Includes the latest products
and tools for DNA Assembly

Molecular Cloning

TECHNICAL GUIDE

NEW ENGLAND BioLabs®

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Molecular Cloning Overview

Molecular cloning refers to the process by which recombinant DNA molecules are produced and transformed into a host organism, where they are replicated. A molecular cloning reaction is usually comprised of two components:

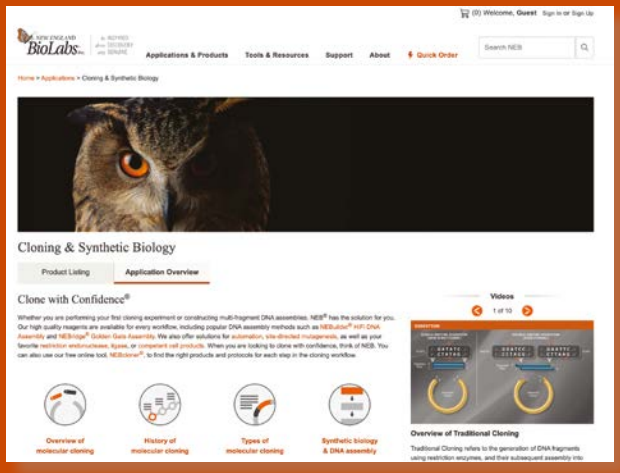
1. The DNA fragment of interest to be replicated.
2. A vector/plasmid backbone that contains all the components for replication in the host.

DNA of interest, such as a gene, regulatory element(s), operon, etc., is prepared for cloning by either excising it out of the source DNA using restriction enzymes, copying it using PCR, or assembling it from individual oligonucleotides. At the same time, a plasmid vector is prepared in a linear form using restriction enzymes (REs) or Polymerase Chain Reaction (PCR). The plasmid is a small, circular piece of DNA that is replicated within the host and exists separately from the host's chromosomal or genomic DNA. By physically joining the DNA of interest to the plasmid vector through phosphodiester bonds, the DNA of interest becomes part of the new recombinant plasmid and is replicated by the host. Plasmid vectors allow the DNA of interest to be copied easily in large amounts, and often provide the necessary control elements to be used to direct transcription and translation of the cloned DNA. As such, they have become the workhorse for many molecular methods such as protein expression, gene expression studies, and functional analysis of biomolecules.

During the cloning process, the ends of the DNA of interest and the vector have to be modified to make them compatible for joining through the action of a DNA ligase, recombinase, or an *in vivo* DNA repair mechanism. These steps typically utilize enzymes such as nucleases, phosphatases, kinases and/or ligases. Many cloning methodologies and, more recently kits have been developed to simplify and standardize these processes.

This technical guide will clarify the differences between the various cloning methods, identify NEB® products available for each method, and provide expert-tested protocols and FAQs to help you troubleshoot your experiments and **Clone with Confidence®**.

Visit CloneWithNEB.com



- Technical tips and FAQs
- Videos and animations
- Much more...

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Online Tools for Cloning

Competitor Cross-Reference Tool

Use this tool to select another company's competent cell product and find out which NEB strain is compatible. Choose either the product name or catalog number from the available selection, and this tool will identify the recommended NEB product and its advantages. A link to the product page where you can also order the product is provided.

DNA Sequences and Maps Tool

With the DNA Sequences and Maps Tool, find the nucleotide sequence files for commonly used molecular biology tools, including plasmid, viral and bacteriophage vectors.

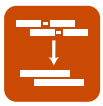
Double Digest Finder

Use this tool to guide your reaction buffer selection when setting up double-digests, a common timesaving procedure. Choosing the right buffers will help you to avoid star activity and loss of product.

Enzyme Finder

Use this tool to select restriction enzymes by name, sequence, overhang or type. Enter your sequence using single letter code, and Enzyme Finder will identify the right enzyme for the job.

NEBridge® Ligase Fidelity Tools



Visualize overhang ligation preferences, predict high-fidelity junction sets, and split DNA sequences to facilitate the design of high-fidelity Golden Gate assemblies.

NEBridge® Golden Gate Assembly Tool

Use this tool to assist with in silico DNA construct design for Golden Gate DNA assembly. It enables the accurate design of primers with appropriate Type IIS restriction sites and overlaps, quick import of sequences in many formats and export of the final assembly, primers and settings. The latest version (v2.1) also incorporates ligase fidelity information.

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. The latest version enables multi-site mutagenesis using NEBuilder HiFi.

NEBcloner®

Use this tool to find the right products and protocols for each step (digestion, end modification, ligation and transformation) of your next traditional cloning experiment. Also, find other relevant tools and resources to enable protocol optimization.

NEBcutter® V3.0

Identify restriction sites within your DNA sequence using NEBcutter. Choose between Type II and commercially available Type III enzymes to digest your DNA. NEBcutter V3.0 indicates cut frequency and methylation sensitivity.

NEBioCalculator®

NEBioCalculator is a collection of calculators and converters that are useful in planning bench experiments in molecular biology laboratories.

NEBuilder® Assembly Tool

NEBuilder Assembly Tool can be used to design primers for your NEBuilder HiFi and Gibson Assembly reaction, based on the entered fragment sequences and the polymerase being used for amplification.

PCR Selector

Use this tool to help select the right DNA polymerase for your PCR setup. Whether your amplicon is long, complex, GC-rich or present in a single copy, the PCR selection tool will identify the perfect DNA polymerase for your reaction.

REBASE®

Use this tool as a guide to the ever-changing landscape of restriction enzymes. REBASE, the Restriction Enzyme DataBASE, is a dynamic, curated database of restriction enzymes and related proteins.

Tm Calculator

Use this tool when designing PCR reaction protocols to help determine the optimal annealing temperature for your amplicon. Simply input your DNA polymerase, primer concentration and your primer sequence and the Tm Calculator will guide you to successful reaction conditions.

MOBILE APPS

NEB Tools for iPhone®, iPad® or Android™

NEB Tools brings New England Biolabs' most popular web tools to your iPhone, iPad or Android devices.

- Use Enzyme Finder to select a restriction enzyme by category or recognition sequence, or search by name to find information on any NEB enzyme. Sort your results so they make sense to you, then email them to your inbox or connect directly to **www.neb.com**.
- Use Double Digest Finder or NEBcloner to determine buffer and reaction conditions for experiments requiring two restriction enzymes.

When using either of these tools, look for rCutSmart™, HF® and Time-Saver™ enzymes for the ultimate in convenience. NEB Tools enables quick and easy access to the most requested restriction enzyme information, and allows you to plan your experiments from anywhere.

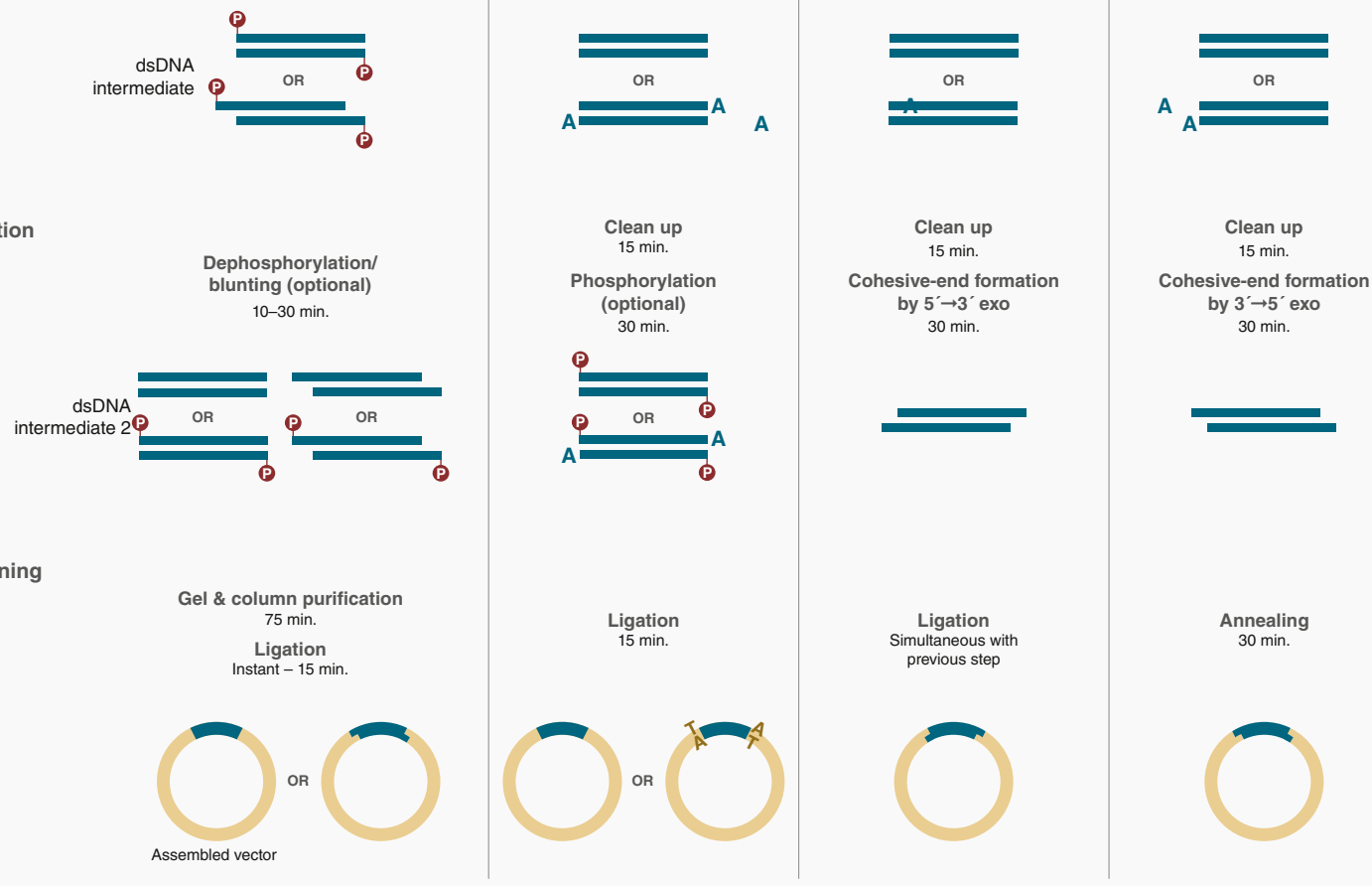
Cloning Workflow Comparison

The figure below compares the steps for the various cloning methodologies, along with the time needed for each step in the workflows.

INSERT PREPARATION

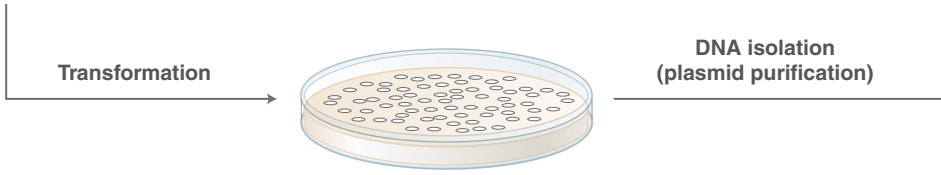
Starting materials

	Traditional Cloning (RE Digestion & Ligation)	PCR Cloning (TA & Blunt-End)	Seamless Cloning (Gene Assembly)	Ligation Independent Cloning (LIC)
DNA preparation	**RE digestion** 60 min. (standard) 5–15 min. (Time-Saver)	**PCR** 90 min.	**PCR** 90 min.	**PCR** 90 min.
DNA end modification	**Dephosphorylation/ blunting (optional)** 10–30 min.	**Clean up** 15 min. **Phosphorylation (optional)** 30 min.	**Clean up** 15 min. **Cohesive-end formation by 5´→3´ exo** 30 min.	**Clean up** 15 min. **Cohesive-end formation by 3´→5´ exo** 30 min.
Vector & insert joining	**Gel & column purification** 75 min. **Ligation** Instant – 15 min.	**Ligation** 15 min.	**Ligation** Simultaneous with previous step	**Annealing** 30 min.
Estimated total time*	1 hr., 20 min. – 3 hr.	2 hr. – 2 hr., 30 min.	2 hr., 15 min.	2 hr., 45 min.



* Note that times are based on estimates for moving a gene from one plasmid to another. If the source for gene transfer is gDNA, add 2 hours to calculation for the traditional cloning method. Total time does not include transformation, isolation or analysis.

** 70 minutes for recombination occurs on second day



Recombinational (Gateway/Creator/Univector)

PCR
90 min.

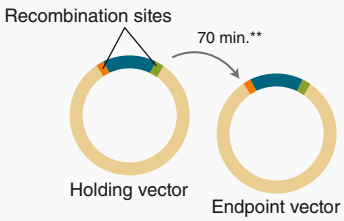
Clean up
15 min.

RE digestion
60 min. (standard)
5–15 min. (Time-Saver)

Clean up
15 min.

Site-specific recombination
60 min.

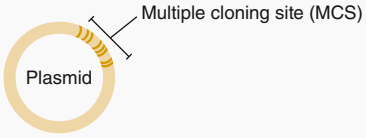
Proteinase K treatment
10 min.



3 hr., 15 min. – 5 hr., 20 min.

VECTOR PREPARATION

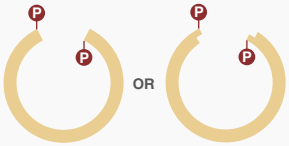
Starting material



Restriction Enzyme (RE) Digestion

DNA preparation

RE digestion
60 min. (standard)
5–15 min. (Time-Saver)



Linear vector

DNA end modification

Dephosphorylation (optional)
10–30 min.

Clean up
15 min.
OR
Gel & column purification
75 min.

Linear vector, ready for joining

PCR

PCR
2 hr.

Clean up
15 min.

T-addition (optional)
1.5 hr.

Estimated total time

20 min. – 2 hr., 25 min.

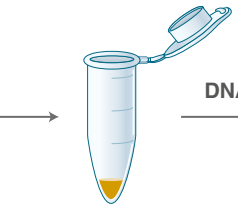
2 hr., 15 min – 3 hr., 45 min.

SELECTION CHARTS & PROTOCOLS

Need help with locating product selection charts & protocols?

NEBcloner®

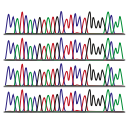
For help with choosing the right product for each step in the cloning workflow, visit **NEBcloner.neb.com**



DNA analysis

RE digest

Colony PCR



Sequencing

Protein expression

Functional analysis

Site-directed mutagenesis

DNA Assembly

DNA assembly refers to a molecular cloning method that physically links together multiple fragments of DNA, in an end-to-end fashion, to acheive a designed, higher-order assembly prior to joining to a vector. This process is the cornerstone of the synthetic biology field and allows the construction of novel biological systems and devices using defined components. These techniques are carried out *in vitro* and are typically enzymatically driven with the final constructions being maintained in microbial host cells.

To help select the best DNA assembly method for your needs, please refer to our Synthetic Biology/DNA Assembly Selection Chart below.

Synthetic Biology/DNA Assembly Selection Chart

	NEBuilder HiFi DNA Assembly NEB #E2621 NEB #E5520 NEB #E2623	**NEB Gibson Assembly** NEB #E2611 NEB #E5510	**NEBridge Golden Gate Assembly Kits (BsaI-HFv2/BsmBI-v2)** NEB #E1601 NEB #E1602 **NEBridge Ligase Master Mix** NEB #M1100	**USER Enzyme** NEB #M5505 **Thermolabile USER II Enzyme** NEB #M5508
Properties				
Removes 5´ or 3´ End Mismatches	★★★	★	N/A	N/A
Assembles with High Fidelity at Junctions	★★★	★★	★★★	★★★
Tolerates Repetitive Sequences at Ends	★	★	★★★	★★★
Generates Fully Ligated Product	★★★	★★★	★★★	NR
Joins dsDNA with Single-stranded Oligo	★★★	★★	NR	NR
Assembles Low Amounts of DNA with High Efficiency	★★★	★★	★★	★★
Accommodates Flexible Overlap Lengths	★★★	★★★	★	★★
Applications				
2 Fragment Assembly (Simple cloning)	★★★	★★★	★★★	★★★
3-6 Fragment Assembly (one pot)	★★★	★★★	★★★	★★★
7-11 Fragment Assembly (one pot)	★★★	★★	★★★	★★★
12-50+ Fragment Assembly (one pot) [1]	★	★	★★★	NR
Template Construction for *In vitro* Transcription	★★★	★★★	★★★	★★★
Synthetic Whole Genome Assembly	★★★	★	★★★	★
Multiple Site-directed Mutagenesis	★★★	★★	★★	★★
Library Generation	★★★	★★★	★★★	★★
Metabolic Pathway Engineering	★★★	★★	★★★	★★★
TALENs	★★	★★	★★★	★★
Short Hairpin RNA (shRNA) Cloning	★★★	★★	★	★
gRNA Library Generation	★★★	★★	★	★
Large Fragment (> 10 kb) Assembly	★★★	★★★	★★★	★★
Small Fragment (< 100 bp) Assembly	★★★	★	★★★	★★★
Use in Successive Rounds of Restriction Enzyme Assembly	★★★	★	NR	★

[1] Please visit **neb.com/GoldenGate** for more information

KEY	
★★★	Optimal, recommended product for selected application
★★	Works well for selected application
★	Will perform selected application, but is not recommended
N/A	Not applicable to this application
NR	Not recommended

NEBridge Golden Gate Assembly

The efficient and seamless assembly of DNA fragments, commonly referred to as Golden Gate assembly (1,2) refers to multiple inserts being assembled into a vector backbone using only the sequential (3) or simultaneous (4) activities of a single Type IIS restriction enzyme and T4 DNA Ligase. Golden Gate has enabled single inserts, the cloning of inserts from diverse populations enabling library creation, and multi-module assemblies. NEB has made extraordinary improvements that touch every application of the Golden Gate technology.

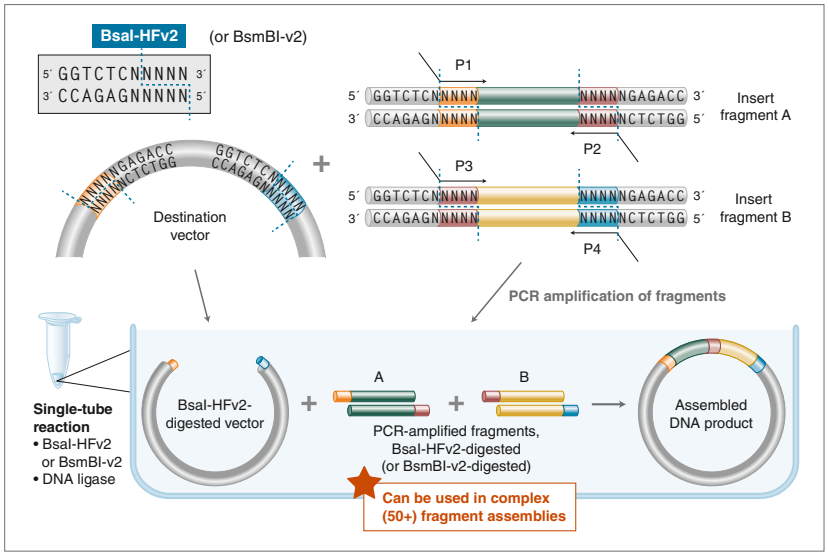
Advances in Ligase Fidelity

Research at NEB has led to increased understanding of ligase fidelity, including the development of a comprehensive method for profiling end-joining ligation fidelity in order to predict which overhangs have improved fidelity (5). This research allows careful choice of overhang sets, which is especially important for achieving complex Golden Gate Assemblies.

Type IIS Restriction Enzymes for Golden Gate Assembly

NEB offers more Type IIS (i.e., recognize asymmetric DNA sequences and cleave outside of their recognition sequence) restriction enzymes than any other supplier, many of which are used in Golden Gate Assembly. These enzymes, along with the ligase fidelity data, allows complex **50+ fragment assemblies** with high efficiency, > 90% accuracy and low backgrounds.

Golden Gate Assembly Workflow for complex assemblies



In its simplest form, Golden Gate Assembly requires a Type IIS recognition site, in this case, BsaI-HFv2 (GGTCTC), or BsmBI-v2 (CGTCTC) added to both ends of a dsDNA fragment. After digestion, these sites are left behind, with each fragment bearing the designed 4-base overhangs that direct the assembly.

RECOMMENDED PRODUCTS

NEBridge Golden Gate Assembly Kits (BsaI-HFv2 or BsmBI-v2) (NEB #E1601, NEB #E1602)

- Seamless cloning – no scar remains following assembly
- Includes destination plasmid with T7/SP6 promoters
- Ordered assembly of multiple fragments (2-50+) in a single reaction
- Can also be used for cloning of single inserts and library preparations
- Efficient with regions with high GC content and areas of repeats
- Compatible with a broad range of fragment sizes (< 100 bp to > 15 kb)

NEBridge Ligase Master Mix (NEB #M1100)

- Optimized for efficient and accurate Golden Gate Assembly
- Convenient 3X Master Mix format
- Use with NEB Type IIS restriction enzymes

Type IIS Enzymes used in Golden Gate

- **BbsI (NEB #R0539)**
- **BbsI-HF (NEB #R3539)**
- **BsaI-HFv2 (NEB #R3733)**
- **BsmBI-v2 (NEB #R0739)**
- **BspQI (NEB #R0712)**
- **BtgZI (NEB #R0703)**
- **Esp3I (NEB #R0734)**
- **PaqCI (NEB #R0745)**
- **SapI (NEB #R0569)**

TOOLS & RESOURCES

Visit www.neb.com/GoldenGate to find:

- Publications and protocols related to ligase fidelity and Golden Gate Assembly
- Access to **NEBridge Golden Gate Assembly Tool**, for help with designing your experiment at **GoldenGate.neb.com**
- Access to the **NEBridge Ligase Fidelity Tools** to facilitate the design of high-fidelity Golden Gate Assemblies
- View our webinar: Listen to DAD when constructing high-complexity Golden Gate Assembly targets
- View our *MoClo Overhang Standards Usage Guidelines* and our tutorial, *Domestication and Golden Gate Assembly*

References:
1. Engler, C. et al. (2008) *PLoS ONE*, 3: e3647.
2. Engler, C. et al. (2009) *PLoS ONE*, 4: e5553.
3. Lee, J.H. et al. (1996) *Genetic Analysis: Biomolecular Engineering*, 13; 139-145.
4. Padgett, K.A. and Sorge, J.A. (1996) *Gene*, 168, 31-35.
5. Potapov, V. et. al. (2018) *ACS Synth. Biol.* DOI: 10.1021/acssynbio.8b00333.

DOWNLOAD THE NEB AR APP*

How does Golden Gate Assembly work?

*see back cover for details

Technical Tips for Optimizing Golden Gate Assembly Reactions

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

Always check your assembly sequences for internal sites before choosing which Type IIS restriction endonuclease to use for your assembly. While single insert Golden Gate assembly has such high efficiencies of assembly that the desired product is obtainable regardless of the presence of an internal site, this is not true for assemblies with multiple inserts. Options include choosing a different Type IIS restriction enzyme to direct your assembly, or eliminating internal sites through domestication. Our tutorial video on Golden Gate Assembly Domestication provides a full description of the many options available for internal site issues. Note the use of a Type IIS restriction enzyme with a 7 base recognition site, such as PaqCI, is less likely to have internal sites present in any given sequence.

2. Orient your primers

When designing PCR primers to introduce Type IIS restriction enzyme sites, either for amplicon insert assembly or as an intermediate for pre-cloning the insert, remember that the recognition sites should always face inwards towards your DNA to be assembled. Consult the Golden Gate Assembly Kit manuals or videos for further information regarding the placement and orientation of the sites.

3. Choose the right plasmid

Consider switching to the pGGAselect Destination Plasmid for your Golden Gate assembly. This versatile new destination construct is included in all Golden Gate Assembly kits and can be used for BsaI-HFv2, BsmBI-v2 or BbsI directed assemblies. It also features T7 and SP6 promoter sequences flanking the assembly site, and has no internal BsaI, BsmBI or BbsI sites. The pGGAselect plasmid can also be transformed into any *E. coli* strain compatible with pUC19 for producing your own plasmid preparation if so desired.

4. Choose the right buffer

T4 DNA Ligase Buffer works best for Golden Gate Assembly with BsaI-HFv2, BsmBI-v2 and PaqCI. However, alternate buffers would be NEBuffer r1.1 for Bsa-HFv2, NEBuffer r2.1 for BsmBI-v2, or rCutSmart™ Buffer for PaqCI, if these buffers are supplemented with 1 mM ATP and 5–10 mM DTT. NEB also offers NEBridge Ligase Master Mix that has been optimized for Golden Gate Assembly with our Type IIS restriction enzymes for Golden Gate.

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

T4 DNA Ligase, BsaI-HFv2, BsmBI-v2 and PaqCI are very stable and continue to be active during extended cycling protocols; an easy way to increase assembly efficiencies without sacrificing fidelity is to increase the total cycles from 30 to 45–65, even when using long (5-minute) segments for the temperature steps.

6. Make sure your plasmid prep is RNA-free

For pre-cloned inserts/modules, make sure your plasmid prep is free of RNA to avoid an overestimation of your plasmid concentrations.

7. Avoid primer dimers

For amplicon inserts/modules, make sure your PCR amplicon is a specific product and contains no primer dimers. Primer dimers, with Type IIS restriction endonuclease sites (introduced in the primers used for the PCR reactions), would be active in the assembly reaction and result in mis-assemblies.

8. Avoid PCR-induced errors

Do not over-cycle and use a proofreading high fidelity DNA polymerase, such as Q5® DNA High-Fidelity Polymerase.

9. Decrease insert amount for complex assemblies

For complex assemblies involving >10 fragments, pre-cloned insert/modules levels can be decreased from 75 to 50 ng each without significantly decreasing the efficiencies of assembly.

10. Carefully design EVERY insert's overhang

An assembly is only as good as its weakest junction. Research at NEB has led to an increased understanding of ligase fidelity, including the development of a comprehensive method for profiling end-joining ligation fidelity in order to predict which overhangs will result in improved accuracy. This ligase fidelity information can be paired with the appropriate Type IIS restriction enzyme chosen to direct your assembly to achieve high efficiencies and accurate complex assemblies. Please use the free NEBridge Golden Gate Assembly Tool to design primers for your Golden Gate Assembly reactions. Predict overhang fidelity or find optimal Golden Gate junctions for long sequences, refer to the NEBridge Ligase Fidelity Tools.

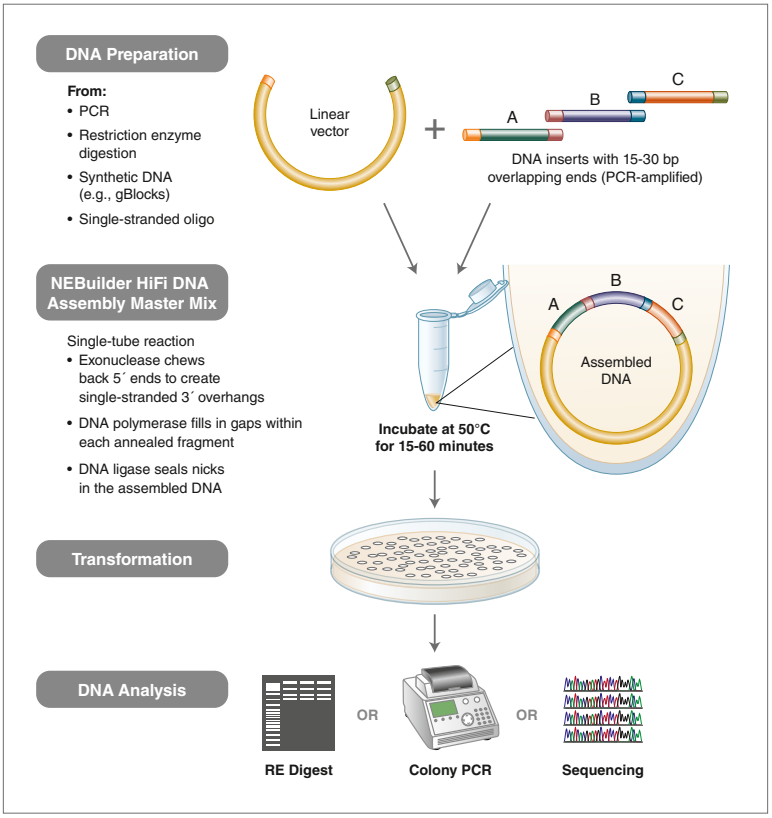
11. If using pre-cloned proven inserts that suddenly become problematic, check for a possible mutational event in your sequence.

Be aware that occasionally a pre-cloned insert/module can become corrupted by an error during propagation in *E. coli*, usually a frameshift due to slippage in a run of a single base (e.g., AAAA) by the *E. coli* DNA Polymerase. This should be suspected if previously functional assembly components suddenly become nonfunctional.

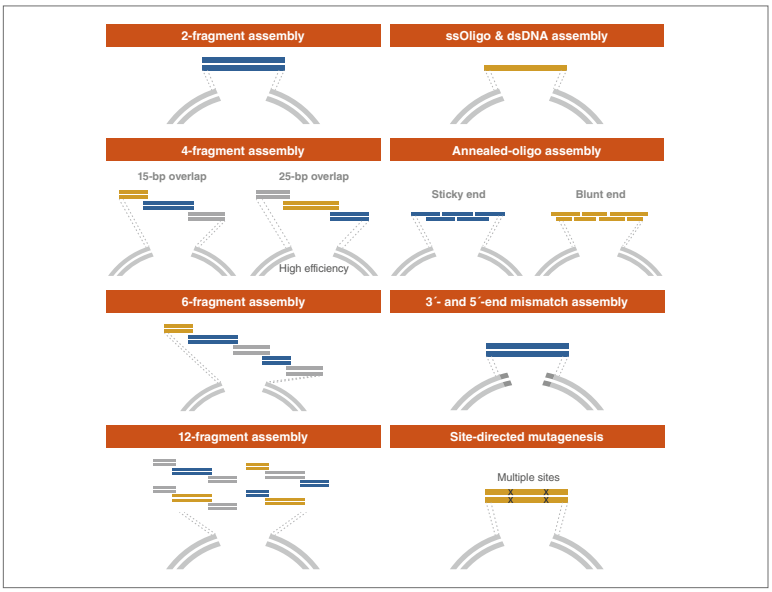
NEBuilder HiFi DNA Assembly

NEBuilder HiFi DNA Assembly enables virtually error-free joining of DNA fragments, even those with 5´- and 3´-end mismatches. Available with and without competent *E. coli*, this flexible kit enables simple and fast seamless cloning utilizing a new proprietary high-fidelity polymerase. Make NEBuilder HiFi your first choice for DNA assembly and cloning.

Overview of the NEBuilder HiFi DNA Assembly cloning method



NEBuilder HiFi DNA Assembly can be used for a variety of DNA assembly methods.



RECOMMENDED PRODUCTS

NEBuilder HiFi DNA Assembly Cloning Kit (NEB #E5520)

NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621)

NEBuilder HiFi DNA Assembly Bundle for Large Fragments (NEB #E2623)

- Simple and fast seamless cloning in as little as 15 minutes
- Use one system for both "standard-size" cloning and larger gene assembly products (up to 12 fragments and 20+ kb)
- DNA can be used immediately for transformation or as template for PCR or RCA
- Adapts easily for multiple DNA manipulations, including site-directed mutagenesis
- Enjoy less screening/re-sequencing of constructs, with virtually error-free, high-fidelity assembly
- Use NEBuilder HiFi in successive rounds of assembly, as it removes 5´- and 3´-end mismatches
- Bridge two ds-fragments with a synthetic ssDNA oligo for simple and fast construction (e.g., linker insertion or gRNA library)
- No PCR cleanup step required
- No licensing fee requirements from NEB for NEBuilder products
- NEBuilder HiFi DNA Assembly Cloning Kit includes the NEBuilder HiFi DNA Assembly Master Mix and NEB 5-alpha Competent *E. coli*
- NEBuilder HiFi DNA Assembly Bundle for Large Fragments includes the NEBuilder HiFi DNA Assembly Master Mix and NEB 10-beta Competent *E. coli* for assemblies larger than 15 kb

TOOLS & RESOURCES

Visit NEBuilderHiFi.com to find:

- Online tutorials to help with assembly and primer design
- Application notes utilizing NEBuilder HiFi
- Access to **NEBuilder Assembly Tool**, our online primer design tool
- Comparison to In-Fusion® Snap Assembly and GeneArt Gibson Assembly®

Optimization Tips for NEBuilder HiFi DNA Assembly

Assembly Reaction

- PCR product purification is not necessary if the total volume of all PCR products is 20% or less of the assembly reaction volume. Higher volumes of unpurified PCR products may reduce the efficiency, so column purification of PCR products is highly recommended when performing assemblies of three or more PCR fragments or assembling longer fragments > 5 kb.
- Carefully follow guidelines as indicated in the protocol regarding total amount of DNA and ratios of insert:vector.
- Vary overlap regions anywhere between 15–30 bp depending on the number of fragments being assembled.

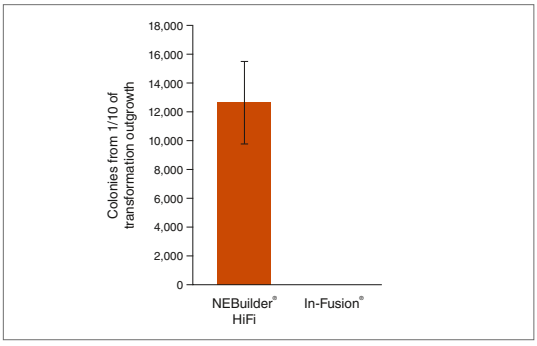
Primer Design

- For help with primer design, we recommend using NEBuilder Assembly Tool at **nebuilder.neb.com**.

Transformation

- The NEBuilder HiFi DNA Assembly Cloning Kit (NEB #E5520) includes NEB 5-alpha Competent *E. coli*. NEB recommends using the competent cells provided with the kit (NEB #C2987) because of their high efficiency. The components of the master mix may inhibit the functionality of competent cells from other companies if not diluted. The NEBuilder HiFi DNA Assembly Bundle for Large Fragments (NEB #E2623) includes NEB 10-beta Competent *E. coli* (NEB #C3019), ideal for assembling larger fragments (> 15 kb).

NEBuilder HiFi DNA Assembly Cloning Kit can assemble a ssDNA oligo with a linearized vector.



One pmol of a ssDNA oligo was assembled with a linearized vector (20 ng, CRISPR Nuclease OFP Reporter) by incubation at 50°C for 15 min. 2 µl of the assembled mix was transformed into to NEB 10-beta Competent E. coli (NEB #C3019). 9 out of 10 colonies selected show correct sequence, while no successful assembled constructs are found using In-Fusion HD.

Gibson Assembly and the Gibson Assembly Cloning Kit

Gibson Assembly enables multiple, overlapping DNA fragments to be joined in a single-tube isothermal reaction, with no additional sequence added (scar-less). The Gibson Assembly Master Mix includes three different enzymatic activities that perform in a single buffer. The assembled, fully-sealed construct is then transformed into NEB 5-alpha competent *E. coli*. The entire protocol, from assembly to transformation, takes just under two hours. Visit **NEBGibson.com** to learn more.

Protocol: Assembly

Before use, thaw and vortex the master mix thoroughly and keep on ice.

1. Set up the following reaction on ice.

	RECOMMENDED AMOUNT OF FRAGMENTS USED FOR ASSEMBLY		
	2–3 Fragment Assembly*	4–6 Fragment Assembly**	NEBuilder Positive Control***
Recommended DNA Molar Ratio	vector:insert= 1:2	vector:insert= 1:1	
Total Amount of Fragments	0.03-0.2 pmol* X µl	0.2-0.5 pmol** X µl	10 µl
NEBuilder HiFi DNA Assembly Master Mix	10 µl	10 µl	10 µl
Deionized H_2O	10–X µl	10–X µl	0
Total Volume	20 µl****	20 µl****	20 µl

* Optimized cloning efficiency is 50–100 ng of vector with 2-fold molar excess of each insert. Use 5-fold molar excess of any insert(s) less than 200 bp. Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%. To achieve optimal assembly efficiency, design 15-20 bp overlap regions between each fragment.

** To achieve optimal assembly efficiency, design 20-30 bp overlap regions between each fragment with equimolarity of all fragments (suggested: 0.05 pmol each).

*** Control reagents are provided for 5 experiments.

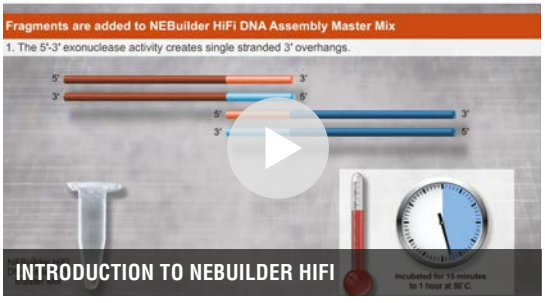
**** If greater numbers of fragments are assembled, increase the volume of the reaction, and use additional NEBuilder HiFi DNA Assembly Master Mix.

2. Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes (when 4–6 fragments are being assembled). Following incubation, store samples on ice or at –20°C for subsequent transformation.

Note: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases (for further details see FAQ section of product pages).

Protocol: Transformation with NEB 5-alpha cells

	STANDARD PROTOCOL
DNA	2 µl
Competent *E. coli*	50 µl
Incubation	On ice for 30 minutes
Heat Shock	Exactly 42°C for exactly 30 seconds
Incubation	On ice for 5 minutes Add 950 µl room temperature SOC 37°C for 60 minutes, with shaking



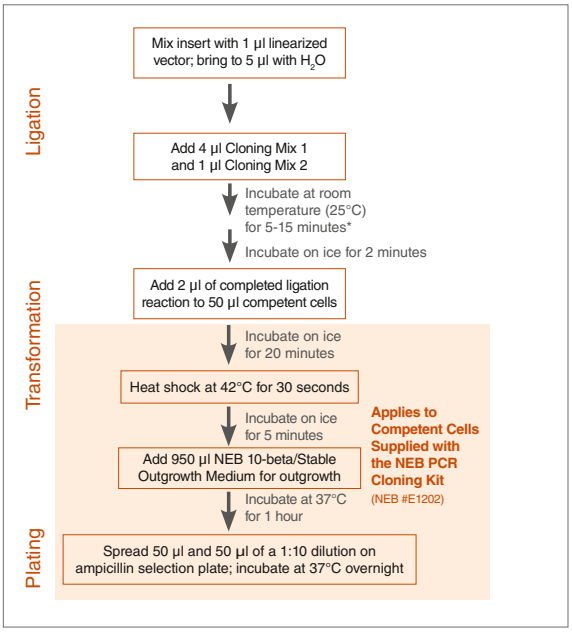
How does NEBuilder HiFi DNA Assembly work?

Cloning & Mutagenesis Kits

NEB PCR Cloning Kit

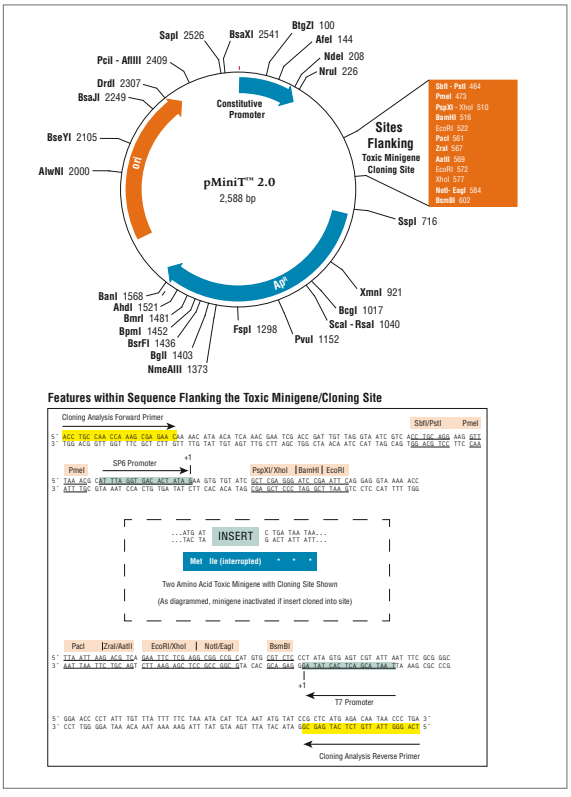
The NEB PCR Cloning Kit enables quick and simple cloning of all your PCR amplicons, regardless of the polymerase used. This kit utilizes a novel mechanism for background colony suppression – a toxic minigene is generated when the vector closes upon itself – and allows for direct cloning from your reaction, with no purification step.

Cloning Kit Protocol Overview



*Note: While 5 minutes is recommended, 15 minutes will increase transformation levels for inserts suspected as being difficult to clone.

Vector Maps and Sequence



Map shown displays the construct formed if no insert is present. Unique restriction sites are shown in bold. Additional restriction sites that can be used for subcloning are also shown. Expanded box below shows location of sequencing primers, restriction sites for subcloning or linearization for in vitro transcription, RNA Polymerase promoter sequences and placement of insertion site within the toxic minigene.

RECOMMENDED PRODUCTS

NEB PCR Cloning Kit (NEB #E1202)

NEB PCR Cloning Kit (without competent cells) (NEB #E1203)

- Easy cloning of all PCR products, including blunt and TA ends
- Fast cloning experiments with 5-minute ligation step
- Simplified screening with low/no colony background and no blue/white selection required
- Save time by eliminating purification steps
- Updated to allow for *in vitro* transcription with both SP6 and T7 promoters
- Flanking restriction sites available for easy subcloning, including choice of two single digest options
- Provided analysis primers allow for downstream colony PCR screening or sequencing
- Ready-to-use kit components include 1 kb control amplicon, linearized cloning vector and optional single-use competent *E. coli*
- Longer shelf life (12 months), as compared to some commercially available products
- Value pricing

TIPS FOR OPTIMIZATION

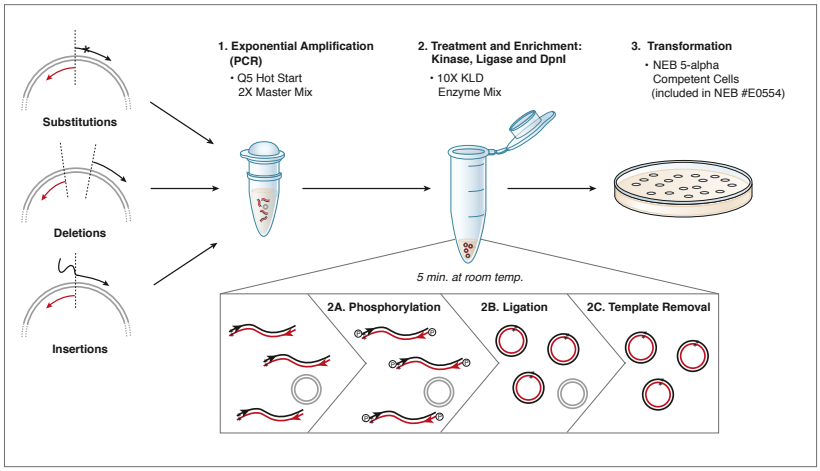
- For first time use of the kit, prepare a positive control reaction containing 2 µl (30 ng) of the 1 kb amplicon cloning control included with the kit
- 3:1 insert:vector ratio is best, but ratios from 1:1 to 10:1 can also be utilized
- Plate 50 µl or less of the 1 ml outgrowth. Plating too much of the outgrowth can increase background, and cause problems with colony PCR
- Important to stop ligations: If you wish to store your ligations to allow transformations at a later time, make sure your freezer is cold enough (-20°C) to freeze the ligations.
- Do not incubate the transformation plates at room temperature. The slow growth rate of the cells at room temperature will increase the number of background colonies.
- Follow the protocol, including the transformation protocol, carefully
- Add the cloning mixes 1 and 2 (which can be mixed together for the day's experiment) to the reaction last

How does the NEB PCR Cloning Kit work?

Q5 Site-Directed Mutagenesis Kit

The Q5 Site-Directed Mutagenesis Kit (with or without competent cells) enables rapid, site-specific mutagenesis of double-stranded plasmid DNA in less than 2 hours. The kit utilizes Q5 Hot Start High-Fidelity DNA Polymerase, along with custom mutagenic primers to create substitutions, deletions and insertions in a wide variety of plasmids. Transformation into high-efficiency NEB 5-alpha Competent *E. coli* cells ensures robust results with plasmids up to, at least, 14 kb in length.

Overview of Q5 Site-Directed Mutagenesis Kit



Protocol: Assembly

Before use, thaw and vortex the master mix thoroughly and keep on ice.

1. Exponential Amplification

	25 µl RXN	FINAL CONC.
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	1X
10 µM Forward Primer	1.25 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	0.5 µM
Template DNA (1–25 ng/µl)	1 µl	1–25 ng
Nuclease-free water	9.0 µl	

2. KLD Reaction

	VOLUME	FINAL CONC.
PCR Product	1 µl	
2X KLD Reaction Buffer	5 µl	1X
10X KLD Enzyme Mix	1 µl	1X
Nuclease-free Water	3 µl	

Protocol: Transformation with NEB 5-alpha

	STANDARD PROTOCOL
KLD Mix	5 µl
Competent *E. coli*	50 µl
Incubation	On ice for 30 minutes
Heat Shock	Exactly 42°C for exactly 30 seconds
Incubation	On ice for 5 minutes. Add 950 µl room temperature SOC 37°C for 60 minutes, with shaking

RECOMMENDED PRODUCTS

Q5 Site-Directed Mutagenesis Kit (NEB #E0554)

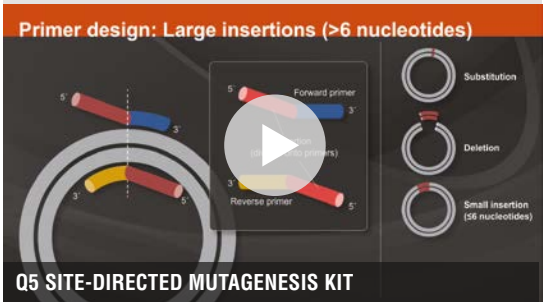
Q5 Site-Directed Mutagenesis Kit (Without Competent Cells) (NEB #E0552)

KLD Enzyme Mix (NEB #M0554)

- 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
- Use of standard primers eliminates need for phosphorylated or purified oligos

TIPS FOR OPTIMIZATION

- For optimal results, use **NEBaseChanger** at **NEBaseChanger.neb.com** to help design the primers for your SDM experiment
- No purification of your plasmid is necessary, either before or after the KLD reaction
- You can expect a high frequency of your desired mutation (> 90%)
- While the Q5 SDM Kit is supplied with high-efficiency, NEB competent *E. coli*, you can use your own chemically competent cells for cloning; results will vary, according to the quality and efficiency of the cells
- KLD Enzyme Mix (NEB #M0554) is available separately for customization
- For multi-site or combinatorial mutagenesis, we recommend NEBuilder® HiFi DNA Assembly products



Learn more about the benefits of the Q5 SDM Kit.

Nucleic Acid Purification

The need for high quality, highly pure DNA and RNA is important for many molecular cloning workflows. These nucleic acids are being purified from a wide variety of sources, such as cells and tissues, enzymatic reactions (e.g., PCR, ligation, digestions), and agarose gel matrices. Purification methods are optimized for various starting materials to ensure excellent recovery, high purity, minimal processing time and compatibility with emerging techniques. The Monarch Nucleic Acid Purification product portfolio addresses the needs upstream and downstream of molecular cloning workflows, including products for isolation of DNA and RNA from biological samples, DNA and RNA cleanup, plasmid purification and gel extraction.

Monarch® DNA & RNA Purification Kits

Designed with sustainability in mind

PRODUCT	APPLICATIONS	FEATURES
Monarch Plasmid Miniprep Kit (NEB #T1010)	Purification of up to 20 µg of plasmid DNA from bacterial culture.	• Elute in as little as 30 µl • Prevent buffer retention and salt carry-over with optimized column design • Includes colored buffers to monitor completion of certain steps • No need to add RNase before starting
Monarch PCR & DNA Cleanup Kit (NEB #T1030)	Purification and concentration of up to 5 µg of DNA from enzymatic reactions.	• Elute in as little as 6 µl • Prevent buffer retention and salt carry-over with optimized column design • Purify oligos and other small DNA fragments with simple protocol modification
Monarch DNA Gel Extraction Kit (NEB #T1020)	Purification of up to 5 µg of DNA from agarose gels.	• Elute in as little as 6 µl • Prevent buffer retention and salt carry-over with optimized column design • Fast, user-friendly protocol
Monarch Total RNA Miniprep Kit (NEB #T2010)	Extraction and purification of up to 100 µg of total RNA from blood, cells, tissues and other sample types.	• Purifies RNA of all sizes, including miRNA & small RNAs > 20 nucleotides • Includes DNase I, gDNA removal columns, Proteinase K, and a stabilization reagent • Protocols also available for RNA fractionation and RNA cleanup
Monarch RNA Cleanup Kits (NEB #T2030, T2040, T2050)	Purification and concentration of RNA after: • Extraction (e.g., TRIzol®) • *in vitro* transcription • Enzymatic reactions • Gel electrophoresis	• Highly pure RNA in as little as 5 minutes • Available in 3 different binding capacities (10 µg, 50 µg and 500 µg) for flexibility in various applications • Simple, user-friendly protocol • Prevent buffer retention and salt carryover with optimized column design
Monarch Genomic DNA Purification Kit (NEB #T3010)	Extraction and purification of genomic DNA from cells, blood, tissues and other sample types.	• Optimized protocols and buffer chemistry for excellent yields from a wide variety of samples • Purifies gDNA with a peak size > 50 kb • Includes RNase A and Proteinase K • Protocol also available for gDNA cleanup

Visit NEBMonarch.com to learn more and request samples.

TIPS FOR OPTIMIZATION

DNA PURIFICATION

- **Ensure that the tip of the column doesn't contact with flow-through after washing:** If in doubt, add a quick spin
- **If working with DNA > 10 kb, heat the elution buffer to 50°C:** Large DNA binds more tightly; heating helps to elute the DNA from the column

PLASMID MINIPREPS

- **Don't use too many cells (culture should not exceed 15 O.D. units):** Using the optimal amount of cells increases lysis efficiency and prevents clogging of the column
- **Lyse cells completely:** In order to release all plasmid DNA, all cells need to be lysed. Resuspend cells completely, and incubate for the recommended time.
- **Don't vortex cells after lysis:** Vortexing can cause shearing of host chromosomal DNA, resulting in gDNA contamination
- **Allow the RNase to do its job:** To prevent RNA contamination, do not skip or reduce the incubation with RNase (which is included in the neutralization buffer)
- **Don't skip any washes:** Proper washes ensure efficient removal of cell debris, endotoxins and salts

GEL EXTRACTION

- **Use the smallest possible agarose plug:** More agarose requires longer melting time and more dissolving buffer (introducing more salts which can co-elute with your sample
- **Minimize exposure to UV light:** UV exposure damages DNA. As long as the excision is done quickly, damage will be negligible
- **Melt the agarose completely:** If the agarose is not completely melted, DNA remains trapped inside and cannot be extracted properly

GENOMIC DNA EXTRACTION

- **Do not reload the same column:** Over-exposure of the matrix to the lysed sample can dislodge the membrane
- **Do not exceed recommended input amounts:** Buffer volumes are optimized for recommended inputs. Exceeding these can result in inefficient lysis and can clog the column.
- **Ensure samples are properly lysed:** Samples should be disrupted and homogenized completely to release all DNA

RNA EXTRACTION & PURIFICATION

- **Inactivate RNases after harvest:** Nucleases in your sample will lead to degradation, so inactivating them is essential. Process samples quickly, or use preservation reagents, and always ensure nuclease-free working environments
- **Do not exceed recommended input amounts:** Buffer volumes are optimized for recommended inputs. Exceeding these can result in inefficient lysis and can clog the column
- **Ensure samples are properly homogenized/ disrupted:** Samples should be disrupted and homogenized completely to release all RNA

cDNA Synthesis

When RNA is used as starting material, a reverse transcriptase can be used to generate cDNA, which can then be used as template for any of the cloning methods listed previously. Depending on which workflow is being followed, the resulting DNA may require a clean-up step. This can be performed using a spin column or by gel extraction.

cDNA Synthesis Selection Chart

cDNA SYNTHESIS	FEATURES
KITS	
LunaScript® RT SuperMix Kit (NEB #E3010/M3010)	• Ideal for cDNA synthesis of shorter fragments • Single tube supermix contains random hexamer and oligo-dT primers, dNTPs, Murine RNase Inhibitor, and Luna Reverse Transcriptase • Visible blue tracking dye for easy reaction setup • Fast 13-minute protocol
LunaScript® RT Master Mix Kit (Primer-free) (NEB #E3025)	• Ideal for first strand cDNA synthesis • Compatible with random primers, oligo dT primers, and gene-specific primers for maximum cDNA synthesis flexibility • 5X master mix contains dNTPs, Murine RNase Inhibitor and Luna Reverse Transcriptase • Visible blue tracking dye for easy reaction setup • Fast 13-minute protocol
ProtoScript® II First Strand cDNA Synthesis Kit (NEB #E6560)	• Generates cDNA at least 10 kb in length • Contains ProtoScript II Reverse Transcriptase, an enzyme with increased thermostability and reduced RNase H activity • Convenient 2-tube kit includes dNTPs, Oligo-dT primer and Random Primer Mix
ProtoScript First Strand cDNA Synthesis Kit (NEB #E6300)	• Generates cDNA at least 5 kb in length • Contains M-MuLV Reverse Transcriptase • Convenient 2-tube kit includes dNTPs, Oligo-dT primer and Random Primer Mix
Template Switching RT Enzyme Mix (NEB #M0466)	• Incorporates a universal adaptor sequence at the 3´ end of cDNA during the RT reaction • Enzyme mix and buffer are optimized for efficient template switching • RT enzyme mix includes RNase Inhibitor • High sensitivity for cDNA amplification – enables transcriptome analysis by RNA-seq from single cells or as low as 2 pg of human total RNA • Robust and simple workflow for 5´ Rapid Amplification of cDNA Ends (RACE) • Retains the complete 5´ end of transcripts for 2nd Strand cDNA Synthesis
STANDALONE REAGENTS	
ProtoScript II Reverse Transcriptase (NEB #M0368) An alternative to SuperScript® II	• RNase H⁻ mutant of M-MuLV Reverse Transcriptase with increased thermostability and reduced RNase H activity • Increased reaction temperatures (37–50°C)
M-MuLV Reverse Transcriptase (NEB #M0253)	• Robust reverse transcriptase for a variety of templates • Standard reaction temperatures (37–45°C)
AMV Reverse Transcriptase (NEB #M0277)	• Robust reverse transcriptase for a broad temperature range (37–52°C) • Can be used for templates requiring higher reaction temperatures
WarmStart RTx Reverse Transcriptase (NEB #M0380)	• Permits room temperature reaction setup • Increased reaction temperatures (50–65°C) • Optimized for RT-LAMP isothermal detection

TIPS FOR OPTIMIZATION

STARTING MATERIAL

- Intact RNA of high purity is essential for generating cDNA for cloning applications.
- Total RNA or mRNA can be used in the reverse transcription reaction. Total RNA is generally sufficient for cDNA synthesis reactions. However, if desired, mRNA can be easily obtained using a Magnetic mRNA Isolation Kit (NEB #S1550).
- The amount of RNA required for cDNA cloning depends on the abundance of the transcript-of-interest. In general, 1 ng to 1 µg total RNA or 0.1–100 ng mRNA is recommended.

PRODUCT SELECTION

- Streamline your reaction setup by using the ProtoScript II First Strand cDNA Synthesis Kit (NEB #E6560). This kit combines ProtoScript II Reverse Transcriptase (NEB #M0360), a thermostable M-MuLV (RNase H⁻) Reverse Transcriptase, and recombinant RNase Inhibitor in an enzyme Master Mix, along with a separate Reaction Mix containing dNTPs. Additionally, the kit contains two optimized reverse transcription primer mixes.

YIELD

- ProtoScript II Reverse Transcriptase is capable of generating cDNA of more than 10 kb up to 48°C. We recommend 42°C for routine reverse transcription.
- You can increase the yield of a long cDNA product by doubling the amount of enzyme and dNTPs.

ADDITIVES

- For most RT-PCR reactions, RNase H treatment is not required. But for some difficult amplicons or sensitive assays, add 2 units of *E. coli* RNase H to the reaction and incubate at 37°C for 20 minutes.

Protocol: cDNA Synthesis

	DENATURATION PROTOCOL
Total RNA	1–6 µl (up to 1 µg)
$d(T)_{23}$ VN (50 µM)	2 µl
Nuclease-free Water	to a total volume of 8 µl
Incubation	65°C for 5 minutes spin briefly and put on ice

	SYNTHESIS PROTOCOL
Denatured RNA	8 µl
Reaction Mix	10 µl
Enzyme Mix	2 µl
Incubation	80°C for 5 minutes store at –20°C

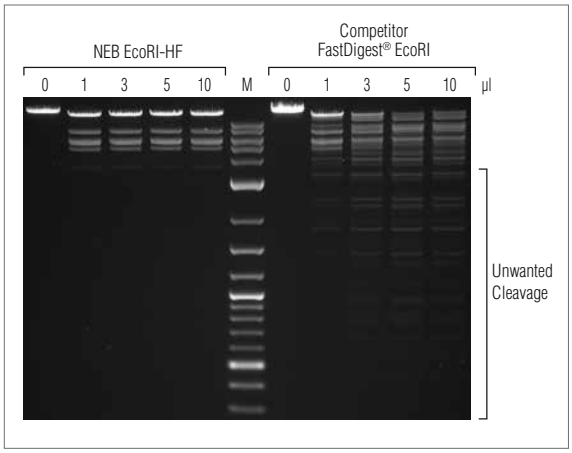
Restriction Enzyme Digestion

Restriction enzyme sites that are unique to both the insert and vector should be chosen. Unidirectional cloning is achieved using two different restriction enzymes, each with unique recognition sites at an end of the insert. Depending on the RE chosen, ends can be blunt or sticky (cohesive). Restriction enzyme digestion is generally used in traditional cloning.

Protocol: Restriction Enzyme Reactions

	STANDARD PROTOCOL	TIME-SAVER® PROTOCOL
DNA	up to 1 µg	up to 1 µg
10X NEBuffer	5 µl (1X)	5 µl (1X)
Restriction Enzyme	10 units*	1 µl
Total Volume	50 µl	50 µl
Incubation Temperature	enzyme dependent	enzyme dependent
Incubation Time	60 minutes	5–15 minutes**

*Sufficient to digest all types of DNAs.
**Time-Saver qualified enzymes can also be incubated overnight with no star activity.



EcoRI-HF (NEB #R3101) shows 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).

TIPS FOR OPTIMIZATION

ENZYME

- Keep on ice when not in the freezer.
- Should be the last component added to reaction.
- Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube. Follow with a quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.
- In general, we recommend 5 – 10 units of enzyme per µg DNA, and 10 – 20 units per µg of genomic DNA in a 1 hour digest.
- Some restriction enzymes require more than one recognition site to cleave efficiently. These are designated with the "multi-site" icon 2*site. Please review recommendations on working with these enzymes at **www.neb.com**.

STAR ACTIVITY

- Unwanted cleavage that can occur when an enzyme is used under sub-optimal conditions, such as:
  – Too much enzyme present
  – Too long of an incubation time
  – Using a non-recommended buffer
  – Glycerol concentrations above 5%
- Star activity can be reduced by using a High-Fidelity (HF) enzyme, reducing the number of units, reducing incubation time, using a Time-Saver enzyme or increasing reaction volume.

DNA

- Should be free of contaminants such as phenol, chloroform, alcohol, EDTA, detergents and salts. Spin column purification readily accomplishes this; extra washes during purification can also help.
- Methylation of DNA can affect digestion with certain enzymes. For more information about methylation visit **www.neb.com/methylation**.

BUFFER

- Use at a 1X concentration.
- Recombinant Albumin is included in NEBuffer r1.1, r2.1, r3.1 and rCutSmart™ Buffer. No additional albumin is needed.

REACTION VOLUME

- A 50 µl reaction volume is recommended for digestion of up to 1 µg of substrate. This helps maintain salt levels introduced by miniprepped DNA low enough that they don't affect enzyme activity.
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol.
- Additives in the restriction enzyme storage buffer (e.g., glycerol, salt), as well as contaminants found in the substrate solution (e.g., salt, EDTA, or alcohol), can be problematic in smaller reaction volumes.

	RESTRICTION ENZYME*	DNA	10X NEBUFFER
10 µl rxn**	1 unit	0.1 µg	1 µl
25 µl rxn	5 units	0.5 µg	2.5 µl
50 µl rxn	10 units	1 µg	5 µl

* Restriction Enzymes can be diluted using the recommended diluent buffer when smaller amounts are needed

** 10 µl rxns should not be incubated for longer than 1 hour to avoid evaporation

INCUBATION TIME

- Incubation time for the Standard Protocol is 1 hour. Incubation for the Time-Saver Protocol is 5–15 minutes.
- Visit **www.neb.com/timesaver** for list of Time-Saver qualified enzymes.
- It is possible, with many enzymes, to use fewer units and digest for up to 16 hours. For more information, visit **www.neb.com**.

STORAGE AND STABILITY

- Storage at –20°C is recommended for most restriction enzymes. For a few enzymes, storage at –80°C is recommended. Visit **www.neb.com** for storage information.
- 10X NEBuffers should be stored at –20°C.
- The expiration date is found on the label.
- Long term exposure to temperatures above –20°C should be minimized whenever possible.

Performance Chart for Restriction Enzymes

New England Biolabs supplies > 210 restriction enzymes that are 100% active in rCutSmart Buffer. This results in increased efficiency, flexibility and ease-of-use, especially when performing double digests.

This performance chart summarizes the activity information of NEB restriction enzymes. To help select the best conditions for double digests, this chart shows the optimal (supplied) NEBuffer and approximate activity in the four standard NEBuffers for each enzyme. Note that Recombinant Albumin is included in all NEBuffers, and is not provided as a separate tube. In addition, this performance chart shows recommended reaction temperature, heat-inactivation temperature, recommended diluent buffer, methylation sensitivity, whether the enzyme is Time-Saver qualified, and whether the enzyme works better in a substrate with multiple sites.

Chart Legend

- **U** — Supplied with a unique reaction buffer that is different from the four standard NEBuffers. The compatibility with the four standard NEBuffers is indicated in the chart.
- **rAlb** — Recombinant
- **e** — Engineered enzyme for maximum performance
- **clock** — Time-Saver qualified
- **2*site** — Indicates that the restriction enzyme requires two or more sites for cleavage
- **dcm** — dcm methylation sensitivity
- **dam** — dam methylation sensitivity
- **CpG** — CpG methylation sensitivity

Activity Notes (see last column)

FOR STAR ACTIVITY

1. Star activity may result from extended digestion, high enzyme concentration or a glycerol concentration of > 5%.
2. Star activity may result from extended digestion.
3. Star activity may result from a glycerol concentration of > 5%.

* May exhibit star activity in this buffer.

\+ For added flexibility, NEB offers an isoschizomer or HF enzyme, supplied with rCutSmart Buffer.

FOR LIGATION AND RECUTTING

a. Ligation is less than 10%

b. Ligation is 25% – 75%

c. Recutting after ligation is < 5%

d. Recutting after ligation is 50% – 75%

e. Ligation and recutting after ligation is not applicable since the enzyme is either a nicking enzyme, is affected by methylation, or the recognition sequence contains variable sequences.

			Enzyme	Supplied NEBuffer	% Activity in NEBuffers r1.1	r2.1	r3.1	rCutSmart	Incub. Temp. (°C)	Inactiv. Temp. (°C)	Dil.	Unit Substrate	Methylation Sensitivity		Notes
rAlb	clock		AatII	rCutSmart	<10	50*	50	100	37°	80°	B	λ DNA		CpG	
rAlb			AbaSI	rCutSmart + DTT	25	50	50	100	25°	65°	C	T4 wild-type phage DNA (fully ghmC-modified)			e
rAlb	clock		Acc65I	r3.1	10	75*	100	25	37°	65°	A	pBC4 DNA	dcm	CpG	
rAlb	clock		AccI	rCutSmart	50	50	10	100	37°	80°	A	λ DNA		CpG	
rAlb	clock		AciI	rCutSmart	<10	25	100	100	37°	65°	A	λ DNA		CpG	
rAlb	clock		AclI	rCutSmart	<10	<10	<10	100	37°	No	B	λ DNA		CpG	
rAlb	clock		AcuI	rCutSmart	50	100	50	100	37°	65°	B	λ DNA			1, b, d
rAlb			AfeI	rCutSmart	25	100	25	100	37°	65°	B	pXba DNA		CpG	
rAlb	clock		AflII	rCutSmart	50	100	10	100	37°	65°	A	ΦX174 RF I DNA			
rAlb	clock		AflIII	r3.1	10	50	100	50	37°	80°	B	λ DNA			
rAlb	clock	e	AgeI-HF	rCutSmart	100	50	10	100	37°	65°	A	λ DNA		CpG	
rAlb	clock		AhdI	rCutSmart	25	25	10	100	37°	65°	A	λ DNA		CpG	a
rAlb		e	AleI-v2	rCutSmart	<10	<10	<10	100	37°	65°	B	λ DNA		CpG	
rAlb	clock		AluI	rCutSmart	25	100	50	100	37°	80°	B	λ DNA			b
rAlb			AlwI	rCutSmart	50	50	10	100	37°	No	A	λ DNA (dam-)	dam		1, b, d
rAlb	clock		AlwNI	rCutSmart	10	100	50	100	37°	80°	A	λ DNA	dcm		
rAlb	clock		ApaI	rCutSmart	25	25	<10	100	37°	65°	A	pXba DNA	dcm	CpG	
rAlb	clock		ApaLI	rCutSmart	100	100	10	100	37°	No	A	λ DNA (HindIII digest)		CpG	
rAlb	clock		ApeKI	r3.1	25	50	100	10	75°	No	B	λ DNA		CpG	
rAlb	clock	e	ApoI-HF	rCutSmart	10	100	10	100	37°	80°	B	λ DNA			
rAlb	clock		AscI	rCutSmart	<10	10	10	100	37°	80°	A	λ DNA		CpG	
rAlb	clock		AseI	r3.1	<10	50*	100	10	37°	65°	B	λ DNA			3
rAlb			AsiSI	rCutSmart	100	100	25	100	37°	80°	B	XhoI digested pXba		CpG	2, b
rAlb	clock		AvaI	rCutSmart	<10	100	25	100	37°	80°	A	λ DNA		CpG	
rAlb	clock		AvaII	rCutSmart	50	75	10	100	37°	80°	A	λ DNA	dcm	CpG	
rAlb	clock		AvrII	rCutSmart	100	50	50	100	37°	No	B	λ DNA (HindIII digest)			
rAlb	clock		BaeGI	r3.1	75	75	100	25	37°	80°	A	λ DNA			

a. Ligation is less than 10%
b. Ligation is 25% – 75%
c. Recutting after ligation is < 5%
d. Recutting after ligation is 50% – 75%
e. Ligation and recutting after ligation is not applicable since the enzyme is either a nicking enzyme, is affected by methylation, or the recognition sequence contains variable sequences.

Performance Chart for Restriction Enzymes (Continued)

Enzyme	Supplied NEBuffer	% Activity in NEBuffers r1.1	r2.1	r3.1	rCutSmart	Incub. Temp. (°C)	Inactiv. Temp. (°C)	Dil.	Unit Substrate	Methylation Sensitivity		Notes
BaeI	rCutSmart	50	100	50	100	37°	65°	A	λ DNA		CpG	e
BamHI	r3.1	75*	100*	100	100*	37°	No	A	λ DNA			3
BamHI-HF	rCutSmart	100	50	10	100	37°	No	A	λ DNA			
BanI	rCutSmart	10	25	<10	100	37°	65°	A	λ DNA	dcm	CpG	1
BanII	rCutSmart	100	100	50	100	37°	80°	A	λ DNA			2
BbsI	r2.1	100	100	25	75	37°	65°	B	λ DNA			
BbsI-HF	rCutSmart	10	10	10	100	37°	65°	B	λ DNA			
BbvCI	rCutSmart	10	100	50	100	37°	No	B	λ DNA		CpG	1, a
BbvI	rCutSmart	100	100	25	100	37°	65°	B	pBR322 DNA			3
BccI	rCutSmart	100	50	10	100	37°	65°	A	pXba DNA			3, b
BceAI	r3.1	100*	100*	100	100*	37°	65°	A	pBR322 DNA		CpG	1
BcgI	r3.1	10	75*	100	50*	37°	65°	A	λ DNA	dam	CpG	e
BciVI	rCutSmart	100	25	<10	100	37°	80°	C	λ DNA			b
BclI	r3.1	50	100	100	75	37°	No	A	λ DNA (dam-)	dam		
BclI-HF	rCutSmart	100	100	10	100	37°	65°	B	λ DNA (dam-)	dam		
BcoDI	rCutSmart	50	75	75	100	37°	No	B	λ DNA		CpG	
BfaI	rCutSmart	<10	10	<10	100	37°	80°	B	λ DNA			2, b
BfuAI	r3.1	<10	25	100	10	50°	65°	B	λ DNA		CpG	3
BglI	r3.1	10	25	100	10	37°	65°	B	λ DNA		CpG	
BglII	r3.1	10	10	100	<10	37°	No	A	λ DNA			
BlpI	rCutSmart	50	100	10	100	37°	No	A	λ DNA			d
BmgBI	r3.1	<10	10	100	10	37°	65°	B	λ DNA		CpG	3, b, d
BmrI	r2.1	75	100	75	100*	37°	65°	B	λ DNA (HindIII digest)			b
BmtI-HF	rCutSmart	50	100	10	100	37°	65°	B	pXba DNA			
BpmI	r3.1	75	100	100	100*	37°	65°	B	λ DNA			2
Bpu10I	r3.1	10	25	100	25	37°	80°	B	λ DNA			3, b, d
BpuEI	rCutSmart	50*	100	50*	100	37°	65°	B	λ DNA			d
BsaAI	rCutSmart	100	100	100	100	37°	No	C	λ DNA		CpG	
BsaBI	rCutSmart	50	100	75	100	60°	80°	B	λ DNA (dam-)	dam	CpG	2
BsaHI	rCutSmart	50	100	100	100	37°	80°	C	λ DNA	dcm	CpG	
BsaI-HFv2	rCutSmart	100	100	100	100	37°	80°	B	pXba DNA	dcm	CpG	
BsaJI	rCutSmart	50	100	100	100	60°	80°	A	λ DNA			
BsaWI	rCutSmart	10	100	50	100	60°	80°	A	λ DNA			
BsaXI	rCutSmart	50*	100*	10	100	37°	No	C	λ DNA			e
BseRI	rCutSmart	100	100	75	100	37°	80°	A	λ DNA			d
BseYI	r3.1	10	50	100	50	37°	80°	B	λ DNA		CpG	d
BsgI	rCutSmart	25	50	25	100	37°	65°	B	λ DNA			d
BsiEI	rCutSmart	25	50	<10	100	60°	No	A	λ DNA		CpG	
BsiHKAI	rCutSmart	25	100	100	100	65°	No	A	λ DNA			
BsiWI	r3.1	25	50*	100	25	55°	65°	B	ΦX174 DNA		CpG	
BsiWI-HF	rCutSmart	50	100	10	100	37°	No	B	ΦX174 DNA		CpG	
BsII	rCutSmart	50	75	100	100	55°	No	A	λ DNA	dcm	CpG	b
BsmAI	rCutSmart	50	100	100	100	55°	No	B	λ DNA		CpG	

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2. Star activity may result from extended digestion.
3. Star activity may result from a glycerol concentration of > 5%.

* May exhibit star activity in this buffer.
+ NEB isoschizomer or HF enzyme supplied with rCutSmart Buffer.

Performance Chart for Restriction Enzymes (Continued)

Enzyme	Supplied NEBuffer	% Activity in NEBuffers r1.1	r2.1	r3.1	rCutSmart	Incub. Temp. (°C)	Inactiv. Temp. (°C)	Dil.	Unit Substrate	Methylation Sensitivity		Notes
BsmBI-v2	r3.1	<10	50	100	25	55°	80°	B	λ DNA		CpG	
BsmFI	rCutSmart	25	50	50	100	65°	80°	A	pBR322 DNA	dcm	CpG	1
BsmI	rCutSmart	25	100	<10	100	65°	80°	A	λ DNA			
BsoBI	rCutSmart	25	100	100	100	37°	80°	A	λ DNA			
Bsp1286I	rCutSmart	25	25	25	100	37°	65°	A	λ DNA			3
BspCNI	rCutSmart	100	75	10	100	37°	80°	A	λ DNA			b
BspDI	rCutSmart	25	75	50	100	37°	80°	A	λ DNA	dam	CpG	
BspEI	r3.1	<10	10	100	<10	37°	80°	B	λ DNA (dam-)	dam	CpG	
BspHI	rCutSmart	10	50	25	100	37°	80°	A	λ DNA	dam		
BspMI	r3.1	10	50*	100	10	37°	65°	B	λ DNA			
BspQI	r3.1	100*	100*	100	100*	50°	80°	B	λ DNA			3
BsrBI	rCutSmart	50	100	100	100	37°	80°	A	λ DNA		CpG	d
BsrDI	r2.1	10	100	75	25	37°	80°	A	λ DNA			3, d
BsrFI-v2	rCutSmart	25	25	0	100	37°	No	C	pBR322 DNA		CpG	
BsrGI-HF	rCutSmart	10	100	100	100	37°	80°	A	λ DNA			
BsrI	r3.1	<10	50	100	10	65°	80°	B	ΦX174 DNA			b
BssHII	rCutSmart	100	100	100	100	50°	65°	B	λ DNA		CpG	
BssSI-v2	rCutSmart	10	25	<10	100	37°	No	B	λ DNA			
BstAPI	rCutSmart	50	100	25	100	60°	80°	A	λ DNA		CpG	b
BstBI	rCutSmart	75	100	10	100	65°	No	A	λ DNA		CpG	
BstEII-HF	rCutSmart	<10	10	<10	100	37°	No	A	λ DNA			
BstNI	r3.1	10	100	100	75	60°	No	A	λ DNA			a
BstUI	rCutSmart	50	100	25	100	60°	No	A	λ DNA		CpG	b
BstXI	r3.1	<10	50	100	25	37°	80°	B	λ DNA	dcm		3
BstYI	rCutSmart	25	100	75	100	60°	No	A	λ DNA			
BstZ17I-HF	rCutSmart	100	100	10	100	37°	No	A	λ DNA		CpG	
Bsu36I	rCutSmart	25	100	100	100	37°	80°	C	λ DNA (HindIII digest)			b
BtgI	rCutSmart	50	100	100	100	37°	80°	B	pBR322 DNA			
BtgZI	rCutSmart	10	25	<10	100	60°	80°	A	λ DNA		CpG	3, b, d
BtsCI	rCutSmart	10	100	25	100	50°	80°	B	λ DNA			
BtsI-v2	rCutSmart	100	100	25	100	37°	No	A	λ DNA			1
BtsIMutI	rCutSmart	100	50	10	100	55°	80°	A	pUC19 DNA			b
Cac8I	rCutSmart	50	75	100	100	37°	65°	B	λ DNA		CpG	b
ClaI	rCutSmart	10	50	50	100	37°	65°	A	λ DNA (dam-)	dam	CpG	
CspCI	rCutSmart	10	100	10	100	37°	65°	A	λ DNA			e
CviAII	rCutSmart	50	50	10	100	25°	65°	C	λ DNA			
CviKI-1	rCutSmart	25	100	100	100	37°	No	A	pBR322 DNA			1, b
CviQI	r3.1	75	100*	100	75*	25°	No	C	λ DNA			b
DdeI	rCutSmart	75	100	100	100	37°	65°	B	λ DNA			
DpnI	rCutSmart	100	100	75	100	37°	80°	B	pBR322 DNA (dam methylated)		CpG	b
DpnII	U	25	25	100*	25	37°	65°	B	λ DNA (dam-)	dam		
DraI	rCutSmart	75	75	50	100	37°	65°	A	λ DNA			

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b. Ligation is 25% – 75%
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e. Ligation and recutting after ligation is not applicable since the enzyme is either a nicking enzyme, is affected by methylation, or the recognition sequence contains variable sequences.

Performance Chart for Restriction Enzymes (Continued)

Enzyme	Supplied NEBuffer	% Activity in NEBuffers r1.1	r2.1	r3.1	rCutSmart	Incub. Temp. (°C)	Inactiv. Temp. (°C)	Dil.	Unit Substrate	Methylation Sensitivity		Notes
DraIII-HF	rCutSmart	<10	50	10	100	37°	No	B	λ DNA		CpG	b
DrdI	rCutSmart	25	50	10	100	37°	65°	A	pUC19 DNA		CpG	3
EaeI	rCutSmart	10	50	<10	100	37°	65°	A	λ DNA	dcm	CpG	b
EagI-HF	rCutSmart	25	100	100	100	37°	65°	B	pXba DNA		CpG	
EarI	rCutSmart	50	10	<10	100	37°	65°	B	λ DNA		CpG	b, d
EciI	rCutSmart	100	50	50	100	37°	65°	A	λ DNA		CpG	2
Eco53kI	rCutSmart	100	100	<10	100	37°	65°	A	pXba DNA		CpG	3, b
EcoNI	rCutSmart	50	100	75	100	37°	65°	A	λ DNA			b
EcoO109I	rCutSmart	50	100	50	100	37°	65°	A	λ DNA (HindIII digest)	dcm		3
EcoP15I	r3.1 + ATP	75	100	100	100	37°	65°	A	pUC19 DNA			e
EcoRI	U	25	100*	50	50*	37°	65°	C	λ DNA		CpG	
EcoRI-HF	rCutSmart	10	100	<10	100	37°	65°	C	λ DNA		CpG	
EcoRV	r3.1	10	50	100	10	37°	80°	A	λ DNA		CpG	
EcoRV-HF	rCutSmart	25	100	100	100	37°	65°	B	λ DNA		CpG	
Esp3I	rCutSmart	100	100	<10	100	37°	65°	B	λ DNA		CpG	
FatI	r2.1	10	100	50	50	55°	80°	A	pUC19 DNA			
FauI	rCutSmart	100	50	10	100	55°	65°	A	λ DNA		CpG	3, b, d
Fnu4HI	rCutSmart	<10	<10	<10	100	37°	No	A	λ DNA		CpG	a
FokI	rCutSmart	100	100	75	100	37°	65°	A	λ DNA	dcm	CpG	3, b, d
FseI	rCutSmart	100	75	<10	100	37°	65°	B	pBC4 DNA	dcm	CpG	
FspEI	rCutSmart + Enz. Activ.	<10	<10	<10	100	37°	80°	B	pBR322 (dcm+) DNA			1, e
FspI	rCutSmart	10	100	10	100	37°	No	C	λ DNA		CpG	b
HaeII	rCutSmart	25	100	10	100	37°	80°	A	λ DNA		CpG	
HaeIII	rCutSmart	50	100	25	100	37°	80°	A	λ DNA			
HgaI	r1.1	100	100	25	100*	37°	65°	A	ΦX174 DNA		CpG	1
HhaI	rCutSmart	25	100	100	100	37°	65°	A	λ DNA		CpG	
HinP1I	rCutSmart	100	100	100	100	37°	65°	A	λ DNA		CpG	
HincII	rCutSmart	25	100	100	100	37°	65°	B	λ DNA		CpG	
HindIII	r2.1	25	100	50	50	37°	80°	B	λ DNA			2
HindIII-HF	rCutSmart	10	100	10	100	37°	80°	B	λ DNA			
HinfI	rCutSmart	50	100	100	100	37°	80°	A	λ DNA		CpG	
HpaI	rCutSmart	<10	75*	25	100	37°	No	A	λ DNA		CpG	1
HpaII	rCutSmart	100	50	<10	100	37°	80°	A	λ DNA		CpG	
HphI	rCutSmart	50	50	<10	100	37°	65°	B	λ DNA	dcm		1, b, d
Hpy166II	rCutSmart	100	100	50	100	37°	65°	C	pBR322 DNA		CpG	
Hpy188I	rCutSmart	25	100	50	100	37°	65°	A	pBR322 DNA	dam		1, b
Hpy188III	rCutSmart	100	100	10	100	37°	65°	B	pUC19 DNA	dam	CpG	3, b
Hpy99I	rCutSmart	50	10	<10	100	37°	65°	A	λ DNA		CpG	
HpyAV	rCutSmart	100	100	25	100	37°	65°		λ DNA		CpG	3, b, d
HpyCH4III	rCutSmart	100	25	<10	100	37°	65°	A	λ DNA			b
HpyCH4IV	rCutSmart	100	50	25	100	37°	65°	A	pUC19 DNA		CpG	
HpyCH4V	rCutSmart	50	50	25	100	37°	65°	A	λ DNA			

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Performance Chart for Restriction Enzymes (Continued)

Enzyme	Supplied NEBuffer	% Activity in NEBuffers r1.1	r2.1	r3.1	rCutSmart	Incub. Temp. (°C)	Inactiv. Temp. (°C)	Dil.	Unit Substrate	Methylation Sensitivity		Notes
I-CeuI	rCutSmart	10	10	10	100	37°	65°	B	pBHS ScaI-linearized Control Plasmid			
I-SceI	rCutSmart	10	50	25	100	37°	65°	B	pGPS2 NotI-linearized Control Plasmid			
KasI	rCutSmart	50	100	50	100	37°	65°	B	pBR322 DNA		CpG	3
KpnI-HF	rCutSmart	100	25	<10	100	37°	No	A	pXba DNA			
LpnPI	rCutSmart + Enz. Activ.	<10	<10	<10	100	37°	65°	B	pBR322 (dcm+) DNA			1, e
MboI	rCutSmart	75	100	100	100	37°	65°	A	λ DNA (dam-)	dam	CpG	
MboII	rCutSmart	100*	100	50	100	37°	65°	C	λ DNA (dam-)	dam		b
MfeI-HF	rCutSmart	75	25	<10	100	37°	No	A	λ DNA			
MluCI	rCutSmart	100	10	10	100	37°	No	A	λ DNA			
MluI-HF	rCutSmart	25	100	100	100	37°	No	A	λ DNA		CpG	
MlyI	rCutSmart	50	50	10	100	37°	65°	A	λ DNA			b, d
MmeI	rCutSmart	50	100	50	100	37°	65°	B	ΦX174 RF I DNA		CpG	b, c
MnlI	rCutSmart	75	100	50	100	37°	65°	B	λ DNA			b
MscI	rCutSmart	25	100	100	100	37°	80°	C	λ DNA	dcm		
MseI	rCutSmart	75	100	75	100	37°	65°	A	λ DNA			
MslI	rCutSmart	50	50	<10	100	37°	80°	A	λ DNA			
MspA1I	rCutSmart	10	50	10	100	37°	65°	B	λ DNA		CpG	
MspI	rCutSmart	75	100	50	100	37°	No	A	λ DNA			
MspJI	rCutSmart + Enz. Activ.	<10	<10	<10	100	37°	65°	B	pBR322 (dcm+) DNA			1, e
MwoI	rCutSmart	<10	100	100	100	60°	No	B	λ DNA		CpG	
NaeI	rCutSmart	25	25	<10	100	37°	No	A	pXba DNA		CpG	b
NarI	rCutSmart	100	100	10	100	37°	65°	A	pXba DNA		CpG	
Nb.BbvCI	rCutSmart	25	100	100	100	37°	80°	A	supercoiled plasmid DNA			e
Nb.BsmI	r3.1	<10	50	100	10	65°	80°	A	supercoiled plasmid pBR322 DNA			e
Nb.BsrDI	rCutSmart	25	100	100	100	65°	80°	A	supercoiled pUC19 DNA			e
Nb.BssSI	r3.1	10	100	100	25	37°	No	B	supercoiled pUC19 DNA			e
Nb.BtsI	rCutSmart	75	100	75	100	37°	80°	A	supercoiled pUC101 DNA (dam-/dcm-)			e
NciI	rCutSmart	100	25	10	100	37°	No	A	λ DNA		CpG	b
NcoI	r3.1	100	100	100	100	37°	80°	A	λ DNA			
NcoI-HF	rCutSmart	50	100	10	100	37°	80°	B	λ DNA			
NdeI	rCutSmart	75	100	100	100	37°	65°	A	λ DNA			
NgoMIV	rCutSmart	100	50	10	100	37°	No	A	pXba DNA		CpG	1
NheI-HF	rCutSmart	100	25	10	100	37°	80°	C	λ DNA (HindIII digest)		CpG	
NlaIII	rCutSmart	<10	<10	<10	100	37°	65°	B	ΦX174 RF I DNA			
NlaIV	rCutSmart	10	10	10	100	37°	65°	B	pBR322 DNA	dcm	CpG	
NmeAIII	rCutSmart	10	10	<10	100	37°	65°	B	ΦX174 RF I DNA			c
NotI	r3.1	<10	50	100	25	37°	65°	C	pBC4 DNA		CpG	
NotI-HF	rCutSmart	25	100	25	100	37°	65°	A	pBC4 DNA		CpG	
NruI-HF	rCutSmart	0	25	50	100	37°	No	A	λ DNA	dam	CpG	
NsiI	r3.1	10	75	100	25	37°	65°	B	λ DNA			

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Performance Chart for Restriction Enzymes (Continued)

Enzyme	Supplied NEBuffer	% Activity in NEBuffers r1.1	r2.1	r3.1	rCutSmart	Incub. Temp. (°C)	Inactiv. Temp. (°C)	Dil.	Unit Substrate	Methylation Sensitivity		Notes
NsiI-HF	rCutSmart	<10	20	<10	100	37°	80°	B	λ DNA			
NspI	rCutSmart	100	100	<10	100	37°	65°	A	λ DNA			
Nt.AlwI	rCutSmart	10	100	100	100	37°	80°	A	pUC101 DNA (dam-/dcm-)	dam		e
Nt.BbvCI	rCutSmart	50	100	10	100	37°	80°	A	supercoiled plasmid DNA		CpG	e
Nt.BsmAI	rCutSmart	100	50	10	100	37°	65°	A	supercoiled plasmid DNA		CpG	e
Nt.BspQI	r3.1	<10	25	100	10	50°	80°	B	supercoiled pUC19 DNA			e
Nt.BstNBI	r3.1	0	10	100	10	55°	80°	A	T7 DNA			e
Nt.CviPII	rCutSmart	10	100	25	100	37°	65°	A	pUC19 DNA		CpG	e
PI-PspI	U + rAlbumin	10	10	10	10	65°	No	B	pAKR7 XmnI-linearized Control Plasmid			
PI-SceI	U + rAlbumin	10	10	10	10	37°	65°	B	pBSvdeX XmnI-linearized Control Plasmid			
PacI	rCutSmart	100	75	10	100	37°	65°	A	pNEB193 DNA			
PaeR7I	rCutSmart	25	100	10	100	37°	No	A	λ DNA (HindIII digest)		CpG	
PaqCI	rCutSmart PaqCI Activator	10	100	10	100	37°	65°	B	λ DNA		CpG	1
PciI	r3.1	50	75	100	50*	37°	80°	B	pXba DNA			
PflFI	rCutSmart	25	100	25	100	37°	65°	A	pBC4 DNA			b
PflMI	r3.1	0	100	100	50	37°	65°	A	λ DNA	dcm		3, b, d
PleI	rCutSmart	25	50	25	100	37°	65°	A	λ DNA		CpG	b, d
PluTI	rCutSmart	100	25	<10	100	37°	65°	A	pXba DNA		CpG	b
PmeI	rCutSmart	<10	50	10	100	37°	65°	A	λ DNA		CpG	
PmlI	rCutSmart	100	50	<10	100	37°	65°	A	λ DNA (HindIII digest) DNA		CpG	
PpuMI	rCutSmart	<10	<10	<10	100	37°	No	B	λ DNA (HindIII digest)	dcm		
PshAI	rCutSmart	25	50	10	100	37°	65°	A	λ DNA		CpG	
PsiI-v2	rCutSmart	25	50	10	100	37°	65°	B	λ DNA			3
PspGI	rCutSmart	25	100	50	100	75°	No	A	T7 DNA	dcm		3
PspOMI	rCutSmart	10	10	<10	100	37°	65°	B	pXba DNA	dcm	CpG	
PspXI	rCutSmart	<10	100	25	100	37°	No	B	λ DNA (HindIII digest)		CpG	
PstI	r3.1	75	75	100	50*	37°	80°	C	λ DNA			
PstI-HF	rCutSmart	10	75	50	100	37°	No	C	λ DNA			
PvuI-HF	rCutSmart	25	100	100	100	37°	No	B	pXba DNA		CpG	
PvuII	r3.1	50	100	100	100*	37°	No	B	λ DNA			
PvuII-HF	rCutSmart	<10	<10	<10	100	37°	No	B	λ DNA			
RsaI	rCutSmart	25	50	<10	100	37°	No	A	λ DNA		CpG	
RsrII	rCutSmart	25	75	10	100	37°	65°	C	λ DNA		CpG	
SacI-HF	rCutSmart	10	50	<10	100	37°	65°	A	λ DNA (HindIII digest)		CpG	
SacII	rCutSmart	10	100	10	100	37°	65°	A	pXba DNA		CpG	
SalI	r3.1	<10	<10	100	<10	37°	65°	A	λ DNA (HindIII digest)		CpG	
SalI-HF	rCutSmart	10	100	100	100	37°	65°	A	λ DNA (HindIII digest)		CpG	
SapI	rCutSmart	75	50	<10	100	37°	65°	B	λ DNA			
Sau3AI	r1.1	100	50	10	100	37°	65°	A	λ DNA		CpG	b
Sau96I	rCutSmart	50	100	100	100	37°	65°	A	λ DNA	dcm	CpG	
SbfI-HF	rCutSmart	50	25	<10	100	37°	80°	B	λ DNA			

1. Star activity may result from extended digestion, high enzyme concentration or a glycerol concentration of > 5%.
2. Star activity may result from extended digestion.
3. Star activity may result from a glycerol concentration of > 5%.

* May exhibit star activity in this buffer.
+ NEB isoschizomer or HF enzyme supplied with rCutSmart Buffer.

Performance Chart for Restriction Enzymes (Continued)

Enzyme	Supplied NEBuffer	% Activity in NEBuffers r1.1	r2.1	r3.1	rCutSmart	Incub. Temp. (°C)	Inactiv. Temp. (°C)	Dil.	Unit Substrate	Methylation Sensitivity		Notes
ScaI-HF	rCutSmart	100	100	10	100	37°	80°	B	λ DNA			
ScrFI	rCutSmart	100	100	100	100	37°	65°	C	λ DNA	dcm	CpG	2, a
SexAI	rCutSmart	100	75	50	100	37°	65°	A	pBC4 DNA (dcm-)	dcm		3, b, d
SfaNI	r3.1	<10	75	100	25	37°	65°	B	ΦX174 RF I DNA		CpG	3, b
SfcI	rCutSmart	75	50	25	100	37°	65°	B	λ DNA			3
SfiI	rCutSmart	25	100	50	100	50°	No	C	pXba DNA	dcm	CpG	
SfoI	rCutSmart	50	100	100	100	37°	No	B	λ DNA (HindIII digest)	dcm	CpG	
SgrAI	rCutSmart	100	100	10	100	37°	65°	A	λ DNA		CpG	1
SmaI	rCutSmart	<10	<10	<10	100	37°	65°	B	λ DNA (HindIII digest)		CpG	b
SmlI	rCutSmart	25	75	25	100	55°	No	A	λ DNA			b
SnaBI	rCutSmart	50*	50	10	100	37°	80°	A	T7 DNA		CpG	1
SpeI-HF	rCutSmart	25	50	10	100	37°	80°	C	pXba-XbaI DNA			
SphI	r2.1	100	100	50	100	37°	65°	B	λ DNA			2
SphI-HF	rCutSmart	50	25	10	100	37°	65°	B	λ DNA			
SrfI	rCutSmart	10	50	0	100	37°	65°	B	pNEB193-SrfI DNA		CpG	
SspI-HF	rCutSmart	25	100	<10	100	37°	65°	B	λ DNA			
StuI	rCutSmart	50	100	50	100	37°	No	A	λ DNA	dcm		
StyD4I	rCutSmart	10	100	100	100	37°	65°	B	λ DNA	dcm	CpG	
StyI-HF	rCutSmart	25	100	25	100	37°	65°	A	λ DNA			
SwaI	r3.1	10	10	100	10	25°	65°	B	pXba DNA			b, d
TaqI-v2	rCutSmart	50	100	50	100	65°	No	B	λ DNA	dam		
TfiI	rCutSmart	50	100	100	100	65°	No	C	λ DNA		CpG	
TseI	rCutSmart	75	100	100	100	65°	No	B	λ DNA		CpG	3
Tsp45I	rCutSmart	100	50	<10	100	65°	No	A	λ DNA			
TspMI	rCutSmart	50*	75*	50*	100	75°	No	B	pBC4 DNA		CpG	d
TspRI	rCutSmart	25	50	25	100	65°	No	B	λ DNA			
Tth111I	rCutSmart	25	100	25	100	65°	No	B	pBC4 DNA			b
WarmStart Nt.BstNBI	r3.1	0	10	100	25	55°	80°	A	T7 DNA			
XbaI	rCutSmart	<10	100	75	100	37°	65°	A	λ DNA (dam-/Hind III digest)	dam		
XcmI	r2.1	10	100	25	100*	37°	65°	C	λ DNA			2
XhoI	rCutSmart	75	100	100	100	37°	65°	A	λ DNA (HindIII digest)		CpG	b
XmaI	rCutSmart	25	50	<10	100	37°	65°	A	pXba DNA		CpG	3
XmnI	rCutSmart	50	75	<10	100	37°	65°	A	λ DNA			b
ZraI	rCutSmart	100	25	10	100	37°	80°	B	λ DNA		CpG	

a. Ligation is less than 10%
b. Ligation is 25% – 75%
c. Recutting after ligation is < 5%
d. Recutting after ligation is 50% – 75%
e. Ligation and recutting after ligation is not applicable since the enzyme is either a nicking enzyme, is affected by methylation, or the recognition sequence contains variable sequences.

1. Star activity may result from extended digestion, high enzyme concentration or a glycerol concentration of > 5%.
2. Star activity may result from extended digestion.
3. Star activity may result from a glycerol concentration of > 5%.

* May exhibit star activity in this buffer.
+ NEB isoschizomer or HF enzyme supplied with rCutSmart Buffer.

Activity of DNA Modifying Enzymes in rCutSmart Buffer

A selection of DNA modifying enzymes were assayed in rCutSmart Buffer, in lieu of their supplied buffers. Functional activity was compared to the activity in its supplied buffer, plus required supplements. Reactions were set up according to the recommended reaction conditions, with rCutSmart Buffer replacing the supplied buffer.

Tech Tip: When supplements are required, one can simply add the supplied buffer of the respective modifying enzyme at 1× concentration to the rCutSmart Buffer to achieve appropriate activity for most applications – no change of buffers needed.

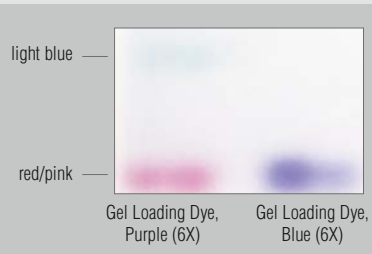
Enzyme	Activity in rCutSmart	Required Supplements
Phosphatases:		
Antarctic Phosphatase	+ + +	Requires Zn^{2+}
Shrimp Alkaline Phosphatase (rSAP)	+ + +	
Quick CIP	+ + +	
Ligases:		
T4 DNA Ligase	+ + +	Requires ATP
Hi-T4™ DNA Ligase	+++	Requires ATP
Salt-T4® DNA Ligase	+	Requires ATP
E. coli DNA Ligase	+ + +	Requires NAD
T3 DNA Ligase	+ + +	Requires ATP + PEG
T7 DNA Ligase	+ + +	Requires ATP + PEG
Polymerases:		
T4 DNA Polymerase	+ + +	
DNA Polymerase I, Large (Klenow) Fragment	+ + +	
DNA Polymerase I	+ + +	
DNA Polymerase Klenow Exo⁻	+ + +	
Bst DNA Polymerase	+ + +	
phi29 DNA Polymerase	+ + +	Requires DTT
T7 DNA Polymerase (unmodified)	+ + +	
Transferases/Kinases:		
T4 Polynucleotide Kinase	+ + +	Requires ATP + DTT
T4 PNK (3´ phosphatase minus)	+ + +	Requires ATP + DTT
CpG Methyltransferase (M. SssI)	+ + +	
GpC Methyltransferase (M. CviPI)	+	Requires DTT
T4 Phage β-glucosyltransferase (T4-BGT)	+ + +	
Nucleases, other:		
DNase I (RNase-free)	+ + +	Requires Ca^{2+}
DNase I-XT	+ + +	Requires Ca^{2+}
Endonuclease III (Nth)	+ + +	
Endonuclease VIII	+ + +	
Exonuclease VII	+ + +	
Exonuclease V (Rec BCD)	+ + +	Requires ATP
Exonuclease III	+ + +	
Exonuclease I	+ + +	
Fpg	+ + +	
Lambda Exonuclease	+ +	
McrBC	+ + +	
Micrococcal Nuclease	+ + +	Requires Ca^{2+}
$RecJ_f$	+ + +	
T5 Exonuclease	+ + +	
T7 Exonuclease	+ + +	
Thermolabile Exonuclease I	+ + +	
Thermolabile USER II Enzyme	+ + +	
Thermolabile USER III Enzyme	+ +	
Thermostable OGG	+ + +	
USER Enzyme, recombinant	+ + +	

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

RECOMMENDED PRODUCT

Gel Loading Dye, Purple (6X) (NEB #B7024)
NEB provides Gel Loading Dye, Purple (6X) with most of our restriction enzymes.

- No UV Shadow, allowing for publication grade images
- Contains Ficoll® for brighter, tighter bands
- Contains SDS for improved band sharpness
- Contains EDTA to stop enzymatic reactions
- Compatible with agarose and non-denaturing polyacrylamide gels
- Contains EDTA to stop enzymatic reactions
- Our Purple Gel Loading Dye sharpens bands and eliminates the UV shadow seen with other dyes. Available also without SDS (NEB #B7025).



COMPARISON OF DYE FRONTS

PCR/Amplification

Amplification can be performed to generate a blunt insert, or to have a 1-base overhang, depending on the polymerase used. Additionally, primers can be used to incorporate RE recognition sites. After amplification, the insert can be used directly or cloned into a holding vector, or RE digestion can be performed to generate cohesive ends. Amplification is often the first step for PCR cloning, seamless cloning, ligation independent cloning and recombinational cloning.

PCR Polymerase Selection Chart for Cloning

For over 40 years, New England Biolabs, Inc. has been a world leader in the discovery and production of reagents for the life science industry. NEB offers a wide range of DNA polymerases, and through our commitment to research, ensures the development of innovative and high quality tools for PCR and related applications. The following table simplifies the selection of a polymerase that best suits your cloning experiment.

	STANDARD PCR		HIGH-FIDELITY PCR		SPECIALTY PCR	
			Highest Fidelity		Long Amplicons	dU Tolerance
	One*Taq*/ One*Taq* Hot Start	*Taq* / Hot Start *Taq*	Q5/Q5 Hot Start	Phusion®(1)/ Phusion(1) Flex	LongAmp®/ LongAmp Hot Start *Taq*	Q5U®
PROPERTIES						
Fidelity vs. *Taq*	2X	1X	~280X(3)	> 50X	2X	ND
Amplicon Size	< 6 kb	≤ 5 kb	≤ 20 kb	≤ 20 kb	≤ 30 kb	app-specific
Extension Time	1 kb/min	1 kb/min	6 kb/min	4 kb/min	1.2 kb/min	2 kb/min
Resulting Ends	3´ A/Blunt	3´ A	Blunt	Blunt	3´ A/Blunt	Blunt
3´→ 5´ exo	Yes	No	Yes	Yes	Yes	Yes
5´→ 3´ exo	Yes	Yes	No	No	Yes	No
Units/50 µl Reaction	1.25	1.25	1.0	1.0	5.0	1.0
Annealing Temperature	Tm-5	Tm-5	Tm+3	Tm+3	Tm-5	Tm+3
APPLICATIONS						
Routine PCR	★	●	●	●	●	
Colony PCR	★	●				
Enhanced Fidelity	●		★	●	●	
High Fidelity			★	●		
High Yield	★	●	★	●		
Fast			★	●		
Long Amplicon			★	●	★	
GC-rich Targets	★		★		●	
AT-rich Targets	★	●	★	●	●	★
High Throughput	●	●	●	●		★
Multiplex PCR	●	★(2)	●	●		
DNA Labeling		★				
Site-directed Mutagenesis			★	●		
Carryover Prevention						★
USER® Cloning						★
FORMATS						
Hot Start Available	●	●	●	●	●	●
Kit		●	●	●	●	●
Master Mix Available	●	●	●	●	●	●
Direct Gel Loading	●	●				

★ indicates recommended choice for application

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

(2) Use Multiplex PCR 5X Master Mix.

(3) Due to the very low frequency of misincorporation events being measured, the error rate of high-fidelity enzymes like Q5 is challenging to measure in a statistically significant manner. We continue to investigate improved assays to characterize Q5's very low error rate to ensure that we present the most robust accurate fidelity data possible (Popatov, V. and Ong, J.L. (2017) PLoS One, 12(1):e0169774. doi 10.1371/journal. pone. 0169774).

GETTING STARTED

- When choosing a polymerase for PCR, we recommend starting with One*Taq* or Q5 DNA Polymerases (highlighted to the left in orange). Both offer robust amplification and can be used on a wide range of templates (routine, AT- and GC-rich). Q5 provides the benefit of maximum fidelity, and is also available in a formulation specifically optimized for next generation sequencing.

TOOLS & RESOURCES

Visit NEBPCRPolymerases.com to find:

- The full list of polymerases available
- FAQs & troubleshooting guides
- Interactive tools to help with experimental design
- Online tutorials for setting up PCR reactions

Why choose Q5 for your PCR?

For additional help with choosing the right polymerase for your PCR, we recommend using our PCR Selector at **PCRSelector.neb.com**.

Protocol: High-Fidelity PCR with Q5

	25 µl REACTION	50 µl REACTION	FINAL CONCENTRATION
5X Q5 Reaction Buffer*	5 µl	10 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM primers (forward and reverse)	1.25 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 1 µg
Nuclease-free water	to 25 µl	to 50 µl	
Q5 High-Fidelity DNA Polymerase**	0.25 µl	0.5 µl	0.02 units/50 µl rxn

* Q5 High GC Enhancer can be used for difficult amplicons.
** For amplicons > 6 kb, up to 2 units/50 µl rxn can be added.

	CYCLES	TEMP.	TIME
Initial denaturation:	1	98°C	30 seconds
Denaturation	30	98°C	5–10 seconds
Annealing		50–72°C*	10–30 seconds
Extension		72°C	20–30 seconds per kb
Final extension:	1	72°C	2 minutes
Hold:	1	4–10°C	

* Tm values should be determined using the NEB Tm calculator **(TmCalculator.neb.com)** Please note that Q5 and Phusion® annealing temperature recommendations are unique.

Protocol: Routine PCR with One*Taq*®

	25 µl REACTION	50 µl REACTION	FINAL CONCENTRATION
One*Taq* Standard 5X Reaction Buffer*	5 µl	10 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM primers (forward and reverse)	0.5 µl	1 µl	0.2 µM
Template DNA	variable	variable	< 1 µg
Nuclease-free water	to 25 µl	to 50 µl	
One*Taq* DNA Polymerase**	0.125 µl	0.25 µl	1.25 units/50 µl rxn

* If reaction buffer is 5X, volume should be doubled.
** Amount of polymerase added will depend on polymerase used. Refer to neb.com for more information.

	CYCLES	TEMP.	TIME
Initial denaturation:	1	94°C	30 seconds
Denaturation	30	94°C	15–30 seconds
Annealing		45–68°C*	15–60 seconds
Extension		68°C	1 minute per kb
Final extension:	1	68°C	5 minutes
Hold:	1	4–10°C	

* Tm values should be determined using the NEB Tm calculator **(TmCalculator.neb.com)**.

TIPS FOR OPTIMIZATION

When switching from a *Taq* product to a high-fidelity polymerase, remember to use:

- Higher annealing temps – check **TmCalculator.neb.com**
- Higher denaturation temps – particularly beneficial for difficult templates
- Higher primer concentrations
- Shorter cycling protocols

DNA TEMPLATE

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

PRIMERS

- Primers should typically be 20–40 nucleotides in length, with 40–60% GC content
- Primer Tm values should be determined with NEB's Tm Calculator **(TmCalculator.neb.com)**
- Primer pairs should have Tm values that are within 5°C
- Avoid secondary structure (i.e., hairpins) within each primer and potential dimerization between the primers
- Higher than recommended primer concentrations may decrease specificity
- When engineering restriction sites onto the end of primers, 6 nucleotides should be added 5´ to the site

ENZYME CONCENTRATION

- Optimal concentration is specific to each polymerase
- Master mix formulations already contain optimal enzyme concentrations for most applications

MAGNESIUM CONCENTRATION

- Most PCR buffers provided by NEB already contain sufficient levels of Mg^{++} at 1X concentrations
- Excess Mg^{++} may lead to spurious amplification; insufficient Mg^{++} concentrations may cause reaction failure

DEOXYNUCLEOTIDES

- Ideal dNTP concentration is typically 200 µM each
- The presence of uracil in the primer, template, or deoxynucleotide mix will cause reaction failure when using archaeal PCR polymerases. Use One*Taq*, *Taq* or Q5U DNA Polymerases for these applications

STARTING REACTIONS

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

DENATURATION

- Avoid longer or higher temperature incubations unless required due to high GC content of the template
- NEB's aptamer-based hot start enzymes do not require additional denaturation steps to activate the enzymes

ANNEALING

- Primer Tm values should be determined using the NEB Tm Calculator **(TmCalculator.neb.com)**
- Non-specific product formation can often be avoided by optimizing the annealing temperature or by switching to a hot start enzyme (e.g., Q5 Hot Start High-Fidelity DNA Polymerase or One*Taq* Hot Start DNA Polymerase)

EXTENSION

- Extension rates are specific to each PCR polymerase. In general, extension rates range from 15–60 s/kb.
- Longer than recommended extension times can result in higher error rates, spurious banding patterns and/or reduction of amplicon yields

Common DNA End Modifications

Modification of the termini of double-stranded DNA is often necessary to prepare the molecule for cloning. DNA ligases require a 5´ monophosphate on the donor end, and the acceptor end requires a 3´ hydroxyl group. Additionally, the sequences to be joined need to be compatible, either a blunt end being joined to another blunt end, or a cohesive end with a complementary overhang to another cohesive end. End modifications are performed to improve the efficiency of the cloning process, and ensure the ends to be joined are compatible.

Phosphorylation

Vectors and inserts digested by restriction enzymes contain the necessary terminal modifications (5´ phosphate and 3´ hydroxyl), while ends created by PCR may not. Typical amplification by PCR does not use phosphorylated primers. In this case, the 5´ ends of the amplicon are non-phosphorylated and need to be treated by a kinase, such as T4 Polynucleotide Kinase (NEB #M0201), to introduce the 5´ phosphate. Alternatively, primers for PCR can be ordered with 5´ phosphate to avoid the need to separately phosphorylate the PCR product with a kinase.

Protocol: Phosphorylation with T4 Polynucleotide Kinase

	STANDARD PROTOCOL
DNA	1–2 µg
10X Polynucleotide Kinase Buffer	5 µl
10 mM Adenosine 5´-Triphosphate (ATP)	5 µl (1 mM final concentration)
T4 Polynucleotide Kinase (PNK)	1 µl (10 units)
Nuclease-free water	to 50 µl
Incubation	37°C, 30 minutes

Dephosphorylation

Dephosphorylation is a common step in traditional cloning to ensure the vector does not re-circularize during ligation. If a vector is linearized by a single restriction enzyme or has been cut with two enzymes with compatible ends, use of a phosphatase to remove the 5´ phosphate reduces the occurrence of vector re-closure by intramolecular ligation and thereby reduces the background during subsequent transformation. If the vector is dephosphorylated, it is essential to ensure the insert contains a 5´ phosphate to allow ligation to proceed. Each double-strand break requires that one intact phosphodiester bond be created before transformation (and *in vivo* repair).

Phosphatase Selection Chart

FEATURES	Recombinant Shrimp Alkaline Phosphatase (rSAP) (NEB #M0371)	Antarctic Phosphatase (AP) (NEB #M0289)	Quick CIP (NEB #M0525)
100% heat inactivation	5 minutes/65°C	2 minutes/80°C	2 minutes/80°C
High specific activity	●		●
Improved stability	●		●
Works directly in NEB buffers	●	●	●
Requires additive		● (Zn^{2+})	
Quick Protocol			●

Protocol: Dephosphorylation using Quick CIP

	STANDARD PROTOCOL
DNA	1 pmol of ends
10X rCutSmart Buffer	2 µl
Quick CIP	1 µl
Nuclease-free water	to 20 µl
Incubation	37°C for 10 minutes
Heat Inactivation	80°C for 2 minutes

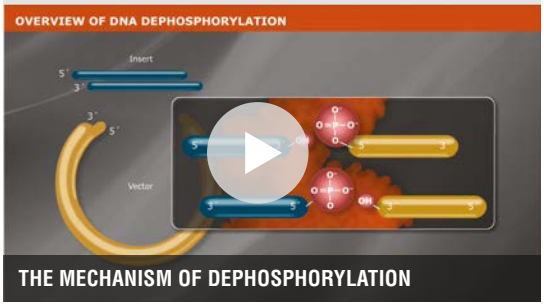
TIPS FOR OPTIMIZATION

ENZYME

- T4 Polynucleotide Kinase (NEB #M0201) and T4 DNA Ligase (NEB #M0202) can be used together in the T4 DNA Ligase Buffer
- T4 Polynucleotide Kinase is inhibited by high levels of salt (50% inhibition by 150 mM NaCl), phosphate (50% inhibition by 7 mM phosphate) and ammonium ions (75% inhibited by 7 mM $(NH_4)_2SO_4$)
- If using T4 Polynucleotide Kinase and working with 5´-recessed ends, heat the reaction mixture for 10 min at 70°C, chill rapidly on ice before adding the ATP (or Ligase Buffer containing ATP) and enzyme, then incubate at 37°C

ADDITIVES

- The addition of PEG 8000 (up to 5%) can improve results



Find an overview of dephosphorylation.

TIPS FOR OPTIMIZATION

ENZYME

- When dephosphorylating a fragment following a restriction enzyme digest, a DNA clean up step is required if the restriction enzyme(s) used is NOT heat inactivatable. We recommend the Monarch PCR & DNA Cleanup Kit (NEB #T1030).
- NEB recommends Quick CIP as a starting point for dephosphorylation
- When working with the Quick CIP (NEB #M0525), rSAP (NEB #M0371) or AP (NEB #M0289), which are heat-inactivatable enzymes, a DNA clean-up step after dephosphorylation is not necessary prior to the ligation step.

ADDITIVES

- AP requires the presence of Zn^{2+} in the reaction, so don't forget to supplement the reaction with 1X Antarctic Phosphatase Reaction Buffer when using other NEBuffers

Blunting/End-repair

Blunting is a process by which the single-stranded overhang created by a restriction digest is either "filled in", by adding nucleotides on the complementary strand using the overhang as a template for polymerization, or by "chewing back" the overhang, using an exonuclease activity. Vectors and inserts are often "blunted" to allow non-compatible ends to be joined. Sequence information is lost or distorted by doing this and a detailed understanding of the modification should be considered before performing this procedure. Often, as long as the sequence being altered is not part of the translated region or a critical regulatory element, the consequence of creating blunt ends is negligible. Blunting a region of translated coding sequence, however, usually creates a shift in the reading frame. DNA polymerases, such as the Klenow Fragment of DNA Polymerase I and T4 DNA Polymerase, included in our Quick Blunting Kit (NEB #E1202), are often used to fill in (5´→3´) and chew back (3´→5´). Removal of a 5´ overhang can be accomplished with a nuclease, such as Mung Bean Nuclease (NEB #M0250).

Blunting Selection Chart

	T4 DNA Polymerase* (NEB #M0203)	DNA Polymerase I, Large (Klenow) Fragment (NEB #M0210)	Quick Blunting Kit (NEB #E1201)	Mung Bean Nuclease (NEB #M0250)
APPLICATION				
Fill in of 5´ overhangs	•	•	•	
Removal of 3´ overhangs	•	•	•	•
Removal of 5´ overhangs				•

* T4 DNA Polymerase has a strong 3´→ 5´ exo activity.

Protocol: Blunting using the Quick Blunting Kit

	STANDARD PROTOCOL
DNA	up to 5 µg
10X Blunting Buffer	2.5 µl
1 mM dNTP Mix	2.5 µl
Blunt Enzyme Mix	1 µl
Nuclease-free water	to 25 µl
Incubation	room temperature; 15 min for RE-digested DNA; 30 min for sheared/nebulized DNA or PCR products*
Heat Inactivation	70°C, 10 minutes

* PCR generated DNA must be purified before blunting by using a purification kit (NEB #T1030), phenol extraction/ethanol precipitation, or gel extraction (NEB #T1020).

TIPS FOR OPTIMIZATION

ENZYME

- Make sure that you choose the correct enzyme to blunt your fragment. The Quick Blunting Kit (NEB #E1201), T4 DNA Polymerase (NEB #M0203) and DNA Polymerase I, Large (Klenow) Fragment (NEB #M0210) will fill 5´ overhangs and degrade 3´ overhangs. Mung Bean Nuclease (NEB #M0250) degrades 5´ overhangs
- T4 DNA Polymerase and DNA Polymerase I, Large (Klenow) Fragment are active in all NEBuffers. Please remember to add dNTPs

CLEAN-UP

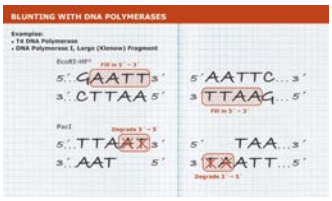
- When trying to blunt a fragment after a restriction enzyme digestion, if the restriction enzyme(s) used are heat inactivable, then a clean up step prior to blunting is not needed. Alternatively, if the restriction enzyme(s) used are not heat inactivable, a DNA clean up step is recommended prior to blunting.
- When trying to blunt a fragment amplified by PCR, a DNA clean up step (e.g., Monarch PCR & DNA Cleanup Kit, NEB #T1030) is necessary prior to the blunting step to remove the nucleotides and polymerase
- When trying to dephosphorylate a fragment after the blunting step, you will need to add a DNA clean up step (e.g., Monarch PCR & DNA Cleanup Kit, NEB #T1030) after the blunting and before the addition of the phosphatase

TEMPERATURE

- When trying to blunt a fragment with Mung Bean Nuclease, the recommended temperature of incubation is room temperature, since higher temperatures may cause sufficient breathing of the dsDNA ends that the enzyme may degrade some of the dsDNA sequence. The number of units to be used and time of incubation may be determined empirically to obtain best results

HEAT INACTIVATION

- Mung Bean nuclease reactions should not be heat inactivated. Although Mung Bean Nuclease can be inactivated by heat, this is not recommended because the DNA begins to "breathe" before the Mung Bean Nuclease is inactivated and undesirable degradation occurs at breathing sections. Purify DNA by phenol/chloroform extraction and ethanol precipitation or spin column purification (NEB #T1030)



The DNA blunting tutorial will teach you how to identify what type of overhang you have, as well as which enzyme will blunt that end, and how.

A-tailing

Tailing is an enzymatic method to add a non-templated nucleotide to the 3´ end of a blunt, double-stranded DNA molecule. Tailing is typically done to prepare a T-vector for use in TA cloning or to A-tail a PCR product produced by a high-fidelity polymerase (not *Taq* DNA Polymerase) for use in TA cloning. TA cloning is a rapid method of cloning PCR products that utilizes stabilization of the single-base extension (adenosine) produced by *Taq* DNA Polymerase by the complementary T (thymidine) of the T-vector prior to ligation and transformation. This technique does not utilize restriction enzymes and PCR products can be used directly without modification. Additionally, PCR primers do not need to be designed with restriction sites, making the process less complicated. One drawback is that the method is non-directional; the insert can go into the vector in both orientations.

TIPS FOR OPTIMIZATION

- If the fragment to be tailed has been amplified with a high-fidelity polymerase, the DNA needs to be purified prior to the tailing reaction. For this we recommend the Monarch PCR & DNA Cleanup Kit (NEB #T1030). Otherwise, any high-fidelity polymerase present in the reaction will be able to remove any non-templated nucleotides added to the end of the fragments.

A-tailing Selection Chart

	Klenow Fragment (3´→5´ exo⁻) (NEB #M0212)	***Taq* DNA Polymerase**
FEATURES		
Reaction temperature	37°C	75°C
Heat inactivated	75°C, 20 minutes	No
Nucleotide cofactor	dATP	dATP

Protocol: A-tailing with Klenow Fragment (3´→ 5´ exo⁻)

	STANDARD PROTOCOL
Purified, blunt DNA	1–5 µg*
NEBuffer 2 (10X)	5 µl
dATP (1 mM)	0.5 µl (0.1 mM final)
Klenow Fragment (3´→5´ exo⁻) (NEB #M0212)	3 µl
H_2O	to 50 µl
Incubation	37°C, 30 minutes

* If starting with blunt-ended DNA that has been prepared by PCR or end polishing, DNA must be purified to remove the blunting enzymes.

Vector and Insert Joining

DNA Ligation

Ligation of DNA is a critical step in many modern molecular biology workflows. The sealing of nicks between adjacent residues of a single-strand break on a double-strand substrate and the joining of double-strand breaks are enzymatically catalyzed by DNA ligases. The formation of a phosphodiester bond between the 3´ hydroxyl and 5´ phosphate of adjacent DNA residues proceeds in three steps: Initially, the ligase is self-adenylated by reaction with free ATP. Next, the adenyl group is transferred to the 5´ phosphorylated end of the "donor" strand. Lastly, the formation of the phosphodiester bond proceeds after reaction of the adenylated donor end with the adjacent 3´ hydroxyl acceptor and the release of AMP. In living organisms, DNA ligases are essential enzymes with critical roles in DNA replication and repair. In the lab, DNA ligation is performed for both cloning and non-cloning applications.

Molecular cloning is a method to prepare a recombinant DNA molecule, an extra-chromosomal circular DNA that can replicate autonomously within a microbial host. DNA ligation is commonly used in molecular cloning projects to physically join a DNA vector to a sequence of interest ("insert"). The ends of the DNA fragments can be blunt or cohesive and at least one must contain a monophosphate group on its 5´ ends. Following the mechanism described above, the covalent bonds are formed and a closed circular molecule is created that is capable of transforming a host bacterial strain. The recombinant plasmid maintained in the host is then available for amplification prior to downstream applications such as DNA sequencing, protein expression, or gene expression/functional analysis.

Recently, NEB has published research on T4 DNA Ligase fidelity. This information enables improved DNA assembly methods (such as Golden Gate). Please visit www.neb.com/GoldenGate to try our free Ligase Fidelity Tools and for more information.

DNA Ligase Selection Chart for Cloning

	Instant Sticky-end Ligase Master Mix (NEB #M0370)	Blunt/TA Ligase Master Mix (NEB #M0367)	ElectroLigase® (NEB #M0369)	T4 DNA Ligase (NEB #M0202)	Quick Ligation Kit (NEB #M2200)	T3 DNA Ligase (NEB #M0317)	T7 DNA Ligase (NEB #M0318)	HiFi *Taq* DNA Ligase (NEB #M0647)	Salt-T4® DNA Ligase (NEB #M0467)	Hi-T4™ DNA Ligase (NEB #M2622)	NEBridge Ligase Master Mix (NEB #M1100)
DNA APPLICATIONS											
Ligation of sticky ends	●●●	●●	●●	●●	●●●	●●	●●	●	●●	●●	●
Ligation of blunt ends	●	●●●	●●	●●	●●●	●●			●●	●●	●
T/A cloning	●	●●●	●●	●●	●●	●	●		●●	●●	
Electroporation			●●●	●●						●●	
Ligation of sticky ends only							●●●				
Repair of nicks in dsDNA	●●	●●	●●	●●●	●●	●●	●●	●●	●●●	●●●	
High complexity library cloning	●●	●●	●●	●●●	●●						
FEATURES											
Salt tolerance (> 2X that of T4 DNA Ligase)						✓			✓		
Ligation in 15 min. or less	✓	✓		✓	✓	✓	✓	✓	✓	✓	
Master Mix Formulation	✓	✓									✓
Thermostable								✓			
Thermotolerant										✓	
Recombinant	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

KEY

- ●●● Recommended product(s) for selected application
- ●● Works well for selected application
- ● Will perform selected application, but is not recommended

GETTING STARTED

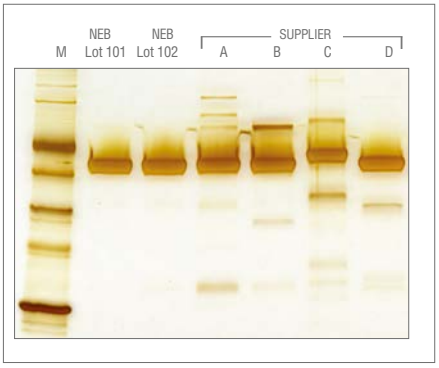
For traditional cloning, follow the ligation guidelines specified by the ligase supplier. If they suggest a 3:1 molar ratio of insert to vector, try this first for the best result. Using a 3:1 mass ratio is not the same thing (unless the insert and vector have the same mass). To calculate how much of your insert and vector to add, use **NEBioCalculator** at **NEBioCalculator.neb.com**. Ligation usually proceeds very quickly and, unless your cloning project requires the generation of a high-complexity library that benefits from the absolute capture of every possible ligation product, long incubation times are not necessary.

TOOLS & RESOURCES

Visit NEBStickTogether.com to find:

- The full list of DNA ligases available
- FAQs
- Videos about ligation and help with setting up ligation reactions

Experience extreme purity with NEB's T4 DNA Ligase

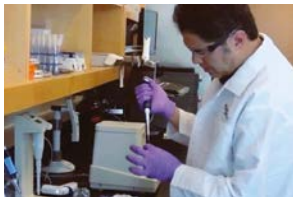
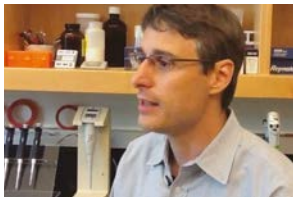
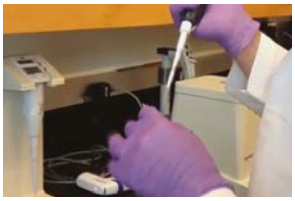


Equivalent amounts of protein were loaded and silver stained using SilverXpress™. Marker M is NEB's Broad Range Protein Marker (NEB #P7702).

Protocol: Ligation

	Quick Ligation Kit (NEB #M2200)	T4 DNA Ligase (NEB #M0202)	Instant Sticky-end Master Mix (NEB #M0370)	Blunt/TA Master Mix (NEB #M0367)
Format	Kit	Enzyme	Master Mix	Master Mix
Vector (4 kb)	50 ng	50 ng	50 ng	50 ng
Insert (1 kb)	37.5 ng	37.5 ng	37.5 ng	37.5 ng
Buffer	2X Quick Ligation Buffer	T4 DNA Ligase Reaction Buffer	5 µl (Master Mix)	5 µl (Master Mix)
Ligase	1 µl	1 µl	N/A	N/A
Nuclease-free water	to 20 µl	to 20 µl	to 10 µl	to 10 µl
Incubation	25°C, 5 minutes	25°C, 2 hrs; 16°C, overnight*	N/A, instant ligation	25°C, 15 minutes

* For sticky-end ligation, the incubation time can be shortened to 25°C for 10 minutes.

For more information on the mechanisms of ligation and tips for optimization, view our videos at **NEBStickTogether.com**

TIPS FOR OPTIMIZATION

REACTION BUFFERS

- T4 DNA Ligase Buffer (NEB #B0202) should be thawed on the bench or in the palm of your hand, and not at 37°C (to prevent breakdown of ATP)
- Once thawed, T4 DNA Ligase Buffer should be placed on ice
- Ligations can also be performed in any of the four standard restriction endonuclease NEBuffers or in T4 Polynucleotide Kinase Buffer (NEB #B0201) supplemented with 1 mM ATP
- When supplementing with ATP, use ribo-ATP (NEB #P0756). Deoxyribo-ATP will inhibit ligation.
- Before ligation, completely inactivate the restriction enzyme by heat inactivation, spin column (e.g., Monarch PCR & DNA Cleanup Kit, NEB #T1030) or Phenol/EtOH purification

DNA

- Either heat inactivate (AP, rSAP, Quick CIP) or remove phosphatase (rSAP) before ligation
- Keep total DNA concentration between 5–10 µg/ml
- Vector:Insert molar ratios between 1:1 and 1:10 are optimal for single insertions
- For cloning more than one insert, we recommend the NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621) or Cloning Kit (NEB #E5520)
- If you are unsure of your DNA concentration, perform multiple ligations with varying ratios

LIGASE

- For cohesive-end ligations, standard T4 DNA Ligase. Instant Sticky-end Ligase Master Mix or the Quick Ligation Kit are recommended.
- For blunt and single-base overhangs the Blunt/TA Ligase Master Mix is recommended
- For ligations that are compatible with electroporation, Electroligase is recommended
- Standard T4 DNA Ligase can be heat inactivated at 65°C for 20 minutes
- Do not heat inactivate the Quick Ligation Kit or the ligase master mixes

TRANSFORMATION

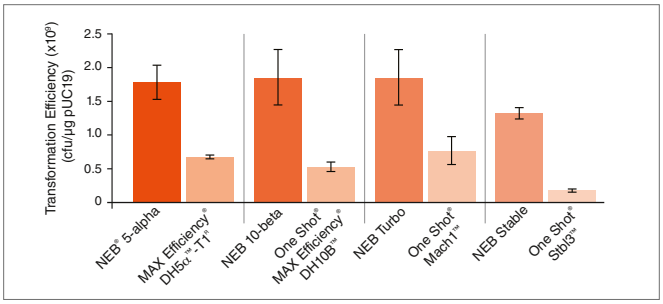
- Add between 1–5 µl of ligation mixture to competent cells for transformation
- Extended ligation with PEG causes a drop off in transformation efficiency
- Electroporation is recommended for larger constructs (> 10,000 bp). Dialyze samples or use a spin column first if you have used the Quick Ligation Kit or ligase master mixes.
- For ligations that are compatible with electroporation, Electroligase is recommended

Find an overview of ligation.

Transformation

Transformation is the process by which an organism acquires exogenous DNA. Transformation can occur in two ways: natural transformation and artificial transformation. Natural transformation describes the uptake and incorporation of naked DNA from the cell's natural environment. Artificial transformation encompasses a wide array of methods for inducing uptake of exogenous DNA. In cloning protocols, artificial transformation is used to introduce recombinant DNA into host bacteria. The most common method of artificial transformation of bacteria involves use of divalent cations (e.g., calcium chloride) to increase the permeability of the bacterium's membrane, making them chemically competent, and thereby increasing the likelihood of DNA acquisition. Another artificial method of transformation is electroporation, in which cells are shocked with an electric current, to create holes in the bacterial membrane. With a newly-compromised cell membrane, the transforming DNA is free to pass into the cytosol of the bacterium. Regardless of which method of transformation is used, outgrowth of bacteria following transformation allows repair of the bacterial surface and selection of recombinant cells if the newly acquired DNA conveys antibiotic resistance to the transformed cells.

Benefit from High Transformation Efficiencies



The transformation efficiencies were compared using manufacturers' recommended protocols. Values shown are the average of triplicate experiments.

Competent Cell Selection Chart

	NEB 5-alpha Competent *E. coli* (NEB #C2987)	NEB Turbo Competent *E. coli* (NEB #C2984)	NEB 5-alpha F´ I^q Competent *E. coli* (NEB #C2992)	NEB 10-beta Competent *E. coli* (NEB #C3019)	*dam*⁻/*dcm*⁻ Competent *E. coli* (NEB #C2925)	NEB Stable Competent *E. coli* (NEB #C3040)
FEATURES						
Versatile	•					•
Fast growth (< 8 hours)		•				
Toxic gene cloning			•			•
Large plasmid/BAC cloning				•		
Dam/Dcm-free plasmid growth					•	
Retroviral/lentiviral vector cloning						•
recA⁻	•		•	•		•
FORMATS						
Chemically competent	•	•	•	•	•	•
Electrocompetent				•		
Subcloning	•					
96-well format	•			•		
384-well format	•					
12 x 8-tube strips	•					

TIPS FOR OPTIMIZATION

THAWING

- Cells are best thawed on ice
- DNA should be added as soon as the last trace of ice in the tube disappears
- Cells can be thawed by hand, but warming above 0°C decreases efficiency

DNA

- Up to 10 µl of DNA from a ligation mix can be used with only a 2-fold loss of efficiency

INCUBATION & HEAT SHOCK

- Incubate on ice for 30 minutes. Expect a 2-fold loss in transformation efficiency (TE) for every 10 minutes this step is shortened
- Both temperature and time are specific to the transformation volume and vessel. Typically, 30 seconds at 42°C is recommended, except when using BL21 (NEB #C2530) which requires exactly 10 seconds

OUTGROWTH

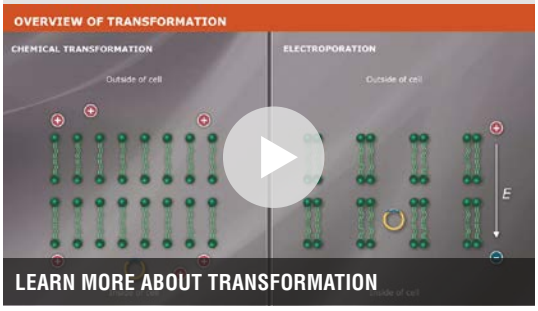
- Outgrowth at 37°C for 1 hour is best for cell recovery and for expression of antibiotic resistance. Expect a 2-fold loss in TE for every 15 minutes this step is shortened.
- SOC gives 2-fold higher TE than LB medium
- Incubation with shaking or rotation results in 2-fold higher TE

PLATING

- Selection plates can be used warm or cold, wet or dry with no significant effects on TE
- Warm, dry plates are easier to spread and allow for the most rapid colony formation

DNA CONTAMINANTS TO AVOID

CONTAMINANT	REMOVAL METHOD
Detergents	Ethanol precipitate
Phenol	Extract with chloroform and ethanol precipitate
Ethanol or Isopropanol	Dry pellet before resuspending
PEG	Column purify (e.g., Monarch PCR & DNA Cleanup Kit) or phenol/ chloroform extract and ethanol precipitate

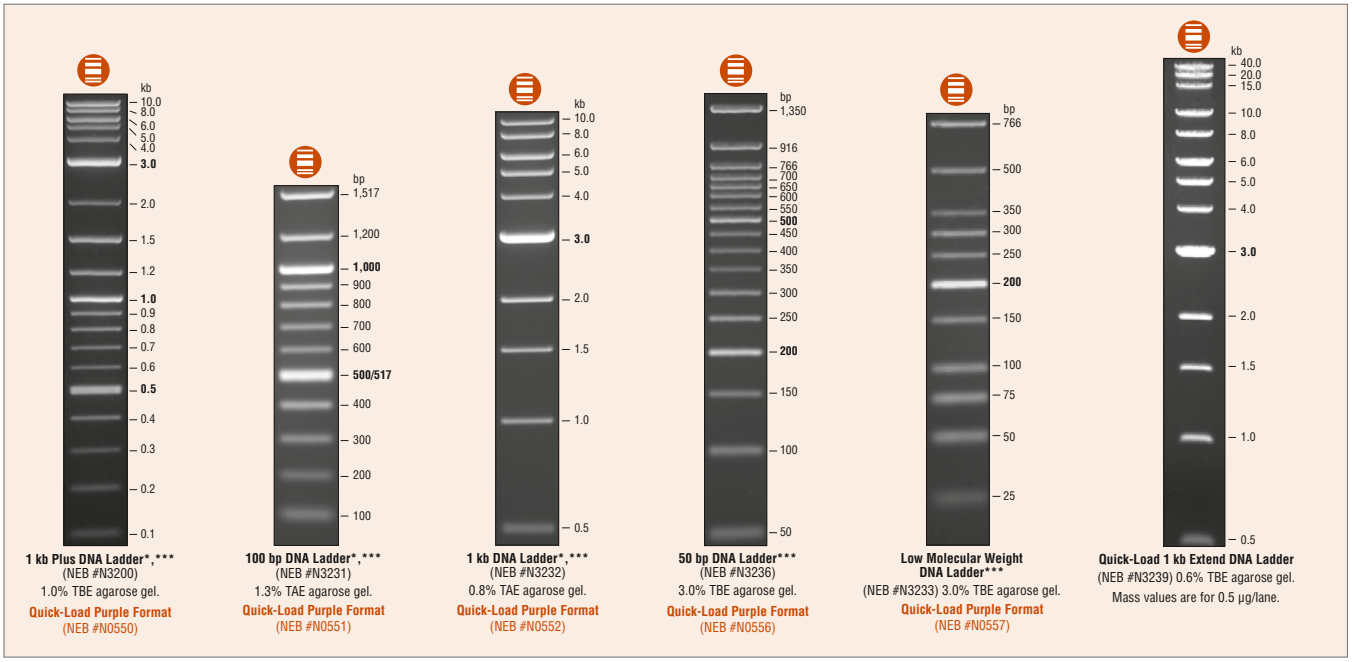


For direct access scan QR code or visit **www.neb.com/nebtv** to find all tutorials available.

DNA Markers and Ladders

Agarose-gel electrophoresis is the standard method used for separation, identification and purification of DNA fragments. DNA is visualized on a gel after soaking or pre-casting the gel with a visualization dye, such as Ethidium Bromide, which is a DNA intercalating agent that fluoresces under UV illumination. DNA markers and ladders are composed of DNA fragments of known sizes and masses which are used as a reference to determine the size and relative mass of the DNA of interest. Bands are visible under UV illumination or under blue light illumination, depending on the visualization dye used. DNA markers and DNA samples have to be combined with loading dyes to give them density in the wells and to track the migration on the gel; some of NEB's ladders come pre-mixed with loading dye for convenience.

Quick-Load and Quick-Load Purple DNA Ladders



1 kb Plus DNA Ladder*,***
(NEB #N3200)
1.0% TBE agarose gel.
Quick-Load Purple Format
(NEB #N0550)

100 bp DNA Ladder*,***
(NEB #N3231)
1.3% TAE agarose gel.
Quick-Load Purple Format
(NEB #N0551)

1 kb DNA Ladder*,***
(NEB #N3232)
0.8% TAE agarose gel.
Quick-Load Purple Format
(NEB #N0552)

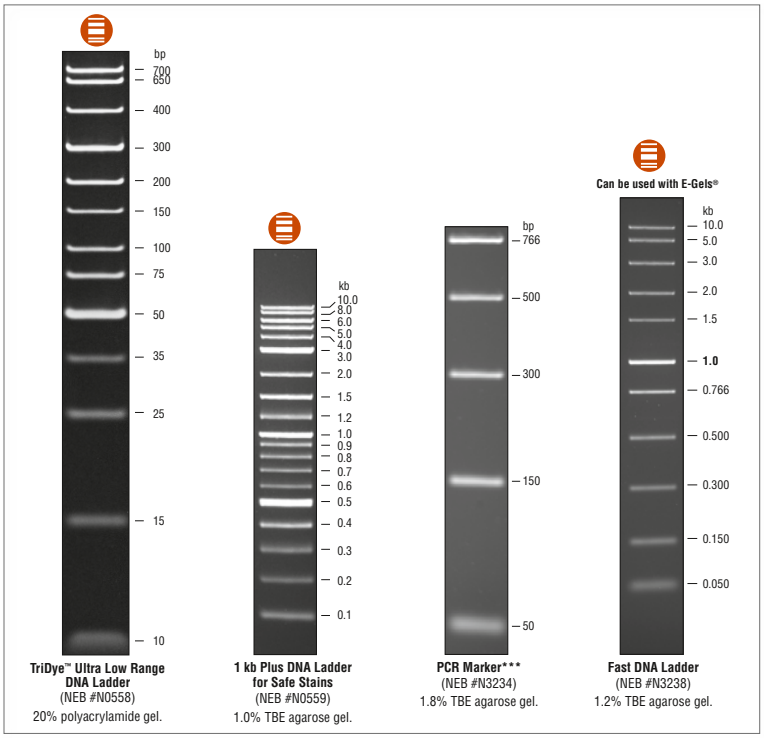
50 bp DNA Ladder***
(NEB #N3236)
3.0% TBE agarose gel.
Quick-Load Purple Format
(NEB #N0556)

Low Molecular Weight DNA Ladder***
(NEB #N3233) 3.0% TBE agarose gel.
Quick-Load Purple Format
(NEB #N0557)

Quick-Load 1 kb Extend DNA Ladder
(NEB #N3239) 0.6% TBE agarose gel.
Mass values are for 0.5 µg/lane.

** Available in Quick-Load® and TriDye™ formats* *Ready-to-Load* **** Free Loading Dye included*

Additional DNA Ladders from NEB



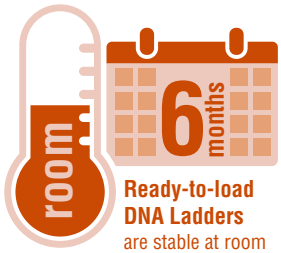
TriDye™ Ultra Low Range DNA Ladder
(NEB #N0558)
20% polyacrylamide gel.

1 kb Plus DNA Ladder for Safe Stains
(NEB #N0559)
1.0% TBE agarose gel.

PCR Marker***
(NEB #N3234)
1.8% TBE agarose gel.

Fast DNA Ladder
(NEB #N3238)
1.2% TBE agarose gel.

- Sharp, crisp bands
- Excellent quality and value
- Convenient 1 kb Plus DNA Ladder available in a variety of formats, including one specifically optimized for safe stains (e.g., GelRed® and SYBR® Safe)
- TriDye Ultra Low Range DNA Ladder ranges as low as 10 bp and is suitable for polyacrylamide gels
- For help with choosing a ladder, visit **www.neb.com/DNAmarkersandladders**



Ready-to-load DNA Ladders
are stable at room temp. for 6 months

Traditional Cloning Quick Guide

Preparation of insert and vectors

Insert from a plasmid source

- Digest plasmid with the 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 Q5 High-Fidelity DNA Polymerase (NEB #M0491)
- Visit **www.NEBPCRPolymerases.com** for additional guidelines for PCR optimization
- Purify PCR product by running the DNA on an agarose gel and excising the band or by using a spin column (e.g., Monarch DNA Gel Extraction Kit, NEB #T1020, Monarch PCR & DNA Cleanup Kit, NEB #T1030)
- Digest with the appropriate restriction enzyme

Standard Restriction Enzyme Protocol

DNA	1 µg
10X NEBuffer	5 µl (1X)
Restriction Enzyme	10 units is sufficient, generally 1 µl is used
Nuclease-free Water	to 50 µl
Incubation Time	1 hour*
Incubation Temperature	Enzyme dependent

* Can be decreased by using a Time-Saver qualified enzyme.

Time-Saver Restriction Enzyme Protocol

DNA	1 µg
10X NEBuffer	5 µl (1X)
Restriction Enzyme	1 µl
Nuclease-free Water	to 50 µl
Incubation Time	5–15 minutes*
Incubation Temperature	Enzyme dependent

* Time-Saver qualified enzymes can also be incubated overnight with no star activity.

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 appropriate enzymes
- Non-phosphorylated oligos can be phosphorylated using T4 Polynucleotide Kinase (NEB #M0201)

Typical Annealing Reaction

Oligo 1	20 µM Final concentration
Oligo 2	20 µM Final concentration
10X NEBuffer r2.1	5 µl
Nuclease-free Water	to 50 µl
Incubation	95°C for 5 minutes, cool slowly to room temp.

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 four products for dephosphorylation of DNA:
- Quick CIP (NEB #M0525), Shrimp Alkaline Phosphatase (rSAP) (NEB #M0371) and Antarctic Phosphatase (AP) (NEB #M0289) are heat-inactivatable phosphatases. They work in all NEBuffers, but AP requires supplementation with Zn^{2+}.

Dephosphorylation of 5´ ends of DNA using Quick CIP

DNA	1 pmol of DNA ends
10X rCutSmart Buffer	2 µl
Quick CIP	1 µl
Nuclease-free Water	to 20 µl
Incubation	37°C for 10 minutes
Heat Inactivation	80°C for 2 minutes

Note: Scale larger reaction volumes proportionally.

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 and chew back a 3´ overhang
- The Quick Blunting Kit (NEB #E1201) is optimized to blunt and phosphorylate DNA ends for cloning in less than 30 minutes
- Analyze agarose gels with longwave UV (365 nM) to minimize UV exposure that may cause DNA damage

Blunting with the Quick Blunting Kit

DNA	Up to 5 µg
Blunting Buffer	2.5 µl
dNTP Mix (1 mM)	2.5 µl
Blunt Enzyme Mix	1 µl
Nuclease-free Water	to 25 µl
Incubation	room temperature; 15 min for RE-digested DNA; 30 min for sheared/nebulized DNA or PCR products*
Heat Inactivation	70°C for 10 minutes

* PCR-generated DNA must be purified before blunting using a purification kit (NEB #T1030), phenol extraction/ethanol precipitation or gel extraction (NEB #T1020).

Getting started with Molecular Cloning:
Simple tips to improve your cloning effeciency.

Traditional Cloning Quick Guide (Cont.)

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). T4 PNK can be inactivated at 65°C for 20 minutes.

Phosphorylation with T4 PNK

DNA (20 mer)	up to 300 pmol of 5´ termini
10X T4 PNK Buffer	5 µl
10 mM ATP	5 µl (1 mM final conc.)
T4 PNK	1 µl (10 units)
Nuclease-free Water	to 50 µl
Incubation	37°C for 30 minutes

Purification of Vector and Insert

- Purify the vector and insert by either running the DNA on an agarose gel and excising the appropriate bands or by using a spin column, such as Monarch DNA Gel Extraction Kit or PCR & DNA Cleanup Kit (NEB #T1020 or T1030)
- 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 (365 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. Use NEBioCalculator to calculate molar ratios.
- If using T4 DNA Ligase (NEB #M0202) or the Quick Ligation Kit (NEB #M2200), thaw and resuspend the Ligase Buffer at room temp. If using Ligase Master Mixes, no thawing is necessary.
- The Quick Ligation Kit (NEB #M2200) is optimized for ligation of both sticky and blunt ends
- Instant Sticky-end Ligase Master Mix (NEB #M0370) is optimized for instant ligation of sticky/cohesive ends
- Blunt/TA Ligase Master Mix (NEB #M0367) is optimized for ligation of blunt or single base overhangs, which are the more challenging type of ends for T4 DNA Ligase
- Following ligation, chill on ice and transform
- DO NOT heat inactivate when using the Quick Ligation Buffer or Ligase Master Mixes, as this will inhibit transformation
- Electroligase (NEB #M0369) is optimized for ligation of both sticky and blunt ends and is compatible with electroporation (i.e., no cleanup step required)
- Improved Golden Gate Assembly can be achieved by selecting high fidelity overhangs [Potapov, V. et al (2018) *ACS Synth. Biol.* 7(11), 2665–2674].

Ligation with the Quick Ligation Kit

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

Ligation with Instant Sticky-end Ligase Master Mix

Vector DNA (3 kb)	50 ng
Insert DNA (1 kb)	50 ng
Master Mix	5 µl
Nuclease-free Water	to 10 µl
Incubation	None

Ligation with Blunt/TA Ligase Master Mix

Vector DNA (3 kb)	50 ng
Insert DNA (1 kb)	50 ng
Master Mix	5 µl
Nuclease-free Water	to 10 µl
Incubation	Room temperature for 15 minutes

Transformation

- To obtain transformants in 8 hrs., use NEB Turbo Competent *E. coli* (NEB #C2984)
- If recombination is a concern, then use the *recA*⁻ strains NEB 5-alpha Competent *E. coli* (NEB #C2987), or NEB-10 beta Competent *E. coli* (NEB #C3019) or NEB Stable Competent *E. coli* (NEB #C3040)
- NEB-10 beta Competent *E. coli* works well for constructs larger than 5 kb
- NEB Stable Competent *E. coli* (NEB #C3040) can be used for constructs with repetitive sequences such as lentiviral constructs
- If electroporation is required, use NEB 10-beta Electrocompetent *E. coli* (NEB #C3020)
- Use pre-warmed selection plates
- Perform several 10-fold serial dilutions in SOC or NEB 10-beta/Stable Outgrowth Medium for plating

Transformation with NEB 5-alpha Competent *E. coli*

DNA	1–5 µl containing 1 pg–100 ng of plasmid DNA
Competent *E. coli*	50 µl
Incubation	On ice for 30 minutes
Heat Shock	Exactly 42°C for exactly 30 seconds
Incubation	On ice for 5 minutes Add 950 µl room temperature SOC 37°C for 60 minutes, with shaking

Troubleshooting Guide for Cloning

We strongly recommend running the following controls during transformations. These controls may help troubleshoot which step(s) in the cloning workflow has failed.

1. Transform 100 pg – 1 ng of uncut vector to check cell viability, calculate transformation efficiency and verify the antibiotic resistance of the plasmid.
2. Transform the cut vector to determine the amount of background due to undigested plasmid. The number of colonies in this control should be < 1% of the number of colonies in the uncut plasmid control transformation (from control #1).
3. Transform a vector only ligation reaction. The ends of the vector should not be able to re-ligate because either they are incompatible (e.g., digested with two restriction enzymes that do not generate compatible ends) or the 5´ phosphate group has been removed in a dephosphorylation reaction (e.g., blunt ends treated with rSAP). This control transformation should yield the same number of colonies as control #2.
4. Digest vector DNA with a single restriction enzyme, re-ligate and transform. The ends of the vector DNA should be compatible and easily joined during the ligation reaction, resulting in approximately the same number of colonies as control #1.

The cloning workflow often benefits from an accurate quantitation of the amount of DNAs that are being worked with. We recommend quantification of DNAs whenever possible.

PROBLEM	CAUSE	SOLUTION
Few or no transformants	Cells are not viable	• Transform an uncut plasmid (e.g., pUC19) and calculate the transformation efficiency of the competent cells. If the transformation efficiency is low (< 10^4) re-make the competent cells or consider using commercially available high efficiency competent cells.
	Incorrect antibiotic or antibiotic concentration	• Confirm antibiotic and antibiotic concentration
	DNA fragment of interest is toxic to the cells	• Incubate plates at lower temperature (25–30°C) • Transformation may need to be carried out using a strain that exerts tighter transcriptional control over the DNA fragment of interest [e.g., NEB-5-alpha F´ I^q Competent *E. coli* (NEB #C2992)]
	If using chemically competent cells, the wrong heat-shock protocol was used	• Follow the manufacturer's specific transformation protocol (Note: going above the recommended temperature during the heat shock can result in competent cell death)
	If using electrocompetent cells, PEG is present in the ligation mix	• Clean up DNA by drop dialysis prior to transformation with Monarch PCR & DNA Cleanup Kit (NEB #T1030) • Try NEB's ElectroLigase (NEB #M0369)
	If using electrocompetent cells, arcing was observed or no voltage was registered	• Clean up the DNA prior to the ligation step • Tap the cuvette to get rid of any trapped air bubbles • Be sure to follow the manufacturer's specified electroporation parameters
	Construct is too large	• Select a competent cell strain that can be transformed efficiently with large DNA constructs [≥ 10 kb, we recommend trying NEB 10-beta Competent *E. coli* (NEB #C3019)] or NEB Stable Competent *E. coli* (NEB #C3040) • For very large constructs (> 10 kb), consider using electroporation
	Construct may be susceptible to recombination	• Select a *recA*- strain such as NEB 5-alpha (NEB #C2987) or NEB 10-beta Competent *E. coli* (NEB #C3019) or NEB Stable Competent *E. coli* (NEB #C3040)
	The insert comes directly from mammalian or plant DNA and contains methylated cytosines, which are degraded by many *E. coli* strains	• Use a strain that is deficient in McrA, McrBC and Mrr, such as NEB 10-beta Competent *E. coli*
	Too much ligation mixture was used	• Use < 5 µl of the ligation reaction for the transformation
	Inefficient ligation	• Make sure that at least one fragment being ligated contains a 5´ phosphate moiety • Vary the molar ratio of vector to insert from 1:1 to 1:10. Use NEBioCalculator to calculate molar ratios. • Purify the DNA to remove contaminants such as salt and EDTA with Monarch PCR & DNA Cleanup Kit (5 µg) (NEB #T1030) • ATP will degrade after multiple freeze-thaws; repeat the ligation with fresh buffer • Heat inactivate or remove the phosphatase prior to ligation • Ligation of single base-pair overhangs (most difficult) may benefit from being carried out with Blunt/TA Master Mix (NEB #M0367), Quick Ligation Kit (NEB #M2200) or concentrated T4 DNA Ligase (NEB #M0202)
	Inefficient phosphorylation	• Purify the DNA prior to phosphorylation with Monarch PCR & DNA Cleanup Kit (5 µg) (NEB #T1030). Excess salt, phosphate or ammonium ions may inhibit the kinase. • If the ends are blunt or 5´ recessed, heat the substrate/buffer mixture for 10 minutes at 70°C. Rapidly chill on ice before adding the ATP and enzyme, then incubate at 37°C. • ATP was not added. Supplement the reaction with 1 mM ATP, as it is required by T4 Polynucleotide Kinase (NEB #M0201) • Alternatively, use 1X T4 DNA Ligase Buffer (contains 1 mM ATP) instead of the 1X T4 PNK Buffer
	Inefficient blunting	• Heat inactivate or remove the restriction enzymes prior to blunting • Clean up the PCR fragment prior to blunting with Monarch PCR & DNA Cleanup Kit (NEB #T1030) • Sonicated gDNA should be blunted for at least 30 minutes • Do not use > 1 unit of enzyme/µg of DNA • Do not incubate for > 15 minutes • Do not incubate at temperatures > 12°C (for T4 DNA Polymerase, NEB #M0203) or > 24°C (for Klenow, NEB #M0210) • Make sure to add a sufficient amount of dNTPs to the reaction (33 µM each dNTP for DNA Polymerase I, Large (Klenow) Fragment, NEB #M0210 and 100 µM each dNTP for T4 DNA Polymerase, NEB #M0203). • When using Mung Bean Nuclease (NEB #M0250), incubate the reaction at room temperature. Do not use > 1 unit of enzyme/µg DNA or incubate the reaction > 30 minutes.

Troubleshooting Guide for Cloning (cont.)

PROBLEM	CAUSE	SOLUTION
Few or no transformants (cont.)	Inefficient A-Tailing	• Clean up the PCR prior to A-tailing. NEB recommends the Monarch PCR & DNA Cleanup Kit (NEB #T1030). High-fidelity enzymes will remove any non-templated nucleotides.
	Restriction enzyme(s) didn't cleave completely	• Check the methylation sensitivity of the enzyme(s) to determine if the enzyme is blocked by methylation of the recognition sequence • Use the recommended buffer supplied with the restriction enzyme • Clean up the DNA to remove any contaminants that may inhibit the enzyme. NEB recommends the Monarch PCR & DNA Cleanup Kit (NEB #T1030). • When digesting a PCR fragment, make sure to have at least 6 nucleotides between the recognition site and the end of the DNA molecule
Colonies don't contain a plasmid	Antibiotic level used was too low	• Increase the antibiotic level on plates to the recommended amount • Use fresh plates with fresh antibiotics
	Satellite colonies were selected	• Choose large, well-established colonies for analysis
Colonies contain the wrong construct	Recombination of the plasmid has occurred	• Use a *recA*⁻ strain such NEB 5-alpha, or NEB 10-beta Competent *E. coli*, or NEB Stable Competent *E. coli*
	Incorrect PCR amplicon was used during cloning	• Optimize the PCR conditions • Gel purify the correct PCR fragment. NEB recommends the Monarch DNA Gel Extraction Kit (NEB #T1020).
	Internal recognition site was present	• Use NEBcutter to analyze insert sequence for presence of an internal recognition site
	DNA fragment of interest is toxic to the cells	• Incubate plates at lower temperature (25–30°C) • Transformation may need to be carried out using a strain that exerts tighter transcriptional control of the DNA fragment of interest (e.g., NEB 5-alpha F´ I^q Competent *E. coli*)
	Mutations are present in the sequence	• Use a high-fidelity polymerase (e.g., Q5 High-Fidelity DNA Polymerase, NEB #M0491) • Re-run sequencing reactions
Too much background	Inefficient dephosphorylation	• Heat inactivate or remove the restriction enzymes prior to dephosphorylation
	Kinase is present/active	• Heat inactivate the kinase after the phosphorylation step. Active kinase will re-phosphorylate the dephosphorylated vector.
	Restriction enzyme(s) didn't cleave completely	• Check the methylation sensitivity of the restriction enzyme(s) to be sure it is not inhibited by methylation of the recognition sequence • Use the recommended buffer supplied with the restriction enzyme • Clean up the DNA to remove contaminants (e.g., too much salt). NEB recommends the Monarch PCR & DNA Cleanup Kit (NEB #T1030).
	Antibiotic level is too low	• Confirm the correct antibiotic concentration
Ran the ligation on a gel and saw no ligated product	Inefficient ligation	• Make sure at least one DNA fragment being ligated contains a 5´ phosphate • Vary the molar ratios of vector to insert from 1:1 to 1:10. Use NEBioCalculator to calculate molar ratios. • Purify the DNA to remove contaminants such as salt and EDTA. NEB recommends the Monarch PCR & DNA Cleanup Kit (NEB #T1030). • ATP will degrade after multiple freeze-thaws; repeat the ligation with fresh buffer • Heat inactivate or remove the phosphatase prior to ligation • Ligation of single base-pair overhangs (most difficult) may benefit from being carried out with Blunt/TA Master Mix, Quick Ligation Kit or concentrated T4 DNA Ligase
The ligated DNA ran as a smear on an agarose gel	The ligase is bound to the substrate DNA	• Treat the ligation reaction with Proteinase K (NEB #P8107) prior to running on a gel
The digested DNA ran as a smear on an agarose gel	The restriction enzyme(s) is bound to the substrate DNA	• Lower the number of units • Add SDS (0.1–0.5%) to the loading buffer to dissociate the enzyme from the DNA or use Gel Loading Dye, Purple (6X) (NEB #B7024)
	Nuclease contamination	• Use fresh, clean running buffer • Use a fresh agarose gel • Clean up the DNA. NEB recommends the Monarch PCR & DNA Cleanup Kit (NEB #T1030).
Incomplete restriction enzyme digestion	Cleavage is blocked by methylation	• DNA isolated from a bacterial source may be blocked by Dam and Dcm methylation • DNA isolated from eukaryotic source may be blocked by CpG methylation • Check the methylation sensitivity of the enzyme(s) to determine if the enzyme is blocked by methylation of the recognition sequence • If the enzyme is inhibited by Dam or Dcm methylation, grow the plasmid in a *dam-/dcm-* strain (NEB #C2925)
	Salt inhibition	• Enzymes that have low activity in salt-containing buffers (NEBuffer r3.1) may be salt sensitive, so clean up the DNA prior to digestion. NEB recommends the Monarch PCR & DNA Cleanup Kit (NEB #T1030). • DNA purification procedures that use spin columns can result in high salt levels, which inhibit enzyme activity. Monarch kits (NEB #T1010, #T1020, #T1030) use columns that have been designed to minimize salt carry over into the eluted DNA, so using them can minimize this issue. To prevent this, DNA solution should be no more than 25% of total reaction volume.
	Inhibition by PCR components	• Clean up the PCR fragment prior to restriction digest. NEB recommends the Monarch PCR & DNA Cleanup Kit (NEB #T1030).
	Using the wrong buffer	• Use the recommended buffer supplied with the restriction enzyme
	Too few units of enzyme used	• Use at least 5–10 units of enzyme per µg of DNA
	Incubation time was too short	• Increase the incubation time
	Digesting supercoiled DNA	• Some enzymes have a lower activity on supercoiled DNA. Increase the number of enzyme units in the reaction.
	Presence of slow sites	• Some enzymes can exhibit slower cleavage towards specific sites. Increase the incubation time, 1–2 hours is typically sufficient.
	Two sites required	• Some enzymes require the presence of two recognition sites to cut efficiently. For more information, visit the table "Restriction Enzymes Requiring Multi-sites" on neb.com.
	DNA is contaminated with an inhibitor	• Assay substrate DNA in the presence of a control DNA. Control DNA will not cleave if there is an inhibitor present. Miniprep DNA is particularly susceptible to contaminants. NEB recommends the Monarch PCR & DNA Cleanup Kit (NEB #T1030). • Clean DNA with a spin column with Monarch PCR & DNA Cleanup Kit (NEB #T1030), resin or drop dialysis, or increase volume to dilute contaminant

PROBLEM	CAUSE	SOLUTION
Extra bands in the gel	If larger bands than expected are seen in the gel, this may indicate binding of the enzyme(s) to the substrate	• Lower the number of units in the reaction • Add SDS (0.1–0.5%) to the loading buffer to dissociate the enzyme from the substrate or add Gel Loading Dye, Purple (6X) (NEB #B7024)
	Star activity	• Use the recommended buffer supplied with the restriction enzyme • Decrease the number of enzyme units in the reaction • Make sure the amount of enzyme added does not exceed 10% of the total reaction volume. This ensures that the total glycerol concentration does not exceed 5% v/v • Decrease the incubation time. Using the minimum reaction time required for complete digestion will help prevent star activity. • Try using a High-Fidelity (HF) restriction enzyme. HF enzymes have been engineered for reduced star activity.
	Partial restriction enzyme digest	• Enzymes that have low activity in salt-containing buffers (e.g., NEBuffer r3.1) may be salt sensitive. Make sure to clean up the DNA prior to digestion. NEB recommends the Monarch PCR & DNA Cleanup Kit (NEB #T1030). • DNA purification procedures that use spin columns can result in high salt levels, which inhibit enzyme activity. Monarch kits (NEB #T1010, #T1020, #T1030) use columns that have been designed to minimize salt carry over into the eluted DNA, so using them can minimize this issue. To prevent this, DNA solution should be no more than 25% of total reaction volume • Clean-up the PCR fragment prior to restriction digest. NEB recommends the Monarch PCR & DNA Cleanup Kit (NEB #T1030). • Use the recommended buffer supplied with the restriction enzyme • Use at least 3–5 units of enzyme per µg of DNA • Digest the DNA for 1–2 hours
No PCR fragment amplified	Used the wrong primer sequence	• Double check the primer sequence
	Incorrect annealing temperature	• Use the NEB Tm calculator to determine the correct annealing temperature (**www.neb.com/TmCalculator**)
	Incorrect extension temperature	• Each polymerase type has a different extension temperature requirement. Follow the manufacturer's recommendations.
	Too few units of polymerase	• Use the recommended number of polymerase units based on the reaction volume
	Incorrect primer concentration	• Each polymerase has a different primer concentration requirement. Make sure to follow the manufacturer's recommendations.
	Mg^{2+} levels in the reaction are not optimal	• Titrate the Mg^{2+} levels to optimize the amplification reaction. Follow the manufacturer's recommendations.
	Difficult template	• With difficult templates, try different polymerases and/or buffer combinations
The PCR reaction is a smear on a gel	If bands are larger than expected it may indicate binding of the enzyme(s) to the DNA	• Add SDS (0.1–0.5%) to the loading buffer to dissociate the enzyme from the DNA
Extra bands in PCR reaction	Annealing temperature is too low	• Use the NEB Tm calculator to determine the annealing temperature of the primers
	Mg^{2+} levels in the reaction are not optimal	• Titrate the Mg^{2+} levels to optimize the amplification reaction. Make sure to follow the manufacturer's recommendations.
	Additional priming sites are present	• Double check the primer sequence and confirm it does not bind elsewhere in the DNA template
	Formation of primer dimers	• Primer sequence may not be optimal. Additional primers may need to be tested in the reaction.
	Incorrect polymerase choice	• Try different polymerases and/or buffer combinations

Selected Products for PCR & Mutagenesis

PRODUCT	NEB #	SIZE
HIGH-FIDELITY DNA POLYMERASES		
Q5 High-Fidelity DNA Polymerase	M0491S/L	100/500 units
Q5 Hot Start High-Fidelity DNA Polymerase	M0493S/L	100/500 units
Q5 High-Fidelity 2X Master Mix	M0492S/L	100/500 reactions
Q5 Hot Start High-Fidelity 2X Master Mix	M0494S/L/X	100/500/500 reactions
Q5 High-Fidelity PCR Kit	E0555S/L	50/200 reactions
Q5U Hot Start High-Fidelity DNA Polymerase	M0515S/L	100/500 units
Phusion High-Fidelity PCR Master Mix with HF Buffer	M0531S/L	100/500 reactions
Phusion High-Fidelity PCR Master Mix with GC Buffer	M0532S/L	100/500 reactions
Phusion Hot Start Flex 2X Master Mix	M0536S/L	100/500 reactions
Phusion High-Fidelity PCR Kit	E0553S/L	50/200 reactions
Phusion High-Fidelity DNA Polymerase	M0530S/L	100/500 units
Phusion Hot Start Flex High-Fidelity DNA Polymerase	M0535S/L	100/500 units
DNA POLYMERASES		
One*Taq* DNA Polymerase	M0480S/L/X	200/1,000/5,000 units
One*Taq* Quick-Load DNA Polymerase	M0509S/L/X	100/500/2,500 units
One*Taq* Hot Start DNA Polymerase	M0481S/L/X	200/1,000/5,000 units
One*Taq* 2X Master Mix with Standard Buffer	M0482S/L	100/500 reactions
One*Taq* Quick-Load 2X Master Mix with Standard Buffer	M0486S/L	100/500 reactions
One*Taq* Hot Start 2X Master Mix with Standard Buffer	M0484S/L	100/500 reactions
One*Taq* Hot Start 2X Master Mix with GC Buffer	M0485S/L	100/500 reactions
One*Taq* Hot Start Quick-Load 2X Master Mix with Standard Buffer	M0488S/L	100/500 reactions
One*Taq* Hot Start Quick-Load 2X Master Mix with GC Buffer	M0489S/L	100/500 reactions
Taq DNA Polymerase with ThermoPol™ Buffer	M0267S/L/X/E	400/2,000/4,000/20,000 units
Taq DNA Polymerase with Standard *Taq* Buffer	M0273S/L/X	400/2,000/4,000 units
Taq DNA Polymerase with Standard *Taq* (Mg-free) Buffer	M0320S/L	400/2,000 units
Taq PCR Kit	E5000S	200 reactions
Quick-Load *Taq* 2X Master Mix	M0271L	500 reactions
Taq 2X Master Mix	M0270L	500 reactions
Taq 5X Master Mix	M0285L	500 reactions
Multiplex PCR 5X Master Mix	M0284S	100 reactions
Hot Start *Taq* DNA Polymerase	M0495S/L	200/1,000 units
Hot Start *Taq* 2X Master Mix	M0496S/L	100/500 reactions
Vent DNA Polymerase	M0254S/L	200/1,000 units
Vent (exo⁻) DNA Polymerase	M0257S/L	200/1,000 units
Deep Vent DNA Polymerase	M0258S/L	200/1,000 units
Deep Vent (exo⁻) DNA Polymerase	M0259S/L	200/1,000 units
LongAmp *Taq* DNA Polymerase	M0323S/L	500/2,500 units
LongAmp Hot Start *Taq* DNA Polymerase	M0534S/L	500/2,500 units
LongAmp *Taq* 2X Master Mix	M0287S/L	100/500 reactions
LongAmp Hot Start *Taq* 2X Master Mix	M0533S/L	100/500 reactions
LongAmp *Taq* PCR Kit	E5200S	100 reactions
PCR CLONING & MUTAGENESIS		
NEB PCR Cloning Kit	E1202S	20 reactions
NEB PCR Cloning Kit (Without Competent Cells)	E1203S	20 reactions
Q5 Site-Directed Mutagenesis Kit	E0554S	10 reactions
Q5 Site-Directed Mutagenesis Kit (Without Competent Cells)	E0552S	10 reactions

Products for cDNA Synthesis

PRODUCT	NEB #	SIZE
KLD Enzyme Mix	M0554S	25 reactions
Deoxynucleotide (dNTP) Solution Set	N0446S	25 µmol of each
Deoxynucleotide (dNTP) Solution Mix	N0447S/L	8/40 µmol of each
AMV Reverse Transcriptase	M0277S/L	200/1,000 units
LunaScript RT SuperMix Kit	E3010S/L	25/100 reactions
LunaScript RT Master Mix Kit (Primer-free)	E3025S/L	25/100 reactions
LunaScript RT SuperMix	M3010L/X/E	100/500/2,500 reactions
M-MuLV Reverse Transcriptase	M0253S/L	10,000/50,000 units
ProtoScript II First Strand cDNA Synthesis Kit	E6560S/L	30/150 reactions
ProtoScript First Strand cDNA Synthesis Kit	E6300S/L	30/150 reactions
Template Switching RT Enzyme Mix	M0466S/L	20/100 reactions
ProtoScript II Reverse Transcriptase	M0368S/L/X	4,000/10,000/40,000 units
WarmStart® RTx Reverse Transcriptase	M0380S/L	50/250 reactions

Products for Restriction Digestion

PRODUCT	NEB #	SIZE
HIGH-FIDELITY (HF®) RESTRICTION ENZYMES		
AgeI-HF	R3552S/L	300/1,500 units
ApoI-HF	R3566S/L	1,000/5,000 units
BamHI-HF	R3136S/L/T/M	10,000/50,000 units
BbsI-HF	R3539S/L	300/1,500 units
BclI-HF	R3160S/L	3,000/15,000 units
BmtI-HF	R3658S/L	300/1,500 units
BsaI-HFv2	R3733S/L	1,000/5,000 units
BsiWI-HF	R3553S/L	300/1,500 units
BsrGI-HF	R3575S/L	1,000/5,000 units
BstEII-HF	R3162S/L/M	2,000/10,000 units
BstZ171-HF	R3594S/L	1,000/5,000 units
DraIII-HF	R3510S/L	1,000/5,000 units
EagI-HF	R3505S/L/M	500/2,500 units
EcoRI-HF	R3101S/L/T/M	10,000/50,000 units
EcoRV-HF	R3195S/L/T/M	4,000/20,000 units
HindIII-HF	R3104S/L/T/M	10,000/50,000 units
KpnI-HF	R3142S/L/M	4,000/20,000 units
MfeI-HF	R3589S/L	500/2,500 units
MluI-HF	R3198S/L	1,000/5,000 units
NcoI-HF	R3193S/L/M	1,000/5,000 units
NheI-HF	R3131S/L/M	1,000/5,000 units
NotI-HF	R3189S/L/M	500/2,500 units
NruI-HF	R3192S/L	1,000/5,000 units
NsiI-HF	R3127S/L	1,000/5,000 units
PstI-HF	R3140S/L/T/M	10,000/50,000 units
PvuI-HF	R3150S/L	500/2,500 units
PvuII-HF	R3151S/L/M	5,000/25,000 units
SacI-HF	R3156S/L/M	2,000/10,000 units
SalI-HF	R3138S/L/T/M	2,000/10,000 units
SbfI-HF	R3642S/L	500/2,500 units
ScaI-HF	R3122S/L/M	1,000/5,000 units
SpeI-HF	R3133S/L/M	500/2,500 units
SphI-HF	R3182S/L/M	500/2,500 units
SspI-HF	R3132S/L/M	1,000/5,000 units
StyI-HF	R3500S/L	3,000/15,000 units
FEATURED GEL LOADING DYE		
Gel Loading Dye, Purple (6X)	B7024S	4 ml
Gel Loading Dye, Purple (6X), no SDS	B7025S	4 ml

For the full list of restriction enzymes available, visit **www.neb.com**.

Products for End Modification

PRODUCT	NEB #	SIZE
Quick CIP	M0525S/L	1,000/5,000 units
Shrimp Alkaline Phosphatase (Recombinant)	M0371S/L	500/2,500 units
Antarctic Phosphatase	M0289S/L	1,000/5,000 units
T4 DNA Polymerase	M0203S/L	150/750 units
DNA Polymerase I, Large (Klenow) Fragment	M0210S/L/M	200/1,000/1,000 units
Quick Blunting Kit	E1201S/L	20/100 reactions
Mung Bean Nuclease	M0250S/L	1,000/5,000 units
T4 Polynucleotide Kinase	M0201S/L	500/2,500 units
Klenow Fragment (3´ → 5´ exo⁻)	M0212S/L/M	200/1,000/1,000 units
β-Agarase I	M0392S/L	100/500 units

Products for Ligation

PRODUCT	NEB #	SIZE
Blunt/TA Ligase Master Mix	M0367S/L	50/250 reactions
Instant Sticky-End Ligase Master Mix	M0370S/L	50/250 reactions
NEBridge Ligase Master Mix	M1100S	50 reactions
ElectroLigase	M0369S	50 reactions
T4 DNA Ligase	M0202S/L/T/M	20,000/100,000 units
Salt-T4 DNA Ligase	M0467S/L	20,000/100,000 units
Hi-T4 DNA Ligase	M2622S/L	20,000/100,000 units
Quick Ligation Kit	M2200S/L	30/150 reactions
T3 DNA Ligase	M0317S/L	100,000/750,000 units
T7 DNA Ligase	M0318S/L	100,000/750,000 units
Taq DNA Ligase	M0208S/L	2,000/10,000 units

Products for Transformation

PRODUCT	NEB #	SIZE
dam-/dcm- Competent *E. coli*	C2925H/I	20 x 0.05 ml/tube/ 6 x 0.2 ml ml/tube
NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987H/I/P/R/U	20 x 0.05 ml/tube/ 6 x 0.2 ml/tube/ 1 x 96 well plate/ 1 x 384 well plate/ 12 x 8 tube strips
NEB 5-alpha Competent *E. coli* (Subcloning Efficiency)	C2988J	6 x 0.4 ml/tube
NEB 5-alpha F´ *I*ᵠ Competent *E. coli* (High Efficiency)	C2992H/I	20 x 0.05/6 x 0.2 ml
NEB 10-beta Competent *E. coli* (High Efficiency)	C3019H/I/P	20 x 0.05 ml/tube/ 6 x 0.2 ml ml/tube 1 x 96 well plate
NEB 10-beta Electrocompetent *E. coli*	C3020K	6 x 0.1 ml/tube
NEB Turbo Competent *E. coli* (High Efficiency)	C2984H/I	20 x 0.05 ml/tube/ 6 x 0.2 ml/tube
NEB Stable Competent *E. coli* (High Efficiency)	C3040H/I	20 x 0.05 ml/tube/ 6 x 0.2 ml/tube
NEB Cloning Competent *E. coli* Sampler	C1010S	8 tubes

For the full list of competent cells available, visit **www.neb.com**.

Products for Nucleic Acid Purification

PRODUCT	NEB #	SIZE
Monarch Plasmid Miniprep Kit	T1010S/L	50/250 preps
Monarch DNA Gel Extraction Kit	T1020S/L	50/250 preps
Monarch PCR & DNA Cleanup Kit (5 µg)	T1030S/L	50/250 preps
Monarch Genomic DNA Purification Kit	T3010S/L	50/150 preps
Monarch Total RNA Miniprep Kit	T2010S	50 preps
Monarch RNA Cleanup Kit (10 µg)	T2030S/L	10/100 preps
Monarch RNA Cleanup Kit (50 µg)	T2040S/L	10/100 preps
Monarch RNA Cleanup Kit (500 µg)	T2050S/L	10/100 preps

Columns and buffers also available separately.

Products for DNA Analysis

PRODUCT	NEB #	SIZE
1 kb DNA Ladder	N3232S/L	200/1,000 gel lanes
TriDye 1 kb DNA Ladder	N3272S	125 gel lanes
Quick-Load 1 kb DNA Ladder	N0468S/L	125/375 gel lanes
100 bp DNA Ladder	N3231S/L	100/500 gel lanes
TriDye 100 bp DNA Ladder	N3271S	125 gel lanes
Quick-Load 100 bp DNA Ladder	N0467S/L	125/375 gel lanes
1 kb Plus DNA Ladder	N3200S/L	200/1,000 gel lanes
1 kb Plus DNA Ladder for Safe Stains	N0559S	50 µg/ml
TriDye 1 kb Plus DNA Ladder	N3270S	250 gel lanes
Quick-Load 1 kb Plus DNA Ladder	N0469S	250 gel lanes
Quick-Load Purple 1 kb Plus DNA Ladder	N0550S/L	250/750 gel lanes
TriDye Ultra Low Range DNA Ladder	N0558S	100 µg/ml
50 bp DNA Ladder	N3236S/L	200/1,000 gel lanes
Quick-Load Purple 50 bp DNA Ladder	N0556S	250 gel lanes
Quick-Load 1 kb Extend DNA Ladder	N3239S	125 gel lanes
Quick-Load Purple 1 kb DNA Ladder	N0552S/L	125/375 gel lanes
Quick-Load Purple 100 bp DNA Ladder	N0551S/L	125/375 gel lanes
Low Molecular Weight DNA Ladder	N3233S/L	100/500 gel lanes
Quick-Load Purple Low Molecular Weight DNA Ladder	N0557S	125 gel lanes
Fast DNA Ladder	N3238S	200 gel lanes
PCR Marker	N3234S/L	100/500 gel lanes

Conventional and PFG markers are also available, visit **www.neb.com/DNAMarkersandLadders**.

Products for Seamless Cloning

PRODUCT	NEB #	SIZE
NEBuilder HiFi DNA Assembly Cloning Kit	E5520S	10 reactions
NEBuilder HiFi DNA Assembly Master Mix	E2621S/L	10/50 reactions
NEBuilder HiFi DNA Assembly Bundle for Large Fragments	E2623S	20 reactions
Gibson Assembly Cloning Kit	E5510S	10 reactions
Gibson Assembly Master Mix	E2611S/L	10/50 reactions
NEBridge Golden Gate Assembly Kit (BsaI-HFv2)	E1601S/L	20/100 reactions
NEBridge Golden Gate Assembly Kit (BsmBI-v2)	E1602S/L	20/100 reactions
NEBridge Ligase Master Mix	M1100S/L	50/250 reactions
BbsI	R0539S/L	300/1,500 units
BbsI-HF	R3539S/L	300/1,500 units
BsaI-HFv2	R3733S/L	1,000/5,000 units
BsmBI-v2	R0739S/L	200/1,000 units
Esp3I	R0734S/L	300/1,500 units
PaqCI	R0745S/L	200/1,000 units
SapI	R0569S/L	250/1,250 units
BtgZI	R0703S/L	100/500 units
BspQI	R0712S/L	500/2,500 units
T4 DNA Polymerase	M0203S/L	150/750 units
Taq DNA Ligase	M0208S/L	2,000/10,000 units
T4 DNA Ligase	M0202S/L/T/M	20,000/100,000 units
T5 Exonuclease	M0363S/L	1,000/5,000 units
Exonuclease V (RecBCD)	M0345S/L	1,000/5,000 units
USER Enzyme	M5505S/L	50/250 units
Thermolabile USER Enzyme II	M5508S/L	50/250 units

Products for Recombinational Cloning

PRODUCT	NEB #	SIZE
Cre Recombinase	M0298S/L/M	50/250 units

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