CLONING INVERSE PCR FRAGMENTS

Having successfully obtained a PCR fragment, our next task is to clone that fragment into a suitable vector (*e.g.,* pBSIISK+). First, it will be necessary to ensure that the fragments are blunt-ended; we will make use of the 3'-5' exonuclease activity of T4 DNA polymerase. Following purification of the blunt-ended fragment, we will ligate it with *Sma*I-cut, dephosphorylated pBSIISK+ plasmid DNA and transform *E. coli* host cells with the ligation mixture. Using X-gal blue/white selection to identify colonies with insert-containing vector, clones will be isolated and DNA from each with be isolated and subjected to restriction tests to confirm the presence of suitable inserts.

Creating blunt-end fragments

1. To remove 3'-protruding ends from your PCR-generated fragments, add to your fragment sample (10 μl), 32 μl water, 5 μl REACT 2 10X buffer mix, 2 μl 2.5 mM dNTP mixture, and 1 μl T4 DNA polymerase. Incubate 15 min. at 12°C.

2. Stop the reaction by incubating 10 min. at 70°C.

3. Extract your sample with 1:1 mixture of phenol/chloroform, then precipitate the DNA with 2.5 volumes of ethanol at -20°C.

4. Collect the precipitate by centrifugation for 20 min; dry in the SpeedVac; dissolve in 5 μl of water.

Ligation with vector

5. To your fragment sample (5 μl), add 2 μl water, 1 μl *Sma*I-digested, dephosphorylated pBSIISK+ (0.1 μg/μl), 1 μl 10X ligase buffer, 1 μl T4 DNA ligase. Mix well and incubate at room temperature for 1 hr.

Bacterial transformation

Transformation competent cells are grown and treated to enhance their ability to directly uptake DNA from their environment. Chemical treatments generally lead to membrane destabilization. Preparation of cells for electroporation is essentially to wash them extensively into distilled water. By either method, once prepared, they are aliquoted and stored at -70°C until thawing immediately before use. We are using E. coli strain DH5α, the genetic constitution of which is given at the end of this protocol.

Transformation of chemically-prepared cells

6. To 200 μl of competent DH5α cells on ice, add 5 μl of your ligation mixture and mix gently. Leave on ice for 15 min.

7. Heat shock your cells by placing them in a 42°C waterbath for 90 sec. Stop the incubation by thrusting the tube into an ice bath.

Transformation by electroporation

8. To 100 μl of competent DH5α cells on ice, add 5 μl of your ligation mixture and mix gently.

9. Load the mixture into an electroporation cells and Zap for the an appropriate pulse.

The following is to be done with cells treated by both methods.

10. Permit your cells to express their new ampicillin-resistance gene by adding 1 ml of LB + glucose medium, mix, and incubate at 37°C for 1 hr.

11. Centrifuge the samples at 5000 rpm for 5 min, then draw up the pelleted cells in 100 μl of supernatant and spreading that onto the surface of an LB+amp+X-gal plate. Incubate plates at 37°C overnight, then store at 4°C.

Picking colonies and plasmid DNA miniprep (Step 11 will be performed in advance of the next lab)

12. Examine your plates; blue colonies represent clones without inserts in the pBS vector While white colonies include clones with inserts. Use a sterile toothpick to dab a white colony and drop it into a tube containing 1.5 ml SB+amp medium. Shake overnight at 37°C.

13. Decant the culture into a microtube and centrifuge at 5000 rpm for 5 min, to pellet the cells. Discard the supernatant.

14. The following DNA isolation protocol utilizes a commercial kit (Sigma GenElute Kit). To each microtube, add 200 μl Resuspension Solution. Vortex to resuspend the cells.

Resuspension solution: 50 mM Tris-HCl, pH 7.5
10 mM EDTA
100 μg/ml RNaseA (store @ 4°C)

15. Add 200 μl Lysis Solution. Mix by inverting the tube several times. *Do not vortex (may shear the chromosomal DNA which we want to keep intact).* The suspension should become translucent and viscous, due to release of bacterial chromosomal DNA.

Lysis Solution: 1% SDS
0.2 M NaOH

16. Add 350 µl Neutralization/Binding Solution. Mix by inverting several times. A potassium dodecylsulfate precipitate will form, trapping with it proteins and chromosomal DNA. Put tubes on ice for 5 min. to promote complete precipitation.

Neutralization/Binding Solution: 0.76 M potassium acetate
2.12 M acetic acid
4 M guanidine hydrochloride

17. Centrifuge for 10 min at max rpm.

18. For each sample, label a new collection tube/spin column combination. Add 500 µl Column Prep Solution to the column and centrifuge 1 min. Discard flowthrough.

19. Very carefully decant each supernatant into a spin column. Centrifuge for 1 min. at max rpm. At high salt, DNA will bind to an affinity resin within the spin column filter.

20. Discard the flowthrough from the bottom of each microtube and return the same spin column to the microtube. Add 750 µl Wash Solution to each spin column. Centrifuge for 1 min. at max rpm. This will wash unbound material through the spin column filter.

Wash Solution: 60 mM potassium acetate
10 mM Tris-HCl, pH 7.5
60% ethanol

21. Centrifuge again for 2 min. at max rpm to eliminate all flowthrough.

22. Transfer each spin filter to a new pop-top microtube. Place 50 µl TE into each spin filter and centrifuge for 1 min. at max rpm. Under this low salt condition, DNA passes through the filter. Discard the spin filter. *The flowthrough contains your plasmid DNA sample in 50 µl.*

Restriction analysis of your cloned DNAs:

23. For each plasmid sample, label a new microtube and prepare a restriction digest using 7 µl water, 1 µl 10X REACT3 buffer, 1 µl DNA, 0.5 µl *Eco*RI, and 0.5 µl *Bam*HI (10 µl final volume). Incubate these reactions in a 37^{o} water bath for 1 hr.

Store your DNA samples at 4^{o}.

24. During incubation of your samples, prepare a 1.6% agarose gel. After the incubation, add 2 µl loading dye to each sample, load the samples into the gel, and carry out electrophoresis.

25. The ethidium bromide-stained image of you gel should show a pBS vector band (3 kb) and an insert band.

***E. coli* strain DH5α:** F- Φ80d*lacZ*ΔM15 Δ(*lacZYA*-argF)U169 *end*A1 *rec*A1 *hsd*R17(R_K- m_K+) *deo*R *thi*-1 *sup*E44 λ-*gyr*A96 *rel*A1

*lacZ*ΔM15: Partial deletion of *lacZ* that allows α-complementation with *lacZ* fragment of pBS

Δ(*lacZYA*-argF)U169: Host chromosome deletion of the *lacZ* region

*end*A1: Endonuclease mutation in host chromosome improves quality of plasmid minipreps

*rec*A1: Recombination deficient host ensures stability of inserts of clones

*hsd*R17(R_K- m_K+): Restriction-negative, modification positive host does not restrict input DNA and synthesizes plasmid copies that efficiently transform R_K+ hosts

*deo*R: Constitutive synthesis of deoxyribose, allows uptake of large plasmids

*rel*A1: Eliminates stringency control, allows high-copy replication of plasmids

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