

Protocol for PCR product cloning using the TOPO TA Cloning Kit for Sequencing

This guide is intended for use in conjunction with the instruction manual provided by Invitrogen. PCR product cloning is a method that aids in sequencing of PCR products. It is a relatively expensive process that requires a significant time commitment. All cloning reactions should be well planned to prevent waste.

Questions to ask before starting:

Calculate how many LB agar plates you will need (generally two plates/fragment cloned).

Check the refrigerator next to the fume hood in room 125 for stocks of LB plates. Be sure to note any antibiotic treatments and their concentrations. The TOPO TA kit calls for plates treated with 50 µg/ml ampicillin or 50 µg/ml kanamycin. DO NOT take plates before asking the maker. If you will need more plates than are available, then make the plates before starting.

Check stock solutions of antibiotics.

If you need to add antibiotic to plates or directly to molten LB agar, first check the stock solutions of antibiotics in the freezer. Solid antibiotics are on the top shelf of the refrigerator in 123 to make fresh stock solutions. After making a stock solution, divide into aliquots and place in the freezer. Many antibiotics are sensitive to refreezing and will no longer be effective when submitted to numerous freeze/thaw cycles.

Check on LB broth stocks.

LB broth is commonly used to grow subcultures for isolating plasmids. The entire lab typically uses the stocks on the top shelf of the refrigerator in room 125. LB is very sensitive to contamination; only dispense LB broth inside of the laminar flow hood. Immediately remove any contaminated stocks from the refrigerator, and autoclave them before pouring down the drain.

Check cloning kit reagents.

Cloning kit reagents are expensive and should be used frugally. TOPO TA cloning kits are divided between the -20 °C freezer left of the fridge in 123 and the -80 °C freezer in room 121. Only 0.5 µl of vector is needed per fragment (1/2 of the recommended volume); briefly centrifuge any vector tubes that appear empty before discarding. Vector is very sensitive to temperature; minimize its time outside of the freezer. In the -80 °C freezer, check for tubes of chemically competent cells. Use 25 µl of competent cells (1/2 the volume of cell suspension in each tube).

How will the fragment be purified?

If more than one fragment is amplified during your PCR reaction, then the individual fragments should be isolated before cloning. One commonly used method is to separate fragments on an electrophoretic gel, extract the individual fragments, and purify them using the QIAquick Gel Extraction kit (Qiagen). Because some loss occurs during this protocol, fragments should be extracted from multiple samples and pooled. Alternatively, the entire PCR product can be cloned and the resulting colonies can be screened using vector primers. An important note, smaller fragments will be preferentially cloned over larger fragments. If a larger fragment is desired, then gel purification is the better method.

When only one fragment is present in the sample, then the PCR product can be used for cloning with no purification. However, a sample of clones should still be screened with the vector primers before growing subcultures and isolating plasmids.

Day 1

- 1) Amplify the fragment(s) of choice using your specific PCR profile. If you will gel purify, prepare at least three 25 μ l samples with the desired template. Taq DNA polymerase adds single deoxyadenosines to the 3' ends of PCR products, which interact with 3' thymidine overhangs in the pCR 4-TOPO vector. The terminal deoxyadenosines are susceptible to cleavage; therefore, samples should be used soon after the PCR profile runs to completion.
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Day 2

- 2) Resolve fragment(s) using agarose gel electrophoresis. Fragments should be well spaced for cutting. Do not run more than 15 μ l of PCR product if that product will subsequently be used for the cloning reaction. This step should always be performed to ensure amplification of the desired fragment occurred and to prevent wasting cloning reagents.
- 3) If desired, cut fragments from the gel and purify DNA (see separate protocol).
- 4) Hold DNA fragment(s) on ice until use
- 5) Prepare for the cloning reaction:
 - i. Equilibrate a non-shaking water bath to 42 °C.
 - ii. Warm a vial of SOC medium to room temperature (found on top shelf of refrigerator).
 - iii. Warm LB agar plates at 37 °C for 30 min. If no antibiotic is present on plates, then the antibiotic may be added after warming. Calculate the amount of antibiotic stock to add (one plate contains ~20 ml of LB) and dispense the amount onto the center of the plate. Spread the antibiotic over the surface using a sterile glass hockey stick. Allow 15 min for the antibiotic to diffuse.
 - iv. **On ice**, thaw one vial of One Shot chemically competent cells per two samples. After thawing the cell suspension, dispense 25 μ l of the mixed suspension into a sterile 1.5 ml microfuge tube. Dispense cells inside of the laminar flow hood using sterile pipette tips.

- 6) Using a 0.5 ml microfuge tube, prepare the TOPO Cloning reaction (ligation of fragment to vector). After adding the water, salt solution and PCR product, dispense the vector directly into solution.
 - 7) Mix the reaction gently by stirring with a pipette tip. Allow the reaction to incubate for at least 5 min but up to 30 min. Longer incubation times generally produce more transformed bacterial colonies.
 - 8) Place the cloning reaction on ice and proceed to the transformation step.
 - 9) Transformation using TOP10 One Shot chemically competent cells
 - i. Add 2 μ l of the chilled cloning reaction to 25 μ l of chemically competent cell suspension and mix gently by stirring with pipette tip.
 - ii. Incubate on ice 5 to 30 min; the length of this incubation does not affect transformant yield.
 - iii. Place the tubes in a microfuge tube float outside of the 42 °C water bath, transfer the rack to the water bath, and incubate for 30 sec.
 - iv. Immediately transfer the tubes to ice for 2 min. The entire microfuge tube float can be transferred to ice to prevent extended incubation at 42 °C.
 - v. Add 250 μ l of room temperature SOC broth, and cap the tubes tightly.
 - vi. Shake cell suspension horizontally (200 rpm) at 37 °C for 1 hour. If half-reactions of cell suspensions were used, incubations may be prolonged to 2 hours total. A shaker/incubator is located in the Hrabak prep room (room 137). A shaker is also located in a warm room on the 2nd floor of Rudman. However, key card access is required for entry to the 2nd floor corridor.
 - 10) Spread 150 μ l and 50 μ l from each transformation onto pre-warmed LB agar plates treated with ampicillin or kanamycin. The density of colonies depends on how much cell suspension is added to the plate. Add more cell suspension to obtain a higher density of colonies. Always plate at least two different volumes to ensure at least one will have well spaced colonies.
 - 11) Incubate the plates at 37 °C for at least 12 hours. Additional incubation time may be required to produce visible colonies depending on antibiotic used and concentration.
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Day 3

- 12) Perform PCR on selected colonies
 - i. Pick 10 round colonies for screening with PCR. Circle and number the chosen colonies using an extra-fine tipped permanent marker.
 - ii. Using vector primers (20 μ M each of either T3 and T7 or M13F and M13R) prepare a PCR master mix.
 - iii. For inside the laminar flow hood: To obtain template DNA from the colonies, use a 0.2-10 μ l pipette tip to gently touch a colony. Do not penetrate the agar growth substrate or smear the colony. Place the tip into 22.5 μ l of PCR master mix and swirl gently. Dispose of used tips in the biohazard burn bag inside of the hood.
 - iv. Transfer tubes to a thermocycler and perform PCR using the COLONY profile (Table 1).

- v. Run amplified fragments on a 2% (w/v) agarose in TBE gel, and screen for desired fragment sizes.

Table 1: COLONY PCR profile

Step	Temperature	Time
1	94 °C	10 min
2	94 °C	1 min
3	53 °C	1 min
4	72 °C	2 min
5	Go to step 2, 29 times	
6	72 °C	10 min
7	4 °C	HOLD

Day 4

- 13) After finding the correct clone, prepare restreaks of the desired colonies on LB plates containing 50-100 µg/ml ampicillin. Multiple colonies can be streaked on a single LB plate, but be sure to clearly identify each division.
- 14) Prepare subcultures of colonies plasmid isolation. For each colony, add 1 ml sterile LB broth containing 50-100 µg ampicillin to a sterile 1.5 ml microfuge tube. Pick the colony of interest with a sterile toothpick or inoculating loop and transfer cells to the LB broth. Place tubes on a shaking 37 °C incubator, and incubate 12-16 hours.
- 15) Plasmids can be purified using Wizard *Plus* SV minipreps DNA Purification System (Promega).
- 16) Store concentrated purified plasmid at -20 °C for long term. Prepare working stocks at low (~1 ng/µl) for analysis with PCR.

Long-term storage and maintenance of colonies

- Restreaks and original plates can be sealed with Parafilm, wrapped in plastic wrap, and stored at 4 °C.
- Glycerol stocks of bacterial cells store
 - a. Isolate a single colony from a restreak, and inoculate into 1 ml of LB broth containing 100 µg ampicillin.
 - b. Grow cells overnight until the broth is saturated (16+ hours).
 - c. Mix 850 µl cell suspension with 150 µl sterile glycerol and transfer to a microfuge tube rated for cryogenic storage. The blue tinted microfuge tubes are available for this purpose.
 - d. Store tubes in the -80 °C freezer in room 121. Tubes and storage container should be clearly labeled with the date prepared, the preparer and fragment contained in plasmids.