The E400/RMMB cryopreservation protocol was developed at the Zebrafish Int’l Resource Center because of a need for a flexible and reliable protocol. Cryopreservation is used extensively at ZIRC as a line management tool to maintain and distribute the ever-growing resource of zebrafish lines. This protocol is easily scalable to research laboratory needs for repository development. The protocol can be performed by a single person or in pairs. Sperm can be collected by stripping or testes dissection from single or pooled males. Sperm can be collected prior to freezing or you can collect and freeze as you go. Optional quality control in the form of cell counts and test thaws can be performed on pooled samples. Post-thaw motility with this method is higher than other methods previously described for zebrafish. Using this protocol, the average percent fertilization from test thaws at ZIRC is slightly more than 50%. Instructions for thawing frozen sperm and in vitro fertilization follow the cryopreservation protocol.

**Sperm Collection**

Sperm can be collected from males by either testis dissection and/or stripping (also referred to as squeezing or abdominal massage). The choice of collection method is often determined by the number of available males. Testis dissection is a terminal procedure but yields greater amounts of sperm per male. These methods will be described in detail below.

**Male Conditioning**

The males that are intended for sperm freezing are “conditioned” by separating them from the females. The males are kept at low density (5-10 males/1 gallon tank) and fed 3-4x/day (1x brine, 2-3x dry food mix). As long as good water quality is maintained you cannot over feed the males. Large males typically give more sperm and survive squeezing better. Males are conditioned for a minimum of 2-4 weeks prior to sperm collection.

**Mouth Pipetting**

Mouth pipetting using an aspirator tube assembly and glass microcapillary is an acquired skill. It is helpful to practice drawing liquid into the capillary and expelling in a controlled manner. The capillary can be shortened by breaking off the top 3-4 cm. A shortened capillary can be easier to maneuver. When collecting sperm, it takes very little suction to start the sperm into the capillary. Capillary action will often take over and no suction is needed. Over aspiration of the sperm up into the capillary is a common problem. When this happens, the meniscus is often lost and the sperm spreads out to a thin layer on the inside of the capillary. If this happens, first try to expel sperm from the capillary into your collection tube. Then rinse the inside of the capillary by drawing up a small amount of E400 into the capillary and then expel and collect in the sample tube. Another common problem is bubbles forming when the sperm is expelled. To prevent bubbles, hold the capillary against the inside of the microcentrifuge tube and expel just above the liquid level. If bubbles do form, hold the capillary a small distance above the sperm solution and expel a soft stream of air to disrupt the bubbles.

**Importance of Osmolality**

Osmolality is defined as the number of dissolved particles in a fluid. Osmolality is important to keep in mind because zebrafish sperm motility is controlled by the osmolality of the external medium. Zebrafish sperm is
immotile in the testes. The osmolality for complete inhibition of motility is ≥ 300 mmol/kg. This corresponds closely to the measured osmolality of zebrafish blood plasma (296 +/- 8 mmol/kg) and the seminal plasma osmolality of other cyprinids (~300 mmol/kg) (Yang et al., 2007; Alavi and Cosson, 2006). Similar to other freshwater fishes, zebrafish sperm is activated by suspending the sperm in a hypotonic solution. Zebrafish sperm is activated below ~280 mmol/kg. After activation, sperm swim from seconds to minutes. The speed and duration of swimming is also dependent on the osmolality. At low osmolality, activated sperm swim faster and for a shorter duration. At higher osmolality, activated sperm swim slower but for longer duration. Motility activation in zebrafish sperm is reversible. Motility can be stopped by raising the osmolality back to >300mmol/kg.

**Urine Contamination in Stripped Sperm**
When squeezing sperm from males it is important to know that the clear watery substance that you often get is urine. Urine from freshwater fish is very dilute and can activate the sperm. This activation can be stopped by raising the osmolality. The sperm extender E400 is a high potassium, buffered salt solution at an osmolality of 400 mmol/kg designed to counteract the activation by urine. It is important to stop the activation as soon as possible. The stripped sperm is stable in the E400 extender for several hours when held on ice. Sperm can be collected in the morning and frozen in the afternoon.

**Rating Stripped Sperm**
A simple rating system is used to communicate the quantity and quality of stripped sperm. The quantity number rating (1-6) is based on a millimeter measurement of sperm collected within the Drummond 10 µL calibrated microcapillary pipet. The quality (neutral or +) is based on the density of the sperm collected. A dense (+) concentration of sperm will appear bright white, whereas a neutral concentration will appear more dilute (see examples below).

**Stripped Sperm Ratings**

<table>
<thead>
<tr>
<th>Rating</th>
<th>Quantity</th>
<th>Quality/Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-3 mm</td>
<td>Neutral</td>
</tr>
<tr>
<td>1+</td>
<td>1-3 mm</td>
<td>Good</td>
</tr>
<tr>
<td>2</td>
<td>4-6 mm</td>
<td>Neutral</td>
</tr>
<tr>
<td>2+</td>
<td>4-6 mm</td>
<td>Good</td>
</tr>
<tr>
<td>3</td>
<td>7-9 mm</td>
<td>Neutral</td>
</tr>
<tr>
<td>3+</td>
<td>7-9 mm</td>
<td>Good</td>
</tr>
<tr>
<td>4</td>
<td>10-12 mm</td>
<td>Neutral</td>
</tr>
<tr>
<td>4+</td>
<td>10-12 mm</td>
<td>Good</td>
</tr>
<tr>
<td>5</td>
<td>13-15 mm</td>
<td>Neutral</td>
</tr>
<tr>
<td>5+</td>
<td>13-15 mm</td>
<td>Good</td>
</tr>
<tr>
<td>6</td>
<td>16-18 mm</td>
<td>Neutral</td>
</tr>
<tr>
<td>6+</td>
<td>16-18 mm</td>
<td>Good</td>
</tr>
</tbody>
</table>

**Pooling Sperm**
Pooling sperm from multiple males is advantageous because it eliminates the variability in sperm concentration and quality that is possible between individual males. Pooled sperm can be collected by stripping or testis dissection. Pooling sperm from available males is the preferred collection method if there are not genetic reasons that require the freezing of individual males. The larger volume of pooled sperm allows for quality control such as concentration determination. Test thaws from pooled samples can also offer greater confidence because the results of a single test thaw represent an entire group of samples.
Collecting Stripped Sperm

Materials for Stripping Sperm
Dissecting microscope with above-stage lighting
10 µL calibrated microcapillary pipettes (Drummond # 2-000-010)
Aspirator tube assembly (included in each pack of microcapillary pipettes)
Millipore forceps (Millipore # XX6200006P) (Rubber tips – electrical shrink tubing)
Plastic spoon for moving fish
Sponge fish holder (in 35 x 10 mm petri dish)
Drawer/shelf liner anti-slip square(s)
Soft paper towels
0.6 mL microcentrifuge tubes (Fisher # 02-681-311)
Ice bucket

Solutions for Stripping Sperm
Tricaine anesthesia
Isotonic PBS rinse
Fish water for recovery
E400 sperm extender

Procedure for Collecting Stripped Sperm
1. Anesthetize male zebrafish in Tricaine solution, briefly rinse (in PBS Isotonic fish rinse or fish water), dry fish by rolling on paper towel, place fish in dorsal recumbency (belly up) in a dampened foam holder.

2. Collect stripped sperm into a 10 µL calibrated microcapillary pipette. Use Millipore forceps to gently stroke the sides of the male fish in an anterior to posterior/dorsal to ventral motion. To collect sperm, place capillary on urogenital opening as sperm is expelled and draw up into capillary. Recover fish in fresh fish water.

3. Without delay, normalize the sperm volume with E400 sperm extender. The normalizing volume can be adjusted for the number of samples desired from single males (1 or 2 samples/male) or for pooling sperm (see Normalizing Volume table below). The 10 µL microcapillary pipettes can be marked at 5 µL (or 25 mm) using a permanent marker. Draw E400 sperm extender into capillary up to the appropriate mark (5 or 10 uL).
Normalizing Volume:

<table>
<thead>
<tr>
<th>Freezing Method</th>
<th>Sample(s)/male</th>
<th>Normalize to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single male freezing</td>
<td>1</td>
<td>5 µL</td>
</tr>
<tr>
<td>Single male freezing</td>
<td>2</td>
<td>10 µL</td>
</tr>
<tr>
<td>Pooled sperm</td>
<td>--</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

4. If freezing from single males, immediately expel sperm into microcentrifuge containing RMMB cryo medium and proceed with freezing (see Freezing from Single Males Procedure below).

5. If pooling sperm from multiple males, expel the collected sperm (+E400) into a microcentrifuge tube on ice. Continue collecting stripped sperm from all available males and pool sperm into the same tube.

Collecting Sperm by Testis Dissection

Materials for Testis Dissection
All materials for sperm stripping
Euthanasia baths (Tricaine anesthesia followed by ice bath)
Micro dissecting scissors (BRI # 11-1390)
Curved serrated forceps (Ted Pella # 5002-53)
Curved retracting tweezers (Ted Pella # 5727)
2 pair fine-tip forceps (Ted Pella # 5360-NM)
1 3/8” mini alligator clip (Radio Shack)
2 x 100 mL polypropylene beakers (Nalgene # 1201-0100, for cleaning instruments between fish)
Klick-fix ® pen holder or poster putty (to support retracting tweezers, art/office supply store)
Weighing paper (Fisher # 09-898-12A)
Filter paper wedges (cut from Whatman grade 3 circles, # 1003-125)
Cotton swabs (Ted Pella # 80911 or alternative)
Kimwipes (Kimberly-Clark # 34155)

Solutions for Testis Dissection
Tricaine anesthesia
Ice slurry bath for euthanasia
Isotonic PBS rinse
E400 sperm extender
Procedure for Collecting Sperm by Testis Dissection
To prevent loss of sperm present in the distal testicular ducts during dissection, males are first stripped and then the testes are removed. The stripped sperm and testes are pooled together in E400 sperm extender on ice.

1. Prepare a microcentrifuge tube (0.6 mL, clear) for sperm collection containing E400 based on the number of males to be dissected (10-15 µL/male) or the number of samples desired (5 µL x # samples). Use whichever is the smaller volume of E400 to keep the sperm concentrated. The sperm can be further diluted if desired prior to freezing. Keep tube on ice.

2. Euthanize male zebrafish just prior to sperm collection in Tricaine solution and/or a crushed ice slurry, dry fish by rolling on paper towel. Position the male on a dissecting microscope stage on a piece of weigh paper in dorsal recumbency (belly up) by grasping the caudal peduncle in a small alligator clip.

3. Dry the urogenital pore with a cotton-tipped swab. Collect stripped sperm into a 10 µL microcapillary pipette. Use Millipore forceps to gently stroke the sides of the male fish in an anterior to posterior/ dorsal to ventral motion. To collect sperm, place capillary on urogenital opening as sperm is expelled and draw up into capillary. Quickly expel the sperm into the E400 in the microcentrifuge tube on ice.

4. Using small serrated forceps and micro-dissecting scissors, pinch the ventral body wall of the cranial abdomen and make a small incision.
5. Insert the tip of the scissors just under the body wall and make two superficial cuts through the body wall encircling the urogenital opening and pelvic fins.

6. Remove the flap of tissue created being careful to not disrupt the internal tissues. Grasp the distal intestine and retract in a cranial direction to expose the bilateral testes.
7. For better exposure, retract the body wall laterally using curved fine-tipped tweezers. The testes are identified as a bilateral, linear white tissue just ventral to the swimbladder. If blood or fluid is present in the body cavity, carefully absorb it with the tip of a Kimwipe or small wedge of filter paper.

8. Use a pair of fine-tipped forceps to remove or gather the fragile testes trying not tear the tissue. Add the dissected testes to the microcentrifuge tube on ice containing the E400 and stripped sperm.
9. If pooling sperm from multiple males continue collecting stripped sperm and testes from available males and pool into the same microcentrifuge tube on ice.

10. Once all the testes have been collected, gently disrupt the tissue by pressing the tissue with forceps against the inside of the microcentrifuge tube. The goal is to gently tear the testes to release the sperm without mashing it. Mix the contents of the tube by flicking it several times and allow to stand on ice for 1-2 minutes. Mix the tube again by flicking and then carefully remove the remaining testis tissue. Blot the tissue on the inside of the microcentrifuge tube to avoid losing the sperm in suspension.
**Cryopreservation of Sperm**

Sperm can be frozen from individual males or as pooled sperm. Both methods will be described below.

**Toxicity of RMMB Cryo Medium**

As with most cryoprotectant media, the RMMB Cryo medium is somewhat toxic to the sperm cells. There is a decrease in sperm post-thaw motility associated with longer equilibration times in the cryo medium prior to freezing. Because of this toxicity, the timing of freezing should be as fast as possible once the RMMB cryo medium is mixed with the sperm. If the final sperm volume is greater than 60 µL or 12 samples, it is recommended to divide the sperm into more than one tube and freeze in batches.

**Powered Dry Ice from Liquid CO₂**

A powdered dry ice is preferred over crushed or pelleted dry ice for consistent and reproducible freeze rates. To produce powered dry ice from CO₂, a fire extinguisher cone is attached to the liquid outlet of a CO₂ Dewer with a siphon tube. It is useful to angle the cone 90° downward (see Image below). Position a Styrofoam box or cooler directly under the cone to catch the dry ice as it exits the cone. For personnel safety, a face shield, ear protection and cryo gloves are recommended. Open the liquid CO₂ outlet valve quickly and adjust to prevent surging and clogging of the outlet. Fill cooler to a minimum depth of 20 cm (approximately 1.5x the length of a 15 mL conical tube with cap). Sublimation to CO₂ gas occurs more rapidly as the container initially cools so filling the cooler slightly higher maybe desired. The dry ice powder can be lightly compacted as the cooler fills. Once filled, simply turn off the CO₂ outlet valve. Smooth the surface with a gloved hand and it is ready to use.
Freeze Rate
Freeze rate is something that is critical for optimal freezing success. The optimum freeze rate for the method is approximately 20°C/min as measured using a controlled rate freezer. To mimic this rate with dry ice, the 2 mL sample cryo vial is placed on top of an empty 2 mL cryo vial (without the cap) inside a 15 mL conical centrifuge tube (see photos below). The configuration for freezing in 0.5-1.0 mL Matrix cryo vials is also shown below. The Matrix cryo vial is placed on top of the 2 mL spacer vial with cap within the conical tube. The conical tubes are capped prior to driving them down into the dry ice. Do not pre-chill the falcon tubes. They should be at room temperature prior to freezing.

Left: Rack of 15 mL conical tubes containing a 2 mL cryo vial (w/o cap) spacers.
Middle: Configurations of conical tubes with sample vials (on top) and spacer vials (on bottom) resulting in approximate 20°C/min freeze rate in dry ice. The falcon tube is capped prior to being driven down into the dry ice. Middle-Left: Configuration for freezing in 2 mL sample cryo vials. Sample cryo vial is placed on top of the 2 mL spacer vial without cap. Middle-Right: Configuration for freezing in 0.5-1.0 mL Matrix cryo vials. Matrix cryo vial is placed on top of the 2 mL spacer vial with cap. Right: Samples in conical tubes being frozen in dry ice (one sample per tube).

Time in dry ice
There is a minimum time in dry ice that is needed (20 minutes) but the total time is not critical but should not exceed 60 minutes. Freeze in dry ice for 20-60 minutes.

Ratio of Sperm (in E400) to RMMB Freezing Medium
In all cases with this protocol, the ratio of sperm in E400 to RMMB Freezing Medium is 1 part sperm to 3 parts RMMB. The sample volume is typically 20 µL, so that equals 5 µL sperm (in E400) + 15 µL RMMB for each sample.

Materials for Sperm Freezing
2 mL cryogenic vials (Corning # 430488, or alternative)
Vial color coders (Nalgene # NNI #15-350-45 to 49, or alternative)
Pipetman and tips
15 mL conical tubes with 2 mL cryo vial spacers
Styrofoam container or cooler (“12” x 12”) for dry ice
Styrofoam container for liquid nitrogen (LN₂) tray
Fiberglass tray (US Plastic Corp # 49273) for LN₂
10x10 Cryobox (Taylor Wharton # R24K-9C44, or alternative)
Liquid nitrogen dewar flask
Cryogloves (Tempshield # 11-394-306)
Solutions for Sperm Freezing
E400 sperm extender
RMMB cryo medium

Single Male Freezing Procedure (2 samples/male) - collect sperm & freeze as you go

Preparations
Prepare 0.5 mL microfuge tubes (colored, green) containing 500 µL E400 each. This will be used for normalizing the sperm volume. Prepare one tube for each line being frozen. Keep chilled on ice until ready to squeeze males.

Prepare 0.5 mL microfuge tubes (clear) containing 30 µL RMMB freezing medium each. Prepare one tube per male. This will result in 2 samples/male.

For best results when freezing two samples/male, a minimum stripped sperm rating of 1+ with 3 mm of sperm is recommended (see Rating Stripped Sperm).

This method can be easily adjusted to freeze one sample/male. In this case, prepare 0.5 mL microcentrifuge tubes (clear) containing 15 µL (instead of 30 µL) RMMB freezing medium. Prepare one tube per male. Mark the 10 µL microcapillary pipettes at 5 µL (or 25 mm) for normalizing the volume for one sample.

Keep tubes containing RMMB freezing medium at room temperature. The raffinose in the RMMB medium can come out of solution if held on ice for extended periods. If this happen, heat tube briefly until the raffinose is back in solution.

Prepare labeled sample cryo vials as needed.

Freezing Procedure for Single Males
1. Anesthetize male zebrafish in Tricaine solution, briefly rinse (in PBS Isotonic fish rinse or fish water), dry fish by rolling on paper towel, place fish in dorsal recumbency (belly up) in a dampened foam or sponge holder.

2. Collect stripped sperm into microcapillary. Use Millipore forceps to gently stroke the sides of the male fish in an anterior to posterior/ dorsal to ventral motion. To collect sperm, place capillary on urogenital opening as sperm is expelled and draw up into capillary. Recover fish in fresh fish water.
3. Without delay, normalize sperm to the appropriate volume (5 or 10 µL for one or two samples, respectively) with E400 (green tube) and then expel the entire contents of the microcapillary (sperm + E400) into one of the 0.5 mL microfuge tubes (clear) containing RMMB freezing medium (15 or 30 µL for one or two samples, respectively). Mix the total solution by pipetting up and down a 1-2 times and then transfer 20 µL of mixture into each cryo vial (20 µL/sample). Quickly cap the cryo vials.

4. Quickly place the sample cryo vials into the 15 mL conical tubes (containing a 2 mL cryo vial spacer) and cap. Drive tubes down into the dry ice until caps are flush with the surface.
5. Freeze samples in dry ice for 20-60 minutes then quickly transfer samples to a cryo box submerged in LN2.

Pooled Sperm Freezing Procedure - collect and dilute sperm prior to freezing

Diluting Pooled Sperm based on NanoDrop Cell Counts.

Diluting Pooled Sperm without Cell Counts.
Whereas cell counts are useful for quality control, they are not necessary for freezing pooled sperm. As previously mentioned, the ratio of sperm in E400 sperm extender to raffinose freezing medium (RMMB) is 1 part sperm (in E400) to 3 parts RMMB. The sample volume is typically 20 µL, so that equals 5µL sperm (in E400) + 15µL RMMB cryo medium for each sample. You can collect sperm in E400, dilute with additional E400 extender to where you are comfortable and then freeze using this 1 part + 3 parts ratio. A conservative guideline for further diluting pooled sperm, based on the possible number of samples/male, is as follows. Always try to freeze samples at the highest possible cell density.

Stripped sperm only:
1-2 samples/male
Final diluted volume = 5-10 uL x # males

Stripped sperm and dissected testes sperm:
2-5 samples/male
Final diluted volume = 10-25 uL x # males
Preparations
Collect pooled sperm into E400 by squeezing and/or testes dissection as described above.

Measure the volume of pooled sperm in E400 with a Pipetman.

Dilute sperm additionally with E400 as described above and note final volume. If the final pooled sperm volume is > 60 uL (12 samples), divide the sperm volume into more than one tube and freeze in batches.

Prepare labeled sample cryo vials as needed prior to freezing.

For each tube of sperm, calculate the volume of RMMB to add as follows:

\[ \text{RMMB Volume} = 3 \times \text{Sperm Volume} \]

Because the RMMB cryo medium is toxic to the sperm cells, timing of freezing should be as fast as possible once the RMMB cryo medium is mixed with the sperm. It is helpful to have a second person at this point to help cap and get tubes into the dry ice.

Freezing Procedure for Pooled Sperm
1. Add calculated volume (3x sperm volume) of RMMB cryo medium to sperm, mix and aliquot immediately to cryo vials (20 µL each).
2. Without delay, cap the cryo vials and place into the 15 mL conical tubes (containing a 2 mL cryo vial spacer). Cap the conical tubes and drive tubes down into the dry ice until caps are flush with the surface.

3. Freeze samples in dry ice for 20-60 minutes then quickly transfer samples to a cryo box submerged in LN₂.
**Thawing Sperm and In Vitro Fertilization (IVF)**

Females should be setup isolated from males in the afternoon prior to egg collection. The collection and fertilization of eggs should be performed first thing in the morning. Zebrafish egg quality will be best in the first couple hours after the lights come on. Both the eggs and the sperm are activated by water. It is very important to use the Isotonic PBS rinse solution when anesthetizing females for egg collection. It is impossible to dry the female completely so the rinse solution insures there is no freshwater present that can activate the eggs. If multiple clutches are being combined, a fine paint brush is an effective and gentle method to move the eggs. Dip and dry briefly fingers and the paint brush in the Isotonic PBS prior to squeezing females or manipulating clutches.

**Materials for Thawing and IVF**
- Plastic spoon for moving fish
- 35 mm Petri dishes
- White Taklon round paint brush, size 2
- Pipetman (P200 and P1000) and tips
- Water bath @ 38°C
- Timer – 2 min. count down
- Embryo medium or fish water

**Solutions for Thawing and IVF**
- MS-222 pre-anesthesia (48 ug/L)
- MS-222 anesthesia (168ug/L)/
  - PBS rinse/Recovery fish water
- Sperm Solution (SS300 +/- 2mg/mL
  - Difco Skim Milk)
- dH2O

**Thawing and IVF Procedure**

1. Place females into pre-anesthesia solution at least 5-10 minutes prior to anesthesia. Females can be held in the pre-anesthesia solution until used for egg collection.

2. Anesthetize female zebrafish in Tricaine solution, rinse fish in PBS isotonic rinse, dry fish by rolling on paper towel, place fish on its side (lateral recumbency) in a 35 mm perti dish. Dampen fingers in PBS fish rinse. Collect eggs from females by applying light digital pressure on the ventral abdomen in an anterior to posterior direction. Eggs will expel easily if the female is ready. Eggs can be moved away from the female using a fine paint brush dampened in the PBS isotonic solution. Transfer the female to a recovery tank. Good eggs will be golden in color, have very little fluid and no opaque or white eggs intermixed. Several clutches of eggs can be pooled together by gently moving eggs to one dish with the paint brush.
3. Remove the sperm sample cryo vial from the LN₂, quickly open cap to vent any LN₂ in the vial.

4. Thaw cryo vial in a 38°C water bath until the frozen pellet is < 3mm (~10-15 sec).

5. Add 150 µL SS300 solution (at room temp.) to the cryo vial. (If thawing sperm that was frozen without milk, add 2 mg/mL Difco Skim Milk (Difco #232100) to the SS300 to help prevent sticking and tangling of the sperm tails.) For motility assessment, a small portion (10-20 µL) of the sperm/SS300 mix can be removed and held in a microcentrifuge tube on ice (see Post Thaw Sperm Motility procedure below).
6. Add 200 uL dH2O to the cryo vial to activate the sperm. Gently mix sperm 1-2x with pipetman, and transfer onto eggs (slide pipet tip along the bottom of petri dish into the pile of eggs and expel the activated sperm into the mass of eggs (don’t just put on top of the eggs).

7. **Do not** mix or swirl the dish at this point - let it sit completely undisturbed. After 2 min, flood the dish with embryo medium.

8. Observe fertilization rate at 3-4 hours post fertilization (high stage). Count embryos and remove the unfertilized.
**Sperm Motility Assessment**

The best way to assess the quality of sperm is to observe its motility. Computer assisted sperm analysis (CASA) software systems provide the most objective and comprehensive quantification of motility parameters but a manual, subjective assessment is sufficient for most sperm freezing applications. A compound microscope is all that is needed. A 20x objective and DIC or dark field is best. If DIC or dark field is not available, contrast can be increased by simply lowering the condenser of the microscope. Osmolality affects both the speed and duration of sperm motility (see Importance of Osmolality in the Sperm Collection section above). Fresh (pre-freeze) sperm will be faster and have a higher percentage of cells motile than post-thaw samples. There is a very strong correlation between sperm motility and fertility.

**Pre-freeze Motility**

For pre-freeze motility, place a 10 µL drop of dH2O on a microscope slide. Add a very small amount (0.5 µL) of the final sperm dilution to the drop, mix and spread quickly with the pipet tip and observer immediately. Examining the remainder of the NanoDrop dilution, if performed, is a good use of sperm. Checking pre-freeze motility confirms the viable and concentration of the sperm being frozen.

**Post-thaw Sperm Motility**

For post-thaw motility, activating the sperm on the slide in the same relative proportions as in the IVF procedure gives a consistent method and provides a good sense of sperm concentration and motility as it is applied to the eggs. The sperm sample is thawed in a water bath as described above. 150 µL of SS300 solution is added to the thawed sperm and gently mixed. At this point, a small portion (10-20 µL) of the sample can be removed and held on ice for motility assessment. The remainder of the sample can be used for IVF as described above. Its best to view motility as soon as possible after thawing but samples are typically stable on ice for 10-20 minutes. For motility observation of post-thaw sperm, place 5 µL dH2O on a slide, add 4.25 µL of your thawed sperm/SS300 solution, briefly mix with pipet tip on the slide and observe immediately.
Solutions for Sperm Collection, Cryopreservation, Thawing & IVF

Sperm Extender ~400 mmol/kg (E400)

- 130 mM KCl
- 50 mM NaCl
- 2 mM CaCl₂
- 1 mM MgSO₄
- 10 mM D-(+)-Glucose
- 30 mM HEPES-KOH (7.9)

Combine the following to make 1L of E400 according to instructions below:
9.70 g KCl
2.92 g NaCl
2.0 mL 1.0 M CaCl₂ (or 0.29 g CaCl₂·2H₂O)
1.0 mL 1.0 M MgSO₄ (or 0.25 g MgSO₄·7H₂O)
1.80 g D-(+)-Glucose
7.15 g HEPES

Add dH₂O to 800 mL
Add dry ingredients, stir to dissolve
Add liquid ingredients, stir to mix
pH with 5N KOH to 7.9
Bring up final volume to 1000 mL with dH₂O
Check osmolality (If made as directed, osmolality should be very close to 400 mmol/kg)
Filter sterilize
Store at 4°C

1M Bicine-NaOH (pH 8.0)

Combine 16.3g Bicine (Sigma-Aldrich B3876) and 80mL dH₂O in a 250 mL beaker
pH to 8.0 with NaOH
Adjust final volume to 100 mL
Filter sterilize

Raffinose Freezing Medium (RMMB)

- 20% (w/v) D-(+)-Raffinose pentahydrate (Sigma R7630)
- 2.5% (w/v) Difco Skim Milk (Difco #232100)
- 6.67% (v/v) Methanol (Acetone-free, Absolute, Certified ACS Reagent Grade, Fisher Scientific A412)
- 30 mM Bicine-NaOH (pH 8.0)

The following is combined to make 100 mL RMMB according to instructions below
20.0 g D-(+)-Raffinose pentahydrate
2.5 g Difco Skim Milk
6.67 mL Absolute Methanol
3.0 mL 1.0 M Bicine-NaOH (pH 8.0) – see below
dH₂O to 100 mL
Combine 20.0 g Raffinose and 70 mL dH2O in a 250 mL beaker. 
Place beaker in a finger bowl or larger beaker containing hot water (~70°C) on a stir plate
Stir mixture until Raffinose is completely dissolved
Add 2.5 g skim milk, stir until completely dissolved
Cool to room temperature
Add 3.0 mL 1M Bicine-NaOH (8.0)
Add 6.67 mL Absolute MeOH (Acetone-Free)
Transfer to 100 mL volumetric flask, adjust final volume to 100 mL with dH2O, mix by inversion 3-4x
Transfer to two 50 mL conical tubes
Centrifuge @15,000 x g for 20 min. at 25°C
Transfer cleared supernatant into clean 50 mL conical tubes or beaker
Aliquot into 1.5 mL microfuge tubes, 1.0 mL each
Store frozen at -20°C until used

For Use:
Thaw aliquot with heating (water bath or heat block 45-50°C) prior to use
Cool to room temp prior to mixing with sperm
Note: Raffinose will precipitate if the RMMB solution is kept on ice for a prolonged time period. If this occurs, heat solution slightly to get it back into solution prior to use.

**Sperm Solution ~300 mmol/kg (SS300)**

- 140 mM NaCl
- 5 mM KCl
- 1 mM CaCl₂
- 1 mM MgSO₄
- 10 mM D-(+)-Glucose
- 20 mM Tris-Cl (pH 8.0)

Combine the following to make 1L of SS300 according to instructions below:

- 8.2 g NaCl
- 5.0 mL 1M KCl (or 0.37 g KCl)
- 1.0 mL 1.0 M CaCl₂ (or 0.15 g CaCl₂-2H₂O)
- 1.0 mL 1.0 M MgSO₄ (or 0.25 g MgSO₄-7H₂O)
- 1.8 g D-(+)-Glucose
- 20 mL 1M Tris-Cl (pH 8.0)

Add dH₂O to 800 mL
Add dry ingredients, stir to dissolve
Add liquid ingredients, stir to mix
Bring up final volume to 1000 mL with dH₂O
Check osmolality (If made as directed, osmolality should be very close to 300 mmol/kg)
Filter sterilize
Store at 4°C
**Sperm Solution ~300 mmol/kg with 2mg/mL Difco Skim Milk (SS300+Milk)**
Used for thawing sperm samples that were frozen with cryo media not containing milk (other protocols). The milk helps prevent sperm tails from sticking and tangling.

Add 100 mg Difco Skim Milk to 50 mL SS300, stir or vortex to dissolve
Aliquot into microcentrifuge tubes and store frozen at -20°C
Thaw and use at room temperature

**0.5X E2 Embryo Medium** - To make 0.5X E2 Embryo Medium see instructions at http://zebrafish.org/documents/protocols/pdf/Fish_Nursery/E2_solution.pdf

- 7.5 mM NaCl
- 0.25 mM KCl
- 0.5 mM MgSO₄
- 0.5 mM CaCl₂
- 75 μM KH₂PO₄
- 25μM Na₂HPO₄
- 0.35 mM NaHCO₃
- 0.5 mg/L Methylene Blue

**4.0 g/L Tricaine Stock Solution** (Tricaine Methanesulfonate, MS-222)
4.0 g Tricaine (Tricane-S, Western Chemical, Inc)
Add dH₂O to 800 mL
Stir to dissolve
Adjust pH with 1M Tris-Cl (pH 9.0) to ~ pH 7.0
Bring up final volume to 1000 mL with dH₂O
Store in an amber bottle at 4°C

**Pre-Anesthesia Working Solution (48 mg/L)**
Used to reduce mortalities when squeezing females. Females are held in pre-anesthetic solution for 5-60 minutes prior to anesthesia for egg collection.

- 6 mL Tricaine Stock Solution (4.0 g/L)
- 500 mL Fish water
Make fresh prior to use

**Anesthesia Working Solution (168 mg/L)**
4.2 mL Tricaine Stock Solution (4.0 g/L)
100 mL Fish water
Make fresh prior to use
**1M Tris-Cl (pH 9.0)**
121.14 g Trizma base (Sigma T6066)  
Add dH₂O to 800 mL  
Stir to dissolve  
pH with concentrated HCl to pH 9.0 (approx. 10 mL)  
Bring up final volume to 1000 mL with dH₂O  
Filter sterilize

**Isotonic PBS Rinse**  
Used as a post-anesthesia rinse for females to help prevent the pre-activation of eggs

Phosphate Buffered Saline (pH 7.4) powder packets (Sigma #P3813)  
Dissolve in 870 mL dH₂O.  
This will give a final osmolality of approximately 305-315 mmol/kg.

Questions or comments on the ZIRC E400/RMMB Cryopreservation and IVF protocols can be addressed to:

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**References:**  