Application Note No. 102

Determination of Sperm Density in Ejaculates from Bulls

Application

This Application Note describes how sperm cell density in ejaculates from bulls can be determined by the NucleoCounter® SP-100™ system.

The system is intended to be used at bull stations and semen collection centers in the production of sperm doses for the artificial insemination of cows. The NucleoCounter SP-100 system determines the sperm cell density in ejaculates with a very high precision and accuracy. The system makes it possible to perform a precise dilution of the semen giving a more well defined end product, with respect to the total cell count in a dose of sperm.

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 1.22 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 with a sample cup holder for 35 ml Sample Cups (CM part no. 929-0006) or a container stand with a sample cup holder for 20 ml Sample Cups (CM part no. 929-0003)
- 2,5-25 ml bottle-top dispenser, Brand Dispensette® III Easy Calibration (CM part no. 911-0010) or a 1-10 ml bottle-top dispenser, Brand Dispensette® III Variabel with filling tube fitted for the Reagent S100 container (CM part no. 911-0003)
- 10-100 µl automatic Finnpipette (ThermoLabsystems no. 4500-110) with tips (ThermoLabsystems no. 9400-260). An equivalent automatic pipette can also be used.
- 35 ml sample cups (CM part no. 911-0007) or 20 ml sample cups (CM part no. 911-0004), both made of polypropylene and with a polyethylene screw cap. A 12-15 ml tube with lid can also be used instead of the 20 ml sample cup.
- Vortex mixer (only used if samples are not fresh)
- 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)
- Analytical balance with a 0,1 mg resolution (for calibration of pipettes and dispenser, and/or routinely weighing of the pipetted ejaculates).

Materials and Equipment (continued)

If a dilution factor (refer to later section) of 401 is chosen together with a 50 µl sample volume the following combination of container stand, dispenser and sample cups should be used:

- Container stand with a sample cup-holder for 35 ml Sample Cups (CM part no. 929-0006)
- 2,5-25 ml bottle-top dispenser, Brand Dispensette® III Easy Calibration (CM part no. 911-0010)
- 35 ml sample cups (CM part no. 911-0007)

If a dilution factor of 401 is chosen together with a 25 µl sample volume the following combination of container stand, dispenser and sample cups should be used:

- Container stand 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)
- 20 ml sample cups (CM part no. 911-0004)

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
In-Vitro part no. EN-482: 35 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 at least a 0,001-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, 20 ml corresponds to 20,1 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 ejaculates 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. Also refer to the section *Pipetting problems* below.

The pipette should be adjusted to dose exactly 25 µl or 50 µl of ejaculate. The 25 µl is used in combination with 10,00 ml Reagent S100 and the 20 ml Sample Cups (or 10-15 ml tubes), while the 50 µl amount is used with 20,00 ml of Reagent S100 and the 35 ml Sample Cups. Both combinations gives a dilution factor of 401, however, the 50 µl amount is recommended due to a higher precision.

The specific gravity of bull semen with 1000 millions cell per ml is approx. 1,025 g/ml at 25°C. Hence, the pipette should be adjusted to give an average dose of 25,6 or 51,3 mg of ejaculate.

For this adjustment a balance with a 0,1 mg resolution shall be used.

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.

Pipetting problems and aspects

Even if calibrated pipettes are used and even if the pipetting step is carried out very carefully it is extremely difficult to obtain a high accuracy in the pipetting step.

An ajaculate with a high cell density is more sticky than an ejaculate with a low cell density. Therefore, the pipetted volume will tend to be relatively low for the high density ejaculate. This is also the case if reverse pipetting is used. The actual pipetted volume of a high density ejaculate can easily be 10-20 % lower than for a low density ejaculate.

There are several ways to overcome or reduce the problem with the pipetting step:

- If there is acces to a balance with a 0,1 mg resolution it is recommended that all dispensed volumes of ejaculates are weighed. The actual weight of the sample is finally used to correct the measured cell density. If the NucleoCounter SP-100 gives 1000 mill./ml and the actual weight if the sample was 24,5 mg then the result shall be corrected to:

  1000 / 24,5 * 25,6 = 1044 mill./ml

  (the 25,6 is the typical weight in mg of 25 µl of ejaculate)
- An auto-diluter may also solve some of the problems with pipetting step. However, ChemoMetec has not investigated the performance of the various types of auto-diluters that are present on the market.
- If the ejaulate is prediluted more than 3 times with extender prior to analysis the generel difference between high and low density ejaculates is being minimized.

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.

In ejaculates from bulls, the sperm cell density is normally within the range of 200-2500 million cells per ml. In the majority of ejaculates, the density is in the range of 800-2000 million cells per ml.

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. As can be seen from the table, a dilution factor of 401 is a good choice as a general dilution factor for bull ejaculates, since the cell density in the majority of the ejaculates lies within the optimal range (marked by dark gray shading).

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						
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1600 mill./ml						
1700 mill./ml						
1800 mill./ml						
1900 mill./ml						
2000 mill./ml						
2100 mill./ml						
2200 mill./ml						
2300 mill./ml						
2400 mill./ml						
2500 mill./ml						

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 is an ejaculate a DF of 401 is recommended. Thus, 1 part of ejaculate should be diluted with 400 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

1. Key in the Sample ID (optional)
2. Check that the Dilution Factor (DF) is correct (DF=401 is recommended)

Now choose one of the four combinations A, B, C or D (A is recommended):

A

3A 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 35 ml sample cup. (*cf. figure 1*)

4A. Place the sample cup in the cup-holder and dispense 20,00 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).* Put on the lid and Vortex the mixture for 10 seconds if the ejaculate is not fresh.

B

3B Put a new tip on the pipette and aspirate a representative volume of 25 µl of the sample. Transfer the sample to the center area of the bottom of a 20 ml sample cup. (*cf. figure 1*)

4B. 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).* Put on the lid and Vortex the mixture for 10 seconds if the ejaculate is not fresh.

C

3C. Dispense 20,00 ml of Reagent S100 in the 35 ml sample cup

4C. Put a new tip on the pipette and aspirate a representative volume of 50 µl of the sample. Clean the outside of the tip with a paper tissue. Transfer the sample to the sample cup with the 20 ml Reagent S100. Put on the lid and invert the cup 5 times or or Vortex the mixture for 10 seconds if the ejaculate is not fresh.

D

3D. Dispense 10,00 ml of Reagent S100 in the 20 ml sample cup or a 12-15 ml tube.

4D. Put a new tip on the pipette and aspirate a representative volume of 25 µl of the sample. Clean the outside of the tip with a paper tissue. Transfer the sample to the sample cup (or the 12-15 ml tube) with the 10 ml Reagent S100. Put on the lid and invert the cup 5 times or or Vortex the mixture for 10 seconds if the ejaculate is not fresh.

Proceed immediately with 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. 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.

Procedure (continued)

Item 4A and 4B 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.

Note! If the ejaculates are not fresh a 10 seconds Vortex is still needed.

Alternative to Vortex mixing

Vortex mixing of the mixture of ejaculate and Reagent S100 is used when the ejaculate is not fresh. If there is not acces to a Vortex mixer the Vortex step can be replaced by a 3 seconds vigorously shaking of the container with lid.

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® system is for research use only. The system is not for human semen diagnostic.

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.

Intended use

The NucleoCounter® system is for research only.

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

NucleoCounter® SP-100™ and SemenView™ are trademarks of ChemoMetec A/S. Dispensette® is a registered trademark of Brand GmbH, Germany. Finnpipette® is a registered trademark of ThermoLabsystems Oy, Finland

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Revision date

Last revision of this document was on December 13, 2019.

Appendix

Illustrations of procedure steps

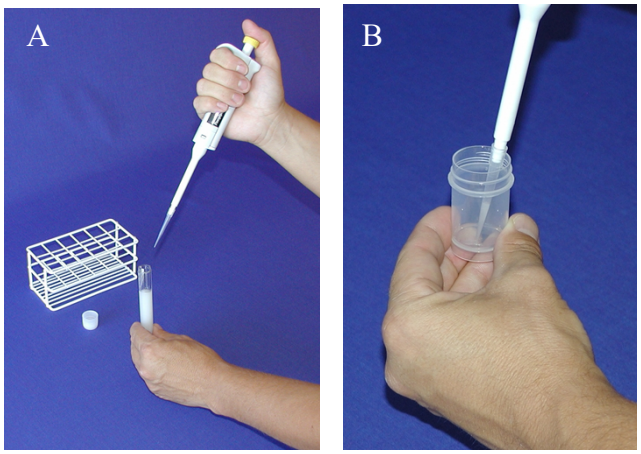

Figure 1. *(A) Pipetting of 25 or 50 µl of sample and (B) transferring sample to center area of bottom of a 20 ml or a 35 ml sample cup*

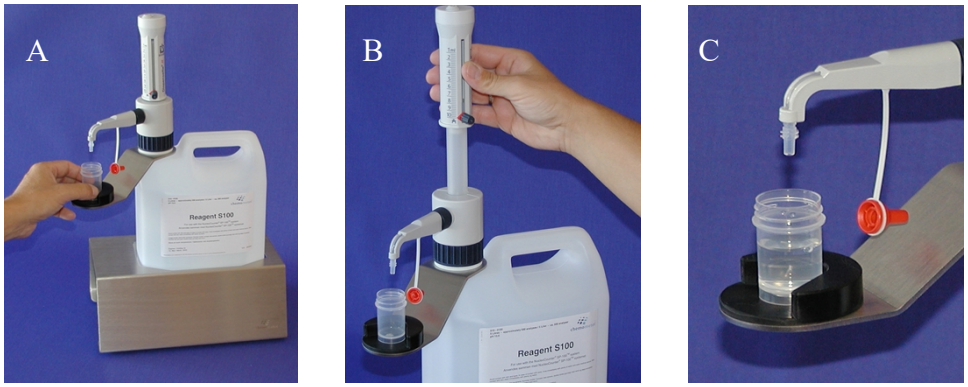

Figure 2. *(A) Placing the sample cup in the cup holder; (B) Dispensing 10 ml or 20 ml of Reagent S100; (C) Sample cup with lyzate mixture.*

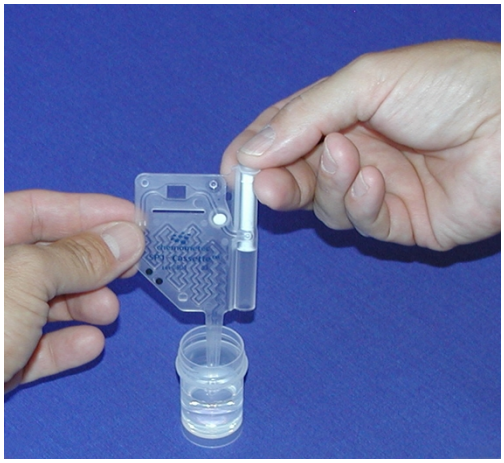

Figure 3*. Loading the cassette with the lyzate mixture*

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

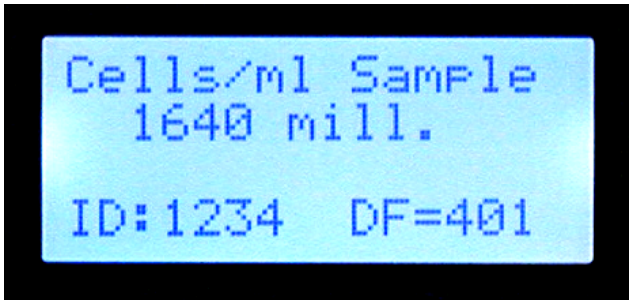

Figure 5*. Result of the analysis shown in the LCD display (in this case the DF was401).*