Polymerase Chain Reaction

Objective

Amplify your insert or verify its presence

Time required

3H

Materials

- Taq platinum PCR mix
- primers
- Ultrapure Water
- PCR tubes
- PCR machine

Procedures

A. Insert amplification

- For insert amplification, use the Taq Platinum HiFi mix. for colonies screening, use the standard one.
- Insert are amplified by PCR in the following conditions:

  For a 50 ul PCR reaction:

  -Taq platinum mix, ----------45ul

  -10-50 ng of template DNA

  -20μM primer 1----------1,25 ul

  -20μM primer 2----------1,25ul

- Run PCR according to the size of your insert for elongation time and annealing temperature according to the Tm (actually, if you start from plasmid, you can

significantly decrease the annealing Temperature without significant background; I actually run most of my PCR @ 55C). Elegation temperature for Taq is 68C

- Amplification is verified by agarose gel electrophoresis, the full sample is run on a 1% Agarose gel or 2% for inserts smaller than 1Kb.

Insert and Cloning vector digestion

Objective

Cut your parts out with restriction enzymes. create accepting sites in the cloning vector

1. Digest with appropriate enzymes @37C
2. Heat inactivate the enzymes 20 min @ 80C

Time required

1 H to 1H30

Materials

- NEB Buffer 2
- 10X NEB BSA
- Ultrapure Water
- PCR tubes
- 37C and 60 C incubators/waterbath (best is a PCR machine where you can program this protocol)

Procedures

- Mix 1-3 ug plasmid with 5ul NEB buffer 2 and 5 ul BSA + ultrapure water to 50ul
- Add 1 ul of each enzyme
- Mix well by pipetting up and down so that you solution is homogenous and glycerol is not in the bottom of the tube
- Incubate for 15 to 60 min @ 37C
- Heat inactivate the enzymes by heating @ 80C for 20 min
- For insert, proceed to gel purification
- For the vector proceed to dephosphorylation (if no ccdb gene inside see part Bba_P1010)

Vector Dephosphorylation

Objective

Prevents self-ligation of mono-digested vector, thereby increasing the yield of positive clones.

Time required

40min to 1 Hour.

Materials

Antarctic Phosphatase and buffer (NEB).

Procedures

1. Add 10X Antarctic phosphatase buffer to your digested vector (5ul for 50ul), mix well.
2. Add 1ul of Antarctic phosphatase, mix well.
3. Incubate @ 37C for 30min to 1 Hour.
4. Heat inactivate @ 65 for 5min.
5. Proceed to purification

More infos: http://openwetware.org/wiki/Phosphatase_treatment_of_linearized_vector

Insert Purification by Gel Extraction

Objective

Get rid of enzymes, salts and get clean products to work with.

3. Run your digested insert on a 0,8 to 1% agarose gel.
4. Cut the band from the gel and purify it.

Time required

1 H 30 to 2 Hours .

Materials

- Loading Buffer
- 100bp DNA ladder
- Agarose gels material
- UV table
- falcons 15ml
- 50C Heating block.
- Qiagen gel extraction kit (please read the protocol)

Procedures

A. Running the insert in the gel

!!! Be careful using ethidium bromide!!!

1. Add 1/6 loading buffer to your digested insert.
2. Place the gel in the tank (DNA will migrate toward the (+) red pole)
3. Add 1X TBE buffer to cover the gel
4. Remove the comb
5. Load your sample on a 1% gel
6. Add the 100bp DNA ladder in a another comb
7. Close the Lid, plug on the generator.
8. Push start and set the voltage around 50 to 90 V.
9. Run for 30/45min: look at the blue marker

10. Take the gel out of the tank place it on a paper towel for transport

B. Cutting the insert out

BE CAREFUL USING THE UV. ALWAYS PROTECT YOUR EYES AND SKIN !!!! Use the screen and wear goggles.

1. Put the gel on the UV table
2. Turn on the table on the low intensity position
3. Locate your band
4. With a clean razor blade, cut the gel around the band (fast)
5. Turn off the UV
6. Take the band off, put it into a 15ml falcon

C. Insert purification

1. Add buffer QG (Orange) to your gel band
2. Heat @ 50C, and vortex every 5min, the gel will dissolve
3. Purify using a mini-elute Qiagen column
4. Elute with 12ul, use 1ul to quantify using the nanodrop.

Vector/PCR products purification (Column purification)

Objective

Get rid of enzymes, salts and get clean products to work with.

11. Dilute your sample on binding buffer PB
12. Purify using Qiagen column

Time required

15 minutes

Materials

- Qiaquick PCR purification kit
- for PCR products, use the mini-elute kit (remember to keep the column at 4C)

Procedures

1. Dilute your sample in 5 vol of binding buffer PB (250ul for 50ul reaction)
2. Mix well
3. Load on the appropriate column
4. Centrifuge 1min @ 13000 RPM
5. Discard flowthrough, add 750ul PE washing buffer
6. Centrifuge 1min @ 13000RPM
5. Discard flowthrough, centrifuge 1min @ 13000 RPM
6. Place column on a clean, labeled tube
7. Add 12ul (mini-elute) or 30ul (standard) of Elution buffer EB on the membrane
8. Let the column stand for 1min on the bench
9. Centrifuge 1min @ 13000 RPM
10. Quantify DNA with the nanodrop

Ligation

Objective

Stick your insert into your vector using the DNA ligase enzyme.

Time required

15min.

Materials

T4 DNA ligase (NEB): -20C

T4 DNA ligase buffer (NEB). Make Aliquots of 10ul as the ATP inside the buffer can degrade after multiple thaw/freeze cycle. -20C

Procedures

1. Calculate your product concentration (DNA= 660 g/mol)

2. For each ligation, a 1:3 to 1:5 vector to insert *molar* ratio is used in each experiment (2nM final vector concentration for 6nM to 10 nM insert) in a 10ul volume. Both ratios generally give good results. Start with 1:3.

3. Perform ligation 10-30 min RT on your bench.

    - Control ligation are performed with H2O instead of insert.

4. Heat inactivate @ 65 for 20min

5. Proceed to transfomation

Bacterial Transformation

Objective

Put you cloned DNA inside bacterial cells that will amplify it.

Time required

1h30 to 2 H.

Materials

Chemically Competent cells: -80C

water bath 42C

Ice

Your ligation reaction

Procedures

Add 1 or 2ul of the ligation reaction to a tube of *E.coli* competent cells.

- Incubate on ice for 30/45 min hour.
- Bacteria are then heat-shocked for 45 sec @ 42°C in a water bath, then returned onto ice for 10 min.
- Add 250 ul SOC medium and incubate cells for 1 hour at 37°C (shake vigorously).
- Plate on LB-Agar plates supplemented with the appropriate antibiotic and incubate overnight at 37°C.
- *Note :* you can often shorten the reaction times to 30/5/30, or even 15/2/30.

Colony screening

Objective

Find the cells containing your cloned DNA

Time required

Materials

PCR machine

Taq platinum PCR mix (not HiFi)

Biobricks primers

Gel material

Procedures

- Ligations generally yield about 50 times more colonies per ligation than on control plates.
- The presence of the insert is assessed by direct PCR of colonies picked from plates, andor by digestion of a DNA miniprep prepared from the isolated colonies.
- Pick a colony, and soak the tip in a PCR reaction mix containing the appropriate primers (biobrick primers OK); then throw the tip in tubes containing LB + antibiotic and grow cells overnight.
- Run PCR samples on Agarose gel to verify the presence of the insert.
- Perform a miniprep from 1ml of bacterial culture grown in LB from an isolated colony; Digest with the restriction enzymes (facultative if PCR OK). The presence of the insert is determined by agarose gel electrophoresis (you should see the band corresponding to the linearized vector and the band corresponding to the insert). Test several colonies for each ligation (start with 2/3 if your negative ligation control is clean).
- Sequence positive clones using appropriate primers.