Simplicon™ RNA Reprogramming Kit

Catalog No. SCR549 & SCR550

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

Various methods utilizing viruses, DNA, RNA, miRNA and protein have been developed to generate integration-free induced pluripotent stem cells (iPSCs). Disadvantages to existing methods include: (1) low reprogramming efficiency (i.e. DNA and protein), (2) a lengthy requirement for negative selection and subcloing steps to remove persistent traces of the virus (i.e. Sendai virus)[1] and (3) for daily transfections of four individual in vitro generated mRNAs over a 14 day period (i.e. mRNA based)[2].

EMD Millipore's Simplicon™ RNA Reprogramming Kit is a safe and efficient method to generate integration free, virus-free human iPS cell using a single transfection step. The technology is based upon a positive strand, single-stranded RNA species derived from non-infectious (non-packaging), self-replicating Venezuelian equine encephalitis (VEE) virus[3]. The Simplicon RNA replicon is a synthetic in vitro transcribed RNA expressing all four reprogramming factors (OKS-iG; Oct4, Klf4, Sox2 and Glis1) in a polycistronic transcript that is able to self-replicate for a limited number of cell divisions.

Advantages of the Simplicon™RNA Reprogramming Kit:

- Integration-free, footprint-free iPS cells. No risk of genomic integration
- Safe, virus free, synthetic polycistronic RNA replicon (all four reprogramming factors in 1 RNA strand)
- Only 1-day transfection required. The RNA replicon is able to self-replicate, elliminating the need for laborious daily transfection of multiple individual mRNAs over a 14 day period.
- Efficient and rapid reprogramming.
- No screening required to ensure viral remnants are not present.
- Controlled elimination of synthetic RNA replicon by removal of B18R protein.
- Validated for reprogramming in feeder-free and feeder-based culture conditions.

EMD Millipore's Simplicon™ RNA Reprogramming Kit contains sufficient material for 20 reactions in a 6-well plate format. Kit components have been validated to efficiently reprogram two lines of human foreskin fibroblasts (HFFs); the slower proliferating BJ and the faster proliferating in-house p6 HFF. The resulting human iPS cells display characteristic ES cell-like morphology, express pluripotent markers and can be rapidly expanded in normal human ES cell culture conditions.

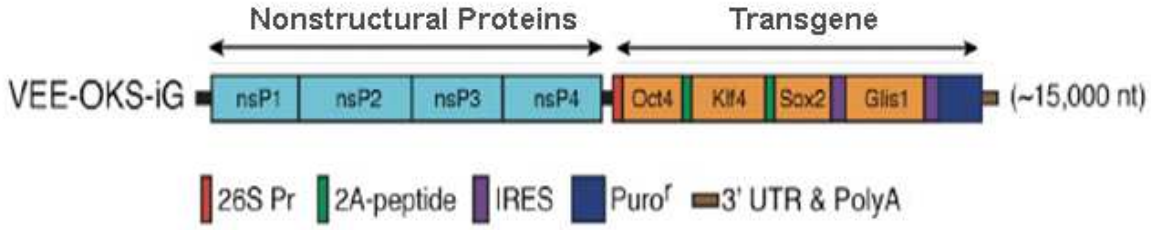

Figure 1. Structure of the Simplicon™ RNA replicon. The RNA replicon encodes four non-structural replication complex proteins (nsPs) as a single ORF in the 5'end of the RNA. At the 3'end, the viral structural proteins ORFs are replaced with the OKS-iG transgenes. Locations of 26S internal, 2A peptide, IRES and Puromycin (Puro)-resistance gene are indicated[3].

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Kit Components (Cat. No. SCR550): Simplicon™ RNA Reprogramming Kit

1. VEE-OKS-iG RNA: (Part No. CS210583) One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80℃.

2. B18R RNA: (Part No. CS210584) One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80℃.

3. Human Recombinant B18R Protein, Carrier-Free: (Part No. GF156) One (1) vial containing 50 µg of 0.5 mg/mL stock of B18R protein. Store at -80℃.

4. Human iPS Reprogramming Boost Supplement II: (Part No. SCM094).

    - TGF-β RI Kinase Inhibitor IV Supplement A-83-01 (1000X), final working concentration 0.5uM: (Part No. CS210445) One (1) vial containing 400 µL of the inhibitor in high quality DMSO. Store at -20℃.

    - Sodium Butyrate Supplement (1000X), final working concentration 0.25mM: (Part No. CS210446) One (1) vial containing 400 µL of the inhibitor in water. Store at -20℃.

    - PS48 Supplement (1000X), final working concentration 5uM: (Part No. CS210447) One (1) vial containing 400 µL of the inhibitor in high quality DMSO. Store at -20℃.

Kit Components (Cat. No. SCR549): Simplicon™ Reprogramming RNA

1. VEE-OKS-iG RNA: (Part No. CS210583) One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80℃.

2. B18R RNA: (Part No. CS210584) One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80℃.

Storage and Stability

- VEE-OKS-iG and B18R RNAs: Stable for 4 months from date of receipt when stored appropriately at -80℃. For best recovery, quick-spin the vial prior to opening. Thaw on ice. While on ice, aliquot into sterile, nuclease-free eppendorf tubes and store at -80℃. Limit repeated freeze-thaw cycles. Use in a sterile RNAse-free environment.

- Human recombinant B18R protein: Stable for 4 months from date of receipt when stored appropriately at -80℃. For best recovery, quick-spin the vial prior to opening. Thaw on ice. While on ice, aliquot B18R protein into sterile, nuclease-free, low protein-binding eppendorf tubes and store at -80℃. B18R protein must be kept on ice in order to avoid degradation. Limit repeated freeze-thaw cycles. Use in a sterile RNAse-free environment.

- Human iPS Reprogramming Boost Supplement II: Stable for 4 months at -20˚C from date of receipt. Upon first thaw, aliquot into smaller working volumes and freeze at -20℃. Upon addition of the small molecule components to the media, filter the supplemented media with a 0.22 µM filtration unit and stored at 2-8˚C. For optimal results, prepare sufficient supplemented media for a 1 week supply of media changes.

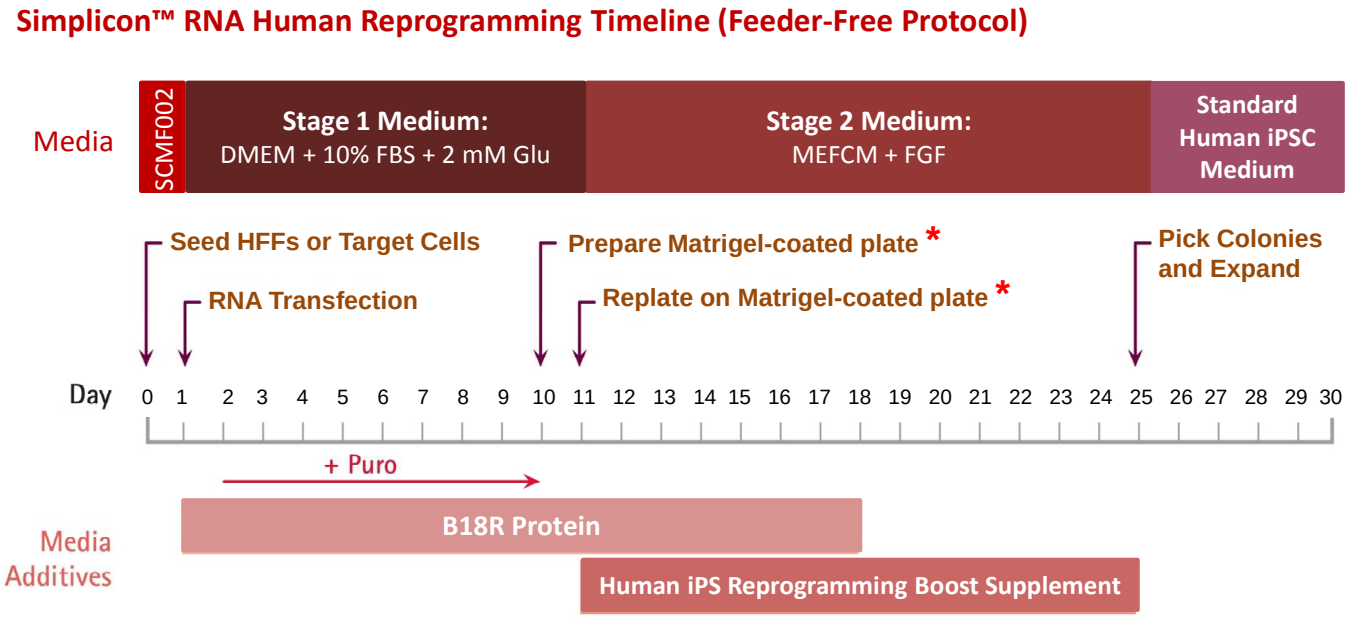
Materials Required But Not Supplied

1. 6-well plates, culture flasks, dishes (TC grade)
2. Nuclease-free, sterile microcentruge eppendorfs
3. Nuclease-free areosol-barrier pipette tips
4. Cell counter / hemocytometer
5. DMEM High-Glucose Medium (EMD Millipore Cat. No. SLM-021-B)
6. Fetal Bovine Serum (EMD Millipore Cat. No. ES-009-B)
7. GlutaMAX (100X) (Life technologies Cat. No. 35050-061
8. Penicillin Streptomycin Solution (100X) (EMD Millipore TMS-AB2-C)
9. Mouse Embryonic Fibroblast (MEF) Conditioned Media (R&D systems, Cat. No. AR005)
10. Recombinant Human FGF-2 (EMD Millipore Cat. No. GF003)
11. FibroGRO™ LS Complete Medium (EMD Millipore Cat. No. SCMF002)
12. FibroGRO™ Xeno-Free Human Foreskin Fibroblasts (EMD Millipore Cat. No. SCC058), optional
13. Accumax™ Cell Detachment Solution (EMD Millipore Cat. No. SCR006)
14. EmbryoMax® 1X Dulbecco's Phosphate-Buffered Saline w/o Ca^{++} or Mg^{++}, 500 mL (Cat. No. BSS-1006-B)
15. PMEF cells, growth-arrested, mitomycin-C treated (EMD Millipore Cat. No. PMEF-CF)
16. EmbryoMax® 0.1% Gelatin Solution (EMD Millipore Cat. No. ES-006-B)
17. RiboJuice™ mRNA Transfection Kit (EMD MilliporeCat. No.TR-1013)
18. Opti-MEM® I Reduced Serum Medium (Life Technologies Cat. No. 31985-062)
19. Human iPS Selection Kit (EMD Millipore Cat. No. SCR502)

Reprogramming Human Somatic Cells

Important note: The following protocol has been optimized using early passage Human Foreskin Fibroblasts (Cat. No. SCC058). The following protocol should only be used as a **reference** to begin optimizing conditions that will enable the generation of iPS cells from other human target cells.

Actual timelines may vary depending upon the cell types and experimental conditions.



* Timing may vary based on cell lines and sensitivity to puromycin. When to replate should be based upon when puromycin-resistant cells are 70-90% confluent.

Note: From time of transfection to replating, media changes should be performed every day. After replating, media changes can be performed every OTHER day.

Before Starting: Determine optimal cell density. Defined as number of cells to achieve 60-80% confluent next day.
Determine optimal puromycin. Defined as ½ concentration to achieve 50% cell death by Day 4-5.

Day 0: Plate cells to achieve 60-80% confluent the next day.
Day 1: Pretreat cells with B18R protein. Transfect with VEE-OKS-iG and B18R RNA.
Day 2: Cells are 80-90% confluent. Apply Puromycin selection (red arrow).
Day 4: Adjust Puromycin based upon cell viability.

- For <20% cell death: Maintain or increase puromycin by 0.5X.
- For 30-60% cell death: Maintain or reduce puomycin by 2X.
- For 80-90% cell death: Reduce puromycin 4X or remove altogether.

Day 7-9: Puromycin resistant cells start to grow back.
Day 11: Change medium to MEF-CM + FGF regardless of whether cells are ready to be replated or not.
Day 11-19: Replate when cells are 70-90% confluent.

Figure 2. Reprogramming timeline using Simplicon™ RNA Reprogramming Kit. Key steps and media requirements are indicated. +Puro signifies addition of puromycin. Stage 1 Medium contains DMEM containing 10% FBS and 1X GlutaMAX. Stage 2 Medium contains MEF conditioned medium (MEFCM) with 10 ng/mL FGF-2. Addition of B18R protein and Human iPS Reprogramming Boost Supplement to Stage 1 or Stage 2 Medium are indicated.

Critical Success Factors in Simplicon™ RNA Reprogramming:

The following guidelines are critical to ensuring success in reprogramming experiments.

- Nucleic acids such as RNAs are subject to degradation by nucleases found in the environment and on human surfaces. Spray work surfaces with 70% ethanol before use and wear powder-free gloves during all procedures. Exercise extreme care in component handling to avoid introduction of nucleases to the Simplicon™ RNAs. Use dedicated nuclease-free sterile eppendorf tubes, sterile nuclease-free arerosol resistant tips and a clean enivronment for sample preparation. Change gloves and tips frequently.

- Appropriate storage and handling of the reagents. Components in the Simplicon™ RNA Reprogramming kit are highly temperature sensitive and prone to degradation if left at room temperature for prolonged lengths of time. Thaw kit components on ice and while on ice, aliquot into smaller volumes for consistency and to eliminate degradation caused by repeated freeze/thaw cycles.

Protocol

Prior to starting the reprogramming experiment, it is critical to establish two key experimental parameters: (1) the optimal plating density and (2) the optimal starting puromycin concentration.

Before Starting Experiment:

1. Determine the optimal plating density of target cells. **The optimal plating density is defined as the number of cells that should be plated at Day 0 in order to have the cells reach 60-80% confluency the next day (Day 1)**. Plate out a range of cell numbers from 1 x 10^5 to 1 x 10^6 cells per well of a 6-well plate. The culture medium should be the same as that used to maintain the target cells in a proliferative state. Volume should be 3 mL per well of a 6-well plate. The number of cells to be seeded at Day 0 will vary depending on the cell types as there are differences in cell sizes, morphology and rate of proliferation. For example, EMD Millipore's FibroGRO Xeno-Free Human Foreskin Fibroblasts plated at 4 x 10^5 cells per well on Day 0 reach 60-70% confluency by Day 1 (Figure 3). Whenever possible, use lower passage target cells as they possess higher proliferative potential than higher passage cells.

2. Determine the optimal starting puromycin concentration. **The optimal starting puromycin concentration is defined as ½ the concentration required to achieve 50% cell death by Day 4-5.** Puromycin sensitivity may vary based on the target cell line and must be determined empirically before starting. Once the effective concentration of puromycin to achieve 50% cell death by Day 4-5 has been determined, use ½ that concentration to account for the cell's sensitivity due to the cellular interferon response to the introduced Simplicon RNAs.

   Day 0: Plate out the optimal plating density of target cell (determined from step 1) into each well of a 6-well plate to reach 60-80% confluency by the next day. Use Stage 1 Medium **without** B18R protein (see formulation below). Incubate the plate in a 37℃, 5% CO_2 incubator.

   Stage 1 Medium without B18R protein (100 mL total volume). Store at 2-8℃ when not in use. Do not use beyond one week.

Component	Quantity	Final Conc.	Cat. No.
DMEM High-Glucose Medium	89 mL		EMD Millipore SLM-120-B
Fetal Bovine Serum	10 mL	10%	EMD Millipore ES-009-B
GlutaMAX (100X)	1 mL	1X	Life technologies 35050-061
Total Volume	**100 mL**		

Day 2: Two days after plating, add varying amounts of puromycin ranging from 0.25 μg/mL to 1 μg/mL into each well of the 6-well plate (For example: 0.25 μg/mL, 0.5 μg/mL, 0.75 μg/mL, 1 μg/mL). Exchange with fresh medium containing puromycin every day for the next 2-3 days (4 days total from time of plating). Monitor daily to assess the effects of puromycin on cell death.

Day 4-5: Determine the puromycin concentration in which there are ~50% cell death. To account for the cell toxicity introduced by the cellular interferon response to the Simplicon™ RNAs, take ½ the concentration as the optimal concentration of puromycin to start with.

For example, EMD Millipore's human foreskin fibroblasts were plated at 4 x 10^5 cells per well of a 6-well plate in 3 mL Stage 1 medium without B18R protein at day 0. This plating density was determined empirically to be the optimal plating density (see step 1 above). By day 4-5, it was determined that 50% cell death occurred at a puromycin concentration of 1.0 μg/mL. Thus our starting puromycin concentration would be 0.5 μg/mL to account for the additional cell toxicity due to the cellular interferon response when the Simplicon RNAs are transfected to the target cells.

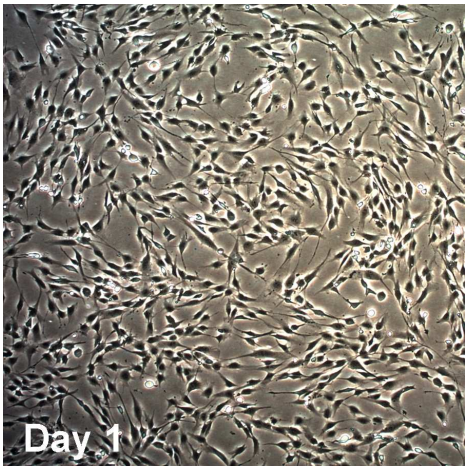


Figure 3. Human foreskin fibroblast cells plated at 4x10^5 cells per well of a 6-well plate. One day after, cells were approximately 60-70% confluent and ready for Simplicon RNA transfection. The image was obtained using 4X objective.

Reprogramming Protocol

Day 0: Plate target cells

Note: The following reprogramming protocol is based upon 1 reaction in one well of a 6-well plate. Scale up accordingly based on the number of reactions being performed.

3. Plate target cells at the optimal plating density (determined from Step 1) in the same culture medium that was used to maintain target cells in the proliferative state. Volume should be 3 mL per well of a 6-well plate. Set aside an untransfected control well to observe the puromycin cell death.

4. Thaw the following kit components on ice and aliquot into sterile nuclease-free eppendorf tubes. Store at -80℃ until ready to use.

   - **VEE-OKS-iG RNA**: Aliquot 3 µL into sterile nuclease-free eppendorf tubes. Store aliquots at -80℃.

   - **B18R RNA**: Aliquot 3 µL into sterile nuclease-free eppendorf tubes. Store aliquots at -80℃.

   - **B18R protein**: Aliquot 3 µL into sterile nuclease-free eppendorf tubes. Store aliquots at -80℃.

Day 1: Pretreat cells with B18R protein
Transfect with Simplicon™ VEE-OKS-iG and B18R RNA

5. Pre-Treatment with B18R Protein: On the day of transfection, cells should be 60-80% confluent (Figure 3). Pre-treat target cells for 20 mins with the B18R protein (Cat. No. GF156) to help suppress the cellular interferon response before transfection with the Simplicon™ RNAs.

   a. Thaw an aliquot of B18R protein (Cat. No. GF156) on ice: For best recovery, quick-spin the vial prior to opening.

   b. Prepare DMEM medium containing 200 ng/mL B18R protein. Below are quantities for one reaction. Scale up accordingly based on the number of reactions being performed.

   For 1 reaction:

Component	Quantity for 1 reaction	Final Conc.	Cat. No.
DMEM High-Glucose	1.0 mL		EMD Millipore SLM-120-B
B18R protein (0.5 mg/mL)	0.4 µL	200 ng/mL	EMD Millipore (GF156)
Total Volume	~1.0 mL		

   Note: Do not use Human ESC Medium as it contains factors that are inhibitory to the RNA transfection.

   c. Mix gently by pipeting up and down.

   d. Aspirate the medium from the cells to be transfected.

   e. Add 1 mL per well of the DMEM medium containing 200 ng/mL B18R protein.

   f. Place the plate in a 37˚C, 5% CO_2 incubator. Incubate for 20 minutes.

   g. Change medium to DMEM+2%FBS containing 200ng/ml B18R protein in the 6-well-plate after 20mins of B18R protein treatment, 2.5ml medium per well, place back to incubator till the RNA-Transfection Reagent complex is ready.

6. Transfect with VEE-OKS-iG and B18R RNAs. Use nuclease-free, aerosol-barrier pipette tips and sterile, nuclease-free eppendorfs.

   a. Prepare the RNA transfection complex. Below is the quantity calculated for one well in a 6-well-plate. Scale up accordingly based on the number of reactions being performed.

b. Thaw an aliquot of the VEE-OKS-iG and B18R RNAs on ice; quickly centrifuge the vial(s) to spin down the contents. Keep RNA vials on ice.

c. Warm RiboJuice™ mRNA Transfection Reagent and RiboJuice™ mRNA Boost Reagent to room temperature and vortex gently.

d. Set up the following reactions in a sterile eppendorf tubes. Store any unused Simplicon™ RNAs at -80℃. Mix gently by pipeting.

RNA Transfection Reaction (1 Rxn):

Component	Eppendorf	Cat. No.
Opti-MEM®	250 µL	Life Technologies (31985-062)
Ribojuice™ mRNA Boost Reagent	4.0 µL	EMD Millipore RiboJuice™ mRNA Transfection kit (TR-1013)
RiboJuice™ mRNA Transfection Reagent	4.0 µL	
VEE-OKS-iG RNA (1ug/ul)	0.5 µL	
B18R RNA (1ug/ul)	0.5 µL	
Total Volume	259 µL	

e. Incubate Eppendorf at room temperature for 2-5 minutes.

f. Add the RNA-transfection reagent complexes (approximately 259µL) **drop wise** into one well of the 6-well plate containing cells pretreated with B18R protein (from step 5g). Total volume is ~2.759 mL.

g. Gently rock the plate from side to side to thoroughly mix the RNA complexes onto the target cells.

h. Incubate the plate in a 37˚C, 5% CO_2 incubator for 4 hrs. Track cell death after 2 hours of culture, stop treatment if a lot of cell death is observed.

7. Prepare Stage 1 Medium containing 200 ng/mL B18R protein: During the 4 hour RNA transfection period, prepare sufficient volume of Stage 1 Medium for 3 days worth of daily media changes. Hence for 1 reaction with a final volume of 2 mL per well: 2 mL/well x 3 days media changes = 6 mL total volume. Scale up based on the number of reprogramming reactions being performed.

Note: It is not recommended to make large volumes of Step 1 medium beyond 3 days worth of daily media changes as the performance of the B18R protein has not been assessed beyond 3 day.

Stage 1 Medium (1 Rxn; 6 mL total volume). Scale up based on the number of reactions being performed. Store at 2-8℃ when not in use. Do not use beyond one week.

Component	Quantity	Final Conc.	Supplier	Cat. No.
DMEM High-	5.4 mL		EMD Millipore	SLM-120-B

Glucose Medium				
Fetal Bovine Serum	0.6 mL	10%	EMD Millipore	ES-009-B
GlutaMAX (100X)	60 µL	1X	Life Technologies	35050-061
B18R protein (0.5 mg/mL)	2.4 µL	200 ng/mL	EMD Millipore	GF156
Total Volume	6 mL			

8. Once the 4 hour RNA transfection is completed, aspirate the medium containing the RNA complex from the well.

   Note: Do not leave the RNA transfection complex medium on the cells for longer than 4 hrs.

9. Add 2 mL per well of Stage 1 Medium containing 200 ng/mL B18R protein to the cells.

10. Place the plate containing the transfected cells overnight in a 37˚C, 5%CO_2 incubator.

Day 2 - Day 4: Apply Optimal Starting Puromycin Concentration

The Simplicon™ replicon contains a puromycin resistance gene (see Figure 1) which is used to select for cells that have taken up the VEE-OKS-iG RNA. During the next several days (Day 2 – 4), it is critical to monitor the cell's response to the Simplicon™ RNA transfection and adjust the amount of puromocyin applied based upon the cell toxicity observed. For this reason, a set amount of puromyocin should not be added to the Stage 1 Medium cocktail, but rather added in fresh with the amounts adjusted based upon the observed cell toxicity.

11. At the time of puromycin application, cells should be approximately 80-90% confluent.

12. Aspirate the medium. Replace with 2 mL of Stage 1 Medium containing 200 ng/mL B18R protein (see step 7 for formulation).

13. Add in the optimal starting puromycin concentration that was determined from step 2. Gently rock the plate from side to side to thoroughly mix the puromycin to the medium.

    Note: The optimal starting puromycin concentration may vary between cell lines and also to some extent between experiments and must be determined empirically.

14. Replace the medium daily with 2 mL Stage 1 medium containing 200 ng/mL B18R protein and fresh puromycin. **Note**: Monitor every day to assess the cell's response to the puromycin.

Days 4 - 11: Adjust puromycin concentration

15. By day 4 – 5, variable levels of cell death may be observed. Adjust the puromycin concentration based upon the following guidelines. Refer to examples for representative images.

    <u>If observed cell death is <20%:</u> Gradually increase the puromycin concentration by small increments of 0.5X. For example, if the optimal starting puromycin concentration is 0.5 µg/mL, then increase to 0.75 µg/mL. Closely monitor the culture every day and if necessary adjust the puromycin concentration.

    <u>If observed cell death is 30-60%:</u> Maintain the optimal starting puromycin concentration (determined from Step 2).

    <u>If observed cell death is 80-90%</u>: Withdraw puromycin altogether.

16. Replace with 2 mL fresh Stage 1 Medium containing 200 ng/mL B18R protein and puromycin every day until day 11. Adjust the puromycin concentration based on the guidelines set forth in step 15.

17. By days 7- 9, puromycin-resistant cells should start to grow back and will begin to proliferate. Replace with fresh Stage 1 Medium containing 200 ng/mL B18R protein and puromycin daily until cells are approximately 70-90% confluent. **Total volume = 2 mL.**

    Note: In cases where there is significant cell death at days 4-5, it may take longer for the puromyocin-resistant cells to recover and proliferate (i.e. 17 – 18 days). Do not discard the culture, but keep maintaining until the cells are 70-90% confluent. **At day 11 onward, change to 2 mL Stage 2 Medium with B18R protein (refer to step 21 for formulation) until cells are ready to be replated.**

18. Cells may be replated when they reach 70-90% confluency.

Examples:

Below are three separate RNA reprogramming experiments performed on different days using EMD Millipore's Human Foreskin Fibroblasts (HFF) (Cat. No. SCC058). For EMD Millipore's HFF, the optimal plating density was empirically determined to be 4 x 10^5 cells per well at day 0 (see step 1) and the optimal starting puromycin concentration was determined to be 0.5 µg/mL (see step 2).

On the day of RNA transfection (day 1), cells were approximately 60-70% confluent. Puromycin (0.5 µg/mL) was applied at day 2. By day 4, varying levels of cell death was observed.

Scenario 1: Cell death is <20% at Day 4. Increase puromycin by 0.5X fold.

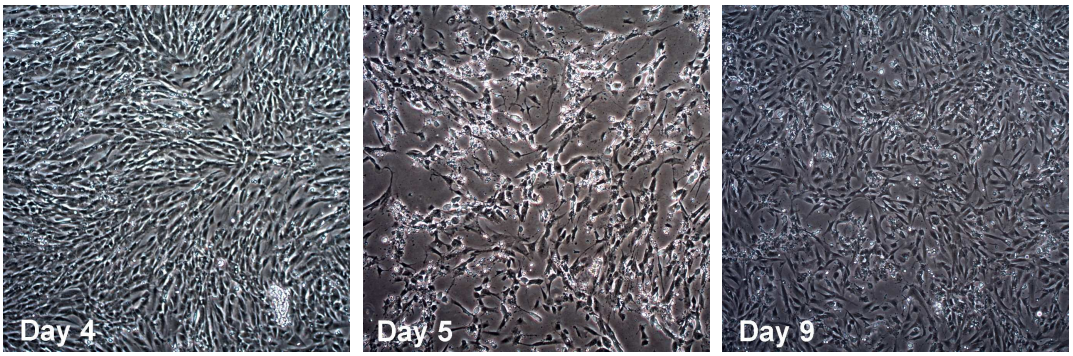


Figure 4. By day 4, minimal cell death was observed. Puromycin was increased to 0.8 µg/mL and maintained at this concentration until day 9 when cells are >80% confluent. Cells were replated at day 9.

Scenario 2: Cell death is 30-60% at Day 4. Maintain puromycin concentration

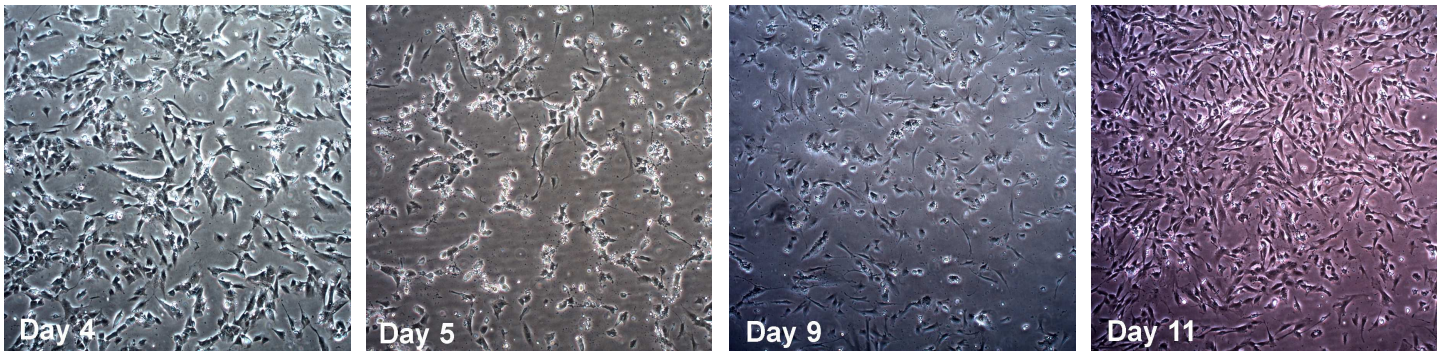


Figure 5. By day 4, approximately 30-60% cell death was observed. Puromycin concentration was maintained at 0.5 µg/mL until day 11 when cells were 75% confluent. Cells were replated at day 11.

Scenario 3: Cell death is 80-90% by Day 4. Withdraw puromycin altogether

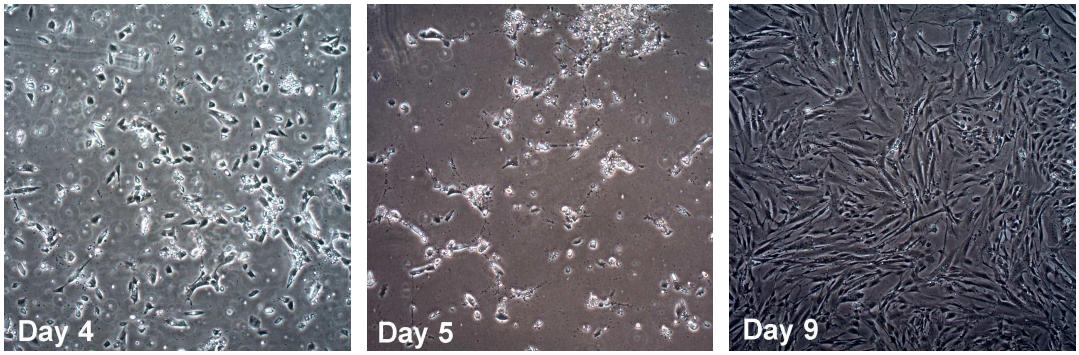


Figure 6. By day 4 and day 5, approximately 80-90% cell death was observed. The puromycin concentration was 0.5 µg/mL on both days. With the significant cell death observed at day 5, puromycin was withdrawn altogether. By day 9, puromycin-resistant cells were 75% confluent. Cells were replated at day 9.

<u>One day before replating:</u>

19. <u>For feeder-free culture:</u> Prepare Matrigel coated 6-well plates:

    a. Thaw Matrigel on ice. Keep on ice and use pre-cooled medium and pipettes to avoid gelling of the ECM gel. **IMPORTANT: Do not thaw Matrigel at temperatures higher than 15ºC to avoid gelling.**

    b. Dilute the Matrigel 1:20 with cold DMEM medium. For example, to every 0.5 mL Matrigel, add 9.5 mL cold DMEM medium for a total volume of 10 mL. Scale according to the volumes required.

    c. Add 1.5 mL of diluted Matrigel to each well. Swirl the culture plates to spread the Matrigel evenly across the surface of the plate. Incubate at room temperature for at least 1 hour or 2 – 8℃ overnight. If not used immediately, store coated cultureware at 2-8˚C until ready to use.

    ***Note**: If not used immediately, Matrigel coated culturewares should be sealed with parafilm to prevent evaporation and can be stored at 2 – 8℃ fo r 2-3 days.*

    <u>For feeder-based culture</u>: Prepare inactivated Mouse Embryonic Fibroblast (MEF) feeder layers to support the cells being reprogrammed as follows.

    a. Coat each well of a fresh sterile 6-well plate with 2 mL of 0.1% gelatin solution (Cat. No. ES-006-B). Incubate for 30 minutes at 37℃. Set a side until ready to receive inactivated MEFs.

    b. Aspirate the 0.1% gelatin coating solution from each well before seeding the inactivated MEFs. Thaw inactivated MEFs (Cat. No. PMEF-CF). Count the number of thawed MEFs and seed 4 x 10^5 cells per well of a 6-well dish. Use MEF Expansion Medium to culture the cells (see below). Total volume per well should be 3 mL. Incubate overnight in a 37℃, 5% CO_2 incubator.

    c. Make up 50 mL MEF Expansion Medium. Sterile filter with 0.22 µm filter

MEF Expansion Medium

Component	Quantity	Final Conc.	Supplier	Cat. No.
DMEM High-Glucose Medium	44 mL		EMD Millipore	SLM-120-B
Fetal Bovine Serum	5.0 mL	10%	EMD Millipore	ES-009-B
GlutaMAX (100X)	0.5 mL	1X	Life Technologies	35050-061
Penicillin Streptomycin Solution (100X)	0.5 mL	1X	EMD Millipore	TMS-AB2-C
Total Volume	**50 mL**			

Replating: When puromycin-resistant cells are 70-90% confluent:

20. When puromycin-resistant cells are 70-90% confluent, they can be replated onto Matrigel coated plates (for feeder-free culture) or onto inactivated MEF feeder layer (for feeder-based culture). This may occur anytime between days 9 - 18 depending upon the cell's response to puromycin (see Figure 8 for examples).

    Note: In the event that puromycin-resistant cells take longer than 11 days to reach 70-90% confluency, (i.e. may take up to 18 days to reach 70-90% confluency), the medium should be changed to 2 mL Stage 2 Medium with B18R protein at **day 11** onward as this medium promotes ES cell proliferation. See step 21 for formulation. Maintain cells in Stage 2 Medium with B18R protein until the cells reach the necessary confluency to be replated. Although the time to reach replating may vary between experiments, the total reprogramming process would still be within 30 days. In instances where replating occurs later (i.e. day 17-18), small ESC-like aggregates may emerge before replating.

21. For 1 reaction, make up 15 mL volume which is sufficient for a week's supply of media changes. Add all components except B18R protein, pass through 0.22 µm filter, and last add B18R protein after filter sterilization. Store at 2-8℃. Scale up accordingly based on the number of reactions being performed.

Stage 2 Medium with B18R protein: 15 mL reaction volume (1 rxn)

Component	Quantity	Final Conc.	Supplier	Cat. No.
MEF conditioned medium	15 mL		R&D Systems	AR005
bFGF (reconstitute to stock concentration 50 µg/mL)	3 µL	10 ng/mL	EMD Millipore	GF003
Human iPS Reprogramming Boost Supplement II (1000X)	15 µL each	1X	EMD Millipore	SCM094
B18 R protein (0.5 mg/mL)	6 µL	200 ng/mL	EMD Millipore	GF156
Total Volume	**15 mL**			

22. Replate RNA transfected cells as follows.

a. For feeder-free cultures: Prior to seeding the cells, bring the Matrigel-coated plates back to room temperature, remove the coating solution and replace with **3 mL** per well of Stage 2 Medium with B18R protein (see formulation above). Set plate aside until ready to receive transfected cells.

   For feeder-based cultures: Remove the medium from the 6-well plate containing inactivated MEF feeder layer (from Step 19). Wash once with 2-3 mL 1X PBS per well. Aspirate the PBS and replace with **3 mL** per well of Stage 2 Medium with B18R protein (see formulation above). Set plate aside until ready to receive transfected cells.

b. Aspirate the medium from the 6-well plate containing the RNA transfected cells (from step 18). Wash once with 3 mL of 1X PBS per well. Aspirate.

c. Add 1 mL of Accumax solution to each well of the plate containing the RNA-transfected cells. Incubate for 3-5 minutes at 37℃ to dissociate the cells. Inspect the plate and ensure the detachment of cells by gently tapping the side of the plate with the palm of your hand.

d. Add 2 mL of Stage 2 Medium without the B18R protein (see table above). As this is a wash step, B18R protein is not added to conserve the protein.

e. Gently swirl the plate to mix the cell suspension. Using a 1000 µL pipetteman, pipette up and down several times to dissociate into a single cell suspension. Transfer the dissociated cells to a 15 mL conical tube.

f. Centrifuge the tube at 800 rpm for 5 minutes to pellet the cells. Discard the supernatant.

g. Resuspend the cell pellet in 1 mL of Stage 2 Medium with the B18R protein (see table above).

h. Count the number of cells using a hemocytometer.

   For feeder-free replating: RNA transfected cells may be replated on matrigel-coated plates in Stage 2 Medium with B18R protein. Seed approximately 5 x 10^4 to 1 x 10^5 of the RNA transfected cells (from step 22h) onto one well of the 6-well matrigel-coated plate. Total volume per well should be 3 mL. It may take longer for iPS colonies to emerge in feeder-free replating conditions.

   For replating on inactivated MEF layer: Seed approximately 3 x 10^4 to 1 x 10^5 of the RNA transfected cells (from step 22h) onto one well of the 6-well plate containing inactivated MEFs. Total volume per well should be 3 mL.

   Note: Puromyocin is no longer required from this point on.

After replating and during period before colonies emerge (Day 12-18)

23. Exchange with 3 mL Stage 2 Medium containing B18R protein every **OTHER** day. It is not necessary to change media every day at this point. Add the medium slowly, using extreme care. Monitor cell morphology daily. Small iPS cell colonies may start to appear around Day 15.

When small hiPS colonies start to emerge (Day 19 – Day 30)

24. When small iPS colonies start to emerge, exchange to 3 mL Stage 2 Medium **WITHOUT** B18R protein. Exchange with 3 mL medium every **OTHER** day.

    Stage 2 Medium without B18R protein: 15 mL reaction volume (1 rxn)

    Sterile filter with 0.22 µm filter.

Component	Quantity	Final Conc.	Supplier	Cat. No.
MEF conditioned medium	15 mL		R&D Systems	AR005
bFGF (reconstitute to stock concentration 50 µg/mL)	3 µL	10 ng/mL	EMD Millipore	GF003
Human iPS Reprogramming Boost Supplement II (1000X)	15 µL each	1X	EMD Millipore	SCM094
Total Volume	**15 mL**			

When colonies are ready to be isolated and expanded (Day 25 – Day 35)

25. Continue to monitor the growth of the human iPSC colonies daily. Look for homogeneous colonies that are compact and have defined borders. When iPSC colonies reach approximately 200 cells or over in size, they are ready to be picked (refer to Figure 8E).

    Note: Monitor the culture daily. Colonies may become large enough to be manually passaged anytime between Day 25 – Day 35; do not let the culture overgrow which can induce differentiation. *Optional: perform live staining using Human iPS Selection Kit (SCR502) to select for Tra-1-60$^+$ SSEA4$^+$ colonies.*

26. One day prior to picking the iPSC colonies:

    a. For feeder-free expansion: Prepare a fresh 6-well plate coated with Matrigel as described in step 19.

    b. For feeder-based expansion: Prepare a fresh 6-well plate with inactivated MEFs as described in step 19.

27. On the day that iPSC colonies are ready to be picked:

    a. For Feeder-free expansion: Aspirate the coating mixture from the Matrigel coated 6-well plate. Add 3 mL of fresh Stage 2 Medium **WITHOUT** B18R protein to each reaction well. Set the plate in a 37℃, 5% CO_2 incubator until the manually passaged iPSC are ready to be plated onto it.

    b. For feeder-based expansion: Aspirate the medium from the 6-well plate containing inactivated MEFs plated from the day before (from step 26). Wash the plate once with 2 mL 1X PBS. Aspirate and add in 3 mL fresh Stage 2 Medium **WITHOUT** B18R protein (to each well of inactivated MEFs. Set the plate in a 37℃, 5% CO_2 incubator until the manually passaged iPSC are ready to be plated onto it.

28. On the day that colonies are to be picked, transfer the 6-well plate containing iPS cell colonies to a tissue culture hood containing a dissecting microscope. Using a 21 gauge needle attached to a 3 mL syringe, cut each iPS colony into 2-5 pieces depending upon the colony size. Using a p200 pipetteman that has been set to 30 µL volume, transfer all the pieces from one well into a

new well of a pre-equilibrated 6-well plate containing inactivated MEFs (from step 27b; for feeder-based culture) or matrigel coated plate (from step 27a; for feeder-free culture). Alternatively, if clonal expansion is desired, small pieces derived from a single colony can be replated onto a pre-equilibrated 4-well plate containing 4 x 10^4 inactivated MEFs (for feeder-based culture) or matrigel coated plate (for feeder-free culture). For a 4-well plate, use 0.5 mL final volume per well.

Note: It is recommended to pick at least 6-8 distinct iPS colonies for expansion and further characterization.

29. Agitate the plates **gently** from side to side and forward and backwards to ensure that iPS clumps are evenly distributed. Place the plate in 37℃, 5 % CO_2 incubator for two days without any media exchanges.

30. DO NOT EXCHANGE MEDIA one day after passaging.

31. On the 2nd day after manual passaging, exchange with 3 mL fresh Stage 2 Medium **WITHOUT** B18R protein to each well of a 6-well plate. Alternatively, if using a 4-well plate, exchange with 0.5 mL fresh Stage 2 Medium **WITHOUT** B18R protein to each well.

32. After 3-4 days, the culture medium may be replaced with any Human ESC medium normally used in the lab (i.e. PluriSTEM™, mTeSR1, KOSR-based medium) Replace daily with 3 mL (for 6-well plates) or 0.5 mL (for 4-well plates) fresh Human ESC Medium. For the first 1-2 passages, colonies may require a longer length of time to grow to sufficient size to be ready for passaging. Monitor iPS colony formation every day to determine optimal time for next passage. By the 3rd passage, iPS cells can be cultured similarly to human ESCs and adapted to other proven ES/iPS cell culture media. We suggest manual passage for the first 5 passages. After the 5th passage, enzymatic passaging (i.e. Dispase II) may be used to maintain and expand the cells.

Representative Results

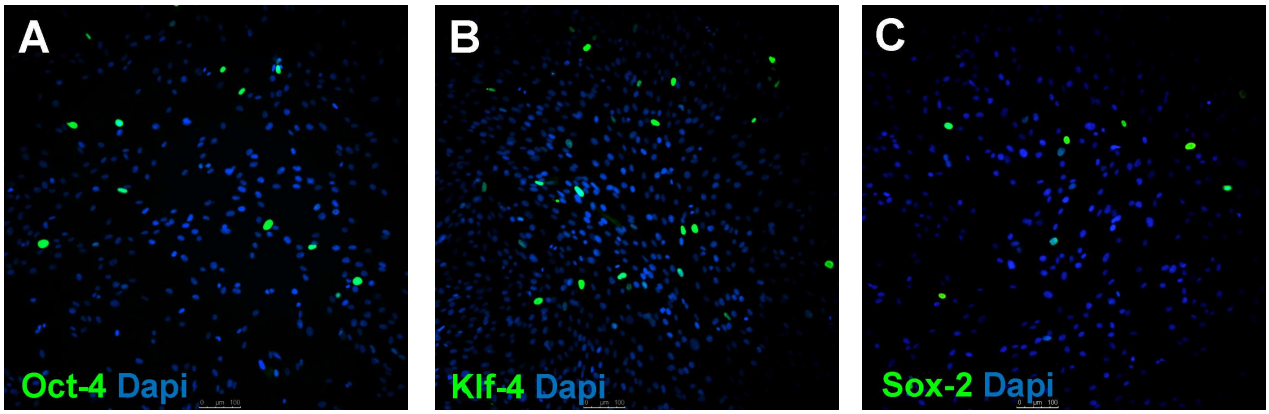
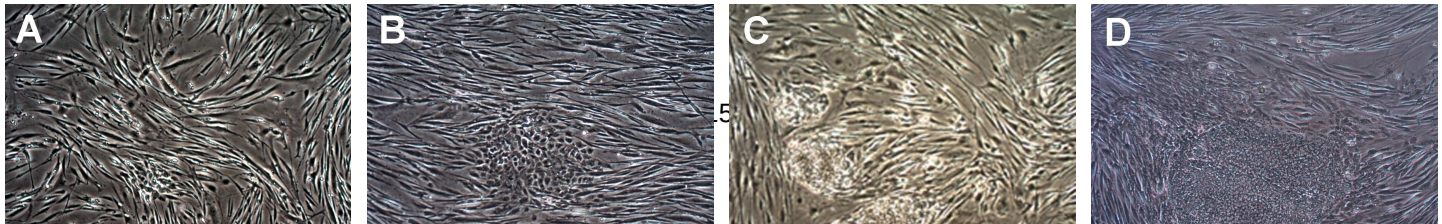


Figure 7. Human foreskin fibroblast cells (HFFs) were transfected with the Simplicon™ RNA Reprogramming Kit (Cat. No. SCR550). 24 hrs after RNA transfection, immunostaining indicated that the transgenes, Oct-4, Sox-2 and Klf-4 were already expressed. **(A-C).** Flow cytometry analysis showed around 15%-20% cells express the Oct-4 (data not shown). HFFs with Simplicon™ RNAs could be enriched by puormycin selection.



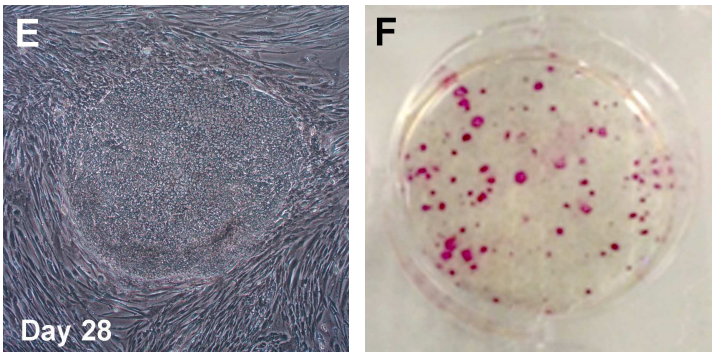


Figure 8. Time course of human iPS colonies generated using Human Simplicon™ RNA Reprogramming Kit (Cat. No. SCR550). The transfected HFFs were replated onto inactive MEFs at Day 10, Colonies start to emerge from Day 15-16 and are more obvious around day 17-20 **(A-C)**. Colonies are ready to be picked at Day 27. Alkaline phosphatase staining of hiPS colonies.

Frequently Asked Questions (FAQ):

1. **How do I determine the optimal plating cell density for my cell type of interest?**
   Refer to step 1 in protocol.

2. **How do I determine the optimal puromycin concentration to start with?**
   Refer to step 2 in protocol.

3. **How much RNA and B18R protein should I use for each transfection?**
   Refer to the protocol section starting on page 8 for detailed instructions. The protocol is based upon 1 reprogramming reaction. Scale up accordingly based upon the number of reprogramming reactions being perfomred.

4. **Do I need to transfect in B18R RNA even though the B18R protein is already included?**
   We have found empirically that cotransfection of VEE-OKS-iG and B18R mRNA in the presence of the B18R protein increased the reprogramming efficiency compared to using VEE-OKS-iG RNA alone.

5. **Is it necessary to add in the small molecules in the Human iPS Reprogramming Boost Supplement II?**
   Yes. The small molecules enhance the efficiency and quality of colony formation.

6. **Can I transfect the RNAs more than once? Will it improve my reprogramming efficiency?**
   Based upon our experience with human foreskin fibroblasts, a single transfection is sufficient. The RNA self-replicates and the puromycin resistance gene will help select for cells that take up the self-replicating RNAs. For other cell types, additional RNA transfections may be helpful but should occur before the replating.

7. **How long will the RNA self-replicate in the cells?**
   Based upon PCR data, the RNA is no longer present at p4.

8. **Do I need to use MEF-CM after replating or can I use another Human ES/iPS Medium?**
   At this time, we have only validated for MEF-CM.

9. **How long can the different media be stored at 2-8C?**
   Prepare just enough medium for 3 days to 1 week usage. Refer to detailed description in protocol.

10. **After application of puromycin, my cells are dying. What should I do?**
    If >80-90% cell death was observed around D4-D5, puromycin should be immediately withdrawn from the medium. Wait for cells to grow back to 70-90% confluency and then do the replating. Refer to step 18, Scenario 3 in protocol.

11. **After application of puromycin, I do not see any cell death. What should I do? Should I increase the puromycin concentration?**
    At day 4-5, if there is <20% cell death, increase the puromycin concentration incrementally by 0.5X. Observe the cell's response daily. Refer to step 18 Scenario 1 in protocol.

12. **It's been over 11 days and my cells are still not proliferating and they are nowhere close to being 70-90% confluent. Should I replate the cells anyways?**
    Exchange with fresh Stage 2 Medium with B18R protein (see step 21 for formulation) at day 11 onwards. Do not replate until cells are approximately 70-90% confluent (optimal time). At a minimum, wait until cells are 50-60% confluent before replating.

13. **Do I need to replate my transfected cells to MEF feeder layer?**
    Transfected cells could be replated on MEF feeder layer or on a matrigel coated plate in Stage 2 Medium with B18R protein. B18R protein may be withdrawn when tiny iPS colonies start to emerge.

14. **Do I need to change the medium every day?**
    Early in the reprogramming process (Stage 1), media changes should be performed every day. Once transfected cells reach 70-90% confluency, they can be replated. Upon replating (Stage 2), medium changes can be performed every **OTHER** day.

15. **Should I use Rock Inhibitor during my replating to increase cell survival?**
    Rock Inhibitor is not required.

16. **Why do I have to adjust my puromycin concentration daily?**
    The Simplicon™ VEE-OKS-iG RNA replicon contains the puromycin resistance gene which is used to select for cells that take up the RNA replicon. Transfected cells are more sensitive to puromycin treatment and thus the culture should be monitored on a daily basis so that a balance between selecting for transfected cells and over-killing is maintained.

References

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