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Challenges in Development of Sperm Repositories for Biomedical Fishes: Quality Control in Small-Bodied Species

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Abstract

Quality control (QC) is essential for reproducible and efficient functioning of germplasm repositories. However, many biomedical fish models present significant QC challenges due to small body sizes (<5 cm) and miniscule sperm volumes (<5 μL). Using minimal volumes of sperm, we used Zebrafish to evaluate common QC endpoints as surrogates for fertilization success along sequential steps of cryopreservation. First, concentrations of calibration bead suspensions were evaluated with a Makler® counting chamber by using different sample volumes and mixing methods. For sperm analysis, samples were initially diluted at a 1:30 ratio with Hanks' balanced salt solution (HBSS). Motility was evaluated by using different ratios of sperm and activation medium, and membrane integrity was analyzed with flow cytometry at different concentrations. Concentration and sperm motility could be confidently estimated by using volumes as small as 1 μL, whereas membrane integrity required a minimum of 2 μL (at 1×10^6 cells/mL). Thus, <5 μL of sperm suspension (after dilution to 30–150 μL with HBSS) was required to evaluate sperm quality by using three endpoints. Sperm quality assessment using a combination of complementary endpoints enhances QC efforts during cryopreservation, increasing reliability and reproducibility, and reducing waste of time and resources.

Keywords: quality control, reproducibility, sperm volume, biomedical model fishes

Introduction

Cryopreservation of fish sperm offers unique opportunities to preserve genetic information through time, allowing the management and regeneration of imperiled fish populations, backup of thousands of lines and strains of biomedical model fishes, hatchery control of stock enhancement, and genetic improvement of cultured species.[1,2] To ensure that thawed sperm satisfies set expectations of reproducibility and fertilization ability, a systematic evaluation of sperm quality is necessary at key steps within the cryopreservation process.[2] This quality assessment should ideally be independent yet parallel to the cryopreservation process itself. Sperm quality evaluation is, unfortunately, typically limited and often assessed only at the end of the process at fertilization or even at the hatching of larvae.

These terminal endpoints represent cumulative changes, and obscure identification of problems in individual steps occurring earlier in the process. This type of sperm evaluation—although currently considered practical—assumes (often without necessary testing) the availability of good quality eggs, and it lacks the resolution that is necessary to ensure quality of cryopreserved samples. It would be the equivalent of having customers or consumers provide the quality control (QC) for commercial products instead of the manufacturer, and, in reality, only serves to shift the costs of QC downstream to storage, thawing, and use steps. A comprehensive QC program, on the other hand, would address sperm quality at collection, after transport or refrigerated storage, after addition of cryoprotectant before freezing (equilibration), and after thawing. In this way, potential problems occurring along the production process could be identified and addressed promptly, saving time and resources,[1] while enhancing the reproducibility of outcomes.

Small-bodied species with short generation times, such as Zebrafish (*Danio rerio*), are widely used for developmental, biomedical, and toxicological research. Zebrafish are easy to breed, individual females can produce 100–200 of eggs at a

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time, fertilization is external, and the embryos can be easily studied due to their relatively large size and transparency.[3] External fertilization allows for artificial spawning, facilitating the production of specific crosses, including thousands of mutant lines (>30,000; http://zebrafish.org/fish/lineAll.php, accessed on September 13, 2016). The generation of new lines demands the availability of culture facilities, leading to high maintenance costs, and the risks associated with maintaining live fish. Gamete cryopreservation offers the possibility of significantly reducing the costs and risks of maintaining genetic resources of different lines through extended periods.[4]

In recent years, reproducibility in science has received increasing attention from major scientific entities (e.g., National Institutes of Health) after growing realization that biomedical studies were not routinely able to be reproduced[5] (www.nih.gov/research-training/rigor-reproducibility, accessed on September 13, 2016). The reproducibility of cryopreservation studies has been especially challenging, mainly due to a lack of standardized approaches and QC strategies.[2] Because of the tremendous research value of biomedical species and the costs of producing and characterizing new lines, high standards for the cryopreservation of their gametes are warranted.

Perhaps the single current greatest limiting factor impeding the development of QC programs for sperm cryopreservation in small-sized fishes is the small volume of sperm that can be obtained. For example, <5 μL of sperm can be acquired by stripping from a Zebrafish male. If a sample is meant to be frozen individually (i.e., not pooled with samples from other males), only 30–150 μL of sperm suspension would be available for QC and freezing after a 1:30 dilution with extender solution (to produce a concentration around 2×10^8 sperm cells/mL). Dealing with small sample volumes limits not only the number of times that sperm can be analyzed for QC along the cryopreservation process but also the quality of the analyses themselves. Nonbiological reasons of concern include, but are not limited to, sample evaporation, insufficient mixing, and pipetting inaccuracies that can lead to irreproducible results. Moreover, known problems can occur in cryopreservation and fertilization when sperm concentration is not measured and controlled.[6]

The goal of this study was to assess the volume requirements for QC of sperm that could be reproducibly monitored along any given cryopreservation protocol despite minimal volumes, and to provide strategies for practical application of QC for biomedical fish models to support development of comprehensive germplasm repositories. For this purpose, different volumes of sperm suspensions were evaluated to determine sample volumes necessary to ensure reproducibility of three of the most common QC endpoints: (1) sperm concentration determination and adjustment, (2) motility analysis, and (3) membrane integrity assessment.

Sperm concentration can be a useful indicator of fish reproductive status,[7] and its control during the cryopreservation process is critical to evaluate and minimize potential damage to sperm.[6] There are several methods used to estimate sperm concentration, including counting chambers, spectrophotometry, computer-assisted sperm analysis (CASA), and flow cytometry. However, detailed procedures, including dilution factors, mixing methods, equipment models, and standard protocols, are generally poorly described in the literature, if mentioned at all.[8]

This is counterintuitive because concentration is one of the easiest factors to estimate and control, yielding great improvements in reproducibility. The Makler® sperm counting chamber (Selfi-Medical Instruments, Haifa, Israel) was selected for this study because counting chambers can be easily adopted due to their high accessibility. Here, a calibration bead suspension with a known concentration range was used as a model for sperm suspension to determine the sample volumes and dilution methods that would reliably yield a concentration within the known concentration range of the calibration beads.

Estimation of motility is the most common evaluation method of sperm quality. Traditionally, motility has been estimated with a microscope by direct observation of activated sperm (by ''naked eye''). This method is subjective and can vary greatly from person to person, limiting reproducibility of results.[9,10] The usage of counting chambers has provided the opportunity to obtain quantitative data and facilitate training. More recently, with the development of CASA software, motility analysis is less subjective, and kinetic information from individual sperm cells within the field of view can be retrieved.[10]

Due to the short-lived nature of peak fish sperm motility (i.e., often <30 s), the methodology for sperm activation and analysis requires special considerations. Because a shift in osmolality is the most common factor triggering sperm activation in fishes,[11,12] characterizing the osmolality range at which sperm cells of a given species become activated is a key initial step for the standardized analysis of sperm motility. For this purpose, Zebrafish sperm suspensions were used to: (1) characterize the relationship between osmolality and activation, and (2) determine the minimum volume of sperm needed to evaluate sperm motility at the most effective activation ratio (sperm suspension to activation medium).

The cell membrane tends to be more vulnerable to cryopreservation than other cell structures.[13,14] It can be affected by osmotic shifts (such of those occurring during sperm activation),[15–18] refrigerated storage, cryoprotectant exposure, and freezing and thawing. Therefore, a systematic analysis of membrane integrity along the cryopreservation process provides important information about sample quality, and the quality of the process itself.[2] Flow cytometry is a powerful tool for the analysis of membrane integrity. It requires a highly diluted sample (around 1×10^6 cells/mL), which makes it suitable for use when limited volumes of sperm are available. Zebrafish sperm suspensions were used to determine the minimum volume of sperm needed to reproducibly evaluate sperm concentration and membrane integrity.

Materials and Methods

Fish husbandry

Protocols for the use of animals in this study were reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee (Baton Rouge, LA). Adult Zebrafish (wild type, AB strain, 6 months and 1 year old) were shipped overnight from the Zebrafish International Resource Center (Eugene, OR) to the Aquatic Germplasm and Genetic Resources Center (AGGRC) at the Louisiana State University Agricultural Center. On arrival,

fish were maintained within a 638-L recirculating system. Target values for water quality parameters were 28.5°C, pH 8.5, and 12-h light:12-h dark photoperiod. Fish were fed to satiation twice daily with a dry food master mix (http://zebrafish.org/documents/protocols/pdf/Fish_Feeding/Flake_Food/Dry_Food_Recipes2015.pdf, accessed on May 10, 2015). Additional water quality parameters that were monitored weekly and maintained within an acceptable range included: ammonia (0–1.0 mg/L), nitrites (0–0.8 mg/L), and nitrates (0–15 mg/L).

Sperm collection

Fish were anesthetized with 0.01% MS-222 (Tricaine methanesulfonate, Western Chemical, Inc. Ferndale, WA). Standard length and body mass were measured. Fish were placed ventral side up on a moist sponge and gently stripped. Sperm was collected into a 10-μL glass capillary tube (Drummond Scientific, Broomall, PA), and it was immediately released into a 1.5-mL microcentrifuge tube containing Hanks' balanced salt solution (HBSS, 0.137 M NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 1.0 mM MgSO4, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 4.2 mM NaHCO3, and 5.55 mM glucose, pH 7.2) with a 301 mOsm/kg osmolality (HBSS301) in a 1:30 ratio (sperm:HBSS301). This adjusted sperm concentration fell within the working range of previously tested cryopreservation protocols.[19]

Sperm concentration

Determining minimum sample volumes. A range of volumes of a calibration bead suspension (Accu-beads, lot# 152807351, Hamilton Thorne, Beverly, MA) with known (manufacturer specified) concentration range ($46 \pm 7 \times 10^6$/mL) was used to evaluate the accuracy and precision of the Makler® counting chamber at estimating bead concentration. The bead suspension was gently vortexed, eight different volumes (1, 2, 4, 5, 10, 20, 40, and 80 μL) were placed on the center of the chamber, and the chamber cover was placed on top. Beads were counted by using a dark-field microscope (200-× magnification; CX41, Olympus Corporation, Tokyo, Japan) on a 2×5-square strip (0.1 mm^2) on the first, second, and third quadrants of the chamber grid. The average count of the three 2×5-square strips was defined as a replicate. Ten replicates were measured per volume tested.

Data were log transformed to comply with normality and homoscedasticity assumptions, and one-way ANOVA with Tukey's multiple-comparisons test were used to compare the concentration of the different volumes of bead suspension. The results were considered statistically significant at $p < 0.05$.

Determining sample dilution effect. A calibration bead suspension (Accu-beads, lot# 152807351) with a known concentration range ($46 \pm 7 \times 10^6$/mL) was used to evaluate the accuracy and precision of the Makler® counting chamber at estimating bead concentration after dilution. Dilutions were prepared in a 1:4 (μL bead suspension: μL water) ratio with different volumes: 1:4, 2:8, and 4:16. To determine whether the dilution technique significantly affected the estimation of sperm concentration, two methods were tested: (1) The sample was directly mixed with water on the counting chamber, and (2) the sample was diluted with water in a microcentrifuge tube and transferred to the counting chamber. Beads were counted by use of a dark-field microscope (200-×) on the entire 100-square grid (1 mm^2). Ten replicates were measured per dilution tested.

One-way ANOVA with Tukey's multiple-comparisons test were used to compare the concentration of the different volumes of bead suspension within each mixing method. An unpaired *t*-test was used to compare the concentration of the bead suspension between mixing methods. The results were considered statistically significant at $p < 0.05$.

Sperm motility

Computer-assisted sperm analysis. A CASA system (HTM-CEROS, version 14 Build 013; Hamilton Thorne Biosciences, Beverly, MA) was used to capture 100 image frames at 60 frames per s. Multiple videos captured using different minimum contrast and minimum cell size values were tested to find a combination that would allow the software to recognize the highest number of correctly identified static sperm cells. Thus, the minimum contrast was set at 50, the minimum cell size was set at 2 pixels, and the default values when less than 5 cells were motile were set at 4 pixels for cell size and 65 for cell intensity.

Ratio of sperm-to-activation medium. To determine the osmolality at which sperm showed highest motility, 22 HBSS solutions across a range of osmolalities (35–269 mOsm/kg) were tested as activation media. Four replicates were analyzed, each consisting of pooled sperm collected from 3 to 6 males (standard length: 24.6 ± 0.9 mm; body mass: 291.1 ± 37.0 mg). The concentration of each replicate was adjusted to 1×10^8 sperm cells/mL using the counting chamber. Total percent motility was estimated by using CASA at 10 s after activation. A replicate for each activation media consisted of the average value of three subsamples of sperm activated in a 1:9 ratio mixed directly on the counting chamber.

Osmolalities were measured with a freezing point osmometer (Model 5010 OSMETTE III™; Precision Systems, Inc., Natick, MA). Final osmolality was defined as the osmolality measured after sperm suspension and activation medium were mixed. Based on that analysis, an activation ratio was selected, and five volumes of sperm were tested: 1, 2, 3, 4, and 5 μL with the corresponding proportional volume of deionized water (activation medium) (Barnstead Nanopure; Thermo Scientific, Dubuque, IA). Five replicates per volume tested were performed.

Because data did not comply with assumptions for normality and homoscedasticity, the Kruskal-Wallis test with multiple comparisons[20,21]was used to compare percent motility among the selected dilution ratios. One-way ANOVA with Tukey's multiple-comparisons test were used to compare the five volumes of sperm tested. The results were considered statistically significant at $p < 0.05$.

Sperm membrane integrity

Sample preparation. Three replicates, each consisting of pooled sperm from 3 to 6 males (standard length: 27.4 ± 0.2 mm; body mass: 391.4 ± 10.8 mg), at a concentration of 1.5×10^8 sperm cells per milliliter were analyzed. To determine the minimum volume required for membrane

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integrity analysis, sperm suspensions were further diluted with HBSS301 (5 μL of sperm into 245 μL HBSS301 [3×10^6 cells/mL], as described in Daly and Tiersch[22]). Four additional sperm volumes were tested: 4 μL of sperm into 246 μL HBSS301 (2.4×10^6 cells/mL), 3 μL of sperm into 247 μL HBSS301 (1.8×10^6 cells/mL), 2 μL of sperm into 248 μL HBSS301 (1.2×10^6 cells/mL), and 1 μL of sperm into 249 μL HBSS301 (6×10^5 cells/mL).

Because data did not comply with normality and homoscedasticity assumptions, the Kruskal-Wallis test with multiple comparisons[20,21] was used to compare total number of cells and percent of cells with intact membranes among the different dilutions. The results were considered statistically significant at $p<0.05$.

Flow cytometry. Sperm plasma membrane integrity was determined by using the fluorescent stains SYBR-14 and propidium iodide (PI) (PI/SYBR-14) (LIVE/DEAD® Sperm Viability Kit, cat. No. L-7011; Molecular Probes, Eugene, OR). The fluorescent dyes were prepared as described in Daly and Tiersch,[22] with the final concentration of SYBR 14 being 100 nM and that of PI being 12 μM. Samples were incubated in the dark for 20 min at room temperature (25°C).

Flow cytometry was performed with an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) equipped with a 488-nm, 50-mW solid-state blue laser. Immediately before analysis, the treated samples in microcentrifuge tubes were suspended with a vortex mixer, and 10 μL of sample was used to set collection gates by use of forward light scatter (FSC) and right-angle side scatter (SSC) at 35 μL/min rate by using CFlow Plus analysis software (version 264.21, BD Accuri; BD Biosciences). The FSC threshold was set at a default value of 80,000 to avoid inclusion of debris in the analysis.

Gating settings for the sperm population (gated events) used to exclude nonsperm events (particles) were based on the FSC and SSC profile of Zebrafish sperm. Gated events were viewed on a scatter plot showing the FL1 versus FL3 fluorescent detectors with fluorescence compensation based on the computed median fluorescence of single-dye control samples to reduce spectral overlap.[22] The PI fluorescence was detected by the FL3 detector (>670 nm), and SYBR-14 was detected by the FL1 detector (533/30 nm). The regions representing the membrane-intact cell population (viable) and the cell population with compromised membranes (nonviable) were set manually.

Results

Sperm concentration

Determining minimum sample volume. Significant differences were not observed among the tested volumes of bead suspension placed on the counting chamber [$F(7,72)=1.60$, $p=0.1493$], and the bead concentration in all the volumes tested fell within the known concentration range of the calibration beads ($4.6\pm0.7\times10^7$/mL) (Fig. 1). By this means, a minimum volume of 1 μL of bead suspension placed on the counting chamber was sufficient to provide an accurate estimation of its concentration.

Determining sample dilution effect. Significant differences in calculated bead concentration were not observed between different volumes when the samples were diluted directly on the counting chamber [$F(2,27)=1.52$, $p=0.2375$], and when the samples were first diluted in a microcentrifuge tube [$F(2,27)=0.51$, $p=0.6039$]. However, when comparing the two mixing methods, significant differences were observed [Unpaired-$t(29)=2.36$, $p=0.0238$] (Fig. 2), with the bead concentration falling outside the known concentration range when the dilution was mixed directly on the counting chamber, suggesting that a more homogeneous dilution was obtained when the mixing took place in a microcentrifuge tube. Also, the lack of significant differences among the different dilution volumes indicated that dilutions could be made with volumes as small as 1 μL of sperm suspension.

Sperm motility

Final osmolalities of around 60 mOsm/kg (as that obtained from a 1:4 activation ratio), and 31 mOsm/kg (obtained from

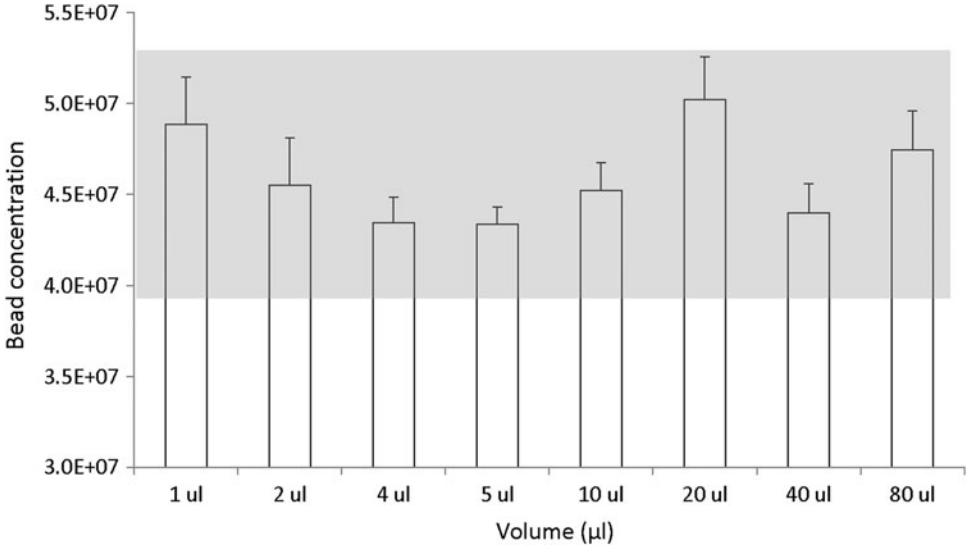

FIG. 1. Concentrations (beads/mL) of a calibration bead suspension estimated by using different volumes in a Makler® counting chamber. Significant differences were not observed among the tested volumes of bead suspension placed on the chamber. The *gray* area represents the known concentration range of the calibration bead suspensions (mean ± SEM). SEM, standard error of the mean.

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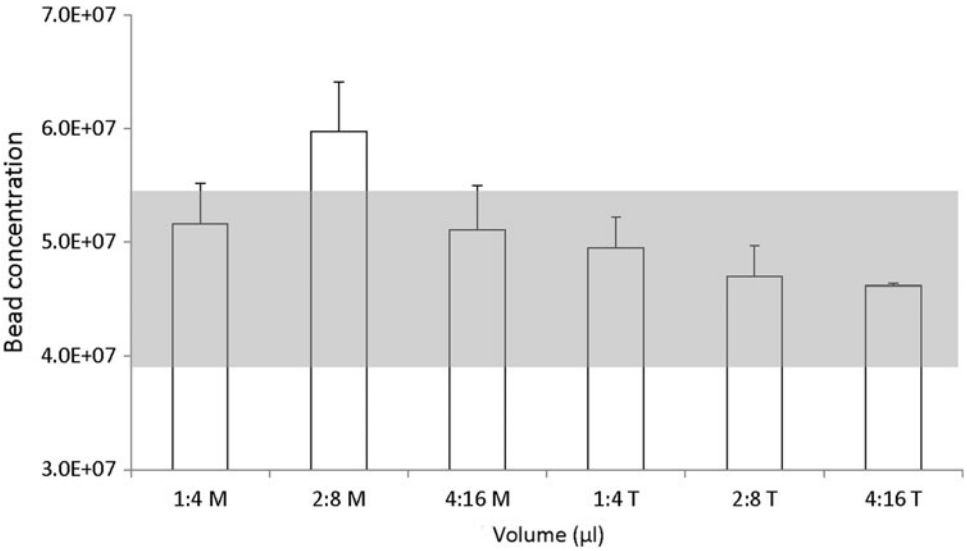

FIG. 2. Concentrations (beads/mL) of a calibration bead suspension estimated by using different dilution methods with dilution performed directly on a Makler chamber (M), or dilution in a microcentrifuge tube (T) before placement in the chamber. Significant differences were observed between the two mixing methods. The *gray* area represents the known concentration range of the calibration bead suspension (mean ± SEM).

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a 1:9 activation ratio) fell on the upper part of the activation curve (Fig. 3). When sperm was activated with media with osmolalities of 39 and 35 mOsm/kg, the final osmolalities (including the sperm sample) were 62 and 61 mOsm/kg. Because these osmolalities were within the range of final osmolality obtained from a 1:4 activation ratio, and the sperm densities obtained from such dilutions (using the original sperm concentration) were suitable for CASA analysis, the two activation media (representing a 1:4 ratio) in addition to the 1:9 ratio were compared. Significant differences among the three activation media were not observed (Table 1) [$H_{(2)} = 2.9921$, $p = 0.2240$]. The 1:4 activation ratio was further analyzed by using different volumes of sperm, and significant differences in percent motility were not observed among the different volumes placed in the counting chamber (Table 1) [$F(4,10) = 0.77$, $p = 0.5680$]. The results suggest that the final osmolality obtained from 1:4 or 1:9 ratios led to a similar degree of sperm activation (expressed as percent of total motility), and that a sperm suspension volume as small as 1 μL (activated with 4 μL of water) was sufficient to reproducibly determine sperm percent motility.

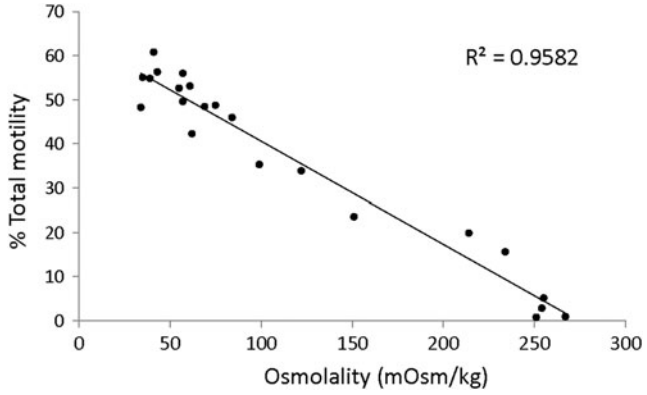

FIG. 3. Percent total motility (proportion of swimming sperm) after activation across a range of osmolalities ($N = 22$) of Hanks' balanced salt solution.

Sperm membrane integrity

Significant differences in the number of cells analyzed were observed among the tested dilutions [$H(4) = 69.0846$, $p < 0.0001$] (Fig. 4). Similarly, significant differences in the percent of cells with intact membranes were also observed among dilutions [$H(4) = 39.5647$, $p < 0.0001$], with the most dilute treatment (1 μL of sperm in 249 μL of HBSS) being significantly lower from the more concentrated treatments (Fig. 4).

As expected, the number of cells analyzed decreased with dilution of the sperm sample. However, the percent of cells with intact membranes was relatively stable in most treatments (Fig. 4). Therefore, the results suggest that when using an initial sperm suspension of 1.5×10^8 sperm cells per mL, only 2 μL of sperm suspension was needed to reproducibly estimate membrane integrity using flow cytometry.

Table 1. Average Percent of Sperm Motility (±Standard Error of the Mean) Obtained After Activation with Media at Different Osmolalities at Two Sperm-to-Deionized (DI) Water Ratios

Sperm-to-DI water ratio	*Activation features Final osmolality (mOsm/kg)*	*Sperm motility (%) (mean ± SEM)* (n = *4*)
1:9	31	55 ± 2
1:4	61	53 ± 4
1:4	62	42 ± 8
	Sperm volume (µL)	(n = *3*)
1:4	1	68 ± 8
	2	64 ± 2
	3	60 ± 3
	4	59 ± 4
	5	60 ± 2

The 1:4 activation ratio was then used to test different sperm volumes.

SEM, standard error of the mean.

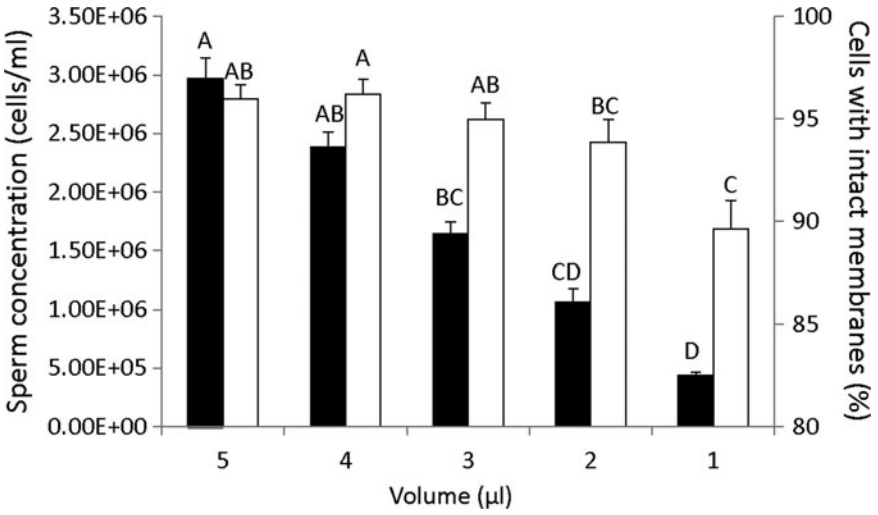

FIG. 4. Sperm concentration and the percentage of cells with intact membranes were analyzed and compared among five different sperm volumes (*y-axis*). Individual bars represent the means for each sperm volume (±SEM). The total number of cells (*left* axis, *black bars*), and percent of cells with intact membranes (*right* axis, *white bars*) are depicted side by side. Sperm volumes showing common *letters* were not significantly different ($p < 0.0001$).

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Discussion

In the past 30 years, several small-bodied fish species have become accepted as biomedical models for human disease. Their small size and reliable fecundity in short reproductive cycles represent advantages in terms of husbandry costs, and they offer the ability to obtain genetically modified lines relatively quickly.[3] However, the high demand for new genetically modified strains has created substantial financial strains in stock centers due to husbandry costs. For example, the Zebrafish International Resource Center catalogs more than 36,000 different genetically modified lines (zirc.org). Without the alternative of germplasm cryopreservation, stock centers would rapidly run out of space and financial resources to accommodate valuable lines.

A recurrent problem in sperm cryopreservation of aquatic species is the low reproducibility of studies due to the lack of standardization and comprehensive QC strategies. Indeed, basic QC endpoints (such as the ones explored in the present study) are rarely performed or reported in the literature; and, on the occasions when they are addressed, the lack of standardized protocols, terminology, and reporting guidelines makes it difficult (if not impossible) to reproduce outcomes.[2]

This problem is even more pronounced when working with small-bodied fishes and their minuscule sperm volumes. This study, however, demonstrated that after the sperm concentration is initially adjusted, it is feasible to repeatedly perform three basic quality analyses (sperm concentration, motility, and membrane integrity) along the cryopreservation process with minimal loss of sample. It is important to emphasize at this point that the information obtained at the different steps of the cryopreservation process for QC purposes provides direct feedback of the process itself while assuring the quality of the individual frozen samples. The value of the information gained and assured quality, therefore, can more than balance out the small volume of sample and time consumed.

In this study, 1 μL of sperm suspension was sufficient to reproducibly estimate sperm concentration and sperm motility, and 2 μL of sperm suspension (1.5×10^8 cells/mL) was sufficient to estimate membrane integrity. When estimating concentration, however, it was observed that diluting the sperm suspension in a microcentrifuge tube before placing it in the counting chamber provided better mixing. Although CASA can simultaneously estimate sperm concentration and motility, the physiology of fish sperm should be considered if attempting to measure both variables simultaneously. For example, if activation takes place in the microcentrifuge tube, sperm concentration estimates might be more accurate, but sperm motility estimates could be affected by the time delay before observation.

Thus, depending on the thresholds of a particular study, decisions of whether to use CASA to measure both variables simultaneously or separately can be made. Thus, 3–4 μL of sperm suspension was required to evaluate sperm quality by using concentration, motility, and membrane integrity as endpoints. This standardized data collection along the cryopreservation process provides the basis for reproducibility and the advancement of research. As such, a scheme of how QC check points could be distributed along the cryopreservation process for protocol validation and for repository production of cryopreserved sperm based on 4 μL of sperm suspension can be proposed (Fig. 5).

Beforehand knowledge of the quantity of sperm that is necessary to perform quality assessments allows for proper planning in terms of the number of fish needed and number of treatments and replicates desired. The limited volume of sperm obtainable from a single Zebrafish can be daunting when planning for sperm quality evaluation along the cryopreservation process. However, once the sperm sample is diluted, especially when multiple samples are pooled, sperm volume is less of a limiting factor as our previous experiences with small-bodied fishes (specifically *Xiphophorus* spp.) have demonstrated.[23]

Although dilution of fresh sperm suspensions (initially with an extender and then with a cryoprotectant) is intuitive, pooling of samples from several fishes is less apparent. Thus, except for cases where the sperm of individual fishes needs to be frozen separately, sperm pooling should be the default path. It is worthy of notice, therefore, that as the number of pooled fish increases, the proportional volume consumed for QC decreases as a percentage of the total volume. Accordingly, quality-controlled batches of pooled sperm can provide a practical approach to address reproducibility, especially when information on fertilization performance of the batches is included in the QC database.

As indicated earlier, there are several endpoints that can be analyzed to estimate sperm quality. Some assays target sperm attributes such as membrane integrity, mitochondrial activity, and DNA integrity. Others analyze sperm function such as

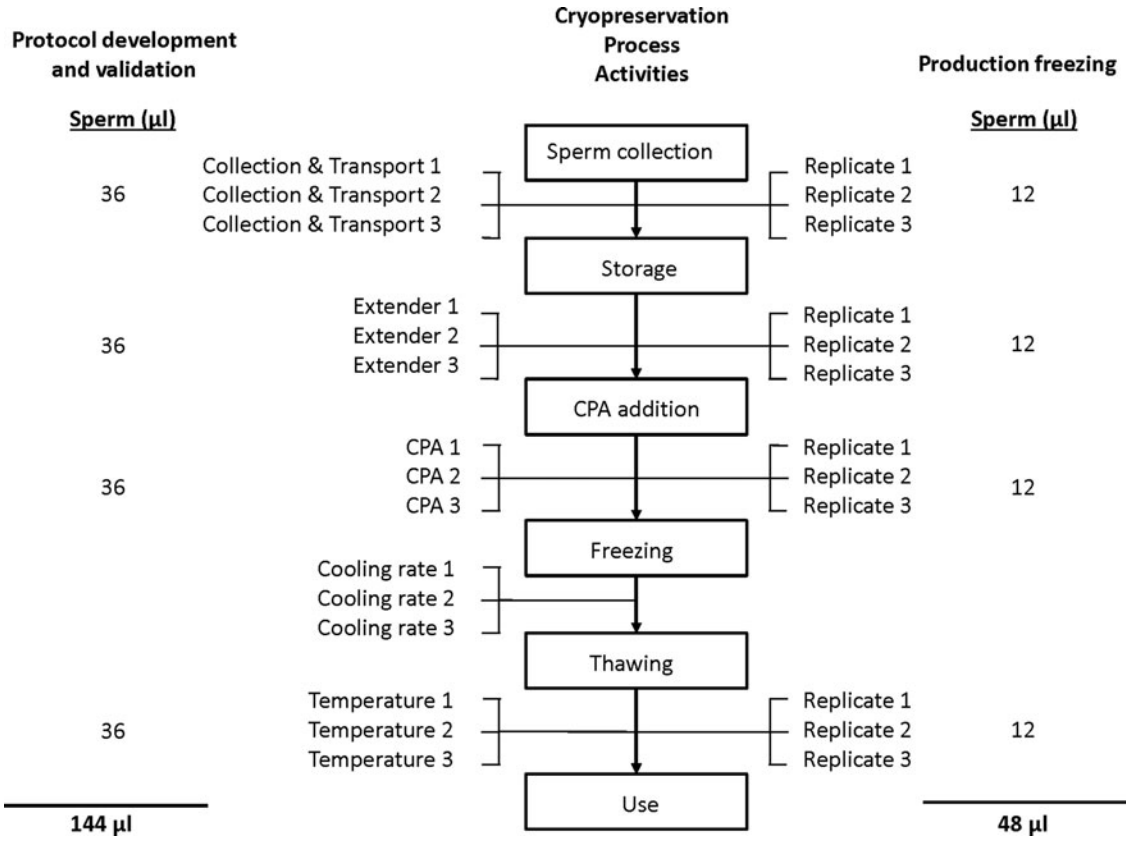

FIG. 5. Schematic of sperm suspension volumes (μL) potentially needed to address sperm concentration, motility, and membrane integrity at each step of the cryopreservation process for use in protocol development and validation (*left*) or in a production freezing scenario (*right*). In each scenario, based on this study, the total volume of sperm used to analyze the three endpoints was 4 μL. For protocol validation, three treatments and three replicates (3 treatments $\times$ 3 replicates $\times$ 4 μL = 36 μL sperm suspension) were proposed for comprehensive analysis of: fresh and refrigerated stored sperm, sperm exposed to cryoprotectants (equilibration), sperm frozen at different cooling rates, and frozen sperm thawed at different temperatures. All treatments included three replicates, leading to 144 μL of total sperm suspension. Based on the conservative assumption that only 2 μL of sperm was obtained per fish, and after a 1:30 initial dilution (which leads to 60 μL of sperm suspension at an approximate concentration of 2×10^8 sperm cells/mL), three fish would be needed to obtain the necessary volume of sperm. In the production freezing scenario, four quality control check points, each with three replicates (3×4 μL = 12 μL sperm suspension), were proposed after sperm collection, storage, cryoprotectant addition, and sperm thawing. A total of 48 μL of sperm suspension would be needed (27% of the total sample if stripping three fish), indicating that (regardless of the number of fish needed in a particular cryopreservation assay) the sperm from one extra fish (60 μL after dilution) would be enough to address sperm quality along the cryopreservation process of pooled samples following the assumption of obtaining 2 μL of sperm per fish.

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motility and fertilization rates. Sperm concentration, when controlled, removes considerable variation that affects QC measurements and the overall success of cryopreservation[6] and fertilization efficiency. Knowledge and control of sperm concentration also enables utilization of a fertilization dose concept (the number of motile sperm needed in a single cryopreservation container unit to fertilize a given relevant number of eggs[24]).

Sperm quality can thereby be assessed by a combination of different endpoints targeting attributes and function, as well as the estimation of sperm concentration. This combination of endpoints increases the chances of identifying and correcting general problems in processing and for individual samples. For example, evaluating post-thaw motility and membrane integrity (function and attributes of surviving sperm) in conjunction with sperm concentration (quality of the cryopreservation process) provides important input that is needed for fertilization planning. As such, a combination of endpoints provides a more comprehensive profile of the sperm sample and its fertilization potential, instead of the limited information that can be obtained from a single endpoint at the end of the process such as percent fertilization, which may be subject to a wide variety of influences other than sperm quality, and be performed years after the actual cryopreservation with data dissociated from the sample information in a repository database. In addition, using percent fertilization as a QC tool would take more sample than the other QC assays combined without providing information that can be used to improve protocols or identify characteristics of samples or batches.

In medicine, the combination of a predetermined group of tests that are used to diagnose a condition is called a test panel. For example, a test panel designed to help diagnose thyroid disorders includes analysis of: thyroid-stimulating hormone, thyroxine, and triiodothyronine. In this study, it was demonstrated that regardless of sperm volume, different endpoints can be analyzed, providing a better understanding and monitoring of the cryopreservation process.

Table 2. List of the Most Common Sperm Quality Endpoints, and the Different Methods That Can Be Used to Analyze Fish Sperm During Cryopreservation

Sperm quality category	*Endpoint*	*Method*
Concentration	Cells/mL	Counting chambers, CASA, spectrophotometer, flow cytometer
Function	Percent total motility	Naked eye, counting chambers, CASA
	Percent progressive motility	Naked eye, counting chambers, CASA
	Average sperm velocity	CASA
	Sperm tail beat frequency	CASA
	Motility duration	Naked eye, counting chambers, CASA
	Fertilization rate	Dissecting microscope
	Hatching rate	Dissecting microscope
Attributes	Morphology	Light microscopy, scanning electron microscopy
	Membrane integrity	Fluorescent microscopy, flow cytometry
	Mitochondrial membrane potential	Flow cytometry
	DNA integrity	Comet assay
	Apoptosis	Flow cytometry
	ATP content	Spectrophotometry
	DNA fragmentation	Flow cytometry

Percent total motility refers to the proportion of swimming sperm, whereas percent progressive motility refers to the proportion of sperm that actively swim cells in a forward direction. When comparing among different techniques, the emphasis of quality control assessment shifts from standardization to harmonization, and it requires a basis of reproducibility for meaningful comparisons. The most fundamental foundation would be control of sperm concentration.

CASA, computer-assisted sperm analysis.

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In this study, we focused on sperm concentration estimated with a counting chamber, motility (sperm function) estimated by using CASA, and membrane integrity (sperm attribute) analyzed with a flow cytometer. However, other methods can be used to analyze each of these endpoints. For example, a novel approach using more stable dyes to analyze membrane integrity is available that would allow laboratories without flow cytometry capabilities to store and ship samples to facilities that can provide flow cytometry services.[25] Also, additional variables could be examined as indicators of sperm function (e.g., sperm average velocity, motility duration, or fertilization) and attributes (e.g., mitochondrial activity, DNA integrity and apoptosis) (Table 2).

The particular endpoints and tests that are used should depend on specific needs or capabilities, and at some point in the future could be set through development of community standards that are relevant to repository development. In the case of single-male freezing, however, it might not always be possible to analyze several parameters due to limited sample volume. Thus, depending on the goals of the study, decisions should be made on which sperm feature provides more information in regards of sperm quality. An estimation of sperm concentration, however, should always be included because the information obtained from this basic QC endpoint cannot be obtained from other types of analysis. Even more important is the adjustment of sperm concentration. Adjusting sperm concentration to a given value not only optimizes the exposure of each sperm cell to the cryoprotectant of choice but also allows for meaningful comparisons across cryopreservation trials. It is essential to ensure reproducibility of cryopreservation and fertilization protocols within and among laboratories.

Preservation of the quality of cryopreserved sperm goes beyond the needs of the biomedical-fish research community. Cryopreservation of aquatic species sperm also allows opportunities for stock enhancement, genetic improvement of commercial species, and the conservation of imperiled species. Therefore, introduction of the test panel concept as a QC strategy for fish sperm—whether the amount of sperm is a limiting factor or not—would help the development of comprehensive QC programs that could be applied in all areas where cryopreservation is used, including in the development of germplasm repositories for biomedical models.

Repositories for livestock and other agricultural species have been long established,[26] but less has been done for aquatic species, in part due to the lack of comprehensive, standardized QC programs that would start with animal screening and address sperm quality in each step of the process.[27] The mission of germplasm repositories includes the commitment to conserve, maintain, and distribute genetic resources and the associated information regarding sample quality and inventory, genetics, phenotypes, locality, and other information about individual fish and populations. Given the myriad of cryopreservation protocols that have been developed for different aquatic species,[28] only through comprehensive QC and quality assurance strategies would it be possible to guarantee the quality of deposited sperm, and the reproducibility of cryopreservation protocols.

Conclusions

Reproducibility of outcomes is one of the most basic principles of science. To assure reproducibility in any laboratory environment, QC strategies and standardized procedures have to be incorporated.[29] These are critical steps that would allow not only the advancement of research but also the eventual transition to community-based and commercial-scale applications, where QC and quality assurance programs are essential. Therefore, making no changes and continuing with the current nonreproducible practices represents significant actual and opportunity costs (in time, personnel, and resources) to laboratories that create mutants, stock centers that accumulate and distribute them, and laboratories that use

these lines. This study demonstrated that the development and application of QC strategies focusing on different aspects of Zebrafish sperm (as surrogates for percent fertilization) are possible even when the sample volumes available are limiting. Practices such as these will greatly assist the development of comprehensive germplasm repositories for aquatic species, especially biomedical model fishes.

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Disclosure Statement

No competing financial interests exist.

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