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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 (4-6), and CRISPR/Cas9 genome editing applications (7-9). 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.

A nascent mRNA, synthesized in the nucleus, undergoes 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 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.

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 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) (10).

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.

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 1). 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.

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 dinucleotide 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.

There are several cap analogs used in co-transcriptional RNA capping (3). 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. 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 CleanCap® Reagent AG co-transcriptional capping

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.

In order to use CleanCap Reagent AG in an *in vitro* transcription reaction the template must contain an AG in place of a GG following the T7 promoter in the initiation sequence.

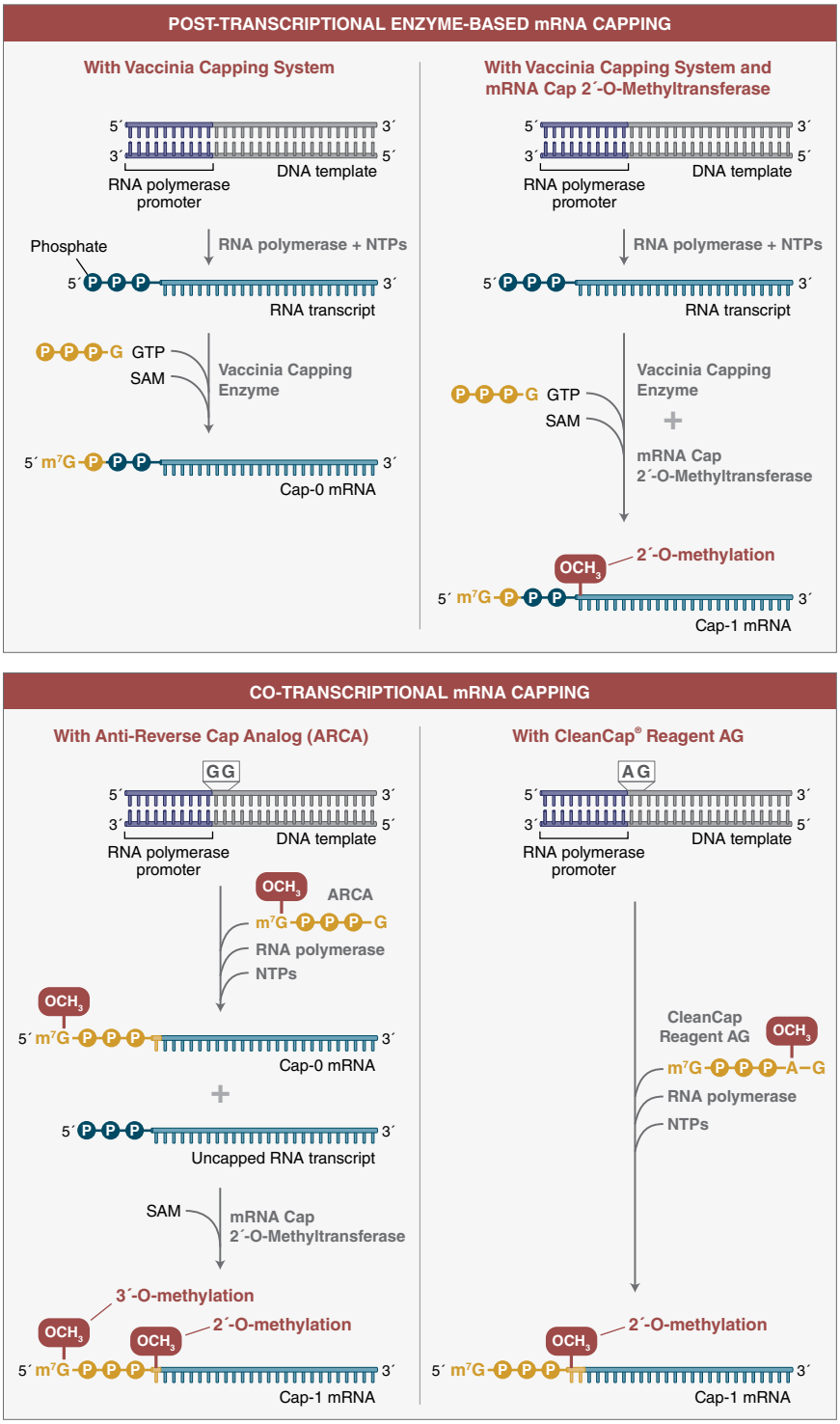
Unlike traditional co-transcriptional capping, reduction of GTP concentration is not required and therefore yield is higher and high capping effiencies, >95%, are achieved.

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 mRNA Kit with CleanCap Reagent AG (NEB #E2080), 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

Figure 1: ***In vitro*** **transcription options based upon capping strategy**



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 co-transcriptional reaction.

"GMP-grade" is a branding term NEB uses to describe reagents manufactured at NEB's Rowley facility. The Rowley facility was designed to manufacture reagents under more rigorous infrastructure and process controls to achieve more stringent product specifications and customer requirements. Reagents manufactured at NEB's Rowley facility are manufactured in compliance with ISO 9001 and ISO 13485 quality management system standards. However, at this time, NEB does not manufacture or sell products known as Active Pharmaceutical Ingredients (APIs), nor does NEB manufacture its products in compliance with all of the Current Good Manufacturing Practice regulations.

by including CleanCap Reagent AG, 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.

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 5´-triphosphate ends of *in vitro* transcripts to m7G-cap (Cap-0) required for efficient protein translation in eukaryotes. The *Vaccinia* Capping System (NEB #M2080) comprises three enzymatic activities (RNA triphosphatase, guanylyltransferase, 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 (SAM). 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 (m7Gppp5´Nm), a natural modification in many eukaryotic mRNAs responsible for evading cellular innate immune response against foreign RNA. This enzyme-based capping approach results in a high 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.

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.

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 modifed nucleotides) with scalability (i.e., ease-of-reaction setup vs. yield of final product).

Products from NEB are available for each step of the RNA synthesis workflow. GMP-grade* reagents suitable for the large scale manufacture of therapeutics mRNA are available through our Customized Solution Group.

References:

1. Warren, L., et al. (2010) *Cell Stem Cell,* 7, 618-630.
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3. Yakubov, E., et al. (2010) *Biochem. Biophys. Res. Commun.* 394, 189.
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9. Bassett, A. R., et al. (2013) *Cell Rep.* 4, 220–228.
10. Wells, S.E., et al. (1998) *Molecular Cell* 2, 135–140.

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NEB has everything you need for your RNA-related workflows.

Featured Application
RNA Synthesis

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.

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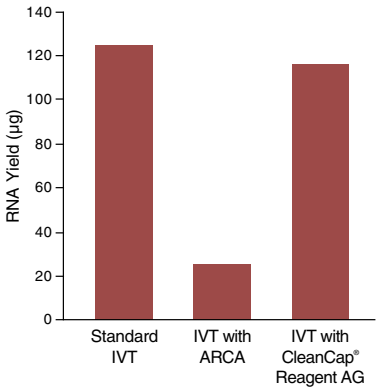
New Product
HiScribe™ T7 mRNA Kit with CleanCap® Reagent AG

The HiScribe T7 mRNA Kit with CleanCap Reagent AG utilizes an optimized RNA synthesis formulation and trinucleotide cap analog technology to co-transcriptionally cap mRNAs containing a natural Cap-1 structure in a single simplified reaction without compromising RNA yield. Using a DNA template with a T7 promoter sequence followed by an AG initiation sequence and an encoded poly(A) tail, mRNAs can be transcribed with a 5´-m7G Cap-1 structure that is polyadenylated, translationally competent and able to evade the cellular innate immune response.

Comparison of RNA Yields from *in vitro* Reagent AG 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 (4mM:1mM). IVT with CleanCap Reagent AG contained 5 mM GTP and 4 mM CleanCap Reagent AG and was performed as described (Standard mRNA Synthesis, HiScribe T7 mRNA Kit with CleanCap Reagent AG). Reactions were incubated for 2 hours at 37°C, purified and quantified by NanoDrop.

Advantages:

- Synthesize and cap mRNA in a single reaction
- Evade immune response with natural Cap-1 structure
- Generate high yields of mRNA, up to > 1.8 mg per kit
- Suitable for full or partial modified nucleotide substitutions

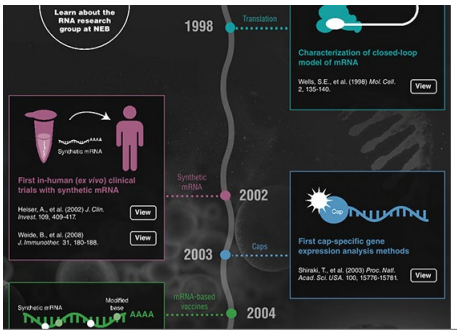
Applications:

- Transfections
- Microinjections
- *In vitro* translation
- Preclinical mRNA therapeutic studies
- RNA structure and function analysis

Ordering information:

Product	NEB #	Size
HiScribe T7 mRNA Kit with CleanCap Reagent AG	E2080S	20 rxns

Discover these RNA Synthesis resources at NEBrna.com



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From research to therapeutic production, NEB's *in vitro* transcription portfolio will meet your needs

NEB's portfolio of research-grade and GMP-grade reagents support 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 for a seamless transition to large-scale therapeutic mRNA manufacturing. To learn more, contact us at custom@neb.com.

ENABLING GRAM-SCALE RNA SYNTHESIS

NEB manufactures and inventories the following enzyme specificities at GMP-grade, meeting customer needs with short lead times:

Product	NEB #	Feature
Vaccinia Capping Enzyme	M2080S	A full system for enzymatic capping based on the Vaccinia virus Capping Enzyme (VCE)
T7 RNA Polymerase	M0251S/L	RNA Polymerase used for *in vitro* mRNA synthesis, and is highly specific for the T7 phage promoter
mRNA Cap 2′-O-Methyltransferase	M0366S	mRNA Cap 2´-O-Methyltransferase 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
RNase Inhibitor, Murine	M0314S/L	RNase Inhibitor, Murine, specifically inhibits RNases A, B and C
Pyrophosphatase, Inorganic (*E. coli*)	M0361S/L	Inorganic pyrophosphatase (PPase) catalyzes the hydrolysis of inorganic pyrophosphate to form orthophosphate
DNase I (RNase-free)	M0303S/L	DNA-specific endonuclease used for removal of contaminating genomic DNA from RNA samples and degradation of DNA templates in transcription reactions
HiScribe T7 High Yield RNA Synthesis Components	E2040S	Separate components available in GMP-grade format

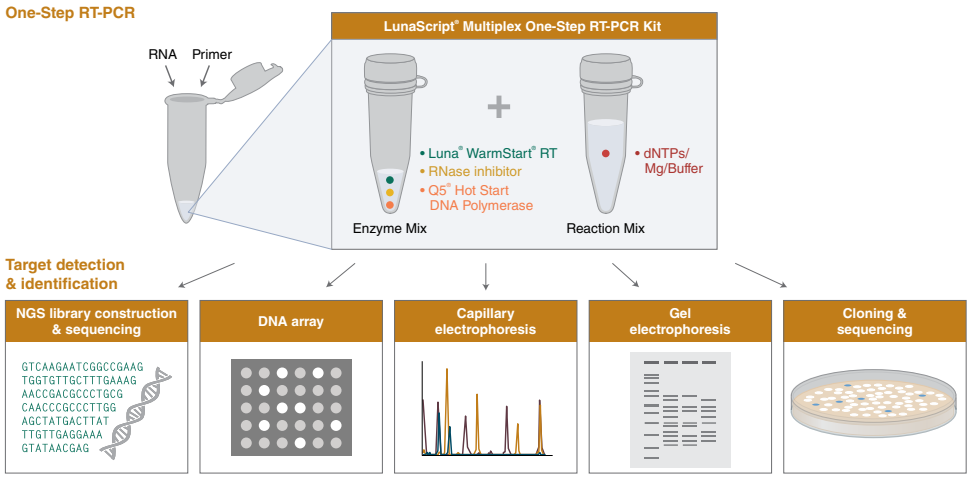
"GMP-grade" is a branding term NEB uses to describe reagents manufactured at NEB's Rowley facility. The Rowley facility was designed to manufacture reagents under more rigorous infrastructure and process controls to achieve more stringent product specifications and customer requirements. Reagents manufactured at NEB's Rowley facility are manufactured in compliance with ISO 9001 and ISO 13485 quality management system standards. However, at this time, NEB does not manufacture or sell products known as Active Pharmaceutical Ingredients (APIs), nor does NEB manufacture its products in compliance with all of the Current Good Manufacturing Practice regulations.

FEATURED PRODUCTS

Lighting the way.

LunaScript® Multiplex One-Step RT-PCR Kit

The LunaScript Multiplex One-Step RT-PCR Kit offers a streamlined protocol for cDNA synthesis and PCR amplification in a single reaction. The 5X reaction mix contains dNTPs and is optimized for robust multiple target detection in a simple workflow. The 25X enzyme mix features Luna WarmStart® Reverse Transcriptase and Q5® Hot Start High-Fidelity DNA Polymerase.



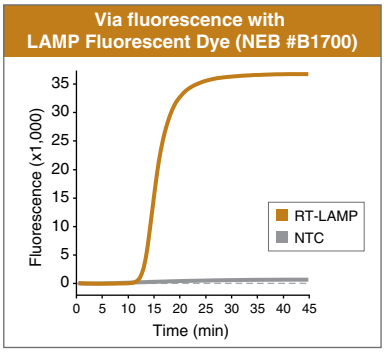
The LunaScript Multiplex One-Step RT-PCR Kit requires only RNA template and gene-specific primers to enable multiplex cDNA target synthesis and amplification in a single reaction. Amplified cDNA products can be detected or identified by downstream applications including next-generation sequencing, DNA arrays, fragment analysis, electrophoresis and traditional cloning/sequencing workflows.

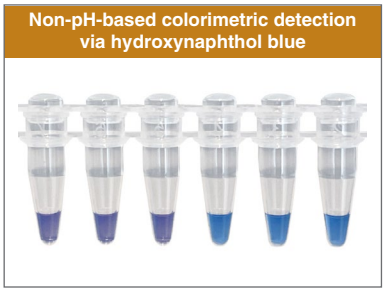
Advantages:

- Closed-tube system with cDNA synthesis and PCR amplification in a single protocol
- Detect as low as 0.01 pg of human total RNA
- Multiplexing capacity supports use in ARTIC workflows
- Aptamer-based enzyme control for room temperature setup and stability up to 24 hours

WarmStart® Multi-Purpose LAMP/RT-LAMP 2X Master Mix (with UDG)

The WarmStart Multi-Purpose LAMP/RT-LAMP 2X Master Mix (with UDG) is fully buffered and compatible with different sample types, enabling multiple detection methods including turbidity detection, real-time fluorescence detection, and end-point visualization such as colorimetric detection via a metal indicator (e.g., hydroxynaphthol blue). For real-time fluorescence detection, the master mix is available as a kit that includes 50X LAMP Fluorescent Dye. The inclusion of dUTP and thermolabile UDG enables carryover contamination prevention.





The WarmStart Multi-Purpose LAMP/RT-LAMP 2X Master Mix (with UDG) is compatible with multiple detection methods.

Advantages:

- Reduce the risk of carryover contamination with thermolabile UDG and dUTP included in the mix
- Set up reactions at room temperature with our unique dual WarmStart formulation
- Optimized performance for real-time fluorescence and endpoint visualization detection methods

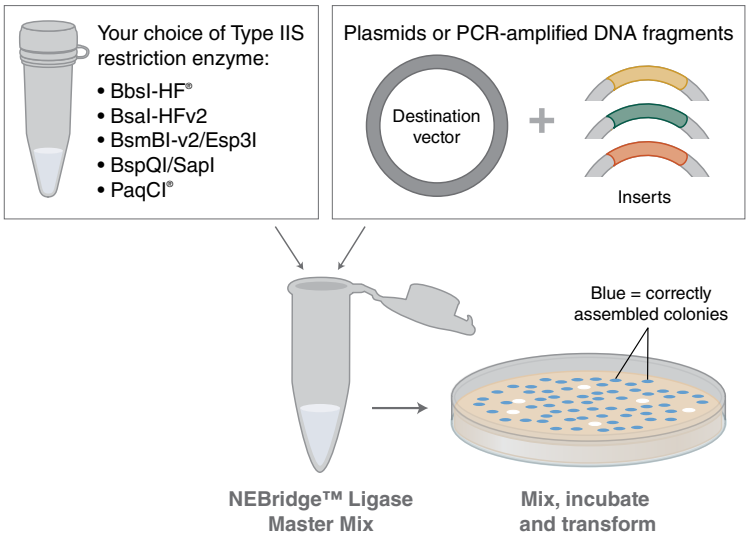
Ordering information:

Product	NEB #	Size
LunaScript Multiplex One-Step RT-PCR Kit	E1555S/L	50/250 rxns
WarmStart Multi-Purpose LAMP/ RT-LAMP 2X Master Mix (with UDG)	M1708S/L	100/500 rxns
WarmStart Fluorescent LAMP/ RT-LAMP Kit (with UDG)	E1708S/L	100/500 rxns

NEBridge™ Ligase Master Mix

Offering flexibility and convenience for users, NEBridge Ligase Master Mix performs high efficiency and high-fidelity Golden Gate Assembly with a broad assortment of NEB Type IIS restriction enzymes.

NEBridge Ligase Master Mix is a 3X master mix for Golden Gate Assembly. Designed for use with NEB Type IIS restriction enzymes, this master mix contains T4 DNA Ligase in an optimized reaction buffer with a proprietary ligation enhancer. Users need only choose their preferred NEB Type IIS restriction enzyme and add DNA substrates to be assembled. Low complexity single-fragment insertions, as well as moderate complexity (3–6 fragment) and high complexity (7–25+ fragment) assemblies, are all supported with this optimized reagent and accompanying protocols.



Advantages:

- Optimized for efficient and accurate Golden Gate Assembly
- Use with NEB Type IIS restriction enzymes
- Use for seamless cloning – no scar remains following assembly
- Ideal for ordered assembly of multiple fragments (2-25+) in a single reaction
- Can also be used for cloning single inserts and library construction
- Design primers with our free tool available at GoldenGate.neb.com
- Try our free Ligase Fidelity Tools (for the design of high-fidelity Golden Gate Assemblies) at www.neb.com/research/nebeta-tools

Ordering information:

Product	NEB #	Size
NEBridge Ligase Master Mix	M1100S	50 rxns

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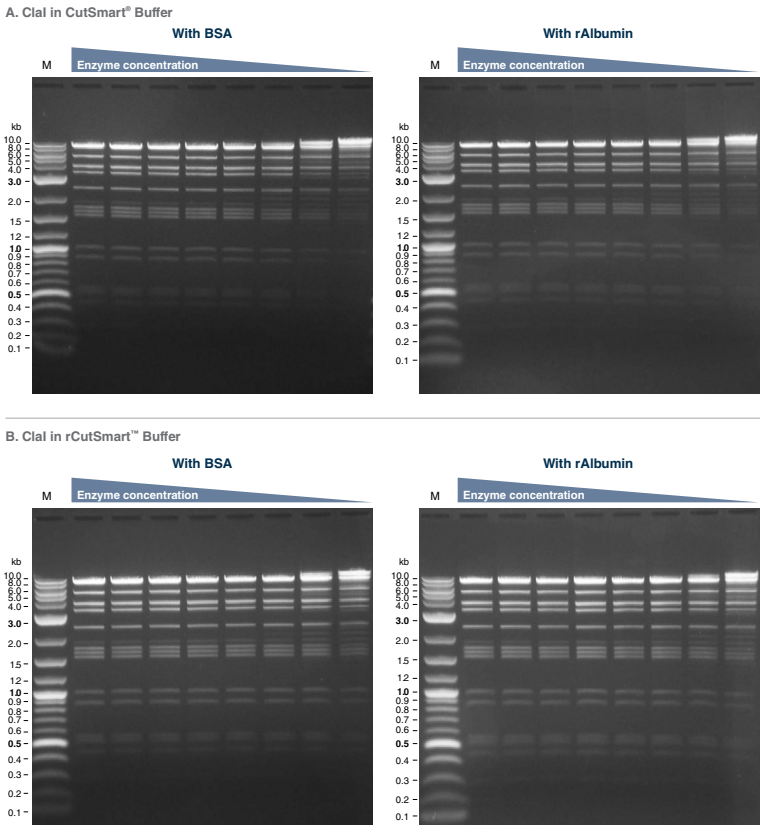
NEB understands that there is an increased need to move away from animal-containing products such as Bovine Serum Albumin (BSA) while maintaining comparable performance. We are excited to announce that NEB has begun transitioning our restriction enzyme formulations and buffers to contain rAlbumin instead of BSA.

We anticipate that the buffer switch will be completed in early 2022. Enzyme formulations will take a little longer and may take several years before all are switched. You can continue to use BSA- or rAlbumin-containing buffers or formulations.

We feel that moving away from animal-containing products is a step in the right direction and can offer this enhancement at the same price.



ClaI (NEB #R0197) restriction endonuclease was formulated with BSA or Recombinant Albumin (rAlbumin). Each of these formulations was tested using either A. CutSmart (contains BSA) or B. rCutSmart (contains rAlbumin) Buffer. Serial dilutions of ClaI were tested using Lambda DNA and the recommended assay conditions, followed by analysis using agarose gel electrophoresis. M is marker 1 kb Plus DNA Ladder (NEB #N3200). ClaI activity is the same in all formulation conditions tested. These representative data demonstrate equal performance between BSA and rAlbumin when used in both enzyme formulation and reaction buffer.

FAQ: What is Recombinant Albumin (rAlbumin)?

Recombinant Albumin (rAlbumin) (NEB #B9200) is a non-bovine derived albumin that can serve as an alternative to Bovine Serum Albumin (BSA). Like BSA, it has been shown to prevent adhesion of enzymes to reaction tubes and pipette surfaces. It also stabilizes some proteins during incubation. Choose rAlbumin when there is a need to avoid BSA.

Enzyme Formulation Changes:

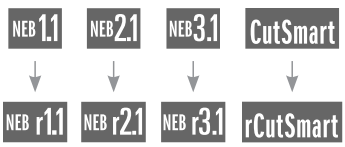
- Use of rAlbumin in enzyme formulations enables them to be used in situations where BSA-free reagents are required
- The catalog number will not change when a restriction enzyme is reformatted with rAlbumin
- There is no difference in enzyme performance when using an enzyme formulated with BSA versus rAlbumin, either can be used in your reaction
- Certificates of Analysis, Certificates of Origin and Specifications will be updated to reflect the change

Find out which enzyme formulations contain rAlbumin by visiting the product page of your enzyme of interest. A message will be included at the top of the product webpage of your enzyme of interest and in the "Specifications and Change Notification" tab - the message will indicate the starting lot number that contains rAlbumin. Please note that all higher lot numbers will also contain rAlbumin.

Learn more about restriction enzyme formulation changes by visiting
www.neb.com/BSA-free2

Buffer Changes:

NEBuffer names will change to include a lowercase "r" in front of the buffer. You can easily tell which version you have by looking at the name on the buffer vial.



Learn more about buffer changes by visiting
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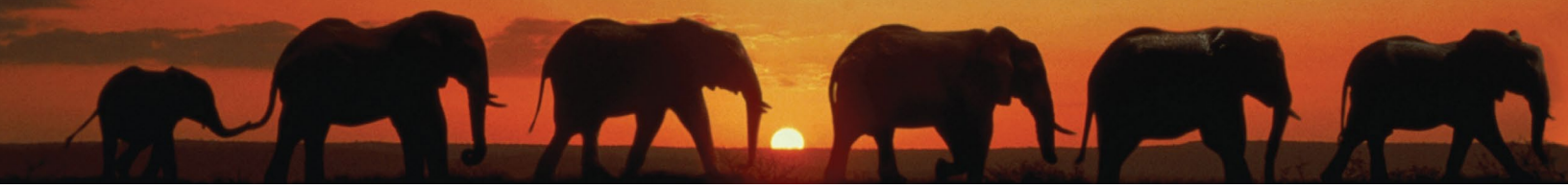

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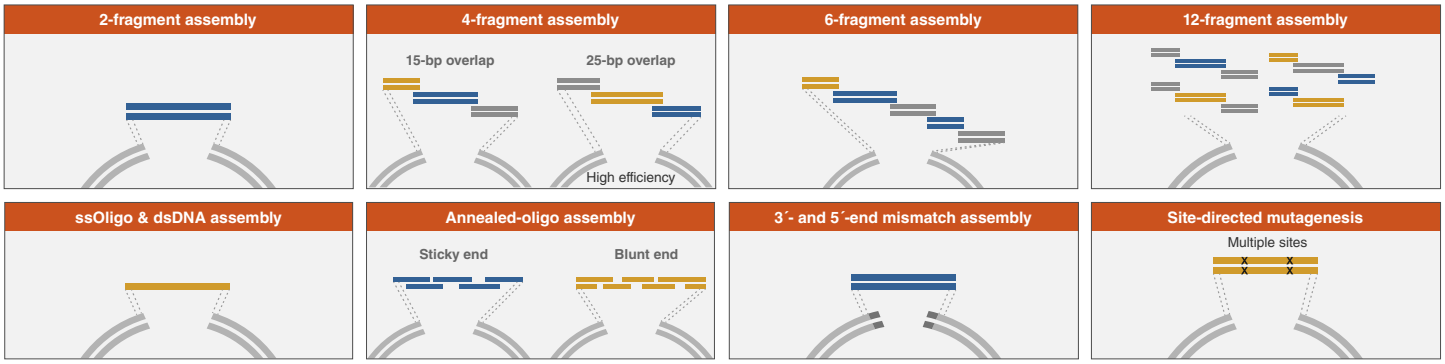
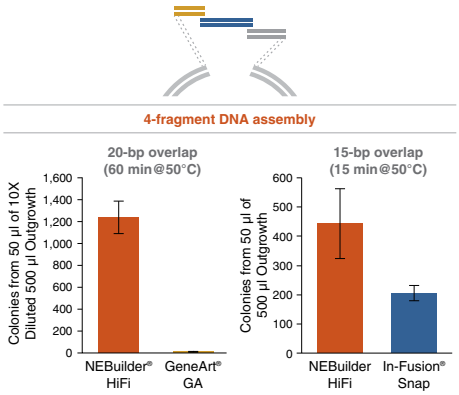
Assembling DNA fragments is a key part of both synthetic biology techniques and cloning. NEBuilder HiFi DNA Assembly enables virtually error-free joining of DNA fragments, even those with 5´- and 3´-end mismatches. This flexible kit enables simple and fast seamless cloning utilizing a proprietary high-fidelity polymerase. Find out why NEBuilder HiFi is the next generation of DNA assembly and cloning.

Not your average DNA assembly reagent

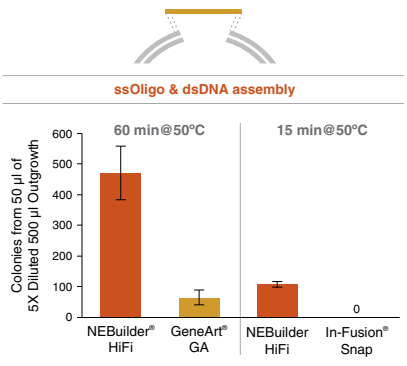


NEBuilder HiFi DNA Assembly offers improved efficiency in 4-fragment assembly reactions



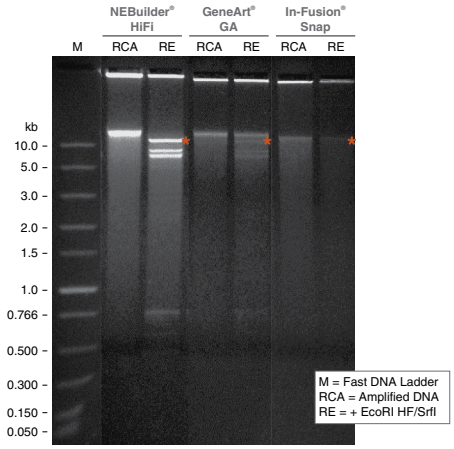
Four fragments (~20 fmol) with 20 bp overlap were assembled using NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621), GeneArt® Gibson Assembly® Mix (Thermo Fisher #A46627) and In-Fusion® Snap Assembly Master Mix (Takara Bio USA #638947) to create a pUC19 vector. 2 µl of each assembled mix was transformed into NEB 5-alpha Competent E.coli (NEB#C2987) and spread on LB/Amp plates with IPTG and X-Gal. Blue colonies that indicated correct assembly were counted.

NEBuilder HiFi DNA Assembly offers improved efficiency in assembly of a ssDNA oligo with a linearized vector



One pmol of ssDNA oligos were assembled with a linearized vector (30 ng of CRISPR Nuclease Reporter DNA) using NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621), GeneArt Gibson Assembly Mix (Thermo Fisher #A46627) and In-Fusion Snap Assembly Mix (Takara Bio USA #638947). 2 µl of the assembled mix was transformed into NEB 5-alpha Competent E.coli NEB #C2987). 20 colonies were further screened by PCR to confirm the presence of inserts. Greater than 95% of colonies tested from NEBuilder HiFi and GeneArt Gibson Assembly reactions contained proper inserts, although GeneArt Gibson Assembly yielded fewer colonies. In-Fusion Snap did not yield any successful colonies.

In vitro enrichment of 20 kb assembled DNA by phi-29 DNA Polymerase



A 3-fragment assembly reaction was set up to construct a 20 kb dsDNA. 1 µl was removed from assembled DNA reactions respectively (NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621), GeneArt Gibson Assembly Mix (Thermo Fisher #A46627) and In-Fusion Snap Assembly Mix (Takara Bio USA #638947) and used as templates in rolling circle amplification (RCA). Amplified DNA was further purified by SPRIselect™ beads followed by restriction enzyme digestion to confirm the insert. Red star indicates the presence of insert when the amplified DNA was digested with EcoRI-HF (NEB #R3101) and SrfI (NEB #R0629). Amplified DNA can be detected from all three assembly mixes followed by phi-29 amplification protocol. However, only the amplified product using templates from NEBuilder HiFi mix yielded a specific DNA band corresponding to the size of insert (~12 kb) after EcoRI-HF/SrfI digestion.

Ordering information:

Product	NEB #	Size
NEBuilder® HiFi DNA Assembly Master Mix	E2621 S/L/X	10/50/250 rxns
NEBuilder HiFi DNA Assembly Cloning Kit	E5520S	10 rxns
NEBuilder HiFi DNA Assembly Bundle for Large Fragments	E2623S	20 rxns

GMP-grade reagent production eases customers' transition from the lab bench to therapeutic production

An interview with Breton Hornblower, Portfolio Manager, Regulated Markets at New England Biolabs, Inc.

For decades, NEB has been involved in manufacturing research-grade reagents for RNA synthesis and RNA modification. We have a portfolio of RNA reagents that continues to grow to this day. These catalog-based products undergo rigorous quality control testing to ensure they are highly pure and of exceptional quality.

To meet the needs of all of our customers, in 2017, we expanded our manufacturing capabilities by building a state-of-the-art, 43,000 ft^2, GMP-grade (Good Manufacturing Practices-grade) facility in Rowley, MA, not far from our main campus in Ipswich.

This GMP grade-dedicated facility is where we manufacture our reagents under stringent cleanroom controls and quality control conditions designed to minimize the risks involved in the production of reagents for regulated markets, such as pharmaceuticals. This allows our customers to move from the early development stages at the research bench to the production of therapeutics such as vaccines without changing reagents or suppliers.

Why did NEB decide to open a GMP-grade facility?

We had been watching the nucleic acid therapeutics market for some time. While the COVID-19 pandemic has brought mRNA vaccines to the forefront of people's minds and become routine dinner-time conversation, mRNA-based therapeutics have been on the horizon for many years, primarily with a focus on oncology. Another example is genome editing-based therapies where a Cas nuclease may be delivered as mRNA. In order to bring these to market, companies must comply with FDA regulations, including the selection of the highest-quality materials available. Rather than retrofit our current headquarters in Ipswich to produce these reagents, we invested in a new facility in 2017, located in Rowley, MA, which is about 15 minutes from our Ipswich, MA headquarters.

"While the COVID-19 pandemic has brought mRNA vaccines to the forefront of people's minds and become routine dinner-time conversation, mRNA-based therapeutics have been on the horizon for many years."

What makes a product GMP-grade?

For a product to be GMP-grade, it must be manufactured in our Rowley facility under more rigorous infrastructure and process controls to achieve more stringent product specifications. Like our Ipswich manufactured products, these products are manufactured under ISO 9001 and ISO 13485 quality management system standards. We also voluntarily adhere to the relevant guidelines from the Code of Federal Regulations Title 21 Part 820. These products have (but are not limited to) attributes such as having been made using processes that do not contain any animal-derived components, using Quality Assurance reviewed and approved batch records, and passing additional release specifications such as bioburden and endotoxin, which are newer requirements for GMP-grade reagents having therapeutic applications.

How is a GMP-grade product different than a diagnostic product?

Our GMP-grade products are designed to be used by our customers to manufacture their active pharmaceutical ingredient (API) or diagnostic device. It is important to be clear that we do not manufacture APIs or finished medical devices ourselves. *In vitro* diagnostics are classified as reagents, instruments and systems intended to diagnose a disease or other condition. These products undergo a different international regulatory approval and registration process with different requirements for enzymes and other reagents used to manufacture them. We can and do supply to this market as well.

What products does NEB currently manufacture at GMP-grade?

Our current product portfolio primarily addresses the mRNA therapeutics market. We manufacture all of the individual components for *in vitro* transcription, including T7 RNA Polymerase and other enzymatic components, and post-transcriptional modifications such as capping enzymes. We also formulate these into reagent mixes that provide easier workflows based on our HiScribe™ kit portfolio. New products continue to be added to the GMP-grade family due to customer requests and market analysis.

How has NEB's GMP-grade facility enabled us to support vaccine production?

The current global pandemic has resulted in the shortest timeline ever achieved for developing and deploying a vaccine. This is largely due to the mRNA platform on which it is based. However, the mRNA vaccines brought to market to address the pandemic are the first approved vaccines based on this platform. As such, the manufacturing infrastructure, including the scale-up of reagents and components to make them, was developed extremely quickly. Fortunately, our new GMP-grade facility was built with expansion in mind and allowed us to scale-up reagent supply faster than we ever have. We are continuing to invest in capital expansion and hiring staff to support the many new and existing therapeutic or vaccine platforms being developed by NEB's customers.

"We manufacture all of the individual components for in vitro transcription, including T7 RNA polymerase and other enzymatic components, and post-transcriptional modifications such as capping enzymes."

To learn more, visit **www.neb.com/GMP**.

Endangered in New England: Using science to restore a depleted river

By Patrick J. Lynch, Ipswich River Watershed Association

An Endangered River

Pandas. Right whales. Those cute little pangolins. All of them are listed as endangered. And, as of April 2021, you can add New England's iconic Ipswich River to the list.

Yep, that's right, rivers can be endangered too. Each year, the national group American Rivers designates ten Most Endangered Rivers in the United States. While the process is different than the method for listing endangered flora or fauna, the intent is the same: mobilizing action to save something that we, as humans, should care about.

Rivers are now making the list. The U.S. has a long history of tapping into its free-flowing rivers to fuel the economy. In the early 1800s, New England's rivers became the birthplace of the Industrial Revolution. Their relatively narrow channels and reliable year-round flows were perfect locations to set up the first mills and factories.

Today, the mills are long gone, old factories are being converted into lofts, and hundreds of dead dams are on the way to come down. Now a larger threat looms: water withdrawals.

I Drink Your Milkshake

In rivers like the Endangered Ipswich, existing water withdrawals have had severe consequences on the ecosystem. Due to a patchwork system of managing local water supplies and exemptions at the state level, 90% of all water withdrawals in the Ipswich basin are exempt from restrictions during drought. Picture thousands of straws, drinking from the same milkshake, even when there's no shake left to be had. Even during non-drought years, excessive withdrawals can drain the water table below the point where natural ecosystems can thrive.

Climate change is making things worse. Across the country, the climate crisis is putting stress on freshwater supplies. While high-profile cases like the Colorado basin are keeping drought on the front page, Massachusetts has also experienced two severe droughts in the last five years. When even in wet, forested regions like New England are suffering from drought, we know we have a problem.

How do we save an endangered river? While people can understand the steps needed to save a species from the brink of extinction, saving an entire ecosystem gets trickier. And, as with elsewhere in the country, many of the solutions have to deal with whether different communities are willing to come together to protect their water supplies.

Focus on the Science

Thankfully, data-driven solutions are popping up that incorporate scientific research into local policy making. Working together, we can help local leaders craft more resilient water policies that keep our communities safe and save water when it matters most.

In the Endangered Ipswich, groups like the Ipswich River Watershed Association are taking a science-based approach to improving water policies in Massachusetts. Our hope is to use research to craft viable solutions so our cities and towns can protect their water supplies and the ecosystems we depend on for survival.

One policy making tool that can benefit from a more science-based approach is the Safe Yield, which is the amount of water that can be safely withdrawn from a basin. In Massachusetts, the state Department of Environmental Protection (DEP) is responsible for calculating Safe Yield for each basin. City and Town planners then use this Safe Yield to determine where they can access future water supplies as their communities grow.

Traditionally, DEP has only used the formula to determine Safe Yield for larger basins, averaging out the impacts in 1,400 smaller sub-basins across the state. These more localized basins—often the source of both public and private water supplies—quickly become depleted when too much water is withdrawn. Safe Yield figures also don't shift based on the time of year a withdrawal occurs, despite big swings in water levels. For anyone who's experienced the snowy winters of New England, it's easy to understand why an annualized Safe Yield doesn't establish the right baseline. Increased variability due to climate change has bolstered the realization that current Safe Yield figures are anything but safe.

To demonstrate this need, the Ipswich River Watershed Association recently commissioned a hydrology assessment for a chronically-depleted sub-basin, using the state's own methodology. The study shows the Safe Yield for that particular sub-basin should actually be three times lower than what's currently being used by nearby communities. This new hydrologic analysis gives an objective assessment using publicly available data, and helps explain why one waterway now runs dry for as much as six months per year.

Think Locally, Act Locally

Going forward, state regulators could help communities figure out how much water they can safely withdraw by calculating Safe Yield at the sub-basin level. Regulators could also revise the Safe Yield methodology to differentiate between monthly flows and adjust levels when flows are lowest, in the summer and early fall months. As local communities start to reexamine the resiliency of their water supplies, a stronger (Safer?) Safe Yield can encourage them to work together.

Our best chance at creating more resilient communities is working together. By sharing information and methodologies about what works across watersheds and states, we can better inform the decision makers as they try and figure out how to react to shifts in water availability due to climate.

Photo Credit:
Ipswich River Watershed Association

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