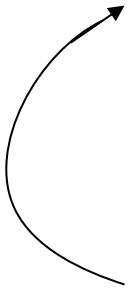
Workflow:



1. Miniprep/Nanodrop
2. Digest
3. Gel
4. Ligation
5. Transformation, Plate
6. Colony PCR (Screening)
7. Gel
8. Inoculate correct colony to a liquid culture

Materials:

Miniprep: grown culture, microcentrifuge, 2 1.5mL microcentrifuge tubes, mini column and collection tube, Solution I, Solution II, Solution III, HBC Wash Buffer, DNA Wash Buffer, Elution Buffer, micropipette and tips

Nanodrop: nanodrop machine, miniprepped DNA, Kimtech wipes, micropipette and tips

Digest: miniprepped DNA, dH_2O, 10X RE-Mix, standard restriction enzyme, micropipettes and tips

Gel: agarose gel (make one if necessary), 1X TAE Buffer, power supply, chamber and electrodes, ladder, micropipette and tips, DNA

Ligation: vector, parts 1 and 2, ligase buffer, ligase, Antarctic phosphatase, microcentrifuge tube, ice, micropipette and tips

Chemical Transformation: ice, ligation mixture, competent cells, incubator, LB media, microcentrifuge tubes, micropipette and tips

Electroporation: electrocompetent cells, DNA (plasmid, ligation mixture), electroporation cuvette, LB media, microcentrifuge tubes, micropipette and tips

Plate: agar plate, micropipette and tips, beads

Colony PCR: dH_2O, buffer, VF_2, VR, Q5 polymerase, dNTP, DNA dilution, micropipette and tips, PCR tubes, thermocycler, ice

Gel: agarose gel (make one if necessary), 1X TAE Buffer, power supply, chamber and electrodes, ladder, micropipette and tips, DNA

Inoculate: LB media, dilution, micropipette and tips

1. **Miniprep (using Omega protocol)**
   - 1.1. Grow 1-5mL culture overnight in a 10mL-20mL culture tube.
   - 1.2. Centrifuge at 2500xg for 5 minutes at room temperature. Decant or aspirate and discard the culture media.
     - *1.2.1. Original protocol called for 10,000xg for 1 minute, but the speed and time above seemed to produce better results.*
   - 1.3. Add 250uL of Solution I mixed with RNase A (pre-added). Vortex to mix thoroughly. Transfer the suspension into a new 1.5mL microcentrifuge tube.
   - 1.4. Add 250uL of Solution II. Invert several times until you get a clear lysate.
     - 1.4.1. Once Solution II is added, do not let it sit for more than 5 minutes!
   - 1.5. Add 350uL of Solution III. Invert several times until a white precipitate forms. Centrifuge at 13,000xg or 17,900rcf for 10 minutes. A compact white pellet should form at the bottom of the tube.
   - 1.6. Insert a mini column into a 2mL collection tube.
   - 1.7. Transfer the clear supernatant into the mini column using a micropipette. Centrifuge at the maximum speed (13,000xg) for 60 seconds. Discard the filtrate and reuse the collection tube.
     - 1.7.1. Be careful not to get any parts of the pellet! Tilt at an angle with the pellet at the top when micropipetting is advisable.
     - 1.7.2. Think about what you are discarding versus what you want to keep!
   - 1.8. Add 500uL of the HBC Wash Buffer diluted in isopropanol. Centrifuge at maximum speed (13,000xg) for 60 seconds. Discard the filtrate and reuse the collection tube.
     - 1.8.1. All wash buffers will be centrifuged for 1 minute.
   - 1.9. Add 700uL of the DNA Wash Buffer diluted in ethanol. Centrifuge at maximum speed (13,000xg) for 60 seconds. Discard the filtrate and reuse the collection tube.
   - 1.10. Centrifuge the empty mini column at the maximum speed (13,000xg) for 2 minutes to remove the ethanol.
   - 1.11. Transfer the mini column to a nuclease-free 1.5mL microcentrifuge tube.
   - 1.12. Add 50uL of Elution Buffer (or sterile deionized water). Let it sit in room temperature for 60 seconds. Centrifuge at maximum speed (13,000xg) for 60 seconds.
   - 1.13. Store eluted DNA at -20°C.
2. **Nanodrop**
   - 2.1. Vortex before nanodrop.
   - 2.2. Wipe down the nanodrop machine with Kimtech wipes to make it sterile.
   - 2.3. Set the program to analyze nucleic acids [because you are dealing with plasmid DNA].
   - 2.4. Do a blank test to ensure that the platform is sterile.
   - 2.5. Load 1uL of the miniprepped DNA onto the platform.
     - 2.5.1. Have steady hands. The sample needs to be in the center for best results.
   - 2.6. Click "measure" on the nanodrop for analysis.
   - 2.7. Write down measurements for the concentration of DNA (in ng/uL), A260, A280, 260/280 (should be around 1.8), and 260/230 (should be around 2.1).
3. **Digest**
   - 3.1. Dilute up to 1ug DNA to 17uL with dH_2O.

3.1.1. Take concentration of DNA from nanodrop and convert from ng/uL to ug/uL. Next, set up a proportion to find out how many uL you need to get 1 ug of DNA.

3.1.2. 20uL (total reaction) - 2uL RE-Mix - 1uL standard enzyme = uL dH_2O

3.2. Use a microcentrifuge tube to put the reaction in. Put in the contents in this order: water, DNA, enzymes.

3.2.1. Add 2uL of the 10X RE-Mix and 1uL of the standard enzyme.

3.2.1.1. E and X = 10X RE-Mix

3.2.1.2. S and P = standard enzymes

3.3. Incubate at 37°C for 1 hour for standard enzymes, then at 80°C for deactivation.

4. Gel

4.1. Set up the chamber and put in the gel. Make sure the wells of the gel is at the end of the chamber so that the DNA runs to red.

4.2. Pour the TAE buffer evenly to completely cover the gel.

4.3. Using a micropipette, put 3uL of DNA in each well and 6uL for the ladder [if using a thin gel]. Thicker gels will require more DNA to be put in each well.

4.4. Connect the electrodes by closing the box and connecting them to the power supply. Make sure the power supply is set for 120 volts and 60 minutes.

4.5. Turn on the power supply and make sure bubbles are rising on the sides of the chamber.

5. Ligation

5.1. Use Antartic phosphatase on the backbone to increase the likelihood of part insertion and decrease backbone closure.

5.2. Make calculations using a 3:1 molar ratio of insert to backbone. Refer to the two tables below.

5.2.1. 20uL total reaction = 1uL Vector + ____uL Part 1 + ___uL Part 2 + 2uL Ligase Buffer + 1uL Ligase + ___uL dH_2O

Parts	Length of Insert	Length of Vector	Total	~[DNA] = 1/total length in kilobases (kb)	uL
Vector					1
Part 1					([DNA vector]/[DNA Part 1]) x 3
Part 2					([DNA vector]/[DNA Part 2]) x 3

5.2.2. EXAMPLE: 20uL total reaction = 1uL Vector + 3.4uL Part 1 + 4.9uL Part 2 + 2uL Ligase Buffer + 1uL Ligase + 7.7uL dH_2O

Parts	Length of Insert	Length of Vector	Total	~[DNA] = 1/total length in kilobases (kb)	uL

Vector IC3	1000	2200	3200	0.31	1
Part 1 BW pLac IAT3	200	3450	3650	0.27	(0.31/0.27) x 3 = 3.4
Part 2 ClpXP IAK3	2000	3200	5200	0.19	1w

5.3. Put in each component in a microcentrifuge tube while on ice. They should be pipetted into the tube in this order: water, DNA, ligase buffer, ligase.

5.3.1. The ligase buffer should be thawed and resuspended at room temperature.

5.4. Gently mix by pipetting up and down and microfuge briefly.

5.5. Incubate at room temperature for 1 hour at 37°C

6. **Transformation, Plate**

6.1. Thaw materials on ice for 5 minutes.

6.2. Put 10uL of ligation mixture into 100uL competent cells in a microcentrifuge tube.

6.3. Flick the tube to mix.

6.4. Put on ice for 30 minutes.

6.5. Heat shock at 42°C for 30 seconds.

6.6. Sit on ice for five minutes.

6.7. Add 950uL of SOC media.

6.8. Incubate at 37°C for one hour (along with correct plates).

6.9. Plate 150uL of cells onto a plate. Make sure plate has the correct antibiotic (based on vector backbone)!

6.10. Grow overnight.

7. **Electroporation**

Take 50 ul of electrocompetant cells, and 10-100 ng of PCR product (don't add more than 2ul) for knockouts, 0.1-10 ng of plasmid

Flick to mix

Transfer to chilled electroporation cuvette

Electroporate (machine in Anton's lab downstairs)

Ec-1 (first setting), target time constant: greater than or equal to 5 ms

Add 1ml of prewarmed LB to cuvette

Transfer cell/LB mixture to microcentrifuge tube

Recover with shaking in incubator for 1.5 hr

Colony PCR

7.1. Pick colonies with a combination of phenotypes i.e. large/small, red/white. Dilute each colony in 40uL dH_2O, 1uL DNA from ligation if transformation is successful.

7.1.1. If necessary, do a quick spin to make sure all the liquid is at the bottom.

7.2. Make the following master mix on ice in this order (this is for 4 tubes): 63uL dH_2O, 20uL buffer, 5uL VF_2 primer, 5uL VR primer, 2uL dNTP, 1uL Q5 polymerase.

7.3. Aliquot the master mixes into PCR tubes, then add 1uL of the DNA dilution.

7.3.1. Make sure PCR tubes are labeled properly and carefully!

7.4. Transfer the PCR tubes to a PCR machine and begin thermocycling.

7.4.1. Initial Denaturation: 98℃ for 30 seconds

7.4.2. 25-35 Cycles: 98℃ for 5-10 seconds, 50-72℃ for 10-30 seconds, 72℃ for 20-30 seconds/kb

7.4.3. Final Extension: 72℃ for 2 minutes

7.4.4. Hold 4-10℃

8. Gel

8.1. Set up the chamber and put in the gel. Make sure the wells of the gel is at the end of the chamber so that the PCR samples run to red.

8.2. Pour the TAE buffer evenly to completely cover the gel.

8.3. Using a micropipette, put 3uL of PCR samples in each well and 6uL for the ladder [if using a thin gel]. Thicker gels will require more PCR sample to be put in each well.

8.4. Connect the electrodes by closing the box and connecting them to the power supply. Make sure the power supply is set for 120 volts and 60 minutes.

8.5. Turn on the power supply and make sure bubbles are rising on the sides of the chamber.

9. Inoculate correct colony to liquid culture

9.1. Get the remaining 39uL of colony dilution.

9.2. Get LB media and make sure to use the appropriate antibiotic resistance.

9.3. Mix the colony dilution into the media.

9.4. Grow overnight.

Sequencing

1. 800ug of DNA needed for sequencing
2. 0.8uL primer

Toehold Switch Workflow

1. Hydrate
2. PCR
3. PCR Purification/Nanodrop
4. Gibson Assembly
5. Transformation
6. Miniprep

I.gBlock Gene Fragment Hydration Protocol-

1. Centrifuge for 3-5 seconds at a minimum of 3000 x g to ensure gBlock DNA is at the bottom of the tube.
2. Resuspend in mqH20 to the recommended concentration (usually to 10 ng/uL)
3. Briefly vortex and centrifuge (gently vortex to prevent DNA breakage)

II. PCR Protocol- https://www.neb.com/protocols/1/01/01/pcr-protocol-m0530

III. PCR Purification-

https://www.neb.com/protocols/2012/12/11/gibson-assembly-protocol-e5510