

Research paper

Development and evaluation of three real-time immuno-PCR assemblages for quantification of PSA

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Abstract

Real-time PCR is a very sensitive technique to measure DNA concentrations. In real-time immuno-PCR, it is used as the detection system for quantification of proteins. Many ways to perform and assemble real-time immuno-PCR are possible. We have tested three different approaches for the detection of prostate specific antigen (PSA) and compared them with each other and with ELISA. We also demonstrate the applicability of real-time immuno-PCR to classify clinical PSA samples. Assemblage I is performed stepwise attaching the capture antibody to the vessel surface by adsorption and having the DNA-label linked to the detection antibody through biotin and streptavidin. In assemblage II, capture antibody is also adsorbed to the vessel surface but the detection antibody is pre-conjugated to the DNA-label. Assemblage III uses the pre-conjugated detection antibody/DNA-label but binds the capture antibody through biotin to surface immobilized streptavidin. We found assemblage II to be the most sensitive, with a detection limit of 4.8×10^5 PSA molecules. This can be compared to the detection limit of the ELISA, which is 5.7×10^7 molecules. Assemblage III was the most reproducible with a standard deviation (SD) of 0.21 cycles, while assemblage I was the least reproducible (SD=0.45 cycles). The SD of assemblage II was 0.25 cycles. We conclude that using pre-conjugated detection antibody/DNA-label enhances both the sensitivity and the reproducibility of real-time immuno-PCR. Measurements of PSA in serum samples using real-time immuno-PCR correlated well with measurements performed with ELISA. The real-time immuno-PCR measurements were more sensitive and the dynamic range was larger than with the ELISA. © 2005 Elsevier B.V. All rights reserved.

Keywords: Real-time immuno-PCR; Real-time PCR; PSA

Abbreviations: PSA, prostate specific antigen; SD, standard deviation; BSA, bovine serum albumin; Ct, threshold cycle; BC, background control; NTC, no template control.

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1. Introduction

Immunoassays have been used for the quantification of proteins since the 1960s and are the basis of many diagnostic applications. The diversity of antibodies has made it possible to raise antibodies for almost any protein and use them for the specific

recognition of antigens in immunoassays. A number of ways to generate and detect signal from analyte bound antibodies have been developed (Yalow and Berson, 1960; Engvall and Perlman, 1971; Chan et al., 1987). During recent years, real-time PCR has become one of the most popular methods to quantify nucleic acids. The very high sensitivity of real-time PCR, which under optimal conditions detects a single molecule, makes it excellent for diagnostic purposes. So far no method developed for protein analysis has reached this sensitivity. In 1992, Sano et al. described a new technique using PCR to detect specific proteins, which they called immuno-PCR. Immuno-PCR combines the molecular specificity of antibodies with the sensitivity of PCR. Antibodies specific for the protein target are immobilized to the surface of a vessel. Sample is added and the targeted proteins are bound by the immobilized antibody molecules. A second specific antibody, coupled to a DNA molecule, is then added. This so-called detection antibody binds to a second epitope on the immobilized protein target. After careful washing to remove all unbound reagents, the DNA is amplified. During the exponential phase of the PCR, the amount of product formed reflects the amount of target protein that was bound by the antibodies.

When the above technique was first developed, the amount of PCR product was assessed either by gel electrophoresis (Sano et al., 1992; Zhou et al., 1993; Furuya et al., 2001) or by PCR-ELISA (Niemeyer et al., 1997). These rather laborious and insensitive post-PCR analytical methods limited the quantification range of immuno-PCR and thereby its applicability. Using real-time PCR instead, the amount of DNA could be quantified with high sensitivity and accuracy over a wide concentration range. This was first shown by Sims et al. (2000), who used real-time immuno-PCR to quantify vascular endothelial growth factor (VEGF). Eliminating the post-PCR step also reduced the assay time and risk of contamination.

Here we use real-time immuno-PCR to quantify prostate specific antigen, PSA. PSA is a well-known tumor marker for prostate cancer and is widely used to detect, stage, and monitor the disease (Ablyn, 1997). It is a 32 kDa glycoprotein serine protease that is produced in the prostate gland. PSA levels in the blood are usually low, and elevated serum concentrations indicate disease of the prostate. We have tested the

PSA real-time immuno-PCR assay on clinical samples and found that it can be used to classify samples for diagnostic purposes.

There are different ways to assemble immunoassays. Most common are sandwich assays where two specific antibodies are used to identify the protein target. This results in higher specificity than when a single antibody is used. The capture antibody can be adsorbed to the tube surface or attached through biotin to streptavidin coated microtiter plates. The detection antibody can be linked by different means to an enzyme (ELISA) or to DNA (immuno-PCR). In the procedures of Sims et al. (2000) and McKie et al. (2002), a covalent conjugate of detection antibody and DNA-label is used, while the methodologies of Adler et al. (2003), Niemeyer et al. (2003), Gofflot et al. (2004), and Barletta et al. (2004) use biotin–streptavidin coupling to attach the DNA. The Adler and Niemeyer procedures use streptavidin and bis-biotinylated DNA-label together with biotinylated detection antibody to obtain a non-covalent conjugate that is prepurified on a gel filtration column. The Gofflot method premixes streptavidin and biotinylated DNA before addition to the reaction wells. In the Barletta protocol, each assay reagent is added stepwise. In all assays other than Sims et al. (2000), the capture antibody or the antigen is directly adsorbed to the well surface. The Sims procedure uses streptavidin coated tubes to which biotinylated capture antibody is bound.

In this work, we compare three ways to assemble the real-time immuno-PCR detection system for the quantification of PSA. We test the sensitivity, reproducibility, and dynamic range. All three assemblages are sandwich assays. They differ in the way the capture antibody is attached to the surface and how the DNA is linked to the detection antibody. The assemblages are optimized separately. In assemblage I (Fig. 1a), the capture antibody is passively adsorbed to the PCR tube surface and the other assay components are added stepwise, one at a time, with incubation and washing in between. Streptavidin is used to couple biotinylated DNA-label to biotinylated detection antibody. This assemblage requires a total of six washing steps. In assemblage II, the capture antibody is also passively adsorbed to the surface, but here, the DNA is chemically conjugated to the detection antibody and premixed with the protein sample before addition to the well (Fig. 1b). This setup requires three

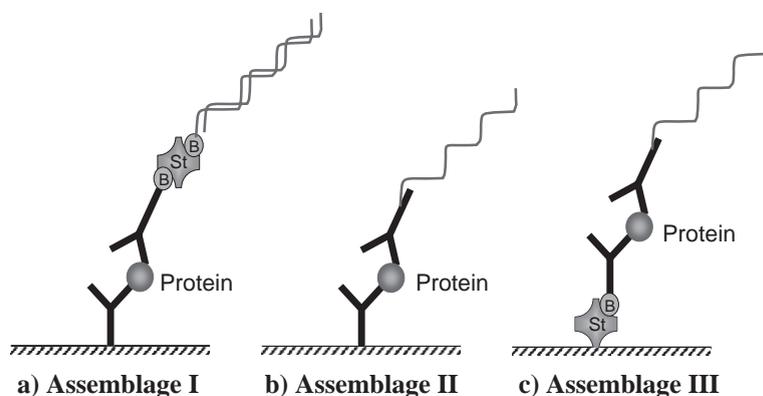


Fig. 1. Schematic representation of the three assemblages.

washing steps. Assemblage III makes use of streptavidin coated PCR microtiter plates and a biotinylated capture antibody (Fig. 1c). The DNA is covalently linked to the detection antibody as in assemblage II. Capture antibody, test sample, and detection antibody/DNA-label conjugate are mixed and incubated before addition to the wells. For this assemblage, two washing steps are sufficient. The three real-time immuno-PCR assemblages are compared with each other and also with classical ELISA.

2. Materials and methods

2.1. Antibodies, protein and DNA-label

Capture antibody (anti-PSA10) and detection antibody (anti-PSA66) were provided by CanAg Diagnostics (www.canag.com). For assemblages I and III, antibodies were biotinylated using biotinamido-caproate-*N*-hydroxysuccinimide ester (BNHS). BNHS in DMSO was added in five times molar excess to a 2.5 mg/ml solution of antibody. One tenth of the antibody solution volume of 1 M NaHCO₃, pH 8.5, was added, and the sample was incubated at room temperature for 2 h. The biotinylated antibody was then purified on a PD-10 gel filtration column (Amersham Biosciences). Pure PSA provided by CanAg Diagnostics was used as antigen. For assemblage I, biotinylated DNA-label was produced by PCR amplification of a 1098 base pair long segment of the *E. coli* β -glucuronidase gene using 5'-biotinylated forward primer (biotin-AACTATGCCGGAATCCATCG-3') and un-

modified reverse primer (5'-ACATATCCAGCCATGCACAC-3'). The DNA product was purified with QIAquick PCR purification kit (Qiagen).

2.2. Chemical conjugation of detection antibody and DNA-label

For assemblages II and III, a detection antibody/DNA-label conjugate was prepared. A 67 bases long DNA-label with amino-modification at the 5'-end was linked covalently to anti-PSA66 using the heterobifunctional cross-linking agent succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate, SMCC (Sigma). The DNA-label sequence was 5'-TGCCCTGCGT TTATCTGCTC TCGCATGTCG CAAGCCTCAT AGTTTAGGAA CATTACATTG ACGCAGG-3' (MWG-Biotech). Anti-PSA66 was activated with thiol groups by mixing 4.8 mg of anti-PSA66 in TSE (0.1 M triethanolamine, 0.1 M sodium chloride, 1 mM EDTA, pH 8.5) with 1.5 mM 2-iminothiolane (Traut's reagent, Sigma) diluted in TSE pH 12.9, in a total volume of 1 ml. After 20 min at room temperature, the reaction was terminated with 30 μ l of 1 M glycine pH 7.3. The amino modified DNA was at the same time activated with SMCC. 40 nmol of DNA in 0.1 M TS (0.1 M triethanolamine, 1 M sodium chloride pH 7.7) was mixed with 2 mM SMCC in a total volume of 650 μ l and incubated for 20 min at room temperature. The reaction was terminated with 13 μ l of 1 M glycine pH 7.3. The activated anti-PSA66 and the activated DNA-label were purified with NAP-10 columns (Amersham Biosciences) and eluted with 1.5 ml of TSE pH 7.3. The two solutions were then

mixed and incubated, first for 1 h at room temperature and then overnight at 4°C. The conjugate was first purified using an ion exchange column (Resource® Q 1 ml, Amersham Biosciences). The buffer used was 10 mM Tris pH 8.0 and the conjugate was eluted with a 0 to 1.5 M NaCl gradient. Free antibody was well separated from the other components. Fractions containing the antibody/DNA conjugate and unreacted DNA-label were pooled and concentrated to about 0.3 ml using a Centricon YM-100 (Millipore). The conjugate was finally separated from the free DNA-label by gel filtration using Superdex 200 HR 10/30 gel filtration column (Amersham Biosciences). The buffer used was 50 mM Tris, 1 mM EDTA, and 0.15 M NaCl, pH 8.0. The fractions containing the antibody/DNA conjugate were pooled. The amount of DNA in the conjugate was estimated to be about 10^{12} molecules per μl by regular real-time PCR.

2.3. Assemblage I

In the first assemblage, the capture antibody was adsorbed directly to the surface of a polypropylene PCR-plate (AB-0600 or AB-0900, ABgene). The assay reagents were then added stepwise following the scheme in Fig. 1a.

After extensive optimization of experimental conditions, we found the following protocol to work best. 25 μl of capture antibody (62.5 nM in 0.2 M NaH_2PO_4) was added to the wells of a microtiter plate and allowed to adsorb to the walls overnight. The wells were washed three times with 200 μl of wash buffer (0.154 M NaCl, 5 mM Tris pH 7.75, 0.005% Tween 20 and 0.1% Germall II) and the surface was blocked from further adsorption with incubation buffer (phosphate-buffered saline also containing 1% BSA (Sigma) and 0.05% Tween 20 (BioRad)) for 1 h at 37 °C. The wells were washed with 200 μl of wash buffer. 25 μl of a dilution series of PSA standard in incubation buffer was then added, and the plate was incubated at room temperature for 1 h. The wells were washed three times with 200 μl of wash buffer, and 25 μl of biotinylated detection antibody (2.3 nM in incubation buffer) was added, followed by an incubation period of 1 h at room temperature. The wells were washed six times with 200 μl of wash buffer and incubated with 25 μl of streptavidin (5 nM in incubation buffer) for 30 min at room

temperature. Then 25 μl of biotinylated DNA-label (0.7 pM in incubation buffer) was added and the plate was incubated for 1 h at room temperature. The wells were washed six times with 200 μl of wash buffer and ten times with 200 μl of Milli-Q water. The DNA was finally quantified by real-time PCR.

2.4. Assemblage II

In assemblage II, the same PCR-plates as in assemblage I were used and the capture antibody was adsorbed. However, in this case, the detection antibody was covalently bound to the DNA-label (Fig. 1b).

The following protocol was found to work best with assemblage II. Capture antibody was added to the wells of the microtiter plate in 25 μl (12.5 nM in 0.2 M NaH_2PO_4) and incubated overnight. The wells were washed three times with 200 μl of wash buffer and the surface was blocked from further adsorption with incubation buffer for 1 h at 37 °C. In the meantime, 5.5 μl of PSA standard or serum sample for each well was mixed with 22 μl of chemically conjugated anti-PSA66 and DNA-label (diluted 1/8000 from the stock) and incubated at room temperature for 1 h. The blocked wells were washed three times with wash buffer, 25 μl of the PSA/conjugate-mix was added and the samples were incubated for 30 min at room temperature. The wells were finally washed six times with 200 μl of wash buffer and ten times with 200 μl of Milli-Q water before the bound DNA was quantified by real-time PCR.

2.5. Assemblage III

In assemblage III, the same covalently conjugated detection antibody/DNA-label was used as in assemblage II except that in this case, the capture antibody was biotinylated and bound to streptavidin coated PCR-plates (AB-1224/0600, ABgene) (Fig. 1c).

The following protocol was found to work best for this setup. Streptavidin coated microtiter plates were blocked with 200 μl of incubation buffer for 1 h at 37 °C. 5.5 μl of PSA standard were mixed in separate tubes with 11 μl of chemically conjugated anti-PSA66 and DNA-label (diluted 1/10 000 from the stock), and 11 μl of biotinylated anti-PSA10 at a concentration of

16 nM. The mix was incubated for 1 h at room temperature, and then 25 μ l was added to the blocked wells that had been washed three times with 200 μ l of wash buffer. The wells were incubated for 30 min at room temperature, washed six times with wash buffer and ten times with Milli-Q water. The DNA was finally quantified by real-time PCR.

2.6. Real-time PCR

The amounts of immobilized DNA in the experiments were quantified by real-time PCR using the Bio-Rad iCycler iQ system. Primers for assemblage I were 5'-GTTAGCCGGGCTGCACTC-3' and 5'-ACATATCCAGCCATGCACAC-3' which were used to produce a 71 base pair long product. For assemblages II and III, the primers were 5'-CCCTGCGTTTATCTGCTCTC-3' and 5'-CCTGCGTCAATG- TAATGTTC-3'. These amplified a 65 base pair long product. Real-time PCR mixtures contained PCR buffer (10 mM Tris-HCl pH 8.3 and 50 mM KCl, Sigma), 4 mM MgCl₂ (Sigma), 200 μ M dNTPs (Sigma), 0.7 U JumpStart Taq polymerase (Sigma), 200–300 nM of each primer, and 0.5 X SYBR Green I (Molecular Probes Inc.) in 25 μ l. Cycling conditions were 95 °C for 2 min followed by 45 cycles of 95 °C for 20 s, 58 °C (assemblage I) or 63 °C (assemblages II and III) for 20 s and 72 °C for 25 s. The fluorescence was read at the end of the 72 °C step.

2.7. ELISA

ELISA was performed with the CanAg PSA EIA kit (CanAg Diagnostics, www.canag.com). It was based on the same antibodies as used in the real-time immuno-PCR assays (capture antibody anti-PSA10 and detection antibody anti-PSA66). It comprised a direct sandwich assay and the detection antibody was chemically conjugated to horse radish peroxidase (HRP) for the colorimetric reaction. The assay was performed according to the manufacturer's instructions. In brief, 25 μ l of a PSA containing sample was mixed with 100 μ l of biotinylated capture antibody and HRP/detection antibody conjugate in streptavidin coated microtiter plate wells. The samples were incubated for 1 h at room temperature. Each well was washed six times with wash buffer supplied with the kit. Thereafter 100 μ l of 3, 3', 5, 5'

tetramethyl-benzidine (TMB) was added and incubated for 30 min. Absorbance values at 620 nm were measured using a microtiter plate reader.

2.8. Serum samples

30 serum samples were tested using assemblage II real-time immuno-PCR and ELISA. Ten samples were from healthy men, ten from women, two from men with benign prostatic hyperplasia, and eight from men with prostate cancer.

3. Results

The sensitivity of real-time immuno-PCR is typically limited by background signal caused by non-specific adsorption of assay reagents, such as detection antibody, streptavidin, biotinylated DNA-label, and the detection antibody/DNA conjugate, to the vessel surface. Adsorption can be reduced by blocking the surface with blocking agents after the adsorption of capture antibody. We tested several blocking agents including BSA, milk powder, herring sperm DNA, and detergents such as Tween 20, and found a combination of 0.5–1% BSA and 0.05% Tween 20 to give best results in our assays. We then compared three different ways to assemble the real-time immuno-PCR (Fig. 1) and tested one of them on serum samples.

3.1. Characteristics of the assemblages

Real-time immuno-PCR standard curves were constructed from a dilution series of purified PSA. The threshold cycle (Ct) was determined by setting a fluorescence threshold in the exponential phase of the amplification curves, reading out the fractional cycle number at which the amplification curve crosses the threshold. Along with the standard samples, a background control (BC) containing all assay components except PSA was run. The signal response from the BC was due to non-specific binding of assay components (in particular of the biotinylated DNA and of the detection antibody/DNA-label conjugate) to the reaction vessel. A no template control (NTC), containing the real-time PCR mastermix only, was also included. The NTC reflects primer-dimer forma-

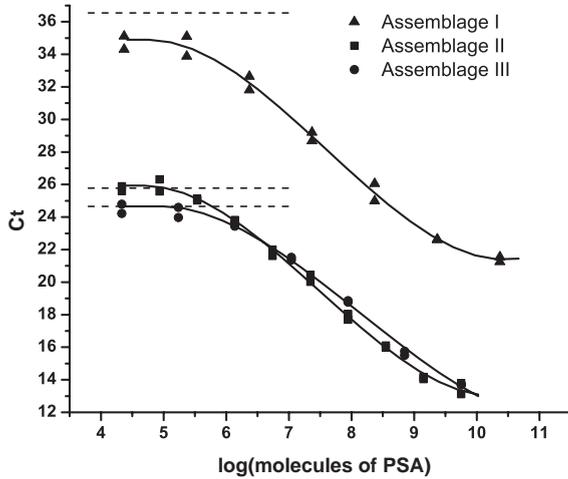


Fig. 2. Standard curves measured with the three assemblages. The horizontal lines indicate the background levels of BC samples.

tion in the real-time PCR assay. All samples were run in duplicate.

Standard curves based on the three assemblages are shown in Fig. 2. The Ct values are plotted versus the logarithm of the number of PSA molecules in the samples. The standard deviations of duplicate samples were 0.45, 0.25 and 0.21 cycles for assemblages I, II, and III, respectively (Table 1). The standard deviation of duplicate DNA samples analyzed by the real-time PCR assay only was about 0.16 cycles (data not shown). The BC samples gave rise to fluorescence amplification curves similar to those of the positive samples (Fig. 3), suggesting that the BC reaction generates the same products as the PSA positive samples. This is due to residual DNA not removed in the washing steps. The BC signal limits the sensi-

Table 1
Dynamic range and reproducibility of real-time immuno-PCR and ELISA for PSA detection

	Dynamic range ^a (molecules)	SD ^b (cycles)
Assemblage I	$1.7 \times 10^6 - 1.0 \times 10^{10}$	0.45
Assemblage II	$4.8 \times 10^5 - 5.6 \times 10^9$	0.25
Assemblage III	$1.8 \times 10^6 - 5.6 \times 10^9$	0.21
ELISA	$5.7 \times 10^7 - 2.8 \times 10^{10}$	— ^c

^a Detection limit is expressed as mean value+three SD (ACS Committee on Environmental Improvement, 1980) of 18 BC samples.

^b Standard deviation of Ct calculated from at least 16 duplicate samples with differing PSA concentrations.

^c Not defined.

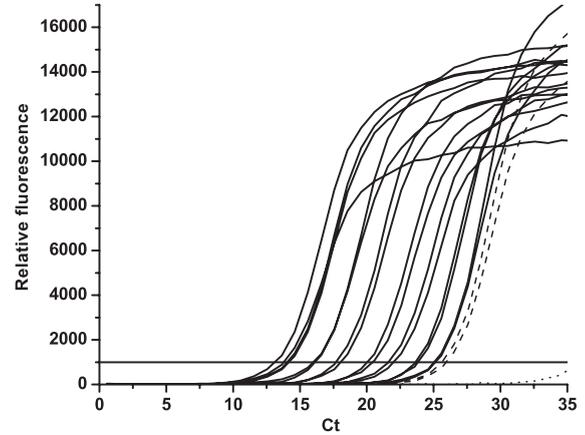


Fig. 3. Amplification curves obtained with assemblage II. Dilution series of PSA (duplicate samples) ranging from 3.5×10^5 to 5.6×10^9 molecules per reaction (—), background control (BC) (- - -), and no template control (NTC) (· · · · ·). NTC does not cross the threshold until after 35 cycles.

tivity of the assay and should be minimized. When comparing the assemblages, we defined assay sensitivity as the mean of the apparent concentrations plus three standard deviations (ACS Committee on Environmental Improvement, 1980) of 18 BC samples (Table 1). We used the same definition for the lower limit of the dynamic range. As seen, assemblage II was about three times more sensitive than the other two assemblages, and it spanned a range of more than four orders of magnitude. Compared to ELISA, the sensitivity of the real-time immuno-PCR assay was more than 100 times higher.

3.2. Classification of serum samples

PSA levels were measured in 30 serum samples by the real-time immuno-PCR assemblage II, to test its applicability for diagnostics. The samples were analyzed along with standard samples of PSA for calibration.

All female samples gave signals that could not be distinguished from background indicating very low PSA concentrations (< 0.005 ng/ml). Samples from men with prostate cancer had higher PSA levels than samples from healthy males, except for one healthy male sample, which had a very high PSA concentration. The two samples from men with benign prostatic hyperplasia had similar PSA levels as those samples from healthy males. PSA levels in samples from

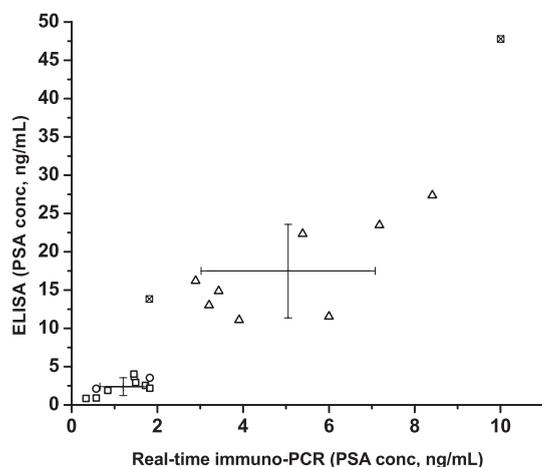


Fig. 4. PSA concentrations in serum samples measured with ELISA and with real-time immuno-PCR. Serum samples were from healthy males (□), males with prostate cancer (△), and males with benign prostatic hyperplasia (○). PSA levels in all female samples were below the detection limit of the techniques. The bars indicate mean \pm 1 SD for samples from males with prostate cancer and for samples from healthy males, as measured with ELISA and real-time immuno-PCR. Two outlier samples from healthy males were omitted from the calculation of averages (⊗).

healthy males and samples from men with prostate cancer were compared by ANOVA and found to be different at the 95% confidence level ($P=0.05$).

The serum samples were also analyzed with a commercial EIA kit from CanAg Diagnostics. Fig. 4 compares the measured PSA levels with real-time immuno-PCR and with ELISA in a scatter plot. The correlation between the two methods was good although the real-time immuno-PCR generally predicts lower concentrations than the ELISA, when compared with standard samples.

4. Discussion

We have compared three ways to assemble the real-time immuno-PCR system: a stepwise assembly that requires six incubations and washing steps (assemblage I), and two assemblages that use a covalent detection antibody/DNA conjugate which reduces the number of washing steps. In one of them, the capture antibody is passively adsorbed to the microtiter plate well which requires a total of three washing steps (assemblage II), and in the other, biotinylated

capture antibody is bound to streptavidin coated PCR microtiter plates resulting in only two washing steps (assemblage III).

The three real-time immuno-PCR assemblages exhibited similar dynamic ranges (Fig. 2). For all assemblages, the standard curves were observed to level off at both high and low PSA concentrations. At high PSA concentration, quantification is limited by the amount of assay components, which in our case is the capture antibody (data not shown). At low analyte concentration, either background from non-specific binding of assay components or the antibody-antigen affinity is limiting (Jackson and Ekins, 1986). In our PSA system, the rather low Ct values of BC samples suggest that non-specific binding is limiting (Fig. 2).

Several of the assay components may contribute to the BC signal. Biotinylated detection antibody, streptavidin, DNA-label, and antibody/DNA-label conjugate can all bind non-specifically to surfaces. In assemblage I, biotinylated detection antibody that binds to the plastic surface and is not removed by washing will be bound first by streptavidin and then by biotinylated DNA-label, which generates PCR signal. Likewise, streptavidin bound non-specifically to the surface will bind biotinylated DNA-label and give rise to PCR signal also in absence of analyte. Finally, the biotinylated DNA-label itself may adsorb to the surface generating non-specific PCR signals. In assemblages II and III, it is the detection antibody/DNA-label conjugate that binds to vessel surfaces generating non-specific PCR signal. In all assemblages, blocking agents were used to reduce non-specific binding. After extensive optimization, we found that BSA combined with Tween 20 gave the best results. We also tested to out-compete adsorption by adding herring sperm DNA to the incubation buffers, but this did not improve sensitivity (data not shown).

Assemblage II was the most sensitive of the tested assays. It can positively detect 4.8×10^5 molecules (mean+3 SD of 18 BC samples). This makes it about three times more sensitive than assemblages I and III. The lower sensitivity of assemblage I is most certainly due to the larger number of washing steps needed, and the larger number of components that can bind non-specifically to the vessel surface. Assemblage III requires one washing step less than assem-

blage II but is less sensitive. Possibly the immobilized streptavidin or the biotinylated capture antibody gives rise to higher non-specific background (Fig. 2). Background signal generally increases with the amount of antibody/DNA-label conjugate added (data not shown). When comparing assemblage III with assemblage II, we noted that assemblage III had somewhat higher backgrounds even though less antibody/DNA-label conjugate was used.

Although the sensitivity of real-time immuno-PCR is high compared to ELISA, it is still way below the sensitivity of regular real-time PCR for nucleic acid detection, which typically detects some ten DNA molecules positively. This is due to the homogeneous nature of the real-time PCR assay. Not relying on adsorption and washing, background is essentially zero, and assay sensitivity is only limited by sample handling and primer-dimer formation (the NTC signal). A corresponding homogeneous assay for proteins was recently developed based on proximity probes (Gullberg et al., 2004). Although highly promising, it requires two antibodies to be labeled with oligonucleotides without interfering with antigen binding, which may be difficult. Furthermore, inhibition of the PCR is expected to be a more serious problem because contaminants are not removed by washing prior to PCR.

The reproducibilities of assemblages II and III were substantially higher (SD~0.2 cycles) than was the case with assemblage I (SD~0.5 cycles) (Table 1). In assemblage I, the amount of DNA used was about 100 times less than the amount of antibody/DNA-label conjugate used in assemblages II and III. This results in higher Ct values (Fig. 2). But since there was no correlation between Ct and SD in the dilution series, it cannot explain the higher experimental variation of assemblage I compared to assemblages II and III. Instead the high SD of assemblage I is most certainly due to the larger number of incubation and washing steps needed. This in turn is a consequence of attaching the DNA-label to the detection antibody in the course of the assembly process. Hence, attaching the DNA-label to the detection antibody first and purifying the antibody/DNA conjugate prior to assembly improves reproducibility. Instead of using a chemical conjugate, one can premix the streptavidin and the DNA as suggested by Gofflot et al. (2004). This also reduces the number of washing steps but not as

much. The somewhat higher standard deviation of assemblage II compared to assemblage III is most likely due to the extra incubation and washing step required with assemblage II.

We used assemblage II for the quantification of PSA in clinical serum samples. We found an excellent correlation between the results of the PSA real-time immuno-PCR assay and the PSA ELISA (Fig. 4), suggesting that the real-time immuno-PCR assay also performs well when using more complex samples. Serum samples from female donors did not contain measurable PSA levels. By ELISA, PSA levels in female samples were below 0.1 ng/ml (25 μ l sample), which is the detection limit of this technique, whereas with real-time immuno-PCR PSA levels were below 0.005 ng/ml (5 μ l sample). One sample from a healthy male was found to have a PSA level similar to those of males with prostate cancer as measured with real-time immuno-PCR. This result was confirmed by ELISA, by which unusually high PSA level was found also in a second healthy male sample (Fig. 4). This may not be due to inherent errors in the assay, since PSA serum concentrations differ substantially among healthy males and some healthy individuals have natural levels similar to those typical of disease (Ablin, 1997). In general, real-time immuno-PCR predicted lower PSA levels than ELISA when comparing serum samples with standard samples based on purified PSA. This is a matrix effect caused by substances present in the serum but not in the standards that interfere differently with the real-time immuno-PCR and ELISA chemistries. The matrix effect can be calibrated for. The error bars in Fig. 4 indicate the mean \pm 1 SD of the PSA levels as measured by ELISA and by real-time immuno-PCR, of samples from healthy males and of samples from males with prostate cancer. The two samples from healthy males with elevated PSA levels were considered outliers and were omitted in the calculation. Both techniques clearly distinguish negative from cancer positive samples (95% confidence as estimated by ANOVA).

Compared to the ELISA based on the same antibodies, the real-time immuno-PCR assay (assemblage II) is about 100 times more sensitive (Table 1). This large improvement is due to the fundamentally different signal generation and detection processes. In ELISA, the detection antibody is coupled to a detec-

tion system that generates a linear signal increase with time, while in real-time immuno-PCR, the detection antibody is part of a PCR system that generates an exponential signal growth in time. Furthermore, the ELISA signal is read out as intensity value, while in real-time PCR, the number of amplification cycles needed to reach a particular signal is registered. These differences have major implications for the dynamic range and reproducibility of the two techniques. The difference is reflected in Fig. 5, where the standard curve based on real-time immuno-PCR (assemblage II) is compared with the standard curve based on ELISA. Both curves are shown using a log-scale. It is clear that the dynamic range of the real-time immuno-PCR, which for this particular system ranges from 4.8×10^5 to 5.6×10^9 molecules, is much larger than that of the ELISA, which ranges from 5.7×10^7 to 2.8×10^{10} molecules. This difference is primarily a consequence of the real-time readout in real-time immuno-PCR compared to the endpoint readout in ELISA. The reproducibility of the ELISA is, however, higher than that of real-time immuno-PCR (Fig. 5), which is a consequence of its linear response compared to the exponential response in PCR.

When comparing the three real-time immuno-PCR assemblages, assay time is an important factor. Not counting the overnight incubation with capture antibody needed with assemblages I and II, assay time for assemblage I is about 7 h, while for assemblages II

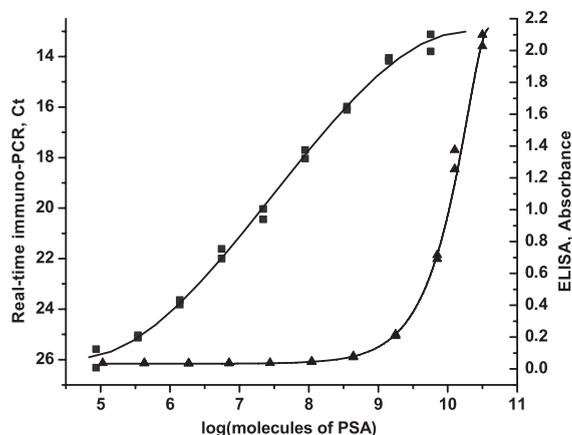


Fig. 5. Real-time immuno-PCR (assemblage II) (■) and ELISA (▲) readouts of standard samples (logarithmic scale).

and III, it is about 3 1/2 h. The time saved in assemblages II and III is due to the smaller number of incubation steps, which is possible because of the pre-conjugated detection antibody/DNA-label used. Also, it is easier to optimize new assays based on the conjugate because there are fewer reaction components. The only drawback is that conjugation, as we perform it, requires rather large amounts of antibody. It is also important to purify the conjugate stringently. If free DNA-label contaminates the conjugate the assay will lose sensitivity. When testing a new assay, the stepwise assemblage may be an option because it is cheaper and rather straightforward to set up. But for any routine work, assemblages based on detection antibody/DNA-label conjugates are recommended. Assemblage II has somewhat higher sensitivity than assemblage III, while the reproducibility of assemblage III is somewhat better (Table 1). Another advantage of assemblage III over assemblage II is that it does not require overnight incubation since all ingredients are added at the same time. This simplifies the procedure. However, assay costs are higher because streptavidin coated plates are five to ten times more expensive than regular plates.

There is an increasing interest in approaches able to analyze more than one analyte in the same reaction mixture by multiplexing. Assemblages based on pre-conjugated detection antibody/DNA-label are very suitable for multiplex protein quantification, because optimal DNA sequences to bind sequence specific probes can be used for each of the different detection antibodies and, hence, for each analyte. It may be an advantage to use streptavidin coated plates because the binding of capture antibodies is expected to be more homogeneous through biotin-streptavidin coupling than through adsorption only. Assemblage I cannot be multiplexed because of its stepwise assembly.

In summary, the real-time immuno-PCR assemblage that performed best was assemblage II, which is based on surface adsorption of capture antibody and preformed detection antibody/DNA-label conjugate. It had the highest sensitivity and also high reproducibility. Compared to ELISA, the real-time immuno-PCR was much more sensitive with a much wider dynamic range. These are major advantages in applications with a wide spread in target protein concentrations.

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