Plexor™ Technology vs. SYBR® Green

A Comparison of Plexor™ and SYBR® Green Chemistries

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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

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).

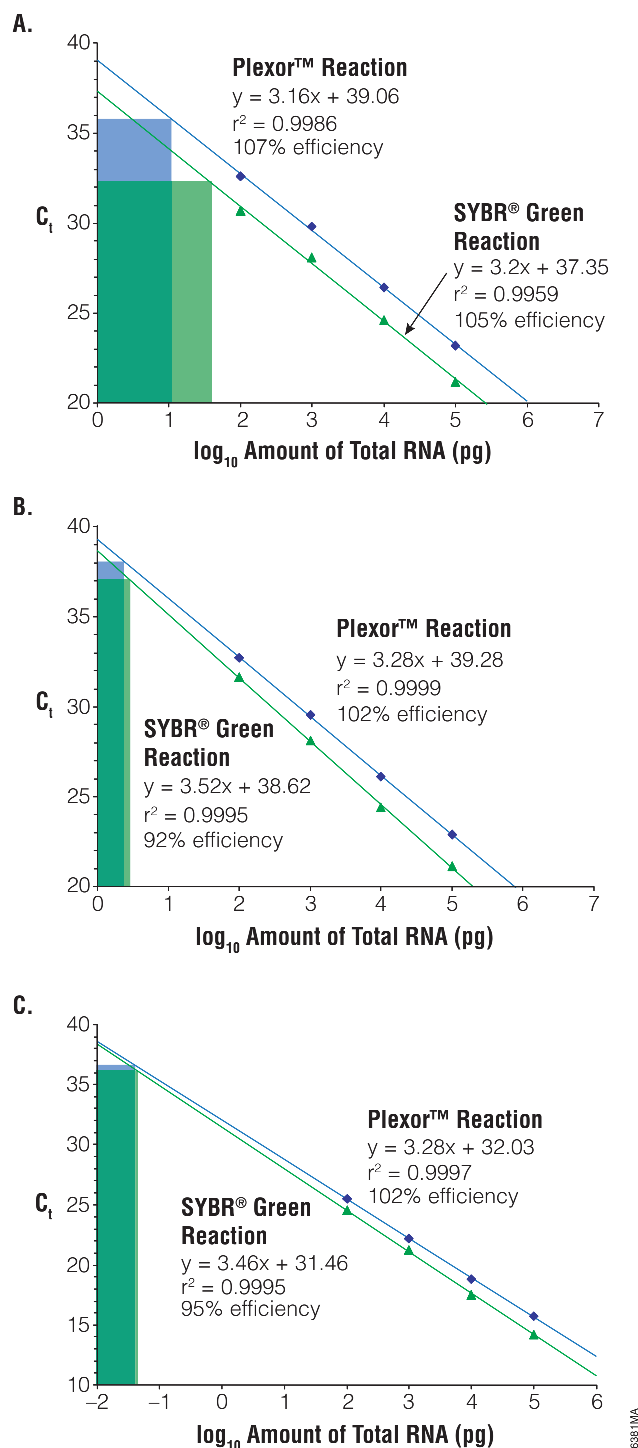

Figure 1. qRT-PCR analysis of total RNA samples. Plots of C_t values generated with SYBR® Green and Plexor™ chemistries using the indicated concentrations of human total RNA. The cycle at which nonspecific amplification products cross the cycle threshold in the no-template controls is indicated by either 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 glyceraldehyde-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

[1]Nonspecific amplification observed in 1 of 4 no-template control reactions.
[2]Nonspecific 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).

References

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