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July 2022

Friday, July 1, 2022

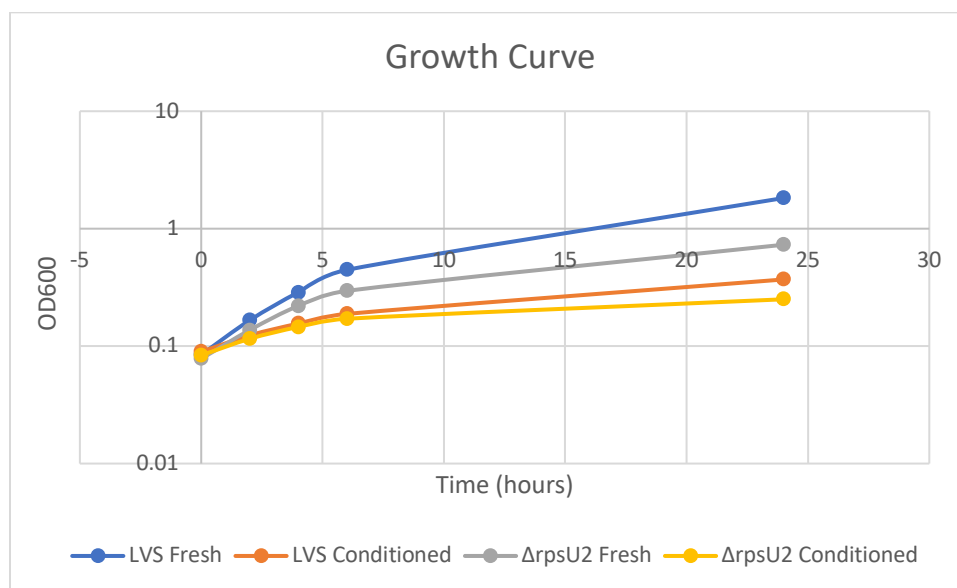
To Do:

- ~~Analyze growth data~~

Side Project

I took out the overnight cultures at 10 am and checked their OD600s. The summary of all the OD600s can be seen below:

Sample	0 (hours)	2 (hours)	4 (hours)	6 (hours)	24 (hours)
LVS Fresh	0.0845	0.166	0.287	0.446	1.824
LVS Conditioned	0.0895	0.1235	0.1555	0.188	0.3695
Δ rpsU2 Fresh	0.078	0.137	0.22	0.2955	0.73
Δ rpsU2 Conditioned	0.0835	0.1155	0.1455	0.171	0.2505



Generation Times	0-2 (hours)	2-4 (hours)	4-6 (hours)
LVS Fresh	131.0987546	154.0235688	223.0153148
LVS Conditioned	271.1895492	337.591975	820.3827353
Δ rpsU2 Fresh	145.3063624	191.7151694	234.795012
Δ rpsU2 Conditioned	271.5490397	341.1966109	523.3159576

The cells grew well again, as seen by the generation times. These results also match the most recent growth experiment and validate that Δ rpsU2 loses its growth defect in conditioned media.

The next step will be to figure out a way to isolate and purify siderophore so we can add it to fresh media and determine if Δ rpsU2 loses its growth defect.

Tuesday, July 5, 2022**To Do:**

- ~~1. Ribosome assay~~

Delta Ribosome Kit Protocol

Today I will prepare my first ribosome assay using controls to validate whether the kit works with my plasmid. I used the PURExpress® Δ Ribosome Kit and followed the modified version of the protocol which Hannah made.

1. Thaw all components on ice. Pulse-spin in microfuge to collect solutions to bottom of tubes. If Solution A has precipitates, mix well prior to assembling reaction
2. Dilute ribosomes to 2.666 pmol/uL in H10M10A50 buffer, enough for a final volume of 15 uL per reaction and 40 pmol per reaction.
3. Dilute DNA in 0.1xEB to a concentration of 125 ng/uL
4. Add components in the following order:

Solution A	10 uL
Factor Mix	3 uL
Ribosomes	15 uL
Template DNA	2 uL

Total 30 uL

5. Mix gently and pulse-spin in microfuge to collect mixture at the bottom of the tube
6. Incubate at 37C for 2-4 hours (incubator is preferred to water bath)
7. Stop the reaction by placing tubes on ice.

To dilute the DNA I mixed 4.81 uL of 0.1X EB with 3.19 uL of phenol:chloroform purified pKR144 to make 8 uL.

I used Hannah's diluted ribosomes.

Tube	DNA	Ribosomes
1	Phenol:chloroform purified pKR144	Wild-type <i>E. coli</i>
2	Phenol:chloroform purified pKR144	Wild-type <i>F. tularensis</i>
3	Phenol:chloroform purified pKR144	Nuclease-free water

I incubated the reactions in the thermocycler for 4 hours.

Nano-Glo® Luciferase Assay Protocol

Next, I prepared the Nano-Glo assay following the protocol below:

1. Thaw Nano-Glo® Luciferase Assay Buffer and Nano-Glo® Luciferase Assay Substrate on ice – mix by pipetting
2. Prepare appropriate volume of reconstituted reagent by combining one volume of substrate with 50 volumes of buffer – mix by pipetting
 - a. Example: for 150 uL of reagent mix 150 uL of buffer with 3 uL of substrate
3. Pipet PURExpress ribosome kit reactions into Costar 3789A plate wells

- a. Separate reactions by at least 2 wells in all directions
4. Add equal volume of assay substrate to reaction volume (30 μ L) – mix by pipetting
5. Let stand for 3 mins
6. Measure luminescence using plate reader
 - a. Acquisition settings: select luminescence; set wavelength to all; select plate as 96 well Costar 3789A
 - b. Hit read
 - c. Export data as text file

Note: Ensure luminescence values remain within the linear range of 10^3 and 10^8

Well	Sample
B1	Wild-type <i>E. coli</i> ribosomes + substrate
A2	Wild-type <i>F. tularensis</i> ribosomes + substrate
B3	Water + substrate
A4	H10M10A50 Buffer + substrate
A5	H10M10A50 Buffer

Added 3 μ L of substrate to 150 μ L buffer to make 150 μ L reagent

1	2	3	4	5	6
42132	134193	1799	1404	1390	1354
32396028	46462	2256	1401	1392	1346
52326	7738	1652	1485	1446	1429
1924	1651	1471	1427	1435	1367
1471	1467	1449	1513	1469	1379

This is a small snapshot of the plate. The wells with reactions in them are bright yellow while lighter yellow wells represent values above the background. When discussing with Hannah, we determined that this is probably due to the reaction's luminescence being strong enough to impact the wells directly surrounding them. To combat this, in the future I should spread the reactions out more, especially surrounding the *E. coli* ribosomes since they have such a strong signal.

The results indicate that the plasmid does work with the kit! The *E. coli* ribosomes had the highest luminescence while the *F. tularensis* ribosomes still had a significantly higher value than the negative control (59X stronger). There was also no difference between empty wells and the two buffer negative controls which means the substrate doesn't interact with the buffer.

Wednesday, July 6, 2022**To Do:**

- ~~1. Ribosome assay~~

Ribosome Assay

Today I will do another ribosome assay, this time comparing the three rpsU ribosomes. My hypothesis is that the *E. coli* ribosomes will have the highest luminescence, followed by wild-type *F. tularensis* and rpsU2, which should have approximately the same luminescence values. rpsU1 and rpsU3 should have the lowest amount of luminescence. I also used buffer as a negative control.

Tube	DNA	Ribosomes
1	Phenol:chloroform purified pKR144	Wild-type <i>E. coli</i>
2	Phenol:chloroform purified pKR144	Wild-type <i>F. tularensis</i>
3	Phenol:chloroform purified pKR144	<i>F. tularensis</i> Tn7::rpsU1-V
4	Phenol:chloroform purified pKR144	<i>F. tularensis</i> Tn7::rpsU2-V
5	Phenol:chloroform purified pKR144	<i>F. tularensis</i> Tn7::rpsU3-V
6	Phenol:chloroform purified pKR144	H10M10A50 Buffer

To dilute the DNA I mixed 8.41 uL of 0.1X EB with 5.59 uL of phenol:chloroform purified pKR144 to make 14 uL.

I also had to dilute more *E. coli* ribosomes (2 uL control ribosomes + 8 uL H10M10A50 buffer) and mixed it with Hannah's diluted ribosomes. For all the other ribosomes I used the ones Hannah already had diluted.

I then incubated the samples in the thermocycler for 4 hours.

After incubation, I put the samples on ice to stop the reactions. I then removed 5 uL of each sample and put it in a new set of PCR tubes to be used for a future Western blot.

Added 3.5 uL substrate to 175 uL buffer to make 175 uL reagent.

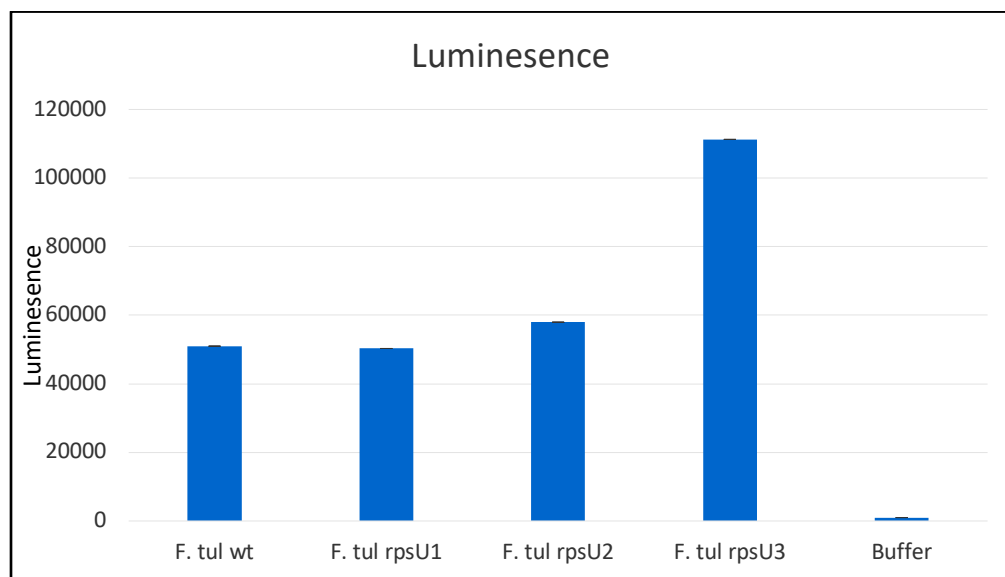
I then added 25 uL of reagent to each sample and mixed by pipetting. Let sit for 3 mins. at room temperature. Next, I added 15 uL of each sample to the wells in triplicate and measured the luminescence using the plate reader.

Well	Sample
A1	Wild-type <i>E. coli</i> ribosomes + substrate A
A2	Wild-type <i>E. coli</i> ribosomes + substrate B
A3	Wild-type <i>E. coli</i> ribosomes + substrate C
A6	Wild-type <i>F. tularensis</i> ribosomes + substrate A
A7	Wild-type <i>F. tularensis</i> ribosomes + substrate B
A8	Wild-type <i>F. tularensis</i> ribosomes + substrate C
E1	<i>F. tularensis</i> Tn7::rpsU1-V ribosomes + substrate A
E2	<i>F. tularensis</i> Tn7::rpsU1-V ribosomes + substrate B
E3	<i>F. tularensis</i> Tn7::rpsU1-V ribosomes + substrate C

D6	<i>F. tularensis</i> Tn7::rpsU2-V ribosomes + substrate A
D7	<i>F. tularensis</i> Tn7::rpsU2-V ribosomes + substrate B
D8	<i>F. tularensis</i> Tn7::rpsU2-V ribosomes + substrate C
H1	<i>F. tularensis</i> Tn7::rpsU3-V ribosomes + substrate A
H2	<i>F. tularensis</i> Tn7::rpsU3-V ribosomes + substrate B
H3	<i>F. tularensis</i> Tn7::rpsU3-V ribosomes + substrate C
G6	Water + substrate A
G7	Water + substrate B
G8	Water + substrate C
D12	H10M10A50 Buffer

The average luminescence of each sample, with its standard deviation, can be seen below:

Averages		St Dev	
E. coli	13775570.3	E. coli	88869.1511
F. tul wt	50989.3333	F. tul wt	378.863212
F. tul rpsU1	50302.6667	F. tul rpsU1	178.746562
F. tul rpsU2	57978.6667	F. tul rpsU2	202.013201
F. tul rpsU3	111281.333	F. tul rpsU3	706.286297
Buffer	988.333333	Buffer	9.86576572



As seen by the graph, wild-type, rpsU1, and rpsU2 ribosomes have pretty similar luminescence. However, rpsU3 ribosomes have over 2X the luminescence. This is not what we expected, and a Western blot should be performed to measure the levels of tul4 across all the samples. We can then calculate an NLuc/tul4 ratio for each sample to more accurately determine any differences.

Western Blot

1. Add 5 uL of 2X SLB to each 5 uL sample (saved from ribosome assay) - Mix by pipetting
2. Heat in thermocycler at 95C for 10 mins
3. Store in -20C freezer box

Monday, July 11, 2022**To Do:**

- ~~1. Western Blot day 1~~

Western Blot Protocol Day 1

- Boil samples at 95C for 5 mins
- Assemble gel chamber
 - Use pre-cast NuPAGE 4-12% Bis-Tris gel
 - Make running buffer – 400 mL
 - 380 mL ddiH₂O
 - 20 mL 20x MES
 - 1 mL NuPAGE antioxidant
- Use pipet to wash wells of gel (use 200 uL set to 200 uL)
- Load 10 or 12 uL of each sample
 - Use 5 uL of WesternSure for the ladder (thaw and vortex to mix)
- Run at 150V until the blue dye front reaches the bottom ridge of the gel. (~40 mins)

Well	Sample	Volume (uL)
1	1XSLB	10
2	WesternSure Ladder	5
3	1XSLB	10
4	1XSLB	10
5	1XSLB	10
6	1- Wild-type <i>F. tularensis</i>	10
7	2- <i>F. tularensis</i> Tn7::rpsU1-V	10
8	3- <i>F. tularensis</i> Tn7::rpsU2-V	10
9	4- <i>F. tularensis</i> Tn7::rpsU3-V	10
10	5- H10M10A50 Buffer	10
11	1XSLB	10
12	1XSLB	10
13	1XSLB	10
14	1XSLB	10
15	1XSLB	10

Wet transfer

Make transfer buffer in a 500 mL graduated cylinder

425 mL ddiH₂O

50 mL methanol

25 mL NuPAGE 20x transfer buffer

Store in freezer to chill until the gel has stopped running

When the gel has about 10 minutes remaining, begin setting up the transfer; make sure you have ice on hand.

1. Remove transfer buffer from freezer and add 500 uL NuPAGE antioxidant
2. Activate PVDF membrane (must use Millipore Immobilon-FL #IPFL00010) in ethanol (can do in a tip box). For a full gel, cut to approximately 6 cm x 8 cm using pre-made guide.
3. In a large container (9"x9" Pyrex baking dish, for example), presoak membrane, 2 filter papers cut to membrane size, and 4 sponges in transfer buffer. Use a roller to push bubbles out of the sponges.
4. Open gel case, cut off wells and at the bottom ridge on the gel and place wet sheet of filter paper on the gel.
5. Peel gel and filter paper off and place wet membrane on gel.
6. Place other filter paper on membrane and roll out bubbles
7. Dunk the transfer cassette halves in the transfer buffer and begin assembling sandwich:
2 sponges
filter/membrane/gel/filter sandwich so that **the membrane is on top of the gel**
2 more sponges
8. Close transfer apparatus and clamp into the gel box.
9. Fill the inside chamber with transfer buffer, fill the outer chamber with dH₂O. Place the gel box into a large ice bucket. Close the lid, seal the hole in the lid with tape, and cover the whole gel box with ice. Clear off the top of the box to remove the tape.
10. Run at 20V for 1 hour.

Blocking and probing

Block the membrane with Li-Cor Odyssey Blocking Buffer (PBS) diluted 1:5 in PBS. Use ~25 mL or enough to cover the membrane and rock **overnight** at room temperature. DO NOT add any detergents (Surfact-Amps, Tween, SDS) to the blocking buffer.

Store diluted blocking buffer at 4C.

Tuesday, July 12, 2022**To Do:**

1. ~~Western Blot day 2~~

Western Blot Protocol Day 2

Make primary antibody solution:

- 4.5 uL anti-VSVG (bS21)
- 10 uL anti-tul4
- 10 mL diluted blocking buffer
- Put in 50 mL tube and invert to mix

Rotate for 1 hour at room temperature

Meanwhile, make wash buffers:

1. 100 mL PBS-T
 - a. 10 mL 10X PBS
 - b. 90 mL ddiH2O
 - c. 500 uL NP-40
2. 30 mL PBS
 - a. 3 mL 10X PBS
 - b. 27 mL ddiH2O

Wash 4x on rotator for 10 minutes each, using 10 mL wash buffer per wash

Use 10 mL diluted blocking buffer and block again, for 15 min

Make secondary antibody solution: (keep in black box – light sensitive)

- 1 uL red anti-rabbit (bS21)
- 1 uL green anti-mouse (tul4)
- 10 mL PBS-T
- 10 uL 10X SDS
- Put in 50 mL tube and invert to mix

Rotate for 1 hour at room temperature

Wash 4x on rotator for 10 minutes each, using 10 mL wash buffer per wash

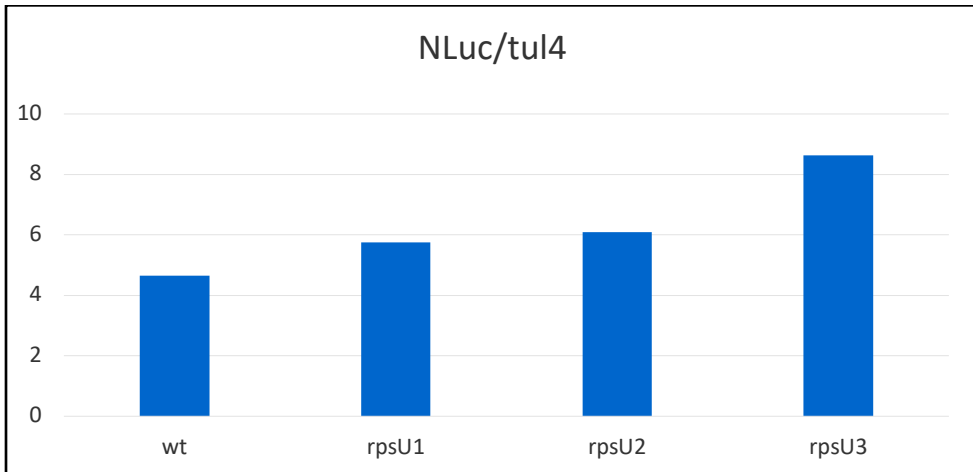
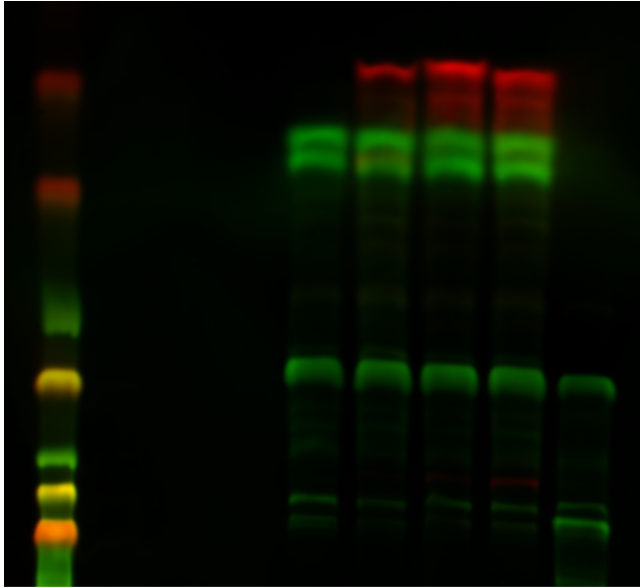
Wash 2x on rotator for 10 minutes each, using 10 mL of wash buffer WITHOUT detergent

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

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.

Blot	Protein	kDa	Primary Dilution	Secondary Dilution
1	bS21-VSVG (anti-VSVG)	9	1:2222	1:10,000 anti-rabbit, 680 CW (red)
	Tul4	17	1:1000	1:10,000 anti-mouse, 800 CW (green)



As seen above, the NLuc/tul4 ratio for each ribosome follows the same pattern as the NLuc graph. Most notably, rpsU3 has the highest expression, while rpsU1 and rpsU2 are similar to wild-type levels.

Monday, July 18, 2022**To Do:**

- ~~1. Ribosome assay~~

Ribosome Assay

Today I will repeat the ribosome assay to validate my previous results. I will modify the experiment slightly and compare the three wild-type ribosomes which were purified on three different dates.

Tube	DNA	Ribosomes
1	Phenol:chloroform purified pKR144	Wild-type <i>F. tularensis</i> 11/10/21 - 1
2	Phenol:chloroform purified pKR144	Wild-type <i>F. tularensis</i> 3/22/19
3	Phenol:chloroform purified pKR144	Wild-type <i>F. tularensis</i> 11/13/20 (used the ones diluted 6/25/21)
4	Phenol:chloroform purified pKR144	<i>F. tularensis</i> Tn7::rpsU1-V
5	Phenol:chloroform purified pKR144	<i>F. tularensis</i> Tn7::rpsU2-V
6	Phenol:chloroform purified pKR144	<i>F. tularensis</i> Tn7::rpsU3-V
7	Phenol:chloroform purified pKR144	H10M10A50 Buffer

To dilute the DNA I mixed 9.60 uL of 0.1X EB with 6.40 uL of phenol:chloroform purified pKR144 to make 16 uL.

I diluted the ribosomes following the chart below. There was already a tube of diluted WT from 11/13/2020.

Genotype	Volume of ribosomes	Volume of buffer
Tn7::rpsU1	30.4	2.6
Tn7::rpsU2	14.1	18.9
Tn7::rpsU3	23.2	9.8
WT (11/13/2020)	-	-
WT (3/22/2019)	9	24
WT (11/10/2021 - 1)	8.2	6.8

I then incubated the samples in the thermocycler for 4 hours.

After incubation, I put the samples on ice to stop the reactions. I then removed 5 uL of each sample and put it in a new set of PCR tubes to be used for a future Western blot.

Added 4 uL substrate to 200 uL buffer to make 200 uL reagent.

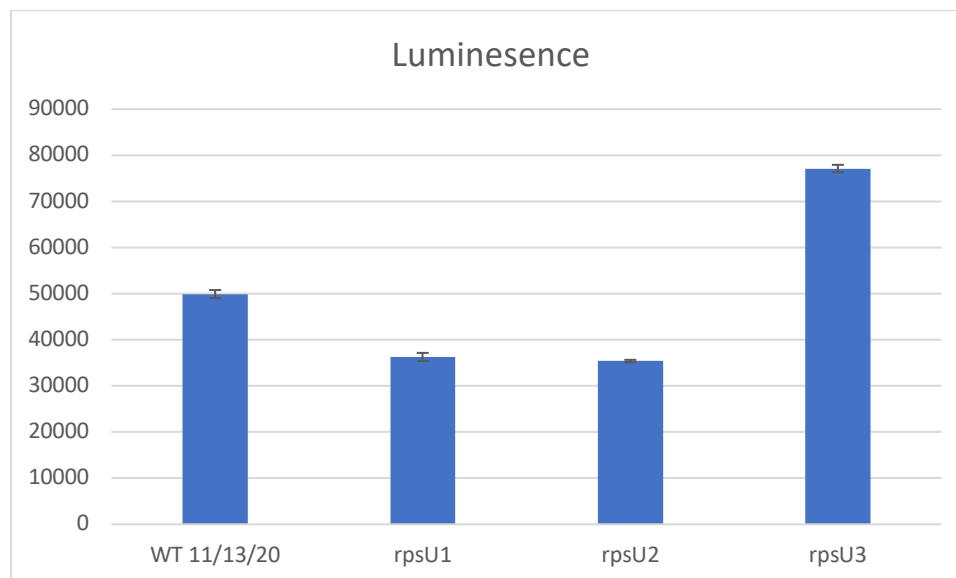
I then added 25 uL of reagent to each sample and mixed by pipetting. Let sit for 3 mins. at room temperature. Next, I added 15 uL of each sample to the wells in triplicate and measured the luminescence using the plate reader. I then removed 6 uL from the first well of each sample and put it in a new set of PCR tubes to be used for the Western blot. This will help determine if there

is a difference in the Western blot before and after the luciferase assay. If there isn't, it will make the protocol much easier.

Well	Sample
A1	Wild-type <i>F. tularensis</i> ribosomes 11/10/21 + substrate A
A2	Wild-type <i>F. tularensis</i> ribosomes 11/10/21 + substrate B
A3	Wild-type <i>F. tularensis</i> ribosomes 11/10/21 + substrate C
A6	Wild-type <i>F. tularensis</i> ribosomes 3/22/19 + substrate A
A7	Wild-type <i>F. tularensis</i> ribosomes 3/22/19 + substrate B
A8	Wild-type <i>F. tularensis</i> ribosomes 3/22/19 + substrate C
D1	Wild-type <i>F. tularensis</i> ribosomes 11/13/20 + substrate A
D2	Wild-type <i>F. tularensis</i> ribosomes 11/13/20 + substrate B
D3	Wild-type <i>F. tularensis</i> ribosomes 11/13/20 + substrate C
D6	<i>F. tularensis</i> Tn7::rpsU1-V ribosomes + substrate A
D7	<i>F. tularensis</i> Tn7::rpsU1-V ribosomes + substrate B
D8	<i>F. tularensis</i> Tn7::rpsU1-V ribosomes + substrate C
G1	<i>F. tularensis</i> Tn7::rpsU2-V ribosomes + substrate A
G2	<i>F. tularensis</i> Tn7::rpsU2-V ribosomes + substrate B
G3	<i>F. tularensis</i> Tn7::rpsU2-V ribosomes + substrate C
G6	<i>F. tularensis</i> Tn7::rpsU3-V ribosomes + substrate A
G7	<i>F. tularensis</i> Tn7::rpsU3-V ribosomes + substrate B
G8	<i>F. tularensis</i> Tn7::rpsU3-V ribosomes + substrate C
A11	H10M10A50 Buffer + substrate A
B11	H10M10A50 Buffer + substrate B
C11	H10M10A50 Buffer + substrate C
F12	H10M10A50 Buffer

The average luminescence of each sample, with its standard deviation, can be seen below:

Sample	Averages	St. dev
WT 11/10/21	53803.3	546.027
WT 3/22/19	208403	4223.30
WT 11/13/20	49895	875.851
rpsU1	36273.3	884.269
rpsU2	35388	260.397
rpsU3	77116.6	804.595



Western Blot

1. Add an equal volume of 2X SLB to each sample (saved from ribosome assay) - Mix by pipetting
2. Heat in thermocycler at 95C for 10 mins
3. Store in -20C freezer box

Tuesday, July 19, 2022**To Do:**

- ~~1- Western blot day 4~~

Western Blot Day 1

Today I will repeat the western blot to measure the levels of tul4 for each of the samples used in the assay yesterday.

Well	Sample	Volume (uL)
1	WesternSure Ladder	5
2	1- Wild-type <i>F. tularensis</i> 11/10/21 Before	10
3	2- Wild-type <i>F. tularensis</i> 3/22/19 Before	10
4	3- Wild-type <i>F. tularensis</i> 11/13/20 Before	10
5	4- <i>F. tularensis</i> Tn7::rpsU1-V Before	10
6	5- <i>F. tularensis</i> Tn7::rpsU2-V Before	10
7	6- <i>F. tularensis</i> Tn7::rpsU3-V Before	10
8	7- H10M10A50 Buffer Before	10
9	1- Wild-type <i>F. tularensis</i> 11/10/21 After	10
10	2- Wild-type <i>F. tularensis</i> 3/22/19 After	10
11	3- Wild-type <i>F. tularensis</i> 11/13/20 After	10
12	4- <i>F. tularensis</i> Tn7::rpsU1-V After	10
13	5- <i>F. tularensis</i> Tn7::rpsU2-V After	10
14	6- <i>F. tularensis</i> Tn7::rpsU3-V After	10
15	7- H10M10A50 Buffer After	10

*Before denotes samples taken before adding the luciferase assay reagent

Left the blot in blocking solution on the rocker overnight.

Wednesday, July 20, 2022**To Do:**

1. ~~Western blot day 2~~

Western Blot Day 2

Make primary antibody solution:

- 4.5 uL anti-VSVG (bS21)
- 10 uL anti-tul4
- 10 mL diluted blocking buffer
- Put in 50 mL tube and invert to mix

Rotate for 1 hour at room temperature

Meanwhile, make wash buffers:

1. 100 mL PBS-T
 - a. 10 mL 10X PBS
 - b. 90 mL ddiH2O
 - c. 500 uL NP-40
2. 30 mL PBS
 - a. 3 mL 10X PBS
 - b. 27 mL ddiH2O

Wash 4x on rotator for 10 minutes each, using 10 mL wash buffer per wash

Use 10 mL diluted blocking buffer and block again, for 15 min

Make secondary antibody solution: (keep in black box – light sensitive)

- 1 uL red anti-rabbit (bS21)
- 1 uL green anti-mouse (tul4)
- 10 mL PBS-T (wash w/ detergent)
- 10 uL 10X SDS
- Put in 50 mL tube and invert to mix

Rotate for 1 hour at room temperature

Wash 4x on rotator for 10 minutes each, using 10 mL wash buffer per wash

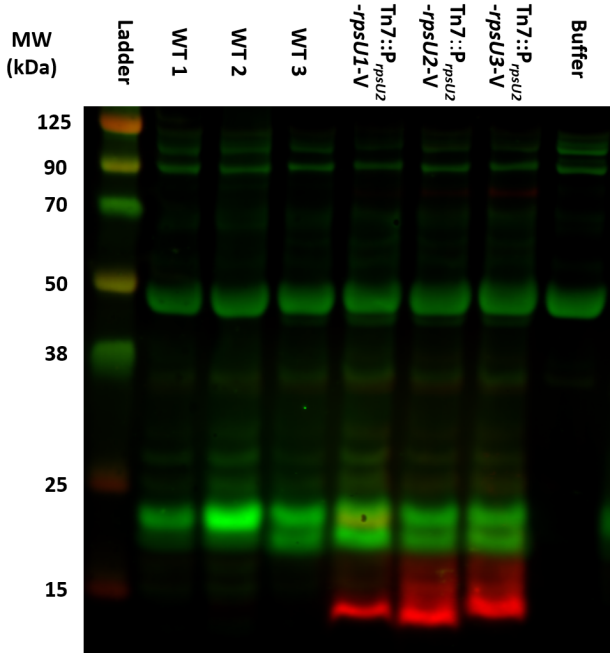
Wash 2x on rotator for 10 minutes each, using 10 mL of wash buffer WITHOUT detergent

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

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.

Blot	Protein	kDa	Primary Dilution	Secondary Dilution
1	bS21-VSVG (anti-VSVG)	9	1:2222	1:10,000 anti-rabbit, 680 CW (red)
	Tul4	17	1:1000	1:10,000 anti-mouse, 800 CW (green)



Thursday, July 21, 2022

To Do:

- ~~1. Work on poster~~

Friday, July 22, 2022

To Do:

- ~~1. Work on poster~~

Monday, July 25, 2022

To Do:

- ~~1. Make hemoglobin~~
- ~~2. Overnight cultures~~
- ~~3. Add primers to reaction tubes~~

Transformation Analysis / Overnight Cultures

Today I checked the transformation plates Hannah prepared last week of pKR127. This plasmid is the exact same backbone as mine (pKR144) but instead of the *tul4* gene it has another luciferase gene. Therefore, we can use the dual glo luciferase assay to measure the levels of translation from both the *pdpA* and *tul4* UTRs using the luminometer instead of having to perform an additional Western blot.

There were a ton of colonies present on the positive control plate (pKR144) and no colonies on the negative control plate. For the ligation plates, Hannah did both a regular ligation and a calculated one. The colony counts and ratios for each are below:

Backbone: 30
Regular: 105 (3.5)
Calculated: 312 (10.4)

Since the ratios were good for both ligations, I will prepare overnight cultures using one small colony from the regular plate, one big colony from the calculated plate, and two small colonies from the calculated plate. Hannah will use these tomorrow to miniprep and prepare for sequencing.

1. Add 5 mL of LB broth to a sterile glass culture tube
2. Add 5 uL of carbenicillin (Carb) to the tube
3. Vortex briefly to mix
4. Warm in shaking incubator
5. Using a stick, pick the isolated colony from the plate and inoculate the tube
6. Place in the 37°C shaking incubator overnight. ~16-22 hours

Put in the incubator at 3:20 pm.

- 1: small colony from regular late
- 2: big colony from calculated plate
- 3: small colony from calculated plate
- 4: small colony from calculated plate

Tuesday, July 26, 2022

To Do:

1. ~~Miniprep~~
2. ~~Sequence reactions~~
3. ~~Make MHB~~

MHB Protocol

1. Add 10.5 g MHB and 500 mL ddiH₂O to 1 L orange-topped jar
2. Shake to mix
3. Autoclave on 30 min. liquid cycle

Hannah minipreped the overnight cultures I made yesterday and prepared sequence reactions. Once we get the results back we can determine which miniprep has the correct sequence and proceed from there.

Thursday, July 28, 2022**To Do:**

- ~~1. Prepare SOC media~~
- ~~2. Transform pKR147~~
- ~~3. Isolate siderophore~~

SOC Media Protocol

1. Add 95 mL dH₂O to 250 mL orange-topped jar
2. Add and dissolve the following:
 - a. 2.0 g tryptone
 - b. 0.5 g yeast extract
 - c. 1 mL 1M NaCl
 - i. 1 mL 5 M NaCl + 4 mL dH₂O
 - d. 0.25 mL 1M KCl
3. Adjust to pH 7.0 using pH probe in INBRE
 - a. Add HCl or 5 N NaOH depending on starting pH
4. Autoclave in instapot for 30 mins
5. Cool in water bath to temperature below 60C
6. Add 1 mL filter-sterilized 2M Mg⁺²
 - a. Equal volumes sterile 1 M MgCl + sterile 1 M MgSO₄
 - b. Used Hannah's MgCl
 - c. MgSO₄: 40 mL dH₂O + 9.8 g MgSO₄
7. Add 1 mL filter-sterilized 2M glucose
 - a. 40 mL dH₂O + 14.45 g glucose
8. Add sterile dH₂O to a final volume of 100 mL

NEB 5-alpha Competent *E. coli* Transformation Protocol

Today I will transform more pKR147 so I will have enough minipreps to phenol-chloroform purify next week. Hannah checked the sequences of the previous minipreps we sent in and one out of the four looked good, so I will use that miniprep (miniprep 1) to proceed. The protocol is below:

Reaction table

Tube number	Purpose	DNA	Volume of DNA	Final volume to plate	Number of carbenicillin-containing plates
1	(+) control	pKR147	1 uL	20 uL, 100 uL	2
2	(-) control	None	0	20 uL, 100 uL	2
Total number of plates					4

1. Check to be sure you have enough LB plates with appropriate antibiotic. If plates were stored at 4°C, warm at 37°C until needed
2. Obtain DNA and thaw on ice if necessary
3. Thaw appropriate number of competent cell tubes (1 per reaction) on ice for 10 mins.
4. Add appropriate amount of DNA to each tube. Flick 4-5 times to mix. Do not vortex
5. Incubate cells on ice for 30 minutes. During incubation, find or set heat block to 42°C. Do not mix

6. Place tubes with cells and DNA onto 42°C heatblock for 30 seconds (heat shock step). Do not mix
7. After heat shock, place tubes back on ice for 5 mins. Do not mix
8. Using aseptic technique, add 950 uL of room temperature SOC media to each microfuge tube
9. Using autoclave tape, tape microfuge tubes down in shaking incubator set to 37°C.
10. Allow cells to recover for 1 hour at 37°C, shaking. Place in a rack after shaking (NOT back on ice).
11. Using aseptic technique, plate indicated amount of cells on appropriate antibiotic plates (LB-Carb), spreading until plates look dry. For “remaining” volume, spin tubes at max speed in benchtop centrifuge for 30 seconds. Remove 800 uL of media. Using 200 uL pipette, resuspend cells at bottom of tube and plate all the remaining culture.

Left plates on bench at room temperature over the weekend.

Fractionating Siderophore from Conditioned Media Protocol

1. Set up StrataX 3 mL column on vacuum manifold
2. Run 2 column volumes of methanol through column
 - a. Collect in waste bucket
3. Run 4 column volumes of water through column
 - a. Collect in waste bucket
4. Run sample through column (initial test with 12 mL)
 - a. Collect flow through in medium vial
5. Run 2 column volumes of water through column
 - a. Collect in waste bucket
6. Wash column with 2.5 mL methanol
 - a. Collect in small vial
7. Remove column from vacuum (confirm with Dr. Rowley for disposal)
8. Run small sample vial in Speedvac on manual run for 1-1.5 hours (until methanol is fully gone)
9. Filter-sterilize remaining conditioned media (collected in medium vial) – save in 4C fridge

Obtained approximately 125 uL of siderophore from the ~13 mL conditioned media. The OD600 of the conditioned media I used (from June 8th, 2022) was approximately 1.46.

Assuming that the siderophore bound to the membrane in the column, the remaining conditioned media should only contain any other nutrients that may be present. I left both this remaining media and the siderophore in the 4C fridge.

4 mL cultures (split siderophore 4 ways)

Measure 600 uL at time 0 (before supplementing)

Determine proportion of siderophore to media and make a blank (1 mL)

Start cultures above 4 mL, after taking OD600 should end up at 4 mL, then supplement

Save blanks for next day (put parafilm on cuvette and put in fridge)

Do 600 uL if below 0.1

Do 300 uL if above 0.1 and dilute with 300 uL MHB

(take 300 uL of supplemented blank and 300 uL MHB for blank)

125 uL siderophore:

28 uL to each culture

7 uL to blank

August 2022

Monday, August 1, 2022

To Do:

1. ~~Streak out Δ rpsU2~~

Growth Experiment

Today I streaked out Δ rpsU2 on a non-antibiotic CHAH plate and left it in the standing incubator. This will be used for the growth experiment on Wednesday.

Transformation Analysis

I also checked the transformation plates I had left on my bench over the weekend. There were no colonies on the negative control plates and there was growth on the two positive plates but the colonies were still very small. This was probably due to the AC being on all weekend so they did not grow very well. I put them in the 30C incubator overnight to allow them to grow.

Tuesday, August 2, 2022

To Do:

1. ~~Streak out LVS~~
2. ~~Prepare overnight cultures~~

Growth Experiment

Today I streaked out LVS on a non-antibiotic CHAH plate and left it in the standing incubator overnight. I also made preparations for the growth experiment.

Transformation Analysis / Overnight Cultures

The transformation plates looked good, with lots of growth on both positive control plates. In the future I should use 5 uL instead of 100 uL since it was difficult to pick isolated colonies.

I prepared 8 overnight cultures using LB-Carb media and left them in the shaking incubator set to 30C overnight at 2:55 pm.

Wednesday, August 3, 2022

To Do:

- ~~1. Growth experiment~~
- ~~2. Miniprep~~

Growth Experiment

Today I will perform a growth experiment to test if the addition of siderophore results in the loss of the growth defect in $\Delta rpsU2$.

I will grow 8 cultures using different media and different cells and compare their growth:

- 2 cultures of LVS in fresh MHB
- 2 cultures of LVS in supplemented fresh MHB
- 2 cultures of $\Delta rpsU2$ in fresh MHB
- 2 cultures of $\Delta rpsU2$ in supplemented fresh MHB

I prepared the cultures by first scraping the cells into their own tubes and diluting 1:20 in a cuvette using fresh MHB.

LVS: OD600 = 0.208 (added 88.4 μ L to each 4.6 mL culture tube)

$\Delta rpsU2$: OD600 = 0.190 (added 96.8 μ L to each 4.6 mL culture tube)

I then checked the starting OD600s of each culture (600 μ L).

I then added 26 μ L of siderophore to half of the 4 mL cultures to make supplemented fresh MHB.

Started at 10:32 am.

I then checked the OD600s every 2 hours for 6 hours total.

As a blank for the supplemented fresh MHB I added 6.5 μ L of siderophore to 1 mL of fresh MHB. I stored it in the fridge covered in parafilm throughout the day and overnight.

Left in the shaking incubator to grow overnight starting around 4:50 pm.

Miniprep Protocol

Today I will miniprep the overnight cultures of pKR147. I noticed that the overnight cultures had very little growth. This is probably a result of how small the colonies were as well as growing them at a lower temperature. Also, I dropped culture 1 when taking them out of the incubator so now there are only 7 minipreps.

1. Pellet bacterial overnight culture by centrifugation (max speed for 3 min.) at room temp.
2. Resuspend pelleted bacterial cells in 250 μ L Buffer P1 (in fridge) and transfer to micro centrifuge tube
3. Add 250 μ L Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear
4. Add 350 μ L Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times
5. Centrifuge for 10 min. at 13000 rpm

6. Apply 800 uL supernatant from previous step to QIAprep 2.0 spin column by pipetting
7. Centrifuge for 1 min and discard flow through
8. Wash column with 0.5 mL Buffer PB
9. Centrifuge for 1 min and discard flow through
10. Wash column with 0.75 mL Buffer PE
11. Centrifuge for 1 min and discard flow through (wash with PE 3x)
12. Centrifuge for 3 min.
13. Place column in 1.5 mL microcentrifuge tube; add 50 uL 0.1X Buffer EB to center and let stand 1 min.
14. Centrifuge for 1 min.
15. Save in -20 fridge

I then checked the concentrations using the nanodrop:

- 2: 3.5 ng/uL
- 3: 10.1 ng/uL
- 4: 4.9 ng/uL
- 5: 5.5 ng/uL
- 6: 7.4 ng/uL
- 7: 9.5 ng/uL
- 8: 5.4 ng/uL

Since all of the concentrations were extremely low, I decided not to continue on with the phenol-chloroform purification. I will prepare 8 new overnight cultures using the same transformation plate, which I had saved on my bench, using LB-Carb media. I also prepared an LB-Carb blank. Left them in Dr. Rowley's shaking incubator set to 30C at 1 pm.

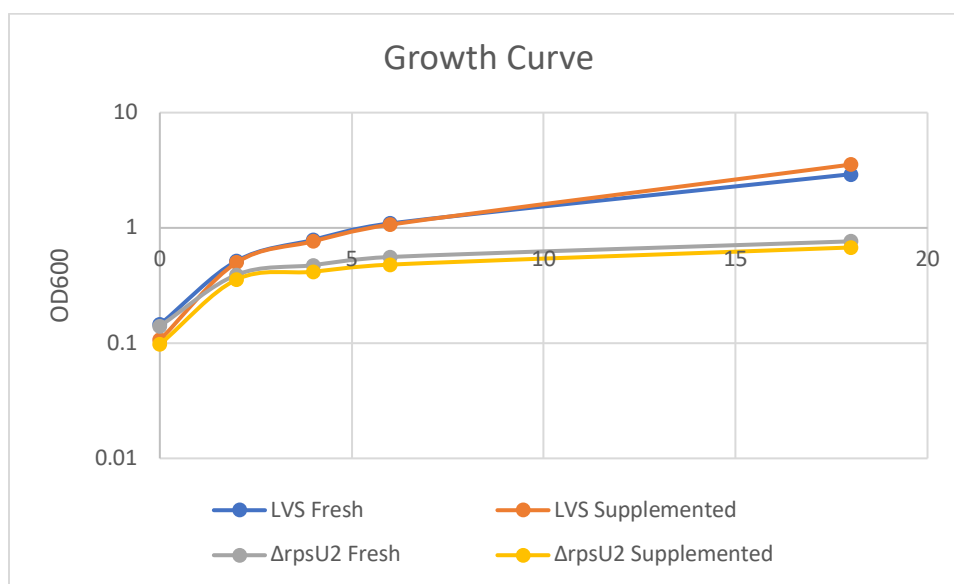
Thursday, August 4, 2022

To Do:

1. ~~Finish growth experiment~~
2. ~~Miniprep~~

Growth Experiment

I took out the culture tubes at 10:40 am and checked their final OD600s. All four $\Delta rpsU2$ tubes visibly looked like they grew less than LVS. Also, the two LVS in supplemented media had precipitate.



When viewing the graph above, you can see that there is no difference between the strains grown in fresh MHB and siderophore supplemented MHB. $\Delta rpsU2$ has a clear growth defect compared to LVS, which was not altered when grown in supplemented media. This means that either it is not siderophore which leads to the loss of the growth defect as seen in previous experiments, or what we fractionated from the conditioned media was not actually siderophore. Alternatively, we might not have supplemented the fresh MHB with enough of the fractionate to result in a noticeable difference.

Miniprep

The overnight cultures look like they barely grew again. They were only slightly cloudier than the LB control I used.

I took out cultures 3 and 7 from the incubator at 11 am and left the rest of the cultures to grow. I then miniprepmed the two cultures, this time eluting in 30 μ L of 0.1X EB. When I added the buffer to the membrane to elute, I let it sit for 1 min. and then spun down. I then pipetted the elution back onto the membrane and spun it again.

I then checked the concentrations using the nanodrop:

3: 15.3 ng/ μ L

7: 11.9 ng/ μ L

Friday, August 5, 2022

To Do:

- ~~1. Clean out lab~~

I had left the overnight cultures from Wednesday in the shaking incubator to see if they would every grow. They did, however I decided not to miniprep them since whatever is growing most likely doesn't contain the plasmid since it would have had time to develop antibiotic resistance.