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Breaking through the limitations of Golden Gate Assembly – The co-evolution of test systems, engineered enzymes and understanding ligase fidelity

by Rebecca Kucera, M.Sc. and Eric Cantor, Ph.D., New England Biolabs, Inc.

Golden Gate Assembly is a molecular assembly technique that utilizes simultaneous digestion with Type IIS restriction enzymes and ligation by a DNA ligase to enable the scarless, ordered assembly of multiple fragments (1). Embraced by the synthetic biology community, as well as the general molecular biology community, it is commonly used to assemble 2-10 inserts, or modules, in a single "one-pot" reaction to form complex, multi-insert modular assemblies that enable biosynthetic pathway engineering and optimization. Recent advances in Golden Gate Assembly at New England Biolabs have results in unprecedented improvements in efficiencies for both higher-complexity assemblies, as well as single-insert cloning, that enables library creation.

Current best practices for assemblies of more than 10 modules often relies on two-step hierarchical approaches using different Type IIS restriction enzyme specificities at each step. In the past, factors such as enzyme efficiency, stability and buffer compatibility have placed practical limits on single- or two-step assemblies, and large scale downstream screening is required to find correct assemblies – a highly undesirable outcome. The constraints of this assembly approach called for further development. As a result, NEB has been able to reduce those limitations through ongoing engineering efforts of Type IIS restriction enzymes and careful choice of junction sequences guided by experimentally-derived DNA ligase fidelity data. Our work demonstrates that it is now possible to achieve 20+ fragment assemblies with both robust efficiency and accuracy.

DEVELOPMENT OF GOLDEN GATE ASSEMBLY TEST SYSTEMS

New England Biolabs has been committed to further developing Golden Gate protocols and enzymes for a number of years, a commitment that has enabled the development of a variety of test systems with increasing complexities. These more difficult assemblies have allowed us to identify and implement improvements in Golden Gate assembly that could not be detected with simpler 1–10 insert test systems. Our research focused on three different levels of assembly:

- high efficiency and accurate single-insert assembly
- intermediate 5- or 12-fragment assembly, mirroring the commonly perceived "upper limit" for assembly
- more complex 24-fragment assembly

Table 1 (page 3) illustrates the breadth of test systems used by NEB to address this range of usage for Golden Gate as assembly approaches have evolved.

Before assembly optimization, each test system was evaluated in a variety of ways. Single insert cloning based on the acquisition of a selectable antibiotic marker allowed fast throughput testing of efficiencies. This cloning was also compared to a similar-sized lambda amplicon to indicate any possible bias towards suppression of background by antibiotic selection. Screening of transformants by colony PCR confirmed the insertion of the lambda insert at the same high frequencies. The 5-, 12- and 24-assembly systems are based on the ability to correctly assemble a *lacI/lacZ* cassette (designed by NEB for use in optimization of assembly systems) to produce a blue color phenotype upon growth on LB/Cam/X-gal/IPTG agar plates, indicating successful reconstruction of the coding sequence for beta-galactosidase in the *lacI/lacZ* cassette.

Additional confirmation of accurate assemblies was achieved by the sequencing of plasmids isolated from blue or white colonies. Sequencing of blue colonies showed the expected complete sequence for the *lacI/lacZ* genes (2), while sequences of white colonies revealed a mixture of mis-assemblies and very occasional uncut or cut/re-ligated pGGA destination plasmids. A final validation of the 5-, 12- or 24-fragment test systems was performed by setting up assembly reactions in which a single component was purposefully omitted. Since any assembly is dependent on the presence of every module, destination construct and functioning Type IIS restriction enzyme and DNA ligase, any single omission should completely block the formation

FIGURE 1:
Golden Gate Assembly workflow for complex assemblies

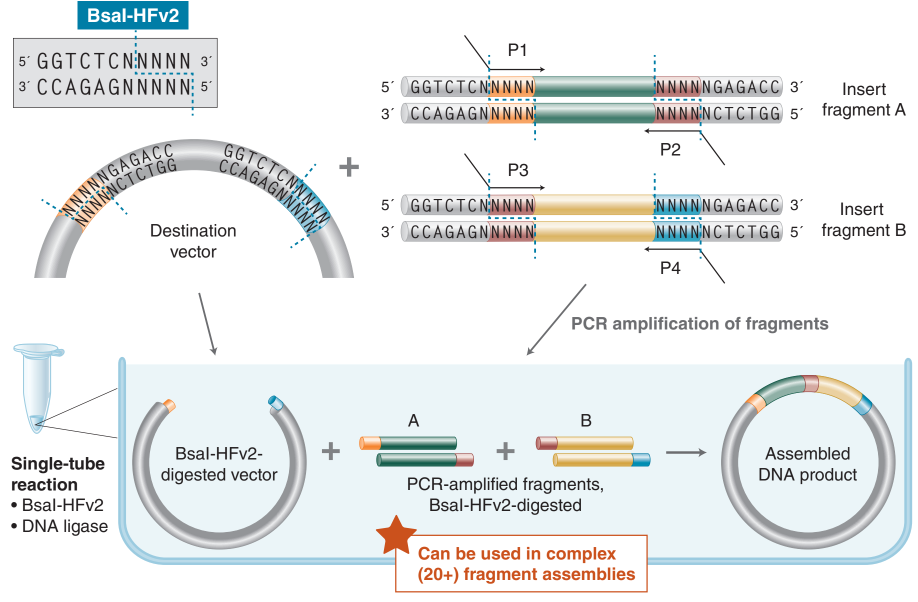


TABLE 1:
Assembly test systems of increasing complexities designed at New England Biolabs

All inserts self-assemble into the pGGA destination construct. Reactions were incubated at 37°C for 5-min. or 60-min. for single inserts, and 30 cycles of (5 min. 37°C → 5 min. 16°C) for the 5-, 12- and 24-fragment assemblies. In both cases, a terminal soak of 5 min. at 55°C was used to complete the assemblies at a temperature that favors cutting of any uncut/re-formed pGGA to reduce background. Single-insert assemblies used purified amplicon inserts at a 2:1 insert:vector ratio, while the *lacI/lacZ* cassettes used precloned inserts at equimolar levels to pGGA.

NUMBER OF INSERTS	GOLDEN GATE ASSEMBLY TEST SYSTEM	SIZE OF INDIVIDUAL INSERTS	SIZE OF ASSEMBLED INSERTS	INDICATION OF CORRECT ASSEMBLIES INTO pGGA (Cam^R) DESTINATION PLASMID
1	Amp^R, Kn^R, or Lambda amplicon	~1 kb	~1 kb	Growth on Cam/Amp, Cam/Kn or Cam/colony PCR
5	*lacI/lacZ* Cassette	~1 kb	~5 kb	Blue colony on Cam/X-gal/IPTG plates
12	*lacI/lacZ* Cassette	~300–600 bp	~5 kb	Blue colony on Cam/X-gal/IPTG plates
24	*lacI/lacZ* Cassette	~100–300 bp	~5 kb	Blue colony on Cam/X-gal/IPTG plates

of a complete assembly that would result in a blue phenotype. Indeed, this was seen in all *lacI/lacZ* assembly test systems; no blue colonies were obtained if any single component was omitted.

All Golden Gate assemblies feature an inverse proportionality between the complexity of the assembly (number of inserts or modules) and the resulting efficiency of assembly (number of transformants); the greater the number of inserts, the lower the number of transformants. This is often compensated for by plating greater volumes of the outgrowth on the selection plate to achieve enough transformants for downstream screening. Figure 2 shows representative transformation plates obtained from 1-, 12- and 24-fragment assemblies of the *lacI/lacZ* cassette, and illustrates how the volume of the 1 ml outgrowth spread on each transformation plate can be manipulated to result in appropriate levels of colony plating densities.

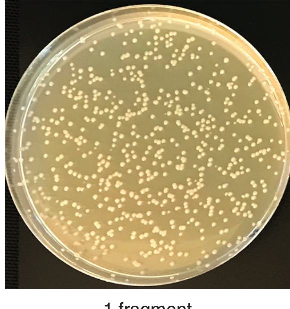
BREAKING THROUGH THE LIMITS OF GOLDEN GATE ASSEMBLY

Five fragment *lacI/lacZ* cassette assembly was easily achievable with high levels of transformants and low backgrounds – so much so that there was little range for detectable improvements in the methodology. The decision was made to re-design the test system for 12 and 24 fragments. This was guided by both advances in the re-engineering of the original BsaI-HF® Type IIS restriction enzyme and the completion of T4 DNA Ligase fidelity studies conducted by Potapov, et al. at NEB (1,2). While T4 DNA Ligase, the mainstay of most biotechnological cloning efforts for over 50 years, prefers ligation of Watson-Crick base pair substrates, it can show significant activity on some mismatch-containing pairings. During Golden Gate Assembly, ligation of mismatched pairs of overhangs can lead to incorrect assemblies, so care must be taken to minimize this possibility. Recently, NEB researchers profiled the comprehensive fidelity of cohesive end ligation by this enzyme for all 3- and 4-base overhang sequences under standard reaction conditions. This data set allows quantitation of sequence dependent ligation efficiency and identification of mismatch-prone pairings. Using the 4 bp overhangs, accurate "high-fidelity" junction sets for both the 12- and 24-fragment versions of the *lacI/lacZ* cassette were designed and synthesized. In conjunction with BsaI-HFv2 (NEB #R3733), re-engineered to provide improved Golden Gate performance, a series of optimization experiments for these more complex assemblies were performed. It was found that high efficiencies and accurate assembly levels were indeed possible, with correct, in-frame assembly proceeding in 99% of 12-fragment assemblies and over 90% for 24-fragment assemblies (Figure 3, Table 2, page 4). The fidelity data can be applied to derive similar high fidelity overhangs for any Golden Gate assembly design. Additionally, the stability of the enzymes allowed use of a greater number of cycles, pushing the efficiency levels even higher than afforded by the standard 30 cycles for those wishing maximal transformation levels. Extended reaction cycling is only successful when using highly stable enzymes that maintain activity beyond the standard 5 hours required for 30 cycles, utilizing 5-minute stages at temperatures favoring digestion (37°C) and ligation (16°C).

FIGURE 2:
Representative transformation plates of Golden Gate Assemblies featuring increasing complexities

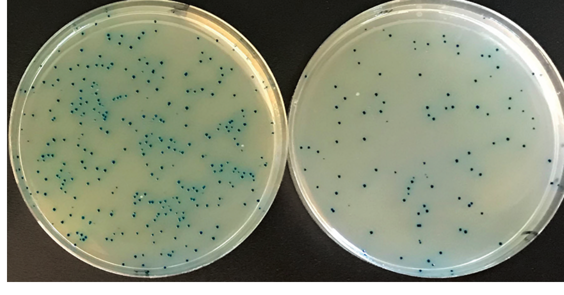
Assembly reactions were transformed into competent *E. coli* strains NEB 10-beta (#C3019) (1 fragment) and T7 Express (#C2566) (12 and 24 fragments) and incubated for 16 hours at 37°C. While many strains support assembly protocols, and NEB 10-beta is routinely recommended due to its ability to stably maintain large construct plasmid sizes, the non-alpha complementing T7 Express cell strain was used for the *lacI/lacZ* cassette testing to avoid any possibility of alpha-fragment LacZ complementation.

Amp^R amplicon



1 fragment
(2.5 µl outgrowth)

***lacI/lacZ* gene cassette fragments**



12 fragments
(5 µl outgrowth)

24 fragments
(100 µl outgrowth)

continued on page 4...

LOOKING TO THE FUTURE

DNA assembly methods are important tools for many areas of science, and researchers continue to test the limits of DNA assembly approaches with increasingly complex experimental conditions. The ability to construct more complex, multi-fragment assemblies, as shown in this work, will fuel additional efforts to push the technique forward. Our research and development efforts continue to focus on providing optimized reaction components for a wider number of type IIS specificities and substantial improvements to the methodology, with the goal of enabling routine, efficient and accurate assembly of 50 fragments in a single tube in the not-so-distant future.

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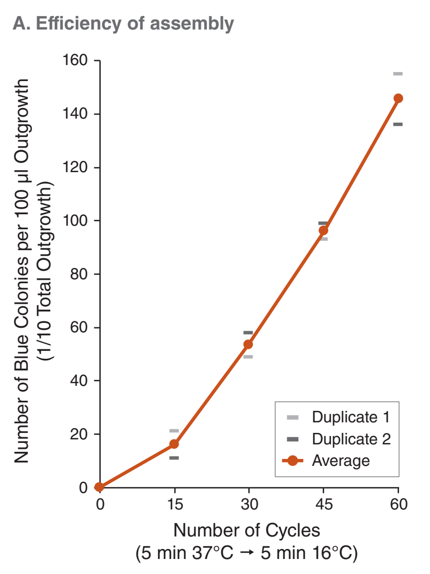
1. Potapov, V., et al. (2018) *Nucleic Acids Research*, gky303; doi: https://doi.org/10.1093/nar/gky303
2. Potapov, V., et al. (2018) *bioRxiv*, 322297; doi: https://doi.org/10.1101/322297

Visit **www.neb.com/GoldenGate** to access the protocol for 20+ fragment assembly using BsaI-HFv2 and T4 DNA Ligase

FIGURE 3:
Golden Gate Assembly of 24 fragments can be achieved with high efficiency and accuracy

Twenty-four fragment assemblies of the *lacI/lacZ* cassette were performed using the protocol available at www.neb.com/GoldenGate. While 30 cycles is sufficient to achieve 24 fragment assemblies, the stability of the BsaI-HFv2 and T4 DNA Ligase allows continued assembly through 45 and 60 cycles with a low background. (a) Efficiency of assembly and (b) accuracy of assembly versus cycle number. This continued functionality past the traditional 30 cycles of assembly indicates a high level of enzyme stability.



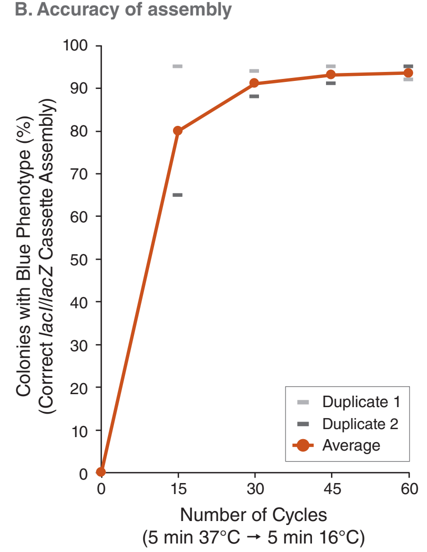


TABLE 2:
Yields and fidelities for Golden Gate Assemblies with BsaI-HFv2 and T4 DNA Ligase

Efficiency of assemblies per plate using outgrowth volumes described in Figure 3, with calculated yields from entire outgrowth built from 2 µl of the assembly reaction, and from the entire assembly reaction. All assembly protocols had a 5 min, 55°C terminal soak before transformation.

NUMBER OF FRAGMENTS ASSEMBLED	GOLDEN GATE ASSEMBLY PROTOCOL* (VOLUME OF 1 ml OUTGROWTH PLATED)	CORRECT ASSEMBLIES PER PLATE	FIDELITY OF ASSEMBLY (PERCENT CORRECT)	CALCULATED COLONY TOTALS	
				PER 2 µl ASSEMBLY REACTION	PER FULL ASSEMBLY REACTION**
1	5 min., 37°C (2.5 µl)	687	100%	274,200	2,742,000
1	60 min., 37°C (2.5 µl)	1,623	100%	649,200	6,492,000
12	(5 min., 37°C → 5 min. 16°C) x 30 (5 µl)	245	99.5%	48,900	489,000
24	(5 min., 37°C → 5 min. 16°C) x 30 (100 µl)	78	90.7%	783	9,792

* *All assembly reactions had a 5 minute terminal soak after either 37°C incubations or cycling.*
** *Assembly reaction volumes were 20 µl (1, 12 fragments) or 25 µl (24 fragments).*

FEATURED PRODUCTS

Push the limits –

20+ fragment assembly now achievable with high efficiency and accuracy!

With constant advances both in the development of new enzymes (e.g., BsaI-HFv2) and research on maximizing enzyme functionality (e.g., ligase fidelity), NEB has become the industry leader in pushing the limits of Golden Gate Assembly and related methods such as MoClo, GoldenBraid, Mobius Assembly and Loop Assembly. NEB has all the products and information you need to perform complex assemblies, as demonstrated with 20+ fragment assemblies exhibiting high efficiencies, high accuracy (>90%) and low backgrounds (see pages 2–4).

TYPE IIS RESTRICTION ENZYMES

NEB offers more Type IIS restriction enzymes (that recognize asymmetric DNA sequences and cleave outside of their recognition sequence) than any other supplier, many of which are used in Golden Gate Assembly. NEB is pleased to introduce two new restriction enzymes for use in Golden Gate: Esp3I, an isoschizomer of BsmBI that is recommended for use at 37°C, stored at -20°C, and is supplied with CutSmart® Buffer, and the improved BsaI-HFv2, which is optimized for Golden Gate Assembly.

NEB GOLDEN GATE ASSEMBLY MIX

The NEB Golden Gate Assembly Mix incorporates digestion with BsaI and ligation with T4 DNA Ligase into a single reaction, and it can be used to assemble up to 12 fragments in a single step.

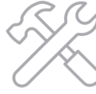
ORDERING INFORMATION

PRODUCT	NEB #	SIZE
NEB Golden Gate Assembly Mix	E1600S/L	15 rxns
BsaI	R0535S/L	1,000/5,000 units
BsaI-HFv2	R3733S/L	1,000/5,000 units
BbsI	R0539S/L	300/1,500 units
BbsI-HF	R3539S/L	300/1,500 units
BsmBI	R0580S/L	200/1,000 units
Esp31	R0734S/L	300/1,500 units
T4 DNA Ligase	M0202S/L/T/M	20,000/100,000 units
T7 DNA Ligase	M0318S/L	1,000/5,000 units
NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987H/I/P	20 x 0.05 ml/6 x 0.2 ml/ 1 x 96 well plate
NEB 10-beta Competent *E. coli* (High Efficiency)	C3019H/I	20 x 0.05 ml/6 x 0.2 ml

Advantages

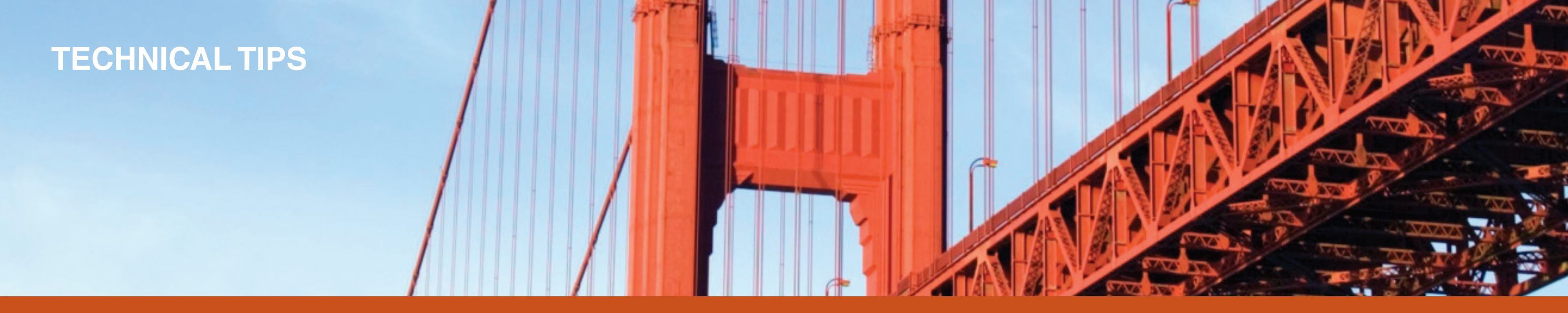
- Seamless cloning – no scar remains following assembly
- Fast (5 minute) protocols for routine, single-insert cloning
- High efficiencies for cloning during library creation
- Ordered assembly of multiple fragments (20+) in a single reaction
- Efficient with high GC regions and areas of repeats
- Compatible with a broad range of fragment sizes (<100 bp to >15 kb)

Learn more and request a sample of our new BsaI-HFv2 at **www.neb.com/GoldenGate**



Try the NEB Golden Gate Assembly Tool at **GoldenGate.neb.com** for help with designing your experiment

View our webinar: *Fidelity and bias in end-joining ligation: Enabling complex multi-fragment Golden Gate DNA Assembly* at **www.neb.com/NEBTVwebinars**



10 Tips for Optimizing Your Golden Gate Assembly:

Looking to assemble multiple DNA fragments in a single reaction?
Here are some tips to keep in mind when planning your Golden Gate Assembly experiment.

1 Check your sequences

Check for internal recognition sequences before choosing your Type IIS restriction endonuclease. Internal sites should be eliminated by site-directed mutagenesis. The Q5® Site-Directed Mutagenesis Kit (NEB #E0554S) and the online tool NEBaseChanger® (**NEBaseChanger.neb.com**) work well for this purpose.

2 Orient your primers

When you design your PCR primers to introduce the Type IIS restriction enzyme sites that will flank the sequences to be cloned, the recognition sites should be oriented in opposing directions. Consult the Golden Gate Assembly Mix (NEB #E1600) manual for further information regarding the placement and orientation of the sites.

3 Choose the right plasmid

The pGGA destination plasmid has the T7 and SP6 promoters flanking the assembly sites, and it is free of BsaI sites. It can also be transformed into any *E.coli* strain for assembly generation or propogation of the plasmid. pGGA is included with the Golden Gate Assembly Mix (NEB #E1600).

4 Choose the right buffer

T4 DNA Ligase Buffer is the buffer of choice for Golden Gate Assembly. However, NEBuffer 1.1, supplemented with 10 mM DTT and 1 mM ATP, also supports successful assembly for BsaI/BsaI-HFv2-based Golden Gate protocols.

5 Make sure your plasmid prep is RNA-free

Contaminating RNA can result in an overestimation of your plasmid concentration. Ensure that your plasmid prep is RNA-free with the use of RNase A-containing buffers used in spin column-based purification protocols.

6 Increase your complex assembly efficiency by increasing the Golden Gate cycling levels

While the standard 30 cycles of alternation between temperatures best for cutting and ligation of DNA results in high levels of accurate assemblies with low backgrounds, even for 24-fragment assemblies, the stability of BsaI-HFv2 and T4 DNA Ligase continue to function up to 45–60 cycles, if desired.

7 Decrease insert amount for complex assemblies

For complex assemblies involving >10 fragments, the amount of precloned insert can be decreased from 75-100 ng each to 50 ng each without significantly decreasing the efficiency of assembly.

8 Increase enzyme amount for complex assemblies

For complex assemblies involving >10 fragments, the amount of BsaI-HFv2 and T4 DNA Ligase can be increased 2-fold to enhance the efficiency of assembly.

9 Carefully design EVERY insert's overhang

Assembly efficiency is only as good as its “weakest link”. Ensure that every precloned insert/module or amplicon has well designed overhangs that are unique, nonpalindromic, and are known to lead to high fidelity ligation by T4 DNA Ligase (1).

10 Check for a sequence error if your assembly becomes non-functional

Sometimes, a precloned insert can become corrupted with a sequence error during propagation in *E.coli* for plasmid prep generation. This is usually a frameshift caused by *E.coli* DNA Polymerase slipping *in vivo,* in a run of single bases (e.g., AAAA). This should be suspected if a previously functional assembly suddenly becomes nonfunctional. Sequence your precloned insert stocks to indentify the mutated insert.

References:
1. Potapov, V., et al. (2018) bioRxiv, 32297; doi: https://doi.org/10.1101/322297

RESEARCH SPOTLIGHT

Lessons from the sea: Sea urchins as models for aging and cancer research

Andrea Bodnar, Ph.D., Science Director,
Gloucester Marine Genomics Institute

NEB has long recognized the potential of the marine environment as a source for new discovery and the need to protect and preserve the ocean's vast biodiversity for the benefit of humankind. The unique adaptations of marine organisms have made them valuable models for biomedical research, provided novel therapeutics for human disease and uncovered new tools to advance biotechnology. Located just 12 miles from NEB's headquarters in Ipswich MA, a new marine biotechnology institute, Gloucester Marine Genomics Institute (GMGI), is applying innovative genomic technologies to marine science for discoveries that impact human health, biotechnology and fisheries. In February, GMGI's Science Director, Dr. Andrea Bodnar, visited NEB to provide an overview of the research programs at GMGI and to present some of her work using sea urchins as models to unlock the secrets of living a long and healthy life.

Understanding Extreme Longevity

The oceans are home to many of the Earth's longest-lived animals with several non-colonial marine invertebrates and vertebrates documented to live for more than 100 years (Table 1) (1–8). Many of these animals grow and reproduce throughout their lifespans with no apparent functional decline, no increased incidence of disease or increase in mortality rate with age. A better understanding of the mechanisms by which these animals achieve their extraordinary life histories may reveal exceptionally effective defenses against the destructive process of aging and suggest novel avenues to prevent or treat human age-related degenerative diseases.

The red sea urchin is among the Earth's longest-lived animals, estimated to live for more than 200 years without evidence of age-related decline and no reported cases of cancer (6,7,9). Sea urchins have served as model organisms for scientific research for more than a century and provide a unique opportunity to investigate the mechanisms underlying extreme longevity and negligible aging. Sea urchins have contributed to our understanding of important biological processes including fertilization, the role of chromosomes in inheritance and the gene regulatory networks that guide embryonic development. The fact that cell division is synchronized in early sea urchin development facilitated Tim Hunt's Nobel prize winning discovery of cyclins, proteins that play a key role in controlling cell cycle. Part of their value as a model organism is their close genetic relationship with humans; as non-chordate deuterostomes, sea urchins are one of our closest invertebrate relatives. This makes them ideal models to investigate the cellular pathways contributing to longevity and disease resistance with direct relevance to human health.

In addition, sea urchins are commercially fished and therefore considerable data are available regarding their growth, survival, longevity, susceptibility to disease and reproductive patterns as this information is essential for effective fisheries management (10). From these data it has been noted that different species of sea urchins exhibit very different natural lifespans in the wild. While the red sea urchin (*Mesocentrotus franciscanus*) is reported to be very long-lived, the purple sea urchin (*Strongylocentrotus purpuratus*) has an estimated maximum life expectancy of more than 50 years and the variegated sea urchin (*Lytechinus variegatus*) has an estimated life expectancy of about 4 years (6,7,11-13). Comparisons between long-, intermediate- and short-lived species provide an excellent model to understand mechanisms of lifespan determination and can provide insight into how these animals avoid the process of aging.

Aging is a complex and multifactorial process and there have been many theories proposed to explain this phenomenon at the molecular, cellular, systemic and evolutionary levels (14). Human aging is accompanied by the shortening of telomeres (caps that protect the ends of chromosomes), accumulation of cellular oxidative damage, and reduced ability to repair and replenish damaged tissues. In contrast, sea urchins maintain their telomeres(15,16), have little accumulation of oxidative damage (17) and maintain the ability to continually regenerate lost or damaged appendages throughout their lives (18). In most animals, there is a delicate balance between promoting cell renewal and regeneration for maintaining healthy tissues, and the danger of unchecked, abnormal cell growth that defines cancer. Notably, there are no documented cases of cancer in sea urchins (19,20). The ability of sea urchins to continually grow and regenerate while apparently resisting cancer holds great promise for discovering naturally occurring cancer prevention mechanisms.

Initial studies with sea urchins have demonstrated that the circulating cells (coelomocytes) are highly resistance to DNA damaging agents, invoke a robust DNA repair response and can effectively repair DNA damage (21,22). However, more study is required to determine if the high resistance to DNA damage in sea urchin cells contributes to the low incidence of cancer and to understand the cellular mechanisms protecting DNA.

At GMGI, our goal is to use sea urchins and other long-lived marine animals as models to identify the key genes and cellular pathways involved in long-term maintenance of tissues and resistance to cancer. Insight gained from studying exceptional longevity in sea urchins may reveal novel strategies to slow the destructive process of aging and identify new avenues for prevention or treatment of age-related diseases such as cancer.

Dr. Bodnar would like to thank Dr. Barton Slatko for the invitation to participate in NEB's seminar series.

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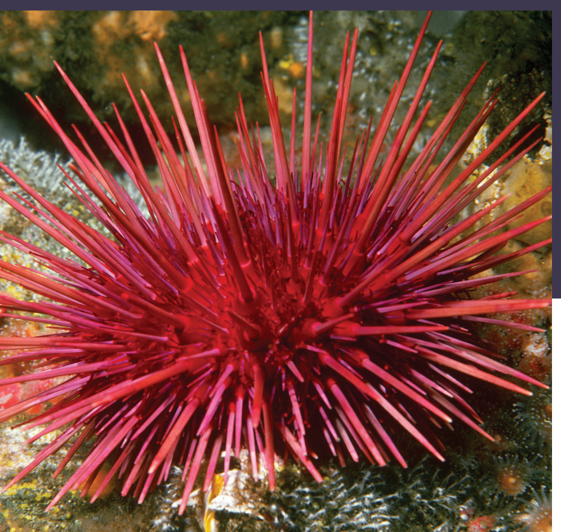


< The red sea urchin (*Mesocentrotus franciscanus*) is one of the earth's longest-lived animals, potentially living for more than 200 years with no signs of aging and no reported cases of cancer.

LONGEVITY IN NON-COLONIAL MARINE ANIMALS

Common Name	Species	Oldest Recorded Lifespan (Years)
Ocean quahog clam	*Arctica islandica*	507[1]
Greenland shark	*Somniosus microcephalus*	392[2]
Marine tubeworm	*Lamellibrachia sp.*	250[3]
Bowhead whale	*Balaena mysticetus*	211[4]
Rougheye rockfish	*Sebastes aleutianus*	205[5]
Red sea urchin	*Mesocentrotus franciscanus*	200[6,7]
Geoduck clam	*Panopea abrupta*	168[8]

Founded in 2013, Gloucester Marine Genomics Institute is a 501(c)3 with a mission to conduct world class marine biotechnology research which expands the regional economy.

NEW PRODUCTS

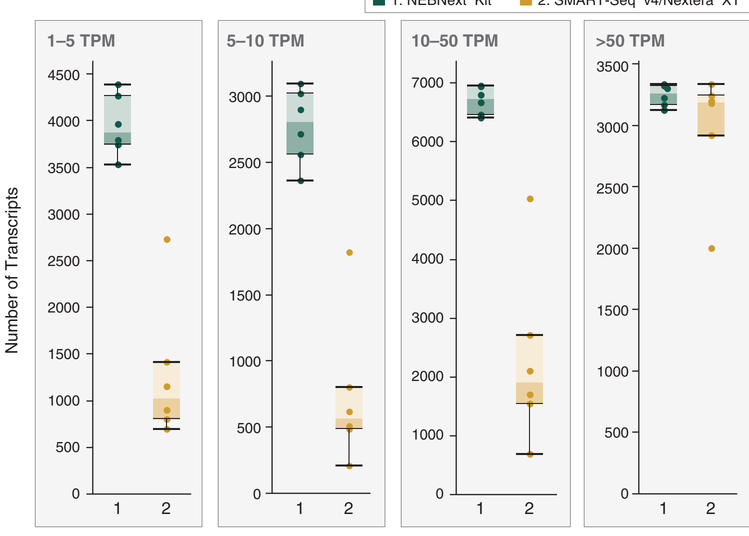
NEBNext® Single Cell/ Low Input RNA Library Prep Kit for Illumina®

This unique workflow meets the demand for a highly sensitive, yet robust method that consistently generates high-quality, full-length transcript sequencing libraries from single cells or ultra-low input RNA.

Optimized cDNA synthesis and amplification steps incorporate template switching, with a unique suite of reagents. The resulting sensitivity means that even low-abundance transcripts are represented in the high yields of cDNA obtained. Subsequent library construction incorporates the NEBNext Ultra™ II FS enzymatic DNA fragmentation/end repair/dA-tailing mix in a simple and efficient workflow.

Increased transcript detection with the NEBNext Single Cell/Low Input RNA Library Prep Kit

Sequencing libraries were generated from Jurkat single cells (6 replicates) using the NEBNext Single Cell/Low Input RNA Library Prep Kit, or the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech # 634891) plus the Nextera XT DNA Library Prep Kit (Illumina #FC-131-1096). Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). TPM = Transcripts per Kilobase Million. Each dot represents the number of transcripts identified at the given TPM range, and each box represents the median, first and third quartiles per replicate and method. Salmon 0.6 was used for read mapping and quantification of all GENCODE v25 transcripts. Panels show the number of transcripts detected within the following TPM ranges: 1-5, 5-10, 10-50 and >50 TPM. Increased identification of low abundance transcripts is observed with the NEBNext libraries.



Advantages

- Generate the **highest yields** of **high-quality, full-length transcript sequencing libraries** from single cells, or 2 pg – 200 ng total RNA
- Experience **unmatched detection of low abundance transcripts**
- Rely on **consistent transcript detection** for a wide range of input amounts and sample types
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Visit **NEBNext.com** for more information on our NEBNext portfolio of products for sample prep

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PRODUCT	NEB #	SIZE
NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina	E6420S/L	24/96 rxns
NEBNext Single Cell/ Low Input cDNA Synthesis & Amplification Module	E6421S/L	24/96 rxns
NEBNext Multiplex Oligos for Illumina (Index Primers Set 1, 2, 3, 4)	E7335, E7500, E7710, E7730S/L	24/96 rxns
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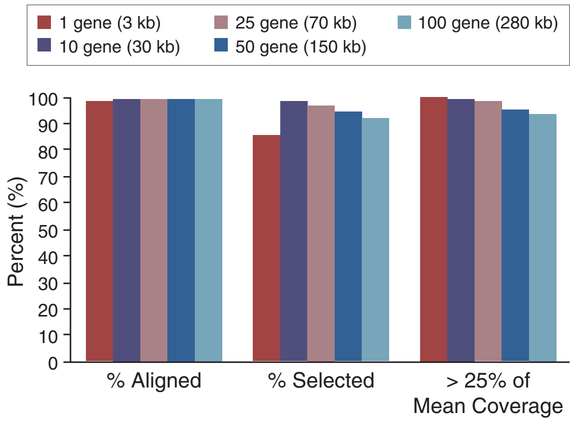
NEBNext Direct Custom Ready Panels demonstrate optimum performance across a wide range of panel sizes

Key target enrichment metrics demonstrate consistent performance across a range of panel sizes. 100 ng of DNA was tested against panels of 1, 10, 25, 50 and 100 genes, and sequenced using Illumina paired-end 150 bp sequencing. Larger panels included all genes present in smaller panels.



Learn more about NEBNext Direct in our white paper: *Overcoming the challenges of applying target enrichment for translational research* at **NEBNextDirect.com**

View our webinar: *High-quality accessible pipelines for germline and somatic variant discovery using NEBNext Direct target enrichment* at **www.neb.com/NEBTVwebinars**

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PRODUCT	NEB #	SIZE
NEBNext Direct Custom Ready Panels	E6631S/L/X	8/24/96 rxns

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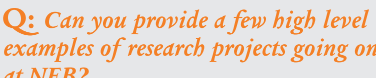
The Importance of Basic Research at NEB

In our latest podcast episode, host Lydia Morrison sits down with Tom Evans, Ph.D., the Executive Director of Research at NEB, to discuss the goals of NEB's Research Department.



Q: Can you provide a few high level examples of research projects going on at NEB?

A: The difficult part of that question is choosing only a couple of projects. About a fourth of the company is involved in research and that translates into scores of projects. I can't talk about all of these so I'll choose three.

The first is an on-going project to understand how T4 DNA Ligase joins DNA *in vitro*. A great deal is known about ligase mechanisms, but even so there was more to be learned; especially for T4 DNA Ligase. We started by asking foundational questions such as how fast does it react with ATP? What is the rate limiting step? Does it prefer to bind DNA ends? We published what we learned. At some point we asked a simple question, how well does T4 DNA Ligase tolerate mismatches in a 4-base pair overhang ligation. Simple question, difficult to answer. By applying the unique properties of a PacBio sequencing instrument and performing a ligation reaction containing DNA oligos with all 256 possible 4-base overhangs we were able to determine the preferences and mistake profile of T4 DNA Ligase. Basic enzyme information, but information that has significant impact on DNA assembly reactions and by extrapolation synthetic biology and pathway engineering.

The second project involves identifying unknown open reading frames. We have all heard that around half of all the ORFs in *E. coli* don't have a known function or activity, even less for less well studied organisms. A short time ago we constructed a metagenomics library and it was one of these instances where everything fell into place and the diversity ended up being excellent. To give the library a test drive we screened for two activities: exosialidase and AP-endonuclease. Two very different activities, but we ended up finding enzymes that belong to previously unknown protein families. Hopefully, this is only the beginning.

The final project feels retro, but current. Retro because NEB has been selling restriction enzymes, proteins that prevent phage infection in bacteria, for decades. The project looks not at the restriction enzymes in bacteria, but instead the phage responses to them; specifically modifications in the phage DNA that allow them to become immune to the immunity system. Phages are amazingly diverse and when we looked, we found two new base modifications in phages that infect *Pseudomonas* or *Salmonella*. What made this particularly exciting was that the researchers involved reconstituted the pathway that generates one of the modifications, thus identifying more proteins that were previously unknown open reading frames. One of these proteins, a kinase, is being sold as an Enzyme for Innovation. (You can learn more about Enzymes for Innovation at **www.neb.com/EnzymesforInnovation**.)

Q: Do NEB researchers actively publish their work?

A: In fact, we have published over 1,100 peer-reviewed articles since our inception. We also have Master's students, Ph.D. students and post-doctoral associates. These scientists are expected to perform research that will lead to publications.

Q: What does having a successful research program offer a biotech company?

A: Well, a successful research program, especially one that is not simply laser focused on the current market leading product, offers a number of advantages; some obvious and some not so obvious. Some of the obvious advantages are a direct product improvement pipeline, staying current and creating intellectual property. Product improvements are critical in a competitive marketplace, and understanding enzymes and protein engineering methodologies allows us to improve upon our already excellent product offerings.

Q: Can you share a couple of examples of how enzyme-based research led to product improvement?

A: Two examples that come to mind are our line of high fidelity (HF®) restriction enzymes and Luna® Reverse Transcriptase. Another aspect of product development is the insight that we gain as not only producers of reagents, but also as consumers of those reagents for use in our own research programs. Firsthand experience with our reagents gives us a better perspective on how to improve them, or what new enzymes or workflows would make our research more effective. If it makes our research more effective, it will probably make other research programs more effective too.

Q: What are the less obvious benefits of a research program?

A: That's a good question. I think some of the less obvious benefits of a diverse and publication-oriented research program is that it allows us to develop something entirely new and without a specific commercial directive. Such discoveries or technology advances allow a strong IP position and allow NEB to be first to market. Furthermore, basic research has a much higher potential to create whole new areas of research and therefore new markets.

Q: Beyond the product portfolio benefits, are there any other benefits to the company or the community?

A: Well, outside of the standard commercial implication there is an even more valuable benefit - it results in an innovative and exciting place to work. For example, basic research has a significant and positive impact on the company culture. Thinking broadly and creatively, as is needed to do basic research, generates a culture of innovation and of asking the big questions. This results in a company that is adaptable, not only being able to change with changing technology, but thriving on change. There is also the satisfaction of contributing to the world.

Q: *What is the mission of the NEB research program?*

A: That is a great question. I suppose in a very broad sense I feel the mission of research at NEB is to generate knowledge. Knowledge about the amazing world around us. However, that is only one part of our goal, simply knowing something is not enough, that information needs to be disseminated.

Q: *And how do NEB scientists go about sharing that information?*

A: Well, we share our information in a number of ways, we publish in peer reviewed journals, that's probably our biggest and most important route, but we also present posters at scientific conferences, and when appropriate we turn the information into cutting edge or superior products. I suppose the short answer is that we do research to learn and then pass along that information to the general community, which, of course, is the goal of many research programs.

Actually, perhaps the most interesting aspect of that question is NOT what the mission is. Our mission is not confined to short-term R&D to create products. In fact, it is not even confined to long-term R&D to create products. Of course, product-related research is pursued and is vital to being competitive in the market landscape of biotechnology, but we also do research from which we do not anticipate immediate commercial upsides.

Q: *Can you think of an example, where research has not led to a product for the company?*

A: In the 1980's, when NEB had been profitable for a number of years, our founder Don Comb felt very strongly that he wanted to give back to the general community. He chose to do this not only by donating to charitable organizations, which NEB did and still does, but also by generating knowledge that would magnify the effect. Rich Roberts, currently our Chief Scientific Officer, helped organize discussions on areas that NEB could do research that would have the greatest impact. They chose to do research in the area of filarial nematodes. These worms cause terrible diseases such as river blindness and elephantiasis in areas of the world that can least afford either the research investment or the impact of these diseases. Our hope is that someday these parasites will be brought under control, but the goal was not just to generate a product.

Q: *As the director of NEB's research program, where would you like to see the program in the next five years?*

A: NEB's core strengths have been enzymes and enzyme-based technologies. We need to find new enzyme activities, characterize our enzymes more fully, and engineer enzymes when needed to fit the ever-changing requirements of life science research. We spoke about some of our initiatives to find new enzymes, but we are also investigating how machine learning and deep learning can accelerate enzyme discovery.

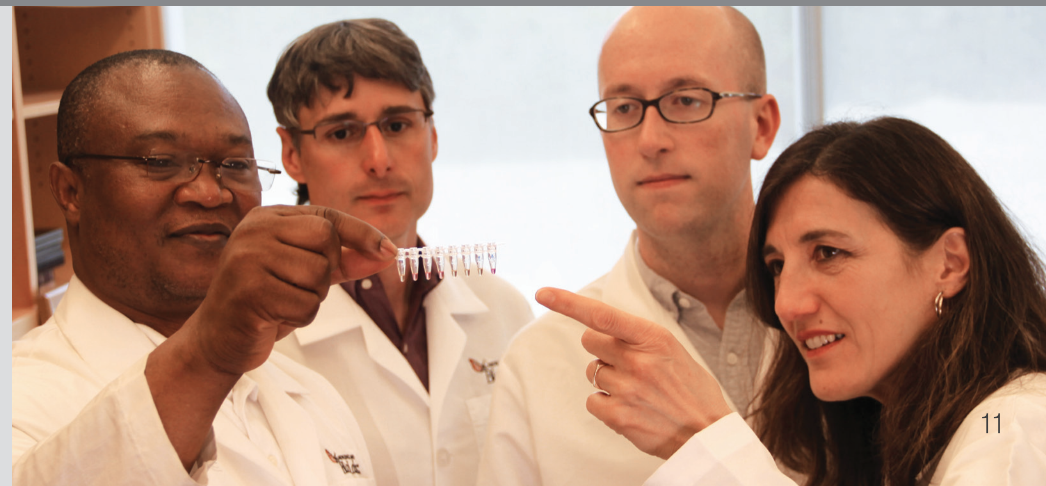
Q: *How does machine learning or artificial intelligence apply to biological discovery?*

A: It will lead to better and importantly, higher throughput analysis methods and will allow us to extract more information from the volumes of data currently being generated. The T4 DNA Ligase project we talked about is just an example of how improved understanding of even a core enzyme can have dramatic effects. There is no perfect enzyme, but knowing the capabilities of an enzyme will permit far more effective workflows to be developed. Finally, what we learn can be harnessed to engineer ever faster, more stable, and reliable enzymes. As we learn, we will publish our findings and also use this knowledge to expand the repertoire of enzymes available to the scientific community.

Listen to the full podcast and find other interesting stories at **www.neb.com/nebpodcast** Lessons from Lab & Life is also available at iTunes® and Stitcher®.

Interested in learning more?

To learn more about NEB's research program, including our Parasitology Program and Enzymes for Innovation initiatives mentioned above, visit **www.neb.com/research**



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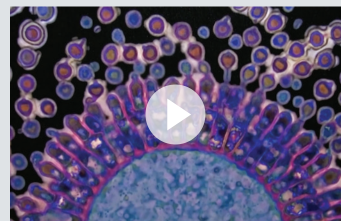

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A Closer Look at Microbial Life

Episode 20:
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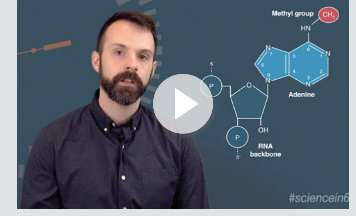

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RNA Modifications

Episode 22:
Technical Support at NEB