User Manual
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Product Information

Simplicon® OKSG RNA
Simplicon® OKSG-cMyc RNA
Simplicon® OKSG-cMyc TagRFP RNA

SCR549, SCR550, SCR703, SCR714

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures. Not for human or animal consumption.

Product Overview

Many kinds of tools including viruses, DNA, RNA, mRNA, and protein, have been developed to generate integration-free induced pluripotent stem cells (iPSCs). Disadvantages to existing methods include: (1) low reprogramming efficiency (For example, DNA and protein), (2) a lengthy requirement for negative selection and subcloning steps to remove persistent traces of the virus (For example Sendai virus),[1] (3) daily transfections of cells using four synthetic mRNAs over 14 days (For example mRNA based),[2] and (4) the inability to directly assess transfection or viral transduction efficiency of the introduced reprogramming factors.

Simplicon® OKSG, OKSG-cMyc, and OKSG-cMyc TagRFP RNA use 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 Venezuelan equine encephalitis (VEE) RNA species that expresses the combination of reprogramming factors (RF) ORFs3,4 (Oct4, Klf4, Sox2, Glis1, and cMyc). The Simplicon® RNA replicon is a synthetic polycistronic VEE-RF RNA 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 (For example slower proliferating cells or aged somatic cells). The OKSG-cMyc TagRFP allows the visualization of RFP positive cells during the iPSC generation.

The B18R RNA (human codon optimized for B18R) is a synthetic mRNA that encodes vaccinia virus B18R protein. When it is co-transfected with the Simplicon® RNA, it efficiently suppresses the IFN responses. The B18R RNA (Cat. No. SCR723) and the B18R plasmid (Cat. No. SCR728) are also available individually to prepare the B18R conditioned medium that works as well as the recombinant B18R protein.

Advantages of the Simplicon® OKSG, OKSG-cMyc and 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).
- Only one single transfection is required. The RNA replicon is able to self-replicate, eliminating the need for additional daily transfections of multiple individual mRNAs over 14 days.
- Efficient and rapid reprogramming with OKSG-cMyc RNAs for aged somatic cells.
- No screening is 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.
- The TagRFP reporter gene in OKSG-cMyc TagRFP RNA allows visualization and quantification of the transfection efficiency.

Simplicon® OKSG, OKSG-cMyc, and OKSG-cMyc TagRFP RNA contain 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 rapidly expand under normal human ESC culture conditions.

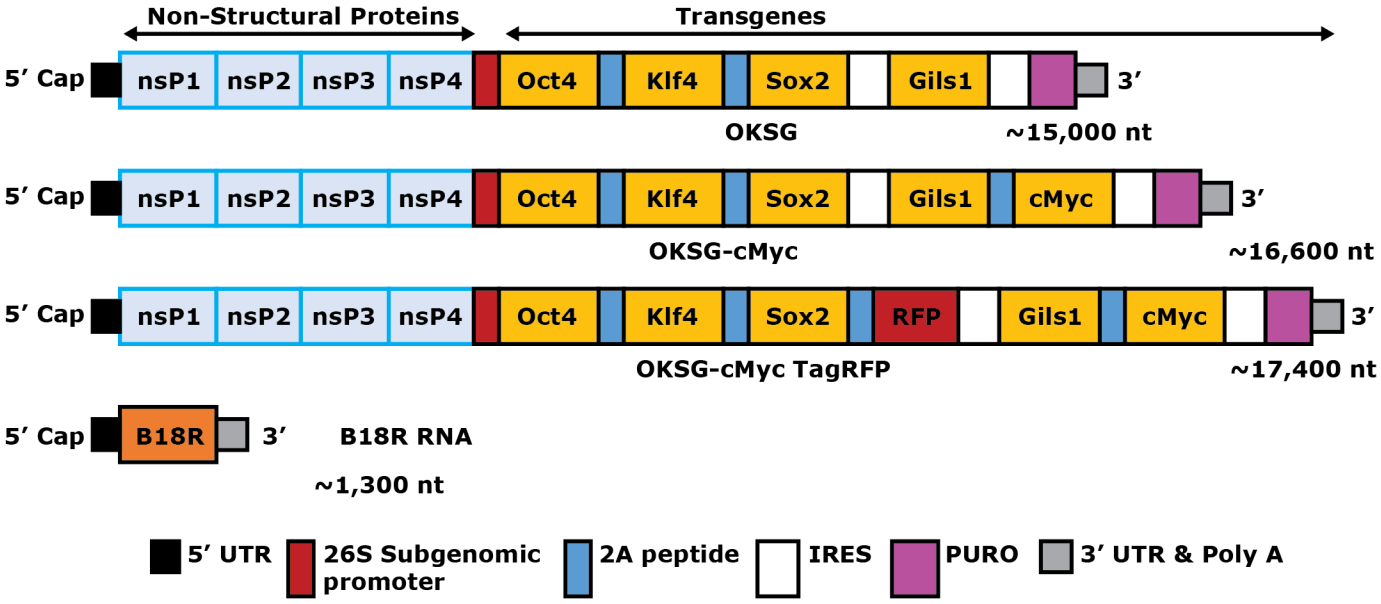


Figure 1. Structure of the Simplicon® OKSG, OKSG-cMyc, OKSG-cMyc TagRFP RNA replicons and B18R RNA. The RNA replicon encodes four non-structural replication complex proteins (nsPs) as a single ORF at the 5′ end of the RNA. At the 3′ end, the viral structural proteins ORFs are replaced with the OKSG, OKSG-cMyc, or OKSG-cMyc TagRFP transgenes. The B18R RNA is a synthetic mRNA that provides B18R protein derived from a vaccinia virus. B18R protein suppresses the IFN responses caused by RNA transfection. Locations of the 26S subgenomic promoter, 2A peptides, IRES and Puromycin (Puro)-resistance gene are indicated.

Materials Required (Not Included)

- **Vaccinia Virus B18R protein, recombinant expressed in HEK 293 cells, Carrier-Free** (Cat. No. GF197)
  One (1) vial containing 50 µg of 0.3-0.7 mg/mL stock of B18R protein. This protein has been shown to inhibit all IFN-α subtypes and IFN-β, but not type II or III IFNs. For best recovery, quick-spin the vial after thawing on ice before opening. Aliquot B18R protein into sterile, RNase-free tubes on ice and store at -80 °C. B18R protein must keep on ice in order to avoid degradation. Limit repeated freeze-thaw cycles. Use at 50-200 ng/mL.

  Note: SCR550 contains one vial of Human Recombinant B18R protein (GF156).
- **Human iPS Reprogramming Boost Supplement II** (Cat. No. SCM094)
  Stable for 4 months at -20 °C from the date of receipt. Upon the first thaw, aliquot into smaller working volumes and freeze at -20 °C. Upon adding 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.
- **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.

  Note: CSR550 contains Human iPS Reprogramming Boost Supplement II (Cat. No. SCM094).

Accessories

- Human Foreskin Fibroblasts (Cat. No. SCC058)
- EmbryoMax® DMEM (1x) (Cat. No. SLM-120-B), a DMEM High-Glucose Medium
- Advanced DMEM
- PluriSTEM® Human ES/iPS Cell Medium (Cat. No. SCM130) or PluriSTEM®-XF Human ES/iPS Cell Medium (Cat. No. SCM132)
- Mouse Embryonic Fibroblast (MEF) Conditioned Media
- Fetal Bovine Serum (Cat. No. ES-009-B)
- Ala-Gln (Cat. No. G8541), a stable glutamine
- Penicillin Streptomycin Solution (100X) (Cat. No. TMS-AB2-C)
- Recombinant Human FGF-2 (Cat. No. GF003)
- ROCK Inhibitor (Y-27632) (Cat. No. SCM075)
- Accutase™ cell detachment solution (Cat. No. SCR005)
- EmbryoMax® 1X Dulbecco's Phosphate-Buffered Saline w/o Ca++ or Mg++, 500 mL (Cat. No. BSS-1006-B)
- ECMatrix™-511 Silk E8 Laminin Substrate (Cat. No. CC161-1050UG)
- EZ-LiF T™ Stem Cell Passaging Reagent (Cat. No. SCM139), a superior reagent for cloning of iPSC colonies
- PMEF cells, growth-arrested, mitomycin-C treated (Cat. No. PMEF-CF)
- EmbryoMax® 0.1% Gelatin Solution (Cat. No. ES-006-B)
- Lipofectamine™ MessengerMAX™ Transfection Reagent
- Human iPS Selection Kit (Cat. No. SCR502)
- Nuclease-free, sterile microcentrifuge tubes
- Nuclease-free aerosol-barrier pipette tips

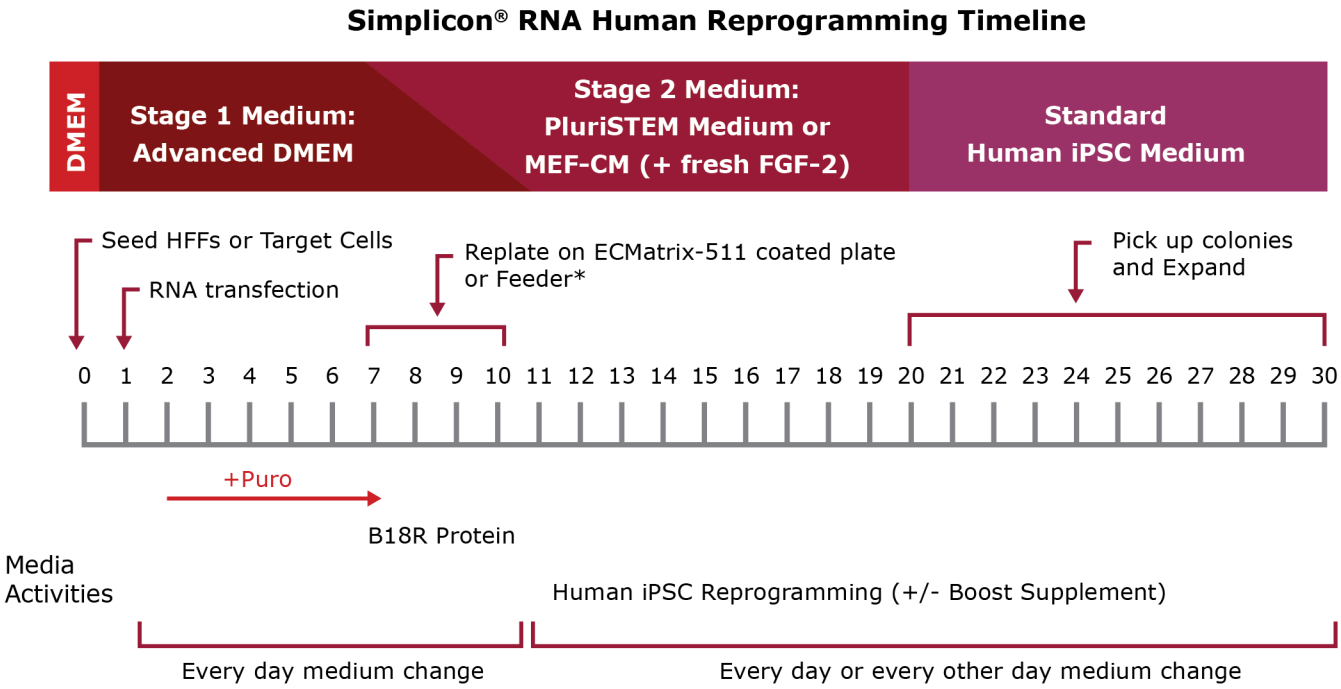
Storage and Stability

VEE-OKSG, OKSG-cMyc, OKSG-cMyc TagRFP and B18R RNAs: Stable at least for 4 months from the 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, RNase-free tubes on ice and store at -80 °C. Limit repeated freeze-thaw cycles. Use in a sterile RNase-free environment.

Protocol

Overview for iPSC Generation of Human Fibroblasts

The following protocol has been optimized using early passage Human Foreskin Fibroblasts (Cat. No. SCC058) and adult human fibroblasts such as BJ and should use as general guidelines. Modifications of the protocol may be needed to enable the optimized generation of iPSCs from other types of target 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, OKSG-cMyc, and OKSG-cMyc TagRFP RNAs. Key steps and media requirements are indicated. +Puro signifies the addition of Puromycin. Stage 1 Medium contains Advanced DMEM with 10% FBS and Ala/Gln (1x).

Note: From the time of transfection to replating, everyday media change is recommended. After replating, media changes can be performed every OTHER day.

Simple Protocol

Day 0	Plate cells to achieve 80-100% confluent on the next day.
Day 1	Co-transfection with Simplicon® 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 the 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 ECMatrix™-511 coated plate or feeder plate using a 2-6 split ratio. For example, one 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. The Boost Supplement is optional. After replating: Use Stage 2 Medium containing B18R protein (50-200 ng/mL). Change media every day. The Boost Supplement is optional.
Day 11-20	Change medium every other day until iPSC colonies are generated.
Day 21-30	Pick up colonies and expand.

Quick Transfection Protocol for One Well of 6-Well Plate

Transfection with MessengerMAX™. Transfection protocol is optimized for Simplicon® RNA.

Step 1	Wash cells once with DMEM (no serum and no antibiotics) and add 1 mL/well of DMEM (no serum and no antibiotics). Option: Depending on cell types, add 1-10% serum and B18R protein (200 ng/mL).		
Step 2	Prepare RNA mixture in Tube 1: Dilute RNAs in DMEM by pipetting. No vortex!		
	Total amount of RNAs (Simplicon® RNA & B18R RNA):	**1 µg**	**2 µg**
Tube 1	DMEM (no serum, no antibiotics)	10 µL	20 µL
	B18R RNA (1.0 µg/ µL)	0.5 µL	1 µL
	Simplicon® RNA (1.0 µg/ µL)	0.5 µL	1 µL
	Total volume:	**11 µL**	**22 µL**
Step 3	Prepare MessengerMAX™ dilution mixture in Tube 2 and quickly add into Tube 1. Incubation of the diluted MessengerMAX™ solution significantly decreases the transfection efficiency.		
Tube 2	DMEM (no serum, no antibiotics)	40 µL	80 µL
	MessengerMAX™ transfection reagent*	3 µL	6 µL
	Total volume	43 µL	86 µL
	Total RNAs amount in a tube after mixing:	**1 µg/54 µL**	**2 µg/108 µL**
Step 4	Incubate for 5-10 minutes at room temperature.		
Step 5	Add the RNA-transfection reagent complex dropwise into one well.		
Step 6	Incubate for 4-6 hrs, and then replace the medium to Stage I Medium containing B18R protein (200 ng/mL).		

*Increasing the transfection amount may increase the cytotoxicity. See Figure 3.

Option: The B18R-E3L RNA (Cat. No. SCR722) is available instead of B18R RNA. The B18R-E3L RNA can increase the transfection efficiency.

Critical Success Factors in Simplicon® RNA Reprogramming

The following guidelines are critical for ensuring success in reprogramming.

- RNAs are subject to degradation by RNase 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 the introduction of RNase to the Simplicon® RNAs. Use dedicated RNase-free tubes, aerosol resistant tips, and a clean environment for sample preparation.
- Appropriate reagents storage and handling. Components in the Simplicon® RNAs are temperature-sensitive and prone to degradation if left at room temperature for prolonged lengths of time. Thaw the RNAs on ice and aliquot into smaller volumes to minimize degradation caused by the repeated freeze-thaw cycles.
- B18R protein. During the self-replication of Simplicon® RNA, host cells produce IFNs to shut down the Simplicon® RNA. The B18R protein works for neutralizing IFN responses and allows the continuous replication of Simplicon® RNA. A continuous supplement of fresh B18R protein is essential.
- Puromycin selection. The self-replication of Simplicon® RNA is high in the beginning, and then eventually stabilizes at a low level in 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.

Preparation of Media and Reagents Protocol for Human iPSC Generation (Step-by-Step)

- DMEM High-Glucose Medium: No serum and no antibiotics.
- Stage 1 medium
  Advanced DMEM with 1x Ala/Gln (stable glutamine), 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 fresh at each use.
- Stage 2 medium
  Thaw the PluriSTEM® Human ES/iPS Cell Medium (Cat. No. SCM130 or 132) or MEF-CM 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 for both media. The PluriSTEM® medium contains the FGF-2 but additional FGF-2 is recommended for iPSC generation.

  Option: Add Human iPS Reprogramming Boost Supplement II and 1x Penicillin/Streptomycin if required. Sterile the medium using a 0.22 µm filter (Cat. No. SCGPU01RE) when Human iPS Reprogramming Boost Supplement II is added. The medium after filtration is suitable for two weeks at 4 °C.

1. Make aliquots of reagents upon first time thawing to minimize the repeated freeze-thaw cycles.
2. Simplicon® RNA
   Aliquot 2-3 µL into RNase-free tubes. Store aliquots at -80 °C.
3. B18R RNA
   Aliquot 2-3 µL into sterile nuclease-free Eppendorf® tubes. Store aliquots at -80 °C.
4. B18R protein (not provided, available separately)
   Aliquoting is recommended to escape the repeated freeze thaw cycles. Store aliquots at -20 °C for a few months. For long term storage, store at -80 °C.

iPSCs Generation with Human Fibroblasts

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
2. Day 0: Plate target cells
3. Plate target cells at the optimal plating density (80-100% confluency) in the same culture medium used to maintain target cells. Set aside an untransfected control well to observe the Puromycin cell death.

Option: Pre-treatment with B18R Protein

This step is optional. Pre-treatment with B18R protein (Cat. No. GF197 or GF156) will support the inhibition of interferon responses that occur by the RNA transfection. When cells were co-transfected with Simplicon® RNA and B18R RNA, this step can omit in most of cell types. When cells were transfected with Simplicon® RNA only (no B18R RNA), pre-treatment with B18R protein is essential.

Day 1: Pretreat cells with B18R protein

1. Thaw an aliquot of B18R protein on ice: For best recovery, quick-spin the vial before opening.
2. Prepare a 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 (0.3~0.7 mg/mL)	0.3-0.7 µL	200 ng/mL	GF197
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 the serum dependency of the target cells, it is possible to perform the transfection in the presence of 1-10% serum.

3. Mix gently by pipetting up and down.
4. Aspirate the medium and wash cells once with 2 mL DMEM (no serum and no antibiotics).
5. Add 1 mL per well of the DMEM medium containing 200 ng/mL B18R protein.
6. Place the plate in a 37 °C, 5% CO_2 incubator. Incubate for 10-20 minutes.

Co-Transfection with Simplicon® RNA and B18R RNA

Day 1: Transfect with Simplicon® RNA

Transfection of Simplicon® RNAs has been validated using the Lipofectamine™ MessengerMAX™ Transfection Reagent with modified transfection protocol. Follow the modified transfection protocol for the Simplicon® RNA transfection. Depending on the target cells, amounts of RNAs and MessengerMAX™ reagents can be changed. Use RNase-free, aerosol-barrier pipette tips and sterile, RNase-free tubes.

1. Aspirate the medium and wash cells once with 2 mL DMEM (no serum and no antibiotics). When B18R protein is pretreated, use the pre-treatment medium containing B18R protein without medium change.
2. Add 1 mL per well of the DMEM medium (no serum and no antibiotics).

   Note: Transfection efficiency will be maximized if the medium does not contain serum and antibiotics. However, depending on the serum dependency of the target cells, it is possible to perform the transfection in the presence of 1-10% serum.
3. Thaw an aliquot of the Simplicon® RNA and B18R RNA on ice; quickly centrifuge the vial(s) to spin down the contents. Keep RNA vials on ice.
4. Set up the following reactions in 1.5 mL tubes. Store any unused Simplicon® RNA at -80 °C. Mix gently by pipetting.

Total amount of RNAs (Simplicon® RNA & B18R RNA):		**1 µg**	**2 µg**
Step 1	Prepare RNAs dilution mixture in Tube 1.		
Tube 1	DMEM high glucose (no serum, no antibiotics)	10 µL	20 µL
	B18R RNA (1.0 µg/µL)	0.5 µL	1 µL
	Simplicon® RNA (1.0 µg/µL)	0.5 µL	1 µL
	Total volume:	**11 µL**	**22 µL**
Step 2	Prepare MessengerMAX™ dilution mixture in Tube 2 and quickly add into Tube 1. Incubation of the diluted MessengerMAX™ solution significantly decreases the transfection efficiency.		
Tube 2	DMEM high glucose (no serum, no antibiotics)	40 µL	80 µL
	MessengerMAX™ transfection reagent*	3 µL	6 µL
	Total volume:	**43 µL**	**86 µL**
	Total RNAs amount in a tube after mixing:	**1 µg/54 µL**	**2 µg/108 µL**

*Increasing the transfection amount may increase the cytotoxicity. See Figure 3.

Option: The B18R-E3L RNA (Cat. No. SCR722) is available instead of B18R RNA. The B18R-E3L RNA can increase the transfection efficiency.

5. Incubate the RNA transfection reagent complex at room temperature for 5-10 minutes.
6. Add the RNA-transfection reagent complex dropwise into one well of the 6-well plate containing target cells.
7. Gently rock the plate from side to side to thoroughly mix and apply the RNA complexes onto the target cells.
8. Incubate the plate in a 37 °C, 5% CO_2 incubator for 4-6 hrs.
9. **Option:** When medium contains 2% serum, overnight incubation is possible in fibroblasts.
10. Prepare the Stage 1 Medium containing B18R protein.
11. During the 4-6 hrs RNA transfection period, prepare Stage 1 Medium containing 200 ng/mL B18R protein (see below).

    Option: This step can omit in most human fibroblasts when cells were co-transfected with Simplicon® RNA and B18R RNA.

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 and Reagents for formulation)	2 mL	
B18R protein (0.3-0.7 mg/mL)	0.6-1.4 µL	200 ng/mL
Total Volume:	**~ 2 mL**	

1. Once the 4-6 hrs RNA transfection is completed, aspirate the transfection medium.
2. Add 2 mL per well of Stage 1 Medium or Stage 1 Medium containing B18R protein.
3. Place the plate containing the transfected cells overnight in a 37 °C, 5% CO_2 incubator.

Start Puromycin Selection

1. Aspirate the medium. Replace with 2 mL of Stage 1 Medium containing 200 ng/mL B18R protein and 0.5 µg/mL Puromycin (see Preparation of Media and Reagents for formulation).
2. Note: The optimal starting Puromycin concentration may vary among cell types. 0.5 µg/mL Puromycin works in most of human fibroblasts. For highly sensitive cells, start at 0.1 µg/mL Puromycin.

   Note: When high cytotoxicity is observed after the transfection, replace with a medium that does not contain Puromycin (add the B18R protein only), and wait one day more to start the Puromycin selection.
3. Monitor every day to assess the status of Puromycin selection. Replace with 2 mL fresh Stage 1 Medium containing 200 ng/mL B18R protein and Puromycin every day.

Days 5-10: Adjust Puromycin concentration if necessary

4. When mock-transfected cells are dead by Puromycin selection, while 30~80% transfected cells are survived, stay at 0.5 µg/mL Puromycin. If most of mock-transfected cells are survived, increase the Puromycin up to 0.8-1.0 µg/mL until the mock-transfected cells are dead.
5. When 80-90% transfected cells are dead, withdraw Puromycin. Keep culture for several days without Puromycin. See FAQ 11.
6. Replace with 2 mL fresh Stage 1 Medium containing 200 ng/mL B18R protein with or without Puromycin depending on selection condition, every day.
7. By days 7-10, cells should start to grow back and begin to proliferate. Replace with fresh Stage 1 Medium containing 200 ng/mL B18R protein with or without Puromycin daily until cells are approximately 70-100% confluent.
8. 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%.
9. Prepare ECMatrix™-511 (aka iMatrix-511) coated well or feeder cells.

One day before replating

For feeder-free culture: Prepare ECMatrix™-511 Silk E8 Laminin Substrate (Cat. No. CC161-1050UG) coated 6-well plates.

1. Dilute the ECMatrix™-511 Silk with PBS (-) at 2.5 µg/mL.
2. Add 1.5-2 mL of diluted ECMatrix™-511 Silk to each well. Incubate overnight at 4 °C.

   Note: ECMatrix™-511 Silk 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.

1. 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 allow feeder cells to remain adherent for up to 2-3 weeks.
2. Makeup 50 mL MEF Culture 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**		

3. Aspirate the 0.1% gelatin-coating solution from each well and add 1 mL MEF Culture Medium. Do not allow the plates to dry.
4. Thaw the inactivated MEFs (Cat. No. PMEF-CF) and resuspend in MEF culture Medium. Count the number of thawed MEFs and seed 4 x 10^5 cells (~ 1 mL) per well of a 6-well dish. Incubate overnight in a 37 °C, 5% CO_2 incubator.

Replating: When Puromycin-resistant cells are 70-100% confluent

When Puromycin-resistant cells become 70-100% confluent, they can be replated onto ECMatrix™-511 Silk coated plates for feeder-free culture or inactivated MEF feeder layer. This may occur anytime between days 7-10, depending on the status of Puromycin selection.

Note: If Puromycin-resistant cells do not reach 70-100% confluency by day 10, replate all cells into a fresh well without further cell dilution.

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

   Note: One confluent well may be replated into 2-8 wells. As reprogramming efficiency may vary between cell types, it is recommended that users set up different 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 (0.3-0.7 mg/mL)	2-10 µL	50-200 ng/mL	GF197
Rock inhibitor (Y-27632) (10 mM)	15 µL	10 µM	SCM075
Total Volume:	**~15 mL**		

For feeder-free cultures: Before seeding the cells, bring the ECMatrix™-511 Silk coated plates back to room temperature, remove the coating solution, and replace it with 2 mL per well of Replating Medium containing B18R protein and Rock inhibitor. Do not allow the plates to dry. Set the 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 and replace with **2 mL** per well of Replating Medium containing B18R protein and Rock inhibitor. Set the plate aside until ready to receive the RNA-transfected cells.

1. Aspirate the medium from the 6-well plate containing the RNA-transfected cells. Wash once with 2 mL of 1X PBS per well. Aspirate.
2. Add 0.5 mL of Accutase™ solution per well and incubate for 3-5 minutes at 37 °C to dissociate the cells. Ensure the detachment of cells under the microscope.
3. Add 1-2 mL of Replating medium and resuspend well. Split into 2-8 wells of ECMatrix™-511 Silk coated or MEF feeder plate.

   Option: Count the cell number using a hemocytometer and seed approximately 5 x 10^4 to 1 x10^5 of the RNA-transfected cells onto one well of the 6-well plate.

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

Replace Stage 1 medium to Stage 2 Medium

After replating and during the period before colonies emerge (Day 8-Day 20):

1. Thaw PluriSTEM® Human ES/iPS Cell Medium (Cat. No. SCM130 or 132) or MEF-CM at room temperature, and store at 4 °C until use.
2. Prepare Stage 2 Medium containing B18R protein (50-200 ng/mL) and FGF-2 (10 ng/mL). Do not warm up at 37 °C. It is possible to change the 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 14.

   Option: 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 suitable for up to 2 weeks at 4 °C.

   Note: It is recommended to add B18R protein and FGF-2 fresh at each use. The PluriSTEM® medium contains the FGF-2 but additinal FGF-2 is recommended for iPSC generation.

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

Component	Quantity	Final Conc.	Cat. No.
PluriSTEM® Human ES/iPS Cell Medium or MEF-CM	18 mL		SCM130 or SCM132
FGF-2 (reconstitute to stock concentration 50 µg/mL)	3.6 µL	10 ng/mL	GF003
B18R protein (0.3-0.7 mg/mL)	2-15 µL	50-200 ng/mL	GF197
	Total Volume:	**~ 18 mL**	

When small iPSC colonies start to emerge (Day 14-Day 20)

3. 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.

Isolation of iPSC colonies and expansion of iPSCs

When colonies are ready to be isolated and expanded (Day 21-Day 30)

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 4H-4J).

Note: Monitor the culture daily. Colonies may become large enough to be manually passaged anytime between Day 21-Day 30; do not let the culture overgrow, which can induce differentiation.

Option: Perform live-cell staining using the Human iPS Selection Kit (SCR502) to select the Tra-1-60+ SSEA4+ colonies.

One day before picking the iPSC colonies

For feeder-free expansion: Prepare ECMatrix™-511 Silk coated well as described in step 5. 24-6-well plate size will work.

For feeder-based expansion: Prepare inactivated MEFs as described in step 5. 24-6-well plate size will work.

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 the medium. Simplicon® RNA will become undetectable with several passages in B18R free medium.

For feeder-free expansion: Aspirate the coating mixture from the ECMatrix™-511 Silk coated well plate. Add Stage 2 Medium containing 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 plate containing inactivated MEFs plated from the day before (from step 8a). Aspirate and add the Stage 2 Medium (MEF-CM) 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.

1. Pick up iPSC colonies. Using a P200 Pipetman® that has been set to 30 µL volume, pick up colonies under a dissecting microscope, and transfer into a new well coated with the ECMatrix™-511 Silk or MEF feeders. Alternatively, the well can be treated with the EZ-LiFT™ (Cat. No. SCM139) for 5-10 min and then pick up iPSC colonies. The treatment with EZ-LiFT™ will help the viability of iPSCs after picking up and/or cloning.

   Note: It is recommended to pick at least 6-8 distinct iPSC colonies for expansion and further characterization.
2. Place the plate in a 37 °C, 5% CO_2 incubator for two days without any media exchanges.
3. DO NOT EXCHANGE MEDIA on the day following passaging.
4. On the 2nd day after manual passaging, exchange with Stage 2 Medium WITHOUT B18R protein to each well.
5. After 3-4 days, the culture medium may be replaced with any Human ESC medium generally used in the lab (For example PluriSTEM®, mTeSR1, KOSR-based medium). Replace daily with 3 mL (for 6-well plates) or 0.5 mL (for 24-well plates) fresh Human ESC Medium. For the first 1-2 passages, colonies may require a longer time to be ready for passage. Monitor iPSC colony formation every day to determine the optimal time for the next passage. By the 3rd passage, iPSCs can be cultured similarly to human ESCs and adapted to other proven ESC/iPSC culture media. The EZ-LiFT™ Stem Cell Passaging Reagent (Cat. No. SCM139) is recommended for the first 5 passages. After the 5th passage, enzymatic solutions (For example, Accutase™) can use to harvest and expand the cells.

Data Analysis

Representative Results

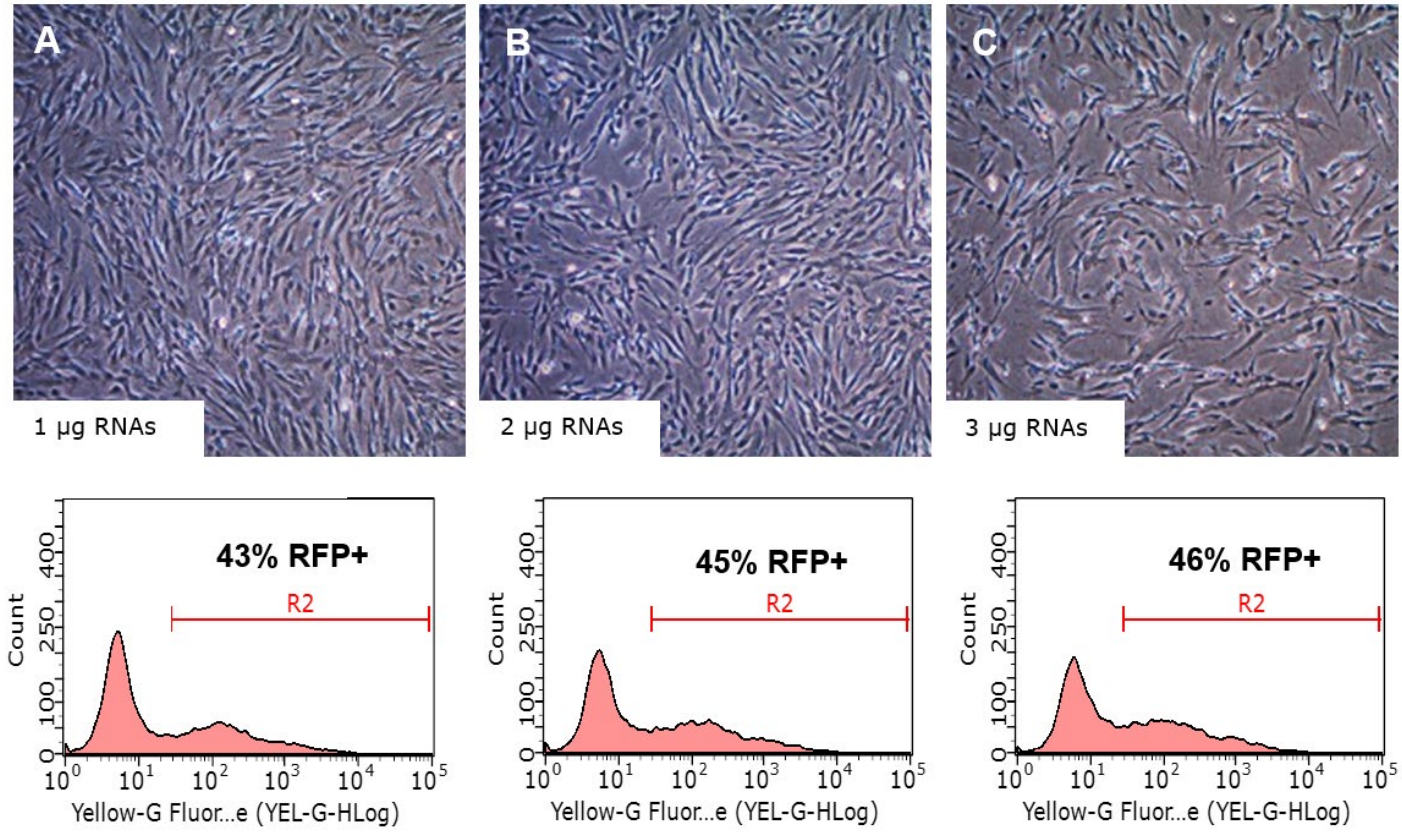


Figure 3. Effects of increasing transfected RNA concentrations on cell viability & cytotoxicity. For each condition, equal concentrations of Simplicon® RNA and B18R RNA were co-transfected (For example, 0.5 µg Simplicon® + 0.5 µg B18R RNA = 1 µg RNA) with 5 µL MessengerMAX™ Reagent. Brightfield images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

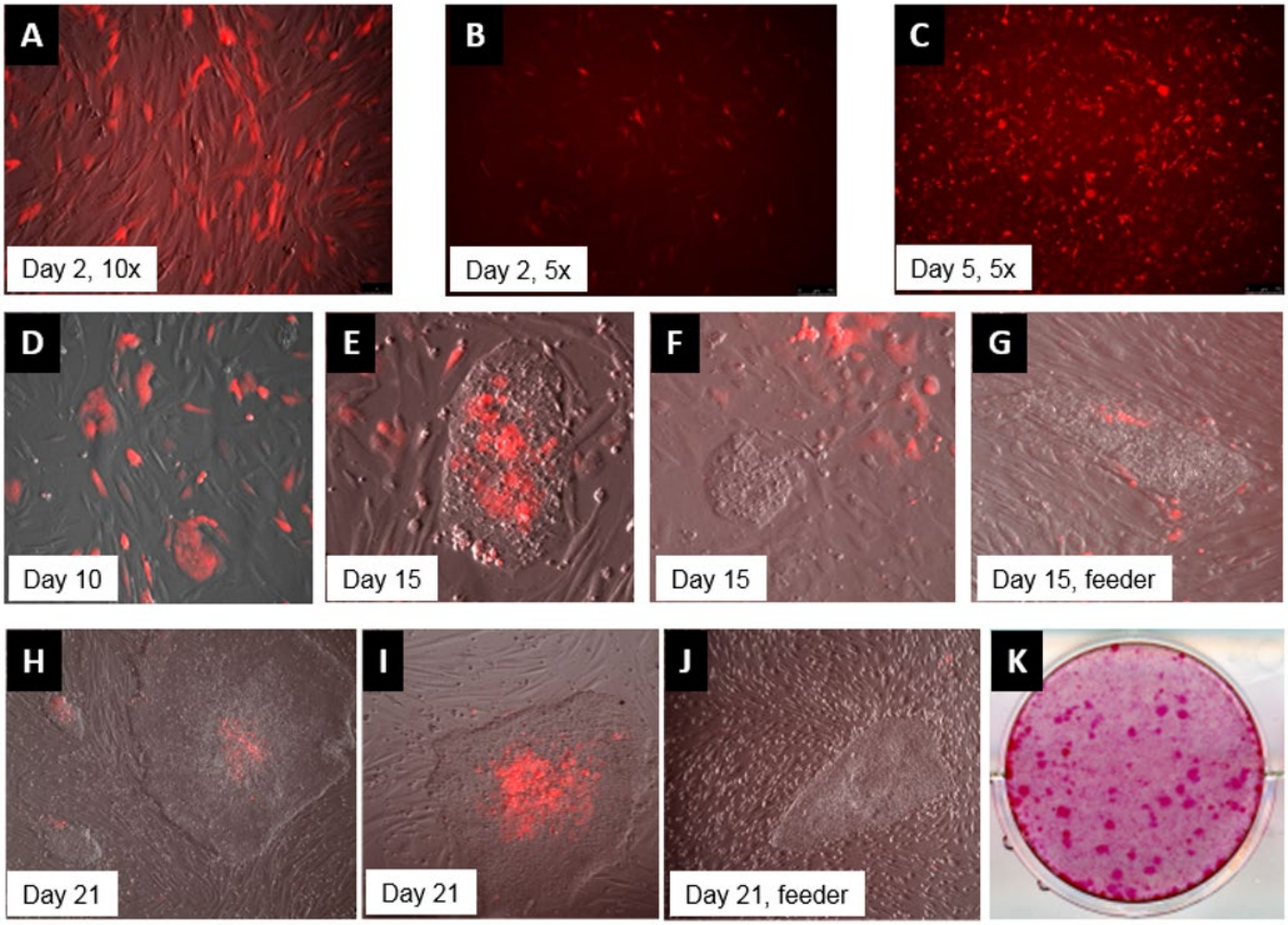


Figure 4. Time course of human iPSC colonies generated using Human Simplicon® OKSG-cMyc TagRFP RNA (Cat. No. SCR714). BJ human foreskin fibroblasts were transfected with Simplicon®-OKSG-cMyc TagRFP and B18R RNAs. One day after transfection (Day 2), approximately 37% cells are RFP positive (A, B) and 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).

Troubleshooting

Frequently Asked Questions (FAQ)

How do I determine the optimal plating cell density for my cell type of interest?

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

How do I determine the optimal Puromycin concentration to start with?

Set up the Puromycin concentration that works in 4 days treatment, not 2 days treatment. Most of human fibroblasts work at 0.5 µg/mL Puromycin. We recommend the test to evaluate the Puromycin sensitivity for other types of cells before starting the experiment.

How much RNAs 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 one reprogramming reaction in a 6-well plate. Scale up and down accordingly based on the number of reprogramming reactions and plate size being performed.

Do I need to transfect in B18R RNA even though the B18R protein is already included?

Co-transfection with Simplicon® RNA and B18R RNA worked good with many types of cells in our experience. The pre-treatment with B18R protein will work, but the protein expression is less than the co-transfection method.

Will the TagRFP fluorescent signal stay on throughout the reprogramming process?

No, TagRFP is used to monitor the transfection efficiency of the Simplicon® OKSG-cMyc TagRFP. The TagRFP fluorescence will be downregulated during the iPSC generation. Wide variations in TagRFP fluorescence may be observed between different hiPSC colonies. In our experiences with human fibroblasts iPSC reprogramming, TagRFP fluorescence was easily detectable by ~ day 10, while most typical iPSC colonies have lost TagRFP fluorescence by day 21. Some colonies may have all cells within the colony expressing TagRFP, while other colonies may have only some cells expressing the TagRFP (Figure 4E, H, I) or not at all (Fig. 4F, G, J).

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. The Boost supplements enhace the efficiency of iPSC generation in fibroblasts but it will be varied in cell types.

Can I transfect the RNAs more than once? Will it improve my reprogramming efficiency?

Based upon our experience with human foreskin fibroblasts, we observed 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.

How long will the RNA self-replicate in the cells?

Based on PCR data, the RNA is no longer present at P4.

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.

How long can the media be stored at 2-8 °C?

3-day usage is maximal for B18R protein containing media. Two weeks is maximal for the Human iPS Reprogramming Boost Supplement II containing media.

After the application of Puromycin, my cells are dying. What should I do?

- Puromycin concentration is too high.
    - If > 80-90% cell death was observed around Day 4-5, Puromycin should be immediately withdrawn from the medium. Wait for cells to grow back to 70-90% confluency and then do the replating. If morphological change is observed, there is a chance to get iPSCs after replating.
    - Repeat the experiment with a lower concentration of Puromycin such as 0.1 µg/mL Puromycin. Determine the optimal Puromycin concentration that works in 4 days selection.
- Transfection efficiency is no good.
    - The Simplicon® RNA transfection works well in early passaged fibroblasts but it decreased in late passaged fibroblasts. Use early passaged cells as possible.
    - Follow the Simplicon® RNA transfection protocol.
    - Simplicon® RNA transfection protocol is different from the manufacturing protocol described in the MessengerMax™ transfection reagent. Follow our protocol for the Simplicon® RNA transfection.
    - Try the different lot number of MessengerMax™ transfection reagent.
    - In our experience, some lot numbers showed the low transfection efficiency. Try the different lot number. Note that the MessengerMax™ reagent should be stored at 4 °C or room temperature during the preparation of transfection complex. Put it on ice is not recommended.
    - The Simplicon® RNA transfection worked in most of the tested cells but keratinocytes are difficult. We recommend the early passage (P0 to P2) for adult keratinocytes and 3 to 4 passages for neonatal keratinocytes for the Simplicon® RNA transfection (no transfection observed in P1 neonatal keratinocytes).
    - The B18R-E3L RNA (Cat. No. SCR722) is available for maximizing the transfection efficiency. Use the B18R-E3L RNA instead of the B18R RNA minimizes the IFN responses and increases the transfection efficiency.

After the application of Puromycin, I do not see any cell death. What should I do? Should I increase the Puromycin concentration?

When transfection efficiency 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, it may need to increase the Puromycin concentration incrementally by 0.5X. Observe the cell's response daily.

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 is OK to replate all cells into a fresh well (ECMatrix™-511 Silk coated well or feeder) without cell dilution when cells are ~ 20% confluency. When cells are below ~ 20% confluency, it may try to exchange with fresh Stage 2 Medium with B18R protein at day 10 and wait a few days for replating until cells are ~ 20% confluent.

Do I need to replate my transfected cells to MEF feeder layer?

Transfected cells could be replated on MEF feeder layer or ECMatrix™-511 Silk coated well in Stage 2 Medium with B18R protein. B18R protein may be withdrawn when tiny iPSC colonies start to emerge.

Do I need to change the medium every day?

In the early 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.

Should I use Rock Inhibitor during my replating to increase cell survival?

Rock Inhibitor is preferred to use, but not essential.

References

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3. Yoshioka, N., et. al. (2013). Efficient Generation of human iPSCs by a synthetic self-replicative RNA. Cell Stem Cell 13(2):246-254.
4. Yoshioka, N and Dowdy SF. (2015). Enhanced generation of iPSCs from older adult human cells by a synthetic five-factor self-replicative RNA. PLoSONE 12(7): e0182018. https://doi.org/10.1371/journal.pone.0182018.

Product Ordering

Purchase products online at SigmaAldrich.com.

Description	Catalogue Number
Simplicon® OKSG RNA Components	
• VEE-OKS-iG RNA Part No. CS210583 One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80 °C. • B18R RNA Part No. CS224516 One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80 °C	SCR549 SCR550
Simplicon® OKSG-cMyc RNA Components	
• VEE-OKSG-cMyc RNA Part No. CS 221303 One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80 °C. • B18R RN Part No. CS224516 One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80 °C.	SCR703
Simplicon® OKSG-cMyc TagRFP RNA Components	
• VEE-OKSG-cMyc TagRFP RNA Part No. CS222750 One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80 °C. • B18R RNA Part No. CS224516 One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80 °C	SCR714M

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