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Quantitative Polymerase Chain Reaction Based on a Dual-Analyte Chemiluminescence Hybridization Assay for Target DNA and Internal Standard

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We have developed a dual-analyte chemiluminescence hybridization assay for quantitative polymerase chain reaction (PCR). The method allows simultaneous determination of both amplified target DNA and internal standard (IS) in the same reaction vessel. The target DNA from the sample (233 bp) was coamplified with a constant amount of a recombinant DNA IS that had the same size and primer binding regions as the target DNA, differing only by a 24-bp sequence, centrally located. Biotinylated PCR products from target DNA and IS were captured on a single microtiter well coated with streptavidin. The amplified target DNA was hybridized with a digoxigenin-labeled specific probe, and the hybrids were determined by using antidigoxigenin antibody labeled with aequorin. The amplified DNA IS was hybridized, in the same well, with a fluorescein-labeled probe, and the hybrids were determined by using an antifluorescein antibody conjugated to alkaline phosphatase. Aequorin was measured by adding a Ca^{2+}-containing light-triggering solution. Alkaline phosphatase was measured by using a dioxetane chemiluminogenic substrate. The ratio of the luminescence values obtained from the target DNA and IS amplification products was linearly related to the number of target DNA molecules present in the sample prior to amplification. The linear range extended from 430 to 315 000 target DNA molecules. Average CVs ranged from 7 to 17%. The proposed system is expected to facilitate the automation and routine use of quantitative PCR.

The polymerase chain reaction (PCR) entails the selective and exponential amplification of nucleic acid sequences to levels that are several orders of magnitude higher than those in the starting material.[1] Conventional methods of nucleic acid analysis, such as Southern and Northern blots, are not sensitive enough to detect DNA or RNA in small biological samples or in samples containing low copy numbers of the sequences of interest. Because of its superior sensitivity, PCR has rapidly become one of the most important analytical techniques in all aspects of biological research and molecular diagnostics.

In recent years, several methods have been developed for analysis of PCR products. These include electrophoretic separation of the amplified DNA, high-performance liquid chromatography,[2] capillary electrophoresis,[3] and hybridization followed by radioactive or nonradioactive detection of the hybrids. Nonradioactive hybridization assays based on fluorescent, chemiluminescent, or enzyme labels have been developed. The replacement of conventional chromogenic substrates with alternatives that allow monitoring of enzyme activity by chemiluminescence or time-resolved fluorescence has further improved the sensitivity.[4−6]

Despite the advances described above, the exponential nature of PCR poses serious difficulties in its utilization for determining the starting quantity of target DNA. Quantification requires the establishment of a reproducible relationship between the analytical signal obtained from the amplification product and the number of target DNA molecules in the sample prior to amplification. The amount (P) of product accumulated after n cycles is given by the equation $P = T(1 + E)^n$, where T is the initial amount of target DNA and E is the average efficiency. As a consequence, small sample-to-sample variations in E lead to dramatic changes in the amount of product and large errors in estimating the initial quantity of the target (especially when n is large).

The variability of the efficiency can be circumvented by coamplifying, in the same reaction tube, the target with an internal standard and relating the ratio of the two amplification products to the initial amount of target in the sample.[7−10] The IS should have the same primer binding sites as the target DNA. Moreover, it must be possible to analyze the amplification products from target DNA and IS in the PCR mixture without interference from each other. In the majority of reports on quantitative PCR, the IS is designed to contain a deletion or insertion large enough to allow electrophoretic separation of the products.[7−10] The fragments may then be quantified by scanning densitometry. Alter-

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natively, the IS has the same size as the target but contains a new restriction site, allowing product digestion prior to electrophoresis. The use of HPLC may facilitate the separation and quantification of the two products.[11] Furthermore, the target DNA and the IS may be analyzed by hybridization to specific probes.[12−14] The latter methods offer significant advantages because they allow confirmation of the amplified sequences and provide much higher sensitivity.

Until now, hybridization-based quantitative PCR methodologies required that the hybridization assays for amplified target DNA and IS be performed separately by splitting each sample in different reaction vessels (e.g., microtiter wells). To facilitate automation of quantitative PCR, we have developed a dual-analyte chemiluminescence hybridization assay for simultaneous determination of both amplification products in the same sample. Aequorin and alkaline phosphatase (ALP) are used as reporter molecules. Hybrids of target DNA and IS with their specific probes are linked to aequorin and alkaline phosphatase through the digoxigenin (Dig)/antidigoxigenin and fluorescein/antifluorescein interaction, respectively. First, we proved that the dual-analyte approach is valid by using mixtures of target DNA and IS. Then, the sensitivity, linear range, and reproducibility of the hybridization assays were assessed. Finally, we developed a quantitative PCR methodology based on the proposed dual-analyte assay.

EXPERIMENTAL SECTION

Instrumentation. Luminescence measurements were carried out using a microplate luminometer, model MLX, with two injectors, from Dynex Technologies (Chantilly, VA). Polymerase chain reaction experiments were carried out in the 48-well Perkin-Elmer Cetus (Norwalk, CT) DNA thermal cycler. The microtiter plate washer model EAW II was from SLT-Lab Instruments (Salzburg, Austria). Hybridization assays were performed using the Amerlite shaker/incubator (Amersham, Oakville, ON, Canada).

Reagents. A covalent conjugate of aequorin with antidigoxigenin antibody (Fab fragments) was purchased from Sealite Sciences (Atlanta, GA). Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenylphosphate (CSPD) and the chemiluminescence enhancer Sapphire II were purchased from Perkin-Elmer (Mississauga, ON, Canada). Sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin) was from Pierce (Rockford, IL). Ultrapure 2′-deoxyribonucleoside 5′-triphosphates (dNTPs) and Sephadex G-25 purification columns (Nap-5) were from Pharmacia-LKB (Montreal, PQ, Canada). Fluorescein-5(6)-carboxamidocaproyl-[5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate] (fluorescein-dUTP), terminal deoxynucleotidyl transferase, digoxigenin-11−2′-deoxyuridine 5′-triphosphate (Dig-dUTP), alkaline phosphatase-labeled antifluorescein antibody (Fab fragments from sheep), and blocking reagent (Catalog No. 1096 176) were purchased from Boehringer Mannheim Biochemica (Laval, PQ, Canada). White, flat-bottom microtiter wells, Microlite 2, were from Dynex Technologies. Streptavidin was from Sigma (St. Louis, MO). BDH (Toronto, Canada) supplied all other general laboratory chemicals used in buffer preparation.

The oligonucleotides used in this work were synthesized by Biosynthesis Inc. (Lewisville, TX). The 20mer 5′-(NH_2)-CTCT-CGTGGCAGGGCAGTCT-3′ was used as an upstream primer. The primer was biotinylated by using NHS-LC-biotin according to the procedure described in ref 15. The 20mer 5′-GGTCGTGGCTG-GAGTCATCA-3′ was used as a downstream primer.The 24mer 5′-ATCACGCTTTTGTTCCTGATGCAG-3′ was used as the target-specific probe (p_1). The 24mer 5′-CTTGCTGAACTTCTGACTAC-GACT-3′ was used as an internal standard-specific probe (p_2).

A 233-bp DNA fragment was used as a target. The target DNA was synthesized by amplifying the prostate-specific antigen (PSA) mRNA using reverse transcriptase PCR as described in detail elsewhere.[16] Briefly, total RNA was isolated from human prostate adenocarcinoma cells (LNCaP cells), and the PSA mRNA was reverse transcribed using the downstream primer shown above. Subsequently, the synthesized cDNA was amplified by PCR using the upstream and downstream primers to generate a 233-bp product,[16] which was used as the target DNA throughout this work. The target DNA concentration was determined by scanning densitometry of the negatives prepared from pictures of ethidium bromide-stained agarose gels. Solutions with various target DNA concentrations were prepared by diluting the stock in 20 mmol/L ammonium sulfate, 75 mmol/L Tris-HCl (pH 8.8), 0.1% Tween-20, and 2 mmol/L $MgCl_2$.

A recombinant DNA internal standard was constructed by replacing a 24-bp segment of the target DNA (spanning the region 67−90) with a different sequence of the same size (5′-AGTCG-TAGTCAGAAGTTCAGCAAG-3′) using PCR as a synthetic tool, as described previously.[12]

The phosphate-buffered saline (PBS) contained 0.14 mol/L NaCl, 10 mmol/L sodium phosphate, and 1.7 mmol/L potassium phosphate, pH 7.4. The wash solution consisted of 50 mmol/L Tris, pH 7.4, 0.15 mol/L NaCl, 1 mL/L Tween-20, and 2 mmol/L EGTA. The blocking solution contained 10 g/L blocking reagent, 0.1 mol/L maleic acid, 0.15 mol/L NaCl, and 2 mmol/L EGTA (final pH 7.5).

Labeling of Probes with Fluorescein and Digoxigenin.[17] The IS probe (p_2) was tailed with multiple fluorescein molecules. The tailing reaction was performed in a total volume of 20 μL, which consisted of 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl (pH 6.6), 0.25 g/L bovine serum albumin, 5 mmol/L $CoCl_2$, 50 μmol/L fluorescein-dUTP, 0.5 mmol/L dATP, 25 units of terminal deoxynucleotidyl transferase, and 100 pmol of probe. The reaction was carried out at 37 °C for 60 min.

Probe p_1 (specific for the target DNA) was tailed with digoxigenin exactly as above using Dig-dUTP instead of fluorescein-dUTP. The labeled probes were used without purification.

Microtiter Well-Based Dual-Analyte Chemiluminescence Hybridization Assay. Opaque polystyrene wells were coated overnight at room temperature with 50 μL of 1.4 mg/L streptavidin diluted in PBS. Prior to use, the wells were washed three times with wash solution. PCR products containing amplified target DNA and DNA IS, both biotinylated at their 5′ end, were diluted

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10 times in blocking solution, and then 50 μL was pipetted into each well. The DNA fragments were allowed to bind to streptavidin for 30 min with shaking. The wells were washed three times as above, and then the non-biotinylated strand was dissociated by incubating for 20 min with 50 μL of 0.2 mol/L NaOH. The wells were washed, and 50 μL of a solution containing fluorescein-labeled probe p_2 and Dig-labeled probe p_1 (each at a concentration of 7 nmol/L diluted in blocking solution and preheated at 42 °C) was pipetted into each well. The immobilized single-stranded amplification products for target DNA and IS were allowed to hybridize simultaneously with their specific probes for 30 min with shaking at 42 °C. The wells were washed three times to remove the unbound probes, and then 50 μL of a solution containing 75 munits/L ALP-labeled antifluorescein antibody and 10 μg/L aequorin-labeled antidigoxigenin antibody (diluted in blocking solution) was added into each well. The labeled antibodies were allowed to bind simultaneously to their corresponding haptens for a period of 30 min at room temperature, and then the excess reagent was removed by washing the wells. The wells were then placed in the luminometer, and 50 μL of the aequorin luminescence-triggering solution (25 mmol/L $CaCl_2$ and 20 mmol/L Tris, pH 7.5) was injected into each well. Subsequent light emission was integrated for 3 s. This was immediately followed by the addition of 50 μL of alkaline phosphatase substrate solution (0.8 mmol/L CSPD, 0.2 mol/L diethanolamine, 2 g/L Sapphire II, 2 mmol/L $MgCl_2$, pH 9.5). The enzymic dephosphorylation reaction was allowed to proceed for 20 min, after which light emission was integrated for 10 s.

Quantitative Polymerase Chain Reaction. PCRs were performed in a total volume of 100 μL containing (final concentrations) 20 mmol/L ammonium sulfate, 75 mmol/L Tris-HCl (pH 8.8), 0.1% Tween-20, 2 mmol/L $MgCl_2$, 50 μmol/L dNTPs, 50 pmol of each of the biotinylated upstream primer and the downstream primer, 2.5 units of polymerase (Ultratherm DNA polymerase, Bio/Can Scientific, Mississauga, ON, Canada), a constant amount of internal standard (in the range of 10 000−40 000 molecules), and target DNA varying from 430 to 315 000 molecules. The hot-start protocol[18] was followed, in which the reaction mixture was heated to 95 °C for 5 min and then the primers were added. PCR was carried out for 25 cycles of denaturation at 95 °C for 30 s, annealing (65 °C, 30 s), and extension (72 °C, 1 min). Finally, the mixtures were incubated at 72 °C for 10 min and then cooled to 4 °C until their analysis by the dual-analyte chemiluminescence hybridization assay protocol described above.

RESULTS AND DISCUSSION

The proposed dual-analyte chemiluminescence hybridization assay allows the determination of PCR products from both target DNA and DNA IS in the same reaction vessel. The two biotinylated PCR products are captured on streptavidin-coated wells, and one strand is dissociated with NaOH and removed by washing. The immobilized single-stranded target DNA and IS are allowed to simultaneously hybridize with their specific probes, that is, a Dig-labeled probe p_1 and a fluorescein-labeled probe p_2, respectively. Aequorin-labeled antidigoxigenin and alkaline phosphatase-labeled antifluorescein antibodies, in the same mixture, are then allowed to bind to their corresponding haptens. Injection of a Ca^{2+}-containing solution triggers the aequorin chemiluminescent reaction. The light emission from the aequorin bound to the target DNA hybrids reaches a peak at about 1 s and is complete in 10 s. The signal from this flash-type reaction is integrated for the first 3 s. This is followed by the addition of the CSPD substrate solution (without prior washing of the wells). Dephosphorylation of this substrate by alkaline phosphatase bound to the IS hybrids is allowed to proceed for 20 min, upon which the light emission is integrated for 10 s. Figure 1 shows the time course of light emission for both the aequorin and the alkaline phosphatase reactions.

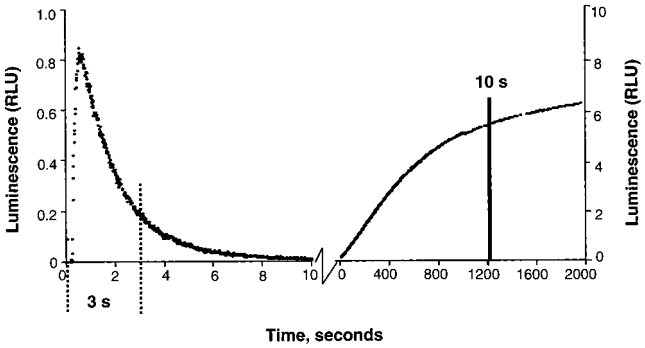

Figure 1. Time course of light emission for both the aequorin and alkaline phosphatase reactions. Aequorin serves as a reporter molecule for the determination of amplified target DNA, and alkaline phosphatase is the reporter molecule for quantification of amplified DNA IS. Following the addition of Ca^{2+}, light emission from aequorin reaches a peak at about 1 s, and the luminescence is integrated for the first 3 s. Subsequently, CSPD substrate solution is injected, and, after 20 min, the luminescence is integrated for 10 s.

To assess the ability of the dual-analyte hybridization assay to provide accurate determination of the two DNA fragments in a mixture, we prepared two pools containing a low target DNA concentration in the presence of a high concentration of IS and vice versa. Pool 1 contained 15 and 500 pmol/L of amplified target DNA and IS, respectively. Pool 2 contained 500 and 15 pmol/L of amplified target DNA and IS, respectively. The pools were first analyzed by single-assay protocols. In the single-assay protocol, the hybridization solution contained only one labeled probe, and the hybrids were reacted with the corresponding labeled antibody. The wells were washed, and Ca^{2+}-containing solution and CSPD substrate solution were injected. The dual protocol was carried out with mixtures of probes, antibodies, and detection reagents as described in the Experimental Section. The results are presented in Table 1. It is observed that the dual-analyte assay protocol does not interfere with the determination of either DNA fragment. Apparently, the fluorescein moieties that are immobilized in the wells do not interfere with the aequorin emission. Also, the alkaline phosphatase reaction was not inhibited by the presence of the Ca^{2+}-containing light-triggering solution for aequorin, thus allowing the two reporter molecules to be determined in the same well.

The sensitivity and the linear range of the hybridization assays were established as follows. We first prepared a stock solution of biotinylated amplification product by pooling several PCRs of the target DNA and determined its concentration by scanning densitometry, as described above for the target DNA. A stock solution of biotinylated PCR product for the DNA IS was also

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Table 1. Comparison between Single- and Dual-Analyte Hybridization Assays for Target DNA and DNA Internal Standard

analyte	assay type	luminescence pool 1	luminescence pool 2
target DNA	single	30.0 ± 1.4[a]	904 ± 20
	dual	36.3 ± 4.7	832 ± 96
IS	single	37 003 ± 4167	2674 ± 182
	dual	40 986 ± 1706	2714 ± 251

[a] The mean values and standard deviations are given ($n = 4$).

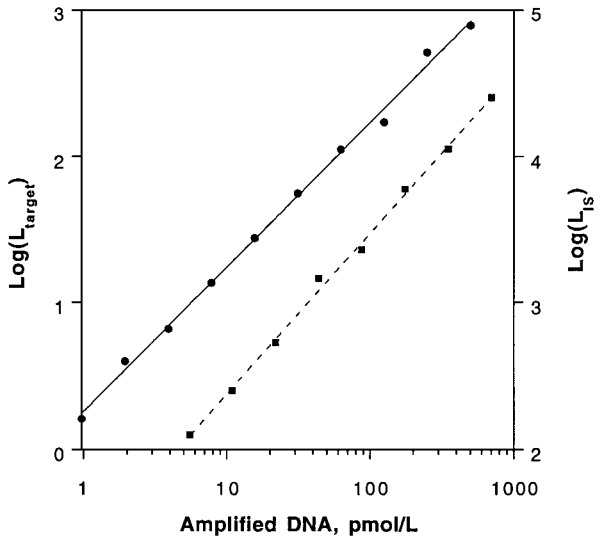

Figure 2. Luminescence as a function of the concentration of amplified target DNA (solid line) and DNA IS (dashed line). Biotinylated PCR products from target and IS were captured on microtiter wells coated with streptavidin. The amplified target DNA was hybridized with a digoxigenin-labeled specific probe, and the hybrids were determined by using antidigoxigenin antibody labeled with aequorin. The amplified DNA IS was hybridized with a fluorescein-labeled probe, and the hybrids were determined by using an antifluorescein antibody conjugated to alkaline phosphatase. Aequorin was measured by adding a Ca^{2+}-containing light-triggering solution. Alkaline phosphatase was measured by using a dioxetane chemiluminogenic substrate.

prepared in the same manner. After quantification, various dilutions of each stock solution were analyzed by hybridization to probes p_1 or p_2. In Figure 2, the luminescence (corrected for the background) was plotted as a function of the amplified DNA in the well. The background is defined as the luminescence obtained when a sample containing no amplification product was analyzed. Each data point represents the average of two assays. We observe that concentrations as low as 0.97 pmol/L (48.5 amol/well) of amplification product for the target DNA were determined with a signal-to-background (S/B) ratio of 3.7. The linearity extends up to 500 pmol/L. Also, 5.5 pmol/L (267 amol/well) of the amplification product for DNA IS can be determined with a S/B ratio of 2.3, and the linearity extends up to 700 pmol/L.

The results presented in Figure 2 show that the dual-analyte assay provides higher sensitivity for the amplified target DNA compared to the IS. The reasons for this difference were investigated as follows. To directly compare the detectabilities of the two chemiluminescence detection systems, we analyzed

Table 2. Study of the Two Detection Systems Used in the Dual-Analyte Hybridization Assay for Target DNA and DNA Internal Standard

analyte	concn, pmol/L	anti-Dig-aequorin (S/B)[a]	anti-Dig-ALP (S/B)[a]	anti-F-ALP (S/B)[a]
target DNA	20	66	19	7.5
	60	203	53	24
	180	679	168	91
DNA IS	20			13
	60			38
	180			96

[a] The values represent the average of two measurements.

solutions containing 0, 20, 60, and 180 pmol/L of biotinylated amplification product from target DNA. After hybridization with Dig-labeled probe p_1, the hybrids were reacted either with antidigoxigenin-aequorin (anti-Dig-aequorin) or with antidigoxigenin-alkaline phosphatase (anti-Dig-ALP, Boehringer) conjugates. The results, expressed as S/B ratios, are presented in Table 2. It is observed that the aequorin-based detection system provides a 3.5-fold improvement of the S/B ratio compared to the alkaline phosphatase-based assay. Since the proposed hybridization assays use hybrids linked to their corresponding reporter molecules through a hapten/antibody interaction, we studied the effect of digoxigenin/antidigoxigenin and fluorescein/antifluorescein linking systems on the sensitivity of the assay. The target DNA solutions were analyzed by hybridization to probe p_1 labeled with either digoxigenin or fluorescein (the probe p_1 was tailed with fluorescein-dUTP as described for probe p_2 in the Experimental Section). The hybrids were then reacted with either anti-Dig-ALP or antifluorescein-alkaline phosphatase (anti-F-ALP) conjugates, respectively. The S/B ratios were 2 times higher with the digoxigenin/antidigoxigenin system (Table 2). Also, in Table 2, data are included from the parallel analysis of solutions containing 0, 20, 60, and 180 pmol/L of DNA IS using the fluorescein/antifluorescein-alkaline phosphatase system.

To summarize, the higher sensitivity observed with the target DNA is due to the combined use of aequorin as a reporter molecule and the Dig/anti-Dig interaction as a linker.

The reproducibility of the dual-analyte hybridization assay was tested by analyzing three pools, each containing both target DNA and the IS at the following concentrations: pool 1, 15 pmol/L of each; pool 2, 100 pmol/L of each; and pool 3, 300 pmol/L of each DNA. The pools were analyzed five times with the dual-analyte hybridization assay. The %CVs obtained for target DNA and IS were 6.7 and 7.7 (pool 1), 4.9 and 2.9 (pool 2), and 6.1 and 5.9 (pool 3), respectively.

The quantitative PCR assays were carried out by coamplifying samples containing target DNA concentrations varying from 430 to 315 000 molecules with a constant amount of DNA IS (in the range of 10 000−40 000 molecules). Following amplification, the products were determined by the dual-analyte hybridization assay using probes p_1 and p_2. For each standard curve, a negative was prepared (PCR mixture containing IS but no target DNA), amplified, and measured by the same protocol. The luminescence values obtained, L_{target} and L_{IS}, reflect the concentrations of amplification products from target DNA and DNA IS, respectively. In Figure 3, the luminescence (corrected for the background) was

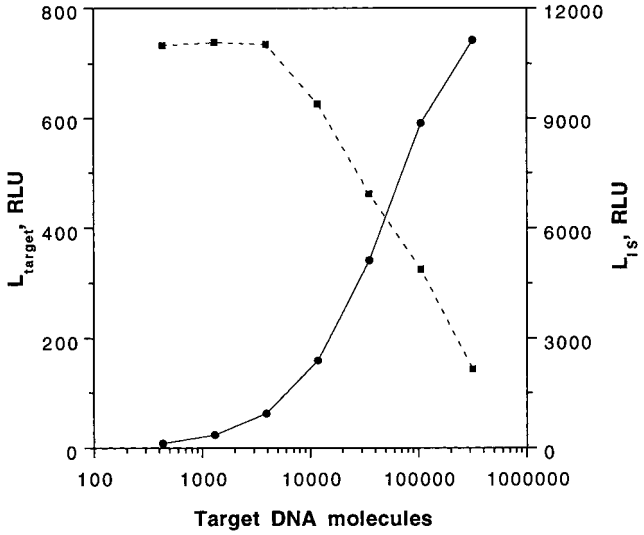

Figure 3. Study of the variation in luminescence as the target DNA corresponding to 430−315 000 molecules was coamplified with a constant amount of DNA IS (40 000 molecules). The amplification products were determined by the dual-analyte chemiluminescence hybridization assay. The solid and dashed lines represent the signals obtained from target DNA and DNA IS, respectively.

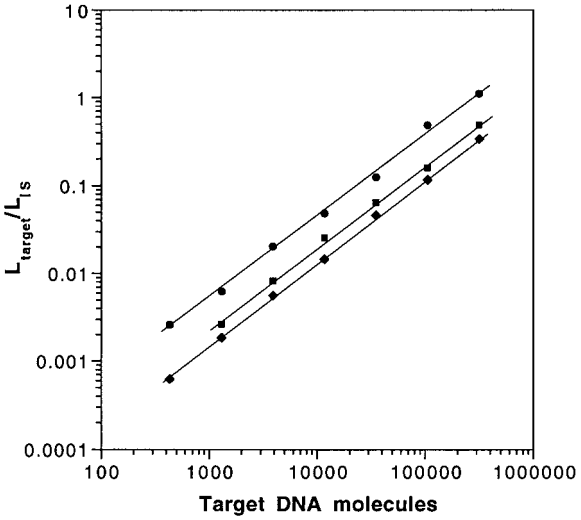

Figure 4. Study of the linearity and sensitivity of the proposed quantitative PCR assay. The ratios of luminescence signals (L_{target}/L_{IS}) obtained from the target DNA and IS were plotted against the initial number of target DNA molecules in the sample prior to amplification. The three lines correspond to assays performed in the presence of 10 000 (●), 20 000 (■), and 40 000 (◆) molecules of DNA IS.

plotted as a function of the number of target DNA molecules in the sample prior to amplification. The background is defined as the luminescence obtained from the PCR negative. We observe that the luminescence corresponding to the DNA IS remains constant for a small number of target DNA molecules. However, as the target DNA increases and the PCR enters its plateau phase,[19] the amplification efficiency drops for both DNA fragments, and the total amount of product remains practically constant, regardless of the starting amount of target DNA. As a consequence, a further increase of target DNA results in suppression of the amplification of IS.

In Figure 4, the ratio L_{target}/L_{IS} of the luminescence values obtained for the target DNA and DNA IS was plotted against the initial number of target DNA molecules present in the sample prior to amplification. A linear relationship is observed which extends over 3 orders of magnitude (from 430 to 315 000 molecules). Because of the close resemblance in the structures of target DNA and DNA IS, the amplification factors for the two fragments (i.e., the factor by which the concentration of each fragment increases during PCR) are the same regardless of the phase of PCR (exponential phase or plateau phase). Consequently, the ratio of the amplification products (represented here by the ratio of the luminescence signals) is linearly related to the ratio of the amounts of target DNA and IS in the sample prior to amplification. Because the amount of IS is kept constant, the ratio of the luminescence signals is a linear function of the amount of target DNA. Furthermore, Figure 4 demonstrates the effect of the amount of IS on the calibration graphs. The three lines correspond to quantitative PCR assays performed in the presence of 10 000, 20 000, and 40 000 molecules of DNA IS. In theory, the ratios of the luminescence signals are inversely related to the number of DNA IS molecules used. This is reflected by the parallel shift of the double logarithmic plot to higher L_{target}/L_{IS} values as the amount of IS decreases. The signal-to-background ratios observed at 430 molecules of target DNA were 4.4, 2.4, and 2.7, respectively. Because only 5% of the initial PCR mixture was used in the dual-analyte hybridization assay, the luminescence signal was essentially obtained from amplification product corresponding to 22 target DNA molecules.

The overall reproducibility of the proposed quantitative PCR assays (including the PCR step and the dual-analyte hybridization assay) was tested by analyzing samples containing 1300, 13 000, and 130 000 target DNA molecules. To each sample was added 40 000 molecules of DNA IS, and the PCRs were performed on different days. The %CVs obtained for the L_{target}/L_{IS} ratios were 7.3, 8.9, and 17.6, respectively ($n = 4$).

The most widely used internal standards for quantitative PCR share the same primers with the target but contain a deletion or insertion to enable electrophoretic separation of the amplification products from target DNA and IS. Sequence length, however, is also a major determinant of the amplification efficiency. It has been shown[20] that the efficiency is inversely related to the size of the DNA. In the present work, the target DNA and the DNA IS not only share a common set of primers but also have identical sizes. Moreover, the IS closely resembles the target DNA, since it differs only by a 24-bp segment (10% of its size). This DNA IS has been prepared by using PCR as a synthetic tool,[12] a simple procedure that is complete in 1 day, thus avoiding tedious and time-consuming cloning techniques which involve culturing bacteria, plasmid purification, digestion, and ligation.

It has been observed that coamplification of DNA fragments that share considerable sequence homology leads to the formation of heteroduplexes during PCR, even if their sizes are different.[20] During the annealing phase of a PCR cycle, a fraction of the single-stranded fragments of target DNA will hybridize with the

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complementary strand from the IS and vice versa. Upon electrophoresis, the heteroduplexes may migrate between the target DNA and the IS, causing errors in the determination of the products, especially if they cannot be resolved from the homoduplexes. In the case of an internal standard having the same size as the target, but differing only in a restriction site, the heteroduplexes interfere because they are resistant to digestion. In the proposed dual-analyte hybridization assay, both amplified fragments are captured in the well, followed by alkaline denaturation and washing of the strands. The subsequent hybridization essentially measures immobilized single-stranded DNA. Therefore, heteroduplex formation is not a concern for our assays.

Recently, methods have been developed for continuous determination of the products generated during PCR, i.e., for real-time monitoring of DNA amplification.[21−23] One such approach uses the compound SYBR Green I, whose fluorescence increases upon binding to double-stranded DNA. The co-determination of undesired products represents a serious limitation of this method. An alternative strategy employs an oligonucleotide probe labeled with a donor fluorescent molecule (6-carboxyfluorescein) at the 5′ end and an acceptor molecule (6-carboxytetramethylrhodamine) at the 3′ end. The close proximity between donor and acceptor allows for energy transfer to occur, and, therefore, no fluorescence from the donor is observed. During annealing, the probe hybridizes to the template DNA at a position flanked by the primers. However, because of the 5′ exonuclease activity of Taq DNA polymerase, the hybridized probe is degraded during the extension phase of each cycle, and the fluorescence increases. Specific probes labeled with different donor molecules and the same acceptor molecule may be used for determination of amplified target DNA and DNA IS. Real-time quantitative PCR methods are advantageous in that they do not require post-PCR sample processing. However, the detectability of the fluorescent label is in the nanomoles per liter range, and the fluorescence is an indirect measure of the amplification product. It has also been reported[23] that hybridization is a necessary but not sufficient condition for probe hydrolysis; i.e., all probes are not cleaved efficiently. In contrast, the proposed chemiluminescence assay allows confirmation of the final amplification product after PCR and offers at least 1000-fold higher sensitivity, since it detects 1 pmol/L of amplification product (see Figure 2). Also, the labeling of probes in the proposed system is accomplished by enzymic tailing, which is much easier than the conjugation techniques required for double-labeling of the probes in real-time PCR methods.

In conclusion, we have developed a quantitative PCR methodology based on a dual-analyte chemiluminescence hybridization assay for amplified target DNA and DNA IS. The assay is highly sensitive, and, because it is performed in microtiter wells, it is suitable for automation and high-throughput analysis. The proposed method may find wide applications in a variety of circumstances in which it is necessary not only to confirm the presence of a specific nucleic acid sequence but also to determine its concentration. For instance, the elimination of an infectious agent (e.g., HIV) or a tumor's response to chemotherapy can be monitored by measuring the concentration of a characteristic abnormal DNA (or RNA) sequence in successive clinical samples.

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