Published in final edited form as:

J Immunol Methods. 2011 June 30; 369(0): 74–80. doi:10.1016/j.jim.2011.04.006.

Immunoassay for the discrimination of free prostate-specific antigen (fPSA) forms with internal cleavages at Lys₁₄₅ or Lys₁₄₆ from fPSA without internal cleavages at Lys₁₄₅ or Lys₁₄₆

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Abstract

Total levels of circulating prostate-specific antigen (tPSA) are strongly associated with prostate cancer (PCa) risk and outcome but benign prostate disease is the most frequent cause of a moderately elevated PSA level. Free PSA (fPSA) forms are independently associated with PCa risk and contribute modest diagnostic enhancements above and beyond tPSA alone. We developed an immunoassay for fPSA subfractions containing internal cleavages at Lys₁₄₅ or Lys₁₄₆ (fPSA-N). The assay was based on blocking intact single-chain fPSA (fPSA-I) with antibody 4D4 which does not detect PSA containing internal cleavages at Lys₁₄₅ or Lys₁₄₆. We also measured fPSA-N in blood from healthy volunteers and in anti-coagulated plasma from 76 men with or without evidence of PCa at biopsy. The analytical and functional detection limits of this assay were 0.016 ng/mL and 0.10 ng/mL, respectively. The median recovery of male fPSA-N from female plasma was 95.0 %. All 12 female samples (average age 28 years) had fPSA-N concentrations at or below the analytical detection limit. The median fPSA-N concentration (0.050 ng/mL) in 9 healthy male volunteers (age < 40 years) was below the functional detection limit, 0.420 ng/mL in 27 patients with benign prostate conditions and 0.239 ng/mL in 49 patients with PCa. Deming regression analysis of the patient samples showed that the measured fPSA-N concentrations were generally 23% lower than the previously calculated (fPSA minus fPSA-I) concentrations, likely due to differences in the antibody combinations used. In conclusion, we have developed a sensitive,

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specific and direct immunoassay for fPSA-N which can be used to study the clinical relevance of this PSA isoform.

Keywords

prostate-specific antigen; free PSA isoform; internally cleaved PSA; nicked PSA; immunoassay; prostate cancer

1. Introduction

During the past two decades, measurements of prostate-specific antigen (PSA) levels in blood have become widely used and shown to strongly associate with both risk and outcome of prostate cancer (Catalona et al., 1991; Lilja et al., 2008; Thompson et al., 2004; Vickers et al., 2010b). However, despite evidence from large randomized population-based trials that PSA-based prostate cancer screening reduces mortality from prostate cancer controversy remains regarding the value of PSA-testing as there is evidence that PSA-testing leads to considerable overdetection and consequential overtreatment (Crawford et al., 2011; Hugosson et al., 2010; Schroder et al., 2009). Also, most men with moderately elevated PSA do not have evidence of prostate cancer biopsy (Catalona et al., 1991; Hugosson et al., 2010; Schroder et al., 2009), and many men with PSA-levels below common biopsy cut-points harbour prostate cancer (Thompson et al., 2004; Vickers et al., 2010b). Benign prostate disorders such as nodular hyperplasia (BPH) or inflammation are frequent causes of a moderate PSA-elevations and reduces cancer-specificity of the PSA testing (Lilja et al., 2008; Schroder et al., 2009; Thompson et al., 2004). Based on findings that PSA in blood occurs both as free PSA (fPSA) and in a stable complex with alpha-1-antichymotrypsin (PSA-ACT), sensitive and specific assays were developed to measure each individual form (Lilja et al., 1991). This was critical to show that the ratio of free-to-total PSA was independently associated with prostate cancer risk (Christensson et al., 1993), and that measurements of fPSA (or PSA-ACT) enhanced cancer specificity compared to testing of total PSA (tPSA) alone (Catalona et al., 1998; Lilja et al., 2008). Commercialized immunoassays for free and complexed PSA are widely used in current clinical practice.

Circulating fPSA has been concluded to be non-catalytic since catalytically active PSA released into circulation rapidly forms complexes with the huge excess of protease inhibitors of the serpin-type such as ACT and alpha-2-macroglobulin (Piironen et al., 2001; Zhang et al., 1998). Isoforms of fPSA have been identified that have maintained some or all of the pro-peptide sequence (Mikolajczyk et al., 2004a; Peter et al., 2001), and the pro-peptide has been suggested as a mechanism of retaining inactivity (Lovgren et al., 1997; Vaisanen et al., 1999). In addition, internal cleavages of the protein backbone cause reduction in enzyme activity (Zhang et al., 1995). Of these, the internal cleavage at Lysine145 (Lys₁₄₅) (Christensson et al., 1990) but also other internal cleavages e.g. at Lys₁₈₂ (benign PSA or BPSA) most likely disrupt the protein conformation so that they render PSA inactive (Chen et al., 1997; Mikolajczyk et al., 2000). Subsequently, selective immunodetection of molecular fPSA isoforms in the circulation has been suggested as a novel approach to improve early detection of PCa. This has led to the development and

clinical evaluation of several immunoassays of these fPSA derivatives (Linton et al., 2003; Mikolajczyk et al., 2004a; Mikolajczyk et al., 2004b; Nurmikko et al., 2001; Steuber et al., 2002). We have developed an immunoassay for the single chain isoform that do not contain an internal cleavage between Lysines₁₄₅₋₁₄₆ or the “intact” PSA (fPSA-I) (Nurmikko et al., 2001). In a recent study fPSA-I was successfully used in a statistical model together with tPSA, fPSA and human kallikrein-related peptidase 2 (hK2) to predict the prostate biopsy outcome (Vickers et al., 2010a; Vickers et al., 2008).

We previously targeted on internally cleaved multi-chain fPSA in the circulation defined as “nicked” free PSA (fPSA-N), which encompasses protein formats that contain a characteristic clip behind Lys₁₄₅ alone, or in combination with, cleavage behind Lys₁₈₂ (Nurmikko et al., 2001; Steuber et al., 2005; Steuber et al., 2002). Clinical testing demonstrated a strong correlation of systemic circulating fPSA-N levels with increasing prostate volume in a large population of men referred for prostate biopsy due to a suspicious PSA level. In addition, we found fPSA-N determination to be useful in distinguishing men with PCa from those with an elevated PSA level caused by benign prostatic alteration. In these our initial findings fPSA-N was indirectly obtained by calculation from two separately determined immunoassays (fPSA-I subtracted from total fPSA). Direct immunodetection of fPSA-N would improve the analytical performance and expand the possibilities to study its clinical value as an independent marker in the statistical methods. Therefore, in this study we report the development of a sensitive and specific immunoassay for the direct measurement of fPSA-N.

2. Materials and methods

2.1. Purified proteins, monoclonal antibodies, other reagents and instrumentation

The monoclonal antibodies (Mab) H117, 5A10, 4D4, 2C1 and recombinant Fab 5A10 have been described elsewhere (Eriksson et al., 2000; Lilja et al., 1991; Nurmikko et al., 2001; Nurmikko et al., 2000; Pettersson et al., 1995). Monoclonal antibodies were labeled with Europium and biotinylated as previously described (Vaisanen et al., 2006). Microtiter plates coated with streptavidin, assay buffer and wash buffer concentrate were from Kaivogen, Turku, Finland. Enhancement solution was prepared as previously (Pettersson and Soderholm, 1990). The Victor 1420 Multilabel Counter was from Perkin-Elmer Life Sciences, Wallac, Turku, Finland.

2.2. Assay procedures

2.2.1. fPSA-N immunoassay—The specificity of the new immunoassay is based on the unique reactivity of the antibody 4D4 that does not recognize a PSA molecule with cleavage between Lys₁₄₅₋₁₄₆ (Nurmikko et al., 2000). Hence, this antibody is used to block all intact PSA isoforms (not cleaved at Lys₁₄₅₋₁₄₆) from participating in the sandwich formation which results in the detection of nicked PSA, fPSA-N, as the remainder portion.

The assay principle is shown in the Figure 1 A. We used a biotinylated fPSA specific recombinant Fab-fragment 5A10 as a capture antibody that recognizes all fPSA isoforms equally (Eriksson et al., 2000; Pettersson et al., 1995). The capture Fab (150 ng/well) was

immobilized to streptavidin coated wells in 25 μ L of assay buffer. After washing, 50 μ L assay buffer containing 25 mg/L of denatured mouse IgG and 50 μ L sample were incubated for one hour at room temperature. Plates were again washed and Mab 4D4 (5000 ng/50 μ L) was added to block all non-cleaved, intact PSA present in the sample. The blocking step was performed at + 4° C as an overnight incubation. The plate was sealed with a tape (Tarrax, Naantali, Finland) to minimize evaporation. Without a wash step, Europium-labeled Mab 2C1 (100 ng/well in 50 μ L volume) was added to detect all unblocked PSA and the incubation was continued for 4 hours at +4° C. The plates were washed before adding the enhancement solution (200 μ L/well). Plates were incubated for 30 min at room temperature before the time-resolved fluorescence measurement of the Europium signal. The affinity of 2C1 towards intact PSA (recombinant proPSA forms) and seminal plasma PSA (containing both intact and nicked PSA forms) is approximately the same (data not shown). The epitope of 2C1 is partly overlapping with that of 4D4 and thus it cannot bind to intact PSA that is already bound by 4D4 (Nurmikko et al., 2000).

2.2.2. fPSA, tPSA and fPSA-I immunoassays—In combination with the internally cleaved PSA assay, an otherwise identical assay but without the 4D4 blocking in the overnight incubation was performed (Figure 1 B.). This assay measures all free PSA forms regardless of internal cleavage sites and thereby only assay buffer without the Mab 4D4 is added. This non-blocking reaction was used to standardize the two assays identically. To measure comparable total PSA concentrations a protocol similar to fPSA immunoassay was used. The capture Fab 5A10 was changed to tPSA specific biotinylated Mab H117 which was used 300 ng/well in 25 μ L volume, otherwise the steps were identical to the fPSA assay. We also performed fPSA-I immunoassay as described previously (Vaisanen et al., 2006) with analytical and functional detection limits of 0.01 and 0.13 ng/mL, respectively.

2.2.3. Calibration—For calibration, we used affinity-purified recombinant precursor form of PSA, proPSA, which was produced in a baculovirus expression system as described previously (Rajakoski et al., 1997) and purified with affinity chromatography (Nurmikko et al., 2000; Vaisanen et al., 1999). The concentration of the recombinant proPSA was calibrated against the World Health Organization (WHO) standard (Rafferty et al., 2000). The proPSA dilutions (0.006–570 ng/mL) were made in Tris-saline-azide buffer (50 mmol/L Tris, 150 mmol/L NaCl, 0.5 g/L NaN₃, pH 7.5) containing 1 g/L bovine serum albumin and used as triplicates in the immunoassays.

Since we used the unblocked assay for standardization, we were able to use single-chain, intact PSA as a standard material, which offered an advantage due to its ease of availability. In addition, we could control the performance of 4D4 blocking of intact PSA and calculate the efficiency of blocking at different PSA concentrations. The specific signal of each calibrator dilution in fPSA-N assay was divided by the corresponding signal in the unblocked fPSA assay, the ratio was then subtracted from 1 and multiplied with 100 % to obtain the blocking efficiency for the particular calibrator dilution in each run. The average blocking efficiency of all calibrator dilutions was used to correct the fPSA-N concentrations for the cross-reaction with fPSA-I.

2.3. Clinical sample material

EDTA plasma of 12 apparently healthy female and 9 male volunteers were collected at the Department of Biotechnology, University of Turku in April 2007. The average age of both females and males was 28 years (range for females 23–50 years and for males 19–39 years). The 8 random unidentifiable female heparin plasma samples used for recovery study were obtained from Lund University, University Hospital UMAS (Malmö, Sweden) according to institutional review board approved procedures.

We also used samples from a total of 76 consecutive patients that underwent clinical work-up with digital rectal examination and transrectal ultrasound-guided prostate biopsy due to their symptomatic prostate condition at the Department of Urology at Turku University Hospital between October 2001 and February 2006. EDTA or heparin anti-coagulated plasma was retrieved from the patients prior to prostate biopsy or initiation of treatment by centrifugation within 3 hours from blood draw and then stored at -20°C until analysis. Median patient age was 64 years (range 43–80 years). There were 49/76 patients diagnosed with prostate cancer at biopsy and 27 patients with no evidence of prostate cancer at biopsy. Of the patients without evidence of prostate cancer, 9 patients had benign prostatic hyperplasia, 14 had prostatitis and 3 patients had both. One patient had atypical small acinar proliferation. There were 20 patients with organ confined cancer (pathological TNM classification, (Sobin LH, 1997)), 20 patients with locally advanced, non-organ confined cancer and 9 patients with metastasized cancer. In addition, heparin plasma samples of another 5 patients were used for the recovery study.

The samples were stored at -20°C before immunoassay measurements which were done during July 2009 at the Department of Biotechnology, University of Turku. The storage time was from 2 to 8 years. Based on prior data from the measurements of fPSA in anticoagulated plasma after 20 years of storage (Ulmert et al., 2006) and from the BPSA and fPSA-I measurements after short term storage (Linton et al., 2003; Nurmikko et al., 2001) the storage conditions were not expected to importantly influence the accuracy or precision of the measurements of different fPSA forms in anti-coagulated plasma. The Ethical Committee of the Turku University Hospital approved the study.

2.4. Assay evaluations

2.4.1. Analytical recovery—Five male heparin plasma samples were used to study the recovery of fPSA-N, fPSA and tPSA. The original fPSA-N concentrations were 1.211–6.486 ng/mL, fPSA concentrations 2.323–11.380 ng/mL and tPSA concentrations 10.350–36.020 ng/mL. A 1/20 volume of the male sample was added to a 19/20 volume of female heparin plasma pool without detectable PSA concentration. The samples were let stand at room temperature for 1.5 hours before measurement with the assays. The recovery was calculated by dividing the measured analyte concentration by the expected concentration.

2.4.2. Measurement of different seminal plasma PSA forms—PSA pools A, B, C, D and E purified from seminal plasma (Zhang et al., 1995) were a kind gift from Professor U-H. Stenman (Department of Clinical Chemistry, University of Helsinki, Helsinki, Finland). The pools contain different amounts of internally cleaved PSA as described by

Zhang et al. The pools were diluted to approximately 20 ng/mL (1 ng/well) in Tris-saline-azide buffer with bovine serum albumin. fPSA-N, fPSA and tPSA concentrations of the diluted pools were measured and the fPSA-N and fPSA concentrations were compared to tPSA concentrations.

2.5. Comparison to previously used assay methods

The tPSA and fPSA concentrations and calculated fPSA-N concentrations of the 76 patient samples had been determined previously (Peltola et al., 2011) with in-house immunoassay methods described elsewhere (Vaisanen et al., 2004; Vaisanen et al., 2006). These concentrations were compared to the concentrations measured with the assays described here.

2.6. Statistical analysis

MultiCalc software version 2.52 (Perkin-Elmer Life Sciences) was used to calculate the unknown concentrations based on the calibrator dilutions. fPSA-N concentrations were corrected by multiplying with the calculated blocking efficiency (see 2.2.3). The analytical detection limit was calculated as concentration corresponding the mean + 2 SD of the calibration diluent signal and the functional detection limit was determined as the lowest sample concentration with coefficient of variation (CV) <20 %. Deming regression analysis to compare the calculated and directly measured fPSA-N concentrations was performed using GraphPad Prism 4 version 4.03 (GraphPad Software, La Jolla, USA).

3. Results

3.1. Analytical performance

The fPSA-N assay was calibrated with intact proPSA in the fPSA assay, thus the assays had a common standard curve. This and tPSA assay standard curve and precision profiles (mean of five different runs) are shown in the Figure 2. The curves were linear in the range 0.059–55 ng/mL and the blocking efficiency in fPSA-N assay was stable 88 % at this range (average value of six calibrator dilutions in five different runs). The blocking efficiency at different calibrator concentrations is shown in Figure 2. Thus, we applied a correction factor 0.88 to the measured fPSA-N concentrations to correct for the 12 % cross-reaction with fPSA-I.

The analytical detection limits (based on 21 replicas of calibration diluent) were 0.016 ng/mL for fPSA and fPSA-N and 0.006 ng/mL for tPSA. The functional detection limits were 0.100 ng/mL for both fPSA-N and fPSA and approximately 0.05 ng/ml for tPSA (mean CV for male samples was 2.1 %, range 0–9.2 %).

Median fPSA-N recovery of the five male heparin plasma samples was 95.0 % (range 89.6–145.3 %). The median recoveries (range) for fPSA and tPSA were 90.7 % (84.7–101.4%) and 94.8 % (87.9–108.1%), respectively.

3.2 Detection of different seminal plasma PSA forms

The ratios of fPSA-N and fPSA to tPSA in the seminal plasma PSA pools A, B, C, D and E are shown in Figure 3. The proportion of fPSA ranged from 96 % to 121 % while fPSA-N proportion increased from pool A (9 %) to pool E (73 %).

3.3. fPSA forms in clinical material

3.3.1. Healthy volunteers—The fPSA-N, fPSA and tPSA as well as fPSA-I concentrations of the 12 female samples from healthy volunteers were all at or below the analytical detection limits of the assays. In the 9 healthy male volunteers the median (interquartile range) fPSA-N concentration (0.050 ng/mL, 0.033–0.064 ng/mL) was below the functional detection limit of the assay (Figure 4). Median concentration (interquartile range) of tPSA was 0.649 ng/mL (0.432–0.821 ng/mL) of which 38.5 % (35.2–40.8%) was in free form. Of the fPSA, 19.9 % (18.1–22.4%) was fPSA-N and 70.8 % (68.2–71.3%) was fPSA-I (median values with interquartile range).

3.3.2. Patient samples—The median (interquartile range) fPSA-N, fPSA and tPSA concentrations in the 27 patients with benign prostate conditions were 0.420 ng/mL (0.153–0.786 ng/mL), 1.385 ng/mL (0.651–2.771 ng/mL) and 10.338 ng/mL (4.375–19.564 ng/mL), respectively. For the 49 prostate cancer patients the median (interquartile range) concentrations for fPSA-N, fPSA and tPSA were 0.239 ng/mL (0.131–0.452 ng/mL), 0.855 ng/mL (0.628–1.761 ng/mL) and 5.464 ng/mL (4.758–10.743 ng/mL), respectively. The distribution of fPSA-N concentrations in different patient groups is shown in Figure 4.

3.3.3. Comparison to previously used methods—The directly measured and calculated fPSA-N concentrations in the 76 patient samples are plotted on Figure 5. The Deming (model II) regression analysis was done to compare the agreement between the methods and the regression line is shown in the Figure 5. The slope was 1.230 (95% confidence interval 1.157–1.304) and the y-axis intercept when $x=0$ was 0.0285 (95% confidence interval –0.0476–0.1047).

The measured fPSA-N concentrations were on average 91 % (SD 64 %, median 77 % interquartile range 62–96 %) of the calculated values. With the new tPSA assay (antibody combination bio-H117/2C1-Eu) the concentrations were on average 75 % (SD 14 %, median 73 %, interquartile range 67–82 %) of the previously used assay (antibody combination bio-H117/H50-Eu) (Peltola et al., 2011). The concentrations measured with the new fPSA assay (antibody combination bio-5A10-Fab/2C1-Eu) were on average 128 % (SD 51 %, median 112 %, interquartile range 95–145 %) of the concentrations measured with the assay used previously (antibody combination bio-H117/5A10-Eu).

4. Discussion

We have developed an immunoassay for measuring fPSA-N, a free PSA isoform with internal cleavage site at Lys₁₄₅. Previously we have measured the fPSA-I form which is not cleaved at Lys₁₄₅ and subtracted its concentration from the fPSA concentrations to calculate the amount of the fPSA-N. Developing a direct assay for fPSA-N improves the analytical

performance and enables the use of fPSA-N as an individual marker in different statistical models.

Internally cleaved free PSA forms (fPSA-N and BPSA) have been associated with BPH (Stephan et al., 2009; Steuber et al., 2005) while the single-chain intact free PSA forms (fPSA-I and proPSA) seem to be more cancer related (Jansen et al., 2010; Steuber et al., 2002). These forms have been used in many studies which have aimed at developing (statistical) methods to improve PCa diagnostics in different clinical situations. Serum fPSA-I and calculated fPSA-N have been used in a statistical model to predict which of the PSA screened men would benefit from biopsy (Gupta et al., 2010; Vickers et al., 2010a; Vickers et al., 2008). The number of biopsies could be reduced by 49–60 % and the few cancers that were missed were mostly low-grade cancers. The direct fPSA-N immunoassay described here might improve further the diagnostic power of this or other statistical models either in combination with or replacing the other markers used.

The developed assay construct for fPSA-N uses the same fPSA-I specific antibody 4D4 as applied in the fPSA-I assay (Nurmikko et al., 2000), but in the fPSA-N assay 4D4 is used to block all fPSA-I away instead of using it as a reporter molecule. The reporter antibody 2C1 in the fPSA-N assay has an epitope partly overlapping with that of 4D4, and thus cannot bind to fPSA-I simultaneously with 4D4. In order to achieve stable blocking, careful optimization of the labeling reaction of Mab 2C1 was required as well as a large excess of Mab 4D4 in the assay. However, as demonstrated with our 88 % blocking efficiency, the binding of 4D4 and 2C1 is not completely mutually exclusive. The blocking degree could be further increased by increasing the amount of 4D4 per well but we did not consider it to be beneficial anymore since the amount of Mab consumed would become too high. Also, enhancing the affinity of 4D4 would likely improve the blocking efficiency and we are currently modifying the recombinant Fab fragment of 4D4 in order to apply an high-affinity binder to the assay. The stable 88 % blocking efficiency was considered satisfactory at this stage.

Some concern was caused by the finding that with the fPSA-N assay we measured generally 5–40 % lower fPSA-N concentrations compared to the calculated values. Based on the Deming (model II) regression analysis the difference seem to be proportional rather than constant as the slope (1.23) is indicating the calculated values to be generally 23% higher than the measured concentrations while the y-axis intercept is close to zero. This difference may merely reflect the different tracer antibodies and the assay constructs (Kort et al., 2006; Stephan et al., 2006) or the slightly low affinity of 4D4 antibody affecting the blocking degree. The recoveries of PSA forms in male heparin plasma spiked into female heparin plasma pool were acceptable (100% $\pm$ 20%) except for one sample which had fPSA-N recovery of 145.3%. This may be due to some factor in the male sample that interferes with the blocking or a random error.

Zhang et al. (1995) have purified seminal plasma PSA into five pools (from A to E) which contain different proportions of fPSA-I and fPSA-N. The measured proportions of fPSA-N in these pools were as reported except for the pools D and E which contained 40% and 73% of fPSA-N instead of the reported 90 % and 95% of nicked PSA, respectively. Both the

patient samples and the pools may also contain other forms of nicked PSA which are not recognized by our fPSA-N assay.

Our assay is calibrated with recombinant intact proPSA in the fPSA assay without the blocking step. This calibrator was compared to known dilutions of pool E PSA and no difference between the two calibrators in the measured fPSA-N concentrations of 60 patient samples was observed (data not shown). We consider the current calibration method accurate until we have developed an efficient method to purify fPSA-N for calibration purpose

For the future, the described fPSA-N in-house assay would benefit from further assay improvements in order enable a simplified and automated assay protocol. Also, the present standardization of the PSA-N assay using single-chain recombinant preparation without blocking needs to be replaced by a direct standardization with Lys₁₄₅₋₁₄₆ internally clipped material. We are currently addressing several aspects of the assay to arrive at a simplified automated protocol suitable for implementation into routine testing.

Acknowledgements

The work on this research was funded by the Finnish Academy (project numbers 78541 and 206690), the National Technology Agency of Finland (TEKES), European Union 6th Framework contract LSHC-CT-2004-503011 (P-Mark), the National Cancer Institute (grant number P50-CA92629), Swedish Cancer Society [3455], the Sidney Kimmel Center for Prostate and Urologic Cancers, and David H. Koch through the Prostate Cancer Foundation. We thank Veikko Wahlroos and Pirjo Pietilä for their excellent technical assistance.

Non-standard abbreviations

ACT	alpha-1-antichymotrypsin
BPH	benign prostatic hyperplasia
BPSA	benign prostate-specific antigen
fPSA	free prostate-specific antigen
fPSA-I	intact free prostate-specific antigen
fPSA-N	internally cleaved “nicked” prostate-specific antigen
hK2	human kallikrein-related peptidase 2
PCa	prostate cancer
proPSA	pro-form of prostate-specific antigen
PSA	prostate-specific antigen
PSA-ACT	complex of prostate-specific antigen and alpha-1-antichymotrypsin
tPSA	total prostate-specific antigen
WHO	World Health Organization

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Fig. 1.
A The nicked free PSA (fPSA-N) immunoassay principle and B the free PSA (fPSA) immunoassay principle.

Fig. 2. Standard curves (closed symbols) and imprecision profiles (open symbols) for fPSA and fPSA-N assay (rectangle) and tPSA (square). The same standard curve was used for both fPSA and fPSA-N assay due to proPSA standardization. Blocking efficiency of f...

Fig. 3. Proportions of fPSA (circle) and fPSA-N (square) to tPSA in PSA pools purified from seminal plasma (Zhang et al., 1995).

Fig. 4. Distribution of fPSA-N concentrations in different patient groups. The horizontal line represents the median value.

Fig. 5. Concentration of fPSA-N determined by measuring directly with fPSA-N immunoassay or by calculation (fPSA minus fPSA-I) in 76 men with benign or cancerous prostate condition. The line represents the Deming (model II) regression analysis.