

# Detection of prostate-specific antigen mRNA and protein in breast tumors

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We have developed reverse transcription-polymerase chain reaction (RT-PCR) methods for detecting prostate-specific antigen (PSA) mRNA. Using these methods, and a highly sensitive immunofluorometric assay for measuring PSA protein, we have assessed the concentrations of PSA mRNA and PSA protein in 30 primary breast tumors and a few other control tissues. We found good agreement between presence of PSA protein and PSA mRNA in breast tumors. We thus propose that, in women, detection of PSA protein or PSA mRNA in tissues and tumors offers equivalent information. Because PSA protein is present in male blood and thus could contaminate extracts from tumors and tissues from men, we propose that the RT-PCR methods we describe be used to assess nonprostatic expression of the PSA gene in men.

**INDEXING TERMS:** gene expression • polymerase chain reaction • immunofluorometric assay

Prostate-specific antigen (PSA) is a 33-kDa glycoprotein thought to be produced exclusively by the prostate epithelial cells.<sup>1</sup> PSA is currently used as a marker for screening, diagnosis, and monitoring of prostate cancer [1-3]. Immunohistochemical detection of PSA in tumors of nonprostatic origin has been reported as a rare event [4]. Since the recent description by our group of the production of PSA protein by breast tumors [5], we have demonstrated that PSA is present in 30-40% of female breast tumors [6], more rarely in many other tumors [7], in normal breast tissue [8], and in biological fluids including breast milk [9], breast cyst fluid [10], breast discharge fluid, and amniotic fluid [11]. DNA sequence analysis of reverse transcrip-

tion-polymerase chain reaction (RT-PCR) amplified PSA mRNA has demonstrated that PSA mRNA derived from breast tumors is identical to PSA mRNA from prostatic tissue [12].

Many techniques are currently available for monitoring changes in gene expression, including Northern blots, RNase protection assays, in situ hybridization, and RT-PCR. Recently, PCR-based methods have been applied to the detection of micrometastasis or minimal residual malignant disease [13]. In the present study, we describe the development of sensitive RT-PCR assays capable of detecting PSA mRNA expression in breast tumors, using various nonisotopic detection systems. We have used these methods in parallel with a highly sensitive immunological method for measuring PSA protein [14] to study PSA expression at the mRNA and protein level in 30 breast tumor tissues.

## Materials and Methods

### MATERIALS

**PSA cDNA.** DH5 $\alpha$  *Escherichia coli* competent cells were transfected with a recombinant pA75 plasmid (a gift from J. Trapman, University of Texas, M.D. Anderson Cancer Center, Houston, TX). This plasmid contains the complete sequence of PSA cDNA [15, 16]. After transfection, the DH5 $\alpha$  cells were grown on Luria-Bertani (LB)-ampicillin agar plates at 37 °C overnight. Isolated colonies were used to inoculate 10-mL portions of LB-ampicillin media. Cultures were incubated for 4 h at 37 °C with shaking and then used to inoculate larger volumes of fresh media, which were incubated at 37 °C for 24 h. The pA75 plasmid was isolated and purified with use of the Magic Maxiprep kit (Promega, Madison, WI). The concentration and purity of the plasmid were determined spectrophotometrically.

A plasmid containing the complete  $\beta$ -actin cDNA was obtained from Clontech Labs., Palo Alto, CA.

**Cell lines.** The PSA-expressing human prostatic carcinoma cell line LNCaP and the non-PSA-expressing human breast carcinoma cell line BT-20 (both lines from the American Type Culture Collection, Rockville, MD) were used in control experiments. The cell lines were cultured in flasks at 37 °C and 50 mL/L CO<sub>2</sub>-enriched air in RPMI 1640 medium supplemented with bovine insulin (200 IU/L), glutamine (29 g/L), and fetal

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<sup>1</sup> Nonstandard abbreviations: PSA, prostate-specific antigen; RT-PCR, reverse transcription-polymerase chain reaction; DIG-dUTP, digoxigenin-labeled deoxyuridine triphosphate; dNTPs, deoxynucleoside triphosphate mix; and SSC, saline sodium citrate (1 $\times$  SSC is 8.765 g of NaCl + 4.41 g of sodium citrate per liter, pH 7.0).

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calf serum (100 mL/L). When the cells were grown to near confluency, they were washed with isotonic saline, detached by trypsin-EDTA treatment, and counted. Samples of  $10^7$  cells were kept frozen at  $-70^\circ\text{C}$  until their RNA was extracted.

**Tissue specimens.** Thirty breast cancer specimens were obtained from women undergoing surgery for primary breast cancer. The breast tumor tissues were stored in liquid nitrogen immediately after surgical resection, transported to the laboratory, and then stored at  $-70^\circ\text{C}$  until protein and RNA extractions were performed. All tumors were primary lesions except for one ovarian carcinoma, which was metastatic from a primary breast cancer. We also used as control tissues one lung carcinoma that was metastatic from a primary prostate cancer, one primary liver cancer, and one primary prostate cancer. All tissues were leftovers from routine pathological examinations.

**Tumor cytosol extracts.** Tissue from each tumor ( $\sim 0.2$  g) was pulverized manually with a hammer to a fine powder at  $-80^\circ\text{C}$ . The cells were lysed for 30 min on ice with 1 mL of lysis buffer (50 mmol/L Tris buffer, pH 8.0, containing 150 mmol/L NaCl, 5 mmol/L EDTA, 10 g/L Nonidet NP-40 surfactant, and 1 mmol/L phenylmethylsulfonyl fluoride). The lysates were centrifuged at 15 000g at  $4^\circ\text{C}$  for 30 min and the supernates (cytosolic fractions) were analyzed for PSA and total protein.

#### PROCEDURES

**Measurement of PSA and total protein.** A time-resolved immunofluorometric PSA assay was used to measure PSA concentration in the cytosolic extracts. Each extract was measured in duplicate. The PSA assay, described in detail elsewhere [14], has a detection limit of 10 ng/L. Total protein in each sample was measured in duplicate with the bicinchoninic acid commercial method (Pierce Chemical Co., Rockford, IL). PSA concentration in all cytosols is expressed as nanograms of PSA per gram of total protein.

**Isolation of total RNA.** For total RNA isolation from tissues and cell lines, we used the TRIzol reagent and method (Gibco BRL, Gaithersburg, MD), following the instructions of the manufacturer. The integrity of the RNA was checked electrophoretically and its amount and purity by spectrophotometry.

**RT-PCR.** We synthesized cDNA from the isolated total RNA with a first-strand cDNA synthesis kit and SuperScript II reverse transcriptase (Gibco BRL). In brief, 1–5  $\mu\text{g}$  of RNA and 500 ng of oligo(dT)<sub>12–18</sub> primers (see below) were first denatured for 10 min at  $70^\circ\text{C}$ , chilled on ice for 1 min, and then incubated for 5 min at  $42^\circ\text{C}$  in 19  $\mu\text{L}$  of reaction mixture: PCR buffer,  $\text{MgCl}_2$ , dithiothreitol, and deoxynucleoside triphosphates (dNTPs) as per the Gibco BRL protocol. Then, 200 U (1  $\mu\text{L}$ ) of SuperScript II reverse transcriptase was added to the reaction mixture, which was then incubated for 50 min at  $42^\circ\text{C}$ , terminated at  $70^\circ\text{C}$  for 15 min, and chilled on ice. Before proceeding to amplification of the target cDNA, we treated the mixture with 1  $\mu\text{L}$  of RNase H (Gibco BRL) for 20 min at  $37^\circ\text{C}$ . Negative

control reactions for RT-PCR were performed by using all reagents but the SuperScript II.

We used two oligonucleotide primers to amplify a 754-bp region of PSA cDNA. The primers, originally described by Deguchi et al. [13], have the following sequences:

PSA A1: 5'-TGCGCAAGTTCCACCCTCA-3'

PSA B1: 5'-CCCTCTCCTTACTTCATCC-3'.

We have further developed another two primers for nested primer PCR as follows:

PSA N1: 5'-CTGTGTGCTGGACGCTGG-3'

PSA N3: 5'-ACCTCACACCTAAGGACA-3'.

For actin cDNA amplification, we used the following primers, previously published [17]:

ACT 1: 5'-ACAATGAGCTGCGTGTGGCT-3'

ACT 2: 5'-TCTCCTTAATGTCACGCACGA-3'.

PCR with primers A1/B1 yields a 754-bp fragment; with primers N1/N3 for nested primer PCR, a 303-bp fragment; and with ACT1/ACT2, a 372-bp fragment.

PCR was performed in 0.2-mL thin-walled MicroAmp reaction tubes on a Perkin-Elmer (Palo Alto, CA) Gene Amp 2400 system. Total volume was 50  $\mu\text{L}$ . The reaction mixture contained PCR buffer (50 mmol/L KCl, 10 mmol/L Tris buffer, pH 8.3, 1.5 mmol/L  $\text{MgCl}_2$ , 10 mg/L gelatin), 200  $\mu\text{mol/L}$  of dNTPs, 1  $\mu\text{mol/L}$  of PCR primers, 2.0 U of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN), and 5  $\mu\text{L}$  of cDNA target (added last). The PCR was performed with 1 cycle at  $94^\circ\text{C}$  for 5 min; 30 cycles with denaturation at  $94^\circ\text{C}$  for 30 s, annealing at  $60^\circ\text{C}$  for 30 s, and extension at  $72^\circ\text{C}$  for 30 s; and 1 cycle at  $72^\circ\text{C}$  for 7 min. Portions (20  $\mu\text{L}$ ) of the PCR reaction products were electrophoresed on 2% agarose gels and visualized with ethidium bromide staining. Negative controls did not contain template DNA.

The procedure for nested primer PCR was the same as for the above PCR protocol except that 25 cycles were used in the first PCR, primers N1/N3 were used in the nested primer PCR for 20 cycles, and 1  $\mu\text{L}$  of the first PCR product was added as target.

PSA cDNA plasmid was linearized with *Hind*III enzyme digestion and labeled with the random primer method by incorporation of digoxigenin-labeled deoxyuridine triphosphate (DIG-dUTP). We used the DIG-DNA labeling kit from Boehringer Mannheim and the protocol recommended by the manufacturer.

**Gel electrophoresis, Southern transfer, and hybridization.** Aliquots of PCR products (20  $\mu\text{L}$ ) were electrophoresed at 100 V for 45 min on 2% agarose minigels containing ethidium bromide. The gels were then Southern transferred onto positively charged nylon membranes (Boehringer Mannheim) by overnight alkali capillary blotting with use of 0.4 mol/L NaOH. The membranes were then baked for 15–30 min at  $120^\circ\text{C}$ . For hybridization, the membranes were placed in tubes with 20 mL of hybridization buffer per 100  $\text{cm}^2$  of membrane. We used a commercial hybridization solution (DIG Easy Hyb; Boehringer Mannheim). Prehybridization was at  $68^\circ\text{C}$  for 1 h. The solution was then replaced with 5 mL (per 100  $\text{cm}^2$  membrane) of hybridization buffer containing freshly denatured labeled PSA probe (50

$\mu\text{g/L}$ ). Hybridization was carried out for 12–16 h at 42 °C. Filters were subsequently washed twice with  $2\times$  SSC (saline sodium citrate;  $1\times$  contains 8.765 g of NaCl + 4.41 g of sodium citrate per liter, pH 7.0) containing 1 g/L sodium dodecyl sulfate at room temperature (5 min per wash) and twice with  $0.1\times$  SSC containing 1 g/L sodium dodecyl sulfate at 68 °C (15 min per wash). Detection was accomplished by using an antibody against digoxigenin, labeled with alkaline phosphatase and the substrate 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD) (Boehringer Mannheim). We followed the instructions of the manufacturer and captured the chemiluminescence on x-ray film, using exposure times of 15–30 min.

**Direct incorporation of DIG-dUTP during PCR.** We used the same PCR protocol to amplify PSA cDNA but with a dNTP mixture that contains, in addition to the four nucleoside triphosphates, DIG-11-dUTP. The final concentrations of dNTPs and DIG-11-dUTP in the PCR reaction were 200 and 0.7  $\mu\text{mol/L}$ , respectively. PCR products were electrophoresed on 2% agarose gels, transferred, and fixed as described. The DIG-11-dUTP incorporated was detected by chemiluminescence, as described in the previous section.

**Detection limit of PCR.** We used total RNA extracted from  $10^7$  LNCaP prostate carcinoma cells as a reference preparation for determining the limits of detection of the methods. This total RNA was reverse-transcribed and then diluted successively to give cDNA amounts corresponding to a certain number of cells. We also used purified plasmid pA75 containing full-length PSA cDNAs to calculate the detection limits. Plasmid and LNCaP cDNAs were diluted in a 10 mmol/L Tris buffer, pH 7.80, containing 1 g/L salmon sperm DNA to avoid losses from adsorption to tubes. Negative controls were included as necessary with the diluent used as target.

## Results

The detection limit of our PCR method was first determined by amplification of plasmid pA75 containing the full-length PSA cDNA. Ethidium bromide staining of the PCR product on a 2% agarose gel revealed a detection limit of  $\sim 1000$  copies of PSA cDNA (data not shown). Amplification of a plasmid containing  $\beta$ -actin cDNA with  $\beta$ -actin-specific primers gave a detection limit for actin cDNA (ethidium bromide staining) of 200 copies. In both PCRs, for PSA cDNA and actin cDNA, the PCR products were of the expected length, i.e., 754 and 372 bp, respectively.

Total RNA was extracted from the human prostatic carcinoma cell line LNCaP, which is known to express PSA, and from the human breast carcinoma cell line BT-20, which is steroid hormone receptor-negative and does not express PSA protein. The total RNA from both cell lines was reverse-transcribed, and the cDNA was diluted serially and amplified by PCR. The BT-20 cDNA did not yield any amplification products at any dilution, confirming the absence of PSA mRNA in this cell line. The LNCaP cDNA yielded the expected 754-bp

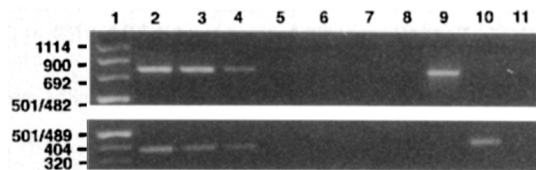


Fig. 1. RT-PCR of PSA mRNA (top) and  $\beta$ -actin mRNA (bottom), as detected on agarose gels by ethidium bromide staining.

Lane 1: molecular mass markers, with sizes (base pairs) indicated; lanes 2–8: mRNA extracted from LNCaP cells in dilutions corresponding to 20 000, 2000, 200, 20, 2, 0.2, and 0.02 cells, respectively; lane 9: control PCR with 9000 molecules of plasmid pA75, containing full-length PSA cDNA, as target; and lanes 10 and 11: mRNA extracted from, respectively, 20 000 or 2000 BT-20 cells, which are negative for PSA mRNA but positive for  $\beta$ -actin mRNA. The detection limit of the PSA mRNA RT-PCR is equivalent to the quantity in 2 LNCaP cells.

amplification product at cDNA amounts equivalent to 2 LNCaP cells or more. Actin cDNA was successfully amplified from both cell lines. These data are presented in Fig. 1.

Similar experiments were performed by the nested primer protocol. With this method, we could detect  $<10$  molecules of plasmid pA75 and cDNA equivalent to 0.02 LNCaP cells (data not shown). These data suggest that the nested primer PCR method is  $\sim 100$ -fold more sensitive than the regular PCR protocol, as determined with use of ethidium bromide-stained gels for detection of PCR products.

We have also developed a third highly sensitive PCR procedure for PSA cDNA, using one PCR reaction in which DIG-11-dUTP is directly incorporated into the PCR product. DIG-11-dUTP is added in minute amounts in the PCR mix along with the other dNTPs. The DIG-11-dUTP-labeled PCR product is first run on agarose gels, then transferred by Southern transfer to nylon membranes, and subsequently detected by using antidigoxigenin antibodies labeled with alkaline phosphatase and chemiluminescence. This method can detect  $\sim 10$  molecules of pA75 plasmid and 0.2 LNCaP cells (Fig. 2).

A fourth method for detection of PSA cDNA is based on a single PCR reaction, agarose gel electrophoresis of the product, Southern transfer to nylon membranes, hybridization with a

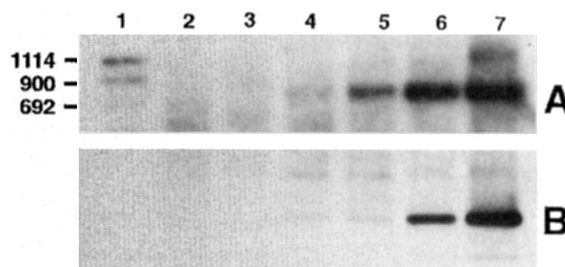


Fig. 2. RT-PCR protocol for PSA mRNA with direct incorporation of DIG-11-dUTP and chemiluminescence detection.

(A) The target is pA75 plasmid. The number of pA75 molecules per lane is as follows: (2) 0, (3) 1, (4) 10, (5) 100, (6) 1000, and (7) 10 000; lane 1 contains biotinylated molecular mass markers. The detection limit of the assay is 10 pA75 molecules. (B) The target is LNCaP mRNA equivalent to the following number of cells per lane: (2) 0, (3) 0.02, (4) 0.2, (5) 2, (6) 20, and (7) 200. The detection limit is 0.2 LNCaP cells.

**Table 1. Detection of PSA protein and PSA mRNA in 30 primary breast tumors.**

Specimen no.	PSA, ng/g of total protein	ER/PR, pmol/g of total protein <sup>a</sup>	PSA mRNA method <sup>b</sup>		
			1	3	4
1	269	181/80	-	+	+
2	1102	2/4	+	+	+
3	8281	451/661	-	+	ND
4	1106	235/515	+	+	+
5	3417	71/16	+	+	+
6	2287	8/4	-	+	+
7	104	143/421	+	+	+
8	1415	16/253	+	+	+
9	7241	34/19	-	+	+
10	181	190/368	-	+	+
11	64	0/5	-	-	-
12	8	4/1	-	-	+
13	24	36/73	-	+	+
14	24	742/296	-	+	+
15	12	390/307	-	+	+
16	79	5/95	-	-	-
17	52	2/42	-	+	-
18	57	32/379	-	+	+
19	20	70/118	-	+	+
20	86	22/57	-	-	-
21	0	58/53	-	-	-
22	0	266/5	-	-	-
23	0	99/128	-	-	-
24	0	97/85	-	-	+
25	0	0/0	-	-	-
26	0	278/582	-	-	-
27	0	0/1	-	-	-
28	0	1/0	-	-	-
29	0	189/245	-	-	-
30	0	63/2	-	-	-

<sup>a</sup> Concentration of estrogen receptor/concentration of progesterone receptor.

<sup>b</sup> Method 1 is one PCR, agarose gel electrophoresis, and ethidium bromide staining; method 3 is based on 11-dUTP incorporation; method 4 is based on Southern blot and hybridization.

ND, not done.

digoxigenin-labeled PSA cDNA probe, and detection by chemiluminescence. This method also detects ~10 molecules of pA75 plasmid and 0.2 cells of LNCaP (data not shown).

To study PSA mRNA expression in breast tumors, we initially screened 240 breast tumor cytosolic extracts for PSA protein by using a highly sensitive immunological assay [14]. We then classified these tumors into three categories according to the amount of PSA expressed: i.e., highly positive tumors (PSA >100 ng/g of total protein), weakly positive tumors (15 ng/g < PSA <100 ng/g), and negative tumors (PSA <15 ng/g). We

then selected 30 tumors for further study by PCR, hoping to quantify PSA mRNA. The results are shown in Table 1. We did not use the nested primer method for tumor RNA analysis because we found that this assay could not be completely controlled for PCR contamination.

Of the 10 tumors that were highly positive for PSA protein, 5 were positive by PCR method 1 (one PCR, ethidium bromide staining), 10 were positive by method 3 (DIG-dUTP incorporation), and 9 were positive by method 4 (probe hybridization); 1 sample was not evaluated by method 4. Of the 10 tumors that were weakly positive for PSA protein, none was positive with method 1, 6 were positive by method 3, and 6 were positive by method 4; 2 of the 6 positives (12 and 17) gave discrepant results between methods 3 and 4. Of the 10 tumors that were negative for PSA protein, none was reported to contain PSA mRNA by any of the methods, except sample 24, which was weakly positive by method 4 (Table 1).

Results of PCR analysis of some breast tumors are shown in Fig. 3. We also tested control tissue from prostate (positive by all methods), a lung cancer metastatic from a prostate primary lesion (positive by all methods), and one primary liver cancer (negative by all methods).

### Discussion

A serine protease present at very high concentrations in seminal plasma, PSA has been suggested to be involved in semen liquefaction postejaculation. PSA is considered a highly specific biochemical marker of the prostate gland and is currently used for diagnosis, prognosis, and management of patients with prostate cancer [1-3].

Our group has recently demonstrated that ~30% of female breast tumors produce a 33-kDa protein with striking similarities to seminal plasma PSA [5, 6]. This immunoreactive PSA is associated with the presence of steroid hormone receptors, earlier disease stage, and younger patient age [6]. Preliminary data suggest that PSA may be a new favorable prognostic indicator in breast cancer [18]. Immunoreactive PSA has also been detected in ovarian, colon, lung, and parotid tumors; however, its expression in these tumors is infrequent and involves much lower amounts than in breast tumors [7]. Recently, PSA mRNA isolated from a few PSA-positive breast tumors was demonstrated to have a sequence identical to that of the PSA cDNA derived from prostate tissue [12].

A very sensitive procedure for in vitro amplification of DNA sequences, PCR has gained widespread acceptance in many areas of molecular biology—particularly in tumor biology and clinical medicine, and more recently in routine diagnostic applications. RT-PCR-based methods for analyzing mRNA expression specific for neoplastic cells have been applied to the

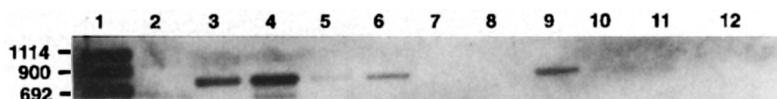


Fig. 3. Detection of PSA mRNA by RT-PCR and hybridization (method 4) in breast tumors weakly positive for PSA protein.

Lane 1, biotinylated molecular mass markers; lanes 2-11, samples 11-20 from Table 1; lane 12, negative control. All samples were positive for actin PCR (data not shown).

detection of micrometastasis or of minimal residual disease [13, 19].

In this study, we developed several sensitive RT-PCR methods for detecting PSA mRNA. Among the variations tested, the nested primer assay was the most sensitive; however, it was difficult to control for contamination and thus was not used to assay the breast tumors. The simple PCR assay in which PCR products are detected by ethidium bromide staining was also found to be inadequate, giving negative PSA mRNA results for many PSA protein-positive tumors. The other two modifications, in which the PCR products are detected with chemiluminescence and with use of digoxigenin as label, were found to be satisfactory. Data generated with these two methods were in good agreement with the immunoassay results for PSA protein. The few discrepancies seen probably reflect tumor heterogeneity because not all tumor cells in PSA-positive tumors produce PSA; as shown by immunohistochemical localization, the immunoreactivity is focal and restricted to clusters of cells (data not shown). Tumor heterogeneity is probably also the reason why we did not observe a consistent correlation between the concentrations of PSA protein and intensity of the PCR bands: Two different tumor sections were used for cytosol extraction and PSA protein analysis and for mRNA extraction and PCR.

In the past few years, many other groups have developed sensitive PCR procedures for PSA mRNA. Deguchi et al. [13] developed a PCR assay with detection limit of between 0.1 and 1 LNCaP cell and used it to detect prostatic carcinoma tumor cells invading the regional lymph nodes. Moreno et al. [20] developed a PCR assay of unspecified sensitivity and used it to detect prostate cancer cells in the circulation of patients with advanced cancer. Katz et al. [21] developed a PCR assay for PSA mRNA that incorporated DIG-11-dUTP in the reaction products and could detect 1 LNCaP cell admixed with  $10^5$  human blood lymphocytes. Using their method to assay human blood, they found that 78% of prostate cancer patients with metastatic disease and 39% with localized disease had cancer cells in the circulation. More recently, Jaakkola et al. [22] developed a nested primer PCR assay with the ability to detect  $\sim 2$  LNCaP cells admixed with  $10^6$  leukocytes. They detected no PSA mRNA in the blood of patients with benign prostatic hyperplasia, nonprostatic types of cancer, or localized prostate cancer, but 50% of the patients with metastatic disease tested positive by their method.

A universal finding among these studies [20–22] is that none of the numerous blood specimens from healthy subjects, male or female, tested positive for PSA mRNA. However, Smith et al. [23], using a highly sensitive nested primer protocol, reported 100% positivity among blood samples from seven healthy men and six healthy women. Given that nested primer procedures are prone to contamination, we recommend caution before concluding that blood samples contain traces of PSA mRNA until the data of Smith et al. are reproduced by others.

The data presented here support the view that assessment of PSA production by breast tumors can be studied by either highly sensitive immunoassays for PSA protein [14] or highly sensitive RT-PCR assays for PSA mRNA, like those described here. We

anticipate that the RT-PCR-based assays will be useful to assess PSA mRNA concentrations in tumors other than those of the breast and in nonprostatic tumors and tissues from male patients. Although the PSA protein assay is extremely sensitive, it is severely limited by the fact that male blood contains substantial amounts of PSA, which could contaminate tumor or tissue extracts from male subjects. Detection of PSA mRNA would effectively circumvent this potential problem.

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