Highly multiplexed *EGFR* mutation detection from liquid biopsy samples using the 6-color Crystal Digital PCR™

GUSTAVE ROUSSY CANCER CAMPUS GRAND PARIS #75

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Abstracts

Liquid biopsies are a minimally invasive sampling approach to overcome the heterogeneity of tumors and represent a valuable source of circulating tumor DNA (ctDNA) for oncological biomarker analysis. ctDNA measurements require a highly sensitive and reliable detection technology to quantify often low-level genetic aberrations within a high background of wild-type sequences. Digital PCR has emerged as a powerful technology for the next-generation analysis of liquid biopsies. Stilla Technologies' 6-color naica® system is an ultrasensitive, easy-to-use digital PCR platform capable of simultaneously and precisely quantifying high numbers of biomarkers in a single reaction. Non-small cell lung cancer (NSCLC) is a leading cause of cancer mortality worldwide. Epidermal growth factor receptor (*EGFR*) is frequently mutated in NSCLC. A handful of anti-EGFR Tyrosine Kinase Inhibitor therapies are approved by the FDA; however, most patients develop resistance overtime to these treatments. Both monitoring primary *EGFR* mutations to predict treatment response and precociously detecting resistance mutations to adapt treatments promptly through ctDNA analysis stand to improve the effectiveness of NSCLC patient management.

Stilla has developed a highly multiplexed 6-color Crystal Digital PCR™ *EGFR* kit detecting more than 90% of *EGFR* mutations described in NSCLC from ctDNA. The assay detects 32 common and rare somatic *EGFR* mutations in exons 18, 19, 20, and 21, including both activating and resistant mutations. In this work, we evaluated the sensitivity, precision and specificity of the *EGFR* 6-color Crystal Digital PCR™ assay and analyzed ctDNAs from NSCLC clinical samples. We showed that the *EGFR* 6-color Crystal Digital PCR™ assay is highly specific and sensitive for the detection of *EGFR* mutations, with a Limit of Detection in a high background of wild-type DNA ranging from 0.30 to 0.46 cp/µL (with observed MAFs ranging from 0.06 to 0.09%). Moreover, a high concordance was observed between those *EGFR* results obtained for NSCLC ctDNA samples using 6-color Crystal Digital PCR™ and other technologies. With a rapid time to results and straightforward ctDNA analysis workflow, Crystal Digital PCR™ on the naica® system promises ultrasensitive, highly multiplexed 6-color mutation detection, maximizing the information obtained from precious samples.

Introduction

A liquid biopsy is the sampling and analysis of a non-solid biological specimen, for example blood. Liquid biopsies are a minimally invasive and straightforward sampling approach that can overcome the heterogeneity of tumors, and thus represent a valuable source of ctDNA for oncological biomarker analysis.

ctDNA measurements require a highly sensitive and reliable detection technology to quantify often low-level genetic aberrations within a high background of wild-type sequences. Digital PCR has emerged as a powerful technology for the next-generation analysis of liquid biopsies.

Stilla® Technologies' 6-color naica® system is an ultrasensitive digital PCR technology capable of simultaneously and precisely quantifying high numbers of biomarkers in a single sample, simplifying the detection process, minimizing detection variability, and significantly reducing the hands-on-time to results.

Single assay detection of more than 90% of described *EGFR* mutations in NSCLC

Non-small cell lung cancer (NSCLC) is a leading cause of cancer mortality worldwide. Epidermal growth factor receptor (*EGFR*) is frequently mutated and is a well-known genetic aberration in NSCLC. The highly multiplexed, ready-to-use *EGFR* 6-color Crystal Digital PCR™ kit allows the detection from circulating free DNA (cfDNA) of more than 90% of *EGFR* mutations described in NSCLC, including 32 common, rare, activating, and resistant somatic *EGFR* mutations in exons 18, 19, 20 and 21 **(Table 1).**

Exon	Mutation	Base changes	Cosmic ID
Exon 18	p.G719A	c.2156G>C	COSM6239
	p.G719C	c.2155G>T	COSM6253
	p.G719S	c.2155G>A	COSM6262
Exon 19 deletion Drop-off	p.E746-A750del	c.2236_2250del	COSM6225
	p.E746_T751del	c.2236_2253del	COSM12728
	p.E746-T751delinsA	c.2237_2251del	COSM12678
	p.E746_T751delinsI	c.2235_2252delinsAAT	COSM13551
	p.E746_S752delinsA	c.2237_2254del	COSM12367
	p.E746_S752delinsD	c.2238_2255del	COSM6220
	p.E746-S752delinsV	c.2237_2255delinsT	COSM12384
	p.E746-P753delinsVS	c.2237_2257delinsTCT	COSM18427
	p.L747-E749del	c.2239_2247del	COSM6218
	p.L747-A750delinsS	c.2240_2248del	COSM4170221
	p.L747-A750delinsP	c.2239_2248delinsC	COSM12382
	p.L747-A750delinsP	c.2238_2248delinsGC	COSM12422
	p.L747-T751del	c.2240_2254del	COSM12369
	p.L747-T751delinsP	c.2239_2251delinsC	COSM12383
	p.L747-T751delinsQ	c.2238_2252delinsGCA	COSM12419
	p.L747-A751delinsS	c.2240_2251del	COSM6210
	p.L747-S752del	c.2239_2256del	COSM6255
	p.L747-P753delinsS	c.2240_2257del	COSM12370
	p.L747-P753delinsQ	c.2239_2258delinsCA	COSM12387
Exon 20	p.T790M	c.2369C>T	COSM6240
	p.C797S	c.2389T>A	COSM6493937
	p.C797S	c.2389G>C	COSM5945664
Exon 20 insertion drop-off	p.D770_N771insG	c.2310_2311insGGT	COSM12378
	p.H773-V774insH	c.2319-2320insCAC	COSM12377
	p.H773-V774insPH	c.2319-2320insCCCCAC	COSM28944
	p.H773-V774insAH	c.2319-2320ins-CCCACG	COSM1238028
	p.H773-V774insNPH	c.2319-2320insAAACCCCAC	COSM18491
Exon 21	p.L858R	c.2573T>G	COSM6224
	p.L861Q	c.2573T>G	COSM6213

Table 1. *EGFR* mutations detectable with the *EGFR* 6-color Crystal Digital PCR™ kit

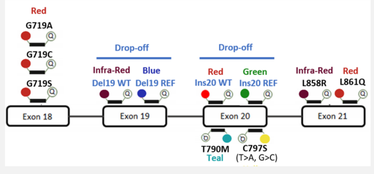


Figure 1: *EGFR* 6-color Crystal Digital PCR™ kit detection design. TaqMan™ probe positions on each exon and fluorescent color code are noted.

The *EGFR* 6-color Crystal Digital PCR™ kit enables highly multiplexed cfDNA analysis on the naica® system

The *EGFR* 6-color Crystal Digital PCR™ kit uses TaqMan™ probe technology to detect eight point mutations, as well as twenty-four Exon 19 deletion and Exon 20 insertion alterations using drop-off detection **(Figure 1).**

A major advantage of a drop-off digital PCR assay is the simplified detection of numerous proximal genetic lesions (including deletions, insertions and nucleotide substitutions) within a short genomic interval using minimal reagents. Indeed, a drop-off assay requires only two TaqMan™ probes targeting the same amplicon: a wild-type probe that spans the genetic lesion site but is uniquely complementary to the wild-type sequence, and a reference probe that hybridizes adjacent to the mutation site and is thus complementary to both the mutant and the wild-type alleles. In the presence of a wild-type allele, both the wild-type and reference probes will hybridize with their targets, leading to a double positive population **(Figure 2A and 2C).** Whereas in the presence of a mutant allele, only the reference probe anneals to its target leading to an additional simple positive population. **(Figure 2B and 2D).** The custom analysis template provided with the *EGFR* 6-color Crystal Digital PCR™ kit combined with Crystal Miner software allows the automated generation of 2D-plots and sample quantification.

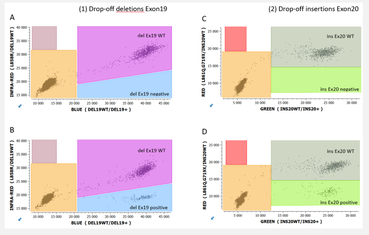


Figure 2. Representative 2D-plots of wild-type (WT) deletion Exon 19 (A) and deletion Exon 19 positive cfDNA samples (B). Representative 2D-plots of WT insertion Exon 20 (C) and insertion Exon 20 positive cfDNA samples (D). The x- and y-axis correspond to the fluorescence units of the indicated color channel.

The *EGFR* 6-color Crystal Digital PCR™ kit is optimized for ultrasensitive detection on the naica® system

The *EGFR* 6-color Crystal Digital PCR™ kit is compatible with the 6-color naica® system using highly sensitive Sapphire chips. To determine the sensitivity of detection of each *EGFR* target, the Limit of Blank (LoB) and the Limit of detection (LoD) were determined following the Clinical and Laboratory Standards Institute (CLSI) EP17-A2 standard (Protocols For Determination Of Limits Of Detection And Limits Of Quantitation; Approved Guideline). The LoB for *EGFR* mutation detection in the *EGFR* 6-color Crystal Digital PCR™ kit ranges from 0.06 to 0.17 copies per µL (cp/µL), depending on the target, whereas the LoD ranges from 0.30 to 0.46 cp/µL, depending on the target **(Table 2).**

To evaluate the sensitivity of the *EGFR* 6-color Crystal Digital PCR™ kit, a linear titration was performed targeting *EGFR* exon 19 deletions, *EGFR* exon 20 insertions, *EGFR* L858R, *EGFR* G719A, *EGFR* L861Q, *EGFR* T790M, *EGFR* C797S (GC) and *EGFR* C797S (TA) mutations from 64 cp/µL down to 0.25 cp/µL **(Figure 3)** in a background of 120 cp/µl of wild-type *EGFR* DNA (equivalent to 3,000 copies per 25µl Sapphire chip reaction). The *EGFR* 6-color Crystal Digital PCR™ kit displayed ultrasensitive and reliable detection for all mutations.

Target	LoB95	LoD95
DEL19+	0.11	0.37
L858R	0.09	0.38
INS20+	0.17	0.46
L861Q, G719X	0.09	0.37
T790M	0.12	0.46
C797S	0.06	0.30

Table 2. *EGFR* mutations detectable with the *EGFR* 6-color Crystal Digital PCR™ kit

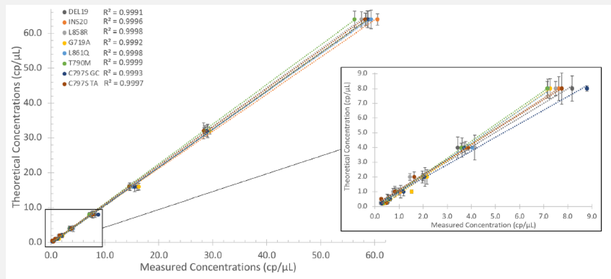


Figure 3. Titrations of theoretical concentrations of 64cp/µl, 32 cp/µl, 16 cp/µl, 8 cp/µl, 4 cp/µl, 2 cp/µl, 1 cp/µl, 0.5 cp/µl and 0.25 cp/µl of *EGFR* exon 19 deletion, *EGFR* exon 20 insertion, *EGFR* L858R, *EGFR* G719A, *EGFR* L861Q, *EGFR* T790M, *EGFR* C797S (GC) and *EGFR* C797S (TA) DNAs, in a background of 120 cp/µl of wild-type EGFR (3,000 copies per 25µl reaction). Reactions were performed using synthetic Ultramer™ mutant DNA oligonucleotides templates ranging in size from 97 to 124 nucleotides, and wild-type human genomic DNA. Shown are the averages of a minimum of triplicate measurements with standard errors for each target.

The *EGFR* 6-color Crystal Digital PCR™ kit is optimized for ultrasensitive and highly specific detection on the naica® system

The *EGFR* 6-color Crystal Digital PCR™ kit showed robust inter-run repeatability, with concentration RSDs (relative standard deviations) ranging from 4.5 to 8.6% (N = 12), depending on the target **(Figure 4).**

In addition, the *EGFR* 6-color Crystal Digital PCR™ kit showed high specificity, with no false positives obtained **(Table 3).**

Targets	DEL19+	INS20+	L858R	L861Q, G719X	T790M	C797S
WT hgDNA	NEG	NEG	NEG	NEG	NEG	NEG
DEL19+ DNA	**POS**	NEG	NEG	NEG	NEG	NEG
INS20+ DNA	NEG	**POS**	NEG	NEG	NEG	NEG
L858R DNA	NEG	NEG	**POS**	NEG	NEG	NEG
L861Q DNA	NEG	NEG	NEG	**POS**	NEG	NEG
G719A DNA	NEG	NEG	NEG	**POS**	NEG	NEG
G719C DNA	NEG	NEG	NEG	**POS**	NEG	NEG
G719S DNA	NEG	NEG	NEG	**POS**	NEG	NEG
T790M DNA	NEG	NEG	NEG	NEG	**POS**	NEG
C797S-GC DNA	NEG	NEG	NEG	NEG	NEG	**POS**
T790M/C797S-TA DNA	NEG	NEG	NEG	NEG	**POS**	**POS**

Table 3. Specificity results of Crystal Digital PCR™ using the *EGFR* 6-color Crystal Digital PCR™ kit. Reactions were performed in duplicate using synthetic Ultramer™ mutant DNA oligonucleotides and wild-type human genomic DNA as templates.

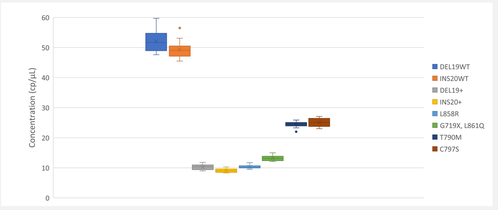
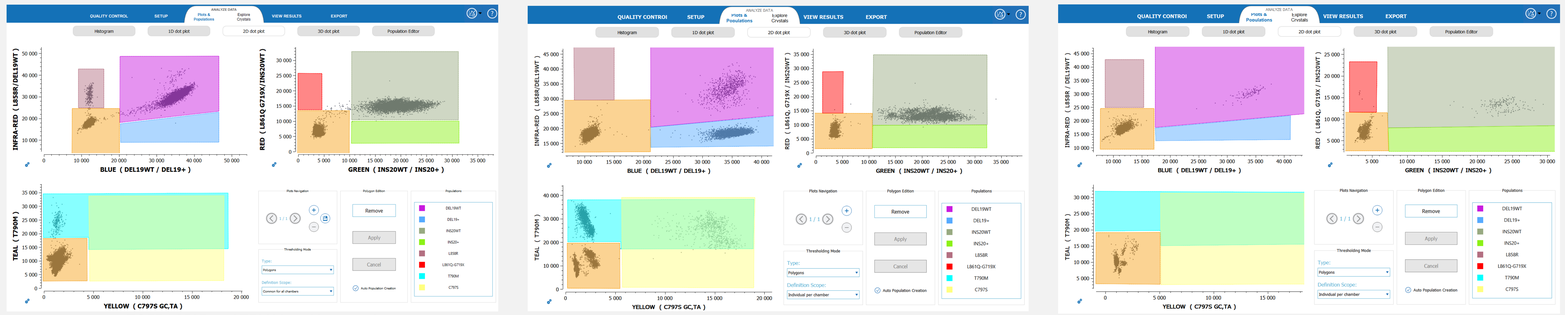


Figure 4. Inter-run repeatability of the *EGFR* 6-color Crystal Digital PCR™ kit. The inter-run repeatability was measured by comparing the results obtained on one positive control sample chamber in 12 different runs (N = 12) with the *EGFR* 6-color Crystal Digital PCR™ kit.

Analysis of a mutated NSCLC clinical samples using the *EGFR* 6-color Crystal Digital PCR™ kit on the naica® system

The analysis template provided with the *EGFR* 6-color Crystal Digital PCR™ kit combined with Crystal Miner software allow for the straightforward generation of 2D-plots **(Figure 5)** and the rapid obtention of highly concordant results.



EGFR L858R and T790M positive sample | *EGFR* Deletion exon19, T790M and C797S positive sample | *EGFR* L861Q or G719X positive sample

Figure 5. A representative example of the analysis of a positive NSCLC clinical samples using Crystal Miner software and the custom *EGFR* 6-color Crystal Digital PCR™ kit analysis template. The x- and y-axis correspond to the fluorescence units of the indicated color channel. The samples showed a perfect concordance between the expected and measured results.

Poster Highlights

Ready-to-use | Intuitive | Rapid time to results | High multiplexing | High sensitivity | ctDNA compatible

- The *EGFR* 6-color Crystal Digital PCR™ kit reliably detects 32 common, rare, activating, and resistant somatic *EGFR* mutations in a single assay.
- The *EGFR* 6-color Crystal Digital PCR™ kit is optimized for ultrasensitive and highly robust *EGFR* mutation detection on the naica® system with LoDs ranging from 0.30 to 0.46 cp/µL, depending on the target using Sapphire chips.