

Protocols:

Stock solutions

S200 Buffer

- 50mM Tris, 150mM NaCl, 5mM MgCl₂ hexahydrate, 2mM ATP, 2mM 2-Mercapto Ethanol, 12.5% Glycerol, pH 7.6

APS (store in fridge):

- 2.5g in 10mL water

10X running buffer SDS - bring to 1 Litres:

- 144g glycine
- 30g Tris base
- 10g SDS Dissolve SDS to completion first. Heat at low after you put glycine and Tris base in. Don't need pH.

4x separating buffer bring to 100mls

- 18.17g Tris base
- 4.0 mls 10% SDS pH to 8.8 using concentrated HCl, then add dH₂O to make up volume.

4x stacking gel buffer

- 6.06g Tris-base
- 10% SDS 2.0ml
- 100mL H₂O pH to 6.8 using conc H₂O

Buffer for gel baths

- 1L cylinder. 900mLs of distilled H₂O. 100mLs of TBE Buffer (10x)

40% acrylamide

- 200g acrylamide
- 10g bis
- Total volume 500mL ddH₂O
- (wear a mask, don't heat)

0.5M EDTA pH 8.0-8.5

- 186.12g of EDTA + ddH₂O 800mL
- Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with sodium hydroxide (~20g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving. Label with actual pH value.

Coomassie Blue Stain

- For 1L, add
- 2.5g Coomassie blue
- 450mLs methanol
- 90mLs acetic acid
- 460mLs dH₂O
- Dissolve stain completely in methanol before adding water. Then filter through whatman #1 after coomassie blue has been completely dissolved before adding H₂O.

100mg/mL chloramphenicol

- Weigh 1g chloramphenicol
- Dissolve in 95% ETOH 10mL
- Sterilize through the filter
- Do not freeze, put fridge

40mg/mL Kanamycin

- 400mg Kan, 10ul water, filter sterilize. Freeze in aliquots

100mg/mL Amp

- 1g Amp, 10mL water. Filter sterilize, freeze in aliquots.

Destaining solution

- 10% methanol, 10% acetic acid.
- 100mL methanol, 100mL acetic acid, 800mL H₂O for 1L

Dialysis solution

- 20mLs 5M NaCl
- 50mLs 1M Tris pH 7.5
- 0.2 mLs 0.5 M EDTA
- (In a liter flask, make 1L total)

TE Buffer

- 10mM Tris
- 1mM EDTA
- 100mL H₂O + 1ml 1M Tris pH8 + 0.2mL 0.5M EDTA

1M IPTG

- 2.38 grams in 10mLs, sterile ddW, filter sterilize with Millipore filters.

pH Machine

- Adjust pH to 7.0 with buffer. Rise off probe with ddH₂O. Uncover hole on top (push down the rubber band, be very careful).
- Place probe in solution and adjust pH with proper chemical.

Preparation of Competent BL21 and Origami Cells

- Thaw an aliquot of cells (without any plasmid in them) on ice
- To 200 mL of sterile LB, add 50ul aliquot of the thawed cells: remember, this LB does not have any antibiotic in it, so work as sterile as possible (i.e. autoclave all solutions and use sterile pipettes)
- Grow cells in the shaker at 37°C until they reach an OD = 0.3-0.4 @600nm (1cm pathlength cell). This usually takes 3-6 hours.
- Aliquot into sterile 50mL tubes and Spin down at 2700xg for 10 minutes at 4°C; discard supernatant and let drain upside down on a paper towel for ~1 minute.
- Ice down sterile 100mM CaCl₂ and 100mM MgCl₂ solutions during centrifugation.
- Gently resuspend each pellet in 8mL 0.1M MgCl₂ and 2mL 0.1 M CaCl₂ and combine into 2 tubes (this should take 1-2 minutes per tube)
- Centrifuge 2700xg for 10 minutes and discard supernatant.
- Resuspend each pellet on ice in 4 mLs of ice cold CaCl₂ and combine into one tube
- Add 2.7mL sterile 60% glycerol and mix gently
- Aliquot 100uL and/or 250uL volumes into sterile (autoclaved), cold eppendorf tubes, freeze rapidly in liquid nitrogen and store at -80°C
- Test for (1) contamination by plating untransformed cells on Amp plate and (2) competence with a transformation

Preparation of Competent DH5-alpha cells

- Materials:
 - o LB Buffer
 - o 50 mL polypropylene tubes
 - o TB Buffer
 - o DMSO
 - o DH5-alpha colonies or freshly thawed cells
- TB Buffer (DH5-alpha competent cell buffer)
 - o 5mLs of 1M Hepes Buffer (final concentration 10mM)
 - o 1.1 grams of CaCl₂ · 2H₂O (final concentration 15mM)
 - o 9.32 grams KCL (final concentration 250mM)
 - o Bring to volume 300 mL; pH to 6.7 with 5M KOH
 - o Add 5.44 grams MnCl₂ · 4H₂O (final concentration 55mM)
 - o Bring volume to 500mL and sterile filter into sterile bottle

- Start 5mL overnight culture in LB from single DH5-alpha colony.
- Inoculate 200mL of LB with 2mL of overnight culture and grow at 25°C until OD = 0.5-0.55 (Alternatively, inoculate 200mL LB with 50uL freshly thawed DH5-alpha cells and grow at 25°C). Note – if you do it this way, it may take a loooooong time (more than 10 hours) to read OD = 0.5.
 - o Note from 11 August 2010: Using a single colony ON growth followed by inoculation of 200mL with 2mL of ON growth, it took 8.5 hours to reach OD = 0.5.
 - o Notes from 11 August 2011: Used 50uL of freshly thawed cells to start 50mL ON growth. Using 2 mL of ON growth to inoculate 200mL, it took 8h and 45 minutes to reach OD = 0.503. Using 4mL of ON growth, the OD > 0.55 after 7h45min; a closer monitoring of the OD at this stage could shorten the prep by at least 1 hour.
- Transfer to 6 sterile, disposable ice-cold 50mL polypropylene tubes (~35mL per tube). Store on ice for 10 minutes.
- Harvest cells by centrifugation at 2500xg for 10 minutes at 4C.
- Pour off medium and stand upside down on paper towel for 1 minute to remove remaining medium
- Resuspend pellets by gently pipetting or swirling in 60mL (total) of ice cold TB buffer. Combine re-suspended cells into a total of two tubes.
- Centrifuge at 2500xg for 10 minutes at 4°C to recover cells.
- Pour of medium and stand upside down on a paper towel for 1 minute to remove remaining medium.
- Resuspend pellets by gently pipetting or swirling in 20mL (total) of ice cold TB buffer. Combine cells into a single tube.
- Add 1.5mLs of DMSO and mix by swirling. Store cells on ice for 10 minutes.
- Pipette 250 and/or 100ul aliquots into chilled eppendorf tubes. Make sure to close tightly and then flash freeze in liquid nitrogen and store at -80°C.
- Test for (1) contamination by plating untransformed cells on Amp plate and (2) competence with a transformation.

Transformation

1. Thaw competent cells on ice
2. Aliquot 50ul of cells into two prechilled 15mL culture tubes (one for control, one for actual transformation).
3. Add 1-5ng in 1-5uL of DNA. Swirl gently with pipette.
4. Incubate tubes on ice for 30 minutes
5. Heat pulse tubes in 42°C water bath for 30-40 seconds.
6. Incubate on ice for 2 minutes
7. Add 500uL of LB broth to each tube and incubate for an hour at 37°C with shaking.

8. Spread 100uL of each culture on an LB agar plate containing the appropriate antibiotics and incubate overnight at 37°C (spread using beads).

Miniprep

- Centrifuge sample in Eppendorf tube approximately 1.5 mL at a time, draining off supernatant after each spin and adding more cell solution
- Resuspend pelleted bacterial cells in 250 µL of Buffer P1.
- Add 250 µL Buffer P2 and mix thoroughly by inverting the tube 4-6 times. Add 350 µL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
- Centrifuge for 10 min at 13,000 rpm (approximately 17,900 times g) in a table-top microcentrifuge.
- Apply the supernatant (from step 5) to the QIAprep spin column by decanting or pipetting.
- Centrifuge for 30-60 seconds. Discard flow-through.
- Wash the QIAprep spin column by adding 0.5 mL Buffer PB and centrifuging for 30-60 seconds. Discard the flow-through.
- Wash QIAprep spin column by adding 0.75 mL Buffer PE and centrifuging for 30-60 seconds.
- Discard the flow-through, and centrifuge for an additional 1 minute to remove residual wash buffer.
- To elute DNA, place the QIAprep column in a clean 1.5 mL microcentrifuge tube. Add 30 µL of water to the center of each QIAprep spin column, let stand for 1 minute, and centrifuge 1 minute.
- Rinse column by adding 10 µL of water to the center, letting it stand for one minute, and centrifuging for another minute.

Gel Extraction

- Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
- Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg is approximately 100 uL). If the color of the mixture is orange or violet, add 10 uL of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
- Incubate at 50 degrees Celsius for 10 minutes (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 minutes during the incubation. For gels greater than 2% agarose, increase incubation time.
- After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to the Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 uL of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
- Add 1 gel volume of isopropanol to the sample and mix.
- Place a QIAquick spin column in a provided 2 mL collection tube.

- To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 minute. Discard flow-through and place the QIAquick column back into the same tube. The maximum volume of the column reservoir is 800 μ L. For sample volumes of more than 800 μ L, simply load and spin again.
- Recommended: Add 0.5 mL of buffer QG to QIAquick column and centrifuge for 1 minute. Discard flow-through and place the QIAquick column back into the same tube. This step is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.
- To wash, add 0.75 mL of Buffer Pe to QIAquick column and centrifuge for 1 minute. Discard flow-through and place the QIAquick column back into the same tube. Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2-5 minutes after addition of Buffer PE, before centrifuging.
- Centrifuge the column in a 2 mL collection tube (provided) for 1 min at 17,900 times g (13,000 rpm).
- Place QIAquick column into a clean 1.5 mL microcentrifuge tube.
- To elute DNA, add 50 μ L of Buffer Ed (10 mM Tris*Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 minute. Alternatively, for increased DNA concentration, add 30 μ L elution buffer to the center of the QIAquick membrane, let the column stand for 1 minute, and then centrifuge for 1 minute.
- If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

PCR Purification

- Add 5 volumes of Puffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.
- Place a QIAquick spin column in a provided 2 mL collection tube.
- To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60 seconds.
- Discard flow-through. Place the QIAquick column back into the same tube.
- To wash, add 0.75 mL Buffer PE to the QIAquick column and centrifuge for 30-60 seconds.
- Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 minute. IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
- Place QIAquick column in a clean 1.5 mL microcentrifuge tube.

- To dilute DNA, add 40 μ L Buffer EB or water to the center of the QIAquick membrane, let stand for one minute, then centrifuge the column for 1 minute. Repeat with 15 mL to elute remaining DNA.

Bacterial Plates and Liquid Culture

- For 1L plates = 2 sleeves, add 25g of LB Broth (Miller), 15g agar, and 1L water. Autoclave on liquid cycle (50 mins). Cool mixture to 42°C and add 1mL of 1000x antibiotic. Pour into plates and let sit overnight. Refrigerate next morning.
- For liquid cultures, follow same protocol, except do not add agar.

Bacterial Glycerol Stocks

- Put 0.5mL bacterial culture in a sterile eppendorf tube.
- Add 0.5mL of sterile 80% (v/v) glycerol solution
- Freeze in liquid nitrogen and add to -80°C freezer
- To recover, scrape frozen surface of culture with sterile inoculating needle, and then streak onto LB agar plate containing appropriate antibiotics, or inoculate liquid culture containing appropriate antibiotics.

Protein Expression

- Subculture at 1/40 dilution (i.e. 25mL overnight into 1L of broth) and grow until an OD(600) of 0.5-1.0. This is done at 37°C. Liquid culture containing Amp is inoculated with glycerol stock of RiAFP-GFP
- Add 1mM IPTG, and grow overnight (12-16 hours) at 22°C.
- Harvest cells in centrifuge at 6,000 rpm for 20 minutes (we use Modis Lab or Steitz Lab centrifuge, 1L flasks).
- Pellets should be green. Discard supernatant. Scrape pellets from tubes into 50mL falcon tubes, flash freeze in liquid nitrogen, and store at -80°C until use.

Protein Purification

- Add 10mLs of lysis buffer per 1L of bacterial juice spun into pellet.
- Lysis buffer: 50mM NaH_2PO_4 , 300mM NaCl. 1mM PMSF added before lysis.
- The pellet is dissolved in lysis buffer (by stirring, vortexing, mashing together).
- Lysis buffer + pellet mixtures are combined into a 50mL falcon tube, and 1mM PMSF is added. PMSF is in DMSO.
- Sonicate (using Spiegel lab sonicator) with 1s on/4s off for 5 total minutes of sonication. Keep cold. Amplitude 50%.
- While this is happening, equilibrate 1mL packed Ni-NTA columns (Modis lab) with lysis buffer spiked with imidazole. We want 10mM imidazole in the equilibration buffer. To equilibrate, pass 10mLs of lysis buffer + imidazole mixture through the column, either

using the syringe or the peristaltic pump. Do this a few times to ensure equilibration. Try not to get air through the column. Go at a rate of 1 drop per second, not faster. (Syringe method takes longer, but both are fine). Also, make sure that the column is not exposed to air for too long (i.e. make sure it is capped with the black stopper when not in use). Before you inject the equilibration buffer through the column, add a few drops to the place that you screw in the pump, and then fully screw it in, then push down on syringe to wash fluid through.

- Take the sonicated lysis buffer and pass it through a Millipore 0.22 μM filter to get rid of junk. Note that the filter gets pretty clogged so you need to change filters every once and a while. After this syringe filtering is done, add flow through into another syringe to pass through the His-6 column. Make sure that the rate at which flow through is coming out of the column is 1 drop per second. Collect flow through to run on a gel.
- Once everything is run through the column, it is time to elute. Elution is done with the following buffers: 500mM imidazole (0), 250mM imidazole (-1), 125mM imidazole (-2), 62.5mM imidazole (-3), 31.25mM imidazole (-4). Collect 5 1mL fractions with each buffer, starting with the lowest concentration of imidazole. The protein should come off around the 16th fraction collected. The protein comes off green, which is awesome.
- Run everything on a gel to check for purity / rough amounts.
- Pool fractions that have pure protein / a lot of protein, and concentrate using a 10K molecular weight cutoff filter. Spin at 13k for 5-10 minutes, until desired volume is reached. Concentrating to 3mLs is optimal. Do not concentrate too much or protein will aggregate and precipitate out of solution if spun. (this happened to me once when I got a sample that was 21mgs/mL).
- For crystallization studies, greater purity is required. Proceed to size exclusion chromatography.
- To isolate RiAFP from the fusion protein, incubate overnight with TEV protease (Modis Lab). Use 1mg of TEV protease for 100mg of protein. This is usually around 40uL of TEV protease.
- Check concentration using nanodrop A280 or Bradford Assay.

Size Exclusion Chromatography

- Combine all elutions from metal affinity chromatography that contain samples after visualizing on Coomassie SDS-PAGE gel
- Spin concentration using Millipore conc. to a volume of 3 mL
- Load 3 mL of S200 buffer on Hi-Load size exclusion chromatography columns and run program to equilibrate column (takes ~3 hours)
- Load 3 mL of sample on Hi-Load column and run program to collect fractions on FPLC
- Run Coomassie SDS-PAGE gel on all samples, keep the pure ones and spin concentrate

Bradford Assay

- You cannot use the Nanodrop A280 to determine protein concentration of RiAFP. This is because it lacks a certain type of residue (tryptophan and tyrosine). But A280 is fine for RiAFP-GFP fusion protein.
- To do a Bradford assay, have three tubes. One tube has 1mL of Bradford Assay Reagent. One tube has 999uL Bradford Assay Reagent and 1uL Buffer. Final Tube has 999uL Bradford Assay Reagent and 1uL Buffer + Protein. The Bradford Assay Reagent is 5x so you must dilute it with water before use.
- Look at absorbance at 1 lambda at 595 nm. Add to curvette.
- Compare to standard curve chart that is on the wall in Modis Lab. If it gives you 6uL/mL, for example, you have to multiple by 1/6 in 1000 = 1 mg/mL.
- Other possibility is calculating concentration of GFP fusion using the UV-Vis setting on the Nanodrop 2000c spectrophotometer (ϵ for eGFP at 488nm = $55,000 \text{ M}^{-1} \text{ cm}^{-1}$).

TCA Precipitation

- Prepare Stock solution 100% w/v Trichloroacetic acid (TCA).
 - o Dissolve 500g TCA (as shipped) into 350mL dH₂O, store at RT.
- Add 1 volume of TCA stock to 4 volumes of protein sample (In a 1.5ml tube with maximum vol, add 250ul TCA to 1.0mL sample)
- Incubate 10min at 4C.
- Spin tube in microcentrifuge at 14K rpm, 5 min. Remove supernatant, leaving protein pellet intact. Pellet should be formed from whitish, fluffy ppt.
- Wash pellet with 200ul cold acetone. Spin tube in microcentrifuge at 14K rpm, 5 min.
- Repeat steps 4-6 for a total of 2 acetone washes.
- Dry pellet by placing tube in 95°C heat block for 5-10min to drive off acetone.
- For SDS/PAGE, add 2X or 4X sample buffer (with or without BME) and boil sample for 10 min at 95C heat block before loading sample into Polyacrylamide gel.

Running SDS PAGE Gels

- Loading samples into gel:
 - o If you have a pellet, add 1mL loading dye per 50mL spun down pellet, redissolve, and load onto gel.
 - o If you have liquid sample (say, from elution off of a His-6 column), add 3ul sample and 9ul loading dye. Mix the loading dye with DTT before use (final concentration of DTT in sample must be 200mM).
 - o Boil sample for 10 minutes at 95°C.
 - o Load 10ul to lanes.
- Casting a gel:
 - o Do minimum 2 at a time, can store them in fridge in plastic wrap for about a month, add wet tissue to edge of the gel to keep moist).

- Add grey strips. Scrub glass holder and surface glass with wire/warm water. Dry spacers, ethanol, dry again. Put clean face of surface glass on glass. Have biorad facing you at top. Make sure that there is a tight seal, do on hard surface. Close the press while the two are sealed together. Then make separating buffer from stock solutions. Use 1/2 listed volumes to make 2 gels! After 10mls added add 40 microlitres of APS, then add 20ul TEMED (starts to polymerize right away). Add with pasteur pipet until it is right above the line. Stop below green. Then add water saturated butanol to the top, and let sit 50 mins
- Then prepare stacking gel. Make 10mls from stock solutions (2.5mL of 4X stacking buffer, 1.25mls acrylamide, and 6.25mls of water), split in half, then add 20ul APS and 10ul Temed. Add stacking gel, then set comb.
- Running gel:
 - Run for 90 minutes at 100V.

Bacterial Survival Assay

- Liquid culture of RiG is are grown to OD ~0.5, then split, and one tube is induced for 4 hours at 25°C with 1mM IPTG. Controls of just T7 vector are also prepared.
- 10 fold serial dilutions are prepared after induction. Serial dilutions are done in LB. Before freezing, plate 10^{-5} , 10^{-6} and 10^{-7} dilutions. Then freeze original tubes. Then redo serial dilution the next morning (12-16 hours later), and plate again for cell viability count.
- Calculate relative freezing tolerance by determining number of viable cells before and after freezing.

Western Blots

- Gels were transblotted onto nitrocellulose membranes with Invitrogen iBlot Dry Blotting.
- Perform according to manufacturer's protocol.

Sequencing Sample Submissions

- (<http://medicine.yale.edu/keck/dna/protocols/tube/index.aspx>):
- 500-600 ng ds plasmid DNA template
- 2 microliters of 4 micromolar primer
- fill up to 18 microliters water

Ice Recrystallization Inhibition Assay

Splat Assay

- 7ul of a 30% sucrose solution containing a known concentration of sample is sandwiched between two sterile glass slides.

- Sandwiches are sealed with plastic polymer, tape and clips, and are placed in an ethanol beaker submerged in liquid nitrogen.
- The sealed samples are placed in a -10°C freezer and imaged at 30 min, 5 hours, and 18 hour intervals.
- Samples are transferred to a Nikon optical microscope on a super-cooled NaCl ice bed, and imaged using a plane polarized filter at 50x unless otherwise noted.

Capillary method

- Samples diluted in lysis buffer are loaded into capillary tubes and snap frozen in liquid nitrogen. After snap freezing, the samples are immersed in a jacketed beaker at -10°C.
- For pre-recrystallization, the samples are imaged immediately after snap freezing. Samples are incubated for 30 minutes, 5 hours, and 18 hours.

PCR Amplification

- We use Phusion Hi Fidelity Polymerase:

Table 1. Pipetting instructions (in order).

Component	Volume / 50 µl reaction	Volume / 20 µl reaction	Final conc.
H ₂ O	add to 50 µl	add to 20 µl	
5x Phusion HF Buffer*	10 µl	4 µl	1x
10 mM dNTPs	1 µl	0.4 µl	200 µM each
primer A**	x µl	x µl	0.5 µM
primer B**	x µl	x µl	0.5 µM
template DNA	x µl	x µl	
(DMSO***, optional)	(1.5 µl)	(0.6 µl)	(3 %)
Phusion DNA Polymerase	0.5 µl	0.2 µl	0.02U/µl

- For short fragment and colony PCR, we use GoTaq Flexi polymerase:

Component	Final Volume	Final Concentration
5X Green or Colorless		
GoTaq® Flexi Buffer ¹	10µl	1X
MgCl ₂ Solution, 25mM ¹	2–8µl	1.0–4.0mM
PCR Nucleotide Mix, 10mM each	1µl	0.2mM each dNTP
upstream primer	Xµl	0.1–1.0µM
downstream primer	Yµl	0.1–1.0µM
GoTaq® DNA Polymerase (5u/µl)	0.25µl	1.25u
template DNA	Zµl	<0.5µg/50µl
Nuclease-Free Water to	50µl	

¹Thaw completely and vortex thoroughly prior to use.

- Thermal cycling conditions for GoTaq DNA Polymerase were as follows:

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	2 minutes	1 cycle
Denaturation	95°C	0.5–1 minute	
Annealing	42–65°C*	0.5–1 minute	25–35 cycles
Extension	72°C	1min/kb	
Final Extension	72°C	5 minutes	1 cycle
Soak	4°C	Indefinite	1 cycle

*Annealing temperature should be optimized for each primer set based on the primer T_m.

Restriction Digest

- The following online tools were helpful:
- <http://www.promega.com/techserv/tools/biomath/calc06.htm>
- <http://www.neb.com/nebecomm/DoubleDigestCalculator.asp>
- We used NEB restriction enzymes and followed the manufacturer's protocols.

Ligation

- We used NEB T4 DNA ligase and followed the manufacturer's protocols. Four different vector:insert ratios were used (1:1, 1:3, 3:1, 1:8)
- Set up the following reaction in a microcentrifuge tube on ice. (*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
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10X T4 DNA Ligase Buffer*	2 μ l
Vector DNA (3 kb)	50 ng (0.025 pmol)
Insert DNA (1 kb)	50 ng (0.076 pmol)
Nuclease-free water	to 20 μ l
T4 DNA Ligase	1 μ l

- * *The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.*
- Gently mix the reaction by pipetting up and down and microfuge briefly.
- Incubate at room temperature for 10 minutes.
- Chill on ice and transform 1-5 μ l of the reaction into 50 μ l competent cells.

Colony PCR

- Touch a colony with a sterile pipet tip. Then smear the pipet tip in the bottom of an eppendorf tube to get some colony in the bottom of the tube. You don't want too much colony, a little is fine.
- Then eject the sterile pipet tip in a culture tube containing LB and the appropriate antibiotic.
- Label tubes appropriately!
- Conduct PCR with GoTaq. After running a gel, stain with EtBr, and if the PCR is successful go back to the appropriate culture tube and eventually miniprep it.

DNA Agarose Gels

- Weigh out 0.5g of agarose into a 250mL conical flask. Add 50mL of 0.5x TBE, swirl to mix.
- Microwave until dissolved. Do not let boil over, stop and swirl every minute. Wear gloves.
- Leave it to cool to about 60°C
- Add 1 μ L of ethidium bromide (10mg/mL) and swirl to mix). Handle with extreme caution. Dispose of contaminated tip into a dedicated ethidium bromide waste container.
- Pour gel slowly into the tank. Push bubbles away using a pipet tip. Insert the comb, make sure it is correctly position.
- Let cool in cold room for 1 hour.
- Pour 0.5X TBE buffer into the gel tank to submerge the gel. This is the running buffer.

Lambda Red Recombination Protocol

- Perform o/n PCR to amplify regions containing antibiotic and gene that you would like to integrate.
- Perform cross over PCR on the two PCR-purified constructs (see below).
- Run 1% agarose gels to screen PCR reactions. Include a negative control.

- Pick 3 colonies of EcNR2 strain, grow in 2x 1mL and 1x 2mL tubes of LB at 30°C → grow until mid-log growth, OD 0.6-0.8.
- Once cells reach mid-log growth (about 4 hours), immediately transfer to shaking water bath (42°C, 250rpm for 15 minutes).
- Immediately transfer to ice and leave on ice until DNA is ready for electroporation/recombination.
- Add 1.5uL of DpnI enzyme to XO PCR product and incubate at 37°C for 30 minutes.
 - o This step digests methylated DNA which originates from the template that was grown in cells and subsequently miniprep. Only DNA amplified by PCR will remain intact.
 - o Critical step, eliminates possibility of false positive, contaminating DNA during transformation step.
- Prepare Gel, run PCR product post DpnI digest, stain with SYBR-safe. Cut out lanes. Post-stain with EtBr.
- Perform gel purification with Qiagen kit.
- After purification, check concentration of DNA.
- Setting-up recombination/electroporation step:
 - o Will recombine 50ng of amplified DNA.
- Do everything from this step on in the cold room and on ice.
- For cells on ice, also add cells on ice that were not subjected to 42C lambda red induction step → negative controls.
- Wash cells in 2x or 1x volume of cold ultra pure distilled water.
- 1mL worth of cells / electroporation.
- For each mL of culture, resuspend in 50uL of cold dwater.
- Transfer each cell aliquot to pre-chilled (on ice) 0.2cm cuvettes.
- Electroporation step:
 - o Keep on ice.
 - o Wipe side dry prior to electroporation.
 - o Electroporate at the following settings:
 - 2.5kV
 - 200 ohms resisatnce
 - 25uF
 - Immediately add 1mL LB
- Incubate cultures at 30C in rotator for 50-60 minutes.
- Transfer LB-cell suspension to 1.5mL eppendorf tubes.
- Plate samples on LB with antibiotic overnight. Allow cells to absorb into agar plates for 15 minutes prior to flipping upside down and placing in 30°C incubator overnight.

Cross Over PCR

- For crossover PCRs, set-up your reactions such that each of the four templates exist at the same relative concentration. Thus, do the following:
 1. Calculate difference in size between the four templates
 2. Based on size differences, for a given number of molecules estimate the stoichiometric ratios of each oligo.
 3. Use the measured concentration (via Nanodrop) of each template to determine how much volume of each should be added to the crossover reaction
- MW of a dsDNA molecule = (# of bp) X (650 daltons/bp)
- Source: New England Biolabs (www.neb.com)
- Also look at: http://molbiol.edu.ru/eng/scripts/h01_07.html

Performing 12 identical crossover PCR reactions at varying annealing temperatures: **Gradient PCR**

Using BD-Clontech's Advantage 2 PCR:

ONLY use the BD Advantage 2 PCR Buffer (green top)

Do not use the BD Advantage 2 SA PCR Buffer (yellow top)

NOTE The Advantage 2 PCR polymerase has hotstart activity

Perform a control PCR, which does not contain any template DNA.

Therefore, perform 13 total PCR reactions, one being a negative control.

Crossover PCR rxns:

1. Make the master mix. Include everything but your template mix (4 PCR products from above table).
2. Aliquot the negative control into a PCR tube.
3. Add the 12 uL volume of template mix to the master mix.
4. Mix well by pipeting up-and-down 5-10 times.
5. Aliquot identical reactions into 12 separate tubes.

Components	Volume	Master Mix (x13)
Template	1	-
10x BD Buffer	5	65
10 mM dNTPs	1.5	19.5
20 μM Primer 1 (F)	1.	13
20 μM Primer 2 (R)	1.	13
dH₂O	40	520
Advantage 2	0.5	6.5

Total (μL)	50	
----------------------------------	----	--

PCR Cycle:

Step	Temperature ($^{\circ}$ C)	Time
1	95	4'
2	94	30"
3	Gradient: 58-72	30"
4	68	8.5
5	Go to Step 2	22x
6	68	10'
7	4	Forever

Ice Recrystallization Assay

All the images exhibiting rounded crystals similar to those of this paper: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1220261/pdf/10333479.pdf> are made using the following procedure.

A modified splat assay was employed by loading 2-3 μ L of sample onto 2 sterile gas slides with buffer in different dilutions. The sample contained either lysed bacteria expressing protein or purified protein based on the particular assay. After loading, the sample was squashed between the two slides and excess liquid was removed. The slides were sealed with epoxy. The sandwich was dropped into a bath of heptane cooled by liquid NO₂ for 30 s, and then transferred to an constructed chamber with cooled ethanol. The sandwich with loaded sample was maintained between 6- 10 degrees C for the period of freezing. Samples were imaged with a Nikon multi-function optical microscope with cross-polarizing and plane-polarizing filters.

For the images that appear to be individual crystals (i.e. the clear hexagonal shapes, etc):

Samples are loaded into 1 mm diameter glass "wells" using approximately 10 μ L of sample. The well tubes are directly snap frozen in liquid nitrogen by placement sample-side upward into a container with liquid nitrogen on the bottom, such that the liquid sample never comes in contact with the liquid nitrogen. A glass cover slip may be placed on top, if desired. The samples are then incubated between 6-10 degrees C and imaged as discussed above.

For the green ones, this is 4 μ L/mg of AFP imaged in a 470-480 fluorescence light filter in a multi-function Nikon optical microscope after 30 minutes of incubation following procedure 1.

Bacterial Cyro-survivability Assay

1. Grow overnight culture of Escherichia coli K12 in Luria Broth at 37 $^{\circ}$ C with shaking (220 rpm)

2. Dilute culture (1:50) and grow to OD600 of 0.500
3. Dilute culture 10⁻⁴ in LB (all dilutions were done in triplicate)
4. Do final culture dilution to 10⁻⁵ into experimental conditions (LB + Protein Buffer, 50:50 LB/Glycerol, 400 ug/ml BSA + buffer, 400 ug/ml RiAFP + buffer)
5. Plate 100uL of each dilution on LB petri dishes and grow overnight at 37°C. Count number of colonies the next day
6. Place final 10⁻⁵ dilutions at -20°C
7. After 48 hours, remove frozen dilutions and thaw at room temperature.
8. Plate 100uL of dilutions on LB petri dishes and grow overnight at 37°C. Counter number of colonies the next day

PP01 Cyro-survivability Assay

1. Placed 10ul of bacteriophage PP01 (z x 10⁹ pfu/ml) in 990ul of experimental conditions (LB + protein Buffer, 50:50 LB/Glycerol, 400 ug/ml BSA + buffer, 400 ug/ml RiAFP + buffer)
2. Place viral solutions at -20°C
3. After 48 hours, remove frozen tubes and thaw at room temperature.
4. Dilute frozen viruses to 10⁻⁶ and titer on Escherichia coli O157:H7

Physiological structure assay

3mm cubes of rat (Sprague-Dawley, Charles River Laboratories) liver were removed from a euthanized, saline-perfused animal. Tissue samples were immediately immersed in one of four solutions: 1) 9 mg/mL RiAFP, 2) 4.5 mg/mL RiAFP, 3) 0 mg/mL RiAFP, 4) 0.9% regular saline. The immersed samples were subsequently frozen at -20 C for 12 hours. Upon thawing, the tissue was then fixed in 4% paraformaldehyde via immersion at 4 C for 24 hours. Fixed samples were paraffin blocked, sectioned, and stained with hematoxylin and eosin for further histological analysis.

Primer Designs

For cloning:

Prefix RBS Coding sequence

Suffix terminator stop coding sequence

GFP-TmAFP (and for GFP-TEV-TmAFP):

Forward primer

GTTTCTTCGAATTCGCGGCCGCTTCTAGAGTtaaggaggtaaaaaaATGGGCAGCAGCCATCATCA

Reverse primer

CTACCGGCTGCCCGGGTCACATAAcgcaaaaaccccgcttcggcggggtttttcgcTACTAGTAGCGGCCGCTGCAGGAAGAAAC

Reverse complement:

GTTTCTTCCTGCAGCGGCCGCTACTAGTAgcgaaaaaccccgccgaagcggggtttttcgcTTAGTGACCCGGGCAGCCGGTAG

His-TEV-TmAFP:

Forward primer (degenerate His forward primer)

GTTTCTTCGAATTCGCGGCCGCTTCTAGAGTtaaggaggtaaaaaaATGCATCATCATCATCATCAT

RiAFP

Generic forward primer for RiAFP only

GTTTCTTCGAATTCGCGGCCGCTTCTAGAGTtaaggaggtaaaaaaATGTATTCCTGTCGTGCTGTTGG

Reverse primer (with His-tag added)

ACTACCACCGTTACCGCAAGCCATCATCATCATCATCATCATATAAcgcaaaaaccccgcttcggcggggtttttcgcTACTAGTAGCGGCCGCTGCAGGAAGAAAC

His tag

Reverse complement:

GTTTCTTCCTGCAGCGGCCGCTACTAGTAgcgaaaaaaccccgccgaagcgggggtttttgcgTTAGTGA
TGATGATGATGATGGCTTGCGGTAACGGTGGTAGT

Forward primer for RiAFP starting with eGFP:

GTTTCTTCGAATTCGCGGCCGCTTCTAGAGTtaaggaggtaaaaaaaATGGTCTCCAAGGTG
AAGAGC

For integration into genome:

Recombination sites for MAGE primers:

78110, 1415470, 2428900, thrBC

78110 forward primer:

AGGCGCTTTTTTGCTATTCAGGCATCCTCAATTTCACTTTGTAAACCTGAXXXX

78110 reverse primer:

CTAATTCCACCGACATAGAGTTGCTTGCACAGTAAGCTCTGACGATGTCAXXXX

1415470 forward primer:

CCTCAACTCAGATTAAAATTCGTTTTGTTTCAGTGAATGATCTTGCCGGATXXXX

141570 reverse primer:

GAAATCTGAAAGAAATAGCCTGCGTATGGCGCAGGCTATGAACAGTGTGTXXXX

2428900 forward primer:

TTTGCGTAGGGATTTCCCTCCCGCGCATCAATAAAAATGGCGCTGAAAAAXXXX

2428900 reverse primer:

ACGCATTGCCCGATGCCGCAAAGGCATAAAAAGTCGATGGCGTTGAATATXXXX

thrBC forward primer:

CTGATCTGCTACGTACCCTCTCATGGAAGTTAGGAGTCTGACATGXXXX

thrBC reverse primer:

GCATAAAAGCAAACCCGGCCTGATTGAGATAATGAATAGATTTTXXXX

78110 and 141 are same replichore

78110-RiAFP-overhang:

Forward Primer (integration homology for site 78110 followed by RiAFP):

AGGCGCTTTTTTGTCTATTCAGGCATCCTCAATTTCACTTTGTAAACCTGAtaatacactcact
ataggaatacaa

Reverse primer for crossover with Kan (RiAFP terminator end part and Kan homology):

aagtgatcttccgtcacagggcgaaaaaaccccgccga

Overhang-kanamycin-78110

Forward Primer (start of Kanamycin):

Cctgtgacggaagatcactt

Reverse Primer (all of 78110 rev, followed by end of kanamycin reverse complement)

CTAATTCCACCGACATAGAGTTGCTTGACAGTAAGCTCTGACGATGTCAaaccagcaata
gacataagcgG

141 RiAFP Forward:

CCTCAACTCAGATTA AAAATTCGTTTTGTTCAGTGAATGATCTTGCCGGATtaatacactcac
tataggaatacaa

141-KanR

GAAATCTGAAAGAAATAGCCTGCGTATGGCGCAGGCTATGAACAGTGTGTaaccagcaat
agacataagcgG

MAGE Primers:

MAGE Oligos to Target Ice-Binding Sites of RiAFP

Nucleotide and protein sequence for RiAFP:

atgtattcctgctgctgtgtggcgtagacggcgtgcagtaacggatatccagggtacc
M Y S C R A V G V D G R A V T D I Q G T
tgccacgctaagccaccgggtgccccgcatggcgtccggcacctccgaaccaggtcc
C H A K A T G A G A M A S G T S E P G S
acctccaccgcgaccgcaacgggcccgtggcgccactgctcgtagcacctccaccggcgt
T S T A T A T G R G A T A R S T S T G R
ggtactgctaccaccactgcaactggtaccgcatctgcgacctccaacgccatcggtcag
G T A T T T A T G T A S A T S N A I G Q

ggtaccgcaaccacgactgcgacgggttccgcaggtggccgtgcaaccggtagcgcgact
 G T A T T T A T G S A G G R A T G S A T
 acgagcagctccgcgagccagccgaccagactcaaaccattaccggcccgggtttcag
 T S S S A S Q P T Q T Q T I T G P G F Q
 actgcgaaaagctttgctcgtaacactgcaactaccaccgtaccgcaagc
 T A K S F A R N T A T T T V T A S

Ice-binding			Hydrophilic		
#	beta-strand	turn	#	beta-strand	turn
1	YSCRAVGV	DG	2	RAVTDIQ	G
3	TCHAKAT	GAGA	4	MASGTSE	PGS
5	TSTATAT	GRGA	6	TARSTST	GRG
7	TATTTAT	G	8	TASATSN	AIGQG
9	TATTTATGSAGGRA		10	TGSATTSSSASQP	
11	TQTQTIT	GPG	12	FQAKSF	ARN
13	TATTTVTAS				

Figure 10. Predicted segmentation pattern for *RiAFP*. The *RiAFP* sequence is segmented on the basis of the *iwAFP* folding scheme. The sequence is arranged as β -strand segments based on the Thr-Ala/Ser dipeptide repeat pattern. The putative ice-binding face and hydrophilic face are categorized and labeled. Shorter regions in which this pattern is disrupted, often by Pro or Gly, are shown to the right of the putative strands. Outward-pointing residues are highlighted in gray.

Targets (assuming AFP in Replichore 1 via recombination at 1415470 site):

Insertion of additional Tx repeat in every occurrence:

1. TSTATAT->TSTATAT**XT**

acctccaccgacgaccgcaacg -> acctccaccgacgaccgcaacg**nnnacn**

Oligo (33-27-30mer):

gcatggcgctccggcacctccgaaccaggtcc**acctccaccgacgaccgcaacgnnnacn**ggccgtggcgccactgctcgtacacctcc

Reverse complement (for lagging strand target):

ggaggtgctacgagcagtgggcggccacggccggtnnncgttgcggctgcgggtggaggtggaacctgggtcggaggtgccggacgccatggc

2. TATTTAT->TATTTAT**XT**

actgctaccaccactgcaact -> actgctaccaccactgcaact**nnnacn**

Oligo (33-27-30mer):

gccaactgctcgtacacctccaccggctcgtggt**actgctaccaccactgcaactnnnacn**ggtagccatctgcgacctccaacgccatc

Reverse complement (for lagging strand target):

gatggcgttgagggtcgcagatgcgggtaccngtinnnagttgcagtggtgtagcagtagcaccagaccgggtggaggtgctacgagcagtggc

3. TATTTAT->TATTTAT**XT**

accgcaaccacgactgcgacg -> accgcaaccacgactgcgacg**nnnacn**

Oligo (33-27-30mer):

gcatctgcgacctccaacgccatcggtcagggt**accgcaaccacgactgcgacgnnnacn**ggttccgcaggtggccgtgcacccgtagc

Reverse complement (for lagging strand target):

gctaccgggtgcacggccacctgcggaaccngtinnncgtcgcagtcgtggttgcgggtaccctgaccgatggcgttggaggtcgcagatgc

4. TQTQTIT->TQTQTIT**XT**

accagactcaaaccattacc -> accagactcaaaccattacc**nnnacn**

Oligo (33-27-30mer):

agcgcgactacgagcagctccgcgagccagccg**accagactcaaaccattaccnnaacn**ggcccgggtttcagactg
cgaaaagcttt

Reverse complement (for lagging strand target):

aaagcttttcgagctctgaaaaccgggccngtnnnggtaatggtttgagtctgggtcggctggctcgcggagctgctcgtagt
cgcgct

5. TATTTVT->TATTTVT**NT**

actgcaactaccaccgttacc -> actgcaactaccaccgttacc**nnaacn**

Oligo (33-27-30mer; note: end from His tag+STOP+Term):

ggttttcagactgcaaaaagctttgctcgtaac**actgcaactaccaccgttaccnnaacn**gcaagccatcatcatcatcatcac
taacgc

Reverse complement (for lagging strand target):

gcgtagtgatgatgatgatgatggcttgcngtnnnggtaacgggtgtagttgcagtgttacgagcaaagcttttcgagctctgaa
aacc

Deletion of Tx repeat in every occurrence:

1. TSTAT**AT**->TSTAT

acctccaccgacgacc**gcaacg** -> acctccaccgacgacc

Oligo (37-15-38mer):

gggcgccatggcgtccggcacctccgaaccaggtcc**acctccaccgacgacc**ggccgtggcgccactgctcgtagcacctc
caccggtcg

Reverse complement (for lagging strand target):

cgaccggtggaggtgctacgagcagtggcgccacggccggtcgcggtggaggtggaacctggttcggaggtgccggacgc
catggcgccc

2. TATTT**AT**->TATTT

actgctaccaccact**gcaact** -> actgctaccaccact

Oligo (37-15-38mer);

tggcgccactgctcgtagcacctccaccggtcgtgg**actgctaccaccact**ggtaccgcatctgcacctccaacgccatcg
gtcaggg

Reverse complement (for lagging strand target):

ccctgaccgatggcggtggaggtcgcagatgcggtaccagtgggtgtagcagtaccacgaccggtggaggtgctacgagcag
tggcgcca

3. TATTT**AT**->TATTT

accgcaaccacgact**gcgacg** -> accgcaaccacgact

Oligo (37-15-38mer):

taccgcatctgcacctccaacgccatcggtcaggg**accgcaaccacgact**ggtccgcaggtggccgtgcaaccggtagc
gcgactac

Reverse complement (for lagging strand target):

gtagtcgcgctaccggtgcacggccacctgcggaaccagtcgtggttcggtaccctgaccgatggcgtggaggtcgcaga
tgcgta

4. TQTQT**IT**->TQTQT

accagactcaaacc**attacc** -> accagactcaaacc

Oligo (37-15-38mer):

cggtagcgcgactacgagcagctccgcgagccagccg**accagactcaaacc**ggcccgggtttcagactgcgaaaagctt
tgctcgtaa

Reverse complement (for lagging strand target):

ttacgagcaaagcttttcgagctctgaaaaccgggcccggtttgagtctgggtcggctggctcgcggagctgctcgtagtcg
ctaccg

5. TATTT**VT**->TATTT

actgcaactaccacc**gttacc** -> actgcaactaccacc

Oligo (37-15-38mer; note: end from His tag+STOP+Term):

cccgggttttcagactgcgaaaagctttgctcgtaac**actgcaactaccacc**gcaagccatcatcatcatcactaacgcaa
aaacc

Reverse complement (for lagging strand target):

ggtttttgcgtagtgatgatgatgatggcttgcgggtgtagtgcagtggttacgagcaaagcttttcgagctctgaaaaccg
gg

Deletion of entire TxT segments

1. Delete **TCHAKATGAGAMASGTSEPGS**

Oligo (45-45mer):

cgtgctgttggcgtagacggtcgtgcagtaacggatatccagggt**acctgccacgctaaggccaccggcggggccatgg
cgccggcacctccgaaccagggtcc**acctccaccgcgaccgcaacgggcccgtggcggccactgctcgtagc ->

cgtgctgttggcgtagacggtcgtgcagtaacggatatccagggtacctccaccgcgaccgcaacgggcccgtggcggccactg
ctcgtagc

Reverse complement (for lagging strand target):

gctacgagcagtggcggccacggcccgttgcggtcgcggtggaggtacctggatatccgtactgcacgaccgtctacgcca
cagcag

2. Delete **TSTATATGRGATARSTSTGRG**

Oligo (45-45mer):

accggtgcggggccatggcgtccggcacctccgaaccagggtcc**acctccaccgcgaccgcaacgggcccgtggcggccact
gctcgtagcacctccaccggcgtggt**actgctaccaccactgcaactggtaccgcatctgcgacctccaac ->

accggtgcggggccatggcgtccggcacctccgaaccagggtccactgctaccaccactgcaactggtaccgcatctgcgac
ctccaac

Reverse complement (for lagging strand target):

gttgaggtcgcagatgcggtaccagttgcagtggtgtagcagtggaacctggtcggaggtgccggacgccatggcggcc
gcaccggt

3. Delete **TATTTATGTASATSNAIGQG**

Oligo (45-45mer):

acgggcccgtggcggccactgctcgtagcacctccaccggtcgtggt**actgctaccaccactgcaactggtaccgcatctgcgac
ctccaacgccatcggtcagggt**actgctaccaccactgcaactggtaccgcatctgcgacctccaac ->

acgggcccgtggcggccactgctcgtagcacctccaccggtcgtggtactgctaccaccactgcaactggtaccgcatctgcgac
ctccaac

Reverse complement (for lagging strand target):

gttgaggtcgcagatgcggtaccagttgcagtggtgtagcagttaccacgaccggagggtgctacgagcagtgggccca
cggcccgt

4. Delete **TATTTATGSAGGRATGSATTSSSASQP**

Oligo (45-45mer):

gcaactggtaccgcatctgacacctcaacgccatcgggcagggt**accgcaaccacgactgcgacgggtccgcaggtggccgtgcaaccggtagcgcgactacgagcagctccgcgagccagccg**accagactcaaaccattaccggcccgggtttcagactgcgaaa ->

gcaactggtaccgcatctgacacctcaacgccatcgggcagggtaccagactcaaaccattaccggcccgggtttcagactgcgaaa

Reverse complement (for lagging strand target):

tttcgagtctgaaaaccgggccgtaatggtttgagctcgggtaccctgaccgatggcgttgagggtcgagatgcggtaccagttgc

5. Delete **TQTQTITGPGFQTAKSFARN**

Oligo (45-45mer):

cgtgcaaccggtagcgcgactacgagcagctccgcgagccagccg**accagactcaaaccattaccggcccgggtttcaga**ctgcaactaccaccgttaccgcaagccatcatcatcatcac ->

cgtgcaaccggtagcgcgactacgagcagctccgcgagccagccgactgcaactaccaccgttaccgcaagccatcatcatcatcac

Reverse complement (for lagging strand target):

gtgatgatgatgatgatggcttgcggtaacgggtgtagttgcagtcggctggctcgcggagctgctcgtagtcgcgctaccggttgcacg

6. Delete **TATTTVTAS**

Oligo (45-45mer):

attaccggcccgggtttcagactgcgaaaagctttgctcgtaac**actgcaactaccaccgttaccgcaagc**atcatcatcatcatcactaacgcaaaaaaccccgttcggcggg ->

attaccggcccgggtttcagactgcgaaaagctttgctcgtaacatcatcatcatcatcactaacgcaaaaaaccccgttcggcggg

Reverse complement (for lagging strand target):

cccgccgaagcgggggtttttgcggttagtgatgatgatgatgatggttacgagcaaaagctttcgcagtctgaaaaccgggccgtaaat

Replacement of Tx repeats with degenerate TXTXTXT

1. TCHAKAT->TXTXTXT

acctgccacgctaaggccacc -> accnnnacnnnacnnnac

Oligo (35-21-34mer):

gcgtagacggctgcagtaacggatatccagggtaccnnnacnnnacnnnacgggtcggggcgccatggcgtccggcaccctccgaac

Reverse complement (for lagging strand target):

gttcggaggtgccggacgccatggcgcccgaccggtnnnngtnnnngtnnnggtaccctggatatccgttactgcacgacgtctacgc

2. TSTATAT->TXTXTXT

acctcaccgcgaccgcaacg -> accnnnaccnnnaccnnnacg

Oligo (35-21-34mer):

gcgccatggcgtccggcacctccgaaccaggtccaccnnnaccnnnaccnnnacgggccgtggcgccactgctcgtagcaccctccaccg

Reverse complement (for lagging strand target):

cggaggaggtgctacgagcagtgggccacggccggtnnnngtnnnngtnnnggtggaacctggtcggaggtgccggacgccatggcgcc

3. TATTTAT->TXTXTXT

actgctaccaccactgcaact -> actnnnaccnnnactnnnact

Oligo (35-21-34mer):

gcgccactgctcgtagcacctccaccggctcgggtactnnnaccnnnactnnnactgggtaccgcatctgcgacctccaaccctccatcggtc

Reverse complement (for lagging strand target):

gaccgatggcgttgagggtcgcagatcgggtaccagtnnnagtnnnngtinnnagttaccacgaccgggtggaggtgctacgacagtggtggcgcc

4. TATTTAT->TXTXTXT

accgcaaccacgactgcgacg -> accnnnaccacgactnnnacg

Oligo (35-21-34mer):

ccgcatctgcgacctccaacgccatcggtcagggtagctaccnnnaccagactnnnacgggtccgaggtggccgtgcaacc
gtagcgcga

Reverse complement (for lagging strand target):

tcgcgctaccggttgcacggccacctgcggaaccgtnnnagtcgtgtnnnggtaccctgaccgatggcgttgagggtcgc
agatgcgg

5. TQTQTIT->T**X**T**X**T**X**T

accagactcaaaccattacc -> accnnnactnnnaccnnnacc

Oligo (35-21-34mer):

gtagcgcgactacgagcagctccgcgagccagccgtagctaccnnnactnnnaccnnnaccggcccgggttttcagactgcgaa
aagctttgctc

Reverse complement (for lagging strand target):

gagcaaagcttttcgactctgaaaaccggccggtnnngttnnnagtnnnnggtcggctggctcgcggagctgctcgtagt
cgcgctac

6. TATTTVT->T**X**T**X**T**X**T

actgcaactaccaccgttacc -> actnnnactnnnaccnnnacc

Oligo (35-21-34mer; note: end from His tag+STOP+Term):

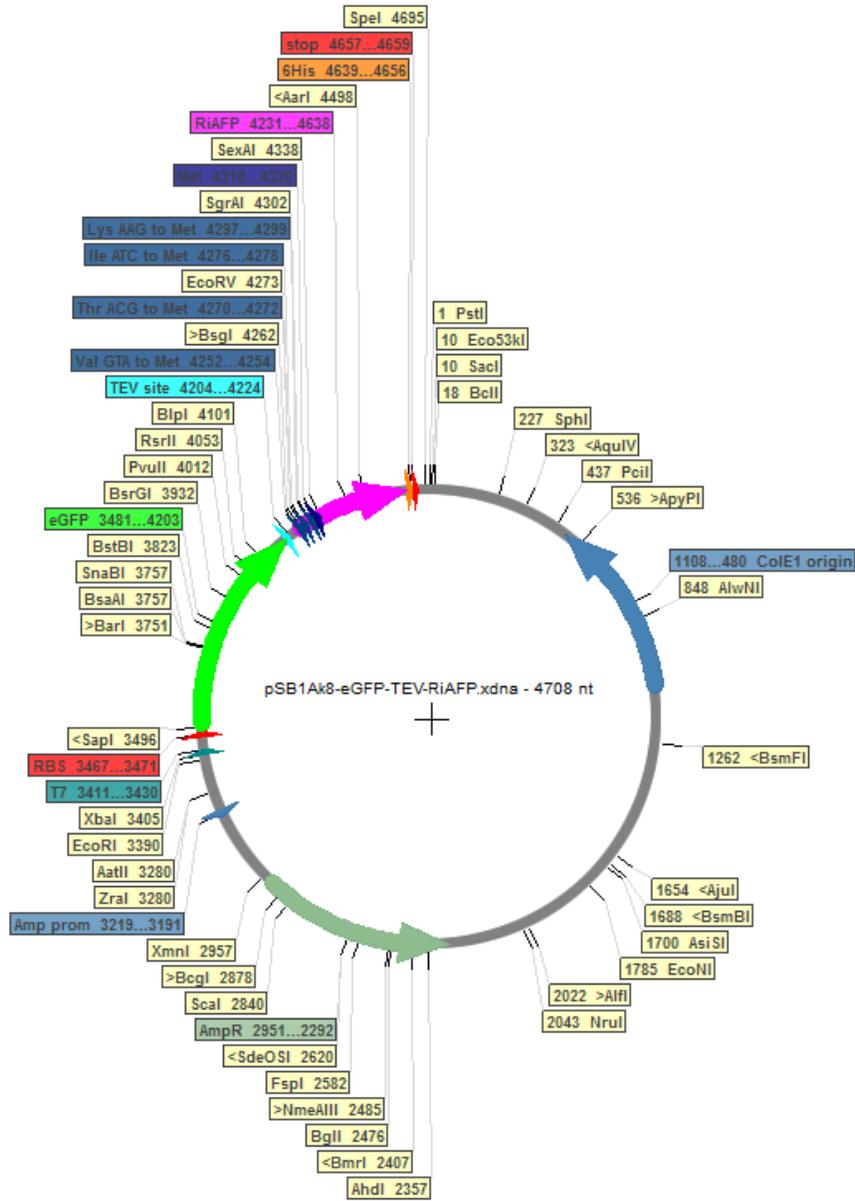
cgggttttcagactgcgaaaagctttgctcgtaacactnnnactnnnaccnnnaccgcaagccatcatcatcatcactaa
cgcaaaa

Reverse complement (for lagging strand target):

ttttgcgttagtgatgatgatgatgatggcttgcggtnnngttnnnagtnnnagtttacgagcaaagcttttcgactctgaaaa
cccg

Se-Methionine Quick Change Primer Design

Restriction map of pSB1Ak8-eGFP-TEV-RiAFP.xdna - 4708 nt
<Serial Cloner V2.1> -- <Fri, Sep 16, 2011 2:49 PM>



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BlpI
|
G CTG AGC AAA GAC CCG AAC GAA AAA CGC GAT CAC ATG GTT CTG CTG GAG TTC GTA ACT GCC GCA GGC ATC ACT CTG GGC ATG GAT GAA CTG TAT AAA GGT < 4200
L S K D P N E K R D H M V L L E F V T A A G I T L G M D E L Y K G
C GAC TCG TTT CTG GGC TTG CTT TTT CGG CTA GTG TAC CAA GAC GAC CTC AAG CAT TGA CCG GGT CCG TAG TGA GAC CCG TAC CTA CTT GAC ATA TTT CCA
4110 4120 4130 4140 4150 4160 4170 4180 4190

                                     >Ile ATC to Met
                                     >Thr ACG to Met
                                     EcoRV
                                     >Lys AAG to Met
                                     >Val GTA to Met
                                     >BagI
                                     >TEV site
GGT GAA AAC CTG TAC TTC CAG GGT GGC GGT TAT TCC TOT CGT GCT GTT GGC GTA GAC GGT CGT GCA GTA ACG GAT ATC CAG GGT ACC TGC CAC GCT AAG G < 4300
G E N L Y F Q G G G G Y S C R A V G V D G R A V T D I Q G T C H A K A
CCA CTT TTG GAC ATG AAC CTC CCA CCG CCA ATA AGG ACA GCA CGA CAA CCG CAT CTG CCA GCA CGT CAT TGC CTA TAG GTC CCA TGG ACC GTG CGA TTC C
4210 4220 4230 4240 4250 4260 4270 4280 4290

SgrAI                                     >Met                                     SexAI
|                                     |                                     |
CC ACC GGT GCG GGC GCC ATG GCG TCC GGC ACC TCC GAA CCA GGT TCC ACC TCC ACC GCG ACC GCA ADG GGC CGT GGC GCC ACT GCT CGT AGC ACC TCC AC < 4400
T G A G A M A S G T S E P G S T S T A T A T G R G A T A R S T S T
GG TGG CCA CCG CCG CCG TAC CCG AGG CCG TGG AGG CTT GGT CCA AGG TGG AGG TGG CCG TGG CGT TGC CCG GCA CCG CCG TGA CGA GCA TCG TGG AGG TG
4310 4320 4330 4340 4350 4360 4370 4380 4390

                                     >RiAFP
                                     |
C GGT CGT GGT ACT GCT ACC ACC ACT GCA ACT GGT ACC GCA TCT GCG ACC TCC AAC GCC ATC GGT CAG GGT ACC GCA ACC ACG ACT GCG ACG GGT TCC GCA < 4500
G R G T A T T T A T G T A S A T S N A I G Q G T A T T T A T G S A
G CCA GCA CCA TGA CGA TGG TGG TGA CGT TGA CCA TGG COT AGA CCG TGG AGG TTG CCG TAG CCA GTC CCA TGG CGT TGG TGC TGA CCG TGC CCA AGG CGT
4410 4420 4430 4440 4450 4460 4470 4480 4490

                                     <AarI
                                     |
GGT GGC CGT GCA ACC GGT AGC GCG ACT ADG AGC AGC TCC GCG AGC CAG CCG ACC CAG ACT CAA ACC ATT ACC GGC CCG GGT TTT CAG ACT GCG AAA AGC T < 4600
G G R A T G S A T T S S A S Q P T Q T Q T I T G P G F Q T A K S F
CCA CCG GCA CGT TGG CCA TCG CCG TGA TGC TCG TCG AGG CCG TCG GTC GCG TGG GTC TGA GTT TGG TAA TGG CCG GGC CCA AAA GTC TGA CCG TTT TCG A

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Features :

- T7 : [3411 : 3430 - CW]
- ColE1 origin : [1108 : 480 - CCW]
- AmpR : [2951 : 2292 - CCW]
- Amp prom : [3219 : 3191 - CCW]
- 6His : [4639 : 4656 - CW]
- TEV site : [4204 : 4224 - CW]
- RBS : [3467 : 3471 - CW]
- RiAFP : [4231 : 4638 - CW]
- eGFP : [3481 : 4203 - CW]
- stop : [4657 : 4659 - CW]
- Met : [4318 : 4320 - CW]
- Thr ACG to Met : [4270 : 4272 - CW]
- Ile ATC to Met : [4276 : 4278 - CW]
- Lys AAG to Met : [4297 : 4299 - CW]
- Val GTA to Met : [4252 : 4254 - CW]

Se Methionine Quick Change Primer Design:

Val GTA to Met (nts 4252-4254)	
Forward:	
original sequence	GCCGCAGGCATCACTCTGGGCATGGATGAACTGTATAAAGGTGGTGAAA ACCTGTACTTCCAGGGTGGCGGTTATTCTGTCGTGCTGTTGGC GTA G
Oligo to order: (5' to 3')	GCCGCAGGCATCACTCTGGGCATGGATGAACTGTATAAAGGTGGTGAAA AACCTGTACTTCCAGGGTGGCGGTTATTCTGTCGTGCTGTTGGCATGG
Tm (degC)	73.2
#nts	97
Reverse:	
orig reverse complement	GGTGCCGGACGCCATGGCGCCCGCACCGGTGGCCTTAGCGTGGCAGGT ACCCTGGATATCCGTTACTGCACGACCGTCT ACGC
Oligo to order: (5' to 3')	GGTGCCGGACGCCATGGCGCCCGCACCGGTGGCCTTAGCGTGGCAGGT ACCCTGGATATCCGTTACTGCACGACCGTCTCATGC
Tm (degC)	76.7
#nts	83
Ile ATC to Met (nts 4276-4278)	
Forward:	
original sequence	CCTGTACTTCCAGGGTGGCGGTTATTCTGTCGTGCTGTTGGCGTAGACG GTCGTGCAGTAACGGATAT CC
Oligo to order: (5' to 3')	CCTGTACTTCCAGGGTGGCGGTTATTCTGTCGTGCTGTTGGCGTAGAC GGTCGTGCAGTAACGGATATGC
Tm (degC)	72.8
#nts	71
Reverse:	
orig reverse complement	GCCGGACGCCATGGCGCCCGCACCGGTGGCCTTAGCGTGGCAGGTACCC TG G ATATC
Oligo to order: (5' to 3')	GCCGGACGCCATGGCGCCCGCACCGGTGGCCTTAGCGTGGCAGGTACC CTGCATATC
Tm (degC)	77.7
#nts	57

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3097 | c a a a a t g c c g c a a a a a g g g a a t a a g g g c g c a c g g a a a t g t t g a a t a c t c a t a c t c t t c c t t t t t c a a t a t t a t t g a a g c a t t t a
3183 | t c a g g g t t a t t g t c t c a t g a g c g g a t a c a t a t t t g a a t g t a t t t a g a a a a a t a a a c a a a t a g g g g t t c c g c g c a c a t t t c c c c g a a
3269 | a a g t g c c a c c t g a c g t c t a a g a a a c c a t t a t t a t c a t g a c a t t a a c c t a t a a a a a t a g g c g t a t c a c g a g g c a g a a t t t c a g a t a a
3355 | a a a a a t c c e t t a g e t t t e g c t a a g g a t g a t t t c t g g a a t t c G C G G C C G C T T C T A G A t a a t a c g a c t c a c t a t a g g g a a t a c a a g c t
3441 | a c t t g t t c t t t t t g c a a c t a g a g T t a a g g a g g t a a a a a a a a A T G G T C T C C A A A G G T G A A G A G C T G T T T A C G G C G T T G T C C G A T C C
3527 | T G G T T G A A C T G S A T G S T G A T G T A A A C G G C C A C A A A T T C T C T G T T T C T G G T G A A G G T G A G G G T G A C G C C A C T T A C G S T A A A C T G A C C
3613 | C T G A A A T T C A T T T G C A C C A C C G G C A A A C T G C C G S T A C C A T G C C G A C T C T G G T T A C G A C C C T G A C C T A C G C G T T C A S T G C T T C T C
3699 | T C G C T A C C C G G A T C A C A T G A A A C A G C A C G A C T T T T T C A A G T C C C G A T G C C G A A G G T T A C G T A C A A G A A C G T A C C A T C T T T T T C A
3785 | A G G A T G A C G S T A A C T A T A A A A C C C G C G G A A G T T A A A T T C G A A G G C G A C A C T C T G G T T A A T C G T A T C G A G C T G A A A A G G T A T C G A T
3871 | T T T A A A G A G G A T G S T A A C A T C C T G S G C C A C A A A C T G S A A T A T A A C T A C A A C A G C C A C A C G T G T A C A T C A T G S C T G A C A A A C A G A A
3957 | G A A C G G C A T C A A A G T T A A T T T T A A A A T C C G T C A C A A C A T C G A A G A C G G T T C T G T C C A G C T G G C T G A C C A T T A T C A G C A G A A C A C T C
4043 | C G A T C G G T G A C G S T C C C G T T C T G C T G C C G G A T A A C C A T T A C C T G T C T A C T C A G T C C C G C G T G A G C A A A G A C C C G A A C G A A A A C G C
4129 | G A T C A C A T G S T T C T G C T G G A G T T C G T A A C T G C C G C A G C A C A C T C T G G G C A T G G A T G A A C T G T A T A A A G G T G G T G A A A A C C T G T A
4215 | C T T C C A G G G T G G C G G T T A T T C C T G T C G T G C T G T T G G C G T A G A C G G T C G T G C A G T A A C G G A T A T C C A G G T A C C T G C C A C G C T A A G G
4301 | C C A C C G G T G C G G G C G C C A T G C G T C C G G C A C C T C C G A A C C A G G T T C C A C C T C C A C C G C G A C C G C A A C G G G C C G T G G C G C C A C T G C T
4387 | C G T A G C A C C T C C A C C G G T C G T G G T A C T G C T A C C A C C A C T G C A A C T G G T A C C G C A T C T G C G A C C T C C A A C G C C A T C G G T C A G G G T A C
4473 | C G C A A C C A C G A C T G C G A C G G G T T C C G C A G G T G G C C G T G C A A C C G G T A G C G C G A C T A C G A G C A G C T C C G C G A G C C A G C C G A C C C A G A
4559 | C T C A A A C C A T T A C C G G C C C G G G T T T C A G A C T G C G A A A A G C T T T G C T C G T A A C A C T G C A A C T A C C A C G T T A C C G C A A G C C A T C A T

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Complete Plasmid Sequence:

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