## Materials

- frogs
- incubator at 16°C
- clinical centrifuge
- DuPont RC-5 centrifuge
- HB-4 rotor with rubber adapters
- Beckman ultraclear SW50 tubes, 2.5 ml syringe
- 18 and 20G needles
- glass Pasteur pipettes.
- Pregnant mare serum gonadotropin Intergonan was purchased from Vemie Veterinaer
- ATP (Cat. No. A-2383) from Sigma
- Bovine serum albumin (Cat. No. A-9647) from Sigma
- L-cysteine (Cat. No. C-7755) from Sigma
- Cytochalasin D (Cat .No. C-8273) from Sigma
- EGTA (Cat .No. E-4378) from Sigma
- Human Chorionic Gonadotropin (Cat. No. CG-10) from Sigma
- 4.9 M MgCl2 (Cat. No. 104.20) from Sigma
- Lysolecythin (Cat. No. 4129) from Sigma
- Phenylmethylsulfonyl fluoride (Cat. No. P-7626) from Sigma
- Spermidine trihydrochloride (Cat. No. L-2501) from Sigma
- spermidine tetrahydrochloride (Cat .No. L-1141) from Sigma
- CaCl2 (Cat .No. art.2383) from Merck
- KCl (Cat .No. 1.04936.1000) from Merck
- NaCl (Cat .No. 1.06404.1000) from Merck
- Sucrose (Cat. No. 1.07653.1000) from Merck
- Chymostatin (Cat .No. E16) from Chemicon
- Leupeptin (Cat. No. E18) from Chemicon
- Pepstatin (Cat. No. E19) from Chemicon
- HEPES from Biomol
- Creatine phosphate (Cat. No. 127 574) from Boehringer Mannheim.

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## Solutions

**Pregnant mare serum gonadotropin (PMSG) 100 units/ml.**

Disslove in 500 units in 5 ml sterile distilled water. Store at 4°C.

**Human chorionic gonadotropin (HCG)**

500 units/frog (0.5 ml). Dissolve10000 units in 10 ml sterile distilled water. Store at 4°C

**MMR**

100 mM NaCl, 2 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 0.1 mM EDTA, 5 mM HEPES pH 7.8. Prepare 500 ml 1x MMR from 10x stock by combining 50 ml 10x and 450 ml distilled water. To prepare 1 liter of 10x stock, combine 58.44 g NaCl, 1.49 g KCl, 2.04 ml 4.9 M MgCl2 stock solution, 2.94 g CaCl2, 372 mg EDTA and 11.91 g HEPES and 900 ml distilled water. Adjust pH to 7.8 with 6 M NaOH and bring volume to 1 liter. Autoclave and store at room temperature.

**Dejellying solution**

2% L-cysteine, pH 7.8. To prepare 250 ml, dissolve 5g cysteine in 250 ml water. Adjust pH to 7.8 with NaOH. Always preepare fresh just before use.

**Protease inhibitors (LPC)**

10 mg/ml solution of leupeptin, pepstatin, chymostatin in DMSO. To prepare 1 ml, dissolve 10 mg of each leupeptin, pepstatin and chymostatin in 1 ml DMSO. Store in 50 µl aliquots at -20°C.

**Cytochalasin D**

10 mg/ml in DMSO. To prepare 1 ml, dissolve 10 mg cytochalasin D in 1 ml DMSO. Store in 50 µl aliquots at -20°C.

**XB**

100 mM KCl, 0.1 mM CaCl2, 1 mM MgCl2, 50 mM sucrose, 10 mM K-HEPES pH 7.7. Prepare fresh from stock solutions of 20x XB salts, 1 M HEPES and 1.5 M sucrose. To prepare 20x XB salts (2M KCl, 20 mM MgCl2, 2 mM CaCl2), dissolve 74.56 g KCl, 147 mg CaCl2 and 2.04 ml of 4.9M MgCl2 in 500 ml of distilled water. Filter sterilize and store at 4°C. To make 1M HEPES, dissolve 59.6 g HEPES in 200 ml distilled water,. Adjust pH to 7.8 with concentrated KOH and volume to 250 ml. Filter sterilize and store in 5 ml aliquots at -20°C. To prepare 1.5 M sucrose, dissolve 256.72 g sucrose in distilled water. Store in 15 ml aliquots at -20°C. For an extract prep to make spindles, prepare 250 ml of XB: add 12.5 ml 20x XB salts, 2.5 ml 1 M HEPES and 14 ml of 1.5 M sucrose to 200 ml of distilled water. Adjust pH to 7.7 with KOH if necessary and bring volume to 250 ml.

**CSF-XB**

100 mM KCl, 0.1 mM CaCl2, 2 mM MgCl2, 5 mM EGTA, 50 mM sucrose, 10 µg/ml LPC, 10 mM HEPES pH 7.7. Prepare by adding MgCl2, EGTA and LPC stock solutions to XB solution (above). To prepare 0.5 M EGTA, dissolve 19.02 g EGTA in 100 ml distilled water, and adjust pH to 7.7 with 10 M NaOH. Store in 1 ml aliquots at -20°C. To make 50 ml of CSF-XB, add 10.2 µl of 4.9M MgCl2, 0.5 ml of 0.5M EGTA, and 50 µl of 10 mg/ml LPC to 49.5 ml of XB. Check the pH and adjust if necessary to 7.7 with 1 M KOH.

**Energy Mix**

7.5 mM creatine phosphate, 1 mM ATP pH 7.7, 1 mM MgCl2. Prepare stock solutions of 100 mM ATP, 750 mM creatine phosphate and 100 mM MgCl2. To prepare 0.1 M ATP, dissolve 275.5 mg of ATP in 5 ml distilled water. To prepare 750 mM creatine phosphate, dissolve 245.4 mg in 1 ml distilled water. For 100 mM MgCl2, combine 20.4 µl 4.9 M MgCl2 and 979.6 µl distilled water. Store in aliquots at -20°C

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## Steps

1. Inject 2 to 4 frogs subcutaneously with 0.5 ml (50 units) PMSG each using 1 ml syringes and 27 gauge needle at least 4 days before planning to make an extract. They should be used within 2 weeks after the priming injection. The number of frogs required depends on the quantity and quality of eggs.

2. 12-18h before use, inject frogs subcutaneoulsy with 0.5 ml (500 units) HCG. Place the frogs in individual boxes containing 500 ml MMR at 16°C.

3. Prepare all solutions before starting. Rinse all glassware with distilled water (eggs stick to plastic dishes). Cut the end of a glass pasteur pipette and fire-polish it to make a wide mouth pipette.

4. Collect laid eggs in MMR. Frogs can also be squeezed which often gives the highest quality eggs. Keep eggs from different frogs in separate batches in 400 ml beakers. Discard batches of eggs containing more than 5% of lysed, bad looking or stringy eggs.

5. Pour off MMR and add 50-100 ml dejellying solution. When laid, eggs are enveloped in a transparent jelly coat and do not pack closely together. Swirl the beaker frequently, and change the cysteine solution 2-3 times. After removal of the jelly coat, eggs pack together. This takes about 5 minutes. Eggs left for too long in cysteine will lyse.

6. Pour off the cysteine solution and add 50-100 ml MMR. Repeat the rinse several times. After removal of the jelly coat, the eggs become fragile. They lyse easily, and can activate if in contact with air. They must always remain immersed in buffer. Remove all bad looking eggs, white and puffy, flattened, activated ones (darker pole retracted) and those with mottled pigmentation. Wash again in MMR.

7. Wash 3 times with 50-100 ml XB.

8. Remove as much buffer as possible keeping all eggs immersed. Wash twice with CSF-XB.

9. Transfer eggs to SW50 tubes containing 1 ml CSF-XB plus 10µl cytochalasin D (100 µg/ml). Always immerse the pipette tip in solution before expelling eggs to prevent contact with air. Transfer the SW50 tubes to 12 ml polypropylene adapter tubes (Sarstedt), which contain 0.5 ml of water to prevent the tubes from collapsing.

10. Centrifuge in a clinical centrifuge at 150g, 30 seconds, then at 700g, 30 seconds at 16°C. Then remove all excess buffer from the top of the packed eggs. Removal of buffer is critical to obtain a concentrated cytoplasm.

11. Place the tubes in an HB-4 rotor containing rubber adapters. Centrifuge at 10,000 rpm in a sorvall for 15 minutes at 16°C to crush the eggs.

12. Place the tubes on ice. A yellow lipid layer is at the top of the tube, Underneath is the cytoplasmic layer, then heavy membranes and yolk particles at the bottom of the tube. Wipe the sides of the tubes with a tissue before piercing with a 20G needle at the bottom of the cytoplasmic layer. Slowly and carefully remove it.

13. Add LPC and cytochalasin D to 10 ug/ml (1:1000 dilution of stocks). Add energy mix (1:100 dilution of stocks). Mix gently. The extract can be kept on ice up to 6 hours before use.