

Practicalities and Pitfalls of Semen Evaluation

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■ Introduction

Current artificial insemination (AI) protocols use 2.5-3.0 billion sperm cells per insemination dose. However, despite this number of cells, there are many factors that can influence fertility with AI. These factors can include undefined fertility factors associated with the boar, the volume inseminated, the interval from insemination to ovulation, sperm motility, the percent abnormal sperm, contaminants within the dose, and even the amount of sperm cell agglutination. Final sperm numbers in the AI dose are critical, since the numbers of sperm can be adjusted to compensate for infertile sperm. Yet despite the numerous measures for semen quality, assessment for semen concentration is the most common test performed. This is done as insurance against, or for investigation into the causes of, poor reproduction.

There are growing industry concerns about how many sperm cells are actually provided in an AI dose. When considering the number of sperm cells required for optimal farrowing rate and litter size, numbers appear to be closely linked to interval from insemination to ovulation and also to the number of inseminations. For example, when using a single AI of 2 billion sperm, litter size and farrowing rates are optimal with semen that was less than 36 h old, and AI was performed less than -28 h prior to ovulation (Nissen et al., 1997). Even with multiple inseminations, Watson and Behan (2001) reported that when performing AI at 0 and 24 hours and using semen <48 hours old, 1 billion sperm cells resulted in lower farrowing rates and smaller litter sizes when compared to 2 and 3 billion sperm. Most studies indicate that 2 billion cells will not limit reproduction, but fewer cells reduces performance and higher cell numbers provide little or no advantage (Steverink et al., 1997). This may be related to transport and reservoir establishment since these are similar with sperm in the AI dose at 50-500 million sperm/mL (range: 1-10 billion sperm, Baker et al., 1968).

Successful AI must supply enough sperm to allow establishment of a viable sperm reservoir. Adequate or even excessive numbers of sperm cells in a dose may help to keep a sperm reservoir functional for longer periods and even compensate for increased intervals from insemination to ovulation, and possibly even compensate for lower quality semen. A good model for the effects of low numbers of viable sperm is the use of frozen sperm, since more sperm must be used (5 billion) to achieve the same reproductive rates when compared to non-frozen-thawed sperm. Additionally, insemination must occur even closer to time of ovulation (within 4 hours before ovulation, Waberski et al., 1994). When using frozen-thawed sperm, the 5 billion sperm used are intended to compensate for loss of fertile sperm, and provide ~1.5 billion motile sperm (Hofmo and Grevle, 2000). Collectively, these studies indicate that when using conventional AI, 2 billion fertile sperm can reliably result in good fertility. However, when conditions are less than optimal, lowered sperm numbers put fertility is at risk.

■ Assessment of Sperm Concentrations

It is clear that a quick and accurate method to evaluate sperm concentration in an ejaculate and an AI dose is required. The two methods commonly used are the cell-counting chamber (hemocytometer) and analysis by spectrophotometry. Use of the technologies of computer assisted analysis and flow cytometry, although quite expensive, are on the rise, and have shown much promise, but will be discussed only for comparison with the less expensive and more common analysis methods.

Within the swine industry, it is not uncommon for those associated with the operation of the breeding farm, to question the reliability of the estimate of sperm numbers within the AI dose. What is not clear is whether the estimate of the number of sperm is correct or incorrect due to the method of evaluation (or evaluator), or due to factors associated with the ejaculate itself. Methods of concentration estimation have been compared and with any method used >95% of the variation can be explained by the ejaculate itself, while the remainder of variation is explained by random and operator effects (Hansen et al., 2002). If the ejaculate itself causes the greatest variation in the estimate, what causes variation in the ejaculate? Factors that influence boar sperm production have been reviewed (Clark and Knox, 2003) and although many factors are involved, it appears that both boar age and collection frequency have the greatest impact. Obviously, these cannot be controlled, so it is worthwhile to focus on what range of concentrations are expected and how this will impact the method of estimation.

■ Counting Chambers

The standard method (although not the most common) for estimating sperm cell number is by microscopic determination of cell concentration using a hemocytometer. It is considered somewhat challenging, since basic lab and microscope skills are required. However, it does have the advantage of visual assessment of the sperm that are being counted. From start to finish, the time required takes about 10 minutes, which has been determined as being too slow for line-speed evaluation. Despite the time limitation, the method is relatively accurate and low cost. The equipment costs US ~\$1200 and other routine costs are between US \$0.05-\$1.00 per sample. It is not uncommon to observe a 5 to 20% variation in the final concentration estimate using this method (Hansen et al., 2002; Knox et al., 2002). In **Table 1**, the relative outcome in final sperm in the AI dose from any range of errors (and also from the photometer and any other method) is shown. It can be seen that estimation in the 5 to 20% range result in sperm cells ranging from 4.0 to 2.5 billion sperm per dose. For an average ejaculate, errors in this range should have minimal effects on fertility. However, when the concentrations are far below or above average, and the fertility of the semen is less than optimal, the seriousness of the estimation errors will likely impact performance.

Table 1. Relative outcome in final sperm in the AI dose

Count Error	Concentration (x 10 ⁶)	Final Sperm in AI Dose (x 10 ⁹)
-50%	125.0	6.0
-25%	187.5	4.0
-10%	200.0	3.8
-5%	237.5	3.16
0	250.0	3.0
+5%	262.5	2.9
+10%	300.0	2.5
+25%	310.0	2.4
+50%	375.0	2.0

*assumes a 200 mL ejaculate of 250 x 10⁶ / mL and 3.0 billion sperm cells desired in 80 mL (375 x 10⁵ / mL).

Errors with this methodology typically occur through improper sub-sampling, improper pipetting, improper filling of counting chambers, under or over dilution of the sample (for counting 50 to 100 sperm), and consistent errors in counting. In our lab, the same semen sample diluted at 1:25 and at 1:100 resulted in estimates that were within 5% of each other (188 versus 196 ± 12 million sperm/mL, respectively). This error will have little impact on fertility. However, counting errors when determining the number of sperm in an AI dose have far greater implications. A 20% error in estimation in concentration can cause interpretation of a dose that actually has 3 billion sperm to be estimated to have as little as 2.4 billion or as much as 3.6 billion sperm. This type of error has serious implications for breeding farm, the semen supplier, and the evaluator.

■ Optical Density Systems

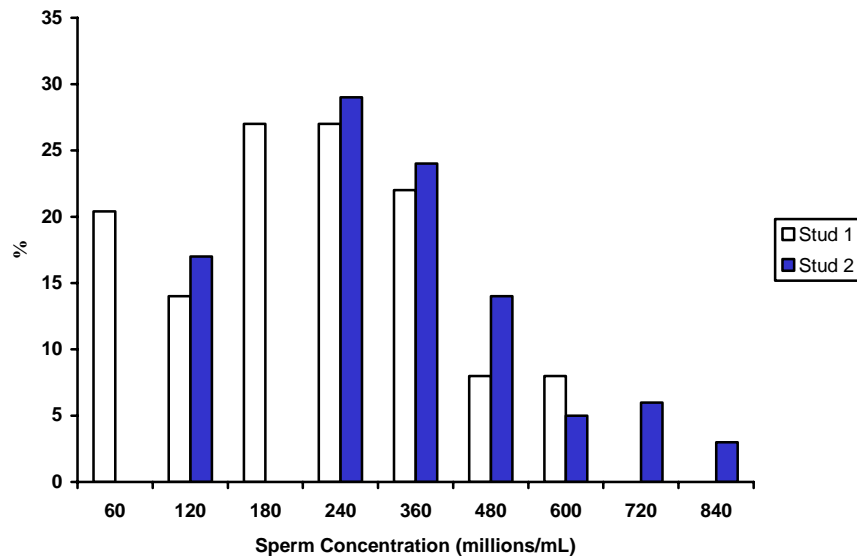
Today the use of optical density to determine concentration (reviewed by Knox et al., 2002) is probably the most common and practical method used for commercial semen production. Spectrophotometers (variable wavelengths) or photometers (single wavelengths) are used because of their ease of use and speed of estimation. The estimation of concentration is highly related to estimates from the hemocytometer, computerized, and cell-counting methods. Photometry measures the amount of light that is transmitted through a sample. During passage through the sample, the light is absorbed, scattered, and transmitted, depending upon the wavelength and number of sperm. On the opposite side of the sample, a detector receives the light and produces an electrical signal proportional to the amount of light, and converts this into a reading. The wavelengths are often set individually for pieces of equipment, but for white suspensions, wavelengths for sperm appear most sensitive in the range of 550 to 576 nm (Foote, 1972). The equipment measures the relative amount of light transmitted or absorbed, which are proportional to the concentration (Absorbance = absorptivity of sample \times wavelength \times concentration). Photometers and spectrophotometers are priced between US \$1,500-\$6,000. Many of the available photometers have predetermined curves that calculate the concentration of sperm/mL while others provide a chart for conversion of the reading to sperm/mL. The accuracy for each machine is based on a regression equation, which is used to "predict" sperm cell numbers based on the readings.

When using this method there are several sources of error. These errors can originate from improper sampling, pipette error, an improper sample holder (misaligned, damaged, wrong material), incorrect diluent, failure to zero to the blank, incorrect standard curve, and reading out of the range of the photometer accuracy. It appears the accuracy (-20 to +30%) is affected by light scattering due to differences in concentration and also due to seminal plasma. However, dilution rate may be used to partially control for this problem. There is no

standard dilution rate and some manufacturers require certain dilution levels while others do not. This is important, since improper dilution will result in readings near the upper and lower limits of detection, which have high degrees of reading inaccuracy (<20% and >80% for transmittance).

Evaluation of boar semen at concentrations between 54-287 million sperm/mL using a photometer indicated that the error was $\pm 16.2\%$. Paulenz et al. (1995) reported that the hemocytometer was the most variable (12% CV), compared to the photometer (2.9%) and the cell counter (2.3%). Yet values from all three were highly related. The authors observed that both the photometer and the cell counter both over- and under estimated the concentration at high and low concentrations. In our lab, in a study involving 29 boar ejaculates, a high correlation between the concentration and transmittance was observed ($r = 0.98$). However, for any machine, the primary factor limiting this relationship appears to be when readings occur outside of the optimal range.

Knowledge of the equipment accuracy range and the concentration of the ejaculate could allow a single dilution to be performed that would provide optimal readings within the limits of the spectrophotometer for a wide range of ejaculates. Data from our lab indicates that studs may have quite large differences in average concentrations (**Figure 1**).



Stud 1 averaged 179 million sperm/mL (range: 47-375 million/mL) while stud 2 averaged 349 million sperm/mL (range: 100-840 million/mL). Samples evaluated in triplicate at dilutions within 1:10-1:50 showed low variability (5 to

7%). Interestingly, for stud 1 with lower output, the best predictive dilution rate was 1:5 while for stud 2 with higher output, there was no difference between 1:10 to 1:25 dilution range for prediction of concentration ($R^2 = 0.75$). However, at high concentrations, the 1:5 dilution was not predictive. Our evaluation of two different photometers at similar dilution rates, (Micro-Reader I, Hyperion, Inc. Miami FL and Spectronic 401, Spectronic Instruments, Inc. Rochester, NY) resulted in different values but did not produce different estimates for concentration. Our interpretation of these data suggest that 1) the hemocytometer and photometers were accurate for measuring sperm concentrations, 2) a standard curve may be necessary for each individual machine, and 3) average sperm concentrations can differ by the hundreds of millions between studs, indicating optimal dilution rates may be stud specific.

■ Expected Variation in Sperm Concentrations

Determining the range of semen concentrations range that will be encountered in the lab could help choose the lab's standard dilution rate and give warning when expected values are out of the expected range and reading accuracy for the equipment. Larsson (1986) reported that ejaculate volumes ranged between 100 to 500 mL, with total sperm produced in the range of 10 to 100 billion, and with concentrations in the range of 5 to 1000 million sperm per mL. Others have indicated average values for an ejaculate; both Crabo (1986) and Colenbrander et al. (1993) reported that the average normal ejaculate contains 25 to 50 billion sperm, similar to estimates by Garner and Hafez (1993), who indicated that average volume was between 150 to 200 mL, concentration was 200 to 300 million sperm/mL, and total output was 30 to 60 billion sperm per ejaculate. In contrast Rutten et al., (2000) showed an average ejaculate output of 82 billion (estimate of ~370 million sperm at 220 mL) when boars are collected approximately once per week (expected volume ~220 mL and concentration of ~370 million sperm/mL). Similarly, Paulenz et al. (1995) reported ranges of 250 to 400 million/mL, while Hansen et al. (2002) observed ranges of 150 to 700 million/mL, Marin-Guzman et al. (1997) 800 million/mL (a total of 128 billion sperm / ejaculate) and Ciereszko et al. (2000) reported ~400 million/mL with 90 billion sperm per ejaculate. These data are not dissimilar to observations by Paulenz et al. (1995) and Knox et al. (2002) who found that ejaculate concentrations were higher than expected and that alternative dilution rates for optimal reading were needed.

It appears that average concentrations today are consistently higher compared to some earlier reports. It is possible that this due to lower collection frequencies, selection for testes size, greater maturity (age), and better health, nutrition and housing. This is supported by reports that lower sperm collection frequency can cause ejaculation output to exceed 100 billion, while high collection frequencies can result in ejaculates containing less than 5 billion

sperm (Crabo, 1993; Levis, 1997). Colenbrander et al. (1993) also observed that with collection frequencies averaging 1.6 times per week, the average ejaculate contains 62 billion sperm, with about ~100 billion sperm produced per week. Sperm concentration is linked to age of the boar and differences of tens of billions of sperm have been observed (Clark et al., 2003). Yet regardless of the reason, it appears that concentrations are highly variable, and that some studs have quite different average outputs when compared to others. The importance of this may be that at lower or higher concentrations, the readings that are made will most likely be out of the accuracy range of the photometer. This will likely result in over or underestimate of sperm in the AI dose and improper number of doses produced.

It could be beneficial to produce a matrix grid for ejaculate volumes and concentrations for certain ages of boars (under the standard collection frequency for the stud). Additionally, standard curves should be generated for the optimal reading range of the equipment (see Knox et al. 2002). When expected values fall outside of the pre-determined concentration range based on the grid, investigation into reasons for this (collection frequency), alternative counting methods or alternative dilution rates should be considered. This could serve as a method for internal quality control and protect the interests of both the stud and the breeding farms and provide confidence in sperm numbers in AI doses produced.

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