

Stallion semen evaluation and interpretation

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Introduction: The evaluation of stallion semen quality has historically relied on the evaluation of sperm motility, which includes the immediate evaluation of raw and extended motility; longevity of motility; evaluation of morphologic features; and determination of sperm numbers. These techniques have been previously outlined and described (Kenney et al, 1983). The intent of evaluating these various semen parameters is to render an opinion as to the potential fertility of a particular stallion and describe management strategies to maximize fertility. Based on the levels of these semen parameters as well as physical characteristics (testicular size), stallions are categorized to reflect the level of potential fertility when bred to a certain number of mares (120 using artificial breeding, 45 by natural cover). These categories (Satisfactory, Questionable, Unsatisfactory) give the practitioner a reference point from which to make management recommendations as to how a particular stallion should be managed. The intent of applying different levels is not to categorize stallions as fertile, subfertile or sterile since these classifications can only be applied when management effects have been completely eliminated.

Since the introduction of the Stallion Manual (Kenney et al, 1983), there have been several important changes in the breeding industry which include:

- 1) Increase in the mare book of the prominent Thoroughbred stallions. A traditional full mare book for a Thoroughbred stallion was 45 mares, however, this has changed to approximate 60-90 mares, with some stallions breeding upwards of 150 mares. This change has raised the question of what the upper limit of their reproductive capability might be for the average stallion. In addition, it is unclear how the recommendations and parameters set forth in the stallion manual might apply to the increased mare books.
- 2) Introduction of preserved semen techniques (cooled-shipped and frozen-thawed) semen to the Standardbred and Quarterhorse breeds. While other breeds have allowed the use of these techniques prior to acceptance by these two breed registries, none have applied it in such a widespread manner. While these techniques have proven to be useful and have been widely accepted and applied, there appear to be problems that result in reduced fertility in a subset of stallions, that is not seen when these stallions are bred with fresh semen that is not stored or processed. In some cases, similar to the Thoroughbreds, overall mare books have increased. In addition, instead of most mares being housed and bred on the farm where the stallion is located, semen is cooled and shipped to other locations and is

often 24 hours old when the mare is inseminated. Shipped semen often includes double the usual insemination dose of 500 million progressively motile sperm. This increased need for sperm has the effect of doubling the mare book for each mare that is bred using shipped semen. Therefore, while a stallion may be booked to 125 mares, if 50% of these are bred using shipped semen he requires enough sperm to breed 188 mares. Therefore, not only is there an effect of increased mare books, but mares bred with shipped semen are, in some cases getting poorer quality sperm. This situation has heightened the need for diagnostic tests that will identify sperm that will tolerate the cooling process and in addition the clinician must be able to differentiate between a primary sperm deficiency (sperm that is poor to start with) and a management induced problem such as poor semen handling or the shipment of sperm numbers that are below that needed to maximize fertility.

Both of the above situations have increased the breeding intensity that each stallion is exposed to during the breeding season, which in return requires the farm manager and clinician to have a more refined understanding of the limitations that each stallion may encounter.

From a diagnostic point of view the clinician would like to be able to evaluate all compartments of the sperm, such as the integrity of the acrosome, head membrane, chromatin, as well as mitochondrial viability. It would be ideal to perform these tests using multiple probes on the same sperm, but tests often only evaluate one compartment at a time and therefore separate tests must be performed on the same sample.

Many of these tests use fluorescent probes that identify specific aspects of the sperm and therefore requires either a fluorescent microscope or a flow cytometer for evaluation. Flow cytometry has the advantage of being able to identify, evaluate and enumerate large numbers (5-10,000) of sperm in a short time period (30-60 seconds). This is an advantage over fluorescent microscopy because of the larger number of cells evaluated, but also because there is less likelihood of artifactual fluorescent changes (due to the stability of the dye used) occurring due the extended period of time required to prepare and evaluate the sperm sample. Acridine orange would be an example of a dye that bleaches out quickly. When this happens the red fluorescence that occurs when it binds to single-stranded DNA turns to green and therefore does not accurately represent the condition of sperm DNA.

Following are some of the diagnostic tests that can be used to define the level of sperm quality that a stallion has, as well as the fertility that would be expected from that sperm, assuming management of the mares is not a major factor in reducing the stallions fertility.

Sperm morphology: Evaluation of the shape of the sperm is perhaps the most objective test that can be performed on the semen sample. While iatrogenic effects can alter some morphologic features, this is less likely than with sperm motility, which requires immediate evaluation. The raw sample can be preserved and evaluated at a later time. There are certain abnormalities (abnormal heads, abnormal midpieces, proximal and

distal droplets) that can be detected by morphologic evaluation, but may not influence the level of initial motility. These samples may give the impression of “good” motility if that is the only parameter evaluated, but may exhibit poor longevity because of the abnormalities present.

There are several methods that can be used to prepare the appropriate fixed semen sample. The one we prefer uses the liquid fixative, buffered-formol saline (BFS). This solution can be prepared and placed in 2-3 ml aliquots in 4-5 ml tubes. Raw semen is added to create a slightly cloudy solution. This sample is left at room temperature until evaluation or can be shipped for referral and consultation. Although slides of semen smears can also be stained with Eosin-Nigrosin and evaluated with light microscopy at a later date, the visualization of certain sperm defects using this method is inferior to that obtained when sperm are preserved in BFS and examined with phase-contrast or Nomarski optics. Microscopic evaluation of sperm morphology should employ a high quality microscope equipped with phase-contrast or Nomarski optics. Use of 100x magnification under oil immersion maximizes resolution and identification of abnormalities. Lesser quality optics increases the likelihood of overlooking important abnormalities such as roughened midpieces.

A minimum of one hundred sperm should be counted. Some sperm may have more than one abnormality, in which case ***all the abnormalities*** present should be counted, but only one sperm should be added to the total count. Therefore, when the evaluation is finished and a total of 100 separate sperm have been counted, the total of all abnormalities and normal sperm will be greater than 100. If only one abnormality is counted per sperm, the evaluator is assuming that the abnormality recorded is more important than the ones that were not. In addition, we are interested in the ***prevalence of each abnormality*** in the sample as well as the number of normal sperm. If an abnormal midpiece is ignored because the same sperm also has an abnormal head we are unable to determine the prevalence (% in the population) of abnormal midpieces in the sample. Determining the prevalence of all abnormalities is important, since certain types of abnormalities and combinations of abnormalities appear to have a graver prognosis with regards to fertility than do others (Love et al, 2002). These include abnormal heads, detached heads, abnormal midpieces (usually roughened midpieces), coiled tails, and premature germ cells.

Morphologic evaluation of a stallion's sperm involves the determination of the percentage of normal sperm, but also uses the types of abnormalities present to further define the extent of semen quality. In effect, this method recognizes the fact that all abnormalities are not equal in their impact on fertility. An extreme example would be two stallions, both of which have 35 % normal sperm. If the types of abnormalities are ignored the clinician would interpret these two stallions to be essentially of equal fertility. However, if the types of abnormalities are taken into account and it is determined that one stallion has 35% distal droplets, while the other has 20 % abnormal heads, 20% midpiece abnormalities, and 10 % detached heads, the fertility of these two individuals would be completely different assuming all other aspects of surrounding the breeding of these stallions is similar.

In a group of Thoroughbred stallions it was determined that certain abnormalities were associated with a reduction in fertility. These abnormalities included abnormal heads, abnormal midpieces, detached heads, coiled tails, and premature germ cells. In addition, these abnormalities reduce fertility when they occur in combination. The clinician should be particularly cautious when a high percentage of one of these abnormalities occurs alone. Examples of this might occur when a high percentage of abnormal heads, detached heads, or abnormal midpieces are identified and occur by themselves without the presence of large amounts of other abnormalities. One possible reason for a singularly high percentage of abnormally heads could relate to the normally heterogeneous nature of the head shape of stallion sperm. In particular, certain individuals may exhibit considerable variability in the shape of their sperm heads. For the clinician familiar with the uniformity of bull, boar, or ram sperm, the ejaculate from a stallion such as this may appear to have a high percentage of abnormal heads. These stallions, if evaluated on a routine basis are usually fertile animals. The second example would be an elevated level of detached heads in the absence of other abnormalities. In many instances this is caused by the accumulation of sperm in the ampullae (plugged ampullae), which results in deterioration of the midpiece and subsequent separation of the head and midpiece. This condition is reversible and in most cases the stallions are fertile when the accumulated sperm are removed. Detached heads may also occur, usually in smaller percentages (5-15%), in combination with other abnormalities such as abnormal heads, abnormal midpieces, coiled tails and premature germ cells. In this case the detached head is possibly a result of poor testicular function (the neck was not made correctly) rather than deterioration.

The proximal droplet is a common abnormality that can occur in varying frequencies within as well as between stallions. It has historically been thought to be a result of epididymal dysfunction resulting in an immature sperm, since the droplet should descend proximally next to the head, distally and eventually be shed in the epididymis. In addition, they have been reported to be associated with a reduction in fertility in one study in Standardbreds (Jasko et al, 1990), but did not reduce fertility in the above-mentioned study in Thoroughbreds (Love et al, 2000). A possible reason for the discrepancy could be due to the difference in the breeding methods used (artificial breeding vs. natural cover). Since larger numbers of sperm are deposited in the uterus of the Thoroughbred mare following natural cover compared to the Standardbred in which a *minimum insemination dose* is used, the reduction in fertility seen in the Standardbred may be *compensated* for by the increase in sperm numbers in the Thoroughbred. Saacke et al, introduced the concept of *compensable* defects in bull sperm, whereby there are certain abnormalities that do not make it to the site of fertilization and therefore promote reduced fertility because the semen does not have a threshold level of normal sperm to maximize fertility. In this case, fertility can be maximized, or *compensated*, by simply increasing the total number of sperm in the uterus, such that the number of normal sperm is at or above that needed for optimum fertility. This is in contrast to *uncompensable* abnormalities that have the ability, like normal sperm, to fertilize and initiate early embryonic development, but subsequently result in embryonic loss. These abnormalities cannot be overcome (or compensated for) by increasing sperm numbers, because they have the ability to fertilize like the normal sperm.

Sperm Chromatin Structure Assay (SCSA): This assay uses the metachromatic dye, acridine orange to evaluate the ratio of single (abnormal) and double-stranded (native) DNA present in individual sperm. The dye intercalates into the DNA and fluoresces red when bound to single-stranded DNA and green when bound to double-stranded DNA. An increase in the amount of single-stranded DNA in fresh-frozen sperm has been associated with reduced fertility in a variety of species including humans (Evenson, 1980), boars (Evenson, 1994), bulls (Ballachey, 1987), and stallions (Love, 1998). In addition, some stallions that exhibit a have a high level of compromised chromatin in fresh semen also have an accelerated rate of decline in chromatin quality when the sperm are chilled and stored over time (Love, 2002). Therefore, the SCSA can be used to evaluate fresh sperm as well as evaluate the quality of semen that is to be cooled and shipped. As a general rule the DNA of sperm that have been collected, processed and handled properly should not show any signs of deterioration after 46 hours of storage at 5°C. Stallions that exhibit a decline in DNA quality after storage should be investigated and a cause should be identified. In many cases the causes can be traced to improper handling and extension or processing of the semen.

Acrosome Dysfunction: In most cases of reduced fertility in the stallion, a thorough breeding soundness evaluation in conjunction with a careful review of the breeding records and management can identify the cause(s). However, there appears to be a subset of stallions that exhibit characteristics (good semen quality and sperm numbers, normal testicular size) that are consistent with a fertile stallion, yet impregnate mares at reduced rate (10-30% per cycle pregnancy rates). These stallions are unique because of the discrepancy between the high quality of their reproductive parameters and low fertility. Recently, the function of the acrosomes of these stallions has been investigated at the electron microscope level (Varner 2002). Acrosome function was tested by challenging the sperm with calcium ionophore, A23187, a promoter of the acrosome reaction. Following ionophore exposure, the acrosome reaction from subfertile stallions was minimal (~6%), while fertile stallions exhibited a high percentage (84%) of reacted acrosomes. The discrepancy between the fertile and subfertile groups was dramatic and appears to describe a specific compartmental sperm defect that is present in combination with “normal” reproductive parameters (motility, morphology, chromatin, sperm numbers and testicular size).

Sperm Viability: Tests of sperm “viability” have been introduced that measure the integrity of the sperm membrane. One of the more recent viability tests utilize SYBR-14 and propidium iodide (PI), which penetrate the head membranes of intact and disrupted membranes, respectively and bind to the DNA and fluoresce either green (SYBR-14) or red (PI). The obvious difference in color makes evaluation of membrane integrity straightforward using fluorescent microscopy or flow cytometry (Figure 1). Recently, Garner et al, introduced the use of a third dye, JC-1, used in combination with SYBR-1 and PI, that allows for the evaluation of the sperm midpieces (Figure 2). This dye specifically evaluates mitochondrial membrane potential. It is unique in that dye *aggregates* are formed when a high membrane potential is present resulting in orange fluorescence, but dye *monomers* result in a green fluorescent. This combination of dyes

allows for a simultaneous evaluation of the membrane integrity of the sperm head as well as the membrane potential of the mitochondrial membranes of the midpieces. The majority of sperm with intact sperm head membranes (green fluorescence) also have midpieces with mitochondria with high membrane potentials (orange fluorescence). Nonviable sperm stain red due to propidium iodide with midpieces that show little fluorescence at all.

In the extended sperm from stallions of high fertility, sperm motility and percent viable sperm should be similar after at least 48 hours of storage at 5°C. This does not appear to be the case in stallions that do not have adequate sperm motility when cooled as sperm heads go through transition phases in which the head goes from green to orange and the midpieces changes from orange to green and eventually loses most fluorescence. The membrane integrity these individuals appear to be maintained long after sperm motility has declined. It is not clear at this time the diagnostic significance of this finding. It may indicate that even though they sperm lack motility they are still able to maintain the capability of renewed motility if environmental conditions are changed. While good motility is associated with a high membrane potential, it appears that the high membrane potential is maintained for a period of time after motility declines

The use of the SYBR-14/PI/JC-1 triple stain combination has the potential of providing important diagnostic information on sperm from stallions that are used for shipping as well as sperm that is frozen and thawed.

The ability to describe those semen characteristics that are consistent with stallions of high, medium, and low fertility as well as those that are subfertile is an important diagnostic tool when attempting to identify the cause of reduced fertility. As stallion's reproductive capabilities are challenged by increased book sizes as well as increased use of stored semen, some stallions are showing a reduction in fertility not due to poor semen quality, but due to a combination of too little sperm and inadequate management considering the number of mares in his book as well as the method of breeding used. Oftentimes, these stallions are considered "normal" based on conventional measures of semen quality, such as motility, morphology, as well as the SCSA. It is often concluded that these semen tests are inadequate because they indicate the stallion is "normal", yet clinically he is "subfertile". The semen tests are in fact telling an accurate story in these cases and should encourage, rather than frustrate, the clinician to investigate whether the stallions is being overbred and whether the management of the mares is adequate. Good semen quality in the absence of good fertility oftentimes indicates a primary mare or management problem that may be related to overuse of the stallion.

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Table 1. Show sperm values based on different levels of stallion fertility.

Fertility groups	1	2	3
Seasonal pregnancy rate	93 ^a	86 ^b	73 ^c
% Pregnant/cycle	82 ^a	55 ^b	39 ^c
% Total motile	84 ^a	74 ^b	63 ^c
% Morphologically normal	69 ^a	46 ^b	46 ^b
% COMP- α t	12 ^a	17 ^b	25 ^c

Superscripts in rows are different (p<0.05).
Data from Love and Kenney.

Table 2. Shows those morphologic features related to fertility in a group of Thoroughbred stallions.

Fertility groups	High	Medium	Low
% pregnant / cycle	66	51	37
normal	66	56	23
Abnormal heads	7	12	26
Detached heads	1.4	2.6	4
Midpieces abnormalities	2.9	3.7	11
Coiled tails	1.0	2.1	5.4
Premature germ cells	0.2	0.3	1.2

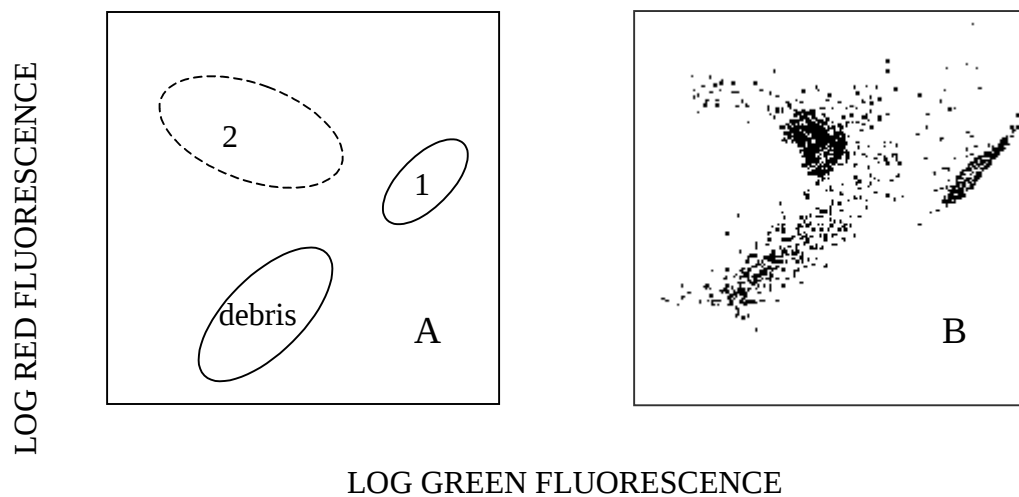


Figure 1. Schematic (A) and actual scattergram (B) showing the sperm populations for the SYBR-14/PI stain combination. Population 1 corresponds to viable sperm (heads fluorescence green), and population 2 includes non-viable sperm (head fluorescences red)

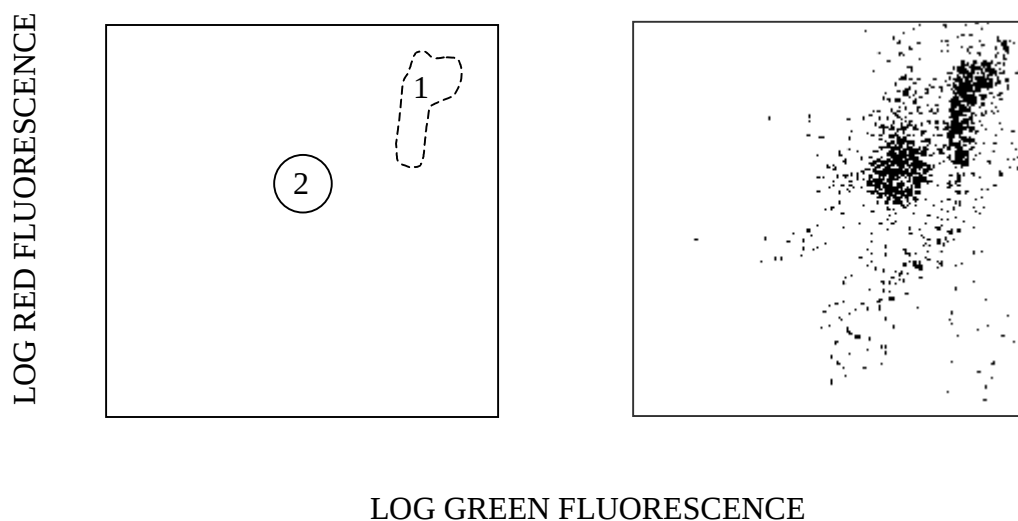


Figure 2. Schematic (A) showing the sperm populations measured and scattergrams for the SYBR-14/JC-1/PI stain combination. Population 1 corresponds to viable sperm (head fluorescence green, midpiece fluorescences orange or green); population 2 to non-viable sperm (head fluorescences red)