

A novel immuno-polymerase chain reaction protocol incorporating a highly purified streptavidin-DNA conjugate.

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Abstract

We have developed and tested an immuno-PCR format which includes a highly purified STV-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. The procedure involves a 90-min coating step, two (indirect) or three (capture) immunoassay steps followed by elution and real-time PCR. Thus, the assay can be completed in a single day using standard laboratory equipment. We show that the immuno-PCR is reproducible, with a larger dynamic range and a sensitivity several orders of magnitude greater than the corresponding conventional ELISA. The performance of this immuno-PCR was similar to that with 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. These characteristics should contribute to the adoption of immuno-PCR by research and clinical laboratories.

Keywords: Immuno-PCR, STV, biotin, monoclonal antibody, D-dimer, canine heartworm.

1. Introduction

The detection and quantitation of analytes such as hormones, toxins, cytokines and pathogens is a major activity of biomedical research and 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 (Bobrow et al. 1989)

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. (1992) and has been modified by several other workers to follow a standard ELISA protocol, except that the

read-out system involves detection by PCR amplification of a DNA template linked to the ligand, usually a monoclonal antibody. The advent of real-time PCR assay technology has increased the sensitivity and ease of use of immuno-PCR, and allowed accurate quantification of the analyte (Sims et al. 2000). The linking of the DNA template to the antibody has been achieved through direct conjugation (Sano et al., 1992 , Lind and Kubista, 2005) and indirectly through, for example, secondary antibodies and STV (STV) – biotin bridges (Adler et al., 2003, Niemeyer et al., 2003). The advantage of the indirect systems is their generic utility for different ligand-analyte combinations. 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 conjugation of thiol-modified DNA template to STV and purification through size exclusion and affinity chromatography. When used in detection assays for two antigens, D-dimer and canine heartworm antigen (CHA) routinely assayed in human and veterinary clinical laboratories respectively, we show that the sensitivity of the immuno-PCR is up to 10,000 fold that of conventional ELISA, with an estimated detection level of 50 fg. This was achieved with standard ELISA washing protocols. The system employs a real-time PCR using an intercalating dye, rather than a template-specific probe, which further increases the generic utility the system.

2. Materials and methods

2.1 Monoclonal antibodies and antigens

The monoclonal antibodies (mAb) and antigens were supplied by Agen Biomedical Ltd. and are those used in their commercial diagnostic 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 (Masci et al., 1985).

2.2 Synthesis of 5' sulfhydryl-modified reporter DNA

A 602-base pair DNA template was synthesized using PCR from mouse leukaemia virus genomic DNA isolated from the mouse myeloma cell line NS0 (Nikbakht et al., 1987). 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 5' (thiol -S-S-)-5'-CCCCACCATCAGGCTTAG-3' and 5'-GGCTTTATTGGGAGCACGG-3', respectively (Sigma Genosys, Sydney). The primers were chosen such that an *NheI* site is 3 bp downstream of the forward primer binding site, which allows for release of most of the template DNA by *NheI* digestion. The PCR was conducted in a total volume of 25 μ l, comprising 1.0 U Taq DNA

polymerase ('JumpStart', Sigma Genosys, Sydney), 1 X supplied PCR buffer, 200 μ M dNTPs, 1.0 mM of $MgCl_2$, 40 nM each primer and 1 ng of 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 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.

2.3 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 μ L of sulfo-SMCC solution was mixed and reacted with 100 μ L of 10 mg/mL STV (Sigma) in 50 mM sodium phosphate buffer (pH7.0) at room temperature (RT) for 2 h. The STV-SMCC was passed through a 'HiTrap' desalting column (Amersham Biosciences, USA), equilibrated with 50 mM phosphate buffer (pH7.0), to remove unreacted sulfo-SMCC (Fig. 1a). The concentration of STV-SMCC was determined by absorbance at 280 nm.

2.4 Conjugation of DNA to STV

The purified STV-SMCC (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 Iminobiotin column (Pierce, USA), which was equilibrated with binding buffer (50 mM ammonium carbonate buffer with 0.5 M NaCl, pH11.0). After washing with 10 mL of binding buffer to remove unreacted DNA and proteins, the STV-DNA conjugate and unreacted STV were eluted with 0.1 M acetic acid as shown in Fig. 1b.

To recover the STV-DNA conjugate free of unreacted 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, pH6.5. Control STV was passed through the column which showed that the elution time of unreacted STV was greater than 9 min, Fig 1c). The STV-DNA conjugate was eluted from 5.5 - 6.2 min.

2.5 Biotinylation of mAb.

The mAb solution, 2 ml of 5 mg/ml in 50 mM phosphate buffer, pH7.0, was 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).

2.7 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 indirect assays, the respective antigens were coated onto the plates in ten-fold serial dilutions in 50 μ L coating buffer (50mM Na₂CO₃ buffer, pH9.66) 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). The biotinylated mAb (50 μ L at 1 μ g/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 μ L at 10ng/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 μ L comprising 1U *Nhe*I, 1 μ L 10X restriction enzyme buffer, 1 μ L BSA at 1 mg/mL and 8 μ L water) for 60 min at 37 °C. The restriction enzyme and buffer were supplied by New England Biolabs (Mass., USA). A 1 μ L sample of each well was removed and assayed by quantitative PCR as follows. The reaction was conducted in a total volume of 25 μ L as described above (section 2.2) except that the forward primer sequence (primer 3, Fig. 1 a) is 5'- ATAGAGGTGCACAGTGCTCTGGC-3', and 0.5X Sybr Green™ (Invitrogen Molecular Probes, USA) was included in the reaction mix. Amplification conditions were as above. The reactions were performed in a 'Rotor-Gene' thermal cycler (Corbett Research, Sydney) and the supplier's software was used to estimate the Ct values. The 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 mAb 4D2 (CHA) in 50ul at 1 µg/mL in phosphate buffer (pH7.0) and incubated for 90 min at RT. The protocols were 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. The rest of the assay was as described above.

2.8 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 uL of 1 in 500 dilution in PBS-T with 2%BSA) was added to the plates at RT for 60 min and the plates were washed three times with PBS-T. The substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added and the plates were incubated for 20 min at RT. The reaction was stopped by adding an equal volume of 1M H₂SO₄. The absorbance at 450nm was determined by an automatic plate reader.

3. Results

3.1. 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 602bp 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 was prepared as described in the Materials and Methods using sulfo-SMCC. Unused sulfo-SMCC was separated from the maleimide-modified STV and unreacted 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 unreacted STV from other reaction components (Fig. 1b). The STV-DNA conjugate was separated from unconjugated STV by size exclusion HPLC (Fig. 1c). The concentration of the final conjugate was estimated by HABA reagent (Pierce, USA).

3.2. Indirect D-dimer assay

We compared the sensitivity 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 MAb3B6. 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. 3 a) indicate that the sensitivity of the immuno-PCR was estimated to be at least 1pg/mL (50 fg per well), compared to a sensitivity of about 1ng/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.

3.3 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, are 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 therefore compared the sensitivity of immuno-PCR using STV-DNA conjugate in a capture assay format 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, the wells were blocked with 2% BSA in PBS for 60 mins at RT
- the biotinylated MAb1D2 was added in 2% fat-free milk in PBS
- the STV-DNA conjugate was added in 0.1% BSA in PBS

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 1ng/mL (50pg 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.

3.4 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. 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.

3.5 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 initial experiments contained a modification to the basic protocol, in that all reagents, except the capture MAb, were added in 5% BSA in PBS-T, together with a blocking step of 5% BSA in PBS-T for 60 mins at RT after addition of the capture MAb. The results (Fig. 3c) showed that the sensitivity was similar to conventional ELISA. We suspected that non-specific binding of the STV-DNA complex was contributing to the Ct value obtained with negative control (0 ng/mL) and thus the low Δ Ct values at the low concentrations of antigen. We addressed this by adding human genomic DNA (1 ng / mL) to the STV-DNA complex.

The results obtained with this protocol were similar to those obtained for D-dimer. The sensitivity of the conventional ELISA was between 0.1 to 1 ng / mL, whereas the capture immuno-PCR was at least 0.1 pg / ml, which represents a 1,000- to 10,000-fold increase in sensitivity.

3.6 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 negative control samples 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). We also performed a melt-curve analysis following PCR. The results of the analysis for the indirect D-dimer assay are shown in Figure 4, but similar results were observed for all assays. There is a small peak at about 83 °C for the sample removed from a well to which no STV-DNA conjugate had been added (no template control), compared to the large peaks observed at about 91 °C to 92 °C for the sample eluates. (The threshold shown in Figure is set at 0 and is not related to the cut-off point used to determine Ct values.) The results suggest that a single product was produced during the PCR reaction 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.

4. Discussion

The results presented here demonstrate the utility of a highly purified STV-DNA conjugate in an immuno-PCR format in detecting and quantitating two different analytes in both capture and indirect formats. The performance of this immuno-PCR was similar to that with 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 level of detection of immuno-PCR has not approached that of PCR which can detect a few DNA molecules (e.g., Hobson-Peters et al., 2007), for two main reasons. The first is the 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 in our experiments 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 case, 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 try 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.

The second factor which decreases 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.

Amplification of the signal in traditional ELISA is achieved by the use of secondary reagents in the indirect ELISA format, in which more than one reporter molecule is bound to the ligand. One example is the use of conjugated anti-immunoglobulin antibody. These secondary reagents have the additional advantage of genericity, such that a single conjugate can be used for an array of analyte – ligand types. However, they can also contribute to decreased sensitivity through non-specific binding, as discussed above. Thus, the predominant disadvantages of the use of secondary reagents is the increase in the number of washing steps and the time taken to perform an assay, the latter being of especial importance in clinical laboratories. It can be seen that the use of secondary reagents in immuno-PCR reflects a balance between the advantages in signal amplification and genericity, against the increase in background signal due to non-specific attachment of reagents, more washing steps and time taken to perform the reaction.

Various methods have been employed to link the detector ligand to the DNA template. A recent study which compared three types of 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 compromising biotinylated mAb and DNA template linked by a STV bridge in an indirect or capture format (Lind et al., 2005). As mentioned above, one disadvantage of this format is the use of a non-generic reagent produced by several types of chemical modification and three purification steps. The direct conjugation of DNA to protein is difficult to control, although recent reports using intein-mediated, expressed protein ligation should decrease the variability of production of DNA-MAb conjugates (Lovrinovic et al., 2005, Burbulis et al., 2005).

We have examined an alternative system in which 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 and the use of an intercalating dye in the real-time PCR.

We assessed the performance of the STV-DNA conjugate in both indirect and capture formats. Although the indirect assay involves fewer steps, it can suffer from 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, it 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 compared to the indirect format is the presence of the capture ligand as another biological entity for potential non-specific binding of the detector molecules. We observed much lower delta Ct 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.

Acknowledgements

The authors are very grateful to Jody Hobson-Peters for her expert technical assistance in establishing the PCR methodology and to Dr. Mike Gerometta for his insightful advice on the D-dimer and canine heartworm antibodies and antigens.

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Figure 1.

a. Purification of maleimide-modified 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. STV-SMCC conjugates were collected from 14 to 26 seconds and unreacted sulfo-SMCC was eluted after 34 seconds as shown.

b. Purification of unconjugated STV and DNA-conjugated STV 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 and STV-DNA were eluted with 0.1 M acetic acid (peak 2).

c. Separation of DNA-STV conjugate and unreacted STV by HPLC. Samples were loaded on a size exclusion HPLC column (Phenomenex, Bio-SEC-3000 U.S.A.) equilibrated with 0.1 M phosphate buffer, pH 6.5, containing 200mM 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).

Fig. 1a.

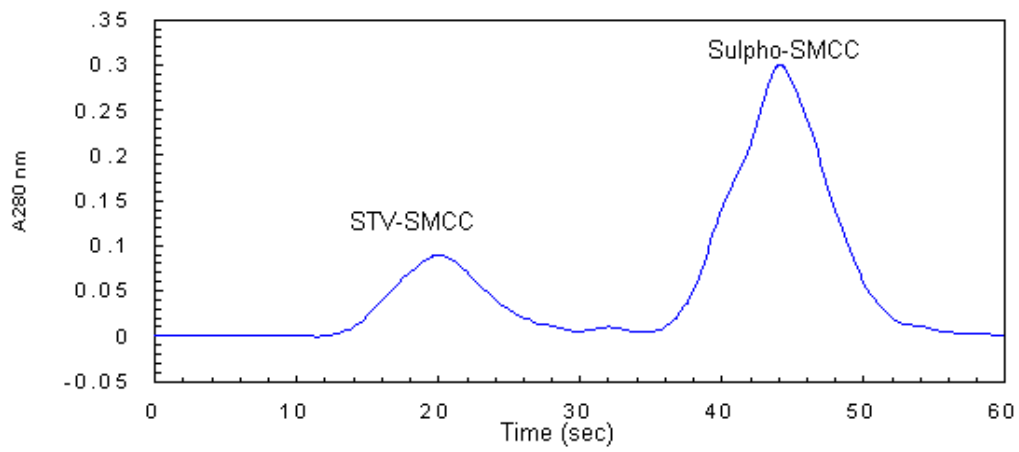


Fig. 1b.

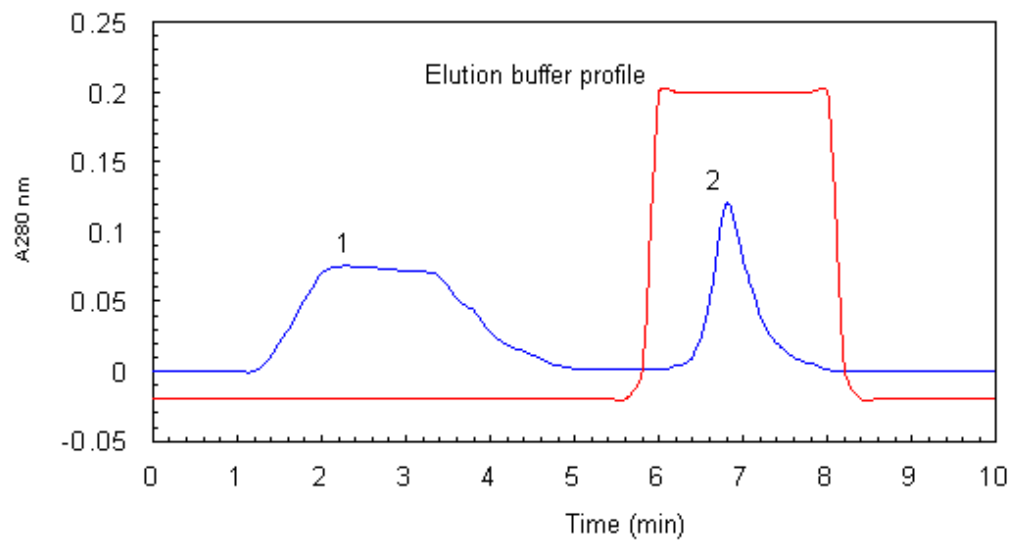


Fig. 1c.

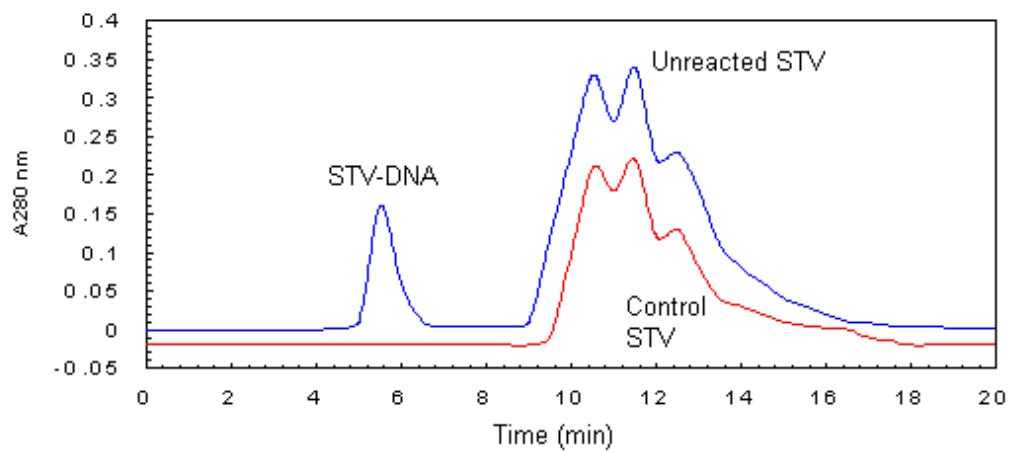


Figure 2.

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 *NheI* and amplified with primers 3 and 2.

b and c. Schematic representation of the indirect (b) and capture (c) immuno-PCRs.

Fig. 2a.

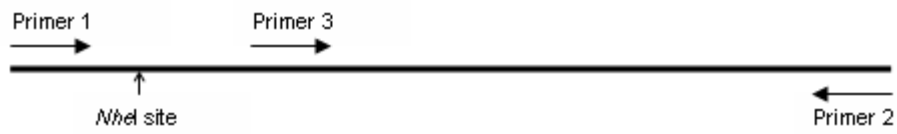


Fig. 2b.

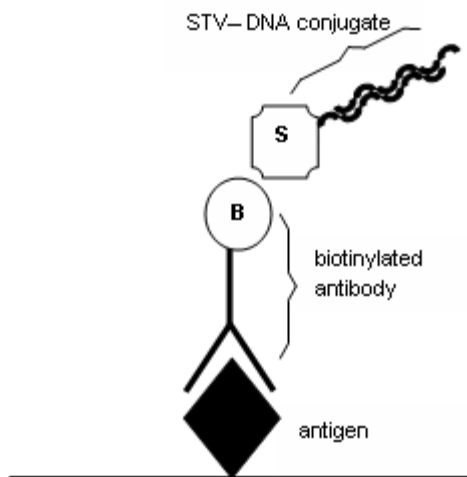


Fig. 2c.

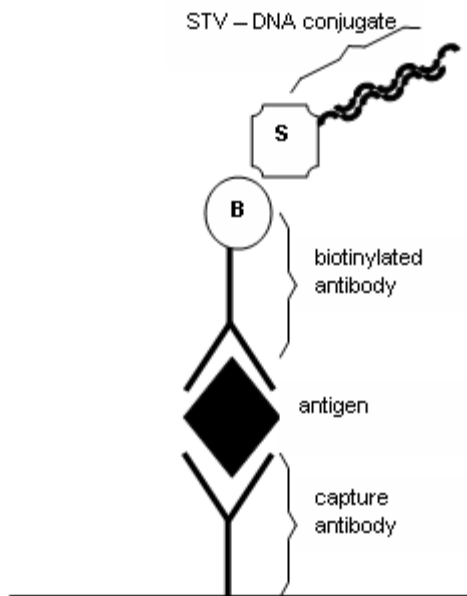


Figure 3.

- a. Comparison of indirect D-dimer immuno-PCR (■) and conventional ELISA (◆).
- b. Comparison of capture D-dimer immuno-PCR (■) and conventional ELISA (◆).
- c. Comparison of a capture CHA immuno-PCR with (■) and without (▲) genomic DNA blocker, and conventional ELISA (◆).

In all figures, the left axis represents the difference in Ct value obtained with the negative control (0 ng antigen) and the value obtained with the respective antigen concentration. The right axis represents the absorbance at 450nm obtained in conventional ELISA.

Fig. 3a.

Indirect D-dimer

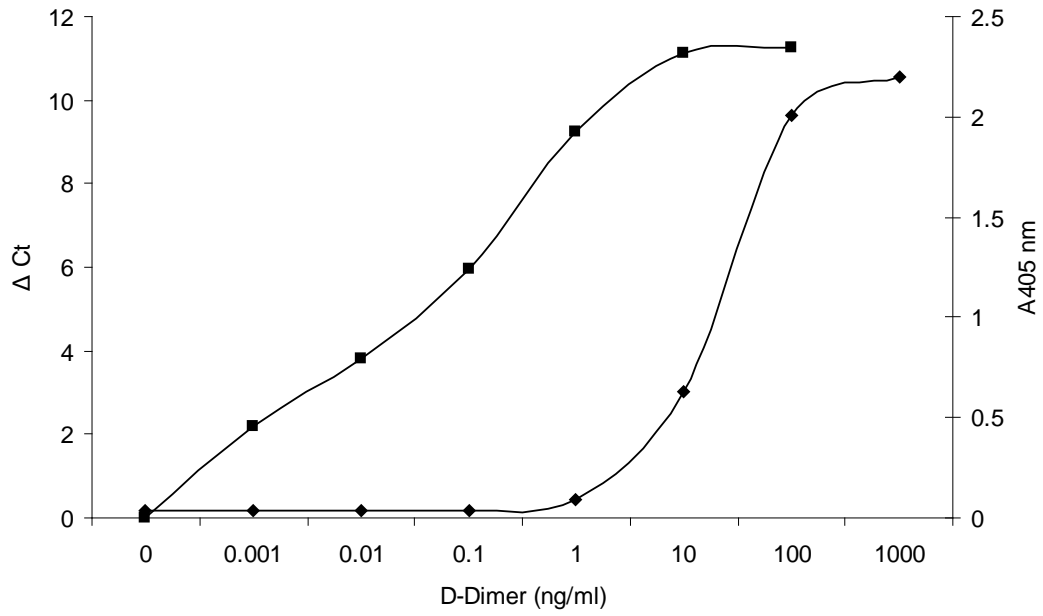


Fig. 3b.

Capture D-dimer

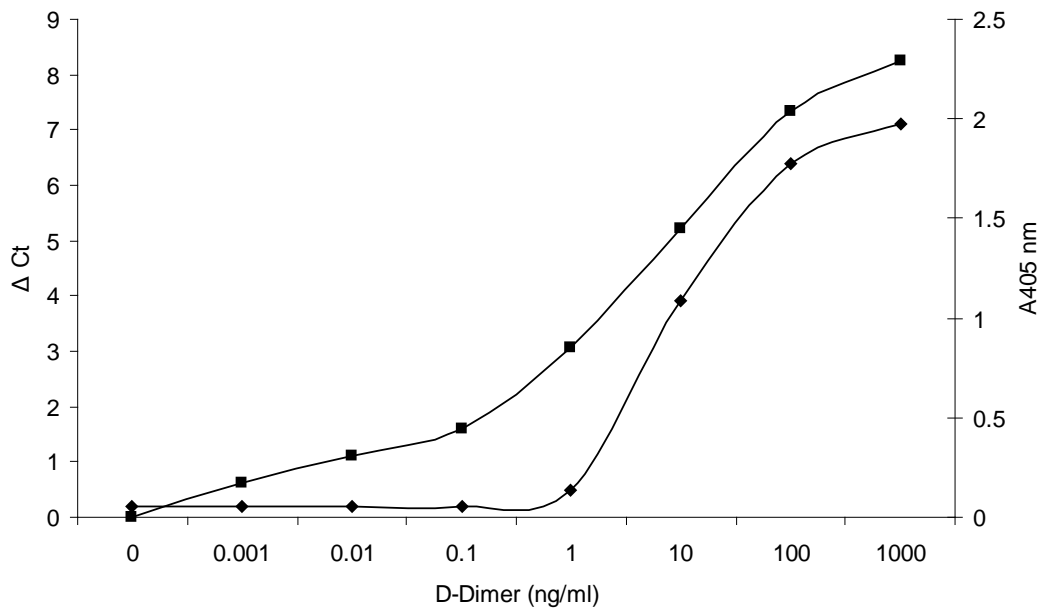


Fig. 3c.

Capture CHA

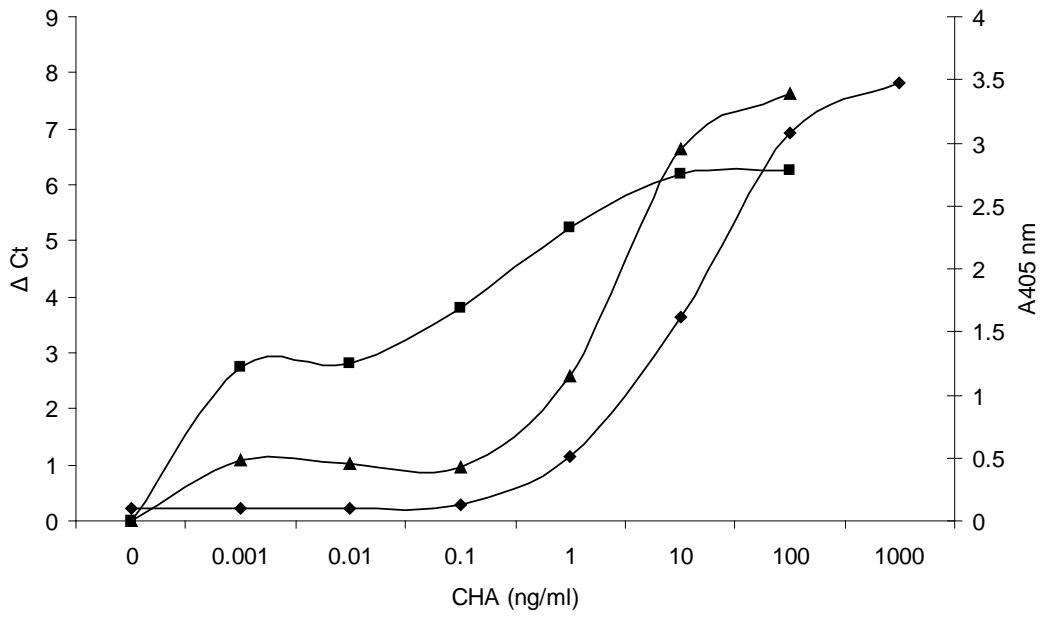


Figure 4.

Melt curve analysis of the PCR products generated during indirect D-dimer assay. The samples eluted from wells to which STV-DNA had been added showed melt curve peaks of about 91 °C to 92 °C, whereas that from the well to which no STV-DNA had been added (NTC - no template control) 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).

Fig. 4.

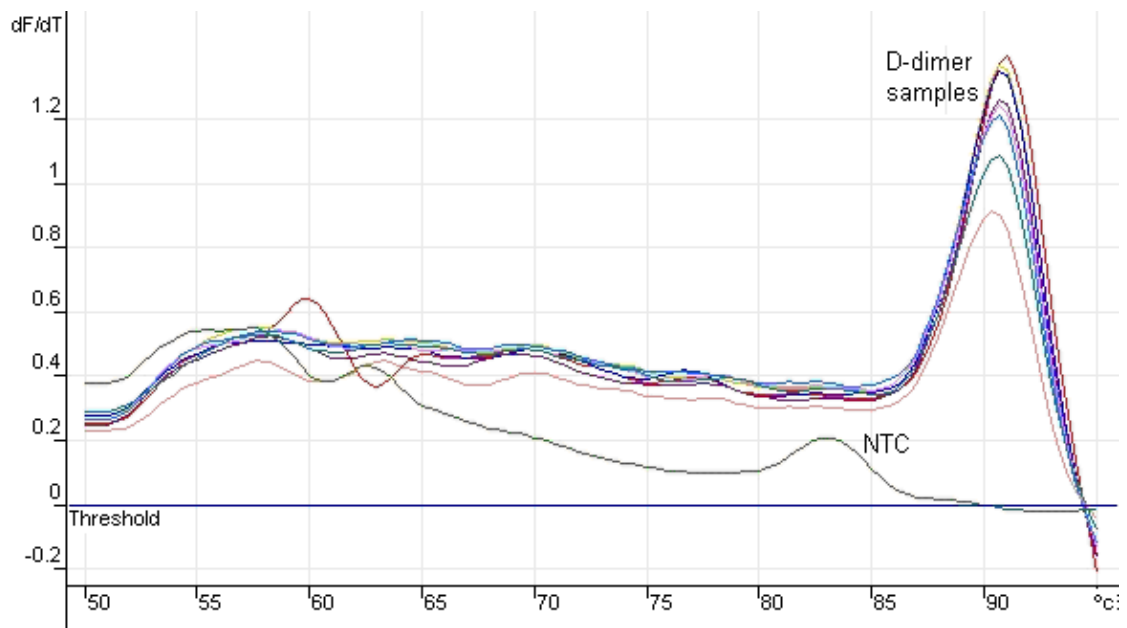


Table 1. Reproducibility of indirect (a) and capture (b) immuno-PCR for D-dimer.

a.

D-dimer (pg / ml)	100	10	1	0
Replicate 1	14.47 ^a	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.

D-dimer (pg / ml)	100	10	1	0
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