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September 2021

Wednesday, September 8, 2021

To Do:

1. Patch out primary integrants
2. Prep CHAH (ATC+NAT) + 10% sucrose plates, CHAH (ATC+NAT) plates, and CHAH (ATC+NAT) + Kan
3. Pour plates

Results and Data:

Patch Plate Observations	
E1-5	Decent growth, moved forward
E1-6	No growth

Note on Original CHAH recipe. No longer use a manufactured mix of cystine heart agar, instead a mix is made in house. Additionally, when autoclaving place the flasks in a tub with water, like a Bain-Marie. This will help reduce the likelihood of boiling over. (CHAH agar boils over quite easily). Can leave stir bar in the flask when autoclaving. Autoclave program: page 3, third item; liquid for 30'.

Note on plate pouring technique. Use Electronic Pipette Controller and disposable, sterile pipettes to dispense 24 mL of agar solution into plates. Clear pipette tip clear of plate lids. For now, use a 25 mL pipette and individually dispense, perhaps later move to the standard 50 mL pipette and dispense two plates at a time.

Patching Primary Integrants from Frozen Stock

1. Prepare work area: turn on flame, retrieve sticks, have plates ready under foil, etc.
2. Retrieve Primary Integrant from the -80°C freezer, paying attention to freezer map and location.
3. Spray gloves with ethanol and allow to dry before moving to work near the flame.
4. Wipe outside of the tube to remove frost.
5. Unscrew sticks container, retrieve stick and remove lid on frozen stock. Take care to leave lid facing up.
6. Scrape out a large amount of frozen stock, and patch in the center of the plate. This plating technique is not being used for isolation.

Reagents

CHAH Agar with ATC and NAT (+ Kan)

Mix 30.6 g cystine heart agar (lab mix) with 300 mL type I ddiH₂O; add stir bar and let stir on a 90°C hot plate for 10 minutes.

Add flask to a tub with water and autoclave 30' liquid culture program.

While autoclaving, warm 300 mL of 2% hemoglobin in water bath at 55°C

Place CHA flask in 55°C water bath

Wipe down flask and bottles with EtOH and with sterile technique, pour hemoglobin into CHA flask, stir to mix

Add 120uL of ATC and 30uL of Nat, and stir to mix. Pour 14 plates.

Add 24uL of Kan, stir to mix, and pour the last 10 plates.

Label plates and cover in foil

CHAH Agar with 10% Sucrose (+ATC and NAT)

Mix:

5 g Beef Heart Infusion

5 g Protease Peptone

5 g Glucose

0.5 g L-Cystine

7.5 g Agar

with 150 mL type I ddiH₂O.

Stir on 90°C heat for about 10 minutes.

Add flask to a tub with water and autoclave 30' liquid culture program.

While autoclaving, warm 250 mL of 2% hemoglobin and sterilized 50% sucrose in water bath at 55°C

Place CHA flask in water bath at 55°C

Wipe down flask and bottles with ethanol and using sterile technique, pour hemoglobin into CHA flask

Stir until mixed

Add 100 mL 50% sucrose to CHA-hemoglobin flask, stir to mix

Add 100 uL ATC and 25 uL NAT and stir to mix

Use sterile pipette, pour ~24 mL media per plate

Label plates and cover in foil

Calculations

	Stock Concentrations	Final Concentrations
kan	50 mg/mL	5 ug/mL
nat	100 mg/mL	5 ug/mL
ATc	500 ug/mL	100 ng/mL

(C₁V₁ = C₂V₂); Final volume: 500 mL

Sucrose Plates (500 mL total volume)

Sucrose | $\frac{(500 \text{ mL})(10\% \text{ sucrose})}{(50\% \text{ sucrose})} = 100 \text{ mL } 50\% \text{ sucrose}; 150 \text{ mL CHA}; 250 \text{ mL } 2\% \text{ hemoglobin}$

nat | $\frac{(500 \text{ mL})(5 \text{ ug/mL})}{(100,000 \text{ ug/mL})} = 0.025 \text{ mL} = 25 \text{ uL}$

ATc | $\frac{(500 \text{ mL})(100 \text{ ng/mL})}{(500,000 \text{ ng/mL})} = 0.1 \text{ mL} = 100 \text{ uL}$

12 CHA Plates + 10 CHA+Kan Plates (600 mL total)

nat | $600 \text{ mL} \times \frac{(25 \text{ uL})}{(500 \text{ mL})} = 30 \text{ uL}$

ATc | $600 \text{ mL} \times \frac{(100 \text{ uL})}{(500 \text{ mL})} = 120 \text{ uL}$

kan | $240 \text{ mL} \times \frac{(25 \text{ uL})}{(500 \text{ mL})} \times 2 = 24 \text{ uL}$

Thursday, September 9, 2018**To Do:**

- ~~1. Conduct sucrose selection on primary integrants~~

Results and Data:**Table 1:** Sucrose selection plate observations from the following Monday, 9/13/2021.

Dilution Plate	Observation/Colony Count Estimate
10^{-7} (Not sucrose)	Good, looks as would be expected; nice colonies
10^{-7} (Sucrose)	No colonies
10^{-6} (Sucrose)	Small colonies, <50 (to pick from this plate)
10^{-5} - 10^{-2} (Sucrose)	Small colonies, crowded; >50

Sucrose Selection

1. Scrape up small loop of cells and resuspend in 1X PBS
2. Dilute culture 1:10 in sterile PBS to 1×10^{-7}
3. Perform first dilution 1:100 so first dilution tube is 1×10^{-2} , adding 10 uL of resuspended cells to 990 ul 1x PBS
4. Perform subsequent dilutions 1:10, diluting 25 uL cells into 225 uL 1X PBS
5. Plate 100 uL of last dilution (1×10^{-7}) onto CHAH plates.
6. Plate 100 μ L of each dilution 10^{-2} – 10^{-7} on CHAH + 10% sucrose plates in duplicate
7. Incubate plates at 37°C for 4 days.

Monday, September 13, 2021.**To Do:**

- ~~2. Cross patch colonies from sucrose selection~~
- ~~3. Patch out biological replicant cells for Western Blot protocol~~

Cross Patch on ATc+nat and ATc+nat+kan Plates

1. Label two plates of ATc+nat and ATc+nat+kan each
2. Use stick to patch from dilution sucrose selection plate onto ATc+nat followed by ATc+nat+kan.
3. Do this for 16-20 colonies.

Patching Biological Replicants

1. From frozen stocks, use a stick to patch a heavy amount of replicants on half of a plate.

Tuesday, September 14, 2021**To Do:**

- ~~1. Prepare cells in the correct concentrations of ATc, checking ODs and attempt to start cultures at same OD~~
- ~~2. Pellet cells after growth and resuspend in 1x Sample Loading Buffer in preparation for next steps in Western Blot protocol~~

Results and Data:**Table 2: OD₆₀₀ readings of BR cultures during 9/14 experiment.**

Sample	Starting OD ₆₀₀	OD ₆₀₀ at ~4 hours	Final OD ₆₀₀ (4.66 hours)
1 – MHB; BR-1	- 0.007	0.026	0.038
2 – MHB; BR-2	0.048	0.237	0.292
3 - 0.5 ATc; BR-1	0.042	0.195	0.235
4 – 0.5 ATc; BR-2	0.043	0.256	0.318
5 - 1 ATc; BR-1	0.030	0.198	0.235
6 - 1 ATc; BR-2	0.044	0.270	0.322
7 - 5 ATc; BR-1	0.028	0.168	0.200
8 – 5 ATc; BR-2	0.033	0.198	0.238
9 - 50 ATc; BR-1	0.025	0.148	0.174
10 – 50 ATc; BR-2	0.001	0.054	0.074

Preparing Liquid Cultures of ATc Titrations for Western Blot Protocol**Experimental Concentrations:**

- 50 ng/mL; 5 ng/mL; 1 ng/mL; 0.5 ng/mL; 0 ng/mL
1. Create dilutions of 500 ug/mL ATc stock by adding 5 uL of stock to 45 uL of MHB to get 50 ng/mL and making two 10-fold dilutions to get 5 and 0.5 ng/mL. From the 5 ng/mL dilution take 10 uL and add to 40 uL of MHB to create the 1 ng/mL dilution tube.
 2. Add 12 uL of each dilution into 5 mL of MHB each in 10 labelled glass tubes.
 3. Take patch plates of biological replicant **pkrm ΔRPU cells and resuspend, with loop, in 0.5 mL of MHB**
 4. **Use 50 uL diluted in 950 uL MHB to check OD₆₀₀ of each biological replicant.**
 5. Calculate the volume of sample to add to each concentration using the OD₆₀₀ (see [Calculations](#)),

6. Add tubes to 37°C shaker for ~4 hours (ideally reaching OD₆₀₀ = 0.3-0.4)
7. Check OD's then take 1 mL of culture and place in centrifuge tubes, then spin in mini centrifuge at 15,000 rpm for 3 minutes
8. Take off supernatant and resuspend in 1 OD per 1 mL 1x Sample Loading Buffer.
9. Boil in 95°C heat block for 5-10 minutes, then place in -20°C freezer.

Calculations

	Stock Concentration	Final Concentration
BR-1	3.04 OD ₆₀₀	0.05 OD ₆₀₀
BR-2	2.76 OD ₆₀₀	0.05 OD ₆₀₀

(C1V1 = C2V2); Final volume: 5 mL

Volume of Resuspended BR Cells

$$\text{BR-1} \mid \frac{(5000 \text{ uL})(0.05 \text{ OD})}{(0.152 \text{ OD}) \times 20} = 82.24 \text{ uL}$$

$$\text{BR-2} \mid \frac{(5000 \text{ uL})(0.05 \text{ OD})}{(0.138 \text{ OD}) \times 20} = 90.57 \text{ uL}$$

Wednesday, September 15, 2021

To Do:

1. ~~Western Gel and transfer~~
2. ~~Protein quantification~~

Results and Data:

Table 3: 9/15 Gel key; correlates with Table 2 numbering system.

Western Blot Gel Loading Diagram														
BioRad Ladder	1x slb	MHB 1	MHB 2	MHB 3	MHB 4	MHB 5	MHB 6	MHB 7	MHB 8	MHB 9	MHB 10	1x slb	1x slb	Prestained Ladder

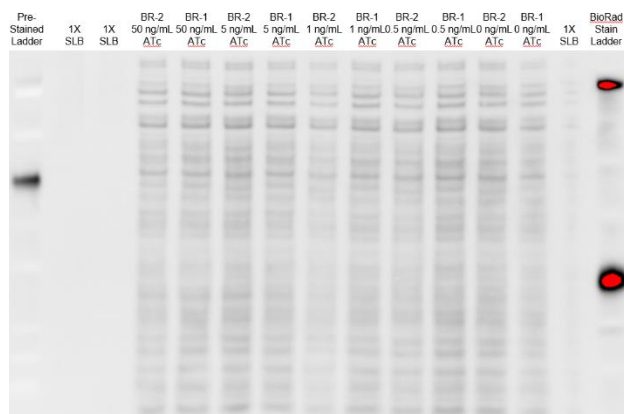


Figure 1: Total Protein Quantification of 9/15 gel.

Running the Gel

1. Heat samples after thawing for an additional 5-10 minutes.
2. Assemble gel chamber
 - Use 15-well pre-cast NuPAGE 4-12% Bis-Tris gel
3. Adding Running Buffer to gel chamber (front and back)
4. Use pipet to wash wells of gel
5. Load 7 uL of each sample
6. Use 2 uL of 1:10 diluted BioRad Precision Plus Dual Color Protein Ladder (#161-0374) for the ladder.
9. Run at 144V for 45 minutes.

Wet Transfer

1. Make transfer buffer and store in freezer to chill until the gel has stopped running.
2. Grab a bucket full of ice from the third floor ice room, then proceed to set up for the transfer.
 - a. Grab the Pyrex dish and materials from under the cabinet near Hannah's desk and cut filter paper and membrane.
 - i. Make sure to use tweezers and scissors that have been sprayed with ethanol and wiped clean when handling the membrane
 - ii. Membrane has two pieces of paper on either side, be careful
3. Activate PVDF membrane (must use Millipore Immobilon-FL #IPFL00010) in ethanol from chemical storage cabinet
4. Remove transfer buffer from freezer and add 0.5 mL NuPAGE antioxidant
5. Presoak three sponges and filter paper in Pyrex dish with transfer buffer; roll air bubbles out of sponges, then presoak membrane in transfer buffer
6. Open gel case, cut off wells and at the bottom ridge on the gel and place wet sheet of filter paper on the gel.
7. Peel gel and filter paper off and place wet membrane on gel.
8. Place other filter paper on membrane and roll out bubbles
9. Dunk the transfer cassette halves in the transfer buffer and begin assembling sandwich:
 - a. Thickest sponge
 - b. Filter/Membrane/Gel/Filter sandwich so that **the membrane is on top of the gel**
 - c. 2 more sponges
10. Close transfer apparatus and clamp into the gel box.
11. Place the gel rig into a large ice bucket. Fill the gel chamber with transfer buffer. Close the lid and pack ice around the sides of the active gel chamber.
12. Run at 21V for 1 hour.

Total Protein Quantification

1. Prepare No-Stain labeling buffer by diluting 20x stock to 1x (9.5mL ddiH₂O+0.5mL 20x buffer).
2. Thaw Activator and Derivatizer to room temperature, then add 20 uL of activator followed by 20 uL of derivatizer to 10 mL of 1X buffer. Mix.
3. Pre-wash the blot twice with water, for 2 minutes each, on a nutator.
4. Add 10 mL of No-stain labeling solution to the blot, and nutate for 10 minutes.
5. Post-wash with water for 2 minutes, three times.

6. Image using the Bio-Rad imager with protocol Blot->No Stain.
7. Quantify using LiCor Image Studio Lite.

Blocking and Probing

1. Block membrane with Li-Cor Odyssey Blocking Buffer (PBS) diluted 1:5 in PBS overnight.

Reagents

1X Loading Buffer

Mix together:

- 250 uL NuPage LDS sample buffer (4x, room temp)
- 100 uL 0.5 M DTT (-20C)
- 650 uL dH₂O

Running Buffer (400 mL | 1 gel)

Mix together:

- 1 x MOPS for large proteins
- 1x MES for <50 kD proteins
 - 380 mL ddiH₂O
 - 20 mL 20x MES
 - 1 mL NuPAGE antioxidant

Transfer Buffer (500 mL | 1 gel)

Mix together:

- 50 mL methanol
- 25 mL NuPAGE 20x transfer buffer
- Water to 500 mL

Thursday, September 16, 2021

To Do:

1. ~~Antibody Incubation and Membrane Washes~~
2. ~~Imaging~~

Results and Data:

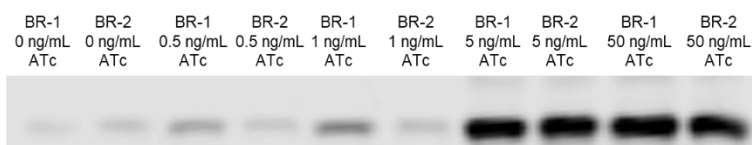


Figure 2: Western Blot of MHB ATc titrations.

Table 4: Normalized Total Protein for MHB ATc titration.

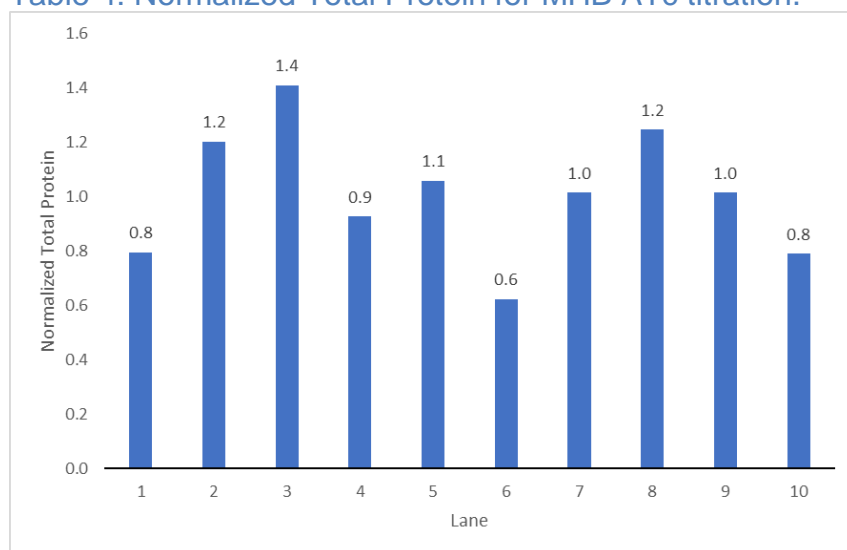
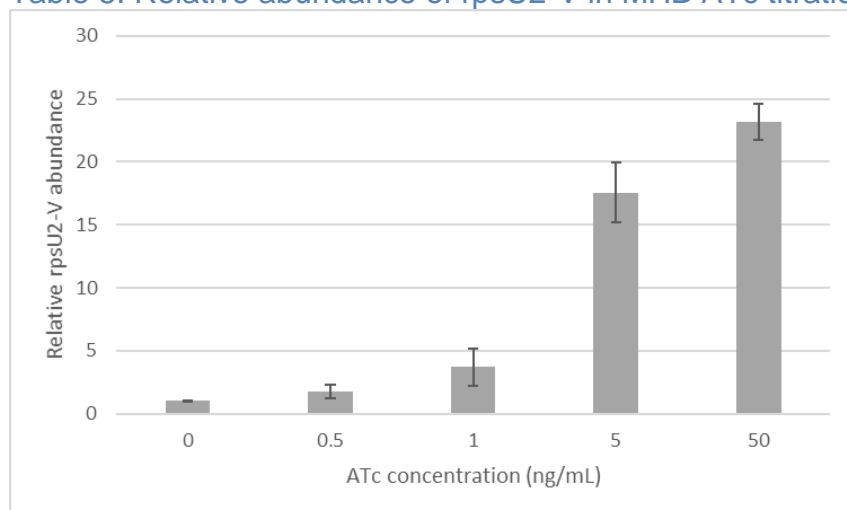


Table 5: Relative abundance of rpsU2-V in MHB ATc titration.



Antibody Incubation and Membrane Washes

1. Add antibody (anti-VSVG) in 15 mL of blocking buffer. Rotate for 1 hour at room temperature.
2. Prepare 1X Wash Buffer
3. Wash (w/detergent) 4x on rotator for 10 minutes each..
4. Use diluted blocking buffer and block again, for 20 min.
5. Transfer membrane to black box and add 1 uL antibody (anti-rabbit) to 10 mL wash buffer (w/detergent). **Also add 0.01% SDS to the wash buffer (10 uL of 10% SDS).**
6. Label for 1 hour on rocker at room temperature.
7. Wash 4x on rotator for 10 minutes each.
8. Wash 2x on rotator for 10 minutes each with wash buffer WITHOUT detergent.
9. If faint bands are expected, do a quick methanol rinse by dunking the blot in methanol for 2 seconds then letting air dry for 2 minutes prior to imaging.

Imaging

1. Leave the membrane in the box containing the final wash buffer. Bring the box, gloves, forceps, and a timer to the imager in the INBRE facility.
2. Open the Li-Cor and cleaned the surface of the scanning apparatus.
3. Place membrane face down in the bottom left hand corner. Note the size of the membrane using the grid (usually about 8x8 or smaller).
4. Set Image Quality to High and press 'Start'
5. Adjust the brightness and contrast on each channel until image appears the best. Using the black bands on white background setting is typically best.
6. Save image as Image Work File and export as Tiff
7. Remove your membrane and spray down the glass surface of the scanner with ethanol and kimwipes (provided there).
8. For quantification, click on Analyze. Either use "Add Rectangle" or "Draw Rectangle" features to instantly quantify the density of each band.

Reagents

1X Wash Buffer (Scaled down to 130 mL)

Mix together:

- 13 mL 10X PBS
- 117 mL dH2O

Separate into two graduated cylinders, 100 mL and 30 mL. To the 100 mL graduate cylinder add 0.52 mL of Sufact-Amps (Stored in 4° fridge)

Monday, September 20, 2021**To Do:**

- ~~1. Patch 4 single colonies onto 4 separate plates.~~
- ~~2. Make ATc plates with varying concentrations (2 per concentration)~~

Results and Data:

N/A.

Pouring ATc Plates with Varying Concentrations

Follow CHAH recipe for 600 mL

1. Weigh out 30.6g of cystine heart agar into 1L flask (non-baffled; 10.2g/100mL)
2. Add 300mL of ddiH₂O (type I)
3. Add stirbar to flask
4. Heat on low, stirring, for about 10 minutes (media should be totally dissolved)
5. Autoclave on 30' liquid cycle, filling the water bin up to the height of the media
6. Cool down (ideally to ~55C)
7. Separately (before), prepare hemoglobin 2% solution
8. Add 6g freeze-dried hemoglobin to 300mL of ddiH₂O (type I)
9. Autoclave on 20' liquid cycle with water in the bin
10. Cool down (ideally to ~55C)
11. Using sterile technique, pour hemoglobin into CHA
12. Using a 50mL pipet, aliquot 24mL of CHAH mixture into each 100mm plate (should make approximately 25 plates) Try to avoid bubbles!

Divide into four portions of 100 mL + leftover.

Using serial dilutions of ATc create plates with 0.05, 1.0, 5.0, and 50.0 ng/mL ATc. Additionally add NAT.

Tuesday, September 21, 2021**To Do:**

- ~~1. Make single use aliquots of biological replicants~~
- ~~2. Patch out double mutants onto ATc plates~~

Results and Data:

N/A

Making Single Use Aliquots

1. Prepare 2.4mL of MHB in a 50mL conical (adjust if you are also making single use stocks)
2. Take at least half of a thickly spread plate and add cells to the MHB tube
3. Resuspend until there are no clumps in the MHB
4. Add 600ul of 75% glycerol to the 2.4mL mix by pipetting
5. Aliquot 1mL per cryovial, freeze at -80
6. For single use stocks follow the same protocol but pipet 50ul of solution to tubes

Wednesday, September 22, 2021**To Do:**

- ~~1. Prepare CHA ATc Induction Titration Plates for Gel and Transfer~~

Results and Data:

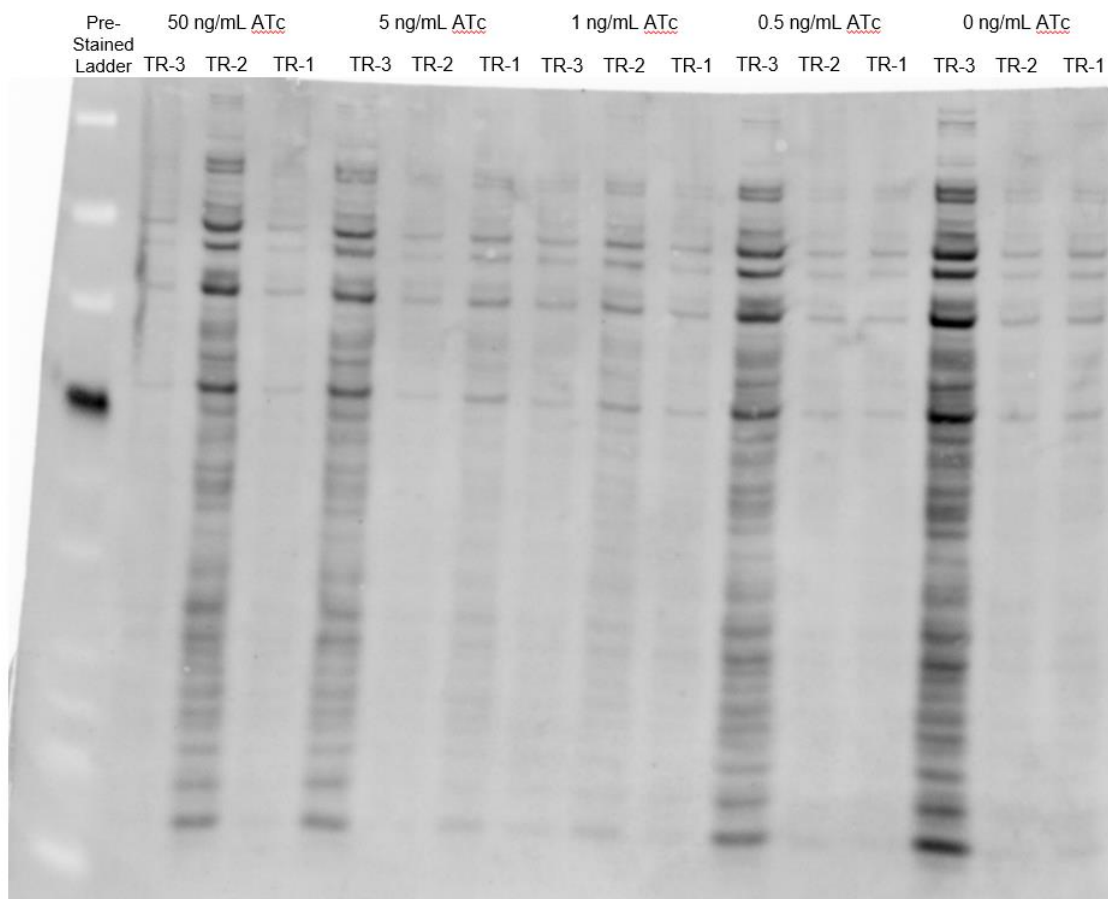
N/A

Prepare CHA ATc Induction Titration Plates

1. Scrape up each plate and resuspend in individual tubes of MHB.
2. Check OD's, and calculate the actual OD for each
3. Centrifuge at max for 1' and resuspend in 1 OD: 1 uL 1X SLB.
4. Place on 95°C heat block for 10-15 minutes
5. Store in -20°C freezer

Thursday, September 23, 2021**To Do:**

- ~~1. Gel and transfer of CHA ATc Induction Plates~~

Results and Data:

Running the Gel

1. Heat samples after thawing for an additional 5-10 minutes.
2. Assemble gel chamber
 - a. Use 15-well pre-cast NuPAGE 4-12% Bis-Tris gel
3. Adding Running Buffer to gel chamber (front and back)
4. Use pipet to wash wells of gel
5. Load 7 uL of each sample
6. Use 2 uL of 1:10 diluted BioRad Precision Plus Dual Color Protein Ladder (#161-0374) for the ladder.
10. Run at 144V for 45 minutes.

Wet Transfer

1. Make transfer buffer and store in freezer to chill until the gel has stopped running.
2. Grab a bucket full of ice from the third floor ice room, then proceed to set up for the transfer.
 - a. Grab the Pyrex dish and materials from under the cabinet near Hannah's desk and cut filter paper and membrane.
 - i. Make sure to use tweezers and scissors that have been sprayed with ethanol and wiped clean when handling the membrane
 - ii. Membrane has two pieces of paper on either side, be careful
3. Activate PVDF membrane (must use Millipore Immobilon-FL #IPFL00010) in ethanol from chemical storage cabinet
4. Remove transfer buffer from freezer and add 0.5 mL NuPAGE antioxidant
5. Presoak three sponges and filter paper in Pyrex dish with transfer buffer; roll air bubbles out of sponges, then presoak membrane in transfer buffer
6. Open gel case, cut off wells and at the bottom ridge on the gel and place wet sheet of filter paper on the gel.
7. Peel gel and filter paper off and place wet membrane on gel.
8. Place other filter paper on membrane and roll out bubbles
9. Dunk the transfer cassette halves in the transfer buffer and begin assembling sandwich:
 - a. Thickest sponge
 - b. Filter/Membrane/Gel/Filter sandwich so that **the membrane is on top of the gel**
 - c. 2 more sponges
10. Close transfer apparatus and clamp into the gel box.
11. Place the gel rig into a large ice bucket. Fill the gel chamber with transfer buffer. Close the lid and pack ice around the sides of the active gel chamber.
12. Run at 21V for 1 hour.

Total Protein Quantification

1. Prepare No-Stain labeling buffer by diluting 20x stock to 1x (9.5mL ddiH₂O+0.5mL 20x buffer).
2. Thaw Activator and Derivatizer to room temperature, then add 20 uL of activator followed by 20 uL of derivatizer to 10 mL of 1X buffer. Mix.
3. Pre-wash the blot twice with water, for 2 minutes each, on a nutator.
4. Add 10 mL of No-stain labeling solution to the blot, and nutate for 10 minutes.
5. Post-wash with water for 2 minutes, three times.
6. Image using the Bio-Rad imager with protocol Blot->No Stain.

7. Quantify using LiCor Image Studio Lite.

Blocking and Probing

1. Block membrane with Li-Cor Odyssey Blocking Buffer (PBS) diluted 1:5 in PBS overnight.

Reagents

1X Loading Buffer

Mix together:

- 250 uL NuPage LDS sample buffer (4x, room temp)
- 100 uL 0.5 M DTT (-20C)
- 650 uL dH₂O

Running Buffer (400 mL | 1 gel)

Mix together:

- a. 1 x MOPS for large proteins
- b. 1x MES for <50 kD proteins
 - 380 mL ddiH₂O
 - 20 mL 20x MES
 - 1 mL NuPAGE antioxidant

Transfer Buffer (500 mL | 1 gel)

Mix together:

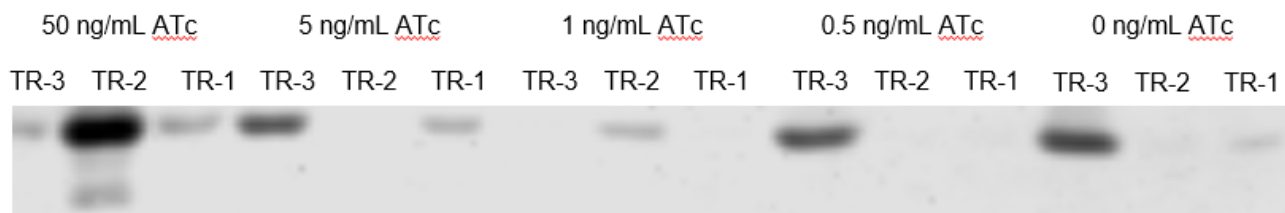
- c. 50 mL methanol
- d. 25 mL NuPAGE 20x transfer buffer
- e. Water to 500 mL

Friday, September 24, 2021

To Do:

1. ~~Blocking and probing pt. 2 of double mutant ATc titration on CHA plates~~
2. ~~Imaging blot~~

Results and Data:



Antibody Incubation and Membrane Washes

1. Add antibody (anti-VSVG) in 15 mL of blocking buffer. Rotate for 1 hour at room temperature.
2. Prepare 1X Wash Buffer
3. Wash (w/detergent) 4x on rotator for 10 minutes each..
4. Use diluted blocking buffer and block again, for 20 min.

5. Transfer membrane to black box and add 1 μ L antibody (anti-rabbit) to 10 mL wash buffer (w/detergent). **Also add 0.01% SDS to the wash buffer (10 μ L of 10% SDS).**
6. Label for 1 hour on rocker at room temperature.
7. Wash 4x on rotator for 10 minutes each.
8. Wash 2x on rotator for 10 minutes each with wash buffer WITHOUT detergent.
9. If faint bands are expected, do a quick methanol rinse by dunking the blot in methanol for 2 seconds then letting air dry for 2 minutes prior to imaging.

Imaging

1. Leave the membrane in the box containing the final wash buffer. Bring the box, gloves, forceps, and a timer to the imager in the INBRE facility.
2. Open the Li-Cor and cleaned the surface of the scanning apparatus.
3. Place membrane face down in the bottom left hand corner. Note the size of the membrane using the grid (usually about 8x8 or smaller).
4. Set Image Quality to High and press 'Start'
5. Adjust the brightness and contrast on each channel until image appears the best. Using the black bands on white background setting is typically best.
6. Save image as Image Work File and export as Tiff
7. Remove your membrane and spray down the glass surface of the scanner with ethanol and kimwipes (provided there).
8. For quantification, click on Analyze. Either use "Add Rectangle" or "Draw Rectangle" features to instantly quantify the density of each band.

Reagents

1X Wash Buffer (Scaled down to 130 mL)

Mix together:

- 13 mL 10X PBS
- 117 mL dH₂O

Separate into two graduated cylinders, 100 mL and 30 mL. To the 100 mL graduate cylinder add 0.52 mL of Sufact-Amps (Stored in 4° fridge)

Tuesday, September 28, 2021**To Do:**

- ~~1. Patch out double mutants onto ATc plates~~

Results and Data:

N/A

Wednesday, September 29, 2021**To Do:**

- ~~1. Prepare CHA ATc Induction Titration Plates for Gel and Transfer~~

Results and Data:

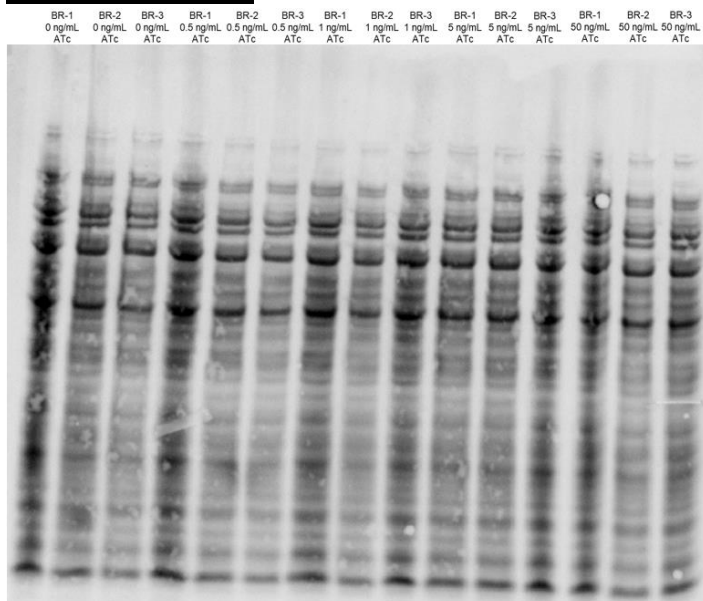
N/A

Prepare CHA ATc Induction Titration Plates

6. Scrape up each plate and resuspend in individual tubes of MHB.
7. Check OD's, and calculate the actual OD for each
8. Centrifuge at max for 1' and resuspend in 1 OD: 1 uL 1X SLB.
9. Place on 95°C heat block for 10-15 minutes
10. Store in -20°C freezer

Thursday, September 30, 2021**To Do:**

- ~~1. Gel and transfer~~
- ~~2. Total Protein Quantification~~

Results and Data:

Running the Gel

1. Heat samples after thawing for an additional 5-10 minutes.
2. Assemble gel chamber
 - a. Use 15-well pre-cast NuPAGE 4-12% Bis-Tris gel
3. Adding Running Buffer to gel chamber (front and back)
4. Use pipet to wash wells of gel
5. Load 7 μ L of each sample
6. Use 2 μ L of 1:10 diluted BioRad Precision Plus Dual Color Protein Ladder (#161-0374) for the ladder.
11. Run at 144V for 45 minutes.

Wet Transfer

1. Make transfer buffer and store in freezer to chill until the gel has stopped running.
2. Grab a bucket full of ice from the third floor ice room, then proceed to set up for the transfer.
 - a. Grab the Pyrex dish and materials from under the cabinet near Hannah's desk and cut filter paper and membrane.
 - i. Make sure to use tweezers and scissors that have been sprayed with ethanol and wiped clean when handling the membrane
 - ii. Membrane has two pieces of paper on either side, be careful
3. Activate PVDF membrane (must use Millipore Immobilon-FL #IPFL00010) in ethanol from chemical storage cabinet
4. Remove transfer buffer from freezer and add 0.5 mL NuPAGE antioxidant
5. Presoak three sponges and filter paper in Pyrex dish with transfer buffer; roll air bubbles out of sponges, then presoak membrane in transfer buffer
6. Open gel case, cut off wells and at the bottom ridge on the gel and place wet sheet of filter paper on the gel.
7. Peel gel and filter paper off and place wet membrane on gel.
8. Place other filter paper on membrane and roll out bubbles
9. Dunk the transfer cassette halves in the transfer buffer and begin assembling sandwich:
 - a. Thickest sponge
 - b. Filter/Membrane/Gel/Filter sandwich so that **the membrane is on top of the gel**
 - c. 2 more sponges
10. Close transfer apparatus and clamp into the gel box.
11. Place the gel rig into a large ice bucket. Fill the gel chamber with transfer buffer. Close the lid and pack ice around the sides of the active gel chamber.
12. Run at 21V for 1 hour.

Total Protein Quantification

1. Prepare No-Stain labeling buffer by diluting 20x stock to 1x (9.5mL ddiH₂O+0.5mL 20x buffer).
2. Thaw Activator and Derivatizer to room temperature, then add 20 μ L of activator followed by 20 μ L of derivatizer to 10 mL of 1X buffer. Mix.
3. Pre-wash the blot twice with water, for 2 minutes each, on a nutator.
4. Add 10 mL of No-stain labeling solution to the blot, and nutate for 10 minutes.
5. Post-wash with water for 2 minutes, three times.
6. Image using the Bio-Rad imager with protocol Blot->No Stain.

7. Quantify using LiCor Image Studio Lite.

Blocking and Probing

1. Block membrane with Li-Cor Odyssey Blocking Buffer (PBS) diluted 1:5 in PBS overnight.

Reagents

1X Loading Buffer

Mix together:

- 250 uL NuPage LDS sample buffer (4x, room temp)
- 100 uL 0.5 M DTT (-20C)
- 650 uL dH₂O

Running Buffer (400 mL | 1 gel)

Mix together:

- a. 1 x MOPS for large proteins
- b. 1x MES for <50 kD proteins
 - 380 mL ddiH₂O
 - 20 mL 20x MES
 - 1 mL NuPAGE antioxidant

Transfer Buffer (500 mL | 1 gel)

Mix together:

- c. 50 mL methanol
- d. 25 mL NuPAGE 20x transfer buffer
- e. Water to 500 mL

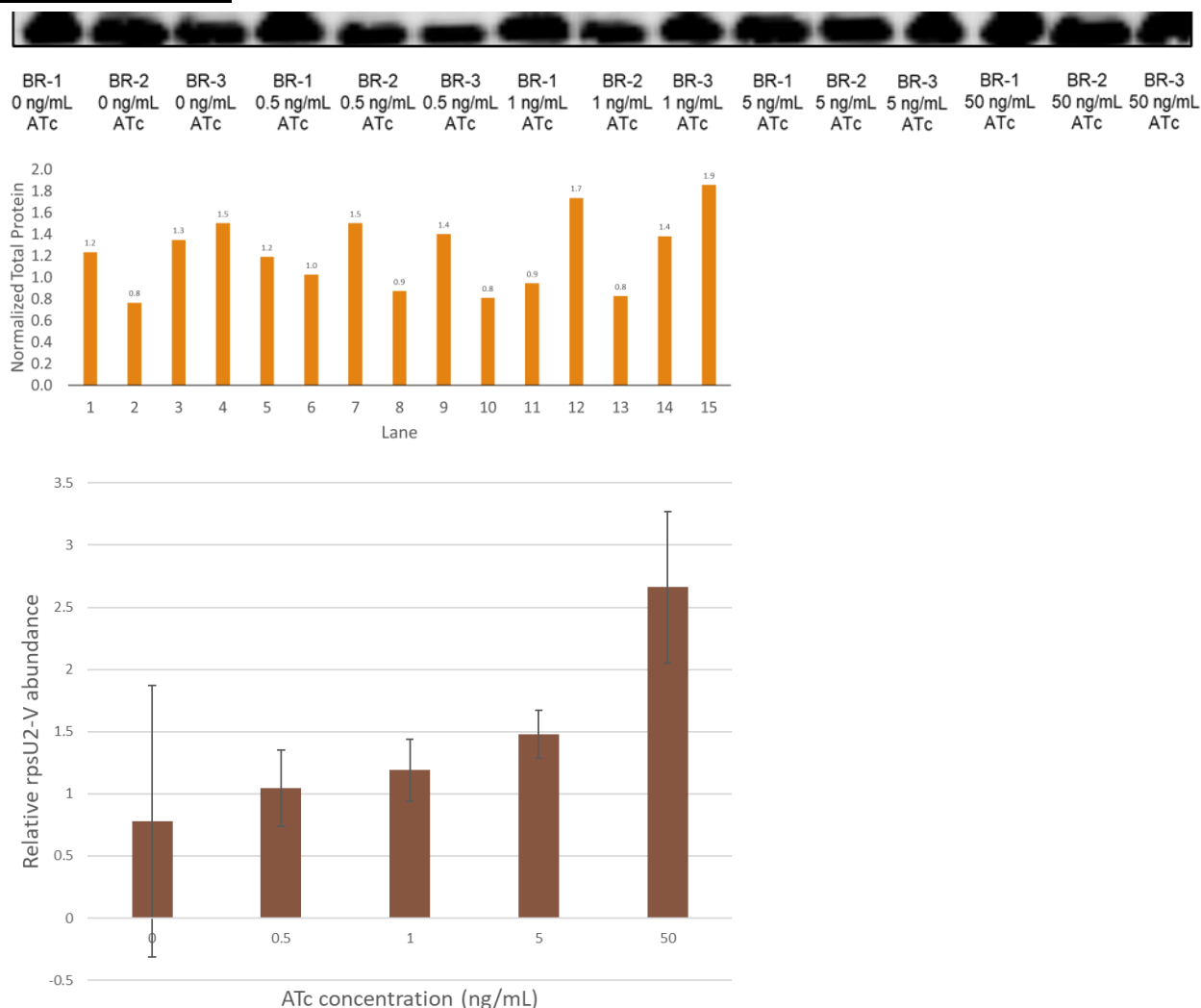
October 2021

Friday, October 1, 2021

To Do:

1. ~~Blocking and probing pt. 2 of CHA ATc Induction Titration on CHA~~
2. ~~Image blot~~

Results and Data:



Antibody Incubation and Membrane Washes

1. Add antibody (anti-VSVG) in 15 mL of blocking buffer. Rotate for 1 hour at room temperature.
2. Prepare 1X Wash Buffer
3. Wash (w/detergent) 4x on rotator for 10 minutes each..
4. Use diluted blocking buffer and block again, for 20 min.
5. Transfer membrane to black box and add 1 μ L antibody (anti-rabbit) to 10 mL wash buffer (w/detergent). **Also add 0.01% SDS to the wash buffer (10 μ L of 10% SDS).**
6. Label for 1 hour on rocker at room temperature.

7. Wash 4x on rotator for 10 minutes each.
8. Wash 2x on rotator for 10 minutes each with wash buffer WITHOUT detergent.
9. If faint bands are expected, do a quick methanol rinse by dunking the blot in methanol for 2 seconds then letting air dry for 2 minutes prior to imaging.

Imaging

1. Leave the membrane in the box containing the final wash buffer. Bring the box, gloves, forceps, and a timer to the imager in the INBRE facility.
2. Open the Li-Cor and cleaned the surface of the scanning apparatus.
3. Place membrane face down in the bottom left hand corner. Note the size of the membrane using the grid (usually about 8x8 or smaller).
4. Set Image Quality to High and press 'Start'
5. Adjust the brightness and contrast on each channel until image appears the best. Using the black bands on white background setting is typically best.
6. Save image as Image Work File and export as Tiff
7. Remove your membrane and spray down the glass surface of the scanner with ethanol and kimwipes (provided there).
8. For quantification, click on Analyze. Either use "Add Rectangle" or "Draw Rectangle" features to instantly quantify the density of each band.

Reagents

1X Wash Buffer (Scaled down to 130 mL)

Mix together:

- 13 mL 10X PBS
- 117 mL dH₂O

Separate into two graduated cylinders, 100 mL and 30 mL. To the 100 mL graduate cylinder add 0.52 mL of Sufact-Amps (Stored in 4° fridge)

Bibliography

Ramsey, K. M. and Dove, S. L. (2016) ' A response regulator promotes *Francisella tularensis* intramacrophage growth by repressing an anti-virulence factor ', *Molecular Microbiology*. doi: 10.1111/mmi.13418.