

High multiplex, ultrasensitive *EGFR* detection using the *EGFR* 6-color Crystal Digital PCR™ kit



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

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

A liquid biopsy is the sampling and analysis of a non-solid biological specimen, most often the case 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 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 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.

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**, next page).

Application Note Highlights

- 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.
- The Crystal Miner software and custom *EGFR* 6-color analysis template enable fast and straightforward analysis of cfDNA.

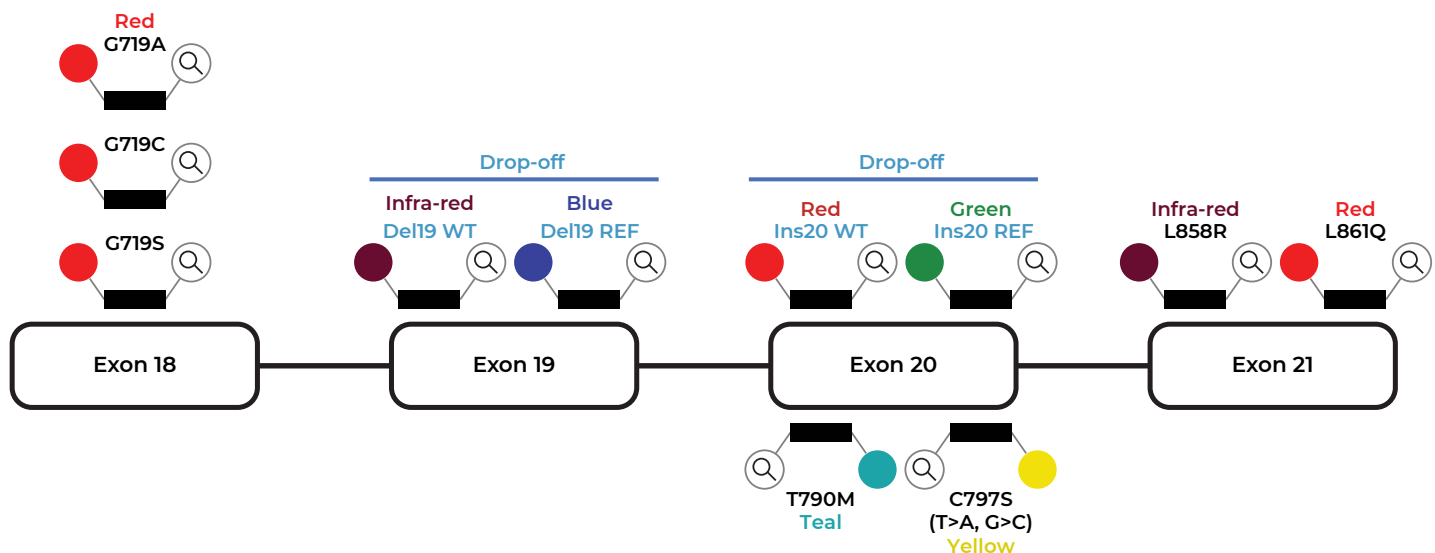
The *EGFR* 6-color Crystal Digital PCR™ kit enables highly multiplexed cfDNA analysis on the naica® system

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**, next page).

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

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	COSM6252
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

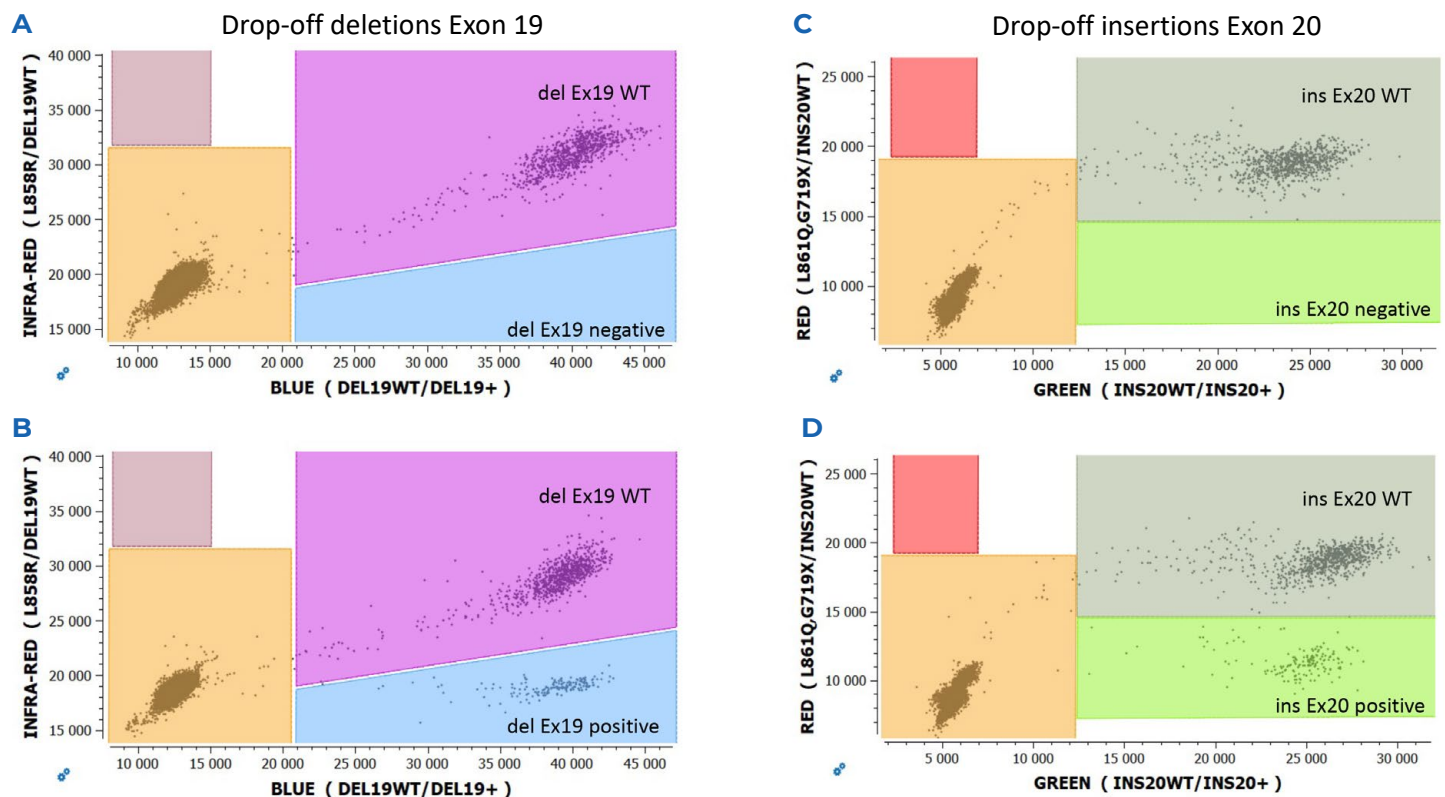
Figure 1 | *EGFR* 6-color Crystal Digital PCR™ kit detection design. TaqMan™ probe positions on each exon and fluorescent color code are noted.



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 | **A:** Representative 2D-plots of wild-type (WT) deletion Exon 19 and **B:** and deletion Exon 19 positive cfDNA samples **C:** Representative 2D-plots of WT insertion Exon 20 and **D:** and insertion Exon 20 positive cfDNA samples. 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 and highly specific detection on the naica® system.

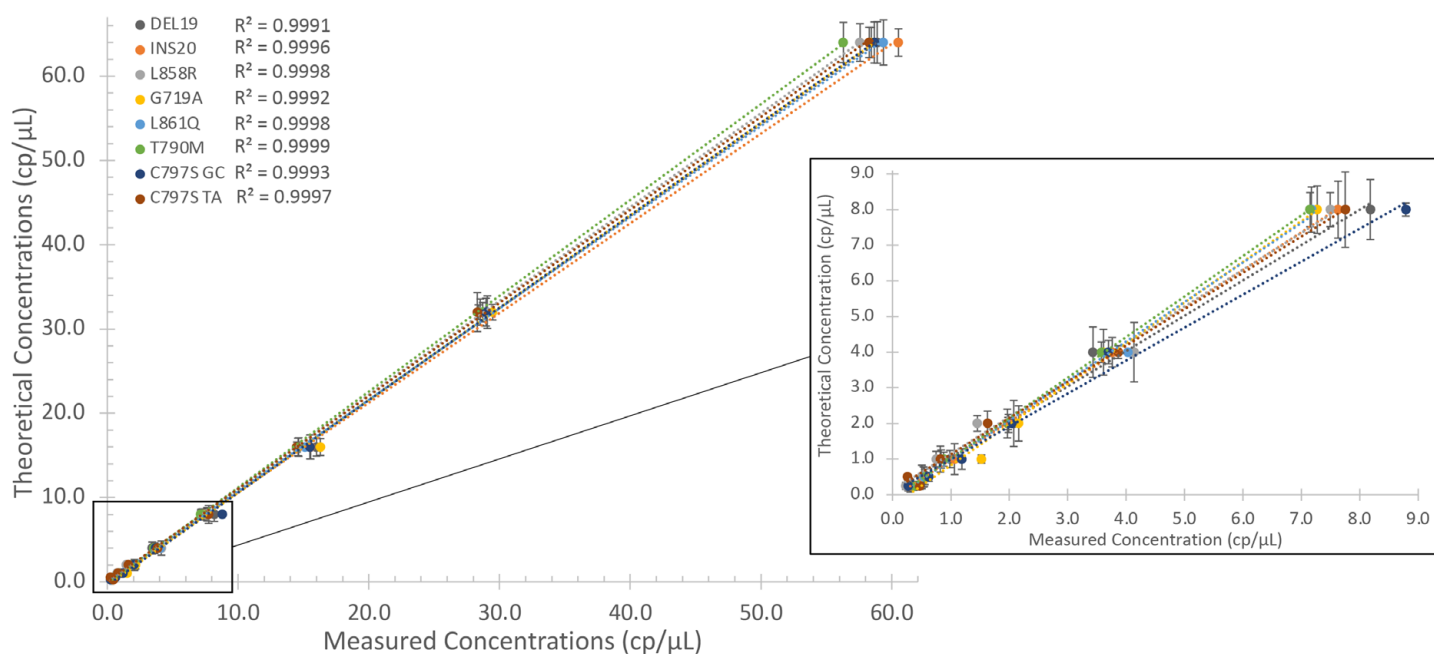
The *EGFR* 6-color Crystal Digital PCR™ kit is compatible with the 6-color naica® system using highly sensitive Sapphire chips. To determine the sensitivity of detection of each *EGFR* target, the Limit of Blank (LoB) and the Limit of detection (LoD) were determined following the Clinical and Laboratory 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).

Table 2 | LoB and LoD of the *EGFR* 6-color Crystal Digital PCR™ kit determined following the CLSI EP17-A2 standard (Sapphire chip well concentrations in cp/ μL).

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

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 25 μL Sapphire chip reaction). The *EGFR* 6-color Crystal Digital PCR™ kit displayed ultrasensitive and reliable detection for all mutation.

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 25 μL 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 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**).

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 1 positive control sample chamber in 12 different runs (N = 12) with the *EGFR* 6-color Crystal Digital PCR™ kit

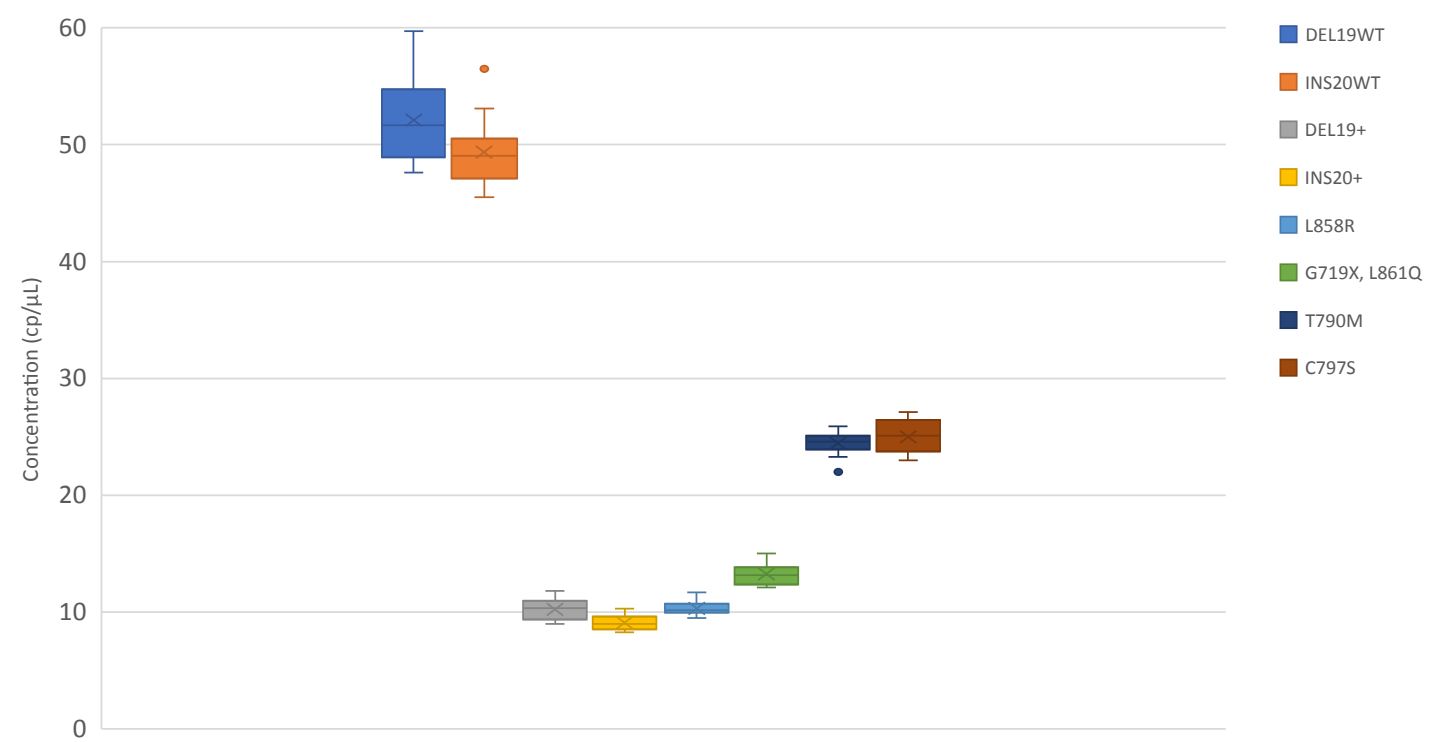


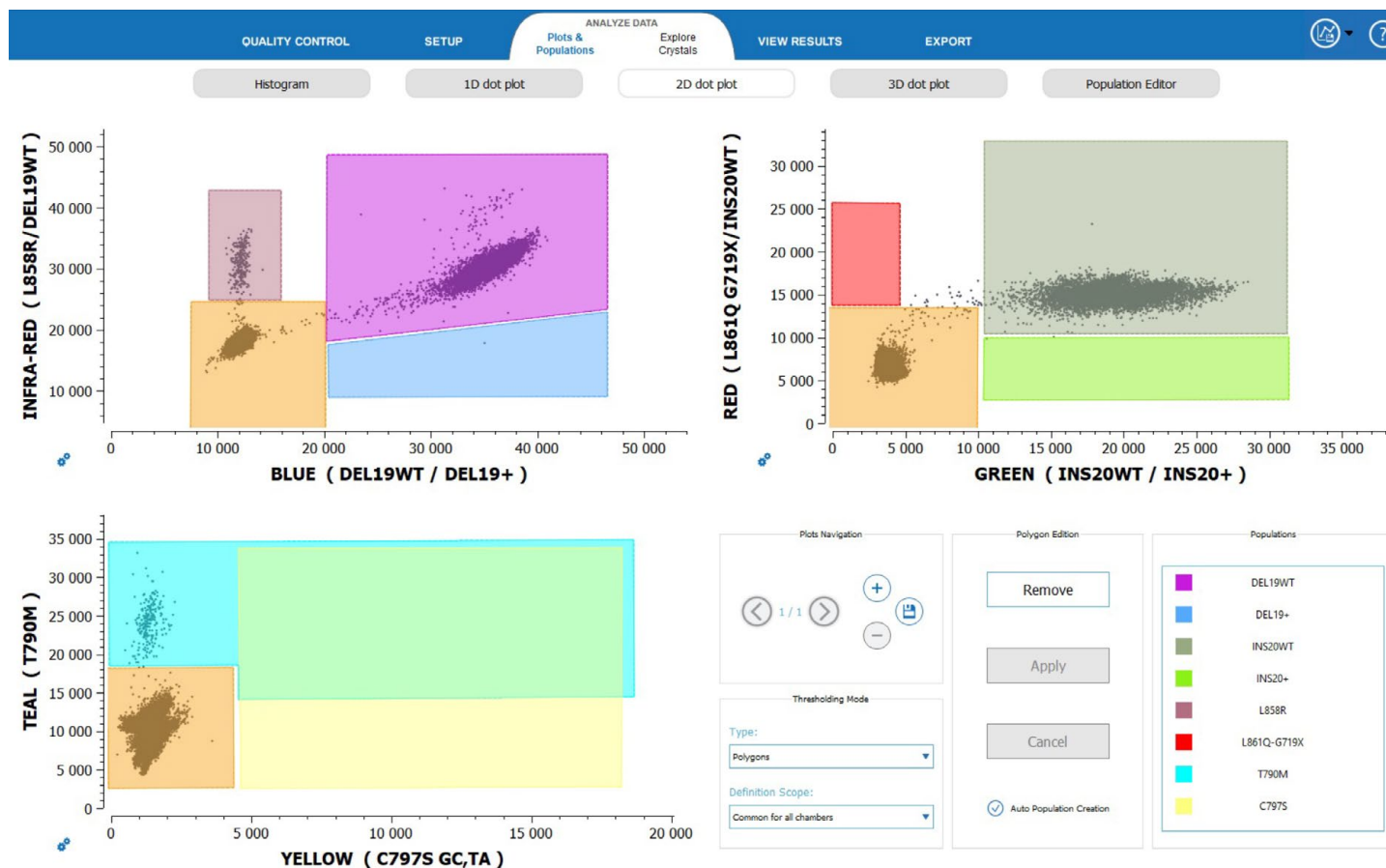
Table 3 | Specificity results of Crystal Digital PCR™ using the *EGFR* 6-color Crystal Digital PCR™ kit. Reactions were performed in duplicate using synthetic Ultramer™ mutant DNA oligonucleotides and wild-type human genomic DNA as templates.

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

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.

Figure 5 | A representative example of the analysis of a L858R and T790M positive NSCLC clinical cfDNA sample using Crystal Miner software and the custom *EGFR* 6-color Crystal Digital PCR™ kit analysis template. The cfDNA sample showed a perfect concordance between the expected and measured results. The x- and y-axis correspond to the fluorescence units of the indicated color channel.



To learn more about digital PCR, please visit Stilla Technologies' Learning Center at www.gene-pi.com