Plasmid Subcloning using low melt ligation

Design Considerations

General

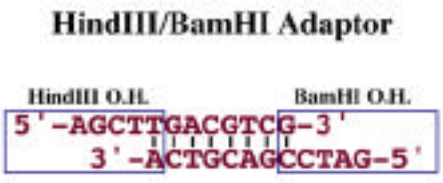
1) We much prefer directional cloning (since it usually works better and takes less time) and we have found that with the help of adaptors, directional cloning is almost always possible.
2) We have found that low melt ligation is not only easier than the old extraction methods but it gives much better results.

Use of Adaptors

Adaptors are an excellent tool that greatly facilitates the design of cloning strategies. Using such a strategy, only one compatible end is required for cloning and the other end is made compatible using an adaptor.

Notes on Adaptor use:

1) We accumulate a library of adaptors as cloning experiments require new ones (keep a good data base of all adaptors made).
2) If new adaptors are needed, order new oligos that will form 8bp duplex in addition to the desired overhangs on each end (see below for annealing conditions). We usually design the annealing nucleotide that is next to the overhang so that the corresponding restriction site is regenerated following ligation.



Example of HindIII/BamHI Adaptor Design

3) Oligos do not have to be kinased. In fact, we never kinase adaptors so that adaptor cannot form multimers during ligation reactions.
4) Since only 1-2 ng of adaptor is used for each experiment, these adaptors will last a life time!
5) We have used adaptors successfully in up to 5 part ligation experiments.
6) You cannot clone using an adaptor at each end of the insert (unless you kinase oligos).

Adaptor Annealing:

- We try to hybridize oligos at a concentration of between 0.25 and 1ug/ul total oligo (the working concentration for ligations is much lower and we dilute later).
- Oligos are hybridized at equal molar amounts. So, for oligo pairs that are the same length, its easy, just use equal weights. For oligo pairs of different size, be sure to compensate by adding a certain amount of one oligo and then multiplying this amount by the ratio of the size difference to obtain the amount of the second oligo to add.
- Salt helps stabilize the duplex so we hybridize in 1X Medium Restriction Buffer (1X MRB).

Example of Annealing Rxn:
Starting material
1ug/ul Oligo 1 (12-mer)
1ug/ul Oligo 2 (16-mer)

Mix
25ul Oligo 1
33ul Oligo 2 [=25ul (Oligo1)x16/12]
10ul 10X MRB
32ul H_2O
100ul = Total (Final concentration is 0.58ug/ul but to make dilutions easy, we would label it as 0.5ug/ul)

1) Add ingredients together and mix.
2) Put eppendorf tube in a rack in a 68°C water bath. We usually put a container in 68°C water bath and let it fill with the 68°C water, then we put the rack with the tube in the water that is in the container. Keep tube at 68°C for 10 minutes.
3) After 10 minutes at 68°C, take container with water and tube out of water bath and let cool slowly until it reaches room temperature.
4) Keep this stock frozen but also make a dilution to a working concentration of 1ng/ul in 1X MRB (e.g. for above example, add 2ul of adaptor to 50ul 10X MRB + 450ul H_2O).

Protocol

I. Plasmid Restriction Digestion

	Vector Plasmid	Insert Plasmid
DNA	0.5ul (0.5ug)	1-5ul (1-5ug)
10XRB (usually 10X HRB)	3ul	3ul
Enzyme 1	1.5ul	1.5ul
Enzyme 2	1.5ul	1.5ul
RNase	3ul (if mini prep DNA)	3ul (if mini prep DNA)
H_2O	20-23ul	16-23ul
Total	30ul	30ul

Note: We try to keep the ratio of fragments around equimolar (although fairly significant differences will also work). We always digest 0.5 ug of vector [this lower amount helps prevent overloading of the gel and therefore better separation from low amounts of single cut and supercoiled plasmid (which cause significant background)]. The amount of insert plasmid digested depends on the size of the insert and is primarily a consideration for detection issues. If the insert is between 150-800bp, we will digest around 5ug of plasmid. If the insert is 800-2000bp, we will digest about 2ug. If the plasmid is bigger than that, we will digest 0.5-1ug.

II. Agarose Gel Electrophoresis

Notes:

1) We always use 1% Low Melting Point (LMP) Agarose unless the insert size is less than 300bp (in which case, we use 1.5% LMP).
2) We always use TAE Buffer (we never use TBE – may affect ligation, but we don't know for sure).
3) Separate fragments fairly well. Separation of the vector is most important in order to isolate from minute amounts of either single cut or supercoiled DNA which can cause significant background.
4) After running gel, isolate gel piece containing fragments of interest (using a clean razor blade) with somewhat little amounts of surrounding agarose (minimizing the amount of excess agarose tends to increase the concentration of fragment and provides better purity) and put each gel piece into a separate eppy tube.
5) Of course, try to minimize exposure of fragments to UV light.

III. Ligation Reaction

- Put eppy tubes containing gel pieces into a 68°C water bath for 5 minutes (if temp is slightly below 68°C, gel pieces will not melt well – you can also use 70°C water bath).
- When gel pieces are melted, take tubes out one at a time, mix briefly with finger and immediately dole out appropriate amount (see below) to the CNTL and INSERT tubes. You may save remaining gel pieces for future experiments if you wish (keep at –20°C).
- Put CNTL and INSERT tubes back into 68°C water bath for 3-5 minutes.
- While incubating CNTL and INSERT tubes at 68°C, prepare Ligation Reaction Mix (see below).
- After CNTL and INSERT tubes have been at 68°C for 3-5 minutes and the Ligation Reaction Mix has been prepared, transfer all of these (CNTL, INSERT, and Ligation Reaction Mix) to 37°C water bath. Keep at 37°C for 5 minutes.
- Keeping all tubes in 37°C water bath, pipette up 10ul of ligation mix. Then pick up CNTL tube and immediately transfer the ligation mix into the CNTL tube. Mix immediately with finger and then leave at room temperature.
- Similarly, transfer 10ul of ligation mix to the INSERT tube and leave at room temperature.

Note: Let two part ligations go for between 3hrs – overnight. Three or more part ligations must incubate overnight.

For two part ligations:
CNTL tube = 10ul of vector gel piece
INSERT tube = 5ul of vector gel piece + 5ul of insert gel piece

For two part ligation with an adaptor:
CNTL tube = 5ul of vector gel piece + 5ul of insert gel piece
INSERT tube = 5ul of vector gel piece + 5ul of insert gel piece + 1ul of Adaptor**

**Important: Adaptor should be added <u>immediately after adding ligation mix</u> to ligation fragments (do not add adaptor at the same time that other fragments are added because this mixture is too warm and the adaptor will unanneal).

For three part ligations:
CNTL tube = 5ul of vector gel piece + 5ul of one of the insert gel pieces
INSERT tube = 4ul of vector gel piece + 3ul of each of the two insert gel pieces

Ligation Reaction Mix:

Per Reaction	3X	5X	7X	9X	
6ul H_2O	18ul	30ul	42ul	54ul	H_2O
2ul 10X Lig Buff (with New England Biolabs Ligase**)	6ul	10ul	14ul	18ul	10X Lig Buff
2ul Ligase (New England Biolabs Cat# 202L, 400u/ul)	6ul	10ul	14ul	18ul	Ligase
10ul					

**It is important to warm Ligase buffer in 37°C water bath for a few minutes to get DTT into solution.

IV. Transformation of competent bacteria

1) After ligation, put tubes at 68°C for 5-10 minutes.
2) Take tubes out individually and immediately add 180ul of Transformation Buffer (kept at 4°C) – pipette up and down 4-5 times and put on ice.
3) After adding Transformation Buffer to each ligation, let sit on ice for 15 minutes to cool to 4°C.
4) Add 100ul competent cells and mix well with finger (because of the diluted agarose, the solution is semi-solidified. But this can be disrupted by mixing well with finger after adding competent cells).
5) Incubate on ice for 30 minutes.
6) Heat shock at 37°C for 2.5 minutes and then transfer back to ice bucket.
7) Transfer the mixture to a sterile tube containing 1ml of 2xYT, mix briefly with finger, and incubate in 37°C water bath for 45-60 minutes.
8) Plate 300ul onto an amp containing 100mm 2xYT plate.
9) Incubate plate overnight at 37°C (note: 300ul of soln is generally too much to absorb well into agar so we do not invert plate during overnight incubation – this allows the plate to dry slightly and allows the soln to soak in better).
10) Next day, pick colonies and innoculate cultures for mini prep analysis.

Notes:
We always do a negative control (CNTL). However, if you get similar numbers of colonies on the Insert and Control plates (or even if you see less colonies on the Insert plate relative to the Control plate), you experiment still may have worked! The main reason we include a control in these experiments is to allow us to gauge how many mini preps to do for each cloning experiment. For example, if we have 50 times more colonies on the Insert plate relative to the Control plate, we may only need to do a couple minis or we may even go directly to a maxi-prep and verify the cloning using the maxi prep DNA. If we have a few fold more colonies on the Insert plate relative to the Control plate, we may do 4-6 minis. If we have similar numbers or fewer colonies on the Insert plate, we generally do 10-12 minis. If all of these are negative, this suggests that the strategy is either incorrect or very inefficient. In this case, we generally develop another strategy instead of testing more minis.

Solutions

We find that the standard restriction buffers described in Maniatis give more consistent results than those supplied by vendor.

10X Low Restriction Buffer (10XLRB)	10ml of 10X LRB
100 mM Tris-Cl (pH 7.5)	1ml 1M Tris (pH 7.5)
100mM $MgCl_2$	1ml 1M $MgCl_2$
10mM DTT	100ul 1M DTT

10X Medium Restriction Buffer (10XMRB)	10ml of 10X MRB
100 mM Tris-Cl (pH 7.5)	1ml 1M Tris (pH 7.5)
500 mM NaCl	1ml 5M NaCl
100mM MgCl	1ml 1M $MgCl_2$
10mM DTT	100ul 1M DTT

10X High Restriction Buffer (10XHRB)	10ml of 10X HRB
500 mM Tris-Cl (pH 7.5)	5ml 1M Tris (pH 7.5)
1 M NaCl	2ml 5M NaCl
100mM $MgCl_2$	1ml 1M $MgCl_2$
10mM DTT	100ul 1M DTT

Ligase Buffer
We use the buffer that is supplied by New England Biolabs Ligase (Cat# 202L, 400u/ul).

Transformation Buffer
10mM Hepes (pH 7.7)
50mM $CaCl_2$
10% Glycerol

50X TAE
1 L
242g Tris Base
57.1 ml Glacial Acetic Acid
100 ml 0.5M EDTA (pH 8.0)

2xYT	1L
tryptone	16g
yeast extract	10g
NaCl	5g