Transgenesis in Xenopus

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1. Sperm Nuclei Preparation

A. Stock Solutions

10X Marc's Modified Ringers (MMR): 1 M NaCl; 20 mM KCl; 10 mM $MgCl_2$; 20 mM $CaCl_2$; 50 mM HEPES, pH 7.5. Sterilize by autoclaving and store at room temperature.

1.5 M Sucrose: filter sterilize and store 8.33 ml aliquots at -20°C.

1 M HEPES: titrate with KOH so that when diluted to 15 mM the pH is 7.7, should require about 5.5 ml of 10 N KOH for 100 ml; filter sterilize and store 0.75 ml aliquots at -20°C.

10 mM spermidine trihydrochloride: filter sterilize and store 2.5 ml aliquots at -20°C.

10 mM spermine tetrahydrochloride: filter sterilize and store 1 ml aliquots at -20°C.

100 mM Dithiothreitol: filter sterilize and store 0.5 ml aliquots at -20°C.

500 mM EDTA pH 8: stored at room temperature.

10% Bovine Serum Albumin (BSA; fraction V): in H_20; store 3.5 ml aliquots at -20°C.

100% glycerol: sterilize by autoclaving and store at room temperature.

10 mg/ml Hoechst No. 33342: in H_20; store in a light-tight vessel at -20°C.

Sperm Dilution Buffer (SDB): 250 mM sucrose; 75 mM KCl; 0.5 mM spermidine trihydrochloride; 0.2 mM spermine tetrahydrochloride; add about 80μl of 0.1N NaOH per 20mls solution to titrate to pH 7.3-7.5; store 1 ml aliquots at -20°C.

B. Solutions for Sperm Nuclear Preparation.

On the day of sperm nuclei preparation, make up the following:

50 ml of 1X MMR:
45 ml H_20
5 ml 10X MMR

store on ice
25 ml of 2X Nuclear Preparation Buffer (NPB)

	2 X Final concentration
11.8 ml H_20	
8.33 ml 1.5 M sucrose	500 mM
0.75 ml 1 M HEPES	30 mM
2.5 ml spermidine trihydrochloride	1 mM
1 ml spermine tetrahydrochloride	0.4 mM
0.5 ml 100mM Dithiothreitol	2 mM
0.1 ml 500mM EDTA	2 mM

store on ice

30 ml of 1X NPB:
15 ml H_20
15 ml 2X NPB
store on ice

100 μl of 10 mg/ml Digitonin (Sigma D-5628); dissolve in DMSO at room temperature immediately before use.

5 ml of 1X NPB + 0.3% BSA:
2.35 ml H_20
2.5 ml 2X NPB
0.15 ml 10 % BSA
store on ice

0.5 ml 1X NPB + 30% glycerol + 0.3% BSA (Sperm Storage Buffer)
85 ul H_20
250 ul 2X NPB
15 ul 10 % BSA
150 ul 100 % glycerol
store on ice

C. Sperm Nuclei Preparation.

The procedure is for nuclei preparation from one testis. Increase the solution volumes accordingly when process more testes.

1) Anaesthetize a male by immersion in 0.05% benzocaine, cut through the ventral body, remove the testes with dissecting scissors and place them in a 35mm tissue culture dish containing cold 1X MMR.

2) Rinse the testes in three changes of cold 1X MMR.
3) Using fine forceps, remove any remaining fat body and excess blood.

4) Place the cleaned testis in another 35mm tissue culture dish with 5 ml of cold 1X NPB for 2 to 5 minutes.

5) Transfer the testis to a dry 35mm tissue culture dish, and macerate the tissue well with a pair of clean forceps.

6) Resuspend the macerated testes in 2 mls of cold 1X NPB by pipetting the mixture up and down through a sterile, disposable 5 ml pipette.

7) Squirt the sperm suspension through two-four thicknesses of cheesecloth placed into a funnel and collect the solution into a 14 ml sterile culture tube. Rinse the dish with an additional 3 ml of cold 1XNPB and add to the cheesecloth. After adding 5 ml more (10 mls total) of cold 1XNPB use a gloved hand to fold the cheesecloth and squeeze any remaining liquid through the funnel into the 14 ml tube. We usually end up with 9 ml of sperm suspension in the tube.

8) Centrifuge the sperm suspension at 3,000 rpm for 10 min. at 4°C. During the spin, allow 1 ml of 1XNPB to equilibrate to room temperature.

9) Decant the supernatant and resuspend the sperm pellet in 9 ml of cold 1XNPB and repellet by centrifugation at 3,000 rpm, 10 min., 4°C. During this spin dissolve 1 mg of Digitonin in 100 ul (10mg/ml) of DMSO at room temperature.

10) Decant the supernatant and resuspend the sperm pellet in the 1 ml of 1XNPB that has equilibrated at room temperature and add 50μl of 10mg/ml Digitonin. Mix gently and incubate for 5 minutes at room temperature.

11) Add 5 ml cold 1XNPB + 0.3% BSA, mix gently by pipetting up and down, and centrifuge at 3,000 rpm, 10 min., 4°C.

12) Decant the supernatant and resuspend the pellet in 250μl of Sperm Storage Buffer and transfer suspension into a 1.5 ml eppendorf tube. Store at 4°C and use for transgenesis for up to 48 hours.

13) Cut the tip of a yellow tip and mix the sperm nuclei suspension by pipetting up and down. Remove 2 μl and dilute into 200 ul of sperm dilution buffer (i.e. 1:100 dilution). Add 2 μl of a 1:100 diluted Hoechst stock and transfer the diluted sperm nuclei to a hemacytometer for counting. Visualize the sperm nuclei under a fluorescence microscope using a DAPI/Hoechst filter set. For a 1:100 dilution of our sperm nuclei stock, we typically obtain counts of 125-200 ($X10^4$ nuclei/ml). At this concentration, the undiluted stock contains 125-200 nuclei/nl. We store the fresh nuclei overnight at 4°C and after extensive mixing by pipetting up and down with a cut yellow tip, freeze 10 μl aliquots at -20°C , and then transfer to -80°C for storage. One aliquot is thawed for each day of transgenesis.

2. Preparation of DNA, Needles and Equipment

A) Preparation of linearized DNA.
Digest DNA using standard conditions, run the DNA on an agarose gel, isolate the appropriate band and purify using the Geneclean II Kit by MP Biomedicals. Elute the DNA in H_2O at a final concentration of 150-250 ng/μl.

B) Preparation of nuclear transplantation needles.
30 μl Drummond micropipets (Drummond Scientific Company, cat. #: 1-000-0300) are used to make the needles. We use a Sutter Instrument Co Puller (Model P-80/PC) for pulling our needles with a setting of Heat=805;Pull=47;Ver.=95;Time=5. Clip the needle with forceps to produce a tip of diameter of around 70 μm, and then beveled to 80-100um with a 45 degree angle (using a Narishiga Beveller, model EG-44).

C) Agarose-coated injection dishes
Pour 1% agarose into around 10 60mm petri dishes. Before the agarose solidifies, place small weigh boats on the agarose so that as the agarose solidifies, a square depression in the agarose remains. The depression will accommodate ~500 eggs. Wrap the dishes and store at 4°C until use. 1% agarose dishes without this weigh boats work as well.

D) Transplantation Apparatus
We use a Harvard Apparatus infusion syringe (5ml) pump (Model 22; Cat.# 55-2222) to introduce sperm nuclei into eggs, with flow speed set to 40 μl /hour.

3. Transgenesis by sperm nuclear transplantation into unfertilized eggs

1) The night before eggs are needed for transplantations, inject adult female frogs in the dorsal lymph sac with 500-800U/each frog HCG and incubate at 18°C for 10-12 hours.

2) Remove a 1ml aliquot of Sperm Dilution Buffer (SDB) from the freezer and allow it to warm to room temperature.

3) Make up 100ml of 2.5% Cysteine in 1X MMR, adjust pH to 8.0 (with 4ml 5N NaOH).

4) Fill agarose coated injection dishes with 0.4X MMR + 6% Ficoll.

5) Set up a reaction:
2 μl sperm stock (~2 X 10^5 nuclei)
2μl linearized plasmid (100 ng/μl)
Incubate 5min at room temperature.
Add 0.5ul of 1:30 diluted enzyme (in water) to 11μl SDB, mix and add it to the sperm/DNA mixture at the end of the 5min incubation. Mix the reaction by gentle pipetting (using a clipped yellow tip). Incubate 15 min. at room temperature.

6) While sperm are swelling in reaction mixture, collect eggs from the frogs and dejelly them in the 2.5% cysteine prepared in step 3. Wash the eggs thoroughly in 1X MMR. Transfer dejellied eggs into agarose coated dishes containing 0.4X MMR with 6% ficoll. After about 5 minutes in 0.4X MMR + 6% Ficoll the eggs will pierce easily.

7) After the 15 minute incubation, mix the sperm nuclei gently by pipetting up and down with a cut of yellow tip. Then transfer 5 μl of mixture into 150 μl of SDB that is already at room temperature, allow the sperm nuclei to slowing equilibrate with the SDB over the span of a few minutes.

8) Using a piece of Tygon tubing attached to a yellow tip, draw up about 25ul of the dilute sperm suspension, and backfill the need. Detach the needle and attach it to the tygon tubing filled with mineral oil that is connected to the Harvard Apparatus infusion pump.

9) Transplant sperm nuclei into unfertilized eggs.
Start the flow in the infusion pump and begin injecting eggs, keeping the needle inside each egg for approximately one second. Move the needle fairly rapidly from egg to egg, piercing the plasma membrane of each egg with a single, sharp motion then drawing the needle out more slowly.

10) When the embryos have reached the 4-cell stage, gently separate them from uncleaved eggs and transfer them to a separate agarose-coated dish of 0.1X MMR + 6%Ficoll +50μg/ml gentamycin. When embryos are around stage 12, media is replaced with 0.1X MMR + 50μg/ml gentamycin without Ficoll, until the stage desired for collection.

4. References

Kroll, K.K. and Amaya, E. (1996) Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 122:3173-3183.
Murray, A.W. (1991). Cell cycle extracts. In Methods in Cell Biology, (B. K. Kay, and H. B. Peng), ed., Vol. 36, pp. 581-605. San Diego: Academic Press, Inc.