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LABEL LICENSE: The purchaser of this product is hereby licensed to practice U.S. Patent No. 5,897,988 only with respect to the process for determining the proportion of mature sperm in a sperm sample. The purchaser is not authorized to use the product to practice the process for selecting mature sperm by removing bound sperm from the periphery of the substrate. This license limits the purchaser to single use of each of the assay chambers of a slide in practicing the licensed process. Those who wish to obtain a separate license to practice the process of determining the proportion of mature sperm in a sperm sample under U.S. Patent No. 5,897,988, without purchasing this product, should contact Biocoat, Inc., 211 Witmer Road, Horsham, PA 19044. Patents: US Patents 5,897,988: 5,349,436; 5,037,677: 5,023,114: 4,801,475: RE 35,589 European Patent No. 1056336 DBP/69924472.2

Instructions for Use:
HBA®
Sperm-Hyaluronan Binding Assay

Proprietary and Established Names:
HBA® Sperm-Hyaluronan Binding Assay

Indications for Use:
The HBA® Sperm-Hyaluronan Binding Assay is indicated for use:
1. As a component of the standard analysis of semen in the diagnosis of suspected male infertility.
2. As a component of analyses for determining the proper course of IVF treatment of infertility.

Intended Use:
The HBA Assay is a qualitative assay for the maturity of sperm in a fresh semen sample. It is intended to provide additional information to professionals for the evaluation of an infertile couple. It is not intended to be a single diagnostic indicator of potential fertility. The assay is based on the ability of mature, but not immature, sperm to bind hyaluronan, the main mucopolysaccharide of the *cumulus oophorus* matrix and a component of human follicular fluid.

Summary:
In natural fertilization mature sperm bind to hyaluronan, the main component of the *cumulus oophorus* matrix; immature sperm do not bind (3, 9, 13, 14, 19, 21).

Mature sperm also bind to hyaluronan chemically attached to a support (9, 10) such as the hyaluronan-coated glass slides that make up the HBA® kit. Viewed in the microscope, bound sperm are differentiated from unbound sperm by their beating tails with heads that make no progressive movement.

Sperm unable to bind hyaluronan share many aspects of immaturity: they retain cytoplasm and histones (9), they show a higher frequency of aberrant morphology (18, 22, 23) and they have a lower genomic integrity than hyaluronan binders – hyaluronan binders have many fewer DNA single strand breaks than non-binders (24) and a 4-to-6 fold lower frequency of chromosomal aneuploidies (10, 11). Sperm motility is stimulated upon binding hyaluronan (6, 7, 14). Thus, the ability to bind hyaluronan designates a mature sperm and the proportion of mature sperm indicates the maturity of sperm in a semen sample.

The HBA® Slide:
The HBA® slide contains two identical assay chambers. Each chamber has a thin layer of hyaluronan bound to it. With cover slip in place, the chamber depth is uniform and will be between 14-24 microns. By counting the numbers of motile bound and unbound sperm in a common area, the proportion of binders in the motile sperm population can be calculated.

The Physiological Basis for the Assay:
Hyaluronan binding indicates the successful completion of spermiogenic events including: meiotic segregation of one complete haploid chromosomal complement; stabilization of the chromosomes by substitution of protamines for histones; repair of DNA damage; extrusion of cytoplasm; synthesis of midpiece, tail and acrosome; and remodeling of the plasma membrane to incorporate hyaluronan and zona binding receptors. The success of these events is directly linked to the timing and expression of a critical chaperone protein, HspA2, during spermiogenesis (2, 8). The chaperone is a component of the synaptonemal complex (1) and like other chaperones in the Hsp70 family, it fosters intracellular transport and membrane remodeling. In the absence of the chaperone, deficits in sperm maturation may occur, including deficits in the critical functions listed above and in hyaluronan binding.

Specimen Collection and Preparation:
Semen should be obtained by masturbation, preferably following 2-3 days of abstinence. It should be collected in a clean, dry container and kept at room temperature (20-28ºC) for 30 minutes to liquefy. A specimen is considered liquefied if it can be drawn into a pipette tip and discharged cleanly, i.e., without lumps blocking the tip. If the specimen cannot be cleanly pipetted, wait an additional 30 minutes and test it again. If it still appears non-liquefied, dilute an aliquot of semen with an equal volume of a sperm dilution medium such as human tubal fluid (HTF), and mix by drawing the mixture up and down in the pipette tip. This will produce a specimen that can be applied to the HBA® slide.

Perform the assay on the semen specimen within 3 hours of collection.

Warnings and Precautions:
1. *Semen is a hazardous fluid and must be handled and disposed of using normal precautions for infectious, biohazardous materials. In particular, wear gloves, eye protection and a laboratory coat. Disinfect all semen waste and dispose of it in a properly labeled biohazard container.*
2. *Do not use HBA® slides for selecting sperm for ICSI or IVF! The slides are not sterile, they contain endotoxins and may be embryotoxic. Sperm selection on HBA® slides is prohibited by the License.*

Storage:
HBA® slides should be stored in a dry environment in its original packaging or in a clean, dry, dust-free environment at room temperature (20-28°C).

Performing the Assay:
Materials Provided:
HBA® slides
Cell-Vu® gridded cover slips
Product Instructions for Use

Required Materials not Provided:
Phase contrast microscope with at least 400x magnification
Disposable, sterile capped test tubes
Mechanical counting device
Human tubal fluid (HTF, Irvine Scientific, Santa Ana, CA) or other acceptable sperm diluent containing >0.50 mg/mL protein
Disposable specimen handling gloves
Timer/clock
Pipette to deliver 2-10 µL, disposable pipette tips

Useful Accessories Not Provided:
Microscope reticle delineating area
Toluidine Blue O (Aldrich Chemical Co.)
Urea (Aldrich Chemical Co.)

Step-by-step Procedure:
1. Perform the assay at 20-30° C. Do not exceed 30° C.

2. Load an assay chamber with the semen sample.

   Immediately before use, mix the sample and pipette a drop, 7-10 µL in volume, onto the center of the assay chamber.

3. Immediately install the Cell-Vu® gridded cover slip: avoid entrapping air bubbles.

   The cover slip provides a grid of 100 squares, 0.1 x 0.1 mm, within a viewing circle for counting sperm. Install the cover slip, with the name "Cell-Vu®" and the viewing circle facing upward, in the following manner to avoid air bubbles: first, contact one edge of the cover slip to the edge of the chamber, forming an angle like two arms of a hinge (Figure 1); second, slowly lower the opposite edge of the cover slip onto the slide, closing the hinge – when the cover slip is quite close to the slide, it will contact the sample drop (Figure 2); continue to slowly lower the cover slip without releasing it, observing the droplet of semen as it is pushed down by the cover slip and spreads out into the assay chamber circle; next, when the assay chamber is almost full with sample and the viewing circle is completely covered with sample, the cover slip may be released to cover the assay chamber (Figure 3); finally, center the viewing circle over the assay chamber and gently press the edges of the cover slip to seat it.

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Figure 1
Contact cover slip to the slide:

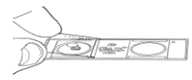

Figure 2
Contact cover slip to the sample:

Figure 3
Cover slip in place:

4. Incubate the chamber for at least 10 and not more than 20 minutes. Count the unbound, motile sperm and the bound, motile sperm in the same number of grid squares.

   A minimum of 10 minutes is needed for all of the sperm to contact and bind to the immobilized hyaluronan layer. After 20 minutes weak motile sperm may begin to lose motility.

   The indication that a motile sperm has bound to hyaluronan is a cessation of progressive movement of the sperm head accompanied by active tail movement. Bound, motile sperm are differentiated from non-motile sperm which show no tail movement. Motile sperm unable to bind to hyaluronan behave as freely swimming sperm.

   Good assay precision will be achieved when the count of bound plus unbound motile sperm is between 100-200. A quick visual inspection of the chamber will show whether the bound or the unbound motile sperm predominate. Count the predominant class (bound or unbound) of motile sperm until you have counted at least 100 sperm or 100 grid squares. Immediately, repeat the count in exactly the same number of grid squares, counting the other class of motile sperm.

   It will not always be possible to find 100 motile sperm in a semen sample, particularly in oligospermic samples.

5. Calculate the percent hyaluronan-binding sperm.

   The percent of sperm binding to the hyaluronan layer is calculated:

$$\%\ Bound = 100\ X\ \frac{Bound\ Motile\ Sperm}{Bound\ Motile\ Sperm + Unbound\ Motile\ Sperm}$$

   Example 1:
   A count of 115 bound, motile sperm was obtained by counting 22 grid squares. Immediately after, a count of 9 unbound, motile sperm was obtained from the same 22 grid squares.

$$93\ \%\ Bound = 100\ X\ \frac{115\ Bound\ Motile\ Sperm}{115\ Bound\ Motile\ Sperm + 9\ Unbound\ Motile\ Sperm}$$

   Example 2:
   In a different sample, a count of 89 unbound, motile sperm was obtained by counting 100 grid squares. Immediately after, a count of only 27 bound, motile sperm was obtained from all 100 grid squares.

$$23\ \%\ Bound = 100\ X\ \frac{27\ Bound\ Motile\ Sperm}{27\ Bound\ Motile\ Sperm + 89\ Unbound\ Motile\ Sperm}$$

Precision of the Assay:
The precision of the assay is related to the numbers of sperm counted. When the total count of bound and unbound motile sperm is between 100-200, the coefficient of variation (CV) of the result is typically about 5%. Higher variances result when fewer sperm are counted. Results are acceptable when at least 30 total motile sperm are counted.

In a study of variability within and among three production lots of slides, single semen specimens were assayed in replicates of assay slides, all within 3 hours of semen collection. Three different individuals read the slides. Table 1 shows the results for a normal semen specimen; Table 2 shows results for an abnormal, oligospermic specimen.

Table 1

Inter- and Intra-lot Precision with a Normal Specimen						
HBA® slide lot	Number of Replicates	Mean Percent Bound	Std. Dev.	CV	Mean Count* Bound	Mean Count* Unbound
A	20	86.9	3.9	4.5	164	23.6
B	10	85.5	4.5	5.3	182	29.9
C	10	85.0	4.1	4.8	160	27.2

*On average, the sperm in nine grid squares were counted.

Table 2

Inter- and Intra-lot Precision with an Abnormal, Oligospermic Specimen						
HBA® slide lot	Number of Replicates	Mean Percent Bound	Std. Dev.	CV	Mean Count▲ Bound	Mean Count* Unbound
A	20	59.0	10.9	18	21.5	14.4
B	10	55.8	19.0	34	20.1	16.0
C	10	58.4	10.1	17	23.1	16.3

▲The sperm in all 100 grid squares were counted.

Quality Control:
Fresh mature semen can be used to demonstrate the sperm-hyaluronan binding reaction; however, fresh semen controls are not commercially available. Users are encouraged to establish their own sources of fresh control semen and to develop an associated history of normal and abnormal HBA® scores.

The sperm-hyaluronan binding reaction is not altered by freezing and thawing of sperm (16, 20, 25). Similarly, sperm processed either by swim up or by gradient preparation bind normally to hyaluronan.

The presence of the hyaluronan layer can be confirmed by flooding the assay chamber for a minute with a solution of 0.10% Toluidine Blue O in 8 *M* urea then rinsing with water. A purple stain denotes the presence of hyaluronan (12). Do not use the stained chamber for assay; the dye inhibits sperm motility.

Expected Results:
The percent of hyaluronan-bound sperm ranges from essentially zero to essentially 100%. In a study of 157 semen samples from patients presenting for diagnosis of suspected infertility, the upper half of the samples showed an average HBA® score of 93 ± 2.6% binding. The lower half had HBA® scores from 88% to zero binding, the average was 70 ± 18% binding. Approximately 1% of these samples showed no detectable binding to hyaluronan.

HBA® scores on prepared sperm may differ from those of raw semen.

Limitations and Troubleshooting:
Use only fresh semen, less than three hours old.

The sample should permit at least 30 motile sperm to be counted per 100 grid squares. If there are too few motile sperm to reach this number, replicate assays should be conducted or a microscope reticle should be installed to permit inspection of an area large enough to count a minimum of 30 motile (bound plus unbound) sperm.

If all the sperm in a sample appear either unbound or immotile, check that the sample has been applied to the coated side of the slide. When the coated side is up, the label, "Cell-Vu®/HYDAK HBA®" can be clearly read. Otherwise, the label appears upside down and/or backwards. Application of sperm and cover slip to the uncoated side of the slide will give the appearance of no sperm binding. If the cover slip has been pressed down hard, it can cause the sperm to appear immotile. **Whenever no binding is observed, repeat the assay on the sample and confirm the result on a different slide.**

Install the cover slip so that the Cell-Vu® trademark can be clearly read. When installed upside down, the counting grid will be out of the plane of focus of the sperm sample. Defective cover slips occur rarely, such that the grid is etched on the top of the slip instead of in contact with the sample. When this occurs, repeat the assay with a new cover slip. Contact your distributor for replacements.

If an air bubble blocks counting within the cover slip counting grid, the assay must be repeated using a fresh chamber and cover slip.

Interpretation and Significance of the Results:
The HBA® assay is based on the concept that a low level of sperm binding to hyaluronan demonstrates a low proportion of mature sperm in the semen sample and therefore predicts infertility. Hyaluronan-binding sperm, in contrast, are competent in the interaction with the oocyte complex and hyaluronan binding is also associated with high genomic integrity (11, 14, 21, 24), which improves the quality of the paternal contribution to the zygote. Thus, hyaluronan-binding differentiates high and low functional integrity and fertilizing potential.

In the prediction of fertility, it is not necessary that all sperm should be capable of binding hyaluronan, but the proportion of hyaluronan binders must reach an effective level in order to achieve a good likelihood of fertility. Binding above the effective level probably does not further improve fertility. Based on correlations with normal sperm morphology, the Hyaluronan Binding Assay level differentiating higher from lower expectation of fertility is estimated to be approximately 80%.

Table 3

HBA® Score Results Interpretation	
HBA® Score (% binding)	Interpretation
≥80 % binding	Normal maturity and physiological function
<80 % binding	Diminished maturity and physiological function

Performance Characteristics:
A study comparing HBA® score results to normal sperm morphology (strict criteria) was carried out with semen samples from three IVF or andrology laboratories in three states (CT, CA and PA), with patients presenting for diagnosis of suspected infertility. At least 50 samples were obtained from each site and the HBA® assay performed on site according to the Instructions for Use. Morphology slides were prepared on site and sent to a central location where they were scored by a single reader.

The data were analyzed by a two-way contingency table using morphology (classified as subfertile if <5% normal morphology, otherwise fertile (4, 5, 15, 17)) and HBA® score (classified as subfertile if <80% binding, otherwise fertile). Significance was tested with the Pearson Chi-squared statistic using SPSS for Windows software. The analysis showed that HBA® score was significantly related to morphology for data from the combined data (all three sites), Table 4.

The combined data showed good specificity and positive predictive values, although sensitivity was only 40%. Therefore, at a cutoff of <80% binding, the HBA® assay detects less than half of semen samples with truly low morphology, but among those detected as low HBA® score there is a strong prediction (>80%) of poor morphology.

Table 4

Significance of HBA® Score to Morphology					
Study Site	N	Sensitivity	Specificity	Positive Predictive Value	p =
PA	50	43.2	76.9	84.2	0.198 (NS)
PA-r◊	50	51.3	90.9	95.2	0.012
CA	52	54.2	78.6	68.4	0.015
CT	55	26.5	100	100	0.01
Combined	157	40.0	85.5	80.9	0.001

◊Site data recalculated with cutoffs of ≤ 5% for morphology and ≤ 80% for HBA®.

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