Duplexing in quantitative real-time PCR with TaqMan Gene Expression Assays

Summary

Applied Biosystems™ TaqMan® Gene Expression Assays provide excellent duplex qPCR performance, with results that are highly correlated with those obtained from the same assays run in singleplex.

Introduction

Duplexing in quantitative real-time PCR (qPCR) is the simultaneous amplification and quantification of two target sequences using two qPCR assays in a single well. Its key benefits come from combining experimental and control reactions to minimize the impact of pipetting errors and maximize experimental efficiency by increasing sample throughput, preserving limited samples, and saving reagent costs. Endogenous controls are often used as the second assay in a duplex reaction. TaqMan Gene Expression Assays enable duplex qPCR by offering a choice of two reporter dyes (either FAM™ or VIC™ fluorescent dye) for their TaqMan probes. These different reporter dyes are detected independently on qPCR instruments with excitation sources and emission filters in the respective wavelengths for FAM and VIC dyes.

Multiplex qPCR has inherent challenges. Optimization of primer designs, reaction components, and conditions has often been required to obtain results similar to those of each individual assay performed in singleplex. In duplex RT-qPCR, the amplification of a more highly expressed transcript target would consume a higher proportion of reaction components (enzyme and dNTPs) and may attenuate or eliminate the ability to detect the less abundant transcript. In addition, enzyme, buffer, and dNTP formulations that are not optimized may contribute to poor duplex performance. However, previous studies have shown that duplexing of two assays in a single well is much more straightforward and has a higher likelihood of success than traditional multiplexing of three or more assays in the same well [1].

Here we describe results from duplexing VIC dye–labeled and FAM dye–labeled TaqMan Gene Expression Assays. We show that the assays provide excellent duplex performance, with duplex C_t values showing very high correlation to singleplex values for both FAM and VIC dye–labeled assays.

Methods

Assay selection and sample preparation

A set of 96 TaqMan Gene Expression Assays was chosen that represents a wide range of expression levels in Stratagene™ Universal Human Reference (UHR) RNA. For each of the 96 assays, four formulations were generated: FAM dye–labeled MGB probe, both non–primer-limited (1X = 900 nM each primer) and primer-limited (1X = 150 nM each primer); and VIC dye–labeled MGB probe, both non–primer-limited and primer-limited. The singleplex FAM dye–labeled MGB formulations were analyzed alongside the VIC dye–labeled MGB assays as a baseline for the duplexing experiment. UHR RNA and human brain total RNA were reverse-transcribed using the Applied Biosystems™ High Capacity cDNA Reverse Transcription Kit to generate cDNA.

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For singleplex characterization, all 96 assays of each primer formulation were run at 1X concentration with 10 ng of cDNA (UHR and/or brain) and 1X TaqMan Gene Expression Master Mix in a 10 µL reaction under universal cycling conditions (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min) on an Applied Biosystems™ 7900HT Fast Real-Time PCR System. For duplex reactions, we included the VIC dye–labeled MGB (primer-limited) assays run at 1X concentration. All reactions were run in quadruplicate, and C_t values were averaged across replicates.

Results

Singleplex characterization

To characterize the effect of reporter dyes and primer concentrations, we compared the four formulations for the 96 assays in singleplex reactions. Comparing FAM and VIC dyes, our data indicated an average C_t difference of ~1.4 in UHR cDNA (Figure 1) and ~1.2 in brain cDNA (data not shown), with the FAM dye–labeled assays having the earlier C_t values. Moreover, primer-limited and non–primer-limited formulations did not differ significantly in C_t when assays with the same dye label were compared (data not shown). Figure 1 shows the high correlation of the C_t difference across the 96 assays. These data suggest that the experimental design must ensure that the choice of dye for each target be consistent within a single analysis. For instance, calculating the ΔC_t between a FAM label assay for target 1 and a VIC label assay for target 2 is valid; however, it cannot be directly compared with a ΔC_t calculated for a VIC assay for target 1 and a FAM assay for target 2.

Performance verification of duplexing

To demonstrate successful duplexing of FAM and VIC dye–labeled assays, 32 assays (C_t range 20–30) were used to generate 24 duplex pairs. The assays were paired following the recommendations in the Thermo Fisher Scientific application note "Factors influencing multiplex RT-PCR" [2]. If the non–primer-limited assay is targeting the more abundant target, reagents may become depleted, impacting the measurement of the less abundant target. Thus, the recommended pairing has the more abundant target detected by the VIC primer-limited assay and the less abundant target detected by the FAM non–primer-limited assay when possible. The 24 duplex pairs were separated into four sets: one set comprised targets with a C_t difference of ≥3 (7 assays); two sets had C_t differences of <3 (4 assays with C_t 1 to <3; 7 assays with C_t <1); and one set contained targets with similar expression levels (high, medium, and low) (6 assays). The 32 assays were run in singleplex reactions across a series of six 10-fold dilutions with UHR cDNA, plus a no-template control. In parallel, the 24 duplex pairs were also run using the same 10-fold dilution series and a no-template control. For analysis, we used a C_t cutoff of 35 to ensure we were detecting real targets and to minimize potential variability. Across the 10-fold dilution series, the duplex assay pairs showed no performance differences compared to singleplex assays. The performance of duplex assays for combinations of high and low expressors was similar to singleplex assays, as was the case for assay pairs with similar expression levels (C_t difference <1) (Figure 2).
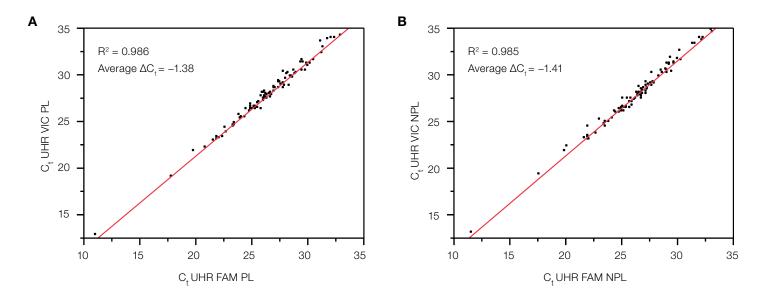

Figure 1. UHR cDNA correlation plots (signals in FAM non–primer-limited assays filtered for C_t <35). A C_t shift is seen when the dye is changed, and from (A) primer-limited to (B) non–primer-limited, but the correlation is still high among the 96 assays.
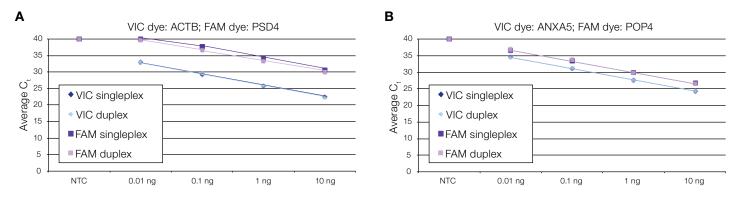
Figure 2. Assays run in singleplex or duplex show comparable performance in determining C_t for the VIC or the FAM dye–labeled assay. **(A)** Sample data for assays with large (>3) difference in C_t. **(B)** Sample data for assays with small (1–3) difference in C_t. NTC: no-template control.

An additional 91 pairs were run using the recommended pairing conditions. Figure 3 shows the distribution of the absolute C_t differences for these assays run in singleplex and duplex and the correlation of the C_t values across the range of expression levels. As shown, the same assay run in either singleplex or duplex showed high C_t correlation, regardless of using VIC or FAM dye label.

The same 32 single assays and 24 duplex pairs were also run with 10 ng of human brain cDNA and compared to the 10 ng UHR single and duplex data, respectively. The $\Delta\Delta C_t$ correlation results show that the relative expression values obtained for UHR and brain did not change for an assay that is run in duplex or in singleplex ($R^2 = 0.9859$), and the difference between the $\Delta\Delta C_t$ values was <1.5 for each assay comparison (Figure 4).
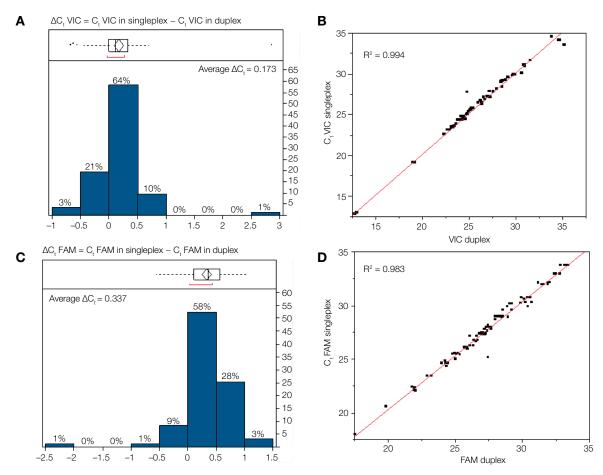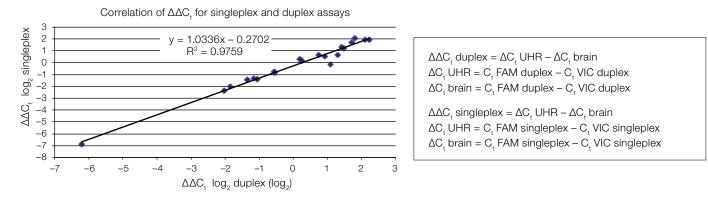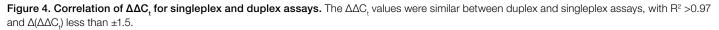

Figure 3. Duplexing TaqMan Gene Expression Assays does not alter C_t values significantly compared to singleplex assays. (A, C) Absolute C_t differences across both FAM and VIC channels are small for assays run in singleplex or duplex. For VIC duplex pairs (A), 86% (77 pairs) had ΔC_t between –0.5 and 0.5, and 98% (89 pairs) had ΔC_t between –1 and 1. For FAM duplex pairs (C), 67% (60 pairs) had ΔC_t between –0.5 and 0.5, and 96% (90 pairs) had ΔC_t between –1 and 1. **(B, D)** Singleplex vs. duplex assay correlations. In B, the VIC singleplex data were filtered for C_t <35.

Figure 4. Correlation of ΔΔC$_t$ for singleplex and duplex assays. The ΔΔC$_t$ values were similar between duplex and singleplex assays, with $R^2 > 0.97$ and $\Delta(\Delta\Delta C_t)$ less than ±1.5.

Since it is not always known if a particular target is a high expressor or not, we wanted to see if there would be a greater C$_t$ difference for the VIC dye–labeled primer-limited assays compared to singleplex ones if we reversed the pairing and used the FAM non–primer-limited assay formulation on the high expressor. We ran duplex reactions on 49 additional pairs with reversed assay targeting: the FAM non–primer-limited assay targeting the more abundant transcripts, and the VIC primer-limited assay targeting the low-abundance transcript. We compared these results to singleplex and duplex reactions run under recommended pairing conditions. The results showed that only 3 pairs had ΔC$_t$ >1 between singleplex and duplex reactions for the VIC dye–labeled primer-limited assay. In addition, the FAM dye–labeled assay for these 3 duplex pairs had ΔC$_t$ ≤1 between the singleplex and duplex assays. The remaining 46 duplex pairs showed ΔC$_t$ ≤1 between singleplex and duplex for both the VIC and FAM dye–labeled assays (data not shown).

These data suggest that pairing assays is feasible even when it is not known which transcript is the more (or less) abundant one. If the duplex pair is reversed (high expressor as the FAM dye–labeled non–primer-limited assay and the low expressor as the VIC dye–labeled primer-limited assay), this assay pairing may still be relatively robust. Since this may not be true for all assay duplex pairs, it is recommended that a duplex reaction be verified to confirm that the same results would be obtained if the reactions were performed individually. Please note that when verifying duplex qPCR assays, dilutions of each target should be tested against each other to determine the dynamic range of the duplex—it may be different from those of the two singleplex assays.

For QuantStudio systems or Connect platform users, the C$_{rt}$ (relative threshold) setting may be tested. In some cases, this has improved duplex qPCR results. For more information on C$_{rt}$, read the application note [3].

Conclusion

In the past, development and utilization of duplex qPCR assays has been hampered by the complexity of reaction design and multiple rounds of optimizing duplex reactions for high similarity to singleplex reactions. Thermo Fisher Scientific has greatly simplified the process by offering a total qPCR solution comprising optimized TaqMan Gene Expression Assays labeled with FAM or VIC dye, TaqMan Gene Expression Master Mix or Applied Biosystems™ TaqMan® Fast Advanced Master Mix, and a user protocol.

We have demonstrated that duplexing with FAM and VIC dye–labeled TaqMan Gene Expression Assays produces results that are highly correlated with those obtained for the same assays run in singleplex. For researchers interested in duplexing, comparable singleplex and duplex results are paramount for maintaining the accuracy of gene signatures. It is recommended that, on the first run, the results obtained from a duplex reaction be verified to confirm that the same results would be obtained if the reactions were performed in singleplex. Using our recommended protocol, researchers with large studies or those who routinely use a defined set of gene expression assays can take advantage of the benefits that duplex qPCR has over standard singleplex qPCR—higher throughput, less sample usage, and potential cost savings in master mix. For researchers in fields such as translational and clinical research, duplexing with TaqMan Gene Expression Assays allows gaining more information with high efficiency.

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Ordering information

Product	Quantity	Cat. No.
TaqMan Gene Expression Assay, FAM dye	250 reactions, inventoried **Additional sizes available**	4331182
TaqMan Gene Expression Assay, VIC dye, primer-limited	360 reactions, made to order Additional sizes available	4448484
TaqMan Gene Expression Assay, VIC dye	360 reactions, made to order Additional sizes available	4448489
TaqMan Fast Advanced Master Mix	1 x 5 mL Additional sizes available	4444557
TaqMan Gene Expression Master Mix	1 x 5 mL Additional sizes available	4369016
High-Capacity cDNA Reverse Transcription Kit	200 reactions Additional sizes available	4368814
Human Brain Total RNA	100 µg	AM7962
Universal Human Reference RNA	10 µg	QS0639

References

1. Hein AE, Bodendorf U (2007) Real-time PCR: duplexing without optimization. *Anal Biochem* 360:41–46.
2. Thermo Fisher Scientific. Application note: Factors influencing multiplex RT-PCR (https://assets.thermofisher.com/TFS-Assets/LSG/Application-Notes/cms_076529.pdf).
3. Thermo Fisher Scientific. Application note: C_{rt}, a relative threshold method for qPCR data analysis on the QuantStudio 12K Flex system with OpenArray technology (https://assets.thermofisher.com/TFS-Assets/LSG/brochures/CO28730-Crt-Tech-note_FLR.pdf).

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