iGEM Lab Manual 2015

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Colony PCR

Protocol:

Template Preparation

First, one must have a *monoclonal* E Coli colony source. This may be one of the following three things:

(1) A glycerol stock made from a SINGLE colony on an agar plate
(2) An overnight culture in LB media (+ antibiotics if appropriate) started from a SINGLE colony on an agar plate
(3) A SINGLE colony on an agar plate

Label a sterile eppendorf with the strain number and assign a unique colony number for each colony picked. Pipet 20 uL of sterile water into the tube. If the e coli is coming from sources (1) or (3), use a clean, sterile pipet tip to scrape a tiny bit of bacteria (even the very smallest amount is okay) and then pipet up and down into the waiting 20 uL. If the e coli is coming from source (2), pipet 1 uL of the LB culture into the water. Mix well.

This water/colony mixture will be the "template" for your PCR reaction. Next, set up your reaction mixture and run the cycler protocol. Follow this up by running a gel to examine the PCR amplicon lengths.

Reaction Preparation

Reaction Mixture (20 uL):

1 uL Template
10 uL 2x Taq Master Mix (Long term storage in freezer, will last one month at 4C)
1 uL FWD Primer (@ 10 uM)
1 uL REV Primer (@ 10 uM)
7 uL H2O

Cycler Protocol

The cycler protocol is fixed except for the extension time. Set the extension time for 1 min per kb of desired amplicon at 68C, plus a little bit of buffer time. For example, for a 2 kb amplicon, set the extension time to 2 min 30 seconds.

95ºC for 6 minutes
30x [95ºC for 30 sec, 55ºC for 30 sec, 68ºC for 1 min/kb amplicon]
68ºC for 20:00 min
Hold at 4ºC

DNA Digestion

Reaction Mixture Preparation

Reaction Mixture (50 uL reaction volume):

5 uL 10X Digest Buffer
1 uL of each digestion enzyme up to maximum of 2.5 uL
2000 ng of desired cut fragment up to max volume of 50 - enzyme volume (cutsmart+digestion enzyme volumes)
Fill with milliQ water to 50 uL

Digestion Protocol

Incubate the reaction at 37ºC for 1 hour.

Notes:

(1) The digest buffer is specific to the enzyme and it stated on the enzyme product description - look it up on NEB's webpage for each enzyme. It is generally "Cutsmart." For digests with multiple enzymes, use NEB's tool to identify the right buffer.
(2) Enzymes are stored in glycerol, but glycerol can inhibit digestion reactions. To keep the volume to 2.5 uL, use 1 uL of 2 enzymes. For three enzymes, consider using just 0.5 uL of the most active one or reducing the volume of all three to 0.8 uL.
(3) You want there to be at least 2000 ng of the fragment you want to isolate in the end. This is because DNA digestion products must be purified, a process that is only about 50% efficient. Isolating 2000 ng ensures you have enough DNA in the end for cloning. To calculate the volume, use the DNA concentration and the relative fraction of the plasmid that is the fragment you want. For example: for a 10 kb plasmid at 1000 ng/uL with a 1kb fragment I want to cut out, I'd want to do the following calculation:

    (1kb /10kb)*(1000 ng/uL) = 100 ng/uL fragment I want
    2000 ng/(100 ng/uL) = 20 uL

    In this case, I'd digest 20 uL of plasmid to get 2000 ng of the fragment I want in the end.

(4) Some enzymes cut quickly and only require a 5 min digestion - see NEB's website for details. In general, I leave everything for 1 hour to ensure completeness.

DNA Gel Electrophoresis

Pouring the Gel:

(1) Make up the agarose gel mixture - 1% agarose seems to work well for constructs between 0.5-3 kb. Typically make 50 mL (mix 500 mg of solid agarose in 50 mL of TAE Buffer)
(2) Heat in the microwave for 5-10s bursts until the agarose has completely dissolved. In between microwave bursts, mix well by gently swirling. Careful - the glass may get quite hot. Do not heat too much such that the mixture boils and spills out of the vessel
(3) Once the agarose has completely dissolved, add SYBR SAFE DNA gel stain at a 1:10,000 ratio - i.e., for a 50 mL gel, add 5 uL. Gently swirl until the dye is uniformly distributed (should be a pinkish color).
(4) Pour the liquid agarose mixture into a gel mold with a comb in place (choose between different well arrangements). Careful not to introduce bubbles. If bubbles do form, pop or move them out of the way with a pipet tip.
(5) Allow the gel to cool until it solidifies - generally takes 30-45 min. Gels may be wrapped in saran wrap and stored at 4C for a short time before use.

Running the Gel:

(1) Mix each DNA sample with 6x loading buffer. Mix well.
(2) Carefully arrange plastic gel bed (with gel) in the gel running setup. Fill to the line with 1x TAE buffer.
(3) Carefully and slowly pull out the comb. Can gently wiggle back and forth to help - watch out for ripped wells!
(4) Load the gel by slowly pipetting the DNA into each well. The DNA sample should sink to the bottom of the well and be clearly visible thanks to the loading buffer. Do not overfill wells or else they will spill into neighboring wells. Generally, 8-well combs leave wells that can accommodate 50 uL DNA and 15-well combs leave wells that can accommodate 20 uL DNA.
(5) Set the voltage to 120V and the time to 35 min. Once the bands have migrated ⅔-¾ of the way down the gel, stop it and place the gel on a blue-light imager.
(6) Take a picture for your notebook with the gel camera.
(7) For preparative gels - use a razor to cut out desired bands and put them in eppendorf tubes (no more than 400 mg of gel in each tube).

Gel extraction:

Follow the Qiagen QiaQuick DNA Gel Extraction protocol that accompanies their kits. Below are the essential steps:

(1) Add 3 volumes of QG buffer per 100 mg of agarose gel (i.e. add 450 uL of QG buffer for a 150 mg agarose gel chunk)
(2) Heat at 50ºC for ~10 minutes until the gel completely dissolves. Vortex to mix.
(3) Add 1 volume of isopropanol per 100 mg of agarose gel (i.e. add 150 uL of Isopropanol to a 150 mg agarose chunk dissolved in 450 uL of QG buffer)

(4) Vortex to mix.
(5) Add up to 700 uL isopropanol-QG-DNA-Agarose mixture to a gel extraction column (purple with lids). Spin down at 13000 rpm for 1 minute. Discard the flow through.
(6) Repeat step 5 until you have spun down all the dissolved gel you have
(7) Pipet 750 uL of PE buffer (**Make sure 200 proof Ethanol has been added) onto the column
(8) Spin down at 13000 rpm for 1 min. Discard the flow through.
(9) Air dry by spinning down at 13000 rpm for 1 min.
(10) Move the column to a fresh eppendorf tube. Pipet 35 uL of EB buffer above the column's membrane - careful not to puncture it!
(11) Let the EB buffer sit on the membrane for 1 min.
(12) Spin at 13000 rpm for 1 min to elute the purified DNA into the eppendorf tube.
(13) Consider measuring concentration on the nanodrop (see separate protocol)

DNA Purification (aka PCR cleanup)

(1) Add 5 volumes of PB (binding) buffer to the DNA sample you'd like to purify. For example, if you have a 50 uL completed PCR reaction to cleanup, start by adding 250 uL of PB buffer and mixing well.
(2) Add the PB + DNA mixture to a PCR cleanup column (purple columns, same as gel extraction). Spin at 13000 rpm for 1 min. Discard the flow through.
(3) Pipet 750 uL of PE buffer onto the column. Spin at 13000 rpm for 1 min. Discard the flow through.
(4) Air dry the column by spinning at 13000 rpm for 1 min. Transfer the dried column to a fresh eppendorf tube.
(5) Add 35 uL of EB buffer to the column and allow it to stand for 1 min.
(6) To elute, spin at 13000 rpm for 1 min. The solution that flows into the eppendorf collection tube is your purified DNA. Consider measuring its concentration with the nanodrop.

Option: Skip the gel extraction, and just run a digest with DpnI.

- DpnI template digest
  - cuts methylated DNA sequence at every GATC
  - minimalizes contamination of template plasmid that will also give transformed bacteria antibiotic resistance

- 2000 ng Backbone (x uL) ****2000 ng MAX***
  - If you need any more, do double
  - (40 uL of DNA) ^
  - 1 uL RE #1
  - 1 uL RE #2
  - 5 uL buffer (usually CutSmart)
  - 0.5 uL of dPn1

- 42.5-x uL H_2O
- TOTAL: 50 uL

E-Gel Electrophoresis

Protocol:

(1) Unwrap a new E-Gel and snap it into the E-Gel Holder. Make sure the gel holder lights up to indicate that the E-Gel is properly connected.
(2) For the 1.2% 12 well E-Gels, add 15 uL of H_2O to each well that will receive a sample, and add 5 uL of sample (for a total of 20 uL). You may load all 20 uL for a colony PCR reaction to ensure bright bands for even the smallest of amplicons. NO loading buffer is required - do not add any!
(3) Add 20 uL of 2-log DNA Ladder (at 1 ug/20 uL) to one lane
(4) Run for 15 minutes-30 minutes and visualize on the system itself (SAFETY: Only visualize the gel when the orange protective cover is on! Do not look directly into the blue light)
(5) Capture image on gel camera for long-term preservation of the result

Glycerol Stock Preparation

Protocol:

(1) Grow a saturated culture of a bacterial strain of interest in LB (plus antibiotics if appropriate)
(2) Pipet 560 uL of the saturated culture into a sterile eppendorf tube
(3) Pipet 140 uL of sterile filtered 50% glycerol into the tube
(4) Be sure to clearly label the tube with the strain ID and date you made the glycerol stock
(5) Mix well by pipetting or vortexing
(6) Store at -80ºC and avoid freeze/thaws!

Inoculating Cultures

Protocol:

(1) Obtain a sterile SNAP top culture tube and label it with the strain ID of the bacteria you'd like to grow
(2) Fill with 3-5 mL of LB (plus antibiotic if appropriate) using a sterile serological pipetter and sterile technique

If inoculating from a glycerol stock:

(3) Put a sterile pipete tip on a P200 pipetter as if you were to pipet a liquid. Touch the tip to the surface of the glycerol stock so a tiny bit of it is on the tip. Minimize time the glycerol stock is out of the -80ºC freezer to prevent freeze/thaw.

If inoculating from a single colony on an agar plate:

(3) Put a sterile pipete tip on a P200 pipetter as if you were to pipet a liquid. Touch the tip to the surface of the colony so a tiny bit of it is on the tip. I find holding the plate above a dark surface helps to visualize the colony and reduce glare. It is optimal to come at the plate with the tip from a roughly 45° angle and to just touch the surface of the colony without gouging the agar underneath.

Then for both glycerol stock/agar plate colony cultures:

(4) Pipet up and down with the bacteria-stabbed tip to mix.
(5) Replace Snap Cap top on culture top such that it is down a single notch (snap cap tops may be pushed to two stops. The first maintains the top on, but allows it to wiggle up and down. The second stop, achieved by pushing the cap further down, locks it into place). You want to have to cap on, but NOT in the locked position, such that the culture may be aerated.
(6) Place the tube in a shaking incubator set to 250 rpm and your growth temperature. For most E Coli work, 37ºC is appropriate for growing cultures. See the storage and experimental temperatures section for more detail.

Ligation

Protocol:

Reaction Mixture:

To calculate the volumes to be used, try this ligation calculator.

5 fmols backbone
30 fmols insert
1 uL 10x T4 ligation buffer
0.5 uL T4 ligase
Fill to 10 uL with MilliQ Water

Mix everything together.
*If you get a negative value for water, add 2 uL of ligase buffer and 1 uL ligase and make a 20 uL ligation reaction. Add 2 uL of ligation product for transformation
Reaction Conditions:

Quick Ligation

Incubate mix at 37ºC for 30 min

Chill to 4ºC until ready to use

Overnight Ligation

Incubate mix at 16ºC overnight

Chill to 4ºC until ready to use

Making LB Media

Goal: *Prepare sterile liquid growth media for E Coli cultures*

Protocol:

(1) Measure out 25 grams of LB broth powder and add it to a 1L vessel
(2) Fill to 1L with RO water
(3) Shake and allow to sit until most of the LB broth dissolves
(4) Loosen cap and mark with autoclave tape
(5) Autoclave on the liquid setting (“9” on floors 3,4 in Skirkanich)
(6) Tighten cap and allow to cool to room temperature before use

Making M9 Media

For 1L of media

1. 500ml 2xM9 salts ∗
2. 30ml 10 mg/ml thiamine hydrochloride
3. Dissolve 10 mg per ml of H_2O
4. Filter-sterilize using a 0.22µm filter
5. Light-sensitive: store covered
6. 10ml 40% glycerol ∗
7. 20ml 10% casamino acids ∗
8. 20ml 0.1M $MgSO_4$ ∗
9. 200µl 0.5M $CaCl_2$ ∗
10. 419.8ml sterile deionized H_2O ∗
11. Set up a sterile filter column and add each component separately into a 1 L bottle
12. Add antibiotic as appropriate and store at 4˚C
13. ∗ Can be obtained from the media room
14. *Add 1 ml of desired antibiotic to media

Measuring DNA Concentration

(1) Squirt some water onto the nanodrop pedestal and wipe with a kimwipe
(2) Open the nanodrop software and chose the “Measure DNA” option
(3) Pipet 1.5 uL of EB buffer onto the nanodrop pedestal and hit the “blank” button to subtract off the background absorbance
(4) Pipet 1.5 uL of the EB buffer onto the pedestal and hit the “measure” button. The

concentration should be between 0-2 ng/uL. If not, repeat steps 1-3 until the concentration is between 0-2 ng/uL.

(5) Vortex your sample and briefly spin in a minifuge. Pipet 1.5 uL of your DNA sample onto the nanodrop pedestal. Hit the "measure" button. See your yield and 260/280 ratio - which should ideally be between 1.8-2.0

Miniprep

(1) Grow 3-5 mL of saturated culture. The larger the volume, the more plasmid you'll get in the end. For a normal cloning strain, grow the culture for ~12-16 hours. For NEB Turbo, 5-8 hours is sufficient, with yields increasing towards 8 hours.
(2) Spin the tubes at 3000 x g for 5 min or until all bacterial has pelleted and the remaining culture is clear. Discard the supernatant (spent media). You may do this by fully inverting the tube - the pellet will remain secure in the bottom of the tube. Note that spent media should be bleached before being disposed of in the sink.
(3) Pipet 250 uL of P1 buffer (make sure RNAse buffer has been added) into the tube and pipet up and down to resuspend the bacterial pellet. Return the P1 buffer to 4C storage. Transfer the resuspended pellet to a fresh eppendorf tube.
(4) Pipet 250 uL of P2 buffer into the tube. Invert 5-6 times to mix. Contents should become gooey.
(5) Pipet 350 uL of N3 buffer into the tube. Invert 5-6 times to mix. White precipitate should form.
(6) Spin at 13000 rpm for 10 min. White precipitate should form a pellet or deposit along the sides of the tube.
(7) Pipet the supernatant onto a miniprep spin column, careful not to dislodge any of the white precipitate. Discard the white precipitate pellet.
(8) Spin the column at 13000 rpm for 1 min. Discard the flow through by inverting the collection tube.
(9) Pipet 750 uL of PE Buffer (Make sure 200 proof ethanol has been added) onto the column. Spin the column at 13000 rpm for 1 min. Discard the flow through.
(10) Air dry by spinning at 13000 rpm for 1 minute. Transfer the column to a fresh eppendorf tube.
(11) Pipet 35 uL of EB buffer onto the column. Allow the column to stand for 1 minute.
(12) Spin the column for 13000 rp m at 1 min to elute the DNA. Consider measuring the concentration by nanodrop.

PCR

Taq Polymerase: General all-purpose polymerase that is VERY robust
Phusion Polymerase: For amplifying a sequence with high fidelity and very quickly
Pfu Turbo Cx Polymerase: For amplifying uracil-containing sequences with high fidelity

Taq PCR Protocol:

Taq Reaction Mixture (50 uL):

25 uL 2x Taq Master mix
2.5 uL Forward Primer (10 uM)
2.5 uL Reverse Primer (10 uM)
1 uL DNA template @ 10 ng/uL
19 uL H_2O

Taq thermocycler conditions:

95ºC for 3 minutes (or 6 min for colonies)
Repeat 30 cycles of:
 95ºC for 5 seconds
 55ºC for 30 seconds
 68ºC for x seconds (should be 60s per kb of desired amplicon, i.e. 2 min for a 2 kb amplicon) Sometimes 72º, check for the enzyme/ manufacturer that you're using.
68ºC for 20 minutes
Hold at 4ºC

Phusion PCR Protocol

Phusion Reaction Mixture (50 uL):

0.5 uL Phusion Polymerase (Note: polymerase and other enzymes should be stored on ice until immediately before adding. Once polymerase has been added to the tube, it should also be set on ice.)
10 uL 5X HF Phusion Buffer
2.5 uL Forward Primer (10 uM)
2.5 uL Reverse Primer (10 uM)
1 uL 10 mM dNTPs
1 uL DNA template (@ 10 ng/uL)
32.5 uL H_2O

Phusion thermocycler conditions:

98ºC for 30 seconds (or 6 minutes for colonies)
Repeat 30 cycles of:
 98ºC for 10 seconds
 58ºC for 30 seconds
 72ºC for x seconds (should be 30s per kb of desired amplicon, i.e. 1 min for a 2 kb amplicon)
72ºC for 10 minutes
Hold at 4ºC

Preparing selective plates

Protocol:

First, you need to determine your antibiotic working concentration. You may find working concentrations for several commonly used E Coli antibiotics here. With that number, calculate how much of your concentrated stock solution you need to add to the plate such that it is at the correct final concentration. You may assume that the plain LB agar plates have a volume of 25 mL. Feel free to use the dilution calculator to help you.

Reaction mixture:

x uL concentrated antibiotic stock
Fill to 150 uL with Sterile Water

Example - Kanamycin comes from the cell center in a concentrated stock of 10 mg/mL. It is used at a working concentration of 20 ug/mL. This is a 500 fold dilution. (10 mg/mL)*(x mL) = (0.02 mg/mL)*(25 mL). I'll need to add 0.05 mL or 50 uL. Note, this is also 25 mL/500 = 50 uL. My reaction mixture will be 50 uL conc. kanamycin stock and 100 uL sterile water.

Chloramphenicol- (Working concentration of chloram: 25 ug/ml; stock concentration chloram: 34 mg/ml; 10-15 mL plates from stock center)

LB chlor: 1 mL per 1000 mL LB
LB Kan: 2 mL per 1000 mL LB
LB Amp: 3 mL per 1000 mL LB
LB Amp + Chlor:
Spreading protocol:

(1) Prewarm an LB agar plate at 37ºC for at least 30 min
(2) Spread all 150 uL of the reaction mixture on the prewarmed LB agar plate
(3) Allow to dry face up (agar side down) for 1 hour
(4) Once dry, the plate is ready for transformation

Pouring protocol (for 500 mL of LB-Agar):

(1) Weigh out 12.5 g of LB powder + 8 g of Agar
(2) Fill to 500 mL with water
(3) Autoclave on liquid setting (remember to loosen cap before autoclaving)
(4) After removing from autoclave, cool to 55ºC by placing in a water bath at 55ºC
(5) Add chloramphenicol from 34 mg/mL stock to 25 ug/mL final working concentration by diluting 1,360 fold [i.e. add 367.6 uL of chlor stock at 34 mg/mL to 500 mL LB agar cooled to 55ºC]

(6) Swirl to mix
(7) Pour or use a serological pipet to transfer ~20- 25 mL of lb agar + antibiotics into an empty petri dish [try to maintain as sterile of an environment as possible - ideal to do this in a sterile hood, or by a flame/bunsen burner, bench is okay if you've sprayed down with ethanol first)
(8) Allow plates to cool until solidified (30-60 min). Invert the plates and let sit overnight/several more hours.
(9) Store in plastic bags at 4C.

Plasmid Transformation & Strain Choice

Transformation Protocol

(1) Obtain chemically competent cells from the -80ºC freezer. These MUST be kept on ice since their membranes are very very fragile. Do not vortex! Allow to completely thaw while on ice.
(2) Add 100 ng of DNA or 1 uL of ligation product to a labeled eppendorf tube that will be used for transformation.
(3) Pipet competent cells onto the DNA. For commercially made cells (i.e. NEB Turbo), I'd use 10 uL of cells. For homemade cells, I'd use 50 uL of cells. For BL21, homemade cells, use 100uL of cells.
(4) Mix the DNA with the cells by gently flicking the tube.
(5) Incubate the DNA/cells on ice for 30 minutes.
(6) Heat shock the sample by placing it in a float and lowering it into a water bath at 42ºC for exactly 30 seconds.
(7) Place the tube back on ice for an additional 2 minutes.
(8) For commercially made cells, add 100 uL of SOC media to the tube. For homemade cells, add 200 uL.
(9) Incubate at 37ºC while gently shaking for 60 minutes.
(10) Plate the entire reaction on an LB-Antibiotic plate.

Back Dilution

Protocol:

1. Start with an overnight culture inoculated from a glycerol stock (See: Inoculating cultures)
2. Depending on the volume of bacteria you need for your experiment, fill either a SNAP-top culture tube or sterilized, baffled culture flask with the appropriate media and antibiotic using sterile technique.
3. After the media is warm, pipet a small volume of your overnight culture into the new tube/flask. The amount of overnight culture you add is dependent on the volume of the tube/flask, and also the time at which you want to do your experiment.