Protocol: Stemgent® StemRNA™ 3rd Gen Reprogramming Kit for Reprogramming Adult and Neonatal Human Fibroblasts

Overview

This protocol describes procedures for reprogramming adult and neonatal human fibroblasts in a complete xeno-free culture environment using non-modified RNAs (NM-RNAs) to generate induced pluripotent stem cells (iPSCs). This kit supports the reprogramming of **nine wells** in a standard 6-well plate format using the Stemgent StemRNA 3rd Gen Reprogramming Kit (Cat. No. 00-0076).

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

> **Note:** This protocol describes using iMatrix-511 (a laminin substrate) and NutriStem® hPSC XF Culture Medium to provide a complete xeno-free reprogramming environment. Alternatively, other substrates such as Corning® Matrigel® can be used (**see Appendix A, page 12**).

StemRNA 3rd Gen 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 hypoxic (5% O_2) conditions, often yielding 2 to 5 times as many iPSC colonies (Yoshida *et al.* 2009).

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

> **Note: This protocol describes reprogramming of human fibroblasts only.** Protocols for reprogramming blood-derived endothelial cells (EPCs) and urine-derived epithelial cells using the Stemgent StemRNA 3rd Gen Reprogramming Kit (Cat. No. 00-0076) are also available. These protocols require different conditions for reprogramming. Please contact Stemgent Technical Support (techsupport-us@reprocell.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 fibroblasts derived from human tissue. 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

NM-RNA	Non-modified RNA
OSKMNL	Oct4, Sox2, Klf4, cMyc, Nanog, Lin28 reprogramming factors
EKB	E3, K3, B18 immune evasion factors
FBS	Fetal bovine serum
PBS	Phosphate-buffered saline
A-DMEM	Advanced DMEM
EDTA	Ethylenediaminetetraacetic acid
EPCs	Human blood-outgrowth endothelial progenitor cells

Required Reagents

PRODUCT DESCRIPTION	CAT. NO.	FORMAT	STORAGE
StemRNA 3rd Gen 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
iMatrix-511	Stemgent NP892-011	350 µg	4 °C
NutriStem® hPSC XF 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	
StainAlive™ TRA-1-60 (Dylight™ 488), mouse anti-human	Stemgent 09-0068	100 µL	4 °C
Human serum	Sigma H4522	Per manufacturer's instructions	
Advanced-DMEM	ThermoFisher Scientific 12491015	500 mL	4 °C
Glutamax™ Supplement	ThermoFisher Scientific 35050061	Per manufacturer's instructions	
DPBS, Calcium-free, Magnesium-free	ThermoFisher Scientific 14190144	Per manufacturer's instructions	
CryoStem™ Freezing Medium	Stemgent 01-0013-50	50 mL	4 °C
Trypsin-EDTA (0.05%), phenol red	ThermoFisher Scientific 25300054	Per manufacturer's instructions	
Standard tissue culture laboratory supplies and equipment.			

Optional Reagents & Equipment

PRODUCT DESCRIPTION	CAT. NO.	FORMAT	STORAGE
RNase Zap™	Ambion AM9780	Per manufacturer's instructions	
Multi-gas incubator (equilibrated to 5% O_2) ("hypoxic incubator (5% O_2)")	–	–	

Related Protocols

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: https://www.reprocell.com/resources/protocols.html , or by contacting REPROCELL Technical Support (techsupport-us@reprocell.com)

Timeline

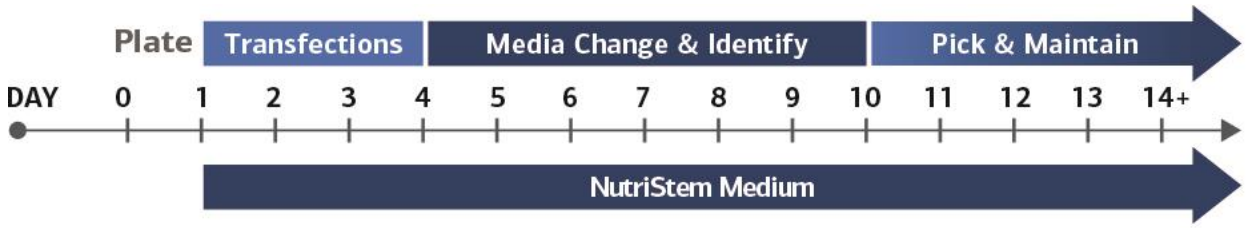

FIGURE 1. Fibroblast Reprogramming Timeline

Day 0: Plate Fibroblasts in Fibroblast Expansion Medium.
Day 1: Media switch to NutriStem Medium.
Day 1-4: Daily NM-RNA cocktail overnight transfections.
Optional on Day 8-14: Identify emerging iPSC colonies by TRA-1-60 live stain.
Day 10-14: Pick primary Fibroblast-NM-RNA-iPSC colonies, replate in NutriStem Medium.

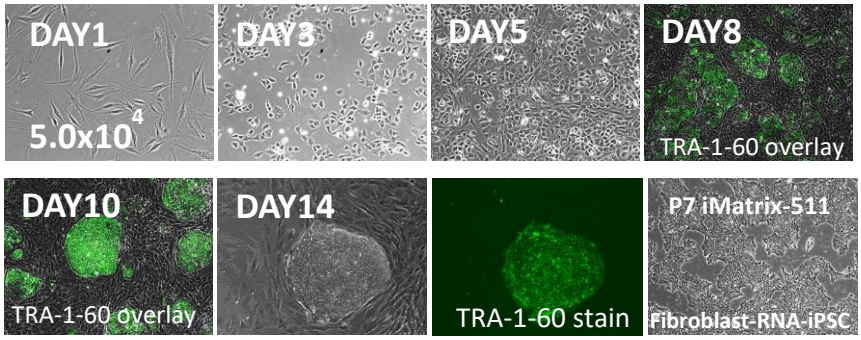

FIGURE 2: Primary reprogramming culture morphology progression, resulting from the reprogramming of adult fibroblasts with the StemRNA 3rd Gen Kit on iMatrix-511 in NutriStem hPSC XF Culture Medium. Day 8, 10 and Day 14 primary Fibroblast-RNA-iPSC colonies were identified using Stemgent StainAlive TRA-1-60 antibody and can be isolated from the primary culture between Day 10-14. Fibroblast-RNA-iPSCs were expanded on iMatrix-511 in NutriStem hPSC XF Culture Medium.

Reprogramming Protocol

Step 1: Material Preparation

1.1 Preparation of Fibroblast Expansion Medium

1. Add 5 mL human serum and 0.5 mL Glutamax Supplement to 44.5 mL A-DMEM (10% human serum final V/V).
2. Store at 4 °C for up to 2 weeks.

   Note: For alternative serum supplementations see **Appendix A (page 11).**

1.2 Preparation of NM-RNA-Reprogramming Cocktail for Fibroblast Reprogramming

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

Note: This protocol and the calculations below are based on reprogramming one well of fibroblasts in a 6-well plate format. Please scale appropriately for larger experiments (e.g. different densities).

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. Prepare **NM-RNA reprogramming cocktai**l to reprogram one well of a 6-well plate of neonatal or adult fibroblasts for a total of four transfections by combining the following in a sterile, **RNase-free** microcentrifuge tube:

OSKMNL NM-RNA:	**32.0 µL**
EKB NM-RNA:	**24.0 µL**
NM-microRNAs:	**5.6 µL**
Total NM-RNA reprogramming cocktail:	**61.6 µL**

   The daily **NM-RNA reprogramming cocktail** is composed of 0.8 µg OSKMNL NM-RNA, 0.6 µg EKB NM-RNA (total mRNA= 1.4 µg), and 0.4 µg NM-microRNAs per transfection per well (6-well plate format).

4. Divide the mixture into four 15.4 µL single-use 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 the reprogramming of **9 wells** (a total of thirty-six 15.4 µL NM-RNA reprogramming cocktail aliquots) in a standard 6-well plate format. Please prepare and freeze reprogramming aliquots accordingly.

Step 2: Prepare Target Cells

2.1 Preparation of Fibroblasts

Prepare a T25 flask of exponentially growing neonatal or adult fibroblasts. Fibroblast can be thawed and expanded on a T25 flask prior to seeding for the reprogramming experiment in **Fibroblast Expansion Medium**.

> **Note:** Fibroblasts with lower passage numbers (P2-P6) usually have higher proliferation potential and therefore are easier to transfect with RNA.

2.2 Day 0: Plating of Fibroblasts for Reprogramming Experiment

1. Plate 1 mL per well of 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 (see iMatrix-511 protocol for details).
2. Remove the culture medium from the T25 flask of exponentially growing fibroblasts 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 flask.
5. Add 6 mL **Fibroblast Expansion 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 × g.
8. Remove the supernatant and resuspend the pellet in 1 mL **Fibroblast Expansion Medium**.
9. Count the cells and calculate the live cell density.
10. To each well of the iMatrix-511-coated plate, add **5.0×10^4 – 7.5×10^4** cells per well in 2 mL of **Fibroblast Expansion Medium.**

    > **Note:** Depending on the proliferation potential of your cells, **$2.5x10^4$ to $1.0x10^5$** fibroblasts per well can be seeded. See also **Appendix A, page 11** for more information about fibroblast seeding densities.

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

Step 3: Transfections

3.1 Day 1: NM-RNA Reprogramming Cocktail (Overnight Transfection, with Medium Change)

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

1. Warm up **NutriStem Medium** in a 37 °C water bath.
2. Remove the old medium from the wells in the reprogramming plate. Add 2 mL **NutriStem Medium** to each well.
3. Incubate in hypoxic incubator at 37 °C, 5% CO_2, 5% O_2.
4. Thaw one **NM-RNA Reprogramming Cocktail** single-use aliquot at room temperature, then immediately place on ice. Label as tube "**A** (RNA + Opti-MEM)".
5. Label a sterile, RNase-free 1.5 mL microcentrifuge tube "**B** (RNAiMAX+ Opti-MEM)".
    - To tube A, add 234.6 µL Opti-MEM (tube A already contains 15.4 µL NM-RNA Reprogramming Cocktail).
    - To tube B, add 6 µL RNAiMAX transfection reagent to 244 µL Opti-MEM (see Figure 3).

FIGURE 3. NM-RNA Reprogramming cocktail set-up

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 reprogramming plate by tilting the plate and pipetting drop-wise into medium. Mix by rocking in the X- and Y-directions.
9. Return the reprogramming plate to a hypoxic incubator (5% O_2) overnight.

3.2 Days 2-4: NM-RNA Reprogramming Cocktail (Overnight Transfection, with Medium Change)

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

1. **At the beginning of the day,** Warm **NutriStem Medium** in a 37 °C water bath.
2. Remove the old medium from the wells in the reprogramming plate. Add 2 mL **NutriStem Medium** to each well.
3. Incubate in hypoxic incubator at 37 °C, 5% CO_2, 5% O_2 for at least 6 hr.

Note: Adding some recovery time before adding the next transfection complex decreases the cell toxicity.

4. **At the end of the day,** thaw **NM-RNA Reprogramming Cocktail** single-use aliquot at room temperature, then immediately place on ice. Label as tube "**A** (RNA + Opti-MEM)".
5. Label a sterile, RNase-free 1.5 mL microcentrifuge tube as "**B** (RNAiMAX + Opti-MEM)".
    - To tube A, add 234.6 µL Opti-MEM (tube A already contains 15.4 µL NM-RNA Reprogramming Cocktail).
    - To tube B, add 6 µL RNAiMAX transfection reagent to 244 µL Opti-MEM (see Figure 3).
6. Pipette gently three to five times to mix.
7. Using a pipettor, transfer the entire content 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 of NM-RNA transfection complex solution to the well in the reprogramming plate by tilting the plate and pipetting drop-wise into medium. Mix by rocking in the X- and Y-directions.
9. Return the reprogramming plate to a hypoxic incubator (5% O_2) for 15-18 hr.

3.3 Days 5-10 (-14): NutriStem Medium Media Changes

1. Warm **NutriStem Medium** in a 37 °C water bath.
2. Remove the medium from each well in the reprogramming plate and exchange with 2 mL fresh **NutriStem Medium.**
3. Return the reprogramming plate to a hypoxic incubator (5% O_2) overnight.

Optional: To determine whether the cells have become pluripotent, stain the colonies using the StainAlive™ TRA-1-60 antibody, following the protocol: "**Protocol: Immunocytochemistry of Live Cells**." Alternatively, the StainAlive TRA-1-81 Antibody or the StainAlive SSEA-4 Antibody can also be used. Maintain sterility in ongoing reprogramming cultures.

Note: If the reprogrammed cells show mature iPSC morphology, and the colonies are big enough in size and express TRA-1-60, the picking of Fibroblasts-NM-RNA-iPSC colonies can be done as early as day 10 (see Step 4 and Figure 2).

Step 4: Pick and Passage Fibroblast-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 10-14: 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 of 2.4 µg/mL iMatrix-511 in PBS and incubate at 37 °C for 1 hour prior to picking.
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 Medium.
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 fibroblasts 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 fibroblasts.

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. 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.

11. 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.

12. Continue to culture the reprogramming (6-well) plate until the picked colonies are established.

13. 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 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 EDTA only or 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)

Licensing

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 different fibroblast expansion conditions and substrate choices

Fibroblast Expansion Medium	Recommended Plating Densities for Reprogramming on One Well of a 6-Well Plate (Cells per well)		Reprogramming Efficiency*
	iMatrix-511	Corning® Matrigel®	
A-DMEM + Glutamax **10% human serum**	$5.0\times10^4 - 7.5\times10^4$	$5\times10^4 - 1.0\times10^5$	~1-2%
A-DMEM + Glutamax **10% Hyclone FBS**	$5.0\times10^4 - 7.5\times10^4$	$5.0\times10^4 - 7.5\times10^4$	~2-4%

Note: Reprogramming efficiency is highly dependent on the primary fibroblast culture.

* Reprogramming efficiencies are based on fibroblast passage number between P2-P5. After passage 7 the reprogramming efficiency can drop below 1%.