BAC Microinjection Protocol

Pre-injection Checklist:

Reagents:

1. 1/9x Modified Ringer's solution (1/9x MR)
2. 1/20x MR with Gentamicin (100 microgram/ml)
3. 3% Cysteine by volume in 1/9x MR (pH 8.0)
4. 1/9xMR+3% Ficoll
5. Injection buffer: 88 mM NaCl
   10 mM Hepes (pH 6.8)
6. Purify BAC DNA using Qiagen Large-Construct Kit and resuspend it in the injection buffer or 10 mM Tris-Cl, pH 8.5 and leave it at RT O/N. Once resuspended, keep BAC DNA at 4ºC (i.e., Don't freeze BAC DNA).

   Note: BAC DNA needs to be handled very carefully and always use a clipped pipet tip to transfer.
7. 1 μg/μl ϕC31 integrase protein (Home made)

Instruments:

1. Micropipette puller
2. Picospritzer Microinjector
3. Mesh coated injection dish
4. Agarose-coated petri dishes
5. Eppendorf tube

Injection Steps:

Step 1: Collect fertilized eggs from a naturally mating pair in a rectangular tank.

Fertilized eggs tend to float on top of water and very easy to collect them using a mesh.

Step 2: Cysteine Embryos.

To de-jelly the embryos, prepare a 3% cysteine solution, using 1/9x MR. Insure that the pH of your solution is 8.0 by adding NaOH. Free base cysteine rather than cysteine-HCl can be used which requires less NaOH to bring to neutral pH. This appears to improve the firmness of the eggs (E. Amaya).

Place the embryo-collected mesh in a petri dish on the tabletop and pour the cysteine solution. Gently shake the mesh using a forceps so that embryos fall to the bottom of dish.

Let sit for 3 minutes. Gently stir the embryos. Avoid vigorous swirling or the embryos will develop secondary axes. The embryos should become loose and separated. Wash 3

times with 1/9x MR after transferring the embryos to a 250 mL beaker. Once the embryos are adequately de-jellied, transfer them to an agarose-coated petri dish containing 1/9xMR+3% Ficoll using a wide-bore plastic Pasteur pipette.

Step 3: Prepare Injection mixture and Needles.

Injection mixture:

1) X μl of BAC DNA (This amount should be determined beforehand and it should **not** result in transgenesis, when injected alone!)

2) 1.5 μl of ϕC31 integrase protein

3) X μl of injection buffer to make the total volume of 20 μl

A 2 nanoliter injection is ideal for a one or two cell *tropicalis* embryo. Back-load the injection mixture into the needle using a pipetman and a clipped pipet tip. Place your needle on the picospritzer. Under the microscope using a forceps break the tip of the needle. Lower the needle into a petri dish filled with mineral oil and apply pressure with the picospritzer. A small bubble will form in the oil, which will allow you to determine the volume of your injection. Calibrate the pressure and the size of the needle accordingly until one has a 2 nanoliter injection.

Step 4: Microinjection.

Fill a mesh-bottomed dish with 1/9x MR+3% Ficoll. Under the microscope, select embryos for injection and transfer them to the mesh-bottomed dish. Fill the dish from the center out. Avoid placing embryos too close to the wall of the dish. Using a used injection needle gently nudge and rotate embryos into the isolated wells of the mesh until the are all aligned and ready to be injected. Inject in an organized manner-row by row or column by column. This will allow you to keep track of what you injected with minimal effort. At the end of each row or column check that the needle is not clogged.

After all embryos are injected transfer the injected embryos to an agarose-coated petri dish containing 1/9xMR+3% Ficoll. Some injected embryos may need to be discarded due to leakage of cytoplasm, which will become apparent shortly after injecting. After 30 min to a few hours (before gastrulation begins), transfer the healthy embryos to an agarose-coated dish with 1/20xMR+gent. Raise the embryos in agarose covered dishes (E. Amaya) which greatly prevents the embryos from sticking to the dishes.

Step 5: Store embryos in incubator at 22-23ºC overnight. After 24 hours, it is safe, and even preferable to transfer embryos to 25-28 ºC for raising.