

SEMEN QUALITY ASSESSMENT

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Semen Collection

Semen can be collected from bulls by a variety of means including per-rectal massage, the use of an artificial vagina and by electro-ejaculation. The latter method is the one most commonly employed with range-type bulls. Method of collection should be noted as it may affect qualitative aspects of the ejaculate.

Electroejaculators and Probes.

Commercially available electroejaculators are available with power being provided by AC current, by internal rechargeable batteries, or by 12v automobile batteries. Electroejaculation requires the stimulation of pelvic nerves controlling not only the emission of semen into the penile urethra but also those controlling erection and ejaculation. Newer probe designs have full-length longitudinal electrodes which stimulate all functions simultaneously.

Preparation and Stimulation.

The bull's rectum should be emptied of feces before the probe is inserted. The lubricated probe is inserted so that the anal sphincter closes behind the main body of the unit. It is helpful to determine the lowest current level at which the animal first shows an obvious physical response. This initial response may be subtle e.g. a twitch of the tail, a tightening of the anal sphincter, or a tensing of the gluteal muscles. This stimulus "threshold" provides the starting point for subsequent stimulations which should be conducted with a smooth routine to which the bull can easily adapt.

With Bos taurus breeds, a typical approach is to deliver a smooth increase in probe current from zero to the desired level over a duration of 1 to 2 seconds, followed by a more rapid reduction to zero current and a rest period of approximately one second before the next stimulation. Once the bull is settled into the routine, 5 to 7 stimulations are given at each succeeding voltage step until erection and ejaculation occur. For machines which do not have separate voltage and current controls, the same stimulation pattern is employed except that the single control is used to generate incremental increases in probe voltage and current until ejaculation occurs. During the early stages of stimulation and erection, clear seminal plasma is often passed which is not generally collected. When the ejaculate turns cloudy, the subsequent jets of semen are collected. It is important to continue stimulation until the ejaculate starts to become clear again. Failure to proceed to this point can lead to errors of interpretation in the spermogram as the initial portion of the ejaculate may contain large numbers of degenerating spermatozoa, especially in bulls which have been sexually quiescent for some time. For the same reason, if an ejaculate shows substandard motility in the absence of an obvious physical cause, the collection of a second sample within a short

period of time (e.g. 5 to 10 minutes) can often result in improvement.

Variations on these stimulus patterns occur with different machines and operators. With difficult bulls some experimentation might well be necessary. In all situations, however, the welfare of the animal is paramount and stimulation should be discontinued if either undue stress is being caused or physical injury to the bull might occur.

Collection Devices.

Semen is collected into a prewarmed insulated or jacketed tube through a funnel or cone. All surfaces coming into contact with semen should be clean, warm, dry and free of spermatoxic agents. Because "cold shock" causes irreversible damage to spermatozoa, efforts to maintain semen at 30-35°C until the "on-site" evaluation procedures are complete is an important consideration for successful semen assessment.

Semen Evaluation

Initial Impressions.

Volume, density, and gross characteristics of the ejaculate are not "front-line" BSE assessments because they have not been shown to be related to fertility. Space on the score sheet is, however, provided for the recording of such information. Likewise, the assessment of spermatozoal concentration is not a routine part of the BSE; the measurement of scrotal circumference provides a better estimate of sperm production in range-type bulls which are subject to infrequent examinations. However, informal recording of such information may help to monitor the success of semen collection and to interpret gross motility estimation.

Other gross characteristics which may be noted include evidence of contamination, hemorrhage or inflammatory material. If the ejaculate contains sufficient purulent material for this to be obvious to the naked eye, then that bull should not be classified as satisfactory at least until a benign cause is found. Debris or contamination from the sheath may be regarded as being less serious unless it represents active infection.

Motility should be assessed microscopically. Two methods of assessing sperm motility are traditionally employed; gross motility (or mass activity) and individual motility (or percent progressive motility). It is good procedure to use both methods as can they differ somewhat in interpretation and precision. With all motility estimations it is important to protect semen against adverse effects (e.g cold shock) and to do the estimation as soon as possible after semen collection.

Gross motility, or the amount of swirling (or wave motion) present in an undiluted semen sample, is a function of both sperm concentration and individual motility. Under field conditions, gross motility is typically assessed by placing a drop of raw semen on a warmed slide and observing it at 100 magnifications (10X eyepiece and 10X objective). With the condenser properly adjusted, mass action or "swirl" can be observed in samples which have adequate numbers of motile spermatozoa. The rankings for this estimate are as follows:

Mass Activity (Gross Motility)	Rating
<i>Rapid Swirling</i>	<i>Very Good (VG)</i>
<i>Slower Swirling</i>	<i>Good (G)</i>
<i>Generalized Oscillation</i>	<i>Fair (F)</i>
<i>Sporadic Oscillation</i>	<i>Poor (P)</i>

THE MINIMUM RECOMMENDED THRESHOLD FOR GROSS MOTILITY IS FAIR (F).

Individual progressive motility of spermatozoa is assessed under a brightfield or phase-contrast microscope preferably equipped with a warm stage or other means of preventing cold shock of spermatozoa. Coverslipped specimens are usually examined at a total magnification of 400x. In dense samples (milky or creamy) the sample should be diluted for proper observation of individual spermatozoa. Sodium citrate or skim milk based semen extenders are serviceable diluents; physiological sterile saline (PSS) may be used although readings should not be delayed when it is used. The percentage of active, progressively motile cells is estimated. This procedure takes more practice than does the gross motility estimation, but is probably more accurate in experienced hands. Individual motility ratings are as follows:

Percent Progressive Motility	Rating
<i>≥70%</i>	<i>Very Good (VG)</i>
<i>50 - 69%</i>	<i>Good (G)</i>
<i>30 - 49%</i>	<i>Fair (F)</i>
<i><30%</i>	<i>Poor (P)</i>

THE MINIMUM RECOMMENDED THRESHOLD FOR INDIVIDUAL MOTILITY IS 30%.

Observation of the semen sample at 400x can also help to identify the presence of abnormal numbers of other cells (e.g. squamous epithelial cells, inflammatory cells or spheroids) within the sample. The identification of aberrant cellular material can benefit from the staining of a semen smear with Dif-Quik, New Methylene Blue, or other differential blood cell stains, while bacteria are best categorized using a Gram stain.

Morphology of spermatozoa (differential counts of normal and abnormal cells) is assessed either by phase microscopy (using preparations "fixed" in e.g. formal-buffered-saline or PBS-glutaraldehyde) or by using brightfield microscopy of stained smears. Common stains used for this purpose include nigrosin-eosin, William's stain, modified Giemsa and even India-ink. The Society for Theriogenology (SFT) recommends the nigrosin-eosin stain for its combination of ease and utility. Although this stain is a "supravital" stain (i.e. sperm which are "alive" at staining will not absorb stain while those that are "dead" will partially or completely absorb the red eosin color), here it is used for its ability to depict sperm morphology only.

With nigrosin-eosin staining of spermatozoa, the most common method is to mix a fraction of a drop of semen with a drop of background or "negative" stain and spread the mixture over the surface of a glass slide which is allowed to air-dry. Care should be taken during the smearing process to avoid trauma to sperm. It is also helpful to vary the thickness of the smear to provide a variety of background densities to the stain from which an area can be picked for best microscopic examination.

Brightfield microscopy of stained smears is best done at 1000X with an oil immersion lens. At least 100 spermatozoa should be observed in different fields and classified for normality or abnormality. Normal sperm should be at least 70% of the ejaculate for the bull to pass the BSE (see below).

In the older SFT BSE system, sperm abnormalities were classified as being either "primary" or "secondary" with the underlying assumptions being that primary abnormalities (considered to be caused during spermatogenesis) were more serious than secondaries (caused subsequent to sperm release into the extragonadal system). More recent knowledge has cast doubt upon these assumptions. In the meantime a system using "major" and "minor" abnormalities was created to more accurately reflect sperm abnormalities for which fertility data was available. It was apparent that the lists of sperm abnormalities in routine use for both systems were essentially indistinguishable (except perhaps for proximal cytoplasmic droplets). Thus, as the primary/secondary scheme is currently widely used, this was retained as the reference point in the present system (see appendix for categories of abnormalities). Although total abnormalities only are employed as the threshold in the new BSE system, "primary" and "secondary" abnormalities can be collated to arrive at this number. The recording of specific abnormalities (or their category as primary or secondary) can also be useful for the monitoring of bulls and their progress.

**THE MINIMUM RECOMMENDED
THRESHOLD FOR SPERM
MORPHOLOGY is 70% NORMAL
SPERMATOZOA.**

It should be noted that a system describing compensable and uncompensable sperm defects, has been proposed (Saacke et al 2000), which shows much promise in helping to categorize sperm defects in manner which reflects their function and significance. This system is, however, still evolving.

Advances in Semen Assessment

To be able to accurately predict fertility by using a rapid, economic assessment of a single semen sample remains an elusive goal for researchers and clinicians alike. This is because fertility is a multifactorial trait, and also because the semen assay attempts to predict future performance from past events. Difficulty exists in defining which semen or sperm traits are most associated with fertility. For example, although motility undoubtedly plays a role in sperm transport and penetration of the zona pellucida of the oocyte, results with ICSI (intracytoplasmic sperm injection) show that it is not an essential prerequisite for actual fertilization to occur. In addition, although significant morphological abnormalities can prevent sperm from reaching the site of fertilization, this does not appear to be true for more

subtle abnormalities. However, there is some evidence that the latter group might contribute to lowered embryonic viability. In general, the best approach for semen assessment is to use a combination of several seminal quality attributes (Garner 1997), choosing those which reflect different aspects of sperm function (eg motility and morphology).

Sperm Movement

Visual assessment of sperm movement using a bright-field microscope is still the most common method employed. Although rapid and relatively inexpensive, this method is susceptible to subjectivity and environmental insult, resulting in a relatively low allowable threshold for this parameter in the current SFT bovine BSE system (30%). Accuracy improves with the use of phase microscopy in controlled environments and appropriate dilution of concentrated samples. More objective methods have included the use of time-lapse micro-photography, photo-electric systems (e.g. Optibreed) and computer assisted semen analysis (CASA). Some of these show considerable promise, as well as providing a wealth of additional information concerning sperm movement attributes. Cost is a constraint for routine field use of some of these systems. For such use, reasonable repeatable results may be obtained by a trained observer using a good microscope in a controlled environment.

Sperm Morphology

Sperm morphology represents one of the more important aspects of semen assessment (Garner 1997), as it directly affects fertility. Difficulties arise in determining which abnormalities are most problematic, and what are their acceptable levels of tolerance. Sperm morphology classification systems have attempted to categorize abnormalities in terms of their assumed origin (primary/secondary), significance (major/minor) and presumed functional contribution to infertility (compensable/uncompensable). Another approach is to assess the number of viable sperm which are free of abnormalities in relation to a estimated fertility "threshold" - the route followed by the Society for Theriogenology with its breeding soundness guidelines for bulls (Chenoweth et al 1991).

Traditionally, sperm morphology assessments have been conducted by observing stained semen samples via a bright-field microscope. Commonly used stains have included nigrosin-eosin, eosin-congo red, Williams stain and Giemsa. If these are not available, Dif-Quik or even India ink can provide adequate staining. Sperm morphology assessment is best done at a magnification of 1000 plus X, which usually implies use of an oil-immersion objective. When done with care, using a good microscope, the evaluation of 100 sperm in random fields has been shown to be sufficient for routine assessment purposes.

Improvements in sperm morphology assessment have been attributed to the use of different forms of phase-contrast microscopy, with differential-interference contrast (DIC) microscopy being regarded as the "gold standard" for certain types of abnormalities, particularly those involving the acrosome (Garner 1997), and for depicting subtle abnormalities of the sperm head or midpiece (Chenoweth et al 1994). With subtle sperm head abnormalities, DIC has allowed identification of a stereotyped spectrum of sperm abnormalities following insult, which is first observable as the classic sperm "diadem defect". Phase microscopy has an advantage that it may be used with "fixed" semen samples, thus avoiding possible structural damage to sperm which may occur with traditional stained smears. However, it represents increased expense and sophistication, both of which reduce its attractiveness for routine use. The electron microscope (EM) represents the ultimate

method for depiction of individual sperm abnormalities. However, this option is not regarded as practical for routine use as the process is complex, expensive and difficult to quantify.

Automated high resolution image analyzers can identify subtle differences in head shape and size, with the added advantage that they can process sperm at high speed, and thus provide quantifiable results (Gravance et al 1998). Difficulties encountered in their ability to differentiate between sperm and non-sperm particulate matter can be circumvented with the use of fluorochromes. Despite this, their applicability for routine use in domestic animals has yet to be established.

Fluorescence " Flow Cytometry

Fluorochromes can be used to assess specific functional aspects of sperm (such as live/dead) or sperm organelles (such as the acrosome or mitochondria). A number of stains have been employed to assess factors such as sperm DNA/nucleic acids (eg. propidium iodide, acridine orange, DAPI, Hoechst 33258), sperm membrane integrity (eg. CFDA, BCECF), intact acrosomes (PSA-FITC), functional mitochondria (rhodamine 123, JC-1, MITO) and sperm capacitation (CTC). The combination of fluorochrome staining of sperm with flow cytometry allows large numbers of sperm to be accurately categorized in a short period of time. Combinations of stains have also proven to be useful, with SYBR-14/PI being used in our laboratory to differentiate between living and dead sperm. Such methods have shown good relationships with other bovine sperm viability measures in our laboratory (Figure 1), as well as with other species elsewhere (Garner and Johnson 1994; Ferrara et al 1997; Pena et al 1998).

Although the use of fluorochromes in conjunction with flow cytometry provides rapid and accurate results, it does have several disadvantages including the extra expense and logistics involved. However, flow cytometry in association with cell sorting has the added advantage of being able to not only identify sperm with certain characteristics, but also to separate them. This method is being used to successfully separate X and Y spermatozoa based on differences in DNA content between the two populations (Garner 1997), although limitations are imposed by both cost and by the relatively low numbers of separated sperm which become available. However, indications are this technique will be commercially available before long.

K-State Andrology Laboratory

An Andrology Laboratory within the College of Veterinary Medicine at K-State provides male evaluation capabilities and services, including an evaluation service for semen samples sent in by practitioners. This is available for all species and for semen which is fresh as well as stored (frozen, chilled, extended). The tests available are shown after the references section as an example of andrology services available.

Selected References

Bellin ME, Hawkins HE and Ax RL (1994). Fertility of range bulls grouped according to the presence or absence of heparin-binding proteins in sperm membranes and seminal fluids.

- JAnimSci 72:2441-
- Chenoweth PJ, Spitzer JC and Hopkins FM (1992). A new bull breeding soundness evaluation form. Proc. Society for Theriogenology AGM, pp63-70.
- Chenoweth PJ, Risco CA, Larsen RE, Velez J, Tran T and Chase CC Jr (1994). Effects of dietary gossypol on aspects of semen quality, sperm morphology and sperm production in young Brahman bulls. Theriogenology 42:1-13.
- Barth, A.L. and Oko, R.J. *Abnormal Morphology of Bovine Spermatozoa*. Iowa State University Press, Ames, Iowa. 1989
- Blom, E.: The Ultrastructure of Some Characteristic Sperm Defects and a Proposal for a New Classification of the Bull Spermogram. Atti del VIII. Simposio Int. di Zootechnia. Milan. pp 125-139 (1972).
- Carroll, E.J., Ball, L. and Scott, J.A.: Breeding Soundness in Bulls - a Summary of 10,940 Examinations. J.A.V.M.A. 142:1105-1111 (1963).
- Lagerlof, N.: Changes in the Spermatozoa and in the Testes of Bulls with Impaired or Enhanced Fertility. Acta. Path. et Microbiol. Scand. Suppl. 19. 254 pp. (1934).
- Larsen, R.E. and Chenoweth, P.J.: Diadem/Crater Defects in Spermatozoa from Two Related Angus Bulls. Molec. Repro. and Devel. 25:87-96 (1990).
- Ferrara F, Daverio R, Mazzini G, Bonini P and Banfi G (1997). Automation of human sperm cell analysis by flow cytometry. ClinChem 43:801-897.
- Fitzpatrick LA, Fordyce G, McGowan M, Holroyd R, Bertram JD, Miller RG, Jayawardhana GA, Doogan VJ and De Favert J (1998). Bull selection and use in northern Australia. 4. Semen traits. In Proceedings XX World Buiatrics Congress (Sydney), Volume 1. ppp407-412. Garner DL (1997). Ancillary tests of semen quality. In VetClinNAmer:Food Animal Practice. Vol13(2):313-330.
- Garner DL and Johnson LA (1995). Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biology of Reproduction 53:276-284.
- Gravance CG, Champion ZJ and Casey PJ (1998). Computer-assisted sperm head morphometry analysis (ASMA) of cryopreserved ram spermatozoa. Theriogenology 49:1219-1230.
- Lunstra DD and Coulter GH (1997). Relationship between scrotal infrared temperature patterns and natural-mating fertility in beef bulls. JAnimSci. 75:767-774.
- Pena AI, Quintela LA and Herradon PG (1998). Viability assessment of dog spermatozoa using flow cytometry. Theriogenology 50:1211-1220.
- Saacke, R.G., De Jarnette, J.M., Nebel, R.L. and Nadir, S.: Assessing Bull Fertility. Proc. Soc. Theriogenology AGM, San Diego, California. pp 56-69 (1991).
- Vogler, C.J.: Effects of Elevated Testicular Temperature on Viability of Cryopreserved Semen and Morphological Characteristics of Ejaculated Spermatozoa. M.S. Thesis, Virginia Polytechnic Inst., Blacksburg, Va. 1990.

Fresh
Frozen

**KANSAS STATE UNIVERSITY
COLLEGE OF VETERINARY MEDICINE
ANDROLOGY LABORATORY**

Case #.
Date.
Species.
Breed.
Age.

Owner: _____ Address _____ Ph _____

Submitted by: _____ Address _____ Ph _____

History/Exam Details:

(Attach copy of BSE form if available)

Ejaculate Details: Method of Collection - Massage, AV, EEJ, Other.

Vol: _____ Color: _____ Density: DDDD, DDD, DD, D Comments:

Gross Motility. VG, G, F, P, na Individual Motility (%).

Tests requested:

Fresh - stain, extender, "fixed"

Live/dead %
Morphology (stain)
Morphology (phase/DIC)
Sperm Concentration
PIA
Gram stain

Frozen

Post-thaw motility (15 min & 2h)
Live/dead %
Morphology (phase/DIC)
Sperm Concentration
PIA
Gram stain

Other (including fluorochromes, flow cytometry) - by request.

Results

% Live

Morphology Stain Phase DIC
Fluoro/Other

Normal
Head
Midpiece
Tail 1⁰
2⁰
Prox. Drop
Dist. Drop
Acrosome 1⁰
2⁰
Detached Hd
Other

Concentration:

RBC
WBC
Other

1⁰ Sperm Defects
2⁰ Sperm Defects

Other tests

Frozen Semen
% Live

Motility

15min 2h

Concentration:

Fluoro/Flow Cytometry
"live/dead"

Chromatin
Other

Other Tests

HOS

CASA (IVOS):

NOTES
