Flowchart Cassette Mutagenesis (Steps 0. A-L)

0. Original plasmid pT7-αHL-RL3-D8

In our strategy, for double-mutant E111C-K147C, we will make first K147C by a couple of oligo-pairs shorter than 40 bp (so that, they do not require PAGE purification). Then, we will construct the mutant E111C in K147C to make the double-mutant. Short and long oligos should be dissolved in water to a stock concentration of 10 pmol/μl (excepting for PCR at 50 pmol/μl). Longer oligos (>40 nucl) have to be PAGE purified by IDT (Integrated DNA Technologies, Coralville, IA, use PO#5514). The pellet should be re-suspended first in TE8 buffer (10 mM Tris-Cl, 1 mM EDTA, p8.0), then diluted in water at a final concentration of 10 pmol/μl.

For instance, if 10 pmol/μl stock concentration is necessary (for cassette), then in a total volume of 50 μl, we need to add x (μl)=0.5*M_w(ssDNA)/C(ng/μl from IDT). If 50 pmol/μl stock concentration is necessary (for PCR) in the same volume of 50 μl, then we need to add 2.5M_W/C.

We have to avoid a high concentration in EDTA for DNA solution, as this will affect the enzymatic reactions later on. However, a small concentration is necessary to chelate the metal ions present in solution. The vector pT7-αHL-RL3-D8 was engineered by Steve- (actually by inserting the silent mutations within WT background with a D8 tail linked at the C terminus. This plasmid is engineered/provided by Steve.

A. Vector Preparation (Double-Diggestion)

1. Vector pT7-αHL-RL3-D8/*StuI*/*XhoI* (for mutant single cysteine mutant K147C)

0.5 μl 100X BSA (to obtain a final concentration of 100 μg/ml or 0.1 μg/μl)
25 μl RL3-D8 (400 μg/ml; 10 μg total)
5 μl 10X NEB2
12.0 μl ddH_2O
5 μl *StuI* (10 units/μl)
2.5 μl *XhoI* (20 units/μl)
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50 μl Total

Incubate at 37 °C overnight.

2. Vector pT7-αHL-RL3-D8/*HpaI*-*AflII* (for mutants T117C-S141C and N123C-L135C)

0.5 μl 100X BSA (Bovine Serum Albumine) to obtain a final concentration of 100 μg/ml
25 μl RL3-D8 (400 μg/ml; 10 μg total)
5 μl 10X NEB4
12.0 μl ddH_2O
5 μl *HpaI* (10 units/μl) (5-fold enzyme excess)
2.5 μl *AflII* (20 units/μl) (5-fold enzyme excess)
****
50 μl Total

Incubate at 37 °C overnight.

3.Vector pT7-αHL-RL3-D8/*SacII-StuI* (for mutant T115C-T117C)

25 µl RL3-D8 (400 µg/ml; 10 µg total)
5 µl 10X NEB4
12.5 µl ddH_2O
5 µl *SacII* (10 units/µl)
2.5 µl *StuI* (20 units/µl)
****

50 µl Total

Incubate at 37 °C overnight.

4. Vector pT7-αHL-RL3-D8-K147C/*SacII-HpaI* (for mutant E111C) (only 10-fold excess reaction)

12.5 µl RL3-D8-K147C (400 µg/ml; 5 µg total)
5 µl 10X NEB4
20 µl ddH_2O
2.5 µl *SacII* (20 units/µl; total 50 units)
10 µl *HpaI* (5 units/µl; total 50 units)

50 µl Total

Incubate at 37 °C for 1 hour or all day.

1 unit of restriction endonuclease enzyme activity is defined as the amount of enzyme required to completely digest 1 µg of substrate DNA in a total reaction volume of 50 µl in one hour using the NEBuffer provided. Double-check the conditions of reactions for the enzymes (temperature, concentration in units, the buffer, whether does it need BSA or not, star activity etc.). As models, you can have the above-mentioned reactions. One problem with the enzymes is the star activity. To avoid the star activity as much as we can, we have to reduce drastically the glycerol concentration. So, we have to use lower enzyme concentration and longer incubation times. It is recommended that double-digestion be made for 10-fold in excess, otherwise the DNA may be jeopardy. Always keep the enzymes for short times in ice, and the most in –20 °C.

B. Purification of the double-digested fragment by DNA *Qiaquick Gel extraction Kit* (QIAquick Gel Extraction Kit Cat#28704) and its quantification

Take the 50 µl digested fragment product and run on a 1.2 % preparative Agarose DNA Gel and extract the main band from the gel, then purify it by *Qiaquick Gel Extraction Kit* (Cat#28704). You should see only one band following the double-digestion reaction, as the other fragment is quite short (about 60 bp). Follow the protocol given by *Qiaquick Gel Extraction Kit* (**cat#28704 (50) or cat#28706 (250)**) at the page 23: "*QIAquick Gel Extraction Kit Protocol*" using a microcentrifuge (steps 1-13). Take 5 µl out of that and run on a second quantification agarose DNA gel (so that including λ DNA-*Hind III* Diest Marker), therefore you have the remaining 45 µl purified double-digested vector for further use. The plasmid DNA extraction yield from one prep to another varies drastically, even in similar conditions of the experiment. Therefore, the quantification agarose gel at this point of the experiment is critical.

C. Phosphorilation Reaction (we are trying to phosphorilate both together)

Mutant K147C

1 µl (10 pmol/µl) LM23 (E111C-K147C ascending anti-sense I)
1 µl (10 pmol/µl) LM24 (E111C-K147C ascending sense II)
1 µl 10X PNK buffer (Polynucleotide Kinase (PNK) buffer)
2 µl ATP (final of 2 mM) from stock of 10 mM
4 µl ddH_2O
1 µl PNK (10 units/µl)
****
10 µl Total

Mutant T117C-S141C

1 µl (10 pmol/µl) LM09 (T117C-S141C descending anti-sense)
1 µl (10 pmol/µl) LM10 (T117C-S141C ascending sense)
1 µl 10X PNK buffer (Polynucleotide Kinase (PNK) buffer)
2 µl ATP (final of 2 mM) from stock of 10 mM
4 µl ddH_2O
1 µl PNK (10 units/µl)
****
10 µl Total

Mutant N123C-L135C

1 µl (10 pmol/µl) LM13 (N123C-L135C descending anti-sense)
1 µl (10 pmol/µl) LM14 (N123C-L135C ascending sense)
1 µl 10X PNK buffer (Phosphonucleotide Kinase (PNK) buffer)
2 µl ATP (final concentration of 2 mM); stock of 10 mM
4 µl ddH_2O
1 µl PNK (10 units/µl)
****
10 µl Total

Mutant T115C-T117C

1 µl (10 pmol/µl) LM17 (T115C-T117C descending anti-sense)
1 µl (10 pmol/µl) LM18 (T115C-T117C ascending sense)

1 µl 10X PNK buffer (Phosphonucleotide Kinase (PNK) buffer)
2 µl ATP (final concentration of 2 mM); stock of 10 mM
4 µl ddH_2O
1 µl PNK (10 units/µl)
****
10 µl Total

Phosphorilation reaction takes place at 37 °C for 1 hour. Stop phosphorilation reaction by **raising the temperature at 75 °C for 10-15 minutes** (Set up the temperature for the water bath well in advance of the phosphorylation end).*Use 1 µl of each oligo from stock (10 pmol/µl stock) to get a final concentration of 1 pmol/µl.

D. Ligation reaction

Mutant K147C

1 µl LM22 (1 pmol/µl)
1 µl (LM23-P + LM24-P)
1 µl LM25
1 µl vector (pT7-RL3-D8-(*StuI/XhoI*)
1 µl 10X Ligase Buffer
4 µl ddH_2O
1 µl T4 DNA Ligase (400 units/µl)
****
10 µl Total

Mutant T117C-S141C

1 µl LM08 (1 pmol/µl)
1 µl (LM09-P + LM10-P)
1 µl LM11
1 µl vector (pT7-RL3-D8-(*HpaI-AflII*)
1 µl 10X Ligase Buffer
4 µl ddH_2O
1 µl T4 DNA Ligase (400 units/µl)
****
10 µl Total

Mutant N123C-L135C

1 µl LM12 (1 pmol/µl)
1 µl (LM13-P + LM14-P)
1 µl LM15
1 µl vector (pT7-RL3-D8-(*HpaI-AflII*)
1 µl 10X Ligase Buffer
4 µl ddH_2O
1 µl T4 DNA Ligase (400 units/µl)
****
10 µl Total

Mutant T115C-S117C

1 µl LM16 (1 pmol/µl)
1 µl (LM17-P + LM18-P)
1 µl LM19
1 µl vector (pT7-RL3-D8-(*SacII-StuI*)
1 µl 10X Ligase Buffer
4 µl ddH_2O
1 µl T4 DNA Ligase (400 units/µl)
****
10 µl Total

Mutant K147C-E111C

1 µl LM20 (1 pmol/µl) (descending sense)
1 µl LM21 (1 pmol/µl) (descending anti-sense)
1 µl vector (pT7-RL3-D8-K147C-*SacII/HpaI*)
1 µl 10X Ligase Buffer
5 µl ddH_2O
1 µl T4 DNA Ligase (400 units/µl)
****
10 µl Total

Ligation reaction takes place at 4 °C overnight. Take later on 5 µl of each product for transformation XL-10 Gold Competent Cells, then grow the cells on LB-Amp plates in order to have colonies (for DNA MiniPreps).

E. Transformation (late in the afternoon, about 5:00 pm)

LB/amp plate should be kept at 37 °C in the incubator, for 1 h in advance of the transformation. Always keep the LB plates upside down. To dry the remaining water on the caps, you have to keep them opened/upside down in the incubator. Prepare the water bath at 42 °C for heat-shock. Take 1-10 µl of the ligated product WT-RL3-D8-Mutant and re-suspend in the 200 µl competent cells. Mix the tube gently. Do not vortex the cells. Then, place the mixture in ice for about 40 minutes. Place the tube at 42 °C for about 45 seconds, then bring it back on ice for a few minutes. The cells should be spread out on the plate with care (keep everything sterile and clean).

F. Growing the cells overnight on LB/Amp plate

In the morning take a look at the colonies on the LBA plates and chose one for Quigen Mini-Preps. Usually you can take 4 to 6 colonies from the LBA plate to prepare analytical samples for Qiagen MiniPreps. In extreme situations (like that with our mutants 123-135, 117-141 and 147, you can take about 18 colonies for minipreps for a single mutant. Protocol for LB and LBA medium/plates is provided in a separate folder

regarding competent cells/culture media (Competent Cells_LBPlates.doc). The colonies could be kept at the 4 °C fridge for maximum one-two weeks without any problem. Do not worry about additional mutations in the DNA plasmid.

G. Growing the cells in LB/Amp Media (between 14-18 hours)

Take a part of a colony and re-suspend in a volume of 10 ml LB_Amp medium for the MiniPreps. Allow growing the cells between 12 to 16 hours (never more than 18 hours).

H. Mini-Prep

Start Mini-Preps. Follow the protocol given by *QiaPrep Spin MiniPrep Kit*, **Cat#27104 (50) and Cat#27106 (250)**. Centrifugate the lysate for 40 minutes at 14000 rpm in a table top microcentrifuge. Save 1 ml of each sample from the MiniPreps to further use for the Maxi- or Midi-Preps in order to avoid an additional transformation. In addition, this 1 ml culture could be useful, if an additional Mini's Prep run is required. To do a second mini-prep, you need to take only 2 µl cultured cells (equivalent to a colony) from the previous grow/run (kept at 4 degrees) and re-suspend in 10 ml LB_Amp. Then allow them growing for 12 to 16 hours, but not more than 18 hours. The plasmid DNA yield from Mini's should be about **100 nl/µl.** Out of that between 10-20 µl should be sent out for sequencing, if it passes the knocked out enzyme test.

I. Screening the knocked out restriction sites and sending the samples off for sequencing***

Make a mix of n+1 (6+1) reactions for the screened restriction site. Prepare the analytical SDS DNA-gels for running the screened sites. Following that, send for sequencing 10-20 µl vector (100 ng/µl) from Qiagen Miniprep screened with Afl II (or your respective enzyme) and 25 µl screening primer SC002 (at concentration of 2 pmol/µl). The bottom lanes (that migrated faster within the gel) are more compact and not cut by the restriction endonuclease enzyme (so that the restriction site is replaced and the restriction enzyme is knocked out). Those bottom bands are sent off for sequencing to Lone Star (Houston).

Mutant K147C: *AflII* knocked out
For instance: decide for 6 samples
Make a mixture of 6+1=7 reaction mixes.

One reaction
0.5 µl 100X BSA
2.5 µl ddH_2O
1.0 µl NEB2
1.0 µl *AflII*
5.0 µl DNA
10.0 µl Total
Run 1 hour at 37 °C

7 reactions mix

3.5 µl 100X BSA
17.5 µl ddH_2O
7 µl NEB2
7 µl *AflII*

take 5 µl plasmid DNA + 5 µl mix >>>>> for 1 hour at 37 °C

Mutant T115C-T117C: *HpaI* knocked out by mutation in position 117

For instance: decide for 6 samples
Make a mixture of 6+1=7 reaction mixes.

One reaction

2.0 µl ddH_2O
1.0 µl NEB4
2.0 µl *HpaI* (5 units/µl)
5.0 µl DNA
10.0 µl Total
Run 1 hour at 37 °C

7 reactions mix
14.0 µl ddH_2O
7 µl NEB4
14 µl *HpaI*

take 5 µl plasmid DNA + 5 µl mix >>>>> for 1 hour at 37 °C

Mutant N123C-L135C: *HpaI* knocked out by silent mutation in the oligonucleotide Descending
Similar procedure like that for T115C-T117C

Mutant T117C-S141C: *HpaI* knocked out by mutation in position 117
Similar procedure like that for T115C-T117C

Mutant E111C-K147C (after previously cloned K147C): *HpaI* knocked out by mutation in position 117
Similar procedure like that for T115C-T117C

Run 10 µl digestion reaction on an analytical 1 % DNA gel. Also add λ-Hind III Marker lanes for right quantification of the DNA plasmid.

J. Follow the gene sequence using the software Chroma2 from the site:***
Chromas2 could be downloaded for free trial (60 days) from either Technelysium (Australia) or LoneStar Labs (Houston, TX). Version1 is free from Technelysium.
www.technelysium.com.au/chromas.html

Sequencing is performed very well by Lone Star Laboratories:
www.lslabs.com
It is provided in two days.

K. Recombination of the mutated plasmid DNA (the same could be done with a WT protein)***

If the sequences are correct (follow the β-barrel part the most), then proceed with the recombination and Midi's or Maxi's. Keep in mind that recombination is much more efficient than transformation. For midi's/maxi's take the plasmid DNA from the the right sample confirmed by sequencing. Then dilute to a factor of at least 1/100 to maximum of 1/500!!!! Mix 10 µl diluted (at least 1/100) and mutated plasmid DNA with 150-200 µl competent cells and plate them for overnight growing at 37 °C (LB_Amp plates).
Then repeat the transformation step (E.), as written above.

In the next morning, take one colonies and re-suspend in 10 ml LB_Amp in a 50 ml tube, then place in the shaker incubator at 37 degrees and 250 rpm for 8 hours.

In the same day, late in the afternoon, take 1 ml of cells grown in LB_Amp and dissolve in 60 ml LB_Amp (for Midi's) or 200 ml (for Maxi's), then introduce again in the shaker incubator (37 °C and 250 rpm) for overnight growing (between 12-16 hours). Be careful to autoclave the LB_Media in the Glass Erlemayer, then add Ampicillin to the required concentration of 50 µg/ml.

K. Midi's or Maxi's

If the sequence from Lone Star Labs is OK (www.lslabs.com), then we continue with Midi's or Maxi's for preparation the final product of the mutant plasmid. Follow the instructions from *Qiagen Plasmid Midi and Maxi Purification Kit* (**Cat#12143 (25) and Cat#12145 (100) for Midi's, or Cat#12162 (10) and Cat#12163 (25) for Maxi's**). The protocols for Midi's and Maxi's by Qiagen are identical, while only the culture volumes vary. Optimal culture volumes are 50-60 ml for Midi's and 130-150 ml for Maxi's, respectively. First, take a colony and re-suspend in 10 ml LB_Amp, allow growing the cells for 8 hours, then take 1 to 5 ml and re-suspend in the required LB_Amp volume for Midi's or Maxi's and allow growing the cells between 12 and 16 hours (see above). If you saved 1 ml cells from Mini's, then take 2 µl of cells from Mini's and proceed by re-suspending it in 10 ml LB_Amp and continue the Midi's or Maxi's Preps.

L. Precipitate, Quantify and Alliquote the mutated plasmid DNA

Use TE8.5 buffer, NaAcetate, and Ethanol for precipitation procedure (Use either a complex protocol from Lakmal – Via George, or simplified protocol wit direct dissolving in TE8.0 Buffer – via Steve)

Alternative construct for N123C-L135C following a combination cassette mutagenesis/PCR