Retrieving DNA by Gap Repairing

Day One: This step can be done at the end of a day.

1. Inoculate a single colony of EL350 cells containing a BAC from a fresh agar plate into a 14-ml tube containing 5 ml of LB medium. Incubate the culture overnight at **32ºC** in a rotary shaker (250 rpm).

Day two: It starts in the morning and takes about ~ hours. Have these solutions ready: prewarmed 1 liter LB medium, pre-cooled 500 ml of Ultra-pure water, 10% glycerol, and GYT medium.

2. Inoculate 20 ml of prewarmed LB medium in a 500-ml flask with 1 ml of the overnight bacterial culture (OD_{600}~1.2). Incubate the flasks at **32ºC** with agitation (180 rpm in a rotary shaker). Measure the OD_{600} of the growing bacterial cultures every 20 min.
   This density is usually achieved after ~2.5 hours of incubation for DH5a (or DH10B?). It is essential that the density does not exceed OD_{600}, 0.4.
3. When the OD_{600} of the cultures reaches 0.5 (~2 hr), incubate the flask at **42ºC** for 15 min.
4. Immediately transfer the flasks to an ice-water bath, swirl the culture occasionally to ensure that the temperature drops as fast as possible. Incubate on ice for an additional 5 min. In preparation of the next step, place the centrifuge bottles in an ice-water bath, and pre-cool the rotor and chamber of centrifuge to 4ºC.
   For maximum efficiency of transformation, it is crucial that the temperature of the bacteria not rise above 4ºC at any stage in the protocol.
5. Transfer the cultures to an ice-cold 50-ml Falcon tube. Harvest the cells by centrifugation at 1,000g for 20 min at 0ºC in a bench-top centrifuge. Decant the supernatant, resuspend the cell pellet in 20 ml of ice-cold pure H_2O.
6. Harvest the cells by centrifugation at 1,000g for 10 min at 0ºC. Decant the supernatant and resuspend the cell pellet in 10 ml of ice-cold 10% glycerol.
   This should be done immediate after the centrifugation finishes. Take care when decanting because the bacterial pellets lose adherence in 10% glycerol.
7. Harvest the cells by centrifugation at 1,000g for 20 min at 0ºC. Decant the supernatant and resuspend the cell pellet in 2 ml of ice-cold 10% glycerol, transfer to two microfuge tubes.
8. Harvest the cells by centrifugation at 1,000g for 5 min at 0ºC. Carefully decant the supernatant and use a Pasteur pipette attached to a vacuum line to remove any remaining drops of buffer. Resuspend the pellet in 50 μl of ice-cold GYT medium.
   This is best done by gentle swirling rather than pipetting or vortexing.

9. Add 1-2 μl of linearized retrieving vector (200-500 ng) to the cells and electroporate.

Solutions:

Glycerol (10% v/v)

GYT Medium

10% (v/v) glycerol
0.12% (w/v) yeast extract
0.25% (w/v) tryptone
Sterilize the medium by passing it through a prerinsed 0.22-μm filter. Store in 2.5 ml aliquots at 4ºC.