A streamlined workflow for liquid biopsy sample extraction and highplex Crystal Digital PCR™ analysis using the Maxwell® RSC system and the 6-color naica® system

INTRODUCTION

Liquid biopsies, such as blood samples, can harbor a wealth of genetic information from both healthy and unhealthy cells to inform disease diagnosis and treatment. Rigorously qualified pre-analytical protocols are vital to ensure the performance of downstream liquid biopsy workflows and thus high-quality results. Circulating cell free DNA (cfDNA), extracted from liquid biopsy samples, are an established sample type for characterizing oncology targets. Nevertheless, cfDNA measurements require a highly sensitive and reliable detection technology to quantify, often low-level, genetic aberrations within a complex background of wild-type sequences.

This technical note details a flexible method for automated plasma sample extraction that seamlessly integrates into a straightforward and sensitive digital PCR genetic analysis workflow. Combining the Maxwell® RSC 48 instrument for automated cfDNA extraction with Crystal Digital PCR™ on the naica® system allows ultrasensitive high-plex detection from liquid biopsy samples. By bridging the two technologies, 32 common and rare somatic *EGFR* mutations in exons 18, 19, 20, and 21, representing more than 90% of *EGFR* mutations described in non-small-cell lung carcinoma (NSCLC) are sensitively and precisely detected. The combination of the two technologies also allowed for the detection of a set of *PIK3CA* mutations from cfDNA samples.

The combined workflow of automated plasma sample extraction using the Maxwell® RSC 48 and the high-plex and sensitive target detection with Crystal Digital PCR™ thus represents a streamlined full solution from sample-to-answer that can benefit cancer researchers across the biomarker testing landscape.

MATERIAL AND METHODS

Plasma-like samples (SensID reference material ref SID-000002, SID-000016, SID-000089) and human K2EDTA plasma samples, collected from healthy donors, were extracted with the Promega Maxwell® RSC ccfDNA LV Plasma Kit (Promega, ref AS1840). Before extraction, all samples were spiked with a known quantity of an exogenous extraction control DNA (EC), and after extraction, aliquots of the human plasma samples were spiked with known amounts of synthetic mutant DNA. For comparison to the automated sample extraction methodology, plasma samples were also manually extracted with the QIAamp circulating nucleic acid kit (QIAGEN, ref 55114) according to supplier recommendations.

The extracted cfDNA samples were then analyzed by Crystal Digital PCR™ on the 6-color naica® system with two independent multiplexed cancer detection assays, a 6-plex and a 33-plex.

Stilla Technologies defines plex as the number of targets detected in a Crystal Digital PCR™ assay.

A custom **6-plex Rectal Cancer assay** detecting six targets: *PIK3CA* wild-type, four *PIK3CA* mutations (p.E542K, p. E545K, p.H1047L, p. H1047R) and the EC.

The EGFR **6-color Crystal Digital PCR™ kit** (Stilla Technologies®, Ref R30006), an off-the-shelf 33-plex digital PCR kit detecting *EGFR* wild-type and the 32 most common non-small cell lung cancer *EGFR* mutations[1].

Maxwell® RSC 48 Instrument

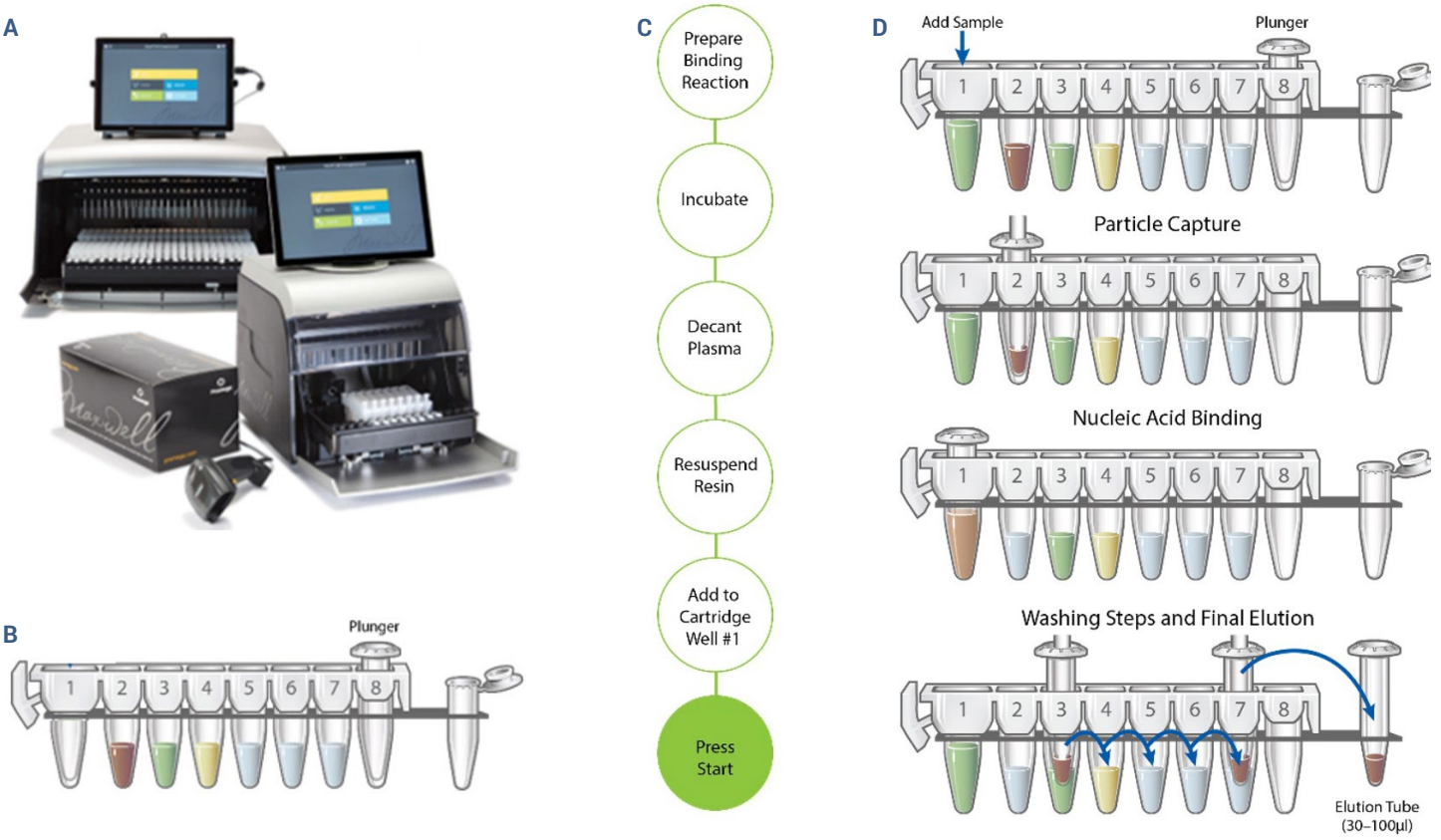

Figure 1 | Maxwell® system workflow. A) Maxwell® RSC 48 (top) and Maxwell® RSC 16 (bottom) instruments, B) the Maxwell® extraction methods start with prefilled cartridges ready for the samples. C) Sample preparation for the use of Maxwell® RSC ccfDNA Plasma Large volume Kit extraction D) After sample addition, the instrument moves the particles and associated nucleic acids through a series of automated steps, ultimately yielding highly pure nucleic acids.

The Maxwell® RSC 48 Instrument **(Figure 1A)** is a compact, automated nucleic acid purification platform that processes up to 48 samples simultaneously. Using Maxwell® cartridges, prefilled with purification reagents and paramagnetic particles **(Figure 1B),** the Maxwell® RSC 48 Instrument enables consistent high-throughput purification of DNA or RNA from a variety of sample types. The Maxwell® RSC 48 Instrument's intuitive graphical interface makes the instrument easy to use. The integrated vision system with its large LED indicator reduces the potential for user error by detecting proper cartridge placement. This informs the user of any issues before the run starts. An integrated bar code reader makes it easy to track sample information along the extraction workflow.

Because the Maxwell® RSC instruments are magnetic particle movers, not liquid handlers, they offer advantages over other automated nucleic acid extraction systems. There is minimal risk of cross-contamination because no liquid handling or splashing occurs during sample processing. With no clogs and fewer breakdowns, there are fewer disruptions to the nucleic acid extraction workflow. High-quality nucleic acid purification, with minimal steps and hands-on time, can be obtained with a wide range of available extraction kits, including the Large Volume (LV) ccfDNA plasma extraction kit used for this study **(Figure 1C and 1D).**

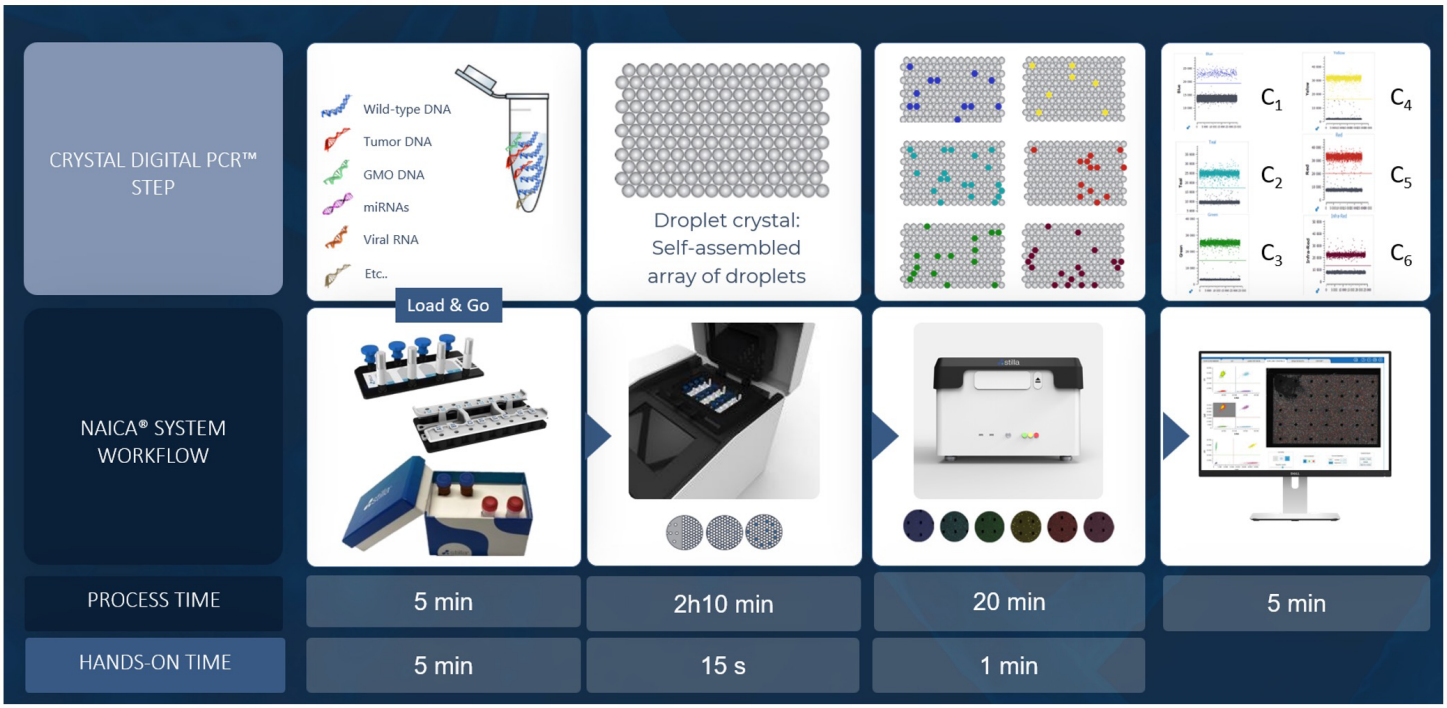

Figure 2 | The 6-color naica® system: absolute quantification of multiple genetic targets in a single run. The 6-color naica® system is an easy-to-use digital PCR platform whose cutting-edge microfluidic technology automatically integrates the digital PCR workflow into a ready-to-use single consumable chip. With no moving parts, Crystal Digital PCR™ partitions the sample into a 2D array of thousands of individual droplet reaction compartments that individually 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.

Crystal Digital PCR™ on the 6-color naica® system

The 6-color naica® system Crystal Digital PCR™ workflow **(Figure 2)** enables high multiplexing capacity in a single reaction, saving both time and precious samples, and provides ultra-sensitivity and increased low-level detection of multiple reactions in parallel. By automatically partitioning sample reactions into a 2D array of tens of thousands of droplets, Crystal Digital PCR™ technology can be used for absolute nucleic acid quantification in a wide range of applications. For example, Crystal Digital PCR™ can be used for oncological analysis applications, including copy number variation, mutation detection, rare event detection, and therapeutic monitoring. The accompanying Crystal Miner software measures the concentrations of targeted nucleic acids, providing automatic identification of positive and negative Droplet Crystals for the selected fluorescence channels. With intuitive visuals for image analysis, users can easily explore their multiplex data and directly inspect the Droplet Crystals in different fluorophore channels. This visual inspection allows unparalleled peace of mind for quality control.

RESULTS

Integration of the Maxwell® RSC LV kit and the 6-color naica® system into a compatible sample-to-answer workflow

To evaluate the Droplet Crystal stability and compare the sample concentrations of cfDNA using two different extraction procedures, cfDNA from plasma-like samples were extracted automatically with the Maxwell® RSC LV kit on the Maxwell® RSC 48 system (Promega) and manually with the QIAamp circulating nucleic acid kit (Qiagen). The samples were processed by Crystal Digital PCR™ on the 6-color naica® system using the *EGFR* 6-color Crystal Digital PCR™ kit. All samples showed full compatibility for Droplet Crystal stability (**Figure 3A**). Furthermore, a comparison of the total DNA concentration **(Figure 3B)** and mutant DNA concentrations **(Figure 3C)** revealed highly similar results across the two extraction procedures.

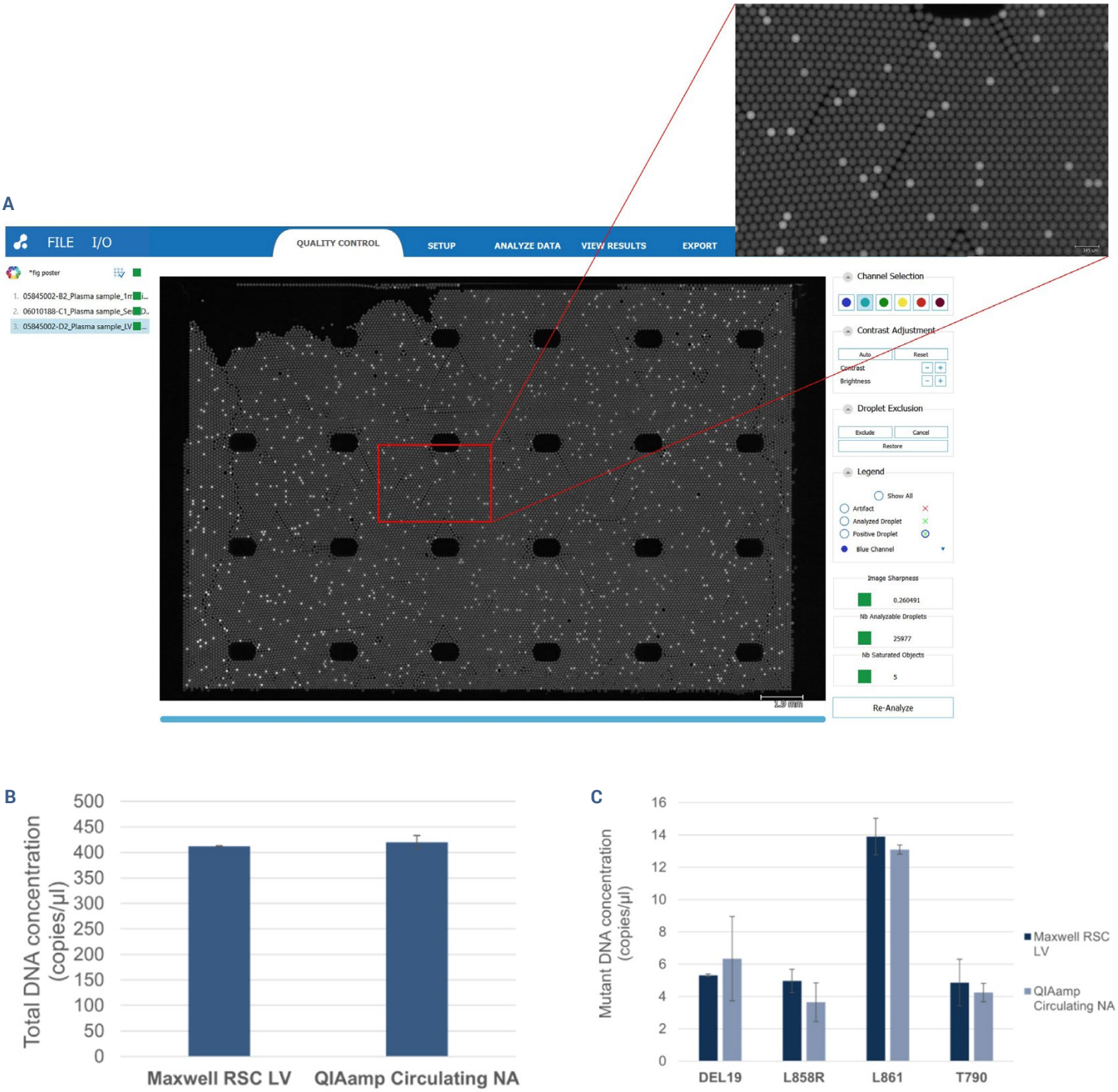

Figure 3| A. Comparing automated Maxwell RSC LV extraction with another manual extraction method. A. Direct visualization of droplets containing amplicons amplified from cfDNA extracted with the Maxwell® RSC system. Droplets show a stable 2D Droplet Crystal characteristic of high-quality Crystal Digital PCR™ data. Inset: a zoom of the Droplet Crystal structure with positive and negative droplets clearly distinguishable. **B.** Total DNA concentrations (copies/µL) of circulating cfDNA samples extracted from 2mL of plasma-like samples with the Maxwell® RSC LV kit and the QIAamp circulating nucleic acid kit. **C.** *EGFR* mutant DNA concentrations (copies/µL) of cfDNA samples extracted from 2mL of plasma-like samples by the Maxwell® RSC LV kit (dark blue) and the QIAamp circulating nucleic acid kit (light blue).

Mutation detection in plasma and plasma-like samples

Extracted cfDNA from both plasma and plasma-like samples were analyzed by two multiplexed detection assays customized for Crystal Digital PCR™ workflow: a custom designed 6-plex Rectal Cancer assay and the commercially available *EGFR* 6-color Crystal Digital PCR™ kit. Quantification of the EC allowed to calculate the extraction yield for each sample. The mean yield obtained for eight samples (three plasma-like and five plasma) was 74.5%. All mutations known to be present in the samples were detected **(Table 1).**

		6-plex Rectal Cancer assay							*EGFR* 6-color Crystal Digital PCR™ kit				
		Extraction control DNA			Measured concentrations (copies /µL)								
	Mutants	Expected Concentration (copies/µL)	Measured Concentration (copies/µL)	**Extraction yield (%)**	WT DNA	*PIK3CA* E545K	*PIK3CA* H1047R	*PIK3CA* H1047L	WT DNA	*EGFR* Del exon 19	*EGFR* L858R	*EGFR* L861Q, G719S	*EGFR* T790M
Plasma-like WT	No mutant	60.0	47.3	**78.8**	**735.6**	0.0	0.0	0.0	N.A	N.A	N.A	N.A	N.A
Plasma-like *PIK3CA*	*PIK3CA* E545K, H1047R	60.0	38.0	**63.3**	**642.0**	**2.7**	**6.0**	0.0	N. A	N.A	N.A	N.A	N.A
Plasma-like *EGFR*	*EGFR* Deletion exon19, L858R, L861Q, G719S, T790M	60.0	47.0	**78.3**	N.A	N.A	N.A	N.A	**411.3**	**5.3**	**5.0**	**14.0**	**4.8**
Plasma WT	No mutant	60.0	46.0	**76.6**	**175.3**	0.0	0.0	0.0	N.A	N.A	N.A	N.A	N.A
Plasma *PIK3CA* E545K	*PIK3CA* E545K	60.0	38.2	**63.6**	**190.4**	**12.9**	0.0	0.0	N.A	N.A	N.A	N.A	N.A
Plasma *PIK3CA* H1047L	*PIK3CA* H1047L	60.0	46.7	**77.8**	**116.6**	0.0	0.0	**33.1**	N.A	N.A	N.A	N.A	N.A
Plasma *PIK3CA* H1047R	*PIK3CA* H1047R	60.0	44.6	**74.3**	**109.0**	0.0	**46.5**	0.0	N.A	N.A	N.A	N.A	N.A
Plasma *EGFR*	*EGFR* Deletion exon19	60.0	50.0	**83.3**	**122.0**	0.0	0.0	0.0	**105.0**	**4.8**	0.0	0.0	0.0
			Mean Yield (%)	***74.5***					N.A. Not applicable				

Table 1 | Crystal Digital PCR™ 6-color naica® system results: Two assays, 6-plex Rectal Cancer assay and *EGFR* 6-color Crystal Digital PCR™ kit, were used with the 6-color naica® system to quantify wild-type and mutant DNA from cfDNA extracted from plasma or plasma-like samples with the Maxwell® RSC LV kit. Concentrations are in copies/µL. Extraction yield was calculated by dividing the measured EC DNA concentrations by the expected concentrations.

Example 1D-dot plots generated by the Crystal Miner software resulting from the detection by the 6-plex Rectal Cancer assay are shown in **Figure 4.**

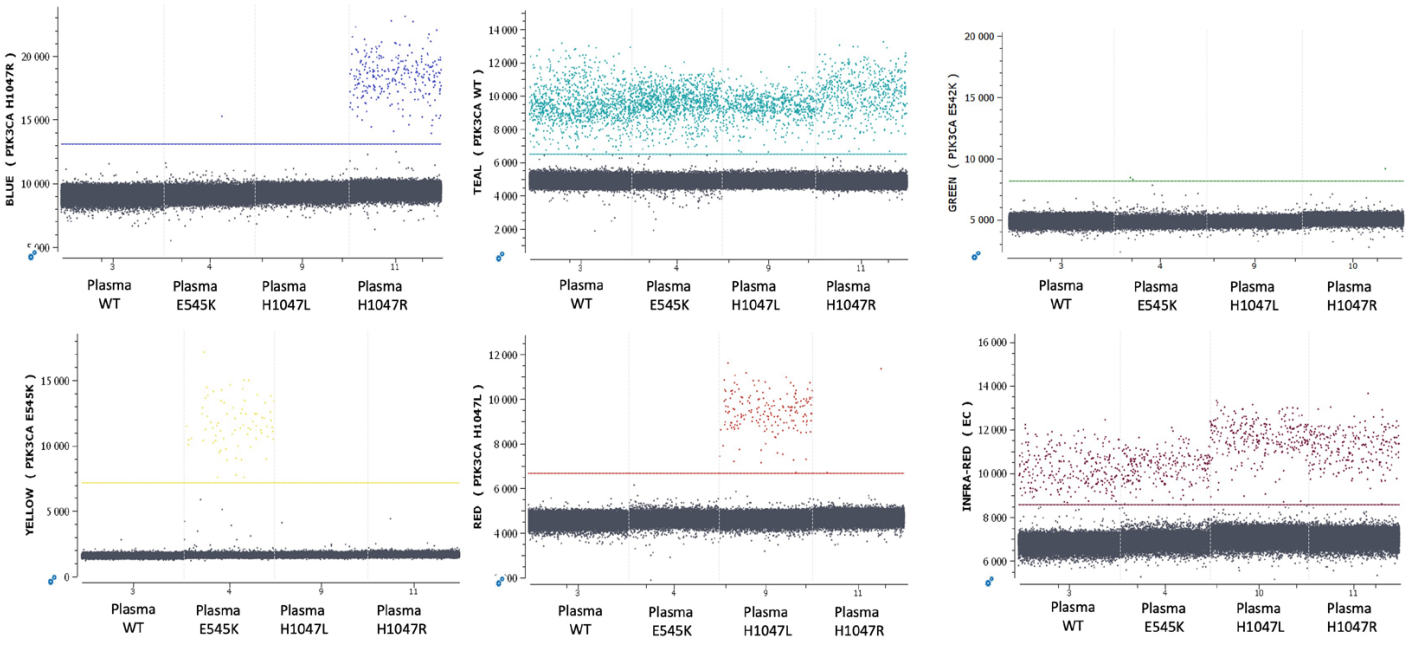

Figure 4 | 6-plex Rectal Cancer assay analysis with Crystal Miner software. 1D dotplots generated by Crystal Miner software after Crystal Digital PCR™ partitioning, amplification and scanning using the 6-plex Rectal Cancer assay of DNA extracted from 2mL of clinical plasma with the Maxwell RSC LV kit. Detected alleles, are the *PIK3CA* wild-type in the teal channel, the *PIK3CA* H1047R, E542K, E545K and H1047L mutations in the blue, green, yellow and red channels respectively. The IR channel quantifies the EC. Sample names are shown on the X-axis and fluorescence intensities for each color channel on the Y-axis. Threshold lines are automatically generated to separate the negative and positive clusters of droplets.

Furthermore, 2D-dot plots resulting from the detection by the *EGFR* 6-color Crystal Digital PCR™ kit show clear separability between the positive and negative clusters. **(Figure 5)**.

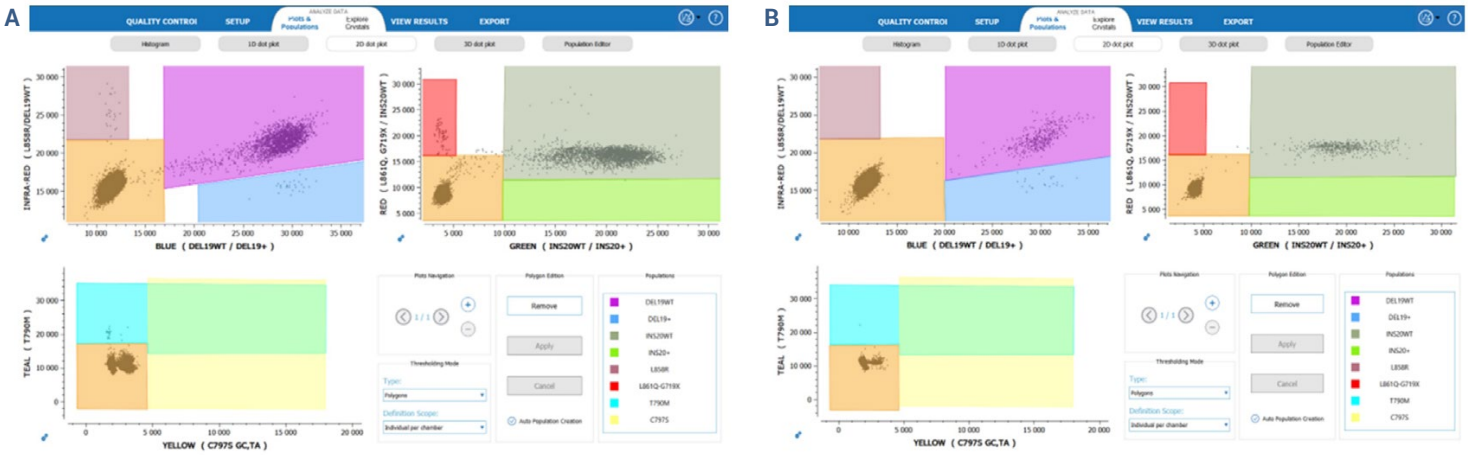

Figure 5 | *EGFR* 6-color Crystal Digital PCR™ kit analysis with Crystal Miner software. Examples of 2D dotplots, generated by Crystal Miner software, of the fluorescence intensities of each indicated color channel after Crystal Digital PCR™ partitioning, amplification and scanning of two cfDNA (A and B), extracted from 2mL of plasma-like sample with the Maxwell® RSC LV kit. Colored polygon thresholds separate the negative (orange polygon) and positive clusters as indicated on the axes.

CONCLUSIONS

This study demonstrates compatibility between the automated Maxwell® RSC cfDNA extraction system and high-plex detection from liquid biopsy samples by Crystal Digital PCR™ on the 6-color naica® system. This full solution workflow enables largely automated highly sensitive detection and quantification of the main somatic *EGFR* mutations described in NSCLC and a set of *PIK3CA* mutations in breast and rectal cancers. Both technologies produce results quickly with minimal hands-on-time, enabling a complete sample-to-result workflow in less than a day. This proof-of-concept workflow creates the foundation for the further development of streamlined sample-to-answer protocols for high multiplex assays that will benefit cancer researchers across the biomarker testing landscape.

HIGHLIGHTS

- The Maxwell® RSC cfDNA extraction system and Crystal Digital PCR™ on the 6-color naica® system for a seamless, largely automated, highly sensitive detection and quantification end-to-end workflow.
- Demonstrated detection and quantification of the main somatic EGFR mutations described in NSCLC and a set of PIK3CA mutations described in breast and rectal cancers.
- Minimal hands-on-time, enabling a complete full solution sample-to-result workflow in less than a day.

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 further information on Maxwell Extraction systems and technologies, contact Maxwell-dPCR@Promega.com

Reference:

[1] Harrison PT, Vyse S, Huang PH, Rare epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer, Seminars in Cancer Biology (2019), doi: https://doi.org/10.1016/j.semcancer.2019.09.015