TagGFP2 Simplicon™ RNA (E3L) Kit

Synthetic RNA

Cat. # SCR720

Pack size: 10 µg

Store at -80 ºC

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.
NOT FOR HUMAN OR ANIMAL CONSUMPTION.

Data Sheet

Background

Simplicon™ is a novel system to effect immediate high sustained protein expression of multiple genes into transfected cells without the risk of genome integration. The technology employs a single, synthetic, polycistronic, self-replicating RNA based on the Venezuelan equine encephalitis (VEE) genome[1,2,3]. 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 removed and replaced with the transgenes of interest.

Introduction and replication of the Simplicon™ RNA is expected to elicit a strong interferon response in transfected cells. To suppress the IFN responses, a Vaccinia virus protein[4], B18R, is used for the original Simplicon™ technology. Recently, we found that another Vaccinia virus protein[4], E3L, also suppresses the IFN responses in Simplicon™ RNA expression. B18R neutralizes type I interferons by direct binding, while E3L inhibits the cytoplasmic signaling pathways of IFN responses. Therefore, B18R and E3L are both employed in the Simplicon™ Expression System and work collaboratively to suppress IFN responses. As a result, there is increased cell viability during RNA transfection and increased expression of the transgenes. The Simplicon™ Expression System works in human cells and is not expected to work in mouse cells. This is because the B18R does not effectively neutralize mouse interferon (IFN)-β.

The TagGFP2 Simplicon™ RNA (E3L) Kit was developed to enable the evaluation of the Simplicon™ Expression System in targeted cells. E3L[4] incorporation in Simplicon™ System strongly suppresses interferon responses and enables increased expression of the target gene(s). TagGFP2 Simplicon™ RNA 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 483/506 nm, respectively[5, 6].

Storage & Handling of Kit Components

1. TagGFP2 Simplicon™ RNA (E3L): (Part No. CS224549) One (1) vial containing 10 µL of RNA (1 µg/µL). Store at -80°C.

2. B18R-E3L RNA (human codon optimized for B18R and E3L): (Part No. CS224503) 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.
- 40-60% GFP-positive human foreskin fibroblasts (HFFs) with FACS analysis.

Representative Data

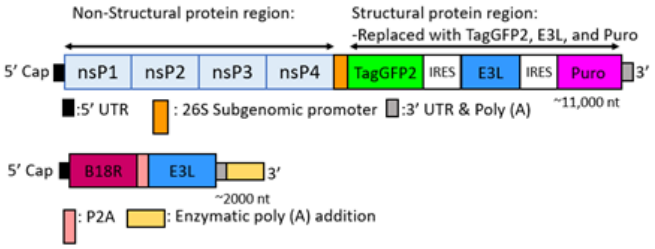

Figure 1. Structure of the TagGFP2 Simplicon™ RNA (E3L) and B18R-E3L RNA. The RNA replicon encodes four non-structural replication complex proteins (nsPs) as a single ORF at the 5' half of the RNA. At the 3' half, the viral structural proteins ORFs are replaced with the TagGFP2, E3L, Puro and IRES.

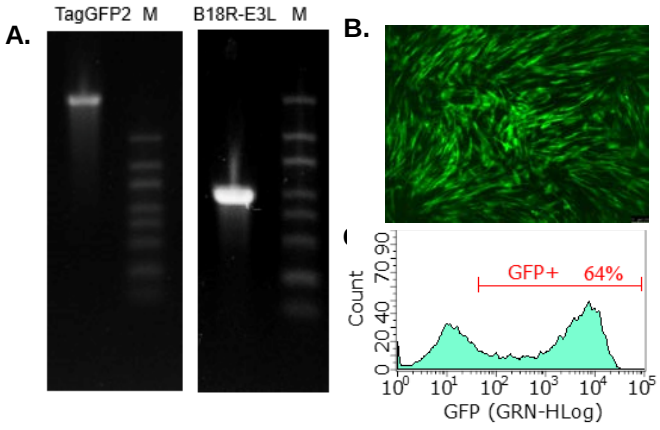

Figure 2. RNA gels and TagGFP2 expression in HFFs.
(A) RNA gels (Left): TagGFP2 Simplicon™ RNA (E3L), (Right): B18R-E3L RNA. Marker: 6, 4, 3, 2, 1.5, 1, 0.5, 0.2 kb. **(B, C)** TagGFP2 Simplicon™ RNA (E3L) and B18R-E3L RNA were co-transfected into HFFs with MessengerMax™ transfection Reagent. One day after transfection, GFP expression was analyzed by fluorescence microscopy and FACS.

Rev.1.0/2018-04-20/SCR720/VC

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Transfection Protocols

Transfection of Simplicon™ RNA has been validated using the RiboJuice™ mRNA Transfection Kit (**Part No. TR-1013)** and Lipofectamine® MessengerMAX™ Transfection Reagent **(ThermoFisher LMRNA001)**. Amounts of RNAs and transfection reagents may vary depending on the target cells. Set up different RNA: transfection reagent ratios. B18R is always required for the expression of Simplicon™ RNA. B18R is provided as B18R-E3L RNA or B18R RNA for co-transfection with Simplicon™ RNA.

Forward Transfection Protocol

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).

   Option 1: *Add 200 ng/mL of B18R protein (Cat. No. GF156 or GF197) in medium. Pre-treatment of B18R protein may support the neutralization of IFN responses. Incubate cells in a 37°C, 5% CO_2 incubator for 10-20 minutes with B18R protein.*

   Option 2: *No serum condition increases the transfection efficiency. However, it is possible to use 1-10% serum depending upon cell types.*

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 MessengerMAX™ Transfection Reagent

Total RNA Amount		**1 µg**	**2 µg**
1)	Prepare RNA mixture in Tube 1.		
Tube 1	DMEM (no serum, no antibiotics)	50 µL	100 µL
	B18R-E3L RNA (1 µg/ µL)	0.5 µL	1 µL
	TagGFP2 Simplicon™ RNA (E3L) (1 µg/ µL)	0.5 µL	1 µL
	Total volume	51 µL	102 µL
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™	3 µL	6 µL
	Total volume	53 µL	106 µL
3)	Quickly add tube 2 into tube 1.		
Total volume in a tube		104 µL	208 µL

If using RiboJuice™ mRNA Transfection Kit

Component	**Vial**	**Cat. No.**
Opti-MEM®	250 µL	Thermo (31985-062)
B18R-E3L RNA (1 µg/µL)	0.5 µL	
TagGFP2 Simplicon™ RNA (E3L) (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**	

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 hrs incubation using MessengerMAX™ reagent in human fibroblasts.

6. Aspirate the transfection medium and add 2 mL per well of culture medium for target cells. Incubate in a 37°C, 5% CO_2 incubator overnight.

   Option: Add 200 ng/mL B18R protein. Addition of B18R protein may support the neutralization of IFN responses.

7. Next day, aspirate and exchange with fresh culture medium containing 200 ng/mL B18R protein and puromycin (0.2-1 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.

9. 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™ TagGFP2, after a week, it is possible to transition to media changes every other day and to reduce the amounts of puromycin (0.1-0.5 µg/mL) and B18R protein (50-200 ng/mL).

10. 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 (i.e. HepG2) reverse transfection may be more efficient.

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

On the 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 and resuspend cells in normal culture medium containing 2% serum (no antibiotics). 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.*

   Option: *Add 10-20% of B18R-CM or 200 ng/mL B18R protein. Addition of B18R protein may support the neutralization of IFN responses.*

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.

Electroporation Protocol for Human Primary T Cells

Electroporation is an alternative way to introduce Simplicon™ RNA into difficult to transfect cells such as primary human T cells ((activated and expanded from peripheral blood mononuclear cells (PBMCs)). Using this protocol, it is possible to achieve 20-70% electroporation efficiency of Simplicon™ TagGFP2 RNA to primary human T cells.

1. Prepare healthy growing primary human T cells. We generally use CD3/CD28 beads (ThermoFisher 11131D) and rIL2 (MilliporeSigma IL002) for activation and expansion of primary human T cells.

2. Using a Bio-Rad electroporation unit, set up the following program: Exponential pulse condition of 160V and 950μF.

3. Prepare 1x10^6 cells in Ingenio® Electroporation Solution (Mirus Bio MIR 50111) in 100 µL total volume. Transfer the cell mixture to an electroporation cuvette (0.2 cm-gap). Store on ice.

4. Prepare the RNA mixture: 5 μg of Simplicon™ RNA plus 5 μg of B18R-E3L RNA.

5. Add the RNA mixture to the cells. Electroporate as soon as possible.

6. Put on ice for a few minutes, and then transfer cells into the appropriate wells of a 48 well plate with 1 mL of cell culture medium containing 200 ng/mL B18R protein. Incubate at 37°C in a 5% CO_2 incubator.

Troubleshooting

Low or no GFP or RFP expression observed in target cells.

1. Transfection efficiencies may differ between cell types. For example, Simplicon™ RNA transfection efficiency was very low with neonatal human keratinocytes while efficiency was higher with adult human keratinocytes. We recommend RiboJuice™ mRNA Transfection Kit (MilliporeSigma TR-1013) and MessengerMAX™ Transfection Reagent (ThermoFisher LMRNA001) for most cell types. However, other transfection reagents for DNA or mRNA may work better depending on the target cells.

   Please note that Lipofectamine® RNAiMAX Transfection Reagent (ThermoFisher 13778030) does not work well for Simplicon™ RNA transfection.

2. Increase the amount of transfection reagent per μg RNA. Determine empirically the optimal amount of transfection reagent to apply while still minimizing cytotoxicity.

Many dead cells observed after transfection.

1. Reduce the amount of RNAs to be transfected.
2. Try a different transfection reagent for mRNA and DNA.
3. Pretreat with B18R protein before and after transfection (see the options in the protocol).

No cells survived after puromycin selection.

1. Reduce the puromycin concentration. Simplicon™ RNA expression will be high for a few days (overexpression level). Over time, expression levels are expected to diminish and stabilize to 1/5~1/10 the levels initially observed and may be close to physiological levels after one week. We recommend setting the puromycin at very low concentrations (0.2-1 µg/mL, mostly 0.2-0.4 µg/mL). Low puromycin concentration will take ~ 5 days for selection.

2. B18R protein is required for continuous expression of the Simplicon™ RNA. Change cell culture medium every day and freshly supply B18R protein for at least one week after transfection. B18R protein is also available as B18R conditioned medium. For detailed information, please refer to the User Guide for the Simplicon™ Expression System on our website (www.emdmillipore.com).

References

1. Yoshioka N, et al. 2013 *Cell Stem Cell*. **13** (2): 246-254.
2. Yoshioka N, et al. 2017 *PLoS One*. **12**(7): e0182018.
3. Pegg G, et al. 2018 *Drug Metabolism and Pharmacokinetics* 33 (1): S33-S34.
4. Perdiguero B, et al. 2009 *J Interferon Cytokine Res*. **29**(9): 581-98.
5. Mertzlyak EM, et al. 2007 *Nat. Methods* 4: 555-557.
6. Subach OM, et al. 2008 *Chem. Biol.* 15: 1116-1124.

RELATED PRODUCTS

Cat #	Description
SCR721	TagRFP Simplicon™ RNA (E3L) Kit
SCR722	B18R-E3L RNA (human codon optimized for B18R and E3L)
SCR723	B18R RNA (human codon optimized)
SCR724	Simplicon™ Cloning Vector (E3L)
SCR725	TagGFP2 Simplicon™ Plasmid (E3L)
SCR726	TagRFP Simplicon™ Plasmid (E3L)
SCR727	B18R-E3L Plasmid (human codon optimized for B18R and E3L)
SCR728	B18R Plasmid (human codon optimized)
GF156	B18R protein (produced from insect cells)
GF197	B18R protein (priduced from HEK 293 cells)

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