Plexor™ Technology: A New Chemistry for Real-Time PCR

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Introduction

The power of the polymerase chain reaction (PCR) is greatly enhanced by techniques that accurately, sensitively and reproducibly quantify DNA samples. These real-time or quantitative PCR[a] (qPCR) techniques rely on the ability to detect the PCR product at each cycle during the exponential phase. Here we describe the Plexor™ technology, a novel technique for real-time PCR that requires only two primers for sensitive and specific quantification.

The Plexor™ chemistry is a powerful new technology for real-time PCR assays.

Primer Design

Plexor™ technology[a,b] takes advantage of the highly specific interaction between two modified nucleotides for qPCR analysis (1–3). These two novel bases, isoguanine (iso-dG) and 5′-methylisocytosine (iso-dC), form a unique base pair when incorporated in double-stranded DNA and pair only with each other (Figure 1). In Plexor™ reactions, one PCR primer is synthesized with an iso-dC residue and a fluorescent label at the 5′-end. The second PCR primer is unlabeled. Iso-dGTP nucleotides, modified to include dabcyl as a quencher, are included in the reaction mix. During the amplification reaction only dabcyl-iso-dGTP can be incorporated at the position complementary to the iso-dC residue. Incorporation of the dabcyl-iso-dGTP in close proximity to the fluorescent label effectively quenches the fluorescent signal (Figure 2).

The simplicity of primer design is a distinct advantage of the Plexor™ Systems. A web-based primer design program for use with the Plexor™ Systems will be available to design primers for single and multiplex qPCR reactions. The Plexor™ Primer Design software will also select the appropriate fluorescent labels for single and multiplex PCR or quantitative reverse-transcription PCR (qRT-PCR) using several real-time instruments. A number of oligonucleotide suppliers have been licensed to provide primers with an iso-dC residue. Convenient links to these suppliers will be included with the Plexor™ Primer Design software.

Quantitative Amplification

Using this innovative, quantitative technology, the accumulation of amplification product results in a reduction in fluorescence that is proportional to the quantity of the input DNA template (Figure 3). Real-time instrumentation, which couples fluorescence detection and thermal cycling, measures the change of signal (in Relative Fluorescent Units, RFU) at every cycle. Amplification results from Plexor™ reactions present a characteristic three-phase curve (Figure 3). Results obtained during the exponential phase of amplification give the best estimate of the amount of starting material. An amplification threshold is set within the early exponential phase. The cycle number at which the amplification curve crosses this threshold is the cycle threshold (C_t) of the sample. A standard curve can be generated using the C_t values from a dilution series of a sample with a known DNA or RNA quantity.

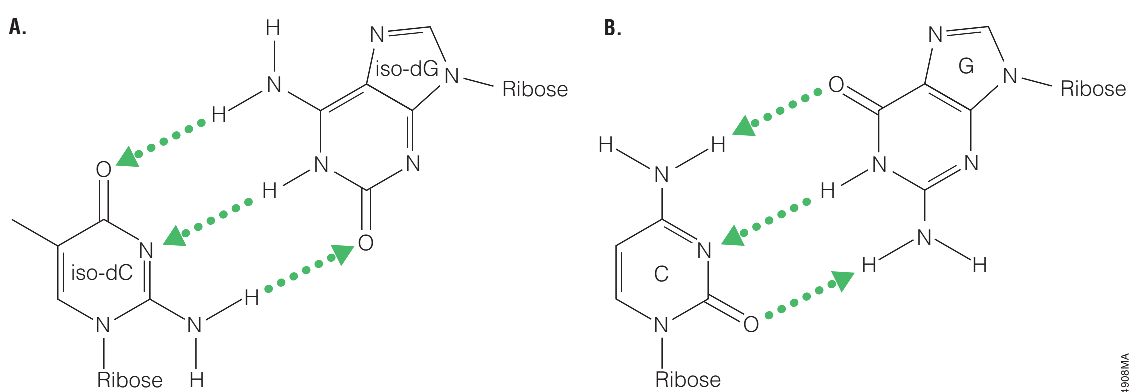

Figure 1. Comparison of base pairing. Panel A. Isoguanine (iso-dG) paired with 5′-methylisocytosine (iso-dC). **Panel B**. Deoxyguanosine paired with deoxycytidine.

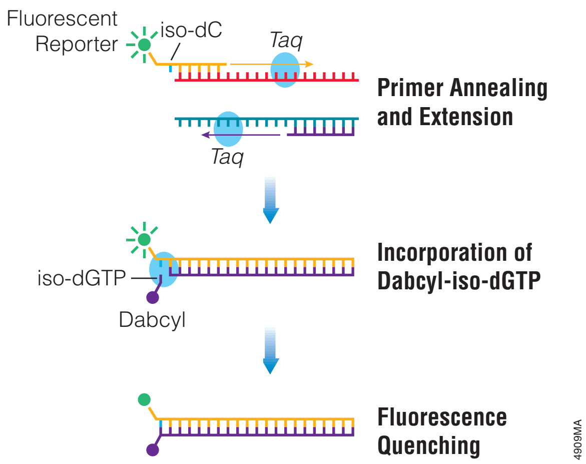

Figure 2. Quenching of the fluorescent signal by dabcyl during product accumulation.

A.

B.

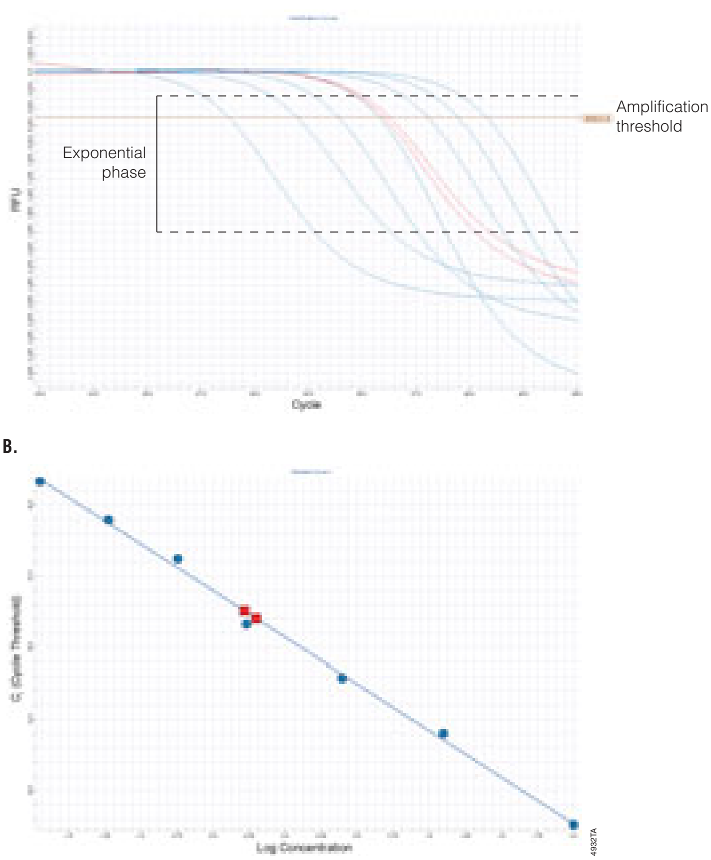

Figure 3. Amplification and standard curves. Panel A. A representative amplification curve, which shows the Relative Fluorescence Units (RFU) at each cycle of the reaction. The amplification threshold is indicated by a horizontal line across the graph. This threshold is used to establish the cycle threshold (C_t), the cycle at which the amplification curve crosses the amplification threshold, for each sample. **Panel B.** A standard curve generated from the amplification curve data shown in Panel A. The blue circles represent standard samples, and the red squares represent unknown samples. Data was collected on a BioRad iCycler® instrument and analyzed using the Plexor™ Analysis Software.

In Plexor™ reactions, the quenching of the fluorescent label by dabcyl is a reversible process. When the product is double-stranded, the dabcyl and fluorescent label are in close proximity and fluorescence is quenched. Denaturing the double-stranded product separates the label and quencher, resulting in an increase in fluorescent signal. Thermal melt (T_m) curves can be generated and used to determine the melting temperature of the amplification products (Figure 4). A T_m profile can be produced by starting at a low temperature (approximately 60°C) and slowly increasing the temperature up to denaturing levels (approximately 95°C). Product length and sequence impact T_m, and consequently, the melt curve characterizes the homogeneity of the amplicons. This is useful for assessing the specificity of the reaction since nonspecific amplification products can be identified by multiple or broad peaks in the melt curve attributed to amplicons with different T_m values. The ability of the melt curve to distinguish specific and nonspecific amplification products validates that only one product is being made.

Applications of the Plexor™ Technology

The broad dynamic range and sensitivity of the Plexor™ technology, as well as the ability to perform multiplex reactions without a loss of sensitivity, make it ideal for a variety of real-time PCR applications. Coupled (one-step) and uncoupled (two-step) RT-PCR can be performed for gene expression studies as well as identification and quantification of viral or other RNAs. The Plexor™ chemistry can also be used for identification and quantification of specific DNA sequences in genomic DNA, mitochondrial DNA, cDNA or viral DNA samples. Finally, single nucleotide polymorphism (SNP) assays can be performed to distinguish between alternative bases at a specific site in a known DNA sequence.

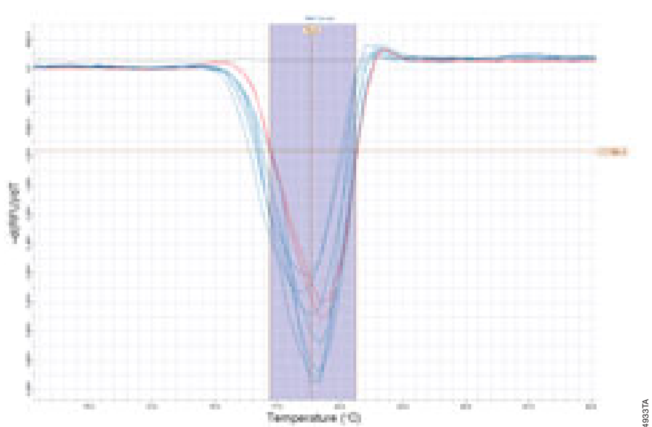

Figure 4. Thermal melt curve. The melting temperature was empirically determined from the data shown in Figure 3 by plotting the change in fluorescence with temperature (–dRFU/dT) versus temperature and calculating the temperature at which the biggest change in fluorescence occurs. Data was collected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

Multiplex Reactions

Two important features enhance the power of the Plexor™ chemistry: the ability to multiplex reactions and the compatibility with a wide range of real-time instrument platforms. In multiplex reactions, one of the primers for each target must have a different fluorescent label. The types and number of fluorescent labels that can be used depend upon the detection capabilities of the real-time instrument used.

Data Analysis and Instrument Compatibility

The Plexor™ Analysis Software will be available to visualize amplification data from various instrument platforms, plot standard curves and calculate DNA concentrations of unknowns. The analysis software will be distributed free-of-charge and allows users to import data from their preferred instrument. Instruments that are compatible with this software are the ABI PRISM® 7000 and 7700 sequence detection systems, Applied Biosystems 7300, 7500 and 7900HT 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® system and the Stratagene real-time PCR systems.

References

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2. Johnson, S.C. *et al.* (2004) *Nucl. Acids Res.* **32**, 1937–41.
3. Moser, M.J. and Prudent, J.R. (2003) *Nucl. Acids Res.* **31**, 5048–53.

Additional Information

For information about how to bring the the Plexor™ technology to your lab, contact Promega at: plexor@promega.com

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