INSTRUCTION MANUAL

NEW ENGLAND BioLabs Inc. | be INSPIRED drive DISCOVERY stay GENUINE

HiScribe® T7 ARCA mRNA Kit (with tailing)

NEB #E2060S

20 reactions

Version 4.0_7/23

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The HiScribe T7 ARCA mRNA Kit (with tailing) Includes:

All kit components should be stored at –20°C. Each kit contains sufficient reagents for 20 reactions of 20 µl each. Each standard reaction yields up to 20 µg of capped mRNA from 1 µg control template. Up to 25 µg capped and tailed mRNA can be obtained after poly(A) tailing and purification by LiCl precipitation.

ARCA/NTP Mix (2X)

T7 RNA Polymerase Mix

CLuc Control Template (0.25 µg/µl)

DNase I (RNase-free) (2 units/µl)

E. coli Poly(A) Polymerase

Poly(A) Polymerase Reaction Buffer (10X)

LiCl Solution (7.5 M LiCl, 10 mM EDTA)

Dithiothreitol (DTT) (0.1 M)

Required Materials Not Included:

DNA Template

Thermocycler or 37°C incubator.

Nuclease-free water

Buffer- or water-saturated phenol:chloroform

Ethanol

3 M Sodium acetate, pH 5.2

5 M Ammonium acetate

Spin columns (see Monarch® RNA Cleanup Kits, NEB #T2040 or #T2050)

Gels, running buffers and gel box

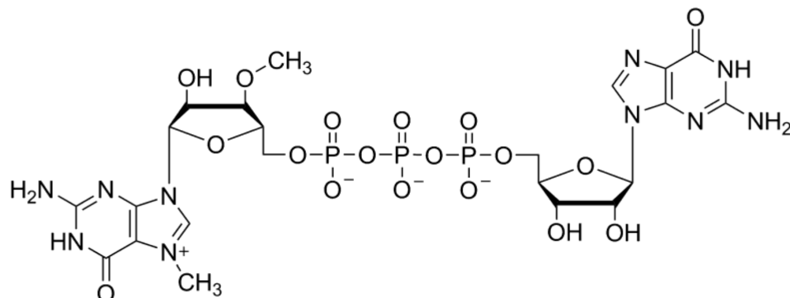
Equipment for RNA analysis

Introduction

Most eukaryotic mRNAs require a 7-methyl guanosine (m7G) cap structure at the 5´ end and a poly(A) tail at the 3´ end to be efficiently translated. The HiScribe T7 ARCA mRNA Kit (with tailing) is designed for quick production of ARCA capped and poly(A) tailed mRNA in vitro. Capped mRNAs are synthesized by co-transcriptional incorporation of Anti-Reverse Cap Analog (ARCA, NEB #S1411) using T7 RNA Polymerase. The transcription reaction can be set up easily by combining the ARCA/NTP mix, T7 RNA Polymerase Mix and a suitable DNA template. The kit also allows for partial incorporation of 5mCTP, Pseudo-UTP and other modified nucleotides into mRNA. After a brief DNase I treatment to remove the template DNA, capped mRNA is poly(A) tailed with Poly(A) Polymerase. mRNAs synthesized with the kit can be used for cell transfection, microinjection, *in vitro* translation and RNA vaccines.

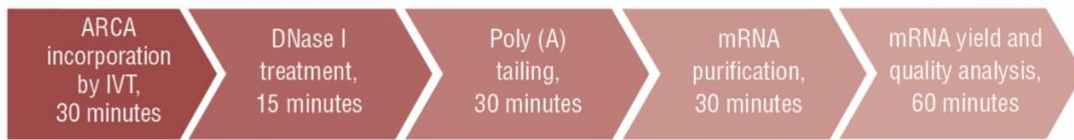
ARCA is incorporated into mRNA exclusively in the correct orientation, generating capped mRNA that is more efficiently translated. Standard cap analogs can be incorporated in either direction resulting in only 50% of capped mRNA that is functional in protein translation.

Figure 1. Structure of Anti-Reverse Cap Analog (ARCA, NEB #S1411)



Methylation at the 3´ position of 7mG forces the cap structure to be attached to mRNA in the correct orientation.

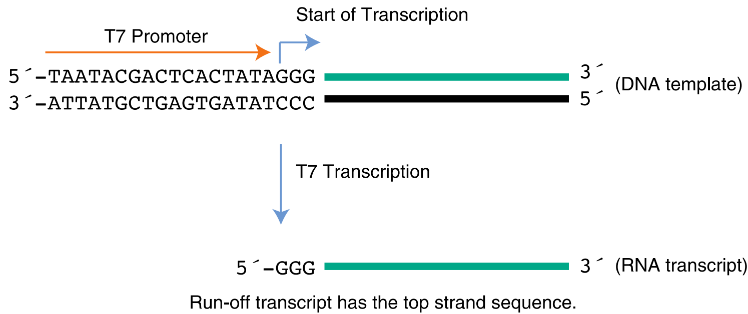
Figure 2. Overview of mRNA synthesis workflow with the HiScribe T7 ARCA mRNA Kit (with tailing)



DNA Template Preparation

Linearized plasmid DNA and PCR product containing a T7 promoter can be used as template for *in vitro* transcription with the HiScribe T7 ARCA mRNA Kit. Figure 3 illustrates the minimal T7 promoter sequence, as well as a run-off transcript after T7 transcription.

Figure 3. Transcription by T7 RNA Polymerase



Plasmid Templates

Plasmid DNA templates to be used with the HiScribe T7 ARCA mRNA Kit must be highly purified. Any purification method may be used, as long as the product is predominately supercoiled and free of contaminating RNase, proteins and salts.

To produce an RNA transcript of defined length, plasmid DNA must be completely linearized with a restriction enzyme, downstream of the insert to be transcribed. NEB offers a large selection of restriction enzymes for this purpose; we recommend selecting restriction enzymes that generate blunt ends or 5´overhangs.

After linearization, the template DNA can be purified by phenol:chloroform extraction:

1. Extract DNA with an equal volume of 1:1 phenol:chloroform mixture, and 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 Fragment containing a T7 promoter in the correct orientation can be transcribed using the kit. PCR product should be purified and run on an agarose gel to confirm amplicon size prior to its use as a template in the T7 ARCA mRNA transcription reaction. 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). Generally, 0.1–0.5 µg of PCR fragments can be used in a 20 µl *in vitro* transcription reaction. NEB supplies a variety of DNA polymerases for PCR. We recommend using hot-start high fidelity polymerases such as Q5® Hot Start High-Fidelity DNA Polymerase (NEB #M0493).

Figure 4. PCR Primer Design for T7 RNA Polymerase Templates

mRNA Synthesis Protocols

Please wear gloves when setting up RNA transcription reactions. Be sure to use nuclease-free tubes and reagents to avoid RNase contamination. Transcription 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.

   Assemble the reaction at room temperature in the following order:

REAGENT	AMOUNT	FINAL CONCENTRATION
Nuclease-free water	to 20 µl	
2X ARCA/NTP Mix	10 µl	1 mM GTP, 4 mM ARCA, 1.25 mM CTP, 1.25 mM UTP, >1.25 mM ATP final
Template DNA	X µl	1 µg
DTT (0.1M)*	1 µl	5 mM
T7 RNA Polymerase Mix	2 µl	
Total Reaction Volume	**20 µl**	

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

2. Mix thoroughly and pulse-spin in a microfuge. Incubate at 37°C for 30 minutes.

   Do not heat the reaction. Do not purify the RNA. Proceed to DNase treatment and Poly(A) tailing steps. Or you can store the reaction at –20°C for a few days.

   Reaction time depends on template amount, quality and RNA transcript length. For reactions with transcripts longer than 0.5 kb, 30 min incubation should give you the maximum yield.

   For reactions with short RNA transcripts (< 0.5 kb), incubation time of 1 hour or longer is necessary to achieve good yield. It is safe to incubate the reaction for 16 hours (overnight).

   For reaction times of 60 minutes or less, a water bath or heating block may be used; for reaction times longer than 60 minutes, please use a dry air incubator or PCR machine.

3. DNase treatment to remove template DNA. Add 2 µl of DNase I, mix well and incubate at 37°C for 15 minutes.

   DNase treatment is optional if the template does not interfere with downstream experiment. If left untreated, DNA template containing eukaryotic promoters may produce a background in mRNA transfection experiments.

4. Save 1 µl for gel analysis if desired. Do not heat the reaction or purify the RNA. Proceed to tailing reaction.

5. Poly(A) tailing. Set up the tailing reaction as below. The unpurified IVT reaction contains enough ATP, no extra ATP is necessary for the tailing reaction. Standard tailing reaction volume is 50 µl. Tail length is slightly longer in a 100 µl reaction volume for some transcripts.

REAGENT	AMOUNT	AMOUNT
Nuclease-free water	20 µl	65 µl
IVT Reaction	20 µl	20 µl
Poly(A) Polymerase Reaction Buffer (10X)	5 µl	10 µl
Poly(A) Polymerase	5 µl	5 µl
Total Reaction Volume	**50 µl**	**100 µl**

6. Mix thoroughly and pulse-spin in a microfuge. Incubate at 37°C for 30 minutes. Save 1 µl for analysis if necessary.

   A 30 min tailing reaction will give 150 nt or longer poly(A) tail for majority of mRNAs. Due to the nature of the tailing reaction, tail length will vary depending on RNA sequence, structure, yield, length, etc. For very short RNA (< 300 nt), tailing time can be extended to 1 hour to achieve sufficient tailing.

   The 3´ end of RNA must be exposed for efficient tailing. If the 3´ end is buried inside the RNA structure, it will not be available for tailing. Redesigning the mRNA 3´ end sequence may resolve the problem.

7. Proceed with mRNA purification. For purification, we recommend the Monarch RNA Cleanup Kits (NEB #T2040 or #T2050).

mRNA Synthesis with Modified Nucleotides

Modified mRNAs containing 5mCTP and Pseudo-UTP have been shown to suppress RNA-mediated innate immune activation *in vivo*. The HiScribe T7 ARCA mRNA Kit (with tailing) is capable of incorporation of 5mCTP and Pseudo-UTP, into mRNA. Up to 2.5 mM total modified nucleotides can be added into the transcription reaction without impacting the mRNA yield significantly. Other modified UTP or CTP may also be used but RNA yield will vary depending on the properties of the nucleotides. Modified GTP and ATP should not be used because they will interfere with capping and tailing efficiency. Please note modified nucleosides are not supplied with the kit.

The protocol below uses 1.25 mM 5mCTP and 1.25 mM Pseudo-UTP, generating mRNA containing 50% 5mCTP and 50% Pseudo-UTP.

1. Thaw the necessary kit components, mix and pulse-spin in microfuge to collect solutions to the bottoms of tubes. Assemble the reaction in the following order:

REAGENT	AMOUNT	FINAL CONCENTRATION
Nuclease-free water	to 20 µl	
ARCA/NTP Mix (2X)	10 µl	1 mM GTP, 4 mM ARCA, 1.25 mM CTP, 1.25 mM UTP, > 1.25 mM ATP final
5mCTP, 10 mM	2.5 µl	1.25 mM 5mCTP final
Pseudo-UTP, 10 mM	2.5 µl	1.25 mM Pseudo-UTP final
Template DNA	X µl	1 µg
DTT (0.1M)*	1 µl	5 mM
T7 RNA Polymerase Mix	2 µl	
Total Reaction Volume	**20 µl**	

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

2. Follow standard mRNA synthesis protocol.

mRNA Purification

Synthesized mRNA can be purified by LiCl precipitation, phenol:chloroform extraction followed by ethanol precipitation, or by using a spin column based method (e.g., Monarch RNA Cleanup Kits, NEB #T2030, #T2040 or #T2050).

LiCl Precipitation

The kit includes LiCl solution for quick recovery of the synthesized mRNA. LiCl precipitation of RNA is effective in 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. LiCl purified mRNA is suitable for transfection and microinjection experiments.

1. To the 50 µl tailing reaction, add 25 µl LiCl solution and mix well.
2. Incubate at –20°C for 30 minutes.
3. Centrifuge at 4°C for 15 minutes at top speed to pellet the RNA.
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. Heat the RNA at 65°C for 5-10 minutes to completely dissolve the RNA. Mix well.
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.

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 Purification

Spin columns will remove unincorporated nucleotides, proteins and salts. Please follow the manufacturer's instructions. Extra column washes before elution may help in minimizing binding reagent carry over.

Gel Purification

Because poly(A) addition by Poly(A) Polymerase is distributive, the tailed mRNA is heterogeneous in length, gel purification of tailed mRNA is not recommended.

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. Free nucleotides from the transcription reaction must be removed before the RNA concentration can be quantified. RNA solution can be read directly on a Nanodrop™ Spectrophotometer. The Nanodrop Spectrophotometer can directly read RNA concentrations from 10 ng/µl to 3000 ng/µl. 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
   a. Denaturing agarose gel:
      To make a 100 ml 1% denaturing agarose gel, add 1 gram agarose powder to 72 ml nuclease-free water. Melt the agarose and 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
   a. Mix 0.2–1 µg RNA sample with 5-10 µl 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. Visualize RNA by staining the gel with SYBR® Gold or ethidium bromide.

mRNA Quality Analysis by Bioanalyzer or Capillary Electrophoresis

Choose appropriate assay chips and follow the instructions carefully.

Figure 5. Analysis of mRNAs before and after tailing on Agilent 2100 Bioanalyzer

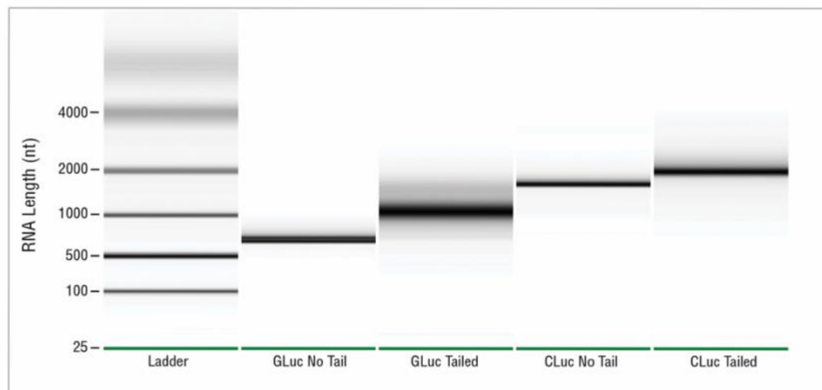
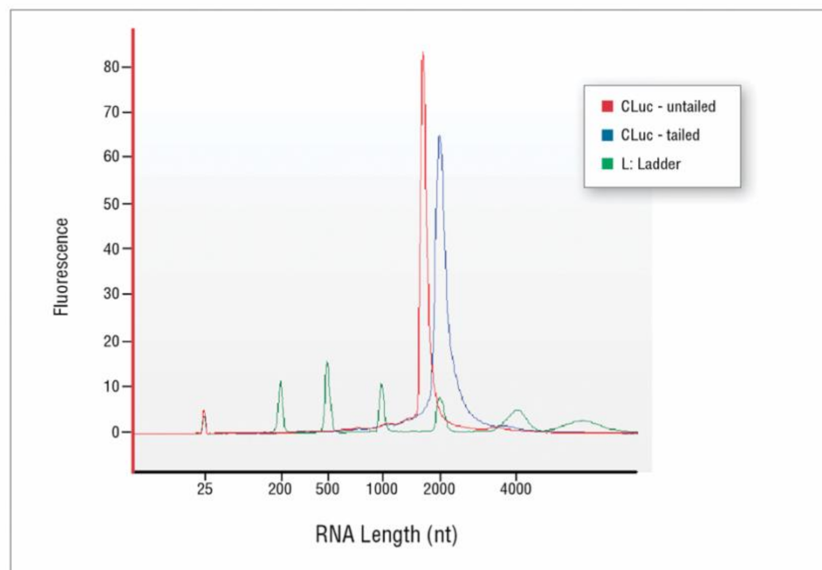


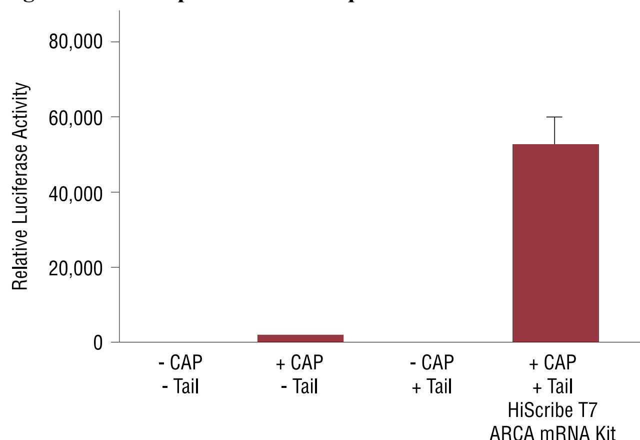
Figure 6. Analysis of CLuc mRNA before and after tailing on Agilent 2100 Bioanalyzer



mRNA Functional Analysis by Cell Transfection

Cell transfection experiment shows CLuc mRNA synthesized with the kit is efficiently expressed in U2OS cells (Figure 7). Both cap and tail are required for mRNA function in cell transfection.

Figure 7. Both cap and tail are required for mRNA function in cell culture



Luciferase expression in U2OS cells. Purified Cypridina luciferase RNA produced as indicated was co-transfected into U2OS cells with purified Gaussia luciferase mRNA. mRNAs produced using the HiScribe T7 ARCA mRNA Kit (With Tailing) are 5´- capped and have 3´- poly(A) tails. After 16 hours incubation at 37°C, cell culture supernatants from each well were assayed for CLuc and GLuc activity and luminescence values were recorded. Relative luciferase activity was calculated using the equation:

Relative Luciferase Activity = [CLuc activity(x)/GLuc activity(x)] / [CLuc activity(no CAPno TAIL) /GLuc activity(no CAP no TAIL)]

Data are presented as mean +/- SEM of 2 or more independent experiments.

Troubleshooting

Control Reaction

The CLuc control template DNA is a linearized plasmid containing the *Cypridina luciferase* gene under the transcriptional control of the T7 promoter. The size of the runoff transcript is 1.76 kb. The control reaction should yield ≥ 15 µg RNA transcript in 30 minutes.

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 all precautions 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 “pCMV-CLuc 2”. The pCMV-CLuc 2 control template is generated by linearizing the plasmid with restriction enzyme Xba I.

Low Yield of Full-length RNA

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

Addition of DTT

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

Low Yield of Short Transcript

High yields of short transcripts (< 0.3 kb) are achieved by extending incubation time and increasing the amount of template. Incubation of reactions up to 16 hours (overnight) or using up to 2 µg of template will help to achieve maximum yield. Alternatively, clean up the DNA template using a spin column based method, Monarch PCR & DNA Cleanup Kit (5 µg), NEB #T1030.

RNA Transcript Smearing on Denaturing Gel

If the RNA appears degraded (e.g., smeared) on denaturing agarose or polyacrylamide gel, the DNA template is likely 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, plasmid DNA may be incompletely digested. Even small amounts of undigested circular plasmid 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. Sequences with resemblance to 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.

Tailing Length Control

A standard 30 min tailing reaction can add a poly(A) tail at least 150 nt in length to an average size mRNA generated from the IVT reaction. Short RNA may require longer incubation time for sufficient tailing.

No Tailing or Partial Tailing

3´ end of the mRNA must be exposed for efficient tailing. Because T7 RNA Polymerase tends to generate 3´ end heterogeneity by adding extra bases, a small percentage of the mRNA may adopt alternate structures which may not be suitable for tailing. The following tips may help with successful tailing.

- Run the whole mRNA synthesis work flow without freezing the RNA between steps.
- To avoid preferential tailing, pre-incubate tailing mix at 37°C for 3 minutes before adding Poly(A) Polymerase. Mix well immediately.
- Tailing reaction should be at 37–40°C. Lower temperatures are not recommended.
- If still no tailing, redesign the DNA template with different sequences at the 3´ end.

mRNA Not Functional

- Verify the mRNA is intact, capped and tailed.
- Be sure the mRNA is clean, free from any inhibitors of downstream experiments.
- Follow instructions carefully with appropriate controls.
- Verify the DNA template has the correct sequence.

Ordering Information

NEB #	PRODUCT	SIZE
E2060S	HiScribe T7 ARCA mRNA Kit (with tailing)	20 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
E2040S	HiScribe T7 High Yield RNA Synthesis Kit	50 reactions
E2050S	HiScribe T7 Quick High Yield RNA Synthesis Kit	50 reactions
E2065S	HiScribe T7 ARCA mRNA Kit	20 reactions
T2040S/L	Monarch RNA Cleanup Kit (50 µg)	10/100 preps
T1030S/L	Monarch PCR & DNA Cleanup Kit (5 µg)	50/150 preps
B0363S	RNA Loading Dye (2X)	4 x 1 ml
M0307S/L	RNase Inhibitor, Human Placenta	2,000/10,000
M0314S/L	RNase Inhibitor, Murine	3,000/15,000
M0303S/L	DNase I (RNase-Free)	1,000/5,000 units
M0493S/L	Q5 Hot Start High-Fidelity DNA Polymerase	100/500 units
N0362S	ssRNA Ladder	25 gel lanes
N0364S	Low Range ssRNA Ladder	25 gel lanes
S1411S/L	3´-O-Me-m^{7}G(5´)ppp(5´)G RNA Cap Structure Analog	1/5 µmol
S1405S/L	m^{7}G(5´)ppp(5´)A RNA Cap Structure Analog	1/5 µmol
S1406S/L	G(5´)ppp(5´)A RNA Cap Structure Analog	1/5 µmol
S1407S/L	G(5´)ppp(5´)G RNA Cap Structure Analog	1/5 µmol
S1404S/L	m^{7}G(5´)ppp(5´)G RNA Cap Structure Analog	1/5 µmol
E3320S	RNase Contamination Assay Kit	50 reactions
M2080S	Vaccinia Capping System	400 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
N0450S/L	Ribonucleotide Solution Set	10/50 µmol of

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	3/15
1.1		10/16
2.0		1/19
3.0	Applied new manual format.	4/20
3.1	Corrected number of reactions on front page from 50 to 20.	2/22
4.0	Updated to included addition of DTT. Updated location of control plasmid sequence on page 8. Also updated table formatting and legal footer.	7/23

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