Protocol

High-Yield Synthesis of RNA Using T7 RNA Polymerase and Plasmid DNA or Oligonucleotide Templates

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When a large amount (milligram quantities) of RNA is desired (e.g., for making RNAs for RNA interference studies or for structural analyses), it is advantageous to use optimized transcription conditions. The method described here uses high millimolar concentrations of rNTPs, pyrophosphatase, and a specially formulated high-yield transcription buffer. These conditions prevent product (pyrophosphate) inhibition of RNA polymerase, which permits the accumulation of large quantities of RNA.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

ATP, CTP, GTP, and UTP stocks (100 mM each, pH ~7.0)
Dithiothreitol (DTT) (0.5 M)
High-yield transcription buffer (10×) <R>
Inorganic pyrophosphatase (IPP) in storage buffer <R>
$MgCl_2$ (1 M)
RNasin RNase inhibitor (40 units/µL; Promega)
RQ1 RNase-free DNase (1 unit/µL; Promega)
T7 RNA polymerase, purchased commercially or prepared in the laboratory (see **Expression and Purification of Active Recombinant T7 RNA Polymerase from *E. coli*** [Rio 2013])
Template DNA

Use cleaved plasmid DNA (0.5 mg/mL), gel-purified PCR product (0.1 mg/mL), with sequence of interest adjacent to a T7 promoter, or annealed synthetic oligonucleotides (20 µM). For hybridizing oligonucleotides to prepare templates 10–80 nucleotides in length, make a 20-µM mix of T7 top strand (17-mer) and the oligonucleotide sequence of interest in TE buffer containing 0.1 M NaCl. Heat the mixture for 2 min to 90°C and then leave it at room temperature until it cools to <37°C.

Equipment

Air chamber incubator or room at 37°C
Desalting column

Adapted from *RNA: A Laboratory Manual*, by Donald C. Rio, Manuel Ares Jr, Gregory J. Hannon, and Timothy W. Nilsen. CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Dry ice
Heat block (set at 90°C)
Ice
Microcentrifuge tubes
Preparative gel (optional; see Step 4)

METHOD

1. Combine the following in a microcentrifuge tube in the order listed (add the RNA polymerase enzyme last). Keep the components for the reaction on ice, but add them to a reaction tube at room temperature.

High-yield transcription buffer (10×)	10 µL
H_2O	44.4 µL
DTT (0.5 M)	2 µL
$MgCl_2$ (1 M)	2.6 µL
ATP (100 mM)	7.5 µL
CTP (100 mM)	7.5 µL
GTP (100 mM)	7.5 µL
UTP (100 mM)	7.5 µL
RNasin RNase inhibitor (40 units/µL)	2 µL
IPP (0.5 unit/µL)	2 µL
Template DNA (use annealed synthetic oligonucleotides [20 µM], cleaved plasmid DNA [0.5 mg/mL], or gel-purified PCR product [0.1 mg/mL])	5 µL
T7 RNA polymerase (500 units/µL)	2 µL
Total	100 µL

Add $MgCl_2$ only if the 100 mM NTP stocks do not contain magnesium. The $MgCl_2$ concentration should be 6 mM greater than the total NTP concentration.

The 10× high-yield transcription buffer contains spermidine, which can form an insoluble complex with the template DNA if the reaction is set up on ice. Therefore, it is critical that the components for the reaction (which are kept on ice) be added to a reaction tube at room temperature.

The reaction can be scaled up many-fold if necessary.

2. Incubate the reaction in an air chamber incubator or room for 8 h to overnight at 37°C. Do not use a temperature block.
3. Add 5 µL of 1 unit/µL RQ1 RNase-free DNase and incubate for 30 min at 37°C.
4. Purify by using a desalting gel filtration column and then a preparative gel, if necessary.

   See Troubleshooting.

5. Quantify the RNA products, if desired. (See **Determining the Yield of RNA Synthesized In Vitro** [Nilsen 2013].)

   This requires the setup of a trace-labeled reaction (i.e., with [α-^{32}P]UTP or [α-^{32}P]GTP) in parallel with the unlabeled reaction in Step 1. If 20% of the nucleotides are incorporated, you should have made 200 µg per 100-µL reaction. See Troubleshooting.

TROUBLESHOOTING

Problem (Step 4): Products are shorter than expected.

Solution: Either the transcription reaction produced truncated RNAs (which can occur when the enzymes pause at A/T-rich stretches in the template) or RNase contamination was introduced at some point during the procedure. Often, if this is the case, it is better to prepare all new reagents and begin again, rather than individually test each component separately. Create an RNase-free

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environment and ensure that researchers are free of RNases; this will go a long way to eliminate frustration caused by degraded RNA preparations.

Problem (Step 5): The yield of RNA product is low.

Solution: A common reason for this problem is inaccurate quantification of the template DNA. Usually, it is better to quantitate DNA on a gel rather than use OD_{260}. The yields of synthetic RNAs from oligonucleotide templates are often much lower per template molecule than those with cleaved plasmid DNA templates. If this is the case and a partial duplex oligonucleotide template is being used, such as that described in Milligan et al. (1987), try using a full-length top-strand oligonucleotide instead of just a top-strand promoter oligonucleotide.

RECIPES

High-Yield Transcription Buffer (10×)

Reagent	Quantity (for 1 mL)	Final concentration (10×)
HEPES–KOH (2 M, pH 7.5)	0.5 mL	1 M
$MgCl_2$ (1 M)	0.1 mL	0.1 M
Spermidine–HCl (1 M)	20 µL	0.02 M
DTT (2 M)	0.2 mL	0.4 M
Bovine serum albumin (BSA), RNase-free (20 mg/mL)	50 µL	1 mg/mL
H_2O	130 µL	

Store for up to 1 wk at 20°C.

Inorganic Pyrophosphatase in Storage Buffer

Reagent	Quantity (for 10 mL)	Final concentration
HEPES–KOH (1 M, pH 7.5)	500 µL	50 mM
DTT (2 M)	5 µL	1 mM
Glycerol, autoclaved	5 mL	50%
H_2O	4.45 mL	

Resuspend the IPP (Sigma I1891; 1000 units/mg, 100 µg/vial) in the solution above at a concentration of 0.5 unit/µL. Store it for up to 1 yr at 20°C.

REFERENCES

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