IVD Technology
For In Vitro Diagnostics Development and Manufacturing

# Developing and validating a lateral-flow cassette for fertility diagnostics in bulls

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An IVD detects the presence of a fertility-associated protein in bull semen to predict fertility.

The fertility of cattle is more important to profitability than any other factor. For example, in 2001, 38 million calves were born on approximately 800,000 ranches in the United States. With a profit margin of $100 per calf, a 1% increase in the birth rate would translate into an additional $38 million earned. Moreover, if each ranch weaned one additional calf, that would generate $80 million in added profit.[1] Since approximately 60–65% of cows bred in the United States produce a calf, a significant opportunity exists to raise fertility rates, which could improve efficiency and create additional beef on the market.

Prospective herd bulls can be qualified as satisfactory breeders by undergoing a breeding soundness evaluation. One component of this evaluation involves a semen analysis. However, while semen from different bulls may be identical in quality in terms of physical characteristics, the semen can vary widely in actual fertility whether mated naturally or through artificial insemination. In fact, a sterile bull may produce semen that appears acceptable based on a physical evaluation. Researchers have been working on developing biochemical markers in semen that can serve as diagnostic indicators for fertility potential.[2]


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**A diagnostic cassette manufactured by Midland Bioproducts (Boone, IA) provides chute-side detection of a seminal protein related to the fertility of bulls.**

## Determining Sperm Fertility

In order to acquire its fertilizing ability, sperm must first reside in the female reproductive tract for about 6–8 hours. This time requirement is called capacitation, which involves diluting sperm of inhibitory compounds in seminal plasma.[3,4] After capacitation, the fertilizing sperm undergoes an exocytotic event known as an acrosome reaction on the egg's surface *(zona pellucida)*. This acrosome reaction involves fusion of the sperm plasma membrane with the outer acrosomal membrane, which leads to a release of acrosomal contents, including enzymes that are utilized for binding, penetrating, and fertilizing the egg.

Developing procedures for in vitro fertilization (IVF) of bovine oocytes required the ability to mimic capacitation outside the reproductive tract. A biochemical analysis of female reproductive tract mucus confirmed the presence of heparin-like carbohydrates that cause capacitation of bull sperm in a dose-dependent manner.[5,6] Thereafter, heparin became the reagent commonly used

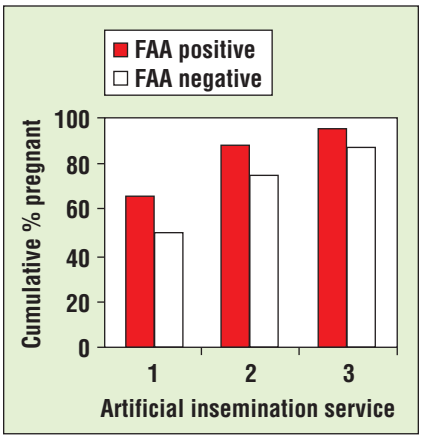


**Figure 1. Actual first service pregnancy outcomes from artificial insemination (AI) and projected cumulative pregnancies after second and third AI, expressed as a percentage, if fertility within bull remained constant. Adapted from Sprott et al.[21]**

to capacitate bull sperm used for IVF, a practice that remains in effect today.[7] In addition, sperm from higher- versus lower-fertility bulls was found to have more-frequent acrosome reactions in response to a particular dose of heparin-like material.[8–10] The fertility of a bull's sperm can be determined in vitro by evaluating the frequency of acrosome reactions that are caused by compounds chemically similar to female reproductive tract secretions.

## Heparin Binding to Surface-Associated Sperm Proteins

A number of kinetic studies have evaluated heparin-sperm interactions. For example, using a fluorescent-labeled preparation of heparin, microscopic examinations revealed binding across an entire sperm cell.[11] When radiolabeled heparin was utilized, binding to bull and monkey sperm was found to be saturable, specific, reversible, and dependent on temperature, pH, and buffer osmolarity.[11] Similar results were also observed with human sperm.[12]

In another study, when sperm from bulls of varying fertility were compared for their ability to bind with heparin-like sugars commonly found in the female reproductive tract, the binding affinities corresponded to fertility.[13] The lower dissociation constants, or higher association constants, were computed for sperm from higher-fertility bulls. Each ejaculated sperm cell contains approximately one million sites on which to bind with heparin, regardless of fertility. Based on binding affinities, at a given dose of radiolabeled heparin, greater binding of heparin to sperm will be observed in high- versus low-fertility bulls.

Sperm leaving the epididymis at ejaculation are devoid of heparin-binding proteins (HBP). These peptides are produced by the seminal vesicles, prostate, and Cowper's glands. They are also testosterone dependent and bind to sperm during ejaculation as sperm traverse the male reproductive tract.[14] Sperm that have been exposed to seminal plasma possess more binding sites for heparin than epididymal sperm and are able to undergo capacitation and *zona pellucida*–induced acrosome reactions.[15,16]

To investigate the role of HBPs in sperm, affinity chromatography was utilized to isolate those proteins from seminal plasma. Five different protein families were fractionated based on affinity elution characteristics. Adding each of those complexes at physiological concentrations to epididymal bull sperm rendered the sperm susceptible to the capacitating effects of heparin.[17] Furthermore, the fertility of range bulls corresponded to the presence of HBPs with the highest binding affinity detected on sperm.[18]

A monoclonal antibody that was produced against the family of HBPs with the greatest affinity for heparin targeted an epitope on three proteins that ranged in size from 21–31 kilodaltons (kDa). Western blot experiments effectively segregated the semen samples in terms of identifying those bulls with different surface-associated proteins on sperm cells. In particular, the presence of a 31-kDa HBP on sperm cells corresponded to higher fertility when cows were bred to bulls with that detectable sperm-associated antigen.[19] This protein is called fertility-associated antigen (FAA).[19]

Based on the above observations, the chemical identities of unique seminal proteins related to fertility in bull sperm have been sought. After heparin-affinity chromatography was used as an enrichment step, reverse-phase high-performance liquid chromatography was used to purify and obtain partial amino acid sequences of FAA. FAA is a nonglycosylated protein that is unique. GenBank searches using the N-terminal amino acid sequence and two internal amino acid peptides revealed that FAA shared a 90% identity with a deduced peptide sequence from a cDNA for a molecule similar to human DNase-I.[20]

## Relation of FAA Presence to Fertility

Since 1992, studies identifying bulls according to their fertility potential have utilized immunodetection of seminal proteins. In all breeding trials, bulls were subjected to a breeding soundness evaluation. The semen from those bulls that passed the evaluation were immediately frozen, and the samples were shipped to the University of Arizona (Tucson, AZ) where Western blot analyses were performed. Without a monospecific antibody, Western blots were routinely performed to sort the genotypes of bulls based on profiles of surface-associated sperm proteins recognized by the monoclonal antibody.[19]

This procedure involved extraction of the surface-associated proteins and conduction of one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE). The samples were electrotransferred onto nitrocellulose so FAA could be visualized utilizing the monoclonal antibody. By December 2003, more than 6000 samples from bulls around the world had been assayed, and the percentage of bulls categorized as FAA positive was 85%. This population represents bulls from progressive elite herds, including all major beef breeds, crossbreeds, and composite breeds. Across this population, the incidence of FAA-negative bulls ranged from 0 to 50% within a herd.

In field trials, the fertility of bulls was determined by a pregnancy diagnosis of cows that were artificially inseminated, or were allowed to comingle with bulls for natural mating in pastures where the

ratio of bulls to cows was 1:25.[19,21,22] After a 60-day breeding period, the bulls were removed; the cows' rectums were then palpated 60 days later to determine their pregnancy status. In the initial set of breeding trials, every cow had given birth to a calf when bulls were introduced into the pastures. Therefore, all cows were presumed to be fertile in order to avoid biased results from subfertile or infertile females.

To date, more than 10,000 cows have been bred to bulls categorized as either FAA positive or negative. While bulls with sperm lacking FAA were still fertile, they were 17% lower in fertility compared with their herdmates classified as FAA positive. Approximately two dozen samples from known sterile bulls have been assayed and have tested both positive and negative for FAA. However, twice as many FAA-negative samples resulted in that subpopulation of sterile bulls. Thus, FAA status is not an indicator of sterility, but rather a discriminator for high or low fertility potential overall. Obviously, other components in semen interact with FAA that ultimately affect sperm function and corresponding fertility. Nonetheless, every year in every replicate pasture, the pregnancy rates were higher in cows bred to FAA-positive bulls.

Not only are FAA-positive bulls more fertile than their FAA-negative herdmates, they are also more efficient in getting cows pregnant sooner. For example, when bulls were subcategorized on the basis of sperm-associated FAA and libido (or serving capacity), the high serving capacity bulls that were FAA positive yielded more pregnancies in cows during the first 21 days of the estrous cycle than FAA-negative bulls.[19] With artificial insemination, the bull's libido is not a factor because cows are inseminated when in estrus. In that scenario, semen from FAA-positive bulls still led to 16% more pregnancies than semen from FAA-negative bulls after a single insemination (see Figure 1).[21]

In addition, when the daughters of cows originally bred to FAA-positive bulls were also bred to FAA-positive bulls, they gave birth to 18% more calves during the first 21 days of the calving season.[23] In other words, these pregnancies occurred with a higher incidence in the first 21 days of the breeding season nine months earlier. That outcome supports a genetic relationship between FAA and fertility, which could be expected since FAA is a unique protein that is encoded by a unique gene. The results from the selection experiment also reinforce the findings cited from the serving capacity and artificial insemination field trials.[19,21] Overall, FAA-positive bulls would be expected to get more cows pregnant at their first opportunity for breeding compared with FAA-negative bulls.

## On-Site Fertility Diagnostics

Diagnostic identification of the 31-kDa FAA in the above studies required SDS-PAGE and Western blotting to identify the individual sperm proteins by molecular weight. However, that technology is burdensome, time-consuming, and laborious. It also requires specialized equipment to detect the presence of FAA and is not applicable for widespread commercial use. Moreover, presumably due to its extreme hydrophobic nature, neither purified native FAA nor synthetic peptides of specific FAA sequences could elicit an immune response when attempting to generate a new panel of monospecific monoclonal antibodies.

At the same time, parallel studies centered on sequencing the gene and producing recombinant FAA isotypes. One recombinant form that spanned amino acid 73-269 was much less hydrophobic than the native FAA protein. This recombinant fragment was expressed in *E. coli* and used to generate rabbit polyclonal antisera. This recombinant FAA was also immunogenic, and the antisera were monospecific for FAA (see Figure 2a). In addition, immunolocalization

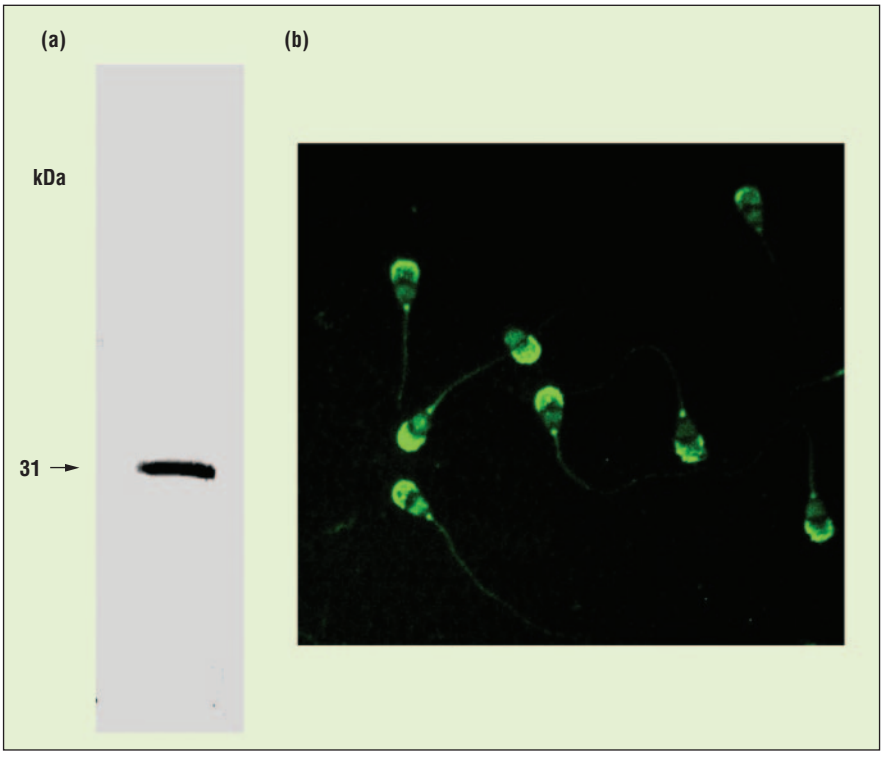


**Figure 2. Characterization of anti-FAA polyclonal antisera. a) Bovine sperm proteins were separated by SDS-PAGE, transferred to nitrocellulose and Western blots were probed with rabbit polyclonal antisera produced against recombinant FAA. The product recognized is a 31-kDa sperm protein, identical to the expected molecular mass of FAA. b) Indirect immunofluorescence was performed on bovine sperm using anti-FAA antisera and FITC-conjugated goat anti-rabbit secondary antibody. Results demonstrated specific localization of FAA to the acrosome of bull sperm. Preimmune sera used as control displayed no immunoreactivity (data not shown).**

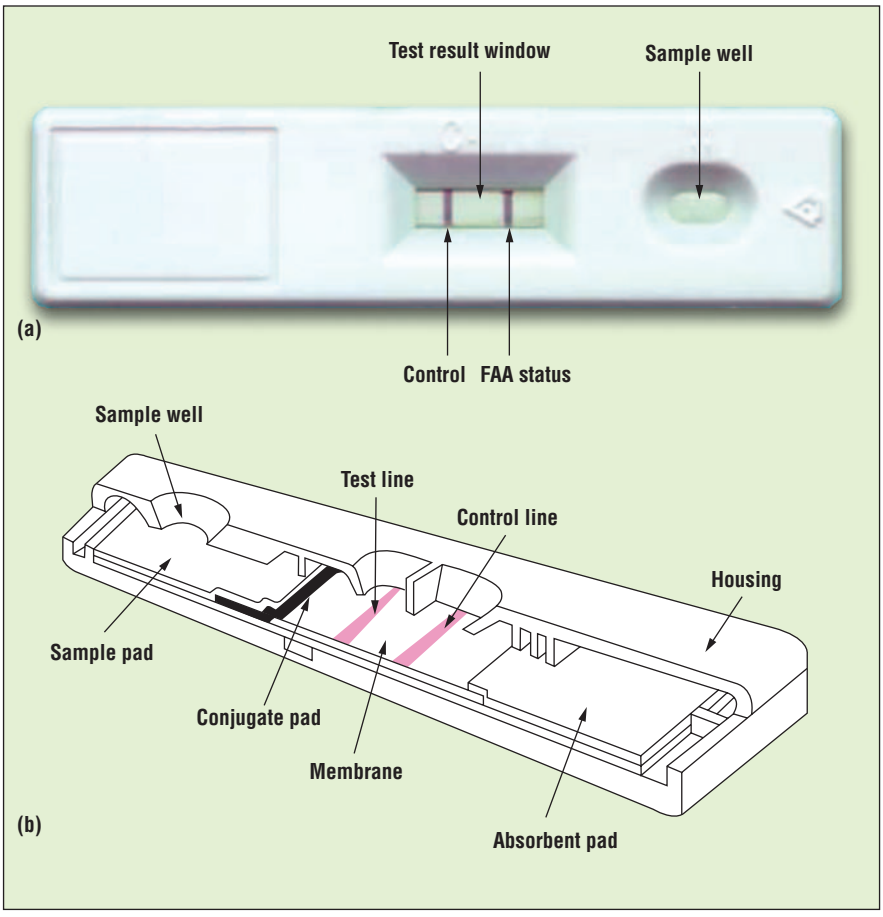


**Figure 3. Lateral-flow cassette for detection of fertility-associated antigen (FAA) in bull semen developed by Midland BioProducts (Boone, IA). a) Picture of a lateral-flow cassette displaying the colorimetric development of control and test lines. b) Schematic showing the internal design present within the housing of the cassette. The sample migrates by capillary action over the conjugate pad before reaching the test and control lines, respectively.**

performed with the antisera revealed specific binding of FAA to the acrosomal region on the head of bull sperm (see Figure 2b).

After biochemical characterization, the antisera was immobilized on a suitable support matrix and incorporated in a rapid immunodetection lateral-flow cassette to allow on-site fertility analysis. The detection sensitivity was determined to be 20 ng/ml FAA. With Western blots, sperm proteins were extracted to ensure that sperm-associated FAA was analyzed. That approach is not feasible with a lateral-flow cassette because sample preparation considerations would discourage users. The lateral-flow cassette measures FAA in whole semen, making comparisons with previous Western blot data irrelevant.

Midland Bioproducts (Boone, IA) manufactured such a device during spring 2003 (see Figure 3). With this device, a sample of diluted semen is exposed to the labeled anti-FAA antibody. The sample and labeled antibody conjugate migrate along the membrane via capillary action and are exposed to immobilized anti-FAA antibody at a test position. The presence of FAA in the sample is evidenced by binding of the FAA and labeled antibody to the immobilized antibody, resulting in colorimetric visualization of the label at the test position. A control reagent that serves as a substrate for unbound conjugate is applied to the membrane at the control position spaced downstream from the test position. Visualization of the control band verifies that the cassette performed correctly, regardless of the presence or absence of FAA in the sample.

In order to validate this technology, in 2003 the lateral-flow cassette was used to assay the FAA status of 914 bulls in 18 commercial herds across the Midwest and Southwest of the United States. Once the semen samples were collected and diluted in buffer, 100 µl of the diluted samples were applied to the sample well of the cassette. Within approximately five minutes, the results were determined based on colorimetric development of the test and control bands. Overall, across a random and diverse subpopulation of purebred and composite bulls, 26% of the animals scored negative for FAA. The difference between the incidence of FAA-negative bulls assayed by Western blot versus lateral-flow cassette may be due to a number of reasons, including different extraction methods and detection sensitivity.

Another fertility trial was also conducted to validate further the utility of the lateral-flow cassettes. A cooperative project involving researchers at California State University, Chico (Chico, CA), tested 62 bulls that were bred to cows for 60 days during two consecutive breeding seasons in 2001 and 2002. Similar to earlier field trials, the ratio of bulls to cows was 1:25. Every calf born had a blood sample drawn, and DNA genotyping verified the sire and dam of each animal. Semen samples from each bull were collected and frozen when the breeding soundness evaluations were conducted. These samples were thawed and then assayed using the lateral-flow cassettes.

After testing these samples, 81% of the bulls were classified as FAA positive. These results fall between those previously summarized for Western blots (85%) and the 18 herds screened in 2003 (74%). In this fertility trial at Chico, the FAA-positive bulls sired 75% more calves than their FAA-negative cohorts, or 17.5 calves per 25 cows versus only 10 calves per 25 cows.

A particularly significant observation from this study was that a high proportion of bulls that produced FAA-negative semen in an individual herd could be traced back genetically to a common sire or small group of sires.

## Other FAA Issues

A genetic component governing FAA status is apparent and should be exploited as the FAA gene is studied, sequenced, and annotated for mutations that may hold diagnostic value. For example, one mutation has been identified near the carboxy terminus of FAA in bulls that tested negative for FAA by Western blots. This observation implies that the epitope resides in that region of the protein. Structural prediction software illustrates the mutation results in a different folding of the carboxy terminus. Therefore, FAA-negative bulls may produce an aberrant form of the protein that interacts differently, or not at all, with sperm, resulting in the observed lower fertility rates.

The lateral-flow cassette may also screen semen from other male species besides bulls. Positive results have been obtained with boar, ram, dog, and human semen. However, FAA has not been detected in stallion semen, and it cannot be immunolocalized on stallion sperm using the polyclonal antisera. Future research will focus on obtaining fertility data in those other species, paralleling the approach that was described with bulls.

Finally, a logical question is whether fertility can be improved if FAA is used to fortify semen. Studies on this matter are ongoing. Preliminary findings with a recombinant FAA point to stabilization of acrosomal membranes if FAA is added prior to freezing bull semen in a commercial extender. Since the percentage of intact acrosomes three hours postthaw has a 92% correlation with the fertility of semen used for artificial insemination, FAA may hold promise as a therapeutic product besides being an important diagnostic indicator of fertility potential.

## Conclusion

The criteria to accurately select bulls exhibiting the highest fertility have been poorly defined. An objective of the IVD industry has been to find proteins that either alone or in combination are adequate screening elements to identify bulls likely to be higher in fertility. A critical need also exists to develop new methods and tools to predict bulls with enhanced fertility from large populations of suitable livestock candidates.

A reliable and reproducible method of predicting the fertility of bulls relates to the detection of FAA. Rapid immunodetection of FAA in semen is now possible with the recent development of an FAA-specific lateral-flow cassette applicable to on-site fertility testing. This lateral-flow device is capable of replacing cumbersome Western blot procedures to identify higher-fertility bulls and may be applicable across other mammalian species.

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