**Embryo Production By In Vitro Fertilization**

(Source: C. Walker and G. Streisinger from Zebrafish Book 5th Edition)

**Overview of in vitro fertilization**

Large numbers of synchronously developing embryos can be obtained by *in vitro* fertilization. Maintain breeding males and females on a daily schedule as described in the Zebrafish Breeding Schedule for Maximal Embryo Production. Gametes are expressed from breeding adults by gentle pressure. The sperm are maintained in Hank's saline. The gametes are mixed together in a Petri dish. When water is added to the egg-sperm mixture, fertilization takes place very rapidly in 20 to 60 seconds. After 1 minute, the sperm are no longer active. The clutch of embryos is fertilized essentially synchronously and develops synchronously during the early cleavage stages if maintained at constant temperature.

1. Transfer the males and females into separate holding tanks during the late afternoon of the day before beginning the *in vitro* fertilization.
2. One half hour before "dawn" for the females, collect sperm from the males.
   a. Anesthetize the male fish by immersion in tricaine (see RECIPES, Chapter 10). They can be lifted out of the anesthetic with a plastic spoon.
   b. Rinse in fish water, and place belly up in a slit in a damp sponge.
   c. Gently blot the genital region with a Kimwipe so that no water is present (very important: water activates sperm).
   d. Stroke the sides of the fish gently but firmly with smooth (Millipore) forceps.
   e. As the milky sperm come out of the genital pore, collect them in a microcapillary using gentle suction.
   f. Pool the sperm from several males in ice-cold, full-strength Hank's saline. Sperm from 5-10 males is adequate for fertilization of several hundred eggs. Sperm in cold Hank's will continue to fertilize eggs efficiently for up to 90 minutes. "Estimate" the concentration of sperm, collecting enough to make a cloudy suspension.
3. When the normal light cycle begins in the morning, start collecting eggs.
   a. Anesthetize a female in tricaine.
   b. Rinse in fish water and blot "damp-dry" on a paper towel. Excess water will swell the eggs and prevent fertilization.
   c. Place the female in a 35 mm plastic Petri dish and with damp (not wet) fingers, press gently but firmly on the belly. If she is prepared to lay eggs, they will come out quite easily.
   d. Gather the eggs with a spatula and return the female to water. Good eggs are a yellowish, translucent color, whereas eggs that have remained in the female too long are white and watery. To ensure getting good eggs, collect them during the first 90 minutes after "dawn".
4. Fertilize the eggs.
   a. Add 30-50 l of the sperm suspension in Hank's to the eggs.
   b. Mix gently.
   c. Add about 0.5-0.75 ml of system water and after 1-2 minutes, add 2 ml more water. The actual time of fertilization is when the first water is added to the eggs. Typically, 60-90% of the eggs are fertilized in an *in vitro* cross.

Depending on health or unknown factors, females will produce:

1. **good eggs** (slightly yellowish, granular-looking eggs);
2. **bad eggs** (eggs already broken down, whitish and rather like baby cereal);
3. **mixed eggs** (some good eggs mixed with bad eggs); and
4. **no eggs**

It is possible that females produce and reabsorb their eggs every day and the majority of females are synchronized to lay their eggs at "dawn".

After being used in an *in vitro* experiment, males should be rested for 3 weeks and females rested for 4 weeks before being squeezed again. They may be used for natural crosses during that rest time.

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. Also, extra care should be taken not to damage the gills. Handling fish "front-to-back" when using spoons and spatulas is helpful. One characteristic of sick fish is that their gills hemorrhage easily and the fish die after exposure to tricaine.

**General procedures and overview**

*Why Do We Squeeze?*

- To obtain large numbers of synchronously developing embryos. (Average clutch size: 100 embryos)
- To obtain haploid embryos, such as when identifying heterozygous mutants.
- To obtain embryos for Early Pressure.
- To obtain embryos for Heat Shock.

*Setting Up For A Squeezing Experiment*

1. Set up is done after 3:30 p.m. the afternoon before the experiment.
2. Tanks of prospective fish for squeezing are chosen by:
   a. **Appearance of fish:**
      i. Females should look large and fat in the belly.
      ii. Males should look yellow and spry.
   b. **Elapsed time since fish have been squeezed:**
      i. Fish should not be squeezed more than once/month.
      ii. Longer intervals between squeezing are better.
3. Separate fish during the night in holding tanks. Put males into tanks on a light cycle running ½ hour earlier than females to allow time for obtaining sperm in the morning.
4. **Materials to gather**

#### Male squeezing station

a. i. Place on 2 paper towels the following:
   1. 2 - 300 ml beakers for tricaine
   2. 1 - 10 ml beaker for Hank's solution
   3. 1 - finger bowl for rinsing fish
   4. 1 - plastic spoon
   5. 1 - pair of forceps for squeezing males
   6. 2 - plastic dishes with a (red) sponge with a slit to hold fish while squeezing
   7. 1 - small "single burst" test tube

b. Female squeezing station (per person squeezing)

   i. Place on 2 paper towels the following:
      1. 1 - finger bowl for rinsing fish (beakers of tricaine from males can also be used for females)
      2. 1 - 50 l Wiretrol
      3. 1 - spatula or 2, if dividing clutches
      4. 1 - plastic spoon
         a. 1 - “Sharpie” marker pen

Place a liter beaker of fish water at the fertilizing station with a 25 ml pipette and 2-1 ml pipettes in it.

Make sure there is Hank’s Premix solution, and tricaine in the refrigerator and premeasured sodium bicarbonate near the fertilizing station. If not, see RECI PES, Chapter 10.

#### Morning of the Squeeze

1. Get ice in the ice bucket.
2. To premeasured sodium bicarbonate (0.35 g), add 10 ml dH₂O.
3. Measure 9.9 ml of Hank’s Premix solution into a clean test tube with a screw cap.
   a. Add 0.1 ml fresh bicarbonate solution to the Hank’s Premix.
   b. Put the Hank’s completed solution on ice.
4. Cut "single burst" test tube for sperm. If doing UV sperm, make 2 test tubes.
   a. Label the top of one cork for UV sperm.
   b. Put empty corked test tubes on ice.

#### Squeezing males

**Materials Needed**

- Tricaine solution in two 250 ml beakers
- Stereo microscope
- Sperm collection apparatus
- 20 ml beaker of Hank’s solution
- Finger bowl with fish water
- Lamp
- Kimwipes®
- Male fish
- A fish net

**Preparation**

1. Estimate the number of egg clutches to be obtained.
2. Measure 0.05 ml of Hank's for every clutch of eggs anticipated.
3. Put the measured Hank's in the small test tube, cover with cork and put in ice.
4. Put some of the remaining Hank's solution into the 10 ml beaker.
5. Put the capillary tube of the sperm collecting apparatus into the Hank's solution in the 10 ml beaker when not collecting sperm during the procedure.

**Procedure**

1. Remove two fish from the plastic holding container with net and place into the 250 ml beaker containing the tricaine solution. Repeat this for the other 250 ml beaker.
2. When gill movement has slowed, remove one fish with the plastic spoon.
3. Rinse this fish in the water in the finger bowl and place it upside down in the small sponge in the plastic dish.
4. Gently wipe the region of the anal fin with the corner of a Kimwipe®.
5. Place the dish with the fish under the objective of the microscope, with the light illuminating the fish, especially the region of the anal fin.
6. With the capillary tube of the sperm collecting apparatus, gently push aside the anal fins to expose the anus.
7. Using the forceps gently squeeze the sides of the fish at a point just anterior to the anal fins, collecting the sperm with the capillary tube. When finished return the fish to the finger bowl or recovery container.
8. When you have collected sperm from 23 fish, add them to the Hank’s solution in the small test tube in the ice bucket.
9. Repeat until you have collected the required amount of sperm.
10. Keep the small test tube with the sperm and Hank’s solution in the ice. This helps prolong the viability of the sperm.

#### Squeezing females

**Materials needed**

- Tricaine solution in two 250 ml beakers
- Sperm in Hank’s solution
- One package, 35 mm Petri dishes
- 50 l micropipettes (Drummond Wiretrol)
- Plungers for micropipettes
• Egg Water
• 2-18mm x 150mm test tubes
• 2-1 ml pipettes and a 25 ml pipette in a liter beaker of fish water
• Clean tank water
• Female fish

**Preparation**

1. Fill the large test tubes with egg water.
2. Put one plunger into each test tube.
3. Fill finger bowls with clean tank water.

**Procedure**

1. Place two females into the tricaine solution in each of the two 250 ml beakers.
2. When gill movement has slowed, remove one of the fish with the plastic spoon.
3. Rinse the fish in the water in the finger bowl.
4. Gently place the fish on a paper towel to dry briefly.
5. Using the spoon, transfer the fish into a small plastic dish.
6. Slightly dampen your fingers.
7. Place one finger of one hand on the dorsal side of the fish.
8. Using one finger of the other hand express the eggs by gently pressing on the ventral side of the fish, starting just behind the pectoral fins and moving toward the tail. Only gentle pressure is needed. If the fish has eggs, they will come out easily. If gentle pressure fails to produce eggs, do not continue to squeeze harder. Extra squeezing may injure the fish.
9. If eggs are obtained, use the metal spatula to gently move them away from the fish's body. Then slide the fish out of the dish.
10. Put the fish into a recovery container to revive.
11. When eggs are obtained by squeezing, cover them with the lid to the dish.
12. Repeat this for the remaining fish.

**In vitro fertilization**

**Procedure**

1. Move to the fertilization station and remove a plunger from the water in the test tube. Remove a capillary tube from its container and insert the plunger into the end of the capillary with the green mark.
2. Push the plunger down almost to the end of the tube, leaving about 0.5 cm of air space.
3. Remove the cork from the test tube containing the sperm and insert the capillary tube with the plunger into the sperm.
4. Draw the plunger part way out of the tube to draw sperm up to the black line on the capillary. Be sure to leave air space between the end of the plunger and the sperm.
5. Expel the sperm onto the eggs by pushing the plunger all the way through the capillary so that the tip of the plunger extends out of the end of the capillary.
6. Gently mix the sperm and eggs with the tip of the plunger.
7. Using the 1 ml pipette, add 1 ml of egg water to the egg/sperm mixture. This activates the sperm so that they can fertilize the eggs. The time of fertilization occurs when the WATER is added, not when the sperm is added.
8. Cover the dish with its lid.
9. Allow a few minutes for fertilization to complete, then add more egg water, approximately 2 ml.

**Experiment clean up**

When fish are returned to their tanks, they should be marked with a sticker indicating the date used. Rinse spatulas, Wiretrol, and spoons with tap water. Put away all materials in original places. Enter the number and quality (G,B,M,N) of eggs obtained in *Experiment Done* notebook.

**Follow up of embryos**

Embryos should be left alone for one hour after fertilization. Then:

1. Count fertile embryos.
2. Remove dead and infertile embryos.
3. Transfer groups of 25 embryos into 300 ml beakers with 100 ml of fish water each.

**Morning Following Fertilization**

1. Sort, count, and record the numbers of AA/BB, B/CD and dead embryos.
   - **AA** = Embryos are perfect diploids.
   - **BB** = Diploid embryos that have small imperfections such as a bent tail or one eye.
   - **B** = Very abnormal embryos that have a head, body axis and some sort of tail, but obviously will not survive.
   - **C/D** = Embryos that are yolks with masses of cells on them.
2. Screen according to particular experiment or mutation.