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The Plexor™ Systems Provide Accurate Quantitation in Multiplex qPCR and qRT-PCR

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Introduction

The power of the polymerase chain reaction (PCR) is greatly enhanced by techniques that provide accurate, sensitive and reproducible quantitative data. These real-time or quantitative PCR (qPCR) techniques determine the amount of input template based on the accumulation of PCR product during the exponential phase of an amplification reaction. In this article we introduce the Plexor™ Systems, which use a novel technique for real-time PCR that that requires only two primers for sensitive and specific quantitation.

The Plexor™ technology uses a novel approach to accurate, sensitive and reproducible monoplex or multiplex quantitative PCR and RT-PCR.

Plexor™ Technology

Plexor™ technology[a,b] takes advantage of the highly specific interaction between two modified nucleotides for qPCR[b] analysis (1–3). These two novel bases, isoguanine (iso-dG) and 5´-methylisocytosine (iso-dC), form a unique and specific base pair when incorporated in double-stranded DNA (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 dabcyl-iso-dGTP is preferentially 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).

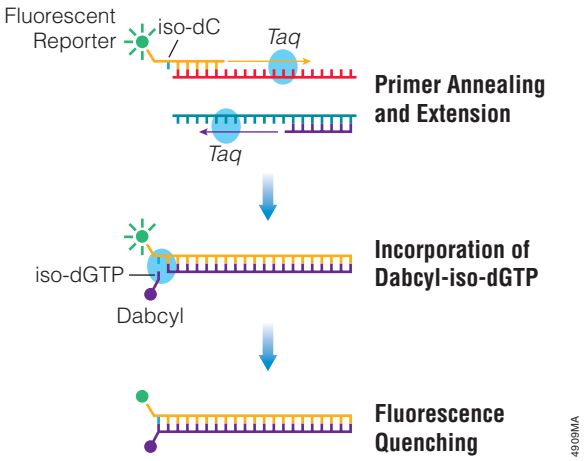

Figure 2. Quenching of the fluorescent signal by dabcyl during product accumulation.

Using the Plexor™ technology, the accumulation of amplification product is associated with a reduction in fluorescence that is proportional to the quantity of the input template (Figure 3). Real-time PCR 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.

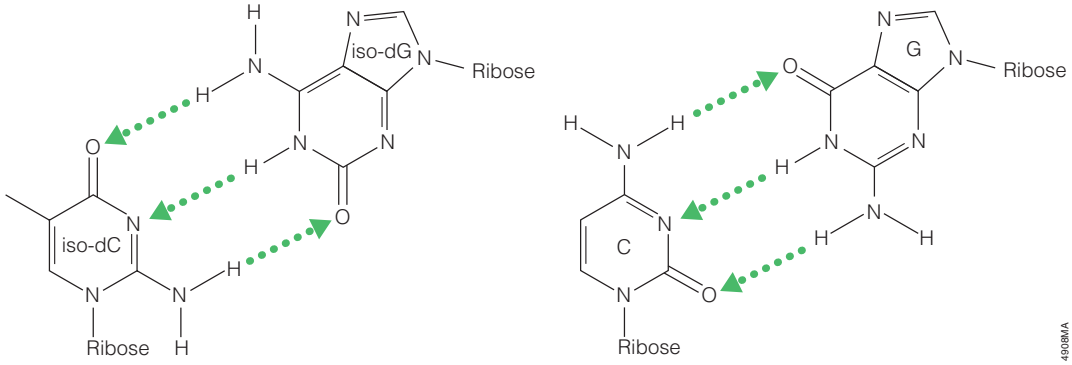

Figure 1. Comparison of base pairing. Panel A. Isoguanine (iso-dG) paired with 5´-methylisocytosine (iso-dC). **Panel B.** Deoxyguanosine paired with deoxycytidine.

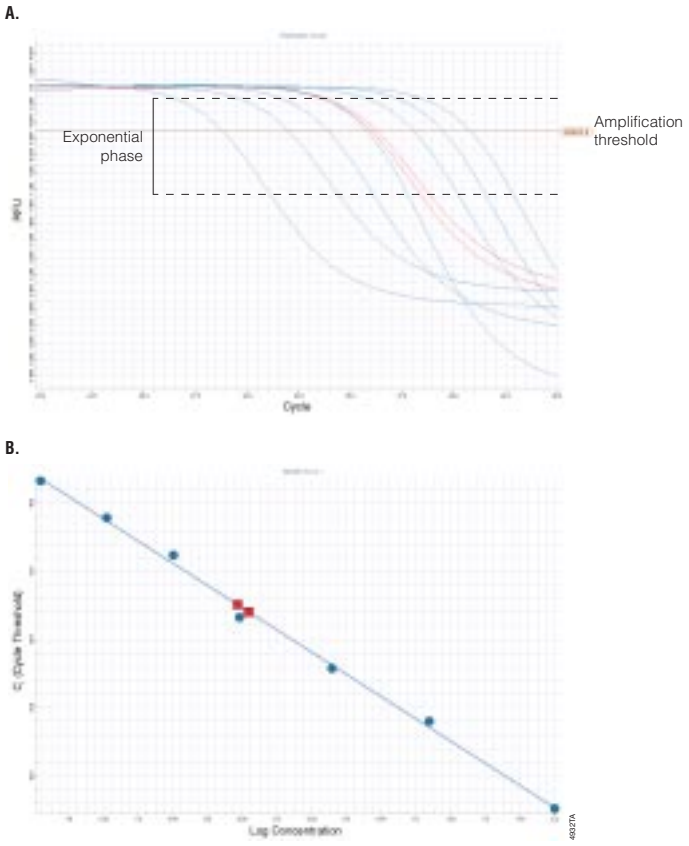

Figure 3. Amplification and standard curves. Panel A. A representative amplification curve, which shows the relative fluorescence units (RFU) at each cycle of the reaction. The amplification threshold is indicated by a horizontal line across the graph. This threshold is used to establish the cycle threshold (C_t), the cycle at which the amplification curve crosses the amplification threshold. **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 Bio-Rad iCycler® instrument and analyzed using the Plexor™ Analysis Software.

In Plexor™ reactions, 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 curves are used to determine the melting temperature (T_m) of the amplification products (Figure 4). Following the amplification reaction, a T_m profile is 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. Nonspecific amplification products can be identified by multiple or broad peaks in the melt curve. This ability to distinguish specific and nonspecific amplification is useful for assessing the specificity of a reaction.

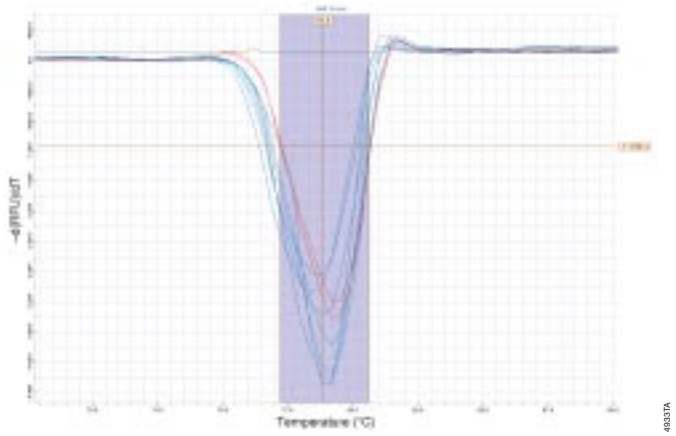

Figure 4. Thermal melt curve. The melting temperature was empirically determined from the data shown in Figure 3 by plotting the change in fluorescence with temperature (–dRFU/dT) versus temperature and calculating the temperature at which the biggest change in fluorescence occurs. Data was collected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

Data Analysis and Instrument Compatibility

An important feature of Plexor™ technology is its compatibility with a wide range of real-time instruments. This is facilitated by the Plexor™ Analysis Software. The Plexor™ Analysis Software allows users to visualize amplification data and melt curves from various instrument platforms, plot standard curves and calculate DNA concentrations of unknowns. Instruments compatible with this software include the ABI PRISM® 7000 and 7700 sequence detection systems, the Applied Biosystems 7300, 7500 and 7900HT real-time PCR systems, the Roche LightCycler® 1.0 and 2.0 instruments, the Bio-Rad iCycler® real-time PCR detection system, the MJ Research DNA Engine Opticon® 2 fluorescence detection system, the Cepheid SmartCycler®, and the Stratagene Mx3000P™ and Mx3005P™ real-time PCR systems and Mx4000® multiplex quantitative PCR system. The Plexor™ Analysis Software can be downloaded free of charge from: **www.promega.com/plexorresources/**

Applications of the Plexor™ Technology

The broad dynamic range and sensitivity of the Plexor™ technology make it ideal for a variety of real-time PCR applications. Using the Plexor™ One-Step qRT-PCR System, both reverse transcription (RT) and amplification are performed in a single reaction. The RT reaction uses ImProm-II™ Reverse Transcriptase, which is included in the Plexor™ One-Step qRT-PCR System. In the Plexor™ Two-Step qRT-PCR System, cDNA is generated using ImProm-II™ Reverse Transcriptase and amplification of the cDNA occurs in a separate reaction. While the Plexor™ One-Step qRT-PCR System provides the convenience of a single reaction, the Plexor™ Two-Step qRT-PCR System is useful when the amount of RNA is

limiting or when many different amplifications will be performed with the same RNA sample. Both the Plexor™ One-Step and Two-Step qRT-PCR Systems can be used for gene expression studies as well as for identification and quantitation of viral or other RNAs.

The Plexor™ qPCR System can be used for the identification and quantitation of specific DNA sequences in genomic DNA, mitochondrial DNA, cDNA or viral DNA samples. The Plexor™ qPCR System can also be used to perform single nucleotide polymorphism (SNP) assays to distinguish between alternative bases at a specific site in a known DNA sequence.

Primer Design

The simplicity of primer design is a distinct advantage of the Plexor™ Systems. A web-based primer design program is available to design primers for monoplex and multiplex qPCR. The Plexor™ Primer Design software will select the appropriate fluorescent labels for monoplex and multiplex qPCR or qRT-PCR on a variety of real-time instruments. Convenient links to oligonucleotide suppliers that have been licensed to provide primers with an iso-dC residue can be found on the Plexor™ Primer Design Web site at: **www.promega.com/plexorresources/**

Multiplex Reactions

The ability to multiplex reactions greatly enhances the power of the Plexor™ technology. In multiplex reactions, the labeled primer for each amplification 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.

The ability of Plexor™ technology to accurately quantitate the expression of three different genes in a single sample is demonstrated in Figure 5. Plexor™ One-Step qRT-PCR System reactions containing increasing amounts of human total RNA were used to quantitate fibroblast growth factor receptor 1 (FGFR1), matrix metalloproteinase 1 (MMP1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA in reactions using one, two or three sets of Plexor™ primer pairs. The Plexor™ Analysis Software was used to generate standard curves for each gene analyzed in single, duplex or triplex reactions. The C_t values of single, duplex and triplex reactions are strikingly similar for each target analyzed.

A significant concern when performing multiplex reactions for quantitation is the potential for the amplification of high-copy templates to affect the amplification of lower-copy templates. This issue was addressed for multiplex reactions using Plexor™ technology (Figure 6). The Plexor™ Two-Step qRT-PCR System was used to analyze human total RNA for the expression of four different genes in reactions containing

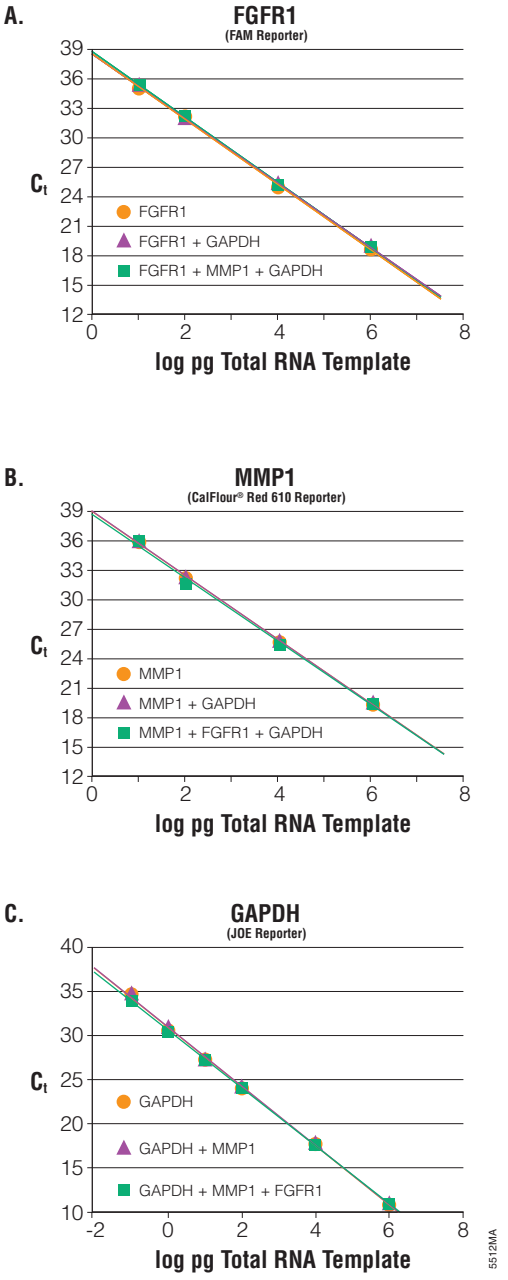

Figure 5. Accurate quantitation of three targets in monoplex, duplex and triplex reactions using the Plexor™ One-Step qRT-PCR System. The indicated quantity of human total RNA was assayed for fibroblast growth factor receptor 1 (FGFR1), matrix metalloproteinase 1 (MMP1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Reactions were set up with one, two or three Plexor™ primer sets as indicated in each panel. Average C_t values were plotted against log picograms of template. **Panel A:** Data from FGFR1 primers labeled with FAM. **Panel B:** Data from MMP1 Plexor™ primers labeled with CalFluor® Red 610. r^2 values ranged from 0.999–1.000 with amplification efficiencies from 99–102%. **Panel C:** Data from GADPH Plexor™ primers labeled with JOE. r^2 values ranged from 0.995–0.999 with amplification efficiencies of 101–102%. r^2 values ranged from 0.999–1.000 and amplification efficiencies ranged from 99–102%. FGFR1 and MMP1 pimers were used at 200nM final concentration. GADPH primers were used at 100nM final concentration. Data were generated with an Applied Biosystems 7500 Real-Time PCR System and analyzed with the Plexor™ Analysis Software. The JOE-labeled primer was synthesized at Promega. The FAM and CalFluor® 610-labeled primers were obtained from Biosearch Technologies.

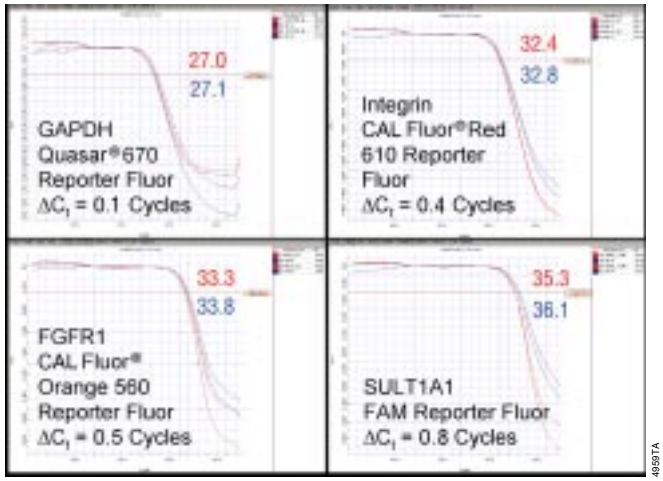

Figure 6. Accurate quantitation of low-copy targets in the presence of high-copy targets using the Plexor™ Two-Step qRT-PCR System. The cDNA generated from 0.1ng of total RNA was assayed as a monoplex (red curves) or in a four-color multiplex (blue curves). The average C_t value is indicated for each target. The C_t value for monoplex versus multiplex reactions varied by less than one cycle, proving that low-copy targets can be assayed accurately in the presence of high-copy targets. Data were generated on the Applied Biosystems 7500 Real-Time PCR System and analyzed with the Plexor™ Analysis Software. Primers were obtained from Biosearch Technologies.

a single Plexor™ primer pair or four Plexor™ primer pairs. The four genes analyzed, GAPDH, FGFR1, integrin and sulfotransferase (SULT1A1), varied greatly in level of expression. For each gene analyzed in this experiment, the C_t values for the quadriplex reactions differed by less than one cycle from the C_t values for the monoplex reactions. This experiment clearly demonstrates the power of the Plexor™ technology for multiplex quantitation.

Summary

The Plexor™ technology uses a novel approach to provide accurate, sensitive and reproducible monoplex or multiplex qPCR and qRT-PCR, and is compatible with a variety of real-time instruments. The Plexor™ Analysis Software will generate amplification and melt curves, plot standard curves and calculate the DNA concentration of unknowns from data generated on various instrument platforms. Plexor™ assay design is facilitated by the web-based Plexor™ Primer Design software for monoplex or multiplex primer design. The Plexor™ Primer Design software will also assist in the selection of fluorescent labels that can be detected by the various real-time instruments.

References

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3. Moser, M.J. and Prudent, J.R. (2003) *Nucl. Acids Res.* **31**, 5048–53.

Additional Information

For information about how to bring the Plexor™ technology to your lab, contact Promega at:
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