfaces (gloves, reagent bottles, biosafety cabinet surfaces, pipettors, etc.) with RNAse Zap.

1. Warm **EPC Reprogramming Medium** in a 37 °C water bath.
2. Prepare fresh **B18R-supplemented EPC Reprogramming Medium** according to the following:

EPC Reprogramming Medium	**4000.0 µL**
B18R (0.5 mg/mL)	**1.6 µL**
Final Volume:	**4001.6 µL**
Final B18R Concentration	*200 ng/mL*

3. Remove the EPC Reprogramming Medium containing the microRNA Transfection Complex from each well of the EPC reprogramming plate and replace with 2 mL of **B18R-supplemented EPC Reprogramming Medium**.
4. Equilibrate MessengerMax and DMEM to room temperature for 15 minutes.
5. Thaw OKSiM-srRNA supplied in the StemRNA-SR kit at room temperature, then immediately place on ice.
6. Label 2 sterile, RNAse-free 1.5 mL microcentrifuge tubes “C” and “D”. Tube C will be used for DMEM plus OKSiM-srRNA. Tube D will be used for DMEM plus MessengerMax.

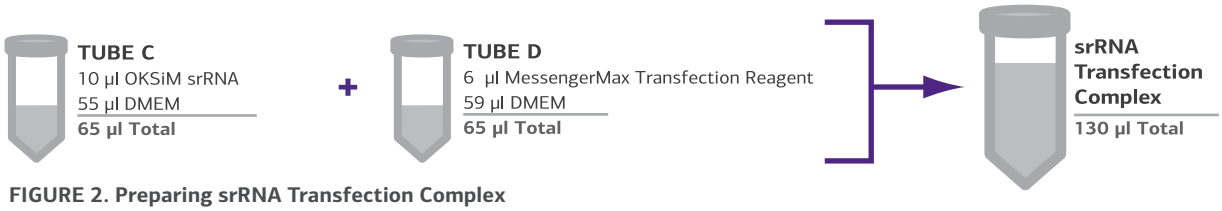


FIGURE 2. Preparing srRNA Transfection Complex

7. To Tube C add 55 µL DMEM, then 10 µL OKSiM-srRNA.
8. To Tube D add 59 µL DMEM, then 6 µL MessengerMax transfection reagent.
9. Mix by tapping the bottom of the tubes with your finger.
10. Using a pipettor, transfer the entire contents of Tube D to Tube C to make the **srRNA Transfection Complex**.
11. Mix by tapping the tube as before, then incubate at room temperature for 15 minutes.
12. Add 130 µL of srRNA Transfection Complex to a single well of cells in the EPC reprogramming plate by tilting the plate and pipetting drop-wise into the existing medium. Mix by rocking in the X- and Y-directions.
13. Incubate 4 hours in hypoxic incubator (5% O_2).
14. After the incubation, aspirate the medium containing the srRNA Transfection Complex and replace with 2 mL pre warmed **B18R-supplemented EPC Reprogramming Medium**. Incubate overnight in a hypoxic incubator (5% O_2).

3.3 Puromycin Addition – DAYS 3-7

Beginning on day 3, add puromycin to the medium, changing the puromycin-supplemented medium every day thereafter.

1. Warm EPC Reprogramming Medium in a 37 °C water bath.
2. Prepare **Puromycin/B18R-supplemented EPC Reprogramming Medium** according to the following:

B18R (0.5 mg/mL)	**0.8 µL**
Puromycin (1 mg/mL)	**0.8 µL**
EPC Reprogramming Medium	**2000.0 µL**
Final Volume	**2001.6 µL**
Final B18R concentration	*200 ng/mL*
Final puromycin concentration	*0.4 µg/mL*

3. Remove used medium from the single well of the EPC reprogramming plate and replace with 2 mL per well freshly prepared **Puromycin/B18R-supplemented EPC Reprogramming Medium**.
4. Incubate overnight in a hypoxic incubator (5% O_2).
5. Repeat media change with freshly prepared **Puromycin/B18R-supplemented EPC Reprogramming Medium** every day through Day 7.

3.4 bFGF Addition – DAYS 8-13

Beginning on day 8, in addition to the puromycin and B18R, supplement the medium with bFGF. Change the bFGF/Puromycin B18R supplemented medium every day thereafter.

1. Warm EPC Reprogramming Medium in a 37 °C water bath.
2. Prepare **bFGF/Puromycin/B18R-supplemented EPC Reprogramming Medium** according to the following:

B18R (0.5 mg/mL)	**0.8 µL**
Puromycin (1 mg/mL)	**0.8 µL**
bFGF (4 µg/mL):	**10.0 µL**
EPC Reprogramming Medium:	**2000.0 µL**
Total:	**2011.6 µL**
Final B18R concentration	*200 ng/mL*
Final puromycin concentration	*0.4 µg/mL*
Final bFGF concentration	*20 ng/mL*

3. Remove used medium from one well of the EPC reprogramming plate and replace with 2 mL **bFGF/Puromycin/B18R supplemented EPC Reprogramming Medium**.
4. Incubate overnight in hypoxic incubator (5% O_2).
5. Repeat media change with freshly prepared **bFGF/Puromycin/B18R-supplemented EPC Reprogramming Medium** every day through Day 13.

3.5 Culturing without Puromycin – DAYS 14-19

Beginning on Day 14, remove puromycin from the medium, continuing to culture with B18R and bFGF. Change the medium every day.

1. Warm EPC Reprogramming Medium in a 37 °C water bath.
2. Prepare **bFGF/B18R-supplemented EPC Reprogramming Medium** according to the following:

B18R (0.5 mg/mL)	**0.8 µL**
bFGF (4 µg/mL)	**10.0 µL**
EPC Reprogramming Medium	**2000.0 µL**
Final Volume	**2010.8 µL**
Final B18R concentration	*200 ng/mL*
Final bFGF concentration	*20 ng/mL*

3. Remove used medium from a single well of the EPC reprogramming plate and replace with 2 mL of **bFGF/B18R-supplemented EPC Reprogramming Medium**
4. Incubate overnight in a hypoxic incubator (5% O_2).
5. Repeat media change with freshly prepared **bFGF /B18R-supplemented EPC Reprogramming Medium** every day through Day 19.

3.6 Culturing with Supplemented NutriStem Medium – DAYS 20-23

Beginning on Day 20, switch to **B18R/bFGF-supplemented NutriStem Medium**. Change the medium every day thereafter.

1. Warm NutriStem in a 37 °C water bath.
2. Prepare **B18R/bFGF-supplemented NutriStem Medium** according to the following table:

B18R (0.5 mg/mL):	**0.8 µL**
bFGF (4 µg/mL):	**10.0 µL**
NutriStem Medium:	**2000.0 µL**
Total:	**2010.8 µL**
Final B18R concentration	*200 ng/mL*
Final bFGF concentration	*20 ng/mL*

3. Remove used medium from a single well of the EPC reprogramming plate and replace with 2 mL of **B18R/bFGF-supplemented NutriStem Medium**.
4. Incubate overnight in hypoxic incubator (5% O_2).
5. Replace medium in reprogramming plate with freshly prepared **B18R/bFGF-supplemented NutriStem Medium** daily.

3.7 Day 24: Culture without B18R

Beginning on Day 24, remove B18R from medium and continue to culture with bFGF-supplemented NutriStem. Change the medium every day thereafter.

1. Warm NutriStem in a 37 °C water bath.
2. Prepare **bFGF-supplemented NutriStem Medium** according to the following:

bFGF (4 µg/mL)	**10 µL**
NutriStem Medium	**2000 µL**
Final Volume	**2010 µL**
Final human bFGF concentration	*20 ng/mL*

3. Remove medium from the reprogramming plate and replace it with 2 mL per well of freshly prepared **bFGF-supplemented NutriStem Medium**.
4. Incubate overnight in hypoxic incubator (5% O_2).
5. Replace medium in reprogramming plate with freshly prepared **bFGF-supplemented NutriStem Medium** daily.

Step 4: Pick and Passage iPSCs

When colonies reach sufficient size and are TRA-1-60 positive they should be picked and replated into individual wells of a Corning Matrigel-coated 12-well plate.

Picking can be performed with a stereo microscope in either a horizontal flow hood (positive pressure) or a static enclosure. Glass picking tools can be made from 9" Pasteur pipettes pulled to a closed, angled end over the controlled flame of an alcohol burner or by using a 10 µL sterile-filter pipette tip.

NOTE: All steps going forward must be performed in a sterile environment.

4.1 Pick and Replate Primary iPSC Colonies – DAYS 26-30

At this point cells can be cultured in **NutriStem Medium** only. No supplementation is required.

NOTE: Pick and replate no more than 6 colonies at one time to avoid keeping the cells out of the incubator for extended periods of time. To maintain clonal lines, transfer all of the pieces of each individual colony into a separate well of a 12 well plate. Change Pasteur pipettes/pipet tips with each new colony to be transferred to avoid cross-contamination of clonal lines.

1. Coat the appropriate number of wells of a 12-well plate with Corning Matrigel according to the manufacturer's instructions.
2. Add 1 mL pre-warmed **NutriStem Medium** to each well of the Corning Matrigel-coated 12-well plate.
3. Aspirate the medium from each well of the primary EPC reprogramming plate and replace with 2 mL pre warmed **NutriStem Medium**. Using a phase-contrast or stereo microscope, locate iPSC colonies based on morphology and pluripotency marker expression.
4. Using a sterile glass picking tool or a 10 µL sterile-filter pipette tip, gently separate the colony from the surrounding EPCs by circling the area to be picked.
5. Using the glass picking tool/pipette tip, gently divide the colony into approximately 3-8 pieces. It is important to break the colony into smaller cell aggregates, but not into single cells.

NOTE: Try to pick the inside of the colony without isolating the surrounding remaining non-reprogrammed EPCs.

6. Using the sterile glass picking tool/pipette tip, gently and completely detach the colony pieces from the tissue
   culture plate so that the cell aggregates are freely suspended in the medium.
7. Using a 20 µL large-bore sterile-filter pipette tip, transfer the detached colony pieces out of the reprogramming well and into an individual well of the prepared 12-well plate. Transfer all of the pieces from one colony into a single well of the 12-well plate.
8. Repeat the picking and re-plating process for each iPSC colonies. Pick one colony at a time, and transfer the cell aggregates of each colony to a different well of the Matrigel-coated 12-well plate prepared in Step 4.1.1.
9. After 6 iPSC colonies have been picked and replated, return both the 12-well plate and the primary EPC reprogramming plate to the hypoxic incubator (5% O_2).

Optional: Repeat Steps 1 through 9 in Section 4.1 in increments of 6 iPSC colonies at a time until the desired

number of colonies have been picked.

10. Continue to culture the original 6-well EPC reprogramming plate in **NutriStem Medium** until the picked colonies are established.

11. Change **NutriStem Medium** in both the 6-well EPC reprogramming plate and the 12-well coated passaging plate every day thereafter.

Step 5: Maintaining iPSC Cultures

Human iPSC cultures should be monitored and cared for every day, as the overall quality of the culture can change rapidly. Human iPSCs are generally passaged every 4 to 7 days in culture, but the actual passaging schedule and split ratio for each passage will vary depending on the cell culture's quality and growth rate.

Within the first few days of each passage, the proliferating cells grow easily in a monolayer colony. Once the colony becomes large, the proliferating cells begin to pile up, sometimes causing unwanted spontaneous differentiation to occur.

NOTE: It is important to passage the cells before the cultures become overgrown.

For maintenance and expansion, the iPSCs should be cultured in NutriStem Medium on hESC-qualified Matrigel Matrix. Between passages, the cell culture medium must be exchanged every day to provide necessary growth factors for the maintenance of human iPSCs

For the first few passages after picking colonies from the primary reprogrammed cultures, the cells should be passaged manually using the EDTA passaging method at low split ratios to build dense cultures.

Optional: After passages 3 to 4, when sufficient cells are available to both analyze and continue passaging, Stemgent recommends that the cells be tested by RT-PCR for clearance of srRNA. Typically, cells do not retain the srRNA after passages 3-4. See Appendix A for protocol.

References

1. Yoshida Y; Takahashi K; Okita K; Ishisaka T; Yamanaka S. "Hypoxia enhances the generation of induced pluripotent stem cells." *Cell Stem Cell* 5:237-41 (2009).

Appendix A: Optional Protocol: Endpoint RT-PCR Assay for srRNA Clearance

Required Materials

PRODUCT DESCRIPTION	CAT. NO.*	AMOUNT	STORAGE
RNeasy™ Mini Kit	Qiagen 74104	Per manufacturer's instructions	
iScript™ cDNA Synthesis Kit	Bio-Rad 170-8891	Per manufacturer's instructions	
HiFi HotStart ReadyMix	KAPA Biosystems kk2601	Per manufacturer's instructions	
GAPDH Primers: **F: AGG TCG GAG TCA ACG GAT TTG** **R: GTC ATG GAT GAC CTT GGC CAG**		Resuspend at 10 µM in H_2O	
NSP4 Primer: **F: CCA CAA TAC GAT CGG CAG TG** **R: ATG TCC TGC AAC ATA TTC AAA**		Resuspend at 10 µM in H_2O	

Procedure

A.1 RNA Isolation and Quantification

1. Suspend the cells from one 80% confluent well of a 6-well plate of cells in NutriStem Medium. Centrifuge one-half of the cells from each sample for RT-PCR.

NOTE: The remaining cells can be plated in a fresh plate for further passaging.

2. Process each cell pellet using the RNeasy Mini Kit. Follow the directions (for harvesting less than 1 million total cells) that come with the kit.

3. Quantitate the final elution using a micro-volume UV-Vis spectrophotometer (such as Thermo Scientific NanoDrop).

A.2 cDNA Synthesis

1. Dilute isolated RNA to 50 ng/µL with nuclease-free water.

2. Use the iScript cDNA Synthesis Kit to generate cDNA. Prepare reaction according to the following table. Use the reaction protocol supplied in the kit.

5x Reaction Mix	**4 µL**
Reverse Transcriptase	**1 µL**
H_2O	**5 µL**
RNA (50 ng/ µL)	**10 µL**
Total:	**20 µL**

A.3 RT-PCR:

1. Add 30 µL of H_2O to the cDNA product to dilute to 10 ng/µL (based on total RNA input).
2. To quantify GAPDH and NSP4, set up end point RT-PCR reactions using the following volumes for each sample.

HiFI HotStart ReadyMix	**25 µL**
Forward Primer	**1 µL**
Reverse Primer	**1 µL**
H_2O	**18 µL**
cDNA product	**5 µL**
Total:	**50 µL**

3. Perform RT-PCR according to the following protocol:

   i. 95 °C for 2 minutes
   ii. 94 °C for 25 seconds
   iii. 56 °C for 25 seconds
   iv. 68 °C for 30 seconds
   v. Repeat steps ii through iv 35 more times.
   vi. 72 °C for 5 minutes
   vii. End

A.4 Analysis

1. Analyze 20 µL of the PCR samples on a 2% agarose gel. GAPDH is approximately 500 bp, NSP4 is approximately 300 bp.

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