

# The Precision and Accuracy of Six Different Methods to Determine Sperm Concentration

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**ABSTRACT:** The development of new technologies and software that are routinely used in laboratories has now allowed for a more diverse novel range of methods to determine sperm concentrations more rapidly. The aim of this study was to compare 3 such novel methods developed in our laboratory, including a new flow cytometry approach, image analysis, and a fluorescent plate reader, with more conventional methods (hemocytometry, spectrophotometry, and Microcell analysis). Fifteen ejaculates were collected from 13 bulls at an artificial insemination center. The semen samples were analyzed for sperm concentration using a spectrophotometer, hemocytometry, and a novel flow cytometry technique based on counting a fixed volume of fluid. The raw ejaculate was also diluted fivefold in a long-term diluent and sent overnight to another laboratory, where sperm numbers were assessed using Microcells, an image analysis system, and a fluorescent plate reader. Each ejaculate was assessed

5 times using each of the methods described in order to determine the coefficient of variation for each method. Comparisons between methods were determined using correlation and limits of agreement. The flow cytometry results showed the lowest coefficient of variation (2.3%), with the plate reader showing the highest coefficient of variation (20.0%). There was no significant difference between any of the methods used, and none of them consistently over- or underestimated numbers when compared against each other. It is concluded that flow cytometry showed the highest repeatability of results. However, the method employed by each laboratory should be determined based on a range of factors, including cost, convenience, sample size, and number of ejaculates to be assessed.

Key words: Cytometry, hemocytometry, microcells, spectrophotometry, plate reader.

**J Androl 2006;27:257–262**

The invention of hemocytometry was a great breakthrough for physiologists, and since its development, it has often been referred to as the “gold standard” for counting sperm numbers. Several different hemocytometers have been developed, all based on the same principle of loading a sample onto a grid of a known depth and counting the number of sperm within the grid. Despite our faith in the hemocytometer, there are several limitations, such as the variation between different hemocytometer designs (Christensen et al, 2005) and operators (Brazzil et al, 2004b). Furthermore, a recent study has demonstrated that counting a relatively high number of sperm (approximately 300–400) is required in order to achieve a high level of precision (World Health Organization, 1999; Christensen et al, 2005), which is not time efficient. In addition, previous methods for determining sperm numbers, such as spectrophotometer counts, have been based on calibration using hemocytometers. It is therefore

important that in order to determine the accuracy and precision of sperm counts, novel methodologies need to be explored that reduce human error and that are time effective.

In this study we aim to explore methods developed in our laboratory using novel techniques (flow cytometry, fluorescent plate reading, and image analysis) not reported in the literature and to compare these methods to routine methods such as hemocytometry, spectrophotometry, and Microcells (a disposable counting chamber). Although sperm concentration has been determined using flow cytometry, previously reported methods have always involved a reference (ie, fluorescent microspheres; Hansen et al, 2002) and have not determined concentration in a fixed volume of fluid. The use of a fixed volume is a novel technique in flow cytometry, and no comparative studies using this method have been reported in the literature. With the introduction and the increased use of fluorescence probes, novel techniques can now also be employed to determine sperm concentrations. We have developed two such methods: a protocol for a fluorescence plate reader and the automated use of image analysis to determine sperm numbers on a hemocytometer. The protocol for the fluorescent plate reader was developed to allow large numbers of ejaculates to be analyzed

Financial and intellectual support for this project provided by Genus Breeding Ltd.

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Received for publication June 21, 2005; accepted for publication September 6, 2005.

DOI: 10.2164/jandrol.05112

Table 1. Dilution of semen for hemocytometer counts determined by spectrophotometry

Estimated Spermatozoa Concentration	Volume of Raw Semen ( $\mu\text{L}$ )	Volume of Diluting Fluid ( $\mu\text{L}$ )
300–700 $\times 10^6$	100	1900
700–1200 $\times 10^6$	50	1950
1200–2000 $\times 10^6$	25	1975
2000+ $\times 10^6$	20	2380

at the same time. The image analysis system was created using Image Pro Plus (Media Cybernetics; San Diego, Calif) software and was developed to allow sperm numbers to be counted on a hemocytometer more rapidly and less subjectively than using the conventional method.

## Materials and Methods

Fifteen ejaculates were collected from 13 bulls at a commercial artificial insemination center (Genus Freezing Unit, Ruthin, Wales, United Kingdom). A subsample of each ejaculate was diluted by adding 500  $\mu\text{L}$  of the raw ejaculate in 2000  $\mu\text{L}$  of Eqcellsire (IMV L'Aigle, France) and was sent overnight to London, where samples were analyzed for sperm concentration using Microcells (Conception Technologies, San Diego, Calif), image analysis (Image Pro Plus), and a fluorescent plate reader (SpectraMax, San Diego, Calif). At the artificial insemination center samples were analyzed for sperm concentrations using a hemocytometer (Thoma, Hawksley, London, United Kingdom), flow cytometry (Cyflow Space, Partec, Munster, Germany), and spectrophotometry (Genesys 20, ThermoSpectronic, New York, NY). Prior to experimentation, all pipettes were calibrated gravimetrically by repeat pipetting onto a 0.001-g balance.

### Spectrophotometer Counts

Prior to commencing the experiment, the spectrophotometer was calibrated at 685 nm using 10 different ejaculates. For calibration, each ejaculate was counted using a hemocytometer (3 preparations, 2 counts of each), and a serial dilution was carried out ranging from 2000 to 200  $\times 10^6$  sperm/mL in a counting solution (1% sodium lauryl sulfate [wt/vol] and 1.9% sodium tetraborate) to construct a standard curve ( $r^2 = 0.965$ ). During the experimental period, the raw ejaculates were diluted 100-fold in the counting solution. Each ejaculate was diluted and counted 5 times.

### Hemocytometer Counts (Thoma)

Following ejaculation, the concentration of spermatozoa was estimated using spectrophotometer counts. The spermatozoa were then diluted in a counting medium (3.2% sodium citrate with 1% formaldehyde) in order to produce a concentration of approximately 20–30  $\times 10^6$  sperm/mL based on the spectrophotometer counts (Table 1). Diluted semen samples were then loaded onto a hemocytometer by moistening the pillars of the hemocytometer prior to applying the cover slip in order to observe Newton's rings: the sperm suspension was loaded to fill the hemocytometer counting chamber exactly. A total of 5 "large"

squares were counted on the hemocytometer, each square being 200  $\mu\text{m}^2$  and 10  $\mu\text{m}$  in depth. Each ejaculate was diluted and counted 5 separate times.

### Flow Cytometry (Partec)

Following ejaculation, raw ejaculates were diluted to give a concentration of approximately 250 000 cells/mL (based on the spectrophotometer count) in Eqcellsire; 500  $\mu\text{L}$  of the diluted ejaculate was pipetted into a tube (Sarstedt 55.484, Numbrecht, Germany) and vortexed. The sperm population was gated on a side vs forward scatter plot (size and granularity) using a solid-state laser at an excitation wavelength of 488 nm. The flow cytometer was set to count the gated particles in a volume of 200  $\mu\text{L}$  of the solution. This was done using 2 electrodes placed at a set distance within the tube attachment assembly, with the flow rate set at 4  $\mu\text{L}/\text{s}$ . Each sample was diluted and counted 5 times.

### Microcells

Each sperm suspension previously diluted 1:5 in Eqcellsire was further diluted 1:10 to give a final 50-fold dilution. Sperm samples were loaded into a Microcell slide (20- $\mu\text{m}$  depth), and the total number of sperm observed in 100 squares in the graticule mounted in the eyepiece was estimated. The concentration of spermatozoa was calculated using a factor (F) determined by calculating the size of each square in the eyepiece based on measurements using a slide graticule (Microcell Product booklet).

The equation to determine sperm concentration using a Microcell follows:  $C = N \times F$ , where C equals concentration in millions/mL; F equals factor; and N equals the average number of spermatozoa per square equals the number of spermatozoa counted/number of squares. F was determined by calculating the size of each square in the eyepiece based on measurements using a slide graticule (Microcell Product booklet).

### Automatic Image Analysis

The image analysis protocol was determined using a range of sperm concentrations (ranging from 0 to 30  $\times 10^6$  sperm/mL). The most appropriate dilution rate of the raw semen was determined in order to produce the lowest coefficient of variation (CV) for the widest range of sperm concentrations. In the current study, semen samples diluted 1:5 in Eqcellsire were further diluted 1:40 in a 1% Triton X (Sigma Chemical Co, Poole, United Kingdom) solution in phosphate-buffered saline (PBS; Dulbecco, Poole, United Kingdom) containing 24  $\mu\text{M}$  propidium iodide (Molecular Probes, Paisley, United Kingdom), resulting in a final 200-fold dilution. Samples were incubated at room temperature for 5 minutes and loaded onto a hemocytometer, and an image was captured on an Olympus IX70 fluorescent microscope (Tokyo, Japan) using a 10 $\times$  objective and an Evolution MP digital camera (Media Cybernetics) with Image Pro-Plus software. The images were saved and spermatozoa were counted using a macro developed within Image Pro Plus. The macro was designed to draw 5 boxes (each box 200  $\mu\text{m}^2$ ) and to count the spermatozoa within the squares (excluding spermatozoa touching the "North" and "East" sides of each square). Spermatozoa that were touching each other were distinguished as 2 cells using the "auto split function" that automatically analyzes every counted object and

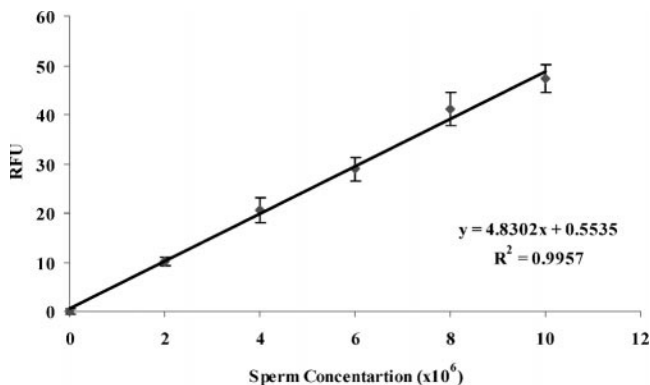


Figure 1. The calibration curve on the fluorescence plate reader indicating the relative fluorescent units (RFUs) for 5 separate sperm concentrations using 10 different ejaculates.

splits any detected clusters into individual objects. Five replicates of each ejaculate were carried out.

Fluorescence Plate Reader

In order to establish a protocol for estimating sperm concentration using a fluorescence plate reader, a variety of sperm concentrations (based on hemocytometer counts) were diluted using a range of propidium iodide concentrations. The highest regression coefficient ( $r^2 = 0.99$ ; Figure 1) was established using 10 ejaculates with sperm concentrations serially diluted from  $10 \times 10^6$  to  $2 \times 10^6$  sperm/mL ( $10 \times 10^6$ ,  $8 \times 10^6$ ,  $6 \times 10^6$ ,  $4 \times 10^6$ , and  $2 \times 10^6$  sperm/mL) using 12  $\mu$ M propidium iodide. Semen samples previously diluted 1:5 in Eqcellsire were further diluted 1:25 in PBS. Diluted semen (100  $\mu$ L) was added to 100  $\mu$ L of PBS containing 1.0% Triton X and 12  $\mu$ M propidium iodide in a well in a 96-microwell plate (NUNC, Rochester, NY), giving a final 250-fold dilution of the original ejaculate. The diluted samples were incubated at 37°C for 30 minutes in the dark prior to using fluorescence at an excitation wavelength of 525 nm and an emission wavelength of 625 nm. Each ejaculate was diluted and counted 5 times.

Statistics

Statistical analysis was carried out using Minitab 3.1 (Minitab Inc, Coventry, United Kingdom). Repeatability of each method was established by calculating the CV for the 5 repeats of each ejaculate. Comparisons between methods were initially carried out using a 1-way analysis of variance (ANOVA), and direct comparisons between treatments were compared with reference to the least significant difference. Since there was no significant difference between methods, further analysis between samples was carried out by correlation. The limits of agreement were also deduced by calculating 2 times the standard deviation (SD) of the differences between the 2 methods (Petrie and Watson, 1999), and scatter diagrams of the difference between the methods (y-axis) and the average of 2 of the methods (x-axis) were plotted to visualize the data.

Table 2. A correlation matrix indicating the r value for each method compared

Flow*	*					
Hem	0.99	*				
Image	0.90	0.90	*			
Micro	0.96	0.96	0.86	*		
Plate	0.91	0.91	0.86	0.83	*	
Spec	0.99	0.99	0.90	0.97	0.88	*
	Flow	Hem	Image	Micro	Plate	Spec

\* Flow indicates flow cytometry; Hem, hemocytometry; Image, image analysis; Micro, Microcells; Plate, plate reader; and Spec, spectrophotometer.

Results

The repeatability of each method is illustrated by the average CV across all 5 repeats; these values increased as follows: flow cytometry (2.3%), spectrophotometer (4.1%), hemocytometer (7.8%), Microcells (10.7%), image analysis (14.9%), and fluorescent plate reader (20.0%).

A 1-way ANOVA demonstrated no significant differences between any of the methods compared. These data were supported by high correlation coefficients between each of the different methods ( $P < .001$ ; Table 2). Data plotted between the average of the 2 means and the difference between the 2 means (eg, Figures 2 and 3) illustrated that the differences were not related to the size of the measurements (ie, all the plots fall around the mean) and comparisons did not consistently over- or underestimate sperm concentrations; this was consistent for all of the methods compared. Table 3 demonstrates the relationship between each pair of methodologies. The closest relationship between the limits of agreement was that observed between the hemocytometer and the spectrophotometer (Figure 2), followed by the one between the hemocytometer and the flow cytometer. The lowest limits of agreement were between the Microcells and the plate reader (Figure 3). The closest relationship to the image

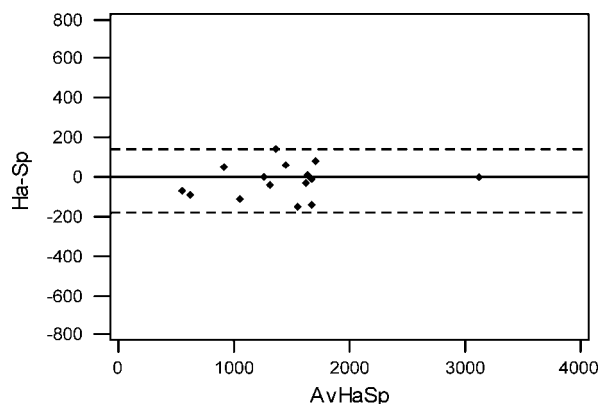


Figure 2. The difference between the counts of sperm using a hemocytometer and a spectrophotometer plotted against their average.

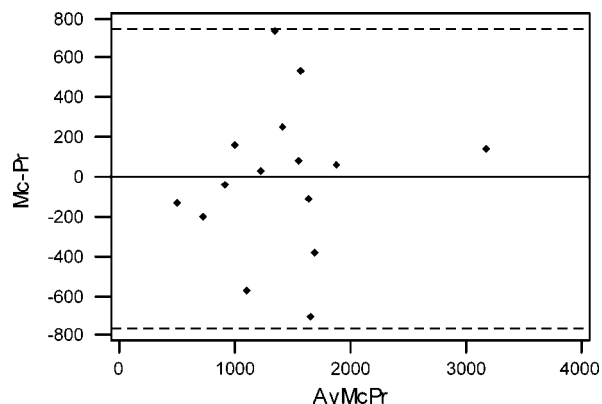


Figure 3. The difference between sperm using a Microcell and plate reader (y-axis) plotted against the average of the 2 counts (x-axis). The diagram illustrates the size of differences between the 2 methodologies and the distribution around 0.

analysis methodology was the spectrophotometer, and the closest relationship to the plate reader was the hemocytometer (determined by  $2 \times SD$ ).

## Discussion

The current study was designed to compare 6 separate means of determining sperm concentration, including 3 novel methods; a fluorescent plate reader, image analysis, and a novel flow cytometric approach. The lowest CV, indicative of the highest precision of all the methods used, was the flow cytometer. This method was also independent of all the other methods studied, as was the Microcells. Because of the nature of this and similar studies, the majority of techniques used were not totally independent of each other. The spectrophotometer and the plate reader were each calibrated by reference to the hemocytometer, and the image analysis system is essentially a development of hemocytometer counts (hence, the high correlation and level of agreement).

In the current study, the actual concentration of spermatozoa in each ejaculate was unknown. This is a common problem across all studies attempting to estimate accuracy of sperm counts. Some authors have attempted to overcome this problem using latex beads (Seaman et al, 1996; Brazil et al, 2004a) of a known concentration to calibrate or measure sperm concentrations by combining the sperm sample with the beads. The advantage of latex beads is that numbers can be determined more accurately, since they can be measured gravimetrically as long as the properties of the beads are uniform and constant (National Physical Laboratory, personal communication). In addition, latex beads can be stored for prolonged periods and are therefore available for repeated use over several months, allowing comparisons of different ejaculates to be measured with reference to a consistent bead number

Table 3. The limits of agreement of each combination of methods used for all the ejaculates

Variable*	Mean	Standard Deviation	Limits of Agreement		Range of Limits
Ia-Pr	-37.1	318.2	-673.5	599.3	1273
Ia-Mc	-27.6	350.0	-727.6	672.4	1400
Ia-Ha	30.3	270.8	-511.3	571.9	1083
Ia-Sp	50.4	256.8	-463.2	564.0	1027
Ia-Fc	55.2	314.5	-573.8	684.2	1258
Pr-Sp	13.3	296.0	-578.7	605.3	1184
Pr-Fc	18.1	286.7	-555.3	591.5	1147
Pr-Ha	-6.8	258.6	-524.0	510.4	1034
Pr-Mc	-9.6	379.2	-768.0	748.8	1517
Ha-Mc	2.8	185.6	-368.4	374.0	742
Ha-Sp	-20.1	82.6	-185.3	145.1	330
Ha-Fc	-24.9	119.3	-263.5	213.7	477
Fc-Mc	27.7	189.6	-351.5	406.9	758
Fc-Sp	4.8	134.5	-264.2	273.8	538
Sp-Mc	22.8	178.0	-333.2	378.8	712

\* Ia indicates image analysis; Pr, plate reader; Mc, Microcells; Ha, hemocytometer; Sp, spectrophotometer; and Fc, flow cytometer.

(Accubead, Hamilton Thorne, Beverly, Mass). This partially overcomes the problem of determining variation in cell numbers, but variability does nevertheless exist in bead numbers between batches ( $18 \times 10^6 \pm 2.5 \times 10^6$  beads/mL, or  $35 \times 10^6 \pm 5 \times 10^6$  beads/mL; Accubead). In a study carried out by Mahmoud et al. (1997), it was demonstrated that when sperm cells were mixed with a bead suspension and counted using an improved Neubauer hemocytometer, a higher CV was noted for both the bead and sperm concentrations; an underestimation of the sperm concentration was also noted. Therefore, beads are not an ideal standard to develop new counting systems. With these problems evident, we decided not to use beads to calibrate new methodologies and compare instruments but rather to use several ejaculates and repeat each sample several times. Although this approach would not allow us to determine the accurate numbers of spermatozoa, it would allow comparisons between each of the different methodologies and a determination of the repeatability (precision) of each of them. None of the methods produced results that were statistically exceptional, and none consistently overestimated or underestimated sperm concentration. From this it can be concluded that all the methods were similar, including the novel flow cytometric approach and the 2 methods established in our laboratory (image analysis and the fluorescent plate reader).

Sperm counts using flow cytometry have been documented by several authors and show results consistent with those obtained in our study (ie, lower CV for flow cytometry than for use of a hemocytometer [Eustache et al, 2001] or spectrophotometry [Evenson et al, 1993; Hansen et al, 2002]). The novelty in the present study is that sperm concentrations were determined using a constant volume of fluid (ie, 200  $\mu$ L; Absolute Volumetric

Counting, Partec, Germany) instead of the traditional reference to fluorescent beads; to our knowledge, this has not previously been reported. The results from this study demonstrated the high repeatability of this method (CV = 2.3%), which is comparable with that obtained with the methods using fluorescent beads reported previously (Eustache et al, 2001; Hansen et al, 2002). The next most repeatable results came from using spectrophotometry. This method is routinely used in a large number of artificial insemination laboratories to determine sperm concentrations. In the current study we showed that it is a highly repeatable technique (CV = 4.1%) that accords with other studies (CV = 6.3%; Hansen et al, 2002). It also has the advantage of being carried out rapidly, but does require constant calibration and maintenance of the equipment. Because these instruments are designed to measure color in solutions, not opacity due to particulates, the absorption reading with suspensions of cells varies rapidly with time. The point at which the reading is taken is critical. This introduces an element of subjectivity, particularly where repeat samples from the same ejaculate are processed, and this may have had a positive effect on the CV in this study.

The long-established “gold standard” to determine sperm counts, as with numerous cells types, has been the use of the hemocytometer. Although widely used, previous studies have indicated that use of the hemocytometer results in high variation and imprecision (Seaman et al, 1996), as well as large variations among the different types of hemocytometers used (Mahmoud et al, 1997). We opted for the Thoma hemocytometer, since a previous study demonstrated a lower CV with it than with other hemocytometers (Christensen et al, 2005). Although our CV was generally lower than that reported in most studies, it still yielded the third most imprecise method to determine sperm concentrations. It is laborious for routine use and subject to human error (Cooper et al, 1992). In an attempt to automate the use of hemocytometers, and at the same time reduce variability as a result of human error, a macro was written for Image Pro Plus allowing images captured from a hemocytometer loaded with fluorescent-labeled spermatozoa to be used for counting. Use of the image analysis program generated a higher CV than the standard hemocytometer counts. These results were initially surprising, but they probably resulted from the lower numbers of sperm cells counted. Prior to carrying out this study, the protocol was optimized by varying the sperm numbers loaded onto the hemocytometer. The optimized concentration of sperm appeared to be at around  $2.5 \times 10^6$  sperm/mL for counts. Using the same concentrations as those used for the hemocytometer alone ( $20\text{--}30 \times 10^6$  sperm/mL), the program was unable to distinguish adequately between cells within clusters and therefore became inaccurate. However, use of the software was

more rapid than hemocytometer counting. The higher CV for the image analysis system in retrospect may be improved by increasing the area used for cell counting rather than increasing the concentration of the cells to be counted.

Although some initial work has been carried out on the fluorescent plate reader by previous authors (Gravance et al, 2000), there are no published data illustrating the use of this methodology for counting spermatozoa. It was envisaged that the use of a plate reader would allow counts to be carried out on several ejaculates simultaneously in artificial insemination centers with high throughput. A fluorescence plate reader could also be used as a low-cost alternative to flow cytometry, allowing large numbers of cells to be counted from a large number of ejaculates. Despite the high levels of agreement with hemocytometer counts, this technique showed the highest CV of any of the methods used, despite the fact that the result were not significantly different. Further development is required for this method, since it may have the potential of assessing sperm numbers in conjunction with the proportion of viable cells in the same assay. A protocol has also been established to determine the proportion of live cells in a given population (Prathalingam et al, 2002).

In this study, the CV was higher for Microcells than for hemocytometer counts, a finding that has been reported in previous studies (Tomlinson et al, 2001; Brazil et al, 2004b). However, in accordance with a previous study (Sokol et al, 2000), we demonstrated a close relationship between Microcells and hemocytometer counts ( $r = 0.88$ ). In contrast, another study (Tomlinson et al, 2001) reported significantly lower counts than were obtained using a hemocytometer ( $P = .011$ ). Although Microcells did not produce a lower CV in the current study, they have the added advantage of allowing sperm motility to be observed at the same time that sperm numbers are being determined.

In this study we used bull spermatozoa to address the generic problems in determining sperm concentration. However, caution should be used when extrapolating these results to different species, including humans. Differences between human and bull ejaculates include lower concentrations in humans, slower motility, and more debris in human ejaculates.

In conclusion, this study demonstrates the differences among each of the methodologies used. The flow cytometer was the most precise method used, but it does require a preliminary sperm count assessment by a different method to ensure that the semen is diluted to the correct extent (close to 250 000 sperm/mL) for accurate counting. It is essential that concentration and flow rate are adjusted to ensure the absence of “missed events.” The spectrophotometer was the second most precise method assessed and is easy to use for bull, boar, and ram studs. However,

it is probably inappropriate for human studies because of the low ejaculate concentration and volumes reported for this species. Important factors and constraints to consider when standardizing a laboratory procedure for spermatozoa counts are species, frequency of use, size of sample required, the number of samples routinely assessed, and the cost.

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