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.

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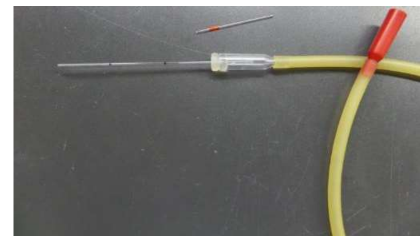
1. Introduction to Sperm Collection

Sperm is obtained from males by stripping (also referred to as squeezing or abdominal massage) and collected into a glass microcapillary pipette. After collection and dilution in sperm extender E400, sperm can be held on ice for several hours, providing sufficient time for quality analysis. When sperm is collected by stripping, males can be recovered from anesthesia and, if necessary, used again for sperm collection after a couple weeks of rest. Testis dissection can also be used but it has a much greater risk of pathogen contamination and is a terminal procedure. Testis dissection is described in a separate document (available upon request).

1.1 Male Conditioning

To increase the amount of sperm produced by males, they are conditioned prior to sperm collection. For conditioning, males are separated from females, held at a low density (~8 males/3.6L tank), and fed an additional midday feeding (3 feedings/day). ZIRC typically starts conditioning at 4.5 months of age and continues it for 4-6 weeks prior to collecting sperm.

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, if any, to collect the sperm with the capillary. Usually, capillary action will 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 it 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 at the liquid level. If bubbles do form, cap the collection tube, and give it a quick downward flick while holding the tube firmly between the thumb and index fingers.

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). Like 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

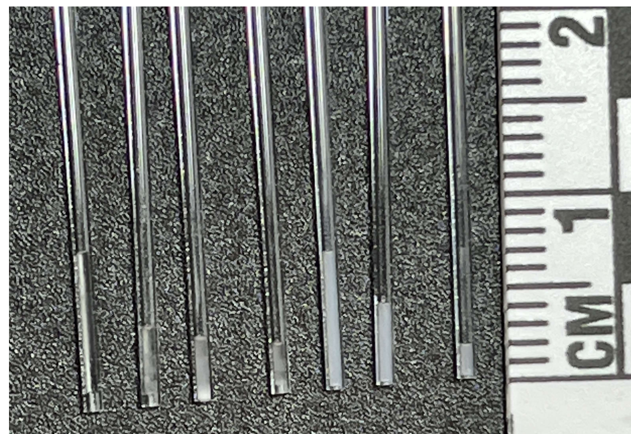
When stripping sperm, it is important to know that the clear, watery substance that is often collected is urine. Freshwater fish excrete a large amount of very dilute urine. Both the distal mesonephric duct and the spermatic duct terminate at the urogenital sinus and ultimately release both urine and sperm through the urogenital pore. If urine is collected with sperm, it can activate the sperm. This activation can be stopped by raising the osmolality with the sperm extender, E400. You will often see urine as the first liquid collected in the microcapillary. If you get just urine, stop collecting and expel it into a Kimwipe or paper towel and then continue collecting sperm. The opacity of freshly stripped sperm can vary greatly from male to male, suggesting varying amounts of sperm in the collected fluid. Concentrated zebrafish sperm appears very white within the capillary. A cloudy appearing sample is likely mixed with urine and activated. It is important to stop the activation as soon as possible by mixing with E400. The stripped sperm is stable in the E400 extender for several hours when held on ice.

1.5 Fecal Contamination in Stripped Sperm

The zebrafish intestinal tract terminates at the anus which is located just anterior to the urogenital pore. Fecal contamination of sperm must be avoided. Food should be withheld from fish for 18-24 hours prior to collecting sperm. Tanks should also be checked for food debris and cleaned if food is present. If feces is collected with sperm, the sperm and microcapillary should be discarded. Sperm with fecal contamination will contain bacteria and is at greater risk of agglutination (clumping).

1.6 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 is based on a millimeter measurement of sperm collected within a Drummond 10 µL microcapillary pipet. The opacity of the collected sperm (+ or neutral) provides a rough indication of concentration. Highly concentrated sperm will appear bright white (+ rating, capillaries on right in photo), whereas a neutral concentration will appear clearer (left/center capillaries in photo). Transparent or very dilute samples contain mostly urine and should not be used (far left capillary in photo).



1.7 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 group of samples.

1.8 Starting Volume of E400 for Pooling Sperm

When pooling sperm from multiple males, it is most efficient to start with a volume of E400 in a microcentrifuge tube and add the sperm to it. The starting volume is based on the number of available males being stripped for sperm. Because you often don't get sperm from all the males, a conservative calculation is shown below. This volume can later be diluted depending on how well the fish give and the number of samples desired. Because long-fin zebrafish typically give less sperm, the starting volume is slightly less.

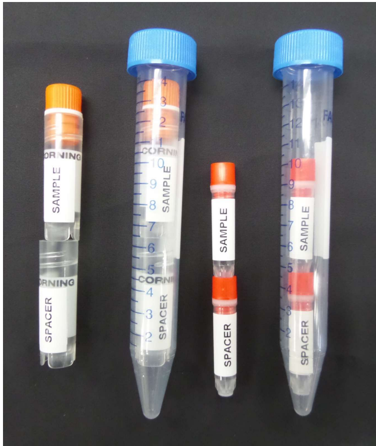
E400 Starting Volume for Pooled Sperm:
Short-fin zebrafish: Starting E400 volume (µL) = (# of males -2) x 5 µL
Long-fin zebrafish: Starting E400 volume (µL) = (# of males -4) x 5 µL

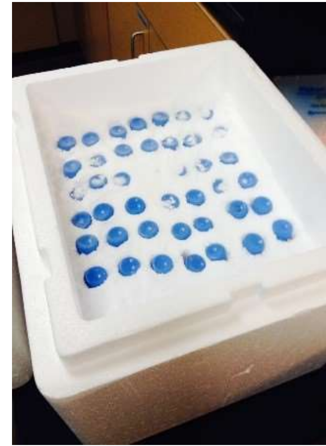
2. Introduction to Sperm Cryopreservation

Cryopreservation is the use of very low temperatures to preserve structurally intact living cells. Cryoprotective agents are typically added to reduce the osmotic shock and physical stresses that cells undergo during the freezing process. The optimal rate of cooling is important and depends on many factors including the specific cell type, cryoprotective agent(s) and other solution components. At optimum 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 result in cell damage. Maximum cell viability is obtained by cooling at a rate in which the combined effect of both of these mechanisms is minimized (Cryo Bio System, 2006). Once at liquid nitrogen temperatures (−196 °C or −321 °F), any cell metabolism which might cause damage to the sperm is effectively stopped. Appropriately cryopreserved sperm, when stored in liquid nitrogen and handled with care, should be viable indefinitely. Cryopreservation of sperm cells is a highly efficient method of preserving genetic resources.

2.1 Optimum Cooling Rate

For zebrafish sperm and the E400/RMMB cryopreservation method, the optimum cooling rate was determined using a controlled rate freezer to be approximately 10 to 15°C/min. To achieve this rate, sperm is initially frozen in dry ice (to approximately −80 °C) and then transferred to liquid nitrogen (-196 °C) for long-term storage. For most laboratories, freezing samples in dry ice is advantageous because it is readily available and offers flexibility in workflow and the timing of sample freezing compared to a controlled rate freezer. To mimic the 10 to 15°C/min 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 (see Section 2.6). Do <u>not</u> pre-chill the falcon tubes. They should be at room temperature prior to freezing to reproduce the optimal cooling rate. Sample/spacer configuration, a rack of 15 mL conical tubes containing cryovial spacers and tubes in dry ice are shown below.





2.2 Toxicity of RMMB Cryoprotective Medium
The cryoprotective medium (CPM) used is named RMMB based on its components. RMMB consists of a mixture of both non-penetrating (**R**affinose and **M**ilk) and penetrating (**M**ethanol) cryoprotective agents. It also includes a buffer (**B**icine, pH 8.0) that is stable at low temperatures. 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 RMMB CPM prior to freezing. Because of this toxicity, freezing should happen as fast as possible once the RMMB is mixed with the sperm. To minimize solution toxicity, samples should be aliquoted and ready to freeze in dry ice within 5 minutes. As soon as the RMMB is mixed with the sperm, the mixture is quickly aliquoted to cryovials, cryovials are capped, placed into the conical tubes (containing a spacer vial) and then the conical tubes are capped and driven into the dry ice. Because this process takes some time, if more than 12 samples will be frozen, the sperm is divided and frozen in batches to minimize RMMB solution toxicity. The diluted sperm aliquots should be 60 µL or less, resulting in a maximum of 12 samples/batch.

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

2.4 Number of Samples Needed to Preserve a Line
When deciding how many samples to freeze for a particular line of fish, a number of factors should be considered. These factors may be specific for the numerous zebrafish lines and zebrafish research labs or facilities. Sperm can be frozen from individual males or pooled from multiple males. ZIRC typically cryopreserves 15-50 samples per line for distribution based on the criteria below. For backup of lines without distribution, preserving 10 samples per line (with the samples stored in two separate freezers, if possible), from a minimum of 8 males would be recommended for mono-allelic lines. Additional samples may be necessary for backup of multi-allelic lines.

<u>Preserved Sample Quantity Considerations</u>

- Freezer space
- On-site and off-site sample inventory
- Number of available males
- Number of alleles in line
- Line identification ability
- Transgenic line stability (when functional expression relies on multiple integrations)
- Line distribution and expected order frequency (line popularity)

2.5 Diluting Pooled Sperm without Cell Counts

Whereas cell counts are useful for quality control, they are not necessary for freezing pooled sperm. Several things should be considered as you prepare to collect and dilute sperm prior to freezing.

1. What you want. This is the number of samples desired. This should be decided prior to sperm collection. Keep in mind that more than one freeze event may be necessary to get all the desired samples.

2. What you have. This is the number of males available for sperm collection. More males will result in more sperm. Conditioning males prior to sperm collection also helps increase the amount of sperm. The number of males also determines the starting volume of E400 for pooling sperm.

3. What you get. This is the quantity and quality of the collected sperm. The resulting pooled sperm is what you have to work with as you decide how far to dilute the sperm. The volume will be measured with a Pipetman. The density of the sperm is evaluated by visual inspection. The opacity of the pooled sperm directly correlates to concentration. Your eye will get better at judging sperm concentration with some experience.

4. Additional volume add-ons.
   a. *Test thaw*. It is recommended to add an additional sample for a test thaw when freezing pooled sperm.
   b. *Pipetting loss*. In the process of aliquoting pooled sperm into multiple cryogenic vials, a small amount of the material will be lost due to its adherence to the pipet tip, even when low-retention plastic is used. Therefore, for each batch of up to 12 samples, an additional sample is calculated into the dilution and RMMB formulas.

The resulting pooled sperm can be diluted prior to freezing based on the on the number of samples desired or on the number of samples/male (see calculations and examples below). Once the final dilution volume is calculated, additional E400 is added accordingly. Always try to freeze samples at the highest possible cell density.

2.5.1 Dilution based on desired number of samples.

This is the most common method:

Dilution based on desired number of samples:

Samples = desired samples + 1 (test thaw)

If Samples = (5 to 12), add 1 sample
= (13 to 24), add 2 samples
= (25 to 36), add 3 samples (*etc*.)

Dilution vol = total samples x 5 µL/sample

2.5.2 Dilution based on the number of samples/male.

A conservative estimate of the number of frozen samples that can be acquired from a single male zebrafish is 1-2 samples/male. If you are pooling sperm from multiple males and get sperm with a high visual density or a

large volume, this can be stretched to 3-4 samples/male. Due to the recommended inclusion of one test thaw per line frozen and pipetting loss additions, this nominal per-male sample count serves as a guideline rather than an actual outcome.

Dilution based on the number of samples/male:

Samples = [# males x samples (1 to 4)/male] + 1 (test thaw)

If Samples = (5 to 12), add 1 sample
= (13 to 24), add 2 samples
= (25 to 36), add 3 samples (*etc.*)

Dilution vol = total samples x 5 µL/sample

2.5.3 Pooled Sperm Dilution Examples:

2.5.3.1 Example of dilution based on the desired number of samples.

We want to freeze sperm from 12 males of a particular short-fin line.

Our goal is 14 samples plus a test thaw, 15 samples.

Starting E400 volume (short-fin males) = (# of males -2) x 5 µL= (12 males -2) x 5 µL = 10 x 5 µL = 50 µL

We collect sperm from all males, but one fish did not give any sperm. Our final measured sperm volume is 57 µL from 11 males.

We want 15 samples. Because this is more than 12 samples, we will divide the sperm into two aliquots for freezing. To account for pipetting loss we add one sample volume for each aliquot, so 17 samples total volume.

This is 17 samples/11 males = 1.5 samples/male.

Dilution vol = total samples x 5 µL/sample = 17 samples x 5 µL sperm/sample = 85 µL final volume.

85 µL final diluted volume – 57 µL measured volume = 28 µL of additional E400 to add

The sperm (17 samples) will be divided into two unequal aliquots (85 µL/2). Because the final sample number is an odd number, the sperm is divided based on whole samples of 40 µL (8 samples) and 45 µL (9 samples).

RMMB Volume for Aliquot 1 (40 µL) = (Sperm Volume) x 3 = 40 µL x 3 = 120 µL
RMMB Volume for Aliquot 2 (45 µL) = (Sperm Volume) x 3 = 45 µL x 3 = 135 µL

2.5.3.2 Example of dilution based on the number of samples/male.

We have 7 short-fin males from an imported line. Before we retire these males, we would like to freeze as many samples as possible in one freeze event.

Starting E400 volume (short-fin males) = (# of males -2) x 5 µL= (7 males -2) x 5 µL = 5 x 5 µL = 25 µL

We collect relatively good sperm from all the males resulting in 30 µL of moderately dense sperm.

We decide to dilute for 3 samples/male plus a test thaw.
Samples = [# males x 3 samples/male] + 1 (test thaw) = [7 males x 3 samples/male] + 1 = 21 + 1 = 22

22 samples will need to be frozen in two batches so we will divide the sperm into two aliquots. To account for pipetting loss, we add one sample volume for each aliquot, so 24 samples total.

With the additional volume add-ons (for test thaw and pipetting loss), this results in an actual per-male yield of 24 samples/7 males = 3.4 samples/male.

Dilution vol = total samples x 5 µL/sample = 24 samples x 5 µL sperm/sample = 120 µL final volume.

120 µL final diluted volume – 30 µL measured volume = 90 µL of additional E400 to add.

The sperm (24 samples) will be divided into two equal aliquots (120 µL/2) of 60 µL (12 samples) each.

RMMB Volume = (Sperm Volume) x 3 = 60 µL x 3 = 180 µL for each batch of samples

2.6 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 conical tube 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 9 inches or 23 cm (approximately 2x 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 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.

2.7 Time in Dry Ice

Freeze in dry ice for 20-45 minutes. The sample tube assembly should remain in dry ice for a minimum of 20 minutes, but not longer than 45 minutes. The exact time is not critical apart from observing the minimum and maximum times.

3. Step-by-Step Protocols

3.1 Protocol for Collecting Stripped Sperm

3.1.1 Materials for Stripping Sperm

- Dissecting microscope with incident lighting
- 10 µL calibrated microcapillary pipettes (Drummond # 2-000-010)
- Aspirator tube assembly (Sigma A5177-5EA, one also included in each pack of Drummond microcapillary pipettes)
- Millipore filter forceps (Millipore # XX6200006P) (Rubber tips – electrical shrink tubing)
- Plastic spoon for moving fish (1/8" holes drilled in tip for draining water)
- Soft foam rubber 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 low-retention microcentrifuge tubes (colorless, Fisher 02-681-311)
- Pipetman and tips
- Ice bucket (with 1000 µL pipet tip box insert or similar tube rack driven into ice to hold 0.6 mL tubes)

3.1.2 Solutions for Stripping Sperm

- Anesthesia solutions
    - Tricaine anesthesia (168 mg/L)
    - Isotonic PBS rinse
    - Fish water for anesthesia recovery
- E400 sperm extender

3.1.3 Preparations for Collecting Stripped Sperm

Withhold food and separate males from females 18-24 hours prior to sperm collection.

3.1.4 Procedure for Collecting Stripped Sperm

1. Anesthetize male in Tricaine solution (168 mg/L), briefly rinse fish (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 (with PBS or fish water) 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 cotton-tipped swab or the end of the microcapillary can be used to move the pelvic fins out of the way if necessary. Use Millipore filter 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 very 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. To stop any inadvertent sperm motility from urine contamination, the sperm should be immediately expelled into a tube containing E400 sperm extender for pooled sperm (see Section 3.2 Cryopreservation of Pooled Sperm). Alternatively, the sperm volume can be normalized with E400 within the capillary and then expelled into RMMB CPM to freeze immediately (see Section 3.3 Cryopreservation of Sperm from Individual Males)

3.2 Protocol for Cryopreservation of Pooled Sperm

3.2.1 Materials for Cryopreservation of Pooled Sperm

- Materials for Stripping Sperm (see Section 3.1.1)
- Cryogenic vials and caps (Thermo Scientific 0.5 mL Matrix #3744, or 2-mL Corning # 430488, or similar)
- Pipetman and tips
- 15 mL conical tubes (Falcon 352096 or similar) with appropriate cryovial spacers (see Section 2.1 Optimum Cooling Rate
- Styrofoam container or cooler (~12" x 12") for dry ice
- Timer set to count up (Fisher 06-662-47)
- Styrofoam container for liquid nitrogen (LN_2) tray
- Fiberglass tray for LN_2 (Molded Fiberglass Toteline Nesting Tote 922108 - 9-3/4"L x 6-1/8"W x 2-1/8"H)
- Appropriate cryogenic vial storage box
- Liquid nitrogen Dewar flask (Thermo Scientific™ 41502000 or similar)
- Cryogloves (Tempshield # 11-394-306)

3.2.2 Optional Materials for Cryopreservation

- Cryovial labels and printer (GA International, Labtag.com)
- Thermo Scientific 8-Channel Screw Cap Decapper for Matrix screw top vials
- LN_2 freezer racks - Custom BioGenic Systems, (800) 523-0072, Customerservice@custombiogenics.com

3.2.3 Solutions for Cryopreservation of Pooled Sperm

- Anesthesia solutions (see Section 3.1.2 Solutions for Stripping Sperm)
- E400 sperm extender
- RMMB CPM – thaw aliquot with heating (45-50℃), cool to room temperature prior to use

3.2.3 Preparations for Cryopreservation of Pooled Sperm

Prepare labeled sample cryovials as needed prior to sperm collection.

Prepare a 0.6 mL microcentrifuge tube (low retention, colorless) containing E400. Calculate the volume of E400 based on the number of males as follows. Prepare one tube for each line being frozen. Keep chilled on ice.

E400 Starting Volume for Pooled Sperm:
Short-fin zebrafish: Starting E400 volume (µL) = (# of males -2) x 5 µL
Long-fin zebrafish: Starting E400 volume (µL) = (# of males -4) x 5 µL

3.2.4 Procedure for Cryopreservation of Pooled Sperm

1. Collect pooled sperm from fasted males by squeezing and expel into E400 as described above.

2. Continue collecting stripped sperm from all available males and pool into the same 0.6 microfuge tube on ice.

3. Measure the volume of pooled sperm in E400 with a Pipetman or similar air-displacement pipettor. Set the Pipetman at or slightly more than the starting E400 volume. Draw the sperm (in E400) into the pipet tip and adjust the pipette volume until all the solution just fills the tip and no solution remains in the tube. Expel the sperm back into the tube and record the estimated volume.

4. Dilute sperm additionally with E400 based on the number of desired samples or the number of samples/male as described in Section 2.5 Diluting Pooled Sperm without Cell Counts.

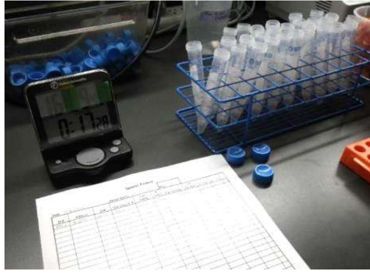
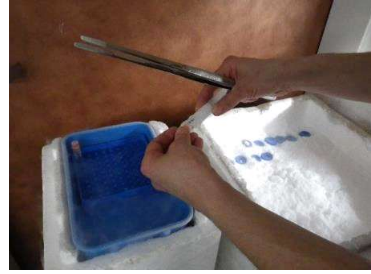
5. If the final pooled sperm volume is > 60 µL (12 samples), divide the sperm volume into more than one tube and freeze in batches.

6. For each tube of sperm, calculate the volume of RMMB to add: RMMB Volume = 3 x Sperm Volume

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

8. 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. Start timer.

9. Freeze samples in dry ice for 20-45 minutes, then quickly transfer samples to a cryovial storge box submerged in LN_2.

3.3 Protocol for Cryopreservation of Sperm from Individual Males (1 or 2 samples/male)

3.3.1 Materials for Cryopreservation of Sperm from Individual Males

- Materials for Stripping Sperm (see Section 3.1.1)
- Cryogenic vials and caps (0.5 mL Thermo Scientific Matrix #3744, or 2 mL Corning # 430488, or similar)
- 0.6 mL microcentrifuge tubes (clear, Fisher 02-681-311 and green, Fisher 05-408-124)
- Pipetman and tips
- 15 mL conical tubes (Falcon 352096 or similar) with appropriate cryovial spacers (see Section 2.5 Optimum Cooling Rate)
- Styrofoam container or cooler (~12” x 12”) for dry ice
- Timer set to count up (Fisher 06-662-47)
- Fiberglass tray for liquid nitrogen (LN_2) (Molded Fiberglass Toteline Nesting Tote 922108 - 9-3/4"L x 6-1/8"W x 2-1/8"H)
- Styrofoam container for LN_2 tray
- Appropriate cryogenic vial storage box
- LN_2 Dewar flask (Thermo Scientific™ 41502000 or similar)
- Cryogloves (Tempshield # 11-394-306)

3.3.2 Optional Materials for Cryopreservation

- Cryovial labels and printer (GA International, Labtag.com)
- LN_2 freezer racks - Custom BioGenic Systems, (800) 523-0072, Customerservice@custombiogenics.com

3.3.3 Solutions for Cryopreservation of Sperm from Individual Males

- Anesthesia solutions (see Section 3.1.2 Solutions for Stripping Sperm)
- E400 sperm extender
- RMMB CPM: Thaw aliquot with heating (45-50℃), cool to room temperature prior to use

3.3.4 Preparations for Cryopreservation of Sperm from Individual Males (1 or 2 samples/male)

Prepare labeled sample cryovials as needed.

Prepare 0.6 mL microcentrifuge tubes (colored, green) containing 500 µL E400 each. Prepare one tube for each line being frozen. This will be used for normalizing the sperm volume. Keep chilled on ice until ready to squeeze males.

Prepare 0.6 mL microcentrifuge 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 “3 mm +” is recommended (see Section 1.6 Rating Stripped Sperm).

This method can be easily adjusted to freeze one sample/male. In this case, prepare 0.6 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. If precipitation is noted in the RMMB CPM, heat the tube briefly until the raffinose is back in solution.

3.3.5 Procedure for Cryopreservation of Sperm from Individual Males

1. Anesthetize fasted male zebrafish and collect stripped sperm into microcapillary. Transfer fish to system water for recovery.

2. Without delay, normalize sperm to the appropriate volume (5 or 10 µL for one or two samples, respectively) with E400 (green microcentrifuge tube) in the microcapillary and then expel the entire contents of the microcapillary (sperm + E400) into one of the 0.6 mL clear microcentrifuge tubes 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 labeled cryovial (20 µL/sample). Quickly cap the cryovials.

3. Immediately 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. Record the time samples are placed into dry ice.

4. Keep samples in dry ice for 20-45 minutes, then quickly transfer samples to a cryovial storage box submerged in LN_2.

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

Females should be isolated from males and food should be withheld 18-24 hours 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. Female zebrafish can be sensitive to tricaine anesthesia (168 mg/L). Holding females in a lower dose of tricaine (Tricaine Pre-anesthesia Solution, 48 mg/L) for a minimum of 10 minutes prior to anesthesia helps reduce gill hemorrhage and anesthesia mortality. Females can be held in the pre-anesthetic solution until anesthetized or until the morning egg collection is completed. Eggs, like sperm, are activated by water. It is 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 rinse solution prior to squeezing females or manipulating eggs. Minimize manipulation of the eggs as much as possible.

4.1 Protocol for Sperm Thawing and IVF

4.1.1 Materials for Sperm Thawing and IVF

- Plastic spoon for moving fish
- 60 x 15 mm Petri dishes
- White Taklon round paint brush, size 2
- Pipetman and tips
- Water bath @ 38°C
- Timer: 2 min. count-down

4.1.2 Solutions for Sperm Thawing and IVF

- Tricaine Pre-Anesthesia Working Solution (48 mg/L)
- Tricaine Working Solution (168 mg/L)
- PBS Isotonic Rinse and fish water for anesthesia recovery
- Sperm Solution SS300 (if thawing samples frozen without milk, use SS300+Milk)
- dH_20
- 0.5X Embryo Medium

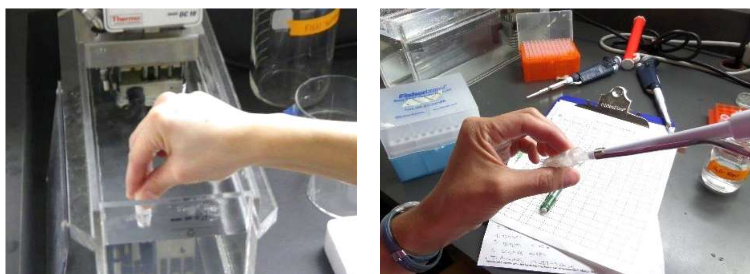
4.1.3 Procedure for Sperm Thawing and IVF

1. Place female zebrafish into pre-anesthesia solution (typically 500 mL) 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. Place cover on petri dish while thawing sperm.

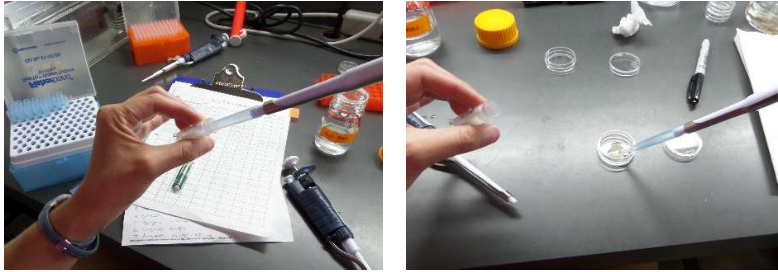
3. Remove the sperm sample cryovial from the LN_2 and quickly open cap to vent any LN_2 in the vial.

4. Thaw cryovial in a 38°C water bath until the frozen pellet is < 2 mm (~10-15 sec).

5. Add 200 µL room-temperature SS300 solution (or SS300+Milk) to the cryovial.



6. Transfer sperm into the egg mass (slide pipet tip along the bottom of petri dish into the pile of eggs and expel sperm into the egg mass, not just on top of the eggs).



7. Add 320 µL dH2O to the eggs to activate sperm and eggs. Start a count-down timer set for 2 minutes.

8. **<u>Do not</u>** mix or swirl the egg dish at this point - let it sit completely undisturbed. After 2 minutes, flood the dish with 0.5X Embryo Medium.

9. Observe fertilization rate at 3-4 hours post-fertilization (high stage). Count embryos and remove the unfertilized. The fertilization rate is calculated as the number of viable embryos divided by the total number of eggs in the dish. Percent fertilization = [# fertilized/(# fertilized + # unfertilized)] x 100

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.

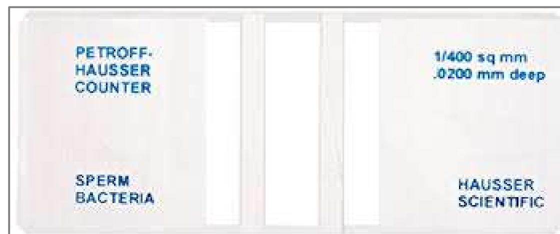
<u>5.1.1 Cell Density Assessment by Counting Chamber or Hemocytometer</u>

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
https://www.irvinesci.com/media/IrvineScientific/Resources/m/a/makler_counting_chamber-rev0.pdf
(Available from Irving Scientific: Makler® Counting Chamber Kit, Catalog ID: IS-363

https://www.irvinesci.com/makler-counting-chamber-kit.html)

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. 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 ($R^2 = 0.989$) between data and fitted curve resulting from a third-order polynomial equation.

$$\text{cells/mL} = (6\text{x}10^8)(A_{OD400})^3 - (4\text{x}10^8)(A_{OD400})^2 + (4\text{x}10^8)(A_{OD400})$$

The equation is used in an Excel calculator to determine sperm concentration. It is for use with OD_{400} measurements for stripped sperm in E400 extender with a NanoDrop 2000 Spectrophotometer. Some variation can exist between spectrophotometers so a calibration curve should ideally be developed for each specific instrument (Tan et al.,2010).

ZIRC NanoDrop 2000 Calibration Curve and Sperm Density Calculator
https://zebrafish.org/wiki/protocols/cryo

Additional information on diluting pooled sperm based on NanoDrop cell counts can be found on the ZIRC website: https://zebrafish.org/wiki/protocols/cryo

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 Section 1.3 Importance of Osmolality). 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 9 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 observe immediately. Examining the remainder of the NanoDrop dilution, if performed, is a good use of the sperm. Checking pre-freeze motility confirms the viability 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 (Section 4.1 Protocol for Sperm Thawing and IVF). 200 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 5.8 µL dH_20 on a slide, add 4.0 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 RMMB Cryoprotective Medium (CPM)
20% (w/v) D-(+)-Raffinose pentahydrate (Sigma-Aldrich 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 recipe above
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 to a clean beaker
Aliquot into 1.5 mL microfuge tubes, 1.0 mL each (or other convenient volume)
Store frozen at -20°C until used

For Use:
Thaw aliquot with heating (water bath or heat block 45-50°C) prior to use.
Make sure all raffinose is in solution prior to use.
Cool RMMB CPM to room temperature prior to mixing with sperm.

Note: Raffinose will precipitate if the RMMB CPM is held on ice. If this occurs, heat solution again to get it back into solution. Hold RMMB CPM at room temperature 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^oC

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^oC
Thaw and use at room temperature

6.6 0.5X E2 Embryo Medium
To make 0.5X E2 Embryo Medium see instructions at https://zebrafish.org/wiki/protocols/nursery

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 – see recipe below
Bring final volume up to 1000 mL with dH_2O
Store in an amber bottle at 4°C

6.8 Tricaine 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 Tricaine 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 powdered 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)

CO_2 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)

CO_2 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

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Yang H, Carmichael C, Varga ZM, et al. (2007) Development of a simplified and standardized protocol with potential for high-throughput for sperm cryopreservation in zebrafish Danio rerio. *Theriogenology* 68(2): 128-136.

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