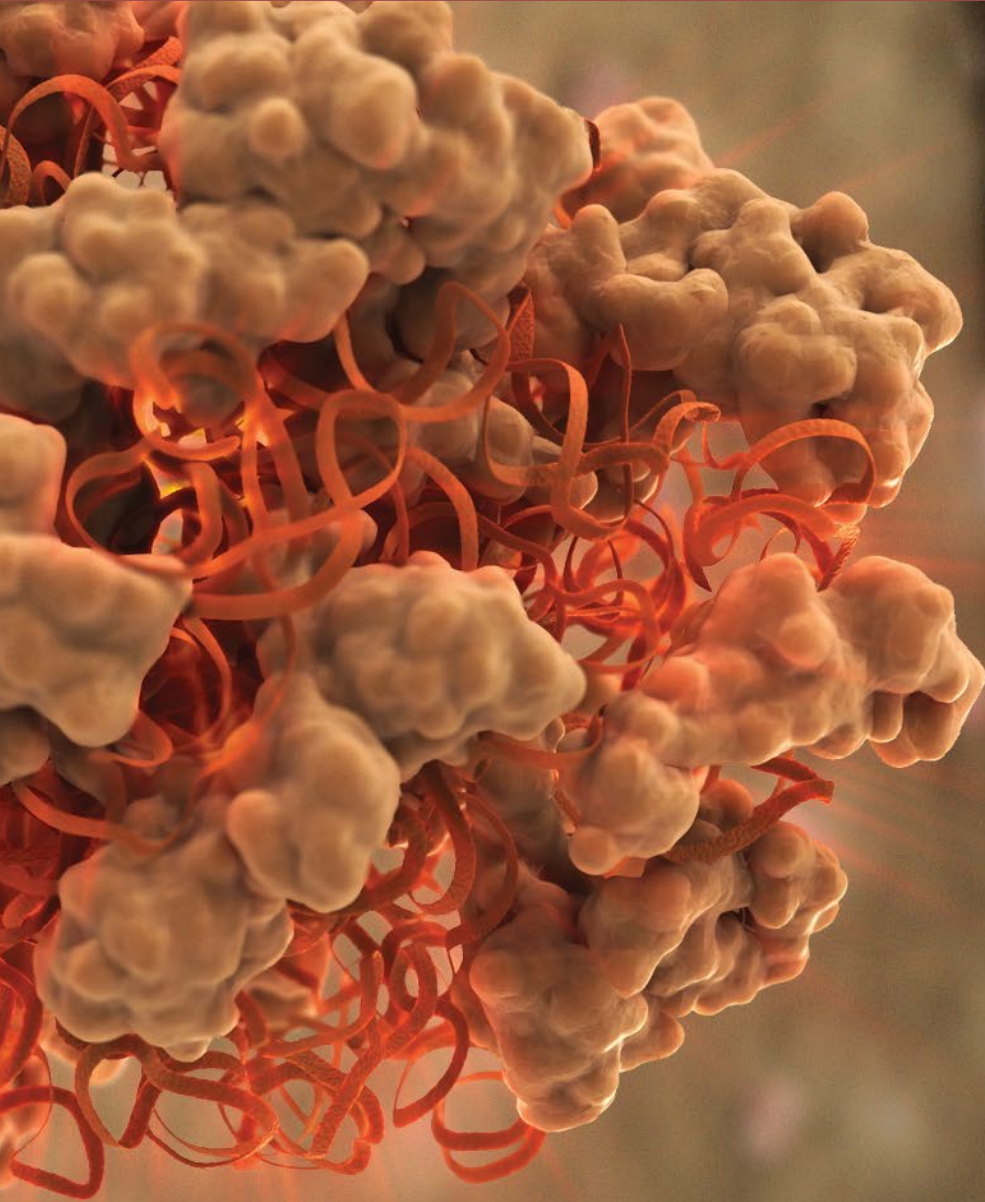




Now includes  
Faustovirus Capping Enzyme

# RNA Synthesis

FROM TEMPLATE TO TRANSCRIPT



NEW ENGLAND  
**BioLabs**<sup>®</sup> Inc.

be INSPIRED  
drive DISCOVERY  
stay GENUINE

# RNA Synthesis:

## Tools to Take You from Template to Transcript

For almost 50 years, NEB® has been a world leader in the discovery and production of reagents for the life science industry. New England Biolabs' enzymology expertise allows us to supply reagents for the synthesis of high-quality RNA – from template generation and transcription, to capping, tailing, and cleanup after synthesis. These products are designed and manufactured by building upon decades of molecular biology experience, so that you can be confident they will work for your application.

*In vitro* synthesis of single-stranded RNA molecules is a widely used laboratory procedure that is critical to RNA research, as well as to RNA biopharmaceuticals. This technique is versatile in that it allows the researcher to tailor synthesis and introduce modifications to produce a transcript. Downstream applications include biochemical and molecular characterization of RNA for RNA-protein interactions and structural analyses, generation of RNA aptamers, synthesis of functional mRNAs for expression, and generation of small RNAs for alteration of gene expression (e.g., guide RNAs, RNAi). Furthermore, the use of *in vitro* synthesized RNA has been instrumental in the development of RNA vaccines and CRISPR/Cas9 genome editing tools, generation of pluripotent stem cells, screening of RNA inhibitors, as well as development of RNA amplification-based diagnostics.

High-yield robust reactions require optimization of each reaction component. NEB offers five *in vitro* RNA synthesis kits, all of which have been optimized to generate reproducible yields of quality RNA. Additionally, individual components can be purchased for *in vitro* transcription (IVT) and mRNA capping.

NEB's portfolio of research-grade and GMP-grade\* reagents enables bench-scale to commercial-scale mRNA manufacturing. Our optimized HiScribe kits enable convenient *in vitro* transcription (IVT) workflows. When it is time to scale up and optimize reaction components, our standalone reagents are readily available in formats matching our GMP-grade offering, enabling a seamless transition to large-scale therapeutic mRNA manufacturing.



Learn more about products for your RNA-related workflow, including mRNA synthesis at [NEBrna.com](https://www.neb.com/NEBrna.com)

\* "GMP-grade" is a branding term NEB uses to describe reagents manufactured or finished at our Rowley, MA facility, where we utilize procedures and process controls to manufacture reagents under more rigorous conditions to achieve more stringent product specifications, and in compliance with ISO 9001 and ISO 13485 quality management system standards. NEB does not manufacture or sell products known as Active Pharmaceutical Ingredients (APIs), nor do we manufacture products in compliance with all of the Current Good Manufacturing Practice regulations.

### mRNA synthesis workflow example & available NEB products

TEMPLATE GENERATION	IN VITRO TRANSCRIPTION	RNA CAPPING	POLY(A) TAILING	RNA PURIFICATION	
Q5® High-Fidelity DNA Polymerase	HiScribe® T7 mRNA Kit with CleanCap® Reagent AG		<i>E. coli</i> Poly(A) Polymerase	Monarch® RNA Cleanup Kit (10 µg)	
	HiScribe T7 ARCA mRNA Synthesis Kit (with tailing)				
dNTP solution mixes	HiScribe T7 ARCA mRNA Synthesis Kit			Monarch RNA Cleanup Kit (50 µg)	
BspQI*	HiScribe T7 High Yield RNA Synthesis Components	Faustovirus Capping Enzyme <small>COMING SOON</small>		Monarch RNA Cleanup Kit (500 µg)	
DNA Assembly • NEBuilder HiFi DNA Assembly • Golden Gate Assembly	HiScribe T7 Quick High Yield RNA Synthesis Kit	Vaccinia Capping System		Lithium Chloride	
	HiScribe SP6 High Yield RNA Synthesis Kit	mRNA Cap 2'-O-Methyltransferase			
	T3 & SP6 RNA Polymerases	ARCA and other mRNA cap analogs			
	T7 RNA Polymerase				
	Hi-T7 RNA Polymerase				
	<b>Companion Products</b>				<b>Companion Products</b>
	RNase inhibitor (Murine)				Monarch RNA Cleanup Binding Buffer
	RNase Inhibitor (Human Placental)				Monarch RNA Cleanup Wash Buffer
	Pyrophosphatase, Inorganic ( <i>E. coli</i> )				Nuclease-free Water
	Pyrophosphatase, Inorganic (Yeast)				
	DNase I (RNase-free)				
	NTPs				

= available in GMP-grade

\* NEB can offer large-scale preparations of restriction enzymes using Recombinant Albumin (BSA-free)





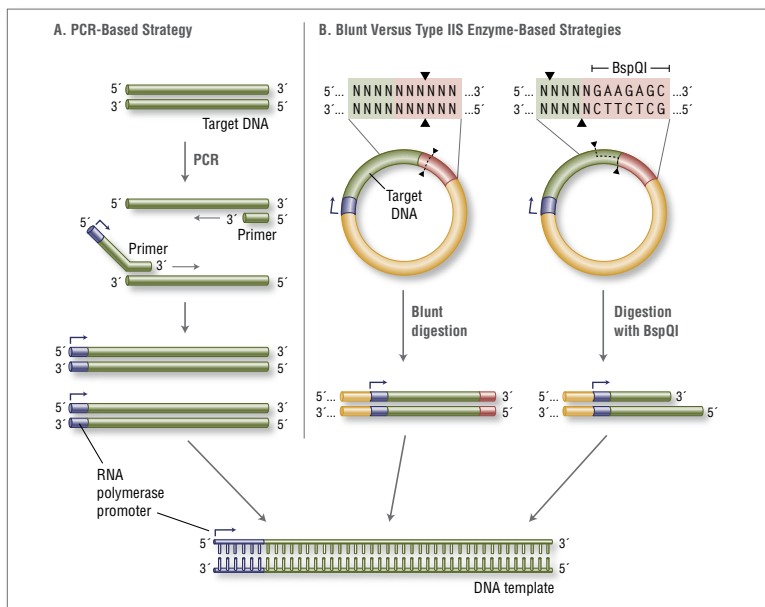
# Template Generation

Effective *in vitro* transcription starts with high-quality template. The quality of the template DNA affects transcription efficiency, as well as the integrity of the synthesized RNA. Yield is highly dependent upon template purity. Any purification method may be used, as long as the product is free of contaminating RNase, protein and salts.

Whether your template is linearized plasmid or PCR-amplified DNA, NEB can supply the reagents for high-quality template generation that help you maximize RNA synthesis yields.

- Ensure that your template is error-free by choosing a robust, high-fidelity DNA polymerase for amplification, such as Q5® High-Fidelity DNA Polymerase (NEB #M0491). Reduce non-specific amplification by choosing a hot start formulation, such as Q5 Hot Start High-Fidelity DNA Polymerase (NEB #M0493). PCR product should be purified and analyzed to estimate concentration, and to confirm amplicon size prior to its use as template in the transcription reaction.
- When using plasmid DNA, it is important to completely linearize the input DNA downstream of the insert with an appropriate restriction enzyme, such as SapI (NEB #R0569) or BspQI (NEB #R0712). The use of type IIS enzymes generates scarless templates, as shown in the figure below. This ensures that the synthesis reaction produces an RNA transcript of defined length, and may help prevent ‘template switching’ products. NEB offers a number of suitable restriction enzymes for this purpose.
- If you need to introduce changes to your template, the Q5 Site-Directed Mutagenesis Kit (NEB #E0554) enables rapid, site-specific mutagenesis of double-stranded plasmid DNA in less than 2 hours.

Figure 1. Transcription Template Generation



(A) PCR can be used to amplify target DNA prior to transcription. A polymerase promoter can be introduced via the upstream primer. Choose a high-fidelity polymerase to ensure that your template is error free.

(B) When using plasmid DNA as a template, linearize with an enzyme that produces blunt or 5'-overhanging ends. Using a type IIS restriction enzyme (e.g., BspQI) allows RNA synthesis with no additional 3'-nucleotide sequence from the restriction site.

## RECOMMENDED PRODUCTS

### NEB Restriction Enzymes

- Visit [NEBRestrictionEnzymes.com](http://NEBRestrictionEnzymes.com) for the full list

### Q5 and Q5 Hot Start High-Fidelity DNA Polymerases (NEB #M0491 & #M0493)

- ~ 280X fidelity of *Taq* DNA Polymerase
- High specificity and robust yields with minimal optimization
- Superior performance for a broad range of amplicons (from high AT to high GC)
- Hot start formulation allows room temperature reaction setup and reduces non-specific amplification

### Monarch PCR & DNA Cleanup Kit (5 µg) (NEB #T1030)

- Fast and simple purification in 5 minutes
- Highly pure DNA eluted in as little as 6 µl

### Q5 Site-Directed Mutagenesis Kit (NEB #E0554)

- Rapid site-specific mutagenesis of double-stranded plasmid DNA in less than 2 hours

## TOOLS & RESOURCES

Visit [NEBPCRPolymerases.com](http://NEBPCRPolymerases.com) & [NEBRestrictionEnzymes.com](http://NEBRestrictionEnzymes.com) to find:

- FAQs and troubleshooting
- Interactive tools to help with experimental design
- Online tutorials for setting up a PCR or RE reaction



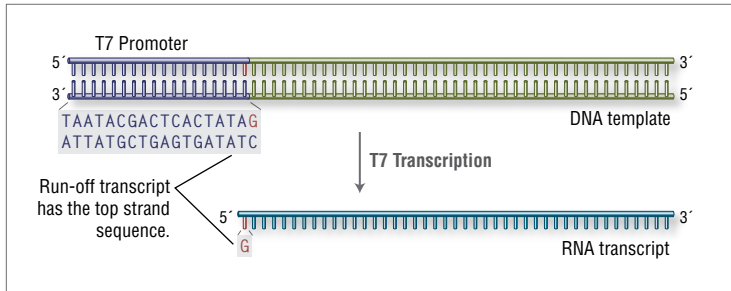
LEARN HOW TO AMPLIFY GC-RICH DNA



# In vitro Transcription

*In vitro* RNA synthesis requires DNA template, enzymes, nucleotides and buffer components. High-yield robust reactions require optimization of each reaction component. NEB offers six *in vitro* RNA synthesis kits, all of which have been rigorously formulated to provide reproducible high yields of quality RNA.

Figure 2. T7 Transcription

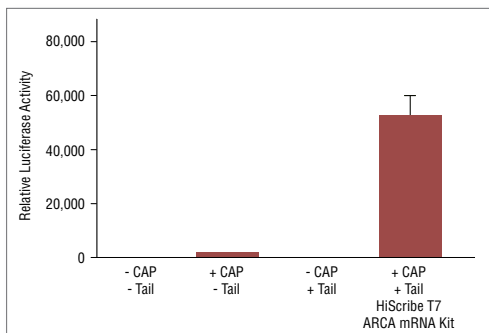


A transcription template contains a T7 promoter sequence followed by the sequence of interest. The T7 promoter is required for transcription to occur. The sequence of the transcript is the same as the top strand of the DNA template and initiates from G residues encoded in the optimal T7 RNA polymerase promoter.

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 mRNA Kit with CleanCap Reagent AG (NEB #E2080) provides the highest yields of mRNA with a natural Cap-1 structure for a variety of applications. Tailing can be accomplished co-transcriptionally by using a DNA template encoding a poly(A) stretch or enzymatically using *E. coli* Poly(A) Polymerase (NEB #M0276). Additionally, The HiScribe T7 ARCA mRNA Synthesis Kit with Tailing (NEB #E2060) is available for co-transcriptional incorporation of Anti-Reverse Cap Analog, ARCA, using T7 RNA Polymerase resulting in a Cap-0 structure. A poly(A) tail is then added by *E. coli* Poly(A) Polymerase. A separate version of the kit (NEB #E2065), without *E. coli* Poly(A) Polymerase, is available. These kits also include DNase I and LiCl for DNA template removal and quick mRNA purification.

The HiScribe T7 High Yield RNA Synthesis Kit (NEB #E2040) delivers robust RNA synthesis for a wide range of template sizes (Figure 4). Flexible protocols ensure that performance is maintained, even under demanding conditions, such as extended reaction time using very low amounts of template (Figure 6). Protocols are included for partial or complete incorporation of modified or labeled nucleotides in the transcript body, and cap analogs at the RNA 5' end.

Figure 3. 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. Luminescence values were recorded and used to calculate relative luciferase activity.

## RECOMMENDED PRODUCTS

### HiScribe T7 mRNA Kit with CleanCap Reagent AG (NEB #E2080)

- Streamlined workflow with single-step co-transcriptional capping
- CleanCap Reagent AG trinucleotide cap technology results in a natural Cap-1 structure, maximizing translatability and minimizing immune response from synthetic mRNA
- High capping efficiency
- Optimized for high yields
- Suitable for full or partial modified nucleotide substitution

## TOOLS AND RESOURCES

- Visit [www.neb.com/RNAanalysis](http://www.neb.com/RNAanalysis) to find more information, including our JoVE video demonstrating *in vitro* synthesis of capped and uncapped RNA (1).



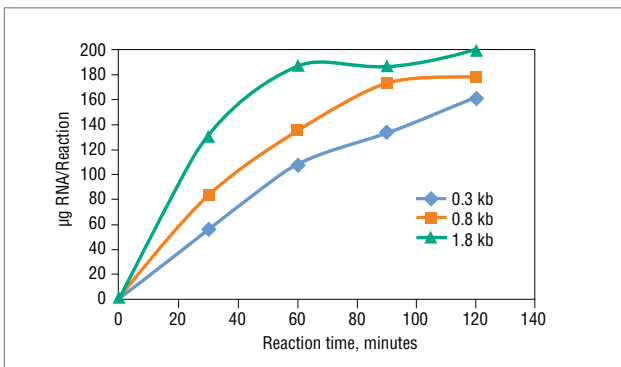
HIGH YIELD *IN VITRO* SYNTHESIS OF CAPPED AND UNCAPPED RNA



The HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB #E2050) utilizes a master mix format, allowing for faster reaction setup. All that is required is the addition of two master mix reagents to your DNA template and water, reducing pipetting errors. DNase I and lithium chloride are included for DNA template removal and quick RNA purification. Alternatively, our Monarch RNA Cleanup Kits can be used for quick cleanup of transcripts (see page 11).

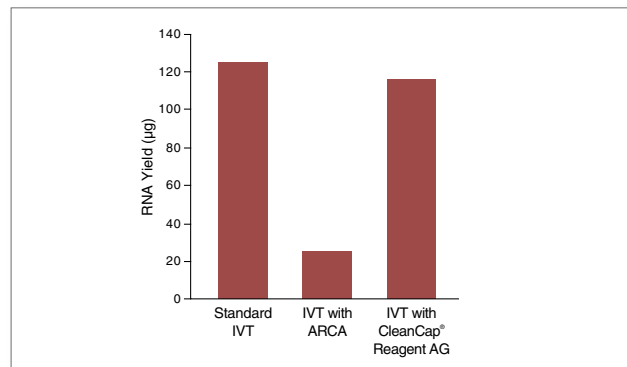
The HiScribe SP6 RNA Synthesis Kit (NEB #E2070) is designed for the *in vitro* transcription of RNA using SP6 RNA Polymerase. This kit is suitable for synthesis of high yield RNA transcripts and for incorporation of cap analogs (not included) or modified nucleotides (not included) to obtain capped, biotin-labeled or dye-labeled RNA. The kit is also capable of synthesizing high specific activity radiolabeled RNA for use as probes or targets.

**Figure 4. Robust RNA Synthesis from a Variety of Template Sizes using the HiScribe T7 High Yield RNA Synthesis Kit**



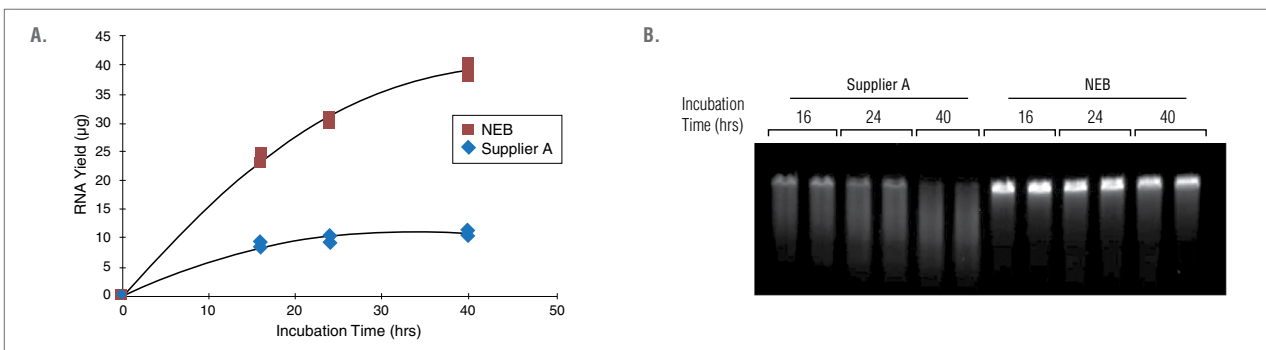
Time course of standard RNA synthesis from three DNA templates of different sizes using HiScribe T7 High Yield RNA Synthesis Kit. Reactions were assembled according to the protocol and incubated at 37°C for the indicated time. Transcripts were purified over spin columns and quantified on a NanoDrop™ Spectrophotometer to calculate reaction yield.

**Figure 5. 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.

**Figure 6. Improved RNA Yield and Integrity from Low Template Amount and Extended Duration Transcription Reactions using the HiScribe T7 High Yield RNA Synthesis Kit**



Reactions were assembled, in duplicate, according to the manufacturer's suggested protocols using 3 ng of dsDNA template encoding a 1.8 kb RNA, and incubated at 37°C for 16, 24 and 40 hours. At each time point, the corresponding tubes were transferred to -20°C to stop the reaction.

(A) Transcript yield – After column purification, RNA concentration was measured using a NanoDrop spectrophotometer and total RNA yield was calculated. These data demonstrate that a substantially higher yield of RNA was synthesized using the HiScribe T7 High Yield RNA Synthesis Kit as compared to the competitor's kit.

(B) Transcript integrity – 150 ng of column purified RNA was run a 1.2% denaturing agarose gel, stained with ethidium bromide and visualized by UV fluorescence. The data demonstrate greatly improved transcript integrity after extended duration RNA synthesis reactions using the HiScribe T7 High Yield RNA Synthesis Kit, as compared to the competitor's kit.



# In vitro Transcription (Cont'd)

## Recommended HiScribe RNA Synthesis Kits by Application

The HiScribe High Yield RNA Synthesis Kits are ideal for numerous downstream applications. Use the guide below to determine which kit is best suited for your application.

APPLICATION	T7 KITS					SP6 KITS
	HiScribe T7 High Yield RNA Synthesis Kit (#E2040)	HiScribe T7 Quick High Yield RNA Synthesis Kit (#E2050)	HiScribe T7 ARCA mRNA Kit (#E2065)	HiScribe T7 ARCA mRNA (with Tailing) (#E2060)	HiScribe T7 mRNA Kit with CleanCap Reagent AG (#E2080)	HiScribe SP6 RNA Synthesis Kit (#E2070)
<b>Probe labeling</b>	Fluorescent labeling: FAM, Cyanine (Cy) dyes, etc. • Fluorescent <i>in situ</i> hybridization (FISH)		✓			✓
	Non-fluorescent labeling: Biotin, Digoxigenin • <i>In situ</i> hybridization • Blot hybridization with secondary detection • Microarray		✓			✓
	High specific activity radiolabeling • Blot hybridization • RNase protection	✓				✓
<b>mRNA &amp; RNA for transfection</b>	Streamlined mRNA synthesis with ARCA co-transcriptional capping and enzymatic poly(A) tailing • Transfection • Microinjection • <i>In vitro</i> translation				✓	
	Streamlined ARCA capped RNA synthesis • Template encoded poly(A) tails • Non polyadenylated transcripts • Transfection • Microinjection • <i>In vitro</i> translation			✓		
	Co-transcriptional capping with alternate cap analogs • Transfection • Microinjection • <i>In vitro</i> translation		✓			✓
	Co-transcriptional capping with CleanCap Reagent AG • Transfection • Microinjection • <i>In vitro</i> translation				✓	
	Post-transcriptional capping with Faustovirus Capping Enzyme or Vaccinia Capping System • Transfection • Microinjection • <i>In vitro</i> translation	✓	✓			✓
	Complete substitution of NTPs: 5-mC, pseudouridine, etc.	✓				✓
	Partial substitution of NTPs: 5-mC, pseudouridine, etc.	✓	✓	✓	✓	✓
	Unmodified RNA		✓			✓
	Hairpins, short RNA, dsRNA • Gene knockdown		✓			✓
<b>Structure, function, &amp; binding studies</b>	Complete substitution of NTPs • Aptamer selection • Isotopic labeling	✓				✓
	Partial substitution of one or more NTPs • Aptamer selection • Structure determination		✓			✓
	Unmodified RNA • SELEX • Structure determination		✓			✓



# mRNA Modifications: Capping

Capping and tailing are key steps in producing active synthetic mRNA for use in functional studies; these modifications prevent degradation and facilitate translation in eukaryotic cells.

## RNA Capping

Most eukaryotic cellular mRNAs are modified at their 5' ends by the addition of a 7-methyl guanosine ( $m^7G$ ) residue in a 5'  $\rightarrow$  5' triphosphate linkage to the first encoded nucleotide of the transcript. The mRNA cap structure engages critical translation factors to recruit ribosomes to mRNAs, promoting translation.

Cap structures can be added to *in vitro* transcripts in two ways:

- After transcription by using capping enzymes, GTP and S-adenosyl methionine (SAM)
- During transcription by including cap analogs

### RECOMMENDED PRODUCTS

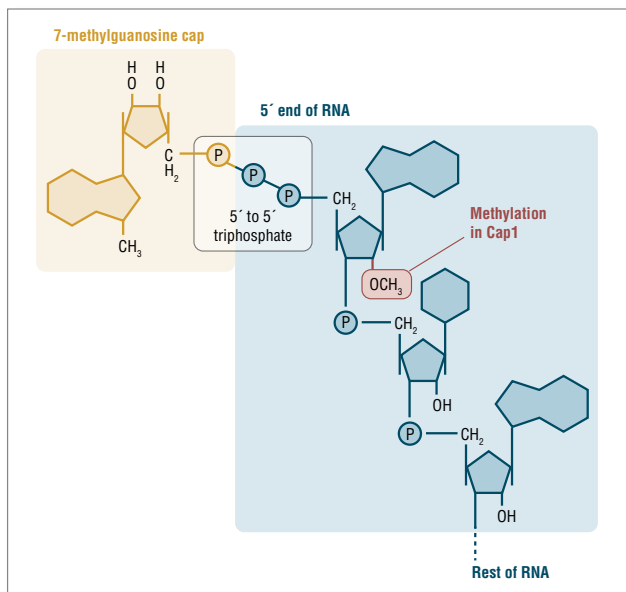
#### Faustovirus Capping Enzyme (NEB #M2081)

- Experience improved capping efficiency, even on difficult substrates
- Achieve robust capping with less enzyme
- Set up reactions under a broad temperature range, for added flexibility

#### mRNA Cap 2'-O-Methyltransferase (NEB #M0366)

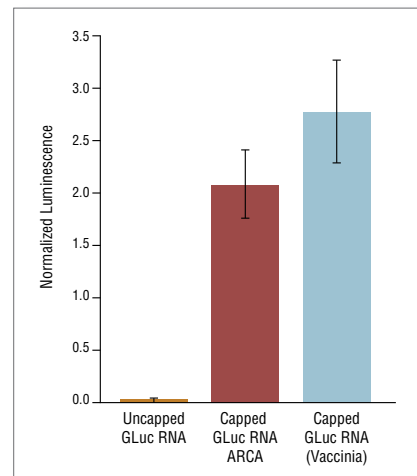
- Enhances translation of mRNA
- Improves mRNA expression *in vivo*

Figure 7. 5' Cap Structure



Schematic representation of mRNA 5' cap structure indicating the 7-methylguanosine, shown in yellow, and the 5' end of the mRNA, shown in blue. The 2'-O-methyl group present in Cap-1 structures is shown in red.

Figure 8. GLuc Expression



Purified Cap-0 and uncapped GLuc mRNA were transfected into HeLa cells and incubated overnight (16 hrs.) at 37°C. Cell culture supernatants from each well were assayed for GLuc and CLuc activity and luminescence values were recorded. The GLuc luminescence values were normalized to the luminescence values of Cap-0 CLuc RNA.

## Post-transcriptional Enzymatic mRNA Capping

Highest efficiency mRNA capping is achieved using the Faustovirus Capping Enzyme (NEB #M2081). This system has three enzymatic activities (RNA triphosphatase, guanylyltransferase, guanine methyltransferase); all are necessary for the addition of a complete Cap-0 structure,  $m^7Gppp5'N$ . *In vitro* transcripts can be capped in less than one hour in the presence of the capping enzyme, reaction buffer, GTP and the methyl donor, SAM. All capped structures are added in the proper orientation for recognition by the translational machinery, unlike co-transcriptional addition of some cap analogs (2).

### ADVANTAGES

- Natural caps
- Cap-0 to Cap-1 possible in same reaction using 2'-O-Methyltransferase





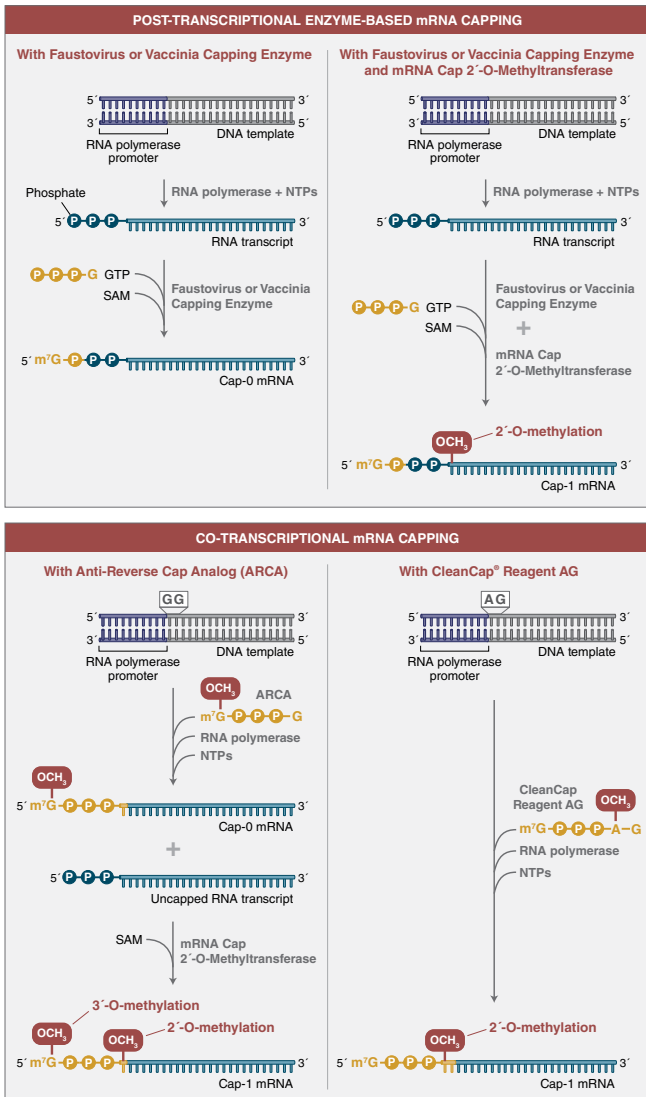
# mRNA Modifications: Capping (Cont'd)

## Co-transcriptional Capping with Dinucleotide Cap Analogs

Anti-Reverse Cap Analog (ARCA) [3'-O-Me-m<sup>7</sup>G(5')ppp(5')G RNA Cap Structure Analog, (NEB #S1411)] is the preferred cap analog for co-transcriptional capping. Transcription with ARCA produces 100% translatable capped transcripts, because it can only incorporate in the 'correct' orientation, where the N7-methylguanosine is at the terminus [m<sup>7</sup>G(5')pppG-RNA] (3,4).

In contrast, the standard cap analog [m<sup>7</sup>G(5')ppp(5')G RNA Cap Structure Analog (NEB #S1404)] can be incorporated in either orientation [m<sup>7</sup>G(5')pppG-RNA] or [G(5')pppm<sup>7</sup>G-RNA], resulting in a mixture of transcripts (3,5). mRNAs with cap analog incorporated in the incorrect orientation are not efficiently translated, resulting in lower protein yields (2). The RNA products are a mixture of 5'-capped and 5'-triphosphorylated transcripts. This may necessitate purification or treatment with a phosphatase in order to avoid unintended immune stimulation by 5'-triphosphorylated RNA.

Figure 9. Schematic representation of alternative mRNA synthesis workflows



Enzyme-based capping (top) is performed after *in vitro* transcription using 5'-triphosphate RNA, GTP, and S-adenosyl- methionine (SAM). Cap-0 mRNA can be converted to Cap-1 mRNA using mRNA cap 2'-O-methyltransferase (MTase) and SAM in a subsequent or concurrent reaction. The methyl group transferred by the MTase to the 2'-O of the first nucleotide of the transcript is indicated in red. Conversion of ~100% of 5'-triphosphorylated transcripts to capped mRNA is routinely achievable using enzyme-based capping.

Co-transcriptional capping (bottom) uses an mRNA cap analog, shown in yellow, in the transcription reaction. For ARCA (anti reverse cap analog) (left), the cap analog is incorporated as the first nucleotide of the transcript. ARCA contains an additional 3'-O-methyl group on the 7-methylguanosine to ensure incorporation in the correct orientation. The 3'-O-methyl modification does not occur in natural mRNA caps. Compared to reactions not containing cap analog, transcription yields are lower. ARCA-capped mRNA can be converted to Cap-1 mRNA using mRNA cap 2'-O-MTase and SAM in a subsequent reaction. CleanCap Reagent AG (right) uses a trinucleotide cap analog that requires a modified template initiation sequence. A natural Cap-1 structure is accomplished in a single reaction.

### RECOMMENDED PRODUCTS

#### HiScribe T7 mRNA Kit with CleanCap Reagent AG (NEB #E2080)

- Streamlined workflow with single-step co-transcriptional capping
- CleanCap Reagent AG trinucleotide cap technology results in a natural Cap-1 structure, maximizing translatability and minimizing immune response from synthetic mRNA
- High capping efficiency
- Optimized for high yields
- Suitable for full or partial modified nucleotide substitution

#### HiScribe T7 ARCA mRNA Synthesis Kit (with tailing – NEB #E2060) (without tailing – NEB #E2065)

- Faster workflow takes you from IVT and capping to purification in 2 hours
- Enables incorporation of modified bases
- High efficiencies with correctly-oriented ARCA caps
- All-inclusive kit provides reagents for twice the reactions than competitors' kits





## Cap-1 Modification and Co-transcriptional Trinucleotide Capping

The Cap-1 structure has been reported to enhance mRNA translation efficiency (6) and hence may help improve expression in mRNA transfection and in microinjection experiments.

Cap-0 transcripts can be enzymatically converted to cap-1 *in vitro*. mRNA Cap 2'-O-Methyltransferase (NEB #M0366) adds a methyl group at the 2'-O position of the first nucleotide adjacent to the cap structure at the 5' end of the RNA. The enzyme utilizes S-adenosylmethionine (SAM) as a methyl donor to methylate capped RNA (Cap-0) resulting in a Cap-1 structure. Alternatively, Cap-1 mRNA can be synthesized co-transcriptionally with a trinucleotide cap analog such as CleanCap Reagent AG. The use of CleanCap reagent AG results in significant advantages over traditional dinucleotide co-transcriptional capping. CleanCap Reagent AG is a trinucleotide with a 5'-m7G joined by a 5'-5' triphosphate linkage to an AG sequence. The adenine has a methyl group on the 2'-O position. The incorporation of this trinucleotide in the beginning of a transcript results in a Cap-1 structure.

Figure 10. Molecular structure of CleanCap Reagent AG

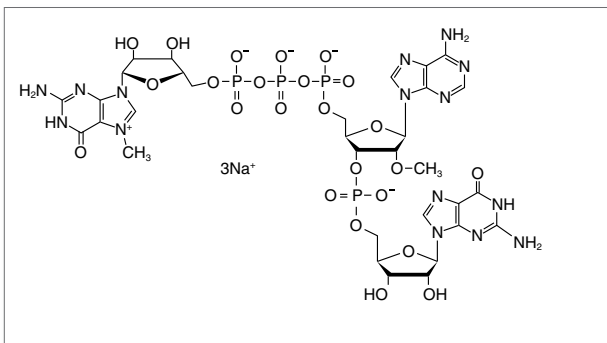
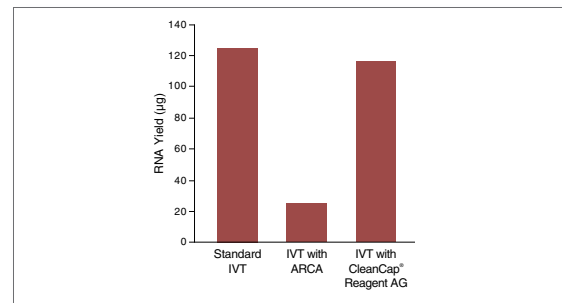


Figure 11. Comparison of RNA Yields from *In Vitro* Transcription Reactions with no cap analog, ARCA, or CleanCap Reagent AG



## RNA Cap Analog Selection Chart

The 5' terminal m7G cap present on most eukaryotic mRNAs promotes translation, *in vitro*, at the initiation level. For most RNAs, the cap structure increases stability, decreases susceptibility to exonuclease degradation, and promotes the formation of mRNA initiation complexes. Certain prokaryotic mRNAs with 5' terminal cap structures are translated as efficiently as eukaryotic mRNA in a eukaryotic cell-free protein synthesizing system. Splicing of certain eukaryotic substrate RNAs has also been observed to require a cap structure.

PRODUCT	APPLICATION
<b>HiScribe T7 mRNA Kit with CleanCap Reagent AG*</b> *kit only	<ul style="list-style-type: none"> <li>High yield</li> <li>Natural Cap-1 structure</li> <li>Produces 100% translatable transcripts</li> <li>Highest efficiency capping</li> </ul>
<b>Anti-Reverse Cap Analog 3'-O-Me-m7G(5') ppp(5')G</b>	<ul style="list-style-type: none"> <li>Produces 100% translatable capped transcripts</li> <li>Co-transcriptional capping with T7 (NEB #M0251), Hi-T7 (NEB #M0658), SP6 (NEB #M0207) and T3 RNA polymerases (NEB #M0378)</li> <li>Synthesis of m7G capped RNA for <i>in vitro</i> splicing assays</li> <li>Synthesis of m7G capped RNA for transfection or microinjection</li> </ul>
<b>Standard Cap Analog m7G(5')ppp(5')G</b>	<ul style="list-style-type: none"> <li>Co-transcriptional capping with T7, Hi-T7, SP6 and T3 RNA polymerases</li> <li>Synthesis of m7G capped RNA for <i>in vitro</i> splicing assays</li> <li>Synthesis of m7G capped RNA for transfection or microinjection</li> </ul>

PRODUCT	APPLICATION
<b>Unmethylated Cap Analog G(5')ppp(5')G</b>	<ul style="list-style-type: none"> <li>Co-transcriptional capping with T7, Hi-T7, SP6 and T3 RNA polymerases</li> <li>Synthesis of unmethylated G capped RNA</li> </ul>
<b>Methylated Cap Analog for A +1 sites m7G(5')ppp(5')A</b>	<ul style="list-style-type: none"> <li>Co-transcriptional capping with T7 RNA polymerase from the phi2.5 promoter that contains an A at the transcription initiation site</li> <li>Synthesis of m7G capped RNA for <i>in vitro</i> splicing assays</li> <li>Synthesis of m7G capped RNA for transfection or microinjection</li> </ul>
<b>Unmethylated Cap Analog for A +1 sites G(5')ppp(5')A</b>	<ul style="list-style-type: none"> <li>Co-transcriptional capping with T7 RNA polymerase from the phi2.5 promoter that contains an A at the transcription initiation site</li> <li>Synthesis of unmethylated G capped RNA</li> <li>Synthesis of A capped RNA</li> </ul>



# mRNA Modifications: Tailing

## Poly(A) Tailing

Poly(A) tailing of RNA is another key step that has been found to increase the stability and translation efficiency of transfected *in vitro*-transcribed RNA. *In vivo*, poly(A) tails recruit poly(A) binding proteins to mRNAs, conferring stability by inhibiting exonucleases. In addition, poly(A) binding protein interactions with the cellular translation machinery figure prominently in the determination of translation efficiency.

Obtaining *in vitro* transcripts with 3'- poly(A) tails can be achieved in 2 ways:

- By enzymatic polyadenylation after transcription
- By encoding a poly(A) stretch in the transcription template

Poly(A) tails can be added after transcription using *E. coli* Poly(A) Polymerase (NEB #M0276) and ATP. Poly(A) tail addition is template independent in this case.

Poly(A) stretches in transcription templates can be encoded in plasmid templates. However, it is advisable to carefully design the template so that restriction enzyme linearization of the plasmid yields a template that encodes a poly(A) tail with no extra nucleotides. We recommend placing a BspQI (NEB #R0712) site immediately downstream of the poly(A) stretch to achieve this.

Alternatively, a poly(A) stretch can be added during PCR-based generation of transcription templates. In this approach, a reverse primer that contains a poly(dT) (e.g., dT120) stretch at the 5' end is used (7).

### RECOMMENDED PRODUCTS

#### *E. coli* Poly(A) Polymerase (NEB #M0276)

- Enhances translation of RNA transfected into eukaryotic cells

#### HiScribe T7 ARCA mRNA Synthesis Kit (with tailing) (NEB #E2060)

- Poly(A) Polymerase included for enzymatic polyadenylation

#### HiScribe T7 mRNA Kit with CleanCap Reagent AG (NEB #E2080)

- For template-encoded poly(A) tailing

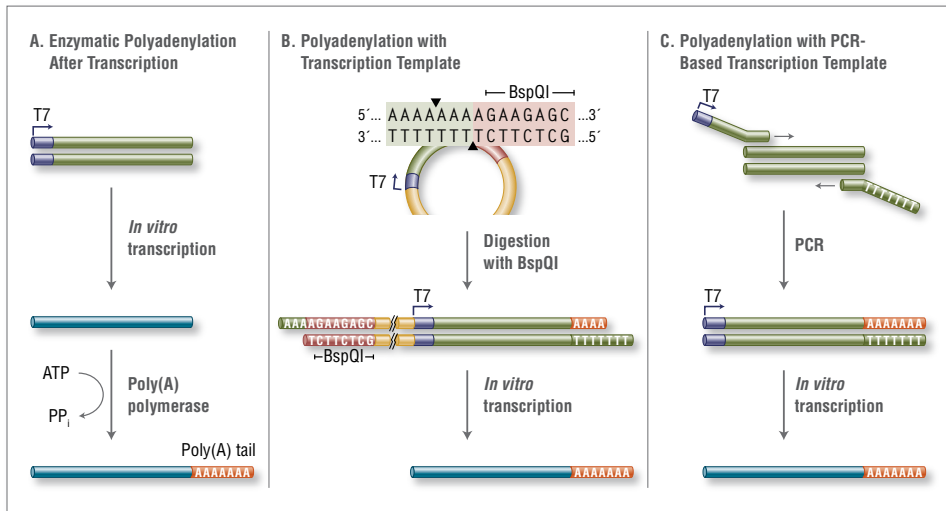
### ADVANTAGES

- No additional steps with template-encoded poly(A) tails
- No introduction of poly(A) tracts into templates required for enzymatic polyadenylation with *E. coli* Poly(A) Polymerase

### DISADVANTAGES

- Template generation can be difficult when using template-encoded poly(A) tails
- Enzymatic polyadenylation with *E. coli* Poly(A) Polymerase requires an additional step and produces a range of A-tail lengths

Figure 12. Polyadenylation Strategies



Polyadenylation can be done several ways: (A) enzymatically after transcription, (B) digestion of desired template with BspQI followed by transcription, or (C) by adding the tail to a template via PCR.



# Purification after RNA Synthesis

Purification of RNAs from enzymatic synthesis or modification reactions is essential before use. After RNA synthesis by *in vitro* transcription, unincorporated nucleotides, short aborted transcripts, enzymes and buffer components must be removed before using the RNA for downstream applications including transfection, microinjection or RNP formation. Complete removal of small molecule and enzyme reaction components is also necessary after protocols such as RNA labeling, DNase I treatment, Proteinase K treatment, and mRNA capping.

RNA can be purified in various ways, including phenol/chloroform extraction and ethanol precipitation, lithium chloride precipitation, or by gel purification. Silica-based columns are a popular and user-friendly method for fast RNA cleanup. Column-based cleanup methods also provide an easy way to concentrate purified RNA by using low elution volumes. NEB is proud to offer a family of high performance and easy to use RNA cleanup kits for all your RNA workflows.

The Monarch RNA Cleanup Kits provide a fast and simple column-based solution for RNA cleanup and concentration after *in vitro* transcription or other reactions. For convenience and flexibility, Monarch RNA Cleanup Kits are available in three different binding capacities: 10 µg, 50 µg and 500 µg, each containing unique columns designed to prevent buffer retention, ensure no carryover of contaminants and to prevent elution of silica particles, a common annoyance in RNA cleanup applications. The simple bind-wash-elute protocol can be completed in minutes and is simplified by the inclusion of a single wash buffer.

## Specifications:

MONARCH RNA CLEANUP KIT	NEB #T2030 (10 µg)	NEB #T2040 (50 µg)	NEB #T2050 (500 µg)
Binding Capacity	10 µg	50 µg	500 µg
RNA Size Range	≥ 25 nt (≥ 15 nt with modified protocol)		
Typical Recovery	70–100%		
Elution Volume	6–20 µl	20–50 µl	50–100 µl
Purity	$A_{260/280} > 1.8$ and $A_{260/230} > 1.8$		
Protocol Time	5 minutes of spin and incubation time		10–15 minutes of spin and incubation time
Common Downstream Applications	RT-PCR, RNA library prep for NGS, small RNA library prep for NGS, RNA labeling	RT-PCR, RNA library prep for NGS, formation of RNP complexes for genome editing, microinjection, RNA labeling, transfection	RT-PCR, RNA library prep for NGS, RNA labeling, RNAi, microinjection, transfection

## RECOMMENDED PRODUCTS

**Monarch RNA Cleanup Kit (10 µg)**  
(NEB #T2030)

**Monarch RNA Cleanup Kit (50 µg)**  
(NEB #T2040)

**Monarch RNA Cleanup Kit (500 µg)**  
(NEB #T2050)

## ADVANTAGES

- Three binding capacities for versatility in any applications
- Clean up RNA in 5–10 minutes with simple bind/wash/elute protocol, using a single wash buffer
- Prevent buffer carryover and elution of silica particles with optimized column design
- High yields – 70–100% recovery
- High purity –  $A_{260/280}$  and  $A_{260/230} \geq 1.8$
- Bind up to 500 µg of RNA (NEB #T2050)
- Elute in as little as 6 µl (NEB #T2030) or 20 µl (NEB #T2040)
- Enjoy the flexibility of columns and buffers available separately (see page 12)

## TOOLS AND RESOURCES

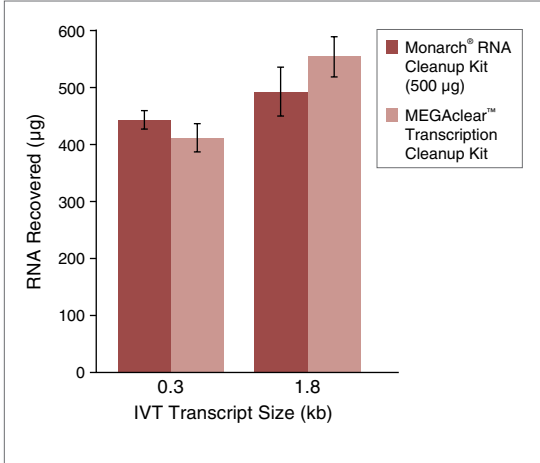
Visit [www.neb.com/MonarchRNACleanup](http://www.neb.com/MonarchRNACleanup) to find:

- Protocol and optimization tips videos
- Performance data
- Troubleshooting guide for RNA cleanup, also shown on page 14



# Purification after RNA Synthesis (Cont'd)

Figure 12. The Monarch RNA Cleanup Kit (500 µg) cleans up large-scale *in vitro* transcription reactions and generates yields consistent with other large-scale RNA cleanup kits

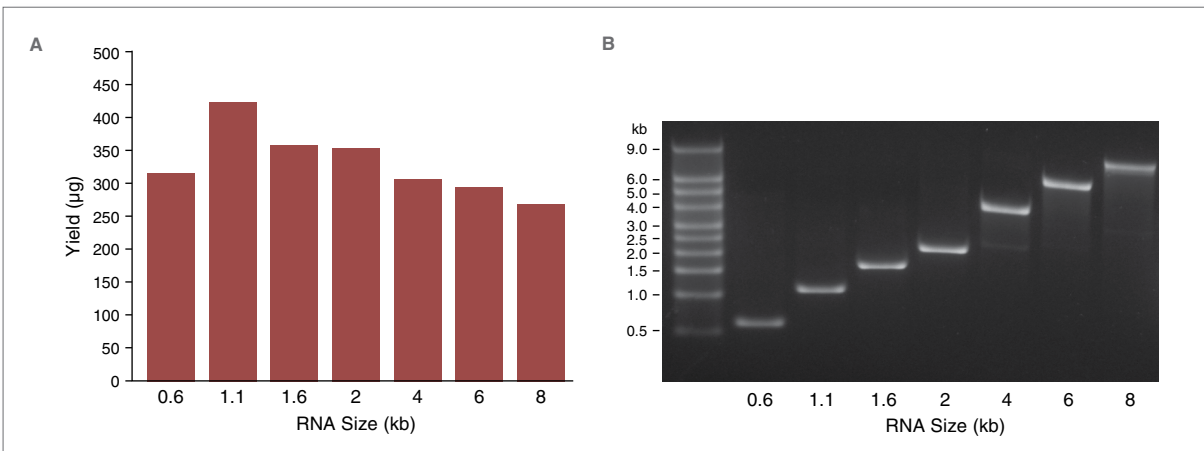


0.3 kb and 1.8 kb RNAs were transcribed using the HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB #E2050). Following DNase I treatment (4 U DNase I, 37°C, 15 min), transcription reactions were pooled and 200 µl were cleaned up using either the NEB Monarch RNA Cleanup Kit (500 µg, dark red) or the MEGAClear Transcription Clean-Up Kit (Thermo Fisher Scientific). *In vitro* transcribed RNA was eluted twice with 100 µl of nuclease-free water following a 5-minute on-column incubation (room temperature for Monarch and 65° C for MEGAClear). Recovery of the synthesized RNA transcript was calculated from the resulting A<sub>260</sub> as measured using a Trinean DropSense™ 16. The Monarch RNA Cleanup Kit (500 µg, NEB #T2050) produces similar RNA yields as the MEGAClear Kit for large-scale *in vitro* transcription reactions, without high temperature elution.

**COMPANION PRODUCTS**

- Monarch RNA Cleanup Columns (10 µg) (NEB #T2037)
- Monarch RNA Cleanup Columns (50 µg) (NEB #T2047)
- Monarch RNA Cleanup Columns (500 µg) (NEB #T2057)
- Monarch RNA Cleanup Binding Buffer (NEB #T2041)
- Monarch RNA Cleanup Wash Buffer (NEB #T2042)

Figure 13. The Monarch RNA Cleanup Kit (500 µg) is suitable for cleaning up large quantities (>250 µg) of RNA from *in vitro* transcription reactions



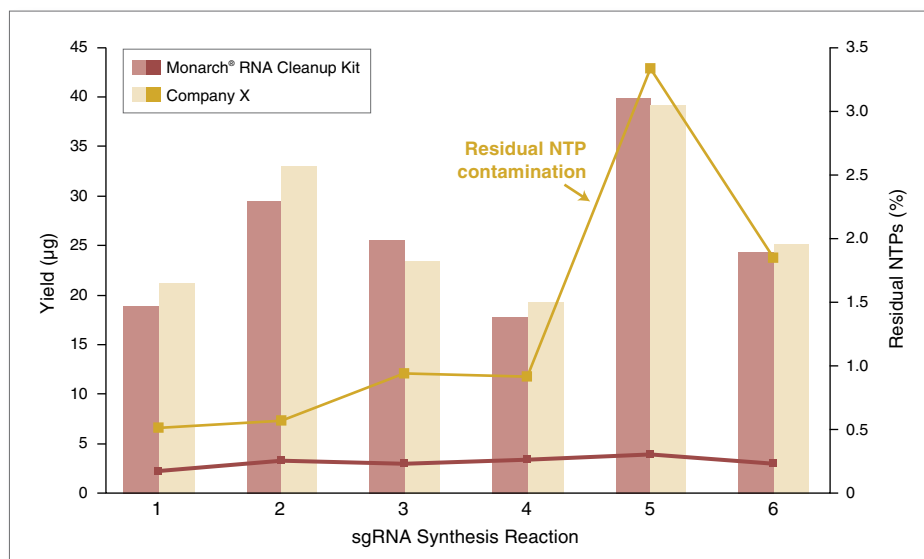
A. RNA transcripts of varying sizes (0.6-8 kb) were synthesized using the HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB #E2050). 40 µl of each *in vitro* transcription (IVT) reaction was cleaned up using the Monarch RNA Cleanup Kit (500 µg) (NEB #T2050). RNA yields were calculated from the resulting A<sub>260</sub>, measured using a Nanodrop™ spectrophotometer and ranged from 268–425 µg of RNA per IVT reaction.

B. RNA integrity (200 ng/lane) was assessed on a 1% agarose-TBE gel stained with SYBR® Gold.





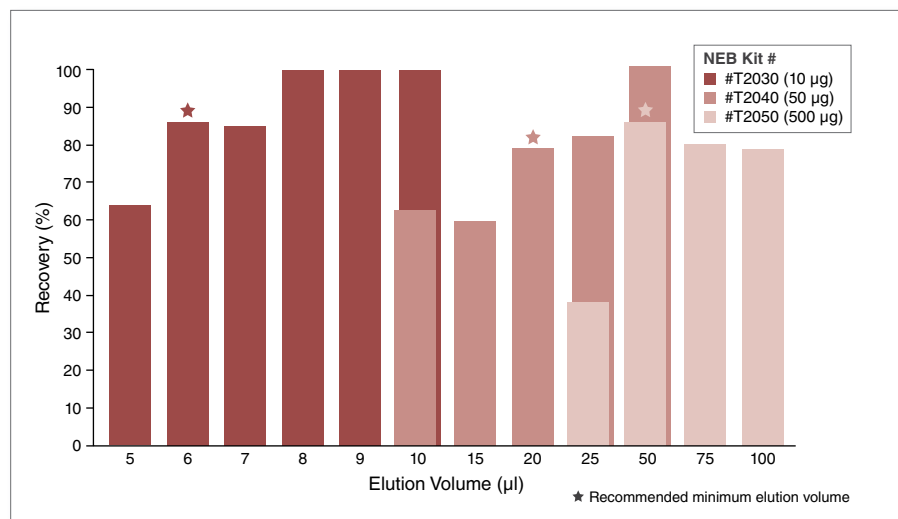
Figure 14. The Monarch RNA Cleanup Kit (50 µg) produces sgRNA yields consistent with other competitor RNA cleanup kits and with lower residual NTP contamination



Six different sgRNA synthesis reactions from the EnGen® sgRNA Synthesis Kit, *S. pyogenes* (NEB #E3322) were cleaned up using either the Monarch RNA Cleanup Kit (50 µg, NEB #T2040) or a competitor kit (according to manufacturer's recommendations) and eluted in 50 µl nuclease-free water. sgRNA yield was calculated from the resulting  $A_{260}$ , measured using a Trinean DropSense 16. The Monarch RNA Cleanup Kit produced sgRNA yields consistent with other commercially available RNA cleanup kits.

Following cleanup, residual nucleotides (NTPs) were measured by LC-MS and are reported as percent area NTPs ( $rATP+rCTP+rGTP+rUTP$ )/percent area sgRNA). The NEB Monarch RNA Cleanup Kit consistently outperforms other commercially available RNA cleanup kits in the removal of residual NTPs from sgRNA synthesis reactions.

Figure 15. Recovery of RNA from Monarch RNA Cleanup Kits with Varying Elution Volumes



10, 50 or 500 µg of RNA (6S and 23S Ribosomal Standard from *E. coli*, Sigma) was purified using a Monarch RNA Cleanup Kit (10 µg, NEB #T2030) (50 µg, NEB #T2040) (500 µg, NEB #T2050). Nuclease-free water was used to elute the RNA. The percent recovery of the RNA was calculated from the resulting  $A_{260}$ , as measured using a Trinean DropSense 16. ~80% of RNA can be efficiently recovered in 6 µl from the Monarch RNA Cleanup Kit (10 µg, NEB #T2030), 20 µl from the Monarch RNA Cleanup Kit (50 µg, NEB #T2040), and 50 µl from the Monarch RNA Cleanup Kit (500 µg, NEB #T2050).



## RNA Cleanup Troubleshooting Guide

PROBLEM	PROBABLE CAUSE(S)	SOLUTION(S)
<b>Low RNA Yield</b>	Reagents added incorrectly	Check protocol to ensure correct buffer reconstitution, order of addition of buffers and ethanol, and proper handling of column flow-through and eluents.
	Insufficient mixing of reagents	Ensure the ethanol is thoroughly mixed with RNA sample and RNA Cleanup Binding Buffer before applying the sample to the RNA Cleanup Column.
	Incomplete elution during prep	Ensure the nuclease-free water used for elution is delivered directly to the center of the column so that the matrix is completely saturated. Larger elution volumes, multiple elutions, and longer incubation times can increase yield of RNA but will dilute the sample and may increase processing times. For typical RNA samples, the recommended elution volumes and incubation times should be sufficient.
	High degree of RNA secondary structure	Binding and elution of smaller RNAs (< 45 nt) can be affected by secondary structure of the RNA molecules. If poor yield of a small RNA is observed, we recommend diluting your sample with 2 volumes of ethanol instead of one volume in Step 2 of the protocol.
<b>Purified RNA is Degraded</b>	RNase contamination	In order to avoid RNase contamination during RNA cleanup, make sure to work on a clean lab bench, wear gloves and use disposable RNase-free pipet tips and microfuge tubes (not provided). Keep all kit components tightly sealed when not in use.
	Improper storage of RNA	Purified RNA should be used immediately in downstream applications or stored at -70°C.
<b>Low A<sub>260/230</sub> Ratios</b>	Residual guanidine salt carry-over	Ensure wash steps are carried out prior to eluting sample. Use care to ensure the tip of the column does not contact the flow-through. If unsure, repeat centrifugation. When reusing collection tubes, blot the rim of the tube on a Kimwipe prior to reattachment to the column to remove any residual wash buffer.
<b>Low Performance of RNA in Downstream Steps</b>	Salt and/or ethanol carry-over	Ethanol and salt remaining after the washes may inhibit downstream applications. Use care to ensure that the tip of the column does not come into contact with the flow-through. If in doubt, re-centrifuge for 1 minute to ensure traces of salt and ethanol are not carried over in the eluted RNA.
	DNA contamination	DNA removal may be necessary for certain applications. Incubate RNA sample with DNase I (NEB #M0303) and cleanup RNA using the Monarch RNA Cleanup Protocol.

## Ordering Information

	PRODUCT	NEB #	SIZE
<b>Template generation</b>	Q5 High-Fidelity DNA Polymerase	M0491S/L	100/500 units
	Q5 Hot Start High-Fidelity DNA Polymerase	M0493S/L	100/500 units
	Q5 Hot Start High-Fidelity 2X Master Mix	M0494S/L	100/500 reactions
	Deoxynucleotide (dNTP) Solution Set	N0446S	25 µmol each
	Deoxynucleotide (dNTP) Solution Mix	N0447S/L	8/40 µmol each
	BspQI	R0712S/L	500/2,500 units
	SapI	R0569S/L	250/1,250 units
<b>In vitro transcription</b>	T3 RNA Polymerase	M0378S	5,000 units
	T7 RNA Polymerase	M0251S/L	5,000/25,000 units
	Hi-T7® RNA Polymerase	M0658S	50,000 units
	SP6 RNA Polymerase	M0207S/L	5,000/25,000 units
	Pyrophosphatase, Inorganic (yeast)	M2403S/L	10/50 units
	Pyrophosphatase, Inorganic ( <i>E. coli</i> )	M0361S/L	10/50 units
	Ribonucleotide Solution Set	N0450S/L	10/50 µmol each
	Ribonucleotide Solution Mix	N0466S/L	10/50 µmol each
	RNase Inhibitor, Murine	M0314S/L	3,000/15,000 units
	RNase Inhibitor, Human Placenta	M0307S/L	2,000/10,000 units



## Ordering Information Cont'd

	PRODUCT	NEB #	SIZE
<b>In vitro transcription &amp; mRNA synthesis kits</b>	EnGen sgRNA Synthesis Kit, <i>S. pyogenes</i>	E3322S	20 reactions
	HiScribe T7 mRNA Kit with CleanCap Reagent AG	E2080S	20 reactions
	HiScribe T7 ARCA mRNA Kit (with tailing)	E2060S	20 reactions
	HiScribe T7 ARCA mRNA Kit	E2065S	20 reactions
	HiScribe T7 High Yield RNA Synthesis Kit	E2040S	50 reactions
	HiScribe T7 Quick High Yield RNA Synthesis Kit	E2050S	50 reactions
	HiScribe SP6 RNA Synthesis Kit	E2070S	50 reactions
<b>Capping &amp; tailing</b>	Faustovirus Capping Enzyme	M2081S/L	500/2,500 units
	Vaccinia Capping System	M2080S	400 units
	mRNA Cap 2'-O-Methyltransferase	M0366S	2,000 units
	m <sup>7</sup> G(5')ppp(5')G RNA Cap Structure Analog	S1404S/L	1.0/5.0 μmol
	3'-O-Me-m <sup>7</sup> G(5')ppp(5')G Cap Structure Analog (ARCA)	S1411S/L	1.0/5.0 μmol
	m <sup>7</sup> G(5')ppp(5')A Cap Structure Analog	S1405S/L	1.0/5.0 μmol
	G(5')ppp(5')A Cap Structure Analog	S1406S/L	1.0/5.0 μmol
	G(5')ppp(5')G Cap Structure Analog	S1407S/L	1.0/5.0 μmol
	<i>E. coli</i> Poly(A) Polymerase	M0276S/L	100/500 units
	Adenosine-5' Triphosphate (ATP)	P0756S/L	1.0/5.0 μmol
<b>RNA Purification</b>	Monarch RNA Cleanup Kit (10 μg)	T2030S/L	10 preps / 100 preps
	Monarch RNA Cleanup Kit (50 μg)	T2040S/L	10 preps / 100 preps
	Monarch RNA Cleanup Kit (500 μg)	T2050S/L	10 preps / 100 preps
	Monarch RNA Cleanup Columns (10 μg)	T2037L	100 columns + tubes
	Monarch RNA Cleanup Columns (50 μg)	T2047L	100 columns + tubes
	Monarch RNA Cleanup Columns (500 μg)	T2057L	100 columns + tubes
	Monarch RNA Cleanup Binding Buffer	T2041L	80 ml
	Monarch RNA Cleanup Wash Buffer	T2042L	40 ml
<b>Cleanup</b>	DNase I (RNase-free)	M0303S/L	1,000/5,000 units
<b>Other RNA reagents</b>	RNA Loading Dye (2X)	B0363S	4 x 1.0 ml
<b>RNA markers</b>	ssRNA Ladder	N0362S	0.05 ml
	Low Range ssRNA Ladder	N0364S	0.05 ml

### References

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