

RNA ENZYMES & GENE ANALYSIS

HiScribe™ T7 *In Vitro* Transcription Kit

Instruction Manual

NEB #E2030S
Store at -20°C

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Kit Components:

All Kit components should be stored at –20°C.

Transcription Buffer (10X)	200 µl
Ribonucleotide Solution Mix (20X)	100 µl (80 mM each)
HMW (20X)	100 µl
T7 RNA Polymerase (500 units/µl)	100 µl
Stul Linearized LITMUS 28iMal Control Plasmid (1 mg/ml)	20 µl

Introduction:

The HiScribe T7 *In Vitro* Transcription Kit is a system for the *in vitro* synthesis of large amounts of single-stranded (ss) or double-stranded (ds)RNA. This RNA can be used in numerous applications including RNA structural studies, ribozyme biochemistry, *in vitro* translation, RNA-protein interactions, anti-sense technology, aptamer discovery (SELEX), and RNA interference (RNAi) experiments.

The HiScribe T7 *In Vitro* Transcription Kit is designed for the production of RNAs that are 0.3 kb and longer (Protocol 1) or for small RNAs 50–300 nt in length (Protocol 2).

The kit contains sufficient reagents for synthesis of up to 3 mg RNA in 2 ml of total reaction volume.

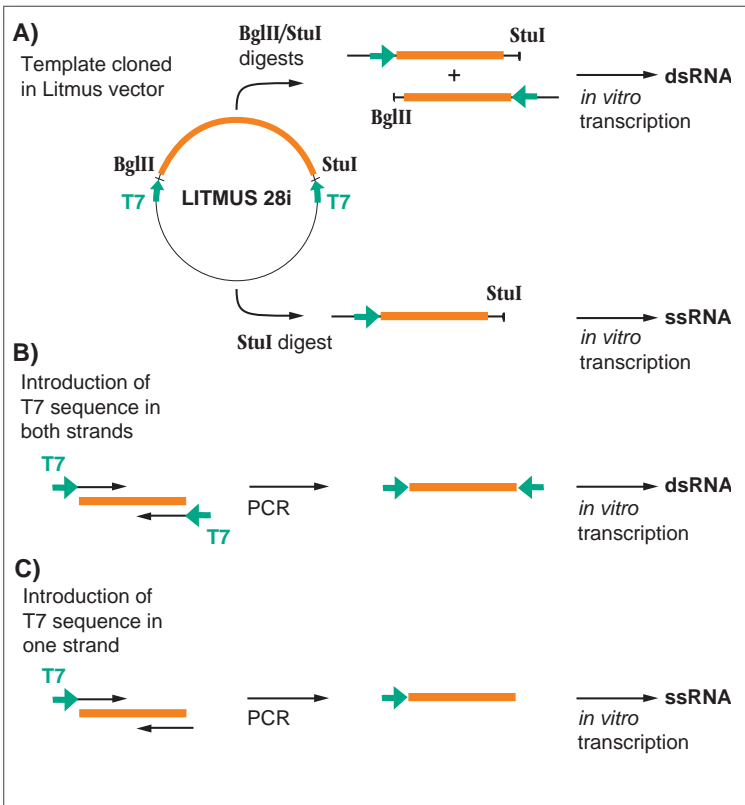
DNA Template Preparation:

The sequence to be transcribed can be cloned in a plasmid downstream of a T7 promoter (e.g. LITMUS 28i or LITMUS 38i – see companion products) or generated by PCR (Figure 1).

Plasmid Templates

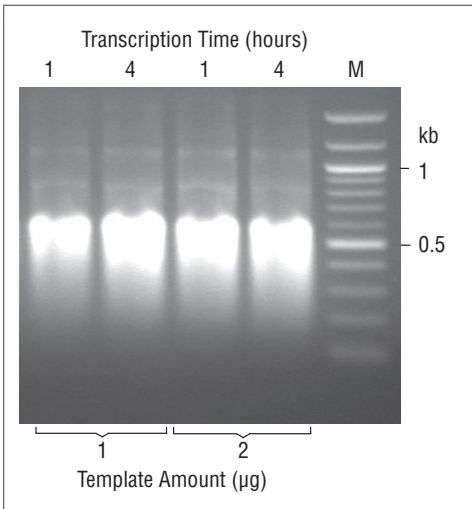
1. Plasmid DNA containing the insert to be transcribed should be purified by a CsCl gradient, or with commercially available chromatographic methods (e.g., Wizard or Qiagen columns). Crude alkaline lysis miniprep DNA contains unacceptable levels of ribonuclease and should not be used for *in vitro* transcription.
2. For ssRNA: Plasmid templates should be linearized by digesting with a restriction enzyme that will cut the plasmid at a site downstream of the insert to be transcribed.
3. For dsRNA: For simultaneous transcription of both strands encoded by a DNA insert, the plasmid containing T7 promoters on both sides of the polylinker should be linearized in separate reactions. The linearized plasmids should then be pooled prior to transcription.
4. The restriction enzyme used to linearize the plasmid should leave blunt ends or 5' overhangs (linearization of template with an enzyme that produces a 3' overhang will result in aberrant transcripts)(1).
5. The quantity of DNA to be digested depends on the scale of the subsequent transcription reaction (see protocols for *in vitro* transcription). As a rough guide, 30 µg of plasmid DNA should be digested per milliliter of transcription reaction. DNA should be digested in a minimum volume of 10 µl per µg of DNA, using the buffer and temperature recommended for each enzyme.

Figure 1.



In vitro transcription with different templates (A) The Litmus 28iMal Control Plasmid containing a portion of *malE* gene is linearized by *StuI* & *BglIII* in two separate restriction digest reactions followed by inactivation of REs: Both linearized DNAs are combined and used as the template to generate double-stranded (ds)RNA or only *StuI* linearized DNA is used as template to generate single-stranded (ss)RNA. (B) When the target template lacks T7 sequence, it can be amplified using T7 containing primers. The PCR product is used as template to generate dsRNA; (C) When the T7 sequence is included in only one primer, the PCR product is used as template to generate ssRNA.

Figure 2.



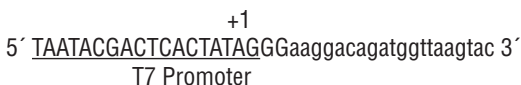
In vitro transcription using either 1 µg or 2 µg of the *StuI* Linearized LITMUS 28iMai Control Plasmid. Transcription reactions were incubated at 42°C for either 1 or 4 hours. One µl of each 40 µl reaction was prepared in formamide containing loading buffer and loaded on a 1% agarose gel. Note: Transcribed ssRNA product (~800 nt) appears smaller than the dsDNA template. The marker lane (M) contains (non-denatured) 100 bp DNA Ladder (NEB #N3231).

6. Confirm digestion by agarose gel electrophoresis and estimate quantity of DNA by comparison of band intensity to a known quantity of linearized marker DNA. The presence of small amounts (< 5%) of undigested plasmid will have little effect on RNA yield.
7. Linearized DNA can be used straight from the digestion reaction if desired, with only a slight reduction of yield (< 10%). However, digestion reactions must be heat-denatured at 65°C for 20 minutes prior to transcription. Template linearized DNA from restriction digestion reaction should not constitute more than 10% of the total transcription volume.

PCR Templates

1. The template can be amplified by PCR using a primer containing the T7 promoter sequence. *In vitro* transcription of the PCR product will produce single-stranded, or double-stranded RNA directly if both PCR primers contain the T7 promoter sequence.
2. When designing the T7 promoter sequence containing primers, it is recommended to add two Gs after the T7 promoter sequence. Transcription yields have been shown to be reduced if these two Gs are absent (2).

An example of an amplification primer with T7 promoter (underlined) at the 5' end preceding the gene-specific (lower-case) sequence is shown below. The first nucleotide of the transcript is indicated (+1).



3. The PCR reaction should be analyzed by gel electrophoresis in order to confirm amplification and to provide a estimate of quantification.
4. The PCR product can be used directly as a template for transcription, without purification. Alternatively, purify the PCR product by phenol/chloroform extraction and ethanol precipitation, or equivalent chromatographic method (e.g., QiaQuick), and resuspend in TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, prepared with Milli-Q water or equivalent] to a final concentration of ~500 µg/ml.

Protocol 1

In Vitro Transcription of Long Templates (> 0.3 kb):

This protocol may be used for any large-scale transcription reaction. Yields of ~1.5 mg per ml of transcription reaction should be easily obtainable using the HiScribe T7 *In Vitro* Transcription Kit. A 40 µl pilot reaction should be carried out initially to test the quality of DNA template and transcription reagents. Reactions can then be scaled up accordingly as required by the particular application. Pilot reactions should be carried out side-by-side with reactions containing the *Stul* Linearized LITMUS 28iMal Control Plasmid.

Transcription Reaction

1. Thaw the 10X transcription buffer and 20X NTP mix at room temperature for the minimum amount of time required for complete thawing. Do not thaw at 37°C. If precipitant is evident following thawing, vortex briefly to resuspend. Keep the 20X High Molecular Weight Component (HMW) Mix and T7 RNA Polymerase at -20°C until needed.
2. Combine the following, in order, taking caution to avoid ribonuclease contamination. Set up separate reactions for each plasmid of interest, as well as the *Stul* Linearized LITMUS 28iMAL Control Plasmid.

RNase-Free Water	X µl
10X Transcription Buffer	4 µl
20X Ribonucleotide Solution Mix	2 µl
Template(s) (1–2 µg)	X µl
20X HMW Mix	2 µl
T7 RNA Polymerase (500 units/µl)	2 µl
Total reaction volume	40 µl

3. Incubate at 42°C for 2–4 hrs.
4. For analysis of transcription run 1 μ l of transcription reaction in a 1% agarose gel. The expected length of the transcript from the control template (StuI Linearized LITMUS 28iMal Control Plasmid) is 901 nt.

Large Scale Transcription Reaction

1. If the yield of RNA from the pilot reaction is sufficient, scale-up the reaction appropriately. Use no more than 500 μ l per tube; use multiple tubes if necessary.
2. The RNA product can be purified either by phenol/chloroform extraction and ethanol precipitation, isopropanol precipitation or by using a commercial kit for RNA purification.

Protocol 2

In Vitro Transcription of Short Templates (50-300 nt):

For short templates we recommend an increase in template concentration, polymerase concentration and reaction time, since transcription of small RNAs is largely dependent on the number of initiation events.

RNase-Free Water	X μ l
10X Transcription Buffer	4 μ l
20X Ribonucleotide Solution Mix	2 μ l
Template (0.3–0.5 μ g)	X μ l
20X HMW Mix	2 μ l
T7 RNA Polymerase (500 units/ μ l)	4 μ l
Total reaction volume	<hr/> 40 μ l

Incubate at 42°C for at least 3–4 hrs. Overnight incubations will result in higher yields.

Notes:

Transcription yields increase linearly for the first 90 minutes (approximately) and reach maximum after 2–3 hours. Reactions can be carried out overnight if desired, but yields will not be higher (except when transcribing RNA shorter than 0.5 kb). Double-stranded RNA is stable upon prolonged incubation at 42°C.

The volume of template used in the transcription reaction depends on the method of purification. For unpurified, heat-killed restriction digests, include no more than 13 μ l of template per 40 μ l reaction. For unpurified PCR product, include no more than 7 μ l per 40 μ l reaction. In all cases, the amount of added template DNA should not exceed 2 μ g per 40 μ l reaction, as RNA yields will not be higher at template concentrations greater than this.

While all of the components in the HiScribe T7 In Vitro Transcription Kit are RNase-free, it is possible to introduce ribonucleases into the transcription reaction from the laboratory environment. This can be avoided by following some simple precautions: 1) always wear gloves when working with RNA, 2) use either a dedicated set of pipettors for RNA work or aerosol-resistant (barrier) pipette tips, 3) use ultrapure water (Milli-Q or equivalent) and autoclave all solutions if possible, and 4) use disposable plasticware instead of glassware whenever possible. It should NOT be necessary to treat solutions and equipment with diethyl pyrocarbonate (DEPC). DEPC can inhibit subsequent reactions if it is not completely inactivated following treatment.

Frequently Asked Questions:

Why cloned templates have to be linearized prior to transcription? Why can't I simply transcribe the circular plasmid containing my target sequence?

T7 RNA Polymerase is an extremely processive enzyme, and will continue to transcribe around a circular template multiple times without disassociating, producing a transcript much longer than the plasmid. Not only will the transcript from each promoter contain mostly vector-derived sequence, but the long length makes coherent annealing into a fully double-stranded product very difficult. Linearization at a point downstream from the cloned target sequence, either by restriction digestion or PCR, will produce an RNA transcript with a length defined by the 3' end of the template (3), and the resulting RNA duplex will contain only the target sequence flanked by a small amount of vector polylinker sequence.

What is the shelf-life of the supplied reagents?

The T7 RNA Polymerase and High Molecular Weight (HMW) Mix should be stable for at least 1 year when stored at -20°C . The 10X Transcription Buffer/NTP Mix should be stable for up to 6 months, but stability will vary greatly depending on how often it is thawed and refrozen. For maximum stability, aliquot and store at -70°C .

Why is the yield for my transcription reaction lower than expected, and/or the bands are smeary?

Be sure to carry out a control pilot reaction with the *StuI* Linearized Litmus 28iMal Control Plasmid. If the yield from your plasmid is substantially lower than that from the control reaction, it is possible your plasmid has RNase contamination. This problem can be solved either by further purifying your plasmid DNA, carrying out phenol extraction and ethanol precipitation steps. If yields from reactions containing your plasmid and the control plasmid are equally low, double-check the concentration of your template DNA, make sure your gel loading buffer does not contain SDS, and confirm that both the gel and tank buffer were prepared using ultrapure water (Milli-Q or equivalent). It is also possible that the linearization method used (restriction digestion or PCR) introduced RNase into both templates; again, phenol extraction followed by ethanol precipitation prior to transcription should solve this problem.

Additional Protocols:

Purification of dsRNA by Isopropanol Precipitation:

This RNA purification protocol is adequate for some applications such as RNaseIII digestions for siRNA production

1. Dilute transcription reaction by adding 1 volume (1 V) of RNase-free water.
2. Add an equal volume of isopropanol.
3. Add 1/10 V of 3M Sodium Acetate (pH 5.2)
4. Incubate at room temperature for 5 minutes
5. Centrifuge for 10 minutes at 16,000 g
6. Remove supernatant and add 1V of 70% ETOH
7. Incubate at room temperature for 2–5 minutes
8. Centrifuge for 5 minutes at 16,000 g
9. Remove supernatant and allow the RNA pellet to air dry
10. Resuspend RNA RNase-free water or TE

RNA Quantitation:

UV Absorbance: 1 A_{260} unit = 40 $\mu\text{g/ml}$ RNA

Note: Nucleotide removal by G25 spin column is essential for accurate quantitation.

References:

1. Schenborn, E.T. and Mierendorf, R.C. (1985) *Nucl. Acids Res.*, 13, 6223–6236.
2. Milligan, J.F. et al. (1987) *Nucl. Acids Res.*, 15, 8783–8798.
3. Sampson, J.R. and Uhlenbeck, O.C. (1988) *Proc. Natl. Acad. Sci. USA*, 85, 1033–1037.

Appendix:

Supplied Components:

10X Transcription Buffer, 200 μ l

400 mM Tris-HCl (pH 8.0)

190 mM MgCl₂

50 mM DTT

10 mM spermidine

20X Ribonucleotide Solution Mix (rNTPs), 100 μ l

20 mM Tris-HCl (pH 7.8)

10 mM DTT

80 mM each NTP

20X High Molecular Weight (HMW) Mix, 100 μ l

20 mM Tris-HCl (pH 8.0)

83 units/ml inorganic pyrophosphase (yeast)

20,000 units/ml murine ribonuclease inhibitor

40% glycerol

Ordering Information

PRODUCT	NEB #	SIZE
HiScribe™ T7 <i>In Vitro</i> Transcription Kit	E2030S	50 Reactions
COMPANION PRODUCTS		
LITMUS 28i Vector	N3528S	20 μ g
LITMUS 38i Vector	N3538S	20 μ g
T7 Minimal Primer (19-mer)	S1272S	0.5 A ₂₆₀ units
Biotinylated T7 Primer (25-mer)	S1273S	0.5 A ₂₆₀ units
Ribonucleotide Solution Mix	N0466S/L	8/40 μ mol of each
ShortCut RNase III	M0245S/L	130/650 units
dsRNA Ladder	N0363S	25 gel lanes
Low Range ssRNA Ladder	N0364S	25 gel lanes
ssRNA Ladder	N0362S	25 gel lanes
siRNA Marker	N2101S	100 gel lanes
microRNA Marker	N2102S	100 gel lanes

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