Standard Operating Procedures
Chen Laboratory Zebrafish Facility
CCSR 3151

Updated May 27, 2015

General Rules

1. Before and after handling fish, system water, or equipment that may come in contact with fish or system water (tanks, nets, etc.), hands must be washed with soap and water.

2. Water and fish from the Quarantine system (*indicated with YELLOW labels*) must never be introduced to the Main system (*indicated with WHITE labels*).

3. Separate fish nets should be used for the Quarantine and Main systems. *Green fish nets* are used for the Quarantine system, and *white fish nets* are reserved for the Main system. The nets should be placed in corresponding Virkon S disinfectant buckets when not in use.

4. Fish should only be exposed to system water as tap water contains chlorine or chloramines, which kills fish.

5. Watch for signs of overfeeding: fat fish, uneaten food on the bottom of the tank, clogged screens in baby tanks. The rule of thumb is to give the fish as much as they will eat in about 5 minutes.

6. Watch for signs of underfeeding: skinny fish, really hungry fish, slow growth of young fish.

7. Maintain a fish density of between 5-15 fish per liter.

8. Do not enter the fish room when the lights are out (between 9 PM and 7 AM).

Fish Health

Health Assessment:

1. Remove any dead fish or fish that display symptoms of disease, and record these data in the fish facility log. If pathology services are warranted, sick fish should be kept alive and shipped to either the Stanford Dept. of Comparative Medicine staff or the Zebrafish International Resource Center's pathology services. Dead fish should be placed in a cooler at $+4^{o}$ C or fixed in paraformaldehyde (or Bouin's solution) for necropsy analysis.

2. Symptoms of nematode infection: skinny, arched body or a failure to mate or feed.

3. Symptoms of TB infection: raised scales, open lesions, skin ulcers, and wound spots.

4. For more extensive descriptions and illustrations of zebrafish symptoms, please refer to the illustrative chart in the zebrafish facility.

Euthanization:

1. Adult fish that exhibit symptoms of illness or infection should be either provided to the Stanford Dept. of Comparative Medicine staff or the Zebrafish International Resource Center's pathology services.

2. Adult fish that require euthanization due to infertility, age, or experimental need should be anesthetized with lethal dose of Tricaine (200-300 mg/L) in system water or E3 solution containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$, buffered to a final pH of 7 using sodium bicarbonate.

3. Tricaine used for this purpose must be pharmaceutical grade (e.g. Finquel from Argent Labs). Always wear nitrile gloves, lab coat, and safety glasses when handling Tricaine (work in fume hood when using Tricaine powder). Stock solutions can be frozen and stored for up to six months and must be labeled with an expiration date.

4. Fish should then be submerged in ice water (5 parts ice/1 part water, 0-4^{o} C) for at least 20 minutes following cessation of opercular (i.e., gill) movement from Tricaine treatment.

Water Quality Maintenance

The fish facility includes two independent, recirculating systems (Quarantine and Main systems), manufactured by Pentair Aquatic Habitats. Fish facility water is obtained directly from the CCSR building's reverse osmosis water system and stored in a 90-gal holding tank. A ten percent water exchange occurs daily for each recirculating system (Quarantine and Main systems) using an automatic timer. Water in the racks is passed through charcoal and 50 micron mechanical filters and irradiated with ultraviolet light in a continuous cycle. The aquaculture systems also have automatic pH/conductivity sensors that measure and dose at regular time intervals.

Daily Water Quality Testing:

1. At 10 AM, collect 30 mL of Q and M system water into two 50 mL Falcon tubes labeled accordingly. For both Quarantine and Main, fill 6 glass test tubes with 5 mL sample water for testing.

2. Use API test kits to monitor pH, nitrite, ammonia, nitrate, general hardness (GH), and carbonate hardness (KH). **All solutions should be shaken well before using, and when administering drops, the bottle should be held vertically to ensure consistent drop size. When matching the color of the sample to the provided color chart, hold sample against the white background of the card in a well-lit area.** The table on the following page provides details instructions and normal ranges for each test. Record all results in the daily log book.

Test	**Instructions**	**Normal Range**	**If reading falls outside of normal range…**
pH	Add 3 drops and invert tube several times. Also record the sensor's pH reading for both Q and M in the log book.	6.5-7.5	If the system's dosing equipment is not working properly, adjust with the acid/base buffer. If the pH is >7.4, use the High Range pH test kit instead.

Test	**Instructions**	**Normal Range**	**If reading falls outside of normal range…**
Conductivity	There is no manual conductivity test, but record the sensor reading for both Q and M	700-900 uS	If the system's dosing equipment is not working properly, adjust with Equilibrium salt solution.
Nitrite	Add 5 drops and invert tube several times. Wait 5 minutes before reading.	0-1 ppm	Increase water exchange to 20% to introduce fresh system water to dilute this contaminant
Ammonia	Add 8 drops from bottle #1, then add 8 drops from bottle #2, and invert tube several times. Wait 5 minutes before reading	0-2 ppm	Increase water exchange to 20% to introduce fresh system water to dilute this contaminant
Nitrate	Add 10 drops from bottle #1, then cap tube and invert several times. Shake bottle #2 for 30 seconds (IMPORTANT!), then add 10 drops. Invert the tube for 1 min to mix.	0-40 ppm	Increase water exchange to 20% to introduce fresh system water to dilute this contaminant
General Hardness (GH)	Record number of drops needed to change solution from orange to green. Convert this to units of ppm using the provided conversion chart	3-6 drops (53.7 - 107.4 ppm)	
Carbonate Hardness (KH)	Record number of drops needed to change solution from blue to yellow. Convert this to units of ppm using the provided conversion chart	1-2 drops (17.9-53.7 ppm)	

System Maintenance

Pad Filters:

1. The square pad filters located near the sump should be switched out every 3 days for both Q and M systems, or sooner if the accumulated waste is inhibiting water flow.

Mechanical Filters:

The 50 micron mechanical filters for both Q and M systems should be changed once every 120 hours (5 days).

Protocol for Quarantine System:

1. Stop the system using the touch screen interface.

2. Slowly screw the old filter loose and replace with a new 50 micron filter.

3. Turn the water flow back on and reset the 50 micron filter change alarm on the sensor.

Protocol for Main System:

1. Cut off water flow to the pH and conductivity sensors, and put the TGP sensor on bypass.

2. Stop the system using the touch screen interface.

3. Cut off the main water valve to the fish tanks, and cut off any water flow to or from the mechanical filter.

4. Take a new 50 micron Main system mechanical filter and wash thoroughly with MilliQ water. Replace the old filter, open up valves to the filters, restart the system, and slowly open the water line to the fish tanks.

5. Turn back on the pH, conductivity, and TGP sensors, and reset the 50 micron filter alarm on the touchscreen.

Carbon Filters:

Carbon media (activated charcoal) is used to trap and filter out any carbon based impurities and chlorine. It is recommended that carbon media be changed every 2-3 weeks. Both systems are set up to be alarm every 262 hours (~ 11 days). To replace the carbon media:

1. Pre-soak the carbon in the designated system bucket in RO water for at least 24 hpf.

2. Shut off the system and remove old carbon media.

3. Fill the appropriate container and rinse with RO water until the water runs clear.

4. Replace the carbon media and restart the system.

Pentair's Required System Maintenance:

Regular maintenance on the physical system is outlined by Pentair's guidelines and warranty agreement. Below is a table of the regular maintenances required and the frequency with which they should be completed. There is a yearly maintenance log on the fish room door that needs to be signed after the completion of each maintenance cycle.

Maintenance	**System**	**Frequency**
pH Sensor Calibration	BOTH	Every 2 weeks
Conductivity Calibration (Monthly)	BOTH	Monthly
Clean Air Manifold (2 weeks)	BOTH	Every 2 weeks
Level Sensor (monthly)	BOTH	Monthly
Lubricate O-rings (monthly)	MAIN	Monthly
TGP Calibration (2 weeks)	MAIN	Monthly
Water Exchange Calibration (2 weeks)	BOTH	Monthly

Water Exchange Clean (monthly)	BOTH	Monthly
Pressure relief valve (monthly)	MAIN	Monthly
Pump Bolts (monthly)	BOTH	Monthly
Sensaphone Batteries (monthly)	--	Monthly
Clean heater (monthly)	BOTH	Monthly
Piston Filters (6 months)	BOTH	Every 6 months
Clean Flow Sensor (6 months)	MAIN	Every 6 months
UV Bulb (yearly)	BOTH	Yearly
UV Sleeves (yearly)	BOTH	Yearly
Dosing Tubing (yearly)	BOTH	Yearly

Fish Feeding

Brine Shrimp Production

Decapsulation:

Decapsulation removes the chorion (outer shell) from Artemia cysts via bleach treatment. This eliminates the need to separate nauplii from their hatching shells before feeding and is especially useful when high quality Artemia cysts are unavailable.

Materials Needed:

Bleach (100%): 8.7 L bleach (1 gal = 3.785 L) ~2.3 gal bleach or 3 x ¾ gal bottles

Buffered salt solution: (25 ppt salt; 2.5% NaOH) and premake in a plastic gallon container: 100 g NaCl, 100 g NaOH, 4.25 L MilliQ water

Sodium Thiosulfate (1.0%): 60 g sodium thiosulfate of 94.8 g sodium thiosulfate pentahydrate, 6 L MilliQ water

Saturated Brine Solution: 2.4 kg NaCl (use a bag to weight this out), 8.0 L tap water (make this directly in the hatchery while dehydrating overnight)

Protocol:

Cyst Hydration:

1. Add 2 cans of unbleached Artemia cysts to 10 L of MilliQ water in the hatching cone specified for decapsulation (located near the Chen Lab weigh station).

2. Aerate for 1 hour at room temperature.

3. After 1 hour has passed, examine cysts under a microscope. At this the cysts should be completely spherical, not like a deflated basketball. If not, continue hydration for up to 2 hours and check every 15 minutes.

4. Filter cysts through a medium sized brine shrimp bag (1000-µm mesh) and rinse with tap WATER). This can be done with the water outlet in the chemical hood.

5. Add 4 L of Buffered Salt Solution to the cone and transfer rinsed cysts back to cone to aerate.

Decapsulation:

1. Turn aeration down and add 8.7 L chilled bleach (or whatever vol. will fit) to the cone with a funnel. Cysts should begin to change color, first turning from brown to gray and then from gray to orange. Check constantly, this color change will happen very quickly. Note that over-bleaching will prevent the cysts from hatching.

2. As soon as 90% of the cysts turn orange, stop the reaction by quickly transferring cysts into 100-µm mesh bag and rinsing with cool tap water.

Neutralization of residual chlorine:

1. Transfer mesh bag into a large container and pour 1% sodium thiosulfate. Soak cysts in sodium thiosulfate for 1 min, then rinse with MilliQ water.

Dehydration for long-term storage:

1. Transfer cysts to a normal hatchery cone with 8 L saturated brine solution and aerate for 18-24 hours (**overnight**).

2. While keeping the aeration in, fill 1 L with resuspended cysts.

3. Store at 4 °C in saturated brine solution for up to 6 months.

Feeding Adult Fish

Fish are fed three times a day on weekdays. On weekends, once a day will suffice. Brine shrimp is the primary adult fish food. The preferred brand is Hatching Shell-Free Brine Shrimp E-Z Egg, sold by Brine Shrimp Direct. Argentemia's Platinum Grade Brine Shrimp will also suffice if E-Z Egg is unavailable. Add enough brine shrimp that can be consumed in 5 minutes.

Adult fish should also be fed supplemental food (Ziegler's Adult Zebrafish Complete Diet) once a day. Add enough food that can be consumed in 5 minutes. Please note that all supplemental food products that do not have a specified expiration date should be disposed of six months after opening. Always add a label to containers specifying the open date and expiration date. Both brine shrimp and supplement are stored at 4 °C.

On weekdays, feed the fish brine shrimp in the morning and evening (10 AM and 5 PM). For the afternoon feeding (2 PM), give supplement to the adult fish, and brine shrimp to fish less than 2 months old. *Do not feed fish in the mating tanks*—these will be fed upon their return to the Quarantine or Main system racks.

On weekends, feedings occur once a day at 11AM. Feed the equivalent of both a morning and evening culture.

When feeding, avoid spilling shrimp or supplement on the tank lids, as this can promote bacterial growth. Use the Q and M squirt bottles for dispensing brine shrimp and the designated pipette feeder devices for dispensing supplement.

<u>Weekday Morning (10 AM) and Evening (5 PM) Feeding:</u>

1. Pull out the aeration tube from the 12 L culture and let shrimp settle for 10-15 minutes.
2. Drain the shrimp into a brine shrimp net and wash thoroughly with system water to remove salt.
3. Dilute the brine shrimp into 1 L of system water, and distribute between Main and Quarantine feeding bottles.
4. Feed fish an amount that they can consume in 5 minutes.

5. Wash the hatchery and tubing immediately after feeding. Scrub with a white bristle brush and rinse with system water. Make sure extra shrimp and slime is removed from the sides.The hatchery should not feel slippery or slimy to the touch. Unclog any residual salt buildup in the aerator tube.

6. Restart the culture with 12 L system water, 300 g marine salt (Coralife Marine Salt Mix), and 60 mL brine shrimp.

Weekday Afternoon (2 PM) Feeding:

1. Drain and wash the 8 L afternoon brine shrimp culture as described in the Weekday Morning and Evening Feeding Procedure. Distribute the shrimp between Main and Quarantine feeding bottles, and feed only the fish that are less than 2 months old, enough that they can consume in 5 minutes.

2. Feed the adult fish supplement (Ziegler's Adult Zebrafish Complete Diet), enough that they can consume in 5 minutes.

3. Wash the hatchery and tubing, as described in the Weekday Morning and Evening Feeding Procedure.

4. Restart the culture with 8 L system water, 200 g marine salt (Coralife Marine Salt Mix), and 30 mL brine shrimp.

Weekend (11 AM) Feeding:

1. Drain, rinse, and feed **two** 12 L shrimp cultures, according to the Weekday Morning and Evening Feeding Procedure.

2. Wash and scrub both hatcheries thoroughly, as described above, and restart each with 12 L system water, 300 g marine salt, and 60 mL brine shrimp.

Rotifer Production and Maintenance

Rotifers (*Brachionus plicatilis;* L-type rotifer) are maintained as a continuous culture at a density of ~ 20-100 rotifers per mL 15 ppt marine salt (Coral Life)

solution in a 15 L conical hatching jar (CCH10; Aquatic Habitats) at 26.5 °C in the fish room.

Starting a new culture:

In event of a culture crash, fresh rotifers can be purchased from Reed Mariculture (Marine Rotifers [L-Type]; 1 million). Fresh rotifers can be kept in 4 °C for up to two days before beginning continuous culture.

1. Fill conical hatching jar with 15 L MilliQ water and 225 g marine salt.
2. While leaving rotifers in delivery bag, place rotifers in conical hatching jar to allow temperature equilibration for 30 minutes.
3. Cut open bag and release rotifers into hatching jar
4. Rotifers are then fed RotiGrow Plus (Reed Mariculture; aliquot into 50 ml falcon tubes and store in -20 °C) NOTE: Add RotiGrow Plus dropwise until water is tinted green.
5. Three days after starting a new culture, the culture undergoes an entire water change and hatchery cleaning.
6. Keep water tinted green with RotiGrow Plus. Feed Rotifers 2x a day (1 mL each time) with RotiGrow Plus. NEVER ALLOW CULTURE TO APPEAR CLEAR OR ROTIFERS WILL STARVE!

Daily maintenance of continuous culture:

To maintain fresh rotifer production, at least 25% culture volume must be replaced with fresh 15 ppt marine salt solution when density increases. Rotifer feeding with 1 ml RotiGrow Plus is required once in the morning and once in the afternoon.

1. Determine rotifer density by pipetting 1 ml culture into a glass vial and counting the number of rotifers present. (If this number is <75, it may be best not to perform a water change that day)
2. If there are larval fish to raise, see "**Feeding Baby Fish"**.
3. Drain 25% of rotifer culture into the sink and refill to 15 L with fresh 15 ppt marine salt solution (60 g marine salt in 4 L MilliQ water)

4. Feed rotifers 1 ml Rotigrow Plus. NOTE: On weekends, a single feeding of 2 ml RotiGrow Plus is sufficient. However, water changes are mandatory unless otherwise instructed.

5. Every week, the rotifer culture undergoes an entire water change and hatchery cleaning. In short, collect all rotifers in compound net (see below) and replace entire 15 L of 15 ppt water.

Feeding Baby Fish

Zebrafish larvae are raised until 5 dpf in a 28.5 °C incubator. At 5 dpf, larvae are fed rotifers until 10 - 12 dpf (5 - 7 days of rotifer feeding) and then are introduced into the adult system and fed brine shrimp until adulthood. Rotifers are cultured at 15 ppt salinity; however, they are co- cultured with zebrafish larvae at 5 ppt salinity. It is not the rotifers themselves that provide nutrition to the larvae but rather the food the rotifers eat. Starved rotifers will do no good for raising baby fish!

1. Transfer 5 dpf larvae into approximately 200 ml volume of 5 ppt water (~ 30-40 larvae per 3 L tank).

2. After checking rotifer density, drain 10 - 25% (depending on density) of rotifer culture into a compound collection net composed of a 150 μm mesh (N2150A; Aquatic Habitats) within a 25 μm bag (N1025; Aquatic Habitats). Large debris are filtered out through the 150 μm mesh and the rotifers are retained within the 25 μm bag. (See *Daily maintenance of continuous culture* for instructions of how to maintain culture.)

3. Resuspend collected rotifers in 5 ppt water

4. Feed enough rotifers to the zebrafish larvae to create a “blizzard” of rotifers (increases chance that larvae will find and consume the rotifers)

5. Add enough RotiGrow Plus to the co-culture (larvae + rotifers) to tint the water green.

6. The next morning, replace co-culture water with fresh 200 ml 5 ppt water and re-dose with freshly collected rotifers.

7. Repeat until the larvae are are 10-12 dpf and introduce larvae into the adult system.
8. Once larvae are 45+ dpf, move larvae into larger tanks to provide space for growth

Fish Mating

Setting up Crosses

1. Set up mating crosses about one hour after the second feeding.

2. Assemble Aquatic Ecosystems breeding tanks with plastic partitions and fill with system water.

3. Place 1-5 female fish in one compartment and an equal number of male fish in the other.

4. Keep the segregated fish in the climate- and light-controlled fish room to allow mating the next morning.

5. Remove the plastic partitions to allow the fish to mate as soon as possible after the light comes on the next morning (7 AM).

6. Check for eggs every 30 minutes or so and collect them in separate Petri dishes.

7. Wash eggs extensively with egg water, using a mesh sieve to remove debris.

8. Place eggs in new Petri dishes, approximately 50 embryos/10-cm dish with about 30 mL of egg water.

9. Incubate eggs at the external 28.5 °C incubator to ensure properly staging. Remove dead or contaminated eggs regularly to ensure maximal survival rates.

Bleaching Embryos for Main System Aquaculture

Embryos to be transferred to the Main System *must* be bleached in a 50 ppm bleach solution prior to their introduction. Procedures for this process can be found in Nüsslein-Volhard's Zebrafish book, which is located in the laboratory.

Prepare a 50 ppm sodium hypochlorite solution, 0.5 g/L sodium thiosulfate solution, and 0.5X E2 embryo medium. First, remove dead eggs, rinse thoroughly with system water, then transfer eggs to hypochlorite solution and swirl for 10 minutes. Quench in thiosulfate solution by swirling for 1 minute, then transfer to the embryo medium and swirl for an additional minute. Finally, place the eggs into a clean sterile petri dish containing methylene blue working solution and label appropriately.

Tank Labeling

All aquaculture tanks containing zebrafish larvae and adult fish should be clearly labeled using removable tape. Wildtype fish and transgenic fish should be segregated (except for crosses) and should be housed in separate aquaculture tanks. Tank labels should include information on the fish line (wildtype, transgenic, or mutant strain) and the date of birth. Please note that the use of transgenic fish is restricted by the California Department of Fish and Game. No transgenic fish should ever leave the lab without proper authorization.

Tank Sanitization

Aquaculture and mating tanks should be sanitized between uses, particularly in preparation for Main System fish. The tanks should be soaked for 24 hours in a 0.6% solution of sodium hypochlorite and soaked for at least one hour in a 0.3% solution of sodium thiosulfate (to quench any residual sodium hypochlorite). The tanks should then be transferred to the Chen Lab dishwasher located in CCSR 3141 and washed using the normal cycle on sterilization mode. Sterilized aquaculture tanks should be stored in the designated laboratory shelves. Sterilized mating tanks should be stored on the carts located in the fish facility itself. Sodium hypochlorite and sodium thiosulfate baths should be changed monthly.

Fish Net Sanitization

Fish nets should be sanitized between each use, and care should be taken to segregate nets used with Main System versus Quarantine System fish. The nets should be soaked for at least 30 minutes in Virkon S disinfectant solution (1

tablet/500 mL) and then washed with copious amounts of MilliQ water prior to use. This step is especially important since trace amounts of disinfectant can kill the fish. The Virkon S cleaning solutions should be changed weekly.

Emergency Preparedness

In the event that either the main or quarantine system water flow shuts off, the Sensaphone monitoring system will alarm and call the following phone numbers in this order until someone answers: the laboratory telephone, Robert Pearce (CSB Operations Manager), and James Chen (Principal Investigator. If the Sensaphone alarms, proceed immediately into the fish facility and turn off the alarm. Assess the situation and turn the water flow back on if it is safe to do so.