Thursday, 6/21

- Since PCR reactions did not work, we are reconsidering procedures using this webpage: https://docs.google.com/viewer?a=v&pid=gmail&attid=0.1&thid=1380f492712905cf&mt=application/pdf&url=https://mail.google.com/mail/?ui%3D2%26ik%3D8f0fa5e08d%26view%3Datt%26th%3D1380f492712905cf%26attid%3D0.1%26disp%3Dsafe%26realattid%3Db75bb8ed85086e6a_0.1%26zw&sig=AHIEtbR3X3ml7yEny1CFObLmCwedtlfNUg
- Looked up alternative protocols for SDM PCR reactions
- Shadowed Natalie in Isaacs lab
- Worked on setting up the project defense
- Streaked a plate of XL1-Blue competent E. coli
- Finished rough primer designs for disabling threonines to alanines in RiAFP
- Received the sequencing of RIG

Friday, 6/22

- Scavenged and acquired equipment from Rhodes Lab
- Inoculated a colony of XL1 Blue in 100ml of LB
- Made 6 L of LB in various flasks
- Shadowed Natalie running an EtBr gel
- Looked up protocols for preparation of XL1 blue and decided to go with the Inoue method:
  1. When OD at 600 nm reaches .55 of XL1 blue in 220 ml SOB medium, transfer the cell solutions into four 50 ml falcon tubes.

     (SOB: 20.0g tryptone
     5.0g yeast extract
     .5g NaCl
     1L DIW
     Autoclave
     Add 10ml 1M MgCl2 and 10ml 1M MgSO4 through a filter sterilizing system.
     SOC: - prepare only immediately before use
     Add 2ml of filter-sterilized 20% glucose or 1ml filter-sterilized 2M glucose to autoclaved SOB for a total volume of 100 ml
     Or

SOB

     0.5 g NaCl
     5 g yeast extract
     20 g tryptone
     2.5 mM KCl pH to 7.0 with NaOH
     autoclavebefore use
     add sterile MgCl2 ad 10 mM (from unused SOB one may prepare

SOC

     for later transformations by adding sterile glucose for 0.4%))

2. Begin chilling Inoue buffer to become ice-cold while cooling the cell media for at least 10 minutes in an ice bath.
3. Harvest cells by centrifugation at 2500g (3900rpm) at 4C or lower for 10 min.
4. Pour off the medium and invert the tubes on kimwipes for about 2 minutes to dry the pellets from the medium as much as possible.
5. Gently resuspend the pellets in 10 ml of ice-cold Inoue buffer each, while placing the falcon tubes back on ice. (Try using a sterile disposable pipet to break up the pellets.)
6. Combine the contents into 2 falcon tubes and harvest the cells in the same manner as in steps 3 and 4.
7. Repeat step 5, but be as gentle as possible.
8. Add 1.5 ml DMSO (can chill on ice, but make sure that DMSO does not freeze)
   NOTE: Do all steps in conditions as cold as possible.
9. While the suspension is stored on ice for about 10 min, prepare the prechilled (in -20C freezer) sterile eppendorf tubes for aliquoting (110 and/or 215 microliters recommended) by placing the also prechilled open bottom racks on ice.
10. Working as quickly as possible, aliquot the cells, snap freeze in liquid nitrogen, and store in the -80 freezer. If liquid nitrogen is unavailable, can freeze cells in -80C directly, but make sure to place the tubes in the coldest, best ventilated part of the freezer in a way so that the tubes are freeze as quickly as possible – in open bottom racks or spread out on a pre-frozen tray.)

- Prepared the Inoue buffer:

1. Make .5M PIPES by
   A. Dissolving 15.1 g of PIPES in 80 ml of pure H2O (Milli-Q, or equivalent).
   B. Adjust the pH of the solution to 6.7 with 5 M KOH
   C. Add pure H2O to bring the final volume to 100 ml.
   D. Sterilize the solution by filtration through a disposable prerinsed Nalgene filter (0.45-µm pore size) and divide into aliquots.
   E. Store frozen at -20°C
2. Filter sterilize after dissolving the following:

Reagent	**Amount per 200mL**	**Amount per liter**	**Final concentration**
MnCl2•4H2O	2.2 g	10.88 g	55 mM
CaCl2•2H2O	0.44 g	2.20 g	15 mM
KCl	3.73 g	18.65 g	250 mM
PIPES (0.5 M, pH 6.7)	4 ml	20 ml	10 mM
H2O	To 200 ml	to 1 liter	

- Plated freeze-thaw assay samples by adding 200 microliters of each sample per plate + spreading with beads
- minipreped the potential 15-16 mutants
- partially prepared the SOB medium

Saturday, 6/23

- prepared competent XL1 blue cells
- made kanamycin plates

Sunday, 6/24

- checked primer sequence – VR displayed the complete correct sequence or RiAFP, except for presence of "ggt" (glycine) as the first amino acid rather than atg, the start codon.
- Ligated 3 previously DPN1 digested PCR reactions, and 2 nondigested reactons.
- Transformed XL1 blue with 5+6 and 21+22 previously digested PCR reactions using SOB medium and plated the hopeful mutants.
- Made 10mM DNTP mix by diluting 40 microliters (10 microliters of each triphosphate) in 60 microliters of Tris (pure DIW or solution as described in dNTP manual can be used instead)

Monday, 6/25

- Finalized design of last primers
- Useful site: http://www.finnzymes.fi/tm_determination.html
- Diluted and loaded a gel cell with TBE buffer.
- Remade an EtBr gel
- Loaded gel with 4 PCR reactions and RiG plasmid for comparison: (RiG, 5+6, 7+8, 9+10, 29+30, RiG)
    1. Aliquot 5 microliters of loading dye onto the waxy side of parafilm
    2. One sample at a time, including the comparisons like the ladder, add 5 microliters of the DNA and mix thoroughly by pipetting up and down without creating bubbles.
    3. Load the 10 microliters of DNA into wells, and run at 100-120 milliamperes until the DNA has traveled about 2/3 down

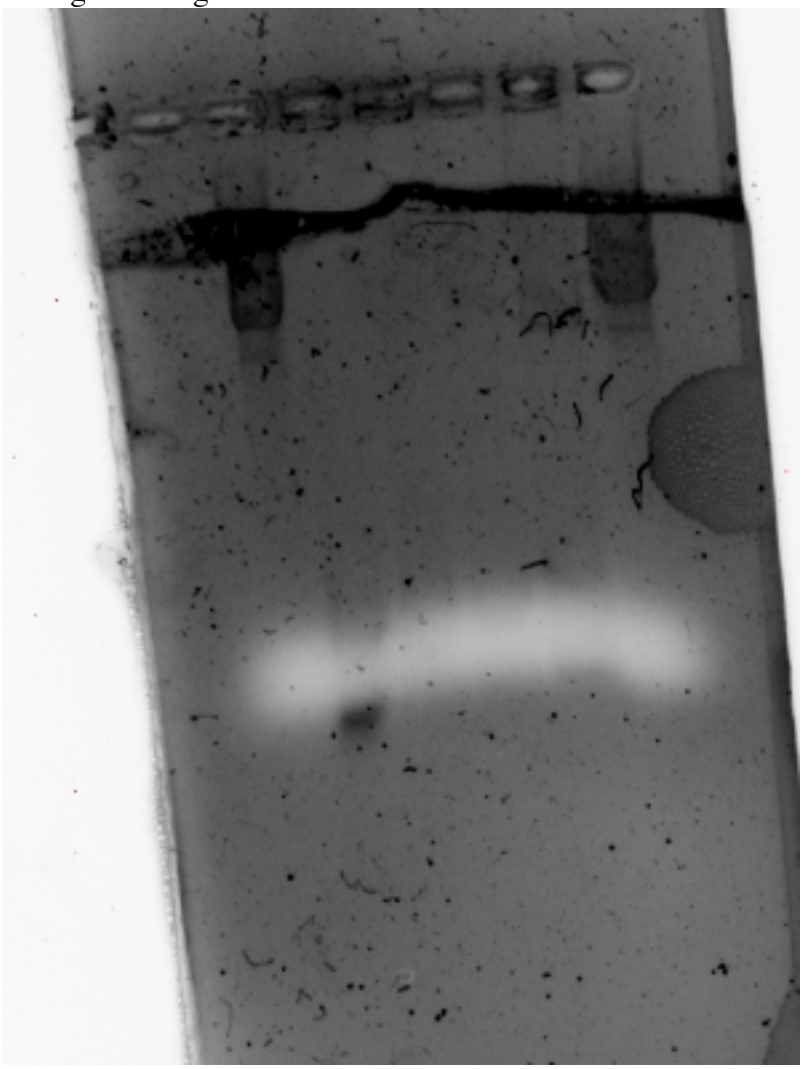
Useful Protocol:

http://www.biologie.uni-hamburg.de/b-online/library/bryophytes/agarose.pdf

PROTOCOL FOR AGAROSE GEL ELECTROPHORESIS

1. Prepare gel solution as outlined under Recipes section.
2. Pour the gel when the agarose has cooled to about 55˚C. Insert the proper comb for the particular gel rig. The gel should be allowed to cool until it has set (it will turn whitish and opaque when ready). The amount of agarose depends on the size of the gel rig. Gels should be fairly thin, approximately 1/4 to 1/2 inch.
3. Carefully remove the comb and place the gel in the gel rig with the wells closest to the cathode (black) end. Cover the gel with 1X TAE running buffer.
4. Cut a piece of parafilm and place a 5 µl drop of glycerol loading dye onto the waxy side for each sample to be loaded.
5. Keeping samples on ice, pipette up 5 µl of a sample, wipe the excess oil from the pipette tip with a Kimwipe and add the sample to one of the drops of loading dye.
6. Switch the pipette tip to another pipette set for 10 µl. Mix the sample and loading dye by filling and emptying the pipette a few times then load the mixture into a well.
7. Continue loading the rest of the samples, placing 5 µl of 1 Kb ladder at both ends of the series of samples and between every 10 samples.
8. Place the cover on the gel rig and run the samples towards the anode (red) end. For a small gel, we set the power pack to about 60 ma. For a large gel, we use about 120 ma.

Milliamperage increases during the run, so check it periodically. Stop the run before the bromophenol blue loading dye front exits the gel.
9. Turn off the power pack, remove the gel and place it in a stain box with 40 µl ethidium bromide: 200 ml 1X TAE for approximately 45 minutes. NOTE: Ethidium bromide is light sensitive and must be stored in darkness.
10. Visualize with U.V. light (take proper precautions!) and photograph with a polaroid Photo documentation camera.
11. Dispose of the gel properly. (check to see how your facility handles disposal of ethidium bromide).
- Imaged EtBr gel:



Analysis: 5+6 reaction worked, but not sure if we want the seen bigger piece (cut it out and a plasmid anyway). The 29+30 reaction might have some plasmids, but the others do not.

- rechecked primer concentrations, and primers are present in sufficient quantity.
- Transformed the remaining ligated mutants: 25+26, 21+22, 7+8, and used remainder of T7 and plated on Amp/Kan plates

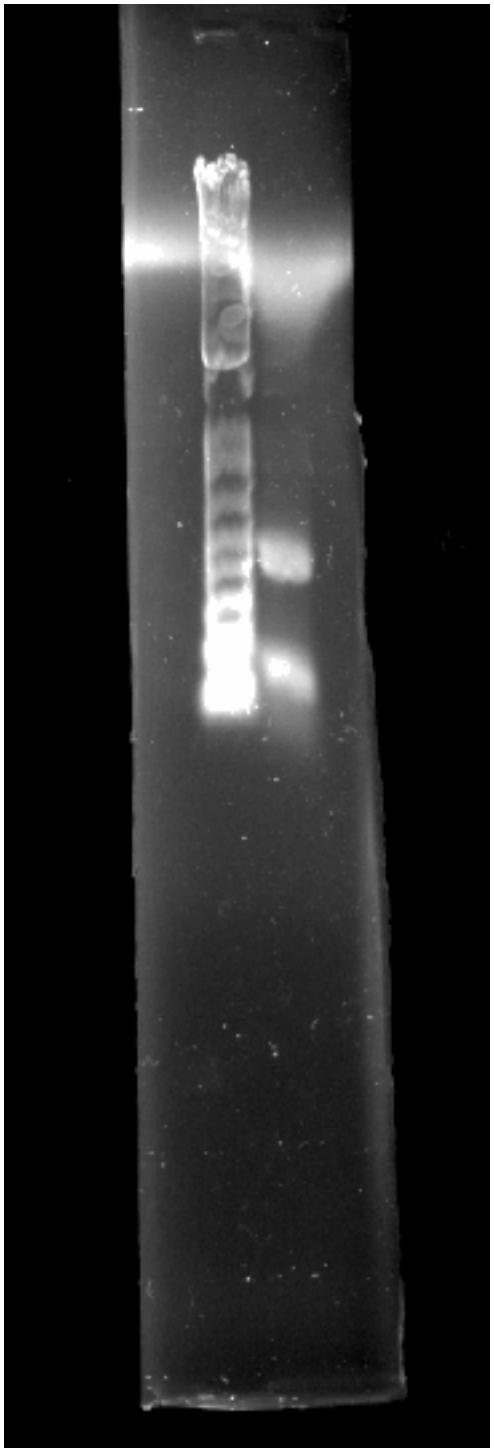
Tuesday, 6/26

- Figured out that the way to get hopefully mutant colones after transformation is to

use competent XL1 Blue prepared using the Inoue method, SOC, and after ligating the products. (Use full 5ul of PCR product for each ligation).
- Restreaked the 15+16 and 21+22 plate to ensure one plasmid type in future miniprep product
- Started growing the previous day's plated colonies
- Made kanamycin plates
- Ligated the remaining 2 PCR reactions/possible mutants and performed transformations and plated them.
- Ran the RiA reaction with a DNA ladder, but this was unsuccessful due to addition of too much dye 1:1, instead of 1:9. Also, need to make new stock gel with EtBr because the consistency is not right=gel is too brittle.

Wednesday, 6/27
- Minipreped possible mutants 7+8, 23+24, 25+26 and T7 plasmid
- Measured the concentrations of these products
- Made appropriate solutions with these and possible 15+16 mutants and submitted them for sequencing.
- Unsuccessfully tried remaking the EtBr gel yet again
- Digested the 2 remaining PCR reactions with DPN1

Thursday, 6.28
- Reran the gel with 5+6 and a 1kb ladder
- Checked out the NCBI Primer Blast website, but could not figure out how to use it for wanted purposes



- Double digested T7 plasmids and 5+6 RiA PCR product according to the following protocol:

1.Combine the following in 2 separate PCR tubes:
A.

Upstream Part Plasmid (T7):	500 ng

PstI:	1 µl
SpeI:	1 µl
10X NEBuffer 2:	5 µl
100X BSA:	0.5 µl
H_2O:	to 50 µl

B.

Downstream Part (5+6PCR rxn.):	500 ng
XbaI:	1 µl
PstI:	1 µl
10X NEBuffer 2:	5 µl
100X BSA:	0.5 µl
H2O:	to 50 µl

2. Incubate the reactions using a PCR machine at 37C for 10 minutes, and then heat kill at 80C for 20 minutes.

- Ligated the double digestion products according to the following protocol:

    1. Combine the following in a microcentrifuge tube:

(T4 DNA Ligase should be added last. Note that the table shows a ligation using a molar ratio of 1:3 vector to insert.)

COMPONENT	**20 µl REACTION**
10X T4 DNA Ligase Buffer*	2 µl
Vector DNA (3 kb)	50 ng (0.025 pmol)
Insert DNA (1 kb)	50 ng (0.076 pmol)
Nuclease-free water	to 20 µl
T4 DNA Ligase	1 µl

** The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.*

2. Gently mix the reaction by pipetting up and down and microfuge briefly.

3. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes. For blunt ends or single base overhangs (as in this case), incubate at 16°C overnight or room temperature for 2 hours *(alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation)*.

4. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

- Transformed DH5 alpha with the recombinant plasmid.

Friday, 6/29

- Minipreped three more possible mutants for sequencing, measured the obtained plasmid concentration, and sent the samples to Keck
- Ordered 20 more primers for threonine row and column disabling by substitution to alanines
- Went to pick up more equipment and supplies from the Roader lab

- Analyzed returned sequences from Keck to find out that the 4 samples have been unsuccessful
- Repeated 15+16 mutation PCR according to the following protocol:
    1. Combine the following for a 12.5 microliter reaction:
        A. 2.5ul 5x Phusion HF buffer
        B. .25ul 10mM dNTP mix
        C. x forward primer for a final concentration of .5uM
        D. x reverse primer for a final concentration of .5uM
        E. x DNA template for fin
        F. .4ul DMSO
        G. .125ul phusion DNA polymerase

    2. Thermocycling Conditions:
        A. Initial denaturation: 98C for 30 seconds
        B. 30 cycles of the following:
            a. Denaturation: 98C for 10 seconds
            b. Annealing: 10C lower than calculated TM for 30 sec.
            c. Extension: 72C for 30sec./kb
        C. Final Extension: 72C for 10 minutes
        D. Hold at 4C infinitely

- digested 10 microliters of the 7 previous PCR reactions with DPN1 again, but this time for 2 hours at 37C, without heat kill
- Transformed XL1 blue with 5 microliters of each of the redigested solutions
- Transformed Bl21 and Origami with RiG for Jae Seong's tests (including controls)
- Plated the cells after incubating with shaking for 100 min at 34C

Saturday, June 30

- DPN1 digest of the 15+15(2) PCR for 2 hours at 37C without heat kill
- Colony PCR using Taq DNA Polymerase:

Reaction setup from New England Biolabs:

- We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation **temperature (95°C).**

Component	**25 µl reaction**	**50µl reaction**	**Final Concentration**
10X Standard *Taq* Reaction Buffer	**2.5 µl**	**5 µl**	**1X**
10 mM dNTPs	**0.5 µl**	**1 µl**	**200 µM**
10 µM Forward Primer	**0.5 µl**	**1 µl**	**0.2 µM (0.05–1 µM)**
10 µM Reverse Primer	**0.5 µl**	**1 µl**	**0.2 µM (0.05–1 µM)**
Template DNA	**variable**	**variable**	**<1,000 ng**
***Taq* DNA Polymerase**	**0.125 µl**	**0.25 µl**	**1.25 units/50 µl PCR**

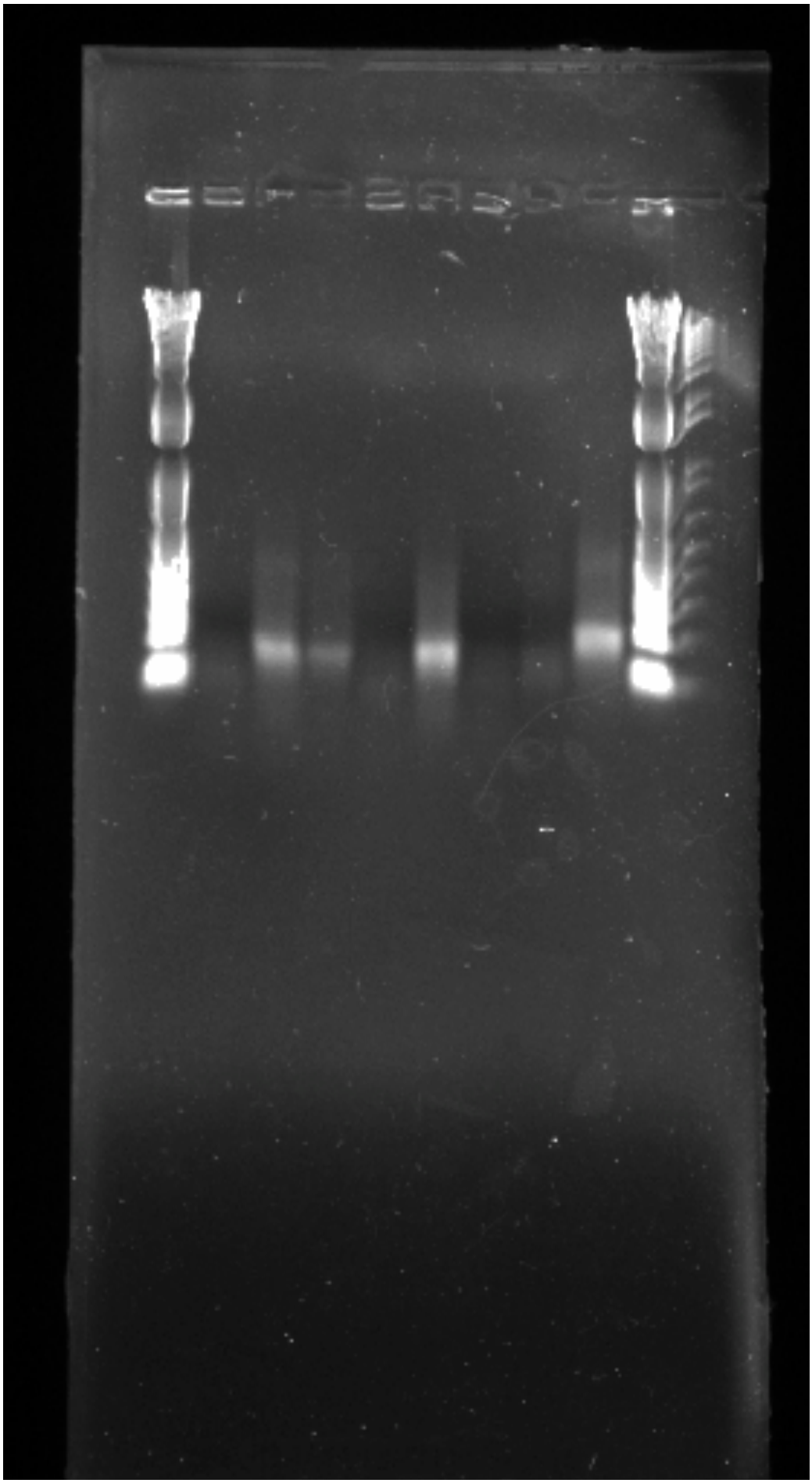
Nuclease-free water	**to 25 µl**	**to 50 µl**	

Notes:
* Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.
* Transfer PCR tubes from ice to a PCR machine with the block preheated to 95°C and begin thermocycling:

Thermocycling conditions:

A. Initial denaturation: 95°C for 30 seconds
B. 30 cycles:
    1. 95°C for 15-30 seconds
    2. 45–68°C 15-60 seconds (used 50C)
    3. 68°C with 1 minute per kb (5 min)
C. Final extension: 68°C for 5 minutes
D. Hold: 4–10°C indefinitely

- Made amp/kan plates
- Made an EtBr gel for analysis of the colony PCR
- Dry-cycle autoclaving
- Ran the gel and imaged the result:
  (ladder is in lanes 1 and 9, sample 2 is in lanes 3+4)
  Analysis: Band between the 100 and the 200 bp markers indicate self-annealed primers rather than presence of the desired insert



Sunday, 7/1

- Observed colonies on 4/7 double-digest plates: 9+10, 15+16, 21+22, 29+30, and began overnight cultures. Replated 7+8, and added more transformation liquid to other 3.
- Began overnight culture of transformed and control BL21 and Origami (in amp/kan and plain media)

Monday, 7/2

- PCR (5+6) purification
- Redid Colony PCR:
- Double digested T7 plasmids (this time also with Cip (2.2 microliters) and the purified 5+6 RiA PCR reaction. Also, incubate the reactions using a PCR machine at 37C for 90 minutes, rather than 10, and then heat kill at 80C for 20 minutes.

Tuesday, 7/3

- Ligated the 5+6 double-digest sample with the double-digested T7 vector (incubate a PCR tube for 150 min at 37C, and then store at -20C)
- Took overnight culture of RIG BL21 for protein test to main campus
- Transformed XL1 Blue cells with the ligation product (note: heat shock at 37C for an hour rather than at 42C for 45 sec. if using thick tubes – any other tubes than PCR tubes. Also, for future transformations, do not aliquot cells but carry out transformation in the same test tube if using small amounts of DNA or DNA that is not readily taken up. Finally, do not pipet up and down to mix, but rather add and swirl the DNA because competent cells are very sensitive and are damaged very easily.)
- Plated the hopefully T7 RIA transformants
- Diluted the received primers with 200 ul of Tris, and used about 2 microliters of the resulting solution to make further primer dilutions using 100ul of nuclease-free water.
- Measured the concentrations of resulting dilutions

Useful link: http://www.promega.com/techserv/tools/biomath/calc06.htm

Interesting Paper about introducing multiple mutations:
http://www.cellbio.duke.edu/faculty/soderling/labpage/Protocols/Site-DirectedMutagenesis(SupportProtocol1).pdf