QUANTITATIVE PCR

Development of a Novel, Fluorescent, Two-Primer Approach to Quantitative PCR

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The first predesigned assays will allow quantitation of total human and male-specific DNA in a two-color multiplex assay, with the potential for further multiplexing.

INTRODUCTION

Multiplexed short tandem repeat (STR) analysis has become the dominant technology in DNA-based human identification. Although highly informative, these assays require a defined range of template quantities to produce optimal results. Thus, the ability to accurately assess the extracted DNA quantity and quality has become increasingly important. Quantification of male-specific DNA has also become necessary with the advent of forensic Y-STR testing. All of these issues can be addressed in a quantitative PCR system.

Many STR megaplexes used in forensic applications require 0.5–1ng of DNA template. Using template quantities below the suggested range increases the likelihood of allelic imbalance and partial amplification. Excessive template can lead to signal saturation during analysis, nonspecific amplification and product imbalance for the different amplified loci. Precise quantification of template DNA conserves resources by reducing the need to reamplify samples that are not interpretable due to insufficient or excessive template.

Common hybridization-based quantification methods produce increased levels of false-negative results (due to lack of sensitivity) or subjective conclusions (due to visual comparison of band intensities). Amplification-based quantification methods provide a high level of sensitivity, and real-time PCR methods can deliver a dynamic range that exceeds that of other methods. The numerical output of real-time quantitative PCR also increases the objectivity of data interpretation.

Here we describe a new technology for real-time quantitative PCR. The background of this chemistry is presented here, and an article in the next issue of *Profiles in DNA* will describe the development of a human-specific and male-specific quantitation system based on this technology.

THE PLEXOR™ TECHNOLOGY

This new real-time PCR[a] method takes advantage of the specific interaction between two modified nucleotides to achieve quantitative analysis (1–3). As shown in Figure 1, two novel bases, isoguanine (iso-dG) and 5´-methylisocytosine (iso-dC),

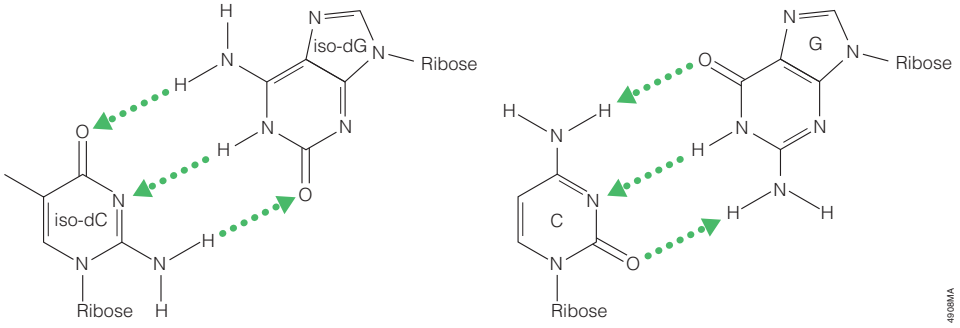

Figure 1. Base pairing between isoguanine (iso-G) and 5´-methylisocytosine (iso-C).

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form a unique base pair in double-stranded DNA (2). To perform fluorescent quantitative PCR using this new technology, one primer is synthesized with an iso-dC residue as the 5´-terminal nucleotide and a fluorescent label at the 5´-end; the second primer is unlabeled. During PCR, this labeled primer is annealed and extended, becoming part of the template used during the next round of amplification. During subsequent rounds of amplification, the complementary iso-dGTP, which is available in the nucleotide mix as dabcyl-iso-dGTP, pairs specifically with iso-dC. When the dabcyl-iso-dGTP is incorporated, the close proximity of dabcyl and the fluorescent label on the opposite strand effectively quenches the fluorescent signal. This process is illustrated in Figure 2.

By directly coupling fluorescence detection and thermal cycling, real-time PCR measures the change of fluorescent signal (in relative fluorescent units, RFU) at every cycle. The initial fluorescence level of the labeled primers is high in Plexor™ System[(b)] reactions. As amplification product accumulates, the signal decreases. Amplification data present a characteristic three-phase curve (Figure 3, Panel A).

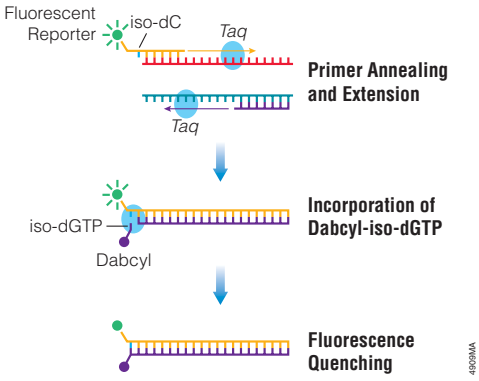

Figure 2. Quenching of the fluorescent signal by dabcyl during product accumulation.

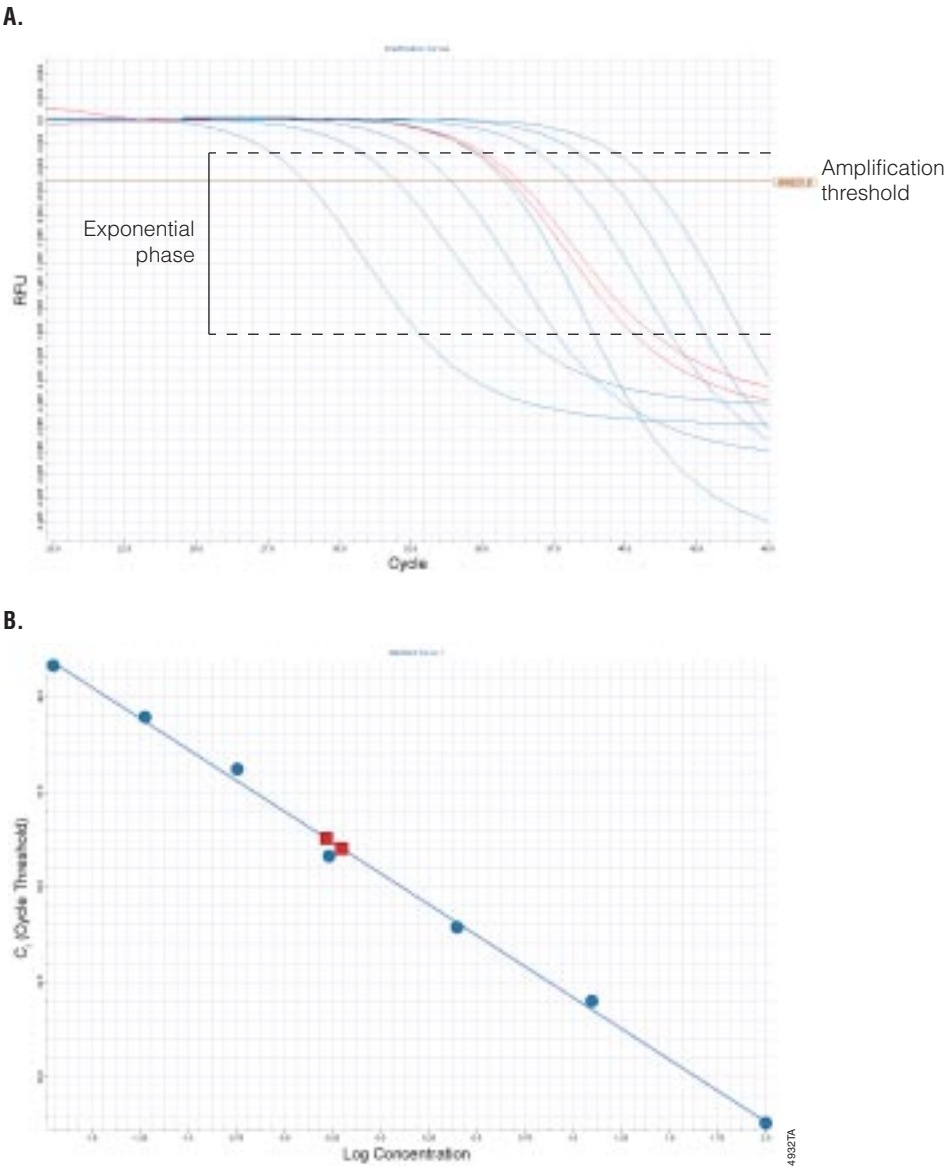

Figure 3. Panel A. A representative amplification curve, which shows the relative fluorescence units (RFU) at each cycle of the reaction. The amplification threshold is indicated by a horizontal line across the graph. This threshold is used to establish the cycle threshold, the cycle at which the amplification curve crosses the amplification threshold, for each sample. The blue circles represent standard samples, and the red squares represent unknown samples. **Panel B.** A standard curve generated from the amplification curve data shown in Panel A.

The part of the curve with the biggest signal change is the exponential phase. The exponential phase is the most consistent phase and is used to estimate the quantity of starting material. An amplification threshold is set within the exponential phase at a fluorescence level where all amplification curves exhibit the most significant signal decrease. The point at which an amplification curve crosses that threshold is the cycle threshold (C_t) of the sample. C_t values for a dilution series of a sample of known

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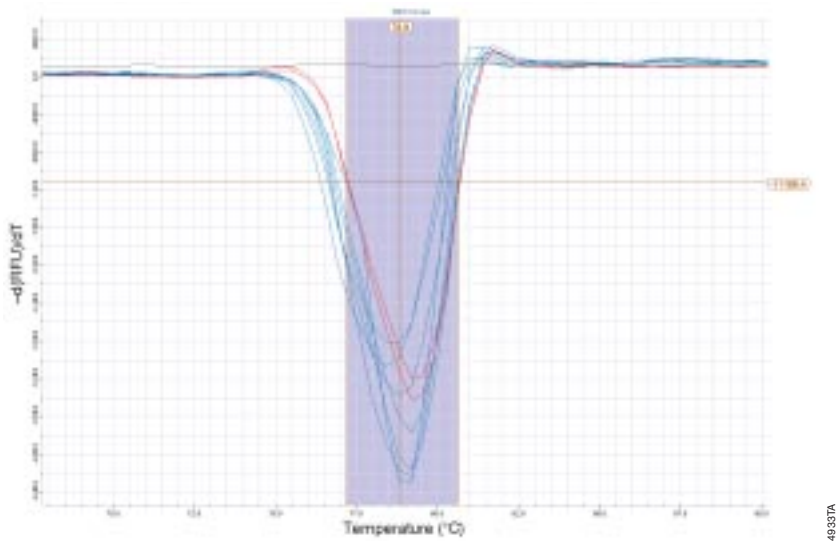

Figure 4. Thermal melt curve. The melting temperature was empirically determined from the data shown in Figure 3 by plotting the change in fluorescence with temperature (–dRFU/dT) versus temperature and calculating the temperature at which the biggest change in fluorescence occurs.

DNA quantity are used to generate a standard curve, which is used to quantify samples with unknown amounts of DNA (Figure 3, Panel B).

Quenching of the fluorescent label by dabcyl is a reversible process. Fluorescence is quenched when the product is double-stranded due to the close proximity of dabcyl and the fluorescent label. Denaturing the product separates the label and quencher, resulting in an increased fluorescent signal. Consequently, thermal melt curves can be generated by allowing all product to form double-stranded DNA at a lower temperature (approximately 60°C) and slowly ramping the temperature to denaturing levels (approximately 95°C).

Figure 4 illustrates a "melt curve" with the empirically derived melting temperature (T_m). The product length and sequence impact T_m, so the melt curve is used to characterize amplicon homogeneity in the selected wells. Nonspecific amplification can be identified by broad peaks in the melt curve or peaks with different T_m values. By distinguishing specific and nonspecific amplification products, the melt curve adds a quality control aspect during routine use of validated or predesigned assays.

A benefit of the Plexor™ technology over detection using simple DNA-binding dyes, such as SYBR®Green, is the capacity for multiplexing. The labeled primer can be tagged with one of many common fluorescent labels used in fluorescent PCR, allowing two- to five-color multiplexing, depending on the instrument used. The simplicity of primer design for the Plexor™ technology is a distinct advantage over probe-based quantitative PCR approaches.

DATA ANALYSIS AND INSTRUMENT COMPATIBILITY

The Plexor™ Analysis Software has been developed to analyze amplification data from a variety of real-time instruments, plot standard curves and calculate DNA concentrations of unknown samples. The software, which will be distributed free of charge, allows freedom of choice for instrument use. Currently, data can be imported from the ABI PRISM®7000, 7700 and 7900HT sequence detection systems, Applied Biosystems 7500 Real Time PCR System, Roche LightCycler®1.0 and 2.0 instruments, Bio-Rad iCycler® thermal cycler, MJ Research DNA Engine Opticon®2 fluorescence detection system, Cepheid SmartCycler®II system and the Stratagene Mx3000 and Mx3000P™ real-time PCR systems.

APPLICATION OF THE PLEXOR™ TECHNOLOGY

Plexor™ System protocols are being developed for quantitative PCR, quantitative RT-PCR and genotyping. Users of the Plexor™ Systems can design and order fluorescently labeled, iso-dC-containing primers specific for their assays.

Predesigned assays using this technology are in development. The first predesigned assays will allow quantitation of human-specific and male-specific DNA. We have demonstrated simultaneous quantitation of total human and male-specific DNA in a two-color multiplex assay, with the potential for further multiplexing.

Stay tuned for more information about this application of the Plexor™ technology in the next issue of *Profiles in DNA.*

The Plexor™ technology is licensed from EraGen Biosciences.

REFERENCES

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