In Vitro Fertilization Experimental Protocol

For production of large groups of synchronous embryos, propagation of uncooperative, or interbreeding of isolated stocks (i.e. quarantine):

Materials needed:
Dissecting microscope with overhead light source
Millipore Filter forceps
Sponge with groove
Tygon tubing (1/32" ID x 3/32" OD)
WPI glass capillaries (1.0 MM ID, 3", thin walled)
Plastic spoon with drilled holes
Pipet (20 or 200 ul) with unfiltered tips
Razor blade
Tricaine solution (see recipe below); *keep on ice*
Plastic beaker, 250 ml
Ice bucket
Hank's Buffered Saline (see recipe below); *keep on ice*
Strips or individual PCR tubes
Petri dishes
Paper towels
Plastic bulb transfer pipet
Marker
Methylene Blue water (see recipe below)

On the night before the experiment:

1. Set up the fish in crossing cages. It is important for the males and females to share the same water to ensure that the fish are chemically primed (pheremones) for breeding, yet remain physically separated until the morning of the experiment.
For a "mouse-cage"-style crossing cage, fill the bottom with system water. Place up to 4-5 males into this compartment. Fit the meshed-lined insert onto the bottom and net up to 4-5 females into the top compartment.

2. Place a lid on the crossing cage and label with the appropriate information, and position the cage in front of a light source (on a timed cycle) for maximum stimulation.

During the morning of the experiment:

Preparing your work area:

3. Get your work area prepped for collecting sperm from the males.
Turn on overhead light source to your dissecting microscope. Take out forceps, sponge, tubing, capillaries, and spoon for this portion of the protocol.

For the sperm extraction tube, take an unfiltered pipet tip and cut off ~1/4 to 1/3 inch of the end. Place the cut end of the tip into a piece of Tygon tubing (~12 inches long)- this will be your mouthpiece. In the other end of the tubing, place a glass capillary.

4. Prepare the working concentration for the tricaine solution: Measure out 4 ml of tricaine stock into 100 ml of system water in a plastic beaker.

5. Measure 10-15 ul of the Hank's saline into a strip or individual PCR tubes and place on ice. They can be numbered for ease of use.

6. Separate the males and females into two different tanks, and set aside a couple of spare tanks for recovery post-extraction.
Anesthetize 1 or 2 male fish by immersion in the Tricaine solution. The males should be fairly heavily anesthetized since they will be out of water for up to 1.5 minutes.
When the fish are initally placed into the tricaine, the respiration will be elevated, and then will gradually decline, as evidenced by the movement of the operculum (gill cover). The fish should be taken out of the Tricaine with a spoon when ocular movement and respiration has slowed to almost negligible levels (~1 to 1.5 minutes total time in Tricaine).

7. Blot the fish on paper towel, especially around the area of the anal or urogenital pore. If this area is not properly dried, then the collected sperm sample quality will be compromised. **WATER WILL ACTIVATE THE SPERM!**

8. Place the fish belly up into the groove of a moistened sponge. Separate the pelvic fins that surround the area of the anal pore, and blot again with a piece of paper towel. Apply gentle pressure with the forceps to the abdomen and stroke towards the tail of the fish, while simultaneously applying gentle suction with the sperm extraction tube. Make sure that the glass capillary is held at roughly a 45-degree angle to maximize the integrity of the seal around the pore.

9. The milky sperm will collect into the glass capillary, and it is important that the applied suction is controlled to keep the same in the end of the capillary. Once the entire sample has been collected, place the end of the capillary into the tube containing the Hanks saline. Expel the sperm into the saline, and draw up and down a few times thereafter to ensure that the entire sample has been transferred. The sperm sample can be kept on ice for up to 1 to 1.5 hours.

10. Place the male fish into a recovery tank filled with system water, and monitor its health status to ensure proper recovery.
Occasionally fish die in an in vitro experiment. Care should be taken not to leave fish in tricaine for too long. Sometimes fish can be revived from too long exposure to tricaine by irrigating their gills with water.

11. Once all sperm samples have been collected, it is now time to extract the eggs from the female fish:
Place 1-2 females into the Tricaine solution. Note that for the females, they do not have to be as heavily anesthetized since they will be out of water for a shorter amount of time.

12. Blot the females on the paper towels in the same manner as the males. **WATER WILL CAUSE THE EGGS TO WATER-HARDEN PREMATURELY AND PREVENT FERTILIZATION!!**

13. Position the female between your thumb and index finger, and apply gentle pressure to the lower abdomen of the fish. If the female is healthy, you should not have to squeeze too hard for the eggs to begin to come out. Collect the eggs into a petri dish.

14. Assess egg quality. If a female has not been crossed in a while, has been kept sexed, overfed, or is old, the quality of the eggs might be suboptimal. Signs of healthy eggs are color (should have a yellow to orange color), translucency, and uniform size. If the eggs have any signs of opacity, fat globules, or are runny in consistency, then they will not become fertilized.

15. Pipet the sperm sample onto the clutch of eggs, and tilt the plate to ensure that the sample is evenly distributed. Wait for 30 seconds.
Add 1 ml of system water with a plastic pipet to the clutch- this will activate the sperm, and hopefully result in fertilization!
Typically, up to 60-90% of good quality eggs and sperm will result in fertilization!

After the experiment:

16. After 1.5 to 2 minutes, add methylene blue water to fill the dish. Label the dish and place in a 28.5-degree incubator.
The dish should be checked in the afternoon of the experiment. Unfertilized eggs and other debris should be removed, and the methylene blue water should be refreshed.

17. Check on the status of the fish. Place back into system tanks if the fish appear healthy. After being used in an in vitro experiment, males and females rested for 3 weeks before being squeezed again.

RECIPES (obtained from the Zebrafish Book):

1. Tricaine:
Tricaine (3-amino benzoic acid ethy lester also called ethyl m-aminoboenzoate) comes in a powdered form from Sigma (Cat.# A-5040). It is also available as Finquel (Part No. C-FINQ-UE)

from Argent Chemical Laboratories, Inc. Make tricaine solution for anesthetizing fish by combining the following in a glass bottle with a screw cap:

400 mg tricaine powder
97.9 ml DD water
2.1 ml 1 M Tris (pH 9).

Adjust pH to ~7. Store this solution in the freezer. (Buy the smallest amount possible because tricaine gets old.) To use tricaine as an anesthetic combine 4 ml into 100 ml of system water.

2. Hanks Buffered Saline:
0.137 M NaCl
5.4 mM KCl
0.25 mM $Na_2H PO_4$
0.44 mM $KH_2 PO_4$
1.3 mM $CaCl_2$
1.0 mM Mg SO_4
4.2 mM $NaH CO_3$
in RO water

3. Methylene Blue water for embryos:
20 ml of 0.1% methylene blue stock solution
1.2 g of Instant Ocean
20 L of RO water