Zebrafish sperm cryopreservation with N,N-dimethylacetamide

John P. Morris IV, Stephane Berghmans, David Zahrieh,
Donna S. Neuberg, John P. Kanki, and A. Thomas Look
Dana-Farber Cancer Institute, Boston, MA, USA

BioTechniques 35:956-968 (November 2003)

*High fecundity, rapid generation time, and external development of optically clear embryos make the zebrafish (*Danio rerio*) a convenient vertebrate model for genetic, developmental, and disease studies. Efficient sperm cryopreservation enhances the zebrafish model system by optimizing productive use of facility space, extending the reproductive lifetime of males, providing an alternative to live stocks for strain recovery, and ensuring the survival of valuable mutant lines. Here we identify a cryoprotective medium, 10% N,N-dimethylacetamide (DMA) (v/v) diluted in buffered sperm motility-inhibiting solution (BSMIS), as well as parameters for zebrafish sperm cryopreservation that enhance cryopreservation efficiency and significantly increase the yield of live embryos from archived stocks. Our experiments emphasize the effect of the ratio of sperm and medium volume and the use of large egg clutches to maximize the recovery of viable embryos.*

INTRODUCTION

The zebrafish (*Danio rerio*) is a convenient model organism for genetic and developmental studies because of its high fecundity, rapid generation time, and the external development of optically clear embryos (1,2). These qualities in a vertebrate make zebrafish an excellent animal to genetically model human diseases (3).

While the small size and fecundity of the zebrafish make it amenable to large-scale genetic studies, limited animal facility capacities can pose a rate-limiting obstacle to experiments (4,5). Efficient sperm cryopreservation can help circumvent this problem by reducing the number of live fish in a system while maintaining their reproductive capacity (5). Sperm cryopreservation also provides "genetic insurance" to recover strains if living stocks are lost (6) and extends the functional reproductive lifetime of males as long as sperm samples remain viable in storage (7). The technique has also been exploited experimentally in a novel reverse genetic mutagenesis study in which a cryopreserved sperm library was used to recover heterozygote mutant zebrafish of interest (8).

Sperm cryopreservation protocols currently available for zebrafish con-

sist of collecting sperm via abdominal massage or by dissecting fish to remove testes and freezing samples by incubation on dry ice and immersion in liquid nitrogen in a medium containing methanol and powdered milk diluted in Ginzburg Ringer solution (4,9). Harvey and colleagues (4) presented a protocol that yields a single low-volume frozen sperm stock collected from each male via abdominal massage. Artemis Pharmaceuticals GmbH has published a revised version of this protocol in which 2–4 samples (20 μ L total) are frozen from sperm collected by homogenizing testes in freezing medium (9). Efficient recovery of embryos from frozen stocks has been reported from both the original and updated protocols; however, we were unable to reproducibly obtain adequate yields of embryos using either protocol. Furthermore, both protocols require complex manipulations of samples in microcapillaries containing small total volumes.

Successful sperm cryopreservation has been reported in other fish species, such as salmon, catfish, cyprinids, and carp, with various cryoprotective media (6,7,10–12). Fish sperm freezing medium contains two components: at least one cryoprotectant and a diluent (13). Cryoprotectants are a class of chemicals that protect cells from damage

during freezing and thawing (14,15). Internal, or permeating, cryoprotectants permeate the cell membrane and regulate cellular osmotic environment during freezing, eliminating intracellular ice formation and helping to reduce the drastic changes in cell volume and pH that occur when supercooled water leaves the cell (7,15). Diluents, or extenders, are buffered solutions that maintain sperm in an immotile state and are used to collect sperm and to dilute cryoprotectants that are toxic to sperm at high concentrations (12,16). Experiments to evaluate cryoprotective media other than those containing methanol and powdered milk diluted in Ginzburg Ringer solution have not been reported for zebrafish.

Here we describe a method for zebrafish sperm cryopreservation using a cryoprotective medium containing N,N-dimethylacetamide (DMA) diluted to 10% (v/v) in buffered sperm motility-inhibiting solution (BSMIS) (10). This method yields four 50- μ L frozen sperm samples per male, which results in significantly higher in vitro fertilization rates than samples frozen using freezing medium that has been previously described (4,5,9). To achieve increased cryopreservation efficiency, we compared sperm collection techniques, evaluated the ability of a battery of cryoprotective media to maintain sperm viability during freezing and thawing, and investigated the effect of increasing the volume of the freezing medium on the fertilization capacity of cryopreserved sperm samples.

MATERIALS AND METHODS

Sperm Collection via Dissection

All male zebrafish used for this study were healthy wild-type fish from the AB strain that had reached sexual maturity but were not older than one and a half years. Testes were collected by dissection from males that were anesthetized with tricaine (Sigma, St. Louis, MO, USA) (5), thoroughly dried with lint-free wipes, decapitated, and the tail removed. Under a dissecting microscope (Leica MZ 12.5; Leica Microsystems, Buffalo, NY, USA), the abdomen was opened, and the testes were

removed and placed in 1.5-mL sterile Seal-Rite™ microcentrifuge tubes (USA Scientific, Ocala, FL, USA) containing a selected diluent or cryoprotectant solution and homogenized using microcentrifuge pestles (Kontes Glass Company, Vineland, NJ, USA).

Sperm Collection via Abdominal Massage

Male zebrafish were anesthetized and dried as described above. The fish were placed on their backs on a foam holder under the dissecting microscope. Gentle finger pressure was applied to the abdomen, and sperm was collected with a 2-μL Microcaps® microcapillary (Drummond Scientific, Broomall, PA, USA) directed to the anal pore. Collected sperm was expelled and mixed with diluent in a 1.5-mL sterile microcentrifuge tube.

Sperm Motility and Fertilization Correlation Analysis

The testes from 14 fish were removed and homogenized. Sperm was incubated in 100 μL of (i) Ginzburg Ringer solution (GRS) (0.11 M NaCl, 4 mM KCl, 2.7 mM CaCl₂, and 77 mM NaHCO₃) (*n* = 6) (4) or (ii) Hank's solution (HS) (0.14 M NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, and 4.2 mM NaHCO₃) (*n* = 8) (5). The sperm was incubated for 25 min before percent motility was assayed by activating 10 μL of the sperm dilution with 90 μL egg water (5) and examining 10 μL of the activated solution at 100× magnification (Eclipse TE2000; Nikon, Melville, NY, USA) on a hemacytometer (Bright-line Hemacytometer; Hauser Scientific, Buffalo, NY, USA). Percent motility was defined as the number of active sperm divided by the number of total sperm. Immediately after determining motility, eggs were collected in Petri dishes from one female fish (5). Three sperm samples incubated in GRS and four samples incubated in HS were diluted 1:10 (10 μL sperm:90 μL GRS or HS, respectively), and the remaining three samples incubated in GRS and four samples incubated in HS were diluted 1:100 (1 μL sperm:99 μL GRS or HS,

respectively) and each applied to an egg clutch. Fertilization capacity was recorded as percent fertilization, defined as the number of fertilized eggs divided by the total number of eggs per clutch. The eggs were fertilized with diluted sperm previously described (5). Percent fertilization was determined 18 hours post-fertilization.

Cryoprotectant Evaluation

We evaluated the post-thaw percent motility of sperm samples frozen in medium containing 5%, 10%, 15%, or 20% (v/v) cryoprotectant diluted in HS. Five cryoprotectants with a range of cell permeability were tested: DMA, dimethyl sulfoxide (DMSO), ethylene glycol, glycerol, and methanol (all reagent grade; Sigma). Permeability was reported as permeability coefficient, which was defined as a measure of the ability of a solute to pass through a membrane over time (17). Sperm samples were frozen from three males per cryoprotectant concentration using the standard freezing technique described previously (4), with the following modifications. The sperm was collected by homogenizing testes removed by dissection in 20 μL of freezing medium, and the samples were incubated on dry ice for 30 min before being immersed in liquid nitrogen. Post-thaw percent motility was assayed by thawing one 10-μL sample from each male fish at room temperature and then activating the sperm by adding 90 μL of egg water and examining motility as described above.

The *in vitro* fertilization of samples frozen using the protocol described above (4) was performed as described previously (5), using 20 μL of sperm frozen from individual males. To normalize the number of eggs being fertilized, egg clutches were pooled from three females (300–600 eggs). The established freezing medium was prepared as previously described (4) using 10% methanol and 15% powdered skim milk diluted in GRS, and the samples frozen using this medium were incubated on dry ice for 20 min before immersion in liquid nitrogen.

Diluent Evaluation

The effect of five diluent solutions

on prefreeze sperm percent motility was evaluated: (i) BSMIS (75 mM NaCl, 70 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, and 20 mM Tris, pH 8.0) (10); (ii) GRS; (iii) HS; (iv) Mounib's solution (MS) (125 mM sucrose, 6.5 mM reduced glutathione, and 100 mM KHCO₃) (18); and (v) sperm-extender solution (SES) (80 mM KCl, 45 mM sodium acetate, 0.4 mM CaCl₂, 0.2 mM MgCl₂, and 10 mM HEPES, pH 7.7) (16). Sperm from six males was used to evaluate each diluent. The sperm from each male was collected via testes dissection and homogenized in 100 μL of diluent. Percent motility was assayed at 5, 15, 45, and 60 min post-dilution by activating 10 μL of diluted sperm with 90 μL of egg water as described above.

Biostatistical Analysis

Averages of replicate experiments are reported as the mean ($\bar{x} \pm \text{SD}$). The variation in percent motility among different groups of sperm frozen in experimental cryoprotectants and incubated in experimental diluents was analyzed by analysis of variance (ANOVA) (19). F-tests were used to test for differences in variances or equality of means among two or more groups (19). The Wilcoxon rank-sum test, or *t* test if appropriate (19), was used for testing differences among clutch sizes as well as among the following sperm sample characteristics: average total sperm count, average percent motility, percent fertilization, and recovered embryos. The correlations between percent motility and percent fertility and among clutch size, recovered embryos, and fertilization capacity were assessed by Pearson's correlation coefficient (20). *P* values less than or equal to 0.05 were deemed statistically significant. No statistical adjustment was made for performing multiple tests.

RESULTS AND DISCUSSION

Zebrafish sperm may be collected directly by abdominal massage (4,5,9,21) or from testes removed by dissection and homogenized in diluents appropriately buffered to inhibit sperm motility (9,21). To determine which collection

Table 1. Cryopreserved Sample Post-Thaw Motility

Cryoprotectant	Permeability Coefficient (cm/s)	Post-Thaw Percent Motility (n = 3)				Average Post-Thaw Percent Motility (n = 12)
		5%	10%	15%	20%	
DMA	$14.7 \times 10^{-5} \pm 0.37 \times 10^{-5}$	8.8 ± 1.2	11.6 ± 5.8	12.1 ± 4.9	7.2 ± 0.6	9.9 ± 3.9
Ethylene Glycol	$3.4 \times 10^{-5} \pm 0.1 \times 10^{-5}$	7.7 ± 1.3	9.0 ± 6.8	7.4 ± 2.6	6.8 ± 0.4	7.6 ± 3.3
Methanol	$11.4 \times 10^{-5} \pm 0.4 \times 10^{-5}$	3.1 ± 2.4	8.9 ± 4.5	9.9 ± 2.2	9.3 ± 2.2	7.5 ± 3.7
DMSO	$1.30 \times 10^{-5} \pm 0.1 \times 10^{-5}$	8.0 ± 2.1	6.0 ± 1.0	7.6 ± 2.2	6.8 ± 0.8	7.1 ± 1.6
Glycerol	$0.58 \times 10^{-5} \pm 0.04 \times 10^{-5}$	5.7 ± 0.6	7.4 ± 0.2	8.4 ± 1.8	5.3 ± 0.4	6.7 ± 1.5

Cryoprotectant permeability coefficients are from Naccache et al. (17). Post-thaw percent motility is presented as the mean ($\bar{x} \pm sd$) for sperm frozen in medium that consisted of each cryoprotectant diluted to the listed concentration in Hank's solution. Average post-thaw percent motility represents sperm frozen with all four concentrations of each cryoprotectant. DMA, N,N-dimethylacetamide; DMSO, dimethyl sulfoxide.

technique provided the most reliable source of high-quality sperm samples, we recorded the average percent motility and average total sperm count of the samples collected using each method.

Abdominal massage was performed on 26 males, and the sperm was diluted in 100 μ L HS. Sperm was successfully collected from 13 fish (50%), with an average volume of $0.75 \mu\text{L} \pm 0.4$. Samples collected by abdominal massage had a mean percent motility of $32.3\% \pm 13.6$, with an average total sperm count of $1.3 \pm 1.2 \times 10^7$ sperm per male.

Testes were collected from 13 males via dissection and homogenized in 100 μ L HS. Viable sperm was collected from all 13 dissected males, with a mean percent motility of $23.0\% \pm 8.7$ and an average total sperm count of $2.5 \pm 1.2 \times 10^7$ sperm per male. Significantly more sperm was collected per male via dissection ($P = 0.05$) than with abdominal massage, although mean percent motility did not show a statistical difference ($P = 0.1$). Because dissection yielded large viable sperm samples from all fish while abdominal massage yielded sperm from only half of all fish tested, we conducted all further assays using sperm collected via dissection.

Fertilization is considered the best measure of sperm quality in fish (11),

but fertilization assays require large numbers of females available for egg collection. Sperm motility following activation with water has been shown in some fish to be correlated with fertilization capacity (10,11). Harvey et al. (4) showed that the percent motility of zebrafish sperm is positively correlated with fertilization capacity, which indicates that percent motility may be an appropriate measure of zebrafish sperm quality.

To compare percent motility with fertilization, the percent motility of sperm incubated in either HS or GRS for 25 min was recorded, and the sperm was immediately used for the in vitro fertilization of freshly collected eggs. Preliminary data indicated that sperm incubated in GRS possesses lower percent motility than sperm incubated in HS (data not shown), allowing a range of percent motilities to be analyzed. The sperm was diluted before being activated to eliminate the possibility of saturating eggs with viable sperm, which could have masked the effect of motility differences upon fertilization capacity (7). A significant positive correlation was observed between percent fertilization and percent motility of sperm that was diluted 1:10 (Figure 1) ($r = 0.9$, $P = 0.004$) and 1:100 ($r =$

0.9 , $P = 0.04$; data not shown). Thus, sperm percent motility was shown to be a reasonable measure of the quality of sperm subjected to different conditions and an alternative to fertilization assays, requiring fewer fish and less time to preliminarily evaluate experimental cryopreservation conditions.

We used sperm percent motility as an assay to evaluate various freezing media for use in zebrafish sperm cryopreservation. An optimal freezing medium minimizes toxicity to sperm while providing maximum protection from freezing damage. We evaluated the ability of five internal cryoprotectants over a wide range of cellular permeability to maintain sperm viability during cryopreservation by using each agent at four different concentrations and recording post-thaw motility of frozen sperm (Table 1).

Sperm frozen in 10% and 15% DMA displayed the highest overall values of post-thaw percent motility (Table 1) ($11.6\% \pm 5.8$ and $12.1\% \pm 4.9$, respectively). The average sperm post-thaw percent motility across all cryoprotectant concentrations was also highest in DMA (Table 1) ($9.9\% \pm 3.9$). Our experiments possessed 90% and 89% power to detect differences between post-thaw percent motility

Table 2. In Vitro Fertilization with Cryopreserved Sperm Samples

Protocol (n = 8)	Freezing Medium	Clutch Size	Recovered Embryos	Fertilization (%)
Capillary/20 μ L	10% Methanol and 15% Powdered Milk/GRS	442.1 $\pm$ 172.8	1.0 $\pm$ 1.2	0.2 $\pm$ 0.2
Capillary/20 μ L	10% DMA/HS	169.6 $\pm$ 124.6	27.9 $\pm$ 32.3	14.0 $\pm$ 10.1
Capillary/20 μ L	15% DMA/HS	312.1 $\pm$ 142.7	26.0 $\pm$ 15.9	10.7 $\pm$ 12.2
Protocol 1/200 μ L	10% DMA/BSMIS	478.4 $\pm$ 192.2	38.6 $\pm$ 10.6	10.2 $\pm$ 4.1
Protocol 1/200 μ L	15% DMA/BSMIS	436.6 $\pm$ 147.0	47.6 $\pm$ 42.4	9.3 $\pm$ 5.5

In vitro fertilization with sperm frozen using the standard freezing protocol derived from Harvey et al. (4), designated "capillary/20 μ L," was performed with the entire 20- μ L sample collected from one male. Fertilization with sperm frozen using Protocol 1, designated "protocol 1/200 μ L," was performed with one 50- μ L aliquot of 200 μ L samples frozen from individual males (n = 8 for all trials). Data are presented as the mean ($\bar{x} \pm$ sd). GRS, Ginzburg Ringer solution; DMA, N,N-dimethylacetamide; HS, Hank's solution; BSMIS, buffered sperm motility-inhibiting solution.

due to cryoprotectant and concentration, respectively, but we were unable to determine a statistically significant optimal combination ($P = 0.09$) at the 95% confidence level. Because our analysis possessed significant power to detect differences and we observed that DMA had the highest overall estimate of post-thaw percent motility at any concentration, the highest estimate of post-thaw percent motility at three of the four concentrations tested, and the highest overall average post-thaw percent motility estimate, we decided to select 10% and 15% DMA as our cryoprotectant concentrations for further optimization of other aspects of the cryopreservation protocol.

To determine if the results of our motility assay had identified a better cryoprotectant solution, we tested the fertilization capacity of sperm frozen using 15% or 10% DMA versus that of sperm frozen using a cryoprotectant medium that has been previously described (4). Testes from 24 males were collected, diluted in different freezing media, and frozen using the standard cryopreservation technique derived from Harvey et al. (4). Sperm from eight males per group were frozen using either 20 μ L of 10% (v/v) DMA diluted in HS, 15% DMA (v/v) diluted in HS, or 10% methanol (v/v) and 15% powdered milk diluted in GRS (4). Samples frozen using 10% DMA re-

sulted in an average fertilization rate of 14% $\pm$ 10.1, while samples frozen in 15% DMA resulted in an average fertilization rate of 10.7% $\pm$ 12.2 (Table 2). Samples frozen using the established medium resulted in an average fertilization rate of 0.2% $\pm$ 0.21, which was significantly less than that of samples frozen using 15% and 10% DMA diluted in HS (Table 2) ($P = 0.04$ and 0.006, respectively). This indicates that DMA diluted in HS provides significantly higher protection from freezing damage than methanol and powdered milk diluted in GRS. The addition of powdered milk, a nonpermeating or external cryoprotectant (15), may contribute to the low post-thaw fertilization capacity of the sperm frozen using the control freezing medium. As a cell impermeable agent, powdered milk may disrupt the osmotic environment of sperm, leading to cellular damage irrespective of that produced by the freeze-thaw process. Several external cryoprotectants have been evaluated for their ability to enhance the efficiency of freezing medium in other fish, such as cyprinids and northern pike (*Esox lucius*) (10,22), and could be evaluated as alternatives to powdered milk for enhancing zebrafish sperm cryopreservation efficiency.

We investigated the toxicity of five diluents by analyzing the percent motility of sperm incubated in the solutions. We analyzed both the average percent motility and decrease in percent motility over time. We chose BSMIS as our

Figure 1. Zebrafish sperm percent motility is correlated with sperm fertilization capacity. Sperm samples were incubated in both Ginzburg Ringer solution (GRS) and Hank's solution (HS) as described (see Materials and Methods). Figure 1 depicts sperm that was diluted at a ratio of 1:10 (see Results and Discussion).

diluent because it provided a high level of average sperm percent motility and a low and stable decrease in percent motility over time (data not shown).

High concentrations of sperm in frozen samples have been reported to decrease fertilization capacity (13). Therefore, we investigated the fertilization capacity of sperm samples frozen in a relatively high volume of medium. Sperm was frozen from eight males using both 10% and 15% DMA diluted in BSMIS and diluted by a factor of 10, compared to the volume used in our preliminary cryoprotectant evaluation assays and that used in a published protocol (9), which provided four separate 50- μ L cryopreserved sperm samples from each male (Protocol 1). In vitro fertilization was performed using one of the 50- μ L aliquots (Protocol 1). Eight 50- μ L sperm samples frozen in 10% DMA medium exhibited capacities of $10.2\% \pm 4.1$ in fertilization assays, while eight 50- μ L samples frozen in 15% DMA medium resulted in a fertilization rate of $9.3\% \pm 5.5$ (Table 2). Percent fertilization, clutch size, and the number of embryos recovered from each sample used for fertilization were not significantly different ($P = 0.99, 0.63, \text{ and } 0.57$, respectively). We observed significantly greater variance in the number of embryos recovered from fertilizations performed with sperm samples frozen with 15% DMA versus those frozen with 10% DMA ($P < 0.001$). This analysis led us to choose 10% DMA diluted in BSMIS as our cryoprotectant solution because the 10% DMA solution produced more consistent results and higher probability of embryo recovery.

The samples frozen in four 50- μ L aliquots using 10% or 15% DMA diluted in BSMIS exhibited significantly higher fertilization capacity when compared to samples frozen with methanol and powdered milk diluted in GRS in the standard freezing technique ($P = 0.006 \text{ and } 0.002$, respectively). Thus, we found that although increasing the freezing medium volume did not adversely affect sample fertilization capacity, it has the advantage of increasing the number of available cryopreserved samples that result from each male. Because increasing the freezing medium volume 10-fold maintains ef-

Protocol 1. Zebrafish Sperm Cryopreservation with 200 μ L 10% DMA/BSMIS and In Vitro Fertilization with Frozen Samples

Preparation

1. Prepare freezing solutions.

BSMIS: 75 mM NaCl, 70 mM KCl, 2 mM CaCl_2 , 1 mM MgSO_4 , and 20 mM Tris, pH 8.0. Store at 4°C. For each male fish from which sperm will be cryopreserved, aliquot 20 μ L of BSMIS into a microcentrifuge tube and place on ice.

Freezing Medium Stock: 11.2% DMA in BSMIS. Vortex mix for 10 min. DMA/BSMIS stock solution should be made fresh before each round of cryopreservation.

2. Fill a large styrofoam box (at least 10 in $\times$ 10 in $\times$ 10 in) with dry ice. For each male fish from which sperm will be cryopreserved, insert two 50-mL conical centrifuge tubes (Falcon®, Becton Dickinson, Franklin Lakes, NJ, USA) into the dry ice until just the cap is visible.

Sperm Collection and Freezing

1. Anesthetize the male fish, dissect, and remove the testes. Place the testes in 20 μ L chilled BSMIS.
2. Homogenize testes by grinding with a pestle.
3. Add 180 μ L of 11.2% freezing medium stock and mix until uniformly cloudy. [Note that for samples frozen in 15% DMA (see Results and Discussion), 180 μ L of a 16.75% DMA stock are added to homogenized sperm in BSMIS to achieve a final DMA concentration of 15% DMA.]
4. Aliquot 50 μ L of the sperm solution into 4 labeled 1.5 mL cryotubes (Neptune™; Continental Lab Products, San Diego, CA, USA).
5. Insert two cryotubes each into the 50-mL centrifuge tubes on dry ice.
6. Incubate for 30 min on dry ice.
7. Quickly remove samples from 50-mL centrifuge tubes and immerse directly in liquid nitrogen. Transfer the samples to a liquid nitrogen freezer for long-term storage.

In Vitro Fertilization

1. Preheat BSMIS to 37°.
2. Collect eggs from the females in a Petri dish. Pool egg clutches from at least three females (300–600 eggs).
3. Remove one 50- μ L sample from the liquid nitrogen and open the cap. Add 1 mL 37°C BSMIS and mix until the sample is completely thawed. Apply the thawed sperm to the eggs.
4. Immediately add 1 mL of egg water.
5. After chorions separate from the egg mass, fill the Petri dish with egg water and incubate the embryos at 28.5°C.

DMA, N,N-dimethylacetamide; BSMIS, buffered sperm motility-inhibiting solution.

ficient fertilization capacity, increasing the dilution to yield even more aliquots per male may be an option for laboratories that have adequate liquid nitrogen storage space and want to further maximize the reproductive capacity of archived stocks.

Cryopreservation with DMA-containing medium has been shown to

lead to increased ATP concentrations in European catfish sperm (23). Because the loss of ATP due to the freeze-thaw process could reduce post-thaw motility, DMA may have the same affect on zebrafish sperm, potentially contributing to the observed trend toward higher post-thaw quality than treatment in other cryoprotectants. Interestingly,

ATP content in European catfish sperm frozen with DMSO, methanol, and glycerol (23) followed the trend of post-thaw motility of sperm frozen in those cryoprotectants observed in our study. In addition, DMA possessed the highest cell permeability of the evaluated cryoprotectants (Table 1). We observed a general trend of increasing post-thaw percent motility with respect to increased cryoprotectant cell permeability (data not shown). This may indicate that cryoprotectants that readily pass through the zebrafish spermatozoa membrane may better control the equilibrium between supercooled intracellular water and extracellular ice, reducing freezing damage. Thus, other cryoprotectants with high cell permeability and the ability to increase intracellular ATP concentration may be the best candidates for future study.

Although the effect of adding ATP to freezing medium on post-thaw sperm quality has not been evaluated, the ability of other agents to enhance post-thaw sperm viability has been tested in some species. Methylxanthine phosphodiesterase inhibitors such as caffeine and theophylline have been shown to increase both intracellular cyclic AMP concentration as well as the fertilization capacity of low-quality sperm samples from salmonids and trout (22), and their effect could be evaluated in the zebrafish model as a strategy to increase cryopreservation efficiency.

Increasing the initial quality of samples collected by fish dissection may further optimize our protocol. We observed that sperm samples collected by dissection possessed a significantly higher total sperm count than those collected by abdominal massage but did not exhibit significantly higher percent motility. Treating male zebrafish with agents that induce spermatozoa maturation, such as 17 α , 20 β -dihydroxy-4-pregnen-3-one, a hormone that has been shown to induce spermatogenesis in teleosts, may provide a way to exploit the large inactive sperm population observed in homogenized samples by increasing the number of mature sperm (24). Other variables such as incubation time during slow freezing on dry ice before immersion in liquid nitrogen, different freezing techniques and rates, and novel cryo-

protectants and additives as discussed above may be investigated in future studies to further enhance zebrafish sperm cryopreservation efficiency. Methods for recording sperm quality used in other models that may provide a more precise assay for evaluating cryopreservation conditions could be tested for use in the zebrafish as a part of further studies. For example, sperm viability in other fish species has been reliably determined by computer-aided sperm analysis (CASA) (10,11), and fluorescent staining has been used to determine the viability of dog sperm samples (25).

We performed our fertilizations with large egg clutches pooled from at least three females. As a teleost, zebrafish fertilization depends on the ability of active sperm to successfully reach the micropylar chorion passage and penetrate the egg mass (26). Because this is a stochastic process, we used relatively large clutch sizes in these assays to increase the probability that viable sperm could reach the eggs. Clutch size was positively correlated with fertilization rate when samples frozen using Protocol 1 with relatively high medium volumes of both 10% and 15% DMA diluted in BSMIS were used for in vitro fertilization ($r = 0.82$ and 0.98 , respectively). In addition, the number of recovered embryos was positively correlated with clutch size when samples frozen using the standard freezing technique with 10% DMA diluted in HS and samples frozen using Protocol 1 with 15% DMA diluted in BSMIS were used for in vitro fertilization ($r = 0.81$ and 0.98 , respectively). However, we did not observe these correlations in all sets of fertilization experiments; thus, these data indicate that although large egg clutches may not necessarily increase post-thaw fertilization capacity, post-thaw sperm quality is best exploited if large egg clutches are in vitro fertilized, thus maximizing embryo recovery from cryopreserved sperm stocks.

ACKNOWLEDGMENTS

We thank Doris Dodson for assistance with manuscript preparation and John-Paul Hezel with manuscript editing. This work was supported in part by National Institutes of Health grant

nos. CA68484 (A.T.L.) and CA21765 (A.T.L. and S.B.). Stephane Berghmans is a fellow of the Lymphoma Research Foundation. J.P.M. and S.B. contributed equally to this work.

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Received 18 April 2003; accepted 13 August 2003.

Address correspondence to A. Thomas Look, Department of Pediatric Oncology, Mayer-630, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA. e-mail: thomas_look@dfci.harvard.edu

Impact of RNA extraction from limited samples on microarray results

Hanna Ojaniemi^{1,3}, Birgitta Evengard¹, Daisy R. Lee², Elizabeth R. Unger², and Suzanne D. Vernon²

¹Karolinska Institutet at Huddinge University Hospital, Stockholm, Sweden,

²Centers for Disease Control and Prevention, Atlanta, GA, USA, and

³Royal Institute of Technology, Stockholm, Sweden

BioTechniques 35:968-973 (November 2003)

To move microarray technology into the diagnostic realm, the impact of technical parameters, such as sample preparation and RNA extraction, needs to be understood and minimized. We evaluated the impact of two RNA extraction methods, DNase treatment and the amount of hybridized cDNA probe, on the outcome of microarray results. The results for both RNA extraction methods were comparable, although one method resulted in residual DNA that slightly affected the microarray results. As little as one microgram of total RNA could be used to synthesize a cDNA probe and resulted in a gene expression profile that was similar to one produced using 5 µg total RNA, even though the overall signal intensity was lower. These experiments illustrate that microarray technology holds great promise for the use of limited clinical samples in the diagnostic setting.

INTRODUCTION

Microarray gene expression technology is an increasingly important tool in biomarker discovery. There are numerous potential clinical applications that could refine diagnosis and therapeutic monitoring as well as improve disease prevention through risk assessment and early detection. To move these platforms into the realm of in vitro diagnostics and to improve the efficiency of marker discovery, investigators need to understand and minimize the impact of biologic and technical variations on data interpretation. The numerous and potentially interrelated biologic variables in the microarray protocol include sample acquisition, extraction, labeling, hybridization, and detection. Technical variables include array design and fabrication, image acquisition, background correction, and data processing. In addition, the limited nature of the clinical samples presents challenges that must be overcome to move microarray technology into the diagnostic setting. The reproducible and reliable interpretation of microarray results depends on the use of high-quality RNA, which is invariably linked to factors such as sample processing and labeling (1).

It is universally accepted that RNA quality and quantity are of paramount

importance for assuring the reliability and reproducibility of microarray results. The current gold standard for assessing total RNA quality is the ratio of 28S:18S rRNA, which is observed following denaturing agarose gel electrophoresis. With appropriate standards, this method is also quantitative and is the only method that indicates the presence of contaminating DNA. Many different total RNA extraction methods are commercially available and each offers advantages. However, given the different extraction chemistries involved, we wanted to know if using the total RNA extraction method would impact the microarray gene expression results in ways that were unanticipated. To test this, we examined two of the most commonly used total RNA extraction chemistries and the utility of each of these to generate cDNA probes from limited samples for use with microarrays.

MATERIALS AND METHODS

Cellular Sample

Whole blood from the Centers for Disease Control and Prevention (CDC) donor services was collected into 8-mL citrate BD Vacutainer™ tubes (BD Vacutainer Systems, Franklin Lakes, NJ, USA). Peripheral blood mononuclear cells