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Early Identification of KRAS and PIK3CA Mutations using Multiplex Digital PCR Compatible with Liquid Biopsy Samples to Support Tumor Progression Surveillance Research

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INTRODUCTION

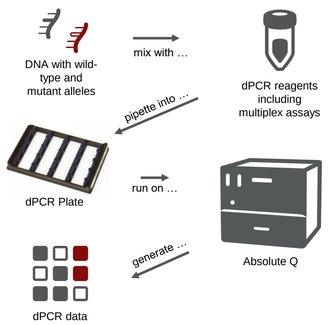
Kirsten rat sarcoma viral oncogene homolog (KRAS) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) are oncogenic genes which are frequently mutated across many types of solid tumors, thus making them high interest targets in oncology research. Ongoing research to improve personalized treatments often focus on trying to measure biomarkers that may be associated with therapeutic response or possible recurrence. In the field of liquid biopsy research, it is therefore crucial to measure these mutation targets which may be linked to residual tumor burden and emerging resistance with high sensitivity and specificity.

Here, we present three KRAS and two PIK3CA mutation multiplex panels (For Research Use Only. Not for use in diagnostic procedures.) compatible with liquid biopsy samples on the Applied Biosystems™ QuantStudio™ Absolute Q™ digital PCR (dPCR) system. This platform provides a simplified workflow, throughput flexibility, multiplex capability, and a fast turnaround time. We demonstrate that the multiplex panels for KRAS and PIK3CA can identify multiple mutations simultaneously with high sensitivity (0.1 % mutant allele frequency, MAF) and low false positives on the Absolute Q system, providing an advantage in liquid biopsy cancer research for future early detection and monitoring applications.

MATERIALS AND METHODS

We developed three KRAS (Table 1) and two PIK3CA (Table 2) dPCR multiplex panels for Absolute Q targeting oncogenic variants on each target gene. The KRAS common mutations panel consists of one wildtype probe labelled with JUN and mutant probes labelled with FAM, VIC, and ABY. The two KRAS identification panels have a single VIC wild type probe with the mutation of interest to be distinguished in FAM and the rest of the mutation in ABY. Each PIK3CA multiplex panel has two wild type probes in VIC and JUN with the mutant probes in FAM and ABY. All probes were ordered from Thermo Fisher Scientific as TaqMan™ minor groove binder (MGB) or QSY probes. To emulate the cell free DNA (cfDNA) template, synthetic DNA fragments with length similar to cfDNA fragments were purchased from Thermo Fisher Scientific (*Cat #*: 815010DE) and combined to have 1%, 0.1%, and 0% (wild type, WT) MAF. All multiplex panels were tested with these synthetic controls on the Absolute Q dPCR system. The PIK3CA panels were also tested with cfDNA liquid biopsy samples.

Figure 1. Applied Biosystems QuantStudio Absolute Q workflow



Simplified workflow for performing dPCR and quantifying mutant and wild-type alleles.

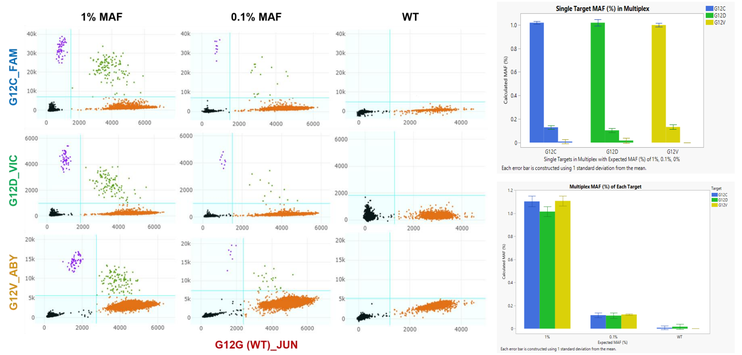
RESULTS

Table 1. KRAS multiplex panels

Panel	Mutation	Dye
KRAS G12C + G12D + G12V	G12C	FAM
	G12V	ABY
	G12D	VIC
	G12G	Cy5
KRAS G12C Identification Panel	G12C	FAM
	G12S	ABY
	G12R	
	G12V	
	G12D	
	G12A	
	G12G	VIC
KRAS G12D Identification Panel	G12D	FAM
	G12C	ABY
	G12S	
	G12R	
	G12V	
	G12A	
	G12G	VIC

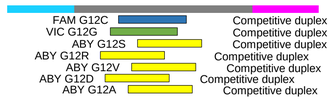
KRAS G12C and G12D are frequent mutations associated with cancer, and they have been linked to potential treatment options. Therefore, accurately identifying and differentiating them from other KRAS variants is valuable for cancer research. Therefore, the identification panels in the table above use a specific probe to either G12C or G12D mutations labelled in FAM, allowing for their distinction from other KRAS variants. The remaining mutant probes are labeled with the same fluorophore, ABY, solely for the purpose of identifying their presence.

Figure 3. Two-dimensional dPCR scatterplots and calculated MAF (%) results show that the KRAS G12C, G12D, and G12V multiplex panel is sensitive (0.1%) and specific for each mutation.



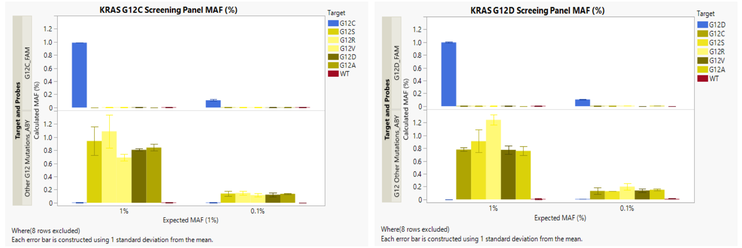
Two-dimensional scatter plots (left image) from the Absolute Q dPCR software (version 6.2.1) and the calculated MAF (%) (right figures) shows that each mutant probe of the multiplex panel is specific for each mutation with high sensitivity (0.1%) in multiplex. The multiplex panel performance is consistent whether only one KRAS G12 mutation is present (top right) or all G12 mutations are present (bottom right).

Figure 2. Example schematic of the G12C Identification panel



This panel consists of one amplicon and 7 probes in "duplex". A "duplex" is characterized by whether the mutant probe sequences overlap with the wild-type probe and if it is located on the same amplicon as the wild-type probe (Whale et al. 2016). The example schematic indicates that higher specificity can be achieved by unique probe design.

Figure 4. Calculated MAF (%) of the KRAS Identification panels compared to expected MAF (%)



Calculated MAF (%) of each target in the KRAS G12C (left) and G12D (right) identification panel shows high specificity to the target of interest. FAM signal is only observed with the target of interest with high sensitivity (top row of each figure, 0.1%). Other G12 mutations are identified using the identification panels even when low amount (0.1%) is present (bottom row of each figure).

RESULTS

All multiplex panels were optimized to specifically identify mutant targets with high sensitivity at 0.1% MAF for each mutation using synthetic controls emulating cfDNA. The PIK3CA multiplex panels were able to identify each mutant targets at 0.1% MAF in cfDNA samples with low false positives (data not shown).

CONCLUSIONS

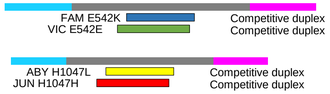
We developed a comprehensive multiplex mutant identification method with high sensitivity and specificity. This study demonstrates the strong potential of Absolute Q dPCR as a powerful multiplex platform for early mutation detection and tumor progression surveillance, providing an advantage in liquid biopsy cancer research using liquid biopsy samples.

Table 2. PIK3CA multiplex panels

Panel	Mutation	Dye
PIK3CA E542K + H1047L	E542K	FAM
	E542E	VIC
	H1047L	ABY
	H1047H	JUN
PIK3CA E545K + H1047R	E545E	FAM
	E545K	VIC
	H1047R	ABY
	H1047H	JUN

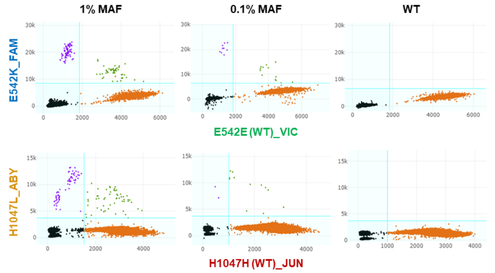
Each panel consists of duplex probes of FAM/VIC and ABY/JUN for the two targets.

Figure 5. Example schematic of the PIK3CA E542K + H1047L panel



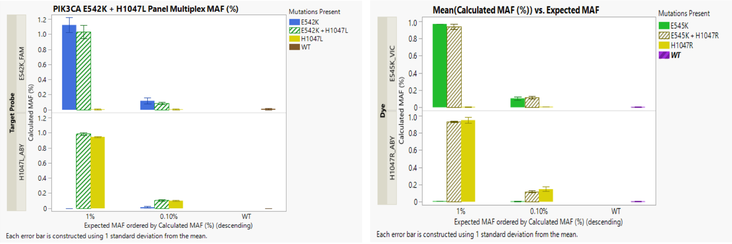
This panel consists of two amplicons (top and bottom) for each target with a competitive duplex probes.

Figure 6. Two-dimensional dPCR scatterplots indicating the high sensitivity (0.1%) and specificity of the PIK3CA E542K+H1047L multiplex panel.



Two-dimensional scatter plots of PIK3CA E542K+H1047L multiplex panel from the Absolute Q dPCR software (version 6.2.1).

Figure 7. Calculated MAF (%) of the two PIK3CA multiplex panels compared to the expected MAF (%).



Calculated MAF (%) of each targets in multiplex shows that both PIK3CA multiplex panels are sensitive (0.1%) and specific for each mutations.

TRADEMARKS/LICENSING

REFERENCES

1. Whale, Alexandra S., et al. "Fundamentals of Multiplexing with Digital PCR." *Biomolecular Detection and Quantification*, Elsevier, 27 May 2016

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