

Multianalyte quantitative competitive PCR on optically encoded microspheres for an eight-gene panel related to prostate cancer

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Abstract Nucleic acid-based tests have a profound impact in every medical discipline. Because multigene tests offer higher diagnostic accuracy and lower overall cost than single assays, they are especially useful for diseases, like prostate cancer, that present variability at the molecular level and diversity of available therapeutic interventions. We have developed a quantitative competitive PCR for an eight-gene panel, related to prostate cancer, that includes five genes of the human tissue kallikrein family (*KLKs*), prostate-specific membrane antigen (*PSMA*), prostate cancer antigen 3 (*PCA3*), and *HPRT1* as a reference gene. Using PCR as a synthetic tool, a competitor was prepared for each target sequence containing the same primer binding sites as the target but differing in a short segment to enable discrimination by hybridization. The assay involves multiplex amplification of targets and competitors followed by a multiplex hybridization assay for the 16 amplification products. The assay was performed on optically

encoded microspheres with oligonucleotide probes attached to their surface. The microspheres were analyzed rapidly (1 min) by flow cytometry. The signal ratio of the target and cognate competitor is a function of the target copy number in the sample prior to amplification. The multiplexing potential of the proposed method is much higher than real-time PCR and other end-point methods since there are 100 sets of commercially available microspheres.

Keywords Multianalyte · Quantitative competitive PCR · Fluorescent microspheres · Flow cytometry

Introduction

Nucleic acid-based tests have already widespread applications and a significant impact in every specialty of medicine. DNA/RNA markers provide a unique approach to diagnosis and prognosis of disease, prediction of the response to treatment, and testing for susceptibility to a disease [1]. The most common nucleic acid markers include mutations, single nucleotide polymorphisms, gene amplifications, or differentially expressed RNA sequences (up- or down-regulated). The methodology of nucleic acid analysis comprises two main directions, the discovery tools and the screening tools. DNA sequencing and high density planar microarrays enable the interrogation of hundreds of thousands of target sequences (high sequence-throughput) in a single or a few samples at a time and are suitable for large-scale association studies to discover new biomarkers for a particular disease. The outcome of this discovery phase is the selection of a limited number (a panel) of biomarkers that are tested per patient on a routine basis in the clinical laboratory. Consequently, screening tools that offer high sample-throughput along with a moderate multiplexing (multianalyte) ability are more suitable for

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the routine diagnostic practice. Compared to single assays, multiplex nucleic acid assays offer higher diagnostic specificity and sensitivity, lower overall cost, and timely diagnosis and therapeutic intervention. In general, diseases (such as prostate cancer) that present variability at the molecular level along with a diversity of available therapeutic interventions call for the development of nucleic acid-panel tests [2].

Exponential amplification of target sequences, by the polymerase chain reaction or an isothermal reaction, is an essential step to achieve high detectability for molecular diagnosis. In quantitative real-time PCR, multiplexing can be accomplished by spectral discrimination of the reporter fluorophores [3]. However, the number of spectrally distinguishable fluorophores is small because of the broad emission peaks of molecular fluorescence and the relatively short Stokes shifts, thereby limiting the number of quantifiable target sequences. Spatial discrimination of the assays provides another approach of multiplexing [4, 5].

In the present work, we have developed a multiplex quantitative competitive PCR with both a much higher multiplexing potential than real-time PCR and a high sample-throughput. The determination of amplification products is performed by a multianalyte hybridization assay, in a single tube, on a suspension of optically encoded microspheres with specific oligonucleotide probes anchored to their surface. There are 100 sets of commercially available fluorescence-encoded polystyrene microspheres enabling the parallel determination of up to 100 nucleic acid sequences. A single fluorophore, phycoerythrin, serves as a reporter for hybridization. Multiplexing is achieved by spatial resolution. Contrary to positional encoding of planar microarrays, the suspension of microspheres offers fluorescence encoding and solution kinetics and the microspheres are analyzed rapidly (in 1 min) by flow cytometry.

To this end, a quantitative competitive PCR was developed for an eight-gene panel, related to prostate cancer, that comprises five genes of the human kallikrein family, prostate-specific membrane antigen (PSMA), prostate cancer gene 3 (PCA3), and hypoxanthine phosphoribosyltransferase 1 (HPRT1) as a reference gene. Human tissue kallikreins or kallikrein-related peptidases (KLKs) comprise a subgroup of 15 homologous, secreted serine proteases, encoded by a clustered multigene family located on the genomic region 19q13.3-4 [6]. The deregulated expression of *KLK* genes is often associated with the progression of various types of cancer and patients' clinicopathological characteristics and prognosis [7, 8]. Most of them were found to be involved in prostate cancer prognosis [9–12].

The exploitation of the power of exponential amplification for quantification of target sequences requires compensation for sample-to-sample variation of the amplification efficiency because even small variations lead to profound changes in the amount of amplification product. A 5% drop

in the efficiency causes an almost 50% decrease of the amplification product (for 25 cycles). To compensate for the variation of the amplification efficiency, we prepared eight DNA competitors (internal standards) using PCR as a synthetic tool. The DNA competitors share the same primer binding sites and same sequence as their corresponding target genes but differ in a short (24-nucleotide) segment that allows discrimination by hybridization. Because of the resemblance between targets and competitors, any variation of the efficiency affects both sequences equally and the ratio of the amplification products is unaffected. In a typical assay, the eight target sequences are coamplified by multiplex PCR with a mixture of the eight competitors. The 16 amplification products are determined by hybridization using 16 sets of microspheres and analyzed by flow cytometry. The ratio of the fluorescence values for target and competitor is a function of the number of copies of the target sequence in the sample prior to amplification. The method offers remarkable versatility because the gene panel can be easily extended with new sequences by simply adding more sets of microspheres, thus increasing the multiplexity.

Materials and methods

Apparatus and reagents

PCR was performed in the TP 600 Gradient thermal cycler obtained from Takara (Otsu, Shiga, Japan). An ultrasonic cleaner (Branson Ultrasonics, Danbury, CT) was used for keeping the microspheres in suspension. The Luminex 100 IS flow cytometer (Luminex, Austin, TX) was used for fluorescence measurements of individual microspheres. Multiplex PCR was carried out by using the FastStart PCR master mix from Roche (Mannheim, Germany). *Taq* DNA polymerase was from New England Biolabs (Beverly, MA). Deoxynucleoside triphosphates (dNTPs) and DNA markers (Φ X174 DNA-HaeIII Digest) were from HT Biotechnology (Cambridge, UK). NucleoSpin Extract II for PCR clean-up/gel extraction was obtained by Macherey-Nagel (Düren, Germany). ExoSAP-IT was purchased by USB (Cleveland, OH). Different groups (maximum 100) of fluorescent polystyrene microspheres (5.6 μ m in diameter) that contained carboxyl groups on their surface and two fluorescent dyes inertly in different ratios were purchased from Luminex. 1-Ethyl-3,3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was from Pierce Chemicals (Rockford, IL), while tetramethylammonium chloride (TMAC) was from Fluka Chemie (Buchs, Switzerland). 2-(N-morpholino)ethanesulfonic acid (MES) and ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) were from Sigma (St. Louis, MO). Streptavidin-R-phycoerythrin conjugate (SA-PE) was from Molecular Probes (Eugene, OR). Oligonucleotides

used as primers and probes (Table S1, see Electronic Supplementary Material (ESM)) were synthesized by VBC Biotech (Wien, Austria). All reverse primers were biotinylated at the 5'-end, while all oligonucleotide probes used for the detection carried a primary amino group at 5'-end.

Preparation of oligonucleotide-functionalized microspheres

The 5'-NH₂-oligonucleotide specific probes were conjugated to carboxylated microspheres using carbodiimide chemistry. More specifically, a volume containing 2.5×10^5 microspheres was sonicated for 10 min and then centrifuged for 1 min at $10,000\times g$ followed by washing of the microspheres once with 50 μL of 0.1 M MES buffer, pH 4.5, and resuspended in the same buffer. An amount of 100 pmol of oligonucleotide probe was then added and the mixture was sonicated for 5 min. Afterwards, 2.5 μL of 10 g/L EDC solution freshly prepared with water was added and the mixture was left for 30 min at room temperature in the dark. At the end of this period, a 2.5- μL aliquot of 10 g/L EDC was added again followed by another 30-min incubation and centrifugation at $10,000\times g$ for 1 min. The pellet was washed once with 100 μL of 0.2 mL/L Tween-20 and once with 100 μL of 1 g/L sodium dodecyl sulfate (SDS). Finally, the microspheres were resuspended in 100 μL of $1\times$ TE buffer (10 mM Tris-HCl and 1 mM EDTa, pH 8.0).

RNA extraction and cDNA synthesis

The human prostate cancer cell lines LNCAP, PC3, and DU145 were from the American Type Culture Collection (ATCC) (Manassas, VA). The cell lines were maintained according to the ATCC instructions, in an atmosphere of 95% air/5% CO₂, with 100% humidity, at 37 °C. The prostate tissue specimen was obtained from a prostate cancer (PCa) patient who underwent radical retropubic prostatectomy. The tissue sample of approximately 100 mg was sectioned from the peripheral zone of prostate gland based on the preoperative features of the biopsy and on macroscopic findings. The sample was thereafter divided into two mirror-image specimens, one of which was tested by a pathologist, while the other one was immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Our studies were approved by the ethics committee of the National and Kapodistrian University of Athens Hospital named "Laiko General Hospital."

Total RNA was extracted from prostate tissue and cell lines using the TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) protocol following the manufacturer's instructions. The quality and integrity of all total RNA extracts were checked using agarose gel electrophoresis. Agarose gels (1.5% w/v) were stained with ethidium bromide and visualized

under ultraviolet light. 28S and 18S RNA bands without any smear were observed for all total RNA extracts. Validation experiments led to the optimization of the cDNA synthesis reaction conditions; consequently, the efficiency of cDNA synthesis reactions was optimal with regard to all samples. The human prostate cell lines LNCAP, PC3, and DU145 were used for this work. After spectrophotometric and gel electrophoresis (1% w/v) evaluation of isolated total RNA concentration, purity, and integrity, 2 μg was reverse transcribed using 1 μg oligo(dT)₁₈ primer, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM of each dNTP, 200 U M-MuLV reverse transcriptase RNase H⁻, 40 U RNase Inhibitor (Finnzymes, Espoo, Finland), and diethylpyrocarbonate-treated (DEPC) water to a total volume of 20 μL . The reaction mixture was incubated at 37 °C for 1 h.

Construction of competitors (internal standards)

Eight DNA competitors (internal standards) were synthesized by replacing a 24-bp segment of each target DNA sequence with a different sequence of the same size. PCR was employed here as a synthetic tool. The procedure involved two separate PCRs (PCR-A and PCR-B), using the target DNA as a template, in order to create two short products, A and B, that contained the new inserted segment of 24 bp. The PCR mixture (total volume, 50 μL) consisted of 10 mM (NH₄)₂SO₄, 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 0.1% Triton X-100, 2 mM MgSO₄, 200 μM of each dNTP, 10 μM of each primer (upstream and downstream), 1 μL of DNA template (PCR product), and 0.5 units of *Taq* DNA polymerase. The cycling parameters were as follows: 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. After the completion of 30 cycles, samples were incubated at 72 °C for 10 min and cooled down to 4 °C. The short products A and B were then isolated by extraction of the DNA fragment from the agarose gel and purification with the commercial kit NucleoSpin Extract II, according to the manufacturer's instructions. A quantity of 5 μL of each purified short product was then separated by agarose gel electrophoresis (2%) and quantified by scanning densitometric analysis using ethidium bromide. Equimolar amounts of products A and B were mixed in a pool similar to the PCR solution described above without primers or DNA template and subjected to an amplification reaction. The cycling conditions were as follows: denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min for 40 cycles to obtain the DNA internal standard (IS). Purification of PCR products from primer dimers and excess of oligonucleotides prior to re-amplification of internal standards was performed by adding 2 μL of ExoSAP-IT enzyme mixture to 5 μL of PCR product. The resulting mixture was incubated at 37 °C for 15 min and afterwards the temperature was set at 80 °C for another 15 min to inactivate the enzymes. Subsequently, the purified competitor (5 μL) was re-amplified

for 35 cycles, using oligo (a) and biotinylated oligo (d) as upstream and downstream primers, respectively. The DNA competitors were finally quantified by 2% agarose gel electrophoresis and ethidium bromide staining.

Multianalyte quantitative competitive PCR

The multianalyte quantitative competitive PCR (MQC-PCR) involved simultaneous amplification of 16 DNA sequences, i.e., 8 target DNA sequences and 8 DNA competitors (namely, KLK3, KLK3_C, KLK4, KLK4_C, KLK5, KLK5_C, KLK11, KLK11_C, KLK15, KLK15_C, PSMA, PSMA_C, PCA3, PCA3_C, HPRT1, and HPRT1_C). The reaction volume was 50 μ L containing 1 \times FastStart PCR master mix, 0.05 μ M for KLK3 primer, 0.1 μ M each for KLK4, KLK11, KLK15, and HPRT1 primers, and 0.2 μ M each for KLK5, PCA3, and PSMA primers (eight pairs of primers for the eight targets and their corresponding competitors) and 100 ng of cDNA. Moreover, the PCR mixture contained a constant amount of 5×10^4 copies of each of the eight competitors. The thermal cycling parameters were as follows: initial denaturation at 95 $^{\circ}$ C for 4 min, followed by 35 cycles at 95 $^{\circ}$ C for 30 s, 50 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 45 s, and a final extension step at 72 $^{\circ}$ C for 7 min.

Multianalyte hybridization assay on fluorescent microspheres

The amplification products of the previous step were detected by a multianalyte hybridization assay performed on oligonucleotide-functionalized fluorescent microspheres. A 5- μ L aliquot of the PCR mixture was mixed with 5 μ L of PCR buffer and 15 μ L of a suspension containing 16 sets of fluorescent microspheres coupled to specific oligonucleotide probes (2.5×10^3 microspheres of each set). The mixture was incubated for 5 min at 98 $^{\circ}$ C and 15 min at 52 $^{\circ}$ C. Subsequently, 30 μ L of hybridization buffer (50 mM Tris-HCl pH 8.0, 4 mM EDTA pH 8.0, 1 g/L SDS, and 3 M TMAC) was added and the mixture was incubated for 90 min at 45 $^{\circ}$ C with occasionally stirring. The microspheres were then centrifuged at 13,400 \times g for 2 min and washed once with 50 μ L of hybridization buffer. The microspheres were resuspended in 50 μ L hybridization buffer and sonicated for a few seconds to keep the microspheres in suspension. Then, 12 μ L of SA-PE (10 mg/L) was added and the mixture was incubated for 20 min at ambient temperature. Subsequently, the microspheres were washed once with 50 μ L hybridization buffer, resuspended in 50 μ L of the aforementioned buffer, and the emitted fluorescence was finally measured using the Luminex IS 100 flow cytometer.

Results and discussion

A MQC-PCR based on optically encoded microspheres for the detection of amplified products has been developed. The overall method includes (a) RNA isolation and cDNA synthesis, (b) multiplex quantitative competitive PCR, and (c) detection of amplified products by a multianalyte hybridization assay on fluorescent microspheres. The method has been applied for the simultaneous quantification of eight target genes, namely, KLK3, KLK4, KLK5, KLK11, KLK15, PSMA, PCA3, and HPRT1 (accession numbers are given in Table S2, see ESM) in parallel with their corresponding DNA competitors. HPRT1 was used as a reference gene. The 16 amplified fragments (KLK3, KLK3_C, KLK4, KLK4_C, KLK5, KLK5_C, KLK11, KLK11_C, KLK15, KLK15_C, PSMA, PSMA_C, PCA3, PCA3_C, HPRT1, and HPRT1_C) were simultaneously detected by a suspension of 16 sets of fluorescent microspheres. The principle of the hybridization assay is schematically presented in Fig. 1. Oligonucleotide probes specific to the eight targets and the eight competitors that carry a -NH₂ group at the 5'-end were covalently coupled to 16 different carboxylated microsphere sets using carbodiimide chemistry. The PCR products from the 16 DNA fragments were biotinylated at one end during PCR by using a biotinylated primer. The products were hybridized, after heat denaturation, on a suspension of the oligonucleotide-functionalized microspheres. The detection of the hybrids was accomplished by a streptavidin-phycoerythrin conjugate. The microspheres were finally analyzed rapidly one-by-one within a flow (Fig. 1). The matrix of each microsphere is stained with two spectrally distinguishable fluorescent dyes. Optical encoding is achieved by changing the molar ratio of the dyes. The different ratios of the two dyes result in 100 sets of distinguishable microspheres. During flow cytometric analysis, the fluorescent dyes are excited by a single wavelength (laser line at 635 nm) and their respective emissions are measured at 658 and 712 nm, thereby enabling classification of the microspheres. The oligonucleotide probes are attached to the surface of each microsphere. The maximum number of quantifiable sequences (via hybridization) is 100 (50 target sequences and 50 DNA competitors). The classification results for the 16 different microsphere sets that were coupled with oligonucleotide probes specific to the selected targets and respective competitors are presented in Fig. 2. The flow cytometer classifies the microspheres into different sets that are represented by the red circles within prespecified limits. The events measured outside the circles are due to broken beads or other particles that exist in the solution (e.g., dust) and are excluded from the analysis. A green laser beam at 532 nm was used at the same time for the excitation of phycoerythrin, the reporter fluorescent dye. The emitted fluorescence intensity at 578 nm was a linear function of the concentration of the interrogated PCR products

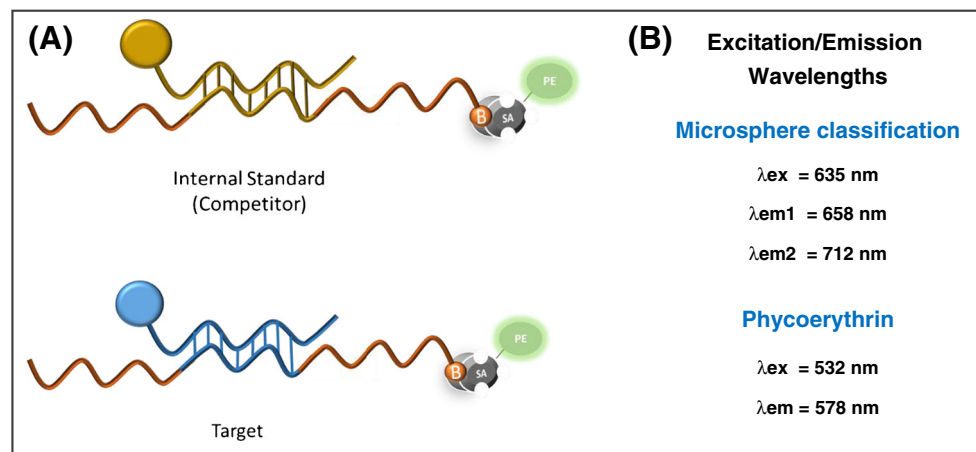


Fig. 1 (A) Principle of the multianalyte hybridization assay for target sequences and their corresponding competitors on the surface of optically encoded microspheres. The biotinylated amplification products are hybridized, after heat denaturation, to a suspension of 16 different sets of microspheres that are coupled with specific oligonucleotide probes.

Streptavidin-Phycoerythrin is used for the detection of the hybrids. B, biotin; SA, streptavidin; PE, phycoerythrin. (B) Summary of excitation and emission wavelengths of the fluorescent dyes of the microspheres and of the hybridization assay reporter phycoerythrin

hybridized on the surface of the microsphere. At least 100 microspheres of each group of microspheres are analyzed in a single run. The fluorescence measurements were performed within 1 min, while the entire multiplex hybridization assay was complete in less than 2 h (excluding the PCR amplification step).

The PCR products for KLK3, KLK4, KLK5, KLK11, KLK15, PSMA, PCA3, and HPRT1 have sizes of 108, 144, 135, 278, 225, 229, 299, 216, and 151 bp, respectively, which were confirmed by agarose gel (2%) electrophoresis and ethidium bromide staining. For MQC-PCR, eight DNA competitors were constructed, one for each target DNA. The synthetic competitors have the same size with their respective DNA targets but are different only in a 24-bp sequence. The

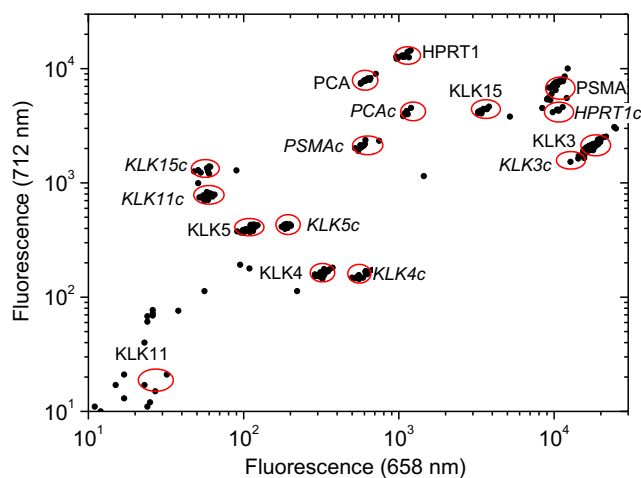
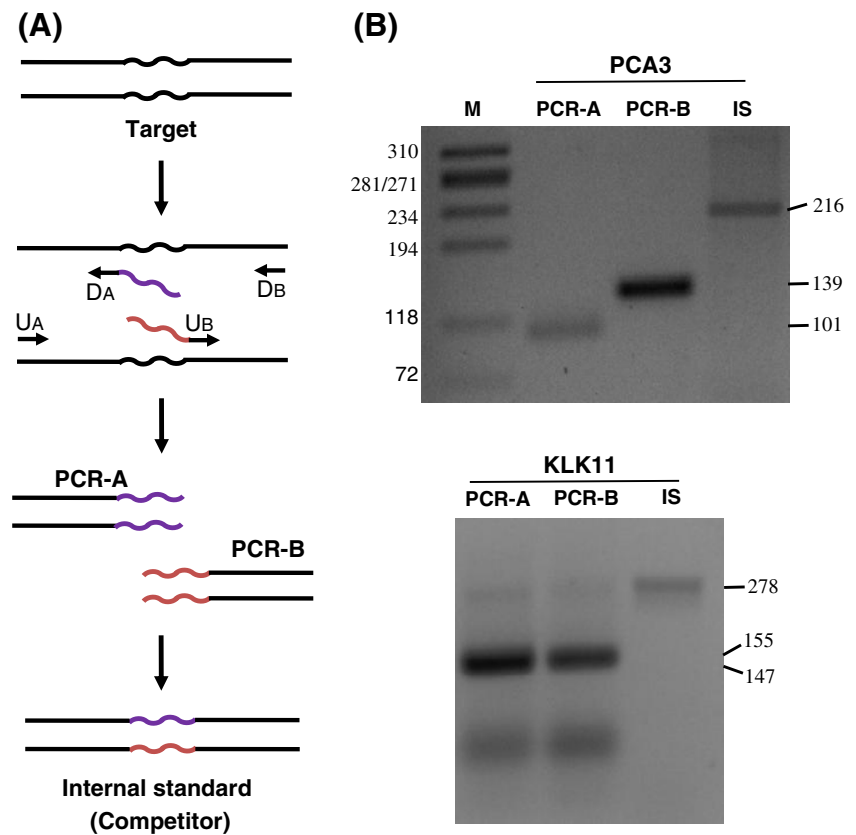


Fig. 2 Classification results for the 16 sets of microspheres used in the present method as a solid support for the capture probes. Following excitation at 635 nm, the emitted fluorescence, at 658 and 712 nm, of the two fluorescent dyes, entrapped in the microspheres are used for classification. The red circles delimit the distinct sets of the 16 microspheres

DNA targets and their cognate competitors are discriminated via hybridization assay with specific probes anchored on the surface of the microspheres. We estimate that in order to achieve both efficiency and specificity of hybridization assay we need oligonucleotide probes longer than 17 bp. Figure 3 presents an outline of the procedure of the synthesis of DNA competitors (internal standards). The first step involves two PCRs (PCR-A and PCR-B) that amplify the left and the right segment of the target DNA, respectively, excluding a 24-bp central sequence. The 5'-ends of the downstream primer of PCR-A and the upstream primer of PCR-B have been designed to contain new, complementary 24-nucleotide segments. As a result, the PCR-A and the PCR-B products contain double stranded (24-bp) extensions. The DNA internal standard is assembled by mixing equimolar amounts of gel purified PCR-A and PCR-B products, in the presence of DNA polymerase and dNTPs, and performing a thermal cycling process in which PCR-A and PCR-B strands serve as primers and templates to each other. To obtain larger amounts of competitor, the assembled DNA is purified and amplified by PCR using the outer primers. The entire process of construction of a DNA competitor can be completed within a day. The size of the DNA competitors was confirmed by 2% agarose gel electrophoresis and ethidium bromide staining. Also, in Fig. 3, typical electropherograms of the short products, PCR-A and PCR-B, as well as the assembled DNA competitor for the PCA3 and KLK11 genes are presented. The confirmation of the 24-bp distinct sequence of each DNA competitor was confirmed by using specific oligonucleotide probes in hybridization assay as described below.

The specificity of the 16 oligonucleotide probes (8 for the targets and 8 for the competitors) to their corresponding sequences was initially assessed by performing cross-hybridization studies. Separate solutions containing each

Fig. 3 **A** Schematic presentation of the competitor (internal standard) synthesis and relative positions of the primers. Primers U_A and D_A are used for the synthesis of short product PCR-A and primers U_B and D_B are used for short product PCR-B. The final amplification of target sequence and its corresponding competitor is accomplished by primers U_A and D_B . **B** Electropherograms of short products A and B and the competitors (internal standards, IS) for PCA3 and KLK11. For PCA3, the short products A and B have sizes 101 and 139 bp, respectively, while the competitor (IS) has a size of 216 bp. For KLK11, short product A, short product B, and synthesized IS are 147, 155, and 278 bp, respectively. M, DNA molecular markers



amplified DNA sequence (target or competitor) at a very high concentration (1000 pM) were hybridized with a suspension of all 16 sets of oligonucleotide-functionalized microspheres, containing 10^3 microspheres of each set. The fluorescence intensities obtained from the cross-hybridization assays are given in Fig. 4. The cross-hybridization for all 16 specific probes was below 14%. It should be noted that the GC content is not a critical parameter in our hybridization assays because the hybridization buffer contains TMAC and not NaCl for ionic strength adjustment. It is well established in the literature [13, 14] that in TMAC-containing buffers, the T_m of the oligonucleotides is independent of the base composition. This is due to the fact that TMAC binds selectively to A:T base pairs and stabilizes them.

The optimum number of microspheres in the hybridization assay was subsequently determined to obtain the maximum fluorescence intensity. For this study, a mixture containing eight amplified DNA targets (KLK3, KLK4, KLK5, KLK11, KLK15, PSMA, PCA3, and HPRT1) at a concentration of 1000 pM was hybridized with a suspension containing from 10^3 to 5×10^3 microspheres of each set. As shown in Fig. 5, the increased number of microspheres (above 2.5×10^3) resulted to a decrease of the fluorescence intensity for all eight DNA targets, probably due to the fact that the ratio of target molecules hybridized per microsphere decreases. A number of 2.5×10^3 microspheres from each set was finally selected for both a high

signal and easy handling of the microspheres (pipetting and centrifugation of the suspension of microspheres).

Moreover, calibration graphs of the multianalyte hybridization assay for all 16 DNA sequences were constructed. Mixtures containing all 16 biotinylated DNA fragments were prepared in different concentrations ranging from 10 to 1000 pM using PCR buffer as a diluent. Upon completion of the hybridization of each mixture with the suspension of 16 sets of microspheres, containing 2.5×10^3 microspheres of each set, the fluorescence intensity was plotted versus the concentration of each DNA sequence. The results are presented in Fig. 6. We observe that the multianalyte hybridization assays have analytical ranges from 10 to 1000 pM for all 16 sequences. As low as 10 pM of each DNA sequence was detected, corresponding to 500 attomoles, taking into consideration that the sample volume was 50 μ L.

Calibration graphs for the multiplex quantitative competitive PCR for determination of the number of copies of the eight targets (KLK3, KLK4, KLK5, KLK11, KLK15, PSMA, PCA3 and HPRT1) were constructed as follows. Mixtures of all eight targets containing from 5×10^2 to 5×10^6 copies of each along with a constant amount of 5×10^3 copies of each competitor were prepared and subjected to multiplex quantitative competitive PCR. The serial dilutions of the mixtures of the eight targets were prepared freshly in Tris-EGTA buffer (100 mM Tris, 10 mM EGTA, pH 8.0).

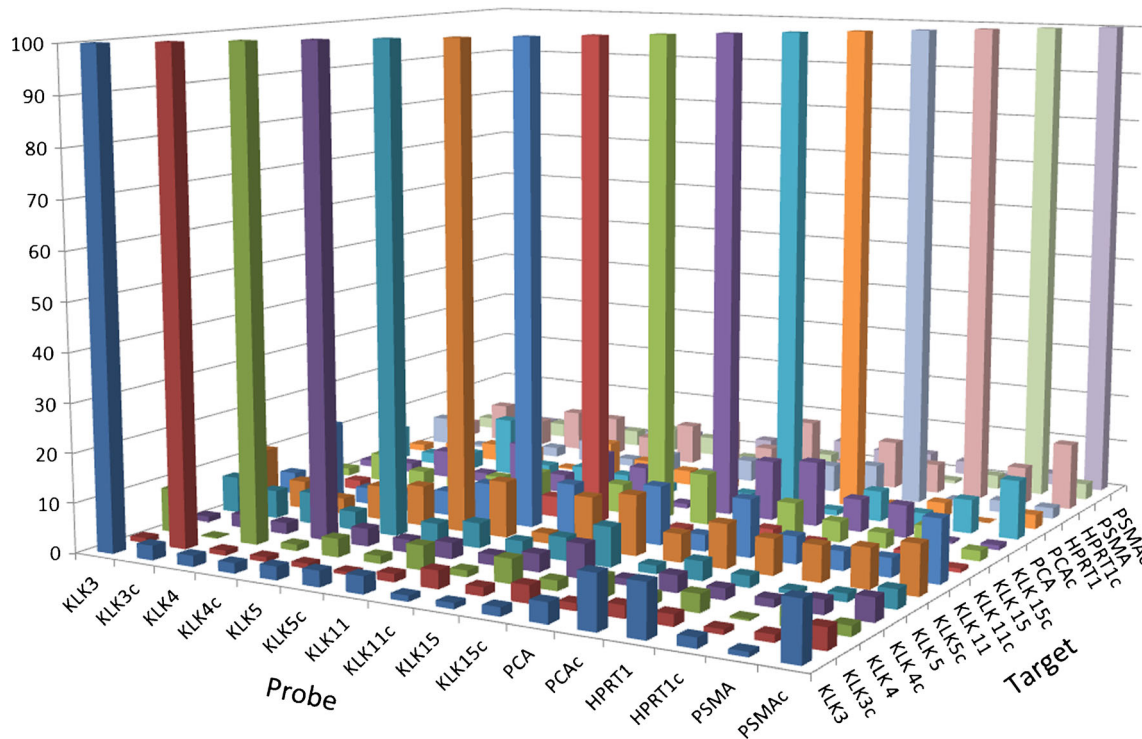


Fig. 4 Cross-reactivity study of the multianalyte (16plex) hybridization assay for targets and competitors. A very high concentration of 1000 pM of each biotinylated sequence was hybridized with a suspension of all 16 sets of oligonucleotide-functionalized fluorescent microspheres that

contained 10^3 microspheres of each group. The fluorescence intensities are expressed as percentages of the maximum fluorescence value. The cross-reactivity for all 16 specific probes was < 14%

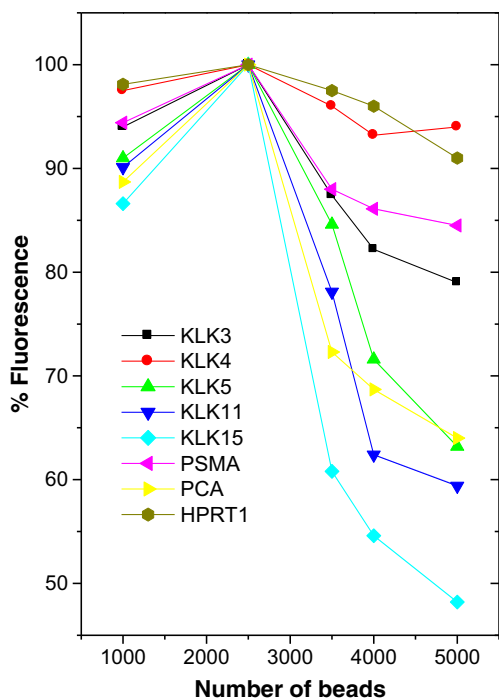
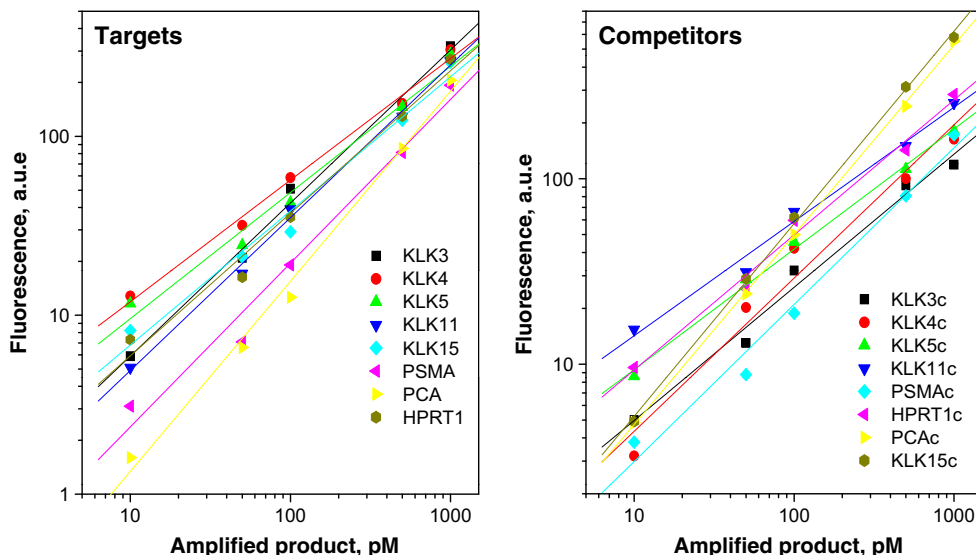


Fig. 5 Optimization study of the number of microspheres used in the multianalyte hybridization assay. A mixture of the eight DNA targets (1000 pM each) was hybridized with a mixture of 10^3 to 5×10^3 microspheres of each set. The % normalized fluorescence value is plotted versus the number of microspheres. The normalized fluorescence value of 100 equals to the maximum fluorescence intensity of each set

All competitors were added to the PCR mixture as a single pool. Following a 10-fold dilution, PCR products were then tested by the multianalyte hybridization assay. The ratio F/F_C of the fluorescence intensities of each target and the cognate competitor was plotted against the initial target copies present in the sample prior to MQC-PCR (Fig. 7). As low as 500 copies of each target sequence can be quantified by the proposed method. This corresponds to 100 copies of the target since only 10 μ L of the PCR product was used for hybridization and flow cytometry.

The overall detectability of any quantitative PCR method (including amplification and detection) depends on (a) the amount of accumulated amplification product for a certain number of target copies (i.e., the amplification factor) and (b) the detectability of the hybridization assay used for determination of amplified DNA. Besides the multiplexity, the proposed hybridization assay offers a high detectability of amplified DNA to levels as low as 10 pM due to laser-induced fluorescence detection on the microspheres. However, regardless of the detection approach (real-time or end-point), when the multiplexity of the PCR step increases the amplification factor decreases compared to the PCR of a single target for an equal number of cycles. Consequently, optimization of the primer sequences, the composition of the PCR mixture, the thermal cycling conditions, and the number of amplification cycles is required in order to achieve the desired overall detectability.

Fig. 6 Calibration graphs of the multianalyte hybridization assay for the eight targets and the eight competitors. The fluorescence intensity is plotted as a function of the concentration of amplified products. A mixture containing all 16 sequences at different concentrations (0–1000 pM) was hybridized with the mixture of 16 sets of microspheres



The reproducibility of the entire MQC-PCR method was assessed by analyzing samples containing 6×10^2 , 5×10^3 , and 12×10^4 DNA copies from all eight target sequences ($n = 5$) along with 5000 copies of each competitor. The values of the coefficient of variations (%CV) are presented in Table 1. All %CVs were below 20%.

Conclusions

It is well established that quantitative competitive PCR enables precise quantification of nucleic acid sequences

[15–19]. Several quantitative competitive PCR methods have been reported previously [20–23]. Dual and quadruple analyte quantitative competitive PCR have been developed by combining chemiluminescent reporters [20, 21]. Although chemiluminometric hybridization assays offer higher detectability compared to fluorometric ones, their multiplexing potential is limited because of the small number of chemiluminescent reporters that can be distinguished either by the emission wavelength or by the emission kinetics. Spatial resolution of the assays [22, 23] enhances greatly the potential for multianalyte quantification.

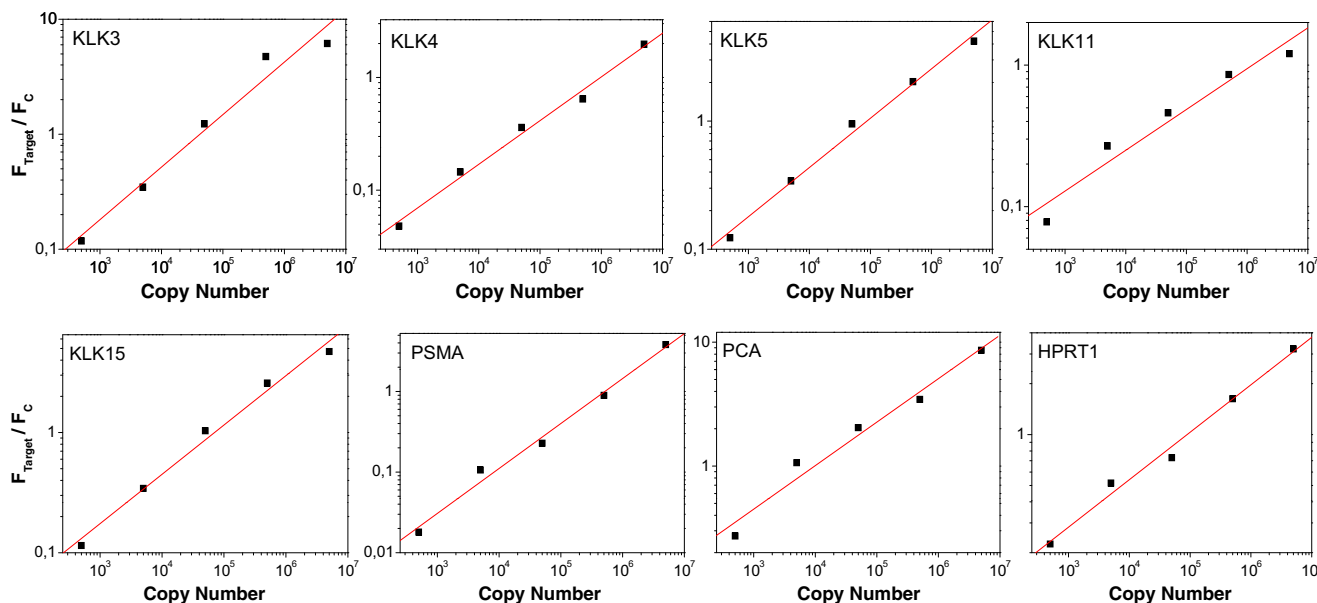


Fig. 7 Calibration graphs for the multiplex quantitative competitive PCR (MQC-PCR). The ratio of the fluorescence intensity of the target to the respective competitor (internal standard) (F_{Target}/F_c) is plotted against the initial copies of the target prior to amplification. Mixtures of the eight

DNA targets containing from 5×10^2 to 5×10^6 copies of each along with a constant amount of 5×10^3 copies of each competitor were subjected to MQC-PCR and multianalyte hybridization assay with 16 sets of oligonucleotide-functionalized microspheres

Table 1 Reproducibility (%CV, $n = 5$) of the entire MQC-PCR method. Various copy numbers of the eight targets along with a constant number (5000 copies) of each of the eight competitors were subjected to multiplex PCR and multiplex hybridization assay for all 16 DNA sequences

Copy number		Gene							
		<i>KLK3</i>	<i>KLK4</i>	<i>KLK5</i>	<i>KLK11</i>	<i>KLK15</i>	<i>PSMA</i>	<i>PCA</i>	<i>HPRT1</i>
600	Target	8.7	7.7	2.4	14.0	4.5	1.7	7.8	16.3
	Competitor	4.8	12.2	8.0	7.1	2.2	2.2	7.3	12.1
5000	Target	10.4	7.7	4.9	5.7	2.8	4.6	3.0	9.1
	Competitor	2.4	4.8	2.7	5.3	5.7	4.2	4.1	3.3
120,000	Target	7.1	17.2	4.7	11.2	2.1	7.3	12.8	10.7
	Competitor	11.8	32.1	4.8	18.3	6.3	3.2	6.2	13.4

In the present work, a novel multiplex quantitative competitive PCR for an eight-gene panel was developed. To compensate for the variation in the amplification efficiency between samples, we prepared and characterized eight competitor sequences that were added to the amplification mixture. The PCR products from targets and competitors were determined by a multianalyte (16plex) hybridization assay on spectrally discrete microspheres, in a single tube. The proposed method is suitable for all applications that require co-determination of a panel of specific genes. An excellent example is early diagnosis, prognosis, and monitoring of patient's response to treatment in prostate cancer, in which biomarkers consisting of prostate cancer-related genes are determined compared to a reference gene (i.e., a housekeeping gene such as *HPRT1*). The proposed assay is highly flexible because the biomarker panel can easily be expanded by adding more sets of microspheres. Considering that there are up to 100 different sets of optically encoded microspheres, a number of 50 target sequences with their respective 50 competitors can be simultaneously determined by the hybridization assay (upper limit of the multiplexing ability of the hybridization assay). To achieve such a high multiplexity for the quantitative competitive PCR step, the design of primers and the optimization of the cycling temperatures is a prerequisite. Consequently, the great advantage of the proposed method is the enhanced multiplexity of MQC-PCR.

DNA chips (high density DNA microarrays) enable the interrogation of hundreds of thousands of target sequences (high sequence-throughput) in a single or a few samples at a time and are suitable for large-scale association studies to discover new biomarkers for a particular disease. However, this is a very costly methodology with a low sample-throughput. In the routine clinical laboratory, a small number (a panel) of markers are tested per patient and a high sample-throughput is required along with a medium multiplexity. Quantitative real-time PCR ($\Delta\Delta$ approach) has very limited multiplexing ability (five to six analytes) due to the small number of spectrally distinguishable fluorophores. The proposed method offers both a high sample-throughput and a much higher multiplexing ability than real-time PCR because it combines spatial discrimination of up to 100 analytes and

solution kinetics for hybridization. Given the development of the multiplex hybridization assay, what limits the ultimate multiplexing ability is the multiplex PCR amplification step itself. For a successful multiplex exponential amplification, care must be taken with the primer design in order to avoid interactions that result in inhibition of amplification of certain target(s).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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