Molecular Cloning

TECHNICAL GUIDE

UPDATE 2018/19

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.

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.

NEB 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.

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.

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® V2.0

Identify restriction sites within your DNA sequence using NEBcutter. Choose between Type II and commercially available Type III enzymes to digest your DNA. NEBcutter V2.0 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 Gibson Assembly reaction, based on the entered fragment sequences and the polymerase being used for amplification.

PCR Selection Tool

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.

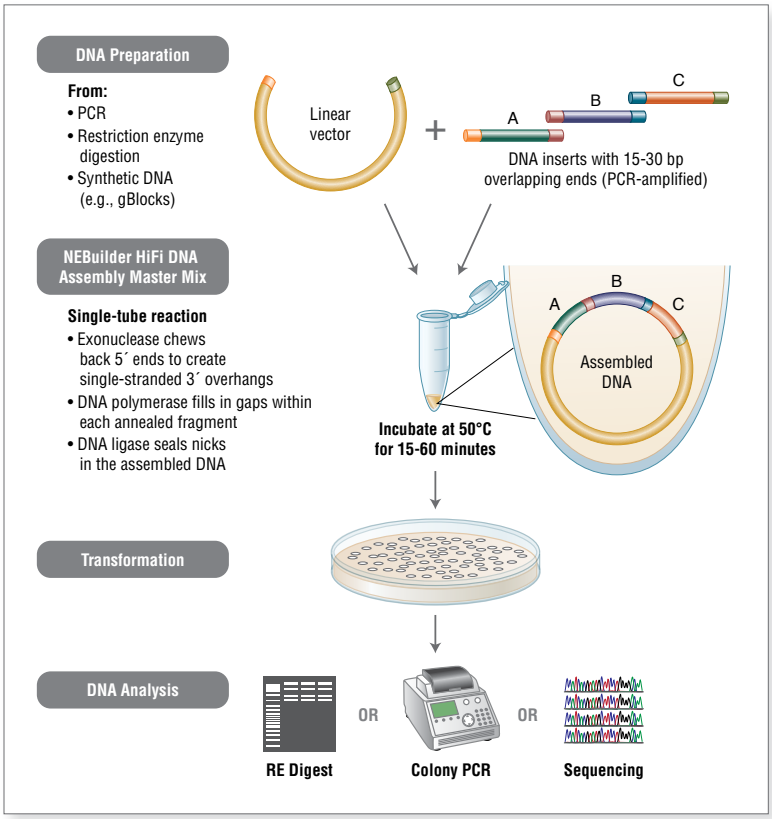
When using either of these tools, look for CutSmart®, HF® and Time-Saver™ enzymes for the ultimate in convenience. NEB Tools enables quick and easy access to the most requested restriction enzyme information, and allows you to plan your experiments from anywhere.

Cloning & Mutagenesis Kits

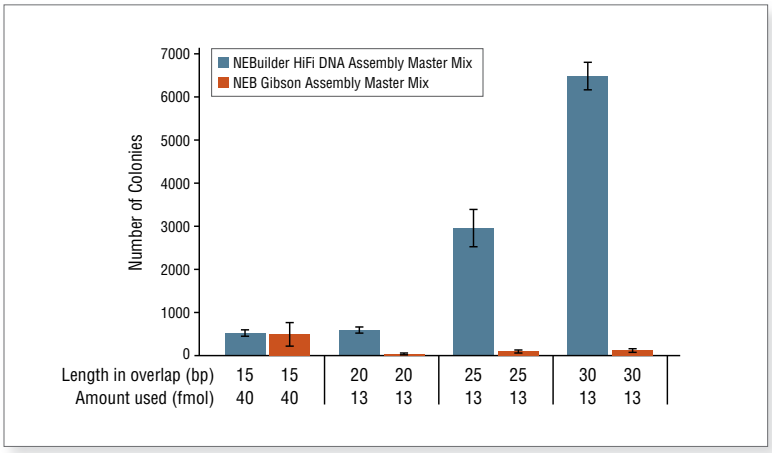
NEBuilder HiFi DNA Assembly

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 offers improved efficiency and accuracy with lower amounts of DNA by increasing overlap length



Reactions were set up in a 4-fragment assembly reaction according to recommended reaction conditions. Amount of DNA and size of overlap is shown.

RECOMMENED 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 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*

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

For help with designing primers, try NEBuilder Assembly Tool at **NEBuilder.neb.com**

Cloning & Mutagenesis Kits (Cont.)

Optimization Tips for NEBuilder HiFi DNA Assembly

Assembly Reaction

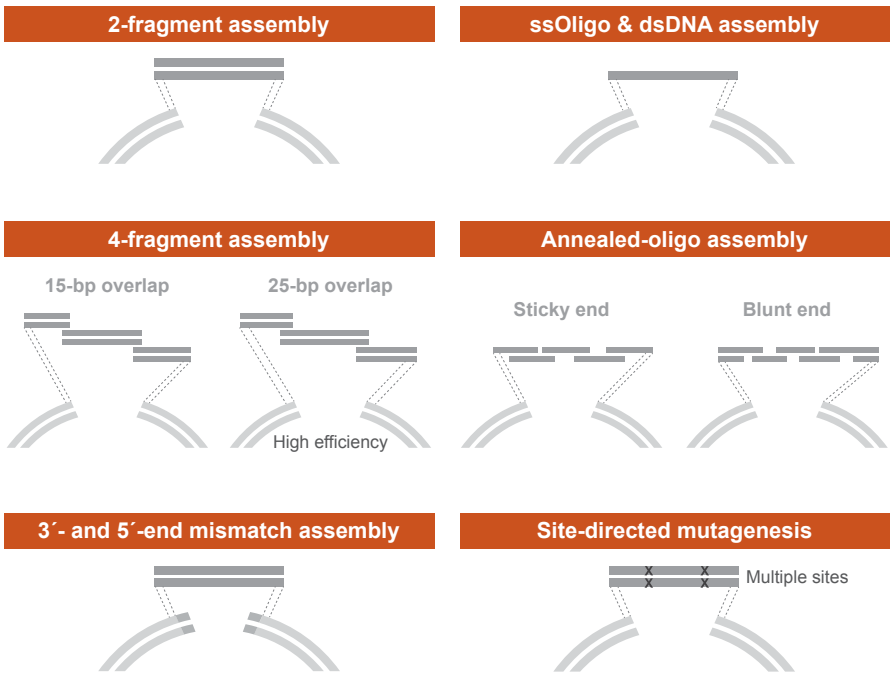
- When directly assembling fragments into a cloning vector, the molar concentration of assembly fragments should be 2–3 times higher than the concentration of vector.
- For multiple (4–12) fragment assembly, design 25–30 bp overlap regions between each fragment to enhance assembly efficiency. Use 0.05 pmol of each fragment in the assembly reaction.
- For assembly of 1–3 fragments, 15 minute incubation times are sufficient. For assembly of 4–6 fragments, 60 minute incubation times are recommended. Reaction times less than 15 minutes are generally not recommended.

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) and the Gibson Assembly Cloning Kit (NEB #E5510) include 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 includes NEB 10-beta Competent *E. coli* (NEB #C3019), ideal for assembling larger fragments (>15 kb).



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**	Positive Control***
Recommended DNA Molar Ratio	vector:insert= 1:2	vector:insert= 1:1	
Total Amount of Fragments	0.03-0.2 pmols* X µl	0.2-0.5 pmols** 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 excess of inserts. Use 5 times more insert if size is less than 200 bp. Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%.

** To achieve optimal assembly efficiency, design ≥ 20 bp overlap regions between each fragment with equimolarity (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).

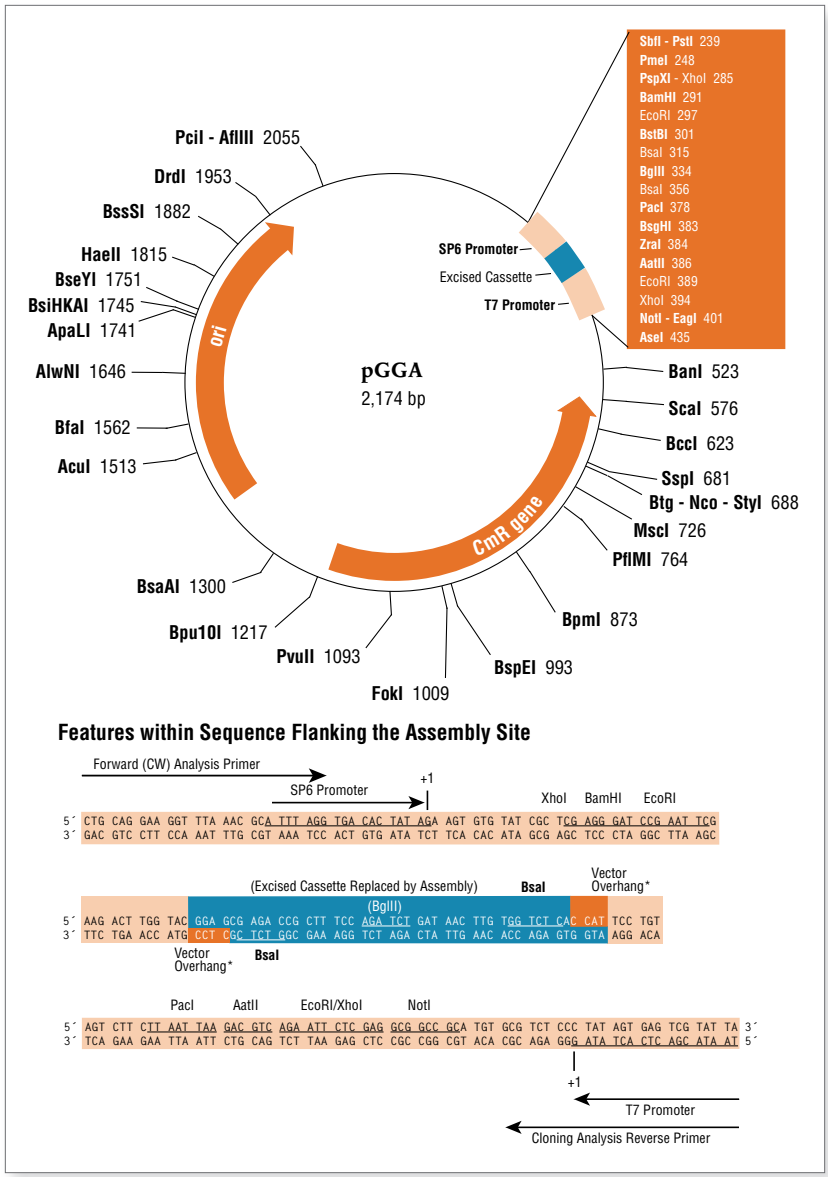
Protocol: Transformation with NEB 5-alpha cells

	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

NEB Golden Gate Assembly

The efficient and seamless assembly of DNA fragments, commonly referred to as Golden Gate assembly (1,2), has its origins in 1996 when, for the first time, it was shown that multiple inserts could be 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. This method can be accomplished using Type IIS restriction enzymes, such as BsaI-HF v2, BbsI-HF or Esp3I and can also be used for the cloning of single inserts. The method is efficient and can be completed in one tube in as little as 5 minutes for single inserts, or can utilize cycling steps for multiple inserts (see page 33 for workflow).

The NEB Golden Gate Assembly Mix incorporates digestion with BsaI-HF v2 (resp. BbsI-HF or Esp3I) and ligation with T4 DNA Ligase into a single reaction, and can be used to assemble up to 10 fragments in a single step.



RECOMMENDED PRODUCTS

NEB Golden Gate Assembly Mix (NEB #E1600)

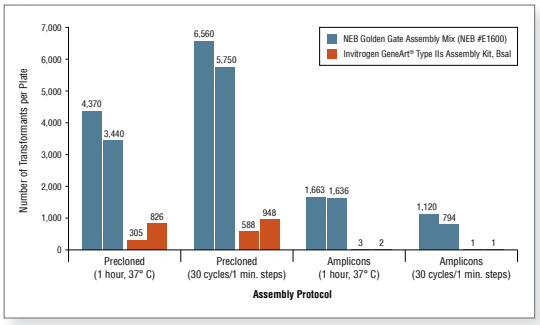
- Seamless cloning – no scar remains following assembly
- Ordered assembly of up to 10–20 fragments in a single reaction
- Efficient with regions with high GC content and areas of repeats
- Compatible with a broad range of fragment sizes (< 100 bp to > 15 kb)
- pGGA destination plasmid included

TOOLS & RESOURCES

Visit www.neb.com/GoldenGate to find:

- Publications and protocols related to Golden Gate Assembly
- Access to **NEB Golden Gate Assembly Tool**, our online assembly tool

NEB Golden Gate Assembly Mix offers improved assembly



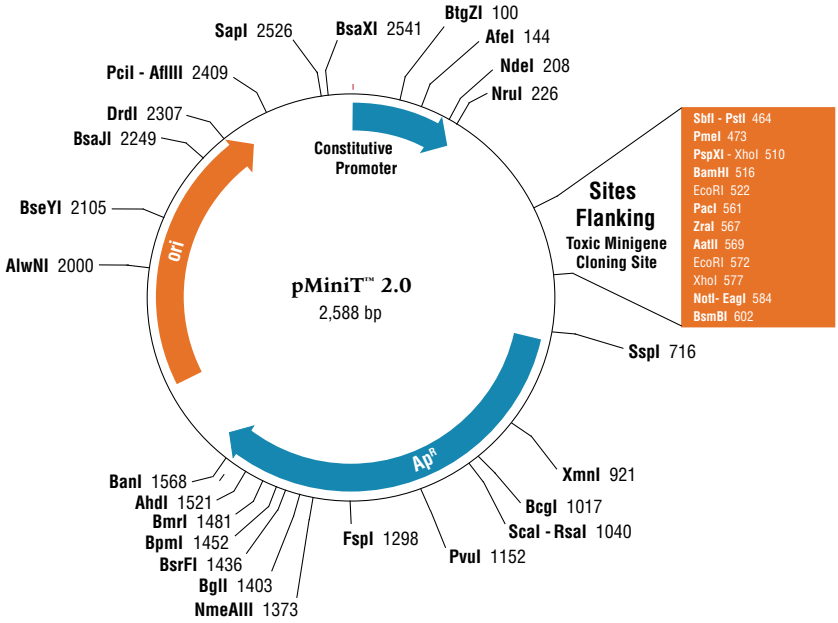
Assembly reactions were set up using either precloned inserts or PCR amplicons directly. Reaction conditions were set up according to manufacturer, and are shown above. Two separate experiments are shown for each reaction type.

Speed up your experimental design with our online assembly tool at **GoldenGate.neb.com**

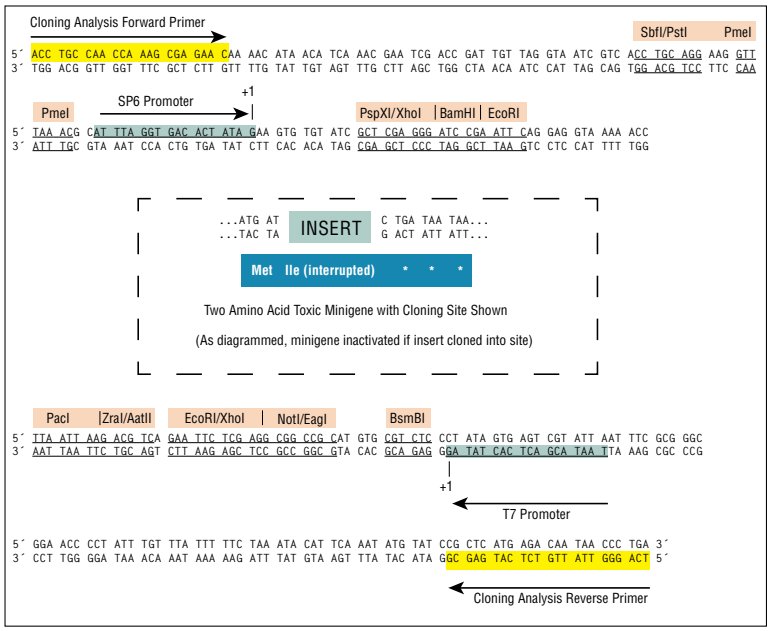
References:
1. Engler, C. et al. (2008) *PLoS ONE*, 3: e3647.
2. Engler, C. et al. (2009) *PLoS ONE*, 4: e5553.
3. Lee, J.H. et al. (1996) *Genetic Analysis: Biomolecular Engineering*, 13; 139-145.
4. Padgett, K.A. and Sorge, J.A. (1996) *Gene*, 168, 31-35.

NEB PCR Cloning Kit

The NEB PCR Cloning Kit [with (NEB #E1202) or without (NEB #E1203) competent cells] 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. The NEB PCR Cloning Kit is supplied with the pMiniT 2.0 vector, which allows *in vitro* transcription from both SP6 and T7 promoters, features more unique restriction sites for subcloning (including four 8-base cut sites) and can be used for Golden Gate Assembly as the plasmid has no internal BsaI sites.



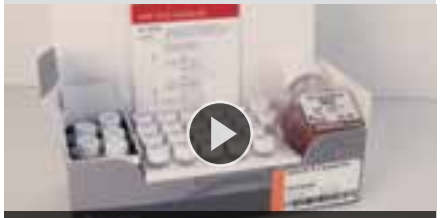
Features within Sequence Flanking the Toxic Minigene/Cloning Site



Top map shown above 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 cloning analysis primers for cloning PCR or sequencing, restriction sites for subcloning or linearization for in vitro transcription, RNA Polymerase promoter sequences and placement of insertion site within the toxic minigene.

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



HOW DOES THE NEB PCR CLONING KIT WORK?

Protocol: Ligation

	STANDARD PROTOCOL
Linearized pMiniT 2.0 Vector (25 ng/µl)	1 µl
Insert + H_2O	4 µl
Cloning Mix 1	4 µl
Cloning Mix 2	1 µl
Incubation	5–15 minutes, 25°C

Protocol: Transformation

	STANDARD PROTOCOL
Ligation Reaction	2 µl
Competent *E. coli*	50 µl
Incubation	On ice for 20 minutes
Heat Shock	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

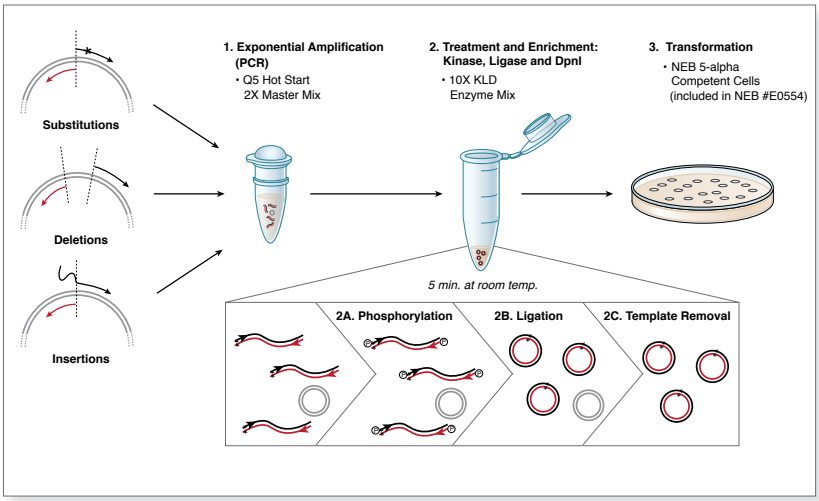
Protocol: Plating

1. Mix cells thoroughly by flicking or inversion and spread 50 µl of the 1 ml outgrowth onto 37°C pre-warmed agar plates containing 100 µg/ml ampicillin. If a 15 minute ligation time was used, also plate 50 µl of a 1:10 diluiton prepared with SOC.
2. Invert plate and incubate overnight at 37°C or for 24 hours at 30°C. Do not use room temperature growth as the slow growth rate will interfere with selection of constructs with inserts.
3. After colonies appear, use the plate with well separated colonies for screening.

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)

- 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

OVERVIEW OF THE Q5 SDM KIT

DNA Assembly Selection Chart

New England Biolabs now offers several products that can be used for DNA assembly and cloning. Use this chart to determine which product would work best to assemble your DNA.

	NEBuilder HiFi DNA Assembly (NEB #E2621) (NEB #E5520) (NEB #E2623)	Gibson Assembly (NEB #E5510) (NEB #E2611)	NEB Golden Gate Assembly Mix (NEB #E1600)	USER® Enzyme (NEB #M5505)
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 with High Efficiency with Low Amounts of DNA	★★★	★★	★★	★★
Accommodates Flexible Overlap Lengths	★★★	★★★	★	★★
APPLICATIONS				
Simple Cloning (1-2 Fragments)	★★★	★★★	★★★	★★★
4-6 Fragment Assembly	★★★	★★★	★★★	★★★
>6 Fragment Assembly	★★★	★★	★★★	★★★
Template Construction for *In vitro* Transcription	★★★	★★★	★★★	★
Synthetic Whole Genome Assembly	★★★	★	★	★
Multiple Site-directed Mutagenesis	★★★	★★	★★	★★
Library Generation	★★	★★	★★	★★
Pathway Engineering	★★★	★★	★★	★★★
TALENs	★★	★★	★★★	★★
Short Hairpin RNA Cloning (shRNA)	★★★	★★	★	★
gRNA Library Generation	★★★	★★	★	★
Large Fragment (> 10 kb) Assembly	★★★	★★★	★★★	★★
Small Fragment (< 100 bp) Assembly	★★★	★	★★★	★★★
Use in Successive Rounds of Restriction Enzyme Assembly	★★★	★	NR	★

KEY

★★★	Works best for selected application.	N/A	Not applicable to this application.
★★	Suitable for selected application, but other product(s) perform better.	NR	Not recommended.
★	Will perform selected application, but is not recommended.		

Nucleic Acid Purification

Purification of nucleic acids is an important part of cloning workflows. Once plasmids containing a desired gene of interest are generated. They can be propagated using competent cells to increase the quantity of the desired DNA. Following this, plasmids need to be recovered from their bacterial hosts using plasmid purification methods. It is important in these processes to separate the plasmid effectively from the RNA and the genomic DNA of the host cells. For downstream reactions like ligation and restriction digestion, it is important that DNA be free from contaminating salts for optimal enzyme activity. It is also important that the DNA be present in a concentration amendable to use in those small-volume reactions. This is where efficient DNA cleanup and gel extraction becomes vital, and where low-volume elutions facilitate cloning workflows. RNA purification is an important first step in successful cDNA synthesis.

Monarch Nucleic Acid Purification Kits

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 carryover with optimized column design • Includes colored buffers to monitor completion of certain steps • No need to add RNase before starting
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 carryover with optimized column design • Fast, user-friendly protocol
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 carryover with optimized column design • Purify oligos and other small DNA fragments with simple protocol modification
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.	• Works with a variety of samples • 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

Visit NEBMonarch.com for protocol videos and other usage guidelines

TIPS FOR OPTIMIZATION

DNA PURIFICATION

- **Ensure that the tip of the column doesn't come into contact with the flow-through after washing:** If in doubt, a quick additional spin is a good idea
- **Heat the elution buffer for large DNA fragments or plasmids:** Large DNA binds more tightly; heating the elution buffer helps to more efficiently release the DNA from the column matrix

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 ensures that cell debris does not clog column
- **Lyse cells completely:** In order to release all plasmid DNA, ALL of the 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:** Do not skip or reduce the incubation with RNase (which is included in the neutralization buffer), otherwise you may observe RNA contamination
- **Don't skip any washes:** Proper washes ensure the removal of cell debris, endotoxins and salts

GEL EXTRACTION

- **Use the smallest possible agarose plug:** More agarose requires longer melting time and more buffer to dissolve it (introducing more salts which can co-elute with your sample).
- **Minimize exposure to UV light:** Exposure to UV light 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

RNA EXTRACTION & PURIFICATION

- **Inactivate RNases after harvesting your sample:** 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
- **For sensitive applications, ensure proper gDNA removal:** use gDNA removal column and DNase I treatment. Off-column DNase I treatment can also be used.

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.

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

cDNA Synthesis Selection Chart

cDNA SYNTHESIS	FEATURES
KITS	
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
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

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 PolyA Spin mRNA Isolation Kit (NEB #S1560) or 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 are 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.

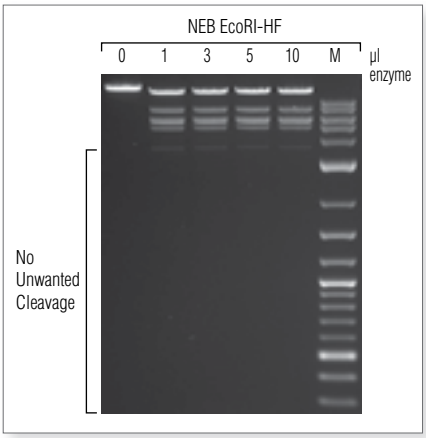
Restriction Enzyme Digestion

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

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 effect digestion with certain enzymes. For more information about methylation visit **www.neb.com/methylation**.

BUFFER

- Use at a 1X concentration
- BSA is included in NEBuffer 1.1, 2.1, 3.1 and CutSmart Buffer. No additional BSA is needed.
- Restriction enzymes that do not require BSA for optimal activity are not adversely affected if BSA is present in the reaction

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 a single buffer, CutSmart. 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 BSA is included in all NEBuffers. In addition, this performance chart shows recommended reaction temperature, heat-inactivation temperature, recommended diluent buffer, methylation sensitivity, whether the enzyme is Time-Saver qualified (cleaves substrate in 5–15 minutes under recommended conditions, and can be used overnight without degradation of DNA), 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.	**SAM**	Supplied with a separate vial of S-adenosylmethionine (SAM). To obtain 100% activity, SAM should be added to the 1X reaction mix as specified on the product data card.
R	Recombinant	dcm	dcm methylation sensitivity
⏱	Time-Saver qualified	CpG	CpG methylation sensitivity
e	Engineered enzyme for maximum performance	2*site	Indicates that the restriction enzyme requires two or more sites for cleavage
dam	dam methylation sensitivity		

NEBuffer Compositions (1X)

NEBuffer 1.1	10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 100 µg/ml BSA (pH 7.0 @ 25°C).
NEBuffer 2.1	10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 100 µg/ml BSA (pH 7.9 @ 25°C).
NEBuffer 3.1	50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 100 µg/ml BSA (pH 7.9 @ 25°C).
CutSmart	20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 100 µg/ml BSA (pH 7.9 @ 25°C).
Diluent A	50 mM KCl, 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 200 µg/ml BSA (pH 7.4 @ 25°C).
Diluent B	300 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 500 µg/ml BSA, 50% glycerol (pH 7.4 @ 25°C).
Diluent C	50 mM KCl, 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.15% Triton X-100, 200 µg/ml BSA 50% glycerol (pH 7.4 @ 25°C).

TOOLS & RESOURCES

Visit NEBRestrictionEnzymes.com to find:

- The full list of HF restriction enzymes available
- The latest activity/performance chart
- Videos for setting up restriction enzyme digests, double digestions and troubleshooting reactions

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 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 1.1	2.1	3.1	CUTSMART	INCUB. TEMP. (°C)	INACTIV. TEMP. (°C)	DILUENT	UNIT SUBSTRATE	METHYLATION SENSITIVITY		NOTE
R ⏱	AatII	CutSmart	< 10	50*	50	100	37°	80°	B	Lambda		CpG	
R	AbaSI	CutSmart	25	50	50	100	25°	65°	C	T4 wt Phage			e
R ⏱	AccI	CutSmart	50	50	10	100	37°	80°	A	Lambda		CpG	
R ⏱	Acc65I	3.1	10	75*	100	25	37°	65°	A	pBC4	dcm	CpG	
R ⏱	AciI	CutSmart	< 10	25	100	100	37°	65°	A	Lambda		CpG	d
R ⏱	AclI	CutSmart	< 10	< 10	< 10	100	37°	No	B	Lambda		CpG	
R ⏱	AcuI	CutSmart + SAM	50	100	50	100	37°	65°	B	Lambda			1, b, d
R	AfeI	CutSmart	25	100	25	100	37°	65°	B	pXba		CpG	
R ⏱	AflII	CutSmart	50	100	10	100	37°	65°	A	phiX174			
R	AflIII	3.1	10	50	100	50	37°	80°	B	Lambda			
R	AgeI	1.1	100	75	25	75	37°	65°	C	Lambda		CpG	
R ⏱ e	AgeI-HF	CutSmart	100	50	10	100	37°	65°	A	Lambda		CpG	
R ⏱	AhdI	CutSmart	25	25	10	100	37°	65°	A	Lambda		CpG	a
R	AleI	CutSmart	< 10	< 10	< 10	100	37°	80°	B	Lambda		CpG	
R ⏱	AluI	CutSmart	25	100	50	100	37°	80°	B	Lambda			b
R	AlwI	CutSmart	50	50	10	100	37°	No	A	Lambda dam-	dam		1, b, d
R ⏱	AlwNI	CutSmart	10	100	50	100	37°	80°	A	Lambda	dcm		
R ⏱	ApaI	CutSmart	25	25	< 10	100	25°	65°	A	pXba	dcm	CpG	
R ⏱	ApaLI	CutSmart	100	100	10	100	37°	No	A	Lambda HindIII		CpG	
R ⏱	ApeKI	3.1	25	50	100	10	75°	No	B	Lambda		CpG	
R ⏱	ApoI	3.1	10	75	100	75	50°	80°	A	Lambda			

ENZYME	SUPPLIED NEBUFFER	% ACTIVITY IN NEBUFFERS 1.1	2.1	3.1	CUTSMART	INCUB. TEMP. (°C)	INACTIV. TEMP. (°C)	DILUENT	UNIT SUBSTRATE	METHYLATION SENSITIVITY		NOTE
ApoI-HF	CutSmart	10	100	10	100	37°	80°	B	Lambda			
AscI	CutSmart	< 10	10	10	100	37°	80°	A	Lambda		CpG	
AseI	3.1	< 10	50*	100	10	37°	65°	B	Lambda			3
AsiSI	CutSmart	50	100	100	100	37°	80°	B	pXba (Xho digested)		CpG	2, b
AvaI	CutSmart	< 10	100	25	100	37°	80°	A	Lambda		CpG	
AvaII	CutSmart	50	75	10	100	37°	80°	A	Lambda	dcm	CpG	
AvrII	CutSmart	100	50	50	100	37°	No	B	Lambda HindIII			
BaeI	CutSmart + SAM	50	100	50	100	25°	65°	A	Lambda		CpG	e
BaeGI	3.1	75	75	100	25	37°	80°	A	Lambda			
BamHI	3.1	75*	100*	100	100*	37°	No	A	Lambda			3
BamHI-HF	CutSmart	100	50	10	100	37°	No	A	Lambda			
BanI	CutSmart	10	25	< 10	100	37°	65°	A	Lambda	dcm	CpG	1
BanII	CutSmart	100	100	50	100	37°	80°	A	Lambda			2
BbsI	2.1	100	100	25	75	37°	65°	B	Lambda			
BbsI-HF	CutSmart	10	10	10	100	37°	65°	B	Lambda			3
BbvI	CutSmart	100	100	25	100	37°	65°	B	pBR322			3
BbvCI	CutSmart	10	100	50	100	37°	No	B	Lambda		CpG	1, a
BccI	CutSmart	100	50	10	100	37°	65°	A	pXba			3, b
BceAI	3.1	100*	100*	100	100*	37°	65°	A	pBR322		CpG	1
BcgI	3.1 + SAM	10	75*	100	50*	37°	65°	A	Lambda	dam	CpG	e
BciVI	CutSmart	100	25	< 10	100	37°	80°	C	Lambda			b
BclI	3.1	50	100	100	75	50°	No	A	Lambda dam-	dam		
BclI-HF	CutSmart	100	100	10	100	37°	65°	B	Lambda dam-	dam		3
BcoDI	CutSmart	50	75	75	100	37°	No	B	Lambda		CpG	
BfaI	CutSmart	< 10	10	< 10	100	37°	80°	B	Lambda			2, b
BfuAI	3.1	< 10	25	100	10	50°	65°	B	Lambda		CpG	3
BfuCI	CutSmart	100	50	25	100	37°	80°	B	Lambda		CpG	
BglI	3.1	10	25	100	10	37°	65°	B	Lambda		CpG	
BglII	3.1	10	10	100	< 10	37°	No	A	Lambda			
BlpI	CutSmart	50	100	10	100	37°	No	A	Lambda			d
BmgBI	3.1	< 10	10	100	10	37°	65°	B	Lambda		CpG	3, b, d
BmrI	2.1	75	100	75	100*	37°	65°	B	Lambda HindIII			b
BmtI	3.1	100	100	100	100	37°	65°	B	pXba			2
BmtI-HF	CutSmart	50	100	10	100	37°	65°	B	pXba			
BpmI	3.1	75	100	100	100	37°	65°	B	Lambda			2
Bpu10I	3.1	10	25	100	25	37°	80°	B	Lambda			3, b, d
BpuEI	CutSmart + SAM	50*	100	50*	100	37°	65°	B	Lambda			d
BsaI	CutSmart	75*	75	100	100	37°	65°	B	pXba	dcm	CpG	3
BsaI-HF v2	CutSmart	100	100	100	100	37°	80°	B	pXba	dcm	CpG	
BsaAI	CutSmart	100	100	100	100	37°	No	C	Lambda		CpG	
BsaBI	CutSmart	50	100	75	100	60°	80°	B	Lambda dam-	dam	CpG	2
BsaHI	CutSmart	50	100	100	100	37°	80°	C	Lambda	dcm	CpG	
BsaJI	CutSmart	50	100	100	100	60°	80°	A	Lambda			
BsaWI	CutSmart	10	100	50	100	60°	80°	A	Lambda			
BsaXI	CutSmart	50*	100*	10	100	37°	No	C	Lambda			e
BseRI	CutSmart	100*	100	75	100	37°	80°	A	Lambda			d
BseYI	3.1	10	50	100	50	37°	80°	B	Lambda		CpG	d
BsgI	CutSmart + SAM	25	50	25	100	37°	65°	B	Lambda			d
BsiEI	CutSmart	25	50	< 10	100	60°	No	A	Lambda		CpG	
BsiHKAI	CutSmart	25	100	100	100	65°	No	A	Lambda			
BsiWI	3.1	25	50*	100	25	55°	65°	B	phiX174		CpG	
BsiWI-HF	CutSmart	50	100	10	100	37°	No	B	phiX174		CpG	3
BsII	CutSmart	50	75	100	100	55°	No	A	Lambda	dcm	CpG	b

ENZYME	SUPPLIED NEBUFFER	% ACTIVITY IN NEBUFFERS 1.1	2.1	3.1	CUTSMART	INCUB. TEMP. (°C)	INACTIV. TEMP. (°C)	DILUENT	UNIT SUBSTRATE	METHYLATION SENSITIVITY		NOTE
BsmI	CutSmart	25	100	< 10	100	65°	80°	A	Lambda			
BsmAI	CutSmart	50	100	100	100	55°	No	B	Lambda		CpG	
BsmBI	3.1	10	50*	100	25	55°	80°	B	Lambda		CpG	
BsmFI	CutSmart	25	50	50	100	65°	80°	A	pBR322	dcm	CpG	1
BsoBI	CutSmart	25	100	100	100	37°	80°	A	Lambda			
Bsp1286I	CutSmart	25	25	25	100	37°	65°	A	Lambda			3
BspCNI	CutSmart + SAM	100	75	10	100	25°	80°	A	Lambda			b
BspDI	CutSmart	25	75	50	100	37°	80°	A	Lambda	dam	CpG	
BspEI	3.1	< 10	10	100	< 10	37°	80°	B	Lambda dam-	dam	CpG	
BspHI	CutSmart	< 10	50	25	100	37°	80°	A	Lambda	dam		
BspMI	3.1	10	50*	100	10	37°	65°	B	Lambda			
BspQI	3.1	100	100	100	100	50°	80°	B	Lambda			3
BsrI	3.1	< 10	50	100	10	65°	80°	B	phiX174			b
BsrBI	CutSmart	50	100	100	100	37°	80°	A	Lambda		CpG	d
BsrDI	2.1	10	100	75	25	65°	80°	A	Lambda			3, d
BsrF$^{\alpha}$I	CutSmart	25	25	0	100	37°	No	C	pBR322		CpG	
BsrGI	2.1	25	100	100	25	37°	80°	A	Lambda			
BsrGI-HF	CutSmart	10	100	100	100	37°	80°	A	Lambda			
BssHII	CutSmart	100	100	100	100	50°	65°	B	Lambda		CpG	
BssS$^{\alpha}$I	CutSmart	10	25	< 10	100	37°	No	B	Lambda			
BstAPI	CutSmart	50	100	25	100	60°	80°	A	Lambda		CpG	b
BstBI	CutSmart	75	100	10	100	65°	No	A	Lambda		CpG	
BstEII	3.1	10	75*	100	75*	60°	No	A	Lambda			3
BstEII-HF	CutSmart	< 10	10	< 10	100	37°	No	A	Lambda			
BstNI	3.1	10	100	100	75	60°	No	A	Lambda			a
BstUI	CutSmart	50	100	25	100	60°	No	A	Lambda		CpG	b
BstXI	3.1	< 10	50	100	25	37°	80°	B	Lambda	dcm		3
BstYI	2.1	25	100	75	100	60°	No	A	Lambda			
BstZ17I-HF	CutSmart	100	100	10	100	37°	No	A	Lambda		CpG	
Bsu36I	CutSmart	25	100	100	100	37°	80°	C	Lambda HindIII			b
BtgI	CutSmart	50	100	100	100	37°	80°	B	pBR322			
BtgZI	CutSmart	10	25	< 10	100	60°	80°	A	Lambda		CpG	3, b, d
Bts$^{\alpha}$I	CutSmart	100	100	25	100	55°	No	A	Lambda			
BtsIMutI	CutSmart	100	50	10	100	55°	80°	A	pUC19			b
BtsCI	CutSmart	10	100	25	100	50°	80°	B	Lambda			
Cac8I	CutSmart	50	75	100	100	37°	65°	B	Lambda			b
ClaI	CutSmart	10	50	50	100	37°	65°	A	Lambda dam-	dam	CpG	
CspCI	CutSmart + SAM	10	100	10	100	37°	65°	A	Lambda			e
CviAII	CutSmart	50	50	10	100	25°	65°	C	Lambda			
CviKI-1	CutSmart	25	100	100	100	37°	No	A	pBR322			1, b
CviQI	3.1	75	100*	100	75*	25°	No	C	Lambda			b
DdeI	CutSmart	75	100	100	100	37°	65°	B	Lambda			
DpnI	CutSmart	100	100	75	100	37°	80°	B	pBR322		CpG	b
DpnII	U	25	25	100*	25	37°	65°	B	Lambda dam-	dam		
DraI	CutSmart	75	75	50	100	37°	65°	A	Lambda			
DraIII-HF	CutSmart	< 10	50	10	100	37°	No	B	Lambda		CpG	b
DrdI	CutSmart	25	50	10	100	37°	65°	A	pUC19		CpG	3
EaeI	CutSmart	10	50	< 10	100	37°	65°	A	Lambda	dcm	CpG	b
EagI	3.1	10	25	100	10	37°	65°	B	pXba		CpG	
EagI-HF	CutSmart	25	100	100	100	37°	65°	B	pXba		CpG	
EarI	CutSmart	50	10	< 10	100	37°	65°	B	Lambda		CpG	b, d
EciI	CutSmart	100	50	50	100	37°	65°	A	Lambda		CpG	2

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.

ENZYME	SUPPLIED NEBUFFER	% ACTIVITY IN NEBUFFERS 1.1	2.1	3.1	CUTSMART	INCUB. TEMP. (°C)	INACTIV. TEMP. (°C)	DILUENT	UNIT SUBSTRATE	METHYLATION SENSITIVITY	NOTE
Eco53kI	CutSmart	100	100	< 10	100	37°	65°	A	pXba	CpG	3, b
EcoNI	CutSmart	50	100	75	100	37°	65°	A	Lambda		b
EcoO109I	CutSmart	50	100	50	100	37°	65°	A	Lambda HindIII	dcm	3
EcoP15I	3.1 + ATP	75	100	100	100	37°	65°	A	pUC19		e
EcoRI	U	25	100*	50	50*	37°	65°	C	Lambda	CpG	
EcoRI-HF	CutSmart	10	100	< 10	100	37°	65°	C	Lambda	CpG	
EcoRV	3.1	10	50	100	10	37°	80°	A	Lambda	CpG	
EcoRV-HF	CutSmart	25	100	100	100	37°	65°	B	Lambda	CpG	
Esp3I	CutSmart	100	100	10	100	37°	65°	B	Lambda	CpG	
FatI	2.1	10	100	50	50	55°	80°	A	pUC19		
FauI	CutSmart	100	50	10	100	55°	65°	A	Lambda	CpG	3, b, d
Fnu4HI	CutSmart	< 10	< 10	< 10	100	37°	No	A	Lambda	CpG	a
FokI	CutSmart	100	100	75	100	37°	65°	A	Lambda	dcm CpG	3, b, d
FseI	CutSmart	100	75	< 10	100	37°	65°	B	pBC4	dcm CpG	
FspI	CutSmart	10	100	10	100	37°	No	C	Lambda	CpG	b
FspEI	CutSmart	< 10	< 10	< 10	100	37°	80°	B	pBR322	dcm	2, e
HaeII	CutSmart	25	100	10	100	37°	80°	A	Lambda	CpG	
HaeIII	CutSmart	50	100	25	100	37°	80°	A	Lambda		
HgaI	1.1	100	100	25	100	37°	65°	A	phiX174	CpG	1
HhaI	CutSmart	25	100	100	100	37°	65°	A	Lambda	CpG	
HincII	3.1	25	100	100	100	37°	65°	B	Lambda	CpG	
HindIII	2.1	25	100	50	50	37°	80°	B	Lambda		2
HindIII-HF	CutSmart	10	100	10	100	37°	80°	B	Lambda		
HinfI	CutSmart	50	100	100	100	37°	80°	A	Lambda	CpG	
HinP1I	CutSmart	100	100	100	100	37°	65°	A	Lambda	CpG	
HpaI	CutSmart	< 10	75*	25	100	37°	No	A	Lambda	CpG	1
HpaII	CutSmart	100	50	< 10	100	37°	80°	A	Lambda	CpG	
HphI	CutSmart	50	50	< 10	100	37°	65°	B	Lambda	dam CpG	b, d
Hpy99I	CutSmart	50	10	< 10	100	37°	65°	A	Lambda	CpG	
Hpy166II	CutSmart	100	100	50	100	37°	65°	C	pBR322	CpG	
Hpy188I	CutSmart	25	100	50	100	37°	65°	A	pBR322	dam	1, b
Hpy188III	CutSmart	100	100	10	100	37°	65°	B	pUC19	dam CpG	3, b
HpyAV	CutSmart	100	100	25	100	37°	65°		Lambda	CpG	3, b, d
HpyCH4III	CutSmart	100	25	< 10	100	37°	65°	A	Lambda		b
HpyCH4IV	CutSmart	100	50	25	100	37°	65°	A	pUC19	CpG	
HpyCH4V	CutSmart	50	50	25	100	37°	65°	A	Lambda		
I-CeuI	CutSmart	10	10	10	100	37°	65°	B	pBHS ScaI-linearized		
I-SceI	CutSmart	10	50	25	100	37°	65°	B	pGPS2 NotI-linearized		
KasI	CutSmart	50	100	50	100	37°	65°	B	pBR322	CpG	3
KpnI	1.1	100	75	< 10	50	37°	No	A	pXba		1
KpnI-HF	CutSmart	100	25	< 10	100	37°	No	A	pXba		
LpnPI	CutSmart	< 10	< 10	< 10	100	37°	65°	B	pBR322		2, e
MboI	CutSmart	75	100	100	100	37°	65°	A	Lambda dam-	dam CpG	
MboII	CutSmart	100*	100	50	100	37°	65°	C	Lambda dam-	dam	b
MfeI	CutSmart	75	50	10	100	37°	No	A	Lambda		2
MfeI-HF	CutSmart	75	25	< 10	100	37°	No	A	Lambda		
MluI	3.1	10	50	100	25	37°	80°	A	Lambda	CpG	
MluI-HF	CutSmart	25	100	100	100	37°	No	A	Lambda	CpG	
MluCI	CutSmart	100	10	10	100	37°	No	A	Lambda		
MlyI	CutSmart	50	50	10	100	37°	65°	A	Lambda		b, d
MmeI	CutSmart + SAM	50	100	50	100	37°	65°	B	phiX174	CpG	b, c
MnlI	CutSmart	75	100	50	100	37°	65°	B	Lambda		b
MscI	CutSmart	25	100	100	100	37°	80°	C	Lambda	dcm	

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 1.1	2.1	3.1	CUTSMART	INCUB. TEMP. (°C)	INACTIV. TEMP. (°C)	DILUENT	UNIT SUBSTRATE	METHYLATION SENSITIVITY		NOTE
MseI	CutSmart	75	100	75	100	37°	65°	A	Lambda			
MslI	CutSmart	50	50	< 10	100	37°	80°	A	Lambda			
MspI	CutSmart	75	100	50	100	37°	No	A	Lambda			
MspA1I	CutSmart	10	50	10	100	37°	65°	B	Lambda		CpG	
MspJI	CutSmart	< 10	< 10	< 10	100	37°	65°	B	pBR322			2, e
MwoI	CutSmart	< 10	100	100	100	60°	No	B	Lambda		CpG	
NaeI	CutSmart	25	25	< 10	100	37°	No	A	pXba		CpG	b
NarI	CutSmart	100	100	10	100	37°	65°	A	pXba		CpG	
Nb.BbvCI	CutSmart	25	100	100	100	37°	80°	A	pUB			e
Nb.BsmI	3.1	< 10	50	100	10	65°	80°	A	pBR322			e
Nb.BsrDI	CutSmart	25	100	100	100	65°	80°	A	pUC19			e
Nb.BssSI	3.1	10	100	100	25	37°	No	B	pUC19			
Nb.BtsI	CutSmart	75	100	75	100	37°	80°	A	phiX174			e
NciI	CutSmart	100	25	10	100	37°	No	A	Lambda		CpG	b
NcoI	3.1	100	100	100	100	37°	80°	A	Lambda			
NcoI-HF	CutSmart	50	100	10	100	37°	80°	B	Lambda			
NdeI	CutSmart	75	100	100	100	37°	65°	A	Lambda			
NgoMIV	CutSmart	100	50	10	100	37°	No	A	pXba		CpG	1
NheI	2.1	100	100	10	100	37°	65°	C	Lambda HindIII		CpG	
NheI-HF	CutSmart	100	25	< 10	100	37°	80°	C	Lambda HindIII		CpG	
NlaIII	CutSmart	< 10	< 10	< 10	100	37°	65°	B	phiX174			
NlaIV	CutSmart	10	10	10	100	37°	65°	B	pBR322	dcm	CpG	
NmeAIII	CutSmart + SAM	10	10	< 10	100	37°	65°	B	phiX174			c
NotI	3.1	< 10	50	100	25	37°	65°	C	pBC4		CpG	
NotI-HF	CutSmart	25	100	25	100	37°	65°	A	pBC4		CpG	
NruI	3.1	< 10	10	100	10	37°	No	A	Lambda	dam	CpG	b
NruI-HF	CutSmart	0	25	50	100	37°	No	A	Lambda	dam	CpG	
NsiI	3.1	10	75	100	25	37°	65°	B	Lambda			
NsiI-HF	CutSmart	< 10	20	< 10	100	37°	80°	B	Lambda			
NspI	CutSmart	100	100	< 10	100	37°	65°	A	Lambda			
Nt.AlwI	CutSmart	10	100	100	100	37°	80°	A	pUC101 dam-dcm-	dam		e
Nt.BbvCI	CutSmart	50	100	10	100	37°	80°	A	pUB		CpG	e
Nt.BsmAI	CutSmart	100	50	10	100	37	65°	A	pBR322		CpG	e
Nt.BspQI	3.1	< 10	25	100	10	50°	80°	B	pUC19			e
Nt.BstNBI	3.1	0	10	100	10	55°	80°	A	T7			
PacI	CutSmart	100	75	10	100	37°	65°	A	pNEB193			
PaeR7I	CutSmart	25	100	10	100	37°	No	A	Lambda HindIII		CpG	
PciI	3.1	50	75	100	50*	37°	80°	B	pXba			
PflFI	CutSmart	25	100	25	100	37°	65°	A	pBC4			b
PflMI	3.1	0	100	100	50	37°	65°	A	Lambda	dcm		3, b, d
PI-PspI	U	10	10	10	10	65°	No	B	pAKR XmnI			
PI-SceI	U	10	10	10	10	37°	65°	B	pBSvdeX XmnI			
PleI	CutSmart	25	50	25	100	37°	65°	A	Lambda		CpG	b, d
PluTI	CutSmart	100	25	< 10	100	37°	65°	A	pXba		CpG	
PmeI	CutSmart	< 10	50	10	100	37°	65°	A	Lambda		CpG	
PmlI	CutSmart	100	50	< 10	100	37°	65°	A	Lambda HindIII		CpG	
PpuMI	CutSmart	< 10	< 10	< 10	100	37°	No	B	Lambda HindIII	dcm		
PshAI	CutSmart	25	50	10	100	37°	65°	A	Lambda		CpG	
PsiI	CutSmart	10	100	10	100	37°	65°	B	Lambda			3
PspGI	CutSmart	25	100	50	100	75°	No	A	T7	dcm		3
PspOMI	CutSmart	10	10	< 10	100	37°	65°	B	pXba	dcm	CpG	
PspXI	CutSmart	< 10	100	25	100	37°	No	B	Lambda HindIII		CpG	

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.

ENZYME	SUPPLIED NEBUFFER	% ACTIVITY IN NEBUFFERS 1.1	2.1	3.1	CUTSMART	INCUB. TEMP. (°C)	INACTIV. TEMP. (°C)	DILUENT	UNIT SUBSTRATE	METHYLATION SENSITIVITY		NOTE
PstI	3.1	75	75	100	50*	37°	80°	C	Lambda			
PstI-HF	CutSmart	10	75	50	100	37°	No	C	Lambda			
PvuI	3.1	< 10	25	100	< 10	37°	No	B	pXba		CpG	
PvuI-HF	CutSmart	25	100	100	100	37°	No	B	pXba		CpG	
PvuII	3.1	50	100	100	100*	37°	No	B	Lambda			
PvuII-HF	CutSmart	< 10	< 10	< 10	100	37°	No	B	Lambda			
RsaI	CutSmart	25	50	< 10	100	37°	No	A	Lambda		CpG	
RsrII	CutSmart	25	75	10	100	37°	65°	C	Lambda		CpG	
SacI	1.1	100	50	10	100	37°	65°	A	Lambda HindIII			
SacI-HF	CutSmart	10	50	< 10	100	37°	65°	A	Lambda HindIII		CpG	
SacII	CutSmart	10	100	10	100	37°	65°	A	pXba		CpG	
SalI	3.1	< 10	< 10	100	< 10	37°	65°	A	Lambda HindIII		CpG	
SalI-HF	CutSmart	10	100	100	100	37°	65°	A	Lambda HindIII		CpG	
SapI	CutSmart	75	50	< 10	100	37°	65°	B	Lambda			
Sau3AI	1.1	100	50	10	100	37°	65°	A	Lambda		CpG	b
Sau96I	CutSmart	50	100	100	100	37°	65°	A	Lambda	dcm	CpG	
SbfI	CutSmart	50	25	< 10	100	37°	80°	A	Lambda			3
SbfI-HF	CutSmart	50	25	< 10	100	37°	80°	B	Lambda			
ScaI-HF	CutSmart	100	100	10	100	37°	80°	B	Lambda			
ScrFI	CutSmart	100	100	100	100	37°	65°	C	Lambda	dcm	CpG	2, a
SexAI	CutSmart	100	75	50	100	37°	65°	A	pBC4 dcm-	dcm		3, b, d
SfaNI	3.1	< 10	75	100	25	37°	65°	B	phiX174		CpG	3, b
SfcI	CutSmart	75	50	25	100	37°	65°	B	Lambda			3
SfiI	CutSmart	25	100	50	100	50°	No	C	Adenovirus-2	dcm	CpG	
SfoI	CutSmart	50	100	100	100	37°	No	B	Lambda HindIII	dcm	CpG	
SgrAI	CutSmart	100	100	10	100	37°	65°	A	Lambda		CpG	1
SmaI	CutSmart	< 10	< 10	< 10	100	25°	65°	B	Lambda HindIII		CpG	b
SmlI	CutSmart	25	75	25	100	55°	No	A	Lambda			b
SnaBI	CutSmart	50	50	10	100	37°	80°	A	T7		CpG	1
SpeI	CutSmart	75	100	25	100	37°	80°	C	Adenovirus-2			
SpeI-HF	CutSmart	25	50	10	100	37°	80°	C	pXba			
SphI	2.1	100	100	50	100	37°	65°	B	Lambda			2
SphI-HF	CutSmart	50	25	10	100	37°	65°	B	Lambda			
SrfI	CutSmart	10	50	0	100	37°	65°	B	pNEB193-SrfI		CpG	
SspI	U	50	100	50	50	37°	65°	C	Lambda			
SspI-HF	CutSmart	25	100	< 10	100	37°	65°	B	Lambda			
StuI	CutSmart	50	100	50	100	37°	No	A	Lambda	dcm		
StyD4I	CutSmart	10	100	100	100	37°	65°	B	Lambda	dcm	CpG	
StyI	3.1	10	25	100	10	37°	65°	A	Lambda			b
StyI-HF	CutSmart	25	100	25	100	37°	65°	A	Lambda			
SwaI	3.1	10	10	100	10	25°	65°	B	pXba			b, d
TaqαI	CutSmart	50	75	100	100	65°	80°	B	Lambda	dam		
TfiI	CutSmart	50	100	100	100	65°	No	C	Lambda		CpG	
TseI	CutSmart	75	100	100	100	65°	No	B	Lambda		CpG	3
Tsp45I	CutSmart	100	50	< 10	100	65°	No	A	Lambda			
TspMI	CutSmart	50*	75*	50*	100	75°	No	B	pUCAdeno		CpG	d
TspRI	CutSmart	25	50	25	100	65°	No	B	Lambda			
Tth111I	CutSmart	25	100	25	100	65°	No	B	pBC4			b
XbaI	CutSmart	< 10	100	75	100	37°	65°	A	Lambda HindIII dam-	dam		
XcmI	2.1	10	100	25	100	37°	65°	C	Lambda			2
XhoI	CutSmart	75	100	100	100	37°	65°	A	Lambda HindIII			b
XmaI	CutSmart	25	50	< 10	100	37°	65°	A	pXba		CpG	3
XmnI	CutSmart	50	75	< 10	100	37°	65°	A	Lambda			b
ZraI	CutSmart	100	25	10	100	37°	80°	B	Lambda		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.

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.

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* or *Taq* 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

PCR Polymerase Selection Chart for Cloning

For almost 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
	One *Taq*/ One *Taq* Hot Start	*Taq* / Hot Start *Taq*	Q5/ Q5 Hot Start	Phusion®(1) / Phusion(1) Flex	LongAmp®/ LongAmp Hot Start *Taq*
PROPERTIES					
Fidelity vs. *Taq*	2X	1X	~280X(4)	> 50X	2X
Amplicon Size	< 6 kb	≤ 5 kb	≤ 20 kb	≤ 20 kb	≤ 30 kb
Extension Time	1 kb/min	1 kb/min	6 kb/min	4 kb/min	1.2 kb/min
Resulting Ends	3´ A/Blunt	3´ A	Blunt	Blunt	3´ A/Blunt
3´→ 5´ exo	Yes	No	Yes	Yes	Yes
5´→ 3´ exo	Yes	Yes	No	No	Yes
Units/50 µl Reaction	1.25	1.25	1.0	1.0	5.0
Annealing Temperature	Tm^{-5}	Tm^{-5}	Tm^{+3}	Tm^{+3}	Tm^{-5}
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)	●	●	
Site-directed Mutagenesis			★	●	
FORMATS					
Hot Start Available	●	●	●	●	●
Kit		●	●	●	●
Master Mix Available	●	●	●	●	●
Direct Gel Loading	●	●(3)			

(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) Use Quick-Load 2X *Taq* Master Mix
(4) We continue to investigate improved assays to characterize Q5's very low error rate to ensure that we present the most accurate fidelity data possible (Potapov, V. and Ong, J.L. (2017) PLoS ONE. 12(1): e0169774).

★ indicates recommended choice for application
ND indicates not determined

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

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

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

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
- 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

THE MECHANISM OF DNA PHOSPHORYLATION

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).

Protocol: Dephosphorylation using Quick Dephosphorylation Kit

	STANDARD PROTOCOL
DNA	1 pmol of ends
10X CutSmart 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

- 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).
- When working with the Quick Dephosphorylation Kit (NEB #M0508), 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. However, when using CIP (NEB #M0290), a clean-up step (e.g., Monarch PCR & DNA Cleanup Kit, NEB #T1030) prior to ligation is necessary.

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

Phosphatase Selection Chart

	Recombinant Shrimp Alkaline Phosphatase (rSAP) (NEB #M0371)	Antarctic Phosphatase (AP) (NEB #M0289)	Alkaline Phosphatase Calf Intestinal (CIP) (NEB #M0290)	Quick Dephosphorylation Kit (NEB #M0508)
FEATURES				
100% heat inactivation	5 minutes/65°C	2 minutes/80°C	No	2 minutes/80°C
High specific activity	●		●	●
Improved stability	●			●
Works directly in NEB buffers	●	●	●	●
Requires additive		● (Zn^{2+})		
Quick Protocol				●

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).

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).

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.

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.

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).

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.

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.

A-tailing Selection Chart

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

Activity of DNA Modifying Enzymes in CutSmart Buffer

A selection of DNA modifying enzymes were assayed in CutSmart Buffer and functional activity was compared to the activity in their supplied buffers. Reactions were set up according to the recommended reaction conditions, with CutSmart Buffer (plus required supplement) 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 CutSmart Buffer to achieve appropriate activity for most applications – no change of buffers needed.

ENZYME	ACTIVITY IN CUTSMART	REQUIRED SUPPLEMENTS
Alkaline Phosphatase (CIP)	+++	
Antarctic Phosphatase	+++	Requires Zn^{2+}
Bst DNA Polymerase	+++	
CpG Methyltransferase (M. SssI)	+++	Requires SAM
DNA Polymerase I	+++	
DNA Polymerase I, Large (Klenow) Fragment	+++	
DNA Polymerase Klenow Exo⁻	+++	
DNase I (RNase free)	+++	Requires Ca^{2+}
E. coli DNA Ligase	+++	Requires NAD
Endonuclease III (Nth), recombinant	+++	
Endonuclease VIII	+++	
Exonuclease III	+++	
GpC Methyltransferase (M. CviPI)	+	Requires DTT
McrBC	+++	

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

ENZYME	ACTIVITY IN CUTSMART	REQUIRED SUPPLEMENTS
Micrococcal Nuclease	+++	Requires Ca^{2+}
phi29 DNA Polymerase	+++	
$RecJ_f$	+++	
Shrimp Alkaline Phosphatase (rSAP)	+++	
T3 DNA Ligase	+++	Requires ATP + PEG
T4 DNA Ligase	+++	Requires ATP
T4 DNA Polymerase	+++	
T4 Phage β-glucosyltransferase (T4-BGT)	+++	
T4 Polynucleotide Kinase	+++	Requires ATP + DTT
T4 PNK (3´ phosphatase minus)	+++	Requires ATP + DTT
T7 DNA Ligase	+++	Requires ATP + PEG
T7 DNA Polymerase (unmodified)	+++	
T7 Exonuclease	+++	
USER™ Enzyme, recombinant	+++	

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.

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 (3 kb)	50 ng	50 ng	50 ng	50 ng
Insert (1 kb)	50 ng	50 ng	50 ng	50 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

- Use DNA free from contaminants such as EDTA and salts. We recommend Monarch PCR & DNA Cleanup Kit (NEB #T1030).
- Either heat inactivate (AP, rSAP) or remove phosphatase (CIP, BAP or SAP) 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 most ligations (blunt or cohesive), the Quick Ligation Kit (NEB #M2200) or the master mixes are recommended
- For large inserts, reduce insert concentration and use concentrated ligase at 16°C overnight
- T4 DNA Ligase (NEB #M0202) can be heat inactivated at 65°C for 20 minutes
- Do not heat inactivate if there is PEG in the reaction buffer because transformation will be inhibited
- Electroporation is recommended for large constructs (> 10,000 bp). If planning to electroporate, we recommend ElectroLigase (NEB #M0369) for your ligation step.

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
- Do not heat inactivate when using the Quick Ligation Buffer or Ligase Master Mixes, as this will inhibit transformation

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)	*Taq* DNA Ligase (NEB #M0208)
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								✓
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

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.

Protocol: High Efficiency Transformation

	STANDARD PROTOCOL
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

* Follow specific heat shock recommendations provided for the *E. coli* competent cell strain being used.

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						•
FORMATS						
Chemically competent	•	•	•	•	•	•
Electrocompetent	•	•		•		
Subcloning	•					
96-well format*	•					
384-well format*	•					
12 x 8-tube strips*	•					

* Other strains are available upon request. For more information, contact NEBsolutions@neb.com.

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

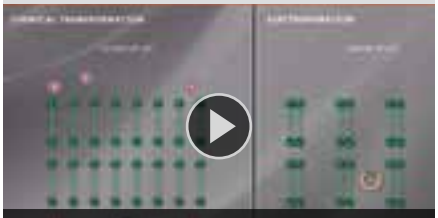
INCUBATION & HEAT SHOCK
- Incubate on ice for 30 minutes. Expect a 2-fold loss in transformation efficiency (TE) for every 10 minutes this step is shortened.
- Both temperature and time are specific to the transformation volume and vessel. Typically, 30 seconds at 42°C is recommended.

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

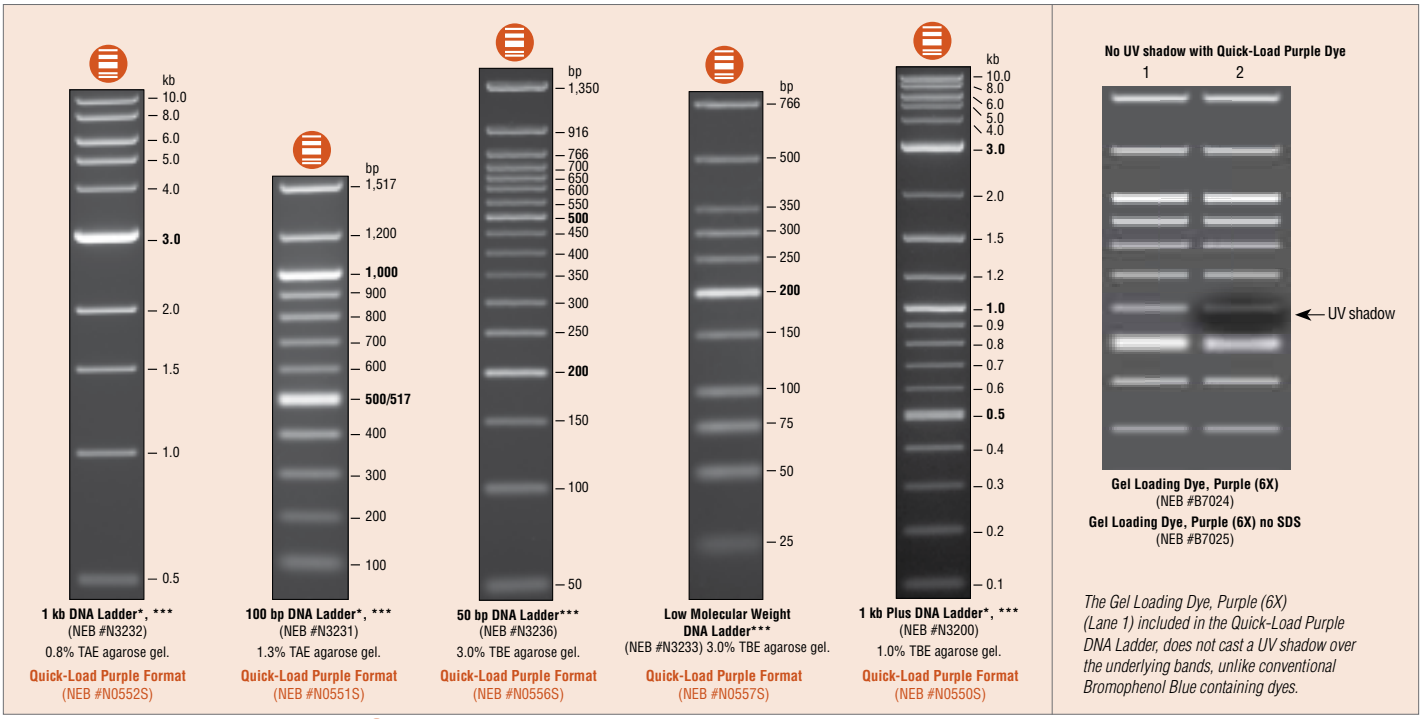


LEARN MORE ABOUT TRANSFORMATION

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.

The Following DNA Ladders are Now Available in Quick-Load Purple Format



1 kb DNA Ladder*, ***
(NEB #N3232)
0.8% TAE agarose gel.
Quick-Load Purple Format
(NEB #N0552S)

100 bp DNA Ladder*, ***
(NEB #N3231)
1.3% TAE agarose gel.
Quick-Load Purple Format
(NEB #N0551S)

50 bp DNA Ladder***
(NEB #N3236)
3.0% TBE agarose gel.
Quick-Load Purple Format
(NEB #N0556S)

Low Molecular Weight DNA Ladder***
(NEB #N3233) 3.0% TBE agarose gel.
Quick-Load Purple Format
(NEB #N0557S)

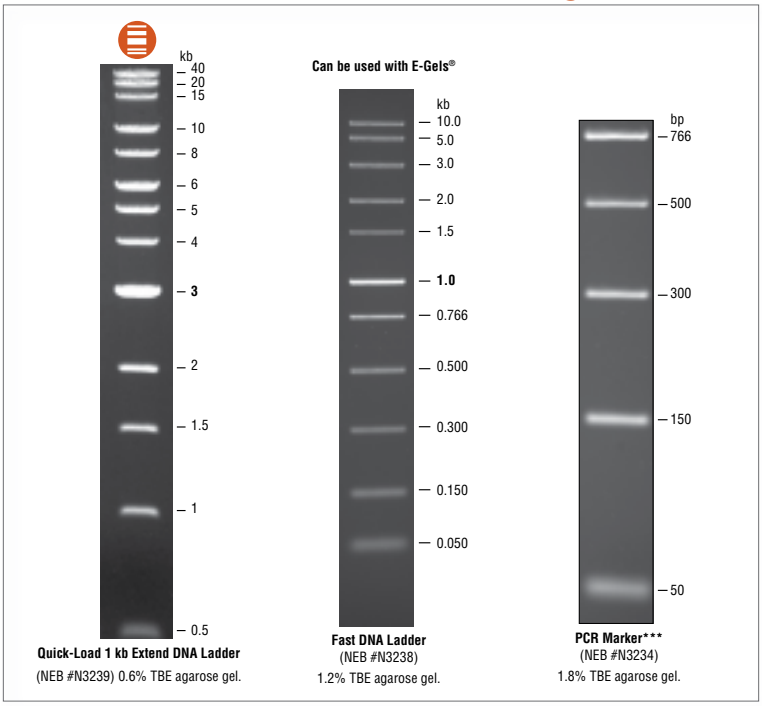
1 kb Plus DNA Ladder*, ***
(NEB #N3200)
1.0% TBE agarose gel.
Quick-Load Purple Format
(NEB #N0550S)

Gel Loading Dye, Purple (6X)
(NEB #B7024)
Gel Loading Dye, Purple (6X) no SDS
(NEB #B7025)

The Gel Loading Dye, Purple (6X) (Lane 1) included in the Quick-Load Purple DNA Ladder, does not cast a UV shadow over the underlying bands, unlike conventional Bromophenol Blue containing dyes.

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

Additional DNA Ladders from New England Biolabs



Quick-Load 1 kb Extend DNA Ladder
(NEB #N3239) 0.6% TBE agarose gel.

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

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

**** Free Loading Dye included*

View all available DNA Ladders, including traditional DNA and PFG markers, at https://international.neb.com/marker-chart

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

Primer	1 µg
10X T4 Ligase Buffer	5 µl
Nuclease-free Water	To 50 µl
Incubation	85°C for 10 minutes, cool slowly (30-60 min.)

Vector

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

Dephosphorylation

- Dephosphorylation is sometimes necessary to prevent self ligation. NEB offers four products for dephosphorylation of DNA:
- The Quick Dephosphorylation Kit (NEB #M0508), 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+}. The Quick Dephosphorylation Kit is optimized for fast and robust dephosphorylation in 10 minutes, and is heat inactivated in 2 minutes.
- Calf Intestinal Phosphatase (CIP) (NEB #M0290) will function under many different conditions and in most NEBuffers. However, CIP cannot be heat inactivated and requires a purification step (such as with Monarch PCR & DNA Cleanup Kit, NEB #T1030) before ligation.

Dephosphorylation of 5´ ends of DNA using the Quick Dephosphorylation Kit

DNA	1 pmol of DNA ends
10X CutSmart 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 (360 nM) to minimize UV exposure that may cause DNA damage

Blunting with the Quick Blunting Kit

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

* PCR-generated DNA must be purified before blunting using a commercial purification kit, phenol extraction/ethanol precipitation or gel electrophoresis (e.g., Monarch PCR & DNA Cleanup Kit, NEB #T1030).

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)

Phosphorylation With T4 PNK

DNA (20 mer)	1–2 µg
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 (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 (360 nM) to minimize UV exposure that may cause DNA damage

Ligation of Vector and Insert

- Use a molar ratio of 1:3 vector to insert
- 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)

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 tranformants 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 #3040)
- 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 5-alpha Electrocompetent *E. coli* (NEB #C2989) or NEB 10-beta Electrocompetent *E. coli* (NEB #C3020)
- Use pre-warmed selection plates
- Perform several 10-fold serial dilutions in SOC 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 • 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)] • For very large constructs (> 10 kb), consider using electroporation
	Construct may be susceptible to recombination	• Select a Rec A- 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 • Purify the DNA to remove contaminants such as salt and EDTA • 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) • Test the activity of the ligase by carrying out a ligation control with Lambda-HindIII digested DNA (NEB #N0312)
	Inefficient phosphorylation	• Purify the DNA prior to phosphorylation. 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

PROBLEM	CAUSE	SOLUTION
Few or no transformants	Inefficient blunting	• Heat inactivate or remove the restriction enzymes prior to blunting • Clean up the PCR fragment prior to blunting • 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.
	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* (NEB #C3040)
	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 • 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 • Test the activity of the ligase by carrying out a ligation control with Lambda-HindIII digested DNA
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
	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 3.1) 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. 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 3–5 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 supercolied DNA. Increase the number of enzyme units in the reaction.

Troubleshooting Guide for Cloning (cont.)

PROBLEM	CAUSE	SOLUTION
Incomplete restriction enzyme digestion	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
	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. Mini prep DNA is particularly susceptible to contaminants. NEB recommends the Monarch PCR & DNA Cleanup Kit (NEB #T1030). • Clean DNA with a spin column, resin or drop dialysis, or increase volume to dilute contaminant
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
	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 3.1) 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. 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

Cloning Workflow Descriptions

There are several methods that can be used to generate DNA constructs, each of which is described below. A comparison of the various workflows discussed can be found on page 6.

Seamless Cloning/Gene Assembly

The group of cloning methods we refer to as "seamless cloning" typically combine attributes from more established cloning methods to create a unique solution to allow sequence-independent and scarless insertion of one or more DNA fragments into a plasmid vector. Various commercial systems, such as NEBuilder HiFi DNA Assembly, NEB Gibson Assembly and In-Fusion® employ PCR to amplify the gene of interest, an exonuclease to chew back one strand of the insert and vector ends, and either a ligase, recombination event, or *in vivo* repair to covalently join the insert to the vector through a true phosphodiester bond. The ability to quickly join a single insert to a plasmid at any sequence in the vector, without a scar, makes these technologies very appealing cloning methods. Additionally, the ability to join 5–10 fragments in a predetermined order, with no sequence restrictions or scars, provides a powerful technique for synthetic biology endeavors, such as moving whole operons for metabolic engineering or whole genome reconstructions.

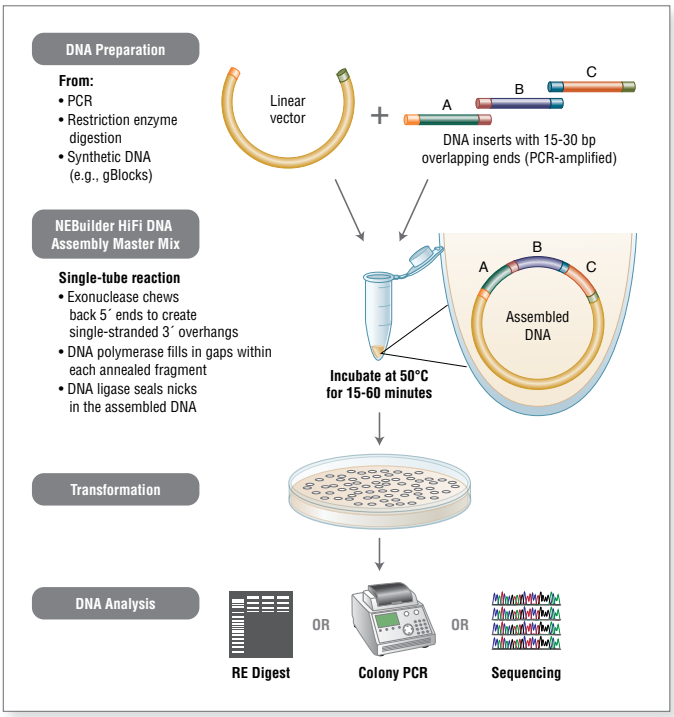
ADVANTAGES

- No sequence constraints
- Efficient assembly of multiple fragments
- High cloning efficiency
- Exquisite control of higher-order gene assembly

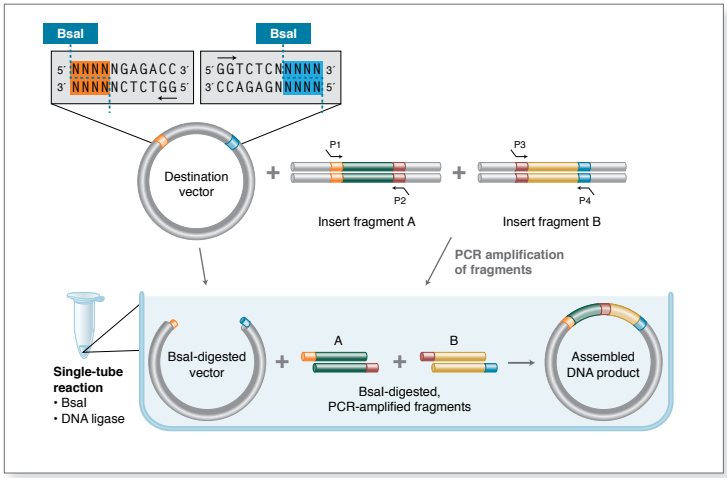
DISADVANTAGES

- Cost, relative to traditional methods
- PCR primers for vector and insert must be designed and ordered

Overview of the NEBuilder HiFi DNA Assembly cloning method



NEB Golden Gate Assembly workflow



In its simplest form, Golden Gate Assembly requires a Type IIS recognition site, in this case, BsaI (GGTCTC), 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.

Golden Gate Assembly is another method of seamless cloning that exploits the ability of Type IIS restriction enzymes (such as BsaI) to cleave DNA outside of the recognition sequence. The inserts and cloning vectors are designed to place the Type IIS recognition site distal to the cleavage site, such that the Type IIS restriction enzyme can remove the recognition sequence from the assembly. The advantages of such an arrangement are three-fold: 1. the overhang sequence created is not dictated by the restriction enzyme, and therefore no scar sequence is introduced; 2. the fragment-specific sequence of the overhangs allows orderly assembly of multiple fragments simultaneously; and 3. the restriction site is eliminated from the ligated product, so digestion and ligation can be carried out simultaneously. The net result is the ordered and seamless assembly of DNA fragments in one reaction. The full list of Type IIS enzymes, including some that are recommended for use in Golden Gate Assembly, can be found at **www.neb.com/TypeIIS**.

Traditional Cloning

Traditional Cloning usually refers to the use of restriction endonucleases to generate DNA fragments with specific complementary end sequences that can be joined together with a DNA ligase, prior to transformation. This typically involves preparing both a DNA fragment to be cloned (insert) and a self-replicating DNA plasmid (vector) by cutting with two unique restriction enzymes that flank the DNA sequence, and whose cut sites are present at the preferred site of insertion of the vector, often called the multiple cloning site (MCS). By using two different REs, two non-compatible ends are generated, thus forcing the insert to be cloned directionally, and lowering the transformation background of re-ligated vector alone. Directional cloning is useful to maintain open reading frames or another positional requirement with *cis*-acting regulatory elements. Non-directional cloning can also be performed with compatible ends generated by a single restriction enzyme; in this case the clones will need to be screened to determine that the gene orientation is correct. Typically the vector needs to be de-phosphorylated to prevent self-ligation, which directly competes with the insert and lowers the efficiency of the cloning reaction.

In the early years of cloning, genomic DNA was often cloned into plasmid vectors using DNA adaptors to add the required restriction sites to a sequence of interest, prior to ligation. Additionally, genes or other DNA elements were swapped between vectors using compatible ends contained by both vectors. More recently, PCR is used as an upstream step in a cloning protocol to introduce the necessary restriction sites for directional cloning prior to preparation of the vector and insert by restriction digests, followed by fragment purification, fragment ligation, and transformation into an *E. coli* cloning strain for plasmid amplification. Transformed colonies, now resistant to an antibiotic due to a resistance gene harbored by the plasmid, are screened by colony PCR or restriction digest of plasmid DNA for the correct insert. Direct sequencing of the recombinant plasmid is often performed to verify the sequence integrity of the cloned fragment.

ADVANTAGES

- Low cost
- Versatile
- Many different vector choices
- Directional cloning can be easily done

DISADVANTAGES

- Possible sequence constraints due to presence and/or translation of restriction site

LEARN MORE ABOUT TRADITIONAL CLONING

PCR Cloning

PCR cloning differs from traditional cloning in that the DNA fragment of interest, and even the vector, can be amplified by PCR and ligated together without the use of restriction enzymes. PCR cloning is a rapid method for cloning genes, and is often used for projects that require higher throughput than traditional cloning methods can accommodate. It also allows for the cloning of DNA fragments that are not available in large amounts. Typically, a PCR reaction is performed to amplify the sequence of interest and then it is joined to the vector via a blunt or single-base overhang ligation prior to transformation. Early PCR cloning often used *Taq* DNA Polymerase to amplify the gene. This results in a PCR product with a single template-independent base addition of an adenine (A) residue to the 3´ end of the PCR product, through the normal action of the polymerase. These "A-tailed" products are then ligated to a complementary T-tailed vector using T4 DNA Ligase, followed by transformation. High-fidelity polymerases are now routinely used to amplify DNA sequences with the PCR product containing no 3´ extensions. The blunt-end fragments are joined to a plasmid vector through a typical ligation reaction or by the action of an "activated" vector that contains a covalently attached enzyme, typically Topoisomerse I, that facilitates the vector:insert joining. PCR cloning with blunt-end fragments is non-directional. Some PCR cloning systems contain engineered "suicide" vectors that include a toxic gene into which the PCR product must be successfully ligated to allow propagation of the strain that takes up the recombinant molecule during transformation. A typical drawback common to many PCR cloning methods is that a dedicated vector must be used. These vectors are typically sold by suppliers, like NEB, in a ready-to-use, linearized format and can add significant expense to the total cost of cloning. Also, the use of specific vectors restricts the researcher's choice of antibiotic resistance, promoter identity, fusion partners, and other regulatory elements.

ADVANTAGES

- High efficiency, with dedicated vectors
- Amenable to high throughput

DISADVANTAGES

- Higher cost
- Multi-fragment cloning is not straight forward
- Directional cloning is difficult

LEARN MORE ABOUT PCR CLONING

Ligation Independent Cloning (LIC)

Ligation Independent Cloning (LIC) is a technique developed in the early 1990s as an alternative to restriction enzyme/ligase cloning. Inserts are usually PCR amplified, and vectors are made linear either by restriction enzyme digestion or by PCR. This technique uses the 3´→5´-exo activity of T4 DNA Polymerase to create overhangs with complementarity between the vector and insert. Incorporation of only dGTP in the reaction limits the exonuclease processing to the first complimentary C residue, which is not present in the designed overlap, where the polymerization and exonuclease activities of T4 DNA Polymerase become "balanced". Joined fragments have 4 nicks that are repaired by *E. coli* during transformation. This technique allows efficient creation of scarless recombinant plasmids at many, but not all, positions in a vector.

More recently, the technique has evolved to include many useful variations. One in particular, Sequence and Ligation Independent Cloning (SLIC), has been adopted by many researchers. In this variation, all dNTPs are initially excluded from the reaction with T4 DNA Polymerase. This allows the exo activity of T4 DNA Polymerase to proceed and generate the complementary overlaps between insert and vector. After the overlap is generated, dCTP is added back to the reaction, which shifts the enzyme back into a polymerase. It then stalls due to the lack of a complete set of dNTPs in the buffer, and the complementary overlap is retained. The product contains 4 nicks, just like the original LIC product, and is repaired by *E. coli* during transformation. This modification of the protocol allows a scarless and sequence-independent insertion into nearly any vector.

ADVANTAGES

- Low cost
- Many different vector choices

DISADVANTAGES

- Some types of sequence modifications not possible

Recombinational Cloning

Recombinational cloning became popular with the introduction of three cloning systems: Gateway®, Creator™, and Echo Cloning™ systems. These systems use a site-specific recombinase (Integrase in Gateway and Cre Recombinase in Creator and Echo) to allow the reliable transfer of a fragment from one vector to another without using restriction enzymes and ligases. Typically, a researcher would clone a sequence of interest into a holding vector ("Entry" for Gateway and "Donor" for Creator) using traditional cloning methods. Once the new clone is made, it is easily shuttled to many different "destination" or "acceptor" vectors that contain the appropriate sequence recognized by the recombinase (attachment sites *attB* and *attP* with Gateway and *loxP* with Creator/Echo). Higher throughput is possible with these systems and they have become a useful tool for screening many different expression hosts for protein expression projects or for multiple reporter vectors for functional analysis studies. At this time, only the Gateway system is still commercially supported, although NEB does sell Cre Recombinase (NEB #M0298), an essential reagent for the *in vitro* recombination step used by the Creator and Echo Cloning systems.

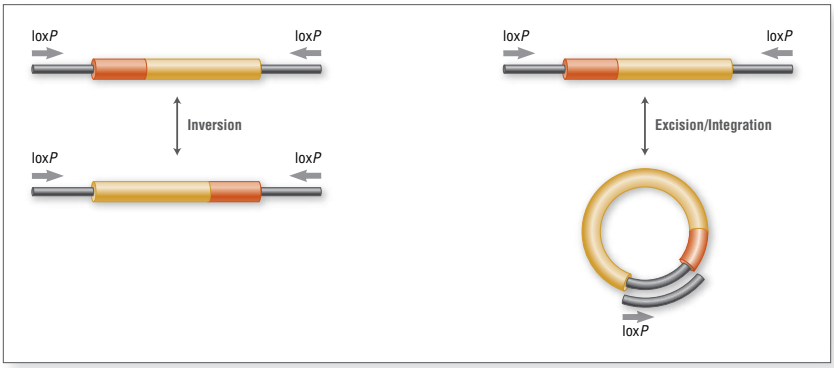
ADVANTAGES

- Allows high-throughput vector creation
- Widely available ORF collections

DISADVANTAGES

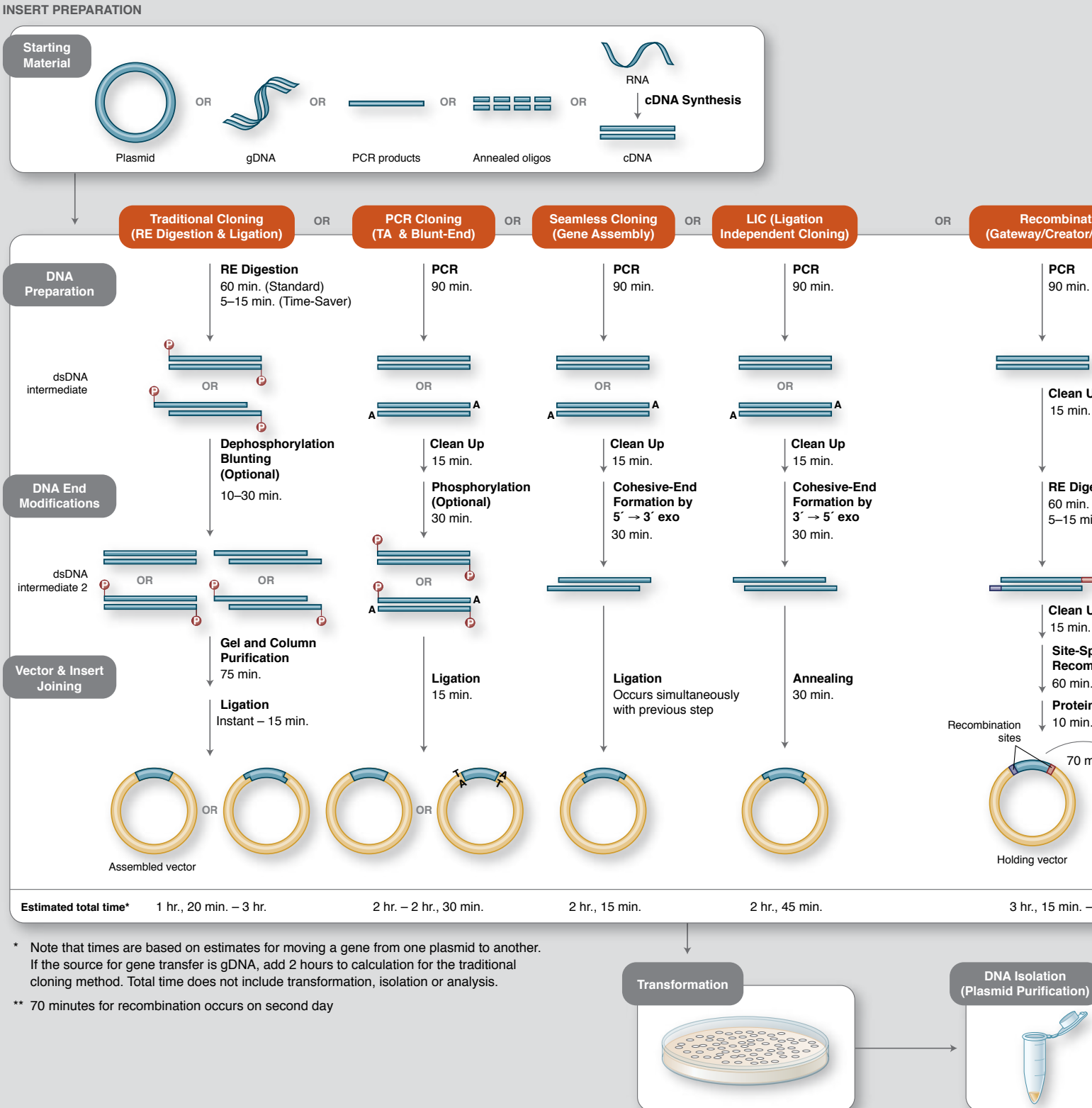
- Cost relative to traditional methods
- Vector sets typically defined by supplier
- Proprietary enzyme mixes often required

Cre/loxP Site-specific Recombination



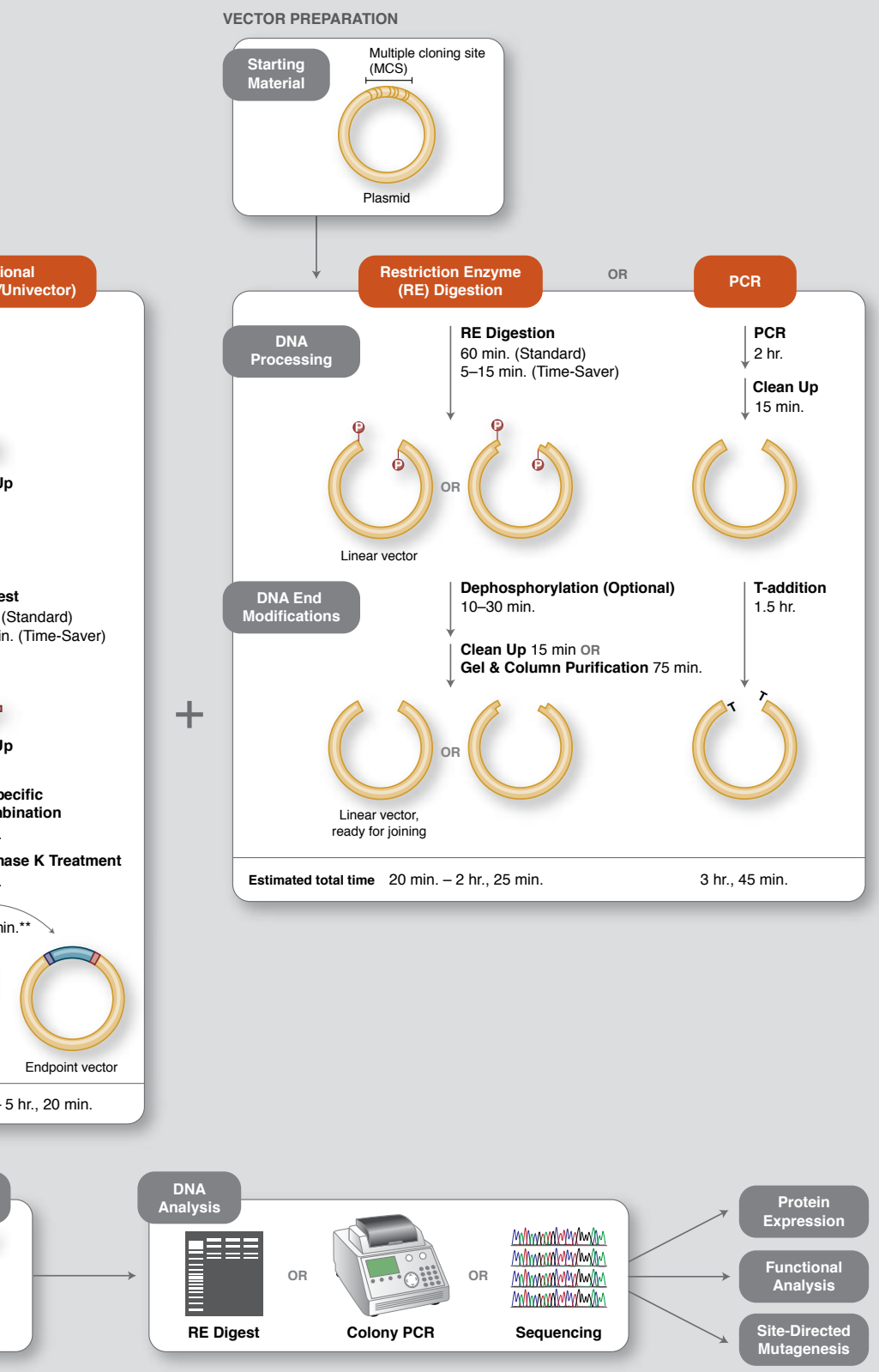
Cloning Workflow Comparison

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



* 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



SELECTION CHARTS & PROTOCOLS

Need help with locating product selection charts & protocols?

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	100/500 reactions
Q5 High-Fidelity PCR Kit	E0555S/L	50/200 reactions
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* 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
$Vent_R$ DNA Polymerase	M0254S/L	200/1,000 units
$Vent_R$ (exo-) DNA Polymerase	M0257S/L	200/1,000 units
Deep $Vent_R$ DNA Polymerase	M0258S/L	200/1,000 units
Deep $Vent_R$ (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
KLD Enzyme Mix	M0554S	25 reactions

Selected Products for PCR & Mutagenesis (Cont.)

PRODUCT	NEB #	SIZE
dNTPs		
Deoxynucleotide (dNTP) Solution Set	N0446S	25 µmol of each
Deoxynucleotide (dNTP) Solution Mix	N0447S/L	8/40 µmol of each

Products for cDNA Synthesis

PRODUCT	NEB #	SIZE
ProtoScript II First Strand cDNA Synthesis Kit	E6560S/L	30/150 reactions
ProtoScript First Strand cDNA Synthesis Kit	E6300S/L	30/150 reactions
ProtoScript II Reverse Transcriptase	M0368S/L/X	4,000/10,000/40,000 units
M-MuLV Reverse Transcriptase	M0253S/L	10,000/50,000 units
AMV Reverse Transcriptase	M0277S/L/T	200/1,000/500 units

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-HF v2	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
OTHER POPULAR RESTRICTION ENZYMES		
AscI	R0558S/L	500/2,500 units
AvrII	R0174S/L	100/500 units
BglII	R0144S/L/M	2,000/10,000 units
BsaI	R0535S/L	1,000/5,000 units
BsmBI	R0580S/L	200/1,000 units
DpnI	R0176S/L	1,000/5,000 units

Products for Restriction Digestion (Cont.)

PRODUCT	NEB #	SIZE
OTHER POPULAR RESTRICTION ENZYMES (CONT'D)		
MluI	R0198S/L	1,000/5,000 units
NcoI	R0193S/L/T/M	1,000/5,000 units
NdeI	R0111S/L	4,000/20,000 units
NheI	R0131S/L/M	1,000/5,000 units
PacI	R0547S/L	250/1,250 units
PmeI	R0560S/L	500/2,500 units
SmaI	R0141S/L	2,000/10,000 units
SpeI	R0133S/L/M	500/2,500 units
XhoI	R0146S/L/M	5,000/25,000 units
XbaI	R0145S/L/T/M	3,000/15,000 units
XmaI	R0180S/L/M	500/2,500 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 Dephosphorylation Kit	M0508S/L	100/500 reactions
Shrimp Alkaline Phosphatase (Recombinant)	M0371S/L	500/2,500 units
Antarctic Phosphatase	M0289S/L	1,000/5,000 units
Alkaline Phosphatase, Calf Intestinal (CIP)	M0290S/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
ElectroLigase	M0369S	50 reactions
T4 DNA Ligase	M0202S/L/T/M	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 Electrocompetent *E. coli*	C2989K	6 x 0.1 ml/tube
NEB 5-alpha F´ I^q Competent *E. coli* (High Efficiency)	C2992H/I	20x0.05/6x0.2 ml
NEB 10-beta Competent *E. coli* (High Efficiency)	C3019H/I	20 x 0.05 ml/tube/ 6 x 0.2 ml ml/tube
NEB 10-beta Electrocompetent *E. coli*	C3020K	6 x 0.1 ml/tube

Products for Transformation (Cont.)

PRODUCT	NEB #	SIZE
NEB Turbo Competent *E. coli* (High Efficiency)	C2984H/I	20 x 0.05 ml/tube/ 6 x 0.2 ml/tube
NEB Turbo Electrocompetent *E. coli*	C2986K	6 x 0.1 ml/tube
NEB Stable Competent *E. coli*	C3040H/I	20 x 0.5 ml/tube/ 6 x 0.1 ml/tube

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

For the list of components available separately, visit **www.NEBMonarch.com**.

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
2-Log DNA Ladder (0.1 - 10.0 kb)	N3200S/L	200/1,000 gel lanes
TriDye 2-Log DNA Ladder	N3270S	250 gel lanes
Quick-Load 2-Log DNA Ladder	N0469S	250 gel lanes
Quick-Load Purple 2-Log DNA Ladder	N0550S/L	250/750 gel lanes
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	1.25 ml
Quick-Load Purple 100 bp DNA Ladder	N0551S	1.25 ml
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

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
NEB Golden Gate Assembly Mix	E1600S	15 reactions
BioBrick® Assembly Kit	E0546S	50 reactions
BbsI	R0539S/L	300/1,500 units
BsaI	R0535S/L	1,000/5,000 units
BsaI-HF	R3535	1,000/5,000 units
BsmBI	R0580S/L	200/1,000 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
USER Enzyme	M5505S/L	50/250 units

Products for Recombinational Cloning

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

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