

Research Note

Quantitative Multiplex Real-Time PCR Assay for Shrimp Allergen: Comparison of Commercial Master Mixes and PCR Platforms in Rapid Cycling

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MS 14-203: Received 2 May 2014/Accepted 10 July 2014

ABSTRACT

Real-time PCR has been used widely in numerous fields. In food safety, it has been applied to detection of microbes and other contaminants, including food allergens. Interest in rapid (fast) cycling real-time PCR has grown because it yields results in less time than does conventional cycling. However, fast cycling can adversely affect assay performance. Here we report on tests of commercial master mixes specifically designed for fast real-time PCR using a shrimp allergen assay we previously developed and validated. The objective of this work was to determine whether specialized commercial master mixes lead to improved assay performance in rapid cycling. Real-time PCR assays were carried out using four different master mixes and two different rapid cycling protocols. Results indicated that specialized master mixes did yield quality results. In many cases, linear ranges spanned up to 7 orders of magnitude, R^2 values were at least 0.95, and reaction efficiencies were within or near the optimal range of 90 to 110%. In the faster of the two rapid cycling protocols tested, assay performance and PCR amplification were markedly better for the shorter PCR product. In conclusion, specialized commercial master mixes were effective as part of rapid cycling protocols, but conventional cycling as used in our previous work is more reliable for the shrimp assay tested.

Methods based on a real-time PCR are nearly ubiquitous and are used in diverse and numerous fields, including applied research in food safety, environmental science, biodefense, and clinical diagnostics. In the area of food safety, the real-time PCR method has been applied to the detection of food allergens. Food allergen detection requires methods that can specifically detect trace amounts of the target allergenic food against a complex background of a nontarget food matrix, and the real-time PCR method is well suited to this task (14). PCR-based allergen detection is not optimal for some allergenic foods, but in those cases in which it is appropriate, PCR has important advantages. The most significant advantage is that the PCR method is not adversely affected by food processing or complex food matrices as are the enzyme-linked immunosorbent assays often used in allergen detection (14).

Compared with end-point PCR methods, the real-time PCR method is more sensitive and more specific and can be used for quantitation, whereas end-point PCR methods cannot. Real-time PCR methods do not require postrun processing, thus reducing the chance of contamination and providing faster results (17, 19). The real-time PCR approach is well suited to simultaneous detection of multiple targets. Such multiplexing can increase laboratory throughput and allows internal confirmation or control

targets to be assayed in the same sample as the allergen targets. Sample preparation, DNA extraction, and PCR can readily be carried out on 24 samples at a time by a single analyst. There is significant interest in rapid cycle (fast) real-time PCR methods in which denaturation, annealing, and extension steps are shortened dramatically and PCR run times are decreased from 2 to 3 h to 30 to 40 min (8, 12, 15). Although rapid cycling decreases total run time and can increase laboratory throughput, it can also adversely affect assay performance (5, 8). Although conventional longer cycling may increase the time needed to obtain a result, it does not require a greater time commitment for the laboratory analyst because the PCRs simply remain in the instrument for a longer time.

Results of the fast real-time PCR can be affected by PCR product length, reaction composition, and platform. Results of our previous work suggest that fast cycling has a greater adverse effect on longer PCR products than on shorter PCR products (5). The importance of the reaction composition in PCR amplification has long been appreciated; concentrations of common reaction components such as free Mg^{2+} and K^+ influence PCR amplification through their effects on DNA melting, hybridization, and secondary structure (13, 16, 18). Reaction composition can be expected to become especially relevant during fast cycling, in which amplification must be optimal for targets to be copied efficiently in the short time allowed. Numerous manufacturers sell prefabricated master mixes specially

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formulated and optimized for use in fast real-time PCR assays. Although published studies comparing such commercial master mixes are limited, tests have shown that these mixes work on numerous platforms, can improve results compared with conventional reagents, and can yield high reaction efficiencies and high R^2 values in fast real-time PCR assays (3, 12). Variations in performance between real-time PCR platforms likely arise from discrepancies between the instrument's target temperature and the actual temperature of the sample. Sample temperature often lags behind instrument temperature, and differences can be up to 5°C. The effects of such differences are exacerbated in fast cycling and can have a significant impact on results (9, 15).

Several studies have been conducted to compare commercial master mixes for rapid cycle PCR assays (2, 3, 8). In previous studies of fast reaction mixes used in real-time PCR assays, a single product was amplified, and standard curves were generated via dilution of target DNA rather than by spiking (3, 8). Spiking into food matrices is important for allergen detection methods because these methods must detect trace amounts of an allergenic food against a complex background of a nonallergenic food matrix. Our previous work with a real-time PCR assay for shrimp (a crustacean shellfish allergen) has revealed that conventional cycling produced substantially better results than did fast cycling when conventional reagents were used (5). The work reported here was done to determine whether altering the reaction conditions would improve assay performance with rapid cycling. Tests were conducted with (i) four commercial real-time PCR master mixes designed for rapid cycling, (ii) two different real-time PCR platforms, and (iii) two different rapid thermal profiles. Here, we report (i) the linear range, R^2 , and reaction efficiencies for all conditions, (ii) standard curves generated via both dilution of target DNA in buffer and spiking of the allergenic food (shrimp) into a nontarget matrix, and (iii) the use of a multiplex PCR assay that includes two target PCR products of different lengths and an internal control. This report of the effects of fast cycling on real-time PCR assay performance is more thorough than those published previously.

MATERIALS AND METHODS

Assay design, sample preparation, DNA extraction, and PCR were carried out as previously described (1, 5). For the tests done using shrimp DNA in buffer, shrimp meat was homogenized and directly subjected to DNA extraction followed by 10-fold serial dilution of DNA in Tris-EDTA (TE) buffer. Shrimp DNA was used in PCRs at 0.005, 0.05, 0.5, 5, 50, 500, and 5,000 pg per reaction. For the spiking experiments, raw salmon was spiked with shrimp meat at 0.1, 1, 10, 100, 1,000, 10^4 , and 10^5 ppm before homogenization and DNA extraction. Spiked samples were mixed with 2% sodium dodecyl sulfate buffer (4 ml/g) and homogenized in a blender for 2 min. DNA was extracted from the homogenate using a combined salt extraction (1) and the DNeasy kit (Qiagen, Valencia, CA) as previously described (5). DNA from spiking experiments was used in PCRs at 50 ng of total DNA per reaction for all samples, which corresponds to 0.005 pg (0.1 ppm), 0.05 pg (1 ppm), 0.5 pg (10 ppm), 5 pg (100 ppm), 50 pg (1,000 ppm), 500 pg (10^4 ppm), and 5,000 pg (10^5 ppm) of shrimp DNA per

reaction. Oligonucleotide primers and probes were designed using Allele ID software (Premier Biosoft, Palo Alto, CA) and targeted conserved regions of the 12S and 16S mitochondrial gene sequences from six species of penaeid shrimp (5). A single primer pair and a single probe were used for each target (12S or 16S); sequences were previously published (5). All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). The internal amplification control (IAC) developed by Deer et al. (4) was incorporated into PCRs as previously described (5). Four commercial master mixes were tested: Brilliant III Ultra-Fast (cat. 600880, Agilent Technologies, Santa Clara, CA), QuantiFast Multiplex (cat. 204752, Qiagen), TaqMan Fast Advanced (cat. 4444556, Applied Biosystems, Carlsbad, CA), and iTaq Fast (cat. 172-5105, BioRad, Hercules, CA). PCRs were set up for each master mix according to the manufacturer's instructions in 12.5 μ l of total reaction volume. Primers and probes for each target sequence and the IAC were used at 200 nM (5). Two different real-time PCR platforms were used: the Mx3005P (Agilent Technologies) and the 7500 Fast (Life Technologies, Grand Island, NY). All master mixes were tested on both platforms using the same two rapid cycling thermal profiles (Fast 1 [Table 1] and Fast 2 [Table 2]) for a direct comparison under identical cycling conditions. The manufacturer-recommended thermal profile for each master mix was tested on the 7500 Fast platform using shrimp DNA diluted in buffer (Fig. 1). Data analysis for each real-time system was conducted using the software provided by its manufacturer. Results presented here are the combined data from two independent PCR runs.

RESULTS AND DISCUSSION

The real-time PCR assay provided cycle threshold (C_T) values that were plotted as a function of picograms of shrimp DNA (for pure shrimp DNA experiments) or ppm (for spiking experiments). The best-fit line through the data points was used to generate linear standard curves, which were analyzed for linear range, R^2 (how well the points fit the line), and reaction efficiency (slope of the line) (19). The optimal real-time PCR assay has reaction efficiencies of $100\% \pm 10\%$, an R^2 value of 0.98 to 1.00, and a linear range spanning six to eight orders of magnitude. Standard curves for pure shrimp DNA diluted in TE buffer, using the manufacturer's recommended thermal profile for each master mix, yielded the expected negative slopes as a function of shrimp DNA quantity (Fig. 1). The IAC yielded a horizontal line as expected; the same amount of IAC template was spiked into each PCR, and therefore C_T values were similar in all samples regardless of the amount of shrimp DNA. More thorough tests were carried out to compare master mixes under identical cycling conditions. Linear range, reaction efficiency, and R^2 values resulting from these tests are reported for both pure shrimp DNA diluted in TE buffer and shrimp spiked into salmon using the Fast 1 (Table 1) and Fast 2 (Table 2) thermal profiles. Results from both the Fast 1 and Fast 2 thermal profiles revealed that data obtained using shrimp spiked into salmon (bottom of Tables 1 and 2) are comparable to data obtained using pure shrimp DNA in buffer (top of Tables 1 and 2). Thus, the results of fast cycling were not adversely affected by high levels of nontarget background (salmon) DNA in the spiking experiments. Specific differences with respect to

TABLE 1. *Fast 1 data*^a

		16S			12S			IAC	
Platform	Master mix	Range ^b	Efficiency (%)	R ²	Range	Efficiency (%)	R ²	Range	C _T ± SD
Shrimp DNA diluted in TE buffer									
7500 Fast	Brilliant III	0.05–500	88.1	0.81	0.005–500	88.1	0.83	0.005–500	23.41 ± 0.26
	iTaq Fast	0.05–50	82.2	0.99	0.05–5,000	93.8	0.93	0.005–50	22.86 ± 0.13
	QuantiFast	0.05–50,000	84.7	0.86	0.005–50,000	88.5	0.60	0.005–500	23.74 ± 0.57
	TaqMan Fast	0.05–50,000	90.0	0.57	0.005–500	104.2	0.98	0.005–500	23.83 ± 2.02
Mx3005P	Brilliant III	0.05–500	83.2	0.98	0.005–500	101.5	0.97	0.005–500	22.79 ± 1.11
	iTaq Fast	0.05–500	106.1	0.89	0.005–500	94.5	0.95	0.005–500	24.76 ± 2.05
	QuantiFast	0.005–500	119.7	0.91	0.005–500	110.9	0.93	0.005–500	21.73 ± 0.34
	TaqMan Fast	0.05–500	85.6	0.96	0.005–500	99.5	0.99	0.005–50,000	21.47 ± 1.18
Shrimp spiked into salmon									
7500 Fast	Brilliant III	10–10 ⁵	96.9	0.65	1–10 ⁵	130.9	0.74	0.1–10 ⁵	23.56 ± 0.09
	iTaq Fast	1–10 ⁵	85.4	0.99	0.1–10 ⁵	97.6	1.00	0.1–10 ⁵	22.88 ± 0.10
	QuantiFast	0.1–10 ⁵	84.2	0.92	0.1–10 ⁵	100.4	0.95	0.1–10 ⁵	23.77 ± 0.28
	TaqMan Fast	10–10 ⁵	81.0	0.97	0.1–10 ⁵	119.0	0.97	0.1–10 ⁵	28.64 ± 2.51
Mx3005P	Brilliant III	1–10 ⁵	115.9	0.88	0.1–10 ⁵	133.3	0.92	0.1–10 ⁵	23.24 ± 1.46
	iTaq Fast	10–10 ⁵	109.8	0.99	0.1–10 ⁵	113.4	0.97	0.1–10 ⁵	24.41 ± 1.75
	QuantiFast	0.1–10 ⁵	101.9	0.97	0.1–10 ⁵	125.5	0.97	0.1–10 ⁵	22.88 ± 0.96
	TaqMan Fast	0.1–10 ⁵	106.4	0.88	0.1–10 ⁵	118.6	0.97	0.1–10 ⁵	22.79 ± 0.16

^a Fast 1 thermal cycling parameters: 95°C for 3 min followed by 45 cycles of 95°C for 10 s, 55°C for 20 s, and 72°C for 20 s. Total run times were 65 min on the 7500 Fast and 81 min on the Mx3005P.

^b Ranges for shrimp DNA diluted in buffer are given in picograms of DNA per reaction; ranges for shrimp in salmon are given in ppm.

PCR product, reaction composition (master mix), and platform are discussed in greater detail below.

PCR product. Using thermal profiles recommended by the manufacturer of each master mix, reaction efficiencies

for the 12S PCR product were generally in the optimal ranges, but efficiencies and R² values for the 16S product were lower (Fig. 1). Calculations using the standard error of the slopes for each standard curve indicate that differences between the 12S and 16S PCR products were significant for

TABLE 2. *Fast 2 data*^a

		16S			12S			IAC	
Platform	Master mix	Range ^b	Efficiency (%)	R ²	Range	Efficiency (%)	R ²	Range	C _T ± SD
Shrimp DNA diluted in TE buffer									
7500 Fast	Brilliant III	NA			0.05–500	100.0	0.43	0.005–50	24.04 ± 0.14
	iTaq Fast	NA			0.005–5,000	92.8	0.92	0.005–50	22.27 ± 0.79
Mx3005P	QuantiFast	MA			0.005–500	80.3	0.94	0.005–500	24.86 ± 3.42
	TaqMan Fast	5–500	67.7	0.88	0.005–500	127.7	0.70	0.005–500	24.92 ± 2.25
	Brilliant III	NA			0.05–500	82.3	1.00	0.005–500	22.66 ± 1.03
	iTaq Fast	NA			0.5–500	87.6	0.96	0.005–50	25.15 ± 3.81
	QuantiFast	NA			0.05–500	113.8	0.68	0.005–500	22.72 ± 1.23
	TaqMan Fast	0.5–500	64.7	0.80	0.005–500	102.1	0.97	0.005–5,000	24.23 ± 1.58
Shrimp spiked into salmon									
7500 Fast	Brilliant III	NA			0.1–10 ⁵	98.1	0.99	0.1–10 ⁵	24.84 ± 0.27
	iTaq Fast	MA			0.1–10 ⁵	93.8	0.94	0.1–10 ⁵	22.63 ± 1.36
	QuantiFast	NA			1–10 ⁵	69.9	0.99	0.1–10 ⁵	24.70 ± 1.69
	TaqMan Fast	1–10 ⁵	89.5	0.99	0.1–10 ⁵	122.9	0.79	0.1–10 ⁵	25.11 ± 2.30
Mx3005P	Brilliant III	MA			0.1–10 ⁵	122.4	0.93	0.1–10 ⁵	24.35 ± 0.62
	iTaq Fast	MA			0.1–10 ⁵	101.8	0.99	0.1–10 ⁵	25.16 ± 2.66
	QuantiFast	NA			0.1–10 ⁵	100.1	0.94	0.1–10 ⁵	23.54 ± 0.77
	TaqMan Fast	1–10 ⁵	75.2	0.78	0.1–10 ⁵	104.8	0.90	0.1–10 ⁵	23.30 ± 1.87

^a Fast 2 thermal cycling parameters: 95°C for 3 min followed by 45 cycles of 95°C for 1 s and 60°C for 20 s. Total run times were 38 min on the 7500 Fast and 54 min on the Mx3005P.

^b Ranges for shrimp DNA diluted in buffer are given in picograms of DNA per reaction; ranges for shrimp in salmon are given in ppm. NA, no amplification; MA, miscellaneous amplification.

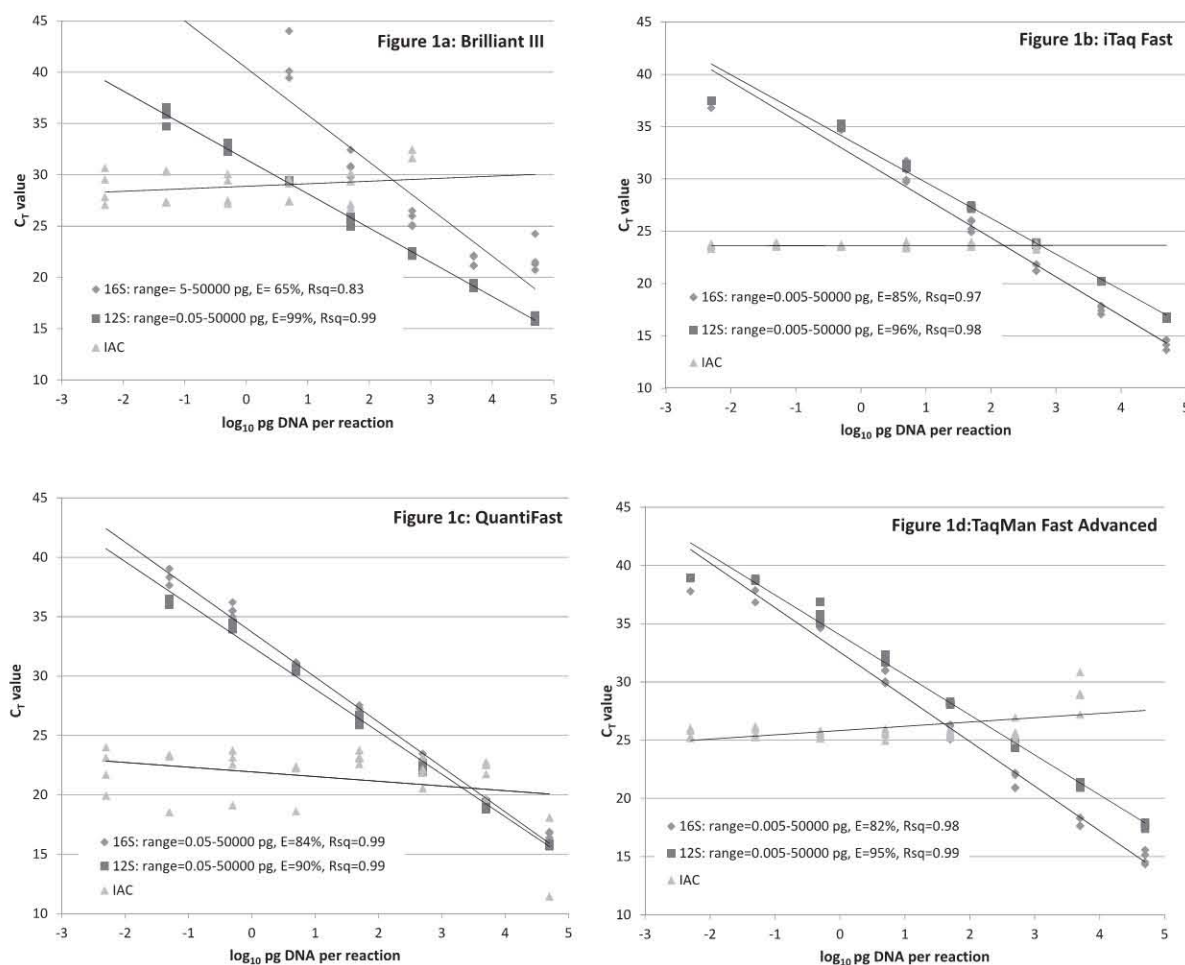


FIGURE 1. Data obtained with the 7500 Fast using the manufacturer's recommended thermal cycling parameters for (a) Brilliant III, (b) iTaq Fast, (c) QuantiFast, and (d) TaqMan Fast Advance master mixes.

the Brilliant III, QuantiFast, and TaqMan FastAdvance (Fig. 1a, 1c, and 1d) but not for the iTaq Fast (Fig. 1b). For the Fast 2 thermal profile, marked differences were observed between the 16S and 12S PCR products; the 16S product did not amplify at all in several cases (Table 2). This lack of amplification may be a result of PCR product length or of other sequence-specific aspects of the 16S target. The 16S PCR product (176 bp) is more than twice as long as the 12S PCR product (79 bp). Because the Fast 2 profile is especially short, even compared with the Fast 1 profile, there may not have been sufficient time for the longer 16S target to amplify. Using Fast 2 cycling conditions, some researchers have reported a slight adverse effect on reaction efficiencies and R^2 values with increasing PCR product length up to 121 bp (7). In other reports of Fast 2 with PCR products up to 177 bp, full reaction efficiency data were not reported (10, 11). Differences between the two PCR products is the most striking result in our data set, and these data suggest that shorter targets are better suited to fast real-time PCR assays.

In addition to the 12S and 16S shrimp targets, this assay included an IAC as previously described (4, 5), which is designed to detect PCR inhibition and consists of exogenous template DNA and its associated primers and probe. The IAC amplified consistently, with average C_T values of 22 to

25 and standard deviations of less than 2.50 (relative standard deviations of less than 10%) in nearly all cases (Fig. 1 and Tables 1 and 2). The IAC amplified well even under conditions at which the 16S target was not amplified at all (Table 2). Therefore, consistent amplification of the IAC was not necessarily indicative of consistent or efficient amplification of assay targets. The IAC was designed to detect inhibitors in PCRs. Although the PCRs reported in this study were clearly not inhibited, amplification of the 16S target was adversely affected—even completely eliminated—under certain conditions. These results underscore the importance of thoroughly optimizing and validating PCR methods for all targets.

Reaction composition. Reaction composition influences PCR results primarily through the effects of mono- and divalent cations on DNA melting and hybridization (13, 16). Although manufacturers provide limited details concerning the exact compositions of fast master mixes, most are optimized for high specificity, efficiency, and multiplexing in rapid cycling. Commercial master mixes used in this work contained Mg^{2+} , K^+ , and/or NH_4^+ salts and proprietary components that (i) favor rapid, specific binding of primers to template DNA, (ii) stabilize primer and probe duplexes with template, or (iii) facilitate polymerase activity. Compared

with the results of our previous work, the commercial master mixes did improve the results obtained with fast cycling. However, our previously reported standard thermal cycling procedure with conventional PCR reagents yielded the most consistent and reliable results (5, 6).

Platform. In addition to the contents of the PCR itself, instrument performance is important and can vary by platform. Relevant factors include time required to reach the target temperature, over- and undershooting the target temperature, and temperature fluctuation during holding periods (9, 15). Such factors are especially important during fast PCR assays and can significantly affect results. The two real-time PCR systems we tested use different methods of detection; the Mx3005P uses a photomultiplier tube, and the 7500 Fast uses a charge coupled device camera. These differences in the optical system can theoretically affect final results. Substantial differences between PCR platforms were not observed in the data obtained in this study.

In summary, fast master mixes did yield quality results in rapid cycling assays but did not outperform conventional reagents and conventional cycling as used in our previous work.

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