Lance's Subcloning procedures

These are in as modular a format as possible my preferred methods for subcloning. They are not in anyway the only way to proceed but if they are followed with precision I will vouch for their efficacy.

General notes of strategy (possibly the most IMPORTANT section)

We subclone to tailor reagents to experiments. This simple thought must govern all else. Subcloning is not an experiment that will generate its own results. Therefore as little time as possible should be spent doing it. (Note I know this because I have failed to follow this rule to my peril L).

DOGMA (things I've said and heard more than I care to remember)

1. RULE ONE OF SUBCLONING: Pick one person's methods and stick with them. A given set of methods are far more likely to be internally consistent than using one person's PCR method, another's sample handling, another's ligation etc.

2. RULE TWO OF SUBCLONING: Follow Pamela's tube labeling rules, note I know this also from failure to adhere.

3. FUNDAMENTAL SUBCLONING STRATEGY:

A. PCR gene of interest in quantity

B. Double-digest 5-10 ug of the plasmid of interest.

C. Ethanol precipitate the PCR reaction(s) and the double-digested plasmid to concentrate them.

D. Run concentrated PCR reactions and double-digested plasmid on a gel to isolated the amplified insert and double-cut backbone DNA respectively.

E. Cut out the desired bands from gels and isolate the DNA from the agarose.

F. Phenol/Chloroform extract the double-cut backbone DNA isolated from the agarose.

G. Double-digest the PCR amplified fragment isolated from the agarose.

H. Phenol/Chloroform extract the double-digested PCR amplified insert isolated from the agarose.

I. Ethanol precipitate the Phenol/Chloroform extracted double cut backbone and PCR amplified isert.

J. Ligate the insert to the backbone.

K. Transform the ligation.

L. Screen 4-5 colonies per ligation. Efficiency of insertion will be between 40-80%.

4. WHEN NOT TO USE THE FUNDAMENTAL SUBCLONING STRATEGY

When thinking about designing a reagent for an experiment it is tempting to try to anticipate all possible eventualities and develop a sub-cloning strategy that satisfies all these constraints. It may be tempting to add your own multiple cloning site or to try the latest blunt-end ligation kit I would urge strongly that you stick to the above simple strategy and make the simplest, easiest, clone possible in all but the most compelling cases.

5. SITE-DIRECTED MUTAGENESIS (the other protocol you will need)

PCR driven commercially available site-directed mutagenesis kits are excellent, fast and tremendously efficient. Thanks to Randall Bass I became aware of the Stratagene kit which is essentially perfect. The one mutagenesis reaction that this method is not ideal for is the large loop-out mutagenesis. However, you may use these kits to accomplish a large loop out mutagenesis indirectly by mutating the same restriction site at the 5' and 3' end of the section that you wish to remove. After isolating this double mutant you digest the plasmid, isolate the large fragment on a gel and ligate the ends together. The resulting construct will have the loop out section replaced by the restriction site.

Methods listed:

A. PCR of gene of interest
B. Digests
C. Ligations
D. Transformations
E. Ethanol precipitations
F. Phenol/Chloroform extractions
G. Isolation of DNA from agarose gels
H. Site-directed mutagenesis
I. Notes on DNA isolation for mammalian cell transfection
J. Quantification of nucleic acids

A. PCR gene of interest

Use the thin walled PCR tubes - set up 4 identical reactions 20 ul each

6.8 ul H2O
2 ul 10 X Rxn buffer
0.4 ul dNTP (2mM (of each) stock - 400uM final concentration)
4 ul Q buffer from Qiagen, excellent for long fragments and generally makes the product more digestible.
2 ul 5' primer (62.5 ng/ul for oligos of 30-60 bases in length. If longer add in stoichiometric amounts).

2 ul 3' primer (62.5 ng/ul)
2 ul template (40ng of template is enough copies when the vector is 12kb, and not too much when the vector is 2 kb).
0.8 ul Polymerase (I use Vent, except when using the site-directed mutagenesis kit)

Program

1. 95 degrees C 2min
2. 95 degrees C 30 sec
3. 55 degrees C 1 min
4. 72 degrees C 2min/kb of desired fragment length
5. return to step 2, 15 times
6. 72 degrees C 7 min
7. 4 degrees ad infinitum

Comments: I am aware of people optimizing the [Mg2+] or the annealing temperature. Using this protocol I have never had to bother. There is one other thing I often do if the desired fragment is > 1.5kb. I will spike the reaction with additional enzyme and dNTPs after 10 cycles.

2.6-3 ul H2O
0.5 ul 10 X Rxn buffer
0.1 ul dNTP (2mM (of each) stock - 400uM final concentration)
1 ul Q buffer from Qiagen.
0.2-0.4 ul Vent.

B. Double digests

Use 5-10 ug of plasmid or all of the purified product from a PCR reaction set.

74 ul H20
10 ul 10 X buffer (Chose according to NEB or Boehringer catalog specs)
1 ul 100 X BSA
5 ul DNA
5 ul Enzyme 1 (add 50 units, usually the enzymes are 10U/ul)

Comments: The large reaction volume is to accommodate the large excess of enzyme. The enzyme stock solutions must be diluted 10x in the reaction to avoid non-specific endonuclease activity, aka star activity.

Clearly if your DNA is more dilute or if you have more volume from a PCR reaction then you will add more volume of DNA and less volume of water.

C. Ligations

Use thin walled tubes

13 ul H2O

2 ul 10 X ligase buffer
2 ul insert (200-1000ng)
2 ul backbone (20-200ng)
1 ul Ligase

Run reaction for 20-30 minutes at room temperature.
Negative Controls:
Reaction without insert -> if colonies then backbone is not completely double cut
Reaction without insert and ligase -> if colonies then backbone is not completely single cut
Reaction without template and ligase -> if colonies then insert is contaminated with PCR template

Comments: Ligations are the most frustrating step in the entire sub-cloning process. There is no easy way to directly monitor reaction progress, and success is only verified after colonies have been picked and screened (~2 days later). The control ligations I have suggested may indicate sooner the success of the reactions. In practice I rarely see no colonies on the negative control plates. Colonies from the experimental ligation are likely to contain the insert of interest if the negative control plates have <10 colonies and the experimental ligation plate has 5-10 times as many colonies.

In my experience failure to acquire the desired clone is always the result of inadequately prepared DNA fragments. If your reactions fail, improve the backbone or the insert preparation. The methods I have outlined here are the best I have. They are very clean and thorough, though not perfect.

D. Transformations

Use the chemically competent XL-1 blue cells from Stratagene, and the manufacturer's protocol.

In the absence of funds use the protocol for preparing and transfecting either chemically or electrocompetent cells in the Current Protocols in Molecular Biology.

E. Ethanol precipitation

This is one of the most useful and underused standard procedures.

Use standard clear polypropelene eppendorf tubes

To 180 ul DNA

Add 18 ul 3 M NaAcetate (not pH'd)
Add 600 ul punctillious alcohol

If a gossamer white precipitate does not form at room temp then chill the tubes for 20 min to 1 hr at -20.

Spin at 14krpm, 4 degrees C, in an eppendorf 5415C microfuge for 15-30 minutes.

Carefully remove the supernatent, pellet may be barely or not visible, that is ok.

Add 180 ul of 70% ethanol, without disturbing pellet.

Spin at 14krpm, 4 degrees C, in an eppendorf 5415C microfuge for 5-10 minutes.

Carefully remove the wash, without disturbing pellet (or where it might be).

Allow to air dry at room temperature or in a chemical fume hood.

Resuspend pellet in 20 ul H2O or 10 mM Tris-Cl pH 8.0.

F. Phenol/Chloroform extraction

This is the ugly step-child of molecular biology, nobody likes it. But it works, and this version is easy to do.

1. To 180 ul DNA

Add 180 ul Phenol/Chloroform/Isoamyl Alcohol 25:24:1 v:v:v
Vortex and shake until many small bubbles

2. Spin at 14krpm, room temperature, in an eppendorf 5415C microfuge for 5minutes.

3. IMMEDIATELY remove the aqueaous (top) phase.

This is done with a P200 micropipettor set to 200 ul. Remove the liquid slowly. At some point the aquaeous layer will bump and become surrounded by the P/C/I. After this happens stick the pipette tip into the top of the aquaeous bubble and keep removing liquid until about 10-20 ul are left behind.

For some applications it is useful to add 180 ul Chloroform and repeat 2 and 3. Generally this aquaeous phase maybe used for ethanol precipitation directly.

G. Isolation of DNA from agarose gels

In general existing kits are good for extracting fragments up to 2-3 kb. This method works on well on those and on large fragments (12kb id done routinely) where the kit yield is somewhat dicey.

1. Cut out the gel slice from a 1% low melt agarose gel (high melt may be used, the yields will be lower, I recommend using the low melt if it can be afforded)

2. Freeze the slice.

3. Crush the frozen slice with a P1000 tip.

4. Add 200 ul of TAE with EtBr (EtBr is not necessary but allows monitoring the extraction)

5. Vortex

6. Spin at 14krpm, room temperature, in an eppendorf 5415C microfuge for 1minute.

7. Remove the buffer and save.

8. Repeat 4-7.

9. You may then heat the agarose and the 400ul pooled TAE to 70 degrees C.

10. When the gel has melted add 1ml of Wizard resin to both the agarose and the buffer.

11. Add resin-DNA mixtures to syringe barrel column setup, pack the column.

12. Wash the column with 80% Isopropanol

13. Spin column dry, at 14krpm, room temperature, in an eppendorf 5415C microfuge for 2minutes.

14. Elute column with 50-100ul 10 mM Tris pH 8.0 heated to 90 degrees C.

15. DNA is ready for Phenol/Chloroform extraction.

Comments: The TAE with the extracted DNA (generated in step 7) may be used directly for the P/C extraction, the yields will be lower but a kit is not needed.

H. Notes on DNA isolation for mammalian cell transfection

In general the following rule is useful:

A transfection depends on the DNA isolation method, the transfection reagent, and the type of cell being transformed.

I know the following combinations work:

Wizard prepped and ethanol pptd DNA : Superfect : Cos 7 cells

Qiagen prepped and ethanol pptd DNA : Lipofectamine 2000 : CHO cells

Qiagen prepped and ethanol pptd DNA : Lipofectin : CHO cells

CsCl prepped and ethanol pptd DNA : Lipofectin : CHO cells

CsCl prepped and ethanol pptd DNA : Lipofectamine 2000 : CHO cells

CsCl prepped and ethanol pptd DNA : Lipofectamine 2000 : MDCK cells

In general I use the manufacturers' transfection protocol and isolate clones by limiting dilution.

This means if you transfect a 10 cm dish of cells:

You should split the cells into 2-5 96-well plates.

J. Quantification of nucleic acids

The rules are covered on page E5 of volume 3 of Sambrook et al (aka Maniatis)

The only thing I have to add is dilutions which make the Absorbance at 260 nm equal to the concentration in g/L.

The small quartz cuvette holds 130 ul

For dsDNA (plasmids & PCR products) 6.5 uL of concentrated DNA and 123.5 ul of Tris pH 8.0.

For ssDNA (oligos) 2.6 uL of concentrated DNA and 127.4 ul of Tris pH 8.0.