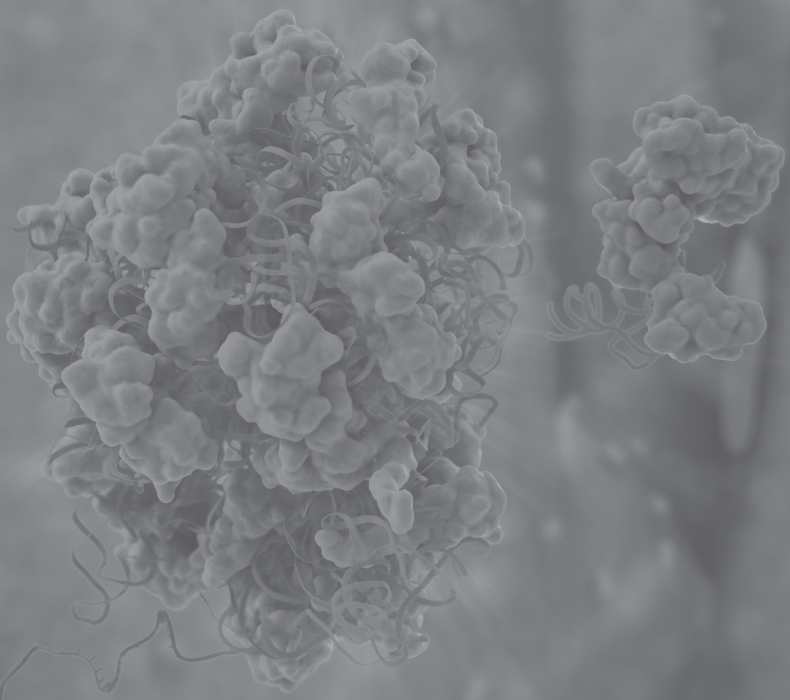
RNA ENZYMES & GENE ANALYSIS

HiScribe™ T7 Quick High Yield RNA Synthesis Kit

Instruction Manual



NEB #E2050S
50 reactions
Version 2.1 1/17

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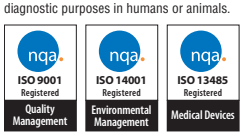

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Kit Components:

All kit components should be stored at –20°C. The kit contains sufficient reagents for 50 reactions of 20 µl each. Each standard reaction yields up to 180 µg of unmodified RNA from 1 µg control template.

NTP Buffer Mix (20 mM each NTP)
T7 RNA Polymerase Mix
FLuc Control Template (0.5 µg/µl)
DNase I (2 units/µl)
LiCl Solution (7.5 M LiCl, 10 mM EDTA)

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

Gels, running buffers and gel box

Introduction:

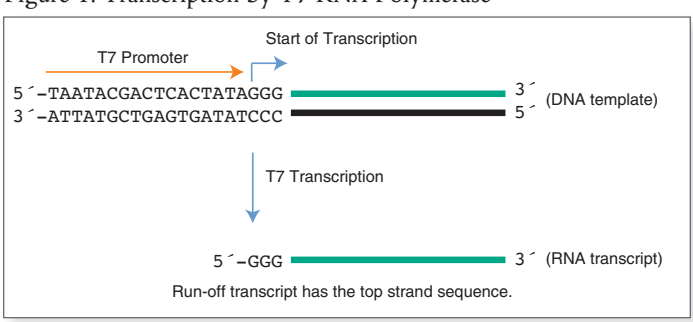
The HiScribe T7 Quick High Yield RNA Synthesis Kit is designed for quick set-up and production of large amounts of RNA *in vitro*. The reaction can be set up conveniently by combining the NTP buffer mix, T7 RNA Polymerase mix and a suitable DNA template. The kit also allows for capped RNA or dye-labeled RNA synthesis by incorporation of cap analog (ARCA, NEB #S1411) or dye-modified nucleotides. RNA synthesized with the kit can be used for RNA structure and function studies, ribozyme biochemistry, as probes for RNase protection assays and hybridization based blots, anti-sense RNA and RNAi experiments, microarray analysis and microinjection, as well as *in vitro* translation and RNA vaccines.

To synthesize high specific activity radioactive RNA probes or RNA with 100% substitution of one or more modified nucleotides we recommend using the T7 High Yield RNA synthesis kit (#E2040), in which the four nucleotides are supplied separately.

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 Quick 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, as well as a run-off transcript after T7 transcription.

Figure 1. Transcription by T7 RNA Polymerase



Plasmid Templates

It is of the utmost importance to begin the HiScribe T7 Quick High Yield RNA Synthesis Kit with highly purified, completely linearized plasmid template. Quality of the template DNA affects transcription efficiency, as well as the integrity of the RNA synthesized. Yield is commensurate with template purity. Any purification method may be used, as long as the product is predominately supercoiled and free of contaminating RNase, protein, RNA 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. In contrast, 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 for this purpose; we recommend selecting restriction enzymes that generate blunt ends or 5´-overhangs.

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

1. Extract DNA with an equal volume of 1:1 phenol:chloroform mixture, 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 products containing a T7 promoter in the correct orientation can be transcribed. While PCR mixture may be used directly, better yields will be obtained with purified PCR products. PCR products should be run on an agarose gel to estimate concentration and to confirm amplicon size prior to its use as a template in the T7 Quick RNA transcription reaction. Generally, 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 T7 Quick transcription reaction. In general, the yields are relatively

low and also variable depending upon the sequence, purity and preparation of the synthetic oligonucleotides.

RNA Synthesis Protocols:

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

Standard RNA Synthesis

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

Nuclease-free water	X µl	
NTP Buffer Mix	10 µl	10 mM each NTP final
Template DNA	X µl	1 µg
T7 RNA Polymerase Mix	2 µl	
Total reaction volume	20 µl	

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

Reaction time depends on template amount, quality and RNA transcript length. For reactions with transcripts longer than 0.3 kb, 2 hour incubation should give you the maximum yield. For reaction times of 60 minutes or less, a water bath or heating block may be used; for reaction times longer than 60 minutes, we recommend using a dry air incubator or a thermocycler to prevent evaporation of the sample.

For reactions with short RNA transcripts (< 0.3 kb), we recommend an incubation time of 4 hours or longer. It is safe to incubate the reaction for 16 hours (overnight). For example, we have achieved good yield with only 0.2 µg plasmid template encoding a 50-mer RNA by incubating the reaction overnight at 37°C.

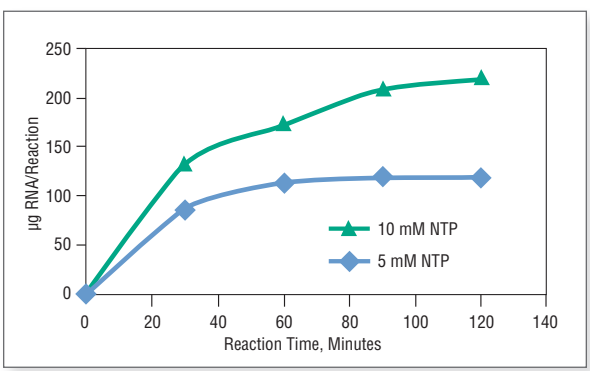
Reaction set up for short transcripts (< 0.3 kb):

Nuclease-free water	X µl	
NTP Buffer Mix	10 µl	6.7 mM each NTP final
Template DNA	X µl	1 µg
T7 RNA Polymerase Mix	2 µl	
Total reaction volume	30 µl	

Compared to the standard reaction, this reaction uses 10 µl more water. The volume of NTP Buffer Mix and T7 RNA Polymerase Mix, however, remains the same. The kit contains sufficient materials for 50 reactions.

Note that the amount of NTP Buffer Mix in a standard 20 µl reaction can vary from 2 to 10 µl. The final yield is proportional to the amount of input nucleotides, meaning that the nucleotide incorporation efficiency remains the same when different amounts of NTP are used. Figure 2 shows the time course of standard RNA synthesis from 1 µg control DNA template coding for a 1.8 kb RNA transcript with the T7 Quick Kit using 10 µl and 5 µl NTP Buffer Mix in a 20 µl reaction.

Figure 2. RNA synthesis with different amounts of NTP.



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

4. Optional: DNase treatment to remove DNA template. Standard reactions are capable of generating 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 to each 20 µl reaction, followed by 2 µl of DNase I (RNase-free), mix and incubate for 15 minutes at 37°C.
5. Proceed with purification of synthesized RNA or analysis of transcription products by gel electrophoresis.

Capped RNA Synthesis

The kit formulation allows for efficient capped RNA synthesis using cap analog (ARCA). The recommended ratio of cap analog to GTP is 4:1. 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 reaction. Cap analogs are sold separately. Please refer to the ordering information section or www.neb.com for more information.

1. Prepare 40 mM cap analog. Cap analog (ARCA, NEB #S1411) is supplied in a lyophilized form of 1 µmol per tube. Dissolving it in 25 µl nuclease-free water will yield a concentration of 40 mM.

2. Thaw the necessary kit components, mix and pulse-spin in a microfuge to collect solutions to the bottoms of tubes.

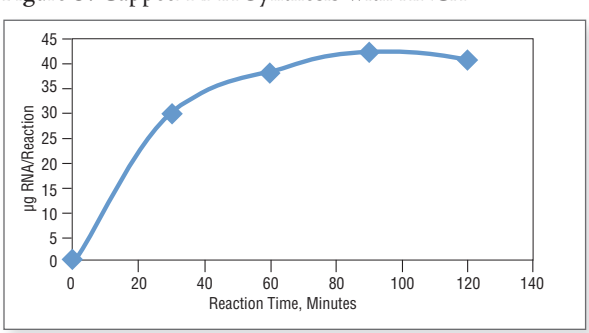
3. Assemble the reaction at room temperature in the following order:

Nuclease-free water	X µl	
NTP Buffer Mix	2 µl	2 mM each NTP final
Cap Analog (40 mM)	4 µl	8 mM final
Template DNA	X µl	1 µg
T7 RNA Polymerase Mix	2 µl	
Total reaction volume	20 µl	

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

The yield per reaction is 30–40 µg RNA with approximately 80% capped RNA transcripts. Figure 3 shows the time course of capped RNA synthesis from 1 µg control template. Most reactions will be complete in 1 hour.

Figure 3. Capped RNA Synthesis with ARCA



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

5. *Optional:* To remove template DNA, add 2 µl of DNase I (RNase-free), mix and incubate at 37°C for 15 minutes.
6. Proceed with purification of synthesized RNA or analysis of transcription products by gel electrophoresis.

RNA Synthesis with Modified Nucleotides

The kit is capable of synthesizing biotin- or dye-modified RNA with the following protocol. The recommended molar ratio of modified NTP (Biotin-, Fluorescein-, Digoxigenin-, or Aminoallyl-NTP) to standard NTP is 1:2. The following reaction set-up assumes modified UTP is used. Please note that Dye- or Biotin-NTPs are not supplied with the kit.

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

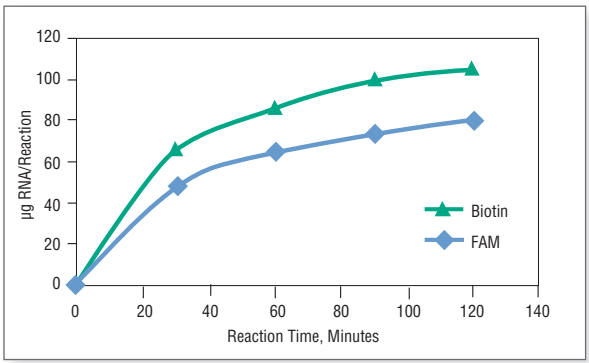
Nuclease-free water	X µl	
NTP Buffer Mix	5 µl	5 mM each NTP final
Modified UTP (10 mM)	5 µl	2.5 mM final
Template DNA	X µl	1 µg
T7 RNA Polymerase Mix	2 µl	
Total reaction volume	20 µl	

3. Mix thoroughly, pulse-spin and incubate at 37°C for 2 hours. For short (< 300 nt) transcripts incubate at 37°C for 4–16 hours.

   Note that the ratio of modified nucleotide to standard nucleotide can be adjusted by varying the amount of the NTP Buffer Mix and modified nucleotide. For complete modified nucleotide substitution we recommended using the T7 High Yield RNA Synthesis Kit (NEB #E2040), in which the four nucleotides are supplied separately. Figure 4 shows the time course of labeled RNA synthesis using 1 µg control template with Biotin-16-UTP and Fluorescein-12-UTP following the above reaction setup.

   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 an insignificant effect on yields, while lower yields can be expected for transcription reactions containing Fluorescein-NTP or Cy-NTP. In addition, transcripts containing modified ribonucleotides have reduced electrophoretic mobility due to higher molecular weight.

Figure 4. RNA synthesis with modified nucleotides



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

4. *Optional:* To remove template DNA, add 30 µl nuclease-free water and 2 µl of DNase I (RNase-free), mix and incubate at 37°C for 15 minutes
5. Proceed with purification of synthesized RNA or analysis of transcription products by gel electrophoresis.

Purification of Synthesized RNA:

Synthesized RNA 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.

LiCl Precipitation

The kit includes LiCl solution for quick recovery of the synthesized RNA. 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 RNA is suitable for cap addition with NEB's Vaccinia Capping System (NEB #M2080) and Poly(A) tailing with NEB's Poly(A) Polymerase (NEB #M0276).

1. Adjust the reaction volume to 50 µl by adding nuclease-free water.
2. 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 and rinse the pellet with 500 µl of ice cold 70% ethanol.
6. Resuspend the RNA in 50 µl of 0.1 mM EDTA. 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 to a new tube.
3. Precipitate the RNA by adding 2 volumes of ethanol. Incubate at –20°C for at least 30 minutes and collect the pellet by centrifugation.
4. Remove the supernatant and rinse the pellet with 500 µl of ice cold 70% ethanol.
5. Resuspend the RNA in 50 µl 0.1 mM EDTA. Store the RNA at –20°C or below.

Spin Column Purification

Spin columns will remove unincorporated nucleotides, proteins and salts. Adjust the volume of the reaction mixture to 100 µl by adding nuclease-free water and mix well. Purify the RNA by following the spin column manufacturer's instructions. Each reaction produces up to 200 µg of RNA, which may exceed column capacity, thus requiring additional columns.

Gel Purification

When high purity RNA transcript is desired, we recommend gel purification of the transcription product.

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. A 1:200 dilution of a sample of the purified RNA should give an absorbance reading in the linear range of a spectrophotometer. RNA dilution may not be necessary if using a NanoDrop Spectrophotometer. A NanoDrop Spectrophotometer can 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 of non-radiolabeled RNA

   a. Mix 0.2–1 µg RNA sample with an equal volume of RNA Loading Dye (2X, NEB #B0363).

   b. Denature the RNA sample and an aliquot of RNA marker by heating at 65–70°C for 5–10 minutes.

   c. Pulse-spin prior to loading onto gel.

   d. Visualizing RNA by staining the gel with SYBR® Gold or ethidium bromide.

Troubleshooting:

Control Reaction

The FLuc control template DNA is a linearized plasmid containing the firefly luciferase gene under the transcriptional control of the T7 promoter. The size of the runoff transcript is 1.8 kb. The control reaction should yield ≥ 150 µg RNA transcript in 2 hours.

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

The control plasmid sequence can be found at www.neb.com. The FLuc control template is generated by linearizing the plasmid with StuI.

Low Yield of Full-length RNA

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

Low Yield of Short Transcript

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

RNA Transcript Smearing on Denaturing Gel

If the RNA appears degraded (e.g., smeared) on denaturing agarose or polyacrylamide gel, 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). We recommend evaluating the plasmid DNA template with the RNase Contamination Assay Kit (NEB #E3320) before plasmid DNA template is used in the HiScribe T7 Quick High Yield RNA Synthesis Kit. 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 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.

Ordering Information

PRODUCT	NEB #	SIZE
HiScribe T7 Quick High Yield RNA Synthesis Kit	E2050S	50 reactions
COMPANION PRODUCTS		
RNA Loading Dye (2X)	B0363S	4 x 1 ml
RNase Inhibitor, Human Placenta	M0307S/L	2,000/10,000 units
RNase Inhibitor, Murine	M0314S/L	3,000/15,000 units
DNase I (RNase-Free)	M0303S/L	1,000/5,000 units
Q5 Hot Start High-Fidelity DNA Polymerase	M0493S/L	100/500 units
ssRNA Ladder	N0362S	25 gel lanes
Low Range ssRNA Ladder	N0364S	25 gel lanes
3´-O-Me-m^{7}G(5´)ppp(5´)G RNA Cap Structure Analog	S1411S/L	1/5 µmol
m^{7}G(5´)ppp(5´)A RNA Cap Structure Analog	S1405S/L	1/5 µmol
G(5´)ppp(5´)A RNA Cap Structure Analog	S1406S/L	1/5 µmol
G(5´)ppp(5´)G RNA Cap Structure Analog	S1407S/L	1/5 µmol
m7G(5´)ppp(5´)G RNA Cap Structure Analog	S1404S/L	1/5 µmol
RNase Contamination Assay Kit	E3320S	50 reactions
Vaccinia Capping System	M2080S	400 units
mRNA Cap 2´-O-Methyltransferase	M0366S	2,000 units
E. coli Poly(A) Polymerase	M0276S/L	100/500 units
Ribonucleotide Solution Mix	N0466S/L	10/50 µmol of each
Ribonucleotide Solution Set	N0450S/L	10/50 µmol of each

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