A Fast Semi-Automated Quantitative PCR Method for Monitoring Differential Gene Expression for Drug Target Discovery

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Tech Note

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Abstract

Here we describe a fully integrated, semi-automated quantitative PCR workflow incorporating steps from preamplification and amplification to automated plate handling and data processing. This workflow is designed for researchers performing differential gene expression analysis as part of drug discovery and development. The workflow provides results 5 hours faster with a 50% reduction in hands-on time.

Introduction

The ability to assess the differential expression of many genes in a large number of patient samples simultaneously plays an important role in understanding complex diseases. In drug discovery and development this analysis is used to identify new drug targets, monitor a patient's response to therapeutics, and identify biomarkers. In addition, characterizing changes in gene expression patterns during disease progression can aid in the development of better disease prognostics and will help drive toward more personalized treatment regimens.

We developed a semi-automated workflow (Figure 1) that was applied to analysis of the differential expression of genes in the anti-apoptotic TNFs/NF-κB/Bcl-2 pathway across samples obtained from patients with various stages of prostate cancer. This pathway was chosen because the evasion of apoptosis is fundamental to tumorigenesis (Wong 2011) and its role in prostate cancer progression is well documented (McDonnell et al. 1992).

The CFX Automation System II, a plate handling system that automates plate loading and data collection on CFX Real-Time PCR Detection Systems, was used in conjunction with PrimePCR™ Pathway Plates to streamline the qPCR workflow. This semi-automated workflow led to a total reduction of 50% in hands-on time, 78% less time spent in the laboratory, and results achieved 5 hours earlier than when the workflow was performed manually. This workflow can be applied across multiple stages of drug discovery and development, from early discovery applications such as target identification and validation to biomarker studies that span multiple stages.

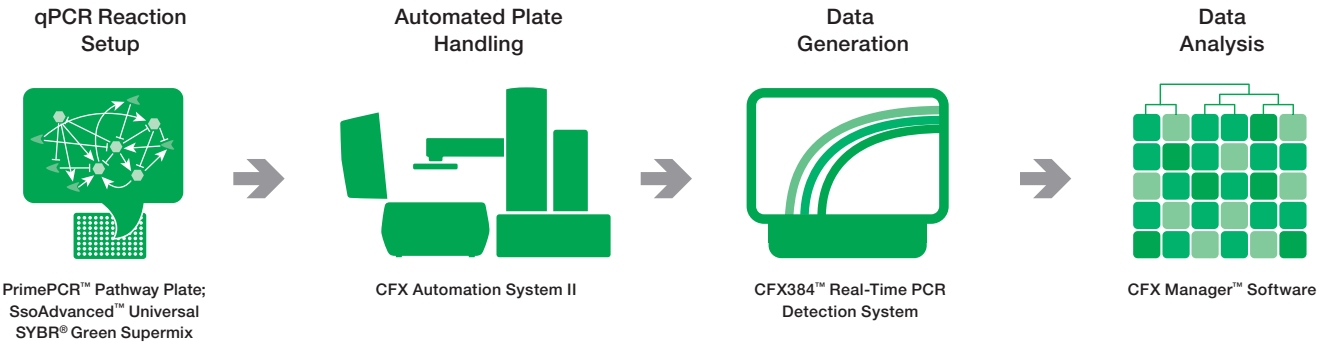

Fig. 1. Semi-automated gene expression analysis workflow.

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Methods

Study Design

Gene expression profiles were analyzed in prostate samples from 39 patients with prostate cancer and nine healthy adults using the PrimePCR Anti-Apoptotic TNFs/NF-κB/Bcl-2 Pathway H384 (human, 384-well) Plate (Table 1). This plate provides preloaded primer sets for the analysis of 40 genes of interest in the TNFs/NF-κB/Bcl-2 pathway along with three reference genes (*HPRT1*, *GAPDH*, and *TBP*) for analysis of up to eight samples. Because only small amounts of each sample were available (2–3 ng cDNA), all samples were preamplified prior to qPCR to ensure that enough sample was available for analysis of all the genes of interest.

Sample Source

cDNA from nine healthy and 39 cancerous prostates were obtained from OriGene Technologies.

cDNA Preamplification

To obtain sufficient amounts of cDNA, preamplification was performed using SsoAdvanced™ PreAmp Supermix and PrimePCR PreAmp Assays. The 43 PrimePCR PreAmp Assays were pooled and diluted 100-fold with PCR-grade water to create the preamplification assay pool. A master mix sufficient for 192 reactions (48 samples with four replicates each) was prepared such that each 50 µl preamplification reaction contained 25 µl SsoAdvanced PreAmp Supermix, 5 µl preamplification assay pool, and 20 µl nuclease-free water. Two hundred microliters of master mix were dispensed into each lyophilized cDNA sample and mixed well. The resulting reaction mixes were split into four replicate 50 µl preamplification reactions in 96-well PCR plates and sealed with Peelable Foil Heat Seals using the PX1™ PCR Plate Sealer. The reactions were cycled in a T100™ Thermal Cycler according to the SsoAdvanced PreAmp Supermix protocol using 12 preamplification cycles (95°C, 3 min; 12 cycles of [95°C, 15 sec; 58°C, 4 min]; 4°C hold) for a 4,096-fold theoretical target amplification. Preamplified cDNA was then diluted fivefold using TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8) prior to its use in the qPCR reaction.

Quantitative PCR

After preamplification, a qPCR master mix was prepared for each of the 192 preamplified samples such that a single 10 µl reaction contained 5 µl SsoAdvanced™ Universal SYBR® Green Supermix, 1 µl preamplified cDNA, and 4 µl nuclease-free water. SsoAdvanced™ Universal SYBR® Green Supermix was chosen for its superior stability at room temperature. Ten microliters of the master mix were dispensed into each well of the PrimePCR Anti-Apoptotic TNFs/NF-κB/Bcl-2 Pathway H384 Plates. PrimePCR Plates were sealed with Optically Clear Heat Seals using the PX1 PCR Plate Sealer. Sealed qPCR plates were then centrifuged at 4,000 x g for 4 min and loaded into the CFX Automation System II.

The CFX Automation System II was configured with two CFX384™ Real-Time PCR Detection Systems for this experiment. The PrimePCR run file containing gene targets and control assay information for the PrimePCR Anti-Apoptotic TNFs/NF-κB/Bcl-2 Pathway H384 Plate was downloaded from the PrimePCR website and imported into the automation system software. Reactions were cycled using a standard protocol (95°C, 2 min; 40 cycles of [95°C, 5 sec; 60°C, 30 sec]; melt curve analysis: 65–95°C, 0.5°C increments, 5 sec hold). The automation system was started and proceeded to run 24 plates over an 18.2 hour period.

Data Analysis

Data analysis was conducted using CFX Manager™ Software. Each file was annotated with its corresponding sample name and the individual data files were imported into a CFX Manager gene study file, where the results across all 24 PrimePCR Plates were compiled.

The clustergram module in CFX Manager Software was used to analyze the anti-apoptotic gene expression profiles for this set of prostate tissue samples. The clustergram assembles samples and/or gene targets into hierarchies based on similarities in gene expression and is appropriate for use in relative quantification (ΔCq) experiments consisting of unpaired samples.

Table 1. Gene targets assayed using the PrimePCR Anti-Apoptotic TNFs/NF-κB/Bcl-2 Pathway Plate.

BCL2	*IRAK2*	*RELB*	*TNFRSF4*
BCL2A1	*NFKB1*	*RIPK1*	*TNFSF11*
BCL2L1	*NFKB2*	*SQSTM1*	*TNFSF12*
CD40	*NFKBIA*	*TBP*	*TNFSF13*
CD40LG	*NFKBIB*	*TNF*	*TNFSF13B*
CHUK	*NFKBIE*	*TNFRSF11A*	*TNFSF4*
GAPDH	*NGF*	*TNFRSF12A*	*TRADD*
HPRT1	*NGFR*	*TNFRSF13B*	*TRAF2*
IKBKB	*PRKCZ*	*TNFRSF17*	*TRAF3*
IKBKG	*REL*	*TNFRSF1A*	*TRAF6*
IRAK1	*RELA*	*TNFRSF1B*	

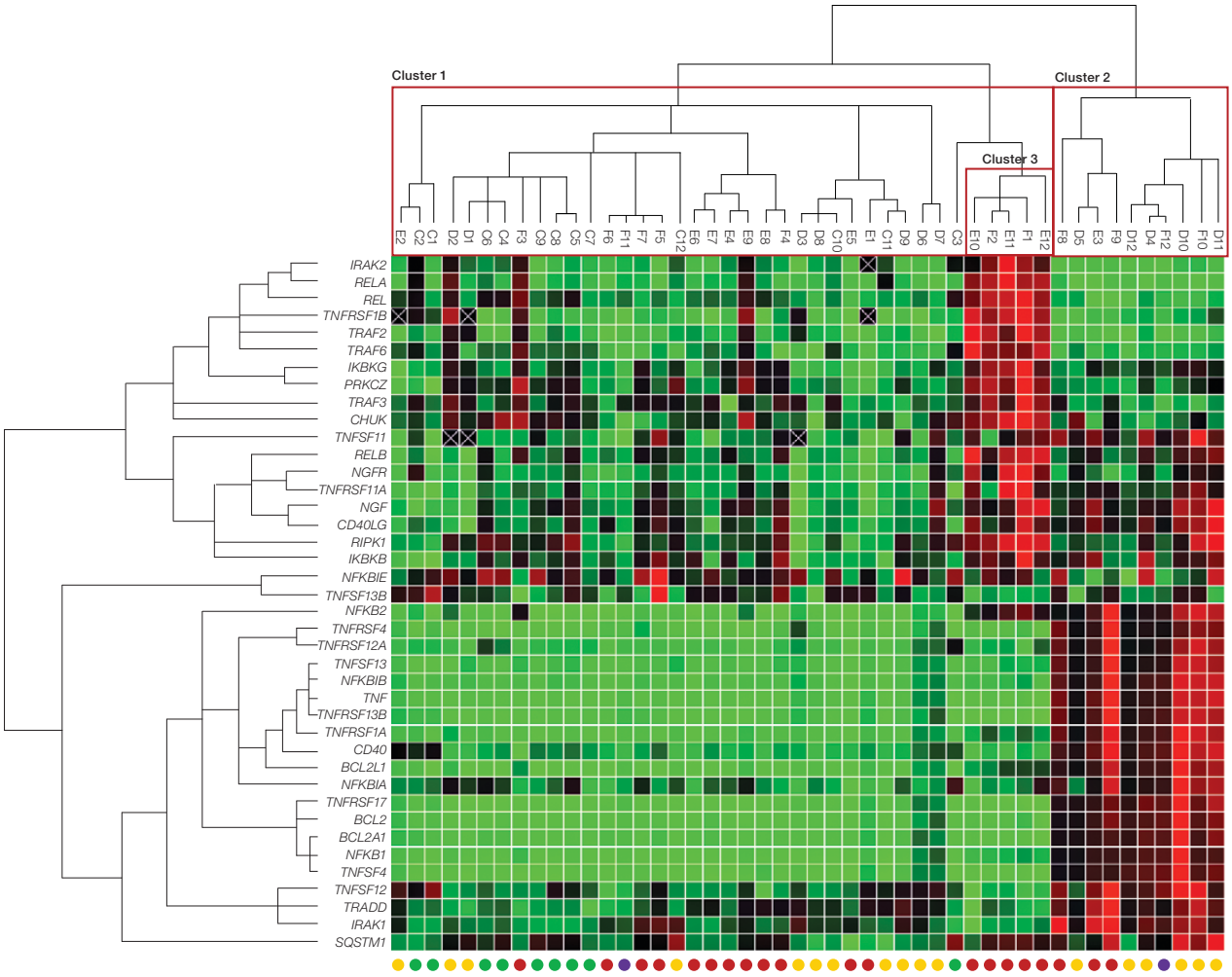

Fig. 2. Gene expression patterns in normal and cancerous prostate tissues. Red squares indicate upregulated genes, green squares downregulated genes, and black squares genes that showed no change in expression. An X indicates that no transcript was detected. Circles at the bottom of each column indicate cancer status of each prostate tissue sample: normal (●); stage II (●); stage III (●); stage IV (●).

Results

Inspection of the qPCR results clustered by sample reveals that two primary clusters of samples exist: one consisting of normal and cancerous prostate tissues (Cluster 1 in Figure 2) and another that is dominated by stage II–IV prostate cancer samples (Cluster 2 in Figure 2). This second cluster shows the upregulation of *BCL2* and associated genes, indicating the inhibition of apoptosis mediated by the Bcl-2 pathway (Cory et al. 2003). The clustering analysis also revealed a secondary expression profile (Cluster 3 in Figure 2) specific to a subset of the stage III prostate cancer samples within Cluster 1. This expression profile is marked by the upregulation of *RELA*, *REL*, *TRAF2*, and *RIPK1*, likely indicating activation of NF-κB and, ultimately, inhibition of apoptosis mediated by XIAP, c-IAP1, and c-IAP2 (de Almagro and Vucic 2012). While not performed here, expression changes in this pathway could be further explored using the PrimePCR Anti-Apoptotic TNFs/NF-κB/IAP Pathway Plate.

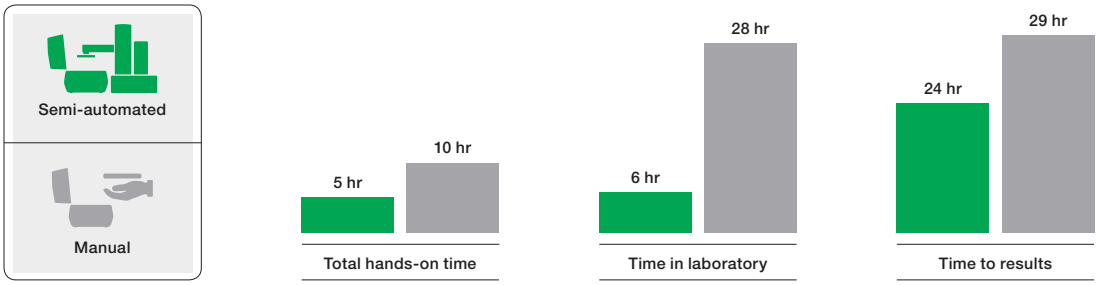

Fig. 3. Time savings with Bio-Rad's semi-automated gene expression analysis workflow for 24 qPCR plates. Total hands-on time is cut in half because PrimePCR Plates are preloaded with primers and do not have to be manually loaded into the real-time PCR detection system. Since the plates are loaded automatically by the CFX Automation System II, the total time spent in the laboratory is reduced by 78%. The 5 hour reduction in time to results is a consequence of the reduction in hands-on time.

Conclusions

As the requirement for qPCR throughput in drug discovery and development grows, the complexity of and time requirement for qPCR setup rises proportionally. In this study, we created a semi-automated qPCR workflow that reduces the experimental complexity and setup time and in turn provides data faster than manual methods (Figure 3). This new workflow enabled us to obtain 9,216 data points across 48 patient samples in 29.3 hours, approximately 5 hours faster than with a conventional workflow, and with approximately 50% less hands-on time.

Three components are central to this faster method:

- PrimePCR Pathway Plates removed a liquid handling step during reaction setup because lyophilized primer sets are provided preloaded in qPCR plates
- The CFX Automation System II paired with two CFX384 Real-Time PCR Detection Systems permitted collection of qPCR data for all twenty-four 384-well plates in 18.2 hours (506 data points per hour) while leaving the instruments unattended, resulting in 78% less time spent in or near the laboratory over the course of the experiment
- SsoAdvanced™ Universal SYBR® Green Supermix ensured the integrity of assembled qPCR reactions until they were processed because it is stable at room temperature for up to 48 hours

If required, this workflow can easily be converted into a fully automated process through the addition of a liquid handling system, which would further increase reproducibility and permit even higher throughput analysis of gene expression patterns for all phases of research and development.

References

Cory S et al. (2003). The Bcl-2 family: Roles in cell survival and oncogenesis. Oncogene 22, 8,590–8,607.

de Almagro MC and Vucic D (2012). The inhibitor of apoptosis (IAP) proteins are critical regulators of signaling pathways and targets for anti-cancer therapy. Exp Oncol 34, 200–211.

McDonnell TJ et al. (1992). Expression of the protooncogene Bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 52, 6,940–6,944.

Wong RS (2011). Apoptosis in cancer: From pathogenesis to treatment. J Exp Clin Cancer Res 30, 87.

Related Information

Bureeva S et al. (2012). PrimePCR™ pathway analysis: Pathway curation and real-time PCR panel design strategy. Bio-Rad Bulletin 6263.

Hellemans J et al. (2012). PrimePCR™ Assays: Meeting the MIQE guidelines by full wet-lab validation. Bio-Rad Bulletin 6262.

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