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A rapid and highly-sensitive surface plasmon resonance (SPR)-based immunoassay procedure for human fetuin A

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Method Article

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Abstract

A rapid and highly-sensitive procedure has been developed for surface plasmon resonance \(SPR)-based immunoassay \(IA) for human fetuin A \(HFA). It employs a highly-simplified antibody \(Ab) immobilization strategy, which only involves sequentially the dispensing of anti-HFA capture Ab diluted in 1% \(v/v) 3-aminopropyltriethoxysilane \(APTES) onto a potassium hydroxide \(KOH)-treated gold \(Au)-coated SPR chip followed by its incubation for 30 min. The devised Ab immobilization strategy enables the leach-proof binding of capture Ab, resulting in highly reproducible and cost-effective HFA IAs. The SPR IA detected HFA with a dynamic range, limit of detection, and analytical sensitivity of 0.3-20 ng mL^{-1}, 0.7 ng mL^{-1} and 1 ng mL^{-1}, respectively. The detecting chip can be easily regenerated after each analyis by treatment with 10 mM glycine-HCl, pH 2.0. The Ab-bound SPR chip can be stored at 4°C for up to 4 months without any significant loss of its functional activity. The developed IA procedure has been employed to detect HFA spiked in diluted human whole blood and plasma. Moreover, the developed SPR IA has high analytical precision as its results are correlated well with those of conventional HFA sandwich ELISA.

Introduction

Surface plasmon resonance \(SPR) has been the most widely used optical technique for the real-time and label-free detection of biomarkers based on their specific biomolecular interactions with the bound antibodies \[1-3]. The main SPR-based instruments are the Biacore systems from GE Healthcare, which have been widely used by researchers during the last two decades for a plethora of applications in academia, healthcare and industry. The conventional microtiter plate \(MTP)-based IA formats, such as enzyme-linked immunosorbent assay \(ELISA), chemiluminescent IA and fluorescent IA, have prolonged assay duration of several hours, which hinders their application for rapid diagnostic applications. However, SPR-based IAs provide critically shortened assay duration of just a few minutes. A wide range of Ab immobilization strategies \[4-10] have been employed for SPR IAs, which employ commercial SPR chips that are pre-functionalized with carboxymethyl dextran, streptavidin, nitriloacetic acid, long chain alkanethiol molecules or lipophilic groups \[11]. A variety of fragment crystallizable \(Fc)-binding proteins has been considered \[9, 12, 13], i.e. protein A, protein G and protein A/G, for the oriented immobilization of capture Ab. However, these Ab immobilization strategies require several hours and involve a large number of process steps. We have developed a novel and highly-simplified Ab immobilization strategy, which leads to the leach-proof binding of capture Ab on the Au-coated SPR chips in just 30 min \[14]. It only involves the dispensing of Ab diluted in APTES onto a cleaned Au SPR chip, where the carboxyl groups of Abs are bound by ionic binding to the amino groups on APTES molecules that bind simultaneously to the hydroxyl groups on the KOH-treated Au SPR chip. The developed Ab immobilization strategy enables rapid and cost-effective SPR IAs as it obviates the need for multiple process steps, additional chemicals required for Ab immobilization, and the costly pre-functionalized SPR chips. The Ab-bound SPR chips are evaluated for the detection of HFA in buffer, diluted human whole blood and plasma, with respect to bioanalytical performance, reusability and storage stability. The bioanalytical

performance of the developed SPR IA procedure will also be compared with our previously-developed and commercial CM5 dextran-based SPR IA procedures \[9].

Reagents

• Human Fetuin A ELISA kit \(R & D Systems, Cat. No. DY1184e) **\!CAUTION** Store reconstituted Ab and antigen at 4°C if they are to be used within a month. Otherwise make the aliquots and store them at -80°C if they are to be used within 6 months. The kit contains • Mouse anti-HFA capture Ab \(720 μg mL^{-1}) • HFA \(20 ng mL^{-1}) • Biotin-labeled goat anti-HFA detection Ab • HRP-conjugated streptavidin **\!CAUTION** Store in dark as streptavidin is light-sensitive. • Blocker bovine serum albumin \(BSA) in PBS \(10X), pH 7.4, 10% \(w/v) \(Thermo Scientific, Cat. No. 37525) **CRITICAL** Filter prior to use to avoid clumping and any microbial contamination. • KOH pellets \(99.99%), semiconductor grade \(Sigma Aldrich, Cat. No. 306568) **\!CAUTION** Avoid contact with skin and eyes as it can cause severe burns. Use personal protective equipment \(PPE) and handle it only in a safety cabinet. **CRITICAL** The concentration of KOH used must be 1% \(w/v) in autoclaved deionized water \(DIW, 18 Ω) as it may affect the surface properties of the Au surface, which can significantly affect the antibody immobilization. • 3-aminopropyltriethoxysilane \(3-APTES) \(Sigma Aldrich, Cat. No. A3684) **\!CAUTION** Avoid contact with skin and eyes as it is an irritant. Handle it only in a safety cabinet. The higher concentrations are potentially toxic. **CRITICAL** Prepare it in autoclaved DIW \(18Ω), see **REAGENT SETUP**. • BupH Phosphate Buffered Saline Packs \(0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) \(Thermo Scientific, Cat. No. 18372) **\!CAUTION** Avoid direct inhalation. **CRITICAL** Prepare it in autoclaved DIW \(18Ω), see **REAGENT SETUP**. • HBS-EP \(0.01 M 4-\(2-hydroxyethyl)-1-piperazineethanesulfonic acid \(HEPES) pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) \(GE Healthcare, UK, Cat No. BR-1001-88). • Glycine-HCl \(10 mM, pH 2.0) \(GE Healthcare, UK, Cat. No. BR-1003-55). • Deionized water \(18 Ω DIW). \(Direct-Q® 3 Water Purification System, Millipore, USA) • SigmaPlot software bundle version 11.2 from Systat • Eppendorf® microtubes \(1.5 mL; Sigma Aldrich, Cat. No. Z606340) **REAGENT SETUP** **PBS** Add a BupH PBS pack containing 0.1M phosphate and 0.15M NaCl to 100 mL of autoclaved DIW. Dissolve well and make the volume up to 500 mL with autoclaved DIW. Each pack makes 500 mL of PBS at pH 7.2 that can be stored at RT for a week and at 4ºC for up to four weeks. **APTES** The commercially-available APTES solution has a purity of 98%. Reconstitute in autoclaved DIW to make an effective 1% \(v/v) solution just prior to mixing with capture anti-HFA Ab.

Equipment

• -70ºC freezer \(operating range -60 to -86ºC) \(New Brunswick) • 2-8ºC refrigerator \(Future, UK) • Biacore™ 3000 SPR instrument \(GE Healthcare, Uppsala, Sweden) • SIA Kit Au \(GE Healthcare, Cat. No. BR-1004-05)

Procedure

Surface cleaning TIMING ~ 8 min 1. The Au-coated SPR chip was cleaned by incubating with 90 µL of 1% \(w/v) KOH for 5 min followed by extensive washing with DIW. This treatment will generate the hydroxyl groups on the Au surface. **Antibody immobilization and blocking TIMING ~ 35 min** 2. The capture anti-HFA antibody \(200 µg mL^{-1}) was mixed with 1% APTES in the ratio of 1:1 v/v. Ninety microliters of this capture anti-HFA Ab solution, with a final concentration of 100 µg mL^{-1} in 0.5% APTES, was dispensed onto the Au chip and incubated for 30 min at RT in the fume hood. The anti-HFA antibody-bound Au SPR chip was washed extensively with 10 mM HBS, pH 7.4. It was then docked into Biacore 3000 and primed. **? TROUBLESHOOTING** 3. Twenty microliters of 1% \(w/v) BSA was then injected over all four flow cells of anti-HFA antibody-bound Au SPR chip at a flow rate of 10 µL min^{-1}. The procedure blocked the non-specific binding sites on the anti-HFA Ab-bound Au SPR chip. **CRITICAL STEP** Use filtered BSA or filter the BSA solution prior to use to remove any microbial or other contaminants. **? TROUBLESHOOTING** **HFA-detection TIMING ~ 15 min per concentration** 4. Fifty microliters of the running buffer \(10 mM HBS, pH 7.4) was passed through all flow cells before HFA capture and the resultant changes in SPR response units \(RU), corresponding to blanks, were recorded. 5. Fifty microliters of HFA at seven different concentrations \(0.3, 0.6, 1.2, 2.5, 5.0, 10.0 and 20.0 ng mL^{-1}) were passed through the flow cells of an Ab-bound SPR chip in consecutive HFA detection cycles, where the Ab-bound SPR chip surface was regenerated after each analysis by treatment with 10 mM glycine-HCl, pH 2.0. The RU values obtained for blanks were subtracted from the RU values for HFA detected in the respective flow cells. 6. The SPR-based HFA detection curves were plotted with SigmaPlot software, version 11.2 using standard curve analysis based on a four parameter logistic function. The analytical parameters such as EC50 and Hillslope were generated by the software analysis report.

Timing

Steps 1, Surface cleaning: 8 min Steps 2-3, Antibody immobilization and blocking: 35 min Step 4-6, HFA detection: 15 min for each HFA concentration

Troubleshooting

Troubleshooting advice is provided in Table 1.

Anticipated Results

The developed SPR IA procedure detected HFA \[**Fig. 1**] in the dynamic range of 0.3-20 ng mL^{-1} with a limit of detection \(LOD) of 0.7 ng mL^{-1}, analytical sensitivity of 1 ng mL^{-1}, and a maximal half-effective concentration \(EC50) of 3.8 ng mL^{-1} \[14] \[**Fig. 2a,b**]. It is the most sensitive SPR IA procedure for HFA. The intra-assay variability, calculated on the basis of five assay repeats \(in triplicate) in a single day, was in the range of 1.2-5.8, while the inter-assay variability, determined by five assay repeats \(in triplicate) on five consecutive days, was in the range of 2.1-8.6. The developed SPR IA had an anti-HFA immobilization density of 182.6±1 ng cm^{-2}, which was better than that of our previously developed

covalent \(153.6±1 ng cm^{-2}) and commercial CM5 dextran chip based Ab immobilization procedures \(172.8±1.2 ng cm^{-2}) \[9] \[**Table 2**]. It detected HFA spiked in diluted human whole blood and plasma \[**Fig. 2a**] with precision similar to that of conventional HFA sandwich ELISA, as shown by the direct agreement of results in the linear range of 1.2-20 ng mL^{-1} \[**Table 3**]. The Ab-bound SPR chip was reused for 35 consecutive HFA IAs to detect 5 ng mL^{-1} HFA \[**Fig. 2c**]. The leach-proof binding of anti-HFA capture Ab to the Au SPR chip was observed by the highly reproducible detection of HFA in consecutive IAs on the same chip. The Ab-bound SPR chip was regenerated after each HFA detection cycle by treatment with 20 μL of 10 mM glycine-HCl, pH 2.0. The storage stability of the anti-HFA Ab-bound SPR chip stored at 4°C was also determined, where the Ab-bound chip was used after every two weeks for the detection of 5 ng mL^{-1} HFA. The Ab-bound chips were highly stable for 2 months with only an 18 % decrease in HFA response after 4 months \[**Fig. 2d**]. The developed Ab-immobilization strategy is highly cost-effective as it enables the preparation of Ab-bound Au chips in just 30 min before their intended use, which will lead to significantly increased bioanalytical performance, obviating the advanced preparation and storage requirements of the Ab-bound Au sensing chip. It is 5-fold more rapid than the conventional Ab immobilization strategies that are being used for the development of SPR IAs \[9]. The developed SPR IA procedure is generic and can be used for the detection of biomarkers and other analytes in industrial, healthcare and other bioanalytical settings.

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Figures

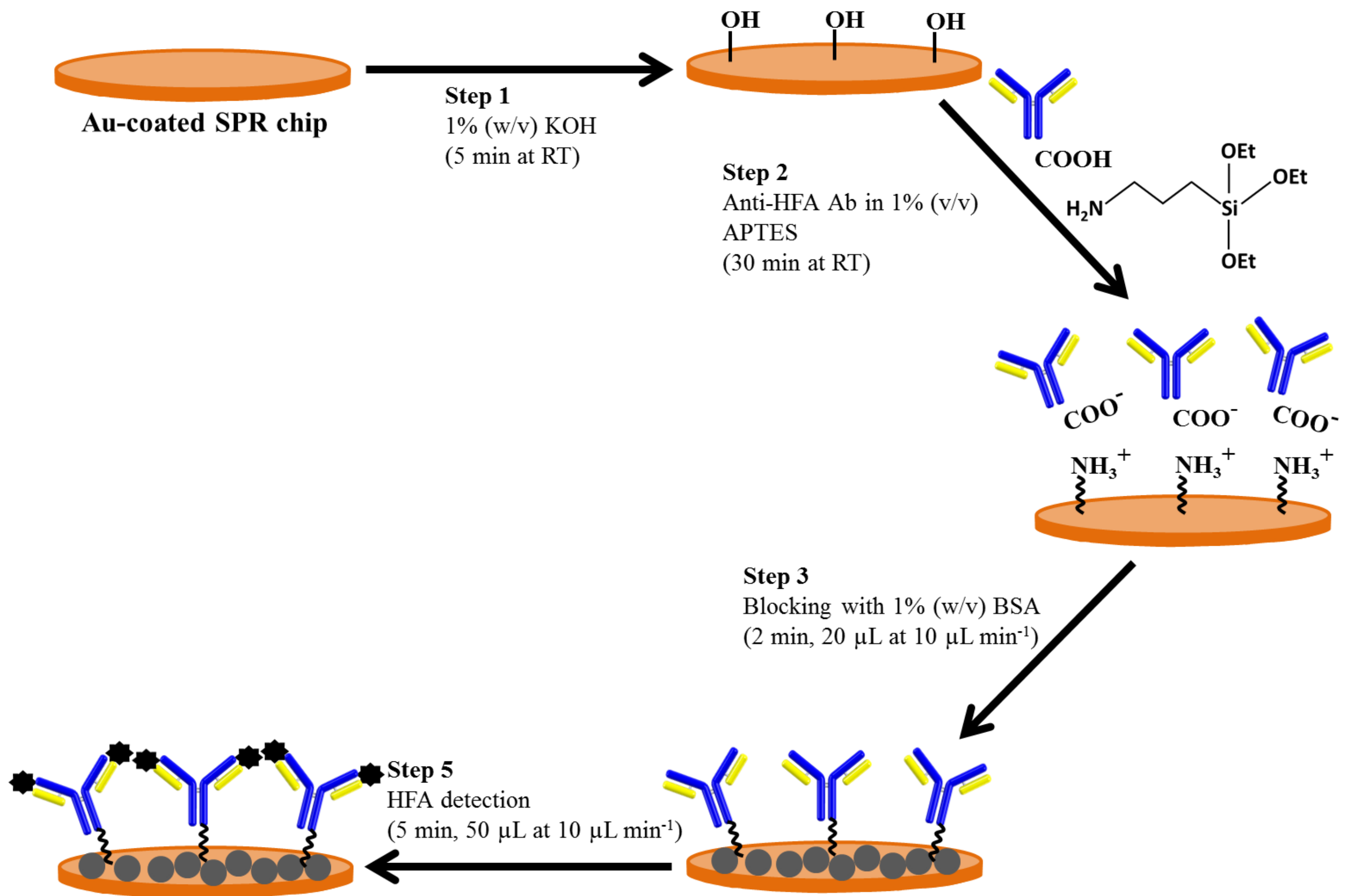

Figure 1

Schematic of the developed procedure Schematic representation of the developed SPR-based IA procedure for the detection of HFA. The gold surface is treated with 1% KOH to generate the hydroxyl groups, which is followed by their binding to the alkoxy groups of APTES in a solution comprising of anti-HFA capture Ab in 1% APTES. The anti-HFA Ab binds to APTES by ionic interactions. The Ab-bound chip was then blocked with 1% BSA and subsequently used for the detection of HFA.

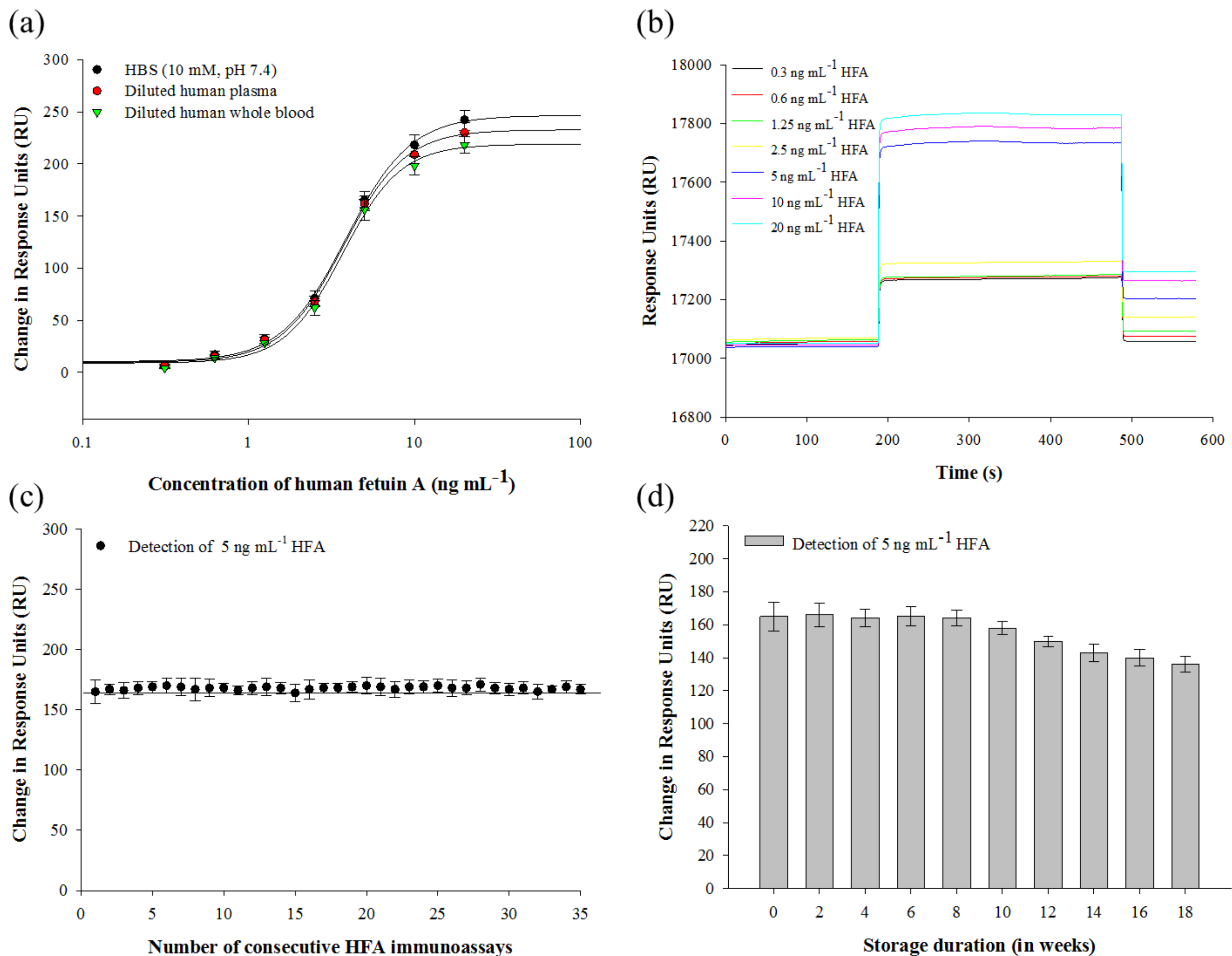

Figure 2

Bioanalytical performance of the developed SPR-based HFA IA procedure (A) Detection of HFA spiked in HBS (10 mM, pH 7.4), diluted human whole blood and diluted human plasma. (B) Sensorgrams for the detection of various HFA concentrations in HBS, 10 mM, pH 7.4. (C) Multiple consecutive IAs for the detection of 5 ng mL^-1^ HFA using the same anti-HFA Ab-bound SPR chip, which is regenerated after each IA using glycine-HCl (10 mM, pH 2.0). (D) Stability of the anti-HFA Ab-bound SPR chip stored at 4°C, as determined by the detection of 5 ng mL^-1^ HFA. All experiments were done in triplicate with the error bars representing the standard deviation.

Step	Problem	Probable reason	Solution
Step 2	No binding of antibody	Au chips may not have been cleaned properly. If the problem is not resolved, proceed to the next troubleshooting step.	Clean the Au chip again.
Step 2	No binding of antibody	APTES may have been hydrolyzed/ degraded.	Prepare a fresh APTES solution from a different batch.
Step 3	High background	Check the BSA quality. BSA may have been contaminated.	Prepare a fresh BSA solution using a different stock.
Step 5	No HFA detection	Anti-HFA antibody may have become non-functional. If the problem is not resolved, proceed to the next troubleshooting step.	Use another stock of anti-HFA antibody. As proteins are highly susceptible to storage conditions, store them properly and avoid repeated freezing-thawing cycles.
Step 5	No HFA detection	HFA may have become non-functional.	Use another stock of HFA.

Figure 3

Table 1 Troubleshooting Table

Antibody Immobilization Procedure	Immobilization of anti-HFA antibody			HFA***			EC_{50}
	ΔRU	Mass density* (ng cm^{-2})	Molecular density** (molecules cm^{-2})	ΔRU	Mass density* (ng cm^{-2})	Molecular density** (molecules cm^{-2})	(ng mL^{-1})
Developed	1826 .0± 9.9	182.6 ± 1.0	7.3 $x10^{11}$	165.0 ± 11	16.5 ± 1.1	(2,4 ± 0.2) $x10^{11}$	3.8
Covalent	1536.0 ± 10.4	153.6 ± 1.0	6.1 $x10^{11}$	149.0 ± 9.6	14.9 ± 1.0	(2.1 ± 0.1) $x10^{11}$	3.8
Covalent CM5 dextran	1728.0 ± 12.4	172.8 ± 1.2	6.9 $x10^{11}$	138.0 ± 10.4	13.8 ± 1.0	(1.9 ± 0.1) $x10^{11}$	4.1

ΔRU: Change in resonance units (RU) caused by binding

*Calculated using the commonly used conversion factor, i.e. 1000 RU=100 ng cm^{-2} [15-19].

**Calculated by [Mass density (ng cm^{-2}) / Molecular weight (in ng)]. Molecular weight of anti-HFA antibody and HFA were 150 kDa and 43.5 kDa, respectively. In order to calculate molecular weight in SI units, the conversion factor 1 kDa = 1000 Da = 1000 g was used. The molecular weight of anti-HFA antibody and HFA is $24.9x10^{-11}$ng and $7.0x10^{-11}$ng, respectively.

***Calculations were performed for the detection of 5 ng mL^{-1} of HFA i.e. the concentration just above the EC_{50}.

Figure 4

Table 2 Determination of molecular densities Determination of molecular densities of immobilized anti-HFA Ab and the detected amount of HFA, when different SPR immunoassay formats, based on various antibody immobilization strategies, were employed.

Sample matrix	Spiked HFA Conc. (in ng mL^{-1})	Detected HFA Conc.	
		Developed SPR IA	Commercial sandwich ELISA
Diluted human whole blood	20	20.3±0.8	19.8±1.1
	10	10.2±0.3	9.9±0.4
	5	5.2±0.2	5.3± 0.3
	2.5	2.4±0.1	2.5±0.2
	1.2	1.1±0.1	1.3±0.2
Diluted human plasma	20	20.1±0.6	20±0.4
	10	10.3±0.4	10.1±0.5
	5	4.9± 0.3	4.8± 0.4
	2.5	2.5±0.2	2.7±0.3
	1.2	1.3±0.1	1.2±0.1

Figure 5

Table 3 Technology Correlation Determination of spiked HFA concentrations in diluted human whole blood and plasma by the developed SPR IA and the commercial sandwich ELISA. The experiments were performed in triplicate with the results presented as mean ± S.E.