The Art of Molecular Cloning

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This is meant to be a short introduction to molecular cloning. If you are interested in a very comprehensive manual on the topic I recommend Molecular Cloning by Sambrook and Russell. I compiled this document utilizing material from a number of resources, in particular the molecular biology workshop at UBC (HTTP://BIOTEACH.UBC.CA), invitrogen, NEB, my lab notebook, Molecular Cloning: A Laboratory Manual, CSH protocols and notes from Russell Monds and Tiffany Vora. If you find any typos or inconsistencies please email me at tropini@stanford.edu so that I can keep this document up to date and as useful as possible.

1 Cloning? The general concept

Molecular cloning was first reported in the 1970's, as a way to controllably isolate and replicate DNA sequences in cells. To first order, to do cloning you require a DNA sequence and a host cell. We are going to be discussing plasmid cloning in *E. coli* cells.

1.1 Plasmids

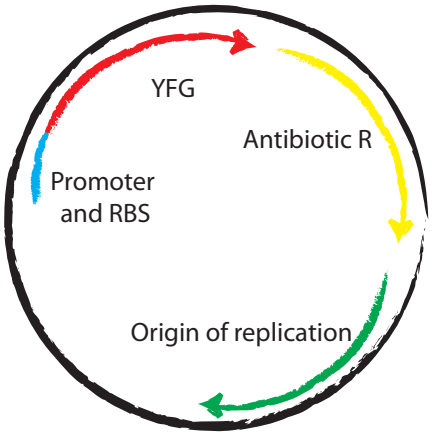
Plasmids are extrachromosomal DNA molecules. We will be using double-stranded, circular plasmids 1-10 kb in size (although they can go up to more than 200 kb).

A plasmid has 4 features of interest to us. 1) YFG is your favorite gene or DNA sequence, the one that you want (expressed and) replicated in cells. 2) In order to express YFG in the cell you need a promoter and ribosome binding site. 3) Naked DNA does not preserve itself. To propagate it, you need an origin of replication, or a sequence telling your cells that this DNA should be replicated. Different origin sequences will lead to different mean number of plasmids per cell. 4) Cells don't want to carry extra DNA, you need to (peer) pressure them into carrying your plasmid. To do so you treat cells with an antibiotic and have on the plasmid a gene that confers resistance to this drug, so that only cells with the plasmid will grow. This is called a selectable marker.

The plasmid used to shuttle your favorite DNA sequence is commonly called a vector.

1.2 Inserting YFG into the plasmid

If you are attempting to clone a novel gene you will be starting from genomic DNA, getting YFG via PCR, inserting it into a plasmid and getting *E. coli* to replicate it. As you will be doing PCR on your gene you will need to add at the end of each primer some sequence to allow you to insert it easily into your vector of interest. Once you have these extra "tails" at the end of your gene you can rely on base pairing and DNA ligation to join YFG to the plasmid. We will be covering two ways of creating our final pladmid: by restriction enzyme and by enzymatic assembly cloning.



1.2.1 Restriction Enzymes

Restriction enzymes (REs) are proteins that cut DNA in a very specific manner (they recognize specific 5-10 bp sequences). There are several classes and kinds, but in general we distinguish them depending on how they cut DNA. They can either leave overhangs (sticky ends) or blunt ends in the DNA. We will be using sticky ends restriction enzymes and take advantage of the complementarity of DNA base pairs to selectively insert our DNA of interest. To stop the reaction some enzymes can be denatured with heat or other compounds but the safest way is to purify the DNA. After the overhangs are formed, ligase is used to seal the fragments together after the compatible ends have annealed.

RE, beware!

A couple of general metrics about REs: they require special buffers to be active, they need specific temperatures to operate (generally 37^{o}C) and they need to stay cold (-20^{o}C) unless they are being used; it generally takes 1 hour to cut and 1 unit of enzyme each µg of DNA but this might depend on the enzyme and on the particular reaction. Consult the data worksheet of the enzyme that you are using to make sure you are giving your reaction the best possible chance to work, and remember that some enzymes interfere with one another (they might give rise to the same compatible ends)! Also make sure that your sequence contains no more number of restriction sites than how many you want to cut. If you are starting from a PCR product you can insert the restriction sites upstream and downstream of YFG and also add 5 bp at each end to facilitate cutting. If you need to cut with more than one enzyme make sure that they can be used in the same buffer, otherwise you might have to do the restriction separately and purify the DNA in between, which loses a lot of it! Some enzymes also have "star activity", meaning that they can cut unspecifically, particularly if they are left in the reaciton for too long or under less than optimal conditions (temperature, salt concentration, pH). Also make sure to have small amounts of glycerol in your reaction (enzymes are kept in glycerol). Methylation will also affect the ability to be cleaved. This is why most e. coli strains that you work with have dam- and dcm- nomenclature. This means that these methylation enzymes have been knocked out so that they won't affect the ability to cut DNA isolated from these bacteria.

1.2.2 Enzymatic assembly cloning

Restriction enzymes work well but at times you might have a DNA sequence that already contains the site recognized by a lot of REs. However, we can take advantage of the 5' to 3' specificity of T5 exonuclease to expose a DNA overhang at the ends of the fragments that you want to assemble. These regions of complimentarity are built into the PCR products by adding 35-40 bp tails. DNA polymerase fills in the gaps of the annealed fragments and ligase is used to seal the fragments. The first rounds of PCR are the most important ones, after those work there is such a large region of complementarity that it won't be a problem to amplify. If you are having problems with the amplification you can try to have 5 rounds of PCR with lower annealing temperature, just to match the primers themselves (~Tm -5^{o}C, usually about 55^{o}C) and then continue with 30 rounds of annealing at higher temperature (around 60^{o}C ballpark). Also, it is of course important that the fragments do not have high similarity as to ensure that other kinds of ligations might occur. Importantly, even without the addition of exonuclease the reaction will produce closed plasmids (this is a good control to compare with +Exo and to estimate how many clones to propagate and send for sequencing) but they might have illegitimate recombination events.

1.3 Getting your new plasmid into *E. coli*

This process is called a transformation if you are working with bacteria. There are two main techniques to do this, one is to heat shock the cells and the other is to electroporate them. Either way it means to create large pores into the membrane, large enough for the cells to take up a whole plasmid! Amazingly the cells survive this (at least enough do). We will be performing heat shock transformations.

Cells that are able to take up DNA are called competent. To prime them into accepting DNA, cells are treated with CaCl2, which is quite harsh on the membranes. Cells are kept on ice to allow DNA to bind and then during the heat shock the membrane pores become even bigger/weaker (it's not really known how it works) and take up the DNA. Then to let cells recover we grow them without antibiotic in SOC (a high nutrient medium) for at least an hour and then plate them. We will be using DH5α cells to propagate our plasmids. This strain transforms with high efficiency and has a number of mutations that make miniprepping more efficient and eliminates homologous recombination.

1.4 Getting your plasmid out of *E. coli*

This step is necessary both prior to cloning (to have a plasmid to put YFG into) and after, to check the molecular cloning worked. To do this you will need to miniprep 1-5 ml of liquid culture grown for 12-16 hours. For sequencing have primers that span no more than about 7-800 bp so that the reads are clean.

2 RESTRICTION CLONING

1. DIGESTION OF VECTOR AND INSERT: ***1.5 hours***

VECTOR: 4200 bp; **INSERT**: GFP 750 bp; (want to start with 2-3+ µg of DNA for gel purification)

- Buffer 3, 10X; (BSA, 100 X)
- XhoI 0.05 µl/µg of DNA present
- BglII 0.05 µl/µg of DNA present
- 1 hour at 37^{o}C (if restriction sites are very close cut with XhoI first for 1hr, 37^{o}C then heat inactivate at 65^{o}C for 20 mins then digest with the other enzyme for 1 hr)

2. RUN GEL: ***1 hour***

- 100 mL of SB buffer + 1.5g Agarose, bring to boil
- Add 10 µl of Ethidium Bromide/1 µl of gel stain when mixture is cool enough to touch, pour into gel boat and add spacers
- After gel solidifies add SB on top, pipet in 5 µl 1kb DNA ladder/100bp ladder diluted 1:10 from stock
- Add to DNA 5x solution of loading dye
- Run for 20-30 mins at 150-180V

3. CUT GEL AND PURIFY DNA WITH QUIAQUICK GEL EXTRACTION KIT: ***0.5-1 hours***

- Quantify with nanodrop

4. LIGATION (Don't forget vector controls!)**:** ***40 mins***

- Molar ratio= nanodrop ng/µl /(660g/mol *bp) = x mM = x * 10^6 nM
- Want 2 nM final vector and 10 nM insert
- Add ligase buffer 10X , T4 ligase ~ 0.5 µl (2 units+/0.12 µM DNA): 30 mins incubation
- Take out DH5α and leave on ice, turn on water bath to 42^{o}C

5. TRANFORMATION: ***2 hours***

- Add 1-2 µl ligated DNA to 100µl DH5α cells
- Incubate on ice for 20 min
- Heat shock in 42^{o}C water bath for 1.5 mins (make sure water bath is at exactly 42^{o}C)
- Incubate on ice 2 min
- Add 900 µl growth medium (LB or SOC) and shake 1 hour at 37^{o}C
- Plate about 200 µl (on LB+antibiotic plates)

6. GROW PLATES: ***Overnight***

7. GROW LIQUID CULTURE: ***Overnight***

8. MINIPREP: ***1 hour***

9. Send to sequence with correct primers

3 ENZYMATIC ASSEMBLY CLONING

1. PCR : ***1-3 hours***

PCR (50 µl total volume/rxn)	Thermocycling	Thermocycling conditions with tails
28.5 µl of H20	98^{o}C denaturation 30s	98^{o}C denaturation 30s
10 µl PHUSION 5X buffer	40 cycles:	
5 µl dNTP (final 200 µM)	98^{o}C denaturation (d) 5s	5 cycles: 98^{o}C d. 5s; 55^{o} a. 10s; 72^{o}C e. 15s/kb
2.5 µl Fw primer @ 10 µM	55^{o}C annealing (a) 10s	
2.5 µl Rv primer @ 10 µM	72^{o}C extension (e) 15s/kb	30 cycles: 98^{o}C d. 5s; 60^{o} a. 10s; 72^{o}C e. 15s/kb
0.5 µl of Phusion Taq NEB		
1 µl template (1:10 dilution)	72^{o}C 5 mins, 4^{o}C Hold	72^{o}C 5 mins, 4^{o}C Hold

- Run gel to visualize the PCR product, PCR purify.

2. ENZYMATIC ASSEMBLY: ***45 mins***

- 4 µL 5x Isothermal reaction buffer (see appendix)
- 2 µL Taq Ligase (NEB)
- 0.5 µL Phusion DNA Pol (NEB/Finnzymes)
- 1:1 stoichiometry vector to insert DNA
- Make up to 19 µl with DNA fragments to assemble and water
- Add 1 µL 1:25 dilution of T5 Exonuclease (Epicenter) (see appendix for dilution buffer)
- Make also control without T5 Exonuclease
- Mix gently with pipet and incubate at 50^{o}C for 30 mins
- Take out DH5α and leave on ice, turn on water bath to 42^{o}C

3. TRANFORMATION: ***2 hours***

- Add 1-2 µl ligated DNA to 100µl DH5α cells
- Incubate on ice for 20 min
- Heat shock in 42^{o}C water bath for 1.5 mins (make sure water bath is at exactly 42^{o}C)
- Incubate on ice 2 min
- Add 900 µl growth medium (LB or SOC) and shake 1 hour at 37^{o}C
- Plate about 200 µl (on LB+antibiotic plates)

4. GROW PLATES: ***Overnight***

5. GROW LIQUID CULTURE: ***Overnight*** with correct antibiotic

6. MINIPREP: ***1 hour***

7. Send to sequence with correct primers

4 Reagents, media recipes and material specs

- T5 Exonuclease: Epicenter Cat # T5E4111K
- Taq DNA ligase: NEB Cat# M0208S
- Phusion polymerase: NEB Cat# F-530S
- XhoI restriction enzyme: NEB Cat#R0146L
- BglII restriction enzyme: NEB Cat#R0144L

4.1 Competent Cell protocol

Preparation of competent cells

1. Pick one colony (DH5α cells) for O/N growth in 5ml media (LB) @37^{o}C
2. Inoculate 500ml media w/5ml of O/N culture
3. shake until OD600 ~ 0.25-0.4 @ 37^{o}C
4. Chill culture on ice 15 min to slow cell growth
5. pellet cells by centrifugation @ 2000-3000g, 4^{o}C for 15-20min
6. decant supernatant
7. resuspend pellet in 1/5 volume of original culture of cold 0.1M CaCl2 (for 500ml culture, resuspend in 100ml)
8. incubate cells on ice for 20min
9. repeat step 5 and 6
10. resuspend cells in 1/25 original volume of 0.1M CaCl2/10% glycerol (for 500ml original culture, resuspend in 20ml solution)
11. add aliquots to prechilled eppendorfs (100-500 µl depending on use in experiment)
12. snap freeze w/liquid N and store tubes at -80^{o}C

4.2 SOC media

For 1L of media:

- 5 g yeast extract
- 20 g tryptone
- 0.584 g NaCl
- 0.186 g KCl
- 2.4 g MgSO4
- Sterilize by autoclaving
- After cooling medium to less than 50°C, add 20 ml filter sterilized 20% glucose solution
- Adjust to pH 7.5 prior to use. This requires approximately 25 ml of 1M NaOH per liter.
- water

4.3 5x Isothermal buffer (1mL)

- 0.5mL Tris HCL pH 7.5 (1M) Final conc. 500mM
- 50 µL MgCl2 (1M) Final conc. 50mM
- 10 µL each dNTP (100mM) Final conc. 1mM
- 50 µL DTT (1M) Final conc. 50mM
- 0.25 g PEG-8000 Final conc. 25%
- 50 µL NAD (100mM) Final conc. 5mM

Notes:
PEG-8000 is difficult to dissolve and is very viscous. Hot water helps to dissolve it with vigorous mixing.
Scale up as necessary, buffer can be stored at -20°C till needed.

4.4 Dilution buffer for T5 exonuclease

- 50% glycerol 50 mM Tris-HCl (pH 7.5)
- 0.1 mM EDTA,
- 1 mM DTT,
- 0.1 M NaCl,
- 0.1% Triton® X-100.

4.5 SB buffer

SB is a sodium borate buffer with lower conductivity, (and hence can be run at higher speeds) than can gels made from TBE buffer or TAE buffer (5–35 V/cm as compared to 5–10 V/cm).
20X (100 mM) stock solution.

- 38.17 g Sodium Borate decahydrate
- 33 g Boric Acid
- Bring to 1L with dH2O
- Dilute to 1X and use to make gel and running buffer.

4.6 Web links to other useful resources

- Video on key steps of molecular cloning
- Nanodrop tutorial video
- Restriction enzyme lab tutorial
- A free Plasmid Editor (ApE)

- BioPerl module for restriction enzymes
- EnzymeX for restriction enzymes (Mac)
- NEBCutter for enzymes
- Primer3Plus for primer design
- PrimerBLAST for primer checking
- PCR virtual lab