

TOPO TA Cloning for community analysis Instructions Modified for Firestone Lab

1. **PCR:** Perform a standard PCR reaction using Ex *Taq* polymerase (Takara).
Include a 72° extension step for 7 to 30 minutes at the end of the reaction to ensure that all products are full length and 3' adenylated. Check results on a gel and proceed immediately to the cloning reaction unless excessive primer dimers or non-specific products are formed. In this case perform a PCR clean up step using a kit or gel purify your band of interest with the GeneClean Turbo kit. DO NOT FREEZE PCR PRODUCT (freezing will remove the A overhangs and reduce ligation efficiency).

If you gel-purify your PCR product after amplification you will need to replace some A overhangs that may be lost. After purification, add Ex *Taq* polymerase buffer, 0.25 µl 10 mM dATP, and 0.5 unit of *Taq* polymerase. Incubate the reaction for 10-15 minutes at 72°C (using either a heatblock, waterbath or thermocycler) and use 4 µl in the TOPO Cloning reaction.

2. **Prepare for the Transformation Reaction.**
 - a. Set water bath to 42° C.
 - b. Warm SOC medium (Aliquoted – 250 µl in -20°C freezer) to room temperature.
 - c. Warm pre-poured LB agar plates containing 50 µg/ml ampicillin at 37° for 30 minutes. Spread 40 µl X-gal (40 mg/ml in DMF) onto the LB plates and allow to air dry for 5 min in laminar flow cabinet (note: IPTG is not necessary when using TOPO 10 cells). Replace into incubator until ready to use.
 - d. Thaw 1 vial of One Shot cells (**on ice**) for each transformation.

3. **Set up Cloning Reaction:** The ligation reaction mixture is as follows:

Fresh PCR product	3 μ l	(from Step 1)
Salt Solution	1 μ l	(in kit in -20°C freezer)
<u>TOPO Vector</u>	<u>1 μl</u>	(in kit in -20°C freezer)
FINAL VOLUME	5.00 μ l	

4. **Incubate:** Gently mix by tapping the tube (*do not* use a pipettor!) and incubate at room temperature for 30 minutes. After incubation, place the reaction on ice (or store overnight at -20°C).

5. **Transform into Competent Cells:**

- To one vial of One Shot Chemically Competent *E. coli* cells add 2 μ l of the cloning reaction and mix gently. DO NOT MIX BY PIPETTING.
- Incubate on ice for 5 minutes
- Heat-shock the cells for 30 seconds at 42°C (in waterbath) without shaking.
- Immediately transfer the tubes to ice.
- Add 250 μ l of the room-temperature SOC medium.
- Incubate tubes for 1 hour in the 37° shaking incubator at 200 rpm.
- Using sterile technique, spread 25, 50, 75 and 100 μ l from each transformation (4 plates) onto prewarmed LB plates. Let stand right-side up for about 5 minutes to allow the cells to adhere to the agar, then invert and incubate overnight at 37°C.

6. **Select and Analyze Colonies:** Pick white and very light blue colonies for analysis. We analyze colonies via PCR as follows: First prepare sufficient microplates (both PCR type and culture type). In the PCR plate, aliquot 5 μ l of

sterile water per well; in the culture type add 100 μ l of LB (containing 50 μ g/ml ampicillin) – all of this should be performed in the laminar cabinet.

Using sterile toothpicks picks transfer white/light blue colonies into PCR plate wells, disperse cells in the 5 μ l water, then dip the same toothpick into the corresponding well in the culture plate (this will be the master plate for future use). Always leave at least one well uninoculated per plate to serve as a negative PCR/sterile control. Label plate with Name; Plate Number; Gene; and Origin of sample. Incubate the culture plate at 37° overnight (18-24 h) in a sealed whirlpack bag with a dampened tissue under the plate (prevents the media evaporating).

Continue setting up the PCR reaction (20 μ l final volume) for each plate according to the optimal conditions for the primers used. Typically the first screening will be performed with the M13 primer pair.

PCR reactions should contain:

Component	Volume (μL) for 1 sample	Mastermix for 96 samples (μL) (make enough for 100 to allow for loss)
10X PCR buffer	2.0	200
25mM MgCl ₂	1.6	160
Forward primer (3 μ M)	2.0	200
Reverse primer (3 μ M)	2.0	200
dNTP mix (10mM)	0.4	40
Promega Taq DNA polymerase in storage buffer B (5 U/ μ L)	0.2	20
ddH ₂ O	6.8	680
Totals	15	1500

Cycling conditions are: 95°C for 10 min; 35 cycles of 95°C – 1 min, 50°C – 1 min, 74°C - 1.5 min; a final extension of 74°C – 10 min; hold at 4°C. The program is called TOPO 2 under my profile (Eoin).

Run the PCR products on a 1 % agarose gel in 1X TAE buffer at 90V for 45 - 60 min. Take a picture and record results in a spreadsheet with the following data: Name (Your name); Project (e.g. NABIR-U); Plate number; Gene (e.g. 16S); Origin of sample.

Then record presence or absence of correct band size, noting any unusual banding patterns (i.e. more than one band).

Plate and well reference numbers should be retained for future identification of clones (e.g. EB146.A1 – Eoin Brodie Plate 146 Well A1).

The following day check the incubation plate to make sure all wells (apart from the uninoculated control have grown) then add sterile glycerol to a final concentration of 15% (usually 50 µl of 45% glycerol in water) mixing well and freeze at -80°C (make sure to keep upright – place caution notice and name on box containing plates). **CORRECT ASEPTIC TECHNIQUE IS VITAL!**

7. Screening: If screening is required (you may want to sequence all clones) then only screen PCR products that yield full length inserts. This can be performed by restriction digestion of the amplified DNA using a couple of 4-base recognition endonucleases (i.e. those that have 4 base recognition sequences, e.g. *MspI*, *Sau3AI*, *HhaI*, *AluI*).

This can be performed in a PCR microplate as follows:

To remaining 10 µl PCR product add 2.3 µl of a mastermix containing (per reaction) 2 µl 10X buffer (buffer #2 if using *MspI* and *AluI* from NEB), 0.2 µl *AluI* (2U), 0.1 µl *MspI* (2U). Incubate at 37°C overnight.

The next day add 2 μ l 6X loading buffer to each well and run contents on a 3.5% agarose gel for 1h 20 min at 95V. Photograph and compare banding patterns either by eye or using software. Choose representatives of each pattern type (OTU) for sequencing.