**General Molecular cloning Protocols**

**(Subcloning a 300bp fragment into a 5kp vector)**

Design Primers

1. 18-25 bp overlapping with the desired PCR fragment, with 5-6 extra base pairs and the DNA restriction enzyme recognition sequence flanking the overlapping sequences
2. Each overlapping A/T can be approximately counted as 2oC and G/C as 4oC for calculating Tm (annealing temperature)
3. If possible, primers should end with 1-3 G/C.
4. GC>50%, Tm~66-72oC, both primers should have similar GC percentile and Tm.
5. ΔG (free energy) for hairpin and dimmer close to Zero.
6. Dissolve the primers in ddH2O for the stock concentration 20 M and keep in -20C.

PCR Reaction

5x Phusion buffer 20 l

10 mM dNTP 2 l

20 M primer 1 2.5 l

20 M primer 2 2.5 l

Phusion 0.5 l

DNA plasmids 0.2 l (from miniprep)

Water 72 l

Total 100 l

Note: Keep the reaction mixture in ice all the time until the lid and slots are heated before putting the reaction tubes into the PCR machine to decrease the chance of non-specific annealing.

It is recommended to use 8 strip tubes, USA scientific, temp assure PCR 8-tube strips, Dove Caps, Natural

PCR program:

1. 95oC 30 sec
2. 95oC 30 sec
3. 62oC 20 sec
4. 72oC 30 sec
5. 2-4, repeat for 30 cycles
6. 72oC 5 min
7. 4oC forever

DNA Electrophoresis

1. For 300 bp DNA product, prepare 50 ml 2% Agarose Gel with 2.5 l EB, microwave to dessolve
2. Pull the gel with 2-well comb,
3. Add 10X or 5X DNA loading buffer, mix and load onto the wells together with appropriate DNA ladder (e.g. 300bp DNA fragment can use 100 bp Ladder)
4. Run 20 min with 120V in 1X TAE buffer,
5. Take out the gel and take a picture for record
6. Cut the gel with the desired band, weight the gel extraction and mark it on the tube wall

Gel Extraction

1. For >2% gel, 6X QG buffer, otherwise, 3X QG buffer, for example, if the weight of the extracted gel is 200 g, then 3X QG buffer is 600 l, incubated in 50oC for 10 min until completed desolved,
2. Add 1X Isopropanyl, mix and load on to the spin column,
3. Centrifuge for 30 sec top speed, discard the flow through,
4. add 750 L QG buffer or (QG+Isopropanol for small size DNA fragments), 30 sec centrifuge top speed, discard the flow through
5. Add 750 L PE with ethanol to the column,
6. Centrifuge for another 30 sec, discard the flow through,
7. Centrifuge another 1 min top speed,
8. Transfer the column to 1.5 ml Eppendorf tubes,
9. Add 30-50 L ddwater, wait for 1 min,
10. Centrifuge 1 min 8k rpm to collect the DNAs.

Insert Fragment and Vector Digestion

1. Insert Fragment Digestion using KpnI HF (high fidelity) and XhoI:

10x buffer 4 5 l +0.5 l 100XBSA

KpnI HF 2.5 l

XhoI 2.5 l

DNA 30 l from gel extraction product

H2O 10 l

Total 50 l

Digestion for 6 hr at 37oC

1. Vector Digestion using KpnI HF and XhoI, and subsequent dephosphorylation:

10x buffer 4 5 l +0.5 l 100XBSA

KpnI 2.5 l

XhoI 2.5 l

DNA plasmid from miniprep 15 l

H2O 25 l

Total 50 l

Digestion for 6 hr at 37oC

Then add 3 l Antarctic Phosphotase (Biolab M0289S)

 1 l H2O

 6 l 10 x buffer (Biolab B0289S)

Incubate for 1 hr at 37oC to dephosphorylate the DNAs

Then incubate in 65ºC for 5 min to deactivate Antarctic phosphatase

Note: phosphatase is quite robust, and hence no need to clean the digestion reaction buffer before the dephosphorylation reaction

Gel Extraction and purification for the vector,

PCR cleaning kit purification for the insert fragment digestion

Using 50 L ddwater to elute for each group

For PCR cleaning kit purification:

1. Add 5X PBI (PB buffer with isopropanol)
2. Mix and load into spin column
3. Spin for 30 sec top speed, Discard the flow through
4. add 750 PE buffer with Ethanol
5. Spin for 30 sec top speed, discard the flow through
6. Spin for another 1 min top speed, transfer the columns to 1.5 ml Eppendorf tubes
7. Add 30-50 L ddwater, wait for 1 min, centrifuge 30 sec top speed to elute.

Measure the DNA concentration for the purified vector and insert fragments

Nanodrop 2000 measurement for DNA concentration

1. 70% Ethanol to spray the Kimwipe tissue paper, wipe the tip
2. Select Type Nucleic Acid
3. load 1.5 L elution solution on the tip, EB buffer or water depending on the elution solution
4. click Blank
5. Load 1.5 L Sample, and click Measure

DNA Ligation

For 20 l reaction volume, it is good to use Vector around 100-150 ng,

For Insert fragments, 5X or 10X in moles

So for 5 kp vector and 300 bp insert,

insert=5\*(300bp/5kp)\* (100-150ng) vector=5\*(6-9)ng=30-45ng

The concentration of pYD1 vector measured is: 72.7 ng/l, size 5kb

 That of Src-SH2 insert fragment is : 62.7 ng/l, size 300 bp

So prepare the reaction mixtures in ice bucket as following:

Groups 1 2 3

Vector 2 l 2 l 2 l

PCR insert 0.5l 1 l 0 l

10 x buf 2 l 2 l 2 l

T4 1 l 1 l 1 l

H2O 14.5 l 14 l 15 l

Total 20 20 20

Ligation buffer is very sensitive, so mark the usage times of the ligation buffer after each use, typically, ligation buffer should be aliquoted for the first time and not to be used more than 3-4 times

Ligate at 16oC for overnight, or PCR using the following program:

1. 37oC 30 sec
2. 10oC 30 sec
3. 16oC 10 min
4. 1-3, repeat for 30 cycles
5. 16oC 1 hr
6. 4oC forever

Note: If nonspecific recombination events are the concern, add 1 L plasmid safe ATP-dependent nuclease (from Epicenter) and 1 L ATP (from Epicenter) to the reaction mixture after ligation, incubate for 30 min at 37oC to digest all non-ligated products.

Transformation

Chemical Competent Cells:

1. Get an ice bucket, put in chemical competent DH5 cells, wait for the cells to thaw, typically 5-10 min, add the ligation mixture tubes in ice as well.
2. Add 1 L ligation mixture into the competent cells, wait for 30 min in ice.
3. Heat shock at 42oC for 45 sec
4. Chill on ice for 2 min
5. Add 1 ml pre-warmed 37oC SOC medium (Soc medium from Invitrogen, 15544-034) to the competent cells, immediately transfer to 15 ml culture incubation tubes
6. Incubate at 37oC for 1 hr

ElectroCompetent Cells:

1. DNA cleaning and concentratorTM-5 kit (DNA cleaning kit, Zymo Research, D4013)
2. 4x binding buffer, so 80 l for 20 l ligation production, mix
3. Load on the column, Spin 30 sec, 6K rpm, discard the flow through
4. add 200 l washing buffer, spin 30 sec, discard the flow through
5. spin another 1 min top speed
6. transfer to 1.5 ml collection tubes
7. Add 6 l water to the column to elute, wait for 1 min, Spin 30 sec top speed to elute
8. Get an ice bucket, put in electrocompetent cells, wait for the cells to thaw, typically 5-10 min, add the electroporation cuvettes and ligation mixture tubes in ice as well.
9. Add all the 6 L purified ligation mixture into the competent cells, wait for 1 min
10. Take the ice bucket to the BioRad electroporation machine,
11. Configure the machine to set Voltage 2.5 KV, Resistant 200 om, 25 capacity, 5 mSecond duration,
12. pulse first to charge, hear the beeping sound with around 4.78-5.23 display
13. add the competent cells into 0.2 mm cuvettes,
14. load and push in the cuvettes in the electroporation machine, electroporate and pulse, wait for the beeping sound and display
15. Add 1 ml pre-warmed Soc medium (Soc medium from Invitrogen, 15544-034) immediately to cuvette, transfer to 15 ml culture incubation tubes for amplification in 37oC 1 hr.

Plating

1. Pre-warm the agar+Ampcillin plates, 10-15 min at 37oC
2. Add autoclaved beads into each plate, add 100 L competent cells (or 10 L competent ells + 90 L LB mediaum if efficiency is too high, or if the efficiency is low, centrifuge down the cells with 6000rpm for 5 min, aspirate 900 l supernatant, resuspend the cells in the remaining 100 l LB medium) into each plate
3. Shake to spread the cells,
4. Wait 3-5 min so that the cells are absorbed into the plates, discard the beads
5. incubate at 37oC overnight
6. In this case, chemical competent cells, 1:5 vector:insert molar ratio gives several hundred colonies, while 1:10 vector:insert molar ratio gives 50-60 colonies.

Colony PCR

5X buffer 4 l

10 mM dNTP 0.4 l

20 M primer 1 0.5 l

20 M primer 2 0.5 l

Phusion 0.1 l

DNA colony

H2O 14.5 l

Total 20 l

Run PCR and gel electrophoresis

Pick several colonies, inoculate into 5 ml LB+1:1000 Amp, 37oC overnight

Purify the DNA plasmids with Qiagen Miniprep (be careful to make sure that the mixing are complete after each steps of P1, P2, and N3 before centrifuge)

Run digestion and Gel electrophoresis to check the DNA plasmid qualities.

Select several DNA plasmids with correct digested patterns and send for sequencing

**Yeast transformation:**

EBY100 streaks on SC-trp plates, pick one colony, add into 3 ml YPAD medium

1X YPAD: Yeast extract peptone dextrose medium (1 liter)

1% Bacto yeast extract

2% bacto peptone

Adenine hemisulfate 80 mg/L

2% dextrose (D-glucose)

1. Dissolve the following in 900 ml water

10 g yeast extract

20 g of peptone

1. Optional: add 20 g agar, if making plates
2. Autoclave for 20 min on liquid cycle and cool to 55-60oC.
3. Add 100 ml of 20% dextrose (filter-sterilized)

Store medium at room temperature for 1-2 month

Vortex the YPAD medium, incubate, shaking 250 rpm, 30oC overnight

Measure yeast density, add 10 ul yeast culture into 1ml water, mix and measure at 600 nm (1X106 cells/ml give OD600 of 0.1). reading is 1.6, so times 10, the actual culture reading should be 16, so 1.6\*108 cells/ml of the culture.

Add 1/1.6 ml (1\*108 cells) to 50 ml pre-warmed 2xYPAD, OD reading should be 0.2 now, 2\*106 cells/ml

Shaking 30oC, 200 rpm until titer is 2X107 cells/ml, OD reading should be about 1, usually takes 4 hr

Transfer to 50 ml tube, 4000 rpm, centrifuge 5 min, (eppendorf, 5810R)

Wash 3X with 50ml water

Resuspend in 1 ml sterile water, transfer to a 1.5 ml eppendorf tube,

30 sec top speed spin down, resuspend in 1 ml water,

Aliquot 100ul/tube, centrifuge to spin down, some for frozen

Frozen medium for yeast: 10% glycerol, 5% DMSO, 85% H2O

Boil ssDNA (2mg/ml), 5 min, chill in ice water,

Transformation mix components:

PEG 3350 (50% w/v) 240 ul

LiAc 1.0M 36 ul

ssDNA (2mg/ml) 50 ul

plasmid DNA plus sterile water 34 ul

total 360 ul

mix to the yeast pellet, vortex, heat shock 42OC, 30 min

(optional) spin down, 0.5 min top speed, resuspend in 300 ul water

add 1 ml YPAD into the transformation tube, vortex to mix and resuspend

incubate for 2-3 hr at 30oC to ensure good expression

plate 2, 20, 200 ul of the cell suspension into SC-trptophan plates

SC selection medium for yeast:

SC-X 600 ml

3g (NH4)SO4 Ammonium Sulfate (Fisher Chemical, A702-500)

1.02 g Difco Yeast invitrogen bases w/o amino acids (Becton Dickinson, BD, 291940)

12 g glucose (sugar, Sigma G8270)

0.5 g SC-x mix (e.g. CSM-TRP, MP4511-022)

60 mg Adenine Hemisulfate Salt (Sigma, CAS 321-30-2)

Agar plate, add Bacto-Agar 10 g (BD 214010)

Incubate the plates for 3-4 days and counts the colonies to calculate the efficiency

Pick one colony, grow in 10 ml SC-W (2% glucose), overnight 30oC with shaking in flasks

Next day, OD reading should be between 2-5, a little over should be okay

Centrifuge, 4000rpm 10 min at RT (20-25oC)

Discard, the supernatant, 1X wash with SC-W, 2%galactose without glucose

Add 10 ml SC-W, 2%galactose without glucose

Incubate overnight (not to over 48 hr), reading for staining

1X wash with 100 ul PBS+0.5% BSA, for each group, 50 ul will be enough

Resuspend in 100 ul 1st Ab (1:100) in 0.5BSA-PBS, for 1 hr RT or overnight 4oC (e.g. mouse anti-V5, Invitrogen 46-0705)

1x wash with 0.5BSA-PBS, 2nd Ab (1:100) in 0.5BSA-PBS, for 1 hr RT or overnight 4oC, (e.g. biotinylated anti-mouse)

1x wash with 0.5BSA-PBS, PE-streptavidin (1:200) in 0.5BSA-PBS, for 0.5 hr RT (e.g. Strep-PE, BD 554061)

3X wash with 0.5BSA-PBS, resuspend in 500 ul PBS, ready for flow cytometry

Click scatter plot, 1. Fwd (FSC-DSSC), 2 PE-A/EGFP

2 histogram plot, 1. X-axis, PE, 2. X-axis, EGFP

Binding buffer is better than PBS in allowing the binding of phosphorylated tyrosine peptides to SH2 domain