

Cloning (using the Invitrogen TA Cloning Kit)

What's needed:

- Good PCR products (e.g., bright, hopefully single bands as visualized on an agarose gel)
- Ligation reagents (H₂O, 10X ligation buffer, PCR2.1 vector, ligase – from kit)
- 0.2 ml and 1.5-1.8 mL centrifuge tubes.
- PCR machine for incubating ligations at 14°C
- LB/amp plates (recipe at end of procedure)
- SOC (from kit) (Kept in freezer near gel bench).
- Ampicilin
- Competent cells (KEEP ON ICE PRIOR TO USE)
- Heating block set to 42°C
- Ice bucket with ice
- Shaking incubator set to 37°C
- Stationary incubator set to 37°C (should always be at this temperature)
- X-gal (recipe at end of procedure)
- 95-100% ethanol
- spreading stick
- Bunsen burner

Useful tips:

- Cloning involves bacteria. Wear gloves. Always dispose of any material (tips, tubes, plates, etc.) that have come into contact with the bacteria into special cloning-waste containers that should be autoclaved when full.
- Label all tubes and plates prior to use.

PROCEDURE

Ligation (Day 1)

1. Place 0.5 uL of PCR product into 0.2 ml tube. [**IMPORTANT: Only use “post-PCR” pipettes for transferring the PCR product or any solution containing a PCR product.**]
2. Add to tube containing PCR product:
 - 1.25 uL H₂O
 - 0.25 uL 10X buffer
 - 0.5 uL PCR2.1 vector
 - 0.25 uL ligase (**KEEP IN FREEZER OR ON ICE ALWAYS!**)

The above reagents should be made as a cocktail for use with more than one sample, particularly because the volumes of most are so small. When preparing the cocktail always add the ligase right before aliquoting the cocktail to each of the PCR products. Make up cocktail for N+0.5 (N<5) or N+1 (N>5) for the number of samples to be ligated (=N).

3. Incubate ligations overnight at 14°C (in PCR machine that holds 0.2 mL tubes).

Transformation (Day 2)

1. Place tubes containing competent cells on ice. [The cells are kept in the -80°C freezer. Never place thawed cells back into the freezer – they lose their competency with thawing!] Each tube contains 50 μL of competent cells. Although Invitrogen recommends using the entire 50 μL , transformations work just fine using only 16 (1/3 reaction) to 25 μL (1/2 reaction) of the cells. Thus you will only need 1/2 or 1/3 as many tubes of cells for the number of ligations you want to transform. [**IMPORTANT: Only use “post-PCR” pipettes when working with any solutions that contain cells/bacteria or ligation.**]
2. Once cells are thawed, aliquot 25 μL (or 16 μL) of cells into a 1.5-1.8 mL centrifuge tube. You can certainly leave 25 μL (or 16 μL) of cells in the tube the cells come in for use in the following.
3. Add 2 μL of the ligation to the cells. Tap tube gently to mix. Place tube on ice for 30 minutes.
4. While waiting for the 30 minute incubation to finish, place the SOC (kept in the -20°C freezer) in the incubator at 37°C and verify that the heating block (with holes filled with water) is set to 42°C .
5. Heat shock: After the 30 minute incubation, place cells/ligation in the heating block at 42°C for 30-45 seconds. Then place on ice for 90 seconds.
6. Add 125 μL of SOC to cells/ligation mix. Place SOC tube back in freezer.
7. Incubate tubes in shaking incubator at 37°C for 1 hour at 225 rpm.
8. While cells are incubating, place LB/amp plates in the stationary incubator to warm.
9. A few minutes before the 1 hour incubation is complete, remove the X-gal from the -20°C freezer and place on benchtop to thaw or warm up. Also, remove the LB plates from the incubator, place on benchtop, and label.
10. After incubation, add 12.5 μL of X-gal to cell/ligation mix.
11. Add all of the cell/ligation/X-gal solution to the LB plate and spread evenly with sterile spreading stick over plate.
12. Let plate set on benchtop for 10-15 minutes so that the solution absorbs into the agar.
13. Place plates upside down in incubator for 18 hours. After incubation there should be many (>100) colonies evenly distributed on the surface of the agar in each plate. There should be blue ones, white ones, white ones with blue dots in the middle, and whitish-blue ones. The white colonies are usually the good ones (i.e., with an insert), but often so are the others except the all-blue ones.

Screening of Colonies (Day 3)

1. Prepare PCR cocktail with M13 and M13R primers. Label colonies on plate to screen.
2. Make grid on warmed LB/amp plate (for the numbers of colonies to be screened).
3. Add cocktail to tubes/PCR plate.
4. The template for the amplification are the DNA present in the colonies (precisely, the vectors in the bacteria). To add a small amount of the bacteria to the PCR reagents, barely touch the tip of a toothpick (autoclaved or not, apparently it doesn't matter) to a colony, touch the colony end of the toothpick onto the gridded LB/amp plate, and then gently dab the toothpick into the tube containing the PCR reagents. Be careful – too much of the cells and the PCR just won't work!

5. Run the PCR at 94C/30 seconds, 55C/30 seconds, and 72C/30 seconds for 30 cycles (extending the annealing stage to 45-60 seconds if screening for large (>500 bp) inserts. It will be done in about 1 hour and 15 minutes or so – you can get the agarose gels ready now!
6. After reaction is complete, run samples on 1.8% agarose/TBE gel. Amplifications of good inserts will be approximately 180 bp larger than the insert size (due to the amplification of part of the vector).

RECIPES

LB/amp plates (for 20-25 plates)

1. Into 500 mL flask add the following:

| | | |
|----------------------|----------|--------------------|
| 2.5 g tryptone | -- or -- | 5 g Bacto LB broth |
| 1.25 g yeast extract | | 3.5 g agar |
| 3.5 g agar | | |
| 2.5 g NaCl | | [no need to pH] |
2. Add H₂O to a final volume of 250 mL
3. Add about 30-40 drops of 1 N NaOH until pH of solution is 7 (IF USING FIRST RECIPE – see above).
4. Cover top with foil and autoclave (with liquid/solutions setting) for 15 minutes.
5. Remove from autoclave and let cool on benchtop. You want the temperature of the solution to be high enough so that the agar doesn't solidify but low enough so that the ampicillin that you will add in the subsequent step isn't rendered inactive. If you can hold the flask on the palm of your hand for 15 seconds or so it has reached the proper temperature (~55°C).
6. Dissolve 25 mg of ampicillin (kept in the refrigerator) in 1 mL of H₂O and add to agar broth.
7. Pour LB/amp into plastic plates. If you are frugal (i.e., only pour enough LB/amp to fill about 2/3 of the bottom of the plate), you will be able to prepare 20-25 plates. Gently swirl the plate to enable the solution to completely cover the bottom of the plate.
8. Let plates set on benchtop until the agar solution has completely solidified (30-60 minutes)
9. Place LB/amp plates in bag and put so that plates are upside down into the refrigerator.

X-gal

Make up X-gal as 50 mg/mL stock solutions with dimethyl formamide (doesn't freeze in freezer) or DMSO (dimethyl sulfoxide) (freezes in freezer).

Aliquot only about 100-200 uL per tube of working solution. Frequent freeze/thaws may render the X-gal inactive and so working stocks should be kept at small volumes.