NEB EXPRESSIONS

A scientific update

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Key considerations for optimal lyophilized reagent development

by Martin A. Lee, Ph.D., Steven G. Chiu, Diane R. Lee, B.Sc. & M.Sc., Nicole M. Nichols, Ph.D.

A wholly-owned subsidiary of New England Biolabs (NEB®), Fluorogenics™ Limited (FGL) works in concert with NEB to provide lyophilized nucleic acid amplification reagents to the research, applied and diagnostics markets.The acquisition of FGL enables NEB to combine its expertise in enzyme manufacturing and assay development with FGL's expertise in lyophilization, providing a complete solution. The purpose of this paper is to explain the lyophilization development process, as well as costs, capabilities, and supply options associated with customized lyophilized projects, in order to streamline your product development.

When is lyophilization recommended?

Although frozen reagents are an acceptable and reasonable solution for most laboratory applications, they require a robust cold chain and may be susceptible to impaired performance due to improper transport and storage. The effect of temperature changes and defrosting is dependent on the functional specification of the chosen frozen product; reverse transcriptases, for example, are more labile than thermostable DNA-dependent DNA polymerase enzymes. When designed well, lyophilized products are far more robust to transient changes in temperature and offer an ideal solution for low-cost transport as they do not require a cold chain or special handling at ports of entry or upon receipt. For applications that benefit from reagent robustness over long time periods, such as extended trials or diagnostics, lyophilized products can provide greater uniformity, will offer a longer shelf-life and can be customized for numerous workflows.

Lyophilization Requirements

The lyophilization process requires different protectants and stabilizers to those used in frozen or liquid mixes. Indeed, many cryo-protectants used in frozen formulations (e.g., glycerol) are inhibitory to the freeze-drying processes used to create a dry master mix product. For this reason, it is not typically possible to freeze-dry most liquid master mixes directly. However, most lyo providers work with commercially available reagents and process enzymes and other raw materials from external suppliers without the benefit of compositional details or sufficient support. This is feasible, but unnecessarily challenging.

Lyophilization of Custom Products

The enzyme expertise of NEB and the lyophilization capabilities of FGL have been combined to create lyophilized products that do not sacrifice the high-performance qualities of their liquid counterparts. NEB provides core lyophilized formulations through its catalog business which can be used for a variety of purposes, including early-stage testing in the research and molecular diagnostics space, lowering the barrier to initial proof-of-principle studies or iterative development trials.

Where customization is needed, FGL can also build upon these core reagents to include primers, probes and other solutes to make your lyophilized product a 'ready-to-use' solution. This greatly simplifies testing and removes the need for automation while also eliminating human and operational errors. By building upon reagents that have already been optimized and lyophilized, the process of creating a custom formulation is simplified, reducing the time it will take to create modified versions.

When moving from the catalog offering to a custom product, members of our technical and project management teams will meet with you to understand your project, goals, and timelines, as well as your detailed requirements. Information collected during this discovery phase will include details about your intended application (target market, desired price points, end user workflow), reagent requirements (necessary enzyme properties, buffers, salts, other solutes), assay design (reagent volumes, sample inputs, primers/probes), and desired packaging formats (glass vials, PCR tubes, plates, custom cartridges). Providing as much detail as possible early in the discovery phase will enable us to design and produce a product that will meet your performance and manufacturing needs. In areas of design flexibility, we will offer assistance to ensure compatibility with scale-up and manufacturing.

Raw Materials for Custom Products

Our team can provide a variety of materials and biologics to meet functional and cost requirements for each custom project. We can manage the supply chain for your enzymes and other components as needed. Custom products may draw on the NEB portfolio of enzymes and biologics, and the combined expertise across our organization to provide your specification lyophilized from a single provider.

The Lyophilization Process

Lyophilization, or freeze-drying, involves freezing a reagent mixture containing cryo-protectants (also known as excipients) at low temperatures (~ -40°C) and pressures (~100 mTorr) such that the water within the mixture sublimes from the solid to vapor phase. Excipients typically replace water and are chosen to protect biologics such as enzymes.

Product lyophilization at FGL is carried out in a freeze dryer – a dedicated instrument that automates the drying process. Freeze dryers are batch-based devices, typically containing multiple shelves. Dryer capacity is defined by the number of shelves and the density of the material that can be added per shelf. The primary packaging of a reagent (vial, plate, cartridge, etc.) must maintain contact with the shelf to ensure a good thermal contact to facilitate drying, therefore the shape and form of the packaging should be considered when designing a workflow, container or consumable device. For custom containers, we can design custom tooling to enable shelf contact and achieve an optimally dried product. The freeze-drying development process includes carefully optimized step-changes in both temperature and pressure to thoroughly dry a product while ensuring that full reagent activity can be maintained upon rehydration. This process may vary with reagent formulations, so research and development is often needed to optimize the drying of new enzymes/components.

Environmentally Friendly Global Distribution: Lyophilized products are not constrained by supply logistics.

FGL has extensive experience freeze drying some of NEB's most popular amplification products, effectively reducing the research and development timelines of custom products based on these reagents. State-of-the-art analytical tools and processes allow us to quickly optimize process parameters. In addition, NEB's use of engineered enzymes with enhanced stability and performance plus aptamers that control room temperature enzyme activity simplifies the lyophilization process, extending the stability of formulations through the key step of product dispensing prior to lyophilization.

The Dry Formulation

When the right excipients are used, the active materials in a product (e.g., enzymes) will be protected at ambient temperatures in the same way that they are in a frozen product. When lyophilization is done well, there is no need to increase the amount of active ingredients (e.g., enzymes) in a dried product to achieve a desired activity. Spending time to get a formulation correct early can bring significant long-term savings over the life of a project/product.

Over the years, FGL has evaluated numerous excipients and developed an in-depth understanding of their benefits and limitations. Additionally, FGL has proprietary excipient formulations that enable excellent protection of biologicals while also permitting instant resuspension. The most popular excipients exhibit desiccating properties such that instant dissolution of the product is maintained even after exposure to high humidity environments. This is an invaluable feature when using lyophilized products in automated workflows and in integrated systems. FGL can also offer an excipient formulation that will enable your product to adhere to plasticware, if desired.

Primary Packaging & Product Format

As noted previously, the amount of material that can be dried in a single freeze dryer is dependent upon the specifications of the dryer, but also by the primary packaging footprint. Product height plays a lesser role, provided it does not impact the number of dryer shelves that can be used. Packaging with a higher volume-to-shelf surface area will produce more product per unit shelf area and will therefore be less expensive to develop and manufacture.

FGL manufactures nucleic acid amplification-based products in crimped glass vials, screw capped vials, 96-well plates, PCR strips and in a variety of integrated platform consumables. Containers that can be closed within the dryer, such as butyl-stoppered glass vials with crimped or screw caps, are an ideal choice for lyophilized reagents. These glass vials are highly resistant to both moisture and oxygen ingress, allowing for an extended product shelf life at room temperature. Glass vials are also an ideal size for running high-density dryer batches, and thus offer the most straightforward and cost-effective product formatting.

Plastic containers such as PCR plates are made from polypropylene, which are pervious to both moisture and oxygen. For most projects, PCR plates and strips require secondary packaging to provide a seal that protects against moisture and oxygen – for example metalized foil pouches. Other vials that do not close within the dryer may also require secondary packaging to exclude moisture and oxygen. FGL has custom packaging cabinets to apply secondary packaging in an inert atmosphere, providing longevity for your products.

QC & Quality Assurance

Depending upon your product needs, FGL can carry out functional and materials testing. Functional testing of custom products verifies post-drying performance. FGL has thermocyclers and lab automation tools to qualify your products as soon as drying is completed. Materials testing may include residual moisture determination using Karl Fischer analysis. This provides important information about the success of the research and development phase and provides an important measurement for high value product QC and release.

A typical freeze dryer

LyoPrime Luna™ Probe One-Step RT-qPCR Mix with UDG (NEB #L4001)

During the research and development of your products, FGL takes a stepwise approach, first formulating and lyophilizing in vials (if not already complete), then perfecting formulation and confirming dryness. Analytical development tools such as freeze-drying microscopy can identify key drying process parameters. In-process analysis such as "pressure rise" and "transition" analysis assist with process optimization and scale-up. This ensures both concrete science and value for your development program with reduced risks at each stage.

NEB and FGL operate under an ISO 13485: 2016 compliant quality management system. This provides the assurance for manufacturers of IVD products that our products are positioned to deliver products that meet all necessary requirements. Quality audits are routinely performed by our customers or other external agencies, as required.

Development & Manufacturing costs

Understanding your specific project requirements, timelines and predicted volumes will enable the creation of detailed costing proposals for your product development program. Increasing scale, simplifying formats, designing and employing more automation and tooling, and providing purchasing commitments are all potential ways to reduce product costs. Typically, the most cost-effective projects will be those that build upon the development work that has already been incorporated into commercially available lyophilized products. Customization of these products is often a straightforward and low-risk process. Additionally, packaging that enables higher throughput in the dryer and automated sealing (e.g., glass vials) will often represent an economical choice.

For *in vitro* diagnostics customers, if necessary, license agreements can be crafted to transfer process and/or provide dried product intermediates that can be used to assemble final products in your own facilities.

Capacity & Distribution

The FGL facilities are located in the UK, approximately 2 hours from London. Delineated areas keep quality control processes separate from research and development and manufacturing processes. The facility has the capacity to deliver millions of assays and can work with you to meet your needs at higher volumes. FGL is further supported by the NEB global network with headquarters in the US (near Boston, MA) and hubs in Europe and Asia Pacific (Frankfurt, Singapore, China, Japan) to ensure that your product is shipped wherever you need it.

Experience the benefits of working with a combined organization that will simplify the process of lyophilizing nucleic acid amplification reagents.

To get started, contact us at **www.neb.com/CustomContactForm**.

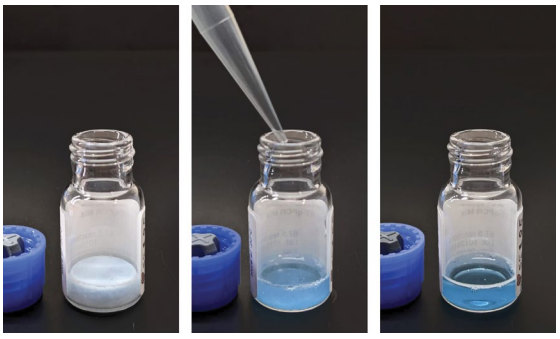
Glass vials are ideal for reagent manufacture: glass is impervious to both oxygen and moisture

Interested in learning more about whether lyophilization is the right product format for you?
View a recording of our recent webinar, "Lyophilizing reagents: requirements, challenges and considerations for assay developers", by clicking the link at
www.neb.com/webinar/neb-tv-webinar-series

LyoPrime Luna® Probe One-Step RT-qPCR Mix with UDG

Supplied as a lyophilized cake, the LyoPrime Luna Probe One-Step RT-qPCR Mix with UDG enables sensitive detection of target RNA sequences in a room temperature-stable format. This product contains the same versatile features and strong performance as the liquid version: Luna® Probe One-Step RT-qPCR 4X Mix with UDG (NEB #M3019).

LyoPrime Luna offers a robust, versatile RT-qPCR option in a shelf-stable lyophilized format



Advantages:

- Simply add nuclease-free water for rapid rehydration
- Multiplex up to 5 targets to increase throughput
- Increase reaction specificity and robustness with our unique pairing of Luna WarmStart® RT and Hot Start *Taq*
- Eliminate pipetting errors with non-fluorescent blue tracking dye
- Use with a variety of instrument platforms, including those that require a high or low ROX reference signal
- Developed in collaboration with Fluorogenics™ Limited, a wholly owned subsidiary of New England Biolabs, Inc.®

Ordering information:

Product	NEB #	Size
LyoPrime Luna Probe One-Step RT-qPCR Mix with UDG	L4001S	120 rxns

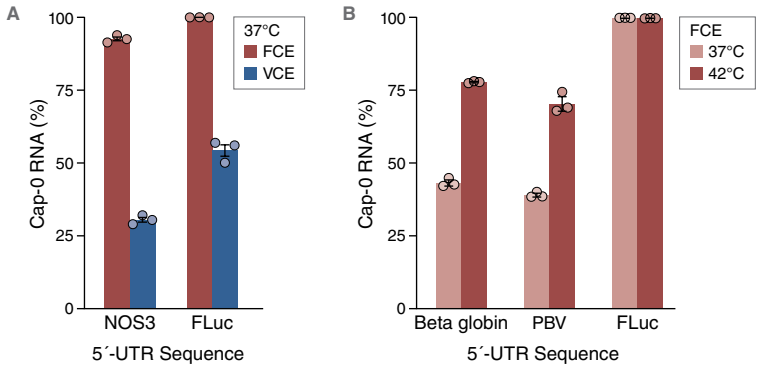
Capping it off

Faustovirus Capping Enzyme (FCE)

Looking for a robust, scalable capping solution for your mRNA manufacturing process?

Faustovirus Capping Enzyme (FCE) catalyzes the addition of N7-methylguanosine cap (m7G) to the 5´ end of triphosphorylated and diphosphorylated transcripts, producing Cap-0 RNA (1). FCE is a single-subunit enzyme that combines the three activities necessary to produce the Cap-0 structure – triphosphatase, guanylyltransferase, and (guanine-N7)-methyltransferase. FCE retains significant capping activity at low temperatures and tolerates reaction temperatures up to 55°C. In many cases, 1 µl of FCE (25 units) can cap over 100 µg of RNA in 1 hour at 37°C. GTP and S-adenosylmethionine (SAM) are required for capping activity and are included with the enzyme.

FCE offers increased capping efficiency and workflow optimization



A. mRNA capping by FCE and Vaccinia Capping Enzyme (VCE) at 37°C. 200 µg (~350 picomoles, 7 µM) of a 1.77 kb FLuc transcript having 5´-UTR sequences as indicated were treated with a limiting amount of FCE (25 units, 1 picomole, 20 nM in 50 µl) or VCE (10 units, 1 picomole, 20 nM in 50 µl) for 1 hour at 37°C. Note that this is less than our recommended amount of enzyme highlighting the increased capping efficiency of FCE vs VCE and the potential benefits of workflow optimization.

B. mRNA capping by FCE at 37°C and 42°C. 200 µg (~350 picomoles, 7 µM) of a 1.77 kb FLuc transcript having 5´-UTR sequences as indicated were treated with a limiting amount (25 units, 1 picomole or 20 nM) of FCE for 1 hour at 37°C or 42°C. Note that this is less than our recommended amount of enzyme highlighting the potential benefits of workflow optimization. All capping reactions were performed in 50 µl reactions containing 0.1 mM SAM, and 0.5 mM GTP, 1X FCE Capping Buffer for FCE reactions or 1X Capping Buffer for VCE reactions. Following capping reactions, mRNA capping was measured using targeted RNase H cleavage and LC-MS.

Advantages:

- Experience improved capping efficiency, even on difficult substrates
- Achieve robust capping with less enzyme
- Set up reactions under a broad temperature range, for added flexibility
- Choose as an alternative to Vaccinia Capping System, with minimal optimization required
- Enable one-pot Cap-1 synthesis, as FCE is compatible with mRNA Cap 2´-O-Methyltransferase
- Benefit from no licensing fees from NEB for the use of FCE

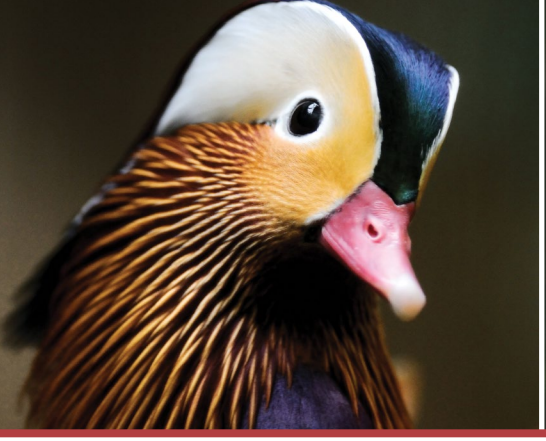
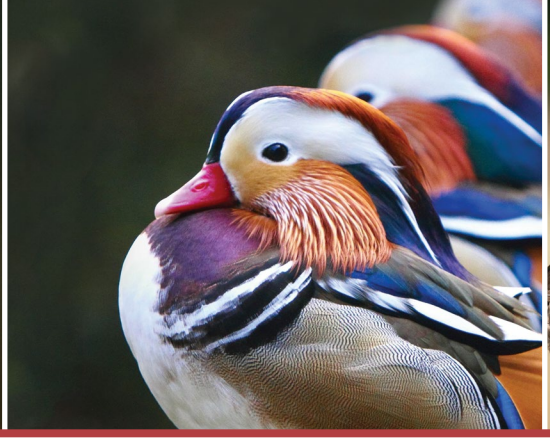
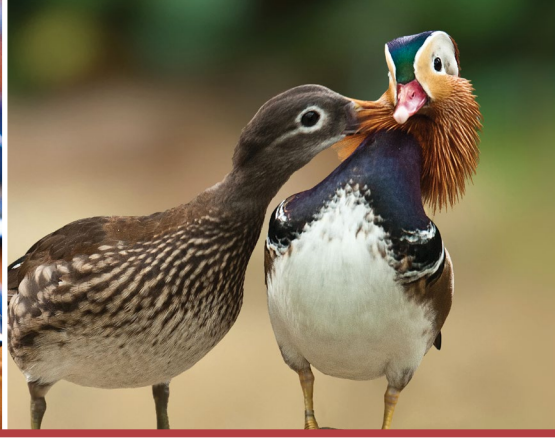
Ordering information:

Product	NEB #	Size
Faustovirus Capping Enzyme (FCE)	M2081S/L	500/2,500 rxns

Reference:

1. Ramanathan, A., et al. (2016) *Nucleic Acids Res.* 44(16), 7511–7526.

To learn more, visit **www.neb.com/m2081**

The finest in fidelity – for over **10** years

How time flies! It has been over 10 years since the release of Q5® High-Fidelity DNA Polymerase. In that time, it has set the standard for performance and fidelity (>280 times higher fidelity than Taq). Its unique buffer system provides superior performance for a broad range of amplicons, regardless of GC content.

Q5 is featured in multiple products to support a range of applications, and we are proud to announce our newest release for direct sample amplification: ***Q5 Blood Direct 2X Master Mix****.*

Trust Q5

FOR ALL YOUR HIGH-FIDELITY NEEDS

PCR

Q5 High-Fidelity DNA Polymerase (NEB #M0491)

Q5 Hot Start High-Fidelity DNA Polymerase (NEB #M0493)

Q5U Hot Start High-Fidelity DNA Polymerase (NEB #M0515)

Q5 High-Fidelity 2X Master Mix (NEB #M0492)

Q5 Hot Start High-Fidelity 2X Master Mix (NEB #M0494)

Q5 High-Fidelity PCR Kit (NEB #E0555)

NGS LIBRARY AMPLIFICATION

NEBNext Ultra™ II Q5 Master Mix (NEB #M0544)

NEBNext Q5U® Master Mix (NEB #M0597)

ARTIC VIRAL SEQUENCING

One-Step
LunaScript® Multiplex One-Step RT-PCR Kit (NEB #E1555)

Two-Step
LunaScript RT SuperMix (NEB #M3010)

Q5 Hot Start High-Fidelity 2X Master Mix (NEB #M0494)

DIRECT SAMPLE AMPLIFICATION

Q5 Blood Direct 2X Master Mix (NEB #M0500)

MUTAGENESIS

Q5 Site-Directed Mutagenesis Kit (NEB #E0554)

Q5 Site-Directed Mutagenesis Kit (Without Competent Cells) (NEB #E0552)

View the full product portfolio and request your free sample at [Q5PCR.com](Q5PCR.com)

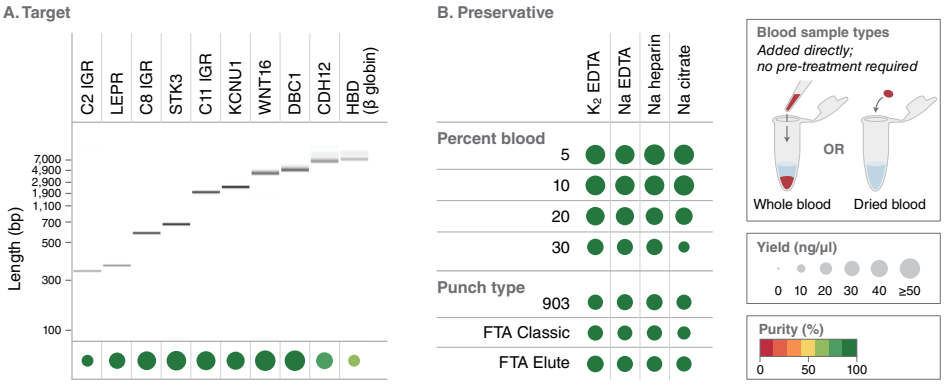
PCR direct from blood with Q5® Blood Direct 2X Master Mix

Our Q5 portfolio has just expanded to include Q5 Blood Direct 2X Master Mix. This mix can amplify a wide variety of targets directly from dried blood spots or up to 30% whole human blood, skipping DNA purification. The master mix includes Q5 Hot Start High-Fidelity DNA Polymerase and dNTPs in an optimized buffer that delivers increased resistance to inhibitors in blood, anti-coagulants, and chemicals on filter papers. It is capable of amplifying products up to 7.5 kb from human whole blood cells preserved with sodium EDTA, potassium EDTA, sodium citrate and sodium heparin, as well samples stored on common preservative filter papers.

Advantages:

- Skip DNA purification and amplify direct from human whole blood cells or dried blood spots
- Utilize Q5 Hot Start High-Fidelity DNA Polymerase for accurate and efficient PCR
- Benefit from increased resistance to inhibitors and chemicals with our optimized buffer system
- Set up reactions at room temperature with aptamer-based enzyme control

Q5 Blood Direct 2X Master Mix enables robust amplification directly from whole or dried blood



PCR was performed using Q5 Blood Direct 2X Master Mix under standard recommended conditions with 35 cycles of amplification. Yield and purity were quantitated by microfluidic LabChip® analysis and are indicated by dot size and color, respectively, with a large, dark green dot representing the strongest performance.

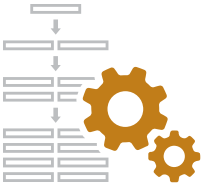
A. Amplification of a variety of human genomic amplicons, 0.3 to 7.5 kb in length, from 10% EDTA-preserved human whole blood. Results are shown as both a virtual gel (top) and corresponding dot plot (bottom). Q5 Blood Direct 2X Master Mix performs well across a broad range of amplicon sizes.

B. Amplification of a 604 bp human genomic amplicon from whole blood (top) or blood dried on filters (bottom). Human whole blood comprised 5-30% of the total reaction volume (50 µl) as indicated. Untreated 1 mm punches from dried blood spots were added directly to 25 µl reactions (one punch per reaction), even where pre-treatment of the punch was recommended by the manufacturer. Q5 Blood Direct 2X Master Mix shows broad tolerance to varying blood volumes, preservatives and punch types.

Ordering information:

Product	NEB #	Size
Q5 Blood Direct 2X Master Mix	M0500S/L	100/500 rxns

To request a sample, visit www.neb.com/m0500



General Guidelines for **PCR Optimization**

New England Biolabs offers a diverse group of DNA Polymerases for PCR-based applications. Specific recommendations for PCR optimization can be found in the product literature or on the individual product webpages. However, these general guidelines will help to ensure success using our PCR enzymes.

Setup Guidelines

DNA Template

- Use high quality, purified DNA templates whenever possible. Please refer to specific product information for amplification from unpurified DNA (e.g., colony PCR or direct PCR).
- For low complexity templates (e.g., plasmid, lambda, BAC DNA), use 1 pg–10 ng of DNA per 50 µl reaction
- For higher complexity templates (e.g., genomic DNA), use 1 ng–1 µg of DNA per 50 µl reaction
- Higher DNA concentrations tend to decrease amplicon specificity, particularly for high numbers of cycles

Primers

- Primers should typically be 20–30 nucleotides in length, with 40–60% GC Content
- Primer Tm values should be determined with NEB's Tm Calculator (TmCalculator.neb.com)
- Primer pairs should have Tm values that are within 5°C
- Avoid secondary structure (e.g., hairpins) within each primer and potential dimerization between the primers
- Higher than recommended primer concentrations may decrease specificity
- When engineering restriction sites onto the end of primers, 6 nucleotides should be added 5´ to the site
- Annealing temperatures should be determined according to specific enzyme recommendations. *Please note that Q5® and Phusion®* annealing temperature recommendations are unique.*
- Final concentration of each primer should be 0.05–1 µM in the reaction. Please refer to the more detailed recommendations for each specific enzyme.
- When amplifying products > 20 kb in size, primers should be ≥ 24 nucleotides in length with a GC content above 50% and matched Tm values above 60°C
- To help eliminate primer degradation and subsequent non-specific product formation, use a hot-start enzyme (e.g., One*Taq*® Hot Start DNA Polymerase or Q5 Hot Start High-Fidelity DNA Polymerase)

Magnesium Concentration

- Optimal Mg^{2+} concentration is usually 1.5–2.0 mM for most PCR polymerases
- Most PCR buffers provided by NEB already contain sufficient levels of Mg^{2+} at 1X concentrations.
- NEB offers a variety of Mg-free reaction buffers to which supplemental Mg^{2+} can be added for applications that require complete control over Mg^{2+} concentration
- Further optimization of Mg^{2+} concentration can be done in 0.2-1 mM increments, if necessary. For some specific applications, the enzyme may require as much as 6 mM Mg^{2+} in the reaction.
- Excess Mg^{2+} may lead to spurious amplification; Insufficient Mg^{2+} concentrations may cause reaction failure

Deoxynucleotides

- Ideal dNTP concentration is typically 200 µM of each, however, some enzymes may require as much as 400 µM each. Please refer to specific product literature for more detailed recommendations.
- Excess dNTPs can chelate Mg^{2+} and inhibit the polymerase
- Lower dNTP concentration can increase fidelity, however, yield is often reduced
- The presence of uracil in the primer, template, or deoxynucleotide mix will cause reaction failure when using archaeal PCR polymerases. Use One*Taq* or *Taq* DNA Polymerases for these applications.

Enzyme Concentration

- Optimal enzyme concentration in the reaction is specific to each polymerase. Please see the product literature for specific recommendations.
- In general, excess enzyme can lead to amplification failure, particularly when amplifying longer fragments

Starting Reactions

- Unless using a hot start enzyme (e.g., One*Taq* Hot Start DNA Polymerase or Q5 Hot Start High-Fidelity DNA Polymerase), assemble all reaction components on ice
- Add the polymerase last, whenever possible
- Transfer reactions to a thermocycler that has been pre-heated to the denaturation temperature. Please note that pre-heating the thermocycler is not necessary when using a hot start enzyme (e.g., One*Taq* Hot Start DNA Polymerase or Q5 Hot Start High-Fidelity DNA Polymerase).

Cycling Guidelines

Denaturation

- Optimal denaturation temperature ranges from 94°–98°C and is specific to the polymerase in the reaction. Please refer to product information for recommended conditions.
- Avoid longer or higher temperature incubations unless required due to high GC content of the template
- For most PCR polymerases, denaturation of 5–30 seconds is recommended during cycling
- NEB's aptamer-based hot start enzymes do not require additional denaturation steps to activate the enzymes

Annealing

- Primer Tm values should be determined using the NEB Tm Calculator (TmCalculator.neb.com)
- For PCR polymerases other than Q5 High-Fidelity DNA Polymerase or Phusion High-Fidelity DNA Polymerase*, annealing temperatures are usually set at 2°–5°C below the lowest Tm of the primer pair
- When using Q5 High-Fidelity DNA Polymerase or Phusion High-Fidelity DNA Polymerase*, annealing temperatures should be set at 0°–3°C above the lowest Tm of the primer pair. Please refer to the product literature for detailed recommendations.
- Non-specific product formation can often be avoided by optimizing the annealing temperature or by switching to a hot start enzyme (e.g., One*Taq* Hot Start DNA Polymerase or Q5 Hot Start High-Fidelity DNA Polymerase)
- Annealing temperatures can be optimized by doing a temperature gradient PCR, starting at 5°C below the lowest Tm of the primer pair
- Ideally, primer Tm values should be less than the extension temperature. However, if Tm values are calculated to be greater than the extension temperature, a two-step PCR program (combining annealing and extension into one step) can be employed.

Extension

- Extension temperature recommendations range from 65°–72°C and are specific to each PCR polymerase. Please refer to the product literature for specific recommendations.
- Extension rates are specific to each PCR polymerase. In general, extension rates range from 15–60 seconds per kb. Please refer to the recommendations for each specific product.
- Longer than recommended extension times can result in higher error rates, spurious banding patterns and/or reduction of amplicon yields

**Phusion DNA Polymerase was developed by Finnzymes Oy, now a part of Thermo Fisher Scientific. This product is manufactured by New England Biolabs, Inc. under agreement with, and under the performance specifications of Thermo Fisher Scientific. Phusion® is a registered trademark and property of Thermo Fisher Scientific.*

New England Biolabs partners with Reforest the Tropics on a biodiverse reforestation project

by Joanne Gibson, Ph.D.

About four years ago, New England Biolabs began to explore ways to go carbon neutral. We have always considered ourselves stewards of the environment and tried to minimize our greenhouse gas emissions, but in this time of climate action urgency, it was clear that more needed to be done.

The international scientific community has set a target for CO_2 emissions at 350 parts per million (ppm) to limit the more dramatic effects of a changing climate. Currently, at about 415 ppm, we are not on a good trajectory to meet that target. Additionally, when we think of CO_2 in the atmosphere, we also need to think about the residency rate – how long it persists without intervention; while the range is hard to calculate, we know that getting to zero emissions is not enough – we are cumulatively adding about three ppm each year. Negative emission technologies, such as carbon capture and storage, are being explored by many. Right now, though, it's costly, and there are some safety concerns.

Alternatively, we can plant forests. It is less expensive and has several ancillary benefits beyond carbon capture. Historically, there are two primary approaches: the natural forest analog, where the forest design copies the natural forest, and many different tree species are planted, or tree farming with a single species (specifically *Gmelina arborea*). The natural forest analog is ideal from an ecological standpoint. Still, it's inefficient at carbon capture and doesn't work well on privately held land due to a lack of profit potential for the landowner. The single species/monoculture projects that are taking place worldwide capture carbon much more efficiently and generate income for the landowners (through regular clear-cutting); however, this is not an ideal approach for a more comprehensive global strategy toward reforestation. What is needed is a balance between planting trees specifically for carbon capture while at the same time promoting biodiversity of both flora and fauna to create a long-term stable ecosystem.

After much research, NEB turned to Reforest the Tropics (RTT). Founded in the 1960s, RTT's mission is to create a reforestation model that is improved over the currently available options. They operate in Costa Rica (with a plan to expand to other tropical regions) because of its long history of environmental stewardship. The Costa Rican farmers who partner with RTT support, and have a good understanding of, the project's goals. This is essential for the project's long-term success; the farmers' needs must be met regarding financial incentives. Their goal is to make forestry a competitive land use activity, generating similar income potential to cattle farming. Additionally, RTT is committed to creating forest models that are more environmentally and ecologically responsible; diverse mixed-species models that maximize carbon capture while providing critical habitat to maintain species native to Costa Rica.

The NEB Costa Rican Forest

The NEB Costa Rica reforestation project contains 30 tree species planted on 100 hectares at three different sites. The model incorporates two exotic species because of their unique and necessary roles. The remaining tree species in the project are all native to Costa Rica. Each tree species is carefully chosen and has a specific purpose:

1) **Long-term carbon capture** – RTT has experimented with several models for decades. They incorporate the non-native tropical conifer *Araucaria hunsteinii* (Klinky) – a juggernaut of carbon capture that allows them to double the amount of carbon sequestered compared to most other reforestation models. It has a narrow crown that allows for more trees in a space.

2) **Generating profit for the landowner** – the other non-native species they incorporate is eucalyptus to provide early income for the farms. The eucalypts are short-term residents in their projects and are phased out within 15 years of the 100-year project. There are other fast-growing species in Costa Rica, but unlike the eucalypts, they have dense crowns that prevent light from hitting neighboring trees and are disruptive to the project. After the eucalypts are phased out, the established forest project generates about $500/ha/year for the farmer.

3) **Encouraging wildlife** – The largest of the three NEB sites in Costa Rica is 71 hectares. It is RTT's most biodiverse project to date, and it has 16 hectares dedicated to wildlife – fruit trees such as bananas, apple, guava and avocado are planted. This is not related to carbon capture but encourages the wildlife to find habitat there. Camera traps have provided evidence of jaguars, macaws, toucans, and endangered tapirs. Another of the NEB properties in Costa Rica has five hectares dedicated to wildlife that abuts a major river, an essential attraction for wildlife.

RTT measures the amount of carbon captured annually. They also go through an independent third-party verification process. Their model sequesters, on average, double what most forestry projects capture – about 25 metric tons of carbon dioxide equivalent/hectare/year, compared to the industry standard of 12-15 metric tons/ hectare/ year. Once there is evidence that the trees are starting to compete and there is a deceleration of annual, incremental growth, they thin about 15% of the volume. This immediately stimulates new growth. The carbon is recouped within 12-18 months, which gives about four years of carbon accumulation before the subsequent thinning. Growing trees under a primary canopy is extremely challenging. RTT has also been researching species that are good candidates to create a secondary canopy, and these trees, after 50-60 years, will become meaningful in the overall matrix. Additionally, RTT is working with researchers to measure soil carbon and use drones to measure and monitor the forests.

Reforestation is a very efficient, cost-effective way to extract carbon dioxide from the atmosphere. It's not the only tool we need, but it is essential, not just for the carbon capture aspect but for creating balanced ecosystems that support a wealth of biodiversity. RTT has developed a model that works. They maintain an open-source information-sharing philosophy because they look at the big picture of addressing our lofty environmental goals.

Learn more about Reforest the Tropics in our "Lessons from Lab & Life" Podcast at www.neb.com/podcasts

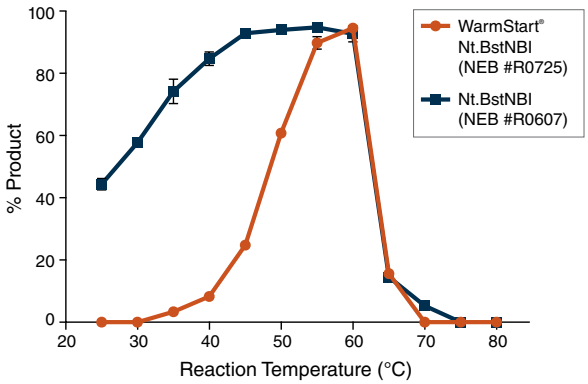
PROJECT OUTCOMES

- The project will sequester at least 50,000 mt of CO_2 equivalent
- Six permanent full-time jobs are created
- An estimated $1,125,000 will be infused into communities
- Many native fauna species will reside in these forests
- Forest design, soil carbon, and drone measuring studies will contribute to the global body of knowledge
- Water and air quality, recreational opportunities, national goals

New innovation: WarmStart® Nt.BstNBI

NEB continues to innovate in the area of restriction enzymes with the introduction of WarmStart Nt.BstNBI. This site-specific nicking endonuclease recognizes the site site GAGTC(4/-5). It is formulated with a reversibly-bound aptamer, which inhibits its nicking activity at temperatures below 40°C. WarmStart Nt.BstNBI catalyzes a single-stranded break 4 bases beyond the 3′ side of the recognition sequence, and is suitable for strand displacement amplification (SDA).

Reaction temperature profile of WarmStart Nt.BstNBI



Nicking activity of WarmStart Nt.BstNBI (NEB #R0725) and Nt.BstNBI (Non-WarmStart NEB #R0607) on a fluorescently-labeled DNA duplex was measured at various temperatures (25°C to 80°C, 5°C intervals). Reactions containing 0.125 unit WarmStart or non- WarmStart Nt.BstNBI, 100 nM DNA duplex (5′ 6-FAM labeled) in 20 µl NEBuffer r3.1 were incubated for 30 min at various temperatures. The average and the standard deviation of % product (Y-axis) plotted with temperatures (X-axis) was taken from triplicate reactions.

Conclusion: WarmStart Nt.BstNBI shows less than 10% activity below 40°C and is undetectable below 30°C, thereby enabling reaction setup at room temperature with no unintended conversion of substrate.

Advantages:

- WarmStart technology inhibits enzyme activity below 40°C
- Generates DNA molecules that are "nicked", rather than cleaved
- Allows for room temperature setup, facilitating strand displacement amplification (SDA)

To learn more, visit
www.neb.com/r0725

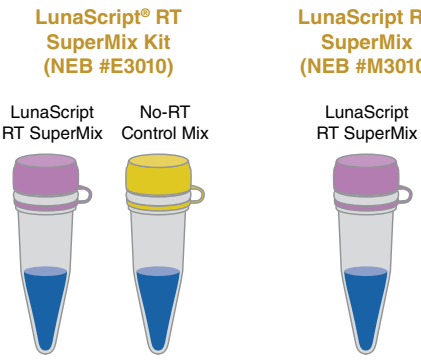
Ordering information:

Product	NEB #	Size
WarmStart Nt.BstNBI	R0725S	1,000 units

LunaScript® RT SuperMix

With a 13-minute protocol, LunaScript RT SuperMix enables efficient first strand cDNA synthesis in applications such as amplicon sequencing or two-step RT-qPCR. LunaScript RT SuperMix is a streamlined, supermix-only offering and does not include a No-RT control mix or nuclease-free water. LunaScript RT SuperMix is now also available in large, bulk-size formats for high throughput users.

Comparison of LunaScript products



	LunaScript® RT SuperMix Kit (NEB #E3010)	LunaScript RT SuperMix (NEB #M3010)
RT SuperMix included	✓	✓
No-RT Control Mix included	✓	✗
Nuclease-free water included	✓	✗
Bulk size available	✗	✓

Advantages:

- Simplify reaction setup with convenient supermix format
- Less than 15 minute first-strand cDNA synthesis protocol
- Featured in the ARTIC SARS-CoV-2 sequencing workflow and a component in the NEBNext® ARTIC SARS-CoV-2 kits
- Eliminate pipetting errors with non-interfering, visible tracking dye

To request a sample, visit
LUNAqPCR.com

Ordering information:

Product	NEB #	Size
LunaScript RT SuperMix Kit	E3010S/L	25/100 rxns
LunaScript RT SuperMix	M3010L/X/E	100/500/2,500 rxns
Luna Universal qPCR Master Mix	M3003S/L	200/500 rxns
Luna Universal Probe qPCR Master Mix	M3003S/L	200/500 rxns

Fantastic Ocean Creatures and Where to Put Them

by Rosie Poulin, Hannah Appiah-Madson, and Dan Distel, Ocean Genome Legacy Center

Around the world, every day, researchers collect and document samples from a mind-blowing variety of fantastic ocean creatures. But what happens to these samples after they've been collected?

Eventually, most of them will end up lost in the back of a freezer or simply discarded. But those same samples may contain DNA with tremendous value beyond the purpose for which they were collected. DNA is full of information. It can reveal an organism's evolutionary origins and history, capabilities and characteristics, reproductive strategies, and lifestyle. It can also be used to evaluate needs for species and ecosystem protection, sustainably regulate fisheries, identify seafood mislabeling, monitor the spread of invasive and genetically modified species, and contribute to the discovery of new medicines and biotechnologies.

The great news is that these underutilized samples can now be put to good use. Researchers can give these samples a second life by contributing them to a publicly accessible biological repository, like the Ocean Genome Legacy Center (OGL). Originally established by NEB's founder, Donald Comb, OGL is a public, nonprofit, open-access biological collection located at Northeastern University's Marine Science Center. OGL is comprised of well-documented and curated samples of genomic DNA and DNA-containing tissues representing a broad cross section of the diversity of life in the ocean. OGL works by collaborating with researchers, institutions, and individual collectors to acquire marine DNA and DNA-containing tissues and making them available to researchers around the world to support new discoveries.

The OGL collection is large, diverse, and growing. Since its inception in 2012, OGL's collection has grown to encompass over 28,000 DNA extracts and 18,000 tissue samples from over 3,600 species. These samples and DNA extracts were collected from approximately 5,000 locations across 91 countries and all ocean basins. OGL has distributed over 8,000 biological samples to the research community.

To support this mission, OGL has recently begun reaching out to retiring researchers, aiming to rescue their samples and data from an untimely fate. To date, OGL has collaborated with three retiring researchers, Dr. Bill Detrich (Northeastern University), Dr. Charles Fisher (Penn State University) and Dr. Marian Litvaitis (University of New Hampshire) and added samples from over 3,200 marine organisms to OGL's collection. These collections include rare and mysterious creatures from deep-sea hot springs miles below the sea surface, Antarctic icefish that thrive in freezing waters because of antifreeze in their blood, and dazzlingly colored marine flatworms that grace coral reefs around the world.

While these collections are specialized and represent the specific scientific interests of their original collectors, the samples and data can now be used by other researchers to ask important new questions.

But sample collection is just the first step. A critical part of OGL's mission is to make samples easily accessible to support amazing new research. This reduces the need for new field sampling that may be environmentally and economically costly. OGL also ensures that these samples are used ethically and fairly by complying with international standards established by the Nagoya Protocol on Access and Benefits Sharing of the Convention on Biological Diversity. In this way, researchers can be confident that samples from OGL are well-documented, legally collected, properly permitted, and that any benefits that arise from new research are shared with the countries of origin. Researchers can find all OGL samples via the OGL online catalog (https://ogl.northeastern.edu/catalog/) and the Global Genome Biodiversity Network (GGBN).

It's not just physical materials that are important. Their associated data can also have great value for tackling new research questions. OGL shares its taxonomic and biogeographic location data with world-wide biodiversity databases such as the Ocean Biodiversity Information System (OBIS) and the Global Biodiversity Information Facility (GBIF). To date, OGL data has been downloaded more than 40,000 times!

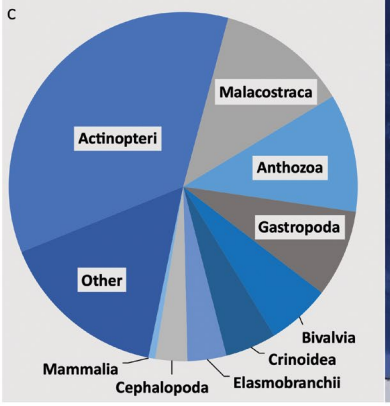
A

Stored Objects	**47,076**
Nucleic Acid Extracts	28,615
Other Sample Types	18,461
Collection Locations	**4,967**
Collection Events	**4,462**
Depth Range (m)	**0-5,983**
Distributed Biological Samples	**8,148**
Publications that Cite OGL	**181**
GBIF Records	**28,299**
OBIS Records	**23,002**

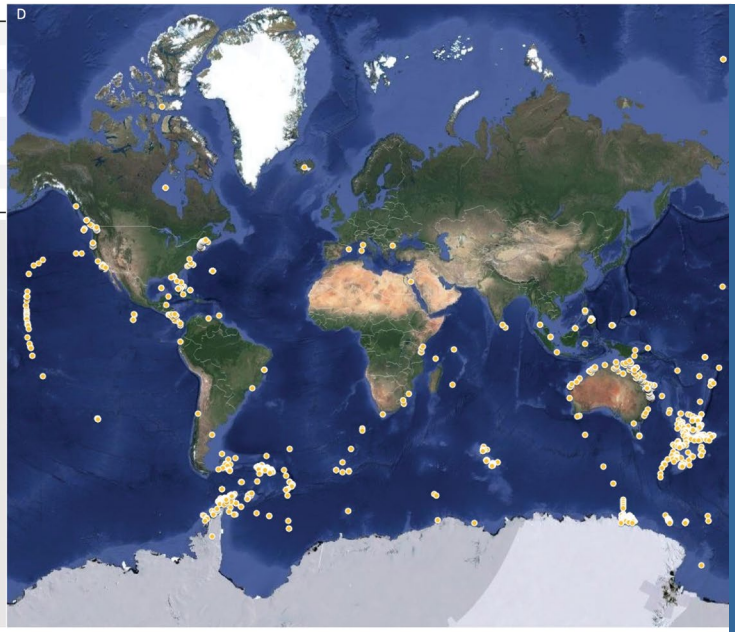
B

Taxonomic Breakdown	
Kingdoms	4
Phyla	29
Classes	72
Orders	284
Families	1,165
Genera	2,628
Species	3,621

C



D



The Ocean Genome Legacy (OGL) Genomic Resource Collection. (A) Description of OGL holdings and products, including distributed biological materials and data. (B) Taxonomic ranks represented by stored objects held in the OGL collection. (C) Proportion of stored objects in the OGL collection by class. (D) Map displaying collection locations associated with stored objects held in the OGL collection. All data reflects the collection as of October 22, 2021.

OGL's mission may sound like an immense undertaking. And it is! But with the support of New England Biolabs and many other generous donors, OGL continues to find new homes and uses for the DNA of fantastic ocean creatures.

To find out more about OGL, visit our website at **ogl.northeastern.edu**. Also, please feel free to download OGL's latest publication "The Ocean Genome Legacy: A Genomic Resource Repository for Marine Life" to learn more about OGL's mission, collection, policies, and some of the ways we serve our constituents.

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