AGOURON COURSE 2012
MOLECULAR BIOLOGY PROTOCOLS

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I. WATER SAMPLING

1) Collect seawater sample from the CTD rosette via sterile (acid washed) 4 L polycarbonate or polypropylene containers equipped with tubing and spigot.
2) Using a peristaltic pump and sterile (acid washed & autoclaved) silicone tubing, prime the tubing with ca. 100 ml of raw sample water. After priming, filter the seawater sample through a 25 mm diameter, 0.2 µm pore-sized Supor (Supor-200) membrane in a clean swinnex filter holder. Keep track of the total volume filtered through the membranes by collecting the filtrate in a collection container, and measure with a graduated cylinder. [NOTE: a prefiltration step is optional: 25 mm diameter, 1.6 µm pore-sized GF-A glass fiber filter loaded in a swinnex filter holder. This is not really necessary in the oligotrophic North Pacific]
3) Once filtration is complete (i.e. filtration rate decreases to a drip), remove the swinnex from the pump tubing and blow out the remaining liquid in the swinnex holders using a sterile disposable syringe.
4) Using ethanol-cleaned and/or flamed forceps, place the membrane filter into a 2 ml screw-cap tube containing appropriate storage buffer (500 µl volume): DNA lysis buffer for DNA, and Qiagen buffer RLT for RNA (with β-mercaptoethanol added at a concentration of 10 µl per ml of RLT*)*. The sample tubes for RNA extraction should also be pre-loaded with autoclaved zirconia beads (0.1 g, 0.1 mm diameter, BioSpec cat. # 11079101z). Good tubes are: Sarstedt, 2 ml, cat. # 72.693.005.
5) Store tubes at -80ºC or in liquid nitrogen until extraction.
6) Bottles, tubing, and filter holders should be rinsed thoroughly with MQ water soon after sampling is complete.

   To make 200 ml total of DNA lysis buffer (20 mM Tris HCL, pH 8.0, 2 mM EDTA, pH 8.0, 1.2% Triton X and 20 mg ml^{-1} lysozyme), add the following to 180 ml ultrapure water:

   4 ml 1M Tris (pH 8.0)
   800 µl 0.5M EDTA
   2.4 g Triton X
   4 g Lysozyme

   Bring up to 200 ml with ultrapure water
   Aliquot 500 µl per screwcap tube
   Store buffer at -20^{o}C

II. NUCLEIC ACID EXTRACTION

A) Genomic DNA Extraction

Important notes: this protocol is adapted from the Qiagen DNeasy Tissue Kit (Qiagen Cat. #69504) instructions. Be sure that you check the particular instructions that come with your kit in order to confirm that the volumes listed below remain accurate. The "Gram-Positive Bacteria protocol" is used here. All reagents are supplied with the kit except for molecular biology grade ethanol and ultrapure water.

1) Place ultrapure water at 70^{o}C (200 µl per sample).
2) Remove the sample filters from -80^{o}C or liquid nitrogen and place at 37^{o}C for 1 hr, vortexing every 15 min.
3) After 1 hr, remove the samples from the incubator and add 70 µl of proteinase K and 556 µl of buffer AL to each tube. Vortex and place the tubes at 56^{o}C for 30 min.
4) After incubation, add 556 µl molecular biology grade ethanol to each tube and vortex.
5) Transfer the samples into DNeasy spin columns and centrifuge at 8000 rpm for 1 min in a microcentrifuge. Discard the flow-thru and repeat with whatever sample volume remains, in order to get all of the sample volume onto the column. Place the spin columns in new collection tubes (provided).
6) Add 500 µl of buffer AW1 (with ethanol already added per kit instructions) to each tube and centrifuge at 8000 rpm for 1 min. Discard the flow-thru.
7) Add 500 µl of buffer AW2 (with ethanol already added per kit instructions) to each tube and centrifuge at 13,300 rpm for 3 min.
8) Discard the collection tubes and place the spin columns in labeled 1.5 ml microcentrifuge tubes. Optional - allow tubes to dry with caps open at 70^{o}C for up to 10 min.
9) To elute the genomic DNA, add 100 µl of preheated ultrapure water (or buffer AE) to each tube. Incubate at room temp. for 1 min.
10) Centrifuge at 8000 rpm for 1 min.
11) Repeat steps 9 and 10 in order to obtain a final elution volume of 200 µl.
12) Discard the spin columns, close the cap, and label and store the genomic DNA samples at -20^{o}C.

B) RNA Extraction

Important notes: this protocol is adapted from the Qiagen RNeasy Mini Kit (Qiagen Cat. #74106) instructions. Be sure that you check the particular instructions that come with your kit in order to confirm that the volumes listed below remain accurate. All reagents are supplied with the kit except for β-mercaptoethanol, molecular biology grade ethanol and ultrapure water. Samples should already be at -80˚C and contain zirconia beads.

1) Prepare the DNase I stock solution by dissolving the solid DNase I by adding 550 µl RNase-free water and inverting the tube several times. Do not vortex.
2) Prepare DNase Master Mix in a microcentrifuge tube by adding 10 µl of DNase I stock solution to 70 µl Buffer RDD per sample. Account for pipetting error (e.g. prepare 11 for 10 samples).
3) Remove sample tubes from the freezer and place on ice.
4) Place sample tubes in bead beater and beat at the highest speed setting (6.0 setting on the Biospec Fast Prep bead beater) for 30 seconds.
5) Place sample tubes on ice for ~5 min or until cool.
6) Repeat beating and cooling for a total of 3x times.
7) With samples on ice, add 250 µl of 100% molecular biology grade ethanol to each sample. Mix by pipetting and then transfer the sample onto the Qiagen RNeasy mini column with 2 ml collection tube. Be careful not to transfer any of the beads. This may need to be done over two steps due to the volume of sample.
8) Centrifuge for 15 seconds at 14,000 rpm. Discard the flow through. Repeat until the entire sample has been transferred into the mini columns.
9) Pipette 350 µl of buffer RW1 onto the RNeasy mini column.
10) Centrifuge for 15 seconds at >14,000 rpm to wash. Discard flow through.
11) Pipette 80 µl of the DNase Master Mix onto each RNeasy column.
12) Incubate on the benchtop for approximately an hour.
13) Pipette an additional 350 µl of buffer RW1 onto the mini column.
14) Centrifuge for 15 sec at 14,000 rpm. Discard flow through and collection tube.
15) Add 500 µl of buffer RPE onto column.
16) Centrifuge for 15 seconds at 14,000 rpm. Discard flow through.
17) Add another 500 µl buffer RPE onto column.
18) Centrifuge for 2 mins at 14,000 rpm to dry the column.
19) Place mini columns into pre-labeled 1.5 ml microcentrifuge tubes, and pipette 50 µl of RNase-free water (*supplied with kit*) onto the spin column. Incubate 2 min.
20) Centrifuge for 1 minute at 14,000 rpm to elute RNA.
21) Store samples in -80˚C freezer.

III. POLYMERASE CHAIN REACTION (PCR)

A) PCR of 16S rDNA for Cloning

Note: Platinum Taq DNA Polymerase (Invit# 10966-034) is used in this protocol. Other DNA polymerases will work fine as well, but might require slight tweaking of the protocol. Also, as written the protocol calls for using genomic DNA extracts as template that have not been quantified. Ideally, we are shooting for 10-50 ng of genomic DNA in each reaction. Based on previous experience, that is ca. 1-2 µl of extraction from surface waters. Deep water may require more template.

1) Fill out a PCR worksheet, including all volume calculations per sample and the master mix. In general, for each 20 µl reaction you will need:

	Volume per rxn	Final conc.
dNTPs (10 mM stock)	0.4 µl	0.2 mM each
10X PCR buffer	2 µl	1X
Magnesium chloride (50 mM)	0.6 µl	1.5 mM
Primer 27F-B (10 µM)	0.4 µl	0.2 µM
Primer 1492R (10 µM)	0.4 µl	0.2 µM
Platinum Taq enzyme (5 U/µl)	0.2 µl	1 unit
Template gDNA	1.0 µl	10-50 ng
Ultrapure water	to 20 µl total	

NOTE: Remember to calculate for a positive and negative control as well.

2) On Ice, thaw ultrapure water, dNTPs, 10X buffer, $MgCl_2$, and primers.

3) In a separate ice bucket, place the extracted genomic DNA samples to thaw.

4) To make your master mix, pipette the appropriate amount (according to your worksheet calculations) of the above reagents into a sterile microcentrifuge tube, in the order that they appear at the bottom of the PCR worksheet.

5) Making sure to keep the DNA polymerase enzyme on ice as much as possible, add the appropriate volume of Platinum Taq to the master mix and vortex.

6) Centrifuge the master mix briefly, and then aliquot 19 µl of master mix into each PCR tube (thin-walled) and label.

7) Once thawed, pipette 1 µl of each genomic DNA sample into the appropriate PCR tube. Also prepare a positive (1 µl of any genomic DNA known to amplify previously) control and a negative (1 µl additional ultrapure water) control.

8) Flick PCR tubes gently to mix and load them into the thermocycler. Run with the following cycling conditions.

   a. 94°C for 2 min (1 cycle)
   b. 94°C for 0.5 min, 51°C for 1 min, 72°C for 2 min (34 cycles)
   c. 94°C for 0.5 min, 51°C for 1 min, 72°C for 20 min (1 cycle)
   d. 4°C hold

9) Upon completion, transfer PCR tubes to 4°C, or immediately analyze in an agarose gel.

B) PCR of Proteorhodopsin

Note: Platinum Taq DNA Polymerase (Invit# 10966-034) is used in this protocol. Other DNA polymerases will work fine as well, but might require optimization of the protocol. Also, as written the protocol calls for using genomic DNA extracts as template that have not been quantified. Ideally, we are shooting for 10-50 ng of genomic DNA in each reaction. Based on previous experience, that is ca. 1-2 µl of extraction from surface waters. Deep water may require more template.

The primers used in this assay originate from Sabehi et al., (2005), and target a ca. 330 bp region of the proteorhodopsin gene. The degenerate primers are: RYIDWLfwd, (5' -MGNTAYATHGAYTGGYT-3') and GWAIYPrev, (5' -GGRTADATNGCCCANCC-3'), targeting the conserved RYIDWL and GWAIYP regions in proteorhodopsin proteins, respectively.

1) Fill out a PCR worksheet, including all volume calculations per sample and the master mix. In general, for each 30 µl reaction you will need:

	Volume per rxn	Final conc.
dNTPs (10 mM stock)	0.6 µl	0.2 mM each
10X PCR buffer	3.0 µl	1X
Magnesium chloride (50 mM)	1.5 µl	2.5 mM
Primer *RYIDWLfwd* (10 µM)	2.4 µl	0.8 µM
Primer *GWAIYPrev* (10 µM)	2.4 µl	0.8 µM
Platinum Taq enzyme (5 U/µl)	0.3 µl	1 unit
Template gDNA	2.0 µl	10-50 ng
Ultrapure water	to 30 µl total	

NOTE: Remember to calculate for a positive and negative control as well.

2) Place ultrapure water, dNTPs, 10X buffer, magnesium chloride, and both primers on ice to thaw.

3) In a separate ice bucket, place the extracted genomic DNA samples to thaw.

4) To make your master mix, pipette the appropriate amount (according to your worksheet calculations) of the above reagents into a sterile microcentrifuge tube. NOTE: add reagents in the order that they appear in the master mix calculation at the bottom of the PCR worksheet.

5) Making sure to keep the DNA polymerase enzyme on ice as much as possible, add the appropriate volume of Platinum Taq to the master mix and vortex.

6) Centrifuge the master mix briefly, and then aliquot 28 µl of master mix into each PCR tube (thin-walled) and label.

7) Once thawed, pipette 2 µl of each genomic DNA sample into the appropriate PCR tube. Also prepare a positive (2 µl of any genomic DNA known to amplify previously) control and a negative (2 µl additional ultrapure water) control.

8) Flick PCR tubes gently to mix and load them into the thermocycler. Run with the following cycling conditions.

    e. 94^{o}C for 4 min (1 cycle)

f. 94°C for 0.5 min, 52°C for 1 min, 72°C for 2 min (34 cycles)
g. 94°C for 0.5 min, 52°C for 1 min, 72°C for 10 min (1 cycle)
h. 4°C hold

9) Upon completion, transfer PCR tubes to 4°C, or immediately analyze in an agarose gel.

References

Sabehi G, Loy A, Jung KH, Partha R, Spudich JL, Isaacson T et al. (2005). New insights into metabolic properties of marine bacteria encoding proteorhodopsins. PLoS Biol 3: e273.

Sabehi, G., Kirkup, B. C., Rozenberg, M., Stambler, N., Polz, M. F., & Béjà, O. (2007). Adaptation and spectral tuning in divergent marine proteorhodopsins from the eastern Mediterranean and the Sargasso Seas. ISME J, 1: 48–55.

RYIDWLfwd (5'-3')

MGN	TAY	ATH	GAY	TGG	YT
A A	T	A	T		T
C T	C	T	C		C
G		C			
C					

GWAIYPrev (5'-3')

GGR	TAD	ATN	GCC	CAN	CC
A	A	A		A	
G	G	T		T	
	T	G		G	
		C		C	

C) Reconditioning PCR for 16S rDNA cloning

This is an extra step used when cloning and sequencing 16S rDNA amplification products. Do not use for T-RFLP.

1) Fill out PCR worksheet, including all volume calculations per sample and the master mix. NOTE: for this amplification, you want to perform duplicate reactions per initial PCR reaction, and include a negative control. In general, for each 50 µl reaction you will need:

	Volume per rxn	Final conc.
dNTPs (10 mM stock)	1.0 µl	0.2 mM each
10X PCR buffer	5.0 µl	1X
Magnesium chloride (50 mM)	1.5 µl	1.5 mM
Primer 27F-B (10 µM)	1.0 µl	0.2 µM
Primer 1492R (10 µM)	1.0 µl	0.2 µM
Platinum Taq enzyme (5 U/µl)	0.5 µl	1 unit
Ultrapure water	35 µl	

2) Place ultrapure water, dNTPs, 10X buffer, magnesium chloride, and both primers on ice to thaw.

3) To make your master mix, pipette the appropriate amount (according to your worksheet calculations) of the above reagents into a sterile microcentrifuge tube. NOTE: add reagents in the order that they appear in the master mix calculation at the bottom of the PCR worksheet.

4) Making sure to keep the DNA polymerase enzyme on ice as much as possible, add the appropriate volume of Platinum Taq to the master mix and vortex.

5) Centrifuge the master mix briefly, and then aliquot 45 µl of master mix into each PCR tube (thin-walled) and label.

6) Flick PCR products from initial reaction (to be used as template here) and pipette 5 µl of each sample into duplicate PCR tubes. Also prepare a negative (5 µl additional ultrapure water) control.

7) Flick PCR tubes gently to mix and load them into the thermocycler. Run with the following cycling conditions based on Platinum Taq and the primers listed above:

   94^{o}C for 2 min (1 cycle)
   94^{o}C for 0.5 min, 51^{o}C for 1 min, 72^{o}C for 2 min (2 cycles)
   94^{o}C for 0.5 min, 51^{o}C for 1 min, 72^{o}C for 20 min (1 cycle)
   4^{o}C hold

8) Upon completion, transfer PCR tubes to 4^{o}C until you are able to run an agarose gel using the protocol described previously.

Reference

Thompson et al. (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. Nucleic Acids Res 30: 2083–88.

IV. PCR PRODUCT ANALYSIS

A) Agarose Gel Electrophoresis

This protocol calls for TAE as the running buffer, and SYBR Safe (Invitrogen cat. # S33102) as the fluorescing agent used to visualize the DNA inside the gel. There are many other variants in both running buffer and gel type and grade, and gel stain.

1) Determine the correct amount of agarose and the correct volume of 1X TAE, depending on gel size and concentration. 1% w/v is common, so an example calculation would be: 0.4 g agarose + 40 ml 1X TAE.

2) Pour the agarose powder into an erlenmeyer flask of an appropriate size (at least twice as large as your final volume---i.e. use 250 ml flask for 100 ml gel).

3) Add the 1X TAE and swirl to mix. Microwave until boiling bubbles are first visible (ca. 30 sec.) and remove flask to swirl. Repeat the microwave step until no crystals can be seen during swirling. Avoid boiling over as this will alter the gel concentration and cause a mess in the microwave. Set the flask on the bench top to cool to ca. 55^{o}C (cool enough to hold in hand and no visible steam escaping).

4) During cooling, prepare gel to be poured by rinsing the comb and gel bed and ensuring that the gel box is level and the combs are set correctly.

5) Once cool enough to hold (but still warm), add SYBR Safe to the flask and swirl to mix. SYBR Safe comes as a 10,000x concentrate, so 1 µl per 10 ml of gel.

6) Pour the gel carefully and slowly to minimize bubble formation. Use a sterile pipette tip to pop any bubbles that form or to move them toward the sides of the gel box.

7) Once the gel cools (turns opaque), rock the combs back and forth gently as you pull them straight up and out of the gel. Remove tape or re-orient the gel inside the gel box so it is ready to be run.

8) Pour 1X TAE into gel box to the fill line (or until it just covers the top of the gel).

9) Aliquot 2 µl of loading buffer onto parafilm for each sample. Pipette 5 µl of each PCR product into a drop of loading buffer and pipette up and down to mix. Orient the pipette tip so that it is just inside the first well of the gel and expel the liquid, being careful not to puncture the bottom of the well. Repeat this process with all of the samples, and a DNA mass ladder. Be sure to keep track of which well contains which sample.

10) Put the lid on and plug in the positive and negative connections to the voltage box. Remember that DNA “runs toward red” (assuming your connections are correct!).

11) Set desired voltage and time of run (i.e. 100 volts for 35 min) and start the current. Check to be sure bands are migrating in the proper direction and not out of the gel.

12) After the run ends, carefully remove gel and place it in gel-doc system (or equivalent). Turn off white light and turn on the UV source to expose the DNA bands. Be careful of skin exposure to UV light. Use the camera’s zoom, aperture, and focus controls to take a picture of the gel for archival purposes.

B) PCR Purification

This protocol is adapted from the QIAquick PCR Purification Kit (Qiagen # 28104) instructions. Be sure that you check the instructions that come with your kit in order to confirm that the volumes listed below are accurate, because kit instructions change fairly frequently. All of the reagents are supplied with kit except for molecular biology grade ethanol and ultrapure water.

1) Combine the remaining 45 µl from each duplicate PCR product after reconditioning into 1 tube of 90 µl for each sample.
2) Add 5 volumes of buffer PB to 1 volume of PCR product (i.e. 450 µl buffer PB to 90 µl PCR product) and vortex.
3) Transfer the samples to a labeled QIAquick spin column and centrifuge at 13,300 rpm for 1 min. Discard the flow-thru and place the spin column back in the same collection tube.
4) Add 750 µl buffer PE (with ethanol already added per kit instructions) and centrifuge at 13,300 rpm for 1 min. Discard the flow-thru and place column in the same collection tube.
5) Repeat the above centrifugation (13,300 rpm) for 1.5 min to dry column completely.
6) Place each column in a sterile, labeled 1.5 ml tube and add 30 µl of ultrapure water to the center of each column. Let stand 1 min at room temp., then centrifuge at 13,300 rpm for 1.5 min.
7) Discard the columns, cap the 1.5 ml tubes and store at -20^{o}C.
8) Run a gel of the purified PCR products using the protocol described previously. Use the gel to quantify the purified products.

C) Gel Extraction

This protocol is adapted from the QIAquick Gel Extraction Kit (Qiagen # 28704) instructions. Be sure that you check the instructions that come with your kit in order to confirm that the volumes listed below are accurate, because kit instructions change fairly frequently. All of the reagents are supplied with kit except for molecular biology grade ethanol, 100% isopropanol, and ultrapure water.

1) Before starting, ensure that molecular biology grade ethanol has been added to Buffer PE (see tube for volume), and turn the water bath to 50°C.
2) While limiting your exposure to UV light, excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
3) Weigh the gel slice in a clear tube.
4) Add 3 vol buffer QG to 1 vol gel (100 mg gel = 100 µl). The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, add 6 vol buffer QG.
5) Incubate at to 50°C for 10 min, or until the gel slice has dissolved completely. Vortex every 2-3 min.
6) After the gel has dissolved, check that the color of the mixture remains yellow. If it is orange or violet, add 10 µl of 3M sodium acetate, pH 5.0.
7) Add 1 vol isopropanol to the sample and mix.
8) Transfer the samples to a labeled QIAquick spin column and centrifuge at 13,000 rpm for 1 min. Discard the flow-thru and place the spin column back in the same collection tube. For large volume samples, load and spin the column again.
9) Add 750 µl buffer PE (with ethanol already added per kit instructions) and centrifuge at 13,000 rpm for 1 min. Discard the flow-thru and place column in the same collection tube.
10) Repeat the above centrifugation (13,000 rpm) for 1 min to dry column completely.
11) Place each column in a sterile, labeled 1.5 ml tube and add 50 µl of ultrapure water to the center of each column. Let stand 1 min at room temp, then centrifuge at 13,000 rpm for 1 min. (can let stand up to 4 min to increase yield).
12) Discard the columns, cap the 1.5 ml tubes and store at -20^{o}C.
13) Purified products can be run again on a gel using the protocol described previously in order to quantify the purified products.

V. CLONING

A) Ligation Reaction

This protocol employs the TA Cloning kit (Invitrogen # K2040-40).

1) Before starting, equilibrate a water bath at 14°C.

2) Use the formula below to estimate the amount of PCR product needed to ligate with 50 ng (20 fmoles) of pCR2.1 vector in a 1:1 molar ratio of insert:vector:

$$\text{X ng PCR product} = \frac{(\text{Y bp PCR product})(50 \text{ ng pCR}^{®}2.1 \text{ vector})}{(\text{size in bp of the pCR}^{®}2.1 \text{ vector: } \sim 3900)}$$

Where X ng is the amount of PCR product of Y base pairs to be ligated for a 1:1 vector:insert molar ratio. Use sterile water to dilute your PCR sample if necessary.

3) Allow all tubes to thaw on ice. Centrifuge one vial of pCR2.1 to collect all the liquid in the bottom of the vial.

4) Set up 10 µl ligation reactions as follows:

Fresh PCR product	X µl (determined above)	
10X ligation buffer	1 µl	
pCR2.1 vector (25 ng/µl)	2 µl	
Sterile water - to a final volume of	9 µl	(Note: water + PCR product = 6 µl)
T4 DNA ligase (4.0 Weiss units)	1 µl	

5) Flick ligation tubes to mix and incubate at 14°C for a minimum of 4 hrs (preferably overnight).

B) Transformation Reaction

This protocol employs the Invitrogen One Shot TOP10 Competent Cells.

1) Before starting, equilibrate a water bath to 42°C and an incubator to 37°C, set the SOC out and bring to room temp, and equilibrate LB plates containing 100 µg/ml ampicillin to 37°C. Make sure that the plates are incubated upside down (agar side up).
2) After 30 min, spread each LB plate with 40 µl of 40 mg/ml X-Gal and return to incubator (again, upside down).
3) Centrifuge tubes containing the ligation reactions briefly to collect contents.
4) Remove the appropriate number of 50 µl tubes of One Shot TOP10 competent cells from the freezer and thaw them on ice (ca. 5 minutes). DO NOT VORTEX. Flick gently to mix.
5) Pipette 2 µl of each ligation reaction directly into a vial of competent cells. Gently pipette up and down to mix – DO NOT VORTEX.
6) Incubate the vials on ice for 30 min. Freeze the remaining ligation at -20^{o}C.
7) Heat shock the transformation reaction at 42^{o}C for 30 sec without shaking. Quickly chill the reaction by immediately placing the vials back on ice for 2 min.
8) Add 250 µl of room temp SOC medium to each vial.
9) Shake the vials horizontally at 37^{o}C for 1 hr at 225 rpm.
10) Spread 10-50 µl (perhaps try 20 and 50) of each transformation reaction onto a room temperature LB plate containing ampicillin and X-Gal. Be sure to label the bottom of each plate. Dry the plates right-side up at 37^{o}C with the lids cracked for 15-20 min. Close the lids, invert the plates and incubate them overnight hour at 37^{o}C.
11) Once visible colonies are formed, shift the plates to 4^{o}C for colony color development. Plates can then be sealed with parafilm and stored at 4^{o}C.

C) Culture Growth and Processing

1) Dispense 1 ml LB broth containing 100ug/ml ampicillin (e.g. add 100 µl ampicillin (100 mg/ml) to 100 ml LB) into each well of a sterile deep-well 96-well microtiter plate.
2) Using a sterile toothpick, touch the tip of one white colony and transfer to the corresponding well. Gently swirl the toothpick in the well. Repeat until all wells are full but one. Pick one blue colony for the last well as a control.
3) Seal the deep well plate with a porous adhesive cover and incubate overnight (or ca. 12 hours) at 37°C while shaking. Store at 4°C after incubation if not processing immediately.

VI. T-RFLP

A) PCR Reaction for T-RFLP

Note: this protocol is very similar to the PCR protocol used for cloning, with the following exceptions: the forward primer is end-labeled with a flourochrome; the reverse primer is changed to provide a shorter amplicon, the reaction conditions call for fewer amplification cycles in order to try to limit PCR bias. This reaction should be prepared in subdued lighting.

1) Fill out PCR worksheet, including all volume calculations per sample and the master mix. In general, for each 50 µl reaction you will need:

	Volume per rxn	Final conc.
dNTPs (10 mM stock)	1.0 µl	0.2 mM each
10X PCR buffer	5.0 µl	1X
Magnesium chloride (50 mM)	1.5 µl	1.5 mM
Primer 27F-B* (10 µM)	1.0 µl	0.2 µM
Primer 519R (10 µM)	1.0 µl	0.2 µM
Platinum Taq enzyme (5 U/µl)	0.5 µl	1 unit
Ultrapure water	35 µl	

NOTE: Remember to calculate for a positive and negative control as well.

2) Place ultrapure water, dNTPs, 10X buffer, magnesium chloride, and both primers on ice to thaw.

3) In a separate ice bucket, place the extracted genomic DNA samples to thaw.

4) To make your master mix, pipette the appropriate amount (according to your worksheet calculations) of the above reagents into a sterile microcentrifuge tube. NOTE: add reagents in the order that they appear in the master mix calculation at the bottom of the PCR worksheet.

5) Making sure to keep the DNA polymerase enzyme on ice as much as possible, add the appropriate volume of Platinum Taq to the master mix and vortex.

6) Centrifuge the master mix briefly, and then aliquot 45 µl of master mix into each PCR tube (thin-walled) and label.

7) Once thawed, pipette 5 µl of each genomic DNA sample into the appropriate PCR tube. Also prepare a positive (5 µl of any genomic DNA known to amplify previously) control and a negative (5 µl additional ultrapure water) control.

8) Flick PCR tubes gently to mix and load them into the thermocycler. Run with the following cycling conditions.

PCR program for Platinum Taq and the primers listed above:
94°C for 2 min (1 cycle)
94°C for 0.5 min, 51°C for 1 min, 72°C for 2 min (34 cycles)
94°C for 0.5 min, 51°C for 1 min, 72°C for 20 min (1 cycle)
4°C hold

9) Upon completion, wrap the PCR tubes in foil and transfer to 4^{o}C, or immediately analyze in an agarose gel.

10) Purify successful reactions with the QIAquick PCR Purification kit and protocol described previously.

B) Restriction Digest

1) Add the appropriate volumes of the following reagents to create a restriction master mix depending on the number of reactions.

Per reaction, combine:

Ultrapure H_2O	3.3 µl
Multicore 10x Buffer	1.0 µl
BSA (10mg/ml)	0.2 µl
*Hae*III (10 U/µl)	0.5 µl

2) Be sure to keep the *Hae*III at $^{-}20^{o}C$ as long as possible.

3) Vortex the above master mix and aliquot 5 µl into labeled 0.5 ml eppendorf tubes.

4) Add 5 µl of purified PCR product to the appropriate tubes and vortex.

5) Incubate restrictions at 37ºC for 6.5 hr. and then transfer tubes to $4^{o}C$.

C) Restriction Clean-up

Note: This protocol is adapted from the instructions included with the QIAquick Nucleotide Removal kit (Qiagen # 28304). Be absolutely sure that you check the instructions that come with your kit in order to confirm that the volumes listed below are accurate – kit instructions do change fairly frequently. All reagents are supplied with kit except for molecular biology grade ethanol and ultrapure water.

1) Add 10 volumes of buffer PN to 1 volume of restriction digest (i.e. 100 µl buffer PN to 10 µl restriction digest) and vortex.
2) Pipette the samples into QIAquick spin columns and centrifuge at 6,000 rpm for 1 min. Discard the flow-thru and place the columns into the same collection tubes.
3) Add 750 µl buffer PE (with ethanol already added per kit instructions) and centrifuge at 6,000 rpm for 1 min. Discard the flow-thru and place column in the same collection tube.
4) Centrifuge the columns at 13,300 rpm for 1.5 min to dry the columns.
5) Place each column in a sterile, labeled 1.5 ml tube and add 30 µl of ultrapure water to the center of each column. Let stand 1 min at room temp and then centrifuge at 13,300 rpm for 1.5 min.
6) Discard the columns, cap the 1.5 ml tubes and store at $4^{o}C$ for as little time as possible.
7) Quantify the restriction digest spectrophotometrically, and then dilute or concentrate the samples to an empirically determined target concentration based on trial and error at your sequencing facility. In our case, we concentrate to ca. 12 ng/µl, and submit 2 µl of each sample to be run for fragment analysis on a genetic analyzer.

NO.:_____

PCR WORKSHEET

Name: _____ Date: _____

Thermocycler: _____ Program: _____

Denat: _____ Anneal: _____ Exten: _____ Cycles: _____

Rxn	Template	vol	10X	10 mM dNTPs	MgCl2	50% Acet.	H2O	10 µM prim.F	vol	10 µM prim.R	vol	DNA poly.
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												
17												
18												

Mix:

H2O	_____	_____	_____
Aceta	_____	_____	_____
dntps	_____	_____	_____
10X	_____	_____	_____
BSA	_____	_____	_____
Mg	_____	_____	_____
prmA	_____	_____	_____
prmB	_____	_____	_____
polym	_____	_____	_____
total	_____	_____	_____
per tube	_____	_____	_____

Block setup:

___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___

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