

EGFR 6-color Crystal Digital PCR™ kit

Instructions for Use

For Research Use Only. Not for use in diagnostic procedures.

For use by qualified personnel trained on the 6-color naica® system.

Product Name

EGFR 6-color Crystal Digital PCR™ kit (R30006)

Number of reactions: 48 reactions

Samples: cell-free DNA

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Intended Use

The *EGFR* 6-color Crystal Digital PCR™ kit is intended for the detection and quantification of 32 somatic *EGFR* mutations in exons 18, 19, 20 and 21 associated with non-small cell lung cancer (NSCLC) using the Sapphire Chip on the 6-color naica® system.

This kit can be used on cell-free DNA (cfDNA) extracted from plasma.

Please note:

- ✓ The extraction method used and sample purity might have an influence on sample compatibility.
- ✓ **Individual sample-type and extraction method compatibilities for digital PCR applications may require a dedicated assay validation by the end user.**
- ✓ Only compatible with Sapphire Chips.

Table 1. Mutations Table

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	Exon 19 Deletions = All deletions between the bases c.2235 to c.2250 including:		
	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.2390G>C	COSM5945664
	Exon 20 insertions = All insertions between the bases c. 2310 to c. 2320 including:		
	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.2319-2320insAAACCCAC	COSM18491
	Exon 21	p.L858R	c.2573T>G
p.L861Q		c.2582T>A	COSM6213

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 through the use of drop-off detection (Figure 1). 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 (please refer to technical note “[How to quantify Drop-Off digital PCR Assays with Crystal Miner](#)”).

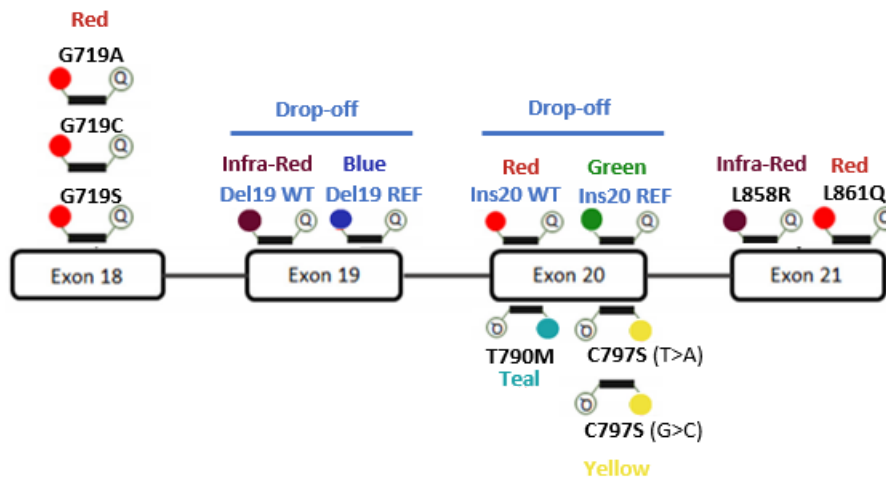


Figure 1. *EGFR* 6-color Crystal Digital PCR™ kit detection design.

Crystal Reader software and Crystal Miner software version 3.0 and above are required to analyze the results of the *EGFR* 6-color Crystal Digital PCR™ kit. A custom scanning and analysis template is provided (see section “Image acquisition with the Prism6 instrument”).

Resources

The technical resources for the *EGFR* 6-color Crystal Digital PCR™ kit are available at Stilla Technologies website: <https://www.stillatechnologies.com/technical-resources/naica-system-prism6/>.

➤ Before using the *EGFR* 6-color Crystal Digital PCR™ kit for the first time:

PCR step:

Download the **PCR Program** of *EGFR* 6-color Crystal Digital PCR™ from the technical resources section on a USB key and transfer the program to the Geode to import it to the PCR programs (Please refer to Geode User Manual for how to import a program from an USB key).

Image acquisition step:

Download the latest version of the **Scanning Template** of *EGFR* 6-color Crystal Digital PCR™ from the technical resources section on the naica® PC of the 6-color naica® system.

Interpretation of sample results step:

Request the **Certificate of Compliance** (CoC) of the *EGFR* 6-color Crystal Digital PCR™ from the Technical Support Department. The CoC contains the accepted concentration range for all targets in the positive control. As it is a batch number-related information, this step should be repeated for each new batch of *EGFR* 6-color Crystal Digital PCR™ kit.



Composition

The *EGFR* 6-color Crystal Digital PCR™ kit contains six tubes:

Name	Cap Color	Initial Concentration	Volume	Description
Buffer A	● Blue	10X	140 µL	naica® multiplex PCR MIX
Buffer B	● Red	50X	50 µL	Reaction enhancer solution
Buffer C	● Yellow	8X	175 µL	Ready to use primer and probe solution
Positive control	● Purple	5X	100 µL	Synthetic mutated DNA pool (concentrations defined in the Certificate of Compliance - CoC)
Negative control	○ White	5X	100 µL	N/A
Nuclease free water	Transparent	N/A*	1 mL	N/A

*N/A: not applicable

Storage

- All reagents provided in the *EGFR* 6-color Crystal Digital PCR™ kit must be stored between -25°C and -15°C in the original tubes until the expiration date indicated on the respective packaging.
-  Buffer A and Buffer C contain a photosensitive fluorescent dye and are thus provided in opaque tubes to protect them from light. Store tubes protected from light sources.
-  Store tubes at all times in an upright position. Tubes can be stored in the provided cardboard box if desired.

Under these conditions, the *EGFR* 6-color Crystal Digital PCR™ kit is stable for 12 months from the date of production. The respective expiration dates are specified on the *EGFR* 6-color Crystal Digital PCR™ kit packaging and on the product labels.

Reagents can be thawed up to 10 times. Do not aliquot reagents in alternative tubes.

Conditions for Use

Ensure to thaw completely each *EGFR* 6-color Crystal Digital PCR™ kit reagent prior to reaction mix preparation.

Throw away the kit as soon as one of the tubes is empty.

Materials and Equipment Required

The *EGFR* 6-color Crystal Digital PCR™ kit can only be run on the 6-color naica® system with the Prism6 instrument (N20001.6).

Specific Crystal Reader scanning configuration template for the *EGFR* 6-color Crystal Digital PCR™ kit is available on the Technical Resources webpage.

Additional materials required but not provided in the *EGFR* 6-color Crystal Digital PCR™ kit:

- 0.5 mL and 1.5 mL microcentrifuge tubes
- Micropipettes and tips (from 1 to 200 µL)
- Benchtop vortex
- Benchtop mini-spin centrifuge
- Sapphire Chips (C14012)

Assay Protocol

It is recommended to quantify the DNA concentrations of the samples before performing Crystal Digital PCR™. The *EGFR* 6-color Crystal Digital PCR™ kit is compatible with a range of DNA concentrations. However, users must not exceed 40 ng of DNA per reaction for optimal performance.

Note: Do not perform an overnight run with the EGFR 6-color Crystal Digital PCR™ kit.

Table 2: EGFR 6-color Crystal Digital PCR™ kit reaction mix assembly per reaction

Name	Initial Concentration	Final Concentration	Vol (µL) for 1 reaction
● Buffer A	10X	1X	2.6
● Buffer B	50X	1X	0.5
● Buffer C	8X	1X	3.3
● <i>diluted clinical sample or</i> ● <i>diluted positive control or</i> ○ <i>diluted negative control</i>			19.6
Total volume (µL)			26

It is recommended to perform a full run of three Sapphire Chips as one positive and one negative control has to be included for each Geode amplification run, irrespective of the number of clinical samples analyzed (from 1 to 10 cfDNA samples).

- Thaw completely each *EGFR* 6-color Crystal Digital PCR™ kit reagent (equilibrate to room temperature), vortex all tubes (ensure to vortex tube A for at least 10 seconds), and spin briefly in a mini-spin centrifuge to collect the material at the bottom of each tube.
- Assemble the reagents as shown in Table 2.
 - Start by preparing the reaction mix for **X+1** reactions.

- (X = n samples + 1 negative control + 1 positive control)

Table 3: Reaction mix setup for 1 reaction

Name	Vol (µL) for 1 reaction
Buffer A ●	2.6
Buffer B ●	0.5
Buffer C ●	3.3
Total volume (µL)	6.4

- Vortex and spin the tube containing the reaction mix including Buffers A, B and C. Split the content of this reaction mix into X tubes of 6.4 µL each. Label tubes Neg Ctl, Pos Ctl and Samples (from 1 to 10 cfDNA samples). Stilla Technologies recommends following the order indicated below:

a) Preparation of the negative control reaction:

- Label one of the tubes containing 6.4 µL of reaction mix “Neg Ctl”, add 5.2 µL of negative control sample from the provided tube with the white cap and add additionally 14.4 µL of the provided nuclease free water.

b) Preparation of the sample reaction:

- Add the desired sample volume to each of the individual sample tubes “Sample X” (1 to 10) containing 6.4 µL of reaction mix and add additionally nuclease free water to reach a final volume of 26 µL.

Note: Do not exceed a total DNA amount of 40 ng per reaction.

c) Preparation of the positive control reaction:

- Label one of the tubes containing 6.4 µL of reaction mix “Pos Ctl”, add 5.2 µL of positive control sample from the provided tube with the purple cap and add additionally 14.4 µL of the provided nuclease free water. It is recommended to end the preparation of the PCR mixes with the positive control reaction to avoid any risk of contamination of the clinical samples.

- Vortex each tube thoroughly to mix all reagents and briefly centrifuge to collect the content at the bottom of the tube.
- Load 25 µL of the reaction mix into 1 chamber of the Sapphire Chip (Please refer to the Sapphire Chip IFU for instructions to load a Sapphire Chip).

Note: Stilla Technologies recommends loading first the negative control reaction, then loading the samples and ending with the positive control. Do not pipet directly into the oil phase. Make sure to change tips after each pipetting.

Table 4: Example of recommended Sapphire Chip layout plan

	Chip 1	Chip 2	Chip 3
A	Neg Ctl.	Sample 4	Sample 8
B	Sample 1	Sample 5	Sample 9
C	Sample 2	Sample 6	Sample 10
D	Sample 3	Sample 7	Pos Ctl.

Perform the PCR:

- Place the Sapphire Chips in the Geode and close the lid.
- Select the *EGFR* 6-color Crystal Digital PCR™ kit **PCR Program** “PartitioningProgram_Geode_SapphireChip_EGFR-6color-Kit_V1.js” from the main menu of the Geode screen and press “Play” to start the program (Please refer to the Geode User Manual).


Table 5: *EGFR* 6-color Crystal Digital PCR™ kit PCR program

Step	Temperature	Duration
Enzyme activation	95 °C	3 min
20 cycles		
Denaturation	95 °C	15 sec
Elongation	65 °C	30 sec
40 cycles		
Denaturation	95 °C	15 sec
Elongation	64 °C	30 sec

- The total duration of the *EGFR* 6-color Crystal Digital PCR™ kit Geode program is 2 hours and 30 min.
- At the end of the run, the message “PCR completed successfully, touch the screen to continue” appears. Recover Geode “Live Logs” on a USB stick (Please refer to the Geode user manual for how to recover Geode Live Logs). Open the Geode lid and transfer the Sapphire Chips to the Prism6 instrument.

Image acquisition with the Prism6 instrument:

Please refer to Prism6 and Crystal Reader software User Manual for instructions on how to acquire images with the Prism6 instrument.

- Ensure the Prism6 instrument is turned on. Launch the Crystal Reader software application by double-clicking on the “Crystal reader” icon  on the monitor.
- Click on “Open tray”, place the Sapphire Chips in the Prism6 instrument and click on “Close the tray”.
- Click on the “NEW EXPERIMENT” button to create a new experiment with Sapphire Chips and browse the latest version of the *EGFR* 6-color Crystal Digital PCR™ kit Scanning Template.

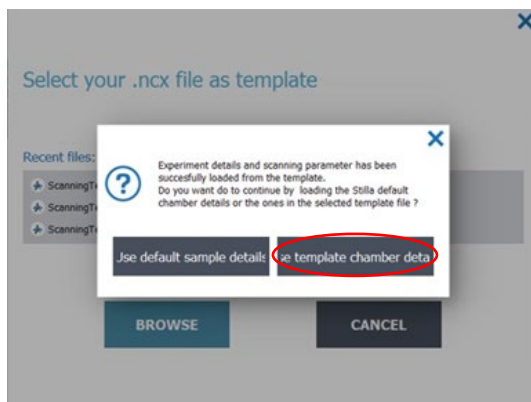


Figure 2: Home menu prompt for sample configuration selection.

- A prompt appears, select “Use template chamber detail.”
- Name your experiment (Fill in the “New Experiment” case): it is recommended to enter the experiment name by indicating at least the experiment type as a prefix (i.e., “EGFR-6-color”). Note that date (e.g., YYMMDD) and chip IDs (e.g., XXXXXXXX-XXXXXXX-XXXXXXXX) can also be indicated in the experiment name as good practice for file searchability.
- Enter the Chip ID (either manually or with an external barcode reader).
- Click on “Save” and save the “.ncx” file in the desired folder.
- In the “Chamber Details” section, for each sample:
 - Modify the sample name (NEG CTL, SAMPLE X, POS CTL)
 - Add the dilution factor value for the 6 channels (Blue, Teal, Green, Yellow, Red, And Infra-Red) for **positive and negative controls**. If the guidelines described in the Assay protocol section above “Preparation of the positive control reaction” were followed for the reaction mix assembly, the dilution factor is **5**)
 - Add the dilution factor value for the 6 channels (Blue, Teal, Green, Yellow, Red, And Infra-Red) for **samples**. This allows to quantify properly the final DNA concentration in the sample. If the guidelines described in the Protocol section “Preparation of the samples reaction” were followed for the reaction mix assembly, the dilution factor can be calculated using the below equation:

$\text{Dilution factor} = \text{total volume of 1 reaction (26 } \mu\text{L)} \div \text{sample volume (X* } \mu\text{L)}$ $= 26 \div X$

** Sample volume (X) can be up to 19.6 μL .*

- Click on “scan” (Please refer to the Prism6 and Crystal Reader software User Manual for detailed instructions). The total duration of the *EGFR* 6-color Crystal Digital PCRTM kit Prism6 scan is approximately 55 min for up to 12 Sapphire Chip chambers. The total workflow time is about 3 hours and 25 min for three Sapphire Chips.
- Once a chamber is scanned, a 3-level quality flag appears in the chamber rectangle. (Please refer to Prism6 and Crystal Reader software User Manual for detailed instructions for checking image quality).

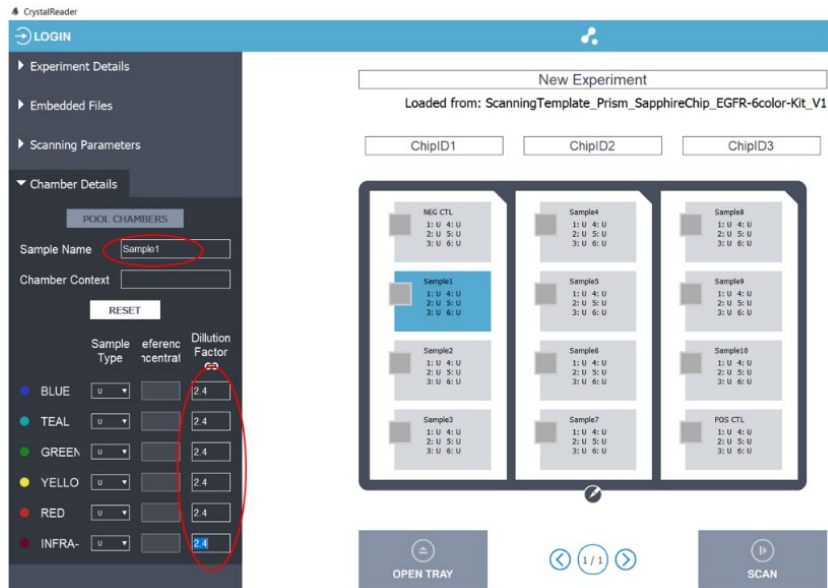


Figure 3: Edit “Chamber Details” for the required fields.

Note: In case dust particles are present, clean the Sapphire Chip and rescan the chamber (Please refer to the Prism6 & Crystal Reader software User Manual for instructions on how to clean a Sapphire Chip)

- At the end of the run, place the chips at 4°C in fridge. If stored at 4°C, chips can be scanned/rescanned up to 48h after the end of the run. If stored at room temperature, chips can be scan/ rescanned up to 6h after the end of the run.

Data Analysis with Crystal Miner Software

- Open the “.ncx” file generated by the Crystal Reader software in the Crystal Miner software after the scanning step by double-clicking the file.
- Go to the menu “ANALYZE DATA > Plots & Populations > 2D dot plot”. If needed, double click on the graph to adjust the axis scales, and if not sufficient click on the blue gear below the 2D graph (indicated by the red arrow in Figure 4 below) to manually enter the desired value.

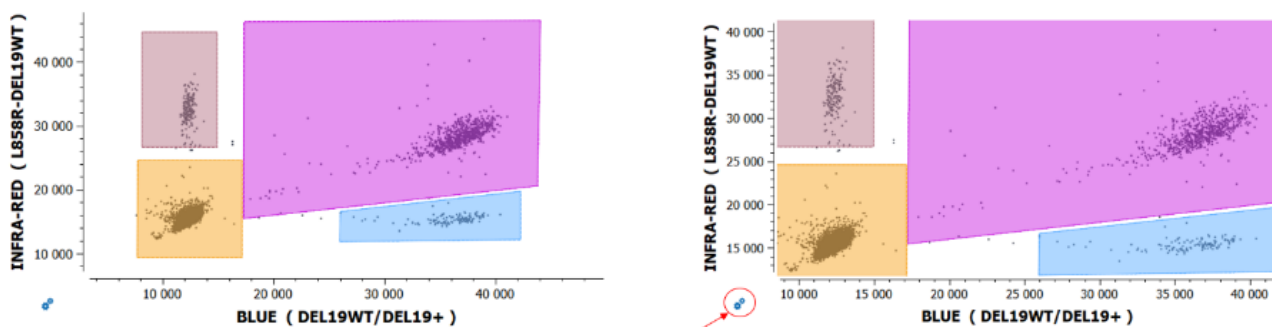


Figure 4: The Crystal Miner software 2D dot plot for data visualization and analysis.

- Check or manually adjust the position of the polygons for each target population listed in the “Populations” widget for the **positive control** of the run (refer to the example in Figure 5 below).
 - To translate a polygon, left-click on an edge of the polygon and drag and drop it, then click on the “Apply” button to confirm.
 - To move the vertex of a polygon, left-click on the vertex of the polygon and drag and drop it, then click on the “Apply” button to confirm.
 - To cancel polygon adjustment, click on the “Cancel” button.

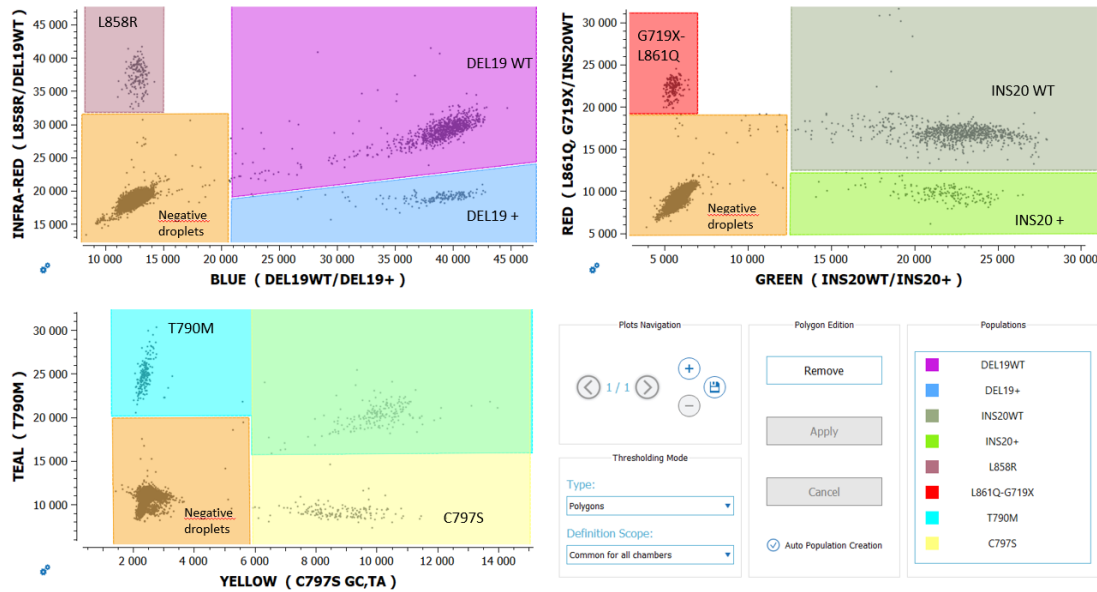


Figure 5: An example illustrating correct placement of the polygons on the positive control in the Crystal Miner software.

- In the thresholding mode, select “individual per chamber” for the definition scope.
- Check or manually adjust the position of the polygons for each target population listed in the “Populations” widget for each sample. Polygons should now be correctly placed if the previous step is followed.
- In case of **rare events** (<10 positive droplets) for a population of interest, confirm in the “Explore Crystals” submenu that each droplet classified as positive inside a polygon is a real positive droplet. If not, exclude it (Please refer to Crystal Miner software User Manual for how to exclude droplets).
- Go to “FILE > Save” to save the experiment in “.ncr” format (under the same name as the experiment name).
- Go to “VIEW RESULTS > Result Table” to access quantification results for each target population.
- Optional: Go to the “EXPORT” menu and click on the “Browse” button to select the output folder, then click on the “Export” button to export the data in the “.xlsx” format (under the same name as the experiment name).

Analytical performances:

The Limit of Blank (LoB) and Limit of Detection (LoD) were determined following the Clinical and Laboratory Standards Institute (CLSI) EP17-A2 standard recommendations (*Protocols For Determination Of Limits Of Detection And Limits Of Quantitation; Approved Guideline*).

Table 6: EGFR 6-color Crystal Digital PCR™ kit Limit of Blank (LOB) and Limit of Detection (LOD) in one Sapphire chamber (copies/μL).

Target	LOB	LOD
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

This table indicates the LOB/LOD concentrations in one Sapphire chamber.

To be able to compare them to your sample concentrations, multiply these LOB/LOD values by the sample dilution factor previously calculated - DNA sample volume divided by 26μL (total reaction volume).

Interpretation of the sample results:

In the excel export file, in the “Results” tab, look at the total droplet number (“Nb Droplets”) and target concentration columns.

Table 7: Decision table

Target X concentration value	Interpretation
$C[X] \geq LOD$	Sample mutated for X
$LOB < C[X] < LOD$	Undetermined*
$C[X] \leq LOB$	Sample non-mutated for X

*For undetermined results, Stilla Technologies advises to retest the sample in a new run and, if possible, increase the tested volume of the sample.

VAF (Variant Allele Frequency) Calculation:

$C[X]$ = target X concentration (copies/μL)

$C[DNA_{tot}]$ = Concentration of total DNA in the sample

$$= (C[Del19WT] + C[Del19+] + C[Ins20WT] + C[Ins20+]) / 2$$

$$X \text{ VAF} = C[X] * 100 / C[DNA_{tot}]$$

Run data is validated if:

- Total number of droplets in each chamber is > 12,900 droplets
- Negative control: Concentrations of all targets is ≤ LOB
- Positive control: Concentration of all targets must be within the range of concentrations defined in the CoC (Certificate of Compliance).
- Measured amount of total DNA in a reaction (= C[DNA_{tot}] x initial volume of sample) is less than 40 ng (~13.000 copies).

If measured amount of DNA_{tot} > 40ng, results are non-interpretable. Perform a new run with a decreased initial volume of sample. The concentrations measured in the first run should be used to adapt the volume of sample to be loaded in the reaction mix.

Quality Control

Each batch of *EGFR* 6-color Crystal Digital PCR™ kits is functionally tested against a reference batch.

Precautions and Warnings

The reagents included in the *EGFR* 6-color Crystal Digital PCR™ kit are not classified as dangerous substances according to Regulation (EC) No. 1272/2008 [CLP].

Appropriate personal protection equipment for handling this product, including lab coat, disposable gloves, and safety goggles, is required. Wear additional personal protection equipment when needed. Wash hands before breaks and after work. Remove contaminated, saturated clothing.

In case of exposure:

General information: when in doubt or if symptoms are observed, get medical advice.

Following inhalation: no special measures are necessary. Provide fresh air.

Following skin contact: wash with soap and water.

Following eye contact: in case of eye irritation consult an ophthalmologist. Rinse immediately, carefully, and thoroughly with eyebath or water.

Following ingestion: if swallowed: Rinse mouth. Do NOT induce vomiting.

Self-protection of the first aider: no special measures are necessary.

For further information, please refer to the material safety data sheet available on the technical resources webpage.

Disposal Considerations

Waste can be considered as a biohazardous waste and must be disposed of according to applicable domestic legislation.

For recycling, please contact the manufacturer.

Technical Support Contact Information



Stilla Technologies

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For help and technical advice, please contact the Technical Support Department at Stilla Technologies.

For European Customers:

Phone: (+33) 09 82 27 47 47

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For North American Customers:

Phone: 1-833-888-0150 ext.1

Email: support@stilla-inc.com

Online Technical Support is also available at:

<https://www.stillatechnologies.com/technical-support/>

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