Dual-Label Detection of Amplified Products in Quantitative RT-PCR Assay Using Lanthanide-Labeled Probes

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ABSTRACT

Quantitative RT-PCR (QRT-PCR) enables the sensitive and specific detection of mRNA with a small copy number. We used the QRT-PCR method and dual-label analysis of amplification products for the detection of prostate-specific antigen (PSA) mRNA. The QRT-PCR assay employed a PSA-like internal standard (IS) mRNA, which was used to quantify the PSA mRNA copies and to control the variations during the whole assay procedure from the RNA extraction to the detection of QRT-PCR amplification products by hybridization assay. After co-amplification, the PSA and IS products were detected in a microplate using Eu³⁺ chelate-labeled PSA and Tb³⁺ chelate-labeled IS hybridization probes. The detection probes allowed the simultaneous and dual-label detection of PSA and IS products in the same microtiter well. Compared to the single-label assay, the dual-label detection improved the within- and between-assay cv% from 21.7 to 7.5 and from 36.0 to 30.3, respectively. The between- and within-assay variation of the dual-label assay was further studied using PSA-producing LNCaP cells. The cells were found to express 980 ± 170 (mean $\pm$ SD) copies of PSA-mRNA with the within-assay cv% of 17.7 and 890 ± 220 (mean $\pm$ SD) copies of PSA-mRNA with the between-assay cv% of 25.0. The methodology developed may help in future studies to obtain reliable quantification of PSA mRNA generated by circulating prostate cancer cells.

INTRODUCTION

Time-resolved fluorometry (TRF) with lanthanide chelates is a very sensitive technique that utilizes the long decay time and the large shift between the excitation and emission wavelengths (Stokes shift) of the lanthanide chelates (18,34). The long decay time allows the detection of the specific fluorescence after a time delay, during which the rapidly decreasing background fluorescence from the sample dies out. We have previously utilized TRF with single-label detection of the products amplified in quantitative RT-PCR (QRT-PCR) for prostate-specific antigen (PSA) mRNA (44). The use of different lanthanide chelates permits the detection of multiple analytes in the same reaction because of the sharp emission peaks and the different decay times of the lanthanides (Figure 1). The TRF technology and the detection of multiple analytes have been widely used in both hybridization assays for DNA and immunoassays (13,14,20,29,31,38,43). One of the most multiplexed assays has employed seven colors in TRF-based hybridization analysis (30).

RT-PCR is an important method for the determination of gene expression (1,3,41). RT-PCR assays have been used for the detection of specific tumor- or tissue-associated markers to monitor metastatic tumor cells in the blood, lymph nodes, or other organs (17,19,32). For the detection of prostate cancer cells in blood and lymph node samples, a widely studied target is PSA mRNA (8,15,16,21,40). However, the data from different studies seem to disagree with the power of RT-PCR assays for PSA mRNA to predict the micrometastatic spread and clinical stage and to monitor the treatment efficacy

(6,33,35). Some studies suggest the development of more standardized and reproducible QRT-PCR assays to further study the utility of this technique in the molecular staging of prostate cancer (7,26). There are several quantitative methods utilizing different approaches to detect the target mRNA. Some of the studies have used an endogenous calibrator for the relative quantification of the target mRNA, while others have employed an exogenous control and/or an external calibration curve for the quantification (1–3, 23,27,28,38,39,41,42,44). The endogenous and exogenous calibrators or controls can be either internal (to be amplified in the same reaction tube with the target) or external (to be amplified in a separate tube from the target).

Our study was designed to develop a dual-analyte hybridization assay for the quantitative and nonradioactive detection of RT-PCR-amplified PSA and internal standard (IS) products and to determine whether the reproducibility of the assay could be improved by this simultaneous detection. The assay describes a TRF and lanthanide-labeled, oligonucleotide-based detection of QRT-PCR amplification products. The QRT-PCR assay for PSA mRNA contains a novel exogenous IS mRNA and an external calibration curve. The IS mRNA has only a 2-bp deletion with respect to the wild-type PSA mRNA, and purified IS and PSA mRNAs are used to generate the calibration curve for each experiment. In addition, unlike in the existing assays in which the IS RNA is added to the sample at the beginning of cDNA synthesis, in our assay, a known amount of the IS mRNA is added into each sample at the beginning of RNA extraction. This permits us to quantify the PSA mRNA copies in the denatured

cell samples and to control variations from the beginning of the RNA extraction to the detection of the amplification products by hybridization. The PSA and IS mRNA are co-amplified in QRT-PCR with the same primers, and the amplification products are detected on a streptavidin-coated microtiter well with specific hybridization probes. The PCR products are immobilized on the microtiter well. After denaturation into a single-stranded form, the Tb³⁺ chelate-labeled IS and the Eu³⁺ chelate-labeled PSA probes are allowed to hybridize simultaneously. The signals of the bound probes are measured by TRF technology, which gives numerical values and allows the calculation of the PSA mRNA copies in the sample by comparing the PSA-to-IS fluorescence ratio of the sample to the ratio in the calibration curve. The developed assay may enable more quantitative and accurate detection of PSA mRNA in prostate cancer cells shed into the blood, lymph nodes, or bone marrow.

MATERIALS AND METHODS

Cell Lines

The PSA-expressing human prostatic carcinoma cell line LNCaP and the non-PSA-producing mouse myeloma cell line SP2/0 were obtained from the ATCC (Manassas, VA, USA). The cell

lines were cultured in flasks containing DMEM (Life Technologies, Rockville, MD, USA) with 100 mL/L fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and maintained in a 5% CO₂ incubator at 37°C. The medium for LNCaP culture was supplemented with 1 nM synthetic hormone methyltrienolone (R1881) (New England Nuclear, Boston, MA, USA). The cells were grown until near confluency, detached, washed with PBS, and counted. Unlike the SP2/0 cells, the LNCaP cells were detached by trypsin-EDTA treatment. The SP2/0 samples of 2.5×10^6 cells and the dilutions of LNCaP cells were stored at -70°C until RNA extraction.

Synthesis and Labeling of Oligonucleotides

The synthesis of PCR primers, detection probes, and DNA targets, along with the biotinylation of the 3' PCR primer and the DNA targets, was carried out as reported previously (Table 1) (44). The detection probes for the hybridization assay contained additional diaminohexanedeoxycytidines (modC) to be labeled with lanthanide chelate as described earlier (5,12,36). The detection probe for PSA was labeled with the Eu³⁺ chelate, and the probe for the IS was labeled either with the Eu³⁺ chelate for use in the single-label assay or with the Tb³⁺ chelate for use in the dual-label assay.

RNA Extraction

Total RNA was extracted from the pelleted LNCaP and SP2/0 cells using a TRIzol[®] reagent (Life Technologies). This method is based on the acid guanidinium thiocyanate-phenol-chloroform isolation (4). The RNA extraction was performed according to the manufacturer's instructions. Briefly, LNCaP and SP2/0 cells were denatured with the TRIzol reagent and combined to have a calculated amount of 1, 5, 10, 50, 100, 500, and 1000 denatured LNCaP cells in 2.5×10^6 SP2/0 cells. A constant amount of IS mRNA (5×10^4 molecules) was added into each sample after denaturation of the cells. The full-length PSA-like IS mRNA was produced in vitro and purified as described earlier (44). After TRIzol and 1-Bromo-3-Chloropropane (Sigma, St. Louis, MO, USA) treatment, RNA in the aqueous phase was precipitated once with isopropanol. The RNA pellet was washed twice with cold 700 mL/L ethanol, air dried and dissolved in 80 µL diethylpyrocarbonate (DEPC)-treated water. The RNA preparations were stored at -70°C until they were analyzed with RT-PCR assay.

RT-PCR Amplification

The cDNA synthesis was carried out with a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) (44). Each cDNA synthesis contained samples for the calibration curve using in vitro produced and purified PSA and IS mRNA diluted in 0.2 g/L *E. coli* tRNA (Roche Molecular Biochemicals, Mannheim, Germany) solution, which was used as an inert carrier solution. After the synthesis, a 5-µL cDNA sample was amplified in a 100-µL PCR, which consisted of 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton[®] X-100, 3.5 mM MgCl₂, 200 µM each dNTP, 0.2 µM 5' primer a, 0.035 µM biotinylated 3' primer b, 0.065 µM unlabeled 3' primer c, and 1 U DynaZyme[™] II Recombinant DNA Polymerase (Finnzymes Oy, Espoo, Finland). Before amplification the PCRs were kept on ice. The PCR amplifications were performed in a PTC-200[™] DNA Engine (MJ Research, Watertown, MA, USA) using a program of 94°C for 30 s

Figure 1. Principle of time-resolved fluorometry. Emission spectra and decay profiles of Tb³⁺, Eu³⁺ and Sm³⁺ chelates.

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Table 1. Names, Sequences, Labels, and Degrees of Labeling of Primers and Probes Used in This Study

Oligonucleotide	Name	Sequence from 5' to 3' End	Label	Labels/ Oligonucleotide
PCR				
5' primer	a	TGAACCAGAGGAGTTCTTGAC	— ^a	—
3' primer	b	(modC)CCCAGAATCACCCGAGCAG ^b	Biotin	1
3' primer	c	(modC)CCCAGAATCACCCGAGCAG	—	—
Hybridization Assay				
PSA probe	d	(modC) ₂₀ GCGCAAGTTCACCCTCA	Eu ³⁺	17
IS probe	e	(modC) ₂₀ GTGCGCAATCACCTC	Eu ³⁺	15
IS probe	f	(modC) ₂₀ GTGCGCAATCACCTC	Tb ³⁺	22
PSA target DNA	g	(modC)CCTTCTGAGGGTGAAGTTGCGCACACA	Biotin	1
IS target DNA	h	(modC)CCTTCTGAGGGTGATTGCGCACACACG	Biotin	1

^a—, no label.
^bmodC, diaminohexanedeoxycytidine.

(3 min for the first cycle), 61.5°C for 30 s, and 72°C for 45 s (10 min 45 s for the last cycle) for 30 cycles.

Hybridization

The products of the PCR amplification were analyzed either with single- or dual-label assay formats as shown in Figure 2. A 10- μ L aliquot of PCR product and 50 μ L DELFIA® Assay Buffer containing 1 M NaCl were added into each streptavidin-coated microtiter well (PerkinElmer Life Sciences Wallac, Turku, Finland). In the single-label assay, each PCR product was added into four wells, the first two replicas were detected with the Eu³⁺-labeled PSA hybridization probe and the second two replicas were detected with the Eu³⁺-labeled IS probe. In the dual-label assay, each PCR product was added into two wells, and the PSA and IS products were detected from the same wells with the Eu³⁺-labeled PSA and Tb³⁺-labeled IS probes, respectively. PCR amplification and the biotinylated 3' primer (b) resulted in biotinylated PCR products, which were captured onto the wells by incubating at room temperature with slow shaking for 30 min. After the capture reaction, the wells were washed three times with DELFIA Wash Solution, and 100 μ L 50 mM NaOH were added into each well for the denaturation of the double-stranded PCR products. Denaturation was carried out by incubating at room

temperature with slow shaking for 5 min; the denatured DNA strand was then removed by washing three times as described above. The captured DNA strand was detected by adding 100 μ L hybridization solution containing a detection probe or detection probes in DELFIA Assay Buffer containing 0.1% non-fat milk powder and 1 M NaCl. The hybridization solutions of the single-label assay contained 20 pg/ μ L detection probe (Table 1, d or e), and that of the dual-label assay contained 20 pg/ μ L PSA probe (d) and 10 pg/ μ L IS probe (f). After hybridization at 50°C for 2 h, the wells were washed six times with 55°C DELFIA Wash Solution. Then, 200 μ L DELFIA Enhancement Solution were added into each well, and after shaking for 30 min at room temperature, the Eu³⁺ fluorescence was measured with 1420 Victor™ Multilabel Counter (Perkin Elmer Life Sciences Wallac). In the dual-label assay after the measurement of Eu³⁺ fluorescence, 50 μ L DELFIA Enhancer was added into each well, and after a 5-min incubation, the Tb³⁺ fluorescence was measured.

RESULTS

Assay Design

The dual-label hybridization assay was developed to detect both PSA and IS amplification products simultane-

ously from the same microtiter well. For the dual-label assay, the detection probe for IS (f) was labeled with the Tb³⁺ chelate instead of with the Eu³⁺ chelate, which was used for labeling the IS probe in the single-label assay. The Eu³⁺-labeled PSA probe (d) and the sequences of the PSA and IS detection probes were the same in both the single- and the dual-label assays.

Synthetic DNA targets (g and h) were used to optimize the amount of Tb³⁺-labeled IS hybridization probe and the simultaneous detection of PSA and IS targets in the same microtiter well. In the dual-label assay, the highest efficiency in hybridization was obtained with hybridization solution containing 10 pg/ μ L Tb³⁺-labeled IS probe (f) and 20 pg/ μ L PSA probe (d). The other hybridization conditions for the dual-label assay were the same as those for the single-label assay (see Materials and Methods).

The emission peaks of the Tb³⁺ and Eu³⁺ chelates are sharp (Figure 1), and the fluorescence signals can be clearly distinguished by measuring the signals at different wavelengths. However, the relative fluorescence intensity of the Tb³⁺ chelate is smaller than that of the Eu³⁺ chelate; therefore, the fluorescence signals (counts per second) obtained with the Tb³⁺-labeled IS probe are lower than those obtained with the Eu³⁺-labeled IS probe. Because of the lower relative Tb³⁺ fluorescence, the amount of IS mRNA was raised from 1

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$\times 10^3$ to 5×10^3 copies for the calibration curve and for the quantification of PSA mRNA in the samples. The use of 5×10^3 IS mRNA copies gave good linearity (from 50 to 10^6 PSA mRNA copies) for the calibration curve, which was similar to the curve generated with the single-label assay and 10^3 IS mRNA copies (Figure 3). For the quantification of PSA mRNA in the samples, 5×10^4 copies of IS mRNA were added into each sample at the beginning of the RNA extraction, and the RNA pellet was dissolved into 80 μ L DEPC-treated water. One tenth (8 μ L) of each sample (corresponding to 5×10^3 copies of IS mRNA in theory) was then analyzed with the QRT-PCR assay.

Precision and Detection Limit of the Assay

Precision of the optimized dual-label hybridization assay was compared with that of the single-label assay using the QRT-PCR amplified samples for the calibration curve and two replicas of each sample in each of the five independent analyses. The within-assay precision over the calibration range (50– 10^6 PSA mRNA copies, 5×10^3 IS mRNA for the dual-label and 10^3 IS mRNA for the single-label assay in each) was 2.5%–21.7% for the single-label assay and 0.8%–7.5% for the dual-label assay (Figure 4A). The between-assay precision over the calibration range was 8.9%–36.0% for the single-label assay and 11.8%–30.3% for the dual-label assay (Figure 4B). With both the single- and the dual-label assays, the smallest detectable number of PSA mRNA was 50 copies, and the lin-

Figure 2. Principle of single- and dual-label assays. A known amount of IS mRNA is added into a mixture of denatured cells, and then PSA and IS mRNAs in the sample are co-extracted in total RNA isolation. PSA and IS mRNAs in the RNA sample or calibrator are co-amplified by RT-PCR (primers a and b). One of the PCR primers (b) is biotinylated (B), which results in biotinylated amplification products. The amplification products are captured on streptavidin (SA)-coated microtiter wells. In the single-label hybridization assay, the PSA and IS PCR products are detected separately with specific europium chelate-labeled detection probes (d and e) (44). In the dual-label assay, PSA and IS PCR products are detected with europium and terbium chelate-labeled probes (d and f), respectively.

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Table 2. Comparison of Different Q&T-PCR Assays Utilizing Exogenous Calibrators or Controls

Analyte mRNA ^a	Calibrator/Control ^b	Quantification Method ^c	Overall Precision, cv%		Reference
			Within-assay	Between-assay	
A					
CFTR	Homologous IS RNA	PAGE, EtBr staining and laser densitometer	— ^d	13 (n = 5)	1.7–177.7 amol IS RNA (appr. 10 ⁵ –1.1 × 10 ⁸ molecules) (23)
c-erbB-2	Nonhomologous IS DNA	Fluorescent PCR products, PAGE and automated DNA sequencer	10.6 (n = 6)	4.07 (n = 3)	0.007–0.105 amol of target cDNA (appr. 4.2 × 10 ³ –6.3 × 10 ⁴ molecules) (27)
B					
PTHrP	Homologous IS DNA, EOC with target cDNA	Single-label solution hybridization assay, TRF	15, 17, 13, and 10 for input of 50, 2 × 10 ² , 2 × 10 ³ , and 5 × 10 ⁴ copies, respectively (n = 6)	<11 (n = 4)	1–10 ⁵ target cDNA copies (28)
EGFR	Nonhomologous IS RNA, EOC with total liver RNA	AGE, EtBr staining and computer scanning	—	—	0.19–4.20 amol target mRNA/μg total liver RNA (appr. 1.14 × 10 ⁵ –2.53 × 10 ⁶ molecules) (2)
PSA	Homologous IS DNA, EOC with target DNA	Dual-label chemi-luminescence solution hybridization assay	—	7.3, 8.9, and 17.6 for 1.3 × 10 ³ , 1.3 × 10 ⁴ , and 1.3 × 10 ⁵ target DNA molecules, respectively (n = 4)	4.3 × 10 ² –3.15 × 10 ⁵ target DNA molecules (38)
PSA	Homologous IS RNA, EOC with LNCaP cells	Single-label solution hybridization assay, TRF	—	19.1, 19.1, and 17.0 for 31, 391, and 1826 LNCaP cells, respectively (n = 4)	4–3000 LNCaP cells (39)
Tg	No IS, EOC with total thyroid RNA	AGE with EtBr staining and Quantitative PCR with real-time detection of the accumulation of PCR products, Taqman chemistry	<1 for threshold cycle of calibrators and amounts of >3.2 pg thyroid RNA	1.6 (n = 6) for threshold cycle of calibrators 24 (n = 10)	3.2–10000 pg total thyroid RNAe (42)
PSA	Homologous IS mRNA, EOC with target mRNA	Single-label solution hybridization, TRF	13, 2.5, and 14 for 50, 10 ⁴ , and 10 ⁵ target mRNA copies, respectively (n = 5)	—	50–10 ⁵ target mRNA copies (44)
PSA	Homologous IS mRNA, EOC with target mRNA	Dual-label solution hybridization, TRF	7.5, 5.2, 0.8, 1.4, 4.7, and 6.4 for 50, 10 ³ , 10 ⁴ , 10 ⁵ , and 10 ⁶ target mRNA copies, respectively (n = 2)	30.3, 24.7, 16.7, 11.8, 17.5, and 12.4 for 50, 10 ³ , 10 ⁴ , 10 ⁵ , and 10 ⁶ target mRNA copies, respectively (n = 5)	50–10 ⁶ target mRNA copies Current paper

Table 2. Comparison of Different QRT-PCR Assays Utilizing Exogenous Calibrators or Controls (continued)

^a CFTR, Cystic fibrosis transmembrane conductance regulator; EGFR, epidermal growth factor receptor; PTHrP, parathyroid hormone-related protein; Tg, thyroglobulin; PSA, prostate-specific antigen.
^b IS, internal standard; EOC, external calibration curve.
^c PAGE, Polyacrylamide gel electrophoresis; EtBr, ethidium bromide; AGE, agarose gel electrophoresis; TRF, time-resolved fluorometry.
^d -, not reported.
^e Amount of molecules not reported.
Part A includes the assays in which different dilutions of IS calibrator are amplified with a constant amount of sample. The quantification of the target mRNA is carried out by establishing the equivalency point between the calibrator and the target. Part B includes the assays in which a constant amount of sample is amplified with or without a constant amount of IS. The quantification of the target mRNA is done by comparing the signal of the sample to the signals of an external calibration curve or by comparing the target-to-IS ratio of the sample with the calibrator-to-IS ratios of an external calibration curve.

ear range was up to 10^6 copies. The low detection limits correspond to a signal of two times the mean of zero dose. The dual-label detection was used in the following experiments.

PSA-Producing LNCaP Cells

The number of PSA mRNA copies in LNCaP cells ($1-10^3$ cells) mixed with 2.5×10^6 SP2/0 cells was determined using nine replicas of each LNCaP dilution in three independent analyses. The PSA-negative SP2/0 cells did not yield amplification product. The number of PSA mRNA copies in $1-10^3$ LNCaP cells ranged within the assay from 1300 ± 300 to $830\,000 \pm 13\,000$ (mean $\pm$ SD) and between the assays from 1300 ± 700 to $680\,000 \pm 210\,000$ (mean $\pm$ SD) (Figure 5, A and B). The mean number of PSA mRNA per one LNCaP cell was 980 ± 170 (mean $\pm$ SD) copies with the within-assay CV% of 17.7, and 890 ± 220 (mean $\pm$ SD) with the between-assay CV% of 25.0. The dual-label QRT-PCR method for PSA detected one LNCaP cell in the presence of 2.5×10^6 SP2/0 cells.

DISCUSSION

QRT-PCR assays have been developed to contain either endogenous or exogenous controls or calibrators, which can be either external or internal. Originally, Chelly et al. (3) used an endogenous internal calibrator for the relative quantification of the target mRNA. In this method, the calibrator and target were amplified with different primers. Furthermore, some of the studies used an exogenous target-like IS RNA or DNA using dilutions of the IS for the quantification of the target mRNA (1,23,27,41). More recently, the quantification of the target mRNA has been carried out using a constant amount of IS in each sample and an external calibration curve consisting of the dilutions of calibrator and a constant amount of IS in each ($2,28,38,39,42,44$).

Table 2 summarizes some studies ($2,23,27,28,38,39,42,44$) that report the precision or the detection range of their QRT-PCR assays and that utilize an exogenous IS (panel A) and/or an external calibration curve (panel B). There are

many RT-PCR assays each utilizing its own principle, which makes the direct comparison of different assays quite difficult. However, there are some facts that need to be taken into account when developing a QRT-PCR assay with an exogenous IS and/or an external calibration curve. For the quantification of the target, it is a prerequisite that the target and the IS are amplified with the same efficiency. The assays in Table 2 employ different types of IS, which are either DNA or RNA, and either homologous or non-homologous with respect to the target. The same amplification efficiency can be achieved by developing an IS with similar sequence and size with respect to the target so that they can be co-amplified with the same primers and result in amplification products of similar size. Furthermore, to obtain selective detection of the amplification products, the IS should contain a small but distinguishable difference in its amplified sequence with respect to the target, and there should be a sophisticated detection method to quantify the target and the IS PCR products.

The IS DNA and IS RNA control different steps, to some extent, during the quantification of target mRNA by RT-PCR. The use of IS DNA makes it possible to quantify the target cDNA and controls variations from the PCR step to the detection of amplification

Figure 3. Comparison of calibration curves generated with single- and dual-label assays. Squares indicate the curve for the single-label assay containing $50-10^6$ initial PSA mRNA molecules with 10^3 IS mRNA in each. Circles indicate the curve for the dual-label assay containing $50-10^6$ initial PSA mRNA molecules with 5×10^3 IS mRNA in each.

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products (27,28,38). IS RNA enables quantification of the target from RNA level and controls the variations from the beginning of RT-PCR to the detection of amplification products (2,23, 39). However, the RNA extraction step should be also controlled. In our current and previous work (44), the target-like IS mRNA is added into the cell samples at the beginning of the RNA extraction, and the assays are controlled from the RNA extraction to the hybridization assay used for the detection of the amplification products. Therefore, the amount of target mRNA represents the number of mRNA copies in the denatured cell pellet before the RNA extraction. However, no assays exist that control the collection of the target cells from the biological material, which cannot provide information whether there are a few cells that contain many target mRNA copies or many cells that contain a few target mRNA copies.

There are two main principles in the use of an exogenous IS for the quantification of the target mRNA. The first

principle is to amplify serial dilutions of IS (or sample) with a constant amount of sample (or IS) and quantify the target mRNA by establishing an equivalency point between the IS and the target (23,27). This method requires many tubes for the analysis of one sample and therefore limits the number of

samples that can be analyzed in one experiment. The second principle is to amplify a constant amount of IS in each sample and in each of the calibrators covering a certain concentration range of the target (2,28,38,39,44). The concentration of the target mRNA in the sample can then be calculated from the

Figure 4. Within- and between-assay reproducibility of single- and dual-label hybridization assays over the calibration range (50–10⁶ PSA mRNA copies, 5×10^3 IS mRNA for the dual-label, and 10³ IS mRNA for the single-label assay in each). Panel A represents the within-assay reproducibility, and panel B represents the between-assay reproducibility. Closed and open triangles indicate the calibration curve and its cv values for the single-label assay, respectively. Closed and open squares indicate the calibration curve and its cv values for the dual-label assay.

calibration curve by comparing the target-to-IS ratio in the sample to the target-to-IS ratio in the calibration curve. The analysis of one sample needs only one tube, and the number of target copies can be calculated using the external calibration curve. However, in addition to the development of the IS, this method needs preparation of the calibrator material. At the same time, this method allows the analysis of multiple samples in the same experiment.

We described here a TRF-based dual-label hybridization assay combined with the QRT-PCR assay for the quantification of PSA mRNA. Our QRT-PCR assay was based on the use of an exogenous IS mRNA and an external calibration curve generated with PSA mRNA and IS mRNA (covering the range of $50\text{--}10^6$ PSA mRNA copies). The quantification of PSA mRNA copies in the sample was carried out by calculating the fluorescence ratio of PSA-to-IS in the sample and by comparing the ratio to that in the calibration curve. Each assay contained the external calibration curve and one tube for the analysis of one sample. The PSA-like IS mRNA contained only a 2-bp difference with respect to the PSA mRNA. The IS mRNA was added to the sample at the beginning of RNA extraction; thus, the IS controlled the variations from the RNA extraction to the hybridization assay and could be used for the quantification of PSA mRNA copies in the sample. We have shown earlier that PSA mRNA and IS mRNA are amplified with the same efficiency and that the two amplification products are selectively detected by the hybridization assay (44). The present dual-label hybridization assay was developed to detect the amplified PSA and IS products simultaneously from a single microtiter well and to improve the reproducibility of the PSA mRNA quantification. The hybridization assay utilized PSA- and IS-specific detection probes labeled with different lanthanide chelates, which had different emission wavelengths and sharp emission peaks, allowing separation of the signals and the simultaneous detection of the PSA and IS amplification products. The analyses of a large number of samples using dual-label TRF and microplate-based method was easier to perform,

more rapid, and more sensitive than using the conventional analyses based on gel electrophoresis, Southern blot, and membrane hybridization. The numerical results produced by the TRF method were useful for the exact quantification of PSA mRNA. The QRT-PCR assay with the dual-label detection improved the within-assay precision over the calibration range ($50\text{--}10^6$ PSA mRNA copies, 5×10^3 IS mRNA for the dual-label and 10^3 IS mRNA for the single-label assay in each) from 21.7% to 7.5% when compared to the single-label assay. Correspondingly, the between-assay precision was slightly improved from 36.0% to 30.3% using the dual-label assay. The dual-label detection of the amplification products was more reproducible and obviously a lot simpler to carry out than the single-label assay; therefore, the dual-label assay was evaluated in the following experiments. The within- and between-assay CVs for the dual-label assay including all the steps from the RNA extraction to the hybridization were studied using LNCaP cells. One LNCaP cell was found to express 980 ± 170 (mean $\pm$ SD) copies of PSA mRNA with the within-assay CV% of 17.7, and 890 ± 220 (mean $\pm$ SD) copies of PSA mRNA with the between-assay CV% of 25.0. The single- and dual-label assays had the same detection limit, linear assay range and ability to detect one LNCaP cell in 2.5×10^6 non-PSA-producing cells. The number of PSA mRNA copies in LNCaP cells is in accordance with our previous results when PSA mRNA copies were determined in four replicas within one run. However, the present study reports more extensive determination of PSA mRNA in LNCaP cells. To our knowledge, we are the first to report the exact number of PSA mRNA copies in LNCaP cells. Additionally, the developed assay offers a suitable tool for further studies to study cell lines expressing PSA mRNA.

Lately, novel label technologies have contributed to the development of new methods that are applicable for the detection of accumulating PCR product directly from a closed tube (11,22,24,25,37). The use of such a real-time detection of PCR product from a closed tube decreases the risk of carry-over contamination because the amplification and the detection steps take place in

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the same tube. Therefore, there is no need to open the tubes after PCR. In addition, this approach simplifies the assays by eliminating the post-PCR analysis and allows the analysis of a large number of samples simultaneously. These methods can be used in both quantitative PCR and QRT-PCR assays (9,10,42). A commonly used approach for the quantification of the target is to amplify several samples and an external calibration curve generated with target-like DNA or RNA in the same run (42). The real-time detection of amplification products is performed at the exponential phase of PCR when the accumulating PCR product gives a signal above the threshold cycle. This method gives very reproducible results, as shown by

Wingo et al. (Table 2) (42). However, in RT-PCR assays, it is also important to control the pre-PCR steps, which are usually carried out by amplifying the target transcript and an endogenous housekeeping gene transcript in the same PCR. The number of target mRNA copies can then be normalized with the mRNA expression of the housekeeping gene. This kind of relative quantification is possible to carry out because the signals are measured at the exponential phase of PCR while the amplification efficiencies are at their highest and adjustable by optimization to be the same for both the target and the housekeeping gene transcript. Nevertheless, even after careful optimization of the PCR step, it can be difficult to use abundantly expressed housekeeping mRNA for the quantification and normalization of the number of infrequently expressed target mRNA. This problem was overcome when target-like IS mRNA was used in addition to an external calibration curve. The new technologies allow the real-time detection of PCR products from a closed tube, which will be the method of choice for quantitative PCR and RT-PCR analysis in routine use and diagnostic purposes.

In conclusion, the assay described here possesses a wide linear detection range of five orders of magnitude, which is similar to the detection range of the assays reported by Rong et al. (28) and Verhaegen et al. (38,39) (Table 2). Furthermore, the simultaneous detection of PSA and IS amplification products from one microtiter well gives reasonably good within- and between-assay reproducibility comparable to other end-point RT-PCR assays. In our assay, the PSA-like IS mRNA allows variations to be controlled from the beginning of RNA extraction to the detection of amplification products, which is different from other assays utilizing IS RNA or IS DNA for the control of variations from RT-PCR or from PCR steps to the detection of amplification products. The present dual analyte assay has been shown to be applicable for accurate detection of PSA mRNA copies in LNCaP cell samples and, therefore, can be used in further studies to quantify PSA mRNA in cultured PSA-producing cells or in circulating prostate cancer cells. More broadly, a similar

approach should prove useful for monitoring other types of metastasizing cancer cells such as those associated with breast cancer.

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Figure 5. Within- and between-assay reproducibility of number of PSA mRNA copies in LNCaP cells. Panel A represents the within-assay reproducibility of the mean number of PSA mRNA copies (closed triangles), and the error bars show the SD of nine replicates. Panel B represents the between-assay reproducibility of the mean of PSA mRNA copies (closed triangles), and the error bars show the SD of three independent analyses. Closed diamonds in both panels indicate the mean number of PSA mRNA copies per LNCaP cell.

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