Optimal Generation of Short RNAs Using the T7 RiboMAX™ Express RNAi System

ABSTRACT The T7 RiboMAX™ Express RNAi System is an in vitro transcription system designed for the efficient synthesis of milligram amounts of short interfering RNAs (siRNAs) or hairpin siRNAs (shRNAs) for mammalian RNAi studies. Previous work by others has shown that the synthesis of short RNAs is dependent on transcription initiation by T7 RNA polymerase and that the sequence of the +1 nucleotide can dramatically affect the yield of RNA. Studies were conducted to extend this information to shRNAs for use in RNAi experiments.

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INTRODUCTION

The T7 RiboMAX™ Express RNAi System[a,b,c] is designed to synthesize siRNAs or hairpin siRNAs (shRNAs) in vitro quickly and efficiently for use in mammalian RNAi studies. In addition, this system can also synthesize longer dsRNAs used in most non-mammalian RNAi systems. The buffering system, NTP concentration, T7 RNA Polymerase, inorganic pyrophosphatase and magnesium levels of the T7 RiboMAX™ Express RNAi System have been optimized for increased RNA yield compared to standard in vitro transcription reactions (1).

Synthesis of siRNAs or shRNAs using the T7 RiboMAX™ Express RNAi System uses two DNA oligonucleotides that are annealed to form the duplex DNA template. Generally 20pmol of duplex oligonucleotides is required per 20µl in vitro transcription reaction. Using the combination of the RiboMAX™ Express T7 Buffer and Enzyme Mix allows efficient synthesis of RNA in as little as 30 minutes. The annealed DNA oligonucleotide template is removed by a DNase digestion step, and the separate small RNA strands are annealed to form siRNA. In the case of short hairpin siRNA, the single small RNA is allowed to anneal to itself to form the hairpin. The siRNA is precipitated using sodium acetate and isopropanol. The resuspended product can be analyzed on polyacrylamide gels for size and integrity. Quantitation of the siRNA can be accomplished by using gel analysis with SYBR® green II staining.

Previous work by Milligan and Uhlenbeck demonstrated that G at the +1 position is optimal for transcription of small RNAs (2), as it enhances transcription initiation.

TEMPLATE DESIGN AND CONSIDERATIONS

Generating small RNAs using in vitro transcription requires a DNA oligonucleotide duplex template. A T7 RNA polymerase promoter sequence is engineered into the top- and bottom-strand oligos to allow transcription. Previous work by Milligan and Uhlenbeck demonstrated that G at the +1 position is optimal for transcription of small RNAs (2), as it enhances transcription initiation. Based on that data, we typically recommend that the target sequence contain the sequence 5´-GN_{17}C-3´ to allow efficient transcription initiation by T7 RNA polymerase and to incorporate the recommended 19–nucleotide length.

However, these earlier experiments were not performed using transcription conditions that are comparable to those recommended with the RiboMAX™ Express System. The T7 RNA polymerase used was not commercially available, and the short RNA product synthesized was only 21 bases in length, compared to shRNAs, which are typically ~48–50 bases in length. In addition, only three of the four possible nucleotides at the +1 position were tested. To determine if these sequences would be optimal for use with the RiboMAX™ system, we conducted experiments using the four possible bases at the +1 position and investigated the effect on shRNA yield.

EXPERIMENTAL METHOD AND RESULTS

Four oligonucleotides were synthesized such that they contained all of the necessary sequences for in vitro transcription of a *Renilla* luciferase shRNA. The oligonucleotides were identical except for the +1 position in the T7 RNA Polymerase promoter region (i.e., G, C, A or T; see Figure 1). The complementary oligonucleotides were synthesized, and the oligos were annealed and used as template DNA in triplicate reactions with the T7 RiboMAX™ Express RNAi System, as described in the *T7 RiboMAX™ Express RNAi System Technical Bulletin*, TB316. The resulting shRNAs were analyzed on a 2.5% agarose gel, followed by staining with SYBR® green II dye. Scanning and quantitation was performed on a Molecular Dynamics Storm® gel blot imaging system.

The results are shown in Table 1 and demonstrate that a G at the +1 position is optimal, and substitution by C, A or T result, in a 2.3X, 3.4X or 8.5X decrease in yield of shRNA, respectively. These results are similar to those seen by Milligan and Uhlenbeck (2). In addition, further studies showed that either a G or A at position +2 yields comparable results and that the +2 position does not have a dramatic effect on transcription initiation and thus overall short RNA yield.

G: 5'-
GGATCC**TAATACGACTCACTATA**GCGGCCTCTTCTTATTTATTTCAAGAGAA
TAAATAAGAAGAGGCCGC-3'

C: 5'-
GGATCC**TAATACGACTCACTATA**CCGGCCTCTTCTTATTTATTTCAAGAGAA
TAAATAAGAAGAGGCCGC-3'

A: 5'-
GGATCC**TAATACGACTCACTATA**ACGGCCTCTTCTTATTTATTTCAAGAGAA
TAAATAAGAAGAGGCCGC-3'

T: 5'-
GGATCC**TAATACGACTCACTATA**TCGGCCTCTTCTTATTTATTTCAAGAGAA
TAAATAAGAAGAGGCCGC-3'

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Figure 1. Top-strand oligonucleotide sequence used for synthesis of *Renilla* luciferase shRNAs. The oligonucleotide sequences differed only at the +1 nucleotide position (highlighted in red). The bottom-strand oligonucleotide used to form the double-stranded molecule was complementary to the sequence listed. The T7 RNA polymerase promoter is in bold, the *Renilla* luciferase sequence is in blue and the loop sequence for the shRNA is in green.

Table 1. Comparison of shRNA Yield with Different Nucleotides at the +1 Position of the T7 RNA Polymerase Promoter Region. The sequences of the oligonucleotides used are shown in Figure 1. The results are the average of triplicates. The yield of shRNA was determined by gel analysis with fluorescent staining.

Nucleotide at Position +1	Yield of *Renilla* luciferase shRNA (RFU)	Yield of *Renilla* luciferase shRNA Compared to "G" at +1 (%)
G	17,790,219 ± 1,236,063	100%
C	7,852,422 ± 508,337	44.1%
A	5,254,836 ± 271,241	29.5%
T	2,081,763 ± 861,234	11.7%

The sequence requirements for transcription by T7 RNA polymerase were not different under the conditions recommended for the T7 RiboMAX™ Express RNAi System.

CONCLUSION

We determined that the sequence requirements for transcription by T7 RNA polymerase were not different under the conditions recommended for the T7 RiboMAX™ Express RNAi System, confirming earlier findings indicating that optimal transcription by T7 RNA polymerase requires a G at the +1 position. Further, replacement of this G with other nucleotides results in reduced RNA yields or short RNAs, including shRNAs. In addition to short RNAs, this effect of the nucleotide at the +1 position is probably true for longer RNA transcripts as well.

REFERENCES

1. Betz, N. *et al.* (2003) *Promega Notes* **84**, 7–11.
2. Milligan, J. and Uhlenbeck, O. (1989) *Methods Enzymol.* **180**, 51–62.

PROTOCOL

- *T7 RiboMAX™ Express RNAi System Technical Bulletin* #TB316, Promega Corporation.
(www.promega.com/tbs/tb316/tb316.html)

ORDERING INFORMATION

Product	Size	Cat.#
T7 RiboMAX™ Express RNAi System	50 × 20µl reactions	P1700
T7 RiboMAX™ Express Large Scale RNA Production System*	50 × 20µl reactions	P1320

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[c] U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.

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