Traditional Cloning Quick Guide

Preparation | Blunting | Phosphorylation | Purification | Ligation | Transformation

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
- Digest with the appropriate restriction enzyme

Standard Restriction Enzyme Protocol

Restriction Enzyme	10 units is sufficient, generally 1µl is used
DNA	1 µg
10X NEBuffer	5 µl (1X)
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

Restriction Enzyme	1µl
DNA	1 µg
10X NEBuffer	5 µl (1X)
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 the 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 the 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 three products for dephosphorylation of DNA:
  - Shrimp Alkaline Phosphatase (rSAP) (NEB #M0371) and Antarctic Phosphatase (AP)(NEB #M0289) are heat-inactivated phosphatases. Both work in all NEBuffers, but AP requires that the reaction be supplemented with Zn^{2+}
  - Calf Intestinal Phosphatase (CIP) (NEB #M0290) is a robust enzyme that will function under many different conditions and in most NEBuffers. However, CIP cannot be heat inactivated and requires a purification step before ligation.

Dephosphorylation of 5' ends of DNA Using Shrimp Alkaline Phosphatase (rSAP)

rSAP Reaction Buffer (10X)	2 µl
DNA	≥ 1 pmol of DNA ends (about 1 µl of 3 kb plasmid)
rSAP (1 unit/ µl)	1 µl
Nuclease-free Water	To 20 µl
Incubation	37°C for 30 minutes
Heat Inactivation	65°C for 5 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

Blunting Buffer (10X)	2.5 µl
DNA	Up to 5 µg
dNTP Mix (1mM)	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

Phosphorylation

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

Phosphorylation With T4 PNK

T4 PNK	1 µl (10 units)
10X T4 PNK Buffer	5 µl
10 mM ATP	5 µl (1 mM final conc.)
DNA (20 mer)	1-2 µg
Nuclease-free Water	To 50 µl
Incubation	37°C for 30 minutes

Purification of Vector and Insert

- Purify the vector and insert before ligation by either running the DNA on an agarose gel and excising the appropriate bands or using a spin column
- DNA can also be purified using β-Agarase I (NEB #M0392) with low melt agarose or an appropriate spin column or resin

- Analyze agarose gels with longwave UV (360 nM) to minimize UV exposure that may cause DNA damage

Ligation of Vector and Insert

1. Use a molar ratio between 1:1 and 1:10 of vector to insert (1:3 is typical). Use NEBioCalculator to calculate molar ratios.
2. If using T4 DNA Ligase (NEB # M0202) or the Quick Ligation™ Kit (NEB #M2200), thaw and resuspend the Ligase Buffer at room temperature. If using Ligase Master Mixes, no thawing is necessary.
3. The Quick Ligation™ Kit (NEB #M2200) is optimized for ligation of both sticky and blunt ends
4. Instant sticky-end Ligase Master Mix (NEB #M0370) is optimized for instant ligation of sticky/cohesive ends
5. 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
6. Following ligation, chill on ice and transform
7. DO NOT heat inactivate when using the Quick Ligation Buffer or Ligase Master Mixes as this will inhibit transformation
8. If planning to electroporate, then use ElectroLigase (NEB #M0369) for your ligation step

The following three tables show ligation using a molar ratio of 1:3 vector to insert for the indicated DNA size. Use NEBioCalculator to calculate molar ratios.

Ligation with The Quick Ligation™ Kit

Quick T4 DNA Ligase	1 µl
2X Quick Ligation Buffer	10 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	37.5 ng (0.060 pmol)
Nuclease-free Water	20 µl (mix well)
Incubation	Room temperature for 5 minutes

Ligation with Instant Sticky-end Ligase Master Mix

Master Mix	5 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	50 ng
Nuclease-free Water	To 10 µl
Incubation	None

Ligation with Blunt/TA Ligase Master Mix

Master Mix	5 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	50 ng
Nuclease-free Water	To 10 µl
Incubation	Room temperature for 15 minutes

Transformation

- To obtain transformants in 8 hrs., use NEB Turbo Competent *E. coli* (NEB #C2984)
- If recombination is a concern, then use the RecA$^-$ strains NEB 5-alpha Competent E. coli (NEB #C2987) or NEB-10 beta Competent *E. coli* (NEB #C3019)
- NEB-10 beta Competent *E. coli* works well for constructs larger than 5 kb
- 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