Diluting Pooled Sperm based on NanoDrop® Cell Counts

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Sperm concentration determination using a NanoDrop® 2000 spectrophotometer and an Excel Pooled Sperm Worksheet for dilution and cryopreservation of pooled zebrafish sperm samples.

1. Protocol for Sperm Concentration Determination Using a NanoDrop® 2000

Using a micro-volume spectrophotometer such as the NanoDrop® 2000 (Thermo Scientific), a relatively common laboratory instrument, is a quick and practical method for routine measurement of zebrafish sperm concentration (Tan et al., 2010). ZIRC's method for determining sperm concentration prior to freezing is described below. Depending on how concentrated the sperm visually appears, a 1:5 or 1:10 sperm dilution is recommended for the NanoDrop measurements. Your eye will get better at estimating concentration and what dilution to use after some experience. It's easier to visualize and estimate sperm concentration when it is in a colorless tube.

1.1 Materials and Solutions for Sperm Concentration Determination Using a NanoDrop® 2000

0.6 mL microcentrifuge tubes (yellow, Fisher 02-681-311)
Pipetman and tips
E400 Sperm extender
dH2O and Kimwipes for cleaning NanoDrop after use

1.2 Procedure for Sperm Concentration Determination Using a NanoDrop® 2000

1. Collect and pool sperm from available males as previously described (see ZIRC E400_RMMB Sperm Cryopreservation & IVF Protocol 04.24.2023.pdf at https://zebrafish.org/wiki/protocols/cryo)

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

3. Immediately remove 1.0 µL for the NanoDrop dilution. A 1:5 or 1:10 dilution is typically made, depending on how concentrated the sperm sample appears, based on its opacity. Prepare a 0.6 mL microcentrifuge tube containing either 4 or 9 µL E400 for the NanoDrop dilution. Add the 1.0 µL of sperm to the dilution. Mix by flicking the tube and hold on ice. To easily distinguish the diluted samples, a colored (yellow) microcentrifuge tube is typically used for the NanoDrop dilutions.

4. Open the NanoDrop software and choose the Cell Cultures option and set the Cursor Absorbance to 400 nm. Blank the spectrophotometer using the E400 diluent. After blanking, take a measurement of the blanking solution to confirm calibration. A tolerance of ± 0.004 is acceptable. If the blank solution reads outside of this margin, repeat the blanking step.

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

6. Clean the NanoDrop pedestal according to manufacturer's instructions.
Note: If there is any sperm left over from the NanoDrop dilution, take a quick look at it under a microscope to assess concentration and pre-freeze motility (place 9 µL dH2O on a microscope slide, add 1 µL of diluted sperm, quickly mix with pipet tip and observe with 20X objective and DIC or dark field).

1.3 NanoDrop Calibration Curve and Sperm Density Calculator

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

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

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

ZIRC utilizes a hemocytometer-generated standard curve. The best fit ($R^2 = 0.989$) between data and fitted curve resulted from a third-order polynomial equation:

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

This equation is used in an Excel calculator to determine cell density based on absorption. To the right of the graph there is a location to enter the A_{OD400} for a sample and the embedded formula will calculate the estimated concentration according to the curve. The resulting concentration should be multiplied by the dilution factor to calculate the concentration of the original sample.

1.4 Pooled Sperm Dilution Worksheet for Use with NanoDrop A_{OD400} Measurements

The third order polynomial curve equation from the NanoDrop calibration curve is also used in an Excel worksheet to optimize sperm cell dilutions for cryopreservation. The worksheet has embedded formulas that populate automatically once some basic information (grey and blue cells) is filled in. Refer to the following blank form to view the self-populating fields.

Pooled Sperm Dilution Worksheet - Blank.xlsx
https://zebrafish.org/wiki/protocols/cryo
This is the blank worksheet for collection and dilution of stripped zebrafish sperm.

A completed example worksheet has been provided below to help explain its use. The notes on the right side of the worksheet walk you through the significant steps to dilute and cryopreserve sperm samples. The light grey fields contain information that can be completed in advance and can be altered to the specific needs of individual laboratories. Information filled into the light blue fields is used in formulas built into the worksheet.

2023_04_01 Pooled Sperm Freeze Data — RMMB Lot: 8/10/2022

General Info	
User Initials	JLM
Fish Line ID	ZS
Fish Line ID	ZL
# Males pulled	14
Starting Vol (uL) E400	60
Number of Samples Desired	15
Number of Test thaw(s)	1
Total Samples Desired	16
Total Samples to Prepare	18

Pooled Sperm	
# Males giving sperm	13
Pooled Vol (uL) - Measured	67
Nanodrop dilution: uL pooled sperm	1
uL E400	9
NanoDrop Dilution Factor	10
Nanodrop Average OD_{400}	0.400
Cells/ml as read =	1.34E+08
Cells/ml Undiluted =	1.34E+09
Final Undiluted Vol (uL) - Calculated	66

Final Sperm Dilution	AS IS	Diluted
Final Cell Count (cells/sample)	6.72E+06	4.93E+06
Total # of samples to prepare	13.2	18.0
Samples/Male	1.02	1.38
Final Dilution volume (µL)	66.0	90.0
uL E400 to add (must be Positive No.)	0.0	24.0
Total µL RMMB to add (15µL/sample)	198.00	270.00
Conc of sperm in 5uL aliquot (cells/mL)	1.34E+09	9.86E+08
Final Conc of sperm as frozen (cells/mL)	3.36E+08	2.46E+08
Final Cell Count (cells/sample)	6.72E+06	4.93E+06

Spreadsheet Notes

User should complete all grey and blue fields.
Grey fields are inforation only; Blue fields are used in formulas
White and yellow fields contain formulas and will populate automatically
Enter number of samples and test thaw(s) desired
Total Samples to Prepare will self-populate (+1 or +2 to account for pipetting loss)

Method Notes

Suggested starting volume of E400: Short-fin zebrafish = (# males - 2) x 5 µL
Long-fin zebrafish = (# males - 4) x 5 µL
Collect and pool sperm in a microcentrifuge tube containing E400 held on ice
Measure volume of pooled sperm with Pipetman (just prior to making NanoDrop dilution)
Prepare NanoDrop dilution (typically 1:10 or 1:5)
Measure Nanodrop OD_{400} and calculate average OD_{400}

Final Sperm Dilution

"AS IS" cell count is the exisiting sperm concentration without additional dilution
"Diluted" cell count adjusts based on the total number of samples desired
For best results, a final cell count of 2.0E+06 to 8.0E+06 cells/sample is recommended
For final sperm dilution, add the indicated volume of E400 from table: uL E400 to add

Freezing

If preparing >12 samples, split sperm into two tubes
Enter divided sperm volume into table below to calculate RMMB volume

			# of Samples	
Final Dilu Volume	Divided Vol	Vol RMMB	To Prepare	To Freeze
90.00	45	135	9	8

Add RMMB to sperm, aliquot to cryovials (20 µL each), freeze in dry ice
Transfer vials to LN2 after 20-45 min.

Follow the entries in the spreadsheet as the steps are described below. Before starting a new spreadsheet, the blank spreadsheet should be "Saved As" a new file. In the example below, the file was saved as "2023_04_01 Pooled Sperm Freeze Data. The file name is included in the header of the spreadsheet. The lot of RMMB utilized for freezing is also recorded in the header area.

The user initials and fish identification information are entered.

In this example, we wanted to freeze pooled sperm from 14 males of a particular shortfin line. The starting E400 volume for sperm collection was calculated to be 60 µL as follows:

For short-fin zebrafish, E400 starting volume = (# of males -2) x 5 µL= (14 - 2) x 5 µL = 12 x 5 µL = 60 µL

Our goal was 15 samples plus a test thaw, 16 samples total. The number of samples to prepare automatically populates to account for freezing in batches and pipetting loss. We want 16 samples. Because this is more than 12 samples, we will divide the sperm into two aliquots for freezing. To allow for pipetting loss we add one sample volume for each aliquot, so 18 samples total are prepared.

We collected sperm from all males, but one fish did not give any sperm. Our final measured sperm volume was 67 µL from 13 males.

The collected sperm appeared rather concentrated, so we prepared a 1:10 dilution for NanoDrop measurements by adding 1 µL of the sperm to 9 µL of E400 in a separate (yellow) 0.6 mL microfuge tube.

NanoDrop measurements were made and the average A_{OD400} was calculated to be 0.400.

After the average A_{OD400} is entered into the spreadsheet, the sperm dilution fields automatically populate. The sperm concentration "AS IS" is shown in the first column. The "AS IS" column is used if the sperm is already relatively dilute, and it will not be diluted additionally. The second column shows the concentration based on the number of samples to prepare. It is recommended that the final cell count be 2.0E+06 to 8.0E+06 cells/sample (or 4.0E+08 to 1.6E+09 cells/mL in the 5µL prefreeze sperm aliquot). If necessary, the number of samples can be lowered or increased to get the final cell count into this range.

The final dilution volume (90 µL) and amount of additional E400 to add (23 µL) for the dilution is shown in the table.

To prepare for sperm freezing, there's one additional table on the lower right side. If more than 12 samples are being frozen, the sperm needs to be divided into two aliquots. Once the divided volume is entered into the table, the amount of RMMB to add and the resulting number of samples are calculated. If you have a large sperm volume, additional aliquots may be needed for freezing additional batches.

References

Tan, E., Yang, H., Tiersch, T.R., 2010. Determination of sperm concentration for small-bodied biomedical model fishes by use of microspectrophotometry. Zebrafish 7, 233-240.