MOLab Standard Operating Protocol (SOP) for Molecular Cloning

Updated by TCH 08/29/2016

Vector Preparation:

1. Digest 0.5-1.0ug vector DNA or 5-10ul of miniprep DNA with 20-40 units of appropriate enzyme in 100ul volume, 30 min. See the Large Scale DNA Digestion Protocol.
   NOTE: Larger amount of vector DNA will only increase your ligation background; Longer digestion just increases chewing in of ends.

2. Perform ethanol precipitation/washing.

3. Dissolve the DNA pellet in 20-30ul ddH_2O. Use 1.0ul per ligation reaction (see below).
   NOTE: try to avoid gel purification for the vector whenever possible.

Insert Preparation:

If you isolate a fragment from another plasmid:

1. Digest 1-5ug of insert plasmid DNA or 15-30ul of miniprep DNA using the Large Scale DNA Digestion Protocol. Perform stepwise digestions whenever possible.

2. At the end of the digestion(s) (reaction volume = 100ul), add 20ul 6x GSB (DNA sample buffer), and load to **0.6%-0.8% agarose gels** (i.e., **6-well minigel**s, usually thicker than regular gels. You will need 70-100ml to pour such a gel). The gel should be resolved at 60-70V for 1~2 hours, depending on insert sizes.

3. Isolate the desired DNA fragment from the gel using our homemade Magic Column.

4. PC-8 extraction twice → ethanol precipitation/washing → dissolve the pellet in 12ul ddH2O. Use 3ul per ligation reaction (see below).

If you PCR amplify a fragment for cloning:

1. Perform a large scale PCR reaction (i.e., 80-100ul reaction mix divided into 8-10 tubes).
   NOTE: 1) Hi-Fi PCR is recommended, but if Hi-Fi PCR fails to amplify, you have to use our regular PCR (also called DMSO PCR protocol) to amplify at low PCR cycle# (usually <15 cycles); **2)** You should always perform a 10-20ul pilot run to determine if Hi-Fi PCR works first; **3)** If you amplify a fragment from cDNA or genomic DNA samples, you should always perform the touchdown protocol.

2. Check 5-10ul of your PCR product on agarose gel. If the product looks correct, pool all PCR reactions (it's even okay if you have some mineral oil in your samples), and perform PC8 extraction, followed by ethanol precipitation/washing.

3. Digest the PCR product with desired enzymes using the Large Scale DNA Digestion Protocol.
   NOTE: **1)** You should always **perform enzyme digestions first,** whenever possible, and then gel purification; **2)** Stepwise digestions are always preferred.

4. At the end of the digestion(s) (reaction volume = 100ul), add 20ul 6x GSB (DNA sample buffer), and load to **0.6%-0.8% agarose gels** (i.e., **6-well minigels**, usually thicker than regular gels. You will need 70-100ml to pour such a gel). The gel should be resolved at 60-70V for 1~2 hours, depending on insert sizes.

5. Isolate the desired DNA fragment from the gel using our homemade Magic Column.

4. PC-8 extraction twice → ethanol precipitation/washing → dissolve the pellet in 12ul ddH2O. Use 3uls per ligation reaction (see below).

Ligation Reaction and DH10B Transformation:

1) Set up the ligation reactions as follows:

Reaction (in ul)	**Ligation**	Vector only (optional)	Insert only (optional)
5x Ligase buffer (Invitrogen)	**3.0**	3.0	3.0
Vector (~20 -50ng/ul)	**1.0**	1.0	0.0
Insert DNA	**3.0**	0.0	3.0
ddH_2O	**7.0**	10.0	8.0
T4 DNA Ligase (Invitrogen)	**1.0**	1.0	1.0

2) Incubate reactions at **Room Temperature** for 1-4 hours (overnight for blunt-end ligations or large inserts, but it is usually unnecessary: 1-hr ligation gives ~1/3 colonies vs. 4hrs; 4-hr ligation gives> 1/2 colonies vs. overnight).
NOTE: For most cloning, 1~2h ligation is more than sufficient as you should have >70% of ligation reactions completed by then.

3). **Reduce background by cutting ligation products**: Take **7.5ul** of the ligation product, add 10ul of 10x enzyme buffer + 81.5ul ddH_2O + **1ul enzyme**, incubate at **37°C for 10-20min.**
NOTE: **1)** Please carefully choose the enzyme to cut background. It should not cut your insert and choose best NEB enzyme possible (remember the guiding principle: the cheaper the better!); **2)** Do not add too much enzyme and/or digest too long, or you risk losing your DNA; **3)** Do not try to precipitate your ligation products prior to enzyme digestion as it's unnecessary and hurtful.

4a). Perform ethanol precipitation/washing. Let the pellet air dry, and dissolve it in 30ul ddH_2O.

4b). To the remaining 7.5ul ligation product (i.e., uncut control), add 192.5ul ddH_2O and perform ethanol precipitation/washing. Let the pellet air dry, and dissolve it in 30ul ddH_2O.

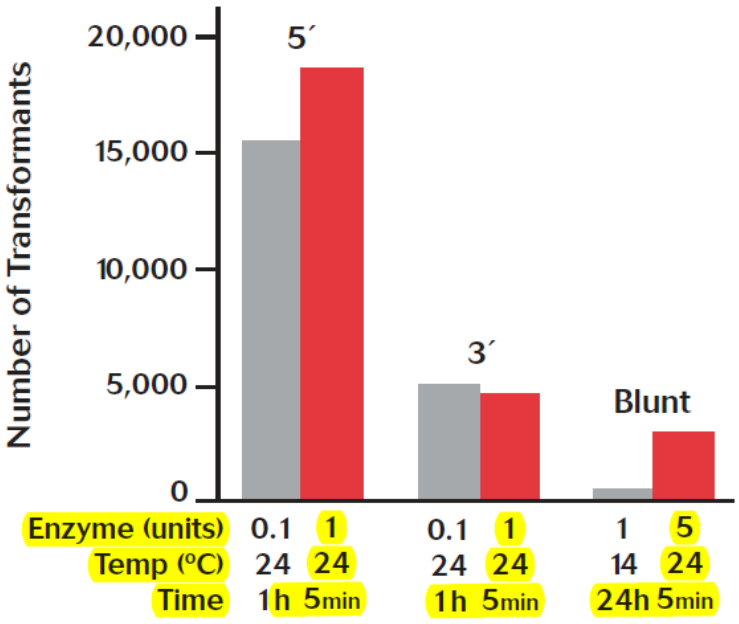
5) Use **10-15ul** of the ligation products (cut or uncut samples) to transform electrocompetent DH10B (we usually buy this from NEB) (see the Bacterial Transformation protocol).
NOTE: 1) You do not need to use all 30ul of your digested or uncut ligation product to

transform DH10B. If you do not get anything from ½ or 1/3 of your ligation products, you probably will get nothing even use all 30ul. Furthermore, higher DNA concentrations could reduce electroporation efficiency! **2)** It's critical to keep the DNA/DH10B mix containing **cuvettes on wet ice**, and zap the cuvette one at a time. Any warm-up of the cuvettes would drastically reduce electroporation efficiency.

6) Add **500ul LB medium** (without any antibiotics) using the individually-wrapped plastic **Pasteur transfer pipets** and mix well, and transfer to 1.5ml Eppendorf tubes.
NOTE: **1)** You **should NEVER use your p1000 pipette** to add LB and flash out the cell mix. This is the best way to contaminate your cloning products! **2)** You should not try to use a smaller volume of LB to rinse out the bacterial cells as this will not help you on getting what you want.

7) Immediately plate **100-200ul** of the transformation mix (i.e., 500ul LB) to LB/antibiotic agar plates. You can incubate the rest of the mix at **37°C for 10-20min**, and then plate 100-200ul on LB/antibiotic plates.
NOTE: 1) There is no need to plate >200ul of the mix onto one LB agar plate; **2)** 37°C incubation will help you to get more colonies, but will sadly increase the background as well. Thus, your priority should always focus on the colonies from direct plating.

Good Luck!



Comparison of standard and rapid ligation protocols. Inserts were ligated into pUC19 treated with *Hind* III (5′ overhang, 1.1-kb insert), *Kpn* I (3′ overhang, 1.0-kb insert), and *Eco*R V (blunt ends, 1.1-kb insert) using the standard protocol (■) or the rapid ligation protocol (■). Time and amount of T4 DNA Ligase were as shown, all other parameters were as describd in the rapid protocol. MAX EFFICIENCY DH10B™ cells were transformed and incubated overnight on LB agar with ampicillin and X-gal. White colonies were counted. **Note:** For the blunt-end ligation, 1 U of ligase for 5 min at 24°C resulted in 480 colonies.

General Cloning Tips from UCMOLab

(http://www.boneandcancer.org/UCMOLab_Cloning%20tips_4-6-04.pdf)

LIGATION REACTIONS AND TRANSFORMATION

1) Vector Preparation
- a. use 0.5-1.0 ug DNA (>1ug leading to high background)
- b. do stepwise digestions (in 100ul reaction) whenever possible
- c. avoid gel purification (reducing ligation efficiency) whenever possible

2) Insert Preparation
- a. use 5-10ug DNA (or 15-20ul miniprep DNA)
- b. do stepwise digestions whenever possible
- c. usually need gel purification
- d. may PCR amplify inserts (always digest PCR DNA first, then gel purify; any PCR amplified fragments need to be sequenced)

3) Ligation Setup
- a. inserts are always in excess (e.g., vector =10-20ng/Rx, insert = 50-200ng/Rx)
- b. always include a vector control ligation (insert control may be desirable in some cases).
- c. incubation at 16oC x 1-4 hrs (O/N may only be necessary when extremely large and/or blunt-ended inserts are involved)

4) Electroporation/Transformation
- a. require ethanol precipitation/wash to remove salts
- b. cool cuvettes on fresh ice
- c. zap at 1.8KV/mm gap (bigger plasmids use lower voltage, 1.2-1.3KV/mm)
- d. direct plating is good for low background, but 10-20 min incubation at 37oC is needed for inoculating to liquid culture. If you do not which way is suitable for your ligation/transformation, you can plate 100-200ul of the E. coli mix immediately after transformation, and plate the rest after a 20-40min incubation).

SCREENING FOR AND CONFIRMATION OF POTENTIALLY CORRECT CLONES

1) Direct miniprep/diagnostic digestions (low background)
2) PCR screening (moderate background)
3) Colony-hybridization (high background /challenging cloning)

TROUBLE-SHOOTING GUIDELINES

1) No Colonies
- a. check insert and vector on gel
- b. check proper antibiotic selection
- c. check electrocompetent cells
- d. check electroporation procedure
- e. check cloning strategies
- f. re-do ligation with more vector and/or insert
- g. re-do vector and insert preparation

2) High Background (and/or too Many Colonies)
- a. direct plating after electroporation
- b. use less vector for digestion and/or ligation
- c. treat vector with CIAP (calf intestinal alkaline phosphatase) (but not on both insert & vector; not on vectors when unkinased oligo cassettes are used as inserts)
- d. cut ligation mix with a linker enzyme that is not present in the final construct.
- e. use inserts from different antibiotic selection marker.