ORIGINAL PAPER

Evaggelia Emmanouilidou · Penelope C. Ioannou
Theodore K. Christopoulos

High-throughput chemiluminometric determination of prostate-specific membrane antigen mRNA in peripheral blood by RT-PCR using a synthetic RNA internal standard

Abstract A quantitative reverse transcriptase polymerase chain reaction (RT-PCR) method, employing internal standard (IS) RNA and a simplified chemiluminometric hybridization assay, is described for the determination of prostate-specific membrane antigen (PSMA) mRNA. The recombinant RNA IS has the same binding sites and size as the amplified PSMA mRNA. Biotinylated PCR products (263 bp) from PSMA mRNA and RNA IS are captured in microtiter wells coated with streptavidin, and hybridized with alkaline phosphatase-conjugated probes. The bound alkaline phosphatase (AP) is measured by using a chemiluminogenic substrate. The ratio of the luminescence values obtained for PSMA mRNA and the RNA IS is a linear function of the initial amount of PSMA mRNA present in the sample before RT-PCR. The linear range extends from 500 to 5,000,000 PSMA mRNA copies and the overall reproducibility of the assay, including RT-PCR and hybridization, ranges from 7.4 to 16.6%. Samples containing total RNA from PSMA-expressing LNCaP cells give luminescence ratios linearly related to the number of cells in the range 0.5–5,000 cells.

Keywords Quantitative RT-PCR · PSMA mRNA · Peripheral blood · Chemiluminescence · Hybridization

E. Emmanouilidou · P. C. Ioannou (✉)
Department of Chemistry, University of Athens,
15771 Athens, Greece
E-mail: ioannou@chem.uoa.gr
Tel.: +30-21-07274574
Fax: +30-21-07274750

T. K. Christopoulos
Department of Chemistry, University of Patras,
26500 Patras, Greece

T. K. Christopoulos
Institute of Chemical Engineering and High Temperature Chemical Processes, P.O. Box 1414, 26500 Patras, Greece

Introduction

Prostate-specific membrane antigen (PSMA) is a 100-kDa transmembrane glycoprotein with peptidase activity that also acts as a folate hydrolase [1, 2]. The gene encoding PSMA is located on the short arm of chromosome 11 and consists of 19 exons spanning about 60 kbp of genomic DNA. The coding sequence of PSMA gene is 2.65 kbp [3, 4]. Because its expression is restricted to the prostate, several groups have studied the usefulness of PSMA in the diagnosis and management of prostate cancer [5–7]. DNA microarray studies on prostate tissue specimens have recently shown that the level of PSMA mRNA is substantially higher in prostate cancer compared with benign prostate hyperplasia (BPH) [8, 9]. The expression of PSMA remains unchanged or is upregulated under conditions of androgen deprivation, which is the most effective therapy for prostate cancer. Thus, PSMA might be a more informative indicator of tumor progression after androgen deprivation therapy than prostate-specific antigen (PSA), whose expression is suppressed during androgen deprivation [10, 11]. A significant diagnostic application of PSMA entails the use of ^{111}In-labeled anti-PSMA monoclonal antibody that recognizes PSMA on the cell-surface, for the detection of extraprostatic spread of the disease by radioimmunoscintigraphy [12].

Accurate staging is a major concern in the management of prostate cancer, because organ-confined disease is potentially curable by radiation or radical prostatectomy whereas patients with metastatic disease are offered systemic treatment to slow down the progression of the malignancy. However, the sensitivity of current staging methods (e.g. computed tomography scans, transrectal ultrasonography, and magnetic resonance imaging) is insufficient and understaging is common. As a result, a significant percentage of patients who undergo prostatectomy are found to have metastasis subsequent to surgery [13]. Reverse transcriptase polymerase chain reaction (RT-PCR) has enabled the development of

"molecular staging" methods that involve the detection of small numbers of circulating tumor cells in the bloodstream. This is achieved by monitoring tumor-specific mRNA sequences in the presence of mRNA from a large excess of normal cells. Because of its tissue specificity PSMA mRNA is currently being investigated as a candidate marker for molecular staging of prostate cancer [14–23]. The RT-PCR assays reported so far for PSMA mRNA in peripheral blood are, however, qualitative, i.e. they detect the presence or absence of the specific mRNA without indication of its quantity. Moreover, to achieve high detectability the assays are based on nested PCR protocols. The PCR products are analyzed by agarose gel electrophoresis. The specific amplified sequence is then verified by Southern blot, restriction enzyme digestion, or sequencing.

The use of nested PCR with numerous amplification cycles has led to the detection of PSMA mRNA in blood samples from healthy individuals, cell lines used as negative controls, and patients with localized prostate cancer [6, 14–23]. The need for a quantitative assay for PSMA mRNA in blood has already been emphasized [6]. A quantitative assay will enable the establishment of a relationship between the concentration of PSMA mRNA and metastatic potential, aggressiveness of tumor, and clinical outcome.

In this work we have developed a high-throughput quantitative RT-PCR method for determination of PSMA mRNA in peripheral blood. The method is based on coamplification of the target mRNA with a recombinant RNA internal standard (IS). The IS has the same sequence as that of the PSMA mRNA amplification product but differs only in a 24-bp centrally located segment. The RT-PCR products are determined by a rapid chemiluminometric hybridization assay which is performed in microtiter wells. We have prepared two conjugates of oligonucleotide probes with alkaline phosphatase (AP) and employed these as detection reagents for the amplification fragments of the PSMA mRNA and the IS. It is demonstrated that the ratio of the luminescence values for PSMA and IS is linearly related to the number of PSMA mRNA copies initially present in the sample.

Materials and methods

Reagents

The PSMA-expressing human prostatic carcinoma cell line LNCaP was obtained from the American type culture collection. All cell culture reagents were purchased from Invitrogen (Paisley, UK). AMV reverse transcriptase and Dynazyme EXT DNA polymerase were from Finnzymes (Espoo, Finland). T7 RNA polymerase and T4 DNA ligase were from MBI Fermentas (Vilnius, Lithuania). Restriction enzymes were from Takara (Shiga, Japan). RQ1 RNase-free DNase was from Promega (Lyon, France). RNase inhibitor and dNTPs were from HT Biotechnology (Cambridge, UK). RNA isolation reagent, RNAwiz, was purchased from Ambion (Austin, Texas). N-Succinimidyl-S-acetylthioacetate (SATA) and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1 carboxylate (sulfo-SMCC) were from Pierce (Rockford, IL, USA). Escherichia coli tRNA, blocking reagent, and AP for enzyme immunoassay (grade I, from calf intestine) were from Roche Diagnostics (Mannheim, Germany). Microspin Sephadex G-25 columns were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Centricon-30 microconcentrators were purchased from Millipore (Billerica, MA, USA). Opaque Microlite 2 polystyrene microtiter wells were from Dynex Technologies (Chantilly, VA, USA). Lumiphos substrate for AP was from Mediators PhL (Vienna, Austria). Streptavidin was from Sigma (St Louis, MO, USA). All other chemicals were purchased from Sigma or Fluka (Buchs, Switzerland).

Oligonucleotides

All oligonucleotides used in this study were synthesized by MWG Biotech (Ebersberg, Germany); their sequences are shown in Table 1. The upstream (b-up) and downsteam (d) primers used in PCR amplification were designed to prime to different exons of the PSMA gene (Gene Bank accession number M99487). The upstream primer is biotinylated at the 5′ end and is homologous with a sequence in exon 14. The downstream primer is complementary to a sequence in exon 16. The specific probe for the amplified PSMA mRNA (PSMA-probe) was designed to span the exon 15/exon 16 junction in the PSMA mRNA with a complementary sequence to the last 12 bases of exon 15 and to the first 12 bases of exon 16. The oligonucleotide IS-probe was used as a specific probe for amplified RNA IS. Both PSMA- and IS-specific probes were labeled with an –NH$_2$ group at the 5′ end to allow conjugation with AP.

Oligonucleotides UO, DO, UI, and DI (Table 1) were used in the creation and subcloning of RNA IS. Primers UO and DO are homologous to sequences of exons 4 and 16, respectively. The underlined sequences in UO and DO correspond to the restriction sites for KpnI and XbaI, respectively. Primers UI and DI are designed to prime to exons 15 and 16, respectively. The sequences shown in bold represent the new sequences to be introduced in the IS and are complementary to each other.

Preparation of alkaline phosphatase–oligonucleotide conjugates

Alkaline phosphatase was first activated with maleimide groups by reacting with the heterobifunctional cross-linking reagent sulfo-SMCC. A 14 μL aliquot of 10 mg mL^{-1} AP (1 nmol) was mixed with 11 μL of 2 mg mL^{-1} sulfo-SMCC (50 nmol) solution in 0.1 mol L^{-1} MOPS, pH 7.5, and 3.5 μL of 0.5 mol L^{-1} MOPS, pH 7.5. The reaction was allowed to proceed for 1 h at

Table 1 Oligonucleotide sequences used as primers and probes in this study

Oligonucleotide	Name	Sequence	Size (mer)	Label
IS synthesis				
Upstream outside primer	UO	TTTT GGTACCGAATGCCAGAGGGCGATCTA	30	–
Upstream inside primer	UI	**TGATCTGAATCACAAGTTTCTACA**TTCAGCGGCTATCCA	39	–
Downstream inside primer	DI	**TGTAGAAACTTGTGATTCAGATCA**ATACCGTGCTCTGCC	39	–
Downsteam outside primer	DO	TTTT TCTAGAAACACCATCCCTCCTCGAACC	31	–
Subcloning				
Upstream primer	Clu-PSMA	TTTT GGATCCGAATGCCAGAGGGCGATCTA	30	
Downstream primer	Cld-PSMA	TTTT TCTAGAAACACCATCCCTCCTCGAACC	31	–
PCR				
Upstream primer	b-up	GTCCTTCCCCAGAGTTCAGT	20	5′-biotin
Downstream primer	d	CCTGGGCCACAGTGAGGTGA	20	–
AP conjugates/hybridization assay				
PSMA-probe	PSMA-probe	TTTGTTTGTTTCCCAATTTTTAGT	24	5′-NH₂
IS-probe	IS-probe	TGTAGAAACTTGTGATTCAGATCA	24	5′-NH₂

ambient temperature in the dark. Excess sulfo-SMCC was removed by ultrafiltration by using the Centricon-30 centrifugal filter device. Prior to ultrafiltration, the reaction mixture was diluted 70× with 0.1 mol L^{-1} MOPS, pH 7.5, and centrifuged at 5,000×g for 30 min at 4°C. This step was repeated and the retentate was collected by centrifugation at 1,000×g for 2 min at 4°C (retentate volume about 50 μL). Next, a sulfhydryl group was introduced into the amino-modified oligonucleotide probe by using SATA. A 28-μL aliquot of PSMA-probe or IS-probe solution (11.2 nmol) was mixed with 17 μL of 15 mg mL^{-1} SATA (1.12 μmol) solution in 50% DMF–50% NaHCO₃ 0.1 mol L^{-1}, pH 9, and 9 μL 0.5 mol L^{-1} NaHCO₃, pH 9. Following 1 h incubation at ambient temperature in the dark, excess SATA was removed by gel filtration using a Sephadex G-25 Spin Pure column (pre-washed twice with sterile water). Finally, the reaction between SATA-derivatized oligonucleotide probes and maleimide-activated AP was initiated by the addition of hydroxylamine. A 50-μL aliquot of maleimide-activated AP was mixed with SATA-derivatized oligonucleotide, 14 μL of 0.1 mol L^{-1} NH₂OH solution and 28 μL of 0.1 mol L^{-1} MOPS buffer solution, pH 7.5, containing 5 mmol L^{-1} EDTA. The mixture was incubated for 18 h at 4°C. The conjugate was purified from the excess of oligonucleotide by using the Centricon-30 centrifugal device. Prior to ultrafiltration, the reaction mixture was diluted with 2 mL of 3 mol L^{-1} NaCl, 0.03 mol L^{-1} Tris-HCl, 0.1 mol L^{-1} ZnCl₂ and 0.05% NaN₂, pH 7.5. Ultrafiltration was performed by centrifuging the sample at 5,000×g for 30 min at 4°C. This step was repeated twice and the retentate was collected by centrifugation at 1,000×g for 2 min (retentate volume about 50 μL) and stored at 4°C. The oligo-AP conjugate was used in hybridization assays without further purification.

Cell culture

LNCaP cells were cultured in RPMI 1640 medium (with L-glutamine) containing 100 mL L^{-1} fetal bovine serum, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin.

For RNA extraction, cells were grown until near confluency, detached by trypsin–EDTA treatment, washed with PBS, and counted.

Synthesis of recombinant RNA internal standard

cDNA encoding PSMA (1,272 bp) was amplified by RT-PCR from total RNA isolated from the human prostatic carcinoma cell line, LNCaP. Reverse transcription was performed using oligo(dT)₂₀ primer. PCR for 1272-bp PSMA cDNA fragment was performed using the Clu-PSMA and Cld-PSMA primers. The PSMA fragment was then subcloned into the T7 promoter-bearing vector, pcDNA3 (Invitrogen) using the introduced *Bam*HI and *Xba*I restriction sites. Plasmid pcDNA3.PSMA (Fig. 1a, left panel) was used as a template for the synthesis of fragments A (1143 bp) and B (153 bp) by PCR employing primer pairs UO, DI and UI, DO, respectively (Fig. 1a, right panel). A new sequence was introduced into each fragment during PCR. PCRs for both fragments A and B were carried out in a total volume of 50 μL containing 50 mmol L^{-1} Tris-HCl (pH 9.0), 15 mmol L^{-1} (NH₄)₂SO₄, 0.1% Triton X-100, 2.5 mmol L^{-1} MgCl₂, 0.2 mmol L^{-1} of each dNTP, 25 pmol of each primer, 10^{10} molecules of pcDNA3.PSMA plasmid, and 1 U of Dynazyme EXT DNA polymerase. All PCR reactions were performed in a MJ Research PTC-150 MiniCycler (Watertown, MA, USA). The cycling conditions for fragment A were 95°C, 30 s (1 min 30 s for the first cycle); 65°C, 30 s, and 72°C, 1 min (10 min for the last cycle) for 30 cycles. The cycling conditions for fragment B were 95°C, 15 s (75 s for the first cycle), 60°C, 15 s, and 72°C, 30 s (10 min for the last cycle) for 30 cycles. Agarose gel-purified fragments A and B were then mixed in a 1:1 molar ratio and joined by a PCR-like reaction (40 cycles) in the absence of primers producing a 1,272-bp DNA fragment (AB). The cycling parameters for the joining reaction were 95°C, 30 s, 55°C, 30 s, and 72°C, 70 s (10 min for the last cycle). Subsequently, 5 μL of fragment AB was amplified by PCR for 35 cycles using primers UO and DO. The cycling program was 95°C, 30 s (1 min 30 s for the first cycle), 60°C, 30 s, and

Fig. 1 a *Left panel* plasmid pcDNA3.PSMA, *right panel* the relative positions of oligonucleotides used as primers for the synthesis of RNA IS and RT-PCR. The sequences for all oligonucleotides are presented in Table 1. **b** Outline of the strategy used for preparation of conjugates of AP to oligonucleotide probes
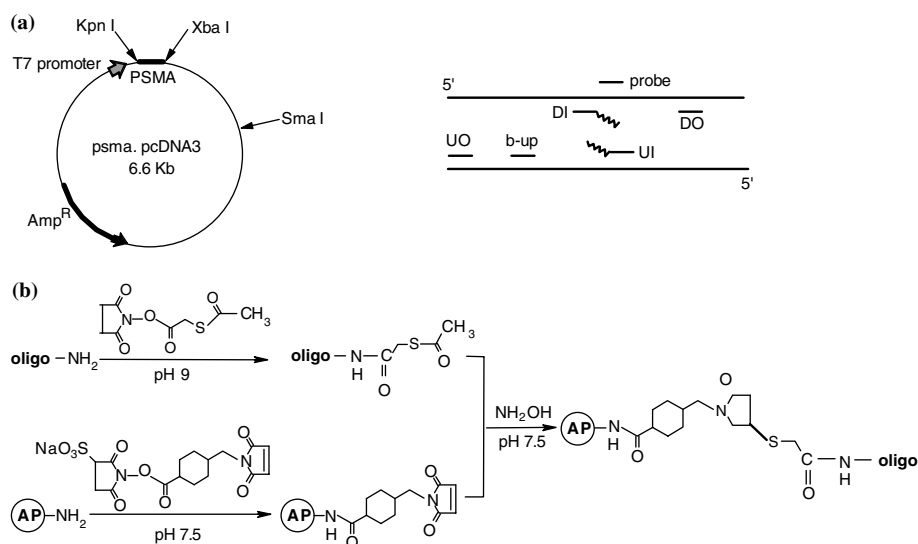
72°C, 70 s (10 min for the last cycle). Following its synthesis, AB DNA fragment was subcloned into vector pcDNA3 downstream of the T7 promoter using the *Kpn*I and *Xba*I sites introduced by the UO and DO primers, respectively. PSMA mRNA and RNA IS were produced by in vitro transcription of pcDNA3.PSMA and pcDNA3.IS constructs, respectively, under the control of the T7 promoter of the vector. The plasmids were first linearized with *Sma*I and then subjected to a transcription reaction in 50 μL total volume consisting of 40 mmol L^{-1} Tris-HCl (pH 7.9), 6 mmol L^{-1} MgCl$_2$, 10 mmol L^{-1} DTT, 5 mmol L^{-1} NaCl, 2 mmol L^{-1} spermidine, 60 U of RNase inhibitor, 2.5 mmol L^{-1} of each rNTP, 15 μL of linearized DNA (approximately 2 μg), and 70 U of T7 RNA polymerase. The reaction was allowed to proceed at 37°C for 90 min. The resulting PSMA and IS mRNAs were treated with the RQ1 RNase-free DNase (1 U DNase μg^{-1} RNA) at 37°C for 30 min. The PSMA and IS RNA stock solutions were then purified with RNAwiz according to the manufacturer's instructions, redissolved in 20 μL RNase-free water, and quantified by spectrophotometry at 260 nm. RNA stock solutions were finally stored at −70°C in the presence of 0.1 g L^{-1} *E. coli* tRNA and 40 U RNase inhibitor. RNA solutions at various concentrations were prepared by diluting the stocks in 0.05 g L^{-1} *E. coli* tRNA.

Blood specimens

Peripheral blood samples were collected (using EDTA as anticoagulant) from healthy female volunteers.

RNA isolation from blood specimens and cell lines

Blood (5 mL) was mixed with an equal volume of 20 g L^{-1} dextran (MW 70,000) in 9 g L^{-1} NaCl. After incubation at room temperature for 45 min the upper yellowish phase containing the leukocytes was transferred to a clean sterile tube and the nucleated blood cells were pelleted by centrifugation at 2,000×g for 10 min at 4°C. The cell pellet was obtained no later than 3 h from venipuncture. If RNA extraction was going to be performed later, cell pellets were snap-frozen in liquid nitrogen and stored at −70°C. The nucleated cells were lysed with 1 mL RNAwiz reagent and total RNA was isolated according to the manufacturer's instructions. Finally, the pellet was air-dried, dissolved in 80 μL RNase-free, water and stored at −70°C. For total RNA isolation from LNCaP cells, the cells were sedimented and lysed with RNAwiz reagent (1 mL/1×10^7 cells). Total RNA was extracted according to the manufacturer's instructions. Following precipitation, RNA was washed once with 75% cold ethanol, air-dried, and dissolved in RNase-free water (30 μL/10^7 cells) containing the appropriate amount of RNase inhibitor (40 U/10^7 cells). The amount of RNA was quantified by spectrophotometry at 260 nm.

RT-PCR

For the reverse transcription reaction, a 16 μL solution containing 10 pmol of the downstream primer, DO, and 5 μL of sample RNA was heated at 70°C for 10 min and then placed on ice for 1–2 min. Subsequently, 4.25 μL of a solution consisting of 2 μL of reverse transcriptase buffer solution (250 mmol L^{-1} Tris-HCl, pH 8.3, 50 mmol L^{-1} MgCl$_2$, 500 mmol L^{-1} KCl, 20 mmol L^{-1} DTT), 2 μL of 10 mmol L^{-1} of each dNTP, and 5 U AMV reverse transcriptase (0.25 μL) was added. The reverse transcription reaction was allowed to proceed for 40 min at 42°C and then the mixture was briefly chilled on ice. cDNA was stored at −20°C until amplification.

A 5-μL aliquot of cDNA was amplified in a total reaction volume of 50 μL containing 50 mmol L^{-1}

Tris-HCl (pH 9.0), 15 mmol L^{-1} $(NH_4)_2SO_4$, 0.1% Triton X-100, 2 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} of each dNTP, 25 pmol of each of the biotinylated upstream primer (b-up) and the downsteam primer (d), and 1 U Dynazyme EXT DNA polymerase. Amplification was carried out for 35 cycles using the following program: 95°C, 15 s (75 s for the first cycle), 60°C, 15 s, and 72°C, 30 s (10 min for the last cycle). PCR mixtures were stored at 4°C until hybridization.

Microtiter well-based chemiluminometric hybridization assay

Polystyrene wells were coated overnight at ambient temperature with 50 μL of 1.4 mg L^{-1} streptavidin solution in PBS. Prior to use, the wells were washed three times with wash solution (50 mmol L^{-1} Tris, pH 7.4, 0.15 mol L^{-1} NaCl, and 1 mL L^{-1} Tween 20) using the WellWash4 microplate washer (Labsystems, MA, USA). PCR products were diluted 20 times in blocking solution (10 g L^{-1} blocking reagent in 0.1 mol L^{-1} maleic acid, 0.15 mol L^{-1} NaCl, pH 7.5) and 50 μL was pipetted into each of four wells. Immobilization of DNA on the well surface (by streptavidin–biotin interaction) was achieved by 30-min incubation at ambient temperature with mechanical shaking. The wells were washed as above and 50 μL of 0.2 mol L^{-1} NaOH was added to denature immobilized double-stranded PCR products. After 10 min incubation at room temperature the non-biotinylated DNA strand was removed by washing. Subsequently, 50 μL of each AP-conjugated

Fig. 2 a Schematic diagram showing the hybridization assay configuration developed in this work. Biotin-labeled DNA products are captured on streptavidin-coated wells. After separation of the two DNA strands, the single-stranded DNA fragments are allowed to hybridize to the specific AP-labeled probes and the hybridization products are directly detected with a dioxetane chemiluminogenic substrate. **b** Study to establish the specificity of the detection probes used for PSMA and IS. PCR products containing no DNA (background), target PSMA or IS only were assayed by hybridization using target specific AP-labeled probes
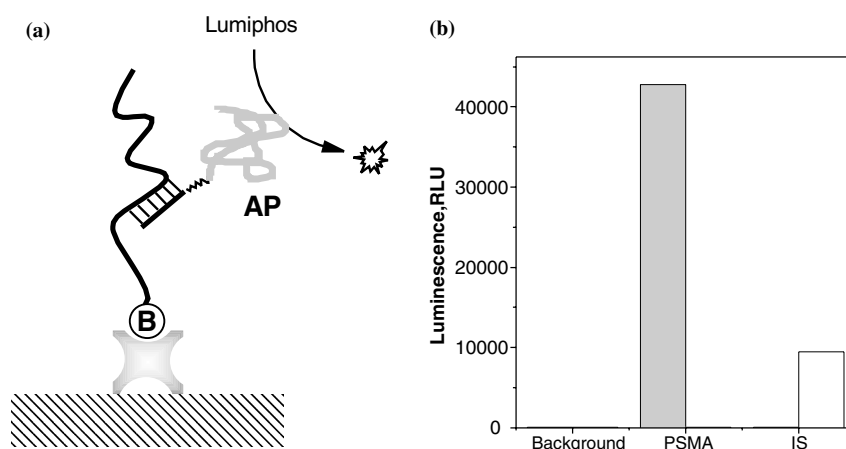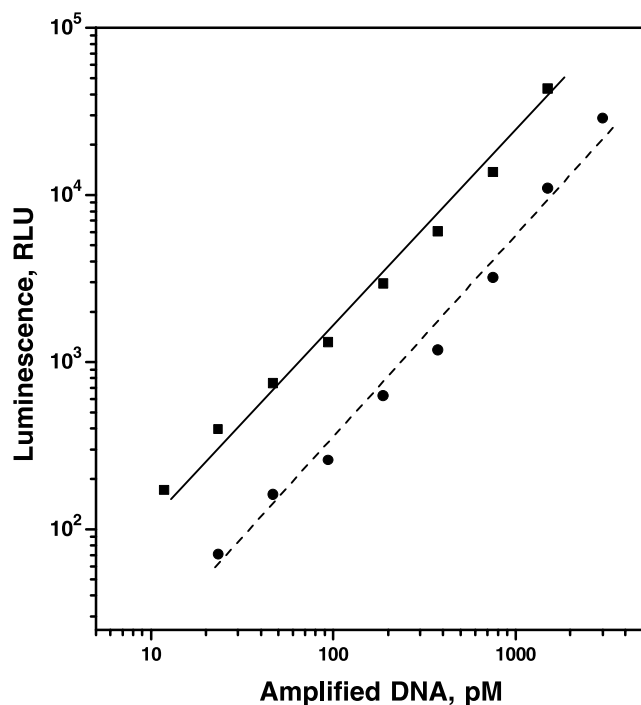
PSMA and IS detection probes, diluted 500 times in blocking solution containing 10% DMSO, was added in duplicate to the wells. Hybridization was carried out for 30 min at 42°C with shaking. The wells were washed and 50 μL of the chemiluminogenic substrate Lumiphos was added to each well. Following incubation for 30 min at 37°C, the chemiluminescence was integrated for 1 s using the PhL microplate luminometer/photometer manufactured by Mediators (Vienna, Austria).

Results and discussion

The principle of the preparation of AP–oligonucleotide conjugates (oligozymes) is illustrated in Fig. 1b. The conjugation strategy involves chemical modification of the amino group of the probe with SATA for introduction of a protected thiol group. Maleimide groups were introduced to AP by reacting with the heterobifunctional cross-linker sulfo-SMCC. Maleimide-activated AP was then mixed with SATA-modified oligonucleotide probe and the reaction was initiated by deprotecting the sulfhydryl group with hydroxylamine. The conjugate was separated from the free oligonucleotide by ultrafiltration and used directly in hybridization assays. The assay configuration is presented in Fig. 2a. PCR products, biotinylated at their 5′ end by PCR, are immobilized on the streptavidin-coated surface of polystyrene wells. After removing one DNA strand with NaOH, single-stranded DNA is hybridized with AP-conjugated target-specific probes. Following hybridization, the enzyme activity is measured by using chemiliminogenic substrate.

The hybridization assays were optimized by using dilutions of biotinylated amplification products from PSMA and IS. The concentration of the stock solutions was determined by densitometry from images of ethidium bromide-stained gels taken with a Kodak DC120 digital camera. The ΦX174 DNA fragments were used as calibrators. Experiments were carried out to test for the presence of any cross-hybridization, i.e. hybridization of the IS-probe with the PSMA-amplified DNA and/or hybridization of the PSMA-probe with the

Fig. 3 Calibration graphs for the hybridization assays for PSMA (*solid line*) and IS (*dashed line*) amplification products

IS-amplified sequence. Solutions containing PSMA and IS DNA (1,000 pmol L^{-1}) were assayed by the proposed hybridization assay and detected with both PSMA- and IS-specific probes. From the results presented in Fig. 2b it is concluded that PSMA-specific probe binds exclusively to PSMA DNA, whereas IS-specific probe binds only to IS DNA. No hybridization occurred when PSMA or IS DNA was measured with IS- or PSMA-probe, respectively. The detectability and linear range of the two hybridization assays were established by preparing serial dilutions of the PSMA and IS stock DNA solutions and performing hybridization assays with the two specific AP-conjugated probes. The results are presented in Fig. 3. As low as 6 pmol L^{-1} of amplified PSMA DNA (0.3 fmol/well) and 23 pmol L^{-1} of amplified IS DNA (1.17 fmol/well) can be detected with a signal-to-background ratio of two. The difference in detectability found for PSMA DNA and PSMA IS is because of the hybridization efficiency of the respective probes. The linear range of the assays extends up to 3,000 pmol L^{-1}. The reproducibility of the hybridization assay was determined by analyzing samples containing 23.5, 188, and 750 pmol L^{-1} amplified products. The coefficients of variation (CVs) were 5.0, 6.9, and 13.4%, respectively ($n=3$). It should be pointed out that the conjugation reaction of AP with the amino-modified oligonucleotides was very reproducible. Conjugates prepared in three separate reactions (on different days) were used to generate calibration graphs in the range 23–750 pmol L^{-1} of target DNA. The CVs obtained for the signals at each target DNA concentration using conjugates from different reactions did not exceed 20%.

We compared the proposed "direct labeling" method with an "indirect labeling" approach in which the PSMA probe was labeled at the 3′ end with multiple digoxigenin moieties and the hybrids were detected by using antidigoxigenin antibody conjugated to AP. The "indirect labeling" approach gave higher luminescence signal. However, the S/B ratios were practically the same. At 750 pmol L^{-1} target DNA, the S/B ratios were 714 and 813 for the "indirect" and "direct" labeling of the probe, respectively. This is due to the lower background obtained by using probes directly conjugated with AP. The two systems offer the same detectability and analytical range.

Quantitative RT-PCR assays were performed with samples containing PSMA mRNA varying from 500 to 5,000,000 copies along with a constant amount (20,000 copies) of RNA IS. Amplified PSMA and IS RNA were detected by hybridization with the two target-specific AP-conjugated probes. The luminescence signals (corrected for the background), L and L_{IS}, which reflect the amount of amplified PSMA RNA and RNA IS, were plotted as a function of the number of input copies of PSMA mRNA (Fig. 4). The background is defined as the signal obtained when no PSMA mRNA or RNA IS are present in the RT-PCR mixture. The variation of the signals for PSMA mRNA and IS RNA versus the number of input PSMA mRNA copies is typical for the competitive nature of the PCR reaction between PSMA and IS DNA templates, especially when PCR enters the
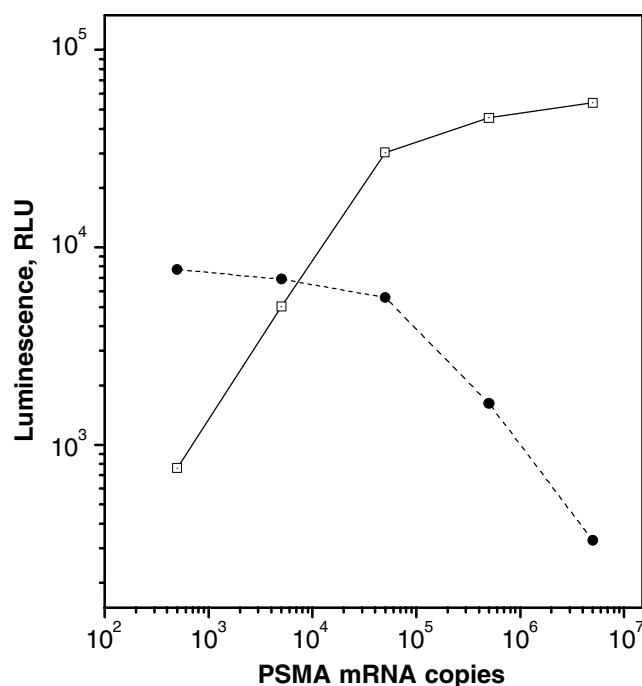

Fig. 4 Variation of luminescence with the number of PSMA mRNA copies. Samples containing 500–5,000,000 PSMA mRNA copies are coamplified with 20,000 copies of RNA IS. The signals obtained for PSMA mRNA and RNA IS are plotted with *solid* and *dashed lines*, respectively
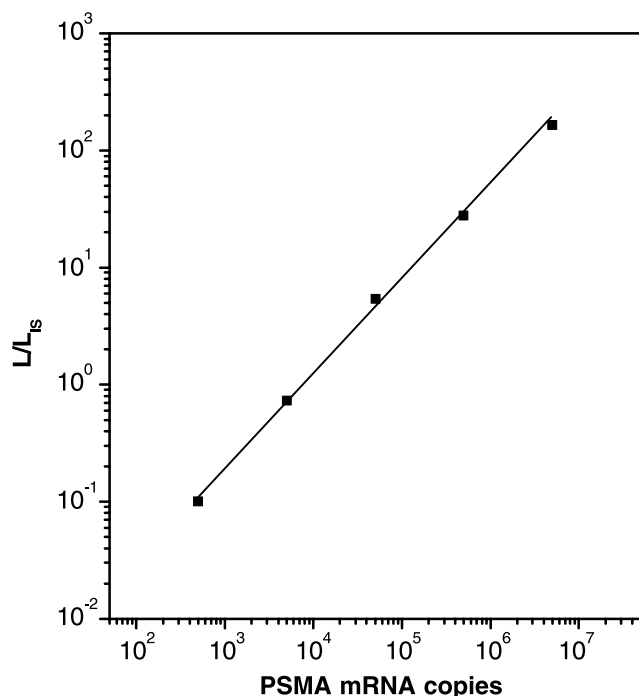
Fig. 5 Calibration graph for the quantification of PSMA mRNA. The ratio, L/L_{IS}, of the luminescence values obtained from PSMA mRNA and RNA IS is linearly related to the number of PSMA mRNA copies found in the sample prior to RT-PCR
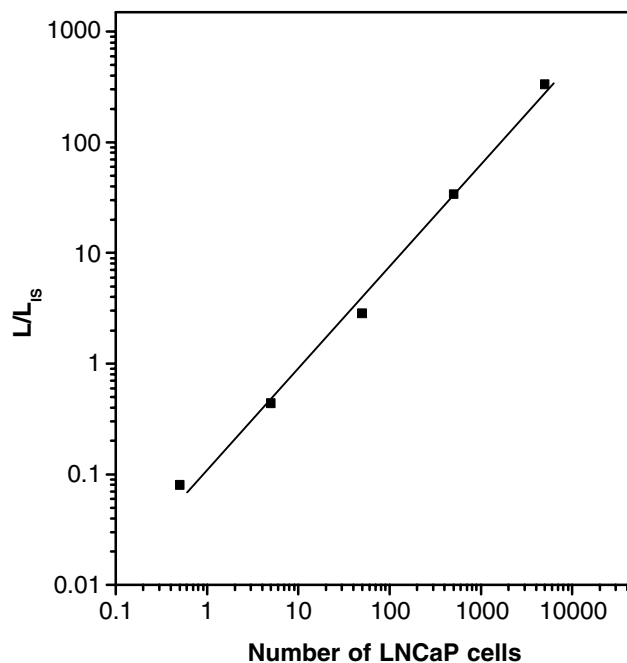

Fig. 6 Variation of the ratio, L/L_{IS}, of the luminescence values obtained from PSMA mRNA and RNA IS with the number of LNCaP cells in the sample

plateau phase. In Fig. 5, the ratio of the luminescence values for PSMA mRNA and RNA IS (L/L_{IS}) is plotted versus the number of PSMA mRNA molecules. Dilutions of mRNA were prepared in 0.05 g L^{-1} of total RNA from healthy leukocytes isolated from whole blood as described in the Materials and methods section. As few as 500 copies of PSMA mRNA were detected with a signal-to-background ratio of five. Because only 5% of the amplified PCR product was used for hybridization, the luminescence signal corresponds to amplification product from 25 PSMA mRNA copies. The linearity of the assay extends up to 5,000,000 PSMA mRNA copies.

The overall reproducibility (within-run) of the proposed assay (including reverse transcription, PCR, and hybridization) was assessed by analyzing samples containing 5,000, 50,000 and 500,000 PSMA mRNA copies in the presence of 20,000 copies of IS RNA ($n = 3$). The CVs obtained for the L/L_{IS} ratios were 13.2, 7.7, and 5.9%, respectively. The corresponding CVs for PSMA mRNA copies calculated from the calibration curve and the L/L_{IS} ratios were 16.6, 9.6, and 7.4%, respectively.

The quantitative RT-PCR method was also validated by analyzing samples containing total RNA from 0.5 to 5,000 LNCaP cells diluted in 0.05 mg mL^{-1} total RNA from healthy leucocytes. The samples were subjected to RT-PCR in the presence of a constant amount of IS RNA (20,000 copies) and subsequently assayed by hybridization assay. In Fig. 6, the L/L_{IS} ratio is plotted as a function of the number of LNCaP cells. It is

observed that the linearity covers the range from 0.5 to 5,000 cell equivalents, i.e. four orders of magnitude. A S/B ratio of four was obtained at the level of 0.5 LNCaP cell equivalents.

The determination of mRNA at low concentrations might be performed either by real-time PCR or by branched DNA technology. In contrast with the proposed method, which is an end-point approach, real-time PCR allows continuous monitoring of DNA amplification either by using an intercalating fluorescent dye (e.g. SYBR Green I) or by exploiting the 5′ exonuclease activity of DNA polymerase in combination with fluorescence resonance energy transfer. The co-determination of non-specific double-stranded DNA fragments is a serious limitation of the former strategy. Real-time PCR involves expensive instrumentation and reagents. Also, the real-time PCR-based methods do not use an IS to compensate for any variation in the reverse transcription reaction. The branched DNA technology (bDNA) [24, 25] is a signal-amplification system. Typically, the mRNA is hybridized with multiple specific probes spanning the entire region of the target sequence. These probes carry characteristic tails that allow capture of the hybrids by immobilized oligonucleotide probes and hybridization with a branched DNA molecule. Each branched DNA is then hybridized with multiple oligonucleotides conjugated to AP. The advantage of this technology arises from elimination of the target amplification step and the associated problems of contamination. This technique also allows direct determination of the specific mRNA in the cell extracts without prior isolation. However, the assay procedure requires 16–

20 h for hybridization of the target with the set of probes followed by two consecutive hybridization steps with the branched DNA and the AP-conjugate (2 h). Also, the assay requires the design and purchase of about 30 oligonucleotides per target mRNA. Moreover, the reported detectability of bDNA technology for mRNA determination (about 10,000 mRNA molecules) does not reach the levels achieved by PCR amplification.

The proposed method has several advantages. Quantitative RT-PCR is based on coamplification of PSMA mRNA with a recombinant PSMA-like RNA IS, thus compensating for any variations in reverse transcription and amplification reactions. The IS has the same primer binding sites as those of the PSMA and the amplification products are of the same size, differing only in a 24-bp centrally located segment. The hybridization assay developed for the analysis of amplification products is simplified by the use of specific oligonucleotide probes directly conjugated with AP. Thus the use of haptens and specific antibodies conjugated with reporter molecules (indirect labeling) is avoided and the assay procedure becomes much shorter. The procedure used for the preparation of probe-AP conjugates is very simple and does not require HPLC purification. Oligonucleotide–AP conjugates are stable at 4°C at least for 6 months. The conjugate obtained from a reaction that uses 1 nmol AP is sufficient for about 500 hybridization assays. The proposed method detects as few as 500 PSMA mRNA molecules.

The applications of the proposed assay for the determination of PSMA mRNA could be:

– to investigate the relationship between the number of circulating cancer cells and tumor grade;
– to identify early tumor spread thereby allowing prediction of the disease progression, especially in patients diagnosed with localized prostate cancer;
– to provide a means of molecular staging particularly for patients who receive androgen ablation therapy or suffer from hormone refractory tumors, because PSMA expression is up-regulated in the absence of androgens;
– to discriminate between BPH and prostate cancer, because PSMA was found to be significantly overexpressed in prostate cancer compared with benign hyperplasia; and
– to provide a powerful research tool in the study of the biology of prostate cancer and the comprehension of the metastatic process.

References

1. Carter RE, Feldman AR, Coyle JT (1996) Proc Natl Acad Sci USA 93:749–753
2. Pinto JT, Suffoletto BP, Berzin TM, Qiao CH, Lin S, Tong WP, May F, Mukherjee B, Heston WD (1996) Clin Cancer Res 2:1445–1451
3. Israeli RS, Powell T, Fair WR, Heston WD (1993) Cancer Res 53:227–230
4. O'Keefe DS, Su SL, Bacich DJ, Horiguchi Y, Luo Y, Powell CT, Zandvliet D, Russell PJ, Molloy PL, Nowak NJ, Shows TB, Mullins C, Vonder Haar RA, Fair WR, Heston WD (1998) Biochim Biophys Acta 1443:113–127
5. Israeli RS, Powell T, Corr JG, Fair WR, Heston WDW (1994) Cancer Res 54:1807–1811
6. Lintula S, Stenman U-H (1997) J Urol 157:1969–1972
7. Kawakami M, Nakayama J (1997) Cancer Res 57:2321–2324
8. Burger MJ, Tebay MA, Keith PA, Samaratunga HM, Clements J, Lavin MF, Gardiner RA (2002) Int J Cancer 100:228–237
9. Stamey TA, Warrington JA, Caldwell MC, Chen Z, Fan Z, Mahadevappa M, McNeal JE, Nolley R, Zhang Z (2001) J Urol 166:2171–2177
10. Wright GL, Grob BM, Haley C, Grossman K, Newhall K, Petrylak D, Troyer J, Konchuba A, Schellhammer PF, Moriarty R (1996) Urology 48:326–334
11. Bostwick DG, Pacelli A, Blute M, Roche P, Murphy GP (1998) Cancer 82:2256–2261
12. Kahn D, Williams RD, Seldin DW, Libertino JA, Hirschhorn M, Dreicer R, Weiner GJ, Bushnell D, Gulfo J (1994) J Urol 152:1490–1495
13. Lu-Yao GL, McIerran D, Wasson J, Wennberg JE (1993) JAMA 269:2633–2655
14. Israeli RI, Miller WH, Su SL, Powell T, Fair WR, Samadi DS, Huryk RF, DeBlasio A, Edwards ET, Wise GJ, Heston WD (1994) Cancer Res 54:6306–6310
15. Lucotte G, Mercier G, Burckel A (1998) Mol Cell Probes 12:421–425
16. Renneberg H, Friedetzky A, Konrad L, Kurek R, Weingartner K, Wennemuth G, Tunn UW, Aumuller G (1999) Urol Res 27:23–27
17. Loric S, Dumas F, Eschwege P, Blanchet P, Benoit G, Jardin A, Lacour B (1995) Clin Chem 41:1698–1704
18. Cama C, Olsson CA, Raffo AJ, Perlman H, Buttyan R, O'Toole K, McMahon D, Benson MC, Katz AE (1995) J Urol 153:1373–1378
19. Llanes L, Ferruelo A, Paez A, Gomez JM, Moreno A, Berenguer A (2002) BJU Int 89:882–885
20. Adsan O, Cecchini MG, Bisoffi M, Wetterwald A, Klima I, Danuser H-I, Studer UE, Thalmann GN (2002) BJU Int 90:579–585
21. Hara N, Kasahara T, Kawasaki T, Bilim V, Obara K, Takahashi K, Tomita Y (2002) Clin Cancer Res 8:1794–1799
22. Grasso YZ, Gupta MK, Levin HS, Zippe CD, Klein EA (1998) Cancer Res 58:1456–1459
23. Gala J, Heusterspreute M, Loric S. Hanon F, Tombal B, Cangh PV, De Nayer P, Philippe M (1998) Clin Chem 44:472–481
24. Zhou L, Cryan EV, Minor LK, Gunnet JW, Demarest KT (2000) Anal Biochem 282:46–53
25. Czerwinski M, Opdam P, Madan A, Carroll K, Mudra DR, Gan LL, Luo G, Parkinson A (2002) Methods Enzymol 357:170–179