Multiplex Applications Using the iCycler iQ™ Real-Time PCR Detection System

BIO-RAD

Abstract

Multiplex PCR involves amplification of more than one DNA target in a single reaction tube. One obvious advantage to multiplexing is that it allows for higher throughput, providing maximum information from minimal sample. It also provides a higher confidence in results when quantitating and comparing different genes (e.g., gene of interest with housekeeping genes) in applications such as detection of genetically modified organisms (GMO) and assessing relative gene expression by RT-PCR. Another example of the power of multiplexing is in the unambiguous assignment of single nucleotide polymorphisms (SNPs) that can be achieved when probes for both wild-type and mutant sequences are present in the same reaction mixture. Real-time PCR using the iCycler iQ detection system allows for amplification and quantitation of up to four DNA targets in the same reaction tube. In this poster, we present results from optimized multiplex real-time PCR experiments conducted with the iCycler iQ. We demonstrate the accurate, simultaneous quantitation of a GMO (Roundup Ready soya) normalized to a control gene (lectin) in soya genomic DNA preparations. We also demonstrate a triplex assay used to quantitate the relative expression of three individual genes in prostate and thymus. Finally, we show results from an SNP assay that distinguishes between four allelic variants in a single multiplex reaction. These data prove that multiplexing is feasible and is a powerful technique for real-time PCR applications.

Genetically Modified Organism (GMO) Testing Using Multiplex Real-Time PCR

Introduction

Multiplex real-time PCR is a method widely used to measure absolute quantities of GMOs present in foods, finished goods, and agricultural products. GMOs are produced by genetically altering plant DNA to introduce beneficial traits. Multiplex real-time PCR is the only method that can simultaneously detect a soya-specific endogenous control gene and a GMO-specific gene, to quantitate the absolute percentage of GMO content in genomic DNA samples. Products containing as little as 0.01% GMO can be quantitated by this method. This level of accuracy has become particularly important due to labeling requirements by the European, Japanese, and Korean governments (>1% GMO must be labeled). This study demonstrates quantitative detection of Roundup Ready soya (Monsanto genetically modified soybeans, tolerant to the herbicide glyphosate) normalized to the lectin gene (specific for soya) by real-time multiplex PCR.

Materials and Methods

The methods used for quantitation of Roundup Ready soya (RRS) were based on a proprietary procedure from Nestlé. A similar procedure can be found in Vaitilingom M et al., J Agric Food Chem 47, 5261–5266 (1999).

Specific primers designed for amplification of the lectin gene (endogenous control gene, specific for soya) and the CP4EPSPS gene (specific for RRS) were obtained from Integrated DNA Technologies, Inc. (IDT). RRS was detected by a specific dual-labeled oligonucleotide probe (IDT) labeled with FAM and quenched by Black Hole Quencher 1. The lectin gene was also detected with a specific dual-labeled oligonucleotide probe (IDT), labeled with HEX and quenched with Black Hole Quencher 2. Standard curves for RRS and soya DNA were generated from dilutions of DNA prepared from a soya bean powder standard (Fluka) containing 2% GMO (2% RRS in 100% soya). The final DNA dilutions for the standard curves were derived from 2–0.02 ng of RRS and 100–1 ng of soya. The DNA for the 1% and 0.1% RRS references were extracted from soya bean powder standards (Fluka). The amounts of RRS and soya in reference and unknown samples were quantitated in multiplex PCR reactions on the same plate. Percentages of RRS in the reference and unknown samples were calculated from the RRS and lectin DNA standard curves using the threshold cycle values (C_T). The amount of RRS DNA was then normalized to the amount of lectin DNA (specific for soya) to determine the final % RRS/soya in each sample.

Results and Conclusions

These results demonstrate that a dynamic range of 2–0.02 ng of Roundup Ready soya can be accurately determined using multiplex real-time PCR. Both the CP4EPSPS gene (specific for RRS) and the lectin endogenous control gene (specific for soya) can be simultaneously amplified and detected in a single, closed-tube multiplexed reaction using specific dual-labeled oligonucleotide probes. Accurate quantitation of both genes in individual or multiplex reactions is only possible with proper primer and probe design and optimization of reaction conditions. The standard curves generated for both genes show excellent correlation coefficients (R>0.991) and equivalent amplification efficiencies (as determined by the slopes of the standard curves). The accuracy of the experimental results was verified by the accurate quantitation of 1% and 0.1% RRS reference standards. These same results have been reproduced with identical reaction conditions and also with a VIC-labeled lectin oligonucleotide probe quenched with TAMRA.

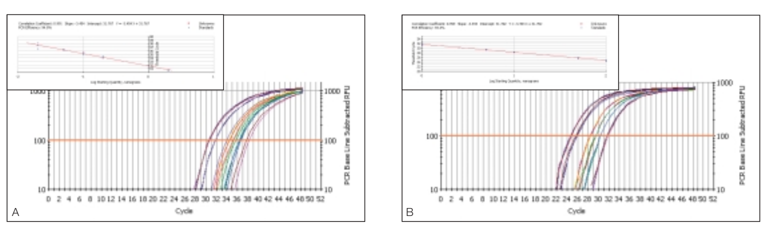


Fig. 1. The CP4EPSPS (A) and lectin (B) genes (specific for RRS and soya, respectively) were amplified in a multiplex reaction using dilutions of DNA extracted from a soya bean powder sample containing 2% RRS. The final dilutions of DNA represented 2.0, 1.0, 0.2, 0.1, 0.05, and 0.02 ng of RRS and 100, 50, 10, 5, 2.5, and 1 ng of total soya. The target genes were detected using specific dual-labeled oligonucleotide probes labeled with FAM and Black Hole Quencher 1 (RRS) or HEX and Black Hole Quencher 2 (lectin). Four replicates were run for each dilution. The inset in each figure is the standard curve generated from the amplification plot. The standard curves had slope values of –3.380 (FAM-RRS) and –3.409 (HEX-lectin), which indicate similar amplification efficiencies of 97.6% and 96.5%, respectively.

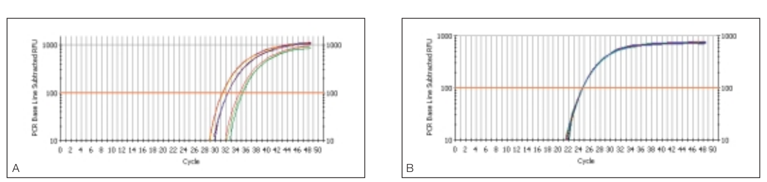


Fig. 2. DNA from the unknown sample as well as the 0.1% and 1% RRS reference standards was amplified (three replicates each) in multiplex reactions to detect the CP4EPSPS gene specific for RRS (A) and the lectin endogenous control gene specific for soya (B). The threshold values determined from these plots were applied to the standard curves from Figure 1 to determine the amounts of RRS and soya DNA in each sample. The table below presents the derived % RRS content of the samples.

Table 1. Quantitation by real-time PCR of RRS content in soya samples.

Sample	FAM-CP4EPSPS (RRS) C_T	HEX-Lectin (Soya) C_T	ng RRS in Sample	ng Soya in Sample	% RRS/ Total Soya	Average ± SD % RRS in Soya	
1% Reference	31.359	24.548	1.311	111.711	1.173	1.174	0.053
	31.573	24.697	1.136	101.260	1.121		
	31.367	24.625	1.304	106.182	1.228		
0.1% Reference	34.635	24.364	0.146	126.117	0.116	0.099	0.015
	35.045	24.401	0.111	123.078	0.090		
	35.265	24.620	0.096	106.533	0.090		
Unknown	32.399	24.527	0.653	113.268	0.577	**0.52–0.58%**	
	32.465	24.431	0.625	120.668	0.518	**RRS in soya**	
	32.415	24.508	0.646	114.696	0.564		

Three-Color Multiplex Real-Time RT-PCR for Simultaneous Gene Expression Analysis

Introduction

A multiplex real-time RT-PCR approach can be used for the simultaneous analysis of gene expression in one reaction tube. We developed a three-color multiplex RT-PCR reaction to analyze the expression levels of three different genes within the same sample material. The assay, which is based on the 5' nuclease technology (1), used cDNA prepared from human thymus and prostate total RNA as template. We chose to quantify the expression of the ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) genes, and the β-actin gene as the reference housekeeping gene. ODC and AdoMetDC are both rate-limiting enzymes in the polyamine biosynthesis pathway (2). The prostate, which produces high levels of polyamines, the end products in the polyamine-biosynthesis pathway, should exhibit higher expression of AdoMetDC and ODC mRNA than the thymus.

Materials and Methods

Chemicals and Reagents

Human prostate and thymus total RNA, SUPERase•In RNase inhibitor, and oligo dT (12- to 18-mer) were purchased from Ambion, Inc. Ultrapure deoxynucleotides were from Clontech (BD Biosciences). SuperScript II reverse transcriptase was obtained from Invitrogen. iTaq™ DNA polymerase was from Bio-Rad Laboratories. Nuclease-free water was from Sigma. Primers and fluorescent probes were from Integrated DNA Technologies, Inc. (IDT), and TriLink Biotechnologies, Inc. (TriLink). All gene targets were detected with dual-labeled oligonucleotide probes. The AdoMetDC probe (TriLink) was labeled with HEX and quenched by Black Hole Quencher 1, the ODC probe (IDT) was labeled with Texas Red and quenched by Black Hole Quencher 2, and the β-actin probe (TriLink) was labeled with FAM and quenched by Black Hole Quencher 1.

Real-Time RT-PCR

First-strand cDNA synthesis used 1 mM oligo dT (12- to 18-mer), 20 U SUPERase•In, and 1.0 µg of total RNA per 20 µl reaction. For validation of the efficiency of the real-time PCR assay, a 5-fold dilution series was generated using cDNA from the synthesis step (Figure 1). For the relative quantitation using the $\Delta\Delta C_T$ method, the equivalent of 0.08 µl of the cDNA synthesis step was used per reaction.

PCR reactions were performed in a 25 µl or 50 µl final volume containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 200 µM each dNTP, 0.5 µM each primer, 0.2 µM each probe, and 3.5 mM $MgCl_2$. For the validation of the PCR efficiency, the dilution series were run using duplicate reactions. For relative quantitation using the $\Delta\Delta C_T$ method, four replicates were used for both tissue types. Singleplex and triplex assays used 0.625 U and 1.875 U, respectively, per 25 µl PCR reaction. PCR was carried out with an initial 3 min denaturation at 95°C followed by 45 cycles of a combined annealing and extension step at 57°C for 30 sec, and denaturation at 95°C for 10 sec. All real-time RT-PCR reactions were run on the iCycler iQ system.

Results

Validation of the PCR Efficiency in a Multiplex System: Generation of Dilution Series and Standard Curve for Single and Multiplex Reactions

In order to use the $\Delta\Delta C_T$ calculations, it is important to verify that all the genes to be multiplexed are amplified with approximately the same efficiency. In addition, the amplification efficiency of the individual targets should not be significantly different between single and multiplex reactions. To determine the amplification efficiency of a particular target gene, PCR reactions are set up to amplify a serial dilution of a cDNA template. After PCR completion, the iCycler iQ software calculates the threshold cycle (C_T) for the various starting amounts of cDNA template, the slope of the curve, the PCR efficiency, and the correlation coefficient. The use of properly designed PCR primers and probes along with optimal reaction conditions should generate PCR efficiencies higher than 90%. If optimized PCR reactions fail to generate an efficiency greater than 90%, primers and probes should be redesigned.

The data shown in Figure 3 demonstrate that there is no significant difference between single-target and three-gene target multiplex PCR assays for the β-actin, AdoMetDC, and ODC genes. The C_T values, the slope, and PCR efficiency remain nearly constant. The correlation coefficients show a value of 0.997 or higher for replicate samples.

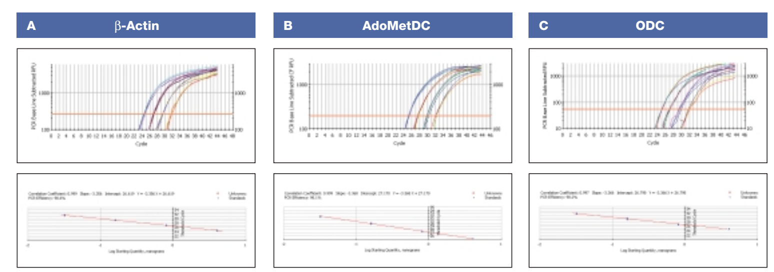


Fig. 3. Single and multiplex reactions for the amplification of β-actin (A), AdoMetDC (B), and ODC (C) cDNA templates. Upper panels show the real-time RT-PCR plots, with single and multiplex reactions overlaid. Lower panels show the standard curves for the plots. The dilution series used to generate the data contained the equivalent of 0.08 µl, 0.016 µl, 3.2×10^{-3} µl, and 6.4×10^{-4} µl of the cDNA synthesis reaction outlined in Materials and Methods.

Relative Quantitation Using the $\Delta\Delta C_T$ Method: Multiplex Analysis of β-Actin, AdoMetDC, and ODC Expression Using Human Thymus and Prostate cDNA

We used cDNA prepared from human thymus and prostate total RNA to perform a comparative gene expression analysis. Thymus was selected as a control due to its lower expression of AdoMetDC and ODC. The β-actin gene was chosen as the reference housekeeping gene. All three gene targets were assayed in the same tube; i.e., in a three-gene target multiplex assay. As seen in Figure 4, the expression of β-actin is nearly identical in both tissue types, whereas AdoMetDC and ODC are expressed at different levels. We used the $\Delta\Delta C_T$ method to analyze the relative difference between thymus and prostate of AdoMetDC and ODC expression (Table 2). The data analysis showed that prostate has a relative increase of 4.72 and 10.70 for AdoMetDC and ODC mRNA, respectively, compared to thymus.

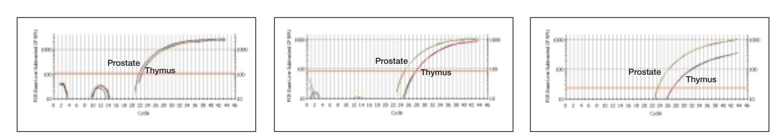


Fig. 4. Real-time RT-PCR plots of multiplex PCR analysis of expression of β-actin (A), AdoMetDC (B), and ODC (C) using cDNA prepared from thymus and prostate total RNA. The corresponding C_T values are shown in Table 2. The equivalent of 0.08 µl of the cDNA synthesis reaction outlined in Materials and Methods was used in the assay.

Table 2. Relative quantitation of AdoMetCD and ODC expression in a real-time multiplex RT-PCR analysis, using the $\Delta\Delta C_T$ method.

	AdoMetDC C_T	ODC C_T	β-Actin C_T	ΔC_T* (AdoMetDC – β-Actin)	ΔC_T* (ODC – β-Actin)	$\Delta\Delta C_T$** AdoMetDC	$\Delta\Delta C_T$** ODC	$AdoMetDC_N$ relative to thymus***	ODC_N relative to thymus***
Thymus	27.49 27.74 27.73 27.76	27.19 27.26 27.30 27.23	22.69 22.57 22.69 22.59	4.80 5.17 5.04 5.17	4.50 4.69 4.61 4.64				
Average				5.04 ± 0.15	4.60 ± 0.07	0.00 ± 0.15	0.00 ± 0.07	1.00 (0.90–1.11)	1.00 (0.95–1.09)
Prostate	24.72 24.75 24.75 24.85	23.15 23.13 23.13 23.41	21.98 21.89 21.99 22.11	2.74 2.77 2.76 2.74	1.17 1.15 1.14 1.30				
Average				2.75 ± 0.01	1.19 ± 0.17	–2.29 ± 0.01	–3.41 ± 0.17	4.72 (4.56–4.92)	10.70 (9.45–11.95)

* ΔC_T values are determined by subtracting the average β-actin C_T value from the average AdoMetDC and ODC C_T values, respectively.
** $\Delta\Delta C_T$ is calculated by subtracting the ΔC_T control (thymus in this case) from ΔC_T AdoMetDC and ODC values.
*** Relative expression is calculated as $2^{-(\Delta\Delta CT)}$.

Conclusions

- PCR primer and probe design and PCR optimization are important factors to generate similar efficiencies for the amplification of a cDNA target in single and multiplex assays.
- We have developed a three-color, multiplex real-time RT-PCR assay for the simultaneous analysis of three different gene targets within a single sample. The three-color assay was applied to quantitate the relative expression of AdoMetDC, ODC, and a reference housekeeping gene in human thymus and prostate. The assay showed higher expression of AdoMetDC and ODC in prostate, as expected.

References

1. Holland PM et al., Detection of specific polymerase chain reaction product by utilizing the 5'→3' exonuclease activity of *Thermus aquaticus* DNA polymerase, Proc Natl Acad Sci USA 88, 7276–7280 (1991)
2. Heby O and Persson L, Molecular genetics of polyamine synthesis in eukaryotic cells, Trends Biochem Sci 15, 153–158 (1990)

Four-Color Multiplex Real-Time PCR Assay for the Simultaneous Detection of Factor V Leiden and Prothrombin G20210A Mutations

Introduction

Multiplex real-time PCR can be used to detect multiple target sequences within a single sample. We show a four-color, multiplex 5' nuclease assay that simultaneously genotypes an individual for both the factor V Leiden (FVL) and prothrombin (PT) G20210A mutations, the two most common known genetic risk factors for venous thrombosis in Caucasians. The multiplex assay, which can be easily adapted to the detection of other SNPs, will be useful for both molecular diagnostic and research laboratories.

Materials and Methods

Factor V Leiden and Prothrombin G20210A Genotyping by the Multicolor Multiplex 5' Nuclease Assay

DNA was prepared from whole blood using standard procedures. Primers were obtained from Integrated DNA Technologies, Inc. (IDT). The multiplex PCR reactions were run in a 25 µl final volume containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 200 µM each dNTP, 0.4 µM each FVL and PT G20210A PCR primer, 0.4 µM each allele-specific oligonucleotide (ASO) probe, and 2.25 U iTaq™ DNA polymerase (Bio-Rad). The ASO probes were labeled with FAM and Black Hole Quencher 1 (FVL wild-type), TET and Black Hole Quencher 1 (FVL mutant), TAMRA and Black Hole Quencher 2 (PT G20210A wild-type), and Texas Red and Black Hole Quencher 2 (PT G20210A mutant). The FAM-labeled FVL wild-type probe was obtained from IDT; all other probes were obtained from BioSource International, Inc. PCR reactions were performed in the iCycler iQ system using 96-well PCR microplates. Following *Taq* polymerase activation and DNA denaturation at 95ºC for 3.5 min, the multiplex amplification-detection of both FVL and PT G20210A mutations was carried out for 50 cycles as follows: annealing at 58ºC for 45 sec, and denaturation at 95ºC for 10 sec.

The fluorescent data generated by the cleavage of the dual-labeled ASO probes were collected during the PCR annealing step. Data analysis was performed by analyzing the real-time amplification plots or with the iCycler iQ software for allele discrimination. The software classifies the different genotypes of a genetic locus by using either end-point fluorescent units (RFU) or threshold cycles (C_T).

Results

Genotyping With the Multicolor 5' Nuclease Assay

Four of the possible outcomes for the genotyping of the FVL and PT G20210A mutations are shown in Figure 5. DNA from a wild-type (WT) individual (Figure 5A) shows a fluorescent signal for both the FVL and PT G20210A WT probes but not for their mutant counterparts. DNA from an individual homozygous for one of the two mutations (Figure 5B) shows a fluorescent signal with the mutant ASO probe complementary to the mutated sequence but not with its normal analog, and another fluorescent signal with the other WT probe but not with its mutant counterpart. DNA from an individual heterozygous for a single mutation generates a fluorescent signal with both the normal and mutant ASO probes specific for that site, and a signal only with the WT probe specific for the other gene (Figure 5C). DNA from a compound heterozygous individual generates fluorescent signals with both mutant probes and both WT probes (Figure 5D).

Assay Performance

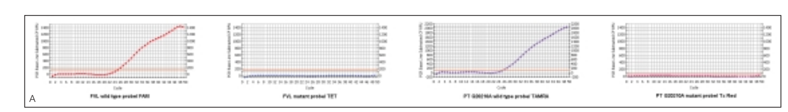
Figure 6 shows the genotyping results generated from 21 DNA samples from individuals with known FVL (16 wild-type, 2 homozygous mutant, and 3 heterozygous) and PT G20210A genotypes (13 wild-type, 5 heterozygous, and 3 homozygous mutant). The allelic discrimination feature of the iCycler iQ software analyzes the signals of the different fluorophores, and results are displayed as a scatter plot. There was a 100% concordance between the data generated with the multicolor real-time approach and the reference method (PCR + restriction fragment length polymorphism) (1). Figure 6A shows four clearly defined clusters. The first cluster consists of samples with FAM signals (x-axis) within the range 730–1,000 RFU that depicts individuals with the factor V Leiden WT genotype. The second cluster includes samples with TET values (y-axis) within the range 750–800 RFU that represent individuals with the factor V Leiden homozygous mutant genotype. The third cluster (factor V Leiden heterozygous individuals) includes samples with FAM and TET values within the range 200–520 RFU. Finally, the last cluster represents samples with very low RFU values for both FAM and TET (no-template controls). Similarly, Figure 6B shows four well-defined clusters that clearly distinguish the different prothrombin G20210A genotypes.

Conclusions

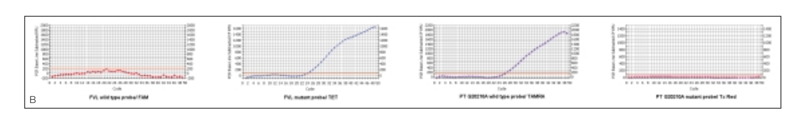
- We have shown the simultaneous detection of four alleles in real time in a single closed tube
- The four-color assay for genotyping the factor V Leiden and prothrombin genes showed 100% specificity and 100% sensitivity compared with the results obtained by a reference method
- The number of allelic variants that can be detected in a 5' nuclease assay depends on the selection of available fluorophores and the design of the assay instrument. The iCycler iQ real-time PCR detection system is well suited for the simultaneous detection of up to four target sequences, using four differentially labeled ASO probes

Reference

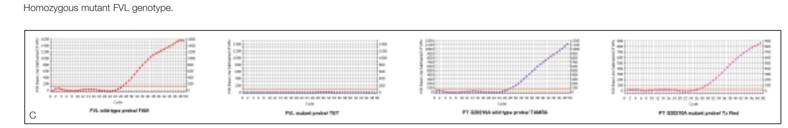
1. Dubreuil Lastrucci RM et al., Development of a simple multiplex polymerase chain reaction for the simultaneous detection of factor V Leiden and prothrombin 20210A mutations, Mol Diagn 4, 247–250 (1999)



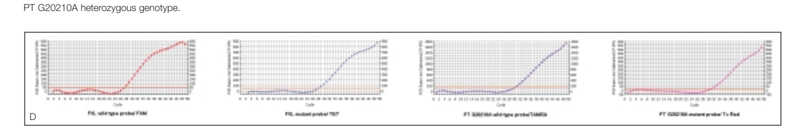
Wild-type genotype.



Homozygous mutant FVL genotype.



PT G20210A heterozygous genotype.



FVL/PT G20210A compound heterozygous genotype.

Fig. 5. Interpretations of the multicolor multiplex 5' nuclease assay results for FVL and PT G20210A genotyping on four samples. Fluorescence (RFU) was measured every cycle and, after normalization, plotted against cycle number.

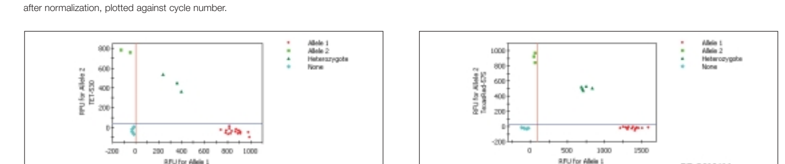


Fig. 6. Genotyping with the multicolor assay. A, scatter plot of fluorescence values for FAM (FVL wild-type probe) and TET (FVL mutant probe); B, scatter plot of fluorescence values for TAMRA (PT G20210A wild-type probe) and Texas Red (PT G20210A mutant probe).

Multiplex Applications Using the iCycler iQ™ Real-Time PCR Detection System

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