Advancing cancer research: A novel digital PCR tool for simultaneous detection of multiple hallmark mutations in *BRAF* and *EGFR*

Claudia Kappmeier[1], Sherina Edward[1], Corinna Hochstein[1], Jette Ellehauge[2], Stina Christensen[2], Tae Jung Kim[3], Dávid Kis[4], Orsolya Biró[4], Ellen Bruske[1], Francesca Di Pasquale[1], Ronny Kellner[1]
[1]QIAGEN GmbH, QIAGEN Strasse 1, 40724, Hilden, Germany; [2]Klinisk Biokemisk Afdeling, Ringstedgade 57, 4700 Næstved, Denmark;
[3]Seoul St. Mary's Hospital, Department of Pathology, 222 Banpo-Daero, Seocho-Gu, Seoul, Korea; [4]Clinomics Europe Ltd., 1094 Budapest, Mihálkovics utca 10, Hungary

Faster and easier monitoring of hallmark cancer mutations

Precision oncology requires the identification of the specific genetic mutations driving cancer. Some hallmark mutations, such as those found in *BRAF* and *EGFR* genes, are important in the analysis of multiple types of cancer. The capability to concurrently and accurately detect multiple hallmark mutations would greatly benefit cancer research.

Digital PCR (dPCR) technology provides an opportunity for highly sensitive, quantitative mutation analysis. Here, we introduce the new and innovative dPCR PanCancer Kits (RUO), which are designed to detect multiple hallmark mutations in *BRAF* or *EGFR*.

Each kit is tailored to target a spectrum of mutations associated with these genes, facilitating comprehensive mutation analysis. The dPCR PanCancer Kit *BRAF* V600 FAM (200) targets 8 *BRAF* V600 mutations in a single assay and the dPCR PanCancer Kit EGFRex19del FAM (200) targets 23 *EGFR* exon 19 deletions. Each PanCancer assay contains a reference assay for the human single-copy gene *AP3B1* to determine the number of genome copies in the sample and to control for dPCR efficiency.

Here, we present our initial data for various sample types, including blood, plasma, stool and FFPE samples. Through a meticulously optimized dPCR setup, we have achieved exceptional sensitivity and specificity, enabling the detection of multiple mutations in a single channel at allelic frequencies below 1%.

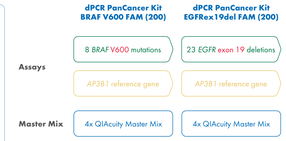
dPCR PanCancer Kit (RUO) assay design and function

dPCR PanCancer Kits (RUO) offer duplex assays to simultaneously detect multiple hallmark mutations in cancer-specific genes and a reference gene. Mutations are detected in the green channel and the reference gene is detected in the yellow channel. Combined with an optimized dPCR Master Mix, these assays reduce the cost and time required for the comprehensive analysis of hallmark mutations.

To comprehensively assess the performance of the dPCR PanCancer Kits (RUO) across varied sample types and methodologies, three reputable institutes conducted rigorous testing. The findings from all three independent evaluations validate the efficacy of the assay design, demonstrating robust compatibility with diverse samples and concordance with testing results from established industry standard methods.

List of mutations targeted by the PanCancer assays

Assay	Mutation aa	Mutation nucleotide	COSMIC ID
dPCR PanCancer Kit BRAF V600 FAM (200)	p.V600K	c.1798_1799delinsAA	COSV56057713
	p.V600R	c.1798_1799delinsAG	COSV56058419
	p.V600E	c.1799_1800delinsAA	COSV56059110
	p.V600E	c.1799T>A	COSV56056643
	p.V600D	c.1799_1800delinsAT	COSV56059623
	p.V600G	c.1799T>G	COSV56080151
	p.V600M	c.1798G>A	COSV56075762
	p.V600R	c.1798_1799delinsCG	COSV56288520
dPCR PanCancer Kit EGFRex19del FAM (200)	p.K745_E749del	c.2233_2247del	COSV51769442
	p.E746_A750delinsIP	c.2235_2248delinsAATTC	COSV51817953
	p.E746_A750del	c.2235_2249del	COSV51765119
	p.E746_T751delinsIP	c.2235_2251delinsAATTC	COSV51782151
	p.E746_T751delinsI	c.2235_2252delinsAAT	COSV51850034
	p.E746_A750del	c.2236_2250del	COSV51765066
	p.E746_T751delinsA	c.2237_2251del	COSV51769364
	p.E746_T751delinsV	c.2237_2252delinsT	COSV51777936
	p.E746_T751delinsVA	c.2237_2253delinsTTGCT	COSV51771891
	p.E746_S752delinsV	c.2237_2255delinsT	COSV51765862
	p.L747_A750delinsP	c.2238_2248delinsGC	COSV51782279
	p.L747_T751delinsQ	c.2238_2252delinsGCA	COSV51863059
	p.E746_S752delinsD	c.2238_2255del	COSV51772418
	p.L747_E749del	c.2239_2247del	COSV51780076
	p.L747_A750delinsP	c.2239_2248delinsC	COSV51765099
	p.L747_T751delinsP	c.2239_2251delinsC	COSV51765856
	p.L747_S752del	c.2239_2256del	COSV51767308
	p.L747_S752delinsQ	c.2239_2256delinsCAA	COSV51778874
	p.L747_P753delinsQ	c.2239_2258delinsCA	COSV51785746
	p.L747_T751delinsS	c.2240_2251del	COSV51768180
	p.L747_T751del	c.2240_2254del	COSV51766247
	p.L747_A750delinsS	c.2240_2248del	COSV51810296
	p.L747_P753delinsS	c.2240_2257del	COSV51767961



Components of the dPCR PanCancer Kit (RUO) assays.

Field test results

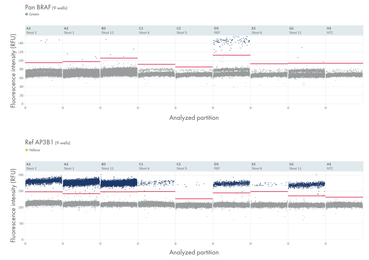
These results from the Klinisk Biokemisk Afdeling are for experiments using the dPCR PanCancer Kits (RUO) with DNA extracted from stool samples and compared to NGS screening of DNA extracted from tumor tissue. DNA extraction for dPCR was performed on collected stool samples from donors with known and unknown status of *BRAF* V600 mutations and *EGFR* exon 19 deletions using extraction kits optimized for stool samples. Donors with known mutation status were previously analyzed for the presence of *BRAF* and *EGFR* mutations by applying NGS to DNA extracted from tumor tissue. In the dPCR reaction, 1–50 ng of extracted stool DNA was used.

Screening results for DNA from stool samples.

ID	Expected mutation	PanCancer Kit result
Stool 1	Unknown	*BRAF* V600 Mut
Stool 2	Unknown	*BRAF* V600 Mut
Stool 4	Unknown	*BRAF* V600 WT
Stool 5	*BRAF* V600 WT	*BRAF* V600 WT
Stool 6	Unknown	*BRAF* V600 WT
Stool 8	*BRAF* V600 Mut	*BRAF* V600 Mut
Stool 11	*BRAF* V600 WT	*BRAF* V600 Mut
Stool 12	Unknown	*BRAF* V600 Mut
Stool 1	*EGFR* WT	n.a.
Stool 3	*EGFR* WT	*EGFR* WT
Stool 5	*EGFR* WT	*EGFR* WT
Stool 6	*EGFR* WT	n.a.
Stool 7	*EGFR* WT	*EGFR* WT
Stool 8	*EGFR* WT	*EGFR* WT
Stool 9	*EGFR* WT	*EGFR* WT
Stool 10	*EGFR* WT	*EGFR* WT
Stool 11	*EGFR* WT	*EGFR* WT
Stool 12	*EGFR* WT	n.a.

Evaluation of positive predictive validity (PPV) and negative PV (NPV) for dPCR PanCancer Kits (RUO).

		Standard method (NGS from tumor tissue) Pos	Neg		
PanCancer Kit (dPCR from stool samples)	Pos	1	1	2	PPV = 50%
	Neg	0	8	8	NPV = 100 %
		1	9		



Examples of 1D scatterplots for individual samples analyzed with the dPCR PanCancer Kit *BRAF* V600 FAM (200).

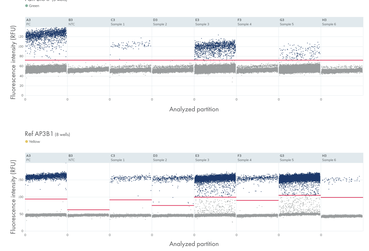
These results from St. Mary's Hospital in Seoul (part of the Catholic University of Korea) are for experiments where the dPCR PanCancer Kits (RUO) and qPCR screening were used on DNA extracted from FFPE samples. DNA extraction for dPCR was performed on collected needle biopsy FFPE samples from donors with known and unknown status of *BRAF* V600 mutations and *EGFR* exon 19 deletions using extraction kits optimized for FFPE tissue samples. Donors with known mutation status were previously analyzed for the presence of *BRAF* and *EGFR* mutations by applying qPCR analysis for the same DNA extractions. In the dPCR reaction, 10–60 ng of extracted FFPE DNA was used.

Screening results for DNA from FFPE samples.

ID	Expected mutation	PanCancer Kit result
Sample 1	*BRAF* V600 Mut	*BRAF* V600 Mut
Sample 2	*BRAF* V600 WT	*BRAF* V600 WT
Sample 3	*BRAF* V600 Mut	*BRAF* V600 Mut
Sample 4	*BRAF* V600 WT	*BRAF* V600 WT
Sample 5	*BRAF* V600 Mut	*BRAF* V600 Mut
Sample 6	*BRAF* V600 WT	*BRAF* V600 WT
Sample 7	*EGFR* exon 19 deletion	*EGFR* exon 19 deletion
Sample 8	Unknown	*EGFR* exon 19 deletion

Evaluation of PPV and NPV for dPCR PanCancer Kits (RUO).

		Standard method (NGS from tumor tissue) Pos	Neg		
PanCancer Kit (dPCR from stool samples)	Pos	5	0	5	PPV = 100%
	Neg	0	3	3	NPV = 100 %
		5	3		



Examples of 1D scatterplots for individual samples analyzed with the dPCR PanCancer Kit *BRAF* V600 FAM (200).

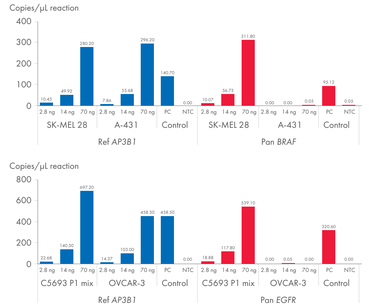
These results from Clinomics Europe are for experiments where the dPCR PanCancer Kits (RUO) were used on DNA extracted from human reference cell lines. DNA extraction for dPCR was performed on four reference cell lines with known status of *BRAF* V600 mutations and *EGFR* exon 19 deletions using extraction kits optimized for fresh tissue samples. Donors with known mutation status were previously analyzed for the presence of *BRAF* and *EGFR* mutations by applying qPCR analysis on the same DNA extractions. In the dPCR reaction, 2.8 ng, 14 ng and 70 ng of extracted gDNA were used.

Screening results for DNA from human reference cell lines.

Cell line	dPCR template input	Expected mutation	PanCancer Kit result
SK-MEL28	70 ng	*BRAF* V600E	*BRAF* V600 Mut
SK-MEL28	14 ng	*BRAF* V600E	*BRAF* V600 Mut
SK-MEL28	2.8 ng	*BRAF* V600E	*BRAF* V600 Mut
A-431	70 ng	*BRAF* V600 WT	*BRAF* V600 WT
A-431	14 ng	*BRAF* V600 WT	*BRAF* V600 WT
A-431	2.8 ng	*BRAF* V600 WT	*BRAF* V600 WT
CS693 P1 mix	70 ng	*EGFR* exon 19 deletion	*EGFR* exon 19 deletion
CS693 P1 mix	14 ng	*EGFR* exon 19 deletion	*EGFR* exon 19 deletion
CS693 P1 mix	2.8 ng	*EGFR* exon 19 deletion	*EGFR* exon 19 deletion
OVCAR-3	70 ng	*EGFR* exon 19 WT	*EGFR* 19 WT
OVCAR-3	14 ng	*EGFR* exon 19 WT	*EGFR* 19 WT
OVCAR-3	2.8 ng	*EGFR* exon 19 WT	*EGFR* 19 WT

Evaluation of PPV and NPV for dPCR PanCancer Kits (RUO).

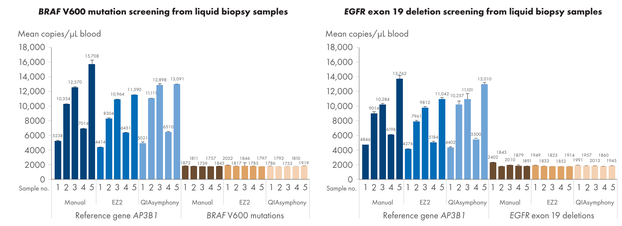
		Standard method (qPCR from cell line DNA) Pos	Neg		
PanCancer Kit (dPCR from cell line DNA)	Pos	2	0	2	PPV = 100%
	Neg	0	2	2	NPV = 100 %
		2	2		



Barplots of concentrations (measured copies/µL) for human DNA samples extracted from reference cell lines. Analyses were performed with the dPCR PanCancer Kits for *BRAF* (top) and *EFGR* (bottom). DNA input was titrated, and synthetic DNA (Gblocks) were used as the positive control (PC). Mean cp/µL values from replicates are given above the bars.

Complete workflow with blood samples

Testing the compatibility of the dPCR PanCancer Kits (RUO) with various DNA extraction protocols and workflows is essential prior to applying it in cancer mutation research. To demonstrate compatibility with one manual and two automated extraction protocols (QIAsymphony® SP, EZ2®), human gDNA was extracted from blood samples from five healthy donors and subsequently analyzed using the dPCR PanCancer Kits (RUO) for *BRAF* (below left) and *EGFR* (below right). The results showed stable DNA recovery across the three extraction methods as measured with the AP3B1 reference genes and consistent amplification and quantification of spiked-in mutation templates for both dPCR PanCancer Kits (RUO). This experiment was performed at the QIAGEN GmbH facilities in Germany.



Detection of somatic mutations in genomic DNA extracted from blood and analyzed using the dPCR PanCancer Kits on the QIAcuity® platform. DNA extraction was performed on collected blood procured from five healthy donors (1–5) employing three distinct methodologies. Blood collection for donors 1 and 2 utilized PAXgene® Blood DNA Tubes, while donors 3–5 underwent blood collection using EDTA blood collection tubes. Manual extraction was executed using the QIAamp® DNA Blood Mini Kit, while automated extractions were conducted using an EZ2 with the EZ1&2™ Blood 350 µL Kit and a QIAsymphony SP with the QIAsymphony DSP DNA Midi Kit. To simulate the presence of mutated DNA content, 2000 copies of synthetic DNA (GBlocks) were spiked into the dPCR reaction, resulting in an expected 2000 copies/µL. In the subsequent dPCR reactions, 1 µL of the eluate served as the input template. This quantity represented 1 µL of blood from the manual preparations, 3.5 µL of blood from the EZ2 preparations, and 2.5 µL of blood from the QIAsymphony preparations. Error bars depict the standard deviation from 2 replicates.

Conclusions

Both dPCR PanCancer Kits (RUO) have potential applications in research prescreening samples, e.g., prior to next-generation sequencing (NGS), or research into monitoring cancer cells. The assays simultaneously assess multiple mutations, reducing time and costs and saving sample material. Additionally, this novel technology is adaptable for other cancer-associated genes, so similar assays can potentially be developed.

Overall, we have demonstrated that our dPCR PanCancer kits (RUO) provide a robust, fast and efficient technology to identify critical mutations, ultimately enhancing our understanding of *BRAF*- and *EGFR*-driven cancers. There is a strong concordance between dPCR PanCancer Kit (RUO) assay results and results from industry standard methods for cancer mutation detection. The assay design is compatible with various sample types.

The dPCR PanCancer Kit is for research use only. Not for the diagnosis, prevention, or treatment of a disease. For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit instructions for use or user operator manual. QIAGEN instructions for use and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services (or your local distributor).

Sample to Insight