mRNAExpress™
mRNA Synthesis Kit
Cat. #MR-KIT-1

User Manual

Check Individual Components for Storage conditions

ver. 1-032312

Contents

I. Product Information

A. Product Description

The SBI mRNAExpress mRNA Synthesis Kit is designed for *in vitro* transcription of mRNAs to be used in transfection of mammalian cells, oocyte micro-injection, *in vitro* translation and other applications. This high-yield kit can produce 20-40 µg of high quality mRNAs in a standard reaction.

The *in vitro* transcription reaction utilizes robust T7 RNA polymerase. Anti-reverse cap analog (ARCA)-modified nucleotides (5-Methylcytidine-5'-Triphosphate and Pseudouridine-5'-Triphosphate) and poly-A tail are incorporated in the transcribed mRNAs to enhance the stability and to reduce the immune response of host cells.

DNase I is provided to digest the DNA template and a phosphatase is provided to remove the 5’ triphosphate at the end of the RNA to further reduce the innate immune response in mammalian cells. This clean-up system yields high recovery of mRNAs that are ready for downstream applications.

B. Precautionary Notes

All components of SBI mRNAExpress mRNA Synthesis Kit are free of detectable RNase activity.

General precautions should be taken when handling mRNA to maintain its integrity.

1. Wear gloves throughout the procedure to protect RNA samples from degradation by RNase.
2. Use decontamination solutions, such as RNaseZap (Invitrogen), to treat bench surfaces, centrifuges and containers.
3. Use commercially available RNase-free pippet tips and other plastic ware.

C. Kit Components (5 Standard Reactions)

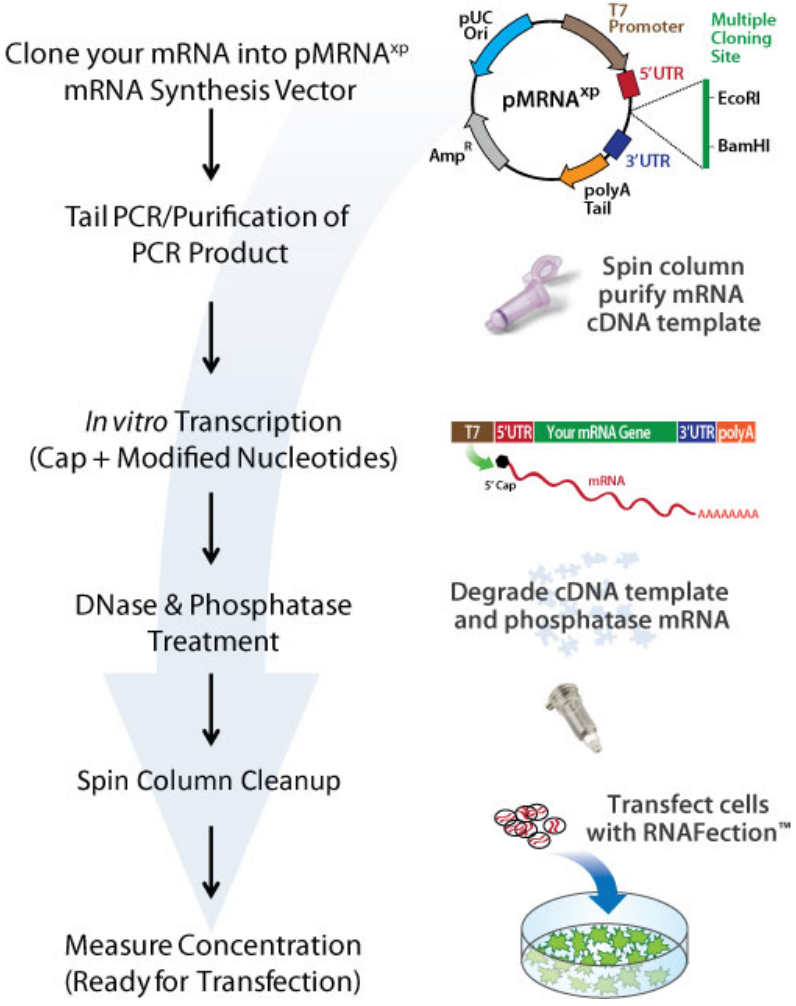
Size	Components	Storage
5 µg	pMRNA^{XP} Plasmid	-20°C
10 µL	Sequencing Primer Forward	-20°C
10 µL	Sequencing Primer Reverse	-20°C
20 µL	Tail PCR Primer Mix	-20°C
50 µL	Nuclease-free Water	any temp
20 µL	5×Reaction Buffer	-20°C
35 µL	NTP/Cap Mix	-20°C
12.5 µL	T7 RNA Polymerase Mix	-20°C
5 µL	RNase-free DNase	-20°C
25 µL	10×Phosphatase Buffer	-20°C
10 µL	RNase-free Phosphatase	-20°C
5	Collection Tubes with Filter Cartridges	room temp
5	Collection Tubes	room temp
2 mL	Binding Buffer	4°C
1 mL	Washing Buffer (5×concentrate)	4°C
1 mL	Elution Buffer	4°C

D. Additional Materials and Instruments Needed

- Table-top centrifuge
- PCR enzymes and buffers
- Customer DNA template
- Restriction Enzymes and Buffer
- ColdFusion Cloning kit (SBI)
- Phusion (from NEB)
- PCR Purification kit (Qiagen)
- Gel Extraction kits (Qiagen)
- Materials and apparatus for DNA and RNA electrophoresis

II. Protocols

A. Flowchart



B. Cloning into pMRNA^{XP} mRNA Synthesis Vector

1. Preparation of Linearized pMRNA^{xp} Vector

Complete linearization of the vector is critical to achieve successful cloning. Incomplete linearization of the vector will result in high background. We recommend digesting 2μg pMRNA^{xp} vector with EcoR1 and BamH1 in a 50μl reaction for 3 hours or even overnight. Use QIAGEN's QIAquick Spin Gel Extraction kit for gel purification and elute the DNA with 30 μl dH_2O. Check the background of your vector by transforming 1μl (10-100ng) linearized and purified vector into competent cells. If the background is high, continue digesting the remaining vector for a longer time after the addition of more restriction enzyme(s).

A typical restriction digestion is shown below

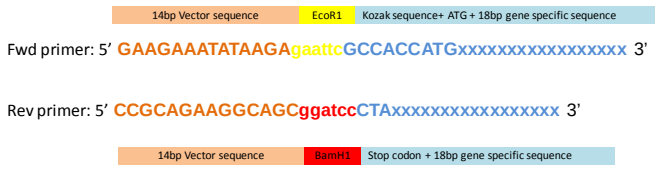
pMRNA^{xp} vector (0.5μg/μl)	4μl*
10X Buffer 4	5μl*
EcoR1 (20U/μl)	0.5μl
BamH1 (20U/μl)	0.5μl
Nuclease-free water	40μl
Total	50μl

2. PCR amplification of target gene

The pMRNA^{xp} lentivector does not contain an ATG initiation codon or a stop codon (TAA, TAG, TGA). If the DNA fragment to be cloned does not have a start or stop codon, please incorporate the ATG and stop codon in the

insert. We also recommend including a Kozak sequence (i.e. GCCACC) before the ATG for optimal translation.

To successfully clone any DNA fragment into the linearized pMRNA^{xp} vector, we recommend using SBI's Cold Fusion cloning kit. Using Cold Fusion cloning kit, PCR primers must be designed to have about 14 bases of homology with the end of the linearized vector. Thus, a primer will consist of a 14-base vector homology sequence at the 5'-end, and restriction site in the middle, and the gene-specific sequence at the 3'-end. The guidelines for primer design are shown in the graph below.



Example: To clone the copGFP open reading frame into pMRNA^{xp} vector, the primers are as follows.

atggagagcgacgagagcggcctgcccgccatggagatcgagtgcc
gcatcaccggcaccctgaacggcgtggagttcgagctggtgggcggc
ggagagggcaccc----------
---
agcacgccttcaagacccccatcgccttcgccagatcccgcgctcagt
cgtccaattctgccgtggacggcaccgccggacccg**gctccaccggat**
ctcgctag

Fwd primer: GAAGAAATATAAGAgaattcGCCACCATGgagagcgacgagagcgg
Rev primer: CCGCAGAAGGCAGCggatccCTAgcgagatccggtggagc

The PCR fragments can be generated by Taq DNA polymerase or other high fidelity DNA polymerase. The melting temperature (T_m) should be calculated based on the 3' (gene-specific) end of the primer, NOT the entire primer.

Primers and primer dimmers produced in PCR reactions are inhibitory to the Cold Fusion cloning reaction. If the

PCR produces a single specific band (from an agarose gel), the PCR product can be purified by simply using a PCR purification kit. If the PCR produces multiple bands, the specific DNA band desired should be purified by a gel purification kit to remove non-specific DNA bands and avoid false-positive clone.

3. Set up Cold Fusion reaction

Set up the following reaction in a 1.5 ml sterile reaction tube by mixing the following reagents gently and then spin down briefly to collect the reagents at the bottom of the tube.

a. Cloning reaction

Linearized pMRNA^{xp} vector (10-100ng/μl)	1μl*
Purified PCR product (20-200ng/μl)	1μl*
dH_2O	6μl
5x master mix	2μl
total	10μl

Positive control reaction (from ColdFusion Cloning kit)

Linearized vector, Amp^R (positive control)	1μl
500bp PCR insert (positive control)	1μl
dH2O	6μl
5x master mix	2μl
total	10μl

Negative Control

Linearized $pMRNA^{xp}$ vector (10-100ng/μl)	1μl*
dH_2O	7μl
5x master mix	2μl
total	10μl

When using the Cold Fusion cloning kit for the first time, we strongly recommend that you perform the positive and negative control reactions in parallel with your Cold Fusion cloning reaction. The positive control 500bp PCR insert and linearized vector provided in the kit has already been purified. There is no treatment needed prior to the cloning reaction.

b. Cold Fusion Reaction incubation

1) 5 minutes at room temperature
2) 10 minutes one ice

c. Transformation

1) Add 50µl competent cells to the cloning mixture
2) Incubate on ice for 20 minutes
3) Heat shock at 42˚C for 50 seconds
4) Transfer on ice for 2 minutes
5) Add 250µl S.O.C medium or LB broth
6) Incubate at 37˚C for an hour
7) Take 100µl culture spread on pre-warmed (37˚C) culture plate containing 50 µg/ ml ampicillin
8) Incubate the plate at 37˚C

4. Identify clones with gene insert

Randomly pick 4 or more well-isolated colonies and grow each clone in 3ml LB Broth with ampicillin at 37˚C overnight with shaking. Purify the constructs using a plasmid purification kit. Use a PCR or enzyme digestion method to check the positive clones containing gene inserts. Confirm identity of the gene insert by sequence analysis of the construct using the sequencing primers provided in the kit. The construct with correct insert sequence can be used as the template of the downstream Tail PCR

C. Tail PCR/ Purification of PCR product

The template for *in vitro* transcription should be generated using a PCR reaction that adds a polyA tail to the end of the DNA template. We recommend a typical setup with Phusion (NEB) (shown below).

Component	Amount/ 20 µL Reaction
5×Buffer	4 µL
dNTP Mix	0.4 µL
Tail PCR Primer Mix	2 µL
Plasmid Template	10 – 50 ng
Phusion DNA Polymerase	0.2 µL
H_2O	to 20 µL

A typical PCR program with Phusion is shown below:

Cycle(s)	Temperature	Time
1	98 °C	30 min
30	98 °C	30 s
	72 °C	30 s/kb
1	72 °C	10 min

Other high fidelity enzymes can also be used for the tail PCR reaction according to the manufacturer's instructions.

The PCR product can be purified using QIAquick PCR Purification Kit from QIAGEN.

D. *In vitro* Transcription

1. Thaw the frozen reagents for IVT at room temperature.
2. The 5×Reaction Buffer and NTP/Cap mix should be briefly vortexed before using.
3. Spin down all reagents briefly before opening to prevent loss or contamination around the rim of the tube.
4. Assemble IVT reaction at room temperature. Add the reagents in the order specified. The following is recommended for one 20 µL reaction.

Amount	Component
7 µL	NTP/Cap Mix
4 µL	5×Reaction Buffer
1 µg	Template DNA
2.5 µL	T7 RNA Polymerase Mix
to 20 µL	Nuclease-free Water
20 µL	Total volume

5. Mix thoroughly by pipetting the mixture up and down or flicking the tube gently. Spin down the mixture briefly with a microcentrifuge.
6. Incubate at 42°C for 2 hours. (Optional: Additional incubation may increase the yield with lower amounts of DNA template.)

E. DNase and PhosphataseTreatment

1. Adjust the IVT reaction to 24 µL with RNase-free water.
2. Add 3 µL of 10×Phosphatase Buffer to the reaction.
3. Add 1 µL of RNase-free DNase I and 2 µL Phosphatase and mix.
4. Incubate for 30 min at 37°C.

F. RNA Purification

1. Bring the sample volume to 100 µL with Elution Buffer. Mix gently by pipetting.
2. Add 350 µL Binding Buffer. Mix gently by pipetting.
3. Add 250 µL 100% ethanol to sample. Mix gently by pipetting.
4. Pipet the RNA mixture (~650 µL) onto a Collection Tube with Filter Cartridge.
5. Centrifuge at 10,000-15,000x*g* (10,000-14,000 rpm) for 1 min.

6. Discard flow-through and insert the filter back to the Collection Tube for the washing steps.
7. Make sure that ethanol has been added to Washing Buffer before use. (Add 4 mL 100% ethanol to 1 mL 5× Washing Buffer.)
8. Apply 500 µL Washing Buffer.
9. Centrifuge at 10,000-15,000x *g* (10,000-14,000 rpm) for 1 min.
10. Discard the Washing Buffer.
11. Repeat steps 8 through 10.
12. Centrifuge for another 10-30 sec to remove the remaining washing buffer.
13. Place the Filter Cartridge to a new Collection Tube (supplied).
14. Add 50 µL Elution Buffer to the center of Filter Cartridge.
15. Close the cap and incubate at 65-70°C for 5-10 min.
16. Collect the eluted mRNA by centrifuging for 1 min at 10,000-15,000x *g* (10,000-14,000 rpm).

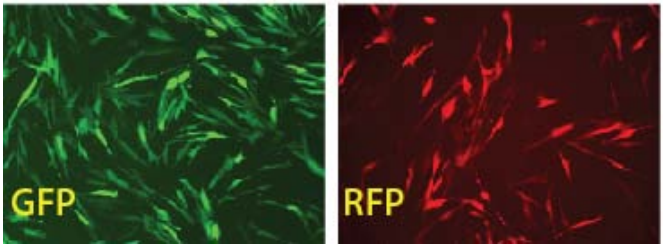
G. Analysis of Transcription Products by Gel Electrophoresis and Quantitation by UV Light Absorbance

The size of the mRNA products from mRNAExpress mRNA Synthesis Kit can be analyzed by running an aliquot of the reaction on formaldehyde agarose gel.

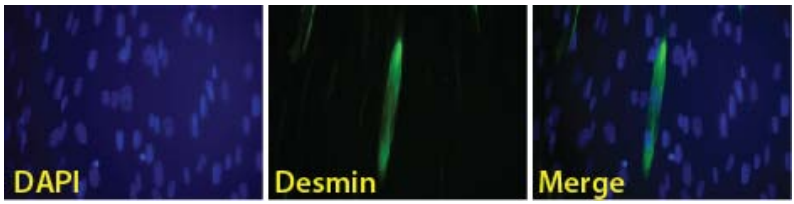
The concentration of the mRNA products can be determined by reading the A_{260} of a diluted aliquot. Typically, a 1:50 dilution will give an absorbance reading in the linear range of the spectrometer. One A_{260} unit corresponds to 40 µg/mL of mRNA.

III. Sample results

A. Transfection of human foreskin fibroblasts with GFP and RFP mRNAExpress transcripts.



B. Transfection of human foreskin fibroblasts with MyoD mRNAExpress transcript. After 3 days transdifferentiation, cells were immunostained with Desmin (myogenic marker) for myotube formation and imaged for nuclei with DAPI.



IV. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

Phone: (650) 968-2200
(888) 266-5066 (Toll Free)
Fax: (650) 968-2277
E-mail:
General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

System Biosciences (SBI)
265 North Whisman Road
Mountain View, CA 94043

V. Licensing and Warranty

Use of the mRNAExpress *in vitro* Transcription Kit (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

The purchaser of the Product is granted a limited license to use the Product under the following terms and conditions:

The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use.

Limited Warranty

SBI warrants that the Product meets the specifications described in the accompanying Product Analysis Certificate. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

SBI's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

SBI is committed to providing our customers with high-quality products. If you should have any questions or concerns about any SBI products, please contact us at (888) 266-5066.