Charlotte Bourg iGEM Journal

6/1/15

Fill in from journal

6/2/15

Make 1.5 liters of Luria-Bertani (LB) agar and 1.5 liters of LB media (in Zhang lab):

* Recipe for 1 liter of total volume of LB:
  + 5 g of yeast extract
  + 10 g of NaCl
  + 10 g of tryptone
  + 15 g of agar (for LB agar only)
* add 2 L of water to a flask with 15 g yeast extract, 30 g NaCl, and 30 g tryptone
* stir until dissolved
* add 500 mL of the dissolved solution to each of 3 bottles with 7.5 mL of agar
* divide the remaining solution between 2 1-L bottles
* autoclave the bottles:
  + loosen the lid of the bottles slightly
  + put autoclave tape crossing from the bottle onto the lid—can move to the lid later
  + place sensor in a flask of water (would leave sensor in holder for hard goods)

Make overnight cultures from frozen stock to make electrocompetent cells the next day (in Moon lab):

* strains: JM109 and MG1655
* from glycerol stock, set up two 50mL cultures in 250-mL flasks
* grow overnight

6/3/15

Electrocompetent prep of JM109 and MG1655 (in Moon lab):

* add 25 mL of each culture (JM109 and MG1655) into 500 mL of LB in a 2 L flask
* grow at 37°C and 250 rpm for ~1 hour
  + started at ~10:33 am, taken out at ~11:35 am
* add a 10x dilution into cuvettes
  + 900 μL of H20 and 100 μL of JM109
  + 900 μL of H20 and 100 μL of MG1655
    - Mix each with the pipetter
  + blank cuvette: 1 mL of water
    - avoid touching the bottom area of the cuvettes
* take the OD600:
  + open i-control application and use file “cuvette 600”
    - specific to Moon lab
  + click “apply blanking” and start blanking, then take measurements of other 2 cuvettes
  + JM109 has OD .287
  + MG1655 has OD .390
  + Desired range: 0.35 to 0.4
    - Put JM109 flask back in incubator for ~15 minutes starting at 11:56 am to try to increase OD
    - Leave MG1655 on bench top (growing more slowly)
  + Second measurement of OD of JM109: 0.375
* In cold (4°C) room, evenly distribute each culture into 2 centrifuge bottles (total of 4 bottles) and place in an ice bucket. Wait for 15-30 minutes.
  + Placed on ice at ~12:30 pm
* Keep on ice and bring bottles to centrifuge. Set at 4°C and 3000 rpm for 25 minutes
* Return to cold room and discard supernatant by pouring quickly and gently
* Suspend with total 500 mL of water (125 mL each bottle) and swirl until pellet disappears
* Centrifuge at 4°C and 3000 rpm for 25 minutes
* In cold room, discard liquid and suspend pellet gently (by swirling) with total 220 mL of 10% glycerol (55 mL each bottle) until you do not see pellet
* Aliquot contents of each bottle into 4 15-mL conical tubes (16 tubes total)
* Centrifuge at 4°C and 3000 rpm for 25 minutes
* In cold room, pour off the liquid and pipette out the remaining liquid
* Resuspend pellets in 1 mL glycerol in each tube, then combine into 1 tube per strain
* Centrifuge at 4°C and 3000 rpm for 25 minutes
* In cold room pour out liquid and pipette of remaining liquid
* Add 1.5 mL of 10% glycerol to each tube and resuspend
* Aliquot 40 μL each into small labeled vials and place in a labeled box in the -80°C freezer

Transformation (in Moon lab):

* Take MG1655 and WM1788 electrocompetent cells out of -80°C and place on ice
* Take PSL2397 (plasmid) out of -80°C and place on ice
* Add 2 μL of PSL2397 directly into MG1655 tube
  + Just add; do not draw back up and down with pipette
* Set pipette over 40 μL and draw up the MG1655 and PSL2397 mixture and place into electroporation cuvette
* Tap cuvette to ensure cells are at bottom of cuvette—should check to see that there are no gaps—and place into electroporator; turn on
* Immediately add 500 μL of LB into the cuvette then pour into culture tube
* Place culture tube into incubator for 1 hour
  + MG1655 placed into incubator at 4:58 pm and had time constant of 5.5 ms
* Repeat with WM1788
  + Placed into incubator at 5:04 pm and had time constant of 5.3 ms

6/4/15

Isolate plasmid (Zhang lab):

* Work within 30 cm of flame and flame lip of glass flask at opening and closing
* Add 50 mL of LB to conical 50 ml tube
* Add 50 μL of chloramphenicol and mix
* Pour into 125 mL flask
* Add 1.5 mL of PA2C-TesA (cells with plasmid to be isolated) with a 1000 μL pipette
* Place flask on shaker in warm room at 250 rpm (200 to 250 rpm in standard for E. coli)
  + Placed on shaker at 9:20 am – leave there until ~ 2 or 3 pm
  + Took out of warm room/off shaker at 2:12pm
* Pour into a 50 mL conical tube
* Centrifuge until supernatant is clear and then pour off supernatant
* Add 2.5 mL of resuspension buffer and pipette in and out to resuspend until no pellet remains
* Add 2.75 mL of lysis buffer and invert a few times to mix
  + Solution is now viscous
* Add 4 mL of neutralization buffer and invert a few times to mix
  + Solution is cloudy
* Centrifuge at 4700 rpm for 15 minutes
  + Supernatant not completely clear after centrifuging, but that is fine because it will be filtered
* Filter into a new conical tube using a syringe with a cotton ball in it
  + Filtered liquid is clear
* Add 2.5 times the volume of the liquid of cold pure ethanol (~10 mL 🡪 ~35 mL total volume
* Put in -20°C for 20 minutes
  + Put in at ~4:00 pm
  + Taken out at ~4:22 pm
* Centrifuge for 25 minutes at 4°C at 4700 rpm, then check for a pellet
* Pour off supernatant
  + Can tap the top of the tube against a paper towel to remove more ethanol
* Add at least 20 mL of 70% EtOH and shake to break up the pellet, then fortex
* Centrifuge at 3000 rpm for 7 minutes at 4°C
* Pour off the supernatant
* Resuspend in 500 μL of TE RNAse A (20 μg/mL)
* Add 5 times that volume (2.5 mL) of BNL buffer (from old miniprep kit) and pipette in and out to mix
* Add 750 μL of the solution to a spin mini column and collection tube
* Balance centrifuge and spin for 1 minute at 12500 rpm; discard flow-through
* Repeat with additional 750 μL until all of the solution has run through the column (centrifuge each time on the same settings and in the same column)
* Spin down for 2 minutes at 12500 rpm to get rid of the rest of ethanol
  + Optional step that was not performed: can put in 50°C room for a few minutes to evaporate the rest of the ethanol
* Add 750 μL of wash buffer; spin and discard the flow-through
* Repeat with additional 750 μL of wash buffer
* Place column into a new Eppendorf tube and place 40 μL off eluent (in this case, water) directly onto the bottom of the column without touching it with the pipette tip
* Wait ~3 minutes
* Centrifuge at 13000 rpm for 2 minutes and label flow-through vial
* Take measurement on nanodrop and label the vial with the concentration

Use SnapGene to design primers for the 14 sequences to be overexpressed:

* Check for EcoRI and XhoI restriction sites within each of the sequences
* Check for directionality on the plasmid: direct or complementary
* If a restriction enzyme does not have sites within the sequence, add a site for that restriction enzyme to the appropriate primer and add 6 adenines beyond the site on the primer so that the restriction enzyme will work properly
* If the restriction enzyme does have a site within the sequence, end the primer at the end of the sequence to be amplified to leave the ends blunt
* If direct, add EcoRI to the 5’ end and XhoI to the 3’ end as appropriate
* If complementary, add XhoI to the 5’ end and EcoRI to the 3’ end as appropriate
* Maintain a Tm for each primer above 60°C and approximately match the Tm of paired primers
* Ensure that there is only 1 binding site on the plasmid for each primer

6/5/15

Continue designing primers as on 6/4 and check for correct amplification and for suggested annealing temperatures on amplifX:

* Add the primers designed in SnapGene to the primers list of AmplifX
* Add the full sequence of the plasmid to the sequence portion of AmplifX
* Select the primers in pairs as they should be used and select the full sequence; then click “PCR” > “Run PCR”
* Click on the amplicon in the window at the bottom to display information on it, then see the “Infos” tab for details
  + Check for expected annealing of primers, correct amplicon (length and portion of plasmid), and take note of information especially suggested annealing temperature

Order primers

6/8/15

Waiting for primers—no wet lab work

6/9/15

Group the 14 amplicons to be overexpressed by size so that similarly sized fragments can be run through PCR together

Primers arrive

Resuspend the dry primers in Tris-EDTA (TE) solution:

* Add TE in μL 10 times the number of nmoles of primer that there are in each vial—just add the solution; do not pipette in and out
* Place the vials in the thermomixer at 65°C for ~10 minutes

Make a 10x dilution of the concentrated primer solution:

* To labeled vials, add:
  + 90 μL of H20 MQft
  + 10 μL of primer solution

Picked one of the groupings of amplicons made earlier to run PCR at the same settings: today, doing hesA, nifB, and nifV. Run 100 μL PCR tubes

Calculate volumes of solutions to add for 100 μL tube, then make 320 μL of solution, without adding primers or polymerase, then complete the PCR solutions in

* 100 μL recipe:
  + 29.9 μL water
  + 20 μL Phusion buffer
  + 16 μL dNTP solution
  + 2 μL forward primer
  + 2 μL reverse primer
  + 2 μL 22.74 ng/μL PSL2397 (plasmid as DNA template)
  + 7.5 μL DMSO
  + 20 μL betaine
  + .6 μL Phusion polymerase
* Make PCR master mix for 320 μL of solution without primers or polymerase, adding:
  + 95.68 μL water
  + 64 μL buffer
  + 51.2 μL dNTP solution
  + 6.4 μL template DNA
  + 24 μL DMSO
  + 64 μL betaine
* Add 95.4 μL of that solution to each of 3 labeled PCR tubes, then add the following to complete the reaction mixtures:
  + 2 μL of 10x diluted appropriate forward primer
  + 2 μL of 10x diluted appropriate reverse primer
  + .6 μL polymerase
* Run the 3 PCR tubes under the following conditions:
  + 98°C for 2 minutes initially
  + Cycle the following 35 times:
    - 98°C for 25 seconds
    - 56°C for 35 seconds
    - 72°C for 45 seconds
  + Hold at 10°C

Prepare a gel:

* Add ~50 mL of 1% agarose (in tris acetic acid EDTA or TAE buffer) to a flask in the ethidium bromide (EtBr) area of the lab
* Add 4 μL of EtBr to the flask and swirl to mix
* Pour the mixture into the gel plate, with a plastic piece inserted to form 3 large wells and a small one on the side
  + Try to remove bubbles—can chase them out with a pipette tip
* Allow the gel to set
* Pour additional TAE buffer into the plate as necessary
* Pipette in and out of wells to clear them of any agarose—want clean wells in the gel

Take samples out of PCR thermocycler and run them in the gel:

* Add 15 μL of 10x DNA loading dye to each and pipette in and out to mix to homogeneity
* Add each mixture to one of the wells in the gel
* Add 1 kb ladder to the small well
* Run the gel at ~82 V for ~20 minutes until 2 different shades of blue bands appear
* Run the gel at ~120 V for an additional ~20 minutes

6/10/15

Run PCR for the remaining amplicons

* Make PCR master mix for 1200 μL of solution without primers or polymerase, adding:
  + 358.8 μL water (add 359 μL with a P1000)
  + 240 μL buffer
  + 192 μL dNTP solution
  + 24 μL 22.74 ng/μL PSL2397 (plasmid as DNA template)
  + 90 μL DMSO
  + 240 μL betaine
* Add 95.4 μL of master mix to each of 11 labeled PCR tubes
* Add 95.4 μL of that solution to each of 11 labeled PCR tubes, then add the following to complete the reaction mixtures:
  + 2 μL of 10x diluted appropriate forward primer
  + 2 μL of 10x diluted appropriate reverse primer
  + .6 μL polymerase
* Thermocycler conditions for largest size group (nifHDK and nifEN):
  + 98°C for 2 minutes initially
  + Cycle the following 35 times:
    - 98°C for 25 seconds
    - 56°C for 35 seconds
    - 72°C for 2 minutes and 30 seconds
  + 72°C for 10 minutes (final extension time)
  + Hold at 10°C
* Thermocycler conditions for smallest size group:
  + 98°C for 2 minutes initially
  + Cycle the following 35 times:
    - 98°C for 20 seconds
    - 55°C for 25 seconds
    - 72°C for 25 seconds
  + 72°C for 10 minutes (final extension time)
  + Hold at 10°C

Prepare gels for PCR products with 1% agarose

Dilute concentrated DNA ladder (.5 μg/μL 1kb plus):

* Combine:
  + 40 μL DNA
  + 40 μL water
  + 40 μL DNA loading dye
* Pipette in and out to mix

Add 12 μL of DNA loading dye to each 100 μL PCR reaction tube

Run gels, cut out bands

Purify the PCR product, using a kit:

* Spin down the bands in vials
* Add BNL buffer to cover the gel
* Place on thermomixer at 65°C for about 10 minutes until the agarose is no longer solid
* Split into 2 tubes per PCR product
* Add 150 μL of isopropanol and invert to mix
* Place one spin column in a collection tube for each DNA gel mixture
* Add 750 μL of DNA mixture to the column and centrifuge at 13000 rpm for 1 minute; discard the flow-through and place back in same collection tube
* Repeat the previous step with addition DNA mix until all the mix has run through the column
* Add 700 μL of washing buffer to the column and spin at 13000 rpm for 1 minute; discard the flow through and replace in collection tube
* Repeat previous step with additional 700 μL of washing buffer
* Centrifuge for 2 minutes at 13,000 rpm
* Place columns in thermomixer for ~3 minutes
* Place columns into a clean 1.5 mL microcentrifuge tube and apply 30 μL of water to the center of the column to elute
* Incubate at room temperature for 1 minute
* Centrifuge for 1 minute at 13,000 rpm
* Measure concentrations on nanodrop with 1.5 μL of each sample

6/11/15

Perform a restriction enzyme digest of PCR products and of the plasmid backbone using 20 μL reactions:

* Recipe specifications:
  + Use 2 μL 10x buffer with dye
  + Enough volume of PCR product to provide 500 ng if possible (300 is enough if the concentration is too low)
  + 1 μL of each restriction enzyme (XhoI and EcoRI as appropriate)
  + Water to reach 20 μL total volume
  + Order of addition: water, buffer, DNA, enzyme
* Centrifuge for 1 minute; incubate at 37°C for ~3 hours

Redo PCR for the gene that did not have a band, using the same recipe and conditions as previously used

Prepare two gels:

* 1 for restriction digest products with 1% agarose
  + Add ladder and plasmid digest
    - Can differentiate between digested and undigested (or linearized) plasmid, but other products have too small of a difference between digested and undigested to tell
* 1 for PCR products of the small gene for which PCR is being redone
  + Make 2% agarose (helpful for smaller bands):
    - Weigh 1 g agarose
    - Add 50 mL TAE buffer
    - Microwave until all agarose is dissolved

Purify the DNA fragments from the other digests (non-plasmid) using the small kit to elute at higher concentration:

* Add 100 μL of DNA binding buffer to each sample
  + 20 μL digest reaction x 5 (multiplier for DNA fragments, according to kit) = 10 μL to add
* Pipette in and out to mix
* Transfer mixture to a spin column in a labeled collection tube
* Centrifuge for 30 seconds; discard the flow-through
* Add 200 μL of DNA wash buffer to the column. Centrifuge for 30 seconds. Repeat the wash step
* Add 10 μL water to the column matrix and incubate at room temperature for ~2 minutes
* Transfer the column to a 1.5 mL microcentrifuge tube and centrifuge for 30 seconds to elute the DNA

Quantify the eluate with the nanodrop using 1 μL of each sample

Run gels for plasmid digest and PCR product

* Plasmid:
  + saw bands for fragment, backbone with fragment cut out, and linearized fragment (hoped to see only the first two)
  + cut out only the band of the backbone with fragment cut out and purified
  + took concentration on nanodrop
* PCR product:
  + Cut and purified band
  + Took concentration on nanodrop

Purify the DNA fragments from non-plasmid digests using a kid for high concentration:

* Add 100 μL of DNA binding buffer to each sample
  + 20 μL sample x 5 (multiplier for DNA fragments, according to kit) = 100 μL
* Pipette in and out to mix
* Transfer the mixture to a spin column in a labeled collection tube
* Centrifuge for 30 seconds; discard the flow-through
* Add 200 μL of DNA wash buffer to the column. Centrifuge for 30 seconds
* Repeat the wash step
* Add 10 μL water to the column matrix and incubate at room temperature for ~2 minutes
* Transfer the column to a 1.5 mL microcentrifuge tube and centrifuge for 30 seconds to elute the DNA
* Quantify concentrations with the nanodrop—1 μL of each sample
* Store at -20°C

Set up 7 μL ligation reactions for the digestion products that were digested by both enzymes:

* Recipe conditions:
  + .7 μL buffer
  + 1 μL enzyme
  + .7 μL digested plasmid
  + 5x more insert DNA than plasmid DNA
  + Water to final volume
* Order of addition: water, DNA, buffer, enzyme
* Set up an additional ligation reaction for negative control: see if the plasmid will ligate to itself with no enzyme
* Cyclic ligation conditions:
  + 37°C for 3 minutes
  + 22°C for 3 minutes

Start an overnight culture in 5 mL of LB at 37°C for transformation of ligated plasmids

6/12/15

Prepare competent cells using the RbCl method:

* Dilute 1 mL of culture into 50 mL of LBMg medium pre-warmed to 37°C
* Grow at 37°C for approximately 2 hours on shaker to OD600 of about 0.6
  + Final OD600 =0.598
* Do not vortex cells after this point or allow them to warm above 4°C
* Incubate for 20 minutes on ice
* Transfer culture to an ice-cold 50 mL conical tube
* Centrifuge for 15 minutes at 3000 rpm and 4°C; pour of the supernatant
* Resuspend in 20 mL of Tbf1 from 4°C fridge
  + Using pipette tips from the freezer, start with less than 20 mL and pipette in and out to resuspend, then add up to 20 mL volume
* Incubate on ice for 25 minutes
* Centrifuge for 10 minutes at 3000 rpm and 4°C; remove the supernatant
* Resuspend in 4 mL of Tbf2 from the fridge
* Aliquot 100 μL into microcentrifuge tubes
  + Work in the hood with cells on ice and place filled tubes into liquid nitrogen

Transform products from yesterday’s ligation reactions into the cells:

* Use PE5A plasmid (with ampicillin resistance) as an additional control
  + Original concentration of plasmid: 375 ng/μL
  + Take 2 μL of PE5A and 98 μL water
  + Take 1 μL of that mixture and add 99 μL water
  + Add 1 μL of that to a vial of competent cells
* Add the ligation reaction products to other labeled vials of competent cells
* Heat shock at 42°C for 1 minute; replace on ice for ~2 minutes
* Add 900 μL of LB to each tube (working within the radius of flame)
* Place on shaker at 37°C for 1 hour

Make plates:

* Melt LB agar in the microwave; incubate on ice for 25 minutes
* Add appropriate antibiotic to LB agar
  + Antibiotic is heat sensitive—wait until cooled on ice
  + Antibiotic stocks in the lab are prepared such that 1 μL is needed per mL of agar
  + Use chloramphenicol for the digests and negative control
  + Use ampicillin for the PE5A plasmid
* Pour ~20 mL of LB agar with antibiotic per plate; pour slowly to avoid bubbles
* Place in hood with lids slightly off to avoid condensation
* Label bottom of plates with antibiotic, gene, and date

Plate cells:

* Take cells out of 37°C room and spin down all but the control for efficiency of transformation (PE5A) for 4 minutes
* Take 100 μL from PE5A control and add to 900 μL of LB
  + Plate 100 μL of that dilution on the amp plate
  + Add sterile glass beads and shake laterally to spread around the culture
  + Dump beads into nonsterile glass beads container
* From centrifuged cultures, pipette off the media to the 100 μL mark and resuspend the pellet in that amount of media
  + Add full resuspended quantity to the correct labeled plate
  + Add beads and shake
* Place all plates in 37°C room

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Take plates out of 37°C room, check for colonies, and place those that grew into the 4°C room for the remainder of the weekend

6/15/15

Start a culture of PA2C-TesA in LB for ~4 hours

Redo PCR using same conditions as previously for the genes that were not successfully transformed

Prepare a gel

Run the PCR products on the gel and cut appropriate bands

Purify the PCR product with a kit, as previously and take concentrations on nanodrop

Purify the PA2C-TesA plasmid as previously and take concentration on nanodrop

Set up 20 μL digest reactions for the newly purified genes using the same specifications as previously

* Place at 37°C for ~2 hours

Prepare a gel

* ~50 mL 1% agarose and 4 μL of EtBr
* Run digested plasmid through the gel
  + First well: undigested plasmid (2 μL 1:10 PA2C-TesA, 2 μL DNA loading dye 10x, and 16 μL water)
  + Second well: digested plasmid (3 μL 10x loading dye and digest product)
  + Last well: 1 kb plus ladder
* Saw no band for digested plasmid and a band in unexpected location for the undigested plasmid🡪 need to redo the digestion of the plasmid