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

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Published online 10 March 2011; doi:10.1038/nprot.2011.304

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.

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.

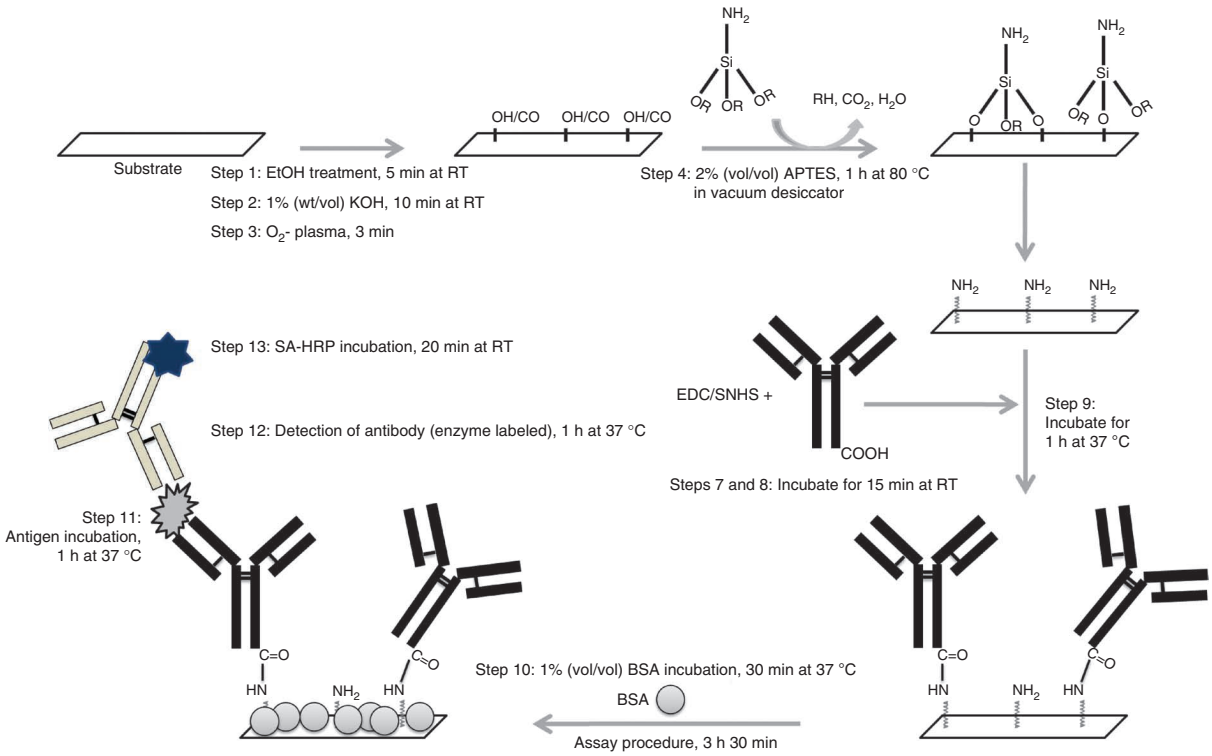

Figure 1 | Schematic representation of the optimized surface modification and antibody immobilization strategy. The method involves 1% (wt/vol) KOH treatment that generates OH/CO groups on the polymeric surfaces, depending on their chemical nature; this facilitates the reaction of amine-terminal silane (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.

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.

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

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

● 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

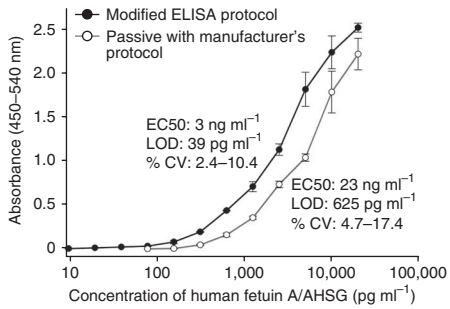

Figure 2 | Assay performance with the developed and conventional ELISA procedure. A concentration range of HFA/AHSG (0–20,000 pg ml^{-1}) was subjected to ELISA on different plates with either covalently immobilized or passively adsorbed antibody. The results shown are the mean ± s.d. The EC_{50} represents 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.

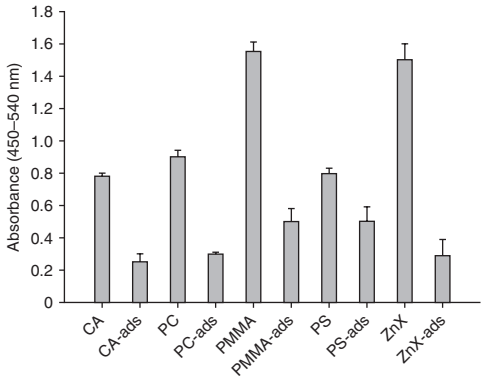

Figure 3 | The performance of the developed procedure on a range of polymeric solid support matrices. The assay was performed at a HFA concentration of 1.25 ng ml^{-1} and a comparison was made. CA, PC, PMMA, PS and ZnX correspond to signals obtained for the assays performed on amine-functionalized cellulose 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.

ACKNOWLEDGMENTS We acknowledge Bristol-Myers Squibb (BMS), Syracuse, USA and Industrial Development Agency, Ireland for the financial support under the Centre for Bioanalytical Sciences (CBAS) project code 116294. This material is based on work supported in part by the Science Foundation Ireland under grant 05/CE3/B754.

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.

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