

# Setting up for transgenesis transplantations

## I. PREPARATION OF REAGENTS AND EQUIPMENT:

- **Agarose dishes for injection:** In a 60 mm plastic petri dish, lay a small hexagonal weigh boat on molten 2.5 % agarose in water to create a depression for filling with eggs. Once agarose has hardened, fill with 0.2XMMR+6% Ficoll and wrap in parafilm. Store at 4°C until use. Make 2-3 dishes for each transgenic reaction you plan to do.
- **Linearized plasmid:** Prepare linearized plasmid at a concentration of 250 ng/ $\mu$ l in sterile, nuclease-free water (we avoid Tris and EDTA-containing buffers, which are somewhat toxic to embryos). The restriction enzyme used to linearize the plasmid does not have to be the same as the one used in the REMI reaction. We usually use NotI for all REMI reactions, regardless of what plasmid is linearized with. Plasmid can be purified in several different ways: we usually use the Qiagen Qiaquick PCR purification kit according to the manufacturers directions. If plasmid is purified using phenol/chloroform extractions and ethanol precipitation, be certain to remove all traces of organics and ethanol.
- **Set up the Harvard Apparatus infusion pump.** We use a 3 cc syringe/needle filled with mineral oil. Blunt the needle tip (to keep it from perforating the tubing) and attach fine Fisher tubing. Set the diameter button on the pump to 8.70 mm and run the pump at 0.1 ml/hour. Pump should be prerun for several minutes prior to starting transgenesis for the day to assure that the plunger for the syringe is flush with the piston and that positive flow of oil out of the tubing is occurring.
- **Make p200 tip-tygon tubing assemblages for backfilling needles.** Using a razor blade or clean scissors, cut the tip off of p200 pipette tips (such that the inner diameter of the tip is at least 200-300 microns). Alternatively, use wide opening p200 pipette tips. Push a 1-3 cm piece of fine tygon tubing (the same stuff used with the infusion pump) onto the end of the clipped pipette tip. Generate several dozen of these for use in backfilling needles for transgenesis.
- **Determine needle puller settings/Prepare needles.** You will need to produce a needle with a long region that clips to about an 80 micron tip (seems to change every year depending on how fresh the filaments are on the Narishige pullers we get--bot horizontal and vertical pullers have worked in previous years)
- **Prepare needles.** See CSH manual chapter for details.
- make cysteine (2.5% IN 1XMMR, NOT water! pH8.0). You will need several hundred mls

- make 0.2XMMR+6%Ficoll+100 $\mu$ g/ml gent for transplantation dishes and recovery of transgenic embryos and 0.2XMMR+100 $\mu$ g/ml gent (no Ficoll) for raising embryos.
- Thaw enough frozen aliquots of SDB (sperm dilution buffer) for the day's transplantations and prepare a 100mM MgCl<sub>2</sub> solution.
- prepare clipped (wider orifice) P-200 pipette tips with tubing attached for loading needles  
(many of these can be prepared beforehand and stored in a large plastic petri dish)
- be sure solutions are not warmer than 18-21°C  
(transgenic embryos should be raised through early cleavages at temperatures between 16 and 21°C, since higher temperatures adversely affect both frequency of transgenesis and embryonic development)
- be sure frogs are laying and eggs are of high quality. Optimally, eggs should have a firm cortex (hold shape after dejellying). We typically use young frogs from NASCO, since these give higher frequency of normal gastrulation.

## II. SETTING UP A TRANSGENESIS REACTION:

**IMPORTANT: Check that solutions, equipment and frogs are all ready before beginning a reaction. Once you begin, you must proceed with the reaction using the timetable described below, since many components do not remain stable for >30minutes. While the sperm nuclei stock is kept on ice, transgenesis reactions (both diluted and concentrated) must always be kept at room temperature.**

1) Very gently (through a clipped tip / taking care not to push tip into bottom of tube) mix the nuclei stock and combine in a 1.5ml eppendorf tube:

5 $\mu$ l nuclei (1.25X10<sup>8</sup> nuclei / ml)

4 $\mu$ l linearized plasmid (150-250ng /  $\mu$ l)

2) INCUBATE 5 MINUTES AT ROOM TEMPERATURE.

3) while incubation is proceeding, thaw a 25 $\mu$ l aliquot of extract and dilute 1 $\mu$ l of restriction enzyme (NotI, diluted 1:10 in water). Note: Aliquot of extract can be thawed and kept on ice for use throughout the day.

4) add to the reaction, in this order:

23  $\mu$ l SDB

2  $\mu$ l high speed extract

2 $\mu$ l MgCl<sub>2</sub> (100mM)

0.5 $\mu$ l diluted restriction enzyme

5) INCUBATE 10 MINUTES AT ROOM TEMPERATURE

6) while incubation is proceeding, squeeze eggs from 2-3 frogs into the cysteine solution. Dejelly the eggs, wash them well (5X) with 1XMMR and use a wide bore pipette to transfer 400-500 dejellied eggs to an agarose well dish containing 0.2X MMR +4% Ficoll.

7) AT 15-20 MINUTES AFTER BEGINNING REACTION, dilute some of the reaction in SDB to obtain the proper concentration for transplantations: VERY gently mix the reaction (nuclei / extract / DNA) with a clipped tip and add about 4-6 $\mu$ l (usually) to 195  $\mu$ l SDB at room temperature. Nuclei in this diluted mixture are stable for about 1hr., whereas they deteriorate more rapidly in the concentrated mixture.

9) Mix the diluted reaction prepared in #7 very gently with a wide orifice pipette tip, then load the needle NO MORE THAN A FEW SECONDS LATER (nuclei will settle rapidly in the tube). Attach needle to pump tubing and begin transplantations of nuclei into eggs. Injections should be rapid, shallow and approximately perpendicular to the egg's plasma membrane to avoid doing damage. At the rate of flow suggested above, you should be able to obtain

approximately 1 nucleus per egg if the needle is in the egg for a fraction of a second. You should complete a dish of transplantations within 1.25 hrs.

After injections, leave the dishes at 18-22°C until embryos have reached the 4-8 cell stage. Sort the cleaving embryos away from their non-cleaving neighbors by transferring (with a glass pasteur pipette with a tip approximately the same diameter as an egg) to a fresh large dish of 0.2XMMR +4% Ficoll. Then subdivide the cleaving embryos into smaller subpopulations and culture in 0.2XMMR (no Ficoll) +100 $\mu$ g/ml gentamycin through early development. We usually put 10-15 into each well of a 6-well plate. Embryos should be checked during gastrulation with any dying embryos removed promptly and the media changed (if contaminated with yolk or lysed embryo). Culture of early embryos at 16°C generally allows us to remove dying embryos before they lyse and compromise healthy embryos in their well.