Application Note No. 103

Version 1.2

Determination of Sperm Density in Bull Semen Doses for AI

Application

This Application Note describes how **sperm cell density in bull semen doses for Artificial Insemination** can be determined by the NucleoCounter® SP-100™ system.

The system is intended to be used for **quality control** of semen doses at bull stations and semen collection centers. The NucleoCounter SP-100 system determines the sperm cell density in semen doses with a very high precision and accuracy. The system can also be used for the determination of the sperm cell density in ejaculates, which have been pre-diluted with extender.

Semen doses diluted with the most widely used extenders (also **egg yolk containing extenders**) can be analyzed with the NucleoCounter SP-100.

The NucleoCounter SP-100 system consists of an instrument (NucleoCounter SP-100), a single use, disposable cassette (SP1-Cassette), a dilution- and lyzing buffer (Reagent S100) and various accessories (please see the Materials and Equipment section below).

Measurement data (sperm cell densities and images) may optionally be transferred to a PC using the SemenView™ software package or a printer.

Principle

Please refer to the users manual for the NucleoCounter SP-100 and Package Inserts for Reagent S100 and SP1-Cassettes.

Materials and Equipment

- NucleoCounter SP-100 with instrument software version 1.21 or later and **settings for bull semen** (CM part no. 900-0100)
- SP1-Cassettes (CM part no. 941-0006)
- Container with Reagent S100 (CM part no. 910-0100)
- Container stand. If 20 ml Sample Cups are used then the container stand should also be equipted with a sample cup holder for 20 ml Sample Cups (CM part no. 929-0003)
- 1-10 ml bottle-top dispenser, Brand Dispensette® III Variabel with filling tube fitted for the Reagent S100 container (CM part no. 911-0003). A 2,5-25 ml bottle-top dispenser, Brand Dispensette® III Easy Calibration (CM part no. 911-0010) can also be used. Use a 100-1000 µl automatic Finnpipette (ThermoLabsystems no. 4500-120) for dispensing Reagent S100 volumes from 500-1000 µL.
- If sample volume < 200 µl: Use a 20-200 automatic Finnpipette (ThermoLabsystems no. 4500-090) with tips (Thermo-Labsystems no. 9400-260) or use an equivalent pipette.
- If sample volume is 100 µl or less: Use a10-100 µl automatic Finnpipette (ThermoLabsystems no. 4500-110) or a 20-200 automatic Finnpipette (ThermoLabsystems no. 4500-090) with tips (Thermo-Labsystems no. 9400-260). An equivalent automatic pipette can also be used.
- If a sample volume > 200 µl is going to be analyzed: Use a 100-1000 µl automatic Finnpipette (ThermoLabsystems no. 4500-120) with tips (ThermoLabsystems no. 9401-100). An equivalent automatic pipette can also be used.

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Materials and Equipment (continued)

- 20 ml sample cup (CM part no. 911-0004) made of polypropylene and with a polyethylene screw cap. A plastic tube with lid can also be used instead of the 20 ml sample cup.
- If a small volume of semen dose is being used (e.g. 100 µl or less) then it is recommended to use a 2.0 mL Eppendorf tube with a round bottom for the dilution of the sample.
- Waste container (e.g. 25L plastic drum or container with lid)
- PC with SemenView (CM part no. 950-0100) installed and connected to the NucleoCounter SP-100 (recommended)

ChemoMetec A/S requests the customers to buy the sample cups from:

In Vitro A/S
Kratbjerg 336, DK-3480 Fredensborg
Phone +45 4847 5070, Fax +45 4847 5775,
e-mail in-vitro@in-vitro.dk
In-Vitro part no. EN-481-8: 20 ml PP sample cup with PE lid

Adjustment and usage of dispenser

The dispenser should be adjusted to dose the exact amount of Reagent S100. It is recommended, that this is being done using a balance with a 001-gram resolution.

The specific gravity of Reagent S100 at 25°C is **1,005 g/ml** (1,006 g/ml at 20°C). At 25°C, the dispenser shall be adjusted to give a volume of an average weight of **1,005 g/ml x target volume in ml** (10 ml corresponds to 10,05 g, 5 ml ≈ 5,03 g, 4 ml ≈ 4,02 g, 2,5 ml ≈ 2,51 g, 2,0 ml ≈ 2,01 g).

The dispenser should be controlled and if necessary adjusted at appropriate intervals.

Please also refer to the manufactures manual regarding dispensing and maintenance of the dispenser.

Adjustment and usage of pipette

Please, be aware that semen samples have a sticky character and a minor part of the sample will always remain in the pipette tip after pipetting. Therefore, it is important to calibrate the pipette using semen samples instead of water.

The pipette should be adjusted to dose the exact volume of sample.

The specific gravity of the sample depends primarily on the specific gravity of the semen extender. The specific gravity of a semen dose can be estimated by weighing a known volume of the sample at a given temperature. When the specific gravity is determined the pipette can be calibrated using a balance with 0,1 mg resolution. If the specific gravity is SG g/ml, 200 µl of the sample should weigh SG x 200 mg, etc.

Please also refer to the manufactures manual regarding pipetting and maintenance of the pipette. Please note that the applied pipetting principle has a substantial influence on the volume that is dispensed. Always apply the same principle.

The pipette should be controlled and if necessary adjusted at appropriate intervals.

Determination of Dilution Factor

All samples have to be diluted with Reagent S100 prior to measurement. How much the sample must be diluted depends on the sperm cell concentration of the sample.

The NucleoCounter SP-100 system has the highest precision, when the cell density in a Reagent S100 diluted semen sample is in the range 0,5-7,0 million cells per ml dilution. This is marked with the gray zones on the table below. The zone with the darkest shading shows the optimal area in which the cell density in the S100 diluted sample is in the range of 1,0-5,0 million cells per ml.

If the semen doses in average contain **25-100 million sperms per ml** it is recommended to use a DF of **21**. This DF can also be used if the dose contains 15-25 million/ml or 100-125 million/ml (light gray zones) but the DF is not optimal and an appropriate DF should be considered if the vast majority of the doses are within concentration marked by the light gray shadings.

If the semen doses in average contain **60-250 million sperms per ml** it is recommended to use a DF of **51**. This DF can also be used if the dose contains 25-60 million/ml or 250-300 million/ml (light gray zones) but the DF is not optimal and an appropriate DF should be considered if the vast majority of the doses are within concentration marked by the light gray shadings.

Use the table below to obtain an appropriate DF for samples containing other sperm cell concentrations (e.g. ejaculates pre-diluted with extender). Use a DF of 401 for ejaculates, refer to Application Note No. 102.

Sperm cell density in the semen sample before dilution with Reagent S100	Choose the dilution factor which best covers the sperm cell density of the semen sample (refer to the left column)					
	DF = 401	DF = 201	DF = 101	DF = 51	DF = 21	DF = 11
5 mill./ml						
8 mill./ml						
10 mill./ml						
15 mill./ml						
20 mill./ml						
25 mill./ml						
30 mill./ml						
35 mill./ml						
40 mill./ml						
45 mill./ml						
50 mill./ml						
60 mill./ml						
70 mill./ml						
80 mill./ml						
90 mill./ml						
100 mill./ml						
125 mill./ml						
150 mill./ml						
175 mill./ml						
200 mill./ml						
250 mill./ml						
300 mill./ml						
400 mill./ml						
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1800 mill./ml						
1900 mill./ml						
2000 mill./ml						
2100 mill./ml						
2200 mill./ml						
2300 mill./ml						
2400 mill./ml						
2500 mill./ml						

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How to obtain a certain Dilution Factor

The Dilution Factor, DF, is calculated as:

(Number of parts of sample + number of parts of Reagent S100)/number of parts of sample

If the sample type is a semen dose with an average sperm cell concentration of 69 million cells per ml a DF of 21 is recommended. Thus, 1 part of ejaculate should be diluted with 20 parts of Reagent S100 prior to measurement.

The tables below specify how a number of standard dilutions may be obtained.

Please note, that a DF below 11 should never be used.

In order not to compromise the precision and accuracy of the analysis it is in general recommended to use at least 50 µl of sample. However, it is often more convenient to use a smaller amount of sample because this saves both sample material and Reagent S100. Thus, there can be a tug-of-war between precision and accuracy on one side and the practical aspects on the other side. If it is chosen to use a 25 µl volume of sample or less is it of outmost importance to pay special attention to the pipetting step.

How to obtain a Dilution Factor of 401:

Sample	50 µl	25 µl	10 µl
Reagent S100	20,0 ml	10,0 ml	4,0 ml

How to obtain a Dilution Factor of 201:

Sample	100 µl	50 µl	25 µl	10 µl
Reagent S100	20,0 ml	10,0 ml	5,0 ml	2,0 ml

How to obtain a Dilution Factor of 101:

Sample	200 µl	100 µl	50 µl	25 µl	10 µl
Reagent S100	20,0 ml	10,0 ml	5,0 ml	2,5 ml	1,0 ml

How to obtain a Dilution Factor of 51:

Sample	400 µl	200 µl	100 µl	50 µl	25 µl	20 µl
Reagent S100	20,0 ml	10,0 ml	5,0 ml	2,50 ml	1,25 ml	1,00 ml

How to obtain a Dilution Factor of 21:

Sample	1000 µl	500 µl	200 µl	100 µl	50 µl	25 µl
Reagent S100	20,0 ml	10,0 ml	4,0 ml	2,0 ml	1,0 ml	0,5 ml

How to obtain a Dilution Factor of 11:

Sample	1000 µl	500 µl	200 µl	100 µl	50 µl	25 µl
Reagent S100	10,0 ml	5,0 ml	2,0 ml	1,0 ml	0,5 ml	0,25 ml

Procedure

Recommended procedure using a DF of 21

1. Key in the Sample ID (optional)
2. Check that the Dilution Factor (DF) is 21

Now choose one of the four combinations A, B, C, D or E:

A

3A Put a new tip on the pipette and aspirate a representative volume of **500 µl** of the sample. Transfer the sample to the center area of the bottom of a **20 ml sample cup**. (*cf. figure 1*)

4A. Place the sample cup in the cup-holder and dispense **10,00 ml** of Reagent S100 into the cup using the dispenser. Dispense the reagent directly onto the semen sample at the bottom of the cup. Use a firm, consistent plunger pressure, so that the plunger moves smoothly. (*cd. figure 2)*

B

4b. Put a new tip on the pipette and aspirate a representative volume of **200 µl** of the sample. Transfer the sample to the center area of the bottom of a **20 ml sample cup** or another suitable container/tube with lid

4B. Place the sample cup in the cup-holder and dispense **4,00 ml** of Reagent S100 into the cup using the dispenser. Dispense the reagent directly onto the semen sample at the bottom of the cup/tube. Use a firm, consistent plunger pressure, so that the plunger moves smoothly. Put on the lid and invert the cup 5 times.

C

3C. Put a new tip on the pipette and aspirate a representative volume of **100 µl** of the sample. Transfer the sample to the center area of the bottom of a **20 ml sample cup** or another suitable container/tube with lid.

4C. Place the sample cup in the cup-holder and dispense **2,00 ml** of Reagent S100 into the cup using the dispenser. Dispense the reagent directly onto the semen sample at the bottom of the cup/tube. Use a firm, consistent plunger pressure, so that the plunger moves smoothly. Put on the lid and invert the cup 5 times.

D

3D. Put a new tip on the pipette and aspirate a representative volume of **50 µl** of the sample. Transfer the sample to the bottom of an **Eppendorf tube** or another suitable container/tube with lid.

4D. Dispense **1,00 ml** of Reagent S100 into the tube using a pipette. Dispense the reagent directly onto the semen sample at the bottom of the tube. Put on the lid and invert the tube 5 times.

E

3E. Put a new tip on the pipette and aspirate a representative volume of **25 µl** of the sample. Transfer the sample to the bottom of an **Eppendorf tube** or another suitable container/tube with lid.

4E. Dispense **0,500 ml** of Reagent S100 into the tube using a pipette. Dispense the reagent directly onto the semen sample at the bottom of the tube. Put on the lid and invert the tube 5 times.

Proceed **immediately** with step 5-9 on page 7:

Procedure (continued)

Recommended procedure using a DF of 51

1. Key in the Sample ID (optional)
2. Check that the Dilution Factor (DF) is 51

Now choose one of the three combinations A, B, C or D:

A

3A Put a new tip on the pipette and aspirate a representative volume of **200 µl** of the sample. Transfer the sample to the center area of the bottom of a **20 ml sample cup**. (*cf. figure 1*)

4A. Place the sample cup in the cup-holder and dispense **10,00 ml** of Reagent S100 into the cup using the dispenser. Dispense the reagent directly onto the semen sample at the bottom of the cup. Use a firm, consistent plunger pressure, so that the plunger moves smoothly. There is no need for further mixing, since the sample and the reagent are thoroughly mixed during the dispensing of the Reagent S100. (*cd. figure 2)*

B

3B. Put a new tip on the pipette and aspirate a representative volume of **100 µl** of the sample. Transfer the sample to the center area of the bottom of a **20 ml sample cup** or another suitable container/tube with lid

4B. Place the sample cup in the cup-holder and dispense **5,00 ml** of Reagent S100 into the cup using the dispenser. Dispense the reagent directly onto the semen sample at the bottom of the cup/tube. Use a firm, consistent plunger pressure, so that the plunger moves smoothly. Put on the lid and invert the cup 5 times.

C

3C. Put a new tip on the pipette and aspirate a representative volume of **50 µl** of the sample. Transfer the sample to the center area of the bottom of a **20 ml sample cup** or another suitable container/tube with lid.

4C. Place the sample cup in the cup-holder and dispense **2,50 ml** of Reagent S100 into the cup using the dispenser. Dispense the reagent directly onto the semen sample at the bottom of the cup/tube. Use a firm, consistent plunger pressure, so that the plunger moves smoothly. Put on the lid and invert the cup 5 times.

D

3D. Put a new tip on the pipette and aspirate a representative volume of **20 µl** of the sample. Transfer the sample to the bottom of an **Eppendorf tube** or another suitable container/tube with lid.

4D. Dispense **1,00 ml** of Reagent S100 into the tube using a pipette. Dispense the reagent directly onto the semen sample at the bottom of the tube. Put on the lid and invert the tube 5 times.

Proceed **immediately** with step 5-9 on page 7:

Procedure (step 5-9)

5. Aspirate a portion of the mixture into an SP1-Cassette. The tip of the cassette should be immersed below the surface of the sample during aspiration. If necessary keep the container at a tilt in order to get the cassette tip immersed in the lyzate mixture. Apply a consistent pressure to the piston and press the piston all the way down until it reaches the cassette. Avoid touching the window (clear area) of the measurement chamber. (*cf. figure 3*)
6. Once the sample has been aspirated into the SP1-Cassette, open the lid and insert the cassette in the NucleoCounter SP-100. Close the lid and press the **"Run"** key on the instrument in order to initiate the analysis. (*cf. figure 4*)
7. After approximately 30 seconds the analysis is completed, and the result is shown on the LCD-display (in millions cells/ml, see *figure 5*) and on the PC (in SemenView) or on the printer if such are connected.
8. Open the lid and remove the used cassette from the NucleoCounter SP-100. Now, the instrument is ready for a new analysis.
9. The used cassette and the used sample cup shall be disposed of. The screw cap of the sample cup should be applied before the cup and sample is disposed of.

Item 4A (both for procedure with DF=21 and DF=51) describes a mixing step, which comprise an addition of Reagent S100 to a semen sample. As long as at least 10 ml of Reagent S100 is dispensed onto the sample there is no need for further mixing, since the sample and the reagent are thoroughly mixed during the dispensing. If less than 10 ml reagent is added then further mixing is necessary by putting on the lid and inverting the cup 5 times.

Handling and storage

For handling and storage of Reagent S100 and SP1-Cassettes, refer to the individual packing labels and Packing Inserts.

Warnings and precautions

For safe handling and disposal of the reagent and cassettes refer to the packing labels, Packing Inserts and the user's guides for the NucleoCounter SP-100, the dispenser and the pipette.

Limitations

The NucleoCounter SP-100 system is for research usage only as well as for production and control of animal semen. The system is not for diagnostic use of human semen.

Refer to the NucleoCounter SP-100 user's guide for instructions and limitations.

The results presented by the NucleoCounter SP-100 system depend on correct use of the reagents, SP1-Cassettes and the NucleoCounter SP-100 instrument.

Disclaimer

ChemoMetec A/S reserves the right to introduce changes in the product to incorporate new technology. This application note is subject to change without notice.

Trademarks

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Revision date

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Appendix Illustrations of procedure steps

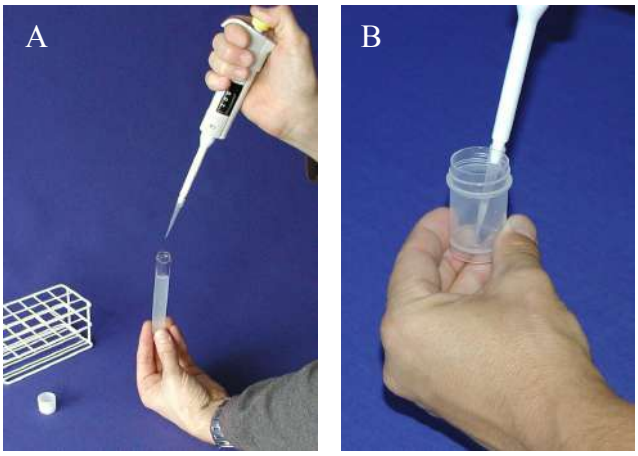

Figure 1. *(A) Pipetting of sample and (B) transferring sample to center area of bottom of a 20 ml sample cup*

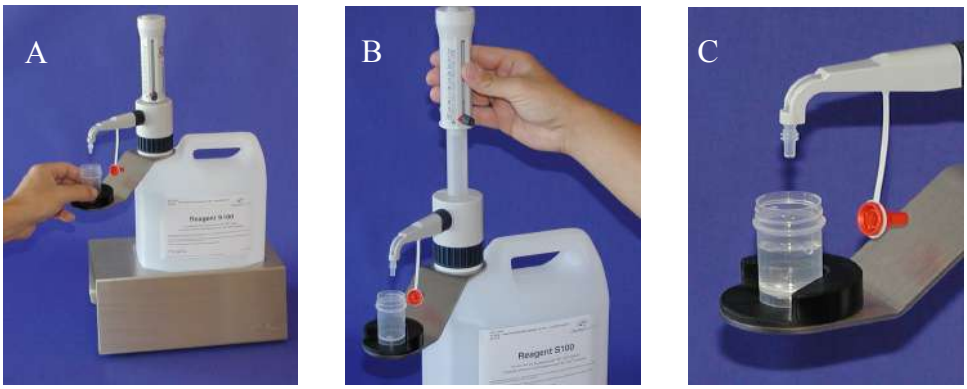

Figure 2. *(A) Placing the sample cup in the cup holder; (B) Dispensing 10 ml of Reagent S100; (C) Sample cup with lyzate mixture.*

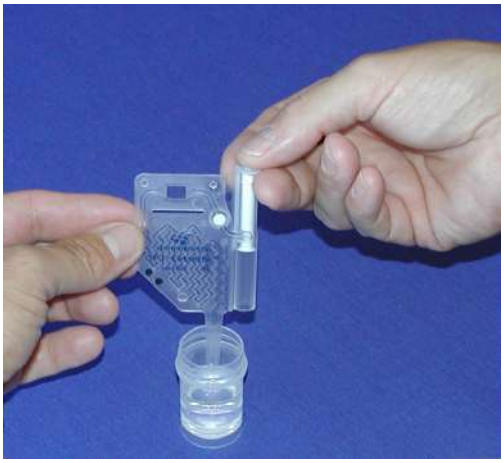

Figure 3*. Loading the cassette with the lyzate mixture*

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Figure 4. *(A) Insertion of cassette; (B) Closing the lid; (C) Pressing the* ***Run*** *button*

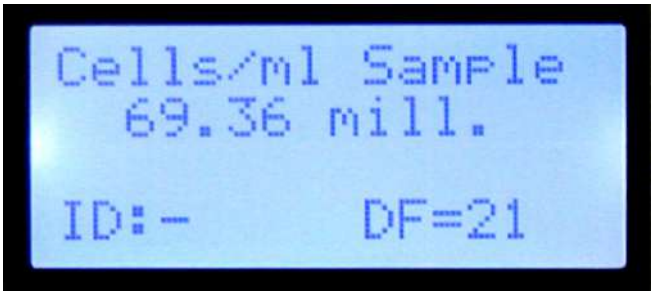

Figure 5*. Result of the analysis shown in the LCD display (in this case the DF was21).*

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