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Ultrasensitive detection of prostate-specific antigen by a time-resolved immunofluorometric assay and the Immulite® immunochemiluminescent third-generation assay: potential applications in prostate and breast cancers

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We report an ultrasensitive time-resolved immunofluorometric assay (TRIFA) for prostate-specific antigen (PSA). The assay is an improvement of our previous report (*Clin Chem* 1993;39:2108–14) and includes the utilization of two monoclonal antibodies and a one-step incubation period, which greatly reduces analysis time. The new method demonstrates a superior lower analytical limit of detection (≤1 ng/L), a wide dynamic range, absence of a hook effect at 10^6 ng/L PSA, and equimolarity for free PSA and PSA-antichymotrypsin complex. Also, we have compared several aspects of our TRIFA with a commercially available third-generation assay (Immulite®). An evaluation of breast tumor cytosol extracts from 315 patients shows PSA immunoreactivity >15ng/g of total protein in 28% and 23% by TRIFA and Immulite analysis, respectively. Both methods demonstrate a significant association between breast tumor PSA immunoreactivity and progesterone and estrogen receptor positivity (P <0.001). Analysis of serum samples obtained for monitoring of postradical prostatectomy patients reveals significant PSA changes at concentrations undetectable by conventional methods. The significance of these results as well as the potential applications of ultrasensitive PSA assays in breast and prostate cancers are discussed.

INDEXING TERMS: tumor markers • clinical chemistry analyzer

Prostate-specific antigen (PSA), a 33-kDa glycoprotein with serine protease activity, is found in copious amounts in the prostate and seminal plasma *[1–4]*.[1] In its physiological role, PSA acts to liquify the seminal clot formed after ejaculation *[5]*. An abnormally increased serum PSA concentration serves as one of the hallmarks of prostatic adenocarcinoma. The determination of serum PSA concentration, in combination with rectal examination, has been proposed as a screening test for prostatic carcinoma *[6, 7]*. While support for this particular application is not unanimous at present *[8]*, investigations into the clinical utility of serum PSA in screening for prostate cancer continue *[9, 10]*. In contrast to the debate surrounding its putative value as a screening tool, PSA is widely accepted and used to monitor and manage patients with medically established prostate cancer *[11–14]*. Serial monitoring of postprostatectomized patients for increased serum PSA is a common approach for the detection of recurrent or metastatic cancer *[15–18]*. Furthermore, it has recently been demonstrated that PSA's potency as a marker for disease monitoring is greatly enhanced when ultrasensitive, as opposed to conventional, assays are used for its determination *[19–21]*. For example, Yu et al. *[21]* estimate that by using a time-resolved immunofluorometric PSA assay system with a detection limit of the order of 10 ng/L, patient relapse could be determined several months or years earlier than by using conventional assays with detection limits of 100 ng/L or higher. Excitement surrounding the power of these ultrasensitive methods has been largely responsible for the appearance of commercially available "third-generation" PSA immunoassays, which have been developed for use on automated analyzers such as Immulite®. Thus, third-generation PSA testing capabilities are

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[1] Nonstandard abbreviations: PSA, prostate-specific antigen; TRIFA, time-resolved immunofluorometric assay; DFP, diflusinal phosphate; BSA, bovine serum albumin; SA-ALP, streptavidin-conjugated alkaline phosphatase; NHS-LC-Biotin, *N*-hydroxysuccinimide ester of biotin; ACT, α_1-antichymotrypsin; ER, estrogen receptor; and PR, progesterone receptor.

now available in an automated platform to any clinical biochemistry service laboratory that wishes to use them.

There is, in addition to prostate epithelial cells, a growing list of fluids, tissues, and (or) cells that have been found to be associated with PSA immunoreactivity. The list now includes breast milk, *[22]*, breast cyst and amniotic fluids *[23]*, parotid glands *[24]*, endometrial tissue *[25]*, normal breast tissue *[26]*, and various tumor tissues *[27]*, including those of the breast *[28, 29]*. In the latter case, PSA immunoreactivity was associated with steroid hormone receptor positivity, suggesting a possible role for PSA as a biochemical marker for prognosis and (or) treatment of breast cancer *[28, 29]*. The PSA concentration in such tissues is relatively low in comparison with that seen in seminal plasma and sera of patients with prostate cancer. It follows, therefore, that a very sensitive yet simple assay system is required for the investigation of the association between PSA and the pathobiochemistry of such tissues.

There exist at least two areas where ultrasensitive PSA assays can be of great value: (*a*) early warning of prostatic carcinoma relapse and (*b*) further elucidation of the association between breast cancer and tumor cytosol PSA concentrations. The first of these is directly related to the clinical setting, whereas the second is currently restricted to research. The requirement for a very sensitive yet simple and rapid assay for PSA has led us to the development of the time-resolved immunofluorometric assay (TRIFA) described herein. This report describes our new ultrasensitive PSA assay and contrasts several aspects of its performance to the commercially available Immulite method.

Materials and Methods

PSA ASSAYS

Instrumentation. Analysis of PSA was performed with the new TRIFA method as well as the Immulite third-generation PSA chemiluminescent enzyme immunoassay system [Diagnostic Products Corp. (DPC), Los Angeles, CA]. The 615™ Immunoanalyzer (CyberFluor, Toronto, ON), a time-resolved fluorometer, was used in our TRIFA analyses. The time-gate settings of this instrument as well as the interference filter used in the emission pathway have been previously described *[30, 31]*. The manufacturers' recommended calibration and maintenance schedules were followed for both instruments.

Reagents and solutions. All reagents for the analysis of PSA by the Immulite were obtained from DPC in kit format (#LKUP5). The reagents used in the preparation of buffers and solutions used in our new TRIFA method were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. The coating solution was 0.5 g/L sodium azide in 50 mmol/L Tris, pH 7.80; wash solution 0.15 mol/L NaCl and 0.5 g/L Tween 20 in 5 mmol/L Tris, pH 7.80; substrate buffer 0.15 mol/L NaCl, 1 mmol/L $MgCl_2$, and 0.5 g/L sodium azide in 0.1 mol/L Tris, pH 9.1; substrate stock solution 10 mmol/L diflusinal phosphate (DFP) in 0.1 mol/L NaOH (available from CyberFluor); development solution 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, and 3 mmol/L EDTA in 1 mol/L Tris base (no pH adjustment); assay buffer 60 g/L bovine serum albumin (BSA), 0.5 mol/L KCl, 0.5 g/L sodium azide, 50 mL/L normal mouse serum, 0.5 g/L Triton X-100 in 50 mmol/L Tris buffer, pH 7.80; and streptavidin-conjugated alkaline phosphatase (SA-ALP) diluent 60 g/L BSA in 50 mmol/L Tris buffer, pH 7.80. SA-ALP was obtained from Jackson Immunoresearch, West Grove, PA.

Antibodies. The new TRIFA was developed with two murine monoclonal anti-PSA antibodies. Both antibodies were obtained from Diagnostic Systems Laboratories (DSL), Webster, TX as 1 g/L solutions. Antibody DSL-01 is used for coating and DSL-11 for detection. The monoclonal DSL-11 was prepared for biotinylation by overnight dialysis against 0.1 mol/L sodium bicarbonate, followed by addition of an equal volume of carbonate buffer (0.50 mol/L, pH 9.1) to a final protein content of ~0.5 g/L. *N*-hydroxysuccinimide ester of biotin (NHS-LC-Biotin) was solubilized in dimethyl sulfoxide (1 mg in 50 μL) before incubation with the antibody (2 h, 25 °C). We used 1 mg of NHS-LC-Biotin per mg of antibody.

Microtiter well preparation. Twelve-well microtiter polystyrene strips were purchased from Dynatech Labs. (Alexandria, VA). These opaque white wells were coated overnight (≥8 h, 25 °C) with monoclonal antibody DSL-01 in coating buffer (100 μL/500 ng antibody per well). After this incubation period, the wells were washed twice with wash solution.

Calibrators. For TRIFA PSA analysis, we prepared purified seminal plasma PSA calibrators by diluting human seminal PSA (a gift from T. Stamey, Stanford University, Palo Alto, CA) in 50 mmol/L Tris buffer (pH 7.80) containing 60 g/L BSA. The concentrations of the preparations used for calibration were 0, 5, 10, 25, 100, 500, 2000, and 10 000 ng/L. Additional calibrators were prepared from a purified PSA-α_1-antichymotrypsin (PSA-ACT) calibration solution, also a gift from T. Stamey. Values for the TRIFA calibrators were assigned on the basis of the exact concentrations of the primary PSA and PSA-ACT preparations and were further checked by analysis on the Immulite. Agreement was within 10%.

The Immulite PSA assay is precalibrated with calibrators prepared by the manufacturer. Each lot-specific calibration curve is entered by barcode wand and further adjusted for the specific analyzer by analysis of two concentrations of commercially prepared PSA solutions.

Assay procedures. The frozen breast cytosol extracts and serum specimens were allowed to thaw by incubation at 5 °C and were subsequently vortex-mixed to ensure homogeneity. For the Immulite: Specimens were analyzed singly (volume ≥150 μL) as per the manufacturer's instructions. For the TRIFA: Calibrators and breast tumor cytosol extracts (50 μL) were added in duplicate to the coated and washed microtiter wells. Into each of these wells was added 50 μL of assay buffer containing diluted biotinylated monoclonal antibody DSL-11 (0.5 mg/L). The wells, containing assay buffer, detection antibody, and either calibrator or specimen, were then incubated for 1 h at 25 °C with shaking. At the end of this incubation period, the plates were washed six times with wash solution by using an automated microtiter plate washer. To each well was then added 100 μL of

SA-ALP conjugate stock solution diluted 1:20 000 with SA-ALP diluent (final quantity of SA-ALP added per well = 5 ng). The wells were incubated with conjugate for 15 min at room temperature with shaking and then washed six times with wash solution. Working substrate (100 μL; stock DFP solution diluted 1:10 in substrate buffer immediately before use to a final concentration of 1 mmol/L) was added to each of the wells and incubated for 10 min at room temperature with shaking. Finally, 100 μL of developing solution was added to each well (containing substrate) and incubated 1 min at room temperature, with shaking, before reading of the Tb^{3+} chelate fluorescence *[30, 31]*. Data reduction is performed automatically by the CyberFluor 615 Immunoanalyzer.

CLINICAL SPECIMENS

Breast tumor cytosols. Primary breast tumor tissue was obtained from female patients at participating hospitals of the Ontario Provincial Steroid Hormone Receptor Program. The tumor tissue was immediately frozen in liquid nitrogen after surgical resection and stored in this manner until extraction. The tissues were pulverized with a hammer under liquid nitrogen before extraction in ice-cold buffer (~0.5 g of pulverized tissue per 10 mL of 10 mmol/L Tris, 5 mmol/L EDTA, and 1.5 mmol/L sodium molybdate, pH 7.40). Extraction was facilitated by solubilization with a 5-s burst of a Polytron homogenizer (Brinkmann Instruments, Des Plaines, IL). The resulting homogenate was then centrifuged (~100 000*g* for 1 h) and the intermediate layer (cytosol extract) was collected. The protein content of the breast tumor cytosol extracts were quantified by the Lowry method *[32]*. The remainder of the cytosol extract was stored at −70 °C until further analysis. Before these analyses, the frozen extracts were allowed to thaw at 5 °C and vortex-mixed to ensure homogeneity.

Breast tumor receptor contents. Quantitative analysis of estrogen and progesterone receptors (ER and PR, respectively) was performed with the Abbott enzyme immunoassay kits (Abbott Labs., N. Chicago, IL) as per the manufacturer's instructions.

Other specimens. Aliquots were taken from separated serum specimens that were received in our laboratory and identified as being from prostate cancer patients, including postprostatectomized patients, as well as healthy female patients. These aliquots were stored at −70 °C until analysis.

Tumor cytosol PSA stability. Five breast tumor cytosol extracts were removed from storage at −70 °C and thawed at room temperature. After vortex-mixing, aliquots were removed from each. PSA analysis was carried out immediately on one aliquot from each of the five extracts and the remaining fractions were stored at −20, 4, 25, and 37 °C. PSA analysis was subsequently performed at 1, 8, and 15 days on a new aliquot from each of the storage groups.

EVALUATION OF IMPRECISION

An evaluation of imprecision was carried out as per NCCLS document EP10-T *[33]* for both the Immulite and our TRIFA third-generation assay systems. Control materials utilized in this evaluation were prepared by adding known quantities of PSA-ACT to 60 g/L BSA. These controls (six concentrations) were run by both methods in quadruplicate on each of six consecutive days.

CHARACTERIZATION OF PSA FRACTIONS

HPLC. PSA present in breast tumor cytosol extracts was evaluated by gel filtration HPLC on a Hewlett-Packard (Palo Alto, CA) Series 1050 system. The mobile phase used was 0.1 mol/L Na_2SO_4 and 0.1 mol/L NaH_2PO_4, pH 6.80. The isocratic runs were maintained at 0.5 mL/min. A 600 × 7.5 mm Bio-Sil SEC-250 gel-filtration column was used (Bio-Rad Labs., Richmond, CA). Column calibration was achieved with a molecular mass calibration solution containing thyroglobulin (670 kDa), IgG (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.4 kDa). Eluent fractions were collected by the Pharmacia (Uppsala, Sweden) FRAC-100 fraction collector and analyzed by both ultrasensitive PSA methods. Sera obtained from male prostate cancer patients were similarly run on HPLC and analyzed by the new TRIFA method as well as our formerly described TRIFA method *[19]*.

STATISTICAL ANALYSIS

All ANOVA, χ^2 tests, and the corresponding probability (*P*) values were calculated with the statistical software SAS (SAS Institute, Cary, NC). Correlation analyses were performed with the LINEST function of Microsoft Excel Version 5.0 (Microsoft Corp., Redmond, WA).

Results

PERFORMANCE CHARACTERISTICS OF THE TRIFA

Assay optimization. The final assay conditions selected were found to be optimal. In brief, we varied factors such as the combinations and quantities of various monoclonal and polyclonal antibodies, the characteristics of the diluents, and the periods of incubation to obtain the most precise and sensitive assay performance. The adoption of a one-step assay approach (i.e., concurrent incubation of specimen and detection antibody) was made only after an evaluation for high-dose hook effect (see below).

Calibration curve. Duplicate 50-μL calibrators (0, 5, 10, 25, 100, 500, 2000, and 10 000 ng/L PSA) exhibit an overall imprecision in fluorescence readings of <5%. The Immulite has a broader dynamic range of 0–20 000 ng/L. We have increased the TRIFA upper dynamic range limit severalfold by decreasing specimen volumes, but at the expense of sensitivity (data not shown).

High-dose hook effect. Given that this version of our PSA TRIFA includes a one-step incubation of analyte and detection antibody, we investigated the possibility of a high-dose hook effect by assaying preparations of free PSA up to 1 000 000 ng/L. As illustrated in Fig. 1, the assay does not exhibit a hook effect at these concentrations of PSA. A similar evaluation was performed

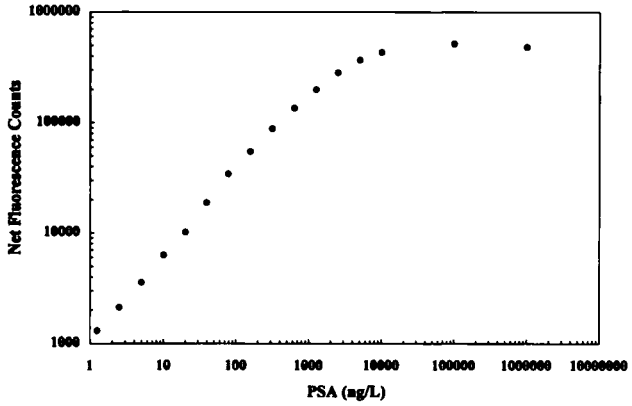

Fig. 1. TRIFA calibration curve.

TRIFA calibration curves are constructed by analyzing, in duplicate, 50 μL of human seminal PSA calibrators at eight concentrations, from 0 to 10 000 ng/L. Net fluorescence counts for each concentration are automatically calculated by the instrument by subtraction of the zero calibrator mean value (typically 1000–1500 arbitrary units). Fluorescence counts of duplicate analyses are routinely within 5% of the mean value. In the above example, the calibration curve was extended to evaluate for a high-dose hook effect.

on the Immulite and, in agreement with the manufacturer's claims, no hook effect was observed at 1 000 000 ng/L PSA.

Lower limit of detection. The lowest limit of detection of the TRIFA was determined by analyzing 11 replicates of the zero seminal PSA calibrator. The PSA concentration, which corresponds to the fluorescence of the zero calibrator plus 2 SD, was calculated to be 1 ng/L. This detection limit corresponds to 50 fg (~10^6 molecules) of PSA per assay. When we modified this assay to incorporate a 100-μL sample volume and include a 200 g/L BSA solution as a SA-ALP diluent (to further lower background), the detection limit dropped to 0.3 ng/L (data not shown). The Immulite PSA assay demonstrated a detection limit of 3 ng/L. The biological detection limits *[34, 35]* of the two assay systems were determined by using the estimation of total imprecision observed at 2 ng/L PSA-ACT (see below). We calculate these to be ~2 and ~4 ng/L for our TRIFA and the Immulite assays, respectively.

Imprecision. The results of our evaluation of imprecision for the Immulite third-generation PSA immunoassay and our own TRIFA method are presented in Table 1. The imprecision was found to be comparable for the two methods over a wide range of concentrations.

Recovery and equimolarity. To evaluate the recovery of free PSA by our TRIFA method, seminal PSA in 60 g/L BSA was used to supplement human sera and BSA (60 g/L) to concentrations of 50 and 1000 ng/L. Mean concentrations of 28 ng/L (57%, n = 3) and 463 ng/L (46%, n = 3) were recovered from supplemented female sera. Recoveries from male sera were similar to those from female sera at mean concentrations of 22 ng/L (44%, n = 3) and 509 ng/L (51%, n = 3), respectively. The low recovery reflects the binding of PSA to α_2-macroglobulin to form a complex that is not measurable by the two PSA assays. As expected, recovery of seminal PSA from BSA was practically complete, with mean values of 50 ng/L (100%) and 940 ng/L (94%), respectively. Similar recoveries were obtained for free PSA by the Immulite assay.

We have further assayed purified preparations of free PSA and PSA-ACT complexes in 60 g/L BSA, at concentrations ranging from 10 to 1000 ng/L. The molar response of the new assay to the two forms of PSA was similar (±10%), confirming the equimolarity of the new assay. Similar results were obtained with the Immulite.

Linearity. The TRIFA was evaluated for linearity over the range 3–400 ng/L by assaying, in triplicate, specimens prepared by mixing human male serum with high PSA concentrations (~410

Table 1. Evaluation of Imprecision of Immulite and TRIFA PSA assays.

	Nominal PSA-ACT, ng/L					
	3000	300	150	30	3	2
Immulite						
Grand mean[a]	3066	290	144	29	4.3	3.0
(SD)	(143)	(15)	(9)	(1.3)	(0.6)	(0.6)
Imprecision, % CV						
Within-run	3.4	5.4	4.9	4.1	14.4	19.9
Between-day[b]	3.4	2.3	3.6	2.0	3.2	6.4
Total	4.8	5.8	6.1	4.5	14.7	20.9
TRIFA						
Grand mean	3230	279	144	27	2.6	1.6
(SD)	(250)	(22)	(11)	(2.9)	(0.3)	(0.3)
Imprecision						
Within-run	6.1	6.3	4.8	9.8	10.4	8.1
Between-day	5.2	5.0	6.5	5.1	8.4	15.8
Total	8.0	8.1	8.0	11.1	13.4	17.7

[a]Grand mean and total SD of 24 determinations; see text for details.
[b]Between-day imprecision was calculated by using the variance of daily means.

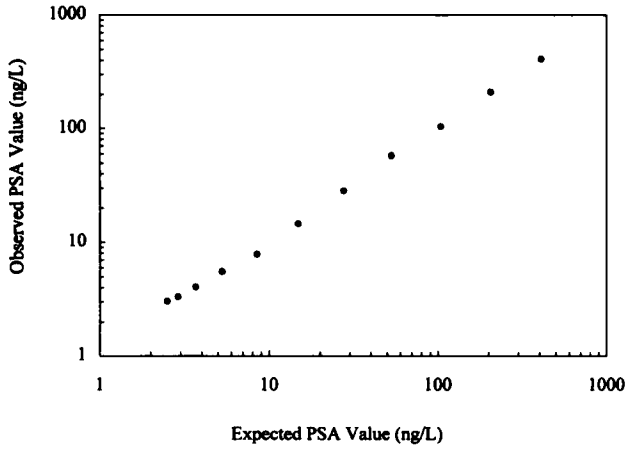

Fig. 2. Evaluation of linearity over the range 2–400 ng/L PSA for the TRIFA.

Data are based on dilution of a male human serum with female serum. Equation: $y = 0.76x + 1.00$, $R^2 = 0.9998$. The SE of the slope and the SE of the intercept were 0.004 and 0.618, respectively. $S_{y|x} = 1.74$.

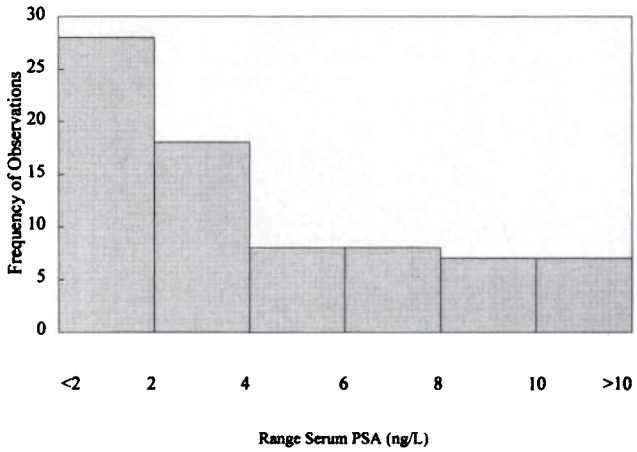

Fig. 3. Frequency histogram of 76 prostate cancer patients' serum PSA concentrations at least 8 weeks after radical prostatectomy.

Bins correspond to <2–2, >2–4, >4–6, >6–8, >8–10, and >10 ng/L PSA as indicated above.

ng/L) and serum with low PSA concentrations (~2 ng/L, obtained from a healthy female patient). The equation of the best-fitting regression line is given with Fig. 2. Another three male sera were also diluted from twofold to 32-fold with female serum and reassayed. These sera contained PSA ~1000, 500, and 400 ng/L. When the found PSA concentrations were plotted against the expected PSA concentrations, as shown in Fig. 2, the slopes of the linear regressions were between 1.00 and 1.02 and the intercepts between 6 and 30 ng/L. The correlation coefficients were >0.99 in all three cases, confirming good dilution linearity of the method.

Correlations with patients' sera. We analyzed 42 sera from post-prostatectomy patients with our new assay and Immulite. The range of values was from 0 to 1000 ng/L. When we plotted the TRIFA values (x) vs the Immulite values (y) and analyzed the data by linear regression, we obtained: $y = 1.18x + 13.6$ ng/L; $R^2 = 0.98$.

PATIENT SPECIMEN STUDIES

Postprostatectomy serum PSA. Serum specimens were collected for analysis by TRIFA from 76 prostatectomized patients at least 8 weeks after surgery. Specimens were chosen arbitrarily from those analyzed during routine service activity and assessed to have PSA concentrations at or below the lower limit of the manufacturer's recommended reportable range of the Immulite (10 ng/L). The median value of PSA was observed to be 2.6 ng/L. Of the 76 specimens, 28 (36%) and 46 (60%) possessed PSA values below the biological detection limits of the TRIFA and Immulite methods, respectively. The distribution of PSA immunoreactivity by the enhanced TRIFA method is shown in Fig. 3. Analyses by both new and old *[19]* TRIFA methods of serum PSA fractionated by HPLC are shown in Fig. 4. The two major peaks correspond to molecular masses of ~100–110 kDa (first peak) and 27–31 kDa (second peak), corresponding to PSA-ACT and free PSA, respectively. This patient's free PSA, as a percentage of total PSA, was ~10% by the new TRIFA method and 20% by our former method. This discrepancy arises from the greater PSA-ACT immunoreactivity detected by our new ultrasensitive method.

In Fig. 5 we present six representative patients who were monitored after radical prostatectomy with the new TRIFA method. These patients were selected to have PSA <100 ng/L after radical prostatectomy, thought to be free of cancer, and are still clinically asymptomatic. Patient a had significant PSA changes by TRIFA 100–200 days after surgery that were not detectable by the Abbott IMx assay. The PSA doubling time of this tumor calculated as described in ref. *21* by using the first three observation points was 32 days. Patient b had no indication of relapse, with values <3 ng/L during the observation period of

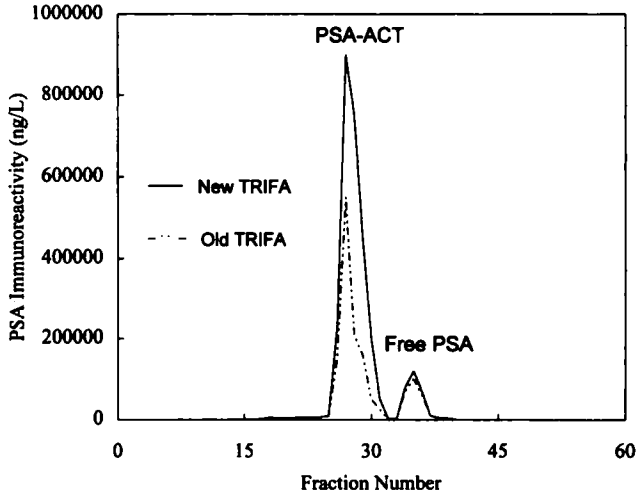

Fig. 4. Male serum PSA fractionation by HPLC and analysis by our former *[19]* and current TRIFA methods.

The first peak of each chromatogram corresponds to PSA-ACT complex (100–110 kDa), whereas the second corresponds to free PSA (27–31 kDa). The new TRIFA is equimolar for free and ACT-bound PSA; the old TRIFA detects free PSA about 2 times more efficiently than PSA-ACT. Both assays were calibrated with seminal plasma PSA.

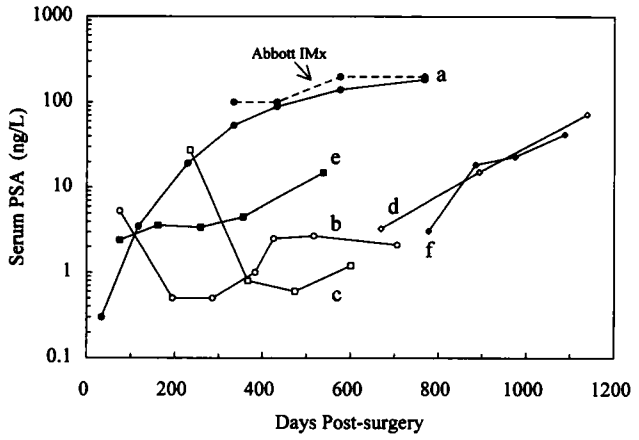

Fig. 5. Monitoring serum PSA changes in serum of six patients (a–f)after radical prostatectomy with the new TRIFA method.
For details see text.

~700 days. Patient c cleared his PSA at ~370 days after surgery and then stabilized his PSA to <1.2 ng/L over the observation period of >600 days, suggesting no relapse. Patient d had a clear PSA increase between 665 and 1095 days after surgery, with a calculated doubling time of 97 days. Patient e showed an abrupt increase in PSA from 4.5 ng/L at 355 days to 14.9 ng/L at 535 days, with a calculated doubling time of 103 days. Patient f had three consecutive PSA increases from a baseline of 3.1 ng/L, reaching PSA of 42 ng/L at 1069 days. His doubling time was 86 days. The clear changes of PSA in patients a, d, e, and f were undetectable by the Abbott IMx assay, which reported PSA <100 ng/L in almost all cases.

Stability of breast tumor extract PSA. Aliquots obtained from five breast tumor cytosol specimens containing PSA values ranging from 11 to 1090 ng/L were stored under various conditions and analyzed by the Immulite and TRIFA methods at 1,8, and 15 days following storage at −20, 4, 25, and 37 °C. A one-way ANOVA failed to uncover a significant effect of storage temperature, time in storage, or initial PSA concentration on the immunoreactive PSA detected by either method ($P > 0.05$). Thus, it appears that PSA immunoreactivity in these breast tumor cytosol preparations is very resistant to decomposition under a wide variety of storage conditions.

Breast tumor analysis. All breast tumor biopsy specimens that were collected and analyzed were from female patients with established breast cancer. The ages of these patients ranged from 27 to 94 years (median age 59 years). Percentile descriptors of specimen PSA and receptor contents are shown in Table 2. None of these three biochemical markers had normally distributed values. The median PSA concentration by third-generation TRIFA was 5 ng/L of cytosol extract, well below the sensitivity of most commercially available PSA assay systems. The values of cytosol extract protein content were normally distributed with a mean value of 1.71 g/L. Of the 315 breast tumor specimens analyzed, 88 (28%) and 73 (23%) had values exceeding our cutoff of 15 ng/g protein by the TRIFA and the Immulite third-generation methods, respectively.

No correlation between tumor cytosol extract PSA content (ng/g protein) and ER or PR content (fmol/mg protein) or age was found by linear regression analysis of the 315 data sets described above. This was true regardless of the method used to quantify PSA. On the other hand, there is a positive linear correlation between ER and PR content ($r = 0.37$, $P < 0.001$) in these specimens. A similarly strong linear relation was observed between the age of the patient and the ER content of the tumor extract ($r = 0.41$, $P < 0.001$). In addition, a weaker linear correlation was observed between patient age and tumor extract PR content ($r = 0.12$, $P = 0.028$). χ^2 analysis of breast tumor cytosol extract PSA concentrations (by both TRIFA and Immulite) and steroid hormone receptor status demonstrated a significant association ($P < 0.0001$) between PSA positivity (cutoff 15 ng/g protein) and both ER and PR positivity (cutoff 5 fmol/mg protein).

Correlation data for breast tumor cytosol extract immunoreactivity by the two ultrasensitive PSA assays are illustrated in Fig. 6. Over all the ranges evaluated, the slope of the best-fitting line is significantly <1, indicating that the TRIFA is estimating more PSA in these specimens than is the Immulite assay (see also Table 2). We characterized the PSA of female breast tumor cytosol extracts by gel filtration HPLC and analysis of PSA

Table 2. Percentile descriptors of breast tumor cytosol PSA and receptor contents.

	PSA, ng/L[a]				PSA, ng/g[c]	
Percentile	Immulite	TRIFA	PR, fmol/mg[b]	ER, fmol/mg[b]	Immulite	TRIFA
5th	0	0	1	0	0	0
10th	0	0	1	1	0	1
25th	0	2	4	4	0	1
50th	3	5	39	42	2	3
75th	18	26	174	147	12	19
90th	187	250	304	279	99	141
95th	762	1077	414	367	381	529
100th	12 700	12 750	999	548	5026	6543

[a]Breast tumor cytosol PSA concentrations observed in extracts.
[b]Breast tumor cytosol extract receptor contents normalized for protein content.
[c]Breast tumor cytosol PSA concentrations normalized for protein content.

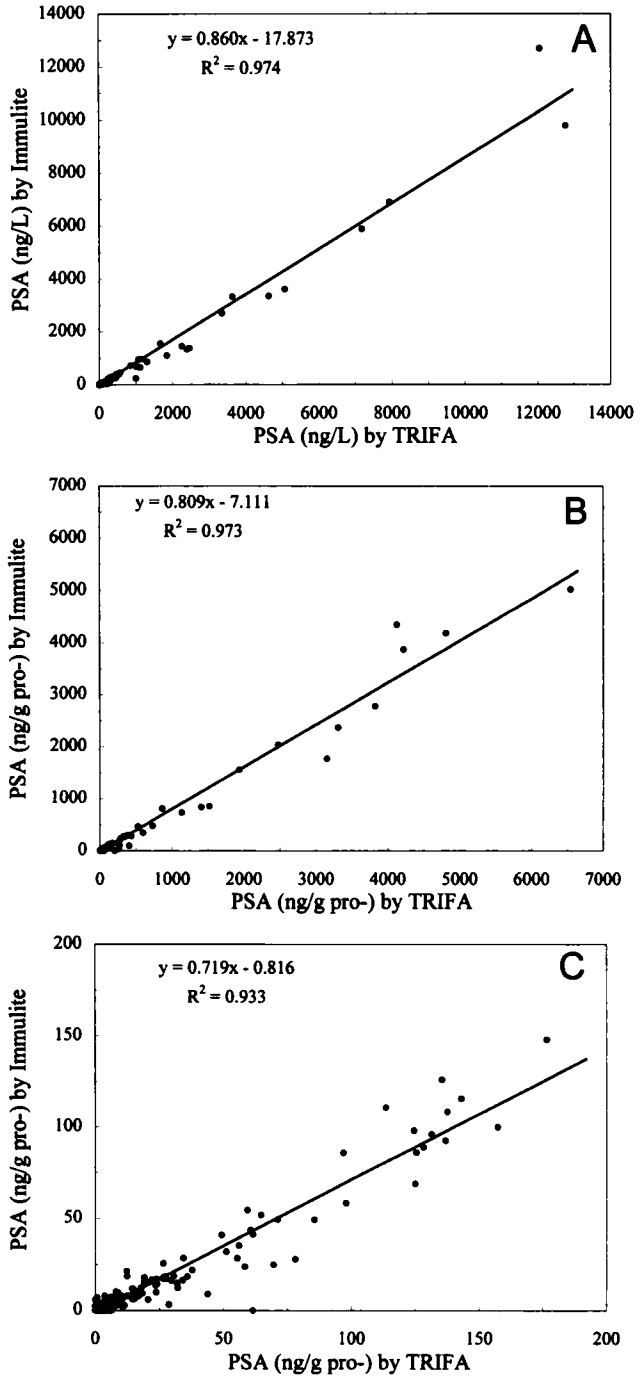

Fig. 6. Correlation of breast tumor cytosol extract PSA concentrations by TRIFA and Immulite methods.

(*A*) Correlation of extract PSA immunoreactivity. SE slope = 0.008, SE intercept = 10.308, $S_{y|x}$ = 179, n = 315. (*B*) Correlation of extract PSA normalized for protein content. SE slope = 0.008, SE intercept = 5.310, $S_{y|x}$ = 91, n = 315. (C) Correlation of extract PSA, normalized for protein content, in the range 0–188 ng/g. SE slope = 0.011, SE intercept = 0.387, $S_{y|x}$ = 5.9, n = 286.

immunoreactivity by both our TRIFA and the Immulite ultrasensitive assays. A typical chromatogram is illustrated in Fig. 7. The first peak of each chromatogram, representing the PSA-ACT complex, accounts for only a small fraction of the total PSA present in the extracts as determined by evaluation by both

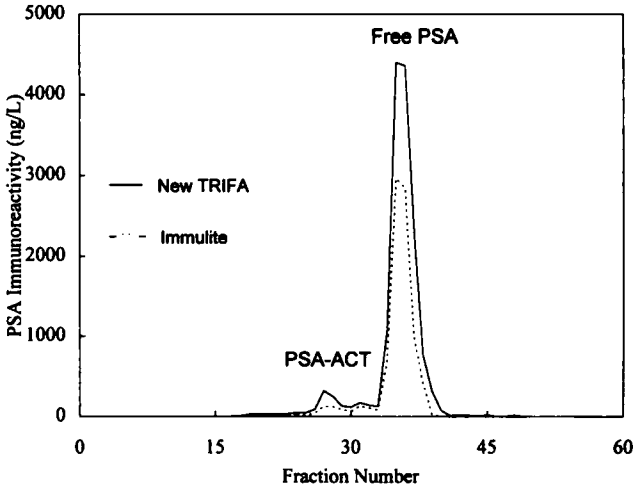

Fig. 7. Breast tumor cytosol PSA fractionation by HPLC and analysis by TRIFA and Immulite methods.

The first peak of each chromatogram corresponds to PSA-ACT complex (100–110 kDa); the second corresponds to free PSA (27–31 kDa).

TRIFA (7%) and the Immulite (5%) methodologies. The degree of immunoreactivity found by the two methods also differs. The total PSA immunoreactivity of the fractions collected (i.e., free + ACT-PSA) as determined by the Immulite is ~60% that determined by our TRIFA method.

Discussion

Ultrasensitive assays for PSA will undoubtedly contribute to opening up new avenues of opportunity in cancer management and research. Many of these opportunities already have been identified. Thus, it appears that such an analytical capability will contribute to the earlier detection of prostate cancer relapse and (or) residual disease in prostatectomized patients as well as the more timely evaluation of response to contemporary therapies (e.g., *[21, 34, 35]*). Furthermore, the utility of ultrasensitive PSA analysis is now extending beyond the realm of prostate cancer to that of breast cancer *[28, 29]* and probably other cancers *[27]*.

It is evident that conventional analytical systems for the determination of PSA do not have the detection limits necessary to quantify the relatively low concentrations of this tumor marker as it occurs in breast tumor cytosols and the sera of postprostatectomized men. For example, two popular assay systems for PSA analysis in Canadian service laboratories, the IMx® and AxSYM®, display analytical detection limits of ~20–30 ng/L *[35, 36]*. It is evident from the data presented here that methods such as the DPC Immulite third-generation PSA assay as well as our own new TRIFA PSA assay are suited to applications that these less sensitive methodologies are not.

The assay conditions related here for the TRIFA were selected for optimal sensitivity. In brief, we varied factors such as the combinations and quantities of various monoclonal and polyclonal antibodies, the characteristics of the diluents, and the periods of incubation steps to obtain the most precise and sensitive assay performance. The optimized assay described in this report differs from our previous assay *[19]* in two important respects. First, we use two monoclonal murine antibodies in the

present assay. Second, the detection antibody and specimen are added together to the capture antibody-coated microtiter wells, which allows a one-step incubation. This one-step approach not only simplifies the previous assay procedure *[19]* but shortens the assay time by at least 2.5 h. The adoption of this one-step approach was made only after an evaluation for high-dose hook effect. It is evident from Fig. 1 that our assay is not susceptible to this phenomenon at PSA concentrations ≤1 000 000 ng/L.

Another important improvement that is realized by the present TRIFA assay is its increased sensitivity. The assay design described previously was also based on enzymatically amplified time-resolved fluorometry with Tb chelate labels and displayed analytical and biological detection limits of 2 and 10 ng/L, respectively. The current optimized method displays even greater sensitivity, with analytical and biological detection limits of 1 and 2 ng/L, respectively, about two times lower than those found for the Immulite. With a greater sample volume (100 μL instead of 50 μL), the detection limit falls to <0.5 ng/L. The significant improvement in the calculated biological detection limit of our new TRIFA arises in part from its improved precision. For instance, within-run imprecision at 16 ng/L PSA was 21.4% with our former TRIFA method *[19]*. Our new optimized method, on the other hand, has only 18% total imprecision at a nominal PSA-ACT concentration of 2 ng/L (Table 1). In comparison, the automated DPC Immulite ultrasensitive assay has a biological detection limit of 4 ng/L PSA and an analytical detection limit of 3 ng/L, a value that is in agreement with the manufacturer's claimed value. Both systems offer significant improvement in sensitivity over most commercially available assay systems, including the Abbott IMx and AxSYM (detection limit = 20 ng/L *[36]*) and the BMI Enzymun-Test (detection limit = 50 ng/L).

Another important advantage of the present method is the equimolar recognition of free and ACT-bound PSA. PSA-ACT is the predominant form of circulating serum PSA (Fig. 4). Thus, an enhanced recognition of this form of PSA should give a greater ability to detect recurrence of prostatic cancer during monitoring than would be provided by a low detection limit for free PSA in and of itself. Fig. 4 illustrates the greater (~twofold) ability of the new method to detect PSA-ACT in comparison with our former TRIFA method. The benefits of equimolar reactivity for free and ACT-complexed PSA forms in terms of standardization have been reviewed by Graves *[37]*.

Of considerable importance is the benefit of such highly sensitive PSA assays for the monitoring of prostate cancer patients after radical surgery. In >50% of these patients, PSA is <10 ng/L (this study and *[21]*)—well below the detection limit of conventional assay systems. In these patients, the accurate postsurgical PSA concentration cannot be determined unless ultrasensitive methods such as the methods described in this report are used. We now possess the sensitivity to detect these concentrations and have the potential to detect recurrence at least one, and possibly as many as three, doubling times (i.e., months to years) sooner than previously possible *[5, 21]*.

In this report we describe six patients whom we monitored for PSA changes over a relatively long period after radical prostatectomy (Fig. 5). We chose patients who had at least one postsurgery PSA value <5 ng/L so that the benefit of monitoring in the ultrasensitive ranges is highlighted. Among the six patients, two (patients b and c) showed no indication of biochemical relapse and their PSA concentrations never exceeded 2.7 ng/L. In contrast, patients a, d, e, and f showed strong evidence of biochemical relapse, because their PSA increased consistently from 0.3 to 185 ng/L (a), 3.3 to 72 ng/L (d), 2.4 to 15 ng/L (e), and 3.1 to 42 ng/L (f). Almost all changes observed could not be seen with conventional assays such as the IMx, which have detection limits of ~20–30 ng/L, at least an order of magnitude inferior to the TRIFA assay described here. We anticipate that our assay, or similar assays developed by companies, will become invaluable tools in detecting early relapse when effective therapies of minimal disease are introduced. Unfortunately, the current capabilities of our assay could not be fully realized because there are no effective therapies to treat early relapsed prostate cancer. When these therapies become available, we will need clinical trials to assess the success rate when the therapy is instituted at the earliest possible time. Until such data become available, we would recommend, as Vessella did *[38]*, that the ultrasensitive assays be used only in research settings.

An important application of ultrasensitve PSA assay methodologies relates to the investigation of the relations between breast tumor pathobiochemistry, steroid hormone receptor status, PSA immunoreactivity, and therapeutic options. Such investigations are very likely to have clinical relevance. For instance, hormonal therapy is routinely used in the treatment of breast cancer and is based in large part on the ER and PR characteristics of the breast tumor. Only a fraction of ER- or PR-positive patients respond to endocrine therapy. Thus, an important goal of breast cancer research is to further define the prognostic power of available markers and to better tailor treatment modalities on the basis of available markers such as ER and PR status and putative markers such as PSA *[28, 29]*. Both our TRIFA assay and the Immulite third-generation PSA assay demonstrate the ability to reliably detect the low concentrations of this potential marker in extracts of breast tumor cytosols. In this study, we found 28% and 23% positivity for PSA immunoreactivity in breast tumor cytosol extracts by our enhanced TRIFA and the Immulite assays, respectively (Table 2). These rates correlate well with that determined previously in this laboratory with our older TRIFA method and a different breast cancer patient series *[28, 29]*. The higher positivity rate obtained by our enhanced TRIFA method presumably arises from its higher readings in comparison with the Immulite by a factor of 20–30%, especially at PSA concentrations <150 ng/mg (Figs. 6 and 7). This bias is unlikely to arise only from standardization bias, since our free PSA calibrators were checked against the Immulite calibrators and they agreed to ~±10% or less. We speculate that the bias arises from matrix differences between breast tumor extracts and serum, for which the Immulite is optimized to measure. In general, breast tumor extracts contain 1–5 g/L protein, whereas serum contains 60–80 g/L protein. In our TRIFA assay, total protein differences were minimized by adding the sample in parallel with the assay buffer, which contains 60 g/L total protein. Interestingly, comparison

of sera with PSA <1000 ng/L has shown that the Immulite assay measures values ~20% higher than TRIFA. Although the absolute values between TRIFA and Immulite for breast cytosols and serum differ, the correlation was excellent in both cases.

In contrast to the situation in male sera (Fig. 4), PSA in female breast tumors exists primarily as the uncomplexed free form (Fig. 7), with the ACT-complexed fraction accounting for only 5% (Immulite) to 7% (TRIFA) of the total PSA in these tumor tissues.

In the series of patients evaluated in this study, ER and PR positivity was associated with women of an older age. Also, breast tumor PSA positivity is preferentially associated with the early stages of breast cancer *[28]*. Furthermore, PR positivity is recognized as being a favorable prognostic indicator in breast cancer. Thus, further investigation into the clinical utility of PSA immunoreactivity as a prognostic marker in women with breast cancer is indicated by these findings. Such studies are currently under way in our laboratory.

In conclusion, we present data related to the performance and potential utility of third-generation PSA immunoassays in breast and prostate cancer treatment and research. Experience with such ultrasensitive assays is just beginning to accrue. Nevertheless, we anticipate that the utility of this methodology will soon be established through studies that are currently under way in our and other laboratories.

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