

SOP-106

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# Plasmid DNA Digestions

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Procedure for digesting DNA using restriction enzymes.

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Heating Block

Restriction Enzyme

UV Transilluminator & UV Eye Protection

Photo Documentation Equipment

Electrophoresis Buffer (TAE)

50X stock solution pH ~8.5

242 g Tris base

57.1 ml glacial acetic acid

37.2 g  $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$

Add distilled water to 1 liter, make 0.5 ug / ml Ethidium bromide

DNA Ladder

1 kb DNA ladder (New England Biolabs)

Agarose

10X Loading Buffer

20% (w/v) Ficoll 400

0.1 M  $\text{Na}_2\text{EDTA}$ , pH 8.0

1.0% SDS

0.25% bromophenol blue

0.25% xylene cyanol

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Most cloning experiments involve the insertion of a DNA fragment into a plasmid for downstream protein expression, insertion of a multiple cloning site or the addition of a property to the vector (e.g., a drug resistance marker, a promoter, a signal sequence, etc.). The following tips will help with the design and troubleshooting of cloning experiments.

### **Preparation of Insert and Vectors**

#### **Insert from a plasmid source**

- Digest plasmid with 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.
- To identify appropriate enzymes requires analyzing DNA sequence for restriction sites and then choosing those enzymes that define or flank region of interest. There are many software packages available to perform this type of analysis.

#### **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
- Guidelines for PCR optimization can be found on internet
- Purify PCR product by running the DNA on an agarose gel and excising the band or by using a spin column
- Digest with appropriate restriction enzyme

#### **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

### Typical Annealing Reaction

Primer	1 µg
10X Ligase Buffer	5 µl
Total Volume	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. This enzyme modification is used to reduce background signals in ligation reactions.
- Calf Intestinal Phosphatase (CIP) is a robust enzyme that will dephosphorylate DNA.
- Antarctic Phosphatase (AP) can carry out all the same functions as CIP and can be heat inactivated. It has a strict requirement for zinc.

### Typical Dephosphorylation Reaction

Antarctic Phosphatase	1 µl (5 units)
DNA	1-5 µg
10X Buffer	2 µl
Total Volume	20 µl
Incubation	37°C for 15 minutes (5' extensions/blunt ends) or 60 minutes (3' extensions)
Heat Inactivate	65°C for 5 minutes

### Blunting / “Polishing Ends”

In some instances the ends of the insert or vector require blunting or removal of overhanging ends.

- PCR with a proofreading polymerase will leave a predominantly blunt end
- T4 DNA Polymerase or Klenow will fill in a 5' overhang (e.g., EcoRI) and chew back a 3' overhang (e.g., PstI)

- 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  $\beta$ -Agarase I 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

## Procedure

1. Analyze DNA sequences for restriction sites. Cloning and expression plasmids (vectors) usually have an associated map of restriction sites as well as list of defined sequences important for the biology of the plasmid. Generally a plasmid may have a multiple cloning site (MCS) that consists of some DNA sequence with a high frequency of unique enzyme restriction sites. Your DNA sequence for cloning (target) must have complimentary enzyme restriction sites.
2. Select appropriate enzymes for digestion. Based on vector and target maps count number of DNA base pairs between restriction sites. This data defines the size of DNA fragments that result from the digestion.
3. Using manufacture's recommendations for buffer, BSA, temperature and time define reactions in laboratory note book as well as anticipated outcomes.

Note: generally want to keep the volume of enzyme used to about 1/10 of totally reaction volume. This prevents glycerol used in storage of enzyme from interfering with reaction

## For example:

*Restriction Enzyme:* BamHI (Manufacture: NEB)

Recognition sequence

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5'...GGATCC...3'
3'...CCTAGG...5'

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Temperature: 37 C

Buffer: NEB 2-4 (100%)

BSA: Required

Methylation Sensitivity: None

*Vector:* pHT43 (see Figure 1) 1 cut, 8057 bp.

In one eppendorf tube add:

Distilled H <sub>2</sub> O	10 ul
NEB Buffer (10x)	2 ul
BSA (1:10 dilution)	2 ul
pHT43	5 ul
<u>BamHI</u>	<u>1 ul</u>
Total volume	20 ul

Incubate at 37 C for 60 minutes.

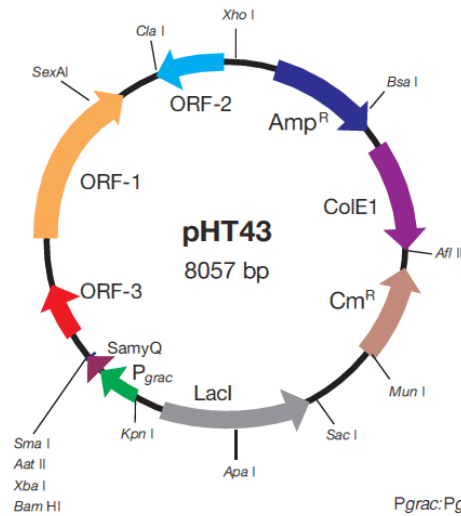
4. Analyze digestion on 1% agarose gel. To the eppendorf tube add 3 ul DNA loading buffer to whole digestion reaction. Prepare agarose gel for electrophoresis (see SOP 105).

Expected outcome: Single band DNA migrating at 8057 bp when compared to DNA ladder.

### **Gel extraction**

Agarose gels can be used for separation and extraction of DNA fragments, for example, a specific DNA fragment from a PCR or restriction digestion reaction. Ensure that the percentage of agarose used for the gel allows good separation of DNA fragments for easy excision. Electrophoresis agarose gels for DNA extraction at a low voltage. This will enable efficient separation of DNA bands without smearing, facilitating excision of the gel slice. Excise the fragment quickly under low-strength UV light to limit DNA damage.

DNA fragments can be extracted quickly and efficiently from agarose gels using silica-gel-based purification. Silica-gel-based methods typically result in higher and more reproducible recoveries than other gel extraction methods, such as electroelution, and require no phenol extraction or ethanol precipitation. In a typical silica-gel-based purification procedure, the agarose gel slice is first solubilized. DNA is then bound to the silica-gel material in the presence of high concentrations of chaotropic salts. A wash step removes impurities, and DNA is then eluted in low-salt buffer.



*Pgrac*: *Pgrac* promoter (consisting of the *groE* promoter; the *lacO* operator and the *gsiB* SD sequence)  
*ColE1 ori*: *ColE1* origin  
*Amp<sup>R</sup>*: ampicillin resistance  
*lacI*: *lacI* gene (*lac* repressor)  
*Cm<sup>R</sup>*: chloramphenicol resistance  
*SamyQ*: *amyQ* signal sequence

Complete DNA sequence is available on request

**GCGGATAACAATT****Tc****ccaatta****aaaggaggaaggatca**  
**lacO** **RBS**  
 atgattcaaaaacgaaagcggacagtttcgttcagacttgtgcttatg  
 M I Q K R K R T V S F R L V L M  
 tgcacgctgttattttgtcagtttgcgattacaaaaacatcagccg  
 C T L L F V S L P I T K T S A  
**taggatecctctagagtcgacgtccccggggcagcc**  
**BamHI XbaI AatII SmaI**

**Figure 1 Plasmid Map of Vector pHT43 and MCS**

Bacillus subtilis Expression Vectors, Product Information and Instructions, (November 2005)

Short Protocols in Molecular Biology. 4<sup>th</sup> Edition. Editors Frederick M. Ausubel, Roger Brent, Robert E. Kingston, David D. Moore, J.G. Seidman, John A Smith and Kevin Struhl. John Wiley & Sons, Inc. 1999

<http://www.neb.com/>

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