Stemgent® StemRNA™-NM Reprogramming Kit for Reprogramming Blood-Derived EPCs

Overview

This protocol describes procedures for reprogramming human blood-outgrowth endothelial progenitor cells (EPCs) in a xeno-free environment using non-modified RNAs (NM-RNAs) to generate induced pluripotent stem cells (iPSCs). This kit supports the reprogramming of three wells in a standard 6-well plate format using the Stemgent StemRNA-NM Reprogramming Kit (Cat. No. 00-0076).

Note: This protocol describes using iMatrix-511 (a laminin substrate) and human serum to provide a protocol using xenofree reprogramming reagents. Alternatively, other substrates and/or sera such as Corning® Matrigel® and FBS can be used by switching out one component or both (see Appendix A, see page 14).

This protocol describes the procedure for reprogramming one well of EPCs in a 6-well tissue culture plate. Please scale appropriately for larger experiments.

StemRNA-NM reprogramming experiments can be successfully performed under both atmospheric conditions (21% O_2) and decreased oxygen levels (5% O_2). However, the reprogramming process has proven to be more efficient under lower oxygen levels, often yielding 2 to 5 times as many iPSC colonies (Yoshida *et al.* 2009).

Note: Stemgent recommends using a 5% O_2 hypoxic incubator for increased efficiency in reprogramming experiments.

Note: This protocol describes only the reprogramming of blood-derived EPCs. *Derivation of EPCs is described in separate protocols "EPC Derivation from Whole Blood" using 50 mL of blood or "EPC Derivation from 10 mL of Whole Blood". Protocols for reprogramming fibroblasts and urine-derived epithelial cells (UDCs) using the Stemgent StemRNA-NM Reprogramming Kit #00-0076 are also available. These protocols require different conditions for reprogramming. Please contact Stemgent Technical Support (Tech.Support@ReproCELLUSA.com) to obtain these protocols.*

Reading and understanding the entire protocol prior to beginning your experiments is highly recommended. 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 institutional 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.

Abbreviations

EPCs	Human blood-outgrowth endothelial progenitor cells
OSKMNL	Oct4, Sox2, Klf4, cMyc, Nanog, Lin28 reprogramming factors
EKB	E3, K3, B18R immune evasion factors
FBS	Fetal bovine serum
PBS	Phosphate-buffered saline
NM-RNA	Non-modified RNA
UDCs	Human urine-derived epithelial cells

Required Reagents

PRODUCT DESCRIPTION	CAT. NO.	FORMAT	STORAGE
StemRNA-NM Reprogramming Kit OSKMNL NM-RNA EKB NM-RNA NM-microRNAs	Stemgent 00-0076 Part No. 05-0040 Part No. 05-0041 Part No. 05-0042	30 µg, 100 ng/µL 22 µg, 100 ng/µL 15 µg, 285 ng/µL	-80 °C
NutriStem™ XF/FF Culture Medium	Stemgent 01-0005	500 mL	-20 °C
Lipofectamine® RNAiMAX™ Transfection Reagent	ThermoFisher Scientific 13778030	Per manufacturer's instructions	
Opti-MEM® Reduced Serum Medium	ThermoFisher Scientific 31985062	Per manufacturer's instructions	
DPBS, Calcium-free, Magnesium-free	Life Technologies 14190144	Per manufacturer's instructions	
StainAlive™ TRA-1-60 (Dylight™ 488), mouse anti-human	Stemgent 09-0068	100 µL	4 °C
EGM™ Bullet Kit	Lonza CC-3162	Per manufacturer's instructions	
iMatrix-511	Stemgent NP892011 NP892012	350 ug 1050 ug	4 °C
Human serum	Sigma H4522	Per manufacturer's instructions	
CryoStem™ Freezing Medium	Stemgent 01-0013-50	50 mL	4 °C
Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units, 500 mL; Pore Size: 0.20 µm	Thermo Scientific 569-0020	Per manufacturer's instructions	
Trypsin-EDTA (0.05%), phenol red	Life Technologies 25300054	Per manufacturer's instructions	
Standard Tissue Culture laboratory supplies and equipment.			

Substrate and Serum

This protocol describes using iMatrix-511 (a xeno-free laminin-511 E8 fragment substrate) and human serum to provide a protocol using xeno-free reprogramming reagents. Alternatively, other substrates and/or sera can be used by switching out one component or both (see Appendix A, see page 13).

Optional Reagents & Equipment

PRODUCT DESCRIPTION	CAT. NO.	FORMAT	STORAGE
RNase Zap™	Ambion AM9780	Per manufacturer's instructions	
Low oxygen incubator	-	-	

Related Protocols

For derivation and culturing of EPCs:

- "EPC Derivation from Whole Blood" and "EPC Derivation from 10 mL of Whole Blood"

For reprogramming of neonatal and adult fibroblasts:

- "Stemgent® StemRNA™-NM Reprogramming Kit for Reprogramming Neonatal and Adult Fibroblasts"

For reprogramming of urine-derived cells:

- "Stemgent® StemRNA™-NM Reprogramming Kit for Reprogramming Urine-derived Cells".

For live staining with StainAlive TRA-1-60 antibody to verify pluripotency:

- "General Protocol for ICC Staining of Live Cells".

These protocols can be obtained on the REPROCELL website: www.reprocell.com under "Stemgent Protocols"

Timeline

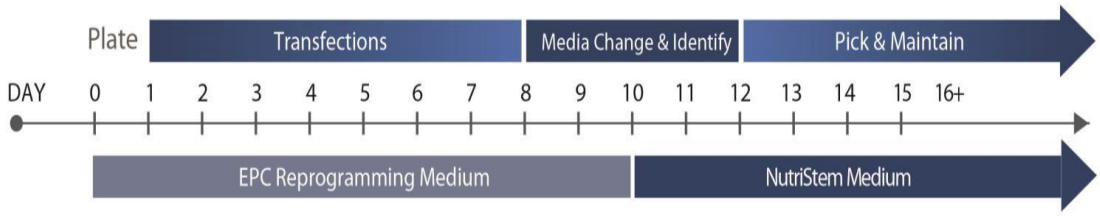


FIGURE 1A. EPC Reprogramming Timeline

Day 0: Plate EPCs.
Day 1-8: Daily NM-RNA cocktail transfection overnight.
Day 10: Switch to NutriStem medium.
Optional: Identify emerging iPSC colonies by TRA-1-60 live stain
Day 12: Continue culture in NutriStem medium
Day12-16: Pick primary EPC-NM-RNA-iPSC colonies, replate in NutriStem Medium.

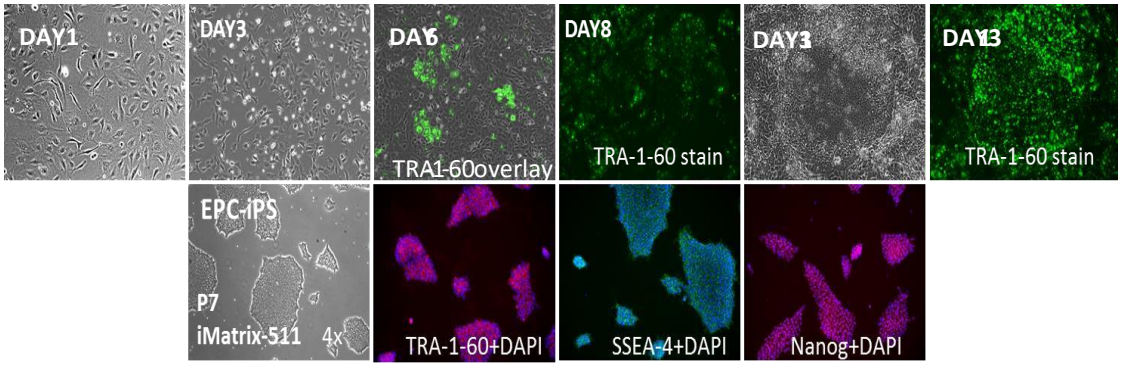


FIGURE 1B: Primary reprogramming culture morphology progression resulting from the reprogramming of EPCs with StemRNAiPSC colonies were identified using Stemgent StainAlive TRA-NM Kit on iMatrix-511 and EPC-Reprogramming M-1edium containing human serum. Day 6, 8, 13 primary EPC-60 antibody and can be isolated from the primary culture by -RNA - Day 12-14. EPC-RNA-iPSCs were expanded on iMatrix-511 in NutriStem XF/FF and stained for pluripotency associated genes at P7 by ICC.

Note: *These timelines are appropriate for most EPC lines tested. For more difficult EPC lines (higher passage number or low proliferation potential) with longer reprogramming timelines, please* **see Figure 3, page 9.**

Reprogramming Protocol

Step 1: Material preparation

1.1 Preparation of EPC Reprogramming Medium

Note: This protocol describes using iMatrix-511 (a laminin substrate) and human serum to provide a protocol using xeno-free reprogramming reagents. Alternatively, other substrates and/or sera such as Corning® Matrigel® and FBS can be used by switching out one component or both (see Appendix A, see page 13).

1. Thaw human serum in refrigerator overnight.
2. To 450 mL EBM-2 basal medium, add EGM-2 SingleQuots (excluding the FBS and heparin) supplied with the EGM Bullet Kit. Filter medium using 0.2 µm filter unit. Then add 50 mL human serum to make complete EPC Reprogramming Medium.

Note: Do not filter human serum. Do not use the FBS and heparin supplied in the SingleQuot kit.

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

3. Store EPC Reprogramming Medium at 4 °C for up to four weeks.

Note: 20 mL of EPC Reprogramming Medium is sufficient to reprogram one well of a 6-well plate using the StemRNA-NM kit. An additional 25 mL of EPC Reprogramming Medium is required for thawing and culturing of EPCs prior to the reprogramming experiment.

Note: It is highly recommended that the EPC Reprogramming Medium is aliquoted and stored in the fridge. The aliquots should be prepared that each one gets warmed up prior to medium change up to 3 times.

1.2 Preparation of NM-RNA-Reprogramming Cocktail for Blood-derived EPCs

1. Thaw the 3 vials of NM-RNAs provided in the kit (OSKMNL NM-RNA, EKB NM-RNA, and NM-microRNAs) on ice. Once thawed, keep the vials on ice at all times.
2. Briefly centrifuge the vials to collect the contents at the bottom of the tube.
3. The daily RNA reprogramming cocktail is composed of 1.2 µg OSKMNL NM-RNA, 0.6 µg EKB NM-RNA (total mRNA = 1.8 µg), and 0.4 µg NM-microRNAs per transfection per well (6-well plate format).
4. Prepare reprogramming cocktail in a sterile, RNase-free microcentrifuge tube by combining the volumes below to reprogram one well of a 6-well plate of blood-derived EPCs.

OSKMNL NM-RNA:	96.0 µL
EKB NM-RNA:	48.0 µL
NM-microRNAs:	11.2 µL
Total RNA reprogramming cocktail:	155.2 µL

5. Divide the mixture into **eight 19.4 µL aliquots** in sterile, RNase-free microcentrifuge tubes. Store the aliquots at -80 °C for up to three months. Avoid additional freeze thaw cycles.

Note: *This Kit supports reprogramming of 3 wells in a standard 6-well plate format (**a total of twenty-four 19.4 µL NM-RNA reprogramming cocktail aliquots**). Please prepare and freeze reprogramming aliquots accordingly.*

Step 2: Prepare Target Cells

2.1 Preparation of EPCs

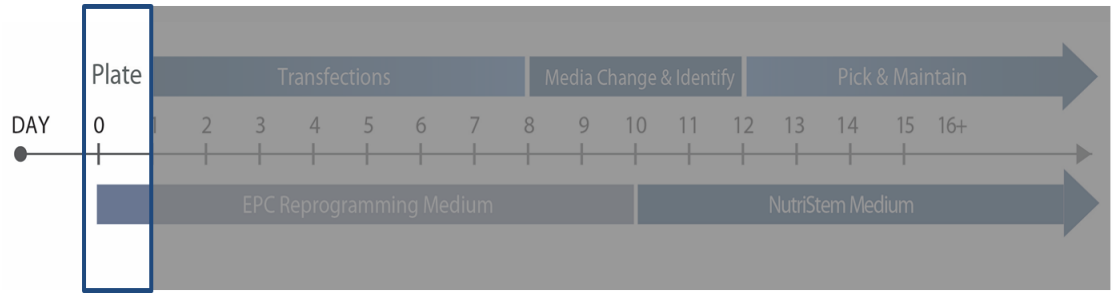
Prepare a T25 flask of exponentially growing EPCs as described in the accompanying protocols "Whole Blood Processing for EPC Derivation" (using 50 mL or more whole blood, using FBS or human serum) or "EPC Derivation from 10 mL of Whole Blood" (using human serum only).

Note: *Derivation and culturing of EPCs from whole blood will take approximately 18-21 days before they are ready to be plated for the reprogramming experiment. Please plan accordingly.*

Note: *Use one well of a 6-well plate of cells for a typical reprogramming experiment.*

Note: *EPCs with lower passage numbers (P2-P4) usually have higher proliferation potential and therefore show superior susceptibility for RNA transfection.*

2.2 Day 0: Plating of EPCs for Reprogramming Experiment



1. Plate 1 mL per well 2.4 µg/mL iMatrix-511 substrate (diluted in PBS) on an appropriate number of wells of a 6-well plate and incubate at 37 °C for 1 hour prior to seeding cells.

2. Remove the culture medium from the T25 flask of exponentially growing EPCs to be harvested. Add 5 mL PBS to the culture surface of the flask to wash. Aspirate the PBS.
3. Add 3 mL 0.05% Trypsin/EDTA to the culture surface of the flask and incubate for 3 to 5 min at 37 °C and 5% CO_2.
4. Tap the flask to completely detach the cells from the culture surface.
5. Add 6 mL EPC Reprogramming Medium to the flask to neutralize the Trypsin/EDTA.
6. With a 5 mL pipette, transfer the harvested cell suspension from the flask to a 15 mL conical tube. Pipette up and down gently to disrupt the cell aggregates.
7. Centrifuge the cells for 5 minutes at 250 x g.
8. Remove the supernatant and resuspend the pellet in 1 mL EPC Reprogramming Medium.
9. Count the cells and calculate the live cell density.
10. To each well of the iMatrix-511-coated 6-well plate, add $7.5x10^4$ EPCs per well in 2 mL EPC Reprogramming Medium.

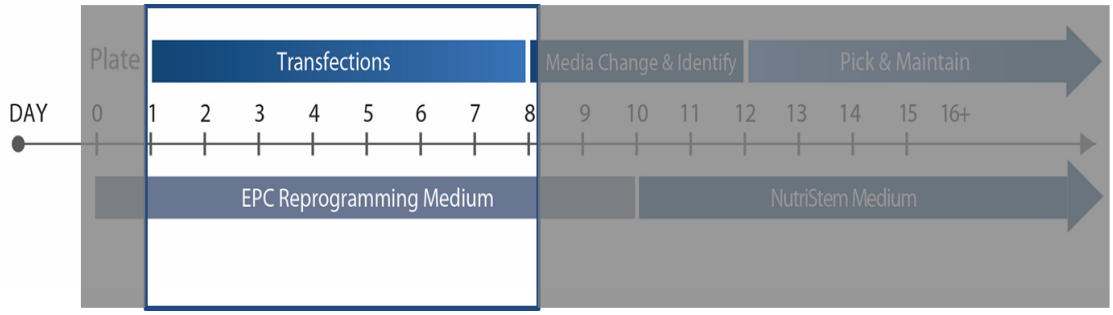
Note: Depending on the proliferation potential of your cells and if cells were grown and reprogrammed in human serum containing or FBS containing Reprogramming Medium, 2.5x10⁴ to 1x10⁵ EPCs per well can be seeded. See also Appendix A on page 13 for more information about EPC seeding densities.

11. Incubate the cells overnight at 37 °C, 5% CO_2 and 21% oxygen incubator.

Step 3: Transfections

Note: This protocol is based on reprogramming one well of EPCs in a 6-well plate format. Please scale appropriately for larger experiments.

3.1 Day 1-8: Daily NM-RNA Reprogramming Cocktail Transfections (Overnight Transfection)



Recommended: Wipe down all working surfaces (gloves, reagent bottles, biosafety cabinet surfaces, pipettors, etc.) with RNase Zap. **Note:** Stemgent recommends using a hypoxic incubator (5% O_2) starting on day 1 of the reprogramming protocol.

1. Warm **EPC Reprogramming Medium Aliquot** in a 37 °C water bath.
2. Remove the medium from the wells in the EPC plate. Add 2 mL **EPC Reprogramming Medium** to each well. Return the plate to the incubator while carrying out steps 3.1.3 to 3.1.6.
3. Equilibrate RNAiMAX transfection reagent and Opti-MEM at room temperature for at least 30 min.
4. Thaw NM-RNA-Reprogramming cocktail aliquot at room temperature and immediately place on ice. Label as Tube "**A** (RNA + Opti-MEM)".
5. Label one sterile, RNase-free 1.5 mL microcentrifuge tube as "**B** (RNAiMAX + Opti-MEM)".

- To Tube A, add 230.6 µL Opti-MEM to the 19.4 µL NM-RNA cocktail aliquot in the tube.
- To Tube B, add 8 µL RNAiMAX transfection reagent to 242 µL Opti-MEM.

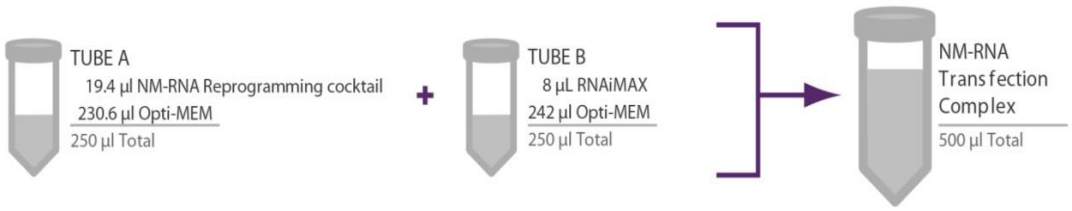
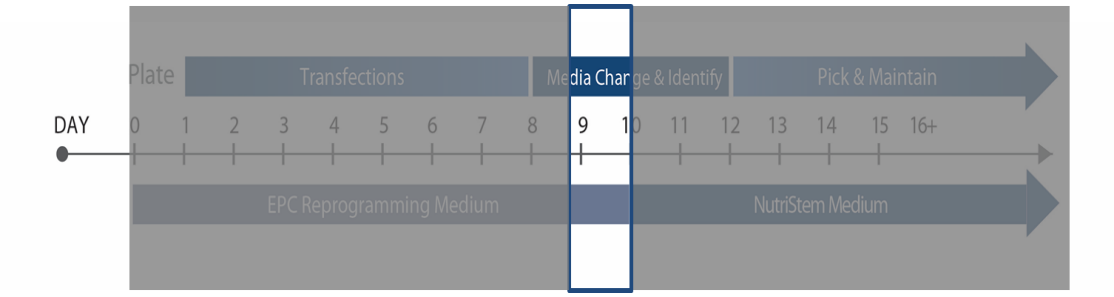


FIGURE 2: Pipetting Scheme

6. Pipette gently three to five times to mix.
7. Using a pipettor, transfer the entire contents of Tube **B** to Tube **A** drop-wise at meniscus level. Mix by tapping the bottom of the tube. Incubate at room temperature for 15 min.
8. Add 500 µL NM-RNA transfection complex solution to the well in the EPC reprogramming plate by tilting the plate and pipetting drop-wise into medium. Mix by rocking in the X- and Y-directions.
9. Incubate EPC reprogramming plate overnight in hypoxic incubator (5% O_2).
10. Repeat Steps 1-9 for following daily transfections on Day 2-8.

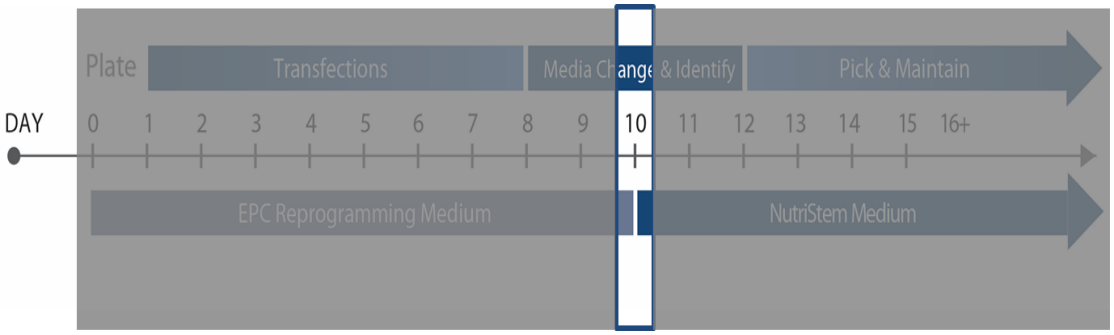
Note: For best results, change medium as early as possible in the day and add the new transfection complex as late in the day as possible. Adding some recovery time before adding the next transfection complex decreases the cell toxicity.

3.2 Days 9: Media change only



1. Warm EPC Reprogramming Medium in a 37 °C water bath.
2. Remove medium from reprogramming plate, replace with 2 mL per well **EPC Reprogramming Medium.**
3. Return the EPC reprogramming plate to a hypoxic incubator (5% O_2) overnight.

3.3 Day 10: Switch to NutriStem XF/FF Culture Medium



Optional: On Day 10, check for TRA-1-60 positive cells by using the StainAlive TRA-1-60 antibody, following the protocol: "Protocol: Immunocytochemistry of Live Cells". Maintain sterility in ongoing reprogramming cultures. Regardless of detection of positive or negative TRA-1-60 emerging colonies the reprogramming plate should be switched to NutriStem medium on Day 10.

1. Warm NutriStem in a 37 °C water bath.
2. Remove medium from EPC reprogramming plate, replace with 2 mL per well NutriStem Medium.
3. Return the plate to a hypoxic incubator (5% O_2) over night.
4. Replace medium in EPC reprogramming plate with NutriStem Medium daily until iPSC colonies are ready to be picked.

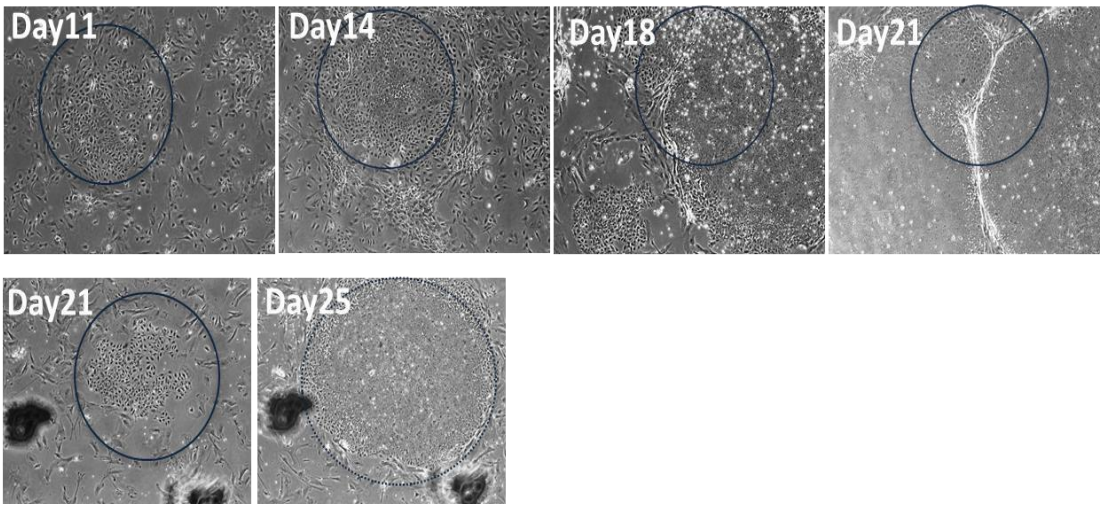


FIGURE 3: Examples of more difficult to reprogram EPC lines with longer reprogramming time lines.

Shown are 2 different EPC lines with longer reprogramming time lines compared to other EPC lines tested. EPCs were cultured in NutriStem Medium from day10 until pick date (day 21/25).

EPC line #1 (upper panel): Immature iPSC colonies developed late on day 11 but were ready to be picked between day 1821. EPC line #2 (lower panel): iPSCs were still immature on day 21 but were ready to be picked on day 25.

Step 4: Pick and Passage EPC-NM-RNA-iPSCs

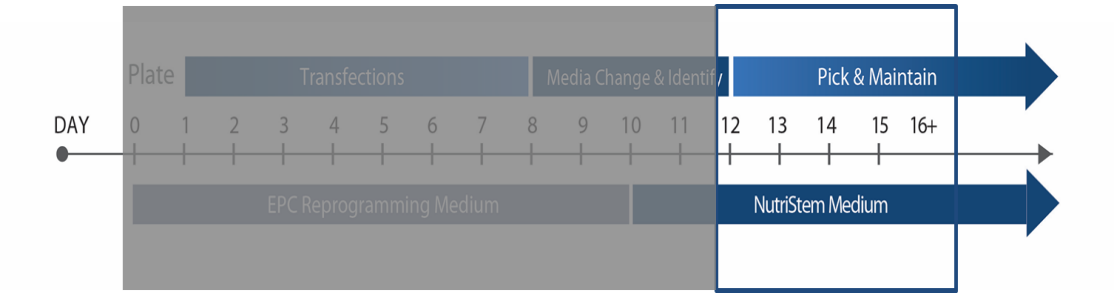
When colonies reach sufficient size and are TRA-1-60 positive they should be picked and replated into individual wells of an iMatrix-511-coated 12-well plate ("Passaging Plate").

Note: *All procedures in this picking protocol must be performed in a sterile environment.*

Note: *Alternatively, other substrates such as Corning Matrigel can be used*.

Picking can be performed with a stereo microscope in either a horizontal flow hood (positive pressure) or a static enclosure. Picking can be done using glass tools 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 pipette tip.

4.1 Days 12-18: Pick and Replate Primary iPSC Colonies



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 ("Passaging Plate") with 0.5 mL 2.4 µg/mL iMatrix-511 in PBS and incubate at 37 °C for 1 hour prior to picking.

Note*: NM-RNA-iPSC colonies derived on iMatrix-511 can also be picked and maintained on Corning Matrigel-coated 12-well dishes*.

2. Aspirate the medium from 6 wells of an iMatrix-511-coated 12-well Passaging Plate.
3. Add 1 mL prewarmed NutriStem medium to each of these 6 wells of the Passaging Plate.
4. Aspirate the medium from each well of the primary 6-well reprogramming plate and replace with 2 mL prewarmed NutriStem.
5. Using a phase-contrast or stereo microscope, locate iPSC colonies based on morphology and pluripotency marker expression.
6. Using a glass picking tool or a 10 µL pipette tip, gently separate the colony from the surrounding EPCs by circling the area to be picked.
7. 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.

8. Using the 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.
9. Using a 20 µL pipettor with a sterile, large-bore tip, transfer the detached colony pieces out of the reprogramming well and into an individual well of the prepared 12-well Passaging Plate. Transfer all of the pieces from one colony into a single well of the Passaging Plate.
10. Repeat the picking and replating process for each iPSC colony. Pick one colony at a time and transfer the cell aggregates of each colony to a different well of the prepared 12-well Passaging Plate.
11. After 6 iPSC colonies have been picked and replated, place both the 12-well Passaging Plate and the primary reprogrammed colonies in the hypoxic incubator (5% O_2) to re-equilibrate.
12. Repeat the process (Steps 1 through 11) in increments of 6 iPSC colonies at a time until the desired number of colonies has been picked.
13. Continue to culture the reprogramming (6-well) plate until the picked colonies are established.
14. Change NutriStem Medium in both the reprogramming (6-well) and the Passaging Plate (12-well) every day thereafter.

Step 5: Maintain 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. It is important to passage the cells before the cultures become overgrown.

For maintenance and expansion, the iPSCs should be cultured in NutriStem Culture Medium on iMatrix-511 or adapted to other proven human iPSC culture conditions. 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. The cells can be split using an enzymatic protocol for routine culture once there are a large number of human iPSC colonies in the well(s).

References

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).

Poleganov MA; Eminli S; Beissert T; Herz S; Moon JI; Goldmann J; Beyer A; Heck R; Burkhart I; Barea Roldan D; Türeci Ö; Yi K; Hamilton B; Sahin U. Efficient Reprogramming of Human Fibroblasts and Blood-Derived Endothelial Progenitor Cells Using Non-modified RNA for Reprogramming and Immune Evasion. *Human Gene Therapy* 26:751 (2015)

Appendix A. Matrix and Serum Options

TABLE A1. Alternative Products

PRODUCT DESCRIPTION	CAT. NO.	FORMAT	STORAGE
Matrix: Corning ® Matrigel® hESC-Qualified Matrix, *LDEV-free	Corning 354277	Per manufacturer's instructions	
Serum: FBS, mESC qualified, defined	GE Healthcare Hyclone™ SH30070.03E	Per manufacturer's instructions	

TABLE A2. Plating conditions for matrix/serum combinations

SERUM + SUBSTRATE COMBINATION	RECOMMENDED PLATING DENSITY	REPROGRAMMING EFFICIENCY UP TO
iMatrix-511+ human serum	$7.5x10^4$	-0.15%-4% (~100-1000 iPSC colonies)
iMatrix-511 + Hyclone FBS	$5.0x10^4$	0.2%-4% (~100-1000 iPSC colonies)
Matrigel + Hyclone FBS	$7.5x10^4$	0.15%-4% (~100 iPSC colonies)
Matrigel + human serum	$7.5x10^4$	0.15% (~100 iPSC colonies)

Note: The recommended plating densities in the table reflect the optimal density for most EPC lines at P4 under the specific conditions. Stemgent recommends plating between $2.5x10^4$-$1.0x10^5$ EPCs per 6-well plate. The higher the proliferation potential and the lower the passage number the lower is the required seeding density. The reprogramming efficiency is highly dependable on the EPC line.

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