MOLECULAR CLONING

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E. COLI TRANSFORMATION

1. Thaw competent *E. coli* cells on ice (should take around 10min).
2. Add 1-5ul DNA. Mix gently. Do not pipette up-and-down.
3. Incubate on ice for 30min.
    a. During this incubation, make plates with the proper antibiotics if necessary.
4. Heat shock at 42°C for 30s.
5. Cool on ice for 2min.
6. Add 250ul sterile LB media.
7. Incubate shaking at 200rpm and 37°C for 1hr.
8. Pellet cells by centrifuging at 14,000rpm for 1min.
9. Discard supernatant and re-suspend pellet in ~20ul of LB media.
10. Spread re-suspended cells onto a plate with the proper antibiotics.
11. Incubate overnight at 37°C. Next day, the plate may be parafilmed and stored at 4°C if desired.
12. To make bacterial stocks, transfer a colony from the plate into 5ml of LB media supplemented with the appropriate antibiotic. Incubate shaking at 200rpm and 37°C overnight. Next day, take 500ml of the bacteria growing in media and mix with 500ml of 80% glycerol. Aliquot into a freezer tube and store at -80°C.

DNA DIGESTION AND LIGATION

1. We use the Thermo Scientific FastDigest restriction enzymes, which are both robust and fast.
    a. These enzymes all have 100% activity in the same FastDigest buffer
2. I usually do a 10ul reaction:
    a. 6ul of dH_2O
    b. 1ul of 10× buffer
    c. 1ul of DNA fragment to be digested
    d. 1ul each of restriction enzymes (usually two different enzymes for cloning and one enzyme for restriction digestion checks)
3. Digest at 37°C for 10min.
4. More DNA to be digested can be added (in place of dH_2O) as needed. Add 10min for each additional microgram of DNA to be digested.
5. The products of the digestion may be isolated using either a PCR purification kit or by gel purification.
6. Digested products with complementary sticky ends can be ligated using T4 ligase.
7. I use an insert to vector ratio of 6:1. And a total reaction volume of 10ul with T4 ligase buffer.
8. The total amount of DNA to be ligated should not exceed 1pmol.

9. Ligation may proceed at either 15min at room temperature or overnight at 16°C as desired. Ligation with blunt ends will require double the amount of T4 ligase and at least 2h of incubation at room temperature.

GEL ELECTROPHORESIS

1. Making the gel:
    a. For the smaller gel tank
        i. 0.5g of agarose in 50ml TAE or TBE buffer
        ii. Heat until uniformly liquid in microwave
        iii. Wait until not too hot to touch, then add 2.5ul RedSafe
    b. For the larger gel tank
        i. 1g of agarose in 100ml TAE of TBE buffer
        ii. Heat until uniformly liquid in microwave
        iii. Wait until not too hot to touch, then add 5ul RedSafe
    c. Cast gel, wait ~20min to solidify
2. Running the gel:
    a. 5ul of DNA ladder
    b. 5-10ul of product (with loading dye added)
    c. 30-45min at 120V
3. For gel purification, cast gels with the large-toothed combs, do not use ladder and run for 30min at 120V.

STANDARD PCR

1. Primer design:
    a. Melting temperature should be approximately 60°C.
    b. Ideally, the 3'-end of the primer should end with a G or C.
    c. When designing primers for cloning, don't forget to add in restriction sites, start codons, Kozak sequences, etc. to the 5'-end of the primers. These sequences do not factor into the calculation of primer melting temperature.
    d. If a restriction site is to be placed right at the terminus 5'-end of a primer, an additional AA should be added before the restriction site to help restriction enzymes bind to the site.
2. Reaction conditions:
    a. I usually do a 50ul reaction. If reagents are limited, this can be scaled down by half to a 25ul reaction.
    b. For a 50ul reaction:
        i. 31ul of dH_2O
        ii. 5ul of 10× buffer
        iii. 5ul of 2mM dNTPs
        iv. 3ul of 25mM $MgSO_4$
        v. 2ul each of 10pmol/ul primers
        vi. 1ul of 10ng/ul DNA template
        vii. 1ul of DNA polymerase
    c. For templates with a high GC content, the reaction may be supplemented with 1ul of DMSO to improve reaction efficiency.
3. Cycling conditions:

a. Will be dependent on several factors: DNA polymerase used, size of expected product and melting temperature of primers used
b. I usually use KOD polymerase for standard PCR. KOD polymerase is nice because it is has a fast extension rate and proof-checking capacity.
    i. The choice of polymerase will influence denaturing temperature and time as well as extension temperature and time since each polymerase has slightly different optimal conditions. Refer to manufacturer's instructions for optimal conditions.
c. Cycling conditions with KOD polymerase:
    i. 94°C for 5min
    ii. 94°C for 15s
    iii. Tm - 5°C for 30s
    iv. 72°C for 1min × # of kb of product
    v. Run ii to iv for 30 cycles
    vi. 72°C for 10min
    vii. Hold at 4°C

SITE-DIRECTED MUTAGENESIS

1. Primer design:
    a. The mutation site should be centered in the middle of the primers
    b. The primers must be mostly complementary
    c. The melting temperature of the primers should be at least 78°C
    d. I would not try to mutate more than three adjacent sites at once
    e. PrimerX is an excellent tool for designing SDM primers
2. PCR reaction conditions:
    a. Approximately 10ng of DNA template should be used in the reaction
    b. The annealing temperature should be around 60°C
    c. The extension time is: 1min × # of kb
3. DpnI digestion:
    a. Digest 10-20ul of the PCR reaction with 1ul of DpnI for 1h at 37°C
4. Cloning:
    a. Transform 2ul of digestion products directly into competent *E. coli*
    b. Check for success of mutation by sequencing
    c. I usually sequence 3-5 colonies for SDM

OVERLAP DELETION PCR

1. Primer design:
    a. Primers are designed to amplify two fragments on either side of the sequence to be deleted. These fragments must also contain sections that are complimentary to each other. For an illustration of this , see: http://www.molecularinfo.com/MTM/B/B2/B2-1.html
    b. Thus, two primers must be designed to have two parts each, one which is complimentary to the fragment to be amplified, and one which is complimentary to the other fragment.
    c. Each of these parts must have a melting temperature of approximately 60°C.

    d. In addition, there must be two additional “end” primers that complete the primer set needed to amplify each fragment.
2. Extension PCR:
    a. Two separate reactions to amplify each fragment.
    b. Standard PCR reaction and cycling conditions may be used.
    c. Ideally, a proofreading enzyme such as KOD polymerase should be used.
3. The products from the extension PCR should be cleaned up by gel purification to isolate the correct fragments.
4. Purification PCR:
    a. One reaction to join the two fragments generated in the extension PCR step.
    b. An equal mix of both fragments should be used as the template.
    c. The “end” primers should be used for amplification.
    d. Standard PCR cycling conditions may be used.
5. Run a gel purification to isolate the finished product.
6. Overlap PCR can also be adapted to add insertions into genes. See: http://openwetware.org/wiki/PCR_Overlap_Extension for details.