Chapter 2

Generation and Care of *Xenopus laevis* and *Xenopus tropicalis* Embryos

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Abstract

Robust and efficient protocols for fertilization and early embryo care of *Xenopus laevis* and *Xenopus tropicalis* are essential for experimental success, as well as maintenance and propagation of precious animal stocks. The rapid growth of the National *Xenopus* Resource has required effective implementation and optimization of these protocols. Here, we discuss the procedures used at the National *Xenopus* Resource, which we found helpful for generation and early upkeep of *Xenopus* embryos and tadpoles.

Key words *Xenopus laevis*, *Xenopus tropicalis*, In vitro fertilization, hCG, oLH

1 Introduction

The strengths of *Xenopus laevis* and *Xenopus tropicalis* as experimental model systems include the ability to produce a large number of eggs year-round using injection of gonadotropic hormones to induce ovulation and the entirely external synchronous, stereotypical development following fertilization [1–4]. The fertilization protocols are straightforward and robust, and can be performed via in vitro fertilization (IVF), when a large number of synchronously developing siblings are required, or through natural mating if the parental male being used is precious and needs to be preserved for further experiments. During the first 2 weeks following fertilization, careful attention to culture density and cleanliness as well as frequency and amount of food provided is necessary to assure normal development and to promote animal survival.

The frog colony at the National *Xenopus* Resource (NXR), a national repository and distribution center for a large number of different *Xenopus* stocks, has grown in size from zero animals in 2010 to a current combined total of over 8000 *X. laevis* and *X. tropicalis*. This rapid growth has required us to optimize protocols necessary for efficient and rapid embryo production. Since many of the animals we stock have been derived from external sources, we have

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incorporated procedures for embryo surface sterilization into our standard operating procedures to limit the potential of introducing pathogens into our colony. In this chapter, we present the techniques we use for efficient embryo generation in *X. laevis* and *X. tropicalis*, embryo care during the first 2 weeks following fertilization, and the embryo surface sterilization protocol.

2 Materials

2.1 Animals

Wild type outbred *X. laevis* and *X. tropicalis* can be purchased from a variety of commercial vendors, including Nasco, *Xenopus* One, and *Xenopus* Express. These companies are an excellent resource for obtaining large quantities of outbred *Xenopus* and they work closely with each researcher to provide animals at all stages. More specialized strains and lines of *Xenopus*, such as the inbred *X. laevis* J strain and the Nigerian *X. tropicalis* strain, are available for purchase through regional stock centers, including the National *Xenopus* Resource (NXR, RRID:SCR_013731, http://www.mbl.edu/xenopus), the European *Xenopus* Resource Centre (EXRC, RRID:SCR_007164, https://xenopusresource.org), the *Xenopus laevis* Research Resource for Immunobiology (XLRRI, https://www.urmc.rochester.edu/microbiology-immunology/xenopus-laevis.aspx) and the *Xenopus tropicalis* National BioResource Project of Japan (http://home.hiroshima-u.ac.jp/amphibia/xenobiores_en/iweb_en/Top.html). In addition, these stock centers distribute transgenic and mutant lines for both *X. laevis* and *X. tropicalis*. Detailed information about the various suppliers, including contact information, can be found online at Xenbase (RRID:SCR_003280, http://www.xenbase.org/other/obtain.do).

2.2 Food

1. Sera Micron Growth Food (sera North America, Montgomeryville, PA, USA).

2.3 Hormones

1. Pregnant Mare Serum Gonadotropin (PMSG) (e.g., BioVendor, Asheville, NC, USA): make a stock of 100 U/mL in 1× PBS and store at −20 °C.
2. Ovine Luteinizing Hormone (oLH) (e.g., National Hormone and Peptide Program, Los Angeles, CA, USA): make stock of 0.4 mg/mL in 1× PBS and store at −20 °C.
3. Human Chorionic Gonadotropin (hCG) (available from several sources including: National Hormone and Peptide Program, Los Angeles, CA, USA; Sigma-Aldrich, St. Louis, MO, USA; Chorulon brand, Merck Animal Health, Madison, NJ, USA): make a stock of 1000 U/mL in 1× PBS.

2.4 Anesthesia and Euthanasia Reagents

1. Ethyl 4-aminobenzoate, 98%; aka. Benzocaine (CAS# 94-09-7): make 10% stock in 95% Ethanol and store at room temperature.
2. Tricaine Methanesulfonate; aka. Tricaine-S, aka. MS 222 (CAS# 886-86-2).

2.5 Embryo and Tadpole Culture Solutions

1. Marc's Modified Ringer's Solution (MMR).
   Make 10× stock: 1 M NaCl, 20 mM KCl, 10 mM $MgSO_4 \cdot 7H_2O$, 20 mM $CaCl_2 \cdot 2H_2O$, 50 mM HEPES free acid; adjust the pH to 7.4–7.8 with NaOH; autoclave or filter sterilize (*see* **Note 1**).
   Dilute the 10× MMR stock with autoclaved ultrapure water to make culture solutions of required concentration. No additional pH adjustment is necessary (*see* **Note 2**).
2. Modified Barth's Saline (MBS).
   Make 10× stock: 800 mM NaCl, 10 mM KCl, 10 mM $MgSO_4 \cdot 7H_2O$, 50 mM HEPES free acid (*see* **Note 3**), 25 mM $NaHCO_3$; adjust the pH to 7.8 with NaOH; autoclave or filter sterilize.
   Make 0.1 M stock of $CaCl_2$.
   To make culture solutions dilute 10× MBS stock with autoclaved ultrapure water and add $CaCl_2$ stock. The final concentration of $CaCl_2$ in 1× MBS should be 0.7 mM.
3. Gentamicin Reagent Solution, 10 mg/mL in distilled water (e.g., ThermoFisher Scientific, Waltham, MA, USA).
   Where required use the 10 mg/mL solution as a 1000× stock (*see* **Note 4**).
4. L-Cysteine free base (CAS# 52-90-4) dejelly solution (*see* **Notes 5** and **6**).
   Make a 2% for *X. laevis* or 2.5% for *X. tropicalis* solution of L-Cysteine in 0.1× MMR, adjust the pH to 7.8–8.0 using NaOH.
5. Thimerosal (CAS# 54-64-8).
   Make minimum of 100 mL solution. Add 0.01 g thimerosal to 100 mL 0.1× MMR. Shake to dissolve. Store at 4 °C up to 7 days.
6. Phosphate Buffered Saline (PBS) tablets, 100 g.
   Make 1× PBS by dissolving the tablets in ultrapure water. Autoclave to sterilize. Use to resuspend hormones.

2.6 Egg Laying Solutions

1. 1× MMR works well as egg laying solution for *X. laevis*. Two alternative egg laying solutions are listed below.
2. 10× egg laying solution stock: 1.20 M NaCl, 24 mM KCl, 24 mM $CaCl_2 \cdot 2H_2O$, 12 mM $MgCl_2 \cdot 6H_2O$, 18 mM HEPES free acid; adjust the pH to 7.6 with NaOH; autoclave or filter sterilize.

Use autoclaved ultrapure water to dilute when making 1× egg laying solution.

3. 8× RAL's Ca_{2+} free High Salt Egg Laying Buffer for *X. laevis*: 880 mM NaCl, 120 mM Tris base, 16 mM KCl, 4 mM Na_2HPO_4, 16 mM $NaHCO_3$; adjust pH to 7.6 with glacial acetic acid; add 8 mM $MgSO_4{\cdot}7H_2O$.

   Use autoclaved ultrapure water to dilute when making 1× egg laying solution. Use fresh made 1× solution (*see* **Note 7**).

3 Methods

3.1 Generating Embryos

Both *X. laevis* and *X. tropicalis* are capable of generating well over a 1000 embryos per mating [2]. In both, injection of mammalian gonadotropins is used to stimulate ovulation and the male mating behavior, with the main difference between the two species being the amount of the hormone used. The experimental goals as well as the potential rarity of the parental male line used will influence whether the embryos are generated via a natural mating or through IVF. Finally, when generating embryos from parental individuals that have been kept in quarantine and might have an uncertain health record, cleavage stages are a good developmental period for producing a clean population of animals by performing a surface sterilization protocol.

3.2 Sexing Adults

Several sexually dimorphic phenotypic traits aid in distinguishing males and females. Same traits can be used for sexing both *X. laevis* and *X. tropicalis* (*see* **Note 8**).

1. Body size and shape is sufficient in most cases to distinguish male from female. In sexually mature individuals of the same age females are larger than males. Females have a pear-shaped body with large adult individuals showing a pair of distinct lateral ridges parallel to the anteroposterior body axis in the dorsal posterior region of the body.
2. The females have prominent cloacal labia. During mating these become reddish and engorged.
3. Sexually mature males have keratinized nuptial pads on the ventral surface of the forearm which they use to clasp onto the females back during amplexus. These pads tend to be darker in color than the surrounding epidermis. In individuals in which the color difference is not very prominent, running a finger along the ventral surface of the forearm can be used as an additional identification method; on male frogs this region of the skin will have a rough texture.

3.3 Gonadotropic Hormone Injection to Promote Ovulation in Females

A number of mammalian gonadotropic hormones have been identified as effective in inducing ovulation in females and mating behavior in males. These are generally injected subcutaneously into the dorsal lymph sacs of the animal, using a 27G–30G needle. Typically, females will be given two injections with the initial priming injection followed by a later boosting injection that induces ovulation (*see* **Note 9**). Boosting males seems to be of particular importance during natural mating as it seems to increase the likelihood of amplexus. Historically, human chorionic gonadotropin (hCG) has been used for boosting injections, but recent work has shown that both human and ovine luteinizing hormones can be used as well [2].

1. In large adult wild type *X. laevis* females, first prime them with 50 U of PMSG or 50 U of hCG between 24 h to 7 days before the boosting injection (priming is not essential for induction of ovulation). To induce ovulation boost them with 2 μg of oLH per 1 g of body mass (~200 μg of oLH) or with 500 U of hCG; some larger frogs may require greater amounts of hCG.
2. Inbred J strain *X. laevis* females are smaller than wild type and require lesser amounts of hormone. Prime them with 30 U of PMSG or 30 U of hCG and boost with 140 μg of oLH or 350 U of hCG.
3. *X. laevis* females kept at 18 °C should begin ovulating approximately 8–9 h following the boosting shot. Ovulation can be delayed by keeping the females at a lower temperature (*see* **Note 10**).
4. For *X. tropicalis,* prime the females with 20 U of PMSG or 10 U of hCG between 12 h to 2 days before the boosting injection. Boost the females with 5 μg of oLH per 1 g of body mass (~80 μg of oLH) or with 200 U of hCG.
5. *X. tropicalis* females kept at 25 °C will begin ovulating 3–5 h after the boosting injection.
6. Between ovulations females should be allowed to rest to let their oocytes mature. *X. laevis* require 3 months of rest and *X. tropicalis* require 2 months.
7. Frogs kept on a regular ovulation and rest schedule tend to produce high quality eggs.
8. Recently some labs have reported successful use of superovulation in *X. laevis* females to increase the number of eggs extracted from a single female. To do this, following ovulation let the female rest 1 week before giving it a priming hormone injection and then a boosting hormone injection 2 days later. After the second ovulation have the female rest 3 months.
9. After ovulation, the females should be transferred to a clean container with system water and allowed to recover for 24 h before being returned back into the system.

3.4 In Vitro Fertilization (IVF)

IVF allows generation of large numbers of simultaneously fertilized embryos, which is particularly useful for a number of experimental designs. The main drawback is that it requires the parental male to be euthanized which may be problematic if the male comes from a stock that is limited in number and will be used again (*see* **Note 11**).

1. The male needs to be euthanized prior to extraction of the testes. Put it in a bath of 0.15% benzocaine in frog water, or 0.5% of MS222 in water buffered to pH 7.0 with $NaHCO_3$. After 15–30 min check that the male is unresponsive to foot pinching and does not have a swallowing reflex when its throat is rubbed. Use scissors to perform cervical section and sever the spine immediately posterior to the skull as a secondary euthanasia method on the insensate male. Using dissection scissors cut through the skin and the abdominal muscle to open the lower abdominal cavity on the ventral side. The testes are found on the dorsal side of the cavity, on either side of the aorta. It may be necessary to move the fat bodies out of the way to make the testes visible. Cut the testes out and trim away any attached fat and viscera. Store the testes in 1× MMR with 10 μg/mL of gentamicin added to prevent the sperm from activating and limit growth of contaminants.
2. If stored at 4 °C, *X. laevis* testes will maintain their viability for at least a week.
3. *X. tropicalis* testes will remain viable for at least 2 days if stored at 25 °C–27 °C.
4. IMPORTANT: it is extremely important that the *X. tropicalis* testes are never kept on ice or at 4 °C. Even brief exposures to low temperatures cause *X. tropicalis* sperm to lose activity.
5. To collect eggs, the females need to be either squeezed to physically entice them to ovulate directly into an empty collection dish, or they can be placed into an egg laying bath of high salt and the eggs collected over time. Eggs ovulated directly into frog water lose viability shortly after having been laid and should not be used.
6. Eggs can be acquired from both *X. laevis* and *X. tropicalis* by squeezing. To do this grab the female with the dominant hand (the description given assumes the right hand as dominant, reverse sides if using left hand). The back of the female should face the palm of the hand with the head directed toward the wrist and the index finger between the legs. The index finger should be on the left side of the cloaca, with the left leg squeezed between the index and the middle fingers and pushed as close to the body as possible. The little, ring, and middle fingers wrap around the left side of the animal with the tips of the fingers resting along the ventral midline of the animal. The

thumb goes around the right side. Use the left hand to restrain the right leg, pulling it away from the cloaca along the right side of the animal. It may be necessary to let the animal relax before being able to cause a maximal leg extension. With legs braced against the body wall the frog is essentially immobilized in a joint lock, unable to get away. While holding the female, orient her vertically, with the cloaca over a dish to collect the oocytes, this will help prevent the eggs from running along the body of the female instead of directly into the collection dish. In *X. tropicalis* the female flexing against this hold is typically sufficient for egg expulsion through the cloaca. If that is not sufficient or if handling *X. laevis* it may be necessary to apply pressure to squeeze the eggs out. Use the right hand ring and middle finger tips to apply pressure to the belly with the thumb firmly around the left side. The thumb of the left hand should apply the pressure to the back of the animal. Shift the pressure in an anterior toward posterior direction as if squeezing paste out of a tube. Keep both index fingers as far from the cloaca as possible to prevent the eggs from collecting on fingers instead.

7. Egg laying baths (1× MMR or equivalent) work well with *X. laevis*. Frogs are kept in a bath at 18 °C–22 °C. The eggs are collected every 30–60 min and fertilized. Egg laying baths keep the eggs viable for at least 1 h following ovulation. Recipes for two alternative egg laying solutions are given in Subheading 2.6.
8. We have not heard of anyone using egg laying baths to collect eggs from *X. tropicalis*, and although the females can be comfortably kept in 1× MMR for at least 2 days we are not sure how long the eggs laid into 1× MMR would remain viable (*see* **Note 12**).
9. Fertilization of *X. tropicalis* eggs is relatively straightforward. Collect eggs into an empty petri dish. If any frog water dripped into the dish, remove as much of it as possible. Using a plastic transfer pipette try to spread the eggs into a monolayer. Place a half to a whole testis in a 1.5 mL microcentrifuge tube with 0.5 mL of 1× MMR. Use a plastic microcentrifuge tube pestle to crush the testis tissue in the tube and release the sperm. Using a transfer pipette apply the testis solution to the eggs and mix it in. Let it sit for 3 min, flood the dish with 0.1× MMR, and use a transfer pipette to mix again.
10. To fertilize *X. laevis* eggs collect them into an empty petri dish. Remove as much frog water or egg laying solution as possible. Put an eighth to a quarter of a testis into a 1.5 mL microcentrifuge tube with 1 mL of 1× MMR and use a plastic microcentrifuge pestle to crush it and release sperm. Using a transfer pipette add the sperm solution to the eggs and mix. Let it sit

for 5 min, then flood the dish with 0.1× MMR, and mix again with a transfer pipette. Alternatively, the testes piece can be placed in a glass dish and macerated using forceps; add a small amount of 0.1× MMR (5–10 mL) to the dish and then use a pipette and transfer the larger chunks into a 1.5 mL microcentrifuge tube; use a plastic microcentrifuge pestle to crush the larger pieces and release more sperm. Transfer the solution to the glass dish with the original macerated testes and add this solution to the laid eggs; let it sit for 5 min then flood with additional 0.1× MMR (*see* **Note 13**).

11. For smaller cultures, it may be fine to let the embryos develop in the jelly coat. However, removing the jelly helps maintain the cleanliness of the cultures and is absolutely necessary for microinjection, microsurgery, or small molecule treatments. To dejelly the embryos, make a 2% (*X. laevis*) or 2.5% (*X. tropicalis*) solution of L-Cysteine in 0.1× MMR, with the pH adjusted to 7.8–8.0 using NaOH. As early as 15 min after the embryos have been flooded with 0.1× MMR, decant all the 0.1× MMR from the dish and flood the dish with the L-Cysteine dejelly solution. Let it sit at room temperature occasionally gently moving the embryos around with a transfer pipette or by gently swirling the dish. Five to ten minutes after adding the dejelly solution they should be able to clump together, indicating dissolution of the jelly coat. Pipet or pour out as much of the cysteine as possible then wash with 0.1× MMR three to five times. These steps can be performed in the same petri dish or alternatively following the dissolution of the jelly coat the culture can be poured into a beaker and then gently washed with the 0.1× MMR. Following the final wash transfer the embryos to a clean petri dish with 0.1× MMR and 10 μg/mL gentamicin.
12. *X. tropicalis* embryos need a little bit extra care after removing the jelly coat. For the first 2 days following the fertilization or at least until they have hatched out of the vitelline envelopes and are sporadically swimming they should be kept in a dish coated with 2% noble agar (or agarose) in 0.05× MMR, or coated with 0.1% sterile BSA, or kept in 0.05× MMR with added BSA for a final concentration of 0.1%. *X. laevis* do not require this and can be kept in uncoated dishes without any BSA.
13. Adding dejelly solution less than 15 min after flooding with 0.1× MMR will result in abnormal development. Waiting 15 min is usually sufficient but the washes need to be done gently. Waiting 20 min may be better to assure maximal normal development.

14. Dejelly solution should be made fresh, as it will lose potency over time.
15. Embryos can tolerate a range of temperatures during early development with lower temperatures resulting in slower development. *X. laevis* can be kept at 15° C–23° C and *X. tropicalis* can be kept at 23° C–29° C. Middle of the range assures most consistently normal development especially during the more sensitive stages of gastrulation and neurulation and we recommend culturing *X. laevis* at 18°C and *X. tropicalis* at 23°C.

3.5 Natural Mating

When dealing with males that are from a limited and precious stock it may be prudent to generate embryos via natural mating. The main advantage is that the male is not sacrificed in the process and can be used repeatedly. The drawback is that the embryos produced this way will not be fertilized simultaneously and some sorting for embryos of a particular stage may be necessary depending on the experimental purpose they are being used for.

1. To induce the male mating behavior, inject the male with priming and boosting shots following that same schedule as for the female and outlined in Subheading 3.1, **step 2**. For the boosting injection use half the amount of hormone when injecting the male as that specified for the female.
2. After the boosting shot, put male and female together in a tank with system water. We generally use mouse breeding cages as these are easy to handle and make collecting embryos simple. Put the mouse cage in the dark (either covered on a bench top or in an incubator with no lights).
3. After approximately 4 h for *X. tropicalis* or 9 h for *X. laevis* it should be fine to begin checking on whether the frogs are in amplexus and if there are any eggs or embryos in the tank. Checking too early or too often can spook the animals preventing the male from going into amplexus or causing it to release.
4. Depending on the experimental design embryos can be collected at regular intervals as frequent as 30 min or the animals may be allowed to mate undisturbed for an extended period of time and all the embryos collected together at the end of the procedure.
5. If using these embryos for microinjection it may be useful to work in a pair with one person collecting, dejellying, sorting, and staging the embryos while the other one is solely dedicated to doing the injections.
6. After the mating is complete, the frogs should be transferred to a clean container with system water and allowed to recover for 24 h before being returned back to the system. Males and

females should be kept separate after mating is completed to avoid prolonged amplexus.

3.6 Surface Sterilization

When breeding quarantined animals in order to bring the new generation into the clean facility the following embryo surface sterilization protocol can be followed to limit the likelihood of introducing any pathogens into the colony. This protocol was originally devised for isolation of *Xenopus* neurons and muscle cells but has been adopted for use with embryo culture (Marilyn Fisher and Robert Grainger personal communication) [5]. The same procedure can be used for both *X. laevis* and *X. tropicalis*.

1. Before starting the procedure make the following solutions.
   (a) 70% Ethanol in 0.1× MMR.
   (b) 2.5% L-Cysteine free base.
   (c) 0.1 mg/mL thimerosal in 0.1× MMR.
   (d) 0.1% BSA in 0.1× MMR.
   (e) Coat five 10 cm petri dishes with 0.1% BSA per fertilization.
   (f) 1 L 0.1× MMR per fertilization.
2. The embryos can be generated either through natural mating or via IVF, however if any "clean" animals are required, for example males to provide the testes, they should be handled and processed first before handling any of the quarantined animals.
3. Begin the surface sterilization protocol before the onset of gastrulation to assure no pathogens present on the surface of the embryo are internalized, and in an isolated area to limit spread of any contaminants.
4. Change gloves and pipettes at each step.
5. Collect the embryos in a petri dish and drain any remaining culture solution. If they are clumped in a mass try to spread it out as much as possible without damaging the embryos.
6. Add 70% Ethanol solution and incubate for 10 s.
7. Remove 70% Ethanol and rinse three times with 0.1× MMR.
8. Dejelly to completion with 2.5% L-Cysteine, approximately 5–10 min at room temperature.
9. Remove the cysteine and wash embryos 8–10 times with 0.1× MMR. After the final wash place the dejellied embryos into a clean 0.1× MMR filled BSA coated petri dish.
10. Sort out any unfertilized eggs and dead embryos.
11. Transfer the clean embryos with as little fluid as possible to a BSA coated petri dish filled with thimerosal solution.

12. Incubate for 15 min with gentle agitation.
13. While the embryos are incubating fill the remaining three BSA coated dishes with 0.1× MMR with BSA and coat 3 transfer pipettes with BSA.
14. After the 15 min incubation is complete use a transfer pipette to move the embryos with as little fluid as possible into first of the three dishes. Change pipette and gloves and repeat two more times to move the embryos to the second and then the third dish.
15. Once the embryos are in the third dish they are considered sterile and can be grown in an incubator in the clean facility.
16. Sterilize all the surfaces used with 10% bleach and then 70% ethanol.
17. Following the protocol do not handle any clean animals or interact with any clean systems without showering and having a complete change of clothes first.

3.7 Embryo and Early Tadpole Care

Embryos and early tadpoles require attention and care. Following the guidelines listed here will help assure normal and healthy development.

1. Dejellied embryos should be kept in 0.1× MMR with 10 μg/mL gentamicin until they begin to feed after stage 45. At that point, they should be switched to gentamicin free 0.1× MMR.
2. Keep *X. tropicalis* embryos in 0.05× MMR with gentamicin, instead of 0.1× MMR, for the first day following fertilization.
3. Crowded cultures lose synchronous development over time and have a higher chance of showing abnormal development and death. We recommend keeping not more than 100 embryos younger than stage 35/36 and not more than 50 embryos stage 35/36 and older per 10 cm petri dish for *X. laevis* and *X. tropicalis*. As the embryos grow it may be necessary to transfer them to even larger plates, beakers, or flasks.
4. Cultures need to be checked every day. Dead or abnormal embryos should be removed; failure to remove the dead embryos may adversely affect the growth of other embryos. For the first 2 days of development if the cultures are particularly dirty it may be necessary to transfer all the healthy embryos to a clean dish with fresh 0.1× MMR. After that, once the embryos hatch, simply replacing the 0.1× MMR may be enough to keep the culture growing.
5. For the first 2 days following fertilization, or essentially until they begin swimming, *X. tropicalis* embryos need to be kept in dishes coated with 2% Noble agar (or agarose) in 0.1× MMR, or 0.1% BSA, or in 0.1× MMR with BSA added to a final concentration of 0.1%.

6. Begin feeding tadpoles after stage 45 by mixing Sera Micron into the culture medium [6]. At this point it may be useful to transfer the embryos into a larger container, like a beaker or a 15 cm petri dish. Once the embryos are in the beaker either add fresh 0.1× MMR to the beaker every day or do a 50% volume exchange. Use a pipette to clean out food and excretion solids accumulated on the bottom.
7. At 2 weeks post fertilization transfer the tadpoles to a nursery tank and follow the feeding directions outlined in the accompanying chapter 1.

4 Notes

1. Original formulation of 10× MMR also includes 10 mM EDTA. We omit it from our stock with no noticeable effect.
2. The pH of MMR will likely change following dilution of the 10× stock with ultrapure water, for example in our lab 0.1× MMR will usually have pH 6.95–7.00 when made from 10× MMR pH 7.4. Readjusting the pH of the diluted MMR is not necessary and we typically do not even trouble with checking it following dilution.
3. When making 1× MBS, HEPES sodium salt can be used instead of HEPES free acid.
4. 10 μg/mL of gentamicin in an embryo culture solution is sufficient to limit bacterial growth. Also, gentamicin is highly thermostable and can be added to solution prior to autoclaving if necessary [7].
5. When making dejelly solution we recommend using the L-Cysteine free base since it works efficiently in both *X. laevis* and *X. tropicalis*. Dejelly solution made with L-Cysteine hydrochloride salt works fine with *X. laevis* but is inefficient at degrading *X. tropicalis* jelly coat. A 3% solution takes as much as 12 min to remove the *X. tropicalis* jelly sufficiently for the embryos to be ready for microinjection.
6. We recommend making dejelly solution fresh. Although, it will work for several days when stored in a sealed bottle, it loses potency over time and will require longer incubations to completely remove the jelly coat. Eventually, a precipitate will form at which time the solution should no longer be used.
7. 8× stock of RAL's Ca_{2+} free High Salt Egg Laying Buffer can be stored at 4 °C for up to 8 weeks.
8. When sexing immature individuals in both *X. laevis* and *X. tropicalis*, and sometimes even in sexually mature *X. tropicalis*, the cloacal labia and nuptial pads may be somewhat incon-

spicuous. Priming and boosting such animals with gonadotropins may induce prominence of these features.

9. The priming gonadotropin injection aids in consistent production of a high number of mature eggs; however, females given only a boosting hormone injection are likely to lay large numbers of eggs as well.
10. Many labs do not feed the females during the period between the priming hormone injection and ovulation. The intent is to prevent excretion of solid waste concurrent with ovulation which may reduce oocyte durability and embryonic culture quality. Furthermore, frogs that are fed prior to ovulation often regurgitate the food.
11. Before IVF some labs prime, or prime and boost, the males with gonadotropic hormones following the same injection schedule but using half the amount of hormone as for the females. Evidence as to whether this in any way affects the quality of the testes, sperm viability, or fertilization efficiency is anecdotal.
12. Do not use 1× RAL's Ca_{2+} free High Salt Egg Laying Buffer with *X. tropicalis*, it will cause twitching and seizures within 30 min.
13. When making the testis solution and flooding the eggs and sperm mix during IVF, some labs substitute 1× MMR with 1× MBS and 0.1× MMR with 0.1× MBS. We exclusively use MMR as in our experience it works great for both *X. laevis* and *X. tropicalis* and unlike 0.1× MBS, 0.1× MMR does not need to be made fresh or have its pH frequently rechecked. The pH of 0.1× MBS tends to fall over time due to weak buffer strength.

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