RNA ENZYMES & GENE ANALYSIS

# HiScribe™ T7 High Yield RNA Synthesis Kit

Instruction Manual

NEB #E2040S
50 reactions
Version 3.0 1/19

be INSPIRED
drive DISCOVERY
stay GENUINE

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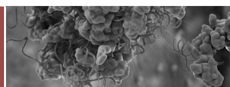

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## Kit Components:

All kit components should be stored at –20°C. Each kit contains sufficient reagents for 50 x 20 µl reactions.

| Component | Amount |
|---|---|
| Reaction Buffer (10X) | 100 µl |
| ATP (100 mM) | 100 µl |
| GTP (100 mM) | 100 µl |
| UTP (100 mM) | 100 µl |
| CTP (100 mM) | 100 µl |
| FLuc Control Template (0.5 µg/µl) | 10 µl |
| T7 RNA Polymerase Mix | 100 µl |

## 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: | [$\alpha$-$^{32}$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.

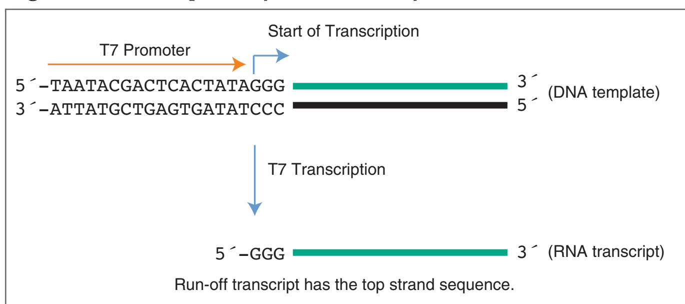
RNA synthesized from the kit is suitable for many applications including RNA structure and function studies, ribozyme biochemistry, probes for RNase protection assays and hybridization based blots, anti-sense RNA and RNAi experiments, microarray analysis, microinjection, and *in vitro* translation and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

The kit contains sufficient reagents for 50 reactions of 20 µl each. Each standard reaction yields up to 180 µg of RNA from 1 µg control template. Each kit can yield up to 9 mg RNA. For $^{32}$P labeling, the kit contains enough reagents for 100 reactions of 20 µl each.

# 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.
3. Precipitate the DNA by adding 1/10th volume of 3 M sodium acetate, pH 5.2, and two volumes of ethanol. Incubate at –20°C for at least 30 minutes.
4. Pellet the DNA in a microcentrifuge for 15 minutes at top speed. Carefully remove the supernatant.
5. Rinse the pellet by adding 500 µl of 70% ethanol and centrifuging for 15 minutes at top speed. Carefully remove the supernatant.
6. Air dry the pellet and resuspend it in nuclease-free water at a concentration of 0.5–1 µg/µl.

## 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 µg of PCR fragments can be used in a 20 µl *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 µl but can be scaled up as needed. Reactions should be assembled in nuclease-free microfuge tubes or PCR strip tubes.

## Standard RNA Synthesis

1. Thaw the necessary kit components, mix and pulse-spin in microfuge to collect solutions to bottom of tubes. Keep on ice.

2. 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 µl per reaction.

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

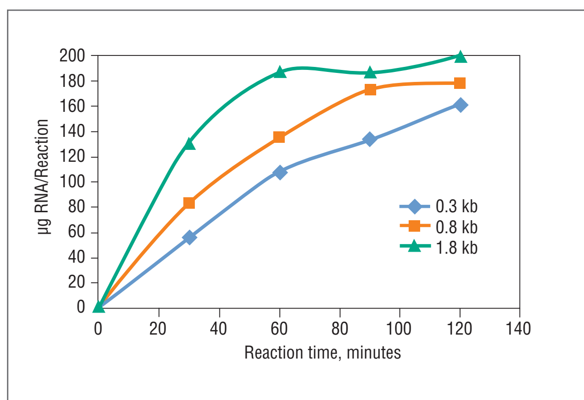
| | | |
|---|---|---|
| Nuclease-free water | X µl | |
| 10X Reaction Buffer | 2 µl | |
| ATP (100 mM) | 2 µl | 10 mM final |
| GTP (100 mM) | 2 µl | 10 mM final |
| UTP (100 mM) | 2 µl | 10 mM final |
| CTP (100 mM) | 2 µl | 10 mM final |
| Template DNA | X µl | 1 µg |
| T7 RNA Polymerase Mix | 2 µl | |
| Total reaction volume | 20 µl | |

4. Mix thoroughly, pulse-spin in microfuge. Incubate at 37°C for 2 hours. The yield will not be compromised if the incubation temperature is within the range of 35–40°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 µg linearized plasmid DNA templates coding for 0.3 kb, 0.8 kb and 1.8 kb RNA transcripts with the HiS-

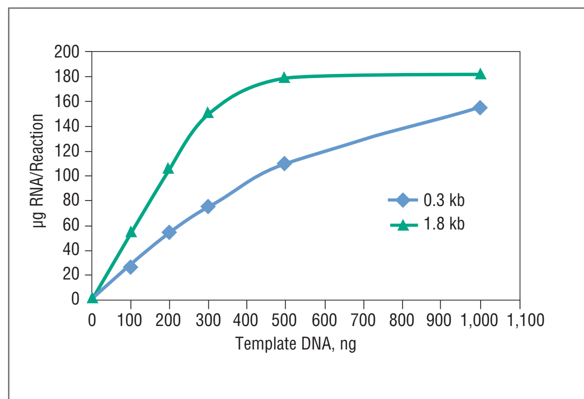
cribe T7 High Yield RNA Synthesis Kit. For reactions with transcripts longer than 0.3 kb, 2 hour incubation should give you the maximum yield. Figure 3 shows DNA template titrations for 0.3 kb and 1.8 kb RNA transcripts with the HiScribe T7 High Yield RNA Synthesis Kit at 37°C for 2 hours.

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):

| | | |
|---|---|---|
| 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 |
| T7 RNA Polymerase Mix | 1.5 µl | |
| Total reaction volume | 20 µl | |

With this set up, the kit contains sufficient materials for 65 reactions.

5. *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.
6. 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 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 µl of 100 mM GTP and 8 µl of nuclease-free water. Extra 20 mM GTP can be stored at –20°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:

| | | |
|---|---|---|
| Nuclease-free water | X µl | |
| 10X Reaction Buffer | 2 µl | |
| ATP (100 mM) | 2 µl | 10 mM final |
| UTP (100 mM) | 2 µl | 10 mM final |
| CTP (100 mM) | 2 µl | 10 mM final |
| GTP (20 mM) | 2 µl | 2 mM final |
| Cap Analog (40 mM) | 4 µl | 8 mM final |
| Template DNA | X µl | 1 µg |
| T7 RNA Polymerase Mix | 2 µl | |
| Total reaction volume | 20 µl | |

5. Mix thoroughly, pulse-spin and incubate at 37°C for 2 hours.

The yield per reaction is 40–50 µg 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 (µg) 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 µl of DNase I (RNase-free), mix and incubate at 37°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:

| | | |
|---|---|---|
| Nuclease-free water | X µl | |
| 10X Reaction Buffer | 1.5 µl | 0.75X final |
| ATP (100 mM) | 1.5 µl | 7.5 mM final |
| GTP (100 mM) | 1.5 µl | 7.5 mM final |
| CTP (100 mM) | 1.5 µl | 7.5 mM final |
| UTP (100 mM) | 1 µl | 5 mM final |
| Modified UTP (10 mM) | 5 µl | 2.5 mM final |
| Template DNA | X µl | 1 µg |
| T7 RNA Polymerase Mix | 1.5 µl | |
| Total reaction volume | 20 µl | |

   With this set up, the kit contains sufficient materials for 65 reactions. 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 µl nuclease-free water to each 20 µl reaction, 10 µl of 10X DNase I Buffer, and 2 µl 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/µg.

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

   We recommend using [α-$^{32}$P] UTP or CTP at 800–6000 Ci/mmol and ≥ 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 [α-$^{32}$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 µM UTP if [α-$^{32}$P] UTP is used.

   a. Prepare 400 µl of 1 mM UTP by combining 4 µl of 100 mM UTP and 396 µl of nuclease-free water. Extra 1 mM UTP solution can be stored at –20°C for future use.

   b. Prepare 100 µl of 40 µM UTP by combining 4 µl of 1 mM UTP and 96 µl of nuclease-free water.

4. Prepare master mix. For accurate pipetting we recommend preparing 15 µl master mix at a minimum, which is enough for 5 labeling reactions. The master mix contains reaction buffer, ATP, GTP and CTP.

| | |
|---|---|
| Nuclease-free water | 10 µl |
| 10X Reaction Buffer | 2 µl |
| ATP (100 mM) | 1 µl |
| GTP (100 mM) | 1 µl |
| CTP (100 mM) | 1 µl |
| Total volume | 15 µl |

   Use 3 µl master mix for each labeling reaction. Extra master mix can be stored at –20°C for future use.

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

| | | | |
|---|---|---|---|
| Nuclease-free water | X µl | | |
| Master Mix | 3 µl | 1 mM each A, G and C, | final |
| UTP (40 µM) | 2 µl | 4 µM | final |
| [α-$^{32}$P] UTP | X µl | 0.2 to 1 µM | final |
| Template DNA | X µl | 0.1 to 1µg | |
| T7 RNA Polymerase Mix | 1 µl | | |
| Total reaction volume | 20 µl | | |

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 µM 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 $^{32}$P-UTP will be incorporated and the specific activity will be higher.

Table 2. Concentration of [α-$^{32}$P] NTP in a transcription reaction

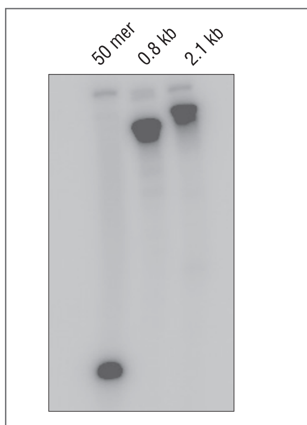
| SPECIFIC ACTIVITY (Ci/mol) | CONCENTRATION (mCi/ml) | VOLUME USED PER 20 µl REACTION | CONCENTRATION IN 20 µl REACTION (HOT LABEL) |
|---|---|---|---|
| 800 | 10 | 1 µl | 0.63 µM |
| 800 | 20 | 1 µl | 1.25 µM |
| 800 | 40 | 1 µl | 2.5 µM |
| 3000 | 10 | 1 µl | 0.17 µM |
| 3000 | 20 | 1 µl | 0.33 µM |
| 3000 | 40 | 1 µl | 0.67 µM |
| 6000 | 40 | 1 µl | 0.33 µM |

6. Mix thoroughly; pulse-spin in microfuge and incubate for 10 minutes. Incubation temperature is not crucial for labeling efficiency. Room temperature to 40°C can be used.

7. *Optional:* To remove template DNA, add 2 µl of DNase I (RNase-free) (NEB #M0303), mix and incubate for 15 minutes at 37°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 $^{32}$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 µl by adding 160 µl nuclease-free water. Add 20 µl of 3 M sodium acetate, pH 5.2 or 20 µl 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°C for at least 30 minutes and collect the pellet by centrifugation.
4. Remove the supernatant and rinse the pellet with 500 µl of cold 70% ethanol.
5. Resuspend the RNA in 50 µl of 0.1 mM EDTA. Store the RNA at –20°C or below.

## Spin Column Purification

Spin columns will remove unincorporated nucleotides, proteins and salts. Adjust the volume of the reaction mixture to 100 µl by adding 80 µl nuclease-free water, mix well. Purify the RNA by following the manufacturer's instructions. Each reaction could produce up to 180 µg 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 $A_{260}$ is equivalent to RNA concentration of 40 µg/ml. The RNA concentration can be calculated as follows:

$A_{260}$ x dilution factor x 40 = __ µg/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 $^{32}P$ 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 ≥ 150 µg 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 at www.neb.com. 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).

## 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 µg 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

| PRODUCT | NEB # | SIZE |
|---|---|---|
| HiScribe T7 High Yield RNA Synthesis Kit | E2040S | 50 reactions |
| COMPANION PRODUCTS | | |
| Monarch RNA Cleanup Kit (50 µg) | T2040S/L | 10/100 preps |
| Monarch RNA Cleanup Kit (10 µg) | T2030S/L | 10/100 preps |
| Monarch RNA Cleanup Kit (500 µg) | T2050S/L | 10/100 preps |
| RNA Loading Dye (2X) | B0363S | 4 x 1 ml |
| RNase Inhibitor, Human Placenta | M0307S/L | 2,000/10,000 units |
| RNase Inhibitor, Murine | M0314S/L | 3,000/15,000 units |
| DNase I (RNase-Free) | M0303S/L | 1,000/5,000 units |
| Q5 Hot Start High-Fidelity DNA Polymerase | M0493S/L | 100/500 units |
| ssRNA Ladder | N0362S | 25 gel lanes |
| Low Range ssRNA Ladder | N0364S | 25 gel lanes |
| 3´-O-Me-$m^7G$(5´)ppp(5´)G RNA Cap Structure Analog | S1411S/L | 1/5 µmol |
| $m^7G$(5´)ppp(5´)A RNA Cap Structure Analog | S1405S/L | 1/5 µmol |
| G(5´)ppp(5´)A RNA Cap Structure Analog | S1406S/L | 1/5 µmol |
| G(5´)ppp(5´)G RNA Cap Structure Analog | S1407S/L | 1/5 µmol |
| m7G(5´)ppp(5´)G RNA Cap Structure Analog | S1404S/L | 1/5 µmol |
| RNase Contamination Assay Kit | E3320S | 50 reactions |
| Vaccinia Capping System | M2080S | 400 units |
| mRNA Cap 2´-O-Methyltransferase | M0366S | 2,000 units |
| *E. coli* Poly(A) Polymerase | M0276S/L | 100/500 units |
| Ribonucleotide Solution Mix | N0466S/L | 10/50 µmol of each |
| Ribonucleotide Solution Set | N0450S/L | 10/50 µmol of each |

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