Detection of SMAD4, MAP2K4 and RB1 Gene Deletions in Human Tumor Cells by Multiplex qPCR

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ABSTRACT

Gene duplications and deletions are critical to understanding cancer biology. Deletion in SMAD4, MAP2K4 and RB1 genes are known to play important roles in certain types of cancer or cancer states. Characterization of such mutations in different tumor cells provide insight into the study of novel biomarkers of cancer susceptibility, initiation, progression and metastases and help identify them as risk factors for various types of cancer. We demonstrated a quantitative method to detect multiple gene deletions in human tumor cells by multiplex PCR. We designed four TaqMan® Copy Number Assays to interrogate common deletion mutations in SMAD4, MAP2K4, and RB1 genes and performed multiplex PCR alongside with endogenous controls on representative human tumor and normal cells in different tissues. Since the detection of multiple deletion targets were performed in one single reaction, we were able to profile multiple deletion mutations in one single sample. This method can potentially be applied to the study of Copy Number Variations (CNV) on purified biological samples, as well as enables the characterization of multiple targets on samples in exactly the same condition and state. Feasibility using purified DNA samples from FFPE was also briefly investigated.

INTRODUCTION

Current analyses of cell and tissue functionality require extracting as much information as possible from materials that are often limiting. In particular, patient samples, such as blood draws and tumor biopsies, are difficult to collect and usually yield only a small amount of usable nucleic acid. qPCR has been the gold standard for analyzing clinical samples on the nucleic acid level, and has been invaluable in extending the limits of biological knowledge for more than a quarter century. However, the limited amount of sample obtained from clinical specimens often forces choices to be made about how best to utilize these precious samples and maximize the amount of information that can be extracted from the sample. Furthermore, solid tumors often shows high heterogeneity. Even adjacent areas of the tumor can harbor different mutations, thus making it hard to study associations between two targets when separate samples are used. Finally, the additional time and materials required to set up multiple single-assay reactions could increase the expense of a complex project significantly.

Multiplex qPCR analysis of nucleic acids, a strategy where more than one target is amplified and quantified from a single sample aliquot, is an attractive solution to these problems. In multiplex PCR, a sample aliquot is queried with multiple, different fluorescence dyes probes assays in a single PCR reaction. This increases the amount of information that could be extracted from that sample. With multiplex qPCR, significant savings in sample and materials can be realized.

We described a multiplex qPCR solution for Copy Number detection of three of the more commonly used targets in cancer research: SMAD4, RB1, and MAP2K4 . We then compared the results with that from the traditional duplex approach, which includes one target and one reference assay.

To enable multiplex, we developed a 4 reporter-dye strategy with a new passive reference dye, MUSTANG PURPLE®, that excites and emits at longer wavelengths. ABY®-QSY® and JUN®-QSY® are new reporter dye-quencher probe options that complement the current FAM™-MGB and VIC®-MGB probes for multiplexing (Life Technologies, Waltham, MA). Their spectral profiles are shown in Figure 1.

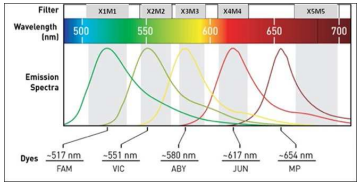

Figure 1. Fluorescence emission spectra of different dyes used for multiplex PCR.

MATERIALS AND METHODS

24 DNA samples were used for our multiplex experiment: 19 genomic DNA samples with confirmed diploid copy number of the four loci (Coriell Cell Repository, Camden, NJ), 3 genomic DNA samples from cancer cell lines SW620, MDA-MB-468, PANC-03-27, which have verified deletions of one of more of the four loci (ATCC, Manassas, VA) (Table 1), and 2 DNA samples extracted from AcroMetrix® MultiMix FFPE control MultiMix C and E (Thermo Fisher Scientific, Waltham, MA). The concentration of each sample was measured by A260/280 using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) and normalized to 5ng/uL using 1X TE Buffer. 10ng of DNA was used in each 10uL qPCR reaction. Each reaction was run in four replicates.

Cell Line	ATCC ID	Source	Documented Deletion
PANC-03-27	CRL-2549	Pancreas	SMAD4[3], MAP2K4
MDA-MB-468	HTB-132	Breast	SMAD4[1], RB1[4], MAP2K4
SW620	CCL-227	Colon	SMAD4[2], RB1

Table 1. Documented deletions of SMAD4, RB1 and MAP2K4 genes in various cancer cell lines

The CNV multiplex reaction consists of three targets and one reference gene, as shown in Table 1. SMAD4 (FAM™-MGB), RB1 (VIC®-MGB) and MAP2K4 (JUN®-QSY®) assays were combined with RNaseP reference (ABY®-QSY®). The primer and probe concentrations of each target assay are 900nM and 250nM in final reaction, respectively.

For comparison purpose, the traditional TaqMan® CNV duplex qPCR reactions were performed on the same samples using TaqMan® Genotyping Master Mix and its recommended thermal cycling conditions. In each set, FAM™-MGB assay was used for the target (SMAD4, RB1 or MAP2K4), while VIC®-TAMRA™ was used for the reference (RNaseP).

CNV Multiplex reactions were run in 384-well format on Applied Biosystems® QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific) using TaqMan Multiplex Master Mix. The thermal cycling conditions are as follow: 95ºC 20s, [95ºC 5s + 60ºC 30s] x 40 cycles

Data analysis was based on threshold of 0.2 with auto-baseline. One of the Coriell gDNA sample, NA17230, with which two copies of each of the genes being studied are expected, was used as the reference sample for manual Copy Number Variation calculation, as shown as follow:

$$dCt = Ct_{target} - Ct_{RnaseP}$$

$$ddCt = dCt_{sample} - dCt_{reference\ sample}$$

$$Copy\ Number = 2^{(2^{(-ddCt)})}$$

Results

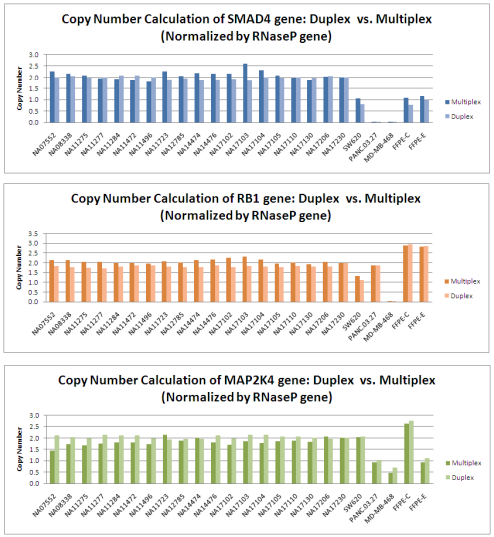

Figure 2. Comparison of copy number calculation for SMAD4, RB1 and MAP2K4 in 24 DNA samples in Duplex (FAM™/VIC®) and Multiplex (FAM™/VIC®/ABY®/JUN®) Copy Number qPCR

CONCLUSIONS

We demonstrated the feasibility of detecting multiple deletion targets quantitatively using multiplex qPCR (Figure 2). Our results indicated a high concordance in copy number results compared to the traditional duplex TaqMan® Copy Number qPCR Assays, which is often regarded as the golden standard for analyzing samples on the nucleic acid level. We were able to simulltaneously profile SMAD4, RB1 and MAP2K4 deletion mutations in one single sample. Our results serve as a proof –of-concept for a potentially powerful tool that enables the characterization of multiple targets on samples in exactly the same condition and state, while at the same time, saves time and reagents cost. While the multiplex chemistry was initially designed for gene expression and genotyping applications, the results from our initial attempt for Copy Number analysis were encouraging. The study should be extended to explore the requirements for combining different copy number targets in a single qPCR reaction, as well as to optimize the reaction to further improve the precision of the results.

Since DNA purification from FFPE samples often requires multiple reagents and steps, we are interested in learning whether any carry over from the purification procedures would impact multiplex chemistry differently from the traditional duplex PCR. Of the three targets we studied in this poster, we did not see a significant change in copy number results for the two FFPE samples used. Further investigation using patients' samples with validated copy number would be informative.

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TRADEMARKS/LICENSING