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April 2024

April 2024

Monday, April 08, 2024

To Do:

1. cDNA generation on RNA samples from April 5th.

Protocol: Generate cDNA (half protocol)*

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

1. Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
$(NS)_5$ Primer (250 ng/ul)	1.5 ul	25 g/ul
RNase-free water	up to 15 ul	

2. To normalize all the cDNA samples to the same DNA mass, dilute with RNase-free water in PCR strip tubes.

Table : RNA volumes to add to each tube

	Sample	Patch	Template type	Date Isolat	RNA conc	RNA	H2O	Total RNA
1	LVS pF (KRLVS 120) A	A	LVS pF	4/5/2024	362.4	5.2	8.3	1888.65
2	LVS pF (KRLVS 120) B	B	LVS pF	4/5/2024	274	6.9	6.6	1888.65
3	LVS pF (KRLVS 120) E	E	LVS pF	4/5/2024	214.3	8.8	4.7	1888.65
4	LVS Δ rpsU2 pF (KRLVS 121) C	C	LVS Δ rpsU2 pF	4/5/2024	139.9	13.5	0.0	1888.65
5	LVS Δ rpsU2 pF (KRLVS 121) D	D	LVS Δ rpsU2 pF	4/5/2024	231.2	8.2	5.3	1888.65
6	LVS Δ rpsU2 pF (KRLVS 121) E	E	LVS Δ rpsU2 pF	4/5/2024	210.8	9.0	4.5	1888.65
7	LVSΔrpsU2 pF - rpsU2-V (KRLVS 123) A	A	LVSΔrpsU2 pF - rpsU2-V	4/5/2024	249.4	7.6	5.9	1888.65
8	LVSΔrpsU2 pF - rpsU2-V (KRLVS 123) D	D	LVSΔrpsU2 pF - rpsU2-V	4/5/2024	205.8	9.2	4.3	1888.65
9	LVSΔrpsU2 pF - rpsU2-V (KRLVS 123) E	E	LVSΔrpsU2 pF - rpsU2-V	4/5/2024	235.3	8.0	5.5	1888.65

*made total RNA 1888 this time instead of 3000 so all could be equal starting amount. If it was 3000, there wouldn't be enough of sample 4. Im slightly nervous as this is less than the recommended amount of RNA but I'm hoping it will be okay. If not, I will do again for the rest of the samples.

Note the total volume of cDNA and water is 13.5 uL.

3. Add the 1.5 uL $(NS)_5$ oligo (stored in small aliquots at -80°C; take your own aliquot to use and move it to your own stock box; minimize freeze/thaw cycles) to the tubes.

4. Incubate using program JSScDNA1 in the thermocycler:
   a. Step Temp Time

b. 1 70°C 10'
c. 2 25°C 10'
d. 3 4°C hold

5. While waiting, prepare the cDNA synthesis reaction in master mix format. The number of reactions you have plus 1.5 should be the factor you multiply by.

	Master mix for cDNA synethesis reaction		# Reactions
Component	Final Conc	Volume (uL)	10.5
5x 1st strand buffer	1x	6	63
Rnase-free water		2.87	30.135
100mM DTT	10mM	3	31.5
10mM dNTPs	0.5mM	1.5	15.75
Superscript III (200U/uL)	10.8U/uL	1.63	17.115
	total	15	157.5

*Buffer, DTT, and dNTPs should be from the cDNA reagents box (they are RNase free!)

6. Aliquot 15 ul of master mix per reaction into each PCR tube from the first reaction (total volume now 30ul)

7. Incubate using program JSScDNA2
   a. Step Temp Time
   b. 1 25°C 10'
   c. 2 37°C 60'
   d. 4 42°C 60'
   e. 5 70°C 10'
   f. 6 4°C hold

*potential stopping point- samples can be stored at -80°C if necessary

8. Remove RNA from sample by degrading with sodium hydroxide:

9. Add 10 ul of 1N NaOH
10. Incubate 65°C for 30'
11. Neutralize with 10 ul of 1N HCl
12. Final volume is 50 ul
13. Purify cDNA using Qiagen PCR clean-up column (be very sure there is no ethanol carry-over!) THIS IS THE PCR PURIFICATION PROTOCOL

14. Elute in 60 ul of 0.1x EB

15. Check concentration by Nanodrop

16. Store cDNA at -80°C

Table: Nanodrop results after cDNA generation

#	Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
1	1	14.1	ng/µl	0.428	0.238	1.8	0.93
2	2	15.2	ng/µl	0.46	0.255	1.81	0.91
3	3	14.3	ng/µl	0.435	0.26	1.67	0.74
4	4	9.2	ng/µl	0.279	0.151	1.85	1.84
5	5	10.1	ng/µl	0.307	0.161	1.91	0.69
6	6	12.3	ng/µl	0.372	0.217	1.71	0.69
7	7	12.4	ng/µl	0.377	0.207	1.82	0.64
8	8	10.4	ng/µl	0.314	0.165	1.9	0.65
9	9	11.6	ng/µl	0.353	0.2	1.76	0.82

cDNA!!! I'm not sure why the purity values look like this but at this point I'm going to leave them on the bench top for a bit and see if ethanol evaporates and if not just run with it. I'm coming to the conclusion the 260/230 values will neve be fantastic. On the brightside, using less of a starting amount of RNA did not seem like it affected the cDNA concentration too much.

Tuesday, April 09, 2024

To Do:

1. ~~Streak out KRLVS 148~~

Struck out KRLVS 148 for RNA stability assay. Struck out on Chah

Wednesday, April 10, 2024

To Do:

1. ~~Streak out KRLVS 149 for RNA stability assay on CHAH~~
2. ~~RT-qPCR on cDNA from Monday~~

Protocol: Real-Time PCR on cDNA samples

Original protocol by Heather McManus, edited by Kathryn Ramsey

1. Each experiment will need at least one test primer and one control primer for each sample
    a. Example: 3 biological replicates = 3 DNA samples
2. Using two primer pairs = 6 different reactions

3. Each different reaction type must be run in triplicate on the real-time plate
    a. Example: 6 reactions = 18 wells

4. Each Individual reaction = 20 µL
    a. 10 µL PowerUp SYBR Green Master Mix
    b. 1 µL 5uM combined forward and reverse primer
    c. 1 µL cDNA (~1.5 ng/ul, can base off efficiency tests)
    d. 8 µL ddiH_2O

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

6. For each reaction type (primer/DNA combination) set up a master mix equal to 3.5 reactions:
7. Put 3.5µL of DNA type into strip tubes

cDNA Dilutions for each sample to put into strip tubes

	Starting		Want	Need of original
Sample	Conc (ng/uL)	Total volme dilution (uL)	ng/ul	uL
LVS pF (KRLVS 120) B	14.1	100	1.5	10.64
LVS pF (KRLVS 120) A	15.2	100	1.5	9.87
LVS Δ rpsU2 pF (KRLVS 121) A	14.3	100	1.5	10.49
LVS Δ rpsU2 pF (KRLVS 121) E	9.2	100	1.5	16.30
LVS ΔrpsU2 pF - rpsU2-V (KRLVS 123) A	10.1	100	1.5	14.85
LVSΔrpsU2 pF - rpsU2-V (KRLVS 123) D	12.3	100	1.5	12.20
	12.4	100	1.5	12.10
	10.4	100	1.5	14.42
	11.6	100	1.5	12.93

8. 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
    a. Example: 6 DNA samples per primer pair = 6 x 3.5 + 3.5 = 24.5

9. Set up primer master mixes in separate 1.5 mL tubes (make one for each primer pair)
    a. Example:
    b. 10 µL PowerUp SYBR Green Master Mix x 24.5 = 245.0 µL
    c. 1 µL 5µM combined F and R primer x 24.5 = 24.5 µL
    d. 8 µL ddiH_2O x 24.5 = 171.5 µL
    e. TOTAL = 465.5 µL

Set Up of Primers and Plate

Master Mix	Primer Set	Why
1	KROL 504/505	rpsU
2	KROL 63/64	tul4 (control)

Strip Tube DNA + Master Mix KROL 504/505	1	2	3	4	5	6	7	8	9
	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6	Sample 7	Sample 8	Sample 9
Strip Tube DNA + Master Mix KROL 63/64	1	2	3	4	5	6	7	8	
	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6	Sample 7	Sample 8	Sample 9

Master Mix		35
Component	Individual	Master Mix
PowerUp SYBR Green MM	10	350
5uM combined F/R Primer	1	35
DNA	1	
ddiH2O	8	280
Total	20	665

10. Add primer master mix to tubes containing DNA.
11. 3.5 reactions x 20 µL volume = 70 µL. DNA tubes already have 3.5µL of DNA. Add 66.5 µL of primer master mix to each tube

12. Pipette 20 µL of each reaction into 3 separate wells on the 96 well plate using dispense option on multichannel

13. Spin plate down

14. Place in real-time machine and run using the same program used to determine that the primers are appropriately efficient.

    GSC computer login:
    K_Ramsey Lab
    Password:
    Roche480

<u>To make 5uM primer set stocks:</u>

270 µL 0.1x EB
15 µL 100uM combined forward primer
15 µL 100uM combined reverse primer
*Ensure you vortex 100uM stocks vigorously in order to ensure complete thawing

Plate Overview

Plate Set Up	1	2	3	4	5	6	7	8	9	10	11	12
A	MM1 sample 1 rep 1	MM1 sample 2 rep 1	mm1 sample 3 rep 1	mm1 sample 4 rep 1	mm1 sample 5 rep 1	mm1 sample 6 rep 1	mm1 sample 7 rep 1	mm1 sample 8 rep 1	mm1 sample 9 rep 1			
B	MM1 sample 1 rep 2	MM1 sample 2 rep 2	mm1 sample 3 rep 2	mm1 sample 4 rep 2	mm1 sample 5 rep 2	mm1 sample 6 rep 2	mm1 sample 1 rep 2	mm1 sample 8 rep 2	mm1 sample 9 rep 2			
C	MM1 sample 1 rep 3	MM1 sample 2 rep 3	mm1 sample 3 rep 3	mm1 sample 4 rep 3	mm1 sample 5 rep 3	mm1 sample 6 rep 3	mm1 sample 7 rep 3	mm1 sample 8 rep 3	mm1 sample 9 rep 3			
D	MM2 sample 1 rep 1	MM2 sample 2 rep 1	mm2 sample 3 rep 1	mm2 sample 4 rep 1	mm2 sample 5 rep 1	mm2 sample 6 rep 1	mm2 sample 7 rep 1	mm2 sample 8 rep 1	mm2 sample 9 rep 1			
E	MM2 sample 1 rep 2	MM2 sample 2 rep 2	mm2 sample 3 rep 2	mm2 sample 4 rep 2	mm2 sample 5 rep 2	mm2 sample 6 rep 2	mm2 sample 7 rep 2	mm2 sample 8 rep 2	mm2 sample 9 rep 2			
F	MM2 sample 1 rep 3	MM2 sample 2 rep 3	mm2 sample 3 rep 3	mm2 sample 4 rep 3	mm2 sample 5 rep 3	mm2 sample 6 rep 3	mm3 sample 7 rep 3	mm2 sample 8 rep 3	mm2 sample 9 rep 3			
G												
H												

During this, I remade the primers for KROL 504/505 and KROL 63/64 using 0.1x EB.

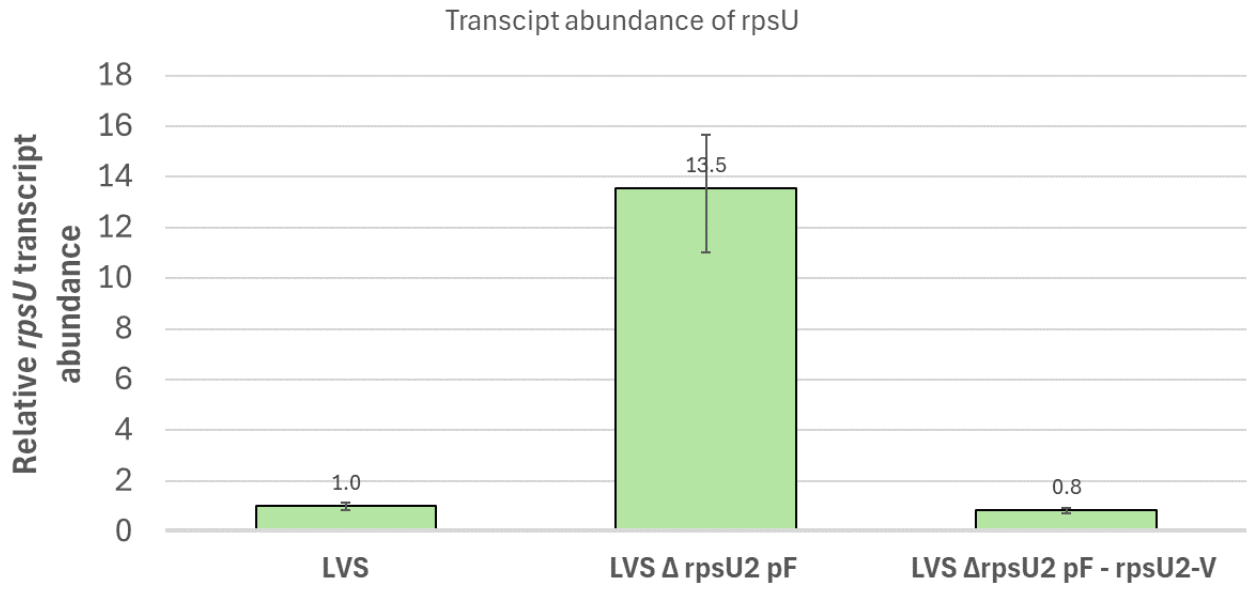


Figure: RT-qPCR Results April 10, 2024

This is consistent with what was seen in Sierra's work, with deletion rpsU2 having much higher transcript abundance than the WT and the complement strand. Shows that rpsU2 somehow regulates itself *in vivo*.

After meeting with Kathryn realized I started my inoculation of KRLVS 148 and 149 too early. Need to start on Friday. Will start over then and on Monday we will do quick walk through of the procedure and on Wednesday we will do the assay.

Friday, April 12, 2024

To Do:

1. ~~Streak out KRLVS 148 on CHAH-Kan~~

Saturday, April 13, 2024

To Do:

1. ~~Streak out KRLVS 149 on CHAH-Kan~~

Monday, April 15, 2024

To Do:

1. ~~Patches on KRLVS 148~~
2. ~~Walkthrough of RNA stability assay with KMR~~
3. Put single colonies of P. ging into liquid culture and let grow

Was going to inoculate *P. ging* liquid culture yesterday but forgot the media needs to be in the anaerobic chamber for a few hours so all the oxygen can dissipate. Will do tomorrow.

Set the shaking incubator in the Nelson lab to 37C and left overnight (after cleaning) so it should be ready for Wednesday

Tuesday, April 16, 2024

To Do:

1. ~~Patches on KRLVS 149 for RNA stability assay~~
2. ~~Supplemented TBHK and out in anaerobic chamber~~
3. Put single colonies of *P. ging* into liquid culture and let grow
4. ~~Label 30 50mL conical tubes for RNA Stability assay.~~

Wednesday, April 17, 2024

To Do:

1. ~~Put single colonies of *P. ging* into liquid culture and let grow~~
2. ~~Start RNA stability assay~~

When scraping up colonies to put into microcentrifuge tubes to pellet, the tubes got opened and flown against wall when becoming anaerobic in vacuum, tried to disinfect and then put back in vacuum before scraping up pellets. Put rest of bacteria into 400uL of TBHK media. When putting single colonies into broth, got 6 colonies of wildtype 83 and 3 colonies of PG0121.

Protocol: RNA Stability Assay

Adapted from protocol sent by Dr. Scarlet Shell, WPI

1. Prior to the day of the experiment, streak strains of interest to single colony and patch onto CHAH-Kan. Takes six days
2. Resuspend patches of strains of interest in 400 uL of MHB and check OD in a 1:20 dilution. Ended up being 1:80 dilution
3. Start cultures at an appropriate OD (0.08 for WT background or 0.10 for strains with a growth defect) in 40 mL of culture and grow to mid-log (OD 0.3-0.4), typically ~4-4.6 hours
4. While cultures are growing, label and prepare 50 mL tubes and prepare 0.5 mg/mL rifampin in DMSO. We did 50mL for more aeration
5. Once OD ~0.3, transfer 7mL of each replicate into appropriately labeled 50 mL tube, one for each time point and allow to shake for an additional 30 minutes

6. After 30 minutes, flash freeze 0' time point in liquid nitrogen, then add rifampin to the tubes to a final concentration of 0.5 ug/mL (7uL of 0.5 mg/mL rif) to each subsequent tube of a given replicate, flashing freezing each 15 mL tube after either 1, 2, 4, or 8 minutes.
7. Repeat for each biological replicate, can start second time course during 4 minute wait, cultures can be stored in the -80 for up to two months
8. When prepared, thaw cultures and proceed with RNA extraction and purification, cDNA synthesis, and qRT-PCR protocols
9. For analysis, preform linear regression on a plot of $-C_T$ over time. Half-life = −1/slope. If there is a delay in decay, the first or second time point can be removed from the linear regression. If there is a plateau after an exponential decay, timepoints after plateau can be removed.

Sample Experimental Configuration																														
Strain	KRLVS 148															KRLVS 149														
Replicate	1					2					3					1					2					3				
rif (min)	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8

*****NOTE: switched timepoints for KRLVS 149 #2 (sample 5) 0 and 1 min. so the one labeled 0 is actually the 1min timepoint and the one labeled 1min is the 0min timepoint.

OD600 of samples for RNA stability assay

Genotype	Measured OD600	Dilution factor	Calculated OD600	Volume to add for	Desired final	uL to add for 0.08 in	notes	10am measured OD600	1:50 measured OD600	2:41 for 4-6, 3:50 for 1- measured OD600
KRLVS 148 A	0.319	80	25.52	0.15674	0.1	157	*originally	0.101	0.24	0.294
KRLVS 148 B	0.304	80	24.32	0.164474	0.1	164		0.096	0.19	0.247
KRLVS 148 E	0.278	80	22.24	0.179856	0.1	180		0.1	0.209	0.265
KRLVS 149 B	0.21	80	16.8	0.190476	0.08	190		0.078	0.274	0.333
KRLVS 149 C	0.162	80	12.96	0.246914	0.08	247		0.073	0.257	0.328
KRLVS 149 E	0.188	80	15.04	0.212766	0.08	213		0.078	0.268	0.331

Friday, April 19, 2024

To Do:

1. ~~Streak out KRLVS 148 on CHAH Kan~~

Saturday, April 20, 2024

To Do:

1. ~~Streak out KRLVS 149 on CHAH Kan~~
2. ~~Checked liquid cultures~~

When checking the liquid cultures of *P. ging* some cultures looked like they had more growth and others had less. I tested two samples one with the most confluence one with a medium amount to see what the OD600s were as I will be pelleting them around OD600.

The *p.ging* mutant culture was at an OD600 of 0.467, the WT culture was at 0.115. They both went back in the incubator.

Monday, April 22, 2024

To Do:

1. ~~Patched of KRLVS 148 on CHAH Kan~~
2. ~~Checked liquid p.ging cultures~~

~~I checked the OD600s of the *p.ging* cultures today. They're all super overgrown now. I have no idea how since on Saturday the reading was 0.115 for the second most confluent.~~

Culture	OD600
PG 0121 – 1	0.974
PG 0121 – 2	0.006
PG 0121 – 3	1.065
WT 1	1.196
WT 2	1.256
WT 3	1.102
WT 4	1.396
WT 5	1.197
WT 6	0.989

Since PG 0121-2 didn't grow, I took that one out and now PG 0121-3 is PG0121-2. I spun down all the grown samples into pellets (about 3mL of culture (1.5mL each spin, max speed, 5mins) and put them at -20C.

Tuesday, April 23, 2024

To Do:

1. ~~Patched of KRLVS 149 on CHAH Kan~~
2. ~~Prepped for stability assay tomorrow~~
3. ~~Made supplemented MHB media~~

Wednesday, April 24, 2024

To Do:

1. ~~RNA Stability assay~~
2. ~~Picked up ethanol from stock180~~

Protocol: RNA Stability Assay

Adapted from protocol sent by Dr. Scarlet Shell, WPI

3. Prior to the day of the experiment, streak strains of interest to single colony and patch onto CHAH-Kan. Takes six days
4. Resuspend patches of strains of interest in 400 uL of MHB and check OD in a 1:20 dilution. Ended up being 1:80 dilution for samples 1,2,3,5 and a 1:40 for samples 4,6
5. Start cultures at an appropriate OD (0.08 for WT background or 0.10 for strains with a growth defect) in 40 mL of culture and grow to mid-log (OD 0.3-0.4), typically ~4-4.6 hours

6. While cultures are growing, label and prepare 50 mL tubes and prepare 0.5 mg/mL rifampin in DMSO. We did 50mL for more aeration
7. Once OD ~0.3, transfer 7mL of each replicate into appropriately labeled 50 mL tube, one for each time point and allow to shake for an additional 30 minutes
8. After 30 minutes, flash freeze 0' time point in liquid nitrogen, then add rifampin to the tubes to a final concentration of 0.5 ug/mL (7uL of 0.5 mg/mL rif) to each subsequent tube of a given replicate, flashing freezing each 15 mL tube after either 1, 2, 4, or 8 minutes.
9. Repeat for each biological replicate, can start second time course during 4 minute wait, cultures can be stored in the -80 for up to two months
10. When prepared, thaw cultures and proceed with RNA extraction and purification, cDNA synthesis, and qRT-PCR protocols
11. For analysis, preform linear regression on a plot of $-C_T$ over time. Half-life = −1/slope. If there is a delay in decay, the first or second time point can be removed from the linear regression. If there is a plateau after an exponential decay, timepoints after plateau can be removed.

Sample Experimental Configuration																												
Strain	KRLVS 148															KRLVS 149												
Replicate	1					2					3					1					2					3				
rif (min)	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8	0	1	2	4	8

OD600 readings for RNA stability assay

									10am	2pm	3:17pm	4:30
Sample	Genotype	Measured OD600	Dilution factor	Calculated OD600	Volume to add for	Desired final	uL to add for 0.08 in	notes	measured OD600	measured OD600	measured OD600	measured OD600
1	KRLVS 148 A	0.293	80	23.44	0.170648	0.1	171	*low so ad	0.099	0.215	0.262	0.313
2	KRLVS 148 B	0.441	80	35.28	0.113379	0.1	113		0.07	0.16	0.195	0.24
3	KRLVS 148 E	0.308	80	24.64	0.162338	0.1	162		0.074	0.165	0.196	0.233
4	KRLVS 149 C	0.243	40	9.72	0.329218	0.08	329	*unsure wl	0.058	0.266	0.364	finished
5	KRLVS 149 D	0.249	80	19.92	0.160643	0.08	161		0.077	0.208	0.291	finished
6	KRLVS 149 E	0.273	40	10.92	0.29304	0.08	293		0.085	0.29	0.385	finished
				0	#DIV/0!	0.08	#DIV/0!					
				0	#DIV/0!	0.08	#DIV/0!					
				0	#DIV/0!	0.08	#DIV/0!					

Thursday, April 25, 2024

To Do:

1. ~~Cleaned up from previous day~~

Friday, April 26, 2024

To Do:

1. ~~Lab Chores~~

Monday, April 29, 2024

To Do:

Tuesday, April 30, 2024

To Do:

1. ~~gDNA Extraction of 4 *P. ging* colonies~~

Worked with George in the morning, extracting gDNA from 4 of the WT *P. ging* samples and 4 of his samples (E2-24, E2-26, E2-27, and KRLVS 294.1) Was able do use the gDNA extraction for LVS protocol, even though I'm working with *P. ging*.

Protocol: gDNA Extraction of LVS

1. Dilute 1uL of Proteinase K into 310uL of Tissue and Cell Lysis Solution for each sample.
2. Scraped up patches of George's samples and resuspended in 500uL MHB. My cells were pelleted already so skipped to step 3 for the *P ging*.
3. Pellet cells by centrifugation and discard the supernatant, leaving approximately 25 uL of liquid.
4. Vortex for 10 seconds to resuspend the cell pellet.
5. Add 300uL of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly (pipette up and down).
6. Incubate at 65°C for 15 minutes, vortex every 5 minutes.
7. Cool the samples to 37°C and add 2ul of 20mg/mL RNase A to the sample, mix thoroughly.
8. Incubate at 37°C for 30 minutes.
9. Place the samples on ice for 5 minutes.
10. Add 150uL of MPC Protein Precipitation Reagent to 200uL of lysed sample and vortex vigorously for 10 seconds. Transferred 200uL of the lysed sample to a new microcentrifuge tube before adding the 150uL MPC Protein PPT Reagent. Solution was almost gelatinous after. Very sticky
11. Pellet the debris by centrifugation at 4°C for 10 minutes at max speed in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25uL of MPC Protein Precipitation Reagent, mix, and pellet the debris again. Pellet was loose, so added the additional 25uL and redid and pellet was no longer loose.
12. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
13. Add 500uL of isopropanol to the recovered supernatant. Invert the tube 40 times.
14. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
15. Carefully pour off the isopropanol without dislodging the DNA pellet.
16. Rinse twice with 70% ethanol (~1mL) being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all the residual ethanol with a pipet and let it air dry. Freshly made the 70% ETOH.
17. Resuspend the DNA in 35uL of 0.1x EB.

Nanodroped the gDNA extractions after. Below are my samples, I put georges in his folder. The extraction seemed to go well. Good 260/280 values, curious 260/230 values but that may be because somehow the solution was still sticky at the end. I do not think that's normal and don't know how it would've happened. We shall see if that's actually an issue later.

Nanodrop of *P. ging* samples after gDNA extraction.

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230
WT - 1	703.2	ng/µl	14.064	7.313	1.92	1.71
WT - 2	1046.6	ng/µl	20.932	10.968	1.91	1.76
WT - 3	746.8	ng/µl	14.935	7.994	1.87	1.61
WT - 4	783.4	ng/µl	15.668	8.329	1.88	1.62

Wednesday, May 1, 2024

To Do:

1. ~~Lab Chores~~

Thursday, May 2, 2024

To Do:

1. ~~gDNA extraction on remaining pellets~~.

Protocol: gDNA Extraction of LVS

1. Dilute 1uL of Proteinase K into 310uL of Tissue and Cell Lysis Solution for each sample.
2. Scraped up patches of George's samples and resuspended in 500uL MHB. My cells were pelleted already so skipped to step 3 for the *P ging*.
3. Pellet cells by centrifugation and discard the supernatant, leaving approximately 25 uL of liquid.
4. Vortex for 10 seconds to resuspend the cell pellet.
5. Add 300uL of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly (pipette up and down).
6. Incubate at 65°C for 15 minutes, vortex every 5 minutes.
7. Cool the samples to 37°C and add 2ul of 20mg/mL RNase A to the sample, mix thoroughly.
8. Incubate at 37°C for 30 minutes.
9. Place the samples on ice for 5 minutes.
10. Add 150uL of MPC Protein Precipitation Reagent to 200uL of lysed sample and vortex vigorously for 10 seconds. Transferred 200uL of the lysed sample to a new microcentrifuge tube before adding the 150uL MPC Protein PPT Reagent. Solution was almost gelatinous after. Very sticky
11. Pellet the debris by centrifugation at 4°C for 10 minutes at max speed in a microcentrifuge. If the resultant pellet is clear, small, or loose, add an additional 25uL of MPC Protein Precipitation Reagent, mix, and pellet the debris again. Pellet was loose, so added the additional 25uL and redid and pellet was no longer loose.
12. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
13. Add 500uL of isopropanol to the recovered supernatant. Invert the tube 40 times.
14. Pellet the DNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
15. Carefully pour off the isopropanol without dislodging the DNA pellet.
16. Rinse twice with 70% ethanol (~1mL) being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all the residual ethanol with a pipet and let it air dry. Freshly made the 70% ETOH.
17. Resuspend the DNA in 35uL of 0.1x EB.

Nanodrop results of gDNA extraction

#	Sample ID	Nucleic Acid	Unit	260/280	260/230
1	WT 5	387.1	ng/µl	1.89	1.6
2	WT 6	851.7	ng/µl	1.85	1.87
3	PG0121 1	770.2	ng/µl	1.85	1.91
4	PG0121 2	820.8	ng/µl	1.83	1.72
5	WT 5 repeat	682.2	ng/µl	1.81	1.63

Going to use the second value for WT5 (the repeat) because when I measured the first time it seemed low and the nanodrop took a while to read it, which made me think something was weird and the nanodrop needed to be warmed up.

Monday, May 6, 2024

To Do:

1. ~~RNA isolation day 1 of samples from KRLVS148 4.17.24~~

Protocol – RNAsnap

Adapted from: Stead et al. (2012) *Nucleic Acids Research*
Updated 10/26/22

Note: All reagents should be RNAse free; use good RNA-technique by cleaning work area, gloves, and instruments with RNAse-away.

Wear safety goggles when handling formamide and dispose of as hazardous waste.

RNA*snap*

1. Grow strains to mid-log
2. Centrifuge 1 to 10 mL of bacterial culture at max speed for 3 (1 MIN) mins. Discard supernatant. Repeat added 1.8ml to tube once, centrifuged, discarded supernatant, then added another 1.8ml to tube and repeated process. (remaining steps will be in hood. Bring in heat block, vortex, and centrifuge into hood. Super sterile)
3. Resuspend cell pellet in 100 µL of **fresh** RES (see recipe below) by vortexing vigorously. If using 10 mL of culture, use 300 µL of RES.
4. Incubate sample at 95°C for 7 minutes.
5. Pellet cell debris by centrifuging at max speed for 5 mins at room temperature. Carefully move the supernatant to a fresh tube without disturbing the gelatinous pellet. We did twice because couldn't see pellet first time. We did this outside of hood because no room in hood.

Sodium acetate/ethanol precipitation

7. BACK TO HOOD. Dilute RNA sample to 300 µL with water. had 60ul of rna sample, Added 240ul of water
8. Add 1/10 volume of 3 M sodium acetate, pH 5.2 (30 µL).
9. Add 2 µL GlycoBlue Coprecipitant (ThermoFisher)

10. Add three volumes of 100% ethanol (900 µL). Mix by vortexing then incubate for at least 60 mins (or overnight) at -80°C. STOPPED HERE 5/6/24.
11. Centrifuge at max speed for 30 mins at 4°C. Carefully remove supernatant. Set up centrifuge to correct temp prior. Started here 5/7/24
12. Wash cell pellet with 250 µL of freshly-diluted 75% ethanol. Make fresh each time.
13. Centrifuge at max speed for 5 mins at 4°C. Carefully remove supernatant.
14. Briefly centrifuge again to allow removal of all remaining ethanol, then air dry the pellet.
15. Resuspend the pellet in 80 µL water. Centrifuge at max speed for 1 minute to pellet any remaining water insoluble proteins. Transfer the RNA-containing supernatant to a fresh tube.
16. Check concentration by Nanodrop (RNA setting).

DNAse Treatment (Promega)

17. Add 10 µL RNase-free DNase buffer and 10 µL RNase-free DNase (Promega, RQ1)
16. Incubate at 37°C for 1 hour. Can be right in incubator doesn't need heat block.
17. Repeat sodium acetate/ethanol precipitation as above (steps 6-15). Resuspend in 50uL this time, NOT 80 (step 14).

RNA isolation tube numbers key

Tube #	Contents	Tube #	Contents	Tube #	Contents
1	KRLVS148 1 0'	6	KRLVS148 2 0'	11	KRLVS148 3 0'
2	KRLVS148 1 1'	7	KRLVS148 2 1'	12	KRLVS148 3 1'
3	KRLVS148 1 2'	8	KRLVS148 2 2'	13	KRLVS148 3 2'
4	KRLVS148 1 4'	9	KRLVS148 2 4'	14	KRLVS148 3 4'
5	KRLVS148 1 8'	10	KRLVS148 2 8'	15	KRLVS148 3 8'

Tuesday, May 7, 2024

To Do:

1. ~~RNA isolation day 2~~

Protocol – RNAsnap

Adapted from: Stead et al. (2012) *Nucleic Acids Research*
Updated 10/26/22

Note: All reagents should be RNAse free; use good RNA-technique by cleaning work area, gloves, and instruments with RNAse-away.

Wear safety goggles when handling formamide and dispose of as hazardous waste.

RNA*snap*

1. Grow strains to mid-log
2. Centrifuge 1 to 10 mL of bacterial culture at max speed for 3 (1 MIN) mins. Discard supernatant. Repeat added 1.8ml to tube once, centrifuged, discarded supernatant, then added another 1.8ml to tube and repeated process. (remaining steps will be in hood. Bring in heat block, vortex, and centrifuge into hood. Super sterile)

3. Resuspend cell pellet in 100 µL of **fresh** RES (see recipe below) by vortexing vigorously. If using 10 mL of culture, use 300 µL of RES.
4. Incubate sample at 95°C for 7 minutes.
5. Pellet cell debris by centrifuging at max speed for 5 mins at room temperature. Carefully move the supernatant to a fresh tube without disturbing the gelatinous pellet. We did twice because couldn't see pellet first time. We did this outside of hood because no room in hood.

Sodium acetate/ethanol precipitation

6. Dilute RNA sample to 300 µL with water. had 60ul of rna sample, Added 240ul of water
7. Add 1/10 volume of 3 M sodium acetate, pH 5.2 (30 µL).
8. Add 2 µL GlycoBlue Coprecipitant (ThermoFisher)
9. Add three volumes of 100% ethanol (900 µL). Mix by vortexing then incubate for at least 60 mins (or overnight) at -80°C. Stopped here 5/6/24.
10. Centrifuge at max speed for 30 mins at 4°C. Carefully remove supernatant. Set up centrifuge to correct temp prior. Started here 5/7/24
11. Wash cell pellet with 250 µL of freshly-diluted 75% ethanol. Make fresh each time.
12. Centrifuge at max speed for 5 mins at 4°C. Carefully remove supernatant.
13. Briefly centrifuge again to allow removal of all remaining ethanol, then air dry the pellet.
14. Resuspend the pellet in 80 µL water. Centrifuge at max speed for 1 minute to pellet any remaining water insoluble proteins. Transfer the RNA-containing supernatant to a fresh tube.
15. Check concentration by Nanodrop (RNA setting).

DNAse Treatment (Promega)

16. Add 10 µL RNase-free DNase buffer and 10 µL RNase-free DNase (Promega, RQ1)
17. Incubate at 37°C for 1 hour. Can be right in incubator doesn't need heat block.
18. Repeat sodium acetate/ethanol precipitation as above (steps 6-15). Resuspend in 50uL this time, NOT 80 (step 14).

Notes:

After finishing step 17, I went to take the remaining reagents out of the 4C and -20C fridge/freezer. In doing so, I saw I wouldn't have enough sodium acetate to finish the protocol. Therefore, the samples went into the -80C until the sodium acetate comes in and I can continue the protocol. Going to pick up at step 18 (so really step 6).

Nanodrop values after day 2

#	Nucleic Acid	Unit	260/280	260/230	Sample Type
1	296.1	ng/µl	1.86	1.13	RNA
2	345.7	ng/µl	1.86	1.13	RNA
3	391.1	ng/µl	1.86	1.14	RNA
4	343.3	ng/µl	1.88	1.14	RNA
5	344.8	ng/µl	1.86	1.13	RNA
6	302	ng/µl	1.87	1.27	RNA
7	376.9	ng/µl	1.31	0.94	RNA
8	259.4	ng/µl	1.89	1.28	RNA
9	277.9	ng/µl	1.91	1.26	RNA
10	269.7	ng/µl	1.88	1.22	RNA
11	284	ng/µl	1.9	1.16	RNA
12	280	ng/µl	1.87	1.13	RNA
13	265.6	ng/µl	1.85	1.12	RNA
14	256.2	ng/µl	1.85	1.09	RNA
15	291.4	ng/µl	1.85	1.08	RNA

Wednesday, May 8, 2024

To Do:

1. ~~Finish coding !!!!~~

All day today was spent finish the coding assignment analyzing the triple mutant to see if we were able to produce the triple mutant. We were not, the results were no triple mutant.

Thursday, May 9, 2024

To Do:

1. ~~RNA isolation day 2.5~~
2. ~~Lab chores~~

Because of the sodium acetate snafu, I will finish day 2 of the RNA isolation today. Starting at step 6 until the overnight step. I don't think it's a good idea to only do the hour in the freezer because I haven't done that yet and don't want to risk messing up the samples even more.

Friday, May 10, 2024

To Do:

1. ~~RNA isolation day 3~~

Nanodrop values after day 3 of isolation

#	Sample ID	Nucleic Acid	Unit	260/280	260/230
1	KRLVS 148 #1 0min	252.3	ng/µl	1.91	1.23
2	KRLVS 148 #1 1min	268.4	ng/µl	1.92	1.34
3	KRLVS 148 #1 2min	433.8	ng/µl	1.57	0.97
4	KRLVS 148 #1 4min	313.2	ng/µl	1.89	1.14
5	KRLVS148 #1 8min	266.2	ng/µl	1.91	1.33
6	KRLV S 148 #2 0min	231.2	ng/µl	1.94	1.35
7	KRLVS 148 #2 1min	246	ng/µl	1.9	1.18
8	KRLVS 148 #2 2min	258.5	ng/µl	1.9	1.08
9	KRLVS 148 #2 4min	235	ng/µl	1.89	1.17
10	KRLVS 148 #2 8min	245.9	ng/µl	1.87	1.12
11	KRLVS 148 #3 0min	263.2	ng/µl	1.85	1.08
12	KRLVS148 #3 1min	255	ng/µl	1.83	1.04
13	KRLVS 148 #3 2min	189.3	ng/µl	1.93	1.43
14	KRLVS 148#3 4min	237.3	ng/µl	1.85	1.07
15	KRLVS 148 #3 8min	262.1	ng/µl	1.84	1.11

Monday, May 13, 2024

To Do:

1. ~~cDNA generation~~
2. ~~Lab chores~~

Protocol: Generate cDNA (half protocol)*

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

1. Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	267 - 333 ng/ ul
$(NS)_5$ Primer (250 ng/ul)	1.5 ul	25 g/ul
RNase-free water	up to 15 ul	

2. To normalize all the cDNA samples to the same DNA mass, dilute with RNase-free water in PCR strip tubes.

cDNA volumes to add to each tube

#	Sample	Conc (ng/uL)	RNA needed	H20 needed	total RNA
1	KRLVS 148 #1 0min	252.3	10.1	3.4	2555
2	KRLVS 148 #1 1min	268.4	9.5	4.0	2555
3	KRLVS 148 #1 2min	433.8	5.9	7.6	2555
4	KRLVS 148 #1 4min	313.2	8.2	5.3	2555
5	KRLVS148 #1 8min	266.2	9.6	3.9	2555
6	KRLV S 148 #2 0min	231.2	11.1	2.4	2555
7	KRLVS 148 #2 1min	246	10.4	3.1	2555
8	KRLVS 148 #2 2min	258.5	9.9	3.6	2555
9	KRLVS 148 #2 4min	235	10.9	2.6	2555
10	KRLVS 148 #2 8min	245.9	10.4	3.1	2555
11	KRLVS 148 #3 0min	263.2	9.7	3.8	2555
12	KRLVS148 #3 1min	255	10.0	3.5	2555
13	KRLVS 148 #3 2min	189.3	13.5	0.0	2555
14	KRLVS 148#3 4min	237.3	10.8	2.7	2555
15	KRLVS 148 #3 8min	262.1	9.7	3.8	2555

Note the total volume of cDNA and water is 13.5 uL.

3. Add the 1.5 uL $(NS)_5$ oligo (stored in small aliquots at -80°C; take your own aliquot to use and move it to your own stock box; minimize freeze/thaw cycles) to the tubes.

4. Incubate using program JSScDNA1 in the thermocycler:
   a. Step Temp Time
   b. 1 70°C 10'
   c. 2 25°C 10'
   d. 3 4°C hold

5. While waiting, prepare the cDNA synthesis reaction in master mix format. The number of reactions you have plus 1.5 should be the factor you multiply by.

	Master mix for cDNA synethesis reaction		# Reactions
Component	Final Conc	Volume (uL)	16.5
5x 1st strand buffer	1x	6	99
Rnase-free water		2.87	47.355
100mM DTT	10mM	3	49.5
10mM dNTPs	0.5mM	1.5	24.75
Superscript III (200U/uL)	10.8U/uL	1.63	26.895
	total	15	247.5

*Buffer, DTT, and dNTPs should be from the cDNA reagents box (they are RNase free!)

6. Aliquot 15 ul of master mix per reaction into each PCR tube from the first reaction (total volume now 30ul)

7. Incubate using program JSScDNA2
   a. Step Temp Time
   b. 1 25°C 10'
   c. 2 37°C 60'
   d. 4 42°C 60'
   e. 5 70°C 10'
   f. 6 4°C hold

*potential stopping point- samples can be stored at -80°C if necessary

8. Remove RNA from sample by degrading with sodium hydroxide:

9. Add 10 ul of 1N NaOH
10. Incubate 65°C for 30'
11. Neutralize with 10 ul of 1N HCl
12. Final volume is 50 ul
13. Purify cDNA using Qiagen PCR clean-up column (be very sure there is no ethanol carry-over!) THIS IS THE PCR PURIFICATION PROTOCOL
14. Elute in 60 ul of 0.1x EB

15. Check concentration by Nanodrop

16. Store cDNA at -80°C

Nanodrop values after cDNA generation

Sample ID	Nucleic Acid	Unit	260/280	260/230
1	12	ng/µl	1.75	2.18
2	13	ng/µl	1.89	1.02
3	9.5	ng/µl	2.03	1.16
4	14.3	ng/µl	1.85	1
5	12.6	ng/µl	1.65	0.97
6	14	ng/µl	1.89	1
7	15	ng/µl	1.79	0.84
8	13.5	ng/µl	1.89	0.89
9	14.4	ng/µl	1.89	1.17
10	14	ng/µl	1.67	1
11	12.9	ng/µl	1.91	0.93
12	15	ng/µl	1.88	1.08
13	13.8	ng/µl	1.63	0.88
14	13.3	ng/µl	1.92	1.03
15	12.5	ng/µl	1.97	1.04

Tuesday, May 14, 2024

To Do:

1. ~~RNA isolation day 1 of next 15 samples RNA stability assay~~
2. ~~Re-autoclave tubes from yesterday~~

RNA isolation on KRLVS 149 samples from 4.17.24. These are the samples were #2 0 and 1 min were switched, so it is noted, and they are numbered properly going forward.

When doing the autoclaving yesterday, I used the first floor autoclave on grav30 to sterilize tip boxes and glass culture tubes. Pulling it out, it was very wet with a lot of condensation. This is weird. Therefore, decided not to use them and the autoclave tape was changed and I reran it in the third floor autoclave today on grav20. They don't have a grav30 option, it should be okay. I am no longer trusting of the first floor autoclave and will be going to the third and fourth floor now until the second floor is done. I told Kira and Ben so they know too.

Protocol – RNAsnap

Adapted from: Stead et al. (2012) *Nucleic Acids Research*
Updated 10/26/22

Note: All reagents should be RNAse free; use good RNA-technique by cleaning work area, gloves, and instruments with RNAse-away.

Wear safety goggles when handling formamide and dispose of as hazardous waste.

RNA*snap*

1. Grow strains to mid-log
2. Centrifuge 1 to 10 mL of bacterial culture at max speed for 3 (1 MIN) mins. Discard supernatant. Repeat added 1.8ml to tube once, centrifuged, discarded supernatant, then added another 1.8ml to tube and

repeated process. (remaining steps will be in hood. Bring in heat block, vortex, and centrifuge into hood. Super sterile)

3. Resuspend cell pellet in 100 µL of **fresh** RES (see recipe below) by vortexing vigorously. If using 10 mL of culture, use 300 µL of RES.
4. Incubate sample at 95°C for 7 minutes.
5. Pellet cell debris by centrifuging at max speed for 5 mins at room temperature. Carefully move the supernatant to a fresh tube without disturbing the gelatinous pellet. We did twice because couldn't see pellet first time. We did this outside of hood because no room in hood.

Sodium acetate/ethanol precipitation

6. BACK TO HOOD. Dilute RNA sample to 300 µL with water. had 60ul of rna sample, Added 240ul of water
7. Add 1/10 volume of 3 M sodium acetate, pH 5.2 (30 µL).
8. Add 2 µL Clear Glycogen Coprecipitant (ThermoFisher)
9. Add three volumes of 100% ethanol (900 µL). Mix by vortexing then incubate for at least 60 mins (or overnight) at -80°C. STOPPED HERE 5/6/24.
10. Centrifuge at max speed for 30 mins at 4°C. Carefully remove supernatant. Set up centrifuge to correct temp prior. Started here 5/7/24
11. Wash cell pellet with 250 µL of freshly-diluted 75% ethanol. Make fresh each time.
12. Centrifuge at max speed for 5 mins at 4°C. Carefully remove supernatant.
13. Briefly centrifuge again to allow removal of all remaining ethanol, then air dry the pellet.
14. Resuspend the pellet in 80 µL water. Centrifuge at max speed for 1 minute to pellet any remaining water insoluble proteins. Transfer the RNA-containing supernatant to a fresh tube.
15. Check concentration by Nanodrop (RNA setting).

DNAse Treatment (Promega)

16. Add 10 µL RNase-free DNase buffer and 10 µL RNase-free DNase (Promega, RQ1)
17. Incubate at 37°C for 1 hour. Can be right in incubator doesn't need heat block.
18. Repeat sodium acetate/ethanol precipitation as above (steps 6-15). Resuspend in 50uL this time, NOT 80 (step 14).

Tube number contents KRLVS 149

Tube #	Contents	Tube #	Contents	Tube #	Contents
1	KRLVS 149 #1 0min	6	KRLVS 149 #2 0min	11	KRLVS 149 #3 0min
2	KRLVS 149 #1 1min	7	KRLVS 149 #2 1min	12	KRLVS 149 #3 1min
3	KRLVS 149 #1 2min	8	KRLVS 149 #2 2min	13	KRLVS 149 #3 2min
4	KRLVS 149 #1 4min	9	KRLVS 149 #2 4min	14	KRLVS 149 #3 4min
5	KRLVS 149 #1 8min	10	KRLVS 149 #2 8min	15	KRLVS 149 #3 8min

Wednesday, May 15, 2024

To Do:

1. ~~RNA isolation day 2~~

Protocol – RNAsnap

Adapted from: Stead et al. (2012) *Nucleic Acids Research*
Updated 10/26/22

Note: All reagents should be RNAse free; use good RNA-technique by cleaning work area, gloves, and instruments with RNAse-away.

Wear safety goggles when handling formamide and dispose of as hazardous waste.

RNA*snap*

1. Grow strains to mid-log
2. Centrifuge 1 to 10 mL of bacterial culture at max speed for 3 (1 MIN) mins. Discard supernatant. Repeat added 1.8ml to tube once, centrifuged, discarded supernatant, then added another 1.8ml to tube and repeated process. (remaining steps will be in hood. Bring in heat block, vortex, and centrifuge into hood. Super sterile)
3. Resuspend cell pellet in 100 µL of **fresh** RES (see recipe below) by vortexing vigorously. If using 10 mL of culture, use 300 µL of RES.
4. Incubate sample at 95°C for 7 minutes.
5. Pellet cell debris by centrifuging at max speed for 5 mins at room temperature. Carefully move the supernatant to a fresh tube without disturbing the gelatinous pellet. We did twice because couldn't see pellet first time. We did this outside of hood because no room in hood.

Sodium acetate/ethanol precipitation

6. BACK TO HOOD. Dilute RNA sample to 300 µL with water. had 60ul of rna sample, Added 240ul of water
7. Add 1/10 volume of 3 M sodium acetate, pH 5.2 (30 µL).
8. Add 2 µL Clear Glycogen Coprecipitant (ThermoFisher)
9. Add three volumes of 100% ethanol (900 µL). Mix by vortexing then incubate for at least 60 mins (or overnight) at -80°C. STOPPED HERE 5/14/24.
10. Centrifuge at max speed for 30 mins at 4°C. Carefully remove supernatant. Set up centrifuge to correct temp prior. Started here 5/15/24
11. Wash cell pellet with 250 µL of freshly-diluted 75% ethanol. Make fresh each time.
12. Centrifuge at max speed for 5 mins at 4°C. Carefully remove supernatant.
13. Briefly centrifuge again to allow removal of all remaining ethanol, then air dry the pellet.
14. Resuspend the pellet in 80 µL water. Centrifuge at max speed for 1 minute to pellet any remaining water insoluble proteins. Transfer the RNA-containing supernatant to a fresh tube.
15. Check concentration by Nanodrop (RNA setting).

DNAse Treatment (Promega)

16. Add 10 µL RNase-free DNase buffer and 10 µL RNase-free DNase (Promega, RQ1)
17. Incubate at 37°C for 1 hour. Can be right in incubator doesn't need heat block.
18. Repeat sodium acetate/ethanol precipitation as above (steps 6-15). Resuspend in 50uL this time, NOT 80 (step 14).

Nanodrop Values Day 1

#	Nucleic Acid	Unit	260/280	260/230
1	481	ng/µl	1.82	1.13
2	631.7	ng/µl	1.87	1.18
3	624.4	ng/µl	1.91	1.18
4	625.1	ng/µl	1.85	1.12
5	641.3	ng/µl	1.85	1.13
6	620.2	ng/µl	1.86	1.14
7	552.7	ng/µl	1.84	1.14
8	527.1	ng/µl	1.85	1.16
9	403.4	ng/µl	1.88	1.19
10	609.6	ng/µl	1.87	1.16
11	502.8	ng/µl	1.85	1.14
12	572	ng/µl	1.84	1.08
13	462	ng/µl	1.85	1.12
14	638.4	ng/µl	1.84	1.08
15	618	ng/µl	1.87	1.11

Notes after meeting with Kathryn:

Thursday, May 16, 2024

To Do:

1. ~~RNA isolation day 3~~

Protocol – RNAsnap

Adapted from: Stead et al. (2012) *Nucleic Acids Research*
Updated 10/26/22

Note: All reagents should be RNAse free; use good RNA-technique by cleaning work area, gloves, and instruments with RNAse-away.

Wear safety goggles when handling formamide and dispose of as hazardous waste.

RNA*snap*

1. Grow strains to mid-log
2. Centrifuge 1 to 10 mL of bacterial culture at max speed for 3 (1 MIN) mins. Discard supernatant. Repeat added 1.8ml to tube once, centrifuged, discarded supernatant, then added another 1.8ml to tube and repeated process. (remaining steps will be in hood. Bring in heat block, vortex, and centrifuge into hood. Super sterile)
3. Resuspend cell pellet in 100 µL of **fresh** RES (see recipe below) by vortexing vigorously. If using 10 mL of culture, use 300 µL of RES.
4. Incubate sample at 95°C for 7 minutes.

5. Pellet cell debris by centrifuging at max speed for 5 mins at room temperature. Carefully move the supernatant to a fresh tube without disturbing the gelatinous pellet. We did twice because couldn't see pellet first time. We did this outside of hood because no room in hood.

Sodium acetate/ethanol precipitation

6. BACK TO HOOD. Dilute RNA sample to 300 µL with water. had 60ul of rna sample, Added 240ul of water
7. Add 1/10 volume of 3 M sodium acetate, pH 5.2 (30 µL).
8. Add 2 µL Clear Glycogen Coprecipitant (ThermoFisher)
9. Add three volumes of 100% ethanol (900 µL). Mix by vortexing then incubate for at least 60 mins (or overnight) at -80°C. STOPPED HERE 5/15/24.
10. Centrifuge at max speed for 30 mins at 4°C. Carefully remove supernatant. Set up centrifuge to correct temp prior. Started here 5/16/24
11. Wash cell pellet with 250 µL of freshly-diluted 75% ethanol. Make fresh each time.
12. Centrifuge at max speed for 5 mins at 4°C. Carefully remove supernatant.
13. Briefly centrifuge again to allow removal of all remaining ethanol, then air dry the pellet.
14. Resuspend the pellet in 80 µL water. Centrifuge at max speed for 1 minute to pellet any remaining water insoluble proteins. Transfer the RNA-containing supernatant to a fresh tube.
15. Check concentration by Nanodrop (RNA setting).

DNAse Treatment (Promega)

16. Add 10 µL RNase-free DNase buffer and 10 µL RNase-free DNase (Promega, RQ1)
17. Incubate at 37°C for 1 hour. Can be right in incubator doesn't need heat block.
18. Repeat sodium acetate/ethanol precipitation as above (steps 6-15). Resuspend in 50uL this time, NOT 80 (step 14).

Nanodrop values final for KRLVS 149

Sample ID	Nucleic Acid	Unit	260/280	260/230
1	345.2	ng/µl	1.99	1.39
2	448.1	ng/µl	1.87	1.35
3	399.8	ng/µl	1.94	1.35
4	410.4	ng/µl	1.94	1.37
5	433.6	ng/µl	1.92	1.37
6	458.8	ng/µl	1.88	1.17
7	430.7	ng/µl	1.9	1.16
8	392.1	ng/µl	1.89	1.29
9	354.9	ng/µl	1.88	1.18
10	438.5	ng/µl	1.93	1.3
11	426.7	ng/µl	1.89	1.15
12	467.9	ng/µl	1.84	1.16
13	433.1	ng/µl	1.86	1.06
14	483.2	ng/µl	1.83	1.13
15	491.8	ng/µl	1.75	1.05

Monday, May 20, 2024

To Do:

1. ~~RNA isolation day 1 of KRLVS148 samples from 4.24.24~~
2. ~~Make agarose gel~~
3. ~~Run isolated RNA on a gel to see bands~~

Protocol – RNAsnap

Adapted from: Stead et al. (2012) *Nucleic Acids Research*
Updated 10/26/22

Note: All reagents should be RNAse free; use good RNA-technique by cleaning work area, gloves, and instruments with RNAse-away.

Wear safety goggles when handling formamide and dispose of as hazardous waste.

RNA*snap*

1. Grow strains to mid-log
2. Centrifuge 1 to 10 mL of bacterial culture at max speed for 3 (1 MIN) mins. Discard supernatant. Repeat added 1.8ml to tube once, centrifuged, discarded supernatant, then added another 1.8ml to tube and repeated process. (remaining steps will be in hood. Bring in heat block, vortex, and centrifuge into hood. Super sterile)
3. Resuspend cell pellet in 100 µL of **fresh** RES (see recipe below) by vortexing vigorously. If using 10 mL of culture, use 300 µL of RES.
4. Incubate sample at 95°C for 7 minutes.
5. Pellet cell debris by centrifuging at max speed for 5 mins at room temperature. Carefully move the supernatant to a fresh tube without disturbing the gelatinous pellet. We did twice because couldn't see pellet first time. We did this outside of hood because no room in hood.

Sodium acetate/ethanol precipitation

6. BACK TO HOOD. Dilute RNA sample to 300 µL with water. had 60ul of rna sample, Added 240ul of water
7. Add 1/10 volume of 3 M sodium acetate, pH 5.2 (30 µL).
8. Add 2 µL Clear Glycogen Coprecipitant (ThermoFisher)
9. Add three volumes of 100% ethanol (900 µL). Mix by vortexing then incubate for at least 60 mins (or overnight) at -80°C. STOPPED HERE 5/15/24.
10. Centrifuge at max speed for 30 mins at 4°C. Carefully remove supernatant. Set up centrifuge to correct temp prior. Started here 5/16/24
11. Wash cell pellet with 250 µL of freshly-diluted 75% ethanol. Make fresh each time.
12. Centrifuge at max speed for 5 mins at 4°C. Carefully remove supernatant.
13. Briefly centrifuge again to allow removal of all remaining ethanol, then air dry the pellet.
14. Resuspend the pellet in 80 µL water. Centrifuge at max speed for 1 minute to pellet any remaining water insoluble proteins. Transfer the RNA-containing supernatant to a fresh tube.
15. Check concentration by Nanodrop (RNA setting).

DNAse Treatment (Promega)

16. Add 10 µL RNase-free DNase buffer and 10 µL RNase-free DNase (Promega, RQ1)
17. Incubate at 37°C for 1 hour. Can be right in incubator doesn't need heat block.
18. Repeat sodium acetate/ethanol precipitation as above (steps 6-15). Resuspend in 50uL this time, NOT 80 (step 14).

Tube contents KRLVS 148

Tube #	Contents	Tube #	Contents	Tube #	Contents
1	KRLVS148 1 0’	6	KRLVS148 2 0’	11	KRLVS148 3 0’
2	KRLVS148 1 1’	7	KRLVS148 2 1’	12	KRLVS148 3 1’
3	KRLVS148 1 2’	8	KRLVS148 2 2’	13	KRLVS148 3 2’
4	KRLVS148 1 4’	9	KRLVS148 2 4’	14	KRLVS148 3 4’
5	KRLVS148 1 8’	10	KRLVS148 2 8’	15	KRLVS148 3 8’

Made 1% agarose gel for lab. 3g agaose, 300mL 1x TAE, boiled, then let cool. Ran 1% agarose gel on previously isolated RNA samples from last week (KRLVS 148 from 5.10.24 and KRLVS149 from 5.16.24) at 110V for 40min. Added total 1ug to gel to run.

KRLVS 148 from 5.10.24

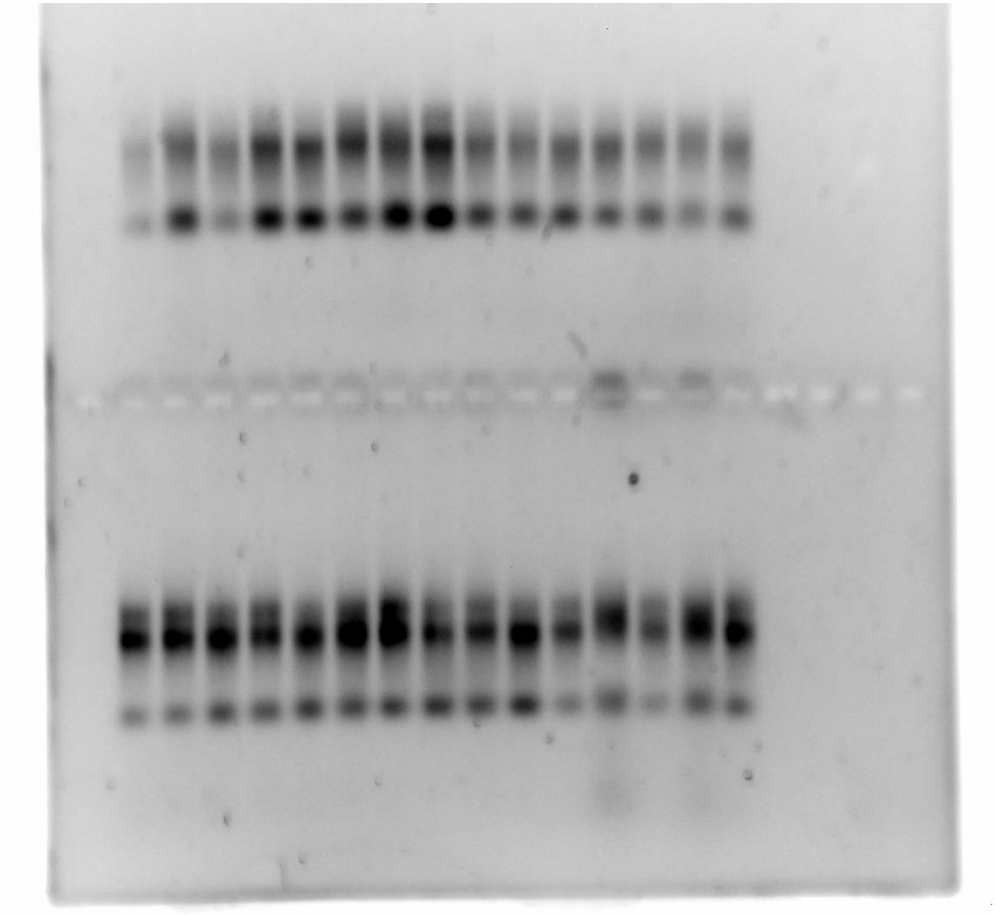
Sample KRLVS 148	RNA to add (uL)	H2O to add (uL)	Total RNA	6x Loading	Total (uL)
1	3.96	6.04	1000	2	12
2	3.73	6.27	1000	2	12
3	2.31	7.69	1000	2	12
4	3.19	6.81	1000	2	12
5	3.76	6.24	1000	2	12
6	4.33	5.67	1000	2	12
7	4.07	5.93	1000	2	12
8	3.87	6.13	1000	2	12
9	4.26	5.74	1000	2	12
10	4.07	5.93	1000	2	12
11	3.80	6.20	1000	2	12
12	3.92	6.08	1000	2	12
13	5.28	4.72	1000	2	12
14	4.21	5.79	1000	2	12
15	3.82	6.18	1000	2	12

KRLVS 149 from 5.16.24

Sample LVS 149	RNA to add	H2O to add	total RNA (ng)	Loading Dye to add	Total per w
1	2.90	7.10	1000	2	12
2	2.23	7.77	1000	2	12
3	2.50	7.50	1000	2	12
4	2.44	7.56	1000	2	12
5	2.31	7.69	1000	2	12
6	2.18	7.82	1000	2	12
7	2.32	7.68	1000	2	12
8	2.55	7.45	1000	2	12
9	2.82	7.18	1000	2	12
10	2.28	7.72	1000	2	12
11	2.34	7.66	1000	2	12
12	2.14	7.86	1000	2	12
13	2.31	7.69	1000	2	12
14	2.07	7.93	1000	2	12
15	2.03	7.97	1000	2	12

Gel image of samples.

First row 1-15 are the KRLVS 148 samples. Row 2 1-15 are the KRLVS 149 samples in order.



I don't know why its so blurry and don't know how to fix it, it looked more clear on the computer. However, you can still see the 5s, 16S, and 23S RNA.

Tuesday, May 21, 2024

To Do:

1. ~~RNA isolation day 2~~
2. ~~Streak out plasmid sent~~

The plasmid was sent in bacteria in a bacterial stab. Took three colonies from stab and streaked it out to isolation on LB plates. Two plates of plain LB one plate of LB – Kan because I wasn't sure if an antibiotic was needed. After talking to Kathryn, she said to do just LB but the plates were already streaked so all three were grown. Put in -37C overnight

Protocol – RNAsnap

Adapted from: Stead et al. (2012) *Nucleic Acids Research*
Updated 10/26/22

Note: All reagents should be RNAse free; use good RNA-technique by cleaning work area, gloves, and instruments with RNAse-away.

Wear safety goggles when handling formamide and dispose of as hazardous waste.

RNA*snap*

1. Grow strains to mid-log
2. Centrifuge 1 to 10 mL of bacterial culture at max speed for 3 (1 MIN) mins. Discard supernatant. Repeat added 1.8ml to tube once, centrifuged, discarded supernatant, then added another 1.8ml to tube and repeated process. (remaining steps will be in hood. Bring in heat block, vortex, and centrifuge into hood. Super sterile)
3. Resuspend cell pellet in 100 µL of **fresh** RES (see recipe below) by vortexing vigorously. If using 10 mL of culture, use 300 µL of RES.
4. Incubate sample at 95°C for 7 minutes.
5. Pellet cell debris by centrifuging at max speed for 5 mins at room temperature. Carefully move the supernatant to a fresh tube without disturbing the gelatinous pellet. We did twice because couldn't see pellet first time. We did this outside of hood because no room in hood.

Sodium acetate/ethanol precipitation

6. BACK TO HOOD. Dilute RNA sample to 300 µL with water. had 60ul of rna sample, Added 240ul of water
7. Add 1/10 volume of 3 M sodium acetate, pH 5.2 (30 µL).
8. Add 2 µL Clear Glycogen Coprecipitant (ThermoFisher)
9. Add three volumes of 100% ethanol (900 µL). Mix by vortexing then incubate for at least 60 mins (or overnight) at -80°C. STOPPED HERE 5/15/24.
10. Centrifuge at max speed for 30 mins at 4°C. Carefully remove supernatant. Set up centrifuge to correct temp prior. Started here 5/16/24

11. Wash cell pellet with 250 µL of freshly-diluted 75% ethanol. Make fresh each time.
12. Centrifuge at max speed for 5 mins at 4°C. Carefully remove supernatant.
13. Briefly centrifuge again to allow removal of all remaining ethanol, then air dry the pellet.
14. Resuspend the pellet in 80 µL water. Centrifuge at max speed for 1 minute to pellet any remaining water insoluble proteins. Transfer the RNA-containing supernatant to a fresh tube.
15. Check concentration by Nanodrop (RNA setting).

DNAse Treatment (Promega)

16. Add 10 µL RNase-free DNase buffer and 10 µL RNase-free DNase (Promega, RQ1)
17. Incubate at 37°C for 1 hour. Can be right in incubator doesn't need heat block.
18. Repeat sodium acetate/ethanol precipitation as above (steps 6-15). Resuspend in 50uL this time, NOT 80 (step 14).

Nanodrop reading after day 2 KRLVS 148

#	Nucleic Acid	Unit	260/280	260/230
1	548.7	ng/µl	1.87	1.15
2	470.5	ng/µl	1.88	1.16
3	554.8	ng/µl	1.9	1.17
4	518.2	ng/µl	1.9	1.15
5	526.7	ng/µl	1.87	1.14
6	419.3	ng/µl	1.89	1.17
7	269.7	ng/µl	1.87	1.2
8	447.1	ng/µl	1.88	1.14
9	540.1	ng/µl	1.91	1.13
10	329.2	ng/µl	1.89	1.21
11	411.6	ng/µl	1.87	1.01
12	496.2	ng/µl	1.75	0.87
13	481.2	ng/µl	1.78	0.94
14	373.6	ng/µl	1.83	1.01
15	346.8	ng/µl	1.8	1.1

Wednesday, May 22, 2024

To Do:

1. ~~RNA isolation day 3 of KRLVS 148 samples from 4.24.24~~
2. ~~RNA isolation day 1 of KRLVS 149 samples from 4.24.24~~
3. ~~Ben RNA extraction?~~

Protocol – RNAsnap

Adapted from: Stead et al. (2012) *Nucleic Acids Research*
Updated 10/26/22

Note: All reagents should be RNAse free; use good RNA-technique by cleaning work area, gloves, and instruments with RNAse-away.

Wear safety goggles when handling formamide and dispose of as hazardous waste.

RNA*snap*

1. Grow strains to mid-log
2. Centrifuge 1 to 10 mL of bacterial culture at max speed for 3 (1 MIN) mins. Discard supernatant. Repeat added 1.8ml to tube once, centrifuged, discarded supernatant, then added another 1.8ml to tube and repeated process. (remaining steps will be in hood. Bring in heat block, vortex, and centrifuge into hood. Super sterile)
3. Resuspend cell pellet in 100 µL of **fresh** RES (see recipe below) by vortexing vigorously. If using 10 mL of culture, use 300 µL of RES.
4. Incubate sample at 95°C for 7 minutes.
5. Pellet cell debris by centrifuging at max speed for 5 mins at room temperature. Carefully move the supernatant to a fresh tube without disturbing the gelatinous pellet. We did twice because couldn't see pellet first time. We did this outside of hood because no room in hood.

Sodium acetate/ethanol precipitation

6. BACK TO HOOD. Dilute RNA sample to 300 µL with water. had 60ul of rna sample, Added 240ul of water
7. Add 1/10 volume of 3 M sodium acetate, pH 5.2 (30 µL).
8. Add 2 µL Clear Glycogen Coprecipitant (ThermoFisher)
9. Add three volumes of 100% ethanol (900 µL). Mix by vortexing then incubate for at least 60 mins (or overnight) at -80°C. STOPPED HERE 5/15/24.
10. Centrifuge at max speed for 30 mins at 4°C. Carefully remove supernatant. Set up centrifuge to correct temp prior. Started here 5/16/24
11. Wash cell pellet with 250 µL of freshly-diluted 75% ethanol. Make fresh each time.
12. Centrifuge at max speed for 5 mins at 4°C. Carefully remove supernatant.
13. Briefly centrifuge again to allow removal of all remaining ethanol, then air dry the pellet.
14. Resuspend the pellet in 80 µL water. Centrifuge at max speed for 1 minute to pellet any remaining water insoluble proteins. Transfer the RNA-containing supernatant to a fresh tube.
15. Check concentration by Nanodrop (RNA setting).

DNAse Treatment (Promega)

16. Add 10 µL RNase-free DNase buffer and 10 µL RNase-free DNase (Promega, RQ1)
17. Incubate at 37°C for 1 hour. Can be right in incubator doesn't need heat block.
18. Repeat sodium acetate/ethanol precipitation as above (steps 6-15). Resuspend in 50uL this time, NOT 80 (step 14).

Nanodrop Reading day 3 KRLVS 148

#	Nucleic Acid	Unit	260/280	260/230
1	408.3	ng/µl	1.96	1.37
2	405.5	ng/µl	1.91	1.26
3	426.5	ng/µl	1.96	1.33
4	409.9	ng/µl	1.94	1.33
5	386.1	ng/µl	1.97	1.5
6	365.8	ng/µl	1.93	1.26
7	235.5	ng/µl	1.91	1.15
8	402.8	ng/µl	1.93	1.26
9	441.9	ng/µl	1.94	1.35
10	274.5	ng/µl	1.95	1.36
11	370.6	ng/µl	1.67	1.03
12	322.6	ng/µl	1.88	1.17
13	372.2	ng/µl	1.86	1.14
14	342.3	ng/µl	1.89	1.13
15	324.6	ng/µl	1.87	1.07

Tube contents KRLVS 149

Tube #	Contents	Tube #	Contents	Tube #	Contents
1	KRLVS 149 #1 0min	6	KRLVS 149 #2 0min	11	KRLVS 149 #3 0min
2	KRLVS 149 #1 1min	7	KRLVS 149 #2 1min	12	KRLVS 149 #3 1min
3	KRLVS 149 #1 2min	8	KRLVS 149 #2 2min	13	KRLVS 149 #3 2min
4	KRLVS 149 #1 4min	9	KRLVS 149 #2 4min	14	KRLVS 149 #3 4min
5	KRLVS 149 #1 8min	10	KRLVS 149 #2 8min	15	KRLVS 149 #3 8min

Thursday, May 23, 2024

To Do:

1. ~~RNA isolation day 2~~
2. ~~Overnight culture of isolated plasmid~~

Protocol – RNAsnap

Adapted from: Stead et al. (2012) *Nucleic Acids Research*
Updated 10/26/22

Note: All reagents should be RNAse free; use good RNA-technique by cleaning work area, gloves, and instruments with RNAse-away.

Wear safety goggles when handling formamide and dispose of as hazardous waste.

RNA*snap*

1. Grow strains to mid-log
2. Centrifuge 1 to 10 mL of bacterial culture at max speed for 3 (1 MIN) mins. Discard supernatant. Repeat added 1.8ml to tube once, centrifuged, discarded supernatant, then added another 1.8ml to tube and repeated process. (remaining steps will be in hood. Bring in heat block, vortex, and centrifuge into hood. Super sterile)

3. Resuspend cell pellet in 100 µL of **fresh** RES (see recipe below) by vortexing vigorously. If using 10 mL of culture, use 300 µL of RES.
4. Incubate sample at 95°C for 7 minutes.
5. Pellet cell debris by centrifuging at max speed for 5 mins at room temperature. Carefully move the supernatant to a fresh tube without disturbing the gelatinous pellet. We did twice because couldn't see pellet first time. We did this outside of hood because no room in hood.

Sodium acetate/ethanol precipitation

6. BACK TO HOOD. Dilute RNA sample to 300 µL with water. had 60ul of rna sample, Added 240ul of water
7. Add 1/10 volume of 3 M sodium acetate, pH 5.2 (30 µL).
8. Add 2 µL Clear Glycogen Coprecipitant (ThermoFisher)
9. Add three volumes of 100% ethanol (900 µL). Mix by vortexing then incubate for at least 60 mins (or overnight) at -80°C. STOPPED HERE 5/15/24.
10. Centrifuge at max speed for 30 mins at 4°C. Carefully remove supernatant. Set up centrifuge to correct temp prior. Started here 5/16/24
11. Wash cell pellet with 250 µL of freshly-diluted 75% ethanol. Make fresh each time.
12. Centrifuge at max speed for 5 mins at 4°C. Carefully remove supernatant.
13. Briefly centrifuge again to allow removal of all remaining ethanol, then air dry the pellet.
14. Resuspend the pellet in 80 µL water. Centrifuge at max speed for 1 minute to pellet any remaining water insoluble proteins. Transfer the RNA-containing supernatant to a fresh tube.
15. Check concentration by Nanodrop (RNA setting).

DNAse Treatment (Promega)

16. Add 10 µL RNase-free DNase buffer and 10 µL RNase-free DNase (Promega, RQ1)
17. Incubate at 37°C for 1 hour. Can be right in incubator doesn't need heat block.
18. Repeat sodium acetate/ethanol precipitation as above (steps 6-15). Resuspend in 50uL this time, NOT 80 (step 14).

Nanodrop readings after day 2 KRLVS 149

#	Nucleic Acid	Unit	260/280	260/230
1	752.6	ng/µl	1.9	1.12
2	941	ng/µl	1.86	1
3	771.6	ng/µl	1.96	1.19
4	847.1	ng/µl	1.9	1.06
5	734.1	ng/µl	1.9	1.1
6	554.4	ng/µl	1.88	1.16
7	602.3	ng/µl	1.87	1.1
8	559.9	ng/µl	1.9	1.1
9	479.6	ng/µl	2	1.29
10	506.2	ng/µl	1.95	1.17
11	790.4	ng/µl	1.92	1.14
12	704.3	ng/µl	1.92	1.17
13	737.9	ng/µl	1.91	1.16
14	743.6	ng/µl	1.98	1.24
15	693.9	ng/µl	1.93	1.18

Overnight culture of plasmid

- Took one isolated colony from each plate and grew up liquid cultures in LB overnight. Incubated at 37C. 3 total cultures

Friday, May 24, 2024

To Do:

1. ~~RNA isolation day 3~~
2. ~~Make glycerol stocks from plasmid overnight cultures~~
3. ~~Pelleted cells from overnight cultures for miniprep on Tuesday~~

Protocol – RNAsnap

Adapted from: Stead et al. (2012) *Nucleic Acids Research*
Updated 10/26/22

Note: All reagents should be RNAse free; use good RNA-technique by cleaning work area, gloves, and instruments with RNAse-away.

Wear safety goggles when handling formamide and dispose of as hazardous waste.

RNA*snap*

1. Grow strains to mid-log
2. Centrifuge 1 to 10 mL of bacterial culture at max speed for 3 (1 MIN) mins. Discard supernatant. Repeat added 1.8ml to tube once, centrifuged, discarded supernatant, then added another 1.8ml to tube and repeated process. (remaining steps will be in hood. Bring in heat block, vortex, and centrifuge into hood. Super sterile)
3. Resuspend cell pellet in 100 µL of **fresh** RES (see recipe below) by vortexing vigorously. If using 10 mL of culture, use 300 µL of RES.
4. Incubate sample at 95°C for 7 minutes.
5. Pellet cell debris by centrifuging at max speed for 5 mins at room temperature. Carefully move the supernatant to a fresh tube without disturbing the gelatinous pellet. We did twice because couldn't see pellet first time. We did this outside of hood because no room in hood.

Sodium acetate/ethanol precipitation

6. BACK TO HOOD. Dilute RNA sample to 300 µL with water. had 60ul of rna sample, Added 240ul of water
7. Add 1/10 volume of 3 M sodium acetate, pH 5.2 (30 µL).
8. Add 2 µL Clear Glycogen Coprecipitant (ThermoFisher)
9. Add three volumes of 100% ethanol (900 µL). Mix by vortexing then incubate for at least 60 mins (or overnight) at -80°C. STOPPED HERE 5/15/24.
10. Centrifuge at max speed for 30 mins at 4°C. Carefully remove supernatant. Set up centrifuge to correct temp prior. Started here 5/16/24
11. Wash cell pellet with 250 µL of freshly-diluted 75% ethanol. Make fresh each time.
12. Centrifuge at max speed for 5 mins at 4°C. Carefully remove supernatant.
13. Briefly centrifuge again to allow removal of all remaining ethanol, then air dry the pellet.
14. Resuspend the pellet in 80 µL water. Centrifuge at max speed for 1 minute to pellet any remaining water insoluble proteins. Transfer the RNA-containing supernatant to a fresh tube.

15. Check concentration by Nanodrop (RNA setting).

DNAse Treatment (Promega)

16. Add 10 µL RNase-free DNase buffer and 10 µL RNase-free DNase (Promega, RQ1)
17. Incubate at 37°C for 1 hour. Can be right in incubator doesn't need heat block.
18. Repeat sodium acetate/ethanol precipitation as above (steps 6-15). Resuspend in 50uL this time, NOT 80 (step 14).

Nanodrop readings after day 3 KRLVS 149

#	Nucleic Acid	Unit	260/280	260/230
1	569.3	ng/µl	2	1.64
2	509.1	ng/µl	1.96	1.46
3	441.5	ng/µl	1.96	1.46
4	546.9	ng/µl	1.97	1.47
5	583.3	ng/µl	1.85	1.16
6	451.6	ng/µl	1.9	1.25
7	420.6	ng/µl	1.91	1.35
8	416.3	ng/µl	1.93	1.32
9	429.1	ng/µl	1.99	1.38
10	419	ng/µl	1.91	1.3
11	485.3	ng/µl	1.92	1.35
12	479.1	ng/µl	1.93	1.41
13	468.5	ng/µl	1.92	1.33
14	495.5	ng/µl	1.92	1.32
15	529.8	ng/µl	1.95	1.38

- Glycerol Stocks
  - Used overnight cultures to make glycerol stocks. 3 tubes total from second individual LB grown plate colony (culture looked most dense) Per tube:
  - 800uL overnight culture
  - 200uL of 75% glycerol
  - Vortex to mix
  - Store at -80C
- Pelleted cells for miniprep
  - 1.6mL culture, spun down max speed 3 mins
  - Discarded supernatant
  - Stored pellet in -20C
  - 4 tubes total, two from culture 1, 1 from culture 2, 1 from culture 3 (colonies grown on LB-kan)

Tuesday, May 28, 2024

To Do:

1. ~~cDNA generation KRLVS149 from 5.16.24~~

Protocol: Generate cDNA (half protocol)*

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

1. Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	268 - 333 ng/ ul
$(NS)_5$ Primer (250 ng/ul)	1.5 ul	26 g/ul
RNase-free water	up to 15 ul	

2. To normalize all the cDNA samples to the same DNA mass, dilute with RNase-free water in PCR strip tubes.

cDNA volumes to add to each tube

#	Sample	Conc (ng/uL)	RNA needed	H20 needed	total RNA
1	KRLVS 149 #1 0min	345.2	8.7	4.8	3000
2	KRLVS 149 #1 1min	448.1	6.7	6.8	3000
3	KRLVS 149 #1 2min	399.8	7.5	6.0	3000
4	KRLVS 149 #1 4min	410.4	7.3	6.2	3000
5	KRLVS149 #1 8min	433.6	6.9	6.6	3000
6	KRLV S 149 #2 0min	458.8	6.5	7.0	3000
7	KRLVS 149 #2 1min	430.7	7.0	6.5	3000
8	KRLVS 149 #2 2min	392.1	7.7	5.8	3000
9	KRLVS 149 #2 4min	354.9	8.5	5.0	3000
10	KRLVS 149 #2 8min	438.5	6.8	6.7	3000
11	KRLVS 149 #3 0min	426.7	7.0	6.5	3000
12	KRLVS149 #3 1min	467.9	6.4	7.1	3000
13	KRLVS 149 #3 2min	433.1	6.9	6.6	3000
14	KRLVS 149#3 4min	483.2	6.2	7.3	3000
15	KRLVS 149 #3 8min	491.8	6.1	7.4	3000

Note the total volume of cDNA and water is 13.5 uL.

3. Add the 1.5 uL $(NS)_5$ oligo (stored in small aliquots at -80°C; take your own aliquot to use and move it to your own stock box; minimize freeze/thaw cycles) to the tubes.

4. Incubate using program JSScDNA1 in the thermocycler:
   a. Step Temp Time
   b. 1 70°C 10'
   c. 2 25°C 10'
   d. 3 4°C hold

5. While waiting, prepare the cDNA synthesis reaction in master mix format. The number of reactions you have plus 1.5 should be the factor you multiply by.

	Master mix for cDNA synethesis reaction		# Reactions
Component	Final Conc	Volume (uL)	16.5
5x 1st strand buffer	1x	6	99
Rnase-free water		2.87	47.355
100mM DTT	10mM	3	49.5
10mM dNTPs	0.5mM	1.5	24.75
Superscript III (200U/uL)	10.8U/uL	1.63	26.895
	total	15	247.5

*Buffer, DTT, and dNTPs should be from the cDNA reagents box (they are RNase free!)

6. Aliquot 15 ul of master mix per reaction into each PCR tube from the first reaction (total volume now 30ul)

7. Incubate using program JSScDNA2
   a. Step Temp Time
   b. 1 25°C 10'
   c. 2 37°C 60'
   d. 4 42°C 60'
   e. 5 70°C 10'
   f. 6 4°C hold

8. Remove RNA from sample by degrading with sodium hydroxide:

9. Add 10 ul of 1N NaOH
10. Incubate 65°C for 30'
11. Neutralize with 10 ul of 1N HCl
12. Final volume is 50 ul
13. Purify cDNA using Qiagen PCR clean-up column (be very sure there is no ethanol carry-over!) THIS IS THE PCR PURIFICATION PROTOCOL
14. Elute in 60 ul of 0.1x EB

15. Check concentration by Nanodrop

16. Store cDNA at -80°C

Wednesday, May 29, 2024

To Do:

1. ~~qRT-PCR on cDNA samples from KRLVS 148 and 149 RNA stability assay #1 (cDNA made on 5.13.24 and 5.28.24)~~

Protocol: Real-Time PCR on cDNA samples

Original protocol by Heather McManus, edited by Kathryn Ramsey

1. Each experiment will need at least one test primer ~~and one control primer~~ for each sample. We don't use control primer for RNA stability samples. So only KROL 504/505 will be used
    a. Example: 3 biological replicates = 3 DNA samples

2. Each different reaction type must be run in triplicate on the real-time plate
    a. Example: 3 reactions = 9 wells

3. Each Individual reaction = 20 µL
    a. 10 µL PowerUp SYBR Green Master Mix
    b. 1 µL 5uM combined forward and reverse primer
    c. 1 µL cDNA (~1.5 ng/ul, can base off efficiency tests)
    d. 8 µL ddiH_2O

4. For each reaction type (primer/DNA combination) set up a master mix equal to 3.5 reactions:
5. Put 3.5µL of DNA type into strip tubes

cDNA Dilutions for each sample to put into strip tubes

	starting	KRLVS 148	Want	Need of original
Sample	conc (ng/uL)	total volume dilution (uL)	ng/ul	uL
1	12	100	1.5	12.50
2	13	100	1.5	11.54
3	9.5	100	1.5	15.79
4	14.3	100	1.5	10.49
5	12.6	100	1.5	11.90
6	14	100	1.5	10.71
7	15	100	1.5	10.00
8	13.5	100	1.5	11.11
9	14.4	100	1.5	10.42
10	14	100	1.5	10.71
11	12.9	100	1.5	11.63
12	15	100	1.5	10.00
13	13.8	100	1.5	10.87
14	13.3	100	1.5	11.28
15	12.5	100	1.5	12.00

	*from 5.29.24 nanodrop			
	starting	KRLVS 149	Want	Need of original
Sample	conc (ng/uL)	total volume dilution (ul	ng/ul	uL
1	22.8	100	1.5	6.58
2	22.4	100	1.5	6.70
3	18.9	100	1.5	7.94
4	21.3	100	1.5	7.04
5	20	100	1.5	7.50
6	18.8	100	1.5	7.98
7	20.5	100	1.5	7.32
8	19	100	1.5	7.89
9	20.1	100	1.5	7.46
10	17.1	100	1.5	8.77
11	17.1	100	1.5	8.77
12	22.6	100	1.5	6.64
13	20.4	100	1.5	7.35
14	15.9	100	1.5	9.43
15	19.3	100	1.5	7.77

6. 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
    a. Example: 6 DNA samples per primer pair = 6 x 3.5 + 3.5 = 24.5

7. Set up primer master mixes in separate 1.5 mL tubes (make one for each primer pair)
    a. Example:
    b. 10 µL PowerUp SYBR Green Master Mix x 24.5 = 245.0 µL
    c. 1 µL 5µM combined F and R primer x 24.5 = 24.5 µL
    d. 8 µL ddiH_2O x 24.5 = 171.5 µL
    e. TOTAL = 465.5 µL

Set Up of Primers and Plate

Master Mix		108.5
Component	Individual	MM
PowerUpSyber Green MM	10	1085
5uM combined F/R Primer	1	108.5
DNA	1	
ddH20	8	868
total		2061.5

148	Strip Tube DNA + MM	1	2	3	4	5	6	7	8
		#1	#2	#3	#4	#5	#6	#7	#8
		9	10	11	12	13	14	15	16
		#9	#10	#11	#12	#13	#14	#15	blank

149	Strip Tube DNA + MM	1	2	3	4	5	6	7	8
		#1	#2	#3	#4	#5	#6	#7	#8
		9	10	11	12	13	14	15	16
		#9	#10	#11	#12	#13	#14	#15	blank

8. Add primer master mix to tubes containing DNA.
9. 3.5 reactions x 20 µL volume = 70 µL. DNA tubes already have 3.5µL of DNA. Add 66.5 µL of primer master mix to each tube

10. Pipette 20 µL of each reaction into 3 separate wells on the 96 well plate using dispense option on multichannel

11. Spin plate down

12. Place in real-time machine and run using the same program used to determine that the primers are appropriately efficient.

    GSC computer login:
    K_Ramsey Lab
    Password:
    Roche480

Plate Overview

	1	2	3	4	5	6	7	8	9	10	11	12
A	148 #1 rep 1	148 #1 rep 2	148 #1 rep 3	148 #9 rep 1	148 #9 rep 2	148 #9 rep 3	149 #1 rep 1	149 #1 rep 2	149 #1 rep 3	149 #9 rep 1	149 #9 rep 2	149 #9 rep 3
B	148 #2 rep 1	148 #2 rep 2	148 #2 rep 3	148 #10 rep 1	148 #10 rep 2	148 #10 rep 3	149 #2 rep 1	149 #2 rep 2	149 #2 rep 3	149 #10 rep 1	149 #10 rep 2	149 #10 rep 3
C	148 #3 rep 1	148 #3 rep 2	148 #3 rep 3	148 #11 rep 1	148 #11 rep 2	148 #11 rep 3	149 #3 rep 1	149 #3 rep 2	149 #3 rep 3	149 #11 rep 1	149 #11 rep 2	149 #11 rep 3
D	148 #4 rep 1	148 #4 rep 2	148 #4 rep 3	148 #12 rep 1	148 #12 rep 2	148 #12 rep 3	149 #4 rep 1	149 #4 rep 2	149 #4 rep 3	149 #12 rep 1	149 #12 rep 2	149 #12 rep 3
E	148 #5 rep 1	148 #5 rep 2	148 #5 rep 3	148 #13 rep 1	148 #13 rep 2	148 #13 rep 3	149 #5 rep 1	149 #5 rep 2	149 #5 rep 3	149 #13 rep 1	149 #13 rep 2	149 #13 rep 3
F	148 #6 rep 1	148 #6 rep 2	148 #6 rep 3	148 #14 rep 1	148 #14 rep 2	148 #14 rep 3	149 #6 rep 1	149 #6 rep 2	149 #6 rep 3	149 #14 rep 1	149 #14 rep 2	149 #14 rep 3
G	148 #7 rep 1	148 #7 rep 2	148 #7 rep 3	148 #15 rep 1	148 #15 rep 2	148 #15 rep 3	149 #7 rep 1	149 #7 rep 2	149 #7 rep 3	149 #15 rep 1	149 #15 rep 2	149 #15 rep 3
H	148 #8 rep 1	148 #8 rep 2	148 #8 rep 3				149 #8 rep 1	149 #8 rep 2	149 #8 rep 3			

Thursday, May 30, 2024

To Do:

1. ~~Analysis of qPCR~~
2. ~~Meeting with Kathryn~~

Meeting with Kathryn:

TO DO over the next few days/week:

- Data analysis of qPCR
- Prep for next set of qPCR
- Miniprep for plasmid

P. ging plasmid

- from the one we got shipped to us we need to fill out the *E.coli* strain sheet and then after doing a miniprep of the plasmid do a restriction enzyme digest to make sure that it is actually the plasmid we said it would be when we ordered it.

Snapgene

- Not bold +6 cutter = not unique. Unique = cuts at single time in plasmid. +6 cutter = enzyme whose recognition site is at least 6bp
- Kpn1 &not1 recognizes 8bp so add 1 more bp to get 3 ala linkers (after SVG tag + stop codon)
- Ignore lac operator etx
- Keep multiple cloning, origin & kan resistance intact
- Won't replicate in Pg because no origin of replication which is good because we don't want it to replicate
- pKR36 on snapgene
- pKR223 = plasmid #

Primers

- we need it to exist in plasmid its going into not one for primers where we create from
- First grab casset with TM = 72
- Then sequence of restriction enzyme Ad1 (make cuts) doesn't anneal, add atat before
- 3' ends in C or G because its stronger (add BTG1 site)
- So get pKR36 digest with (first check if we have enough pKR36 or if I need to get more)

Overall

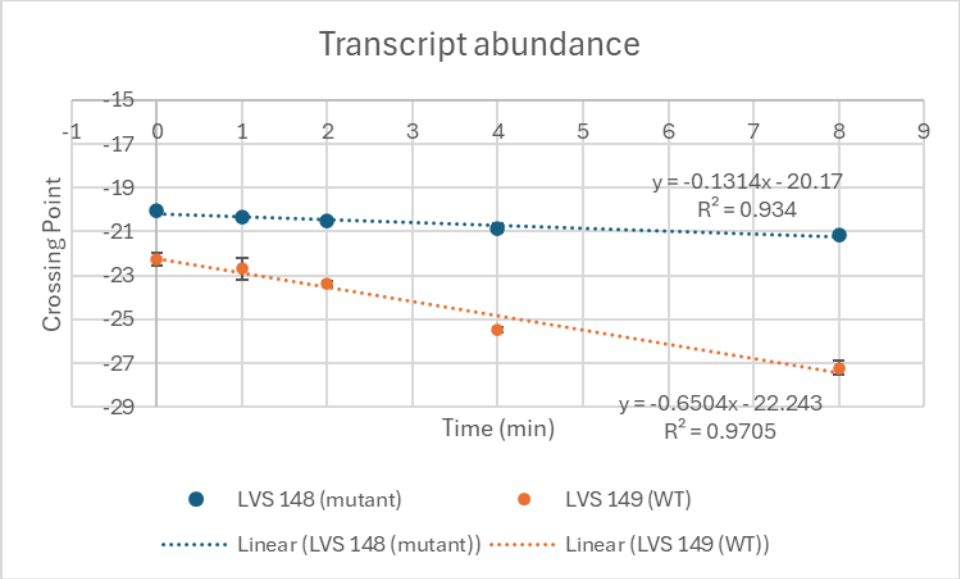
- Get miniprep plasmid pG106K and (after restriction digest) amplify with KROL 700/701
- Digest both pKR36 and pG106K with Ad1 and BTG1
- Order of operations
    - Miniprep pG106K
    - Restriction digest pG106K
    - PCR
    - Gel check
    - Digest gel
    - Gel extract ligaton and transformation
- We need to put pG106K DNA into the plasmid eventually
- Genome info → pG → NC002950

Cloning lab strategies

- Goal: want a plasmid that replicates in e.coli but not p.ging
- Contain ermF gene for selection in p.g
- Selectable marker for e.coli
- Need multiple cloning site and incorporate DNA specifying VSVG tag
- Current plasmid pKR36 has many of these features except ermF resistance. So we are going to clone in the resistance gene from what Mary Ellen sent which will modfy it for use in P.g. (put in ermF cassette)
    - Start first with pKR36
- Broad overview cloning plan. Create plasmid with adding vcvg tags by modifying pKR36 and modifying plasmid to include the ermF gene resistance and then modify further to make plasmids im actually going to use. The plasmid im currently purifying is the one that has the ermF resistance.
- pKR223 is the plasmid I am creating for cloning. Not the pg106K that I am doing the miniprep on rn.
- IN RAMSEY LAB PLASMID LIST pKR223 = pG_VSVG = plasmid to use for single integration in P. gingivalis to add DNA specifying VSVG tag to various genes (purpose). Other notes column is for specified notes. We don't have those yet. Backbone = where are we getting the bit of the plasmid that is allowing for replication. So in this case its PKR36. We digest with Acl1/Bcg1. So the antibiotic selection is kanamycin in e.coli and erythromycin in p.ging. design KRM and ARF. Modification/insert PCR framgemnt amplified from pg106K digested with Acl1/Btg1. Confirmed tells you who confirmed it and if it was made and where it is located.
- Next we have to make sequence file for it.
- Primers: KROL 700. Lowercase letters = letters that are actually on the genome. Capitalized letters = letters that correspond to amplicfication of the cut site. So start with atatCUTSITEdnathatanneals.

qRT-PCR Analysis

Analyzed the samples from the qRT-PCR yesterday. Averaged the runs at different time points together and got the standard deviations of everything. The title and axis' of the graph may be wrong as I wasn't sure what to put



Graph of RT-qPCR results. Shows there was less transcript in the wildtype than in the mutant RNA.

half life	mins (?)
148 (mutant)	7.61035
149 (WT)	1.537515

Half life of the RNA used

Friday, May 31, 2024

To Do:

1. ~~cDNA generation of next LVS148 RNA stability samples 1-15~~
2. ~~make 1.5ng/uL dilutions of the cDNA~~

Protocol: Generate cDNA (half protocol)*

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

1. Combine the first components for primer annealing:

Component	Volume or Amount	Final Concentration
RNA	3 ug	269 - 333 ng/ ul
$(NS)_5$ Primer (250 ng/ul)	1.5 ul	27 g/ul
RNase-free water	up to 15 ul	

2. To normalize all the cDNA samples to the same DNA mass, dilute with RNase-free water in PCR strip tubes.

Volumes to add to each tube

#	Sample	Conc (ng/uL)	RNA need	H20 needed	total RNA
1	KRLVS 148 #1 0min	408.3	7.3	6.2	3000
2	KRLVS 148 #1 1min	405.5	7.4	6.1	3000
3	KRLVS 148 #1 2min	426.5	7.0	6.5	3000
4	KRLVS 148 #1 4min	409.9	7.3	6.2	3000
5	KRLVS148 #1 8min	386.1	7.8	5.7	3000
6	KRLV S 148 #2 0min	365.8	8.2	5.3	3000
7	KRLVS 148 #2 1min	235.5	12.7	0.8	3000
8	KRLVS 148 #2 2min	402.8	7.4	6.1	3000
9	KRLVS 148 #2 4min	441.9	6.8	6.7	3000
10	KRLVS 148 #2 8min	274.5	10.9	2.6	3000
11	KRLVS 148 #3 0min	370.6	8.1	5.4	3000
12	KRLVS148 #3 1min	322.6	9.3	4.2	3000
13	KRLVS 148 #3 2min	372.2	8.1	5.4	3000
14	KRLVS 148#3 4min	342.3	8.8	4.7	3000
15	KRLVS 148 #3 8min	324.6	9.2	4.3	3000

Note the total volume of cDNA and water is 13.5 uL.

3. Add the 1.5 uL $(NS)_5$ oligo (stored in small aliquots at -80°C; take your own aliquot to use and move it to your own stock box; minimize freeze/thaw cycles) to the tubes.

4. Incubate using program JSScDNA1 in the thermocycler:
   a. Step Temp Time
   b. 1 70°C 10'
   c. 2 25°C 10'
   d. 3 4°C hold

5. While waiting, prepare the cDNA synthesis reaction in master mix format. The number of reactions you have plus 1.5 should be the factor you multiply by.

	Master mix for cDNA synethesis reaction		# Reactions
Component	Final Conc	Volume (uL)	16.5
5x 1st strand buffer	1x	6	99
Rnase-free water		2.87	47.355
100mM DTT	10mM	3	49.5
10mM dNTPs	0.5mM	1.5	24.75
Superscript III (200U/uL)	10.8U/uL	1.63	26.895
	total	15	247.5

*Buffer, DTT, and dNTPs should be from the cDNA reagents box (they are RNase free!)

6. Aliquot 15 ul of master mix per reaction into each PCR tube from the first reaction (total volume now 30ul)

7. Incubate using program JSScDNA2
    a. Step Temp Time
    b. 1 25°C 10'
    c. 2 37°C 60'
    d. 4 42°C 60'
    e. 5 70°C 10'
    f. 6 4°C hold

8. Remove RNA from sample by degrading with sodium hydroxide:

9. Add 10 ul of 1N NaOH
10. Incubate 65°C for 30'
11. Neutralize with 10 ul of 1N HCl
12. Final volume is 50 ul
13. Purify cDNA using Qiagen PCR clean-up column (be very sure there is no ethanol carry-over!) THIS IS THE PCR PURIFICATION PROTOCOL
14. Elute in 60 ul of 0.1x EB

15. Check concentration by Nanodrop

cDNA concentrations

Sample ID	Nucleic Acid	Unit	260/280	260/230
1	18.9	ng/µl	1.87	1
2	17.1	ng/µl	1.85	1.18
3	5.3	ng/µl	1.56	0.46
4	17.1	ng/µl	1.8	0.93
5	14.6	ng/µl	1.77	1.53
6	18	ng/µl	1.89	0.83
7	17.7	ng/µl	1.89	0.91
8	17.5	ng/µl	1.78	0.96
9	17.5	ng/µl	1.82	0.95
10	18.7	ng/µl	1.78	1.07
11	14.6	ng/µl	1.82	0.77
12	13.5	ng/µl	1.81	0.88
13	13.6	ng/µl	1.82	0.74
14	14	ng/µl	1.7	0.87
15	15.4	ng/µl	1.81	0.93

**note. Sample 3 was run on the nanodrop multiple times. The first time it said the concentration was around 6.7ng/uL. The second time was 5.3ng/uL. Then I did the rest of the samples, turned the nanodrop off and on again, reblanked it, and got the concentration to be 34ng/uL and then 3.3ng/uL. I ended up using the value of 5.3ng/uL for the actual value because it was in the middle. I ignored the 34ng/uL one.

16. Store cDNA at -80°C

I made 1.5ng/uL stocks of the cDNA generated to be used in the qRT-PCR that I will be doing next week. I figured to make the stocks now while the DNA is out and thawed already.

		KRLVS 148		want final conc	Need of original
Sample	Sample name	starting conc (ng/uL)	total volume	ng/uL	uL
1	KRLVS 148 #1 0min	18.9	100	1.5	7.94
2	KRLVS 148 #1 1min	17.1	100	1.5	8.77
3	KRLVS 148 #1 2min	5.3	100	1.5	28.30
4	KRLVS 148 #1 4min	17.1	100	1.5	8.77
5	KRLVS148 #1 8min	14.6	100	1.5	10.27
6	KRLV S 148 #2 0min	18	100	1.5	8.33
7	KRLVS 148 #2 1min	17.7	100	1.5	8.47
8	KRLVS 148 #2 2min	17.5	100	1.5	8.57
9	KRLVS 148 #2 4min	17.5	100	1.5	8.57
10	KRLVS 148 #2 8min	18.7	100	1.5	8.02
11	KRLVS 148 #3 0min	14.6	100	1.5	10.27
12	KRLVS148 #3 1min	13.5	100	1.5	11.11
13	KRLVS 148 #3 2min	13.6	100	1.5	11.03
14	KRLVS 148#3 4min	14	100	1.5	10.71
15	KRLVS 148 #3 8min	15.4	100	1.5	9.74

END OF NOTEBOOK 2

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.