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 10^2pg 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.

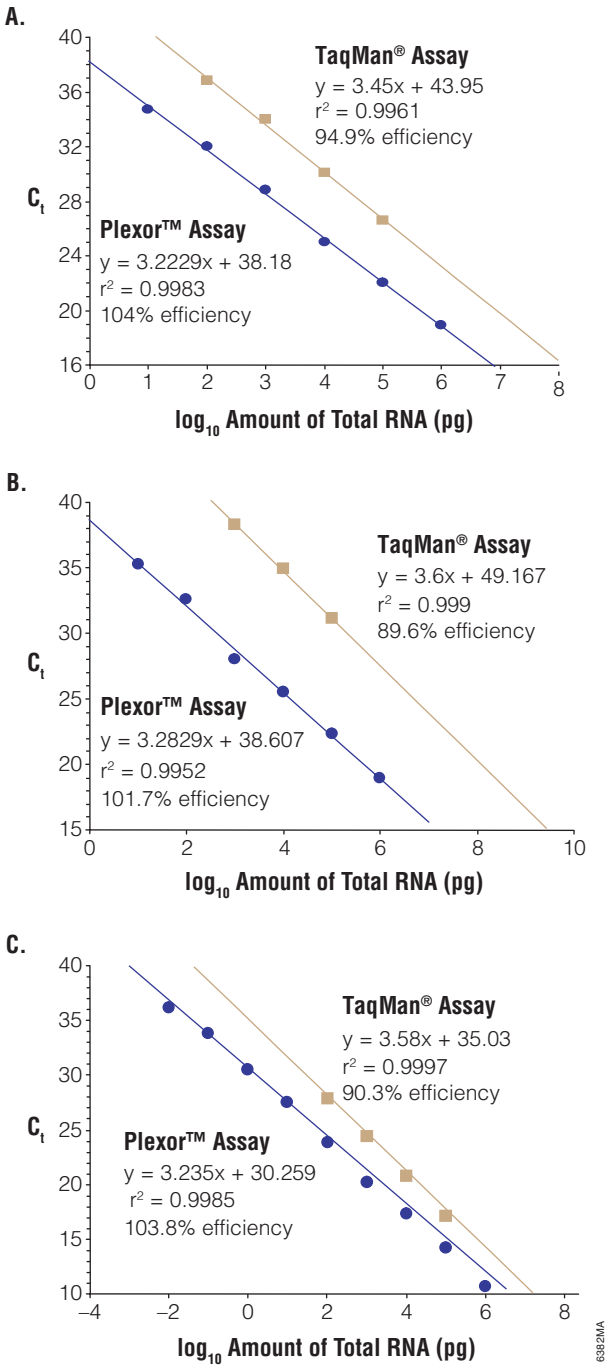

Figure 1. qRT-PCR analysis of samples containing human total RNA. Plots of C_t values generated with Plexor and 5′ nuclease assay chemistries and indicated concentrations of human total RNA. The recommended template range from the Applied Biosystems manual (10^2–10^5pg) was used for all TaqMan® assays. **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 10^2pg 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]

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

References

1. Johnson, S.C. *et al.* (2004) *Nucleic Acids Res.* **32**, 1937–41.
2. Frackman, S. *et al.* (2005) *Promega Notes* **90**, 2–4.
3. Sherrill, C.B. *et al.* (2004) *J. Am. Chem. Soc.* **126**, 4550–6.
4. Moser, M.J. *et al.* (2005) *Antimicrob. Agents Chemother.* **49**, 3334–40.
5. Bustin, S.A. (2004) *A–Z of Quantitative PCR.* International University Line Biotechnology Series, La Jolla, CA
6. Lind, K. *et al.* (2006) *BioTechniques* **40**, 315–18.