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 2^oC and G/C as 4^oC for calculating Tm (annealing temperature)
3. If possible, primers should end with 1-3 G/C.
4. GC>50%, Tm~66-72^oC, 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 ddH_2O for the stock concentration 20 μM and keep in -20^oC.

PCR Reaction

5x Phusion buffer	20 μl
10 mM dNTP	2 μl
20 μM primer 1	2.5 μl
20 μM primer 2	2.5 μl
Phusion	0.5 μl
DNA plasmids	0.2 μl (from miniprep)
Water	72 μl
Total	100 μl

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

DNA Electrophoresis

1. For 300 bp DNA product, prepare 50 ml 2% Agarose Gel with 2.5 μl 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 μg, then 3X QG buffer is 600 μl, incubated in 50^{o}C 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 μL QG buffer or (QG+Isopropanol for small size DNA fragments), 30 sec centrifuge top speed, discard the flow through
5. Add 750 μL 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 μL 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 μl +0.5 μl 100XBSA
KpnI HF	2.5 μl
XhoI	2.5 μl
DNA	30 μl from gel extraction product
H_2O	10 μl
Total	50 μl

Digestion for 6 hr at 37^{o}C

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

10x buffer 4	5 μl +0.5 μl 100XBSA
KpnI	2.5 μl
XhoI	2.5 μl
DNA plasmid from miniprep	15 μl
H_2O	25 μl
Total	50 μl

Digestion for 6 hr at 37^{o}C

Then add 3 μl Antarctic Phosphotase (Biolab M0289S)
1 μl H_2O
6 μl 10 x buffer (Biolab B0289S)

Incubate for 1 hr at 37°C 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 μL 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 μL 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 μL elution solution on the tip, EB buffer or water depending on the elution solution
4. click Blank
5. Load 1.5 μL Sample, and click Measure

DNA Ligation

For 20 μl 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/μl, size 5kb
That of Src-SH2 insert fragment is : 62.7 ng/μl, size 300 bp

So prepare the reaction mixtures in ice bucket as following:

Groups	1	2	3
Vector	2 µl	2 µl	2 µl
PCR insert	0.5µl	1 µl	0 µl
10 x buf	2 µl	2 µl	2 µl
T4	1 µl	1 µl	1 µl
H_2O	14.5 µl	14 µl	15 µl
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 16°C for overnight, or PCR using the following program:

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

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

Transformation

Chemical Competent Cells:

1. Get an ice bucket, put in chemical competent DH5α cells, wait for the cells to thaw, typically 5-10 min, add the ligation mixture tubes in ice as well.
2. Add 1 µL ligation mixture into the competent cells, wait for 30 min in ice.
3. Heat shock at 42°C for 45 sec
4. Chill on ice for 2 min
5. Add 1 ml pre-warmed 37°C SOC medium (Soc medium from Invitrogen, 15544-034) to the competent cells, immediately transfer to 15 ml culture incubation tubes
6. Incubate at 37°C for 1 hr

ElectroCompetent Cells:

1. DNA cleaning and concentrator™-5 kit (DNA cleaning kit, Zymo Research, D4013)
    1. 4x binding buffer, so 80 µl for 20 µl ligation production, mix
    2. Load on the column, Spin 30 sec, 6K rpm, discard the flow through
    3. add 200 µl washing buffer, spin 30 sec, discard the flow through

4. spin another 1 min top speed
5. transfer to 1.5 ml collection tubes
6. Add 6 µl water to the column to elute, wait for 1 min, Spin 30 sec top speed to elute

2. 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.
3. Add all the 6 µL purified ligation mixture into the competent cells, wait for 1 min
4. Take the ice bucket to the BioRad electroporation machine,
5. Configure the machine to set Voltage 2.5 KV, Resistant 200 om, 25 capacity, 5 mSecond duration,
6. pulse first to charge, hear the beeping sound with around 4.78-5.23 display
7. add the competent cells into 0.2 mm cuvettes,
8. load and push in the cuvettes in the electroporation machine, electroporate and pulse, wait for the beeping sound and display
9. 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 37^{o}C 1 hr.

Plating

1. Pre-warm the agar+Ampcillin plates, 10-15 min at 37^{o}C
2. Add autoclaved beads into each plate, add 100 µL competent cells (or 10 µL competent ells + 90 µL LB mediaum if efficiency is too high, or if the efficiency is low, centrifuge down the cells with 6000rpm for 5 min, aspirate 900 µl supernatant, resuspend the cells in the remaining 100 µl 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 37^{o}C 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 µl
10 mM dNTP	0.4 µl
20 µM primer 1	0.5 µl
20 µM primer 2	0.5 µl
Phusion	0.1 µl
DNA	colony
H_2O	14.5 µl
Total	20 µl

Run PCR and gel electrophoresis

Pick several colonies, inoculate into 5 ml LB+1:1000 Amp, 37^{o}C 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
2. Optional: add 20 g agar, if making plates
3. Autoclave for 20 min on liquid cycle and cool to 55-60^{o}C.
4. 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, 30^{o}C overnight

Measure yeast density, add 10 ul yeast culture into 1ml water, mix and measure at 600 nm (1X10^{6} cells/ml give OD_{600} of 0.1). reading is 1.6, so times 10, the actual culture reading should be 16, so 1.6*10^{8} cells/ml of the culture.

Add 1/1.6 ml (1*10^{8} cells) to 50 ml pre-warmed 2xYPAD, OD reading should be 0.2 now, 2*10^{6} cells/ml

Shaking 30^{o}C, 200 rpm until titer is 2X10^{7} 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 42^{O}C, 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 30^{o}C 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 $(NH_4)SO_4$ 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 30^{o}C 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 (20-25^{o}C)
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 4^{o}C (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 4^{o}C, (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