

## Detection of Tumor Markers in Prostate Cancer and Comparison of Sensitivity between Real Time and Nested PCR

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### ABSTRACT

The objective of this study is to investigate and compare the sensitivity in conventional PCR, quantitative real time PCR, nested PCR and western blots for detection of prostate cancer tumor markers using prostate cancer (PCa) cells. We performed conventional PCR, quantitative real time PCR, nested PCR, and western blots using 5 kinds of PCa cells. Prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), and androgen receptor (AR) were compared for their detection sensitivity by real time PCR and nested PCR. In real time PCR, there was a significant correlation between cell number and the RNA concentration obtained ( $R^2=0.9944$ ) for PSA, PSMA, and AR. We found it possible to detect these markers from a single LNCaP cell in both real time and nested PCR. By comparison, nested PCR reached a linear curve in fewer PCR cycles than real time PCR, suggesting that nested PCR may offer PCR results more quickly than real time PCR. In conclusion, nested PCR may offer tumor marker detection in PCa cells more quickly (with fewer PCR cycles) with the same high sensitivity as real time PCR. Further study is necessary to establish and evaluate the best tool for PCa tumor marker detection.

### INTRODUCTION

Prostate cancer (PCa) could be a severe malignancy. Prostate specific antigen (PSA) is well known as a tumor marker for PCa [1], but whether PSA is effective for screening or predicting prognosis is controversial. For instance, PSA screening may result in the detection of insignificant PCa that does not need treatment [2]. In progressive PCa, PSA may not offer appropriate prognosis or work as a good predictive factor [3]. This study compares PSA, prostate specific membrane antigen (PSMA), and androgen receptor (AR) using several PCR detection tools and western blots.

There are several methods for the detection of these markers [4–6]. Zhang et al. stated that PSMA in PCa cells was nicely detected with real time RT-PCR in micrometastatic cells [4]. Nerves et al. reported PSMA and AR measurement by real time RT-PCR using PCa samples from surgical specimens [5]. Joung et al. studied PSMA detection via nested PCR

using PCa samples from surgical specimens [6]. In addition, prostate stem cell antigen mRNA in peripheral blood might be predictive for PCa biochemical recurrence [7]. Our study compared real time PCR and nested PCR using several PCa cell lines to help determine better tumor markers for PCa.

## MATERIALS AND METHODS

### Cell culture

PC-3, C4-2B, LNCaP, DU 145, and 22Rv1, human PCa cell lines were cultured in RPMI-1640 (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with 10% heat-inactivated FBS (fetal bovine serum) at 37°C with 5% CO<sub>2</sub> in a humidified incubator.

### Reverse transcription (RT), real-time PCR and nested PCR

Cultured PCa cell lines were subjected to RNA extraction, reverse transcription, and real-time PCR to analyze mRNA expressions of prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), androgen receptor (AR), or hypoxanthine phosphoribosyltransferase (HPRT) 1, as an internal control for normalizing. In brief, total RNA was extracted with TRIZOL Reagent (Invitrogen, Carlsbad, California, USA) and reverse transcribed from oligo-dT primer with TaqMan reverse Transcription Reagents (Applied Biosystems, Foster City, California, USA) according to the manufacturer's protocol. Twenty ng of the RNA-equivalent cDNA and specific primer set were mixed with Power SYBR Green PCR Master Mix (Applied Biosystems) and semi-quantitative PCR analysis was performed by a real-time PCR instrument (7500 Real-Time PCR System, Applied Biosystems). First PCR amplicons were diluted (1:1,000) and subjected to nested PCR. Primer sequences and thermal conditions are described in Tables 1 and 2. To verify the specificity and purity of the amplified PCR products, a melting curve analysis was performed with the same instrument (data not shown).

Table I. Primer sequences

Target gene	Primer Sequence	Concentration
PSA	(F) 3'-AAGCTGGAGGCACAACGCACC-5'	100 nM
	(R) 3'-CCTCCTGGCTCACAGCCTTC-5'	100 nM
inner	(F) 3'-CCAGACACTCACAGCAAGGATGGA-5'	50 nM
	(R) 3'-CAGTGCCCTCCAGGACAGAGTGG-5'	50 nM
PSMA	(F) 3'-CATAGTGCTCCCTTTGATTGTC-5'	100 nM
	(R) 3'-CTCTCACTGAACCTGGAAAGCAAT-5'	100 nM
inner	(F) 3'-GCTCCCTTTGATTGTCGAGATT-5'	50 nM
	(R) 3'-TGTATGTCTTCATTCTGTGGATG-5'	50 nM
AR	(F) 3'-TGGCTTCCGCAACTTACACGTG-5'	50 nM
	(R) 3'-GGCGAAGTAGAGCATCCTGGAGT-5'	50 nM
inner	(F) 3'-ACGACCAGATGGCTGTCAATTCAAG-5'	50 nM
	(R) 3'-TGAAGGATGCCAGCCCCATG-5'	50 nM
HPRT 1	(F) 3'-TCTTGCTGACCTGCTGGAT-5'	100 nM
	(R) 3'-CGACCTTGACCATCTTGGA-5'	100 nM

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Table II. PCR conditions

Target gene	Primary PCR			Nested PCR		
PSA	95.0 °C	1 sec.	× 40	95.0 °C	15 sec.	× 30
	63.5 °C	1 min.		61.0 °C	1 min.	
PSMA	95.0 °C	1 sec.	× 40	95.0 °C	15 sec.	× 30
	61.5 °C	1 min.		61.0 °C	1 min.	
AR	95.0 °C	15 sec.	× 40	95.0 °C	15 sec.	× 30
	61.0 °C	1 min.		60.0 °C	1 min.	
HPRT1	95.0 °C	15 sec.	× 45			
	63.0 °C	1 min.				

### Western blotting

Total cell lysates and protein extracts were prepared with M-PER Mammalian Protein Extraction Reagent supplemented with a Protease Inhibitor Cocktail Kit, and protein concentrations were measured by Coomassie (Bradford) assay (Thermo Fisher Scientific, Rockford, Illinois, USA) according to the manufacturer's instructions. Subsequently, equal amounts of proteins were separated by sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Massachusetts, USA). The membranes were then blocked and incubated with anti-human PSA monoclonal antibody (mAb) (C-19), anti-human PSMA mAb (Y-PSMA2; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), or anti-human AR mAb (G122-25; BD Biosciences Pharmingen, San Jose, California, USA), followed by horseradish peroxidase (HRP)-conjugated anti-mouse or goat immunoglobulin polyclonal Ab (Santa Cruz Biotechnology Inc.). Finally immunoreactive bands were detected with enhanced chemiluminescence (ECL) Plus Western Blotting Detection Reagents (GE Healthcare, Piscataway, New Jersey, USA) and visualized by an LAS-3000 instrument (Fuji Photo Film, Tokyo, Japan). For confirming the amounts of loaded proteins, the membranes were stripped with stripping buffer [62.5mM Tris-HCl (pH 6.8), 100mM 2-mercaptoethanol and 2% SDS] and re-blotted with anti-beta-actin mAb (C4) (Santa Cruz Biotechnology Inc.).

## RESULTS

### Gene expression profiling of various prostate cancer cell lines

For the detection of potential prognostic markers, it is important to use cell lines that recapitulate the situation in PCa patients. Therefore we initially conducted gene expression profiling on each cell line used. For the precise expression analysis, we compared mRNA by conventional RT-PCR, quantitative real-time RT-PCR, and nested RT-PCR and protein expressions by western blotting.

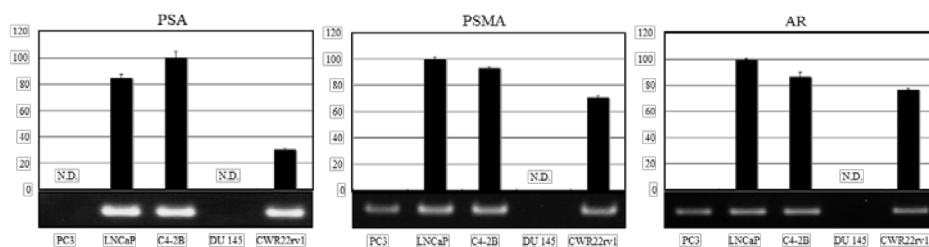
### mRNA expressions

First, we examined the mRNA expressions of PSA, PSMA, and AR in 5 PCa cell lines representing various degrees in progression and AR status. This profiling showed that mRNA expression levels varied significantly. AR mRNA was not expressed in PC-3 and DU145. PSA mRNA was predominantly expressed in LNCaP and C4-2B cells and PSMA mRNA was also predominantly expressed in LNCaP, C4-2B, and CWR22RV1 cells. Interestingly, the expression data for AR mRNA in each cell line was very similar to that of PSMA (Fig. 1A).

### Protein expression

We next conducted western blotting to investigate the protein expressions of the same targets. In this experiment, PSA and PSMA protein expression reflected the results of the mRNA levels in each cell line, and AR expression showed similar trends in all PCa cell lines except CWR22Rv1 cells, which had 2 different bands of AR (Fig. 1B).

**A**



**B**

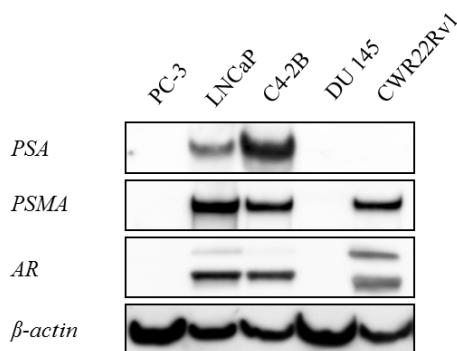


Fig. 1. Expression profiling of the specific biomarkers expressed in human prostate cancer cells.

mRNA and protein expressions of prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), and androgen receptor (AR) in PC-3, LNCaP, C4-2B, DU 145 and 22Rv1 cells were measured by quantitative RT-PCR (A) or analyzed by western blotting (B). mRNA expression was normalized to HPRT1 (encoding hypoxanthine guanine phosphoribosyl transferase 1) expression, and bar graphs show relative expression to maximum for each cell line (arbitrarily set to 100). Data are representative of three independent experiments (error bars (A), S.D. of triplicate samples; beta-actin (B), loading control throughout).

### Comparison of PCR sensitivities and effectiveness between real time PCR and nested PCR

Next, as a comparison study regarding PCR sensitivities and detection efficacy, real time PCR and nested PCR were compared for the possibility of using tumor markers for PCa. Our data showed that even though there was no significant difference in PCR sensitivities for the three markers (PSA, PSMA, and AR) in the 5 tested PCa cell lines, nested PCR demonstrated faster linear RNA expression (reaching a plateau) than real time PCR, especially with small amounts of cells or small RNA concentrations. These data suggest that nested PCR might be a better tool for this purpose, since even 10-20 PCR cycles showed a plateau in RNA concentration that was not apparently different from real time PCR, and the intensities of the PCR bands, especially with a small number of cells or lower RNA concentrations, was higher in nested PCR than real time PCR. This indicates that a very small amount of material (cells or RNAs) could be used for marker detection with nested PCR while real time PCR would require more cell or RNA material to reach the same results (Fig. 2A)

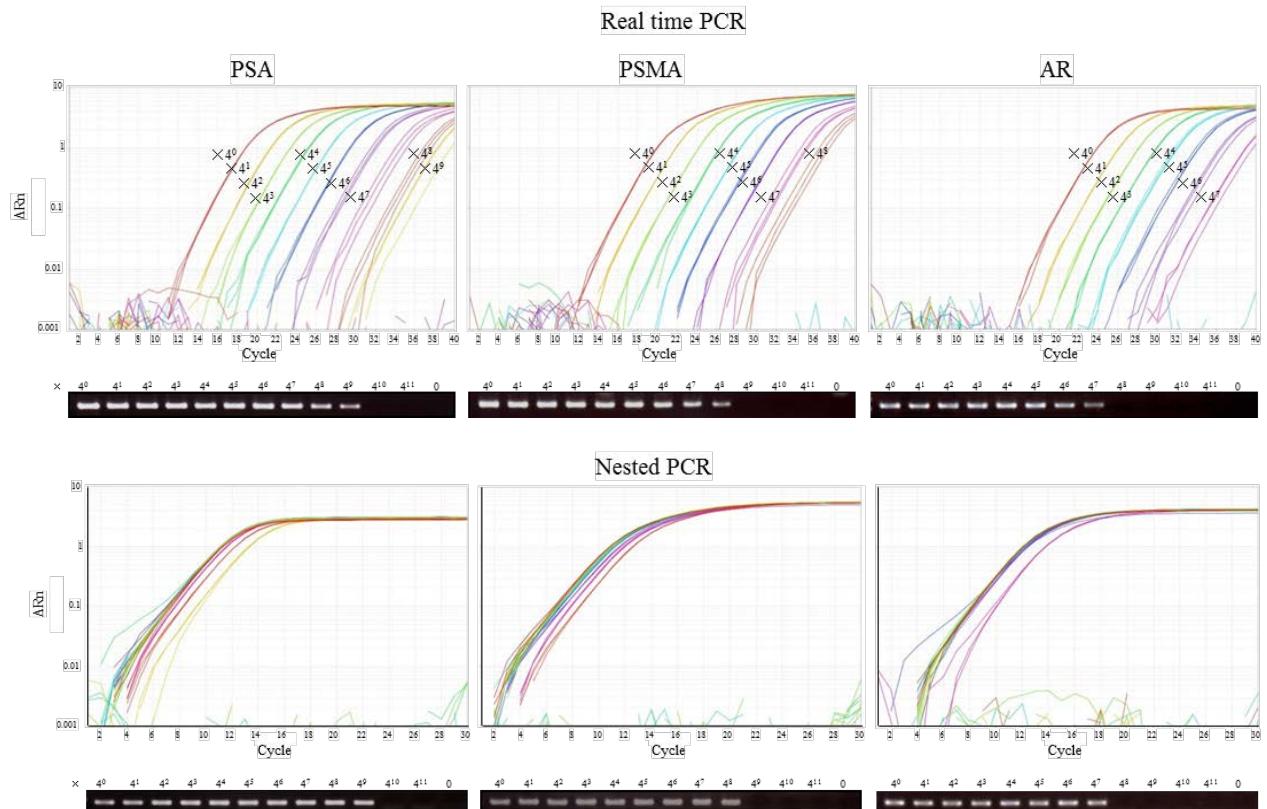


Fig. 2A. Comparison of the sensitivity of real time PCR versus nested PCR for each prostate cancer biomarker, PSA, PSMA, or AR. cDNA (equivalent to 200 ng of total RNA extracted from LNCaP cells) was serially diluted and subjected to real time PCR. The resultant PCR products were diluted (1:1,000) and subjected to nested PCR. Results are presented as amplification plot graphs and gel electrophoresis bands. Data are representative of two to three independent experiments with three replicates.

### Correlation of cell numbers assayed with RNA concentration

We examined the RNA concentrations obtained by RNA extraction using various numbers of cells and found a significant correlation between these 2 factors ( $R^2=0.9944$ ) (Fig. 2B).

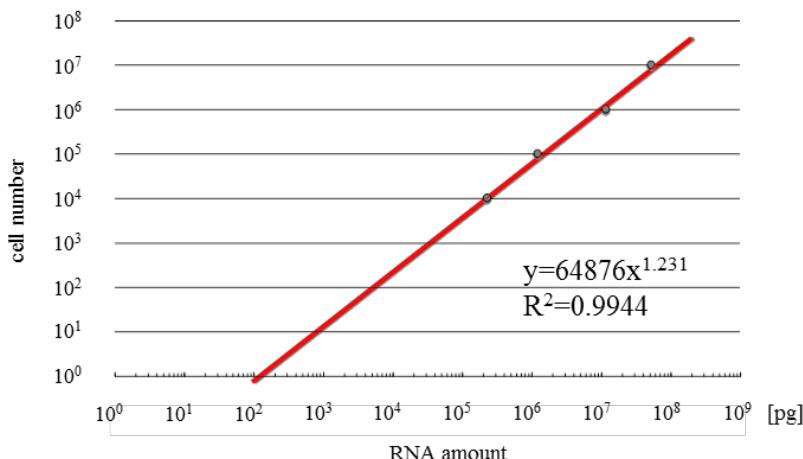


Fig. 2B. Correlation between cell number and total RNA yield of LNCaP cells. Points of logarithmic cell number versus total RNA amount (pg) were plotted, and a fitted curve and its correlation coefficient are shown on the same graph. Results shown are mean and representative of three independent experiments.

### DISCUSSION

The use of PSA as a tumor marker led to a new era in prostate cancer (PCa) research. The PSA marker offered many benefits such as the detection of operable organ-confined disease [8-10]. On the other hand, about 10% of PCa detected is considered insignificant and requires no treatment [11]. Debate continues over the medical and insurance expense of unnecessary therapies [12]. Once treatments for PCa are initiated, PSA marker may continue to be used for the detection or diagnosis of PSA failure, biochemical recurrence or castration refractory disease. However, debate continues over whether PSA reflects the actual disease status [13]. Joung *et al.* stated that PSMA is a typical cell membrane marker of PCa, abundantly and almost universally expressed in prostate carcinoma, and concluded that PSMA mRNA detection by RT-PCR in peripheral blood can be a potent preoperative predictor of biochemical PCa recurrence [6]. Our data revealed that PSMA tended to be more detectable in our tested 3 cell lines than PSA in conventional PCR and western blots. In addition, our nested PCR indicated that the expression of PSMA tended to reach a plateau with fewer PCR cycles than other markers, suggesting that PSMA might be a good marker for detecting androgen-dependent and androgen-independent PCa cells by nested PCR. Currently PSA screening is no longer recommended in the USA because it is felt not to contribute to a better patient prognosis, as mentioned above [14]. Many researchers are seeking new markers for biochemical failure or assuming PCa prognosis as a substitute for PSA [15]. The heterogeneity of PCa is well known and PSA by itself might not adequate to gauge PCa recurrence [16]. We are planning future *in vivo* work to adapt our method of PCR detection of PCa for comparison with clinical data as a predictive marker of patient prognosis.

There are several tools for marker detection of PCa prognosis or biochemical recurrence

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[17, 18]. For instance, Avgeris et al. found that Kallikrein-related peptidase 4 (KLK4) gene was detected quantitatively by real time RT-PCR and was a predictive marker for prostate malignancies [19]. Kurek et al. stated that in their sensitivity assay, mRNA for PSA, Human Kallikrein 2 (hK2) and PSMA from an average of 10 LNCaP cells diluted in  $10^7$  peripheral blood mast cells (PBMCs) could be detected after nested RT-PCR in initially negative control PBMCs from healthy donors [18]. Our nested PCR data showed that PSMA from 3.05 pg of RNA (200 ng RNA diluted by  $4^8$ ) could be detected. This quantity comes within the level of a single LNCaP cell from our fitted curve (Fig. 2B), suggesting that our nested PCR technique for PSMA could show high sensitivity for this purpose. Though Drago et al. stated that real time PCR showed higher sensitivity than nested PCR for the detection of herpes virus DNA [19], O'Neill et al. reported that real time nested PCR (Lightcycler nested multiplex PCR) with agarose gel electrophoresis has the merit of being comparatively easy and reduces the possibility of amplicon contamination [20].

PCa is characteristically heterogeneous as mentioned above [21, 22], which means that PCa cells from the same tumor could show high or low malignant potential. For instance, a prostate may have both high Gleason score and low Gleason score PCa regions [23]. Thus serum PSA or PSA expression from a tumor sample or individual PCa cells cannot necessarily be interpreted as a reliable marker for the exact disease severity or patient prognosis [24]. As to the test materials, this study uses the RNAs from cell culture and our method can be used to assay tumor markers by RNAs from either serum or tissue samples.

Recently, several studies have reported on the efficacy of detecting circulating tumor cells (CTC) to predict cancer malignancy and for clinical prognoses [25], including PCa [26]. As a marker for CTC detection, Scher et al. found that PSA is not suitable as a predictor of cancer progression [27]. Our current study showed that PSMA could be a potential predictive and prognostic marker for CTC using nested PCR as a detection modality.

The limitations of this study include the need for clinical and *in vivo* data using PCa patient specimens and a mouse PCa xenograft model. It would be valuable to investigate the correlation between PSMA expression and PCa recurrence and patient prognosis. In addition, only 3 markers (PSA, PSMA, and AR) were examined. New markers for PCa prognosis and disease severity are being developed [28]. Finally, considering the clinical practice, it would be better to measure tumor markers by chemiluminescent enzyme immunoassay or radioimmunoassay since PCR may be expensive and time consuming. We intend to expand our research with regard to these points in the near future.

## CONCLUSIONS

This study demonstrated that nested PCR may offer tumor marker detection in PCa cells more quickly (with fewer PCR cycles) with the same high sensitivity as real time PCR. Further study is necessary to establish a reliable PCa tumor marker accurately reflecting patient prognosis and disease severity and to determine the best tool for its detection.

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