An ultrasensitive high-plex assay detecting 24 *PIK3CA* mutations using SAGAsafe® technology and 6-color naica® system Crystal Digital PCR™

Miguel ALCAIDE[1], Anthony M GEORGE[1], Yilun CHEN[1], Lao H. SAAL[1*]

[1]SAGA Diagnostics AB, Lund, Sweden

INTRODUCTION

Liquid biopsies, such as blood samples, harbor a wealth of genetic information and they are safe, straightforward to implement, inexpensive, and minimally invasive. Recently, much interest has focused on how the blood can reveal information about cancer, as it can contain circulating tumor DNA (ctDNA) that can be used to inform disease diagnosis, guide therapeutic actions, monitor treatment effectiveness, and quickly detect disease relapse. Starting from a minimally invasive liquid biopsy sample such as a blood sample, one can determine genetic and biological properties of a cancer by quantifying ctDNA. ctDNA has become a popular surrogate for tissue DNA, and advances in its detection and characterization have launched liquid biopsies into the clinic.

The technical challenge of ctDNA however is its scarcity and low titer. Also, measurements require a highly sensitive and precise detection technology to quantify often low-level genetic aberrations within a high background of wild-type DNA. A limitation of traditional PCR approaches, in this context, is the amplification of polymerase copying errors as well as errors from common types of DNA damage. To overcome these limitations, this application note investigated a two-pronged approach, combining high-fidelity SAGAsafe® assay technology and Crystal Digital PCR™ micro-partitioning, to multiplex 24 *PIK3CA* mutations and a wild-type *PIK3CA* reference DNA in a single reaction on the 6-color naica® system. *PIK3CA* encodes the p110-alpha kinase, the target of a recently FDA-approved drug, alpelisib, for metastatic breast cancer. Therefore, knowing the mutation status of *PIK3CA* through ctDNA can guide therapy.

Superb *PIK3CA* mutation detection, specificity and sensitivity, with Limits of Blank (LoB) ranging from 0.003% to <0.001% Mutant Allele Frequency (MAF) and Limits of Detection (LoD) ranging from 0.009% to 0.002% MAF, in a high background of wild-type DNA were observed. Moreover, a high concordance (R^2 > 0.98) was observed between Crystal Digital PCR™ *PIK3CA* SAGAsafe® results and next generation sequencing data on breast cancer biopsies.

Conclusion: by combining the power of Crystal Digital PCR™ and the 6-color naica® system with the high-fidelity SAGAsafe® technology, ultrahigh sensitivity can be maintained even in a complex multiplex oncology assay. This proof-of-concept sets the stage for the future development of ultrasensitive, highly multiplexed cancer detection panels to achieve better patient stratification and monitoring during therapy.

SAGAsafe® Chemistry

SAGA Diagnostics AB is a personalized cancer diagnostics and disease monitoring company focused on molecular genetic analyses of ctDNA with a mission to improve precision cancer medicine, provide more accurate treatment monitoring, and improve patient survival through minimally invasive liquid biopsy cancer testing. SAGAsafe® technology detects and quantifies mutations with unique ultrasensitivity down to 0.001% MAF. SAGAsafe® technology is a patented all-in-one assay technology consisting of two reaction phases: Phase 1 – linear copying of the target strands; Phase 2 – exponential signal generation, allowing the quantification of both the ssDNA and dsDNA fractions. Application of SAGAsafe® greatly reduces sources of detection noise coming from the DNA polymerase itself or from common sources of DNA damage occurring during sample handling and processing. Thereby lowering false positives one hundred-fold and greatly improving limits of detection, particularly in multiplexed assays (**Figure 1**).

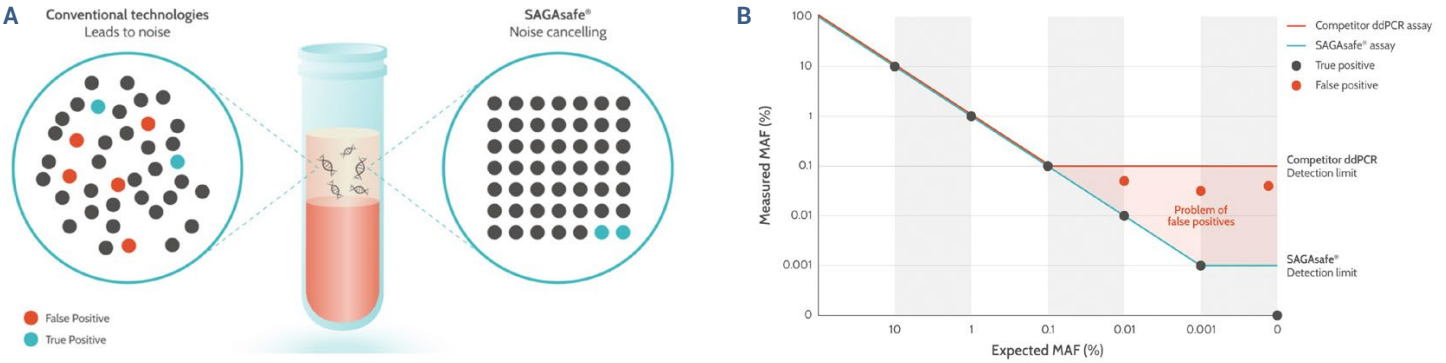

Figure 1 | **SAGAsafe® technology**. **A.** SAGAsafe® chemistry enables superior sensitivity by greatly reducing detection background noise and the risk of false results. **B.** The ultrasensitivity of SAGAsafe® enables accurate detection and quantification of mutant alleles down to frequency of 0.001%.

CRYSTAL DIGITAL PCR™ ON THE 6-COLOR NAICA® SYSTEM

In 2021, Stilla Technologies commercialized the first 6-color digital PCR platform, the naica® system, marking a milestone in digital PCR technology innovation. The 6-color Crystal Digital PCR™ workflow (**Figure 2**) enables a high multiplexing capacity in a single reaction saving both time and precious sample and providing ultrasensitive low-level detection of multiple reactions in parallel. By partitioning sample reactions into a large 2D array of droplets through a confinement gradient, homogeneity in the droplet size is ensured, and the need for oil flow is eliminated. Crystal Digital PCR™ technology can be used for absolute nucleic acid quantification in a wide range of assays including, but not limited to oncology (copy number variation, mutation detection, rare event detection, therapeutic monitoring). Crystal Miner Software measures the concentrations of targeted nucleic acids, providing automatic identification of positive and negative droplets for all fluorescence channels and intuitive image analysis.

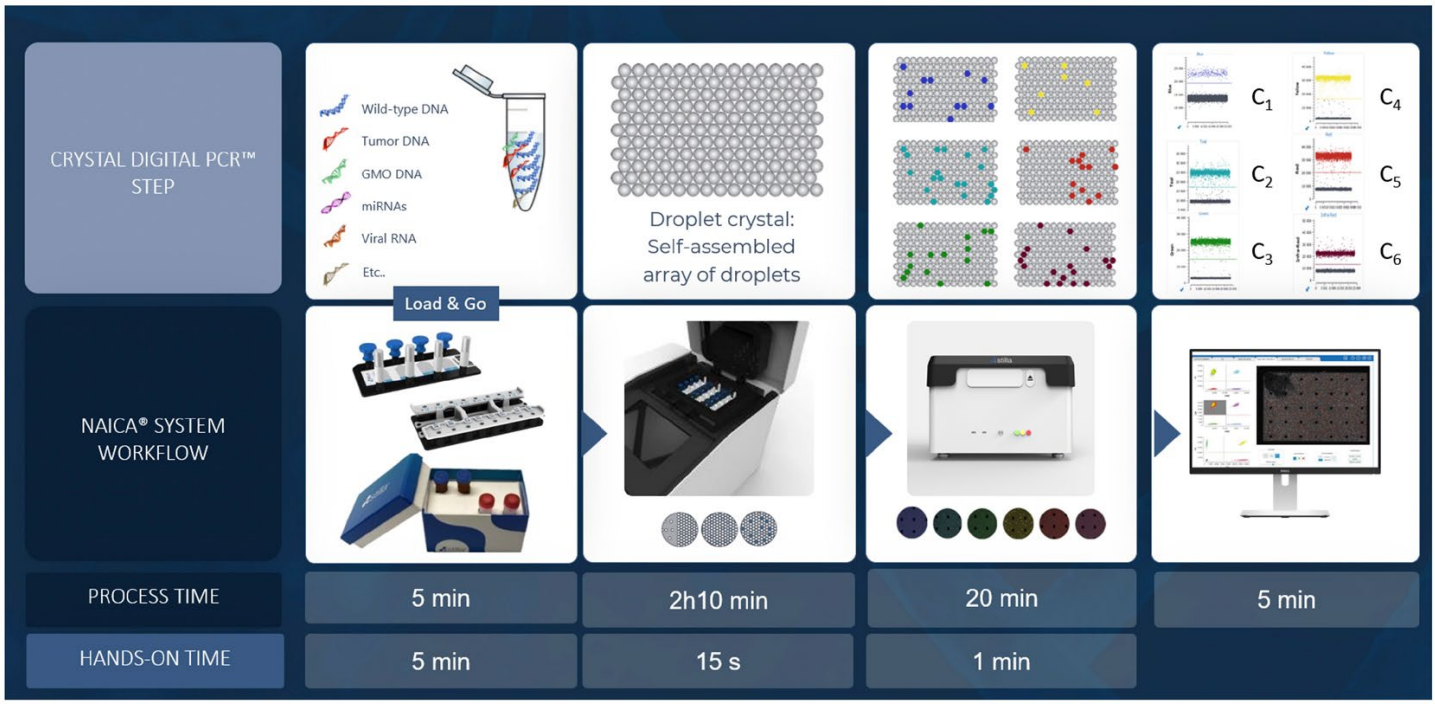

Figure 2 | **The 6-color naica® system: absolute quantification of multiple genetic targets in a single run**. The 6-color naica® system is an easy-to-use digital PCR platform whose cutting-edge microfluidic technology automatically integrates the digital PCR workflow into a ready-to-use single consumable chip. With no moving parts, Crystal Digital PCR™ partitions the sample into a 2D array of thousands of individual droplet reaction compartments that amplify nucleic acid molecules. These reactions are tagged with fluorophores to be read using up to six different fluorescence light channels that can be combined for multiplex detection of dozens of individual target mutations. The 6-color naica® system makes for a fast and simple workflow that can be completed with less than 10 minutes of hands-on time.

RESULTS

All clinical samples are precious, and thus technologies that maximize data output and provide ultrasensitive detection are vital. To maximize the quantity and quality of information obtained from a single assay, the power of SAGAsafe® technology with that of the 6-color naica® system Crystal Digital PCR™ was combined. The resulting 24-plex *PIK3CA* proof-of-concept breast cancer assay detects 24 of the most common oncogenic *PIK3CA* mutations, including hotspot variants such as E542K, E545K, and H1047R (**Table 1**).

Table 1 | **List of the 24 *PIK3CA* mutations**, including their corresponding nucleotide and amino acid alterations, legacy mutation (COSMIC) identifiers and respective 6-color naica® system detection channel.

Mutation #	*PIK3CA* Exon	CDS mutation	AA mutation	Legacy Mutation ID	6-color naica® system detection channel(s)
1	2	c.353G>A	p.G118D	COSM751	GREEN, TEAL
2	4	c. 1030G >A	p.V344M	COSM253279	BLUE
3	4	c.1035T>A	p.N345K	COSM754	YELLOW
4	7	c.1258T>C	p.C420R	COSM757	GREEN
5	9	c.1616C>G	p.P539R	COSM759	BLUE
6	9	c.1624G>A	p.E542K	COSM760	YELLOW
7	9	c.1633G>A	p.E545K	COSM763	INFRARED
8	9	c.1633G>C	p.E545Q	COSM27133	BLUE, INFRARED
9	9	c.1634A>C	p.E545A	COSM12458	GREEN
10	9	c.1634A>G	p.E545G	COSM764	BLUE
11	9	c.1635G>C	p.E545D	COSM27374	BLUE
12	9	c.1636C>A	p.Q546K	COSM766	BLUE
13	9	c.1636C>G	p.Q546E	COSM6147	TEAL
14	9	c.1637A>C	p.Q546P	COSM767	BLUE
15	9	c.1637A>G	p.Q546R	COSM12459	BLUE
16	13	c.2176G>A	p.E726K	COSM87306	YELLOW, BLUE
17	20	c.3132T>A	p.N1044K	COSM12592	TEAL
18	20	c.3132T>G	p.N1044K	COSM27504	TEAL
19	20	c.3139C>A	p.H1047N	COSM5029128	TEAL
20	20	c.3139C>T	p.H1047Y	COSM774	TEAL
21	20	c.3140A>G	p.H1047R	COSM775	BLUE, INFRARED
22	20	c.3140A>T	p.H1047L	COSM776	INFRARED
23	20	c.3141T>A	p.H1047Q	COSM1041524	TEAL
24	20	c.3145G>C	p.G1049R	COSM12597	TEAL
-	9	Wildytpe	Wildytpe	Not applicable	RED

The 24-plex SAGAsafe® Crystal Digital PCR™ assay was tested on nine breast cancer patient samples for which next-generation sequencing data was also available. The two technologies showed excellent concordance with R^2 values of 0.992 (**Figure 3**).

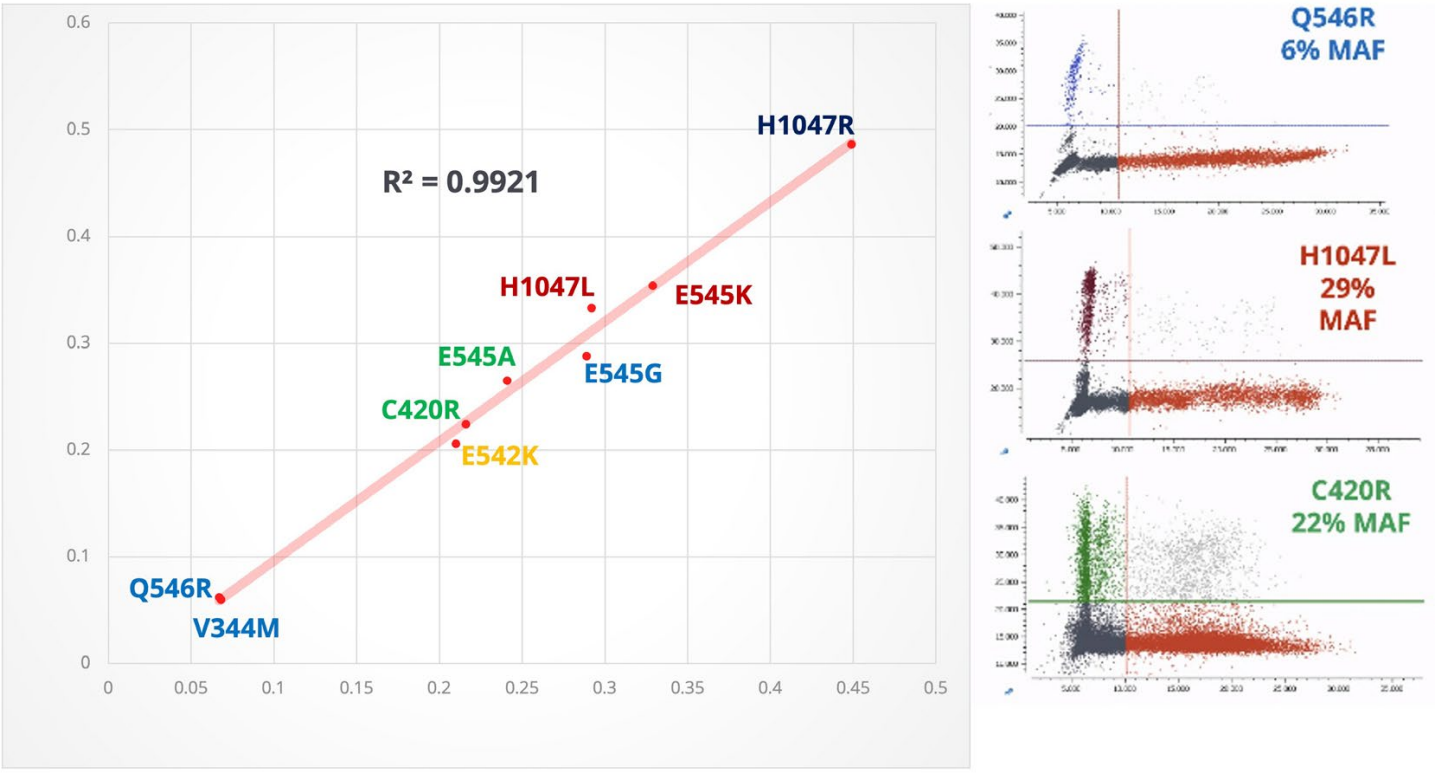

Figure 3 | The 24-plex SAGAsafe® Crystal Digital PCR™ assay. A) Nine breast cancer biopsies previously analyzed by next generation sequencing were analyzed with the 24-plex SAGAsafe® assay using Crystal Digital PCR™ and the Sapphire Chip on the 6-color naica® system. The corresponding obtained average MAFs were plotted for each sample: X axis SAGAsafe® Crystal Digital PCR™; Y axis, Illumina NGS. B): Representative 2D dot-plots, generated by Crystal Miner software, plotting the fluorescence intensities of the *PIK3CA* mutations Q546R, H1047L and C420R detected in the blue, infrared and green color channels, respectively versus wild-type DNA fluorescence intensities detected in the red channel. The thresholds demarcating the positive and negative clusters for each color channel are set just above the ssDNA cluster that is expected with SAGAsafe® assay using Crystal Digital PCR™.To evaluate the capacity of the 24-plex SAGAsafe® Crystal Digital PCR™ assay to perform absolute quantification, serial dilutions of mutant alleles in the presence of a constant amount of wild-type background DNA were made. Analysis of this dilution series revealed accurate linear quantification to below 0.01% MAF with coefficient of determination scores of $R^2 > 0.98$ and $R^2 > 0.99$ depending on the *PIK3CA* mutant target (**Figure 4**).

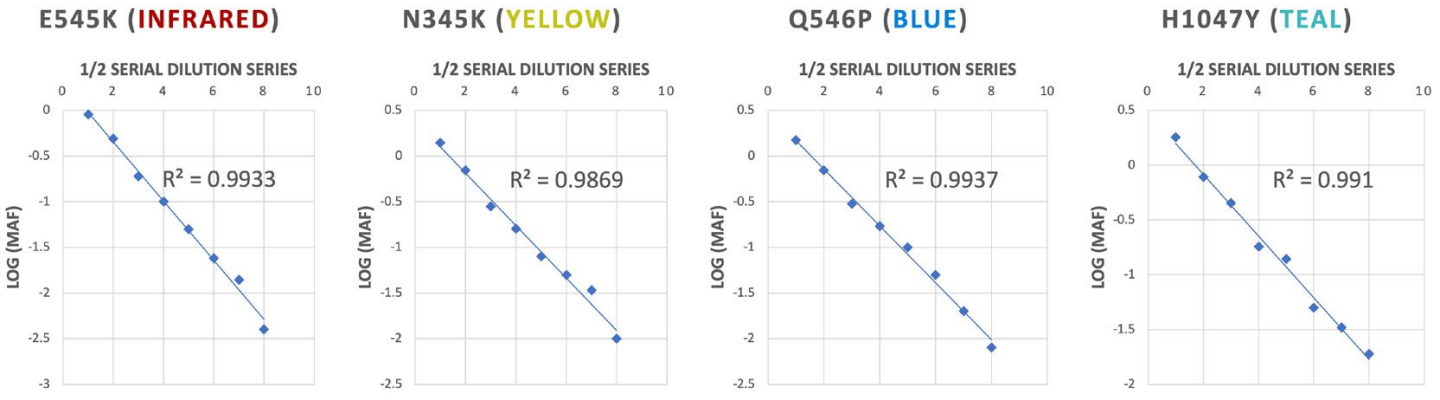

Figure 4 | Highly accurate quantitation. Serial dilution curves of the *PIK3CA* mutations E545K, N345K, Q546P and H1047Y detected by Crystal Digital PCR on the 6-color naica® system. Wild-type copies were maintained at 65k genome copies per 25µL **Crystal Digital PCR™** reaction for each 1/2 serial dilution step. Measurements were performed in duplicate.

Upon analyses of pure wild-type (non-mutant) control DNA, with the 24-plex *PIK3CA* SAGAsafe® Crystal Digital PCR™ assay on the 6-color naica® system, no false positive mutant signals were detected (**Figure 5**), demonstrating the ultra-specific nature of the workflow.

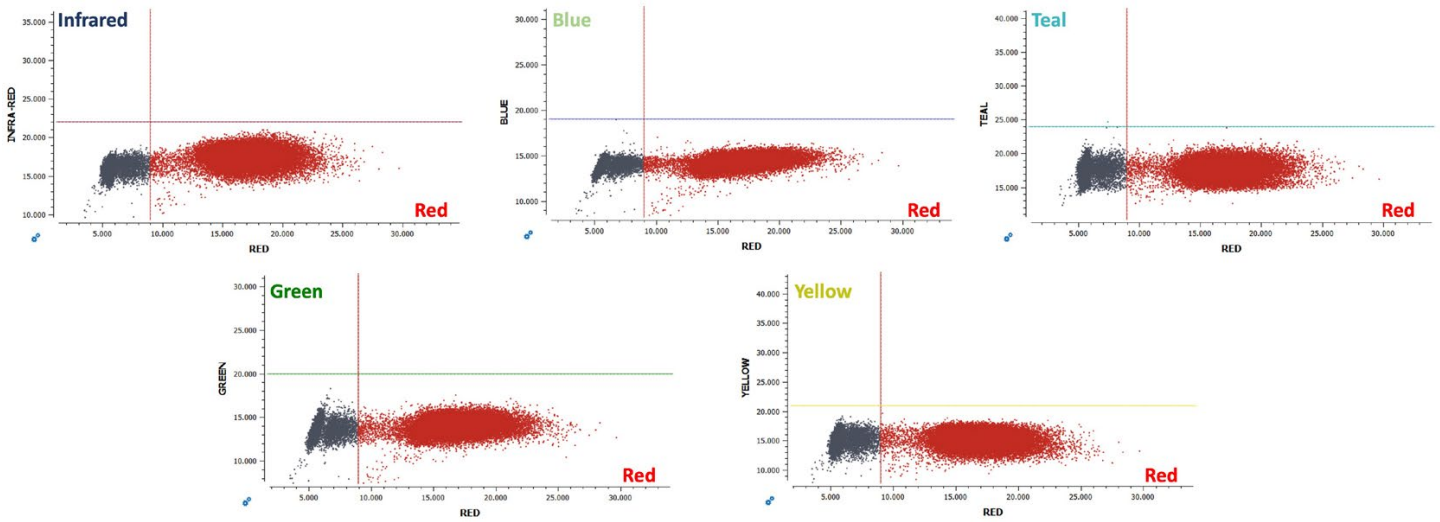

Figure 5 | The ultrasensitive 24-plex *PIK3CA* Crystal Digital PCR™ detection assay. 2D dot-plots of fluorescence intensities generated by Crystal Miner software showing clean fluorescent signals in each of the five *PIK3CA* mutant-dedicated detection channels in a background of pure wild-type control DNA loaded at 65k copies and detected in the Red channel of the 6-color naica® system.

The combined specificity and sensitivity of the SAGAsafe® Crystal Digital PCR™ workflow led to ultralow LoBs, between 0.001% and 0.003%, and mutant allele frequencies (MAFs) and ultrasensitive LoDs between 0.002% to 0.009% (**Table 2**), depending on the mutation and fluorescence channel.

Table 2 | LoB and LoD values of the corresponding *PIK3CA* mutations were calculated from 25 replicates using 65k wild-type genome copies in each replicate.

Channel	PIK3CA mutations	Total wild-type genome copies evaluated	Estimated Limit of Blank (MAF)	Estimated Limit of Detection (MAF)
Blue	Q546 (3), E545(3), V344M, P539R, H1047R	1,368,500	0.003%	0.009%
Teal	Q546E, N1044K(2), G1049R, H1047Q/N/Y	1,498,500	0.003%	0.009%
Green	G118D, C420R, E545A	1,303,500	0.002%	0.006%
Yellow	N345K, E542K, E726K	1,498,500	<0.001%	0.002%
Infrared	E545K, H1047L	1,238,500	0.002%	0.006%
Red	Wild-type exon 9	Many millions	Not applicable	Not applicable

Application Note Highlights

- SAGAsafe® technology is fully compatible with Crystal Digital PCR™ on the naica® system.
- The combination of SAGAsafe® technology and Crystal Digital PCR on the 6-color naica® system achieves superb sensitivity, even in the context of a complex 24-plex assay.
- The SAGAsafe® Crystal Digital PCR™ workflow enables ultra-high multiplex target detection.

Stilla Technologies provides a full set of comprehensive Application Notes and Technical Notes to support the Crystal Digital PCR™ workflow. In case further support is needed, contact sales@stillatechnologies.com

For more information about SAGAsafe® technology, visit SAGA Diagnostics at https://sagadiagnostics.com/