

Isabel's Magical Cloning Protocol

I. Insert amplification:

- 1) Set-up between 2-4x100ul PCR reactions ON ICE accordingly:

Make a “master mix” (on ice) by multiplying each amount below by desired number of reactions, vortex briefly to mix, and aliquot out 100ul to each pre-chilled tube.

100ul PCR reaction:

• ddH ₂ O	76.0ul
• 10x Promega buffer	10.0ul
• dNTP's	5.0ul
• 10-50ng template DNA	2.0ul
• 20 uM primer F	2.5ul
• 20 uM primer R	2.5ul
• <i>Taq</i> polymerase	1.0ul
• <i>Taq</i> Extender	<u>1.0ul</u>
	100ul

Pre-heat thermal cycler to 94 degra. C. and immediately transfer PCR reactions from ice to hot thermal cycler (Hot-start).

- 2) Amplify x 30 cycles. Standard PCR settings (noted below) work for most primers betw/20-80bps and inserts between 0.5bp-1.5Kb. Adjust annealing and extension times and temps. if std. conditions fail (You can try a gradient PCR to determine ideal annealing temp). Any insert >1.5Kb may need longer extension times (Maniatis says 1' extension/1Kb).

Standard PCR program

• Pre-heat	1' @94 degra. C.
• Denaturation	45''@94 degra. C.
• Annealing	45''@60 degra. C.
• Extension	45''@72 degra. C.
• Elongation	3' @72 degra. C.

- 3) Run 5ul of PCR product on a 1% agarose gel (2% for inserts < 1Kb). 5ul should show a whopping product band (about 1ug DNA).

- 4) Purify PCR product with either the Qiaquick gel purification kit or phenol-chloroform extraction and 2-propanol precipitation.

II. **Vector/Insert digestion and Vector dephosphorylation:**

Digest both vector and purified PCR product with same restriction enzyme(s) in corresponding buffer (look this up in NEB chart) at 37 degra. C. Incubation times vary with enzyme. Usually, 1 hr. at 37 degra. C. is enough for most restriction enzymes. If this is not enough, try to plot a time course where you take out an aliquot of your digest reaction every 1/2 hour to determine sufficient incubation time.

There are several possible ways to subclone an insert into a vector. The two most common methods are to subclone in a single restriction site with a single enzyme digest or at two different restriction sites with a double digest.

- 1) . For a **single digest**, set-up digests accordingly:

- a) Insert digest:

- 10ul purified PCR product (5-10ug)
- 10ul 10x buffer
- 10-20U restriction enzyme
- q.s. to 100ul with ddH₂O

- b) Vector digest:

- 5ul vector (2.5-5ug)
- 5ul 10x buffer
- 5-10U restriction enzyme
- q.s. to 50ul with ddH₂O

Incubate in 37 degra.C. incubator for ≥ 1 hr.

- 2) For a **double digest**, follow the following procedure:

- a) First, determine which of the two enzymes has more specific buffer requirements; i.e. some enzymes have their own “unique” buffer (such as EcoRI) or some enzymes have salt concentration requirements (e.g., AatII only cuts effectively in high-salt buffer).

- b) Next, see if your second enzyme can cut 100% in the buffer specific for your first enzyme. Use NEB’s “Double Digest” chart to help determine buffer requirements. If there’s a buffer which is compatible with both enzymes, then you can digest with both enzymes simultaneously. Set-up digests as outlined above and add in equal amounts (in # of units) of your two enzymes.

- c) If no single buffer is compatible for both enzymes, digest sequentially going from low-salt buffer to high-salt buffer. So, start by digesting with the enzyme which cuts in low-salt buffer first. Then, add the enzyme requiring high-salt and its corresponding high-salt buffer and continue incubation.
- 3) Check for digest completion by running an aliquot of each digest next to 1ul undigested DNA on a 1% agarose gel. Run 5ul of insert digest and 2.5ul of vector digest.
- 4) For vector only, dephosphorylate ends by adding 5U Calf Intestinal Alkaline Phosphatase (CIP) and 5ul high-salt CIP buffer**. Continue to incubate at 37 degra.C. for 45'. (**Note: most restriction enzyme buffers have high-salt concentration; therefore, it is only necessary to add CIP buffer if digest buffer is low-salt). For restriction sites with 5' recessed termini (such as SacI), incubate with CIP at 50 degra.C. x 1hr.
- 5) Gel-purify both digested vector and insert with Qiaquick gel purification kit; note: elute vector in 40-50ul and insert in 25-30ul ddH₂O. To gel-purify large volumes, pour a fat-mama gel with wide-tooth combs (e.g., 12-well combs for the medium-size gel) to maximize well size.

III. Ligation:

Most ligations with 5' protruding termini (5' overhang) can be completed at a1:3 or 1:5 vector to insert ratio and with 1-2 hrs. incubation time at room temperature. However, this can vary depending on the restriction site. Blunt ended or 5' recessed ends require ≥ 2 hrs. incubation at room temp.

- 1) Set-up 10ul ligation reactions accordingly:

	DdH ₂ O	10x buffer	T4 DNA Ligase	Vector	Insert
Vector control	7ul	1ul	1ul	1ul	---
1:3	4ul	1ul	1ul	1ul	3ul
1:5	2ul	1ul	1ul	1ul	5ul

- 2) Incubate at room temp. x 1-2 hrs.

IV. Transformation:

For subcloning transformations, use XL10-Gold or XL2-Blue *E.Coli* from Stratagene (not the homemade competent cells). The protocol is as follows:

- 1) Thaw cells on ice x 10'. Flick tube gently to mix; then add 2ul *B*-mercaptoethanol (supplied with cells), flick tube to mix again and incubate on ice for another 10'. **Note:** each tube contains 150ul cell suspension. After thawing cells, aliquot 50ul to 3 pre-chilled tubes. A 50ul aq. is more than enough cells for one transformation.
- 2) Add 1x10ul ligation reaction/50ul competent cells; Flick to mix and incubate on ice x 30'.
- 3) Heat-shock at 42 degra.C. x 30 secs.
- 4) Return to ice and incubate x 2mins. Add 900ul prewarmed LB media (with no antibiotics) to each transformation (prewarm LB at 42 degra.C. while cells are cooling on ice).
- 5) Incubate on rotor-shaker at 37 degra.C. and 250rpm x 45' to recover cells.
- 6) Plate aliquots of 50ul and 500ul on LB-agar plates (pre-warmed at RT) with appropriate selective antibiotic. Note: plates to be used for 500ul aqs. should be prewarmed at 37 degra.C. x 45' while cells are recovering.
- 7) Incubate overnight at 37 degra.C.

V. Colony screening:

A successful subcloning should have about 100-fold more colonies on the ligation plates than on the vector control plate. To screen for positive clones, set-up two sets of colony PCR's as follows:

- 1) Aliquot 10ul ddH₂O to each well on a 96-well PCR plate and 250ul LB+antibiotic to 24-48 wells on a deep-well plate.
- 2) Pick 24 –48 colonies from each ligation. Pick only well-isolated colonies. Inoculate each colony into 2 wells w/10ul ddH₂O (one for insert-primed PCR and the other for vector-primed PCR). After inoculating colony into the PCR wells, inoculate colony into deep well with LB+antibiotic.
- 3) When finished picking colonies, incubate the PCR plate at 94 degra.C. x 2' to lyse cells. Cool on ice > 5' before adding PCR reaction mix.
- 4) Make master mixes on ice as follows:

Insert-primed reaction mix (for 15ul aqs):		<u>x25</u>	<u>x50</u>
• 10x Promega buffer	2ul	50ul	100ul
• dNTP's	1ul	25ul	50ul
• DMSO	1ul	25ul	50ul
• 20uM insert primer F	0.5ul	12.5ul	25ul
• 20uM insert primer R	0.5ul	12.5ul	25ul
• <i>Taq</i> polymerase	0.2ul	5ul	10ul
• DdH ₂ O	9.5ul	237.5ul	475ul

Vector-primed reaction mix:

Set-up is the same as insert-primed reaction mix only with different primers. For subcloning into a single restriction site, you need to check to see if your insert ligated in the correct orientation. To do this, use one vector primer flanking the insert site and one insert primer on the opposite end of the insert. For subcloning into two different sites, simply pick two vector primers, one forward and one reverse, flanking the insert (you don't need to worry about orientation when subcloning into two different sites).

- 5) Keeping the master mix on ice, aliquot 15ul each reaction mix to one of two wells with lysed cells from step one.
- 6) PCR-amplify under standard program. Remember to keep PCR reactions on ice and to "hot-start" the reactions.
- 7) When PCR is complete, run 5ul on a 96-well big-mama agarose gel to identify positives.

VI. Mini-prep positive clones:

- 1) Once positives have been identified from the colony PCR, inoculate 50ul of each positive from the deep-well plate inoculate (V.2. above) into 5mls LB+antibiotic. Grow overnight at 37 degra.C. and 250rpms.
- 2) Pellet cell cultures and mini-prep DNA using the Qiagen mini-prep kit.

VII. Sequencing:

There are two possible points where you can sequence your positives: 1) from vector-primed colony-PCR product or 2) from plasmid preps.

Colony-PCR product sequencing:

- 1) In a Millipore mini-column plate, pass vector-primed colony-PCR product over Sephacryl to purify.
- 2a) Set-up sequencing reactions ON ICE as follows:
 - Purified PCR product 4.0ul
 - DMSO 0.5ul
 - Sequencing primer 0.5ul
 - Sequencing buffer 1.5ul
 - BigDye 1.0ul
 - DdH2O 2.5ul
 - 10ul

Note: Select sequencing primers internal to the vector primers on both ends. For inserts greater 1 Kb, select primers 600bps apart to sequence the full-length. Primers should be in both forward and reverse directions.

Plasmid-prep sequencing:

1) Mini-prep plasmid DNA with Qiagen mini-prep kit.

2b) Set-up sequencing reactions ON ICE as follows:

- Plasmid prep DNA (0.8-1.0ug) 1.5ul
 - DMSO 0.5ul
 - Sequencing primer 0.5ul
 - Sequencing buffer 1.5ul
 - BigDye 1.0ul
 - DdH2O 5.0ul
- 10ul

Note: Select two vector primers which flank the insert in both the forward and reverse directions. The same rules for internal sequencing apply as they do for sequencing PCR product.

- 3) Cycle-sequence with either the “BdSeq” or “CYC1” program on the thermal cycler.
- 2) Purify sequencing reactions over Sephadex on a Millipore mini-column plate.
- 3) Bring volume up to 12ul with ddH2O to sequence on the ABI 3700.
- 4) Sequence on the ABI 3700. Train with Isabel, Steve or Sandy to learn how to set-up the 3700.

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