

Stemgent mRNA Reprogramming System

For faster, integration-free reprogramming
of human fibroblasts

Revision Date January 2012

Table of Contents

mRNA Reprogramming System Overview	3
System Contents and Storage	4
Reprogramming Timeline	5
Getting Started Guidelines	6
Protocol Methods	
Material List	7
Step 1: Material Preparation	8
Step 2: Plating Cells	10
Step 3: Transfecting Cells	12
Step 4: Identifying Cells	17
Step 5: Picking and Passaging Colonies	19
Step 6: Maintaining Cells	21
Appendix	
Appendix A: Single Well Volumes	22
Appendix B: Generating NuFF-conditioned Pluriton™ Reprogramming Medium	25
Appendix C: Thawing and Passaging BJ Fibroblasts	27
Appendix D: Passaging Target Cells	29
Appendix E: Recipes	31
Product List	33
Purchaser Notification	35

mRNA Reprogramming System

mRNA-based Reprogramming

The discovery that induced pluripotent stem (iPS) cells can be generated from somatic cells through the over expression of transcription factors holds the promise of treating diseases, such as diabetes and Parkinson's disease. However, conventional DNA-based reprogramming technologies carry the risk of disrupting the cell's genome and leading it to become cancerous and are therefore not suitable for clinical applications, such as disease modeling, drug discovery, and regenerative medicine.

In November 2010, Warren et al. described the ability to reprogram human cells using modified mRNA with conversion efficiencies and kinetics superior to DNA-based methods. This technology is a significant leap forward in the drive to safely and effectively reprogram mature human cells. The mRNA reprogramming method is the safest and most efficient method currently available, eliminating safety and bio-containment concerns associated with virus as well as the need for the screening of cells to confirm viral remnants no longer remain.

mRNA Reprogramming System

The Stemgent® mRNA Reprogramming System enables non-viral, non-integrating, clinically-relevant reprogramming of human cells. Messenger RNA-based reprogramming completely eliminates the risk of genomic integration and mutagenesis inherent to DNA and viral-based technologies while offering a highly robust and efficient reprogramming system. Stemgent's mRNA Reprogramming System and validated protocol combines everything you need for successful reprogramming of your cells.

Advantages

- Faster reprogramming, generate virus-free iPS cell colonies in as early as 12 days
- Non-integrative technology eliminates the risk of genomic integration
- Safer alternative to viral-based technologies
- Eliminates need of backend screening of colonies to confirm viral remnants no longer remain
- Allows for stoichiometric control of individual reprogramming factors
- Hands-on training and on-site support also available

References

Angel, M., Yanik, M.F. (2010) Innate immune suppression enables frequent transfection with RNA encoding reprogramming proteins. PLoS One 5:e11756.

Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., Daley, D.Q., Brack, A.S., Collins, J.J., Cowan, C., Schläeger, T.M., Rossi, D.J. (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell. 7:618.

Yakubov, E., Rechavi, G., Rozenblatt, S., Givol, D. (2010) Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors. Biochem Biophys Res Commun. 394:189.

System Contents and Storage

System Contents

The Stemgent® mRNA Reprogramming System includes five mRNA reprogramming factors, nuclear GFP (nGFP) marker for monitoring transduction efficiency, the Pluriton™ Reprogramming Medium, B18R Recombinant Protein Carrier-Free, and a validated protocol. The entire system can be purchased as a kit, where all components within the kit have been validated together to reprogram human fibroblasts into iPS cells.

Some components of the mRNA Reprogramming System are also available individually or as a factor set. The B18R protein is offered through Stemgent only as a component of the mRNA Reprogramming Kit (it can be purchased separately through eBioscience). Please see the Appendix section for specific ordering information.

Component	Qty	Amount
Stemgent® Oct4 mRNA, Human	2 vials	20 µg
Stemgent® Klf4 mRNA, Human	1 vial	20 µg
Stemgent® Sox2, Human	1 vial	20 µg
Stemgent® Lin28 mRNA, Human	1 vial	20 µg
Stemgent® c-Myc mRNA, Human	1 vial	20 µg
Stemgent® nGFP mRNA	1 vial	20 µg
B18R Recombinant Protein Carrier-Free	1 vial	40 µg
Pluriton™ Supplement 2500X	1 vial	0.2 ml
Pluriton™ Medium	1 bottle	500 ml

Storage

Store the mRNA factors, B18R protein, and Pluriton™ Supplement at or below -70°C.

Store the bottle of Pluriton™ Medium at -20°C.

All components are stable for a minimum of 3 months from date of receipt, when stored as directed.

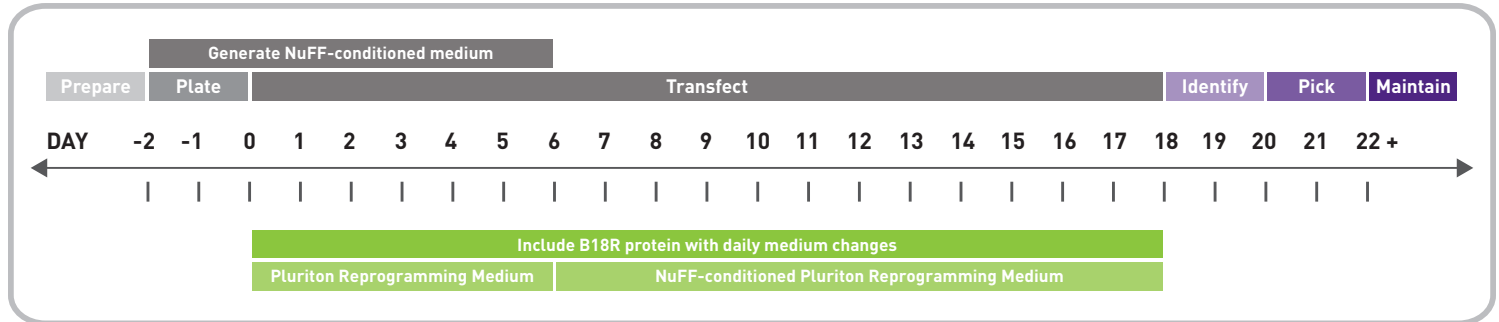
Intended Use

For research use only. Not for use in diagnostic procedures.

Reprogramming Timeline

Workflow

The timeline and table below show the six steps and media changes required for mRNA reprogramming using the Stemgent® mRNA Reprogramming System.



Although Stemgent recommends transfection for 18 days with your first experiment, colonies can begin to appear in as early as 12 days.

Step	Description	Day	Activity	Medium	Page
1	Material Preparation	- 3	Prepare and aliquot materials according to the protocol up to a week prior to beginning the experiment		8
2	Plating Cells	- 2	Plate NuFF cells for the feeder layer on reprogramming plates	NuFF culture medium	10
		- 2	Plate NuFF cells for generation of NuFF-conditioned medium	NuFF culture medium	Appendix B
		- 1	Plate target cells on NuFF feeder reprogramming plates	BJ or target cell culture medium	11
3	Transfecting Cells	0	Transfect cells to begin mRNA reprogramming	Pluriton™ Reprogramming Medium (with B18R protein)	12
		1 to 5	Repeat transfection and change medium daily	Pluriton™ Reprogramming Medium (with B18R protein)	14
		6 to 17	Repeat transfection and change medium daily	NuFF-conditioned Pluriton™ Reprogramming Medium (with B18R protein)	16
4	Identifying Cells	18 to 20	Identify primary iPS cells with a StainAlive™ antibody and change culture medium daily	NuFF-conditioned Pluriton™ Reprogramming Medium (without B18R protein)	17
5	Picking and Passaging iPS Cell Colonies	21	Manually pick and passage primary iPS cell colonies onto 12-well MEF feeder plates	Human iPS Cell Culture Medium	19
6	Maintaining iPS Cell Cultures	22 +	Exchange medium daily and allow iPS cells to expand in culture. When the culture is ready to be passaged, manually pick and passage into new 12-well or 6-well plates.	For maintenance and expansion, the iPS cells can continue to be cultured in Human iPS Cell Culture Medium, Pluriton™ Reprogramming Medium (without B18R protein), or adapted to other iPS cell culture conditions	21

Getting Started Guidelines

Reagents

- Appropriate storage and handling of the reagents is critical to the success of a reprogramming experiment. Aliquot essential components (mRNA cocktail, Pluriton™ Medium, Pluriton™ Supplement, and B18R) into single-use volumes for consistency and to eliminate degradation caused by repeated freeze/thaw cycles.
 - Use a single lot of each reagent throughout the entirety of the experiment.
 - B18R protein is essential to modulate the cells' innate immune response during mRNA transfections. The B18R protein must be supplemented in the cell culture medium to a final concentration of 200 ng/ml during each mRNA transfection in order to maintain healthy cultures throughout the reprogramming experiment.
-

Experimental Parameters

- mRNA reprogramming experiments can be successfully performed under both atmospheric conditions (21% O₂) and decreased oxygen levels (3 to 5% O₂). However, the mRNA reprogramming process has proven to be more efficient under lower oxygen tensions, often yielding 2 to 5 times as many iPS cell colonies than generated under atmospheric conditions. When reprogramming under low oxygen tensions, begin equilibrating the Pluriton Medium in low O₂ conditions prior to the first transfection, and transfer the target cells to a low O₂ incubator during the B18R pretreatment step until the series of transfections is complete.
 - Transfect cells at the same time every day during the reprogramming protocol. Due to the transient nature of the mRNA reprogramming factors, consistent timing of each transfection is critical.
-

Cells

- The feeder layer can greatly affect the health of the culture and the success of reprogramming. Use inactivated NuFF cells (human newborn foreskin fibroblasts) that have been previously established and tested to support somatic cell reprogramming with mRNA.
 - Assess the proliferation rate of your target cells in culture prior to beginning the reprogramming experiment. The cell proliferation rate will help determine the appropriate density to plate target cells for reprogramming.
 - Evaluate the transfection efficiency of your target cell type by transfecting the cells with nGFP mRNA prior to beginning the experiment. Cells that are more refractory to transfection may require additional adjustments to the transfection protocol for successful reprogramming.
-

Evaluating Experiments

- The nGFP control mRNA should be included in the mRNA reprogramming cocktail, as it is useful to assess the transfection efficiency and to track morphological changes throughout the reprogramming process.
- Use a StainAlive™ live-staining antibody to assess the pluripotency marker expression of the emergent iPS cell colonies. An objective marker can be a useful tool to label the areas of the well to be picked and replated.
- Assessing morphological progression and nGFP expression over time can be an invaluable tool to evaluate the progress of a reprogramming experiment. Capture images for reference and monitor the cell cultures on a daily basis to compare the morphologies and mRNA expression levels over the course of the experiment.

Material List

mRNA Factors

Product Description	Vendor	Cat. No.
Oct4 mRNA, Human	Stemgent®	05-0014
Klf4 mRNA, Human	Stemgent®	05-0015
Sox2 mRNA, Human	Stemgent®	05-0016
Lin28 mRNA, Human	Stemgent®	05-0017
c-Myc mRNA, Human	Stemgent®	05-0018
nGFP mRNA	Stemgent®	05-0019

Media and Reagents

Product Description	Vendor	Cat. No.
Pluriton™ Reprogramming Medium (Includes the Pluriton™ Medium and Pluriton™ Supplement (2500X))	Stemgent®	00-0070
B18R Recombinant Protein Carrier-Free	eBioscience	Included in Stemgent mRNA Reprogramming Kit
Stemfactor™ bFGF (Human Recombinant)	Stemgent®	03-0002
Stemolecule™ Y27632	Stemgent®	04-0012
0.05% Trypsin/EDTA	ATCC	PCS-999-003
Trypsin Neutralizing Solution	ATCC	PCS-999-004
EMEM, High Glucose	ATCC	302003
FBS	Atlas Biologicals	F-0500-A
DMEM, High-Glucose	Gibco®	11960-044
Penicillin-Streptomycin(100X)	Gibco®	15070-063
GlutaMAX™	Gibco®	35050-061
Opti-MEM® Reduced Serum Medium	Gibco®	31985-062
Lipofectamine™ RNAiMAX	Invitrogen	13778-075
PBS	Gibco®	14190-144

Cell Lines

Product Description	Vendor	Cat. No.
Human BJ Foreskin Fibroblasts (p6)	Stemgent®	08-0027
USE EITHER/ OR	Human Fibroblasts Mitomycin-C treated, Donor 11 (NuFF cells)	Global Stem GSC-3001M
	Human Fibroblasts Irradiated, Donor 11 (NuFF cells)	Global Stem GSC-3001G
CF-1 Mouse Embryonic Fibroblasts (MEFs) Irradiated	Global Stem	GSC-6201G

Antibodies

Product Description	Vendor	Cat. No.
USE EITHER/ OR	StainAlive™ DyLight™ 488 TRA-1-60	Stemgent® 09-0068
	StainAlive™ DyLight™ 488 TRA-1-81	Stemgent® 09-0069

Other Equipment

- 0.2% gelatin in sterile ddH₂O
- 6-well tissue culture plates
- 12-well tissue culture plates
- T75 tissue culture flasks
- 1.5 ml RNase-free, low protein-binding microcentrifuge tubes
- RNase-free 15 ml conical tubes
- RNase-free 50 ml conical tubes
- RNase-free aerosol-barrier pipet tips

Reprogramming Protocol

STEP 1 Material Preparation

This protocol describes the use of the Stemgent® mRNA Reprogramming System to reprogram 4 wells of human target cells in a 6-well plate format. Reagent and medium volumes reflect the amounts needed to reprogram all 4 wells in one experiment. If reprogramming less than 4 wells at one time, refer to Appendix A for single reaction volumes.

Material preparation should begin several days prior to starting the experiment. All material preparation steps should be performed under sterile conditions in a biological safety cabinet. To begin, aliquot the Pluriton™ Supplement and B18R protein and prepare the mRNA cocktail up to one week prior to Day -2 of the reprogramming protocol. The bottle of Pluriton™ Medium should be thawed at 4°C starting on Day -3 and aliquotted on Day -1. It is also essential to prepare NuFF-conditioned Pluriton™ Medium either prior to beginning the experiment or concurrently beginning on Day -2.

Pluriton™ Supplement

1.1 The 200 µl vial of Pluriton™ Supplement must be aliquotted in single-use vials and frozen at -70°C until use in order to minimize degradation of components in the supplement. One 4 µl aliquot will be used for each daily 10 ml medium preparation.

1. Thaw the 200 µl vial of supplement on ice.
2. Pipet 4 µl of supplement directly into the bottom of 50 sterile, low protein-binding microcentrifuge tubes.
3. Freeze and store the supplement aliquots at -70°C for up to 3 months.

NOTE: Once the single-use aliquots have been thawed they must be used immediately and can not be re-frozen.

B18R Recombinant Protein

1.2 Before beginning the reprogramming experiment, the B18R protein must be aliquotted into single-use vials and frozen at -70°C until use. All vials of the B18R protein must be kept on ice at all times in order to minimize degradation of the protein. One aliquot will be used for each day of transfection.

1. To begin, thaw the 40 µg vial of B18R protein (0.5 mg/ml stock concentration, 80 µl total volume) on ice.
2. Pipet 4 µl of the B18R protein directly into the bottom of 20 sterile, low protein-binding microcentrifuge tubes.
3. Freeze and store the protein aliquots at -70°C for up to 3 months.

NOTE: Once the single-use aliquots have been thawed they must be used immediately and can not be re-frozen.

Reprogramming Protocol

mRNA cocktail

Material Preparation (continued)

1.3 Create a master mRNA cocktail and aliquot the mix into single-use volumes. This can be done up prior to beginning the reprogramming experiment. Combine all mRNA factors according to the volumes in the table below. When reprogramming 4 wells at a time, aliquot the mRNA cocktail into 20 single-use vials, one of which will be used for each day of transfection. The mRNA cocktail, as prepared below, has a molar stoichiometry of 3:1:1:1:1 for the Oct4, Sox2, Klf4, c-Myc, Lin28 and nGFP mRNAs, respectively. Each mRNA factor is supplied at a concentration of 100 ng/μl.

1. Thaw the individual vials containing each mRNA reprogramming factor on ice. Keep mRNA vials on ice at all times.
2. Using RNase-free aerosol-barrier tips, combine the mRNA factors according to the table below in a sterile, 1.5 ml RNase-free microcentrifuge tube on ice.

Oct4 mRNA	385.1 μl
Sox2 mRNA	119.2 μl
Klf4 mRNA	155.9 μl
c-Myc mRNA	147.7 μl
Lin28 mRNA	82.5 μl
nGFP mRNA	110.6 μl
mRNA cocktail mix	1000 μl

3. Pipet the contents of the tube to mix thoroughly.
4. Aliquot 50 μl of the mRNA cocktail into 20 individual sterile, 1.5 ml **RNase-free** microcentrifuge tubes.
5. Freeze and store the aliquots at -70°C.

NOTE: Once the single-use aliquots have been thawed they can not be re-frozen.

Pluriton™ Medium

1.4 The 500 ml bottle of Pluriton™ Medium should be aliquotted as instructed below. Prior to aliquotting, the medium can be supplemented with penicillin/streptomycin (100X). Approximately 220 ml of Pluriton™ Medium will be used during the first week of the protocol and for generating NuFF-conditioned Pluriton™ Medium. The remaining medium should be aliquotted and stored at -20°C until use. After thawing, the shelf-life of Pluriton™ Medium is 2 weeks when stored at 4°C.

1. Thaw the 500 ml bottle of Pluriton™ Medium completely at 4°C.

NOTE: The 500 ml bottle of Pluriton™ Medium may take up to 2 days to thaw completely at 4°C.

2. Once the medium bottle has thawed completely, add 5 ml of penicillin/streptomycin (100X) to the bottle. Pipet thoroughly to mix.
3. Pipet 40 ml aliquots of the medium into seven 50 ml conical tubes (280 ml total).
4. Freeze the 7 medium aliquots at -20°C.
5. Store the remaining 220 ml of medium at 4°C for use during the first week of the protocol and for generating NuFF-conditioned Pluriton™ Medium.

Reprogramming Protocol

STEP 2 Plate Cells

Day -2



IMPORTANT: Begin Conditioned Medium Protocol

NuFF-conditioned Pluriton™ Medium is required to support the reprogramming process starting on Day 6 until the cells are replated onto a new inactivated feeder layer. In order to collect sufficient NuFF-conditioned Pluriton™ Medium by Day 6 of the reprogramming protocol, inactivated NuFF cells should be plated in a separate T75 flask on Day -2. Refer to Appendix B for details on generating NuFF-conditioned Pluriton™ Medium. The NuFF-conditioned Medium Protocol will run concurrently for 7 days alongside the mRNA reprogramming protocol.

2.1 Plate Human NuFF Feeder Cells

On Day -2, NuFF cells should be evenly plated at a density of 2.5×10^5 cells per well of a 6-well plate in a total volume of 2.5 ml of NuFF culture medium per well. The NuFF feeder cells must be allowed to incubate overnight prior to plating target cells for reprogramming. See Appendix E for directions on preparing the NuFF culture medium.

1. Add 1 ml of sterile 0.2% gelatin (in ddH₂O) in each of 4 wells of a 6-well tissue culture plate.
 2. Incubate the plate for a minimum of 30 minutes at 37°C and 5% CO₂.
 3. Thaw one vial of inactivated NuFF cells in a 37°C waterbath until only a small ice crystal remains.
 4. Transfer the contents of the vial of NuFF cells directly to a 15 ml conical tube.
 5. Gradually add 5 ml of NuFF culture medium to the cells while gently agitating the contents of the vial.
 6. Centrifuge the cells for 4 minutes at 200 x g.
 7. Aspirate the supernatant and resuspend the cell pellet in 6 ml of NuFF culture medium.
 8. Count the cells in solution and calculate the live cell density.
 9. Add an appropriate amount of NuFF culture medium to the suspension to bring the cell density to 2.5×10^5 cells/ml.
 10. Aspirate the gelatin solution from the 4 wells of the prepared 6-well plate and add 1 ml of NuFF culture medium to each of the wells.
 11. Evenly distribute 1 ml of the NuFF cell suspension to each of the 4 wells.
- NOTE:** The remainder of the NuFF cells should be plated in a separate T75 flask to be used to generate NuFF-conditioned Pluriton™ Medium. (See Appendix B.)
12. Incubate the cells overnight at 37°C and 5% CO₂.

Expected Results

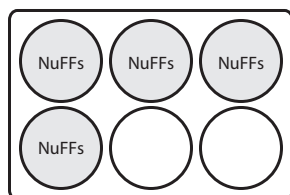


Figure 1. NuFF feeder layer. NuFF cells should be evenly plated at a density of 2.5×10^5 cells per well.

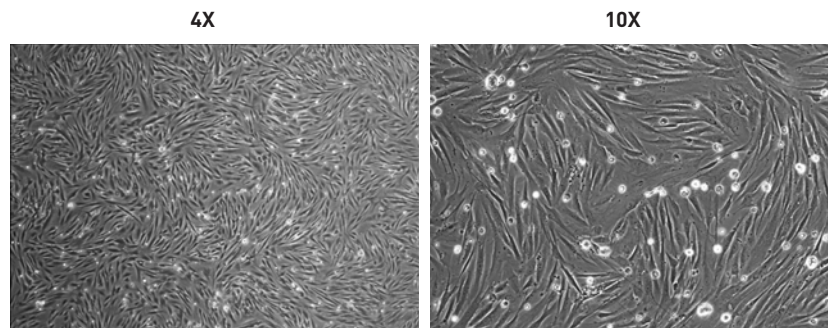


Figure 2. NuFF feeder cells plated at at 2.5×10^5 cells per well on Day -2. Images were captured 24 hours after plating and represent an appropriate NuFF feeder layer density for plating target cells on Day -1.

Reprogramming Protocol

Day -1

Plate Cells (continued)

2.2 Plate Target Cells

- This harvesting procedure is appropriate for BJ fibroblasts in culture in a T75 flask and may not be applicable to all target cell types. For target cells other than BJ fibroblasts, harvest the cells according to an appropriate protocol and plate in the format described below. See Appendix C for protocols for thawing and passaging BJ fibroblasts. See Appendix E for the recipe and preparation of BJ cell culture medium.
1. Remove the culture medium from the T75 flask of cells to be harvested.
 2. Add 10 ml of PBS to the culture surface of the flask to wash. Aspirate the PBS.
 3. Add 3 ml of 0.05% Trypsin/EDTA to the flask.
 4. Incubate the cells for 3 to 5 minutes at 37°C and 5% CO₂.
 5. Tap the flask to completely detach the cells from the culture surface.
 6. Add 6 ml of BJ cell culture medium (or appropriate target cell medium containing serum) to the flask to neutralize the Trypsin/EDTA.
 7. With a 10 ml pipet, transfer the harvested cell solution from the T75 flask to a 15 ml conical tube.
 8. Centrifuge the cells for 5 minutes at 200 x g.
 9. Remove the supernatant and resuspend the pellet in 5 ml of BJ cell culture medium (or appropriate target cell medium).
 10. Count the cells in solution and calculate the live cell density.
 11. Dilute a sufficient volume of the cell suspension to a final density of 4 x 10³ cells/ml in BJ cell culture medium.
 12. Aspirate the NuFF culture medium from one well of the 6-well plate and add 2.5 ml of BJ fibroblasts (at 4 x 10³ cells/ml) to a final density of 1 x 10⁴ BJ cells per well.
 13. Harvest the experimental target cells according to an appropriate protocol and plate the target cells in three independent wells of the NuFF feeder plate at densities of 5 x 10³, 1 x 10⁴, 2.5 x 10⁴ cells per well in 2.5 ml total volume per well.
 14. Incubate the cells overnight at 37°C and 5% CO₂.

Expected Results

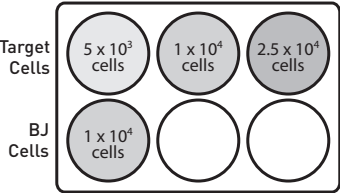


Figure 3. Target cells. Plate target cells in three independent wells and plate BJ fibroblasts in the fourth well as a control

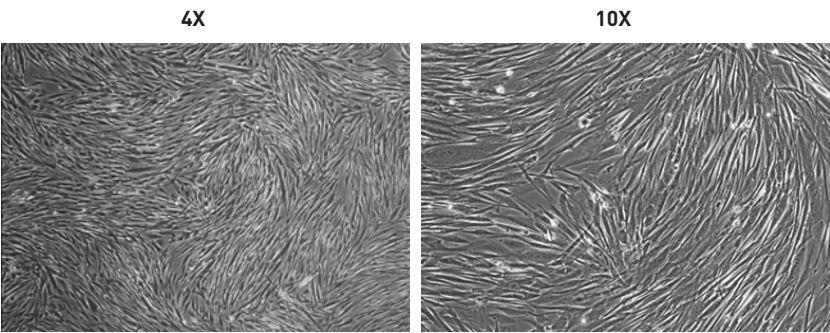


Figure 4. BJ fibroblasts plated at a density of 1 x 10⁴ cells per well on Day -1. Images were captured 24 hours after plating the BJ fibroblasts on a previously prepared NuFF feeder layer.

Reprogramming Protocol

STEP 3 Transfect Cells

Day 0

3.1 Pretreat Target Cells with B18R Protein

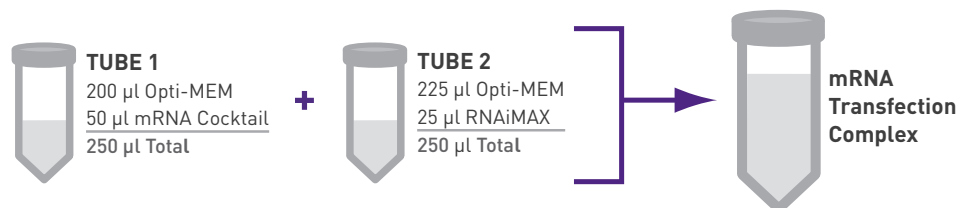
The B18R protein must be present in the culture medium at a concentration of 200 ng/ml during every transfection. A 2 hour pretreatment with the B18R protein is required prior to the first transfection on Day 0 to pre-suppress the cells' interferon response. Subsequent culture and transfections on Day 1 to Day 17 will be carried out in Pluriton™ Reprogramming Medium that is already supplemented with the B18R protein.

1. Add 10 ml of Pluriton™ Medium to a sterile 100 mm dish.
2. Incubate the medium for 2 hours at 37°C and 5% CO₂ to equilibrate the medium to the proper oxygen tension.
NOTE: If reprogramming under low oxygen conditions, the medium should be equilibrated at low O₂ tensions prior to each medium exchange.
3. Just prior to use, thaw one vial of Pluriton™ Supplement and one vial of B18R protein on ice.
4. Add 4 µl of the supplement and 4 µl of the B18R protein to the medium to generate Pluriton™ Reprogramming Medium (with B18R protein).
5. Aspirate the target cell medium from each of the 4 wells to be transfected.
6. Add 2 ml of Pluriton™ Reprogramming Medium (with B18R protein) to each of the 4 wells.
7. Incubate the cells for 2 hours at 37°C and 5% CO₂ prior to transfecting.

NOTE: From this point forward, cells can be cultured in a low O₂ (3 to 5% O₂) incubator.

3.2 Prepare mRNA Transfection Complex

Prior preparation of single-use 50 µl aliquots of the mRNA cocktail ensures that the mRNA is thawed only once prior to each daily transfection. The RNAiMAX® transfection reagent must first be diluted in Opti-MEM® medium before combining with the diluted mRNA cocktail to generate the mRNA transfection complex.



1. Thaw one 50 µl aliquot of the mRNA cocktail on ice (Tube 1).
2. Using RNase-free, aerosol-barrier pipette tips, add 200 µl of Opti-MEM to the tube containing the mRNA cocktail and pipet gently to mix (Tube 1).
3. In a second sterile, RNase-free 1.5 ml microcentrifuge tube, add 225 µl of Opti-MEM (Tube 2).
4. Add 25 µl of RNAiMAX to the Opti-MEM and pipet gently to ensure that all of the transfection reagent is removed from the pipet tip (Tube 2).
5. Using a 1000 µl RNase-free, aerosol-barrier pipet tip, pipet gently but thoroughly to mix the RNAiMAX with the Opti-MEM (Tube 2).
6. Transfer the entire contents of Tube 2 to the mRNA cocktail solution in Tube 1 to generate the mRNA transfection complex and pipet gently 3 to 5 times.
7. Incubate the mRNA transfection complex at room temperature for 15 minutes to allow the mRNA to properly complex with the transfection reagent.

Reprogramming Protocol

Day 0

Transfect Cells (continued)

3.3 Transfect Cells

1. In a dropwise fashion, add 120 µl of the mRNA transfection complex to each of the 4 wells to be transfected.

NOTE: Distribute the mRNA transfection complex uniformly across the 2 ml of Pluriton™ Reprogramming Medium (with B18R protein) already in the wells.

2. Gently rock the 6-well plate from side to side and front back to distribute the mRNA transfection complex evenly across the wells.
3. Incubate the cells for 4 hours at 37°C and 5% CO₂ at the experimental oxygen tension.

3.4 Equilibrate and Prepare Pluriton™ Reprogramming Medium (with B18R Protein)

Pluriton™ Medium must be equilibrated for at least 2 hours to attain the desired oxygen tension. This should be done during the 4-hour transfection. The Pluriton™ Supplement and the B18R protein should be added to the Pluriton™ Medium after equilibration, just prior to exchanging the transfection medium, in order to avoid degradation of these essential components.

1. Add 10 ml of medium to a sterile 100 mm dish.
2. Incubate the medium for 2 hours at 37°C and 5% CO₂ to equilibrate the medium to the proper oxygen tension.
3. Just prior to use, add 4 µl of supplement and 4 µl of the B18R protein to the equilibrated medium to generate Pluriton™ Reprogramming Medium (with B18R protein).

3.5 Remove Transfection Reagent

1. After the target cells have been transfected for 4 hours, aspirate the medium containing the mRNA transfection complex from each well.

NOTE: Do not leave the mRNA transfection complex in the culture medium for longer than 4 hours, as prolonged exposure to the RNAiMAX transfection reagent will result in increased cellular toxicity.

2. Add 2 ml of the equilibrated Pluriton™ Reprogramming Medium (with B18R protein) to each well.
3. Incubate the cells overnight at 37°C and 5% CO₂.

Reprogramming Protocol

Day 1 to Day 5

Transfect Cells (continued)

3.6 Reprogram Cells in Pluriton™ Reprogramming Medium (with B18R Protein)

Cells undergoing reprogramming must be transfected with the mRNA reprogramming factor cocktail every day. It is important to transfect the cells at the same time each day in order to maintain sufficient levels of mRNA transcripts to allow for continual expression of the mRNA factors. A single-use aliquot of each reagent is used to prepare fresh transfection and medium components each day. The transfection procedure must be repeated each day from Day 1 to Day 5 exactly as done on Day 0.

Monitor the cell cultures daily, observing cell proliferation rates, morphology changes, and nGFP expression in each well. Morphological growth patterns indicative of successful transfections can be seen as early as Day 3 or Day 4.

1. Prepare the mRNA transfection complex as described for Day 0.
2. Transfect cells as described for Day 0.
3. Equilibrate Pluriton™ Medium and prepare Pluriton™ Reprogramming Medium (with B18R protein) as described for Day 0.
4. After 4 hours of transfection, remove the medium containing the transfection reagent and add 2 ml of equilibrated Pluriton™ Reprogramming Medium (with B18R protein) to each well, as described for Day 0.
5. Incubate the cells overnight at 37°C and 5% CO₂.

Expected Results

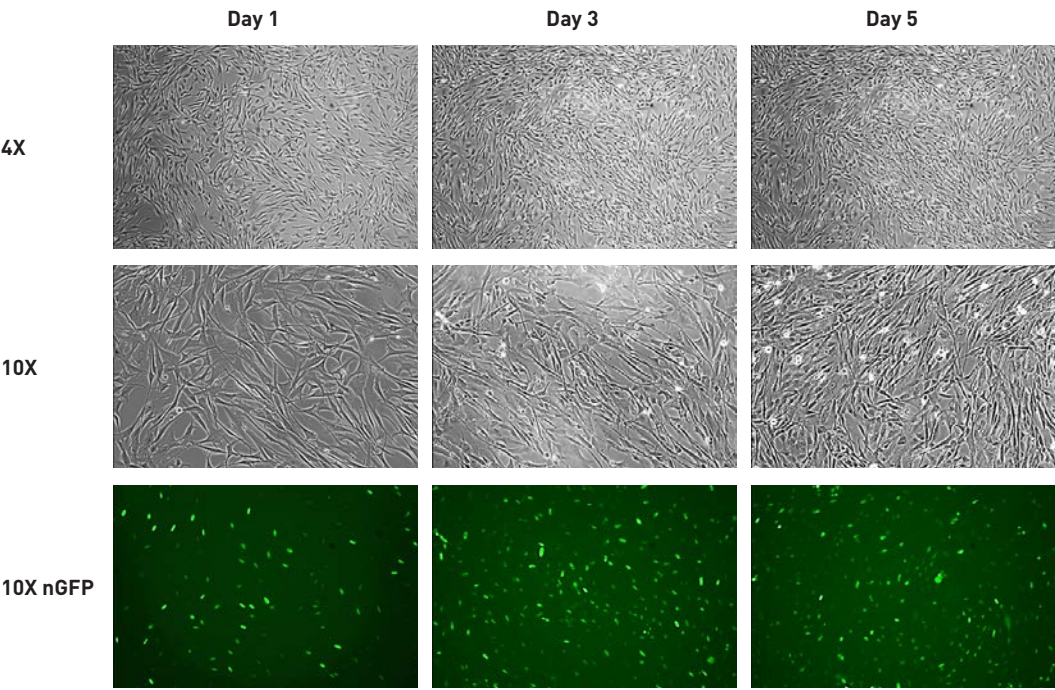


Figure 5. Observe cells in culture daily. Note early morphology changes beginning as early as Day 3. Transfected cells will begin to appear in small clusters with a more compacted morphology relative to the fibroblasts. Early in the experiment, the nGFP expression should appear uniform throughout the transfected wells. In the early stages of reprogramming, the intensity of the observed nGFP expression is often brighter in the initial cell clusters.

Reprogramming Protocol

Day 5



Transfect Cells (continued)

IMPORTANT: Assess proliferation rate and density of target cells

Over-confluent target cell cultures can negatively impact the mRNA transfection efficiency and amount of mRNA entering the cells per cell dose, which can therefore reduce the efficiency of reprogramming. Based on the degree of confluence and the rate of proliferation of the target cells in culture, one or more wells may need to be passaged between Day 6 and Day 8 of the reprogramming protocol in order to be replated at a lower density to support reprogramming.

In order to determine whether target cells should be passaged, compare the experimental target cell densities to the images below to determine if the culture must be passaged. If the target cells have grown close to confluence by Day 6 or Day 7, passage the cells according to the protocol outlined in Appendix D. If the cells do not require passaging at this point, continue with the reprogramming protocol as described.

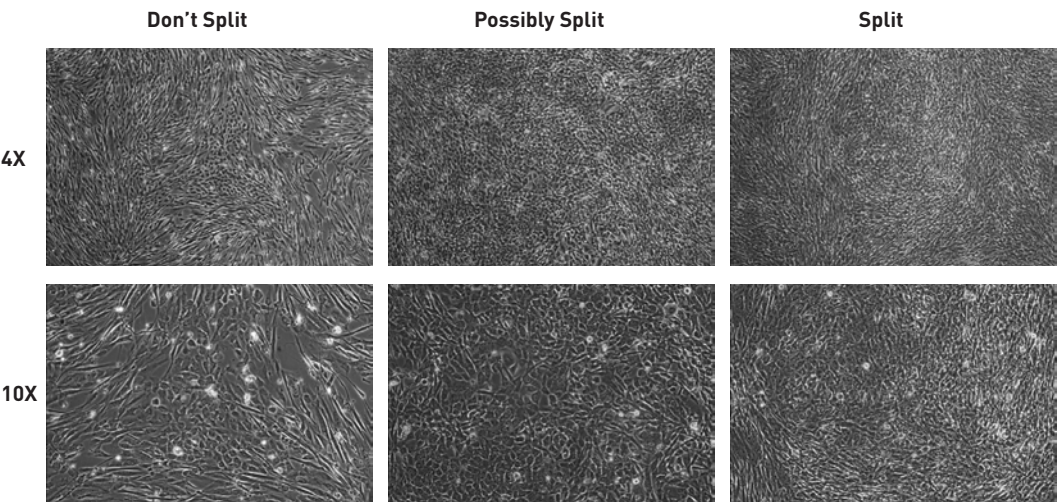


Figure 6. BJ fibroblasts, cultured at varying densities for reprogramming. Images were captured at Day 6 in the reprogramming protocol. The cells in the left panels are at an appropriate density at Day 6 and do not need to be passaged at this point. The culture has adequate space between cells to allow for further proliferation and colony formation to occur. The cells in the middle panels are nearing confluency. If the cells are rapidly dividing, the culture should be passaged to prevent the culture from becoming over-confluent before iPS cell colonies emerge. The cells in the right panels have already become over-confluent by Day 6. This culture must be passaged at this point and replated at a lower density to allow for continual cell proliferation, efficient mRNA delivery, and colony formation to occur.

Reprogramming Protocol

Transfect Cells (continued)

Day 6 to Day 17

3.7 Reprogram Cells in NuFF-conditioned Pluriton™ Reprogramming Medium (with B18R Protein)

Inactivated NuFF feeder cells will support target cells undergoing reprogramming for approximately 7 to 10 days. Starting at Day 6, NuFF-conditioned Pluriton™ Reprogramming Medium must be used in place of Pluriton™ Reprogramming Medium. Transfection of the target cells must be continued as done previously from Day 0 to Day 5. The protocol for generating and preparing NuFF-conditioned Pluriton™ Reprogramming Medium is detailed in Appendix B.

Continue to monitor the cell cultures daily, as morphological changes become more pronounced between Day 6 and Day 17. Emergent colonies can be seen as early as Day 8.

1. Prepare the mRNA transfection complex as described for Day 0.
2. Transfect cells as described for Day 0.
3. Equilibrate NuFF-conditioned Pluriton™ Medium and prepare NuFF-conditioned Pluriton™ Reprogramming Medium (with B18R protein) as described for Day 0.
4. After 4 hours of transfection, remove the medium containing the transfection reagent and add 2 ml of equilibrated NuFF-conditioned Pluriton™ Reprogramming Medium (with B18R protein) to each well, as described for Day 0.
5. Incubate the cells overnight at 37°C and 5% CO₂.

Reprogramming Protocol

STEP 4 Identify Cells

Day 18 to Day 20

4.1 Culture and Monitor iPS Cell Colonies

Once the series of transfections is completed, the primary reprogramming cultures should be maintained in NuFF-conditioned Pluriton™ Reprogramming Medium for for one to three additional days in order to allow iPS cell colonies to expand in size enough to be picked and passaged as primary colonies. During this time the medium must be replaced each day with NuFF-conditioned Pluriton™ Reprogramming Medium. At this stage, it is no longer necessary to pre-equilibrate the medium, and the B18R protein is no longer needed in the cultures after the series of transfections is completed. However, the cell cultures can continue to be maintained under low oxygen tensions until the colonies are picked and passaged.

1. Just prior to use, thaw one aliquot of Pluriton™ Supplement and add 4 µl of the supplement to 10 ml of NuFF-conditioned Pluriton™ Medium to generate NuFF-conditioned Pluriton™ Reprogramming Medium.
NOTE: B18R protein is no longer included in the medium after transfections are completed.
2. Aspirate the culture medium from each well of cells.
3. Add 2 ml of equilibrated NuFF-conditioned Pluriton™ Reprogramming Medium to each well.
4. Incubate the cells overnight at 37°C and 5% CO₂.

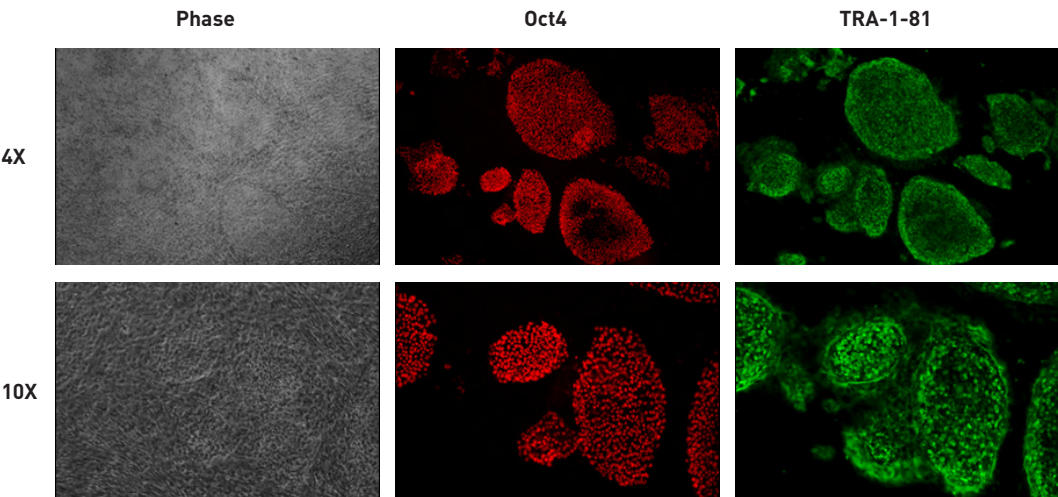


Figure 7. Pluripotency analysis performed on (fixed) iPS cell colonies at Day 18 of mRNA reprogramming derived from BJ fibroblasts plated at 1 x 10⁴ cells per well. Immunostaining of this culture confirms expression of the pluripotency markers TRA-1-81 and endogenous Oct4 in the colonies.

4.2 Identify Colonies using StainAlive™ Antibodies

Prior to manual isolation, the primary iPS cell colonies can be identified using sterile, live-staining antibodies StainAlive™ DyLight™ 488 Mouse anti Human TRA-1-60 or TRA-1-81 to confirm the onset of expression markers associated with pluripotency.

Reprogramming Protocol

Day 18 to Day 20

Identify Cells (continued)

When selecting primary iPS cell colonies for isolation and expansion, preference should be given to those colonies with both proper iPS cell morphology and homogeneous pluripotency marker expression. In cultures with high cell densities or overgrowth of fibroblasts, identification of proper iPS cell morphology with phase-contrast images may be difficult. In these instances, the expression of pluripotency markers should be used as the primary tool for identifying colonies to isolate and expand.

1. Add 6 ml of Pluriton™ Medium to a 15 mL conical centrifuge tube.
2. Add 60 µl of either StainAlive™ DyLight™ 488 TRA-1-60 or StainAlive™ DyLight™ 488 TRA-1-81 antibody to the centrifuge tube containing the medium.

NOTE: This dilution will yield a 5 µg/ml solution of the StainAlive™ antibody.

3. Aspirate the medium from the 4 wells to be stained and add 1.5 ml of the diluted antibody to each well.
4. Incubate the culture for 30 minutes at 37°C and 5% CO₂.
5. Aspirate the Pluriton™ Medium containing the antibody from each well.
6. Add 1.5 ml of Pluriton™ Medium to each well to wash.
7. Aspirate the medium and repeat the wash with an additional 1.5 ml of Pluriton™ Medium per well.
8. Supplement 10 ml of NuFF-conditioned Pluriton™ Medium with 4 µl of Pluriton™ Supplement to generate NuFF-conditioned Pluriton™ Reprogramming Medium.
9. Aspirate the final wash and add 2.5 ml of NuFF-conditioned Pluriton™ Reprogramming Medium to each well.
10. Examine the cultures using a filter set for the appropriate wavelength (maximum excitation wavelength 493 nm, mean emission wavelength 518 nm for DyLight™ 488-labeled antibodies) on a fluorescent microscope.

NOTE: Do not allow the cell cultures to be kept outside of the incubator for extended periods of time to avoid drastic temperature changes and the possibility of contamination.

11. Identify and mark appropriate colonies for picking and expansion with a microscope objective marker.
12. Return the cells to the incubator and continue to culture at 37°C and 5% CO₂.

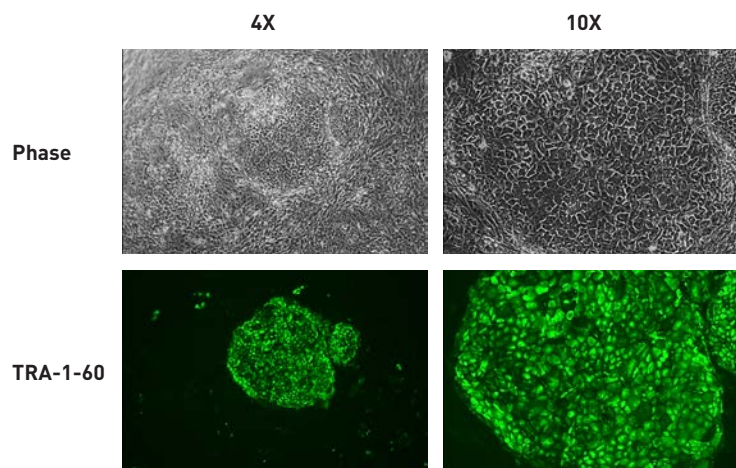


Figure 8. iPS cell colonies at Day 18 of mRNA reprogramming. iPS cell colony morphologies can be seen in the phase images (top panels). Bottom panels represent the same fields of view after incubation with Stemgent™ StainAlive DyLight™ 488 Mouse anti-Human TRA-1-81 antibody. Occasionally colony boundaries may be difficult to visualize due to the high density nature of the culture at this stage in reprogramming. However, the use of live-staining antibodies enables rapid identification of reprogrammed clones prior to colony picking and expansion.

Reprogramming Protocol

Day 21

STEP 5 Pick and Passage iPS Cell Colonies

This step outlines the process of picking and replating primary (passage 0) iPS cell colonies from the reprogrammed culture. Colonies exhibiting proper human iPS cell morphology (compact cells with a colony and defined colony edges) as well as pluripotency marker expression should be given first priority for picking and expansion of cell lines. All procedures in this protocol must be performed in a sterile environment. Picking can be performed with a stereo microscope in either a horizontal flow hood (positive pressure) or a static enclosure. Change pipet tips with each new colony to be transferred to avoid cross-contamination of clonal cell lines.

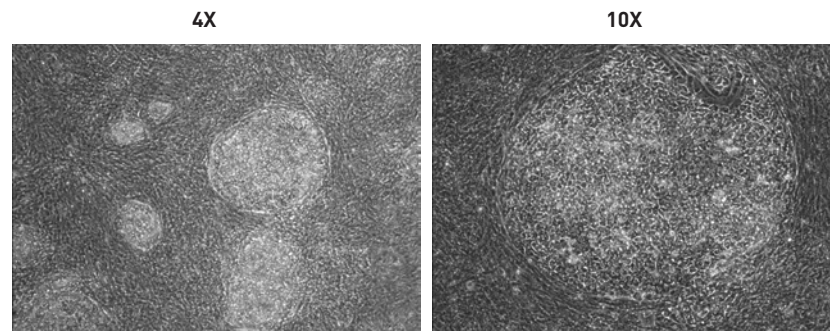


Figure 9. Primary iPS cell colonies with proper human iPS cell morphology. The large colonies are ready to be picked and clonally replated on a new feeder layer for continued growth and expansion.

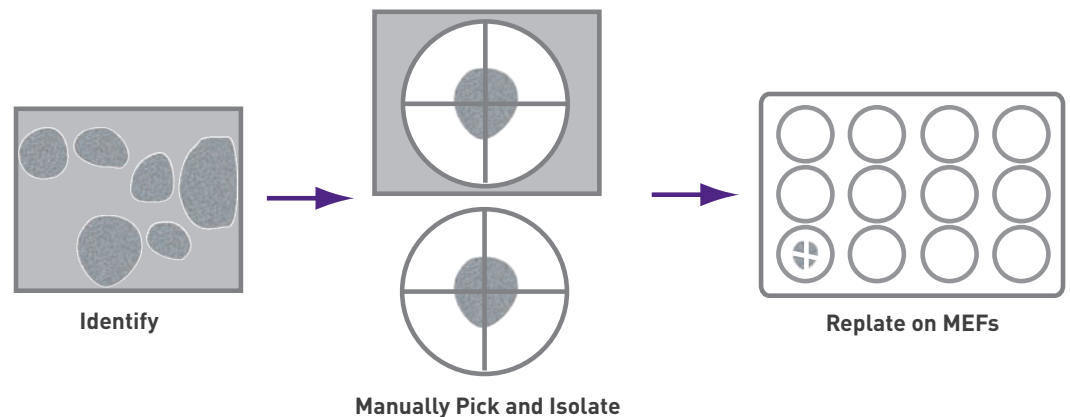


Figure 10. Pick and Passage iPS Cells. Pick and replate no more than 6 colonies at one time to avoid keeping the cells out of the incubator for extended periods of time. To maintain clonal lines, transfer all of the pieces of each individual colony to a individual well of a 12-well plate with a newly plated feeder layer. At this point in the protocol, an inactivated MEF feeder layer can be used for culture in place of the inactivated NuFF feeder cells. The MEF feeder layer should be plated at a density of 1×10^5 cells per well of the 12-well plate. Glass picking tools can be made from 9" Pasteur pipettes pulled to a closed, angled end over the controlled flame of an alcohol burner.

Reprogramming Protocol

Day 21

Pick and Passage iPS Cell Colonies (continued)

5.1 Pick and Replate Primary iPS Cell Colonies

1. Thaw one aliquot of Pluriton™ Supplement on ice and add 4 µl of the supplement to 10 ml of Pluriton™ Medium to generate Pluriton™ Reprogramming Medium.
2. Aspirate the MEF culture medium from 6 wells of a 12-well MEF feeder plate.
3. Add 1 ml of PBS to each well to rinse. Aspirate the PBS.
4. Add 1 ml of Human iPS Cell Culture Medium to each of the 6 rinsed wells.
6. Aspirate the medium from the well of the 6-well plate that the primary iPS cells will be picked from.
7. Add 2 ml of Pluriton™ Reprogramming Medium to the well of iPS cells to be picked.
8. Using a phase-contrast or stereo microscope, locate iPS cell colonies based on morphology and pluripotency marker expression.

NOTE: Live staining antibodies for pluripotency markers are excellent tools identifying reprogrammed colonies. Mark colonies to be picked while evaluating staining (Day 18 to Day 20).
9. Using a glass picking tool, gently separate the colony from the surrounding fibroblasts by “circling” the area to be picked.
10. Using the glass picking tool, gently divide the colony into approximately 4 to 8 pieces.

NOTE: It is important to break up the colony into smaller cell aggregates, but not into single cells.
11. Using the glass picking tool, gently and completely detach the colony pieces from the tissue culture plate so that the cell aggregates are freely suspended in the medium.
12. Using a pipettor with a sterile 10 µl pipet tip, transfer the detached colony pieces out of the reprogramming well and into an individual well of the prepared 12-well plate.

NOTE: Transfer all of the pieces from one colony into a single well of the 12-well plate.
13. Repeat the picking and replating process for the next iPS cell colonies. Pick one colony at a time and transfer the cell aggregates of each to a new well of the prepared 12-well inactivated MEF feeder plate.
14. After 6 iPS cell colonies have been picked and replated, return both the 12-well plate and the primary reprogrammed colonies on the 6-well plate to the incubator at at 37°C and 5% CO₂.
15. After allowing the cells to incubate for at least 30 minutes, an additional 6 primary iPS cell colonies can be picked and replated on a new prepared 12-well MEF feeder plate. Repeat this process (steps 1 to 12) in increments of 6 iPS cell colonies at a time until a sufficient number of colonies have been picked.

Suggested Culture Volumes (per Well)

Culture Vessel	Surface Area	Culture Volume	MEF Density
24-well plate	2 cm ²	0.5 ml	50,000
12-well plate	3.8 cm ²	1 ml	100,000
6-well plate	9.6 cm ²	2 ml	200,000

Reprogramming Protocol

STEP 6 Maintain iPS Cell Cultures**Day 22 +**

Human iPS cell cultures should be monitored and cared for every day, as the overall quality of the culture can change rapidly. Human iPS cells 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. 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, often causing unwanted spontaneous differentiation to occur. It is important to passage the cells before the culture becomes overgrown.

For maintenance and expansion, the iPS cells can continue to be cultured in Human iPS Cell Culture Medium, Pluriton™ Reprogramming Medium (without B18R Protein), or adapted to other proven iPS cell culture conditions. Between passages, the cell culture medium must be changed every day to provide necessary nutrients and growth factors for the maintenance of human iPS cells. If the cells were reprogrammed under low O₂ conditions, the cultures can be transitioned to normal O₂ conditions within the first passage.

For the first few passages after a picking from the reprogrammed cultures, the cells should be passaged manually (without enzymes or centrifugation) at low split ratios to build robust, dense cultures. The cells can be split using an enzymatic protocol for routine culture once there are a large number of human iPS cell colonies in the well(s).

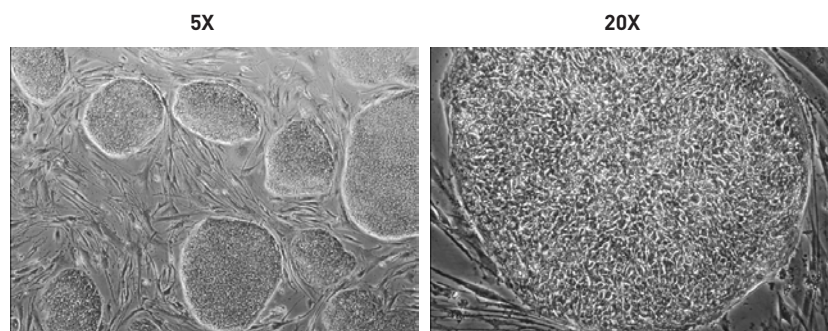


Figure 11. Maintenance of iPS Cells. Healthy human iPS cell colonies. Note the defined colony edges and the uniform and compact iPS cells within the colonies.

Appendix A

Single Well Volumes

The mRNA reprogramming protocol, as written, describes the use of the Stemgent® mRNA Reprogramming System to reprogram 4 wells of human target cells in a 6-well plate format. Reagent and medium volumes throughout the protocol reflect the amounts needed to reprogram all 4 wells in one experiment. If reprogramming less than 4 wells at one time, refer to the volumes needed per individual well below. Prepare aliquots of all reagents in single-use volumes, based on the number of wells used in a single reprogramming procedure.

A.1 Pluriton™ Supplement

Aliquot the Pluriton™ Supplement in the volumes required for a single day. The Pluriton™ Supplement must be distributed into single-use aliquots that can not be re-frozen once thawed.

1. Thaw the 200 µl vial of supplement on ice.
2. Pipet 1 µl of the supplement per well to be reprogrammed directly to the bottom of a microcentrifuge tube.
3. Freeze and store the supplement aliquots at -70°C for up to 3 months.

NOTE: To minimize degradation of essential components, once a single-use supplement aliquot is thawed, it must be used immediately and can not be re-frozen.

A.2 Pluriton™ Medium

The Pluriton™ Medium must be aliquotted into volumes that will be used within 2 weeks when stored at 4°C.

1. Thaw the 500 ml bottle of Pluriton™ Medium completely at 4°C.
2. Once the medium has thawed completely, add 5 ml of penicillin/streptomycin (100X) to the bottle. Pipet thoroughly to mix.
3. Pipet 40 ml of medium into individual conical tubes.
4. Freeze the medium aliquots at -20°C.

NOTE: Medium that will be used within the first week of the reprogramming protocol and for generating NuFF-conditioned Pluriton™ Medium can be stored at 4°C without re-freezing.

A.3 B18R Recombinant Protein

Aliquot the B18R protein in the volumes required for a single day. The B18R protein must be distributed into single-use aliquots which can not be re-frozen once thawed.

1. Thaw the 40 µl vial of B18R protein (0.5 mg/ml stock concentration) on ice.
2. Pipet 1 µl of B18R per well to be reprogrammed directly to the bottom of a low protein-binding microcentrifuge tube.
3. Freeze and store the B18R aliquots at -70°C for up to 3 months.

NOTE: To minimize degradation of the protein, once a single-use aliquot is thawed, it must be used immediately and can not be re-frozen.

Appendix A (continued)

Single Well Volumes

A.4 Pluriton™ Reprogramming Medium (with B18R Protein)

For each well of target cells maintained during the reprogramming experiment, prepare 2.5 ml of Pluriton™ Reprogramming Medium (with B18R Protein) according to the table below. The Pluriton™ Medium must be first equilibrated for 2 hours at 37°C and 5% CO₂ prior to adding the supplement and B18R protein. Exchange the culture medium daily after each 4-hour transfection with 2 ml/well of Pluriton™ Reprogramming Medium.

Pluriton™ Reprogramming Medium (with B18R Protein)

2.5 ml	Pluriton™ Medium (Equilibrated)
1 µl	Pluriton™ Supplement
1 µl	B18R Protein (at 0.5 mg/ml)

A.5 mRNA Cocktail

Create a master mRNA cocktail as described below using a molar stoichiometry of 3:1:1:1:1:1 for the Oct4, Sox2, Klf4, c-Myc, Lin28 and GFP mRNA's, respectively. Aliquot the mRNA cocktail mix into single-use volumes, based on the number of wells to be transfected each day.

1. Thaw the individual vials containing each mRNA reprogramming factor on ice. Keep mRNA vials on ice at all times.
2. Using RNase-free aerosol-barrier tips, combine the mRNA factors according to the table below in a sterile, 1.5 ml RNase-free microcentrifuge tube on ice.

Oct4 mRNA	385.1 µl
Sox2 mRNA	119.2 µl
Klf4 mRNA	155.9 µl
c-Myc mRNA	147.7 µl
Lin28 mRNA	82.5 µl
nGFP mRNA	110.6 µl
mRNA cocktail mix	1000 µl

1. Pipet the contents of the tube to mix thoroughly.
2. Aliquot 12.5 µl of the mRNA cocktail per well to be transfected into individual 1.5 ml RNase-free microcentrifuge tubes.
3. Freeze and store the aliquots at -70°C.

NOTE: Once single-use aliquots have been thawed they can not be refrozen.

Appendix A (continued)

Single Well Volumes

A.6 mRNA Transfection Complex

Prior aliquotting of single-use vials of the mRNA cocktail ensures that the mRNA is freshly thawed and prepared for each daily transfection. The instructions below outline the preparation of the mRNA transfection complex to transfect a single well of a 6-well plate. If transfecting more than one well, scale as appropriate based on the volume of the mRNA cocktail aliquots.

mRNA Transfection Complex

Tube 1: 50 μ l Opti-MEM
12.5 μ l mRNA cocktail
62.5 μ l total volume Tube 1

Tube 2: 56.25 μ l Opti-MEM
6.25 μ l RNAiMAX
62.5 μ l total volume Tube 2

Prepare mRNA Transfection Complex

1. Thaw one 12.5 μ l aliquot of the mRNA cocktail on ice (Tube 1).
2. Using RNase-free, aerosol-barrier pipet tips, add 50 μ l of Opti-MEM to the tube containing the mRNA cocktail and pipet gently to mix (Tube 1).
3. In a second sterile, RNase-free microcentrifuge tube, add 56.25 μ l of Opti-MEM (Tube 2).
4. Add 6.25 μ l of RNAiMAX to the Opti-MEM and pipet gently to ensure that all of the transfection reagent is removed from the pipet tip (Tube 2).
5. Using a 200 μ l RNase-free, aerosol-barrier pipet tip, pipet gently but thoroughly to mix the RNAiMAX with the Opti-MEM (Tube 2).
6. Transfer the entire contents of Tube 2 to the mRNA cocktail solution in Tube 1 and pipet gently to mix to generate the mRNA transfection complex.
7. Incubate the mRNA transfection complex at room temperature for 15 minutes to allow the mRNA to properly combine with the transfection reagent.

Transfect Cells

8. To transfect one well of cells, add 120 μ l of the mRNA transfection complex drop-wise to the 2 ml of Pluriton[™] Reprogramming Medium (with B18R protein) already in the well.
9. Gently rock the plate from side to side and back and forth to distribute the mRNA transfection complex across the well.
10. Incubate the cells for 4 hours at 37°C and 5% CO₂.

Appendix B

Generating NuFF-conditioned Pluriton™ Medium

Generating NuFF-conditioned Pluriton™ Medium

Inactivated NuFF feeder cells will support cells undergoing reprogramming for approximately 7 to 10 days. After this point, the culture medium must be switched to medium that has been pre-conditioned with healthy inactivated NuFF cells to continue the reprogramming process.

Cells undergoing reprogramming must be cultured in NuFF-conditioned medium starting at Day 6 of the protocol until the iPS cell colonies are picked and replated on a new inactivated feeder layer. If the cells are passaged at Day 6 onto a new inactivated NuFF feeder layer, the culture will require NuFF-conditioned medium starting on Day 12 of the reprogramming protocol.

Preparation of NuFF-conditioned medium must be initiated by Day -2 in order to have sufficient medium by Day 6 and to continue the reprogramming protocol. The protocol for generating NuFF-conditioned medium is described below should be followed concurrently with the mRNA reprogramming protocol.

Day -2

NuFF-conditioned Pluriton™ Medium Protocol

B.1 Plate NuFF Feeder Cells

One vial of inactivated NuFF cells contains between 4 and 5 x 10⁶ cells. On Day -2 of the reprogramming protocol, 1 x 10⁶ NuFF cells will be plated in 4 wells for the reprogramming experiment. The remainder of the NuFF cells in the vial (approximately 4 x 10⁶ cells) should be plated in one T75 flask to be used to generate NuFF-conditioned Pluriton™ Medium.

1. Thaw one vial of inactivated NuFF cells following the NuFF thawing procedure outlined on Day -2 in the reprogramming protocol.
2. Add 10 ml of NuFF culture medium to a T75 tissue culture flask.
3. Add up to 16 ml of NuFF cells in suspension at a density of 2.5 x 10⁵ cells/ml in the T75 flask.
NOTE: 3 to 4 x 10⁶ NuFF cells should be plated per T75 flask for efficient NuFF-conditioned medium generation with 25 ml of medium.
4. Incubate the cells overnight at 37°C and 5% CO₂.

Day -1

B.2 Exchange Medium

After allowing the inactivated NuFF cells to attach overnight, the culture can be washed and prepared to generate NuFF-conditioned Pluriton™ Medium. The total number of cells plated in the flask will determine the volume of Pluriton™ Medium that can be effectively conditioned each day. If 3 x 10⁶ to 4 x 10⁶ NuFF cells have been plated in the T75 flask, 25 ml of Pluriton™ Medium can be conditioned each day. If less than 3 x 10⁶ cells were plated in the flask, add 2 ml of Pluriton™ Medium per 2.5 x 10⁵ cells plated. A minimum of 2.25 x 10⁶ NuFF cells (18 mL medium) should be used in one T75 flask.

1. Supplement 25 ml of Pluriton™ Medium with 25 µl of bFGF (to a final bFGF concentration of 4 ng/mL).
2. Aspirate the NuFF culture medium from the T75 tissue culture flask.
3. Add 10 ml of PBS to the cells to wash. Aspirate the PBS.
4. Add 25 ml of Pluriton™ Medium supplemented with bFGF to the T75 flask.
5. Incubate the cells overnight at 37°C and 5% CO₂.

Generating NuFF-conditioned Pluriton™ Medium

Day 0 to Day 5

Appendix B (continued)

B.3 Collect and Freeze NuFF-conditioned Pluriton™ Medium

After each 24 hour incubation, the medium in the T75 flask can be collected as NuFF-conditioned Pluriton™ Medium. Each day the conditioned medium should be collected and replaced with fresh Pluriton™ Medium supplemented with bFGF to a final concentration of 4 ng/ml. Each daily collection of 25 ml of NuFF-conditioned Pluriton™ Medium should be frozen and stored at -20°C until the media can be pooled together on Day 6. Repeat the collection and exchange of medium daily through Day 6.

1. With a 10 ml pipet, transfer the NuFF-conditioned Pluriton™ Medium from the T75 flask to a sterile 50 ml conical tube.
2. Freeze the NuFF-conditioned Pluriton™ Medium at -20°C.
3. Add 25 ml of Pluriton™ Medium supplemented with bFGF to the NuFF cells in the T75 flask.
4. Incubate the cells overnight at 37°C and 5% CO₂.

Day 6

B.4 Collect, Pool, and Re-aliquot NuFF-conditioned Pluriton™ Medium

1. Thaw all aliquots of previously-collected NuFF-conditioned Pluriton™ Medium at 4°C.
2. Collect final 25 ml of NuFF-conditioned Pluriton™ Medium from the NuFF cells in the T75 flask.
3. Pool all thawed NuFF-conditioned Pluriton™ Medium and filter using a 0.22 µm pore size, low protein-binding filter.
4. Dispense filtered NuFF-conditioned Pluriton™ Medium into 40 ml aliquots and re-freeze at -20°C until use.

Day 6+

B.5 Using NuFF-conditioned Pluriton™ Medium

1. Thaw a 40 ml aliquot of NuFF-conditioned Pluriton™ Medium 4°C.
2. Transfer 10 mL of NuFF-conditioned Pluriton™ Medium to a 100 mm culture dish.
NOTE: The remaining 30 ml of NuFF-conditioned Pluriton™ Medium can be stored at 4°C and used over the next 3 days.
3. Equilibrate the 10 ml of Pluriton™ Medium for 2 hours in a 100 mm dish incubated at 37°C and 5% CO₂.
4. Thaw one aliquot of Pluriton™ Supplement and one aliquot of B18R protein on ice.
NOTE: Supplementing the reprogramming medium with B18R protein is only necessary from Day 0 to Day 18 when the cells are undergoing daily transfections.
5. Just prior to use, add 4 µl of the Pluriton™ Supplement add 4 µl of B18R protein (if needed) to the 10 ml of equilibrated NuFF-conditioned Pluriton™ Medium to generate NuFF-conditioned Pluriton™ Reprogramming Medium.
6. Exchange medium and culture cells as directed in the reprogramming protocol.

Appendix C

Thawing and Passaging BJ Fibroblast Cells

Thawing and Passaging BJ Fibroblast Cells

Target cells for reprogramming should be healthy and actively proliferating. If the target cells are frozen, it is best to allow the cells to recover from the thaw prior to plating for a reprogramming experiment. The following protocol outlines the thawing and passaging procedure for one vial of BJ fibroblast cells. One vial of BJ foreskin fibroblasts (p6) can be thawed into one T75 tissue culture flask and passaged approximately 3 to 4 days later. When cells are harvested during the passage, a portion of the cells can be replated on to the wells for reprogramming. The remaining cells can be further expanded in T75 flasks and/or frozen for later use.

C.1 Thaw vial of BJ Cells

1. Thaw one vial of BJ foreskin fibroblasts (p6) in a 37°C waterbath until only a small ice crystal remains.
 2. Transfer the contents of the vial of BJ cells directly to a 15 ml conical tube.
 3. Slowly add 5 ml of BJ culture medium to the cells while gently mixing the contents of the vial.
 4. Centrifuge the cells for 5 minutes at 200 x g.
 5. Aspirate the supernatant and resuspend the cell pellet in 10 ml BJ culture medium.
 6. Add 10 ml of BJ culture medium to a T75 tissue culture flask.
 7. Add the 10 ml of the BJ cell suspension to the T75 flask.
 8. Incubate overnight at 37°C and 5% CO₂.
-

C.2 Exchange Medium and Assess BJ Cell Health and Proliferation

For optimal health and proliferation of the BJ cells, replace the cell culture medium in the flasks every other day.

1. Aspirate the medium in the T75 flask.
2. Add 20 ml of fresh BJ cell culture medium to the T75 flask.
3. Incubate cells overnight at 37°C and 5% CO₂.

Appendix C (continued)

Thawing and Passaging BJ Fibroblast Cells

C.3 Passage BJ Cells

BJ cells should be passaged approximately every 4 days in culture. Early passage, healthy BJ cells will double approximately every other day. Cell cultures should be split at a 1:4 split ratio after 4 days in culture, and replated at a density of 1×10^6 cells per T75 flask. BJ cells can be continued in maintained and expanded through passage 10 or cryopreserved for later use.

1. Remove the culture medium from the BJ cells cultured in a T75 flask.
2. Add 10 ml of PBS to the culture surface of the flask to wash. Aspirate the PBS.
3. Add 3 ml of 0.05% Trypsin/EDTA to the flask.
4. Incubate the cells for 3 to 5 minutes at 37°C and 5% CO₂.
5. Tap the flask to completely detach the cells from the culture surface.
6. Add 6 ml of BJ cell culture medium to the flask to neutralize the Trypsin/EDTA.
7. With a 10 ml pipet, transfer the harvested cell solution from the T75 flask to a 15 ml conical tube.
8. Centrifuge the cells for 5 minutes at 200 x g.
9. Remove the supernatant and resuspend the cell pellet in 5 ml of BJ cell culture medium.
10. Count the cells in solution and calculate the live cell density.
11. Plate 1×10^6 BJ cells in each of 4 new T75 flasks.
12. Add a sufficient amount of BJ cell culture medium to bring the total volume to 20 ml in each T75 flask.
13. Incubate the cells overnight at 37°C and 5% CO₂.

Appendix D

Targeting Cell Passaging Protocol

Targeting Cell Passaging Protocol

The protocol below outlines passaging 1 well of target cells between Day 5 and Day 8 of the mRNA reprogramming protocol and replating the target cells on a new NuFF feeder plate. This step is done in order to replate the target cells at a lower density to allow for further proliferation for the remainder of the reprogramming protocol. The replating density is dependent on the target cell culture. Replating densities can range between 5×10^4 to 1×10^5 cells per well, depending on the health and proliferation rate of each target cell type.

Although it may be a necessary step in some cases, passaging cells during of a reprogramming experiment does introduce additional variables to the experiment. Some cell types may react differently to being passaged while undergoing the reprogramming process and may not attach to the new feeder plate or may become refractive to transfections. Maintain at least one well of the target cells on the original reprogramming culture plate for the duration of the reprogramming experiment.

Prepare new inactivated NuFF feeder plate as directed on Day -2 of the mRNA reprogramming protocol, one day prior to passaging the target cells. Use a ROCK inhibitor to improve attachment of the cells on the new feeder plate. When passaging cells at this point, Y27632 is added to the Pluriton™ Reprogramming Medium to a final concentration of 10 μ M. Because the cells will be transferred to a new inactivated NuFF feeder plate, the cells may continue to be reprogrammed in Pluriton™ Reprogramming Medium, or can be cultured in NuFF-conditioned Pluriton™ Reprogramming Medium.



IMPORTANT: If the target cells require this passaging step during the reprogramming process, begin the passaging protocol after the 4 hour transfection and medium exchange. Allow the cells to incubate in fresh equilibrated Pluriton™ Reprogramming Medium with B18R for 1 to 2 hours at 37°C and 5% CO₂ before beginning this passaging protocol.

D.1 Transfect and Incubate Cells

1. Transfect cells as described for Day 0 in the reprogramming protocol.
2. Equilibrate medium and prepare Pluriton™ Reprogramming Medium (with B18R protein) as described for Day 0 in the reprogramming protocol.
3. After the 4 hour transfection, remove the medium containing the transfection reagent and add 2 ml of equilibrated Pluriton™ Reprogramming Medium (with B18R protein) to each well, as described for Day 0 in the reprogramming protocol.
4. Incubate the cells for 1 to 2 hours at 37°C and 5% CO₂.

D.2 Equilibrate and Prepare Pluriton™ Reprogramming Medium (with B18R protein and Y27632)

1. Add 10 ml of Pluriton™ Medium to a sterile 100 mm dish.
2. Incubate the Pluriton™ Medium for 2 hours at 37°C and 5% CO₂ to equilibrate the medium to the proper oxygen tension.
3. Just prior to use, thaw one aliquot of Pluriton™ Supplement and add 4 μ l to the 10 ml of equilibrated Pluriton™ Medium to generate Pluriton™ Reprogramming Medium.
4. Thaw one aliquot of B18R protein on ice and add 4 μ l of the B18R protein to the Pluriton™ Reprogramming Medium.
5. Thaw one aliquot of Y27632 (10 mM) and add 10 μ l of the compound to the Pluriton™ Reprogramming Medium (with B18R protein).

Appendix D (continued)

Targeting Cell Passaging Protocol

D.3 Passage Target Cells

1. Warm a 0.5 ml aliquots of 0.05% Trypsin/EDTA and a 0.5 ml aliquot of Trypsin Neutralizing Solution in a 37°C waterbath.
2. Add 1 ml of PBS to the well of cells to be passaged. Aspirate the PBS.
3. Add 0.5 ml of the warmed 0.05% Trypsin/EDTA to the well. Gently tip the plate to evenly distribute the enzyme across the culture surface of the well.
4. Incubate the cells for 5 to 10 minutes at 37°C and 5% CO₂.
NOTE: The appropriate incubation time will depend on the target cell type and density.
5. Remove the cells from the incubator and gently tap the side of the plate to completely detach the cells from the culture surface.
6. Add 0.5 ml of the warmed Trypsin Neutralizing Solution to the well.
7. Using a 1000 µl pipet tip, carefully pipet the cell solution in the well to gently rinse the cells from the culture surface of the well.
8. Remove a 10 µl aliquot of the cell suspension in the well and count the cells in solution.
9. Calculate the live cell density of the remaining 1 ml of cells in the well.
10. Calculate the microliter volume of cell suspension required to plate the desired number of cells.
11. Add 2 ml of Pluriton™ Medium to a 15 ml conical tube.
12. Transfer the calculated volume of cells to the tube containing Pluriton™ Medium.
13. Centrifuge the cells for 2 minutes at 200 x g.
14. Carefully remove the supernatant from the tube and resuspend the cells in 2 ml of equilibrated Pluriton™ Reprogramming Medium (with B18R protein and Y27632).
15. Aspirate the NuFF culture medium from one well of a new inactivated NuFF feeder plate.
16. Add 1 ml of PBS to the well to wash. Aspirate the PBS.
17. Add the 2 ml of cell suspension in Pluriton™ Reprogramming Medium (with B18R protein and Y27632) to the well of the prepared NuFF feeder plate.
18. Incubate the cells overnight at 37°C and 5% CO₂.

Continue to transfect the new well(s) of passaged cells together with the target cells remaining on the original plates. NuFF-conditioned Pluriton™ Reprogramming Medium (with B18R protein) can be used to culture target cells on both reprogramming plates throughout the remainder of the experiment.

Recipes

Appendix E

Recipes

NuFF Culture Medium

90 ml	DMEM
10 ml	FBS
1 ml	GlutaMAX
1 ml	Penicillin/Streptomycin (100X)

1. Filter-sterilize using a 0.22 µm pore size, low protein-binding filter.
 2. Store medium for up to 2 weeks at 4°C.
-

BJ Culture Medium

90 ml	EMEM
10 ml	FBS
1 ml	Penicillin/Streptomycin (100X)

1. Filter-sterilize using a 0.22 µm pore size, low protein-binding filter.
 2. Store medium for up to 2 weeks at 4°C.
-

Pluriton™ Reprogramming Medium

10 ml	Pluriton™ Medium (equilibrated)
4 µl	Pluriton™ Supplement 2500X

1. Thaw one aliquot of Pluriton™ Supplement on ice.
2. Transfer 10 ml of pre-equilibrated Pluriton™ Medium to a 15 ml conical tube.
3. Add 4 µl of the Pluriton™ Supplement to a final concentration of 1X just prior to use.

NOTE: Penicillin/streptomycin can be added to the stock bottle of Pluriton™ Medium to a final concentration of 1X prior to use, as outlined in the Material Preparation section.

Y27632 (10 mM)

2 mg	Y27632 (lyophilized)
624 µl	DMSO

1. Briefly centrifuge the vial of lyophilized Y27632 compound. Reconstitute the lyophilized powder in DMSO to make a 10 mM concentrated stock solution.

NOTE: Y27632 at 10 mM is a 1000X stock solution.

2. Aliquot into appropriate working volumes and store at -20°C.

Recipes

Appendix E (continued)

bFGF Solution (4 µg/ml)

50 µg Stemfactor™ Fibroblast Growth Factor-basic (bFGF)
12.5 ml 0.1% BSA in PBS

1. Briefly centrifuge the lyophilized vial of bFGF.
 2. Use 500 µl of 0.1% BSA in PBS to reconstitute the lyophilized bFGF in the vial.
 3. Transfer the reconstituted bFGF to a 15 ml conical tube.
 4. Add 12 ml of 0.1% BSA in PBS to yield a 4 µg/ml working solution.
 5. Filter-sterilize using a 0.22 µm pore size, low protein-binding filter.
 6. Aliquot into single-use volumes in sterile, low protein-binding microcentrifuge tubes and store at -20°C for up to 6 months.
-

Human iPS Cell Culture Medium

400 ml DMEM/F-12
100 ml Knockout™ Serum Replacement
5 ml Non-Essential Amino Acids (100X)
5 ml L-glutamine (200 mM)
1 ml bFGF (at 10 µg/ml; 20 ng/ml final concentration)
500 µl β-mercaptoethanol (1000X)
5 ml Penicillin/Streptomycin (100X) (optional)

1. Filter-sterilize medium using a 0.22 µm pore size, low protein-binding filter.
 2. Store medium for up to 2 weeks at 4°C.
-

MEF Culture Medium

90 ml DMEM
10 ml FBS
1 ml GlutaMAX
1 ml Penicillin/Streptomycin (100X)

1. Filter-sterilize medium using a 0.22 µm pore size, low protein-binding filter.
2. Store medium for up to 2 weeks at 4°C.

Product List

mRNA Factors	Vendor	Qty	Cat. No	Miltenyi Cat. No.
mRNA Reprogramming Kit	Stemgent®	1 Kit	00-0071	130-067-191
mRNA Reprogramming Factors Set: h0SKML	Stemgent®	1 Set	00-0067	130-096-528
Oct4 mRNA, Human	Stemgent®	20 µg	05-0014	130-096-524
Klf4 mRNA, Human	Stemgent®	20 µg	05-0015	130-096-526
Sox2 mRNA, Human	Stemgent®	20 µg	05-0016	130-096-527
Lin28 mRNA, Human	Stemgent®	20 µg	05-0017	130-096-525
c-Myc mRNA, Human	Stemgent®	20 µg	05-0018	130-096-523
nGFP mRNA	Stemgent®	20 µg	05-0019	130-096-522

Cell Line	Vendor	Qty	Cat. No	Miltenyi Cat. No.
Human BJ Foreskin Fibroblasts (p6)	Stemgent®	1 x 10 ⁶ cells/vial	08-0027	130-096-726
Human Fibroblasts Mitomycin-C treated, Donor 11 (NuFF cells)	Global Stem	4 to 5 x 10 ⁶ cells/vial	GSC-3001M	
Human Fibroblasts Irradiated, Donor 11 (NuFF cells)	Global Stem	4 to 5 x 10 ⁶ cells/vial	GSC-3001G	
CF-1 Mouse Embryonic Fibroblasts (MEFs) Irradiated	Global Stem	4 to 5 x 10 ⁶ cells/vial	GSC-6201G	

Media and Reagents	Vendor	Qty	Cat. No	Miltenyi Cat. No.
Pluriton™ Reprogramming Medium	Stemgent®	1 Set	00-0070	130-096-820
Stemfactor™ bFGF (Human Recombinant)	Stemgent®	50 µg	03-0002	
Stemfactor™ Y27632	Stemgent®	2 mg	04-0012	130-095-563
EMEM, High Glucose	ATCC	500 ml	30-2003	
Trypsin EDTA for Primary Cells	ATCC	100 ml	PCS-999-003	
Trypsin Neutralizing Solution	ATCC	100 ml	PCS-999-004	
FBS	Atlas Biologicals	500 ml	F-0500-A	
B18R Recombinant Protein (Carrier Free) 40 micrograms of B18R protein is an included component of the Stemgent mRNA Reprogramming Kit*	eBioscience	500 µg	34-8185-85	
DMEM, High Glucose	Gibco	500 ml	11960-044	
DMEM/F-12	Gibco	500 ml	11330-032	
GlutaMAX™	Gibco	100 ml	35050-061	
L-Glutamine (200 mM, 100X)	Gibco	100 ml	25030-081	
Non-Essential Amino Acids	Gibco	100 ml	11140-050	
Opti-MEM® Reduced Serum Medium	Gibco	100 ml	31985-062	
PBS	Gibco	500 ml	14190-144	
Penicillin-Streptomycin (100X)	Gibco	100 ml	15070-063	
β-mercaptoethanol (1000X)	Gibco	50 ml	21985-023	
0.05% Trypsin/EDTA	Gibco	100 ml	25300-054	
Knockout™ Serum Replacement	Invitrogen	500 ml	10828-028	
Lipofectamine™ RNAiMAX Transfection Reagent	Invitrogen	0.75 ml	13778-075	
Gelatin, Type A, Powder	Sigma-Aldrich	100 g	G1890-100G	

Product List (continued)

Antibodies	Vendor	Qty	Cat. No	Miltenyi Cat. No.
StainAlive™ DyLight™ 488 TRA-1-60	Stemgent®	50 µg (0.5 mg/ml)	09-0068	130-095-669
StainAlive™ DyLight™ 488 TRA-1-81	Stemgent®	50 µg (0.5 mg/ml)	09-0069	130-095-670

Supplemental Reagents	Vendor	Qty	Cat. No	Miltenyi Cat. No.
eGFP mRNA	Stemgent®	20 µg	05-0020	130-096-812
Stemfect™ RNA Transfection Kit	Stemgent®	1 Kit	00-0069	130-096-529
Stemfect™ Enhanced hESC DNA Transfection Kit	Stemgent®	1 Kit	00-0063	130-095-975
NutriStem™ XF/FF Culture Medium	Stemgent®	500 ml	01-0005	130-095-543
NutriStem™ XF/FF Culture Medium	Stemgent®	100 ml	01-0005-100	130-095-544
Alkaline Phosphatase Staining Kit II	Stemgent®	50 assays	00-0055	130-095-616
CryoStem™ Freezing Medium	Stemgent®	5 x 10 ml	01-0013	130-095-847
hES Cell Cloning & Recovery Supplement	Stemgent®	5 x 100 µl	01-0014	130-095-690
Purified Rabbit anti-Mouse Oct4	Stemgent®	100 µl	09-0023	130-095-635
Purified Rabbit anti-Mouse Sox2	Stemgent®	100 µl	09-0024	130-095-636
Purified Rabbit anti-Mouse/Human Nanog	Stemgent®	100 µl	09-0020	130-095-632
StainAlive™ DyLight™ 488 Mouse IgM _k (Isotype Control)	Stemgent®	100 µl	09-0072	130-095-673
Trypan Blue (0.4%)	Gibco	100 ml	15250-061	
ES-Qualified, Defined FBS	HyClone	500 ml	SH300.70.03E	
Collagenase IV	Invitrogen	1 g	17104-019	
Dispase	Invitrogen	5 g	17105-041	
DMSO	Sigma-Aldrich	5 x 5 ml	D2650-5X5ML	

Equipment	Vendor	Cat. No
4-well culture plates	NUNC	12-565-72
6-well culture plates	NUNC	14-832-11
6-well culture plates	BD Falcon	08-772-1B
12-well culture plates	BD Falcon	353043
24-well culture plates	BD Falcon	353047
100 mm culture dish	BD Falcon	351029
9" Pasteur pipette	Fisher	13-678-20D
1.5 ml cryovials	Nalgene	50001020
Isopropanol freezing container	Nalgene	5100-0001
500 ml filter flask (0.22 µm pore size)	Millipore	SCGPU05RE
500 ml filter flask (0.22 µm pore size)	Nalgene	09-741-02
50 ml filter flask (0.22 µm pore size)	Nalgene	09-741-88
50 ml filter flask (0.22 µm pore size, PVDF)	Millipore	SE1M179M6
5 ml glass pipets	Fisher Scientific	13-678-27E
Object Marker	Nikon	MBW10010
C-OA 15mm Objective Adapter	Nikon	MXA20750

Purchaser Notification

mRNA Reprogramming System

This product is intended for research purposes only. It may not be used for (i) any human or veterinary use, including without limitation therapeutic and prophylactic use, (ii) any clinical use, including without limitation diagnostic and prognostic use, (iii) any use in delivery to and/or modification of cells that are intended for clinical, diagnostic or medicinal use, including without limitation, cell-based therapy, (iv) any commercial purposes, including without limitation the performance of contract research or provision of services to a third party and the manufacture of products for general sale. Any use of this product for any of the abovementioned purposes requires a license from the Massachusetts Institute of Technology.

Technical Support

To obtain the latest technical support information, please visit www.stemgent.com. You can have 24-hour access to the following information:

- Access to telephone and fax information for order support
 - Search through the Stemgent protocol video gallery
 - Search product spec sheets, protocols, applications notes and product support docs
 - Obtain information on customer training and workshops
 - Submit a question directly to Technical Support at tech.support@stemgent.com
-

Ordering Information

For the latest products and services available through Stemgent, please visit www.stemgent.com.

U.S. Orders **Stemgent**

One Kendall Square, Suite B6201
Cambridge, MA 02139 USA

T: 617.245.0000

F: 617.812.5900

E: orders@stemgent.com

International Orders **Miltenyi Biotec**

Friedrich-Ebert-Straße 68
51429 Bergisch Gladbach, Germany

T: +49 2204 8306-0

F: +49 2204 85197

E: macs@miltenyibiotec.de

Trademarks

Opti-MEM, GlutaMAX and Lipofectamine are trademarks of Life Technologies.

Stemgent, Stemgent logo, and all other trademarks unless otherwise noted are the property of Stemgent, Inc.