



Simplicon™ Expression System:

“Designing, Cloning and RNA Synthesis for Expression of Self-Replicative RNA”

Catalog No. SCR724, SCR725, SCR726, SCR727, SCR728, SCR729

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Introduction:

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^{1,2,3,4}. 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 (Figure 1A). The Simplicon™ RNA is a synthetic RNA generated from the Simplicon™ Cloning Vector (E3L) plasmid.

Introduction and replication of the Simplicon™ RNA is expected to elicit a strong interferon (IFN) response in transfected cells. To suppress the IFN responses, a Vaccinia virus protein⁵, B18R, is used for the original Simplicon™ technology. Recently, we found that another Vaccinia virus protein⁵, 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, expression of transgenes, cell proliferation and viability during the RNA transfection and replication are improved. 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 .

One day after transfection of the Simplicon™ RNA, a spike in the levels of transgenes can be observed. The expression levels are maintained by addition of B18R, E3L and the selective agent, puromycin throughout the duration of the experiment. 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. Expression levels and duration may change depending upon the cell types, transgenes and media conditions used. The Simplicon™ technology has been successfully utilized for efficient human iPSC generation through the sustained expression of critical reprogramming factors^{3,4} and in the creation of cell lines that express and retain the metabolic activities of five cytochrome P450 enzymes⁶.

In the Simplicon™ Expression System, B18R and E3L are provided as a B18R-E3L RNA (Cat. No. SCR722) for the suppression of IFN responses at RNA transfection (Figure 1B). For sustained transgene expression, recombinant B18R protein (Cat. No. SCR156 or SCR197) or B18R conditioned medium (B18R-CM) can be used. E3L is continuously provided from the Simplicon™ RNA itself. B18R-CM can be produced from B18R-E3L RNA or B18R RNA synthesized using the B18R-E3L plasmid (Cat. No. SCR727) or B18R plasmid (Cat. No. SCR728), respectively.

The Simplicon™ Cloning Vector (E3L) (Cat. No. SCR724) is designed for expression of the transgenes of interest as a Simplicon™ RNA. The vector encodes four non-structural replication complex proteins (nsPs) as a single ORF at the 5' half of the RNA, and multiple cloning site, E3L and puromycin resistance gene at the 3' half of the RNA. Simplicon™ Cloning Vector (E3L) can accommodate transgene cassettes up to a total size of 8,300 bp. The presence of E3L in the Simplicon™ RNA works to suppress the IFN responses for sustained expression of the Simplicon™

RNA. For suppression of IFN responses at RNA transfection, we recommend B18R-E3L RNA (SCR722) prepared from the B18R-E3L plasmid (SCR727).

Overview of Simplicon™ Expression System:

Simplicon™ RNA and B18R-E3L RNA are co-transfected into cells. Soon after transfection, the non-structural proteins (nsP1-4) of Simplicon™ RNA are translated and together with cellular proteins form the replication complexes. These replication complexes begin the replication and generation of the minus strand RNA intermediate as well as transcription of the transgenes-E3L-Puro from 26S promoter (Figure 1C). The RNA transcripts are then translated using the cellular machinery (Figure 1C). RNA transfection and replication are known to elicit IFN responses. To inhibit IFN responses, B18R-E3L RNA is co-transfected with Simplicon™ RNA into cells. B18R-E3L RNA is quickly translated and produces B18R protein and E3L protein. B18R protein is secreted into culture medium, and then neutralize IFNs by direct binding, while E3L inhibits the intracellular signaling pathways for IFN responses.

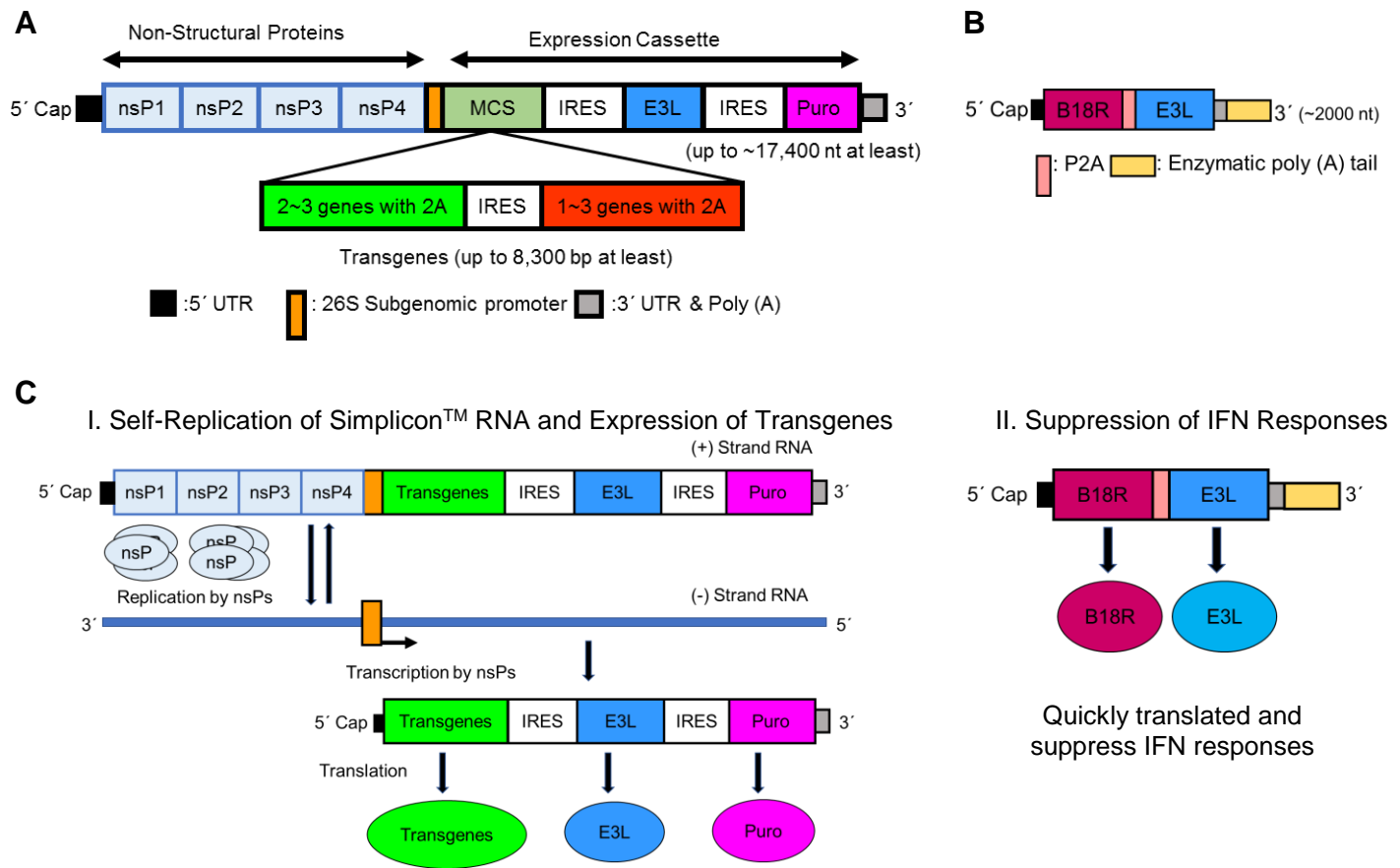


Figure 1. Schemes of Simplicon™ Expression System. **(A)** Structure of the Simplicon™ RNA . The Simplicon™ RNA encodes four non-structural replication complex proteins (nsPs) as a single ORF at the 5′ end of the RNA. At the 3′ end, the viral structural proteins ORFs are replaced with the transgenes of interest. Simplicon™ Cloning Vector (E3L) (Cat. No. SCR724) can accommodate transgene cassettes up to a total size of 8,300 bp by combination with 2A peptides and IRES. E3L is included to protect the resulting RNA from interferon responses caused by RNA replication. Locations of the 26S Subgenomic promoter, E3L, IRES and Puromycin (Puro)-resistance gene are indicated. **(B)** Structure of B18R-E3L RNA. B18R and E3L inhibit interferon responses caused by RNA transfection. B18R-E3L is co-transfected with Simplicon™ RNA and enables expression of transgenes from Simplicon™ RNA. **(C)** Self-replication of Simplicon™ RNA and Expression of Transgenes. Simplicon™ RNA is a (+) strand RNA.

Note on nomenclature:

The prefix “Simplicon™” refers to self-replicating RNA. RNAs that do not have the Simplicon™ prefix (i.e. B18R-E3L RNA) refers to mRNA transcripts that contain a 5´ cap and poly A tail but **can not self-replicate**.

Simplicon™ System Components (Not provided as a kit; available separately):

- **Simplicon™ Cloning Vector (E3L) (Cat. No. SCR724):** Simplicon™ Cloning Vector is designed for cloning of transgene(s) to synthesize the custom Simplicon™ RNA. It encodes nonstructural proteins (nsP1-4) that are responsible for replication of Simplicon™ RNA (genomic RNA) and transcription of subgenomic RNA (transgenes, E3L and puromycin).
- **B18R-E3L Plasmid (human codon optimized for B18R and E3L) (Cat. No. SCR727):** Plasmid encoding human codon optimized B18R and E3L. B18R-E3L plasmid is used as a DNA template for the synthesis of B18R-E3L RNA (Cat. No. SCR722). The B18R-E3L RNA is a synthetic polycistronic mRNA and is used for co-transfected with the Simplicon™ RNA to suppress the IFN responses. B18R-E3L RNA can also be used to prepare B18R-CM by transfection into HFFs (Cat. No. SCC058). B18R-E3L RNA is also available for co-transfection of any kinds of mRNA to suppress the IFN responses.
- **B18R Plasmid (human codon optimized) (Cat. No. SCR728):** Plasmid encoding human codon optimized B18R. B18R Plasmid is used as a DNA template for the synthesis of B18R RNA (Cat. No. SCR722). B18R RNA is used in original research papers for iPSC generation^{3,4}. B18R-E3L will work more efficiently with Simplicon™ Expression System. When B18R plasmid is used, please follow all protocols for B18R-E3L.
- **Puromycin (Sigma 508838):** An antibiotic applied to select for cells that have taken up the Simplicon™ RNA. Puromycin must be applied throughout the duration of the experiment in order to maintain expression of the proteins of interest.
- **B18R Protein (Cat. No. GF156 or Sigma GF197) or B18R-E3L Conditioned Medium:** Recombinant B18R protein or B18R conditioned medium must be applied throughout the duration of the experiment to protect the Simplicon™ RNA from degradation due to interferon responses caused by Simplicon™ RNA transfection and replication. B18R conditioned medium can be produced using B18R-E3L RNA (please refer to pg. 25-27 for detailed protocols).
- **TagGFP2 Simplicon™ Plasmid (E3L) (Cat. No. SCR725) or RNA Kit (Cat. No. SCR720):** Plasmid or RNA encoding a self-replicating RNA with the TagGFP2 fluorescent protein. Used to optimize for transfection conditions in hard to transfect somatic or primary cells.
- **TagRFP Simplicon™ Plasmid (E3L) (Cat. No. SCR726) or RNA Kit (Cat. No. 721):** Plasmid or RNA encoding a self-replicating RNA with the TagRFP fluorescent protein. Used to optimize for transfection conditions in hard to transfect somatic or primary cells.

Please visit our website (www.emdmillipore.com) for a complete listing of Simplicon™ related products.

Materials Required but not Supplied:

Linearization of Plasmids:

1. Mlul-HF: New England Biolabs R3198S or R3198L
2. BamHI-HF: New England Biolabs R3136S or R3136L
3. Phenol:Chloroform:Isoamyl Alcohol 25:24:1: Sigma P3803-100ML
4. Chloroform:Isoamyl Alcohol 24:1: Sigma C0549-1PT

RNA Synthesis:

1. RNaseZap™ RNase Decontamination Wipes: ThermoFisher AM9786
2. Posi-Click 1.7 mL microcentrifuge tube: Denville Scientific C2170
3. Ammonium Acetate Solution, 5 M: Sigma 09691-100ML (Store at 4°C)
4. RiboMAX™ Large Scale RNA Production Systems – T7: Promega P1300
5. CleanCap™ Reagent AG: TriLink N-7113
6. ScriptCap™ m7G Capping System: CellScript C-SCCE0625
7. ScriptCap™ 2'-O-Methyltransferase Kit: CellScript C-SCMT0625
8. A-Plus™ Poly(A) Polymerase Tailing Kit: CellScript C-PAP5104H
9. Antarctic Phosphatase: New England BioLabs M0289S
10. ScriptGuard™ RNase Inhibitor: CellScript C-SRI6310K
11. RNA Storage Solution: ThermoFisher AM7000
12. RiboRuler High Range RNA Ladder: Thermofisher SM1823
13. E-Gel™ Agarose Gel, 0.8% and 2%: ThermoFisher G501808, G501802

RNA Transfection & Electroporation:

1. Lipofectamine™ MessengerMAX™ Transfection Reagent: ThermoFisher LMRNA001
2. RiboJuice™ mRNA Transfection Kit: MilliporeSigma TR-1013
3. Ingenio® Electroporation Solution: Mirus MIR 50111

Other Reagents:

1. FibroGRO™ Xeno-Free Human Foreskin Fibroblasts: MilliporeSigma SCC058
2. TagGFP2 Simplicon™ RNA (E3L) Kit: MilliporeSigma SCR720
3. TagRFP Simplicon™ RNA (E3L) Kit: MilliporeSigma SCR721
4. B18R-E3L RNA: MilliporeSigma SCR722
5. B18R, Human Recombinant Carrier-Free Protein (purified from insect cells): MilliporeSigma GF156
6. B18R, Human Recombinant Carrier-Free Protein (purified from HEK 293 cells), Sigma GF197

Workflow:

- Designing of Simplicon™ plasmid with target transgene(s) (pg. 6-8)
- Cloning transgene(s) into Simplicon™ Cloning vector (E3L) (pg. 8)



- Linearization of Plasmids (Simplicon™ plasmid and B18R-E3L plasmid) (pg. 8-9)
- In vitro RNA synthesis of Simplicon™ RNA (pg. 12-18)
- In vitro RNA synthesis of B18R-E3L RNA (pg. 18-21)
- Make B18R -E3L CM (if not using B18R recombinant protein) (pg. 25-27)



- Co-transfection of Simplicon™ RNA and B18R-E3L RNA (pg. 28-31)



- Sustained expression: Add B18R protein or B18R-CM and Puromycin



- Analyze protein expression of target genes by flow analysis or ICC

Methods:

Step 1: Designing of Simplicon™ Plasmid with Transgene(s)

How to design the expression cassette of transgene(s) is one of key points for the success of Simplicon™ Expression System especially for expression of multiple transgenes. Please refer bellows for designing of expression cassette.

1. Use the Kozak sequence for 1st Methionine (ATG) site

Translation of mRNA will be affected on strength of Kozak sequence⁷. Use of Kozak consensus sequence such as “gccaccATGG” will help increase of protein expression of transgene(s) from Simplicon™ RNA.

2. Multiple Cloning Site of Simplicon™ Cloning Vector contains NdeI site (CATATG)

NdeI site contains “ATG” and it will work as a 1st methionine without Kozak sequence (Figure 2A). Please design 5′ UTR of transgene appropriately. Figure 2B and C show the typical examples of designing of 5′ UTR.

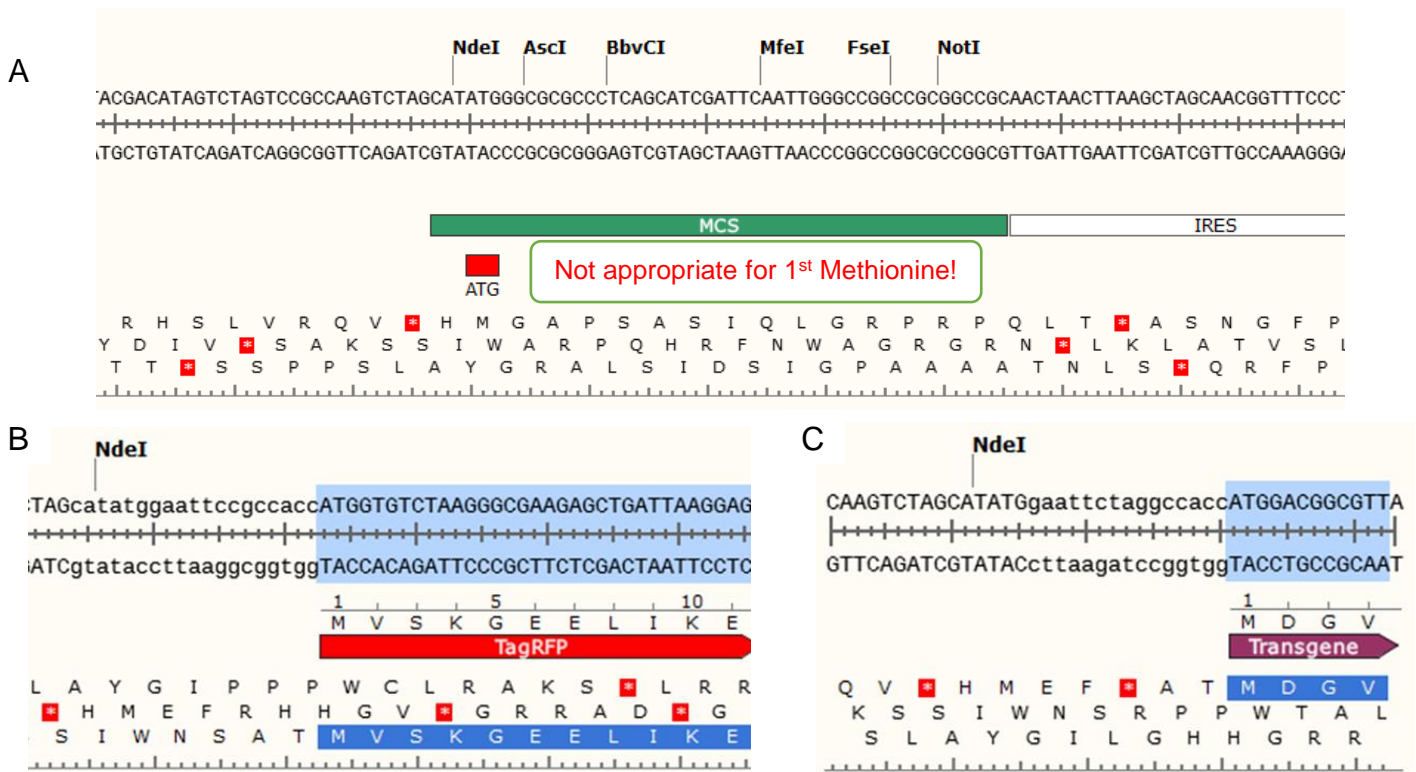


Figure 2. Design of 5′ UTR of transgene. **(A)** NdeI site has a ATG codon. **(B)** Kozak sequence is inserted into different frame with the NdeI site ATG. The frame of NdeI site ATG has a stop codon with second frame of TagRFP, so no protein expression from the NdeI site ATG. **(C)** Kozak sequence and stop codon are inserted with the NdeI site ATG in-frame. The second and third frames of transgene are largely open (more than 50 amino acids, not shown), so the stop codon is inserted after NdeI site ATG with Kozak sequence in-frame to escape the translation of different frame.

3. Expression of Multiple transgenes

Co-expression of two kinds of Simplicon™ RNAs such as Simplicon™ TagGFP2 and TagRFP works only a few days, and then most cells express either TagGFP2 or TagRFP. Therefore, if you are planning to express multiple transgenes, all transgenes need to clone into the Simplicon™ Cloning

Vector and express as a single polycistronic RNA. Please refer belows for the success of polycistronic transgenes expression from the Simplicon™ RNA.

A) Gene ordering

For expression of multiple genes, the order of genes in the expression cassette is important. The first position gene will express most efficiently and thus we recommend placement of the most important genes at the first position.

B) 2A peptides and/or IRES

The 2A peptides^{8,9} and/or IRES¹⁰ are useful to express multiple genes from Simplicon™ Expression System^{3,4,6}. The 2A peptides technology allows the polycistronic expression of proteins from a single mRNA while the expression level will be decreased depending on position and number of genes⁹, and efficiency of 2A peptide cleavage will depend on genes even the addition of GSG linker. Liu *et al.* performed systematic comparison of 2A peptides and showed the different efficiency of direct cardiac reprogramming by positioning of 2A peptides and reprogramming genes⁹. Please note that the protein upstream of the 2A peptide is attached the 2A peptide sequence except for the C-terminal proline, so some proteins may be affected on their activity by the addition of 2A peptide. On the other hand, the internal ribosomal entry site (IRES) from encephalomyocarditis virus (EMCV) is also widely used for polycistronic expression system and can express proteins without adding peptide¹⁰. If you are planning to express more than three genes, we recommend plasmid constructs with not only 2A peptides, but also a combination of 2A peptides and IRES for the test of expression. Figure 3 shows the typical examples for tri- and quad-cistronic constructs. At least, we don't recommend quad-cistronic construct with only 2A peptides in our experience.

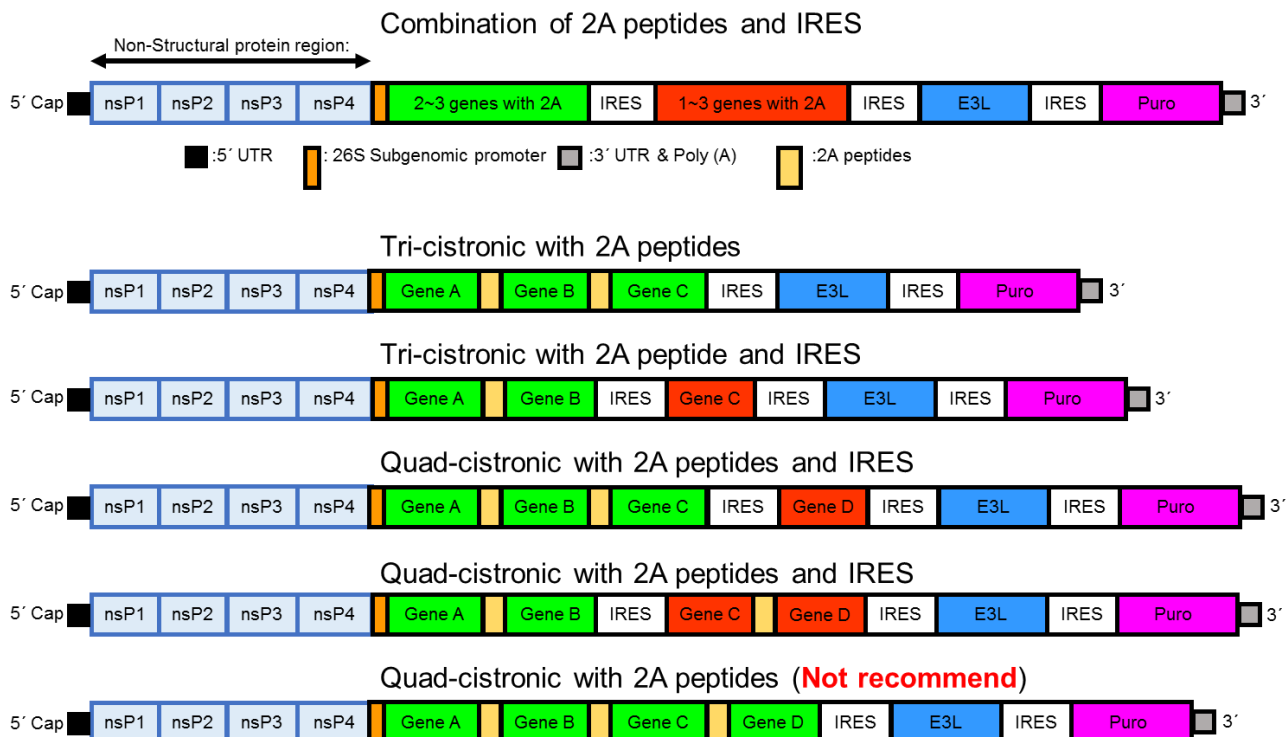


Figure 3. Typical examples of polycistronic constructs for Simplicon™ RNA.

C) Check the availability of MluI or XbaI restriction enzyme site

To synthesize Simplicon™ RNA, Simplicon™ plasmid should be linearized by appropriate restriction enzyme (5' overhang enzyme). For Simplicon™ Cloning Vector, MluI and XbaI are available for linearization. When these enzymes are not available, please add an available restriction enzyme site by oligo DNA or edit the DNA sequence of insert before cloning. Of note, the PacI (located next to the XbaI site) produces 3' overhang, so it is not available for linearization.

Step 2: Cloning of Transgene(s) in Simplicon™ Cloning Vector (E3L)

Use standard molecular cloning techniques such as restriction enzyme cloning and Gibson assembly to clone inserts into the Simplicon™ Cloning Vector (E3L). DH5α, DH10B or equivalent competent cells may be used for transformation and amplification of the plasmids. Transfection-grade plasmid purification is recommended for RNA synthesis.

Step 3: Linearization of Plasmid Templates for RNA Transcription

Linearize the DNA plasmids by restriction enzyme digestion. Follow the restriction enzyme manufacturer's recommended buffer and incubation conditions for the enzymes.

For Simplicon™ plasmid: Use MluI or XbaI (refer to plasmid map).

For B18R-E3L plasmid and B18R plasmid: Use BamHI (refer to plasmid map).

1. Set up the following DNA digestions; scale up or down accordingly.

Note: We generally use 100 Units of restriction enzyme for 20-40 µg of plasmid digestion in 200 µl volume. The DNA pellet after ethanol precipitation can be easily detected without glycogen carrier when more than 20 µg plasmid is used. Glycogen carrier may have a risk of contamination in the final product (RNA).

Simplicon™ Plasmid

36 µL Simplicon™ Plasmid (1 µg/µL)
20 µL CutSmart Buffer (10X)
139 µL Sterile distilled water
5 µL MluI-HF (10,000 units/mL, NEB)
200 µL Total Volume

B18R-E3L Plasmid

36 µL B18R-E3L Plasmid (1 µg/µL)
20 µL CutSmart Buffer (10X)
139 µL Sterile distilled water
5 µL BamHI-HF (20,000 units/mL, NEB)
200 µL Total Volume

Incubate digestion mixtures at 37°C for 3 hours.

2. Verify that the DNA plasmids are completely linearized by gel electrophoresis using a few microliters of digestion mixture. Ensure the presence of a clean (non-degraded) DNA fragment of the correct size.

Simplicon™ Plasmid Expected Size: More than 12,000 bp, depending on insert size.

B18R-E3L Plasmid Expected Size: 4,536 bp

3. Purify plasmids with organic extraction and ethanol precipitation:

- 3.1. Add the same volume (200 μ L) of phenol/chloroform/isoamyl alcohol (25:24:1) to each eppendorf tube containing the digestion mixture.
- 3.2. Vortex the eppendorf tubes for 60 seconds. Centrifuge at high speed (14,000 rpm) for 5 minutes.
- 3.3. Transfer each aqueous phase (top layer) into new eppendorf tubes.

Note: *Simplicon™ plasmid is a large sized DNA and may be trapped in the interphase layer with the proteins. When the interphase is sticky (DNA is trapped), collect the interphase layer along with the aqueous layer.*

- 3.4. To remove the excess phenol, add an equal volume (200 μ L) of chloroform/isoamyl alcohol (24:1) to each eppendorf tubes.
- 3.5. Vortex the eppendorf tubes for 60 seconds and centrifuge at high speed (14,000 rpm) for 5 minutes.
- 3.6. Transfer each aqueous phase (top layer) into new eppendorf tubes.
- 3.7. Add 1/10 volume (20 μ L) of 3 M sodium acetate, pH 5.2 (this can be found in the RiboMAX Large Scale RNA Production kit) and 2.5 X volume (500 μ L) of 100% ethanol to each eppendorf tubes.
- 3.8. Vortex and place at -80°C for 5 minutes or store at -20°C until use.

Note: *If there are no plans to proceed to RNA synthesis on the same day, stop at this step. DNA will be stable for several months at -20°C.*

- 3.9. Centrifuge at high speed (14,000 rpm) for 10-15 minutes at 4°C to pellet the DNA plasmid. Remove the ethanol carefully with a pipet tip, being careful not to dislodge the DNA pellet.
- 3.10. Gently add 1 mL 70% ethanol to wash the DNA pellet. Centrifuge at high speed at 4°C for 1 minute. Remove the ethanol carefully with a pipet tip, being careful not to dislodge the RNA pellet.
- 3.11. Centrifuge shortly, and carefully remove any visible residual ethanol with a P20 pipette. Drying the DNA pellet is not necessary. In general, approximately 20% of plasmid may be lost during purification.
- 3.12. Resuspend the DNA pellet in a small volume (15~20 μ L, depending on starting amount) of DNase & RNase-free water before measuring the concentration.
- 3.13. Measure the DNA concentration of each linearized DNA plasmid. Adjust the concentration to 1 μ g/ μ L.

Note: DNA that is resuspended in water even when stored at -20°C is not stable for long term storage. Purified linearized DNA plasmids stored at -20°C should be used within a week. If the DNA will not be used for RNA synthesis within a week, stop at step 3.8 (ethanol precipitation). DNA will be more stable for a longer length of time in the ethanol precipitation state.

Step 4: RNA Synthesis

Overview of RNA Synthesis:

RNA synthesis is generally composed of three different reactions. 1) *In vitro* transcription, 2) 5' Capping, and 3) Poly (A) tailing. We introduce two methods for Simplicon™ RNA and one method for B18R-E3L RNA as shown in Workflow for RNA Synthesis.

For in vitro transcription: We recommend the T7 RNA Polymerase from Promega, Cat. No. P1300. Other T7 RNA polymerases will be difficult for the synthesis of Simplicon™ RNA because of limited accessibility to the Simplicon™ T7 promoter and large sized RNA.

For 5' Capping: Cap analog with CleanCap Reagent AG (N-7113, TriLink) and enzymatic capping methods are available. We recommend the CleanCap Reagent for the beginners (One-Step RNA Synthesis). Enzymatic capping method is required for three-step reaction while it minimizes the IFN responses by combination with phosphatase treatment⁴.

Poly (A) Tailing Reaction:

- **Simplicon™ RNA:** A long poly (A) tail sequence is already incorporated in the Simplicon™ Cloning Vector (E3L) so polyadenylation is not required.
- **B18R-E3L RNA and B18R RNA:** A long poly (A) tail sequence is **not** pre-incorporated in the B18R-E3L and B18R plasmids so polyadenylation is required.

Prevention of RNase contamination:

All standard precautions should be taken to minimize RNase contamination. Wipe the bench area and all instruments with RNase inactivation reagents (RNase Zap wipes, Ambion AM9786). These could include tabletop centrifuges, heat blocks, vortex, pipettes, tube racks, pipette racks and anything that will touch the eppendorf tubes containing the RNA. Rinse with MilliQ water soaked tissue paper. Only use certified nuclease-free eppendorf tubes (i.e. Posi-click tubes from Denville C-2172). Use fresh boxes of pipette filter tips to avoid contamination. Use nuclease-free water from Promega or Cellscrip kits.

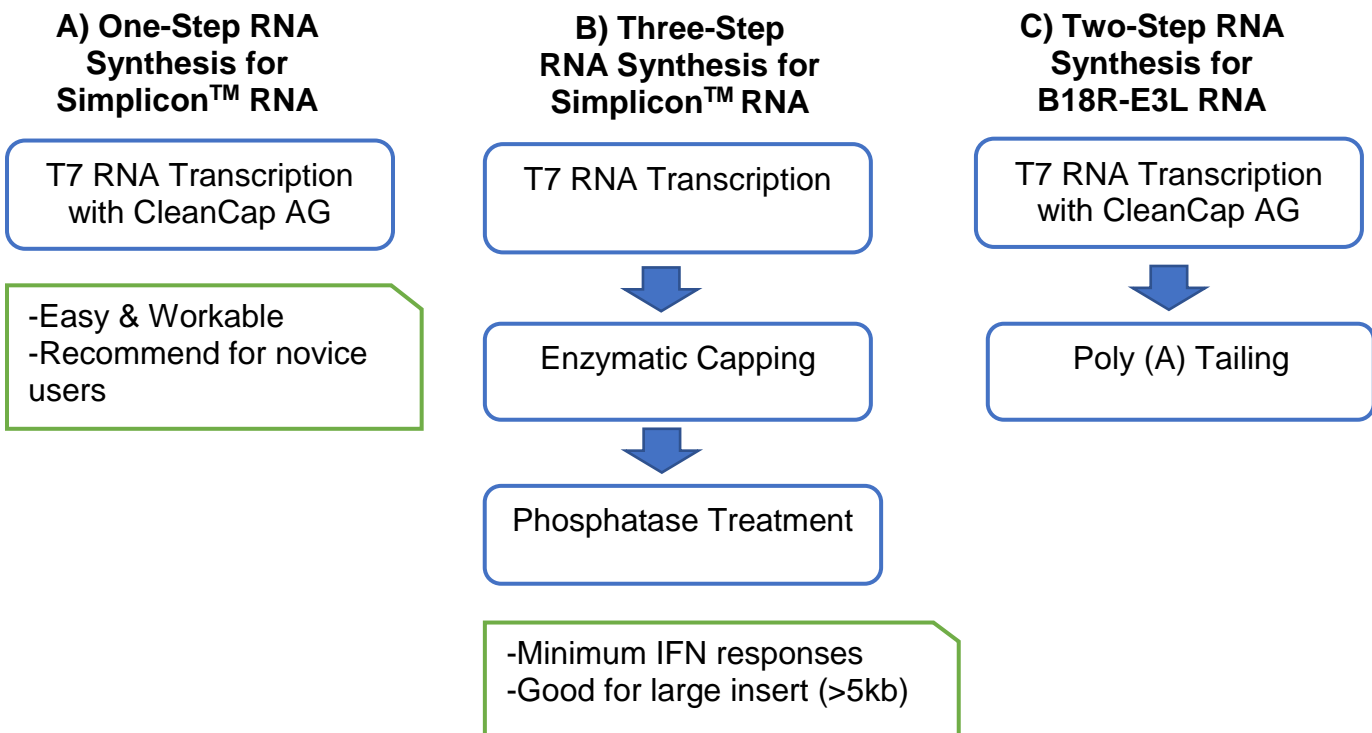
Kits for RNA Synthesis:

RNA synthesis is carried out with several kinds of kit and reagent provided from different companies. We optimized protocols for the synthesis of Simplicon™ RNA and B18R-E3L RNA using these kits and reagents. Please follow our protocols for the synthesis of Simplicon™ RNA and B18R-E3L RNA.

Common Tips for RNA Synthesis:

- 1) **2.5 M ammonium acetate precipitation:** RNA purification method. This method selectively precipitates RNA while leaving most of the DNA, protein, and unincorporated NTPs in the supernatant. Add an equal volume of 5 M ammonium acetate (final 2.5 M) into RNA solution, incubate on ice, and then centrifuge to precipitate RNA. Wash the RNA pellet with 70 % ethanol. Washed RNA pellet is stable for several months at -80°C, so the reaction can be stopped in this step.
- 2) **RNA suspension:** Simplicon™ RNA is large size, so no vortex. Gentle pipetting is recommended for suspension. When RNA pellet is large (more than several hundreds micrograms), make small clumps of RNA by pipetting after addition of water or RNA storage solution, and then incubate for a few minutes at room temperature. The RNA pellet becomes transparent and then, start resuspension of RNA by pipetting. Repeat incubation and pipetting for complete suspension of RNA.

Workflow for RNA Synthesis:



A) One-Step RNA Synthesis for Simplicon™ RNA:

RNA capping with CleanCap AG is performed in the reaction of T7 RNA transcription.

1. Set up the following reaction at room temperature. Keep reagents on ice after thaw except for T7 Transcription Buffer 5X. Add the reaction components in the order shown. After all the components are added, mix by gentle pipetting.

<ul style="list-style-type: none"> • RiboMAX™ Large Scale RNA Production Systems-T7 (Promega P1300) • CleanCap™ AG (TriLink N-7113) 		
Component	Amount (1X)	Amount (____X)
T7 Transcription Buffer 5X	20 µL	
100 mM ATP	7.5 µL	
100 mM GTP	7.5 µL	
100 mM CTP	7.5 µL	
100 mM UTP	7.5 µL	
CleanCap AG, 100 mM	7.5 µL	
DNA plasmid (1 µg/µL)	10 µL	
Sterile distilled water	22.5 µL	
Enzyme Mix (T7)	10 µL	
Total Volume	100 µL	

2. Incubate at 37°C for **2 hrs.**

Note: Do not incubate beyond 2 hours at 37°C as a longer incubation period may result in increased RNA degradation.

Note: Do not freeze the completed transcription reactions.

3. Add 10 µL RQ1 RNase-free DNase (Promega M6101) for 10 µg of template DNA (use 1 µL per µg DNA template). Gently mix by pipetting up and down. Incubate for 15 minutes at 37°C.
4. Purify RNA with 2.5 M ammonium acetate precipitation by adding an equal volume (110 µL) of 5 M ammonium acetate to a reaction mixture (Final: 2.5 M). **Do not add alcohol.** Mix gently by pipetting up and down.

Note: Do not vortex as large RNAs break easily.

Note: The RNA reaction mixture becomes cloudy after the addition of ammonium acetate. 2.5 M ammonium acetate precipitates RNA while leaving most of the DNA, protein, and unincorporated NTPs in the supernatant.

DO NOT perform the organic extraction of RNA as described in the manufacturer's protocol. The Simplicon™ RNA may be trapped in the interphase layer with proteins as white pellets, and may be difficult to get into solution.

5. Store on ice for 10 minutes. No more than 20 minutes. Long time incubation will degrade the RNAs.
6. Centrifuge at high speed (~14,000 rpm or 10,000 x g) for 15 minutes at 4°C to precipitate the RNA.
7. Remove the supernatant and wash the RNA pellet with 1 mL 70% ethanol to remove the salts.
8. Remove the ethanol carefully with a pipet tip, being careful not to dislodge the RNA pellet. If the next step will not proceed on the same day, store the RNA pellet at -80°C. RNA in this state is stable for at least a month.
9. Centrifuge at maximum speed (~14,000 rpm) for 3 minutes at 4°C. Remove any visible remaining ethanol with a P20 pipet. Drying the RNA pellet is not necessary.
10. Resuspend RNA pellet in 100-200 µL of RNA storage solution (ThermoFisher Scientific, AM7000). Store the eppendorf tube on ice after resuspension.

Note: RNA pellet will be large (~500 µg of RNA). Please refer the RNA suspension described in "Common Tips for RNA Synthesis (pg. 11)". RNAs may also be sticky and transparent after addition of water, so make sure that there are no RNAs on the pipette tip before discarding the tip.

11. Measure the RNA concentration by spectrophotometer at 260 nm and adjust the final concentration to 1 µg/µL. Yield of the RNA at this reaction scale is typically 300-500 µg.
12. Aliquot (10~50 µL) and store the Simplicon™ RNA at -80°C until use. Repeated free-thaw will degrade Simplicon™ RNA. Save a few micrograms for RNA gel electrophoresis and functional quality check.

B) Three-Step RNA Synthesis for Simplicon™ RNA:

Enzymatic RNA capping is performed after T7 RNA transcription. Phosphatase treatment is required.

B1. T7 RNA transcription:

1. Set up the following reaction at room temperature. Keep reagents on ice after thaw except for T7 Transcription Buffer 5X. Add the reaction components in the order shown. After all the components are added, mix by gentle pipetting.

• RiboMAX™ Large Scale RNA Production Systems-T7 (Promega P1300)		
Component	Amount (1X)	Amount (____X)
T7 Transcription Buffer 5X	20 µL	
100 mM ATP	7.5 µL	
100 mM GTP	7.5 µL	
100 mM CTP	7.5 µL	
100 mM UTP	7.5 µL	
DNA plasmid (1 µg/µL)	10 µL	
Sterile distilled water	30 µL	
Enzyme Mix (T7)	10 µL	
Total Volume	100 µL	

2. Incubate at 37°C for 2 hrs.

Note: Do not incubate beyond 2 hours at 37°C as a longer incubation period may result in increased RNA degradation.

Note: Do not freeze the completed transcription reactions.

3. Add 10 µL RQ1 RNase-free DNase (Promega M6101) for 10 µg of template DNA (use 1 µL per µg DNA template). Gently mix by pipetting up and down. Incubate for 15 minutes at 37°C.
4. Purify RNA with 2.5 M ammonium acetate precipitation by adding an equal volume (110 µL) of 5 M ammonium acetate to a reaction mixture (Final: 2.5 M). **Do not add alcohol.** Mix gently by pipetting up and down.

Note: Do not vortex as large RNAs break easily.

Note: The RNA reaction mixture becomes cloudy after the addition of ammonium acetate. 2.5 M ammonium acetate precipitates RNA while leaving most of the DNA, protein, and unincorporated NTPs in the supernatant.

DO NOT perform the organic extraction of RNA as described in the manufacturer's protocol. The Simplicon™ RNA may be trapped in the interphase layer with proteins as white pellets, and may be difficult to get into solution.

5. Store on ice for 10 minutes. No more than 20 minutes. Long time incubation will degrade the RNAs.
6. Centrifuge at high speed (~14,000 rpm or 10,000 x g) for 15 minutes at 4°C to precipitate the RNA.
7. Remove the supernatant and wash the RNA pellet with 1 mL 70% ethanol to remove the salts.
8. Remove the ethanol carefully with a pipet tip, being careful not to dislodge the RNA pellet. If the next step will not proceed on the same day, store the RNA pellet at -80°C. RNA in this state is stable for at least a month.
9. Centrifuge at maximum speed (~14,000 rpm) for 3 minutes at 4°C. Remove any visible remaining ethanol with a P20 pipet. Drying the RNA pellet is not necessary.
10. Resuspend RNA pellets in 100-200 µL in DNase-RNase free water. Store the eppendorf tube on ice after resuspension.

Note: RNA pellet will be large (~500 µg of RNA). Please refer the RNA suspension described in "Common Tips for RNA Synthesis (pg. 11)". RNAs may also be sticky and transparent after addition of water, so make sure that there are no RNAs on the pipette tip before discarding the tip.

11. Measure the RNA concentration by spectrophotometer at 260 nm and adjust the final concentration to 2 µg/µL. Yield of the RNA at this reaction scale is typically 300-500 µg. Save a few micrograms for RNA gel electrophoresis.
12. Take 60-600 µg RNA for enzymatic capping reaction. Residual RNA can be saved as a pellet at -80°C by 2.5 M ammonium acetate precipitation.

B2. Enzymatic Capping (Cap-1) Reaction:

ScriptCAP™ 2'-O-Methyltransferase Kit (CellSCRIPT Cat. No. C-SCMT0625) and ScriptCap™ m⁷G Capping System Cellscript: (CELLSCRIPT C-SCCE0625) are used to provide the 5' cap which is important for binding translation initiation factors and contributes to mRNA stability.

The protocol below is designed for use with 60 µg of starting RNA in a 100 µL total reaction volume (1x scale). Reaction scale can be up to 10x scales in an eppendorf tube.

- Denaturing of RNA.
Prepare 60 μg RNA from Step B1-11 in 67 μl RNase-free water and incubate at 65 $^{\circ}\text{C}$ for 5 minutes, then transfer on ice. For more than 5x scales, incubate for 8 min. Cool on ice at least for 3 minutes.
- While the heat-denatured RNA is cooling on ice, prepare enzymatic capping reagents on ice.
- Set up the following reaction **on ice** in the order shown:

<ul style="list-style-type: none"> Standard ScriptCap 2'-O-Methyltransferase Kit Reaction (CellScript, C-SCMT0625) Standard ScriptCap m7G Capping Reaction (CellScript C-SCCE0625) 		
Component	Amount (1X)	Amount (___X)
Heat-denatured RNA (60 μg)	67 μL	
10X ScriptCap Capping Buffer	10 μL	
10 mM GTP	10 μL	
20 mM SAM	2.5 μL	
ScriptGuard RNase Inhibitor	2.5 μL	
ScriptCap Capping Enzyme (10 U/ μL)	4 μL	
ScriptCap 2'-O-Methyltransferase (100 U/ μl)	4 μL	
Total Volume	100 μL	

- Mix gently by pipetting and incubate at 37 $^{\circ}\text{C}$ for **45** minutes.
- Purify RNA with 2.5 M ammonium acetate precipitation by adding an equal volume of 5 M ammonium acetate (for a 1X scale, this would be 100 μL).
- Store the eppendorf tubes on ice for 10 minutes. No more than 20 minutes. Long time incubation will degrade the RNAs.
- Centrifuge at maximum speed ($\sim 14,000$ rpm or 10,000 x g) for 10 minutes at 4 $^{\circ}\text{C}$ to precipitate the RNAs. Remove the supernatant, being careful not to dislodge the RNA pellet.
- Wash the RNA pellet with 1 mL 70% ethanol to remove the salts.
- Remove the ethanol carefully with a pipet tip, being careful not to dislodge the RNA pellet. **When the next step, phosphatase treatment, is not going to proceed on the same day, store the RNA pellet at - 80 $^{\circ}\text{C}$.** RNA in this state is stable for at least several months.

10. Centrifuge at maximum speed (~14,000 rpm) for 1 minutes at 4°C. Remove any visible remaining ethanol with a P20 pipet. Drying the RNA is not necessary.
11. Resuspend the RNA pellet in 77.5 µl RNase-free water for 60 µg of starting RNA. Store the eppendorf tube on ice.

Note: Some small amount of RNA will be lost during the purification.

B3. Phosphatase Treatment:

Enzymatic Capping will not work at 100% efficiency for Simplicon™ RNA especially for large insert size (>5kb). The phosphatase treatment removes 5' phosphate of non-capped RNA, and improves the quality of Simplicon™ RNA⁴. Use Antarctic Phosphatase (NEB, M0289S).

The protocol below is designed for use with ~60 µg RNA (75.5 µl) in a 100 µL total reaction volume (1X scale).

1. Set up the following reaction on ice in the order shown. Scale up according to the number of reactions required.

• Antarctic Phosphatase (NEB, M0289S)		
Component	Amount (1X)	Amount (___X)
~60 µg of RNA in water (Step B2-11)	77.5 µL	
Phosphatase Buffer 10X	10 µL	
ScriptGuard RNase inhibitor (40 U/µl)	2.5 µL	
Antarctic Phosphatase (5 U/µl).	10 µL	
Total Volume	100 µL	

2. Incubate at 37°C for 30 minutes. Do not incubate longer than 30 minutes as longer incubation time may result in increased RNA degradation.
3. Purify RNA with 2.5 M ammonium acetate precipitation by adding an equal volume of 5 M ammonium acetate (for a 1X scale, this would be 100 µL).
4. Store the eppendorf tubes on ice for 10 minutes. No more than 20 minutes. Long time incubation will degrade the RNAs.
5. Centrifuge at maximum speed (~14,000 rpm or 10,000 x g) for 10 minutes at 4°C to precipitate the RNAs. Remove the supernatant, being careful not to dislodge the RNA pellet.

6. Wash the RNA pellet with 1 mL 70% ethanol to remove the salts.
7. Remove the ethanol carefully with a pipet tip, being careful not to dislodge the RNA pellet. **When the next step is not going to proceed on the same day, store the RNA pellet at – 80 °C.** RNA in this state is stable for at least several months.
8. Centrifuge at maximum speed (~14,000 rpm) for 1 minutes at 4°C. Remove any visible remaining ethanol with a P20 pipet. Drying the RNA is not necessary.
9. Resuspend RNA pellet in ~40 µL of RNA storage solution (ThermoFisher Scientific, AM7000). Store the eppendorf tube on ice after resuspension.
10. Measure the RNA concentration by spectrophotometer at 260 nm and adjust the final concentration to 1 µg/µL. About 10-20% of RNA will be lost from starting amount.
11. Aliquot (10~50 µL) and store the Simplicon™ RNA at -80°C until use. Repeated free-thaw will degrade Simplicon™ RNA. Save a few micrograms for RNA gel electrophoresis and functional quality check.

C) Two-Step RNA Synthesis for B18R-E3L RNA and B18R RNA:

C1. T7 transcription with CleanCap™ AG:

1. Set up the following reaction at room temperature. Keep reagents on ice after thaw except for T7 Transcription Buffer 5X. Add the reaction components in the order shown. After all the components are added, mix by gentle pipetting.

<ul style="list-style-type: none"> • RiboMAX™ Large Scale RNA Production Systems-T7 (Promega P1300) • CleanCap™ AG (TriLink N-7113) 		
Component	Amount (1X)	Amount (____X)
T7 Transcription Buffer 5X	20 µL	
100 mM ATP	7.5 µL	
100 mM GTP	7.5 µL	
100 mM CTP	7.5 µL	
100 mM UTP	7.5 µL	
CleanCap AG, 100 mM	7.5 µL	
DNA plasmid (1 µg/µL)	10 µL	
Sterile distilled water	22.5 µL	
Enzyme Mix (T7)	10 µL	
Total Volume	100 µL	

Option: It is possible to replace 25% of CTP and UTP with 5-methyl-CTP (cat# N-1014, TriLink) and Pseudo-UTP (cat# N-1019, TriLink), respectively. More than 25% of replacement, the expression of protein will be decreased. RNA reaction table is available in Appendix.

2. Incubate at 37°C for 2 hrs.

Note: Do not incubate beyond 2 hours at 37°C as a longer incubation period may result in increased RNA degradation.

Note: Do not freeze the completed transcription reactions.

3. Add 10 µL RQ1 RNase-free DNase (Promega M6101) for 10 µg of template DNA (use 1 µL per µg DNA template). Gently mix by pipetting up and down. Incubate for 15 minutes at 37°C.
4. Purify RNA with 2.5 M ammonium acetate precipitation by add an equal volume (110 µL) of 5 M ammonium acetate to a reaction mixture (Final: 2.5 M). **Do not add alcohol.** Mix gently by pipetting up and down.

Note: Do not vortex.

Note: The RNA reaction mixture becomes cloudy after the addition of ammonium acetate. 2.5 M ammonium acetate precipitates RNA while leaving most of the DNA, protein, and unincorporated NTPs in the supernatant.

5. Store on ice for 10 minutes. No more than 20 minutes. Long time incubation will degrade the RNAs.
6. Centrifuge at high speed (~14,000 rpm or 10,000 x g) for 15 minutes at 4°C to precipitate the RNA.
7. Remove the supernatant and wash the RNA pellet with 1 mL 70% ethanol to remove the salts.
8. Remove the ethanol carefully with a pipet tip, being careful not to dislodge the RNA pellet. If the next step will not proceed on the same day, store the RNA pellet at -80°C. RNA in this state is stable for at least a month.
9. Centrifuge at maximum speed (~14,000 rpm) for 3 minutes at 4°C. Remove any visible remaining ethanol with a P20 pipet. Drying the RNA pellet further is not necessary.
10. Resuspend RNA pellets in 100-200 µL in DNase-RNase free water. Store the eppendorf tube on ice after resuspension.

Note: RNA pellet will be large (~500 µg of RNA). Please refer the RNA suspension described in “Common Tips for RNA Synthesis (pg. 11)”. RNAs may also be sticky and transparent after addition of water, so make sure that there are no RNAs on the pipette tip before discarding the tip.

11. Measure the RNA concentration by spectrophotometer at 260 nm and adjust the final concentration to 2 µg/µL. Yield of the RNA at this reaction scale is typically 300-500 µg. Save a few micrograms for RNA gel electrophoresis.

12. Take 60-600 µg RNA for poly (A) tailing reaction. Residual RNA can be saved as a pellet at -80°C by 2.5 M ammonium acetate precipitation.

C2. Poly (A) tailing Reaction for B18R-E3L RNA and B18R RNA:

A long poly (A) tail sequence is **not** pre-incorporated in the B18R-E3L and B18R plasmids, so polyadenylation is required. Addition of optimal length of poly (A) tail (~200 bases in total) will result in several-fold increase of protein production. Use A-Plus™ Poly(A) Polymerase Tailing Kit (CellScript, C-PAP5104H).

The protocol below is designed for use with 60 µg of RNA (75.5 µL) in a 100 µL total reaction volume (1x scale).

1. Prepare 60 µg RNA from Step C1-12 in 75.5 µl RNase-free water on ice.
2. Set up the following reaction on ice in the order shown. Scale up according to the number of reactions required.

• Use A-Plus™ Poly(A) Polymerase Tailing Kit (CellScript, C-PAP5104H)		
Component	Amount (1X)	Amount (____X)
60 µg of RNA in water	75.5 µL	
Poly (A) tailing Buffer 10X	10 µL	
10 mM ATP	10 µL	
ScriptGuard RNase inhibitor (40 U/µl)	2.5 µL	
Poly(A) Polymerase (4 U/µl)	2 µL	
Total Volume	100 µL	

3. Mix gently by pipetting. Incubate at 37°C for **30 minutes. Do not incubate longer than 30 minutes.** Poly(A) tail addition is dependent on incubation time and units of poly(A) enzyme. 30 min incubation will add 100-200 bases of poly A tail. More than 200 bases addition of poly A tail will decrease the RNA stability.
4. Purify RNA with 2.5 M ammonium acetate precipitation by adding an equal volume of 5 M ammonium acetate (for a 1X scale, this would be 100 µL).
5. Store on ice for 10 minutes. No more than 20 minutes. Long time incubation will degrade the RNAs.
6. Centrifuge at maximum speed (~14,000 rpm or 10,000 x g) for 10 minutes at 4°C to precipitate the RNAs. Remove the supernatant, being careful not to dislodge the RNA pellet.

7. Wash the RNA pellet with 1 mL 70% ethanol to remove the salts.
8. Remove the ethanol carefully with a pipet tip, being careful not to dislodge the RNA pellet. **When the next step is not going to proceed on the same day, store the RNA pellet at – 80 °C.** RNA in this state is stable for at least several months.
9. Centrifuge at maximum speed (~14,000 rpm) for 1 minutes at 4°C. Remove any visible remaining ethanol with a P20 pipet. Drying the RNA is not necessary.
10. Resuspend RNA pellet in ~40 µL of RNA storage solution (ThermoFisher Scientific, AM7000). Store the eppendorf tube on ice after resuspension.
11. Measure the RNA concentration by spectrophotometer at 260 nm and adjust the final concentration to 1 µg/µL. About 10-20% of RNA will be lost from starting amount.
12. Aliquot (10~50 µL) and store the Simplicon™ RNA at -80°C until use. Repeated free-thaw will degrade Simplicon™ RNA. Save a few micrograms for RNA gel electrophoresis and functional quality check.

Quality Check I: RNA Electrophoresis

Check RNA quality by gel electrophoresis

1. Prepare 1-2 μg RNA from each steps and RNA marker (Thermofisher, RiboRuler High Range RNA marker, SM1821) in 10-25 μL of RNase free water depending on gel electrophoresis equipment system.
2. Add the same volume of 2x RNA loading Dye *, and incubate at 65 °C for 5 min, then place on ice.

*2X RNA loading Dye: Use the 2X RNA loading dye that is available with RiboRuler High Range RNA marker, #SM1821. Alternatively, 2X RNA loading dye may be prepared with 95% formamide (deionized), 0.025% SDS, and 0.5 mM EDTA (Store at -20 °C). Addition of 0.025% bromophenol blue, 0.025% xylene cyanol FF and 0.025% ethidium bromide is optional.

3. Incubate each RNA sample at 65 °C for 5 min, and then transfer to ice.
4. Prepare a 1% agarose denaturing gel containing with 1x MOPS buffer and 2.2 M formaldehyde as bellow.

1% agarose denaturing gel:
6.5 mL 10X MOPS
55.0 mL sterile distilled water
0.65 g Agarose
3.5 mL 37% Formaldehyde
~65 mL total volume

Note: Add the formaldehyde to agarose melted solution after cooling at ~60 °C in the chemical hood.

Alternatively, Pre-Cast agarose gels (ThermoFisher, E-Gel Precast Agarose Gel, G501808 & G501802) are available for RNA electrophoresis.

5. Load appropriate amount (0.4~2 μg) of RNAs on 1% agarose denaturing gel and run the gel (10 V/cm) with 1x MOPS buffer.
6. Stain RNA with the appropriate dyes (Ethidium bromide, SYBR safe etc.). Check RNA band under UV light. Simplicon™ RNA is large sized, so some degradation of the RNA is generally observed. B18R-E3L RNA will show a slight increased size after poly(A) tailing.

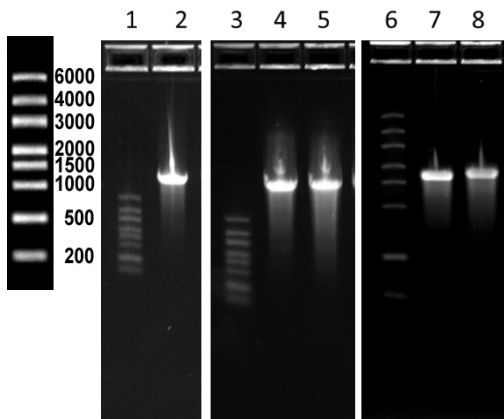


Figure 4. Electrophoresis of Simplicon™ RNA (~14kb) with 0.8 % E-gel and B18R-E3L RNA (~2kb) with 2% E-gel.

Lane 1, 3, 6: RNA Marker
Lane 2: Simplicon™ RNA with CleanCap™
Lane 4: Simplicon™ RNA, T7 transcribed
Lane 5: Simplicon™ RNA, T7 transcribed, Enzymatic capping, phosphatase treated
Lane 7: B18R-E3L RNA with CleanCap™, before poly (A) tail addition
Lane 8: B18R-E3L RNA with CLeanCap™, after poly (A) tail addition

Quality Check II: RNA Transfection

The quality of the RNAs generated may be assessed by transfection into a control cell line. For best results, we recommend using the following tested reagents:

- Human foreskin fibroblasts (HFF), MilliporeSigma SCC058
 - Simplicon™ E3L Reporter Controls:
 - TagGFP2 Simplicon™ RNA (E3L) Control, MilliporeSigma SCR720
 - TagRFP Simplicon™ RNA (E3L) Control, MilliporeSigma SCR721
 - B18R-E3L RNA (human codon optimized for B18R and E3L), MilliporeSigma SCR722
 - MessengerMAX™ Transfection Reagent: ThermoFisher LMRNA001
1. Plate 400,000 Human foreskin fibroblasts (HFF) cells per well of a tissue culture treated 6-well plate in DMEM-high glucose medium containing 10% FBS. Incubate at 37°C in a CO₂ incubator.
 2. On the day of transfection, cells should be 80-100% confluent.
 3. Wash cells once with DMEM (no serum, no antibiotics) and add 1 mL/well of DMEM (no serum, no antibiotics). The absence of serum increases the transfection efficiency. However, it is possible to use 1-10% serum depending upon cell types. Incubate cells at 37°C in a CO₂ incubator (10-20 minutes).
 4. Set up the following transfection reactions in sterile eppendorf tubes. **Do not vortex.**

Step 1		Prepare RNA Mixture in Tube 1			
		Negative Control	Positive Control	TagRFP-E3L Simplicon™ Control + Your B18R-E3L RNA	Your Simplicon™ RNA + B18R-E3L Control
Tube 1	DMEM (no serum, no antibiotics)	50 µL	100 µL	100 µL	100 µL
	B18R-E3L RNA (1 µg/µL)	0 µL	1 µL B18R-E3L control	1 µL Your B18R-E3L RNA	1 µL B18R-E3L control
	Simplicon™ RNA (1 µg/µL)	1 µL Your Simplicon™ RNA	1 µL TagRFP-E3L Simplicon™ control	1 µL TagRFP-E3L Simplicon™ Control	1 µL Your Simplicon™ RNA
	Total Volume	51 µL	102 µL	102 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	100 µL	100 µL
	MessengerMAX™ Transfection Reagent	3 µL	6 µL	6 µL	6 µL
	Total Volume	53 µL	106 µL	106 µL	106 µL
Step 3		Quickly add tube 2 into tube 1. Do not vortex.			
Total RNA amount in tube		1 µg/ 104 µL	2 µg/ 208 µL	2 µg/ 208 µL	2 µg/ 208 µL

5. Incubate at room temperature for 5 minutes and add the RNA-transfection reagent complex **dropwise** into the 6-well plate containing cells.
6. Incubate the plate at 37°C in a CO₂ incubator for 4 hrs.
7. Aspirate the transfection medium and add 2 mL per well of culture medium. Incubate at 37°C in a CO₂ incubator overnight.
8. Analyze Simplicon™ RNA expression one day after transfection using flow analysis if your Simplicon™ RNA contains a fluorescent protein reporter. Transgene expression can also be confirmed by Western blotting etc.

Note: For continuous cell culture, B18R protein is required in culture medium and puromycin selection is recommended.

Interpretation of Results:

- **Positive Control:** Strong RFP expression should be observed due to the presence of B18R-E3L RNA control.
- **Negative Control:** Weak transgene or RFP expression is expected as there is no B18R-E3L RNA control to protect the Simplicon™ RNA.
- **TagRFP-E3L Simplicon™ RNA control + Your B18R-E3L RNA:** If your B18R-E3L RNA is of sufficient high quality, you should obtain similar RFP expression levels as the positive control. Weak RFP expression is indicative of poor B18R-E3L RNA quality. If weak RFP expression is observed, redo RNA synthesis. Poor quality is generally derived from efficiency of capping or length of poly (A) tailing.
- **Your Simplicon™ RNA + B18R-E3L RNA control:** If your Simplicon™ RNA is of sufficient high quality, you should obtain higher transgene expression levels than the negative control (i.e. does not contain B18R-E3L RNA). If your Simplicon™ RNA is of poor quality, the transgene expression levels will be similar to the negative control. Poor quality is generally derived from degradation of RNA, capping efficiency, and others. Please see “Troubleshooting” for details.

Preparation of B18R Conditioned Medium (B18R-CM):

B18R protein and the E3L gene product work to inhibit the IFN responses caused by RNA transfection and RNA replication, and are thus critical to conferring protection to the Simplicon™ RNA. Conditioned medium containing B18R protein is an efficient and cost-effective alternative to recombinant B18R protein. B18R-CM can be produced by transfection of the B18R-E3L RNA or B18R RNA to human foreskin fibroblasts (HFF). One day after transfection, the conditioned medium is collected and filter sterilized using a 0.22 µm filter. B18R-CM can be produced with a wide variety of commonly used basal medium such as DMEM, with 1-10% serum supplementation, Opti-MEM, Keratinocyte SF-Medium and also conventional ES culture medium. B18R-CM can be stored at -20°C for several months and be used experimentally at a 10-20% final dilution. B18R is a secretory protein, so it will be secreted into culture medium soon after the translation, while E3L is cytoplasmic protein, so it will remain in cells. Therefore, B18R-CM from B18R-E3L RNA contains only B18R protein.

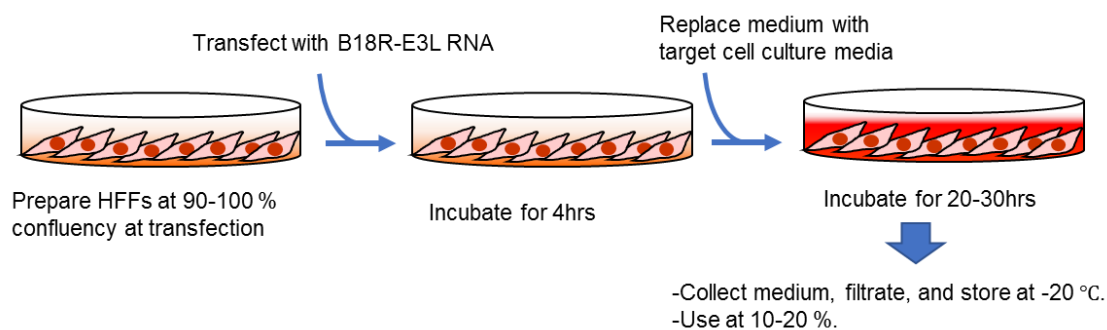


Figure 5. Scheme for production of B18R-CM.

The following protocol is based on transfection of a 10-cm dish of cells. Scale up or down accordingly depending upon the dish or flasks used.

1. Plate Human Foreskin Fibroblasts (MilliporeSigma SCC058) or equivalent human fibroblasts on tissue culture plates at a cell density that would allow the cells to reach 90-100% confluency the next day. Highly proliferative fibroblasts are recommended.
2. On the day of transfection, aspirate the medium and add 5 mL DMEM (no serum, no antibiotics) to the 10-cm dish of cells.
3. Prepare 6 µg B18R-E3L RNA with 300 µl DMEM (no serum, no antibiotics) in a 1st eppendorf tube and prepare 18 µl MessengerMax transfection reagent with 300 µl DMEM (no serum, no antibiotics) in a 2nd eppendorf tube. Mix together as soon as possible after dilution, and incubate for 5 min at room temperature.
4. Add B18R-E3L RNA transfection complexes (600 µl/10 cm dish) dropwise to the 10-cm plate of cells. Incubate for 4 hours at 37 °C in a CO₂ incubator.
5. After 4 hours, aspirate the medium and add 10 mL of the basal medium that will be used to culture the target cells (i.e. DMEM, Opti-MEM, ES culture media, etc). Incubate for 20-30 hr at 37 °C in a CO₂ incubator.

6. One day after transfection, collect the B18R-CM (approximately 10 mL). Filter sterilize using a 0.22 μm low protein binding membrane (MilliporeSigma SCGP00525, SCGPU05RE). Aliquot depending on your experimental scale and store at -20°C until used. B18R-CM is stable at -20°C for several months and for several days at 4°C . Avoid repeated freeze-thaw of the B18R conditioned medium.

Quality Check of B18R-CM:

The quality of the B18R-CM can be assessed by transfection with Simplicon™ TagGFP2 or TagRFP RNA into HFFs. For best results, we recommend using the following tested reagents:

- Human foreskin fibroblasts (HFF), MilliporeSigma SCC058
 - Simplicon™ Reporter Controls:
 - TagGFP2 Simplicon™ RNA (E3L) Control, MilliporeSigma SCR720
 - TagRFP Simplicon™ RNA (E3L) Control, MilliporeSigma SCR721
 - Recombinant B18R protein: MilliporeSigma GF156 or Sigma GF197
 - MessengerMAX™ Transfection Reagent: ThermoFisher LMRNA001
1. Plate Human Foreskin Fibroblasts (MilliporeSigma SCC058) into a 24-well plate at a cell density that would allow the cells to reach 90-100% confluency on the day of transfection.
 2. On the day of transfection, set up serial dilution of the B18R-CM with 0.5 mL total volume per well. Dilute B18R-CM with DMEM containing 5% serum. Set up wells to receive 0%, 5%, 10% and 20% dilution of B18R-CM:
0% Dilution = 500 μL DMEM containing 5% FBS
5% Dilution = 25 μL B18R-CM + 475 μL DMEM containing 5% FBS
10% Dilution = 50 μL B18R-CM + 450 μL DMEM containing 5% FBS
20% Dilution = 100 μL B18R-CM + 400 μL DMEM containing 5% FBS
Positive Control = 200 ng/mL B18R protein (MilliporeSigma GF156 or Sigma GF197) + 500 μL DMEM containing 5% FBS
 3. Aspirate medium from each well. Add the serial B18R-CM dilutions prepared in Step 2 to the appropriate wells. Be sure to include the positive control. Incubate for 15-30 minutes at 37°C in a CO_2 incubator before starting the transfection.

Note: Start the treatment of B18R-CM before Simplicon™ RNA transfection.

4. Prepare the transfection mixture of Simplicon™ TagRFP with MessengerMax transfection reagent. Use 0.2 μg Simplicon™ TagRFP per well (24-well plate). For 10 wells-transfection in total, prepare 2 μg Simplicon™ TagRFP with 100 μL DMEM (no serum, no antibiotics) in 1st tube and 6 μL transfection reagent with 100 μL DMEM (no serum, no antibiotics) in 2nd tube. Mix together as soon as possible after dilution, and incubate for 5 min at room temperature.

Note: This is the test for B18R-CM, so do not co-transfection of B18R-E3L RNA.

5. Incubate at room temperature for 5 minutes and add the transfection complex (20 μL /well) **dropwise** into cells.

6. Incubate the plate overnight at 37°C in a CO₂ incubator.
7. Analyze Simplicon™ TagRFP expression one day after transfection using a fluorescent scope or flow analysis. B18R-CM treated cells and positive control wells should contain 30-60% RFP positive cells, while the negative control cells (0% B18R CM) will have little to no RFP expression.

Note: *B18R-CM and recombinant protein can work at transfection of Simplicon™ RNA, but it is not strong compared to the co-transfection with B18R-E3L RNA.*

Simplicon™ RNA Transfection and Electroporation Methods:

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

A) Forward Transfection Protocol

1. Plate target cells to reach 50-90% confluency at time of transfection in 6-well plate. 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 (MilliporeSigma GF156 or Sigma GF197) in medium. Pre-treatment of B18R protein may support the neutralization of IFN responses. Incubate cells at 37°C in a CO₂ 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 (ThermoFisher LMRNA001):

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
	Simplicon™ RNA (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 (Part No. TR-1013):

Component	Vial	Cat. No.
Opti-MEM®	250 µL	ThermoFisher (31985-062)
B18R-E3L RNA (1 µg/µL)	0.5 µL	
Simplicon™ RNA (1µg/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 at 37°C in a CO₂ 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 10-20% of B18R-CM or 200 ng/mL B18R protein. Incubate at 37°C in a CO₂ incubator overnight.
7. Next day, aspirate and exchange with fresh culture medium containing 10-20% of B18R-CM or 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.
8. Change medium every day for sustaining expression of Simplicon™ RNA. Add fresh 10-20% of B18R-CM or 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™ RNA 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 10-20% of B18R-CM or B18R protein (50-200 ng/mL).
9. Analyze cells depending on experiments.

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.

B) 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 the time of transfection.
2. On the day of transfection, 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 a 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 at 37°C in a CO₂ incubator while you prepare the RNA transfection mixture as previously outlined in step 3 of the Forward Transfection Protocol.
5. Follow Steps 4 – 9 of the Forward Transfection Protocol.

C) Electroporation for primary T cells (human)

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 950 µF.
3. Prepare 1x10⁶ cells in Ingenio® Electroporation Solution (Mirus Bio MIR50111) in 100 µL total volume. Transfer cell mixture to an electroporation cuvette (0.2 cm-gap). Store on ice.
4. Prepare RNA mixture: 5 µg of Simplicon™ RNA plus 5 µg of B18R-E3L RNA.
5. Add RNAs mixture to cells. RNAs are not stable after mixing with cells.
6. Do electroporation as soon as possible.
7. Put on ice for a few minutes, and then transfer the cells into appropriate wells of a 48 well plate with 1 mL of cell culture medium containing 200 ng/mL B18R protein.
8. Incubate at 37°C in a CO₂ incubator overnight.
9. Assay protein expression of Simplicon™ RNA using flow analysis or ICC.
10. For continuous expression of the Simplicon™ RNA, B18R protein or B18R-CM and puromycin should be present throughout the duration of the cell culture experiment. B18R protein and B18R-CM and puromycin should be added fresh during media changes. 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.

Representative Data of Transfection Experiments:

In the Simplicon™ RNA Expression System, a new IFN suppressor, E3L, was introduced as a polycistronic B18R-E3L RNA to suppress the IFN responses at RNA transfection and also incorporated into Simplicon™ RNA itself to suppress the IFN responses during RNA replication. B18R-E3L RNA worked better than B18R RNA at the transfection with Simplicon™ TagGFP2 (Figure 6A). Addition of E3L into Simplicon™ RNA increased the expression of Simplicon™ TagRFP in sustained expression (Figure 6B). Simplicon™ RNA can be introduced with RNA transfection or RNA electroporation in many types of cells as indicated (Figure 6C).

B18R-E3L RNA is also available for mRNA transfection to suppress the IFN responses. B18R-E3L RNA worked better than B18R-RNA in mRNA transfection (Figure 7A). Suppression of IFN responses at mRNA transfection enables the repeated transfection of mRNA (Figure 7B).

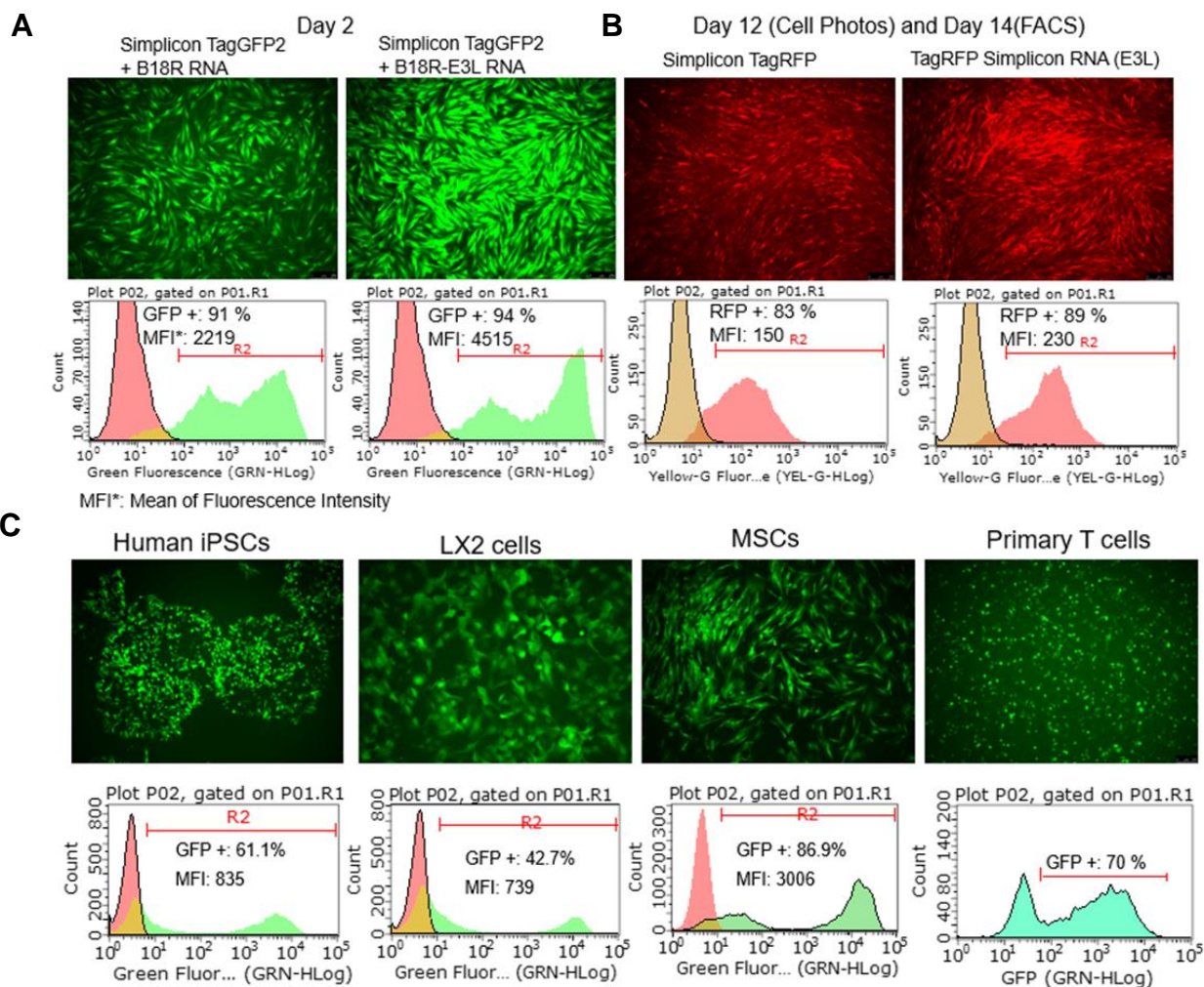


Figure 6. (A) E3L increased Simplicon™ RNA expression levels. BJ human foreskin fibroblasts were co-transfected with Simplicon™ TagGFP2 and B18R RNA or B18R-E3L RNA. (B) E3L worked for continuous expression of Simplicon™ RNA. Simplicon™ TagRFP or TagRFP Simplicon (E3L) was co-transfected with B18R-E3L RNA and cultured with medium containing B18R protein and puromycin for 14 days. RFP expressing cells were imaged on Day 12, and analyzed by FACS on Day 14. (C) Simplicon™ RNA can be transfected into a wide variety of cell types. Simplicon™ TagGFP2 RNA and B18R-RNA were co-transfected with Human iPSCs, LX2 human hepatic stellate cell line (MilliporeSigma SCC064), human mesenchymal stem cells (MSCs, MilliporeSigma SCC034) by MessengerMAX™ transfection reagent. For human primary T cells (PBMCs stimulated with CD3/CD28), electroporation method was used.

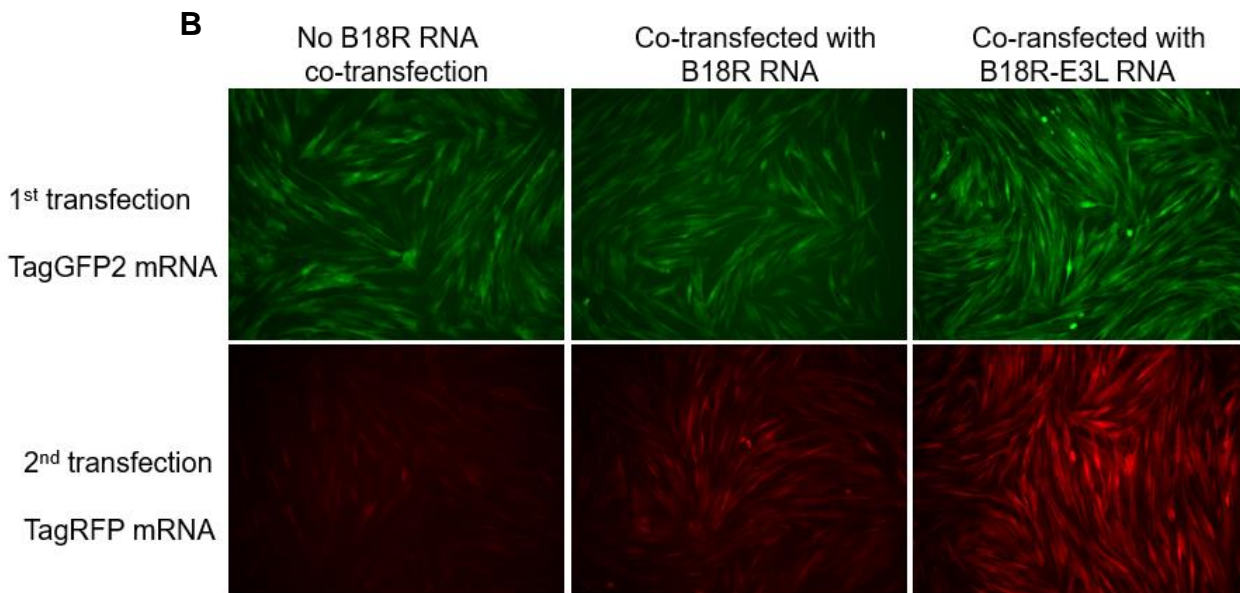
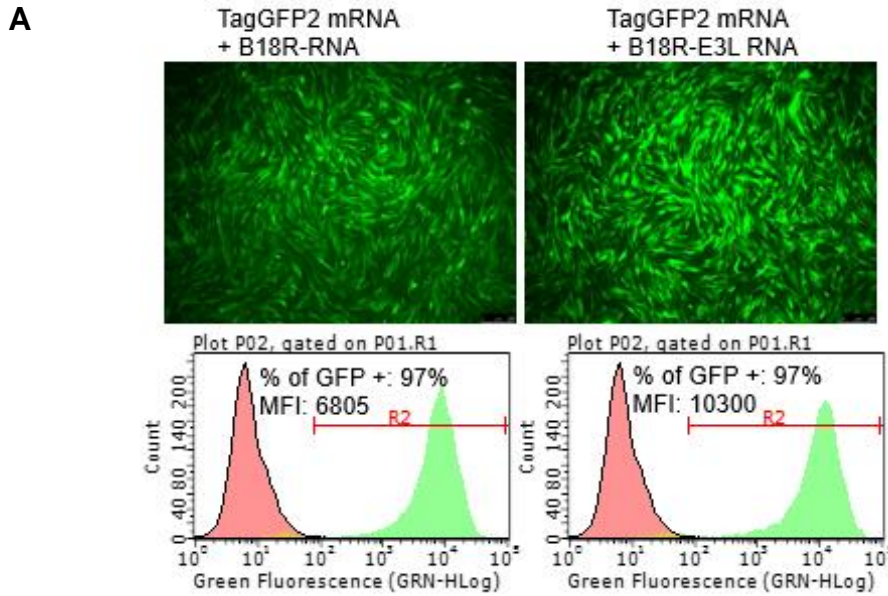


Figure 7. B18R-E3L or B18R RNA for mRNA transfection. **(A)** BJ human foreskin fibroblasts were co-transfected with TagGFP2 mRNA and B18R RNA or B18R-E3L RNA. **(B)** B18R or B18R-E3L RNA co-transfection enables repeated transfection of mRNA. BJ cells were transfected with TagGFP2 mRNA Plus/Minus B18R RNA or B18R-E3L RNA at 1st transfection (upper panel, 1st transfection). Next day, TagRFP mRNA was transfected into the same cells, and imaged for RFP expression one day after the transfection (bottom panel, 2nd transfection). The mRNAs for TagGFP2 and TagRFP were synthesized without modified nucleotides.

Appendix

Troubleshooting:

No or Low RNA yield

1. Check DNA template: Check the amount, quality and size of the linearized DNA (single digestion). Contamination of phenol & chloroform will reduce the activity of the T7 RNA polymerase. Wash DNA pellet with 70% ethanol to remove salts and impurities.
2. Check transcription reaction mixture: Make sure that all of the components are included in the RNA transcription mixture. 5x buffer may have a precipitate after thawing; ensure that 5x buffer is fully resuspended and that the correct amount is added to the transcription reaction mixture.
3. T7 RNA polymerase: The T7 promoter sequence in the Simplicon™ plasmid has been optimized for expression of Simplicon™ RNA. Not all T7 RNA Polymerase will work effectively in the Simplicon™ RNA synthesis. We recommend the use of T7 RNA polymerase from Promega (Cat. No. P1300).

RNA degradation is observed

1. Use RNase-free reagents and tips.
2. Use high quality (transfection grade) DNA plasmid and freshly prepared DNA template for the RNA transcription reaction.
3. Because Simplicon™ RNA is a large sized RNA, some amount of degradation is expected as shown in the RNA electrophoresis gel on pg. 22.
4. Reaction time of RNA transcription: 2 hours reaction time is optimal for minimizing RNA degradation while still yielding high amounts of RNA. One hour incubation may be tried, but RNA yields may be lower.
5. Use RNA storage solution (ThermoFisher AM7000) for long-term storage of RNA at -80°C. TE buffer or water should not be used.

Significant RNA loss after 2.5 M ammonium acetate precipitation

1. A 10-20% loss of RNA may be expected with 2.5 M ammonium acetate precipitation.
2. The quality of 5 M ammonium acetate may affect the RNA yields. Store 5 M ammonium acetate at 2-8°C to maintain high quality.
3. Alternatively, RNA purification columns are available for RNA purification.

Transfection Problems

1. MessengerMax (ThermoFisher LNRNA001) can work for a broad range of cell types. While adult keratinocytes can be transfected using MessengerMax, neonatal keratinocytes are more difficult to transfect. Different type of transfection reagents and method may be used such as RiboJuice™ mRNA Transfection Kit (MilliporeSigma TR-1013,), or electroporation with Ingenio® Electroporation Solution (Mirus Bio MIR 50111). In general, lipid based DNA transfection reagent such as Lipofectamine2000 can work 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.
3. In general, the size of transgenes along with the number of transgenes to be expressed will have an impact on expression levels and transfection efficiency. The smaller size of the transgenes will get the higher the expression levels and transfection efficiency.
4. Dead cells are observed after transfection.
 - a. Reduce the amount of RNAs to be transfected
 - b. Try a different transfection reagent.
 - c. Pretreat with B18R protein before and after transfection.

No protein expression

1. B18R-E3L RNA is required for expression of Simplicon™ RNA at transfection. Do quality check of B18R-E3L RNA by co-transfection with Simplicon™ TagGFP2 (MilliporeSigma SCR720) or TagRFP (MilliporeSigma SCR721).
2. Check DNA sequence of Simplicon™ plasmid: Confirm DNA sequence of insert gene(s) and 26S subgenomic promoter region that is required for transcription of insert gene(s).
3. In general, the size of transgenes along with the number of transgenes to be expressed will have an impact on expression levels and transfection efficiency. The smaller size of the transgenes will get the higher the expression levels and transfection efficiency.

No continuous expression

1. Do puromycin selection: It is better to remove un-transfected cells by puromycin selection. High doses of puromycin selection may kill all the cells. Simplicon™ RNA expression is high for a few days after transfection, and becomes stable at low levels in a week. Puromycin is located after the second IRES and the last gene of the Simplicon™ RNA. Therefore, puromycin selection can be performed at very low amount of puromycin (0.2-1 $\mu\text{g}/\text{mL}$; mostly 0.2-0.4 $\mu\text{g}/\text{mL}$) for 5-7 days selection.
2. B18R is needed for continuous expression. Change cell culture medium every day and freshly supply B18R conditioned medium (CM) or B18R protein. If you are using a recombinant B18R protein, a recombinant B18R protein from HEK293 (Sigma GF197) will work for long term sustained expression.

3. Continuous expression is dependent on cell types, insert genes, and culture conditions.

No good quality of B18R conditioned medium

1. Highly proliferative fibroblasts such as Human Foreskin Fibroblasts (MilliporeSigma SCC058) is recommended for production of B18R-CM. At least, HFFs (MilliporeSigma SCC058) is more proliferative and expressed more proteins compared to BJ fibroblasts.
2. B18R-CM can work for expression of Simplicon™ RNA, but weaker compared to the co-transfection with B18R-E3L RNA.
3. Check the quality of B18R-E3L RNA as described in “Quality Check II: RNA Transfection”.
4. Passage HFFs one day before the B18R-E3L RNA transfection. Condition of HFFs is important to get high transfection efficiency and protein production. Transfection efficiency of HFFs will decrease in a few days after passaging.

Q&A:

What is the maximum size of inserts that can be cloned into the Simplicon™ Cloning Vector (E3L)?

The maximum size of inserts that can be accommodated is ~8,300 bp **at least**. For example, Human OKSG-cMyc TagRFP Simplicon™ plasmid (Cat# SCR729) contains 8,301 bp of insert in Simplicon™ plasmid. The expected Simplicon™ RNA size of Human OKSG-cMyc TagRFP Simplicon™ is ~17.5 kb.

How much and how long does Simplicon™ RNA express?

In general, small sized insert can express much more proteins. Expression of the transgenes will decrease depending on size and numbers of insert gene(s). Duration of expression depends on cell types, insert genes, and culture conditions. In our experience, Human OKSG-cMyc TagRFP Simplicon™ RNA can continuously express the TagRFP in human foreskin fibroblasts with DMEM/10% FBS medium (HFF culture medium) containing B18R proteins and puromycin, while most iPSCs colonies generated with Human OKSG-cMyc TagRFP Simplicon™ RNA (ES culture medium containing B18R and puromycin) lose the TagRFP expression in 15 days. If you are using a recombinant B18R protein, a recombinant B18R protein from HEK293 (GF197) will work for long term sustained expression.

Is phosphatase treatment required for Simplicon™ RNA?

Phosphatase treatment is required for Simplicon™ RNA with three-step RNA synthesis (Enzymatic capping method). If Simplicon™ RNA is generated with one-step RNA synthesis using CleanCap AG reagent, it is not necessary.

How long can a Simplicon™ RNA remain stable?

1. Simplicon™ RNA is stable for at least 1 year in RNA storage solution at -80 °C. Avoid freeze thaws.
2. The RNA pellet after 2.5 M ammonium acetate precipitation is stable for several months at -80°C. Therefore, it is possible to stop the reaction of RNA synthesis after 2.5 M ammonium acetate. Always wash the RNA pellet with 70% ethanol to remove residual ammonium acetate, and then store the RNA pellet at -80°C until restart.

Are modified nucleotides required for RNA synthesis?

It is not necessary to use modified nucleotides such as 5-Methyl-CTP and Pseudo-UTP for either the Simplicon™ and B18R-E3L RNA syntheses. When modified nucleotides are used, do not replace more than 25% of CTP and UTP. 100 % replacement of CTP and UTP with modified nucleotides will lose the expression of Simplicon™ RNA. Higher replacement will decrease the protein expression of B18R-E3L RNA.

Does the Simplicon™ System work in mouse cells?

No. At this time, the Simplicon™ System does not work on mouse cells. This is because the B18R gene products do not effectively neutralize the mouse interferon (IFN-β) response.

Related Products:

SCR720	TagGFP2 Simplicon™ RNA (E3L) Kit
SCR721	TagRFP Simplicon™ RNA (E3L) Kit
SCR722	B18R-E3L RNA (human codon optimized for B18R and E3L)
SCR723	B18R RNA (human codon optimized)
SCR729	Human OKSG-cMyc TagRFP Simplicon™ Plasmid

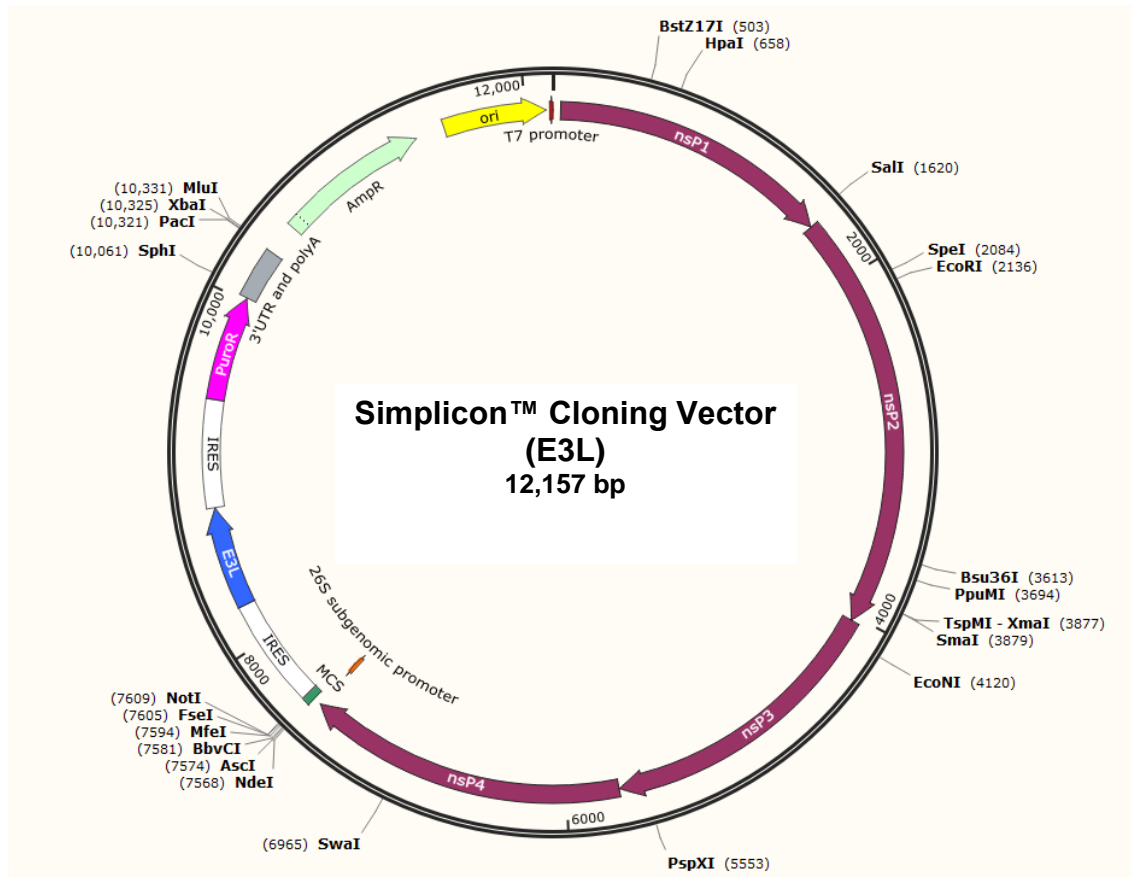
References:

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Plasmid Maps:

Simplicon™ Cloning Vector (E3L), (Cat. No.SCR724)



T7 promoter: Partial promoter for bacteriophage T7 RNA polymerase. Allows *in vitro* transcription of the Simplicon™ RNA.

Non-structural genes (nsP1-4): Encodes four nonstructural proteins (nsP1-4) that are responsible for replication of Simplicon™ RNA (genomic RNA) and transcription of subgenomic RNA (your genes, E3L and puromycin).

26S Subgenomic Promoter: Promotes the transcription of subgenomic RNAs with nsP proteins.

Multiple Cloning Site (MCS): Restriction enzyme sites for cloning of your gene(s)

IRES: Internal Ribosome Entry Site. Allows for translation of E3L and Puromycin genes.

E3L: Encodes Vaccinia virus E3L gene. Human codon optimized.

PuroR: Encodes puromycin resistance gene. Confers resistance to puromycin.

3' UTR: Partial 3' UTR from VEE RNA replicon.

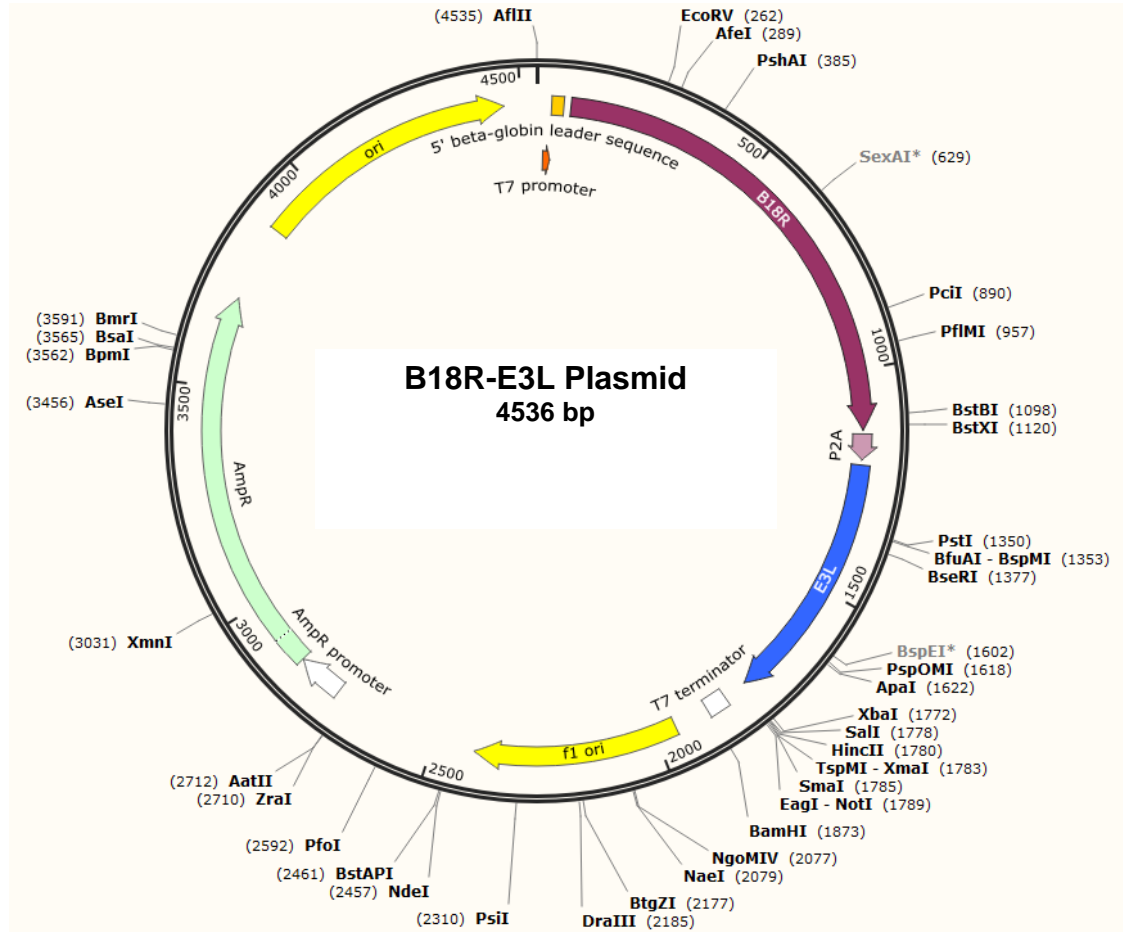
Poly (A): Long poly (A) tail (175 nts) is incorporated into the cloning vector and thus the poly (A) adenylation reaction is no longer required.

AmpR: Ampicillin resistance gene. Confers resistance to ampicillin in *E. coli*.

Ori: high-copy-number ColE1/pMB1/pBR322/pUC origin of replication in *E. coli*.

Full DNA sequence is available from website (www.emdmillipore.com)

B18R-E3L Plasmid (human codon optimized for B18R and E3L): (Cat. No. SCR727)



T7 promoter: Minimum promoter for bacteriophage T7 RNA polymerase. Allows *in vitro* transcription of the B18R-E3L-RNA.

5' β -globin leader sequence: Increases the translation of several genes for more rapid initiation of translation.

B18R: Encodes Vaccinia virus B18R gene. Human codon optimized.

P2A: P2A peptide from porcine teschovirus-1 polyprotein. Allows the polycistronic expression of B18R and E3L proteins.

E3L: Encodes Vaccinia virus E3L gene. Human codon optimized.

T7 terminator: transcription terminator for bacteriophage T7 RNA polymerase

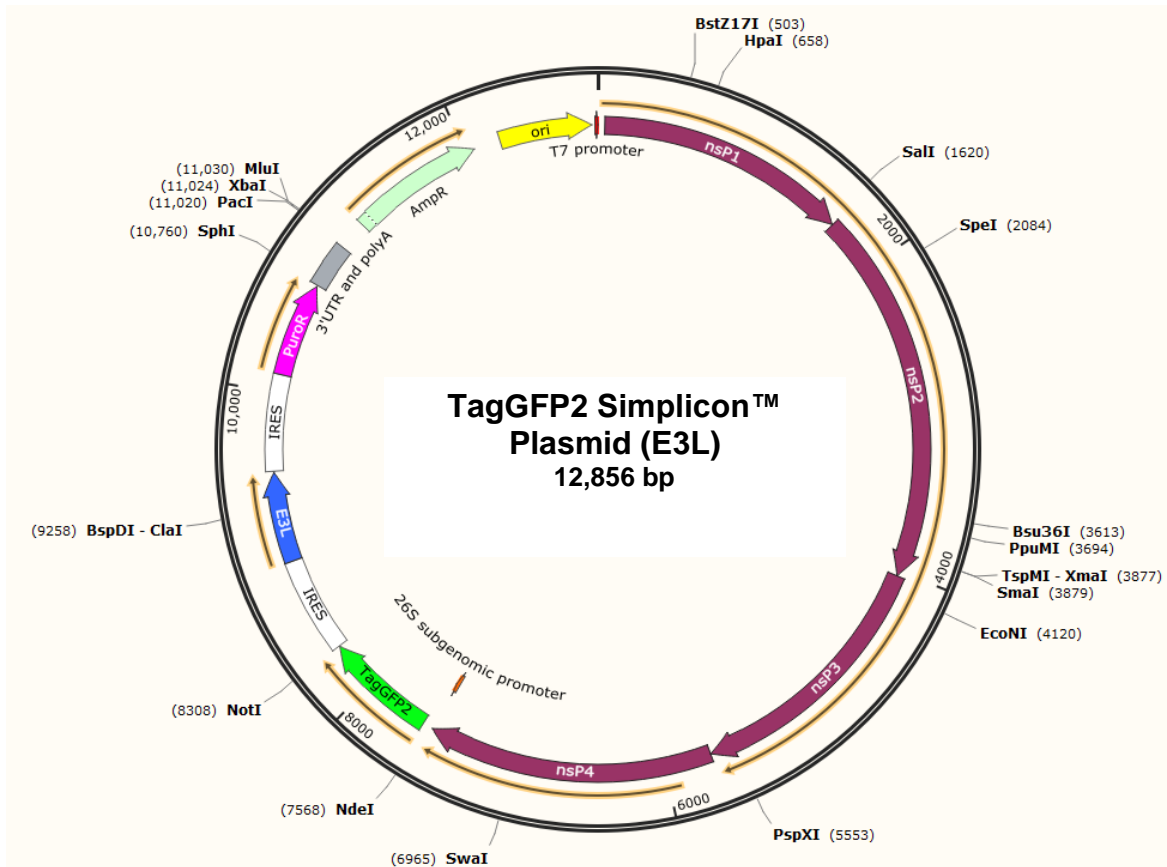
Poly (A) tail: 30 bases of poly A tail has been added in plasmid backbone. 30 bases of poly A is not enough length to stabilize RNA. Poly (A) adenylation reaction is required.

AmpR: Ampicillin resistance gene. Confers resistance to ampicillin in *E. coli*.

Ori: high-copy-number ColE1/pMB1/pBR322/pUC origin of replication in *E. coli*.

Full DNA sequence is available from website (www.emdmillipore.com)

TagGFP2 Simplicon™ Plasmid (E3L) (Cat. No. SCR725)



T7 promoter: Partial Promoter for bacteriophage T7 RNA polymerase. Allows *in vitro* transcription of the Simplicon™ RNA.

Non-structural genes (nsP1-4): Encodes four nonstructural proteins (nsP1-4) that are responsible for replication of Simplicon™ RNA (genomic RNA) and transcription of subgenomic RNA (your genes, E3L and puromycin).

26S Subgenomic Promoter: Promotes the transcription of subgenomic RNAs with nsP proteins.

TagGFP2: Encodes TagGFP2 gene.

IRES: Internal Ribosome Entry Site. Allows for translation of E3L and Puromycin genes.

E3L: Encodes Vaccinia virus E3L gene. Human codon optimized.

PuroR: Encodes puromycin resistance gene. Confers resistance to puromycin.

3' UTR: Partial 3' UTR from VEE RNA replicon.

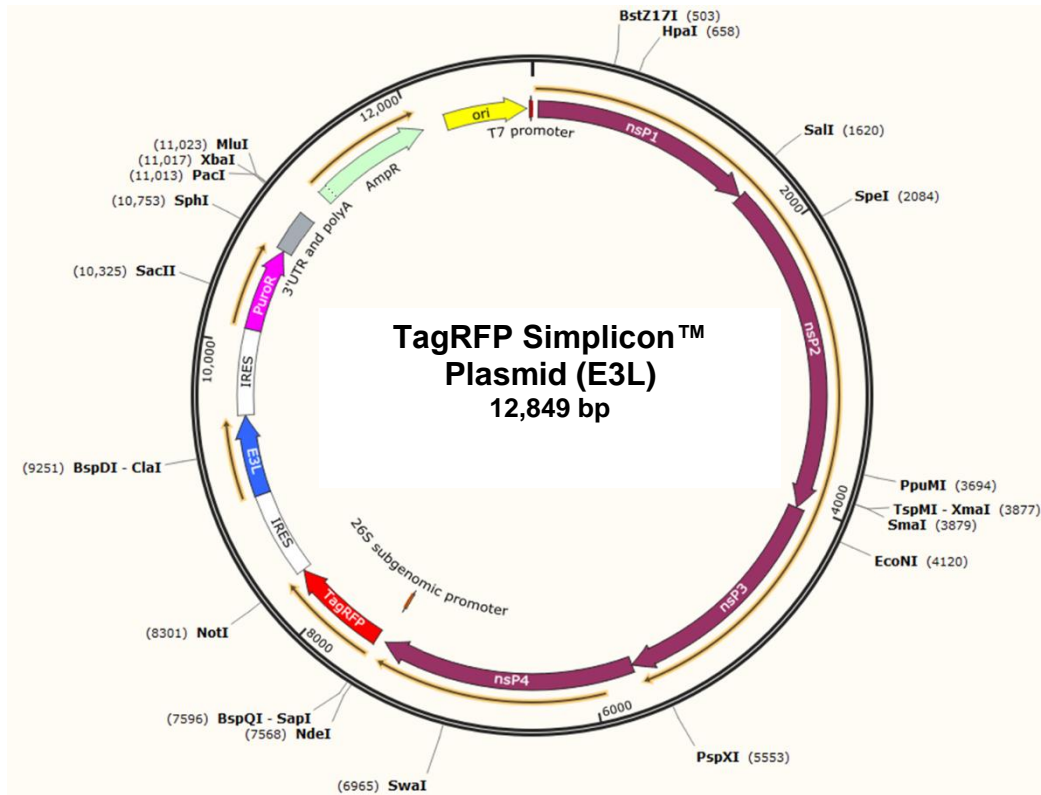
Poly (A): 50 bases x3 (total 150 bases) of poly (A) tail was added in the original poly (A) tail (25 bases) of backbone vector. Addition of poly (A) tail allows to omit the poly (A) tail reaction at RNA synthesis.

AmpR: Ampicillin resistance gene. Confers resistance to ampicillin in *E. coli*.

Ori: high-copy-number ColE1/pMB1/pBR322/pUC origin of replication in *E. coli*.

Full DNA sequence is available from website (www.emdmillipore.com)

TagRFP Simplicon™ Plasmid (E3L) (Cat. No. SCR726)



T7 promoter: Partial Promoter for bacteriophage T7 RNA polymerase. Allows *in vitro* transcription of the Simplicon™ RNA.

Non-structural genes (nsP1-4): Encodes four nonstructural proteins (nsP1-4) that are responsible for replication of Simplicon™ RNA (genomic RNA) and transcription of subgenomic RNA (your genes, E3L and puromycin).

26S Subgenomic Promoter: Promotes the transcription of subgenomic RNAs with nsP proteins.

TagRFP: Encodes TagRFP gene.

IRES: Internal Ribosome Entry Site. Allows for translation of E3L and Puromycin genes.

E3L: Encodes Vaccinia virus E3L gene. Human codon optimized.

PuroR: Encodes puromycin resistance gene. Confers resistance to puromycin.

3' UTR: Partial 3' UTR from VEE RNA replicon.

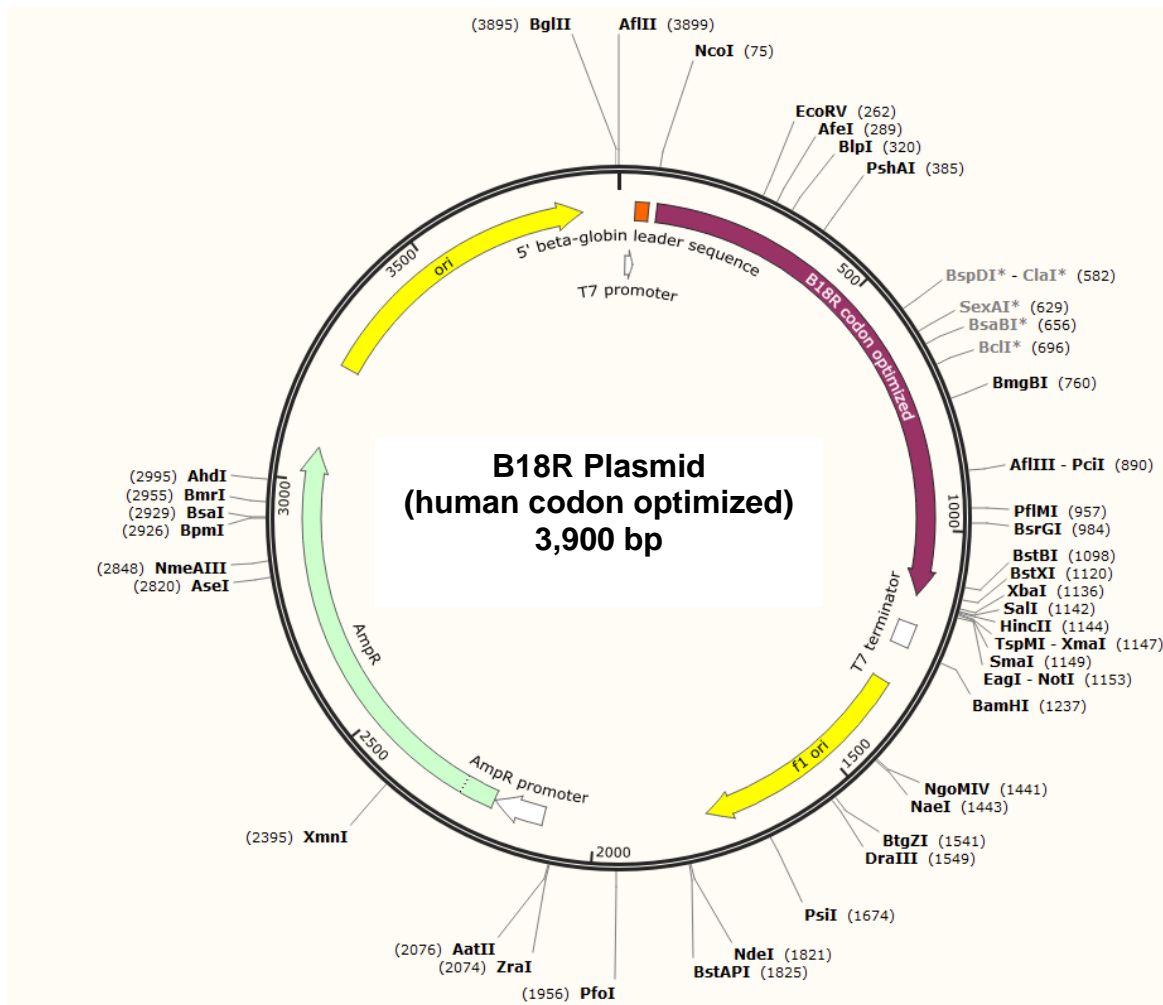
Poly (A): 50 bases x3 (total 150 bases) of poly (A) tail was added in the original poly (A) tail (25 bases) of backbone vector. Addition of poly (A) tail allows to omit the poly (A) tail reaction at RNA synthesis.

AmpR: Ampicillin resistance gene. Confers resistance to ampicillin in *E. coli*.

Ori: high-copy-number ColE1/pMB1/pBR322/pUC origin of replication in *E. coli*.

Full DNA sequence is available from website (www.emdmillipore.com)

B18R Plasmid (human codon optimized): (Cat. No. SCR728)



T7 promoter: Minimum Promoter for bacteriophage T7 RNA polymerase. Allows *in vitro* transcription of the B18R-E3L-RNA.

5' β -globin leader sequence: Increases the translation of several genes for more rapid initiation of translation.

B18R: Encodes Vaccinia virus B18R gene. Human codon optimized.

T7 terminator: transcription terminator for bacteriophage T7 RNA polymerase

Poly (A) tail: 30 bases of poly A tail has been added in plasmid backbone. 30 bases of poly A is not enough length to stabilize RNA. Poly (A) adenylation reaction is required.

AmpR: Ampicillin resistance gene. Confers resistance to ampicillin in *E. coli*.

Ori: high-copy-number ColE1/pMB1/pBR322/pUC origin of replication in *E. coli*.

Full DNA sequence is available from website (www.emdmillipore.com)

RNA Synthesis Tables:

T7 Transcription with CleanCap-AG and Poly (A) Tailing:

RNA transcription with CleanCap Reagent

1x scale (100 µL)		
Component	Amount (1X)	Amount (____X)
T7 Transcription Buffer 5X	20 µL	
100 mM ATP	7.5 µL	
100 mM GTP	7.5 µL	
100 mM CTP	7.5 µL	
100 mM UTP	7.5 µL	
100 mM CleanCap-AG	7.5 µL	
DNA plasmid (1 µg/µL)	10 µL	
Sterile distilled water	22.5 µL	
Enzyme Mix (T7)	10 µL	
Total Volume	100 µL	
Incubate for 2 hrs at 37 °C.		
Add RQ1 (DNase)	10 µL	
Incubate for 15 min at 37 °C.		
Purify with 2.5 M ammonium acetate precipitation.		

Poly (A) tailing reaction

1x scale (60 µg of RNA with 100 µL)		
Component	Amount (1X)	Amount (____X)
60 µg of RNA in water	75.5 µL	
Poly (A) tailing Buffer 10X	10 µL	
10 mM ATP	10 µL	
ScriptGuard RNase inhibitor (40 U/µl)	2.5 µL	
Poly(A) Polymerase (4 U/µl).	2 µL	
Total Volume	100 µL	
Incubate for 30 min at 37 °C.		
Purify with 2.5 M ammonium acetate precipitation.		

RNA transcription with CleanCap Reagent (25% of modified nucleotides)

1x scale (100 µL)		
Component	Amount (1X)	Amount (____X)
T7 Transcription Buffer 5X	20 µL	
100 mM ATP	7.5 µL	
100 mM GTP	7.5 µL	
100 mM CTP	5.625 µL	
100 mM UTP	5.625 µL	
100 mM 5-Methyl-CTP	1.875 µL	
100 mM Pseudo-UTP	1.875 µL	
100 mM CleanCap-AG	7.5 µL	
DNA plasmid (1 µg/µL)	10 µL	
Sterile distilled water	22.5 µL	
Enzyme Mix (T7)	10 µL	
Total Volume	100 µL	
Incubate for 2 hrs at 37 °C.		
Add RQ1 (DNase)	10 µL	
Incubate for 15 min at 37 °C.		
Purify with 2.5 M ammonium acetate precipitation.		

T7 Transcription, Enzymatic Capping and Phosphatase Treatment:

RNA transcription without capping

1x scale (100 µL)		
Component	Amount (1X)	Amount (____X)
T7 Transcription Buffer 5X	20 µL	
100mM ATP	7.5 µL	
100mM GTP	7.5 µL	
100mM CTP	7.5 µL	
100mM UTP	7.5 µL	
DNA plasmid (1 µg/µL)	10 µL	
Sterile distilled water	22.5 µL	
Enzyme Mix (T7)	10 µL	
Total Volume	100 µL	
Incubate for 2 hrs at 37 °C.		
Add RQ1 (DNase)	10 µL	
Incubate for 15 min at 37 °C.		
Purify with 2.5 M ammonium acetate precipitation.		

Phosphatase treatment

1x scale (60 µg of RNA with 100 µL)		
Component	Amount (1X)	Amount (____X)
60 µg of RNA in water	77.5 µL	
Phosphatase Buffer 10X	10 µL	
ScriptGuard RNase inhibitor (40 U/µl)	2.5 µL	
Antarctic phosphatase (5 U/µl)	10 µL	
Total Volume	100 µL	
Incubate for 30 min at 37 °C.		
Purify with 2.5 M ammonium acetate precipitation.		

Enzymatic Capping (Cap-1)

1x scale (60 µg of RNA with 100 µL)		
60 µg of RNA in water	67 µL	
Denature at 65 °C for 5 min. For more than 5x scales, denature for 8 min.		
Put on ice and cooling down.		
Add reagents in this order .		
Component	Amount (1X)	Amount (____X)
10X ScriptCap Capping Buffer	10 µL	
10 mM GTP	10 µL	
20 mM SAM	2.5 µL	
ScriptGuard RNase Inhibitor (40 U/µl)	2.5 µL	
ScriptCap 2'-O-Methyltransferase (100 U/µl)	4 µL	
ScriptCap Capping Enzyme (10 U/µL)	4 µL	
Total Volume	100 µL	
Incubate for 45 min at 37 °C.		
Purify with 2.5 M ammonium acetate precipitation.		

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