

Large Scale Transcription Kits

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About the Kits

Large Scale T7 Transcription Kit	69256-3
Large Scale SP6 Transcription Kit	69206-3

Description

The Large Scale Transcription Kits are designed for convenient and consistent *in vitro* production of large amounts of RNA from sequences cloned in vectors containing T7 or SP6 promoters. The transcription conditions have been optimized to provide maximal yields of full-length RNA that can be used in a variety of applications, including *in vitro* translation, structural analysis, splicing, anti-sense, and ribozyme synthesis. Under these conditions, more than 700 moles of transcript can be synthesized per mole of template (1). The nucleoside triphosphates are provided in separate vials to allow substitution of modified or labeled NTPs, under limiting conditions, for probe synthesis. The kits also include a positive control template to monitor the performance of test DNAs.

Components

The kits include enough reagents to perform 100 synthesis reactions (100 µl vol), each of which will produce more than 50 µg of RNA from 5 µg template in 60 minutes under non-limiting conditions. The kit contains the following components:

- 10,000 U T7 RNA Polymerase
- or 10,000 U SP6 RNA Polymerase
- 2 × 1 ml 5X T7 Transcription Buffer (400 mM HEPES, 60 mM MgCl₂,
50 mM NaCl, pH 7.5)
- or 5X SP6 Transcription Buffer (400 mM HEPES, 80 mM MgCl₂,
50 mM NaCl, 10 mM spermidine, pH 7.5)
- 1 ml 20 mM ATP solution
- 1 ml 20 mM CTP solution
- 1 ml 20 mM GTP solution
- 1 ml 20 mM UTP solution
- 0.1 ml 1 M DTT
- 4 × 1.5 ml Nuclease-free Water
- 1 ml 3 M Na Acetate, pH 5.2
- 5 µg Control Template (0.5 µg/µl)
- 1000 U RNase-free DNase I

Storage

Store all components at -20°C.

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Avoiding Ribonuclease Contamination

Ribonucleases present in the environment, and within your sample, can rapidly degrade RNA, resulting in low yield and poor quality. To avoid RNA degradation, several precautions should be observed:

- All materials coming in contact with the sample must be sterile and RNase-free. Use sterile disposable pipets, pipet tips, and tubes. Use sterile technique at all times.
- Wear gloves during the entire procedure to avoid introducing RNase contaminants to the sample from your hands.
- The 70% ethanol solution used for rinsing DNA and RNA pellets should be made with diethyl pyrocarbonate (DEPC) treated water. DEPC water is made by adding DEPC to Milli-Q, or equivalent, deionized water, to a final concentration of 0.1% (0.001 vol), mixing well, and autoclaving or heating to 70°C for 1 h. Observe proper safety precautions, including use of a fume hood. DEPC is a powerful acylating agent and forms ethyl carbamate, a potent carcinogen, when exposed to ammonia.
- Reserve reagents exclusively for RNA work and store them separately. Avoid using any reagent that may have been used for other work. If possible, separate any laboratory procedures that require the use of RNase, such as plasmid preps, from your RNA work area.

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Template Preparation

In cases where the target gene is in a plasmid vector, the plasmid is usually linearized slightly downstream of, or within, the desired probe sequence prior to transcription. Obtain, or subclone, the sequence of interest into a vector suitable for *in vitro* RNA transcription. For optimal performance, template must be free of nucleases and salts.

If the restriction enzyme used for linearization leaves 3' overhangs, use a treatment to prepare blunt ends prior to transcription to prevent the generation of aberrant transcripts caused by transcription of the non-template DNA strand (2). This can be achieved by treatment with Klenow DNA polymerase. If the restriction digest is performed in a compatible buffer containing 5–10 mM MgCl₂, the procedure involves adding 1 U Klenow/μg DNA and 25 μM of all four dNTPs directly to the reaction upon completion, and incubating at room temperature for 5 min.

Note: Alternatively, target DNA can be converted to a blunt, phosphorylated form in a single, brief reaction using a Perfectly Blunt® Cloning Kit. See User Protocol TB183 for additional information.

The following is a list of some of the restriction enzymes that can be problematic because they produce 3' overhangs of two or more bases:

Aat II	AlwN I	Apa I	ApaB I	Ban II	Bce83 I	Bcg I	Bgl I	Bpm I	BseR I
Bsg I	BsiE I	BsiHKA I	Bsl I	Bsm I	Bsp12681 I	Bsp24 I	Bsr I	BsrD I	BstX I
Dra III	Drd I	Eco57 I	Fse I	Hae II	Hha I	Kpn I	Mme I	Mwo I	Nla III
Nsi I	Nsp I	Pac I	PfIM I	Pst I	Pvu I	RleA I	Sac I	Sac II	Sfi I
Sgf I	Sph I	Sse8387 I	Tai I	Taq II	Tth111 II				

- Linearize recombinant plasmid using an appropriate restriction enzyme. For example, combine:

20 μg	plasmid in TE buffer
20–100 U	appropriate restriction enzyme
10 μl	10X enzyme buffer (as recommended or supplied by manufacturer)
X μl	deionized water
100 μl	total volume

 Incubate at least 2 h at the appropriate temperature.
- Confirm complete digestion by analyzing a 2 μl sample of the reaction on an agarose gel alongside the uncut plasmid.
- Extract DNA twice with 1 vol (100 μl) TE-buffered phenol/CIAA (1:1; CIAA is 24 parts chloroform and 1 part isoamyl alcohol). Each time, vortex sample 30 s. Spin in microcentrifuge for 1–2 min.
- Extract DNA once with 1 vol CIAA. Vortex sample 30 s. Spin in microcentrifuge for 1–2 min.
- Transfer final top aqueous phase to fresh tube. Add 0.1 vol 3 M Na acetate, pH 5.2, and 2 vol ethanol. Place at –20°C for 1 h.
- Collect precipitate by centrifugation at 12,000 × g for 5 min. Carefully remove supernatant with pipet. Rinse pellet with 70% ethanol.
- Remove supernatant and dry pellet briefly under vacuum.
- Resuspend DNA in 20 μl TE buffer (final DNA concentration is approximately 0.5–1 mg/ml). Read absorbance of an appropriate dilution (e.g., 5 μl DNA + 300 μl water) at 260 nm to determine DNA concentration (1 A₂₆₀ U = 50 μg/ml). Store at –20°C.

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Transcription

Standard Reaction

The standard reaction uses 5 µg linearized plasmid in a 100 µl reaction. The reaction can be scaled up or down proportionately.

1. Thaw 5X Transcription buffer, 1 M DTT, NTPs, Nuclease-free Water, and 3 M Na Acetate at room temperature. Immediately after thawing, place all components on ice. Gently vortex or flick tubes to ensure proper mixing of components. It may be necessary to heat the 1 M DTT briefly at 37°C and vortex to ensure all material is resuspended.
2. Assemble the following components, in the order given below, at room temperature in a sterile, RNase-free 1.5-ml tube:

x µl	Nuclease-free Water
y µl	(5 µg) linearized DNA in TE buffer
10 µl	20 mM ATP
10 µl	20 mM CTP
10 µl	20 mM GTP
10 µl	20 mM UTP
20 µl	5X Transcription Buffer
1 µl	DTT
<u>1 µl</u>	<u>T7 RNA Polymerase or SP6 RNA Polymerase (100 U/µl)</u>
100 µl	total volume
3. After adding the enzyme, gently stir with pipet tip to mix. Incubate at 37°C for 2 h.
4. (Optional template removal) Add 5 µl (5 U) RNase-free Dnase I. Incubate at 37°C for an additional 10 min.
5. Extract once with 100 µl TE-buffered phenol (without CIAA). Vortex vigorously for 30 s. Centrifuge at 12,000 × g for 1 min. Carefully remove bottom organic phase, leaving aqueous phase and any interface material.
6. Extract once with 100 µl phenol/CIAA (1:1). Remove organic phase, as above.
7. Extract once with CIAA. Transfer top aqueous phase to fresh 1.5-ml tube, leaving any remaining interface material behind.
8. Add 10 µl 3 M Na Acetate. Mix by vortexing. Add 66 µl (0.6 vol) isopropanol. Mix by vortexing. Incubate at room temperature for 5 min. Centrifuge at room temperature for 5 min. Unincorporated nucleotides do not precipitate under these conditions.
9. Carefully decant supernatant. Drain briefly. Rinse pellet in 70% ethanol. Dry under vacuum. Resuspend RNA in RNase-free water or TE. Alternatively, follow the 70% ethanol wash with a 100% ethanol wash, and air dry.
10. The isopropanol precipitation effectively removes unincorporated nucleotides and allows a reasonably accurate estimation of RNA concentration by reading the absorbance at 260 nm. Read a 1:3000 dilution in water. An A_{260} of 1 equals 40 µg/ml RNA; the A_{260}/A_{280} ratio should be about 2.0 for RNA.
11. Store the RNA at -20°C.

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Notes:

The above conditions should produce a minimum of 50 µg RNA transcript from properly prepared DNA templates. If even greater yields are desired, the following modifications can be made.

- a. Add more RNA polymerase (100 U) after 1 h incubation.
- b. Add 15 µl of each NTP (instead of 10 µl), and add 30 µl of 5X Transcription Buffer (instead of 20 µl). This will increase the NTP concentration to 3 mM each.
- c. Precipitate RNA with 2 volumes ethanol instead of isopropanol. This precipitation will give an artificially inflated yield of RNA when measured with a spectrophotometer (due to precipitation of unincorporated nucleotides), but will maximize recovery of authentic transcripts.

Positive Control Reaction

To verify the performance of the transcription reaction, carry out a control reaction using the supplied Control Template. The Control Template is supplied, ready for use, as a linearized plasmid containing the appropriate transcription promoter. The control can be run as a full-scale 100 µl reaction, using 5 µg DNA. It is also possible to scale the reaction down to conserve reagent. The following conditions for a 20 µl reaction require dilution of the polymerase and DTT prior to their addition.

1. Prepare 1:5 dilution of T7 or SP6 polymerase in 1X Transcription Buffer. Add 3 µl Nuclease-free Water to tube, followed by 1 µl 5X Transcription Buffer and 1 µl enzyme. Stir with pipet tip to mix. Place on ice.
2. Prepare a 1:5 dilution of 1 M DTT. Add 1 µl 1 M DTT to 4 µl Nuclease-free Water in tube. Place on ice.
3. Assemble the following components, in the order shown, at room temperature

4 µl	Nuclease-free Water
2 µl	Control Template
2 µl	20 mM ATP
2 µl	20 mM CTP
2 µl	20 mM GTP
2 µl	20 mM UTP
4 µl	5X Transcription Buffer
1 µl	0.2 M DTT from Step 2
<u>1 µl</u>	<u>Diluted RNA Polymerase from Step 1 (20 U/µl)</u>
20 µl	total volume
4. Incubate and process sample, as described for the standard reaction, at 1/5 scale. Sizes for the Control Template transcripts are 2800 nt (T7) and 846 nt (SP6).

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Quick Gel Analysis of RNA

The quality of RNA preparation can be rapidly assessed by agarose gel electrophoresis, followed by staining with ethidium bromide or SYBR[®] Green II (Molecular Probes). Although denaturing gels (e.g. formaldehyde-agarose, described below) eliminate the effects of RNA secondary structure that can lead to aggregation and anomalous migration, native gels are easier to run. RNA is also stained and photographed with higher sensitivity using native gels.

It is necessary to use an apparatus that is free of RNase. In particular, avoid any apparatus that has been used for analysis of plasmid minipreps, as these usually contain copious amounts of RNase. Always run a control lane of a known intact RNA, such as Novagen Perfect RNA[™] Markers (Cat. Nos. 69924-3, 69946-3, 69211-3) to verify the apparatus and gel are free of RNase contamination.

1. Pour a 1% agarose gel in sterile filtered 1X TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). Use clean glassware and high-quality agarose. Include 0.5 µg/ml ethidium bromide in the gel. For lower background, omit the ethidium bromide from the gel, and post-stain in 0.5 µg/ml ethidium bromide for 10 min.
2. Transfer sample containing 0.5–2 µg RNA in water or TE to sample buffer (90% deionized formamide, 10% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanole). The RNA sample can comprise up to 50% of the volume, although resolution is generally better using less. The final volume should be no more than 20 µl.

Note: Formamide can be deionized using a Dowex[®] resin, or other chelating resins (3). Test the pH using pH paper: deionized formamide should be neutral; non-deionized formamide contains ammonia and is approximately pH 12.

3. Mix sample by gentle pipetting. Heat at 60–65°C for 3 min. Cool to room temperature, and load gel.
4. Run gel at up to 10 V/cm in 1 X TBE. Stain if necessary. Photograph under UV illumination, as with DNA gels.

The presence of significant material greater than the expected size indicates the plasmid was not digested completely with the restriction enzyme, or that the DNA has 3' overhanging ends. Smearing below the expected band indicates RNase activity, or premature transcription termination products. RNase activity can usually be distinguished by the greater severity of smearing, especially in the smaller size ranges. Some degree of premature termination is usually observed, and can be minimized by increasing the NTP concentration in the transcription reaction, or by shortening the time of incubation to 30 min. Although rare, some sequences contain natural pausing sites, or even terminators for T7 or SP6 polymerase, which can be observed as intense bands smaller than the expected size. Modifications that may minimize these effects include raising or lowering the incubation temperature, and adjusting the salt concentration or NTP ratios. If a strong terminator is encountered, it is best to look for another sequence.

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Probe Synthesis

The components of these kits are also suitable for synthesis of labeled RNA for use as hybridization probes or size markers. Novagen offers Perfect RNA™ Marker Template Mix (Cat. No. 69003-3) for preparing labeled markers, ranging from 0.1–1 kb in size, with T7 RNA polymerase. Under these conditions, one of the provided NTPs is usually limited in concentration, to allow efficient incorporation of appropriate nucleotide analog (e.g. radioactive, or biotin-labeled NTP). The reaction volume is usually scaled down to 20 µl, although the reaction can be scaled proportionately, as desired. The following reaction produces high specific activity ³⁵S-UTP labeled transcripts.

1. Prepare 184 µM UTP by adding 1 µl 20 mM UTP stock to 107 µl Nuclease-free Water
2. Assemble the following components, in the order shown, at room temperature:

x µl	Nuclease-free Water
y µl	(1 µg) linearized DNA in TE buffer
2 µl	20 mM ATP
2 µl	20 mM CTP
2 µl	20 mM GTP
2 µl	184 µM UTP (prepared in Step 1)
4 µl	5X Transcription Buffer
2 µl	0.1 M DTT (dilute supplied 1 M DTT 1:10 in Nuclease-free Water)
2–5 µl	[α- ³⁵ S]UTP (1250 Ci/mmol; 20–100 µCi)
0.5 µl	T7 or SP6 RNA Polymerase (100 U/µl)
20 µl	total volume

Note? Alternative labels can be utilized by limiting the appropriate NTP. Eliminating the cold NTP, or using two labeled NTPs and limiting (or eliminating altogether) the two corresponding cold NTPs, allows synthesis of higher specific activity probes. To reduce the occurrence of incomplete transcripts when no cold NTP is used, after the initial labeling reaction, add the appropriate cold NTP for a 30 min "chase" period.

3. Mix by gentle pipetting. Incubate for 1 h at 37°C.
4. The probe can generally be used directly without further processing or template removal. It is usually unnecessary to try and remove unincorporated nucleotides, as nearly all the radioactive label will be incorporated. Incorporation can be quantified using standard methods for precipitation with trichloroacetic acid (TCA). Proper procedures for handling and disposal of radioactive isotopes should be followed.

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