

## HiScribe™ T7 High Yield RNA Synthesis Kit

NEB #E2040S/L

50/250 reactions

Version 7.0\_1/24

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### The HiScribe T7 High Yield RNA Synthesis Kit Includes:

*All kit components should be stored at –20°C. Each kit contains sufficient reagents for 50 x 20 µl reactions (S size) or 250 x 20 µl reactions (L size).*

Reaction Buffer (10X)  
 ATP (100 mM)  
 GTP (100 mM)  
 UTP (100 mM)  
 CTP (100 mM)  
 FLuc Control Template (0.5 µg/µl)  
 T7 RNA Polymerase Mix  
 Dithiothreitol (DTT) (0.1 M)

## Required Materials Not Included:

DNA Template:	The DNA template must be linear and contain the T7 RNA Polymerase promoter with correct orientation in relation to target sequence to be transcribed.
Cap Analogs:	NEB #S1411, #S1405, #S1406 and #S1407
Modified-NTP:	Biotin-, Fluorescein-, Digoxigenin-, or Aminoallyl-NTP
Labeling:	[ $\alpha$ - <sup>32</sup> P] labeled ribonucleotide (800-6,000 Ci/mmol)
General:	37°C incubator or PCR machine, nuclease-free water
DNase I:	DNase I (RNase-free) (NEB #M0303)
Purification:	Buffer- or water-saturated phenol/chloroform, ethanol and 3 M sodium acetate, pH 5.2, spin columns
Gel Analysis:	Gels and running buffers, gel apparatus, power supply

## Introduction

The HiScribe T7 High Yield RNA Synthesis Kit is designed for *in vitro* transcription of RNA using T7 RNA Polymerase. The kit is suitable for synthesis of high yield RNA transcripts and for incorporation of modified nucleotides to obtain biotin labeled, dye labeled or capped RNA. The kit is also capable of synthesizing high specific activity radiolabeled RNA probes.

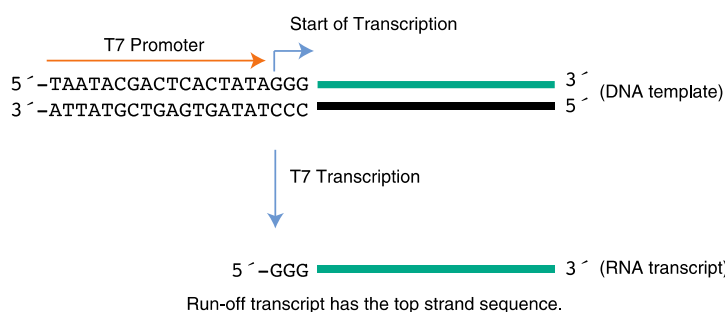
RNA synthesized from the kit is suitable for many applications including RNA structure and function studies, ribozyme biochemistry, probes for RNase protection assays and hybridization based blots, anti-sense RNA and RNAi experiments, microarray analysis, microinjection, and *in vitro* translation and RNA vaccines.

The kit contains sufficient reagents for 50 or 250 reactions of 20  $\mu$ l each. Each standard reaction yields up to 180  $\mu$ g of RNA from 1  $\mu$ g control template. Each kit can yield up to 9 mg RNA (S size) or 45 mg RNA (L size).

## DNA Template Preparation

Linearized plasmid DNA, PCR products or synthetic DNA oligonucleotides can be used as templates for *in vitro* transcription with the HiScribe T7 High Yield RNA Synthesis Kit provided that they contain a double-stranded T7 promoter region upstream of the sequence to be transcribed. Figure 1 illustrates the minimal T7 promoter sequence and the start of transcription as well as a run-off transcript after T7 transcription.

**Figure 1. Transcription by T7 RNA Polymerase**



## Plasmid Templates

Completely linearized plasmid template of highest purity is critical for successful use of the HiScribe T7 High Yield RNA Synthesis Kit. Quality of the template DNA affects transcription yield and the integrity of RNA synthesized. The highest transcription yield is achieved with the highest purity template. Plasmid purified by many laboratory methods can be successfully used, provided it contains mostly supercoiled form, and is free from contaminating RNase, protein, RNA and salts.

To produce RNA transcript of a defined length, plasmid DNA must be completely linearized with a restriction enzyme downstream of the insert to be transcribed. Circular plasmid templates will generate long heterogeneous RNA transcripts in higher quantities because of high processivity of T7 RNA polymerase. NEB has a large selection of restriction enzymes; we recommend selecting restriction enzymes that generate blunt ends or 5'-overhangs.

After linearization, we recommend purifying the template DNA by phenol/chloroform extraction:

1. Extract DNA with an equal volume of 1:1 phenol/chloroform mixture, repeat if necessary.
2. Extract twice with an equal volume of chloroform to remove residual phenol.

- Precipitate the DNA by adding 1/10th volume of 3 M sodium acetate, pH 5.2, and two volumes of ethanol. Incubate at  $-20^{\circ}\text{C}$  for at least 30 minutes.
- Pellet the DNA in a microcentrifuge for 15 minutes at top speed. Carefully remove the supernatant.
- Rinse the pellet by adding 500  $\mu\text{l}$  of 70% ethanol and centrifuging for 15 minutes at top speed. Carefully remove the supernatant.
- Air dry the pellet and resuspend it in nuclease-free water at a concentration of 0.5–1  $\mu\text{g}/\mu\text{l}$ .

### PCR Templates

PCR products containing T7 RNA Polymerase promoter in the correct orientation can be transcribed. Though PCR mixture can be used directly, better yields will be obtained with purified PCR products. PCR products can be purified according to the protocol for plasmid restriction digests above, or by using commercially available spin columns (we recommend Monarch PCR & DNA Cleanup Kit, NEB #T1030). PCR products should be examined on an agarose gel to estimate concentration and to confirm amplicon size prior to its use as a template in the HiScribe T7 High Yield RNA Synthesis Kit. Depending on the PCR products, 0.1–0.5  $\mu\text{g}$  of PCR fragments can be used in a 20  $\mu\text{l}$  *in vitro* transcription reaction.

### Synthetic DNA Oligonucleotides

Synthetic DNA Oligonucleotides which are either entirely double-stranded or mostly single-stranded with a double-stranded T7 promoter sequence can be used in the HiScribe T7 High Yield RNA Synthesis Kit. In general, the yields are relatively low and also variable depending upon the sequence, purity and preparation of the synthetic oligonucleotides.

## RNA Synthesis Protocols

We strongly recommend wearing gloves and using nuclease-free tubes and reagents to avoid RNase contamination. Reactions are typically 20  $\mu\text{l}$  but can be scaled up as needed. Reactions should be assembled in nuclease-free microfuge tubes or PCR strip tubes.

### Standard RNA Synthesis

- Thaw the necessary kit components, mix and pulse-spin in microfuge to collect solutions to bottom of tubes. Keep on ice.
- If you are planning to run many reactions, it is convenient to prepare a master mix by combining equal volumes of the 10X reaction buffer and four ribonucleotide (NTP) solutions. Use 10  $\mu\text{l}$  per reaction.
- Assemble the reaction at room temperature in the following order:

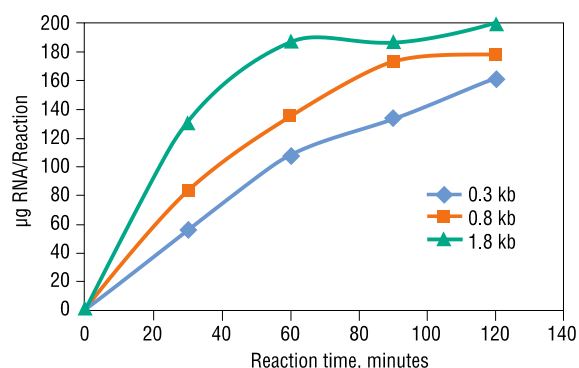
REAGENT	AMOUNT	FINAL CONCENTRATION
Nuclease-free water	X $\mu\text{l}$	
10X Reaction Buffer	2 $\mu\text{l}$	
ATP (100 mM)	2 $\mu\text{l}$	10 mM final
GTP (100 mM)	2 $\mu\text{l}$	10 mM final
UTP (100 mM)	2 $\mu\text{l}$	10 mM final
CTP (100 mM)	2 $\mu\text{l}$	10 mM final
Template DNA	X $\mu\text{l}$	1 $\mu\text{g}$
DTT (0.1M)*	1 $\mu\text{l}$	5 mM
T7 RNA Polymerase Mix	2 $\mu\text{l}$	
<b>Total Reaction Volume</b>	<b>20 <math>\mu\text{l}</math></b>	

\* Addition of DTT to the reaction is optional but recommended. See Troubleshooting section for details.

- Mix thoroughly, pulse-spin in microfuge. Incubate at  $37^{\circ}\text{C}$  for 2 hours. The yield will not be compromised if the incubation temperature is within the range of  $35\text{--}40^{\circ}\text{C}$ .

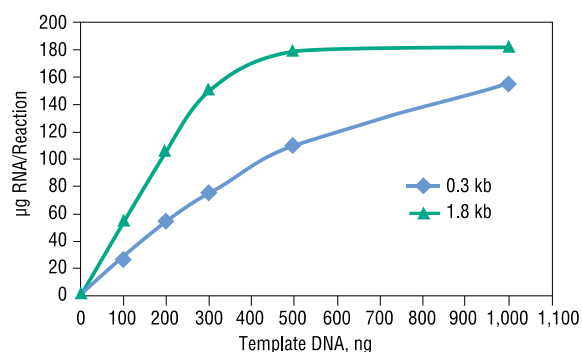
For reaction times of 60 minutes or less, a water bath or heating block may be used; for reaction times longer than 60 minutes, we recommend using a dry air incubator or a PCR instrument, to prevent evaporation. Figure 2 shows the time course of standard RNA synthesis from 1  $\mu\text{g}$  linearized plasmid DNA templates coding for 0.3 kb, 0.8 kb and 1.8 kb RNA transcripts with the HiScribe T7 High Yield RNA Synthesis Kit. For reactions with transcripts longer than 0.3 kb, 2 hour incubation should give you the maximum yield. Figure 3 shows DNA template titrations for 0.3 kb and 1.8 kb RNA transcripts with the HiScribe T7 High Yield RNA Synthesis Kit at  $37^{\circ}\text{C}$  for 2 hours.

**Figure 2. Time course of standard RNA synthesis from three DNA templates**



Reactions were incubated at 37°C in a PCR machine. Transcripts were purified by spin columns and quantified on NanoDrop™ Spectrophotometer.

**Figure 3. Effect of template amount on RNA yield**



Standard reactions were incubated at 37°C in a PCR machine for 2 hours. Transcripts were purified by spin columns and quantified on NanoDrop Spectrophotometer.

For reactions with short RNA transcripts (< 0.3 kb), follow the reaction set up below and incubate the reaction for 4 hours or longer. It is safe to incubate the reaction for 16 hours (overnight).

Reaction set up for short transcripts (< 0.3 kb):

REAGENT	AMOUNT	FINAL CONCENTRATION
Nuclease-free water	X µl	
10X Reaction Buffer	1.5 µl	0.75X final
NTP	1.5 µl each	7.5 mM each final
Template DNA	X µl	1 µg
DTT (0.1M)*	1 µl	5 mM
T7 RNA Polymerase Mix	2 µl	
<b>Total Reaction Volume</b>	<b>20 µl</b>	

\* Addition of DTT to the reaction is optional but recommended. See Troubleshooting section for details.

- Optional:* DNase treatment to remove DNA template. Standard reactions normally generate large amounts of RNA at concentrations up to 10 mg/ml. As a result the reaction mixture is quite viscous. It is easier to perform DNase treatment after the reaction mixture is diluted. To remove template DNA, add 70 µl nuclease-free water, 10 µl of 10X DNase I Buffer, and 2 µl of DNase I (RNase-free), mix and incubate for 15 minutes at 37°C.
- Proceed with purification of synthesized RNA or analysis of transcription products by gel electrophoresis [for purification, we recommend the Monarch RNA Cleanup Kits (NEB #T2040 or #T2050)].

### Capped RNA Synthesis

The recommended ratio of cap analog to GTP is 4:1. Cap analogs are sold separately. Please refer to the ordering information section or [www.neb.com](http://www.neb.com) for more information.

1. Thaw the necessary kit components, mix and pulse-spin in a microfuge to collect solutions to bottom of tubes. Keep on ice.
2. Make 20 mM GTP solution by combining 2  $\mu$ l of 100 mM GTP and 8  $\mu$ l of nuclease-free water. Extra 20 mM GTP can be stored at  $-20^{\circ}\text{C}$  for future use.
3. Prepare cap analog at the concentration of 40 mM.
4. Assemble the reaction at room temperature in the following order:

REAGENT	AMOUNT	FINAL CONCENTRATION
Nuclease-free water	X $\mu$ l	
10X Reaction Buffer	2 $\mu$ l	
ATP (100 mM)	2 $\mu$ l	10 mM final
UTP (100 mM)	2 $\mu$ l	10 mM final
CTP (100 mM)	2 $\mu$ l	10 mM final
GTP (20 mM)	2 $\mu$ l	2 mM final
Cap Analog (40 mM)	4 $\mu$ l	8 mM final
Template DNA	X $\mu$ l	1 $\mu$ g
DTT (0.1M)*	1 $\mu$ l	5 mM
T7 RNA Polymerase Mix	2 $\mu$ l	
<b>Total Reaction Volume</b>	<b>20 <math>\mu</math>l</b>	

\* Addition of DTT to the reaction is optional but recommended. See Troubleshooting section for details.

5. Mix thoroughly, pulse-spin and incubate at  $37^{\circ}\text{C}$  for 2 hours.

The yield per reaction is 40–50  $\mu$ g RNA with approximately 80% capped RNA transcripts. Table 1 shows the effect of varying the ratio of cap analog to GTP on the yield of RNA. Increasing the ratio of cap analog to GTP will increase the proportion of capped RNA transcripts, however it also significantly decreases the yield of the transcription reaction. A ratio of cap analog to GTP of 4:1 is preferably used.

**Table 1. Effect of cap analog:GTP ratios on RNA yield**

CAP ANALOG: GTP RATIO	CONCENTRATION OF CAP ANALOG: GTP (mM)	RNA YIELD ( $\mu$ g) IN 2 HOURS	% CAPPED RNA
0:1	0:10	180	0
1:1	5:5	90-120	50
2:1	6.7:3.3	60-90	67
4:1	8:2	40-50	80
8:1	8.9:1.1	20-25	89

6. *Optional:* To remove template DNA, add 2  $\mu$ l of DNase I (RNase-free), mix and incubate at  $37^{\circ}\text{C}$  for 15 minutes.
7. Proceed with purification of synthesized RNA or analysis of transcription products by gel electrophoresis (for purification, we recommend the Monarch RNA Cleanup Kits (NEB #T2040 or #T2050).

## RNA Synthesis with Modified Nucleotides

Dye or Biotin-NTP is not supplied. The recommended molar ratio of modified NTP (Biotin-, Fluorescein-, Digoxigenin-, or Aminoallyl-NTP) to standard NTP is 1:3 or 1:2. The following reaction set up assumes modified UTP is used.

1. Thaw the necessary kit components, mix and pulse-spin in microfuge to collect solutions to bottom of tubes. Keep on ice.
2. Assemble the reaction at room temperature in the following order:

REAGENT	AMOUNT	FINAL CONCENTRATION
Nuclease-free water	X $\mu$ l	
10X Reaction Buffer	1.5 $\mu$ l	0.75X final
ATP (100 mM)	1.5 $\mu$ l	7.5 mM final
GTP (100 mM)	1.5 $\mu$ l	7.5 mM final
CTP (100 mM)	1.5 $\mu$ l	7.5 mM final
UTP (100 mM)	1 $\mu$ l	5 mM final
Modified UTP (10 mM)	5 $\mu$ l	2.5 mM final
Template DNA	X $\mu$ l	1 $\mu$ g
DTT (0.1M)*	1 $\mu$ l	5 mM
T7 RNA Polymerase Mix	1.5 $\mu$ l	
<b>Total Reaction Volume</b>	<b>20 <math>\mu</math>l</b>	

\* Addition of DTT to the reaction is optional but recommended. See Troubleshooting section for details.

Note the ratio of UTP/modified UTP can be adjusted to meet specific needs. The total amount of UTP can be lowered if higher RNA yield is not necessary. For example, in the above reaction, 3 mM UTP and 1.5 mM modified UTP can be used without affecting the labeling density of the transcript.

3. Mix thoroughly, pulse-spin and incubate at 37°C for 2 hours. For short (< 300 nt) transcripts incubate at 37°C for 4–16 hours.  
Modified ribonucleotides reduce transcription efficiency; therefore lower transcription yields should be expected as compared to transcription using unmodified NTP. In general, Biotin-NTP and Aminoallyl-NTP have an insignificant effect on yields, while 50% or lower yields can be expected for transcription reactions containing Fluorescein-NTP or Cy-NTP. In addition, transcripts containing modified ribonucleotides have reduced electrophoresis mobility due to higher molecular weight.
4. *Optional:* To remove template DNA, add 70  $\mu$ l nuclease-free water to each 20  $\mu$ l reaction, 10  $\mu$ l of 10X DNase I Buffer, and 2  $\mu$ l of RNase-free DNase I, mix and incubate at 37°C for 15 minutes.
5. Proceed with purification of synthesized RNA or analysis of transcription products by gel electrophoresis (for purification, we recommend the Monarch RNA Cleanup Kits (NEB #T2040 or #T2050).

## High Specific Activity Radiolabeled RNA Probe Synthesis

The HiScribe T7 High Yield RNA Synthesis Kit can be used to synthesize high specific activity radiolabeled RNA probes by following the modified protocol below. More than 50% of the label can be incorporated in a 10 minute reaction. The labeled RNA probes have a specific activity of about  $10^8$  cpm/ $\mu$ g.

1. Choosing  $^{32}$ P labeled nucleotide:

We recommend using [ $\alpha$ - $^{32}$ P] UTP or CTP at 800–6000 Ci/mmol and  $\geq$  10 mCi/ml for the synthesis of radiolabeled RNA probes. We do not recommend using radiolabeled ATP or GTP since less label is generally incorporated. RNA labeled with [ $\alpha$ - $^{32}$ P] ATP or GTP appears to be more subject to decomposition during storage.

2. Thaw the necessary kit components, mix and pulse-spin in microfuge to collect solutions to bottom of tubes. Keep on ice.
3. Dilute 100 mM UTP to 40  $\mu$ M UTP if [ $\alpha$ - $^{32}$ P] UTP is used.
  - a. Prepare 400  $\mu$ l of 1 mM UTP by combining 4  $\mu$ l of 100 mM UTP and 396  $\mu$ l of nuclease-free water. Extra 1 mM UTP solution can be stored at  $-20^\circ\text{C}$  for future use.
  - b. Prepare 100  $\mu$ l of 40  $\mu$ M UTP by combining 4  $\mu$ l of 1 mM UTP and 96  $\mu$ l of nuclease-free water.

4. Prepare master mix. For accurate pipetting we recommend preparing 15  $\mu\text{l}$  master mix at a minimum, which is enough for 5 labeling reactions. The master mix contains reaction buffer, ATP, GTP and CTP.

REAGENT	AMOUNT
Nuclease-free water	10 $\mu\text{l}$
10X Reaction Buffer	2 $\mu\text{l}$
ATP (100 mM)	1 $\mu\text{l}$
GTP (100 mM)	1 $\mu\text{l}$
CTP (100 mM)	1 $\mu\text{l}$
<b>Total Reaction Volume</b>	<b>15 <math>\mu\text{l}</math></b>

Use 3  $\mu\text{l}$  master mix for each labeling reaction. Extra master mix can be stored at  $-20^{\circ}\text{C}$  for future use.

5. Assemble the reaction at room temperature in the following order:

REAGENT	AMOUNT	FINAL CONCENTRATION
Nuclease-free water	X $\mu\text{l}$	
Master Mix	3 $\mu\text{l}$	1 mM each A, G and C, final
UTP (40 $\mu\text{M}$ )	2 $\mu\text{l}$	4 $\mu\text{M}$ final
$[\alpha\text{-}^{32}\text{P}]$ UTP	X $\mu\text{l}$	0.2 to 1 $\mu\text{M}$ final
Template DNA	X $\mu\text{l}$	0.1 to 1 $\mu\text{g}$
DTT (0.1M)*	1 $\mu\text{l}$	5 mM
T7 RNA Polymerase Mix	1 $\mu\text{l}$	
<b>Total Reaction Volume</b>	<b>20 <math>\mu\text{l}</math></b>	

\* Addition of DTT to the reaction is optional but recommended. See Troubleshooting section for details.

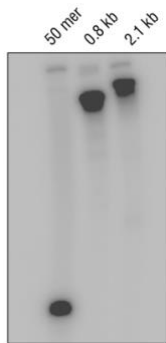
The labeled NTP is present at a limiting concentration and is therefore referred to as the “limiting nucleotide.” (e.g., UTP here). The “limiting nucleotide” is a mixture of both the labeled and unlabeled form of that NTP. There is a trade-off between synthesis of high specific activity probe and synthesis of full-length probe. The higher the concentration of the “limiting nucleotide”, the higher the proportion of full-length transcripts, but if unlabeled nucleotide is used to increase the “limiting nucleotide” concentration, it will lower the specific activity of the transcript. For most labeling reactions, use of 4–5  $\mu\text{M}$  of the “limiting nucleotide” is necessary for full-length probe synthesis with high specific activity. The template sequence will also affect the specific activity of the transcript. For example, if the transcript contains more UTP, more  $^{32}\text{P}$ -UTP will be incorporated and the specific activity will be higher.

**Table 2. Concentration of  $[\alpha\text{-}^{32}\text{P}]$  NTP in a transcription reaction.**

SPECIFIC ACTIVITY (Ci/mol)	CONCENTRATION (mCi/ml)	VOLUME USED PER 20 $\mu\text{l}$ REACTION	CONCENTRATION IN 20 $\mu\text{l}$ REACTION (HOT LABEL)
800	10	1 $\mu\text{l}$	0.63 $\mu\text{M}$
800	20	1 $\mu\text{l}$	1.25 $\mu\text{M}$
800	40	1 $\mu\text{l}$	2.5 $\mu\text{M}$
3,000	10	1 $\mu\text{l}$	0.17 $\mu\text{M}$
3,000	20	1 $\mu\text{l}$	0.33 $\mu\text{M}$
3,000	40	1 $\mu\text{l}$	0.67 $\mu\text{M}$
6,000	40	1 $\mu\text{l}$	0.33 $\mu\text{M}$

6. Mix thoroughly; pulse-spin in microfuge and incubate for 10 minutes. Incubation temperature is not crucial for labeling efficiency. Room temperature to  $40^{\circ}\text{C}$  can be used.
7. *Optional:* To remove template DNA, add 2  $\mu\text{l}$  of DNase I (RNase-free) (NEB #M0303), mix and incubate for 15 minutes at  $37^{\circ}\text{C}$ .
8. Proceed with purification of synthesized RNA or analysis of transcription products by gel electrophoresis (for purification, we recommend the Monarch RNA Cleanup Kits (NEB #T2040 or #T2050).

**Figure 4. Generation of high specific activity  $^{32}\text{P}$ -labeled RNA probes.**



*Reactions were incubated for 10 minutes at room temperature.*

*A small portion of each reaction was run on a 6% PAGE*

*urea gel followed by exposing the gel to a Storage Phosphor Screen (GE).*

### **Purification of Synthesized RNA**

In general, unmodified RNA transcripts from standard RNA synthesis can be purified by phenol-chloroform extraction and ethanol precipitation or by using a spin column based method (e.g., Monarch RNA Cleanup Kits, NEB #T2030, #T2040, #T2050). For capped RNA synthesis, non-radioactively labeled RNA or high specific activity radiolabeled RNA probes, spin column chromatography is the preferred method. If absolute full length RNA is required (e.g., RNA probe for RNase protection assay), we recommend gel purification.

#### **Phenol:Chloroform Extraction and Ethanol Precipitation**

For removal of proteins and most of the free nucleotides, phenol: chloroform extraction and ethanol precipitation of RNA transcripts is the preferred method.

1. Adjust the reaction volume to 180  $\mu\text{l}$  by adding 160  $\mu\text{l}$  nuclease-free water. Add 20  $\mu\text{l}$  of 3 M sodium acetate, pH 5.2 or 20  $\mu\text{l}$  of 5 M ammonium acetate, mix thoroughly.
2. Extract with an equal volume of 1:1 phenol/chloroform mixture, followed by two extractions with chloroform. Collect the aqueous phase and transfer to a new tube.
3. Precipitate the RNA by adding 2 volumes of ethanol. Incubate at  $-20^{\circ}\text{C}$  for at least 30 minutes and collect the pellet by centrifugation.
4. Remove the supernatant and rinse the pellet with 500  $\mu\text{l}$  of cold 70% ethanol.
5. Resuspend the RNA in 50  $\mu\text{l}$  of 0.1 mM EDTA. Store the RNA at  $-20^{\circ}\text{C}$  or below.

#### **Spin Column Purification**

Spin columns will remove unincorporated nucleotides, proteins and salts. Adjust the volume of the reaction mixture to 100  $\mu\text{l}$  by adding 80  $\mu\text{l}$  nuclease-free water, mix well. Purify the RNA by following the manufacturer's instructions. Each reaction could produce up to 180  $\mu\text{g}$  of RNA which may exceed column capacity thus requiring additional columns.

#### **Gel Purification**

When high purity RNA transcript is desired, we recommend gel purification of the transcription product. The Monarch RNA Cleanup Kits (NEB #T2030, #T2040 or #T2050) can be used for RNA gel extraction (see protocol included in NEB #T2030, #T2040 or #T2050 product manual).



## Evaluation of Reaction Products

### Quantification by UV Light Absorbance

RNA concentration can be easily determined by measuring the ultraviolet light absorbance at 260 nm wavelength, however, any unincorporated nucleotides and template DNA in the mixture will effect the reading. Free nucleotides from the transcription reaction must be removed before the RNA concentration can be quantified. A 1:200 dilution of a sample of the purified RNA should give an absorbance reading in the linear range of a spectrophotometer. RNA dilution may not be necessary if using a NanoDrop™ Spectrophotometer. A NanoDrop Spectrophotometer can read RNA concentrations from 10 ng/μl to 3000 ng/μl directly. For single-stranded RNA, 1 A<sub>260</sub> is equivalent to RNA concentration of 40 μg/ml. The RNA concentration can be calculated as follows:

$$A_{260} \times \text{dilution factor} \times 40 = \text{__ } \mu\text{g/ml RNA}$$

### Analysis of Transcription Products by Gel Electrophoresis

To evaluate transcript length, integrity and quantity, an aliquot of the transcription reaction should be run on an appropriate denaturing agarose gel or polyacrylamide gel. Transcripts larger than 0.3 kb can be run on agarose gels, whereas denaturing polyacrylamide gels (5–15%) are necessary for smaller transcripts. The gels should be run under denaturing conditions to minimize formation of secondary structure from the transcript.

1. Preparation of denaturing gels
  - a. Denaturing agarose gel:

To make 100 ml 1% denaturing agarose gel, add 1 gram agarose powder to 72 ml nuclease-free water. Melt the agarose, add 10 ml 10X MOPS buffer. Then in a fume hood, add 18 ml fresh formaldehyde (37%), mix well. Pour the gel.

10X MOPS gel running buffer: 0.4 M MOPS (pH 7.0), 0.1 M Sodium Acetate, 10 mM EDTA
  - b. Denaturing PAGE/Urea Gel:

5–15% PAGE/Urea gel. We recommend using commercially available premade gels. Use standard TBE gel running buffer.

10X TBE buffer: 0.9 M Tris Base, 0.9 M Boric Acid, 20 mM EDTA.
2. Gel electrophoresis of non-radiolabeled RNA
  - a. Mix 0.2–1 μg RNA sample with an equal volume of RNA Loading Dye (2X) (NEB #B0363).
  - b. Denature the RNA sample and an aliquot of RNA marker by heating at 65–70°C for 5–10 minutes.
  - c. Pulse-spin prior to loading onto gel.
  - d. Visualizing RNA by staining the gel with SYBR Gold or ethidium bromide.
3. Gel electrophoresis of radiolabeled RNA
  - a. Mix an aliquot of labeled RNA with an equal volume of RNA Loading Dye (2X) (NEB #B0363).
  - b. Denature the RNA sample by heating at 65–70°C for 5–10 minutes.
  - c. Pulse-spin prior to loading onto gel.
  - d. Visualizing RNA by autoradiography.

Agarose gels should be dried before exposing to X-ray film, but thin (< 1 mM thickness) polyacrylamide gels can be transferred to filter paper, covered with plastic wrap and exposed to X-ray film directly (when <sup>32</sup>P is used). Exposure time could range from 20 minutes to overnight depending on the specific activity of the RNA probe and the type of intensifying screens used. Exposure time could be much shorter if the gels are exposed to Storage Phosphor Screen (GE or equivalent).

## Troubleshooting

### Control Reaction

The FLuc control template DNA is a linearized plasmid containing the firefly luciferase gene under the transcriptional control of T7 promoter. The size of the runoff transcript is 1.8 kb. The control reaction should yield  $\geq 150 \mu\text{g}$  RNA transcript in 2 hours.

If the control reaction is not working, there may be technical problems during reaction set up. Repeat the reaction by following the protocol carefully; take any precaution to avoid RNase contamination. Contact NEB for technical assistance.

The control plasmid sequence can be found within the [DNA Sequences and Maps Tool](#) under the name “FLuc Control Plasmid”. The FLuc control template is generated by linearizing the plasmid with StuI.

### Low Yield of Full-length RNA

If the transcription reaction with your template generates full-length RNA, but the yield is significantly lower than expected, it is possible that contaminants in the DNA template are inhibiting the RNA polymerase, or the DNA concentration may be incorrect. Alternatively, additional purification of DNA template may be required. Phenol-chloroform extraction is recommended (see template DNA preparation section).

### Addition of DTT

Addition of DTT (5 mM final) to the reaction is optional but recommended. The RNA polymerase in the kit is sensitive to oxidation and could result in lower RNA yield over time due to repeated handling etc. Adding DTT to the reaction may help restore the kit performance in such cases. Adding DTT will not compromise the reaction in any situation.

### Low Yield of Short Transcript

High yields of short transcripts (< 0.3 kb) are achieved by extending incubation time and increasing the amount of template. Incubation of reactions up to 16 hours (overnight) or using up to 2  $\mu\text{g}$  of template will help to achieve maximum yield.

### RNA Transcript Smearing on Denaturing Gel

If the RNA appears degraded (e.g. smeared) on denaturing agarose or polyacrylamide gel, DNA template is contaminated with RNase. DNA templates contaminated with RNase can affect the length and yield of RNA synthesized (a smear below the expected transcript length). If the plasmid DNA template is contaminated with RNase, perform phenol/chloroform extraction, then ethanol precipitate and dissolve the DNA in nuclease-free water (see template DNA preparation section).

### RNA Transcript of Larger Size than Expected

If the RNA transcript appears larger than expected on a denaturing gel, template plasmid DNA may be incompletely digested. Even small amounts of undigested circular DNA can produce large amounts of long transcripts. Check template for complete digestion, if undigested plasmid is confirmed, repeat restriction enzyme digestion.

Larger size bands may also be observed when the RNA transcript is not completely denatured due to the presence of strong secondary structures.

### RNA Transcript of Smaller Size than Expected

If denaturing gel analysis shows the presence of smaller bands than the expected size, it is most likely due to premature termination by the polymerase. Some sequences which resemble T7 RNA Polymerase termination signals will cause premature termination. Incubating the transcription reaction at lower temperatures, for example at 30°C, may increase the proportion of full-length transcript, however the yield will be decreased. For GC rich templates, or templates with secondary structures, incubation at 42°C may improve yield of full-length transcript.

If premature termination of transcription is found in high specific activity radiolabeled RNA probe synthesis, increase the concentration of “limiting NTP”. Additional “cold” NTP can be added to the reaction to increase the proportion of full-length transcript, however the improvement in yield of full-length product will compromise the specific activity of the probe.

## Ordering Information

NEB #	PRODUCT	SIZE
E2040S/L	HiScribe T7 High Yield RNA Synthesis Kit	50 reactions

## COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
T2040S/L	Monarch RNA Cleanup Kit (50 µg)	10/100 preps
T2030S/L	Monarch RNA Cleanup Kit (10 µg)	10/100 preps
T2050S/L	Monarch RNA Cleanup Kit (500 µg)	10/100 preps
B0363S	RNA Loading Dye (2X)	4 x 1 ml
M0307S/L	RNase Inhibitor, Human Placenta	2,000/10,000
M0314S/L	RNase Inhibitor, Murine	3,000/15,000
M0303S/L	DNase I (RNase-Free)	1,000/5,000 units
M0493S/L	Q5 Hot Start High-Fidelity DNA Polymerase	100/500 units
N0362S	ssRNA Ladder	25 gel lanes
N0364S	Low Range ssRNA Ladder	25 gel lanes
S1411S/L	3'-O-Me-m <sup>7</sup> G(5')ppp(5')G RNA Cap Structure Analog	1/5 µmol
S1405S/L	m <sup>7</sup> G(5')ppp(5')A RNA Cap Structure Analog	1/5 µmol
S1406S/L	G(5')ppp(5')A RNA Cap Structure Analog	1/5 µmol
S1407S/L	G(5')ppp(5')G RNA Cap Structure Analog	1/5 µmol
S1404S/L	m <sup>7</sup> G(5')ppp(5')G RNA Cap Structure Analog	1/5 µmol
M2080S	Vaccinia Capping System	400 units
M0366S	mRNA Cap 2'-O-Methyltransferase	2,000 units
M0276S/L	<i>E. coli</i> Poly(A) Polymerase	100/500 units
N0466S/L	Ribonucleotide Solution Mix	10/50 µmol of each
N0450S/L	Ribonucleotide Solution Set	10/50 µmol of each

## Revision History

REVISION #	DESCRIPTION	DATE
1.0, 1.1	N/A	7/11, 5/13
2.0, 2.1		6/14, 1/17
3.0		1/19
4.0	Apply new manual format.	4/20
5.0	Updated GTP in the Capped RNA Synthesis Table	9/22
5.1	Updated location to find control plasmid to DNA Sequences and Maps Tool (page 10)	10/22
6.0	Updated protocols and materials to include addition of DTT. Updated UTP concentration (page 7). Also updated table styling and legal footnote.	7/23
7.0	Updated to include new L size.	1/24

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