HiScribe™ T7 mRNA Kit with CleanCap® Reagent AG

NEB #E2080S

20 reactions

Version 2.0_2/22

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The HiScribe T7 mRNA Kit with CleanCap Reagent AG Includes:

All kit components should be stored at –20°C. Each kit contains sufficient reagents for 20 x 20 µl reactions. Each standard reaction yields ≥ 90 µg of Cap-1 unmodified RNA from 1 µg control template.

T7 RNA Polymerase Mix

T7 CleanCap Reagent AG Reaction Buffer (10X)

ATP (60 mM)

GTP (50 mM)

UTP (50 mM)

CTP (50 mM)

CleanCap Reagent AG (40 mM)

CLuc AG Control Template (0.25 mg/ml)

DNaseI (RNase-free) (2,000 units/ml)

LiCl Solution (7.5 M LiCl, 10 mM EDTA)

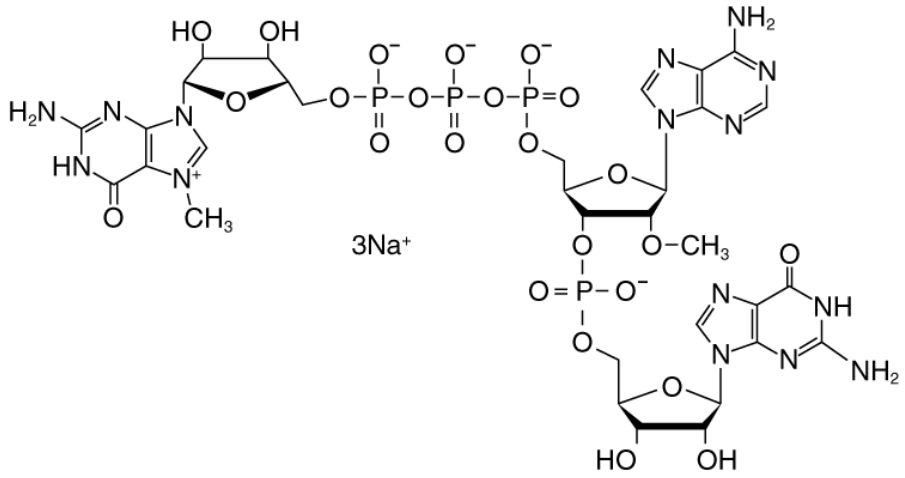
Required Materials Not Included:

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, followed by an AG initiation sequence
Modified-NTP:	Biotin-, Fluorescein-, Digoxigenin-, Aminoallyl-, or Pseudouridine-5–Triphosphate, etc.
General:	Thermal cycler, microcentrifuge, nuclease-free water, nuclease-free tubes and tips
Purification:	Phenol, chloroform, ethanol and 3 M sodium acetate, pH 5.2 or Ammonium Acetate, Monarch® RNA Cleanup Kit (500 µg; NEB #T2050), equipment and reagents for RNA quantitation
Gel Analysis:	Gels, running buffers, loading dye, nucleic acid ladders, gel apparatus, power supply

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 for efficient translation. The HiScribe T7 mRNA kit with CleanCap Reagent AG (Figure 1) utilizes an optimized RNA synthesis formulation and trinucleotide cap analog technology for co-transcriptionally capping mRNAs that contain a natural Cap-1 structure in a single simplified reaction without compromising RNA yield. 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.

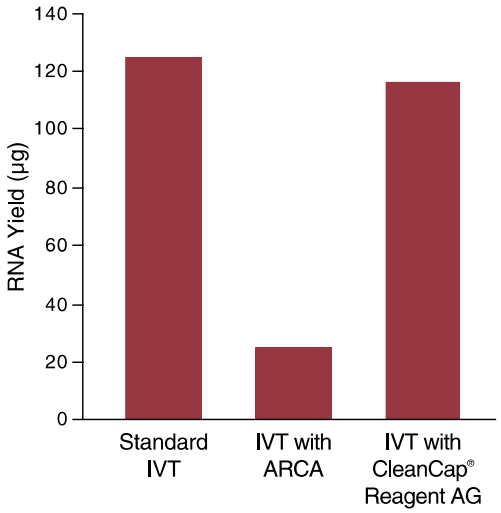
Figure 1. Molecular structure of CleanCap Reagent AG.



The HiScribe T7 mRNA kit with CleanCap Reagent AG is formatted with individual vials of NTPs and CleanCap Reagent AG to allow for partial or complete substitution of modified NTPs. By using a DNA template encoding a poly(A) tail, this kit can be used to generate capped and tailed mRNAs. Cap-1 mRNA synthesized from this kit is suitable for many applications including transfections, microinjections, *in vitro* translation, preclinical mRNA therapeutic mRNA studies as well as RNA structure and function analysis. Unlike *in vitro* transcription reactions that utilize Anti-Reverse Cap Analog (ARCA) for co-transcriptional capping, where expected

yields are significantly decreased (due to lower concentration of GTP to accommodate ARCA incorporation), the HiScribe T7 mRNA kit with CleanCap Reagent AG results in no loss of yield when compared to a standard synthesis reaction where no cap analog is present.

Figure 2. Comparison of RNA Yields from *In Vitro* Transcription Reactions with no cap analog, ARCA, or CleanCap Reagent AG.



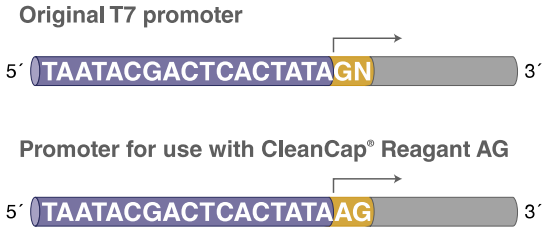
All reactions were performed with 5 mM CTP, 5 mM UTP and 6 mM ATP. Standard IVT reactions included 5 mM GTP and no cap analog. ARCA reactions contained a 4:1 ratio of ARCA:GTP (4 mM:1 mM). IVT with CleanCap Reagent AG contained 5 mM GTP and 4 mM CleanCap Reagent AG and was performed as described below (Standard mRNA Synthesis). Reactions were incubated for 2 hours at 37°C, purified and quantified by NanoDrop®.

This kit contains sufficient reagents for 20 reactions of 20 µl each, and includes DNase I (RNase-free) and Lithium Chloride Solution for template removal and quick mRNA purification. Each standard reaction yields ≥ 90 µg of RNA from 1 µg CLuc AG Control Template DNA. Each kit can yield ≥ 1.8 mg RNA.

DNA Template Preparation

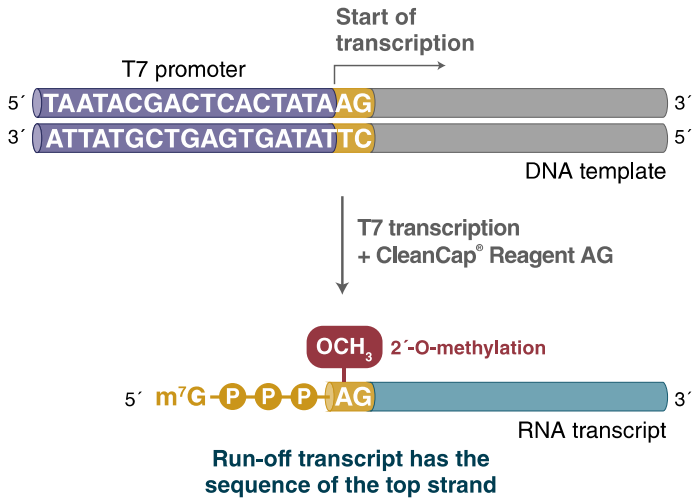
Linearized plasmid DNA, PCR products or synthetic DNA oligonucleotides can be used as templates for *in vitro* transcription with the HiScribe T7 mRNA Kit with CleanCap Reagent AG. These sequences must contain a double-stranded T7 promoter region (5´ TAATACGACTCACTATA 3´), followed by an AG initiation sequence, upstream of the sequence to be transcribed (Figure 3).

Figure 3. T7 promoter sequence and changes to initiating bases for compatibility with CleanCap Reagent AG.



The minimal T7 promoter sequence (5´ TAATACGACTCACTATA 3´), plus AG initiation sequence, as well as the run-off transcript after T7 transcription is illustrated in Figure 4. The +1 A and +2 G are required for co-transcriptional capping using CleanCap Reagent AG.

Figure 4. Co-transcriptional Capping with T7 RNA Polymerase and CleanCap Reagent AG.



Plasmid Templates

Completely linearized plasmid templates of the highest purity are critical for the successful use of the HiScribe T7 mRNA Kit with CleanCap AG Reagent. The quality of the template DNA affects the yield and integrity of the RNA as the highest yield is achieved with the highest purity template.

Plasmids purified by many laboratory methods can be successfully used, provided the preparations contain mostly supercoiled DNA and are free from contaminating RNases, protein, RNA and salts.

Plasmids normally used for *in vitro* transcription with T7 RNA Polymerase using standard NTPs or cap analogs will need to be modified to contain the +1 A and +2 G just downstream of the promoter. We recommend using the Q5® Site-Directed Mutagenesis Kit (NEB #E0554) to create the site-specific mutation(s).

To produce RNA transcripts of 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 due to 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.

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 as 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. Wash 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. Verify concentration using standard nucleic acid quantitation methods.

PCR Templates

PCR products containing the T7 RNA Polymerase promoter with an AG initiation sequence in the correct orientation can be transcribed with the HiScribe T7 mRNA Kit with CleanCap AG Reagent. Though the PCR products can be used directly in a synthesis reaction, better yields can be obtained with purified PCR products (Monarch PCR & DNA Cleanup Kit, NEB #T1030). Templates should be examined on an agarose gel to confirm amplicon size. In general, 0.1–0.5 µg of PCR template can be used in a 20 µl *in vitro* transcription reaction.

Synthetic DNA Oligonucleotides

Synthetic DNA oligos, which are annealed to form entirely double-stranded DNA, can be used with the HiScribe T7 mRNA Kit with CleanCap AG Reagent. Sequence and purity of the oligos may affect the yield of the *in vitro* transcription reactions.

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 mRNA Synthesis

1. Thaw the necessary kit components to room temperature (Reaction buffer, NTPs, CleanCap Reagent AG and Template DNA), mix and pulse-spin to collect solutions to the bottom of the tubes. Keep the Enzyme Mix on ice.
2. If several reactions will be performed, a master mix of Reaction Buffer, NTPs and CleanCap can be prepared according to the volumes per reaction below. Use 12 µl of the Master Mix per reaction
3. Assemble the reaction at room temperature in the following order:

REAGENT	AMOUNT	FINAL CONCENTRATION
Nuclease-free water	X µl	
10X T7 CleanCap Reagent AG Reaction Buffer	2 µl	
ATP (60 mM)	2 µl	6 mM final
GTP (50 mM)	2 µl	5 mM final
UTP (50 mM)	2 µl	5 mM final
CTP (50 mM)	2 µl	5 mM final
Cap Analog (40 mM)	2 µl	4 mM final
Template DNA	X µl	1 µg
T7 RNA Polymerase Mix	2 µl	
Total Reaction Volume	20 µl	

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

   We recommend using a dry air incubator or PCR instrument set to 37°C (with a heated lid) to prevent evaporation. For reactions with transcripts < 0.3 kb incubations up to 16 hours or overnight can be performed.
5. *Optional:* Add 2 µl of DNase I, mix well and incubate at 37°C for 15 minutes.
6. Proceed with mRNA purification (See below).

mRNA Synthesis with Modified Nucleotides

mRNAs containing modified nucleotides (such as 5mCTP and Pseudo-UTP) have been shown to suppress RNA-mediated innate immune activation *in vivo*. The HiScribe T7 mRNA Kit with CleanCap Reagent AG is formulated to allow for the complete substitution of unlabeled NTPs with a modified version of the NTP. The inclusion of most modified NTPs (not included) in the *in vitro* transcription reaction will not affect final RNA yield; however some modified NTPs may impact transcription efficiency. If trying a modified NTP for the first time, we suggest a partial substitution at a molar ratio of 1:3 or 1:2, modified standard NTP.

The reaction conditions in the example below demonstrate a complete substitution of Pseudo-UTP for UTP. If another modified NTP is being used, swap in the modified version for the unmodified version (partial to full substitution) in the final reaction as the stock concentration of modified NTPs may not be the same as the unmodified version provided in the kit.

1. Thaw the necessary kit components to room temperature (Reaction buffer, NTPs, modified NTP, CleanCap Reagent AG and Template DNA), mix and pulse-spin to collect solutions to the bottom of the tubes. Keep the Enzyme Mix on ice.
2. If several reactions will be performed, a master mix of Reaction Buffer, NTPs and CleanCap can be prepared according to the volumes per reaction below. Use 12 µl of the Master Mix per reaction.

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

REAGENT	AMOUNT	FINAL CONCENTRATION
Nuclease-free water	X µl	
10X T7 CleanCap Reagent AG Reaction	2 µl	
ATP (60 mM)	2 µl	6 mM final
Pseudo-UTP (50 mM)	2 µl	5 mM final
GTP (50 mM)	2 µl	5 mM final
CTP (50 mM)	2 µl	5 mM final
Cap Analog (40 mM)	2 µl	4 mM final
Template DNA	X µl	1 µg
T7 RNA Polymerase Mix	2 µl	
Total Reaction Volume	20 µl	

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

   We recommend using a dry air incubator or PCR instrument set to 37°C (with a heated lid) to prevent evaporation. For reactions with transcripts < 0.3 kb incubations up to 16 hours or overnight can be performed.

5. *Optional:* Add 2 µl of DNase I, mix well and incubate at 37°C for 15 minutes.

6. Proceed with mRNA purification (See below).

mRNA Purification

Synthesized RNA can be purified by LiCl precipitation, phenol:chloform extraction followed by ethanol precipitation, or by using a spin column-based method.

LiCl Precipitation

This kit includes LiCl Solution for quick recovery of synthesized mRNA. LiCl precipitation of RNA is effective at removing the majority of unincorporated NTPs and enzymes. However, LiCl precipitation is not effective at isolating RNAs shorter than 300 bases or at concentration lower than 0.1 mg/ml, therefore alternative methods such as those listed below would be more appropriate. mRNA purified by the LiCl-based method is suitable for transfections/electroporations or microinjections.

1. Adjust volume to 50 µl by adding nuclease-free water to the transcription reaction. Add 25 µl of the LiCl Solution, mix well.
2. Incubate at -20°C for a minimum of 30 minutes.
3. Centrifuge at 4°C for 15 minutes at top speed to pellet the RNA.
4. Carefully remove the supernatant.
5. Rinse the pellet by adding 500 µl of cold 70% ethanol and centrifuge at 4°C for 10 minutes.
6. Carefully remove the ethanol. Spin the tube briefly to collect the remaining ethanol to the bottom of the tube.
7. Carefully remove the residual ethanol by using a thin, sharp pipet tip.
8. Air dry the pellet and resuspend in 100 µl 0.1 mM EDTA, RNA storage solution or nuclease-free water.
9. Store the RNA at -20°C or below.

Phenol:Chloroform Extraction and Ethanol Precipitation

For removal of proteins and most of the free nucleotides and CleanCap Reagent AG, phenol: chloroform extraction followed by ethanol precipitation of the RNA transcripts can be performed as follows:

1. Adjust the reaction volumes 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 alone. Collect the aqueous phase and transfer to a new 1.5 ml tube.
3. Precipitate the RNA by adding 2 volumes of ethanol, mix well. Incubate at –20°C for at least 30 minutes and collect the pellet by centrifugation.

4. Carefully remove the supernatant.
5. Rinse the pellet by adding 500 µl of cold 70% ethanol and centrifuge at 4°C for 10 minutes.
6. Remove the residual liquid using a thin, sharp pipet tip.
8. Air dry the pellet and resuspend the RNA in 100 µl of nuclease-free water, 0.1 M EDTA or a suitable RNA storage solution.

Spin Column Purification

Spin columns (we recommend Monarch RNA Cleanup Kit, NEB #T2050 (500 µg capacity) will remove unincorporated nucleotides, proteins and salts. Extra column washes or extra spins following the wash steps may help minimize carryover of ethanol. Make sure to check the binding capacity of the spin columns as RNA yields can be > 90 µg from a 20 µl reaction.

Evaluation of Reaction Products

There are several methods described below to determine the quality and quantity of RNA synthesized using the HiScribe T7 mRNA Kit with CleanCap Reagent AG.

Quantification of RNA Abundance by UV Light Absorbance

RNA concentration can be determined by measuring the ultraviolet (UV) light absorbance at 260 nm, however it is important to keep in mind that any unincorporated NTPs, CleanCap Reagent AG and DNA template present will affect the reading as all can absorb at 260 nm. Free nucleotides and CleanCap Reagent AG leftover from the transcription reaction must be removed before the RNA concentration can be reliably determined (see purification methods above). RNA solution can be read directly on a NanoDrop Spectrophotometer. For single-stranded RNA, 1 A_{260} is equivalent to an RNA concentration of 40 µg/ml and 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 or purified RNA can be run on an appropriate denaturing agarose or polyacrylamide gel. It is necessary to run RNAs under 0.3 kb on denaturing polyacrylamide gels, whereas RNAs > 0.3 kb can be run on denaturing agarose gels. All gels should be run under denaturing conditions to minimize secondary structure formation.

1. Preparation of denaturing gels
    a. Denaturing agarose gels:
    To make a 100 ml 1% denaturing agarose gel, add 1 gram of agarose powder to 72 ml nuclease-free water. Melt the agarose and add 10 ml 10X MOPS buffer. Next, 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

    Follow your institution's recommendations for disposal of formaldehyde waste.

    b. Denaturing PAGE/Urea Gels:
    5–15% PAGE/Urea commercially available gels are recommended depending on the size of the RNA.
    10X TBE buffer: 0.9 M Tris Base, 0.9 M Boric Acid, 20 mM EDTA.

2. Gel electrophoresis
    a. Mix 0.05–1 µg RNA sample with 5–10 µl 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 the RNA sample prior to loading onto gel.
    d. Visualize the RNA by staining with SYBR® Gold or ethidium bromide.

mRNA Quality Analysis by BioAnalyzer or Capillary Electrophoresis

RNA quality can also be assessed by Capillary Electrophoresis, BioAnalyzer or comparable technologies. Please refer to the manufacturers' protocols.

Troubleshooting

Verify that the sequence following the T7 promoter contains the AG initiating sequence.

Control Reaction

The CLuc AG control template is a linearized plasmid containing the *Cypridina lucifierase* gene under the transcriptional control of the T7 promoter. The initiating sequence has been changed to an AG by site-directed mutagenesis to be compatible with CleanCap Reagent AG. The size of the run-off transcript is ~1.76 kb. The control reaction, following the standard reaction protocol, should yield > 90 µg of RNA in 2 hours at 37°C.

If the control reaction is not working, there may be technical issues with the reaction set up. Repeat the reaction following the protocol exactly (thawing specified reagents to room temperature, setting the reaction up at room temperature, and adding the components in the exact order listed in the manual. Take every precaution to avoid RNase contamination.

The control plasmid sequence can be found at www.neb.com. The CLuc AG control template is generated by linearizing the plasmid with the restriction enzyme XbaI.

Low Yield of Full-length RNA

If the transcription reaction generates full-length RNA but yields are significantly lower than expected, it is possible that contaminants in the DNA template are inhibiting the RNA Polymerase or the DNA template concentration may be incorrect or too low. Additional purification of the 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 (< 300 nts) 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 DNA can help achieve maximum yield.

RNA Transcript Smearing on Denaturing Gel

If the RNA appears degraded (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 (smear below the expected RNA length). If the DNA template is contaminated with RNase, we recommend performing phenol:chloroform extraction followed by ethanol precipitation (see template DNA preparation section) and dissolving the DNA in nuclease-free water.

RNA Transcript of Larger Size than Expected

If the RNA transcript appears larger than the expected size on a denaturing gel when compared to a single-stranded RNA ladder, the plasmid DNA that is used for the template may not be completely digested. Even if small amounts of undigested circular plasmid DNA is present, T7 RNA Polymerase can produce large amounts of long transcripts. Check the digestion of the plasmid for complete digestion compared to a sample of undigested plasmid. If undigested plasmid is present repeat the restriction digest.

Alternatively, larger sized bands may be observed when the RNA is not completely denatured due to the presence of strong secondary structure.

RNA Transcript of Smaller Size than Expected

If denaturing gel analysis indicates the presence of smaller bands than expected it is most likely due to premature termination by T7 RNA Polymerase. Sequences that resemble T7 RNA Polymerase termination signals will cause premature termination. For GC-rich templates, or templates with known strong secondary structure, incubation at 42°C may improve the yield of full-length transcript.

Ordering Information

NEB #	PRODUCT	SIZE
E2080S	HiScribe T7 m RNA Kit with CleanCap Reagent AG	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
E2060S	HiScribe T7 ARCA mRNA Kit (with tailing)	20 reactions

B0363S	RNA Loading Dye (2X)	4 x 1 ml
M0307S/L	RNase Inhibitor, Human Placenta	2,000/10,000 units
M0314S/L	RNase Inhibitor, Murine	3,000/15,000 units
M0303S/L	DNase I (RNase-free)	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 reactions
N0362S	ssRNA Ladder	25 gel lanes
N0364S	Low Range ssRNA Ladder	25 gel lanes
E0554S	Q5 Site-Directed Mutagenesis Kit	10 reactions
E0552S	Q5 Site-Directed Mutagenesis Kit (Without Competent Cells)	10 reactions
T1030S/L	Monarch PCR & DNA Cleanup Kit (5 µg)	50/250 preps
T2040S	Monarch RNA Cleanup Kit (50 µg)	10/100 preps
T2050S	Monarch RNA Cleanup Kit (500 µg)	10/100 preps
C2987H/I	NEB 5-alpha Competent *E. coli* (High Efficiency)	20 x 0.05 ml/ 6 x 0.2 ml
M0276S/L	*E. coli* Poly(A) Polymerase	100/500 units
B9020S	SOC Outgrowth Medium	100 ml
T1010S/L	Monarch Plasmid Miniprep Kit	50/250 preps

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	10/21
2.0	Change > 0.3 kb incubations in Step 4 on page 5 and 6 to < 0.3 kb incubations.	2/22

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