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Introduction

Thursday, April 1, 2021

To Do:

1. Prepare cell lysate using bead beating protocol

Methods and Results:

Overnight Culture Setup:

Sample #	Strain	Measured OD600	Dilution Factor	Actual OD600	Desired Volume mL	Desired OD	Volume of Resuspended Cells μ L	Starting OD T0 (1800)	Final OD (1500)
1	Tn7::rpsU2	0.543	10	5.43	70	0.005	64	0.006	0.394
2	Tn7::rpsU3	0.618	10	6.18	70	0.005	57	0.005	0.363

Lysis buffer

Ingredient	Final Conc	Stoc Conc	Volume
Hepes pH 7.6	25 mM	1 M	1250 μ l
NH ₄ Cl	100 mM	5 M	1000 μ l
MgCl ₂	10 mM	1 M	500 μ l
Triton X-100	0.4%	100%	2 ml
NP-40	0.1%	10%	500 μ l
RNase-free DNase	100 U/mL	U/ μ l	1.3 μ l (separate)
			Water up to 50ml
Lysozyme	200 μ g/mL	10mg/ml	

Take 2 ml of lysis buffer and add 400 mg of lysozyme

1. Harvest cells: put 50ml of each culture into a sterile 50ml conical tube and spin at 4°C for 10 minutes at max speed.
2. Remove the supernatant and weigh the pellet
3. Resuspend pellet in 3 ml wash buffer
4. Spin at 4°C for 10 minutes at max speed
5. Move samples into a Styrofoam box and pour liquid N₂ to cool them down.

6. Add 650 ul lysis buffer dropwise to each sample while in liquid N₂, then scrape them into clean 10 mL mixer mill cannisters with beads.
7. Mixer mill pellets 5 times at 15 Hz for 3 minutes, cooling in liquid N₂ between each round
8. Open the cannister and thaw in shallow water bath. Transfer lysate to 1.5 ml tube and add DNase, keep on ice or store in -80°C if not moving to next step.

*Note for the first round of mixer mill we mixed for 1 minute at 10 Hz twice, then proceeded with 4 rounds of 3 minutes mixes at 15 Hz

Tuesday, April 6, 2021

To Do:

1. ~~Prepare cell lysate using bead beating protocol~~
2. Make sucrose solutions for gradients

Methods and Results:

I am getting together the materials I need for the fractionation later in the week. I need to make sucrose solutions that have the appropriate buffer. I will make a 50% and a 10% solution.

Lysis buffer for Sucrose Solutions:

Ingredient	Final Conc	Stoc Conc	Volume
Hepes pH 7.6	25 mM	1 M	12.5 ml
NH ₄ Cl	100 mM	5 M	10 ml
MgCl ₂	10 mM	1 M	5 ml
Triton X-100	0.4%	100 %	20 ml
NP-40	0.1%	10 %	5 ml
Autoclaved Water			Fill up to 500 ml

With this I will make a 50% and a 10% solution. After the addition of the appropriate amount of sucrose, I will filter sterilize the solutions.

50%: 125g sucrose to 250ml buffer

10%: 25g sucrose to 250ml buffer

Thursday, April 8, 2021

To Do:

1. ~~Prepare cell lysate using bead beating protocol~~
2. Make sucrose solutions for gradients

I finished making the sucrose solutions (autoclaved and vacuum filtered, next time no need to autoclave if I'm using autoclaved water). I took the A260 of the bead-beaten lysates and checked the other lysates again after re-spinning them for 10 minutes at 4°C.

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	A	Science	4/8/2021 12:25:08 PM	2755.4	ng/μl	68.886	79.108	0.87	-2.27	RNA	40
2	A10	Science	4/8/2021 12:27:24 PM	105.5	ng/μl	2.638	-2.296	-1.15	8.26	RNA	40
3	B	Science	4/8/2021 12:28:03 PM	3328.6	ng/μl	83.214	74.298	1.12	-1.64	RNA	40
4	B10	Science	4/8/2021 12:28:36 PM	680.7	ng/μl	17.018	17.193	0.99	-7.9	RNA	40
5	1	Science	4/8/2021 12:29:20 PM	119.8	ng/μl	2.994	2.11	1.42	0.16	RNA	40
6	2	Science	4/8/2021 12:30:08 PM	69.4	ng/μl	1.735	-0.459	-3.78	0.1	RNA	40

Sample #	Sample Description	Starting Volume (mL)	Lysis Method	OD600	A260	Notes
1	Tn7:: <i>rpsU2</i>	50	Detergent	0.337	6.6	Lysozyme wasn't fresh
2	Tn7:: <i>rpsU3</i>	50	Detergent	0.307	7.9	
3	Tn7:: <i>rpsU2</i>	50	Bead-beating	0.394	2.6	
4	Tn7:: <i>rpsU3</i>	50	Bead-beating	0.363	17.0	

Saturday, April 10, 2021

To Do:

1. ~~Make sucrose solutions for gradients~~
2. Sucrose gradients and fractionation

Methods and Results:

Making a Sucrose Gradient

1. Have prepared a light solution (i.e. 10% sucrose) and a heavy solution (50% sucrose) that has been autoclaved and filter-sterilized. Sucrose solutions should be in the same buffer as your sample. Also prepare about 205 uL of your sample, diluted to 0.6 ug/uL.
2. Put a tube in the marker block and draw a line at the top ledge

3. With a 30mL syringe, push into light solution and pump the air bubbles out. Draw out light solution (about 8 mL per gradient) and plunge needle to the very bottom of the tube. Lift the needle as solution pours out, be careful to keep the tip of the needle just under the surface of the solution. Stop at just above the line.
4. With a 10mL syringe, push into heavy solution and pump the air bubbles out. Draw out heavy solution, then wipe the needle and push a drop out, dabbing onto a kimwipe (making sure there's no air). Holding the tube with thumb and middle finger, use forefinger as a guide as you plunge needle to bottom of tube, resting the needle along the wall, and push out gently just enough to form a pool. Keep pushing out solution as you draw up the needle, keeping the tip about 1 cm below the interface of the layers until there is about 2-3mm of space at the top. Make sure needle is resting against the wall of the tube as you quickly draw it out. There should be a visible line between the layers.
5. Use light solution to adjust the top layers, making sure all samples are at the same level
6. Cap the tubes, making sure the hole is the last part to seal. Some liquid should be visible inside the cap, if there is visible air or you can't see liquid in cap add some more light solution.
7. Turn on gradient station in back, select "GMST"
8. Use the up and down keys on the gradient station and the level to level the plate. When level press "done".
9. Put tubes in tube holder (no need to balance); Go to exit -> gradient -> recent-> confirm rotor -> use (confirm 10:50) -> run (recent should be the 10-50% sucrose gradient because that's the one we have been using. If you need to find a different one go to recent and scroll through)
10. Put back in tube rack and refrigerate for 45 minutes. Now is a good time to cool down the ultracentrifuge.
11. Take off caps, remove 40uL of top layer and weigh the samples. Using light solution, adjust as necessary until there is no more than .002 difference in weight.
12. Use sample layering device to add 200uL of sample to the top (put tube in tube holder, use both hands for the device, press edge of syringe against farther tube wall and gently push the sample onto the top layer).
13. Put samples on ice and bring to INBRE to spin on ultracentrifuge with rotor SW40 Ti. 40,000 rpm for 4 hours at 4C, using 5 for acceleration and deceleration.
14. After spin, continue with fractionation protocol.

Fractionating

1. Click on Triax software, turn fractionator on via button on side, then set fractionator to "scan"
2. Choose the username, click "single UV OD scan". Channel A Wavelength should be 260 nm
3. On the bottom of the screen fill out Gradients necessary info (i.e. sample volume, gradient type, speed, etc)
4. Under the rotor settings (SW40Ti) select "number of fractions" as the mode to fractionate by
5. On the LED Power screen, make sure Channel A is reading between 800,000 and 900,000. Push water through the cell if it needs to be adjusted
6. Go to scan set up, make sure everything is filled out correctly

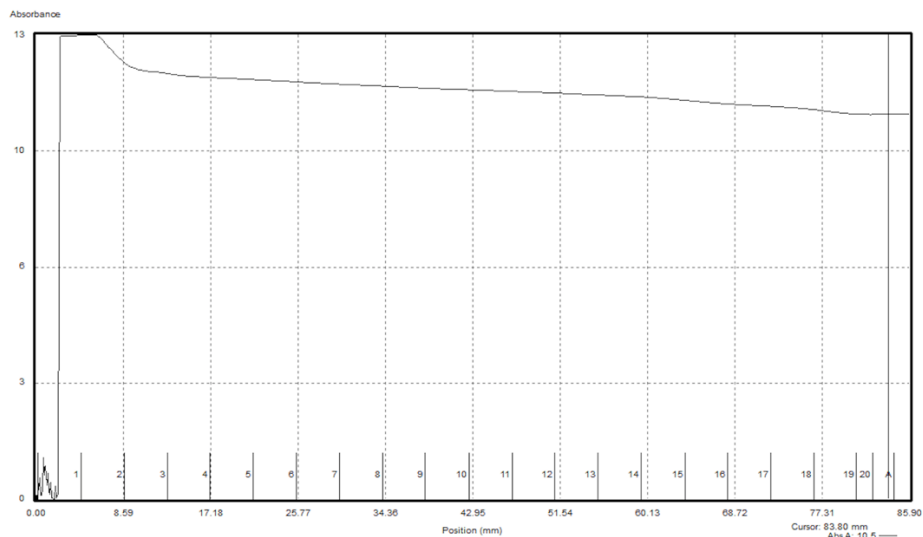
7. Go to graph. Pump water through the cell again to calibrate. Y axis should be close to 0, with at least two zeros following the decimal point (e.g. 0.002)
8. Purge air through the system, then load the first sample. Cap the sample with the flow cell cap by turning and pushing down and put sample in the flow cell, using bottom lock. Slide it on the fractionator then rotate 90°C to fix it in place (window should face front)
9. Load microcentrifuge tubes in rack in the middle two rows, then put the rack on the fractionator. The 29 should face the back. The dispenser will make a U moving back then left then forwards, so load tubes accordingly.
10. On software, hit “start scan”. When all of the tubes are filled, hit “end” to move the tubing to the back. Flush with water for 10 seconds then air for 10 seconds. Repeat for a total of 3 washes. Rinse screw-on cap with DI water.
11. Save run as csv when prompted and graph via file -> save graph image.
12. Repeat Step 9 for the remaining samples.
13. When all of the samples are complete, push water through the flow cell, then flush 5 times with water, 3 times with 70% isopropyl alcohol, and 5 times with air only using the dry syringe. Switch metal lever down to assist with air flushes.
14. Flush the line your sample goes through with DI water.
15. Save fractions at -20C if interested in proteins, -80C if interested in RNA.

Sample Name #	Tube #	A260	DF (10)	Divided by 14.5	C1 (ug/ml)	V2 (ul)	C2 (ug/ml)	V1 (ul)	Vol buffer (ul)	Total Volume (ul)
A (Tn7::rpsU2_bead)	1	2.64	26.4	1.82	1821	205	600	67.6	137.4	205
B (Tn7::rpsU3_bead)	2	17.02	170.2	11.74	11738	205	600	10.5	194.5	205
C (Tn7::rpsU2_standard)	3	6.6	66	4.55	4552	205	600	27.0	178.0	205
D (Tn7::rpsU3_standard)	4	7.9	79	5.45	5448	205	600	22.6	182.4	205

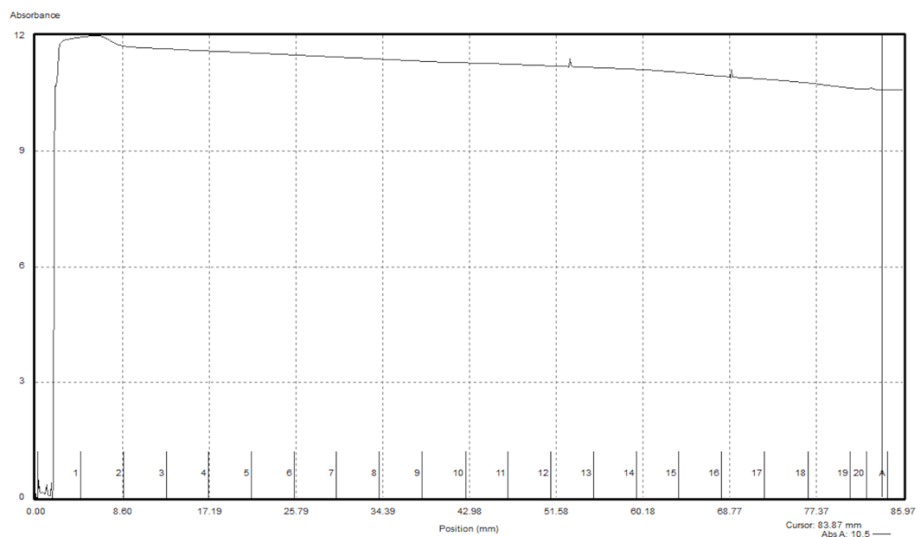
*Actual identities of A and B are unknown due to not properly labeling them during the bead-beating process.

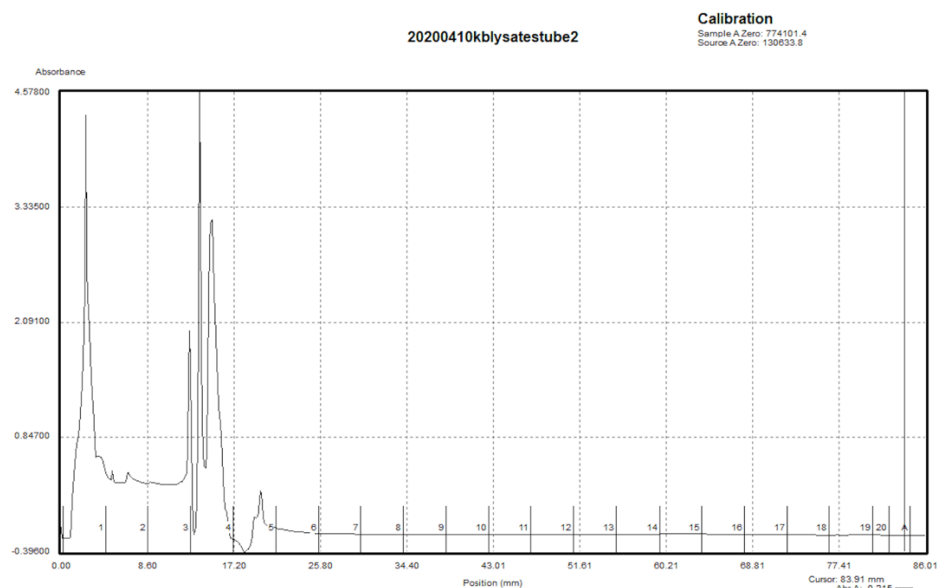
Setting up the gradients was difficult for the bead-beater lysates because the heavy solution was tricky get through the syringe. It took several attempts; I had to deviate from the protocol and use the bigger 30ml syringe instead of the 10ml. I also noticed that the polysome buffer is a lot foamier and prone to bubbling more than the standard buffer.

20200410kblysatestube1

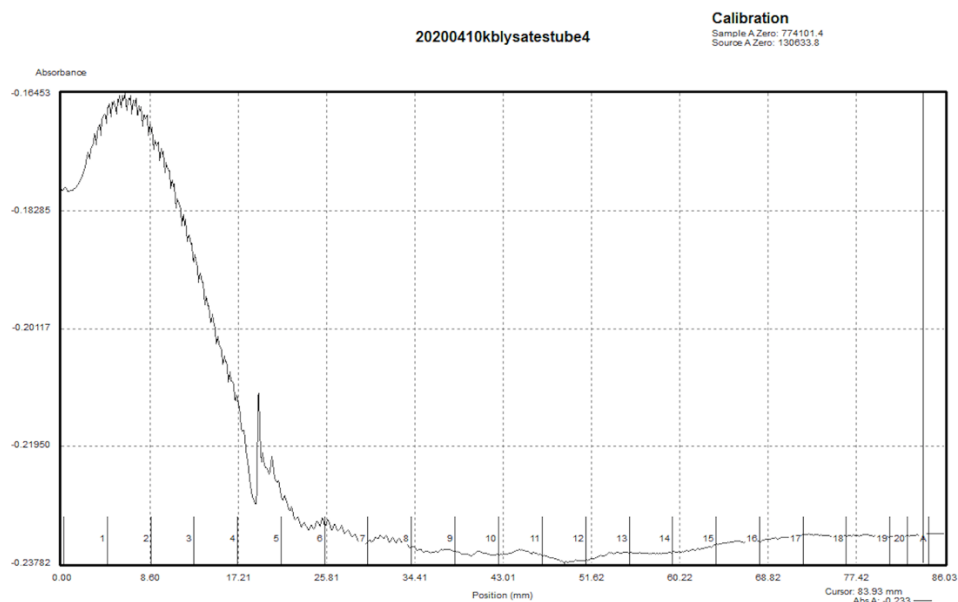
Calibration
Sample A Zero: 785593.0
Source A Zero: 130530.3

20200410kblysatestube2

Calibration
Sample A Zero: 785593.0
Source A Zero: 130530.3



*This is mis-labeled. It is Tube 3.



None of these worked. During the 3rd sample the tubing started leaking and fragments weren't being collected because the sample wasn't getting there. I had a difficult time plugging the tubing back in. I had to hold the whole unit in place. It also made it difficult to flush the machine.

I don't think that was why none of my samples worked though.

Monday, April 12, 2021**To Do:**

- ~~1. Make sucrose solutions for gradients~~
- ~~2. Sucrose gradients and fractionation~~
3. Make a new plan
4. Make new sucrose solutions

After talking with Kathryn, we decided that the Triton X-100 shouldn't be added to the sucrose solutions because it can distort the A260. I will make new sucrose solutions without the detergents and make new lysates. We also talked about how to fix the Triax so the tubing doesn't pop out.

Tuesday, April 13, 2021**To Do:**

- ~~1. Make a new plan~~
- ~~2. Make new sucrose solutions~~
3. Make overnight cultures

Sample #	Measured OD600	Dilution Factor	Actual OD600	Desired Volume mL	Desired OD	Volume of Resuspended Cells μ L
1	0.395	10	3.95	70	0.005	89
2	0.322	10	3.22	70	0.005	109

I started out with suspensions that were too concentrated and not suspended enough. I had some issues with the OD (starting out over 0.6 and forgot to dilute more). I started over taking half (500ul) of the original suspension and adding it to 500ul of MHB. The starting OD was better.

Wednesday, April 14, 2021**To Do:**

- ~~1. Make overnight cultures~~
2. Make lysates

Methods and Results:

Sample #	Measured OD600	Dilution Factor	Actual OD600	Desired Volume mL	Desired OD	Volume of Resuspended Cells μ L	OD After Resuspension T0 1730	OD Next Day 1430	Weight (g)
1	0.395	10	3.95	70	0.005	89	N/A	0.343	0.21
2	0.322	10	3.22	70	0.005	109	N/A	0.299	0.04

Lysis buffer

Ingredient	Final Conc	Stoc Conc	Volume
Hepes pH 7.6	25 mM	1 M	1250 μ l
NH ₄ Cl	100 mM	5 M	1000 μ l
MgCl ₂	10 mM	1 M	500 μ l
Triton X-100	0.4%	100%	2 ml
NP-40	0.1%	10%	500 μ l
RNase-free DNase	100 U/mL	U/ μ l	1.3 μ l (separate)
			Water up to 50ml
Lysozyme	200 μ g/mL	10mg/ml	

Take 2 ml of lysis buffer and add 400 mg of lysozyme

1. Harvest cells: put 50ml of each culture into a sterile 50ml conical tube and spin at 4°C for 10 minutes at max speed.
2. Remove the supernatant and weigh the pellet.
3. Resuspend pellet in 3 ml wash buffer.
4. Spin at 4°C for 10 minutes at max speed, remove supernatant.
5. Move samples into a Styrofoam box and pour liquid N₂ to cool them down.
6. Add 650 μ l lysis buffer dropwise to each sample while in liquid N₂, then scrape them into clean 10 mL mixer mill cannisters with beads.
7. Mixer mill pellets 5 times at 15 Hz for 3 minutes, cooling in liquid N₂ between each round by plunging them into the dewar for 30 seconds.
8. Open the cannister and thaw in shallow water bath (heavy duty plastic bin). Transfer lysate to 1.5 ml tube and add DNase.
9. Spin at 4°C for 10 minutes; transfer supernatant to new 1.5ml tube; take the A260.
10. Store at -80°C if not immediately moving to next step.

*Use the smaller beads

Thursday, April 15, 2021

To Do:

1. ~~Make lysates~~
2. Take A260 of lysates

3. Perform fractionator protocol

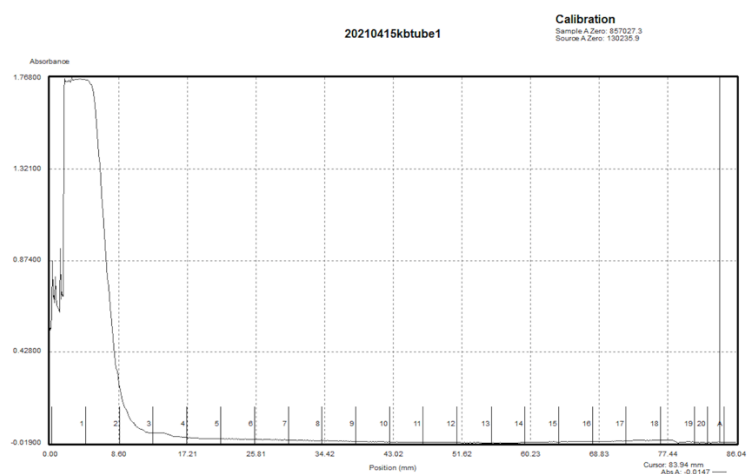
#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	A	Science	4/15/2021 7:29:52 AM	1781.2	ng/μl	44.53	72.875	0.61	3.57	RNA	40
2	A10	Science	4/15/2021 7:30:37 AM	395.3	ng/μl	9.883	16.361	0.6	1.01	RNA	40
3	B	Science	4/15/2021 7:31:18 AM	3421	ng/μl	85.526	132.003	0.65	5.78	RNA	40
4	B10	Science	4/15/2021 7:32:04 AM	500.9	ng/μl	12.522	21.97	0.57	0.19	RNA	40
5	E	Science	4/15/2021 7:32:38 AM	13322	ng/μl	333.049	437.737	0.76	0.68	RNA	40
6	E10	Science	4/15/2021 7:33:09 AM	1043.5	ng/μl	26.089	41.275	0.63	0.55	RNA	40
7	F	Science	4/15/2021 7:33:43 AM	13453.4	ng/μl	336.334	438.136	0.77	0.69	RNA	40
8	F10	Science	4/15/2021 7:34:14 AM	1060.8	ng/μl	26.52	42.8	0.62	0.59	RNA	40

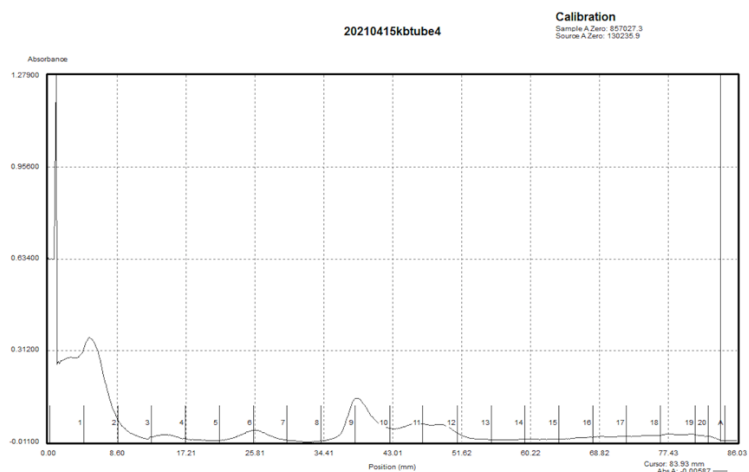
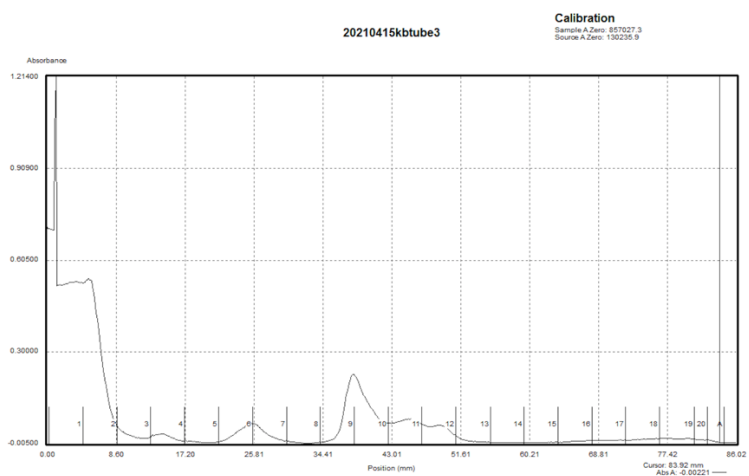
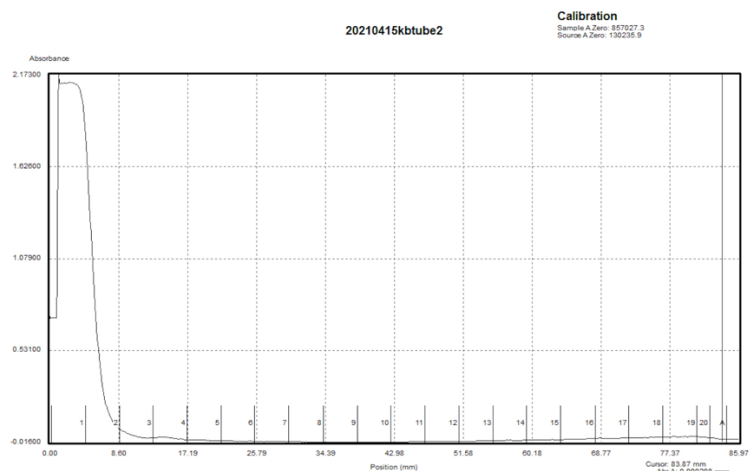
Sample Concentrations:

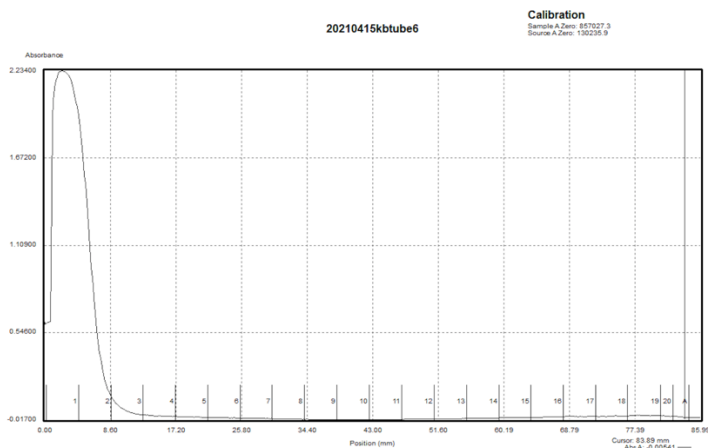
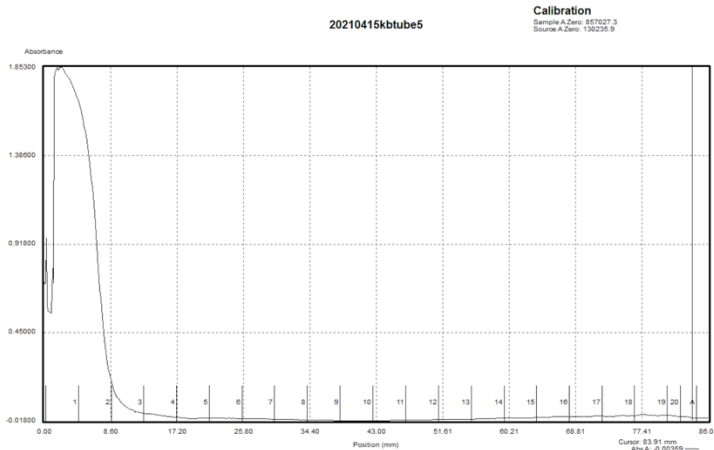
Sample Name #	Tube #	A260	DF (10)	Divided by 14.5	C1 (ug/ml)	V2 (Total Volume ul)	C2 (ug/ml)	V1 (ul)	Vol buffer (ul)
A (Tn7::rpsU2_bead)	1	9.88	98.8	6.81	6814	205	600	18.1	186.9
B (Tn7::rpsU3_bead)	2	12.52	125.2	8.63	8634	205	600	14.2	190.8
C (Tn7::rpsU2_standard)	3	6.60	66	4.55	4552	205	600	27.0	178.0
D (Tn7::rpsU3_standard)	4	7.90	79	5.45	5448	205	600	22.6	182.4
E (Tn7::rpsU2_bead_414)	5	26.09	260.9	17.99	17993	205	600	6.8	198.2
F (Tn7::rpsU3_bead_414)	6	26.52	265.2	18.29	18290	205	600	6.7	198.3

It was a lot easier to set up the sucrose gradients now that we eliminated the detergents from the sucrose solutions.

*Note that I set up all gradients with the solutions that have 10mM MgCl₂ and I should have set up C and D with the 0.6 mM MgCl₂







It looks like using the higher Mg concentrated sucrose solutions had an effect on the standard-prepped lysates. There are some peaks, but very short. It also looks like the bead-beating preparation didn't work for either set of lysates. I'm unsure if it's because of the buffer.

Kathryn and I talked about it and she thinks that I should maybe add more amount to my samples. The set up I used previously was fairly arbitrary, so there is room to tweak. If my sample size of each lysate is 200ul, what is the quantity in the lowest concentration sample? Once I determine that, I will back calculate to figure out what my new sample sizes will be for the others. One thing to know is that the volume of sample dictates how sharp the peaks will be. I should maybe try to load 100 ul.

If I'm able to load a lot of sample and pull 70s, we can see what's there using mass spec. We want to get a lot of protein for mass spec. Determine protein concentration with an assay (ask Hannah). Consider adding RNA inhibitor to the buffer.

Next week I will repeat the experiment using the above outlines, with the previously good result of subunits as a control. I will set up 1,2,3,5, and 6, and use LVS standard lysate as a control.

Thursday, April 22, 2021

To Do:

4. Fractionate

Kathryn and I went over how to calculate the amount of sample I need to put in the loading volume. This table includes the notes of how to do it:

Notes			Measured A260 (1:10 dilution)	Actual A260	Divided by 14.5 multiplied by 1000	(Amt ug/1)(1 ml/conc ug)(1000ul1 ml)	(Vol Sample to Load ul/1)(/ml/1000ul)(Conc ug/1ml)
Sample	Strain	Date Lysed	RNA A260 (Abs)	RNA A260 (Abs)	RNA concentration on ug/ml	Volume Sample to Load (ul)	Total Desired RNA (ug)
A	Tn7::rpsU2 bead	22-Mar	9.88	98.8	6813.79	95.95	653.79
B	Tn7::rpsU3 bead	22-Mar	12.52	125.2	8634.48	75.72	653.79
C	Tn7::rpsU2 standard	17-Mar	0.199	1.99	137.24	200.00	27.45
D	LVS standard	17-Mar	4.74	47.4	3268.97	200.00	653.79
E	Tn7::rpsU2 bead	14-Apr	26.09	260.9	17993.10	36.34	653.79
F	Tn7::rpsU3 bead	14-Apr	26.52	265.2	18289.66	35.75	653.79

Because the concentration of Sample C was so low, we decided to load 200ul of the sample, which is the total volume. Because Sample C was so much lower than the rest of the samples, we then decided that Sample D would be the one to normalize to. However, in the end after running all the calculations, **we decided to just load 200ul of each sample without diluting them.**

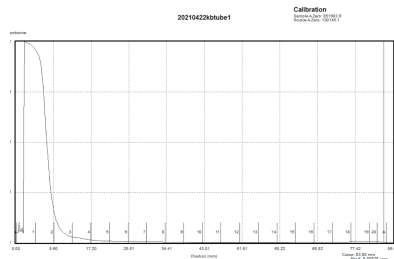
Friday, April 23, 2021

To Do:

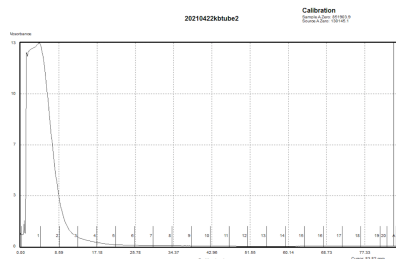
1. ~~Fractionate~~
2. Think about next steps for sucrose gradients

Methods and Results:

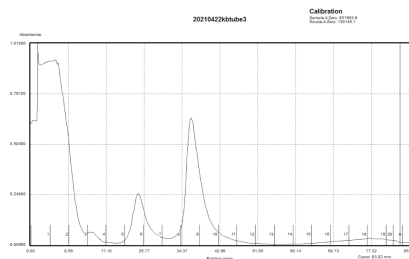
4/22/21



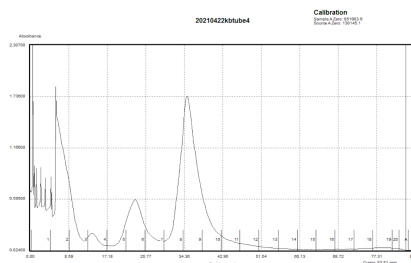
Tn7::rpsU2 bead 4_1



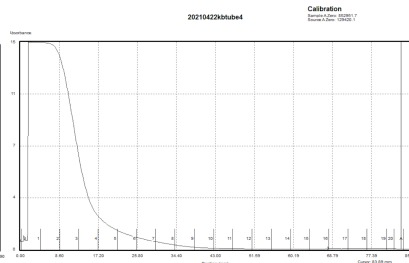
Tn7::rpsU3 bead 4_1



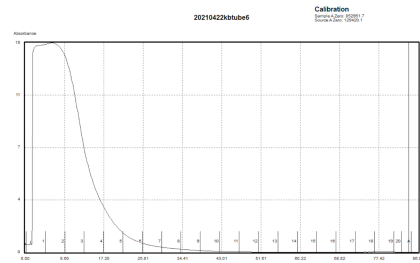
Tn7::rpsU2 standard



LVS standard



Tn7::rpsU2 bead 4_14



Tn7::rpsU3 bead 4_14

The subunit prep worked fine, but the same results happened with the bead-beater preps. We talked about it in the joint lab meeting with the Gregory Lab. It could be that for some reason there's no access to the ribosomes, or that the ribosomes are attaching to something in the cell debris. Steve suggests preparing the lysates in 0.6 mM MgCl_2 using the bead beater method to compare. Kelly previously had issues when she used 10 mM MgCl_2 , and since the 0.6 mM worked on my subunits, I'll use that to make sure there is lysis happening. I'll also potentially use a French Press prep to see if there's a difference.

Tuesday, April 27, 2021**To Do:**

1. ~~Think about next steps for sucrose gradients~~
2. Patch cells for lysates
3. Make media for lysates

Wednesday, April 28, 2021**To Do:**

1. ~~Patch cells for lysates~~
2. ~~Make media for lysates~~
3. Make overnight cultures

Methods and Results:

Because we're doing the French Press method for lysis, I am preparing 500 ml cultures instead of 70 ml.

Sample #	Measured OD600	Dilution Factor	Actual OD600	Desired Volume mL	Desired OD	Volume of Resuspended Cells μ L	OD Next Day 1000
1	0.244	100	24.4	500	0.005	102	0.489
2	0.195	100	19.5	500	0.005	128	0.364

Thursday, April 29, 2021**To Do:**

1. ~~Make overnight cultures~~
2. French Press lysis

Methods and Results:

I am using the swinging bucket rotor (TX-1000) with the 1 liter buckets to pellet the samples. I will add the DNase right before we go to the French Press. I'm not including lysozyme because a: the French Press should be enough to lyse the cells, and b: we don't have enough.

That rotor makes a lot of noise while it is accelerating, it's unnerving.

I washed the pellets in 20ml of buffer, pelleted again and washed in 10ml buffer and then added 20ul DNase to each sample. Each sample went through the French Press 3 times. Afterwards, I spun again and then aliquoted the supernatant in 1 ml tubes.

Monday, May, 3 2021**To Do:**

1. ~~French Press lysis~~
2. Sucrose gradients

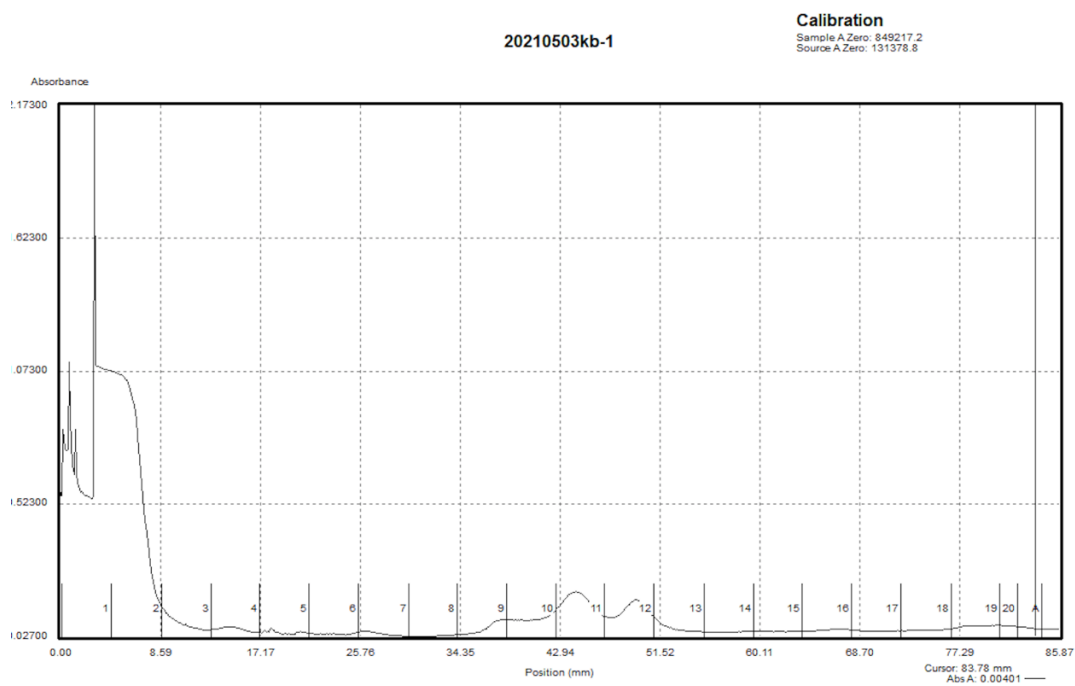
Methods and Results:

I am running sucrose gradients on the two French Press samples.

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	FP1	Science	5/3/2021 8:23:36 AM	1759.9	ng/μl	44.00	59.022	0.75	3.11	RNA	40
2	FP2	Science	5/3/2021 8:24:41 AM	1137.2	ng/μl	28.43	51.948	0.55	2.41	RNA	40
1	FP1 1:10	Science	5/3/21 8:38	187.9	ng/μl	4.70	6.515	0.72	0.28	RNA	40
2	FP2 1:10	Science	5/3/21 8:38	126.8	ng/μl	3.17	5.877	0.54	0.19	RNA	40

Sample Name #	Tube #	A260	DF (10)	Divided by 14.5	C1 (ug/ml)	Total Volume V2 (ul)	C2 (ug/ml)	V1 (ul)	Vol buffer (ul)
FP1	1	4.70	46.99	3.24	3241	205	600	38.0	167.0
FP2	2	3.17	31.71	2.19	2187	205	600	56.2	148.8

I prepared the samples as 600 ug/ml (see chart above), thinking I should just stick to the protocol, but Kathryn mentioned I should maybe have just loaded undiluted 200 ul.



20210503kb-2

Calibration

Sample A Zero: 849217.2
Source A Zero: 131378.8