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## December 2020

### Monday, December 14, 2020

#### To Do:

1. Streak LVS,  $\Delta$ pmrA,  $\Delta$ pmrA(sup)
2. Review RNA isolation protocol
3. Review excel spreadsheet

#### Methods:

2 plates each of LVS,  $\Delta$ pmrA, and  $\Delta$ pmrA(sup) were streaked for isolation.

Going forward throughout the RNA isolation protocol the samples will be known as these numbers:

Sample #	Sample Descriptor	Strain
KB1	LVS WT	-
KB2	LVS WT	-
KB3	$\Delta$ pmrA	KRLVS 40.1
KB4	$\Delta$ pmrA	KRLVS 40.1
	$\Delta$ pmrA(sup	
KB5	)	KMLFT 37.1
	$\Delta$ pmrA(sup	
KB6	)	KMLFT 37.1

### Tuesday, December 15, 2020

#### To Do:

1. ~~Streak LVS,  $\Delta$ pmrA,  $\Delta$ pmrA(sup)~~
2. ~~Review RNA isolation protocol~~
3. ~~Review excel spreadsheet~~
4. Review RNase free
5. Review qPCR
6. Gravity cycle
7. Refill pipette tips

#### Discussion

Met with Kathryn to discuss next steps. Today will be light, mainly lab chores and looking over the protocols for tomorrow. I will scrape only 3 plates tomorrow to set up cultures and then observe Kathryn while she does the RNA isolation.

### Wednesday, December 16, 2020

#### To Do:

1. ~~Review RNase-free~~
2. ~~Review qPCR~~
3. ~~Gravity cycle~~
4. ~~Refill pipette tips~~
5. Set up culture for RNA isolation
6. RNA isolation

#### Methods:

#### Culture growth for RNA isolation

##### Day -3

Streak strains to single colony

##### Day 0

Patch out single colonies in triplicate

##### Day 1 (RNA isolation day)

For each sample, scrape up one patch of cells and resuspend in ~300 uL MHB in sterile 1.5 mL tube  
Add 700 uL more MHB, be sure cells are well-resuspended

Check OD600:

Dilute resuspended cells 1:10 in MHB (100 uL cells, 900 uL MHB)

Check OD600 using 1 mL MHB as blank

Calculate actual OD600 (measured OD600 multiplied by dilution factor [10])\*

Calculate what volume of cells you need to obtain an OD600 of 0.08 in 8 mL\*

For each sample, inoculate one glass culture tube so that the final OD600 is 0.08

Example:

OD600 of resuspended cells: 4.0

$(8 \text{ mL})(0.08 \text{ OD600}) / (4.0 \text{ OD600}) = 0.16 \text{ mL resuspended cells (160 uL)}$

Either:

-Add 7 mL MHB per tube and add 940 uL more MHB and 160 uL resuspended cells

OR

-Add 8 mL MHB per tube, remove 160 uL, and add back 160 uL resuspended cells

Swirl tube to distribute cells

Remove 1 mL and use to measure OD600

Put tubes at 37°C shaking

Check OD600 after ~ 2 hours to be sure cells have come close to doubling (document OD600!\*)

When OD600 reaches between 0.3 – 0.4 (4-6 hours, document OD600!\*):

Transfer 1.8 mL into 2 mL tube

Centrifuge max speed at 4°C for 3 minutes

Resuspend cell pellet in 1 mL TRI-Reagent (step 1 of RNA isolation protocol)

\*Set up an Excel worksheet

As previously discussed with Kathryn, I only set up 3 of the 6 plates for cultures: KB1, KB3, KB5. KB3 was inhibited, and although Kathryn suggested using a 1:20 dilution, I tried it as 1:10 because I had a good loop-ful. Below are the ODs:

Sample #	Measured OD600	Dilution Factor	Actual OD600	Desired Volume mL	Desired OD	Volume of Resuspended Cells $\mu$ L
KB1	0.258	10	2.58	8	0.08	248.06
KB2						
KB3	0.159	10	1.59	8	0.08	402.52
KB4						
KB5	0.192	10	1.92	8	0.08	333.33
KB6						

I set up the cultures by aliquoting 8 mL of MHB, then I removed the necessary volume of the cells, and added that volume of cells to the tube. I re-checked the OD and got 0.076, 0.064, 0.062, respectively. Unfortunately, I dropped KB1 and shattered it on the way to the shaking incubator. I made a new suspension (this time the OD was 0.081!) and put the tubes on the shaker at 10:33am.

At 12:30 pm:

Sample #	Measured OD600	Dilution Factor	Actual OD600	Desired Volume mL	Desired OD	Volume of Resuspended Cells $\mu$ L	Doubling time (2 hours)	Doubling OD
KB1	0.258	10	2.58	8	0.08	248.06	12:30pm	0.113
KB2								
KB3	0.159	10	1.59	8	0.08	402.52	12:30pm	0.092
KB4								
KB5	0.192	10	1.92	8	0.08	333.33	12:30pm	0.101
KB6								

At 2:30 pm:

KB1 0.195

KB3 0.141

KB5 0.187

Final read:

Sample #	Measured OD600	Dilution Factor	Actual OD600	Desired Volume mL	Desired OD	Volume of Resuspended Cells $\mu$ L	Doubling time (2 hours)	Doubling OD	Final Time	Final OD
KB1	0.258	10	2.58	8	0.08	248.06	12:30pm	0.113	4:30pm	0.317
KB2										
KB3	0.159	10	1.59	8	0.08	402.52	12:30pm	0.092	4:30pm	0.218
KB4										
KB5	0.192	10	1.92	8	0.08	333.33	12:30pm	0.101	4:30pm	0.319
KB6										

Although the OD is a little low for KB3 Kathryn says to proceed. I observed her perform the RNA isolation up to step 18, then we stored the samples at  $-80^{\circ}\text{C}$ .

## RNA isolation

### Purify nucleic acids

1. Resuspend cell pellet (1.8 mL - 10 mL) in 1 mL TRI-Reagent
2. Incubate at  $60^{\circ}\text{C}$  for 10 min
3. Spin at  $4^{\circ}\text{C}$  for 10 min at max speed
4. Transfer supernatant to new 2mL tube ( $\sim 950$   $\mu\text{L}$ ).
5. Add equal volume 100% ethanol
6. Pass sample over Directzol column, 600  $\mu\text{L}$  per spin, 30 sec per spin at max speed, discarding flow-through in phenol and methanol waste
7. Place spin column in new collection tube
8. Wash twice with 400  $\mu\text{L}$  RNA PreWash buffer, discarding flow-through in phenol and methanol waste
9. Add 700  $\mu\text{L}$  of Wash buffer, let sit on column for 3 min
10. Spin max speed for 2 min
11. Wash again with 700  $\mu\text{L}$  Wash buffer
12. Spin max speed for 2 min
13. Place column in new collection tube
14. Spin max speed for 3 min
15. Place column in clean 1.5 mL tube
16. Add 90  $\mu\text{L}$  RNase-free water, let sit on column 2 min
17. Spin max speed 1 min

18. Place flow-through on column again, spin 1 min

Store nucleic acids at -80°C if not moving directly to the next step

### DNase treatment

1. Add 10 uL RNase-free DNase buffer and 10 uL RNase-free DNase (Promega, RQ1)
2. Incubate at 37°C for 1 hour
3. Add 300 uL TRI-Reagent
4. Add 400 uL 100% ethanol
5. Go to step 6 in "Purify nucleic acids" protocol above and follow protocol, elute purified RNA in 100 uL RNase-free water.

Check RNA concentration and quality by Nanodrop. The 260/280 ratio should be close to 2.0 and the 260/230 ratio should be greater than 1.9.

**Friday, December 18, 2020**

To Do:

- ~~1. Set up culture for RNA isolation~~
- ~~2. RNA isolation~~
3. DNase treatment

### Methods:

Picking up from Wednesday I am performing the DNase treatment on the 3 samples. At first I took out the incorrect buffer and enzyme, and I was just about to add them to my samples when I thought I should double check to make sure they were correct. Turns out they were not, so after some back and forth with Hannah and Kathryn, I got the right materials. However, my samples were on the bench had started to thaw. I put them back in the -80 while the buffer thaws. I hope they didn't degrade. Went into the incubator at about 12:15.

AT 1:15 I proceeded with the rest of the protocol as normal. I checked concentration on the NanoDrop:

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	KB1	RI-INBRE	12/18/2020 2:28:01 PM	242.2	ng/μl	4.84	2.25	2.15	2.14	DNA	50
2	KB3	RI-INBRE	12/18/2020 2:29:13 PM	184.5	ng/μl	3.69	1.71	2.16	2.28	DNA	50

			12/18/2020								
		RI-	0 2:29:51			6.04	2.76				
3	KB5	INBRE	PM	302.3	ng/μl	7	6	2.19	2.42	DNA	50

I forgot to set the NanoDrop as RNA rather than DNA, so I have to do some math to get the actual concentration. I also didn't record the graphs, have to remember that for next time. But the numbers so far look promising that there is little contamination. I will run them on a gel on Monday.

Actual RNA concentrations of samples: KB1 193.8 ng/ul  
 KB3 147.6 ng/ul  
 KB5 241.8 ng/ul

**Monday, December 21, 2020**

#### To Do:

1. ~~DNase treatment~~
2. Run gel on isolated RNA
3. Patch out 3 strains again
4. Dissolve iron pyrophosphate for MHB tomorrow
5. Pick proteins with Hannah for qRT-PCR project

#### Methods:

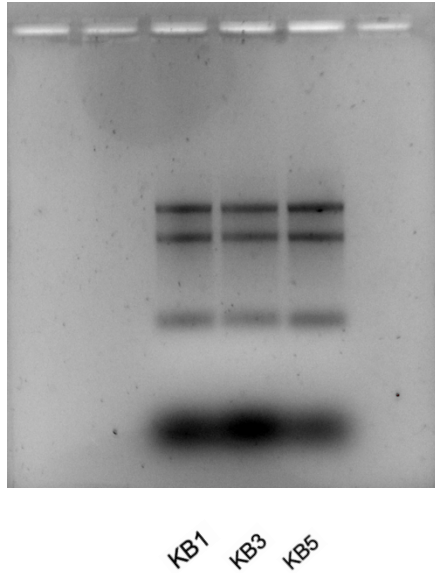
Today I'm running a gel on the RNA to make sure it is pure.

Reaction Table:

Sample #	RNA concentration (ng/μL)	Amount RNA (μg)	Volume RNA (μL)	Volume H2O (μL)	Total volume (μL)
KB1	193.8	1.5	7.74	4.26	12
KB3	147.6	1.5	10.16	1.84	12
KB5	241.8	1.5	6.20	5.80	12

Plus 3 μL loading dye per well.





The gel looks good! No sign of degradation.

Patched the 3 strains again to start RNA isolation on my own tomorrow. I need more MHB, so I dissolved iron pyrophosphate. Hannah and I went over her list of proteomics to choose 3 proteins for me to PCR. So far, we narrowed it down to 6, will consult with Kathryn. Hannah showed me how to order primers.

**Tuesday, December 22, 2020**

**To Do:**

1. ~~DNase treatment~~
2. ~~Run gel on isolated RNA~~
3. ~~Patch out 3 strains again~~
4. ~~Dissolve iron pyrophosphate for MHB tomorrow~~
5. Pick proteins with Hannah for qRT-PCR project
6. Design and order primers
7. RNA isolation

**Discussion:**

There isn't enough growth on my patches to set up RNA isolation today. I'm re-inoculating them till tomorrow. Hannah and I narrowed down our list of proteomics. After consulting with Kathryn we decided on FTL\_0097, FTL\_1181, and FTL\_1883. I designed and ordered the primers, 18 total.

**Wednesday, December 23, 2020**

**To Do:**

1. ~~Pick proteins with Hannah for qRT-PCR project~~
2. ~~Design and order primers~~
3. Supplement MHB
4. Make hemoglobin
5. Autoclave waste
6. Set up cultures for RNA isolation
7. RNA isolation

**Methods:**

I supplemented the MHB with iron pyrophosphate, 10% glucose, and Isovitalex. I set up a sterility check by putting 5 mL of MHB in a culture tube and put in the shaking incubator. I can't leave it overnight, I will check it by OD at end of day. I also made 4X 300 mL of hemoglobin and stored them in the flasks in the fridge.

**Culture Set Up:**

I set up 2 samples each of the patches I made two days ago. LVS grew fine but the other two are inhibited. I was able to scrape enough from each plate to get 2 samples each, although I overdid it for KB3 and had to dilute that 1:20. The rest were diluted 1:10.

Sample #	Measured OD600	Dilution Factor	Actual OD600	Desired Volume mL	Desired OD	Volume of Resuspended Cells $\mu$ L
KB1	0.124	10	1.24	8	0.08	516.13
KB2	0.156	10	1.56	8	0.08	410.26
KB3	0.117	20	2.34	8	0.08	273.50
KB4	0.113	10	1.13	8	0.08	566.37
KB5	0.121	10	1.21	8	0.08	528.93
KB6	0.092	10	0.92	8	0.08	695.65

The 8 mL cultures went in the 37°C shaking incubator at about 10:15am.

I forgot to check the OD before putting them in the incubator.

Sample #	Measured OD600	Dilution Factor	Actual OD600	Desired Volume mL	Desired OD	Volume of Resuspended Cells $\mu$ L	OD After Resuspension	OD at 2 hours 1215	OD at 1445	Final OD at 1600
KB1	0.124	10	1.24	8	0.08	516.13		0.125	0.238	0.327
KB2	0.156	10	1.56	8	0.08	410.26		0.125	0.243	0.334
KB3	0.117	20	2.34	8	0.08	273.50		0.08	0.123	0.164
KB4	0.113	10	1.13	8	0.08	566.37		0.106	0.164	0.211
KB5	0.121	10	1.21	8	0.08	528.93		0.149	0.262	0.367
KB6	0.092	10	0.92	8	0.08	695.65		0.121	0.211	0.279

Even though KB3 is less than 2, Kathryn said it was ok to go to the next step. I performed the RNA isolation protocol. Some notes: the first 3 minute 4°C spin didn't actually get to 4°C, but the 10 minute second one did. At one point, while taking the tubes out of the regular centrifuge, they were too close together and one of them popped out. The column was fine, but if there's contamination, that could be why. That was KB4.

After the samples were eluted with 90 uL RNase free water I stored them at -80°C for the holiday.

## January 2021

### Monday, January 4, 2021

#### To Do:

1. ~~Supplement MHB~~
2. ~~Make hemoglobin~~
3. ~~Autoclave waste~~
4. ~~Set up cultures for RNA isolation~~
5. ~~RNA isolation~~
6. DNase
7. Run gel
8. Primer reconstitution

#### Methods and Results:

##### DNase

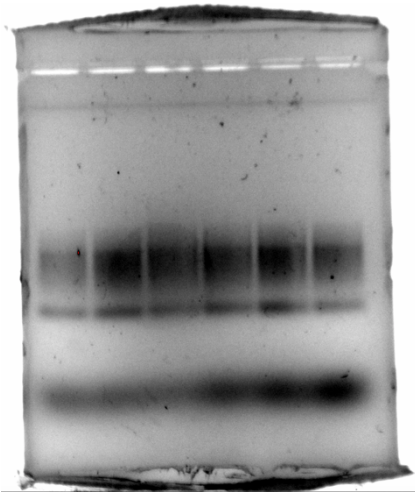
The DNase protocol was followed and I took the concentrations of the samples on the NanoDrop:

Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
KB1	RI-INBR E	1/4/2021 12:31:32 PM	139.4	ng/μl	3.48 5	1.62 8	2.14	2.4	RNA	40
KB2	RI-INBR E	1/4/2021 12:32:19 PM	137.5	ng/μl	3.43 7	1.61	2.14	2.34	RNA	40
KB3	RI-INBR E	1/4/2021 12:32:54 PM	70.2	ng/μl	1.75 5	0.83 6	2.1	2.3	RNA	40
KB4	RI-INBR E	1/4/2021 12:33:30 PM	97.4	ng/μl	2.43 5	1.14 8	2.12	2.4	RNA	40

KB5	RI- INBR	1/4/2021 12:34:10				1.87					
	E	PM	161.6	ng/μl	4.04	5	2.15	2.41	RNA	40	
KB6	RI- INBR	1/4/2021 12:34:47				1.64					
	E	PM	141.6	ng/μl	3.54	6	2.15	2.44	RNA	40	

The  $\Delta$ pmrA has low concentration, but I continued and ran a gel.

Sample #	RNA concentration (ng/ $\mu$ L)	Amount RNA ( $\mu$ g)	Volume RNA $\mu$ L	Volume H2O $\mu$ L)	Total volume $\mu$ L
KB1	139.4	1	7.17	8.83	16
KB2	137.5	1	7.27	8.73	16
KB3	70.2	1	14.25	1.75	16
KB4	97.4	1	10.27	5.73	16
KB5	161.6	1	6.19	9.81	16
KB6	141.6	1	7.06	8.94	16



I consulted with Kathryn and we're not sure why the gel looks like this, she doesn't think it's degraded. I will run a repeat gel on the 2 highest concentration samples; one from each set up.

Sample #	RNA concentration (ng/ $\mu$ L)	Amount RNA ( $\mu$ g)	Volume RNA $\mu$ L	Volume H2O $\mu$ L)	Total volume $\mu$ L
KB5-1	241.8	1.5	6.20	5.80	12
KB5-2	161.6	1.5	9.28	2.72	12

I tried the technique of putting the gel holder into the rig sideways instead of using tape on the sides but it broke as I was trying to change the position so I will not try that again, I'll stick with tape. I'll repeat the gel tomorrow.

### Primer Reconstitution:

The 18 primers that I ordered for qRT-PCR came in. I added the appropriate volume of 0.1x EB to make 100 uMol stocks.

Primer	Concentration (nMol)	Volume Buffer Needed ( $\mu$ L)
KROL377	28.5	285
KROL378	32.1	321
KROL379	29.3	293
KROL380	33.2	332
KROL381	31.3	313
KROL382	31	310
KROL383	33.2	332
KROL384	30.4	304
KROL385	28.8	288
KROL386	27.6	276
KROL387	31.3	313
KROL388	23.5	235
KROL389	31.5	315
KROL390	29.9	299
KROL391	29.5	295
KROL392	27.8	278
KROL393	30.7	307
KROL394	30.4	304

I put them in the 42°C heat block for 5 minutes, then made paired 5uMol stock solutions for qRT-PCR. The 5 uMol stocks are stored at -20 and the 100 uMol stocks are stored at -80.

Primer pairs:

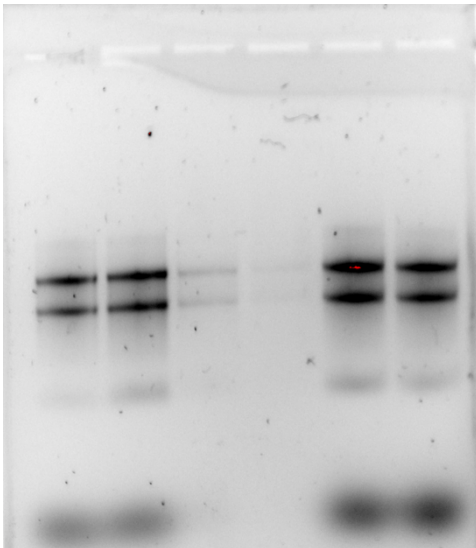
KROL377/KROL378 KROL379/KROL380 KROL381/KROL382

KROL383/KROL384 KROL385/KROL386 KROL387/KROL388

KROL389/KROL390 KROL391/KROL392 KROL393/KROL394

**Tuesday, January 5, 2021****To Do:**

1. ~~DNase~~
2. ~~Run gel~~
3. ~~Primer reconstitution~~
4. Repeat gel
5. cDNA preparation reaction 1 and 2

**Methods and Results:**

tRNA

Gel looks better, now onto cDNA preparation. I am setting up all 9 samples that I've made thus far, so I am renaming my samples. From the first setup KB1, 3, and 5 will be RNA1-3 respectively, and from the second setup KB1-6 will be RNA4-9 respectively.

12/18/20

KB1 -&gt; RNA1

KB3 -&gt; RNA2

KB5 -&gt; RNA3

1/4/21

KB1 -&gt; RNA4

KB2 -&gt; RNA5

KB3 -&gt; RNA6

KB4 -&gt; RNA7

KB5 -&gt; RNA8

KB6 -&gt; RNA9

## Generate cDNA

Adapted from Lory lab microarray protocol

Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	5 - 10 ug	267 - 333 ng/ ul
(NS) <sub>5</sub> Primer (250 ng/ul)	3 ul	25 ng/ul
RNase-free water	up to 30 ul	

Incubate using program JSScDNA1:

Step	Temp	Time
1	70°C	10'
2	25°C	10'
3	4°C	hold

While waiting, prepare the cDNA synthesis reaction in master mix format:

Component	Volume or Amount	Final Concentration
5X 1st strand buffer	12 ul	1x
RNase-free water	5.75 ul	
100 mM DTT	6.0 ul	10 mM
10 mM dNTPs	3.0 ul	0.5 mM
Superscript III (200 U/ul)	3.25	10.8 U/ul

Aliquot 30 ul per reaction

Incubate using program JSScDNA2

Step	Temp	Time
1	25°C	10'
2	37°C	60'
4	42°C	60'
5	70°C	10'
6	4°C	hold

**Remove RNA**

Transfer reactions to 1.5 mL tubes

Add 20 ul of 1N NaOH

Incubate 65°C for 30'

Neutralize with 20 ul of 1N HCl

Final volume is 100 ul

Purify cDNA using Qiagen PCR clean-up column

Elute in 60 ul of 0.1x EB

**Generate cDNA (half protocol)**

**REACTION SIZE CUT IN HALF** from Lory lab microarray protocol

Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
(NS) <sub>5</sub> Primer (250 ng/ul)	1.5 ul	25 ng/ul
RNase-free water	up to 15 ul	

Sample #	Strain	Date Isolated	RNA conc	RNA	H2O	Total Desired	Total Volume	Actual RNA	NS5 Volume	Total Volume
RNA1	LVS	12/18/20	193.8	5.16	8.34	1	13.5		1.5	15
RNA2	ΔpmrA	12/18/20	147.6	6.78	6.72	1	13.5		1.5	15
RNA3	ΔpmrA sup	12/18/20	241.8	4.14	9.36	1	13.5		1.5	15
RNA4	LVS	1/4/21	139.4	7.17	6.33	1	13.5		1.5	15
RNA5	LVS	1/4/21	137.5	7.27	6.23	1	13.5		1.5	15
RNA6	ΔpmrA	1/4/21	70.2	14.25	-0.75	1	13.5	13.5	1.5	15
RNA7	ΔpmrA	1/4/21	97.4	10.27	3.23	1	13.5		1.5	15
RNA8	ΔpmrA sup	1/4/21	161.6	6.19	7.31	1	13.5		1.5	15
RNA9	ΔpmrA sup	1/4/21	141.6	7.06	6.44	1	13.5		1.5	15

Incubate using program JSScDNA1:

Step	Temp	Time
1	70°C	10'



2	25°C	10'
3	4°C	hold

While waiting, prepare the cDNA synthesis reaction in master mix format:

Component	Final Concentration	Volume	x 22.5
5X 1st strand buffer	1x	6	135
RNase-free water		2.88	64.8
100 mM DTT	10 mM	3	67.5
10 mM dNTPs	0.5 mM	1.5	33.75
Superscript III (200 U/ul)	10.8 U/ul	1.63	36.68

Aliquot 15 ul per reaction

Incubate using program JSScDNA2

Step	Temp	Time
1	25°C	10'
2	37°C	60'
4	42°C	60'
5	70°C	10'
6	4°C	hold

The first reaction went smoothly, and for the most part so did the second reaction except for RNA 8, too much master mix ended up in the tube (picked up the wrong pipette after getting a new box of strips). Some of the master mix stayed on top of the tube so I took that out and then set up 8 and 9 with 1x master mix each. 9 should be fine, but 8 probably will not.

**Wednesday, January 6, 2021**

#### To Do:

1. ~~Repeat gel~~
2. ~~cDNA preparation reaction 1 and 2~~
3. Purify cDNA
4. Check cDNA concentration
5. Make cDNA stocks
6. Perform qRT PCR
7. Run qRT PCR plate

#### Methods and Results:

##### Remove RNA (cDNA Purification)

Add 10 ul of 1N NaOH  
 Incubate 65°C for 30'  
 Neutralize with 10 ul of 1N HCl  
 Final volume is 50 ul  
 Purify cDNA using Qiagen PCR clean-up column  
 Elute in 60 ul of 0.1x EB

Check concentration by Nanodrop

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	RNA1	RI-INBRE	1/6/2021 12:37:52 PM	13.6	ng/ul	0.271	0.158	1.72	2.12	DNA	50
2	RNA2	RI-INBRE	1/6/2021 12:38:48 PM	17	ng/ul	0.341	0.194	1.75	1.86	DNA	50
3	RNA3	RI-INBRE	1/6/2021 12:39:27 PM	15.4	ng/ul	0.308	0.182	1.69	1.81	DNA	50
4	RNA4	RI-INBRE	1/6/2021 12:40:01 PM	15.9	ng/ul	0.317	0.193	1.64	1.6	DNA	50
5	RNA5	RI-INBRE	1/6/2021 12:40:45 PM	17	ng/ul	0.34	0.198	1.72	1.87	DNA	50
6	RNA6	RI-INBRE	1/6/2021 12:41:29 PM	16.2	ng/ul	0.323	0.186	1.74	1.97	DNA	50
7	RNA7	RI-INBRE	1/6/2021 12:42:08 PM	13.2	ng/ul	0.265	0.16	1.66	1.59	DNA	50
8	RNA8	RI-INBRE	1/6/2021 12:42:44 PM	8.9	ng/ul	0.178	0.104	1.72	1.37	DNA	50
9	RNA9	RI-INBRE	1/6/2021 12:43:24 PM	12.4	ng/ul	0.249	0.141	1.77	1.46	DNA	50

## Real-Time PCR on cDNA samples

Original protocol by Heather McManus, edited by Kathryn Ramsey

Each ChIP experiment will need at least one test primer and one control primer for both the input and ChIP samples

Example: 3 biological replicates = 3 DNA samples  
 Using two primer pairs = 6 different reactions

Each different reaction type must be run in triplicate on the real-time plate

Example: 6 reactions = 18 wells

Each Individual reaction = 20 µL

10 µL PowerUp SYBR Green Master Mix  
 1 µL 5uM combined forward and reverse primer  
 1 µL cDNA (~1.5 ng/ul, can base off efficiency tests)  
 8 µL ddiH<sub>2</sub>O

The use of master mixes increases the consistency between samples; therefore follow the method below for setting up the plate

For each reaction type (primer/DNA combination) set up a master mix equal to 3.5 reactions:

- 1) Put 3.5µL of DNA type into strip tubes
- 2) To determine the amount of each primer master mix to create, identify the number of reactions for each primer pair (i.e. #DNA samples x 3.5) and add 3.5 to this number to account for additional pipetting error  
 Example: 6 DNA samples per primer pair = 6 x 3.5 + 3.5 = 24.5

- 3) Set up primer master mixes in separate 1.5 mL tubes (make one for each primer pair)

Example:

$$\begin{array}{rcl}
 10 \mu\text{L PowerUp SYBR Green Master Mix} & \times 24.5 & = 245.0 \mu\text{L} \\
 1 \mu\text{L } 5\mu\text{M combined F and R primers} & \times 24.5 & = 24.5 \mu\text{L} \\
 8 \mu\text{L ddiH}_2\text{O} & \times 24.5 & = 171.5 \mu\text{L} \\
 \hline
 \text{TOTAL} & & = 465.5 \mu\text{L}
 \end{array}$$

- 4) Add primer master mix to tubes containing DNA.  
 3.5 reactions  $\times$  20  $\mu\text{L}$  volume = 70  $\mu\text{L}$ . DNA tubes already have 3.5 $\mu\text{L}$  of DNA. Add 66.5  $\mu\text{L}$  of primer master mix to each tube
- 5) Pipette 20  $\mu\text{L}$  of each reaction into 3 separate wells on the 96 well plate using dispense option on multichannel
- 6) Spin plate down
- 7) Place in real-time machine and run using the same program used to determine that the primers are appropriately efficient.

Sample #	Strain	cDNA stock conc (ng/ $\mu\text{L}$ )	cDNA new conc	Total Desired Volume	Volume cDNA	0.1xEB
cDNA1	LVS	13.6	1.5	3	27.2	24.2
cDNA2	$\Delta\text{pmrA}$	17	1.5	3	34	31
cDNA3	sup	15.4	1.5	3	30.8	27.8
cDNA4	LVS	15.9	1.5	3	31.8	28.8
cDNA5	LVS	17	1.5	3	34	31
cDNA6	$\Delta\text{pmrA}$	16.2	1.5	3	32.4	29.4
cDNA7	$\Delta\text{pmrA}$	13.2	1.5	3	26.4	23.4
cDNA8	sup	8.9	1.5	3	17.8	14.8
cDNA9	sup	12.4	1.5	3	24.8	21.8

Note that the sample names are now cDNA1-9. From these I made 1.5 ng/ $\mu\text{L}$  stocks to use in the qRT-PCR protocol and saved the rest. These and the original stock concentrations are stored at  $-80^\circ\text{C}$ .

Plate map:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1			A9			B8					

B	A2	B1	B9	
C	A3	B2		
D	A4	B3		
E	A5	B4		
F	A6	B5		
G	A7	B6		
H	A8	B7		

## Reactions:

A = *priM* (P437 P438)B= *tul4* (KROL63 KROL64)

I depleted the *priM* so I made a new 300 uL stock.

**Thursday, January 7, 2021**

## To Do:

1. ~~Purify cDNA~~
2. ~~Check cDNA concentration~~
3. ~~Make cDNA stocks~~
4. ~~Perform qRT-PCR~~
5. ~~Run qRT-PCR plate~~
6. Analyze PCR
7. Make single-use aliquots of *Tn7:rpsU1*, *Tn7:rpsU2*, *Tn7:rpsU3*
8. Streak strains to single colony for RNA isolation next week  
*LVS*, *Tn7:rpsU1*, *Tn7:rpsU2*, *Tn7:rpsU3*
9. Check efficiencies of new primers for qRT-PCR

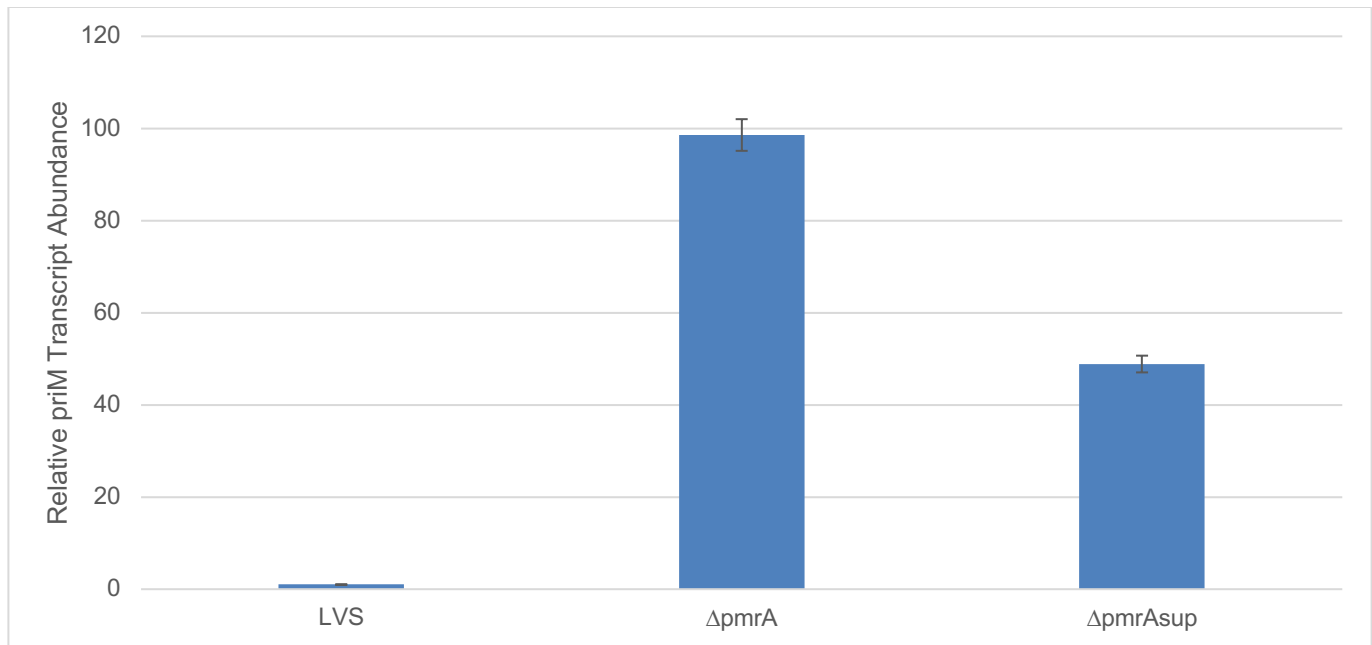
**Methods and Results:****PCR Analysis:**

<b>priM</b>	<b>average</b>	<b>stdev</b>	<b>tul4</b>	<b>average</b>	<b>stdev</b>
LVS1/ <i>priM</i>	27.21	0.031	LVS1/ <i>tul4</i>	19.48	0.004
LVS2/ <i>priM</i>	27.13	0.061	LVS2/ <i>tul4</i>	18.83	0.012
LVS3/ <i>priM</i>	23.85	0.037	LVS3/ <i>tul4</i>	19.17	0.028
$\Delta$ pmrA1/ <i>priM</i>	18.14	0.020	$\Delta$ pmrA1/ <i>tul4</i>	19.00	0.051

$\Delta\text{pmrA2/priM}$	18.51	0.009	$\Delta\text{pmrA2/tul4}$	19.36	0.013
$\Delta\text{pmrA3/priM}$	18.09	0.052	$\Delta\text{pmrA3/tul4}$	19.09	0.016
$\Delta\text{pmrAsup1/priM}$	21.23	0.001	$\Delta\text{pmrAsup1/tul4}$	19.34	0.032
$\Delta\text{pmrAsup2/priM}$	18.74	0.059	$\Delta\text{pmrAsup2/tul4}$	18.87	0.055
$\Delta\text{pmrAsup3/priM}$	18.15	0.024	$\Delta\text{pmrAsup3/tul4}$	19.04	0.016

priM	relative to tul4 $\Delta\text{CT}$	Ave	average $\Delta\text{CT}$	stdev	$\Delta\Delta\text{CT vs control}$	s	$2^{\Delta\Delta\text{CT}}$	$\Delta\text{CT} + / - \text{stdev}$	$2^{\Delta\Delta\text{CT}} + / - \text{stdev}$	error bars
LVS1/priM	7.7340		6.9076	0.0453	0.0000	0.0641	1.00	0.0641	0.963 -	0.0370
LVS2/priM	8.3039							-0.0641	1.038 +	0.0384
LVS3/priM	4.6850									
$\Delta\text{pmrA1/priM}$	-0.8663		-0.9019	0.0383	-7.8095	0.0593	98.52	-7.7501	95.148 -	3.3764
$\Delta\text{pmrA2/priM}$	-0.8473							-7.8688	102.021 +	3.4962
$\Delta\text{pmrA3/priM}$	-0.9921									
$\Delta\text{pmrAsup1/priM}$	1.8940		0.2920	0.0442	-6.6156	0.0633	48.84	-6.5522	47.055 -	1.7843
$\Delta\text{pmrAsup2/priM}$	-0.1303							-6.6789	50.691 +	1.8519
$\Delta\text{pmrAsup3/priM}$	-0.8875									

Error Bars			
priM relative to tul4			
	priM relative to tul4	+	-
LVS	1.00	0.0384	0.0370
$\Delta\text{pmrA}$	98.52	3.4962	3.3764
$\Delta\text{pmrAsup}$	48.84	1.8519	1.7843



It looks like I successfully generated the cDNA and these results confirm that the labeled  $\Delta pmrAsup$  strain is in fact the correct strain.

### Single Use Aliquots and Streaking:

I already have single use aliquots of the strains I need, so I plated them to single colony.

### Real-Time Primer Efficiency Test

Note: Efficiencies should be run on same “type” of DNA as you plan to do the experiment on; i.e. ChIP= sonicated DNA, RNA expression = cDNA or RNA w/ 2-step kit

- 1) Determine concentration of DNA
- 2) Make a dilution that's final concentration is 1.5ng/ul
- 3) Make 1:10 serial dilutions so you have 4 different DNA concentrations (1.5ng/ul, 0.15ng/ul, 0.015ng/ul, 0.0015ng/ul); you will be using 5ul of these DNA dilutions for each rxn so you need 3ul ddH<sub>2</sub>O per reaction instead of 7ul
- 4) Put 17.5ul of each DNA sample into separate tubes (enough for 3.5rxns)
- 5) set up primer master mixes in separate eppendorfs (make one for each primer pair)

Since you have 4 DNA samples you have 14rxns/primer pair (add 3.5 to this for volume error) =

17.5

example:

10ul of SYBR green	x 17.5	= 175	
1ul of 5uM forward primer	x 17.5	= 17.5	
1ul of 5uM reverse primer	x 17.5	= 17.5	*17.5 total not each
3ul ddH2O	x 17.5	= 52.5	* add 17.5uL really should be 70
TOTAL =		262.5	*

- 5) Since 3.5 rxns x 20ul rxn volume = 70; and tubes already have 17.5ul of DNA in them you need to add 52.5ul (70-17.5) of primer master mix to each DNA tube
- 6) Pipette 20ul of each rxn into 3 separate wells on the 96 well plate
- 7) Spin plate down
- 8) Place in real-time machine and run rxn

### Calculations

ChIP enrichment

Primer Efficiency  $^{\wedge}$  (Ct<sub>diff.</sub>)

Ct<sub>diff.</sub> = (control\_ChIP\_Ct – test\_ChIP\_Ct) - (control\_Input\_Ct – test\_Input\_Ct)

Primer Efficiency =  $(10^{(-1/\text{slope})})$

Where the slope is the slope of the standard curve plot

Today I'm only setting up the serial dilution.

I have 9 reactions to prepare plus *tul4* control.

I chose LVS3 as the source DNA because it has the highest stock concentration at 17 ng/uL.

				Total Desire d		
Sample #	Strain	cDNA stock conc (ng/uL)	cDNA new conc (ng/uL)	Volum e of cDNA (x11+)	Volum e cDNA (uL)	Volum e 0.1xEB (uL)
cDNA5	LVS3	17	1.5	250	22.1	228

Tube 1 (250uL) - 25uL □ Tube 2 (250uL) - 25uL □ Tube 3 (250uL) – 25uL □ Tube 4 (250uL)

**Friday, January 8, 2021**

**To Do:**

1. ~~Analyze PCR~~





PCR tubes: (40 total)

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	Rep 2	Rep 3	I1	Rep 2	Rep 3	G2	Rep 2	Rep 3	E3	Rep 2	Rep 3
B	B1	Rep 2	Rep 3	J1	Rep 2	Rep 3	H2	Rep 2	Rep 3	F3	Rep 2	Rep 3
C	C1	Rep 2	Rep 3	A2	Rep 2	Rep 3	I2	Rep 2	Rep 3	G3	Rep 2	Rep 3
D	D1	Rep 2	Rep 3	B2	Rep 2	Rep 3	J2	Rep 2	Rep 3	H3	Rep 2	Rep 3
E	E1	Rep 2	Rep 3	C2	Rep 2	Rep 3	A3	Rep 2	Rep 3	I3	Rep 2	Rep 3
F	F1	Rep 2	Rep 3	D2	Rep 2	Rep 3	B3	Rep 2	Rep 3	J3	Rep 2	Rep 3
G	G1	Rep 2	Rep 3	E2	Rep 2	Rep 3	C3	Rep 2	Rep 3	A4	Rep 2	Rep 3
H	H1	Rep 2	Rep 3	F2	Rep 2	Rep 3	D3	Rep 2	Rep 3	B4	Rep 2	Rep 3
	1	2	3	4	5	6	7	8	9	10	11	12
A	C4	Rep 2	Rep 3									
B	D4	Rep 2	Rep 3									
C	E4	Rep 2	Rep 3									
D	F4	Rep 2	Rep 3									
E	G4	Rep 2	Rep 3									
F	H4	Rep 2	Rep 3									
G	I4	Rep 2	Rep 3									
H	J4	Rep 2	Rep 3									

\*A1, G1, and H1 all had low volume; the third replicate of each did not have 20uL.

**Sunday, January 10, 2021**

**To Do:**

1. Patch out single colonies

**Methods and Results:**

I patched 3 single colonies from each plate.

**Monday, January 11, 2021**

**To Do:**

1. ~~Patch out single colonies~~
2. Check efficiencies of new primers for qRT-PCR
3. Set up cultures for RNA
4. RNA isolation

## **Methods and Results:**

Primer efficiency:

I didn't set the plate up correctly and because I used two plates the data for several of the primer sets are incomplete. For the first 2 primer sets (KROL377/378 and KROL379/380) the efficiencies are 1.861 and 1.930, respectively. I will have to repeat the protocol.

Patches grew well. Cultures were put on the shaker at 11am.

Sample #	Measured OD600	Dilution Factor	Actual OD600	Desired Volume mL	Desired OD	Volume of Resuspended Cells $\mu$ L	Starting OD (1050)	OD at 3 hours (1350)	Final OD at 1600
KB1.11-1	0.119	10	1.19	8	0.08	537.82	0.08	0.178	0.322
KB1.11-2	0.124	10	1.24	8	0.08	516.13	0.073	0.163	0.3
KB1.11-3	0.135	10	1.35	8	0.08	474.07	0.08	0.161	0.296
KB1.11-4	0.174	10	1.74	8	0.08	367.82	0.079	0.158	0.282
KB1.11-5	0.159	10	1.59	8	0.08	402.52	0.077	0.151	0.275
KB1.11-6	0.232	10	2.32	8	0.08	275.86	0.081	0.16	0.285
KB1.11-7	0.22	10	2.2	8	0.08	290.91	0.079	0.159	0.285
KB1.11-8	0.272	10	2.72	8	0.08	235.29	0.077	0.152	0.276

Sample #	Sample
KB1.11-1	LVS WT
KB1.11-2	LVS WT
KB1.11-3	Tn7:rpsU1
KB1.11-4	Tn7:rpsU1
KB1.11-5	Tn7:rpsU2
KB1.11-6	Tn7:rpsU2
KB1.11-7	Tn7:rpsU3
KB1.11-8	Tn7:rpsU3

I completed the RNA isolation and stopped before the DNase. I accidentally incubated the tri-reagent tubes at 95°C instead of 60°. Although I'm sure I cooked the nucleic acid, I'm going to continue the protocol.

**Tuesday, January 12, 2021**

**To Do:**

1. ~~Check efficiencies of new primers for qRT-PCR~~

- ~~2. Set up cultures for RNA~~
- ~~3. Repeat the primer efficiency~~
- ~~4. RNA isolation~~
- ~~5. DNase~~
- ~~6. Run gel on RNA~~
7. Streak strains to single colony for RNA isolation  
LVS, Tn7:*rpsU1*, Tn7:*rpsU2*, Tn7:*rpsU3*

### **Methods and Results:**

I spoke to Kathryn about what happened and she says to throw away the samples and start over. Today I will concentrate on primer efficiency.

Sample #	Strain	cDNA stock conc (ng/uL)	cDNA new conc (ng/uL)	Total Desired Volume of cDNA (x11+)	Volume cDNA (uL)	Volume 0.1xEB (uL)
cDNA2	A $\Delta$ pmr	17	1.5	250	22.1	228

Tube 1 (250uL) - 25uL □ Tube 2 (250uL) - 25uL □ Tube 3 (250uL) – 25uL □ Tube 4 (250uL)

	1	2	3	4	5	6	7	8	9	10	11	12
A	KROL377/K ROL378-1	KROL377/K ROL378-1	KROL377/K ROL378-1	KROL377/K ROL378-2	KROL377/K ROL378-2	KROL377/K ROL378-2	KROL377/K ROL378-3	KROL377/K ROL378-3	KROL377/K ROL378-3	KROL377/K ROL378-4	KROL377/K ROL378-4	KROL377/K ROL378-4
B	KROL379/K ROL380-1	KROL379/K ROL380-1	KROL379/K ROL380-1	KROL379/K ROL380-2	KROL379/K ROL380-2	KROL379/K ROL380-2	KROL379/K ROL380-3	KROL379/K ROL380-3	KROL379/K ROL380-3	KROL379/K ROL380-4	KROL379/K ROL380-4	KROL379/K ROL380-4
C	KROL381/K ROL382-1	KROL381/K ROL382-1	KROL381/K ROL382-1	KROL381/K ROL382-2	KROL381/K ROL382-2	KROL381/K ROL382-2	KROL381/K ROL382-3	KROL381/K ROL382-3	KROL381/K ROL382-3	KROL381/K ROL382-4	KROL381/K ROL382-4	KROL381/K ROL382-4
D	KROL383/K ROL384-1	KROL383/K ROL384-1	KROL383/K ROL384-1	KROL383/K ROL384-2	KROL383/K ROL384-2	KROL383/K ROL384-2	KROL383/K ROL384-3	KROL383/K ROL384-3	KROL383/K ROL384-3	KROL383/K ROL384-4	KROL383/K ROL384-4	KROL383/K ROL384-4
E	KROL385/K ROL386-1	KROL385/K ROL386-1	KROL385/K ROL386-1	KROL385/K ROL386-2	KROL385/K ROL386-2	KROL385/K ROL386-2	KROL385/K ROL386-3	KROL385/K ROL386-3	KROL385/K ROL386-3	KROL385/K ROL386-4	KROL385/K ROL386-4	KROL385/K ROL386-4
F	KROL387/K ROL388-1	KROL387/K ROL388-1	KROL387/K ROL388-1	KROL387/K ROL388-2	KROL387/K ROL388-2	KROL387/K ROL388-2	KROL387/K ROL388-3	KROL387/K ROL388-3	KROL387/K ROL388-3	KROL387/K ROL388-4	KROL387/K ROL388-4	KROL387/K ROL388-4
G	KROL389/K ROL390-1	KROL389/K ROL390-1	KROL389/K ROL390-1	KROL389/K ROL390-2	KROL389/K ROL390-2	KROL389/K ROL390-2	KROL389/K ROL390-3	KROL389/K ROL390-3	KROL389/K ROL390-3	KROL389/K ROL390-4	KROL389/K ROL390-4	KROL389/K ROL390-4
H	KROL63/KR OL64-1	KROL63/KR OL64-1	KROL63/KR OL64-1	KROL63/KR OL64-2	KROL63/KR OL64-2	KROL63/KR OL64-2	KROL63/KR OL64-3	KROL63/KR OL64-3	KROL63/KR OL64-3	KROL63/KR OL64-4	KROL63/KR OL64-4	KROL63/KR OL64-4

[illegible]

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	Rep 2	Rep 3	A2	Rep 2	Rep 3	A3	Rep 2	Rep 3	A4	Rep 2	Rep 3
B	B1	Rep 2	Rep 3	B2	Rep 2	Rep 3	B3	Rep 2	Rep 3	B4	Rep 2	Rep 3
C	C1	Rep 2	Rep 3	C2	Rep 2	Rep 3	C3	Rep 2	Rep 3	C4	Rep 2	Rep 3
D	D1	Rep 2	Rep 3	D2	Rep 2	Rep 3	D3	Rep 2	Rep 3	D4	Rep 2	Rep 3
E	E1	Rep 2	Rep 3	E2	Rep 2	Rep 3	E3	Rep 2	Rep 3	E4	Rep 2	Rep 3
F	F1	Rep 2	Rep 3	F2	Rep 2	Rep 3	F3	Rep 2	Rep 3	F4	Rep 2	Rep 3
G	G1	Rep 2	Rep 3	G2	Rep 2	Rep 3	G3	Rep 2	Rep 3	G4	Rep 2	Rep 3
H	H1	Rep 2	Rep 3	H2	Rep 2	Rep 3	H3	Rep 2	Rep 3	H4	Rep 2	Rep 3

[illegible]

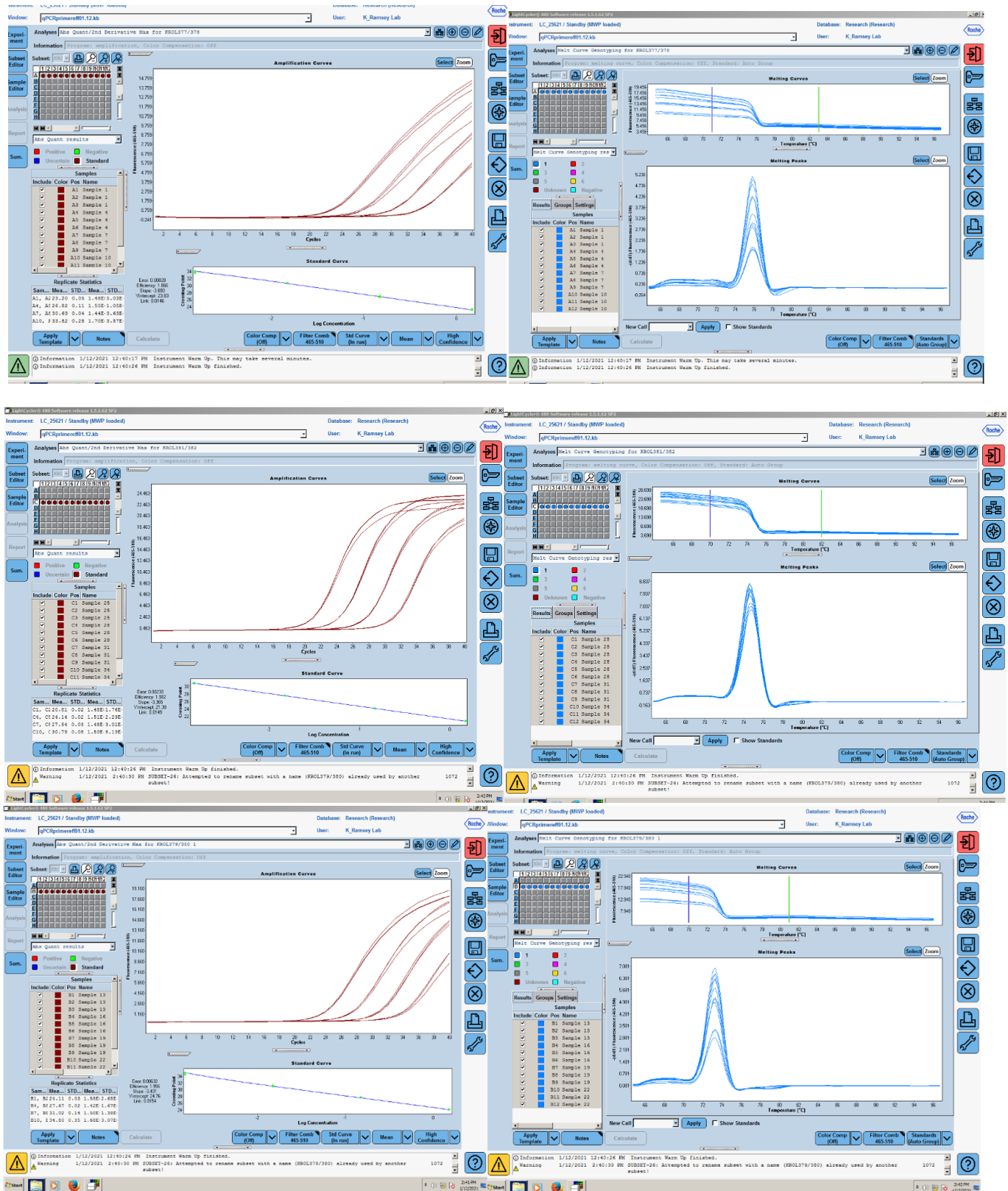
Reagent	Factor	Total volume
10ul of SYBR green	x 17.5	175uL
1ul of 5uM forward/reverse primer	x 17.5	17.5uL
4ul dd H2O	x 17.5	70uL
Tube		262.5uL

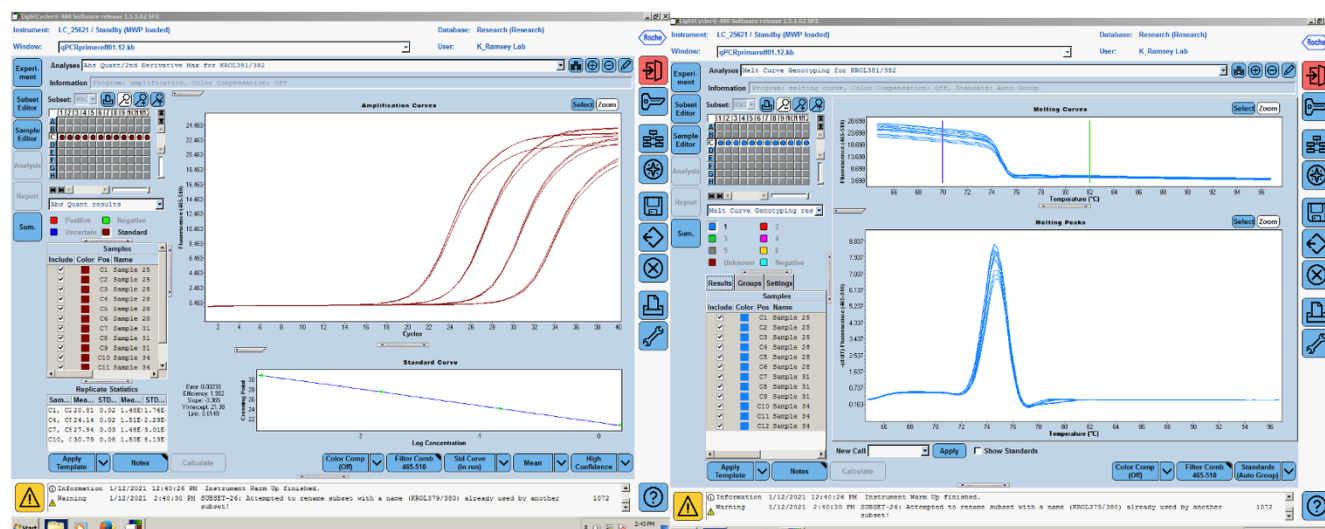
I repeated the primer efficiency and used cDNA2 ( $\Delta$ pmrA) because it has the highest concentration of the group (17ng/uL). I set up my dilution and then made the master mixes. Because I have to use 2 plates, I made two preparations of *tul4*; one for each plate.

The efficiency worked well; every primer set had an efficiency of at least 1.8:

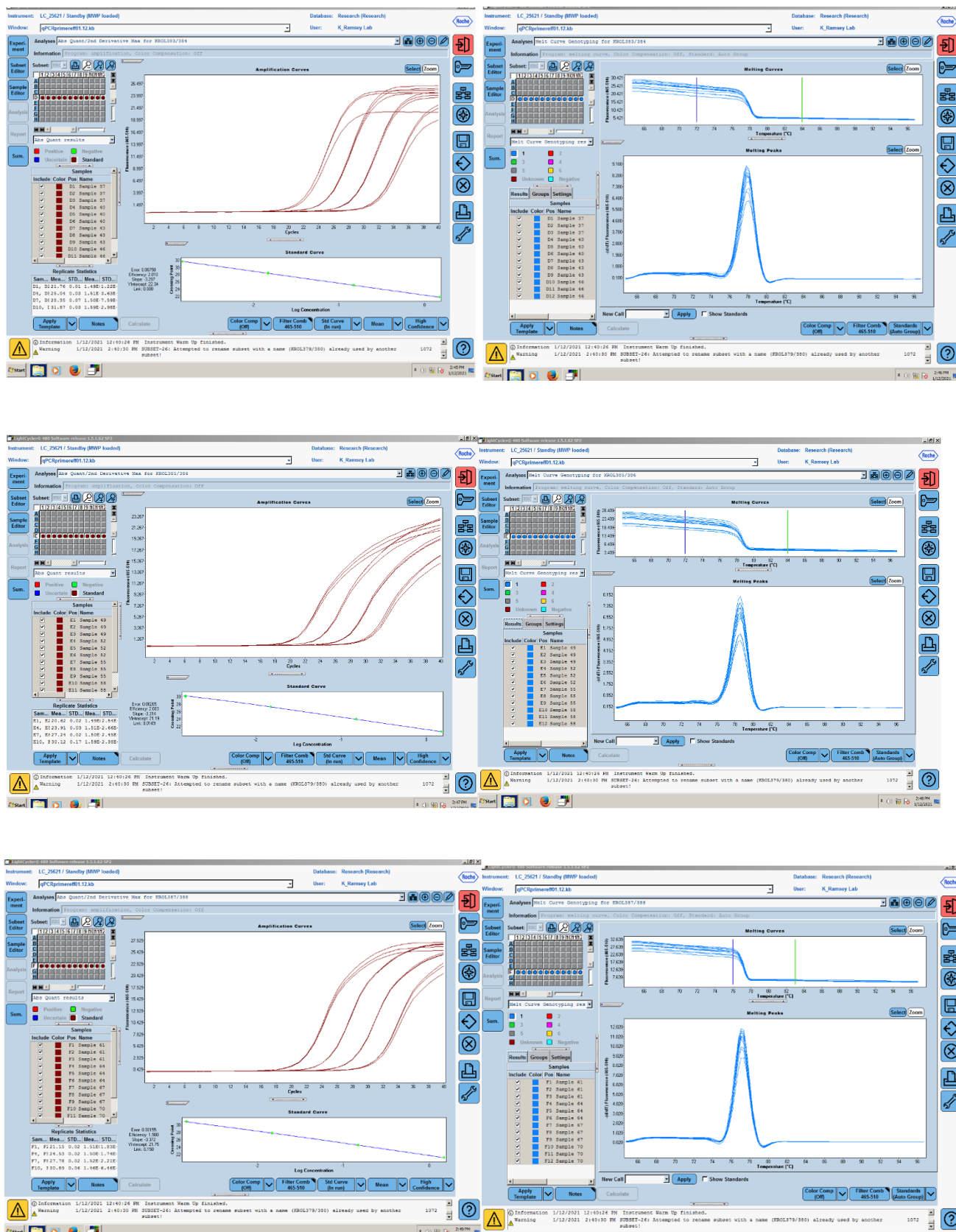
Primer set	Gene	Efficiency
KROL377/378	FTL_0097	1.866
KROL379.380	FTL_0097	1.956
KROL381/382	FTL_0097	1.982
KROL383/384	FTL_1181	2.01
KROL385/386	FTL_1181	2.003
KROL387/388	FTL_1181	1.98
KROL389/390	FTL_1883	2.012
KROL391/392	FTL_1883	1.999
KROL393/394	FTL_1883	1.99
<i>tul4</i>	control	1.972
<i>tul4_2</i>	control	1.982

## Kira Bernabe- Ramsey Lab Notebook Page |



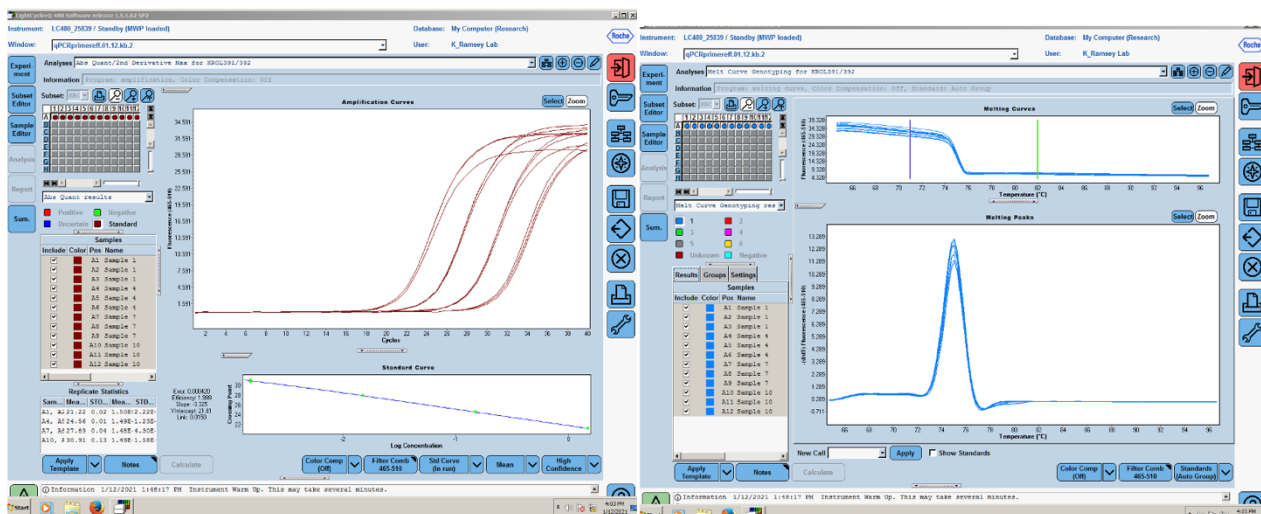
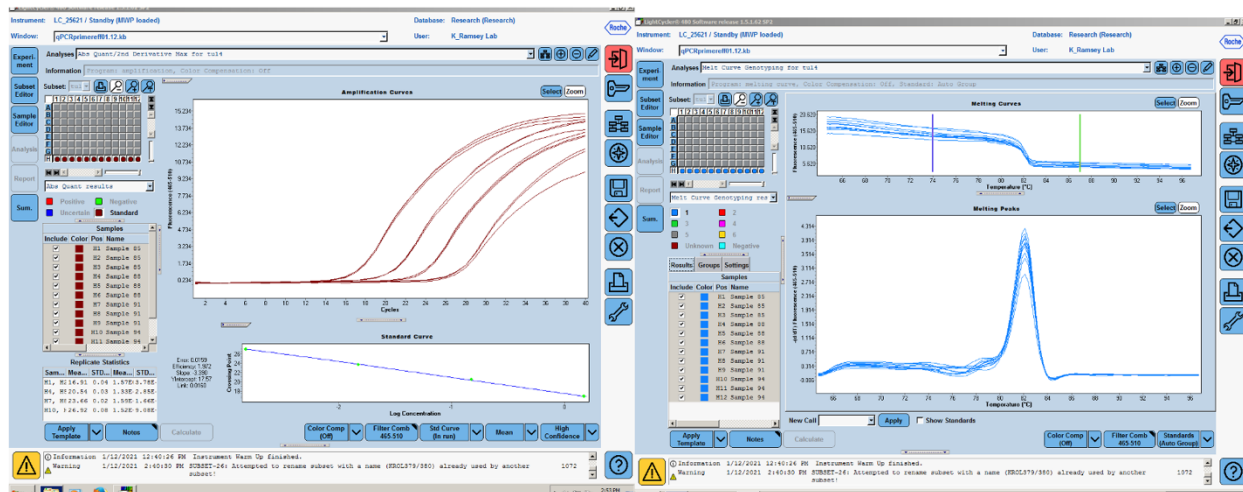


## Kira Bernabe- Ramsey Lab Notebook Page |

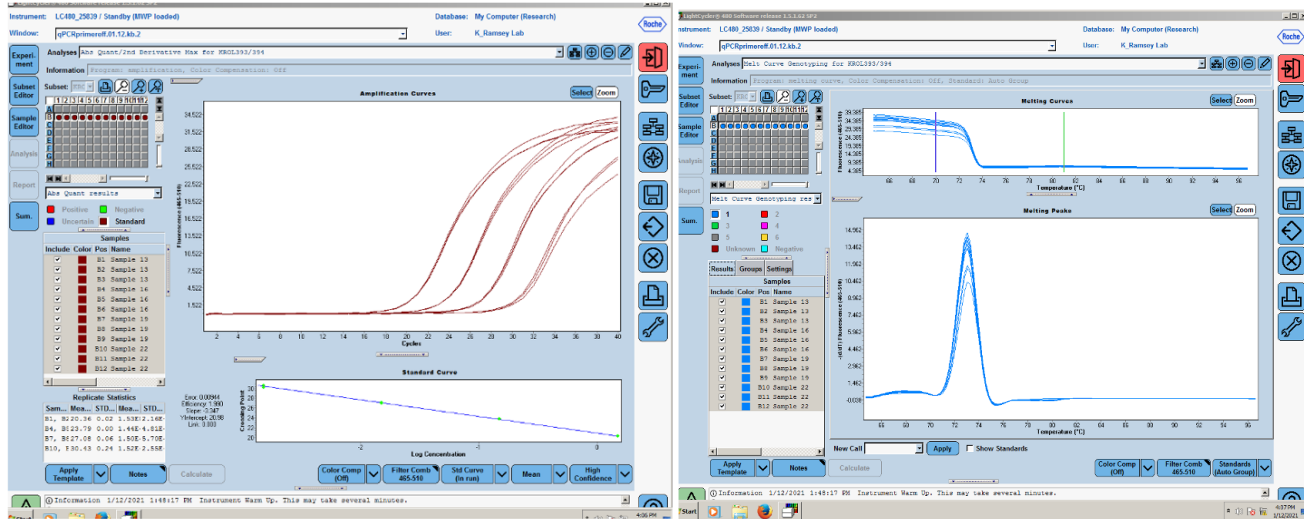




## Kira Bernabe- Ramsey Lab Notebook Page |



## Kira Bernabe- Ramsey Lab Notebook Page |



Wednesday, January 13, 2021

### To Do:

1. Repeat the primer efficiency
2. Grow Tn7:*rpsU1*, Tn7:*rpsU2*, Tn7:*rpsU3* to make single use aliquots
3. Begin prep for T6SS assay

**Methods:**

Single use aliquots:

Took a stick and poked the frozen permanent stock and used that to inoculate a plate, being careful not to let them thaw.

T6SS:

Chris sent me the protocol for his T6SS assay, and I went over it with Kathryn. We will make some modifications to accommodate LVS growth conditions. Depending on what happens with RNA isolation this week I will probably start T6 next week.

**Thursday, January 14, 2021**

**To Do:**

4. Grow *Tn7:rpsU1*, *Tn7:rpsU2*, *Tn7:rpsU3* to make single use aliquots
2. Patch LVS, *Tn7:rpsU1*, *Tn7:rpsU2*, *Tn7:rpsU3* for RNA isolation
3. Make plan for T6SS assay

The strains have not grown enough yet to make aliquots, they will be probably be ready tomorrow.

Similarly, the plates for RNA isolation look good and are showing single colonies but they are pinpoint; I think it's best to restreak from frozen and let them grow over the weekend. Also, *Tn7:rpsU1* has some bigger colonies that I'm concerned might be contamination. I re-streaked them today and will patch them on Sunday.

For the T6SS assay, I will make square CHA plates tomorrow and let them "age" over the weekend. I will patch LVS on Sunday for both the RNA isolation and also T6SS. I will resuspend the cells in 4 mL of PBS and take 1 mL of that (tube 1) and dilute that 1:20, then take the OD. Tube 2 will be 1:1 of PBS and tube 1. I'll check the OD, and continue from there until I have 5 tubes. The tubes will then be put on a 96 well plate where they will undergo further serial dilutions to the -9. Dilutions -4 through -9 will be put on the square CHA plates for the track dilution method.

**Friday, January 15, 2021**

**To Do:**

4. Make plan for T6SS assay
2. Make square CHA plates

I made 19 square CHA plates by pipetting 30mL of CHA into each plate. I will let them age over the weekend. When I come in on Sunday to patch the strains for RNA isolation, I will also patch out strains for single use aliquots again because we need more supplemented MHB, which I will do on Monday.

### Sunday, January 17, 2021

#### To Do:

1. Patch LVS, Tn7:*rpsU1*, Tn7:*rpsU2*, Tn7:*rpsU3* for RNA isolation
2. Grow Tn7:*rpsU1*, Tn7:*rpsU2*, Tn7:*rpsU3* to make single use aliquots
3. Dissolve iron pyrophosphate for MHB

### Monday, January 18, 2021

#### To Do:

1. Patch LVS, Tn7:*rpsU1*, Tn7:*rpsU2*, Tn7:*rpsU3* for RNA isolation
2. Grow Tn7:*rpsU1*, Tn7:*rpsU2*, Tn7:*rpsU3* to make single use aliquots
3. Dissolve iron pyrophosphate for MHB
4. Set up culture for RNA isolation
5. RNA isolation
6. Attempt T6SS assay up to track dilution step (as a monoculture)

### Methods and Results:

#### Culture Set up for RNA:

I had to leave the lab for a bit and didn't get back in time to catch the cultures while they were still in log phase. When I got back at 5:30, the ODs were all above 0.4.

#### RNA Isolation:

I was unable to set up the RNA isolation due to the cultures being overgrown. I streaked new cultures from frozen to be patched on Thursday.

### T6SS Assay Coculture Protocol

Adapted from Chris Schuttert

1. Grow attacking and prey cells under standard conditions\*
  - a. Establish an SOP for growth of these organisms
    - i. Grow LVS overnight on CHA plate as a patch, scrape patch (at ends) and resuspend in 4 mL PBS
    - ii. Dilute 1:20 and check OD, then set up 4 more 1:1 dilutions
2. Set up OD dilutions
  - a. Using a 96 well plate, set up seven serial dilutions of each sample on the plate

- b. Put 180uL PBS in each well rows 1-10.
  - c. Using the multi-channel pipette, add 20uL of sample to first well and mix. Discard tip and move 20 uL of that well to the next well. Repeat until the last well.
  - d. Use multichannel pipette to inoculate 10uL of each dilution of sample to a track plate (X2)
    - i. Total 10 plates (of each strain)
3. Generate an OD-to-CFU curve
  - a. Follow standard growth conditions, calculate OD of 1 based on dilution. Carry out four 1:1 dilutions in growth medium for OD600. You'll determine OD600 and CFU/mL for the 0x, 2x, 4x, 6x and 8x culture dilutions.
    - i. Remember, a 10x dilution this way is a 1 log dilution for reference.
4. Graph with OD600 on the x axis, and CFU/mL on the y axis.
  - a. Generate the  $y=mx+b$  equation and  $R^2$  value for each graph.
    - i.  $R^2 < .95$  is no good

I started with 3 mL of PBS that I inoculated with LVS, and then set up 1:1 dilutions using 700mL of PBS and then adding 700mL of culture from the previous tube. I checked the ODs of each dilution:

Dilution	OD
Tube 1 (0x)	1.293
Tube 2 (2x)	0.765
Tube 3 (4x)	0.383
Tube 4 (6x)	0.193
Tube 5 (8x)	0.095

I then set up ten 1:10 serial dilutions of each sample in a 96 well plate by adding 20uL of sample to 180uL of PBS in each well (serially). I inoculated a track plate of CHA with 10uL of well 4-10, which equals dilution factors  $10^{-3}$ - $10^{-8}$ . I did this in technical duplicate. The plates incubated at 37°C.

**Thursday, January 21, 2021**

#### To Do:

1. ~~Attempt T6SS assay up to track dilution step (as a monoculture)~~
2. Patch LVS, Tn7:*rpsU1*, Tn7:*rpsU2*, Tn7:*rpsU3* for RNA isolation
3. Grow Tn7:*rpsU1*, Tn7:*rpsU2*, Tn7:*rpsU3* to make single use aliquots
4. Count colonies from track dilutions
5. Calculate CFU/mL and make OD-CFU/mL curve

#### Methods and Results:

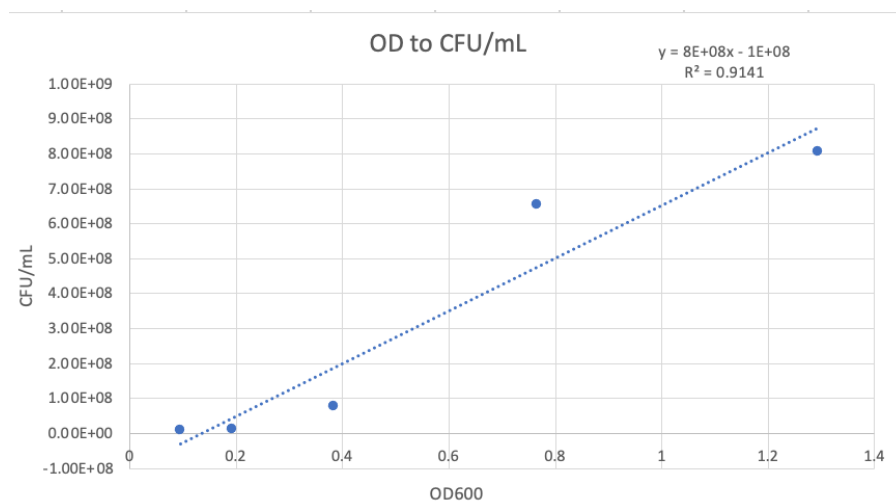
The patches that I streaked on Monday are overgrown, so I re-streaked from frozen today. Tn7rpsU3 appears to be contaminated, so I threw that stock away and I will make another stock of that tomorrow along with the single use aliquots.

The plates grown for RNA isolation had good single colonies; those were patched.

### T6SS:

I counted the colonies and took the average for each pair. I generated the OD to CFU/mL curve:

	Track Plate 1							Track Plate 2									
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	Dilution factor counted	Average Cells	CFU per mL
LVS 0x			77							84					0.00001	81	8.05E+08
LVS 2x			75							56					0.00001	66	6.55E+08
LVS 4x			44							34					0.00001	39	7.80E+07
LVS 6x			32							24					0.00001	28	1.12E+07
LVS 8x			20							19					0.00001	20	7.80E+06
Dilution Fact	0.001	0.0001	0.00001	0.000001				1	0.1	0.01	0.001						



Not a good line or  $R^2$  but now I know what to do for next time.

**Friday, January 22, 2021**

### To Do:

1. Patch LVS, Tn7:rpsU1, Tn7:rpsU2, Tn7:rpsU3 for RNA isolation
2. Grow Tn7:rpsU1, Tn7:rpsU2, Tn7:rpsU3 to make single use aliquots
3. Count colonies from track dilutions
4. Calculate CFU/mL and make OD-CFU/mL curve
5. Set up culture for RNA isolation
6. RNA isolation
7. Make single use aliquots

**Methods and Results:****Single Use Aliquots:**

I already have glycerol stocks, so I am just refreshing my supply of single aliquots for LVS, *Tn7rpsU1*, *Tn7rpsU2*, and *Tn7rpsU3*. I plated these strains from the glycerol stocks yesterday, and today I will inoculate 800uL of MHB, resuspend, then add 200uL of 75% glycerol. Then I will parse each mL out into 50uL aliquots and store at -80°C.

**RNA Culture Set up:**

Sample #	Measured OD600	Dilution Factor	Actual OD600	Desired Volume mL	Desired OD	Volume of Resuspended Cells $\mu$ L	OD After Resuspension (0950)	OD at 2 hours 1145	OD at 4 hours 1400	OD at 1509	OD at 1541	Final OD at 1600
KB1.22-1	0.162	10	1.62	8	0.08	395	0.073	0.129	0.254	0.324	0.358	0.375
KB1.22-2	0.19	10	1.9	8	0.08	337	0.077	0.132	0.259	0.329	0.364	0.384
KB1.22-3	0.207	10	2.07	8	0.08	309	0.077	0.119	0.22	0.276	0.306	0.328
KB1.22-4	0.238	10	2.38	8	0.08	269	0.072	0.107	0.201	0.258	0.284	0.315
KB1.22-5	0.332	10	3.32	8	0.08	193	0.082	0.132	0.251	0.314	0.35	0.372
KB1.22-6	0.234	10	2.34	8	0.08	274	0.080	0.132	0.252	0.316	0.347	0.366
KB1.22-7	0.276	10	2.76	8	0.08	232	0.078	0.128	0.233	0.298	0.329	0.346
KB1.22-8	0.279	10	2.79	8	0.08	229	0.069	0.115	0.212	0.259	0.293	0.31

**Monday, January 25, 2021**

**To Do:**

1. ~~Set up culture for RNA isolation~~

RNA isolation

Make single use aliquots

DNase treatment

Run gel

Make and purify cDNA

Make square CHA plates

**Methods and Results:**

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	KB1.22-1	RI-INBRE	1/25/2021 12:44:54 PM	46	ng/ $\mu$ L	1.15	0.786	1.46	0.63	RNA	40
2	KB1.22-2	RI-INBRE	1/25/2021 12:45:45 PM	26.5	ng/ $\mu$ L	0.66	0.436	1.52	0.63	RNA	40
3	KB1.22-3	RI-INBRE	1/25/2021 12:47:02 PM	32.7	ng/ $\mu$ L	0.82	0.564	1.45	0.62	RNA	40
4	KB1.22-4	RI-INBRE	1/25/2021 12:48:31 PM	35.8	ng/ $\mu$ L	0.9	0.612	1.46	0.62	RNA	40
5	KB1.22-5	RI-INBRE	1/25/2021 12:49:15 PM	5.9	ng/ $\mu$ L	0.15	0.076	1.93	0.58	RNA	40
6	KB1.22-6	RI-INBRE	1/25/2021 12:50:27 PM	20.2	ng/ $\mu$ L	0.51	0.324	1.56	0.61	RNA	40
7	KB1.22-7	RI-INBRE	1/25/2021 12:51:07 PM	17.9	ng/ $\mu$ L	0.45	0.312	1.43	0.58	RNA	40
8	KB1.22-8	RI-INBRE	1/25/2021 12:51:52 PM	15.9	ng/ $\mu$ L	0.4	0.263	1.51	0.61	RNA	40

From the low concentrations as well as the lack of peaks it's likely that these samples are contaminated. I will re-streak from frozen for isolation and then I'll repeat the assay possibly this Friday if the plates grow well. Dan was nice enough to make the square CHA plates.

**Tuesday, January 26, 2021**

**To Do:**

1. DNase treatment
2. Run gel
3. Make and purify cDNA
4. Make square CHA plates
5. Patch LVS for T6SS

There's not much I can do today in the lab besides patch out the LVS for T6SS and routine lab maintenance.

**Wednesday, January 27, 2021**

**To Do:**

- ~~1. DNase treatment~~
- ~~2. Run gel~~
- ~~3. Make and purify cDNA~~
- ~~4. Make square CHA plates~~
- ~~5. Patch LVS for T6SS~~
6. T6SS track dilution for OD to CFU/mL curve

**Methods and Results:**

I scraped the patch of LVS into 3 mL of PBS, then set up five 1:2 dilutions. The first tube had 700uL PBS with 700uL undiluted sample. The next tube had 700uL PBS with 700uL of previous tube added, etc. The tubes are known as 0X, 2X, 4X, 6X, and 8X. I checked the ODs on the spec. The first three were high, so I diluted 0X 1:4 (250uL to 750uL) and serially diluted the next 2 dilutions. The OD readings on the 2X and 4X were within range but 0X was still a little high, so I diluted it 1:8 to get an acceptable OD.

Dilution	Measured OD (1:2)	Measured OD (1:4)	Measured OD (1:8)	Actual OD
Tube 1 (0x)	1.354	0.782	0.435	3.48
Tube 2 (2x)	0.786	0.404		1.616
Tube 3 (4x)	0.406	0.206		0.824
Tube 4 (6x)	0.205			0.41
Tube 5 (8x)	0.106			0.212

I proceeded to the next step of setting up the 1:10 dilutions in the well plate. I added 180uL pf PBS to wells 1-10, then I added 20uL of each sample into the first well and serially diluted down to well ten. I then inoculated 2 square CHA plates with each sample from wells 4-10 at 10uL for the track dilution, and incubated them at 37°C.



**Thursday, January 28, 2021****To Do:**

1. ~~T6SS track dilution for OD to CFU/mL curve~~
2. Patch single colonies for RNA isolation
3. Streak for isolation again

I see some contamination on the single colony plates (LVS and Tn7*rpsU1*), so I will streak new plates today to patch on Sunday and I will also streak new plates tomorrow to patch on Monday so I will have back up in case something goes wrong.

**Friday, January 29, 2021****To Do:**

1. ~~T6SS track dilution for OD to CFU/mL curve~~
2. Streak for isolation again

I will streak for isolation again from frozen today just as a backup in case something goes wrong with either the growth of the organisms over the weekend or the isolation protocol.

I saw some contamination on Tn7*rpsU1*. I will make more aliquots from my glycerol stocks.

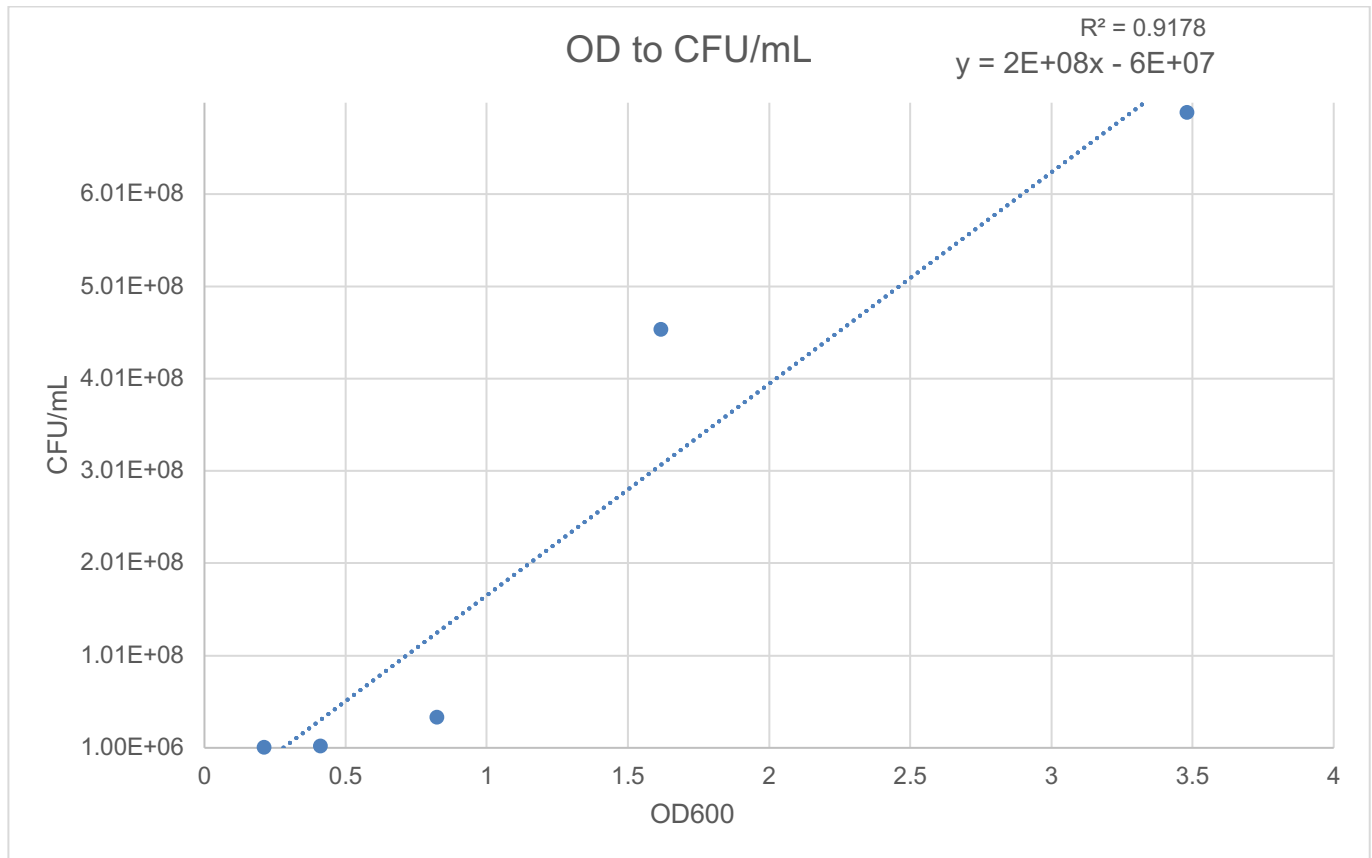
**Saturday, January 30, 2021****To Do:**

1. Streak for isolation again
2. Count colonies on track plates

I streaked my strains for RNA from frozen, and I also streaked from the glycerol stocks to make new single use aliquots.

OD to CFU/mL:

Dilution	Measured OD (1:2)	Measured OD (1:4)	Measured OD (1:8)	Actual OD	CFU per mL
Tube 1 (0x)	1.354	0.782	0.435	3.48	6.90E+08
Tube 2 (2x)	0.786	0.404		1.616	4.55E+08
Tube 3 (4x)	0.406	0.206		0.824	3.42E+07
Tube 4 (6x)	0.205			0.41	3.42E+06
Tube 5 (8x)	0.106			0.212	1.78E+06



Still not good

**Sunday, January 31, 2021**

**To Do:**

1. Streak for isolation again

I streaked from frozen again. The plates from yesterday look good. I'm not coming into the lab on Monday due to weather, so I will most likely set up the RNA on Wednesday.

## 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.