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Protocol

Generation of Transgenic *Xenopus laevis*: I. High-Speed Preparation of Egg Extracts

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INTRODUCTION

Manipulating genes specifically during later stages of amphibian embryonic development requires fine control over the time and place of expression. These protocols describe an efficient nuclear-transplantation-based method of transgenesis developed for *Xenopus laevis*. The approach enables stable expression of cloned gene products in *Xenopus* embryos. Because the transgene integrates into the genome prior to fertilization, the resulting embryos are not chimeric, eliminating the need to breed to the next generation to obtain nonmosaic transgenic animals. The procedure is based on restriction-enzyme-mediated integration (REMI) and can be divided into three parts: (I) high-speed preparation of egg extracts, (II) sperm nuclei preparation, and (III) nuclear transplantation. This protocol describes the method for the high-speed preparation of egg extracts. Briefly, a crude, cytosstatic factor (CSF)-arrested egg extract (i.e., cytoplasm arrested in meiotic metaphase) is prepared. These extracts are driven into the interphase stage of the cell cycle by addition of calcium, and high-speed centrifugation is performed to obtain a purer cytoplasmic fraction. This fraction promotes swelling of sperm nuclei, but does not promote DNA replication. By adding the egg extract to the reaction, the sperm chromatin partially decondenses, facilitating integration of plasmid DNA into the genome.

RELATED INFORMATION

For additional protocols essential to the generation of transgenic *Xenopus laevis*, please refer to [Generation of Transgenic *Xenopus laevis*: II. Sperm Nuclei Preparation](#) and [Generation of Transgenic *Xenopus laevis*: III. Sperm Nuclear Transplantation](#).

MATERIALS


Reagents


1 M CaCl₂

 CSF-XB buffer with protease inhibitors, freshly prepared and stored on ice


  Cysteine solution (1X XB salts containing 2.0% L-cysteine hydrochloride 1-hydrate, pH 7.8), freshly prepared


 Energy mix

 Extract buffer, freshly prepared and stored on ice

 10 mg/mL Hoechst No. 33342, diluted 1:100 prior to use (optional; see Step 21)

1000 U/mL human chorionic gonadotropin (HCG)

 Liquid nitrogen

 10X Marc's modified Ringer's (MMR), diluted to 1X prior to use

100 U/mL pregnant mare serum gonadotropin (PMSG)

  Protease inhibitor mix

Xenopus laevis, adult female

For details on the proper handling of frogs, please refer to [Handling *Xenopus laevis* Adults](#).

Equipment

Beakers for egg collection (see Step 5)

Buckets or containers for holding female frogs (e.g., 4-L plastic beakers with mesh lids)

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)

METHOD

All solutions should be prepared before the extraction process begins. The procedure should be carried through all steps promptly once it is initiated. Optimally, the high-speed centrifugation should begin within 45-60 min of dejelling the eggs.

Collection and Preparation of *Xenopus* Eggs

1. About 3-5 d prior to HCG injection, prime 8-12 adult female *X. laevis* by injecting 50 U of PMSG into the dorsal lymph sac using a 26-gauge needle.
For details on the proper procedure for handling and injection of the frogs, see [Inducing Ovulation in Xenopus laevis](#).
2. The evening before extract preparation, inject each frog with 500 U HCG.
3. Place the frogs into 2 L of 1X MMR (2 frogs/container), and keep them at 15°C-18°C overnight (12-14 h).
Because one frog with lysing or activating eggs can compromise the whole extract preparation, we prefer to separate the frogs into pairs for the ovulation.
4. The next morning, screen the quality of the eggs from each container before mixing all the eggs. Leave out all the eggs released in any container with signs of mottled, lysing, or dying eggs from the extract preparation, as these can affect the quality of the extracts obtained.
5. Gently, manually expel eggs from each frog into large beakers containing 1X MMR.
For details on this procedure, see [Xenopus laevis Egg Collection](#).
6. Collect unbroken eggs with even pigmentation. Good eggs can also be collected from the 1X MMR in the frog buckets.
The total volume of eggs should be >100 mL from the 8-12 females.
7. To dejelly the eggs, remove as much MMR as possible from the eggs. Add a small amount of cysteine solution, and swirl the eggs. Replace with fresh cysteine solution several times during dejelling.
Dejelling should be initiated separately for different batches of eggs. Discard any batches that show breakage or premature egg activation. Egg activation can be recognized by constriction of the animal pole region of the eggs. The rest of the eggs can then be combined.
8. Wash the eggs in ~35 mL of extract buffer, four times.
9. Wash the eggs in 25 mL of CSF-XB with protease inhibitors, twice.
10. Using a wide-bore Pasteur pipette, transfer the eggs into Beckman ultraclear tubes. Allow the eggs to settle. Remove as much CSF-XB as possible.
11. Centrifuge the eggs using a Beckman SW 40 Ti rotor at 1000 rpm (150g) for ~60 sec at 4°C. Remove excess solution from the top of the packed eggs.
This step packs the eggs, but does not crush them yet.

High-Speed Extraction of *Xenopus* Eggs

12. Centrifuge the eggs at 10,000 rpm (16,000g) for 10 min at 4°C to crush the eggs.
The eggs should be separated into three layers: lipid (top), cytoplasm (center), and yolk (bottom).
13. Collect the cytoplasmic layer from each tube with an 18-gauge needle by inserting the needle at the base of the cytoplasmic layer. Transfer the cytoplasm to a fresh ultraclear Beckman tube on ice.
14. Add protease inhibitors to the isolated cytoplasm to a final concentration of 1X. Recentrifuge the cytoplasm at 16,000g for 10 min at 4°C.
15. Collect the clarified cytoplasm as described in Step 13.
Expect to obtain 0.75-1 mL cytoplasm/frog.

16. Add 1/20 of the extract volume of energy mix to the sample. Transfer the cytoplasm into thick-walled polycarbonate tubes. *Tubes hold about 3 mL each and should be at least half full.*

17. Add 1 M CaCl_2 to each tube to a final concentration of 0.4 mM. Incubate the tubes for 15 min at room temperature. *This inactivates CSF and pushes the extract into interphase.*

18. Balance the tubes. Centrifuge them in a Beckman TL-100 ultracentrifuge using a TLA-100.3 rotor at 70,000 rpm for 1.5 h at 4°C. *The cytoplasm will fractionate into four layers, top to bottom: lipid, cytosol, membrane/mitochondria, and glycogen/ribosomes.*

19. Remove the cytosolic layer from each tube (~1 mL if 2-3 mL were loaded into the tube) by inserting a syringe into the top of the tube through the lipid layer.

20. Transfer the cytosolic fraction to fresh tubes. Recentrifuge the samples at 70,000 rpm for 20 min at 4°C.

21. Aliquot the supernatant into 25- μL aliquots in 0.5-mL tubes. Quick-freeze the aliquots in liquid nitrogen. Store them at -80°C until use.

*To test the quality of the extracts, add sperm nuclei to an aliquot of extract and stain with Hoechst to determine whether the nuclei visibly swell (i.e., thicken and lengthen) within 10 min of addition at room temperature (see [Generation of Transgenic *Xenopus laevis*: II. Sperm Nuclei Preparation](#) for more details).*

 **Caution**

Cysteine hydrochloride

Cysteine hydrochloride is an irritant to the eyes, skin, and respiratory tract. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses. Do not breathe the dust.

 **Caution**

General warning

This material contains hazardous components. Please see recipe for full details.

 **Caution**

Hoechst No. 33342

Hoechst No. 33342 see Bisbenzimidide



 **Caution**

Liquid nitrogen (LN_2)

Liquid nitrogen (LN_2) can cause severe damage due to its extreme temperature. Handle frozen samples with extreme caution. Do not breathe the vapors. Seepage of liquid nitrogen into frozen vials that are immersed in liquid nitrogen can cause the vials to explode when they are removed. Use vials with O-rings when possible. Wear cryo-mitts and a face mask. Do not allow the liquid nitrogen to spill onto clothing. Do not breathe the vapors.

 **Recipe**



CSF-XB buffer with protease inhibitors

 5 mM EGTA


10 mM HEPES (pH 7.7)

 1 mM MgCl₂

This is added in addition to the MgCl₂ present in XB salts; the final concentration of MgCl₂ is 2 mM.

  1X Protease inhibitor mix

 50 mM sucrose


 1X XB salts

Recipe

Energy mix

20 mM ATP

150 mM creatine phosphate


 20 mM MgCl₂


Store in 0.1-ml aliquots at -20°C.

Recipe

Extract buffer


10 mM HEPES (pH 7.7)

 50 mM sucrose


 1X XB salts


Recipe


Marc's modified Ringer's (MMR) (10X)



 20 mM CaCl₂

50 mM HEPES (pH 7.5)

 20 mM KCl


 10 mM MgCl₂

 1 M NaCl

  Adjust pH with NaOH to 7.5. Sterilize by autoclaving.


Recipe

Protease inhibitor mix (1000X stock)

 Chymostatin

 DMSO

 Leupeptin

 Pepstatin


Dissolve each of the protease inhibitors (chymostatin, leupeptin, and pepstatin) to a final concentration of 10 mg/mL in DMSO. Store the solution in small aliquots at -20°C.




Recipe

XB salts (20X)

 2 mM CaCl₂

 2 M KCl

 20 mM MgCl₂

Filter-sterilize. Store at 4°C.

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