

EGFR 6-color Crystal Digital PCR® Kit [6-color naica® system]

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 (Sapphire Chip)

Samples: cell-free DNA and FFPE genomic 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) on the 6-color naica® system with the Sapphire Chip.

The *EGFR* 6-color Crystal Digital PCR® Kit is compatible with cell-free DNA (cfDNA) extracted from plasma and genomic DNA isolated from FFPE tissue sections.

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.**
- Compatible with Sapphire Chip consumables on the 6-color naica® system.

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.2319-2320insAAACCCCAC	COSM18491	
	Exon 21	p.L858R	c.2573T>G	COSM6224
		p.L861Q	c.2582T>A	COSM6213

The *EGFR* 6-color Crystal Digital PCR® Kit enables multiplexed DNA analysis on the naica® system. It 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 (refer to technical note “[How to quantify Drop-Off digital PCR Assays with Crystal Miner](#)”).

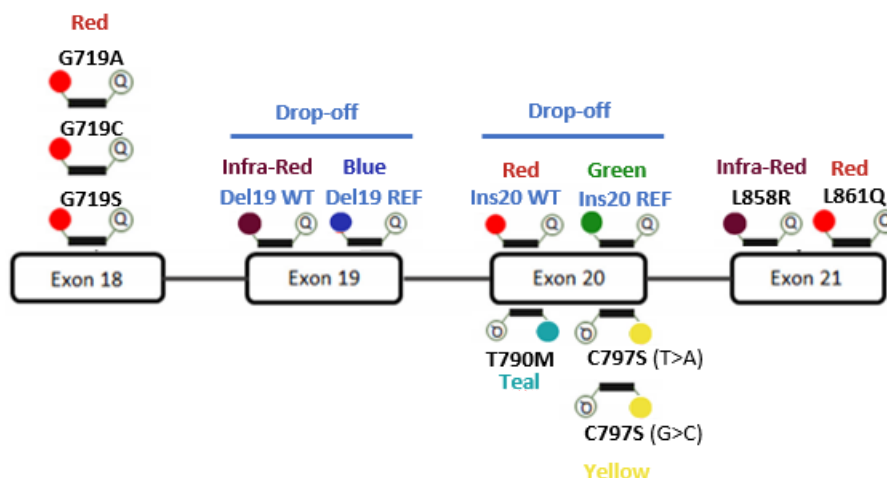


Figure 1. *EGFR* 6-color Crystal Digital PCR® Kit detection design.

Resources

Documents referenced in this Instructions for Use (IFU) can be accessed here:

<https://www.stillatechnologies.com/digital-pcr/naica-system-support/technical-resources/>

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

Partitioning and PCR step:

Download the **PCR Program** of *EGFR* 6-color Crystal Digital PCR® Kit 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 Crystal Reader **Scanning Template** of *EGFR* 6-color Crystal Digital PCR® Kit from the technical resources section on the 6-color naica® system. Crystal Reader software and Crystal Miner software version 4.0 is required for image acquisition and result analysis of the *EGFR* 6-color Crystal Digital PCR® Kit.

Interpretation of sample results step:

Request the **Certificate of Compliance** (CoC) of the *EGFR* 6-color Crystal Digital PCR® Kit 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 is packaged in a sealed box containing a total of six tubes:

Component Name	Cap Color	Initial Concentration	Volume	Description
Buffer A	Blue ●	10X	140 µL	naica® multiplex PCR MIX Buffer A
Buffer B	Red ●	50X	50 µL	naica® multiplex PCR MIX Buffer B
Buffer C	Yellow ●	8X	175 µL	Ready to use primers and TaqMan® probes
Positive Control	Purple ●	5X	100 µL	Synthetic mutated DNA solution (concentrations defined in CoC)
Negative Control	White ○	5X	100 µL	Nuclease-free water for no template control chambers, referred in the assay as “Neg Ctl”.
Nuclease-Free Water	Clear	N/A*	1000 µL	Nuclease-free water for volume adjustment



*N/A: not applicable

Materials Required but Not Provided

- 6-color naica® system
- Standard consumables and equipment for PCR reaction mix preparation:
 - PCR-compliant reaction tubes
 - Centrifuge for microcentrifuge tubes (~700xg)
 - Laboratory mixer - Vortex
 - Micropipettes (from 1 to 200 µL)
 - Micropipette tips
- Sapphire Chip consumables (Reference C14012).
- Antistatic wetted wipes (ACL Staticide®, Reference: SW12).
→ The specific product can be ordered from Stilla® as a spare part (Part number H10000.472) or directly from the supplier Digi-Key using the reference ST1059-ND. [SW12 ACL Staticide Inc | Anti-Static, ESD, Clean Room Products | DigiKey.](#)
- Precision Wipes (Kimtech™ Science, Reference: 7552, 1 ply, 213x114 mm) can be ordered from standard laboratory suppliers.


Storage

Immediately upon reception, inspect package integrity and ensure the correct storage of the *EGFR* 6-color Crystal Digital PCR® Kit at the indicated storage temperatures. In case of doubt on the integrity or correct storage of the *EGFR* 6-color Crystal Digital PCR® Kit upon reception, do not use the kit and contact Technical Support.

- All components of the *EGFR* 6-color Crystal Digital PCR® Kit must be stored at $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ in the original tubes until the expiration date indicated on the respective packaging. Do not aliquot in alternative tubes.
-  Store tubes protected from light sources. It is recommended to store all tubes in the provided cardboard box.
-  Store tubes at all times in an upright position. It is recommended to store all tubes in the provided cardboard box.
- The *EGFR* 6-color Crystal Digital PCR® Kit can be thawed up to 10 times without observable deviations in performance and shelf-life.
- General consideration for reagent storage: all tube caps should be well-closed before stocking.

Under these conditions, the *EGFR* 6-color Crystal Digital PCR® Kit is stable until the expiration date indicated on the external packaging label.

Conditions for Use

- Operate the *EGFR* 6-color Crystal Digital PCR® Kit at a temperature ranging from $+20^{\circ}\text{C}$ to $+25^{\circ}\text{C}$.
- Ensure to thaw completely each *EGFR* 6-color Crystal Digital PCR® Kit reagent prior to reaction mix preparation.
-  Before each use, vortex each component thoroughly at maximum speed for 10 seconds and briefly centrifuge to collect the liquid at the bottom of the tube.
- Discard all *EGFR* 6-color Crystal Digital PCR® Kit reagent components as soon as one reagent component is empty.
- Discard all *EGFR* 6-color Crystal Digital PCR® Kit reagent components as soon as one reagent component is expired. The expiration date of the limiting component is indicated on the external packaging.
- Never combine *EGFR* 6-color Crystal Digital PCR® Kit reagent components from different *EGFR* 6-color Crystal Digital PCR® Kit boxes.

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.

It is recommended to perform a full run of three Sapphire Chip 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).

*Note: Do not perform an overnight run with the *EGFR* 6-color Crystal Digital PCR® Kit.*

Step 1. Reaction Preparation

1. Thaw completely each *EGFR* 6-color Crystal Digital PCR® Kit reagent (equilibrate to room temperature), vortex all tubes, and spin briefly in a mini-spin centrifuge to collect the material at the bottom of each tube.
2. Assemble the reagents as shown in Table 2. Start by preparing the reaction mix for **X+1** reactions of Buffer A, Buffer B, and Buffer C (X = n samples + 1 negative control + 1 positive control)

Table 2: *EGFR* 6-color Crystal Digital PCR® Kit reaction mix assembly per reaction

Component 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>Buffer A, Buffer B, and Buffer C volume (µL)</i>			6.4
○ <i>diluted clinical sample or</i> ● <i>diluted positive control or</i> ○ <i>diluted negative control</i>			19.6
<i>Total reaction volume (µL)</i>			26.0

3. Vortex and spin the tube containing the reaction mix including Buffers A, B and C.
4. Split the content of this reaction mix into **X** tubes of 6.4 µL each.
5. Label tubes Neg Ctl, Pos Ctl and Samples (1 to 10 DNA 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.
 - Add 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.
 - 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:

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.

 - 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.
 - Add additionally 14.4 µL of the provided nuclease-free water.
6. Vortex each tube thoroughly to mix all reagents and briefly centrifuge to collect the content at the bottom of the tube.

7. Load 25 μL of the reaction mix into 1 chamber of the Sapphire Chip (refer to the Sapphire Chip IFU for instructions to load a Sapphire Chip). Recommended chip layout plan shown in Table 4.

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. 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.

Step 2. Partitioning and Amplification

Estimated duration of the EGFR 6-color Crystal Digital PCR® Kit Geode program: 2 hours and 30 min.

1. Place the Sapphire Chip consumables in the Geode and close the lid.
2. Select the EGFR 6-color Crystal Digital PCR® Kit **PCR Program** from the main menu of the Geode screen and press “Play” to start the program (refer to the Geode User Manual for instructions on Geode use).

**Table 5: EGFR 6-color Crystal Digital PCR® Kit PCR program
“PartitioningProgram_Geode_SapphireChip_EGFR-6color-Kit_V1.js”**

Step	Temperature (°C)		Duration
Partitioning & Enzyme activation	40°C		Sapphire partitioning
	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
Release	N/A		Sapphire release

3. 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 (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.

8. Click on “Open tray”, place the Sapphire Chip consumables in the Prism6 instrument and click on “Close the tray”.
9. Click on “Save” and save the “.ncx” file in the desired folder.
10. Click on “Scan”.
11. Once a chamber is scanned, a quality flag appears in the chamber rectangle (refer to Prism6 and Crystal Reader software User Manual for detailed instructions for checking image quality).
12. After the scan, dispose the Sapphire Chip consumables as per recommendations.

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

Step 4. Data Analysis with Crystal Miner

1. Open the “.ncx” file generated by Crystal Reader in the Crystal Miner software after the scanning step by double-clicking the file.
2. Go to the menu “ANALYZE DATA > Plots & Populations > 2D dot plot”. To adjust the axis, double click on the graph or click on the blue gear below the 2D graph (indicated by the red arrow in Figure 4 below) to manually enter the desired value.

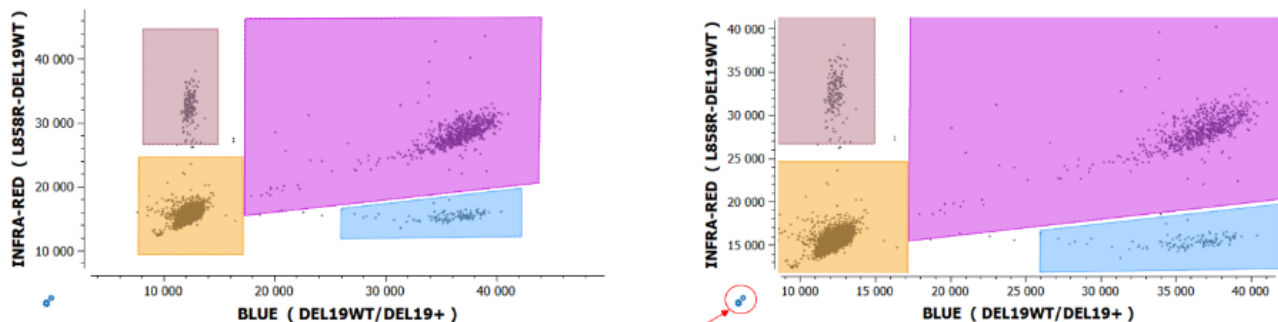


Figure 4: Crystal Miner software 2D dot plot for data visualization and analysis.

3. 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.

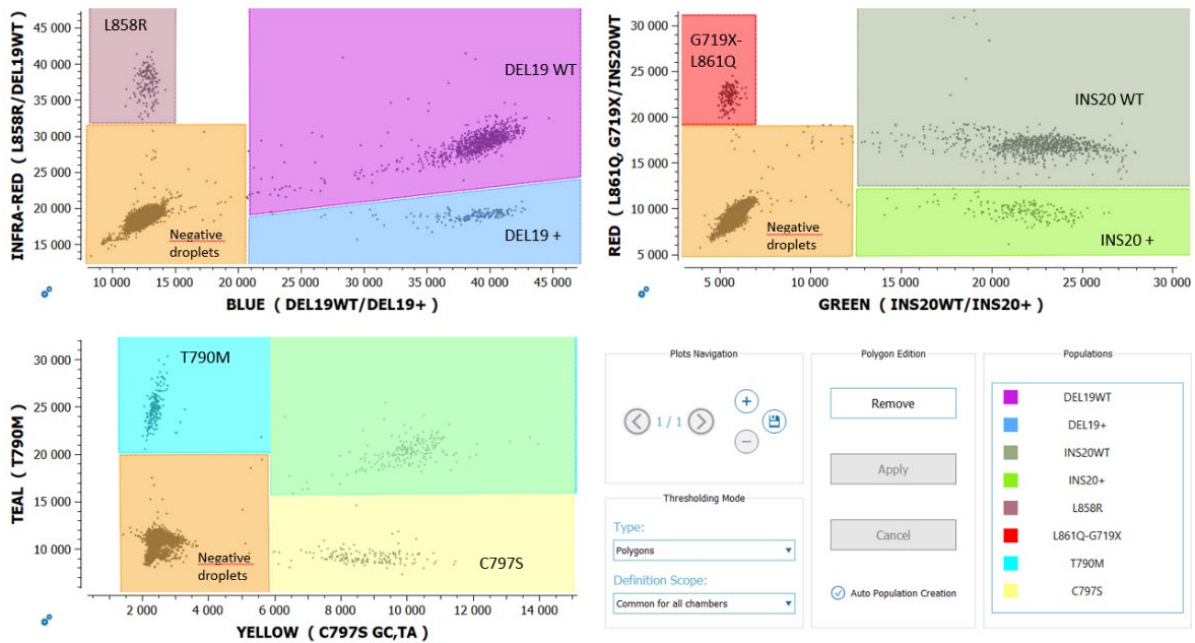


Figure 5: An example illustrating correct placement of the polygons on the positive control in the Crystal Miner software.

4. In the thresholding mode, select “individual per chamber” for the definition scope.
5. 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.
6. 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 (refer to Crystal Miner software User Manual for how to exclude droplets).
7. Go to “FILE > Save” to save the experiment in “.ncr” format (under the same name as the experiment name).
8. Go to “VIEW RESULTS > Result Table” to access quantification results for each target population.
9. 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).

Note: The concentrations exported from Crystal Miner are provided in copies per microliter for the given chamber. To calculate the amount of DNA in copies per microliter in the sample, multiply the exported concentration by the dilution factor.

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

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

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*).

cfDNA samples

Table 6 indicates the LOB/LOD concentrations in one Sapphire chamber for cfDNA samples in copier/ μ L. To compare them to the sample concentrations, multiply the LOB/LOD values by the sample dilution factor previously calculated, DNA sample volume divided by 26 μ L (total reaction volume).

Table 6: EGFR 6-color Crystal Digital PCR® Kit Limit of Blank (LOB) and Limit of Detection (LOD) in one Sapphire chamber (copies/ μ L) for cfDNA samples

Target	LOB	LOD
DEL19+	0.21	0.51
L858R	0.11	0.52
INS20+	0.31	0.77
L861Q, G719X	0.46	0.86
T790M	0.31	0.65
C797S	0.08	0.34

FFPE samples

Table 7 indicates the LOB/LOD concentrations in one Sapphire chamber for FFPE samples in mutant allele frequency (MAF, %).

Table 7: EGFR 6-color Crystal Digital PCR® Kit Limit of Blank (LOB) and Limit of Detection (LOD) in one Sapphire chamber (MAF %) for FFPE samples

Target	LOB	LOD
DEL19+	1.5	2.0
L858R	1.5	2.0
INS20+	1.5	2.0
L861Q, G719X	1.5	2.0
T790M	1.5	2.0
C797S	1.5	2.0

Step 5. Interpretation of Sample Results

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

Table 8: 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.

Mutant Allele Frequency (MAF) Calculation

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

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

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

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

Run Data Validation

The run data with the *EGFR* 6-color Crystal Digital PCR® Kit is validated if:

1. Total number of droplets in each chamber is > 12900 droplets
2. Negative control: Concentrations of all targets is \leq LOB
3. Positive control: Concentration of all targets must be within the range of concentrations defined in the CoC (Certificate of Compliance).
4. 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® Kit is functionally tested on the 6-color naica® system and must perform within the established specifications. The Certificate of Compliance is available upon request from the Technical Support Team.

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 national or domestic legislation.

For recycling of cardboard packaging, please consult local or national regulations.

Technical Support Contact Information

Online Technical Support is available at: <https://www.stillatechnologies.com/technical-support/>

For technical questions or any issue regarding the *EGFR* 6-color Crystal Digital PCR® Kit, contact us:

Monday to Friday, 9:30 AM – 6:30 PM, Central European Time (CET).

Closed on French bank holidays.

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Patents: <https://www.stillatechnologies.com/patents/>

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