Real-Time Quantification of Genomic DNA Using DyNAzyme II DNA Polymerase and SYBR Green I Dye

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Real-Time Detection

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Abstract

Real-time quantification was performed for lambda and human genomic DNA on the DNA Engine Opticon® system (MJ Research). DyNAzyme™ II DNA polymerase was used for amplification, and SYBR Green I fluorescent dye was used for detection. The sensitivity and linear dynamic range of the assay was tested for ranges of 100 to 1x10^8 initial copies of lambda DNA and 75 to 7.5x10^4 initial copies of human DNA. Precision of the assay was measured with lambda template and standard deviations of 0.3 cycles or less were obtained for quadruplicate samples. This assay has been shown to be both reliable and precise for the quantification of genomic DNA templates.*

Introduction

Quantification of DNA and RNA from biological samples can be effectively accomplished using real-time amplification methods[1]. The predominant chemistries are based on either the binding of fluorescent dyes to the double stranded structure of DNA or the hybridization of specific probes, such as TaqMan probes[2] and molecular beacons[3]. The simplicity and directness of using binding dyes such as SYBR Green I (SGI) make this a desirable alternative for many applications[4].

SGI is a dsDNA binding dye that has proven exceptionally useful for assays requiring sensitive nucleic acid detection. The fluorescence of SGI is enhanced approximately 1000-fold upon binding dsDNA[5], making it ideal for detection of amplification products. Because SGI binds to all dsDNA, it does not have to be customized for individual templates. Detection formats utilizing hybridization probes require careful design of template-specific primer sets. SYBR Green I assays require only the two primers needed for amplification—neither of them labeled.

DyNAzyme II DNA polymerase provides superior results in a wide variety of PCR applications. This polymerase, isolated from *Thermus brockianus*, demonstrates better thermal stability than *Taq* DNA polymerase, and the ability to maintain optimal activity over a broad range of reaction conditions. DyNAzyme II is also inherently more resistant than *Taq* to the inhibitory effects of SGI. In this study, we present real-time quantitative PCR results indicating the sensitivity and precision of assays incorporating DyNAzyme II DNA polymerase and SGI for quantitation of starting copy number using both lambda and human genomic DNA templates.

Methods

DyNAzyme II DNA polymerase was from Finnzymes (F-503L). SGI and lambda DNA were from Molecular Probes (S-7567, P-7589); human genomic DNA was from Sigma (D-3160). Reaction components were assembled in low-profile microplates (MJ Research MLL-9651) or strip tubes (MJ Research TLS-0851) and sealed with ultra-clear strip caps (MJ Research TCS-0803). Volumes of individual components and final reaction concentrations are listed in Table 1.

Table 1. Quantitative PCR Reaction Components

Component	Stock Concentration	Volume	Final Concentration
Template	100–1x10^8 copies/µl	1.0µl	100–1x10^8 copies/reaction
Forward Primer	10µM	0.5µl	0.25µM
Reverse Primer	10µM	0.5µl	0.25µM
Mg-free buffer	10X	2.0µl	1X
$MgCl_2$	25mM	1.6µl	2mM
dNTPs	2.5mM each	1.6µl	200µM each
SYBR Green I	10X	1.0µl	0.5X
DyNAzyme II	(2U/µl)	0.2µl	20U/ml
ddH_2O		11.6µl	
Total Volume		20.0µl	

SGI reagents are typically obtained at high concentrations (e.g., 10,000X). We define a 10X SGI solution as one giving 0.4±0.01 O.D. when measured at 495nm. SGI was diluted to a 10X working stock with 0.1X TE buffer.

The following primer set was used in PCR reactions with lambda DNA to generate a 100bp amplicon. The sequences for the primers were: forward primer, 5'-GCA-AGT-ATC-GTT-TCC-ACC-GT-3', and reverse primer, 5'-TTA-TAA-GTC-TAA-TGA-AGA-CAA-ATC-CC-3'.

The β-actin primers used for amplification of human genomic DNA generate a 294bp amplicon. Sequences were: forward, 5'-TCA-CCC-ACA-CTG-TGC-CCA-TCT-ACG-A-3', and reverse, 5'-CAG-CGG-AAC-CGC-TCA-TTG-CCA-ATG-G-3'.

Following reaction assembly, the plates or tubes were transferred to the DNA Engine Opticon real-time system, where cycling was performed according to the program listed in Table 2.

The cycle threshold C(t) line was set using the signal/noise ratio option in the Opticon Monitor™ software (MJ Research) set to 10 standard deviations above the mean fluorescence values for the first 3–7 cycles. This threshold is automatically applied to all wells and allows the

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Table 2. Quantitative PCR Cycling Program

1.	94°C, 2min
2.	96°C, 10sec
3.	63°C, 15sec
4.	72°C, 20sec
5.	78°C, 1sec
6.	Plate read
7.	Go to step 2, 39 more times
8.	72°C, 10min
9.	Melting curve analysis: 65°C to 98°C, 0.2°C/read, 1sec hold
10.	72°C, 10min
11.	10°C, Hold
END	

comparison of standards and samples at a point that provides the most consistent results. It is critical that within any experiment, C(t) values for both standards and samples be determined using the same threshold level.

A concentration series of lambda DNA was prepared from a 0.5µg/µl stock. 1µl of each dilution in 20µl reactions was used to generate the standard curve. The standard curve for the human genomic sequence was prepared using genomic DNA from human placenta. Serial dilutions were made and analyzed to generate the standard curve.

Results

Lambda DNA Template: Linear Range and Reproducibility

Plotting fluorescence signal vs. cycle number (Figure 1a) indicates that the concentration of dsDNA in each sample rose above background fluorescence levels, and then entered a stage of exponential amplification. The C(t) values, the cycle at which the measured fluorescence intersects the cycle threshold line, decrease proportionally with the increase in initial DNA concentration. This trend is expected since higher amounts of initial template more quickly generate the amount of product necessary to be detected with SGI. The linear range extends from 10^8 to 10^2 copies of lambda DNA initially present in the reaction. A plot of log quantity vs. C(t) cycle for the 100bp amplicon is shown in Figure 1b. The regression coefficient is 0.991, indicating a strong linear correlation.

The precision of this assay was measured by calculating the variation in C(t) values across the four replicates at each template concentration. The standard deviation for C(t) values was found to be 0.3 units or less as shown in Table 3.

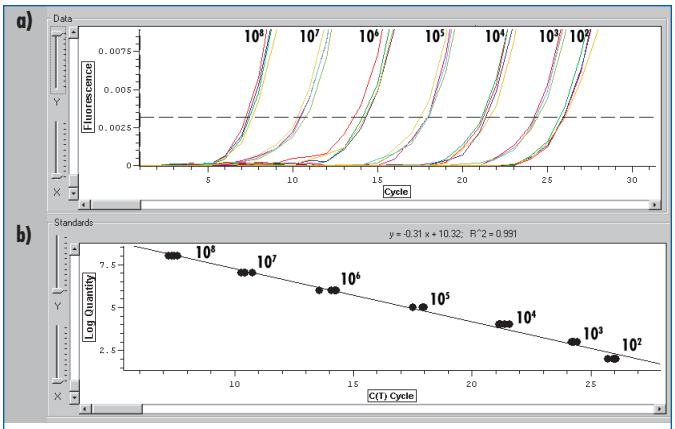

Figure 1. Generating a standard curve for a 100bp PCR amplicon from lambda genomic DNA. a) Quantitation curves obtained for a dilution series of lambda DNA ranging from 10^8 to 10^2 initial template copies per reaction. Four reactions were performed at each starting concentration. b) Standard curve plot of log copy number vs. C(t) value. The regression coefficient is r^2=0.991. (View from Opticon Monitor software)

Table 3. Mean and standard deviation in C(t) values for the four replicates of lambda DNA at each initial template concentration.

Copies	C(t)				Mean	Std. Dev.
$1x10^8$	7.2	7.5	7.4	7.6	7.4	0.2
$1x10^7$	10.3	10.4	10.5	10.8	10.5	0.2
$1x10^6$	13.6	14.1	14.3	14.2	14.1	0.3
$1x10^5$	17.5	17.9	17.9	18.0	17.8	0.2
$1x10^4$	21.2	21.1	21.4	21.6	21.3	0.2
$1x10^3$	24.3	24.2	24.3	24.4	24.3	0.1
$1x10^2$	26.0	25.7	26.0	26.1	25.9	0.1

Figure 2 shows the melting curves for the four replicate lambda DNA standards containing 10^4 copies per reaction. The curve shows the typical smooth decline in fluorescence with an increase in temperature as the strands of dsDNA dissociate and bound SGI is released. The negative first derivative calculation is also shown. Here, the point of inflection for the curve, the melting point (T_m), is clearly seen as a single, common peak at approximately 82°C. This peak corresponds to the predicted melting temperature of the amplicon, indicating amplification of the amplicon of interest. Anomalies due to contamination, primer-dimer, false priming, etc., are not evident in the negative first derivative plot, as indicated by the lack of additional peaks or of abnormal broadening of the single peak.

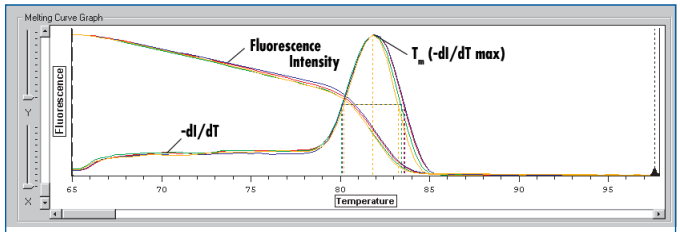

Figure 2. Melting curves depicting the fluorescence intensity and negative first derivative plots for the four replicate lambda DNA standards containing 10^4 copies/ 20µl reaction. (View from Opticon Monitor software)

Human Genomic DNA Template: Patient Samples Quantified Using a Standard Curve

A standard curve generated from human genomic DNA is shown in Figure 3. A linear curve in the range of $7.5x10^4$ copies/reaction to 75 copies/reaction with r^2=0.993 was obtained. Two samples of human genomic DNA were also tested. By interpolation of the standard curve, the first patient sample was determined to have an initial concentration of 254 copies/µl, the second had 357 copies/µl.

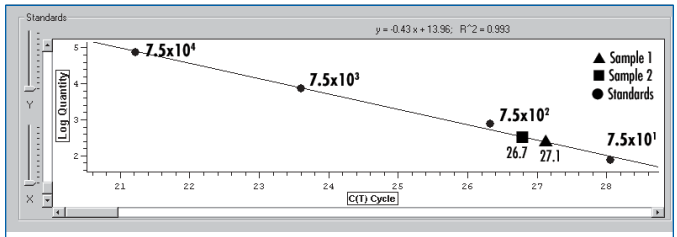

Figure 3. Standard curve generated for human genomic DNA with patient DNA of unknown quantity (samples 1 and 2) plotted according to C(t) value. (View from Opticon Monitor software)

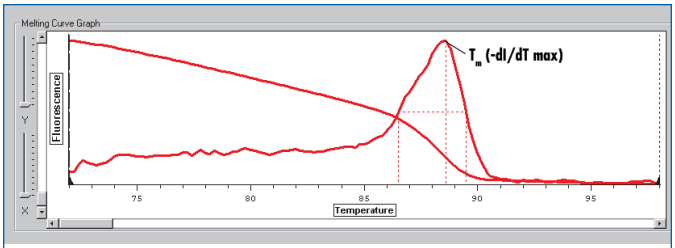

Figure 4. Melting curve and negative first derivative plot for amplified genomic DNA from patient sample 1. (View from Opticon Monitor software)

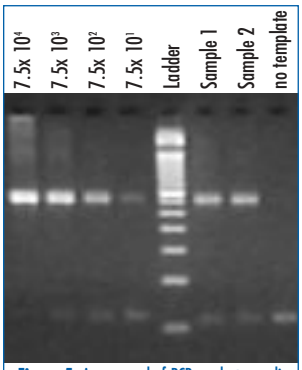

Figure 5. Agarose gel of PCR products amplified with beta-actin primers from serial dilutions (7.5x10^4 to 75 copies/ reaction) of human genomic DNA and two patient genomic DNA samples.

Specificity of the PCR was measured in two ways. First, melting curve analysis was performed on each sample. For example, the melting curve analysis for patient sample 1 revealed a single peak at 88.5°C, which indicates exclusive amplification of the β-actin amplicon as seen in Figure 4. Second, gel electrophoresis was performed to analyze the PCR products. Figure 5 reveals single bands for each standard and sample, indicating specific amplification of the desired product with only minor amplification of a primer-dimer.

Discussion

Broad Dynamic Range for Lambda and Human DNA

DyNAzyme II DNA polymerase can be used successfully with SYBR Green I dye to quantify DNA from small genomes as well as the more complex human genome. Experiments revealed a linear relationship between log starting copy number and C(t) value over at least 6 orders of magnitude for the lambda genomic DNA template, and over at least 3 orders of magnitude for the human genomic DNA template. The broad dynamic range of starting template that can be detected makes this assay particularly valuable when sample DNA concentrations are unknown or vary widely.

In this study, the lower limit of the standard range was 100 copies of lambda DNA, or 75 copies of human genomic DNA per reaction. Other studies have shown linear results below this level. Though not necessary in this application, detection down to several copies of template could be valuable when analyzing small populations of cells or trace amounts of DNA.

Protocol Adaptable for a Wide Variety of Applications

The protocol presented here can be applied to a wide variety of PCR applications by following several guidelines to modify the protocol for work with SYBR Green I. Because SYBR Green I stabilizes dsDNA, the denaturing temperature of the cycling protocol should be raised approximately 2°C in order to ensure complete denaturation of the duplex. In addition, it is recommended that the annealing temperature of PCR primers be optimized for the SGI reaction conditions. This is accomplished by running test reactions with an annealing temperature gradient. The temperature gradient can be programmed into the cycling protocol through the Opticon Monitor software. Melting curves and agarose gel data can be used to evaluate yield and specificity for the target amplicon.

DMSO for the Most Challenging Templates

A wide variety of samples can be analyzed with the protocol described here. The human genomic DNA amplified with β-actin primers represents an optimal sample in terms of purity and ease of amplification. However, amplification of challenging templates may benefit from modification of reaction conditions, such as the addition of DMSO. The destabilizing effect of DMSO on double-stranded DNA reduces the denaturation temperature of the amplicon thereby facilitating the amplification of templates with strong secondary structure. Success has been experienced with the addition of DMSO in concentrations of up to 5% (vol/vol). Caution must be used when adding DMSO to reactions, as the destabilizing effect on dsDNA will result in a decrease of bound SGI, and reduction of the fluorescence signal. Addition of DMSO will also lower the T_m for both the primers and the amplicon. Incremental addition of DMSO is recommended.

Selection of Read Temperatures Enhances Specificity

Melting curve analysis indicates this protocol can be used for analysis of amplified template without complications from side reactions, such as primer-dimer formation. To avoid primer-dimer interference with C(t) value determination, the temperature at which the fluorescence was read during each cycle was adjusted to 78°C, a temperature above the melting point of the primer-dimers. At this temperature, the double stranded primer-dimers should be denatured, releasing bound SGI and diminishing their contribution to the fluorescence signal. At this same temperature, the longer PCR product remains annealed, and is directly correlated to the fluorescence detected. Typically, the read temperature is set three degrees below the T_m of the amplicon. Melting temperatures for both the primer-dimers and the PCR product are determined by analyzing melting curve data.

The results discussed here highlight DyNAzyme II as a robust polymerase that can be used with SYBR Green I dye in real-time PCR applications. The protocol listed here can be used to provide accurate, reproducible quantitation data for both lambda and human genomic DNA templates over a broad concentration range. The discussion of read temperatures, as well as the guidelines for DMSO use and denaturing and annealing temperatures, provides the user with some basic tools for optimizing real-time fluorescence detection of amplification reactions using SYBR Green I.

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6. For more information, please refer to www.finnzymes.fi

Ordering Information

DyNAzyme II DNA polymerase

Cat#	Product	Size
F-501S	250 Units	2U/µl
F-501L	1000 Units	2U/µl

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