# Simplicon™ OKSG-cMyc TagRFP RNA

Catalog No. SCR714

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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], (3) daily transfections of cells using four synthetic mRNAs over a 14 day period (i.e. mRNA based)[2], and (4) the inability to directly assess transfection or viral transduction efficiency of the introduced reprogramming factors.

MilliporeSigma's Simplicon™ OKSG-cMycTagRFP RNA uses a safe and efficient method to generate integration- and virus-free human iPSCs using a single transfection step. The technology utilizes a single self-replicating Venezuelian equine encephalitis (VEE) RNA species that expresses the reprogramming factors (RF) ORFs[3] (OKSG-cMyc; Oct4, Klf4, Sox2, Glis1 and cMyc) along with a red fluorescent protein (TagRFP). The Simplicon™ RNA replicon is a synthetic polycistronic VEE-RF RNA that is capable of self-replicating in a limited number of cell divisions. The OKSG-cMyc transgenes are especially useful for iPSCs generation from somatic cells that are more difficult to reprogram (i.e. slower proliferating cells or aged somatic cells) while the TagRFP provides a rapid assessment of transfection efficiency. Presence of the TagRFP transgene also allows for optimization of the transfection conditions in hard- to- transfect somatic or primary cells.

**Advantages of the Simplicon™ OKSG-cMyc TagRFP RNA:**

- Integration-free, footprint-free iPSCs generation. No risk of genomic integration.
- Safe, virus-free, synthetic polycistronic RNA replicon (all five reprogramming factors in a single RNA strand)
- TagRFP reporter gene construct embedded in the RNA replicon that allows visualization and quantification of the transfection efficiency of the Simplicon™ RNA.
- Only one single transfection required. The RNA replicon is able to self-replicate, eliminating the need for additional daily transfections of multiple individual mRNAs over a 14-day period.
- Efficient and rapid reprogramming.
- No screening required to ensure the absence of viral remnants.
- Controlled elimination of synthetic VEE RNA replicon by the removal of B18R protein.
- Validated for reprogramming in feeder-free and feeder-based culture conditions.

Simplicon™ OKSG-cMyc TagRFP RNA contains sufficient material for 10-20 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 HFFs. The resulting human iPSCs display characteristic ESC-like morphology, express pluripotent markers and can be rapidly expanded under normal human ESC culture conditions.

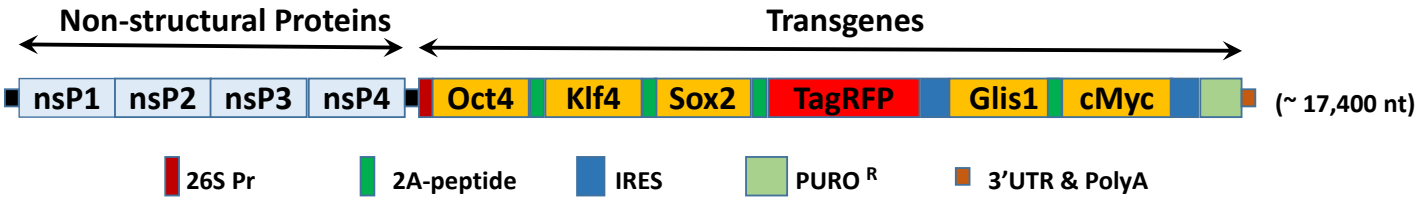


**Figure 1.** Structure of the Simplicon™ OKSG-cMyc TagRFP RNA replicon. The RNA replicon encodes four non-structural replication complex proteins (nsPs) as a single ORF at the 5' end of the RNA. At the 3' end, the viral structural proteins ORFs are replaced with the OKSG-cMyc and TagRFP transgenes. Locations of the 26S internal promoter (Pr), 2A peptides, IRES and Puromycin (Puro)-resistance gene are indicated.

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## Simplicon™ OKSG-cMyc TagRFP RNA Components (Cat. No. SCR714):

1. VEE-OKSG-cMyc TagRFP RNA: (Part No. CS222750) One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80°C.

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

## Critical Materials Required but Not Included in the Kit.

Please note that the following materials are **absolutely** required and critical for the reprogramming reaction. Please ensure that all materials are on hand before initiating the reprogramming reaction. We do not recommend using the Simplicon™ OKSG-cMyc TagRFP RNA without the B18R protein and Human iPS Reprogramming Boost Supplement II. In the event that the Human iPS Reprogramming Boost Supplement II kit is not available, users are recommended to acquire the small molecules individually. Reconstitution instructions and final working concentrations for the small molecules are provided below.

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

2. Human iPS Reprogramming Boost Supplement II: (Cat. No. SCM094).

   - TGF-β RI Kinase Inhibitor IV Supplement A-83-01 (1000X): (Part No. CS210445) One (1) vial containing 400 µL of the inhibitor in high quality DMSO. Store at -20°C. Final concentration of A-83-01 in the reprogramming reaction should be 0.5 µM.

   - Sodium Butyrate Supplement (1000X): (Part No. CS210446) One (1) vial containing 400 µL of the inhibitor in sterile water. Store at -20°C. Final concentration of Sodium Butyrate Supplement in the reprogramming reaction should be 0.25 mM.

   - PS48 Supplement (1000X): (Part No. CS210447) One (1) vial containing 400 µL of the inhibitor in high quality DMSO. Store at -20°C. Final concentration of PS48 Supplement in the reprogramming reaction should be 5 µM.

## Storage and Stability

- VEE-OKSG-cMyc TagRFP and B18R RNAs: Stable for 4 months from date of receipt when stored appropriately at -80°C. For best recovery, quick-spin the vial after thawing on ice before opening. Aliquot into sterile, nuclease-free eppendorf tubes on ice and store at -80°C. Limit repeated freeze-thaw cycles. Use in a sterile RNase-free environment.

- Human recombinant B18R protein (**not provided, but available separately**): Stable for 4 months from date of receipt when stored appropriately at -80°C. For best recovery, quick-spin the vial after thawing on ice before opening. Aliquot B18R protein into sterile, nuclease-free, low protein-binding eppendorf tubes on ice and store at -80°C. 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 (**not provided, but available separately**): Stable for 4 months at -20˚C from date of receipt. Upon first thaw, aliquot into smaller working volumes and freeze at -20°C. 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 no more than two-week supply of supplemented media each time.

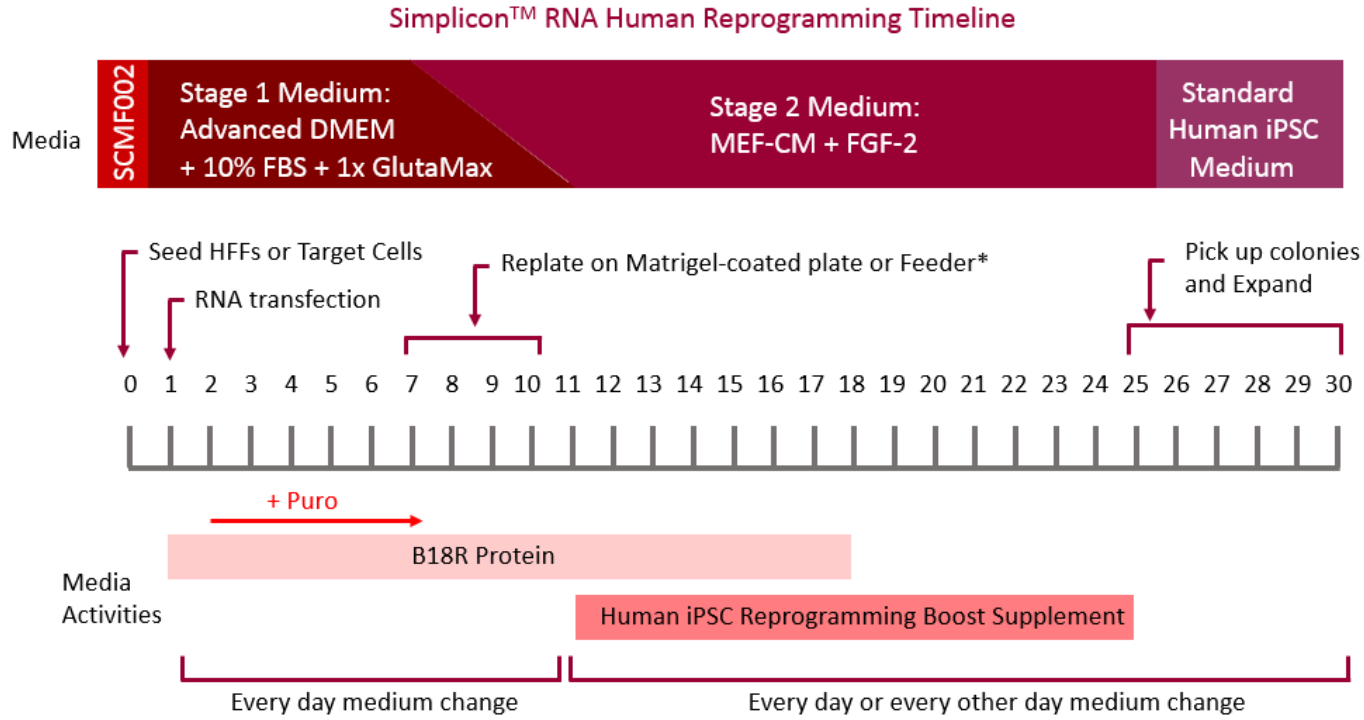
## Accessory Materials Required But Not Supplied

1. DMEM High-Glucose Medium (Cat. No. SLM-120-B)
2. Advanced DMEM (ThermoFisher Scientific, Cat. No. 12491015)
3. Mouse Embryonic Fibroblast (MEF) Conditioned Media (R&D systems, Cat. No. AR005)
4. FibroGRO™ LS Complete Medium (Cat. No. SCMF002)
5. FibroGRO™ Xeno-Free Human Foreskin Fibroblasts ( Cat. No. SCC058), optional
6. Opti-MEM® I Reduced Serum Medium (ThermoFisher Scientific, Cat. No. 31985062)
7. Fetal Bovine Serum (Cat. No. ES-009-B)
8. GlutaMAX™ (ThermoFisher Scientific, Cat. No. 35050061)
9. Penicillin Streptomycin Solution (100X) (Cat. No. TMS-AB2-C)
10. Recombinant Human FGF-2 (Cat. No. GF003)
11. ROCK Inhibitor (Y-27632) (Cat. No. SCM075)
12. Accumax™ Cell Detachment Solution (Cat. No. SCR006) or ACCUTASE™ cell detachment solution (Cat. No. SCR005)
13. EmbryoMax® 1X Dulbecco's Phosphate-Buffered Saline w/o $Ca^{++}$ or $Mg^{++}$, 500 mL (Cat. No. BSS-1006-B)
14. Matrigel® Matrix, hESC-qualified ( Corning, Cat. No. 354277)
15. PMEF cells, growth-arrested, mitomycin-C treated (Cat. No. PMEF-CF)
16. EmbryoMax® 0.1% Gelatin Solution (Cat. No. ES-006-B)

17. RiboJuice™ mRNA Transfection Kit (Cat. No.TR-1013) or
    Lipofectamine® MessengerMAX™ Transfection Reagent (ThermoFisher Scientific Cat. No. LMRNA001)
18. Human iPS Selection Kit (Cat. No. SCR502)
19. 6-well plates, culture flasks, dishes (TC grade)
20. Nuclease-free, sterile microcentruge eppendorfs
21. Nuclease-free areosol-barrier pipette tips
22. Cell counter / hemocytometer

## Protocol overview for iPSC generation of human fibroblasts

***Important note:*** The following protocol has been optimized using early passage Human Foreskin Fibroblasts (Cat. No. SCC058) and adult human fibroblasts, and should be used as general guidelines. Modifications of the protocol may be needed to enable optimized generation of iPSCs from other types of target human cells. Actual timelines may vary depending on the cell types and experimental conditions.




* Timing may vary based on cell lines and sensitivity to puromycin. Replate when puromycin-resistant cells become 70-90% confluent.

**Figure 2.** Reprogramming timeline using Simplicon™ OKSG-cMyc TagRFP RNA. Key steps and media requirements are indicated. +Puro signifies addition of puromycin. Stage 1 Medium contains Advanced DMEM with 10% FBS and GlutaMAX™ (1x). Stage 2 Medium contains MEF conditioned medium (MEF-CM) with 10 ng/mL FGF-2. B18R protein (200 ng/mL) is required to maintain Simplicon™ OKSG-cMyc TagRFP RNA. Human iPS Reprogramming Boost Supplement II enhances the efficieny of iPSC generation. **Note:** From time of transfection to replating, media changes should be performed every day. After replating, media changes can be performed every OTHER day.

### Simple protocol

**Day 0**: Plate cells to achieve 80-100% confluent the next day.

**Day 1:** Transfect with Simplicon™ OKSG-cMyc TagRFP RNA and B18R RNA. 1-2 µg RNAs (Simplicon™ RNA+B18R RNA) works for one well of 6-well plate. See the Transfection Protocols for details.

**Day 2:** Apply Puromycin selection (red arrow). 0.2-0.5 µg/mL Puromycin works for most of human fibroblasts.

**Day 3-7:** Change medium every day. Add B18R protein (200 ng/mL) and puromycin every day fresh.

**Day 7-10**: Puromycin resistant cells start to grow back. When cells are 70-100% confluent, replate to a Matrigel® or feeder containing plate using a 2 – 6 split ratio. For example, 1 confluent well can be passaged into 2 – 6 wells. Use Stage 1 Medium containing B18R protein (200 ng/mL) and Y27632 (10 µM) for replating of cells.
**After replating**: Use Stage 2 Medium containing FGF-2 (10 ng/mL) and B18R protein (200 ng/mL). Change media every day and supply FGF-2 and B18R protein every day fresh.

**Day 11-25:** Change medium every other day until iPSC colonies are generated.

**Day 25-30:** Pick up colonies and expand.

## Quick Transfection Protocol for one well of 6-well plate

1: Transfection with MessengerMAX™ (ThermoFisher Scientific Cat. No. LMRNA001)

| | | | |
|---|---|---|---|
| Step 1 | Wash cells once with DMEM (no serum and no antibiotics) and add 1mL/well of DMEM (no serum and no antibiotics) containing B18R protein (200 ng/mL). No Serum condition increases the transfection efficiency. However it is possible to use 1-10% serum depending on cell types. | | |
| Step 2 | Prepare RNA mixture in Tube 1: Dilute RNAs in DMEM by pipetting. No vortex! | | |
| Amount of Simplicon™ & B18R RNAs | | 1 µg | 2 µg |
| Tube 1 | DMEM (no serum, no antibiotics) | 50 µL | 100 µL |
| | B18R RNA (1.0 µg/ µL) | 0.5 µL | 1 µL |
| | Simplicon™ OKSG-cMyc TagRFP RNA (1.0 µg/ µL) | 0.5 µL | 1 µL |
| | Total volume | 51 µL | 102 µL |
| Step 3 | Prepare MessengerMAX™ dilution mixture in Tube 2. No incubation! Incubation of MessengerMAX™ dilution significantly decreases the transfection efficiency. | | |
| Tube 2 | DMEM (no serum, no antibiotics) | 50 µL | 100 µL |
| | MessengerMAX™ transfection reagent* | 3 - 5 µL | 6 - 10 µL |
| | Total volume | 53 - 55µL | 106 - 110 µL |
| Step 4 | Quickly add tube 2 into tube 1. | | |
| Total RNAs amount in a tube | | 1 µg/104 - 106 µL | 2 µg/208 - 212 µL |
| Step 5 | Incubate for 5 minutes at room temperature. | | |
| Step 6 | Add the RNA-transfection reagent complex dropwise into one well. | | |
| Step 7 | Incubate for 4 hrs, and then replace the medium to Stage I Medium containing B18R protein (200 ng/mL). | | |

*Increasing MessengerMAX™ may increase not only the transfection efficiency, but also cytotoxicity. See Figure 6.

2: Transfection with RiboJuice™ mRNA Transfection Kit (Cat. No. TR-1013)

| | | |
|---|---|---|
| Step 1 | Wash cells once with DMEM (no serum and no antibiotics) and add 1mL/well of DMEM (no serum and no antibiotics) containing B18R protein (200 ng/mL). It is possible to use 1-10% serum depending on cell types. | |
| Step 2 | Prepare RNA mixture in Tube 1: Dilute RNAs in DMEM by pipetting. No vortex! | |
| Amount of Simplicon™ & B18R RNAs (Please follow order of addition) | | 1 µg |
| Opti-MEM® | | 250 µL |
| B18R RNA (1.0 µg/ µL) | | 0.5 µL |
| Simplicon™ OKSG-cMyc TagRFP RNA (1.0 µg/ µL) | | 0.5 µL |
| RiboJuice™ mRNA Boost Reagent | | 4.0 µL |
| RiboJuice™ mRNA Transfection Reagent | | 4.0 µL |
| Total volume | | 259 µL |
| Step 3 | Incubate 5 minutes at room temperature. | |
| Step 4 | Add the RNA-transfection reagent complex dropwise into one well. | |
| Step 5 | Incubate for 4 hrs, and then replace the medium to Stage I medium containing B18R protein (200 ng/mL). | |

## Critical Success Factors in Simplicon™ RNA Reprogramming:

The following guidelines are critical for ensuring success in reprogramming.

- 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, arerosol resistant tips and a clean enivronment for sample preparation. Change gloves and tips frequently.

- Appropriate reagents storage and handling. Components in the Simplicon™ OKSG-cMyc TagRFP 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 to minimize degradation caused by repeated freeze/thaw cycles.

- B18R protein. During the self-replication of RNAs, host cells produce IFNs to shut down the self-replication of RNA. B18R protein works for neutralizing IFN responses and allows the continuous replication of Simplicon™ RNAs. Continous supplement of fresh B18R protein is important.

- Puromycin selection. Self-replication of Simplicon™ RNA is high in the beginning, and then eventually stabilizes to a low level in about a week. Therefore, a low dose of puromycin (0.2-0.5 μg/mL) to draw out the selection for ~5 days benefits the survival of Simplicon™ RNA positive cells.

## Detail Protocol for Human iPSC Generation

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 achieve 80-100% confluency by the next day (Day 1)**. Plate out a range of cell numbers from 1 x $10^5$ to 2 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 due to differences in cell sizes, morphology and rate of proliferation. 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 increased cell sensitivity due to the cellular interferon response to the introduced Simplicon™ RNAs. 0.2-0.5 μg/mL puromycin works for most human fibroblasts.

**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 80-100% confluency by the next day. Culture medium should be the same as that used to maintain the target cells in a proliferative state. Optional: FibroGRO™ LS Complete Medium (Cat. No. SCMF002). Incubate the plate in a 37°C incubator with 5% $CO_2$.

**Day 1**: One day 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 that causes ~50% cell death. To account for the cell toxicity introduced by the cellular interferon response to the Simplicon™ RNAs, use ½ the concentration as the optimal starting concentration.

**For example:** If by day 4-5, it was determined that 50% cell death occurred at a puromycin concentration of 1.0 μg/mL, then the starting puromycin concentration would be 0.5 μg/mL.

3. Optimize the transfection condition using Simplicon™ OKSG-cMyc TagRFP RNA. Prepare target cells in a 24-well plate (about ¼ scale of 6-well plate), and do the co-transfection of Simplicon™ RNA and B18R RNA at 0.125-0.75 μg RNAs/well. Measure the TagRFP expression with fluorescence microscopy and/or flow cytometry one or two days after transfection to determine the optimal transfection condition. The TagRFP fluorecesence becomes stronger two days after transfection.

## Reprogramming Protocol (Step-by-Step)

### Preparation of Media and Reagents:

1. DMEM High-Glucose Medium: No serum and no antibiotics.
2. Stage 1 medium: Advanced DMEM with 1x GlutaMAX™, 10% FBS, 1x Penicillin/ Streptomycin. Store at 4˚C and use at room temperature (do not warm up at 37˚C).

   ***Note**: B18R protein (200 ng/mL) and puromycin (0.2-0.5 μg/mL) should be added separately as required and detailed in the step by step protocol. B18R protein and puromycin should always be added fresh at each use.*

3. Stage 2 medium: Thaw the MEF-CM (R&D systems, Cat. No. AR005) at room temperature one day before use. Store at 4˚C and use at room temperature (do not warm up at 37˚C).

   ***Note:** B18R protein (200 ng/mL) and FGF-2 (10 ng/mL) should be added fresh at each use.*

   ***Optional:*** Add Human iPS Reprogramming Boost Supplement II and 1x Penicillin/ Streptomycin, if required. Sterile filter using a 0.22 μm filter (Cat. No. SCGPU01RE) when Human iPS Reprogramming Boost Supplement II is added. MEF-CM supplemented with Boost Supplement II is good for up to 2 weeks at 4˚C.

4. Make aliquots of reagents upon first time thawing to minimize repeated freeze-thaw.

   a. **VEE-OKSG-cMyc TagRFP RNA**: Aliquot 3 μL into sterile nuclease-free eppendorf tubes. Store aliquots at -80°C.

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

c. **B18R protein** (not provided, available separately**)**: Aliquot 3 μL into sterile nuclease-free eppendorf tubes. Store aliquots at -80˚C.

**iPSCs generation with human fibroblasts:**

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

**1. Prepare target cells**

Day 0: Plate target cells:

a. Plate target cells at the optimal plating density (determined from "Before Starting Experiment", Step 1; 80-100% confluency) 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.

**2. Pre-Treatment with B18R Protein**

Day 1: Pretreat cells with B18R protein:

On the day of transfection, cells should be 80-100% confluent. Pre-treat target cells for 10-20 minutes with the B18R protein (Cat. No. GF156) to help suppress the cellular interferon response after 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 Medium | 1.0 mL | | SLM-120-B |
| B18R protein (0.5 mg/mL) | 0.4 μL | 200 ng/mL | GF156 |
| FBS | 0 - 100 μL | 0 - 10% | ES-009-B |
| **Total Volume** | **~1.0 mL** | | |

***Note:*** *Transfection efficiency will be maximized if the medium does not contain serum and antibiotics. However, depending on serum dependency of the target cells, it is possible to perform the transfection in the presence of 1-10% serum.*

c. Mix gently by pipetting up and down.

d. Aspirate the medium and wash cells once with 2 mL DMEM (no serum and no antibiotics).

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 10-20 minutes.

## 3. Transfect Simplicon™ OKSG-cMyc-TagRFP and B18R RNAs

Day 1: transfect with Simplicon™ RNA:

Tranfsection of Simplicon™ RNAs has been validated using the RiboJuice™ mRNA Transfection Kit and Lipofectamine® MessengerMAX™ Transfection Reagent. Below are detailed transfection protocols for the use of each transfection kit. Use nuclease-free, aerosol-barrier pipette tips and sterile, nuclease-free eppendorfs.

a. Thaw an aliquot of the VEE-OKSG-cMyc TagRFP and B18R RNAs on ice; quickly centrifuge the vial(s) to spin down the contents. Keep RNA vials on ice.

b. Depending upon the transfection kits used, set up the following reactions in sterile eppendorf tubes. Store any unused Simplicon™ RNAs at -80°C. Mix gently by pipetting.

If using RiboJuice™ mRNA Transfection Kit (Part No. TR-1013):

| Component | Eppendorf | Cat. No. |
|---|---|---|
| Opti-MEM® | 250 µL | Life Technologies (31985-062) |
| B18R RNA (1 µg/µL) | 0.5 µL | |
| VEE-OKSG-cMyc TagRFP RNA (1 µg/µL) | 0.5 µL | |
| RiboJuice™ mRNA Boost Reagent | 4.0 µL | RiboJuice™ mRNA Transfection kit (TR-1013) |
| RiboJuice™ mRNA Transfection Reagent | 4.0 µL | |
| **Total Volume** | **259 µL** | |

If using MessengerMAX™ Transfection Reagent (Life Technologies LMRNA001):

The protocol detailed below is slightly different from the manufacturer. For optimal results, please follow the revised protocol below. Amounts of RNAs and MessengerMAX™ reagents required may change depending on the target cells. The transfection conditions were optimized using BJ human fibroblasts.

| Amount of Simplicon™ & B18R RNAs | | 1 µg | 2 µg |
|---|---|---|---|
| Tube 1 | DMEM high glucose (no serum, no antibiotics) | 50 µL | 100 µL |
| | B18R RNA (1.0 µg/ µL) | 0.5 µL | 1 µL |
| | Simplicon™ OKSG-cMyc TagRFP RNA (1.0 µg/ µL) | 0.5 µL | 1 µL |
| | Total volume | 51 µL | 102 µL |
| Step 3 | Prepare MessengerMAX™ dilution mixture in Tube 2. No incubation! Incubation of MessengerMAX™ dilution significantly decreases the transfection efficiency. | | |
| Tube 2 | DMEM high glucose (no serum, no antibiotics) | 50 µL | 100 µL |
| | MessengerMAX™ transfection reagent* | 3 - 5 µL | 6 - 10 µL |
| | Total volume | 53 - 55µL | 106 - 110 µL |
| Step 4 | Quickly add tube 2 into tube 1. | | |
| Total RNAs amount in a tube | | 1 µg/104 - 106 µL | 2 µg/208 - 212 µL |

*Increasing MessengerMAX™ may increase not only the transfection efficiency, but also cytotoxicity.

c. Incubate the RNA transfection reagent complex at room temperature for 5 minutes.

d. Add the RNA-transfection reagent complex **dropwise** into one well of the 6-well plate containing cells pretreated with B18R protein (from step 2).

e. Gently rock the plate from side to side to thoroughly mix and apply the RNA complexes onto the target cells.

f. 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.

g. During the 4 hour RNA transfection period, prepare Stage 1 Medium containing 200 ng/mL B18R protein (see below).

***Note:*** *It is recommended to add B18R protein fresh at each use. However, it is possible to make 3 days medium containing B18R protein for your convenience as the performance of the B18R protein has been assessed for 3 days at 4 ˚C.*

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

| Component | Quantity | Final Conc. |
|---|---|---|
| Stage I medium (refer to "Preparation of Media & Reagents" for formulation) | 2 mL | |
| B18R protein (0.5 mg/mL) | 0.8 μL | 200 ng/mL |
| **Total Volume** | **~ 2 mL** | |

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

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

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

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

## 4. Start Puromycin Selection

At the time of puromycin application, cells should be approximately 70-100% confluent. When high cytotoxicity is observed, replace with medium that does not contain puromycin, and wait one day more to start the puromycin selection.

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-OKSG-cMyc TagRFP RNA. During the next several days (Day 2 –5), it is critical to monitor the cells' response to the Simplicon™ RNA transfection and readjust the amount of puromocyin applied accordingly. For this reason, a set amount of puromyocin should not be added to the Stage 1 Medium cocktail, but rather added in fresh each

time with the amounts readjusted based on the observed cell toxicity. Exchange **daily** with fresh medium containing 200 ng/mL B18R protein and puromycin. Puromycin selection should work within 5 days. Avoid use of high concentrations of puromycin that would kill all Simplicon™ positive cells. In the presence of B18R protein and puromycin, self-replication of the Simplicon™ RNA is expected to spike and then gradually decrease over a period of 7 days. Low expression levels of puromycin resistance gene may be observed at much later time points.

a. Aspirate the medium. Replace with 2 mL of Stage 1 Medium containing 200 ng/mL B18R protein and 0.2-0.5 µg/mL puromycin (see **Preparation of Media and Reagents** for formulation).

   **Note:** The optimal starting puromycin concentration may vary among cell lines and also, to some extent, between experiments and must be determined empirically. 0.2-0.5 µg/mL puromycin works in most human fibroblasts.

b. Monitor every day to assess the cells' response to puromycin. Replace with 2 mL fresh Stage 1 Medium containing 200 ng/mL B18R protein and puromycin every day.

Days 4-10: Adjust puromycin concentration:

c. By day 4-5, variable levels of cell death may be observed. Adjust the puromycin concentration according to the following guidelines. Refer to representative images as examples.

   If observed cell death is <20%: When mock transfected cells are dead by puromycin selection, stay in this concentration of puromycin. If not, 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, readjust the puromycin concentration.

   If observed cell death is 30-60%: Maintain the optimal starting puromycin concentration

   If observed cell death is 80-90%: Withdraw puromycin altogether.

d. Replace with 2 mL fresh Stage 1 Medium containing 200 ng/mL B18R protein and puromycin every day.

e. By days 7-10, 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 70-100% confluent.

f. Cells may be replated when they reach 70-100% confluency.

   ***Note:*** *In cases where there is significant cell death at days 4-5, it may take longer for the puromycin-resistant cells to recover and proliferate. Do not discard the culture, but keep maintaining to day 10, even if cell confluency is below 70%.*

**Examples of puromycin selection in Simplicon™ RNA transfected cells with RiboJuice™**

Below are three separate RNA reprogramming experiments performed on different days using EMD Millipore's Human Foreskin Fibroblasts (HFFs) (Cat. No. SCC058). For this cell line, the optimal starting puromycin concentration was determined to be 0.5 µg/mL.

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. Maintain or Increase (0.5X fold) puromycin.**

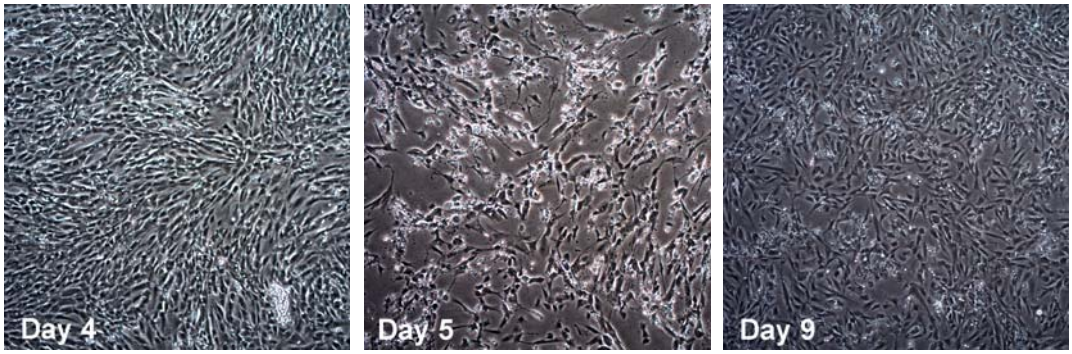



**Figure 3**. By day 4, minimal cell death was observed. Puromycin was increased to 0.8 μg/mL and maintained at this concentration until day 9 when cells were >80% confluent. Cells were replated at day 9.

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

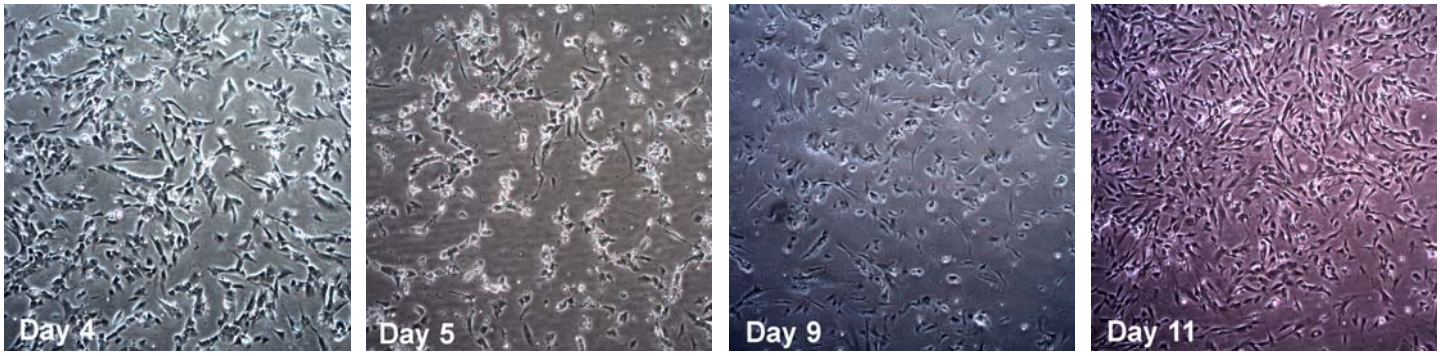



**Figure 4.** By day 4, approximately 30-60% cell death was observed. Puromycin concentration was maintained at 0.5 μg/mL until day 11 when cells were 75% confluent. Cells were replated at day 11.

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

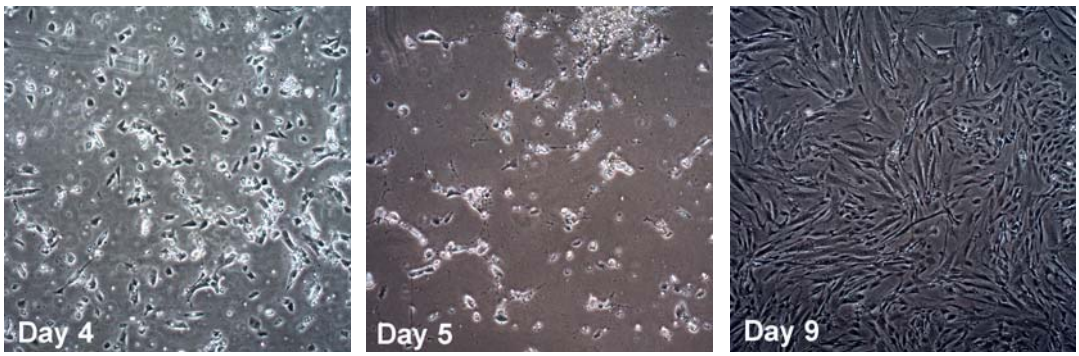



**Figure 5**. By day 4 and day 5, approximately 80-90% cell death was observed. The puromycin concentration was 0.5 μg/mL on both days. With the significant cell death observed at day 5, puromycin was withdrawn altogether. By day 9, puromycin-resistant cells were 75% confluent. Cells were replated at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

**5. Prepare Matrigel® coated well or feeder cells.**

**One day before replating:**

For feeder-free culture: Prepare Matrigel® coated 6-well plates:

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

b. Dilute the Matrigel® 1:20 with cold DMEM/F12 medium. For example, to every 0.5 mL Matrigel®, add 9.5 mL cold DMEM/F12 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 2 – 8°C overnight.

***Note**: If not used immediately, Matrigel® coated culturewares should be sealed with parafilm to prevent evaporation and can be stored at 2 – 8°C for up to one week.*

For feeder-based culture: Prepare inactivated Mouse Embryonic Fibroblast (MEF) feeder layer 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 overnight at 37°C. Overnight incubation of the coating mixture will help allow feeder cells to remain adherent for up to 2-3 weeks.

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

**MEF Expansion Medium**

| Component | Quantity | Final Conc. | Cat. No. |
|---|---|---|---|
| DMEM High-Glucose Medium | 44.5 mL | | SLM-120-B |
| Fetal Bovine Serum | 5.0 mL | 10% | ES-009-B |
| Penicillin Streptomycin Solution (100X) | 0.5 mL | 1X | TMS-AB2-C |
| **Total Volume** | **50 mL** | | |

c. Aspirate the 0.1% gelatin coating solution from each well and add 1 mL MEF Expansion Medium. 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. Total volume per well should be 3 mL. Incubate overnight in a 37°C, 5% $CO_2$ incubator.

**6. Replating: When puromycin-resistant cells are 70-100% confluent**

When puromycin-resistant cells are 70-100% 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 7-10 depending upon the cell's response to puromycin (see Figures 3- 5 for examples).

***Note:** In the event that puromycin-resistant cells does not reach 70-100% confluency by day 10, replate cells directly into a fresh well without further cell dilution.*

a. Prepare the replating medium. 15 mL reaction volume should be sufficient for replating back into 6 wells.

   ***Note:*** *1 confluent well may be replated back into 2-6 fresh wells. As reprogramming efficiency may vary between cell types, it is recommended that users set up cell splitting dilutions to test.*

**Replating Medium with B18R protein (No puromycin):** 15 mL reaction volume (1 rxn). Scale up according to the number of wells required.

| Component | Quantity | Final Conc. | Cat. No. |
|---|---|---|---|
| Stage I medium (refer to "Preparation of Media & Reagents" for formulation) | 15 mL | | |
| B18 R protein (0.5 mg/mL) | 6 μL | 200 ng/mL | GF156 |
| Rock inhibitor (Y-27632) (10 mM) | 15 μL | 10 μM | SCM075 |
| **Total Volume** | **~15 mL** | | |

b. 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 **2 mL** per well of Replating Medium with B18R protein (see formulation above). Set plate aside until ready to receive the RNA-transfected cells.

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

c. Aspirate the medium from the 6-well plate containing the RNA-transfected cells. Wash once with 3 mL of 1X PBS per well. Aspirate.

d. Add 0.5 mL of Accumax solution or Accutase solution to each well of the plate containing the RNA-transfected cells. Incubate for 3-5 minutes at 37°C 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.

e. Add 2 mL of Stage I medium (refer to "Preparation of Media & Reagents" for formulation) without the B18R protein. As this is a wash step, B18R protein is not added to conserve the protein.

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

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

h. Resuspend the cell pellet in 1-3 mL of Replating Medium with the B18R protein depending on dilution scales.

i. Count the number of cells using a hemocytometer or simply split into 2-6 wells of a Matrigel®-coated or MEF feeder layer plate.

For feeder-free replating: RNA-transfected cells may be replated on matrigel® -coated plates in Replating Medium with B18R protein. Seed approximately 5 x $10^4$ to 1 x$10^5$ of the RNA-transfected cells onto one well of the 6-well matrigel-coated plate. It may take longer for iPS colonies to emerge following feeder-free replating. Total volume per well should be >2 mL.

For replating on inactivated MEF layer: Seed approximately 3 x $10^4$ to 1 x $10^5$ of the RNA transfected cells onto one well of the 6-well plate containing inactivated MEFs. Total volume per well should be >2 mL.

**Note: Puromycin is no longer required from this point on.**

**7. Replace Stage 1 medium to Stage 2 Medium**

After replating and during period before colonies emerge (Day 8-18):

a. One day before use, thaw the MEF-CM (R&D systems, Cat. No. AR005) at room temperature, and store at 4˚C.

b. Prepare Stage 2 Medium containing B18R protein (200 ng/mL) and FGF-2 (10 ng/mL). Use at room temperature. Do not warm up at 37˚C. It is possible to change medium every **OTHER** day with **3 mL** volume/well of 6-well plate at this point. Add the medium slowly, using extreme care. Monitor cell morphology daily. Small iPSC colonies may start to appear around Day 16.

***Optional:*** *Add Human iPS Reprogramming Boost Supplement II and 1x Penicillin/ Streptomycin, if required. Sterile filter the MEF-CM supplemented with Boost Supplement II using a 0.22 µm filter (Cat. No. SCGPU01RE). MEF-CM supplemented with Boost Supplement II is good for up to 2 weeks at 4 ˚C.*

***Note****: It is recommended to add B18R protein and FGF-2 fresh at each use. However, it is possible to make 3 days medium containing B18R protein and FGF-2 for your convenience.*

Stage 2 Medium containing B18R protein: 18 mL reaction volume (1 rxn)

| Component | Quantity | Final Conc. | Cat. No. |
|---|---|---|---|
| MEF-CM +/- Human iPS Reprogramming Boost Supplement II (1000X) and Penicillin/Streptmycin | 18 mL | | R&D Systems AR005 |
| FGF-2 (reconstitute to stock concentration 50 µg/mL) | 3.6 µL | 10 ng/mL | GF003 |
| B18R protein (0.5 mg/mL) | 7.2 µL | 200 ng/mL | GF156 |
| **Total Volume** | **~18 mL** | | |

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

c. When small iPSC 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:** 18 mL reaction volume (1 rxn)

| Component | Quantity | Final Conc. | Cat. No. |
|---|---|---|---|
| MEF-CM +/- Human iPS Reprogramming Boost Supplement II (1000X) and Penicillin/Streptomycin | 18 mL | | R&D Systems AR005 |
| FGF-2 (reconstitute to stock concentration 50 μg/mL) | 3.6 μL | 10 ng/mL | GF003 |
| **Total Volume** | **18 mL** | | |

## 8. Isolation of iPSC colonies and expansion of iPSCs

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

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 7H – 7J).

***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 cell staining using Human iPS Selection Kit (SCR502) to select for Tra-1-60$^+$ SSEA4$^+$ colonies.*

a. One day prior to picking the iPSC colonies: See Step 5.

   For feeder-free expansion: Prepare a fresh 6-well plate coated with Matrigel® as described in step 5.

   For feeder-based expansion: Prepare a fresh 6-well plate with inactivated MEFs as described in step 5.

b. On the day that iPSC colonies are ready to be picked: Prepare medium and plate.

   ***Note:*** *To get Simplicon™ RNA free iPSCs, do not add B18R protein into medium. Simplicon™ RNA will become undetectable with several passages in B18R free medium.*

   For feeder-free expansion: Aspirate the coating mixture from the Matrigel®-coated 6-well plate. Add 3 mL of fresh Stage 2 Medium containing FGF-2 (10 ng/mL) and Rock inhibitor (Y-27632, 10 μM). Set the plate in a 37°C, 5% $CO_2$ incubator until the manually passaged iPSCs are ready to be plated onto it.

   For feeder-based expansion: Aspirate the medium from the 6-well plate containing inactivated MEFs plated from the day before (from step 8a). Wash the plate once with 2 mL 1X PBS. Aspirate and add 3 mL of fresh Stage 2 Medium containing FGF-2 (10 ng/mL) and Rock inhibitor (Y-27632, 10 μM) to each well of inactivated MEFs. Set the plate in a 37°C, 5% $CO_2$ incubator until the manually passaged iPSCs are ready to be plated onto it.

*c.* Pick up iPSC colonies. Transfer the 6-well plate containing iPSC 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 matrigel®-coated plate for feeder-free

culture or inactivated MEFs for feeder-based 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 iPSC colonies for expansion and further characterization.*

d. Rock the plates **gently** from side to side and forward and backwards to ensure that iPSC clumps are evenly distributed. Place the plate in a 37°C, 5% $CO_2$ incubator for two days without any media exchanges.

e. DO NOT EXCHANGE MEDIA on the day following passaging.

f. 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.

g. 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 iPSC colony formation every day to determine optimal time for next passage. By the 3rd passage, iPSCs can be cultured similarly to human ESCs and adapted to other proven ESC/iPSC culture media. We suggest manually dislodging the colonies for the first 5 passages. After the 5th passage, enzymatic solutions (i.e. Dispase II) may be used to harvest and expand the cells.

## Representative Results

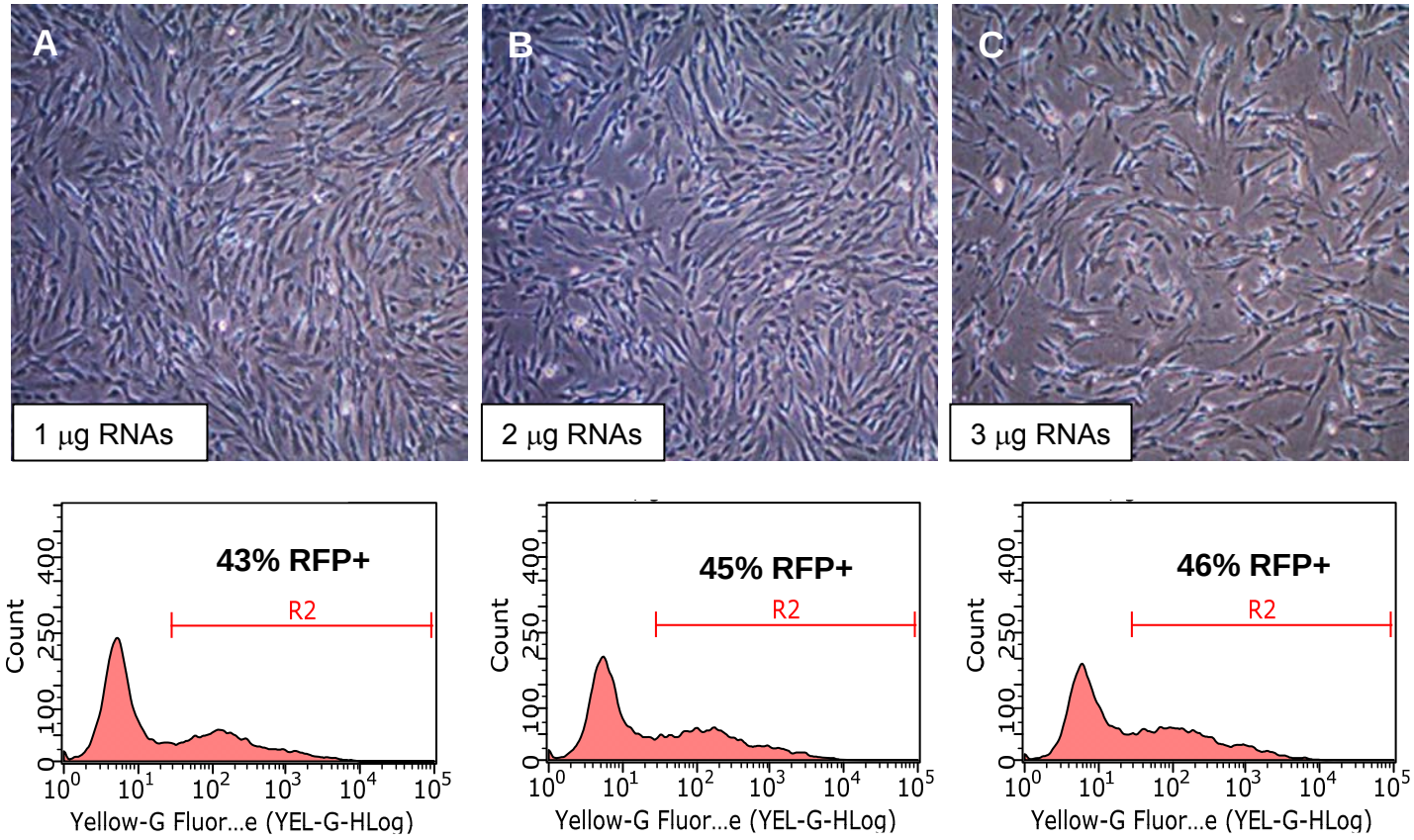



**Figure 6.**

Effects of increasing transfected RNA concentrations on cell viability & cytotoxicity. For each condition, equal concentrations of Simplicon™ RNA and B18R RNA were co-transfected (i.e. 0.5 μg Simplicon™ + 0.5 μg B18R RNA = 1 μg RNA) with 5 μL MessengerMAX™ Reagent. Brightfield images of BJ human fibroblasts one day after transfection (Figure 6A-C). Increasing the total amount of transfected RNAs resulted in increased cell toxicity (Figure 6C). TagRFP positive cells were measured with FACS on day 3.

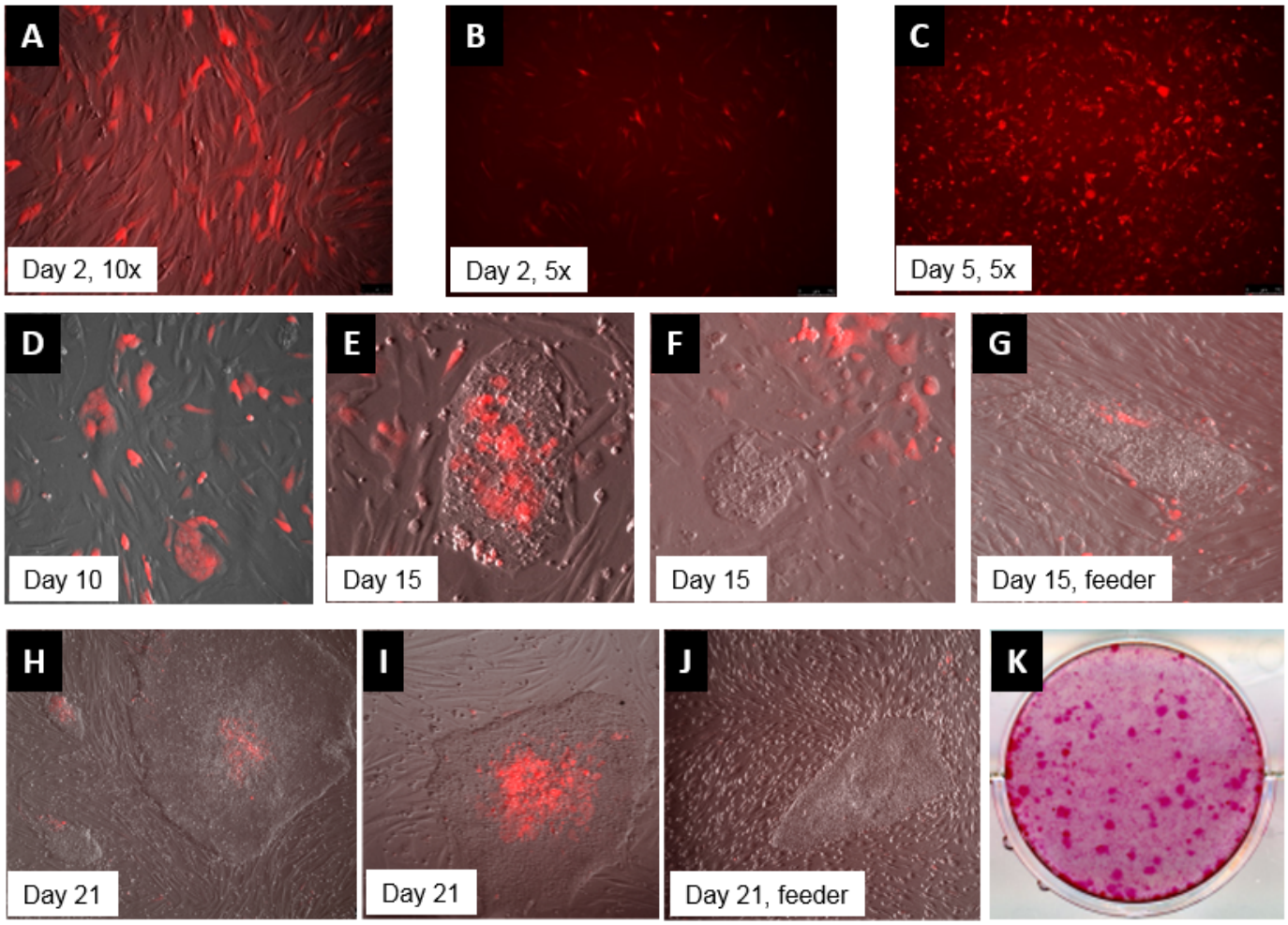



**Figure 7.** Time course of human iPSC colonies generated using Human Simplicon™ OKSG-cMyc TagRFP RNA (Cat. No. SCR714). BJ human foreskin fibroblasts were transfected with Simplicon™-OKSG-cMyc TagRFP and B18R RNAs. One day after transfection (Day 2), approximately 37% cells are RFP positive (**A, B**) and TagRFP expression became strong on Day 5 (**C**). Cells were replated on Day 7. Colonies with TagRFP positive cells on Day 10 (**D**). At later timepoints, colonies may express variable levels of TagRFP, with some expressing partial TagRFP expression (**E, H, I**) or not at all (**F,G,J**). Distinct human iPSC colonies are observed by days 21 (**H, I, J**). Alkaline phosphatase staining of human iPSC colonies **(K)**.

## 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 of "Before Starting Experiment". For human fibroblasts, prepare 100% confluent cells in a 10 cm dish, and then passage all the cells into one 6-well plate one day before the transfection.

2. **How do I determine the optimal puromycin concentration to start with?**
   Refer to step 2 in protocol of "Before Starting Experiment". Most human fibroblasts work with 0.2-0.5 µg/mL puromycin.

3. **How much RNA and B18R protein should I use for each transfection?**
   Refer to the protocol section of "Transfection Protocol" for detailed instructions. The protocol is based on 1 reprogramming reaction in a 6-well plate. Scale up and down accordingly based on the number of reprogramming reactions and plate size 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-OKSG-cMyc TagRFP RNA and B18R mRNA in the presence of the B18R protein increased the reprogramming efficiency compared to using VEE-OKSG-cMyc TagRFP RNA alone.

5. **Will the TagRFP fluorescent signal stay on throughout the reprogramming process?**
   No, TagRFP is **only** used to monitor transfection efficiency of the Simplicon™ RNA. As reprogramming progresses, TagRFP fluorescence may be downregulated. Wide variations in TagRFP fluorescence may be observed between different hiPSC colonies. In our experiences with human fibroblasts iPSC reprogramming, TagRFP fluorecence was easily detectable by ~day 10, while most typical iPSC colonies have lost TagRFP fluorescence by day 21. Some colonies may have all the cells within the colony expressing TagRFP, while other colonies may have only some cells expressing the TagRFP (Figure 7E, H, I) or not at all (Fig. 7F, G, J).

6. **Is it necessary to add in the small molecules in the Human iPS Reprogramming Boost Supplement II?**
   Yes and No. Most human fibroblasts will work without the Boost supplements. However, the small molecules does enhance the efficiency and quality of colony formation. More iPSC colonies will be generated with the Boost supplement, especially for difficult cells such as late passaged fibroblasts or other somatic cells with limited proliferative potential.

7. **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.

8. **How long will the RNA self-replicate in the cells?**
   Based upon PCR data, the RNA is no longer present at P4.

9. **Do I need to use MEF-CM after replating or can I use another Human ES/iPS Medium?**
   The following pluripotent media have been validated to work: MEF-CM, PluriSTEM (Cat., No. SCM130) and mTeSR.

10. **How long can the different media be stored at 2-8C?**
    3-day usage is maximal for B18R protein containing media. Two weeks is maximal for the Human iPS Reprogramming Boost Supplement II containing media. Refer to detailed descriptions in the protocol.

11. **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 4, Scenario 3 and step 6 in the protocol.

12. **After application of puromycin, I do not see any cell death. What should I do? Should I increase the puromycin concentration?**
    When transfection efficiecy is greater than 40%, we do not see significant or detectable cell death during the puromycin selection on day 5. It is important to set up mock transfection to help determine whether puromycin selection is working or not. When mock transfected cells are dead, puromycin selection is working, so it is OK to stay at this concentration. When mock transfected cells are not dying, you may need to increase the puromycin concentration incrementally by 0.5X. Observe the cell's response daily. Refer to step 4 Scenario 1 in protocol.

13. **It's been over 10 days and my cells are still not proliferating and they are nowhere close to being 70% confluent. Should I replate the cells anyways?**
    It's OK to replate cells directly into a fresh well (Matrigel® or feeder) without cell dilution when cells are ~20% confluency. When cells are below ~20% confluecny, you may try to exchange with fresh Stage 2 Medium with B18R protein at day 10 and wait a few days until cells are ~20% confluent for replating.

14. **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 iPSC colonies start to emerge.

15. **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 70-100% confluency, they can be replated. Upon replating (Stage 2), medium changes can be performed every **OTHER** day.

16. **Should I use Rock Inhibitor during my replating to increase cell survival?**
    Rock Inhibitor is preferred to use, but not essential.

17. **Why do I have to adjust my puromycin concentration daily?**
    The Simplicon™ VEE-OKSG-cMyc TagRFP 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


1. Seki, T., et al. (2010). Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell* ***7(1)*****:**11-14.
2. Warren, L., *et al*. (2010). Highly efficient reprogramming to pluripotency and direct differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* **7(5)**:618-630.
3. Yoshioka, N., et. al. (2013). Efficient Generation of human iPSCs by a synthetic self-replicative RNA. *Cell Stem Cell* **13(2)**:246-254.

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