ZIRC E400/RMMB SPERM CRYOPRESERVATION & IVF PROTOCOL

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The E400/RMMB cryopreservation protocol was developed at the Zebrafish International Resource Center (ZIRC) because of a need for a more 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 testis 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 that are representative of an entire freeze event. Post-thaw motility with this method is higher than other methods previously described for zebrafish and the average percent fertilization from test thaws at ZIRC is slightly above 65%. Instructions for thawing frozen sperm and *in vitro* fertilization follow the cryopreservation protocol.

Table of Contents

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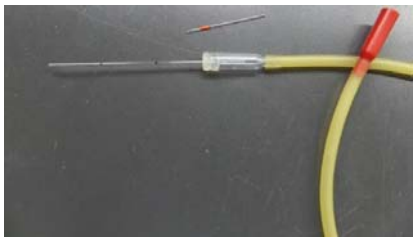
1. Introduction to Sperm Collection

Sperm can be collected from males by 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. Both methods will be described in detail below.

1.1 Male Conditioning

For conditioning, males are separated from females, the density is lowered to 8 males/3.6L tank, and a midday feeding (3 feedings/day, 1x brine shrimp, 2x dry food mix) is added. We start conditioning at 4.5 months and do it for 4 weeks prior to cryopreservation. We recently did a small study on our male conditioning, looking at separation from females, 2 or 3 feedings/day, onset age, density and duration. For our feeding scheme, a density of 8 males/tank worked best. The extra feeding was the most important factor, and the longer the better. Having females mixed with the males was also somewhat beneficial, but you do need to pull the females out the day before sperm collection.

1.2 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. It takes very little suction to collect the sperm with 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 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 sperm collection tube. Then rinse the inside of the capillary by drawing up a small amount of E400 sperm extender into the capillary and then expel into the sperm collection 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.

1.3 Importance of Osmolality

Osmolality is the concentration of a solution expressed as the total number of solute particles per kilogram. 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. For complete inhibition of sperm motility an osmolality of ≥ 300 mmol/kg is required. 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 when exposed to 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 are 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.

1.4 Urine Contamination in Stripped Sperm

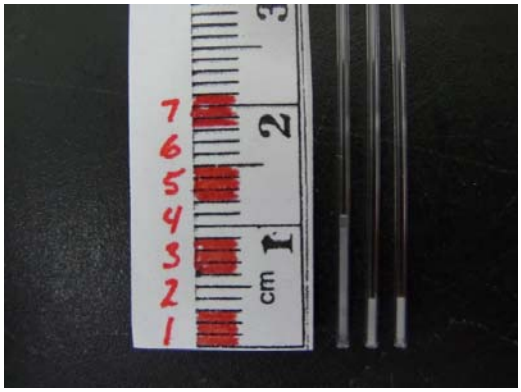
When stripping sperm, it is important to know that the clear, watery substance you often get is urine. Urine from freshwater fish is very dilute and can activate the sperm. This activation can be stopped by raising the osmolality. The sperm extender E400 is a high-potassium, buffered salt solution with an osmolality of 400 mmol/kg, designed to counteract sperm 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.

1.5 Rating Stripped Sperm from Individual Males

A simple rating system is used to communicate the volume and density of stripped sperm collected and frozen from single males. The quantitative number rating (1-6) is based on a millimeter measurement of sperm collected within a Drummond 10 µL calibrated microcapillary pipet. The opacity of the collected sperm (+ or neutral) provides a rough indication of concentration. Highly concentrated sperm will appear bright white, whereas a neutral concentration will appear more clear (see examples below). Transparent or very dilute samples contain mostly urine and should not be used.

Stripped Sperm Ratings

Rating	Quantity	Concentration
1	1-3 mm	Neutral
1+	1-3 mm	Good
2	4-6 mm	Neutral
2+	4-6 mm	Good
3	7-9 mm	Neutral
3+	7-9 mm	Good
4	10-12 mm	Neutral
4+	10-12 mm	Good
5	13-15 mm	Neutral
5+	13-15 mm	Good
6	16-18 mm	Neutral
6+	16-18 mm	Good



1.6 Pooling Sperm

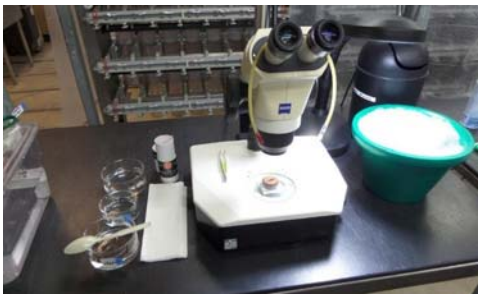
Pooling sperm from multiple males is advantageous because it can average out the variability in sperm concentration and quality that is seen among individual males. Pooled sperm can be collected by stripping or testis dissection. Pooling sperm from available males is the preferred collection method if there are no genetic reasons that require freezing from individuals. The larger volume of pooled sperm allows for quality

assessments such as cell concentration and motility (see Section 5, Quality Control). Test thaws from pooled samples offer greater confidence because the results of a single test thaw represent the entire set of samples.

1.7 Protocol for Collecting Stripped Sperm

1.7.1 Materials for Stripping Sperm

- Dissecting microscope with incident 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), 1-2 inches
- Soft paper towels
- Cotton swab or soft tissue
- 0.6 mL microcentrifuge tubes (Fisher 02-681-311)
- Ice bucket



1.7.2 Solutions for Stripping Sperm

- Tricaine anesthesia
- Isotonic PBS rinse
- Fish water for recovery
- E400 sperm extender

1.7.3 Step-by-step Procedure for Collecting Stripped Sperm

1. Anesthetize male 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. The urogenital pore can be further dried using a cotton tipped swab or soft tissue.

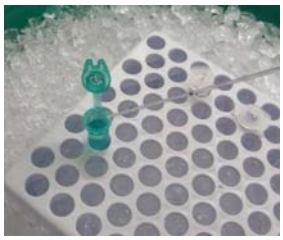
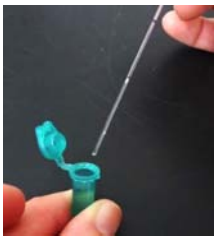
2. To collect sperm, position a 10 µL microcapillary pipette on the urogenital opening. The end of the capillary can be used to move the pelvic fins out of the way if necessary. Use Millipore forceps to apply **gentle** abdominal pressure. Start by positioning the forceps around the sides of the fish, anterior to (just in front of) the urogenital pore. Apply light pressure with the forceps until sperm is seen at the urogenital opening. As sperm is released, collect it into the capillary. Holding the capillary perpendicular and very close to the fish will help start the flow of sperm into the pipet. A small amount of suction may be necessary to initiate the capillary action. The forceps can be slightly repositioned in an anterior to posterior direction to collect additional sperm. Revive the fish in fresh water.

3. Immediately normalize the sperm volume with E400 sperm extender. The normalizing volume can be adjusted for the number of samples desired. The 10 µL microcapillary pipettes can be marked at 5 µL (or 25 mm) using a permanent marker when freezing just one sample per male. To normalize the sperm volume,

draw E400 sperm extender into the capillary up to the appropriate mark (5 or 10 µL). Refer to the following Normalizing Volume Table.

Normalizing Volume:

Freezing Method	Sample(s)/male	Normalize to:
Single male	1	5 µL
Single male	2	10 µL
Pooled sperm	2	10 µL
Pooled sperm	# based on concentration	5 µL

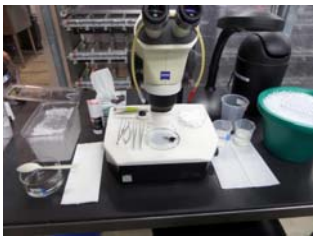
4. If freezing from single males, immediately expel sperm into microcentrifuge tube containing RMMB cryo medium and proceed with freezing (see Protocol for Freezing from Individual Males, Section 2.6).

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, pooling sperm into the same tube. Keep collected sperm on ice. (see Protocol for Freezing Pooled Sperm, Section 2.7).

1.8 Protocol for Collecting Sperm by Testis Dissection

1.8.1 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 dissections)
- 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 (Fisherbrand Cat. No. 23-400-118 or alternative)




1.8.2 Solutions for Testis Dissection

- Tricaine anesthesia
- Ice slurry bath for euthanasia
- Isotonic PBS rinse
- E400 sperm extender

1.8.3 Step-by-step Procedure for Collecting Sperm by Testis Dissection

To salvage the sperm present in the distal testicular ducts, 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 a 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 as described above (1.7.3 Step-by-step Procedure for Collecting Stripped Sperm).


4. The caudal brain stem can be severed using micro-dissecting scissors prior to dissection. Using fine-tipped forceps and micro-dissecting scissors, pinch the ventral body wall of the cranial abdomen and make a small incision.

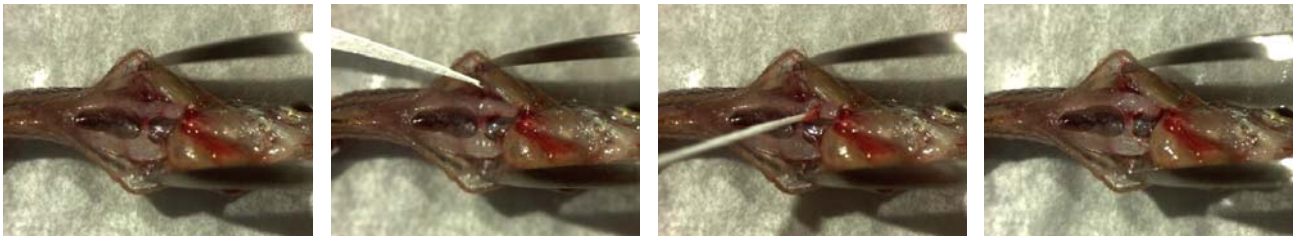

5. Insert the tip of the scissors just under the body wall and make two superficial cuts through the body wall encircling the urogenital opening and pelvic fins.



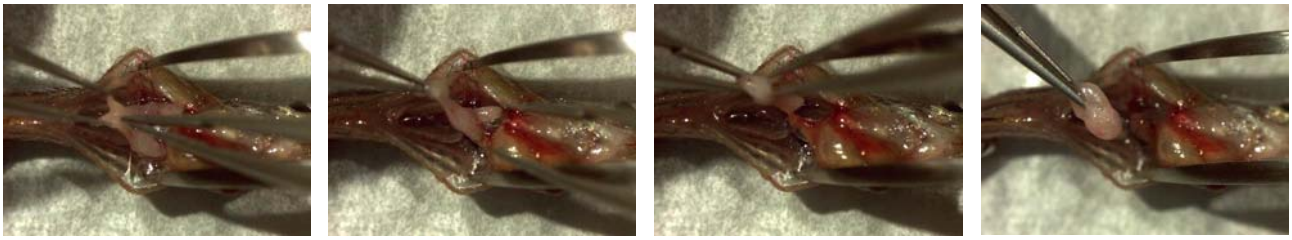

6. Remove the resulting flap of tissue, being careful to not disrupt the internal tissues.

7. Grasp the distal intestine and retract in a cranial direction to expose the bilateral testes.

8. 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 swim bladder. 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.

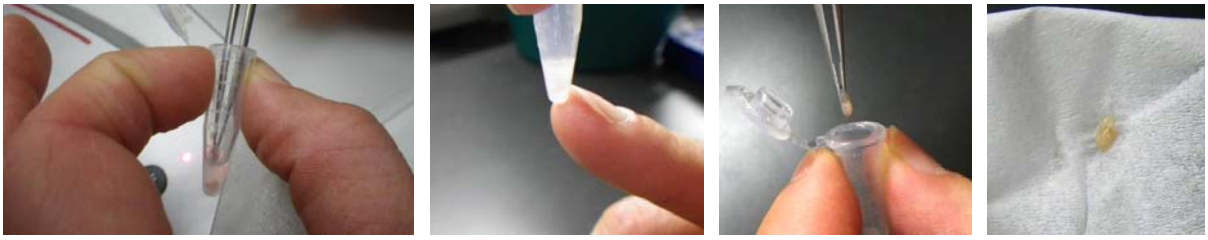


9. Use a pair of fine-tipped forceps to gather and remove the fragile testes, trying not tear the tissue. Add the dissected testes to the microcentrifuge tube on ice containing the E400 and stripped sperm.



10. If pooling sperm from multiple males, continue collecting stripped sperm and testes from available males and pool into the same microcentrifuge tube on ice.

11. 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 crushing it. Mix the contents of the tube by flicking it several times, and then leave it 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.



2. Introduction to Sperm Cryopreservation

Sperm can be frozen from individual males or pooled from multiple males. Both methods are described here.

2.1 Toxicity of RMMB Cryoprotective Media

As with most cryoprotectant media, the RMMB medium is toxic to 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, freezing should happen as fast as possible once the RMMB cryo medium is mixed with the sperm. To minimize solution toxicity, samples should be aliquoted and ready to freeze within 5 minutes. If the final sperm volume is greater than 60 µL (12 samples), dividing the sperm into more than one tube and freezing in batches is recommended.

2.2 Powdered Dry Ice from Liquid CO_2

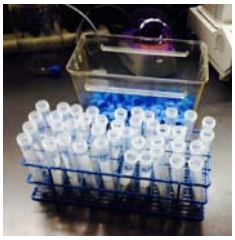
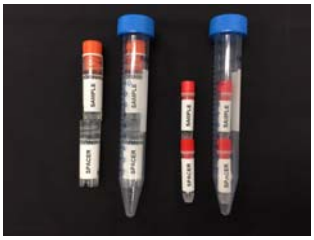
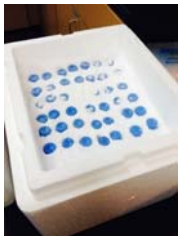
Powdered dry ice is preferred over crushed or pelleted dry ice because better contact is established between the container and the dry ice, which contributes to more consistent and reproducible freeze rates. To produce powdered dry ice from CO_2, a fire extinguisher cone needs to be attached to the liquid outlet of a CO_2 dewar with a siphon tube (See Section 7 for additional information and a parts list to make the fire extinguisher cone attachment). The cone is angled downward at 90° (see image below). Position a Styrofoam box or cooler directly under the cone to catch the dry ice as it exits the cone. For personal safety, a face shield, ear protection, and cryoprotective gloves are recommended. Open the liquid CO_2 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 CO_2 gas occurs more rapidly while the container initially cools, so filling the cooler slightly higher than 20 cm may be desired. The dry ice powder can be lightly compacted as the cooler fills. Once filled, simply turn off the CO_2 outlet valve. Smooth the surface of the dry ice with a gloved hand and it is ready to use.

2.3 Cooling Rate

The optimum rate of cooling depends on many factors including the specific cell type, cryoprotective agent(s) and other solution components. At routine cooling rates, ice seeding and crystallization begins in the extracellular compartment. As ice forms, the extracellular electrolyte concentration increases and results in osmotic dehydration of the cells. If cooling rates are too slow, the resulting increased electrolyte concentration can lead to irreversible cell damage. If cooling rates are too rapid, intracellular ice crystals can form and also result in cell damage. Maximum viability is obtained by cooling at a rate in which the combined effect of both of these mechanisms is minimized (Cryo Bio System, 2006).

For zebrafish sperm and the E400/RMMB cryopreservation method, the optimum cooling rate was determined using a controlled rate freezer to be approximately -15°C/min. To mimic this rate with dry ice, samples are frozen in cryogenic vials within a 15-mL conical centrifuge tube (Falcon, #352096) containing a spacer vial at

the bottom of the tube (see photos below). For freezing with 2 mL Corning vials (Item #430488), an empty 2 mL vial *without* the cap is utilized as a spacer. For freezing with 0.5 mL Matrix Screw Top Storage Tubes (Thermo Scientific, Item #3745-BR), an empty 0.5 mL Matrix vial *with* cap is utilized as a spacer. The sample-containing vial is placed on top of the spacer vial inside the conical tube. The conical tube is then capped and driven into a cooler of powdered dry ice made from liquid CO_2 (Section 2.2). Do not pre-chill the falcon tubes. They should be at room temperature prior to freezing to reproduce the optimal cooling rate. A rack of 15 mL conical tubes containing cryo vial spacers is shown below.

2.4 Time in dry ice

Freeze in dry ice for 20-60 minutes. The sample tube assembly should remain in dry ice for a minimum of 20 minutes, but not longer than 60 minutes. The exact time is not critical apart from observing the minimum and maximum times.

2.5 Ratio of Sperm (in E400) to RMMB Freezing Medium

The ratio of sperm in E400 to RMMB Freezing Medium is always 1 part sperm to 3 parts RMMB. The total sample volume is 20 µL, which equals 5µL sperm (in E400) + 15µL RMMB for each sample.

2.6 Protocol for Freezing from Individual Males (1-2 samples/male)

2.6.1 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 (Falcon 352096) with appropriate cryovial spacers (see Section 2.3, Freeze Rate)
- Styrofoam container or cooler (~12" x 12") for dry ice
- Styrofoam container for liquid nitrogen (LN_2) tray
- Fiberglass tray (US Plastic Corp # 49273) for LN_2
- Cryo vial storage box
- Liquid nitrogen Dewar flask
- Cryogloves (Tempshield # 11-394-306)

2.6.2 Solutions for Sperm Freezing

- E400 sperm extender
- RMMB cryo medium

2.6.3 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 precipitate if held on ice for extended periods. If this happens, heat the tube briefly until the raffinose is back in solution.

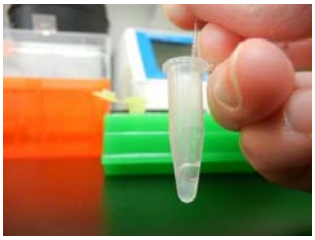
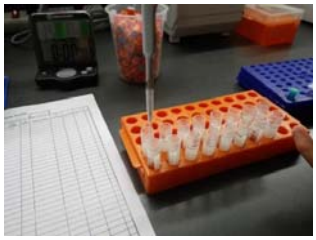
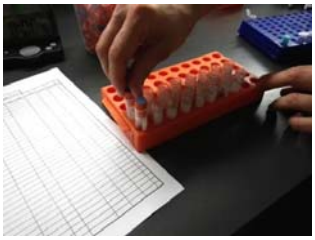
Prepare labeled sample cryovials as needed.

2.6.4 Step-by-step Freezing Procedure for Single Males

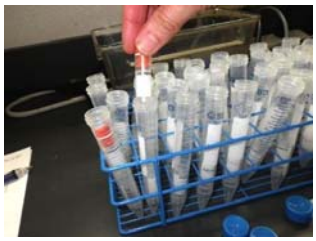
1. Anesthetize male zebrafish in Tricaine solution, briefly rinse (in PBS Isotonic fish rinse or fish water), dry fish by rolling on paper towel, place fish belly-up in a dampened foam or sponge holder.

2. Collect stripped sperm into microcapillary. Use Millipore forceps to apply **gentle** abdominal pressure to the sides of the male fish. To collect sperm, place capillary on urogenital opening as sperm is expelled and draw up into capillary. Revive fish in fresh fish water.

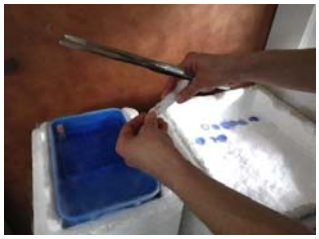

3. Without delay, normalize sperm to the appropriate volume (5 or 10 µL for one or two samples, respectively) with E400 (green tube) and then expel the entire contents of the microcapillary (sperm + E400) into one of the 0.5 mL microfuge tubes (clear) containing RMMB freezing medium (15 or 30 µL for one or two samples, respectively). Mix the total solution by pipetting up and down 1-2 times and then transfer 20 µL of the mixture into each cryovial (20 µL/sample). Quickly cap the cryovials.

4. Quickly place the sample cryovials into the 15 mL conical tubes (containing a cryovial spacer) and cap. Drive tubes down into the dry ice until caps are flush with the surface.




5. Keep samples in dry ice for 20-60 minutes, then quickly transfer samples to a cryo box submerged in LN_2.


2.7 Protocol for Pooled Sperm Freezing - collect and dilute sperm prior to freezing

2.7.1 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, which 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:	**Stripped sperm and dissected testis sperm:**
1-2 samples/male	2-5 samples/male
Final diluted volume = 5-10 µL x # males	Final diluted volume = 10-25 µL x # males

2.7.2 Diluting Pooled Sperm based on NanoDrop® Cell Counts

Sperm Concentration Determination Using a NanoDrop® 2000 Spectrophotometer and the Excel Pooled Sperm Worksheets for use with Nanodrop® Cell Counts are described below.

2.7.2.a Sperm Concentration Determination Using a NanoDrop® 2000 Spectrophotometer

Using a micro-volume spectrophotometer such as the NanoDrop® 2000 (Thermo Scientific), a relatively common laboratory instrument, is a quick and practical method for routine measurement of zebrafish sperm concentration. ZIRC's method for determining sperm concentration prior to freezing is described below.

1. Collect and pool sperm (testes and/or stripped) from available males as previously described.

2. Measure the sperm volume using a pipetman. While measuring, gently pipet the sperm to mix completely. Note the measured volume.

3. Immediately remove 1.0 µL for the NanoDrop dilution. A 1:5 or 1:10 dilution is typically made, depending on how concentrated the sperm sample seems, based on its opacity. Prepare a 0.5 microcentrifuge tube

containing either 4 or 9 µL E400 for the NanoDrop dilution. Add the 1.0 µL of sperm to the dilution. Mix by flicking the tube and hold on ice. Dilute all available samples in a similar manner. To easily distinguish the diluted samples, a colored (yellow) microfuge tube is typically used for the NanoDrop dilutions.

4. Open the NanoDrop Software and choose the Cell Cultures option and set the Cursor Absorbance to 400 nm. Blank the spectrophotometer using the E400 diluent.

5. Mix the sample well using a vortex mixer set at an intermediate speed (~1300 RPM). Immediately load 1.5 µL of the diluted sperm and read the Cursor Absorbance at 400 nm (A_{OD400}). Repeat for several measurements and calculate the average A_{OD400} for all sample dilutions. Occasional errant readings are disregarded.

Note: If there is any sperm left over from the NanoDrop dilution, take a quick look at it under a microscope to assess concentration and pre-freeze motility (place 6uL dH2O on a microscope slide, add 1 µL of diluted sperm, quickly mix with pipet tip and observe with 20X objective and DIC or dark field).

2.7.2.b NanoDrop Calibration Curve and Sperm Density Calculator

The calibration curve developed for the ZIRC NanoDrop 2000 spectrophotometer from hemocytometer cell counts is available on the ZIRC website at:

http://zebrafish.org/zirc/documents/protocols/xls/cryopreservation/zirc_nanodrop_sperm_density_calc.xls

Some variation can exist between instruments so a calibration curve would ideally be developed for every specific instrument. Because the resulting cell count is just an estimate of concentration for sperm freezing purposes, it is reasonable to extend the ZIRC NanoDrop calibration curve to absorbance at 400 nm (A_{OD400}) readings of zebrafish sperm from other NanoDrop 2000 spectrophotometers.

The best fit ($R^2 = 0.982$) between data and fitted curve resulted from a second-order polynomial equation.

$$\text{Cells/mL} = (5\text{x}10^8)(A_{OD400})^2 + (7\text{x}10^7)(A_{OD400}) + 2\text{x}10^7$$

This equation is typically used for the calculation of sperm concentrations. Note that the linear fit is actually a better fit for A_{OD400} of less than 0.300. To the right of the graph there is a location to enter the A_{OD400} for a sample and the embedded formulas will calculated the estimated concentration according to which curve (2nd Order Polynomial or Linear) is selected.

2.7.2.c Excel Pooled Sperm Dilution Worksheets for Use with NanoDrop $A_{OD}400$ Measurements

The second order polynomial curve equation from the NanoDrop calibration is also used in an Excel worksheet to optimize sperm cell dilutions for cryopreservation. There are specific worksheets for stripped and testis sperm. The worksheets have embedded formulas that populate automatically once some basic information (light blue cells) is filled in. Refer to the following blank forms to view the self-populating fields.

Pooled stripped sperm dilution calculator - Blank.xlsx (provide link to document)
This is the blank form for squeezed sperm. The dotted line around the $2\text{x}10^6$ cells/sample column is the desired minimum concentration. Freezing at a lower concentration can still be successful, but having more cells is always better.

Pooled testes sperm dilution calculator - Blank.xlsx (provide link to document)
This is the blank form for testis sperm. Because you get more cells out of testes that aren't necessarily active sperm, testis sperm is typically frozen at a higher concentration

To help explain the spreadsheet some sample data has been filled in. The example shown below is for the collection and dilution of stripped sperm. The method notes in the middle of the worksheet walk you through the significant steps. The light grey cells contain information that can be completed in advance. These fields can be altered to the specific needs of individual laboratories.

Depending on how concentrated the sperm visually appears, a 1:5 or 1:10 dilution for the NanoDrop measurements is recommended. Because the polynomial equation is built into the sperm dilution worksheets, we try to do the NanoDrop dilution so that the average A_{OD400} is greater than or equal to 0.300. For squeezed sperm it is recommended to start with a 1:5 dilution to get a NanoDrop A_{OD400} of >0.300. For pooled testes, a 1:10 dilution is usually safe. Your eye will get better at estimating concentration after some experience. It is also recommended that the dilution is made in a colored 0.5 mL microcentrifuge tube to distinguish it from the undiluted sperm that is collected in a clear 0.5 mL tube. It's easier to visualize and estimate sperm concentration when it is in a colorless tube.

2017_01_13 Pooled Sperm Freeze Data			RMMB Lot: 11/9/2016				Main-rm		Page 1	
1	**Pooled Sperm**		**Method Notes**						**# Samples**	**Test Thaw**
Allele(s) or WT strain	**sa multi**		Mark capillaries at 25 mm (5uL)				**Samples needed:**		20	1
ZL#	8952		Collect sperm from males				**Frozen:**		20	1
ZS# (s)	9931		Normalize with Extender E400 to 5.0 uL ea and pool on ice							
# Males pulled	21		Measure volume of pooled sperm with Pipetman just prior to making nanodrop dilution							
# Males giving sperm	20		Dilute for Nanodrop (1:10 or 1:5)-dilute w/E400						**Test Thaw Results**	
Pooled Vol (uL) - Estimated	100		Determine average A400						**% Motility:**	
Pooled Vol (uL) - Measured	102		Add back E400 to desired concentration						**% Fert:**	
Nanodrop dilution: uL pooled sperm	1		Freeze 20 uL samples (5uL sperm + 15ul CPM)							
uL E400	9		Add RMMB to sperm, aliquot to CVs (20uL each), freeze in dry ice AFastAP							
Dilution Factor	10		CVs frozen in capped 15mL Falcon tube with CV as spacer							
Abs 400 (Ave) =	0.453		Transfer to LN2 after 20-60 minutes							
Cells/ml as read =	1.54E+08		Additional Notes:							
Cells/ml Undiluted =	1.54E+09		Divide final dilution volume if > 12 samples				**Final Dilu Volume**	**Divided Vol**	**Vol RMMB**	**#Samples**
Final Undiluted Vol (uL) - Calculated	101						55	60	165	11
							115	60	180	12
Final Cell Count (#cells/sample)	**AS IS**	**1.00E+06**	**1.50E+06**	**2.00E+06**	**2.50E+06**	**3.00E+06**	**3.50E+06**	**4.00E+06**	**4.50E+06**	**5.00E+06**
Total # of 5 ul aliquots	20.2	155.9	103.9	77.9	62.3	52.0	44.5	39.0	34.6	31.2
Samples/Male	1.01	7.79	5.20	3.90	3.12	2.60	2.23	1.95	1.73	1.56
Final Dilution volume for desired conc.	101	779.29	519.53	389.64	311.72	259.76	222.65	194.82	173.18	155.86
uL E400 to add (must be Positive No.)	0.00	678.29	418.53	288.64	210.72	158.76	121.65	93.82	72.18	54.86
uL RMMB to add (15ul/sample)	303.00	2337.86	1558.58	1168.93	935.15	779.29	667.96	584.47	519.53	467.57
Conc of sperm in 5uL aliquot (cells/mL)	1.54E+09	2.00E+08	3.00E+08	4.00E+08	5.00E+08	6.00E+08	7.00E+08	8.00E+08	9.00E+08	1.00E+09
Final Conc of sperm as frozen (cells/mL)	3.86E+08	5.00E+07	7.50E+07	1.00E+08	1.25E+08	1.50E+08	1.75E+08	2.00E+08	2.25E+08	2.50E+08
Final Cell Count (#cells/sample)	**7.72E+06**	1.00E+06	1.50E+06	2.00E+06	2.50E+06	3.00E+06	3.50E+06	4.00E+06	4.50E+06	5.00E+06
Final Cell Count (#cells/sample)	**5.50E+06**	**6.00E+06**	**6.50E+06**	**7.00E+06**	**7.50E+06**	**8.00E+06**	**8.50E+06**	**9.00E+06**	**1.00E+07**	**6.78E+06**
Total # of 5 ul aliquots	28.3	26.0	24.0	22.3	20.8	19.5	18.3	17.3	15.6	23.0
Samples/Male	1.42	1.30	1.20	1.11	1.04	0.97	0.92	0.87	0.78	1.15
Final Dilution volume for desired conc.	141.69	129.88	119.89	111.33	103.91	97.41	91.68	86.59	77.93	115.00
uL E400 to add (must be Positive No.)	40.69	28.88	18.89	10.33	2.91	-3.59	-9.32	-14.41	-23.07	14.00
uL RMMB to add (15ul/sample)	425.07	389.64	359.67	333.98	311.72	292.23	275.04	259.76	233.79	345.00
Conc of sperm in 5uL aliquot (cells/mL)	1.10E+09	1.20E+09	1.30E+09	1.40E+09	1.50E+09	1.60E+09	1.70E+09	1.80E+09	2.00E+09	1.36E+09
Final Conc of sperm as frozen (cells/mL)	2.75E+08	3.00E+08	3.25E+08	3.50E+08	3.75E+08	4.00E+08	4.25E+08	4.50E+08	5.00E+08	3.39E+08
Final #cells/sample	5.50E+06	6.00E+06	6.50E+06	7.00E+06	7.50E+06	8.00E+06	8.50E+06	9.00E+06	1.00E+07	6.78E+06

In the example, sperm was collected from 20 of the 21 males pulled. The number of males giving sperm is used to calculate an estimated volume of sperm collected. The actual volume of sperm is measured with a Pipetman using the estimated pooled volume as a starting point. The volume of sperm is measured with a Pipetman just prior to making the dilution for the NanoDrop. The sperm has a tendency to settle in the bottom of the microfuge tube. When the sperm is pipetted up and down to make the volume measurement, the settled cells are re-suspended. Immediately after the volume measurement, 1 µL of the sperm suspension is removed for the NanoDrop dilution. The volume of sperm and E400 used for the NanoDrop dilution is entered into the worksheet and the dilution factor is automatically calculated. Finally, the average A_{OD400} from the NanoDrop

readings is entered. Now the cells/sample and number of sample fields will populate. This info can now be used to direct how you will freeze your samples. The number of samples that can be produced at various concentrations and the amount of E400 and RMMB to add is shown. The desired number of samples can also be entered into the field in the bottom right corner.

In the example above we could freeze 77.9 samples at our suggested minimum concentration of $2x10^6$ cells/sample (outlined in blue), but we don't need that many samples. Our sample goal was 21 samples (20 samples plus one test thaw, noted in upper right corner of the worksheet). If we are freezing more than 12 samples, the sperm is divided into batches to minimize the time the sperm cells are exposed to the RMMB CPM prior to freezing. We commonly add a sample or two to account for pipetting error. In the example we needed 21 samples so the sperm was divided and frozen in two batches. One additional sample was added to each batch so the total samples needed was increased to 23. The bottom right-hand corner of the worksheet has a blank field to enter the exact number of samples needed. In the example 23 samples was entered and the remaining fields self-populate. The final dilution volume was 115 µL (after addition of 14 µL of E400 to the 101 µL of undiluted sperm). This was divided into two aliquots (of 55 and 60 µL). The divided volumes were entered into the box on the middle right side and the volume of RMMB to add and the resulting number of samples fields self-populate. Finally, the selected dilution is highlighted in yellow and the worksheet becomes part of the permanent record for the sperm samples.

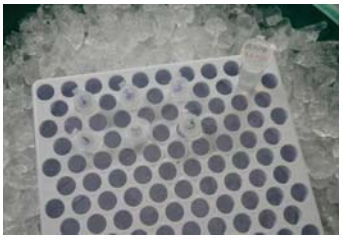
2.7.3 Pooled Sperm Freezing Procedure

1. Collect pooled sperm into E400 by squeezing and/or testis dissection as described above.

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

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

4. Prepare labeled sample cryovials as needed prior to freezing.

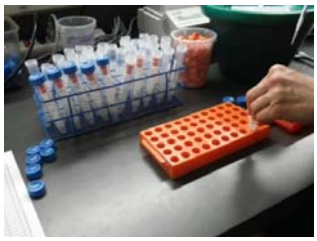


5. For each tube of sperm, determine the volume of RMMB to add from the Excel Pooled Sperm Dilution Worksheet or calculate as follows: RMMB Volume = 3 x Sperm Volume

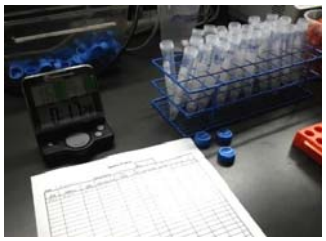
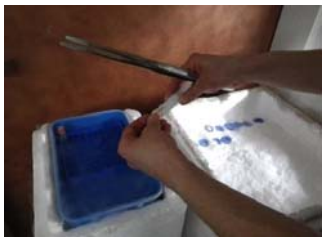
Because the RMMB cryoprotective medium is toxic to the sperm cells, timing of freezing should be as fast as possible once the RMMB is mixed with the sperm. It is helpful to have a second person at this point to help cap and get tubes into the dry ice.

6. Add calculated volume (3x sperm volume) of RMMB cryo medium to sperm, mix and aliquot immediately into cryovials (20 µL each).


7. Without delay, cap the cryovials and place into the 15 mL conical tubes (containing a cryovial spacer). Cap the conical tubes and drive tubes down into the dry ice until caps are flush with the surface.

8. Freeze samples in dry ice for 20-60 minutes, then quickly transfer samples to a cryo box submerged in LN_2.

3. Abbreviated Sperm Freezing Protocols

3.1 Materials & Solutions for Sperm Collection and Freezing

- Dissecting microscope with incident 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), 1-2 inches
- Soft paper towels
- Cotton swab or soft tissue
- 0.6 mL microcentrifuge tubes (Fisher # 02-681-311)
- Ice bucket

- Cryogenic vials (0.5 mL Matrix Screw Top Storage Tubes, Thermo Scientific, Item #3745-BR or 2 mL Corning vials, Item #430488, or alternative)
- Vial color coders (Colored caps or cap inserts specific for vial type)
- Pipetman and tips
- 15 mL conical tubes (Falcon 352096) with cryovial spacers (see Freeze Rate, Section 2.3)
- Styrofoam container or cooler (≈12" x 12") for dry ice
- Styrofoam container for liquid nitrogen (LN_2) tray
- Fiberglass tray (US Plastic Corp # 49273) for LN_2
- Cryo vial storage box (specific for vial type)
- Liquid nitrogen Dewar flask
- Cryogloves (Tempshield # 11-394-306)

- Tricaine anesthesia
- Fish water for recovery
- E400 sperm extender
- RMMB cryo medium

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

3.2.1 Preparations for Freezing from Single Males

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.

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 precipitate if held on ice for extended periods. If this happen, heat tube briefly until the raffinose is back in solution.

Prepare labeled sample vials as needed.

3.2.2 Step-by-Step 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 belly-up in a dampened foam or sponge holder.

2. Collect stripped sperm into microcapillary. Use Millipore forceps to apply **gentle** abdominal pressure to the sides of the male fish. Place capillary on urogenital opening as sperm is expelled and draw up into capillary. Revive fish in fresh fish water.

For best results when freezing two samples/male, collecting a minimum of 3 mm of dense sperm (bright white) within the microcapillary is recommended. Sperm can be collected from a second male into the same capillary if necessary.

3. Without delay, normalize sperm to 10 µL 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 30 µL RMMB freezing medium. Mix the total solution by pipetting up and down 1-2 times with a Pipetman, then transfer 20 µL of the mixture into each cryovial (20 µL/sample). Cap the cryovials.

4. Quickly place the sample cryovials into the 15 mL conical tubes (containing a cryovial spacer) and cap. Drive tubes down into the dry ice until caps are flush with the surface.

5. Keep samples in dry ice for 20-60 minutes, then quickly transfer them to a cryo box submerged in LN_2.

3.3 Pooled Sperm Freezing Procedure (2 samples/male) - collect pooled sperm & then freeze

3.3.1 Preparations for Freezing Pooled Sperm

1. 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.

2. Prepare 0.5 mL microfuge tubes (clear) and hold on ice. Prepare one tube per line being frozen. Pooled sperm will be collected in this tube.

4. This method can be easily adjusted to freeze one sample/male. In this case, sperm will be normalized to 5 µL. Prepare 10 µL microcapillary pipettes marked at 5 µL (or 25 mm) using a permanent marker.

3. Prepare labeled sample cryovials as needed prior to freezing.

3.3.2 Step-by-step Freezing Procedure for Pooled Sperm (2 samples/male)

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 belly-up in a dampened foam or sponge holder.

2. Collect stripped sperm into microcapillary. Use forceps to apply **gentle** abdominal pressure to the sides of the male fish. Place capillary on urogenital opening as sperm is expelled and draw up into capillary. Revive fish in fresh fish water.

3. Without delay, normalize sperm to 10 µL with E400 (green tube) and then expel the entire contents of the microcapillary (sperm + E400) into the 0.5 mL microfuge tube (clear) and hold on ice.

4. Continue collecting stripped sperm from all available males. Normalize with E400 and pool into the same 0.5 microfuge tube on ice.

5. Measure the final volume of pooled sperm and E400 with a Pipetman.

6. If the final pooled sperm volume is > 60 µL (12 samples), divide the sperm into more than one tube and freeze in batches.

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

RMMB Volume = (Sperm Volume) x 3

Note: 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.

8. Add the calculated volume of RMMB cryo medium to the sperm, mix and aliquot immediately into cryovials at 20 µL each.

9. Without delay, cap the cryovials and place into the 15 mL conical tubes (containing a cryovial spacer). Cap the conical tubes and drive tubes down into the dry ice until caps are flush with the surface.

10. Freeze samples in dry ice for 20-60 minutes, then quickly transfer them to a cryo box submerged in LN_2.

4. Introduction to Sperm Thawing and In Vitro Fertilization (IVF)

Females should be isolated from males the afternoon prior to egg collection. 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. Eggs, like sperm, are activated by water. It is very important to use the Isotonic PBS rinse solution with anesthetized females for egg collection. It is impossible to dry the female completely. The isotonic rinse solution ensures there is no water present that can activate the eggs. If multiple clutches are being combined, a fine paint brush is an effective and gentle tool for moving the eggs. Dampen your fingers and the paint brush in the isotonic PBS solution prior to squeezing females or manipulating eggs.

4.1 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

4.2 Solutions for Thawing and IVF

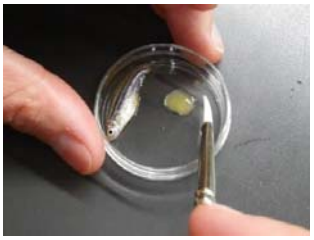
- MS-222 pre-anesthesia (48 µg/L)
- MS-222 anesthesia (168µg/L)/
- PBS rinse/Recovery fish water
- Sperm Solution SS300 (if thawing samples frozen without milk, add 2 mg/mL Difco Skim Milk to the SS300)
- dH_20

4.3 Step-by-Step Procedure for Sperm Thawing and IVF

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

2. Anesthetize females in Tricaine solution, rinse fish in isotonic PBS, dry fish by rolling on paper towel, place fish in lateral recumbency (on its side) in a 35 mm petri dish. Dampen fingers in PBS fish rinse. Obtain eggs from females by applying light digital pressure on the ventral abdomen in an anterior to posterior direction. Eggs will be expelled easily if the female is ready. Eggs can be moved away from the fish 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.

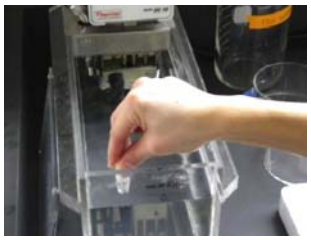





3. Remove the sperm sample cryovial from the LN_2 and quickly open cap to vent any LN_2 in the vial.

4. Thaw cryovial in a 38^{o}C water bath until the frozen pellet is < 3mm (~10-15 sec).

5. Add 150 µL room-temperature SS300 solution to the cryovial. If you are 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 µL dH2O to the cryovial 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 (not just on top of the eggs). Start a count-down timer set for 2 minutes.


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.

5. Quality Control

5.1 Sperm Cell Density

Sperm cell density can be determined by several techniques. These methods are described briefly below. Sperm cell concentrations can be used to determine the number of samples that can be generated from a pool of sperm.

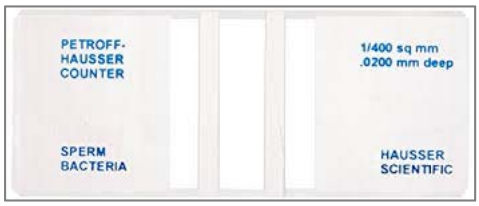
5.1.1 Cell Density Assessment by Counting Chamber or Hemocytometer

A common means of determining sperm concentration is to simply count sperm under a microscope with the aid of a hemacytometer or counting chamber. A counting chamber is a glass slide onto which a precision grid has been etched. They were originally sold for counting blood cells, but work equally well for counting sperm. Since the dimensions of the grid squares and depth of sample chamber are known, it is a simple matter to calculate cell concentration. The depth of the sample chamber will vary depending on the counting chamber. It is important to use the instructions that come with a particular counting chamber to calculate cell concentrations. Using a counting chamber with a shallow depth (10-20 µm) is recommended for sperm cells. The preferred counting chamber for sperm is the Makler Counting Chamber (see link below). Another recommended (and less expensive) counting chamber is also shown below. Zebrafish sperm is immobile when collected in the E400 extender, so it can be used to dilute and/or suspend the sperm for cell counting.

Makler Counting Chamber
http://www.irvinesci.com/uploads/technical-documentations/Makler_Counting_Chamber-Rev0.pdf
(Available from Irving Scientific: Makler® Counting Chamber Kit, Catalog ID: IS-363)

Hausser Scientific Petroff-Hausser Bacteria Counter - Catalog #3900
http://www.hausserscientific.com/products/petroff_hausser_counter.html

(Available from Fisher Scientific, Catalog Number 02-671-13)



5.1.2 Sperm Cell Density Assessment by Optic Density Measurement

Counting sperm using a counting chamber is time consuming and tedious. An alternative technique is to determine sperm concentration using a spectrophotometer. A spectrophotometer measures the amount of light absorbed by a sample, and the more sperm there are in the sample, the more light is absorbed. By generating a standard curve of absorbance versus sperm cell counts, one can quickly and accurately measure sperm concentration without directly counting cells. The Thermo Scientific NanoDrop spectrophotometers are particularly useful for measuring zebrafish sperm concentration because only very small volumes (1-2 µL) are needed for a measurement. Absorbance at 400 nm (A_{OD400}) is measured using the Cell Culture program of the NanoDrop 2000 spectrophotometer (see also Section 2.7). A dilution (typically 1:5 or 1:10 in E400) of each sample is loaded (1.5 µL) and analyzed in triplicate and the results are averaged. The cell concentration is calculated from the average absorbance at 400 nm (A_{OD400}) that had been calibrated with a counting-chamber-generated standard curve and equation (Tan et al., 2010). ZIRC utilizes the best fit ($R^2 = 0.982$) between data and fitted curve resulting from a second order polynomial equation

$$\text{cells/mL} = (5\text{x}10^8)(A_{OD400})^2 + (7\text{x}10^7)(A_{OD400}) + 2\text{x}10^7$$

The equation is used in an excel calculator to determine cell density based on absorption: (zebrafish.org/zirc/documents/protocols/xls/cryopreservation/zirc_nanodrop_sperm_density_calc.xls):

5.2 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 density and 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.

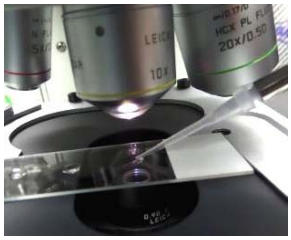
5.2.1 Pre-freeze Motility

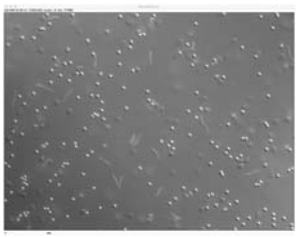
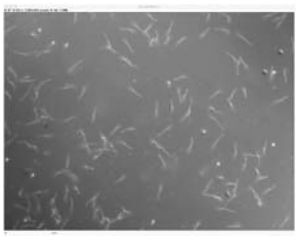
For pre-freeze motility, place a 6 µL drop of dH_20 on a microscope slide. Add a very small amount (0.5-1 µL) of the final sperm dilution to the drop, mix and spread quickly with the pipet tip and observer immediately. Examining the remainder of the NanoDrop dilution, if performed, is a good use of the sperm. Checking pre-freeze motility confirms the viablility and concentration of the sperm being frozen.

5.2.2 Post-thaw Sperm Motility

For post-thaw motility, activating the sperm on a 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. It's 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 dH_20 on a slide, add 4.25 µL of your thawed sperm/SS300 solution, briefly mix with pipet tip on the slide and observe immediately.



6. Solutions for Sperm Collection, Cryopreservation, Thawing & IVF

6.1 Sperm Extender ~400 mmol/kg (E400)

130 mM KCL
50 mM NaCl
2 mM $CaCl_2$
1 mM $MgSO_4$
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 $CaCl_2$ (or 0.29 g $CaCl_2$-$2H_2O$)
1.0 mL 1.0 M $MgSO_4$ (or 0.25 g $MgSO_4$-$7H_2O$)
1.80 g D-(+)-Glucose
7.15 g HEPES

Add dH_2O to 800 mL
Add dry ingredients, stir to dissolve
Add liquid ingredients, stir to mix
Adjust pH with 5M KOH to 7.9
Bring up final volume to 1000 mL with dH_2O
Check osmolality (If made as directed, osmolality should be very close to 400 mmol/kg)
Filter sterilize
Store at 4°C

6.2 1M Bicine-NaOH (pH 8.0)

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

6.3 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
dH_2O to 100 mL

Combine 20.0 g Raffinose and 70 mL dH2O in a 250 mL beaker.
Place beaker in an evaporating dish (Pyrex 3140) or large 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 dH_2O, 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 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.

6.4 Sperm Solution SS300 (~300 mmol/kg)

140 mM NaCl
5 mM KCl
1 mM $CaCl_2$
1 mM $MgSO_4$
10 mM D-(+)-Glucose
20 mM Tris-Cl (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 $CaCl_2$ (or 0.15 g $CaCl_2$-$2H_2O$)
1.0 mL 1.0 M $MgSO_4$) (or 0.25 g $MgSO_4$-$7H_2O$)
1.8 g D-(+)-Glucose
20 mL 1M Tris-Cl (pH 8.0)

Add dH_2O to 800 mL
Add dry ingredients, stir to dissolve
Add liquid ingredients, stir to mix
Bring up final volume to 1000 mL with dH_2O
Check osmolality (If made as directed, osmolality should be very close to 300 mmol/kg)
Filter sterilize
Store at 4°C

6.5 Sperm Solution SS300 with 2 mg/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

6.6 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 $MgSO_4$
0.5 mM $CaCl_2$
75 µM KH_2PO_4
25µM Na_2HPO_4
0.35 mM $NaHCO_3$
0.5 mg/L Methylene Blue

6.7 Tricaine Stock Solution (4.0 g/L)

(Tricaine Methanesulfonate, MS-222, Tricane-S, Western Chemical, Inc.)
4.0 g Tricaine
Add dH_2O 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 dH_2O
Store in an amber bottle at 4°C

6.8 Pre-Anesthesia Working Solution (48 mg/L)

Used to reduce mortalities when squeezing females. Females are held in pre-anesthetic solution for 10-60 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

6.9 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

6.10 1M Tris-Cl (pH 9.0)

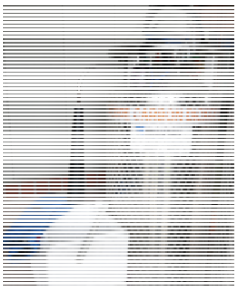
121.1 g Tris base (Fisher Scientific BP152-1)
Add dH_2O to 800 mL
Stir to dissolve
pH with concentrated HCl to pH 9.0 (approx. 10 mL)
Bring up final volume to 1000 mL with dH_2O
Filter sterilize

6.11 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 dH_20.
This will give a final osmolality of approximately 305-315 mmol/kg.

7. CO_2 Fire Extinguisher Cone Attachment for Making Powdered Dry Ice

To make powered dry ice from liquid CO2, a fire extinguisher cone attachment is required. The cone is attached directly to the liquid outlet of a CO_2 Dewar. The cone attachment pipe can be fabricated by a welding shop or simply assembled from common pipe fittings wrapped with teflon (PTFE) tape prior to assembly.



7.1 CO_2 Fire Extinguisher Cone Attachment Parts and Supplier List

Fire Extinguisher Cone: Supplier: Architectural Builders Supply https://absupply.net/
Product Code: Brooks B115
Product Description: Brooks B115 Fire Extinguisher Parts, CO2 Horn, Conical
https://absupply.net/brooks-b115-fire-extinguisher-parts-co2-horn-conical.aspx
Approximate cost: $15.00 (USD)

CO2 Nut: Supplier: TOOLFETCH http://www.toolfetch.com/
Part# 312-CO-2
Product Description: Western Enterprises CO-2 Nut
http://www.toolfetch.com/western-enterprises-co-2-nut.html
Approximate cost: $3.00 (USD)

CO2 Nipple: Supplier: TOOLFETCH http://www.toolfetch.com/
Part# 312-CO-3
Product Description: Western Enterprises CO-3 Nipple

http://www.toolfetch.com/western-enterprises-co-3-nipple.html
Approximate cost: $4.00 (USD)

The remaining brass pipe fittings can be found at most hardware or plumbing supply stores. An on-line supplier is also listed below.

¼ " Brass Pipe Fittings: Supplier: PlumbingSupply.com https://www.plumbingsupply.com/
¼" Brass Coupling (Quantity 1, approximate cost: $3.00 each)
¼" Brass 45° Elbow (Quantity 2, approximate cost: $4.00 each)
https://www.plumbingsupply.com/brass-fittings.html#025
¼" Brass Nipple 3" (Quantity 2, approximate cost: $3.00 each)
¼" Brass Nipple 4" (Quantity 2, approximate cost: $4.00 each)
https://www.plumbingsupply.com/brassnipples.html#14

Acknowledgements

This research was supported by the National Institutes of Health Division of Program Coordination, Planning, and Strategic Initiatives (DPCPSI) and Office of Research Infrastructure Programs (ORIP) awards P40OD011021 and 5R24OD010441-05.

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