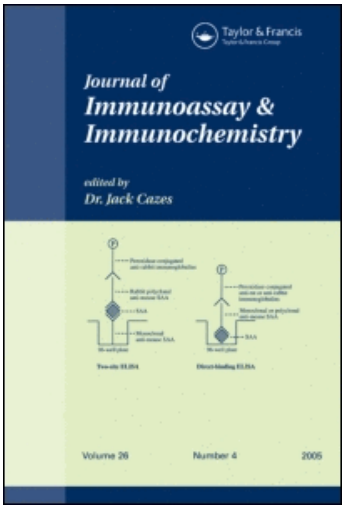

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A Novel Immuno-Polymerase Chain Reaction Protocol Incorporating a Highly Purified Streptavidin-DNA Conjugate

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Abstract: We have developed an immuno-polymerase chain reaction protocol which includes a highly purified streptavidin-DNA conjugate. The protocol comprises standard ELISA methodology and washing buffers together with a real-time PCR read-out system. The conjugate was employed in both indirect and capture assay formats, which can be completed in a single day using standard laboratory equipment. The immuno-PCR is reproducible, with a larger dynamic range and a sensitivity several orders of magnitude greater than the corresponding conventional ELISA. The minimum concentration of analyte detected was of the order of 1 pg/mL These characteristics should contribute to the adoption of immuno-PCR by research and clinical laboratories.

Keywords: Biotin, Canine heartworm, D-Dimer, Immuno-PCR, Monoclonal antibody, Streptavidin

INTRODUCTION

The detection and quantitation of analytes such as hormones, toxins, cytokines and pathogens is a major activity of biomedical research and

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clinical laboratories. The technologies used for this purpose often rely on the ability of detector molecules or ligands to bind very specifically to the analyte under investigation. The ligand is then detected by a more generic read-out system. One very familiar example of this technology is ELISA, in which the analyte-bound ligand, usually an antibody, is detected and quantitated through an enzyme which induces a measurable colour change in an appropriate substrate. The enzyme may be conjugated directly to the antibody, or linked indirectly through another ligand such as anti-immunoglobulin or protein A.

The sensitivity of these assays depends on several interrelated factors, including the avidity of the ligand, the amount of non-specific binding of the ligand to unrelated components in the sample or to the assay platform, and the number of detector molecules required to produce a detectable change in the substrate. Several 'amplification' systems have been introduced to enhance the signal, including the use of indirect ELISA to bind several secondary reporter molecules to each ligand, and nickel enhanced reactions.[1]

The ability of the polymerase chain reaction (PCR) to amplify a few DNA molecules to easily detectable levels offers an alternative means to increase the sensitivity of traditional ELISA technology. This concept, termed immuno-PCR, was first by exploited by Sano et al.[2] and has been modified by several other workers to follow a standard ELISA protocol. The read-out system involves detection by PCR amplification of a DNA template linked to the ligand, usually a monoclonal antibody. The advent of homogeneous or 'real-time' PCR assay technology has increased the sensitivity and ease of use of immuno-PCR, and allowed accurate quantification of the analyte.[3] The linking of the DNA template to the antibody has been achieved through direct conjugation[4] and indirectly through, for example, secondary antibodies and streptavidin (STV) – biotin bridges.[5,6] The advantage of the indirect systems is their generic utility for different ligand-analyte combinations. Conversely, the disadvantages of indirect formats can include the increase in non-specific (background) binding due to the presence of more detection reagents, and decreased sensitivity due to the presence of unconjugated by-products of the conjugation reaction.

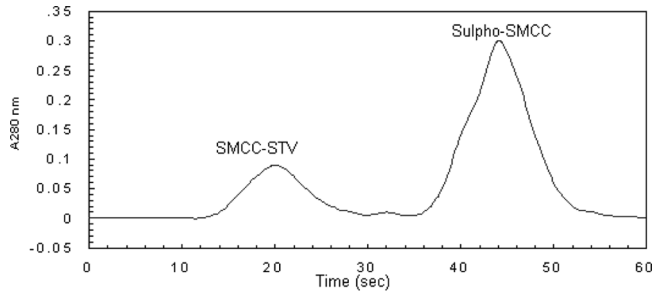
We have developed a novel immuno-PCR reagent system which aims to decrease the number of components in a generic protocol and to reduce the presence of unconjugated by-products. The system employs a STV–DNA conjugate purified by size-exclusion and affinity chromatography. When the conjugate was used in immuno-PCR to detect two different antigens routinely assayed in human and veterinary clinical laboratories (D-dimer and canine heartworm antigen (CHA) respectively), the sensitivity of the immuno-PCR was shown to be up to 10,000 fold that of conventional ELISA, with an estimated detection level of 50 fg, or 1 pg/mL. This was achieved with standard ELISA washing

protocols. The system employs real-time PCR using an intercalating dye, rather than a template-specific probe, which further increases the generic utility the system.

EXPERIMENTAL

Monoclonal Antibodies and Antigens

The monoclonal antibodies (mAb) and antigens were supplied by Agen Biomedical Ltd., and are those used in their commercial diagnostic



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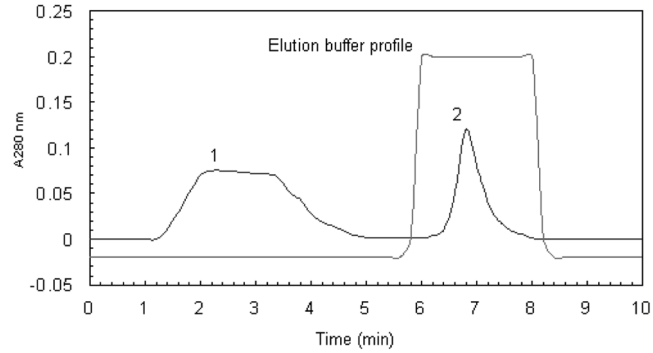

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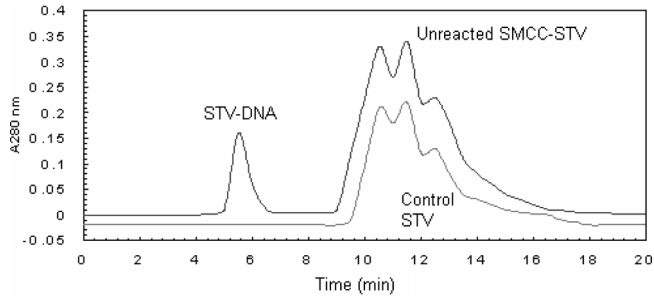


(c)

assays. MAb3B6 and 1D2 are specific for the human thrombosis breakdown product D-dimer, while mAb4D2 and DI16 recognize an antigen from the canine heartworm *Dirofilaria immitis*, which is present in the sera of infected animals. Both antigens are detected in routine diagnostic assays in clinical laboratories. The concentration of the D-dimer was estimated by absorbance at 280 nm, using an extinction co-efficient E_{1cm} 1% of 17.8,[7] whilst that of CHA was determined by a quantitative, in-house ELISA (M. Gerometta, pers. comm.).

Synthesis of 5′ Sulfhydryl-Modified Reporter DNA

A 602-base pair DNA template was synthesized using PCR from mouse leukaemia virus DNA present in genomic DNA from the mouse myeloma cell line NS0.[8] The DNA sequence can be found at GenBank accession no. gb|AC114666.31|, bp 94745–94144. The forward and reverse primer sequences (primers 1 and 2, Fig. 2a) are (thiol S-S)-5′-CCCCACCATCAGGCTTAG-3′ and 5′-GGCTTTATTGGGAGCACGG-3′, respectively. The primers were obtained commercially (Sigma Genosys, Sydney). The forward primer was designed such that an *Nhe*I site is 3 bp downstream of the 3′ end of the forward primer binding site, which allows for release of most of the template DNA by *Nhe*I digestion. The PCR was conducted in a PCR mix (25 µl) comprising 1.0 U Taq DNA polymerase ('JumpStart', Sigma Aldrich, Sydney), 1X supplied PCR buffer, 200 µM dNTPs, 1.0 mM $MgCl_2$, 1.0 µM each primer and 1 ng NS0 genomic DNA. Amplification conditions were: denaturing at 95°C for 15 s, annealing at 57°C for 30 s, and elongation at 72°C for 15 s, for

Figure 1. (a) Purification of SMCC–STV using a desalting column by ion exchange chromatography. The sample was passed through a 'HiTrap' desalting column equilibrated with 50 mM phosphate buffer pH 7.0 at flow rate 5.0 mL/min. The SMCC–STV was collected from 14 to 26 s and unreacted sulpho-SMCC was eluted after 34 s as shown. (b) Purification of STV–DNA conjugate and unreacted STV–SMCC by affinity chromatography. Samples were loaded onto a 2.0 mL 2-iminobiotin column equilibrated with binding buffer (50 mM ammonium acetate with 0.5 M NaCl, pH 11.0). The column was washed with 5 mL of binding buffer at 1.0 mL/min to remove unbound proteins and DNA (peak 1). The STV–DNA and unreacted SMCC–STV were eluted with 0.1 M acetic acid (peak 2). (c) Separation of STV–DNA conjugate from unreacted SMCC–STV by HPLC. Samples were loaded onto a size-exclusion HPLC column (Phenomenex, Bio-SEC-3000 USA) equilibrated with 0.1 M phosphate buffer, pH 6.5, containing 200 mM NaCl. The STV–DNA (upper profile) was eluted between 5.5 and 6.2 min and unreacted STV (upper profile) was eluted after 9 min. As a control, STV was loaded onto HPLC to indicate the elution time of STV (lower profile).

40 cycles. The PCR products were analysed by electrophoresis through a 2% agarose gel to confirm the presence of the appropriately sized fragment using the fluorescent dye 'SYBR-Safe™' (Invitrogen, USA). The PCR product was purified ('QIAquick PCR purification kit', QIAGEN, USA) and the 5′-sulfhydryl DNA was eluted into distilled water. The DNA concentration was determined by absorbance at 260 nm.

Modification of STV with Sulfosuccinimidyl-4-(*N*-maleimidomethyl) Cyclohexane-1-carboxylate (Sulfo-SMCC)

Sulfo-SMCC (Pierce, USA) was dissolved in distilled water at 5.0 mg/mL and 20 μL of sulfo-SMCC solution was mixed and reacted with 100 μL of 10 mg/mL STV (Sigma) in 50 mM sodium phosphate buffer (pH 7.0) at room temperature (RT) for 2 h. The maleimide-modified STV (SMCC-STV) was passed though a 'HiTrap' desalting column (Amersham Biosciences, USA), equilibrated with 50 mM phosphate buffer (pH 7.0), to remove unreacted sulfo-SMCC (Fig. 1a). The concentration of SMCC-STV was determined by absorbance at 280 nm.

Conjugation of DNA to STV

The purified SMCC-STV (10 μg) was reacted with 20 μg 5′-sulfhydryl DNA in 50 mM phosphate buffer containing 5 mM EDTA and 10 mM Tris [2-carboxyethyl] phosphine hydrochloride (TCEP · HCl, Pierce, USA) for 4 h at RT. The reaction mix was loaded onto a 2 mL 2-iminobiotin column (Pierce, USA), which was equilibrated with binding buffer (50 mM ammonium carbonate buffer with 0.5 M NaCl, pH 11.0). After washing with 10 mL of binding buffer to remove unreacted DNA and proteins, the STV–DNA conjugate and unreacted STV-SMCC were eluted with 0.1 M acetic acid as shown in Fig. 1b.

To recover the STV–DNA conjugate free of unreacted SMCC-STV, the sample was loaded onto a size-exclusion HPLC column (Phenomenex Bio-SEC-3000 USA) equilibrated with 0.1 M phosphate buffer containing 200 mM NaCl, pH 6.5. Control STV passed through the column showed that the elution time of unreacted SMCC–STV was greater than 9 min (Fig. 1c). The STV-DNA conjugate was eluted from 5.5–6.2 min. The concentration of the final conjugate was estimated by HABA reagent (Pierce, USA).

Biotinylation of mAb

Solutions of mAb1D2 and DI16, each 2 mL of 5 mg/mL in 50 mM phosphate buffer, pH 7.0, were mixed with 1 mg sulfo-NHS-LC-biotin

(Pierce, USA). After incubation at RT for 3 h, the biotinylated mAb was purified by size-exclusion chromatography (P6DG column, Bio-Rad, USA).

Immuno-PCR Assays

The indirect and capture immuno-PCR assays were performed in 96-well microtitre plates ('MaxiSorp', Nunc) and are represented schematically in Figs. 2b and 2c. For all assays, the plates were agitated during the

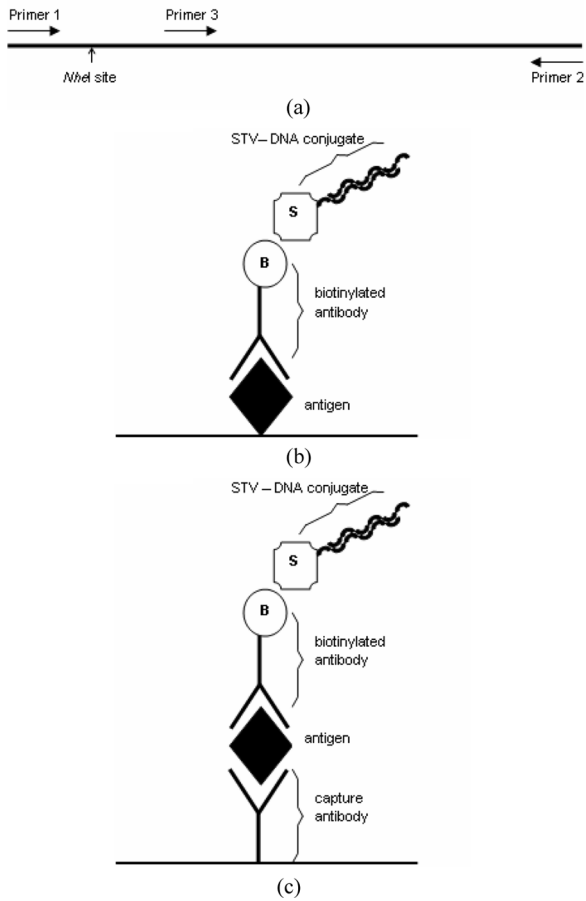


Figure 2. (a) Schematic representation of the DNA template. Primers 1 and 2 were used to generate the template for attachment to STV. DNA for the read-out PCR was released from the bound conjugate by digestion with *Nhe*I and amplified with primers 3 and 2. (b, c) Schematic representation of the indirect (b) and capture (c) immuno-PCRs. In the figures, 'S' indicates streptavidin and 'B' indicates biotin.

incubation steps, except where indicated. In indirect assays, the D-dimer antigen was coated onto the plates in ten-fold serial dilutions in 50 μL coating buffer (50 mM Na_2CO_3 buffer, pH 9.66) and incubated at RT for 90 min. The plates were washed three times with phosphate-buffered saline (PBS) containing 0.5% Tween-20 (PBS-T). Biotinylated mAb1D2 (50 μL at 1 μg/mL) was added to the plates and incubated for 60 min at RT, following which the plates were washed three times with PBS-T. Purified STV-DNA conjugate (50 μL at 10 ng/mL) was added to the wells and incubated for 60 min at RT. The plates were washed a further six times with PBS-T and the bound DNA was released by adding elution buffer (10 μL comprising 1U *Nhe*I, 1 μL 10X restriction enzyme buffer, 1 μL BSA at 1 mg/mL and 8 μL water) for 60 min at 37°C without agitation. The restriction enzyme and buffer were supplied by New England Biolabs (Mass., USA).

A 1 μL sample of each well was removed and assayed by quantitative PCR as follows. The reaction was conducted in a total volume of 25 μL as described above (section 2.2) except that the forward primer sequence (primer 3, Fig. 2a) is 5′-ATAGAGGTGCACAGTGCTCTGGC-3′, and 0.5X Sybr Green™ (Invitrogen Molecular Probes, USA) was included in the reaction mix. Amplification conditions were as above, with fluorescence emission data collected at the end of the extension phase of each cycle. The reactions were performed in a 'Rotor-Gene' thermal cycler (Corbett Research, Sydney) and the supplier's software was used to estimate the threshold cycle (Ct) at which the emitted fluorescence was greater than a cut-off level. The optimal cut-off point for the Ct values was determined by the software. Melt curve analysis was performed immediately after the PCR and consisted of an initial denaturation at 95°C for 60 sec, hybridisation at 55°C for 60 sec and at 50°C for 120 sec, followed by a thermal gradient from 50°C to 95°C, holding for 5 sec at 1°C intervals. Fluorescence emission data were collected at each interval during the thermal gradient. Software supplied with the thermal cycler allowed automatic conversion of the melt curve data to the first derivative (dF/dT) of the fluorescence signals against temperature to produce positive peaks.

For capture assays, the plates were coated with mAb3B6 (D-dimer) or mAb4D2 (CHA) in 50 μL at 1 μg/mL in phosphate buffer (pH 7.0) and incubated for 90 min at RT. The rest of the assay was similar to the indirect assay described above, with modifications to reduce background binding as described in the text in sections 3.3 and 3.5.

Conventional ELISA

Corresponding ELISAs were performed as above except that STV conjugated to horseradish peroxidase (Dako, Denmark) was used rather

than the STV–DNA. The conjugate (50 μL of 1 in 500 dilution in PBS-T with 2% BSA) was added to each well and the plates were incubated at RT for 60 min. The plates were washed three times with PBS-T, and 50 μL of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added. The plates were incubated for 20 min at RT, before the reaction was stopped by adding 50 μL 1 M H_2SO_4. The absorbance at 450 nm was determined by an automatic plate reader.

RESULTS

Generation of STV–DNA Conjugate

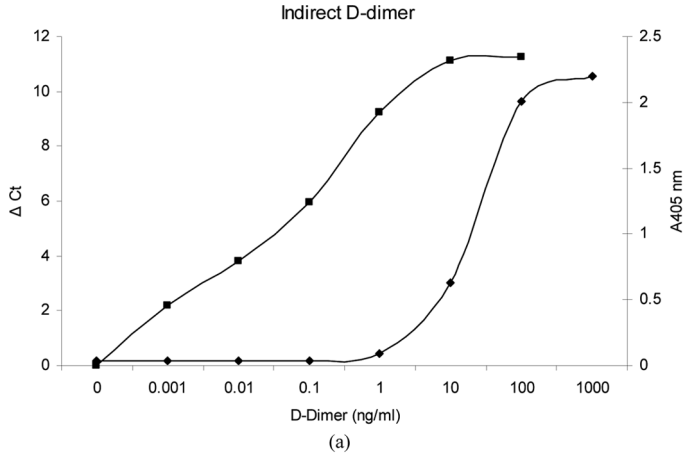
The DNA and STV components were prepared for conjugation by separately generating a thiol S-S-DNA template and maleimide-modified STV. The 602 bp DNA template from murine leukaemia virus was produced by PCR using a thiol S-S-linked primer obtained commercially. The PCR product was separated from unused primers and other reaction components by a standard PCR 'clean-up' kit. The presence and size of the product were confirmed by agarose gel electrophoresis (data not shown).

The maleimide-modified STV (SMCC-STV) was prepared as described using sulfo-SMCC. Unused sulfo-SMCC was separated from the SMCC-STV by ion exchange chromatography ('HiTrap', Fig. 1a).

The two components were conjugated by incubation in the presence of the reducing reagent TCEP · HCl. Affinity chromatography using a 2-iminobiotin column was used to separate the STV-DNA and SMCC-STV from other reaction components (Fig. 1b). The STV-DNA conjugate was separated from SMCC-STV by size-exclusion HPLC (Fig. 1c).

Indirect D-Dimer Assay

We compared the performance of the immuno-PCR using the STV–DNA conjugate with conventional ELISA in an indirect format (Fig. 2b) for the human thrombosis breakdown product, D-dimer. The D-dimer was coated directly onto the plates in ten-fold serial dilutions and probed with biotinylated mAb1D2. The secondary reagents were the STV-DNA conjugate and STV-HRP for immuno-PCR and conventional ELISA, respectively, followed by detection as described. The results (Fig. 3a) indicate that the sensitivity of the immuno-PCR was at least 1pg/mL (50 fg per well), compared to a sensitivity of greater than 1 ng/mL (50 pg per well) by conventional ELISA. This represents at least a 1000-fold difference in sensitivity. The dynamic range of the immuno-PCR assay extended from 10 ng/mL to less than 1 pg/mL, or at least five orders of magnitude, compared with two to three orders of magnitude for the ELISA.



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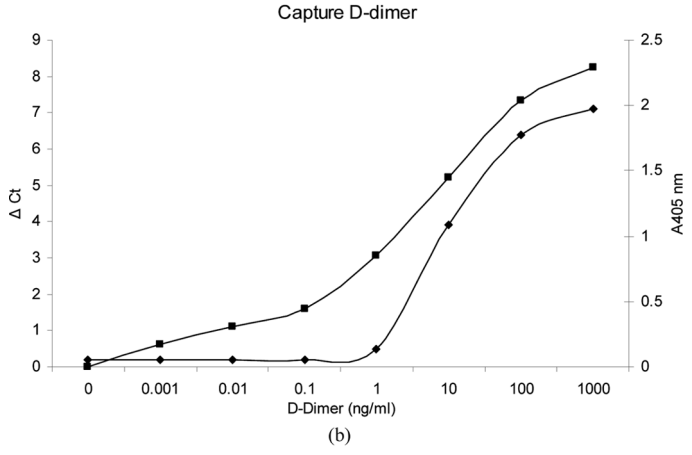

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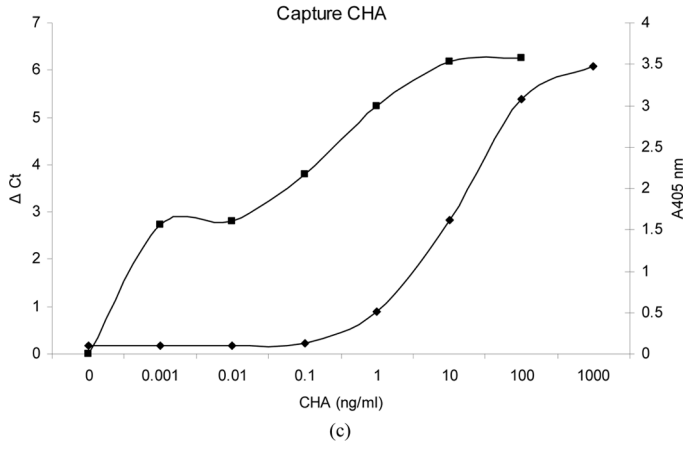

(c)

Capture D-Dimer Assay

A capture format, in which the analyte is 'captured' by a ligand attached to the microtitre plate followed by detection with a second ligand, is commonly employed in diagnostic assays. This format reduces the variability of attachment of the analyte, especially when the analyte is added in a complex biological fluid such as serum.

We compared the sensitivity of immuno-PCR using STV–DNA conjugate in a capture assay to the conventional capture ELISA. Initial experiments showed a higher background, and decreased sensitivity, compared to the indirect assay format. Various changes to the basic protocol described in section 2.7 were assessed, with the following resulting in the best sensitivity:

- after coating with mAb3B6, the wells were blocked with 2% BSA in PBS for 60 min at RT
- the biotinylated mAb1D2 was added in 2% fat-free milk in PBS
- the STV–DNA conjugate was added in 0.1% BSA and plasmid DNA at 1 ng/mL in PBS-T

The D-dimer antigen was diluted in human plasma to mimic samples analysed in clinical laboratories. The results, shown in Fig. 3b, indicate that the sensitivity of the immuno-PCR is at least 1 pg/mL (50 fg per well), compared to a sensitivity of 1 ng/mL (50 pg per well) by conventional ELISA. This represents at least a 1000-fold increase in sensitivity and is similar to the results obtained with the indirect assay. However, we did observe a smaller difference in Ct values with the less concentrated samples compared with those obtained in the indirect format.

Reproducibility

The reproducibility of both the indirect and capture D-dimer immuno-PCR assays was assessed by comparing the Ct values obtained for triplicate samples of D-dimer. The results, which are summarized in Table 1, show highly reproducible Ct values at all levels of detection.

Figure 3. Comparisons of immuno-PCR (■) and conventional ELISA (◆) in indirect D-dimer (a), capture D-dimer (b) and capture CHA (c) assays. In all figures, the left axis represents the difference in Ct value obtained with the respective antigen concentration and that obtained with the negative control (0 ng antigen). The right axis represents the absorbance at 450 nm obtained in conventional ELISA.

Table 1. Reproducibility of indirect (a) and capture (b) immuno-PCR for D-dimer. The values shown are the Ct values observed in the read-out PCR

D-dimer (pg/mL)	100	10	1	0
a				
Replicate 1	14.47	15.85	19.53	23.14
Replicate 2	14.11	16.18	20.62	23.58
Replicate 3	14.02	16.19	19.79	23.26
Mean	14.2	16.07	19.98	23.33
SD	0.24	0.19	0.57	0.23
b				
Replicate 1	21.37[a]	22.56	23.54	24.46
Replicate 2	21.49	22.58	23.54	24.51
Replicate 3	n/d	22.66	23.61	n/d
Mean	21.43	22.60	23.56	24.49
SD	0.085	0.053	0.040	0.035

The discernible difference in Ct values between the minimum D-dimer concentration of 1 pg/mL and 0 pg/mL suggests that the actual detection limit for both assays is less than 1 pg/mL. The greater difference in Ct values for 1 pg/mL and 0 pg/mL obtained for the indirect assay compared to the capture format suggests that there was less non-specific binding in the former assay.

CHA Capture Assay

To confirm the performance of the STV–DNA conjugate in another system, we used the conjugate in a capture immuno-PCR for CHA. The immuno-PCR was compared with the conventional capture ELISA used to detect heartworm antigen in clinical samples.

The following modifications were made to the basic protocol:

- after coating with mAb4D2, the wells were blocked with 5% BSA in PBS-T for 60 min at RT
- the biotinylated mAbDI16 was added in 5% BSA in PBS-T
- the STV-DNA conjugate was added in 5% BSA and non-specific genomic DNA at 1 ng/mL in PBS-T

The results obtained with this protocol are illustrated in Fig. 3c and are similar to those obtained for D-dimer. The sensitivity of the conventional ELISA was between 0.1 to 1 ng/mL, whereas that for the

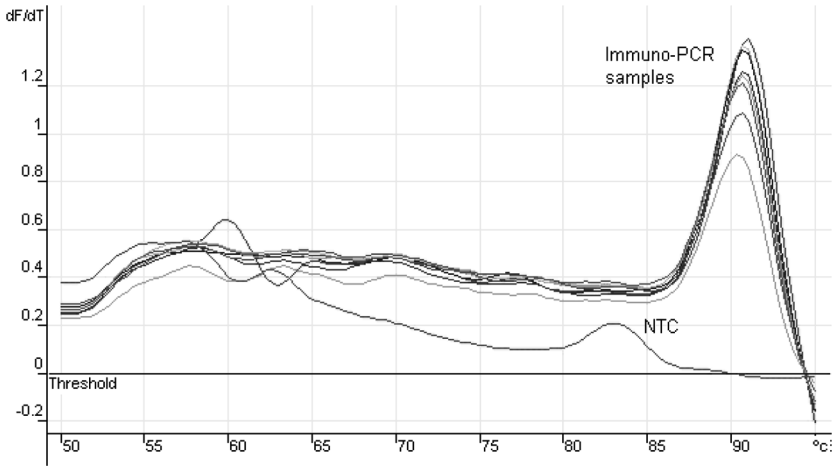

Figure 4. Melt curve analysis of the PCR products generated during indirect D-dimer assay. The samples eluted from wells in the immuno-PCR assay showed melt curve peaks of about 91°C to 92°C, whereas the non-specific products from the NTC sample exhibited a much smaller peak at about 83°C. The fluorescence data are plotted as the first derivative (dF/dT) of the fluorescence signal (vertical axis) against temperature (horizontal axis).

capture immuno-PCR was at least 1 pg/mL, which represents about a 1,000-fold increase in sensitivity.

Nature of the Reporter PCR Product

The use of a DNA-intercalating dye such as Sybr-Green can be problematic if there are non-specific reaction products from the PCR, e.g., from primer-dimer formation. However, we observed that the fluorescence levels recorded from no template control (NTC) samples (i.e., samples comprising the PCR mix with no template DNA) were usually below the level of the cut-off point for Ct estimation, suggesting minimal formation of primer-dimers or other non-specific reaction products (data not shown). To support this observation, we performed melt curve analyses following PCR. The results of the analysis for the indirect D-dimer assay are shown in Fig. 4, but similar results were observed for all assays. There is a small peak at about 83°C for the NTC sample, compared to the large peaks observed at about 91°C to 92°C for the sample eluates. The results suggest that a single product was produced during the PCR with reporter template present, whereas a much smaller amount of a lower-sized product, possibly primer-dimer, was produced in the absence of template DNA.

DISCUSSION

Our aim in these experiments was to develop an immuno-PCR protocol with a minimum number of detection reagents, but which incorporates a generic read-out system applicable to a variety of analyte detection assays. The central feature of the protocol is a highly purified STV–DNA conjugate which is used to detect biotinylated ligand specific for the analyte. The results presented here demonstrate the utility of the STV–DNA conjugate in detecting and quantitating two different analytes in capture and indirect formats. The performance of this immuno-PCR was similar to that of other systems, in terms of minimum concentration of analyte detected (of the order 50 pg/mL) and of increase in sensitivity and dynamic range over conventional ELISA.

The simplest format for immuno-PCR using microtitre plates is to attach the analyte to the surface of the plate and to detect it with a ligand to which the DNA has been conjugated. This format has several deficiencies. Firstly, there is variability in the attachment of analytes to the surface of the microtitre plate. This is especially so when the analyte is present in a complex biological matrix such as serum, where other components compete for attachment to the well surface. The use of a second ligand, often a mAb, to capture the analyte aims to decrease this variability. For commercial assays, this also offers the advantage of obtaining plates to which the capture ligand has been attached, thus reducing the time involved in coating the plate. The disadvantage of the capture assay is the presence of the capture ligand as another biological entity for potential non-specific binding of the detector molecules. We observed much lower ΔCt values at the lower antigen concentrations in the capture assays (Figure 3b and c), presumably due to non-specific interaction with the capture ligand. The use of other more inert ligands, such as aptamers, may resolve this problem.

A second problem with the simplest format is that the direct conjugation of DNA to protein is difficult to control and requires extensive purification to remove chemical modifiers and unconjugated DNA and ligand. Various methods have been employed to link the detector ligand to the DNA template. A recent study which compared three types of capture assay format concluded that detection of analyte with a mAb directly conjugated to the DNA template was more sensitive than use of secondary detection methods comprising biotinylated mAb and DNA template linked by a STV bridge in an indirect or capture format.[9] However, the production of the mAb-DNA conjugate required several chemical modification-conjugation steps and three purification procedures, which highlights the difficulty of generating such assay-specific reagents for a wide range of analytes. Recent reports using intein-mediated, expressed protein ligation should decrease the variability of production of

DNA-ligand conjugates,[10,11] although it requires the availability of DNA encoding the ligand and may be problematic for ligands of more than one protein subunit.

The level of detection of immuno-PCR has not approached that of PCR which can detect a few DNA molecules.[12] One of the main reasons for this is background 'noise' due to non-specific attachment of assay components to each other (e.g., capture to detector antibody), to the analyte or to the assay substrate, such as the microtitre well. This is readily observed as the difference in Ct values obtained from sample wells which contain no analyte (background binding) and those to which no template DNA was added (non-specific DNA amplification). In our experiments, this difference was generally around seven cycles (data not shown), which is greater than the difference in Ct values obtained from samples containing 10 or 0.01 ng/mL D-dimer, or four orders of magnitude (Fig. 3a). To overcome this, immuno-PCR protocols use various protein or DNA blocking reagents to decrease non-specific adsorption, together with extensive washing steps to remove unwanted components. Extensive washing has the disadvantage of removing some authentic detection signal, the extent of which depends on the number and stringency of washing steps and the binding affinity of the analyte–ligand and subsequent detection reagents. It is self-evident that the use of as few reagents as possible will reduce the opportunity for non-specific binding.

The second factor which can decrease the sensitivity of immuno-PCR assays is the presence of unconjugated by-products from the synthesis of conjugated detection reagents. These reagents can increase the background noise or block the available sites of attachment for the corresponding conjugated reagents. Hence, purification of the conjugate away from unreacted reagents is essential for increasing the signal-to-noise ratio.

Thus, it can be seen that the use of secondary reagents in immuno-PCR reflects a balance between the advantage of limiting complicated conjugation reactions to generic reagents applicable to a wide range of assays, against the increase in background signal due to non-specific attachment of reagents, more washing steps and time taken to perform the reaction.

In the immuno-PCR procedure which we have examined, the number of assay components is kept to a minimum whilst employing a generic detection conjugate of STV linked directly to the reporter DNA. The key step in the generation of the STV–DNA was the purification of the conjugate by iminobiotin affinity chromatography. Although the generation of the STV–DNA conjugate involved three chemical reactions and purification steps, this is a generic reagent and a single production run in large quantity can be used for many types of assays. The non-generic (assay-specific) mAb was biotinylated by a simple, two-step conjugation

and purification procedure which facilitates the application of this protocol to different analyte-antibody combinations.

A disadvantage of the system we have developed is that it cannot be applied to the simultaneous detection of several analytes, due to the generic nature of the STV–DNA conjugate.

The detection of the reporter DNA in the original immuno-PCR assays relied on gel electrophoresis and staining with ethidium bromide.[2] The advent of homogeneous or real-time PCR has reduced the time, laboriousness and risk of false positives due to template contamination associated with gel-based DNA detection, and increased the quantitative power of PCR. Real-time PCR uses either DNA-intercalating dyes to detect double-stranded DNA products or fluorescent probes which hybridize to the PCR product. Although intercalating dyes are generic and do not require the design and optimization of assay-specific probes, their promiscuity in binding to non-specific assay products can give rise to misleading results. Thus, it is essential to design PCR primers and conditions which minimise the production of non-specific products, including primer-dimers which can occur especially at low template concentrations. We optimised the PCR conditions initially using gel analysis to confirm a preponderance of specific product. Melt curve analysis subsequent to the detection PCR further indicated that a single, uniform product was generated during detection. The use of an intercalating dye, rather than assay-specific probes, thus increases the general utility of this immuno-PCR protocol.

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REFERENCES

1. Bobrow, M.N.; Harris, T.D.; Shaughnessy, K.J.; Litt, G.J. Catalyzed reporter deposition, a novel method of signal amplification. Application to immunoassays. J. Immunol. Meth. **1989**, *125*, 279–285.
2. Sano, T.; Smith, C.L.; Cantor, C.R. Immuno-PCR: Very sensitive antigen detection by means of specific antibody-DNA conjugates. Science **1992**, *258*, 120–122.
3. Sims, P.W.; Vasser, M.; Wong, W.L.; Williams, P.M.; Meng, Y.G. Immuno-polymerase chain reaction using real-time polymerase chain reaction for detection. Anal. Biochem. **2000**, *281*, 230–232.

4. Hendrickson, E.R.; Hatfield Truby, T.M.; Joerger, R.D.; Majarian, W.R.; Ebersole, R.C. High sensitivity multianalyte immunoassay using covalent DNA-labeled antibodies and polymerase chain reaction. Nucleic Acids Res. **1995**, *23* (3), 522–529.
5. Adler, M.; Wacker, R.; Niemeyer, C.M. A real-time immuno-PCR for routine ultrasensitive quantification of proteins. Biochem. Biophys. Res. Commun. **2003**, *308*, 240–250.
6. Niemeyer, C.M.; Wacker, R.; Adler, M. Combination of DNA-directed immobilization and immuno-PCR: very sensitive antigen detection by means of self-assembled DNA-protein conjugates. Nucleic Acids Res. **2003**, *31* (16), e90.
7. Masci, P.P.; Whitaker, A.N.; Winzor, D.J. A simple chromatographic procedure for the purification of the D dimer fragment from crosslinked fibrin. Anal. Biochem. **1985**, *147*, 128–135.
8. Nikbakht, K.N.; Boone, L.R.; Glover, P.L.; Myer, F. E.; Yang, W.K. Characterization of a molecular clone of RFM/Un mouse chromosomal DNA that contains a full-length endogenous murine leukaemia virus-related proviral genome. J. Gen. Virol. **1987**, *68*, 683–693.
9. Lind, K.; Kubista, M. Development and evaluation of three real-time immuno-PCR assemblages for quantification of PSA. J. Immunol. Meth. **2005**, *304*, 107–116.
10. Lovrinovic, M.; Spengler, M.; Deutsch, C.; Niemeyer, C.M. Synthesis of covalent DNA-protein conjugates by expressed protein ligation. Mol. Biosyst. **2005**, *1*, 64–69.
11. Burbulis, I.; Yamaguchi, K.; Gordon, A.; Carlson, R.; Brent, R. Using protein-DNA chimeras to detect and count small numbers of molecules. Nat. Meth. **2005**, *2* (1), 31–37.
12. Hobson-Peters, J.; O'Loughlin, P.; Toye, P. Development of an internally controlled homogeneous PCR assay for the simultaneous detection and discrimination of herpes simplex virus types 1 and 2 and varicella-zoster virus. Mol. Cell. Probes **2007**, *21*, 24–30.

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