NEB EXPRESSIONS

A scientific update from New England Biolabs

35th Anniversary Issue

CELEBRATING 35 YEARS

NEW ENGLAND BioLabs® Inc.
enabling technologies in the life sciences

Table of Contents

Upcoming Tradeshows

Visit the NEB booth at the following meetings:

- CHI – Next Generation Sequencing – Providence, RI 9/21/2009–9/23/2009
- Society of Neuroscience Annual Meeting – Chicago, IL 10/17/2009–10/22/2009

Follow NEB on Twitter

A Letter from the CEO

Dear Researcher,

New England Biolabs is pleased to be celebrating 35 years in the Life Sciences. In this anniversary edition of NEB Expressions, we share some of the history and corporate philosophy that makes us a unique company—from our founding principles, placing the advancement of science and stewardship of the environment as our highest priorities, to our current research initiatives and sustainability efforts.

It is also an exciting time for product development at New England Biolabs. At NEB, basic research goes hand in hand with a commitment to address the needs of customers. Recently, this has led to the development of engineered restriction enzymes that reduce unwanted star activity, as well as a new line of products that facilitates DNA sample preparation for next generation sequencing technologies.

Above all, we recognize that scientists depend on us to provide exceptional quality products and technical support. We are proud and humbled that our reagents and basic research advance science, help cure disease and bring hope and opportunity to the developing world.

Thank you for your support over the last four decades. We wish you continued success in your research.

Jim Ellard, CEO

New Student Starter Pack

Start the academic year off on the right path with the New Student Starter Pack from NEB! The Starter Pack contains a selection of technical literature, laboratory aids and valuable product samples that aid in your research. Starter packs are available through December 31, 2009 to all new research students.

Visit www.neb.com/starterpack to request your Starter Pack today.

FREE

Starter Pack Includes*:

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- Restriction Enzyme Activity Chart Poster
- Restriction Enzyme Technical Guide
- Ladder/Marker Reference Cards (DNA/RNA/Protein)
- Tips for Using Phusion™ Quick Card
- Double Digest Card
- Floatie
- NEB Sharpie Pen
- D-Cap It Tube Opener

*Contents subject to change. Offer valid while supplies last.

New England Biolabs – Celebrating 35 years in the life sciences

Established in 1974, New England Biolabs is proud to be recognized as a world leader in the discovery, production and supply of reagents for the life science industry. For 35 years, we have been committed to meeting the needs of the scientific community. While our product portfolio and distribution network have expanded, our commitment to our customers remain the same:

- **Set the highest standards for quality and value**
- **Provide our customers with the highest level of support**
- **Maintain a strong basic research program that contributes to the advancement of science**
- **Promote and practice sound ecological practices and environmental sustainability**

Quality Quality has always been the hallmark of NEB. Our state-of-the-art headquarters, which is ISO 9001/14001 and LEED certified, includes a modern fermentation center and laboratories for production, quality control, product development and basic research. With a reliance on recombinant technology and expertise in expression and purification techniques, NEB is able to produce the highest quality products with proven lot-to-lot consistency.

Support Our experienced team of over 140 research and production scientists is available for technical support. The same scientists who purify and quality control our products answer technical questions concerning their products. In addition, our administrative staff in order entry, accounting and marketing are always available to meet our customers needs. This method of technical support was established in 1975 and remains unique to the industry.

Basic Science In addition to applied research, NEB scientists also engage in long-term basic research projects. Over 30 laboratories investigate areas that include structure/function relationships of restriction enzymes and polymerases, parasitology, protein splicing, epigenetics and glycobiology. As a result, NEB scientists have published over 630 articles to date, many of which are in peer-reviewed publications. NEB scientists also supervise post-doctoral associates, student interns and Ph.D. students, whose primary task is research that leads to publication. Members of our research staff are regularly invited to lecture at premiere academic institutions and conferences around the world, and even present at local high schools. NEB hosts a weekly seminar series that brings leading researchers to our facility to share ideas. More information about the basic research program can be found on page 5–6.

Environmental Sustainability One of the founding principles of NEB is a commitment to promoting sound ecological practices and environmental sustainability. For 35 years, we have been looking for new ways to improve our business processes in order to minimize the impact that we have on our environment. We also strive to raise awareness of the need to care for our planet. Additionally, NEB has received ISO 14001 certification, a standard for environmental management systems.

NEB's modern 140,000 square foot research and production facility is LEED certified (Leadership in Energy and Environmental Design). This distinction is given based on a suite of environmentally focused standards that include site sustainability, water efficiency, energy conservation and atmospheric protection, choice of building materials and resources, indoor environmental quality, innovation and building design.

NEB's efforts to reduce waste include a shipping box recycling program, an extensive in-house recycling program and composting of cafeteria waste. In addition, NEB encourages employees to search for alternative ways to commute to work, and provides a van pool for employees commuting from the Boston/Cambridge area.

Most prominent in our conservation efforts is our state-of-the-art Solar Aquatics System®. Housed in a beautiful greenhouse, this system utilizes and accelerates the process found in streams and wetlands to treat the campus' wastewater, making it clean enough for reuse or for ground water recharge. This is described in further detail on page 4.

We are always looking for ways to improve our business practices and welcome any suggestions you may have. Please use our online suggestion form (www.neb.com/nebecomm/suggestion.asp) to share your thoughts or comments.

35th Anniversary Offers

NEB would like to thank its customers for 35 years of support. Join us in celebrating our 35th anniversary by visiting www.neb.com to find 12 months of exciting offers, including significant product discounts and giveaways.

Look for the 35th Anniversary Offers icon on our website to learn about our monthly special offer.

www.neb.com

A History of Sustainability Practices at NEB 1975~2009

As part of our commitment to promoting sound ecological practices, NEB has always been looking for ways to reduce its impact on the environment. For a complete timeline of sustainability practices at NEB, visit www.neb.com/sustainability

1975

The first NEB catalog is released, using 100% recycled paper

1976

NEB initiates a shipping box recycling program

1982

The New England Biolabs Foundation is established to support environmental awareness, education, health and the arts

1987

The first NEB freezer program is established, consolidating product shipments

1992

A comprehensive recycling program is initiated, including paper, plastics, expanded polystyrene, glass, aluminum and batteries

1996

In conjunction with our catalog, NEB begins to support organizations that promote environmental sustainability

2004

Waste water treatment facility is established, treating all of the waste water generated by the NEB campus

2007

NEB Green Committee is established to support a wide array of sustainability projects at NEB

2007

The laboratory facility in Ipswich achieves LEED® Green Building Certification

2008

Ocean Genome Legacy (OGL) is established to explore and preserve the biological diversity of the sea

2008

Composting in the NEB cafe begins; as of 8/09, 56,000 pounds is diverted from landfills

2009

NEB receives ISO 14001 certification

An Unconventional Approach to Wastewater Treatment

Andrew Posner, JTED, Inc.

When NEB headquarters in Ipswich, MA was being designed, the goal was to build a more environmentally sound facility. As such, NEB chose an innovative method to treat wastewater, using a Solar Aquatics® Wastewater Treatment System that is capable of treating up to 27,500 gallons per day. Housed in a beautiful greenhouse abound with tropical plants, the system utilizes and accelerates the processes found in streams and wetlands to purify the water to tertiary standards.

This water treatment process combines proven wastewater treatment practices with the biological components of ecologies found in nature. Water first enters an in-ground blending tank, where it is aerated and bioaugmented with microorganisms that have been recycled from the clarification process described below. The aerobic biological breakdown of organic matter begins here; compounds such as fats, starches and proteins are converted to carbon dioxide and simpler matter that can be utilized by organisms downstream.

The water continues into the greenhouse where it enters several six foot tall translucent tanks. These tanks host a variety of vegetation which provide a habitat for bacteria, zooplankton, snails, and other organisms, while algae grow on the tank walls. This ecosystem works in unison to metabolize nutrients and further break down and remove wastes. Autotrophic bacteria nitrify ammonia by oxidizing it into nitrate while algae and plants directly metabolize nitrate, ammonia and soluble orthophosphates. The bacteria are consumed by rotifers while snails, zooplankton, and worms begin the process of sludge digestion.

Next is the clarification process, where the suspended biological solids are allowed to settle. The biologically rich sediment that forms in the clarifier is recycled back to bioaugment the incoming wastewater stream in the blending tank. Water leaving the clarifier is clear in appearance but still contains microbes and certain nutrients (including nitrates). After clarification, any remaining solids are removed by a sand filter.

Following filtration, water enters the self-contained subsurface flow wetlands, where denitrification is achieved in anoxic conditions. Nitrate is reduced by facultative heterotrophic bacteria to nitrogen gas, hydroxide, and water

Tropical plants growing inside the greenhouse of the Solar Aquatics Wastewater Treatment have a key role in the water treatment process.

in the presence of an electron donor. Certain pathogenic bacteria are destroyed by the action of wetland plants and further nutrient removal is achieved. To keep the wetland biology flourishing, approximately 50 percent of the water that flows to the end is returned back to the beginning of the wetland for bioaugmentation.

The final treatment step is disinfection by ultraviolet light. The tertiary quality water meets stringent requirements, making it clean enough for NEB to discharge for the purpose of groundwater recharge.

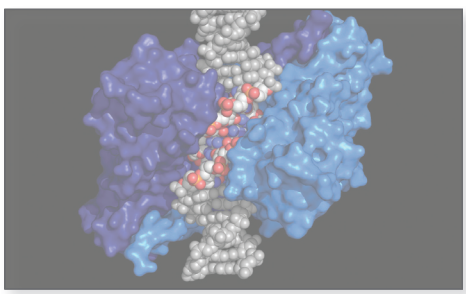
A Passion for Science — Basic Research at NEB

"Established in the mid-1970's as a private cooperative of experienced scientists, New England Biolabs is a world leader in the production of enzymes for molecular biology applications. Through commitment to basic research and ongoing efforts to advance production efficiency, we will continue to provide our customers with the quality, dependability and value they have come to expect."

William E. Jack, Ph.D., Director of Research,
New England Biolabs

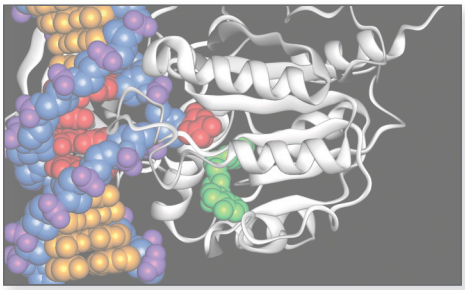
The above statement from the 2009-10 NEB catalog reflects the vision of Don Comb, the founder of New England Biolabs. A number of years ago, Don was quoted in a local newspaper interview saying, "…scientists at New England Biolabs can't wait to get to work each morning to see how their experiments turned out." Although he has retired from active business management at NEB, Don continues to work in the lab exploring his own research interests and encouraging an expansive scientific vision. The fruits of his leadership are evident in the greater than 600 peer-reviewed publications from NEB. Much of this work has been accomplished in collaboration with outside laboratories, furthering a tradition of open collaboration and sharing of materials for basic research purposes. Research at NEB is broad, including not only the characterization of nucleic acid metabolic enzymes, but also exploration of pancreatic beta cell development, small RNA regulatory mechanisms, novel implementations of phage display for epitope generation and microbial fuel cells.

Restriction enzymes The core of NEB's prominence is within the restriction endonuclease (RE) field. NEB has been at the forefront of efforts to discover, clone, and characterize REs from a wide range of environments. Traditional RE discovery involves screening microbial cell lysates for specific DNA cleavage activity. While this method has identified REs in a vast array of microorganisms, the number of new specificities discovered this way has steadily declined. More recent RE discovery has exploited the burgeoning array of genome sequences. Sequence data can be mined to identify and classify potential RE systems.



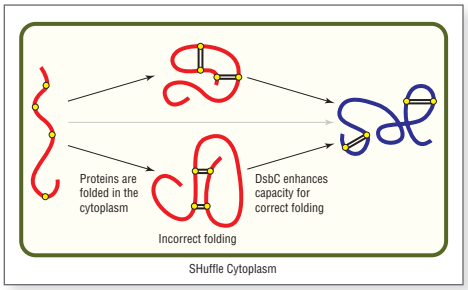
Structural analysis of restriction enzymes has helped to create enzymes with new characteristics (NotI Structure) (20).

At first glance, the REs appear to be a unified group. However, a more careful analysis has resulted in the characterization of alternate subunit configurations, cofactor requirements, juxtaposition of cleavage and recognition domains, dependence on additional recognition sites in cis or trans, and provision for the accompanying methyl transferase. This information has allowed NEB scientists to create enzymes with new characteristics. Most recently, we have engineered enzymes with increased, decreased or altered site-specific recognition. This research has produced site-specific nicking endonucleases (1) and allowed the introduction of High-Fidelity (HF™) restriction enzymes, with dramatically reduced star cleavage (2) (See page 7 for more details). Additionally, key recent research has identified specific amino acid determinants responsible for specific base recognition, and has for the first time allowed specific engineering to create a specificity not previously observed (3).



Methylases bind to DNA, preventing cleavage by the associated restriction enzyme (HhaI Methylase) (21).

***E. coli* expression & methylases** Cloning and overexpression of restriction endonucleases has resulted in the creation of *E. coli* lines that support expression of the potentially toxic endonuclease gene, and also of the accompanying protective methyltransferase (4,5). NEB was at the forefront of discovery and characterization of methyl restriction systems in *E. coli*, the *mcrBC* and *mrr* loci, and engineering of strains expressing methylases. Additional work to create more favorable *E. coli* expression environments is being done in collaboration with Jonathan Beckwith's lab at Harvard. The cytoplasmic environment has been modified to allow disulfide bond formation, and subsequent reshuffling of intramolecular disulfide bridges to the native conformation (6). Remarkable jumps in specific activity have been observed with proteins containing multiple disulfide bonds, a common feature of proteins derived from eukaryotes.

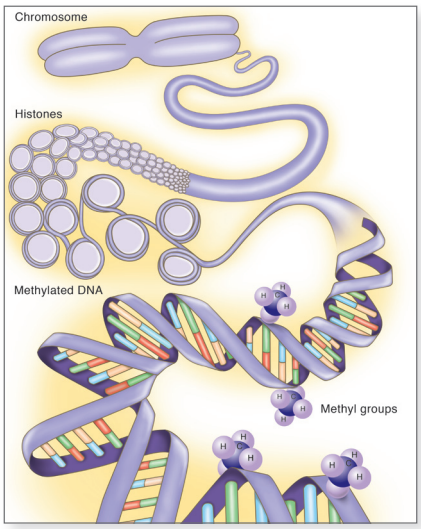


Focus on creating a more favorable cytoplasmic environment for expression led to the release of SHuffle™ Competent E. coli.

NEB has had a natural affinity for methylases as they almost invariably accompany restriction endonucleases. That familiarity made a move into studying methylation in vertebrates a natural succession. While the methylases involved in this modification are homologous to those found in bacterial enzymes, the methylation of cytosine in CpG dinucleotides is involved in gene regulation rather than host chromosome protection. A significant research effort at NEB has mapped the interacting partners for the human CpG methyltransferases and analyzed tissue-specific expression along with coordination of methylation with cell cycle with developmental stages (7,8).

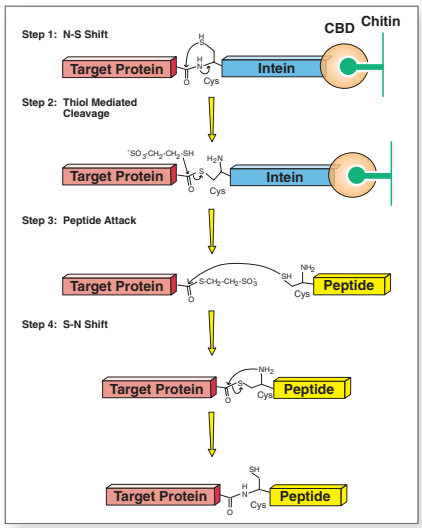
Epigenetics Modification DNA methyltransferases almost invariably accompany restriction endonucleases, acting to protect the host chromosome from the endonuclease digestion. DNA methylation plays a different role in many eukaryotes where DNA hypermethylation is associated with gene silencing and a variety of epigenetic gene regulatory processes. Similarly, histone modifications (methylation, acetylation, phosphorylation, ubiquitination and ADP ribosylation) have been found to modulate the interaction of regulatory and structural proteins, ultimately impacting chromatin structure. Core studies at NEB of human DNA methyl transfer-

ases have provided a foundation for examining the establishment and maintenance of CpG methylation. Continuing studies have examined the further involvement of protein methylation, including histone modification, as a means of signal transduction. A central goal of these ongoing investigations is to understand the influence of cross-talk between proteins, modulated by protein modifications and trans-acting factors, during cellular growth, development, and apoptosis.



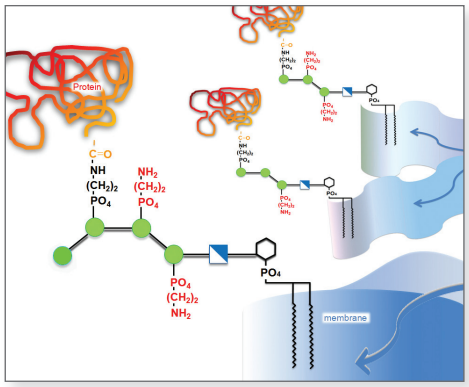
The Epigenetic Code.

Polymerases & inteins NEB pioneered the isolation, characterization and cloning of hyperthermophilic DNA polymerases, enzymes widely used due to their extreme thermostability and high fidelity. Selective mutagenesis, combined with modified nucleotides, has been used to explore substrate discrimination by this family of polymerases (9). In the process of cloning the first of these polymerases, Vent$_R$® DNA Polymerase, it was found that the 180 kDa protein precursor produced in the host archaea underwent two processing events to remove two interior segments, resulting in a mature 90 kDa protein (10). NEB scientists were at the forefront of establishing the reaction mechanism for these protein splicing events (11), and continue to unravel the myriad of both canonical and non-canonical protein splicing events that characterize potential and proven protein splicing events. The phenomenon of protein splicing has been exploited for protein purification and for *in vitro* ligation of protein/peptide segments (12,13).



Intein-mediated Protein Ligation (IPL) uses protein splicing technology to ligate proteins and peptides.

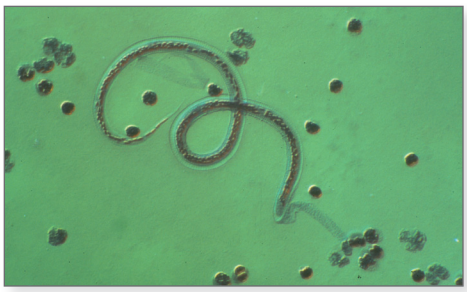
Glycobiology Glycobiology has yearned for the day when its own set of "restriction endoglycosidases" could be used to unravel and modify carbohydrate structure. This has been a particularly daunting task due to the complexity of carbohydrate structure and the heterogeneity of the carbohydrates, even those attached to a protein isolated from a single source. A second challenge has been the lack of rapid, definitive methods to analyze the carbohydrate chains and breakdown products. Current advances in detection, including mass spectrometry and high pressure liquid chromatography instrumentation, have overcome many of the earlier limitations. NEB has responded by identifying and expressing a greater range of novel endo- and exoglycosidases. A significant effort is also being made to characterize the specificity of individual enzymes with a broad goal of exploring the structural motifs responsible for substrate specificity (14).



Etanolamine (red) and mannose (green) extensions affect trafficking of GPI-anchored proteins.

Related glycobiology research is facilitating the elucidation of the pathways for GPI anchoring of proteins, an important type of eukaryotic secretory protein modification (15,16). Currently studies in both yeast and higher eukaryotes are exploring the role specific modifications of the GPI glycan may play in the trafficking of GPI anchored proteins (15,16).

Parasitology After observing the devastating effects of parasitic diseases disproportionately affecting third world countries who could least afford prevention, scientists at NEB established a parasitology research group that continues work on filarial diseases. The current focus of the work is on *Brugia malayi*, the causative agent in lymphatic filariasis, also known as elephantiasis. The discovery and sequencing of an obligate endosymbiont, *Wolbachia*, within *Brugia* has seeded an effort to identify drug targets within *Wolbachia* that may complement missing functions in *Brugia*, and yet are distinct from human counterparts and thus can be specifically targeted without affecting the human host (17,18,19).



Rarasitic nematode worm of the Superfamily filarioidea. Species responsible for lymphatic filariasis include Wuchereria bancrofti, Brugia malayi and Brugia timori. (Provided by HWO/TDR/Stammus)

Basic and applied research continues to play a central and guiding role at NEB. This work not only helps guide product development and testing programs, but also keeps NEB scientists in touch with current trends, techniques, thought, and protocols. NEB scientists, including Don Comb, still can't wait to come into the lab each morning to see how their experiments have worked out!

For more information about ongoing research at NEB, visit www.neb.com/research. For a complete list of publications by NEB scientists, visit www.neb.com/publications.

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High Fidelity Restriction Enzymes

With 35 years of experience in enzyme technology, New England Biolabs leads the industry in the study and improvement of restriction enzymes. NEB is pleased to introduce a line of High Fidelity (HF™) enzymes that have been engineered for reduced star activity. HF enzymes bring a new level of flexibility when choosing reaction conditions, including volume, incubation time and buffer compatibility. Make NEB your first choice and experience the exceptional performance of this next generation in restriction enzyme technology.

- **Selection** – More specificities than any other supplier
- **Convenience** – Optimal activity for over 160 enzymes in a single buffer NEB4
- **Quality** – State-of-the-art production and stringent QCs
- **Innovation** – HF enzymes engineered for reduced star activity
- **Performance** – Guaranteed
- **Value** – Available at the same price as the wild type*

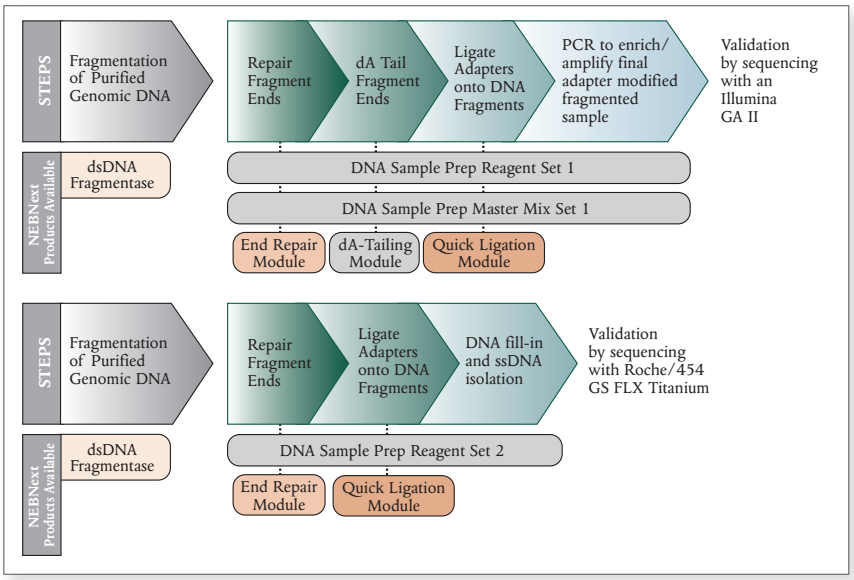
**BamHI-HF is offered at a different $/unit, with a lower cost/vial.*

Visit www.neb.com/HFenzymes for a list of the latest HF enzymes available.

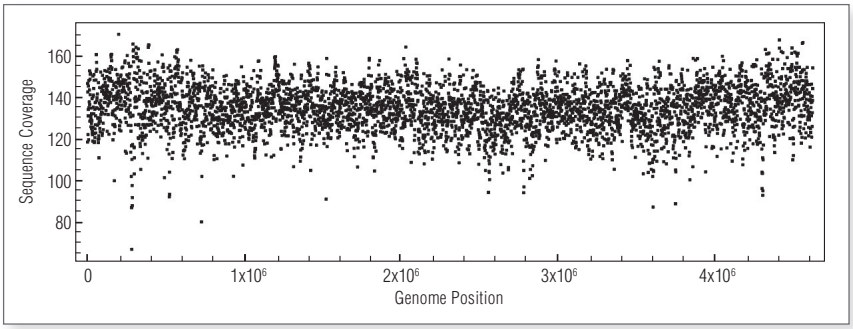
NEBNext™ Reagents for Sample Preparation

Introducing NEBNext™, a series of highly pure reagents that facilitate sample preparation of DNA for downstream applications such as next generation sequencing and expression library construction. Available in sets, master mixes and modules, these robust reagents undergo stringent quality controls and functional validation, ensuring maximum yield, convenience and value.

NEBNext offers a solution for each step of Genomic Library Construction Workflow



Sequencing coverage map of the *E. coli* genome after using NEBNext DNA SPRS1 for sample preparation



E. coli strain MG1655 gDNA was prepared with NEBNext DNA Sample Prep Reagent Set 1 and sequenced on an Illumina Genome Analyzer II.

Advantages

- **Convenient formats** – All of the required enzymes, buffers and nucleotides are included, many available in master mix format. Modules offer the ability to customize sample preparation.
- **Functional Validation** – Each reagent set or module is functionally validated by preparation of a genomic DNA library that is sequenced using the Illumina® GAII and by preparation of an expression library, or sequenced using the Roche/454 GS FLX Titanium™ and preparation of single-stranded DNA.
- **Stringent Quality Controls** – Additional QCs ensure maximum quality and purity.
- **Value Pricing**

GS FLX Titanium is a trademark of Roche.
Illumina is a registered trademark of Illumina, Inc.

Ordering Information

PRODUCT	NEB #	SIZE	PRICE
NEBNext™ DNA Sample Prep Reagent Set 1	E6000S/L	10/50 reactions	$500/2,000
NEBNext™ DNA Sample Prep Reagent Set 2	E6020S/L	10/50 reactions	$400/1,600
NEBNext™ DNA Sample Prep Master Mix Set 1	E6040S/L	10/50 reactions	$350/1,400
NEBNext™ End Repair Module	E6050S/L	20/100 reactions	$85/340
NEBNext™ dA-Tailing Module	E6053S/L	20/100 reactions	$100/400
NEBNext™ Quick Ligation Module	E6056S/L	20/100 reactions	$300/1,200

A complete list of reagents included in each set can be found at www.neb.com.

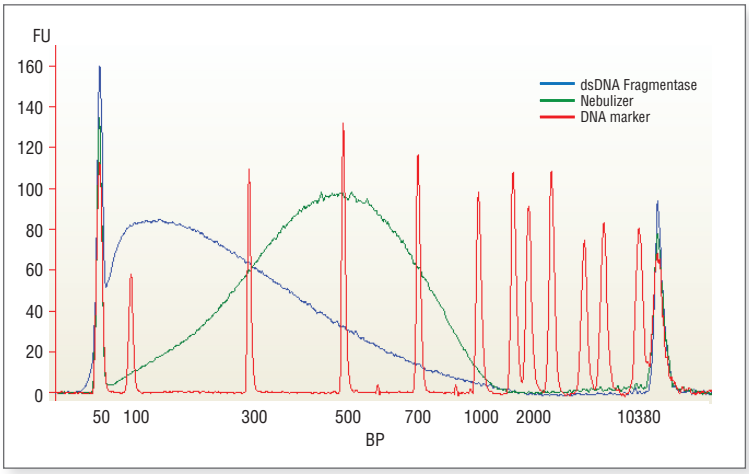
Visit www.neb.com/NEBNext frequently to learn about additional NEBNext products as they become available

For more information on customized solutions please contact NEBNext@neb.com

NEBNext™ dsDNA Fragmentase™

NEBNext™ dsDNA Fragmentase™ is an enzyme-based solution to the fragmentation of DNA. An alternative to nebulization, dsDNA Fragmentase will generate 100–800 bp fragments in a time dependent manner, and is ideal for generation of expression libraries or next generation sequencing.

NEBNext dsDNA Fragmentase generates fragments in the 100–300 bp range more effectively than nebulization



Relative size distribution of E. coli DNA fragments with dsDNA Fragmentase vs. nebulization as seen using the Bioanalyzer 2100. The dsDNA Fragmentase sample was incubated for 30 minutes at 37°C with 0.05 µg of DNA per µl of dsDNA Fragmentase in 1X Fragmentase Reaction Buffer with 100 µg/ml BSA. The Nebulizer sample was prepared by nebulization of DNA in 50% glycerol for 6 minutes at 35 psi.

Advantages

- High throughput compatibility
- Control of fragment size
- Generates fragments in the 100–300 base pair range more effectively than nebulization
- Enzymatic cleavage results in cleaner ends as compared to mechanical shearing
- Value pricing

Ordering Information

PRODUCT	NEB #	SIZE	PRICE
NEBNext dsDNA Fragmentase	M0348S/L	50/250 reactions	$95/380

FAQ Spotlight – NEBNext Reagents for Sample Preparation

Q: Which NEBNext products can I use to prepare libraries for sequencing with a GAII or GAIIx?

A: *NEBNext DNA Sample Prep Reagent Set 1, NEBNext DNA Sample Prep Master Mix Set 1, NEBNext End Repair Module, NEBNext dA-Tailing Module and NEBNext Quick Ligation Module.*

Q: Which NEBNext products can I use to prepare libraries for sequencing with a GS FLX or GS FLX Titanium?

A: *NEBNext DNA Sample Prep Reagent Set 2, NEBNext End Repair Module, and NEBNext Quick Ligation Module.*

Q: The NEBNext product manuals refer to the individual reagents as being "Lot Controlled". What does this mean?

A: *Each set of reagents is functionally validated together through construction of a genomic DNA library and sequenced on the appropriate platform. Once a new lot of any of the reagents is introduced into the set, the set is re-validated by library construction and sequencing.*

Q: Are protocols included with the NEBNext Products?

A: *Protocols are included with the Master Mix Sets, Modules, and dsDNA Fragmentase. Please refer to your specific sample preparation protocol to determine conditions for use with Sample Prep Reagent Sets 1 and 2.*

Cloning Guide

Most cloning experiments involve the insertion of a DNA fragment into a plasmid for downstream protein expression, insertion of a multiple cloning site or the addition of a property to the vector (e.g., a drug resistance marker, a promoter, a signal sequence, etc.). The following tips will help with the design and troubleshooting of cloning.

Preparation of Insert & Vector

Insert from a plasmid source

- Digest plasmid with appropriate restriction enzymes to produce a DNA fragment that can be cloned directly into a vector. Unidirectional cloning is achieved with restriction enzymes that produce non-compatible ends.

Insert from a PCR product

- Design primers with appropriate restriction sites to clone unidirectionally into a vector
- Addition of 6 bases upstream of the restriction site is sufficient for digestion with most enzymes
- If fidelity is a concern, choose a proofreading polymerase such as Phusion (NEB #F-530) or Vent (NEB #M0254) DNA Polymerase.
- Guidelines for PCR optimization can be found in the technical reference section of our website
- Purify PCR product by running the DNA on an agarose gel and excising the band or by using a spin column
- Digest with appropriate restriction enzyme

Insert from annealed oligos

- Annealed oligos can be used to introduce a fragment (e.g., promoter, polylinker, etc.).
- Anneal two complementary oligos that leave protruding 5′ or 3′ overhangs for ligation into a vector cut with the appropriate enzymes
- Non-phosphorylated oligos can be phosphorylated using T4 Polynucleotide Kinase (NEB #M0201)

A "Typical" Annealing Reaction

Primer	1 µg
10X Ligase Buffer	5 µl
Total Volume	50 µl
Incubation	85°C for 10 minutes Cool slowly (30-60 min)

Vector

- Digest vector with appropriate restriction enzymes. Enzymes that leave non-compatible ends are ideal as they prevent vector self-ligation.

Dephosphorylation

- Dephosphorylation is sometimes necessary to prevent self ligation. NEB offers two products for dephosphorylation of DNA.
  - » Calf Intestinal Phosphatase (CIP) (NEB #M0290) is a robust enzyme that will function under many different conditions and in most NEBuffers. However, CIP cannot be heat inactivated and requires a purification step before ligation. To improve DNA purification, do not use more CIP than recommended.
  - » Antarctic Phosphatase (AP) (NEB #M0289) can carry out all the same functions as CIP and can be heat inactivated. It has a strict requirement for zinc but will work in all 4 NEBuffers if zinc is included.

Dephosphorylation With AP

Antarctic Phosphatase	1 µl (5 units)
DNA	1–5 µg
10X Buffer	2 µl
Total Volume	20 µl
Incubation	37°C for 15 minutes (5′ extensions/blunt ends) or 60 minutes (3′ extensions)
Heat Inactivate	65°C for 5 minutes

Blunting

- In some instances the ends of the insert or vector require blunting
- PCR with a proofreading polymerase will leave a predominantly blunt end
- T4 DNA Polymerase (NEB #M0203) or Klenow (NEB #M0210) will fill in a 5′ overhang (e.g., EcoRI) and chew back a 3′ overhang (e.g., PstI)
- Alternatively, the Quick Blunting Kit (NEB #E1201) is optimized to blunt and phosphorylate DNA ends for cloning in less than 30 minutes

Phosphorylation

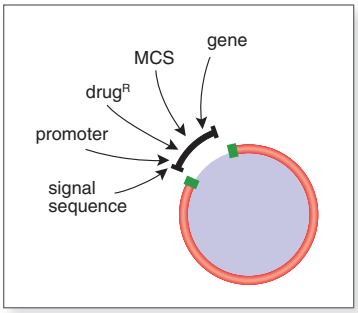
- For ligation to occur, at least one of the DNA ends (insert or vector) should contain a 5′ phosphate
- Primers are usually supplied non-phosphorylated; therefore, the PCR product will not contain a 5′ phosphate
- Digestion of DNA with a restriction enzyme will always produce a 5′ phosphate
- A DNA fragment can be phosphorylated by incubation with T4 Polynucleotide Kinase (NEB #M0201)

Phosphorylation With T4 PNK

T4 PNK	1 µl (10 units)
10X T4 PNK Buffer	5 µl
10 mM ATP	5 µl (1 mM final conc.)
DNA (20 mer)	1–2 µg
Total Volume	50 µl
Incubation	37°C for 30 minutes

Purification of Vector and Insert

- Purify the vector and insert before ligation by either running the DNA on an agarose gel and excising the appropriate bands or using a spin column
- DNA can also be purified using β-Agarase I (NEB #M0392) with low melt agarose or an appropriate spin column or resin
- Analyze agarose gels with longwave UV (360 nM) to minimize UV exposure that may cause DNA damage



Ligation of Vector and Insert

- Use a molar ratio of 1:3 vector to insert
- Thaw and resuspend the Ligase Buffer at room temperature
- Blunt ends often require longer ligation or high concentration ligase
- The Quick Ligation Kit (NEB #M2200) is optimized for ligation of both sticky and blunt ends
- After ligation, chill on ice and transform
- DO NOT heat inactivate when using the Quick Ligation Buffer as this will inhibit transformation

Ligation with the Quick Ligation Kit

Quick T4 DNA Ligase	1 µl
2X Quick Ligation Buffer	10 µl
Vector DNA (3 kb)	50 ng
Insert DNA (1kb)	50 ng
Total Volume	20 µl (mix well)
Incubation	Room temperature for 5 minutes

Troubleshooting a Cloning Experiment

Detailed suggestions can be found on the FAQ pages for individual ligase products at www.neb.com. Often, suitable controls will pinpoint the origin of the problem.

- To confirm that the ligase, ligase buffer and competent cells are functional, repeat the experiment with an uncut plasmid and a single cut plasmid with and without T4 DNA Ligase (NEB #M0202)
- Repeat with fresh buffer as the ATP or DTT may have degraded
- Purify the DNA as the presence of NaCl or EDTA will reduce ligation efficiency
- Confirm there is a 5´ phosphate on either the insert or plasmid
- Ligation produced only linear DNA because the DNA concentration was too high. Keep the total DNA concentration between 1–10 µg/ml.
- Transformation Guidelines and Troubleshooting tips can be found on pages 346–347 of our catalog and in the technical reference section of our website at www. neb. com.

Ordering Information

PRODUCT		NEB #	SIZE	PRICE
Phusion™ High Fidelity DNA Polymerase	RH	F-530S/L	100/500 units	$103/412
Vent® DNA Polymerase	RH	M0254S/L	200/1,000 units	$61/244
T4 Polynucleotide Kinase	RH	M0201S/L	500/2,500 units	$53/212
Alkaline Phosphatase, Calf Intestinal		M0290S/L	1,000/5,000 units	$61/244
Antarctic Phosphatase	RH	M0289S/L	1,000/5,000 units	$58/232
T4 DNA Polymerase	RH	M0203S/L	150/750 units	$58/232
DNA Polymerase I, (Klenow) Lg. Frag.	RH	M0210S/L	200/1,000 units	$53/212
Quick Blunting Kit	RH	E1201S/L	20/100 units	$70/280
β-Agarase I	RH	M0392S/L	100/500 units	$66/264
T4 DNA Ligase	RH	M0202S/L	20,000/100,000 units	$63/252
Quick Ligation Kit	RH	M2200S/L	30/150 reactions	$95/380

RH = recombinant

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