NEB EXPRESSIONS

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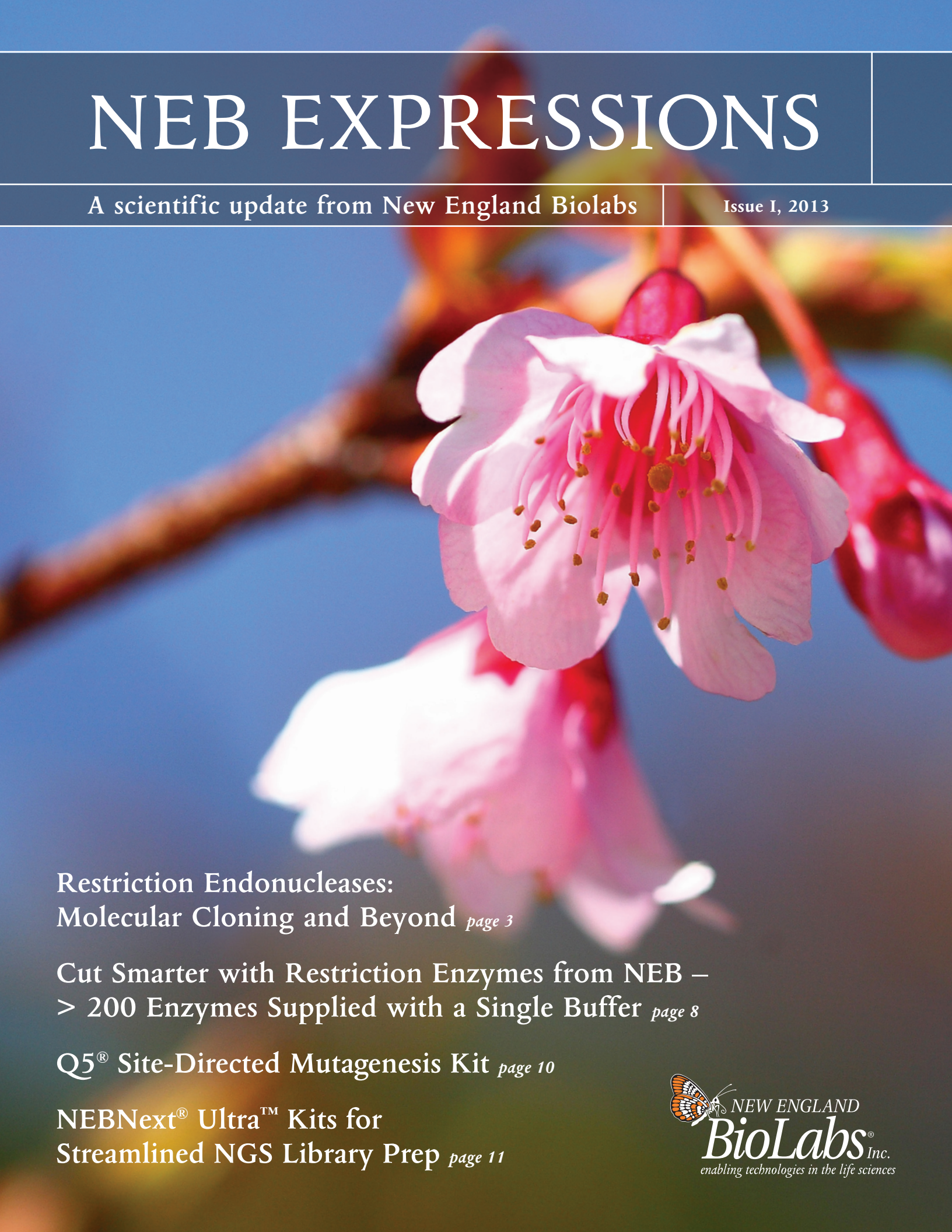

NEW ENGLAND BioLabs® Inc.
enabling technologies in the life sciences

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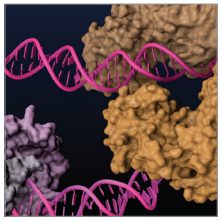
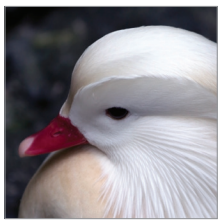
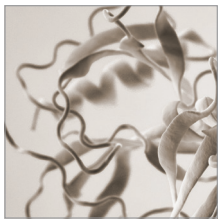
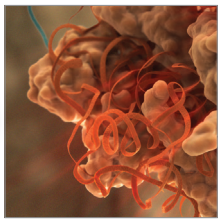








COVER PHOTO

A sure sign of spring, cherry blossoms bloom on this flowering cherry tree (*Prunus okame*).

FEATURE ARTICLE

Restriction Endonucleases: Molecular Cloning and Beyond

The sequence-specific DNA cleavage activity of restriction endonucleases (REases), combined with other enzymatic activities that amplify and ligate nucleic acids, have enabled modern molecular biology. After more than half a century of research and development, the applications of REases have evolved from the cloning of exogenous DNA and genome mapping to more sophisticated applications, such as the identification and mapping of epigenetic modifications and the high-throughput assembly of combinatorial libraries. Furthermore, the discovery and engineering of nicking endonucleases (NEases) has opened the door to techniques such as isothermal amplification of DNA among others. In this review, we will examine the major breakthroughs of REase research, applications of REases and NEases in various areas of biological research and novel technologies for assembling large DNA molecules.

Siu-Hong Chan, Ph.D., New England Biolabs, Inc.

INTRODUCTION

In the 1950s, a phenomenon known as "host controlled/induced variation of bacterial viruses" was reported, in which bacteriophages isolated from one *E. coli* strain showed a decrease in their ability to reproduce in a different strain, but regained the ability in subsequent infection cycles (1,2). In 1965, Werner Arber's seminal paper established the theoretical framework of the restriction-modification system, functioning as bacterial defense against invading bacteriophage (3). The first REases discovered recognized specific DNA sequences, but cut at variable distances away from their recognition sequence (Type I) and, thus were of little use in DNA manipulation. Soon after, the discovery and purification of REases that recognized and cut at specific sites (Type II REases) allowed scientists to perform precise manipulations of DNA *in vitro*, such as the cloning of exogenous genes and creation of efficient cloning vectors. Now, more than 4,000 REases are known, recognizing more than 300 distinct sequences (for a full list, visit REBASE® at rebase.neb.com). With the advent of the Polymerase Chain Reaction (PCR), RT-PCR, and PCR-based mutagenesis methodologies, the traditional cloning workflow transformed biological research in the decades that followed.

ENGINEERING OF RESTRICTION ENZYMES

Traditionally, REases were purified from the native organism. The development of gene cloning vectors and selection methodologies enabled the cloning of REases. Cloning not only allowed the production of large quantities of highly purified enzymes, but also made the engineering of REases possible. Currently, > 250 of the restriction enzymes supplied by New England Biolabs (NEB) are recombinant proteins.

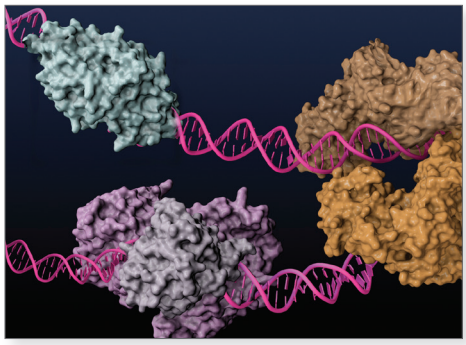
Engineering Improved Performance

Cleavage activity at non-cognate sites (i.e., star activity) had been observed and well-documented for some REases. Of those, some exhibit star activity under sub-optimal reaction conditions, while others have a very narrow range of enzyme units that completely digest a given amount of substrate without exhibiting star activity (4). Through intensive research, scientists at NEB began engineering restriction enzymes that exhibit minimal, if any, star activity with extended reaction times and at high enzyme concentrations. This research enabled the introduction of High Fidelity (HF™) REases that have improved performance under a wider range of reaction conditions (for more information, visit www.neb.com/HF).

Engineering New Sequence Specificities

Attempts to alter the sequence specificities of Type IIP REases have been largely unsuccessful, presumably because the sequence specificity determinant is structurally integrated with the active sites of Type IIP REases. MmeI, a Type IIG REase with both methyltransferase (MTase) and REase activities in the same polypeptide, recognizes the target sequence TCCRAC using the target recognition domain (TRD) within its MTase component. This represented an excellent opportunity to engineer altered sequence specificity into the REase. As an added advantage, the sharing of the TRD between the REase and MTase activities resulted in an equivalent change in MTase activity for any change in target sequence cleavage specificity, protecting the new target site from cleavage in recombinant host cells. Through bioinformatics analysis of homologous protein sequences, scientists at NEB identified the amino acid residues that recognized specific bases within the target sequences and created MmeI mutants with altered sequence specificities (5). Rational design of MmeI mutants and homologs unlocked the potential for the creation of REases with hundreds of new sequence specificities.

Figure 1. Nicking Enzyme Engineering



Type IIS REases, such as FokI (light and dark brown) and BstNBI (isoschizomer of BspD6I, light and dark purple), and homing endonuclease I-AniI (cyan), have been engineered to possess nicking enzyme activities.

TYPES AND ACTIVITIES OF RESTRICTION ENZYMES

Type I
Cleaves DNA at random sites far from its recognition sequence

Type II
Cleaves DNA at defined positions close to or within its recognition sequence

Type IIG
Cleaves outside its recognition sequence with both REase and MTase enzymatic activities in the same protein

Type IIP
Cleaves symmetric targets and cleavage sites

Type IIS
Recognizes asymmetric sequences

Type III
Cleaves outside its recognition sequence and require two sequences in opposite orientations within the same DNA

Type IV
Cleaves modified (e.g., methylated) DNA

Engineering Nicking Endonucleases

Basic research involving REases led to surprising findings about the seemingly straightforward mechanism of cleavage. Prototypical Type IIP REases normally act as homodimers, with each of the monomers nicking half of the palindromic site. Type IIS REases, on the other hand, exhibit a broad range of double-stranded cleavage mechanisms, namely heterodimerization, as by BtsI and BbvCI, and sequential cleavage of the dsDNA as monomer, as by FokI. These properties have been exploited to create strand-specific nicking enzymes (NEases) (for more information about nicking enzymes, see review in (6)).

APPLICATIONS UTILIZING RESTRICTION ENZYMES

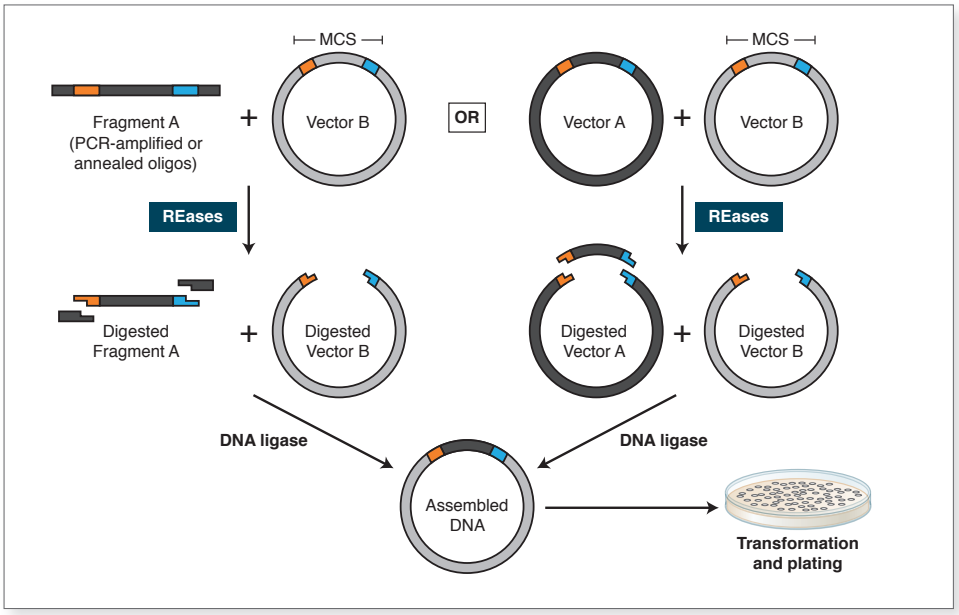
Traditional Cloning

In combination with DNA ligases, REases facilitated a robust "cut and paste" workflow where a defined DNA fragment could be moved from one organism to another (Fig. 2). Using this methodology, Stanley Cohen and his colleagues incorporated exogenous DNA into natural plasmids to create the vehicle for cloning-plasmid vectors that self-propagate in *E. coli* (7). These became the backbone of many present-day vectors, and enabled the cloning of DNA for the study and production of recombinant proteins. Restriction enzymes are also useful as post-cloning confirmatory tools, to ensure that insertions have taken place correctly. The traditional cloning workflow, along with DNA amplification technologies, such as PCR and RT-PCR, has become a mainstream application for REases and facilitated the study of many molecular mechanisms.

DNA Mapping

Armed with only a handful of REases in the early 1970s, Daniel Nathans mapped the functional units of SV40 DNA (8), and commenced the era of "restriction mapping" and comparison of complex genomes. It has since evolved into sophisticated methodologies that allow the detection of single nucleotide polymorphisms (SNP) and insertions/deletions (Indels) (9), driving applications that include identifying genetic disorder loci, assessing the genetic diversity of populations and parental testing.

Figure 2. Traditional Cloning Workflow



Using PCR, restriction sites are added to both ends of a dsDNA, which is then digested by the corresponding REases. The cleaved DNA can then be ligated to a plasmid vector cleaved by the same or compatible REases with T4 DNA ligase. DNA fragments can also be moved from one vector into another by digesting with REases and ligating to compatible ends of the target vector.

Understanding Epigenetic Modifications

REases' sensitivity to the methylation status of target bases has been exploited to map modified bases within genomes. Restriction Landmark Genome Scanning (RLGS) is a 2-dimensional gel electrophoresis-based mapping technique that employs NotI (GC^GGCCGC), AscI (GG^CGCGCC), EagI (C^GGCCG) or BssHII (G^CGCGC) to interrogate changes in the methylation patterns of the genome during the development of normal and cancer cells. Methylation-Sensitive Amplification Polymorphism (MSAP) takes advantage of the differential sensitivity of MspI and HpaII toward the methylation status of the second C of quadruplet CCGG to identify 5-methylcytosine (5-mC) or 5-hydroxymethylcytosine (5-hmC) (10,11). Scientists at NEB further exploited the property of MspI and HpaII on 5-glucosyl hydroxymethylcytosine (5-ghmC) in the EpiMark® 5-hmC and 5-mC Analysis Kit (NEB #E3317S)(12), which differentiates 5-hmC from 5-mC for more refined epigenetic marker identification and quantitation (for more information, visit EpiMark.com). Additionally, the recently discovered REases that recognize and cleave DNA at 5-mC and 5-hmC sites (e.g., MspJI, FspEI and LpnPI), as well as those that preferentially cleave 5-hmC or 5-ghmC over 5-mC or C (e.g., PvuRts1I, AbaSI) (13), are potential tools for high-throughput mapping of the cytosine-based epigenetic markers in cytosine-methylated genomes (14,15).

DEVELOPMENT OF RESTRICTION ENZYMES AND GENE EDITING TECHNIQUES

1952-53
"Host-induced variation of bacterial viruses" first described

1965-68
Biochemical characterization of the first Type I REases

1970
Purification and characterization of the first Type II REases (HindII/HindIII)

1971-73
Mapping of SV40 DNA

1973-74
Construction of the first autonomously replicating plasmids using EcoRI and DNA ligase (the pSC series)

1975
REBASE (Restriction Enzyme Database) launched

1975
Technique: Restriction Fragment Length Polymorphism (RFLP) analysis

In vitro DNA Assembly Technologies

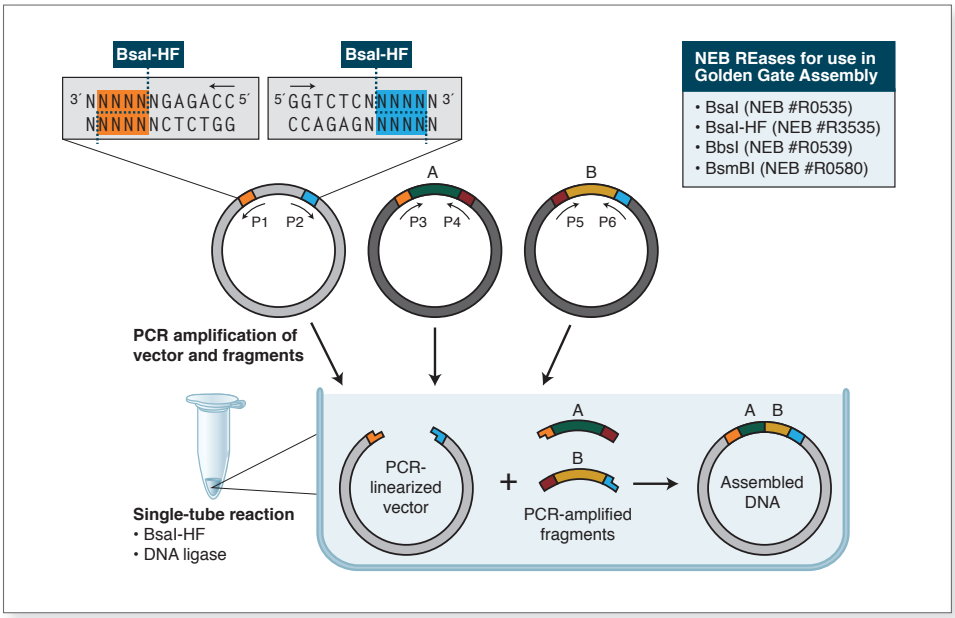
Synthetic biology is a rapidly growing field, in which defined components are used to create biological systems for the study of biological processes and the creation of useful biological devices (16). Novel technologies such as BioBrick™ originally emerged to facilitate the building of such biological systems. Recently, more robust approaches, such as Golden Gate Assembly and Gibson Assembly™, have been widely adopted by the synthetic biology community. Both approaches allow for the parallel and seamless assembly of multiple DNA fragments without resorting to non-standard bases.

BioBrick: The BioBricks community (http://hdl.handle.net/1721.1/21168) sought to create thousands of "standardized parts" of DNAs for rapid gene assembly. With the annual International Genetically Engineered Machines (iGEM) competition (igem.org), the BioBricks community grew and elicited broad interest from many university students in synthetic biology. Based on traditional REase-ligation methodology, BioBrick and its derivative methodologies (BioBrick Assembly Kit, NEB #E0546, and its derivative, BglBricks (17)) are easy to use, but they introduce scar sequences at the junctions. They also require multiple cloning cycles to create a working biological system.

Golden Gate Assembly: Golden Gate Assembly and its derivative methods (19,20) exploit the ability of Type IIS REases to cleave DNA outside of the recognition sequence. The inserts and cloning vectors are designed to place the Type IIS recognition site distal to the cleavage site, such that the Type IIS REase can remove the recognition sequence from the assembly (Fig. 3). The advantages of such an arrangement are three-fold: 1. the overhang sequence created is not dictated by the REase, and therefore no scar sequence is introduced; 2. the fragment-specific sequence of the overhangs allows orderly assembly of multiple fragments simultaneously; and 3. the restriction site is eliminated from the ligated product, so digestion and ligation can be carried out simultaneously. The net result is the ordered and seamless assembly of DNA fragments in one reaction. The accuracy of the assembly is dependent on the length of the overhang sequences. Therefore, Type IIS REases that create 4-base overhangs (such as BsaI/BsaI-HF, BbsI, BsmBI and Esp3I) are preferred. The downside of these Type IIS REase-based methods is that the small number of overhanging bases can lead to the mis-ligation of fragments with similar overhang sequences (21). It is also necessary to verify that the Type IIS REase sites used are not present in the fragments for the assembly of the expected product. Nonetheless, Golden Gate Assembly is a robust technology that generates multiple site-directed mutations (22) and assembles multiple DNA fragments (23,24). As open source methods and reagents have become increasingly available (see www.addgene.org), Golden Gate Assembly has been widely used in the construction of custom-specific TALENs for *in vivo* gene editing (25), among other applications.

Gibson Assembly: Daniel G. Gibson, of the J. Craig Venter Institute, described a robust exonuclease-based method to assembly DNA seamlessly and in the correct order. The reaction is carried out under isothermal conditions using three enzymatic activities: a 5' exonuclease generates long overhangs, a polymerase fills in the gaps of the annealed ss regions, and a DNA ligase seals the nicks of the annealed and filled-in gaps (26) (Fig. 4). Applying this methodology, the 16.3 kb mouse mitochondrial genome was assembled from 600 overlapping 60-mers (26). In combination with *in vivo* assembly in yeast,

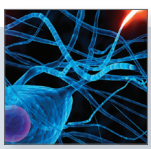
Figure 3. Golden Gate Assembly Workflow



Golden Gate Assembly requires a Type II restriction enzyme with a non-palindromic recognition site. In this example, a BsaI recognition site (GGTCTC) is added to both ends of a dsDNA fragment distal to the cleavage site, such that the BsaI site is eliminated by digestion with BsaI or BsaI-HF (GGTCTC 1/5). Upon cleavage, the overhanging sequences of the adjoining fragments anneal to each other. DNA ligase then seals the nicks to create a new covalently linked DNA molecule. Multiple pieces of DNA can be cleaved and ligated simultaneously.

1978
Over 100 REases identified to-date

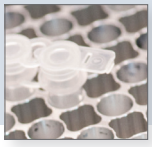
1982
Cloning of PstI



1983
Cloning of cDNA of Nerve Growth Factor precursor from human and mouse

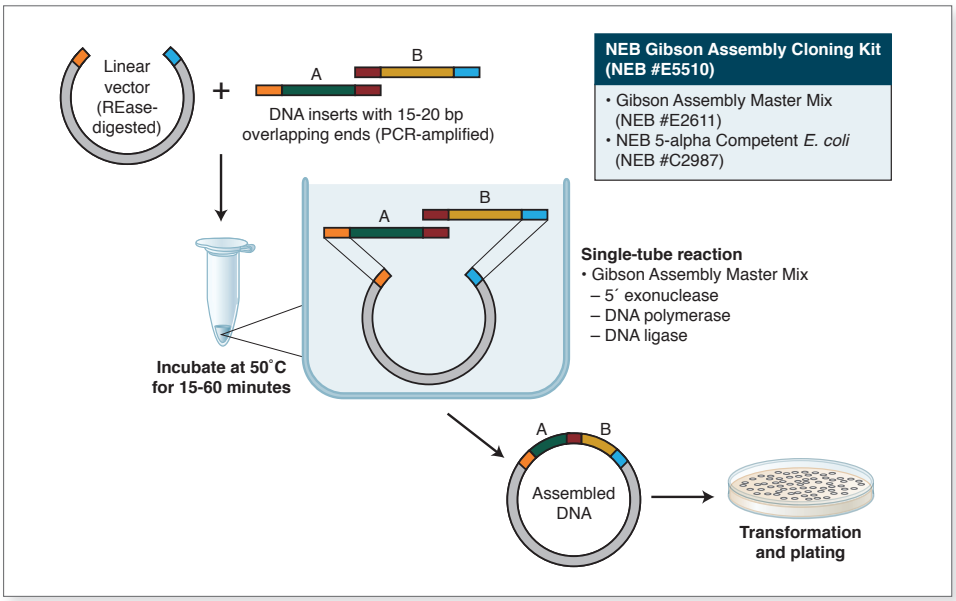
1988
Over 1,000 REases identified to-date

1978
Nobel Prize in Medicine or Physiology awarded to Werner Arber, Daniel Nathans & Hamilton O. Smith "for the discovery of restriction enzymes and their application to problems of molecular genetics"



1983-88
Invention and development of PCR

Figure 4. Gibson Assembly Workflow



Gibson Assembly employs three enzymatic activities in a single-tube reaction: 5´ exonuclease, the 3´ extension activity of a DNA polymerase and DNA ligase activity. The 5´ exonuclease activity chews back the 5´ end sequences and exposes the complementary sequence for annealing. The polymerase activity then fills in the gaps on the annealed regions. A DNA ligase then seals the nick and covalently links the DNA fragments together. The overlapping sequence of adjoining fragments is much longer than those used in Golden Gate Assembly, and therefore results in a higher percentage of correct assemblies. The NEB Gibson Assembly Master Mix (NEB #E2611) and Gibson Assembly Cloning Kit (NEB #E5510S) enable rapid assembly at 50˚C.

Gibson Assembly was used to synthesize the 1.1 Mbp *Mycoplasma mycoides* genome. The synthesized genome was transplanted to a *M. capricolum* recipient cell creating new self-replicating *M. mycoides* cells (27).

Gibson Assembly can also be used for cloning; the assembly of a DNA insert with a restriction-digested vector, followed by transformation, can be completed in a little less than two hours with the Gibson Assembly Cloning Kit (NEB #E5510S, for more information, visit NEBGibson.com). Other applications of Gibson Assembly include the introduction of multiple mutations, assembly of plasmid vectors from chemically synthesized oligonucleotides, and creating combinatorial libraries of genes and pathways.

Construction of DNA Libraries

SAGE (Serial Analysis of Gene Expression) has allowed the identification and quantification of a large number of mRNA transcripts. It has been widely used in cancer research to identify mutations and study gene expression. REases are key to the SAGE workflow. NlaIII is instrumental as an anchoring enzyme, because of its unique property of recognizing a 4-bp sequence CATG and creating a 4 nucleotide overhang of the same sequence. The use of Type IIS enzymes as tagging enzymes that cleave further and further away from the recognition sequence allows for the higher information content of SAGE analyses (e.g., FokI and BsmFI in SAGE (28), MmeI in LongSAGE (29) and EcoP15I in SuperSAGE (30) and DeepSAGE (31)).

Chromosome conformation capture (3C) and derivative methods allow the mapping of the spatial organizations of genomes in unprecedentedly high resolution and throughput (32). REases plays an indispensible role in creating the compatible ends of the DNA cross-linked to its interacting proteins, such that spatially associated sequences can be ligated and, hence, identified through high-throughput sequencing.

Although REases do not allow for the random fragmentation of DNA that most next-generation DNA sequencing technologies require, they are being used in novel target enrichment methodologies (hairpin adaptor ligation (33) and HaloPlex™ enrichment (Agilent)). The long-reach REase, AcuI, and USER™ Enzyme are also used to insert tags into sample DNA, which is then amplified by rolling circle amplification (RCA) to form long, single-stranded DNA "nanoballs" that serve as template in the high density, chip-based sequencing-by-ligation methodology, developed by Complete Genomics (34). ApeKI was also used to generate the DNA library for a genotyping-by-sequencing technology for the study of sequence diversity of maize (35).

Creation of Nicks in DNA

Before NEases were available, non-hydrolyzable phosphorothioate groups were incorporated into a specific strand of the target DNA such that REases can introduce sequence- and strand-specific nicks into the DNA for applications such as strand displacement amplification (SDA), where a strand-displacing DNA polymerase (e.g., *Bst* 2.0 DNA Polymerase, NEB #M0537) extends from the newly created 3'-hydroxyl end, and essentially replicates the complementary sequence (36). Because the nicking site is regenerated, repeated nicking-extension cycles result in amplification of specific single-stranded segments of the sample DNA without the need for thermocycling. NEases greatly streamline the workflow of such applications and open the door to applications that cannot be achieved by REases. Nicking enzyme-based isothermal

DEVELOPMENT OF RESTRICTION ENZYMES AND GENE EDITING TECHNIQUES (CONT'D)

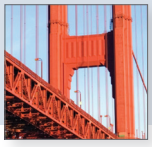
1990-PRESENT
Cloning via PCR amplification followed by REase digestion and ligation

1995
Technique: Amplification Fragment Length Polymorphism (AFLP) analysis

1996
Development of zinc finger nucleases (ZFN)

1997
Technique: methylation-sensitive amplified polymorphism (MSAP) analysis

2000-PRESENT
Gene disruption and insertion induced by ZFNs and engineered meganucleases



2008
Introduction of Golden Gate Assembly and its variants

DNA amplification technologies, such as RCA, NESA, EXPAR and related amplification schemes, have been shown to be capable of detecting very low levels of DNA (37,38). Nicking-based DNA amplification had also been incorporated into molecular beacon technologies to amplify signal (39). The implementation of these sample and/or signal amplification schemes can lead to simple, but sensitive and specific, methods for the detection of target DNA molecules in the field (NEAR, EnviroLogix™). By ligating adaptors containing nicking sites to the ends of blunt-ended DNA, the simultaneous actions of the NEase(s) and strand-displacing DNA polymerase can quickly amplify a specific fragment of dsDNA (40). Amplification by nicking-extension cycling is amenable to multiplexing and can potentially achieve a higher fidelity than PCR. The combined activity of NEases and *Bst* DNA polymerase have also been used to introduce site-specific fluorescent labels into long/chromosomal DNA *in vitro* for visualization (nanocoding) (41). Innovative applications of nicking enzymes include the generation of reporter plasmids with modified bases or structures (42) and the creation of a DNA motor that transports a DNA cargo without added energy (43). A review of NEases and their applications has been published elsewhere (6).

In vivo Gene Editing

The ability to "cut and paste" DNA using REases *in vitro* has naturally led to the quest for performing the art *in vivo* to correct mutations that cause genetic diseases. Direct use of REases and homing endonucleases in Restriction Enzyme Mediated Integration (REMI) facilitated the generation of transgenic embryos of higher organisms (44,45). There is, however, no control over the integration site. The concept of editing genes through site-specific cleavage has been realized using Zinc Finger Nucleases (ZFNs) and Transcription Activator-like Effector Nucleases (TALENs), due to their ability to create customizable double stranded breaks in complex genomes. With the great success of gene editing in model organisms and livestock (46-50), the therapeutic potential of these gene editing reagents is being put to the first test in the Phase I/II clinical trials of a regime that uses a ZFN to improve CD4+ T-cell counts by knocking out the expression of the CCR5 gene in autologous T-cells from HIV patients (ClinicalTrials.gov indentifier NCT00842634) (51). Recent research on CRISPR, the adaptive defense system of bacteria and archaea, has shown the potential of the Cas9-crRNA complex as programmable RNA-guided DNA endonucleases and strand-specific nicking endonucleases for *in vivo* gene editing (52,53).

MOVING FORWARD

Restriction enzymes have been one of the major forces that enabled the cloning of genes and transformed molecular biology. Novel technologies, such as Golden Gate Assembly and Gibson Assembly, continue to emerge and expand our ability to create new DNA molecules. The potential to generate new recognition specificity in the MmeI family REases, the engineering of more NEases and the discovery of ever more modification-specific REases continues to create new tools for DNA manipulation and epigenome analysis. Innovative applications of these enzymes will take REases' role beyond molecular cloning by continuing to accelerate the development of biotechnology and presenting us with new opportunities and challenges.

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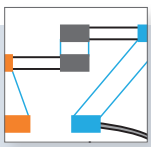
48. Joung, J. K., and Sander, J. D. (2012) *Nat. Rev. Mol. Cell Biol.* 14, 49-55.
49. Collin, J., and Lako, M. (2011) *Stem Cells* 29, 1021-1033.
50. Carlson, D. F., et al. (2012) *PNAS* 109, 17382-17387.
51. Ledford, H. (2011) *Nature* 471, 16.
52. Gasiunas, G., et al. (2012) *PNAS* 109, E2579-2586.
53. Jinek, M., et al. (2012) *Science* 337, 816-821.

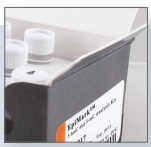
For more information on restriction endonucleases, visit REBASE (The Restriction Enzyme Database), a comprehensive database of information about all known restriction endonucleases and related proteins.

rebase.neb.com.

2009
Phase I clinical trial to remove CCR5 from autologous T-cells using ZFNs begins



2009
Introduction of Gibson Assembly

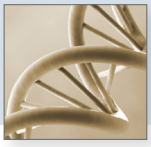


2011
EpiMark 5-hmC and 5-mC Analysis Kit released

2012
Over 4,000 REases experimentally characterized to-date

2009
Identification of the DNA recognition code of TAL effectors

2010-PRESENT
Gene disruption and insertion induced by TALENs in model organisms



2012
Cas9 enables RNA-guided genome editing

Cut Smarter with Restriction Enzymes from NEB – now with CutSmart Buffer

Looking to bring CONVENIENCE to your workflow?

Simplify reaction setup and double digestion with CutSmart Buffer

Over 200 restriction enzymes are 100% active in a single buffer, CutSmart Buffer, making it significantly easier to set up your double digest reactions. Since CutSmart Buffer includes BSA, there are fewer tubes and pipetting steps to worry about. Additionally, many DNA modifying enzymes are 100% active in CutSmart Buffer, eliminating the need for subsequent purification.

For more information, visit **www.NEBCutSmart.com**

Speed up digestions with Time-Saver™ Qualified Restriction Enzymes

Over 180 of our restriction enzymes are able to digest DNA in 5-15 minutes, and can safely be used overnight with no loss of sample. For added convenience and flexibility, most of these are supplied with our new CutSmart Buffer.

For more information, visit **www.neb.com/timesaver**

Keep it simple with our RE-Mix® Restriction Enzyme Master Mixes

RE-Mix Restriction Enzyme Master Mixes are pre-mixed solutions that contain enzyme, buffer, BSA and loading dye. Just add your DNA and water; it's that simple! RE-Mix master mixes are Time-Saver qualified so you can trust your reaction to digest to completion in 15 minutes, or leave it to digest overnight, with no degradation of your final product.

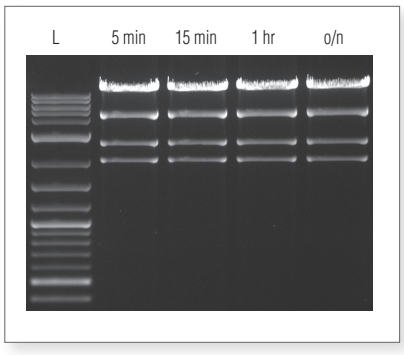
For more information, visit **www.NEBREMix.com**

Activity of DNA Modifying Enzymes in CutSmart Buffer

Enzyme	Activity in CutSmart	Required Supplements
Alkaline Phosphatase (CIP)	+ + +	
Antarctic Phosphatase	+ + +	Requires Zn^{2+}
Bst DNA Polymerase	+ + +	
CpG Methyltransferase (M. SssI)	+ + +	
DNA Polymerase I	+ + +	
DNA Polymerase I, Large (Klenow) Fragment	+ + +	
DNA Polymerase Klenow Exo⁻	+ + +	
DNase I (RNase free)	+ + +	Requires Ca^{2+}
E. coli DNA Ligase	+ + +	Requires NAD
Endonuclease III (Nth), recombinant	+ + +	
Endonuclease VIII	+ + +	
Exonuclease III	+ + +	
GpC Methyltransferase (M. CviPI)	+	Requires DTT
McrBC	+ + +	
Micrococcal Nuclease	+ + +	
Nuclease BAL-31	+ + +	
phi29 DNA Polymerase	+ + +	
$RecJ_f$	+ + +	
T3 DNA Ligase	+ + +	Requires ATP + PEG
T4 DNA Ligase	+ + +	Requires ATP
T4 DNA Polymerase	+ + +	
T4 Phage β-glucosyltransferase (T4-BGT)	+ + +	
T4 Polynucleotide Kinase	+ + +	Requires ATP
T4 PNK (3´ phosphatase minus)	+ + +	Requires ATP
T7 DNA Ligase	+ + +	Requires ATP + PEG
T7 DNA Polymerase (unmodified)	+ + +	
T7 Exonuclease	+ + +	
USER Enzyme, recombinant	+ + +	

+ + + full functional activity + + 50–100% functional activity + 0–50% functional activity

See **NEBCutSmart.com** for full details.



RE-Mix offers maximum convenience.
pXba DNA was digested with EcoRV-HF RE-Mix according to the recommended protocol. Lane L is the TriDye™ 2-Log DNA Ladder (NEB #N3270). Complete digestion, free of unwanted star activity, is seen whether incubated for 5–15 minutes, 1 hour or overnight.

Bring flexibility to your workflow

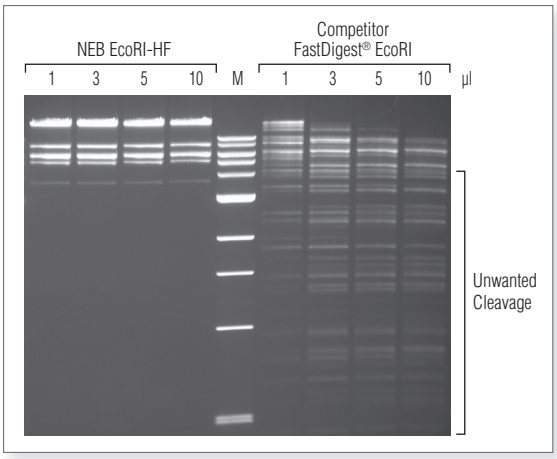
NEB offers the largest selection of restriction enzymes commercially available. With an evergrowing list to choose from, currently 276 enzymes – including traditional restriction enzymes, nicking endonucleases, homing endonucleases and methylation-sensitive enzymes for epigenetics studies – there is no need to look anywhere else.

Looking to optimize PERFORMANCE in your reaction?

Choose a High-Fidelity (HF) Restriction Enzyme

As part of our ongoing commitment to the advancement and improvement of enzymes for the cloning and manipulation of DNA, NEB has developed a line of High-Fidelity (HF) restriction enzymes. These engineered enzymes have the same specificity as the native enzyme, with the added benefit of reduced star activity, rapid digestion (5-15 minutes), and 100% activity in CutSmart Buffer. Enjoy the improved performance of NEB's engineered enzymes at the same price as the native enzymes.

For more information, visit **www.neb.com/HF**



HF enzymes outperform the competition.
EcoRI-HF (NEB #R3101) exhibits no star activity in overnight digests, even when used at higher concentrations. 50 µl reactions were set up using 1 µg of Lambda DNA, the indicated amount of enzyme and the recommended reaction buffer. Reactions were incubated overnight at 37°C. Marker M is the 1 kb DNA Ladder (NEB# N3232).

Benefit from industry-leading quality controls

NEB's reputation as a leader in enzyme technologies stems from the quality and reliability of our restriction enzymes. All of our restriction enzymes undergo stringent quality control testing, ensuring the highest levels of purity and lot-to-lot consistency.

Physical Purity	Enzymes are evaluated by SDS-PAGE and silver-stained gels to ensure the highest levels of purity and the absence of contaminating proteins.
DNA Contamination	Enzymes are screened by qPCR to ensure no contaminating genomic DNA is present. The specification for this assay is less than one *E. coli* genome per sample.
Exonuclease Activity	Using radioactively labelled DNA substrate and/or state-of-the-art capillary electrophoresis-based assays with fluorescently-labelled substrates, NEB is able to detect very low levels of exonuclease activity.
Endonuclease Activity	To ensure that there are no contaminating enzymes that could cause nicking or non-specific nuclease degradation, reagents are incubated with supercoiled plasmid DNA for 4 hours to demonstrate the absence of endonuclease contamination.
Non-Specific DNase Activity	Enzymes are incubated overnight with Lambda DNA to confirm that there is no additional non-specific nuclease activity present.
Cloning QC (Ligation and Re-cutting)	A DNA template is over-digested by the appropriate restriction enzyme and the percentage of DNA fragments ligated and re-cut are determined by agarose gel electrophoresis.
Cloning QC (Blue-white Screening)	A DNA plasmid is over-digested by the appropriate restriction enzyme and the linearized plasmid DNA is ligated and transformed into an *E. coli* strain with greater than 99% correct transformants as determined by a blue-white screen.

The CutSmart Advantage

- > 200 restriction enzymes are supplied in a single buffer, CutSmart Buffer
- 276 restriction enzymes are currently sold by NEB
- > 250 of NEB's restriction enzymes are recombinant
- 238 unique restriction enzymes are available from NEB
- > 180 restriction enzymes are Time-Saver qualified
- 26 High-Fidelity (HF) Restriction Enzymes are currently available

Industry-leading product quality

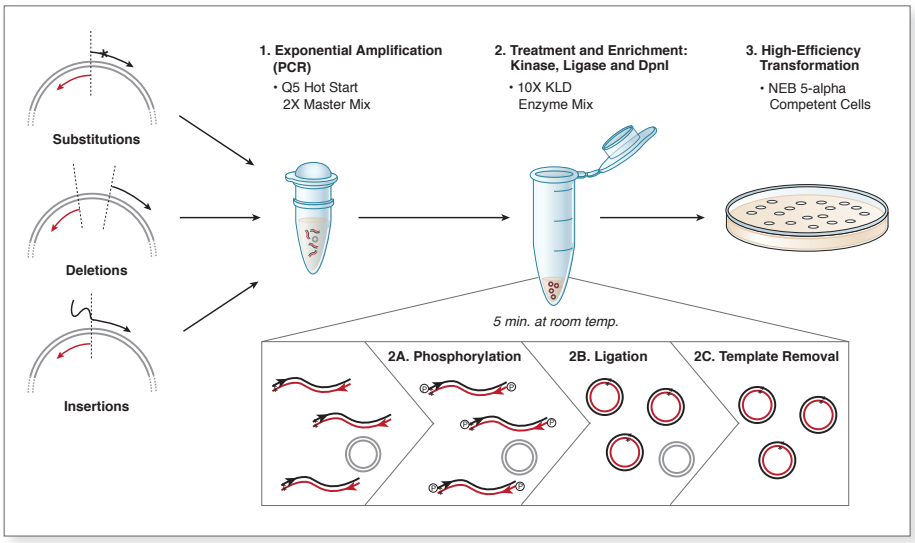
- State-of-the-art production and purification
- Nearly 40 years of experience
- Stringent quality control testing
- Lot-to-lot consistency
- ISO 9001- and 13485-certified

To explore over 200 CutSmart-compatible enzymes, visit **www.NEBCutSmart.com**

Q5 Site-Directed Mutagenesis Kit

The Q5 Site-Directed Mutagenesis Kit enables rapid site-specific mutagenesis of double-stranded plasmid DNA in less than 2 hours. The kit utilizes the robust Q5 Hot Start High-Fidelity DNA Polymerase along with custom mutagenic primers to create substitutions, deletions and insertions in a wide variety of plasmids. After PCR, the amplified material can be added directly to a unique Kinase-Ligase-DpnI (KLD) enzyme mix for rapid (5 minutes) circularization and template removal. Transformation into high-efficiency NEB 5-alpha Competent *E. coli*, provided with the kit, ensures robust results with plasmids up to, at least, 14 kb in length.

Q5 Site-Directed Mutagenesis Kit Overview



This kit is designed for rapid and efficient incorporation of substitutions, deletions and insertions into double-stranded plasmid DNA. The first step is an exponential amplification using Q5 Hot Start High-Fidelity DNA Polymerase. The second step utilizes a unique enzyme mix containing a kinase, ligase and DpnI. Together, these enzymes allow for rapid circularization of the PCR product and removal of the template DNA. The last step is a high-efficiency transformation into chemically competent cells.

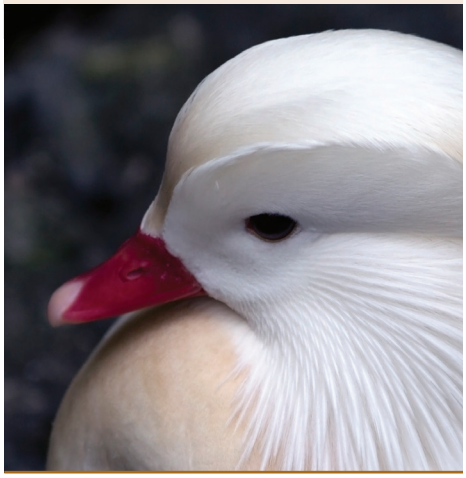
Ordering Information

PRODUCT	NEB #	SIZE
Q5 Site-Directed Mutagenesis Kit	E0554S	10 reactions

For information about other Q5 products, visit **Q5PCR.com**

Advantages

- Generation of mutations, insertions or deletions in plasmid DNA
- Non-overlapping primer design ensures robust, exponential amplification and generates a high % of desired mutations from a wide range of templates
- Intramolecular ligation and transformation into NEB 5-alpha results in high colony yield
- Low error rate of Q5 High-Fidelity DNA Polymerase reduces screening time
- Room temperature reaction setup
- Use of standard primers eliminates need for phosphorylated or purified oligos
- Easy-to-use master mix format with competent cells included



Mandarin Ducks *(Aix galericulata)* are frequently featured in Chinese art and regarded as a symbol of fidelity. The white mandarin's white plumage is caused by a sex-linked, recessive mutation, leading to a disorder in melanin deposition in the feathers, known as leucism.

Introducing NEBaseChanger™

NEBaseChanger™

NEBaseChanger can be used to design primers specific to the mutagenesis experiment you are performing, using the Q5 Site-Directed Mutagenesis Kit. This tool will also calculate a recommended custom annealing temperature based on the sequence of the primers by taking into account any mismatches.

To access this webtool, visit **NEBaseChanger.neb.com**

NEBNext Ultra DNA and RNA Library Prep Kits for Illumina
(Including Directional RNA)

The NEBNext Ultra DNA, Directional RNA and non-directional RNA Library Prep Kits for Illumina have been designed for fast performance and minimal input amounts. Each step has been optimized, resulting in streamlined workflows that robustly produce high-yield, high-diversity libraries with a wide range of input amounts. All Ultra kits are compatible with NEBNext Oligos (NEB #E7335, #E7500 and #E7350).

NEBNext Ultra DNA Library Prep Kit for Illumina

Ultra DNA Library Prep Workflow for 5 ng-1 µg Inputs

STEPS 5 ng–1 µg	Set Up Reaction	End Repair/ dA Tailing	Ligation	Clean Up, Size Select	PCR	Clean Up	~2.5–3 hrs.
HANDS-ON TIME	2 min.	0 min.	1 min.	5 min.	2 min.	5 min.	TOTAL 15 min.
TOTAL TIME	5 min.	60 min.	30 min.	30 min.	0–30 min.	25 min.	150–180 min.

NEBNext Ultra Directional RNA Library Prep Kit for Illumina

The NEBNext Ultra Directional RNA Library Prep Kit robustly produces highly strand-specific libraries through labeling of the second strand cDNA with dUTP, and subsequent excision. Input amounts as low as 10 ng of purified mRNA or rRNA-depleted RNA, or 100 ng Total RNA can be used.

For non-directional RNA libraries, the NEBNext Ultra RNA Kit provides robust performance with as little as 10 ng Total RNA, purified mRNA or rRNA-depleted RNA.

Ultra Directional RNA Library Prep Workflow

STEPS	mRNA Purification and Fragmentation	First Strand cDNA Synthesis	Second Strand cDNA Synthesis with dUTP and Clean Up	End Repair/ dA-Tailing	Adaptor Ligation and Size Selection	PCR Amplification and Clean Up
HANDS-ON TIME	10 min.	1 min.	6 min.	1 min.	6 min.	6 min.	TOTAL 30 min.
TOTAL TIME	35 min.	40 min.	75 min.	60 min.	30 min.	55 min.	4-5 hrs.

Advantages

- **Broad range of sample input amounts**
  - DNA: 5 ng-1 µg
  - RNA:
    - Total RNA: 10 ng-1 µg
    - Purified mRNA: 10 ng-100 ng
    - rRNA-depleted RNA: 10 ng-100 ng
- **Fast workflows with minimal hands-on time**
- **Simple protocols**
- **Minimized GC bias with ultra-high fidelity amplification**
- **Functional validation** – Each kit is functionally validated by preparation of a genomic DNA library followed by Illumina sequencing
- **Stringent quality controls** – Additional QCs ensure maximum quality and purity
- **Value pricing**

Ordering Information

PRODUCT	NEB #	SIZE
NEBNext Ultra DNA Library Prep Kit for Illumina	E7370S/L	24/96 rxns
NEBNext Ultra Directional RNA Library Prep Kit for Illumina	E7420S/L	24/96 rxns
NEBNext Ultra RNA Library Prep Kit for Illumina	E7530S/L	24/96 rxns

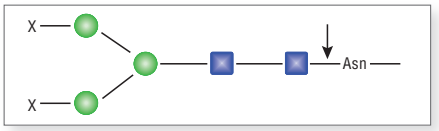
For information about other NEBNext products, visit **NEBNext.com**

Remove-iT Endoglycosidases Bring Convenience to Glycobiology

NEB's Remove-iT products are *N*-glycosidases that have a high specificity for their target residues, and are all tagged with a chitin binding domain (CBD), enabling rapid removal of the endoglycosidase from the reaction. They are supplied glycerol-free for optimal performance in HPLC- and MS-intensive methods. These targeted and time-resolved formulations of PNGase F, Endo D and Endo S are useful additions to your glycobiology investigations.

Remove-iT PNGase F

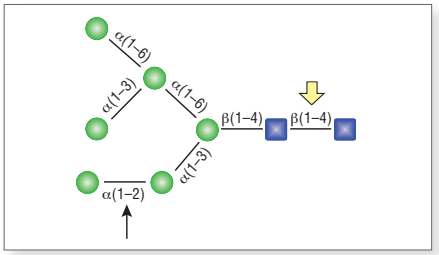
Remove-iT PNGase F is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins.



x = H or oligosaccharide

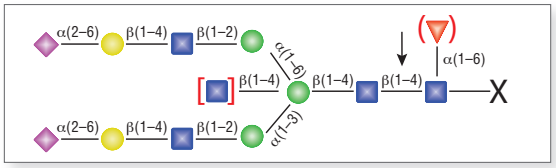
Remove-iT Endo D

Remove-iT Endo D, also known as Endoglycosidase D, is a recombinant glycosidase that cleaves within the chitobiose core of paucimannose *N*-linked glycans, with or without extensions in the antennae.



Remove-iT Endo S

Remove-iT Endo S is an endoglycosidase with a uniquely high specificity for removing *N*-linked glycans from the chitobiose core of the heavy chain of native IgG.



x = peptide, protein, Asn or free glycan

Advantages

Remove-iT PNGase F

- Removal of *N*-linked glycans from glycoproteins

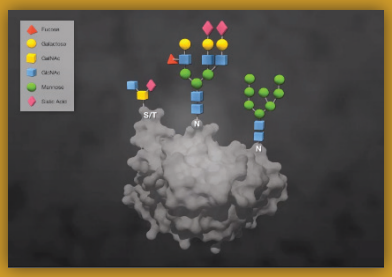
Remove-iT Endo D

- Removal of paucimannose *N*-linked glycans from glycoproteins and glycopeptides
- Useful for determining *N*-glycosylation sites

Remove-iT Endo S

- Removal of *N*-glycans from native IgG
- Useful for determining *N*-glycosylation sites

Find out more…



...at nebglycosidase.com

View a tutorial on *N*- and *O*-linked glycosylation.

Gal Man GlcNAc NeuAc
Glc GalNAc Fuc R = any sugar

Ordering Information

PRODUCT	NEB #	SIZE
Remove-iT PNGase F	P0706S/L	6,750/33,750 units
Remove-iT Endo D	P0742S/L	1,500/7,500 units
Remove-iT Endo S	P0741S/L	6,000/30,000 units
COMPANION PRODUCT		
RNase B	P7817S	250 µg
Chitin Magnetic Beads	E8036S/L	5/25 ml
6-Tube Magnetic Separation Rack	S1506S	6-tube rack

For information about other glycobiology products, visit **NEBGlycosidase.com**

cDNA Synthesis Selection Chart

NEB offers several reagents for cDNA Synthesis for use in applications, including qPCR and qRT-PCR. For your convenience, reagents are available as kits or standalone products, depending on your needs. ProtoScript II Reverse Transcriptase, which is available as a standalone enzyme or in kit format, offers similar performance to SuperScript® II Reverse Transcriptase at a lower price.

cDNA SYNTHESIS	FEATURES	SIZE
Kit		
ProtoScript II First Strand cDNA Synthesis Kit (#E6560S/L)	Convenient 2-tube kit Contains ProtoScript II Reverse Transcriptase Increased thermostability as compared to M-MuLV Reduced RNase H activity Generates cDNA at least 10 kb in length Includes dNTPs, Oligo-dT primer and Random Primer Mix	30/150 rxns
ProtoScript First Strand cDNA Synthesis Kit (#E6300S/L) Formerly ProtoScript M-MuLV First Strand cDNA Synthesis Kit	Convenient 2-tube kit Contains M-MuLV Reverse Transcriptase Generates cDNA at least 5 kb in length Includes dNTPs, Oligo-dT primer and Random Primer Mix	30/150 rxns
AMV First Strand cDNA Synthesis Kit (#E6550S) Formerly ProtoScript AMV First Strand cDNA Synthesis Kit	Convenient 2-tube kit Enables cDNA synthesis form difficult templates requiring higher reaction temps. Generates cDNA at least 10 kb in length Includes dNTPs, Oligo-dT primer and Random Primer Mix	30 rxns
Standalone		
ProtoScript II Reverse Transcriptase (#M0368S/L/X) Formerly M-MuLV Reverse Transcriptase (RNase H$^-$)	RNase H$^-$ mutant of M-MuLV Reverse Transcriptase Increased thermostability Reduced RNase H activity Increased reaction temperatures 37–50°C	4,000/10,000/ 40,000 units
M-MuLV Reverse Transcriptase (#M0253S/L)	Wild-type M-MuLV Reverse Transcriptase Robust reverse transcriptase for a variety of templates Standard reaction temperatures 37–45°C	10,000/ 50,000 units
AMV Reverse Transcriptase (#M0277S/L/T)	Robust reverse transcriptase for a broad temperature range 37–52°C Can be used for templates requiring higher reaction temperatures	200/1,000/ 500 units

More About NEB's Restriction Enzymes

FAQ Spotlight

Q: How is NEB's new buffer system going to help me?

A: *Although the old buffer system worked well, NEB is continuously looking for ways to enhance the convenience and performance of its products for our customers. By adding BSA to the reaction buffer, we were able to offer even more enzymes that cut in a single buffer (> 200). This improves ease of use, especially when performing double digests. In addition, it eliminated the need to add BSA when setting up restriction enzyme digests.*

Q: If I have an old tube of Restriction Enzyme, what NEBuffer should I use?

A: *All NEB Restriction Enzymes have color coded labels for the appropriate NEBuffer; this system can either be used with the previously supplied NEBuffer or with the newly recommended buffer.*

Q: I currently have an old tube of Restriction Enzyme - is it still active in the new buffer?

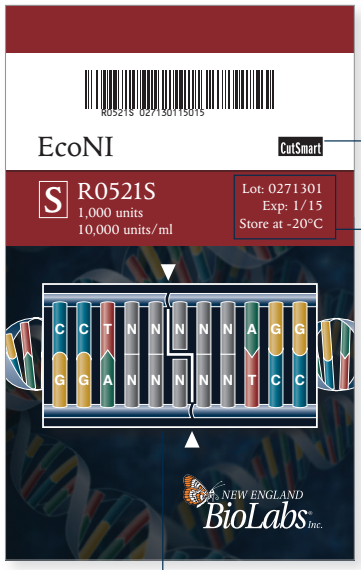
A: *Provided it is still prior to the product's expiry date, yes. The new buffers are mostly identical, except that BSA has been added directly to the buffer and DTT has been removed. BSA will not harm the reaction and may even enhance it in some cases. Extensive testing has shown that DTT was not required.*

Q: The product page on www.neb.com states that my restriction enzyme comes with the new buffer, but when it arrived, it came with the old buffer. Why?

A: *We are currently in the process of transitioning all of our restriction enzymes between the old and new buffer systems. The product pages on neb.com were updated to include the new buffer information. Replacement of product inventory containing the new buffers will happen gradually over the next few months. We are working as quickly as we can to replace stock, but there may be a short period where you may receive an enzyme pack that contains the old buffer. If you would like to receive new buffer, please contact info@neb.com. Information regarding performance in the old buffer system is still available at www.neb.com.*

A Smarter Look

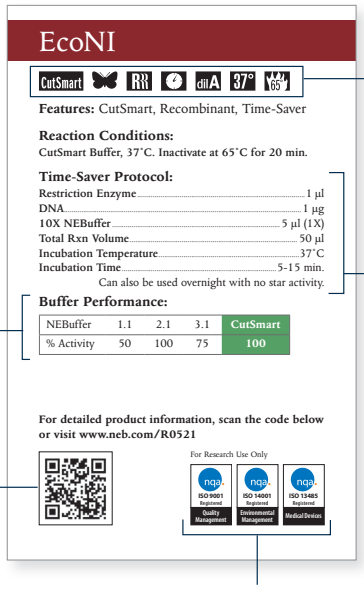
Keep track of your enzyme data with our new, streamlined protocol cards. These collectible cards contain all the information you need for setting up a restriction enzyme digest, and use less paper than a traditional data card. Full product information can always be found by scanning the QR code on the card, or by visiting the indicated product page at neb.com.



If the enzyme is supplied with CutSmart Buffer, it will be noted here.

Lot-specific information, including expiration and storage data.

An expanded cut site diagram gives you information about the enzyme's recognition sequence and restriction site.



At a glance, you'll find icons that detail each enzyme's pertinent properties, including supplied buffer, reaction temperature, heat inactivation temperature and whether the enzyme is Time-Saver qualified or sensitive to methylation.

Here, you'll find the recommended protocol for DNA digestion with this enzyme.

The buffer performance chart lists an enzyme's percent activity in each NEBuffer. The recommended buffer is highlighted.

This unique QR code will bring you directly to detailed information on the enzyme at neb.com.

All of NEB's products, including our restriction enzymes, are manufactured under the strictest of standards, earning ISO registration.

Know Your Restriction Enzyme Icons

Restriction enzyme properties can be easily identified using NEB icons. These icons appear on the trading cards accompanying product, in the NEB catalog and on our website.

The gene encoding this enzyme was cloned at NEB.

RR

This enzyme is purified from a recombinant source.

e

This enzyme has been engineered for maximum performance.

Time-Saver qualified enzymes will digest 1 µg of substrate DNA in 5–15 minutes using 1 µl of enzyme under recommended reaction conditions. These enzymes can also be used overnight with no loss of sample.

Mix

This enzyme is available in RE-Mix Master Mix format.

NEB 1.1 NEB 2.1 NEB 3.1 CutSmart NEB U

Indicates which reaction buffer is supplied with the enzyme for optimal activity. Enzymes with buffer requirements not met by one of the four standard NEBuffers (1.1, 2.1, 3.1 or CutSmart) are supplied with their own unique NEBuffer (NEB U). NEBuffers are color-coded (NEB 1.1-yellow, NEB 2.1-blue, NEB 3.1-red, CutSmart-green) and supplied as 10X stocks with each enzyme.

Epi

This enzyme is EpiMark® validated for epigenetics studies.

SAM

This enzyme is supplied with a separate tube of S-adenosylmethionine (SAM). To obtain 100% activity, SAM should be added to the 1X reaction mix as indicated. When required, a concentrated stock of SAM is supplied with the enzyme.

dam dcm CpG

This restriction enzyme is sensitive to dam, dcm, or CpG methylation. (Note that CpG methylation is applicable to eukaryotic genomic DNA only.)

65° 80° No

Indicates whether or not the enzyme can be heat inactivated. Enzymes are first tested by incubation at 65°C for 20 minutes; any enzyme not inactivated at 65°C is then tested by incubation at 80°C for 20 minutes. If an enzyme can be heat inactivated, the temperature is indicated in the icon.

25° 37° 50° 55° 60° 65° 75°

Indicates the enzyme's optimal incubation temperature.

dilA dilB dilC

Indicates which diluent buffer (A, B or C) is recommended for making dilutions of restriction enzymes.

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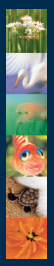

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China, People's Republic
New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
info@neb-china.com

France
New England Biolabs France
Telephone : 0800 100 632
info.fr@neb.com

Germany & Austria
New England Biolabs GmbH
Free Call 0800/246 5227 (Germany)
Free Call 00800/246 52277 (Austria)
info.de@neb.com

Japan
New England Biolabs Japan, Inc.
Telephone: +81 (0)3 5669 6191
info@neb-japan.com

Singapore
New England Biolabs Pte. Ltd.
Telephone +65 6776 0903
sales.sg@neb.com

United Kingdom
New England Biolabs (UK), Ltd.
Call Free 0800 318486
info.uk@neb.com