B. Amphibians

Xenopus

I. Overview of containment measures

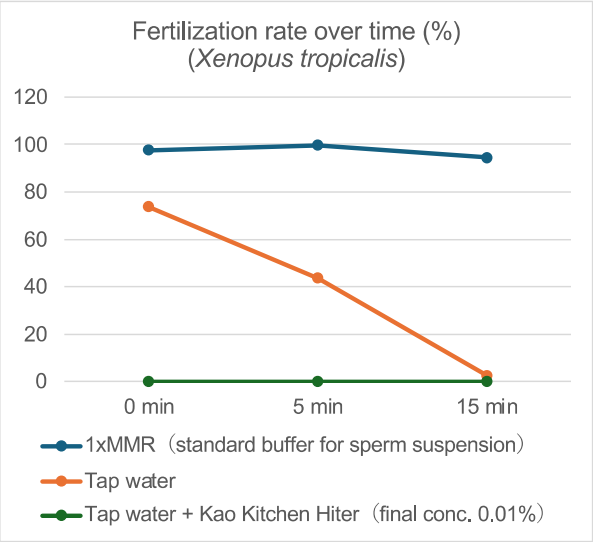
(1) Characteristics of *Xenopus* related to containment measures of recombinant individuals

Xenopus laevis and *Xenopus tropicalis* are the commonly used amphibians in gene modification experiments. In both species, tadpoles and metamorphosed frogs are mainly aquatic, and adults cannot live outside the water for long periods of time. They mate by amplexus, meaning a male will clasp a female and fertilization occurs when the eggs and sperms are released simultaneously. *Xenopus laevis* tolerates a wide range of temperature and can survive winter in Japan. By contrast, *Xenopus tropicalis* requires temperatures between 22°C and 28°C, thus it is highly unlikely that escaped individuals can survive winter in Japan. However, considering the recent climate warming, containment measures to prevent escaping should be considered for both species. Containment measures for the tadpoles can be similar to those for freshwater fish, and for the metamorphosed frogs those for mice, etc., should suffice. If other recombinant amphibians are to be used, containment measures according to their characteristics should be employed. The containment measures for *Xenopus* are basically applicable for Iberian ribbed newt (*Pleurodeles waltl*), which has been developed recently as a new model amphibian species.

(2) Risks of recombinant *Xenopus*

[1] Risk of sperms

The motility of sperms released from males is triggered by osmotic shock in low-salt water (ex. tap water), and is lost within 15 minutes. Therefore, releasing of fertilizable sperms into the natural environment via drainage is highly unlikely. Reference data on the right show fertilization rates in tap water and in tap water with commercial chlorine bleach added in place of hypochlorous acid.



[2] Risk of eggs

After the ovulation, eggs cannot maintain fertility for more than 1.5 hours in low-salt water (ex. tap water). Therefore, releasing of fertilizable eggs into the natural environment via drainage is highly unlikely. Note that eggs can maintain fertility for several hours in water with high salt concentration (water with high salt concentration is never used to raise *Xenopus*).

[3] Risk of fertilized eggs

Fertilized eggs may have more practical risk than sperms or eggs in being released into the natural environment.

[4] Risk of tadpoles

The epidermis of tadpoles is made of 2-3 layers of cells and is very fragile; it is easily damaged by mechanical stimulation. Thus, larvae often die during transport, if not shipped in a special container. Still, the release of tadpoles into the natural environment via drainage should be avoided.

[5] Risk of metamorphosed frogs

Metamorphosed frogs are aquatic and vulnerable to dry conditions. If they escape from the tank, they cannot survive for more than 2-3 days without water.

(3) Overview of containment measures

Doors and windows of the animal breeding room/laboratory should always be closed, and drains of the sinks, aquarium tanks and floor should be covered with mesh or filter to prevent the escape. Pore size of the mesh or filter must be small enough to capture eggs and embryos, if the drains are used to discard water that may contain eggs or embryos. It is recommended to install sterilization tanks with sodium hypochlorite tablets, etc. in drainage channels, if automated water wasting systems are used for the aquarium tanks. After completion of experiments, recombinant frogs and tadpoles should be euthanized before disposal.

Examples provided in this document are containment measures for recombinant *Xenopus* in P1A. Experiments involving the inoculation of recombinant viruses that require P1A, P2A, P3A, or higher levels of containment measures are separately discussed in a different document, since such experiments need the corresponding appropriate containment measures. If containment measures are unclear in the experiment protocol, the application for confirmation by ministers is necessary.

II. Requirements of containment measures

1. Facility requirements (hardware requirements)

(1) Housing for recombinant *Xenopus*

P1A

[Fertilization, embryos, raising tadpoles]

Xenopus sperms lose motility completely within 15 minutes after released from males into low-salt water (ex. tap water). Unfertilized mature eggs lose fertility within 1.5 hours after ovulation in such low-salt water. Water used in mating is discarded after being left more than one hour to ensure that sperms completely lose fertility, and removing eggs and embryos by filtration. Water used for raising tadpoles is discarded after the filtration with mesh or filters. Tadpoles can only live in water, and escaping from routes other than a drainage channel is extremely unlikely.

[1] Animal breeding room/laboratory

- Rooms with doors and windows should be closed.

[2] Drain/water disposal

- All drain ports should be covered with escape-proof traps (mesh or filter). For trapping eggs and embryos, a mesh /filter with a 0.5 mm or smaller grid/pore size is suitable, since the diameters of *Xenopus tropicalis* and

Xenopus laevis eggs are approximately 0.7-0.8 mm and 1.0-1.3 mm, respectively. For trapping tadpoles, the mesh/filter pore size requires adjustment depending on their size and number, since the mesh/filters with too small pores may be easily clogged with the tadpole excreta. It is recommended to install sterilization tanks with sodium hypochlorite tablets, etc. in drainage channels, if automated water wasting systems are used for the aquarium tanks.

- Water that contains sperms is discarded after being left more than a half hour to ensure their complete inactivation, and eggs and embryos trapped on the mesh/filter should be disposed of after freezing. Sperms, eggs and embryos can also be inactivated together by sodium hypochlorite treatment (final concentration should be 60 ppm or more, treatment time should be 30 min or more). Water used to raise tadpoles should be discarded via drains through the mesh/filter traps (and sodium hypochlorite sterilization tanks). The trap with mesh/filters should be checked regularly.

[3] Housing

- An aquarium tank, or a container robust enough to use as an aquarium tank can be used.

[4] Waste disposal system

- Excreta cannot be separated from the water and so should be disposed of together. There is no risk of releasing recombinant animals via the wastewater or excreta, so no special equipment is necessary.

[5] Autoclaves

- Installation of autoclaves in the animal breeding room/laboratory is preferred. If installed outside, recombinant *Xenopus* should be transferred in an escape-proof container. If recombinant *Xenopus* is euthanized by anesthesia or freezing, autoclaving is not necessary.

[Raising metamorphosed frogs]

The risk of metamorphosed frogs escaping into the natural environment is not high as they live in water, but they should be kept in appropriate containers in the designated animal breeding room/laboratory.

[1] Animal breeding room/laboratory

- Closed room with rat guard (frog guard with height 15 cm or higher) should be implemented at the door.

[2] Drain/water disposal

- All drain ports should be covered with mesh/filter traps.
- Water used for raising adults is discarded after leaving more than a half hour through a drain covered by the mesh/filter traps. The water can also be disposed of after the sodium hypochlorite treatment, as with the water used in mating or tadpole breeding. The trap with mesh/filters should be checked regularly. It is recommended to install sterilization tanks with sodium hypochlorite tablets, etc. in drainage channels, if automated water wasting systems are used for the aquarium tanks.

[3] Housing

- Adult frogs should be kept in a tank with a lid (see figure).

[4] Waste disposal system

- Excreta cannot be separated from the water and so should be disposed of together. There is no risk of

releasing-recombinant animals via the wastewater or excreta, so no special equipment is necessary.

[5] Autoclaves

- Installation of autoclaves in the animal breeding room/laboratory is preferable. If installed outside, recombinant *Xenopus* should be transferred in an escape-proof container. If recombinant *Xenopus* is euthanized by anesthesia or freezing, autoclaving is not necessary.

2. Procedure requirements (software requirements)

(1) Housing for recombinant *Xenopus*

P1A

[1] The requirement for P1 animals

[2] If recombinant frogs or tadpoles become unnecessary, discard after euthanasia. For example, 0.05% and 0.2% MS222 (tricaine methanesulfonate) can be used for anesthesia and euthanasia, respectively. After anesthesia, the frogs and tadpoles can be frozen to death.

[3] Housing container should be labeled with signs indicating recombinant frogs or tadpoles are inside. Labels indicating their genetic identities are also required.

[4] When keeping recombinant frogs or tadpoles, the number of individuals should be checked and recorded as needed.

[5] Display signs indicating “Modified animal being bred” on the doors of the animal breeding room/laboratory.

[6] Sodium hypochlorite treatment (final concentration should be 60 ppm or more, treatment time should be 30 min or more) can be used for inactivation of sperms, eggs and embryos.

[7] For transport of recombinant animals during experiments, follow (2)(i) and (ii) of "3. Others" below.

3. Others

(1) Storage

(Cryopreservation techniques have been established for sperms but not for eggs. Thus, only sperm storage methods are discussed).

[1] Label the container to show it contains recombinant frog sperms.

[2] When sperms are stored in a frozen state, the storage freezer or tank with liquid nitrogen should display signs indicating recombinant frog sperms are stored inside.

[3] A record of the storage/usage of sperm must be kept.

(2) Transport

[1] Transport using escape-proof, unbreakable containers. Take appropriate measures to protect the containers from breaking during transport (e.g., put frogs in sealed plastic containers and place the containers in a Styrofoam box).

[2] The outermost container must be labeled clearly to show that it requires handling with care.

[3] When transporting recombinant animals for the purpose of transferring them, transfer records should be kept and managed, and an information sheet regarding the recombinant animal should be attached.

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Exp Anim., 59(4): 395-405, 2010

Hoppler, S. and Vize, P. D.
Xenopus Protocols: Post-Genomic Approaches. Springer, 2012.

Suzuki, N., Watanabe, D., Suzuki, M., Igawa, T., Hayashi, T. and Ogino, H.
Sperm handling as part of containment measures for laboratory amphibian species.
In preparation.

Xenopus resources and reference websites

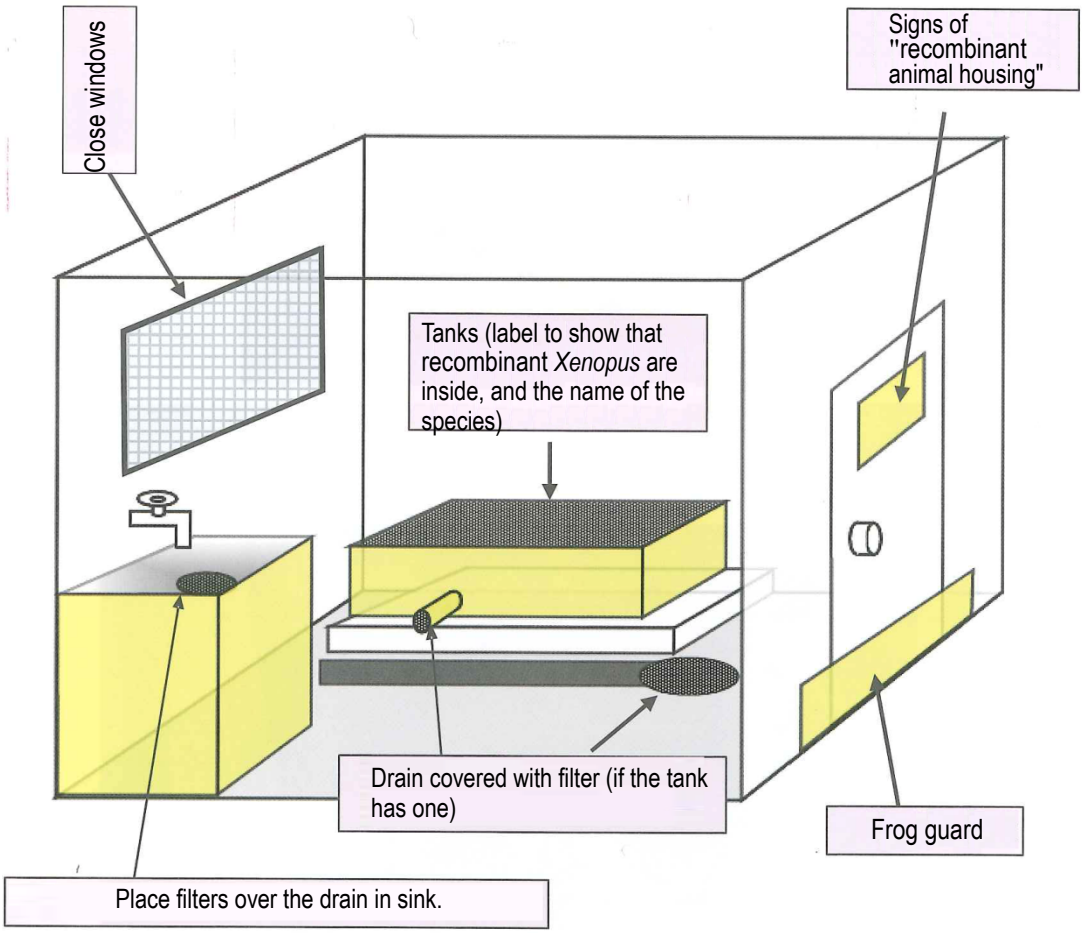
National BioResource Project -*Xenopus tropicalis* (how to raise *X. tropicalis*)
https://xenopus.nbrp.jp/NBRP_Xenopus/NBRP_X._tropicalis_Top_Page_EN.html

Xenopus Genome Project (genome browser for *X. laevis* and *X. tropicalis*)
http://viewer.shigen.info/xenopus/index.php

Harland Laboratory *Xenopus tropicalis* Site (*X. tropicalis* husbandry and protocols)
http://mcb.berkeley.edu/labs/harland/

Xenbase (international database for *X. laevis* and *X. tropicalis* research)
http://www.xenbase.org/entry/

Examples of containment measures for recombinant *Xenopus*



Reference

Examples of housing for recombinant *Xenopus*
(Amphibian Research Center, Hiroshima University)

(A) Door of the breeding room. A sign reading “Breeding recombinant animals (P1)” and a frog trap (blue plastic plate at the bottom) are installed to prevent escape.

(B) Overall view of the water-circulation type multiple tanks system for breeding frogs. The upper two shelves are for water tanks for frogs, and the bottom shelf is for a water filtration tank (gray), water temperature controller, pump, and ultraviolet light (for sterilization of circulating water).

(C) Frog tanks installed in the upper shelf of (B). Information such as the name of the recombinant strain and the number of animals in each tank is displayed directly on the tanks and recorded using barcodes.

(D) A filtration tank (transparent type) at the bottom of the water-circulation type multi-tank frog breeding system. Filtration materials (white) are used for biological filtration by culturing bacteria on them. Unlike the gray filtration tank shown in (A), the transparent filtration tank makes it easy to check the condition of the filtration materials, but algae may grow on its walls.

(E) A chlorination tank is installed between the frog tank system and the drain. Hypochlorite tablets are contained in a tube on the far side. Waste water from the frog tank system is mixed with chlorine at the back, then passes through a mesh filter that divides the center of the tank before being discharged through the tank at the front.