INSTRUCTION MANUAL

HiScribe® T7 High Yield RNA Synthesis Kit

NEB #E2040S/L

50/250 reactions

Version 8.0_8/25

Table of Contents

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:	Dinucleotide Cap Analogs: NEB #S1411, #S1405, #S1406 and #S1407 Trinucleotide Cap Analogs: TriLink's CleanCap Reagent AG or CleanCap Reagent AG (3' OMe)
Modified-NTP:	N1-Methyl-Pseudouridine-5'-Triphosphate (NEB #N0431) 5-Methyl-Cytidine-5'-Triphosphate (NEB #N0432) Pseudouridine-5'-Triphosphate (NEB #N0433) 5-Methoxy-Uridine-5'-Triphosphate (NEB# N0434) 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) (#T2040, or #T2050)
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 is capable of incorporation of cap analogs and base-modified nucleotides, including natural non-canonical nucleotides like pseudouridine or nucleotides with labels like biotin. The kit is also capable of synthesizing high specific activity radiolabeled RNA probes.

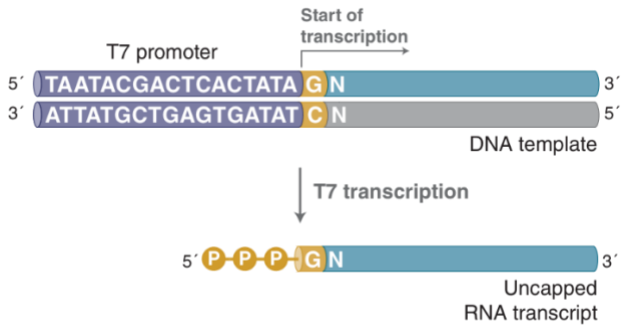
RNA synthesized from the kit is suitable for many applications including RNA structure and function studies, ribozyme biochemistry, probes for RNase protection assays and hybridization based blots, anti-sense RNA and RNAi experiments, microarray analysis, microinjection, and *in vitro* translation and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

The kit contains sufficient reagents for 50 or 250 reactions of 20 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 (S size) or 45 mg RNA (L size). Reactions may be scaled linearly to increase yield proportional to reaction volume.

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. The standard protocol results in 5′ triphosphorylated RNA.

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 the 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. 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 T7 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 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.

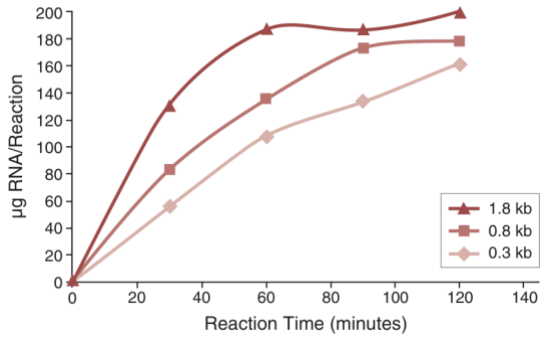
Synthetic DNA Oligonucleotides

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. We do not recommend using synthetic oligonucleotides directly as template for IVT RNA greater than 100 nucleotides.

RNA Synthesis Protocols

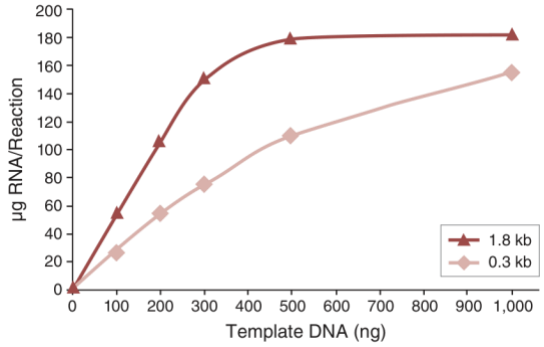
We strongly recommend wearing gloves and using nuclease-free tubes and reagents to avoid RNase contamination. Reactions are typically 20 µl but can be scaled up linearly as needed. Reactions should be assembled in nuclease-free microfuge tubes or PCR strip tubes.

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



For reactions with transcripts longer than 0.3 kb, a 2-hour incubation should result in the maximum yield. Shorter transcripts require longer incubation time to reach saturation. Reactions were incubated at 37°C in a thermocycler. Transcripts were purified by spin columns and quantified on NanoDrop® Spectrophotometer.

Figure 3. Effect of template amount on RNA yield



Shorter RNA requires more template DNA to reach saturation. Standard reactions were incubated at 37°C in a thermocycler for 2 hours. Transcripts were purified by spin columns and quantified on NanoDrop Spectrophotometer.

Standard RNA Synthesis

1. Thaw the necessary components at room temperature. Keep the T7 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	20 µl REACTION	FINAL AMOUNT
Nuclease-free Water	X µl	
10X T7 Reaction Buffer	2 µl	1X
100 mM ATP	2 µl	10 mM
100 mM GTP	2 µl	10 mM
100 mM CTP	2 µl	10 mM
100 mM UTP	2 µl	10 mM
Linear Template DNA*	X µl	1 µg
DTT (0.1M)	1 µl	5 mM
T7 RNA Polymerase Mix	2 µl	

* For reactions that synthesize short transcripts (< 0.3 kb), follow the general reaction above, except add an additional 10 µl of Nuclease-free Water to bring the final reaction volume to 30 µl. Increase template DNA up to 2 µg.

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: 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 30 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 T7 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.

Protocol

1. Thaw the necessary components at room temperature. Keep the T7 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	20 µl REACTION	FINAL AMOUNT
Nuclease-free Water	X µl	
10X T7 Reaction Buffer	1.5 µl	0.75X
100 mM ATP	1.5 µl	7.5 mM
100 mM GTP	1.5 µl	7.5 mM
100 mM CTP	1.5 µl	7.5 mM
100 mM UTP	1 µl	5 mM
10 mM Modified UTP	5 µl	2.5 mM
Linear Template DNA	X µl	1 µg
DTT (0.1M)	1 µl	5 mM
T7 RNA Polymerase Mix	2 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 30 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

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 20 µl reaction yields up to 40-50 µg of RNA with approximately 80% capped RNA transcripts.

Figure 4: T7 RNA Polymerase co-transcriptionally caps RNA with anti-reverse cap analog (ARCA)

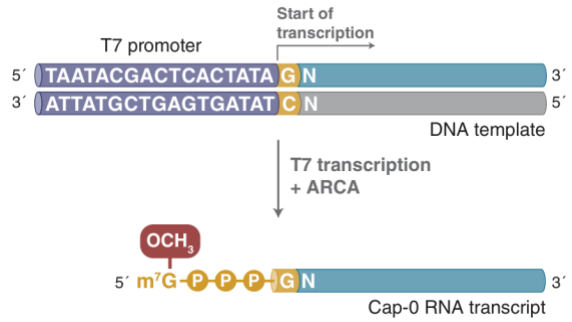


Table 1. Effect of dinucleotide cap analog:GTP ratios on RNA yield

DINUCLEOTIDE CAP ANALOG:GTP RATIO	CONCENTRATION OF DINUCLEOTIDE CAP ANALOG:GTP (mM)	RNA YIELD (µg) IN 2 HOURS	PERCENT CAPPED RNA
0:1	0:10	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%

Protocol

1. Thaw the necessary components at room temperature. Keep the T7 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 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.
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	20 µl REACTION	FINAL AMOUNT
Nuclease-free Water	X µl	
10X T7 Reaction Buffer	2 µl	1X
100 mM ATP	2 µl	10 mM
100 mM UTP	2 µl	10 mM
100 mM CTP	2 µl	10 mM
20 mM GTP	2 µl	2 mM
40 mM Dinucleotide Cap Analog (NEB #S1411, S1405, S1406 or S1407)	4 µl	8 mM
Linear Template DNA	X µl	1 µg
100 mM DTT	1 µl	5 mM
T7 RNA Polymerase Mix	2 µl	

6. 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.

   Optional: To remove template DNA, add 30 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).

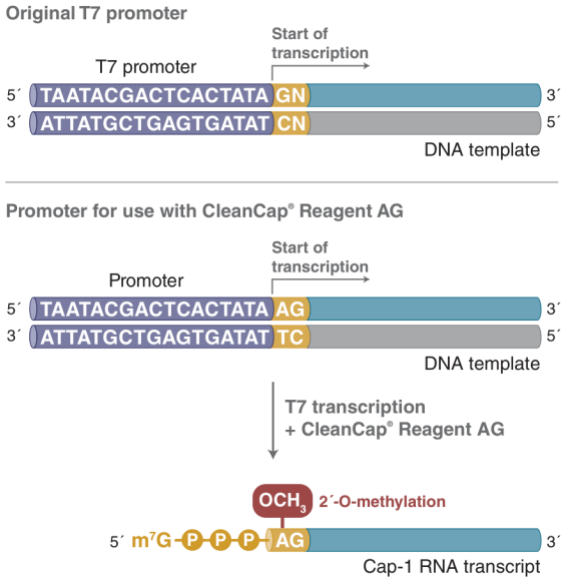
Capped RNA Synthesis Using a Trinucleotide Cap Analog (i.e., TriLink's CleanCap Reagent AG)

This protocol can be used to co-transcriptionally incorporate user-supplied trinucleotide cap analogs, such as CleanCap AG or AG (3´ OMe) from TriLink, into IVT RNA using the HiScribe T7 High Yield RNA Synthesis Kit (NEB #E2040). Unlike standard RNA synthesis with NEB #E2040, reactions with this protocol are typically 40 µl with 5 mM each NTP and will use twice the amount of T7 RNA Polymerase Mix, decreasing the overall number of reactions provided. NEB does offer and recommends using the separate HiScribe T7 mRNA synthesis kit that is supplied with CleanCap Reagent AG (NEB #E2080), which has its own dedicated protocol.

By using a DNA template with a T7 promoter sequence followed by an AG initiation sequence and an encoded poly(A) tail, mRNAs can be transcribed with a 5´-m7G Cap-1 structure that is polyadenylated, translationally competent, and able to evade the cellular innate immune response.

IMPORTANT NOTE ABOUT TEMPLATE SEQUENCE: CleanCap AG requires modification to the sequence immediately downstream of the T7 promoter sequence, replacing the "GG" that follows the T7 promoter sequence with an "AG" as shown in Figure 5 below. The use of CleanCap Reagent AG with templates containing the standard T7 promoter sequence with GG will result in RNA with triphosphorylated 5´ ends. If you need to change your template plasmid DNA sequence to "AG", we recommend using our Q5 Site-Directed Mutagenesis Kit (NEB #E0554).

Figure 5: Co-transcriptional capping with T7 RNA Polymerase and CleanCap Reagent AG requires a modified initiation sequence in the DNA template



Protocol

1. Thaw the necessary components at room temperature. Keep the T7 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 following order:

COMPONENTS	40 µl REACTION	FINAL CONCENTRATION
Nuclease-free Water	X µl	
10X T7 Reaction Buffer	2 µl	0.5X
100 mM ATP	2 µl	5 mM
100 mM GTP	2 µl	5 mM
100 mM UTP	2 µl	5 mM
100 mM CTP	2 µl	5 mM
100 mM CleanCap AG (N-7113) or AG (3′ OMe) (N-7413)	1.6 µl	4 mM
Linear Template DNA	X µl	1 µg
DTT (0.1M)	2 µl	5 mM
T7 RNA Polymerase Mix	4 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.

   Optional: To remove template DNA, add 10 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.

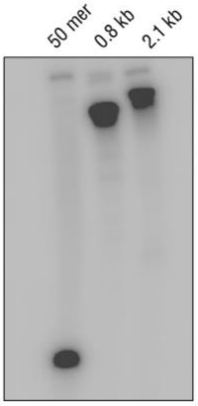
5. Proceed with purification of synthesized RNA and/or evaluation of transcription products yield and/or length.

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

Specific Activity Radiolabeled RNA Probe Synthesis

This protocol can be used to synthesize high specific activity radiolabeled RNA probes from a DNA template containing the T7 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 6. Generation of high specific activity 32P-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).

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 4–5 µM of the "limiting nucleotide" is necessary for full-length probe synthesis with high specific activity.

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	0.63 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

Protocol

1. Thaw the necessary components at room temperature. Keep the T7 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 40 µM UTP working stock:
   a. Combine 4 µl of 100 mM UTP and 396 µl of Nuclease-free Water for 400 µl of 1 mM UTP. 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 the Master Mix. For accurate pipetting, we recommend preparing a minimum of 15 µl master mix, which is enough for 5 labeling reactions. Extra master mix can be stored at -20°C for future use.

MASTER MIX COMPONENTS	15 µl MASTER MIX
Nuclease-free Water	10 µl
10X T7 Reaction Buffer	2 µl
100 mM ATP	1 µl
100 mM GTP	1 µl
100 mM CTP	1 µl

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

COMPONENTS	20 µl REACTION	FINAL AMOUNT
Nuclease-free Water	X µl	
Master Mix from Step 5	3 µl	1 mM each A, G, and C; 0.2X Buffer
40 µM UTP from Step 4	2 µl	4 µM
[α-32P] UTP	X µl	0.2–1 µM
Linear Template DNA	X µl	0.1–1 µg
DTT (0.1M)	1 µl	5 mM
T7 RNA Polymerase Mix	1 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 not provided with this kit (NEB #E2040).

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 µl of 0.1 mM EDTA or a suitable RNA storage solution.
10. Heat the RNA at 65°C for 5-10 minutes to completely dissolve the RNA. Mix well.
11. 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 5 M 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 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 180 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 reading, 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.

1. Preparation of denaturing gels and samples

1a. Denaturing agarose gel:

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. In fact, it has been demonstrated that treatment of RNA samples in a denaturing buffer (outlined below) maintains the RNA molecules in a denatured state for at least 3 hours during electrophoresis. The use of native agarose gels eliminates problems associated with toxic chemicals and the difficulties encountered when staining and blotting formaldehyde gels.

1. Add sample to an equal volume of RNA Loading Dye, (2X) (NEB #B0363) and mix well.
2. Heat at 70°C for 10 minutes to denature the RNA. Cooling prior to loading is optional.
3. Load the samples.

1b. Denaturing PAGE/Urea Gel:

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).

2. Gel electrophoresis of non-radiolabeled RNA

1. Mix 0.2–1 µg RNA sample with 5-10 µl of RNA Loading Dye, 2X (NEB #B0363).
2. Denature the RNA sample and an aliquot of RNA marker by heating at 65-70°C for 5-10 minutes.
3. Pulse-spin prior to loading onto the gel.
4. Visualizing RNA by staining the gel post-electrophoresis with SYBR® Gold (preferred) or ethidium bromide.

3. Gel electrophoresis of radiolabeled RNA

1. Mix an aliquot of labeled RNA with an equal volume of RNA Loading Dye (2X) (NEB #B0363).
2. Denature the RNA sample by heating at 65–70°C for 5–10 minutes.
3. Pulse-spin prior to loading onto gel.
4. 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 32P 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 1.76 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 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 "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 not required but is highly 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 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, 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
T2050S/L	Monarch RNA Cleanup Kit (500 µg)	10/100 preps
T2040S/L	Monarch RNA Cleanup Kit (50 µg)	10/100 preps
T2030S/L	Monarch 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-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, 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. Also updated table styling and legal footnote.	7/23
7.0	Updated to include new L size.	1/24
8.0	Added trinucleotide capping protocol. Updated modified nucleotide protocol with full and partial substitution details. Updated other protocols for consistency. Removed "optional" note about DTT.	8/25