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

#534

Miguel ALCAIDE[1], Anthony M GEORGE[1], Yilun CHEN[1], Cécile JOVELET[2], Sahbi BEN KALANI[2], Lao H. SAAL[1*], Allison C. MALLORY[2*]

[1]SAGA Diagnostics AB, Lund, Sweden; [2]Stilla Technologies, Villejuif, France and Beverly, MA USA; *equal contribution

Abstract Starting from a minimally invasive liquid biopsy sample such as a blood sample, one can determine genetic and biological properties of a cancer by measuring circulating tumor DNA (ctDNA). ctDNA measurements require a highly sensitive and reliable detection technology to quantify often low-level genetic aberrations within a high background of wild-type DNA. The aim of this study was to demonstrate the performance of the patented SAGAsafe® digital PCR chemistry on the 6-color Crystal Digital PCR™ naica® system, by adapting a SAGAsafe® assay for a clinically relevant oncogene, *PIK3CA*. *PIK3CA* encodes the p110-alpha kinase and is the target of a recently approved drug, alpelisib, for metastatic breast cancer. By combining the power of the SAGAsafe® technology with that of 6-color Crystal Digital PCR™, we present a highly multiplexed, ultrasensitive proof-of-concept *PIK3CA* detection assay capable of detecting and quantifying 24 *PIK3CA* mutations in a single digital PCR reaction. We evaluated the sensitivity and performance of the highplex 6-color *PIK3CA* digital PCR assay, and even with its complexity targeting many mutations, the SAGAsafe® assay generates clean, high-quality data. In addition, superb *PIK3CA* mutation detection specificity and sensitivity were observed with Limits of Blank ranging from 0.003% to <0.001% MAF, and Limits of Detection (LoD) ranging from of 0.009% to 0.002% MAF in a high background of wild-type DNA. Moreover, a high concordance was observed between Crystal Digital PCR™ *PIK3CA* results and NGS data on breast cancer biopsies. By combining the power of 6-color Crystal Digital PCR™ with the ultrasensitive SAGAsafe® technology, ultrahigh sensitivity can be maintained even in a complex multiplex dPCR 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.

Introduction

Liquid biopsies, such as blood samples, harbor a wealth of genetic information in the form of circulating tumor DNA that can be used to inform disease diagnosis and guide therapeutic actions, monitor treatment effectiveness and early detection of disease relapse. Liquid biopsies are safe, straightforward to implement, inexpensive, and minimally invasive. The use of ctDNA as a surrogate for tissue DNA has become extremely popular, and advances in the detection and characterization of ctDNA have enabled liquid biopsy assay integration into clinical practice. However, due to their general low abundance, ctDNA measurements require a highly sensitive and precise detection technology to quantify often low-level genetic aberrations within a high background of wild-type DNA. Here, we demonstrate the combined power of SAGAsafe® chemistry and Crystal Digital PCR™ technology to detect 24 *PIK3CA* mutations and a wildtype *PIK3CA* reference DNA in a single reaction on the naica® 6-color system.

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 is based on digital PCR (dPCR) and detects and quantifies mutations with unique ultrasensitivity down to 0.001% MAF (Mutant Allele Frequency). How does it work? SAGAsafe® technology is an all-in-on reaction consisting of two phases: Phase 1 – linear copying of the target strand; Phase 2 – exponential signal generation, allowing the quantification of both the ssDNA and dsDNA fractions **(Figure 1)**. Application of SAGAsafe® greatly reduces sources of detection noise coming from the DNA polymerase itself or from DNA damage, lowering false positives by 100x and greatly improving limits of detection.

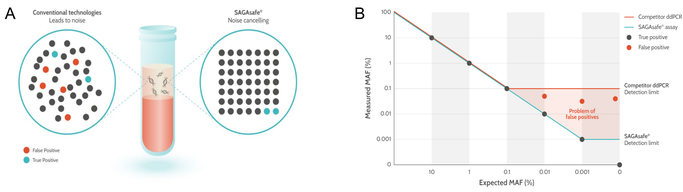


Figure 1. The principle of the SAGAsafe® technology. *A) SAGAsafe® chemistry enables superior sensitivity by greatly reducing detection background noise and the risk of false results. B) The ultrasensitivity of SAGAsafe® enables accurate detection and quantification of point mutations down to 0.001% MAF.*

Crystal Digital PCR™ on the naica® 6-color 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 the highest 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.

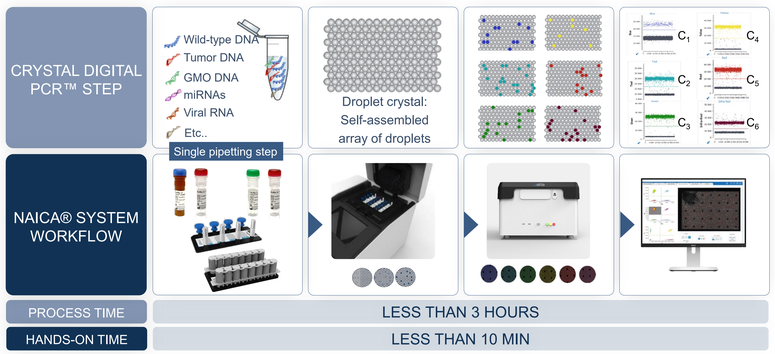


Figure 2. The naica® system: Absolute quantification of multiple genetic targets in a single run*. The 6-color naica® system is an easy-to-use dPCR platform that harnesses cutting-edge microfluidic technology to integrate the dPCR workflow onto a single consumable chip. The technology, known as Crystal Digital PCR™, partitions samples into a large array of thousands of individual droplet crystals – each its own reaction compartment – before amplifying nucleic acid molecules in each droplet crystal. These reactions are tagged with fluorophores to be read using up to six different fluorescence light channels, maximizing multiplexing capacity. The 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 detection assay, we combined the power of SAGAsafe® technology with that of 6-color Crystal Digital PCR™. 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 **(Figure 3A).** The 24-plex SAGAsafe® Crystal Digital PCR™ assay was tested on a range of breast cancer patient samples for which next-generation sequencing data was also available and showed excellent concordance with R^2 values of 0.992 **(Figure 3B).** Moreover, serial dilution experiments **(Figure 4)** showed accurate linear quantification to below 0.01% mutant allele frequency (MAF) with coefficient of determination scores of $R^2 > 0.98$ and $R^2 > 0.99$ depending on the *PIK3CA* mutant target.

A

Mutation #	*PIK3CA* Exon	CDS Mutation	AA Mutation	Legacy Mutation ID	Stilla Prism 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	Wildtype	Wildtype	Not applicable	RED

B

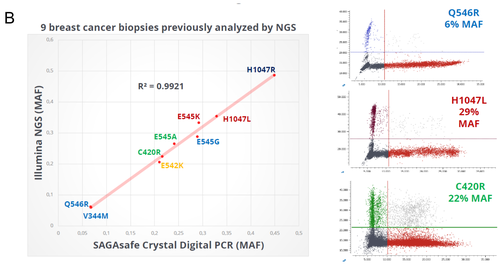


Figure 3. The 24-plex PIK3CA 6-color detection assay. *A) The list of the 24 PIK3CA mutations, their corresponding nucleotide and amino acid alterations and COSMIC identifiers and respective 6-color Crystal Digital PCR™ color detection channel. B) Left: Nine breast cancer biopsies previously analyzed by NGS were analyzed with the 24-plex SAGAsafe® assay using 6-color Crystal Digital PCR™ and the corresponding obtained MAFs were plotted for each sample. Right: Example 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 wildtype DNA fluorescence intensities detected in the red channel.*

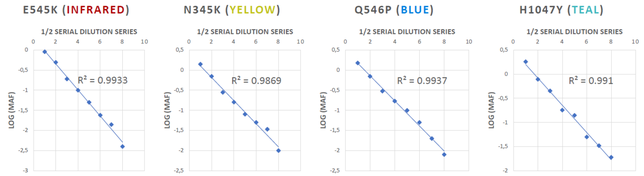
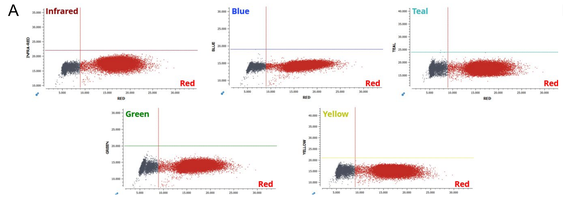


Figure 4. Highly accurate quantitation. *Example serial dilution curves of the PIK3CA mutations E545K, N345K, Q546P and H1047Y detected by 6-color Crystal Digital PCR™. Wild-type copies were kept at a constant amount of 65k wild-type genome copies at each 1/2 serial dilution step. Measurements were performed in duplicate.*

Analyses of pure wildtype (non-mutant) control DNA on the 6-color naica® system, the 24-plex *PIK3CA SAGAsafe®* assay was shown to have extremely clean mutant signals with pure wildtype control DNA (Figure 5A) leading to ultralow limits of blank (LoB) between 0.001% to 0.003% MAF and ultrasensitive limits of detection (LoD) between 0.002% to 0.009 MAF (Figure 5B), depending on the mutation and fluorescence channel. Thus, the combination of SAGAsafe® technology and 6-color Crystal Digital PCR™ achieves superb sensitivity, even with a complex 24-plex assay design.

A



B

Channel	*PIK3CA* mutations	WT 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	Wildtype exon 9	Many millions	not applicable	not applicable

Figure 5. The ultrasensitive 24-plex PIK3CA 6-color detection assay. *A) 2D dot-plots generated by Crystal Miner software showing clean fluorescent signals in each of the five PIK3CA mutant-dedicated detection channels in a background of pure wildtype control DNA loaded at 65k copies and detected in the Red channel. B) LoB and LoD values of the corresponding PIK3CA mutations were calculated from ~25 replicates with wildtype genome at 65k copies in each replicate.*

Conclusions

- SAGAsafe® technology is fully compatible with Crystal Digital PCR™ on the naica® system.
- Six color channels greatly improves the capacity for high multiplex target detection.
- Highplex tests require ultrasensitive assay technology if they are to maintain high performance.
- The combination of SAGAsafe® technology and 6-color Crystal Digital PCR™ on the naica® system achieves superb sensitivity, even with a complex 24-plex assay design.

For more information about 6-color Crystal Digital PCR™ and other naica® products, visit Stilla Technologies at https://www.stillatechnologies.com/ and posters 2930, 527, 75, 2932 and 2942.
For more information about SAGAsafe® technology, visit SAGA Diagnostics at https://sagadiagnostics.com/