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in this issue

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Minding your caps and tails – considerations for functional mRNA synthesis

by Breton Hornblower, Ph.D., G. Brett Robb, Ph.D. and George Tzertzinis, Ph.D., New England Biolabs, Inc.

Applications of synthetic mRNA have grown and become considerably diversified in recent years. Examples include the generation of pluripotent stem cells (1-3), vaccines and therapeutics (4-5), and CRISPR/Cas9 genome editing applications (6-8). The basic requirements for a functional mRNA – a 7-methylguanylate cap at the 5´ end and a poly(A) tail at the 3´ end – must be added in order to obtain efficient translation in eukaryotic cells. Additional considerations can include the incorporation of internal modified bases, modified cap structures and polyadenylation strategies. Strategies for *in vitro* synthesis of mRNA vary according to the desired scale of synthesis. This article discusses options for the selection of reagents and the extent to which they influence synthesized mRNA functionality.

A nascent mRNA, synthesized in the nucleus, undergoes different modifications before it can be translated into proteins in the cytoplasm. For a mRNA to be functional, it requires modified 5´ and 3´ ends and a coding region (i.e., an open reading frame (ORF) encoding for the protein of interest) flanked by the untranslated regions (UTRs). The nascent mRNA (pre-mRNA) undergoes two significant modifications in addition to splicing. During synthesis, a 7-methylguanylate structure, also known as a "cap", is added to the 5´ end of the pre-mRNA, via 5´ → 5´ triphosphate linkage. This cap protects the mature mRNA from degradation, and also serves a role in nuclear export and efficient translation.

FIGURE 1:
Translation initiation complex

A mature mRNA, consisting of the 5´ and 3´ untranslated regions (UTRs) and the open reading frame (ORF), forms a "closed-loop" structure via interactions mediated by protein complexes that bind the cap structure and the poly(A) tail.

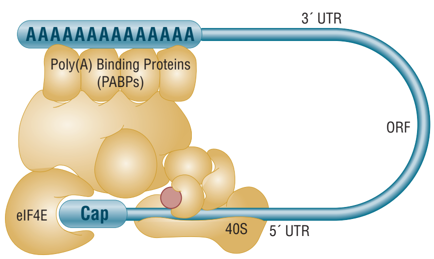
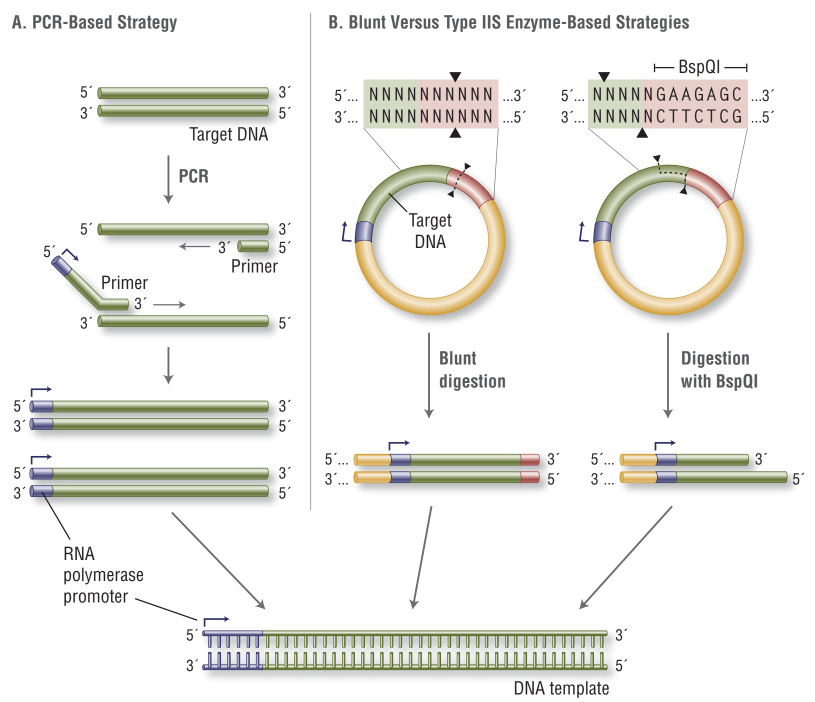


FIGURE 2:
Methods for generating transcription templates

(A) PCR can be used to amplify target DNA prior to transcription. A promoter can be introduced via the upstream primer.

(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.



The second modification occurs post-transcriptionally at the 3´ end of the nascent RNA molecule, and is characterized by addition of approximately 200 adenylate nucleotides (poly(A) tail). The addition of the the poly(A) tail confers stability to the mRNA, aids in the export of the mRNA to the cytosol, and is involved in the formation of a translation-competent ribonucleoprotein (RNP), together with the 5´ cap structure. The mature mRNA forms a circular structure (closed-loop) by bridging the cap to the poly(A) tail via the cap-binding protein eIF4E (eukaryotic initiation factor 4E) and the poly(A)-binding protein, both of which interact with eIF4G (eukaryotic initiation factor 4G), (Figure 1, (8)).

RNA can be efficiently synthesized *in vitro* (by *in vitro* transcription, IVT) with prokaryotic phage polymerases, such as T7, T3 and SP6. The cap and poly(A) tail structures characteristic of mature mRNA can be added during or after the synthesis by enzymatic reactions with capping enzymes and Poly(A) Polymerase (NEB #M0276), respectively.

There are several factors to consider when planning for IVT-mRNA synthesis that will influence the ease-of-experimental setup and yield of the final mRNA product. These are discussed in the following sections.

DNA TEMPLATE

The DNA template provides the sequence to be transcribed downstream of an RNA polymerase promoter. There are two strategies for generating transcription templates: PCR amplification and linearization of plasmid with a restriction enzyme (Figure 2). Which one to choose will depend on the downstream application. In general, if multiple sequences are to be made and transcribed in parallel, PCR amplification is recommended as it generates many templates quickly. On the other hand, if large amounts of one or a few templates are required, plasmid DNA is recommended, because of the relative ease of producing large quantities of high quality, fully characterized plasmids. There are different versions of plasmids available that allow for propagation of homopolymeric A-tails of defined length (1).

PCR allows conversion of any DNA fragment to a transcription template by appending the T7 (or SP6) promoter to the forward primer (Figure 2A). Additionally, poly(d)T-tailed reverse primers can be used in PCR to generate transcription templates with A-tails. This obviates the need for a separate polyadenylation step following transcription. Repeated amplifications should, however, be avoided to prevent PCR-generated point mutations. Amplification using PCR enzymes with the highest possible fidelity, such as Q5® High-Fidelity DNA Polymerase (NEB #M0491), reduces the likelihood of introducing such mutations (2).

The quality of the PCR reaction can be assessed by running a small amount on an agarose gel, and DNA should be purified before *in vitro* transcription using a spin column or magnetic beads (e.g., AMPure® beads). Multiple PCR reactions can be purified and combined to generate a DNA stock solution that can be stored at -20°C and used as needed for *in vitro* transcription.

Plasmid templates are convenient if the template sequence already exists in a eukaryotic expression vector also containing the T7 promoter (e.g., pcDNA vector series). These templates include 5´- and 3´-untranslated regions (UTR), which are important for the expression characteristics of the mRNA.

Plasmid DNA should be purified and linearized downstream of the desired sequence, preferably with a restriction enzyme that leaves blunt or 5´ overhangs at the 3´ end of the template. These are favorable for proper run-off transcription by T7 RNA Polymerase (NEB #M0274), while 3´ overhangs may result in unwanted transcription products. To avoid adding extra nucleotides from the restriction site to the RNA sequence, a Type IIS restriction enzyme can be used (e.g., BspQI, NEB #R0712), which positions the recognition sequence outside of the transcribed sequence (Figure 2B, page 2). The plasmid DNA should be completely digested with the restriction enzyme, followed by purification using a spin column (e.g., Monarch® PCR & DNA Cleanup Kit (5 μg) NEB #T1030) or phenol extraction/ethanol precipitation. Although linearization of plasmid involves multiple steps, the process is easier to scale for the generation of large amounts of template for multiple transcription reactions.

FIGURE 4:
RNA yields from transcriptional capping reactions

Reactions were set up according to recommended conditions for two templates: *Gaussia* luciferase (GLuc) and *Cypridina* luciferase (CLuc). The RNA was quantified spectrophotometrically after purification with spin columns.

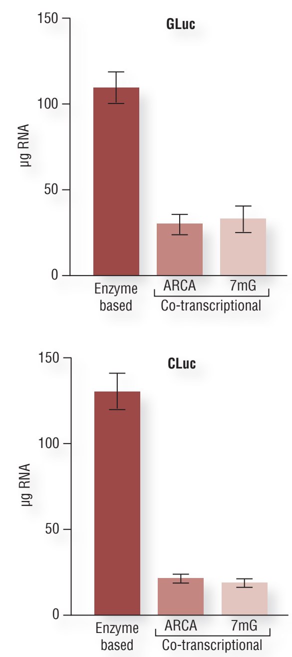
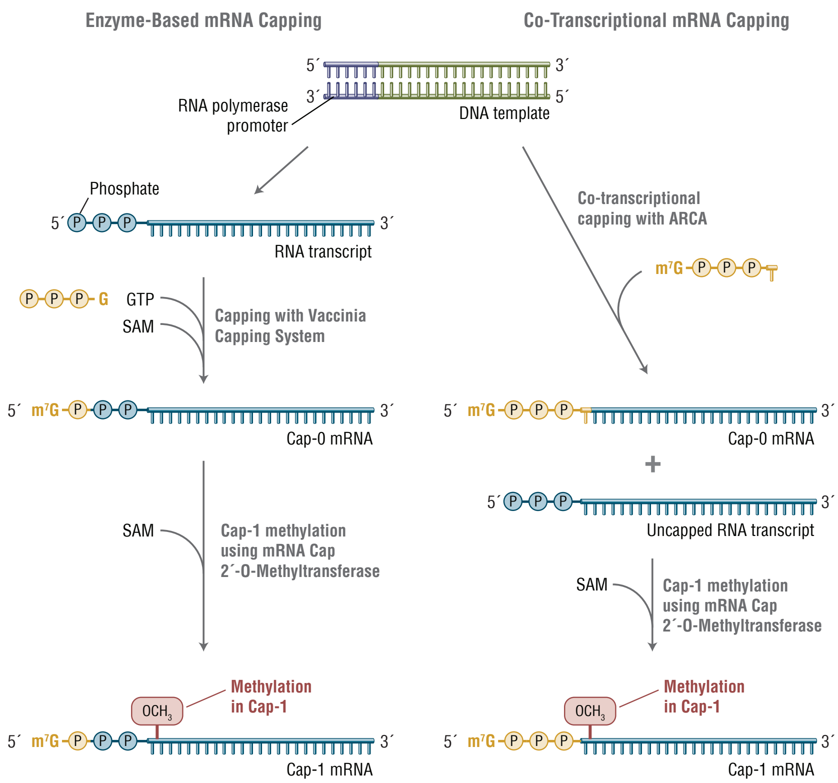


FIGURE 3:
In vitro transcription options based upon capping strategy

Enzyme-based capping (left) is performed after *in vitro* transcription using 5´-triphosphate RNA, GTP, and S-adenosylmethionine (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 (right) uses an mRNA cap analog (e.g., ARCA; anti-reverse cap analog), shown in yellow, in the transcription reaction. 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.



IN VITRO TRANSCRIPTION

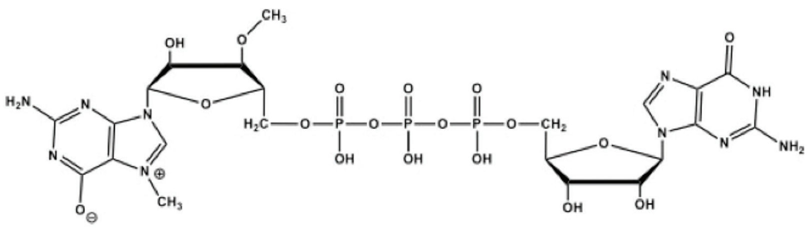
There are two options for the *in vitro* transcription (IVT) reaction depending on the capping strategy chosen: standard synthesis with enzyme-based capping following the transcription reaction (post-transcriptional capping) or incorporation of a cap analog during transcription (co-transcriptional capping) (Figure 3). Method selection will depend on the scale of mRNA synthesis required and number of templates to be transcribed.

TRANSCRIPTION FOR ENZYME-BASED CAPPING (POST-TRANSCRIPTIONAL CAPPING)

Standard RNA synthesis reactions produce the highest yield of RNA transcript (typically ≥100 μg per 20 μl in a 1 hr reaction using the HiScribe™ Quick T7 High Yield RNA Synthesis Kit, NEB #E2050S). Transcription reactions are highly scalable, and can be performed using an all-inclusive kit (e.g., HiScribe kits), or individual

FIGURE 5:
Structure of the anti-reverse cap analog, ARCA

The 3´ position of the 7-methylated G is blocked by a methyl group.



reagents. More information on the HiScribe kits can be found later in the article.

Following transcription, the RNA is treated with DNase I (NEB #M0303) to remove the DNA template, and purified using an appropriate column, kit or magnetic beads, prior to capping. This method produces high yields of RNA with 5´-triphosphate termini that must be converted to cap structures. In the absence of template-encoded poly(A) tails, transcripts produced using this method bear 3´ termini that also must be polyadenylated in a separate enzymatic step, as described below in "Post-transcriptional capping and Cap-1 methylation".

TRANSCRIPTION WITH CO-TRANSCRIPTIONAL CAPPING

In co-transcriptional capping, a cap analog is introduced into the transcription reaction, along with the four standard nucleotide triphosphates, in an optimized ratio of cap analog to GTP 4:1. This allows initiation of the transcript with the cap structure in a large proportion of the synthesized RNA molecules. This approach produces a mixture of transcripts, of which ~80% are capped, and the remainder have 5´-triphosphate ends. Decreased overall yield of RNA products results from the lower concentration of GTP in the reaction (Figure 4, page 3).

There are several cap analogs used in co-transcriptional RNA capping (3,4). The most common are the standard 7-methyl guanosine (m7G) cap analog and anti-reverse cap analog (ARCA), also known as 3´ O-me 7-meGpppG cap analog (Figure 5). ARCA is methylated at the 3´ position of the m7G, preventing RNA elongation by phosphodiester bond formation at this position. Thus, transcripts synthesized using ARCA contain 5´-m7G cap structures in the correct orientation, with the 7-methylated G as the terminal residue. In contrast, the m7G cap analog can be incorporated in either the correct or the reverse orientation.

HiScribe T7 ARCA mRNA Synthesis kits (NEB #E2060 and #E2065) contain reagents, including an optimized mix of ARCA and NTPs, for streamlined reaction setup for synthesis of co-transcriptionally capped RNAs.

TRANSCRIPTION WITH COMPLETE SUBSTITUTION WITH MODIFIED NUCLEOTIDES

RNA synthesis can be carried out with a mixture of modified nucleotides in place of the regular mixture of A, G, C and U triphosphates. For expression applications, the modified nucleotides of choice are the naturally occurring 5´-methylcytidine and/or pseudouridine in the place of C and U, respectively. These have been demonstrated to confer desirable properties to the mRNA, such as increased mRNA stability, increased translation, and reduced immune response in the key applications of protein replacement and stem-cell differentiation (1). It is important to note that nucleotide choice can influence the overall yield of mRNA synthesis reactions.

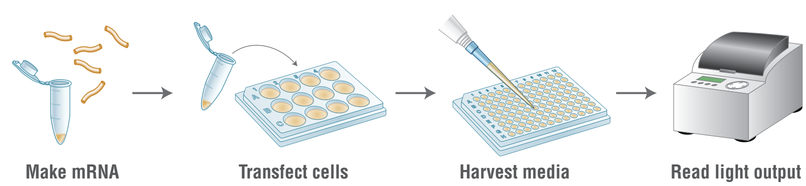
Fully substituted RNA synthesis can be achieved using the HiScribe T7 High-Yield RNA Synthesis Kit (NEB #E2040) or HiScribe SP6 RNA Synthesis Kit (NEB #E2070) in conjunction with NTPs with the desired modification. Transcripts made with complete replacement of one or more nucleotides may be post-transcriptionally capped (see next section), or may be co-transcriptionally capped by including ARCA or another cap analog, as described previously.

If partial replacement of nucleotides is desired, the HiScribe T7 ARCA mRNA Synthesis Kits (NEB #E2060 and #E2065) may be used with added modified NTPs, to produce co-transcriptionally capped mRNAs, as described above. Alternatively, the HiScribe T7 Quick RNA Synthesis Kit (NEB #E2050) may be used to prepare transcripts for post-transcriptional capping (see below).

Analysis of capped RNA function in transfected mammalian cells

(A) Schematic representation of reporter mRNA transfection workflow. (B) Expression of *Cypridina* luciferase (CLuc) after capping using different methods. High activity from all capped RNAs is observed.

A.



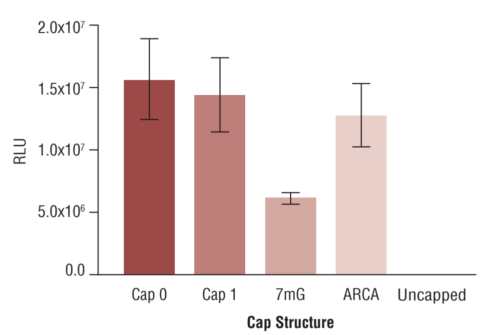
The effect of capping can be studied by delivering the mRNA to cultured mammalian cells and monitoring its translation. Using RNA encoding secreted luciferases (e.g., *Cypridina* luciferase, CLuc) the translation can be monitored by assaying its activity in the cell culture medium (Fig. A).

CLuc mRNA was synthesized and capped post-transcriptionally (Cap 0 or Cap 1) or co-transcriptionally (as described above) using standard (7mG) or anti-reverse cap analog (ARCA). For consistency, the mRNAs were prepared from templates encoding poly-A tails of the same length.

After capping, the mRNA was purified using magnetic beads and quantified before transfection into U2OS cells using the TransIT® mRNA transfection reagent following the manufacturer's protocol. CLuc activity was measured 16 hrs after transfection using the BioLux® *Cypridina* Luciferase Assay Kit (NEB #E3309).

Virtually no luciferase reporter activity was observed in conditions where uncapped RNA was transfected (Fig. B). In contrast, robust activity was detected from cells transfected with RNA capped using the methods described above. As anticipated, lower activity was observed from cells transfected with mRNA capped using the 7mG cap analog as compared to ARCA-capped mRNA.

B.



POST-TRANSCRIPTIONAL CAPPING AND CAP-1 METHYLATION

Post-transcriptional capping is performed using the mRNA capping system from Vaccinia virus. This enzyme complex converts the 5´-triphosphate ends of *in vitro* transcripts to the m7G-cap structures. The Vaccinia Capping System (NEB #M2080) comprises three enzymatic activities (RNA triphosphatase, guanylyl- transferase, guanine N7-methyltransferase) that are necessary for the formation of the complete Cap-0 structure, m7Gppp5´N, using GTP and the methyl donor S-adenosylmethionine. As an added option, the inclusion of the mRNA Cap 2´ O-Methyltransferase (NEB #M0366) in the same reaction results in formation of the Cap-1 structure, which is a natural modification in many eukaryotic mRNAs. This enzyme-based capping approach results in the highest proportion of capped message, and it is easily scalable. The resulting capped RNA can be further modified by poly(A) addition before final purification.

A-TAILING USING *E. COLI* POLY(A) POLYMERASE

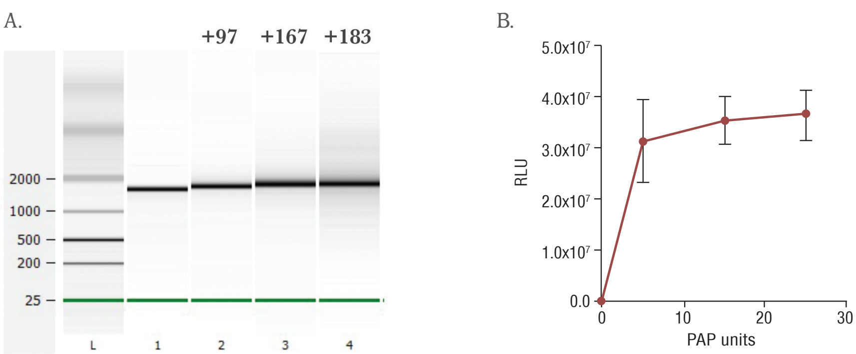
The poly(A) tail confers stability to the mRNA and enhances translation efficiency. The poly(A) tail can be encoded in the DNA template by using an appropriately tailed PCR primer, or it can be added to the RNA by enzymatic treatment with *E. coli* Poly(A) Polymerase (NEB #M0276). The length of the added tail can be adjusted by titrating the Poly(A) Polymerase in the reaction (Figure 6).

The importance of the A-tail is demonstrated by transfection of untailed vs. tailed mRNA. When luciferase activity from cells transfected with equimolar amounts of tailed or untailed mRNAs were compared, a significant enhancement of translation efficiency was evident (Figure 6). HiScribe T7 ARCA mRNA Synthesis Kit (with tailing) (NEB #E2060) includes *E. coli* Poly(A) Polymerase, and enables a streamlined workflow for the enzymatic tailing of co-transcriptionally capped RNA.

For mRNA synthesis from templates with encoded poly(A) tails, the HiScribe T7 ARCA mRNA Synthesis Kit (NEB #E2065) provides an optimized formulation for co-transcriptionally capped transcripts.

FIGURE 6:
Analysis of capped and polyadenylated RNA

(A) Agilent® Bioanalyzer® analysis of capped and polyadenylated RNA. Longer tails are produced by increasing the enzyme concentration in the reaction. Calculated A-tail lengths are indicated over each lane. Lanes: L: size marker,1: No poly-A tail, 2: 5 units, 3 :15 units, 4 : 25 units of *E. coli* Poly(A) Polymerase per 10 µg CLuc RNA in a 50 µl reaction. (B) Effect of enzymatic A-tailing on the luciferase reporter activity of CLuc mRNA.



SUMMARY

In summary, when choosing the right workflow for your functional mRNA synthesis needs, you must balance your experimental requirements for the mRNA (e.g., internal modified nucleotides) with scalability (i.e., ease-of-reaction setup vs. yield of final product).

In general, co-transcriptional capping of mRNA with template encoded poly(A) tails or post-transcriptional addition of poly(A) tail is recommended for most applications. This approach, using the HiScribe T7 ARCA mRNA Synthesis Kits (NEB #E2060 and #E2065), enables the quick and streamlined production of one or many transcripts with typical yields of ≥20 µg per reaction, totaling ~400-500 µg per kit.

Post-transcriptional mRNA capping with Vaccinia Capping System is well suited to larger scale synthesis of one or a few mRNAs, and is readily scalable to produce gram-scale quantities and beyond. Reagents for *in vitro* synthesis of mRNA are available in kit form or as separate components to enable research and large-scale production.

Products from NEB are available for each step of the RNA Synthesis Product Workflow

Template Generation	*In vitro* Transcription	RNA Capping	Poly(A) Tailing
Q5® High-Fidelity DNA Polymerase	HiScribe™ T7 ARCA mRNA Synthesis Kit (with tailing)		
	HiScribe T7 ARCA mRNA Synthesis Kit		*E. coli* Poly(A) Polymerase
dNTP solution mixes	HiScribe T7 High Yield RNA Synthesis Kit	Vaccinia Capping System	
Type IIs restriction enzymes & cloning reagents	HiScribe T7 Quick High Yield RNA Synthesis Kit	mRNA Cap 2´-O-Methyltransferase	
	HiScribe SP6 High Yield RNA Synthesis Kit	ARCA and other mRNA cap analogs	
	T3, T7 and SP6 RNA Polymerases		
	Companion Products		
	RNase inhibitors		
	Pyrophosphatases		
	DNase I		
	NTPs		

Products available from NEB for each step of the functional mRNA synthesis workflow, from template construction to tailing, are shown to the left.

References:
1. Grier, A.E., et al. (2016) *Mol. Ther. Nucl. Acids*, 5, e306.
2. Potapov, V. and Ong, J.L. (2013) *PLOS One*, 12 (7): e0181128.
3. Strenkowska, M., et al. (2016) *Nucl. Acids Res.* 44, 9578–9590.
4. Rydzik, A.M., et al. (2017) *Nucl. Acids Res.* 45, 8661–8675.

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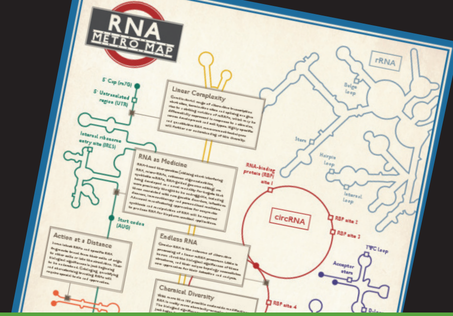

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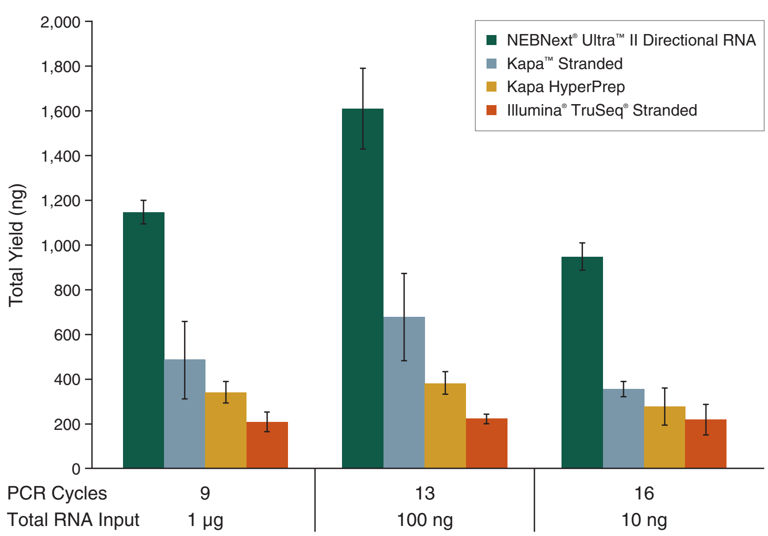
NEBNext Ultra II Directional RNA produces the highest yields, from a range of input amounts

Poly(A)-containing mRNA was isolated from Universal Human Reference RNA (Agilent® #740000) and libraries were made using the NEBNext Ultra II Directional RNA Kit (plus the NEBNext poly(A) mRNA Magnetic Isolation Kit), Kapa Stranded mRNA-Seq Kit, Kapa mRNA HyperPrep Kit and Illumina TruSeq Stranded mRNA Kit. The input RNA amount and number of PCR cycles are indicated. Library yields from an average of three replicates are shown.



1. Parkhomchuck, D., et al. (2009) *Nucleic Acids Res.* 37. e123.
2. Levin, J. Z., et al. (2010) *Nature Methods* 7, 709–715.

Advantages

- **Generate high yield**, high-quality libraries even with limited amounts of RNA (5 ng – 1 µg total RNA)
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- Enjoy the **reliability of the gold standard SPRISelect size selection and clean-up beads**, supplied in just the amounts you need
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NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads	E7765S/L	24/96 rxns
NEBNext Ultra II RNA Library Prep Kit for Illumina	E7770S/L	24/96 rxns
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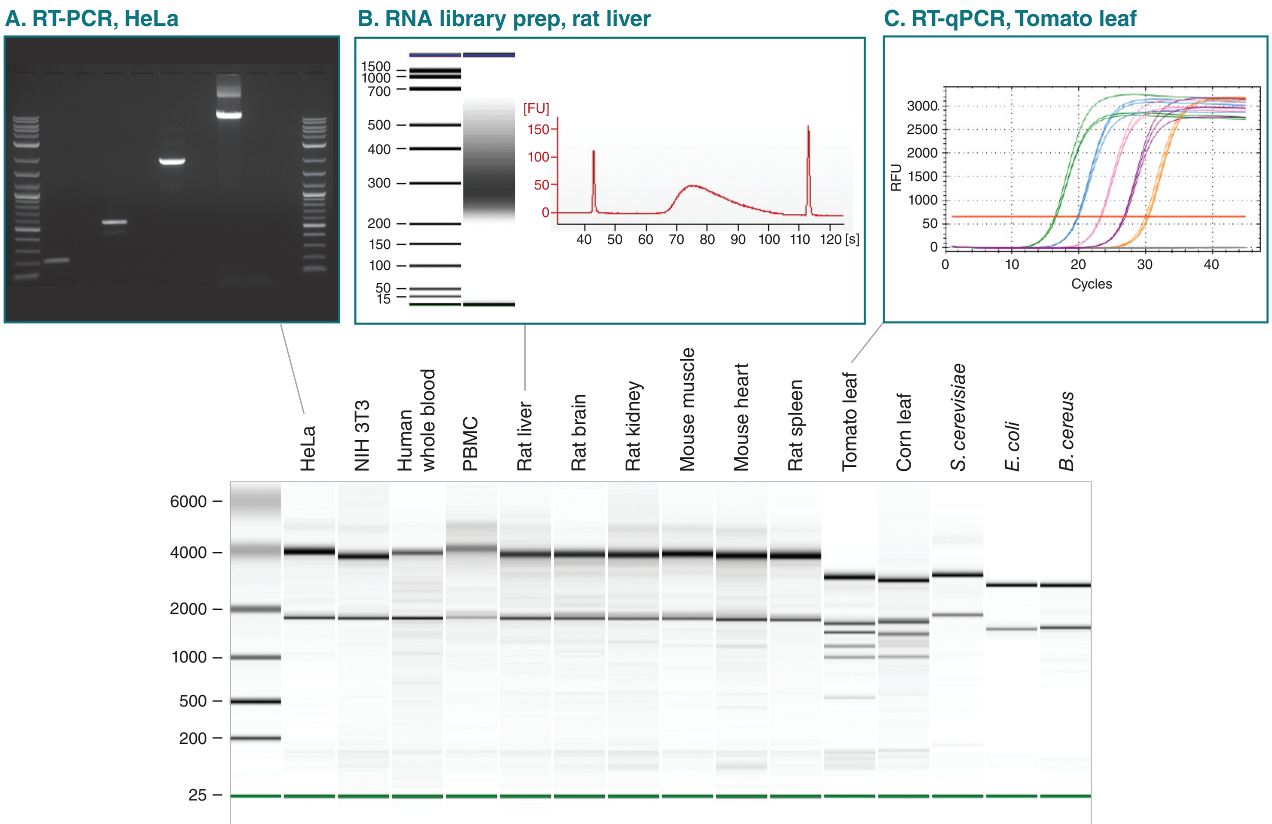
ORDERING INFORMATION

PRODUCT	NEB #	SIZE
Monarch Total RNA Miniprep Kit	T2010S	50 preps
Monarch RNA Purification Columns	T2007L	100 columns

Kit components can also be purchased separately.

Monarch-purified RNA is high-quality and compatible with a wide variety of downstream applications

Total RNA from a broad array of sample types was purified using the Monarch Total RNA Miniprep Kit (NEB #T2010). Aliquots were run on an Agilent® Bioanalyzer® 2100 using the Nano 6000 RNA chip (*S. cerevisiae* RNA was run using a plant Nano assay). RIN values and O.D. ratios confirm the overall integrity and purity of the RNA. To demonstrate compatibility with downstream applications, samples were subsequently used for RT-PCR (+/– RT) (A) for detection of 4 different RNA species using Protoscript® II Reverse Transcriptase (NEB #M0368)/LongAmp® *Taq* DNA Polymerase (NEB #M0323), NGS library prep (B) using NEBNext® Ultra™ II RNA Library Prep Kit (NEB #E7760) and RT-qPCR (C) using Luna® One-Step RT-qPCR Reagents (NEB #E3005).



	HeLa	NIH 3T3	Human whole blood	PBMC	Rat liver	Rat brain	Rat kidney	Mouse muscle	Mouse heart	Rat spleen	Tomato leaf	Corn leaf	*S. cerevisiae*	*E. coli*	*B. cereus*
RIN	9.8	9.8	8.0	7.6	9.2	8.8	8.6	9.4	8.9	9.2	8.0	7.9	9.9	9.8	10
$A_{260/280}$	2.01	2.08	2.04	2.04	2.06	2.07	2.04	2.06	2.09	2.06	2.10	2.09	2.05	2.11	2.11
$A_{260/230}$	2.21	2.23	2.11	2.35	2.07	2.18	2.14	2.22	2.41	2.25	2.38	2.30	2.36	2.41	2.46

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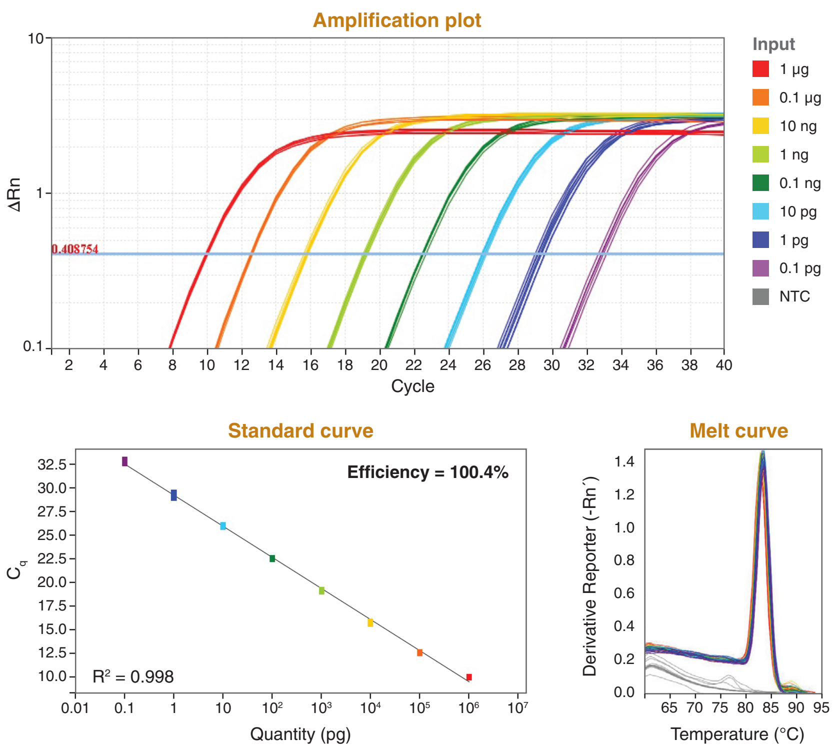
The Luna RT-qPCR kits contain a novel, *in silico*-designed reverse transcriptase (RT) engineered for improved performance. Both the Luna WarmStart Reverse Transcriptase and Hot Start *Taq* DNA Polymerase, included in these kits, utilize a temperature-sensitive, reversible aptamer, which inhibits activity below 45°C. This enables room temperature reaction setup and prevents undesired non-specific activity. Furthermore, Luna WarmStart RT has increased thermostability, improving performance at higher reaction temperatures.

Luna WarmStart Reverse Transcriptase exhibits exceptional sensitivity, reproducibility and RT-qPCR performance

RT-qPCR targeting human GAPDH was performed using the Luna Universal One-Step RT-qPCR Kit, featuring Luna WarmStart Reverse Transcriptase, over an 8-log range of input template concentrations (0.1 pg – 1 µg Jurkat total RNA) with 8 replicates at each concentration. NTC = non-template control.



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- WarmStart RT paired with Hot Start *Taq* increases reaction specificity and robustness

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Luna Universal Probe One-Step RT-qPCR Kit	E3006S/L	200/500 rxns

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		T7 KITS				SP6 KITS
	APPLICATION	**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 SP6 RNA SYNTHESIS KIT** (#E2070)
Probe labeling	Fluorescent labeling: FAM, Cyanine (Cy) dyes, etc. • Fluorescent *in situ* hybridization (FISH)		✓			✓
	Non-fluorescent labeling: Biotin, Digoxigenin • *In situ* hybridization • Blot hybridization with secondary detection • Microarray		✓			✓
	High specific activity radiolabeling • Blot hybridization • RNase protection	✓				✓
mRNA & RNA for transfection	Streamlined mRNA synthesis with ARCA co-transcriptional capping and enzymatic poly(A) tailing • Transfection • Microinjection • *In vitro* translation				✓	✓
	Streamlined ARCA capped RNA synthesis • Template encoded poly(A) tails • Microinjection • Non polyadenylated transcripts • *In vitro* translation • Transfection			✓		✓
	Co-transcriptional capping with alternate cap analogs • Transfection • Microinjection • *In vitro* translation		✓			✓
	Post-transcriptional capping with Vaccinia Capping System • Transfection • Microinjection • *In vitro* translation	✓	✓			✓
	Complete substitution of NTPs: 5-mC, pseudouridine, etc. • Induction of stem cell pluripotency • Modulation of cell fate or phenotype • Post translational capping with Vaccinia mRNA Capping System	✓				✓
	Partial substitution of NTPs: 5-mC, pseudouridine, etc.		✓	✓	✓	✓
	Unmodified RNA		✓			✓
	Hairpins, short RNA, dsRNA • Gene knockdown		✓			✓
Structure, function, & binding studies	Complete substitution of NTPs • Aptamer selection • Isotopic labeling	✓				✓
	Partial substitution of one or more NTPs • Aptamer selection • Structure determination		✓			✓
	Unmodified RNA • SELEX • Structure determination		✓			✓

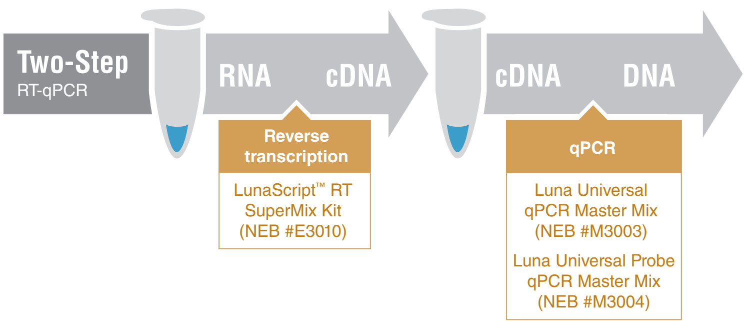
Illuminate your Two-step RT-qPCR

NEW PRODUCT

LunaScript™ RT SuperMix Kit

Our latest addition to the Luna qPCR/RT-qPCR portfolio, the LunaScript RT SuperMix Kit is optimized for first strand cDNA synthesis in the context of a two-step RT-qPCR workflow. It delivers best-in-class performance, user-friendly protocols, and includes a convenient blue dye to track your sample.

The cDNA products generated by LunaScript have been extensively evaluated in qPCR using the Luna® qPCR Master Mixes. In combination, these products provide a two-step RT-qPCR workflow with excellent sensitivity and accurate, linear quantitation.

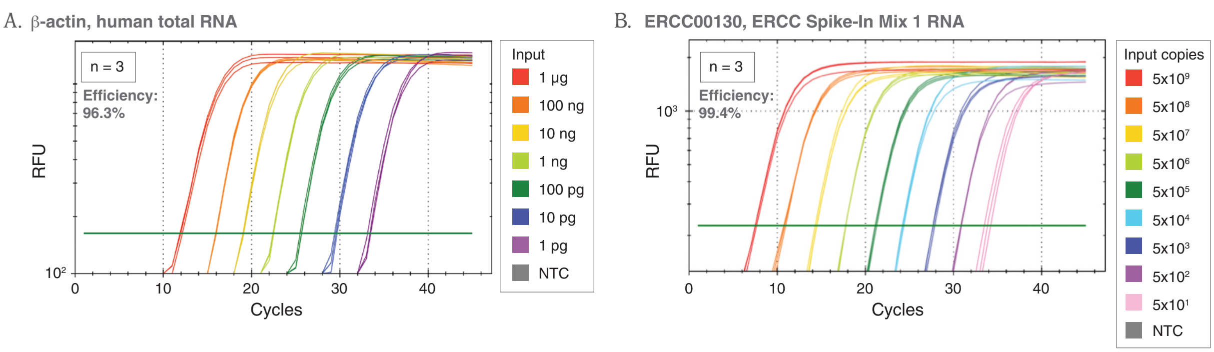


Advantages

- **Simplify reaction setup** with convenient supermix format
- **Eliminate pipetting errors** with non-interfering, visible tracking dye
- Synthesize cDNA in **less than 15 minutes**
- Experience **best-in-class performance**, as all Luna products have undergone rigorous testing to optimize specificity, sensitivity, accuracy and reproducibility
- Enjoy consistent linearity, sensitivity, and capacity for **reliable RNA quantification**

The LunaScript RT SuperMix Kit offers exceptional sensitivity, linearity, and reproducibility in two-step RT-qPCR workflows

RNA was converted to cDNA using the 1X LunaScript RT SuperMix in 20 µl reactions using standard reaction conditions (25°C/2 min, 55°C/10 min, 95°C/1 min). cDNA was then quantitated by qPCR using the Luna Universal qPCR Master Mix (NEB #M3003) and 1 µl of cDNA product as template, with triplicate reactions at each input concentration. A.) A serial dilution of Jurkat total RNA (1 µg – 1 pg) was converted to cDNA and then quantitated by qPCR using a β-actin target. B.) ERCC (External RNA Controls Consortium) mix 1 RNA containing 5x10[9] to 50 copies of ERCC00130 (~10 ng – 10 fg) was converted to cDNA and then quantitated by qPCR.



ORDERING INFORMATION

PRODUCT	NEB #	SIZE
LunaScript RT SuperMix Kit	E3010S/L	25/100 rxns
Luna Universal qPCR Master Mix	M3003S/L	200/500 rxns
Luna Universal Probe qPCR Master Mix	M3004S/L	200/500 rxns

One-step vs. two-step RT-qPCR – which workflow should I choose?

	RT PRIMERS	ADVANTAGES	DISADVANTAGES	IDEAL USES	RECOMMENDED PRODUCTS
One-Step RT-qPCR	• Gene-specific primers	• Quick setup and limited hands-on time • Single, closed-tube reaction, reducing contamination	• Need fresh RNA sample(s) to analyze new targets or repeat experiments	• Assessing many RNA samples • High-throughput applications	*Dye-based detection:* Luna® Universal One-Step RT-qPCR Kit (NEB #E3005) *Probe-based detection:* Luna Universal Probe One-Step RT-qPCR Kit (NEB #E3006)
Two-Step RT-qPCR	• Oligo(dT) primers • Random hexamer primers • Gene-specific primers • A combination of the above	• Choice of RT primers • Flexible reaction optimization (e.g., RNA input, choice of enzyme(s), enzyme amount, and reaction)	• More setup and hands-on time • Greater variation and risk of contamination due to extra open-tube step and pipetting	• Assessing multiple targets from few RNA samples • Saving cDNA product for future re-use	*cDNA synthesis:* LunaScript RT SuperMix Kit (NEB #E3010) *Dye-based detection:* Luna Universal qPCR Master Mix (NEB #M3003) *Probe-based detection:* Luna Universal Probe qPCR Master Mix (NEB #M3004)

Optimization Tips for Luna One-Step RT-qPCR

Successful one-step RT-qPCR is dependent on a number of factors. Controlling variables and giving careful consideration to target selection, primer design and probe design are critical to maximizing your chances of success with Luna One-Step RT-qPCR kits (NEB #E3005, E3006). For more tips and guidelines specific to multiplexing, please visit the Tools and Resources section at neb.com.

Control Variables to Minimize Non-specific Amplification

- Use high quality, purified RNA templates
- Prepare template dilutions in TE or water for each experiment
- Treat with UDG to prevent carryover contamination
- Treat with DNase I to remove any residual genomic DNA
- Follow thermocycler settings best suited to your experiment and reagents
- Due to the dual WarmStart/HotStart feature of the kits, it is not necessary to preheat the thermocycler prior to use
- Reactions should be carried out in triplicate for each sample
- Keep reactions on ice

Target Selection

- Recommended size: 70 to 200 bp
- GC content: 40–60%
- Avoid highly repetitive sequences
- Avoid sequences containing significant secondary structure

Primer Design

- Recommended size: 15–30 nucleotides
- GC content: 40–60%
- Primer Tm: approximately 60°C
  Refer to NEB's Tm Calculator
- Primer pairs should have Tm values within 3° C
- Optimal concentration: 400 mM
- Avoid complementary regions
- Avoid G homopolymer repeats >=4

Hydrolysis Probes

- Recommended size: 15–30 nucleotides
- GC content: 40–60%
- Probe Tm: 5–10°C higher than primer Tms
- Optimal concentration: 200 mM
- Both single- or double-quenched probes can be used
- Avoid 5´-G base

Assay Performance Considerations

- Include No Template and No-RT controls

Data Analysis

- Ensure 90 – 110% PCR efficiency over at least three log10 dilutions of the template
- R2 values >=0.99
- Target specificity should be confirmed by product size, sequencing, or melt-curve analysis

NEB companion products you may be interested in

Monarch RNA Miniprep Kit (NEB #T2010S)

Antartic Thermolabile UDG (NEB #M0372)

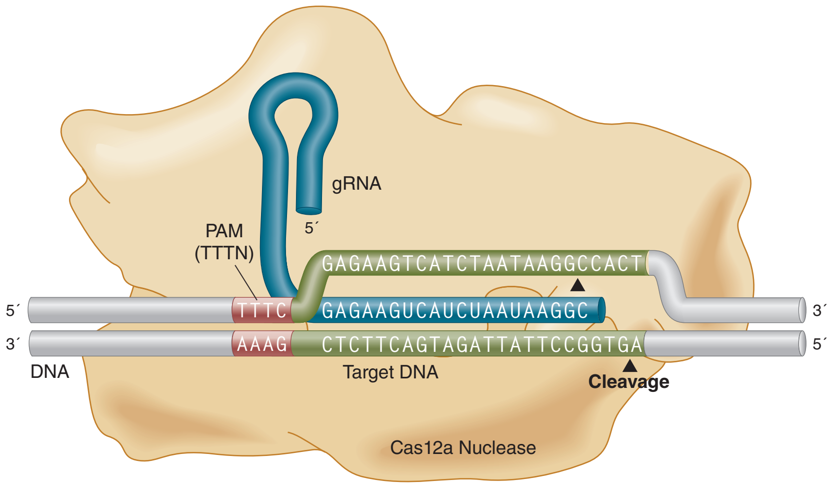
DNase I (NEB #M0303)

Learn more about the **advantages of Luna** on page 8

EnGen® Lba Cas12a (Cpf1)

EnGen Lba Cas12a (Cpf1) is a programmable DNA endonuclease guided by a single guide RNA (gRNA). Targeting requires a gRNA complementary to the target site as well as a 5' TTTN protospacer adjacent motif (PAM) on the DNA strand opposite the target sequence. Cleavage by EnGen Lba Cas12a (Cpf1) occurs ~18 bases 3' of the PAM and leaves 5' overhanging ends.

Schematic representation of Lba Cas12a nuclease sequence recognition and DNA cleavage



Advantages

- TTTN PAM sequence opens up additional genomic regions for targeting
- Shorter, 40–44 base guide RNA
- Two nuclear localization signals for improved transport to the nucleus
- Maintains activity at lower temps. (16–48°C) than the *Acidaminococcus* orthologs, permitting editing in ectothermic organisms, such as zebrafish and Xenopus
- High concentration liquid format can be used for microinjection, electroporation and lipofection

"This is really exciting – the zebrafish community has been waiting eagerly for an orthogonal CRISPR system that works as well as Cas9."

– J.G., Assistant Professor of Biology, University of Utah

ORDERING INFORMATION

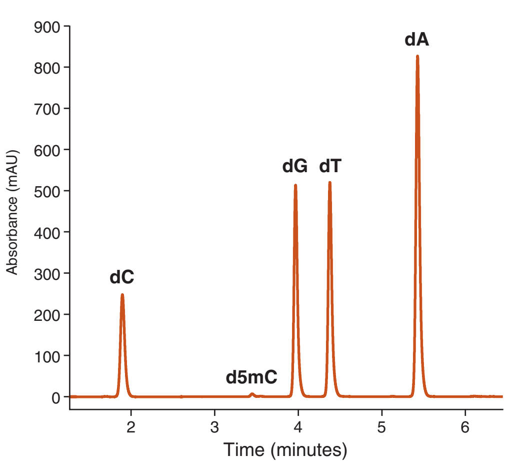
PRODUCT	NEB #	SIZE
EnGen Lba Cas12a (Cpf1)	M0653S/T	70/2,000 pmol

Nucleoside Digestion Mix

The Nucleoside Digestion Mix is an optimized mixture of enzymes that provides a convenient one-step method to generate single nucleosides from DNA or RNA for quantitative analysis by liquid chromatography-mass spectrometry (LC-MS), eliminating the need for sequential multi-step, time-consuming digestion protocols.

Global nucleoside analysis of HeLa DNA following incubation with the Nucleoside Digestion Mix

Representative HPLC chromatogram of individual deoxyribonucleosides obtained from incubation of 1 µg of purified genomic HeLa DNA digested with 1 µl of the Nucleoside Digestion Mix for 1 hour at 37°C. Deoxyribonucleosides were separated by reversed-phase HPLC and detected by UV absorbance at 260 nm.



Advantages

- Convenient one-step protocol
- Digests both DNA and RNA to single nucleosides
- Low-glycerol formulation significantly reduces glycerol-induced ion suppression during MS analysis

"I am very happy with the NEB Nucleoside Digestion Mix. Not only does it provide greater levels of digestion, but it allows me to degrade both DNA and RNA simultaneously."

– R.K., Assistant Professor, University of Pennsylvania

ORDERING INFORMATION

PRODUCT	NEB #	SIZE
Nucleoside Digestion Mix	M0649S	50 rxns

A fast one-step digestion of DNA or RNA for global detection and characterization of nucleotide modifications using the Nucleoside Digestion Mix

Ivan R. Corrêa Jr., Nan Dai and Shengxi Guan, New England BioIabs, Inc.

Introduction

The Nucleoside Digestion Mix (NEB #M0649) is an optimized mixture of enzymes that provides a convenient one-step method to generate single nucleosides for quantitative analysis by liquid chromatography-mass spectrometry (LC-MS). It digests ssDNA, dsDNA, and RNA, and tolerates a wide range of base and ribose modifications (1-11). The Nucleoside Digestion Mix also shows activity towards unnatural nucleobases, as demonstrated by Floyd Romesberg in collaboration with scientists at NEB; this work describes how bases lacking any hydrogen bonds are propagated with high fidelity *in vivo* in *E. coli.* (12). The digestion protocol is fast and very reliable, and has greatly facilitated the characterization and global quantification of DNA and RNA modifications (1-11). Moreover, the low-glycerol formulation (< 1%) significantly reduces glycerol-induced ion suppression during mass analysis.

In this application note, we present examples of how the Nucleoside Digestion Mix has been used to quantify epigenetic DNA modifications, to measure the activity of nucleic acid-modifying enzymes, and to monitor the metabolic incorporation of azido-modified nucleosides into cellular RNA.

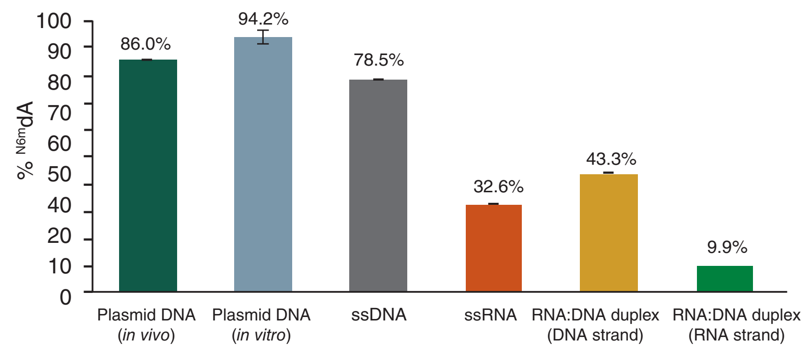
Quantification of Epigenetic Modifications

Epigenetic modification of DNA nucleobases exists in a wide variety of organisms, and plays important roles in both prokaryotes and eukaryotes. The most studied epigenetic modification is DNA methylation, including N6-methyladenosine (N6mA), 5-methylcytosine (5mC), and N4-methylcytosine (N4mC). In prokaryotes, these modifications are involved in protecting bacterial genomes from restriction endonucleases, which target invading bacteriophage DNAs. In eukaryotes, cytosine methylation is reported to play important roles in the control of gene expression, parental imprinting, and developmental regulation in both physiological and pathological conditions. Recent studies have shown that 5mC can be successively oxidized to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) by the ten-eleven translocation (TET) family of enzymes (13).

Several approaches have been utilized to study DNA modifications, including thin layer chromatography (TLC), liquid chromatography-mass spectroscopy (LC-MS), modification-specific antibodies and restriction endonucleases, and chemical labeling. The main challenge with the quantification of modified nucleobases in genomic samples is the relatively low levels of these modifications. The Nucleoside Digestion Mix has enabled the accurate quantitation of cytosine modifications by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with sensitivity down to < 0.002% relative abundance (~ 2 modifications per 100,000 bases, data not shown) (4).

FIGURE 1: **The characterization of the non-specific nature of the adenine DNA methyltransferase M.EcoGII (NEB #M0603) using the Nucleoside Digestion Mix.**

EcoGII Methyltransferase-treated DNA and RNA samples were converted to nucleosides using the Nucleoside Digestion Mix, and analyzed in duplicate using LC-MS. In each sample, the relative percentage the dA bases methylated was calculated as ^{N6m}dA/(^{N6m}dA+dA). Samples analyzed were as follows: *In vivo* assay, from a pRRS:M.EcoGII high-copy replicon that constitutively expresses the EcoGII Methyltransferase introduced into methylation-deficient ER2796 *E. coli* cells; *In vitro* assay, from purified pUC19 plasmid DNA; ssDNA, from single-stranded M13mpl8 bacteriophage DNA; ssRNA, from a 1.8 kb *in vitro* transcribed F-luc RNA; RNA:DNA hybrid duplex, from a a synthetic 48 mer DNA:RNA hybrid oligonucleotide substrate (containing 10 dA bases in the DNA strand and 18 rA bases in the RNA strand, respectively).



Measuring the Activity of Nucleic Acid Modifying Enzymes

Typical methods for assaying the catalytic activity of enzymes that modify DNA and RNA employ radioactivity, fluorescent substrates, or antibody-based immune assays. Direct detection is difficult since nucleic acids are large, highly structured polymers and their modifications are often silent to commonly-used detection techniques, such as PCR and gel electrophoresis. LC-MS is a direct and accurate method for quantification of DNA and RNA modifications such as methylation, oxidation or glycosylation. As an example, the non-specific nature of the adenine DNA methyltransferase M.EcoGII (NEB #M0603) was determined by LC-MS analysis of M.EcoGII-treated DNA and RNA samples digested with the Nucleoside Digestion Mix. This enzyme can catalyze methylation of up to 86% of dA residues of DNA substrates *in vivo* and 96% *in vitro,* thereby rendering them insensitive to cleavage by multiple restriction endonucleases (11). Additionally, M.EcoGII is able to methylate single-stranded RNA and DNA-RNA hybrid substrates (Figure 1).

Monitoring the Metabolic Incorporation of the Azide Functionality into Cellular RNA

Tracking RNA transcription and post-transcriptional regulation is critical to understanding the cellular mechanisms underlying healthy and diseased states. One of the techniques used to interrogate the function of coding and non-coding RNAs is to incorporate modified nucleosides into cellular transcripts. The combination of the Nucleoside Digestion Mix with LC-MS analysis has been used to detect and quantify the incorporation of azido nucleosides into cellular RNA (9). Cells treated with chemically synthesized N^6-ethylazido adenosine (N^6-EtN_3A), N^6-propylazido adenosine (N^6-PrN_3), and 2´-azidoadenosine (2´N_3A) incorporated 0.2-0.3% of these nucleoside analogues relative to canonical adenosine (data not shown). Azidonucleosides are utilized for labeling and real-time imaging of nascent RNA (9).

References:
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12. Malyshev, D. A., et al. (2014) *Nature,* 509, 385-388.
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Interested in learning more?

Visit the literature tab at **www.neb.com/M0649** to **download the full application note**, which includes data and tables not shown.

Cappable-seq for prokaryotic transcription start site (TSS) determination

Cappable-seq is a method for directly enriching the 5´ end of primary transcripts developed at NEB. This is achieved by capping the 5´ triphosphorylated end of RNA with the Vaccinia Capping System (NEB #M2080) and 3´-Desthiobiotin-GTP (NEB #N0761). The primary transcripts are enriched by binding to Hydrophilic Streptavidin Magnetic Beads (NEB #S1421), followed by washing and eluting with biotin. This method enables determination of transcription start sites at single base resolution (1).

Protocol:

Desthiobiotin-GTP capping of prokaryotic RNA:

1. Prepare total RNA from prokaryotic source at a concentration of 300 ng/µl in water or 1.0 mM Tris pH 7.5, 0.1 mM EDTA.

2. Prepare the capping reaction as follows:

Component	Reaction
RNA	10 µl
10X VCE Buffer	5 µl
ddH_2O	25 µl
Total	**40 µl**

3. Incubate reaction for 2 minutes at 70°C.

4. Place the reaction on ice.

5. Add the following to above reaction:

Component	Reaction
5 mM 3'DTB-GTP	5 µl
Vaccinia Capping Enzyme (10 units/µl)	5 µl
Total	**50 µl**

6. Incubate the reaction at 37°C for 30 minutes. Immediately proceed to cleanup.

Note: *We have recently found that capping with DTB-GTP is more efficient in the absence of S-Adenosylmethionine (SAM).*

RNA Cleanup:

1. Purify RNA on a Zymo Research's Clean and Concentrator™-5 column using manufacturer instructions for > 200 nucleotide RNA; with a total of 4 washes with RNA wash buffer.

Note: *wash the sides of the column that may have come into contact with the capping reaction, reducing the carryover of DTB-GTP.*

2. Elute the RNA in 100 µl of 1 mM Tris pH 7.5, 0.1 mM EDTA (low TE).

Note: *it is essential to completely remove unincorporated DTB-GTP (less than 0.01% DTB-GTP remaining). Alternative methods of RNA cleanup such as AMPure beads can also be employed. If the RNA is going to be directly bound to streptavidin before the following fragmentation step, an additional cleanup step should first be employed.*

Enrichment of RNA

1. Fragment desthiobiotin-GTP-capped RNA by setting up the following reaction:

Component	Reaction
10X Polynucleotide Kinase Buffer	2.5 µl
Capped RNA	100 µl

2. Incubate for 5 minutes at 94°C. Put on ice.

3. Clean up RNA as follows:

A) *Bind RNA to AMPure XP beads:*
Add 1.8 volumes of AMPure beads to the eluted RNA volume and add 1.5 volumes of 100% ethanol to the resulting volume of the AMPure/RNA mix (i.e., if volume of RNA is 100 µl, add 180 µl of AMPure beads and 420 µl of ethanol). Incubate the beads on the bench for 5 minutes, then expose to a magnet and wash beads 2 times with 80% ethanol while confined with the magnet. Remove the tube from the magnet and elute the RNA in 75 µl of low TE.

B) *Remove 3´ phosphates from fragmented RNA:*
To 75 µl of the eluted RNA, add the following:

Component	Reaction
10X T4 Polynucleotide Buffer	8.8 µl
ATP-free T4 Polynucleotide Kinase	4 µl
Total	**87.8 µl**

Incubate the reaction at 37°C for 15 minutes. Directly proceed to Streptavidin enrichment.

C) *1st round of streptavidin enrichment:*
Hydrophilic Streptavidin Magnetic Beads (NEB #S1421S) are prepared by washing 2 times with 400 µl of 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA and 2 times with 400 µl of 10 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA and suspended in their original suspension concentration of 4 mg/ml in the 500 mM NaCl wash buffer. Add the DTB-GTP capped RNA from step B to 30 µl of the prewashed streptavidin beads and incubate at room temperature with occasional resuspension for 20 minutes. Wash the beads 2 times with 200 µl of 10 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA, and 2 times with 200 µl of 10 mM Tris-HCl pH 7.5, 50 mM NaCl and 1 mM EDTA to remove unbound material.

D) *Elute RNA from the streptavidin beads:*
Resuspend the beads in 30 µl of 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA and 1 mM biotin. Incubate the beads for 20 minutes at room temperature with occasional resuspension. Collect the biotin-eluted RNA by placing the tube on the magnetic rack.

E) *Clean-up eluted RNA:*
Bind the eluted RNA to AMPure XP beads by adding 1.8 volumes of AMPure beads to one eluted RNA volume. Add 1.5 volumes of 100% ethanol to the resulting volume of the AMPure/RNA mix (i.e., if 30 µl of RNA was recovered from the beads, add 54 µl of AMPure Beads and 126 µl of ethanol). Wash the beads with 80% ethanol 2 times, air dry for 5 minutes on bench, and elute the RNA with 30 µl low TE.

F) *2nd Round of streptavidin enrichment:*
Add 30 µl of the RNA eluate to 30 µl of prewashed streptavidin beads for a second round of enrichment. Wash and elute the streptavidin beads as above. Collect and bind the biotin-eluted RNA to AMPure beads as above, and elute with 30 µl low TE.

G) *Decapping (prior to 5´ end ligation):*
Remove the desthiobiotin cap to leave a 5´ monophosphate terminus by adding 3.3 µl of 10X Thermopol Buffer (NEB #B9004) and 3 µl (15 units) of RppH (NEB #M0356S) and incubate for 60 minutes at 37°C. Terminate the reaction by adding 0.5 ul of 0.5 M EDTA and heat to 94°C for 2 minutes. Bind the RNA to AMPure beads as described above. Wash and elute in 20 µl low TE. The eluted RNA is the starting RNA for library preparation using the NEBNext Small RNA Library Prep Set for Illumina (NEB #E7330).

RNA sequencing library prep:

The NEBNext Small RNA Library Prep Set for Illumina® (NEB# E7330) can be used to generate an Illumina sequencing library. The library is amplified through 15 cycles of PCR. RNA sequencing can be performed on an Illumina MiSEQ® with single reads of 100 bases using V3 illumina platform. Visit **www.neb.com/E7330** to access library preparation protocols.

Learn more about cappable-seq in a publication from NEB scientists:

(1) Ettwiller, L., Buswell, J., Yigit, E. and I. Schildkraut (2016) A novel enrichment strategy reveals unprecedented number of novel transcription start sites at single base resolution in a model prokaryote and the gut microbiome. *BMC Genomics,* 17, 199, doi: 10.1186/s12864-016-2539-z

Practicing sustainable science

New England Biolabs is committed to promoting ecologically sound business practices and environmental sustainability in order to protect our natural resources, both locally and globally. Further, it is our goal to improve our business processes to minimize our impact on the environment. This can be challenging, as bench science often generates a significant amount of plastic waste and can consume a large amount of energy, water and specialized chemicals. NEB has long instituted green business practices into our daily operations, including extensive lab recycling and composting programs, a shipping box recycling program and even incorporating sustainability into our product design.

Danielle Freedman has been a member of NEB's Marketing Team for 11 years and is actively involved in NEB's sustainable initiatives. Below, Danielle offers some simple ideas that can promote sustainability in your lab, without compromising results.

Danielle Freedman, Product Marketing Manager

Reduce, Reuse and Recycle Packaging

When you are ordering direct from NEB, be sure to take advantage of our shipping box recycling program. Simply use the pre-paid return label, and send the shipping container back to NEB at no charge.

Another way to reduce packaging waste from individual lab orders, and potentially maximize your university discount, is to order through an **NEBNow® Freezer Program**, or utilize an onsite supply center. Take advantage of onsite access to products and eliminate those individual shipments. Visit www.neb.com/freezer-programs to see if there is a freezer program at your institution.

Our Monarch Nucleic Acid Purification kit packaging was carefully designed using sustainably-sourced materials. One of the great things about them is that their boxes can be re-used in the laboratory. Customers have shared fun ideas for upcycling their Monarch boxes with us, including a lovely succulent garden to brighten your bench.

View additional Monarch upcycle submissions on our Instagram page @nebiolabs.

Recycle Lab Equipment

Start by looking around your lab. Are there pieces of equipment that your lab no longer uses? Perhaps you recently acquired a new thermocycler, or a recirculating water bath, and the older versions are now taking up valuable space on your lab bench. Why not donate them to scientists and researchers who could put them to use? It's easier than you would think. Organizations such as **Seeding Labs**, (https://seedinglabs.org/) accept donated laboratory equipment and provide it to scientists around the globe along with the training to ensure its utility is realized. And just like that, you helped enable the global research community.

Share Resources

Take a hard look at the chemical shelves in your lab. How many of those chemicals and solutions do you and your labmates use daily? Have you considered setting up a common chemical room for laboratories to share? For example, institutional departments, such as **Harvard's Chemistry and Chemical Biology Department** (https://chemistry.harvard.edu/) have put in place common chemical inventory documents to encourage resource sharing. This translates into a reduction in the amount of chemicals ordered, and ultimately a reduction in the hazardous waste for disposal.

Get Inspired/Informed

The number of great resources for green lab practices are growing. NEB is proud to be the sponsor of **Labconscious**, (http://www.labconscious.com/) a website devoted to sharing stories of sustainability successes, as well as identifying green lab supplies and equipment to avoid toxic materials, reduce energy and conserve water. **My Green Lab** (https://www.mygreenlab.org/) is another resource with a wealth of information on greening your lab.

If you have any suggestions for how to improve sustainability in the laboratory, we would love to hear from you!

labconscious

Labconscious is an open resource website for scientists to share green lab initiatives to reduce lab waste, use green chemistry, conserve water and save energy.

Raise awareness by sharing ideas, protocols, and best practices

Empower scientists to make changes at their bench, in their lab, and across their work space

Inspire scientists to join the movement and teach others

labconscious.com

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