



Institutional Animal Care and Use Committee™

Title: Static Zebrafish Husbandry

SOP Number: 040

Purpose: To maintain and grow juvenile zebrafish. To maintain a reproductively active zebrafish brood stock. To collect naturally fertilized zebrafish eggs and raise until hatching.

HEALTH AND SAFETY

- A. Enroll with Animal Use and Care Committee.
- B. Wear gloves while handling fish or placing hands into tanks/aquaria.
- C. Wear lab coat while working in the laboratory.
- D. Open-toed shoes or shorts should not be worn in the laboratory.
- E. Long hair should be pulled back when working in the laboratory.

PERSONNEL/TRAINING RESPONSIBILITIES

- A. Learn appropriate storage, handling, and disposal procedures for each chemical from SDS or supervisor prior to use. SDS files are located in room 107 of the Fisheries and Wildlife Research Building.
- B. Follow appropriate animal use protocol.
- C. Read and follow Standard Operating Procedure (SOP) for fishless cycling of aquaria (TCFWRU SOP 08-01); use and calibration of balances (SOP IN-4-04); pH meter (TCFWRU SOP 08-03); YSI 85 (SOP ET-4-06-01); chlorine measurement with DR/890 colorimeter (TCFWRU SOP 08-04); and washing procedures for glassware (SOP IN-3-09). These SOPs are located in room 102.
- D. After reading these procedures, have assisted-training from experienced staff.

REQUIRED AND RECOMMENDED MATERIALS

- | | |
|--|--|
| – Gloves and lab coat | – Aerator |
| – Zebrafish adults, eggs and larvae | – Tubing |
| – Aquaria and aquaria glass lids | – Balance |
| – Zebrafish breeding chambers | – Oakton® pH Testr 2 meter |
| – Aquarium-certified silicone | – HCl, NaOH |
| – Glass pipettes, beakers, graduated cylinders, and sample vials | – YSI 85 (temperature, conductivity, salinity, and dissolved oxygen meter) |
| – Ultrapure water | – DR/890 colorimeter |
| – Dechlorinated tap water | – LR Chlorine PourRite Ampule Standard Solution |
| – RO water | – DPD Free Chlorine Powder Pillows |
| – Kent R/O Right buffer salts | – Calcium Lime Rust detergent (CLR) |
| – 70% ethanol | – Alconox/Citranox detergent |
| – Forceps | – Sodium hypochlorite (bleach) |
| – Nets | |
| – Siphon hoses | |

USE OF ZEBRAFISH IN RESEARCH

- A. Current OLAW interpretation of the PHS Policy considers aquatic species as “live, vertebrate animals” at hatching.



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1. Although this is an imprecise stage for zebrafish it can be approximated at 72 hours post fertilization.
2. For purposes of accountability, all stages of development greater than three days of age should be described in an approved animal protocol.
3. An estimate of the number of larval zebrafish from day 4-7 days post fertilization (dpf) should be included in protocol.
4. Since these early stages (4-7 dpf) are not currently considered to feel pain or distress, their numbers should be separated from the zebrafish 8 days of age or older.
 - i. The number of animals used may need to be provided as an estimate, particularly with these young larvae.
 - ii. Consider their size and normal housing conditions.

STOCKING DENSITIES

- A. Stocking densities of up to 5 adult fish/L are allowed
- B. 50-100 eggs, per 30 ml
 1. 0 -7 days post fertilization
- C. 40-50 fish per liter during early larval stage
 1. > 7 – 28 days post fertilization
 2. Reduction should occur after 28 days to achieve 5 adult fish per liter.
- D. Note: Lower fish densities in adult Zebrafish can cause aggressiveness in dominant male and females. In turn, this can lead to reduction in egg production in the non-dominant fish.
- E. High fish density in larval and juveniles can lead to alterations in sex ratio. Therefore, observations should be made throughout rearing of successive generation to determine the optimal density for the tank.

SYSTEM SET UP

- A. Cleaning of glass aquaria and other glass materials
 1. Wear appropriate attire according to SDS for chemicals (gloves, lab coats, face shield as necessary). If glass material (including aquaria) is new, see SOP IN-3-09 in room 102 for specific cleaning procedure. If aquaria are not new and are heavily coated in water residue and lime, place in CLR for 15 minutes to over night prior to continuing washing procedure. Scraping residue with a razor blade may be necessary.
 2. Aquaria or beakers that have previously contained eggs, larva or fish need to be washed and disinfected. For this purpose, wash with detergent and hot water. Rinse 3 or more times with tap water until all visible soap is removed; may rinse with 10% hydrochloric acid (HCl) 2-3 times if necessary by adding small volume (50-100 ml) into tank and gently swirling to wash off the inner walls (to minimize acid waste, may transfer this solution to several additional tanks for rinsing before properly discarding into acid waste container); rinse 2-3 times with house RO water, and 2-3 times with ultrapure water. Finally, to disinfect tanks, spray with 70% ethanol solution and let dry before use. Check silicone seals: if necessary, replace.



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3. Set up aquarium system according to desired configuration. See TCFWRU SOP 08-01 for an example of a 40-gallon fish aquarium coupled to a 10-gallon biofilter.
- B. Preparation of zebrafish water
1. For juvenile fish and broodstock maintenance, zebrafish water is prepared by mixing RO water and dechlorinated tap water in a 2:1 ratio in a large plastic container fitted with a heater to maintain the temperature at 28.5°C, and an air stone to bubble air and help with the conditioning of the water. To a 100-L volume of the water mix, immediately add 5 mL of a 5 mM solution of HCl and 12 hours later add another 6 mL. Add the acid slowly while stirring the water with a paddle. Twenty-four hours later, the water pH should be stable between 7 and 7.5; zebrafish water is now ready for use with fish. (Note: this procedure has been specifically developed when using Lubbock tap water as the source of dechlorinated water.)
 2. For embryo and larval rearing (or in situation when tap water may not be used with older fish), zebrafish water is prepared by mixing RO water with buffer salts (31g of Kent R/O Right for each 100L of RO water). In order to obtain the appropriate pH, may add HCl at 5 mM or NaOH at 5 mM as required. The final pH should be around 7.4, and the water temperature should be maintained at 28.5°C.
 3. When using tap water, must monitor the chlorine concentration in the dechlorinated tap water at least once weekly (TCFWRU SOP 08-04). If chlorine is detected, check if the active-charcoal dechlorination filter is working properly. Change charcoal if necessary.

WATER QUALITY FOR RECIRCULATING SYSTEMS

- A. Temperature
1. 24-30 C, with 28 being the optimal range for rearing adults and breeding.
 2. If fish are not breeding, increase temperature up to 30 C.
 3. Most recirculating systems have a temperature control and monitor probe included in the system.
 4. If the system does not have temp control, then an aquarium heating stick, placed in the water basin, can usually maintain a constant temperature.
- B. pH: 7-8 range, with 7-7.5 more appropriate.
1. Decrease pH;
 - i. Biological filtration (oxidation of ammonia to nitrate),
 - ii. Decomposition (due to excessive feeding),
 - iii. Fish metabolism (release of nitrogenous waste)
 2. Increasing pH:
 - i. Aeration,
 - ii. Water contact with crushed coral, aragonite and sodium carbonate
 3. **As pH increases there is an increase in concentration of un-ionized ammonia (toxic form).
- C. Alkalinity (50-150 mg of CaCO₃ / Liter):
1. This represents the buffering capacity of the water in the system.



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2. High fish density on recirculating systems decrease pH, therefore increased alkalinity is needed to maintain pH.
 3. Although sea salt which is used to increase total hardness will increase alkalinity, adding crushed coral, aragonite or sodium bicarbonate is the easiest way to do this.
- D. Hardness:
1. Fish require a number of ions and addition of CaCO_3 and aquarium sea salt will accommodate this requirement.
 - i. One consideration is that increasing hardness, decreases osmoregulatory stress and decreases toxicity to dissolved metals like copper and zinc.
 - ii. Measurements can be 80- 300 mg/L or as Conductivity (300-1500 microS).
- E. Salinity: 0.5- 2 g/L
- F. Chlorine: 0 mg/L
- G. Ammonia: 0.02 mg/L *
- H. Nitrate: 0 mg/L
- I. Nitrite: < 50 mg/L *
- J. *Assumption is that pH is within 7-8 range.
- K. Recirculating systems with biological filters require a pH between 7-8 and the alkalinity be within 50/ 150 mg CaCO_3 mg/Liter.
1. At TTU, dechlorinated tap water or deionized water—with sea salt and CaCO_3 added—can be used, but the water will have to be “conditioned”.
 - i. Tap water in the Lubbock area is quite alkaline and hard, with a pH in the mid 8 range. To bring the pH down, one needs to add HCl to the system.
 - ii. If fish are on the system, it is not advised to add directly to the system, rather this is done in a large container of water.
 2. Typically, pH-adjusted tap water is aerated while in holding container, which will also increase pH.
 - i. It is advised that before adding fish to any system, a water conditioning protocol be created for lab members to follow to lower pH of aerated tap water.
 3. If deionized water is used, salts will need to be added to water.
 4. This is done by adding Aquarium sea salt and CaCO_3 to the water.
 5. This in turn will increase pH and HCl will need to be added to water to lower pH. As with pH adjusted tap water, it is advised to perform water conditioning in a large container where the water is aerated.
 - i. Again, a water conditioning protocol should be created for the lab to follow.
- L. NOTE: Some Recirculating systems have the option to have Hardness and pH monitoring systems included, where concentrated Sea Salt mixture and/or CaCO_3 can be added dependent on probe readings. These systems should only be used with DI water directly added to system. In many cases, one can set a timer to directly add a



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pre-set volume of DI water to the system and to then have hardness and alkalinity be automatically adjusted.

HUSBANDRY PROCEDURES

A. Receiving zebrafish juveniles and adults

1. Prior the acquisition of new fish, follow the fishless tank cycling procedure to condition the aquaria (TCFWRU SOP 08-01).
2. When introducing new fish into the laboratory, acclimate for at least 2 weeks prior to use in studies. During acclimation the fish will be observed closely to identify unhealthy individuals by their external appearance or abnormal behavior. Unhealthy fish will be isolated and, if necessary, euthanized.
3. Fish loading density will vary depending on tank conditions. If using 40-gallon aquaria with 10-gallon biofilter tanks, 40-50 adult zebrafish may be maintained provided that water quality criteria are met (see 6.2). It is extremely important that nitrogenous waste be monitored very closely during the initial weeks of acclimation to prevent the occurrence of New Tank Syndrome (see 6.2 and TCFWRO SOP 08-01).
4. Small portions of food should be given to the fish during the first few days of acclimation. The amount should increase progressively according to the fish's response until the 'feed to satiation' goal is accomplished.

B. Maintaining zebrafish juveniles and adults

1. In most situations, it is appropriate to set the photoperiod for juvenile and adult zebrafish to 14-h light:10-h dark, and light intensity should be between 540-1080 LUX.
2. To prevent spread of disease among the fish, disinfect gloved hands with 70% ethanol or sodium hypochlorite at 50 ppm, and then rinse several times with distilled water, before placing gloved hands into other tanks.
3. Fish are fed twice daily, in the morning (8-10 am) and the afternoon (4-6 pm). The diet may vary depending on study design.
 - a. For broodstock, fish are fed twice daily. Uneaten food and other debris settling on the bottom of the tanks should be removed daily by siphoning.
 - b. For juveniles, the same feeding regimen as for broodstock may be applied. Alternatively, if cost is of concern or for other reasons related to research, the diet may be based on Tetramin flakes but it is recommended that at least two meals a week be based on frozen brine shrimp. Tanks are also cleaned daily by siphoning.
4. Tank water should be replaced at a rate of no less than 25% of volume per week. However, water should be replaced as often as necessary to maintain quality especially with respect to nitrogenous waste (see 6.2.5 and 6.2.6). (Note: it is better to replace 25% twice a week than 50% at once.)
5. Add Stresszyme® (N-cycle bacteria) after each water exchange at 10 ml per 40 liters of tank water or as recommended by the manufacturer.
6. Water pH should be monitored every day, and especially before and after water exchange; if necessary adjust water pH by adding HCl (5 mM stock) or NaOH (5 mM stock) to tank water (add to biofilter compartment).



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7. Temperature, dissolved oxygen, salinity-conductivity should be measured every other day.
8. In an established aquarium with a fully functional biofilter, free ammonia, nitrite and nitrate may be tested once weekly. However, it is strongly recommended that these measurements be made more often within the first 3-4 weeks of placing fish in a new aquarium – even if the aquarium was pre-cycled. The appropriate volume of water should be replaced if nitrogenous waste levels rise above the recommended limits (see 6.2.9).
9. The following water conditions should be maintained:
 - a. Temperature: 28 to 29°C (preferably constant at 28.5°C)
 - b. pH: 7.0-7.5
 - c. Dissolved oxygen: > 5 mg/L
 - d. Salinity: 0.3-0.5 ‰
 - e. Unionized ammonia: < 0.02 ppm
 - f. Nitrite: 0 ppm
 - g. Nitrate: < 20 ppm (not toxic to fish but causes algal blooms)
10. The walls of the tanks should be periodically scrubbed to avoid algal growth.
11. The Bioballs in the biofilter tank should be cleaned every two weeks in conjunction with a water replacement. For this purpose, remove the Bioballs from the biofilter tank and place them in a plastic bucket, and rinse three or four times with RO water – DO NOT USE CHLORINATED TAP WATER as it will kill the N-cycle bacteria. Also, avoid over-cleaning the Bioballs prevent losing the bacteria.
12. The pipe that connects the pump in the biofilter to the aquarium has to be cleaned every two weeks. For this purpose, disconnect the pump and remove the pipe, rinse the pipe with RO water and remove the waste accumulated in its interior with a brush.
13. None of the maintenance procedures regarding the fish and biofilter tanks (or fish nets) should involve the use of any kind of detergent or soap.
14. Siphon tubes should all be made of glass, silicone, Teflon, or plastic. Disinfect all siphon tubes and nets with 70% ethanol before use in other aquaria and after each use.

BREEDING

- A. For breeding purposes, it is recommended that males and females be kept in separate aquaria at least 1-2 weeks prior to breeding. (Note: sexual maturity in zebrafish is reached at 10-12 weeks of age.)
- B. Spawning
 1. The day before pairing the males and females for breeding purposes, feed the fish frozen brine shrimp in the morning and afternoon to satiation. Fish are not fed on the day of pairing.



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2. Breeding is conducted in zebrafish breeding chambers consisting of two internal compartments. The upper compartment is to place the broodstock and the lower to collect the eggs through a false bottom (Figure 1). Hole in the upper chamber allows for water exchange between the chamber and aquarium in order to maintain water quality within the chamber.



Figure 1. Female and male fish are paired in the breeding chambers immediately before lights-off (artificial dusk). The chambers (containing the fish) are placed within the aquaria and left overnight (A). Spawning will commence upon lights-on the following morning. Typically, eggs are collected 2-3 hours after lights-on. Zebrafish cannibalize their eggs. The two compartments of the chamber are separated by a mesh (B) to allow the eggs to fall into the lower compartment.

1. On the day of pairing, fish are gently transferred to the breeding chambers 5-10 minutes before lights-off. Do not place the fish in the chambers any earlier than this timeframe or else breeding activity may occur before the lights go off (this will yield embryo populations at different stages of development the next day). A 2:1 female: male ratio is recommended for breeding; 8 females and 4 males typically will give optimum results using the breeding chambers just described (Figure 1). The chambers are usually placed in the female tanks. Most breeding activity the next morning will occur within 2-hours after lights-on. Do not disturb the fish during this period.
- C. Collecting eggs
1. Gently remove the breeding chambers from the tanks onto a bench. Remove lid and transfer the adults back into their original tanks. Collect the fertilized eggs from the bottom compartment of the breeding chamber by aspiration with wide-mouth pipette or by gently pouring the water (with eggs) through a fish



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net. If a net is used, gently rinse eggs with zebrafish water (pre-heated to 28.5°C) three times. Gently release eggs from net into a clean beaker with zebrafish water pre-heated to 28.5°C. Remove extraneous materials with clean forceps or pipette.

2. Remove nonviable eggs if present. Nonviable eggs will be whitish, mushy looking; good or viable eggs will be fairly translucent with a slightly yellowish and granular looking yolk.
3. If volume of packed eggs needs to be determined, gently and carefully pour egg slurry into small graduated cylinder (5 ml) for egg volume determination. Wait until eggs settle on bottom of beaker and take volume measurement. Dispose eggs at this time if not needed for embryos or larvae, or else gently pour eggs back into beaker.
4. Disassemble the breeding chambers, rinse them with RO water for several minutes and disinfect them with 70% ethanol, or with 50 ppm sodium hypochlorite solution. Rinse 2-3 times with RO water. Dry and store in appropriate area.

D. Raising embryos

1. For general purposes, count dead eggs (unfertilized) at 8-12 hours after fertilization to determine fertilization rate. Remove as you count. Removal of unfertilized eggs is necessary to prevent fungal growth and infection of healthy embryos. Embryos that die during development must also be removed, twice daily (morning and afternoon).
2. If more accurate estimates of fertilization rate are necessary, fertilization can be determined using a stereomicroscope by monitoring cleavage to the 4-cell stage of embryogenesis after 1 hour of fertilization (unfertilized eggs typically do not progress beyond the 2-cell stage).
3. Fertilized eggs are maintained in glass beakers with zebrafish water (28.5°C), usually at a density of 15-30 eggs per 100 ml zebrafish water in 250 ml beaker, or 60 eggs per 250 ml in 600 ml beaker. Use a larger water volume (beaker) for larger numbers of eggs. Cover the beakers with a lid to help maintain a homogenous temperature.
4. If allowed by study design, add Methylene blue (such as Aquatrol, Inc., Anaheim, CA, USA) at the rate of 4 drops/30 L of zebrafish water to prevent fungal growth. (Note: Some procedures do not allow the use of medication with experimental fish.)
5. If available, use dry incubator to maintain temperature and photoperiod (the one currently available in the laboratory needs to be set at 30.1°C to maintain beaker water temperature at 28.5°C). If an incubator is not available, beakers may be immersed in water bath maintained at 28.5°C.
6. For embryo culture, it is standard to set photoperiod to 12-h light:12-h dark, and light intensity should be between 540-1080 LUX. Hatching may be delayed or will not occur normally if light intensity is too low.
7. Renew one third of the incubation water volume every 24 hours during embryo incubation.



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8. At 28.5°C, hatching will occur between 48 and 72 hours of fertilization. If necessary, determine hatching rate at 72 hours or at the time required by the experimental design.
- E. Raising Larval Fish
1. Static Systems (without biological filter):
 - i. The main challenge to larval health is water quality. It is important in static systems that uneaten food, usually on the bottom be taken out using a pipette and that Ammonia, Nitrite and Nitrate be monitored daily.
 2. Recirculating Systems:
 - i. Flow of water should be kept to a drip rate (1-2 drips per 5 seconds) so larval fish do not use excess energy.
 - ii. The main challenge to larval health is food encounter rates due to depth of tank and flow rates.
 - iii. Low drip rates and food that can stay on top (dry fish food) are helpful in feeding larvae. As the larvae get older, supplanting with Artemia can be done to enrich diet.

EUTHANASIA:

- A. Methods for euthanasia of zebrafish varies depending on life stage. See SOP 031 for an overview of procedure using MS-222.
- B. For zebrafish larvae 3- 7 dpf, euthanasia can be completed using the following method:
 - a. Sodium Hypochlorite (bleach), 1-step method:
 - i. Larvae should remain in this solution at least five minutes prior to disposal to ensure death. *Extreme caution must be used with this method to avoid any possibility of bleach entering the aquatic housing system water.
 - ii. To ensure final concentration is in excess of 1%, verify the sodium hypochlorite concentrations on the stock bottle, as concentrations vary.

Example calculation: 6.15% sodium hypochlorite, add to the culture system water at 1 part bleach to 5 parts system water.
- C. For zebrafish larvae up to 8-15 dpf euthanasia requires a secondary method in order to ensure death. This age group can survive anesthetic overdose and rapid chilling even after prolonged absence of heartbeat. They can revive if returned to water that is within their normal environmental parameters.
 - a. Two step euthanasia method – rapid chilling or general anesthesia followed by an adjunctive method:
 - i. Rapid Chilling- submersion into ice water bath (5 parts ice/1 part water, 0-4° C) for at least 20 minutes to ensure death.
 1. The fish must not come in direct contact with the ice chips; for example, a mesh-bottom inner cage of a breeder tank can be pushed into the ice slurry as a barrier.
 - ii. General anesthesia with pharmaceutical grade buffered MS-222. Note: MS-222 powder must be handled in a chemical fume hood or a ducted



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biosafety cabinet. MS-222 solutions may be handled outside of a chemical fume hood or a ducted biosafety cabinet. Stock solutions may be stored in the dark at 4°C for a maximum of six months.

- iii. Adjunctive methods after fish have been rendered unconscious (rapid chilling or general anesthesia) are freezing or other physical or chemical methods for destroying the brain function.
- D. For zebrafish >15 dpf, euthanasia can be done using the following methods:
- a. Euthanasia by rapid chilling:
 - i. Submersion in ice water bath (5 parts ice/1part water, 0-4° C) for at least 10 minutes after cessation of opercular (i.e., gill) movement.
 1. The fish must not come in direct contact with the ice chips; for example, a mesh-bottom inner cage of a breeder tank can be pushed into the ice slurry as a barrier.
 2. In any fish where it is difficult to visualize opercular movement, fish should be left in the ice water for at least 30 minutes after cessation of all movement to ensure death by hypoxia.
 - ii. Water temperature must be monitored with a thermometer and maintained between 0-4° C to ensure proper euthanasia.
 - b. Overdose by prolonged immersion in buffered pharmaceutical-grade MS-222 in 250-500 mg/L solution.
 - i. Buffering with sodium bicarbonate should result in a pH between 7.0 and 7.5. Non-buffered MS-222 is acidic and causes an aversive reaction in unanesthetized fish.
 - ii. Fish should be left in the solution for at least 30 minutes following cessation of opercular movement.
 - iii. Potency of MS-222 solutions reduce over time. Using a fresh batch of buffered solution for each group of fish is recommended.
 - c. Anesthesia with pharmaceutical grade buffered MS-222 (100-200 mg/L) followed by an adjunctive method after fish have been rendered unconscious prior to their application are decapitation, exsanguination, freezing or other physical or chemical methods for destroying the brain function.

E.

Reference

1. https://oacu.oir.nih.gov/system/files/media/file/2023-08/b17_zebrafish.pdf
2. Panel on Euthanasia. [AVMA Guidelines on Euthanasia: 2020 Edition](#) (2020).

GENERAL MAINTENANCE OF THE ROOM

- A. The broodstock room has to be swept at least once a week. Sweep the floor with bleach, using the concentration suggested by the manufacturer.
- B. To prevent spread of disease among the fish, never place on the floor any tools that are used in aquaria (including the RO and tap water hoses). If for any reason something falls on the floor, be sure to rinse and disinfect properly before reusing.

LITERATURE

- The Zebrafish Handbook, Monte Westerfield, University of Oregon (www.zfin.org)



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- Stocking Densities are based on evidence presented in the paper entitled, “The Effect of Stocking Densities on Reproductive Performance in Laboratory Zebrafish” and the information from E. Herbst the R&D Manager and Senior Biologist for Aquatic Habitats.

SURVEILLANCE PROGRAM JUSTIFICATION

- Established zebrafish colonies that have had recent introduction of outside zebrafish should be surveyed on a regular basis for unhealthy zebrafish.
- Surveillance of zebrafish health will be performed daily when feeding during morning feeding. Fish will be assessed for weight loss evident from observing underbelly, discoloration and bulging eyes as these are symptoms of Mycobacterium infection. Hemorrhages in mouth and ulceration on body will be assessed as they are an indication of Pseudomonas infection. Identified fish will be euthanized.
- Larval, juvenile and adult zebrafish from outside vendors must be quarantined before introduction to recirculating system. Continual introduction of outside zebrafish to the established colony may require testing for Mycobacterium and Pseudomonas.