RNA injection protocol for *Xenopus tropicalis*

09/18/2018

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EQUIPMENT

Pressure microinjector

- HARVARD APPARATUS PLI-100 Pico-Injector
- NARISHIGE IM-300 Microinjector
- NARISHIGE IM-31 Microinjector

IM-31 is the cheapest injector, but appears to be sufficient for RNA injection.

Puller

- NARISHIGE PN-31

Manipulator

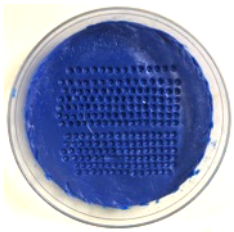
- NARISHIGE M-152
- NARISHIGE MN-151
- NARISHIGE YOU-1

Choose a favorite one.

Injection dish

- Non-toxic Modeling Clay (Van Aken Plastalina)
- 6 cm dish (VALMARK 2901)

Put a layer of the modeling clay in the bottom of a petri dish. Make grooves or holes on its surface to hold fertilized eggs.

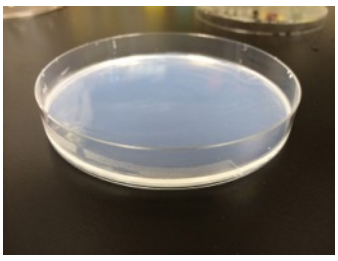


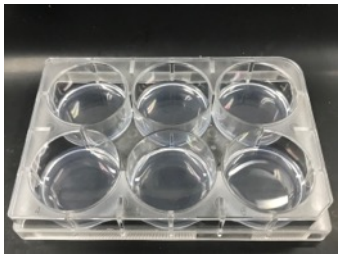
Culture dish

- 6-well dish or a petri dish (100 mm × 15 mm)
- 0.7 – 1 % Agarose in 0.1 x MMR (pH7.5)

Coat the dish bottom with agarose to prevent sticking of embryos.

This agarose-coating is not necessary if 0.1% BSA is included in the culture medium.





Injection needles

- Borosilicate Glass Capillaries (World Precision Instruments (WPI), TW100-4)
- NARISHIGE PN-31 Puller

Puller setting condition

Heater	80
Magnet sub	30
Magnet main	90-100

Injection needle calibration

- Cut the needle tip using a sterile forceps.
- Fill 1 µl of RNase-free water into the needle.
- Set the injection time and pressure (1 second and 10 psi).
- Eject all of the RNase-free water. If it takes about 40 – 60 seconds, you can use that needle for injection into *X. tropicalis* embryos.

Plastic pestle for 1.5 ml microcentrifuge tube (Kontes, 749521-1590)

Air incubator (temperature should be adjustable between 12 –25◦C)

ANIMALS

Researchers can purchase *X. tropicalis* from the "National Bioresource Project of Japan" (http://home.hiroshima-u.ac.jp/amphibia/xenobiores_en/iweb_en/Top.html). The four inbred wild-type strains, Nigerian A, Golden, Nigerian H, and Ivory Coast, are available. The Golden strain is very tough and appear to be most suitable for routine injection experiments

REAGENTS

10 x MMR (1 L)		from stock solutions		in grams	
1 M	NaCl	250 ml	4 M NaCl	58.44 g	NaCl
20 mM	KCl	20 ml	1 M KCl	1.49 g	KCl
10 mM	$MgCl_2$	10 ml	1 M $MgCl_2$	2.03 g	$MgCl_2 \bullet 6H_2O$
50 mM	HEPES	50 ml	1 M HEPES	11.92 g	HEPES
20 mM	$CaCl_2$	20 ml	1 M $CaCl_2$	2.94 g	$CaCl_2 \bullet 2H_2O$

Add distilled water to 1 Liter. Adjust pH to 7.5 by adding approximately 4 ml of 5M NaOH (Nacalai 95539-85). Sterilize by autoclave or filtration if made from dry stocks. Otherwise just combine sterile solutions + sterile H_2O in a sterile bottle. Store at RT.

0.1 x MMR (pH7.5)

- 990 ml Distilled water
- 10 ml 10 x MMR

Re-adjust the pH to 7.5 by adding about 10 ul of 5M NaOH.

0.1 x MMR (pH7.5) + Gentamicin（embryo culture medium）

- 990 ml Distilled water
- 10 ml 10 x MMR
- 1 ml 50 mg/ml Gentamicin Sulfate (Biowest P4020-5GR)
- 100 µl 0.5 % Phenol Red solution (Sigma P0290)

Re-adjust the pH to 7.5 by adding about 20 ul of 5M NaOH (gentamicin decreases the pH).

Dejellying solution (2% L - cysteine in 0.1 x MMR, pH 8.0)

Prepare freshly on the day of injection.

- 50 ml 0.1 x MMR
- 1 g L – cysteine (Wako 326-0615)

Adjust the pH to 8.0 by adding 600 – 700 µl of 5 M NaOH. Such higher pH is crucial for dejellying.

Injection medium (6% Ficoll 400 and 0.1% BSA in 0.1 x MMR, pH 7.5)

- 500 ml 0.1 x MMR, pH7.5
- 30 g Ficoll 400 (GE Healthcare 17-0300-50)
- 5 ml 10% BSA (pH7.5)

Dissolve Ficoll 400 powder slowly (gently swirl one overnight).

The pH may need to be re-adjusted to 7.5.

Make 50 ml aliquots, and store at -20°C.

Anesthetic solution (0.2% MS222 in 0.1 x MMR)

- 990 ml Distilled water
- 10 ml 10 x MMR
- 2 g MS222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma E10521)

Re-adjust the pH if necessary.

Testis storage buffer

Leibovitz L15 medium is convenient. One may keep *X. tropicalis* testes for up to two days at 14°C.

Preparation of the 190% Steinberg solution takes time, but it may be efficient for longer storage.

1. Leibovitz L15 medium (Sigma L4336)

   Follow the manufactures instructions for preparation.

   Supplementing with gentamycin (Final 50 ug / ml) may be effective for longer storage of the testes.

2. 190% Steinberg solution (pH 7.8)

   - 110 mM NaCl
   - 1.29 mM KCl
   - 0.64 mM $Ca(NO_3)_2 \cdot 4H_20$
   - 1.58 mM $MgSO_4 \cdot 7H_2O$
   - 8.78 mM Tris-base

   Add gentamycin (Final 50 ug / ml) and 1/10,000 vol. of 0.5 % Phenol Red solution.

   Adjust the pH to 7.8, then sterilize by filtration. Store at 4°C.

10% BSA (pH7.5)

- 50g BSA (Fraction V, Roche 735094)
- 400 ml Distilled water

Adjust the pH to 7.5, fill up to 500 ml.

Sterilize by filtration, make 50 ml aliquots, and store at -20°C.

Testis homogenizing buffer (0.1% BSA in 1 x MMR)

- 45 ml Distilled water
- 5 ml 10 x MMR
- 0.5 ml 10% BSA

Make 1 ml aliquots, and store at -20°C.

Capped RNAs for injection

One may prepare Capped RNAs by in vitro transcription using a mMESSAGE mMACHINE kit (Thermo Fisher Scientific) or equivalent kits/methods. For details of the RNA preparation method, please see published protocols (Early Development of *Xenopus laevis*, A Laboratory Manual, CSHL press, ISBN 978-087969942-0). The plasmid template may be constructed by introducing your experimental cDNA into the MCS of pCS2+ vector, in which the MCS is flanked by a SP6 promoter and a SV40 poly-A signal sequence (Rupp, R.A., et al., Genes Dev. 8, 1311–1323, 1994).

HCG (Human chorionic gonadotropin) (Aska Pharmaceutical 45254000, or equivalent)

PROCEDURE

1 In the afternoon of the day before the RNA injection, prime *X. tropicalis* females about 20 hr prior to egg laying with 15 units of HCG (15 units/100 ul/frog), and keep them at 25°C in holding tanks.

2 On the morning of the day of RNA injection, boost the females with 100 units of HCG approximately 4 hr prior to the desired time of egg laying. Equilibrate necessary reagents and culture dishes at room temperature (22-25°C).

3 Euthanize *X. tropicalis* males to isolate testes. Pre-warm the anesthetic solution to room temperature, and submerge the males in this solution for between 30 minutes and 1 hour. After euthanasia, dissect out the testes and transfer them to Leibobitz L-15 medium or 190% Steinberg solution. For details of the testis dissection, one should follow standard procedures as described(Early Development of *Xenopus laevis*, A Laboratory Manual, CSHL press, ISBN 978-087969942-0).

CAUTION

Please follow animal protocols of your institute for frog handling.

4 When the female frogs start laying eggs in their containers, start *in vitro* fertilization. Homogenize a testis with 0.5-0.75 ml of the testis homogenizing buffer in a 1.5 ml microcentrifuge tube using a plastic pestle. Gently squeeze 500-600 eggs out of the female in a dry Petri dish, remove all water from the eggs, and then mix them with a part of homogenized testis using a wide-pore pipet. After two minutes, flood eggs with 50 ml of 0.1×MMR (room temperature) in the Petri dish and further incubate them at room temperature.

6 If the fertilization is successful, contraction of the pigmented animal cap occurs after 10-15 minutes. Thirty minutes after the addition of homogenized testis, replace the flooded 0.1×MMR with 50-100 ml of the dejellying solution and swirl eggs until their jelly coat is completely removed (2-5 minutes). After dejellying, wash the dejellied eggs several times with 50-100 ml of 0.1×MMR.

7 Fill injection dishes with the injection medium, and lay the required number of fertilized eggs in the grooves or holes on the dish bottom.

8 Load 1-2 ul of the capped RNA solution into an injection needle. At 1-cell stage, one can inject up to 4 nl per embryo. At 2-cell stage, one can inject up to 2 nl per one blastomere of an embryo. For details

of the microinjection itself, one should follow standard *Xenopus* procedures as described(Early Development of *Xenopus laevis*, A Laboratory Manual, CSHL press, ISBN 978-087969942-0).

9 After the injection, incubate the embryos at 22-25 °C in the injection medium for about 2 hours to let healing.

10 Transfer embryos from the injection dish to Petri dishes filled with 0.1 x MMR (pH7.5) containing gentamycin, and further incubate them at 22-25 °C. Do not place too many embryos in the same dish (up to 100 embryos per Petri dish (100 mm × 15 mm)).

11 Next morning, the embryos will reach stages 20-25. To keep their development, change the culture medium every day, and remove dead embryos.