Quantitative semen analysis using fluorometric lateral-flow assay system for male fertility diagnosis

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Article

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Abstract

About one-third of infertility cases are attributed to male lifestyle-associated factors such as smoking, drug abuse, obesity, and psychological stress. These factors have been suggested as potential contributors to male infertility. High-quality semen is essential for successful fertility, and poor semen quality is a significant hurdle in achieving this outcome. Therefore, it is necessary to evaluate the concentration and quality of semen on-site without visiting a hospital. In this study, we developed two analytical systems using a fluorescence lateral-flow sperm (FLF-sperm) assay for the rapid and quantitative evaluation of sperm concentrations and quality. The sperm-specific protein (SP-10) was chosen as a target analyte to determine sperm counts. An immunostrip was fabricated to obtain quantitative fluorescence signals in a sandwich format. The SP-10 assay provides highly sensitive detection with a detection limit of 1.28 x 10^6/mL and produces a reactive signal proportional to the sperm concentration in clinical semen samples. Additionally, we established a sensing system using enzymatic cleavage to detect sperm quality based on hyaluronidase (HAdase) activity values, covering sperm concentrations from 1.8 x 10^6/mL to 140 x 10^6/mL. These quantitative sperm analysis systems allow users to easily interpret sperm counts and quality on-site for diagnosing male infertility and monitoring treatment progress.

Introduction

Infertility is when a couple, despite having a healthy relationship and not using contraception for a year, struggles to conceive. Male infertility, linked to various factors like lifestyle, misdiagnosis, and delayed treatment, is on the rise[1–3]. According to a Health Insurance Review & Assessment Service survey, infertility cases in Korea surged from 61,903 in 2017 to 86,582 in 2022, marking a 39.97% increase in new cases. About 30–50% of infertility cases are attributed to male factors, and the global prevalence of male infertility has risen to 12%, affecting an estimated 30 million individuals[4].

To conduct fertility diagnostics, semen samples are collected at a medical facility after abstaining for 2–7 days[5]. Following WHO guidelines, samples undergo analysis for volume, sperm count, motility, and shape after liquefaction at room temperature[6]. The normal semen should have at least 16 million sperm per mL (WHO standard value) for a high chance of pregnancy[7]. However, these tests are time-consuming, require careful preparation, and are exclusive to hospitals. Social and cultural stigma can hinder smooth testing, potentially leading to false-positive male infertility results. Consequently, swift on-site detection has the potential to initiate appropriate treatment, improving sperm quality, and augmenting the likelihood of successful conception.

The computer-assisted semen analysis (CASA) test, done in hospitals, analyzes sperm motility and shape, measuring unique motion indexes[8]. However, it requires expensive specialized equipment and a mandatory hospital visit [9]. Commercially available semen test kits, like SpermCheck® and SwimCount®, focus on sperm count and motile sperm count. SpermCheck® measures only sperm counts based on

the semen signal, while SwimCount® detects live sperm counts and motility through color depth, both being time-consuming[10–12]. Specialized tests like Fertell® assess sperm motility by measuring swimming potential through the intestinal wall, simulating a woman's cervix. It differentiates between normal and abnormal results but takes an hour for measurement[13]. Tenga® Men's Loupe and smartphone-based CASA system (SEEM®) observe real-time sperm count and motility using a magnifying lens attached to a smartphone camera[14,15]. These conventional diagnosis methods have the major limiting factor in the timely, accurate, and rapid detection of male infertility. Additionally, they predominantly focus on a single cause, posing challenges in diagnosing male infertility arising from various factors[16–18]. To overcome the limitations of existing analytical methods, this study aimed to provide an easily accessible solution for men to self-diagnose male infertility at home. Furthermore, we sought to establish a dual sperm analysis system by not only evaluating sperm count but also measuring sperm enzyme activity. Unlike the previous detection system of SP-10, which only allowed sperm count analysis, our study demonstrates that through the dual test, a more accurate assessment of sperm quality can be achieved.

We focused on overcoming the limitations of existing methods by creating new FLF-sperm systems for on-site, quantitative assessment of semen counts and quality. Designed to detect sperm concentrations, the SP-10 analysis system offers highly sensitive detection and correlates with the number of sperm measured by commercial instruments. Hyaluronidase (HAdase), found in mature sperm, is crucial for egg penetration during fertilization[19,20]. To accurately measure sperm quality, the HAdase activity was detected using a two-step analysis format with a HA-biotin complex. This approach helps identify causes of male infertility by establishing a cut-off value for HAdase activity in cases of normospermia. These FLF-sperm systems are user-friendly, cost-effective tools for diagnosing male infertility and monitoring treatment progress.

Materials and Methods

Reagents and materials. Monoclonal antibodies to SP-10 (clones MM01 and MM06) were obtained from Sino Biologicals (Beijing, China). Hyaluronic acid was purchased from Creative PEG Works (NC, USA). Goat anti-mouse IgG and streptavidin were from Arista Biologicals Inc. (Allentown, USA) and Prospec-Tany Technogene Ltd. (Israel), respectively. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), NHS (N-hydroxysuccinimide), Alexa Fluor™ 647 NHS and Zeba spin desalting column (MWCO 7K) were obtained from Thermo Fisher Scientific Korea Ltd. Biotin-dPEG4 -hydrazide, HAdase from bovine testes, boric acid, Tween 20, bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were used from Sigma-Aldrich Korea Ltd. Glass fiber for conjugate supply and polyester were supplied by Ahlstrom (Helsinki, Finland). Absorbent pad (17 CHR chromatography grade) and nitrocellulose membrane (90CNPH-N-SS40) were from Whatman (Maidstone, UK) and MDI Membrane Technologies (Ambala Cantt, India), respectively. All reagents used were of analytical grade. Clinical semen samples approved by the Institutional Review Board (IRB) at CHA College of Medicine were used (IRB No. GCI

2019-12-057-002). The informed consent was obtained from all subjects and/or their legal guardian(s). All methods were carried out in accordance with relevant guidelines and regulations.

Labeling with Alexa Flour 647. Alexa Fluor™ 647 NHS was used to prepare the fluorescent conjugates of a SP-10 antibody and a streptavidin. The SP-10 antibody and streptavidin were prepared at concentrations of 1 mg/mL and 5 mg/mL, respectively, and bound with the Alexa Fluor™ 647 NHS in a molar ratio of 20 times. The residual molecules were removed using a Zeba™ spin gel filtration column and stored at 4°C for further use.

Biotinylation of Hyaluronic acid. Hyaluronic acid (HA) was diluted to a concentration of 2.5 mg/mL in 50 mM boric acid (pH 5.3). The HA was conjugated with biotin in a 1:100 molar ratio using a cross-linkage of EDC and NHS. The mixture of EDC and NHS was allowed to react for 1 hour in a ratio of 1:0.5, then applied to HA-biotin reactions for 4 hours. All reactions were carried out at room temperature. The unreacted remaining molecules were removed using a Zeba™ spin gel filtration column and stored at 4°C for further use.

Immunostrip fabrication for detecting sperm protein-10. The SP-10 assay system consists of a sample pad, a conjugate pad, a nitrocellulose membrane, and an absorbent pad (Fig. 1A). The sample pad was 4 mm × 12 mm in size and glass fiber membrane grade 8964 was used. The detection antibody, conjugated with fluorescent tracer, was prepared in a concentration of 50 ug/mL in PB containing 1% BSA, and then 5 µL per strip was immersed on the conjugate pad. In the test and control sections of the nitrocellulose membrane (4 mm × 25 mm), sperm protein-10 (SP-10) antibody and goat anti-mouse IgG were dispensed with 0.5% sucrose at 0.3 mg/mL and 0.5 mg, respectively, dried at 37°C. The absorbent pad, made of 17 CHR, measured 4 mm × 15 mm. The immune strip was assembled, with each prepared component overlapping by about 1 mm, using a backing card.

Analytical procedure of sperm protein-10 measurement. In order to measure the amount of SP-10 present in semen, the experiment was conducted as follows. The clinical semen samples with azoospermia, oligospermia, and normospermia according to WHO standards were used. The samples were diluted with 0.5% casein-PB (pH 7.4) containing 1% Tween-20. We then dispensed 100 µL of each sample into microwells and immersed the assembled strip's sample pad for 15 minutes. The fluorescent signals induced in the sandwich format on the test part of the nitrocellulose membrane were measured using a fluorescence reader. The numerical signal was graphed and analyzed.

Strip fabrication for measuring Hyaluronidase activity. The HAdase activity measurement strip was created by assembling various components, including the buffer pad, conjugate pad, sample pad, nitrocellulose membrane, and absorbent pad (Fig. 1B). We used a glass fiber membrane grade 8964, measuring 4 mm × 6 mm, to provide extra buffer at the bottom. The streptavidin conjugated with fluorescent tracer, was prepared in a concentration of 10 ug/mL in PB containing 1% BSA, and then 2.5 µL per strip was immersed on the conjugate pad. The sample pad, also made of glass fiber membrane grade 8964, was prepared with dimensions of 4 mm × 12 mm nearest to the nitrocellulose membrane. The nitrocellulose membrane's test and control regions (4 mm × 25 mm) were dotted after diluting HA-

biotin and BSA-biotin to 0.1 mg/mL and 0.25 mg/mL in PBS containing 0.5% sucrose, respectively. After dispensing 0.5 μL, the strip was used following drying at 37°C. The absorbent pad (17 CHR) was fashioned in a size of 4 mm × 15 mm. To assemble the strip, each prepared component was overlapped by about 1 mm using a backing card.

Analytical procedure of Hyaluronidase activity detection. To assess the analytical performance of the FLF-sperm system for HAdase activity, we prepared samples representing azoospermia, oligospermia, and normospermia by diluting them with a 1% BSA-sodium acetate solution (pH 5.2) containing 1% Tween20. The pretreated samples (30 μL) were applied to the sample pad, where HAdase moved through the membrane via capillary action, eliminating the biotin molecules through enzyme reactions with the HA-biotin on the membrane surface. The streptavidin labeled with fluorophores (Alexa-SA) was introduced in the second flow from the buffer pad, consisting of a 1% BSA-PB solution (pH 7.4) with 1% Tween20, for 15 minutes. The Alexa-SA tracer facilitated binding reactions with any remaining HA-biotin. We detected reference signals from the control section where BSA-biotin polymer was immobilized, simultaneously with signals from the test area. The fluorescent intensity was then measured using a fluorescence reader, and the numerical signal was graphed and analyzed[21]

Results and discussion

Analytical concept for detecting concentrations and quality of male sperm. In the realm of biosensors specializing in male fertility assessment, a standard semen analysis conventionally examines sperm count, motility, and morphology[22]. Table S1 lists commercialized sperm analysis products to compare their sensing performance, including accuracy and dynamic range, for measuring sperm concentrations. These pivotal parameters provide valuable clinical insights for individuals dealing with infertility. Nevertheless, the direct detection of these parameters for home-based infertility diagnosis poses a challenge. Addressing this, a streamlined and rapid lateral-flow assay (LFA) system emerges as a solution to quantify semen biomarkers. This system facilitates user-friendly, cost-effective self-diagnostics, supplying information on sperm concentration and quality. This, in turn, aids medical professionals in making informed decisions regarding fertility treatments at the early stages of infertility.

Our approach involves the fabrication of fluorometric lateral-flow systems, employing both sandwich and depolymerizing techniques for the quantitative analysis of sperm-specific protein (SP-10) and hyaluronidase (HAdase) activity, recognized as markers for evaluating sperm quality, in clinical semen samples. Leveraging the sensitivity and versatility of fluorescence as a signal tracer in membrane-based assays, we achieve precise measurements of analyte concentrations in clinical samples. The LFA strip comprises a sample pad, a conjugate pad, a nitrocellulose membrane, and an absorption pad. Capillary action propels liquefied semen along the strip, initiating a reaction with capture molecules bound in a dry state on the nitrocellulose membrane. The fluorescently labeled tracer binder, released from the conjugate pad with the semen sample or buffer solution, forms a binding complex with the target on the membrane surface. Detection of the fluorescent polymer at the test site is accomplished using a fluorescent reader, and the resulting signals are digitized for statistical analysis (see Fig. 1C).

Specifically, SP-10 signals are acquired by detecting the antibody-Alexa polymer in a sandwich configuration, correlating with sperm concentrations, as illustrated in Fig. 1A. To assess sperm quality, HAdase activity is measured using a sequential flow strategy. The liquefied semen, containing HAdase, travels along the strip via capillary action, interacting with hyaluronic acid (HA)–biotin complexes fixed on the membrane pad. HAdase catalyzes the degradation of HA, leading to the removal of biotin from the membrane. In a secondary flow, the SA-Alexa polymer, dissolved in a medium solution added to the buffer pad, reacts with the remaining HA-biotin, generating fluorescent signals proportional to the sperm quantity (refer to Fig. 1B).

Construction of SP-10 analysis system for detecting sperm concentrations. The SP-10 protein in semen is a specific acrosomal protein associated with male germ cells[23,24]. It is utilized to identify sperm concentrations, aiding in the diagnosis and observation of treatment effects for male infertility. Figure S1 displays the binding property of monoclonal antibodies specific to the SP-10 analyte. We assessed reactivity by detecting the binding response of the target on the well plate, using sensing signals from the enzyme reaction in ELISA. To quantitatively measure SP-10 concentrations, we created an immuno-strip by immobilizing the selected antibody on the test site of the NC membrane. Fluorescent molecules, serving as a signal tracer, were linked to amine groups of the detection antibody, generating high-intensity signals in a sandwich manner. The labeled antibody was applied in a dry state on the conjugation pad. The different sandwich pairs were evaluated for their specific binding to the SP-10 epitope site (see Fig. 2A), with the antibody pair 1 showing the quantitative binding response to the concentrations of the SP-10 analyte. We assessed the reliability of the specific response using the gold nanoparticle-assisted LFA in clinical semen samples (see Fig. S2).

Signal detection involved measuring emission light intensity in a sensing device using a laser source with a wavelength of 633 nm, tailored to sperm concentrations ranging from 15 x 10^6/mL to 182 x 10^6/mL. Clinical semen samples (IRB No. GCI 2019-12-057-002) were utilized to assess the analytical performance of the SP-10 strip. SP-10 values were correlated with sperm counts determined using the Makler Counting Chamber from Sefi-Medical Instruments. The optimal gain level of the sensing device achieved more than a sixfold enhancement of fluorescent signals in normospermia samples, as shown in Fig. 2B. The strategic combination of specific binding and minimized non-specific binding, achieved through assay design and antibody selection, enables an increase in detector gain to amplify signals related to SP-10 while maintaining low background values in azoospermia samples used as a blank. Figure S3 shows the digitized fluorescent signals plotted together to detect the intensity profiles of the different concentrations along the vertical direction in an overlaid fashion. This demonstrates a significantly increased signal-to-noise ratio within the detection range, ensuring high reliability in detecting clinical semen samples.

Optimization of hyaluronidase activity assay for sperm quality detection. HAdase, found on the sperm surface, is released by the acrosome upon reaching the oocyte, breaking down hyaluronic acid (HA) in the corona radiate[25]. The activity of HAdase is known to be closely linked to the fertilization rate,

especially in dispersing the cumulus-oocyte complex layer[20]. This activity is significantly inhibited under conditions of low ionic strength and at a low ratio of HAdase to HA concentrations[26,27]. To set up the highly sensitive LFA system, our initial focus was on optimizing the pH conditions of the assay buffer to regulate the ionic force concerning HAdase activity. The pH dependency impacts both the inherent HAdase activity and the formations of complexes between HAdase and HA[27]. In Fig. 3A, the enzyme activity of bovine HAdase is displayed in buffer solutions with different pH levels. The HA-biotin complex attached to the microwell plate was disassembled for bovine HAdase concentrations ranging from 1 to 100 U/mL. This led to a decrease in biotin molecules within the HA-biotin complex, and the HAdase activity was measured from the remaining values of HA-biotin through the enzyme reaction involving SA-HRP conjugates. The signals exhibited no noticeable change for varying HAdase doses under basic pH conditions (Fig. 3B). However, the most substantial signal reduction was observed with enhanced HAdase activity in acidic conditions. This pH effect remained consistent across different sperm counts in human semen samples, as depicted in Fig. 3C. The acidic conditions that optimize HAdase activity were subsequently applied in the following assays.

To sensitively detect HAdase activity in the FLF-sperm assay, we initially prepared a capture binder, HA-Alexa, immobilizing the capture molecule onto the membrane surface (see Fig. 4A,). The HAdase samples travel through the strip via capillary action, prompting the breakdown of the fluorophores from HA-Alexa through enzymatic cleavage. The sensing signals decrease with HAdase concentrations as we detect the remaining HA-Alexa (Fig. 4B,). However, obtaining clear dose-response signals for the HAdase dose proved challenging, with only strong signal reductions observed at concentrations over 5 U/mL. The effectiveness of Alexa tracer release from the capture binder digested by HAdase may interfere with the assay's accuracy. To overcome this hurdle, we devised a HA-biotin complex as a capture binder, utilizing biotin molecules to generate sensing signals through avidin-biotin reactions of Alexa-SA tracers (Fig. 4A,). We implemented a two-step method, separating the HA-HAdase reaction from the signal generation process. In the first step, capture binders (HA-biotin) react with the HAdase of samples on the membrane surface, removing biotin components from the binder complex. In the second step, Alexa-SA conjugates were provided with an additional semen-free buffer to generate signals, and the fluorescent signal was detected for the amount of residual sites through avidin-biotin linkage. We obtained the fluorescent signals corresponding to bovine HAdase concentrations, showing a linear dose response for the concentrations ranging from 1 to 50 U/mL (Fig. 4B,). The two-step analysis method enhanced the difference values—calculated by subtracting the sensing signals (B_s) from the intensity of the blank sample (B_0)— by 1.4 times for the maximum concentration of HAdase by effectively removing the decomposed capture binder compared to the one-step format.

We optimized the immobilization concentration of the capture binders (HA-biotin) at 0.1 mg/mL to enhance the signal in the absence of HAdase molecules and to achieve larger difference values directly corresponding to HAdase activity induced by the enzyme dose, as shown in Fig. 5. When using a low concentration (0.05 mg/mL) of capture molecules, the signals tend to be lower, even though the difference values (B_0 - B_s) dramatically increase with enzyme concentrations. These lower intensities

may lead to an increase in sensing errors during HAdase reactions. Conversely, at a high concentration (0.3 mg/mL), binders produce robust signal intensities but result in low difference values at a low HAdase dose. This is attributed to the densely packed capture molecules potentially limiting the molecular accessibility of HAdase by the steric hindrance and impacting enzyme reactions[28,29].

Evaluation of sperm analysis sensors. We assessed the sperm analysis systems for SP-10 and HAdase using clinical semen samples with varying sperm counts. The Makler Counting Chamber (Sefi-Medical Instruments Ltd) was employed to measure the number of semen sperm[30]. In this study, we streamlined the analysis of sperm count by associating it with SP-10 concentrations and assessed sperm quality by detecting HAdase activity. Semen samples representing azoospermia, oligospermia, and normospermia, approved by the Institutional Review Board (IRB) at CHA College of Medicine (IRB No. GCI 2019-12-057-002), were utilized. Figure 6A shows the quantitative dose-response curve for SP-10 concentrations in human semen samples with different sperm counts, demonstrating a proportional correlation. The analytical performance shows a broad dynamic range of 1.8 x 10^6/mL to 140 x 10^6/mL with superior linearity (R^2 = 0.98). The detection limit (LoD) of 1.28 x 10^6/mL was determined based on concentrations corresponding to three times the standard deviation of the blank value. As shown in Table S2, the SP-10 system was found to have an accuracy of 100% compared to a Makler Counting Chamber test. This effectively differentiated azoospermia and oligospermia semen with low sperm counts below 16 x 10^6/mL, indicative of male infertility. The background value, determined with the azoospermia sample, was used to estimate sensing values for non-specific interactions.

Normospermia refers to a condition where a semen analysis reveals normal sperm parameters, including sperm count, motility, and morphology[7]. Despite having normospermia, infertility can stem from various underlying causes, and one aspect worth exploring is low HAdase activity. If HAdase activity is low, it could impact sperm function and contribute to infertility, even when standard semen analysis shows normal parameters[20]. To assess sperm quality, we developed the LFA-sperm system for HAdase activity. The HAdase activity assay exhibits a lower signal as the enzyme activity strengthens, unlike the SP-10 assay. In the case of the azoospermia sample, it shows the highest signal because there are no enzyme activity-reducing binding sites for the fluorescent tracer on the sensing site of the NC membrane. In normospermia, the signals decrease compared to azoospermia as the sperm dose increases, as shown in Fig. 6B. Higher sperm counts typically result in strong HAdase enzyme activity under normal sperm conditions, indicating a robust correlation (R^2 = 0.90) between sperm numbers and enzyme activity. The HAdase assay sensitively detects enzyme activity for sperm counts above 1.8 x 10^6/mL, indicating the cut-off value of HAdase activity based on the fluorescent signals of the sperm counts below 15 x 10^6/mL, as marked by the dashed line in Fig. 6B. These HAdase assays have the potential to pinpoint the root cause of male infertility when assessing cases with a normal sperm count, relying on the established cut-off value.

Conclusion

The LFA-sperm systems for detecting SP-10 concentration and HAdase activity offer promising advancements in male fertility analysis. We achieved highly sensitive detection of the SP-10 analyte with a detection limit of 1.28 x 10^6/mL using a fluorescence tracer to measure quantitative sperm counts in clinical semen samples. The assay system demonstrated excellent linearity (R^2 = 0.98) with concentrations comparable to traditional semen count methods, effectively distinguishing between different semen samples, including azoospermia, oligospermia, and normospermia. Additionally, we established a sensing system using enzymatic cleavage to detect sperm quality based on hyaluronidase (HAdase) activity values, covering sperm concentrations from 1.8 x 10^6/mL to 140 x 10^6/mL, which can be indicative of male infertility. These novel FLF-sperm systems for SP-10 and HAdase enable simple and rapid quantitative detection of sperm concentration and quality, addressing male infertility caused by various factors. In future work, we will fabricate a test cartridge that integrates two test strips for detecting SP-10 and HAdase activity, providing simultaneous analytical information on sperm concentration and quality. The quantitative analysis enables users to interpret the quality of sperm more easily, offering a user-friendly understanding that eliminates arbitrary judgments. This system can serve as a valuable tool in the clinical field for diagnosing male infertility and monitoring treatment progress.

Declarations

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Author contributions

The authors confirm their contributions to the paper as follows: study conception and design: I.H.C.; data collection: J.L.; analysis and interpretation of results: J.H.J. and U.S.K.; draft manuscript preparation: S.H.S. and D.H.K. All authors reviewed the results and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Data availability statement

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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Figures

Figure 1

Schematic diagram of the FLF-sperm systems. (A) SP-10 analyte and (B) hyaluronidase activity detection of FLF-sperm assay was applied to estimate sperm amount and functional quality on-site diagnosis for a male infertility. (C) The fluorescent signals were measured using a fluorescence reader, and the numerical signal was graphed and analyzed.

Figure 2

Optimization of SP-10 analysis system. (A) The specific binding response of the SP-10 analyte was assessed in a sandwich format. (B) The gain value of the sensing device was optimized to enhance the fluorescent signals corresponding to the SP-10 concentrations.

Figure 3

pH effect on HAdase activity assay. (A) The HAdase activity was detected by using the HA-biotin complex bound to the solid matrix of the microwell. The values were obtained through the avidin-biotin interactions of the signal generator. (B) The enzyme activities of bovine HAdase ranging from 1 to 100 U/mL were detected under different pH conditions. (C) The pH effect represented consistent across different sperm counts of human semen samples.

Figure 4

Design of capture binders for HAdase activity detection of FLF system. (A) The FLF-sperm assay was fabricated with the capture binders of HA-Alexa and HA-biotin. The HA-Alexa complex directly produced the fluorescence signals through enzymatic cleavage between HAdase and HA in a one-step method. The HA-biotin binder was applied to separate the HA-HAdase reaction from the signal generation process in a two-step format. (B) The fluorescent signals were detected for bovine HAdase concentrations from 1 to 50 U/mL.

Figure 5

Optimal concentration of capture binder. The concentration of the immobilized capture binder (HA-biotin) was optimized to enhance the fluorescence intensity and to achieve larger difference values (B0 - Bs) in the HAdase activity detection induced by the enzyme dose.

Figure 6

Analytical performance of FLF-sperm systems. (A) The binding response for SP-10 concentrations in human semen samples showed a high correlation (R2 = 0.98) with the sperm counts measured using the Makler Counting Chamber (Sefi-Medical Instruments Ltd.). (B) Elevated sperm counts exhibited robust HAdase enzyme activity under normal sperm conditions, establishing the HAdase activity cut-off value based on the fluorescence intensity for sperm counts below 15 x 10^6/mL (indicated by the dashed line).

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