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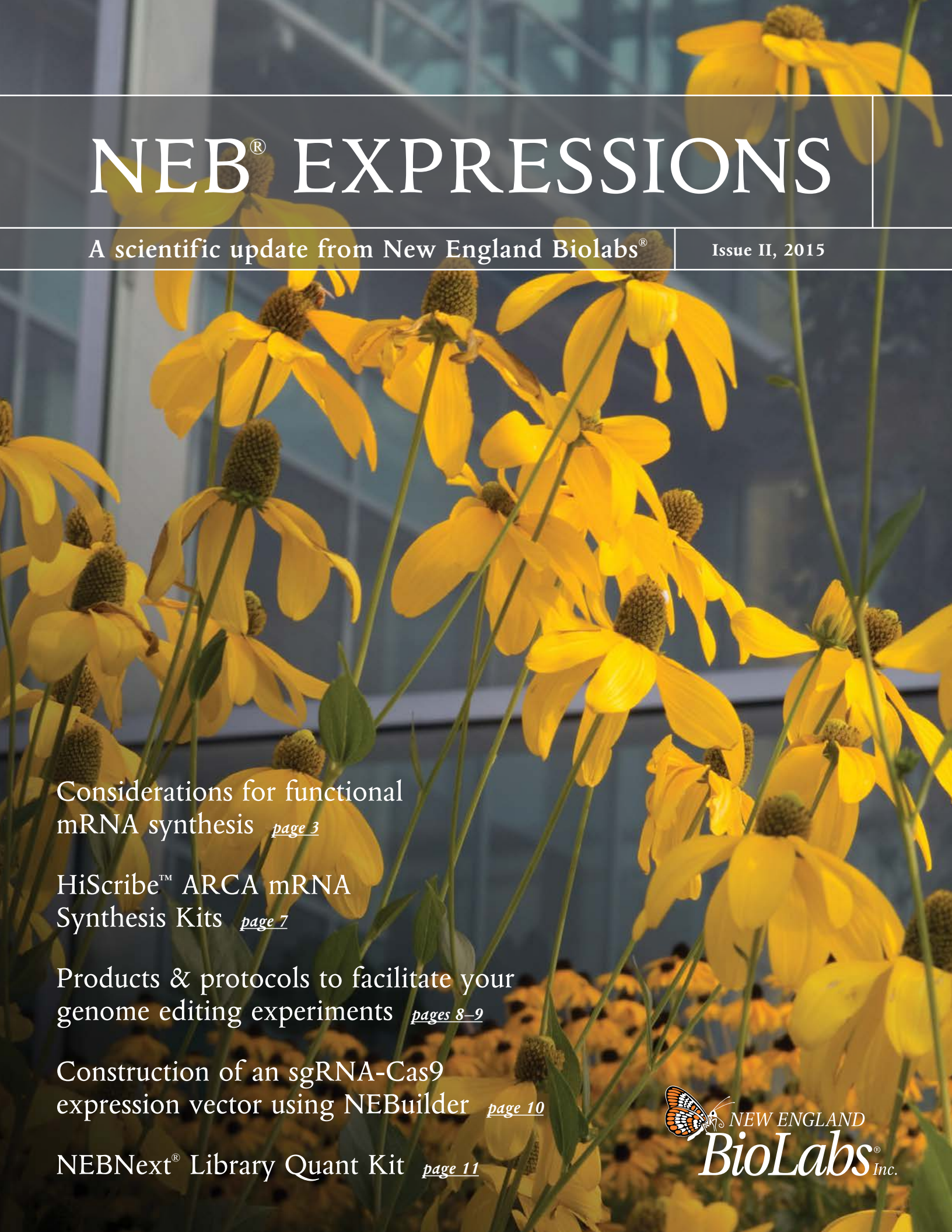
Issue II, 2015



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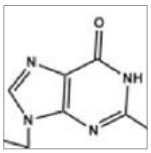
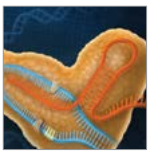
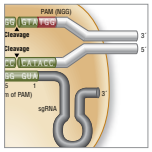
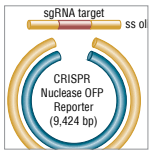








COVER PHOTO

Yellow coneflowers (*Ratibida pinnata*) grow on the NEB campus.

Minding your caps and tails – considerations for functional mRNA synthesis

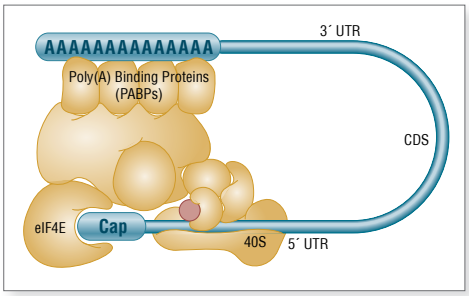
Applications of synthetic mRNA have grown and become considerably diversified in recent years. Examples include the generation of pluripotent stem cells (1-3), vaccines and therapeutics (4), and CRISPR/Cas9 genome editing applications (5-7). The basic requirements for a functional mRNA – a 7-methylguanylate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

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

Before translation in eukaryotic organisms, nascent mRNA (pre-mRNA) receives 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 translation initiation. The second modification is the addition of approximately 200 adenylate nucleotides (a poly(A) tail) to the 3′ end of pre-mRNA by *E. coli* Poly(A) Polymerase. Polyadenylation is coupled to transcription termination, export of mRNA from the nucleus, and, like the cap, formation of the translation initiation complex. The mature mRNA forms a circular structure 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))

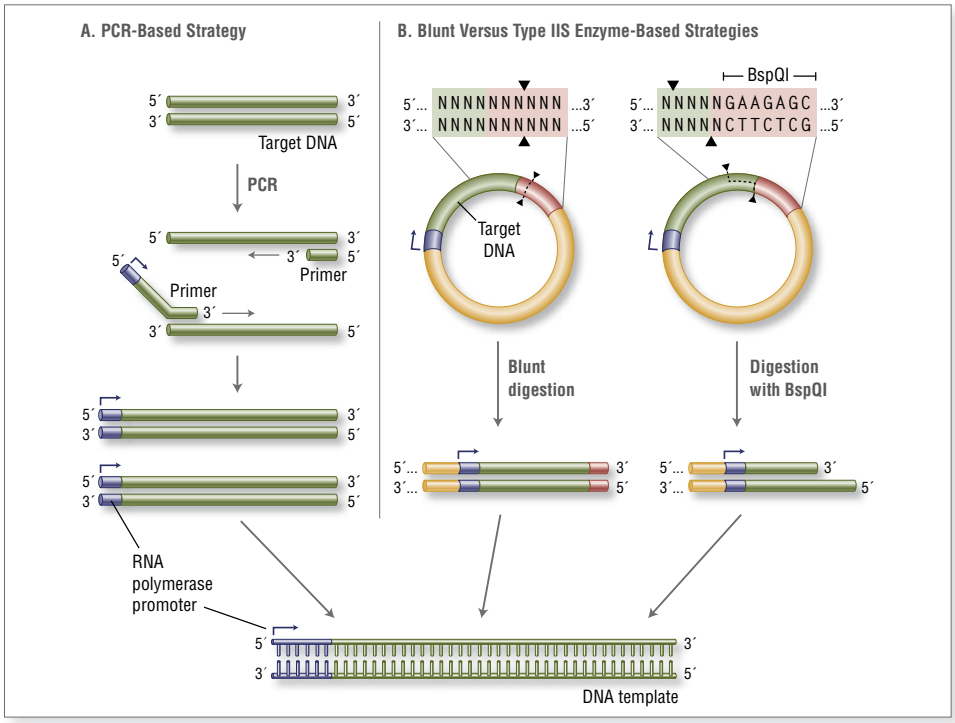
RNA can be efficiently synthesized *in vitro* with prokaryotic phage polymerases, such as T7, T3 and SP6. The cap and poly(A) tail structures characteristic of mature mRNA can be added during or after the synthesis by enzymatic reactions with capping enzymes and Poly(A) Polymerase, respectively.

Figure 1. Translation initiation complex.



The mature RNA forms a circular structure connected by protein complexes that bind the cap structure and 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.

There are several factors to consider when planning for *in vitro* 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.

PCR allows conversion of any DNA fragment to a transcription template by appending the T7 promoter to the forward primer (Fig 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.

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.

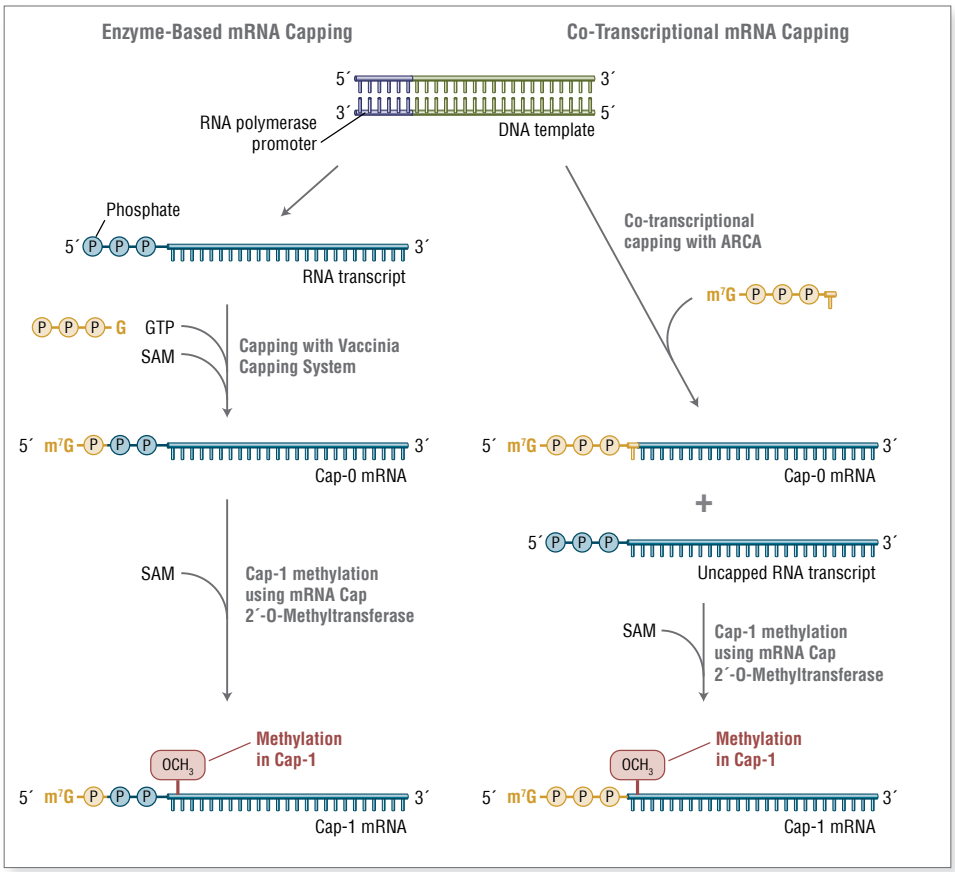
Plasmid templates are convenient if the template sequence already exists in a eukaryotic expression vector also containing the T7 promoter (e.g., pcDNA vector series). These templates include 5´- and 3´-untranslated regions (UTR), which are important for the expression characteristics of the mRNA.

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, 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). The plasmid DNA should be completely digested with the restriction enzyme, followed by purification using a spin column 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.

In vitro transcription

There are two options for the *in vitro* transcription 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) (Fig. 3). Method selection will depend on the scale of mRNA synthesis required and number of templates to be transcribed.

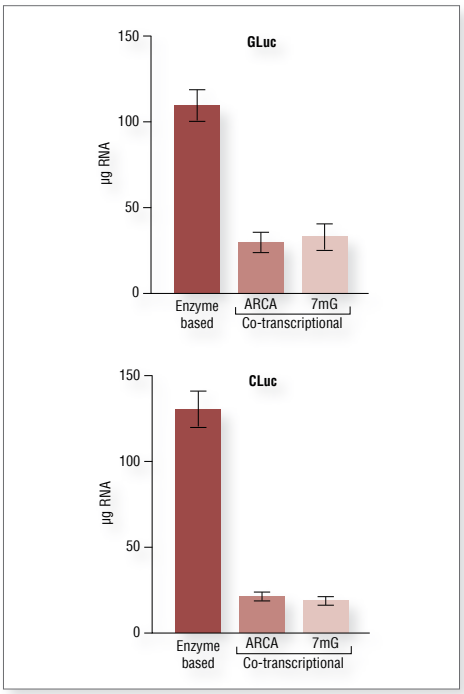
Figure 3. *In vitro* transcription options based upon capping strategy



Enzyme-based capping (left) is performed after in vitro transcription using 5´-triphosphate RNA, GTP, and S-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 (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.

Figure 4. RNA yields from transcriptional capping reactions



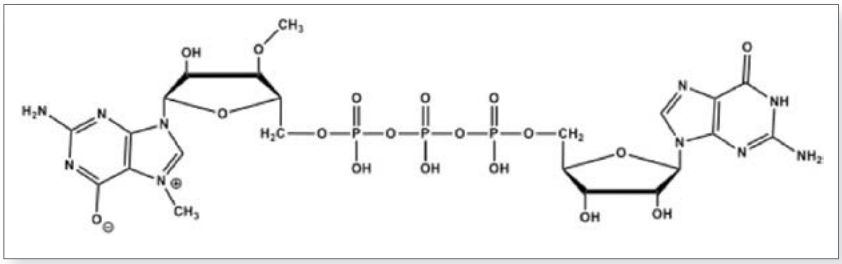
Reactions were set up according to recommended conditions for two templates: Gaussia luciferase (GLuc) and Cypridina luciferase (CLuc). The RNA was quantified spectrophotometrically after purification with spin columns.

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 reagents. More information on the HiScribe kits can be found on page 7.

Following transcription, the RNA is treated with DNase I 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".

Figure 5. Structure of the anti-reverse cap analog, ARCA



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

Transcription with co-transcriptional capping

With 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 (Fig. 4).

There are several cap analogs used in co-transcriptional RNA capping. 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 (Fig. 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 higher expression levels and avoidance of unwanted side effects 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) 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 may be used to produce transcripts for post-transcriptional capping (see below).

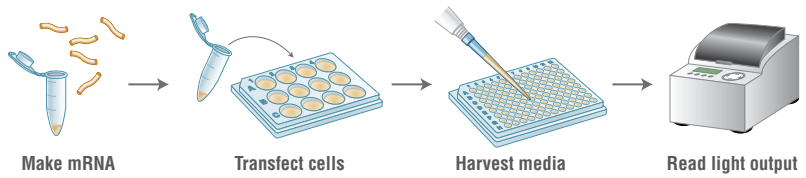
Post-transcriptional capping and Cap-1 methylation

Post-transcriptional capping is often performed using the mRNA capping system from *Vaccinia* virus. This enzyme complex converts the

continued on next page...

Analysis of capped RNA function in transfected mammalian cells

A.



Schematic representation of reporter mRNA transfection workflow.

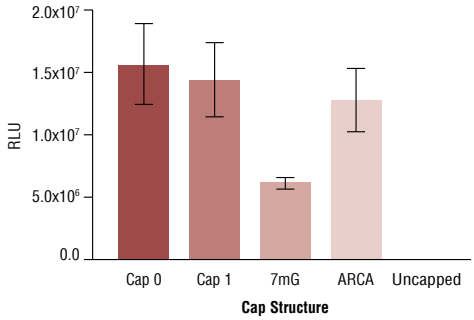
The effect of capping can be studied by delivering the mRNA to cultured mammalian cells and monitoring its translation. Using RNA encoding secreted luciferases (e.g., *Cypridina* luciferase, CLuc) the translation can be monitored by assaying its activity in the cell culture medium (Fig. A).

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.



Expression of Cypridina luciferase (CLuc) after capping using different methods. High activity from all capped RNAs is observed.

5´-triphosphate ends of *in vitro* transcripts to the m7G-cap structures. The Vaccinia Capping System (NEB #M2080) is comprised of 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 PCR-amplified template by enzymatic treatment of RNA with *E. coli* Poly(A) Polymerase (NEB #M0276). The lengths of the added tails can be adjusted by titrating the Poly(A) Polymerase in the reaction (Fig. 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 (Fig. 6). Increasing the length of poly(A) tails did not markedly further enhance reporter activity.

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 and optimized formulation for co-transcriptionally capped transcripts.

Summary

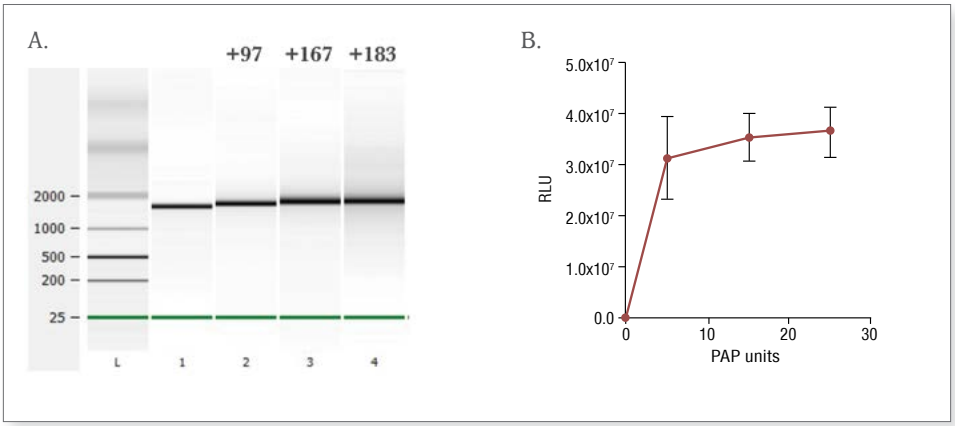
In summary, when choosing the right workflow for your functional mRNA synthesis needs, you must balance your experimental requirements for the mRNA (e.g., internal modified nucleotides) with scalability (i.e., ease-of-reaction setup vs. yield of final product).

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 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 and as separate components to enable research and large-scale production.

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

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

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)		
dNTP solution mixes	HiScribe T7 ARCA mRNA Synthesis Kit		*E. coli* Poly(A) Polymerase
Type IIs restriction enzymes & cloning reagents	HiScribe T7 High Yield RNA Synthesis Kit	Vaccinia Capping System	
	HiScribe T7 Quick High Yield RNA Synthesis Kit	mRNA Cap 2´-O-Methyltransferase	
	T3, T7 and SP6 RNA Polymerases	ARCA and other mRNA cap analogs	

Companion Products

- RNase inhibitors
- Pyrophosphatases
- DNase I
- NTPs

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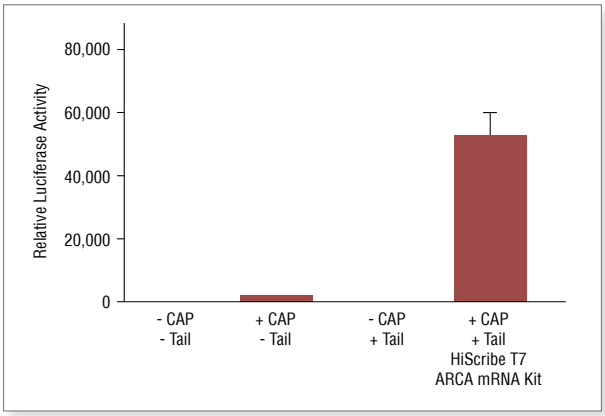
8. Wells, S.E., et al. (1998) *Molecular Cell* 2, 135–140.

HiScribe T7 ARCA mRNA Kits

Available with (NEB #E2060) and without (NEB #2065) tailing

Most eukaryotic mRNAs require a 7-methylguanosine (m7G) cap structure at the 5′ end and a poly(A) tail at the 3′ end to be efficiently translated. The HiScribe T7 ARCA mRNA Synthesis Kit (NEB #E2060S) is designed to synthesize capped and tailed mRNAs for variety of applications. Capped mRNAs are synthesized by co-transcriptional incorporation of Anti-Reverse Cap Analog (ARCA), using T7 RNA Polymerase. A poly(A) tail is then added by *E. coli* Poly(A) Polymerase. A separate version of the kit (NEB #E2065S), without *E. coli* Poly(A) Polymerase, is available for use with DNA templates encoding a poly(A) stretch or not requiring a poly(A) tail. The kits also include DNase I and LiCl for DNA template removal and quick mRNA purification.

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.

ADVANTAGES

- Quicker protocol takes you from capping to purification in 2 hours
- Flexible workflow enables incorporation of modified bases
- Maintain the utmost in mRNA integrity with ultra-high quality components
- Get the best translation efficiencies with correctly oriented ARCA caps
- All-inclusive kit contains all of the reagents you'll need
- Each kit provides reagents for twice the reactions versus competitors' kits

Ordering Information

PRODUCT	NEB #	SIZE
HiScribe T7 ARCA mRNA Kit (with Tailing)	E2060S	20 reactions
HiScribe T7 ARCA mRNA Kit	E2065S	20 reactions

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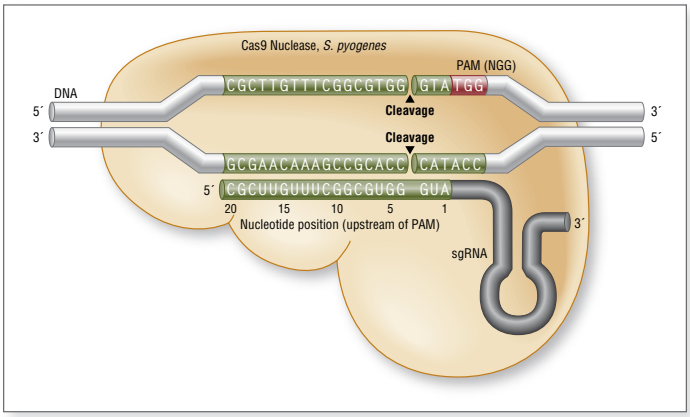
Cas9 Nuclease, *S. pyogenes*

Cas9 Nuclease is a double stranded DNA nuclease and central component of CRISPR (clustered regularly interspaced short palindromic repeat)-based immunity. In bacteria and archea, the CRISPR/Cas9 system functions to protect cells from foreign DNA. CRISPR genomic loci are transcribed and processed into guide RNAs that are incorporated into Cas9 Nuclease. Guide RNAs direct the Cas9 nuclease to its target by complementary base pairing.

Cas9 Nuclease has been adapted for use in genome engineering, because it can be easily programmed for target specificity by supplying guide RNAs of almost any sequence. *In vitro*, Cas9 permits flexible, user-specified, introduction of double stranded breaks at ~20 bp recognition sequences. In cells and animals, genome editing is performed by expressing Cas9 Nuclease and guide RNA (often single-guide RNA – sgRNA) from DNA constructs (plasmid or virus), supplying RNA encoding Cas9 nuclease and sgRNA, or by introducing RNA-programmed Cas9 Nuclease directly. Targeted doubled stranded breaks can be repaired by non-homologous end joining (NHEJ), knocking out gene function and enabling large scale deletions, or homology-directed repair (HDR) in the presence of an HDR template, enabling targeted insertions or substitutions.

Cas9 Nuclease, *S. pyogenes* sequence recognition and DNA cleavage



Visit www.neb.com/GenomeEditing to find our up-to-date listing of products and protocols to support genome editing experiments, and to download the latest Genome Editing brochure.

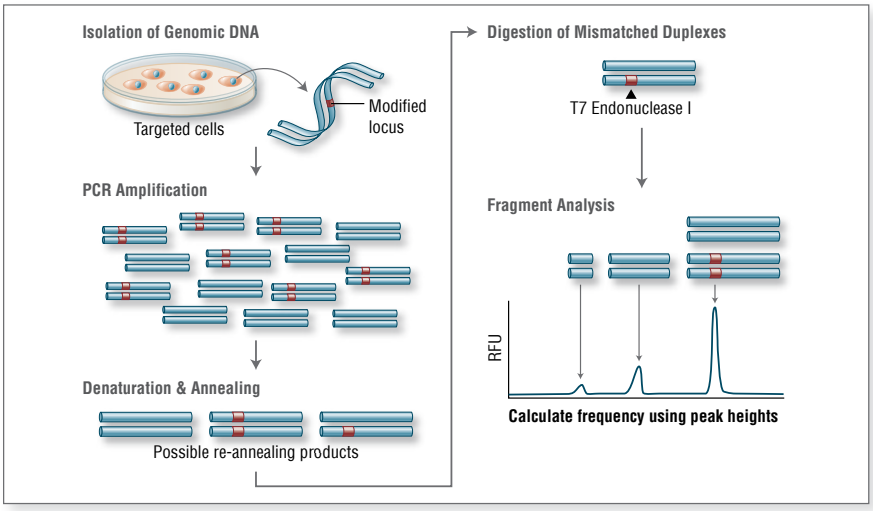
Ordering Information

PRODUCT	NEB #	SIZE
Cas9 Nuclease, *S. pyogenes*	M0386S/L	50/250 pmol
	M0386M	500 pmol
COMING SOON: Cas9 with Nuclear Localization Signal (NLS)		

Evaluating Targeting Efficiency with the T7 Endonuclease I Assay

A widely used method to identify mutations is the T7 Endonuclease I mutation detection assay. This assay detects heteroduplex DNA that results from the annealing of a DNA strand, including desired mutations, with a wild-type DNA strand.

T7 Endonuclease I Targeting Efficiency Assay



Genomic DNA is amplified with primers bracketing the modified locus. PCR products are then denatured and re-annealed yielding three possible structures. Duplexes containing a mismatch are digested by T7 Endonuclease I. The DNA is then electrophoretically separated and fragment analysis is used to calculate targeting efficiency.

Download the full protocol for measuring targeting efficiency at www.neb.com/protocolM0302

Ordering Information

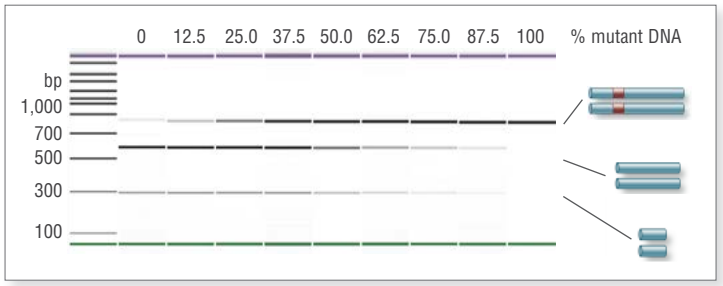
PRODUCT	NEB #	SIZE
T7 Endonuclease I	M0302S/L	250/1,250 units

Protocol for using recombinant Cas9 Nuclease to assess locus modification in genome editing experiments

Introduction

In vitro digestion of PCR amplicons with Cas9 ribonucleoproteins (Cas9 Nuclease) is a sensitive assay for detecting indels at specific loci. Unlike mismatch-detection assays, Cas9 has the additional advantage of determining targeting efficiencies above 50%. This is of value as targeting efficiency in genome editing experiments increases and for the detection of biallelic editing in isolated cell colonies or tissues. Prior to this method, detection was only achievable using specialized PCR or amplicon-sequencing approaches.

Analysis of Cas9 digestion products for locus modification assessment.



Wild-type and mutant PCR Amplicons were mixed as indicated and digested with Cas9 nuclease. The mutant amplicon differed from the wild-type by a 10 bp insertion. Cas9 nuclease was programmed with a sgRNA specific for the wt region. After digestion, the reaction products were resolved on an Agilent 2100 BioAnalyzer instrument using DNA 12000 reagents.

PCR Protocol

1. Set up a 50 µl PCR reaction using ~100 ng of genomic DNA (gDNA) as a template. For each amplicon set up 3 reactions using the following template:
   a. gDNA from targeted cells (e.g., Cas9 or TALEN transfected cells)
   b. gDNA from negative control cells (e.g., non-specific DNA transfected cells)
   c. Water (e.g., no template control)

COMPONENT	50 µL RXN	FINAL CONC.
Q5 Hot Start High-Fidelity 2X Master Mix (NEB #M0494)	25 µl	1X
10 µM forward primer	2.5 µl	0.5 µM
10 µM reverse primer	2.5 µl	0.5 µM
Template DNA	variable	~100 ng
Nuclease-free water	to 50 µl	–

2. Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin, if necessary. Transfer PCR tubes to a PCR machine and begin thermocycling.

STEP	TEMP. (°C)	TIME
Initial denaturation	98	30 sec.
35 cycles	98	5 sec.
	*50-72	10 sec.
	72	20 sec.
Final extension	72	2 min.
Hold	4	–

* Use of NEB's Tm Calculator (TmCalculator.neb.com) is strongly recommended.

3. Analyze a small amount of the PCR product to verify size and appropriate amplification.
4. Measure the concentration of the purified PCR products by the Qubit® dsDNA BR Assay or other relevant system. A yield of >25 ng DNA/µL is sufficient.

Cas9 digestion with sgRNA Protocol

1. Pre-loading of sgRNA to Cas9 Nuclease:

COMPONENT	20 µL REACTION
10X Cas9 Nuclease Reaction Buffer	2 µl
sgRNA (1 µM)	2 µl (100 nM final)
Cas9 Nuclease, *S. pyogenes* (1 µM)	2 µl (100 nM final)
Nuclease-free water	14-x µl
Incubation time & temperature	5-10 minutes at room temp.

2. Digestion of PCR product:

COMPONENT	20 µL REACTION
Reaction from step 1	20-x µl
PCR product	x µl (50-200 ng DNA)*
Incubation time & temperature	30 minutes at 37°C

* <2 µl PCR product per 20 µl is recommended.

Purification is optional. If desired, we recommend either digestion with 1 µl Proteinase K, Molecular Biology Grade (NEB #P8107S) for 15–30 minutes at 37°C or column purification. These steps can be of use if nonspecific binding or high background interfere with fragment analysis.

References:
1. Jinek et al. (2012) Science 337 (6096) 816-821.
2. Larson et al. (2013) Nature Protocol (8) 2180-2196.

BEFORE YOU START:

- We strongly recommend wearing gloves and using nuclease-free tubes and reagents to avoid RNase contamination. For more information, visit www.neb.com/RibonucleaseContamination
- Reactions are typically 20 µl, but can be scaled up as needed. Assemble in nuclease-free microfuge tubes or PCR strip tubes.
- It is essential to keep the molar ratio of Cas9 and sgRNA per target site at or near 10:10:1 to obtain the best cleavage efficiency. We recommend using NEBioCalculator (nebiocalculator.neb.com).
- Prepare 1 µM sgRNA by diluting the stock with nuclease-free water on ice.

NOTES:

- **Primers** – The target site should be offset from the center of the amplicon so that digestion produces easily resolvable DNA fragments. PCR primer design is critical. Please use the NEB Tm Calculator (TmCalculator.neb.com).
- **sgRNA** – sgRNAs can be generated by *in vitro* transcription using the HiScribe T7 Quick High-Yield RNA Synthesis Kit (NEB #E2050S), or by synthesizing RNA oligonucelotides. sgRNAs must contain the target sequences (20 nucleotides) adjacent to the Protospacer Adjacent Motif (PAM, NGG) in the target DNA. (1,2)

ANALYSIS:

1. Analyze the fragmented PCR products by gel electrophoresis or other fragment analysis method.
2. Calculate the estimated gene modification using the following formula:
   % modification = 100 x ([uncut DNA] / [uncut DNA]+[fragment1]+[fragment2])

APPLICATION NOTE

Construction of an sgRNA-Cas9 expression vector via single-stranded DNA oligo bridging of double-stranded DNA fragments

Peichung Hsieh, Ph.D., New England Biolabs, Inc

Introduction

NEBuilder HiFi DNA Assembly Master Mix (NEB# E2621) was developed to improve the efficiency and accuracy of DNA assembly over other DNA assembly products currently available. The method allows for the seamless assembly of multiple DNA fragments, regardless of fragment length or end compatibility. Thus far, it has been adopted by the synthetic biology community, as well as those interested in one-step cloning of multiple fragments due to its ease of use, flexibility and simple master mix format.

CRISPR/Cas9-based gene editing is quickly growing in popularity in the field of genome editing. Due to the size of most commonly used Cas9-containing plasmids, construction of an sgRNA or sgRNA library into a Cas9/sgRNA expression vector can be cumbersome. NEB has developed a protocol to solve this problem, using single-stranded DNA oligonucleotides, a restriction enzyme digested vector and the NEBuilder HiFi DNA Assembly Master Mix.

Protocol

Note: This protocol demonstrates the design of an sgRNA targeting a ~30 kb gene from *H. sapiens*.

1. Scan for a PAM sequence (NGG, in green) in the desired target sequence.

   Example:
   5′GCGAAGAACCTCTTCCCAAGANGG3′

   We suggest using the sgRNA design tool available at: https://chopchop.rc.fas.harvard.edu.

2. Design a 71-base, single-stranded DNA oligonucleotide, containing a 21 nt target sequence (in red), flanked by a partial U6 promoter sequence (in blue) and scaffold RNA sequence (in purple)

   Example:
   5′ATCTTGTGGAAAGGACGAAACACCG
   GCGAAGAACCTCTTCCCAAGA
   GTTTTAGAGCTAGAAATAGCAAGTT3′

   to construct a random library, randomize nucleotides 19–21, as shown below:

   Example:
   5′ATCTTGTGGAAAGGACGAAACACCG
   $N_{19\text{-}21}$GTTTTAGAGCTAGAAATAGCAAGTT3

3. Prepare the ssDNA oligo in 1X NEBuffer 2 to a final concentration of 0.2 µM.

4. Assemble a 10 µl reaction mix with 5 µl of ssDNA oligo (0.2 µM), 30 ng of restriction enzyme-linearized vector* and ddH_2O.

5. Add 10 µl of NEBuilder HiFi DNA Assembly Master Mix to the reaction mix, and incubate the assembly reaction for 1 hour at 50°C.

6. Transform NEB 10-beta Competent *E. coli* (NEB #C3019) with 2 µl of the assembled product, following the protocol supplied with the cells.

7. Spread 100 µl of outgrowth on a plate with ampicillin antibiotic, and incubate overnight at 37°C.

8. Pick 10 colonies to grow, and purify the plasmid DNA for sequencing.

* Researchers can find suitable vectors from Addgene, a non-profit organization. We recommend Addgene plasmid #42230, pX330-U6-Chimeric_BB-CBh-hSpCas9 (for details, see https://www.addgene.org/42230/), although any plasmid containing an sgRNA scaffold under the control of a U6 promotor should be adequate.

Results

Ten colonies were isolated, and plasmid DNA was purified using standard miniprep columns. Insertion of the target DNA sequence was confirmed by DNA sequencing. Of the 10 clones sequenced, 9 contained the target sequence in the correct orientation (data not shown).

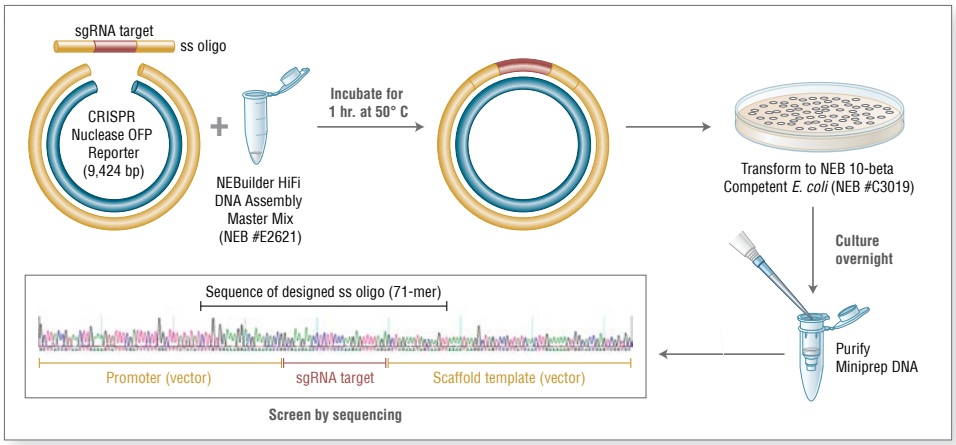
Summary

This Application Note demonstrates the convenience of inserting an sgRNA sequence into a 9.5 kb vector for targeted DNA assembly. Unlike traditional cloning methods, in which two oligos must be synthesized and re-annealed, this new protocol offers a simple way to design an oligo and assemble it with the desired vector.

The NEBuilder HiFi DNA Assembly Master Mix represents a substantial improvement over traditional methods, specifically in time savings, ease-of-use and cost.

Visit the Other Tools and Resources tab at **www.neb.com/E2621** to download the full application note.

Figure 1. sgRNA cloning workflow

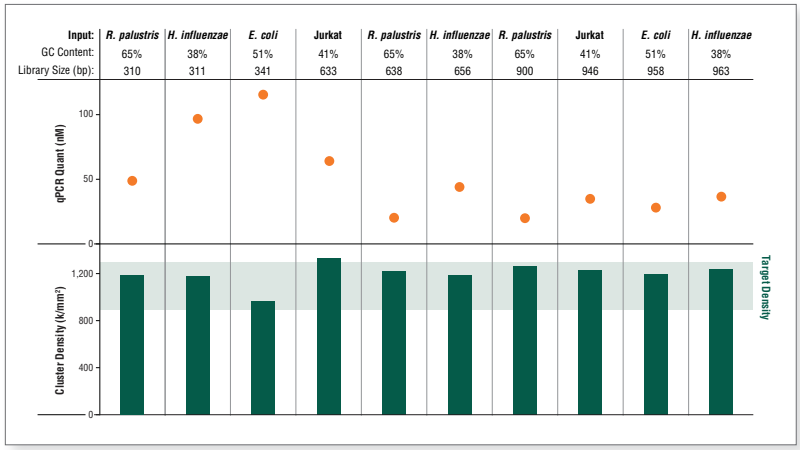


Design an ssDNA oligo containing the target sequence (19-21 bases) of sgRNA flanked by 25 bases of sequence at both ends. Mix the single-stranded oligo, linearized vector DNA and NEBuilder HiFi DNA Assembly Master Mix together, incubate for 1 hour at 50°C and transform into E. coli.

NEBNext Library Quant Kit for Illumina®

Accurate quantitation of next-generation sequencing libraries is essential for maximizing data output and quality from each sequencing run. Furthermore, for Illumina sequencing, accurate quantitation of libraries is critical to achieve optimal cluster densities, a requirement for optimal sequence output. qPCR is considered to be the most accurate and effective method of library quantitation, providing considerably higher consistency and reproducibility of quantitation. Amplification-based methods quantitate only those molecules that contain both adaptor sequences, thereby providing a more accurate estimate of the concentration of the library molecules that can be sequenced. The NEBNext Library Quant Kit delivers significant improvements to qPCR-based library quantitation for next generation sequencing.

With NEBNext, optimal cluster density is achieved from quantitated libraries with a broad range of library size and GC content



Libraries of 310–963 bp from the indicated sources were quantitated using the NEBNext Library Quant Kit, then diluted to 8 pM and loaded onto a MiSeq® (v2 chemistry; MCS v2.4.1.3). Library concentrations ranged from 7–120 nM, and resulting raw cluster density for all libraries was 965–1300 k/mm2 (average =1199). Optimal cluster density was achieved using concentrations determined by the NEBNext Library Quant Kit for all library sizes.

Ordering Information

PRODUCT	NEB #	SIZE
NEBNext Library Quant Kit for Illumina	E7630S/L	100/500 reactions

BENEFITS

- Be confident in your quant values, as our kit provides more accurate and reproducible results than other methods and kits.
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- Get up and running quickly with our easy-to-use kit, containing 4 standards, Library Dilution Buffer, optimized master mix and ROX dye.
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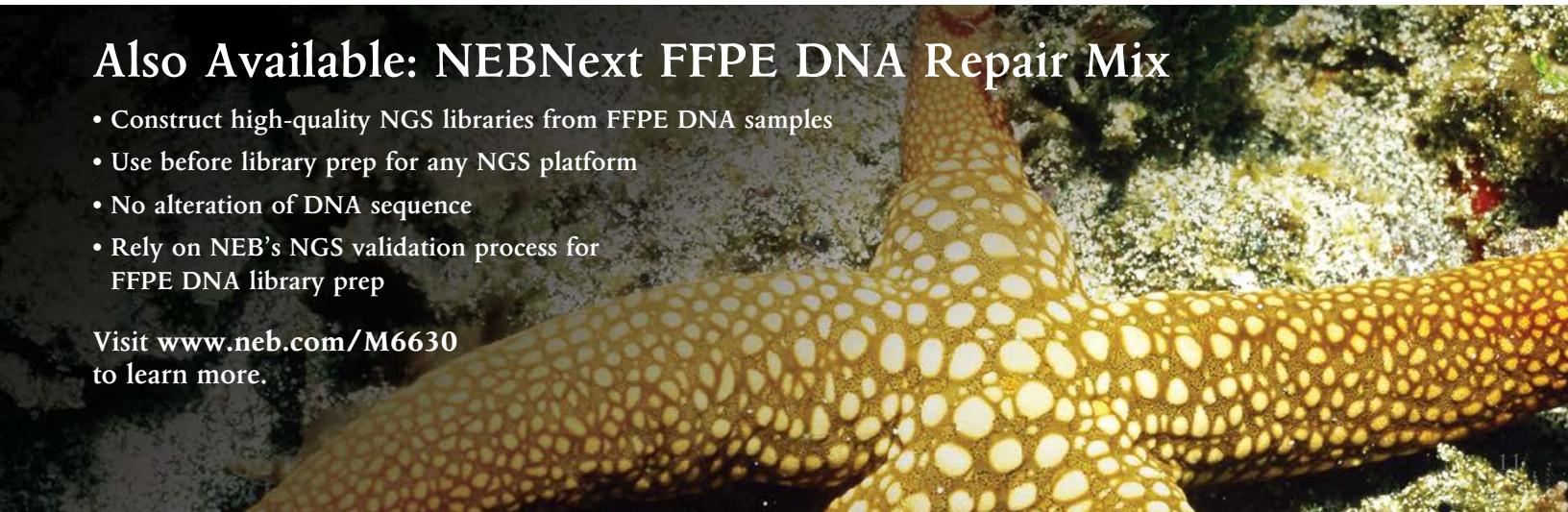

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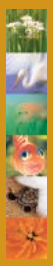

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