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A quality assurance initiative for commercial-scale production in high-throughput cryopreservation of blue catfish sperm[☆]

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ABSTRACT

Cryopreservation of fish sperm has been studied for decades at a laboratory (research) scale. However, high-throughput cryopreservation of fish sperm has recently been developed to enable industrial-scale production. This study treated blue catfish (*Ictalurus furcatus*) sperm high-throughput cryopreservation as a manufacturing production line and initiated quality assurance plan development. The main objectives were to identify: (1) the main production quality characteristics; (2) the process features for quality assurance; (3) the internal quality characteristics and their specification designs; (4) the quality control and process capability evaluation methods, and (5) the directions for further improvements and applications. The essential product quality characteristics were identified as fertility-related characteristics. Specification design which established the tolerance levels according to demand and process constraints was performed based on these quality characteristics. Meanwhile, to ensure integrity throughout the process, internal quality characteristics (characteristics at each quality control point within process) that could affect fertility-related quality characteristics were defined with specifications. Due to the process feature of 100% inspection (quality inspection of every fish), a specific calculation method, use of cumulative sum (CUSUM) control charts, was applied to monitor each quality characteristic. An index of overall process evaluation, process capacity, was analyzed based on in-control process and the designed specifications, which further integrates the quality assurance plan. With the established quality assurance plan, the process could operate stably and quality of products would be reliable.

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Introduction

Cryopreservation can retain the biological function of animal spermatozoa for years or decades under very low temperatures (−196 °C for liquid nitrogen, and usually <−100 °C for vapor phase). Cryopreservation technology has been developed in mammals, birds, amphibians, fishes, and some invertebrate species [23]. The development of fish sperm cryopreservation dates back 60 years [3], and in the past decade alone, there were some 300 publications on fish sperm cryopreservation [34]. With growing interest and applications in cryopreserved fish sperm, laboratory-scale cryopreservation cannot satisfy the demands from aquaculture farms or fish hatcheries. Therefore, cryopreservation of fish sperm at a commercial scale with high-throughput has become an increasingly important research topic [35].

Catfish sperm cryopreservation has been studied for more than 35 years [15] but mostly in the laboratory environment. Previous large-scale trials have been performed in a dairy improvement center using processing specialized for bull semen [21], although these products were not tested at a commercial level. During the past 4 years, automation of the cryopreservation process has been developed at laboratory and commercial scales [18]. A demonstration production line was developed for cryopreserved blue catfish sperm, but was operated without the quality assurance guidelines necessary to produce reliable products at a scale relevant to commercial application.

The quality assurance concept has been integrated previously into aquaculture research. These include qualitative activities, such as sex identification [20] and separation of spawning-ready females after collection from ponds [12], and quantitative activities, such as selective breeding [4]. However, those activities and their details were seldom placed in written form or uniform formats. From an engineering perspective, all criteria used for decision making in those activities could be described as quality characteristics, and all quality assurance can be documented within two aspects: specification design and quality control. Specification design regulates the rejection of materials with certain quality characteristics,

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so that waste and high variation in the process due to less-functional or non-functional components can be avoided [8]. For example, if the quality characteristics were defined by observable body features during selection and hormone injection of females for artificial spawning [12], the specification would be a flat belly (indicating poor potential for spawning); if the quality characteristic is the ultrasonic image features of gonads [25], the specification would be the absence of visible mature eggs. These specifications could help ensure that the final products would meet the basic expectation of farmers (customers). Quality control is used to monitor the variability of products during processing, and to provide suggestions for reduction of variability [24]. In the same example of selecting catfish females, the ultrasound images can provide quantified quality characteristics such as egg size within the ovaries [16]. When statistical quality control is applied, control charts of egg size developed from previous size data can be used to check every new sample and identify samples that exceed the control limits. If the data set exceeds control limits, corrective measurements can be taken to rectify out-of-control products or operation steps by using quality control tools such as a cause-and-effect diagrams [24].

In this study, an initial quality assurance plan for high-throughput sperm cryopreservation of blue catfish (*Ictalurus furcatus*) was developed and evaluated in practice. The main objectives were to identify: (1) the main quality characteristics; (2) the process features for quality assurance; (3) the internal quality characteristics and their specification designs; (4) the quality control and process capability evaluation methods, and (5) the directions for further improvements and applications. This study was the first application of statistical quality control in aquatic species sperm cryopreservation and potentially the first of its kind for any species.

Main quality characteristics

Voice of customers

The “Voice of Customers” represents the demands for product quality which provide the initial point of quality assurance. Properly satisfying the requirements of customers can improve profitability [13]. Typically voice of customers data are collected from large comprehensive surveys, and represent the customer tolerances on the product. Engineers and designers would convert these data into a production engineering matrix using quality function deployment [13]. Commercial-scale high-throughput cryopreservation in blue catfish sperm is a newly developed process. Customers have not yet provided quantitative expectations on its production. Without comprehensive surveys and quality function deployment support, in this study, the voice of customers was generalized as an overall pursuit of high product quality.

Fertility quality characteristics

The product resulting from this process was cryopreserved sperm contained in sealed plastic straws. With the purpose of making hybrid catfish fry, the quality of product was directly related to the fertility of the sperm cells. In commercial practice with fresh sperm, the number of swim-up fry (a free-feeding developmental stage after hatching) is the most commonly used evaluation method [1]. In laboratories, the ratio of the initial number of eggs to different stages of embryo development is often used for evaluations [2,18,19,21]. Although fry and embryo development are reliable indices for sperm fertility, the evaluation takes 1–10 d and introduces other variations such as female condition [11,20] and hatchery management [32]. Specifically, after sperm is used to fertilize eggs, neurulation is assessed after 27–30 h (at 27–29 °C);

embryonic mobility is observed after 48–50 h [18]; hatching percent or sac fry (newly-hatched) numbers are assessed after 5 d, and swim-up fry are counted after 10 d [1]. In addition, channel catfish females only spawn within a strict temperature range [32]. Therefore, collection of high quality eggs for performing fertility evaluations is typically limited to 6–8 weeks per year (during April–June in the southeastern US).

Some of the measureable indices for thawed sperm are motility, membrane integrity and cell concentration. These indices are often used in fish sperm research. Motility represents the progressive sperm swimming behavior by identifying and estimating the proportion within the entire sperm population [29,33]. Membrane integrity represents the overall viability of sperm cells typically measured by flow cytometry [29]. Cell concentration indicates sperm numbers before freezing and after thawing. Although, there may not be significant correlations between these three indices of thawed sperm and the numbers of fry produced (due to a lack of experimental standardization and variations at the hatchery level during development such as water quality), studies have suggested that sperm motility is correlated with embryo development [7,15,18,19,37]. In this study, motility, membrane integrity and cell concentration of thawed sperm were considered as the main quality characteristics for the final product.

Specification design

Catfish culture is the largest foodfish aquaculture industry [36]. Within this industry, hybrid catfish (channel catfish *Ictalurus punctatus* female × blue catfish *Ictalurus furcatus* male) are in high demand due to their fast growth rate and disease resistance [12]. Blue catfish males (D&B strain) used in this study were from Baxter Land Company Fish Farm (Arkansas City, Arkansas; 33°34′58.64″N, 91°15′18.45″W). The males were selected at the farm based on observable secondary sexual characteristics (e.g. a well-muscled head and dark coloration) [1] before transportation. An oxygenated hauler was used for transport from the Baxter Land Company Fish Farm to the Aquaculture Research Station of the Louisiana State University Agricultural Center (Baton Rouge; 30°22′07.32″N, 91°10′27.90″W) in spring of 2009–2011. The males were held for 2–4 weeks in aerated outdoor 0.1-acre ponds and fed commercial broodstock diets (Aquaxcel, CargillTM, 45% protein), before capture and transfer into indoor tanks with a recirculating system 2 d before processing. The system used bubble-washed bead filters that were back-flushed every 2 d. The water quality parameters were: pH 7.0–8.0, total ammonia–nitrogen 0.1–0.8 mg/L, nitrite 0.04–0.30 mg/L, alkalinity 39–125 mg/L, hardness 44–126 mg/L, temperature 28 ± 1 °C, and dissolved oxygen 4.3–6.5 mg/L. Water quality data were obtained throughout the year. With different levels of biomass maintained in the system, the parameters varied but were maintained within the suitable range for catfish. Guidelines from the Institutional Animal Care and Use Committees (IACUC) of Louisiana State University were followed for animal care in this study.

Each year channel catfish and blue catfish spawn during overlapping but different 2-to-3-month windows (i.e., spawning seasons) [16]. For the purposes of this study, all blue catfish that spawned during the same season were considered as a single population designated by the year for quality assurance studies. For specification design, all fish from the first season (2010) were considered as the “normal population” (i.e., representative of this species in all characteristics). The features of this “normal population” initiated the quality assurance plan that was used to monitor the test population of the 2nd season (2011). In future work, both populations (2010 and 2011) could be merged to form a more representative “normal population” which would make the quality assurance plan more comprehensive. Sperm samples from 27 males were cryopreserved individually during the 2010 season

by use of our established high-throughput protocol [18,19], and samples from 59 males were processed in the 2011 season. Thawing of straws was performed in a 40 °C water bath for 20 s. Motility assessment and flow cytometric analysis were performed for the individual thawed samples [9]. The processing period overlapped with the hybrid catfish fry production season (April–June) and the males in this study were representative of the blue catfish male populations used commercially in hybrid catfish hatcheries.

The ideal specifications for industrial processing are often set with the three-sigma rule which equals a 99.7% confidence interval [24]. However, with the biological products in this study, a 95% confidence interval was applied, which equaled to two sigmas (1.96 sigmas above and below the mean value exactly) from the mean value. All data were checked to identify outliers, and tested for normality. The range was calculated according to the data distribution and practical considerations (Table 1).

Because sperm with low post-thaw motility (e.g., $18 \pm 22\%$) can fertilize eggs [18], there were no quantitative studies available to define the acceptable level of post-thaw motility. Based on our practical experience, this study used 10% as a threshold. After discarding values under 10%, Kolmogorov–Smirnov normality testing showed the post-thaw motility followed a normal distribution ($P = 0.127$) with a mean of 25% and standard deviation of 11%. The 95% confidence interval was from 7% to 44%. However, a positive correlation with embryo development suggested that higher motility was better [18], so the adjusted specification for post-thaw motility was from 7% to 100%.

The post-thaw membrane integrity dataset contained a single outlier (statistical extreme value that was outside of the normal data range) based on the data distribution. This outlier was associated with a sample that had incomplete records and poor repeatability among quality characteristics possibly due to human error. After eliminating the outlier, Kolmogorov–Smirnov normality testing showed the data followed a normal distribution ($P = 0.291$) with a mean of 80%, and a standard deviation of 6%. The 95% confidence interval was from 70% to 89%. Because the biological definition of membrane integrity represents viable cells, high membrane integrity was not considered as a negative impact. Therefore, the adjusted specification for post-thaw membrane integrity was 70–100%.

The post-thaw sperm concentration was measured by flow cytometry. For the purposes of the statistical quality control process, the actual (unmodified) data collected were used for analysis. Therefore the dilution (1:249) required for flow cytometry yielded reduced cells counts [10]. These values were used directly as inputs for the quality control analysis. According to statistical analysis of the residual plots of the data distribution, the dataset contained a single outlier. This outlier was associated with a sample that had an extreme value probably due to a pipetting error. After eliminating the outlier, Kolmogorov–Smirnov normality testing showed the data did not follow a normal distribution ($P < 0.005$) with a mean of 1.04×10^5 cells/ml, a standard deviation of 1.34×10^5 /ml, and a skewness of 1.12 and kurtosis of -0.16 . The best fit was 3-parameter Weibull distribution ($P < 0.005$) with shape 0.53, scale of 5.92×10^4 , and a threshold of 4.75×10^3 . A large number of random data (500 data sets) were simulated from the same distribu-

tion and sorted repeatedly. From the probability density function of these simulated data, the 95% confidence interval was from 9,880 to 307,511 cells/ml.

High-throughput cryopreservation process structure

The entire high-throughput cryopreservation process was divided into 11 operations (Table 2). Each operation could be carried out separately as an assembly line; or a single worker could finish the job by executing the operations in sequence. To ensure product quality, detailed quality assurance activities had to be established and documented. The sperm cells were kept in different forms in different operations during the process. The purpose of the process was to transfer the sperm cells from the fish body to a well-controlled artificial condition for long-term preservation. For Operations 1–3, sperm were kept inside the fish body cavity; for Operation 4, sperm were kept inside the extracted gonad; for Operations 5–7, sperm were kept in 50-ml tubes of Hanks' balanced salt solution at 300 mOsmol/kg (HBSS300) with or without cryoprotectant (5% methanol); for Operation 8, sperm were packaged in 0.5-ml straws with HBSS300 and cryoprotectant; for Operations 9–11, sperm were frozen in straws, and for Operation 11 large numbers of straws (e.g., 144 straws) were sorted and grouped into 12-compartment daisy goblets for storage.

The current process had two distinguishing features. First, it was a continuous process specifically designed for processing of blue catfish sperm whenever the process was running. No other fish species were applied in this process, and process setup remained unchanged through time. Secondly, the process was built based on 100% inspection, wherein within each operation, all samples were inspected. The inspection type affected the methods of quality control. For variable (or quantitative) data, this study applied cumulative sum x (CUSUM x) for mean monitoring and cumulative sum x^2 (CUSUM x^2) for variance monitoring [27], and for attribute (or qualitative) data, binomial cumulative sum (CUSUM) [5] has been recommended.

Related internal quality characteristics and specification design

The major quality characteristics of the final products were influenced by the many internal quality characteristics along the operations of processing. As part of the quality assurance plan, identifying and managing these internal quality characteristics helped to control the process and reduce the variation in product quality.

Defects

The high-throughput cryopreservation process was identified as a batch process with several material transformations during the

Table 1
Specifications for the main quality characteristics in blue catfish sperm high-throughput cryopreservation. LSL: lower specification limit; USL: upper specification limit.

Quality characteristic	Unit	LSL	USL
Post-thaw motility	Percent	7	100
Post-thaw membrane integrity	Percent	70	100
Post-thaw concentration	Cells/ml	9,880	307,511

Table 2
List of operations in high-throughput cryopreservation processing for blue catfish sperm.

Operation number	Operation name	Material used
1	Fish handling	Blue catfish males
2	Fish dissection	Blue catfish males
3	Testis processing	Blue catfish testes
4	Filtering sperm suspension	Crushed testes
5	Sample inspection	Sperm suspensions
6	Preparing for freezing	Sperm suspensions
7	Packaging in straws	Sperm suspensions
8	Freezing and off-line inspection	Nonfrozen straws
9	Sorting and storage	Frozen straws
10	Off-line quality check	Frozen straws
11	Storage preparation	Daisy goblets

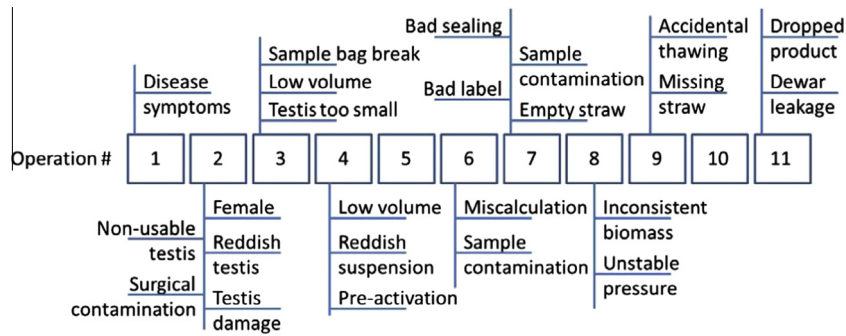


Fig. 1. Common defects along the entire 11-step processing pathway for blue catfish sperm high-throughput cryopreservation.

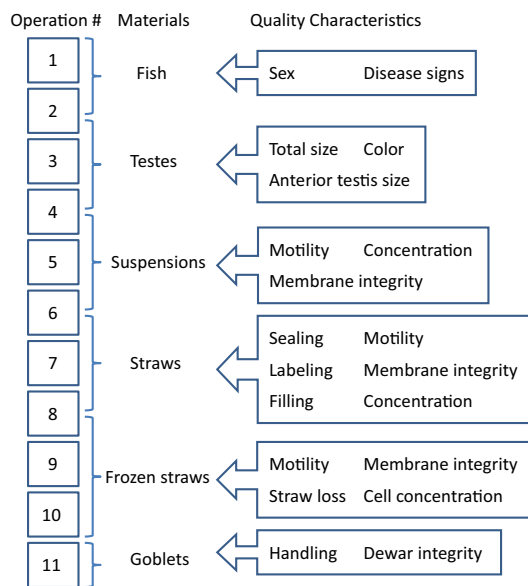


Fig. 2. Key quality characteristics with their target materials along the entire 11-step processing pathway for blue catfish sperm high-throughput cryopreservation.

process (Table 2). Batch processing meant that directly from the fish, materials were handled individually as sperm suspensions. After packaging, the individual materials were distributed into smaller units (straws), but were still identified by their sources as individual fish and were handled together. Throughout the processing, each step of the operation could incur defects that might lead to waste partial batches or low quality (Fig. 1). Some of these defects were related to incoming material quality while others could be caused by operational errors throughout the process.

Identification of quality characteristics

Quality characteristics are identifiable characteristics that can affect product quality. Generally, all possible defects can be considered as quality characteristics. However, tracking of too many characteristics will increase work, and the cost of inspection. Therefore, only key quality characteristics were targeted to be regularly checked. In the process, three inspections were included as routine tasks. Sperm motility, membrane integrity, and sperm concentration were measured through inspections in Operations 5, 8, and 10. Sperm motility and sperm cell membrane integrity reflected sperm quality [29]; concentration reflected sample volumes [33]. Any defects (Fig. 1) that directly led to changes in those three characteristics, which included contamination, low sperm volume,

Table 3

Qualitative specifications for key quality characteristics.

Inspection level	Quality characteristic	Standard for defect	Justification
Fish	Disease	Signs, symptoms	Health issues, biosecurity
Fish	Sex	Female	No sperm
Testis	Total size	No usable testis	No sperm
Testis	Color	Pink or reddish	Undeveloped gonad
Anterior testis	Size	<3 g	Too small to work with
Concentration	Initial concentration	<1 × 10 ⁹ cells/ml	Does not match required density
Motility	Initial motility	<40%	Low motility, low quality
Shipping	Handling	Dropped products	Damaged products
Shipping	Dewar integrity	Leakage	Damaged products

or freezing and sorting errors, were eliminated as secondary characteristics. The independent defects (Fig. 2) along with motility, membrane integrity, and concentration were key quality characteristics used throughout this process (more discussion on these characteristics is provided in following sections).

Key internal qualitative quality characteristics

Among the internal quality characteristics, some were measurable or quantifiable, others were not. In general, those non-measurable quality characteristics were defined by qualitative specifications. Even measurable characteristics can use qualitative specifications according to manufacturer's recommendations [8]. Qualitative specifications set standards for defect identification, such as occurrence of specific phenomena, appearance, or a certain numerical levels. In this study, nine key quality characteristics were applied with qualitative specifications (Table 3).

Key internal quantitative quality characteristics

To further reduce the number of quality characteristics that needed to be inspected, a correlation was performed among quantitative quality characteristics. The sperm samples taken from intact testes were labeled as "intact"; the sperm samples taken from crushed sperm suspension were labeled as "crushed sample"; the sperm samples taken from sperm suspensions after a 30-min equilibration with cryoprotectant were labeled as "equilibration"; and the samples taken from thawed sperm suspensions were labeled as "post-thaw". The membrane integrity measurements used in correlation analysis were from Operation 3 (intact mem-

Table 4

List of indices combinations for correlation testing in blue catfish sperm high-throughput cryopreservation (operation steps and numbers provided in Table 2).

Operation number	Index name	Combined index	New index abbreviation
3	Intact membrane integrity	Intact concentration	Int.live/ml
5	Crushed sample membrane integrity	Crushed sample concentration	Cr.live/ml
5	Initial motility	Initial concentration	Ini.mot/ml
8	Equilibration membrane integrity	Equilibration concentration	Eq.live/ml
8	Equilibration motility	Equilibration concentration	Eq.mot/ml
10	Post-thaw membrane integrity	Post-thaw concentration	Po.live/ml
10	Post-thaw motility	Post-thaw concentration	Po.mot/ml

Table 5

Correlations among combined indices. Int.Live/ml: live cell concentration in testis fractions; Cr.Live/ml: live cell concentration in sperm suspension; Ini.mot/ml: motile cell concentration in sperm suspension; Eq.Live/ml: live cell concentration in equilibrated sperm; Eq.mot/ml: motile cell concentration in equilibrated sperm; Po.Live/ml: live cell concentration in post-thaw sperm; Po.mot/ml: motile sperm concentration in post-thaw sperm.

	Int.Live/ ml	Cr.Live/ ml	Ini.mot/ ml	Eq.Live/ ml	Eq.mot/ ml	Po.Live/ ml
Cr.Live/ml	–0.151 0.387					
Ini.mot/ml	–0.309 0.071	0.436 0.006				
Eq.Live/ml	0.274 0.123	0.725 0.000	0.137 0.407			
Eq.mot/ml	0.014 0.943	0.750 0.000	0.270 0.122	0.837 0.000		
Po.Live/ml	0.161 0.423	–0.193 0.334	–0.260 0.158	–0.016 0.934	–0.166 0.398	
Po.mot/ml	0.078 0.725	–0.046 0.835	–0.245 0.219	0.188 0.369	0.084 0.688	0.807 0.000

brane integrity), Operation 5 (crushed sample membrane integrity), Operation 8 (equilibration membrane integrity), and Operation 10 (post-thaw membrane integrity). The motility measurements used in correlation analysis were from Operation 5 (initial motility), Operation 8 (equilibration motility), and Operation 10 (post-thaw motility). The concentration measurements used in correlation analysis were from Operation 3 by flow cytometer (intact concentration), Operation 5 by spectrophotometer (initial concentration), Operation 5 by flow cytometer (crushed sample concentration), Operation 8 by flow cytometer (equilibration concentration), and Operation 10 by flow cytometer (post-thaw concentration). To reduce the size of the correlation matrix, related indices were combined: because concentration represented the number of live cells in each ml of suspension, concentration combined with motility indicated the number of motile sperm per ml; concentration combined with membrane integrity indicated the number of viable cells (membrane-intact cells) per ml (Table 4). Those new indices were justifiable with biological meanings. All statistics were performed by use of Minitab (version 14.12.0, State College, PA, USA).

A total of 7 combined indices were tested (Table 5). The Int.live/ml (intact membrane integrity divided by intact concentration) had no correlation with the indices. The fractions of testis sampled for this index “intact sample” were largely affected by sampling technique due to small sample size, therefore high var-

Table 6

Internal quality characteristics and specifications in blue catfish sperm high-throughput cryopreservation.

Quality characteristic	Specification type	Reject condition
Disease signs	Qualitative	Symptoms, signs
Sex	Qualitative	Female
Total size	Qualitative	No usable testis
Testis color	Qualitative	Pink or reddish (blood contamination)
Anterior testis size	Qualitative	<3 g
Initial concentration	Quantitative	$<1.23 \times 10^9$ or $>5 \times 10^9$ cells/ml
Initial motility	Quantitative	<40%
Equilibration motility	Quantitative	<32%
Handling	Qualitative	Dropped products
Dewar integrity	Qualitative	Leakage

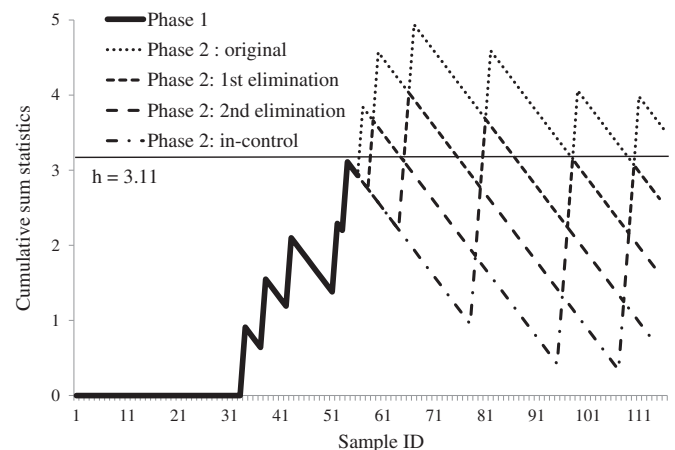


Fig. 3. Binomial cumulative sum control charts for 2010 and 2011 testis weight data. By 100% inspection, all testes that weighed less than 3 g were assigned a value of 1; all others were assigned a value of 0. The chart was set up with 2010 data as Phase 1, and the control limit (straight line) kept all Phase 1 in-control (no data set exceeded the line). The tested 2011 data were Phase 2. The chart presented the approach of eliminating each first out-of-control point until all Phase 2 data were in-control. *h*: control limit.

iation occurred comparing to other inspections later in the process. The Ini.mot/ml (initial motility divided by initial concentration) was correlated with Cr.live/ml (crushed sample membrane integrity divided by crushed sample concentration); however, the equipment required for measuring Cr.live/ml (flow cytometer) was more complex than the use of a microscope or spectrophotometer. In addition, at Operation 5, motility and concentration testing were built as on-line inspections, but flow cytometer testing was an off-line inspection. To reduce inspection costs, the flow cytometer testing at Operation 5 could be optional. The same reasoning applied to Operation 8 off-line inspection. Because Eq.mot/ml (equilibration motility divided by equilibration concentration) and Eq.Live/ml (equilibration membrane integrity divided by equilibration concentration) were correlated, motility test results were sufficient to represent the quality in that operation. Reduction of the flow cytometer inspection at Operation 8 was based on economics. Operation 10 off-line inspection had two correlated indices. To measure the main quality characteristics more accurately, the flow cytometer was recommended. With suggestions from the correlation results, the key internal quality characteristics for quantitative specifications design were therefore initial motility, initial concentration, and equilibration motility.

The calculation of the specifications was performed following the method of the main quality characteristics described previously:

The initial motility data were screened and samples with values lower than 40% were discarded based on qualitative quality characteristics specifications. Kolmogorov–Smirnov normality testing showed that the remaining initial motility data did not follow a normal distribution ($P = 0.007$) with a mean of 56% and a standard deviation of 9%. The best fit distribution was Weibull ($P < 0.010$) with a shape of 7.74, and scale of 60.05. A large number of random data (500 data sets) was simulated from the same distribution and sorted repeatedly. From the probability density function of those simulated data, the 95% confidence interval was from 43% to 70%. In addition, this characteristic was considered to be a one-sided specification, so the adjusted specification was set at 43–100%.

The initial concentration data were screened and values lower than 1×10^9 cells/ml were discarded based on qualitative quality characteristics specifications. Eight outliers based on the data dis-

tribution were identified from the data sets of initial concentration. All outliers were associated with samples that had extreme low values and were eliminated from this study in later steps. After the outliers were eliminated, Kolmogorov–Smirnov normality testing showed that the data did not follow a normal distribution ($P = 0.006$) with a mean of 2.68×10^9 cells/ml, a standard deviation of 1.13×10^9 cells/ml, a skewness of 0.579 and a kurtosis of -0.549 . The best fit distribution was Lognormal ($P = 0.338$) with location of 21.62, and a scale of 0.43. A large number of random data (500 data sets) were simulated from the same distribution and sorted repeatedly. From the probability density function of those simulated data, the tolerance ranged from 1.23×10^9 to 5.00×10^9 cells/ml.

Kolmogorov–Smirnov normality testing showed that equilibration motility followed a normal distribution ($P = 0.231$) with the mean of 48% and standard deviation of 10%. The 95% confidence interval was from 32% to 64%. With initial motility, this characteristic was considered to be a one-sided specification, so the adjusted specification was 32–100%.

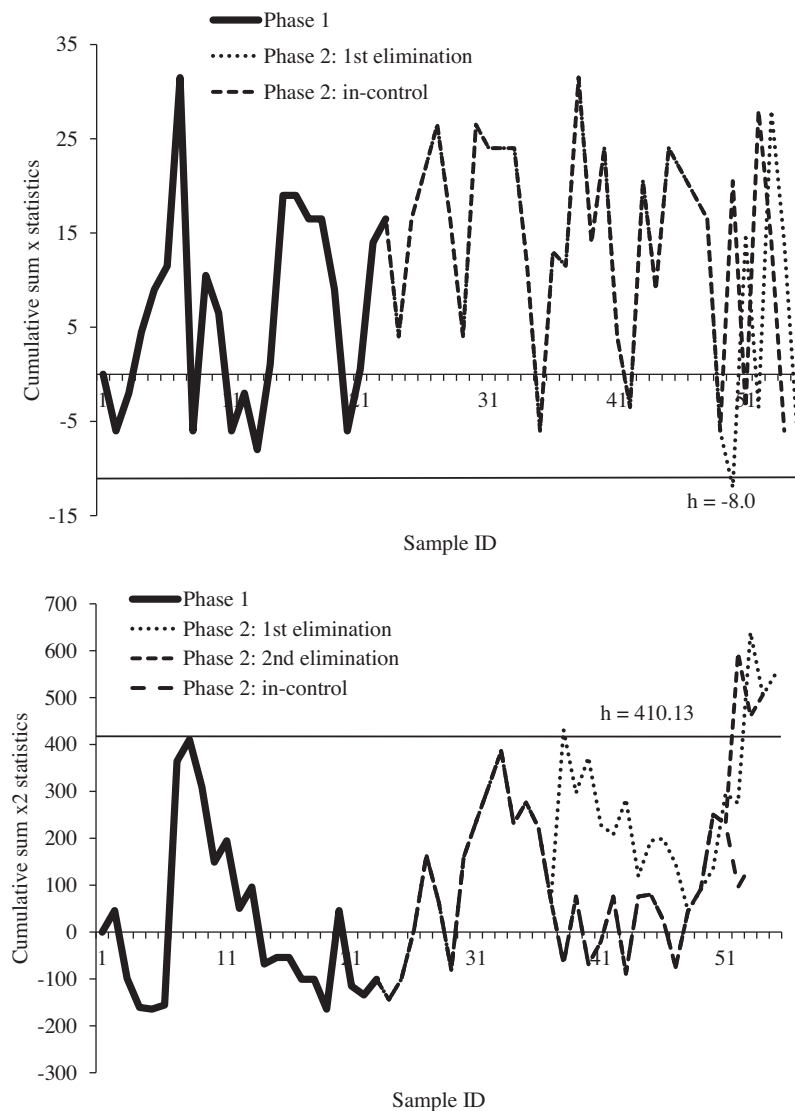


Fig. 4. Cumulative sum X and X square (X^2) control charts for 2010 and 2011 post-thaw motility data. With 100% inspection, the cumulative sum X chart (above) monitored the mean shifts and the cumulative sum X^2 chart (below) monitored the variation changes. The charts were set up with 2010 data as Phase 1, and the control limit (straight line) kept all Phase 1 in-control. The tested 2011 data were Phase 2. The charts presented the process of eliminating every first out-of-control point until all Phase 2 data were in-control. h : control limit.

Design specifications

The specifications for each internal quality characteristic defined the foundations for quality assurance (Table 6). They defined the quality at the accepted level for each operation, such that unnecessary rejection of the final product could be avoided based on the main quality characteristics (Table 1). Those specifications also determined the Quality Control Points for inspections for the key quality characteristics during the process. Therefore quality assurance actions took place at each Quality Control Point to collect and monitor quality characteristics.

Control charts and process capability

Besides designing specifications for quality characteristics, quality control is the other important part of a quality assurance plan. Quality control can monitor each quality characteristic and identify the out-of-control samples based on shifts of mean and variation [22]. Quality control began with the manufacturing industry [24], and was applied to other fields such as food production [14] and hospital management [17,30].

Quality control of a high-throughput cryopreservation process has unique features. Firstly, the biological material has inconsistent quality variation. If the quality characteristics were statistically stable over seasons (years), one quality control

chart generated from a beginning stage could continuously monitor the process. However, if the quality characteristics vary year by year, each year should be monitored on its own by setting a new chart at the beginning of each year. Secondly, this process has a mixture of qualitative and quantitative quality characteristics with 100% inspection. The quality control chart setup depends on the data type and inspection sample size. Use of 100% inspection (rather than use of sampling) is a special case in quality control. Recent research indicates that the cumulative sum (CUSUM) control charts performed better than Shewhart control charts for 100% inspection [27]. Generally, qualitative characteristics should apply binomial CUSUM for analysis [5,30] and quantitative characteristics should apply ordinary or robust CUSUM (insensitive to normal distribution assumptions) [27,28]. In this study, one qualitative characteristic and two quantitative characteristics were selected to demonstrate the use of the above-mentioned control charts using collected data.

Data collection and analysis

High-throughput sperm cryopreservation processing was operated in 2010 and 2011 [18]. Data were collected from inspections throughout the process (Table 2). There were 53 sets of records from 2010, and 59 sets from 2011. The shipping inspection (for

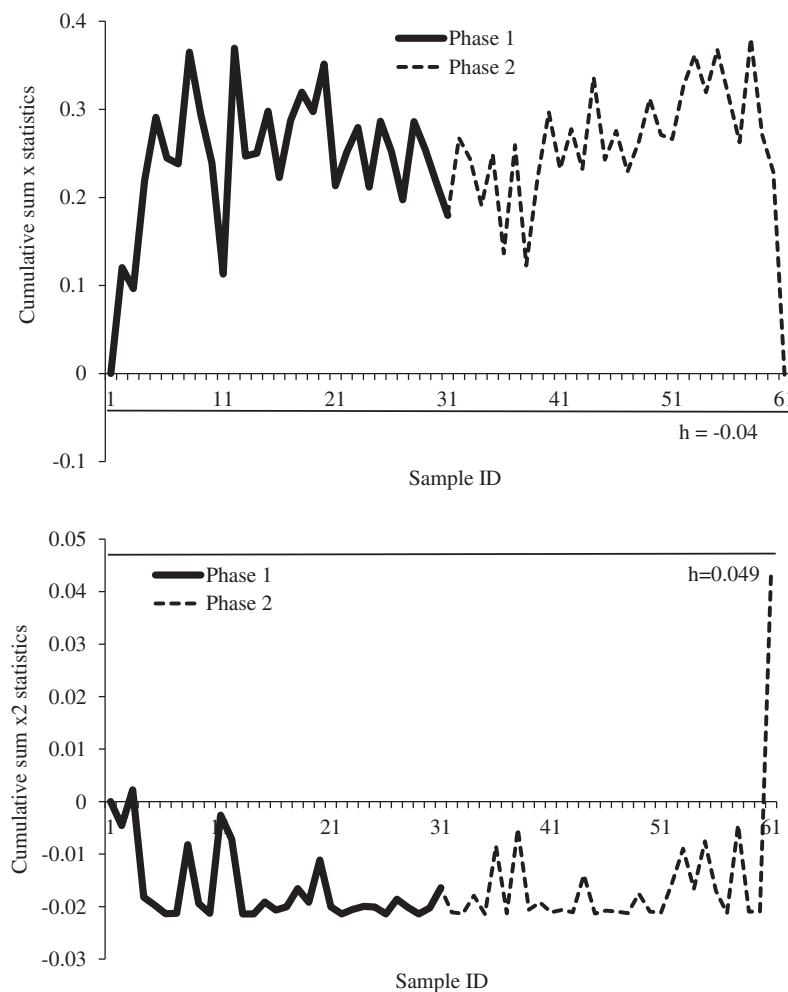


Fig. 5. Cumulative sum X and X square (X^2) control charts for 2010 post-thaw membrane integrity data. With 100% inspection, the cumulative sum X chart (above) monitored mean shifts and the cumulative sum X^2 chart (below) monitored variation changes. The charts were set up with simulated data from the same distribution of the 2010 data as Phase 1, and the control limit (straight line) kept all Phase 1 data in-control. The 2010 data tested were Phase 2. The cumulative sum X chart and the cumulative sum X^2 chart were all in-control. h : control limit.

quality characteristics “handling” and “dewar”) was performed in research separate from this study, so these data were not included in this report. Microsoft Excel 2007 was used for data organization and calculation. The development of quality control charts included two phases: Phase 1 was to use normal group data (in-control data in QC nomenclature) to produce a control chart; Phase 2 was to test the data of interest (e.g., data from processes under monitoring) using the established chart.

Case study: qualitative data

The testes weight data were presented as:

$$V_i = \begin{cases} 1 & \text{if the testis weights less than } 3 \text{ g} \\ 0 & \text{if the testis weights more than } 3 \text{ g} \end{cases}$$

The CUSUM statistics were:

$$S_0 = 0$$

$$S_i = \max(0, S_{i-1} + V_i - k) \quad i = 1, 2, \dots [6],$$

Where $k = -\log(\frac{1-p_1}{1-p_0}) / \log(\frac{p_1(1-p_0)}{p_0(1-p_1)})$, h is the control limit, p_0 is the original occurrence probability, and p_1 is the shifted occurrence probability.

In Phase 1, from the 2010 data, $p_0 = 0.08$. Based on field experience, if one fish of ten males had a testis that was not usable, the population of fish had questionable gonad development. Therefore, assume $p_1 = 0.1$, and $k = 0.090$. Because the 2010 data were considered as a normal group, the highest CUSUM value for the 2010 data was set as the h value at 3.11 (Fig. 3). After the 2011 data were applied to the CUSUM chart in Phase 2, at each out-of-control occurrence, the first out-of-control data point was eliminated and the CUSUM chart was reset (calculated from the first data point again) until all data sets were in-control. After elimination of 3 out-of-control data sets, all processing was in-control. Those three data-sets were identified and the products were separated for further quality inspection and investigation of causes contributing to the out-of-control status. All of the out-of-control datasets were within the lowest 5% of testis weight. Two of these had no detectable testes. The CUSUM chart accurately detected these extreme quality variations.

Case study: quantitative data not different from year to year

Use of t -test showed that the post-thaw motility was not different from 2010 to 2011 ($P = 0.398$). In practice, a value of lower than 10% post-thaw motility was not recommended for production.

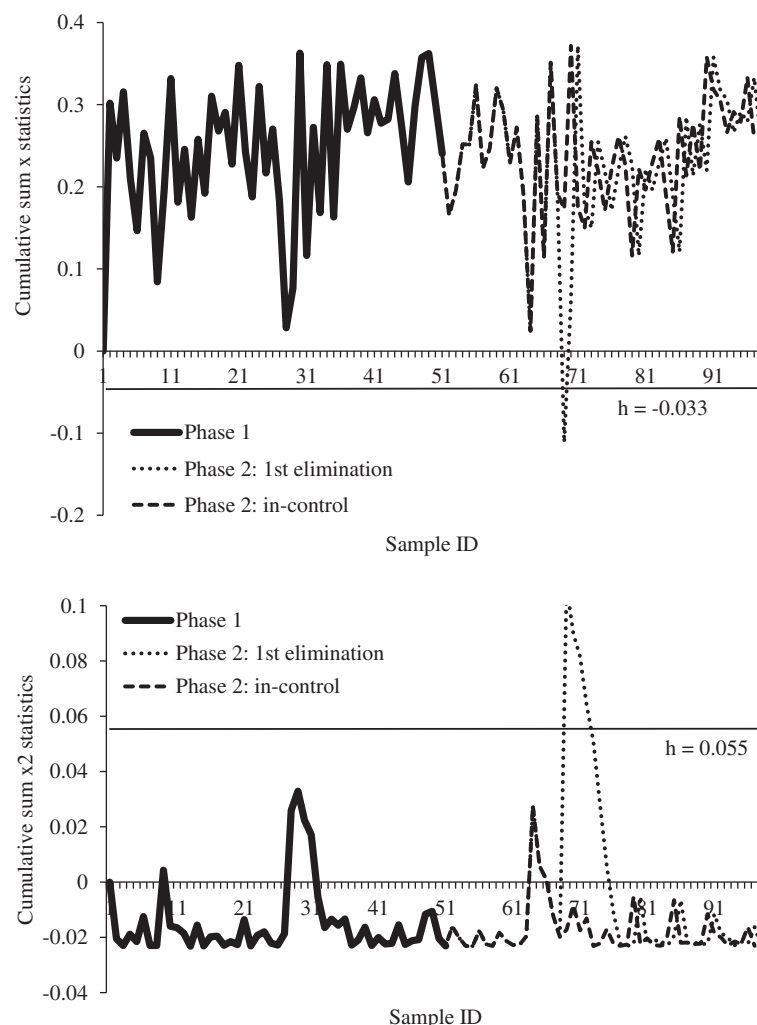


Fig. 6. Cumulative sum X and X square (X^2) control charts for 2011 post-thaw membrane integrity data. With 100% inspection, the cumulative sum X chart (above) monitored mean shifts and the cumulative sum X^2 chart (below) monitored variation changes. The charts were set up with simulated data from same distribution of 2011 data as Phase 1, and the control limit (straight line) kept all Phase 1 in-control. The tested 2011 data were Phase 2. The charts represented the approach of eliminating every first out-of-control point until all Phase 2 in-control. h : control limit.

However, even applying the 10% threshold to both years, there was still no difference ($P = 0.064$). Because high post-thaw motility was not considered to be a negative impact (Table 1), the one-sided CUSUM x chart statistics (C_i^{x-}) were more appropriate:

$$C_i^{x-} = \min\{0, C_{i-1}^{x-}\} + (\bar{X}_i - \mu_0 + \frac{C_{\mu}\sigma_0}{2}), i = 1, 2, \dots, [27,28]$$

Where $C_0^{x-} = 0$, $C_{\mu} = |\mu_1 - \mu_0|/\sigma_0$, and an out-of-control signal will be triggered if $C_i^{x-} < -h_{cx}\sigma_0/\sqrt{n}$;

For variance monitoring, the statistics of CUSUM x^2 chart ($C_i^{x^2}$) were calculated as:

$$C_i^{x^2} = \max\{0, C_{i-1}^{x^2}\} + \left(\sum_{k=1}^n \left(\frac{(X_{ik} - \mu_0)^2}{n}\right) - \left(\frac{2n C_{\sigma}}{1 - C_{\sigma}^2}\right) \sigma_0^2\right), i = 1, 2, \dots, [27,28]$$

Where $C_0^{x^2} = 0$, $C_{\sigma} = \sigma_1/\sigma_0$, $n = 1$ and out-of-control signal will be triggered if $C_i^{x^2} > \frac{h_{cx^2}\sigma_0^2}{n} = h_{cx^2}\sigma_0^2$.

In Phase 1, 2010 data had a mean μ_0 of 27, a standard deviation σ_0 of 10.12. To identify the mean shift to the lowest acceptable value, μ_1 was set at 10, whereas to identify the variation shift that possibly reached the lowest acceptable value, σ_1 was set at 17. To keep all 2010 data in-control (they were a “normal group” by experience), $\min\{C_i^{x-}\} = -h_{cx}\frac{\sigma_0}{\sqrt{n}} = -8$ and $\max\{C_i^{x^2}\} = h_{cx^2}\sigma_0^2 = 410.13$. Therefore, the critical parameters of both control charts, and the control limits, were $h_{cx} = 0.79$ and $h_{cx^2} = 4.00$. After being applied to the 2011 data in Phase 2, one data set was identified as out-of-control in the CUSUM x chart (Fig. 4). In the CUSUM x^2 chart, there were several out-of-control points appearing in the first trial. After eliminating the first out-of-control dataset, there were in total two out-of-control points from the data of 2011, and the rest remained in-control (Fig. 4). The out-of-control dataset in the CUSUM x chart was one of the lowest post-thaw motility values. In addition, referring to other parameters of the same sample, it also had the lowest initial motility, which reinforced the observations of the quality control chart. The two out-of-control points from the CUSUM x^2 chart were the highest values of post-thaw motility. Therefore, both out-of-control signals were not considered to be quality threats.

Case study: quantitative data different from year to year

Analysis by t-test showed post-thaw membrane integrity was different from 2010 to 2011 ($P = 0.000$). Therefore, the 2010 data could serve as the normal group for use in Phase 1, and the data for each year were monitored separately. Because the available data were insufficient to be divided to show Phase 1 and Phase 2 practices, the following strategy was pursued: (1) identify the data distribution using production data early the year, (2) randomly generate data for the quality control chart (Phase 1), and (3) test the production data with an established control chart (Phase 2). With the use of Minitab 14.12.0, the 2010 data were fit into a 3-parameter Weibull distribution ($P = 0.084$) with a shape of 550.73, a scale of 32.31, and a threshold of -31.49 . In Phase 1, 30 new datasets were simulated from the same distribution and a CUSUM x chart was generated based on them. The simulated data had a mean μ_0 of 0.778, a standard deviation σ_0 of 0.074; to detect a 50% change in motility μ_1 was set at 0.278. After calculation, the lower limit for C_i^{x-} was -0.041 . Repeating the same calculation as the last case study, the control limit was $h_{cx} = 0.55$. All simulated data were in-control. In Phase 2, after applying the 2010 data, all data were in-control. For the CUSUM x^2 chart, to detect a large standard deviation in the simulated data σ_1 was set at 0.5, the upper limit for $C_i^{x^2}$ was 0.049 and the control limit was $h_{cx^2} = 8.95$. In Phase 2, all data were in-control (Fig. 5).

The 2011 data was fit into 3-parameter Weibull distribution ($P = 0.266$) with a shape of 486.46, a scale of 32.39, and a threshold

of -31.65 . In Phase 1, 50 new datasets were simulated from the same distribution and a CUSUM x chart was generated based on them. The simulated data had a mean μ_0 of 0.717, and a standard deviation σ_0 of 0.078, to detect a 50% change in motility μ_1 was set at 0.217. After calculation, the lower limit for C_i^{x-} was -0.033 , and the control limit was $h_{cx} = 0.42$. After applying the 2011 data in Phase 2, one data set was out-of-control (Fig. 6). To detect a large standard deviation in simulated data in the CUSUM x^2 chart σ_1 was set at 0.5, the calculated upper limit for $C_i^{x^2}$ was 0.055, and control limit h_{cx^2} was 9.04. In Phase 2, the same dataset that was out-of-control in the CUSUM x chart also appeared out-of-control in the CUSUM x^2 chart (Fig. 6). The out-of-control sample had the lowest membrane integrity as well as the lowest initial and post-thaw motility. According to the results from the quality control chart, this sample in 2011 was discarded due to unreliable quality.

Process capability

There were a total of 13 quality characteristics in the current process, and each had specifications based on customer expectations [8], which was independent to the process operation. Therefore, although the process was in a state of statistical control with the help of quality control [24], it still exceeded specification limits occasionally and did not always produce reliable products. The process capability indicated that the operating process was meeting the specifications by comparing the width of the process variation with the width of the specifications. A higher value of process capability meant a better process with respect to the specifications. For example, in a no-mean-shift normal distribution of quality characteristics, when process capability was one, the process was under a three-sigma level of control, which meant only a 0.3% chance of failure [8].

To carry out process capability analysis, the process must be in-control [24]. If the process is not in control, it is not stable and cannot be described by one distribution. Without a representative distribution, the process variation cannot be predicted during process capability calculations. After the process was in-control, there were different capability indices for different data distributions. For binomial data, a standard normal distribution for historical defect rates was used to test the average defect rate of in-control data and generated the process Z value. For normally distributed data, the short-term process index of process capability

Table 7

Process capability of quality characteristics in blue catfish sperm high-throughput cryopreservation. Pp: long-term capability of process; Process Z: Z-value using standard normal distribution for average defect rate; Cp: short-term process capability.

Quality characteristic	Year*	Data distribution	Index	Value
Post-thaw motility		Smallest extreme value	Pp	1.18
Post-thaw membrane integrity	2010	Weibull	Pp	0.60
	2011	Weibull	Pp	0.53
Post-thaw concentration	2010	Weibull	Pp	0.13
	2011	Logistic	Pp	0.08
Sex**	2011	Binomial	Process Z	1.64
Size		Binomial	Process Z	2.10
Color		Binomial	Process Z	1.24
Anterior testis size		Binomial	Process Z	1.40
Initial concentration	2010	Weibull	Pp	0.16
	2011	Normal	Cp	0.71
Initial motility	2010	Smallest extreme value	Pp	0.76
	2011	Weibull	Pp	1.01
Equilibration motility		Weibull	Pp	1.46

*When 2010 and 2011 data were statistically different, quality control was performed separately.

**2010 data showed no defects, therefore, quality control was only performed on 2011 data.

(Cp) represented the ratio of the specification width (USL - LSL) to the process width ($6 \times$ estimated standard deviation). For other distributions, long-term capability of process (Pp) represented the ratio of the specification width (USL-LSL) to the process width that was the distance between the 99.87th and the 0.13th percentiles in the respective distribution. In general, when the process was under the three-sigma level of control, process Z was equal to 3, Cp was equal to 1, and Pp was equal to 1. With the use of Minitab 14.12.0 the process capabilities of all quality characteristics were calculated when Phase 2 of quality control was in-control (Table 7). Some quality characteristics were not listed because they did not have enough data. Only post-thaw motility, equilibration motility, and 2011 initial motility were able to achieve 3-sigma capability, others required improvement by either increasing the width of specifications if appropriate, or by reducing the width of quality variation.

Conclusions

This project has presented an initiative taken to formulate a quality assurance plan for a newly developed high-throughput cryopreservation process of fish sperm. Key quality characteristics for final and in-process products were identified and their specifications were designed based on data collected during one year of operation (2010, normal group). Different customers could have different quality requirements. For example, use in genetic evaluation programs (e.g., in diallel crosses) would require lower levels of sperm fertility (sufficient only to produce enough offspring for analysis). In such case, the specifications could be less strict than those presented in this report for commercial production scenarios. Even for a particular customer, the requirements could vary on based on specific needs. For example, if all fish were below the specification quality in a certain year (perhaps due to adverse weather conditions) [26], the cryopreservation processor and the customer both face the risk of a complete shut down for that year or season. In such a case, the specifications could be redesigned to allow processing of lower quality material to balance the interests of the processor and customers. Overall, specifications played an important role in process standardization. Development of specifications for biological material processing will present new challenges and ideas for research.

It is recommended that the current high-throughput cryopreservation process be monitored by use of a CUSUM chart for 100% inspection. With proper design of control charts, any out-of-control signals would automatically reject the sample. The control chart could provide more information on operational activities. For example, if out-of-control points appeared in equilibration motility, quality control personnel could check errors during the previous operation (cryoprotectant or filling preparation). A well-developed quality control system can stabilize variation and increase product quality and market competitiveness.

An important feature of this quality assurance plan was the currently intrinsic variation introduced by biological source materials. In this study, five of eight key qualitative quality characteristics were used to select proper materials before cryopreservation. Therefore, the quality of incoming materials (live fish) is extremely important. Catfish processing plants inspect the quality of incoming material by sampling before transport [31]. A similar acceptance-sampling approach is also recommended for high-throughput cryopreservation. If the acceptance sampling can determine the quality of the materials before transport to the cryopreservation processing facility [24], the chance of waste due to the poor quality of biological materials will be reduced.

The outcome of this study indicates that the basic structure of the quality assurance plan developed in this initiative was func-

tional. With the development of aquatic germplasm cryopreservation, more research on process control will be needed in high-throughput processing. This work can serve as a foundation for the expansion of interests in commercialization of cryopreservation processes in other aquatic species, and opens a new vista for ideas in this topic area, including for mammals.

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