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## January 2022

### Monday, January 3, 2022

#### To Do:

- ~~1. Dilute and sterile filter Iron~~
- ~~2. Supplement MHA and pour plates~~
- ~~3. Pour CHAH+Nat Plates~~

**Note.** Hannah patched out LVS and KR LVS 141 on CHAH for tomorrow.

#### Reagents

Mueller Hinton Agar (500 mL; 1% glucose, 0.0125% FePPi, and 2% IsoVitaleX)

To a 1 L flask add:

10.5 g MHB  
6 g agar  
465 mL ddiH<sub>2</sub>O

Autoclave on the 30 minute liquid cycle and then add:

25 mL 20% glucose  
10 mL IsoVitaleX  
5 mL 2.5% FePPi

Pour.

CHAH Agar with Nat (Half sleeve)

Mix 15.3 g cystine heart agar (lab mix) with 150 mL type I ddiH<sub>2</sub>O; add stir bar and let stir on a 90°C hot plate for 10 minutes.

Add flask to Instant Pot and run 30'

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, add 150 mL hemoglobin into CHA flask, stir to mix

Add 15uL of Nat, and stir to mix. Pour.

Label plates and cover in foil

2.5% Iron pyrophosphate

1.25g iron pyrophosphate (cabinet) in 50mL of ddiH<sub>2</sub>O (type 1), dissolved overnight, fresh solution every 2 weeks

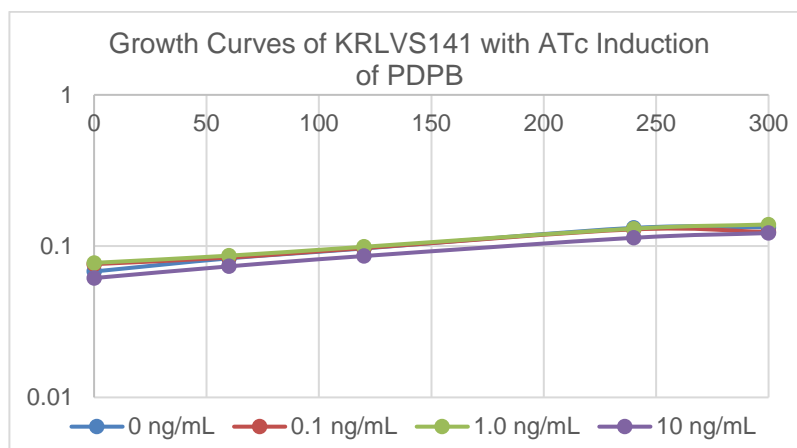
### Tuesday, January 4, 2022

#### To Do:

- ~~1. Disk Diffusion Assay~~
- ~~2. Grow KR LVS 141 cells at increasing ATc concentrations and prepare for Gel and Transfer~~
- ~~3. Create freezer stocks and single use aliquots of KRLVS141 and LVS~~

#### Results and Data:

Treatment	Gen. Time (min)
0 ng/mL	312.03
0.1 ng/mL	427.59
1.0 ng/mL	358.31
10 ng/mL	305.59



### Disk Diffusion Assay

1. Day before experiment, patch cells on CHAH plates and incubate overnight.
2. Resuspend LVS cells in 400  $\mu$ L of MHB. Measure the OD<sub>600</sub>.
3. Dilute cells in centrifuge tubes to an OD<sub>600</sub> of 0.01, 0.02, and 0.1, with a final volume of 1 mL.
4. Add 100  $\mu$ L each of LVS cells at OD<sub>600</sub> 0.01, 0.02, 0.1 to 2 MHA plates each and spread with a spreader. Allow plates to dry.
5. Using sterile tweezers, add filter paper disks to a plate, adding 20  $\mu$ L ddiH<sub>2</sub>O Type 1 or Kasugamycin (Ksg), as appropriate. Allow these disks to dry for 15 minutes.
6. Using sterile tweezers, transfer disks from sterile plate to MHA plates that have been spread with *Francisella* so that there is a water disk and a Ksg disk on each.

### ATc Titration Induction of PDPB and BS21

#### Experimental Concentrations:

1. 10 ng/mL; 1 ng/mL; 0.1 ng/mL; 0 ng/mL
1. Create dilutions (1:100 > 1:10 > 1:10) of 500  $\mu$ g/mL ATc stock in MHB.
2. Add 14  $\mu$ L of the appropriate dilution (1:100 for 10 ng/mL ATc, etc.) into 7 mL of MHB each in 8 labelled glass tubes.
3. Take patch plates of KR LVS 141 cells and resuspend, with loop, in 0.5 mL of MHB
4. Use 50  $\mu$ L diluted in 950  $\mu$ L MHB to check OD<sub>600</sub> of each biological replicant.
5. Calculate the volume of sample to add to each tube for an OD<sub>600</sub> of 0.08
6. Add tubes to 37°C shaker for ~4.5 hours (ideally reaching OD<sub>600</sub> = 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.

Boil in 95°C heat block for 5-10 minutes, then place in -20°C freezer.

**Note.** Maybe check if there's a difference in protein production when using Nat or not in liquid media and maybe try vertexing resuspension before each addition to the tubes, lesser OD at 8?

Sample Number Key							
1	2	3	4	5	6	7	8
TR-1, 0 ng/mL ATc	TR-2, 0 ng/mL ATc	TR-1, 0.1 ng/mL ATc	TR-2, 0.1 ng/mL ATc	TR-1, 1 ng/mL ATc	TR-2, 1 ng/mL ATc	TR-1, 10 ng/mL ATc	TR-2, 10 ng/mL ATc

### Growth Curve

1. The day before, patch out KRLVS141 cells onto CHAH + Nat plates.
2. Resuspend cells in 500 uL of MHB and measure OD<sub>600</sub>, normalizing to an OD of 0.08 in 7mL of MHB (see above for more complete description of tube preparation.)
3. Take 0.5 mL aliquot and measure OD at time 0, followed by subsequent timepoints of 1, 2, and 4 hours, while the previous experiment is running.
  - \* Reduce to 0.25 mL aliquots if tubes begin to run low, should be no lower than 4 mL.
4. Take additional timepoints/final timepoint as the previous experiment runs.

KRLVS141 Growth Curve Data with ATc Induction					
Sample	OD <sub>600</sub> at t=0	OD <sub>600</sub> at t=1	OD <sub>600</sub> at t=2	OD <sub>600</sub> at t=4	OD <sub>600</sub> at t=5
1	0.068	0.088	0.101	0.147	0.150
2	0.068	0.078	0.092	0.117	0.116
3	0.072	0.081	0.095	0.120	0.120
4	0.08	0.087	0.099	0.138	0.128
5	0.073	0.086	0.098	0.125	0.128
6	0.082	0.087	0.100	0.136	0.150
7	0.072	0.087	0.101	0.129	0.154
8	0.051	0.060	0.071	0.098	0.090

### Making Glycerol Stocks

1. Make 2 cryovials for each strain (permanent stocks), label!
2. Prepare 2.4mL of MHB in a 50mL conical
3. Take at least half of a thickly spread plate and add cells to the MHB tube
4. Resuspend until there are no clumps in the MHB
5. Add 600ul of 75% glycerol to the 2.4mL, mix by pipetting
6. Aliquot 1mL per cryovial, freeze at -80
7. Pipet 50ul each of remaining 1mL to centrifuge tubes, freeze at -80

**Wednesday, January 5, 2022**

#### To Do:

- ~~1. Observe Disk Diffusion Assay (shouldn't be ready)~~
- ~~2. Run Gel and Transfer of KRLVS141~~

### Running Gel on ATc Titration Induction of PDPB and BS21 Samples

1. Heat samples for an additional 10 minutes.
2. Assemble gel chamber with 15-well pre-cast NuPAGE 4-12% Bis-Tris gel
3. Add Running Buffer to gel chamber (front and back)
4. Use 200 uL pipet set to 200 to wash wells of gel
  - \* Used 20 uL pipet set to 12 uL for three washes though— smeary ?
5. Loaded 9 uL of each sample
6. Used 5 uL of Western Sure ladder
9. Ran at 144-146V for 40-45 minutes.

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13	Lane 14	Lane 15
SLB	WSL	1	2	3	4	5	6	7	8	SLB	SLB	SLB	SLB	SLB

## 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, 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 20V for 1 hour.

**Note.** Did not image, cut off PDPB band on accident and will re-run the gel and transfer tomorrow. 😞

## Reagents

### 1X Sample Loading Buffer

Mix together:

- 250 uL NuPAGE LDS sample buffer (4x, room temp)
- 100 uL 0.5 M DTT (-20C)
- 650 uL dH<sub>2</sub>O

### Running Buffer (400 mL | 1 gel)

Mix together:

- 1 x MOPS for large proteins
- 1x MES for <50 kD proteins
  - 380 mL ddiH<sub>2</sub>O
  - 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, January 6, 2022**

To Do:

- ~~1. Observe Disk Diffusion Assay (shouldn't be ready)~~
- ~~2. Redo Gel and Transfer of KRLVS 141~~

## **Results and Data:**

**DDA Observations:** OD of: 0.1 has moderate growth, but is still too light for image, 0.02 has some growth but not much, 0.01 has no visible growth. On the OD of 0.1 and 0.02 plates, it does appear that there is sensitivity to Kasugamycin and no sensitivity to the background (water).

————— KRLVS141 —————  
Ladder 0 ng/mL Atc 0.1 ng/mL Atc 1 ng/mL Atc 10 ng/mL Atc

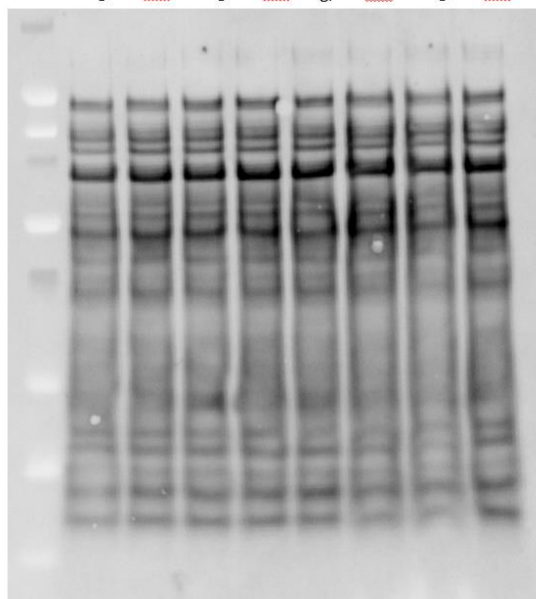


Fig. 1: Total Protein Quantification of KRLVS141 cells grown at 0, 0.1, 1.0, and 10 ng/mL Atc; in technical duplicate.

## **Running Gel on Atc Titration Induction of PDPB Samples**

1. Heat samples for an additional 10 minutes.
2. Assemble gel chamber with 15-well pre-cast NuPAGE 4-12% Bis-Tris gel
3. Add Running Buffer to gel chamber (front and back)
4. Use 200 uL pipet set to 200 to wash wells of gel
  - a. Used 20 uL pipet set to 12 uL for three washes though— smeary ?
5. Loaded 9 uL of each sample
6. Used 5 uL of Western Sure ladder
7. Ran at 144-146V for 40-45 minutes.

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13	Lane 14	Lane 15
SLB	WSL	1	2	3	4	5	6	7	8	SLB	SLB	SLB	SLB	SLB



## 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, 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 20V for 1 hour.

## Total Protein Quantification

1. Prepare No-Stain labeling buffer by diluting 20x stock to 1x (9.5mL ddiH<sub>2</sub>O+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
  - \* In fridge, for potential weekend storage

## Reagents

### 1X Sample Loading Buffer

Mix together:

- 250 uL NuPage LDS sample buffer (4x, room temp)
- 100 uL 0.5 M DTT (-20C)
- 650 uL dH<sub>2</sub>O

## Running Buffer (400 mL | 1 gel)

Mix together:

- 1 x MOPS for large proteins
- 1x MES for <50 kD proteins
  - 380 mL ddiH<sub>2</sub>O
  - 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

## No-Stain Labelling Buffer (10 mL)

Mix together:

- 9.5mL ddiH<sub>2</sub>O
- 0.5mL 20x No-Stain Labelling buffer
- 20 uL Activator followed by 20 uL Derivatizer

**Monday, January 10, 2022**

**To Do:**

1. ~~Blocking and Probing~~
2. ~~Imaging and Analysis of Blots~~
3. ~~Imaging of DDA~~
4. ~~Patch out KRLVS141?~~

**Results and Data:**

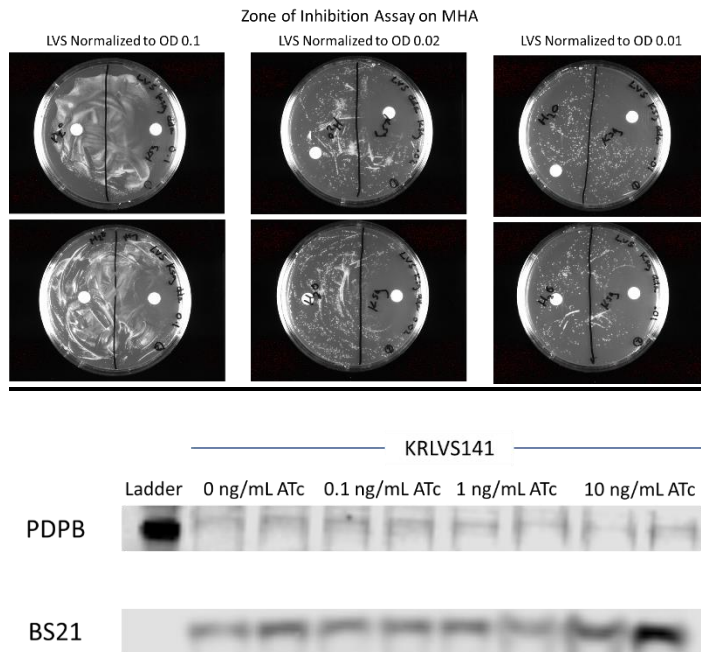


Figure 1: Blot of BS21-2 and PDPB in KRLVS141 (*LVS ΔrpsU1 ΔrpsU2 ΔrpsU3 pFTR3-rpsU2-V\_PrpsL\_tetR E8-1-12B*)

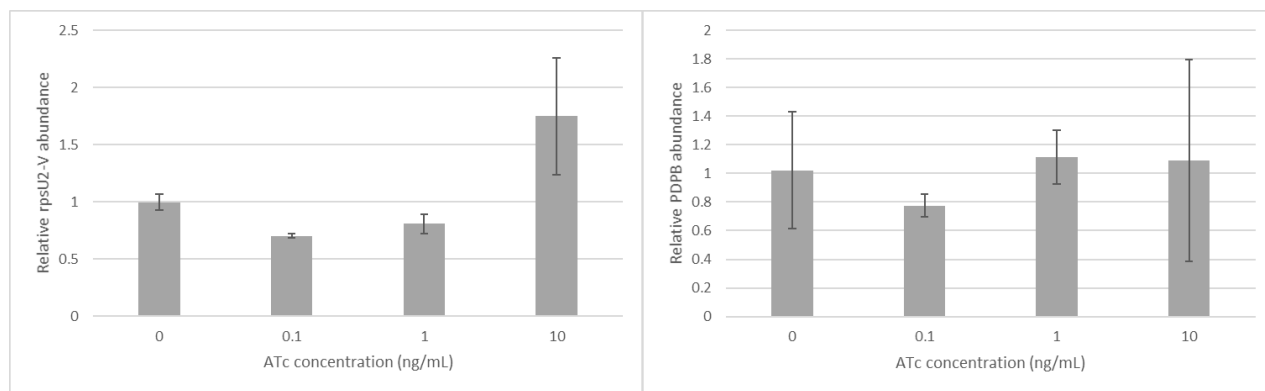


Figure 2: Relative abundance of BS21-2 and PDPB in ATc Induction of KRLVS141 (*LVS ΔrpsU1 ΔrpsU2 ΔrpsU3 pFTR3-rpsU2-V\_PrpsL\_tetR E8-1-12B*)

**Blocking and Probing Pt. 2**

1. Cut the membrane between the PDPB and BS21 bands
2. Add 4.5 uL antibody (anti-VSVG and anti-PDPB) in 10 mL of blocking buffer. Rotate for 1 hour at room temperature.
3. Prepare 1X Wash Buffer
4. Wash (w/detergent) 4x on rotator for 10 minutes each..

5. Use diluted blocking buffer and block again, for 20 min.
6. Transfer membrane to black box and add 1 uL secondary antibody (anti-rabbit for VSVG, anti-mouse for PDPB) to 10 mL wash buffer (w/detergent). Also add 0.01% SDS to the wash buffer (10 uL of 10% SDS).
7. Label for 1 hour on rocker at room temperature.
8. Wash 4x on rotator for 10 minutes each.
9. Wash 2x on rotator for 10 minutes each with wash buffer WITHOUT detergent.
10. 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 Blot

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.

### Imaging Disk Diffusion Assay Plates

1. For imaging MHA plates, place plate lid-side down in imager
2. Switch imager to No Filter (top right of imager)
3. Open Image Lab and select New Protocol>Custom>Disk Diffusion Assay
4. Run protocol and export image as .tif with 600 dpi
  - \* Note: Adjusted 'Low' to 2786 for all

### Reagents

1X Wash Buffer (Scaled down to 130 mL)

Mix together:

- 13 mL 10X PBS
- 117 mL dH<sub>2</sub>O

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, January 11, 2022**

To Do:

1. ~~Make g-glycerol stocks and SUA of KRLVS141~~
2. ~~Analyze DDA Images~~
3. ~~Patch out KRLVS141~~

**Results and Data:**

Disk Diffusion Assay of LVS at Different Starting OD's						
	Sample	ø 1 (mm)	ø 2 (mm)	ø 3 (mm)	Average	Std. Dev.
OD 0.1	1	33.22	34.99	30.79	31.41	2.58
	2	31.85	30.00	27.58		
OD 0.02	1	32.85	34.34	32.67	33.94	1.09
	2	35.63	34.20	33.94		
OD 0.01	1	31.30	35.96	30.51	33.11	2.54
	2	35.87	30.83	34.20		

**Making Glycerol Stocks**

1. Make 2 cryovials for each strain (permanent stocks), label!
2. Prepare 2.4mL of MHB in a 50mL conical
3. Take at least half of a thickly spread plate and add cells to the MHB tube
4. Resuspend until there are no clumps in the MHB
5. Add 600ul of 75% glycerol to the 2.4mL, mix by pipetting
6. Aliquot 1mL per cryovial, freeze at -80
7. Pipet 50ul each of remaining 1mL to centrifuge tubes, freeze at -80

**Wednesday, January 12, 2022****To Do:**

- ~~1. DDA of KRLVS86, KRLVS89, KRLVS91, and LVS~~
- ~~2. Glycerol stocks and SUA of KRLVS86, KRLVS89, and KRLVS91~~

**Making Glycerol Stocks**

1. Make 2 cryovials for each strain (permanent stocks), label!
2. Prepare 2.4mL of MHB in a 50mL conical
3. Take at least half of a thickly spread plate and add cells to the MHB tube
4. Resuspend until there are no clumps in the MHB
5. Add 600ul of 75% glycerol to the 2.4mL, mix by pipetting
6. Aliquot 1mL per cryovial, freeze at -80
7. Pipet 50ul each of remaining 1mL to centrifuge tubes, freeze at -80

**Disk Diffusion Assay**

1. Day before experiment, patch cells on CHAH plates and incubate overnight.
2. Resuspend cells of each strain in 500 µL of MHB. Measure the OD600.
3. Dilute cells in centrifuge tubes to an OD600 of 0.15, with a final volume of 1 mL.
4. Add 100 µL of cells at OD600 0.15 to three MHA plates for each strain and spread with a spreader, sterilized in ethanol. Allow plates to dry.
5. Using sterile tweezers, add filter paper disks to a plate, adding 20 µL ddiH<sub>2</sub>O Type 1 or 50 mg/mL Kasugamycin (Ksg), as appropriate. Allow these disks to dry for 15 minutes.
6. Using sterile tweezers, transfer disks from sterile plate to spread MHA plates so that there is a water disk and a Ksg disk on each.

**Thursday, January 13, 2022****To Do:**

1. Patch out KRLVS141 on two CHAH+Nat plates
2. Pour more CHAH+Nat plates

**Reagents****CHAH Agar with Nat (Half sleeve)**

Mix 15.3 g cystine heart agar with 150 mL t1 ddiH<sub>2</sub>O; let stir on a 90°C hot pate for 10 minutes.

Add flask to Instant Pot and run 30'

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

Place CHA flask in 55°C water bath

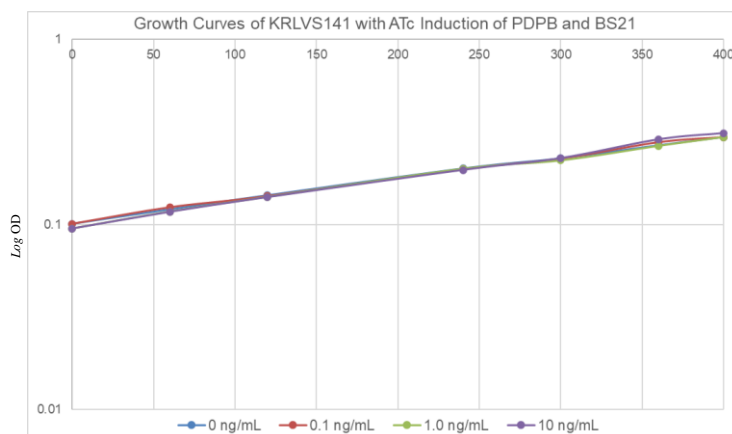
Wipe down flask and bottles with EtOH and with sterile technique, add 150 mL hemoglobin into CHA flask, stir to mix

Add 15uL of Nat, and stir to mix. Pour.

Label plates and cover in foil

**Saturday, January 15, 2022****To Do:**

1. ATc Titration induction of KRLVS141 cells + growth chart
2. Take images of and measure zone of inhibitions for MHA DDA of LVS, KRLVS86, KRLVS89, and KRLVS91

**Results and Data:**

Treatment	Generation Time
0 ng/mL	258.7597034
0.1 ng/mL	259.1645494
1.0 ng/mL	244.8567255
10 ng/mL	235.0281666

Figure 2: Growth curve of KRLVS141 (LVS  $\Delta rpsU1 \Delta rpsU2 \Delta rpsU3$  pFTR3-*rpsU2-V\_PrpsL\_tetR* E8-1-12B)

**ATc Titration Induction of PDPB and BS21-2****Experimental Concentrations:**

10 ng/mL; 1 ng/mL; 0.1 ng/mL; 0 ng/mL

1. Create dilutions (1:100 > 1:10 > 1:10) of 500 ug/mL ATc stock in MHB.
2. Add 28.5 mL into each of two 50 mL conical tubes
3. Take patch plates of KR LVS 141 cells and resuspend, with loop, in 0.7 mL of MHB
4. Use 50 uL diluted in 950 uL MHB to check OD<sub>600</sub>
5. Calculate the volume of sample to add to each tube for an OD<sub>600</sub> of 0.1
6. Check OD's of each then transfer 7mL into each of 8 glass culture tubes
7. Add 14 uL of the appropriate dilution (1:100 for 10 ng/mL ATc, etc.) into 7 mL of MHB each in 8 labelled glass tubes
8. Add tubes to 37°C shaker for ~5 hours (ideally reaching OD<sub>600</sub> = 0.3-0.4)

9. 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
10. Take off supernatant and resuspend in 1 OD per 1 mL 1x Sample Loading Buffer.

Boil in 95°C heat block for 5-10 minutes, then place in -20°C freezer.

Sample Number Key							
1	2	3	4	5	6	7	8
TR-1, 0 ng/mL ATc	TR-2, 0 ng/mL ATc	TR-1, 0.1 ng/mL ATc	TR-2, 0.1 ng/mL ATc	TR-1, 1.0 ng/mL ATc	TR-2, 1.0 ng/mL ATc	TR-1, 10 ng/mL ATc	TR-2, 10 ng/mL ATc

### Growth Curve

1. Two days before, patch out KRLVS141 cells onto CHAH + Nat plates.
2. Resuspend cells in 700 uL of MHB and measure OD600, normalizing to an OD of 0.1 in two aliquots of 28.5 mL of MHB (see above for more complete description of tube preparation.)
3. Take 0.5 mL aliquot and measure OD at time 0, followed by subsequent timepoints of 1, 2, and 4 hours, while the previous experiment is running.
  - a. Reduce to 0.25 mL aliquots if tubes begin to run low, should be no lower than 4 mL.
4. Take additional timepoints/final timepoint as the previous experiment runs.

### Imaging Disk Diffusion Assay Plates

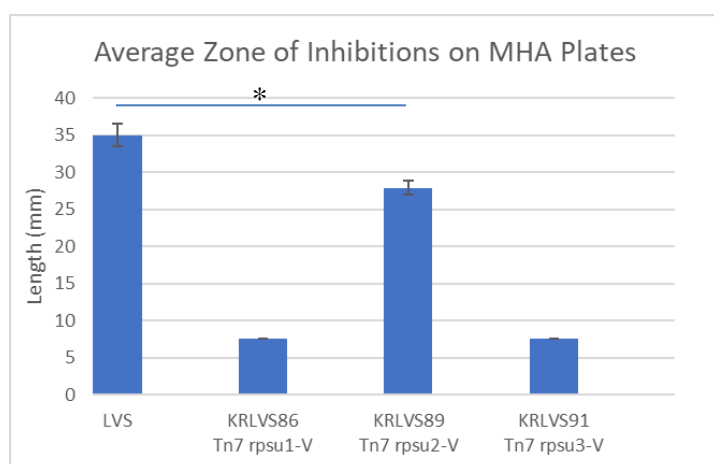
1. For imaging MHA plates, place plate lid down in imager
2. Switch imager to No Filter
3. Open Image Lab and select New Protocol>Custom>Disk Diffusion Assay
4. Run protocol and export image as .tif with 600 dpi
  - \* Note: Adjusted 'Low' to 2786 for all

**Tuesday, January 18, 2022**

To Do:

1. ~~Analyze DDA Images~~

### Results and Data:



### Measuring Zone of Inhibition for Disk Diffusion Assays

1. Open ImageJ
2. File>Open>20201125\_measure.tif
  - \* Note: Changed 'Zoom' to 100% for all images
3. Using "Straight" line tool, measure 10 mm on the ruler, being as precise as possible
4. Analyze>Set Scale...
5. Under 'Known Measurement' type 10.0, under 'Unit' type mm, select the 'Global' checkbox.

For each DDA image:

6. Using Straight Line tool, measure the diameter of the zone of inhibition (draw a line from the edge of inhibition, through the disc, to another edge)
7. Click Analyze>Measure
8. While Measure dialogue is open you can draw two more diameters, clicking Measure after each to record the length
9. Copy lengths into an Excel spreadsheet



**Monday, January 24, 2022****To Do:**

1. ~~Make hemoglobin~~
2. ~~Pour CHAH agar plates~~

**Reagents****2% Hemoglobin**

Add 6 g hemoglobin (kept in fridge) to 500 mL flask (add stir bar)

Add 300 mL Type 1 ddiH<sub>2</sub>O

Stir for at least 10 minutes, until clumps are all dissolved

Autoclave Liquid20 in water bath

**Tuesday, January 25, 2022****To Do:**

1. ~~Gel and Transfer of ATc Induction of PDPB and BS21-2~~

**Results and Data:**

KRLVS141  
Ladder 0 ng/mL Atc 0.1 ng/mL Atc 1 ng/mL Atc 10 ng/mL Atc

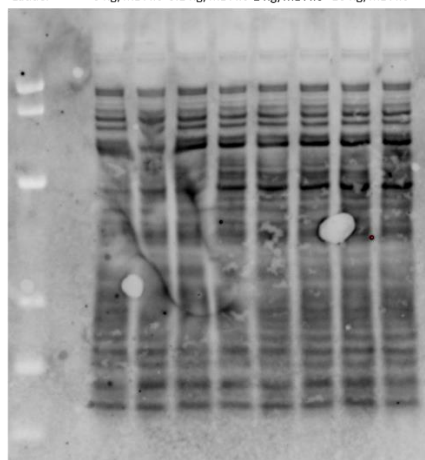


Fig. 1: Total Protein Quantification of KRLVS141 cells grown at 0, 0.1, 1.0, and 10 ng/mL Atc; in technical duplicate.

**Running Gel on ATc Titration Induction of PDPB Samples**

1. Heat samples for an additional 10 minutes.
2. Assemble gel chamber with 15-well pre-cast NuPAGE 4-12% Bis-Tris gel
3. Add Mes Running Buffer to gel chamber (front and back)
4. Use 200 uL pipet set to 200 to wash wells of gel
5. Loaded 9 uL of each sample
6. Used 5 uL of Western Sure ladder
7. Ran at 144-146V for 40-45 minutes.

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13	Lane 14	Lane 15
SLB	WSL	SLB	1	2	3	4	5	6	7	8	SLB	SLB	SLB	SLB

**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, 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 20V for 1 hour.

### Total Protein Quantification

1. Prepare No-Stain labeling buffer by diluting 20x stock to 1x (9.5mL ddiH<sub>2</sub>O+0.5mL 20x buffer).
2. Thaw Activator and Derivatizer, add 20 uL of activator followed by 20 uL of derivatizer
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

### Reagents

Running Buffer (400 mL | 1 gel)

Mix together:

- 1 x MOPS for large proteins
- 1x MES for <50 kD proteins
  - 380 mL ddiH<sub>2</sub>O
  - 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

**No-Stain Labelling Buffer (10 mL)**

Mix together:

- 9.5mL ddiH<sub>2</sub>O
- 0.5mL 20x No-Stain Labelling buffer
- 20 uL Activator followed by 20 uL Derivatizer

**Wednesday, January 26, 2022****To Do:**

1. ~~Blocking and probing of ATe Induction of BS21 and PDPB in KRLVS141~~

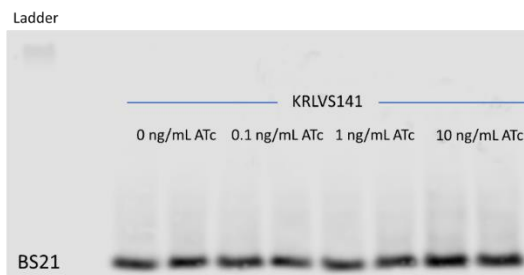
**Results and Data:**

Fig. 2: Western Blot of KRLVS141 cells grown at 0, 0.1, 1.0, and 10 ng/mL Atc; in technical duplicate. Only showing BS21, as PDPB was not visible.

**Blocking and Probing Pt. 2**

1. Cut the membrane between the PDPB and BS21 bands (between fourth and fifth band of ladder)
2. Add 4.5 uL antibody (anti-VSVG and anti-PDPB) in 10 mL of blocking buffer. Rotate for 1 hour at room temperature.
3. Prepare 1X Wash Buffer
4. Wash (w/detergent) 4x on rotator for 10 minutes each..
5. Use diluted blocking buffer and block again, for 20 min.
6. Transfer membrane to black box and add 1 uL secondary antibody (anti-rabbit:VSVG, anti-mouse: PDPB) to 10 mL wash buffer (w/detergent). Add 0.01% SDS to wash buffer (10 uL of 10% SDS).
7. Label for 1 hour on rocker at room temperature.
8. Wash 4x on rotator for 10 minutes each.
9. Wash 2x on rotator for 10 minutes each with wash buffer WITHOUT detergent.
10. For faint bands, dunk blot in methanol for 2 sec then let air dry for 2 minutes prior to imaging.

**Imaging Blot**

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:

26 mL 10X PBS

234 mL diH<sub>2</sub>O

Separate into 200 mL and 60 mL. To the 200 mL add 1 mL of NQ-40

**Thursday, January 27, 2022**

To Do:

- ~~1. Patch out KRLVS156 and KRLVS157~~

**Friday, January 28, 2022**

To Do:

- ~~1. ATc Induction + growth curves of KRLVS156 and KRLVS157~~

## Results and Data:

**ATc Induction.** Did not have enough cells.

**Monday, January 31, 2022**

To Do:

- ~~1. Made and sterile filtered 2.5% iron pyrophosphate~~
- ~~2. Supplemented and poured MHA plates~~
- ~~3. Made and stored two flasks of MHA, non-supplemented~~
- ~~4. Patched out KRLVS156 and KRLVS157~~

## Reagents

2.5% Iron pyrophosphate

1.25g iron pyrophosphate (cabinet) in 50mL of ddiH<sub>2</sub>O (type 1), dissolved overnight, fresh solution every 2 weeks

Mueller Hinton Agar (500 mL; 1% glucose, 0.0125% FePPi, and 2% IsoVitaleX)

To a 1 L flask add:

10.5 g MHB

6 g agar

465 mL ddiH<sub>2</sub>O

Autoclave on the 30 minute liquid cycle and then add:

25 mL 20% glucose

10 mL IsoVitaleX

5 mL 2.5% FePPi

Pour.

2% Hemoglobin

Add 6 g hemoglobin (kept in fridge) to 500 mL flask (add stir bar)

Add 300 mL Type 1 ddiH<sub>2</sub>O

Stir for at least 10 minutes, until clumps are all dissolved

Autoclave Liquid20 in water bath

## February 2022

### Tuesday, February 1, 2022

#### To Do:

- ~~1. Supplemented 500 mL MHB~~
- ~~2. Patched out LVS, KRLVS86, KRLVS89, KRLVS91 for CR~~

## Reagents

### Mueller Hinton Broth (500 mL)

To supplement add:

5mL of 10% glucose

5mL of 2.5% iron pyrophosphate

isovitalex (combine 10mL from liquid vial with solids, add entire volume to broth)

Can keep this supplemented media for 2 weeks, storing at 4 °C

### Wednesday, February 2, 2022

#### To Do:

- ~~1. Patch out double deletion and KRLVS141~~
- ~~2. Make higher concentration of Ksg~~
- ~~3. DDA LVS, KRLVS86, KRLVS89, and KRLVS91 with Caterina~~

## Disk Diffusion Assay

1. Day before experiment, patch cells on CHAH plates and incubate overnight.
2. Resuspend cells of each strain in 500 µL of MHB. Measure the OD<sub>600</sub>.
3. Dilute cells in centrifuge tubes to an OD<sub>600</sub> of 0.2, with a final volume of 1 mL.
4. Add 100 µL of cells at OD<sub>600</sub> 0.2 to three MHA plates for each strain and spread with a spreader, sterilized in ethanol. Allow plates to dry.
5. Using sterile tweezers, add filter paper disks to a plate, adding 20 µL ddiH<sub>2</sub>O Type 1 or 100 mg/mL Kasugamycin (Ksg), as appropriate. Allow these disks to dry for 15 minutes.
6. Using sterile tweezers, transfer disks from sterile plate to spread MHA plates so that there is a water disk and a Ksg disk on each.

## Reagents

### 100 mg/mL Kasugamycin

To 15 mL conical add:

0.5 g of Kasugamycin powder (stored in 4°)

5 mL of diH<sub>2</sub>O Type 1

Pipet up and down/vortex to mix; aliquot and store at -20°

### Friday, February 4, 2022

#### To Do:

- ~~1. ATc Induction of Double Mut. And KRLVS141 Control~~

## ATc Titration Induction of PDPB and BS21-2, KRLVS141 and KRLVS24

### Experimental Concentrations:

10 ng/mL; 0 ng/mL

1. Create dilutions (1:100 > 1:10) of 500 µg/mL ATc stock in MHB.
2. Add 24.5 mL into each of two 50 mL conical tubes
3. Take patch plates of KRLVS141 and DM cells and resuspend, with loop, in 0.7 mL of MHB
4. Use 50 µL diluted in 950 µL MHB to check OD<sub>600</sub>
5. Calculate the volume of sample to add to each tube for an OD<sub>600</sub> of 0.11

6. Check OD's of each then transfer 6mL into each of 4 glass culture tubes per conical tube
7. Add 13 uL of the appropriate dilution (1:100 for 10 ng/mL ATc, etc.) into 7 mL of MHB each in 8 labelled glass tubes
8. Add tubes to 37°C shaker for ~5 hours (ideally reaching OD<sub>600</sub> = 0.3-0.4)
9. 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
10. Take off supernatant and resuspend in 1 OD per 1 mL 1x Sample Loading Buffer.

Sample Number Key							
1	2	3	4	5	6	7	8
24 0 ng/mL	24 0 ng/mL	24 10 ng/mL	24 10 ng/mL	141 0 ng/mL	141 0 ng/mL	141 10 ng/mL	141 10 ng/mL

OD's of Samples at Different Time Points				
	0 min	120 min	290 min	340 min
1	0.103	0.167	0.292	0.327
2	0.103	0.182	0.289	0.332
3	0.103	0.158	0.246	0.272
4	0.103	0.154	0.251	0.276
5	0.133	0.189	0.250	0.299
6	0.133	0.169	0.253	0.282
7	0.133	0.165	0.281	0.299
8	0.133	0.170	0.259	0.293

**Saturday, February 5, 2022**

To Do:

1. Take DDA images for CR

### **Results and Data:**

**Note.** MHA plates were too distorted to take photos or to really see growth. Believe that this was due to not melting MHA at a high enough temperature or for long enough. Will use a high temperature for longer for the next plates.

**Monday, February 7, 2022****To Do:**

1. ~~Make MHA plates~~
2. ~~Show Caterina how to make 2% hemoglobin~~
3. ~~Show Caterina how to Image and Analyze DDA Plates~~
4. ~~Make higher concentration of K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>~~

**Results and Data:**

**DDA Images and Analysis.** Plates appeared ‘wavy,’ probably from not heating up enough and disproportionate distribution of the agar within the media. For future, will stir MHA post-autoclave and then heat up at 300° until melted, then place in water bath prior to supplementation. Additionally, Hannah let me know that the water disks were turning yellow on the edges, needed to filter sterilize the water, but did not realize, will show Caterina how to do this for our next DDA.

**Reagents**

Mueller Hinton Agar (500 mL; 1% glucose, 0.0125% FePPi, and 2% IsoVitaleX)

To supplement:

- 25 mL 20% glucose
- 10 mL IsoVitaleX
- 5 mL 2.5% FePPi

Pour.

2% Hemoglobin

Add 6 g hemoglobin (kept in fridge) to 500 mL flask (add stir bar)

Add 300 mL Type 1 ddiH<sub>2</sub>O

Stir for at least 10 minutes, until clumps are all dissolved

Autoclave Liquid20 in water bath

**Tuesday, February 8, 2022****To Do:**

1. ~~Patch out LVS, KRLVS86, KRLVS89, KRLVS91~~

**Results and Data:**

N/A.

**Wednesday, February 9, 2022****To Do:**

1. ~~Show CR how to filter sterilize (ddiH<sub>2</sub>O)~~
2. ~~Assist CR with DDA of LVS, KRLVS86, KRLVS89, KRLVS91~~

**Results and Data:****Disk Diffusion Assay**

1. Day before experiment, patch cells on CHAH plates and incubate overnight.
2. Resuspend cells of each strain in 500 µL of MHB. Measure the OD<sub>600</sub>.
3. Dilute cells in centrifuge tubes to an OD<sub>600</sub> of 0.2, with a final volume of 1 mL.
4. Add 100 µL of cells at OD<sub>600</sub> 0.2 to three MHA plates for each strain and spread with a spreader, sterilized in ethanol. Allow plates to dry.

- Using sterile tweezers, add filter paper disks to a plate, adding 20  $\mu\text{L}$  filter-sterilized ddiH<sub>2</sub>O Type 1 or 100 mg/mL Kasugamycin (Ksg), as appropriate. Allow these disks to dry for 15 minutes.
- Using sterile tweezers, transfer disks from sterile plate to spread MHA plates so that there is a water disk and a Ksg disk on each.

### Thursday, February 10, 2022

#### To Do:

- ~~Patch out KRLVS156 and KRLVS157~~

#### Results and Data:

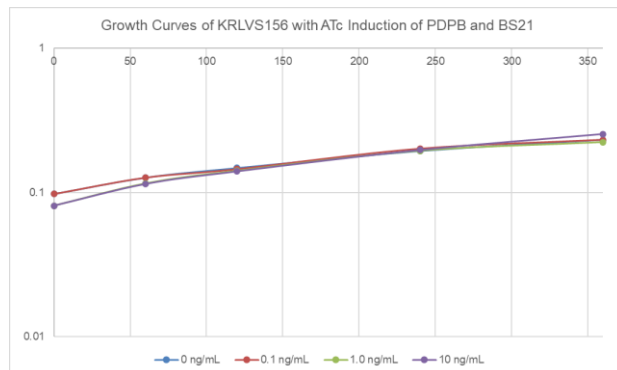
**Note.** Used two single use aliquots of KRLVS157 for one plate.

### Saturday, February 12, 2022

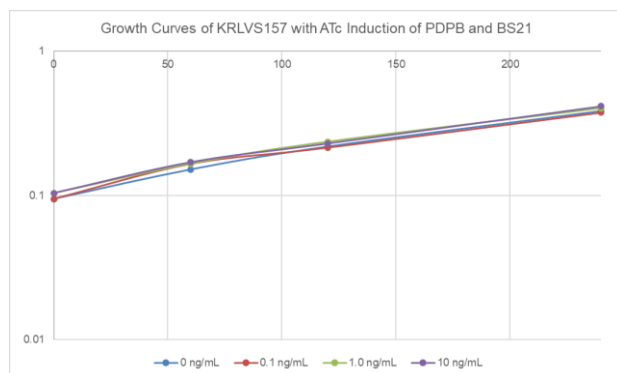
#### To Do:

- ~~Take DDA images for CR~~
- ~~ATc Titration induction of KRLVS156 and KRLVS157 cells + growth chart~~

#### Results and Data:



Treatment	Generation Time
0 ng/mL	291.4827996
0.1 ng/mL	291.4827996
1.0 ng/mL	247.4881196
10 ng/mL	219.7868345



Treatment	Generation Time
0 ng/mL	119.2275079
0.1 ng/mL	121.7265702
1.0 ng/mL	123.5156469
10 ng/mL	120.484567

### ATc Titration Induction of PDPB and BS21-1 or -3 in KRLVS156 and KRLVS157

#### Experimental Concentrations:

10 ng/mL; 1 ng/mL; 0.1 ng/mL; 0 ng/mL

- Create dilutions to  $10^{-4}$  ( $1:100 > 1:10 > 1:10$ ) of 500  $\mu\text{g/mL}$  ATc stock in MHB.
- Add 28.5 mL of MHB into each of four 50 mL conical tubes
- Take patch plates of KRLVS156 and KRLVS157 cells and resuspend, with loop, in 0.7 mL of MHB
- Use 50  $\mu\text{L}$  diluted in 950  $\mu\text{L}$  MHB to check OD<sub>600</sub>, 1:20 dilution.



5. Calculate the volume of sample to add to each conical for an OD<sub>600</sub> of 0.1 and add.
6. Check OD's of each conical then transfer 7mL into each of 8 glass culture tubes
7. Add 14 uL of the appropriate dilution ( $10^{-2}$  for 10 ng/mL ATc, etc.) into 7 mL of MHB each in 8 labelled glass tubes
8. Add tubes to 37°C shaker for ~5 hours (ideally reaching OD<sub>600</sub> = 0.3-0.4)
9. Check OD's then take 1 mL of culture and place in centrifuge tubes and pellet at 15,000 rpm for 3 minutes
10. Take off supernatant and resuspend in 1 OD per 1 mL 1x Sample Loading Buffer.
11. Boil in 95°C heat block for 5-10 minutes, then place in -20°C freezer.

Sample Number Key – KRLVS156							
1	2	3	4	5	6	7	8
TR-1, 0 ng/mL ATc	TR-2, 0 ng/mL ATc	TR-1, 0.1 ng/mL ATc	TR-2, 0.1 ng/mL ATc	TR-1, 1.0 ng/mL ATc	TR-2, 1.0 ng/mL ATc	TR-1, 10 ng/mL ATc	TR-2, 10 ng/mL ATc

Sample Number Key – KRLVS157							
1	2	3	4	5	6	7	8
TR-1, 0 ng/mL ATc	TR-2, 0 ng/mL ATc	TR-1, 0.1 ng/mL ATc	TR-2, 0.1 ng/mL ATc	TR-1, 1.0 ng/mL ATc	TR-2, 1.0 ng/mL ATc	TR-1, 10 ng/mL ATc	TR-2, 10 ng/mL ATc

### Growth Curve

1. Two days before, patch out KRLVS141 cells onto CHAH + Nat plates.
2. Resuspend cells in 700 uL of MHB and measure OD<sub>600</sub>, normalizing to an OD of 0.1 in two aliquots of 28.5 mL of MHB (see above for more complete description of tube preparation.)
3. Take 0.5 mL aliquot and measure OD at time 0, followed by subsequent timepoints of 1, 2, and 4 hours, while the previous experiment is running.
  - a. Reduce to 0.25 mL aliquots if tubes begin to run low, should be no lower than 4 mL.
4. Take additional timepoints/final timepoint as the previous experiment runs.

**Monday, February 14, 2022****To Do:**

1. ~~Make and sterile filter 2.5% iron pyrophosphate~~

**Results and Data:**

N/A.

**Reagents**

2.5% Iron pyrophosphate

1.25g iron pyrophosphate (cabinet) in 50mL of ddiH<sub>2</sub>O (type 1), dissolved overnight, fresh solution every 2 weeks

**Wednesday, February 16, 2022****To Do:**

1. ~~Show CR how to image and measure ZoIA MHA Plates~~
2. ~~Gel and transfer of control ATc Titration Induction of PDPB and BS21-2, KRLVS141 and KRLVS24~~
3. ~~Total Protein Quantification of ATc Titration Induction of PDPB and BS21-2, KRLVS141 and KRLVS24~~

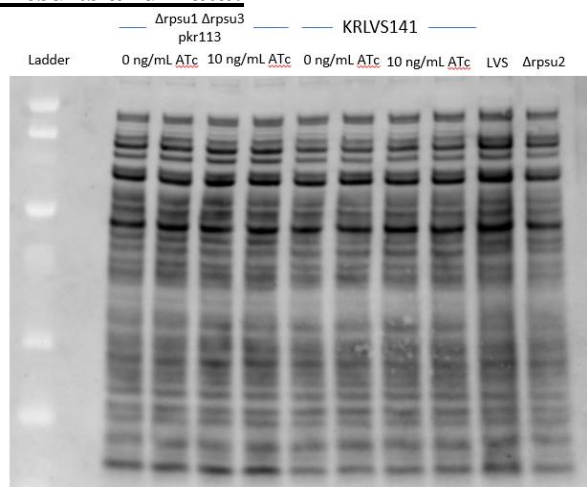
**Results and Data:**

Fig. 1: Total Protein Quantification of KRLVS141 and KRLVS24 cells grown at 0 and 10 ng/mL ATc; in technical duplicate, as well as LVS and  $\Delta$ rpsu2

**Running Gel on ATc Titration Induction of PDPB and BS21-2, KRLVS141 and KRLVS24**

1. Heat samples for an additional 10 minutes.
2. Assemble gel chamber with 15-well pre-cast NuPAGE 4-12% Bis-Tris gel
3. Add Mes Running Buffer to gel chamber (front and back)
4. Use 200 uL pipet set to 200 to wash wells of gel
5. Loaded 9 uL of each sample
6. Used 5 uL of Western Sure ladder
7. Ran at 144-146V for 40-45 minutes.

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13	Lane 14	Lane 15
SLB	WSL	SLB	1	2	3	4	5	6	7	8	LVS	$\Delta$ rpsu2	SLB	SLB

## 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, 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 20V for 1 hour.

## Total Protein Quantification

8. Prepare No-Stain labeling buffer by diluting 20x stock to 1x (9.5mL ddiH<sub>2</sub>O+0.5mL 20x buffer).
9. Thaw Activator and Derivatizer, add 20 uL of activator followed by 20 uL of derivatizer
10. Pre-wash the blot twice with water, for 2 minutes each, on a nutator.
11. Add 10 mL of No-stain labeling solution to the blot, and nutate for 10 minutes.
12. Post-wash with water for 2 minutes, three times.
13. Image using the Bio-Rad imager with protocol Blot->No Stain.
14. Quantify using LiCor Image Studio Lite.

## Blocking and Probing

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

## Reagents

Running Buffer (400 mL | 1 gel)

Mix together:

- 1 x MOPS for large proteins
- 1x MES for <50 kD proteins
  - 380 mL ddiH<sub>2</sub>O
  - 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

## No-Stain Labelling Buffer (10 mL)

Mix together:

- 9.5mL ddiH<sub>2</sub>O
- 0.5mL 20x No-Stain Labelling buffer
- 20 uL Activator followed by 20 uL Derivatizer

**Saturday, February 19, 2022****To Do:**

1. ~~Blocking and probing of control blot~~
2. ~~Image Blot~~
3. ~~Run dishwasher~~

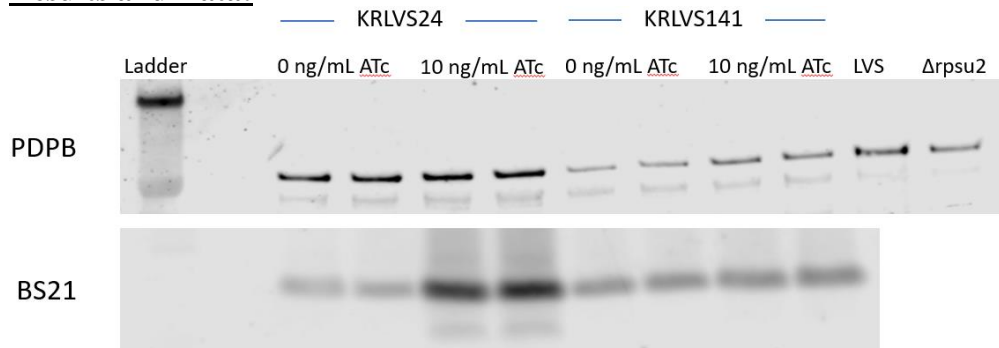
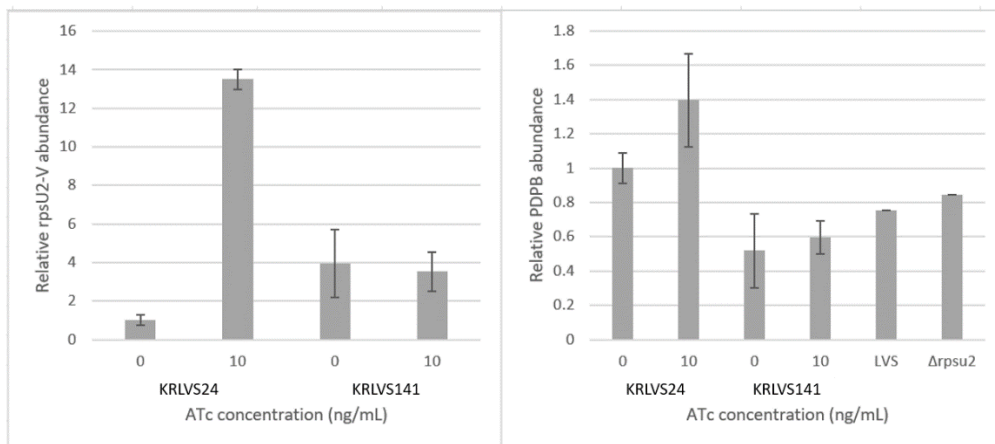
**Results and Data:**

Fig. 2: Western Blot of KRLVS24 ( $\Delta$ rpsu1  $\Delta$ rpsu3 pkr113) and KRLVS141 ( $\Delta$ rpsu1  $\Delta$ rpsu2  $\Delta$ rpsu3 pkr113) cells grown at 0 and 10 ng/mL ATc; in technical duplicate. Additionally, the PDPB of an LVS and  $\Delta$ rpsu2 sample is shown.



## Blocking and Probing Pt. 2

1. Cut the membrane between the PDPB and BS21 bands (between fourth and fifth band of ladder)
2. Add 4.5 uL antibody (anti-VSVG and anti-PDPB) in 10 mL of blocking buffer. Rotate for 1 hour at room temperature.
3. Prepare 1X Wash Buffer
4. Wash (w/detergent) 4x on rotator for 10 minutes each..
5. Use diluted blocking buffer and block again, for 20 min.
6. Transfer membrane to black box and add 1 uL secondary antibody (anti-rabbit for VSVG, anti-mouse for PDPB) to 10 mL wash buffer (w/detergent). Also add 0.01% SDS to the wash buffer (10 uL of 10% SDS).
7. Label for 1 hour on rocker at room temperature.
8. Wash 4x on rotator for 10 minutes each.
9. Wash 2x on rotator for 10 minutes each with wash buffer WITHOUT detergent.
10. 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 Blot

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:

26 mL 10X PBS

234 mL diH2O

Separate into 200 mL and 60 mL. To the 200 mL add 1 mL of NQ-40

**Tuesday, February 22, 2022****To Do:**

- ~~1. Patch out LVS, KRLVS86, KRLVS89, KRLVS91~~

**Results and Data:**

N/A.

**Wednesday, February 23, 2022****To Do:**

- ~~1. Assist CR with supplementing and pouring MHA plates~~
- ~~2. Assist CR with DDA of LVS, KRLVS86, KRLVS89, KRLVS91~~

**Results and Data:**

N/A.

**Disk Diffusion Assay**

1. Day before experiment, patch cells on CHAH plates and incubate overnight.
2. Resuspend cells of each strain in 500  $\mu$ L of MHB. Measure the OD600.
3. Dilute cells in centrifuge tubes to an OD600 of 0.2, with a final volume of 1 mL.
4. Add 100  $\mu$ L of cells at OD600 0.2 to three MHA plates for each strain and spread with a spreader, sterilized in ethanol. Allow plates to dry.
5. Using sterile tweezers, add filter paper disks to a plate, adding 20  $\mu$ L filter-sterilized ddiH<sub>2</sub>O Type 1 or 100 mg/mL Kasugamycin (Ksg), as appropriate. Allow these disks to dry for 15 minutes.
6. Using sterile tweezers, transfer disks from sterile plate to spread MHA plates so that there is a water disk and a Ksg disk on each.

**Reagents**

Mueller Hinton Agar (500 mL; 1% glucose, 0.0125% FePPi, and 2% IsoVitaleX)

To supplement:

25 mL 20% glucose

10 mL IsoVitaleX

5 mL 2.5% FePPi

Pour.

**Saturday, February 26, 2022****To Do:**

- ~~1. Patch out KRLVS156 and KRLVS157~~
- ~~2. Gel and transfer of KRLVS157 ATe Titration Induction~~
- ~~3. Total Protein Quantification of KRLVS157 ATe Titration Induction~~

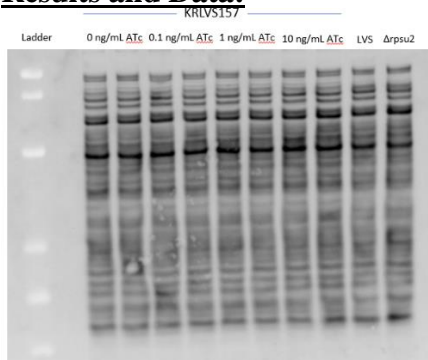
**Results and Data:**

Fig. 1: Total Protein Quantification of KRLVS157 cells grown at 0, 0.1, 1, and 10 ng/mL Ate; in technical duplicate, as well as LVS and  $\Delta$ rpsu2

### Running Gel on ATc Titration Induction of KRLVS157 Samples

1. Heat samples for an additional 10 minutes.
2. Assemble gel chamber with 15-well pre-cast NuPAGE 4-12% Bis-Tris gel
3. Add Mes Running Buffer to gel chamber (front and back)
4. Use 200 uL pipet set to 200 to wash wells of gel
5. Loaded 9 uL of each sample
6. Used 5 uL of Western Sure ladder
7. Ran at 150V for 40-45 minutes.

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 13	Lane 14	Lane 15
SLB	WSL	SLB	1	2	3	4	5	6	7	8	LVS	$\Delta$ rpsu2	SLB	SLB

### 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, 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.
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 20V for 1 hour.

### Total Protein Quantification

1. Prepare No-Stain labeling buffer by diluting 20x stock to 1x (9.5mL ddiH<sub>2</sub>O+0.5mL 20x buffer).
2. Thaw Activator and Derivatizer, add 20 uL of activator followed by 20 uL of derivatizer
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

## Reagents

Running Buffer (400 mL | 1 gel)

Mix together:

- 1 x MOPS for large proteins
- 1x MES for <50 kD proteins
  - 380 mL ddiH<sub>2</sub>O
  - 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

No-Stain Labelling Buffer (10 mL)

Mix together:

- 9.5mL ddiH<sub>2</sub>O
- 0.5mL 20x No-Stain Labelling buffer
- 20 uL Activator followed by 20 uL Derivatizer

**Sunday, February 27, 2022**

To Do:

- ~~1. Blocking and probing of KRLVS157 ATc Titration Induction blot~~
- ~~2. Image Blot~~
- ~~3. Image DDA plates for CR~~

## Results and Data:

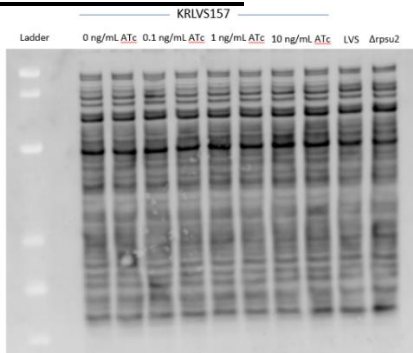
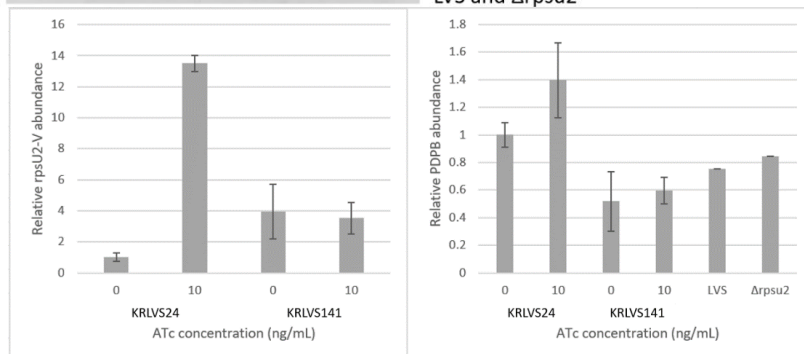


Fig. 1: Total Protein Quantification of KRLVS157 cells grown at 0, 0.1, 1, and 10 ng/mL ATc; in technical duplicate, as well as LVS and Δrpsu2





## Blocking and Probing Pt. 2

1. Cut the membrane between the PDPB and BS21 bands (between fourth and fifth band of ladder)
2. Add 4.5 uL antibody (anti-VSVG and anti-PDPB) in 10 mL of blocking buffer. Rotate for 1 hour at room temperature.
3. Prepare 1X Wash Buffer
4. Wash (w/detergent) 4x on rotator for 10 minutes each..
5. Use diluted blocking buffer and block again, for 20 min.
6. Transfer membrane to black box and add 1 uL secondary antibody (anti-rabbit for VSVG, anti-mouse for PDPB) to 10 mL wash buffer (w/detergent). Also add 0.01% SDS to the wash buffer (10 uL of 10% SDS).
7. Label for 1 hour on rocker at room temperature.
8. Wash 4x on rotator for 10 minutes each.
9. Wash 2x on rotator for 10 minutes each with wash buffer WITHOUT detergent.
10. 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 Blot

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:

26 mL 10X PBS

234 mL diH2O

Separate into 200 mL and 60 mL. To the 200 mL add 1 mL of NQ-40

**Monday, February 28, 2022****To Do:**

1. ~~gDNA prep of KRLVS156 and KRLVS157~~
2. ~~Nanodrop gDNA prep~~
3. ~~Make and sterile filter 2.5% iron pyrophosphate~~
4. ~~Assist CR with analyzing DDA images~~

**Results and Data:**

Sample ID	Nucleic Acid (ng/uL)	A260 (Abs)	A280 (Abs)	260/280	260/230
KRLVS156	611.7	12.234	7.541	1.62	0.53
KRLVS157	342.1	6.842	3.836	1.78	1.13

**gDNA Prep of KRLVS156 and KRLVS157**

1. Dilute 1uL of Proteinase K into 310uL of Tissue and Cell Lysis Solution for each sample.
2. Scraped up patches of KRLVS156 and KRLVS157 and resuspended in MHB
3. Pellet cells by centrifugation and discard the supernatant, leaving approximately 25 uL of liquid.
4. Vortex for 10 seconds to resuspend the cell pellet.
5. Add 300uL of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
6. Incubate at 65°C for 15 minutes, vortex every 5 minutes.
7. Cool the samples to 37°C and add 2uL of 20mg/mL RNase A to the sample, mix thoroughly.
8. Incubate at 37°C for 30 minutes.
9. Place the samples on ice for 3-5 minutes.
10. Add 150uL of MPC Protein Precipitation Reagent to 200uL of lysed sample and vortex vigorously for 10 seconds.
11. Pellet the debris by centrifugation at 4°C for 10 minutes at max speed in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25uL of MPC Protein Precipitation Reagent, mix, and pellet the debris again.
12. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
13. Add 500uL of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
14. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
15. Carefully pour off the isopropanol without dislodging the DNA pellet.
16. Rinse twice with 70% ethanol (~1mL) being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all the residual ethanol with a pipet and let it air dry.
17. Resuspend the DNA in 35uL of 0.1x EB.

**Reagents****2.5% Iron pyrophosphate**

1.25g iron pyrophosphate (cabinet) in 50mL of ddiH<sub>2</sub>O (type 1), dissolved overnight, fresh solution every 2 weeks

## 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.