The T7 RiboMAX™ Express RNAi System: Efficient Synthesis of dsRNA for RNA Interference

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Abstract

The T7 RiboMAX™ Express RNAi System is an in vitro transcription system designed for the efficient synthesis of milligram amounts RNA in as little as 30 minutes. The RNA is then used to generate double-stranded RNA (dsRNA), which can be used for RNA interference (RNAi) assays in nonmammalian systems. The system is quick, flexible and easy to use. In this article, we show that dsRNA generated using the T7 RiboMAX™ Express RNAi System is functional for RNAi using a Drosophila *S2 cell model.*

The T7 RiboMAX™ Express RNAi System is capable of synthesizing large amounts of dsRNA suitable for use in RNA interference studies.

Introduction

The T7 RiboMAX™ Express RNAi System[a,b] is an in vitro transcription system designed to synthesize milligram amounts of RNA in as little as 30 minutes. The buffering system, NTP concentration, T7 RNA Polymerase, inorganic pyrophosphatase and magnesium levels have been optimized for increased RNA yield compared to standard in vitro transcription reactions (1). The RNA produced is used to generate double-stranded RNA (dsRNA) by simple annealing, nuclease digestion and precipitation steps.

RNA interference, in which long double-stranded RNAs specifically suppress expression of a target gene, was originally discovered in *Caenorhabditis elegans* (2) but has since been observed in numerous organisms including *Drosophila* (3), Trypanosomes (4), *Planaria* (5), *Hydra* (6), and Zebrafish (7). The RNAi mechanism appears to be mediated by smaller dsRNA intermediates. The parent, larger dsRNA is processed by ribonuclease III-like enzymes into smaller fragments in vivo, and the resulting small interfering RNAs (siRNAs) direct post-transcriptional, but pre-translational, degradation of the targeted mRNA (8,9). The RNA interference process seems to act nonstoichiometrically and has been observed to spread between cells (2). RNAi is becoming a powerful tool to investigate gene function through the specific suppression of a particular mRNA and thus "knock out/down" phenotypes for a specific protein.

A phenomenon in which siRNAs produce a similar phenotypic result in mammalian cells has also been observed (10,11). In mammalian cells, dsRNA duplexes <30bp in size induce RNAi, while duplexes >30bp lead to induction of the interferon response and global RNA degradation and translational inhibition (12,13).

In this report, the T7 RiboMAX™ Express RNAi System was used to synthesize dsRNA for use in RNAi experiments with a *Drosophila* S2 cell model in which the ERK-A mRNA was targeted for suppression. ERK-A is the *Drosophila* homolog of mitogen-activated protein kinase (MAPK) and is required downstream of *raf* in the sevenless (Sev) signal transduction pathway (14). Work by Clemens and coworkers showing that expression of the ERK-A protein can be inhibited by RNAi (15) was confirmed in this study.

General Protocol and Product Characteristics

The protocol for synthesis of dsRNA using the T7 RiboMAX™ RNAi System is outlined in Figure 1. In vitro transcription using the RiboMAX™ Express T7 Buffer and Enzyme Mix allows efficient synthesis of RNA in 30 minutes. This RNA is annealed to form dsRNA, and the template DNA and any remaining single-stranded RNA are removed with a nuclease digestion step (DNase I and diluted RNase A Solution). Following nuclease treatment, the dsRNA is precipitated using sodium acetate and alcohol and can be analyzed on native agarose gels for size and integrity. A subsequent G25 spin column purification to remove nucleotides allows quantitation by absorbance at 260nm.

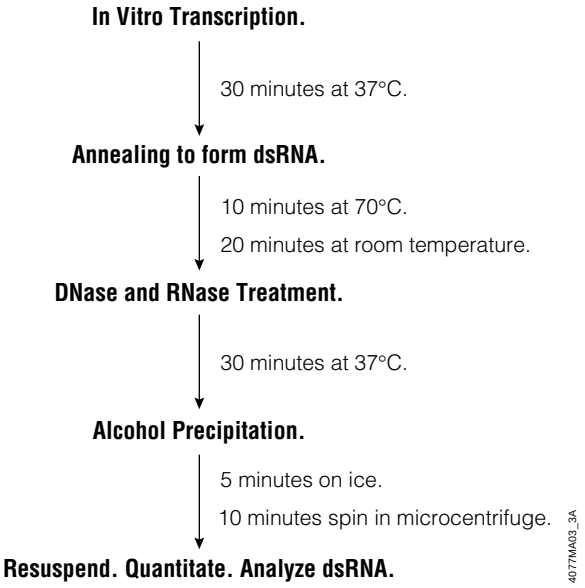

Figure 1. Protocol for the production and purification of dsRNA using the T7 RiboMAX™ Express RNAi System.

The T7 RiboMAX™ Express RNAi System was used to generate dsRNAs in the size range of 180–1,000bp (Figure 2). For most RNAi applications in nonmammalian systems, dsRNAs of 400bp or larger are used (9,16). In general, transcription templates for dsRNA synthesis correspond to most or all of the target message sequence. The yield of dsRNA is template-dependent, but yields of at least 2mg dsRNA/ml of reaction can usually be achieved (Table 1). Longer incubation times during the initial transcription reaction (up to six hours), and incubation at 42°C instead of 37°C, may increase yield, particularly for GC-rich templates. Problematic templates are generally best expressed as single-promoter templates in separate reactions, then pooled prior to annealing.

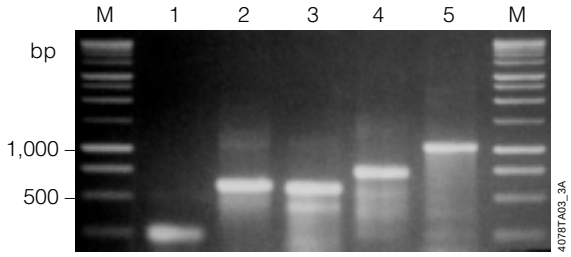

Figure 2. Native gel analysis of different-sized dsRNAs generated using the T7 RiboMAX™ Express RNAi System. Approximately 4×10^{11} molecules of each dsRNA were separated on a 1.8% agarose/1X TAE gel and visualized by staining with 0.5µg/ml ethidium bromide. Lane designations: lane 1, 74ng 180bp ERK-A dsRNA; lane 2, 200ng 500bp *Renilla* dsRNA; lane 3, 200ng 505bp ERK-A dsRNA; lane 4, 312ng 778bp ERK-A dsRNA; lane 5, 400ng 1,000bp *Renilla* dsRNA; lanes M, 1kb DNA Ladder (Cat.# G5711). Note that dsRNA migrates more slowly than double-stranded DNA markers.

Table 1. Yield of Various dsRNAs Produced Using the T7 RiboMAX™ Express RNAi System.

dsRNA Product	Yield (mg dsRNA/ml Reaction)
180bp ERK-A	2.3
500bp *Renilla*	2.8
505bp ERK-A	2.6
778bp ERK-A	2.4
1,000bp *Renilla*	1.7

Results are the average of duplicate reactions using 1µg each of separate, single-promoter templates in the same reaction.

Template Considerations

DNA templates used to generate dsRNA in the T7 RiboMAX™ Express RNAi System must possess a T7 RNA polymerase promoter at the 5′-end of both target sequence strands. This can be accomplished by including T7 promoter sequences in both forward and reverse PCR primers or by using a plasmid vector that includes a T7 promoter (see the *T7 RiboMAX™ Express RNAi System Technical Bulletin* #TB316 for more details). A single template that contains dually opposed T7 promoters will allow the generation of dsRNA. Alternatively, separate single-promoter templates may be used in either the same or separate in vitro transcription reactions.

We have found that the use of separate, single-promoter templates in independent reactions gives the greatest yield of dsRNA (Table 2). Generally, 1–2µg of PCR product template DNA is used per 20µl transcription reaction. We found no difference in the yield of dsRNA between linearized plasmid templates and purified PCR product templates (Figure 3). Up to 3µg of purified, linearized plasmid can be used per 20µl transcription reaction. The transcription reaction volume can be scaled up to 500µl per tube without affecting the yield of dsRNA.

Theoretically, annealing may occur during transcription when using a single, dual-opposed promoter template. However, we have found that, for optimal yield of dsRNA, an annealing step should be performed prior to nuclease treatment for all reactions, regardless of the type of input template DNA (Table 3).

Table 2. Comparison of Yields of 1,000bp *Renilla* dsRNA Obtained Using a Dual-Opposed Promoter Template and Separate Single-Promoter Templates.

Template	dsRNA Yield*
Dual-Opposed Promoter	100%
Separate, Single-Promoter Templates in a Single Reaction (1µg each/20µl reaction)	236%
Separate, Single-Promoter Templates in Two Separate Reactions (1µg/20µl reaction)	323%

* Yield is expressed as a percentage of that achieved using a dual-promoter template. Each synthesis reaction contained the same amount of template DNA for each RNA strand produced.

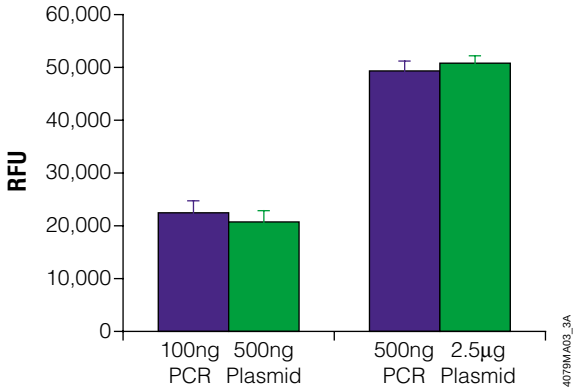

Figure 3. Comparison of dsRNA yields from PCR and plasmid templates. PCR templates (either 100 or 500ng DNA) and an equimolar amount of plasmid template (500ng or 2.5µg) were used to produce dsRNA for the same 778bp ERK-A target. Each reaction contained an equal mix of separate T7 forward and T7 reverse DNAs. The results are the average of duplicate reactions analyzed by agarose gel electrophoresis followed by SYBR® Green II staining. RFU = Relative Fluorescence Units.

Table 3. Effect of Annealing on Yield of dsRNA From Dual-Opposed Templates.

dsRNA Product	Without Annealing	With Annealing*
180bp ERK-A	58%	100%
505bp ERK-A	60%	100%
778bp ERK-A	55%	100%
1,000bp *Renilla*	33%	100%

* Yields obtained with an annealing step were arbitrarily set to 100%.

dsRNA Functional in RNAi

Sense and antisense RNA for the ERK-A target or a nonspecific *Renilla* luciferase target were synthesized using the T7 RiboMAX™ Express RNAi System. *Drosophila* S2 cells were then incubated in the presence or absence of 25µg of ERK-A dsRNA or nonspecific *Renilla* dsRNA for 3 days. Cells were subjected to Western blot analysis for the ERK-A protein (Figure 4). The level of ERK-A protein specifically decreased when the S2 cells were exposed to ERK-A dsRNA but did not decrease upon exposure to the unrelated *Renilla* dsRNA. This is consistent with previous reports of the ERK-A protein decreasing in the presence of ERK-A dsRNA (15).

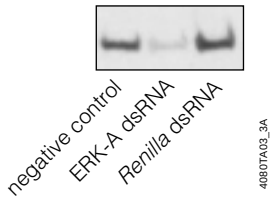

Figure 4. Western blot analysis of the ERK-A (MAPK) protein from S2 cells incubated in the presence or absence of ERK-A 778bp dsRNA or *Renilla* 500bp dsRNA. The blot was generated and analyzed as described in the Methods section. Incubation with the secondary antibody alone showed no bands (data not shown). Lane designations: lane 1, no dsRNA (negative control); lane 2, 25µg ERK-A dsRNA; lane 3, 25µg *Renilla* dsRNA (nonspecific control). Results represent pooled triplicates for each treatment.

Conclusions

The T7 RiboMAX™ Express RNAi System is capable of synthesizing large amounts of dsRNA suitable for use in RNA interference studies in nonmammalian systems. The T7 RiboMAX™ Express RNAi System can be used with plasmid or PCR product template DNA, and can quickly and efficiently generate dsRNA in the range of 180–1,000bp. dsRNA generated against the ERK-A target was able to significantly reduce the levels of ERK-A protein in *Drosophila* S2 cells following incubation of the cells with the dsRNA for 3 days.

Methods

DNA Template Preparation: For ERK-A cloning, *Drosophila* S2 cells were cultured at 26°C in 1X Schneider's *Drosophila* Medium (Invitrogen) containing 12.5% heat-inactivated fetal bovine serum (HyClone), 50 units/ml penicillin and 50µg/ml streptomycin (Invitrogen). Total RNA was purified from the S2 cells using the SV Total RNA Isolation System[c,d] (Cat.# Z3100) according to the protocol provided in the *SV Total RNA Isolation System Technical Manual* #TM048 with approximately 3×10^6 S2 cells/175µl SV RNA Lysis Buffer. The total RNA preparation produced intact 2.2kb (18S) and 2.8kb (28S) ribosomal RNA bands as analyzed by agarose gel electrophoresis. The S2 total RNA (400ng) was used as the template in an RT-PCR reaction for a 778bp region of the *Drosophila* ERK-A mRNA target (see GenBank® accession #M95124; spans from nucleotides 306 to 1,084) using the Access RT-PCR System[b,e] (Cat.# A1250) according to the protocol provided in Technical Bulletin #TB220. The ERK-A RT-PCR product was then reamplified with PCR Master Mix[e,f] (Cat.# M7501) using forward and reverse primers containing a T7 RNA polymerase promoter sequence, either separately or in combination, to produce DNA fragments that contained a T7 RNA polymerase promoter at either or both ends.

PCR products of various sizes were generated either from the 778bp ERK-A target (180bp or 505bp) or from a plasmid containing the *Renilla* luciferase gene (phRL-null Vector[g–j] (Cat.# E6231); 500bp or 1,000bp) using PCR Master Mix.

PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System[k] (Cat.# A9281). The purified DNA was eluted in Nuclease-Free Water and quantitated using absorbance at 260nm.

The 778bp ERK-A product was also cloned into the pGEM®-T Easy Vector[l,m] (Cat.# A1360) in either orientation to the T7 promoter (see the *pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual* #TM042). The vector was then linearized downstream of the insert using *Spe* I, purified using the Wizard® DNA Clean-Up System[k] (Cat.# A7280) and quantitated using absorbance at 260nm.

In Vitro dsRNA Production: The various purified PCR products or linearized plasmids were used as template DNA in the T7 RiboMAX™ Express RNAi System as described in Technical Bulletin #TB316. The protocol is outlined in Figure 1. The precipitated dsRNA was resuspended in Nuclease-Free Water and the concentration determined by gel analysis including comparison to a standard RNA (1.2kb Kanamycin Positive Control RNA[b]; Cat.# C1381). Gels were stained with SYBR® Green II (Molecular Probes), followed by scanning and quantitation using a Molecular Dynamics STORM® machine. In our

experience, neither RiboGreen® nor PicoGreen® analysis is accurate for dsRNA quantitation unless a purified and quantitated dsRNA standard is available.

RNAi in S2 Cells: The purified in vitro-synthesized ERK-A dsRNA was introduced into *Drosophila* S2 cells using the method described in Clemens *et al*. (15). Briefly, 1×10^6 S2 cells in 1ml of *Drosophila* expression system (DES) serum-free medium (Invitrogen) were incubated in triplicate wells of a six-well culture dish in the presence or absence of 25µg of ERK-A dsRNA or nonspecific *Renilla* dsRNA. The S2 cells were incubated at room temperature with the dsRNA for 1 hour, then 2ml of complete growth medium was added and the cells incubated at room temperature for an additional 3 days to allow for turnover of the target protein.

Western Blot Analysis: Following incubation with or without ERK-A dsRNA or *Renilla* dsRNA, the S2 cells were removed from the culture dish. The contents of triplicate wells were pooled and lysed at 2×10^7 cells/ml of RIPA lysis buffer (50mM Tris-HCl [pH 8.0], 150mM NaCl, 0.1% SDS, 1% Triton® X-100, 1% sodium deoxycholate, 0.4mM EDTA, 2µg/ml aprotinin, 2µg/ml Pepstatin A, and 25µg/ml Pefabloc® SC). The lysates were vortexed for 60 seconds to shear genomic DNA. Equal amounts of each cell lysate (12µl) were separated on a 4–12% NuPAGE® Bis-Tris gel (Invitrogen) and transferred to Hybond®-C-supported nitrocellulose (Amersham). After blocking overnight at 4°C in 1X TBS containing 1% blot-qualified BSA, the blot was cut in half, and one half was incubated in 1X TBST containing a 1:5,000 dilution of the Anti-ERK 1/2 pAb, Rabbit (Cat.# V1141) for 2 hours at room temperature with gentle agitation. After three 15-minute washes with 1X TBST, both halves of the blot were incubated with a 1:10,000 dilution of Donkey Anti-Rabbit HRP (Cat.# V7951) in 1X TBST for 1 hour at room temperature with gentle agitation. The blots were washed a further three times (15 minutes each) in TBST and twice for 5 minutes each in TBS. Blots were developed using the HRP detection reagents included in the Transcend™ Chemiluminescent Non-Radioactive Translation Detection System (Cat.# L5080) and exposed to Kodak X-OMAT® film for approximately 60 seconds.

Acknowledgments

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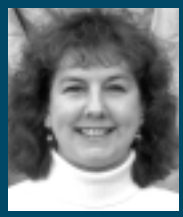
Protocols

- *T7 RiboMAX™ Express RNAi System Technical Bulletin* #TB316, Promega Corporation. (www.promega.com/tbs/tb316/tb316.html)
- *SV Total RNA Isolation System Technical Manual* #TM048, Promega Corporation. (www.promega.com/tbs/tm048/tm048.html)
- *Access RT-PCR System Technical Bulletin* #TB220, Promega Corporation. (www.promega.com/tbs/tb220/tb220.html)
- *pGEM®-T Easy Vector System Technical Manual* #TM042, Promega Corporation. (www.promega.com/tbs/tm042/tm042.html)
- *Wizard® SV Gel and PCR Clean-Up System Technical Bulletin* #TB308, Promega Corporation. (www.promega.com/tbs/tb308/tb308.html)
- *Wizard® DNA Clean-Up System Technical Bulletin* #TB141, Promega Corporation. (www.promega.com/tbs/tb141/tb141.html)



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Ordering Information

Product	Size	Cat. #
T7 RiboMAX™ Express RNAi System[a,b]	50 reactions	P1700
PCR Master Mix[e,f]*	10 reactions	M7501
	100 reactions	M7502
	1,000 reactions	M7505
Access RT-PCR System[b,e]*	20 reactions	A1260
	100 reactions	A1250
	500 reactions	A1280
SV Total RNA Isolation System[c,d]*	10 preps	Z3101
	50 preps	Z3100
pGEM®-T Easy Vector System I[l,m]*	20 reactions	A1360
Blot Qualified BSA*	10g	W3841
Anti-ERK 1/2 pAb, Rabbit	40µl	V1141
Donkey Anti-Rabbit IgG (H+L), HRP*	60µl	V7951
Transcend™ Chemiluminescent Non-Radioactive Translation Detection System*	30 reactions	L5080
Wizard® SV Gel and PCR Clean-Up System[k]*	50 preps	A9281
	250 preps	A9282
Wizard® DNA Clean-Up System[k]*	100 preps	A7280
1.2kb Kanamycin Positive Control RNA[b]*	5µg	C1381
phRL-null Vector[g–j]	20µg	E6231

*For Laboratory Use.

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

[b] U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

[c] U.S. Pat. No. 6,218,531 and other patents pending.

[d] Australian Pat. No. 730718 and other patents and patents pending.

[e] The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this product is recommended for persons that either have a license to perform PCR or are not required to obtain a license.

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