

Protocol: Stemgent® StemRNA™ 3rd Gen Reprogramming Kit for Reprogramming Urine- Derived Progenitor Cells (UPCs)



Overview

This protocol describes procedures for reprogramming human urine-derived progenitor cells (UPCs) 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 3rd Gen Reprogramming Kit (Cat. No. 00-0076).

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, page 12**).

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

StemRNA 3rd Gen reprogramming experiments can be successfully performed under both atmospheric conditions (21% O₂) and decreased oxygen levels (5% O₂). However, the reprogramming process has proven to be more efficient under hypoxic (5% O₂) conditions, often yielding 2 to 5 times as many iPSC colonies (Yoshida *et al.* 2009).

Note: REPROCELL recommends using a hypoxic incubator set to 5% O₂ for increased efficiency in reprogramming experiments.

Note: This protocol describes only reprogramming of UPCs. Derivation of UPCs from a minimum of 30 mL of urine is described in a separate protocol, “Derivation of urine-derived progenitor cells (UPCs) from human urine”. Protocols for reprogramming fibroblasts and blood-derived epithelial progenitor cells (EPCs) using the Stemgent StemRNA 3rd Gen Reprogramming Kit (#00-0076) are also available. These protocols require different conditions for reprogramming. Please contact REPROCELL 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 UPCs derived from human urine. 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, B18R immune evasion factors
UPCs	Human urine-derived progenitor cells
RE	Renal Epithelial
MC	Mesenchymal Cell
FBS	Fetal bovine serum
PBS	Phosphate-buffered saline
bFGF	Basic fibroblast growth factor (FGF-basic; FGF-2)
EGF	Epidermal growth factor
PDGF	Platelet-derived growth factor
HSA	Human serum albumin
DMEM	Dulbecco's Modified Eagle Medium
NEAA	MEM Non-Essential Amino Acids Solution
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	REPROCELL 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	REPROCELL NP892-011	350 µg	4 °C
NutriStem® hPSC XF Culture Medium	REPROCELL 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	REPROCELL 09-0068	100 µL	4 °C
Human serum	Sigma H4522	Per manufacturer's instructions	
REGM™ Bullet Kit	Lonza CC-3190	Per manufacturer's instructions	
FGF-basic, Human Recombinant (bFGF)	REPROCELL 03-0002	50 µg	-80 °C
Human Albumin (HSA)	Sigma A9731	Per manufacturer's instructions	
Primocin*	Invivogen ant-pm-1	Per manufacturer's instructions	

PRODUCT DESCRIPTION	CAT. NO.	FORMAT	STORAGE
Trypsin-EDTA (0.05%), phenol red	ThermoFisher 25300054	Per manufacturer's instructions	
DMEM, high glucose	ThermoFisher Scientific 11965092	Per manufacturer's instructions	
EGF	Peptotech AF-100-15	Per manufacturer's instructions	
PDGF-AB	Peptotech 100-00AB	Per manufacturer's instructions	
NEAA	ThermoFisher Scientific 11140050	Per manufacturer's instructions	
Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units, 500 mL; Pore Size: 0.20 µm	ThermoFisher Scientific 569-0020	Per manufacturer's instructions	
DPBS, Calcium-free, Magnesium-free	ThermoFisher Scientific 14190144	Per manufacturer's instructions	
NutriFreez™ D10 Cryopreservation Medium	REPROCELL 01-0020-50	50 mL	4 °C
*Primocin alternative <ul style="list-style-type: none"> Amphotericin B solution (5 µg/mL) Penicillin-streptomycin-glutamine (100x) 	Sigma A2942 Gibco 10-3780916	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), human serum and NutriStem Medium to provide a protocol using xeno-free reprogramming reagents. Alternatively, other substrates and/or sera can be used by switching out components (**see Appendix A**).

Optional Reagents & Equipment

PRODUCT DESCRIPTION	CAT. NO.	FORMAT	STORAGE
RNase Zap™	Ambion AM9780	Per manufacturer's instructions	
Multi-gas incubator (equilibrated to 5% O₂) ("hypoxic incubator (5% O ₂)")	–	–	

Related Protocols

For derivation and culturing of UPCs:

"Derivation of urine-derived progenitor cells (UPCs) from human urine"

For reprogramming of neonatal and adult fibroblasts:

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

For reprogramming of blood-derived epithelial progenitor cells (EPCs):

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

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/protocols-and-manuals-i65>, or by contacting [REPROCELL Technical Support \(techsupport-us@reprocell.com\)](mailto:techsupport-us@reprocell.com)

Timeline

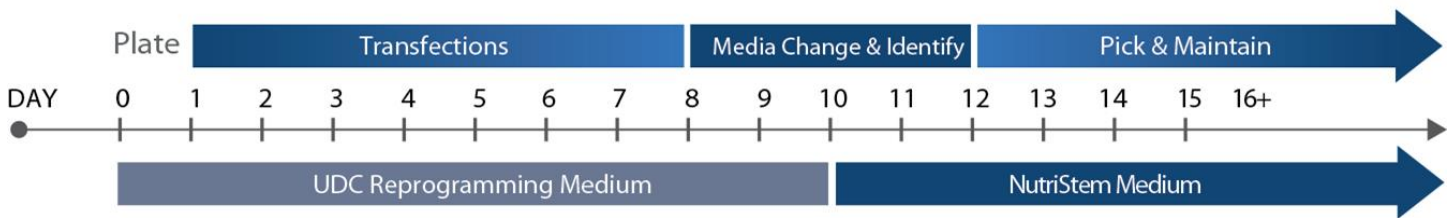


FIGURE 1. UPC Reprogramming Timeline

Day 0: Plate UPCs.

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.

Day 12-16: Pick primary UPC-NM-RNA-iPSC colonies, replating in NutriStem Medium.

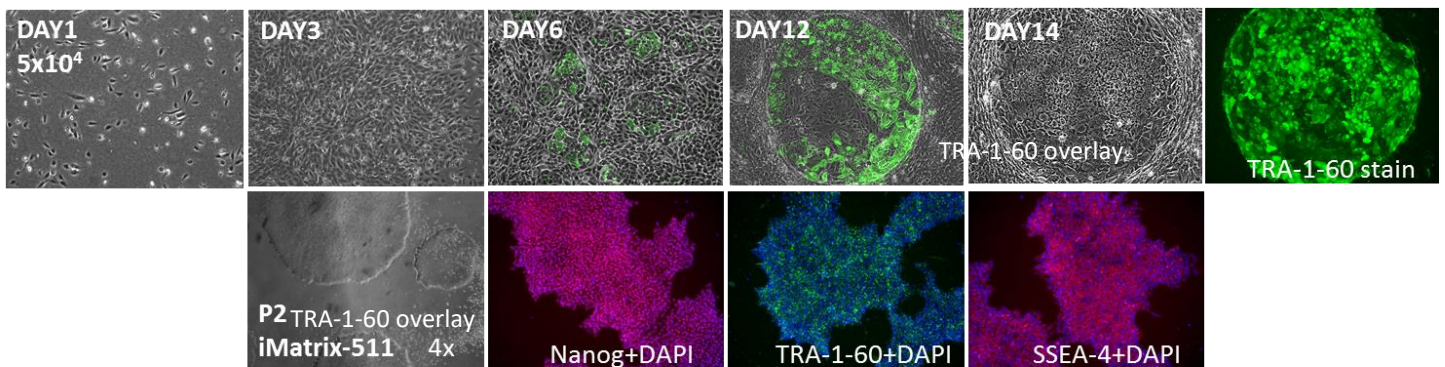


FIGURE 2: Primary reprogramming culture morphology progression, resulting from the reprogramming of UPCs with StemRNA 3rd Gen Kit on iMatrix-511 and UPC-Reprogramming Medium containing human serum. Day 6, 12, 14 primary UPC-RNA-iPSC colonies were identified using Stemgent StainAlive TRA-1-60 antibody and can be isolated from the primary culture by Day 14.

UPC-RNA- iPSC 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 UPC lines tested. For more difficult UPC lines (higher passage number or low proliferation potential) reprogramming timelines need to be extended by continuous culture in NutriStem.

Reprogramming Protocol

Step 1: Material Preparation

1.1 Preparation of UPC Reprogramming Medium (1:1 RE Proliferation Medium + MC Proliferation Medium)

1. Thaw human serum in refrigerator overnight.
2. To make Renal Epithelial (RE) Proliferation Medium add to 450 mL REGM basal medium the REGM SingleQuots supplied with the REGM Bullet Kit. Filter medium using 0.2 µm filter unit. Then add 50 mL human serum.
3. To make Mesenchymal Cell (MC) Proliferation Medium add to 450 mL DMEM: 5 mL NEAA, 1 mL Primocin, 2.5 µg bFGF, 2.5 µg EGF, 2.5 µg PDGF-AB. Filter medium using 0.2 µm filter unit. Then add 50 mL human serum to make MC proliferation medium.
4. Combine 250 mL of RE Proliferation Medium with 250 mL MC Proliferation Medium to make 500 mL of complete **UPC Reprogramming Medium**.

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

5. Store **UPC Reprogramming Medium** at 4 °C for up to four weeks.

Note: 25 mL of the UPC Reprogramming Medium is sufficient to reprogram one well of a 6-well plate using the StemRNA 3rd Gen kit. An additional 25 mL of UPC Reprogramming Medium is required for thawing and culturing of UPCs prior to reprogramming experiment.

1.2 Preparation of NM-RNA-Reprogramming Cocktail for UPCs

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 urine-derived UPCs.

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

- 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 UPCs

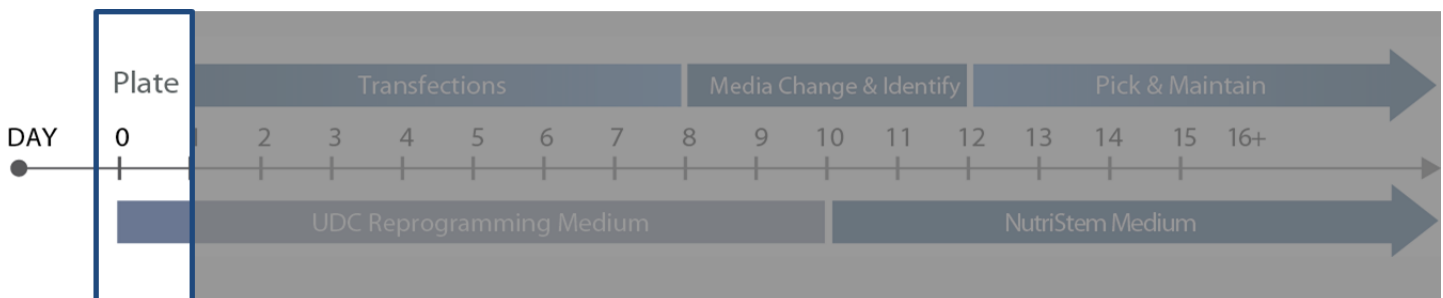
Prepare a T25 flask of exponentially growing UPCs as described in the accompanying protocol “Urine Processing for UPC Derivation.”

Note: Derivation and culturing of UPCs from human urine will take approximately 14-18 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: UPCs 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 UPCs for Reprogramming Experiment



- 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.
- Remove the culture medium from the T25 flask of exponentially growing UPCs to be harvested. Add 5 mL PBS to the culture surface of the flask to wash. Aspirate the PBS.
- 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% CO₂.
- Tap the flask to completely detach the cells from the culture surface.
- Add 6 mL **UPC Reprogramming Medium** to the flask to neutralize the Trypsin/EDTA.
- 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.
- Centrifuge the cells for 5 minutes at 250 x g.

8. Remove the supernatant and resuspend the pellet in 1 mL **UPC 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 5.0×10^4 UPCs per well in 2 mL UPC Reprogramming Medium.

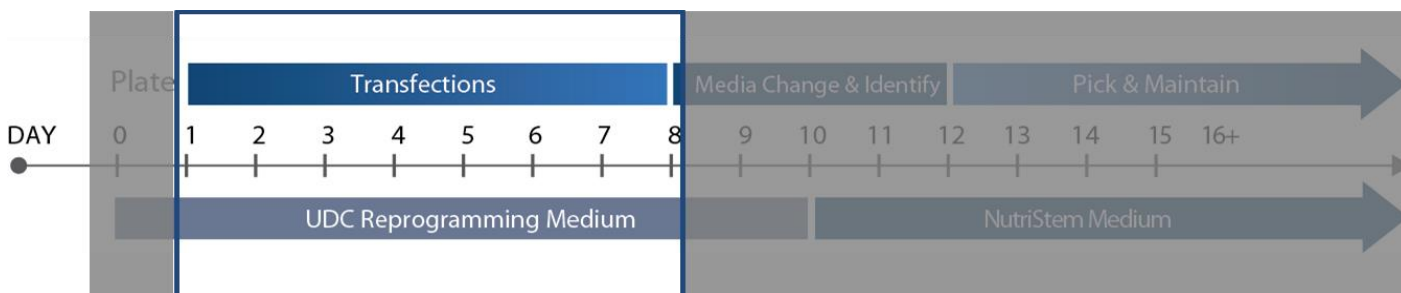
Note: Depending on the proliferation potential of your cells, 2.5×10^4 to 7.5×10^5 UPCs per well can be seeded. See also **Appendix A** for more information about UPC seeding densities.

11. Incubate the cells overnight at 37 °C, 5% CO₂ and 21% oxygen incubator.

Step 3: Transfections

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

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



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

Note: REPROCELL recommends using a hypoxic incubator (5% O₂) starting on day 1 of the reprogramming protocol.

1. Warm **UPC Reprogramming Medium** in a 37 °C water bath.
2. Remove the medium from the wells in the reprogramming plate. Add 2 mL **UPC 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 a sterile, RNase-free 1.5 mL microcentrifuge tube “B (RNAiMAX+ Opti-MEM)”.
 - To tube A, add 230.6 µL Opti-MEM (tube A already contains 19.4 µL NM-RNA Reprogramming Cocktail).
 - To tube B, add 8 µL RNAiMAX transfection reagent to 242 µL Opti-MEM (see Figure 3).

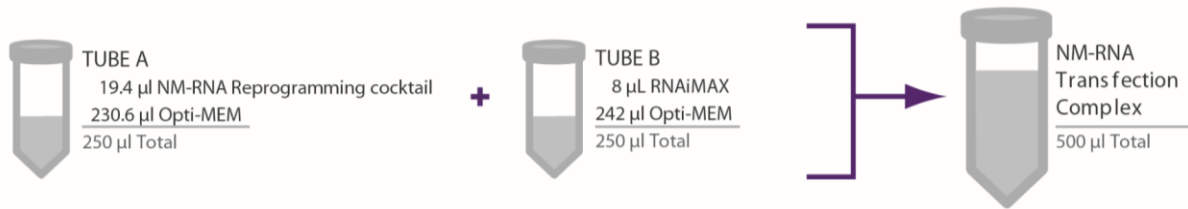
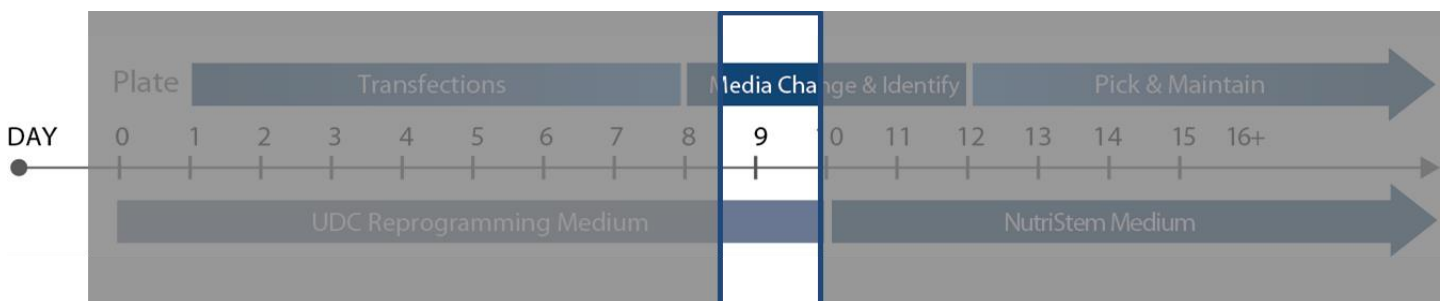


FIGURE 3. NM-RNA Reprogramming cocktail set-up

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% O₂) overnight.
10. Repeat Steps 1-9 for following daily transfections on Day 2-8.

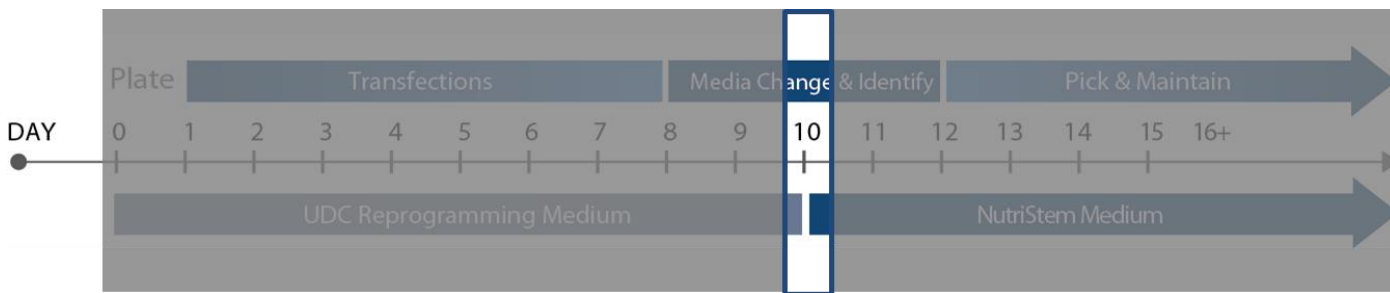
Note: For best results, change medium as early as possible in the day and add the new transfection complex as late in the day as possible. Adding some recovery time before adding the next transfection complex decreases the cell toxicity.

3.2 Day 9: Media change only



1. Warm UPC Reprogramming Medium in a 37 °C water bath.
2. Remove medium from reprogramming plate, replace with 2 mL per well **UPC Reprogramming Medium**.
3. Return the UPC reprogramming plate to a hypoxic incubator (5% O₂) 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 Medium in a 37 °C water bath.
2. Remove medium from UPC reprogramming plate, replace with 2 mL per well **NutriStem Medium**.
3. Return the UPC plate to a hypoxic incubator (5% O₂) over night.
4. Replace medium in UPC reprogramming plate with **NutriStem Medium** daily until iPSC colonies are ready to be picked.

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

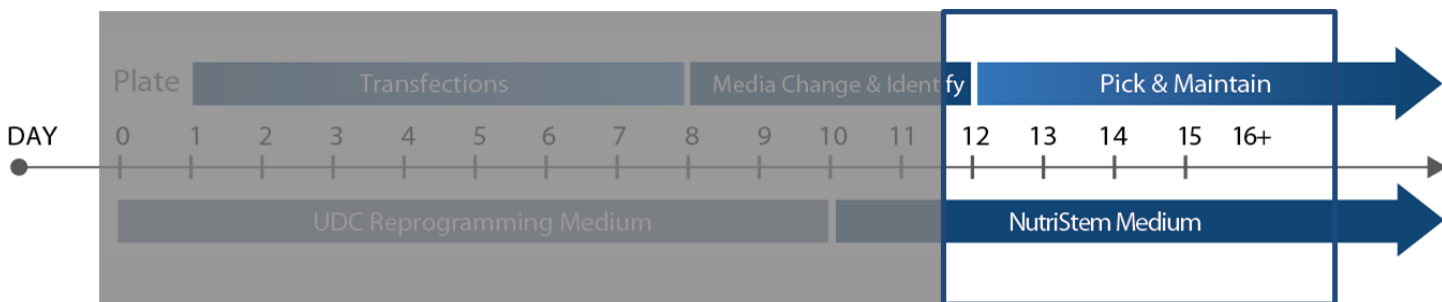
When colonies reach sufficient size and are TRA-1-60 positive they should be picked and replated into individual wells of an iMatrix-511-coated 12-well plate (“Passaging Plate”).

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 of 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 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.
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% O₂) 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 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).

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Zhou T, Benda C, Dunzinger S, Huang Y, Ho JC, Yang J, Wang Y, Zhang Y, Zhuang Q, Li Y, Bao X, Tse H-F, Grillari J, Grillari-Voglauer R, Pei D, Esteban MA. Generation of human induced pluripotent stem cells from urine samples. *Nature Protocols* 7, 2080-2089 (2012)

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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

TABLE A2. Plating conditions for different matrix choices

UPC Expansion Medium Choice	Recommended Plating Density for Reprogramming on One Well of a 6-well Plate		Reprogramming Efficiency up to
	iMatrix511	Matrigel	
UPC Reprogramming Medium with 10% human serum	5.0x10 ⁴	7.5x10 ⁴	~0.1 -1.1%

Note: The recommended plating densities in the table reflect the optimal density for most UPC lines at P2-P4 under the specific conditions. REPROCELL recommends plating between **2.5x10⁴-7.5x10⁴** UPCs per 6-well plate. The higher the proliferation potential and the lower the passage number, the lower the required seeding density. The reprogramming efficiency is highly dependent on the UPC line.



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