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Elene's Lab Notebook

Fish404 Lab Notebook

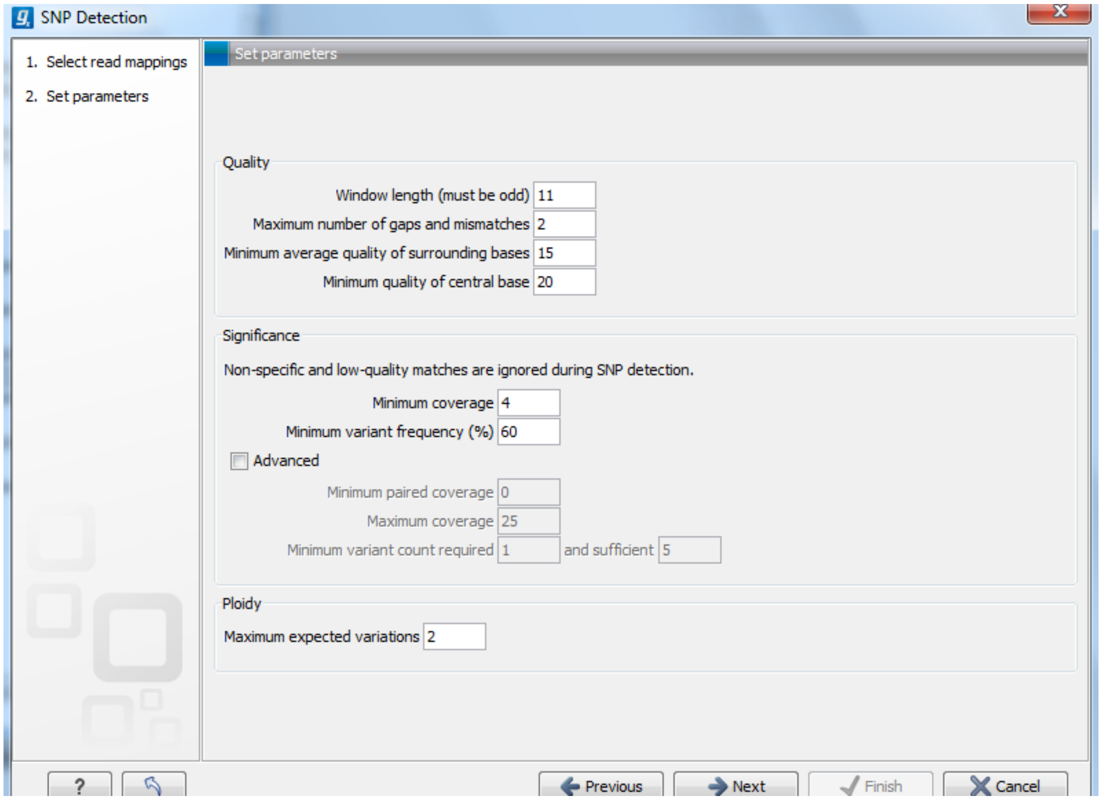
Stats

Thoughts

Evernote

10511 SNP tables from de novo sequence of both Vt strains

Set min variant frequency to 60%
Saved in
SNP tables from de novo consensus of BOTH strains _300bp folder under Assemble to Ref



SNP Detection

1. Select read mappings
2. Set parameters

Set parameters

Quality
Window length (must be odd) 11
Maximum number of gaps and mismatches 2
Minimum average quality of surrounding bases 15
Minimum quality of central base 20

Significance
Non-specific and low-quality matches are ignored during SNP detection.
Minimum coverage 4
Minimum variant frequency (%) 60
☐ Advanced
Minimum paired coverage 0
Maximum coverage 25
Minimum variant count required 1 and sufficient 5

Ploidy
Maximum expected variations 2

10-4-11 Vt de novo assembly BLAST

performed blastP to swissprot DB using: Consensus sequences from denovo BOTH _10530seqs_greaterthan300bp.fa
started at 1:30p

This file is the consensus file de novo assembly of both Vt strains.
These contigs were filtered to only include contigs >300bp in length for ~11k contigs to BLAST (file above).

THE PLAN:
BLAST file
GO terms
stats

SNP tables to be made for each strain using the new file of >300bp contigs for each strain
Stats summaries to the gene level for each table

Creating SNP tables:
Assembly done using trimmed reads from each strain mapped to reference consensus sequence of the de novo file above
Used the assemblies to create SNP tables

10-3-11 Check vtpA assay Lisa vs. Elene

Lisa: 9-14-11_Lisa_vtpA_assay.pdf
Elene: 10-3-11_vtpA_StCv_check_16S_check.pdf
Side by side with Lisa next

9-30-11 VtpR expression of elevated pCO2 samples

16S assay not amplifying. Positive controls did not amplify.
9-30-11_Vt_pCO2_VtpR_16S_expression.pdf

9-30-11_Vt_pCO2_RtqPCR_16S_expression.pdf

9-28-11 RT of DNased samples

Using M-MLV protocol on genefish:M-MLV

I did not normalize concentrations of RNA since the quantities were so low and after talking to SW.

A ratio of 0.25ug of random primers to 1 ug of RNA is needed for each reaction
0.01625/0.5ug = 0.0325 ul primers per reaction

1. 10ul of DNased RNA was used per reaction
2. Up the volume of the primers by diluting them 1:100 and add 3.25 ul of primers into each tube.
3. Add 5 ul of DNase free water to bring volume up to 18.25 ul

Incubate the samples at 70C for 5 min
Ice the samples immediately after incubation.

Create master mix:
Per reaction:
5 ul of 5x MMLV RT Buffer
1.5 ul 10mM dNTPs
0.5ul MMLV RTranscriptase

RT Master Mix	per rxn	rxns needed:	15
5x Buffer (M-MLV RT Buffer)	5.00		75.00
dNTPs (10mM total)	1.25		18.75
M-MLV transcriptase	0.05	(per 100ng RNA)	0.75
Nuclease free water	0.45		6.75
Total	6.75		

Mix well.
Add 6.75uL of master mix to each reaction. (Total reaction volume 25 ul)
Mix well, but do not vortex.
Spot spin.
Incubate @ 37C for 1 hour for random primers.
Heat inactivate @ 95C for 3 min.
Spot spin.
Store @ -20C.

Next step qPCR
For qPCR - used 2 ul template of cDNA for each reaction
Vt_trial 2 thermocycler protocol

9-27-11 DNAse RNA

Pick out the samples with genomic amplification on qPCR and DNase with Ambion kit.

PROCEDURE:
Add to PCR tube:
2.5 ul DNase Buffer
1 ul turbo DNase
20.5 ul RNA sample

TOTAL - 24 ul

Incubate samples for 30min at 37C
After 30 min, add 1 ul of turbo DNase
Incubate samples for 30min at 37C
After 30 min add 2.5 ul Inactivation Reagent
Leave at RT for 2 min, mixing occasionally
Spin down at 10000 ref for 1.5 min
Transfer supernatant to new tube.
Quant and normalize to the lowest RNA value.

Re qPCR these samples to check for leftover genomic carryover. Dilute the RNA 1:4 for PCR template.
9-29-11_DNase_carrryover_check2.pdf
Two samples had carryover: amb1-24 and amb3-24. Re-DNase and qPCR these two.

9-23-11 RNA extraction of Vt mini pCO2 experiment

See protocol on 9711
Qtys:

Sample ID	Average ng/ul
amb1.24	13.99
amb2.24	9.01
amb3.24	7.07
20001-24	16.32
20002-24	12.28
20003-24	13.04
amb1-end	9.05
amb2-end	7.25
amb3-end	13.27
20001-end	11.79
20002-end	12.875
20003-end	14.705

qPCR check for genomic carryover:
9-23-11_Vt_pCO2_RNA_genomic_check.pdf

All samples had carryover. Next step - DNase and RT for gene expression

9-14-11 NGS BLAST + GO term linking on galaxy

Used SR's instructions on table joining on Galaxy:
http://www.evernote.com/pub/sr320/lab#b=00219e0d-d274-4a25-b7a8-26be74c4501f&n=11b5a97f-eab7-4b2b-9c26-c08b54896e98

Joined 19106 BLAST results with SP to uniprot associations table
Then joined that query to GO terms and GO slim
Results were exported

3 pie charts were made from the GO slim terms, one for each component.

Need to BLAST RE22 with SPid to make the same charts

Hand mining contigs. Start with these:
RE22:

Contig	Contig Length	Average Coverage	SNPs per Contig	Count of Amino Acid Change	% SNPs per bp
NODE_366_length_35_cov_278.171417 mapping	35	544	2		0.0571
NODE_359_length_128_cov_321.046875 mapping	128	102	7		0.0547
NODE_640_length_41_cov_315.000000 mapping	41	40	2		0.0488
NODE_362_length_87_cov_617.160950 mapping	87	1207	4		0.0460
NODE_506_length_46_cov_769.913025 mapping	46	62	2		0.0435
NODE_572_length_46_cov_161.130432 mapping	46	7	2		0.0435
NODE_663_length_48_cov_27.020834 mapping	48	14	2		0.0417
NODE_559_length_75_cov_304.586670 mapping	75	489	3		0.0400

ATCC19106:

Contig	Length of Contig	Average Coverage	SNP Count	Count of Amino Acid Change	% SNPs per bp
NODE_334_length_36_cov_740.916687 mapping	36	116	3		0.083
NODE_155_length_64_cov_165.000000 mapping	64	25	5		0.078
NODE_190_length_58_cov_159.741379 mapping	58	27	4		0.069
NODE_627_length_92_cov_164.326080 mapping	92	34	6		0.065
NODE_205_length_48_cov_159.770828 mapping	48	41	3		0.063

NODE_257_length_12170_cov_86.954643 mapping	12170	85	400	55	0.033

9-14-11 Need Vt RNA for gene expression

Setting up mini experiment at elevated pCO2 to get sufficent RNA from Vt since the last extractions were unsuccessful.

Set up:
Triplicate cultures of Vt in 1000ml autoclaved SW
2 treatments - ambient and 2000ppm CO2
25C waterbath
Bubbling 48 hrs before inoculation of flasks with 22um filter on air input to prevent contamination
Inoculation = ~10^4 CFU/ml
cultures will grow 56-72 hrs and 200ml of culture will be sampled at the end of experiment in 50ml conical vials
Conical tubes will be spun at 3600rpm for 30min to pellet. Supernatant removed, and pellets condensed into one microcentrifuge tube per replicate.
Pellets will be flash frozen in liquid nitrogen and held at -80C until RNA extractions can be performed.
pH to be measured before inoculation, 24 hrs post inoculation, and after experiment

Bubbling started on 9-14 at 1030am. pH measurements taken at 24 after bubbling, 48hrs bubbling right before inoculations, and after experiment was ended.
Inoculated ~10^4 CFU/ml into each replicate. Plate counts done to estimate CFU/ml inoculation dose

RESULTS:
Original culture: 2.2 x 10^9 CFU/ml
inoculation = 2.4 x 10^4 CFU/ml
Temp spiked to 30C 24hrs post inoculation. I turned it down a bit.

All cultures grown for 50 hrs at 25C.
Average end of experiment culture CFU estimations:
2.5 x 10^5 CFU/ml at 2000 ppm
2.0 x 10^5 CFU/ml at ambient

9-8-11 reverse transcription of RNA samples from 9711

Using M-MLV protocol on genefish:M-MLV

On re-do, I did not normalize concentrations of RNA since the quantities were so low and after talking to SW.

[start with the RNA sample with the lowest concentration - in this case I used a mid-range value of 6.5 ng/ul since the concentrations are so low.
Normalized all RNA samples to 6.5 ng/ul or 0.0065 ug/ul for a total volume of 10ul. This yields a total volume of 0.065ug in 10ul.
A ratio of 0.25ug of random primers to 1 ug of RNA is needed for each reaction
0.065ug total in each tube x 0.25ug = 0.01625 ug primers needed]

0.01625/0.5ug = 0.0325 ul primers per reaction
Up the volume of the primers by diluting them 1:100 and adding 3.25 ul of primers into each tube.
Add 5 ul of DNase free water to bring volume up to 18.25 ul

Incubate the samples at 70C for 5 min
Ice the samples immediately after incubation.

Create master mix:
Per reaction:
5 ul of 5x MMLV RT Buffer
1.5 ul 10mM dNTPs
0.5ul MMLV RTranscriptase

RT Master Mix	per rxn	rxns needed:	15
5x Buffer (M-MLV RT Buffer)	5.00		75.00
dNTPs (10mM total)	1.25		18.75
M-MLV transcriptase	0.05	(per 100ng RNA)	0.75
Nuclease free water	0.45		6.75
Total	6.75		

Mix well.
Add 6.75uL of master mix to each reaction. (Total reaction volume 25 ul)
Mix well, but do not vortex.
Spot spin.
Incubate @ 37C for 1 hour for random primers.
Heat inactivate @ 95C for 3 min.
Spot spin.
Store @ -20C.

For qPCR - used 2 ul template of cDNA for each reaction
Vt_trial 2 thermocycler protocol

RESULTS:
not good, no amplification of 16S gene in samples. Positive controls OK.
I forgot to add the 5 ul of H2O after adding random primers. Oopsie. But is that the problem?
Will redo the RT and rerun, hopefully with better results.

RT redone, with the same expression results, or lack thereof.

qPCR RESULTS:
9-8-11_Vt_pCO2_16S_resA_gene_express.pdf

Still no amplification of 16S which should come up no prob according to last year's expression results.
Retry subset of samples that amplified rseA gene and re-run with new SYBR and positives.
RESULTS:

9-7-11 RNA extraction from Vt CO2 experiments

Samples used: R-062 - R-069 (2000ppm at 25C experiment) and R-093 - R-100 (750ppm at 25C experiment)
Using Vt saved from each high pCO2 growth trial - 750 and 2000 ppm CO2 - to isolate RNA and quantify gene expression of known virulence factors in Vt.
Samples are from experiments ambient vs 2000ppm performed 5-7-11 and ambient vs 750 ppm performed 7/18/11 both at 25C due to higher CFU/ml content

PROCEDURE:
NEED: ice bath, heat block to 55C, Isopropanol, DEPC H2O, and 75% EtOH + DEPC water

1. Add 500ul of TriReagent to each Vt pellet and vortex for 15 sec.
2. Incubate your homogenized tissue sample tube at room temperature (RT) for 5 mins.
3. In the fume hood, add 200uL of chloroform to your sample and close the tube. the container of chloroform before drawing and chloroform into your pipette tip.
4. Vortex vigorously for 30s. solution becomes a milky emulsion.
5. Incubate tube at RT for 5 mins.
6. Spin tube in refrigerated microfuge for 15 mins. @ max speed.
7. Gently remove tube from microfuge. Be sure not to disturb the tube.
8. Slowly and carefully transfer most of the aqueous phase (the top, clear portion) to a fresh microfuge tube. Do NOT transfer ANY of the interphase (the white, cell debris between the aqueous and organic phase).
9. Close the tube containing the organic and interphase and properly dispose of the liquid inside the tube as well as the tube itself at the end of the lab.
10. Add 500uL isopropanol to the new tube containing your RNA and close the tube.
11. Mix by inverting the tube numerous times until the solution appears uniform. Pay particular attention to the appearance of the solution along the edge of the tube. If mixed properly, it should no longer appear viscous/"lumpy".
12. Incubate at RT for 10 mins.
13. Spin in refrigerated microfuge at max speed for 8 mins. When placing your tube in the microfuge position the tube hinge pointing up, away from the center of the microfuge.
14. A small, white pellet (RNA and salts) should be present. If not, do not fret an continue with the procedure.
15. Remove supernatant.
16. Add 1mL of 75% EtOH to pellet. Close tube and vortex briefly to dislodge pellet from the side of the tube. If the pellet does not become dislodged, that is OK.
17. Spin in refrigerated microfuge at 7500g for 5mins.
18. Carefully remove supernatant. Pellet may be very loose. Make sure not to remove pellet!
19. Briefly spin tube (~15s) to pool residual EtOH.
20. Using a small pipette tip (P10 or P20 tips), remove remaining EtOH.
21. Leave tube open and allow pellet to dry at RT for no more than 5mins.
22. Resuspend pellet in 100uL of 0.1%DEPC-H2O by pipetting up and down until pellet is dissolved.
23. Incubated tube at 55C for 5mins. to help solubilize RNA.
24. Remove tube from heat, flick a few times to mix and place sample on ice. This is the stock RNA sample.
25. Quantitate RNA yield using Nanodrop spectrophotometer.

RNA QUANTIFICATION
NOTE: Always keep your RNA samples on ice!
1. Pipette 2µL of 0.1%DEPC-H20 onto the Nanodrop pedestal and lower the arm.
2. Click "Blank", to zero the instrument. NOTE: steps 1 and 2 only need to be done once for the whole class.
3. Pipette 2µL of your RNA sample onto the Nanodrop pedestal and lower the arm
4. Click "Measure". Record your RNA concentration (ng/µL), A260/280 ratio and A260/230 ratio. NOTE: The Nanodrop uses the Beer-Lambert Law to calculate RNA concentration for you. See Lab 1 notes on RNA extraction for more information on the calculation and how to evaluate RNA purity using A260/280 and A260/A230 ratios.
6. Raise the arm and wipe off you sample with a KimWipe
7. Clearly label your stock RNA sample with concentration in ug/uL.
8. Store samples at -80C.
NOTE: nanodrop needs calibration. +/- 5% off in either direction, but it is thought that ng/ul are underestimated in the readings.

NANODROP RESULTS:

Sample ID	Date	ng/ul	A260	A280	260/280	260/230	**Average ng/ul**

Sample ID	Date	ng/ul	A260	A280	260/280	260/230	**Average ng/ul**
R-62-1	9/7/11	9.57	0.239	0.157	1.52	0.29	**9.22**
R-63-1	9/7/11	7.77	0.194	0.127	1.53	0.21	**7.82**
R-64-1	9/7/11	5.88	0.147	0.108	1.35	0.3	**6.11**
R-66-1	9/7/11	5.57	0.139	0.094	1.49	0.36	**5.765**
R-67-1	9/7/11	5.77	0.144	0.074	1.94	0.33	**5.64**
R-68-1	9/7/11	6.37	0.159	0.104	1.54	0.37	**6.565**
D-405-1	9/7/11	7.45	0.186	0.131	1.42	0.29	**7.62**
D-406-1	9/7/11	8.61	0.215	0.159	1.35	0.25	**8.315**
D-407-1	9/7/11	5.24	0.131	0.096	1.36	0.27	**5**
D-408-1	9/7/11	6.24	0.156	0.098	1.59	0.26	**6.27**
D-409-1	9/7/11	4.34	0.108	0.063	1.71	0.28	**4.54**
D-410-1	9/7/11	2.8	0.07	0.061	1.16	0.39	**2.73**

CHECK FOR GENOMIC CARRYOVER:
qPCR using Vt 16S primers to check for carryover. Master Mix recipe:

		1. rxns =	32
Reagent	ul		
Immomix (2x)	12.5		400
F Primer (10uM)	0.8		25.6
R Primer (10uM)	0.8		25.6
BSA	1.5		48
SYBR	0.5		16
PCR water	7.9		252.8
RNA	1.00		Add Individ
TOTAL	25.0		

Vt_trial2 parameters on desktop.

qPCR RESULTS:
No amplification of RNA samples, although only one positive control replicate amplified.
I am going to re-do this plate again to double check and up the template to 2ul per reaction.

9-8-11_Vt_pCO2_RNA_carrover_check2.pdf

8-11-11 Vt molecular troubleshoot cont'd

qPCR assay using standard curves from (pink) 6-28-10 and 1-12-11 (low curve) to estimate loss in assay sensitivity

PROCEDURE:
Same assay protocol for Vt qPCR - no modifications
Low curve:
~ 500000
50000
5000
500
50

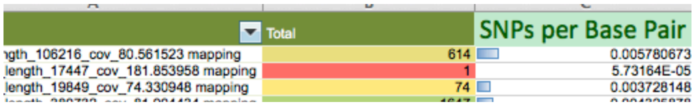
and original standard curve (6-28-10):
-1 = 50,000,000
-2 = 5,000,000
-3 = 500,000
-4 = 50,000
-5 = 5000
-6 = 500
-7 = 50

cells/ml
RESULTS:
8-11-11_Vt_6-28_StCv_retry.pcrd

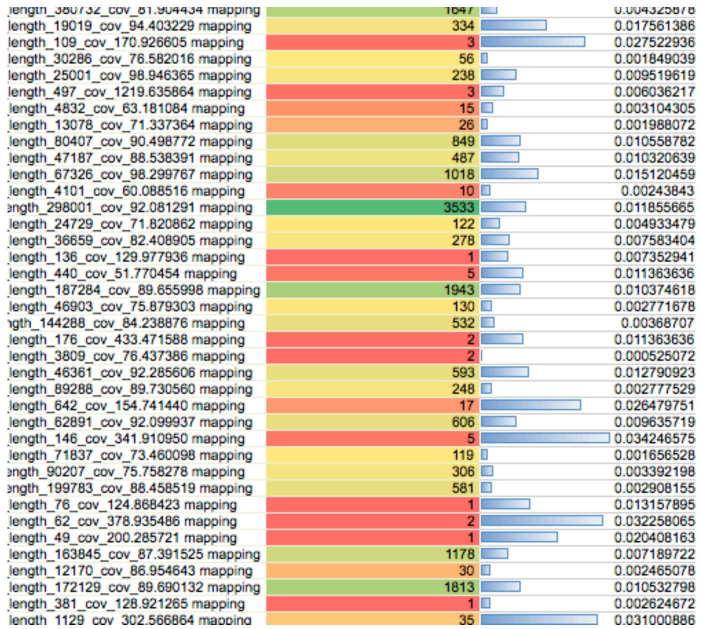
8-10-11

RE22 on publish seq
used to do handmining of text files
Starting with those with the highest SNP/bp.

snapshot!



	Total	SNPs per Base Pair
ngth_106216_cov_80.561523 mapping	614	0.005780673
length_17447_cov_181.853958 mapping	1	5.73164E-05
length_19849_cov_74.330948 mapping	74	0.003728148



length_380732_cov_81.904434 mapping	1647	0.004325878
length_19019_cov_94.403229 mapping	334	0.017561386
length_109_cov_170.926605 mapping	3	0.027522936
length_30286_cov_76.582016 mapping	56	0.001849039
length_25001_cov_98.946365 mapping	238	0.009519619
length_497_cov_1219.635864 mapping	3	0.006036217
length_4832_cov_63.181084 mapping	15	0.003104305
length_13078_cov_71.337364 mapping	26	0.001988072
length_80407_cov_90.498772 mapping	849	0.010558782
length_47187_cov_88.538391 mapping	487	0.010320639
length_67326_cov_98.299767 mapping	1018	0.015120459
length_4101_cov_60.088516 mapping	10	0.00243843
ength_298001_cov_92.081291 mapping	3533	0.011855665
length_24729_cov_71.820862 mapping	122	0.004933479
length_36659_cov_82.408905 mapping	278	0.007583404
length_136_cov_129.977936 mapping	1	0.007352941
length_440_cov_51.770454 mapping	5	0.011363636
length_187284_cov_89.655998 mapping	1943	0.010374618
length_46903_cov_75.879303 mapping	130	0.002771678
ngth_144288_cov_84.238876 mapping	532	0.00368707
length_176_cov_433.471588 mapping	2	0.011363636
length_3809_cov_76.437386 mapping	2	0.000525072
length_46361_cov_92.285606 mapping	593	0.012790923
length_89288_cov_89.730560 mapping	248	0.002777529
length_642_cov_154.741440 mapping	17	0.026479751
length_62891_cov_92.099937 mapping	606	0.009635719
length_146_cov_341.910950 mapping	5	0.034246575
length_71837_cov_73.460098 mapping	119	0.001656528
ength_90207_cov_75.758278 mapping	306	0.003392198
ength_199783_cov_88.458519 mapping	581	0.002908155
length_76_cov_124.868423 mapping	1	0.013157895
length_62_cov_378.935486 mapping	2	0.032258065
length_49_cov_200.285721 mapping	1	0.020408163
length_163845_cov_87.391525 mapping	1178	0.007189722
length_12170_cov_86.954643 mapping	30	0.002465078
length_172129_cov_89.690132 mapping	1813	0.010532798
length_381_cov_128.921265 mapping	1	0.002624672
length_1129_cov_302.566864 mapping	35	0.031000886

FOLLOW UP
Assembled ATCC with 100 bp min
~37k contigs
made consensus
Blasting on inquiry to SP
started 0811- 10:30am

8-4-11 NGS data: Creating SNP tables from contigs and comparing strain differences

PROCEDURE:
Search for node of interest in assembled reference seq - should have decent coverage and somewhere in length of 1000 - 50,000 bp
Make new folder in Fragment section
Go to main reference Vt sequence, search for node, double click it to open
Drag the open sequence to the folder, do not rename
Open the sequence assembly of one strain and find node
Highlight node and Click "Open mapping"
Hightlight the word "consensus" in the open window
right click and select "Copy sequence WITH GAPS"
Drag the new file to the folder
Do the same process to the other strain assembly
Now go to the folder with the 3 sequences
highlight one of the strains and go to Toolbox, Seq Data Analysis, Assemble to Seq and choose the node file from the Vt reference to use as a reference for the assembly
Lower the stringency to 10 and "Low"
Follow this procedure for the other strain
Make sure to keep track of which file belongs to which strain
Highlight the assembly file for one strain and create a SNP detection table (lower the coverage to "1")
Export in csv form and link tables using site of SNP for each strain

7-27-11 Vt SNPs redone

Changing minimum variant frequency from 35% to 60% to see if the alignments will be easier to examine fragment comparisons.
The challenge with the last SNP analysis was the size of the fragments were too large to align to one another.

7-18-11 Vt Growth at 750ppm pCO2 and ambient

Same procedure as 5-4-11 but plating every time point except T0
Inoculation dose also changed:
Adding 100ul of 1:20 dilution Vt strain RE22 grown for 24hrs at RT into 1000ml of autoclaved sterile SW.
Inoculation dose expected to be ~2.0 x 10^3 CFU/ml

Plating 11 timepoints for quantification with parallel qPCR samples taken at each time point.

Original culture:
1.6 x 10^9 CFU/ml average
Actual dosage per flask:
1.6 x 10^3 CFU/ml

7-14-11 Troubleshooting Vt molecular (cont'd)

Redo mini experiment
Procedure same as 6-22-11
Adding more controls to test:

- picked isolated colony used to inoculate broth
- 1 ml broth culture pellet
- colonies grown on agar plates to estimate CFUs during growth

Original culture = 1.6 x 10^9 CFU/ml
Used 100ul of diluted -2 cultureinto 1000ml SW for inoculation.
Culture grew at RT without agitation.
Culture was stirred and sampled at the times below:
T0 = 1.3 x 10^3 CFU/ml
T1 = 2.2 x 10^3 CFU/ml
T2 = 1.5 x 10^3 CFU/ml
T4 = 7.0 x 10^4 CFU/ml

Plating growth at T0 (8:00 am), T1 (4hrs), T2 (6 hrs), T3 (24hrs).
Used 1ml of SW culture to spin at high speed for 12 min and extracted DNA using Qiagen DNeasy kit.

RESULTS:
Used Qiagen extracted DNeasy standard curve made 6-28-10 (green) for additional info.
7-15-11_Vt_test_mini_experiment3.pdf

All colonies were positive and original culture was positive. Standard curve amplified at the higher CFUs but it appears that the assay sensitivity is very low.

7-7-11 LD50 750ppm - #2

Temps:
7-8 = 16C
7-9 = 16C
7-10 = 16C

Inoculation:
162 average colonies at -6 dilution = 1.6 x 10^9 CFU/ml original culture
Dilutions used: 100ul of -1 to -5 for final inoculation doses of:
4.0 x 10^6 - 4.0 x 10^2 CFU/ml

PMEL samples - taken as stated below (6-24). Temperature of samples are stated above.
Salinity = 32ppt for both Ambient and 750ppm
Temperature of samples - 16C
100ul of HgCl2 added to each
A15 - 750
A16 - Ambient

6-30-11 LD50 750 ppm - #1

Temps:
7-1 = 16C - ambient and 17C - 750ppm
7-2 = 16C - ambient and 17C - 750ppm
7-3 = 15C - ambient and 16C - 750ppm

Inoculation:
average of 148 colonies at -6 dilution = 1.5 x 10^9 CFU/ml
Dilutions used - -1 to -5
100ul of each dilution into the corresponding wells.
End dosages = 3.75 x 10^6 - 3.75 x 10^2 CFU/ml

PMEL samples - taken as stated below (6-24). Temperature of samples are stated above.
Salinity = 34 ppt for Ambient and 32 ppt for 750 ppm
A13 - 750
A14 - Ambient
New m-cresol purple indicator dye made 7/1/11.

6-24-11 LD50 2000ppm - #4

Temp:
6-24 = 17C
6-25 = 16C
6-26 =16C

Inoculation:
145 colonies at -6 = 1.5 x 10^9 CFU/ml original culture
Dosages = 3.75 x 10^6 - 3.75 x 10^2 CFU/ml

PMEL samples:
Taken at 16C, added 100ul saturated HgCl2 to each 300ml bottle.Samples taken by siphoning the SW from the bottom of container containing approx 1 liter of SW residing in the chamber through the duration of the experiment. Bottle was overflowed with ~2x the volume of the bottle.
Salinity = 32 ppt for both samples.
A11 - 2000ppm
A12 - Ambient

6-22-11 Redo Mini Vt growth experiment

Use new extraction kit. Same procedure as 6-15-11.
Used streaked plate of the original RE22 isolate in RPH freezer (RE isolate # 99-70-6B-2)
Used isolated colony to inoculate 25 ml 1% tryptone and SW broth
Grew culture for 24 hours on tilty rocker in 236
Plated all samples to estimate CFU/ml on T1N2 agar plates

Samples taken:
T0 - at inoculation (930a)
T1 - 1130am
T2 - 4 pm
T3 - 830a 6/23

RESULTS:
Original culture: 2.0 x 10^9 CFU/ml
Inoculation = 100ul of -2 dilution (1:20) into 1000ml SW = 6.7 x 10^2 CFU/ml

From plate counts:
T0 = 1.5 x 10^3 CFU/ml
T1 = 1.7 x 10^3 CFU/ml
T2 = 3.0 x 10^3 CFU/ml
T3 = 1.7 x 10^5 CFu/ml

qPCR:
little amplification of T2 and T3. No duplicate amplification during analysis. Bad news. Positive controls are still consistent.
Vt_mini_growth_test_6-27-11.pdf

6-20-11 Troubleshooting cont'd

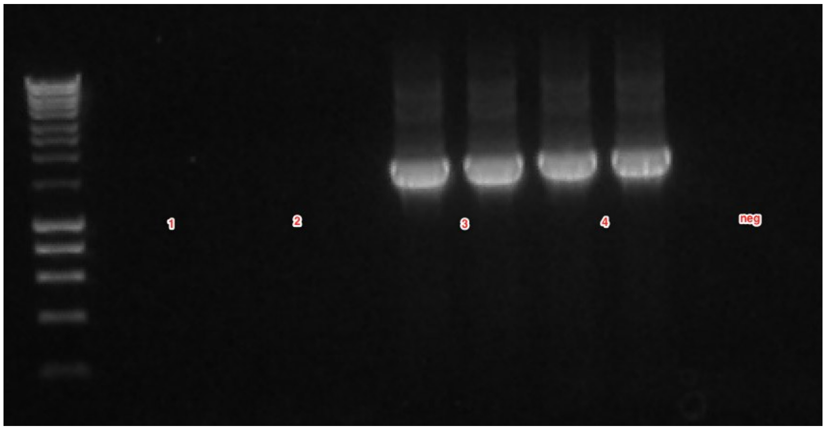
Continuing to PCR to convince myself that bad template due to Qiagen kit is the issue.

1 - Extract a colony of the RE22 used in the mini experiment on 6-15 with the old DNeasy kit
2 - PCR vtpA using 10ul of template for the 2 extractions below that did not amplify the vtpA gene
3 - PCR universal primers used on 4-11-11 using the 2 dud templates and 2 good templates.

Samples used:
1. T1 from mini experiment - did not amplify vtpA gene on 6-17
2. D-234 from Vt pCO2 experiment - neg on qPCR, did not amplify on gel cPCR
3. Culture from mini experiment - vtpA + on gel
4. RE22 culture - extracted on 2/9/11, vtpA + on gel

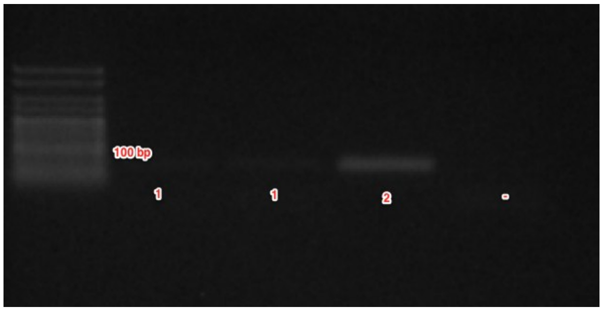
Thermocycle protocol used:
Vt-16S
Vt

RESULTS:
Universal 16S:
1% agarose gel
90v for 45 min, Hyperladder I



Products of 3 and 4 are ~1500 bp

vtpA gene:
2% agarose gel
75v for 65 min, Hyperladder V



faint bands on T1 and clear band on D-234. weird.

CONCLUSIONS:
Looks like template is to blame although I can't explain the vtpA gel and none on the universal primers. The qiagen kit is very suspect.
Redo mini growth experiment using new extraction kit and qPCR.

6-17-11 Troubleshooting qPCR problems with the Vt assay

Task 1 - sequence the templates that are giving me problems
Task 2 - extract using new Qiagen kit

cPCR on the following samples using the following primers:
vtpA
16S tubiashii specific
EUB universal primers

Recipe for each set:

	Vol (ul) per rxn	1. rxns =	18
PCR H20	12.75		229.50
5xBuffer	5.00		90.00
MgCl2	2.00		36.00
BSA	1.00		18.00
dNTPs	0.50		9.00
F Primer (10mM)	0.75		13.50
R Primer (10mM)	0.75		13.50
Taq	0.25		4.50
Template	2.00		Add individually
TOTAL	25.00		

Thermocycler - Vt protocol for vtpA

Thermocycler:	
95 for 3 min	
95 for 15 sec	
55 for 30 sec	} 35 - 40 cycles
72 for 30 sec	
72 for 10 min	
22 for 10 min	

Thermocycler - Vt protocol for tub16S and EUB

95 for 10 min	
95 for 15 sec	
55 for 1 min	} 35 - 40 cycles
72 for 30 sec	
72 for 10 min	
22 for 10 min	

Samples used:

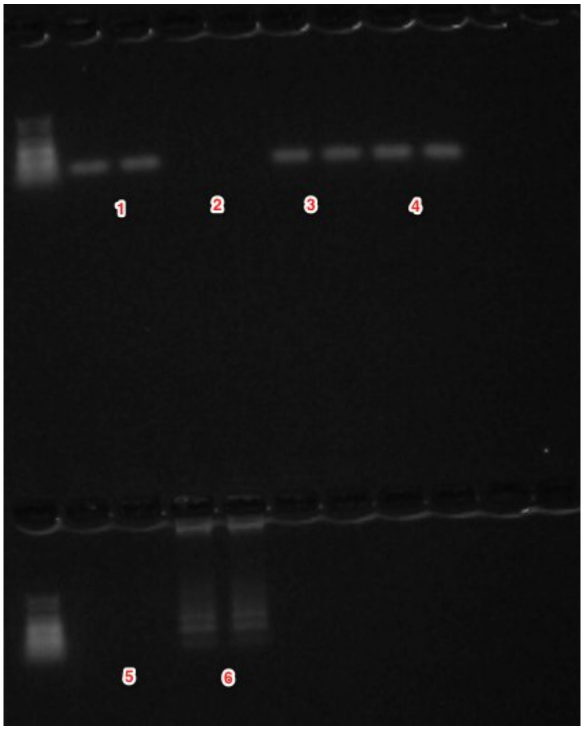
1. RE22 culture ~72 hrs growth, 200ul spun for 10 min at high and extracted using *new* DNeasy kit. Isolate from EDs -80 stock. Have not

tried to qPCR
2. T1 - mini experiment performed 6-15-11, 2 hrs growth (~10^3 CFU/ml) same bacteria as #1, extracted with old DNeasy kit, no amplification on qPCR
3. Positive control RE22 - used on all qPCR plates, always amplifies, very consistent, extracted in Feb
4. RE22 - extracted on 2/9/11 with older kit, I have not tried to qPCR this
5. D-234 - timepoint from pCO2 experiment, should have ~10^5 CFU/ml RE22 from plate counts of that replicate, no amplification on qPCR
6. vtpA plasmid - undiluted sample, no amplification on qPCR, clone made 6/9/11(#5), cPCR positive for vtpA before miniprep (see 5-31-11)

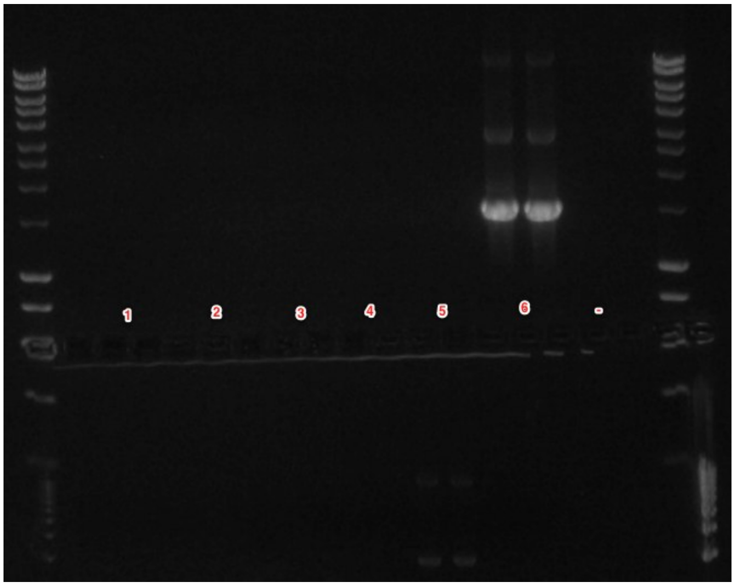
STEPS:
visualize on gel
PCR purification
Sequencing at UW seq fac
Examine sequences

qPCR #1 samples to see if kit is the issue

RESULTS:
vtpA primers:
2% agarose gel
75 v for 40 min, Hyperladder V



EUB primers and Vt16S primers:
120v for 1 hour
1% gel
Top = EUB primers (Hyperladder I); Bottom = Vt 16S (100kb ladder)



Ran the 2nd gel out too long and did not capture 16S bands if there were any. EUB universal primers only amplified plasmid (#6).
Looks as if template problems on the 2 experimental vtpA gels. Possibly too little amplifcation there to capture on the gel.
vtpA plasmid is not amplifying with the primers.

CONCLUSIONS:
Bad clone. Not specific to vtpA gene. Need to start over there.
Qiagen kit may be to blame. The same bacteria was used in samples 1 and 2 pointing to bad kit. Template is still suspect tho, but should amplify. Same "bad" kit was used to extract #5 which should contain plenty of bacteria.

6-15-11 Inoculation dosage and qPCR sensitivity

Inoculated 1000ml of sterile SW with 100ul of 1:20 dilution of Vt grown for 24 hours at RT in 240.
Left to incubate at RT ~22C.
Took two 1ml aliquots of the inoculated SW and spun at high speed for 12 min.
Took off supernatant and immediately started DNeasy B+T kit.
Plated culture on T1N2 plates to double check inoculation dose.
I will take samples of the SW culture throughout the day and plate to double check CFUs/qPCR totals
All aliquots of the culture will be DNA extracted and qty determined by qPCR.

RESULTS:
From Plate Counts:
Starting culture = 2.6 x 10^9 CFU/ml
SW culture inoculated with ~10^3 CFU/ml = 2.2 x 10^3 CFU/ml
T1 (2 hr) = 3.0 x 10^3 CFU/ml
T2 (4 hr) = 2.4 x 10^3 CFU/ml

Re-ran some of the higher quantities of the 25C replicates from the Vt pCO2 experiment. These unknowns should contain ~10^5 CFU/ml of Vt.

qPCR:
Nothing worked except positive controls. No plasmid, no templates.
I am DNA extracting a large amount of DNA to see if I can get the bacteria to amplify at all on qPCR.

6-11-11 SNPs of strains RE22 and 19106

Going through the SNP data -
Node_72_length_44828- interesting - contains metalloprotease genes and others of possible interest.

Tried to assemble onto Vt genome reference but not working. Only one small area aligned well with with RE22.

Procedure for frag comparisons:
Create new folder for node
Open assemblies of both strains and search for node fragment of interest
Open consensus of each
Individually drag and drop into node folder
Should have a double helix icon
To get the reference contig:
Open a sequence assembly for either strain
Search for node of interest
Open mapping
Scroll up on the bottom portion of the screen to reveal contig name
click on contig name and right click
Create a copy of the contig (should have a double helix symbol in the options to copy)
A new tab should appear on the top right
Drag tab into the node folder (should be a double helix symbol)
The contig should be named already with length and coverage

To create a comparison of all three sequences with IDed SNPs:
Toolbox
Sequence Data Analysis
Assemble Sequence to Reference
Select all three sequences in the node folder of both strains to assemble
Select only the Vt genome contig to use as reference
and assemble

Problems:
most nodes are too large to assemble with any usable output.

Tried to assemble node_112_length_19019 and node_72_length_44828 without much success

I realize that the criteria for SNP tables included only a 35% minimum variant frequency (default is 60%)
I may re-run the SNP tables to be a little more stringent with calling SNPs and it may help the assemblies
Possibly??

6-1-11 LD50 D hinge and 1 week old larvae at elevated pCO2

Started bubbling autoclaved SW at 2000ppm and ambient air at 2pm 6/1 in 18C waterbath
Vt failed to grow overnight. I stopped the gas bubbling and capped off the bottles at low CO2.
I will continue the bubbling at 4pm 6/2.

Inoculated tryptone /SW broth at 1pm with RE22 to grow overnight at RT.
New m-cresol purple indicator dye made with pH of 8.04 and A1/A2 of 1.82. I still need to characterize this dye for new slope intercept for pH calculations.
Larvae coming in from the hatchery tonight and will remain in autoclaved SW bubble with ambient air at 18C.
Same experimental design as previous experiments.

Aiming for correct # larvae in each well - 40 larvae
Need modify water chemistry analysis sample procedure. I will work on that tomorrow. I will start with a siphon type tube in a larger reservoir of water to take both the spec samples and the analysis samples for PMEL.

Morning of 6/3:
Spec water samples
Serial dilute Vt and plate for estimation of CFU in original culture
Rinse larvae with appropriate pCO2 level SW
Devy larvae into wells and inoculate with Vt
Fill wells with 4 ml SW total
place into chambers pumped with appropriate pCO2 level.

RESULTS:
Original culture CFU/ml = 3.0 x 10^8 (30 colonies at -6)
Final dosages :
7.5 x 10^4
7.5 x 10^3
7.5 x 10^2
7.5
~0

Control
Unfortunately, original culture was not as high as expected yielding low Vt dosages for this experiment.
I will give this experiment one more go, growing Vt for 24 hours to get the doses higher.
Numbers of larvae per well were manageable, making the counts easier to handle.

Temperature of experiment for all three days = 17C (the ambient SW that was used for pH measurements read at 16C everyday with the laser thermometer, but the culture plates with the larvae read 17C each day)
pH taken every 24 hours by spect
PMEL samples taken in accordance their sampling procedures - Samples (2000 ppm - 17C) A9 and (Ambient - 16C) A10

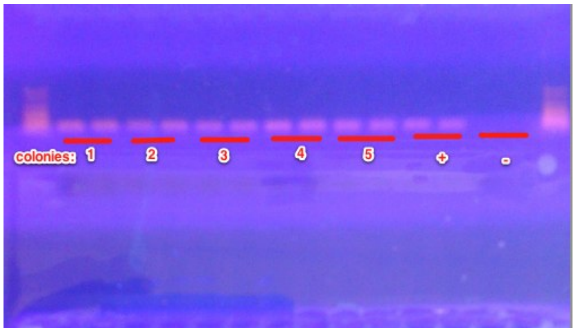
5-31-11 Re-clone vtpA

Plasmids not working on qPCR as of week of 5/9. Re growth of clone culture and miniprep only worked on 1 reaction.

Same procedure as 3-25-11.
Vector used: pCR2.1-TOPO, 3931 bp
100ul of 200X ampicillin spread on LB agar plates prior to incubation.

RESULTS:
Initial DNA template - RE22 grown in tryptone/SW media and DNA extracted using Qigen DNeasy Blood and Tissue kit
PCR product - bands ~50 - 100 bp in size as expected (camera not working)
PCR product purified with Qiagen PCR purification kit and cloning reaction performed on 5/31.

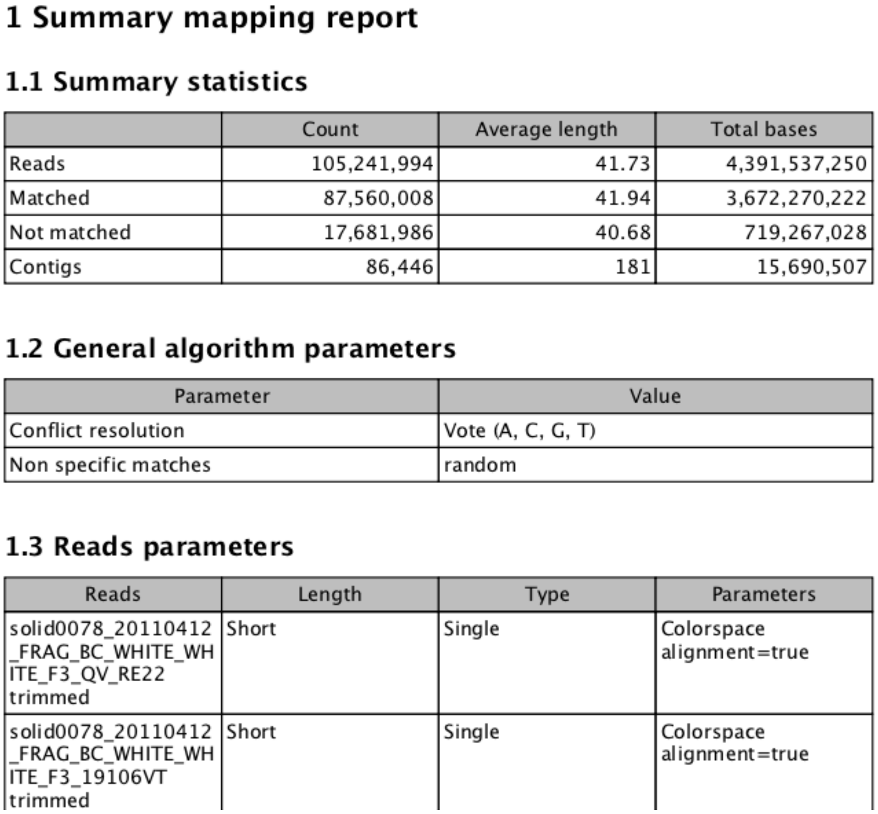
PCR of 5 colonies picked for patch plate and grown in 5ml LB broth with 25ul of 200x amplicillin overnight at 37C.
All product matched the positive control and the initial PCR product used to clone.



Purified plasmid from clones 3 and 5 on 6/2/11. Held at -20 until I can quant and dilute.

5-26-11 NGS data analysis

De novo both RE22 and ATCC
results:



1 Summary mapping report

1.1 Summary statistics

	Count	Average length	Total bases
Reads	105,241,994	41.73	4,391,537,250
Matched	87,560,008	41.94	3,672,270,222
Not matched	17,681,986	40.68	719,267,028
Contigs	86,446	181	15,690,507

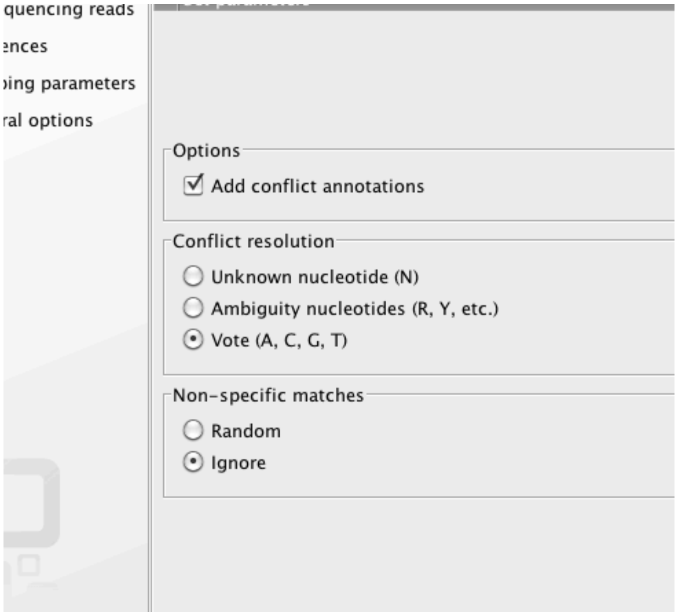
1.2 General algorithm parameters

Parameter	Value
Conflict resolution	Vote (A, C, G, T)
Non specific matches	random

1.3 Reads parameters

Reads	Length	Type	Parameters
solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_QV_RE22 trimmed	Short	Single	Colorspace alignment=true
solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_19106VT trimmed	Short	Single	Colorspace alignment=true

_
Reference RE22 trimmed on ATCC



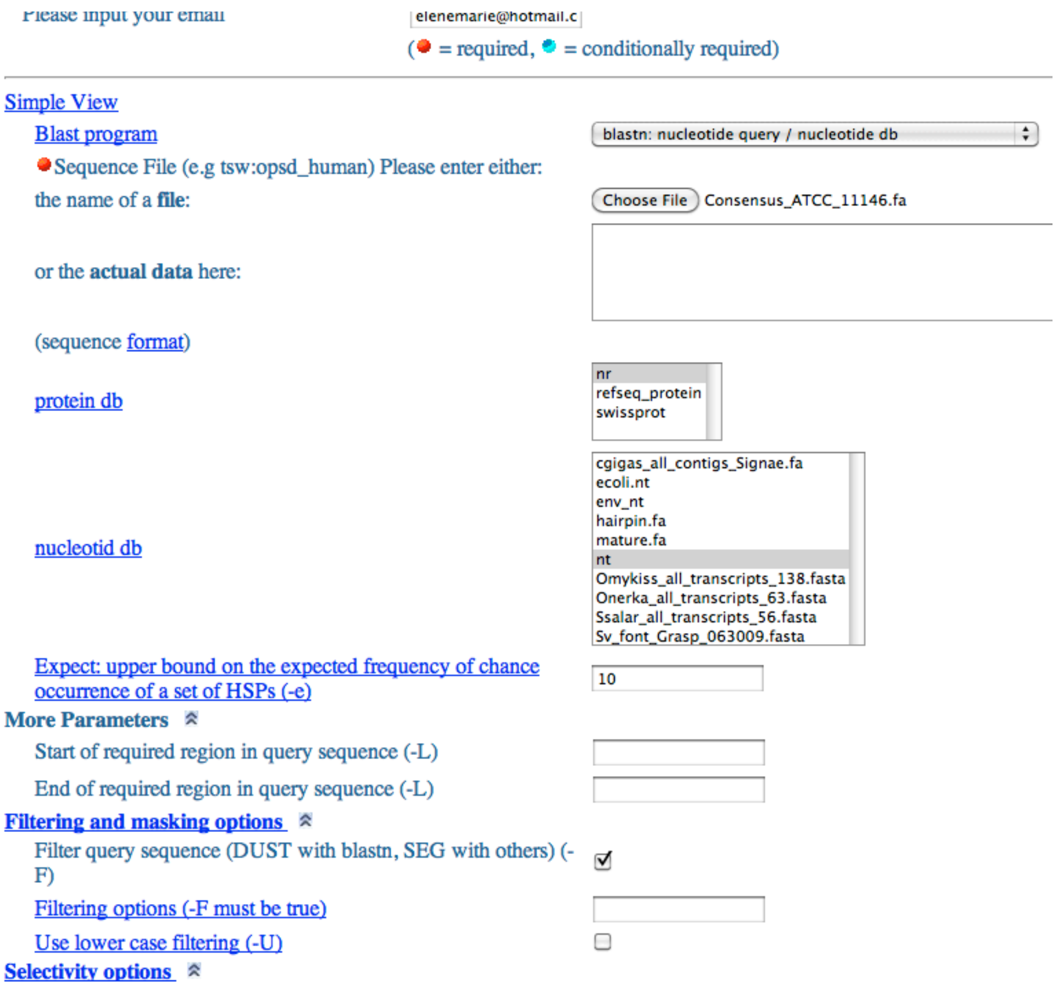
Options
☑ Add conflict annotations

Conflict resolution
○ Unknown nucleotide (N)
○ Ambiguity nucleotides (R, Y, etc.)
● Vote (A, C, G, T)

Non-specific matches
○ Random
● Ignore

and vice versa

Blasting separate consensus



Please input your email elenemarie@hotmail.c

(● = required, ● = conditionally required)

Simple View

Blast program: blastn: nucleotide query / nucleotide db

● Sequence File (e.g tsw:opsd_human) Please enter either:

the name of a **file**: Choose File Consensus_ATCC_11146.fa

or the **actual data** here:

(sequence format)

protein db: nr, refseq_protein, swissprot

nucleotid db: cgigas_all_contigs_Signae.fa, ecoli.nt, env_nt, hairpin.fa, mature.fa, nt, Omykiss_all_transcripts_138.fasta, Onerka_all_transcripts_63.fasta, Ssalar_all_transcripts_56.fasta, Sv_font_Grasp_063009.fasta

Expect: upper bound on the expected frequency of chance occurrence of a set of HSPs (-e): 10

More Parameters

Start of required region in query sequence (-L):

End of required region in query sequence (-L):

Filtering and masking options

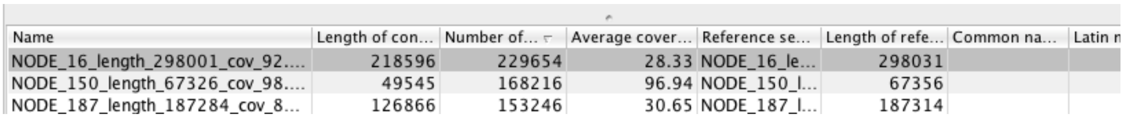
Filter query sequence (DUST with blastn, SEG with others) (-F): ☑

Filtering options (-F must be true):

Use lower case filtering (-U): ☐

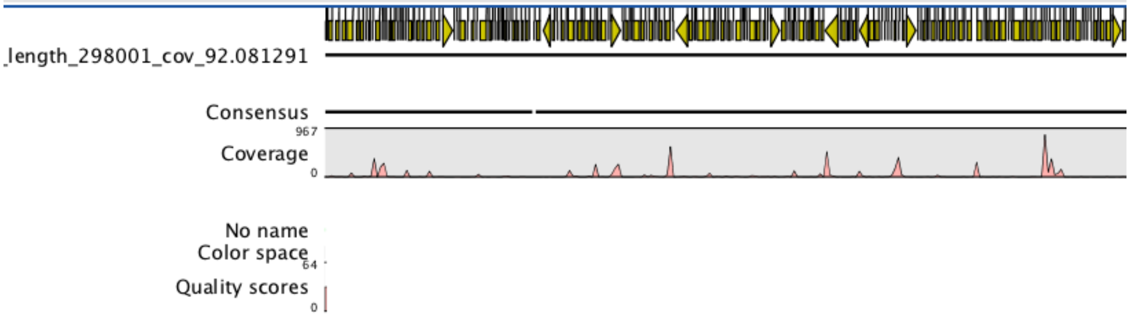
Selectivity options

Vt sequence reference - single gene

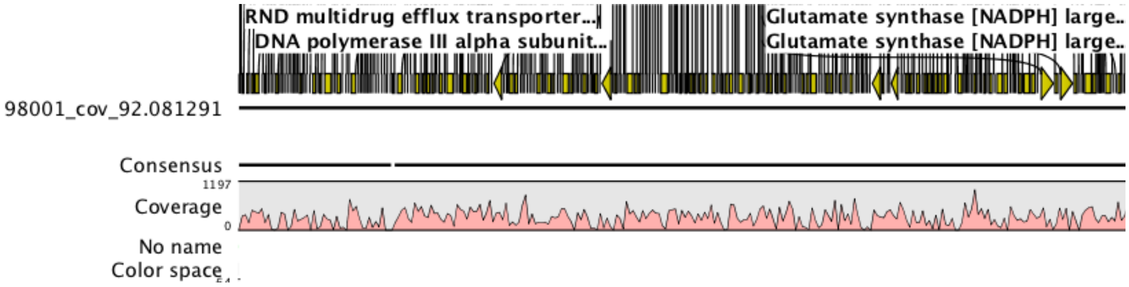


Name	Length of con...	Number of...	Average cover...	Reference se...	Length of refe...	Common na...	Latin n
NODE_16_length_298001_cov_92....	218596	229654	28.33	NODE_16_le...	298031		
NODE_150_length_67326_cov_98....	49545	168216	96.94	NODE_150_l...	67356		
NODE_187_length_187284_cov_8...	126866	153246	30.65	NODE_187_l...	187314		

RE22



ATCC



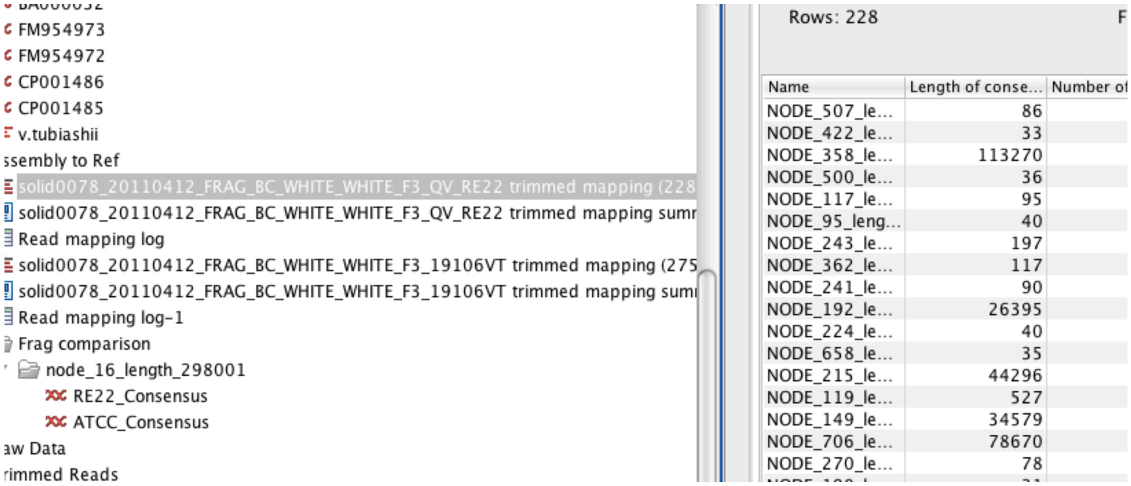
Alignment and Assembly of these two sequences failed.

--

ID SNPs in RE22 assembly on Vt "genome'

ID SNP's in RE22 assembly on VT genome

Pic of contig-

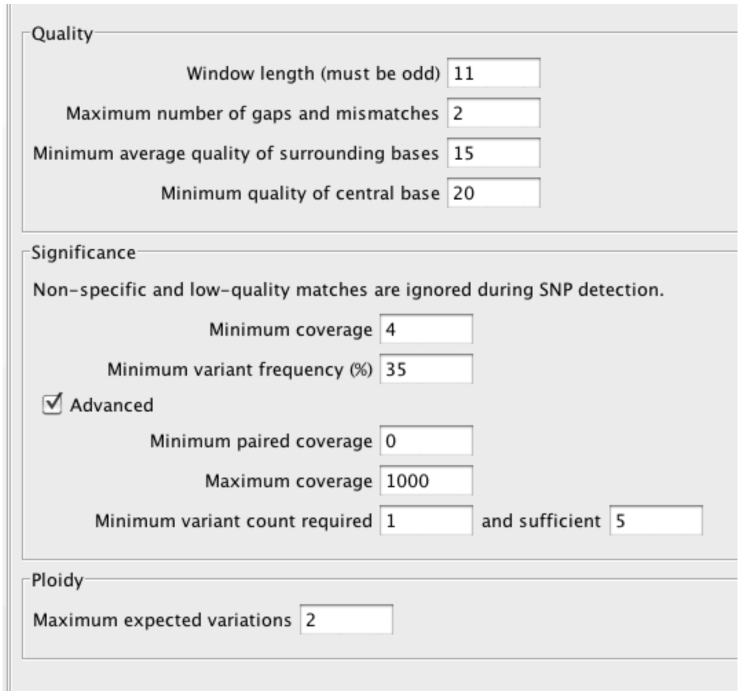


FM954973
FM954972
CP001486
CP001485
v.tubiashii
ssembly to Ref
solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_QV_RE22 trimmed mapping (228
solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_QV_RE22 trimmed mapping sumr
Read mapping log
solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_19106VT trimmed mapping (275
solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_19106VT trimmed mapping sumi
Read mapping log-1
Frag comparison
node_16_length_298001
RE22_Consensus
ATCC_Consensus
aw Data
rimmed Reads

Rows: 228

Name	Length of conse...	Number of
NODE_507_le...	86	
NODE_422_le...	33	
NODE_358_le...	113270	
NODE_500_le...	36	
NODE_117_le...	95	
NODE_95_leng...	40	
NODE_243_le...	197	
NODE_362_le...	117	
NODE_241_le...	90	
NODE_192_le...	26395	
NODE_224_le...	40	
NODE_658_le...	35	
NODE_215_le...	44296	
NODE_119_le...	527	
NODE_149_le...	34579	
NODE_706_le...	78670	
NODE_270_le...	78	

SNP detection

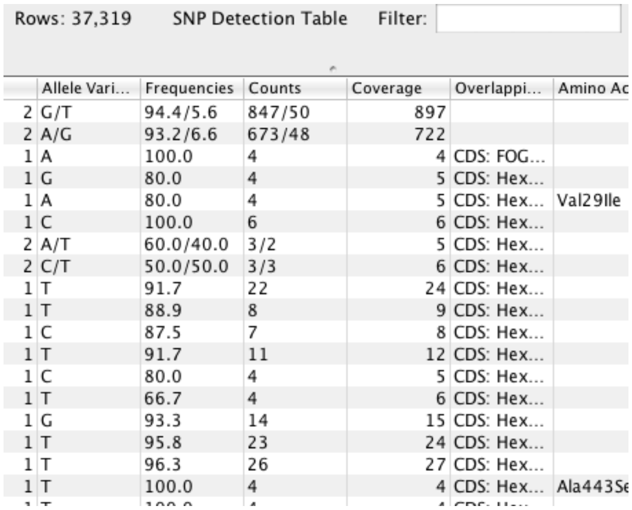


Quality
Window length (must be odd): 11
Maximum number of gaps and mismatches: 2
Minimum average quality of surrounding bases: 15
Minimum quality of central base: 20

Significance
Non-specific and low-quality matches are ignored during SNP detection.
Minimum coverage: 4
Minimum variant frequency (%): 35
☑ Advanced
Minimum paired coverage: 0
Maximum coverage: 1000
Minimum variant count required: 1 and sufficient: 5

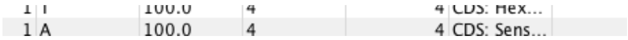
Ploidy
Maximum expected variations: 2

did both RE22 and ATCC
results: very nice!
contains gene / AA info



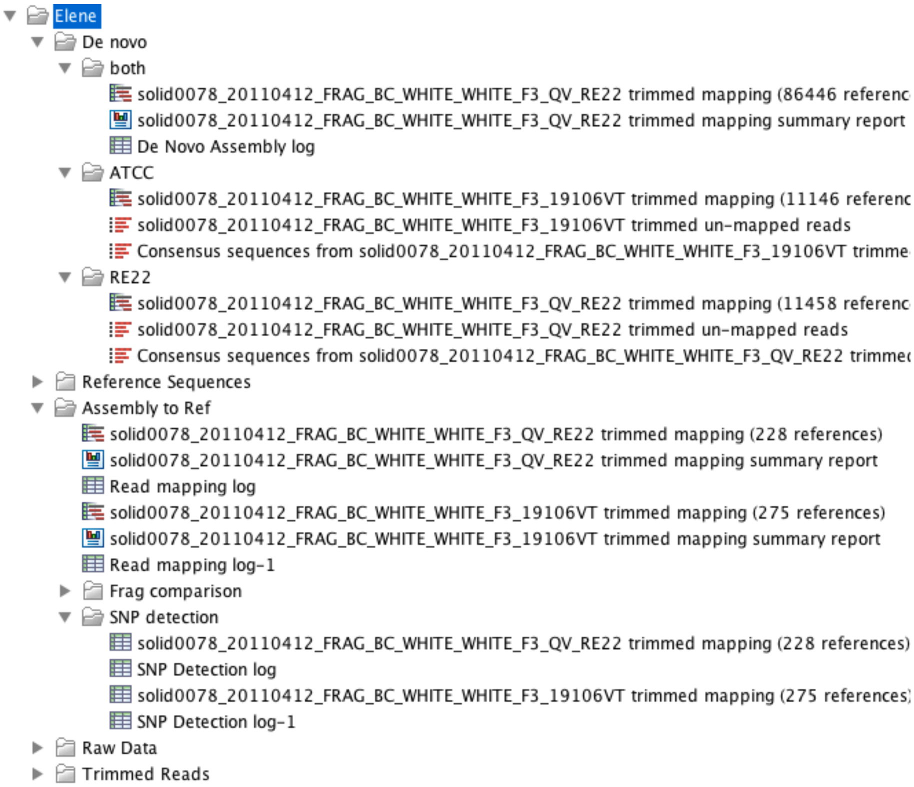
Rows: 37,319 SNP Detection Table Filter:

	Allele Vari...	Frequencies	Counts	Coverage	Overlappi...	Amino Ac
2	G/T	94.4/5.6	847/50	897		
2	A/G	93.2/6.6	673/48	722		
1	A	100.0	4	4	CDS: FOG...	
1	G	80.0	4	5	CDS: Hex...	
1	A	80.0	4	5	CDS: Hex...	Val29Ile
1	C	100.0	6	6	CDS: Hex...	
2	A/T	60.0/40.0	3/2	5	CDS: Hex...	
2	C/T	50.0/50.0	3/3	6	CDS: Hex...	
1	T	91.7	22	24	CDS: Hex...	
1	T	88.9	8	9	CDS: Hex...	
1	C	87.5	7	8	CDS: Hex...	
1	T	91.7	11	12	CDS: Hex...	
1	C	80.0	4	5	CDS: Hex...	
1	T	66.7	4	6	CDS: Hex...	
1	G	93.3	14	15	CDS: Hex...	
1	T	95.8	23	24	CDS: Hex...	
1	T	96.3	26	27	CDS: Hex...	
1	T	100.0	4	4	CDS: Hex...	Ala443Se



1	T	100.0	4	4	CDS: Hex...
1	A	100.0	4	4	CDS: Sens...

Overview of file/folder structure



- Elene
  - De novo
    - both
      - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_QV_RE22 trimmed mapping (86446 referenc
      - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_QV_RE22 trimmed mapping summary report
      - De Novo Assembly log
    - ATCC
      - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_19106VT trimmed mapping (11146 referenc
      - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_19106VT trimmed un-mapped reads
      - Consensus sequences from solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_19106VT trimme
    - RE22
      - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_QV_RE22 trimmed mapping (11458 referenc
      - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_QV_RE22 trimmed un-mapped reads
      - Consensus sequences from solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_QV_RE22 trimmec
  - Reference Sequences
  - Assembly to Ref
    - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_QV_RE22 trimmed mapping (228 references)
    - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_QV_RE22 trimmed mapping summary report
    - Read mapping log
    - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_19106VT trimmed mapping (275 references)
    - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_19106VT trimmed mapping summary report
    - Read mapping log-1
    - Frag comparison
    - SNP detection
      - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_QV_RE22 trimmed mapping (228 references)
      - SNP Detection log
      - solid0078_20110412_FRAG_BC_WHITE_WHITE_F3_19106VT trimmed mapping (275 references)
      - SNP Detection log-1
  - Raw Data
  - Trimmed Reads

5-20-11 LD50 2000ppm (10 day old and D-hinge - 72 hrs)

Inoculation = 1.5 x 10^9 CFU/ml original culture
Dosages = 3.8 x 10^1 - 3.8 x 10^5 CFU/ml
0.01 ml added to 4 ml of SW and larvae in each well.
Two stages of larvae used:

- 10 day old larvae
- D-hinge larvae - 72 hrs old

Autoclaved SW bubbled with ambient air or 2000ppm specialty gas for 24 hrs.
Appropriate water used to clean larvae before aloquoting them into each well (~45 larvae per well)
One temp = 17C
Live/dead larvae counted every 24 hours. Only stationary larvae, at the bottom of the wells were examined every day. At the end of the trial, all larvae were killed by adding bleach and totals per well were counted.
SW pH was measured spectophotometrically every 24 hours after water was initially bubbled.
2 wells from each dosage were sampled to quantify Vt on larvae:
1.5 ml of larvae were pipetted into microcentrifuge tubes, spun at high speed for 10 min.
Supernatant was discarded and samples were flash frozen in liquid nitrogen and stored at -80 until DNA extraction can be performed.

Water chemistry samples were taken for analysis at PMEL.
300 ml bottles were used to take samples after trial was complete (72 hrs)
water was sampled using the same procedure as the Vt pCO2 growth trial (5-4-11)
->**UPDATE** - samples taken for this trial were not taken to PMEL specifications. Samples were tossed and not submitted for analysis.

5-4-11 Vt Growth at 2000ppm pCO2

Same procedure as 3-15-11.
Inoculated cultures at 9am 5-4-11
Spect pH readings performed prior to inoculation

Original culture used for inoculation = 2.0 x 10^9 CFU/ml
25 ul of 1:20 culture was used for inoculating 1000ml of SW of each replicate.
Final inoculation dose = 5.0 x 10^2 CFU/ml

CFU estimations were done via plate counts on T1N2 plates every at 6 hours and 24, 48 and 72 hrs post inoculation.
All DNA samples taken for qPCR will be processed with Qiagen DNeasy kit following manufacturer's instructions.
qPCR protocol will be followed using optimized procedure.

8 samples of seawater sent to PMEL for chemistry analysis:
NOTE to lab: All seawater came from Elliot Bay the week of 4/25/11. Seawater used for this experiment was autoclaved prior to experiment to sterilize. Samples were collected by pouring into sampling bottles carefuly, without bubbles, and overflowed > 2x the volume of the bottles. 200ul of HgCl2 was added to each bottle.

Samples sent:

Internal Sample ID	PMEL Bottle #	Salinity (ppt)	Spectrophotometric pH	Sampling Temp. (C)
12-amb-3	A1	32	7.79	12
12-2000-3	A2	32	7.24	12
12-2000-4	A3	33	7.21	12
12-amb-4	A4	32	7.81	12
25-2000-3	A5	32	7.40	25
25-2000-4	A6	33	7.34	25
25-amb-3	A7	32	7.96	25
25-amb-4	A8	32	7.96	25

UPDATE - 5-31-11 : talked to Simone Alin (PMEL) about discrepancies in the pCO2 data and sample collection techniques. There is some concern about temperature shifts when 12C samples were taken and the process of pouring the water into the bottles. We need to figure out a different method for sample taking without exposing the SW to air when pouring. Suggestions included:
Rigging up a siphon to remove the SW from the replicates in the water bath and filling the sample jars
Doubling the size of the containers and inserting a spiget to the bottom of the flask to sample

The siphon technique may suffice. I need to talk to CF and BV about this option.
Another area of concern is the sampling procedures for the LD50 experiments. This is a tricky one since the chambers are so small.

qPCR RESULTS:
5-10-11_Vt_pCO2-1.pdf
5-11-11_Vt_pCO2-2.pdf
5-12-11_Vt_pCO2-3.pdf
5-12-11_Vt_pCO2-4.pdf

4-27-11 pCO2 LD50 2000ppm

Same procedure as below using only 2000ppm and ambient air.
1 wk old larvae from hatchery
held at 12C
Bubbled gas into two 1L Erlenmeyer flasks for 24 hrs
started Vt culture at 7 pm for a ~12pm inoculation
Using bubbled gas for larvae preparation and bringing up volumes in wells
Ambient air temp -> ~17C

RESULTS:
Original culture used for inoculation was 2.5 x 10^9 CFU/ml
Inoculation dosages were determined to be:
6.25 x 10^4 - 6.25 CFU/ml
Room temperature in the basement all three days - 18C

pH readings:
Inoculation day with 24hrs of bubbling 2000ppm into autoclaved SW : 7.9 pH
Ambient autoclaved SW: 8.5 pH

Plates were placed into the chambers at 12pm 4/27 and piped with corresponding gas (no bubbling).

24 hrs post inoculation:
2000 ppm chamber: 7.7 pH
Ambient chamber: 8.3 pH

48 hours post inoculation:
2000 ppm chamber: 7.56 pH
Ambient chamber: 8.09 pH

72 hours post inoculation:
2000 ppm chamber: 7.55 pH
Ambient chamber: 7.97 pH

4-26-11 pCO2 LD50s

SUMMARY: LD50 challenges of 1 wk old larvae exposed to pCO2 concentrations of 840 and 2000ppm against controls at ambient.

PROCEDURE:
Designer gas used: 2000 and 840 pp, control at ambient
Water will be distributed into open jars in airtight chamber piped with corresponding gas and left to equilibrate for 24 hours
All chambers will be left at RT or ~17C
Vt dosages = 10^2 - 10^6 CFU/ml (5 dosages)

Vt grown as described on 3-29-11
1wk old larvae

6 replicates of each dosage with 6 uninoculated wells at each pCO2 for controls
~40 larvae per well
Larvae will be kept overnight at 14C and rinsed with ambient autoclaved SW before divvying into wells
Wells will be filled with elevated pCO2 water (either 840 or2000) for a final volume of 4 ml in each well
Plates will be transferred to the air chambers and sealed and piped with elevated pCO2 gas for 24 hours

Water pH will be measured using spectrophotometer with m-cresol purple dye
prior to larval input and Vt dosaging
A control jar will be dosed with Vt to simulate pH adjustment with Vt + pCO2 to measure with spect

Larvae will be counted every 24 hours to count live vs. dead in each well.

RESULTS:
pH of SW was not as low as expected after in the chamber for 24 hrs:
2000ppm: 8.04
840ppm: 8.24
ambient: 8.17

The solution may be to bubble the air into larger volumes of SW to place the larvae in, then maintain the pCO2 in the chamber for the duration of the trial. Re-trying with 2000ppm gas.

4-12-11 Linearizing vptA Plasmid DNA

Taking 3 plasmid preps of vtpA and linearizing.
Visualize on gel
Run Antarctic phosphatase reaction
Run three plasmid curves to compare

PROCEDURE:
Run 1/2 reactions due to lower qty of DNA in preps

Digestion reaction:
HALF reaction recipe:
Not1 enzyme 0.25ul
500ng plasmid DNA
NEB Buffer 3 2.5ul
H2O variable
BSA 100ug/ml 0.25ul
Total volume: 25ul

Mix prepared:

Solution	Plas1 - Vol (ul)	Plas2 - Vol (ul)	Plas3 - Vol (ul)
Not1 Enzyme	0.25	0.25	0.25
Plasmid DNA	8.17	6.4	12.75
Neb3 Buff	2.5	2.5	2.5
H20	13.83	15.6	9.25
BSA (100ug/ml)	0.25	0.25	0.25
Total	25	25	25

combine solutions in tube and finger flick
Digest at 37C for 4 hours on a preheated plate warmer, followed by 20 min at 65C

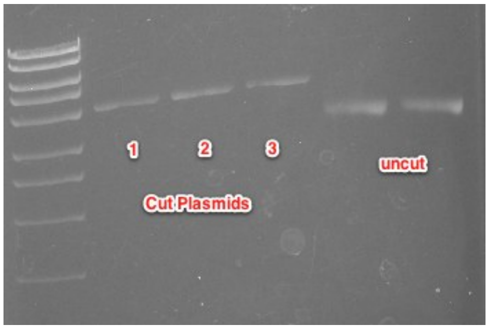
To visualize gel:
SMALL GEL with wide combs
1% agarose gel with 8ul of EtBr
Load gel with each 2ul cut plasmid + 2ul loading buffer per well
Load at least 1 lane of 2ul uncut plasmid dilute to 1:3 to approximately 20ng/ul + 2 ul loading buffer
5ul Hyperladder 1
Run out gel for 2 hours at 120volts

Antarctic phosphatase HALF rxn (500ng DNA):
EACH REACTION:
AP 0.2ul
DNA whatever's left ul
AP rxn buffer 3ul
water 3.4ul

Incubate at 37C for 30 min on preheated plate warmer, followed by 65C for 5 min to inactivate enzyme
Aliquot half of this (~13ul) into another tube and store at -20C for future use.

Quant the other half of the tube and use to make plasmid curve.

RESULTS:



There is a noticeable difference between cut and uncut plasmids on gel.
Next step is AP reaction.

DNA Quanting Results:
vtpA plasmid 1: 44ng/ul
vtpA plasmid 2: 39 ng/ul
vtpA plasmid 3: 41ng/ul

Diluted all plasmids 1:1 for larger pipetting volumes for future plasmid curve dilutions.
Reserved back-stock of plasmids (20ul volume of each) in the -20 240 freezer (4-14-11).
Next step - test sensitivity of plasmid curves. Gharabeh et al. 2008 was only able to detect down to 30 Vt copies.

4-11-11 Making m-cresol purple pH indicator dye

SUMMARY: Concentration: 2mM/L MCP with adjusted pH of 8.0 and approx A1/A2 of 2.1

Added 0.20 g of MCP salt into 250mL of nanopure water with stir rod
Stir for ~15 min
Get initial reading of MCP pH with spec and adjust with NaOH and HCl for a final pH of ~8.0
On 3/8/11 I used this procedure (in drops) for a final pH of 8.1 (A1/A2 2.15)

mcp+1 NaOH (5M)
mcp+2 HCl (1 N)
mcp+ 1HCl (0.5N)
mcp+1HCl (0.5N)
mcp+1HCl (0.5N)

After pH is determined, measurements will be performed to characterize dye to calculate regression curve for this batch of dye
RESULTS:
Added 1 drop 5M NaOH and 2 drops of HCl to get 8.09pH and A1/A2 of 2.03.
Next step - characterization.

4-11-11 Cloning 16S of RE22 and ATCC 19106

Universal primers used to clone:
B27F - AGA GTT TGA TCC TGG CTC AG
U1492R - GGT TAC CTT GTT ACG ACT T
Ref: Universal Bacterial Identification by PCR and DNA Sequencing of 16S rRNA Gene. PCR for Clinical Microbiology, 2010, Part 3, 209-214

PROCEDURE:
Run PCR using universal primers (B27F and U1492R) to amplify strains:

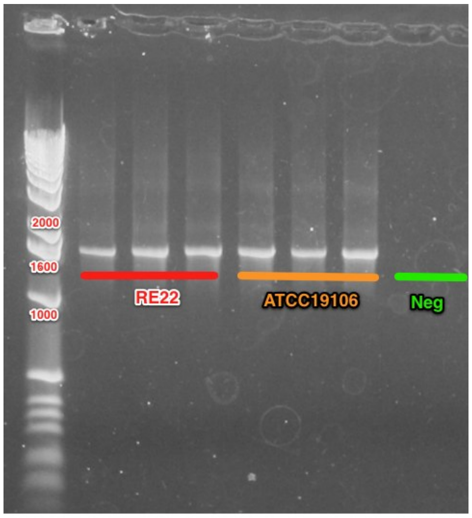
	Vol (ul) per rxn	1. rxns =	9
PCR H20	12.75		114.75
5xBuffer	5.00		45.00
MgCl2	2.00		18.00
BSA	1.00		9.00
dNTPs	0.50		4.50
F Primer (10mM)	0.75		6.75
R Primer (10mM)	0.75		6.75
Taq	0.25		2.25
Template	2.00		Add individually

Thermocycler : Vt-16S in main menu

Strains to clone:
RE22
ATCC 19106

Run on gel to verify ~1500 bp size amplicons of each strain
Purify PCR products of each strain
Clone purified PCR products using method described 3/22 (cloning 1/2 reactions of each strain)
Grow 10 clones of each strain in 5ml LB broth +25ul of ampicillin 200x
Purify all plasmids using Qiagen miniprep kit
Quant clones
(10 plasmids per strain)
Send off for sequencing
200ng/ul of plasmid for sequencing (10ul volume of sample per well)

RESULTS:
Original PCR product (4-11-11):



Product size approx 1500 bp in length.
10 colonies from each strain picked to PCR for product:

Robyn have these sequences done in her MS thesis. Same strains are used. I will develop primers from RE22 16S and run against some RE22 samples to double check. Otherwise, done here.

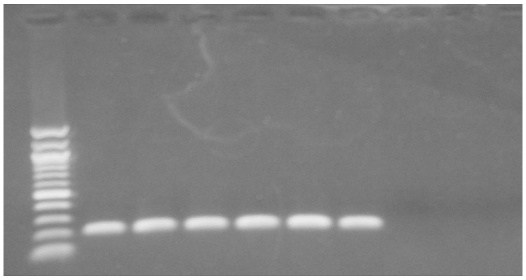
4-8-11 Sequencing 16S rRNA strains

Ran Vt 16S PCR on RE22 and 19106 testing the primers developed by Robert's lab:
F - CAGCCACACTGGAACTGAGA
R - GTTAGCCGGTGCTTCTTCTG

Thermocyler: Vt-16S in main menu

95 for 10 min	
95 for 15 sec	
55 for 1 min	} 35 - 40 cycles
72 for 30 sec	
72 for 10 min	
22 for 10 min	

1% agarose gel, 90v for 40 min:



Lanes 1-3 = RE22; Lanes 4-6 = ATCC 19106 (Vt); Lane 7 = neg control
200bp length as expected.

4-5-11 Prelim LD50 - try 2

First experiment a bust with total larval mortality at 48hrs. Try again, new batch of larvae.
Adjusted the Vt dosages a bit aiming for - 10^6 - 10^2
2 week old larvae

4/4 -
~40 larvae per well, 6 replicates for each dosage and 6 control wells on a separate tissue plate.
2wk old larvae
Inoculated within 24hrs of arrival at UW
Held static for ~72 hrs and larvae live vs. dead to be determined at 24, 48, and 72 hrs.
No water changes
RT for the trial, ambient air.
Larvae was placed into the tissue plates using the method below. Larvae tank was not changed with sterile SW prior to adding larvae into the wells.
Concerned about not placing the larvae in sterile SW prior to experiments.
Determined sterility of Trial 2 experimental set up without changing the water of larvae.
I plated water from the main tank in the basement, loop of control larvae, and water from the larvae tank onto T1N2 agar plates and incubated the plates at 30C overnight to determine bacterial load.
Original culture: 1.7 x 10^9 CFU/ml
Final inoculation dosages = 4.25 x 10^1; 4.25 x 10^2, 4.25 x 10^3, 4.25 x 10^4, 4.25 x 10^5 CFU/ml

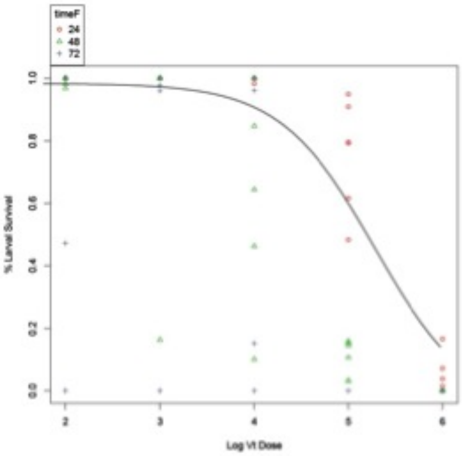
I inoculated a new batch of broth with Vt to start another experiment on Tuesday with the same larvae.

Try 3:
larvae arrived Sunday 4/3, inoculated on tuesday 4/5. Rinsed larvae with sterile SW in 2 changes of water. Larvae was set on a 100um screen with ~1" of SW and removed with sterile transfer pipet. Approx 40 larvae were placed into each well plate. Volume was brought up to 4 ml with sterile SW.
Larvae inoculated with Vt culture grown ~17hrs at RT.
3.0 x 10^9 CFU/ml original culture
Final inoculation dosages = 7.5 x 10^1; 7.5 x 10^2, 7.5 x 10^3, 7.5 x 10^4, 7.5 x 10^5 CFU/ml

After experiment is terminated, larvae will be taken from 3-4 wells to extract and quantify Vt off larvae.
Wells sampled for DNA extraction: A1, B1, B3
Larvae were placed into microcetrifuge tube
Spun at 13000rpm for 1 min
Supernatant extracted before performing DNA extraction
DNA extracted by Qiagen blood and tissue kit
Stored at -20C

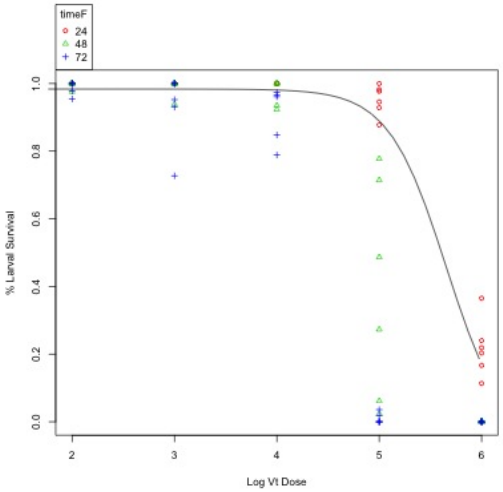
RESULTS:
From 4/4 experimental set-up:
Plates determining bacterial contamination:
Main tank (UV sterilized and jacuzzi filtered) - lots of bacteria
Control larvae of the unchanged water experiment (4/4) - two colonies of bacteria
Larvae holding tank - NG

Trial 2:



LD50 dose:
2.2 x 10^5 CFU/ml incorporating all data and time points

Trial 3:



LD50 dose:
4.3 x 10^5 CFU/ml incorporating all data and time points

3-29-11 Preliminary Larvae LD50

Testing out experimental design of our LD50 experiments with elevated pCO2 at ambient conditions.

PROCEDURE:
(Didn't have time for streak plate and pick colony)
Frozen isolate used to inoculate 25ml 0.25% tryptone and SW media
Overnight shaking at RT
In morning, dilute original culture in 1:10 serial dilutions to estimate CFU/ml and inoculation dosages
2 wk old gigas larvae used from hatchery
Rinsed with 150 um filter with sterile SW and placed in aerated FSW overnight at 14C.

For experiment:
Used 12-well sterile tissue culture plates
5 inoculation dosages of Vt: 10^6, 10^5, 10^4, 10^3, 10^2 + control
6 replicate wells for each inoculation dose
Control placed into a separate plate with lid
~35-40 larvae placed into each well and brought up to 4 ml of SW, with sterile SW
To inoculate:
used 10ul of 1:10 dilutions and original culture for inoculation dosages
left at RT and dead/live larvae will be counted with inverted scope at 24 and 48 hours.

RESULTS:
original culture - 4.1 x 10^9 CFU/ml
Estimates of inoculation dosages were correct - 1 x 10^6, 1 x 10^5, 1 x 10^4, 1 x 10^3, 1 x 10^2
Counting of the larvae were performed under an inverted scope. Dead larvae were viewed under 40x objective and determined to be dead when no ciliary movement was detectable. Swimming larvae were not counted and at the end of the experiment all larvae were killed with EtOH and counted for total number of larvae per well.

48 hrs after inoculation all control larvae died. Ended the experiment there.
Emma will get more larvae from the hatchery sunday and will start again on Monday morning.

3-22-11 Cloning vtpA gene

SUMMARY: vtpA clones to use for standard curve on qPCR

PROCEDURE:
It's been awhile since I've run a cPCR and I forget which method was better so I tried 3:
Instagene DNA extraction of RE22 with this recipe (#1):

	Vol (ul) per rxn	1. rxns =	10
PCR H20	4.75		47.50
5xBuffer	5.00		50.00
MgCl2	2.00		20.00
BSA	1.00		10.00

dNTPs	0.50		5.00
F Primer (10mM)	0.75		7.50
R Primer (10mM)	0.75		7.50
Taq	0.25		2.50
Template	10.00		Add individually
TOTAL	25.00		

95 for 3 min	
95 for 15 sec	
55 for 1 min	} 35 - 40 cycles
72 for 30 sec	
72 for 10 min	
22 for 10 min	

Qiagen extraction of RE22 performed ~2wks ago with 2 ul template added (#2):

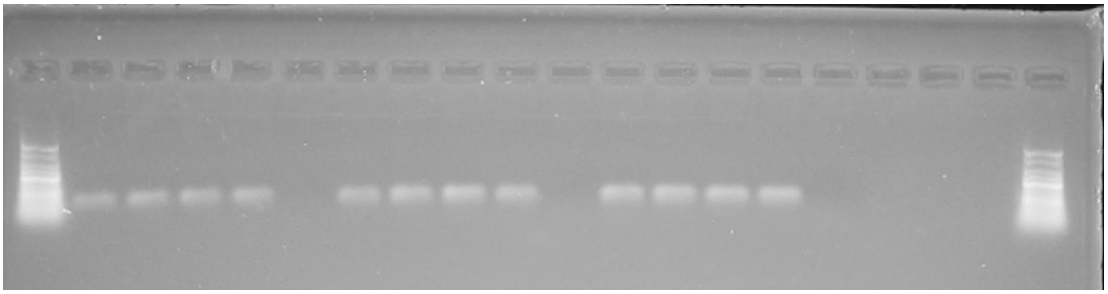
	Vol (ul) per rxn	1. rxns =	10
PCR H20	12.75		127.50
5xBuffer	5.00		50.00
MgCl2	2.00		20.00
BSA	1.00		10.00
dNTPs	0.50		5.00
F Primer (10mM)	0.75		7.50
R Primer (10mM)	0.75		7.50
Taq	0.25		2.50
Template	2.00		Add individually
TOTAL	25.00		

95 for 3 min	
95 for 15 sec	
60 for 1 min	} 35 - 40 cycles
72 for 30 sec	
72 for 10 min	
22 for 10 min	

And the last round used the same recipe above with the Qiagen RE22 DNA but lowered the temp to 55C (#3).

Made LB plates 3/22 left overnight to cool.
Will produce gel of the vtpA products in the morning to see how they look.

RESULTS:
Ended up using PCR product from #2 (Qiagen DNA, 60C annealing) to clone.
Picture of reactions below. All produced a band of correct size - ~63 bp. Ladder is Hyperladder V with smallest band of 25 bp.



CLONING PROCEDURE:
Purified 70ul of PCR product with Qiagen PCR purification kit following manufacturer's instructions.
Stored product in freezer overnight.

Vector used: pCR2.1-TOPO, 3931 bp
· Add 2µl purified PCR product, 0.5µl salt solution, and 0.5µl TOPO vector into a PCR tube
· Mix gently by flicking tube and giving it a brief spin down
· Incubate reaction at room temp for 10 min
After 10 min, place the reaction on ice.
· Thaw **on ice** 1 vial of Top10 *E coli* cells (stored in -80°C freezer) for 5 min

· Thaw **on ice** 1 vial of Top10 *E.coli* cells (stored in -80°C freezer) for 5 min.
· Add 1µl of TOPO cloning reaction (from part A) to ½ vial (25µl) of the *E. coli* cells.
(Refreeze remaining E. coli cells in -80°C and freeze remaining cloning reaction in -20°C)
· Mix gently by flicking tube and giving it a brief spin down
· Incubate on ice for 15 min
· Heat shock cells @ 42°C for 30 sec
· IMMEDIATELY place cells back on ice for 2 min
· Add 125µl of room temp SOC medium (check chemical shelf in 240)
· Cap tube tightly and incubate @ 37°C on shaking platform for 1 h
· Warm LB-amp plates for 30 min @ 37°C
· After the cells have incubated for an hour, spread 30-50µl of product on prewarmed LB-amp plates.
--> 3 plates of 30ul, 1 plate of 50ul product

Incubate plates overnight @ 37°C

3-25-11

· Prewarm more LB-amp culture plates @ 37°C for 30 min for growing single colonies (patch plate)

· Take your plates you incubated overnight which now should have lots of colonies on them and using a pipette tip pick only one colony off of each plate, streak the tip to the fresh patch plate.
· Grow these single colony cultures overnight at 37°C.

PCR the 25 colonies used on patch plate grown to see if the product cloned correctly:

MM Recipe:

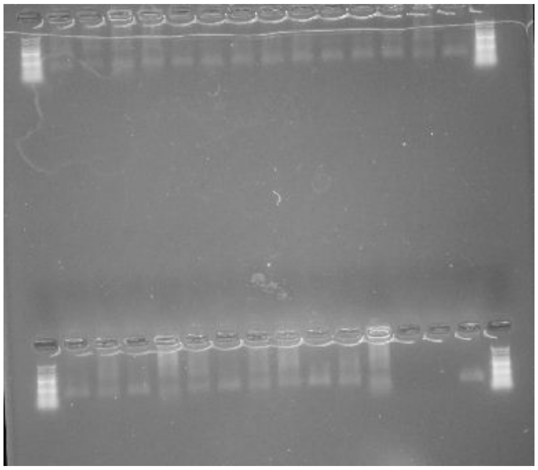
qPCR Protocol SYBR		1. rxns =	30
Reagent	ul		
Immomix (2x)	12.5		375
F Primer vtpA (10uM)	0.8		24
R Primer vtpA (10uM)	0.8		24
BSA	1.5		45
	0		0
MgCl2 (50mM)	0.5		15
PCR water	8.9		267
Template	picked		Add Individ
TOTAL	25.0		

(2 neg control and 1 pos control used)

Thermocycler Protocol:
95C for 10min
35 cycles of:
95C for 10 sec
55 for 15 sec
72 for 10 sec
final extension
72 for 10 min

Run on 2.5% agarose gel with Hyperladder V ladder
90 volts for 15 min.

RESULTS:



Each lane represents a picked colony 1 - 25. The last three lanes represent two negative controls and one positive control at the right.
Colonies 5 - 12, 14, 15, 17, 20, and 23 look like good selections to grow up further.
I will pick four of these re-PCR with a better recipe to double check bands and I will inoculate in 5ml LB broth and miniprep.

3-27-11:

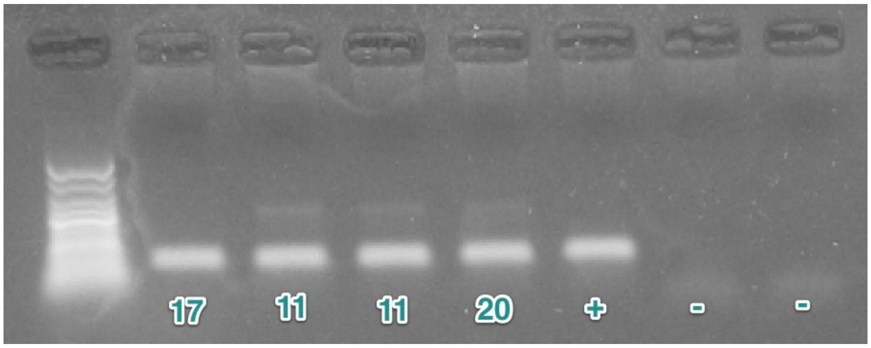
Prepare a 15ml falcon tube with 5ml of LB liquid media and 25µl of 200X stock ampicillin in duplicate.
make 2 tubes for every single colony to culture (one to purify and one for long term storage)
1 LB tube as a blank control.

Cultures failed to grow overnight.
Re-inoculated the cultures from the patch plate using pipet tips this time.
Overnight at 37C.

Re-PCR of the 4 colonies I picked from on the patch plate: 11,14, 17, and 20 with positive and negative controls

	Vol (ul) per rxn	1. rxns =	7
PCR H20	14.75		103.25
5xBuffer	5.00		35.00
MgCl2	2.00		14.00
BSA	1.00		7.00
dNTPs	0.50		3.50
F Primer (10mM)	0.75		5.25
R Primer (10mM)	0.75		5.25
Taq	0.25		1.75
Template	0.00		Add individually
TOTAL	25.00		

Used same thermocycler protocol as the previous week.
RESULTS:



Colony 11: I halved the culture and used 3 ml of each to miniprep. 2 ml was set aside with ~20% glycerol and stored in -80C.
Colony 17: I used 2ml to miniprep and 1 ml to store in 20% glycerol.

- Parafilm and store multiple colony plates in the fridge (4°C)
- Prepare your master mix following your original PCR recipe except substitute 2µl of water in place of your product.
- Aliquot 25µl of your master mix into PCR tubes. Make enough reactions for each colony and a negative control.
- Take your single colony culture and using a pipette tip touch your colony and dip the tip into your PCR tube.
- Run your PCR on the thermocycler and then visualize on a gel to make sure your product is the correct size.
- Parafilm single colony LB-amp plates and store in the fridge (4°C)

- Add 880µl of glycerol to 1 set of cultures in your falcon tubes
- Store at -80°C
- Centrifuge the remaining falcon tube for 10 min @ 2500rpms @ 4°C to pellet cells.
- Pour off LB-amp media
- Followed the Qiagen protocol on pg 22 of the Qiaprep Spin Mini Prep Kit to purify the plasmid

	Nanodrop	Calculated ng/ul =
Unknown	Average ng/ul read	(x + 2.8247)/0.8662
Colony 11	34.59	**43.19406604**
Colony 17	7.83	**12.30050797**

clone 17 ng/ul = 61.23

3-15-11 Elevated pCO2 Vt Growth Trial

Vt Growth curves at ambient and 840ppm CO2 at 2 temperatures: 12 and 25C

PROCEDURE:
Waterbaths used to control temps
Experiment done in triplicate at ea temp and pCO2 level
Control, uninoculated flasks at ea temp and pCO2 level
Sample pH of starting SW before bubbling
Bubble elevated pCO2 or ambient air into respective flasks starting at 9:30am 3/15 for ~24 hrs
streak plate from RE22 frozen isolate, grow out overnight at 30C
inoculate 25 ml 0.25% tryp + SW broth at 3pm 3/15 with isolated colony
grow broth culture in duplicate for 17hrs at RT on shaker

3/16 8 am:
Spect pH of all flasks prior to inoculation
At 9am 3/16 take culture and dilute 1:20
Inoculate each experimental flask with 25ul of diluted culture to achieve ~5 x 10^2 CFU/ml start.
Plate original culture to estimate starting culture
Take 1ml from each flask and place into microfuge tube
Spin for 10 min at full speed
Take off supernatant and flash freeze pellets
Dilute 1ml sample from each flask 1:10 serial dilutions and plate at 6hrs, 12 hrs, 24 hrs and 48 hrs
Spect and check salinity prior to inoculation, 24 hrs, 48 hrs and after experiment is complete

RESULTS:
Beginning pH before bubbling air: 7.9pH
Salinity: 30 ppt

3-8-11 Equilibration experiment and characterization of *m*-cresol purple dye

Need time to equilibration for pCO2 experiments. using 2000ppm CO2 designer gas

PROCEDURE:
Autoclaved SW (as similar to experimental set up as possible)
1000ml poured into 1000ml Erlenmeyer flasks, topped with rubber cork, with gas input and output holes fitted with 1ml serological pipettes
Duplicate flasks used
Took 2 measurements of original SW
Placed both flasks into 25C water bath and bubbled 2000ppm into both flasks at a rate of approx 1 bubble/second
Measured pH of SW intermittently with spectrophotometer using m-cresol purple (mcp) indicator dye
Dye was made the day before the experiment with a pH of ~8.1 and A1/A2 of 2.3 nm
To characterize the new dye, a regression line with a double measurement of mcp into the cuvette
A range of SW pH is necessary to capture the range of pH values for the regression
Values were paste into the "Dye Calibration" worksheet provided by PEML NOAA

RESULTS:
Turns out the older autoclaved SW I've used for the first experiment is lower in pH (~7.6) and ended up around 7.4ish at the end of 8hrs. I will try the equilibration experiment again on 3/9 with freshly autoclaved SW ~8.5 pH to see how long this will take to get down with the 2000ppm CO2.

~17 hrs after autoclaving: 8.41
Bubbling 2000ppm at ~1 bubble/sec...
2 hrs: 8.27
5h30m: 8.06
10h: 7.84
22h: 7.61

Conclusion - after 24hrs of bubbling, pH is still not down to the approx 7.4 pH level although pH started very high after autoclaving. I will bubble the 840ppm (expected to be approx 7.8 pH) for 24 hrs before starting experiment.

3-2-11 Verification of inoculation dose for pCO2 experiments

Aiming for 10^2 CFU/ml dosage for experiment
Double checking inoculation procedure and CFU estimation:

PROCEDURE:
Freezer isolate streaked onto T1N2 plate
Picked isolated colony and grew up in 0.25% trypt+SW broth for ~17 hrs
Diluted culture 1:10 in serial dilutions
Plated duplicate 10^-5 - 10^-7 dilutions on T1N2 plates, grew overnight at 30C

Estimated culture concentration next morning

Performed procedure above again
After serial dilutions and plating as described, I inoculated 100ml SW with 25ul of 1:20 dilution of culture
Plated 10^0 and 10^-1 of the new SW culture
Grew all plates at 30C overnight

RESULTS:
DAY 1:
average 126 colonies from -6 dilution from original culture
original culture 1.26 x 10^9 CFU/ml

DAY 2:
average 102 colonies from -6 dilution of original culture
average 15 colonies from 10^0 SW culture

original culture 1.02 x 10^9 CFU/ml
1000ml SW media 1.5 x 10^2 CFU/ml

Estimations look good and are right on. Will inoculate experimental flasks of 1000ml SW with 25ul of 1:20 culture grown for 17hrs from picked colony to achieve 10^2 CFU/ml

2-8-11: DNA prep for sequencing

RE22 and ATCC 19106

Frozen isolates of each strain streaked onto plates
24hrs at 28C incubation
Picked isolated colony and inoculated 1% tryptone and SW broth at 10am
Approx 22hrs growth sample 1.5 ml from each culture and extract DNA with DNeasy kit
Store at -20C until sequencing.

1-25-11 - DNA extraction / SW sampling methodology testing

SUMMARY: Mini growth experiment to:
1. see if spinning SW samples down in the same tube (1ml) will have a stronger correlation with physical plate counts
2. test DNA extraction methods and DNA yields of 3 methods: DNAzol, Heat/lysis (WS method), Qiagen DNeasy kit with 2 methods: 0.22um PES filter + syringe and spin down 1 ml sample

PROCEDURE:
Inoculate loopful of overnight culture into 25 ml trypt+SW media
Grow for 17hrs
Serial dilutions and plate for estimation of 17hr CFU/ml
3 standard curves made to extract with - WS method, Qiagen Dneasy kit, DNazol
Standard curves for each method include this range:
0 - 400000000
-1 - 40000000
-2 - 4000000
-3 - 400000
-4 - 40000
-5 - 4000
-6 - 400
-7- 40
1:2 - 20
1:2 - 10
1:2 - 5

Inoculate 1000ml autoclaved SW with 25ul of original culture.
Stir bar to aerate, 18C incubation
Actual Inoculation = 1.0 x 10^4 CFU/ml

To sample:
Serial dilutions will be performed to estimate CFU by plate counts on T1N2
1 ml sample of growth culture will be placed into fuge tube and spun at full speed for 10 min to pellet, supernatant discarded, into -80C
1 ml sample will be filtered through millipore 0.22um PES filter attached to a syringe, steriley placed into a microtube and placed into -80C
Samples taken x3 to test DNA extraction methods
Samples will be taken at:
2 hrs
5 hrs
7 hrs
24 hrs
27 hrs

DNA EXTRACTION METHODS:
WS heat/lysis method
See 8/30/10

Qiagen DNeasy kit
Manuf instructions except following modifications:
56C for 1 hr (step 2)
200ul AE buffer incubate at RT for 5 min

DNAzol
Manuf instructions with following modifications:
Step 3 - no spooling, centrifuge sample at 5000rpm for 5 min at -4C
Step 5 - use 200ul of 8mM NaOH (8.0 pH) to store DNA

RESULTS:
T1N2 plate counts:
Original culture: 3.9 x 10^8 CFU/ml
2 hrs = 9.3 x 10^3
5 hrs = 8.6 x 10^3
7 hrs = 1.7 x 10^4
24 hrs = 1.4 x 10^5
27 hrs = 1.75 x 10^5

qPCR results for Qiagen extracted templates comparing
1. filter sampling and spin sampling CFU estimates
2. qPCR estimates to physical plate counts (above)
Report here:1-27-11 - SW and plate count correlation.pdf
Std Crv used = 50,000,000 - 50 cells (extracted 6-28)

Plate Counts	Qiagen ng/ul	Heat Lysis	DNAzol
10^8 CFU/ml	29.9804	17.225	0.1465
10^7 CFU/ml	2.1428	0.5727	NA

Conclusion - looks like samples are too small to be detected on the spect.
Any sample less than 10^7 CFU/ml did not read by quanting. Looks like quiagen is winning the extraction game.

qPCR results for WS method:
1-27-11_Vt_method_test_WS_heat-lysis.pdf
Std Cv used = 400000000 - 400 cells

qPCR results for DNAzol
1-28-11- Vt_method_test_DNAzol.pdf
Std Cv used = 400000000 - 400 cells (used original culture, extracted DNA with DNAzol and serially diluted 1:10 for standard curve)

1-19-11 - Re-run 12C curve qPCR

SUMMARY: Efficiency was high (120%) on the 12C Vt plate performed on 1-14 with pink original curve from 6-28. Re-run plate using same protocol as 1-14.
-1 = 50,000,000
-2 = 5,000,000
-3 = 500,000
-4 = 50,000
-5 = 5000
-6 = 500
-7 = 50
-8 = 5

RESULTS:1-19-11_Vt_12_re-run.pdf

1-18-11 - Vt Baseline 18C Growth vs. Ct

Same procedure as 1-14-11 to run unknowns. Used older curve (pink from 6-28-10):
-1 = 50,000,000
-2 = 5,000,000
-3 = 500,000
-4 = 50,000
-5 = 5000
-6 = 500
-7 = 50
-8 = 5

Results:
1-18-11_Vt_Base_18C_curve.pdf

1-14-11 - Vt Baseline 12C Growth vs Ct using new low curve

SUMMARY - using curved developed on 1/12/11 to re-qPCR the seawater samples taken from the 12C growth curve.

PROCEDURE:

Used MM optimized Vt qPCR protocol:

qPCR Protocol SYBR		1. rxns =	105
Reagent	ul		
Immomix (2x)	12.5		1312.5
F Primer (10uM)	0.8		84
R Primer (10uM)	0.8		84
BSA	1.5		157.5
SYBR	0.5		52.5
MgCl2 (50mM)	0.5		52.5
PCR water	7.4		777
Template	1.00		Add Individ
TOTAL	25.0		

Vt_trial2 parameters on desktop.

RESULTS:
Full report here:
1-13-11_Vt_12C_compare_new_curve.pdf

Correlation graph did not change much.
R2 = 0.69
I realize that the lowest plate counts I have are ~10^4 meaning low curve is not the problem.
I am pretty confident about the higher numbers in the curve, meaning that plate counts may be a better estimate of Vt growth.

1/12/11 - New standard curve for RE22 qPCR

SUMMARY: Making new standard curve with more low standards
PROCEDURE:
Took loopful of 24 hour culture and inoculated 25 ml 0.5% trypt and SW media (4pm)
Incubated culture at RT for 17 hours on shaker (approximately 5.1 x 10^8 CFU/ml)
Vortexed culture and performed serial dilutions to make new curve (10am):
~ 500000
50000
5000
500
50
25
12
3
cells/mL

Plated each dilution to eyeball cell count in the morning.
Plated dilutions to estimate original CFU/ml.
Took 1.5ml of each dilution and placed into microcentrifuge tube
Spun for 10 min at full speed
Discarded supernatant
Immediately started Qiagen DNeasy blood and tissue kit following manufacturer's instructions

Stored curve at -20C until qPCR analysis.
Will use the curve to re-quantify original 12C Vt growth curve and compare to plate counts.

RESULTS:
From plate counts (x3) - estimated CFU/ml = 4.5 x 10^8
Above calculations are almost accurate.
The correct curve:
3
11
22
45
450
4500
45000
450000

12/28/10 - Comparing growth of two Vt strains: RE22 and ATCC 19106

SUMMARY: Abbreviated growth comparison between the two strains to compare growth at 18C.

PROCEDURE: New culture from freezer of both strains started 12/22 and left until 12/27 at RT on a rocker in 25 ml 0.25% tryptone and SW

broth.
Inoculated new 25ml broth with loopful of old culture and left at RT on rocker for 24hrs
After 24 hours, inoculated 1000ml autoclaved SW with 100ul of culture - one with RE22 and one with ATCC19106
Incubate at 18C for approx 60 hrs
Diluted and plated the original cultures to get inoculation dose (I am guessing approx 1.0 x 10 ^ 4CFU/ml)
Used T1N2 plates with 100ul of dilution grown overnight at ~28C
Aliquoted 10ml from each flask into conical tubes and spun at 3600rpm for 25 min.
Removed supernatant and transferred to microcentrifuge tube and spun at full speed for 5 min.
Removed supernatant and froze at -80C for qPCR.
10ml pellets will be saved for qPCR/plate comparisons and new standard curve at each time point.
Aiming for 5-6 timepoints

RESULTS:
Inoculation dose at T0:
19106 = 1.4 x 10^5 CFU/ml
RE22 = 2.14 x 10^5 CFU/ml

T1 (6 hrs):
RE22: 2.35 x 10^5
19106: 1.14 x 10^5

T2 (25 hrs):
RE22:1.14 x 10^6
19106: 1.06 x 10^6

T3 (31 hrs):
RE22: 9.5 x 10^5
19106: 1.21 x 10^6

T4 (48 hrs): NG on plates
RE22:
19106:

T5 (54 hrs): NG on plates
RE22:
19106:

9/16/10 - Vt Disease Challenge with NOAA larvae

SUMMARY: NOAA strip spawned oysters were used in a Vt disease challenge. Estimation of dosage is needed for future experiments and we may gain some mortality data.

PROCEDURE:
Oysters were spawned at NOAA on 9/13, 11am and kept static at 4 pCO2 levels: 280, 380, 750, and 2000ppm for 24 hours at 20C
Six replicates were used for each ppm
After 24 hrs, water was changed using a 20um mesh filter and gently rinsed.
Larvae were then placed on a flow thru system for 24 hours
On day 2, one replicate from each pCO2 level was taken to UW and kept static at 20C with a closed container
The next morning (day 3 - 72 hours old) larvae were homogenized (by plunger) and split into 3 containers containing approx 1600ml each pCO2 and 2/3 containers were inoculated with 205 x 10^4 CFU/ml of a 48 hour Vt culture (RT on shaker for 48 hours in 25 ml 0.25% tryptone and SW media)
One container from each pCO2 level was left un-inoculated to act as a negative control

Vt Preparation:
25 ml 0.25% tryptone and SW media was inoculated with RE22 from freezer stock
Rt for 48 hours on shaker
Diluted 1:10

[Take the original 48 hr culture and dilute 1:10.
Use this dilution and place 1.0 ml into each 10^4 tank.
(1.0ml)(4.0 x 10^7) = 1600ml (x)
x = 2.5 x 10^4]

Used 1 ml of this dilution to inoculate each of the experimentals.

All containers were incubated at 20C for 48 hours.
Samples will be taken at 24 and 48 hrs:
Containers will be homogenized by plunging the larvae and 800ml will be poured into a 50um screen to collect the larvae.
Larvae will be placed in a microcentrifuge tube for RNA isolation
400ml will be filtered onto a 0.2um vacuum filter and flash frozen in liquid nitrogen and used for abundance data
400ml will be filtered the same way and flash frozen to preserve for RNA and gene expression analysis
All samples will be kept at -80C until extraction

RESULTS:

RESULTS:
All larvae dead at 24h. CILIATES.

9/9/10 -RT-qPCR of VtpR

SUMMARY: Using 16S primers as a normalizing gene, perform RT-qPCR to analyze VtpR gene expression.
PROCEDURE:

		1. rxns =	52
Reagent	ul		
Immomix (2x)	12.5		650
F Primer (10uM)	0.8		41.6
R Primer (10uM)	0.8		41.6
BSA	1.5		78
SYBR	0.5		26
PCR water	7.9		410.8
Template	1.00		Add Individ
TOTAL	25.0		

Thermocycling parameters: "Vt_Trial" in Desktop folder

RESULTS:

9/7/10 - Reverse transcription of RNA samples

PROCEDURE:
Use M-MLV protocol on Genefish

Use lowest concentration of RNA sample and normalize all samples to the max amt (17.75ul) (if under 1ng RNA total vol) in PCR tubes
For these samples, all were normalized to 0.00726 ug (7.26 ng)/ul or 0.0726 ng total in 10ul.
A ratio of 0.25ug of Random Primers to 1 ug RNA were added:
For these samples 0.0729ug total x 0.25 ug = 0.018225 ug needed
0.018225/0.5 ug = 0.03645 ul per reaction
Dilute Random primers (500ug/ml) 1:100 and add 3.6ul into each tube
Add 4.65 ul of nuclease free water to bring up volume to 18.25ul in each tube

or -
0.5 ul Primers/1 ug = x/0.0726 ug RNA
x = 0.0363 ul of primer
Dilute primers 1:100 to add 3.63 ul into each reaction

Incubate the samples at 70C for 5 min
Ice the samples immediately after incubating

Mix the master mix:
Per reaction:
5 ul 5x M-MLV RT Buffer
1.25 ul 10mM dNTPs
0.5 ul M-MLV Reverse Transcriptase

For 35 reactions:

RT Master Mix	per rxn	rxns needed:	38
5x Buffer (M-MLV RT Buffer)	5.00		190.00
dNTPs (10mM total)	1.25		47.50
M-MLV transcriptase	0.05	(per 100ng RNA)	1.90
Nuclease free water	0.45		17.10
Total	6.75		

After adding MM, mix well (no vortex) and spin down
incubate at 37C for random primers for 1 hour
Heat inactivate at 95C for 3min
Spot spin
Store at -20C

9/3/10 - qPCR FHL Samples for Vt Abundance

SUMMARY: Run DNA extractions from FHL disease trial for abundance estimation throughout the trials using qPCR.

PROCEDURE:
Master Mix Recipe:(NOTE - addition of BSA reagent into mix)

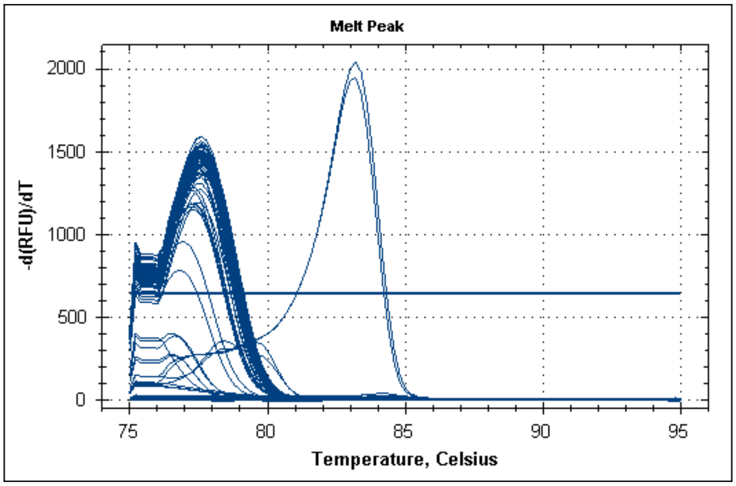
	per rxn	1. rxns =	70
Reagent	ul		
Immomix (2x)	12.5		875
F Primer (10uM)	0.8		56
R Primer (10uM)	0.8		56
BSA	1.5		105
SYBR	0.5		35
MgCl2 (50mM)	0.5		35
PCR water	7.4		518
Template	1.00		Add Individ
TOTAL	25.0		

Using vtpA metalloprotease primers.

Vt_Trial protocol for thermocycling

RESULTS:
Full report here: [9-3-10_FHL_Vt_Abund.pdf]()

16S - version 1 primers worked well and can be used as normalizing gene for gene expression:



9/1/10 - DNase and re-qPCR

Pick out the samples with genomic amplification on qPCR and DNase with Ambion kit.

PROCEDURE:
Add to PCR tube:
2.5 ul DNase Buffer
1 ul turbo DNase
20.5 ul RNA sample
TOTAL - 24 ul

Incubate samples for 30min at 37C
After 30 min, add 1 ul of turbo DNase
Incubate samples for 30min at 37C
After 30 min add 2.5 ul Inactivation Reagent
Leave at RT for 2 min, mixing occasionally
Spin down at 10000 ref for 1.5 min
Transfer supernatant to new tube.
Quant and normalize to the lowest RNA value.

Re qPCR these samples using the same protocol as 9-1-10 to see any leftover genomic carryover. I diluted the RNA 1:4 for PCR template.

RESULTS:

Only the pre-trial samples (R1 and R2) contained genomic carryover. I will re-DNase these samples and PCR them.

9/1/10 - Testing for genomic carryover in RNA samples

SUMMARY: RNA extraction of all samples from FHL disease challenges using TriReagent extraction was performed the week of 8-23-10. Run qPCR to check for genomic contamination.

PROCEDURE:
qPCR Master Mix:
1025 ul Immomix (2x)
65.6 ul F Primer (10mM)
65.6 ul R Primer (10mM)
41 ul SYBR Green
41 ul MgCL2 (50mM)
729.8 ul PCR water

+ 1ul of RNA = 25ul reaction

Duplicate positive and negative controls used on plate.

RESULTS:

qPCR report here: Test_Genom_Contamin_9-1-10.pdf

Next steps: DNase positive samples and re-test for contamination.

8/30/10 - DNA Extraction from filter - testing 2 methods

SUMMARY: Testing two methods of DNA extraction off membrane filters: WS DNA extraction method (heat and vortex) and modified Qiagen kit method.

PROCEDURE:
(Vt filtered from LD50 challenge used for tests)
500ml of SW filtered onto 0.2um Whatcom membrane filter
5 ml of Low TE Buffer was filtered through membrane after water was filtered through
Samples were stored at -80C before extraction

WS DNA extraction off membrane filters:
300ul Low TE buffer added to microcentrifuge tube with rolled filter
Vortex for 30 sec
Incubate at 95C for 5 min
Vortex on high for 2 min
Centrifuge at max speed for 15-30sc
Using sterile pestle, mash and grind filter to the bottom of tube
Incubate at 95C for 5 min
Centrifuge at max speed for 15-30 sc
Store at -20C

Modified from Kahlisch et al 2010 - DNeasy kit extraction modified for filter extraction
Original method:
"In brief, sandwich filters were cut into pieces, incubated with enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2 % Triton X-100; ph 8,0) containing 10 mg/ml lysozym (Sigma) for 60 min in a 37°C water bath. After addition of AL-buffer from the kit, the samples were incubated at 78°C in a shaking water bath for 20 min. After filtration through a polyamide mesh with 250 µm pore size, absolute ethanol was added to the filtrate (ratio filtrate/ethanol 2:1) and the mixture was applied to the spin-column of the kit. After this step, the protocol followed the manufacturer's instruction..."

Modified method:
Add 200 ul of Enzymatic lysis buffer
Incubate at 37C for 1 hr
Mash filter to the bottom of tube with sterile pestle
Add 200 ul AL buffer from kit and incubate at 78c for 20 min vortexing occasionally
Pipette off supernatant and place into new microcentrifuge tube
Add 2:1 ratio of absolute ethanol to the filtrate: 200ul of ethanol
Place everything but the filter into the spin column
Follow manufacturer's instruction starting with step number 4 on page 30

qPCR to quantify:

		1. rxns =	30
MgCl2 (50 mM)	0.5ul		15
Immomix (2x)	12.5		375
F Primer (10uM)	0.8		24
R Primer (10uM)	0.8		24
SYBR	0.5		15

SYBR	0.5		15
PCR water	9.4		282
Template	1.00		Add Individ
TOTAL	25.0		

"Vt_trial 2" saved on desktop in folder "elene"
95C - 7 min
95C - 10 sec
55C - 20 sec
Repeat steps 2-3 x39
75C - 15 sec
95C - end

RESULTS:

Average Ct:				
WS	Mod-Qiagen		One way ANOVA	
35.35	35.47		P = 0.6782	
34.46	32.36			
30.70	33.01			

The Ct values were averaged from the duplicate samples and statistical significance was determined by one way ANOVA. There is no statistical significance between the two methods, although sample size is very small (n=3).

I will continue use the WS DNA extraction method to extract DNA off of membrane filters. This method is significantly cheaper and quicker than the modified Qiagen protocol.

Full qPCR report here:8_31_10_extraction methods_primers.pdf

8-26-10 LD50 challenge

SUMMARY: determine the correct dosage of Vt to inoculate in larval experiments.
PROCEDURE:
Grow Vt at Rt for 48 hours

Design:
Three inoculation doses
Duplicate tanks
Static, not flow-through
Two control tanks uninoculated
Approx 10 larvae/ml = 1.5 million larvae needed for experiment
3-4 day old larvae used
each tank holds approx 1500ml of SW + larvae
To count, homogenize tanks with larvae plunger, and take 10 ml sample. Place into culture wells to bring to scope.
Count to approx mortality every 24 hrs

To count:
take 3 aliquots from each tank and place on depression slide
View at 4x taking 3 counts of dead/live larvae
Look at 10x if necessary to get larval detail

Inoculation:
Shooting for 10^3, 10^5, 10^6 CFU/ml of Vt per 1500ml tank of larvae
48 hrs of growth estimated to be ~4.0 x 10^8 CFU/ml
Duplicate dilution plates (T1N2 agar plates) of -4, -5, -6 will be used to estimate the actual numbers of CFU/ml of the starting culture.

10^6:
Take two 10 ml of original 48 hr culture and spin down to pellet (10 min at 3260rpm)
Take off supernatant and resuspend pellet in 1ml FSW
1ml of this culture will be use to inoculate 2 tanks
This should bring the total CFUs up to 10^9
(1.0 ml)(4.0 x 10^9) = 1500 ml (x)
x = 2.67 x 10^6

10^5:
Take one ml of original 48 hr culture and inoculate each 10^5 tank
(1.0ml)(4.0 x 10^8) = 1500ml (x)
x = 2.67 x 10^5

10^4:
Take the original 48 hr culture and dilute 1:10.

Use this dilution and place 1.0 ml into each 10^4 tank.
(1.0ml)(4.0 x 10^7) = 1500ml (x)
x = 2.67 x 10^4

10^3:
Take the original 48 hr culture and dilute 1:20.
Use this dilution and place 1.0 ml into each 10^3 tank.
(1.0ml)(4.0 x 10^6) = 1500ml (x)
x = 2.67 x 10^3

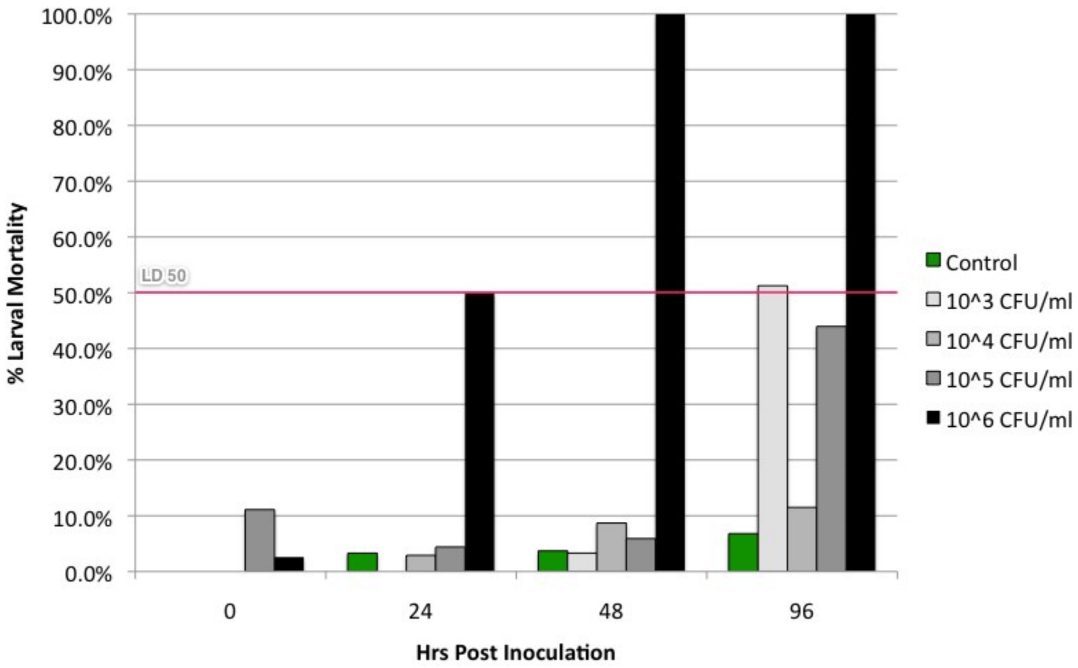
Larvae from each tank were visualized under dissection scope before inoculation to make sure they were alive and happy.

RESULTS:
Plate counts after 24 hrs post inoculation (incubated at 25C) resulted in an average of 4.6 x 10^8 CFU/ml
- average dup plate counts of 46 colonies on -6 plates

Resulting actual inoculations:
3.07 x 10^6 CFU/ml
3.07 x 10^5 CFU/ml
3.07 x 10^4 CFU/ml
3.07 x 10^3 CFU/ml

Tank letters with inoculating dosage:
M - neg control (larvae only, no Vt)
O - neg control (larvae only, no Vt)
C - 10^3
P - 10^3
G - 10^4
J - 10^4
B - 10^5
N - 10^5
D - 10^6
K - 10^6

Mortality results here:LD50 Data
LD50 was achieved approx 4 days at 10^5 after inoculation according to this data. There's some concern that all of the mortality data was not captured due to the design of the chambers. In the future, 10^4 CFU/ml inoculation may be a safe bet in combination with an alternative design of the larval chambers.



8-25-10 Primer Tests

SUMMARY: testing Vt primers for potential use in disease challenges examining gene expression.

PROCEDURE:
cPCR:

	Vol (ul) per rxn	1. rxns =	6	
PCR H20	4.75		28.50	

5xBuffer	5.00		30.00	
MgCl2	2.00		12.00	
BSA	1.00		6.00	
dNTPs	0.50		3.00	
F Primer (10mM)	0.75		4.50	
R Primer (10mM)	0.75		4.50	
Taq	0.25		1.50	per rxn
Template (instagene extraction)	10.00		Add individually	15
TOTAL	25.00			

Thermocycler:		
95 for 3 min		
95 for 15 sec		
55 for 1 min	} 35 - 40 cycles	
72 for 30 sec		
72 for 10 min		
22 for 10 min		

qPCR:

		1. rxns =	6
Reagent	ul		
Immomix (2x)	12.5		75
F Primer (10uM)	0.8		4.8
R Primer (10uM)	0.8		4.8
BSA	1.5		9
SYBR	0.5		3
PCR water	7.9		47.4
Template	1.00		Add Individ
TOTAL	25.0		

Thermocycler parameters:
"Vt_trial 2" saved on desktop in folder "elene"
95C - 7 min
95C - 10 sec
55C - 20 sec
Repeat steps 2-3 x39
75C - 15 sec
95C - end

All DNA template = Vt RE22 extracted following Instagene DNA extraction protocol.

Primers of interest:
ftsZ:
R - CTAAACGCTTTTTGCCTTCG
F - AATACTGATGCTCAGGCGCT
304 bp product
(not tested here - initial primer test, did not yield proper band size)

toxR:
R - acggtacttgagtaagactca
F - ctcaaccctacgtaaaatgctga

16Sv1:
R - GTTAGCCGGTGCTTCTTCTG
F - CAGCCACACTGGAACTGAGA
204 bp product

rseA:
R - GAA GAA CGT GTC AAG CTC ACT GGT
F - GTA TCA CTC GCT GTG ATC TTA GGC

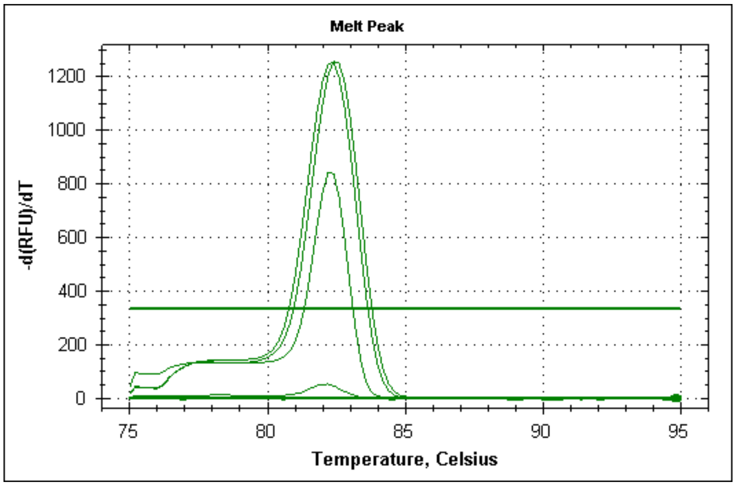
VptR: (NOTE: these primers did not work)

VtpR Forward	AAGCTGGTTGGTACGGTTTG
VtpR Reverse	GTTTGCTCGTCGTGGTATTG ~300 bp product

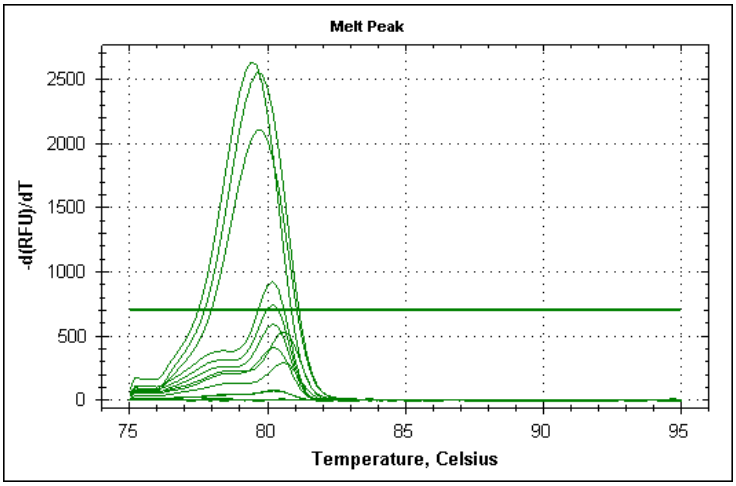
(VtpR - two sets: FHL use and "new" batch of 10mM primers.)

RESULTS:
VtpR primers do not work. After trying 3 times, I am convinced they are not working. I am curious why they were working at FHL.

Good news:
rseA primers, 16S (version1) primers work well and I can use these. I will order new VtpR primers on Tues. and test them on Wed after reverse transcribing my RNA.



Picture: rseA melt curve and on the bottom of same graph, the 2 sets of VtpR primers that don't work.



Above picture: Melt curve for VtpR primer set #4 (3 peaks). The other smaller peaks show the other primer sets not working as well.

VtpR - 4 primer sequence:
F - cagcatgaccaccgcgacca
R - gcgtacgcgtctttcaccaca
~98 bp length product

Used same MM and thermocycler protocol above. NOTE - no MgCl2 added to this recipe.

8-16-10 C. gigas spawning and pCO2 challenge

SUMMARY: Strip spawn Cg under low pCO2 levels and monitor fertilization, larval life stages, and mortality.
PROCEDURE:
4 females stripped gametes into one jar
added 3 ml of female gametes into 6 dishes with approx 57ml of FSW into each (3 for pCO2 of 380 and 3 for 840) (~60ml total)
Water at the appropriate pCO2 level was used for each group
2 males stripped gametes into separtate jar (1:1 mixture using 380 ppm SW)
Added 1ml of sperm into each dish of eggs.
Each pCO2 level contained 3 separate fertilizations, separated into triplicate petri dishes (a total of 18 dishes)
Time of each fertilization was noted for Time 0 - 3:30pm

Each dish was placed into either one of two chambers set up with gas flow of 380 or 840 ppm CO2.

Two control plates of fertilized eggs (one at each ppm) were left out of the chambers in order to monitor development.
Once first cleavage was seen in the control dish, time was noted,, the petri dishes of larvae were counted in order of fertilization to measure % cleave in a 10X objective lens.
Second count was approximately 30 minutes later per dish and measured the same way to calculate proportion cleaved.
A 5-hour count of hatched larvae was taken using 20x objective lens to visualize one field of vision and counting live/dead. This was done in triplicate for each dish. Hatched larvae = swimming. Proportion of hatched vs unhatched was calculated.
2-ml of SW were added to each well to bring up water volume overnight. Corresponding pCO2 water (380 or 840ppm) was added to the appropriate experimental wells.
A 17 hour count was performed using the same procedure for hatched larvae to visualize swimming vs not swimming
A 24-hour count was taken using the same procedure as above.

Total egg and sperm counts:
Sperm counts were performed using hematocytometer at a 1:100 dilution of sperm
Egg counts were performed in duplicate by counting 5ul of larvae at 4x objective lens with 5 ul of EtOH to immobilize larvae
4 dishes from each pCO2 group were used to estimate total eggs

RESULTS:
2.135 x 10^8 ml of sperm used
4.46 x 10^6 eggs/ml
47.9 sperm/egg used

Larval Data Here:
Fertilization Trial FHL
48-hour count was not completed due to invasion of ciliates that killed all larvae in experiment.

8-17-10 Vt larval challenge at high pCO2

SUMMARY: Challenge larvae grown from the last trial (8-15-10) with Vt at ~10^3 CFU/ml for 24-48 hours. Looking at Vt physiology, larval physiology, and disease susceptibility under altered pH.
PROCEDURE:
Used batch of larvae spawned on 8/15.
Larvae were approx 56 hrs old before inoculation and were visualized under dissecting scope to visualize swimming.
They were batched together and maintained at the relative pCO2 until day of inoculation
The larvae were filtered through a 70um mesh screen and rinsed, the water captured
The captured water was then filtered onto a 48um mesh screen , rinsed and placed into approximately 800ml of FSW at the corresponding pCO2 .
Larvae were counted onto a 2ml hematocytometer in triplicate, numbers averaged and estimated larvae/ml of SW.
We wanted to inoculated into 1500ml of SW, so larvae numbers were normalized to contain approx the same amount
Static experiment for 48 hrs

Grow Vt culture for 48 hours on shaker at RT in marine broth
To inoculate: dilute culture of Vt 1:20 and use this to inoculate the experimentals (approx 10^6 CFU/ml)
Use 1000ul of diluted Vt culture to inoculate each tank
Triplicate samples of larvae at 2 pCO2 levels (~840 and 380 ppm)
2 control Vt flasks without larvae to act as Vt control - 840 and 380 ppm
Take four 1 ml aliquots of Vt culture, spin to pellet and take off supernatant
Store pellets in -80C until ready to extract (2 DNA, 2 RNA)

Samples of larvae:
8 hour - take 500ml of water from each experimental and filter onto 0.22um filter, cut into quarters, store at -80C in separate cryovials
24 hour - take all water from each experimental and filter as above

For larvae:
Sample pre-inoculation:, 2hours, and 24 hours
RNA samples collected by filtering larvae through mesh screen (48 um) and bringing larvae back up to a fixed amount
Larvae were then visualized on a 10x objective lens and counted mortality proportions in triplicate.
Take photos for size, % mortality

Vt samples:
Taken at 2, 12, 24 and 48 hours post inoculation.
At each pCO2:
Vt control without larvae
Vt + Cg in duplicate
DNA and RNA samples taken except at T-2 hours due to sampling procedure

RESULTS:
Experiment consisted of:
840:
Estimated larvae: 40/ml of SW
1138 ml SW was added to 362 ml of batch larvae = 1500 ml (in triplicate, including one control)

380:
Estimated larvae: 58/ml of SW
1250 ml of SW was added to 250 ml of batch larve = 1500 ml (in triplicate, including one control)

+ 1500 ml of SW at each pCO2 level
+ 1500ml of Cg not inoculated with Vt

Vt inoculation: Triplicate plates were counted and averaged 40 colonies at 10^-6.
Calculation of CFU/ml = 4.0 x 10^8 CFU/ml of starting culture
The culture was diluted 1:20 to give an ending concentration of 10^6.
1 ml of this dilution was used to inoculate 1500ml of SW and larvae.
The resulting dose was 2.66 x 10^3 per ml of SW in each experimental at 5pm on 8/17.

Sampling :
8/17: 2 hours post inoculation (7pm)
Take 50 ml of stirred larval culture and pass through 48um mesh screen, reserving the filterate for Vt sampling.
The larvae were then rinsed with FSW at the appropriate pCO2 level and placed into 0.5ml of SW.
Approx 20 ul were taken from the filtered larvae and placed into a depression slide with cover slip.
Larvae were viewed under 20x objective lens and proportions of live dead were taken in triplicate for each flask.

Alive = cilia moving, swimming, or internal parts moving
Dead = no cilia moving or undeveloped

Vt sampling:
The filterate taken from each flask was spun down at 3000rpm for 25 min on a Beckman Coulter Allegra X-22 Cetrifuge.
Supernatant was taken off and pellets of bacteria were frozen at -80C until extraction.

8/18: 12 hours post inoculation (5am)
Increased our proportions:
Take 100 ml of stirred larval culture and pass through 48um mesh screen, reserving the filterate for Vt sampling.
The larvae were then rinsed with FSW at the appropriate pCO2 level and placed into 0.25 ml of SW.
Approx 20 ul were taken from the filtered larvae and placed into a depression slide with cover slip.
Larvae were viewed under 20x objective lens and proportions of live dead were taken in triplicate for each flask.
(Still not a drastic change in larvae numbers seen, need to increase numbers)

Vt sampling: same as above, with increased volume/replicates
Plate counts were performed for Vt control and Cg + Vt "A" flasks for each pCO2 level (at 10am, 17hrs post inoculation)
Done on T1N2 agar plates from 1:10 dilution series

RESULTS:
380 Vt only: 3.0 x 10^5 CFU/ml
840 Vt only: 6.0 x 10^4 CFU/ml
380 A: NG
840 A:NG

For unknown reasons I did not get any growth on either Vt experimental. I plated -2 and -3 dilutions of each tank listed in duplicate.
It is possible that human error is to blame since the low dilutions of 840 Vt only did not grow either (-1, -2), but the higher dilution did (-3) indicating possible problem in technique.

8/18: 24 hours post inoculation (5 pm)
Changed protocol once again:
Take 200 ml of stirred larval culture and pass through 48um mesh screen, reserving the filterate for Vt sampling.
The larvae were then rinsed with FSW at the appropriate pCO2 level and placed into 0.25ml of SW.
Approx 20 ul were taken from the filtered larvae and placed into a depression slide with cover slip.
Larvae were viewed under 20x objective lens
Total number of larvae were counted in each aliquot for total larvae at 4x objective
Using 20x objective, live/dead were counted, this was done in triplicate for each flask

Vt sampling: same as above, with increased volume/replicates

8/19: 48 hours post inoculation (5 pm)
Samples were taken by filtering as described above on 8/18 for larvae mortality estimates.
Total larvae were counted in 4 drops and averaged to get # larvae per drop of SW after concentrating
Mortality was estimated counting 3 replicates of one depression slide well three times and averaged.
All filtered water was reserved for Vt samples
The rest of the water from each tank was filtered through 48um mesh to capture remaining larvae
Larvae were stored in RNAlater at -20C overnight and -80C until extraction
Water was filtered through a Millipore GSWP 0.22um mixed cellulose esters, hydrophilic filter using a vacuum pump
Duplicate 500ml SW samples were filtered from each tank, rolled with sterile forceps and placed into microcentrifuge tube at -80C until extraction.
In between filtering samples, apparatus was bleached, rinsed with tap water, then rinsed well with reverse osmosis water.

MORTALITY Results Here:
Disease Trial Full

Disease Trial FHL

Water Chemistry Data:
pH taken with Thermo Scientific Orion 3 Star pH probe:
We could not use the probe on any Vt samples due to FHL policy
pH/Temp - 8/17, 7 pm
380 Cg control: 8.18/22.3
840 Cg control: 7.88/22.3

380: pCO2: 489, Salinity: 29.4
840: pCO2: 968, Salinity: 29.1

pH/Temp - 8/18, 9 am__
380 Cg control: 8.19/20.4
840 Cg control: 7.89/20.4

380: pCO2: , Salinity:
840: pCO2: , Salinity:

8-15-10 Larval Fertilization pCO2 trial

SUMMARY: strip spawn and fertilize oysters at high pCO2 and compare to control at 380 pCO2 and monitor growth through larval life stages.
PROCEDURE:
Oysters: 4 male and 4 female oysters used.
Strip gametes with scalpel and separate eggs and sperm into separate containers with FSW

Experimental design:
Triplicate sterile flasks of experimental pCO2 (~800 ppm) and control (380 ppm) with 2000ml of FSW bubbling into covered containers
1:10 sperm to egg mix was aliquoted into each container: 20ml of egg slurry with 2ml of sperm.
Swirl to mix
Sample from each container and visualize in the microscope for 48 hours noting developmental stage, % mortality, size and water chemistry (temp, salinity, pCO2 and alkalinity).

Sampling timeline:
Hours:
0 - Fertilization
2 - 4th cleavage
4 - Blastula
5 - Hatching cilia
24 - veliger
48 - D-hinge

Homogenize larvae by stirring
10ml samples from each trx
5 pictures at 4x for proportion data
5 pictures at 20x for detailed photos and measurements
Take temp, pCO2, salinity twice daily
On Monday (Moose returns) take pH with spectrophotometer, alkalinity if possible

RESULTS:
Water Chemistry results here:
8/15:
380: pCO2: 440, Salinity: 29.2
840: pCO2: 985, Salinity: 29.5

Flasks:
pH/Temp
840
E: 7.88/23.0
H: 7.90/23.3
L: 7.89/23.3
380
B: 8.15/23.3
I: 8.17/23.4
G: 8.18/23.2

8/16:
380: pCO2: 418, Salinity: 29.4
840: pCO2: 995, Salinity: 29.0

Flasks:
pH/Temp
840

E: 7.59/23.2
H: 7.56/22.8
L: 7.66/22.9
380
B: 7.81/23.4
I: 7.78/23.0
G: 7.84/22.9

We realize this experiment needs to be done differently. The pictures taked cannot accurately measure dead/alive, nor can we measure them accurately on this microscope software. We will strip spawn again this afternoon with a new batch of male/females. This batch will be used for Vt trials and maintained at the corresponding pCO2 until inoculation on 8/17.

NEXT STEPS:
Vt trial with D-hinge larvae. Dosing at ~ 10^3 (due to the early growth stages we are dosing a little low)

8-10-10 12C Baseline Vt Growth - Comparing qPCR results with plate counts

SUMMARY: Used Vt qPCR protocol to re-qPCR the 12C DNA extracts using the standard curve developed on 6-28-10 (green set).
Procedure:
For 96-well plate: (105 rxns)
ImmoMix (2x) - 1312.5 ul
F Primer (10 mM) - 84 ul
R Primer (10 mM) - 84 ul
MgCl2 (50 mM) - 52.5 ul
SYBR - 52.5 ul
PCR water - 934.5 ul

Same Vt_Trial 2 protocol used below.

RESULTS:
Full reports for the 12C growth curve (12-1 and 12-2 -two plates) here:
8-11-10_12C-2_Vt_Base.pdf
8-10-10_Vt_Base_12C.pdf

Used this data to correlate plate counts and qPCR data:

CORRELATION OF vtpA ASSAY Ct VALUES TO CFU DATA:

SUMMARY: correlate qPCR Ct values for *vtpA* and CFU data
PROCEDURE:
Extract 12C baseline growth curve samples using DNeasy Blood and Tissue kit following manufacturer's instructions for Gram negative bacteria.
Run with optimized qPCR assay for vptA gene
Use qPCR master mix recipe and parameters described above.

RESULTS:
Strong correlation...
From regression:
Adjusted R-squared: 0.9097
Ct = 45.7627 - 3.4307(Log CFU)
p-value: < 2.2e-16

Pearson's correlation = -0.9544428

7-30-10 qPCR of Baseline Growth Curves using optimized protocol

The revised protocol is:

- Anneal temp = 55C
- Final Mg2+ = 2.mM (1x Immomix has 1.5mM final Mg2+, so add 0.5uL of 50mM MgCl2 per reaction to achieve a final Mg2+ of 2.0mM).
- Make set of dilutions of known quantities of existing DNA to create a standard curve.

Link to Sam's Friedman Lab notebook: http://friedmanlab.wikispaces.com/Sam%27s+Notebook
SUMMARY: Run a plate of qPCR on the 12C growth curves to get a feel of how well the standard curve works for the unknowns.

PROCEDURE:
Master Mix Recipe Per Reaction:
ImmoMix (2x) - 12.5 ul
F Primer (10 mM) - 0.8 ul
R Primer (10 mM) - 0.8 ul
MgCl2 (50 mM) - 0.5 ul

SYBR - 0.5 ul
PCR water - 8.9 ul
Template - 1 ul
_
Total - 25 ul

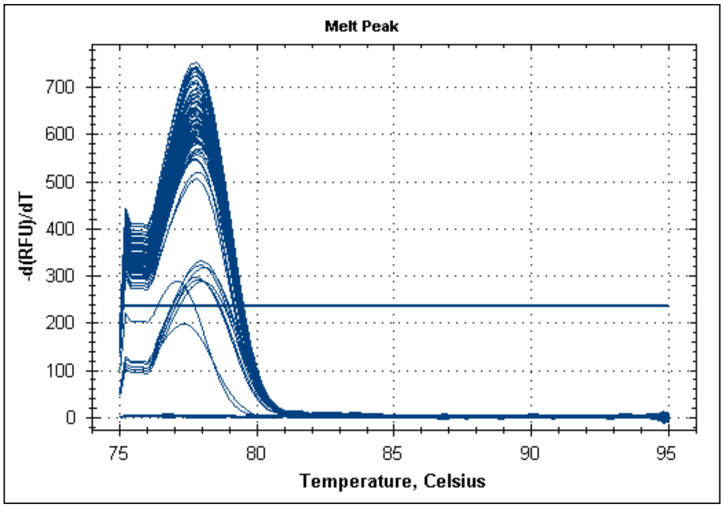
For 96-well plate: (105 rxns)
ImmoMix (2x) - 1312.5 ul
F Primer (10 mM) - 84 ul
R Primer (10 mM) - 84 ul
MgCl2 (50 mM) - 52.5 ul
SYBR - 52.5 ul
PCR water - 934.5 ul

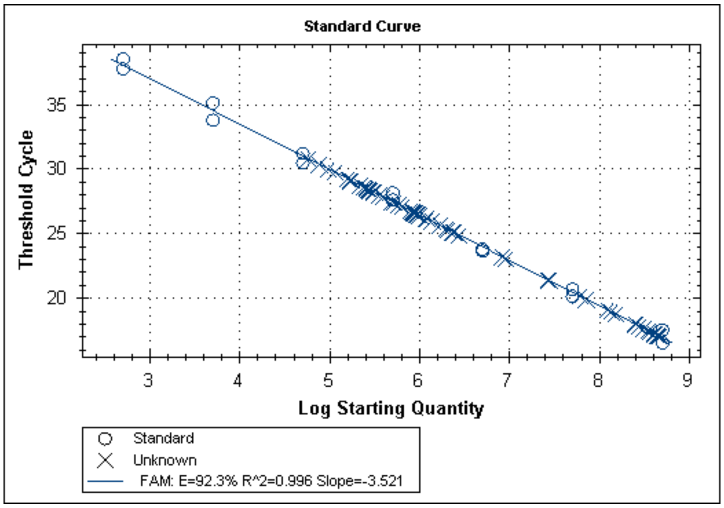
Thermocycler parameters:

"Vt_trial 2" saved on desktop in folder "elene"
95C - 7 min
95C - 10 sec
55C - 20 sec
Repeat steps 2-3 x39
75C - 15 sec
95C - end

Note: trying the multichannel pipette for the first time for MM dispensing.

RESULTS:
Eye-balling the volume of the reactions in each well, I can see that the first row of wells pipetted contains less than the rest of the plate. Unfortunately, these wells contained a standard curve replicate series. I think this was due to keeping the tips sumberged in the row of wells too long. There is a "suck-back" that occurs after the initial quantity is dispensed. The rest of the wells were consistent.





Full report here:

7-30-10_VtBaseline_12.1

7-12-10 Inoculate C. gigas larvae with Vt at 12 and 25C - Round 1

SUMMARY: Challenge Cg with live Vt at 12 and 25C for 72 hours. Samples of Cg larvae and Vt will be taken for gene expression analysis.
PROCEDURE:
Vt inoculation prep:
To inoculate 1500ml of SW + larvae with ~ 10^3 of Vt:
Inoculate 25 ml of 0.25% tryptone and seawater media with Vt (from freezer or loopful of current culture)
Grow at 25C for 24 hours on shaker.
On the morning of larval inoculation, inoculate 25 ml of 0.25% tryptone and seawater media with 100ul of 24 hr culture (grown at 25C) and place into 25ml fresh media
Incubate at 25C for 8 hours on shaker
Inoculate the larvae tanks with 1.5ml of culture into each larval tank

If BOIL KILLING:
After 6 hours, take a few mls and place into glass tube
Set up water bath, turn on high, let water boil
Place culture tube in boiling water bath for 60 mins
Let cool in RT water bath for a few min
Vortex!
Inoculate the larvae tanks with 100ul of the boiled culture

Incubate overnight and take samples in the morning
Plate the culture in duplicate to estimate CFU/ml:
Take 1ml of the 8 hour culture and perform serial 1:10 dilutions in sterile water
Vortex well between samples
Dilute to 10^-5
Take 6 T1N2 agar plates and label with the dilution and date (plates in duplicate, ie -3, -4, -5)
Incubate at 25C overnight
Count colonies and average CFUs next morning

Cg Prep:
Eight tanks approx 1500ml ea in 12C
Eight tanks approx 1500ml ea in 25C
Four tanks at each temp used as control
Static culture - change water every 24 hrs post inoculation and re-inoculate larvae with Vt

Tanks inoculated at 12C:
J, K, C, L

Tanks inoculated at 25C:
A, G, D, H

Cg Mortality Estimation with Neutral Red:
Homogenize larvae by agitating water
Take 50 ml larvae out and place into beaker
Put 50ul of neutral red 1% solution into larvae beaker
Incubate at RT for 6 hours
Take a 40ul aliquot of larvae from each tank and place on depression slide
Photograph larvae (10-20 per chamber) and measure length and width
Note any ciliates if present

Larvae Samples:
Take larvae samples (~1000 larvae) from each tank and place into RNAlater BEFORE inoculation
Take another sample every 24hours post inoculation and before re-inoculating
Cg samples - Tues: 2pm; Wed: 2pm, Thurs: 2 pm, Fri: 2 pm
On last day of trial, filter out larvae on 60um mesh filter and place all the larvae in 1 ml of RNAlater.

Vt Samples:
Take two 1 ml samples of the starting culture - 1 for DNA and 1 for RNA
Dilute and plate samples to estimate starting CFUs and inoculation dose per tank
Inoculate larvae
Take two 15ml samples in conical vials from the out flow of each tank and spin at 3600rpm for 20 min to pellet
Take off supernatant and place into microcentrifuge tube
Spin down again (5min at 12000 rpm) and take off supernatant
1 sample for DNA quantification (store in -80C) and 1 sample for RNA gene expression (store overnight in -4C and longterm at -20C)

Vt Samples - 8 am every morning during trials

RESULTS:

RESULTS:
7/13:
plate counts = 1.0 x 10^5 CFU/ml of starting culture
Inoculated with 100ul of culture into 1500ml of seawater in each tank at 2 pm
End dosage for larvae: ~10cells per ml

Notes: This starting culture amount was too small. We are aiming on inoculating the larvae with 10^3 CFU/ml and this is proving difficult to estimate. Next inoculation will be higher with 2 hours more of incubation at 25C.

7/14:
plate counts = 5.2 x 10^5 CFU/ml of starting culture
Inoculated with 1.5ml of culture into each tank (~1500ml SW) at 4 pm
End dosage for larvae: 5.2 x 10^2 CFU/ml

Notes: This is closer to our target dosage, but still a bit too little Vt. I think that we need to incubate the Vt a little longer, perhaps overnight to get a correct dosage.

7/15:
Plate counts = 1.86 x 10^6
Inoculated with 1.5ml of culture into each tank (~1500ml SW) at 4 pm
End dosage for larvae: 1.86 x 10^3
target dose!

7/16: This morning the 25C tanks were all half empty or more. The larvae was left static after 4 pm and I turned the pumps back on at 8 am the next mornings on all days except Monday night were the pumps were turned on at 9 pm. Not sure why the tanks were not full. I sampled as normal and turned the pumps on at 8am.

The 12C tanks were fine and as full as when I left that afternoon.

Next Steps:
Higher dose of Vt - aim for 10^3 or 10^4
I think we should inoculate some TCBS plates with the control tanks to see if there is any Vibrio growth in future experiments.
I would like to get the experimental tanks and the control tanks farther apart for the disease challenges to eliminate cross contamination.

6-28-10 qPCR of Standard Curve

SUMMARY: Make a graph of standard curve using the extracted DNA from 1:10 dilution series.
This culture was grown at RT over the weekend using 100 ml of 0.25% tryptone and SW media inoculated with a loopful of the previous culture. I stuck the flask of inoculated media into the incubator set up with bubbling airflow (filtered with 0.22um filter) from an airpump, covered this steriley, and left this over the weekend. Started at 3pm Friday and diluted 1:10 to -8 at 10am Monday (67 hours).

PROCEDURE:

		1. rxns =	42
Reagent	ul		
Immomix (2x)	12.5		525
F Primer (10uM)	0.8		33.6
R Primer (10uM)	0.8		33.6
SYBR	0.5		21
PCR water	9.4		394.8
Template	1.00		Add Individ
TOTAL	25.0		

Thermocycler Parameters:
"Vt_trial 2" saved on desktop in folder "elene"
95C - 7 min
95C - 10 sec
60C - 20 sec
Repeat steps 2-3 x39
75C - 15 sec
95C - end

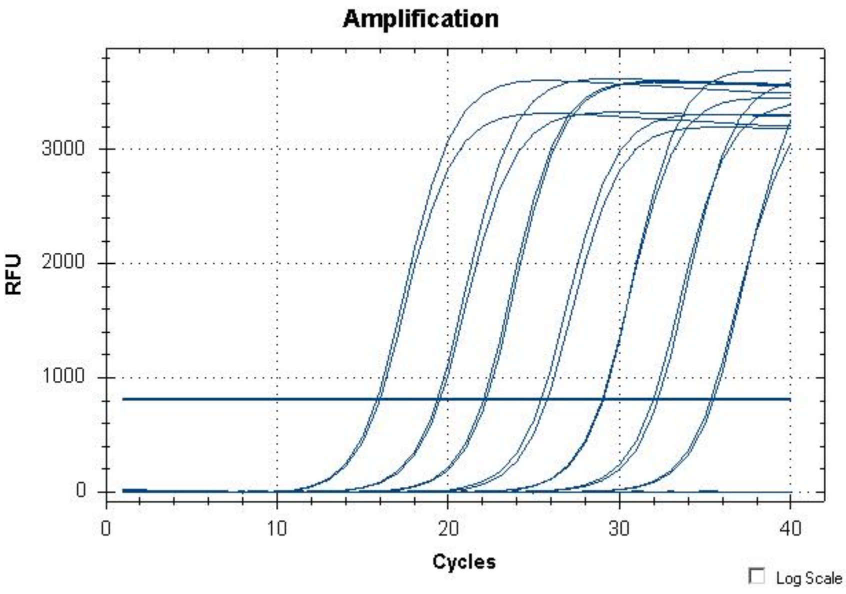
RESULTS:
Counted 500 and 503 colonies on 10^-5 plates

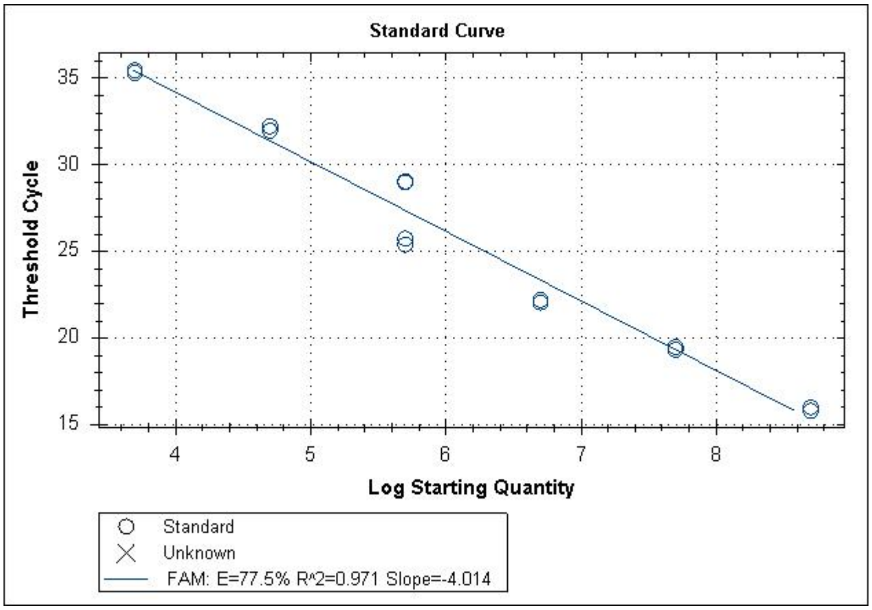
Standard Curve Made on 6/28 : Average = 5.02 x 10^8 CFU per ml starting concentration
Melt curve = 77C
The amplification plot looks pretty good, the replicates could be more accurate. All amplification on the melting plots are at one temp (77C).

I can re-run the qPCR to get better amplification plot.

Next steps: make regression plot of standard curve using the known concentration of starting culture.
Using the starting concentration, the curve is:
10^0 = 5.0 x 10^8
-1 = 50,000,000
-2 = 5,000,000
-3 = 500,000
-4 = 50,000
-5 = 5000
-6 = 500
-7 = 50
-8 = 5

The regression was done in duplicate and the graph that included the four replicates of each dilution had an r^2 of 91%. Not that great. I separated the 2 duplicates and the r^2 did not improve significantly. The higher dilutions, specifically -7 and -8 were off the regression line significantly. The best graph I could make was using the dilutions from 500,000,000 to 500 copies/CFUs and used the second set of replicates (wells C and D, green tubes). This resulted in an r^2 of 97%:





6-24-10 Vt qPCR Standard Curve

SUMMARY: Develop a standard curve for Vt to use for qPCR using serial dilutions of a culture and extracting the DNA.
PROCEDURE:
Inoculate 20ml of 0.25% tryptone and SW media with RE22
Grow at RT for 48 hours on a rotator to aerate
Vortex well and dilute 1:10 to 10^-8
Take one ml of each dilution and extract DNA using the DNeasy Qiagen kit protocol (below)

Quant each extraction
qPCR to get standard curve

Serial Dilutions:
Quantify the starting CFU by plating 1:10 dilutions of this culture.
Dilute to 10^-8
Count plates after 48 hrs growth

RESULTS:
Plates did not grow. This is the third time using these plates that no culture was grown. Need to re-do this procedure and use new set of T1N2 plates for CFU determination. I will re-dilute and re-extract new culture.

NEXT STEPS:
I am concerned that the growth in the conical vials is not as great as growth with an air input from a pump. I am going to do this procedure above using 100ml of media, inoculated with the 48 hour culture, grow for ~48 hours, dilute and extract to see any difference.

6-11-10 DNA extractions: Comparison of recovery from Boil/lysis and Qiagen DNeasy Blood and Tissue kit

SUMMARY: Determining the best extraction method to get the greatest DNA yield. Comparing the boil and lysis method to the DNeasy Qiagen Blood & Tissue kit.
PROCEDURE:
Inoculated a culture of Vt in 0.25% tryptone and SW broth
Incubated on a shaker at RT for 24 hrs
Took one 1ml aliquots into 2 microcentrifuge tubes

DNeasy: (note: these reagents were OLD)
Took one aliquot and spun down at 7500rpm for 10 min
Drew off supernatant
Added 180ul of buffer ATL to pellet and resuspended by vortex
Added 20ul Proteinase K and vortex
Incubate at 56C for 1 hour, vortexing occasionally
VOrtex for 15 sc
Add 200ul buffer AL to sample and vortex
Add 200ul ethanol (100%) and vortex
Pipet contents into 2ml spin column
Centrifuge for 1min at 8000rpm
Discard flowthru and put column into new collection tube
Add 500ul buffer AW1
Centrifuge for 1 min at 8000rpm
Discard flowthru and put column into new collection tube
Add 500ul buffer AW2
Centrifuge for 3 min at 14000rpm
Discard flowthru
Place spin column into new, labeled microcentrifuge tube
Add 200ul of buffer AE into the membrane column see conclusions below
Incubate at RT for 1 min
Centrifuge for 1 min at 8000rpm
(Can repeat the last step again into new microcentrifuge to elute more DNA) - I did the step just to see the quantities

Boil/Cell Lysis:
The other aliquot was spun down at 7500rpm for 10 min
Drew off supernatant
Added 180 ul of Sterile Nanopure water to pellet
Vortexed the pellet to resuspend
Incubate at 56C for 1 hour, vortexing occasionally
Vortex for 15sc
Incubate at 100C for 10 min
Spin down at 8000 for 7 min
Before quanting I vortexed sample well

Quant:
UV plate
Prepare a 1:200 solution of pico green to TE buffer

(For 27 wells, I added 6.75 ul of pico green to 1343 ul of TE)
Grab your standards and label your map of samples
I quanted the boil-extracted DNA, DNeasy DNA, and the second elution DNeasy DNA in triplicate
Standards were done in duplicate
Add 49 ul of TE to each well
Add 1 ul of template into each well
Add 50 ul of picogreen mix to each well and mix with pipet
Throw into quant machine and run, double checking standard entries

RESULTS:
Average:
Boiled DNA: 9837 ng/ul
DNeasy DNA: 13276 ng/ul
DNeasy DNA, second elution: 6963 ng/ul

Conclusions:
DNeasy kit way better quantities of DNA. Moving forward, this looks like the best choice. Second elution resulted in half the quantity of DNA as the first.

  - Talking with Lisa, she recommends using 100ul of the elution buffer AE on the last step and incubating for 5-10 min instead of 1 min as the Qiagen protocol states.

I will give this a try and quant before moving forward with this change.
I diluted this 1:10 to negative 5, grew at RT for 48 hours, but none of the plates showed growth.

10-10 Isolates to use for Vt primer specificity

SUMMARY: Sam is running the VtpA primers against different strains of Vt to compare the specificity of the primers.
Strains to be used:
RE22 - 99-70-6B-2
RE66 - 00-78-3
RE68 - 00-78-5
RE90 - 00-90-9
RE98 - X00-12-1
RE100 -X-00-12-3
RE101 - X00-12-4

PROCEDURE:
Find the isolates
Streak onto T1N2 plates
Label microcentrifuge tube with strain
Add 1 ml sterile water to each tube
Add colony of corresponding strain to tube
Spin down to pellet
Draw off supernatant
Store in -80 for Sam

RESULTS:

5-19-10 SYBR qPCR running standard curve

SUMMARY: produce a standard curve from 48hr RE22 culture.
PROCEDURE:
Grow up Vt RE22 for 48 hrs
Use InstaGene protocol per manufacturer's directions to extract DNA from 0.5ml pellet of bacteria:
spin down to pellet for 5 min at 13k rpm
take off supernatant
add 200ul of Instagene
put on heat block set at 56C for 15-30 min
vortex for 10 sec
put on heat block set at 100C for 8 min
vortex for 10 sec
spin down at 11k rpm for 2 min
freeze whatever is left at -20C

qPCR SYBR			Reactions	20
Reagent	ul			
Immomix (2x)	12.5		250.00	
F Primer (10uM)	0.8		16.00	

R Primer (10uM)	0.8		16.00	
SYBR	0.5		10.00	
PCR water	9.4		188.00	

Take the DNA template from above and make 1:5 serial dilutions to 1:30
Run this dilution set on qPCR using SYBR
Master Mix recipe is above for 20 reactions:
7 serial dilutions + 2 negative controls

Thermocycler Parameters:
Vibrio Protocol saved on desktop in folder "elene"
95C - 7 min
95C - 10 sec
60C - 20 sec
Repeat steps 2-3 x39
75C - 15 sec
95C - end

RESULTS:
This needs to be redone.
I need to grow up a culture for 48 hours, serially dilute 1:10, extract each of these dilutions, qPCR and plate each dilution. Then we should have a standard curve.

4-26-10 VptA Primer test

SUMMARY:Test the published 2008 metalloprotease primers (VtpA) against the different strains of Vt.

VtpA Forward	caaatgctttggctgattgct
VtpA Reverse	ccatctctgcggctgtaactg

PROCEDURE:
Master Mix Recipe:

	Vol (ul) per rxn	1. rxns =	22	
PCR H20	4.75	104.5		
5xBuffer	5.00		110	
MgCl2	2.00		44	
BSA	1.00		22	
dNTPs	0.50		11	
F Primer (10mM)	0.75	16.5		
R Primer (10mM)	0.75		16.5	
Taq	0.25		5.5	per rxn
Template	10.00		Add individually	15
TOTAL	25.00			

For 22 reactions. Strains tested: RE100, 101, 22, 60, 98, ATCC19106, 19109, ATCC 33539 (V. alginolyticus) with 2 negative controls.
Thermocycler Parameters:

95 for 3 min	
95 for 15 sec	
55 for 1 min	} 35 - 40 cycles
72 for 30 sec	
72 for 10 min	
22 for 10 min	

I'm starting with a low annealing temp to start out with and go from there.
1% agarose gel with 100bp ladder.
100v for 1 hour

RESULTS:
Expected band size: 63 bp
Picture:VtpA Primer Test
Order of gel (all strains in duplicate, last 2 lanes are neg controls):

RE 100
RE 101
RE 22
RE 60
RE 98
ATCC 19106
ATCC 19109
ATCC 33539 (TS 24) Photobacterium damselae

Sequence: (RE22 VtpA):

```
     **1 ATGAAACAACGTCAAATGCTTTGGCTGATTGCTGCTGGATTAGGAGTTAGCCTACCAGTT
                    >>>>>>>>>>>>>>>>>>>>>

    61 ACAGCCGCAGAGATGGT**
```

Looks like most of the strains have small bands. I need to increase specificity in order to eliminate the other bands present. I will try to run this again using Annealing temp of 59C with a 2% gel and see what happens.

3-30-10 Growth Trial 25C

SUMMARY:Establish growth curves for Seawater and 0.25% Tryptone and Seawater media at 25C in triplicate with one control for each media type.
PROCEDURE:
Initial inoculation follows these steps:
Tues (3/30): Inoculate 5 ml of 0.25% Tryptone and Seawater media from RE22 Vt stock (RE old stock) at 8 am
Wednesday (3/31): After 24 hours of growth at RT on a rotator, inoculate 100ul of culture to 25ml of 0.25% Tryptone and Seawater media in two conical vials (duplicate) at 8 am Wed morning.
Friday (4/2): At 3 pm Friday afternoon, re-inoculate 25 ml 0.25% Tryptone and Seawater media in duplicate in conical vials with 100ul of Wednesday's culture. Grow this at RT until Monday (4/5) at 6am.
Monday (4/5): At 6 am, inoculate three 0.25% Tryptone and Seawater media and three seawater (63 hours growth) flasks with:

- **Seawater: inoculate with 75ul of culture**
- **0.25% Tryptone and Seawater media: inoculate with 50ul of culture**

Samples will be taken every 4 hours for the first 48 hours and intermittent samples until 72 hours of growth (total of 13 sampling events):

Time Schedule	Time of Sample	Time since inoculation	Dilution Range for Media (#1-3)	Dilution Range for Seawater (#5-7)
T0	6:00 AM	0	1 to 3	-1 to -3
T1	10:00 PM	4	-2 to -4	-1 to -3
T2	2:00 PM	8	-3 to -5	-1 to -3
T3	6:00 PM	12	-4 to -6	-1 to -3
T4	10:00 PM	16	-4 to -6	-2 to -4
T5	6:00 AM	24	-4 to -6	-2 to -4
T6	10:00 PM	28	5 to 7	-2 to -4
T7	2:00 PM	32	5 to 7	-2 to -4
T8	6:00 PM	36	5 to 7	-2 to -4
T9	10:00 PM	40	5 to 7	-2 to -4
T10	2:00 AM	44	5 to 7	-2 to -4
T11	6:00 AM	48	5 to 7	-2 to -4
T12	3:00 PM	56	5 to 7	-2 to -4
T13	6:00 AM	72	5 to 7	-2 to -4

3-17-10 Growth Trial 18C

SUMMARY:Establish growth curves for Seawater and 0.25% Tryptone and Seawater media at 18C in triplicate with one control for each media type.
PROCEDURE:
Same protocol for 12C trial (3/15/10) EXCEPT initial inoculation follows these steps:
Wed (3/17): Inoculate 5 ml of 0.25% Tryptone and Seawater media from RE22 Vt stock (RE old stock) at 8 am
Thursday (3/18): After 24 hours of growth at RT on a rotator, inoculate 100ul of culture to two 25ml of 0.25% Tryptone and Seawater media in conical vials at 8 am Thursday morning.
Friday (3/19): At 5 pm Friday afternoon, re-inoculate each of the two 25 ml 0.25% Tryptone and Seawater media in conical vials with 100ul of Thursday's culture. Grow this at RT until Monday (3/22) at 8am

100ul of Thursday's culture. Grow this at RT until Monday (3/22) at 8am.
Monday (3/22): At 8 am, inoculate three 0.25% Tryptone and Seawater media and three seawater (63 hours growth) flasks with:

- Seawater: inoculate with 75ul of culture
- 0.25% Tryptone and Seawater media: inoculate with 50ul of culture

Samples will be taken every 6 hours for 72 hours (total of 13 sampling events)
Sampling protocol: same as 12C
Original scheduled dilutions and sampling times:

Time Schedule	Time of Sample	Time since inoculation	Dilution Range for Media (#1-3)	Dilution Range for Seawater (#5-7)
T0	8:00 AM	0	-1 to -3	-1 to -3
T1	2:00 PM	6	-1 to -3	-1 to -3
T2	8:00 PM	12	-2 to -4	-1 to -3
T3	2:00 AM	18	-3 to -5	-1 to -3
T4	8:00 AM	24	-3 to -5	-1 to -3
T5	2:00 PM	30	-4 to -6	-1 to -3
T6	8:00 PM	36	-4 to -6	-2 to -4
T7	2:00 AM	42	-4 to -6	-2 to -4
T8	8:00 AM	48	-4 to -6	-2 to -4
T9	2:00 PM	54	-4 to -6	-2 to -4
T10	8:00 PM	60	-5 to -7	-2 to -4
T11	2:00 AM	66	-5 to -7	-2 to -4
T12	8:00 AM	72	-5 to -7	-2 to -4

I changed the dilutions of T6, T7, T8, and T9 to -5 to -7 during the trial.

RESULTS:
Results for 18 and 12 growth trial
Good results, some of the dilutions were too low for the time of growth even though I tried to compare to the last round of growth.
Changes to the dilutions need to be made at T4, T6, T7, T8, and T9 to have one extra in the future.
I revised the soft copy of dilutions at 18C to reflect these changes.

3-15-10: Growth Trial 12C

SUMMARY: Establish growth curves for Seawater and 0.25% Tryptone and Seawater media at 12C in triplicate with one control for each media type.
PROCEDURE:
Inoculate 5 ml of 0.25% Tryptone and Seawater media from RE22 Vt stock (RE old stock)
After 24 hours of growth at RT on a rotator, inoculate 100ul of culture to two 25ml of 0.25% Tryptone and Seawater media in conical vials.
Growth this culture for 48 hours at RT on a rotator
Set up the experimental and control flasks:
Four one-liter erlenmeyer flasks with 500 ml of 0.25% Tryptone and Seawater media
Four one-liter erlenmeyer flasks with 750 ml of seawater
Each flask has a stir bar and a foam stopper.
Autoclave these flasks
After autoclaving, set up each flask with a sterile butterfly catheter (needle in the media and female adapter outside the foam stopper) and 1ml serological pipette using sterile instruments to keep the media sterile.
3 flasks of each media will be experimental (in triplicate) flasks and two will be controls (one seawater and one 0.25% Tryptone and Seawater media).
Turn on incubator to the appropriate temp (12C) at least one day before inoculating
At 8 am on 3/15, inoculate three 0.25% Tryptone and Seawater media and three seawater flasks with:
Seawater: inoculate with 75ul of culture
0.25% Tryptone and Seawater media: inoculate with 50ul of culture
Samples will be taken every 6 hours for 72 hours (total of 13 sampling events)
Sampling protocol: 12C Sample Protocol
Dilutions and sampling times:12C Dilutions and Sampling Times

RESULTS:
Results for 18 and 12 growth trial

3-8-10: 25C Pretrial (3)

SUMMARY: Re-do trial for 25C to get a feel for when stationary growth starts at this temp. Grow sample for 48 hours and start the

samples ~24 hours of growth.
PROCEDURE:
Mon: Inoculate from stock in 5 ml 0.25% tryptone and seawater and grow overnight at RT at 12:30pm
Tues: At 2 pm inoculate 25 ml of same media with 100ul of culture and grow 48 hrs to achieve stationary phase of growth. We will use this culture (hopefully in stationary phase) to inoculate our media on Wed.
Wed: At 2 pm.inoculate 25ml of new media (in dulplicate) with 25ul of culture (1:1000) and let grow at specified temps and select times for sampling. Each set of samples is attached to a rotator to mix samples in incubation.

RESULTS:

Hr	CFU
2	9.5E+05
24	1.14E+09
27.5	9.45E+08
43	6.85E+09

2-26-10 Vt Growth Preliminary Trial take 2

SUMMARY: I need a better idea of where the log growth ends for each temp. CF suggested a mini trial where all are grown at different temps and sample past 24/48 hours. In addition, we need to start off with stationary phase when inoculating the cultures to capture the lag phase in these trials.
PROCEDURE:
Thurs: Inoculate from stock in 5 ml 0.25% tryptone and seawater and grow overnight at RT
Friday: At 4 pm inoculate 25 ml of same media with 100ul of culture and grow 48 hrs to achieve stationary phase of growth. We will use this culture (hopefully in stationary phase) to inoculate our media on Monday.
Monday: At 8am, inoculate 25ml of new media (in dulplicate) with 25ul of culture (1:1000) and let grow at specified temps and select times for sampling. Each set of samples is attached to a rotator to mix samples in incubation.

Game plan and dilution series:
Pretrial Schedule and Results

RESULTS:
See link above for CFUs calculated.
Note: the 25C samples were actually incubated at ~28C (as read on the thermometer in the incubator). This may cause faster growth.

The growth results of the Vt at 25C were troublesome and I didn't end up getting a good range of the growth curve. I will try again next week with a new inoculation next week.

The growth curves for 12 and 18C looked okay and I think I may have enough information to start the 12C trial next week.

Next Steps:
Plan out sampling times for the 12C trial, when to start the inoculations, how much to inoculate and any help, if needed.
Figure out what to do with the old samples, since we have to start the series over.
Make sure the inoculations are at stationary growth. This may be dependent on the 25C pretrial results.

2-16-10 Vt Growth Trial (cont'd)

SUMMARY: Start inoculating for growth trial on Thursday the 19th
PROCEDURE:
Took the *new* stock from the -80 and inoculated 5 ml of 0.25% tryptone and seawater media at 400pm
Will inoculate two 25ml of same media with 100ul of the 24hr culture tomorrow at 400pm
After 10 hours of growth (0200) I will inoculate all my flasks with 50ul of culture on Thursday morning.

RESULTS:
Growth trial went well. The planned dilution series was pretty much on target and I captured the growth from seawater! All in all the experiment went well, but I didn't capture the stationary end of growth on from 0-39 hrs.
18C Growth

2-16-10 RNA Extractions and Quant

SUMMARY:RNA extracting box #1 of the 25C experiment to find out if the sample size was adequate to produce usable RNA for gene expression analysis in the future. I will adjust the sample sizes if necessary in the future growth experiments. I also have samples stored in RNAlater of 0.22um filtered samples vs pellets from overnight cultures in tryptone media that I am interested in comparing RNA quantities for possible future sample processing.

RNA quantities for possible future sample processing.
PROCEDURE:

Turn on the water bath 55C

Add 0.5 mL TriReagent to pellet and mix with pestle.

Add additonal 0.5 mL TriReagent to the same tube.

Vortex the tube for 15 sec.

add 200uL of chloroform to tube and vortex for ~30 sc

incubate at RT for 5min

Centrifuge for 15 min at full speed

Collect aqueous phase of the tube, do not disturb any other layers, and put aqueous phase into new tube

add 500uL of isopropanol to tube containing RNA, invert to mix

Incubate at RT for 10 min

Spin for 8 min at high speed

Remove supernatant, leaving the pellet undisturbed

Add 1 ml 75% EtOH and briefly vortex

Centrifuge 5 min at 7500 g

Remove supernatant, leaving the pellet undisturbed

Spin again briefly and remove any left over EtOH

Leave tube open to dry pellet at RT for no more than 5 min

Resuspend pellet in 100uL of 0.1% DEPC-H2O and pipette up and down to resuspend the pellet

Incubate in 55 degree C water bath for 5 min

Flick a few times and quant sample using spectrophotometer

Place in -80

TO QUANT:

use nanodrop reader in Roberts Lab

Place 2 uL of DEPC-H2O onto the pedestal to blank the spectrophotometer

Pipette 2 uL of RNA sample onto pedestal

Press "Measure" and record findings

Preliminary Quant (NG/UL) Results
before **DNasing:**

DNase Procedure:
Add to PCR tube:
2.5 ul DNase Buffer
1 ul turbo DNase
20.5 ul RNA sample
TOTAL - 24 ul

Incubate samples for 30min at 37C

After 30 min, add 1 ul of turbo DNase

Incubate samples for 30min at 37C

After 30 min add 2.5 ul Inactivation Reagent

Leave at RT for 2 min, mixing occasionally

Spin down at 10000 ref for 1.5 min

Transfer supernatant to new tube.

Quant and normalize to the lowest RNA value.

Results:

I didn't have time to DNAse the samples that I've selected but I the RNA ng/ul are:

RNA subset of Vt Growth

2-10-10 Growth Trial Set up

-SUMMARY: Starting the Vt growth trial and first temp is 25C.
PROCEDURE:
Set up:

- **4 one liter flasks with 500ml 0.25% tryptone and seawater (one control left uninoculated)**
- **4 one liter flasks with 500 ml of straight seawater (one control left uninoculated)**
- **10 full racks of dilution tubes (9ml seawater) dispensed by RH lab aliquoter**
- **Labeled cryovial tubes for each time period**
    - **RNA and DNA samples will be done in duplicate for each flask**
- **400 plates of T1N2 agar media**
- **4 liters of 0.25% tryptone and seawater broth**
- **Labeled tubes for aliquoting samples into the flask**

Samples to be taken from each flask every 2 hours after inoculation for ~24 hrs.
Samples will be aliquoted into cryovial vials for DNA and RNA. One ml will be used for dilution series for plating.
Plates will be grown at RT for 24-48 hrs and read by Image J colony counter.

Samples taken from each flask will be aliquoted like this each sampling period:

	Tryptone Media	Seawater
RNA	2	6

DNA	2	6
Dilutions	1	1
Extra	1	1
TOTAL	6 mL	14 mL

Sampling Protocol: Sampling Protocol - Vt Growth Trials

To inoculate the flasks, Vt RE 22 from the old, original stock by Ralph will be grown for 24 hrs in 5 ml of 0.25% tryptone and seawater broth. 100ul of this broth will be inoculated into 25 ml of 0.25% tryptone and seawater broth after 24 hours. After 24hrs of growth of this second inoculation, 100ul will be inoculated into another 25 ml 0.25% tryptone and seawater broth. This third inoculation will be grown for 8 hours and then be used to inoculate the experimental cultures. This should yield growth of ~1.5 x 10^7 CFUs per ml.

100ul will be taken from the 8 hr growth and dispensed into each experimental flask containing 500ml of 0.25% tryptone and seawater broth or seawater.

Samples will be taken every 2 hours, including time of inoculation (T0). See Protocol above.

To set up flasks:
500ml media or seawater into 1L flask
Stir rod
Foam stopper
Wrap in foil
Autoclave this set up

After autoclaving, sterilely place butterfly catheter and 1 ml serological pipet into the container and put foil back on.
Add tubing with a 0.22um filter attached to the 1 ml pipet (squirt EtoH into the tube connecting the filter and pipet and let dry before attaching)
Run air through the pipet for 24 hours before inoculation.
After 24 hours check for any visual growth in the media.
Plate a couple drops of each media, drawing samples from the butterfly catheter capped end.
Grow for 24 hours to make sure media is not contaminated.
Inoculate and sample!

RESULTS:
First inoculation of the samples was late. I inoculated the 1:250 sample at 4:30pm on Wed. and arrived here at 3 am. That leaves approx 10 hours of growth (not 8 as originally planned). I inoculated each flask with 50ul of culture (not 100ul as originally stated).

I think the inoculation was approx 1.3 x 10^4, may have been more.

Plate Count Data:

2-5-10 Vt Old vs New Stock and Inoculation Calculations

SUMMARY: Determine whether stock of Vt taken at 6 hrs (not 10 as stated below) grows the same as the old stock of RE 22. Also determine best inoculation time for experiments starting on 2/11.
PROCEDURE:
Inoculate duplicate samples of RE22 old stock and new stock (taken 1/30) and perform plate counts to compare at 24hrs, 8 hrs and 16 hrs of growth.

Streak each culture on a plate to examine colony morphology similarity between the two stocks.
Inoculate 5 ml of 0.25% Tryptone and Seawater media with old stock and new stock in duplicate (#1).
Grew for 14 hrs
I took 100ul of culture from each tube and inoculate 25ml of 0.25% Tryptone and Seawater media
Grew this (#2) culture for 10.5 hrs and inoculated 0.25% Tryptone and Seawater media with 100ul of culture (1:250) to make #3.
#3 was grown for 23 hours and inoculated the same way (1:250) with 100ul of culture to 25 ml of 0.25% Tryptone and Seawater media to make #4.

Plate counts were performed on #3 at 24 hours to determine CFUs for 24 hour culture of both stocks.
Plate counts were performed at 8 hours and again at 16 hours for both stocks.
I will use this data to determine how many hours of growth and what quantity to inoculate the experiment with.

RESULTS:
Plate Counts for Old vs. New
Morphology of each plate looked similar.
16hr plates for inoculation #4 did not turn out. They were still wet after 24 hours. I think this may have to do with using wet plates. The liquid was pooled and cells may have continued to grow in the mini-culture before drying. There were TNTC colonies on all the plates at all the dilutions ranging from -6 to -8. I think that the dilutions were too small as well. Maybe try -7 to -9 at 16hrs.

Otherwise the 24 hour culture for 3 turned out well. (Two sets had no growth, don't know why) These plates had ~10^10 - 10^13.

The 8 hour samples (inoculation #4) were the best with all plates showing growth. These quantities ranged from 1.5 x 10^7 and 2.0 x 10^8.
The dilution range was sufficient at -3 to -6

The dilution range was sufficient at -5 to -6.

1-28-10 Vt inoculation and Plate count prep

SUMMARY: verify plate count readings of the 3 media solutions: 0.25, 0.5, and 1% tryptone media
PROCEDURE:
Inoculate 2 tubes of 5 ml of the 3 medias with stock Vt and grow overnight (2pm 1/28)

1/29:
Perform plate counts in duplicate on the overnight culture - up to 10^-12
Take 0.25 ml of this culture and inoculate into 1 conical vial of 24.75 ml media and grow overnight (1/29 at 9am)
Plate 4 hour growth (1pm) in duplicate up to 10^-6
Plate 6.5 hour growth (3:30pm) in duplicate up to 10^-8
Plate 11 hour growth (8 pm) in duplicate up to 10^-11

Each time plate counts are done, 1 ml of culture is put into microvials in duplicate and spun at 13000rpm to pellet.
Supernatant is taken off and pellets are frozen at -80 for qPCR.

1/30
The next day, take 0.25 ml of this culture and inoculate into 2 conical vials of 24.75 ml of each media and grow overnight
Perform serial dilutions on the overnight culture (~20 hrs of growth) to10^-9 and plate out in duplicate
Perform serial dilutions to 10^-5 at 5.5 hours and plate out in duplicate.
Perform serial dilutions on the overnight culture (~18 hrs of growth) to 10^-11.

Read plates in the morning and record.
Plate these in triplicate and try plate counts up to 10^-11.

Read plates in the morning and record.

Start new stock of RE22 with 15% glycerol of the ~10 hr growth

RESULTS:
Pretrial Plate Counts

From this pretrial, it looks like I overshot the dilutions a little because I had too much growth on the first and second inoculations. -7 to -9 were too high of dilutions for the 3 hour mark and -10 to -12 were too high for the 6 hour mark. Looking at the second inoculation data, -4 to -5 were too low for the 3 hour mark and -6 to -7 were too low for the 6.5 hour mark with too many colonies to count on some plates.

For future counts I should aim a little broader with maybe 3-4 dilutions for each one.
For RT samples:
3 hour: try -5 to -7
6 hour: try -7 to -9 or 10
Need samples at:
8 hour (try -9 to -11)
10 hour (try -10 to -12)
12 hour (-11 to -13)
14 hour...maybe

Conclusions about the 0.25% tryptone media with seawater. I think they will do what we want with that dilution. Looks like they do perform a bit slower, but not a drastic amount of change. I wish I had data from the 3rd inoculation at 6 hours, but from the 2nd inoculation, it looks like just a difference of 100 or so colonies between 1% and 0.25% tryptone. The earlier time points look very similar (not surprisingly).

Next steps: set up growth trials at different temperatures.
Gather equipment, sterilize and set up space.

1-25-10 Vt inoculation and Plate count prep

SUMMARY: verify plate count readings of the 3 media solutions: 0.25, 0.5, and 1% tryptone media
PROCEDURE:
Inoculate 2 tubes of 5 ml of the 3 medias with stock Vt and grow overnight
Take 0.25 ml of this culture and inoculate into 1 conical vial of 25ml media and grow overnight (1/26 at 1pm)
The next day, take 0.25 ml of this culture and inoculate into 2 conical vials of 25 ml media and grow overnight (1/27at 9 am)
Perform serial dilutions on the overnight culture (~20 hrs of growth) to10^-9 and plate out in duplicate
Perform serial dilutions to 10^-5 at 5.5 hours (2:30pm) and plate out in duplicate.
Perform serial dilutions on the overnight culture (~18 hrs of growth) to 10^-11.

Read plates in the morning and record.
Plate these in triplicate and try plate counts up to 10^-12
Read plates in the morning and record.

RESULTS:
Overnight culture (~18hrs) (these dilutions were accurate, see note below)
0.25%: 10^-10 - 496; 10^-10 - 24 (both plates) This doesn't seem right

0.25%: 10^-10 - 498, 10^-10 - 24 (both plates) This doesn't seem right
0.5%: 10^-12 - 200 and 165
1%: 10^-10 - 170 and 182

I realized yesterday that some of my dilution tubes had an incorrect amount of diluent. I don't think I trust the plate counts I had performed this week. I will start fresh with a new culture today (1/28).

1-20-10 OD readings test at NOAA

SUMMARY: Grow up bacteria in the different tryptone media and test under NOAA's spectrophotometer to find the best wavelength used for sensitivity in reading the Vt dilutions.
PROCEDURE:
Aliquot 5ml of 0.25, 0.5, and 1% tryptone and seawater media into falcon tubes.
Inoculate the tubes with Vt strain RE22
Grow overnight at RT on a rotator for aeration
Take over to NOAA on 1/21 and meet up with Linda to use spectrophotometer.
Blanks consist of seawater with a dilution series of each media (0.25, 0.5, 1%) up to 10^-9.

RESULTS:

Media Trypt Concentration	Dilution	400	500	600	700	800
0.25	10^0	1.684	1.632	1.393	1.12	0.869
0.5	10^0	1.678	1.681	1.528	1.318	1.061
1	10^0	1.671	1.706	1.59	1.411	1.164
0.25	10^-2	0.078	0.047	0.03	0.021	0.014
0.25	10^-3	0.007	0.006	0.003	0.004	0.004
0.25	10^-4	-0.006	-0.002	-0.004	-0.004	-0.004
1	10^-1	0.564	0.429	0.314	0.24	0.186
1	10^-2	0.069	0.048	0.033	0.022	0.015
1	10^-3	-0.04	-0.032	-0.032	-0.028	-0.028
1	10^-4	-0.005	-0.003	-0.006	-0.004	-0.007

Plate counts 48 hours			
Plate 1	Plate 2	Average:	Original Culture CFUs:
TNTC	TNTC		TNTC
111 (10^-10)	107 (10^-10)	109	1.09 x 10^13
170 (10^-12)	170 (10^-12)	170	1.7 x 10^15

The OD wavelengths were not sensitive enough past 10^-3 or -4, even the lowest one (400). This isn't going to work for our experiments. I think we may need to rely on just plate counts and qPCR.

I also wanted to check the OD readings on the original sample (10^0) and they seem very similar, which is a great thing since I am interested in using the 0.25% tryptone media for my experiments.

Plate Counts:
I performed the plate counts in duplicate but the first set did not turn out. So I ended up with only one set of readable plates and the 0.25% tryptone media wasn't readable at all.
Results for the plate counts:
At 10^-9 for everyone:
1% tryptone media: 609 and 679 colonies for an average of 6.44 x 10^12 cfu
0.5% tryptone media: 654 colonies making it 6.54 x 10^12 cfu
NEXT STEPS:
Solidify the plate counts and concentration of the Vt.

1-19-10 Media Trials

SUMMARY: Testing media pH to see which media recipe will work best for our experiments.
PROCEDURE:
Make 0.25%, 0.5% and 1% tryptone + seawater media, test pH, autoclave and test pH again.
Take subsets of these media and grow RE22, grow for 24 hrs and plate count.

0.25% Tryptone = 2.5g tryptone media added to 1L of seawater
0.5% Tryptone = 5g tryptone media added to 1L of seawater
1% Tryptone = 10g tryptone media added to 1L of seawater

Put stir bar in each bottle and spin until trypone is dissolved
Test pH with the pH probe in 236
Autoclave

RESULTS:
Before autoclaving:
0.25% Tryptone - 7.111 pH
0.5% Tryptone - 7.03 pH
1% Tryptone - 6.927 pH

After autoclaving:
0.25% Tryptone - 8.05 pH
0.5% Tryptone - 7.575 pH
1% Tryptone - 7.225 pH

CONCLUSIONS/NEXT STEPS:
So it looks like 0.25% tryptone +seawater broth is ~the right pH. Tomorrow I will inoculate the 3 concentrations separately and grow overnight. On thurs I will do some plate counts to see if they all grow at approx the same rate. I am also going to NOAA on Thursday afternoon to use the spectophotometer to see which wavelength works best for our experiment.

1-14-10: Clean Room vs. PCR Hood test-

SUMMARY: Make MM in clean room and aliquot and close lids on 5 neg controls (should be neg). Then take MM out to the 240 clean room and aliquot the MM and add in the PCR water in the hood used in the last PCR round.
PROCEDURE:
USE EVERYTHING NEW. Only thing not new used here was primers.

	Vol (ul) per rxn	1. rxns =	12
PCR H20	4.75		57.00
5xBuffer	5.00		60.00
MgCl2	2.00		24.00
BSA	1.00		12.00
dNTPs	0.50		6.00
F Primer (10mM)	0.75		9.00
R Primer (10mM)	0.75		9.00
Taq	0.25		3.00
Template	10.00		Add individually

Same thermocyler parameters as the last round.
Run on 1% agarose gel at 100v for 1 hour to visulalize band ~500-1000 bp.

RESULTS: Contamination everywhere. All lanes have product, some have more than one band.
PCR picture: 1-15-10

CONCLUSION:
Primers were contaminated. Must have been the TE buffer used to dilute the primers. Next time use the clean hood for diluting primers with autoclaved TE.
Order primers again and start over.
Rerun this test again, should be clean.

1-13-10: Re-Try the ITS PCR

SUMMARY: ITS PCR and send out for sequencing of all tubiashii strains I have on hand.
PROCEDURE:
I diluted the new primers in new TE buffer, used new reagents and PCR water.

Recipe for ITS Sequencing:

	Vol (ul) per rxn	1. rxns =	28

PCR H20	4.75		133.00
5xBuffer	5.00		140.00
MgCl2	2.00		56.00
BSA	1.00		28.00
dNTPs	0.50		14.00
F Primer (10mM)	0.75		21.00
R Primer (10mM)	0.75		21.00
Taq	0.25		7.00
Template	10.00		Add individually
TOTAL	25.00		

Thermocycler:	Vt_IGS
94 for 2 min	
94 for 1 min	Step 2-4: 35 cycles
55 **for 1:30 min**	
72 for 2:30 min	
72 for 10 min	
4 for ever	

For 8 strains**:**
19106, 19109, RE22, RE100, RE101, RE98, RE60, RE61
Run on 1% agarose gel at 100v for 1 hour to visulalize band ~500-1000 bp

RESULTS: Still contamination
PCR Picture:1-14-10

NEXT STEPS:
Prepare MM in clean room and compare with MM from PCR hood in 240.

1-12-10: Contamination Isolation

SUMMARY: Find source of contamination
I will reorder the primers
Use new TE for primers
Use new water
It must have happened in the hood. I closed the negatives before exiting the hood.
Potential contamination: all reagents, water, primers, tubes

PROCEDURE:
Run PCR H2O and primer set by themselves and see what lights up.
(Use new water for recipe.)

PCR H20	4.75		28.50
5xBuffer	5.00		30.00
MgCl2	2.00		12.00
BSA	1.00		6.00
dNTPs	0.50		3.00
F Primer (10mM)	0.75		4.50
R Primer (10mM)	0.75		4.50
Taq	0.25		1.50
Template	10.00		Add individually
TOTAL	25.00		

Thermocycler parameters same as ITS run.
Run on 1% agarose gel at 100v for 1 hour to visualize band.

RESULTS:
All bands everywhere!
1-12-10

NEXT STEPS:
Start anew with everything. I reordered the primer and they should be here tomorrow.
Dilute new primers and start new with everything - reagents, water, TE for primer dilution...
Re-do the ITS PCR

1-11-10: DNA isolation, ITS PCR and DNA sequencing

SUMMARY: DNA isolation of Vt strains RE60 and 61
PROCEDURE:
Prepare water bath, incubator (56C) and place InstaGene solution on stir plate
Grew the 2 strains over the weekend and picked isolated colonies
Placed colonies in 1 ml sterile water
Centrifuged for 1 min at 12000rpm
Remove supernatant
Add 200uL of InstaGene to pellet
Incubate at 56C for 15-30 min
Vortex for 10 sec
Place in 100C water bath for 8 min
Vortex for 10 sec
Spin at 11000 rpm for 2-3 min
Freeze stock
Use 20ul template per 50ul rxn

SUMMARY: ITS PCR and send out for sequencing of all tubiashii strains I have on hand.
PROCEDURE:
For ITS Sequencing:

	Vol (ul) per rxn	1. rxns =	28
PCR H20	4.75		133.00
5xBuffer	5.00		140.00
MgCl2	2.00		56.00
BSA	1.00		28.00
dNTPs	0.50		14.00
F Primer (10mM)	0.75		21.00
R Primer (10mM)	0.75		21.00
Taq	0.25		7.00
Template	10.00		Add individually
TOTAL	25.00		

Thermocycler:	Vt_IGS
94 for 2 min	
94 for 1 min	Step 2-4: 35 cycles
55 **for 1:30 min**	
72 for 2:30 min	
72 for 10 min	
4 for ever	

For 8 strains:
19106, 19109, RE22, RE100, RE101, RE98, RE60, RE61
Run on 1% agarose gel at 100v for 1 hour to visulalize band ~500-1000 bp

RESULTS:
Contamination! Need to re-run water and primers solo to isolate source.

PCR Picture:
1-11-10

SUMMARY: Qiagen Qiaquick PCR Clean-up for Sequencing
PROCEDURE:
Add 5:1 volume of Buffer PB to PCR sample (
Check color, make sure it's yellow
Use Qiaspin column to put sample in and centrifuge for 1 min at 13000rpm
Make sure volume of column tube can hold sample (~700ul max!)
Discard flow-thru, put back into the column
Add 750 uL Buffer PE
Spin for 60 sec at 13000 rpm
Discard flowthru and put back into column
Spin for another 1min
Put filter column into clean labeled microcentrifuge tube
Add 30ul of Buffer EB into the column and let stand for 10 min (This is different than specified in the protocol)

Spin for 1 min

SUMMARY: Sequencing Submittal Protocol
PROCEDURE:
Dilute F and R (10uM) primers 1:10
Add 9 ul of TE to 1 ul of each primer
Label top of flip tube in sequential order
Add:
0.8 ul of diluted primer
1 ul of cleaned PCR product
Add PCR H2O to make 12 ul total
See Sequencing Submit Log

1-8-10: ITS PCR using Lee Vt primers cont'd

SUMMARY: Test primers and sequence ITS region of all Vt strains to develop primer for all Vt strains for qPCR.
I ordered ITS primers (forward 16S primers and reverse 23S primers) to isolate the ITS region used in the Lee et al 2002 paper and reconstituted down to 10uM/ul.
16/23S - F: TTGTACACACCGCCCGTC
16/23S - R: CCTTTCCCTCACGGTACTG

PROCEDURE:
Try reducing the MgCl from 2.0 ul to 1.5 ul to lower specificity of the ITS primers used in the last procedure.

	Vol (ul) per rxn	1. rxns =	11
PCR H20	4.75		52.25
5xBuffer	5.00		55.00
MgCl2	1.50		16.50
BSA	1.00		11.00
dNTPs	0.50		5.50
F Primer (10mM)	0.75		8.25
R Primer (10mM)	0.75		8.25
Taq	0.25		2.75
Template	10.00		Add individually
TOTAL	24.50		

Test Strains: 19106 (pos control), RE22 and RE100
Thermocycler parameters are the same as specified on 1-5-10 (Vt-ITS protocol on thermocycler)

RESULTS:
No bands on anything. I don't know why there wasn't even a band on the 19106. I am moving forward with sequencing the ITS region (below) and moving on from there.

PCR picture:
1-8-10

1-6-10:Testing Vt IGS primers from Lee et al 2002 (try 2)

SUMMARY:
I double checked the primer sequences and they are correct on the forms.
I re-diluted the primer set from the 100uM to 10uM/uL and made the master mix as stated above for the 17 rxns.
I changed the thermocycler parameters to match the paper's protocol:

Thermocycler:	
94 for 2 min	
94 for 1 min	Step 2-4: 35 cycles
55 **for 1:30 min**	
72 for 2:30 min	
72 for 10 min	
4 for ever	

Product will be run on a 1% agarose gel at 100v for 1 hour.
Product size should be 394 bp

RESULTS:
Correct band size and good bands (in dup) for ATCC 19106; faint bands (in dup) the correct size for ATCC 19109; No bands present for WT strains RE22, RE100, and RE101.

PCR Picture:
1-6-10

CONCLUSIONS:
Well, looks like these primers work okay with the type strains, but not on the wild strains. I guess I need to develop new primers. Do I need to sequence the WT strains in order to get them to align to develop the primers? Then I would need to cross check them against related species.

1-5-10: Testing Vt IGS primers from Lee et al 2002

SUMMARY:
I skimmed the paper again to look over Tm of the primers. Looking in my notebook, I tried running these primers on a Vt strain a couple months ago with no luck (strain not recorded) so I am trying this again with a lower Tm, specified by the paper, and with 5 Vt strains:

19106
19109
RE22
RE100
RE101

PROTOCOL:
Recipe for 5 strains (17 rxns):

PCR H20	4.75	80.75
5xBuffer	5.00	85
MgCl2	2.00	34
BSA	1.00	17
dNTPs	0.50	8.5
F Primer (10mM)	0.75	12.75
R Primer (10mM)	0.75	12.75
Taq	0.25	4.25
Template	10.00	Add individually
TOTAL	25.00	

Thermocycler:	
95 for 3 min	
95 for 15 sec	
55** for 1 min	} 35 - 40 cycles
72 for 30 sec	
72 for 10 min	
22 for 10 min	

Product will be run on a 1% agarose gel at 100v for 1 hour.

RESULTS:
Didn't work. No bands at all on any of the strains.
CONCLUSIONS/NEXT STEPS:
I will re-mix the original batch of primers (100uM) and dilute to 10uM. I'll try the recipe again to see if maybe it was a problem with the dilution.
Double check the primer sequences
Double check the thermocycler recipe.
Re-order the primer and try again

PCR Picture:
1-5-10