APh162: DNA Science Protocols

Restriction digest:

Digesting the pZS plasmid with KpnI and HindIII:

Double digest for the vector:
The rule of thumb is that you should start with at least 3ug of your original uncut vector. Adding the restriction enzymes should be the very last step. Before that calculate and add the required amount of water.

1. 5ug of pZS plasmid
2. NEB buffer (check the catalog, I think it's NEB2)
3. BSA
4. Complete with water to as small of a volume as possible. This will typically be 100~300ul.
5. 2ul of KpnI
6. 2ul of HindIII
7. Spin down on the tabletop microcentrifuge in order to get all the liquid to the bottom.
8. Pippette up and down in order to mix (watch the glycerol!)
9. Incubate at 37C for 2-3 hours.

Single digest controls for the vectors:
Since we only want this to image it using a gel and EthBr we only need as little as 100ng.

1. 300ng of pZS plasmid
2. NEB buffer (check the catalog, I think it's NEB2)
3. BSA
4. Complete with water to as small of a volume as possible. This will typically be ~30ul.
5. 0.5ul of *either* KpnI or HindIII
6. Spin down on the tabletop microcentrifuge in order to get all the liquid to the bottom.
7. Pippette up and down in order to mix (watch the glycerol!)
8. Incubate at 37C for 2-3 hours.

Double digest for the insert:
The insert is a PCR amplified product. It should be double digested in a similar fashion to the vector. The single digest controls do not make much sense because we cannot distinguish the different fragments easily. After the digestion perform a PCR purification.

Digesting Lambda/HindIII DNA with EcoRI:

1. 3 ul Lambda/HindIII (1.5 ug)
2. 5 ul NEBuffer EcoRI (10x)
3. 40 ul ddH2O
4. 2 ul EcoRI

50 ul Total

PCR amplification of the insert:

PCR cycle:

1. 94C for 2 min – DNAP activation
2. 94C for 15 s – melting
3. 60C for 30 s – anheling
4. 68C for 3.5 min (min/kb) – elongation
5. Go back to 2 for a total of 35 cycles
6. Store at 4C

PCR reaction:

If you're making stocks use 50ul reactions. Check out the composition in AccuPrime II.pdf. The primers to be used are *** (check out APh162 Primers.doc). The template can be any lacZ containing plasmid.

PCR purification:

Protocol is explained in the handbook in QIAquickSpin.pdf. You want QIAquick PCR Purification Kit Protocol using a microcentrifuge on page 18. Elute using EB. In order to increase yield one can use EB at 65C and incubate in the column for 5 minutes.

Preparing and running gels:

Casting a gel:

Gels are 1% agarose in TAE buffer. Make 50x TAE stock solution. Recipe is in the grey protocols book.

Loading a gel:

The smaller combs have wells that can support up to ~20ul without much problems. Chose a volume and mix with 6x loading dye accordingly. Don't forget the ladders!
For the samples we're going to be using 80 minutes at 90V is enough.

Gel extraction:

For these gels use the wider combs, with only 6 lanes:

1. Ladder
2. Uncut plasmid
3. Free (to avoid cross contamination)
4. Double digest
5. Double digest
6. Double digest

Additionally, if you've done the necessary controls already you might choose to use more lines for the double digest. These wells usually cannot take more than 60ul, so be careful with overloading them.

In order to visualize these gels use as long of a wavelength as possible. This supposedly reduces the amount of damage on the DNA. The UV "flashlight" we have might come handy.
Cut all the bands at once with the razorblade. Remember to cut straight! Put the sample in a 15ml centrifuge tube. Weight your sample.
Carry on with the protocol from QIAquick Gel Extraction Kit Protocol using a microcentrifuge (page 23 on QIAquickSpin.pdf). I usually do some optional steps: adding Sodium Acetate in step 4 and step 5. Elute in EB like in PCR purification.

Ligation:
We will use the Roche Rapid Ligation Kit. The protocol is RocheRapidDNALigation.pdf. The exact volumes depend on the vector and insert concentrations, but there are a couple of restrictions (which are also outlined in the protocol):

- The total mass of each reaction should not exceed 200ng
- Your total mixture of vector and insert should have a volume higher than 10ul. Less than that is OK since it can be completed with the provided DNA dilution buffer.

Make four samples:

- 3:1, 1:1 and 1:3 molar ratios between vector and insert
- No insert control

After the ligation is done (this takes only 5 minutes!) you should perform a killer cut. In the case of the starting from one of HG's looping plasmids with YFP it can be either *** or ***.
After the killer cut the samples have to be PCR purified. Otherwise the transformation yield goes down by a lot.

Cell transformation by electroporation:
We'll use previously made electrocompetent cells. DH5alphaZ1 is probably the best choice because of the screening we'll do. Check out the protocol for making competent cells in HG's black folder. Making these usually takes a whole day and they have to be frozen for a day before use, so plan ahead).

- Put 1ml of LB in 14ml Falcon tubes. You need one tube per transformation. Leave them at 37C.
- Prepare LB+Kan plates with X-gal and IPTG. Start by spreading 40ul of 40ng/ul (HG: check) on each plate.
- Pick one vial of electrocompetent cells per ligation reaction form the -80C freezer and put them on ice.

DNA extraction form cells:

Small plasmid quantities:
Regular throughput:
If you want some sample to use as a PCR template this is the way to go.
http://www1.qiagen.com/literature/handbooks/PDF/PlasmidDNAPurification/PLS_QP_Miniprep/1034641_HB_QIAprep_112005.pdf, protocol on page 22.
Higher throughput:
If you wanted to send your samples for sequencing and you're dealing with a low copy plasmid you'll want a modified MiniPrep protocol. Ask HG.

Medium plasmid quantities:
Regular throughput:
If you're doing cloning on a high copy plasmid, the protocol
http://www1.qiagen.com/literature/handbooks/PDF/PlasmidDNAPurification/PLS_HiSpeed_Purification/1034640_HB_HiSpeed_112005.pdf on page 17 (blue protocol corresponding to the MidiKit).
Higher throughput:
If you're doing cloning on low copy plasmid (like HG's looping plasmids) you might need a modified version of the MidiPrep protocol. Ask HG.

In order to get our plasmids into bacterial cells, we must make them electrocompetent – that is, after applying a current across the cells, some holes open up and DNA in the external solution can get in.

-Making electrocompetent cells.
general considerations: keep everything cold, on ice
have 350 mL of 10% sterile glycerol chilled on ice
be gentle
resuspend pellets using sterile plastic pipette.
work quickly
low salt LB has 0.5g NaCl per liter of LB instead of 5g. low salt LB needs to be made from individual components.

Fermentation:
1. Streak single colony from -80 C stock
2. Start 25mL, low salt LB inoculum, 37C, overnight in 50mL conical tube.
3. Tape lid loosely so cells can aerate
4. Using 25mL inoculum per liter of low salt LB
5. Grow at 37C, shate at 200 rpm
6. Grow to 0.6-0.75 OD (600nm)
7. Transfer to ice immediately to chill
Processing:
1. Spin chilled culture at 8000 rpm in 500mL bottles, 10 min.
2. Remove supernatant
3. Resuspend in 5mL of 10% glycerol and transfer to 45 mL conical tube (one conical tube/500mL culture)

4. Add 10% glycerol to each tube to 45mL
5. Spin at 8000 rcf?, 8 min
6. Remove supernatant
7. Resuspend each pellet in 5mL cold 10% glycerol and transfer to one conical tube.
8. Add 10% glycerol to 45mL
9. Spin at 8000 rcf, 8 min.
10. Resuspend pellet in 45mL cold 10% glycerol
11. Spin and repeat x2 (for total of 4 washes)
12. After final spin, add 2mL 10% glycerol.
13. Resuspend with 1mL pipetteman with filter tip
14. Transfer 53 ul to resuspended cells into -80C 1.5mL microcentrifuge tubes
15. Transfer immediately to -80 freezer
16. Freeze overnight.

Qiagen mini prep

2x 5mL cultures, pour into 2 microtubes and spin down
8K 2 min, re pour until culture is gone
resuspend 250 mL cold P1 buffer
add 250 mL P2, invert until clear
*add 350 mL N3, invert until flakes appear, vigorously
put on ice 5 min equilibrate
spin as fast as possible 8 min
*pipette 900 mL off the pellet into column
*after PB wash, let PE wash sit on column for 1-5 min, spin twice and wipe off column
use as an aliquot of filter sterilized EB, heat to 65 C before se
add (30) 60 mL preheated EB, let sit on column 5 min
spin to elute with caps sideways

Sidney's DNA extraction protocol

grow 150 mL in LB + antibiotics
split into 4 50 mL tubes.
Spin at 8K for 8 min
add 5 mL of P1 to tube 1, transfer to 2, etc.
add 5 mL to tube 1 to get the rest, transfer etc.
10 mL P2, invert 10x slowly, let set for 3 min
10 mL P3, invert 10x
spin at 8 K rpm for 8 min
pour lysate, without snotball into a qiafilter midi, let sit for 10 min
prepare midi tip
plunge into midi tip
continue with blue protocol