High sensitivity multianalyte immunoassay using covalent DNA-labeled antibodies and polymerase chain reaction

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ABSTRACT

A multianalyte immunoassay for simultaneous detection of three analytes (hTSH, hCG and β -Gal) has been demonstrated using DNA-labeled antibodies and polymerase chain reaction (PCR) for amplification of assay response. The labeled antibodies were prepared by covalently coupling uniquely designed DNA oligonucleotides to each of the analyte-specific monoclonal antibodies. Each of the DNA oligonucleotide labels contained the same primer sequences to facilitate co-amplification by a single primer pair. Assays were performed using a two-antibody sandwich assay format and a mixture of the three DNA-labeled antibodies. Dose-response relationships for each analyte were demonstrated. Analytes were detected at sensitivities exceeding those of conventional enzyme immunoassays by approximately three orders of magnitude. Detection limits for hTSH, β -Gal and hCG were respectively 1×10^{-19} , 1×10^{-17} and 1×10^{-17} mol. Given the enormous amplification afforded by PCR and the existing capability to differentiate DNA based on size or sequence differences, the use of DNA-labeled antibodies could provide the basis for the simultaneous detection of many analytes at sensitivities greater than those of existing antigen detection systems. These findings in concert with previous reports suggest this hybrid technology could provide a new generation of ultra-sensitive multianalyte immunoassays.

INTRODUCTION

DNA replication of antibody-borne DNA labels has recently greatly extended the sensitivity of immunoassays (1). This hybrid technology, referred to as immuno-PCR (1), combines the versatile molecular recognition of antibodies with the high amplification potential of DNA replication (PCR) (2,3). Using immuno-PCR as

few as 600 molecules of an immobilized antigen (bovine serum albumin) have been detected (1). Sensitive response has also been confirmed in immuno-PCR assays for mouse anti-lipoprotein immunoglobulin G (4) and a human proto-oncogene protein (5).

Antibodies in these examples were indirectly labeled with biotinylated DNA by using different biotin binding proteins. For example, Sano *et al.* (1,6) constructed a protein A-streptavidin chimera protein (7) capable of simultaneously binding antibody and biotinylated DNA label. Ruzicka *et al.* (4) used commercially available avidin to join the biotinylated DNA label and antibody. Zhou *et al.* employed streptavidin to link biotinylated DNA and antibody in this way forming a 'universal' reporter complex (5).

In these approaches, the labeled DNA-antibody complexes are assembled *in situ* during the assay. This can create variable stoichiometry in the assembly of the components and in the attachment of the DNA label. Furthermore, extra steps are required for addition of biotinylated reagents and binding proteins. Numerous wash steps are also needed to remove excess reagents and to free assay components of non-specifically bound reagents. As a consequence immuno-PCR assays have been procedurally complex and have required considerable hands-on time.

We now report on an advancement in immuno-PCR that reduces assay complexity and furthermore, permits multiple analytes to be simultaneously detected. This was achieved through labeling antibody with DNA by direct covalent linkage of the DNA to the antibody. In this approach, the analyte-specific antibody and the 5' amino-modified DNA oligonucleotide are independently activated by means of separate heterobifunctional cross-linking agents. The activated antibody and DNA label are then coupled in a single spontaneous reaction. Specifically, antibodies against human thyroid stimulating hormone (hTSH), human chorionic gonadotropin (hCG), and β -galactosidase (β -Gal), were labeled with specific DNA sequences and incorporated into a new variant of immuno-PCR based on the widely practiced two-antibody sandwich assay format (8,9) (Fig. 1). Immuno-PCR response for each of the three assays was determined over a wide range of analyte concentrations and then compared with ELISA assays developed using the same antibodies. A multianalyte immuno-PCR assay for simultaneous

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Figure 1. Schematic of immuno-PCR sandwich assay for single analytes. The assay uses analyte capture and reporter antibody reagents. The reporter antibody is labeled with a single-stranded DNA by covalently linking an oligonucleotide to the antibody. Test analyte is immunochemically captured using a solid-phase antibody specific for the analyte. A DNA-labeled reporter antibody binds to a second recognition site on the analyte molecule. The resulting molecular sandwich complex of the two antibodies and analyte is washed to remove excess reagents and test materials. The bound DNA label is then replicated using PCR. Amplified DNA products are then analyzed by agarose gel electrophoresis. PCR generates amplified DNA product with length and sequence characteristic of the original DNA label. Therefore, the amplified DNA is indicative of the presence and identity of the analyte. In this way, the amplified DNA functions as a surrogate molecule for recognition and detection of the analyte.

detection of all three analytes was then demonstrated using the sandwich assay format.

These findings, in concert with previous reports (1,4,5) indicate DNA-labeled antibodies can now be used to circumvent current limitations in immunoassay sensitivity and in the number of analytes detectable per test. The immuno-PCR sandwich assays, further, can be accomplished using routine immunoassay procedures.

MATERIALS AND METHODS

Reagents

The test analytes, human thyroid stimulating hormone (hTSH), human chorionic gonadotropin (hCG) and β -galactosidase from *E. coli* (β -Gal) were obtained from Calbiochem Corp. (La Jolla, CA). The murine monoclonal antibodies [anti-hTSH IgG1 (972.3.1), anti-hCG IgG1 (735329.306), and anti- β -Gal IgG (29A51.1)] that were used to covalently couple single-stranded (ss) DNA oligonucleotides to form the reporter conjugates were obtained from the DuPont Co. (Wilmington, DE). Murine monoclonal antibodies [anti-TSH IgG1 (4/46), anti-hCG IgG (735329.302), anti- β -Gal IgG2B (29B1/178.1)] that were used for solid-phase capture were also obtained from the DuPont Co. (Wilmington, DE). Cross-linking reagents *N*-succinimidyl-*S*-acetylthioacetate (SATA) and sulfo-succinimidyl 4-(maleimido-methyl)cyclohexane-1-carboxylate (sulfo-SMCC) were purchased from Pierce Chemical Co. (Rockford, IL). PCR reagents and *Taq* DNA polymerase (Ampli[®]Taq) were obtained from Perkin-Elmer Corp. (Norwalk, CT). The β -cyanoethyl phosphoramidite amino-modifying reagent (Aminolink 2[™]) was purchased from Applied Biosystems (Foster City, CA).

Figure 2. Design of DNA-antibody reporter conjugates for immuno-PCR sandwich assays. DNA-antibody conjugates, composed of an analyte-specific monoclonal antibody (MAB) and a covalently-coupled single-stranded DNA label of discrete size, were designed for three immuno-PCR tests. This diagram illustrates the labeling of three different antibodies with different-sized ssDNA. The β -Gal test conjugate was prepared with a 55 base label, the hTSH test conjugate with an 85 base label, and the hCG test conjugate with a 99 base label. All labels were designed to use the same set of primers. For multianalyte testing, the covalently-linked reporter conjugates were adapted to a multianalyte format. The identification of multiple analytes in an immuno-PCR assay was determined by the size of the PCR products as analyzed by agarose gel electrophoresis.

Primers and DNA labels

Design of primers and DNA labels. The primers and DNA labels were designed with the aid of Oligo[™] 4.0 (National Biosciences Inc., Plymouth, MN). Primers were designed with random base sequences that exhibit specificity, G + C content (~50%), defined duplex stability (T_m , 52°C) and duplex internal stability for PCR primers as described by Rychlik *et al.* (10,11). The DNA labels were designed with three defined sequence regions: the 5' primer sequence, the complement sequence to the 3' primer and a variable 'stuffer' sequence between the primer-defined sequences (Fig. 2). Each label was designed with the same primer sequences to facilitate co-amplification by a single primer pair. Both the primer and label sequences were designed to be free of duplex formation (dimers or hairpins), and to have sequences lacking stretches of homopolymers. Further, the primers were designed so that the sequences comprising the ten 3' terminal bases of each primer exist only within the primer 'binding' sites of the DNA label and nowhere else in the sequences of the label or its complement.

Oligonucleotide synthesis. DNA oligonucleotide primers and reporter labels were prepared using standard β -cyanoethyl phosphoramidite coupling chemistry on controlled pore glass (CPG) supports (12) in automated DNA oligonucleotide synthesizers [DuPont Generator[™] (Wilmington, DE) or Applied Biosystems Model 392 (Foster City, CA)]. The two primer oligonucleotide sequences were: P10: 5'-GGCAGGAAGACAAACA-3' and P20: 5'-ACAGCACCACAGACCA-3'. The DNA label sequences were synthesized and assigned to a specific reporter antibody as follows:

anti- β -Gal: 5'-GGCAGGAAGA CAAACACTGT GCTTGTTA CT GAACTCTAGT GGTCTGTGGTGTGT-3' (55 bases);

anti-hTSH: 5'-GGCAGGAAGA CAAACAATGA TACTGAA TAC TGAATGTACT TACTGAACTC TAGCTCTAGT CTC TCTCTCT GGTCTGTGGT GCTGT-3' (85 bases);

anti-hCG: 5'-GGCAGGAAGA CAAACAATGA TACTGAAT AC TGAATGTACT TACTGAACTC TCTTACTCGT GCAAT AGCTC TAGTCTCTCT CTCTGGTCTG TGGTGCTGT-3' (99 bases).

The 5' terminus of each of these oligonucleotide labels was derivatized using Aminolink 2TM to incorporate a primary aliphatic amine during the final coupling step of the synthesis (13,14). After the deprotection step, the DNA labels were ethanol precipitated. Additional purification steps to remove failure sequences from the preparation were not taken.

Synthesis of the DNA oligonucleotide-antibody reporter conjugates

Synthesis of the DNA-labeled antibody conjugates was accomplished in four phases. In this approach, 5' amino-modified oligonucleotides and analyte-specific antibodies were independently activated by means of separate heterobifunctional cross-linking agents. The activated oligonucleotides and antibodies were then mixed to facilitate spontaneous coupling of the DNA label with the antibody. Specific conditions and protocols for each phase of the synthesis are described below:

Preparation of acetylthioacetyl derivatized DNA. Amino-modified reporter oligonucleotides were reacted with SATA as follows. An aliquot of the amino-modified oligonucleotide preparation, 50–60 nmol, was added to 667 μ l reaction mixture containing 100 mM sodium bicarbonate buffer (pH 9.0), 13.3 mg/ml SATA, and 50% dimethyl formamide (DMF). After 30 min at 25°C, the reaction mixture was immediately applied to a 1 $\times$ 20 cm Sephadex[®] G-25 column (Pharmacia Biotech, Inc., Piscataway, NJ) and eluted at room temperature with 100 mM sodium phosphate buffer, pH 6.5, at a flow rate of ~1 ml/min. The absorbance of the effluent was monitored at 280 nm using a Pharmacia Model 2138 UVICORD S Monitor, and fractions were collected on a Pharmacia Model Frac-100 fraction collector (Pharmacia Biotech, Inc., Piscataway, NJ). Two-milliliter fractions were collected, and those containing the acetylthioacetyl-modified oligonucleotides were pooled. These fractions were concentrated to a final volume of ~1.0 ml using Amicon CentriconTM 3 concentrators (Amicon, Inc., Beverly, MA) and a Sorvall[®] SM-24 rotor in a RC-5B centrifuge (Sorvall[®], DuPont Co., Wilmington, DE), spun at 7500 r.p.m. (7000 g) for 45 min at 20°C. The resulting samples were pooled, and further concentrated using the same procedure in a second set of CentriconTM 3 concentrators. The acetylthioacetyl-modified oligonucleotide concentrate (~1.0 ml) was recovered using the protocol recommended by the manufacturer (Amicon, Inc., Beverly, MA) and was saved at 20°C in the dark until it was needed for the final attachment of DNA label to reporter antibody.

Preparation of maleimide-modified antibodies. The reporter antibodies were derivatized with maleimide groups using sulfo-

SMCC. An aliquot containing 25 nmol of antibody was added to a reaction mixture (2.2 ml) containing 100 mM sodium phosphate buffer (pH 7.0), 1.2 mg/ml sulfo-SMCC, 1.5% DMF. (Note: The antibody modification reaction is started 75 min after beginning the preparation of the acetylthioacetyl-derivatized oligonucleotide. This timing is essential to minimize the deactivation of maleimide groups present in an aqueous solution, prior to the final conjugation reaction.) After the mixture had reacted for 30 min at 25°C, it was immediately applied to a 1 $\times$ 20 cm Sephadex[®] G-25 column and eluted at room temperature with 100 mM sodium phosphate buffer, pH 6.5 at a flow rate of ~1 ml/min. The effluent was monitored and column fractions were collected as previously described for a Sephadex[®] G-25 column. The first peak fractions (2.0 ml/fraction), which contained the maleimide-modified antibody, were pooled (4–6 ml) into one tube. The reaction product was ready for coupling to the modified oligonucleotides.

DNA oligonucleotide-antibody conjugations. The pooled maleimide-modified antibody fraction was immediately added to a 15 ml Falcon[®] 2059 tube (Becton Dickinson, Cockeysville, MD). The concentrated acetylthioacetyl-modified oligonucleotides (~1.0 ml) were added to the same tube and mixed well. The coupling reaction was initiated by adding 75 μ l of 1 M hydroxylamine hydrochloride (Pierce Chemical Co., Rockford, IL), pH 7.0, 50 mM EDTA and mixing well. The reaction mixture was transferred to an Amicon Model 3 mini-ultrafiltration stirred cell fitted with a YM5 membrane filter (Amicon, Inc., Beverly, MA). The cell was connected to a helium source adjusted to 60 p.s.i. The coupling reaction proceeded with stirring at room temperature while the entire vessel was covered with aluminum foil to reduce exposure to light. The reaction mixture was concentrated to ~1.0 ml, removed from the MiniCell apparatus, and transferred to a 4.0 ml amber vial (Wheaton, Inc., Millville, NJ). This vial was incubated in the dark at room temperature on a Lab QuakeTM tube rotator (Labindustries, Inc., Berkeley, CA) until the total reaction time reached 2 h. The reaction was terminated by the addition of 10 μ l of 10 mM *N*-ethylmaleimide in DMF.

Purification of the oligonucleotide-antibody conjugates. The initial step in the purification of the conjugates used gel filtration high pressure liquid chromatography (HPLC). The HPLC system consisted of a Waters Model 600E multisolvent delivery system and Model-991 photodiode array detector (Milford, MA). Separation was accomplished using a mobile phase sodium phosphate buffer (200 mM, pH 7.0) at a flow rate of 1 ml/min through a 9.4 $\times$ 250 mm Zorbax[®] GF-250 column (MAC-MOD Analytical, Inc., Chadds Ford, PA). Injections of the conjugate (200 μ l) were made with a Waters 700 Satellite WISP automated injection system. The first HPLC peak fractions (0.3 ml/fraction) were mixtures of the oligonucleotide-antibody conjugate and the maleimide-modified antibody reaction component that were virtually free of the acetylthioacetyl-modified oligonucleotide precursor peak.

Spectrophotometric scans (320–220 nm) and A_{260/280} ratios measured on a Beckman DU 68 (Fullerton, CA) were used to determine which HPLC fractions contained the oligonucleotide-antibody conjugate. These results were confirmed by 3' end-labeling (terminal deoxynucleotidyl transferase and [α -³²P]-cordycepin, (3'dATP), NEN[®], DuPont, Boston, MA) of the

conjugated oligonucleotide reporter labels followed by gel electrophoresis autoradiography. The conjugate-antibody peak fractions were pooled. The remaining unreacted, free oligonucleotides were removed from the pooled fractions using Microcon™ 100 microconcentrators (Amicon, Inc., Beverly, MA). The recovered conjugates were concentrated using the same procedure and then stored at 4°C.

To determine the purity and average DNA to antibody ratio for each of the conjugates, the conjugate concentrates were characterized by gel filtration HPLC using the conditions previously described. A single peak was observed, comprised of the conjugate and residual unconjugated antibody that was not removed during purification. The average DNA label to antibody ratios for each of the conjugate preparations were determined using the $A_{260/280}$ ratios obtained from absorbance values by the HPLC diode array detector. The ratios were, respectively, 1.09 (anti- β -Gal reporter conjugate), 0.77 (anti-hTSH reporter conjugate) and 0.76 (anti-hCG reporter conjugate).

Immuno-PCR assay for single analytes

The immunoassay protocol. Capture antibody (6 μ g/ml) in 100 mM sodium bicarbonate, pH 9.5, was immobilized on a 96-well, V-bottom, polycarbonate microtiter plate (Concord 25, MJ Research, Inc., Watertown, MA or Thermowell 961, Costar, Corp., Cambridge, MA) by adding 50 μ l/well and incubating overnight (16 h) at 4°C or 1 h at room temperature. Antibody solutions were removed, and the wells were washed three times by adding the assay diluent/wash buffer, TBS/Tween (25 mM Tris, pH 7.4, 50 mM sodium chloride, 0.05% Tween-20), and immediately aspirating the buffer from the wells. The microtiter plate was inverted and slapped vigorously onto absorbent material to remove the residual wash buffer. Non-adsorbed sites in the microtiter wells were blocked with 200 μ l/well of PBS-BLA buffer (10 mM sodium phosphate, pH 7.4, 150 mM sodium chloride, 2% BSA, 10% β -lactose, 0.02% sodium azide) and incubated for 1 h at room temperature. PBS-BLA buffer was removed, and the wells were washed three times as described.

Fifty-microliter aliquots made from serial dilutions of each test analyte were added to the wells of the microtiter plate containing the appropriate capture reagent. Negative control wells received 50 μ l of TBS/Tween buffer. The microtiter plate was incubated at room temperature for 1 h. Analyte solutions were removed and the wells were washed three times as described. Fifty microliters of appropriately diluted oligonucleotide-antibody reporter conjugate were added to the test wells (either a 1:20 000 dilution of the hTSH or hCG DNA-labeled conjugates or a 1:50 000 dilution of the β -Gal DNA-labeled conjugate). The microtiter plate was incubated at room temperature for 1 h. Conjugate solutions were removed and the wells were washed three times as described.

The PCR protocol. The microtiter plate was trimmed for insertion into the 96-well sample block of a Perkin-Elmer GeneAmp™ 9600 thermal cycler (Norwalk, CT). Amplification of the oligonucleotide label conjugated to the assay reporter antibody was performed using the polymerase chain reaction (PCR) (3). The amplification reaction was done in a final volume of 50 μ l containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M dATP, 200 μ M dCTP, 200 μ M dGTP, 200 μ M dTTP, 100 nM each of the amplifying primers, and 1.25 U *Taq* DNA polymerase (Ampli-Taq®, Perkin-Elmer Corp., Norwalk, CT). Thirty microliters of

sterile distilled water were added to each sample well of the microtiter plate. A 5 μ l aliquot of the primer mix was added to the sample wells, followed by a 20 μ l aliquot of liquid wax (Chill-out™, MJ Research Inc., Watertown, MA).

The microtiter plate was inserted into the thermal cycler sample block. The thermal cycler was ramped to 95°C for 5 min (initial denaturation step) and then held at 72°C for a hot start (15). A master mix (3.3 $\times$) containing the reaction buffer, sterile water, MgCl₂, and dNTPs was heated to 72°C and then the *Taq* DNA polymerase was added. A 15 μ l aliquot of master mix at 72°C was added to each test well, dispensing below the liquid wax layer. The microtiter plate was covered and sealed with plate sealing tape (Costar, Inc., Cambridge, MA). A tray assembly was placed over top of the sealed microtiter plate. The tray assembly consisted of a plate weight milled (in-house) to fit inside the Perkin-Elmer MicroAmp™ tray. The heated cover of the thermal cycler was tightened in place to exert even pressure over the plate. Amplification was performed in 40 cycles using the following thermal cycling conditions: reaction volume set for 70 μ l, 94°C for 10 s, 54°C for 15 s and 72°C for 10 s. The final chain extension was made at 72°C for 45 s. The cycler was then ramped to 4°C and held until sample analysis. Note: The 96-well microtiter plate and the modified tray assembly acting as a pressure plate was compared to Perkin-Elmer MicroAmp™ tubes as PCR reaction vessels. They were found to have equivalent performance.

Detection and analysis of PCR products. Amplified products from each assay well were separated on 4% NuSieve® 3:1 agarose (FMC BioProducts, Rockland, ME) in 0.5 $\times$ TBE buffer (Digene Diagnostics, Inc., Silver Spring, MD) containing 0.3 μ g/ml ethidium bromide. An aliquot of 15 μ l from the amplified samples was mixed with 3 μ l of gel loading buffer (30% glycerol and 0.25% bromophenol blue) and loaded onto the agarose gel (8.5 $\times$ 6.0 $\times$ ~0.5 cm:25 ml agarose solution). Gel electrophoresis was carried out by applying 150 V (or 5.9 V/cm) to the gel for 25 min. The ethidium bromide-stained DNA bands were visualized with an UV transilluminator (310 nm wavelength, Model TM-20, UVP, Inc., San Gabriel, CA) and recorded on Polaroid type 665 black and white, positive and negative film (Polaroid Corp., Cambridge, MA). The type 665 negative was scanned using a CCD-50 Xyberion Electronic system and Video Image 1200® software package. The digitized image was further analyzed by using Collage™ software (Fotodyne, Inc., New Berlin, WI) to measure the intensity (pixel intensities per unit area) of the PCR product bands.

Multianalyte immuno-PCR assay

Multianalyte detection was achieved using a combination of DNA-labeled reporter antibodies and solid-phase capture reagents. Reporter reagents were prepared for β -Gal, hTSH and hCG analytes by labeling each of the reporter antibodies with an oligonucleotide 55, 85 and 99 bases in length, respectively (Fig. 2). Each oligonucleotide label was designed with the same primer binding sequences, enabling the DNA labels for each analyte to be co-amplified using a single primer set. The immunoassay protocol was identical to that used for the single-analyte immuno-PCR assays. Solid-phase capture reagent for the assay was prepared with 6 μ g/ml of each of the three analyte-specific antibodies in 100 mM sodium bicarbonate, pH 9.5. The

immobilization procedure was the same as described in the single analyte assay. Non-adsorbed sites in the microtiter wells were blocked by incubating with PBS-BLA buffer as described.

Fifty-microliter aliquots made from serial dilutions of TBS/Tween solutions of the test analytes (individually and in combination with the other two analytes) were added to wells of the microtiter plate containing the capture reagents. Negative control wells received 50 μ l of TBS/Tween buffer. The assay was run as described with one exception. That is, all three reporter antibodies (a reporter cocktail containing 1:20 000 dilutions of the hTSH and hCG DNA-labeled conjugates and a 1:50 000 dilution of the β -Gal DNA conjugate) were used simultaneously in the reporter addition step. The PCR amplification and gel analysis was done as described for the single-analyte assays.

ELISA immunoassays

The immuno-reagents (reporter and capture antibodies) used for ELISA assays for single analytes were the same as those used for immuno-PCR assays, except that the reporter reagents were conjugated to enzymes. The anti-TSH IgG and anti- β -Gal IgG were each conjugated to alkaline phosphatase, and the anti-hCG IgG was conjugated to β -galactosidase (DuPont Co., Wilmington, DE). The ELISA protocols for β -Gal, hTSH and hCG were identical to those used for the immuno-PCR assays, with exception of the signal-generating enzyme used in the assay. For the β -Gal and hTSH ELISA assays, 100 μ l of alkaline phosphatase substrate, para-nitrophenyl phosphate (pNPP), at 1 mg/ml in DEA buffer (1 M diethanolamine, 0.5 mM MgCl_2), was added to each well. After 30 min at room temperature, the absorbance at 405 nm was determined using a microplate reader (Thermo-max, Molecular Devices, Menlo Park, CA). For the hCG ELISA assay, 50 μ l of β -galactosidase substrate, chlorophenol red β -d-galactopyranoside (CPRG), at 0.6 mg/ml in 50 mM HEPES with 1 mM magnesium acetate, was added to each well. After 15 min at room temperature, the absorbance at 562 nm was determined using the same microplate reader.

RESULTS

Immuno-PCR sandwich assays were first investigated in the single analyte format in order to demonstrate the feasibility of the sandwich configuration and to confirm the utility of covalently-coupled, single-stranded oligonucleotide labels. Human thyroid stimulating hormone (hTSH) was selected as the first test analyte because of its clinical importance in the assessment of thyroid function and its requirement for high assay sensitivity. Normal concentrations of hTSH in blood samples are low (5×10^{-12} M, 2.5×10^{-16} mol), generally beyond the detection limit of ELISA (16).

The immuno-PCR sandwich assay for hTSH was performed using microtiter plates coated with anti-hTSH capture antibody. The hTSH reporter antibody was conjugated to an 85 base oligonucleotide DNA label. The assay was performed as described (Fig. 1) and the resulting PCR products were analyzed by agarose gel electrophoresis (Fig. 3). The presence of an 85 base pair (bp) amplification product was detected from wells that contained hTSH. Band intensities of these amplification products increased proportionally with the amount of hTSH in the sample. The lowest level of hTSH detected was 1 fg ($\sim 10^{-19}$ mol) shown in lane 3. PCR products were not observed in negative control samples. These results indicated that the DNA-antibody conjugate formed an effective sandwich complex in response to hTSH.

Figure 3. Gel electrophoretic analysis of hTSH immuno-PCR assay products demonstrating response to increasing concentrations of hTSH. PCR amplification mixtures from hTSH assay (15 μ l) were separated on 4% NuSieve[®] 3:1 agarose (FMC BioProducts, Rockland, ME) and $0.5 \times$ TBE buffer containing 0.3 μ g/ml ethidium bromide. Gel marker (gm): Φ X174 DNA, *Hinf*I marker (Promega Corp., Madison, WI); lanes 1 and 2: immuno-PCR negative controls (no hTSH), lanes 3–8: PCR assay products of samples from assay wells dosed respectively with increasing amounts of hTSH: 10^{-19} mol; 10^{-18} mol; 10^{-17} mol; 10^{-16} mol; 10^{-15} mol; 10^{-14} mol; lane 9: PCR negative control (primers only); lane 10: PCR positive control (1:20 000 dilution of the DNA-labeled conjugate).

Figure 4. Comparison of immuno-PCR dose-response assay to that of ELISA assay using identical antibody reagents. The agarose gel shown in Figure 3 was photographed using Polaroid 665 film, and the negative was scanned and digitized using a CCD-50 Xybion Electronic system and Video Image 1200[®] software package. The PCR product bands were further analyzed using Collage[™] software to integrate them producing values representing pixel intensities per unit area. The response is plotted as band intensity (in arbitrary units) versus mol of analyte per sample. The ELISA dose-response assay for hTSH in a microtiter plate uses the reporter antibody conjugated to alkaline phosphatase. The reporter and capture antibodies were the same as those used with the immuno-PCR assays. The amounts of analyte used in ELISA samples were serial 2-fold dilutions from 1.6×10^{-12} to 10^{-16} mol/test. Negative control received no hTSH. The ELISA color response, developed using *p*-nitrophenyl phosphate as a substrate, is plotted as OD₄₀₅ U versus mol of hTSH per sample.

Further, it was established that a covalently-coupled ssDNA can function as an effective immuno-PCR label.

The sensitivity of the hTSH immuno-PCR assay was compared with a hTSH sandwich ELISA assay (Fig. 4). In both assay formats, dose-response relationships were observed over a range of hTSH concentrations. The detection limit for the ELISA format was ~ 1 pg (10^{-16} mol). Immuno-PCR detected hTSH levels (1 fg, 10^{-19} mol) three orders of magnitude lower than those detected by the ELISA assay.

Similar improvements in sensitivity were observed with immuno-PCR sandwich assays developed for two other clinical analytes, β -galactosidase (β -Gal) and human chorionic gonadotropin (hCG). As with the hTSH assay, label-specific amplification products were observed in response to each analyte. The

Figure 5. Immuno-PCR dose-response assay for three test analytes: hTSH, hCG and β -Gal, in a single analyte format. PCR amplification mixtures from the assays (15 μ l) were separated and the assay response measured as described in Figure 3. Each curve represents an assay response to a series of 10-fold serial dilutions of the specific test analyte: hTSH: 10^{-14} to 10^{-19} mol/test; hCG: 1.3×10^{-13} to 1.3×10^{-17} mol/test, and β -Gal: 10^{-13} to 10^{-16} mol/test and 2×10^{-15} to 2×10^{-17} mol/test. Assay controls were same as those used in Figure 3.

amount of PCR product increased proportionally over a range of concentrations for each analyte (Fig. 5). Immuno-PCR assay detection limits for both β -Gal and hCG were 10^{-17} mol. When testing both analytes in an ELISA format for comparison with the immuno-PCR assays, the detection limits were 10^{-14} mol for β -Gal, and 10^{-15} mol for hCG. These results demonstrate a difference of two to three orders of magnitude higher sensitivity for the immuno-PCR assays over the sensitivity of ELISA assays for both analytes.

The capability to covalently couple different antibodies to unique DNA labels in conjunction with the subsequent formation of label-specific amplification products suggested the possibility of developing an assay that could detect multiple analytes simultaneously. In this approach, antibodies specific to each of the different analytes would be coupled to DNA labels of different lengths. Electrophoresis analysis of the different-sized amplification products from the multianalyte assay would then be used to identify analytes present in test samples. To demonstrate the feasibility of multiple analyte detection, reporter antibodies to β -Gal, hTSH and hCG were respectively conjugated to oligonucleotide labels 55, 85 and 99 bases in length (Fig. 2). Following preparation, reporter antibody conjugates were pooled for use in the multianalyte assay. Solid-phase capture reagents were prepared by coating microtiter plates with a mixture containing equal proportions of β -Gal, hTSH and hCG capture antibodies. Multianalyte testing used protocols similar to those used in the single analyte immuno-PCR assays.

Each of the three analytes was first tested individually and then in combination with the other two analytes. Controls were run to detect cross-reactivity between analytes and nonspecific conjugate responses. Multianalyte test responses for each of the assays are shown in Figure 6. As shown in lanes 2, 3 and 4, PCR products were observed in response to each analyte tested individually, one analyte per test. The appropriate amplification products were observed for each analyte tested, demonstrating both specific analyte response and absence of cross-interference between analytes, capture antibodies, and reporter reagents. The responses

Figure 6. Agarose gel electrophoresis of PCR products from sandwich multianalyte immuno-PCR assays. PCR amplification mixtures from assays of both individual and combinations of analytes (15 μ l) were separated as described in Figure 3. End lanes contained gel markers (gm): Φ X174 DNA, *Hinf*I fragments (Promega Corp., Madison, WI); (samples described as amount of analyte tested). Lane 1: Immuno-PCR negative controls; lane 2: 0.5 ng β -Gal; lane 3: 10 pg hTSH; lane 4: 250 pg hCG; lane 5: 0.5 ng β -Gal + 10 pg hTSH; lane 6: 0.5 ng β -Gal + 250 pg hCG; lane 7: 10 pg hTSH + 5 ng hCG; lane 8: 5 ng β -Gal + 100 pg hTSH + 2.5 ng hCG.

for the combinations of two analytes tested simultaneously are shown in lanes 5–7. In each case, the two specific amplification products corresponding to the specific pair of analytes in the sample were observed. Lane 8 demonstrates simultaneous detection of all three analytes, showing formation of the appropriate PCR product for each of the analytes. As shown in lane 1, amplification products were not observed in absence of analytes. This indicates the absence of detectable nonspecific binding by the three reporter antibody conjugates.

DISCUSSION

Immunoassays are a versatile and highly important means of detecting a variety of analytes, including drugs, hormones, cells, tumor markers, microorganisms, viruses and environmental pollutants (17). Typically, one analyte is detected per assay. Multianalyte testing, however, has been suggested as the next major goal advance in immunoassay technology (18). The emphasis on cost containment, the need to perform groups or panels of tests for accurate clinical assessment, and the need to screen for the presence or absence of multiple agents, all create incentive for technology providing simultaneous detection of multiple analytes.

Multianalyte formats have been explored using combinations of radioisotopic (19), fluorescent (20), enzymatic (18) and chemiluminescent (21) labeled antibodies. However, overlapping signals from different labels and difficulties in discriminating signal intensity at varying analyte concentrations have compromised quantitation and sensitivity. As a consequence, none of these labeling methods has shown practical utility in a multianalyte format (18).

In contrast, DNA provides an ideal molecular label for multianalyte discrimination. Different DNA molecules can be accurately and quantitatively differentiated by both variation in base sequence and molecular size. Consequently, use of nucleic acids provides an almost inexhaustible variety of molecular labels, each capable of detection via similar chemistry, test conditions and measurement approaches.

The simultaneous detection of three analytes (hTSH, β -Gal and hCG) by DNA-labeled antibodies, establishes the feasibility of multiple analyte immuno-PCR assays (Fig. 6). The covalent linkage of DNA label to reporter antibody is essential because it

assures that the surrogate relationship between analyte and amplified product is maintained throughout the assay. Furthermore, covalent coupling facilitates specific labeling of different antibodies each with a unique DNA label. In the present examples, assay discrimination of different analytes was based on differences in size of the PCR products. Given the current capability to perform amplifications with as many as 26 different primers (22) and the ability to accurately detect and differentiate amplified DNA products based on sequence and size differences, it is conceivable that large numbers of analytes could be detected simultaneously in a single assay using this approach. Reagent incompatibility may, however, become apparent when developing tests for use with samples having increased numbers of analytes. Nevertheless, the potential to co-amplify different DNA labels using a single primer set should enhance the probability of extending the technology for simultaneous detection of increased numbers of analytes. These capabilities suggest that limitations in the number of analytes detected per test seemingly, can now be overcome.

The findings presented here, in conjunction with recent reports (1,4-6), demonstrate that immunoassay sensitivity now can be greatly extended. The β -Gal, hTSH and hCG immuno-PCR sandwich assays exhibited a two to three orders of magnitude increase in sensitivity over comparable ELISA assays. Even higher sensitivity was achieved by Sano *et al.* (1), who have detected as few as 600 molecules of BSA. Presently, detection of immuno-PCR products has employed electrophoresis gel analysis and ethidium bromide staining. The sensitivity of the immuno-PCR technology is likely to be extended even further by use of alternative DNA detection methods. As suggested by Sano *et al.* (1), direct detection of ligands or enzyme labels incorporated into the DNA with the use of labeled primers or nucleotides may further increase both the sensitivity and ease of detection.

Optimization of PCR amplification conditions, as well as the type and length of the DNA labels, may also aid in extending immuno-PCR sensitivity. The assays reported here used short (55-99 bases), single-stranded DNA oligonucleotides for labels, both characteristics which may have contributed to a loss of assay sensitivity. We achieved assay sensitivity of 1×10^{-19} mol for hTSH, which is one to two orders of magnitude less sensitive (1×10^{-21} to 1×10^{-20} mol) than those immuno-PCR assays reported using plasmid or PCR-derived double-stranded labels (1,4,5). The reduced molecular size of the shorter PCR products would result in a reduced quantity of DNA produced and in a diminished dye intercalation capacity. Nevertheless, immuno-PCR provides breakthrough potential to extend the detection limit of current immunoassay technology towards single molecule detection.

The immuno-PCR tests described here differ from those in previous reports (1,4-6) in both the nature of the DNA-labeled reporter reagents and the assay format. These differences in approach, while demonstrating the versatility of the technology, directly impact on the procedural complexity, assay duration and ease of use of the technology. The DNA-labeled reporter reagents used in the earlier reports were assembled by affinity binding reactions. Antibodies in these examples were indirectly labeled with biotinylated DNA by using different binding proteins such as a protein A-streptavidin chimera molecule (1,6,7), avidin (4) and streptavidin (5). In each of these processes, assembly of the DNA reporter complex occurred during the assay. This can involve numerous steps for the addition of up to three reporter

reagents. Each process can also include as many as 20 wash steps to remove excess reagents and free the assay of non-specifically bound reagents. These reagent additions and wash steps add complexity and time to the immuno-PCR procedures.

Covalent attachment of the DNA label to the reporter antibody enables reporter reagents to be prepared prior to the assay. Therefore, washes are required only after the addition of sample analyte and after the addition of the DNA-labeled reporter reagent. Further simplification may be possible since analyte and reporter reagents could potentially be added simultaneously in the sandwich format. A wash step to remove excess sample materials may be therefore unnecessary.

In previously reported immuno-PCR tests (1,4-6), analytes were captured by adsorption directly onto the surface of solid supports. This process restricts application of the technology to analytes that have natural affinity for solid test supports. Therefore, analyte detection is subject to potential interference from sample components that may also adsorb onto the surface of the solid test support and possibly exhibit cross-reactivity with reporter antibodies. In contrast, in the immuno-PCR sandwich format, analytes are selectively captured by analyte-specific antibodies. Once captured to the solid phase antibody, analytes can be washed free of most potentially interfering substances. Antibodies generally adsorb strongly onto many test surfaces and can be raised to a wide variety of analytes. Therefore, the sandwich format expands the range of detectable analytes, and adds convenience and utility to the assay.

In conclusion, immuno-PCR technology has been shown to extend the sensitivity of immunoassays and to provide a means of multianalyte detection. Given the enormous amplification capability of PCR and existing capability to differentiate DNA based on size and sequence differences, this hybrid technology could, in principle, provide the basis for a new generation of sensitive multianalyte immunoassays.

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