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# A signal amplification probe enhances sensitivity of antibodies and aptamers based Immuno-diagnostic assays

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ABSTRACT

One major unmet need is improving the sensitivity of immune-diagnostic assays. This is particularly important in the field of biomarker discoveries and monitoring. We have established a novel signal amplification probe system enabling a highly sensitive target detection platform to be used in immuno-assays.
The probe consists of a double stranded DNA that can carry a large number of signaling elements such as biotin or fluorescent molecules. The DNA probe anchors to the recognition unit, whether an antibody or an aptamer, by covalent conjugation or by a simple and rapid molecular association process.
Binding curves obtained by using the DNA amplification probe are dose dependent and linear over a wide range of antigen concentration.
The optimal slopes are characterized by high signals and low background increasing the assay sensitivity and reducing the limit of detection by up to 10-fold compared to biotinylated antibodies commonly used in ELISA systems.
When using aptamers in combination with the amplification probe for antigen recognition, the limit of detection is comparable to that obtained by biotinylated antibodies. Biotin labeled aptamers practically cannot be used for detection of low target levels. The DNA amplification probe system enables to expand the range of diagnostic assays including clinical samples and meet research needs.



## 1. Introduction

Immuno-diagnostic tests are in need for highly sensitive detection of very low levels of antigens including soluble and cell surface bio markers, infectious agents and small molecules (Gomes et al., 2010). Early detection of biomarkers is a crucial step in a successful therapeutic process in cancer, infections and the identification of risk conditions (Solanke et al., 2005; Charbgoo et al., 2012 ). Many of the biomolecules are naturally present in the tested samples at very low levels, therefore the detection process is difficult to achieve, unless highly sensitive systems are used (Salomon and Singh, 2015). Hence, increasing the sensitivity of detection systems has always been considered as one of the major necessities in development of these technologies.

Common diagnostic tests are mainly based on the well establish enzyme immunoassays (Lequin, 2005; Lynch and Howe, 1987), fluorescence based immunoassays and immune-histology (Koivunen and Krogsrud, 2006; Herzenberg et al., 2002). These tests are based on antibodies that are attached to signaling elements and are used extensively as diagnostic tools for recognition and detection of target antigens. In general, signal enhancement is achieved by using Biotin-Streptavidin complex (Gitlin et al., 1990) and fluorescence based analysis. The current technologies of increasing sensitivity all are encountered with imbedded disadvantages. One method of increasing sensitivity is by increasing the number of signaling elements attached to the antibody or increasing antibody concentrations in the assays. This usually results in relatively high background levels, interfering with the assay sensitivity and specificity (Lequin, 2005).

Recent development of nucleic acid aptamers offered the possibility of using aptamers as antibody replacement in immune assays (Kedzierski et al., 2012; Bunka and Stockley, 2006; Song et al., 2012; Jayasena, 1999). Aptamers, also known as chemical antibodies, are short nucleic acid oligos (DNA or RNA, 15–80 bases), which acts like single chain monoclonal antibodies. Aptamers have numerous advantages over antibodies in diagnostics. Their development is independent of the immunogenicity of the target and does not depend on animal experimentations. In addition, their production is fast and fully automated minimizing batch to batch variability. However, aptamers can carry one signaling element limiting the sensitivity of the aptamers based assays. Therefore, the theoretical advantages of aptamers, integrating

*Abbreviations:* ELISA, enzyme-linked immunosorbent assay; PDGF, platelet-derived growth factor; TNF, tumor necrosis factor; SATA, N-succinimidyl *S*-acetylthioacetate; SMCC, succinimidyl 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate; PBST, phosphate buffered saline-tween; HRP, horse radish peroxidase; SD, standard deviation.

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aptamers as replacements for antibodies in the existing ELISA like systems, gave no advantage over antibodies and failed to interest the diagnostic community. In order to increase the sensitivity of aptamers in immune diagnostics assays, they were employed together with detectors that are mainly based on fluorescence methods, fluorescence resonance energy transfer (FRET) or electrochemical biosensor (Song et al., 2012; Han et al., 2010; Mok and Li, 2008). This requires special readout equipment that is not common in diagnostic laboratories, which usually use the conventional ELISA.

Aiming to generate highly sensitive immuno-assays, we have developed a novel amplification probe that provides high signals and sensitive target detection platform with low background levels. This versatile system could be adapted to both antibodies and aptamers based detection platforms. The probe consists of a double stranded DNA that can carry a large number of signaling elements such as biotin or fluorescent molecules and can anchor by covalent conjugation or by molecular association processes to recognition units, such as antibodies or aptamers, to amplify their signals.

In this study we describe the signal amplification ability of the DNA probe when combined with antibodies and aptamers in ELISA formats. We have used antibody based, aptamer based and combination of the two for increasing sensitivity of target detection. The use of the amplification probe in ELISA yields high signals and low background levels, achieving highly sensitive assays that could not be obtained using antibodies or aptamers alone. Moreover, the use of the probe with aptamers enables the establishment of antibody free, aptamers based conventional sandwich ELISA and other immuno-assays.

## 2. Material and methods

### 2.1. Reagents

Rabbit anti-human PDGF-BB (Peprotech, NJ, USA), mouse anti-human TNF-α (Ebioscience, CA, USA), goat anti-mouse IgG (KPL) were used for coating plates and for detection when combined with the DNA probe. Sheep anti-human α-Thrombin (Thermo Fisher, MA, USA) was used only for coating ELISA plates. Biotinylated rabbit anti-human-PDGF (Peprotech), biotinylated mouse anti-human-TNF-α (Ebioscience), biotinylated goat anti-mouse-IgG (Sigma, MO, USA), biotinylated goat anti-rabbit-IgG (Sigma) and biotinylated sheep anti-human α-Thrombin (Thermo-Fisher) were used for antigen recognition and detection.

### 2.2. Aptamers

5′ $NH_2$ - PDGF-T08 (Ahmad et al., 2011) was used for antigen capturing, anti PDGF-BB1 aptamer (Soontornworajit et al., 2010), PDGF-BB2 (Li et al., 2007) and thrombin binding aptamer (Bock et al., 1992) were used for antigen recognition, biotinylated or linked to the probe. All aptamers were synthesized at Ella Biothec DGR (Martinsried, Germany).

The antigens used were human PDGF-BB (Prospec, Rehovot, Israel), human TNF-α (Peprotech), mouse-IgG (Sigma) and human α-Thrombin (MyBioSource.com).

### 2.3. DNA probe

Noncoding synthetic DNA fragment of 3205 bp was inserted into pGMT Easy plasmid into the multiple cloning region between T7 and SP6 at the 5′ end and the 3′ end respectively (Patent No: US 14/565,759 was filed on July 6, 2011, European Patent Application No. 11739164.9 filed on July 6, 2011. "Nucleic acid aptamer-based detection methods characterized by signal enhancement").

### 2.4. Synthesis of DNA probe

The DNA probe was synthesized by PCR reaction using Taq DNA polymerase (Jena Bioscience, Jena, Germany) with homologs primers (see supplement) depending on the selected size of the DNA probe. The PCR reaction includes different ratios of dCTP and biotin labeled dCTP. The PCR product was precipitated using 300 mM sodium acetate and 1:1 isopropanol for at least 2 h at −80 °C. Samples were loaded onto 1% agarose gel containing ethidium bromide. DNA fragments were extracted and purified using zymo-spin™ V-E column (zymo research, CA, USA) according to manufacturer protocol. Concentration of the biotinylated probe was determined by measuring absorbance at 280 and 260 nm.

### 2.5. Conjugation of anchoring elements to antibodies

Conjugation of antibodies to anchoring elements was performed using cross linkers according to manufacturer's protocols. N-succinimidyl *S*-acetylthioacetate (SATA) was attached to the antibody and succinimidyl 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate (SMCC) to the anchoring element. The two components were mixed and incubated overnight (O·N) at RT. Labeled antibody was purified on Hi Trap Q FF (GE life sciences) and eluted with NaCl gradient. Fractions obtained after column separation were tested for IgG content using biotinylated anti-IgG antibody. Fractions containing the labeled IgG were pooled and protein concentration was determined by IgG standard curve in the fractions test.

### 2.6. Attaching aptamers to ELISA plates

96-well ELISA plates (Nunc Inc.) were first coated with 350 μL of 3% BSA in PBST (PBS, 0.05% tween-20) O·N at 4 °C. Aptamers with $NH_2$ terminus were linked to the BSA using Traut's reagent and SMCC according to manufacturer protocols. The plates were washed with PBST.

### 2.7. Antibody based ELISA

96-wells ELISA plates were coated with 50 μL IgG capturing antibodies at 1–5 μg/mL in PBS and incubated 1 h at 37 °C. Plates were then blocked with 3% BSA in PBST (PBS with 0.05% tween-20) O.N. at 4 °C. Plates were washed with wash buffer (PBST containing 0.5% BSA). Antigens at various concentrations (50 μL) were added for 1 h at RT on a rotary shaker and then plates were washed three times. For detection, labeled antibodies (0.5–50 ng per well) or biotinylated antibodies (0.5–250 ng per well) in wash buffer were added for 1 h at RT on a rotary shaker. Plates were washed three times. DNA amplification probe (1 pmol/well) in PVP buffer [20 mg/mL Polyvinylpyrrolidone, 1% salmon sperm (Sigma)] in PBST containing 0.5% BSA was incubated for 1 h at RT on a rotary shaker. After washing, streptavidin HRP (1:10,000 dilution, Sigma) was added and incubated for 30 min at RT on a rotary shaker. After washing 60 μL of TMB (Southern Biotech) were added for 5–30 min. Color development was stopped by adding 60 μL stop solution (Southern Biotech). Absorbance was measured at 450 nm by Epoch Microplate Spectrophotometer (BioTek). Limit of detection was calculated as the antigen levels correspond to twice the background signal in each binding curve. Measurements were done in duplicates ($n = 2$) or in six replicates ($n = 6$) (Shrivastava and Gupta, 2011; Apostol et al., 2012; Armbruster and Pry, 2008). Data represent three independent experiments.

### 2.8. Aptamers based ELISA

The assay was performed using the aptamer coated plates with the same ELISA protocol except PBST was used as a wash buffer and for DNA probe incubation. Labeled aptamers were used at 0.1–50 pmol per well and biotinylated aptamers were used at 3–100 pmol per well.