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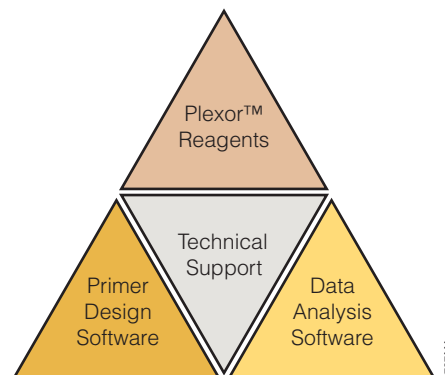
Real Power for Real-Time PCR

An Introduction to the Plexor™ Systems

By Kyle Hooper, Ph.D., Promega Corporation.

The Plexor™ Systems offer easy multiplexing of quantitative PCR (qPCR) and qRT-PCR, increasing productivity and giving you more confidence in your results. With the Plexor™ Systems you can assay relative levels of target and control genes in the same reaction well, increasing accuracy over monoplex reactions by eliminating pipetting differences between target and control wells. Multiplex reactions also increase productivity as less space is used on the plate, leaving more wells for additional samples and allowing generation of more data per plate. In addition, you will use less sample and less master mix, providing an added cost benefit. Here we provide a brief overview of the Plexor™ Systems.

The Plexor™ Systems are not just a set of reagents— they provide a comprehensive approach to real-time PCR beginning with assay design and ending with data analysis.



The Plexor™ Systems provide a comprehensive approach to real-time qPCR and qRT-PCR that begins with assay design and ends with data analysis.

also saves reagents and plate space, allowing you to assay more samples per plate. Multiplexing can be extended to two or three targets and a control gene.

To fully utilize the power of Plexor™ technology, one must design a multiplex assay. Many currently available primer design programs only design primers for monoplex reactions. Therefore, a design engine that takes multiplexing into account during assay design is required. Our free Primer Design Software is available at: www.promega.com/plexorresources/. All you need to do to access this program is register on our web site.

Use of the Primer Design Software is described in more detail in the article “Designing Your Plexor™ Assay” beginning on page 15. Briefly, the following simple steps are required:

1. **Select your instrument.** This allows the software to determine how many possible sequences you could enter based on the detection capabilities of your instrument.
2. **Input your target sequences** and submit the experiment for primer design.
3. **Select primers from the options provided and choose an oligonucleotide supplier.** Currently, Integrated DNA Technologies (www.idtdna.com), Biosearch Technologies (www.biosearch.com) and, in Europe, Eurogentec (www.eurogentec.be) are licensed to provide Plexor™ Primers. Once an oligonucleotide provider is selected, the Primer Design Software determines which fluors must be attached to your primers based on the number of targets, the capabilities of the selected instrument and the fluors available from your chosen supplier.

The Basis of Plexor™ System Reactions

The Plexor™ Systems^(a-c) use standard PCR and are based on the interaction between two novel bases, iso-C and iso-G, which form a unique base pair. One PCR primer is modified to contain an iso-C base and a fluorescent label at the 5' end. The second primer is unlabeled. During PCR, iso-dGTP is incorporated at the position complementary to iso-C. The iso-dGTP in the Plexor™ reaction mix is modified with the quencher, dabcy1. Incorporation of dabcy1-iso-dGTP quenches the fluorescence from the fluor attached to the iso-C base on the primer. Fluorescence quenching is proportional to product accumulation, and the results are displayed as cycle threshold (C_t) values. At the end of the PCR, the reaction products can be heated to separate the strands and measure amplification specificity by monitoring melting temperature (T_m). For more information on Plexor™ chemistry, see the article “Fluorescent Real-Time PCR..Simplified” on page 3.

The Power of Multiplexing

The dabcy1-iso-dGTP used in the Plexor™ Method specifically quenches fluorescence from any fluorescently labeled primer containing an iso-C base. Therefore, more than one labeled primer can be introduced into the reaction, allowing multiplexing of real-time PCR. In multiplex reactions a different fluor is used for each target. Multiplexing can be as simple as combining a target and a control gene in the same reaction, increasing accuracy over separate reactions with the target in one set of wells and the control in another. Multiplexing not only increases accuracy but

Real Power for Real-Time PCR... continued

Three Simple Steps to use the Plexor™ Systems.

Step 1: Design Your Assay

The Plexor™ Primer Design Software provides a simple online tool for design of your multiplex assay. Choose your primer sets, then order from your preferred oligonucleotide provider.

Step 2: Run the Assay

Instruction manuals are available for a wide variety of real-time instruments including those from Applied Biosystems and Roche. All Plexor™ assays are designed to use the same cycling conditions.

Step 3: Analyze Your Data

Export the raw data from your real-time instrument, then import into our free Plexor™ Analysis Software. The Analysis Software converts the quenching data into C_t values, allows you to generate a standard curve, and displays the melt curve(s).

The Power to Choose

Three Plexor™ Reagent Systems are available: The Plexor™ qPCR System, the Plexor™ Two-Step qRT-PCR System and the Plexor™ One-Step qRT-PCR System. All three systems use the same Plexor™ Master Mix for qPCR.

The **Plexor™ qPCR System** can be used for quantitation starting with a genomic DNA template, and including genotyping or gene expression analysis from cDNA generated with your favorite reverse transcription reagents.

Choose the **Plexor™ Two-Step qRT-PCR System** to first create cDNA from your RNA sample then perform qPCR in a separate reaction. Choose the **Plexor™ One-Step qRT-PCR System** to go directly from RNA to qPCR in a coupled reaction. Both of these systems use ImProm-II™ Reverse Transcriptase for cDNA synthesis.

Each Plexor™ System is shipped with a technical manual explaining how to assemble the various reactions. To run the reactions, you will need to download the instruction manual specific for your real-time instrument. Instruments supported at the launch of the Plexor™ Systems are listed in Table 1. Please visit www.promega.com/plexorresources/ for the most up-to-date information on supported instruments.

Plexor™ Analysis Software

You have ordered your Plexor™ primers, set up your reactions and run them on your selected real-time instrument. The analysis software that comes with real-time instruments is designed to analyze gain-of-fluorescence data rather than fluorescence-quenching data. However, all instruments can export the raw fluorescence data. Plexor™ data is analyzed by simply importing the raw data into our free Plexor™ Analysis Software. The software installer is available at: www.promega.com/plexorresources/.

Table 1. Supported Real-Time Instruments.

Instruction manuals for use of Plexor™ Technology on the following instruments are available at www.promega.com/plexorresources/. The list of supported instruments may change as new instruments are added. Please visit the web site for the most up-to-date information.

Supported Instruments

Applied Biosystems 7300 and 7500 Real-Time PCR Systems
ABI PRISM® 7000 Sequence Detection System
ABI PRISM® 7700 Sequence Detection System
Applied Biosystems 7900HT Real-Time PCR System
Roche LightCycler® 1.0 and 2.0 Instruments
Bio-Rad iCycler iQ® Real-Time PCR Detection System
MJ Research DNA Engine Opticon®-2 Fluorescence Detection System
Cepheid SmartCycler® System
Stratagene Mx3000P™ and Mx3005P™ Real-Time PCR Systems
Stratagene Mx4000P® Multiplex Quantitative PCR System

The Plexor™ Analysis Software provides all the tools you need to analyze Plexor™ reaction data. In the Analysis Software interface, each fluor in a multiplex reaction is represented by a tab on the display and is analyzed separately. Output from the Analysis Software includes amplification curves, C_t values, standard curves, melt curves and T_m information. For more details on use of the Plexor™ Analysis Software, see the article “Programmed to Make Analysis Easy” beginning on page 22. The Plexor™ System instrument manuals provide all the information needed to export and analyze the data from your instrument.

Summary

The Plexor™ Systems are not just a set of reagents—they provide a comprehensive approach to real-time PCR that begins with assay design and ends with data analysis. Throughout the process, you can count on expert technical assistance from Promega Technical Services. To bring Plexor™ to your laboratory, please contact your local Promega representative, or e-mail plexor@promega.com or techserv@promega.com if you have further questions.

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Fluorescent Real-Time PCR...Simplified

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

By Susan Frackman, Ph.D., Steve Ekenberg, Ph.D., Katharine Hoffmann, B.S., Benjamin Krenke, M.S., Cynthia Sprecher, B.S., and Douglas R. Storts, Ph.D., Promega Corporation.

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 (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 per target for sensitive and specific quantification.

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

Primer Design

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

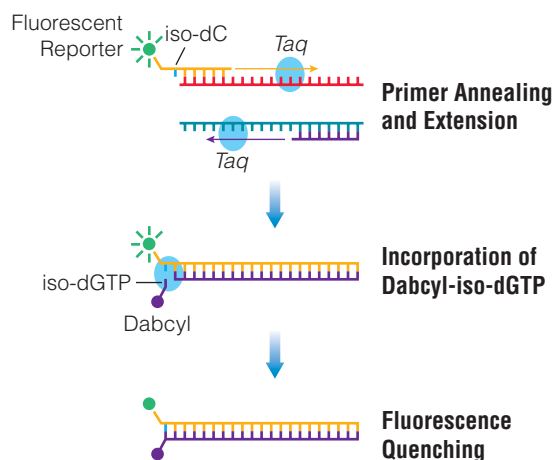


Figure 1. Quenching of the fluorescent signal by dabcyI during product accumulation.

Quantitative Amplification

Using Plexor™ technology, the accumulation of amplification product results in a reduction in fluorescence that is proportional to the quantity of the input DNA template. Real-time instrumentation, which couples fluorescence detection and thermal cycling, measures the change of signal (in relative fluorescence units, RFU) at every cycle. Amplification results from Plexor™ reactions present a characteristic three-phase curve (Figure 2). Results obtained during the exponential phase 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.

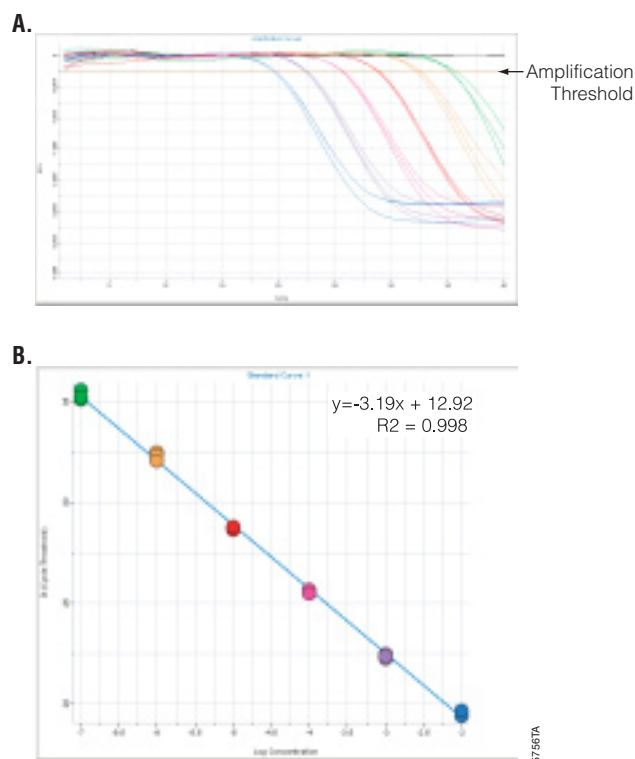


Figure 2. Amplification and standard curves. Panel A. A representative amplification curve, which shows the relative fluorescence units (RFU) at each cycle of the reaction. The amplification threshold is indicated by a horizontal line across the graph. This threshold is used to establish the cycle threshold (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. Data were collected on an Applied Biosystems 7500 Real-Time PCR System and analyzed using the Plexor™ Analysis Software.

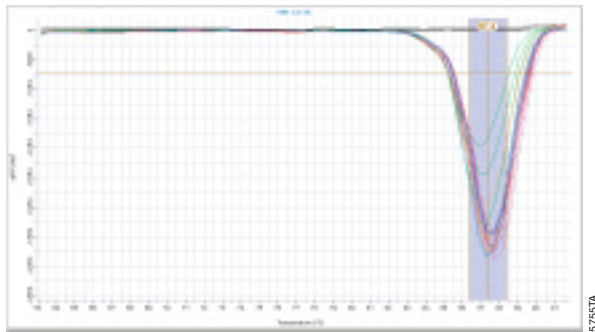


Figure 3. Thermal melt curve. The melting temperature was empirically determined from the data shown in Figure 2 by plotting the change in fluorescence with temperature ($-dRFU/dT$) versus temperature and calculating the temperature at which the biggest change in fluorescence occurs. Data was collected and analyzed as described for Figure 2.

In Plexor™ reactions, the quenching of the fluorescent signal by dabcyil is a reversible process. When the product is double-stranded, the dabcyil 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 3). 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. 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 (4).

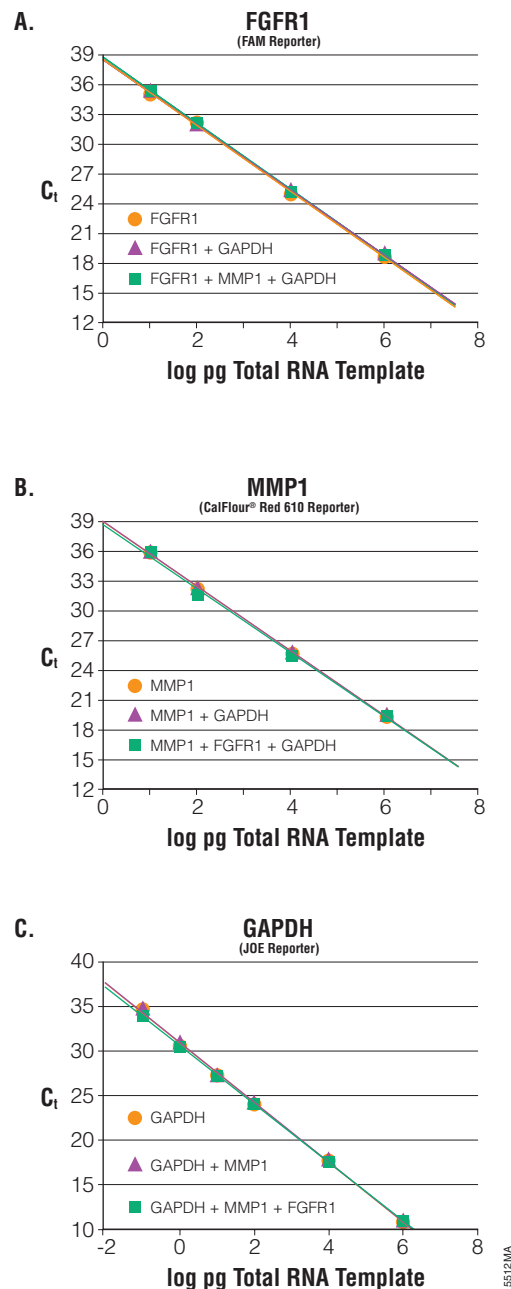


Figure 4. Accurate quantitation of three targets in monoplex, duplex and triplex reactions using the Plexor™ One-Step qRT-PCR System. The indicated quantity of human total RNA was assayed for fibroblast growth factor receptor 1 (FGFR1), matrix metalloproteinase 1 (MMP1) and glyceraldehyde-3-phosphate 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 GAPDH 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 primers were used at 200nM final concentration. GAPDH 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.

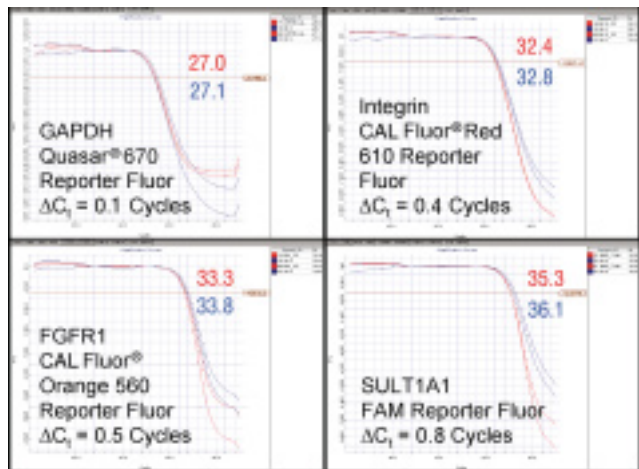


Figure 5. Accurate quantitation of low-copy targets in the presence of high-copy targets using the Plexor™ Two-Step qRT-PCR System. The cDNA generated from 0.1 ng of total RNA was assayed as a monoplex (red curves) or in a four-color multiplex (blue curves). The average C_t value is indicated for each 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.

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 4. 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 5). The Plexor™ Two-Step qRT-PCR System was used to analyze human total RNA for the expression of four different genes in reactions containing 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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Additional Information

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

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Multiplex Reactions for SNP Detection

SNP Genotyping with the Plexor™ qPCR System

By Susan Frackman, Ph.D., and Benjamin Krenke, M.S. Promega Corporation

This article describes how the Plexor™ qPCR System can be used in genotyping assays to distinguish between two alternative bases at a specific site in a known DNA sequence. The multiplexing capabilities of the Plexor™ technology allow distinction of different alleles, such as single nucleotide polymorphisms (SNPs). Plexor™ genotyping reactions use a three-primer method for allele-specific PCR: two labeled primers for allele differentiation, and a common, unlabeled anchor primer. The allele-specific primers are each labeled with a different fluor and the genotype is called, not from the amplification curves, but from the melt curves for each fluor.

The Plexor™ qPCR System provides a convenient method for SNP genotyping. Each genotyping primer is labeled with a different fluorescent dye so that alleles can be easily discriminated.

Introduction

A single nucleotide polymorphism (SNP) is a single base pair substitution of one nucleotide; both genotypes are observed in the general population at a frequency of greater than 1% (1). It is estimated that there are at least 10 million SNPs in the human population—making them the most common type of genomic variation (2). In some cases, specific SNPs have been correlated with disease susceptibility (3). Other SNPs affect drug metabolism and are indicators of individual differences in drug efficacy and the occurrence of side effects (4).

Plexor™ Genotyping Primers

The Plexor™ qPCR System^(a-c) provides a convenient method for SNP genotyping (5). The Plexor™ Primer Design Software (www.promega.com/plexorresources/) is used with the Plexor™ qPCR System to assist in the design of SNP genotyping primers and the selection of the appropriate fluorescent labels for use with different instruments. Genotyping reactions with the Plexor™ qPCR System use three primers in a single allele-specific PCR (Figure 1). There are two genotyping primers, each containing one of the polymorphic bases near the 3' end and an iso-dC at the 5' end. Each genotyping primer is labeled with a different fluorescent dye so that the alleles can be discriminated. The genotyping primers also have a region that is not complementary to the template, and the sequence of this non-complementary region is different for each genotyping primer. The third primer is an unlabeled anchor primer. A PCR product

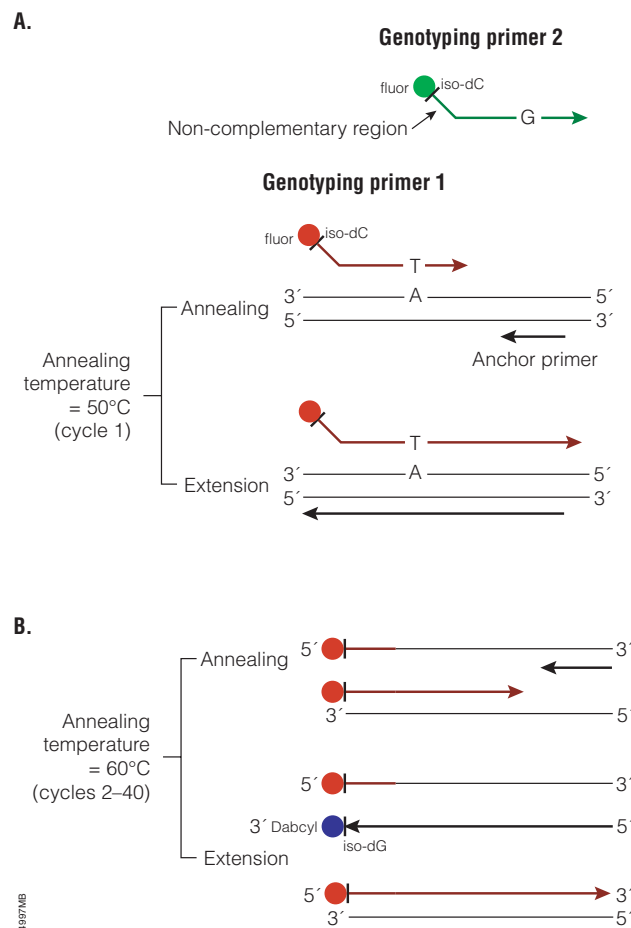


Figure 1. Schematic of a genotyping experiment using the Plexor™ qPCR System. Panel A. First round of amplification at 50°C. **Panel B.** Subsequent rounds of amplification at 60°C.

will be formed in any reaction containing the anchor primer and either genotyping primer when the appropriate template is present.

The design of the genotyping primers enhances amplification specificity. Initially, the two genotyping primers have an annealing temperature lower than that of the anchor primer, and the first amplification cycle is performed at an annealing temperature appropriate for the region of the genotyping primers that is complementary to the template (Figure 1A). During subsequent cycles, the unique, non-complementary, region of the genotyping primer is incorporated into the amplicons, allowing use of a higher annealing temperature (Figure 1B). At the higher annealing temperature only the product from the first round of PCR will be amplified. When the iso-dG with an

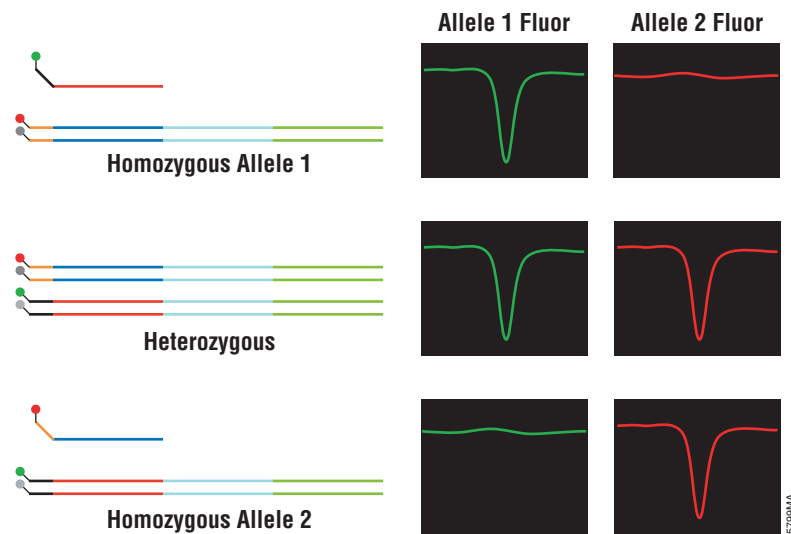


Figure 2. Expected results for a genotyping experiment using the Plexor™ qPCR System.

attached quencher molecule (in the Plexor™ Reaction Mix) base pairs with the iso-dC in the annealed genotyping primer, fluorescence from the PCR product is quenched.

Melt Curve Analysis

Genotype assignments are made by analyzing the melting curves of the amplified products. The melt curve shows the change in fluorescence as temperature increases. As the strands separate the fluorescent dye is no longer quenched, causing an increase in the fluorescent signal. The fluorescence state of the unincorporated primers does not change, so if no product is generated during PCR the melt curve will be flat. Figure 2 shows the expected results for a Plexor™ SNP genotyping reaction with three different templates. Samples that are homozygous for either of the alleles will result in a change in the signal from only one of the two fluorescent dyes. For heterozygous samples, there will be a change in the fluorescence signal from both dyes.

As a model system for use of Plexor™ technology for SNP genotyping, we selected a polymorphism within the hemochromatosis gene *HFE*. Hereditary hemochromatosis, a disorder of iron metabolism that results in iron accumulation in a variety of organs, is a common genetic disease in individuals of Northern European descent (reviewed in reference 6). The recessive C282Y mutation is a G to A transition at nucleotide 845, which results in substitution of tyrosine for cysteine at amino acid position 282. This is the most common *HFE* mutation and has been associated with a severe form of hemochromatosis. Two other mutations have been associated with a milder form of the disease. They are H63D, which is a C to G transversion at nucleotide position 63 and results in replacement of

histidine with aspartic acid, and S65C, which is an A to T substitution at nucleotide position 193 that results in replacement of serine with cysteine.

To demonstrate the utility of the Plexor™ qPCR System for SNP detection, primers were designed for two of the *HFE* mutations, C282Y and H63D. Genotyping primers were designed using the Plexor™ Primer Design Software, and data were analyzed using the Plexor™ Analysis Software. Figure 3 shows the amplification and melt curves from an experiment using three different genomic DNAs previously characterized as wildtype, heterozygous or homozygous for the C282Y mutation. The wildtype primer was labeled with CAL-Fluor® Orange 560 (CFO; Biosearch Technologies, Inc.) and the mutant primer was labeled with FAM™. CAL-Fluor® Orange has comparable spectral properties to HEX™. As expected, in reactions with wildtype DNA amplification only occurred with the CFO-labeled, wildtype primer and was detected in the amplification and melt curves from the CFO channel. Amplification from DNA carrying the homozygous C282Y mutation occurred only with the FAM™-labeled mutant primer and was only detected in the FAM™ channel. Reactions containing DNA heterozygous for the C282Y mutation showed amplification with both the wildtype and mutant primers that was seen in both the CFO and FAM™ channels. There was some nonspecific amplification, probably due to primer-dimer, in the reactions involving the mutant primer that can be seen in both the melt and amplification curves from the FAM™ channel. This nonspecific amplification did not affect genotype determination because the resulting amplification product, as seen in the melt curve, is clearly distinct from the expected product.

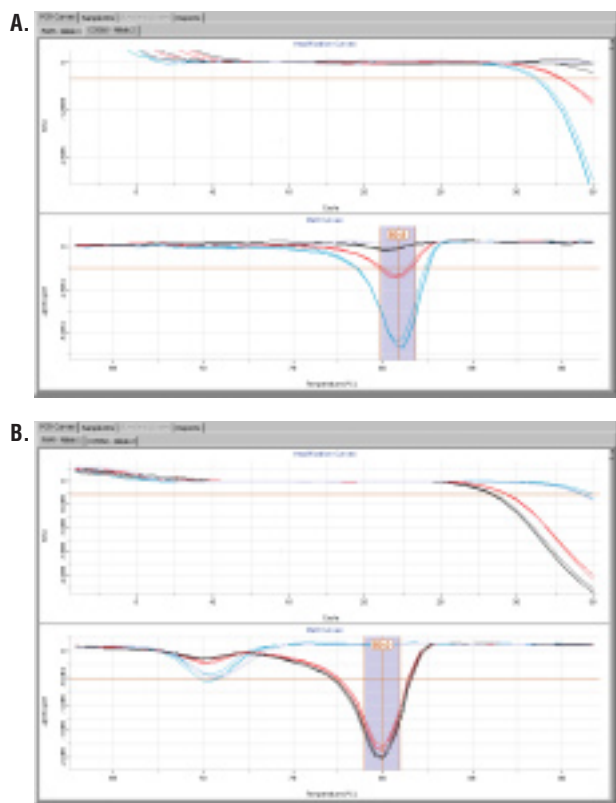


Figure 3. Use of the Plexor™ qPCR System to genotype the C282Y allele of the HFE gene. Plexor™ amplification reactions were performed using 10ng of DNA carrying the wildtype allele (blue), heterozygous for the C282Y allele (red) or homozygous for the CY282 mutant allele (black). The wildtype primer was labeled with CFO and the CY282 primer was labeled with FAM™. **Panel A.** Amplification and melt curves in the CFO channel. **Panel B.** Amplification and melt curves in the FAM™ channel. The Plexor™ Analysis Software was used for data analysis; the scale of the Y axis in the CFO channel was changed from the default setting in order to better visualize the results.

A second example of Plexor™ genotyping is shown in Figure 4. In this example, primers for the HFE H63D mutation were designed. The wildtype primer was labeled with FAM™, and the mutant primer was labeled with CFO. When these primers were used in reactions containing DNA previously known to be wildtype, homozygous or heterozygous for the H63D allele, the predicted pattern of amplification was clearly seen.

Conclusion

The multiplexing capabilities of the Plexor™ qPCR System allow distinction of different alleles, such as single nucleotide polymorphisms (SNPs), in a single reaction. Two allele-specific genotyping primers, each of which is labeled with a different fluor, and an unlabeled anchor primer are used in each reaction. In this article, the Plexor™ qPCR System was used to successfully identify DNA as wildtype, homozygous or heterozygous for a given allele in model system for detection of mutations in the hemochromatosis gene, HFE.

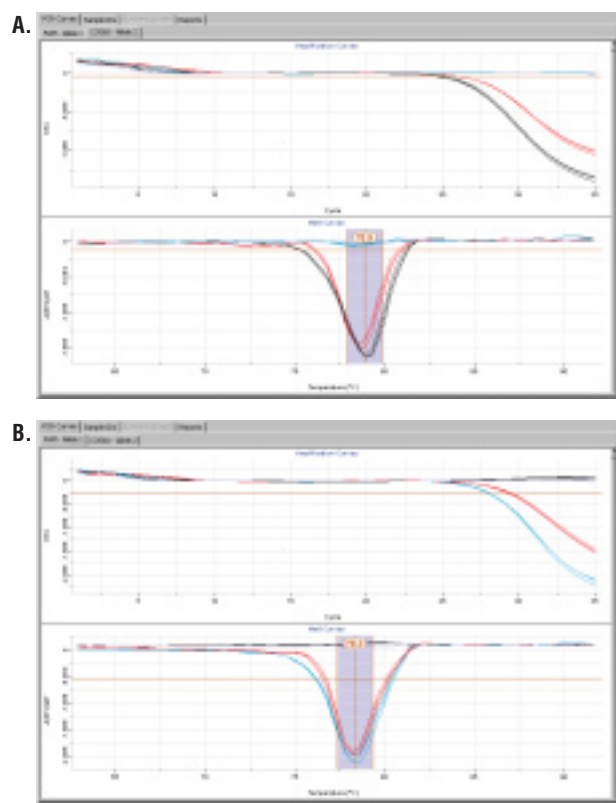


Figure 4. Use of the Plexor™ qPCR System to genotype the H63D allele of the HFE gene. Plexor™ amplification reactions were performed using 10ng of DNA carrying the wildtype allele (blue), heterozygous for the H63D allele (red) or homozygous for H63D mutant allele (black). **Panel A.** Amplification and melt curves in the CFO channel. **Panel B.** Amplification and melt curves in the FAM™ channel. The Plexor™ Analysis Software was used for data analysis.

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Plexor™ Technology vs. SYBR® Green

A Comparison of Plexor™ and SYBR® Green Chemistries

By Benjamin Krenke, M.S., Katharine Hoffmann, B.S., Cindy Sprecher, B.S., Kyle Hooper, Ph.D., and Douglas R. Storts, Ph.D., Promega Corporation.

Real-time PCR methods are based upon a change in fluorescence associated with the accumulation of amplification products. During thermal cycling, the change in fluorescence is monitored in real time. There are different approaches that change the fluorescence including probe cleavage (e.g., TaqMan® chemistry), double-stranded DNA-binding dyes (e.g., SYBR® Green), primer extension (e.g., Molecular Beacons) or incorporation of a fluorescence quencher to reduce the signal generated by a fluorescently-labeled primer (e.g., Plexor™ technology). In this article, we present data on the performance of the Plexor™ technology and the commonly used SYBR® Green approach for real-time, quantitative PCR.

The Plexor™ qRT-PCR Systems offer equivalent sensitivity compared to SYBR® Green dye for real-time PCR detection.

Plexor™ Technology

The Plexor™ Systems^(a-c) are multiplex-capable, real-time amplification systems that use novel base pair chemistry (1–4). Each target is measured directly during the amplification process, in contrast to using a secondary reaction to detect product accumulation (e.g., probe cleavage). Plexor™ reactions require only two primers for each target. Multiplex-assay design is further simplified by the use of the web-based Plexor™ Primer Design Software, which is specifically engineered for multiplex-assay design.

Using the Plexor™ Systems, product accumulation is measured as a reduction in fluorescent signal during amplification. The reaction uses only two primers, one of which contains both a fluorescent tag and a modified base. The other primer is unmodified. As amplification proceeds, fluorescence is reduced by the site-specific incorporation of a fluorescent quencher, which is attached to a modified nucleotide (iso-dG) and inserted opposite the complementary modified base (iso-dC). The quencher is in close proximity to a fluorescent dye located on the 5' end of the primer, resulting in a reduction in the fluorescent signal. After PCR, a melt curve analysis can be performed to expedite troubleshooting during assay development and provide an internal control during routine use of an optimized assay. The system also includes a proprietary reagent to minimize primer-dimer formation.

Many real-time instruments can be used to perform Plexor™ reactions and collect the raw data (for a list of currently supported instruments, visit: www.promega.com/plexorresources/). Because the data analysis software bundled with most instruments is only designed to handle an increase in fluorescence, the software packages cannot directly analyze the Plexor™ data. To analyze the change in fluorescence associated with the Plexor™ chemistry, the raw data collected by the instrument must be imported into the Plexor™ Analysis Software, available for free download at: www.promega.com/plexorresources/

SYBR® Green Technology

SYBR® Green can be used to monitor the accumulation of amplicons in real-time amplification reactions. PCR is performed with two unlabeled primers and SYBR® Green dye. As product accumulates, the dye binds nonspecifically to the double-stranded DNA.

This interaction with the changing level of double-stranded DNA yields an increase in fluorescent signal. As product accumulates, the resulting fluorescent signal increases. At the end of the amplification, the double-stranded DNA products with bound SYBR® Green can be denatured into single strands, liberating the dye with a concomitant decrease in fluorescence intensity. The fluorescence data is used to plot a melt curve and calculate the melting temperature (T_m) for the amplicons in the reaction.

Comparison of Plexor™ and SYBR® Green Chemistries

The Plexor™ and SYBR® Green methods for real-time PCR have many similarities, but there are some significant differences (Table 1). The Plexor™ chemistry directly measures the accumulation of amplicons incorporating a labeled primer. Conversely, SYBR® Green measures any double-stranded DNA generated in the reaction. The Plexor™ chemistry will only measure nonspecific amplicons if they include the labeled primer.

We designed an experiment to directly compare the data generated from both Plexor™ and SYBR® Green chemistries. Target-specific primer sequences used for both reactions were designed with the free Plexor™ Primer Design Software (available at: www.promega.com/plexorresources/). The labeled Plexor™ primer was created by conjugating

Plexor™ Technology vs. SYBR® Green... continued

Table 1. Comparison of the Plexor™ and SYBR® Green Chemistries

	Plexor™ Chemistry	SYBR® Green Chemistry
Mode	Quenching of fluorescence	Gain of fluorescence
Mechanism	Fluorescent label on primer	Binding of dye to double-stranded DNA (dsDNA)
Primers	One labeled primer, one unlabeled primer	Two unlabeled primers
Outputs	Cycle threshold (C _t) value Thermal melt temperature (T _m)	Cycle threshold (C _t) value Thermal melt temperature (T _m)
Multiplex Capability	Yes	No

methylisocytosine (iso-dC) to the 5' end of the upstream PCR primer. The primer also contained a FAM™ label at the 5' end. The SYBR® Green reactions were performed with the same primer sequences except that the upstream primer did not include the FAM™ label and iso-dC. For amplification, we used the Applied Biosystems SYBR® Green RT-PCR Reagents and the Plexor™ One-Step qRT-PCR System. Each amplification reaction contained 200nM primer. The protocol included a 30-minute reverse transcription step, followed by 40 cycles of PCR. Thermal cycling was performed on an Applied Biosystems 7500 Real-Time PCR Instrument. For the SYBR® Green reactions, we performed an extended incubation at 95°C to activate the AmpliTaq Gold® DNA polymerase. The Plexor™ System does not use a hot-start DNA polymerase and, therefore, does not require an activation step. All reactions used dilutions of Universal Human Reference RNA (Stratagene Cat.# 740000) and were tested in quadruplicate at 100pg, 1ng, 10ng and 100ng of total RNA. No-template control reactions were performed to monitor the accumulation of nonspecific amplification products.

SYBR® Green amplification reactions reached the cycle threshold (C_t) earlier than the Plexor™ reactions (i.e., reached the cycle threshold in fewer cycles for a given RNA concentration). However, the Plexor™ reactions produced tighter linear regressions and higher efficiencies (Figure 1). In addition, it is important to note that different data analysis packages were used to analyze the results for each chemistry. This accounts for some of the differences seen in the data. Although the SYBR® Green reactions used a hot-start *Taq* DNA polymerase, each reaction yielded nonspecific amplification products in the no-template controls. The Plexor™ reactions, which contained a primer-dimer inhibitor as opposed to a hot-start DNA polymerase, also generated nonspecific amplification products in the no-template control reactions. Because the appearance of nonspecific amplification products did not occur until lesser amounts of RNA template were used in the Plexor™ reactions, the limit of detection was lower than in the corresponding SYBR® Green reactions (see Table 2).

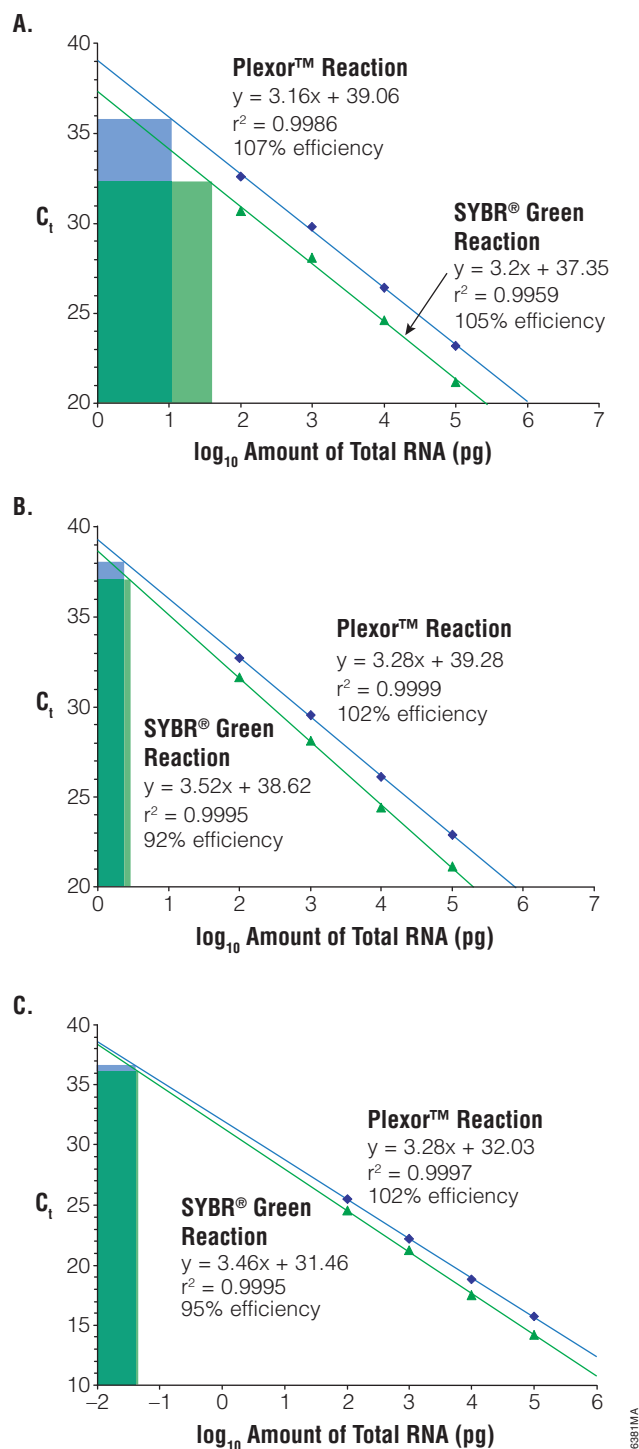


Figure 1. qRT-PCR analysis of total RNA samples. Plots of C_t values generated with SYBR® Green and Plexor™ chemistries using the indicated concentrations of human total RNA. The cycle at which nonspecific amplification products cross the cycle threshold in the no-template controls is indicated by either the green (SYBR® Green Reaction) or blue box (Plexor™ Reaction) within each graph. **Panel A.** Amplification of fibroblast growth factor receptor 1 (GenBank® Accession# NM_000604). **Panel B.** Amplification of matrix metalloproteinase-1 (GenBank® Accession# NM_002421). **Panel C.** Amplification of glycereraldehyde-3-phosphotransferase (GenBank® Accession# NM_002046).

Table 2. Detection Limit of Plexor™ and SYBR® Green Chemistries.

The average C_t value ($n = 4$) of the nonspecific amplification products observed in the no-template controls was determined by linear regression of the plot generated by titrating known amounts of input RNA for each target. This limit-of-detection value was used to determine the corresponding quantity of the total RNA template. Amplification targets were fibroblast growth factor receptor 1 (FGFR1), matrix metalloproteinase-1 (MMP1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Target	Measurement	Plexor™ Assay	SYBR® Green Assay
FGFR1	No-template control (Avg. C_t)	35.7	32.2
	Limit of detection	11.6pg	40.7pg
MMP1	No-template control (Avg. C_t)	38.0 ¹	37.0
	Limit of detection	2.5pg	2.9pg
GAPDH	No-template control (Avg. C_t)	36.6	36.1 ²
	Limit of detection	40fg	46fg

¹Nonspecific amplification observed in 1 of 4 no-template control reactions.

²Nonspecific amplification observed in 3 of 4 no-template control reactions.

For all other reactions, nonspecific amplification was observed in 4 of 4 no-template control reactions.

Conclusions

The Plexor™ and SYBR® Green chemistries offer comparable detection sensitivity, although the limit of detection for the Plexor™ assays was slightly better than that observed in the SYBR® Green assays. The difference in C_t values for both chemistries is largely due to different software evaluations of the raw data and conversion into C_t values, as opposed to differences in amplification efficiency. While this article addressed the comparison of Plexor™ and SYBR® Green chemistries in a monoplex system, one distinct characteristic of the Plexor™ Systems is the ability to multiplex (see the article “Validation of the Plexor™ Primer Design Software” on page 19).

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Plexor™ Technology vs. TaqMan® Assays

A Comparison of Plexor™ and 5' Nuclease Assay Chemistries

By Katharine Hoffmann, B.S., Benjamin Krenke, M.S., Cindy Sprecher, B.S., Kyle Hooper, Ph.D., and Douglas R. Storts, Ph.D., Promega Corporation.

Real-time PCR approaches are based upon a change in fluorescence associated with the accumulation of amplification products. The change in fluorescence is monitored in real time during thermal cycling. Fluorescence changes may be attributed to probe cleavage (e.g., TaqMan® chemistry), double-stranded DNA-binding dyes (e.g., SYBR® Green), primer extension (e.g., Molecular Beacons) or by incorporation of a fluorescence quencher to reduce the signal generated by a fluorescently-labeled primer (e.g., Plexor™ technology). In this article, we present data on the performance of the Plexor™ technology versus the commonly used 5' nuclease assay, the TaqMan® chemistry, for real-time, quantitative PCR.

The Plexor™ qRT-PCR Systems offer similar sensitivity compared to the TaqMan® Systems.

Plexor™ Technology

The Plexor™ Systems^(a-c) are multiplex-capable, real-time amplification systems that use novel base pair chemistry (1–4). Each target is measured directly during the amplification process, not through a secondary reaction. Plexor™ reactions require only two primers for each target. Multiplex-assay design is further simplified by the use of the web-based Plexor™ Primer Design Software, which is specifically engineered for multiplex-assay design.

Using the Plexor™ Systems, product accumulation is measured as a reduction in fluorescent signal during amplification. The reaction uses only two primers, one of which contains both a fluorescent tag and a modified base. The other primer is unmodified. As amplification proceeds, fluorescence is reduced by the site-specific incorporation of a fluorescent quencher, which is attached to a modified nucleotide (iso-dG) and inserted opposite the complementary modified base (iso-dC). The quencher is in close proximity to a fluorescent dye located on the 5' end of the primer, resulting in a reduction in the fluorescent signal. After PCR, a melt curve analysis can be performed to expedite troubleshooting during assay development and provide an internal control during routine use of an optimized assay. The system also includes a proprietary reagent to minimize primer-dimer formation.

All real-time instruments can be used to perform Plexor™ reactions and collect the raw data (for a list of currently supported instruments, visit: www.promega.com/plexorresources/). Because the

data analysis software bundled with most instruments is only designed to handle an increase in fluorescence, the software packages cannot directly analyze the Plexor™ data. To analyze the change in fluorescence associated with the Plexor™ chemistry, the raw data collected by the instrument must be imported into the Plexor™ Analysis Software, available for free download at: www.promega.com/plexorresources/

Overview of the 5' Nuclease Assay

The 5' nuclease assay, exemplified by the Applied Biosystems TaqMan® Systems, relies on hybridization of two PCR primers and a dual-labeled probe to a target (5). The probe contains a fluorescent reporter and a quencher molecule. As a complete probe, the excitation energy absorbed by the fluorophore is transferred to the quencher molecule through fluorescence resonance energy transfer (FRET) due to the close proximity of both molecules on the same oligonucleotide probe. The probe hybridizes to the target along with the PCR primers. The *Taq* DNA polymerase extends the PCR primers, and when the probe is encountered, the 5'→3' exonuclease activity of the polymerase cleaves the probe. This cleavage releases the fluorophore from the influence of the quencher, generating a fluorescent signal. The signal increases as more specific amplicons are synthesized. This probe-based chemistry lends itself to multiplex analysis as different primer/probe combinations can use different fluorescent reporter molecules.

Comparison of Plexor™ and TaqMan® Chemistries

The Plexor™ Systems and 5' nuclease assays differ considerably in their chemistries and experimental outputs (Table 1). The 5' nuclease assay will only produce signal from an amplicon containing sequences complementary to the probe. The Plexor™ chemistry will only measure amplification products that incorporate the labeled primer. The Plexor™ Systems allow post-PCR confirmation of reaction specificity based upon an analysis of the melt curve profile of the resultant amplicons. The 5' nuclease assay destroys the probe, so post-PCR confirmation of reaction specificity of the PCR is not possible.

Experiments were designed to compare the performance of the Plexor™ One-Step qRT-PCR System and the Applied Biosystems TaqMan® qRT-PCR kit. We used Universal Human Reference RNA (Stratagene Cat.# 740000) for all experiments. Dilutions of the RNA were made in a constant background of 10ng of *Escherichia coli*

Table 1. Comparison of the Plexor™ and 5' Nuclease Chemistries

	Plexor™ Chemistry	5' Nuclease Assay Chemistry
Mode	Quenching of fluorescence	Gain of fluorescence
Mechanism	Incorporation of nonnatural base containing a dabcyl quencher opposite a fluorophore-labeled, nonnatural base	5'→3' exonuclease degradation of a probe hybridized to the target molecule, releasing fluorophore from the influence of a quencher molecule
Required Oligonucleotides	One labeled primer, one unlabeled primer	Two unlabeled primers, one dual-labeled probe
Thermal Melt Analysis	Yes	No
Multiplex Capability	Yes	Yes
Primer Design Software for Multiplexes	Yes	No

RNA to minimize nonspecific adsorption of dilute RNA solutions onto the plasticware. The TaqMan® primer/probe reagents were ordered directly from Applied Biosystems using the Gene Expression Assay service with a FAM™ label and recommended quencher chemistry. The Plexor™ primers were designed with the free Plexor™ Primer Design Software (available at: www.promega.com/plexorresources/). One of the Plexor™ primers for each experimental target was synthesized by conjugating methylisocytosine (iso-dC) to the 5' end and then adding a FAM™ label adjacent to the iso-dC. Cycling conditions for both assays were modeled after the recommended protocol for the TaqMan® assay using a 30-minute reverse transcription step followed by 40 cycles of PCR. All experiments were performed on an Applied Biosystems 7500 Real-Time PCR Instrument. TaqMan® primer/probe reagents were used as directed in the Applied Biosystems instructions. Plexor™ primer sets were used at 200nM, as directed in the *Plexor™ One-Step qRT-PCR System Technical Manual #TM263*. The RNA template range for the TaqMan® assays was limited to recommendations in the accompanying literature.

Product accumulation, as evidenced by the C_t values, occurred earlier in the Plexor™ assays (Figure 1). The limit of detection was equivalent between the two chemistries, with the exception of the matrix metalloproteinase assay (Table 2). The TaqMan® chemistry failed to detect the transcript when using 10²pg of total RNA. However, it is important to note that different data analysis software was used to analyze the results for each chemistry. This accounts for some of the differences seen in the data.

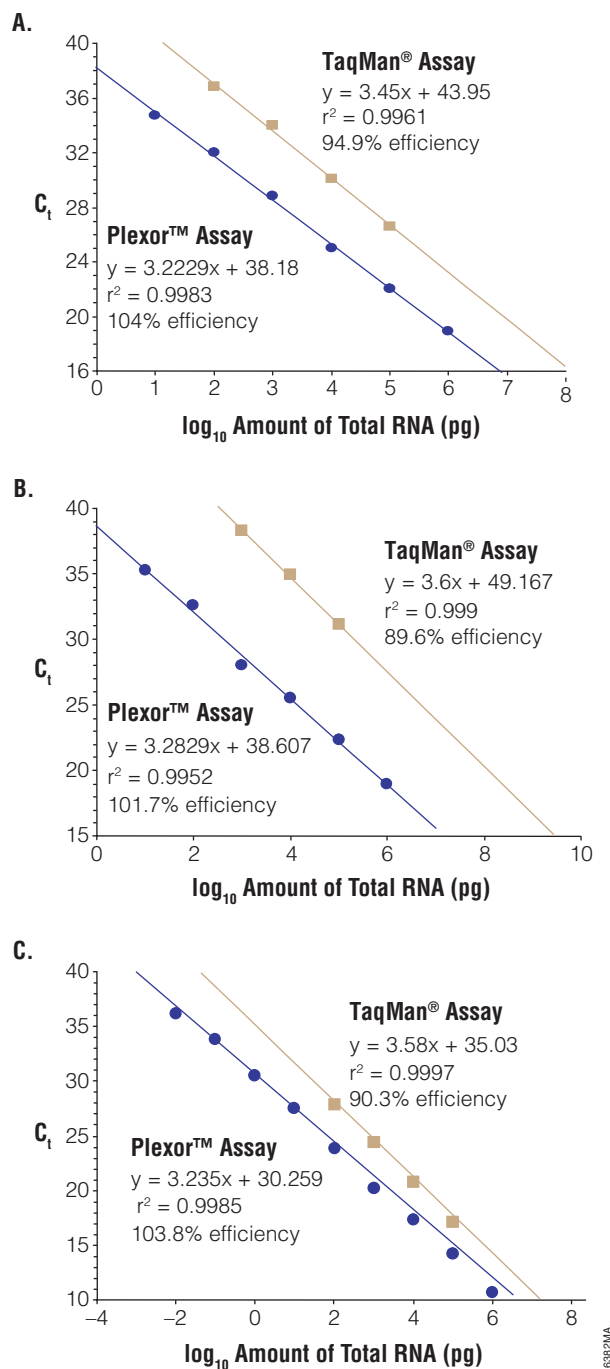


Figure 1. qRT-PCR analysis of samples containing human total RNA. Plots of C_t values generated with Plexor and 5' nuclease assay chemistries and indicated concentrations of human total RNA. The recommended template range from the Applied Biosystems manual (10²–10⁵pg) was used for all TaqMan® assays. **Panel A.** Amplification of fibroblast growth factor receptor 1 (GenBank® Accession# NM_000604). **Panel B.** Amplification of matrix metalloproteinase-1 (GenBank® Accession# NM_002421). The TaqMan® assay failed to react at the 10²pg level in the recommended 40 cycles of amplification. **Panel C.** Amplification of glyceraldehyde-3-phosphotransferase (GenBank® Accession# NM_002046).

Table 2. Detection Limit of Plexor™ and TaqMan® Chemistries.

The average C_t value of the nonspecific amplification products observed in the no-template controls was determined by linear regression of the plot generated by titrating known amounts of input RNA. This limit-of-detection value was used to determine the corresponding quantity of the total RNA template. Amplification targets were fibroblast growth factor receptor 1 (FGFR1), matrix metalloproteinase-1 (MMP1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Target	Measurement	Plexor™ Assay ¹	TaqMan® Assay
FGFR1	No-template control (Avg. C_t)	35.2	–
	Limit of detection	8.4pg	14pg ²
MMP1	No-template control (Avg. C_t)	38.8	–
	Limit of detection	0.87pg	~350pg
GAPDH	No-template control (Avg. C_t)	37.1	–
	Limit of detection	7.7fg	41fg ²

¹Melt curve analysis of the amplification products confirmed that the products observed in the no-template control reactions were aberrant products.

²Theoretical limit of detection, based on 40 cycles of amplification, was derived by using a C_t value of 40 in the TaqMan® linear regression for each assay.

TaqMan® assays have the advantage of virtually no background signal because an internal probe is used to monitor product accumulation. This does not mean that primer-dimer formation and nonspecific amplification do not occur. Reduced amplification efficiency (lower signal) occurs when primer-dimer formation and nonspecific product formation outcompete the desired target in the amplification reaction. Lind, K. *et al.* (6) demonstrated this effect and expressed concerns about false negative readings when nonspecific amplification is excessive. In contrast, nonspecific amplification products are readily distinguished by the thermal melt profile in Plexor™ assays.

Conclusions

Overall sensitivity of detection was essentially equivalent for both Plexor™ and TaqMan® chemistries except for the matrix metalloproteinase assay. While the amplification efficiency of the Plexor™ assays was consistently higher than observed for the TaqMan® assays, both chemistries produced good r^2 values. All experiments were performed using recommended conditions with no optimization of primer or probe concentration. One benefit of the Plexor™ technology, which relies on two primers for amplification, is the ease of designing multiplex reactions. As an added feature, the thermal melt profile, as part of the Plexor™ Systems analysis, gives secondary confirmation of the real-time assay (see the article “Validation of the Plexor™ Primer Design Software” on page 19).

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Designing Your Plexor™ Assay

Using the Plexor™ Primer Design Software

By Ethan Strauss, Ph.D., Promega Corporation

The Plexor™ Primer Design Software is a free, web-based program that designs primers for use in monoplex and multiplex quantitative PCR (qPCR) and qRT-PCR. The Plexor™ Primer Design Software also selects the appropriate fluorescent labels for use in monoplex and multiplex qPCR or qRT-PCR, matching fluorescent reporter choices to your specific real-time instrument and your chosen oligonucleotide manufacturer. Here we describe how to use the Plexor™ Primer Design program to design primers for use in Plexor™ System qPCR and genotyping analyses.

Here we provide step-by-step instructions for designing primers for quantitative PCR and genotyping using the Plexor™ Primer Design Software.

Simple Primer Design

The simplicity of primer design is a distinct advantage of the Plexor™ Systems^(a-c). The Plexor™ Primer Design Software (www.promega.com/plexorresources/) designs primers and selects the appropriate fluorescent labels for monoplex and multiplex qPCR and qRT-PCR on a variety of real-time instruments. Fluorescently labeled oligonucleotides for use as primers with the Plexor™ Systems must be ordered with an iso-C residue adjacent to the 5' fluorescent label. The Primer Design Software includes convenient links to oligonucleotide suppliers that have been licensed to provide primers with an iso-C residue. Registration is required before using the software, as you will be notified by e-mail when your results are available.

The Plexor™ Primer Design Software is easy and intuitive. This article provides a step-by-step overview of the process for designing primers for qPCR and genotyping, and provides the answers to some common questions about the primer design process.

Quantitative PCR Primer Design

1. Select “New Project”, and choose an instrument. The type and number of dyes that can be detected vary between instruments. When a specific instrument is selected, the software limits the number of target and housekeeping gene sequences that you can input based on the number of dyes that can be detected by that instrument. (For example, the software will not design a triplex reaction for an instrument that only has two detection channels.) Following primer design, the primers will include dyes recommended for your instrument.

Select “Other Instrument” if you wish to select the dyes yourself or use a real-time instrument other than those listed. The Plexor™ Analysis Software may not be able to analyze data for instruments that are not listed. Contact Promega Technical Services (techserv@promega.com) for more information.

2. Choose “Quantitative PCR”, and enter your project information as follows:

Project Details: Enter a name for your project (optional), and select the number of solution sets to be returned. Select “Housekeeping Sequences” or “Sequences” to continue.

Housekeeping Sequences: You can include amplicon and primer sequences that have been previously designed and tested. The primer design software will include these sequences in the analysis of false priming and avoid primer-primer interactions within a multiplex reaction.

You may choose from predesigned, prevalidated control primer sets, which can be ordered from your oligonucleotide supplier. The sequences of these primers are proprietary. Alternatively, you may add your own custom housekeeping gene and primer sequences. If you would like to design new primers for a housekeeping or control gene, treat that gene as a target sequence.

Sequences: Target sequences can be typed, pasted in or retrieved from the GenBank® database. To retrieve a sequence, select “Retrieve Sequence”, enter the accession number, then select “Retrieve”. Once the sequence is imported or entered, it can be edited or renamed. You can designate a single base that must be included in each amplicon for that target. This can be done to ensure that the primers span an intron or that they are designed in a region of your sequence that is appropriately conserved or not conserved. Select “Validate”, then “Save”.

A project cannot contain two sequences with identical names. To design primers for different portions of the same sequence, one sequence must be renamed.

The maximum sequence length is 15,000 bases. If the sequence has more than 15,000 bases, only the first 15,000 will be retrieved. To work with a different portion of that sequence, you must retrieve it manually, then paste it into the program.

The “validate” step removes any spaces, numbers, carriage returns and line feeds from the sequence. All characters will be changed to upper case, and all Us converted to Ts.

Occasionally GenBank® sequences contain IUPAC nucleotide ambiguity codes. Any such codes must be removed. The software will prompt you if any such ambiguity is present in your sequence.

3. Select “*Preview*” to display the project details. If desired, you can modify the experiment by selecting “*Edit Experiment*”.
4. Select “*Submit Experiment*” and wait for the results. All projects are queued in the order submitted. It can take a few minutes to several hours for a project to be completed.

An e-mail is sent to the registered address when the project is complete. You can check project status by logging into the Plexor™ Primer Design Software and selecting “*Project Results and Archives*”. Completed projects have a “*View*” button under “*Results*”. Projects not yet complete have “*No*” under “*Results*”.

Viewing Results and Choosing Primers

1. Open the Plexor™ Primer Design Software and select “*Project Results and Archives*”. Select “*View*” next to your project name. A list of solution sets will be displayed. The order indicates how closely the solutions fit the primer design criteria; solution set #1 is theoretically the best, #2 is next, etc.
2. Select “*Click here for a project summary*” to display the information submitted for your project (instrument, sequences, names and required bases). Select “*View*” next to a solution set to view details. Primers for each target, their location on the target, amplicon length, primer lengths, theoretical melting temperatures and a link to a BLAST search for each primer will be displayed.

Use the BLAST link for each primer to check for hybridization with nontarget sequences.

The primer with least chance of mispriming should be labeled with the fluorescent dye. If both primers are equal in this respect, label the shorter of the two. Radio buttons are used to indicate which primer will be labeled with the fluorescent dye and iso-C base.

The primers in a single solution set have been analyzed for primer-dimer formation and cross-hybridization. Do not use primers from different solution sets together. If some of the primers in a solution set are unacceptable, go on to the next set. Once you have chosen a solution set and identified primers in that solution set to label, select “*Continue*”.
3. Choose an oligonucleotide vendor, then select “*Continue*”. All of the selected primer sequences will be displayed, along with fluorescent labels appropriate for the real-time instrument selected and available from the chosen vendor. You can copy this information, or send it to your email address.

Genotyping PCR Primer Design

The Plexor™ Systems genotyping method is based on allele-specific PCR (ASPCR). Follow the steps below to design primers for use in genotyping assays using the Plexor™ Primer Design Software.

1. Select “*New Project*”, and choose an instrument. The type and number of dyes that can be detected vary between instruments. When a specific instrument is selected, the software designs primers for a defined number of targets based on the number of dyes that can be detected by that instrument. Following primer design, the primers will include dyes recommended for your instrument.

Select “*Other Instrument*” if you wish to select the dyes yourself or use a real-time instrument other than those listed. The Plexor™ Analysis Software may not be able to analyze data for instruments that are not listed. Contact Promega Technical Services (techserv@promega.com) for additional information.

2. Choose “*Genotype*”, and enter a name for your project (optional).
3. Select “*Sequences*”, then “*Add Genotype Sequence*”. Enter a name for your sequence.
4. Paste 30–15,000 bases of sequence upstream of your SNP into the “*5' sequence*” input box, select the appropriate SNP from the drop-down box, and paste 30–15,000 bases of sequence downstream of your SNP into the “*3' sequence*” input box.

Note: It is not possible to retrieve sequences directly from the dbSNP database at this time.
5. Select “*Validate*”, then “*Save*”. The “*Validate*” step removes any spaces, numbers, carriage returns and line feeds from the sequence. All characters will be changed to upper case, and all Us converted to Ts. The sequence, other than the SNP itself, must not contain IUPAC nucleotide ambiguity codes. Any such codes must be removed. The software will prompt you if any such ambiguity is present.
6. Select “*Preview*” to display the project details. If desired, you can modify the experiment by selecting “*Edit Experiment*”.
7. Select “*Submit Experiment*” and wait for the results. All projects are queued in the order submitted. It can take a few minutes to several hours for a project to finish.

An e-mail is sent to the registered address when the project is complete. You can check project status by logging into the Plexor™ Primer Design Software and selecting “*Project Results and Archives*”. Completed projects have a “*View*” button under “*Results*”. Projects that are not yet complete have “*No*” under “*Results*”.

Viewing Results and Choosing Primers

1. Open the Plexor™ Primer Design Software, select “Project Results and Archives”, and “View” next to your project name.

One Anchor Primer and two Allele-Specific Primers will be displayed with primer lengths, theoretical melting temperatures and a link to a BLAST search. The allele-specific primers will need to be labeled.

2. Select “Click here for a project summary” to display information (instrument, sequences, names) submitted for your project.

Use the BLAST link for each primer to check for hybridization with nontarget sequences.

3. Select an oligonucleotide vendor, then “Continue”. All selected primer sequences will be displayed, along with fluorescent labels appropriate for the real-time PCR instrument selected and available from the chosen vendor. You can copy this information or send it to your e-mail address.

BLAST Searches

Before ordering primers for qPCR, it is crucial to perform a BLAST search to ensure that the chosen sequences are specific for the target of interest. Project results from the Plexor™ Primer Design Software automatically generate a BLAST link. This link is used to submit the primer sequence to the NCBI (National Center for Biotechnology) BLAST server with parameters to find short, nearly exact matches (NR database, word size = 7, expect = 1000).

Primers that may misprime nontarget sequences from the organism of interest should be avoided. You can limit the BLAST results to show only sequences from your target organism with the “Limit results by...” link in the formatting BLAST page (Figure 1). If your samples contain nucleic acid sequences from a number of different organisms, you should avoid primers that might prime nucleic acid from any of these organisms.

In most cases, it is impossible to completely avoid all problems that might be caused by primer sequence similarity to nontarget sequences. You should strive to minimize this issue as much as possible. The primer with least chance of mispriming should be the one labeled with the fluorescent dye so that, if low-level mispriming does occur, it will not contribute to the signal in the reaction.



Figure 1. The BLAST search formatting page. If the organism you are working with is available, choose it.

The software attempts to identify primer sequences with a T_m of approximately 65°C. If you are attempting to design primers for a GC-rich portion of a gene, the software will return short primer sequences. These short sequences increase the probability of finding significant similarity to undesirable regions of the genome. In addition, GC-rich primers form more stable hydrogen bonds, compounding the problem of nonspecific amplification. We recommend designing primers to a different region of the gene with lower GC content. This can be accomplished by identifying another nucleotide that must be present in the amplicon.

In the near future, we plan to introduce a new BLAST parsing tool to help automate and simplify BLAST analysis and interpretation. Using this new tool, each solution set will have a link to allow simultaneous analysis of all of the primers in the solution set. This link will initiate BLAST analysis of each primer against the standard complete Genbank database (“nr”) as well as against the refseq_rna database (http://www.ncbi.nlm.nih.gov/BLAST/blastcgihelp.shtml#nucleotide_databases).

When the results from these analyses are returned, the BLAST parser will examine them for sequences that are likely to cause problems in Plexor™ analysis. All sequences that cannot anneal to the 3' end of the putative Plexor™ primer and those that have a theoretical annealing temperature more than 10 degrees below that of the perfect match annealing temperature for the primer are discarded.

Any remaining sequences from the RefSeq BLAST analysis are categorized by species and by cognate gene in the gene database (<http://www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pubmed&pubmedid=15608257>). If all the BLAST hits from a species map to the same cognate gene, then the Plexor™ primer is unlikely to prime any other RNA sequences from that species. A report is generated detailing the species where a single gene is likely to be primed, and those where priming of more than one gene is likely. Please note that this analysis is relevant only to RNA samples, for species that are well represented in the refseq database (see Table 1), and for analyses where detection of RNA variants of the same gene is not an issue.

To supplement the analysis of the RefSeq BLAST, the nr (complete) database BLAST is analyzed specifically in the context of a Plexor™ reaction and this analysis is displayed in a table. First, all BLAST hits that are unlikely to cause problems (no 3' end annealing or annealing temperature more than 10 degrees below perfect annealing) are discarded. The remaining hits are displayed with annealing temperature, species, alignment, and Gene match. This allows the user to quickly and accurately see where mispriming might occur and to determine if it is likely to be problematic for their experiment.

Table 1. Examples of Species that are Well-Represented in the refseq Database.

<i>Arabidopsis thaliana</i>	<i>Mus musculus</i> ,
<i>Caenorhabditis elegans</i>	<i>Neurospora crassa</i>
<i>Canis familiaris</i>	<i>Oryza sativa</i>
<i>Danio rerio</i>	<i>Pan troglodytes</i>
<i>Drosophila melanogaster</i>	<i>Rattus norvegicus</i>
<i>Gallus gallus</i>	<i>Saccharomyces cerevisiae</i>
<i>Homo sapiens</i>	<i>Schizosaccharomyces pombe</i>
<i>Magnaporthe grisea</i>	Viruses

Choosing Reverse Transcription Primers

For the Plexor™ One-Step qRT-PCR System, we suggest designing gene-specific primers for reverse transcription. When using the Plexor™ Two-Step qRT-PCR System or the Plexor™ qPCR System with your own cDNA synthesis reagents, you have a variety of choices (gene-specific primers, oligo(dT) and random hexamers). If you are targeting the 5' end of a long transcript, reverse transcription using oligo(dT) may be inefficient.

FAQs Online:

Additional support information, including the answers to Frequently-Asked Questions on the Primer Design Software, is available online at:

www.promega.com/faq/

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Validation of the Plexor™ Primer Design Software

Analysis of 20 Duplex qRT-PCR Assays Designed Using the Plexor™ Primer Design Software

By Benjamin Krenke, M.S., Cindy Sprecher, B.S., Kyle Hooper, Ph.D., and Douglas R. Storts, Ph.D., Promega Corporation

The Plexor™ Primer Design Software, when used in conjunction with the Plexor™ System reagents, offers a robust method to design multiplex, real-time amplification assays. This article details the results of 20 real-time qRT-PCR assays performed on 20 different human mRNA targets. Targets were amplified using the Plexor™ Two-Step qRT-PCR System using primers designed with the Plexor™ Primer Design Software.

Using the Plexor™ Primer Design Software in conjunction with the Plexor™ qRT-PCR reagents to test 20 duplex assays, 70% success (ideal assays) and 95% success (usable assays) was achieved.

Introduction

The Plexor™ qPCR and qRT-PCR Systems^(a-c) are multiplex-capable, real-time amplification systems that use novel base-pair chemistry (1–4). Each target is measured directly during the amplification process, as opposed to using a secondary reaction to detect product accumulation (e.g., probe cleavage). Plexor™ reactions require only two primers for each target. Assay design is further simplified by the use of the web-based Plexor™ Primer Design Software, which is specifically engineered for multiplex assay design.

The Plexor™ Systems work by measuring a reduction in fluorescent signal during amplification. Amplification uses only two primers, one of which contains both a 5'-fluorescent tag and an adjacent modified base. As amplification proceeds, fluorescence is reduced by site-specific incorporation of a fluorescence quencher, which is attached to a modified nucleotide inserted opposite the complementary modified base. The quencher is in close proximity to the fluorescent dye, resulting in a reduction in the fluorescent signal. After PCR, a melt analysis can be performed to expedite troubleshooting during assay development and provide an internal control during routine use of an optimized assay. The system also includes a proprietary reagent to minimize primer-dimer formation.

The Plexor™ Primer Design Software is a freely available, web-based program that designs primers for monoplex and multiplex qPCR. The software will select the appropriate fluorescent labels for monoplex and multiplex qPCR, or qRT-PCR, based upon the user's real-time instrument. Convenient links to oligonucleotide suppliers licensed to provide primers with an iso-dC nucleotide can be found on the Plexor™ Primer Design Web site at: www.promega.com/plexorresources/ (See the article "Designing Your Plexor Assay" (p.15) for detailed information on how to use the Plexor™ Primer Design Software.)

Primer Design

The Plexor™ Primer Design Software was used to design primers for 20 different human mRNA targets. Each primer pair was designed to span an intron, minimizing the potential for generating an amplification product from genomic DNA. A BLAST search was performed against the NCBI database to verify that the primers were specific for the mRNA target of interest. All primer pairs were designed for use in duplex reactions targeting a "housekeeping" gene (glyceraldehyde-3-phosphate dehydrogenase; GAPDH). The GAPDH primer set, labeled with CAL-Fluor® Red 610, was obtained from Biosearch Technologies (Cat.# PLX-3002-1). The other mRNA-specific primers were synthesized by Biosearch Technologies and were labeled with FAM™. Sequences of the target primer sets are available upon request.

Quantitative, Real-Time RT-PCR

Human total RNA (Stratagene Universal Human Reference RNA) was converted to cDNA as directed in the *Plexor™ Two-Step qRT-PCR System Technical Manual* (available at: www.promega.com/plexorresources/). The qPCR reactions were assembled with cDNA generated from 10ng of total RNA. Amplification was performed in 25µl reactions using 200nM of target-specific primers except for the GAPDH primers, which were used at 100nM. Amplification was performed for 40 cycles as recommended in the *Plexor™ Two-Step qRT-PCR System Technical Manual*. Data were collected on an Applied Biosystems 7500 Real-Time PCR System and analyzed using the Plexor™ Analysis Software (also available at www.promega.com/plexorresources/; see the article "Programmed to Make Analysis Easy" (p.22) for further information).

Validation of the Primer Design Software... continued

Table 1. Cycle Threshold (C_t) Values from Assays Producing No Nonspecific Amplification in Duplex with GAPDH.
(No-template controls did not generate a detectable, nonspecific amplification product in 40 cycles).

GenBank® Accession Number	Name	Target C_t (Avg)	GAPDH C_t (Avg)	ΔC_t
NM_000321	Retinoblastoma RB1	26.97	16.96	10.01
NM_000942	Cyclophilin B	24.67	17.11	7.56
NM_001237	Cyclin A2	26.42	17.13	9.28
NM_001838	Chemokine receptor 7	34.82	16.86	17.96
NM_001949	E2F transcription factor 3	28.77	17.08	11.69
NM_002853	RAD1 homolog, transcript variant 1	28.19	17.59	10.60
NM_002895	Retinoblastoma-like 1 RBL1 transcript variant 1	29.85	16.77	13.08
NM_004091	E2F transcription factor 2	30.19	16.61	13.58
NM_004526	MCM2 mini-chromosome maintenance deficient 2	26.51	16.74	9.77
NM_004701	Cyclin B2	27.11	17.09	10.02
NM_005611	Retinoblastoma-like 2 RBL2	28.70	16.61	12.09
NM_005914	MCM4 mini-chromosome maintenance deficient 4	25.83	16.94	8.89
NM_031966	Cyclin B1	24.67	17.44	7.23
NM_033031	Cyclin B3	34.57	17.35	17.22

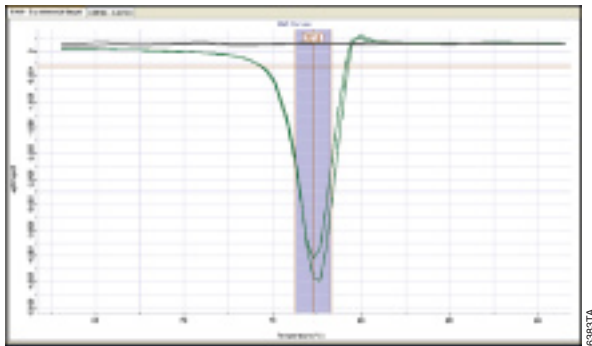


Figure 1. Melt curve of retinoblastoma RB1 (Accession No. NM_000321) amplification product. The assay containing cDNA produced a sharp melt curve (green), indicative of a specific amplification product. No amplification product was observed in the no-template control reactions (black). Data is presented for the FAM™ channel.

Ideal Assays

Nineteen of twenty duplex assays produced usable data at the first design, without optimization. Fourteen assays were ideal, yielding quantitative results over multiple log dilutions of cDNA (data not shown), a single amplification product (as evidenced by a discrete melt curve) and no background amplification in the no-template control reactions (Table 1). The melt curve data for one of these ideal assays, retinoblastoma, clearly demonstrates a single, well-defined melt curve (Figure 1). Comparable melt curves (not shown) were observed for the other targets in this group of assays.

Table 2. Cycle Threshold (C_t) Values From Assays Generating Sharp Melt Curves, But Showing Some Background Amplification in Duplex with GAPDH.

GenBank® Accession Number	Name	Target C_t (Avg)	GAPDH C_t (Avg)	ΔC_t	Target NTC* (Avg)	GAPDH NTC* (Avg)
NM_003914	Cyclin A1	33.78	16.92	16.86	37.16	35.32
NM_004935	Cyclin-dependent kinase 5	26.51	17.04	9.47	34.55	>40
NM_170707	Lamin A/C (LMNA), transcript variant 1	25.49	17.15	8.33	32.32	>40

*NTC = No-template Controls.

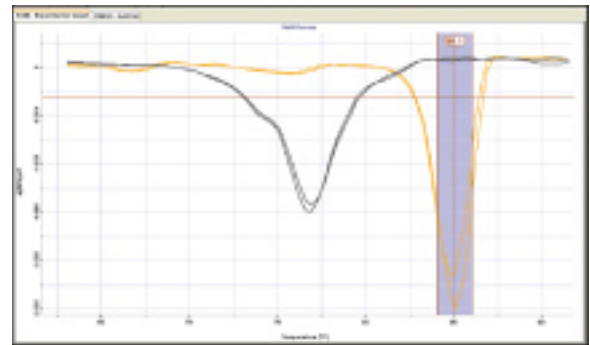


Figure 2. Melt curve of lamin A/C (LMNA) transcript variant 1 (Accession No. NM_170707) amplification product. The assay containing cDNA produced a sharp melt curve (yellow). A nonspecific amplification product was observed in the no-template controls (blue). The assay clearly differentiates specific and nonspecific amplicons. Data is presented for the FAM™ channel.

Usable Assays

Three assay designs produced sharp melt curves, but also yielded aberrant products in the no-template control reactions (Table 2). An example of the results observed for this group of assays is represented by the lamin A/C transcript variant 1 experiment (Figure 2). Although a discrete melt curve attributable to the lamin A/C amplicon was observed, some nonspecific amplification occurred in the no-template control reactions. Two designs were target specific, but had broader melt curves than other assays (Table 3). An example of the results observed for this group of assays (MCM3 mini-chromosome maintenance deficient 3) is shown in Figure 3. The amplicon yields a broad melt curve and an amplification artifact in the no-template control reaction. The artifact can clearly be distinguished from the desired product based upon the melt temperature. The assays that produced broad melt curves may be improved by primer redesign, but may be the result of a heterogeneous target population due to internal polymorphism.

Table 3. Cycle Threshold (C_t) Values From Assays Generating Broad Melt Curves and Varying Levels of Nonspecific Amplification in Duplex with GAPDH.

GenBank® Accession Number	Name	Target C_t (Avg)	GAPDH C_t (Avg)	ΔC_t	Target NTC* (Avg)	GAPDH NTC* (Avg)
NM_001799	Cyclin-dependent kinase 7	27.44	16.79	10.65	>40	>40
NM_002388	MCM3 mini- chromosome maintenance deficient 3	25.80	17.33	8.47	38.62	36.21

*NTC = No-template Controls.

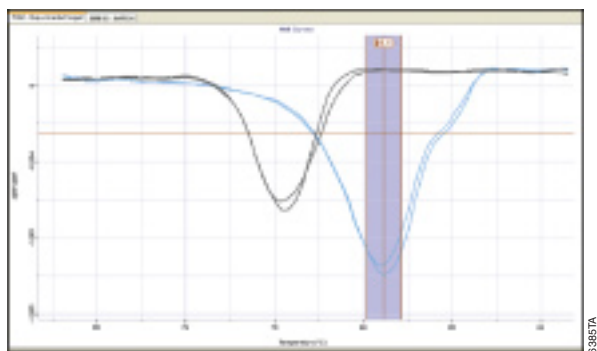


Figure 3. Melt curve of MCM3 minichromosome maintenance deficient 3 (Accession No. NM_002388) amplification product. The assay containing cDNA produced product(s) with a broad melt curve (light blue) and a nonspecific amplification product in the no-template controls (black). The assay clearly differentiates specific and nonspecific amplicons. The broad melt curve may indicate cross-reactivity with a related gene product. The assay is usable, but may benefit from redesign. Data is presented for the FAM™ channel.

Unusable Assay

The assay for cyclin-dependent kinase 3 (Figure 4) did not yield usable data. Two different products, as evidenced by two discrete melt curves, were observed in the experiment. The presence of two discrete products may be attributed to transcript variants, or primers annealing to another RNA target. In addition, a nonspecific amplification product was observed in the no-template control reaction. This assay clearly requires redesign.

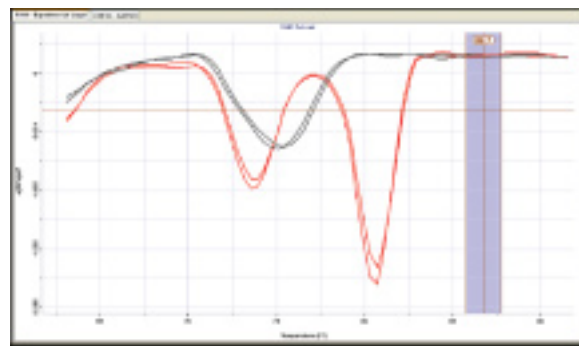


Figure 4. Melt curve of Cyclin-dependent kinase 3 (NM_001258) amplification product. The assay containing cDNA yielded both nonspecific and specific amplification products (red), distinguishable by their melt temperature. The no-template controls yielded a nonspecific amplification product (blue). This is not a usable assay and clearly requires redesign. Data is presented for the FAM™ channel.

Conclusion

The Plexor™ Primer Design Software, when used in conjunction with the Plexor™ qRT-PCR System reagents, offers a robust method to design multiplex, real-time amplification assays. The data show that 70% success for ideal assays and 95% for usable assays can be achieved using this software.

References

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 - Frackman, S. *et al.* (2005) *Promega Notes* **90**, 2–4.
 - Sherrill, C.B. *et al.* (2004) *J. Am. Chem. Soc.* **126**, 4550–6.
 - Moser, M.J. *et al.* (2005) *Antimicrob. Agents Chemother.* **49**, 3334–40.
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Programmed to Make Analysis Easy

Plexor™ Data Analysis Software

By Alyssa TenHarmsel, Ph.D., and Yi Wu, Ph.D., Promega Corporation.

What Is It? Why Use It?

The Plexor™ Data Analysis Software is a program that allows interpretation of fluorescence data generated using the Plexor™ real-time PCR chemistry^(a-c). The Plexor™ chemistry can be used on a wide range of different real-time instruments. However, because the data generated using Plexor™ chemistry reflects quenched fluorescence rather than the increase in fluorescence commonly seen in other real-time PCR systems, most real-time instrument software is unable to analyze the data.

To gain access to the program file, you will need to register with our web site. Registration provides a way for us to contact you when the software is updated.

Software Functions and Output

The Plexor™ Data Analysis Software can be used to generate cycle threshold (C_t) and melt curve data from raw data exported from any of the currently supported real-time instruments (Table 1). The Assay Setup screen allows the user to specify the instrument and fluorescent dyes used in the experiment (Figure 2). The software then prompts the user to import the raw data files and generates a graphical output of the data, displaying C_t values and melt temperatures for each sample in the data set (Figure 3).

The Plexor™ Data Analysis Software is easy to use, has many useful data analysis functions and analyzes Plexor™ data generated on any of the supported real-time instruments.

The Plexor™ Data Analysis Software is designed to take raw fluorescence data from any supported real-time instrument and interpret the data in a conventional format (Figure 1). The Plexor™ Data Analysis Software presents the real-time data in a uniform format regardless of which instrument is used to perform the reactions. This program is available at no charge and can be downloaded from the Plexor™ web site at: www.promega.com/plexorresources/

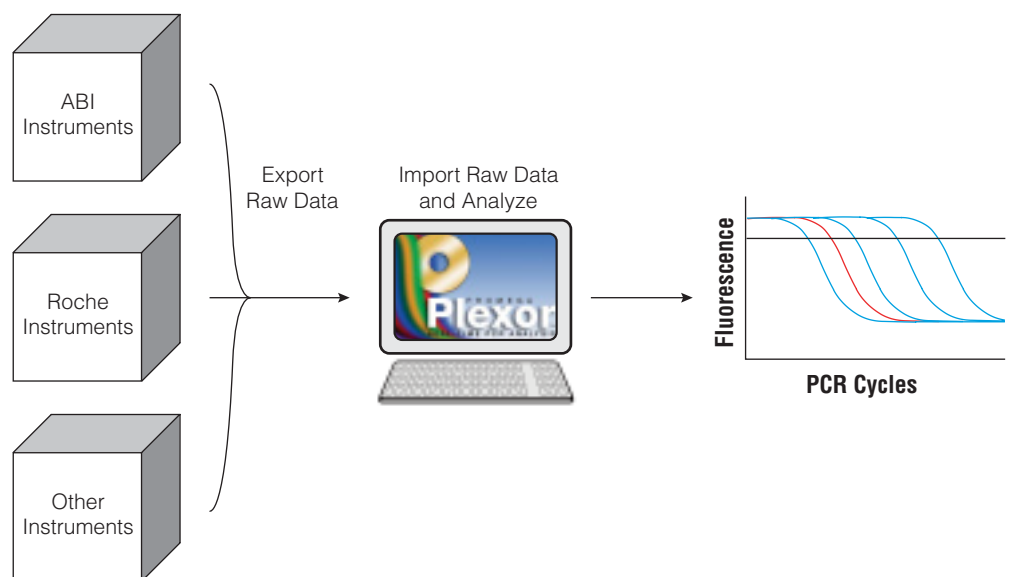


Figure 1. Schematic representation of data analysis using the Plexor™ Data Analysis Software.

Table 1. Real-Time Instruments Currently Supported by the Plexor™ Data Analysis Software.

Instrument	File Type and Number of Files Exported
ABI PRISM® 7700	All data for all dyes are exported in one file (.tab)
ABI PRISM® 7900	All data for all dyes are exported in one file (.txt)
Applied Biosystems 7000, 7300, 7500	One file per curve type (amplification, melt) includes all dyes. (.csv)
Roche LightCycler® and LightCycler® 2	One file per curve type (amplification, melt) per dye type (.txt)
BioRad iCycler® iQ	iCycler® files must be copied into Excel and saved as .txt files. One file per curve type (amplification, melt) per dye type.
Stratagene Mx3000P™, Mx3005™, Mx4000®	All data for all dye types are exported in one file (.txt)
MJ Research DNA Engine Opticon® 2	All data for all dye types are exported in one file (.csv)
Cepheid SmartCycler®	One file for all amplification dyes, one file per dye for melt data (.csv)

The software automatically sets sample baselines, amplification thresholds and melt thresholds based on the raw fluorescence data. The amplification threshold is set at 10 standard deviations below the baseline. The melt threshold is set at 25% of the maximum change in fluorescence for all samples in an experiment. Analyzed data is displayed in the Analysis Desktop window (Figure 3). Amplification curves are displayed in the upper Amplification Curves window of the Analysis Desktop. Amplification baseline regions for each sample are set by the software but can be adjusted manually if necessary. Melt curves are shown in the middle Melt Curves window of the desktop. An expected melt curve range can be specified using the expected melt curve temperature line and associated melt temperature range (specified in blue) within the Melt Curves window. The Well Selector window at the bottom of the Analysis Desktop shows a diagram of the plate or carousel setup. The Graph Legend window on the right side of the desktop shows well number, sample identification, C_t values, melting temperatures, and assigned or calculated concentrations.

The Analysis Desktop also has tabs to display each of the fluorescence channels used in the experiment. These tabs are found at the top of the Amplification Curves window. There is a different tab for each of the fluorescence channels used in the experiment. Selecting a tab for a particular dye will display the amplification data and melt data specific for that fluorescent dye channel.

The software functions include the ability to generate a standard curve from the amplification data. Samples can be designated as standards and assigned specific concentration values using the Assign Standard function.



Figure 2. Plexor™ Assay Setup screen showing instrument and fluorescent dye selection.

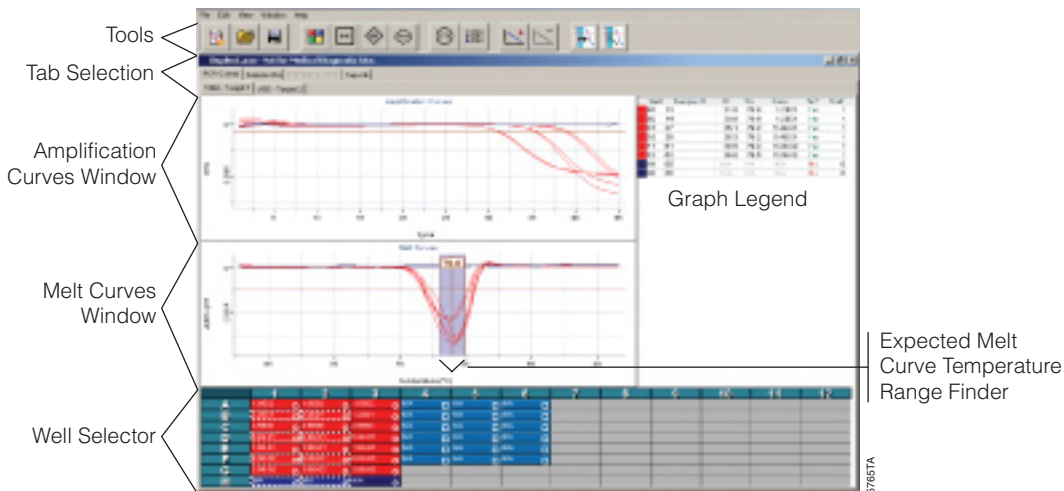


Figure 3. The Plexor™ Analysis Desktop window.

A serial dilution can be assigned using the Create Dilution Series function.



A standard curve is generated by selecting standard wells along with unknown samples to be quantitated in the Well Selector window and adding these samples to a standard curve using the Add Standard Curve function.



The standard curve is graphed as C_t value vs. concentration. The axes can be reversed by selecting “flip standard curve axis” under “Edit”. In this format, a standard formula can be used to calculate the amplification efficiency based on the slope of the standard curve (Figure 4). A standard curve can be generated for each fluorescence channel used in the experiment. Standard curves for each fluorescence channel where a standard curve has been assigned can be viewed by selecting the Standard Curves tab of the Analysis Desktop.

Sample names can be added using the Sample IDs tab. If a particular plate setup is used for multiple experiments, the sample identifications can be saved as a template and applied to future experiments using the Run Template option, which is provided as one of the Advanced Options in the File Import window.

Several report options are available within the software. The Sample Details report shows sample type (standard, unknown, etc.), well location, C_t values, melt temperatures (T_m), calculated concentrations, whether the T_m falls within the expected range for each sample and dye label in a spreadsheet format. The spreadsheets can be printed directly or saved in an Excel worksheet

format for further analysis. Other report functions include Baseline Regions, Thresholds, Add Import Files and Run Info, which includes instrument name, date of data import and the names of raw data files used to create the Plexor™ data analysis file.

Summary

Plexor™ Data Analysis Software is a program for analysis and interpretation of data generated using the Plexor™ chemistry and is available at no cost. The interface is intuitive and easy to use and has many useful data analysis functions. The software allows Plexor™ data generated on any of the supported real-time instruments to be displayed in a uniform format, and this allows direct comparison from experiment to experiment or from instrument to instrument.

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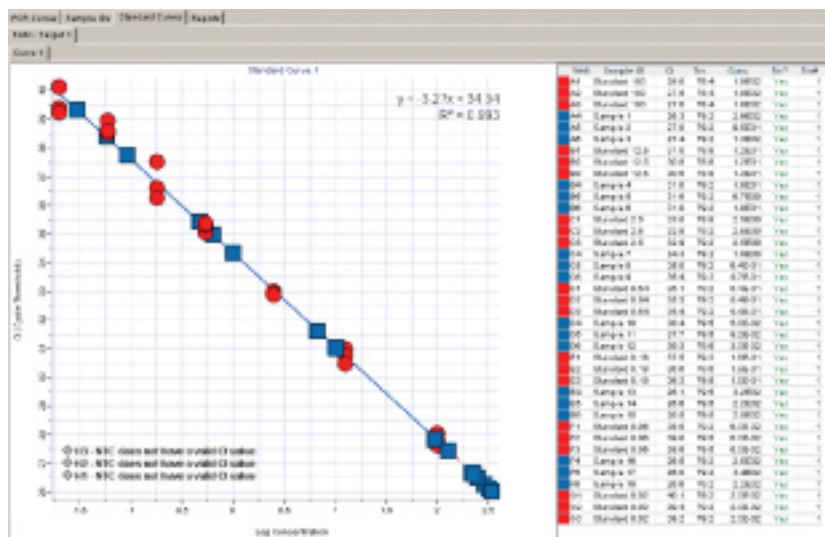


Figure 4. Plexor™ Standard Curve. The cycle threshold is shown on the Y axis and the log concentration on the X axis. The red circles represent standard samples, and the blue squares represent unknown samples.