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PCR

Note: 10X Thermopol Buffer is 10mM KCL, 10mM $(NH_4)2SO_4$, 20mM Tris-HCl, 2mM $MgSO_4$, 0.1% Triton X-100, pH. 8.8

See PCR Buffers recipe to make own PCR buffer

1. Add the following to each sample:

*include controls: NO template and NEG template

Reagent	50 µL reaction	20 µL reaction
20µM primer 1	2.5µL	1µL
20µM primer 2	2.5µL	1µL
2.5mM dNTP's	2.0µL	0.6 µL
10x Buffer (Enzyme-specific – Taq or Pfu)	5µL	
Enzyme – Taq or Pfu	1µL pfu + 5µL DMSO **or** 0.25µL Taq (no DMSO)	0.25µL pfu turbo + 2µL DMSO **or** 0.25µL Taq (no DMSO)
Template DNA (10-100ng)	1.0µL (100ng) cDNA library or genomic DNA **OR** 2.0µL 2ng/µL plasmids w/ cDNA inserts	1.0µL (100ng) cDNA or genomic **OR** 2.0µL 2ng/µL plasmids
18 MΩ dH2O	X (bring up to 50µL)	X (bring up to 20µL)
Final rxn volume	50µL	20µL

2. Mix a cocktail of the constant reagents – multiply reagent amounts by total num. of reactions +1/2
3. Determine annealing temperature of primers: = (4)(# G or C pairs) + (2)(#A or T pairs) – 5 (not including 6 As or restriction sites of hair pin primers)
4. Run PCR at settings:

   Step1) 95°C for 3 min

   Step 2) 95°C for 30 sec

   Step 3) X°C for 30 sec X = annealing temp for specific primers

   Step 4) 72°C for 1 min/KB **OR** 2 min/KB Taq = 1 min/KB pfu = 2 min/KB

   Step 5) 72°C for 5 min

   Step 6) 37°C for 30 sec

   Step 7) 4°C then hold or OFF

*Extend at 64°C for AT rich templates.

5. If using plasmids, do 5 cycles of Steps 2-4 w/ Step 3 at (X-5)°C and 15 cycles with Step 3 at X°C

   If using genomic DNA, do all 20 cycles of Steps 2-4 w/ Step 3 at X°C (Max 25 cycles)

NEXT: DNA Clean-up (phenol-chloroform or glass milk)

NEXT: Run DNA gel (3µL sample + dH2O + loading dye)

Modified: 4/23/15 CRK

DNA CLEAN UP

1. Combine all PCR products with same results.

2. Add an equal part phenol-chloroform (stored in 4°C fridge) to the product. Vortex quickly and spin down samples at max speed for 2 min.

3. Pipette off top layer (has the DNA) into a new tube. Discard phenol-chloroform waste in hazardous waste jar.

4. Add 1/10 vol. 3M NaOAc and 3x vol. ethanol.

5. Let samples incubate at room temperature for 5 min.

6. Spin down at max speed for 15 min.

7. Decant all liquid from tube and discard.

8. Add 200µL 70% ethanol to the tube. Invert a few times & spin down at max speed for 3 min.

9. Decant ethanol from tube and let pellet air dry for 10-15 min.

10. Resuspend pellet in 20µL 18 MΩ dH2O

RESTRICTION DIGEST

Notes: DNA volume should never be more than 25% of total vol. Enzymes volume should never be more than 10% of total vol.

1. Add the following in order:

 1.0µL DNA (up to 5µL total)
 2.0µL enzyme-specific buffer (may include BSA already, ie. Cutsmart)
 2.0µL 10x BSA (if needed for specific buffer)
 1.0µL Enzyme 1
 1.0µL Enzyme 2
13.0µL 18 MΩ dH2O
20µL total reaction volume
*Can make a mastermix of Cutsmart, enzyme 1, enzyme 2 and H_2O.
**Add DNA to each tube then add mastermix volume.

3. Pipette up and down a few times (without introducing air bubbles)

4. Incubate at 37°C for at least 4 hours (Can incubate overnight)

5. Run a 1% agarose gel – See **RUN DNA GEL**

6. Take a picture & cut out desired DNA band using a UV light board and a razor. Store band in a 1.5mL tube at 4°C until ready to use.

GEL PURIFICATION

1. Add 3 vol NaI (sodium iodide) to 1 vol of DNA agarose gel of solution and melt at 45-55°C. Invert tubes a few times to speed up melting process. (Ex. Add 300µL for every 0.1g of gel – for 0.2g sample, add 600µL NaI)

2. Take glass milk stock (stored at room temp) and resuspend beads. Takes about 5 min vortexing.

3. Add 5µL glass milk to each sample. Vortex quickly and incubate at room temperature for 5 min on the rotator. (For amounts greater than 5µg DNA, add an additional 1µL glass milk per µg of DNA over 5µg.)

4. Spin down samples at max speed for 5 sec. Decant supernatant and discard.

5. Resuspend pellet in 1mL New Wash. (stored at room temp) (Combine samples now if needed.)

6. Spin down at max speed for 5 sec. Decant supernatant and discard. Make sure to remove all liquid. *Let air dry for 5 min.*

7. Resuspend pellet in 20µL 18 MΩ dH2O. Incubate at 45-55°C for 5 min. (Removes DNA from beads)

8. Spin down at max speed for 5 sec. Pipette supernatant (has the DNA) into a new tube. Discard remaining pellet. Store at -20°C.

OVERNIGHT DNA LIGATION

1. Thaw 10X T4 DNA Ligase Buffer. Set up 1 control (just vector) and x samples (vector + insert). Want 50-100 ng vector DNA and 250-300 ng insert DNA. Adjust volumes accordingly.

VECTOR + INSERT	**VECTOR ONLY**
3.0µL vector	3.0µL vector
8.0µL insert	
1.5µL 10X T4 DNA Ligase Buffer	1.5µL 10X T4 DNA Ligase Buffer
1.0µL T4 DNA Ligase	1.0µL T4 DNA Ligase
1.5µL 18 MΩ dH2O	9.5µL 18 MΩ dH2O

2. Mix in 1.5mL Eppendorf tube in this order: water, buffer, vector, insert, enzyme. Final Reaction volume should be 15µL.

3. Incubate overnight in a 16°C incubator in cold room.

RAPID DNA LIGATION NOTES

Tube #1 – Ligase Buffer

Tube #2 – DNA Dilution Buffer

Tube #3 – T4 DNA Ligase

Incubate ligation on bench for 5 min.

(More elaborate directions are in rapid ligation kit.)

OVERNIGHT (O/N) INOCULATION

Notes: Numbers are for each sample. Multiply by number of samples, make a cocktail & aliquot. Use glass tubes with metal caps for this.

1. Add 2mL LB + 4µL specified drugs.
   *Want 100µg/mL Amp total, so if using 50mg/mL Amp stock, use 2µL/mL LB
   * Want 34-68µg/mL chloramphenical, so if using 34µg/mL Chloram stock, use 1-2µL/mL LB

   Blares, BGLS, & Rosettas– chloramphenical & ampicillin
   DH5α, STBL2 - ampicillin

2. Pick one colony for each sample using a toothpick or 200µL pipette tip. Just leave the tip in the LB.

3. Label & grow overnight in 30°C or 37°C shaker depending on cells.

4. Using a wire loop, streak an LB plate w/ appropriate drug, with samples because may need later on. O/N inoculations last less than 24 hours. Grow up O/N in 30° or 37° incubator, depending on plasmid & cells. Store in 4°C.

MINI-PREP

1. Pour 1.5mL from each 2mL O/N inoculations into eppendorf tubes. Return stock samples to 4°C.
2. Microfuge tubes for 30 sec - 5 min.
3. Decant & discard supernatant using a Pasteur pipette attached to vacuum (if have a lot of samples).
4. Add 100µL Solution I to each sample. Vortex all samples until pellet is resuspended. (Hold 2 together on vortex for more vibration.)
5. Add 200µL Solution II to each sample. Mix gently by inverting a few times. (For 5mL Sol. II: 100µL 10N NaOH, 4.4mL 18 MΩ dH2O, 500µL 10% SDS)
6. Add 150µL Solution III to each sample. Vortex inverted briefly. Incubate on ice for 5 min.
7. Spin down samples for 10 min. Decant supernatant into a new tube & discard pellet.
8. Add 1mL ethanol to each sample. Invert a few times to mix.
9. Spin down at max speed for 10 min. Discard supernatant.
10. Add 200µL 70% ethanol. Invert gently a few times.
11. Spin down for 2 min. Discard supernatant.
12. Keep tubes turned upside down & let pellets air dry for 10-15 min. (Pellets turn clear when dry)
13. Resuspend pellets in 38µL 18 MΩ dH2O + 2µL 10mg/mL DNase-free RNaseA

DNA (AGAROSE) GELS – MAKING/RUNNING

1. Set up gel container & place in 1 or 2 combs.

2. Pour a 1% agarose gel. If mixture is already made, microwave for about 45 sec. (or until melted), then let cool until can place hand comfortably on glass. If mixture is not made, mix 0.4g agarose with 40mL TAE buffer. Microwave to dissolve & let cool until can touch container with bare hand. **Swirl in 2-3µL EtBR** and then pour gel. Let gel cool at room temperature for 15-20 min. (Pour gel while caster is in 4°C & let set there if want it to set faster.)

3. Set up gel and make sure that there is enough TAE buffer to cover the top of the gel. Place as many dots of gel loading dye as you have samples on a small piece of Para film. Pipette samples up and down to mix in the dye and then load the appropriate lane. Fill lanes – add 2µL 1 KB DNA Ladder to first lane.

4. For PCR products, add 6µL sample. For Restriction Digests, add 20µL sample.

5. If running half of a gel, run the bottom part 1st. Only run for 15 min at 100V. Run the top 1/2 for 20 min. at 100V for best results.

6. Run a full-length gel if really need to spread out the bands. Can run full length for 40 min at 80V.

7. Take a picture of gel using UV light. Cut out desired DNA band if needed.

*Usually add 5µL EtBr to 100mL gel – if making less gel, cut back the EtBr proportionally.

ISOPURE MAXI-PREP

1. Make up LB stock in 1L flasks. For High Copy Plasmid (ex. pBS, pLD1, p90), **use 100mL LB**. For Low Copy Plasmid (ex. pET14B), use 200mL LB. Autoclave & let cool.

2. Add drug as needed for specific plasmid. Add **2μL 50mg/mL stock Ampicillin per mL LB**, so have <u>final conc. of 100μg/mL</u> amp. Add **1μL 34mg/mL stock Choramphenical per mL of LB**, so have <u>final conc. of 34μg/mL</u>. (Ex. For 100mL LB – add 200μL amp and/or 100μL chloram)

3. Add rest of mini-prep O/N Culture (~0.5mL) directly to LB culture. Grow up O/N in 30° or 37° C shaker depending on cells.

Blares: 37°C	Rosettas: 37°C	STBL2: 30°C

4. Make glycerol stock of O/N culture. Add 850μL culture & 150μL glycerol to a 1.5mL screw top tube. Mix. Label well & wrap clear tape over label to ensure that it doesn't fade. Store at -80°C.

5. Pour culture into 250mL white centrifuge tubes and centrifuge at ~10,000 rpm for 10 min with the JA-14 rotor. Decant supernatant & discard.

6. Resuspend pellet in 10mL buffer A by pipetting. (Make sure RNase was added to buffer A.)

7. Transfer to a 50mL centrifuge tube. Add 10mL buffer B. Mix gently by inverting tube 10 times. (Do not vortex!) Let mixture stand at room temp for 5 min. (Should become clear & viscous)

8. Add 13mL buffer C1 to tube. Invert tube 10 times to gently mix. (White precipitation should appear) (Caution: buffer C1 contains chaotropic agent –handle with care.)

9. Centrifuge for 10 min at 13,000 rpm at 4°C.

10. Transfer supernatant to another 50mL centrifuge tube. Keep tube on ice.

11. Transfer ½ of supernatant to the DNA binding column unit. Centrifuge the column at 4,000 rpm for 5 min in clinical centrifuge.

12. Carefully remove DNA binding column from the unit and discard the pass-thru from the collection tube. Reassemble the DNA binding column unit.

13. Repeat steps 11-12 for the remaining supernatant using the same DNA binding column.

14. Add 20mL 70% ethanol to the DNA binding column unit. Centrifuge for 5 min. at 4,000rpm in the clinical centrifuge. Remove the DNA binding column unit, discard the pass-thru, and reassemble.

15. Repeat step 14.

16. Centrifuge the unit (dry spin) for 10 min at 4,000rpm in the clinical centrifuge.

17. Open the cap and let the unit stand at room temp. for 10 min. to dry leftover ethanol.

18. Transfer the DNA binding column to a new 50mL centrifuge tube. Add 1 ml preheated (65-70°C) 18 MΩ dH2O to the center of the DNA binding column and let stand at room temp for 1 min.

19. Elute DNA by centrifuging the unit for 5 min. at 4,000 rpm in the clinical centrifuge.

20. Repeat steps 18-19 for maximum efficiency. (get ~50% more DNA if repeat) Combine eluted DNA in 2mL tube.

21. Dilute 3μL DNA into 597μL 18 MΩ dH2O (= 200 fold dilution) Determine OD_{260} using RNA/DNA program on Spectrometer. Calculate concentration using Beer's Law, as follows:

    $C = [A * df * E] / \lambda$ $C = (A * 200 * 50) / 1$ $C = (10,000 * A)$ dil. factor=200
    A = absorption = OD_{260} $C = (A * 100 * 50) / 1$ $C = (5,000 * A)$ dil. factor=100
    df = dilution factor = 200 or 100 or whatever you dilute by
    E = epsilon = 50μg / mL*cm λ = path length = 1 cm C = concentration =ng/μL

QIAGEN MAXI-PREP

1. Make up LB stock in 1L flasks. For High Copy Plasmid (ex. pBS, pLD1, p90), **use 100mL LB**. For Low Copy Plasmid (ex. pET14B), use 200mL LB. Autoclave & let cool.

2. Add drug as needed for specific plasmid. Add **2µL 50mg/mL stock Ampicillin per mL LB**, so have final conc. of 100µg/mL amp. Add **1µL 34mg/mL stock Choramphenical per mL of LB**, so have final conc. of 34µg/mL. (Ex. For 100mL LB – add 200µL amp and/or 100µL chloram)

3. Add rest of mini-prep O/N Culture (~0.5mL) directly to LB culture. Grow up O/N in 30° or 37° C shaker depending on cells.

   Blares: 37°C Rosettas: 37°C STBL2: 30°C

4. Make glycerol stock of O/N culture. Add 850µL culture & 150µL glycerol to a 1.5mL screw top tube. Mix. Label well & wrap clear tape over label to ensure that it doesn't fade. Store at -80°C.

5. Pour culture into 250mL white centrifuge tubes and centrifuge at ~10,000 rpm for 10 min with the JA-14 rotor. Decant supernatant & discard.

6. Resuspend pellet in 10mL Buffer P1 (Stored at 4°C). Transfer into a 50mL centrifuge tube.

7. Add 10mL Buffer P2 (Stored at room temp.). Invert a few times to mix.

8. Add 10mL Buffer P3 (Stored at room temp.). Invert until turns a cloudy white and "mucus like". (Takes about 15-30 sec.)

9. Incubate on ice for 10 min. Centrifuge at ~15500 rpm (34000G) for 20 min with the JA-17 rotor.

10. Set-up 30mL filter tip on top of 500mL flask. Pour 10mL QBT Equilibrium Buffer into the top & let filter through. (Takes about 10 min.)

11. Pour supernatant through 2 layers of cheesecloth into tip & let filter through. (5-20 min.)

12. Fill tip to top (30mL) QC Wash buffer & let filter through. (Takes about 20 min.)

13. Get new centrifuge tube & add 10 1/2 mL isopropanol (labeled 2-propanol) to it. Once tip is done filtering, place on top of new tube and pour in 15mL QF Elution buffer. Let filter through. (Takes about 10 min.)

14. Incubate in -20°C freezer for a minimum of 2 hours, but can leave overnight.

15. Centrifuge for 30min. at ~17,000 rpm using the JA-20 rotor.

16. Pour off supernatant & add 5mL 70% ethanol. Invert a few times.

17. Centrifuge at ~17,000 rpm for 30 min with the JA-20 rotor.

18. Decant supernatant and let pellet air-dry. (Takes 10 –15 min.)

19. Resuspend pellet in 100-200µL 18 MΩ dH2O. Put into a 1.5mL tube.

20. Dilute 3µL DNA into 597µL 18 MΩ dH2O (= 200 fold dilution)

21. Determine OD_{260} using RNA/DNA program on Spectrometer. Calculate concentration using Beer's Law, as follows:

    C = [A * df * E] / λ   C = (A * 200 * 50) / 1   C = (10,000 * A)   dil. factor=200

    A = absorption = OD_{260} C = (A * 100 * 50) / 1   C = (5,000 * A)   dil. factor=100

    df = dilution factor = 200 or 100 or whatever you dilute by

    E = epsilon = 50µg / mL*cm   λ = path length = 1 cm   C = concentration =ng/µL

PROTEIN (ACRYLAMIDE) GELS

1. Add 2X sample buffer if not already added. Heat shock samples for 30 sec in 100°C heat block. Load appropriate gel (or gels) into container. Use gel dam if only running 1 gel.
2. Fill center area to top with buffer. Fill container w/ ~ 1 inch buffer. Remove combs.
3. Load gels with 5-6µL sample for each column. Load 3µL mwm (molecular weight marker) in 1st column.
4. Double check that buffer covers top of gels.
5. Attach electrodes and run at constant A = 70mA until through stacking gel, then can speed up.
   If running 2 gels – start at 100mA.
   If running 4 gels – start at 140mA

COOMASSIE STAIN

1. Remove gel using razor blade and place in tip cover with about 1/4" coomassie stain.
2. Cover with saran wrap and place on rotator for 1-2 hours.
3. Pour off stain into "Used Coomassie" jar. Rinse gel 1-2x with water.
4. Pour in a little 10% acetic acid, swish around, and pour off.
5. Refill with about 3/4" 10% acetic acid. Wad up a few kimwipes and place in corner to speed up de-staining time. Occasionally refresh destaining solution.
6. Place on rotator until enough stain has seeped out of the gel. (Takes 5-10 hours) Take picture & label. Make sure the gel is facing the right way!

WESTERN BLOT PREP FOR *DICTYOSTELIUM*

1. Resuspend cells in media if plated. Take 6-8mL aliquots depending on density of cells and pipet into 15mL tube. (Scrap ~ 1 1/2 inch colonies from an SM-5 plate and skip to 4.)

2. Take 9.5µL sample from tube and count cells using a hemocytomer. Multiple cell number by volume taken to obtain total cells in the sample. (This final number = **C**, see below)

3. Spin down samples 4,000 rpm for 5 min in microcentrifuge. Remove supernatant.

4. Resuspend cells to appropriate density with 10mM Tris (pH 7.5). You want the final concentration of cells/mL to be around 10^8. Use the equations below to calculate the amount needed.
   →*Multiply cell count by total mL you are spinning down. This equals the total amount of cells.*
   (Cell Count) x (mLs) = Total Cells
   →*Divide this total cells number by 10^8 to determine how many mLs 10mM Tris to resuspend in.*
   (Total Cells) / 10^8 = mL 10mM Tris to use so that your final conc. will be 10^8 cells/mL.

5. Transfer 10 µL from sample tubes into new tubes. These new tubes are now the Cell Lysate Tubes. Freeze in liquid N_2, thaw, freeze again, and thaw again to lyse the cells. Mix well and use 1-3µL in Bradford Assay

6. To the original sample tube – add an equal volume (remembering to subtract the 10µL you just took out) of 2X Sample Buffer and immediately boil for 10min.

7. Do a ***Bradford Assay*** with your Bradford Assay Sample Tubes to get the most accurate protein concentration.

8. Set up a protein gel. Add 3µL mwm to first lane. (See Running a Protein Gel for more details)

BRADFORD ASSAY

1. Thaw tube of 10mg/mL (100x) BSA (stored in -30°C). **First set up your standards.** Take out 7 tubes and label 0, 1.9, 3.75, 7.5, 15, 30, 60. Add 800µL 18 MΩ dH2O to Tube 0. Add 788µL 18 MΩ dH2O to Tube 60. Add 400µL to the other 5 tubes.

2. Add 12µL 10mg/mL BSA to Tube 60 (for a total of 120µg). Vortex quickly. Take 400µL out of tube 60 and add to tube 30. Vortex tube 30 quickly. Now have 60µg total in tube 60. Take 400µL out of tube 30 and add to tube 15. Vortex quickly. Take 400µL out of tube 15 and add to tube 7.5. Vortex quickly. Take 400µL out of tube 7.5 and add to tube 3.75. Vortex quickly. Take 400µL out of tube 3.75 and add to tube 1.9. Vortex quickly. Take 400µL out of tube 1.9 and discard. Now add 400µL dH2O back to all standards tubes **except 0** to raise volume to 800µL.

3. Add 200µL Biorad Assay (Red Dye) (stored in 4°C) to each standard. Invert each one a few times. You should see a colormetric range now.

4. **Now set up your Bradford samples.** Take out a new tube for each sample. Label these tubes and add 800µL dH2O and 200µL Biorad Assay (Red Dye) to each. Add 1-3µL of Cell Lysate. Add the same amount of 10mM Tris to your standard tubes to match. **Record amount added. This amount is now Q in equation below.**

5. Perform spectrophotometer analysis on all tubes. Use λ program with wavelength 595nm. Use Tube 0 to blank spec. Then read Tube 0 as sample. Read rest of standards in order. Then read samples.

6. Print out summary and graph standards using Kaliedagraph. Last two points (30&60) may start saturating and may throw off the line. Discard these points if that is the case. Fit a line to the points and use the equation to solve for concentration of samples. $Y = mx+b \rightarrow X=(Y+b)/m$ Solve for X. This answer is the µg in **Q** µL. Divide by Q to get **M** µg/µL. However, now remember that you diluted the sample by 2 when you added sample buffer. So now divide **M** by 2 to get the concentrations (µg/µL) of your samples that are mixed with sample buffer.

WESTERN BLOT

1. Remove protein gel from plate and place in container with ~ 1/2” 1X SD Transfer Buffer. Place two absorption pads in a different container with same buffer. Soak 10-30 min.

2. Set up Trans-Blot SD. Cut appropriate sized membrane and label front right upper corner with a pencil. Wet in 18 MΩ dH2O water. Place on absorption pad on Trans-Blot. Place membrane labeled side up next. Then place gel on top of membrane and add last absorption pad on top. Follow directions to run the Trans-Blot for designated time. (15V for ~20 min.)

3. Remove membrane, mark mwm bands with a pencil. Throw away gel and blot pads. Place membrane in a container with 50mL 5% milk/PBT. Place on rotator for 2 + hours. (Can leave overnight here or at step 4, if add 30μL 10% 1.54M sodium azide or seal in bag)
(PBT = PBS with 0.2% Tween. 1L PBT = 10mL 20% Tween, 100mL 10X PBS, 890mL dH2O)
(Add 2.5g dry milk to 50mL PBT – mix well. Add 1g dry milk to 20mL PBT)

4. Add determined dilution of primary antibody to fresh 20-50mL milk/PBT solution. Let rotate 1+ hours. If leave O/N, seal in bag.
   For dynacortin: 1:50,000 **suc pAb**- If have 50mL milk/PBT, add 1μL **suc pAb**.
   For myosinII: Add 2μL **myo6Ig** to 20mL milk/PBT.

5. Rinse membrane 3x with PBT. Do 4 - 5min washes with PBT on rotator.

6. Pour off PBT and refill with secondary antibody in 20mL milk/PBT. Let rotate 1+ hours.
   For dynacortin: 1:5,000 dilution of **dαr HRP**. If 20mL milk/PBT, add 4μL.
   For myosinII: Add 2μL **gαm** (goat anti –mouse) to 20mL milk/PBT.

7. Rinse membrane 3x with PBT. Do 4 - 5min washes with PBT on rotator.

8. Shake off excess PBT and wrap membrane in sheet protector. Mix 500μL ECL Reagent 1 with 500μL ECL Reagent 2. Pour mixture onto membrane and wait 1 min. Tilt membrane and pour off excess liquid. Expose using Chemiluminescence setting on VersaDoc.

TRANSFORMATION INTO *DICTYOSTELIUM*

(Use Transformation into Dictyostelium Worksheet)

1. Resuspend enough parent strain cells (combine in 50mL tube if needed). Take cell count for each.

   (Need 1 x 10^7 cells per 350µL for each transformation)

2. Multiply cell count by total mL to get total cell count. Perform calculations below:
   Total Cells / 1x10^7 = X   X * 0.35mL = Y = amt E-Pore buffer needed in Step 6.

3. Spin down cells at 1,500rpm for 5 min. Get a bucket of ice now.

4. Decant supernatant and resuspend pellet in 10mL ice-cold E-Pore Buffer. Keep on ice.

5. Spin down cells at 2,000rpm for 5 min. Label desired number of cuvettes and keep on ice until ready to use. Label 1 petri dish for each cuvette. Chill Enriched + PennStrep Only 1.5X HL-5 media in 4°C.

6. Resuspend in calculated amount of ice-cold E-Pore Buffer (Must have 1x10^7 cells per 350µL E-Pore Buffer) Aliquot 350LµL of cells in E-pore buffer into each cuvette. Keep on ice.

7. Add appropriate amount of DNA or plasmid to each cuvette. Keep on ice.

8. Gather cuvettes, petri dishes, chilled media, & pipets. Go to cell culture room. Add 9mL media to each petri dish. Make sure to use sterile conditions.

9. To electroporate cells: Set cap knob to 3.0. Set voltage @ 1.3kV for orf+ cells, myoII$^-$, 1.1kV for 11-5.1 cells . Hold down both red buttons until hear a beep, release buttons. τ should be between 1.5 – 2.0. Pour contents of cuvette into matching petri dish. Shake back and forth and then side to side gently to spread cells out. Let grow overnight in no-drug media.

10. Pour off media and carefully drip in 9-10mL drug selection media. **Do not resuspend cells.**

11. Change media or split every 2-3 days. Do not shake plate for 1st change, then shake front→back and side→ side a few times for the rest of the changes. (Do not swirl in a circle.)

TRANSFORMATION INTO COMPETENT CELLS

1. Take out an aliquot of competent cells from -80°C and immediately put on ice.

2. Thaw DNA. Use 1 – 3ng DNA per sample depending on design of experiment. Set up enough tubes for total samples plus 1 No DNA control. *(Use 2μL ligation rxn, 1μL pUC)*

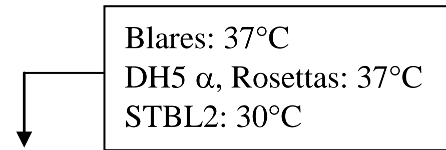
3. Add 50-100μL competent cells to each sample and the control.

4. Add specified amount of DNA to each of the sample tubes. Mix by pipetting up and down a few times.

5. Leave tubes on ice for 30 min. Prepare 42°C heat block by adding a little 18 MΩ dH2O into wells that will be used. Let temp of the water get to 42°C

6. Heat shock tubes at 42°C for 40 sec. (Exceptions: STBL2 for 28 sec, DH5α for 45 sec)

7. Put back on ice for 5 min.



8. Add 1mL LB to each tube. Put all tubes together and, depending on strain, place in 37°C shaker for 45 min or 30°C shaker for 90min.

9. Spin down cells for 30 sec - 5 min at max speed. Remove enough LB so there is 100-200μL left.

10. Resuspend cells in remaining LB & transfer to plates with appropriate drugs.

    pRARE – chloramphenicol resistant
    pakA – amp resistant
    dynaFL, C181, N173, ΔCHD, & C181ΔCHD – amp resistant

    Rosetta cells – no drug resistance
    STBL2 cells – no drug resistance
    BLR(DE3) cells – tet^r resistant, chloramphenicol
    BGL cells – chloramphenicol reistant

11. Grow up overnight in 30°C or 37°C incubator depending on cells.

TESTING EXPRESSION

Notes: Numbers are for each sample. Multiply by number of samples, make a cocktail & aliquot. Use glass tubes with metal caps for this.

1. Add 2mL LB + 4µL amp + 4µL chloramphenical to each tube. (Make sure correct drugs for plasmid – may only need chloram)

2. Pick one colony for each sample using a toothpick or 200µL pipette tip. Just leave the tip in the LB.

3. Label & grow overnight in 37°C shaker.

4. Add 3mL + 6µL amp + 6µL chloramphenical to all new tubes. Make glycerol stocks from overnight cultures by adding 850µL culture + 150µL glycerol. Label & store in -80°C.

5. Inoculate with 100-500µL of the overnight-saturated samples (depending on density).

6. Incubate new samples in 37°C shaker for 1-3 hours. Remove when OD_{600} is between 0.6-1.0. Test this in the Spectrophotometer using a blank of 400µL LB and 400µL of each sample.

7. Add 12µL 100mM IPTG to each 3mL sample. (Want 0.4mM IPTG in sample.)

NEXT: COLLECT TIME POINTS

COLLECT TIME POINTS

1. Collect 200µL of each sample & spin down at max speed for 3 min.
2. Remove LB and resuspend in 40µL 2X Sample Buffer.
3. Boil for 5 min. in 100°C heat block.
4. Freeze samples in -20°C until ready to run a protein gel.

MAKE COMPETENT E. COLI CELLS

1. Make SOB & autoclave. Make TB & store at 4°C. Autoclave ~4-6 bottles. Autoclave tubes, & microfuge tubes as needed.

2. Add 2.5mL 1M $MgCl_2$ to 250mL SOB right before inoculation.

3. For STBL2 – do not add any drug. For Rosetta – add 500µL 34µg/mL chloramphenical For Blares & BGLS – add 500µL 34µg/mL chloramphenical. DH5α – do not add any drug

4. NEED VERY STERILE CONDITIONS: Using a wire loop, transfer 10-12 colonies to a 1mL tube of SOB & pour into beaker with 250mL SOB culture.

5. Grow at 22°C (room temp.) in shaker until A_{600} is between 0.6-0.8. Test this using a blank of 400µL SOB and samples of 400µL in the Spectrometer.

6. When samples are ready, pour broth into sterile centrifuge bottles and put on ice for 10 min. Spin at 2,500g (6,000 rpm) for 10 min. Remove supernatant.

7. Using a 25mL pipette, resuspend pellet in 20mL ice-cold TB. Add 60mL TB to make final vol 80mL

8. Put samples on ice for 10 min. Spin at 2,500g for 10 min. (Get liquid nitrogen now.)

9. Pour off supernatant & resuspend in 20mL TB.

10. Swirl in 1.4mL DMSO to make final [DMSO] = 7% & incubate on ice for 10 min.

11. Aliquot 500µL into 1.5mL tubes & quickly freeze in nitrogen. Store at –80°C. Should last 40 days.

NEXT: TEST COMPETENCY OF E. COLI CELLS

TEST COMPETENCY OF E. COLI CELLS

1. Take out DNA and 1-500μL aliquot of each line of cells testing, thaw and place in ice.

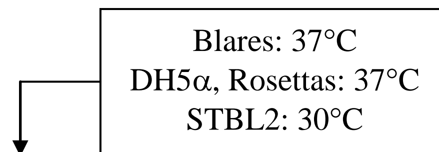
2. Label 1mL tubes for 1 control & 2 tests for each line of cells. Add 1μL DNA to each sample tube. (Not the control tube.)

3. Add 100μL cells to each tube. Mix by pipetting up and down gently.

4. Leave tubes on ice for 30 min. Prepare 42°C heat block by adding a little 18 MΩ dH2O into wells that will be used. Let temp of the water get to 42°C

5. Heat shock tubes for 40 sec. (Exceptions: STBL2 – 28sec, DH5α – 45 sec)

6. Put back on ice for 5 min.



7. Add 1mL LB to each tube. Put all tubes together and, depending on strain, place in 30°C for 90 min. or 37°C shaker for 45 min.

8. Take out samples & dilute by taking 110μL from 1st tube and adding to 1mL LB in a new tube. The first tube becomes [0.9] and the second becomes [0.09]. Mix the second well and pipette 110μL into a new tube with 1mL LB. This third tube is now [0.009]. Mix well.

9. Spin down cells for 5 min at max speed. Remove 850μL LB and discard.

10. Resuspend cells in remaining 250μL LB & transfer to plates with appropriate drugs.

11. Grow up overnight in 30°C or 37°C incubator.

PROTEIN PURIFICATION

Notes: Day 1 – Step 1 Day 2 step 2-12 Day 3 – Steps 12 - 21 or 22

1. Inoculate 250mL LB + 500µL amp + 500µL chloram with 10-12 colonies of desired protein. Let grow up to saturation overnight in 30° or 37°C shaker.

2. Make glycerol stock of the O/N cultures by adding 850µL culture to 150µL glycerol in a screw-top tube. Label well and cover label with clear tape to avoid fading. Store in -80°C freezer.

3. Inoculate as many 2L aliquots of LB + 4mL amp + 4mL chloram as need. Aliquots should be in 6L flasks. Let incubate in 30° or 37°C shaker until in log phase.

4. Test absorbance of cultures until A600 is between 0.6-1.0. When cultures are in log phase, add 0.19g IPTG resuspended in 2mL 18 MΩ dH2O to each 2L culture.

5. Take 200µL samples at T0, & return cultures to incubator.

6. Spin down To samples for 3 min., remove sup, resuspend in 40µL 2X Sample Buffer, boil for 5 min. & store in -30°C until ready to run a protein gel.

7. Remove cultures at designated time (usually T3-T4 is best). (Take time point sample and repeat step 6.) Spin down cultures at 4,000rpm for 10min. in 1L centrifuge bottles using the JLA-8.1 rotor. If have too many samples, spin 6, pour off the supernatant, add more culture, and spin again.

8. Pour off the supernatant, wash pellet quickly in a little 10mM Tris (pH 7.5). Resuspend pellet in 25mL 10mM Tris per Liter of original amount of culture. (Ie. If spun down 2 L of cells in one bottle, add 50mL 10mM Tris.)

9. Switch cells to the 250mL centrifuge bottles if possible & spin down at 12,000 rpm for 10-15min using the JA-14 rotor. (Get liquid nitrogen now. Make lysis buffer now.)

10. Pour off supernatant & resuspend pellets in 30mL Lysis Buffer per Liter of original amount of culture. (See Recipes to make Lysis Buffer.)

11. Freeze 30mL aliquots in small centrifuge tubes in liquid nitrogen. Store in -80°C overnight.

12. Thaw tubes of protein in tub of luke warm water – important that protein does not get above 4°. Keep turning tubes to ensure even thawing. Add fresh protease inhibitors if needed.

13. Pour cells into 1-2 glass beakers and sonicate. Do 2-3 pulses for 30 sec each. Do not touch the sides or bottom of the beaker with the sonicator. Use earphones to protect ears.

14. Pour cells evenly into centrifuge tubes. (Solution should be mucus-like.) Centrifuge for 25 min at 13,000 rpm (~20,000 G).

15. Pour supernatant into a graduated beaker & note amount. Pour into glass beaker with stir bar.

16. Slowly add polyethylenimine (PEI), so that have a 0.2% concentration in supernatant. Must drip in so no local high concentrations. Calculate using:

(5%) (X mL) = (2%) (Y mL)   X = (2*Y) / 5 Y = amt supernatant   X = amt to add

17. Let stir on med-high speed for 5 minutes. (Should see thick white wisps forming.)

18. Centrifuge for ~5-15 min at 13,000 rpm (~20,000 G). Want 100,000g·min. So if spinning at 20,000g, spin for 5 min. Do not want to spin much more – not good for sup. **Supernatant should come out very clear and almost colorless.** If not, spin a little longer.

19. Pour supernatant into a graduated beaker & note amount. Pour into glass beaker with stir bar.

20. Add enough ammonium sulfate to raise concentration to 45%. Must add SLOWLY so that no local high conc. Add while stirring. Let stir for 5 min after having added the whole amount.

[45%] = 277g/L ammonium sulfate ∴ Amt to add (g) = (0.277 g/mL * V mL)   V = amt supernatant

21. Centrifuge for 10 min at 13,000 rpm (~20,000 G). Keep the pellet, but pour off the supernatant into a clean beaker in case the pellet slips.

22. Resuspend pellet in specified amt of No Salt A & run Protein Specific Sizing Column (See Sizing Column Protocol)

dynFL	Resuspend pellet in 8mL No Salt A
	Run on S300 Column (can only load up to 12mL)
N173	Resuspend pellet in 10mL No Salt A / 1 L of original culture
	Run on a SP sepharose column
C181	Resuspend pellet in 10mL No Salt A / 1 L of original culture
	Run on a Nickel Column

23. Run protein gel to identify fractions with the protein if needed.

24. Pool fractions that have the protein & dilute 3 fold. Run Mono S column. (See Mono S Protocol).

NEXT: RUN PROTEIN SPECIFIC COLUMN & PROTEIN DIALYSIS

PROTEIN DIALYSIS

1. Pool desired fractions (run gel to determine fractions if necessary)

2. Cut a piece of dialysis tubing. Clip one end and fill with 18 MΩ dH2O to check for leaks.

3. Carefully pour protein into a dialysis bag. Clip the other end closed.

4. Make up dialysis buffer in a 2L graduated cylinder with a large stir bar at the bottom.

5. Float bag in dialysis buffer for at least 5 hours – better to leave overnight – in the cold room on a stir plate. Check on it a few times before leaving for night because stir bar sometimes gets off track.

6. When protein reaches equilibrium with buffer, collect protein & fill a 50mL conical with the used dialysis buffer. Label & store buffer in 4°C for use in sedimentation experiments.

7. Determine concentration using λ function on the spectrometer. Dilute 4 µL protein in 396µL18 MΩ dH2O for a 100-fold dilution. Dilute 4µL dialysis buffer in 396µL18 MΩ dH2O for a 100-fold dilution. Use 290 as the wavelength and blank against the diluted dialysis buffer. Aliquot protein depending on concentration.

FIXING *DICTY* CELLS W/ PARAFORMALDEHYDE

PREP

1. Thaw 15mL tube of 16% paraformaldehyde.

2. Sterilize cover slips in a beaker of ethanol. Set up and label as many 6 well plates as need.

3. Carefully remove a cover slip with tweezers. Blot a corner on a Kimwipe and put cover slip quickly through a flame to burn off rest of ethanol. Once flame is out, put cover slip into a well. Repeat for as many samples as need.

4. Resuspend cells and add 1mL cells to each well. Add 2mL fresh media to wells to dilute. Let cells sit at room temperature for a few hours to adhere onto coverslip.

5. Make Fix Mix (~2mL for each sample)

   4% paraformaldehyde
   0.1% Triton X-100
   150mM NaCl

   Make Immunofluorescence 1X PBT

   1X PBS
   0.05% Triton X-100
   0.5% BSA

6. For 100mL 1X Immunofluorescence PBT
   10mL 1X PBS
   500µL 10% Triton X-100
   0.5g BSA

FIXING

7. When cells are ready, take off media carefully and add 2mL Fix Mix very gentle to side of well so that the cells are not blasted off of the cover slip.

8. Leave Fix Mix on for 5 min.

9. After 5 min., take off Fix Mix and immediately add 2mL 1X PBS to each well. Take off and add 2mL fresh 1X PBT again. Repeat this wash 4-5 times for each well.

10. Mount cover slips on slides
    Add 10µL 20mM Tris + 90% glycerol to the slide where placing the cover slip.
    Use tweezers to grab the cover slip, blot corner on a Kimwipe and put on slip,
    Make sure that cell side is down.

ACETONE FIX

Add 2mL 100% chilled acetone to each well after removing the media. Put wells on ice for 3 min. Remove acetone and start washes.

FREEZING *DICTYOSTELIUM* CELLS

Need: A bucket of ice and 2mL Freeze Mix per confluent plate.
Each plate of cells makes 4 aliquots to freeze.

1. Mix amount of Freeze Mix needed. mL needed = (Z plates x 2) Invert a few times. Reaction is exothermic, will feel a little warm. Immediately put on ice and chill at least 15 minutes.

2. Label as many screw top 1.5mL tubes as needed. Put clear tape on top of label to keep it from fading. Label top of tube with cap label.

3. Pour media off of plates.

4. Take 2mL freeze mix and resuspend cells quickly.

5. Add 500µL aliquots to each tube and immediately put on ice.

6. Store cells in –80°C freezer.

7. Fill out Storage Log for new tubes.

Freeze Mix

10% DMSO in Bis-Tris HL-5 pH 6.7

To Thaw

1. Remove tube from -80°C freezer.

2. Warm in hand as you walk to work area.

3. Wash exterior of tube with ethanol and wipe with kimwipe.

4. Open tube and pipet contents into a 100mm plate containing 10mL of media.

5. Take 1 mL off and add to new plate with 9mL media to dilute DMSO 10-fold.

Knecht Dicty Genomic DNA MiniPrep

(Use Knecht dicty genomic DNA MiniPrep Worksheet) (protocol from Glen 5/2/95)

1. Pour media off confluent plate of cells. Resuspend cells in 12mL 10mM Tris-HCl, pH 7.5.
2. Spin down at 2,500rpm for 5 min. in tabletop clinical centrifuge. Discard sup.
3. Resuspend pellet in 1mL Nuclei Buffer. Transfer to a 1.5mL eppendorf tube.
4. Add 200uL 20% Triton X-100. Mix by inverting.
5. Incubate on ice for 5 min.
6. Spin at max speed for 5 min. in microcentrifuge. Discard sup.
7. Resuspend cells in 300µL Proteinase K Buffer. Add 10 µL 20mg/mL Proteinase K. Mix by inverting. Incubate at 65ºC for 20 min.
8. Add 200µL TE and 500µL phenolchloroform. Mix by inverting. Will turn cloudy white.
9. Spin at max speed in microcentrifuge until aqueous layer (top layer) turns clear. (~20-30min.)
10. Carefully extract top layer (~400-450µL). Avoid the white and bottom layers! (Discard these layers as hazardous waste.) Can repeat phenolchloroform extraction, steps 8-10, sup should clear faster and only extract 300-350µL the second time.
11. Add 60µL 8M NH4OAc and 800µL EtOH to precipate DNA. Mix by inverting.
12. Spin at max speed 15 min in microcentrifuge. Aspirate sup. (Pellet will be hard to see.)
13. Add 200µL 70% EtOH. Spin down at max speed 3 min. Aspirate sup.
14. Let pellet air dry until clear. (~5-10 min.)
15. Resuspend in 25-50µL TE plus 1-2uL RNAse A.

Nuclei Buffer	To make 10mL
25mM Tris-HCl, pH 7.5	250µL 1M stock
5mM MgOAc	50µL 1M stock
0.5mM EDTA	10µL 0.5M stock
5% sucrose	770µL 65% w/w
	8.99mL dH2O

Proteinase K Buffer	To make 10mL
10mM Tris-HCl, pH 7.5	100µL 1M stock
5mM EDTA	100µL 0.5M stock
1% SDS	1mL 10% stock
	8.8mL dH2O

TE	To make 10mL
10mM Tris-HCl, pH 7.5	100µL 1M stock
1mM EDTA	20µL 0.5M stock
	9.88mL dH2O

PREPARATION FOR FACS SAMPLES

(EMR 09/27/05)

Cells should be healthy and in log phase on day of sort. You will need a fluorescent cell sample and a control non-fluorescent cell sample.

1. Prepare fresh 1X PBS & filter sterilize. Need 25mL per sample.

2. Resuspend cells and take cell count.

3. Spin cells at 2,000 rpm for 3.5 min.

4. Discard media. Wash cells with 10mL 1X PBS.

5. Spin cells at 2,000 rpm for 3.5 min.

6. Discard sup. Resuspend cells in ________ mL 1X PBS so that final conc. Is 1-2 x 10^6 ce''s/mL.

   (________ cells/mL starting) * (________ mL) = _____________ cells total

   (1-2x106 cells/mL) / (__________ cells total) = _____________ mL 1X PBS to resuspend in.

   Need about 3mL of sample.

7. Bring sterile 1X PBS, samples, and 25mL media to FACS facility located in Ross Building, Room 1071. Phone 5-7852.

ISOLATING RNA USING TRIZOL REAGENT

Reagents required:

Chloroform	Isopropyl Alcohol	75% EtOH (in DEPC-treated water)
DEPC-treated water	TRIzol Reagent (Invitrogen)	

HOMOGENIZATION

1. Resuspend cells and take cell count. Determine total number of cells
2. Spin cells down at 2,000rpm for 3min. Discard sup.
3. Lyse cells by adding 1mL TRIzol per $1x10^7$ cells.
4. Transfer cell lysate to autoclaved Falcon tubes.

PHASE SEPARATION

5. Incubate homogenized samples at room temp. for 5 min.
6. Add 1.6mL chloroform per 8ml TRIzol used.
7. Cap tubes securely and shake vigorously by hand for 15 sec.
8. Incubate at room temp. for 3 min.
9. Spin at 3,000 x g for 15 min.
10. Remove top aqueous phase and transfer to 4 eppendorf 1.5mL tubes. This contains the RNA. Discard other layer as hazardous waste.

RNA PRECIPITATION

11. Add isopropyl alcohol (2-propanol) to the top of each tube.
12. Incubate at room temp. for 10 min.
13. Spin at max speed for 10 min in microcentrifuge. Discard sup.
14. Wash pellets once in 1 mL 75% EtOH (25% DEPC-dH2O) per tube.
    Can combine pellets in 1 tube. RNA pellets can be stored in DEPC-EtOH until ready to use.

REDISSOLVING RNA

1. Spin pellets at max speed 3 min. Discard sup.
2. Air-dry pellets until just turn clear. (RNA very hard to dissolve if too dry. Watch closely.)
3. Dissolve RNA in 30 µL DEPC-treated dH2O by pipetting a few times.
   (If sample is too thick, add more DEPC-treated dH2O, so pipets easily)
4. Incubate for 10 min at 55-60ºC.

RT-PCR

1. Combine pellets and REDISSOLVE RNA using protocol on previous page.

2. Determine RNA concentration by adding 3µL sample to 597µL DEPC-treated dH2O. Blank spec using 600µL DEPC-treated dH2O. Use RNA spec mode.

   _________________ (OD_{260}) x 100 x 4/5 = _____________ µg/µL

3. Prepare 1mL of 4ng/µL for the WT RNA sample:

   4ng/µL RNA x 1000µL = ______________ µg/µL RNA x **V**

   V µL + (1mL DEPC-dH2O – **V**) * If necessary, dilute samples so that volume of samples is ≥ 10µL.

4. Prepare serial dilutions of WT sample. Add 500µL DEPC-dH2O to 5 tubes. Label tubes 2, 1, 0.5, 0.25, & 0.125. Take 500µL 4ng/µL sample and add to tube labeled 2. Vortex quickly and take 500 µL from that tube and transfer to tube labeled 1. Do this in succession for tubes 0.5, 0.25, and 0.125. After vortexing tube 0.125, discard 500µL from this tube.

5. Prepare 1 mL of 0.5ng/µL for the unknown sample:

   5ng/µL RNA x 1000µL = ______________ µg/µL RNA x **V**

   V µL + (1mL DEPC-dH2O – **V**) * If necessary, dilute samples so that volume of samples is ≥ 10µL

6. Prepare serial dilutions of the unknown sample. Add 500µL DEPC-dH2O to 5 tubes. Label tubes 2, 1, 0.5, 0.25, & 0.125. Take 500µL 5ng/µL sample and add to tube labeled 2. Vortex quickly and take 500µL from that tube and transfer to tube labeled 1. Do this in succession for tubes 0.5, 0.25, and 0.125. After vortexing tube 0.125, discard 500µL from this tube.

7. Prepare RT-PCR Mix. # sample = 5 WT + 3 per unknown sample

   _____ samples x 25 µL (2X SYBR Green)
   _____ samples x 1.5 µL 10 µM 5' primer
   _____ samples x 1.5 µL 10 µM 3' primer
   _____ samples x 1.0 µL DEPC-dH2O
   _____ samples x 1.0 µL Reverse Transcriptase

8. Aliquot 30µL of RT-PCR mix to PCR tubes. Add 20µL of RNA samples. Put PCR samples on ice until load into PCR machine.

   WT standards: 40ng, 20ng, 10ng, 5ng, 2.5ng Unknown samples: 20ng, 10ng, 5ng, 2.5ng

9. Ask Elizabeth for help in setting up PCR program.

BRADFORD ASSAY – 96-WELL

Steps:

1. Make the protein standards
2. Place 400 ul dH2O in each tube.

BSA stock	10mg/ml	
Tube 1	60 µg/ml	12µL of stock in 788µL H2O
Tube 2	30 µg/ml	Take 400µL of tube 1 and mix with tube 2 (1:2 dilutions)
Tube 3	15 µg/ml	Take 400µL of tube 2 and mix with tube 3
Tube 4	7.5 µg/ml	etc.
	3.75 µg/ml	etc.
	1.8 µg/ml	
	0 µg/ml	400µL of dH2O only

   Place 200µL of each sample in a well (duplicate) of a 96 well plate.
   Will add 50µL dye later to each well (1:4) for a total volume of 250µL/well

3. Add 1µL of your sample to a well (run in duplicate) then 199µL of dH2O
4. Add 50µL of Bradford reagent to each well with samples or standards
5. Read plate at wavelength of 595nm quickly (Room 107 on the Omega machine)
   a. Computer user Cell Bio → password = password
   b. Protocol = "Eric Bradford"

GLASS MILK MINIPREP

Buffers:

A (From Denville maxiprep kit, at 4ºC)

B (From Denville maxiprep kit)

C1: (From Denville maxiprep kit)

WS: 10mM Tris, 100mM NaCl, 80% ethanol, pH 7.8

EB: 10mM Tris, pH 8.5

Glassmilk slurry: Add 5g silicon dioxide (Sigma 55631) to 50 mL H2O in 50mL conical. Let settle for 2 hrs, dump supernatant and remove fine particles, resuspend in 50mL H2O and repeat. Resuspend again in 50mL H2O and store at RT. Resuspend fully by vigourous vortexing before use.

Protocol:

1. Grow up 1-3 mL of E.coli expressing your plasmid of interest.
2. Pellet cells in Eppindorf tubes at max rpm for 1min for E.coli (2,000 rpm for 1min for dicty).
3. Resuspend pellet in 250µL Buffer A
4. Add 250µL Buffer B, invert 10 times to mix
5. Add 300µL Buffer C1, invert 10 times to mix
6. Spin for 10min at max rpm
7. Transfer supernatant to new Eppendorf tubes, add 20µL glassmilk slurry
8. Rotate or rock for 5 min.
9. Spin for 1 min at max RPM
10. Dump supernatant, add 750µL WS buffer and vortex briefly
11. Spin for 1 min at max RPM
12. Aspirate supernatant, trying to remove as much as possible, leave cap open to dry. Drying may be assisted by blowing into tube with air line or placing in incubator or heat block at elevated temp (37-60ºC)
13. Once pellet is dry, add 60µL EB and place on 60-70ºC, mix by vortexing until pellet is thoroughly resuspended.
14. Spin 1min at max RPM
15. Remove supernatant to new tube, attempting not to disrupt pellet. Recover ~55µL at ~0.1-0.2µg/µL.

Modified: 4/23/15 CRK (written by HWF)

CIGARETTE SMOKE EXTRACT

Materials:

- Peristaltic pump with tygon tubing
- U. of Kentucky research cigarettes
- 50mL conicals
- Parafilm
- Sterile glass pipets
- 20µm filter
- Cell-specific media

Steps:

1. Under sterile technique, fill a 50mL conical with 25mL of the specific cell culture media.
2. Flame a glass pipet, place in the conical and parafilm it closed ensuring no areas for leakage
3. Place 1 cigarette in the tubing adaptor. Connect the second tube of the pump to the top of the pipet.
4. Turn on the pump to ensure good bubbling through the media.

   1 cigarette should smoke completely in 6 minutes (~ setting 6 on the current pump)
5. Light the cigarette with a Bunsen burner.
6. The cigarette is complete once it reaches the non-patterned white paper.
7. When done, unhook the pipet and place parafilm over the top of the pipet (sometimes media will begin spilling out if you don't)
8. Filter extract through 20µm filter into autoclaved bottle or sterile conical tube.

The extract made is 100% smoke extract and can then be diluted.

Modified: 4/23/15 CRK

HBE-KT CELL CULTURE

Human Bronchial Epithelial Cells

Cell source

HBE-KT cells: John Minna, M.D. at UT Southwestern

Genotype authentication: complete 12/2014

Mycoplasma testing: 12/2014 - Negative

Cell diameter – 8-10µm

Notes:

These cell lines can be difficult and sensitive - do everything you can to prevent stress to the cells:
Allow 24hr for the cells to adhere to culture vessel surface (they will take ~16 hr to adhere).
Do not split cells more than once in 48hr, preferably in 72hr (generally split twice a week).
Split cells when they are ~80% confluent (never split when less than 60% confluent).

HBECs can be difficult to transfect with lipid-mediated transfection - viral transduction has more success.
Allow the cells an extended time to recover from transfection/transduction (recovery time often takes longer than selective antibiotic time).
Immortalized HBECs (with the suffix 'KT') have G418 and puromycin resistance through the introduction of cdk4 and hTERT.

<u>Materials:</u>

PBS, pH 7.4 (sterile-filtered)

Keratinocyte Serum-Free Media (KSFM) w/ supplements (Invitrogen, #17005042)

KSFM Supplements (come with media): EGF & BPE

Trypsin-EDTA (Invitrogen, #25300)

Trypsin Neutralizing Solution (TNS) (Lifeline, #CM-0018)

HBEC Freezing media: 80% KSFM (w/ supplements), 10% DMSO, 10% FBS

<u>HBEKT Protocol</u>

1. Warm PBS, trypsin, TNS and KSF media (with supplements).

2. Aspirate media and wash cells with PBS. Aspirate PBS.

3. Add 2.5 mL Trypsin (for T75 flask) and incubate in 37°C incubator for 2-8 min. Assist detachment with tapping flask gently.

4. Add 1 volume of TNS as soon as ~75% of cells are detached and pipette thoroughly to ensure single cell suspension. (Do not aim to get >75% of cells detach, you will only kill already detached cells)

5. Spin down cells at 1,000 rpm for 5 min.

Resuspend cell pellet in KSFM and mix.

6. Seed cells as desired (1/3 to 1/5 dilution from 60-80% starting confluency).

1 T75 flask → 5 or 7 new flasks

HBE-KT CELL CULTURE – CONT'D

To freeze cells: Resuspend pelleted cells in freezing media (80:10:10, KSFM:DMSO:FBS) and aliquot to cryovials. 1 T75 flask → 3 cryovials (1mL each). Slowly freeze to -80oC in Mr. Frosty overnight then transfer to liquid nitrogen storage.

To thaw cells: Thaw vials in 37°C waterbath then dilute with KSFM (~1mL) and transfer to 15mL tube. Pellet cells at 1,000 rpm for 3 min, remove supernatant (very important to remove all traces of DMSO and FBS), and resuspend cells in KSFM (~2mL) to seed.

Recommended cell density:

For 8-well glass chamber slides (well area 0.8cm^2, 0.2-0.4mL vol) → 2,500 cells/well
For 6-well plate → 2/5 of one T-75 flask

Modified: 4/23/15 CRK

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING

from Vasudha Srivastava

Image Acquisition

1. Use 63x oil objective for imaging
2. Select Green_Micfac channel
3. In Main Menu, select Bleachin, Time Series and Regions
4. In Lasers tab, turn on the lasers. On LSM510 for Argon laser go to laser properties and increase power to 50%
5. In Channel tab, adjust the following:
   - 5.1. Laser power - 5-20% on LSM510 and 0.6-2% on LSM 780 depending on expression level
   - 5.2. Pinhole – To get 1.5 µm optical section
   - 5.3. Gain (500-800) and Digital offset. In the range indicator you should be able to see few saturated (Red) and zero (Blue) pixels
6. In Acquisition tab, adjust the following
   - 6.1. Image size
     - 6.1.1. Use 512x512 for scanning the field
     - 6.1.2. Use 128x128 or 64x64 for FRAP experiment depending on desired scan speed
   - 6.2. Averaging – Keep at 1 for fast acquisition and reduced photobleaching
   - 6.3. Scan speed – Use 'Max' for fast acquisition. Make sure to hit Max each time you change the image size and zoom
   - 6.4. Increase Depth to 16-bit image
7. In Bleaching tab
   - 7.1. Set Bleach after # of scans to 4
   - 7.2. Set iterations to 20-100 (depending on extent of bleaching)
   - 7.3. Select 488 laser and bleach intensity 100% (reduce if there is significant phototoxicity)
8. In Time series tab
   - 8.1. Set number of images to 150
   - 8.2. Set interval depending on protein
     - 8.2.1. For Dicty cort I, IQGAP2 use 100-150 ms
     - 8.2.2. For Dicty myoII use 300-500 ms
     - 8.2.3. For GFP use 0 ms to go as fast as possible
     - 8.2.4. Might be different for different proteins. Use an interval that gives at least 5-10 points before tau and shows a plateau
9. In regions tab
   - 9.1. Use rectangle tool to mark the bleach region. Try to be consistent in size of ROI across measurements
   - 9.2. Before a new acquisition, delete any saved ROIs
10. Collecting FRAP data
    - 10.1. Acquire and image of the field (512x512)
    - 10.2. Zoom in on cell of interest using Crop function. Reduce size to 128x128 and use max speed. Acquire image using Snap function. When picking a cell to image, choose cells that are moderate expressors with smooth cortex

10.3. Use ROI tool to mark the bleach region
10.4. Start experiment

Data analysis

1. At the end of session, transfer data from Local temp storage to the TempStorage3 server (\\162.129.230.229\TempStorage3)
   User: public, password: pub!@#$
2. Copy the files from the server to your computer/hard drive
3. **Extract time stamp using MATLAB**
   3.1. Copy Scot's MATLAB files to the folder containing your data
   3.2. Open MATLAB and change working directory to your data folder
   3.3. In command window, enter LSM_FRAP (case-sensitive)
   3.4. In the new window that opens, select a file and let it load
   3.5. In the command window, copy the time stamps for each frame and paste in the time column in FRAP template
   3.6. Repeat for all measurements
4. **Measure intensities using ImageJ**
   4.1. Open file in ImageJ
   4.2. In Acquisition->Set measurements select Mean gray value
   4.3. Mark ROI using the freehand tool to mark bleach region. Scroll through the movie to make sure the ROI remains in the bleach region and cell doesn't move
   4.4. In Plugins->Stacks->Measure stack to measure the mean intensity in each frame. Copy to FRAP template
   4.5. Repeat for reference region and background region.
5. **Correction for photobleaching and intensity normalization using KaleidaGraph**
   5.1. Copy the time and background subtracted intensity to KaleidaGraph
   5.2. Use Gallery->Scatter to plot data. Use curve fit->General->FRAP fit to fit an exponential decay
   5.3. Adjust the fitting parameters (m1, m2, m3) to improve fit if needed
   5.4. Double click on parameters to select and copy to FRAP template. Copy the graph to FRAP template.
   5.5. Use the Normalized Intensity for fitting recovery time and immobile fraction. Copy adjusted time and intensity to KaleidaGraph.
   5.6. Use Gallery->Scatter to plot data. Use curve fit->General->FRAP fit to fit an exponential rise. Adjust the fitting parameters (m1, m2, m3) to improve fit if needed
   5.7. Double click on parameters to select and copy to FRAP template. Copy the graph to FRAP template.
   5.8. The recovery time and immobile fraction is calculated and summarized in the Summary worksheet.
6. **Correction for anomalous behavior**
   6.1. If the Reference or Recovery curve doesn't have the characteristic shape, measure intensities again
   6.2. If the Reference curve shows bleach and recovery, move further and re-measure reference intensity. If still not corrected, DISCARD

6.3. If Reference curve shows oscillations, there might be vesicles moving in and out of ROI. Re-measure in a different region that stays uniform
6.4. If Reference curve rises instead of decaying, the cell is contracting. DISCARD
6.5. If Recovery curve does not show good recovery/saturation, increase acquisition interval
6.6. If Recovery curve shows almost immediate recovery, shorten acquisition interval to get enough points in the recovery phase
6.7. If Recovery curve shows biphasic behavior, truncate data to get fit using single exponential.

Modified: 4/26/15 DNR

CYTOSKELETAL FRACTIONATION FOR FCCS

from Vasudha Srivastava

1. Resuspend and count cells. Centrifuge at 2000 rpm for 5 min
2. Resuspend cells in 1 mL 1X PBS and centrifuge 5000g for 5 minutes
3. Discard supernatant and resuspend in lysis buffer to a final concentration of $2x10^8$ cells/mL
4. Vortex briefly and incubate on ice for 10 min with intermittent vortexing
5. Centrifuge at 15,000g for 5 min at 4^0C
6. Transfer supernatant to a fresh tube. Keep on rotator in cold room till needed
7. For imaging, make a 1:5 dilution in triton free buffer and add 200-500 µL solution to imaging chamber

	Lysis Buffer (10 mL)	**Dilution Buffer (10 mL)**
100 mM PIPES pH 6.8 (1M stock)	1000 µl	1000 µl
2.5 mM EGTA (500 mM stock)	5 µl	5 µl
1 mM $MgCl_2$ (1M stock)	10 µl	10 µl
1 mM ATP	6 mg	6 mg
0.2% Triton X-100 (20% stock)	100 µl	-
PI Cocktail (100X stock)	100 µl	100 µl
PMSF (1000X stock)	10 µl	10 µl
TLCK (100X stock)	100 µl	100 µl
Aprotonin (10,000X stock)	1 µl	1 µl
Water	8.7 ml	8.7 ml

Cell line	**Cell density**	**Volume**	**Total Cells**	**Resuspension volume**

FLUORESCENCE CORRELATION SPECTROSCOPY IN CELLS AND LYSATES

from Vasudha Srivastava

Cell lysate prep (Check cytoskeletal fractionation protocol)

Carry out cell lysis at cell density of $2x10^8$ cells/mL. Dilute sample accordingly depending on fluorescence intensity.

Setting up for FCS

1. **Start Zen software.**

2. **Setting up and removing the FCS objective**
   - **2.1.** (Use 40x C-Apochromat (NA 1.2) water objective)
   - 2.2. Center the moveable stage to the center of the objective. Use the colored guides for alignment. Move objective to the load position
   - 2.3. Select 20x objective from the controller and remove objective. Move the cover from 40xW to prevent dust from getting in the system
   - 2.4. Select 40xW from the controller and put the FCS objective on the system. Secure the 20x objective in the safe
   - 2.5. Use syringe to add water to the objective. Make sure there is always enough water between the objective and coverslip. If needed, refill the syringe with DI water
   - 2.6. After your session, center the stage, move to load position, clean the objective and store it in the safe. Put back the 20x objective on the system, and return to 10x

3. **Finding the right imaging plane**
   - 3.1. Use the line scan setting to identify edge of coverslip (Acquisition --> Settings --> FCS_Line scan_VS.
   - 3.2. Starting with the load position, move up in Z-axis using fine focus (clockwise, towards scope). Stop when you see two intensity spikes corresponding to the two edges of the coverslip.
   - 3.3. Move the focus up 200 µm into the solution using FCS->Focus->+200 µm (use Show All option if the +200 button is not visible).

4. **Calibration of the system**
   - 4.1. Select Rhodamine channel (Light path --> pick Standard-488-Rh)
   - 4.2. Click on Count Rate. Adjust laser power to ensure count rates are between 20-150 kHz
   - 4.3. Adjust Correction Collar on the objective while measuring count rates to maximize the count rate. Note that there is a delay between the collar adjustment and visible count rates
   - 4.4. Set the pinhole to 1AU. Adjust the pinhole (Light path ->Adjust pinhole) using both coarse and fine alignment in X and Y directions

5. **Acquisition for Rhodamine standard**
   - 5.1. Make a 50-200 nM Rhodamine 6G solution in lysis buffer
   - 5.2. Acquire every 5 seconds for 10 repetitions.
   - 5.3. Select the model to fit the data -->

     Model: 1triplet_1comp model

     (Gives you immediate readouts, data can be refitted as needed).

     Structure Parameter - 6 (Fixed)

     Triplet state relaxation time – 8 µs (upper limit).
   - 5.4. When using multiple channels, set these parameters for each channel

5.5. Hit start experiment to acquire data. Select new window for each acquisition. Collect about 5 measurements for the standard and 10-15 for experimental samples
5.6. Realign focal plane and pinhole as needed
5.7. The diffusion time for Rhodamine 6G in water should be 20-30 µs depending on temperature. If the measured diffusion time is much larger, re-adjust the correction collar. The diffusion times may vary in buffers

6. **Acquisition of FCS data in solutions**
6.1. Use 200-500 µL sample per well of an 8-well Mattek Imaging chamber. Add samples one at a time to avoid photobleaching from the neighboring wells. For the glass bottom 3.5 mm dishes use 1.5-2 mL sample.
6.2. Select the desired light path. For cross-correlation use the GFP/mCherry cross setting
6.3. Change laser power as required to get high enough counts (Try 0.3-0.5% laser as a start and aim for similar counts in both channels)
6.4. Fix the fitting parameters in the model for each channel
6.5. Collect 5 s acquisitions with 10 repetitions for each measurement. Reduce acquisition time to 2 s if there are too many aberrant curves
6.6. Adjust the sample dilution to control amplitude number of particles (N should be between 0.3-10 for maximum sensitivity). Adjust imaging conditions appropriately to have counts per molecule >1 and correlation >1.

7. **Acquisition of FCS data in cells**
7.1. Acquire image of cell using the acquisition tab (Default Micfac settings work fine). Image dimmer cells but avoid very dim cells
7.2. In FCS menu, check Positions. Shorten acquisition to 2 s. Typically 0.1% laser works decently in cells for GFP (lower gives poor signal and higher results in bleaching and very high counts.
7.3. In Positions->Select, then mark the cross inside the cell in a region that looks uniform. Click Add position in the Position menu and start experiment
7.4. After acquisition, acquire another image of the cell to ensure that the cell did not move out of the imaging volume.
7.5. Export the image of the cell before and after FCS Acquisition using File->Export->Tagged Image file, Contents of the Image window
7.6. Be very quick with the acquisitions to avoid cell from crawling away while you set up. Re-position if there is a delay in starting acquisition

8. **Selection for useable curves**
8.1. Photobleaching – Noticeable downward trending of data -> DISCARD
8.2. Aggregates – Large, broad spikes in the count rate graph -> DISCARD
8.3. Active movement – Significant deviation (>20% from mean) in count rate or general detreding -> DISCARD
8.4. Poor fit – Significant deviation between correlation curve and the fit (large residual curve) -> DISCARD
8.5. Use the average of 3-10 repetitions for each measurement. Discard measurements with fewer good traces.

9. **Calculation of diffusion coefficients**
9.1. Use Rhodamine 6G as standard with D = 426 $\mu m^2/s$ at room temperature
9.2. Calculate the diffusion coefficient for rhodamine at your acquisition temperature and viscosity
9.3. Calculate Diffusion Coefficient of your sample using the equation

$$D_{eff} = \frac{D_{R6} \cdot \tau_{D,R6}}{\tau_D}$$