

RNAi Studies Made Faster, Easier, and at Lower Cost Using the New MessageMuter™ shRNAi Production Kit

Judith E. Meis, EPICENTRE

RNA interference (RNAi) is a powerful technique used to study gene function. In mammalian cells RNAi is mediated by short, double-stranded RNA (dsRNA) and causes the degradation of the specifically targeted mRNA. Short hairpin RNA (shRNA), which can be used as short dsRNA in RNAi, contains a sequence homologous to the target mRNA, a spacer or loop region, and an inverted repeat of the target sequence. The complementary target sequences of the RNA spontaneously base pair to form hairpin RNA (Figure 2C). Whether produced by chemical synthesis^{1,2}, transcribed *in vitro*^{2,3,4} or expressed *in vivo*^{1,2,3}, shRNA has been shown to silence genes as effectively as short dsRNAs. EPICENTRE's new MessageMuter™ shRNAi Production Kit produces transfection-ready shRNA, *in vitro*, faster and easier than other *in vitro* transcription-based RNAi production strategies and at significantly lower cost than chemically synthesized dsRNA. The MessageMuter shRNAi kit provides reagents to produce 10 shRNAs, each in sufficient quantity for 100's of RNAi experiments.

Produce transfection-ready shRNA *in vitro* in about 4 hours

The MessageMuter shRNAi Production Kit uses a simple, unique, 3-step process (Figure 1) that yields transfection-ready shRNA in about 4 hours.

1. Anneal the short T7 Promoter Oligo, provided in the kit, to an oligodeoxynucleotide (DNA oligo) that is designed and provided by the user.

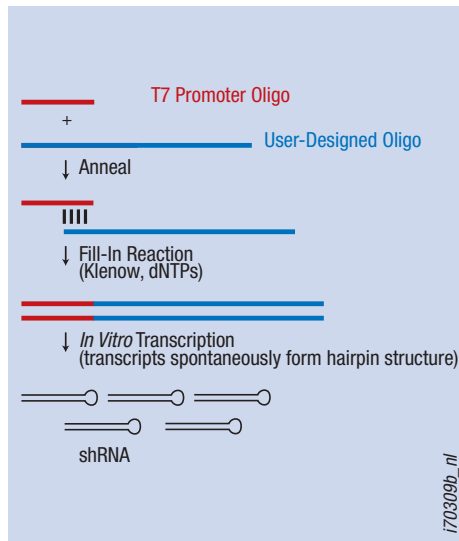


Figure 1. Overview of the method used to produce short hairpin RNA (shRNA) using the MessageMuter™ shRNAi Production Kit. A 60 to 76-base DNA oligo, supplied by the user, is annealed to the T7 Promoter Oligo. A dsDNA template for T7 *in vitro* transcription is produced using Klenow exo-minus DNA Polymerase I. In the final reaction, the shRNA is transcribed from the template. Total time to prepare transfection-ready shRNA for 100's of RNAi experiments is about 4 hours. All reaction components, except the user-designed DNA oligo, are provided in the kit.

2. "Fill-in" the ends of the annealed duplex using the Klenow exo-minus fragment of DNA Polymerase I and dNTPs (both provided in the kit) to generate linear double-stranded DNA (dsDNA), which serves as an *in vitro* transcription template for T7 RNA Polymerase.

3. Transcribe shRNA from the DNA template in a rapid, high-yield *in vitro* transcription reaction using reagents that are provided in the kit. The transcribed RNA spontaneously forms a hairpin structure (shRNA) in solution (Figure 2C). Following clean-up, the shRNA is ready for transfection into cultured cells.

For the complete MessageMuter shRNAi Production Kit procedure, please visit: www.epicentre.com/messagemuter.asp

Prepare shRNA against any mRNA target using a single sequence-specific DNA oligo

The user supplies a single, purified and desalted, DNA oligo of 60 to 76 bases, like the example of an shRNA targeted against firefly luciferase (shRNA-luc) shown in Figure 2. The oligo contains:

- 1) a 5'-sequence of 21 to 29 nucleotides that is identical to the targeted sequence of the mRNA,
- 2) an 8-base "loop" sequence,
- 3) the reverse complement of the first target sequence,
- 4) an 8-base sequence complementary to the kit's T7 Promoter Oligo.

T7 RNA polymerase transcribes most efficiently when the first two nucleotides of the transcript are "GG". This dinucleotide can either be introduced or naturally occurring at the 5' end of the target sequence. The "GG" need not influence the user's selection of a target sequence.^{2,5} When designing the DNA

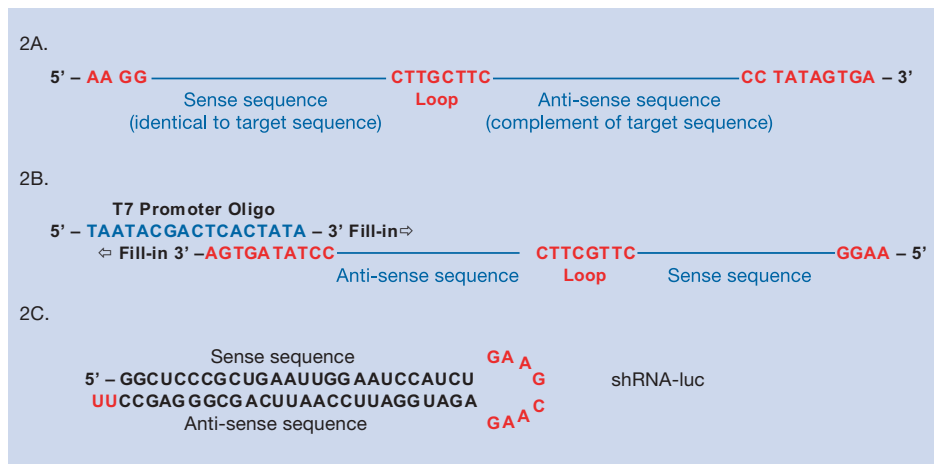


Figure 2. Elements of the single, user-designed DNA oligo used to prepare an shRNA with the MessageMuter™ shRNAi Production Kit. The example shown is a 68-base oligo designed to produce an shRNA targeted against firefly luciferase (shRNA-luc). Standard elements (shown in red) of all user-designed oligos include: 1) the optional "AA", which will be transcribed into a 3'-"UU", 2) the necessary "GG", either at the start of the target sequence or added there, 3) the 8-base loop sequence, and 4) the 8-base sequence complementary to the 3'-end of the kit's T7 Promoter Oligo. **2A.** 5'-3' order of the components in the DNA oligo designed to prepare shRNA-luc. **2B.** The shRNA-luc oligo annealed to the kit's T7 Promoter Oligo. **2C.** Sequence and secondary structure of the shRNA-luc.

oligo for a target sequence that does not begin with "GG", add the dinucleotide immediately 5' to the target sense sequence and add the complementary "CC" to the 3' end of the complementary target antisense sequence, as shown in Figure 2A.

If desired, a 3'-"UU" tail can be added to the shRNA by including an "AA" dinucleotide sequence at the immediate 5'-end of the oligo.

The standard MessageMuter shRNAi reaction uses 50 pmoles of the user-designed DNA oligo. The oligo and the T7 Promoter Oligo are combined, heated at 75°C for 2 minutes, and allowed to anneal at room temperature for 2 minutes. Once annealed, the overlapped oligos are extended using Klenow exo-minus DNA Polymerase I (30 minutes at 37°C) to produce the dsDNA template for T7 *in vitro* transcription (Figure 3). The

in vitro transcription reaction requires only a portion of the prepared dsDNA template; the remainder can be stored at -20°C for future use.

Produce enough shRNA for 100's of RNAi experiments

The *in vitro* transcription reaction uses components of EPICENTRE's new AmpliScribe™ T7-Flash™ Transcription Kit to maximize the yield of the shRNA in the shortest reaction time. (See p. 7 for information about producing short RNA transcripts with the AmpliScribe T7-Flash Transcription Kit). Figure 4 shows a time course for the yield of shRNA-luc produced with the MessageMuter shRNAi Kit. Yield was determined following purification of the shRNA. A 30-minute *in vitro* transcription reaction produced 46.4 µg (2.07 nmoles) of shRNA-luc, which is sufficient for 100's of RNAi experiments performed with cells grown in 24-well plates and transfected with 300 µl of

10 nM shRNA-luc. Unused shRNA can be stored at -70°C for future use.

Produce effective, target-specific shRNA

To demonstrate that the shRNA-luc produced with the MessageMuter kit could knock down the expression of firefly luciferase, the shRNA was tested in HeLa, Cos-7, normal rat kidney (NRK) and mouse embryo fibroblast (NIH/3T3) cells, grown as described previously.⁴ Cells grown in 24-well plates were transfected with 300 µl of Opti-MEM® Media (Invitrogen), 2 µl of TransIT-TKO® Transfection Reagent for shRNA (Mirus) and 1 µl of TransIT® LT1 transfection reagent (Mirus), which contained a firefly luciferase expression vector, a *Renilla* (sea pansy) luciferase expression vector, and 10 nM shRNA-luc. Luciferase assays were performed as previously described.⁴ Figure 5 shows the effect of shRNA-luc on firefly luciferase expression as a ratio of firefly luciferase activity to *Renilla* luciferase

Figure 3.

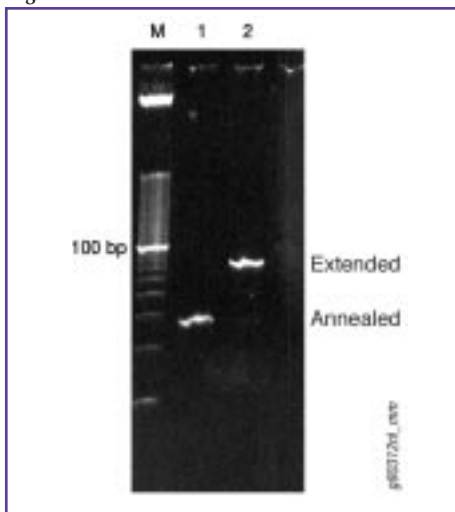
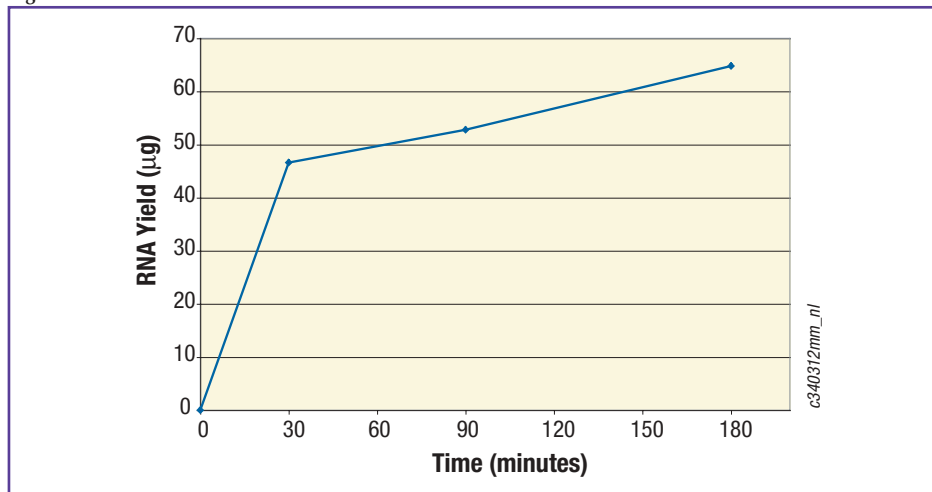


Figure 3. Preparation of the T7 *in vitro* transcription dsDNA template can be monitored by 12% polyacrylamide gel electrophoresis. Lane 1, 68-base DNA oligo, designed to produce a shRNA against firefly luciferase, annealed to the T7 Promoter Oligo. Lane 2, dsDNA template, for *in vitro* transcription, produced by filling in the ends of the annealed oligos using Klenow exo-minus DNA Polymerase I. Lane M, 10-bp marker.

Figure 4. Each reaction of the MessageMuter™ shRNAi Production Kit produces enough shRNA for 100's of RNAi experiments. The dsDNA template for shRNA-luc was transcribed using the AmpliScribe™ T7-Flash™ Transcription Components provided in the kit. The yield of shRNA produced at the indicated times was determined after DNase treatment, ethanol precipitation, and removal of unincorporated NTPs with a spin column. A 30-minute *in vitro* transcription reaction produced enough shRNA to do 100's of RNAi experiments.

Figure 4.



Benefits of the MessageMuter™ shRNAi Production Kit

- ➔ Significantly lower cost per RNAi experiment than chemically synthesized dsRNA.
- ➔ Faster and easier than constructing short dsRNA by other *in vitro* transcription strategies. (No need to anneal sense and antisense RNA strands).
- ➔ Produce transfection-ready, gene-muting shRNA in about 4 hours.
- ➔ High-yield reaction produces enough shRNA for 100's of RNAi experiments.

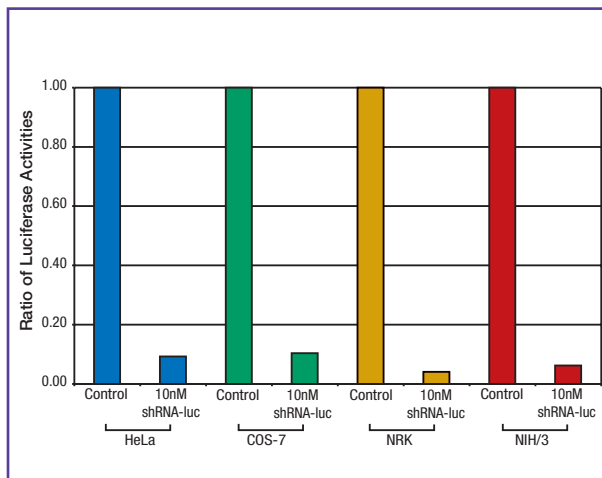
activity in the four cell types tested. At 10 nM, shRNA-luc specifically suppresses firefly luciferase expression by about 90% relative to *Renilla* luciferase expression in all cell types tested. Thus, the MessageMuter shRNAi Production Kit produces effective, target-specific shRNA.

The MessageMuter shRNAi Production Kit prepares short, dsRNA for RNAi studies

The MessageMuter™ shRNAi Production Kit uses *in vitro* transcription to prepare shRNA, which is an effective dsRNA for RNAi studies, in a rapid, simple, and cost-effective process. Each reaction produces enough shRNA, in 4 hours, for 100's of RNAi experiments.

Figure 5. shRNA-luc, produced with the MessageMuter™ shRNAi Production Kit, effectively induced sequence-specific silencing of firefly luciferase in a variety of cultured mammalian cells.

Transfections were performed in triplicate with firefly and *Renilla* (sea pansy) luciferase expression plasmids and 10 nM shRNA-luc, as described in the text. These experiments used the same shRNA-luc depicted in Figure 2C. Cells tested were HeLa (blue), COS-7 (green), Normal Rat Kidney (NRK; yellow) and mouse embryo fibroblast (NIH/3T3; red). Results for all experiments are shown as a ratio of firefly luciferase activity to *Renilla* luciferase activity.



References

- McManus, M.T. (2002) *RNA* **8**, 842.
- Paddison, P.J. et al. (2002) *Gene. Dev.* **16**, 948.
- Yu, J.-Y. et al. (2002) *Proc. Natl. Acad. Sci. USA* **99**(9), 6047.
- Meis, J.E. (2003) *EPICENTRE Forum* **10**(2), 1.
- EPICENTRE, unpublished observations.

www.epicentre.com/messagemuter.asp

MessageMuter™ shRNAi Production Kit

MM031110 10 Reactions

Each kit provides sufficient reagents to prepare 10 different shRNA. User supplies one DNA oligo per reaction.

Contents:
 MessageMuter™ T7 Promoter Oligo, 5X Annealing Buffer, Klenow exo-minus DNA Polymerase I, dNTPs, 10X Klenow Fill-In Buffer, AmpliScribe™ T7-Flash™ Enzyme Mix, AmpliScribe™ T7-Flash™ 10X Reaction Buffer, NTPs, DTT, Control Luciferase Oligo (60 bases), RNase-Free DNase I, Sterile Water.

Improve Your RT-PCR Results Using Thermostable MasterAmp™ Tth DNA Polymerase

In an RT-PCR reaction, secondary structure in the RNA template can cause premature termination and result in low yields of cDNA. MasterAmp™ Tth DNA Polymerase, derived from the thermophilic bacterium *Thermus thermophilus*, is a thermostable enzyme that has both DNA-directed DNA polymerase activity and efficient RNA-directed reverse transcriptase activity. The reverse transcriptase activity does not include RNase H activity. The enzyme has optimal activity at temperatures above 70°C and can be used at temperatures up to 95°C. Using Tth DNA Polymerase and high reaction temperatures minimizes problems due to nonspecific priming and RNA secondary structure. EPICENTRE's MasterAmp Tth DNA Polymerase is highly purified using a procedure that eliminates contaminating bacterial DNA.

MasterAmp Tth DNA Polymerase is supplied with the MasterAmp™ PCR Enhancer (with betaine)* which substantially increases both the ability to obtain

the desired amplification product and reaction-to-reaction consistency.

Applications of MasterAmp™ Tth DNA Polymerase

- High-temperature reverse transcription and RT-PCR.
- PCR amplification of DNA templates.

Benefits of MasterAmp™ Tth DNA Polymerase

- Optimal activity above 70°C eliminates mRNA secondary structure during reverse transcription and subsequent PCR reactions.
- Use the same reaction conditions for both thermostable reverse transcription and PCR. (1-tube, 1 enzyme RT-PCR)
- MasterAmp™ PCR Enhancer (with betaine) is supplied with the enzyme.

*Covered by issued and pending patents. This product is accompanied by a limited license to use in the Polymerase Chain Reaction (PCR) and RT-PCR for life sciences research in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee either by payment to Applied Biosystems or as purchased, i.e., an authorized thermocycler.

www.epicentre.com/tth.asp

MasterAmp™ Tth DNA Polymerase

TTH72100	5 U/μl	100 U
TTH72250	5 U/μl	250 U
TTH72500	5 U/μl	500 U
TTH7201K	5 U/μl	1,000 U
TTH7205K	5 U/μl	5,000 U (5 x 1,000 U)

Contents:
 20X PCR Buffer (without Mg²⁺ or Mn²⁺) plus separate 25 mM solutions of MgCl₂ and MnSO₄ and MasterAmp™ 10X PCR Enhancer.

MasterAmp™ Tth DNA Polymerase (Enzyme Only)

TTH7225N	5 U/μl	250 U
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For use with MasterAmp™ PCR PreMixes.

MasterAmp™ 10X PCR Enhancer

ME81201	1.5 ml
ME81205	5 ml
ME81210	10 ml

25 mM MgCl₂

FB4250	25 ml
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