Multisubstrate-compatible ELISA procedures for rapid and high-sensitivity immunoassays

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This protocol describes an improved and optimized approach to develop rapid and high-sensitivity ELISAs by covalently immobilizing antibody on chemically modified polymeric surfaces. The method involves initial surface activation with KOH and an O_2 plasma, and then amine functionalization with 3-aminopropyltriethoxysilane. The second step requires covalent antibody immobilization on the aminated surface, followed by ELISA. The ELISA procedure developed is 16-fold more sensitive than established methods. This protocol could be used generally as a quantitative analytical approach to perform high-sensitivity and rapid assays in clinical situations, and would provide a faster approach to screen phage-displayed libraries in antibody development facilities. The antibody immobilization procedure is of ~3 h duration and facilitates rapid ELISAs. This method can be used to perform assays on a wide range of commercially relevant solid support matrices (including those that are chemically inert) with various biosensor formats.

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INTRODUCTION

ELISA is a widely used technique and continuous efforts are being made to substantially improve its various applications[1,2]. Adsorption on microtiter plate wells is a commonly used antibody immobilization procedure for ELISAs; however, this strategy has drawbacks associated with loss of antibody functionality, nonuniform distribution of antibody molecules on the surface, poor signal-to-noise ratio, leaching and protein exchange[3–6]. In addition, antibody can only be physisorbed on chemically active hydrophilic and hydrophobic surfaces. Amine-terminated silanes, such as 3-aminopropyltriethoxysilane (APTES), are commonly used for amination of metallic supports for different biosensor applications, and there are also reports on silane-based modification of microtiter plate wells[7,8]. However, these methods use harsh procedures for surface activation, e.g., oxidation of the polymeric surface with strong oxidizing agents, such as sulfuric and nitric acid, in order to generate pendant nucleophilic (hydroxyl or nitro) functional groups. These groups covalently react with the alkoxy groups of silane generating self-assembled layers, but can interfere with the functionality of the target biomolecules if traces remain on the modified surfaces[9,10].

We describe a rapid and high-sensitivity immunoassay procedure that uses covalently immobilized capture antibody on an aminosilane-functionalized microtiter plate[11]. The basic idea of developing a chemically active surface for rapid ELISA-based immunoassay was conceived as an approach for accelerated analysis. The surface activation, which is the primary requirement for silane-based functionalization, was achieved by exposing the desired polymeric surface to KOH[12–14] (1% (wt/vol)) in conjunction with an O_2 plasma[15–21]. This activation procedure induces carbonyl (-CO) and hydroxyl (-OH) groups on the solid matrix, depending on the nature of the polymer[14–18]. These nucleophilic centers on the polymeric surface attract electrophilic alkoxy pockets of APTES, generating silanol bonds (-O-Si-; **Fig. 1**)[14,16]. The developed method was demonstrated using human fetuin A (HFA) as a model antigen system.

The analytical advantages of the approach described in this paper were clearly demonstrated in comparison to conventional methods[11]. Reported techniques using wet methods[7] have certain drawbacks such as generation of a nonspecific pool of oxygen-containing chemical species[9] and uncontrolled surface roughness[10,22]. However, the use of plasma enables considerable control of these aspects. Therefore, our method uses chemical activation using a mild oxidation agent (low KOH concentration) followed by O_2 plasma treatment, enabling the utilization of the combined effects of wet and plasma treatment. It also efficiently increases control over the surface roughness as the acid-based activation is completely replaced by a milder agent, thereby permitting milder functionalization of the surface and covalent crosslinking of antibodies by means of their carboxyl groups. The control over surface roughness in the developed procedure enables the homogeneous distribution of antibody over the surface, resulting in enhancement of analyte detection sensitivity[23], whereas harsh chemical activation generates a non-homogeneous functional layer on the surface with associated non-homogeneous antibody distribution. This could also result in antibody trapping/non-availability for binding in the closely packed network, hence lessening accessibility for the analyte. However, the use of chemicals such as APTES and EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride) introduces additional biosafety requirements, and the need for more sophisticated instrumentation and facilities may also be a restriction.

The described method has enabled us to develop immunoassays on multiple substrates, including inert polymers, either in the same or in separate microtiter plates[18,19,24]. The method has potential applications in various biosensor-based formats. We have used the approach, and modifications thereof, for the development of chemiluminescence, surface plasmon resonance and fluorescence-based microarray assays for highly stable agarose-based chromatographic resins, and it could also be used in methods for screening antibody phage-display libraries (C.K.D., S.K.V. and R.O., unpublished observations). One potential limitation of the approach may be the need for specific conditions and instrumentation for silanization.

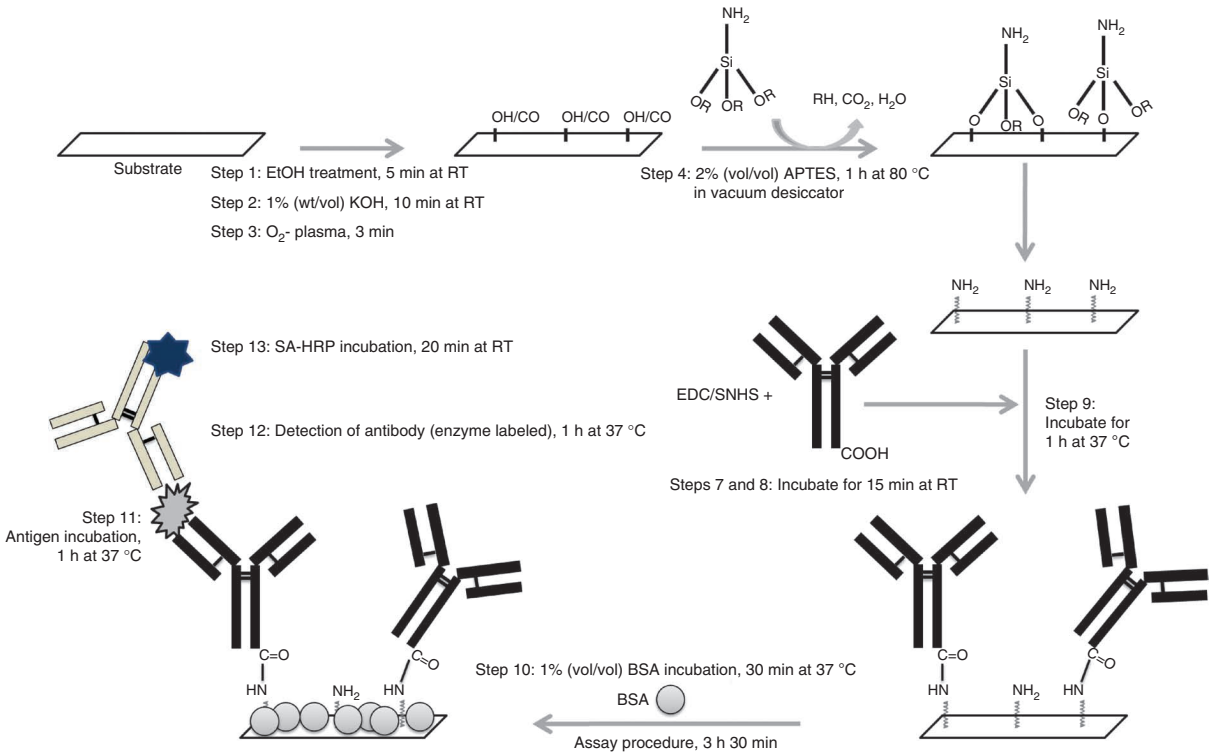

Figure 1 | Schematic representation of the optimized surface modification and antibody immobilization strategy. The method involves 1% (wt/vol) KOH treatment that generates OH/CO groups on the polymeric surfaces, depending on their chemical nature; this facilitates the reaction of amine-terminal silane (APTES). Antibody was conjugated to the amino groups of the functionalized surface. These steps were followed with a standardized ELISA procedure with optimized blocking, washing and incubation steps. SA-HRP, streptavidin-HRP; SNHS, SulfoNHS.

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Nevertheless, silane functionalization can be performed at room temperature (RT; ~25 °C) in a fume hood, thereby obviating the need for use of a vacuum pump and an oven. The use of this alternative approach will certainly affect various analytical parameters such as limit of detection (LOD) and effective concentration for half-maximum response (EC_{50}), but the performance may still be better than the conventional method, particularly when the silanization time is increased.

Experimental design

Silanization. Silanization requires a vacuum desiccator and oven. The oven should be preset to 80 °C for at least 20 min for temperature equilibration, and vacuum should then be applied. It is advantageous to equilibrate the desiccator before generating the vacuum. Various parameters such as APTES concentration and silanization time were also optimized in this study (**Supplementary Fig. 1**). The characterization of APTES functionalization was monitored with Fourier transform infrared (FTIR) spectroscopy (**Supplementary Fig. 2**). The silane has a marked tendency for multimerization, as it contains both nucleophilic and electrophilic centers. This makes the duration of silanization a critical parameter, as formation of randomized multilayers of silane can occur over extended time periods. This will eventually result in random antibody capture, which will directly affect overall immunoassay performance. Reports suggest that APTES forms multilayers after 3 h of incubation[25,26]. Therefore, we fully optimized our protocol, both for the duration of silanization and the concentration of APTES used (**Supplementary Fig. 1**).

Immunoassay optimization. In immunoassay development, all the components were commercially obtained as lyophilized samples and were checked for quality of performance before use. A number of process controls relevant to the reported procedure were included to optimize the ELISA and are shown in **Supplementary Figure 3**. Process controls included were also designed to test the cross-reactivity of kit components. HFA (standard positive control) and standards were supplied with the kit. The relevant analyte (fetuin for this study) obtained from various sources and additional control biomolecules that may present cross-reactivity challenges, such as human fetuin B in the assay developed for HFA, must also be used as controls. In this study, 1% (vol/vol) BSA in 0.1 M PBS was used as a blank. Different HFA dilutions were prepared and subjected to ELISA in the developed and conventional assay formats in order to define various analytical parameters. In addition, a number of assay dilutions were selected such that the lowest and highest concentrations followed the sigmoidal curve and were required to have at least five concentrations in the linear range on the standard curve. An irrelevant analyte, such as albumin, was routinely used as a negative control.

Result analysis. The EC_{50} (ref. 26) is the half-maximal effective concentration of antigen/ligand that induces 50% of the maximum detectable signal on the linear working range of an assay. Therefore, EC_{50} provides quantitative details of the analyte detection ability of an assay. Theoretically, it could be calculated by using four- or five-parameter curve fitting equations[27]. Most of the analysis systems determine the value of EC_{50} using four-parameter logistic-based

standard curve analysis. We have used SigmaPlot 11.0 software to analyze the various parameters such as EC_{50} and Hill slope (**Supplementary Fig. 4**).

LOD or assay sensitivity is the ability of the assay to detect the lowest possible analyte concentration present in the solution. There are many stringent ways to calculate LOD[28]. However, the most common and widely used method to determine LOD is signal of blank ± 3 s.d. The procedure for the calculation of LOD is given in the **Supplementary Methods** section.

Microtiter plate assembly to develop assays on different polymeric supports. The design and assembly of microtiter plates with different solid supports is summarized in **Supplementary Figure 5**. A patterned, double-sided, pressure-sensitive adhesive was designed and developed such that the hole pattern at the bottom of microtiter plate corresponds to that on the adhesive. This adhesive was applied to the pre-blocked bottomless microtiter plates and the desired polymeric support was then attached. Afterward, pressure was applied with a press. More details about this strategy can be found in Vashist *et al.*[24] and also in **Supplementary Figure 5**. Microtiter plates prepared using the described method differ from the commercially available plates in having a different polymeric solid support. Therefore, these plates can be used in the PROCEDURE in the same manner as that of commercial microtiter plates.

MATERIALS

REAGENTS

- Anti-HRP antibody (Sigma-Aldrich, cat. no. P2419-2ML)
- HRP (Sigma-Aldrich, cat. no. P8375)
- Human fetuin A ELISA kit (R&D Systems, cat. no. DY1184e) **! CAUTION** Store the reconstituted antibody and antigen at 4 °C only if they are to be used within 2 months, otherwise, aliquot out and store at −80 °C for up to 6 months. The kit contains mouse anti-HFA capture antibody (720 μg ml^{-1}); HFA/α-2-HS glycoprotein (AHSG) (90 ng ml^{-1}); biotin-labeled goat anti-HFA detection antibody; and HRP-conjugated streptavidin **! CAUTION** Streptavidin is light-sensitive. Store in the dark.
- Blocker BSA in PBS (10×, pH 7.4, 10% (wt/vol); Thermo Scientific, cat. no. 37525) **▲ CRITICAL** Filter before use to remove microbial contamination. This is critical for surface blocking.
- Absolute ethanol (Sigma-Aldrich, cat. no. 02856) **! CAUTION** It is highly flammable. Keep the container tightly closed. Keep away from sources of ignition. Alcohol vapors may be harmful to soft tissues such as the eyes or the respiratory tract. Use in a safety cabinet or a fume cupboard.
- Sulfuric acid (H_2SO_4; Sigma-Aldrich, cat. no. 339741) **! CAUTION** It is strongly corrosive and an irritant. Avoid contact with any part of the body. Wear suitable protective clothing and safety glasses during handling. Handle in a safety cabinet or a fume cupboard. In case of skin contamination, wash immediately with acid neutralizers and seek medical advice immediately.
- KOH pellets (99.99%, semiconductor grade; Sigma-Aldrich, cat. no. 306568) **! CAUTION** It can cause severe burns. Avoid contact with skin and eyes. Wear appropriate protection and handle in a safety cabinet. **▲ CRITICAL** The concentration of KOH must be exactly 1% (wt/vol) in autoclaved deionized water (DIW, 18 Ω). It can affect the surface properties and can affect antibody immobilization.
- 3-Aminopropyltriethoxysilane (3-APTES; Sigma-Aldrich, cat. no. A3684) **! CAUTION** It is a skin and eye irritant. It is potentially toxic to the kidneys. **▲ CRITICAL** Prepare in autoclaved DIW (18 Ω; see REAGENT SETUP).
- BupH phosphate-buffered saline (PBS) packs (0.1 M sodium phosphate and 0.15 M sodium chloride (pH 7.2); Thermo Scientific, cat. no. 18372) **! CAUTION** Avoid inhaling the powder. **▲ CRITICAL** Prepare in autoclaved DIW (18 Ω; see REAGENT SETUP).
- BupH MES-buffered saline packs (0.1 M MES (2-(*N*-morpholino)ethane sulfonic acid), 0.9% (wt/vol) sodium chloride (pH 4.7); Thermo Scientific, cat. no. 28390) **! CAUTION** Avoid inhalation. **▲ CRITICAL** Prepare in autoclaved DIW (18 Ω; see REAGENT SETUP).
- 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Thermo Scientific, cat. no. 22981) **! CAUTION** It is an irritant. Handle inside a fume cupboard. EDC is hygroscopic, absorbs moisture and may lose activity. Equilibrate to RT before opening the container **▲ CRITICAL** Store at recommended temperature (−20 °C). Reconstitute in 0.1 M MES (pH 4.7; see REAGENT SETUP).
- Sulfo-*N*-hydroxysuccinimide (SulfoNHS; Thermo Scientific, cat. no. 24525) **▲ CRITICAL** Store at the recommended temperature (4 °C). Reconstitute in 0.1 M MES (pH 4.7; see REAGENT SETUP).
- TMB substrate kit (Thermo Scientific, cat. no. 34021). The kit contains TMB solution (0.4 g l^{-1}) **! CAUTION** It is a skin, eye and lung irritant. In case of skin contact, wash with plenty of water **▲ CRITICAL** TMB to peroxide ratio is critical for color development. Maintain it in the ratio 1:1; peroxide solution (containing 0.02% (vol/vol) H_2O_2 in citric acid buffer; Thermo Scientific) **! CAUTION** It is a strong oxidizing agent. It is harmful if swallowed and also poses severe risk of damage to eyes. Rinse immediately with plenty of water in case of contact and seek medical attention. Wear suitable protection and work in safety cabinet or fume cupboard.
- Deionized water (18 Ω, DIW)

EQUIPMENT

- Freezer (−70 °C, operating range −60 to −80 °C; New Brunswick)
- Refrigerator (2–8 °C; Future)
- ELISA plate reader (Tecan)
- Mini incubator (Labnet)
- Harrick plasma cleaner (Harrick Plasma; model number PDC-002)
- PVC fume cupboard (Chemflow range; CSC)
- Nunc microwell 96-well polystyrene plates (flat bottom (non-treated), sterile; Sigma-Aldrich, cat. no. P7491)
- PMMA (Poly(methyl methylacrylate)) polycarbonate, cellulose acetate polymer (in the form of 1-mm-thick sheets) and zeonex polymer (1.5-mm-thick slides; Microfluidic ChipShop)
- Desiccator with vacuum control nozzle (Lennox)
- Vacuum source
- Eppendorf microtubes (1.5 ml; Sigma-Aldrich, cat. no. Z 606340)
- SigmaPlot software bundle version 11.0 from Systat for detailed assay analysis

REAGENT SETUP

PBS Each sachet (BupH phosphate-buffered saline (PBS) packs) contains 0.1 M phosphate and 0.15 M NaCl and yields a volume of 500 ml in the pH range 6.8–7.4. Add a sachet to 100 ml of autoclaved DIW and dissolve. Finally, adjust the volume up to 500 ml. This can be stored at RT for 2 weeks. Check pH before use.

MES Each sachet (BupH MES-buffered saline packs) contains 0.1 M MES and 0.9% (wt/wt) NaCl and yields a volume of 500 ml at pH 4.7. Add a sachet to 100 ml of autoclaved DIW and dissolve. Adjust the final volume up to 500 ml. Check the pH and adjust it to 4.7 if necessary. This can be stored at RT for 2 weeks. Check pH before use.

APTES The solution is supplied at 99% purity. Reconstitute in autoclaved DIW to produce an effective 2% (vol/vol) solution. Prepare a fresh solution for each functionalization.

EDC Each pack contains 25 g EDC. Reconstitute in 0.1 M MES buffer (pH 4.7), at a concentration of 8 mg ml^{-1}. Mix it with equal volumes of sulfoNHS solution (22 mg ml^{-1}). Aliquots can be stored effectively for 6 months at −20 °C.

SulfoNHS Each pack contains 5 g sulfoNHS. Reconstitute in 0.1 M MES (pH 4.7), at a concentration of 22 mg ml^{-1}. Mix with equal volumes of EDC (8 mg ml^{-1}). Aliquots can be stored effectively for 6 months at −20 °C.

H_2SO_4 The concentrated solution is 37 N. Add 2.7 ml of 37 N H_2SO_4 to 97.3 ml of DIW to produce a 1 N solution. Reaction of acid and water is exothermic; therefore, acid should always be added to water. This solution can be stored indefinitely at RT.

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PROCEDURE

Surface activation and amine functionalization ● TIMING ~1 h 30 min

1| Incubate the surface of the plate well with 100 μl of absolute ethanol for 5 min and wash extensively with DIW (minimum five times with 300 μl DIW per well). Washing can also be performed with an automatic plate washer or squeeze bottles.

2| Incubate the surface of the plate well with 100 μl of 1% (wt/vol) KOH in DIW for 10 min and wash extensively with DIW (as described in Step 1).
▲ CRITICAL STEP KOH treatment should not be longer than the optimized time, because it may cause strong aberrations in the surface and may change the surface properties.

3| Treat the KOH-activated surface with O_2 plasma in the plasma cleaner set at a plasma strength of 29.6 W. The total input power of the instrument is 200 W. Vacuum pump with a pump speed of 0.83 ft^3 min^{-1} was used to generate a pressure of 0.27 mbar. Monitor the process of plasma development in the chamber.
▲ CRITICAL STEP Flush the plasma cabinet with O_2 to generate an oxidizing environment. Keep the vacuum pump switched on during the whole process. The duration of plasma treatment (3 min) is critical.
? TROUBLESHOOTING

4| Incubate the plasma-oxidized plate well surface with 100 μl of 2% (vol/vol) APTES in DIW.
▲ CRITICAL STEP Place the APTES-added plates in the desiccator and create vacuum. Incubate the vacuum desiccator at 80 °C for 1 h.

5| Equilibrate the desiccator to RT after incubation.
! CAUTION Open the desiccator in a fume cupboard to avoid APTES fumes. Use a fume cupboard and wear protective clothing and safety glasses.
▲ CRITICAL STEP Equilibrating the desiccator stops the APTES vaporization and enhances the uniformity of functionalization.
? TROUBLESHOOTING

6| Wash the APTES-modified plate well surface extensively five times with DIW (as described in Step 1).

Antibody immobilization ● TIMING ~1 h

7| Dissolve 0.4 mg of EDC and 1.1 mg of sulfoNHS in 100 μl of 0.1 M MES (pH 4.7).
▲ CRITICAL STEP The ratio of EDC and sulfoNHS is important for optimal cross-linking. Use the recommended ratios of EDC and sulfoNHS or optimize this ratio.
? TROUBLESHOOTING

8| Incubate 990 μl of the antibody to be immobilized with 10 μl of EDC-sulfoNHS solution for 15 min at RT.

9| Incubate the activated antibody in the APTES-modified plate well surface for 1 h at 37 °C and wash five times with 300 μl of 0.1 M PBS (pH 7.4).
? TROUBLESHOOTING

ELISA ● TIMING ~3 h 30 min (sandwich format)

10| To carry out the HFA ELISA (sandwich format), block the antibody-immobilized surface with 300 μl of 1% (vol/vol) BSA for 30 min at 37 °C; follow this by five washings with PBS, as described in Step 9.
▲ CRITICAL STEP Use filtered BSA or filter the BSA solution before use to remove any microbial or other contaminants.
? TROUBLESHOOTING

11| Add 100 μl of HFA (range from 4.8 pg ml^{-1} to 20 ng ml^{-1}) in triplicate to BSA-blocked wells and incubate for 1 h at 37 °C; follow with five PBS washings, as described in Step 9.
? TROUBLESHOOTING

12| Incubate 100 μl of biotin-conjugated detection antibody in each of the antigen-captured wells for 1 h at 37 °C, and follow by five PBS washings, as described in Step 9.
? TROUBLESHOOTING

13| Add 100 μl of streptavidin-HRP conjugate and incubate for 20 min; follow with five PBS washings, as described in Step 9.
? TROUBLESHOOTING

14| Add 100 μl of TMB-H_2O_2 mixture to each of these wells and incubate for 20 min at RT to develop color.
? TROUBLESHOOTING

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15| Stop the enzyme-substrate reaction with addition of 50 µl of 1 N H_2SO_4 to each well.

16| Record the absorbance at a primary wavelength of 450 nm and secondary wavelength of 540 nm in the Tecan ELISA plate scanner.
▲ **CRITICAL STEP** Determine the absorbance within 10 min of stopping the enzyme-substrate reaction.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
3	No violet color	High O_2 content in the chamber	Restart the plasma etching procedure. Flush less O_2 into the chamber
5	No color development in the enzyme-substrate reaction of the ELISA procedure, whereas the passively adsorbed control plate developed color	Characterize with FTIR spectroscopy to determine whether there is any characteristic amine peaks. If not, then APTES may be degraded (hydrolyzed). If yes, proceed to next troubleshooting step	Use a different batch of APTES
7	Same as in Step 5	Check as in Step 5; if the problem is not resolved after replacing APTES, then the EDC may be degraded/hydrolyzed	Use a new EDC stock. If the problem still persists, check the next trouble-shooting step
9, 11, 12	No color development in the enzyme-substrate reaction of the ELISA procedure	Capture or detection antibody and/ or antigen may have lost functionality	Proteins, such as antibodies or many antigens, are highly susceptible to temperature or storage conditions. Avoid repeated freeze-thaw cycles and store appropriately
10	High background	Check quality of BSA	Use different BSA stock and make up fresh solution in PBS
13	No color development in the enzyme-substrate reaction of the ELISA procedure	Degraded streptavidin	Streptavidin should be stored in the dark. Reconstitute fresh solution and store in the dark
14	Same as in Step 13	TMB or H_2O_2 may be degraded	Use fresh TMB and/or H_2O_2 solution

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● TIMING
Steps 1–6, Surface activation: ~1 h 30 min
Steps 7–9, Antibody immobilization: ~1 h
Steps 10–16, Sandwich format: ~3 h 30 min

ANTICIPATED RESULTS
The developed procedure was demonstrated using HFA as the antigen. With this improved method, a 16-fold increase in assay sensitivity was achieved (**Fig. 2**) in comparison with the conventional assay format. The analytical sensitivity of the developed sandwich ELISA procedure was 39 pg ml^{-1}, with an EC_{50} of 3 ng ml^{-1}, whereas the sensitivity and EC_{50} of the

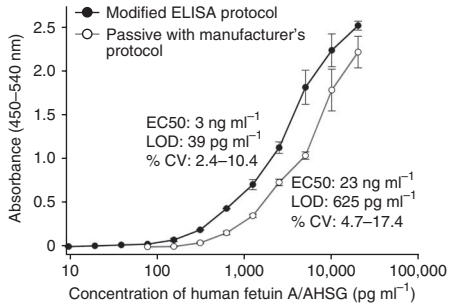

Figure 2 | Assay performance with the developed and conventional ELISA procedure. A concentration range of HFA/AHSG (0–20,000 pg ml^{-1}) was subjected to ELISA on different plates with either covalently immobilized or passively adsorbed antibody. The results shown are the mean ± s.d. The EC_{50} represents the concentration of the analyte/ligand that generates 50% of the maximum signal obtained in the linear working range on a standard curve. This is also known as half-maximum effective concentration. The LOD is the minimum amount of the analyte that is detected when the analyte is subjected to an assay. % CV denotes the variability in measuring a specific analyte concentration in a given number of assay repeats.

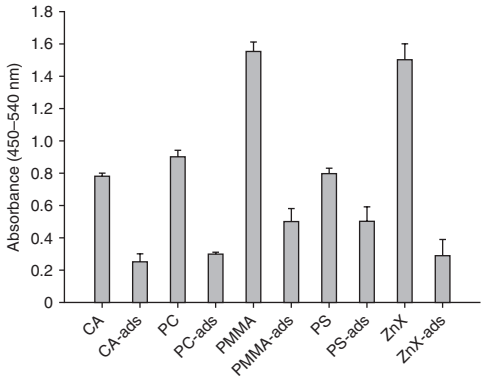

Figure 3 | The performance of the developed procedure on a range of polymeric solid support matrices. The assay was performed at a HFA concentration of 1.25 ng ml^{-1} and a comparison was made. CA, PC, PMMA, PS and ZnX correspond to signals obtained for the assays performed on amine-functionalized cellulose acetate, polycarbonate, polymethyl methacrylate, polystyrene and zeonex, respectively. The suffix '-ads' after the polymer abbreviation (on *x* axis) corresponds to the assay performed with antibody adsorbed on the respective unmodified polymeric matrix, e.g., CA-ads corresponds to signal obtained for the assay performed with antibody adsorbed on unmodified cellulose acetate polymer.

conventional assay were 625 pg ml^{-1} and 23 ng ml^{-1}, respectively. The working/dynamic range for the developed sandwich ELISA was 10–20,000 pg ml^{-1}, whereas for conventional assay it was 150–20,000 pg ml^{-1} (ref. 11). This was mainly because of the higher retention rate of the covalently immobilized antibody, whereas the physisorbed antibody in conventional assays could leach out or be lost because of protein exchanges[3–6]. Another advantage of this strategy is the long-term storage stability of the capture antibody-coated microtiter plates, which can be useful for the development of commercial diagnostics. The developed protocol also enables the utilization of a number of different solid support matrices, irrespective of their chemical nature, as depicted in **Figure 3**. The developed procedure was demonstrated by performing ELISA on different polymeric matrices, such as cellulose acetate, polystyrene, PMMA and polycyclo-olefin (Zeonex), in a modified microtiter plate format[11,24]. Thus, the newly developed procedure has considerable advantages over the existing procedure, which will enable this protocol to find wide applications as an analytical tool in industrial, clinical and research settings.

Note: Supplementary information is available via the HTML version of this article.

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AUTHOR CONTRIBUTIONS C.K.D., S.K.V. and R.O. conceived, designed and refined the method; C.K.D., S.K.V. and R.O. wrote this manuscript; S.K.V., R.O. and B.D.M. supervised the work.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

1. Jia, C.-P. *et al.* Nano-ELISA for highly sensitive protein detection. *Biosens. Bioelectron.* **24**, 2836–2841 (2009).
2. Andrade, G., Barbosa-Stancioli, E.F., Mansur, A.A.P., Vasconcelos, W.L. & Mansur, H.S. Design of novel hybrid organic–inorganic nanostructured biomaterials for immunoassay applications. *Biomed. Mater.* **1**, 221–234 (2006).
3. Ball, V., Huetz, P., Elaissari, A., Cazenava, J.P., Voegel, J.C. & Schaaf, P. Kinetics of exchange processes in the adsorption of proteins on solid surfaces. *Proc. Natl Acad. Sci. USA* **91**, 7330–7334 (1994).
4. Huetz, P., Ball, V., Voegel, J.C. & Schaaf, P. Exchange kinetics for a heterogeneous protein system on a solid surface. *Langmuir* **11**, 3145–3152 (1995).
5. Essa, H., Magner, E., Cooney, J. & Hodnett, B.K. Influence of pH and ionic strength on the adsorption, leaching and activity of myoglobin immobilized onto ordered mesoporous silicates. *J. Mol. Catal. B* **49**, 61–68 (2007).
6. Wang, X., Wang, Y., Xu, H., Shan, H. & Lu, J.R. Dynamic adsorption of monoclonal antibody layers on hydrophilic silica surface: a combined study by spectroscopic ellipsometry and AFM. *J. Colloid Interface Sci.* **323**, 18–25 (2008).
7. Kaur, J., Boro, R.C., Wangoo, N., Singh, K.R. & Suri, C.R. Direct hapten-coated immunoassay format for the detection of atrazine and 2,4-dichlorophenoxyacetic acid herbicides. *Anal. Chim. Acta.* **607**, 92–99 (2008).
8. Mansur, H., Palhares, R., Andrade, G., Piscitelli-Mansur, A. & Barbosa-Stancioli, E. Improvement of viral recombinant protein-based immunoassays using nanostructured hybrids as solid support. *J. Mater. Sci. Mater. Med.* **20**, 513–519 (2009).
9. Gomez-Serrano, V., Acedo-Ramos, M., Valenzuela-Calahorro, C. & Lopez-Peinado, A.J. Regeneration of activated carbon after contact with sulfuric acid solution. *J. Chem. Technol. Biotechnol.* **75**, 835–839 (2000).
10. Vasconcellos, A.S., Oliveira, J.A.P. & Baumhardt-Neto, R. Adhesion of polypropylene treated with nitric and sulfuric acid. *Eur. Polym. J.* **33**, 1731–1734 (1997).
11. Dixit, C.K., Vashist, S.K., O'Neill, F.T., O'Reilly, B., MacCraith, B.D. & O'Kennedy, R. Development of a high sensitivity rapid sandwich ELISA procedure and its comparison with the conventional approach. *Anal. Chem.* **82**, 7049–7052 (2010).
12. Patel, G.N. & Bolikal, D. Single step pre-swelling and etching of plastics for plating. US patent no. 5,049,230 (1991).
13. Patel, G.N. & Patel, S.H. Etching plastics with nitrosyls. US patent no. 5,591,354 (1997).
14. Park, S. & Jung, W. Effect of KOH activation on the formation of oxygen structure in activated carbons synthesized from polymeric precursor. *J. Colloid Interface. Sci.* **250**, 93–98 (2002).
15. Vijayendran, R.A. & Leckband, D.E. A quantitative assessment of heterogeneity for surface-immobilized proteins. *Anal. Chem.* **73**, 471–480 (2001).
16. Park, S., Seo, M., Ma, T. & Lee, D. Effect of chemical treatment of Kevlar fibers on mechanical interfacial properties of composites. *J. Colloid Interface Sci.* **252**, 249–255 (2002).
17. Svarnas, P., Spyrou, N. & Held, B. Polystyrene thin films treatment under DC point-to-plane low-pressure discharge in nitrogen for improving wettability. *Eur. Phys. J. Appl. Phys.* **28**, 105–112 (2004).
18. Laib, S. & MacCraith, B.D. Immobilization of biomolecules on cycloolefin polymer supports. *Anal. Chem.* **79**, 6264–6270 (2007).
19. Raj, J. *et al.* Surface immobilisation of antibody on cyclic olefin copolymer for sandwich immunoassay. *Biosen. Bioelectron.* **24**, 2654–2658 (2009).
20. Boulares-Pender, A., Prager-Duschke, A., Elsner, C. & Buchmeiser, M.R. Surface-functionalization of plasma-treated polystyrene by hyperbranched polymers and use in biological applications. *J. Appl. Polym. Sci.* **112**, 2701–2709 (2009).
21. North, S.H., Lock, E.H., Cooper, C.J., Franek, J.B., Taitt, C.R. & Walton, S.G. Plasma-based surface modification of polystyrene microtitre plates for covalent immobilization of biomolecules. *Appl. Mater. Interface* **2**, 2884–2891 (2010).

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22. Kaur, J., Singh, K.V., Raje, M., Varshney, G.C. & Suri, C.R. Strategies for direct attachment of hapten to a polystyrene support for applications in enzyme-linked immunosorbent assay (ELISA). *Anal. Chim. Acta.* **506**, 133–135 (2004).
23. Lacy, A. *et al.* Rapid analysis of coumarins using surface plasmon resonance. *J. AOAC Int.* **89**, 884–892 (2006).
24. Vashist, S.K., O'Sullivan, S.A., O'Neill, F., Holthofer, H., O'Reilly, B. & Dixit, C.K. A multiwell plate for biological assays. WIPO, Publication no. WO2010/044083 (2010).
25. Song, Y., Hildebrand, H. & Schmuki, P. Optimized monolayer grafting of 3-aminopropyltriethoxysilane onto amorphous, anatase and rutile TiO_2. *Surf. Sci.* **604**, 346–353 (2010).
26. Cass, T. & Ligler, F.S. *Immobilized Biomolecules in Analysis: A Practical Approach* (Oxford University Press, 1998).
27. Armbruster, D.A., Schwarzhoff, R.H., Hubster, E.C. & Liserio, M.K. Enzyme immunoassay, kinetic microparticle immunoassay, radioimmunoassay, and fluorescence polarization immunoassay compared for drugs-of-abuse screening. *Clin. Chem.* **39**, 2137–2146 (1993).
28. Armbruster, D.A., Tillman, M.D. & Hubbs, L.M. Limit of detection (LOD)/limit of quantitation (LOQ): comparison of the empirical and the statistical methods exemplified with GC-MS assays of abused drugs. *Clin. Chem.* **40**, 1233–1238 (1994).

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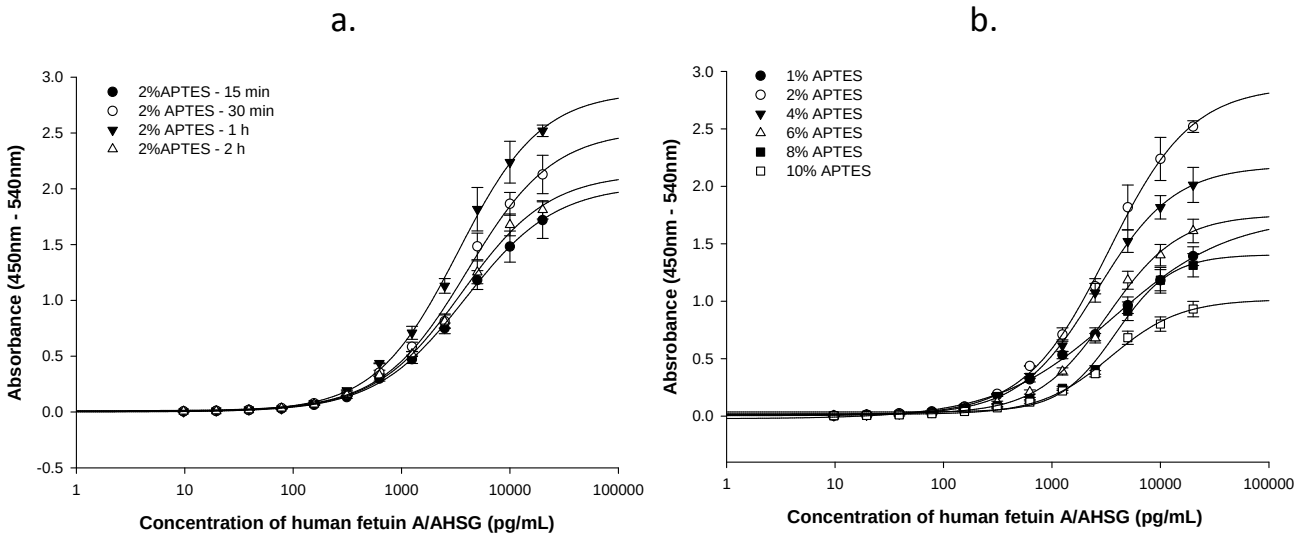
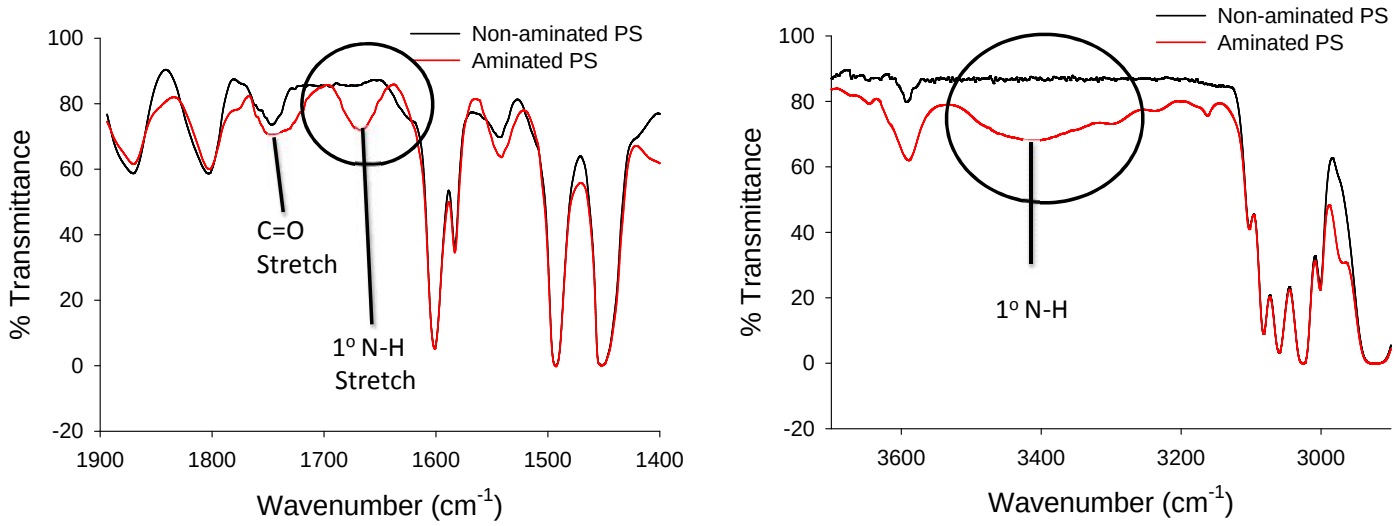
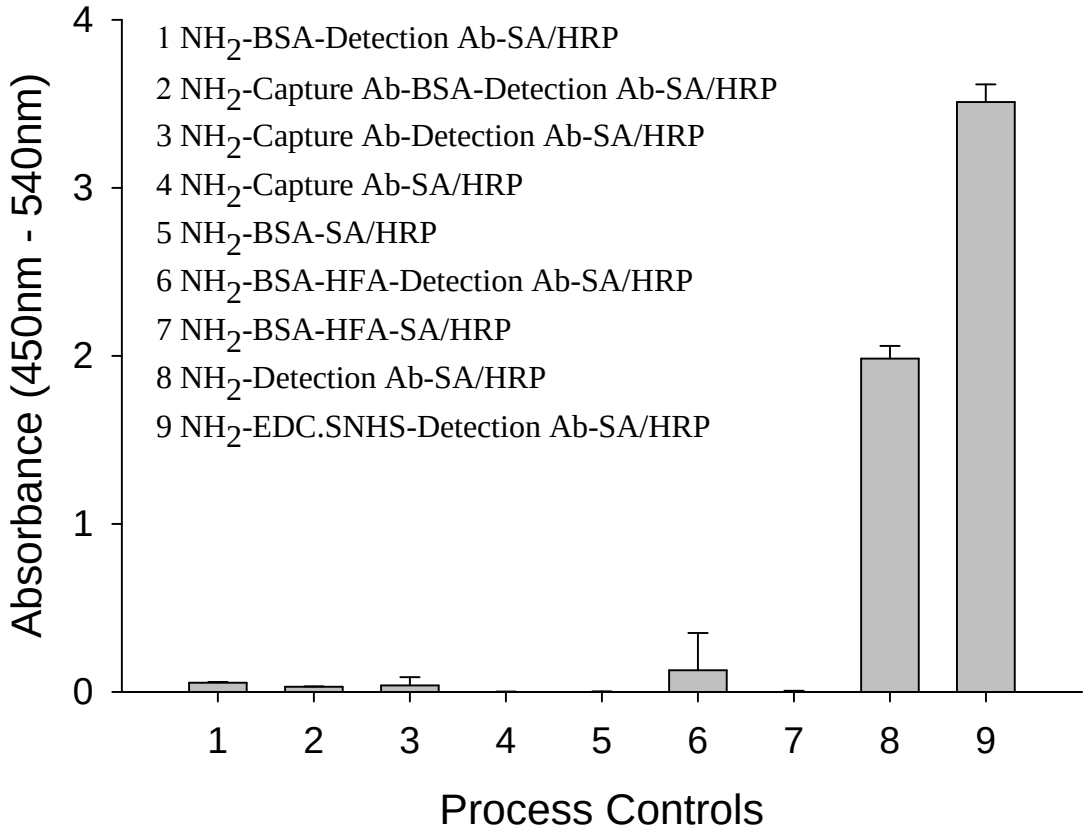


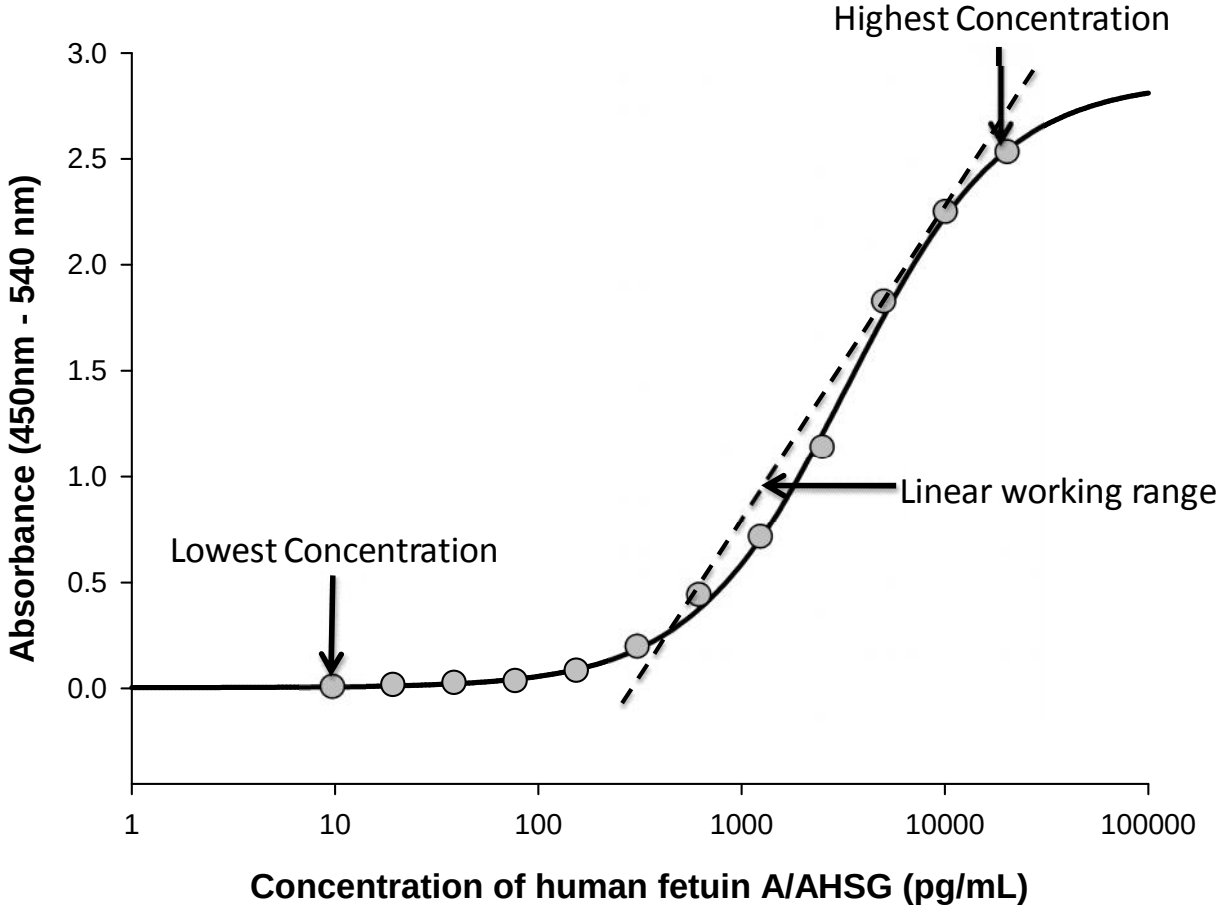
Supplementary Information Fig. 1. Optimization of silanization duration and APTES concentration for the developed protocol. Optimization of silane concentration (b) and silanization time with optimum concentration (a). The selection of optimum concentration and time was based upon the corresponding optimum signals obtained by specific set. Figure 'a' demonstrates that 1hr is the optimum duration for silanization using 2% (v/v) APTES. The figure 'b' shows that 2% (v/v) APTES is the optimum conc. If conc. greater than 2% (v/v) APTES are used, there is a gradual reduction in the assay signal. This may be attributed to the formation of multilayers, which causes decrease in the signal as additional molecules are not correctly oriented and thus their amino groups are not accessible for covalent binding.



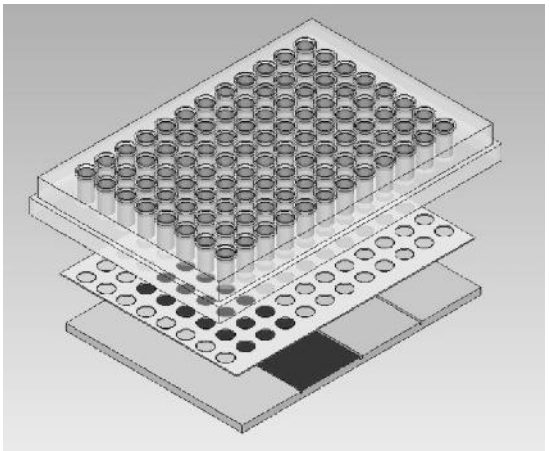
Supplementary Fig. 2. Characterization of amino silane-functionalization of the polystyrene surface. FTIR analysis of APTES-based amine-functionalized polystyrene surface. The Perkin Elmer FTIR instrument was employed to analyze the functionalization. Characteristic N-H stretch and vibrations are visible in the spectra. A ninhydrin test was also performed in addition to contact angle measurement in order to characterize the appearance of amine groups (data not shown).



Supplementary Fig. 3. Relevant process controls employed to develop the reported procedure. To optimize the developed procedure various process controls were employed. These process controls helps in analyzing the cross-reactivity of the various assay components against each other. Since the antibodies and other proteins are developed in animal models, therefore, the probability of cross-reactivity among the biomolecules of animal origin is always a significant consideration. Various process controls employed to monitor the efficacy of each process step. NH_2 corresponds the amine-functionalized microtiter plate. Whereas, all the components in the process controls 1 to 8 were adsorbed on the amine-functionalized plate. Process controls 1-8 were employed to analyze the cross-reactivity of the kit components that is non-specific interaction of these components on plate. Significantly negligible absorbance obtained for controls 1-7 suggests that there is minimum or no non-specific adsorption. Whereas, control 8 suggests high functional efficiency of the detection system that is streptavidin-HRP. In case of control 9, the biotin-conjugated detection antibody was covalently immobilized on the functionalized plate that serves as a positive control and determines the efficacy of the immobilization procedure on the amine-functionalized surface. 'NH_2-BSA' controls were employed to analyze the blocking potential of BSA. Since BSA is used as surface blocker in this study this control also checks the cross-reactivity of kit components to BSA. 'Ab' corresponds to antibody and 'SA/HRP' to streptavidin-HRP conjugate.



Supplementary Figure 4. Standard curve analysis based on a four-parameter logistic in Sigmaplot 11 software. The standard curve plotted using Sigmaplot 11.0 software. An ideal standard curve as represented here is sigmoidal 'S' shaped. The dotted line represent the linear working range. Various analytical parameters such as EC50 and Hill slope can be determined using mathematical calculations for this curve (Supplementary Methods).



Supplementary Fig. 5. Procedure for assembling the modified microtitre plate for performing assays on various polymeric substrates using the developed protocol.

Step 1. The bottomless microtitre plates were preblocked with 1% (v/v) BSA by putting the plate in a container for 30 min.

Step 2. The preblocked bottomless microtiter plates were attached to the double-sided pressure sensitive adhesive, which has the same patterns of holes as the microtiter plate wells.

Step 3. The other side of the double-sided pressure sensitive adhesive attached to the bottomless microtiter plate was then attached to the polymeric substrate(s) in the form of slides or sheets.

Step 4. The assembled modified microtiter plate was then pressed together to form strong attachment of the bottomless microtiter plate and substrate by the pressure sensitive adhesive.

Specific details of the modified microtiter plate are provided in a patent application[1].

1. Vashist, S.K., O'Sullivan, S.A., O'Neill, F., Holthofer, H., O'Reilly, B. & Dixit, C.K. A multiwell plate for biological assays *WIPO,* Publication number WO2010/044083 (2010).

Supplementary Methods.

The data described in this section supplements the main text. It provides information about the determination of analytical information for the modified ELISA protocol using standard curve analysis based on a four-parameter logistic in SigmaPlot 11 software; calculation of LOD; and analytical comparison of the developed protocol with the conventional protocol.

Standard curve analysis based on a four-parameter logistic in SigmaPlot 11 software

The procedure used, as described below, is useful approach for plotting ELISA graphs. The method of calculating EC50 is also specified.

The procedure for creating a standard curve (Supplementary Figure 1) for the modified ELISA protocol using SigmaPlot 11 software is described here:

[Click "Pharmacology" in the Sigmaplot 11 toolbar on top of the screen → click "Standard Curves Analysis" → in "Equation", use "Four Parameter Analysis" and tick the "Log X axis scale" and plot the graph.]

The software also generates a separate report file (as shown below), while plotting the graph, which contains the analytical information pertaining to Hill slope, EC50, and min and max of the plotted curve. The format of the report generated is shown below:

Equation: Standard Curves, Four Parameter Logistic Curve
f1 = min + (max-min)/(1 + (x/EC50)^(-Hillslope))
f = if(x<=0, if(Hillslope>0,min,max), f1)

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.99	0.99	0.99	0.04

	Coefficient	Std. Error	t	P
min	0.0038	0.0215	0.1781	0.8631
max	2.8703	0.1000	28.7068	<0.0001
EC50	3331.2840	270.2485	12.3267	<0.0001
Hillslope	1.1322	0.0727	15.5807	<0.0001

(A similar procedure was used for the ELISAs produced with passive adsorption of antibody)

Calculation of Limit of Detection (LOD).

The calculation of LOD is shown with an example below.

Table depicting the three repeats of a blank and two dilutions of sample 'X'

	Absorbance 1	**Absorbance 2**	**Absorbance 3**	**Average**	**Standard deviation**
Blank	0.0485	0.0397	0.0416	0.0432	0.0039
Sample dil A	0.0496	0.0413	0.0429	0.0446	0.0083
Sample dil B	0.0683	0.0649	0.0701	0.0678	0.0103

The LOD was calculated using the widely used formula

Absorbance corresponding to the LOD = Average absorbance of the blank from all the repeats + 3 standard deviations

For this data, the absorbance corresponding to the LOD will be

= 0.0432 + 3 (0.0039) = 0.0539

Therefore, the concentration corresponding to the absorbance of 0.0539 on the data curve will be the LOD.

The working/ dynamic range of the assay was determined from the standard curve discussed previously.

Analytical comparison of the developed protocol with the conventional protocol [1]

	Developed protocol	Conventional protocol
Detection Range (pg/mL)	4.0-20, 000	151.0-20,000
LOD (pg/mL)	9.0	312.0
Analytical Sensitivity (pg/mL)	39.0	625.0
EC_{50} (pg/mL)	3, 000	23, 000
% CV		
Intra-day (n=5)	2.4-10.4	4.7-17.4
Inter-day (n=3)	1.7-17.6	3.6-20.0
Assay duration (hours)	~6	~20
Assays on various substrates	Yes	No

LOD = Limit of Detection; CV = Coefficient of Variation

Intra-day precision was calculated from the five repeats (n=5) of the same assay at different times on a single day, while inter-day precision was calculated from the three assay repeats on three different days (n=3). All the assays were carried out in triplicate. Analytical sensitivity was calculated using the formula [average absorbance of the blank+3(SD_{blank})].

References:

1. Dixit, C.K., Vashist, S.K., O'Neill, F.T., O'Reilly, B., MacCraith, B.D. & O'Kennedy, R. Development of a high sensitivity rapid sandwich ELISA procedure and its comparison with the conventional approach, *Anal. Chem*. **82**, 7049 (2010).