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Novel homogenous time-resolved fluorometric RT-PCR assays for quantification of PSA and hK2 mRNAs in blood

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Abstract

Objectives: The purpose of this study was to design, validate, and optimize internally standardized real-time quantitative RT-PCR assays and to identify and avoid problems with assay reliability and examine the impact of an exogenous internal standard.

Design and methods: The model system consisted of internally standardized quantitative real-time RT-PCR assays specific for PSA and hK2 mRNA based on time-resolved fluorometric detection of lanthanide chelates.

Results: Reproducibility was best when large copy numbers (>5000 per milliliter blood) were analyzed. Addition of an exogenous target-mimicking internal standard had no significant effect on the reproducibility of the method, but increased the calculated copy numbers by an average of 2-fold.

Conclusions: We developed an internally standardized, specific and reproducible real-time RT-PCR analysis method for PSA and hK2 mRNA in circulating cells in the bloodstream. Both PSA and hK2 assays are sufficiently sensitive to detect two LNCaP cells per milliliter whole blood.

Keywords: Reverse transcriptase polymerase chain reaction; Tissue kallikreins; Reproducibility of results; Neoplasm circulating cells; Sensitivity and specificity

Introduction

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) may be useful for the detection and quantification of circulating cancer cells. However, the lack of robustness and lack of a universal standardization method of the RT-PCR technology are serious impediments to its use for quantification of circulating tumor related cells in routine clinical cancer diagnostics. There is need for better understanding of overall variation of results and the effect of standardization.

The RT-PCR method was first utilized for the detection of circulating tumor cells in the early 1990s [1,2], with great expectations regarding its potential for staging and prognosis in cancer diagnostics. Despite numerous studies in various cancers, the clinical role of RT-PCR-based detection of circulating cells still remains unclear, and reported results are often contradictory. Presently, the ability of RT-PCR to find circulating cancer cells in blood has been demonstrated, particularly in leukemia diagnostics [3].

Cells expressing mRNAs of prostate-specific antigen (PSA) and human kallikrein 2 (hK2) have frequently been found in the blood of patients with various clinical stages of prostate cancer, but very rarely in men with benign prostate disease, according to studies performed by several different research groups [4,5]. However, certain myeloid cells also occasionally contain trace amounts of PSA mRNA [6]. Although PSA in particular has been studied widely, no consensus has been reached so far regarding the utility of PSA mRNA measurements in blood. The lack of agreement may result from different methodologies of blood sampling, sample handling,

Abbreviations: hK2, human kallikrein 2; PSA, prostate-specific antigen; qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction; C_t, threshold cycles.

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template preparation, DNase treatment, cDNA synthesis [7], standardization and normalization methods [8], priming strategies, detection and quantification technologies, reaction chemistries, interpretation of threshold cycles (C_ts) [9], and operator effects [10].

The conditions of PCR chemistry have a crucial role in producing reliable results. For example, false positives can easily be generated if primer–dimers are created in substantial amounts. Furthermore, false negative results may be obtained if the amplification efficiency of the PCR primers is poor. Moreover, multiple transcripts for PSA and hK2 have been demonstrated [11,12]. The multiple transcripts may be detected in a non-uniform manner by different primer sets, and measuring different transcript variants may lead to deviating results. For these reasons, priming strategy is crucial. Furthermore, the variation in standardization methods among different studies certainly contributes to the inconsistency of results in regard to the diagnostic value of various mRNA assays in the detection and monitoring of cancer.

An essential requirement of an RT-PCR assay for analyzing circulating cells is analytical specificity, i.e. false positives and false negatives must be minimal. In addition, analytical reliability – accuracy and reproducibility – needs to be clearly defined to allow the diagnostic and prognostic implications of variable numbers of circulating cells to be evaluated. There is a clear need for assays validated in terms of ease of operation, robustness, reliability, and degree of true quantification. In theory, the addition of a recombinant "target-mimicking" RNA standard as an exogenous internal control, together with an external RNA standard curve, allows absolute quantification by reverse transcription PCR [13–16]. However, only a few papers have described quantitative real-time RT-PCR assays that incorporate exogenous internal standardization [17,18].

We have previously described an end-point RT-PCR hybridization assay for PSA and hK2 mRNA [13,19] and a real-time RT-PCR assay for PSA mRNA [20]. Both assays use time-resolved fluorometry, in which the fluorescence measurement occurs after the excitation and a delay time, allowing the background fluorescence to fade out before the signal measurement and thus improving the sensitivity of the measurements [21]. The end-point versus real-time assays have been compared [14]. Most recently, we developed an internally standardized dual-temperature homogenous quantitative real-time RT-PCR hybridization assay to detect PSA mRNA using separate lanthanide chelate labeled probe and quencher probe [22].

In this paper, we report an internally standardized real-time RT-PCR assay for hK2 mRNA. Particular attention was given to the selection and optimization of the enzymes, primers, reporters, and other essential reagents to provide a protocol of high overall robustness. Furthermore, we describe the reproducibility of the PSA assay and our novel hK2 assay and the effect of the exogenous internal standard on RT-PCR results. A small-scale reproducibility study was performed to define the limits of quantification to be expected when applied to future patient cohorts.

Methods

Blood samples and RNA extraction

Blood samples were collected from a healthy female donor in PAXgene blood RNA tubes (Preanalytix, Switzerland). The contents of four sample tubes, each containing 2.5 mL blood, were pooled and spiked with 0, 2, 20, 200, and 2000 LNCaP cells/mL blood. LNCaP is a cell line derived from prostate cancer metastatic to lymph node; the amounts were chosen to mimic the amounts of prostate cells likely to be found in patients' blood samples. After spiking, each pool was divided into four aliquots of equal volume and processed according to the manufacturer's instructions. RNA was extracted using the PAXgene Blood RNA kit (Preanalytix) according to instructions from Preanalytix. The optional DNase digestion procedure was performed, and mmPSA, the internal standard RNA that mimics PSA mRNA [14], was added after cell lysis (protocol step 5). The quantity and quality of each RNA sample were verified by both spectrophotometry and gel electrophoresis.

cDNA synthesis

The RNAs were reverse transcribed immediately after RNA extraction with High-Capacity cDNA Archive Kit (Applied Biosystems, USA), using random primers included in the kit, according to the manufacturer's instructions. The cDNA products were stored at −20°C until the PCR analysis was performed. 2.5 μL cDNA, representing 39 μL blood, was used as template in 10 μL amplification reactions.

PCR optimization and quantification

The PCR assay involves hybridization of lanthanide-labeled reporter probe to the target amplicon, digestion of probe hybridized to the template by the exonuclease activity of the polymerase at 62–64°C, and quenching of the excess intact lanthanide probe with a separate quencher probe at 35°C for 15 s prior to time-resolved fluorescence measurement (Fig. 1A). This PCR chemistry has previously been described in detail [22].

PCR reaction conditions were optimized for amplification of reverse-transcribed mRNA of either PSA or hK2. Concentrations of primers, dNTPs, polymerase, probe, and magnesium chloride were optimized, with one or two conditions varied at a time. Primers were tested at 0.4, 0.2, 0.15, 0.1, and 0.075 μmol/L concentrations. The reporter probe concentration was optimized with 32, 16, 12, and 8 nmol/L. Two hot-start polymerases, Amplitaq Gold (Applied Biosystems) and HotMaster DNA polymerase (Eppendorf), were tested in identical reactions at 0.050, 0.025, 0.019, 0.016, and 0.013 U/μL. The dNTPs were tested at 0.4, 0.2, 0.1, 0.075, and 0.05 mmol/L. $MgCl_2$ concentrations of 1.5, 2.5, 3.5, and 5 mmol/L were tested in reactions with Amplitaq Gold. After these tests, the best alternatives were combined, and optimal concentrations for all components were verified.