Materials List for:

Production of Transgenic *Xenopus laevis* by Restriction Enzyme Mediated Integration and Nuclear Transplantation

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Materials

A.Sperm nuclei preparation

Reagents:

1. 1X MMR (2mM $CaCl_2$, 5mM HEPES, pH7.5, 2mM KCl, 1mM $MgCl_2$, 100mM NaCl).
2. 0.1% Tricaine Methanesulfonate (MS222, aminobenzoic acid ethyl ester, Sigma A-5040), 0.1% sodium bicarbonate. Dissolve in water.
3. 2X Nuclear Preparation Butter (NPB). On the day of the sperm nuclei preparation, make up 30 ml of 2X NPB from aliquots of the stock solutions stored frozen: 500 mM sucrose (1.5 M stock), 30 mM HEPES (1M stock; titrate with KOH so that pH 7.7 is at 15 mM), 1 mM spermidine trihydrochloride (Sigma S-2501; 10 mM stock), 0.4 mM spermine tetrahydrochloride (Sigma S-1141; 10 mM stock), 2 mM dithiothreitol (Sigma D-0632; 100 mM stock), 2 mM EDTA (500 mM EDTA, pH 8.0).
4. Use the 2XNPB to make a. 30ml 1X NPB, b. 10ml 1XNPB+3%BSA (fraction V, Sigma A-7906), c. 5ml 1XNPB+0.3%BSA.
5. Lysolecithin: 100 µl of 10 mg/ml L-α-lyso-Lecithin, Egg Yolk (Calbiochem, 440154); dissolve at room temperature just before use. Store solid stock at 20 °C. Discard the stock powder if it becomes sticky.
6. Bovine serum albumin (BSA): 10% (w/v) BSA (fraction V, Sigma A-7906) Make up 5 ml in water on the day of the sperm nuclei preparation.
7. Sperm storage buffer (1ml) 1X NPB, 30% glycerol, 0.3% BSA.
8. Sperm dilution buffer: 250 mM sucrose, 75 mM KCl, 0.5 mM spermidine trihydrochloride, 0.2 mM spermine tetrahydrochloride. Titrate to pH 7.3-7.5 and store 0.5-1 ml aliquots at 20 °C.
9. H–chst No. 33342 (Sigma B-2261): 10 mg/ml stock in dH_2O, store in a light tight vessel at 20 °C.

Equipment:

- Swinging bucket rotor and centrifuge
- cheesecloth
- dissection tools (forceps and scissors)
- fluorescence microscope
- funnel
- gloves
- hemocytometer
- needles (26 gauge)
- paper towels
- petri dishes (60 mm)
- pipettes
- plastic (5 and 10 ml)
- Pipetman tips (1 ml and 200µl)
- Syringes (1 ml)
- tubes (14 ml; Falcon, 2059)
- tubes
- microcentrifuge (1.5 ml)

B. Preparation of High Speed Extract

Reagents:

1. 1X Marc's Modified Ringer (MMR): 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.5. Prepare a 10X stock, and adjust pH with NaOH to 7.5.
2. 20X Extract buffer (XB) salt stock: 2 M KCl, 20 mM $MgCl_2$, 2 mM $CaCl_2$, filter-sterilize and store at 4 °C.

3. Extract buffer (XB; freshly prepared and stored on ice): 1X XB salts, 50 mM sucrose,10mM HEPES (1 M stock, titrated with KOH so that pH is 7.7 when diluted to 15 mM; filter-sterilize, and store in aliquots at 20 °C). Prepare about 100 ml.
4. 2% (w/v) L-Cysteine hydrochloride 1-hydrate: Made up in 1X XB salts before use and titrated to pH 7.8 with NaOH. Prepare about 300 ml.
5. CSF-XB: 1X XB salts, 1 mM $MgCl_2$ (in addition to $MgCl_2$ present in XB salts; final concentration 2 mM), 10 mM HEPES, pH 7.7, 50 mM sucrose, 5 mM EGTA, pH 7.7. Prepare 50 ml.
6. Protease inhibitors: Mixture of leupeptin, chymostatin, and pepstatin, each dissolved to a final concentration of 10 mg/ml in dimethyl sulfoxide (DMSO). Store in small aliquots at 20 °C.
7. 1 M $CaCl_2$.
8. Energy mix: 150 mM creatine phosphate, 20 mM ATP, 20 mM $MgCl_2$.
9. Pregnant Mare Serum Gonadotropin (PMSG): 100 U/ml PMSG (P.G.600®, Intervet, Inc., 021825). Dissolve in water and stored at 20 °C.
10. Human Chorionic Gonadotropin (HCG): 1000 U/ml HCG (CHORULON®, Intervet, Inc., 057176). Dissolve in water and stored at 4 °C.

Equipment:

- *Xenopus laevis* females
- Needles (18 and 26 gauge)
- Pasteur pipette
- wide bore
- Syringes (1 mL)
- Tubes, microcentrifuge (0.5 mL)
- Tubes, thick-wall polycarbonate (Beckman, 349622)
- Tubes, ultraclear (14 x 95 mm; Beckman, 344060)
- Ultracentrifuge and rotors (e.g., Beckman TL-100 with rotors SW 40 Ti and TLA-100.3)
- Beakers for egg collection
- Buckets or containers for holding female frogs (e.g., 4-L plastic beakers with mesh lids).

C. Nuclear transplantation.

Reagents:

1. 2.5% agarose in 0.1XMMR (for making injection dishes)
2. 2.5% Cysteine in 1XMMR, pH8.0, prepared on the day of use
3. Ficoll
4. 10 mg/ml gentamycin (1000X stock)
5. high speed egg extract (see above)
6. 100 $MgCl_2$
7. 10X MMR (see above)
8. Restriction enzyme (e.g. NotI from New England Biolabs)
9. Sperm dilution buffer (SDB; see above) and sperm nuclei (see above)
10. Human Chorionic Gonadotropin (HCG) as above
11. mineral oil (Sigma, M8410)
12. Linearized plasmid to be introduced as the transgene: Prepare linearized plasmid at a concentration of about 100 ng/µl 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 d–s not have to be the same as the one used in the nuclear transfer reaction. We usually use NotI for all reactions, regardless of what plasmid is linearized with. Some calibration of the enzyme dilution used in the reaction may be necessary, as too much enzyme can cause adverse effects on post-gastrula development. Plasmid can be purified in several different ways: we usually use the Qiagen Qiaquick PCR purification kit according to the manufacturers directions; purification of a single band from a gel is not necessary. If plasmid is purified using phenol/chloroform extractions and ethanol precipitation, be certain to remove all traces of organics and ethanol.

Equipment:

Agarose dishes for injection: In a 60 mm plastic petri dish, lay a small 35mmX35mm weigh boat on molten 2.5% agarose in water 0.1XMMR to create a depression with an agarose-coated bottom for filling with eggs. Once agarose has hardened, wrap in parafilm and store at 4 °C until use. Make 2-3 dishes in advance for each transgenic reaction you plan to do.

Infusion pump: We use a single syringe infusion pump from Harvard Apparatus, equipped with a 3 cc syringe/needle filled with mineral oil (Sigma M-8410). Blunt the syringe needle tip (to keep it from perforating the tubing) and attach the fine tygon tubing. Run the pump at ~10nl/sec; this assumes that the time the needle is in each egg will be no greater than 1 sec. Pump should be pre-run 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 steady positive flow of oil out of the tubing is occurring.

Needles for nuclear transfers. Using a micropipette puller, generate needles with long, sloping tips. Clip these with a forcep under a dissecting microscope equipped with an ocular micrometer to obtain an ~80 micron opening with a beveled shape.

Other equipment: *Xenopus laevis* females, stereomicroscope, incubator, micromanipulator, microinjection needle puller (e.g. Model P-87, Sutter), syringe needles (26 gauge), glass microinjection needles, ocular micrometer for calibrated clipping of microinjection needle tips to 80µm diameter, petri dishes, weigh boats 35mm, Tygon tubing (ID=1/32 in., OD=3/32 in.)

