INSTRUCTION MANUAL

# HiScribe® SP6 RNA Synthesis Kit

NEB #E2070S

50 reactions

Version 4.0_1/26

## Table of Contents

## The HiScribe SP6 RNA Synthesis Kit Includes:

*All kit components should be stored at –20°C. Each kit contains sufficient reagents for 50 reactions of 25 µl each. Each standard reaction yields ≥ 80 µg of unmodified RNA from 1 µg control template.*

SP6 RNA Polymerase Mix
SP6 Reaction Buffer (10X)
ATP (Tris) (50 mM)
GTP (Tris) (50 mM)
UTP (Tris) (50 mM)
CTP (Tris) (50 mM)
SP6 Control Template (0.25 µg/µl)
DNase I (RNase-free) (2 units/µl)
LiCl Solution (7.5 M LiCl, 10 mM EDTA)
Dithiothreitol (DTT) (0.1 M)

## Required Materials Not Included:

| | |
|---|---|
| DNA Template: | The DNA template must be linear and contain the SP6 RNA Polymerase promoter with correct orientation in relation to target sequence to be transcribed. |
| Cap Analogs: | Dinucleotide Cap Analogs: NEB #S1411, #S1405, #S1406 and #S1407 |
| Modified-NTP: | N1-Methyl-Pseudouridine-5′-Triphosphate (NEB #N0431)<br>5-Methyl-Cytidine-5′-Triphosphate (NEB #N0432)<br>Pseudouridine-5′-Triphosphate (NEB #N0433)<br>5-Methoxy-Uridine-5′-Triphosphate (NEB# N0434)<br>Biotin-, Fluorescein-, Digoxigenin-, or Aminoallyl-NTP |
| Labeling: | [α-$^{32}$P] labeled ribonucleotide (800–6,000 Ci/mmol) |
| General: | 37°C incubator or thermocycler, nuclease-free water |
| DNase: | DNase I (RNase-free) (NEB #M0303) or DNase I-XT (NEB #M0570) |
| Purification: | Buffer- or water-saturated phenol/chloroform, ethanol and 3M sodium acetate, pH 5.2, lithium chloride, or Monarch® RNA Spin Cleanup Kit (50, or 500 µg capacity) (NEB #T2040 or #T2050) |
| Gel Analysis: | Gels and running buffers, gel apparatus, power supply |

## Introduction

The HiScribe SP6 RNA Synthesis Kit is designed for the *in vitro* transcription of RNA using SP6 RNA Polymerase. This kit is suitable for synthesis of high yield RNA transcripts and for incorporation of cap analogs (not included) or base-modified nucleotides (not included) to obtain capped, biotin-labeled or dye-labeled RNA. The kit is also capable of synthesizing high specific activity radiolabeled RNA for use as probes or targets.

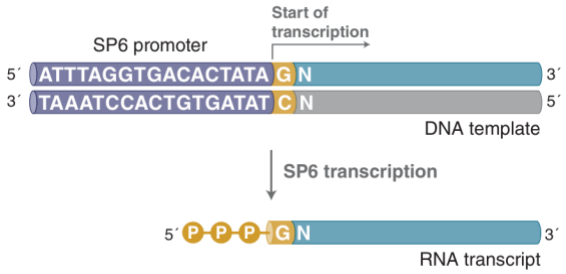
RNA synthesized from this kit is suitable for many applications including RNA structure and function studies, ribozyme biochemistry, probes for RNase protection or gel shift assays, hybridization-based blots, anti-sense RNA or RNAi experiments, microarray analysis, microinjection, sgRNA synthesis and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

This kit contains sufficient reagents for 50 reactions of 25 µl each. Each standard reaction yields ≥ 80 µg of RNA from 1 µg SP6 Control Template DNA. Each kit can yield ≥ 4 mg of RNA.

## DNA Template Preparation

Linearized plasmid DNA, PCR products or synthetic DNA oligonucleotides can be used as templates for *in vitro* transcription with the HiScribe SP6 RNA Synthesis Kit provided that they contain a double-stranded SP6 promoter region upstream of the sequence to be transcribed. Figure 1 illustrates the minimal SP6 promoter sequence and the start of transcription as well as a run-off transcript after SP6 transcription. The standard protocol results in 5′ triphosphorylated RNA.

**Figure 1. Transcription by SP6 RNA Polymerase**




### Plasmid Templates

Completely linearized plasmid template of highest purity is critical for successful use of the HiScribe SP6 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 the high processivity of SP6 RNA polymerase. NEB has a large selection of restriction enzymes; we recommend selecting restriction enzymes that generate blunt ends or 5′-overhangs. If possible, we also recommend using High-Fidelity (HF) restriction enzymes to limit star activity, especially for long RNA.

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 an SP6 RNA Polymerase promoter in the correct orientation can be transcribed. We recommend using Q5® Hot Start High-Fidelity DNA Polymerase (NEB #M0493/M0494). 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 Spin PCR & DNA Cleanup Kit, NEB #T1130). 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 SP6 RNA Synthesis Kit. Depending on the PCR products, 0.1–0.5 µg of PCR fragments can be used in a 25 µl *in vitro* transcription reaction.

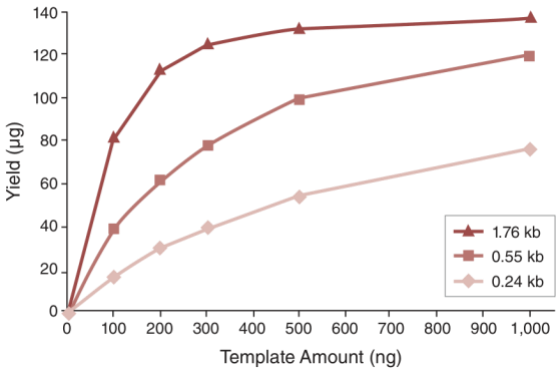
### Synthetic DNA Oligonucleotides

Synthetic DNA Oligonucleotides which are either entirely double-stranded or mostly single-stranded with a double-stranded SP6 promoter sequence can be used in the HiScribe SP6 RNA Synthesis Kit. In general, the yields are relatively low and also variable depending upon the sequence, purity and preparation of the synthetic oligonucleotides. We do not recommend using synthetic oligonucleotides directly as template for IVT RNA greater than 100 nucleotides.

## RNA Synthesis Protocols

We strongly recommend wearing gloves and using nuclease-free tubes and reagents to avoid RNase contamination. Reactions are typically 25 µl but can be scaled up linearly as needed. Reactions should be assembled in nuclease-free microfuge tubes or PCR strip tubes.

**Figure 2. Effect of template amount on RNA yield**




## Standard RNA Synthesis

1. Thaw the necessary components at room temperature. Keep the SP6 RNA Polymerase Mix on ice.
2. Mix and pulse-spin in a microfuge to collect the solutions to the bottom of the tubes.
3. Set up the reaction at room temperature in the order listed in the table below:

| COMPONENTS | 25 µl REACTION | FINAL AMOUNT |
|---|---|---|
| Nuclease-free Water | X µl | |
| 10X SP6 Reaction Buffer | 2.5 µl | 1X |
| 50 mM ATP (Tris) | 2.5 µl | 5 mM |
| 50 mM GTP (Tris) | 2.5 µl | 5 mM |
| 50 mM CTP (Tris) | 2.5 µl | 5 mM |
| 50 mM UTP (Tris) | 2.5 µl | 5 mM |
| Linear Template DNA | X µl | 1 µg |
| DTT (0.1M) | 1.25 µl | 5 mM |
| SP6 RNA Polymerase Mix | 2.5 µl | |

4. Mix thoroughly by pipetting and pulse-spin in a microfuge. Incubate at 37°C for 2 hours in a dry air incubator or thermocycler to prevent evaporation. For reaction times of 60 minutes or less, a water bath or heating block may be used. The yield will not be compromised if the incubation temperature is within the range of 35–40°C.

   Reactions for short RNA transcripts (< 0.3 kb) should be incubated for 4 hours or longer. It is safe to incubate the reaction for 16 hours (overnight).

   Optional: To remove template DNA, add 25 µl nuclease-free water and 2 µl of DNase I (RNase-free) (NEB #M0303), mix, and incubate for 15 minutes at 37°C. Alternatively, 2 µl of DNase I-XT (NEB# M0570) can be added directly to the IVT product and incubated for 15 minutes at 37°C.

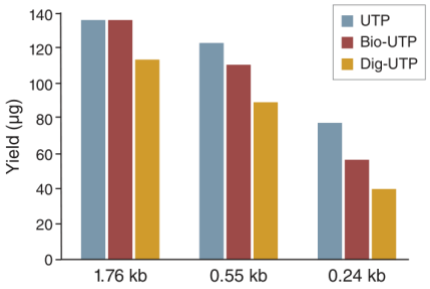
5. Proceed with purification of synthesized RNA and/or evaluation of transcription products yield and/or length.

   Optional: If a poly(A) tail is desired and is not encoded in the template plasmid, you can add one post-transcriptionally with *E. coli* Poly(A) Polymerase (NEB# M0276).

## RNA Synthesis with Modified Nucleotides

Incorporation of chemically modified ribonucleotides can improve the utility of mRNA by increasing stability, reducing immunogenicity, and enhancing translatability. This protocol can be used to synthesize 5′ triphosphorylated RNA containing partial or complete substitutions of canonical nucleotides with base-modified nucleotides from a DNA template containing the SP6 RNA Polymerase promoter sequence immediately followed by guanosine. Modified ribonucleotides reduce transcription efficiency; therefore, lower transcription yields should be expected as compared to transcription using unmodified NTPs. In general, Biotin-NTP and Aminoallyl-NTP have less of an effect on yields, while lower yields can be expected for transcription reactions containing Fluorescein-NTP, Cy-NTP or Digoxigenin-NTP. In addition, transcripts containing modified ribonucleotides have reduced electrophoretic mobility due to higher molecular weight.

**Figure 3. Effect of modified NTPs on RNA yield**




*RNA synthesis reactions using linearized plasmid DNA templates were performed in the presence of unmodified nucleotides or modified nucleotides (Biotin-UTP, Digoxigenin-UTP) at the ratio of 2.3:1 (UTP:modified UTP). Reactions were incubated at 37°C in a PCR machine for 2 hours. Transcripts were treated with DNase I, purified by LiCl precipitation and quantified using a NanoDrop Spectro-photometer.*

**Protocol**

1. Thaw the necessary components at room temperature. Keep the SP6 RNA Polymerase Mix on ice.
2. Mix and pulse-spin in a microfuge to collect the solutions to the bottom of the tubes.
3. Set up the reaction at room temperature in the order listed in the table below:

3a. Follow the Standard RNA Synthesis protocol for complete substitution with a base-modified NTP.

Base-modified nucleotides that NEB has tested that can fully substitute for the canonical NTP:

- N1-Methyl-Pseudo-UTP (NEB #N0431)
- 5-Methyl-CTP (NEB #N0432)
- Pseudo-UTP (NEB #N0433)
- 5-Methoxy-UTP (NEB #N0434)

3b. An example of a 1/3 partial substitution with a base-modified UTP.

Base-modified nucleotides that NEB has tested that require partial substitution:

- Biotin-NTP
- Fluorescein- NTP
- Digoxigenin- NTP
- Aminoallyl-NTP
- N6-methyladenosine

| COMPONENTS | 25 µl REACTION | FINAL AMOUNT |
|---|---|---|
| Nuclease-free Water | X µl | |
| 10X SP6 Reaction Buffer | 2.5 µl | 1X |
| 50 mM ATP (Tris) | 2.5 µl | 5 mM |
| 50 mM GTP (Tris) | 2.5 µl | 5 mM |
| 50 mM CTP (Tris) | 2.5 µl | 5 mM |
| 50 mM UTP (Tris) | 1.75 µl | 3.5 mM |
| 10 mM Modified UTP | 3.75 µl | 1.5 mM |
| Linear Template DNA | X µl | 1 µg |
| DTT (0.1M) | 1.25 µl | 5 mM |
| SP6 RNA Polymerase Mix | 2.5 µl | |

4. Mix thoroughly by pipetting and pulse-spin in a microfuge. Incubate at 37°C for 2 hours in a dry air incubator or thermocycler to prevent evaporation. Reactions for short RNA transcripts (< 0.3 kb) should be incubated for 4 hours or longer. It is safe to incubate the reaction for 16 hours (overnight).

   *Optional:* To remove template DNA, add 25 µl nuclease-free water and 2 µl of DNase I (RNase-free) (NEB #M0303), mix, and incubate at 37°C for 15 minutes. Alternatively, 2 µl of DNase I-XT (NEB# M0570) can be added directly to the IVT product and incubated at 37°C for 15 minutes.

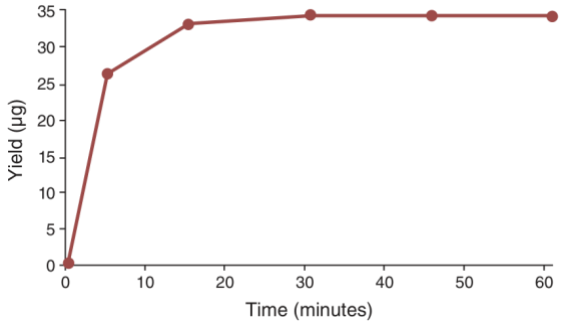
5. Proceed with purification of synthesized RNA and/or evaluation of transcription products yield and/or length.

   *Optional:* If a poly(A) tail is desired and is not encoded in the template plasmid, you can add one post-transcriptionally with *E. coli* Poly(A) Polymerase (NEB# M0276) using this protocol.

## Capped RNA Synthesis Using a Dinucleotide Cap Analog (i.e., ARCA)

This protocol can be used to synthesize Cap-0 RNA utilizing a dinucleotide cap analog, such as ARCA (NEB #S1411); cap analogs are sold separately. The recommended ratio of dinucleotide cap analog to GTP is 4:1. Table 1 below shows the effect of varying the ratio of dinucleotide cap analog to GTP on RNA yield; 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. Each standard 25 µl reaction yields up to 40–50 µg of RNA with approximately 80% capped RNA transcripts.

**Figure 4. Time course of capped RNA synthesis**




*SP6 control template (1.76 kb) was transcribed in the presence of 4 mM ARCA at 37°C in a PCR machine over one hour. Transcripts were treated with DNase I, purified by LiCl precipitation and quantified using a NanoDrop Spectrophotometer.*

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

| DINUCLEOTIDE CAP ANALOG:GTP RATIO | CONCENTRATION OF DINUCLEOTIDE CAP ANALOG:GTP (mM) | RNA YIELD (µg) IN 2 HOURS | PERCENT CAPPED RNA |
|---|---|---|---|
| 0:1 | 0:5 | 100 | 0 |
| 1:1 | 2.5:2.5 | 70 | 50% |
| 2:1 | 3.3:1.7 | 50 | 67% |
| 4:1 | 4:1 | 30 | 80% |
| 8:1 | 4.4:0.56 | 5-15 | 89% |

**Protocol**

1. Thaw the necessary components at room temperature. Keep the SP6 RNA Polymerase Mix on ice.
2. Mix and pulse-spin in a microfuge to collect the solutions to the bottom of the tubes.
3. Prepare a 10 mM GTP solution by combining 1 µl of 10 mM GTP (Tris) and 4 µl of nuclease-free water. Extra 10 mM GTP can be stored at -20°C for future use.
4. Prepare a 40 mM solution of dinucleotide cap analog.
5. Set up the reaction at room temperature in the order listed in the table below:

| COMPONENTS | 25 µl REACTION | FINAL AMOUNT |
|---|---|---|
| Nuclease-free Water | X µl | |
| 10X SP6 Reaction Buffer | 2.5 µl | 1X |
| 50 mM ATP (Tris) | 2.5 µl | 5 mM |
| 10 mM GTP (Tris) (*from step 3*) | 2.5 µl | 1 mM |
| 50 mM CTP (Tris) | 2.5 µl | 5 mM |
| 50 mM UTP (Tris) | 2.5 µl | 5 mM |
| 40 mM Dinucleotide Cap Analog (NEB #S1411, S1405, S1406 or S1407) | 2.5 µl | 4 mM |
| Linear Template DNA | X µl | 1 µg |
| 100 mM DTT | 1.25 µl | 5 mM |
| SP6 RNA Polymerase Mix | 2.5 µl | |

6. Mix thoroughly by pipetting and pulse-spin in a microfuge. Incubate at 37°C for 30 minutes in a dry air incubator or thermocycler to prevent evaporation.

   *Optional:* To remove template DNA, add 25 µl nuclease-free water and 2 µl of DNase I (RNase-free) (NEB #M0303), mix, and incubate at 37°C for 15 minutes. Alternatively, 2 µl of DNase I-XT (NEB# M0570) can be added directly to the IVT product and incubated at 37°C for 15 minutes.

7. Proceed with purification of synthesized RNA and/or evaluation of transcription products yield and/or length.

   *Optional:* If a poly(A) tail is desired and is not encoded in the template plasmid, you can add one post-transcriptionally with *E. coli* Poly(A) Polymerase (NEB# M0276).

## Radiolabeled RNA Probe Synthesis

This protocol can be used to synthesize high specific activity radiolabeled RNA probes from a DNA template containing the SP6 RNA Polymerase promoter sequence immediately followed by a guanosine. 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.

**Figure 5: Generation of high specific activity 32P-labeled RNA probes**


*Labeling 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 exposure of the gel to a Storage Phosphor Screen (GE)*

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 also appears to be more subject to decomposition during storage.

The labeled NTP is present at a limiting concentration and is therefore referred to as the "limiting nucleotide." 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. 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 4-8 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. For example, if the transcript contains more UTP then more $^{32}$P-UTP will be incorporated resulting in a higher specific activity. For longer RNA transcripts > 1 kb it may be necessary to increase the concentration of the unlabeled "limiting nucleotide" to increase the proportion of full-length transcript, however the improvement in yield of full-length transcript will reduce the specific activity of the probe. We recommend increasing the concentration of the unlabeled "limiting nucleotide" to 20 µM.

**Table 2. Concentration of [α-$^{32}$P] NTP in a 20 µl Reaction**

| SPECIFIC ACTIVITY (Ci/mol) | CONCENTRATION (mCi/ml) | VOLUME PER REACTION | HOT LABEL PER REACTION |
|---|---|---|---|
| 800 | 10 | 1 µl | 0.5 µM |
| 800 | 20 | 1 µl | 1 µM |
| 800 | 40 | 1 µl | 2 µM |
| 3000 | 10 | 1 µl | 0.13 µM |
| 3000 | 20 | 1 µl | 0.27 µM |
| 3000 | 40 | 1 µl | 0.53 µM |
| 6000 | 40 | 1 µl | 0.27 µM |

**Protocol**

1. Thaw the necessary components at room temperature. Keep the SP6 RNA Polymerase Mix on ice.
2. Mix and pulse-spin in a microfuge to collect the solutions to the bottom of the tubes.
3. **This protocol will reference labeled UTP as an example.** Prepare a 50 µM UTP working stock:
   a. Combine 4 µl of 50 mM UTP (Tris) and 196 µl of Nuclease-free Water for 200 µl of 1 mM UTP. Extra 1 mM UTP solution can be stored at -20°C for future use.
   b. Prepare 100 µl of 50 µM UTP by combining 5 µl of 1 mM UTP and 96 µl of nuclease-free water.
4. Prepare the Master Mix. For accurate pipetting, we recommend preparing a minimum of 20 µl master mix, which is enough for 4 labeling reactions. Extra master mix can be stored at -20°C for future use.

| MASTER MIX COMPONENTS | 20 µl MASTER MIX |
|---|---|
| Nuclease-free Water | 9 µl |
| 10X SP6 Reaction Buffer | 5 µl |
| 50 mM ATP (Tris) | 2 µl |
| 50 mM GTP (Tris) | 2 µl |
| 50 mM CTP (Tris) | 2 µl |

5. Set up the reaction at room temperature in the order listed in the table below:

| COMPONENTS | 25 µl REACTION | FINAL AMOUNT |
|---|---|---|
| Nuclease-free Water | X µl | |
| Master Mix (*from Step 4*) | 5 µl | 1 mM each ATP, GTP, and CTP; 0.5X Buffer |
| 50 µM UTP (Tris) (*from Step 3b*) | 3 µl | 6 µM |
| [α-$^{32}$P] UTP | X µl | 0.2–1 µM |
| Linear Template DNA | X µl | 0.1–1 µg |
| DTT (0.1M) | 1.25 µl | 5 mM |
| SP6 RNA Polymerase Mix | 2.5 µl | |

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

   *Optional:* To remove template DNA, add 2 µl of DNase I (RNase-free) (NEB #M0303) or DNase I-XT (NEB# M0570) directly to the IVT product and incubate for 15 minutes at 37°C.
7. Proceed with purification of synthesized RNA and/or evaluation of transcription product yield and/or length. The RNA yield will be less than 1 µg at UTP concentrations less than <10 µM. For purification, we recommend the 10 µg capacity Monarch RNA Spin Cleanup Kit (NEB# T2030).

## Purification of Synthesized RNA

In general, RNA synthesized *by in vitro* transcription can be purified by LiCl precipitation, phenol-chloroform extraction followed by ethanol precipitation, or by using a spin column-based method. If absolute full-length RNA is required, we recommend gel purification. For capped RNA, non-radioactively labeled RNA, or high specific activity radiolabeled RNA probes, spin column chromatography is the preferred method.

### LiCl Precipitation

LiCl precipitation can be used for quick recovery of the synthesized RNA and is an effective method for removing the majority of unincorporated NTPs and enzymes. However, RNAs shorter than 300 bases or at concentrations lower than 0.1 mg/ml do not precipitate well. In such cases, other purification methods may be used. A tube of LiCl Solution (7.5 M LiCl, 10 mM EDTA) is provided with this kit.

**Protocol**

1. Adjust the reaction volume to 50 µl by adding nuclease-free water.
2. To the 50 µl tailing reaction, add 25 µl LiCl solution and mix well.
3. Incubate at –20°C for 30 minutes.
4. Centrifuge at 4°C for 15 minutes at top speed to pellet the RNA.
5. Remove the supernatant carefully.
6. Rinse the pellet by adding 500 µl of cold 70% ethanol and centrifuge at 4°C for 10 minutes.
7. Remove the ethanol carefully. Spin the tube briefly to bring down any liquid on the wall.
8. Remove residual liquid carefully using a sharp tip (e.g., loading tip).
9. Air dry the pellet and resuspend the mRNA in 50–200 µl of 0.1 mM EDTA or a suitable RNA storage solution.
10. Store the RNA at –20°C or below.

### 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.

**Protocol**

1. Adjust the reaction volume to 180 µl by adding nuclease-free water. Add 20 µl of 3 M sodium acetate (pH 5.2) or 20 µl of 5M ammonium acetate and 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 it 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 carefully.
5. Rinse the pellet by adding 500 µl of cold 70% ethanol and centrifuge at 4°C for 10 minutes.
6. Remove the ethanol carefully. Spin the tube briefly to bring down any liquid on the wall.
7. Remove residual liquid carefully using a sharp tip (e.g., loading tip).
8. Air dry the pellet and resuspend the mRNA in 50-200 µl of 0.1 mM EDTA or a suitable RNA storage solution.
9. Store the mRNA at –20°C or below.

### Spin Column Chromatography

Spin columns will remove unincorporated nucleotides, proteins, and salts. We recommend using a Monarch Spin RNA Cleanup Kit (10 µg capacity NEB #T2030, 50 µg capacity #T2040, or 500 µg capacity #T2050) with the Monarch® Spin RNA Cleanup Kit Protocol.

Adjust the volume of the reaction mixture to 100 µl by adding nuclease-free water to the IVT product and mix well. Purify the RNA by following the manufacturer's instructions. Be aware that some reactions could produce 100 µg of RNA, which may exceed individual column capacity and require additional columns.

### Gel Purification

When high purity RNA transcript is desired (such as making RNA probe for RNase protection assays), we recommend gel purification of the transcription product. Gel purification of mRNA tailed by Poly(A) Polymerase is not recommended because the tailed mRNA is heterogeneous in length and will result in a smear on the gel. If the poly(A) tail is encoded in the DNA template, the RNA should resolve into a single band for gel purification.

The Monarch Spin RNA Cleanup Kits (NEB #T2030, #T2040 or #T2050) can be used for extraction of RNA from gels, although this is not their primary application, so recoveries may range from 40-70% (see protocol included in NEB #T2030, #T2040 or #T2050 product manual).

## Evaluation of Reaction Products

### Quantification by UV Light Absorbance

RNA concentration can be determined by measuring the ultraviolet light absorbance at 260 nm. However, any unincorporated nucleotides and template DNA in the mixture will affect the measurement, so must be removed before the RNA concentration can be quantified.

A Nanodrop spectrophotometer can directly read RNA concentrations from 10 ng/µl to 3000 ng/µl; it may be necessary to dilute your RNA prior to measurement. For single-stranded RNA, 1 $A_{260}$ is equivalent to an 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 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 structures by the transcript.

**Sample preparation**

1. Prepare denatured samples by mixing 100–200 ng RNA sample with 10–20 µl of RNA Loading Dye, 2X (NEB #B0363).
2. Denature the RNA sample and an aliquot of RNA marker by heating at 70°C for 10 minutes.
3. Pulse-spin prior to loading onto the gel.

**Gel Electrophoresis**

4a. **Denaturing agarose gel:**

Load 100-200ng denatured RNA sample.

It is common practice to electrophorese RNA on a fully denaturing agarose gel, such as one containing formaldehyde. However, in many cases it is possible to run RNA on a native agarose gel and obtain suitable results

4b. **Denaturing PAGE/Urea Gel:**

Load 20-100ng denatured RNA sample.

We recommend using commercially available premade gels and standard TBE gel running buffer (10X TBE buffer: 0.9 M Tris Base, 0.9 M Boric Acid, 20 mM EDTA).

**Visualize RNA**

5. Stain the gel post-electrophoresis with SYBR® Gold (preferred) or ethidium bromide.

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 SP6 control template DNA is a linearized plasmid containing the *Cypridina* luciferase (CLuc) gene under the transcriptional control of SP6 promoter. The size of the runoff transcript is 1.76 kb. The control reaction should yield ≥ 80 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 and supplement with DTT to a final concentration of 5 mM; 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 "pCLuc-SP6". The CLuc control template is generated by linearizing the plasmid with XbaI.

### 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

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 µg of template will help to achieve maximum yield.

### RNA Transcript Smearing on Denaturing Gel

If the RNA appears degraded (e.g. smeared) on a denaturing agarose or polyacrylamide gel, the DNA template may be 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 that resemble SP6 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 |
|---|---|---|
| E2070S | HiScribe SP6 RNA Synthesis Kit | 50 reactions |

COMPANION PRODUCTS

| NEB # | PRODUCT | SIZE |
|---|---|---|
| T2050S/L | Monarch Spin RNA Cleanup Kit (500 µg) | 10/100 preps |
| T2040S/L | Monarch Spin RNA Cleanup Kit (50 µg) | 10/100 preps |
| T2030S/L | Monarch Spin RNA Cleanup Kit (10 µg) | 10/100 preps |
| B0363S | RNA Loading Dye (2X) | 4 x 1 ml |
| M0303S/L | DNase I (RNase-Free) | 1,000/5,000 units |
| M0570S/L | DNase I-XT | 1,000/5,000 units |
| M0493S/L | Q5 Hot Start High-Fidelity DNA Polymerase | 100/500 units |
| M0494S/L | Q5 Hot Start High-Fidelity 2X Master Mix | 100/500 units |
| N0362S | ssRNA Ladder | 25 gel lanes |
| N0364S | Low Range ssRNA Ladder | 25 gel lanes |
| S1411S/L | 3′-O-Me-m7G(5′)ppp(5′)G RNA Cap Structure Analog | 1/5 µmol |
| S1404S/L | m7G(5′)ppp(5′)G RNA Cap Structure Analog | 1/5 µmol |
| S1405S/L | m7G(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 |
| M2080S | Vaccinia Capping System | 400 units |
| M2081S/L | Faustovirus Capping Enzyme | 500/2,500 units |
| M0366S | mRNA Cap 2′-O-Methyltransferase | 2,000 units |
| M0276S/L | *E. coli* 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 |
| N0431S | N1-Methyl-Pseudouridine-5′-Triphosphate | 0.1 ml |
| N0432S | 5-Methyl-Cytidine-5′-Triphosphate | 0.1 ml |
| N0433S | Pseudouridine-5′-Triphosphate | 0.1 ml |
| N0434S | 5-Methoxy-Uridine-5′-Triphosphate | 0.1 ml |

## Revision History

| REVISION # | DESCRIPTION | DATE |
|---|---|---|
| 1.0 | N/A | 12/16 |
| 2.0 | New format applied. | 7/20 |
| 3.0 | Updated to include addition of DTT. Updated LiCl Solution on page 2. Also updated table formatting and legal footer. | 7/23 |
| 4.0 | Reorganized and updated protocols for consistency. Removed "optional" note about DTT. | 1/26 |