THE MULTIPLEX DIGITAL PCR COMPANY

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

#527

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Abstracts

Circulating cell free (cf)DNAs extracted from liquid biopsy samples have become established sample types for characterizing oncology targets. Currently, there are several extraction protocols and genomic platforms for researchers to select when interrogating genetic information. The focus of this work was to identify a flexible method for sample extraction that seamlessly integrates into a straightforward and sensitive genetic analysis workflow from plasma samples. Here, we present a workflow combining the Maxwell® CSC 48 instrument for automated cfDNA extraction and Crystal Digital PCR™ on the naica® system for ultrasensitive high-plex detection from liquid biopsy samples of *EGFR* mutations in non-small cell lung cancer (NSCLC) and *PIK3CA* mutations in breast and rectal cancers. By bridging sample extraction with the Maxwell® system and high-plex digital PCR with the 6-color naica® system, we enabled the detection with high sensitivity and precision of 32 common and rare somatic *EGFR* mutations in exons 18, 19, 20, and 21, representing more than 90% of *EGFR* mutations described in NSCLC. Furthermore, following the same extraction and sample testing workflow, we enabled the detection of a set of *PIK3CA* mutations from cfDNA samples. This proof-of-concept workflow creates the foundation for the further development of streamlined sample-to-answer protocols that will better assist cancer researchers across the biomarker testing landscape.

Introduction

Liquid biopsies, such as blood samples, can harbor a wealth of genetic information from both healthy and unhealthy cells **(Figure 1)** to inform disease diagnosis and treatment. cfDNA measurements require a highly sensitive and reliable detection technology to quantify often low-level genetic aberrations within a high background of wild-type sequences. Rigorously qualified pre-analytical protocols are vital to ensure the performance of downstream liquid biopsy workflows to ensure high-quality sample to results **(Figure 2).** This proof-of-concept study evaluates the performance of the cfDNA extraction protocols of the Promega Maxwell® paired with the detection by 6-color Crystal Digital PCR™ on the naica® system of *PIK3CA* and *EGFR* mutations and external extraction controls.

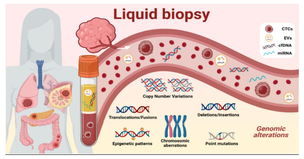

Figure 1. Schematic of the liquid biopsy composition. Liquid biopsy obtained from peripheral blood is composed of different tumoral components such as circulating tumor cells (CTCs), circulating cell-free DNA (cfDNA), extracellular vesicles (EVs), and micro-RNA (miRNA). From Biomedicines, Ayuso-Sacido et al., //doi.org/10.3390/biomedicines9080906

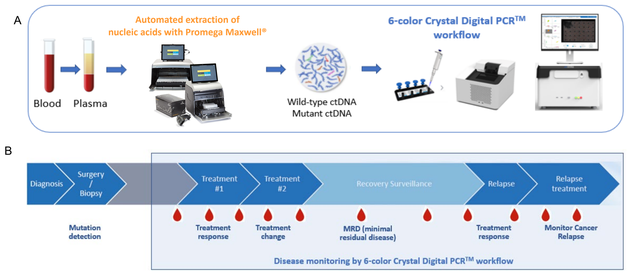

Figure 2. Complete blood sample to results analysis workflow.
A) This workflow combines cfDNA automated extraction using the Maxwell® RSC system and Crystal Digital PCR™ on the naica® system for ultrasensitive high-plex detection. B) cfDNA analysis by the 6-color Crystal Digital PCR™ allows timely and sensitive monitoring of cancer disease (treatment response, MRD, cancer relapse (resistance mutations).

Maxwell® CSC 48 Instrument

The Maxwell® CSC 48 Instrument **(Figure 3A)** 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 3B),** the Maxwell® CSC 48 Instrument brings the same consistent, reliable purification of DNA or RNA from a variety of sample types and a higher throughput. The 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 and lets you know before a run starts if there is an issue. An integrated Bar Code reader makes it easy to track samples.

Because the Maxwell® CSC instruments are magnetic particle movers, not liquid handlers, they offer advantages over other automated systems. There is minimal risk of cross-contamination because no liquid handling or splashing happens during sample processing. With no clogs and fewer breakdowns, there are fewer disruptions to your 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) cfDNA plasma extraction kit used for this study **(Figure 3C).**

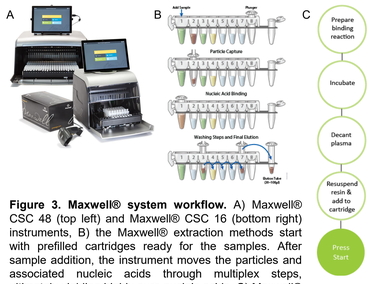

Figure 3. Maxwell® system workflow. A) Maxwell® CSC 48 (top left) and Maxwell® CSC 16 (bottom right) instruments, B) the Maxwell® extraction methods start with prefilled cartridges ready for the samples. After sample addition, the instrument moves the particles and associated nucleic acids through multiplex steps, ultimately yielding highly pure nucleic acids. C) Maxwell® cfDNA plasma kit extraction protocol overview..

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

The 6-color Crystal Digital PCR™ workflow **(Figure 4)** enables the highest multiplexing capacity in a single reaction saving both time and precious sample and providing ultra sensitivity and increased low-level detection of multiple reactions in parallel. By partitioning sample reactions into a large 2D array of droplets, 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 droplet crystals for all fluorescence channels. Through intuitive visuals for image analysis, users can explore their data in multiplex way, including the direct inspection of droplet crystals for quality control.

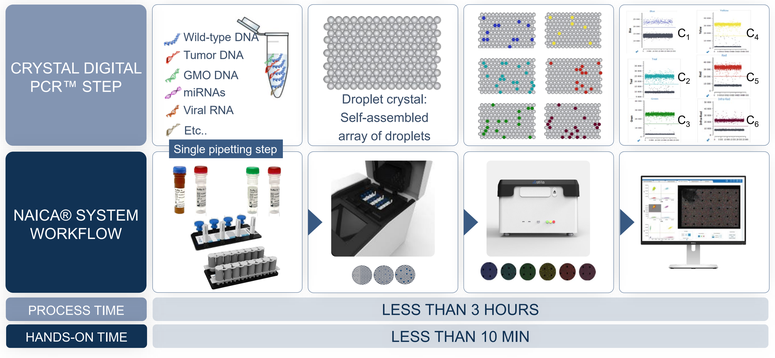

Figure 4. The naica® system: Get absolute quantification of multiple genetic targets in a single run. The 6-color naica® system is an easy-to-use digital PCR platform that harnesses cutting-edge microfluidic technology to integrate the digital PCR workflow onto a single consumable chip. The Crystal Digital PCR™ technology 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.

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 LV cfDNA plasma extraction kit (Promega, ref AS1840), according to supplier recommendations. Before extraction, all samples were spiked with a known quantity of an exogenous Extraction Control DNA (EC), and a portion of the human plasma samples were spiked with known amounts of synthetic mutant DNA. For comparison, plasmas were also extracted with QIAamp circulating nucleic acid kit (QIAGEN, ref 55114) according to supplier recommendations.

cfDNA were analyzed on the naica® 6-color Crystal Digital PCR™ system with two independent 6-color cancer detection assays:
- A custom ***Rectal Cancer 6-color assay*** detecting *PIK3CA* wild-type (WT), 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) detecting *EGFR* WT and 32 *EGFR* mutations (for more information, see poster 75) .

Results

▪ The Maxwell® RSC LV kit and the naica® 6-color system are compatible workflows

We first evaluated the compatibility of the samples extracted with the Maxwell® RSC LV ccfDNA Plasma extraction kit by determining the stability of the droplet crystal using Sapphire chips. Our Crystal Miner software allows a transparent check of the droplet crystal to visualize the individual droplets. All samples extracted with the Promega Maxwell® CSC system were highly compatible with the naica® Crystal Digital PCR™ droplet chemistry **(Figure 5).**

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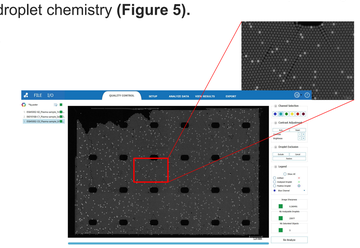

Figure 5. Crystal Miner software Quality Control view of a Sapphire chip chamber. Droplets containing amplicons amplified from cfDNA extracted with the Maxwell® RSC system form a stable 2D droplet crystal characteristic of high-quality Crystal Digital PCR™ data. Inset: a zoom of the droplet crystal structure.

▪ Comparison of automated Maxwell RSC LV extraction to a manual reference method

We compared the concentrations of WT and mutant DNA measured using the *EGFR* 6-color Crystal Digital PCR™ kit on cfDNA extracted from plasma-like samples with the Maxwell® RSC LV kit versus the manual QIAamp circulating nucleic acid kit. Similar WT and mutant concentrations were obtained from samples extracted by the two methods **(Figure 6** and **Figure 7**).

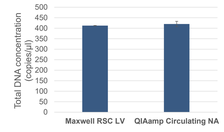

Figure 6. Total DNA concentrations (copies/µL) of ccfDNA samples extracted from 2mL of plasma-like samples by Maxwell® RSC LV kit (Left) and QIAamp circulating nucleic Acid kit (Right)

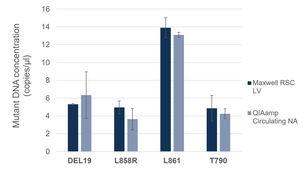

Figure 7. Mutant DNA concentrations (copies/µL) of ccfDNA samples extracted from 2mL of plasma-like samples by the Maxwell® RSC LV kit (Blue) and the QIAamp circulating nucleic Acid kit (Grey)

▪ Mutations detection in plasma-like and plasma samples

We analyzed extracted cfDNA from both plasma-like and plasma samples with each of the two 6-color Crystal Digital PCR™ detection assays, the Rectal Cancer 6-color assay and the *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 (3 plasma-like and 5 plasma) was 74,5%. All mutations known to be present in the samples were detected **(Table 1).** Example 1D-dot plots generated by the naica® system Crystal Miner analysis software resulting from the detection by the Rectal Cancer 6-color assay **(Figure 8),** and 2D-dot plots resulting from the detection by the *EGFR* 6-color Crystal Digital PCR™ kit **(Figure 9)** show clear separability between the positive and negative clusters, with minimum droplet rain.

Mutants		Extraction control DNA			Measured concentrations (copies /µL)								
		Rectal Cancer 6-color assay			Rectal Cancer 6-color assay				*EGFR* 6-color Crystal Digital PCR™				
		Expected Concentrations (copies/µL)	Measured Concentrations (copies/µL)	**Extraction yield (%)**	WT DNA	*PIK3CA* E545K	*PIK3CA* H1047R	*PIK3CA* H1047L	WT DNA	Deletion exon 19	L858R	L861Q, G719S	T790M
Plasma-like WT		60,00	47,25	**78,75**	735,60	0	0	0	0	0	0	0	0
Plasma-like *PIK3CA*	*PIK3CA* E545K, H1047R	60	38,00	**63,3**	641,95	2,69	6,00	0,00	0	0	0	0	0
Plasma-like *EGFR*	*EGFR* Deletion exon19, L858R, L861Q, G719S, T790M	60	47,00	**78,3**	0	0	0	0	411,30	5,30	5,00	14	4,8
Plasma WT		60	45,98	**76,6**	0	0	0	0	0	0	0	0	0
Plasma *PIK3CA* E545K	*PIK3CA* E545K	60	38,17	**63,6**	0	12,9	0	0	0	0	0	0	0
Plasma *PIK3CA* H1047L	*PIK3CA* H1047L	60	46,7	**77,8**	116,6	0	0	33,05	0	0	0	0	0
Plasma *PIK3CA* H1047R	*PIK3CA* H1047R	60	44,55	**74,3**	109	0	46,5	0	0	0	0	0	0
Plasma *EGFR*	*EGFR* Deletion exon19	60	50	**83,3**	122	0	0	0	105	4,8	0	0	0
			Mean Yield (%)	*74,51*									

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

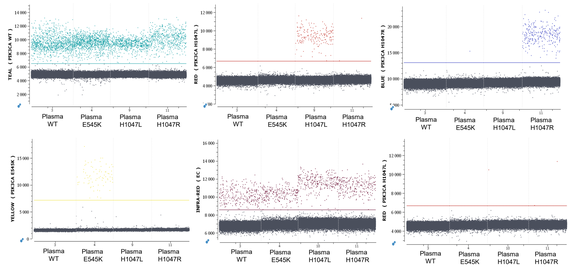

Figure 8. cfDNA analysis with naica® 6-color Crystal Digital PCR™
1D dotplots generated by Crystal Miner software after Crystal Digital PCR™ amplification using the the 6-color Rectal Cancer assay of DNA extracted from 2ml of clinical plasma with the Maxwell RSC LV kit. Fluorescence intensities for each color channel (Y-axis) and sample names (X-axis) are indicated. Threshold lines separate the negative and positive clusters.

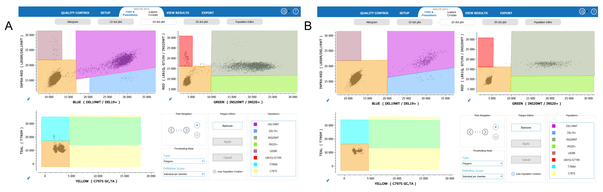

Figure 9. cfDNA analysis with the EGFR 6-color Crystal Digital PCR™
A) 2D dotplots generated by Crystal Miner software of the fluorescence intensities of each color channel after Crystal Digital PCR™ amplification of DNA extracted from 2ml of plasma-like with the Maxwell RSC LV kit. B) 2D dotplots generated after Crystal Digital PCR™ amplification of DNA extracted with Maxwell RSC LV kit from 2ml of plasma spiked with an exon 19 deletion mutant DNA. Colored polygon thresholds separate the various negative and positive clusters.

Conclusions

In this study, we show high compatibility between the automated Maxwell® CSC cfDNA extraction system and high-plex detection from liquid biopsy samples by Crystal Digital PCR™ on the naica® system. This workflow enables the highly sensitive detection and quantification of the main somatic *EGFR* mutations described in NSCLC and a set of *PIK3CA* mutations described in breast and rectal cancers. Both technologies have a fast time-to-result with minimum 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 that will better assist cancer researchers across the biomarker testing landscape.