

Multiplex real-time PCR with four targets using EXPRESS qPCR SuperMix

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Multiplex PCR is a technique in which more than one target is amplified and detected in the same PCR reaction. In real-time PCR, the amplicons are distinguished from one another by the dyes conjugated to the fluorogenic probe or primer. The number of targets that may be amplified in a given reaction is technically limited only by two factors: the number of available spectrally distinct dyes, and the number of dyes that can be excited and detected by the real-time PCR instrument. However, experimental hurdles can be more problematic. In order to achieve successful multiplex PCR results, the primer pairs and/or probes present in the reaction mix must not interact with each other. Typically, it is necessary to spend time optimizing several variables such as the concentrations of Mg^{2+} , dNTPs, enzymes, buffer components, primers, and probes before multiplex PCR can be performed.

Purpose

Here, we demonstrate results from multiplexing experiments using the EXPRESS qPCR SuperMix Universal reagent and the EXPRESS One-Step SuperScript® qRT-PCR Universal reagent from Invitrogen with the Applied Biosystems 7500 Fast Real-Time PCR System, the Applied Biosystems StepOnePlus™ Real-Time PCR System, and the Bio-Rad iQ™5 Real-Time PCR Detection System. The data

demonstrate that even under difficult conditions such as when using the one-step qRT-PCR kits, the EXPRESS SuperMix reagents require little or no reagent optimization, reducing time and costs associated with these methods. General guidelines for successful multiplexing are included.

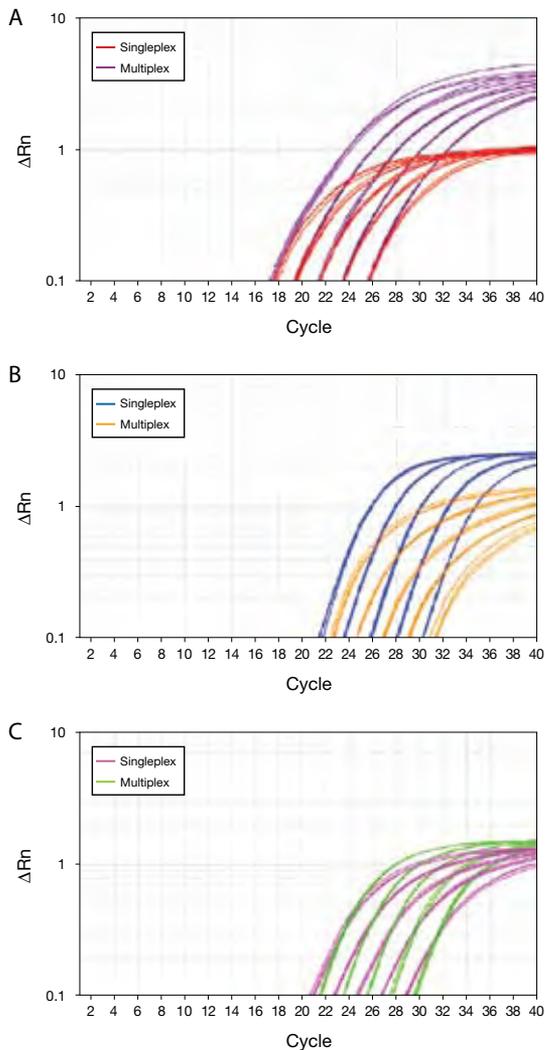


Figure 1—Amplification plots, in singleplex and multiplex, for the three targets in the multiplex assay on the AB 7500 instrument. (A) β -2 microglobulin, (B) CAMK4, and (C) THRA. Standard curves were generated using 4-fold dilutions of cDNA corresponding to 0.39–100 ng RNA, tested in singleplex or triplex. The threshold cycle (C_t) values obtained at each dilution are summarized in Table 2. Over a dilution series of input template, the EXPRESS qPCR SuperMix can successfully and reliably generate up to 3-target multiplex data on the AB 7500 instrument.

Materials and methods

qPCR SuperMix

The EXPRESS qPCR SuperMix Universal reagent (Invitrogen Cat. no. 11785-200) was used for multiplex real-time PCR analysis. The supermix contains all the components necessary for real-time PCR except primers, probe, and template. The EXPRESS One-Step SuperScript® qRT-PCR Universal kit (Invitrogen Cat. no. 11781-200) was used for one-step multiplex experiments.

Assays

TaqMan® assays (Table 1) were designed using the Primer Express® Software Version 3.0 (Applied Biosystems). A concentrated primer/probe mix (4 μ M probe and 10 μ M each primer) was made for each designed assay. The final concentration of each primer and probe in the real-time PCR reaction was 0.5 μ M and 0.2 μ M, respectively.

Template cDNA or RNA

The cDNA used in the multiplex real-time PCR experiments was generated using the SuperScript® VILO® cDNA Synthesis Kit (Invitrogen Cat. no. 11754-250). One microgram of real-time PCR reference RNA (Agilent) was used as the template in the cDNA synthesis, and 100 ng of real-time PCR reference RNA was used in the one-step qRT-PCR reactions.

PCR protocol and data analysis

The PCR experiments were performed according to the protocol and cycling conditions outlined in the EXPRESS qPCR SuperMix kit manual. Data analysis was performed according to the manufacturer’s recommended instructions for each instrument.

Table 1—TaqMan® assays designed for multiplexing qPCR evaluation of EXPRESS qPCR reagents.

Target gene	Fluorophore	Quencher
β -2 microglobulin	TAMRA	BHQ2
CAMK4	FAM	BHQ2
THRA	HEX	BHQ1
Cyclophilin	Quasar® 670	BHQ2

Table 2— C_t values for three targets, β -2 microglobulin (B2M), CAMK4, and THRA, in singleplex or multiplex.

Target tested	Amount of input template per reaction				
	100 ng	25 ng	6.25 ng	1.56 ng	0.39 ng
B2M singleplex	19.0	21.0	23.0	25.1	27.2
B2M multiplex	18.8	20.8	22.8	24.8	26.9
CAMK4 singleplex	22.5	24.6	26.8	29.0	31.2
CAMK4 multiplex	23.6	25.8	28.2	30.4	32.6
THRA singleplex	22.6	24.6	26.6	28.7	30.7
THRA multiplex	22.2	24.2	26.2	28.2	30.4

Results

Three-target multiplex on the AB 7500 using EXPRESS qPCR SuperMix Universal reagent

Figure 1 shows amplification plots of a 3-target multiplex assay. The three targets tested in triplex were β -2 microglobulin, CAMK4, and THRA using the following respective dyes: TAMRA, FAM, and HEX. The data demonstrate that over a dilution series of input template, the EXPRESS qPCR SuperMix Universal reagent can successfully and reliably generate up to 3-target multiplex data on the AB 7500 instrument.

Four-target multiplex on the Bio-Rad iQ™5 instrument using EXPRESS qPCR SuperMix Universal reagent

TaqMan® assays for four targets were tested in single- and 4-plex using the EXPRESS qPCR SuperMix reagent on the Bio-Rad iQ™5 instrument. The targets tested were β -2 microglobulin, cyclophilin, THRA, and CAMK4. The dyes used for each TaqMan® assay were TAMRA, Quasar® 670, HEX, and FAM respectively. β -2 microglobulin and cyclophilin are relatively abundant targets whereas THRA and CAMK4 are mildly abundant targets. The data shown in Figure 2 confirm that EXPRESS qPCR SuperMix robustly amplifies up to four targets using the Bio-Rad iQ™5 instrument.

Multiplex with EXPRESS One-Step SuperScript® qRT-PCR Universal SuperMix on the AB StepOnePlus™ instrument

TaqMan® assays for two targets (β -2 microglobulin and CAMK4) were tested in singleplex and duplex using the EXPRESS One-Step SuperScript® qRT-PCR SuperMix reagent on the AB StepOnePlus™ Real-Time PCR System. The dyes used for each TaqMan® assay were TAMRA and FAM, respectively. Four-fold dilutions starting with 100 ng of qPCR reference RNA were used as template. The standard curves in singleplex and duplex are shown in Figure 3 and demonstrate no difference in efficiency.

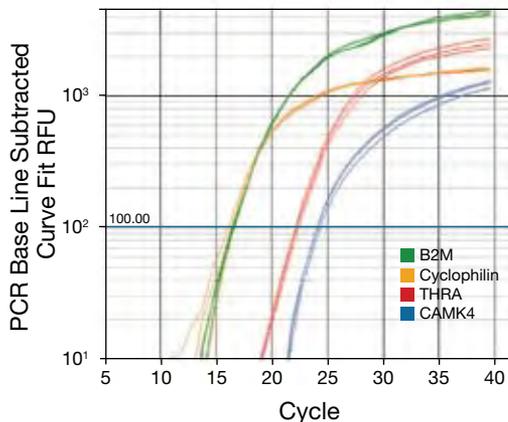
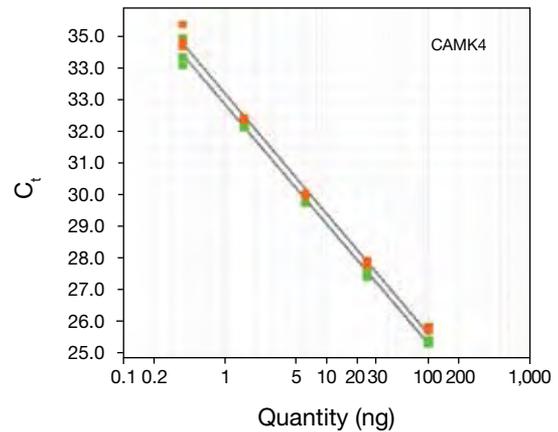
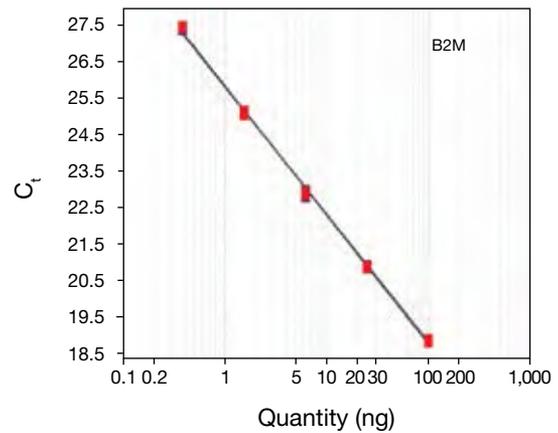


Figure 2—Amplification plots for four targets in a multiplex assay using the Bio-Rad iQ™5 instrument.



	B2M singleplex	B2M multiplex	CAMK4 singleplex	CAMK4 multiplex
Slope	-3.53	-3.49	-3.61	-3.58
R ²	0.998	0.999	0.996	0.996
Y-intercept	25.9	25.9	33.3	32.9

Figure 3—Standard curves for singleplex and duplex assays for β -2 microglobulin (B2M) and CAMK4 using the EXPRESS One-Step SuperScript® qRT-PCR SuperMix with the AB StepOnePlus™ instrument. Without any optimization to the reaction mix, and despite using the challenging one-step qRT-PCR format, the duplex data are comparable to the singleplex data as shown by the R² data for each gene.

Discussion

Our results demonstrate that when using EXPRESS qPCR reagents, optimization of the reaction reagents and cycling conditions are not required for optimal results, saving time and money. Nevertheless, optimization may be necessary with challenging targets such as GC-rich templates or when multiplexing targets with different abundance levels.^{1,2} Some of the key elements to take into consideration for successful multiplexing reactions include:

- **Primer and probe design:**
Primer and probe design is probably the most critical factor in development of multiplex real-time PCR assays. As reaction complexity increases with multiple primer/probe sets, so does the probability that primers and probes will dimerize. General guidelines include using short amplicons (60 to 150 bp), maintaining the primer melting temperatures within 5°C or less of one another, and confirming primer specificity by performing a BLAST search (www.ncbi.nlm.nih.gov/blast).
- **Reagent concentrations:**
Taq DNA polymerase, magnesium, dNTPs, and buffer strength may need to be increased to match the demanding nature of the multiplex reactions.

- **Relative abundance of targets and concentrations of primers/probes:**
Although the data shown in this application note were generated without limiting the primer concentration of the more abundant targets, it is advised to test limiting primer concentrations in order to decrease competition between the highest and lowest abundant genes for the reaction reagents. By limiting the primer concentration of the highest abundant gene, you will limit the amount of this product generated, allowing more reagents to be available for the less abundant genes.
- **Fluorophore/quencher combinations:**
The fluorophore reporter combinations in a multiplex reaction must be spectrally distinct. Choose combinations of reporter dyes and quenchers that are compatible with the detection optics of your instrument. The dye combinations used in a multiplex reaction for a specified instrument should be decided based on the detection capabilities of the instrument.

To increase the chances of easy and successful multiplexing reaction development, we recommend using the EXPRESS qPCR reagents as they are provided in a robust formulation that enable you to go from singleplex assays to multiplex assays without significant reagent optimization.

Ordering information

Product	Quantity	Cat. no.
EXPRESS qPCR SuperMix Universal	5 ml (200 x 50 µl rxns)	11785-200
EXPRESS One-Step SuperScript® qRT-PCR Universal	5 ml (200 x 50 µl rxns)	11781-200

For more information and to order, visit www.invitrogen.com/express.

References:

1. Persson, K. et al. (2005) Four-color multiplex reverse-transcription reaction – overcoming its limitations. *Anal Biochem* 344:33-42.
2. Walker, J.A. et al. (2005) Multiplex polymerase chain reaction for simultaneous quantitation of human nuclear, mitochondrial and male Y chromosome DNA: application in human identification. *Anal Biochem* 337:89-97.