In-Frame

Your Open Reading Guide

Molecular Cloning:
Restriction Cloning Troubleshooting & Tips

GenScript
Make Research Easy

Restriction Cloning

Restriction cloning is one of the most common DNA assembly methodologies used today. Restriction cloning involves the preparation of vector and insert DNA via restriction enzymes, followed by their subsequent ligation to form a new plasmid.

While any vector containing a multiple cloning site (MCS) can be used, the DNA insert is typically generated from an existing plasmid, an amplified PCR product, or via de novo DNA synthesis.

Pros:

Affordable and easy to obtain reagents
Wide array of vectors to choose from

Cons:

- Sequence constraints due to restriction recognition site sequences
- Lower efficiency when cloning multiple inserts

The restriction cloning process consists of 3 major steps:

- Cloning Design
- Restriction Digestion
- Ligation & Transformation

A. Cloning Design

Here are the key factors to consider when planning and designing your cloning experiments:

- **What restriction sites are present in my vector?** Plan to use restriction enzymes that have corresponding restriction sites present in your vector MCS. If you do not have a vector map available, you can use online restriction mapping tools such as RestrictionMapper to help identify and visualize your restriction sites.

  If your vector MCS lacks restriction sites corresponding to a restriction enzyme you plan to use, consider using an alternative vector or modifying your existing vector.

- **What restriction sites are present in my insert?** After identifying the restriction sites available for use in your vector, determine whether your insert DNA contains any internal restriction sites that could result in unwanted cleavage. Use of restriction mapping tools is highly recommended.

  Avoid cloning with any restriction enzymes with corresponding restriction sites within your insert. If this is unavoidable, silent mutations can be introduced via de novo DNA synthesis to modify the internal restriction sites.

- **Will I be conducting a single or double digest?** A double digest is always recommended for directional cloning and preventing self-ligation of the vector. For a double digest, select two unique restriction enzymes from your list that:
  - Share a compatible restriction buffer
  - Maintain at least a 6-12 bp double-stranded spacer between their respective restriction recognition sites

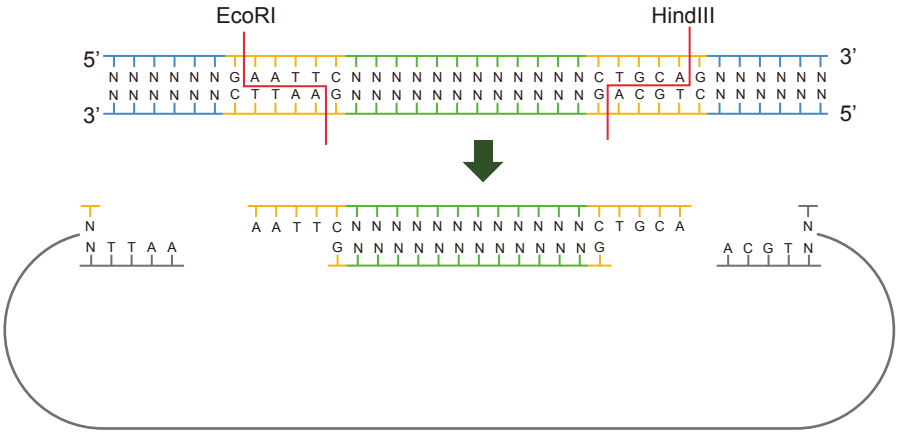

Figure 1: Sample Design & Alignment for a Double Digest

- **Will I be cloning using cohesive-end or blunt-end ligation?**

  Cloning with at least one cohesive-end is always recommended to enhance your ligation efficiency. Watson-Crick base pairing between sticky end overhangs can greatly stabilize the ligation reaction. Blunt-end ligation can be up to 100x less efficient in comparison.

- **Should I optimize my gene expression?**
  Low gene expression may lead to detection and analysis problems when studying your gene of interest. To increase your gene expression, it is recommended to use codon optimization software. Codon optimization modifies your DNA sequence with silent mutations that will enhance the transcriptional and translational efficiency of your gene, while resulting in no changes to the amino acid sequence of the expressed protein.

OptimumGene™ Codon Optimization

GenScript's OptimumGene algorithm takes into consideration an array of critical factors involved in gene expression, including codon usage bias, mRNA structure, and various cis-elements involved in transcription and translation. This proprietary platform provides the ideal sequence for maximized protein expression in multiple host organisms.

After you've reviewed these considerations and completed your cloning design, make sure to review our cloning checklist:

- **Removal of the Poly-A Tail**
  The polyadenylation tail is a post-transcriptional modification of mRNA in eukaryotic cells which does not need to be included in your cloning design. Inclusion of a poly-A tract in your design may lead to sequence complexity issues when generating your insert via PCR amplification or de novo DNA synthesis.

- **Introduce 6-12 bp Spacer Elements to Each End of Your DNA Fragment**
  Unique spacer elements on either end of your restriction recognition sites allow for efficient binding and cleavage by restriction enzymes.

- **Confirm that the Insert is Designed In-Frame**
  After designing your restriction sites and spacer elements, make sure to check that the sequence you are inserting is in-frame with any N- and C-terminal elements. Online tools such as ORFfinder can be useful for confirming your open reading frame.

B. Restriction Digestion

Below is a quick protocol for restriction digestion with our recommended steps:

1 Restriction Enzyme Digestion
Separately digest your vector and insert with restriction enzymes.

Quick Protocol: Restriction Digestion

Reagents	Amount
DNA	Up to 500 ng
10X Restriction Buffer	2 µL
Restriction Enzyme #1	0.5 µL
Restriction Enzyme #2	0.5 µL
dH_2O	To a final volume of 20 µL

Note: Add bovine serum albumin (BSA) to the reaction if not already present in the restriction buffer.

- After adding the reagents, flick the tube to gently mix the contents and briefly spin down the tube contents.
- Incubate the digestion reaction at 37°C for 2 hours.

2 Dephosphorylation of Vector Ends (Optional)
To prevent re-ligation of the linearized vector, remove terminal phosphate groups using a phosphatase, such as calf intestinal alkaline phosphatase (CIP) or shrimp alkaline phosphatase (SAP). This step is essential when cloning using a single digest.

3 Clean-Up (Recommended)
Clean-up the digested vector and insert via gel extraction or column purification. Removal of enzymes and unwanted DNA fragments can enhance cloning efficiency by up to 20%.

If you are having difficulties with your restriction digestion, here are some key factors which may contribute to unexpected or incomplete digestion:

- **Star Activity**
  Star activity refers to the altered specificity of a restriction enzyme under suboptimal conditions. Star activity can result in cleavage at non-canonical restriction recognition sites. Factors which commonly lead to star activity include altered pH levels, salt concentrations, and glycerol concentrations. Make sure to keep glycerol content below 5%, as restriction enzymes are typically supplied in buffers containing 50% glycerol content.

- **DNA Methylation**
  To protect against phage infection, bacteria often utilize DNA methylation to distinguish endogenous DNA from foreign DNA. These DNA modifications may interfere with methylation-sensitive restriction enzymes. To avoid methylation effects, prepare DNA from E. coli that lack Dam and Dcm methyltransferases (dam-/dcm-). Unmethylated control restriction digestions can be performed by utilizing Lambda DNA.

- **Restriction Enzyme Storage**
  Make sure to properly store and use restriction enzymes prior to expiration according to supplier recommendations. In general, restriction enzymes should be storage at -20°C, ideally in small aliquots, to reduce freeze-thaw cycles and maintain activity.

C. Ligation & Transformation

Below is a quick protocol for ligation with recommended extra steps:

1 Heat Inactivation of Restriction Enzymes (Optional)
Heat inactivation of restriction enzymes is required if
clean-up via gel extraction or column purification was not previously conducted.

2 Ligation
Ligate the digested vector and insert using T4 ligase.

3 Transformation
Transform competent cells with the newly ligated plasmid.

Quick Protocol: Ligation

Reagents	Amount
Vector DNA (3-5 kb)	50 ng
Insert (1 kb)	50 ng
10X Ligation Buffer	2 µL
Ligase	1 µL
dH_2O	To a final volume of 20 µL

Note: Add ribo-ATP, DTT,and Mg++ to the reaction if not already present in the ligation buffer.

- After adding the reagents, flick the tube to gently mix the contents and briefly spin down the tube contents.
- Incubate the digestion reaction at 37°C for 2 hours.

Here are key concepts to consider when planning your ligation reaction:

- **Insert-to-Vector Ratios**
  Multiple reactions with varying insert-to-vector ratios are recommended to ensure the ligation of a single insert. An insert-to-vector molar ratio of between 3:1 and 5:1 is recommended to start, but ratios can vary in range from 1:1 to 10:1, with larger ratios of insert-to-vector being recommended for larger inserts and blunt-end cloning. Online applications such as NEBioCalculator can be used to determine how much vector and insert to add.
- **Incubation Times**
  Incubation at higher temperatures will require less time, but may produce lower ligation yield. Lower temperatures will stabilize the DNA annealing interactions.
  - For blunt-end ligation, incubate at 25°C for 2 hours
  - For large inserts, incubate at 16°C overnight

Unexpected Digestion Pattern	Star Activity	Review the restriction protocol and ensure proper reagents, incubation time, and temperature are used Reduce glycerol content to no more than 5%
	Additional Restriction Sites Present	Sequence the DNA to verify sequence fidelity and absence of additional restriction sites
	Restriction Enzyme Contaminant Present	Prepare new reagents
	DNA Contaminant Present	Prepare a new DNA sample
	Gel Shift Due to Restriction Enzyme Binding	Add loading buffer containing 0.2% SDS and heat DNA at 65°C for 10 minutes prior to gel electrophoresis
No/Incomplete Digestion	Incorrect Restriction Cloning Design	Sequence the DNA to verify restriction sites and spacer elements are present
	Restriction Enzyme Quality	Check enzyme expiration date Avoid multiple free-thaw cycles
	DNA Methylation	For methylation-sensitive restriction enzymes prep DNA from methyltransferase-lacking E. coli
	Reagent Contamination	Prepare new reagents
	Sub-optimal Reaction Conditions	Review the restriction protocol and ensure proper reagents, incubation time, and temperature are used
DNA Smear	Poor DNA Quality	Purify the DNA via spin column Prepare a new DNA sample