Chapter 18

Reproducible and Sensitive Assays using 3- and 6-colour Crystal Digital PCR™ for Detecting Point Mutations in Human Breast Cancer

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Reproducible and Sensitive Assays using 3- and 6-colour Crystal Digital PCR™ for Detecting Point Mutations in Human Breast Cancer

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1. Introduction

A brief introduction to the Naica™ System

The Naica System is a digital PCR platform launched in 2016 and consists of the Naica™ Geode and the Naica™ Prism3 instruments, as well as Crystal Reader and Crystal Miner software applications and a single consumable, the Sapphire chip. The features of the Naica System include, but are not limited to, compatibility with a wide range of fluorophores (FAM, Yakima Yellow, Cy3, HEX, ROX, Cy5, etc.), shortest time to result (2h 30 min), easy data visualisation and interpretation, and user-friendly workflow. The Sapphire chip, engineered using microfluidic technology, consists of four microfluidic wells where the PCR reaction sample is divided into up to 30,000 distinct partitions, called droplets, arranged in a monodispersed stable and homogenous crystal layer. This partitioning takes place in the Naica Geode. The number of nucleic acid molecules encapsulated in a droplet follows a Poisson law distribution (see here for Poisson Law), resulting in zero, one or more target molecules per droplet. The utilization of Poisson law for digital PCR is based on the critical assumption that all droplets are homogenous and monodispersed (Pinheiro and Emslie 2018). The Sapphire chips are then imaged by the Naica Prism3 and Crystal Reader software and further analysed using the Crystal Miner software for an easy-to-assess, transparent data output. The Naica System provides automated data processing and the possibility for manual adjustment pertaining to droplet detection and fluorescence thresholding. A descriptive visual of the steps involved in Crystal Digital PCR using the Naica System is available here: https://youtu.be/YYDhRCIPHNk. For a brief overview of the principle of digital PCR and its applications, please see this video: https://youtu.be/Qqdmw3wvMFo.

The Crystal Miner software is intuitive and has a user-friendly interface, allowing users to dynamically visualise droplets in both crystal images and fluorescence dot-plots including 1-3 dimensional graphs. The image processing algorithms include a neural network that automatically excludes artefacts and non-homogenous droplets in crystal images with high reliability. The software also empowers users to accurately analyse the data using adapted statistical methods.

The currently available digital PCR system is the 3-colour Naica System that can detect targets in 3-colour channels and can be easily adapted to various digital PCR applications (Figure 1). A prototype of the Naica System that has the capacity to detect targets in 6-colours was successfully used in a proof-of-concept study for the identification of Epidermal Growth Factor Receptor (*EGFR)* sensitizing and resistance mutations in non-small cell lung cancer (NSCLC) patients (Madic et al. 2019). The Naica System 6-colour solution will be launched soon.

Figure 1. The commercially available Naica System, composed of the Naica Geode, the Naica Prism3 and the Sapphire chips

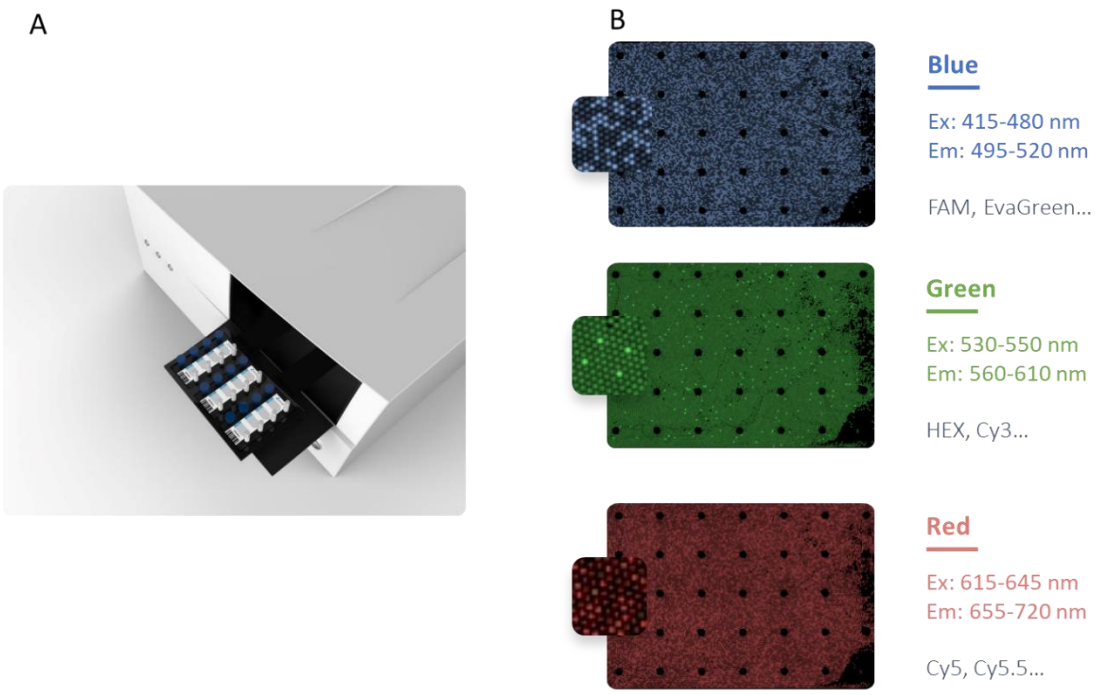


Figure 2. A) The Naica Prism3, a fluorescence reader for crystal droplets, and B) The three detection channels for targets and a few examples of the fluorophores that can be used.

Introduction to Naica System applications

Crystal Digital PCR has many advantages over traditional qPCR, including the lack of reliance on a standard curve, the user-friendliness, as well as robust and specific detection of rare events in a high background of wild-type (WT) DNA.

The Naica System has been optimized for applications in a wide range of nucleic acid quantification and analyses such as molecular and clinical oncology, molecular diagnostics, gene therapy, epigenetics, food safety monitoring, environmental testing, microbial ecology, and infectious diseases. The potential applications include copy number variation, rare event detection, gene editing, whole genome amplification, droplet recovery for downstream applications, NGS library quantification and NGS result validation.

One of the most useful applications of the Naica System is for accurately detecting rare events/mutations in various cancer types. In the case of NSCLC, *EGFR* activating and resistance mutations comprise the most prevalent mutations. Three and six-colour multiplex assays were designed to detect multiple mutations in a single assay, allowing

to rapidly predict patient responses for standard therapy (tyrosine kinase inhibitors). Using a three-colour multiplexing assay, activating *L585R*, *L861Q* and multiple *EGFR* exon 19 alterations, together with the T790M resistance mutation and *EGFR* WT DNA in circulating tumour DNA (ctDNA) NSCLC patient samples were detected using two multiplex assays. The assay design and results are described in detail here: https://www.stillatechnologies.com/color-crystal-tm-digital-pcr-assays-for-egfr-mutation-detection/. Furthermore, using 6-colour multiplexing, a single assay was designed and validated to detect the 19 most prevalent sensitizing and resistance *EGFR* mutations, simultaneously quantifying five sensitizing mutations, 12 indels in exon 19, two resistance mutations and an internal amplification control in tumour tissue and cell-free DNA (cfDNA) from NSCLC patients. To simultaneously detect the 12 most prevalent *EGFR* exon 19 alterations using only two probes, a drop-off assay composed of a drop-off probe spanning the genomic *EGFR* mutation hotspot and a reference probe spanning an adjacent *EGFR* WT genomic region was employed. For more details, please see https://www.ncbi.nlm.nih.gov/pubmed/30647840 (Madic et al. 2019).

Drop-off assays to detect the most prevalent colorectal carcinoma, *NRAS* and *KRAS* proto-oncogene mutations were also developed. The employment of a single amplicon and a limited number of probes to detect any genetic alteration occurring in a short genomic interval allows a rapid and a cost-efficient screening compared to conventional assays that would require many more probes to be designed and optimized. The drop-off assay is especially beneficial for sensitive and robust screening and therapeutic monitoring of clinical samples. The design and results of these three different drop-off assays are described here: https://www.stillatechnologies.com/drop-off-crystal-dpcr-nras-kras-egfr-mutations/.

A complementary technical note detailing the use of the proprietary Crystal Miner software for the analysis of drop-off assays and a step-by-step guide for drop-off quantification can be found here: https://www.stillatechnologies.com/how-to-quantify-drop-off-digital-pcr-assays-with-crystal-miner/

Another advantage of digital PCR over traditional qPCR is that it is less sensitive to the presence of substances naturally present in sample extracts that can inhibit a PCR

reaction. Indeed, many chemicals and substances can act as inhibitors in PCR-based reactions, which may be co-purified with the sample DNA and interfere with the PCR reaction. The presence of inhibitors in a sample can be a limiting factor in obtaining accurate or reliable results using qPCR. By quantifying and comparing the effect of two commonly detected inhibitor molecules, Humic Acid (found in soil samples) and Heparin (often used as a blood anticoagulant), in qPCR vs Crystal Digital PCR assays, it was observed that digital PCR could withstand up to twice the concentration of the inhibitors in the reaction mix as compared to qPCR. For details of the experimental results and more information, see here: https://www.stillatechnologies.com/effect-of-inhibitors-crystal-dpcr-vs-qpcr/

In addition to being compatible with TaqMan™ based PCR detection assays, Crystal Digital PCR is compatible with the use of cham® dye, an inert, non-toxic DNA-binding dye that becomes highly fluorescent when bound to double-stranded DNA (dsDNA) in a sequence-independent manner. EvaGreen offers the advantage of cost-effective probe-less detection using sequence-specific primers. EvaGreen dye can be used with Crystal Digital PCR for absolute quantification at a detection sensitivity of 0.2 copies/µl. For more information regarding EvaGreen dye compatibility with the Naica System see: https://www.stillatechnologies.com/crystal-digital-pcr-using-evagreen/

To ease the transition and augment the adaptability of Crystal Digital PCR to different applications, the Naica System's droplet recovery feature allows the users to recover the droplets for downstream applications such as Next-generation Sequencing (NGS), nest PCR and gel electrophoresis. Users can perform an easy-to-follow protocol described here for recovering the samples:
https://www.stillatechnologies.com/droplet-recovery-with-crystal-digital-tm-pcr/

With recent advances in PCR detection technologies, nucleic acid quantification has now become a standard technique for genetic testing and analysis. In addition, precise and robust genetic testing has opened new frontiers for research and analysis in both food and environmental sample quality monitoring fronts. Digital PCR has been successfully used for detecting genetically modified crops and the composition of food items by several researchers worldwide. A step-by-step detailed tutorial on how to accurately quantify the presence of antibiotic resistance in solid organic waste from

wastewater treatment plants is detailed here: https://www.gene-pi.com/tutorial/introduction-digital-pcr-using-evagreen-as-a-reporting-dye-2/. Organic waste from wastewater treatment plants, such as animal manure and sewage sludge, is known to be rich in antibiotic-resistant bacteria and antibiotic resistance genes. The utilization of organic waste for fertilization of the soil, thus, is a cause of concern as a potential source of infections and resistant genes. In this tutorial, ermB, a gene encoding erythromycin resistance, was detected in several organic waste samples that had previously been subjected to a cleaning process comprised of composting and digestion. A webinar titled “Identification of MRSA using 3-color Crystal Digital PCR” is available here: https://www.youtube.com/watch?v=jq-gUpFvhV8&feature=emb_title. Watch this webinar to explore how 3-colour Crystal Digital PCR can be employed for the detection of methicillin-resistant *Straphylococcus aureus* (MRSA).

For genetic testing and analysis in clinical settings, the amount of sample available is often limiting due primarily to low concentrations of target DNA. Additionally, for liquid biopsies (for example a blood draw or urine sample) and formalin-fixed paraffin-embedded (FFPE) samples, the DNA sample usually consists of a complex mixture of fragmented or ctDNA and WT DNA, demanding a highly sensitive quantification technique. By combining high sensitivity and the possibility to detect multiple targets in a single PCR due to its 3-colour detection technology, the Naica System ensures maximum information output from any precious sample. For example, quantification of Human Epidermal Growth Factor Receptor 2 (*HER2*) amplification, resulting from the overexpression of the *HER2* gene (also known as *ERBB2*, *CD320* and proto-oncogene *neu*) encoding the HER2 receptor, is crucial for anti-HER2 targeted therapy indication in breast cancer patients. Current clinical diagnostic methods for *HER2* gene amplification evaluation in tumour samples are limited to immunohistochemistry (IHC), which may be complemented by fluorescence in-situ hybridization (FISH). However, these techniques are labour-intensive and prone to inter-observer variability (Rodriguez et al. 2016), and so require highly skilled personnel. To simplify the identification of *HER2* amplification in samples with a low tumoral fraction and render it resistant to observer-dependent techniques, a triplex Crystal Digital PCR assay was designed for *HER2* gene quantification. In addition to reducing hands-on time, this assay also allows distinguishing *HER2* amplification from polysomy on chromosome 17, a status that could lead to *HER2* status misinterpretation. DNA samples from seven

breast cancer patients were analysed and the results were compared with standard diagnostic IHC and FISH procedures. For patients 1-4 and patients 6-7, the *HER2* positive and negative status, respectively, determined by Crystal Digital PCR was concordant with the IHC/FISH techniques. Additionally, Crystal Digital PCR detected polysomy on chromosome 17 in patient 6. Interestingly, for patient 5 using standard diagnostic procedures, amplification of *HER2* was detected; however, using Crystal Digital PCR the *HER2* elevation was attributed to chromosome 17 polysomy, a critical factor for the therapeutic consideration of breast cancer patients. Moreover, using this method, mutant fractions as low as 2% were detected, corresponding to an *HER2*/*TSN* (reference gene) normalized ratio of 1.2. Access the application note detailing the results described here: https://www.stillatechnologies.com/detection-of-her2-copy-number-variation-with-crystal-digital-tm-pcr/. Another valuable resource, a webinar, on liquid biopsy workflow and therapeutic monitoring by experts is available here: https://www.youtube.com/watch?time_continue=3&v=dTUUbfUG6mI&feature=emb_title

In the following section, we describe the advantages of multi-colour digital detection for monitoring common breast cancer mutations in liquid biopsy patient samples. Due to factors frequently limiting tumour tissue sampling such as tumour location, size and patient health complications, minimally invasive liquid biopsies represent a valuable source of ctDNA from which the genomic alterations of a patient's tumour can be determined. Indeed, tumours release nucleic acids (DNA or RNA) into the bloodstream, which can be recovered from plasma and used as a surrogate source of tumour DNA (C. Jovelet et al. 2016). This valuable source of genetic material requires sensitive and precise detection methods to quantify a maximum number of genetic alterations. Here, we present the Naica System in both 3-colour and 6-colour detection, as a fast and economical solution for reliable and robust quantification of liquid biopsy-sourced nucleic acid.

2. 6- and 3-colour Crystal Digital PCR assays for screening genomic alterations in breast cancer

Breast cancer subtype characterisation

Breast cancer is the most prevalent cancer among women worldwide with an estimated 1.6 million cases diagnosed in 2012 and remains a major health problem (Ferlay et al. 2015). Breast cancer is a heterogeneous disease and can be categorized into various subtypes based on the expression of molecular receptors and have different prognosis and treatment (Koboldt et al. 2012):

1. Estrogen receptor-positive (ER+)/progesterone receptor-positive (PR+) breast tumours are responsive to endocrine therapies (such as tamoxifen and aromatase inhibitors). These types of hormonal therapies have improved patient outcomes in ER+ metastatic breast cancer (Kurosky et al. 2018).
2. *HER2* positive (HER2+) breast tumours are characterized by the overexpression of the *HER2* gene. It is detected in approximately 25–30% of breast cancers and is often associated with poor prognosis. Trastuzumab has been shown to be an effective treatment for *HER2*+ breast cancer (Slamon et al. 2011).
3. Triple-negative tumours, not displaying any of the three- ER, PR, and *HER2* within the tumour, are associated with high mortality rates and are not responsive to current targeted treatment approaches. The gold-standard approach for this subtype of cancer is systemic chemotherapy.

In breast cancer, the detection of *HER2* amplification in tumour-derived DNA can guide the therapeutic care of patients. IHC and FISH are currently the standard methods to assess *HER2* overexpression. However, nucleic acid quantification technologies, such as digital PCR are highly accurate methods to quantify DNA copy number and a robust solution for *HER2* diagnostics (Page et al. 2011).

With emerging technological advances, digital PCR has become a powerful tool to analyse liquid biopsy samples to quantify genetic alterations that can be associated with treatment decision and outcome. PI3K/AKT is one of the main pathways involved in the oncogenesis of breast cancer. *PIK3CA* is the gene coding for the enzyme (PI3K) playing a role downstream of *HER2*. Recurrent somatic mutations across all breast cancer subtypes were found in *PIK3CA* (36%) (Weisman et al. 2016). Detecting these mutations is important as targeted therapies exist and can improve the patient's Progression-Free Survival (PFS) (André et al. 2016). *PIK3CA* gene mutations are the

most frequent events (40%) associated with hormonal therapy resistance in ER+ breast cancers. Mutations in *PIK3CA* generally occur in one of the two hotspot regions (exon 9: E545K or E542K, or exon 20: H1047R) (Araki and Miyoshi 2018). The early identification of *PIK3CA* mutations in patients undergoing hormone therapy could help better redefine the therapy. Moreover, most of the patients affected by ER+/HER2− tumours presented only bone metastases, a metastatic site that is associated with difficult pathological and molecular analyses. Detection of ctDNA in liquid biopsies is a promising alternative for the global evaluation of all metastatic sites because it eliminates invasive biopsy and the specific difficulties of less accessible bone metastases.

Early detection of relapse and residual disease

Collecting serial liquid biopsy samples to achieve longitudinal monitoring of tumour burden and tumour genetic heterogeneity is essential to evaluate the efficacy of a treatment. Indeed, levels of ctDNA have been shown to correlate with the overall survival of breast cancer patients and provide the earliest measurement of treatment response (Speicher and Pantel 2014; Yu et al. 2019). For example, a recent study examined ctDNA in early-stage before and after therapy and observed drug response showed that relative changes in mutated *PIK3CA* ctDNA levels can be used as a strong predictive tool of response to drug therapy (O'Leary et al. 2018)

Liquid biopsies are also emerging tools for the early detection of relapse in breast cancer patients. Moreover, minimally invasive ctDNA detection can be used to monitor post-operation patients for cfDNA analysis, to search for minimal residual disease (MRD) and to estimate the chances of recurrence. Studies have demonstrated that the follow-up monitoring of genomic alterations in ctDNA (HER2 amplification, *PIK3CA* mutations) enables physicians to detect relapse months before the detection by other follow-up methods including palpable, clinical symptoms and imaging (Olsson et al. 2015). In these studies, target mutations were first identified by tumour sample sequencing followed by ctDNA detection using droplet digital™ PCR (ddPCR™). They further used a digital PCR assay for the analysis of *PIK3CA* mutations in breast cancer patients at different stages of the disease (stages I to III). This study stratified the subjects into two groups with either high or low levels of ctDNA harbouring *PIK3CA* mutations, and subsequently concluded that a shorter recurrence-free survival was

associated with the group with high *PIK3CA* mutations levels in plasma ctDNA (Oshiro et al. 2015).

Multiplex digital PCR assays for screening prevalent genomic alterations in breast cancer

The detection and clinical use of cfDNA have long remained challenging due to the complex mixture of a low level of tumour cfDNA and WT cfDNA. Highly robust and selective techniques are required to detect reliably mutations of clinical relevance. Digital PCR is a proven clinical tool as it combines high sensitivity and absolute quantification with a cost-effective short time-to-results. With the ability to partition samples into thousands of individual reactions, digital PCR enables superior target detection and quantification, allowing the accurate detection of rare mutations in low concentration liquid biopsy ctDNA samples (Madic et al. 2019).

Using the 3-colour Naica Crystal Digital PCR platform, a panel was developed for the detection of both HER2 amplification and chromosome 17 polysomy in a single PCR. Furthermore, using the 6-colour Naica Crystal Digital PCR prototype system, the detection of *HER2* amplification and chromosome 17 polysomy was combined with the simultaneous quantification of the 3 most prevalent *PIK3CA* mutations in breast cancer samples all in a single reaction.

As previously described, the analysis of cfDNA in breast cancer enables the detection and monitoring of molecular abnormalities. The 3-colour and 6-colour assays could provide reliable early monitoring of treatment response and relapse (before other follow-up methods) and detection of resistance mutations. The 6-colour breast cancer panel described here in this chapter is the first digital PCR assay that combines both *HER2* amplification detection and the detection of the main *PIK3CA* mutations in a single assay, maximizing the information output from a single patient sample. Together, these genetic abnormalities account for more than 50% of breast cancers (Slamon et al. 2011; Weisman et al. 2016). The increased number of detection channels with this 6-colour detection platform, combined with the sensitivity of the Naica System, and a multiplexing strategy based on clinical needs, advances the current limits of tumour genotyping and liquid biopsy-based breast cancer monitoring.

Assay design and performance evaluation

Design of a 3-colour HER2 digital PCR panel

A 3-plex digital PCR assay including primers and TaqMan probes was designed to detect both *HER2* amplification and chromosome 17 polysomy, a distinct condition where additional copies of chromosome 17 are present, leading to HER2 status misinterpretation. *HER2* amplification is measured by calculating the ratio of the absolute concentration of the *HER2* gene on chromosome 17 over the absolute concentration of a reference gene, such as the Translin (*TSN*) encoding gene located on chromosome 2. For chromosome 17 polysomy detection, the ratio of Mitochondrial rRNA Methyltransferase 1 (*MRM1*) encoding gene concentration over *TSN* concentration is also measured. The *MRM1* gene is located on chromosome 17 and is not affected by *HER2* amplification but its copy number varies according to chromosome 17 polysomy. Primers for the amplification of *HER2, TSN* and *MRM1* (Figure 3) were selected to generate short amplicons of less than 100bp to ensure detection compatibility in fragmented DNA populations typical of ctDNA extracted from plasma or from FFPE tissues. The annealing temperature, number of cycles and primers concentrations were chosen to ensure optimal amplification, as scored by the distinction between positive and negative partitions, and to avoid nonspecific amplifications. TaqMan probes were labelled with a selection of fluorophores compatible with the Naica Prism3 detection channels: FAM, Cy3 and Cy5 for *HER2, MRM1* and *TSN* genes, respectively (Figure 4). The sensitivity of *HER2* copy number variation detection was measured on serially diluted samples where human genomic DNA was spiked with genomic DNA extracted from the SKBR3 cell line (*HER2*/*TSN* ratio of 10:1). SKBR3 fractions ranged from 3% to 13% and modelled a theoretical range of *HER2*/*TSN* ratios from 1.16 to 2.07. Prior to PCR, samples were fragmented using restriction endonuclease enzyme to ensure separation of the *HER2* copies and assayed in triplicate experiments.

Design of a 6-colour Crystal Digital PCR panel for the detection of HER2 amplification and PIK3CA point mutations:

To assemble the 6-colour Crystal Digital PCR panel, the 3-colour Crystal Digital PCR assay for the detection of *HER2* amplification described above was combined with primers and TaqMan probes targeting the most prevalent *PIK3CA* mutations in breast cancer (Figure 3). *PIK3CA* c.3140A>G (H1047R), c.1624G>A (E542K) and

c.1633G>A (E545K). These mutations were detected using two primer pairs generating short amplicons (<78bp) and three TaqMan probes labelled with the ROX fluorophore. To increase mismatch discrimination, probe length was kept short (<19pb) and a Minor Groove Binder (MGB) group was added to their 3' extremities. The WT *PIK3CA* allele was detected using a 19 base-pair TaqMan-MGB probe labelled with the Yakima Yellow fluorophore and targeting the c.3140A position. The remaining 6th detection channel was used for the detection of PhiX174 bacteriophage DNA as an exogenous internal control using a specific primer pair and an ATTO700 labelled TaqMan probe (Figure 3, Figure 4). The sensitivity of *HER2* amplification detection was evaluated using the 6-colour Crystal Digital PCR panel according to the method described above. *PIK3CA* mutation detection sensitivity was measured on serial dilutions of mutant DNAs in a background of WT DNA. The limit of blank (LOB) was evaluated by running 30 negative control replicates containing 10^4 copies of WT genomic DNA. For more details about setting the LOB and LOD for digital PCR assays, please see https://www.gene-pi.com/item/lob-lod-determination-2/. The decision tree to follow to determine the LOB while setting up an experiment can be viewed in this technical note: https://www.stillatechnologies.com/how-to-characterize-the-limit-of-blank-for-digital-pcr/. An in-depth explanation of the mathematical calculation involved for the determination of LOB is available here: https://www.gene-pi.com/statistical-tools/.

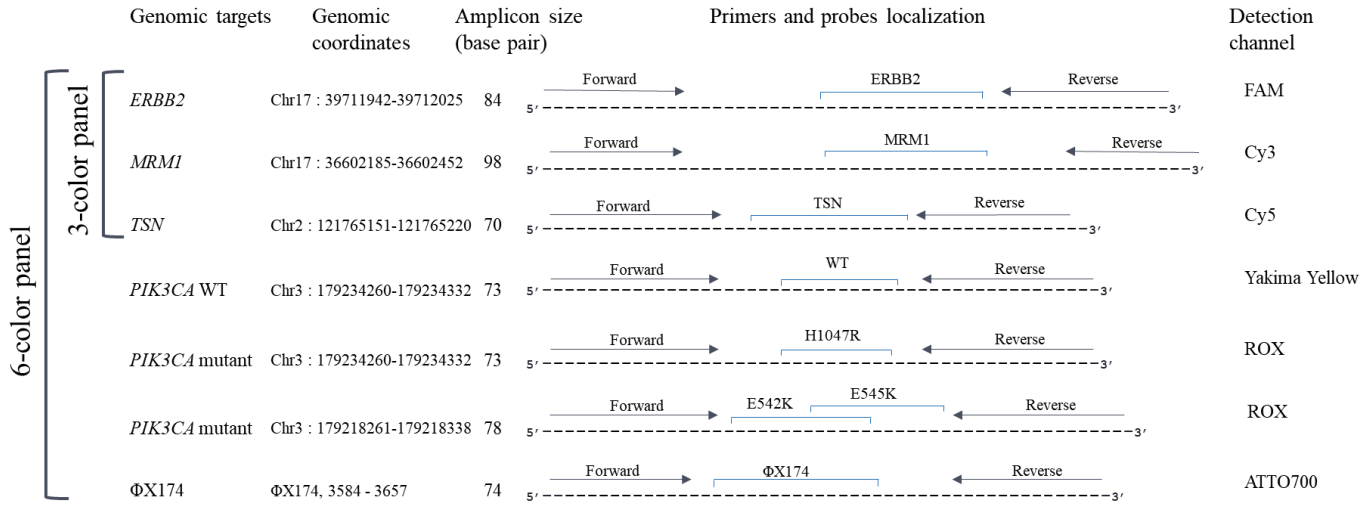


Figure 3: Localisation and design of the 3-colour and the 6-colour Crystal Digital PCR assays for the detection of HER2 copy number variations and the 3 most prevalent PIK3CA mutations in breast cancer.

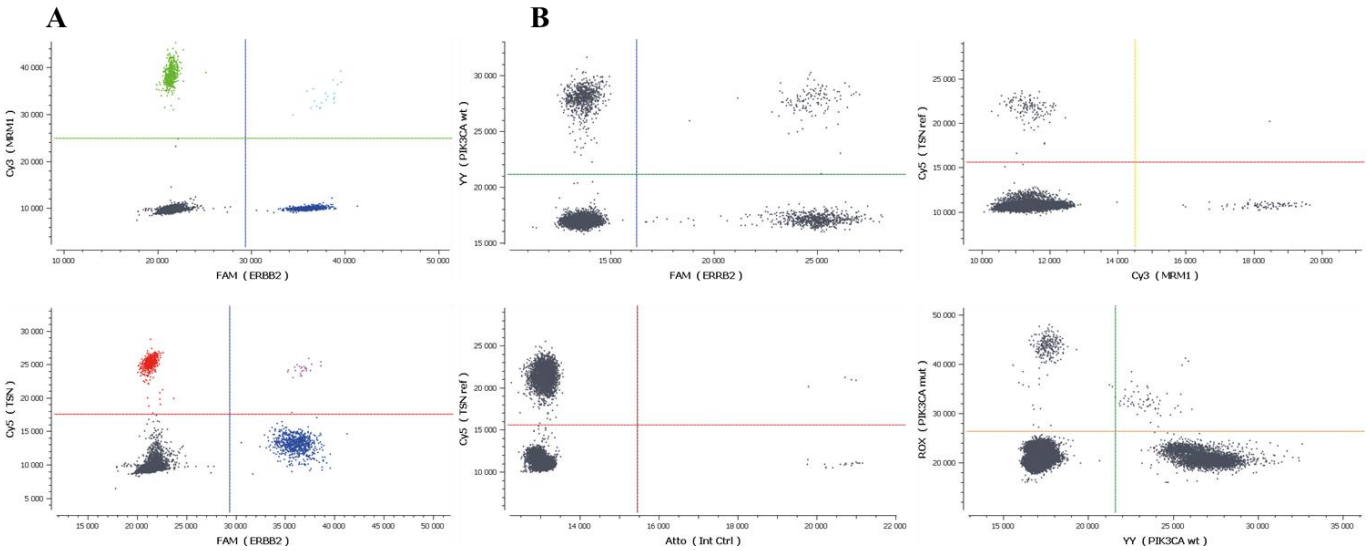


Figure 4: A) 2D dot plots of the 3-colour HER2 digital PCR panel, and B) 2D dot plots of the 6-colour HER2 digital PCR panel. Each target and its respective fluorescent reporter are indicated on the X- and Y-axes.

Results

Evaluation of the 6-colour Crystal Digital PCR panel on reconstruction experiments

Plasma DNAs extracted from breast cancer patients and healthy individuals were quantified and mixed to generate nine composed samples with varying *PIK3CA* mutant fractions and *HER2* copy number variation. The reconstructed samples were blindly tested in triplicate experiments each containing 10 µl of DNA and 500 copies of PhiX174 DNA. Positive and negative control wells containing a known concentration of mutant *PIK3CA* and WT commercial DNA, respectively, were included in each run.

Sensitivity of *HER2* amplification detection using 3-colour Crystal Digital PCR

The experimental *HER2*/*TSN* ratios were obtained by adding a mutant DNA bearing an *HER2*/*TSN* ratio of 10:1 to WT genomic DNA. Samples were assayed in triplicate using the 3-colour Crystal Digital PCR panel and absolute concentrations of *HER2* and *TSN* were calculated. To determine statistically the presence of DNA with *HER2* copy number variation with 95% confidence level (i.e. less than 5% false positives), Welch's

t-test (Whale et al. 2012) was applied on the log-ratio *HER2*/*TSN* (approximated by a Normal distribution) in comparison with 36 negative control replicates. Using this method, a significant presence of mutant DNA in mutant fractions of 3%, corresponding to an *HER2*/*TSN* ratio of 1.16 was observed (Figure 5 A).

Sensitivity of detection of *HER2* amplification and *PIK3CA* mutations using 6-colour Crystal Digital PCR

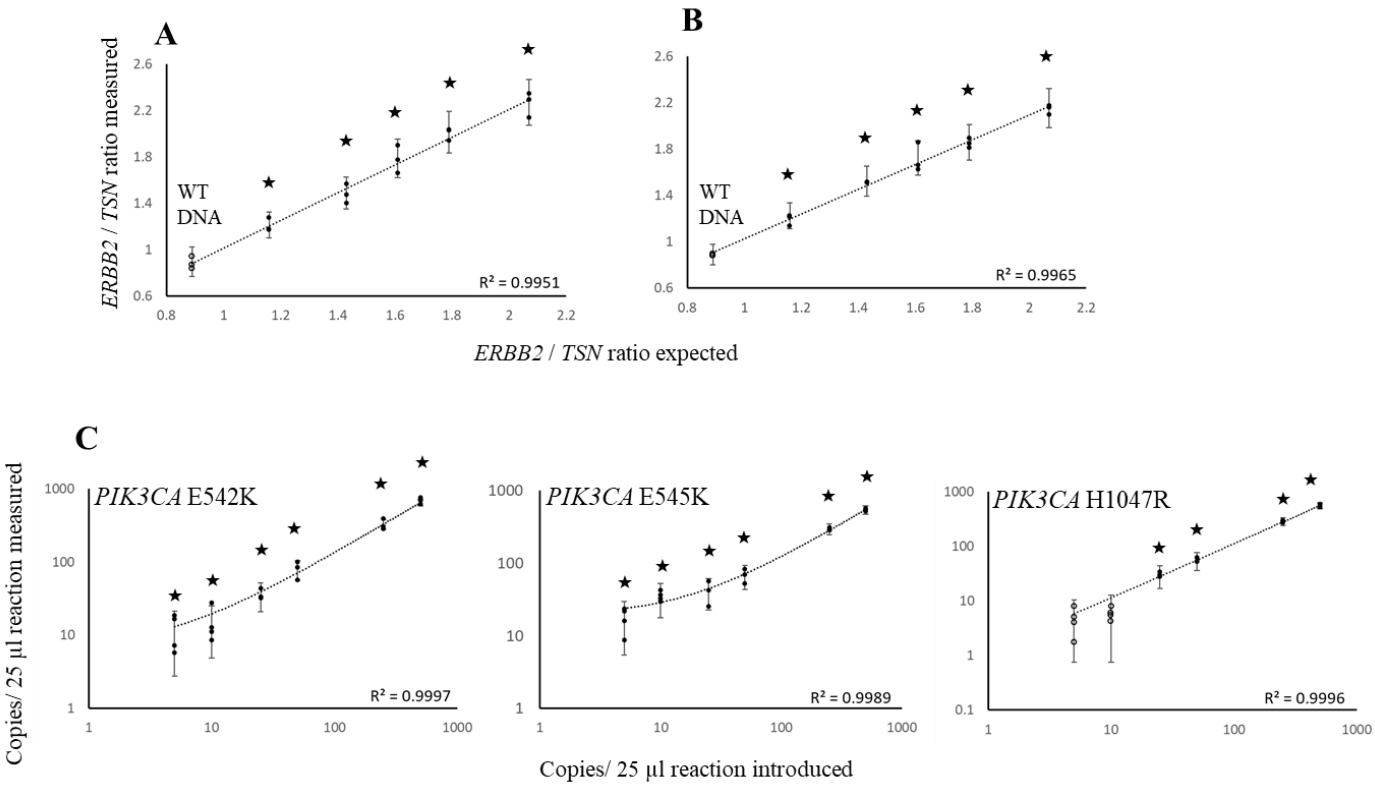
The sensitivity of *HER2* amplification detection using the 6-colour Crystal Digital PCR panel was evaluated using the same approach as described in the 3-colour assays. The 6-colour Crystal Digital PCR panel enabled the detection of mutant fractions as low as 3%, corresponding to an *HER2/TSN* ratio of 1.16 (Figure 5 B). The three most prevalent *PIK3CA* mutations in breast cancer (*PIK3CA* H1047R, E542K and E545K) were detected in the ROX channel of the 6-colour Crystal Digital PCR panel. To calculate the LOB for mutant *PIK3CA* detection, the occurrence of false-positive events observed in negative controls was recorded and the mean (μ) of the false-positive distribution was calculated and corrected using the following formula:

$$\mu corr = \mu + 1.645\ \sigma / \sqrt{R}$$

Where σ is the standard deviation of this distribution and R is the number of negative control replicates. The LOB with a 95% confidence level was determined by fitting the calculated μcorr using Normal Law approximation and Chernoff's inequality. The LOB was thus determined as six false-positive partitions. The sensitivity of detection was determined for each *PIK3CA* mutation using a serial dilution of mutant DNA ranging from 500 to 5 copies per μl in a constant background of 10^4 copies of WT DNA per μl and representing a mutant allele fraction (MAF) ranging from 5% to 0.05%. Each dilution point was assayed in triplicate, except for the lowest mutant concentration that was assayed in quadruplicate. To determine whether the observed mutant quantity was significantly above the LOB, a Bayesian approach based on the probability of occurrence of false positives was used. For each result, the number of positive partitions was corrected by deducting eventual false-positive partitions weighted by their probability distribution. A sample was considered negative when the lower limit of the corrected 95% confidence interval included zero. According to this Bayesian correction, the limit of detection (LOD) at 95% confidence level was experimentally determined as 25 copies per 25 μl reaction for *PIK3CA* H1047R, and 5 copies per 25

µl reaction for E542K and E545K, which represents a minimum detectable MAF of 0.25% and 0.05% respectively in a background of 10^4 WT copies.

Figure 5: A) measure of different HER2/TSN ratios in reconstruction experiments using the 3-colour Crystal Digital PCR assay. B) measure of different HER2/TSN ratios in reconstruction experiments using the 6-colour Crystal Digital PCR assay. C) results obtained using the 6-colour Crystal Digital PCR assay on a serial dilution of mutant PIK3CA in a background of WT DNA (★ indicates that detection is statistically significant, the theoretical 95% confidence intervals are represented as vertical bars, empty circles represent measures which are under the LOB).



Evaluation of the 6-colour Crystal Digital PCR panel on reconstruction experiments

Reconstructed samples (n=9) derived from plasma DNA from breast cancer patients and healthy individuals were assembled to obtain different concentrations of mutant *PIK3CA* and *HER2*/*TSN* ratios. The detection of *PIK3CA* mutations and *HER2* copy number variation was estimated using the statistical tools described earlier. To estimate the *HER2* copy number variation (Figure 5 B), 29 WT DNA controls extracted from plasma were used as controls. The results are shown in Table 1. Sample 9

containing 1.9 mutant *PIK3CA* copies per µl in a background of 348 WT DNA copies per µl (MAF of 0.55%) was detected as positive, whereas Sample 8 containing 0.9 mutant *PIK3CA* copies per µl in a background of 348 WT copies per µl (MAF of 0.26%) was found negative (Figure 5 C). Samples 3, 4 and 6 with expected *HER2*/*TSN* ratios of 1.55, 1.44 and 1.39 were detected positive after digital PCR and statistical analysis. Sample 5, with an expected *HER2*/*TSN* ratio of 1.34 was not considered positive as the lower limit of the 99.5% confidence interval calculated following its testing included zero. No false-positive mutant *PIK3CA* and *HER2* copy number variations were observed in WT DNA only samples.

Table 1: *HER2*/*TSN* ratio and *PIK3CA* mutations detected using the 6-colour Crystal Digital PCR panel in reconstructed samples derived from plasma DNA

Sample no	Mutational status of sample	WT DNA concentration (cp/µl plasma DNA)		*HER2*/*TSN* ratio			*PIK3CA* copies/µl plasma DNA		
		Expected	measured	Expected	Measured	Detected (CL >99.5%)	Expected	measured	Detected (CL>95%)
1	WT	115	92	1.2	1.2	no	0	0	no
2	WT	229	207	1.2	1.3	no	0	0	no
3	*HER2* amplification	229	205	1.55	1.55	yes	0	0	no
4	*HER2* amplification	229	211	1.44	1.56	yes	0	0	no
5	*HER2* amplification	229	208	1.34	1.42	no	0	0	no
6	*HER2* amplification	229	206	1.39	1.42	yes	0	0	no
7	WT	35	32	1.2	1.19	no	0	0	no
8	*PIK3CA* p.E542K	348	352	1.2	1.25	no	0.9	0	no
9	*PIK3CA* p.E545K	348	340	1.2	1.15	no	1.9	0.6	yes

Conclusion

Digital PCR is a promising clinical tool as it combines high sensitivity and absolute quantification with a short time-to-results and a low cost. With the ability to partition samples into tens of thousands of individual reactions, digital PCR allows superior target detection and quantification, enabling accurate detection of rare mutations in low concentration liquid biopsy ctDNA samples and opening new clinical opportunities for this complex mixture of DNA. Here, the 6-colour Crystal Digital PCR technology enabled the monitoring of the main genomic alterations in patients with breast cancer in a first of its kind assay. *PIK3CA* mutations detected were not identified individually in this assay, instead were functionally grouped in separate detection channels based on the current knowledge about treatment efficacy prediction. Although this chapter details a panel for detecting major genomic alterations in breast cancer patients, it can also be used as a model for other oncology analysis developments. The 6-colour

Crystal Digital PCR technology can be flexible and evolutive in terms of assay design. The increase in the number of detection channels and multiplexing strategy, combined with the high sensitivity of Crystal Digital PCR, represents a promising advance for genotyping and tumour monitoring in oncology.

3. Advanced quality control using Crystal Miner

Software description

Crystal Miner is Stilla Technologies' proprietary software component of the Naica System. The Crystal Miner is designed for high-precision genetic data analysis based on Crystal Digital PCR. The Crystal Miner software is a user-friendly and transparent software. It is a powerful tool for statistical analysis of digital PCR data. Additionally, it allows users to visualise the droplets, select the desired droplet populations for analysis and produce reliable and accurate results.

The Crystal Miner software can be downloaded here: https://www.stillatechnologies.com/software/. The software also comes with demo data for first-time users to try different features and analyse data.

Unique characteristics and functions

The Crystal Miner software allows intuitive data visualisation and interpretation. The software offers the droplets to be distinguished in 3-colour channels, and this provides a wide spectrum for the users to visualise the droplets in. The droplets are detected based on the fluorescence emitted by the fluorophores upon hydrolysation or binding to dsDNA. Crystal Miner is compatible with a wide range of fluorescent dyes (non-exhaustive list: FAM, HEX, ROX, Yakima Yellow, Cy3, Cy5, EvaGreen) and accurately detects the targets in the Blue, Green and Red detection channels (Figure 2). The user has the flexibility to choose from these fluorophores and select the ones that seem most compatible with their assay design. The droplets are deemed as "positive" if the fluorescence of the droplet is higher than the defined threshold and "negative" if it is lower. Considering the possibility that a droplet can be either positive or negative in each of the three detection channels, there are $2^3 = 8$ possible ways to classify the droplets. Furthermore, the possibility to visualise the data in 1, 2 or 3-D fluorescence

dot plots (Figures 6, 7 and 8, respectively) allows to accurately analyse the data and interpret it.

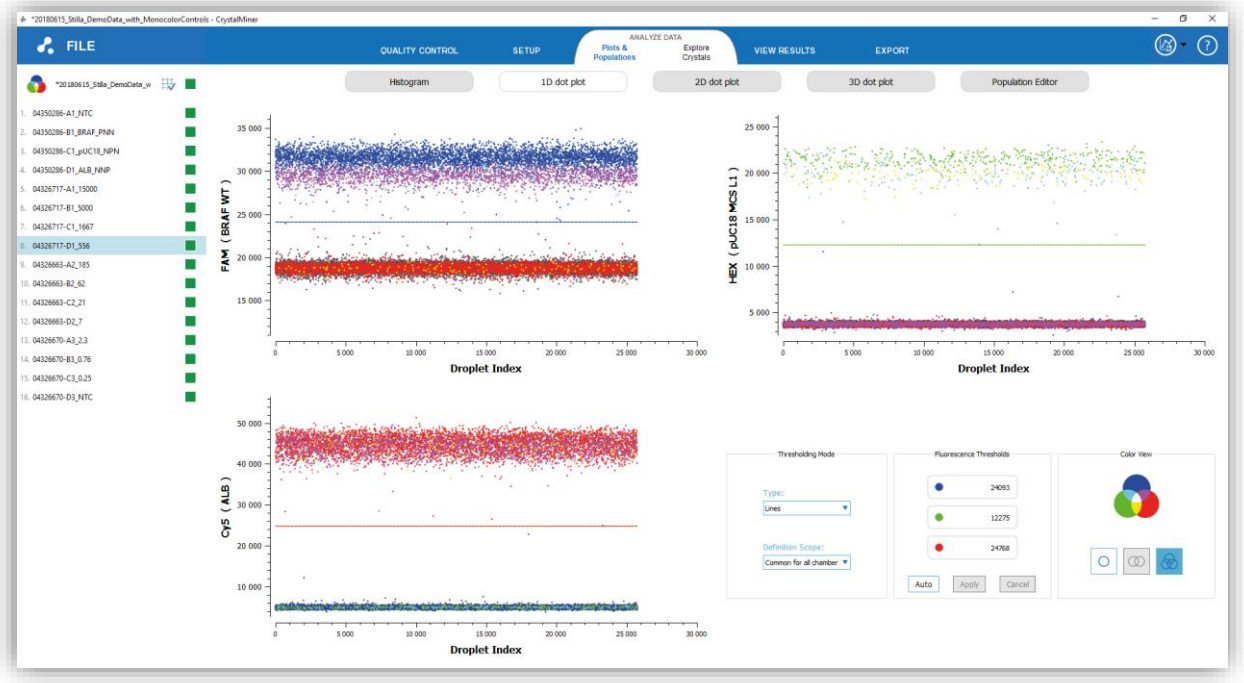


Figure 6: A screenshot of the Crystal Miner software illustrating 1D dot plots in 8-colour mode.

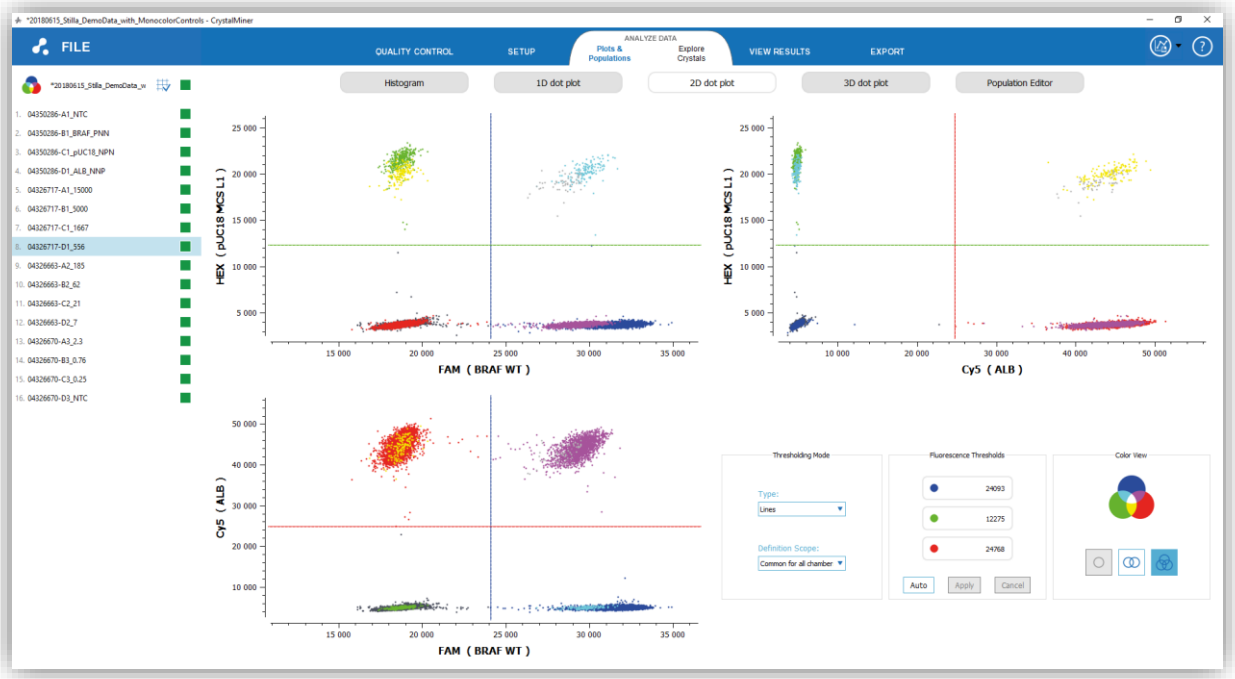


Figure 7: A screenshot of the Crystal Miner software illustrating 2D dot plots in the 8-colour mode.

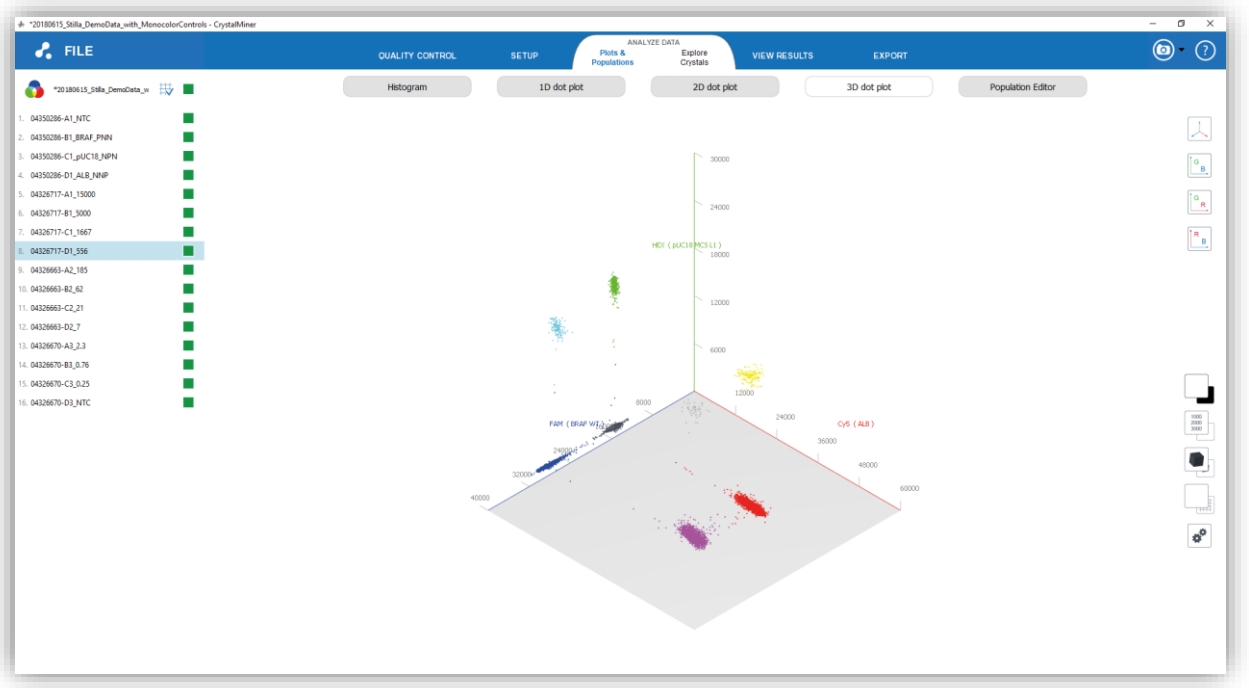


Figure 8: A screenshot of the Crystal Miner software illustrating a 3D dot plot in the 8-colour mode.

Another feature that confers this software reliable is its “white-box” nature with explorable crystal images. This powerful feature aids the user to visualise droplets and confirm that the fluorescence values of the droplets have a homogenous spatial distribution in the crystal layer. The visualisation in different detection channels aids the users to select the droplet populations of interest and analyse them correctly. The software has a range of quality controls in place to ensure high quality and accurate data reporting. For example, quality flags are a feature linked to the white-box nature of Crystal Miner. The quality flags associated with each well are indicative of the quality control at the well level. Ranging from high to moderate and low, they are an instant indication of the quality of the experiment.

Besides, when multiplexing with several hydrolysis probes, it is sometimes possible that a signal from one fluorophore is detected in more than one channel due to spectral overlap, causing a fluorescence spillover or crosstalk. This effect can be seen in the dot plots and may disturb the automatic threshold computation. Using Crystal Miner, fluorescence spillover can be compensated automatically based on mono-colour

controls, easing the thresholds setting. To learn more about spill over compensation see here: https://www.gene-pi.com/item/spill-over-2/.

After compensation (when needed), users can adjust the automatically set thresholds by sliding the corresponding line on the dot plots for the different detection channels, enabling Crystal Miner to distinguish between positive and negative droplets. When a positive population of droplets cannot be separated from the negative population by a line boundary in a 1D dot plot, the Crystal Miner allows the user to define populations by drawing custom zones such as polygons in 2D dot plots. This flexibility to customize populations is another advanced feature of the Crystal Miner software allowing defined populations for specific experimental requirements such as for a drop-off assay, assays based on CRISPR gene-editing, or in cases where multiple targets are colocalised in a single detection channel. Apart from the three default populations, users can choose to define custom populations using the “Zoning” and “Population” defining features. Briefly, using the Zoning feature, the user can divide the 3D fluorescence space into zones, either using the 1D Line threshold or the 2D Polygon threshold modes (Figure 9), and the software automatically calculates the usual results for the populations in these zones. An example where the 2D Polygon thresholding can be used for defining populations is a drop-off assay and a detailed explanation of this thresholding mode can be accessed here:

https://www.stillatechnologies.com/how-to-quantify-drop-off-digital-pcr-assays-with-crystal-miner/

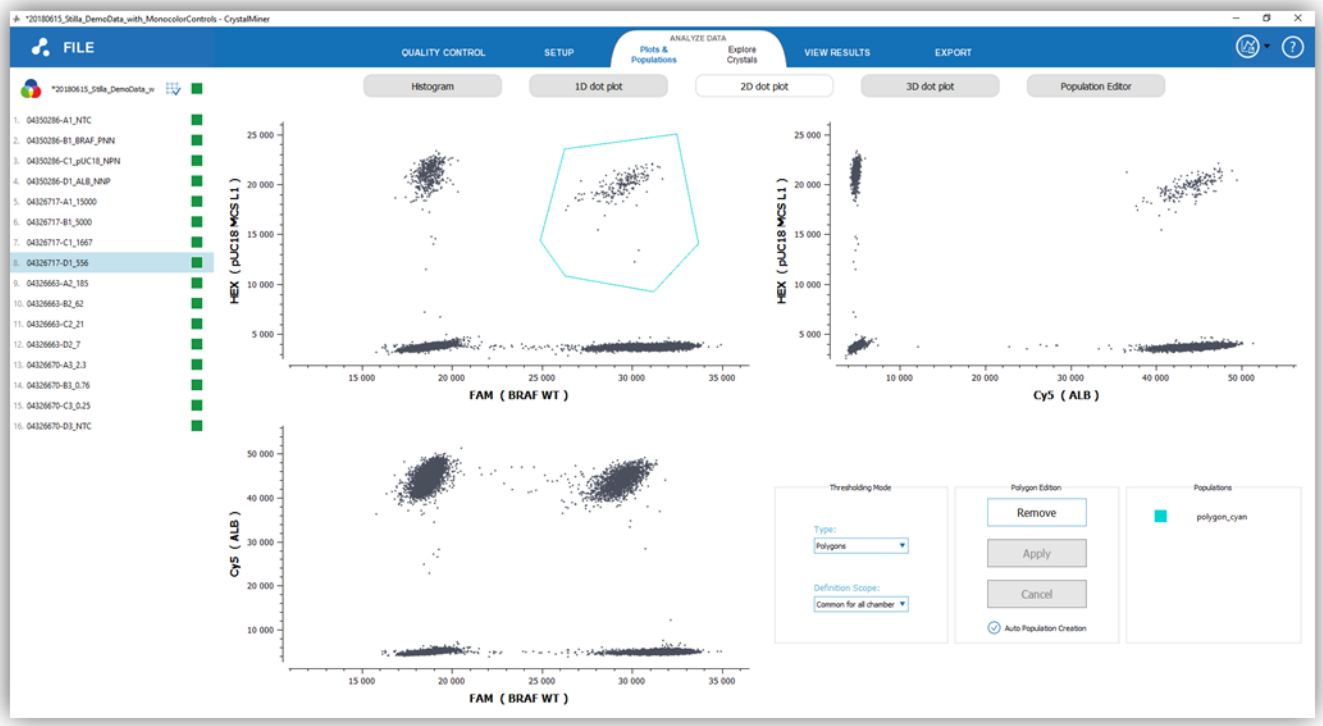


Figure 9. Screenshot of the Crystal Miner illustrating the "Polygon" thresholding mode.

Figure 10. Screenshot of the Crystal Miner illustrating the "population" defining feature.

Further, the "Population" defining feature gives the users the option to manually define complex populations using a building block approach after fluorescence zones have been defined. For example, for a triplex experiment, the user can select or remove the

desired populations, customize the colour of the populations and even add specific negative droplets to be excluded from a population in case of drop-off quantification (Figure 10). The results for the additional populations are then displayed in the Results tab of the Crystal Miner software.

Performance indicators to evaluate the amplification efficiency, quantification precision, and detection sensitivity are also provided by Crystal Miner. Some of the quality control features include the separability score, uncertainty curves and the pooling strategy.

The separability score is an indicator for the quality control and for assay optimization. The separability score is proportional to the distance between the positive and negative droplet clusters and is inversely proportional to the sum of Bessel-corrected standard deviation of each cluster. A higher score points to a good amplification efficiency of the target whereas a lower score indicates that the droplets are ambiguous and have intermediate fluorescence values. A detailed explanation of the mathematical calculations for the separability score is provided in the next section.

Irrespective of the separability score and the thresholding mode, each population is associated to a target or a group of targets whose concentration is estimated based on the ratio of positive and negative droplets for the population. As a result, Crystal Miner provides the estimated concentrations together with their confidence intervals at 95% confidence level. The width of this confidence interval is linked to the uncertainty of the measurement and displayed in the form of uncertainty curves.

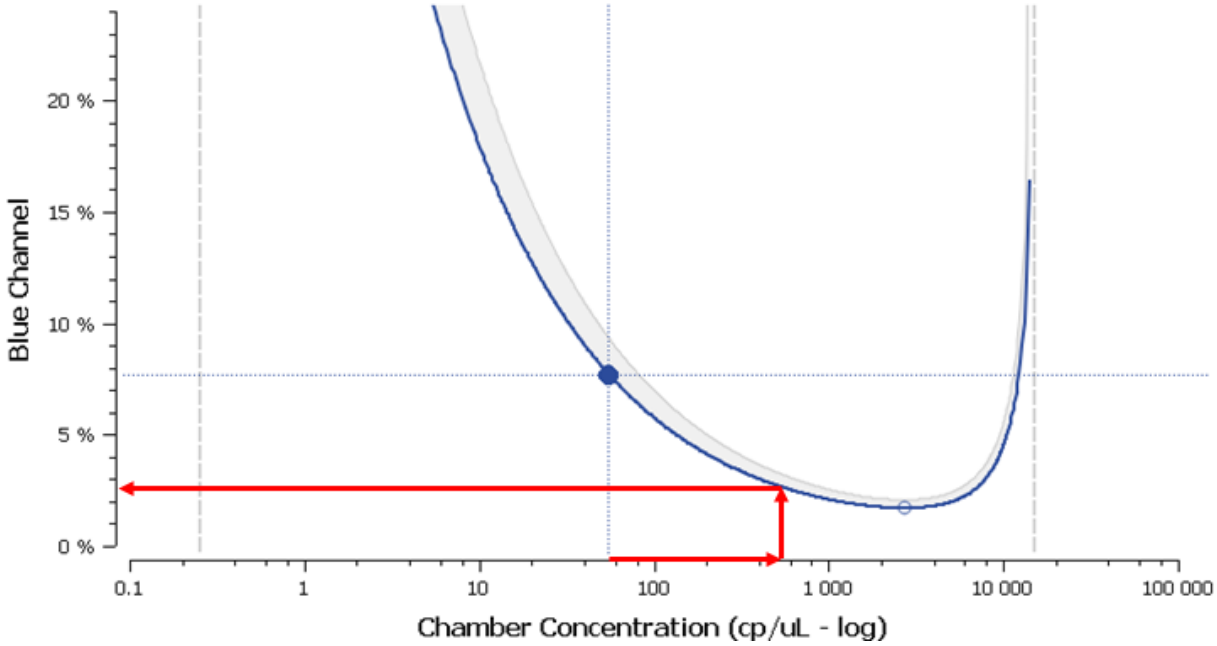


Figure 11: Screenshot of the Crystal Miner illustrating uncertainty curve obtained for a given experiment. In this example, for higher precision, further concentrating the sample would result in a decrease in the uncertainty from 8% to approximately 3%.

Another tool for improving detection sensitivity and quantification precision in case of rare mutations is the possibility to pool wells. Using the pooling feature, users can combine two or more wells containing replicates and increase the analysed volume. The Crystal Miner software automatically calculates the concentration of each target in the pooled wells using the formula based on the sum of positive and negative droplets, briefly:

$$C\ (k\ pooled\ wells) = -\frac{1}{v}\ ln\left(1 - \frac{\sum_{i=1}^{k} p_i}{\sum_{i=1}^{k} N_i}\right).$$

where:

- k refers to the number of pooled wells, assuming that these wells are replicates (e.g. from the same sample)
- v refers to the droplet volume
- N_i refers to the total number of droplets analyzed in well i
- p_i refers to the number of positive droplets observed in well i for the target of interest

An increase in the analysed volume obtained by pooling wells leads to lower sampling and partitioning errors as a consequence of an increase in the total number of droplets analysed, resulting in a lower quantification uncertainty. Assuming the LOB equal to

zero, the formula for LOD indicates that the LOD is inversely proportional to the number of pooled wells. Assuming *N* is the average number of droplets analysed per well, the LOD can be calculated using the formula:

$$LOD\ (k\ pooled\ wells) = \frac{3}{k\ N\ v}$$

4. Guidelines for Digital PCR data analysis

Necessary conditions for a digital PCR experiment to follow Poisson Law

A digital PCR experiment can only be exploited under the assumption that the distribution of the target nucleic acid fragments in the generated partitions follow a Poisson Law, which means that:

- the partition volume v is assumed to be constant;
- the encapsulation of target fragments in the partitions is assumed to occur independent of each other;
- the average number of target fragments encapsulated in a droplet is assumed to be constant and equal to Cv, where C is the expected concentration of target fragments in the digital PCR well.

Checking these assumptions is highly recommended to ensure the reliability of the following Poisson formula which estimates the target concentration in the well:

$$C = -\frac{1}{v}\ ln\left(1 - \frac{p}{N}\right),$$

where N is the number of partitions generated in the well and p is the number of observed positive partitions for the target of interest (i.e. those having encapsulated at least one target fragment).

If the generation of the partitions is performed sequentially through time or in parallel in space, the Poisson Law assumption requires that the fluorescence values of the partition are randomly distributed in time or space, and that the distribution of positive partitions for the target of interest is also random in time or space.

The random distribution assumption can, for example, be compromised in case of fragment sedimentation in the loaded sample, or in case of non-homogeneous temperature heating during the PCR.

The white-box Crystal Miner software allows users to visualise all images of the droplet crystals and to highlight potential droplets associated with rain or unexpected clusters in the dot plot graphs. Such functionalities enable the user to perform quality control for partition volume assumption as well as the random distribution assumption.

Estimation of the theoretical and experimental sources of digital PCR measurement errors

There are two categories of digital PCR measurement errors:

a) The theoretical Poisson error $\varepsilon_{Poisson}$ which cannot be avoided and accounts for both the sampling error and the partitioning error.
b) All the experimental sources of digital PCR measurement errors, which should be mitigated as much as possible. They include the following sources:
    - human manipulation errors (e.g. pipetting errors);
    - biological errors (e.g. target competition, non-specificity, non-optimal amplification efficiency);
    - instrument-related errors (e.g. thermal cycler, fluorescence reader);
    - software-related errors (e.g. partition detection, partition classification by fluorescence thresholding);
    - human interpretation errors (e.g. manual adjustment of data analysis).

The formula for the Poisson error at 95% confidence level is the following:

$$\varepsilon_{Poisson}(95\%) = \frac{C_{max}(95\%) - C_{min}(95\%)}{2\,C}$$

where, using the notations defined in the previous section:

$$C = -\frac{1}{v}\, ln\left(1 - \frac{p}{N}\right)$$

$$C_{min}(95\%) = -\frac{1}{v} \ln\left[1 - \left[\frac{p}{N} - 1.96\sqrt{\frac{\frac{p}{N}\left(1-\frac{p}{N}\right)}{N}}\right]\right]$$

$$C_{max}(95\%) = -\frac{1}{v}\ln\left[1 - \left[\frac{p}{N} + 1.96\sqrt{\frac{\frac{p}{N}\left(1-\frac{p}{N}\right)}{N}}\right]\right]$$

The total variability of digital PCR measurements can be evaluated by performing a sufficiently large number of pure digital PCR replicates (e.g. by loading the same sample in at least $n = 30$ digital PCR wells), by collecting the target concentration values measured by the digital PCR system, and by calculating their coefficient of variation corrected with Bessel's correction:

$$c_v = \frac{\sigma}{\mu}\sqrt{\frac{n}{n-1}}$$

Finally, the experimental relative uncertainty at 95% confidence level can be deduced by subtracting the theoretical Poisson error from the measured total variability:

$$\varepsilon_{Experimental}(95\%) = 1.96\, c_v - \varepsilon_{Poisson}(95\%)$$

Optimization of multiplex digital PCR assays from data processing

Optimization of multiplex digital PCR assays requires maximizing the amplification efficiency of each target of the assay while avoiding target competition and non-specificity issues. If k targets are involved in the multiplex assay, it is recommended to first optimize a simplex experiment for each target, then optimize the possible duplex experiments, triplex experiments, and so forth until the wished k-plex experiment.

The optimization parameters include the choice and the concentration of the primers and probes for each target, as well as the PCR conditions (number of PCR cycles, the duration and temperature for the denaturation step, the annealing step and the elongation step).

One relevant and objective criterion to quantify the amplification efficiency for a target of interest is the separability score between two clusters of points:

- the "positive" cluster of points representing the fluorescence of the positive partitions (i.e. the partitions which have encapsulated at least one target fragment);

- the “negative” cluster of points representing the fluorescence of the negative partitions (i.e. the partitions which did not encapsulate any target fragment).

The formula of the separability score for the target of interest is the following:

$$separability = \frac{\mu_{POS} - \mu_{NEG}}{\sqrt{\frac{p}{p-1}}\,\sigma_{POS} + \sqrt{\frac{q}{q-1}}\,\sigma_{Neg}}$$

where:

- p is the number of positive partitions observed for the target of interest;
- q is the number of negative partitions observed for the target of interest;
- μ_{POS} is the mean fluorescence value of the positive partitions;
- μ_{NEG} is the mean fluorescence value of the negative partitions;
- σ_{POS} is the standard deviation of the fluorescence values of the positive partitions;
- σ_{NEG} is the standard deviation of the fluorescence values of the negative partitions.

In order to optimize a k-plex experiment, the objective is to maximize the separability score for each of the k targets.

Finally, the recommended optimization process is as follows:

- Modify only one parameter at a time;
- In order to determine if the new conditions are better than the current ones, perform at least $n = 30$ pure replicates of the current conditions and at least $n = 30$ pure replicates of the new conditions, then apply for each of the k targets then the Mann–Whitney U test to the separability scores obtained for the targets using the current conditions versus the new conditions (using one-tailed hypothesis and 0.05 significance level);
- If the Mann–Whitney U tests indicate that the new conditions lead to equivalent or statistically higher separability scores for all the targets, then the new conditions do not cause regression and can be kept for a further iteration.

- A separability score of 4 is the lowest acceptable value for any target of interest. We recommend further assay optimization when scores lower than 4 are obtained in order to ensure confident fluorescence threshold placement.

Evaluating the linearity performance of a digital PCR assay:

In order to assess the linearity performance of a digital PCR assay with respect to a given target, it is recommended to perform an m-point dilution range of the target (ideally with $m \geq 5$) along the whole dynamic range of detection specified for the digital PCR system, and to include a negative control point for quality control. Besides, each dilution point should be performed in at least $n = 3$ replicates to ensure a robust evaluation of the linearity.

For example, the Naica System offers a dynamic range of detection from 0.2 cp/µL to 20 000 cp/µl in the digital PCR well, so a suitable linearity study could include the target concentrations 20 000, 2 000, 200, 2, 0.2 and 0 cp/µl.

The standard linear regression, based on the Ordinary Least Squares (OLS) method, assumes homoscedasticity, i.e. the assumption that the variance of the errors (or residues of the regression) is constant across all points in the dilution range. However, this assumption is false for digital PCR, because the variance of the errors is much larger at very low concentrations and at very high concentrations. This non-homogeneity of the variance is called heteroskedasticity.

The recommended methodology consists in applying a robust Weighted Least Squares method (WLS method), which considers as weight for each dilution point the inverse of the variance of the replicates at this dilution point. The higher the coefficient of determination R^2 obtained for the regression, the better the linearity. However, the most important criterion is not the R^2 value but the fact that there is a straight line which passes close enough to each point of the dilution range, with a slope as close as possible to the value 1, and ideally, a bias as close as possible to the value 0.

For more details, please see https://www.stillatechnologies.com/detection-of-her2-copy-number-variation-with-crystal-digital-tm-pcr/. An online calculator to determine CNV from a digital PCR experiment is also available here: https://www.gene-pi.com/statistical-tools/cnv/.

Conclusion

In a nutshell, Crystal Digital PCR technology can be implemented and optimized for accurate and reliable detection of genetic alterations in a broad range of nucleic acid quantification assays. Additionally, the Crystal Miner software, equipped with advanced quality control tools, enables users to robustly identify target mutations in samples of varying origins. Crystal Digital PCR, coupled with the Crystal Miner software, is a high precision nucleic acid quantification tool that can aid researchers working in various nucleic acid testing fields to explore new applications.

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References

André, Fabrice, Sara Hurvitz, Angelica Fasolo, Ling Ming Tseng, Guy Jerusalem, Sharon Wilks, Ruth O'Regan, et al. 2016. "Molecular Alterations and Everolimus Efficacy in Human Epidermal Growth Factor Receptor 2-Overexpressing Metastatic Breast Cancers: Combined Exploratory Biomarker Analysis from BOLERO-1 and BOLERO-3." *Journal of Clinical Oncology* 34 (18): 2115–24. https://doi.org/10.1200/JCO.2015.63.9161. PMID: 27091708

Araki, Kazuhiro, and Yasuo Miyoshi. 2018. "Mechanism of Resistance to Endocrine Therapy in Breast Cancer: The Important Role of PI3K/Akt/MTOR in Estrogen Receptor-Positive, HER2-Negative Breast Cancer." *Breast Cancer*. Springer Tokyo. https://doi.org/10.1007/s12282-017-0812-x. PMID: 29086897

Ferlay, Jacques, Isabelle Soerjomataram, Rajesh Dikshit, Sultan Eser, Colin Mathers, Marise Rebelo, Donald Maxwell Parkin, David Forman, and Freddie Bray. 2015. "Cancer Incidence and Mortality Worldwide: Sources, Methods and Major Patterns in GLOBOCAN 2012." *International Journal of Cancer* 136 (5): E359–86. https://doi.org/10.1002/ijc.29210. PMID: 25220842

Jovelet, C., E. Ileana, M.-C. Le Deley, N. Motte, S. Rosellini, A. Romero, C. Lefebvre, et al. 2016. "Circulating Cell-Free Tumor DNA Analysis of 50 Genes by Next-Generation Sequencing in the Prospective MOSCATO Trial." *Clinical Cancer Research* 22 (12): 2960–68. https://doi.org/10.1158/1078-0432.CCR-15-2470. PMID: 26758560

Jovelet, Cécile, Jordan Madic, Jordi Remon, Aurélie Honoré, Romain Girard, Etienne Rouleau, Barbara André, Benjamin Besse, Magali Droniou, and Ludovic Lacroix. 2017. "Crystal Digital Droplet PCR for Detection and Quantification of Circulating EGFR Sensitizing and Resistance Mutations in Advanced Non-Small Cell Lung Cancer." *PloS One* 12 (8): e0183319. https://doi.org/10.1371/journal.pone.0183319. PMID: 28829811

Koboldt, Daniel C., Robert S. Fulton, Michael D. McLellan, Heather Schmidt, Joelle Kalicki-Veizer, Joshua F. McMichael, Lucinda L. Fulton, et al. 2012. "Comprehensive Molecular Portraits of Human Breast Tumours." *Nature* 490 (7418): 61–70. https://doi.org/10.1038/nature11412. PMID: 23000897

Kurosky, Samantha K., Debanjali Mitra, Giovanni Zanotti, and James A. Kaye. 2018. "Treatment Patterns and Outcomes of Patients With Metastatic ER + /HER-2 − Breast Cancer: A Multicountry Retrospective Medical Record Review." *Clinical*

Breast Cancer 18 (4): e529–38. https://doi.org/10.1016/j.clbc.2017.10.008. PMID: 29199086

Madic, Jordan, Cécile Jovelet, Julien Lopez, Barbara André, Jean Fatien, Isabelle Miran, Aurélie Honoré, et al. 2019. "Correction: *EGFR* C797S, *EGFR* T790M and *EGFR* Sensitizing Mutations in Non-Small Cell Lung Cancer Revealed by Six-Color Crystal Digital PCR." *Oncotarget*. https://doi.org/10.18632/oncotarget.26686. PMID: 30863495

O'Leary, Ben, Sarah Hrebien, James P. Morden, Matthew Beaney, Charlotte Fribbens, Xin Huang, Yuan Liu, et al. 2018. "Early Circulating Tumor DNA Dynamics and Clonal Selection with Palbociclib and Fulvestrant for Breast Cancer." *Nature Communications* 9 (1). https://doi.org/10.1038/s41467-018-03215-x. PMID: 29497091

Olsson, Eleonor, Christof Winter, Anthony George, Yilun Chen, Jillian Howlin, Man-Hung Eric Tang, Malin Dahlgren, et al. 2015. "Serial Monitoring of Circulating Tumor DNA in Patients with Primary Breast Cancer for Detection of Occult Metastatic Disease." *EMBO Molecular Medicine* 7 (8): 1034. https://doi.org/10.15252/EMMM.201404913. PMID: 25987569

Oshiro, Chiya, Naofumi Kagara, Yasuto Naoi, Masafumi Shimoda, Atsushi Shimomura, Naomi Maruyama, Kenzo Shimazu, Seung Jin Kim, and Shinzaburo Noguchi. 2015. "PIK3CA Mutations in Serum DNA Are Predictive of Recurrence in Primary Breast Cancer Patients." *Breast Cancer Research and Treatment* 150 (2): 299–307. https://doi.org/10.1007/s10549-015-3322-6. PMID: 25736040

Page, K., N. Hava, B. Ward, J. Brown, D. S. Guttery, C. Ruangpratheep, K. Blighe, et al. 2011. "Detection of HER2 Amplification in Circulating Free DNA in Patients with Breast Cancer." *British Journal of Cancer* 104 (8): 1342–48. https://doi.org/10.1038/bjc.2011.89. PMID: 21427727

Pinheiro, Leonardo, and Kerry R. Emslie. 2018. "Basic Concepts and Validation of Digital PCR Measurements." In *Methods in Molecular Biology*, 1768:11–24. Humana Press Inc. https://doi.org/10.1007/978-1-4939-7778-9_2. PMID: 29717435

Rodriguez, Claudia, Voichita Suciu, Audrey Poterie, Ludovic Lacroix, Isabelle Miran, Amélie Boichard, Suzette Delaloge, et al. 2016. "Concordance between HER-2 Status Determined by QPCR in Fine Needle Aspiration Cytology (FNAC) Samples Compared with IHC and FISH in Core Needle Biopsy (CNB) or Surgical

Specimens in Breast Cancer Patients." *Molecular Oncology* 10 (9): 1430–36. https://doi.org/10.1016/j.molonc.2016.07.009. PMID: 27555543

Slamon, Dennis, Wolfgang Eiermann, Nicholas Robert, Tadeusz Pienkowski, Miguel Martin, Michael Press, John Mackey, et al. 2011. "Adjuvant Trastuzumab in HER2-Positive Breast Cancer." *The New England Journal of Medicine* 365 (14): 1273–83. https://doi.org/10.1056/NEJMoa0910383. PMID: 21991949

Speicher, Michael R., and Klaus Pantel. 2014. "Tumor Signatures in the Blood." *Nature Biotechnology*. Nature Publishing Group. https://doi.org/10.1038/nbt.2897. PMID: 24811515

Weisman, Paul S., Charlotte K.Y. Ng, Edi Brogi, Rachel E. Eisenberg, Helen H. Won, Salvatore Piscuoglio, Maria R. De Filippo, et al. 2016. "Genetic Alterations of Triple Negative Breast Cancer by Targeted Next-Generation Sequencing and Correlation with Tumor Morphology." *Modern Pathology* 29 (5): 476–88. https://doi.org/10.1038/modpathol.2016.39. PMID: 26939876

Whale, Alexandra S., Jim F. Huggett, Simon Cowen, Valerie Speirs, Jacqui Shaw, Stephen Ellison, Carole A. Foy, and Daniel J. Scott. 2012. "Comparison of Microfluidic Digital PCR and Conventional Quantitative PCR for Measuring Copy Number Variation." *Nucleic Acids Research* 40 (11): e82–e82. https://doi.org/10.1093/nar/gks203. PMID: 22373922

Yu, Dandan, Yan Tong, Xinnian Guo, Lingfang Feng, Zhaoqiang Jiang, Shibo Ying, Junlin Jia, et al. 2019. "Diagnostic Value of Concentration of Circulating Cell-Free DNA in Breast Cancer: A Meta-Analysis." *Frontiers in Oncology*. Frontiers Media S.A. https://doi.org/10.3389/fonc.2019.00095. c