Data Sheet

Simplicon® TagGFP2 RNA Kit

Stem Cell Kit

SCR713

Pack Size 1 kit

Store at -80 °C

FOR RESEARCH USE ONLY

Not for use in diagnostic procedures. Not for Human or Animal Consumption.

Background

Viral and DNA vector-based technologies are used to deliver nucleic acid payloads for targeted gene expressions. Strategies employing retro-, lenti-viral and DNA vectors are able to achieve sustained protein expressions, however their utility is limited by safety concerns associated with insertional mutagenesis. Non-integrative viral and episomal DNA vectors have very low to no risk of integrations but are hampered by the short transient expression of the target gene(s).

Simplicon® is a novel system to effect immediate high sustained protein expression of the target gene(s) without the risk of integration. The technology employs a self-replicating RNA based on the Venezuelan equine encephalitis (VEE) genome.[1] The Simplicon® RNA contains only genes encoding the VEE RNA replication machinery while the structural proteins that are required to make an infectious particle have been completely removed and replaced with the transgene(s) of interest. Because Simplicon® is a synthetic RNA, there is no risk of the transgene(s) integrating into the transfected cell's genome. Moreover, the self-replicating nature of the Simplicon® means that the target protein is continuously expressed in the presence of B18R protein and the selective agent, Puromycin. The Simplicon® technology has been successfully utilized for efficient human iPSC generation through the sustained expression of critical reprogramming factors, OKSG and OKSG-cMyc.[1]

The Simplicon® TagGFP2 RNA Kit was developed to enable the evaluation of the Simplicon® expression system in targeted cell(s) before ordering a custom Simplicon® RNA. Simplicon® TagGFP2 RNA Kit may be used to determine optimal transfection conditions to express the self-replicating RNA in hard-to transfect somatic or primary cells. Simplicon® TagGFP2 expresses an improved variant of the Aequorea macrodactyla GFP-like protein. TagGFP2 exhibits bright green fluorescence comparable to that of EGFP, with excitation/emission maxima at 483 and 506 nm, respectively.

Simplicon® TagGFP2 RNA can maintain GFP expression up to one month in human cells (see Figure 2) when optimal amounts of Puromycin and B18R protein are used. B18R protein is essential to neutralize interferon responses that occur during the self-replication of Simplicon® RNA.

Note: The Simplicon® expression system works primarily in human cells and is not expected to work in mouse cells. This is due to the fact that B18R does not effectively neutralize mouse interferon (IFN)-β.

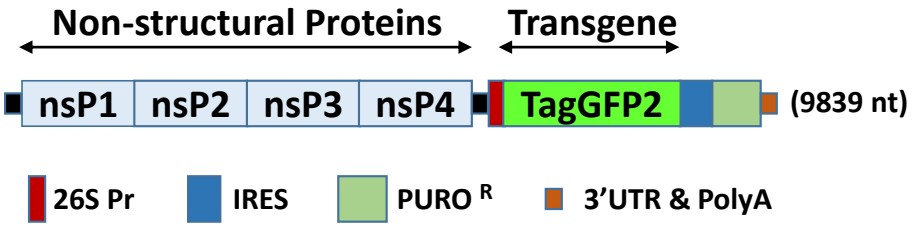

Figure 1. Structure of the Simplicon® TagGFP2 RNA replicon. The RNA replicon encodes four non-structural replication complex proteins (nsPs) as a single ORF at the 5' end of the RNA. At the 3' end, the viral structural proteins ORFs are replaced with the TagGFP2 transgene. Locations of the 26S internal promoter (Pr), 2A peptides, IRES and Puromycin (Puro)-resistance gene are indicated.

Storage & Stability of Kit Components

- TagGFP2 RNA: (Cat. No. CS222751) One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80 °C.
- B18R RNA: (Cat No. CS224516) One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80 °C.

For best recovery, quick-spin the vials after thawing on ice. Do not vortex. Aliquot into sterile, nuclease-free Eppendorf® tubes on ice and store at -80 °C. Limit repeated freeze-thaw cycles.

Quality Control

- Discrete RNA band on RNA gel of appropriate size.
- GFP-positive cells observed one day after transfection.
- 40-60% GFP-positive BJ human fibroblasts with FACS analysis.

Forward Transfection Protocol

Tranfsection of Simplicon® RNAs has been validated using the RiboJuice™ mRNA Transfection Kit and Lipofectamine® MessengerMAX™ Transfection Reagent. Amounts of RNAs and transfection reagents required may vary depending on the target cells. Set up different RNA: transfection reagent ratios.

1. Plate target cells to reach 50-90% confluency at time of transfection. Set aside an untransfected control well to observe the Puromycin cell death. Sensitivity to Puromycin may vary with different cell types.
2. Wash cells once with DMEM (no serum, no antibiotics) and add 1 mL/well of DMEM (no serum, no antibiotics) containing 200 ng/mL B18R protein (Cat. No. GF156). No serum condition increases the transfection efficiency. However, it is possible to use 1-10% serum depending upon cell types. Incubate cells in a 37 °C, 5% CO_2 incubator (10-20 minutes).
3. Set up transfection reactions in sterile Eppendorf® tubes. Follow order of additions. Mix gently by pipetting during each addition of RNA and reagent. **Do not vortex**.

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

Component	**Vial**	**Cat. No.**
Opti-MEM®	250 µL	Thermo (31985-062)
B18R RNA (1 µg/µL)	0.5 µL	
TagGFP RNA (1 µg/µL)	0.5 µL	
RiboJuice™ mRNA Boost Reagent	4.0 µL	RiboJuice™ mRNA Transfection (TR-1013)
RiboJuice™ mRNA Transfection Reagent	4.0 µL	
Total Volume	**259 µL**	

If using MessengerMAX™ Transfection Reagent (ThermoFisher LMRNA001)

Total RNA Amount		1 µg	2 µg
Step1	Prepare RNA mixture in Tube 1.		
Tube 1	DMEM (no serum, no antibiotics)	50 µL	100 µL
	B18R RNA (1 µg/µL)	0.5 µL	1 µL
	TagGFP RNA (1 µg/L)	0.5 µL	1 µL
	Total volume	51 µL	102 µL
Step 2	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	53–55 µL	106-110 µL
Step 3	Quickly add tube 2 into tube 1.		
Total RNAs amount in a tube		1 µg /104-106 µL	2 µg /208-212 µL

4. Incubate at room temperature for 5 minutes and add the RNA-transfection reagent complex dropwise into one well of the 6-well plate containing cells.
5. Incubate the plate in a 37 °C, 5% CO_2 incubator for 2-4 hrs. Maximum transfection efficiency is obtained with 4 hours incubation using MessengerMAX™ reagent in human fibroblasts.
6. Aspirate the transfection medium and add 2 mL per well of culture medium containing 200 ng/mL B18R protein. Incubate in a 37 °C, 5% CO_2 incubator overnight.
7. Next day, aspirate and exchange with fresh culture medium containing 200 ng/mL B18R protein and Puromycin (0.25-1 µg/mL). B18R protein and Puromycin should be added fresh each time. Puromycin selection is used to remove cells that have not taken up the Simplicon® RNA. Sensitivity to Puromycin may vary with different cell types and must be determined empirically.
8. Change medium every day. Add fresh 200 ng/mL B18R protein and Puromycin with each media change. In general Puromycin selection works in 5 days. For long term expression of Simplicon® TagGFP, after a week, it is possible to transition to media changes every other day and also to reduce the amounts of Puromycin (0.1-0.5 µg/mL) and B18R protein (50-200 ng/mL).
9. Analyze and quantify the percentage of GFP-positive cells using FACS.

Note: Some IFNs may not be neutralized by B18R protein and will accumulate in the medium. Cell passaging will remove IFNs more efficiently as compared to media changes and will also help with the long-term expression of the Simplicon® RNA.

Reverse Transfection Protocol

For some cells (for example HepG2) reverse transfection may be more efficient.

1. Prepare target cells to reach 80-100% confluent at time of transfection.

On day of transfection

2. Detach cells with cell detachment solution such as AccuMax, Accutase™ or Trypsin/EDTA to make a single cell suspension. Collect cells in regular cell culture medium.

3. Briefly centrifuge to pellet the cells. Aspirate medium. Resuspend cells in normal culture medium containing 2% serum and 200 ng/mL B18R protein (no antibiotics) and transfer cells to new well to achieve 50-100% the next day.

Note: The percentage of serum in the resuspension medium is dependent on cell types. In general, low serum condition will increase the transfection efficiency.

4. Place newly plated cells in 37 °C, 5% CO_2 incubator while you prepare the RNA transfection mixture as previously outlined in step 3 of the Forward Transfection Protocol.
5. Follow Steps 4-10 of the Forward Transfection Protocol.

Representative Data

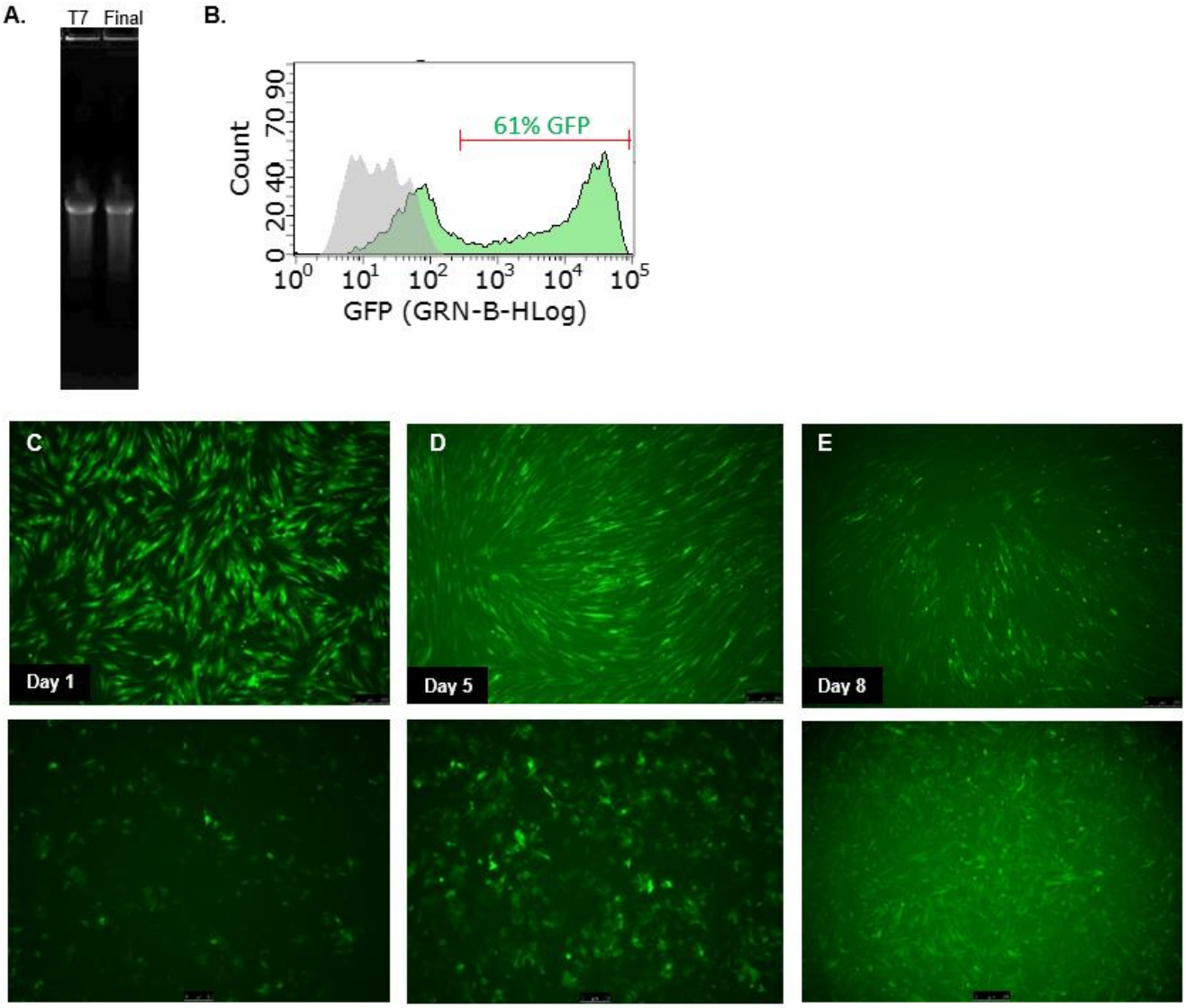

Figure 2. RNA gel of Simplicon® TagGFP2 RNA after the T7 RNA transcription reaction (T7) and after the 5' capping and phosphatase reactions (Final) (**A**). RNA integrity was maintained throughout the RNA manufacturing process. Day 1 (**B, C**), day 5 (**D**) and day 8 (**E**) transfection of BJ human foreskin fibroblasts with 0.2 µg Simplicon® TagGFP2 and 0.2 µg B18R RNA (1:1 ratio) in a 24 well plate using MessengerMAX™ transfection reagent. B18R protein was not added at any time. Day 12 (**F**), day 29 (**G**) transfection of HepG2 cells, and day 29 (**H**) transfection of BJ fibroblasts using similar transfection conditions as in C-E, but in the presence of B18R protein and Puromycin.

References

1. Yoshioka, N., et. al. (2013). Efficient Generation of human iPSCs by a synthetic self-replicative RNA. Cell Stem Cell **13(2)**:246-254.
2. Mertzlyak, E.M. et al. Bright monomeric red fluorescent protein with an extended fluorescence lifetime. Nat. Methods 2007; 4: 555-557.
3. Subach O.M. et al. Conversion of red fluorescent protein into a bright blue probe. Chem. Biol. 2008; 15: 1116-1124.

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