Cloning via Restriction Digest

Materials:

Reagent List: Cloning

Reagent	Catalog Number
Agarose	Sigma #A9539
Agarose, low melting point (LMP)	Sigma #A9414
DNA Ladder, 1 kb	New England BioLabs #N0468S
DNA Ladder, 100 bp	New England BioLabs #N0467S
DNA Polymerase, T4	NEB #M0203S
DNA Polymerase, Klenow	NEB #M0210S
Ethidium Bromide	Sigma #E1385
Ligase, T4	NEB #M0202S
Loading Buffer, 6X	Sigma #G7654
Mung bean nuclease	Promega # M4311
Phosphatase, Calf Intestinal (CIP)	NEB # M0290S
Phosphatase, Shrimp Alkaline (SAP)	Promega # M8201
Qiagen Miniprep kit	Qiagen # 27106
Qiagen Gel Extraction Kit	Qiagen # 28704
Qiagen PCR purification kit	Qiagen # 28104
Restriction enzymes	New England BioLabs
TAE Buffer, 10X	Sigma #T6025

Please note that the catalog numbers given in the list above are only examples, and there are many additional companies that supply these reagents.

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Methods:

Steps for Cloning

1. Prepare DNA. This can be mini-prepped DNA plasmid. It's great if you have 5µg of DNA.

2. Select restriction enzymes for your insert and vector, and determine the appropriate reaction buffers.

3. Combine the following in a microfuge tube (30 uL total volume):

2 ug DNA
1 uL Each Restriction Enzyme
3 uL 10x Buffer
3 uL 10x BSA (if recommended)
x uL H2O (to bring total volume to 30 uL)

Note: If you are using more than one restriction enzyme, depending on the buffers needed or your cloning strategy, you may need to digest with individual enzymes sequentially.

4. Incubate tubes at 37oC for 1 hour.

Tip: Some enzymes require special conditions for digestion, such as a different temperature. Check the manufacturer's instructions.

Note: If you are doing a blunt-end ligation, use T4 DNA Polymerase or Klenow DNA Polymerase I for 3' overhang removal and 5' overhang fill-in. Use mung bean nuclease for both 3' and 5' overhang removal. Follow the manufacturer's instructions.

Note: If you are using blunt ends or a single enzyme to cut the vector, you will need to use a phosphatase to prevent re-circularization of the vector. CIP (calf alkaline phosphatase) or SAP (shrimp alkaline phosphatase) are commonly used. Follow the manufacturer's instructions.

5. If you need to isolate your band of interest from other bands in the digest, proceed to Step 6. Otherwise, purify your DNA and proceed to Step 10 (ligation). Many companies sell kits for purifying DNA from enzymatic reactions (for example, Qiagen sells the QIAquick PCR purification kit or QIAquick Gel Extraction kit).

6. Prepare agarose gel. Low melting point agarose will facilitate DNA extraction at a later step, but regular agarose also works.

1. To make a 0.8% agarose gel, use 0.8 g agarose per 100 mL of 1x TAE. Cover the flask with plastic wrap to prevent boiling over, then microwave the solution for 1-2 minutes to dissolve the agarose.

Tip: Increase the amount of agarose for better resolution of small bands. Decrease the amount of agarose for better resolution of large bands. You want to ensure sufficient separation between your band of interest and neighboring bands.

2. Let agarose solution cool for 5 minutes, then add ethidium bromide to a final concentration of 0.5 ug/mL.

Tip: Run cold water over the outside of the flask for faster cooling.

Safety tip: Ethidium bromide is a known mutagen. Wear a lab coat, eye protection, and gloves when working with this chemical.

In our lab we use Cybr Safe. XXXX

3. Pour the agarose/ethidium bromide solution into a casting tray with the well comb in place. Allow 20 -30 minutes to completely solidify.

Tip: Pour the gel in a 4oC room for faster solidifying. Gels can also be poured in advance, and stored in plastic wrap at 4oC.

7. Gel electrophoresis.

1. Add 6 uL of 6x loading buffer to each of your samples.
2. Place the agarose gel into the gel box (electrophoresis unit) and fill with 1xTAE until the gel is covered.
3. Load a molecular weight marker into the first lane of the gel.
4. In the second lane, load undigested plasmid as a control.
5. Load your samples.
6. Cover the gel box and plug in the electrodes. Black is negative, red is positive. (The DNA is negatively charged and will run towards the positive electrode.)
7. Run the gel at 100V until the dye line is approximately 50-75% of the way down the gel.
8. Carefully remove the gel from the gel box.

8. Extract DNA fragments.

1. Visualize the bands under UV light. Use long-wavelength UV to minimize damage to the DNA.

Safety tip: When using UV light, protect your skin by wearing safety goggles or a face shield, gloves, and a lab coat.

2. Using a clean razor blade, cut the desired DNA fragment from the gel. Turn the gel slice on its side to trim off extra agarose. Place the gel in a microcentrifuge tube.

9. Purify DNA from the agarose gel. Many companies sell kits for isolating plasmid DNA (for example, Qiagen sells the Qiaquick Gel Extraction kit).

Tip: Run 1-2 uL of DNA on a gel to check the concentration of your insert and vector before proceeding.

10. Ligation: Follow the manufacturer's instructions.

Example:

a. Combine the following in a microfuge tube (10 uL total volume):

1 uL Vector DNA
3 uL Insert DNA
1 uL 10x Ligase Buffer
1 uL T4 DNA Ligase
4 uL H20 (to bring total volume to 10 uL)

Tip: Try different vector to insert ratios to optimize ligation reaction.

Tip: Do negative controls: vector only, insert only, no ligase.

b. Incubate at 16oC for 2 hours, or at 4oC overnight (follow the manufacturer's instructions).

11. Transformations: Follow instructions specific to the type of competent cells you are using.

Example:

1. Take competent cells out of -80oC and thaw on ice.
2. Take agar plates out of 4oC to warm up to room temperature.
3. Pipette 2 uL of the ligation and 30 uL of competent cells into each transformation tube.
4. Leave on ice for 20 minutes.
5. Heat shock each transformation tube at 42oC for 45 seconds.
6. Leave on ice for 2 minutes.
7. Add 1 mL LB and grow in 37oC shaker for 45 minutes.

8. Spin down cells at 3000 rpm, and remove all but 100 uL of LB. Resuspend cells in the remaining LB.
9. Spread onto an agar plate containing the appropriate antibiotic.
10. Incubate plates at 37oC overnight.

Tip: If your plasmid has ampicillin resistance and you do not require high transformation efficiencies, save time by skipping the recovery steps (steps #7-8) and directly spreading the 32 uL transformation mixture onto the agar plate.

12. Check colonies! After 16 hours you can usually see colonies.