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Protocol

Generation of Transgenic *Xenopus laevis*: III. Sperm Nuclear Transplantation

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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. 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 a method for the nuclear transplantation in *Xenopus laevis*. Permeabilized sperm nuclei are incubated briefly with linearized plasmid DNA, after which egg extract and a small amount of restriction enzyme are added. The egg extract partially decondenses the chromosomes, and the restriction enzyme stimulates recombination by creating double-strand breaks, facilitating integration of DNA into the genome. Diluted nuclei are transplanted into unfertilized eggs. Because the transgene integrates into the genome prior to fertilization, the resulting transgenic embryos are not chimeric and there is no need to breed to the next generation in order to obtain nonmosaic transgenic animals.

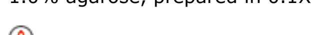
RELATED INFORMATION

For additional protocols essential to the generation of transgenic *Xenopus laevis*, please refer to Generation of Transgenic *Xenopus laevis*: I. High-Speed Preparation of Egg Extracts and Generation of Transgenic *Xenopus laevis*: II. Sperm Nuclei Preparation.

MATERIALS

Reagents

1.0% agarose, prepared in 0.1X MMR

 Cysteine solution (1X MMR containing 2.5% L-cysteine hydrochloride 1-hydrate, pH 7.8-8.0), prepared immediately before use

Ficoll

10 mg/mL gentamycin (1000X stock)

High-speed egg extract (see Generation of Transgenic *Xenopus laevis*: I. High-Speed Preparation of Egg Extracts)

1000 U/mL human chorionic gonadotropin (HCG)

100 ng/µL linearized plasmid

Any enzyme can be used for linearization of plasmid. Digest the DNA using standard conditions, and purify by phenol/chloroform extraction and ethanol precipitation. There is no need to gel-purify the plasmid.

100 mM $MgCl_2$

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

100 U/mL pregnant mare serum gonadotropin (PMSG)

Restriction enzyme

Sperm dilution buffer (SDB)

Sperm nuclei (1×10^5 to 2×10^5 nuclei/µL; see Generation of Transgenic *Xenopus laevis*: II. Sperm Nuclei Preparation)

Xenopus laevis, adult females

For details on the proper handling of frogs, please refer to Handling *Xenopus laevis* Adults.

Equipment

Beaker for egg collection (see Step 14)

Dishes (24 well), coated with agarose (optional; see Step 26)

Forceps

Ice

Incubator

Injection microscope

Micromanipulator

Micropipette puller (Model P-87, Sutter Instruments)

Mineral oil (Sigma, M8410)

Needles (26 gauge)

Needles (30 μL), transplantation (Drummond, Microcaps 1-000-0300)

Ocular micrometer from a dissecting microscope

Parafilm

Pasteur pipettes, wide bore

Petri dishes (60 mm and 10 cm)

Plastic box with lid, empty (approximate dimensions: W 15 cm x L 9 cm x H 5 cm)

Pipetman pipette tips (200 μL), clipped

Syringe pump (Harvard Apparatus, #552222)

Syringes (1 mL)

Syringes, gas-tight (2.5 mL) (Hamilton)

Tubing, plastic (ID = 0.7 mm, OD = 2.4 mm)

Tubing, Tygon (ID = 1/32 in., OD = 3/32 in.)

Weighing boats (35 x 35 mm)

The weighing boats are used as templates during the preparation of injection dishes (see Steps 9-11). This size template holds about 400 X. laevis *eggs.*

METHOD

Preparation of Transplantation System

1. Set the micropipette puller using the following conditions: p = 50, v = 100, and t = 5.

2. Use the puller to produce large transplantation needles with long, gently sloping tips.

3. Using the ocular micrometer from a dissecting microscope for measurement, clip the needles with a forceps to produce a beveled tip with a diameter of 80-100-μm (Fig. 1B).

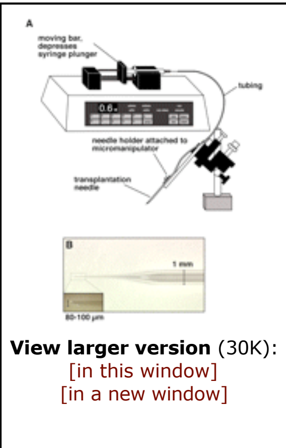


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Figure 1. Nuclear transplantation apparatus. (*A*) This protocol uses an oil-filled injection system for the nuclear transplantations. The syringe and tubing are filled with mineral oil and the infusion pump depresses the syringe plunger, resulting in a constant, desirable flow rate. (*B*) The transplantation needle has a gently sloping tip and is clipped with forceps to produce a beveled 80-100-µm-wide tip.

4. Set up the transplantation apparatus (see Fig. 1A for details). Fill the plastic tubing and gas-tight syringe with mineral oil. Set the flow rate of the syringe pump at 0.6 µL/min. Start the infusion pump before injection to stabilize the flow.

Preparation of Transgenic Nuclei

5. Using a clipped 200-µL pipette tip, mix 4 µL of sperm nuclei (~4 x 10^5 to 8 x 10^5 nuclei) with 1-2 µL of linearized plasmid (100 ng/µL). Incubate for 5 min at room temperature.

6. Dilute 0.5 µL of the restriction enzyme of choice with 4.5 µL of H_2O.

7. Mix 1 µL of the diluted enzyme with 18 µL of SDB, 2 µL of 100 mM $MgCl_2$, and 2 µL of high-speed egg extract.

8. Add the enzyme solution from Step 7 to the sperm/DNA mixture from Step 5. Mix well by gently pipetting using a clipped 200-µL pipette tip. Incubate for 15 min at room temperature.

Preparation of Injection Dishes

These dishes are designed for injection of transgenic nuclear material into Xenopus *eggs (during Steps 16-26) and should be prepared in advance.*

9. Pour the 1.0% agarose solution into 60-mm Petri dishes.

10. Before the agarose solidifies, lay a weighing boat onto each plate as a template.

11. After the agarose solidifies, remove the templates. Wrap the dishes in Parafilm, and store them at 4°C until use.

Preparation of Recipient Eggs

12. About 3-5 d prior to HCG injection, prime two adult female *X. laevis* by injecting 50 U of PMSG into the dorsal lymph sac using a 26-gauge needle fitted to a 1-mL syringe.
For details on the proper procedure for handling and injection of the frogs, see Inducing Ovulation in *Xenopus laevis*.

13. Twelve to fifteen hours before transplantation, inject each frog with 500 U of HCG. Store the frogs overnight at 15°C-18°C.

14. While the enzyme reaction is incubating (Step 8), manually expel the eggs from the frogs. Squeeze the eggs directly into a large dry beaker.
For details on this procedure, see *Xenopus laevis* Egg Collection.

15. Add the cysteine solution immediately to the eggs to ensure egg quality. Dejelly the eggs in the cysteine solution.
For details on this procedure, see Dejellying *Xenopus laevis* Embryos. *This usually takes ~10 min, so by the time the eggs are ready, the enzyme reaction (Step 8) is nearly complete.*

16. Wash the dejellied eggs with 1X MMR at least three times. Use a wide-bore Pasteur pipette to transfer the eggs to injection dishes containing 0.4X MMR supplemented with 6% Ficoll and 10 µg/mL gentamycin. Fill the square space with eggs so that no gap is left between the eggs.

17. Place the injection dish on an empty plastic box half-filled with ice. Place the box and eggs under the injection microscope.
This should provide a transplantation temperature of ~16°C.

Injection of Transgenic Nuclei

18. After incubation with the enzyme (Step 8), mix the sperm nuclei gently by pipetting with a clipped 200-μL pipette tip. Transfer 5 μL of the reaction into 150 μL of SDB equilibrated to room temperature.
Keep decondensed sperm nuclei at room temperature and transplant them within 1 h, but preferably within 30 min.

19. Attach a piece of Tygon tubing to the end of a clipped 200-μL pipette tip (Fig. 2A).

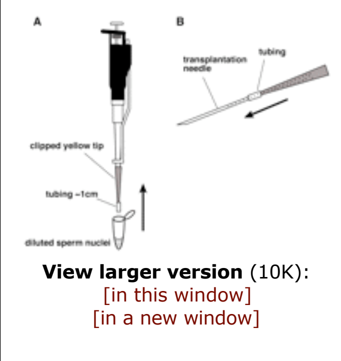

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Figure 2. Loading the transplantation needle. (*A*) The reaction mix (containing diluted, restriction-enzyme-treated sperm nuclei) is drawn into a clipped, 200-μL, yellow pipette tip attached to ~0.5-1cm of Tygon tubing. (*B*) The 200-μL pipette tip containing the Tygon tubing and dilute sperm nuclei is carefully detached from the Pipetman and connected to the blunt end of the transplantation needle using the tubing. The needle is gently loaded with the dilute sperm nuclear reaction by gravity. This is done by slowly increasing the angle of the pipette tip/tubing/needle so that the mixture flows gently into the needle. Once the needle is completely filled, it is detached from the tubing and is ready to connect to the infusion pump. The remaining sperm mixture can be set aside horizontally and used to reload another needle, if two people are injecting simultaneously or if a needle is accidentally damaged or blocked.

20. Using this tip, mix the diluted sperm nuclei well, but avoid making bubbles. Fill the clipped 200-μL pipette tip with the diluted sperm suspension.

21. Carefully detach the clipped 200-μL pipette tip from the Pipetman, keeping the tip horizontal. Attach the end of the Tygon tubing opposite the tip to the blunt end of a transplantation needle.

22. Backfill the transplantation needle by slowly increasing the angle of the pipette tip/tubing/needle so that the mixture flows gently into the needle (Fig. 2B).
You can keep the 200-μL pipette tip with the remaining nuclei by placing it horizontally, in case you need to load another needle.

23. Attach the needle to the mineral-oil-filled tube connected to the syringe in the syringe pump.

24. Check the flow on the pump and start injecting. Keep the needle inside each egg for ~0.5 sec. Move the needle fairly rapidly from egg to egg, piercing the plasma membrane of each egg with a single, sharp motion.
We usually transplant for ~15-20 min.
See Troubleshooting.

Incubation of Transgenic Embryos

25. After injection, transfer the embryos in their injection dishes to an incubator set at 16°C.

26. When the embryos reach the four-cell stage (about 3-4 h after injection at 16°C), use a wide-bore Pasteur pipette to transfer normally dividing embryos gently to a 10-cm Petri dish containing 0.1X MMR supplemented with 6% Ficoll and 10 μg/mL gentamycin.
Alternatively, the embryos can be transferred in groups of up to four or so embryos per well into 24-well dishes.
See Troubleshooting.

27. The next day (when embryos are around stage 12), transfer healthy embryos to a new 10-cm Petri dish (or 24-well dishes) containing 0.1X MMR supplemented with 10 μg/mL gentamycin.
Because of the large needle tip used for transplantations, embryos often develop large blebs at the site of injection. These blebs occur when cells are forced out of the hole left in the vitelline membrane at the injection site. They generally do not affect development, falling off at the neurula or tailbud stages.
See Troubleshooting.

28. Incubate embryos at 14°C-22°C until they reach the stage of interest for your specific experimental needs.
See Troubleshooting.

TROUBLESHOOTING

Problem: The needle is blocked by debris during transplantation.

[Step 24]

Solution: Change the needle or try to fix by pinching the tube or cutting the tip of needle using forceps.

Problem: No cleaving eggs

[Step 26]

Solutions: (1) Make sure that injection needle is not blocked. (2) Check the dilution of the sperm nuclei and/or the injection volume delivered during transplantation to be sure that they are appropriate.

Problem: Many embryos die during gastrulation.

[Step 27]

Solution: Try not to damage sperm nuclei during their preparation or during the enzyme reaction. Decondensed nuclei are very fragile and must be transplanted into eggs very soon. Do not place decondensed nuclei on ice.

Problem: The number of embryos expressing the transgene of interest is low.

[Step 28]

Solutions: (1) Make sure that the enzymes used for either linearization or the transgenic reaction do not digest within your construct. (2) Increase the amount of enzyme used for the reaction by diluting it less.

DISCUSSION

One person can transplant sperm nuclei into several hundred to thousands of eggs in a typical experiment. About 30%-40% of these transplanted eggs typically develop into normally cleaving four-cell stage embryos. About 60%-80% of these embryos proceed through gastrulation normally, while the other 20%-40% exhibit gastrulation abnormalities resulting from chromosomal damage to the sperm nuclei or physical damage to the egg occurring during transplantation. Thus, ~20%-30% of the eggs initially injected with nuclei proceed to post-gastrula stages, and ~10%-50% of these embryos show stable expression of transgenes. Since *Xenopus* embryos can be obtained rapidly, at low cost, and in large numbers, this technique provides a powerful approach for generating transgenic embryos in large numbers.

Transgenesis can be used in many applications: (1) to misexpress genes during development, (2) to label specific structures, using, for example, fluorescent proteins, and (3) to study the regulation of genes. Using this method, the transgene integrates into the genome as a concatemer (5-35 copies; Kroll and Amaya 1996). Therefore, two different constructs mixed in the same reaction cointegrate into the same site of the genome at high frequency (80%-90%; Hartley et al. 2001). To distinguish transgenic embryos, a marker gene (e.g., the γ-crystallin promoter driving GFP) can be co-integrated with a desired transgene. In cases where the effects of misexpression are subtle, it may be difficult to rely solely on F_0 transgenic embryos for the analysis. The reason for this is that each F_0 animal is unique; i.e., each carries a different copy number of transgenes and distinct sites of integration. However, by establishing transgenic lines, this variability can be overcome (Hartley et al. 2002).

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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.

Recipe

Ficoll 400

To prepare a 20% (w/v) of Ficoll 400, dissolve the Ficoll in sterile H_2O and store the solution frozen in 100-µl aliquots at -20°C.

Recipe

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

20 mM $CaCl_2$

50 mM HEPES (pH 7.5)

20 mM KCl

10 mM $MgCl_2$

1 M NaCl

Adjust pH with NaOH to 7.5. Sterilize by autoclaving.

Recipe

Sperm dilution buffer (SDB)

75 mM KCl

0.5 mM spermidine trihydrochloride

0.2 mM spermine tetrahydrochloride

250 mM sucrose

Add ~80 µL of 0.1 N NaOH per 20 mL of solution to titrate the pH to 7.3-7.5. Store 0.5-1 mL aliquots at -20°C.

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