ESR1 Mutation Detection in FFPE and Plasma-Derived DNA From Breast Cancer Research Samples Using the QX700™ HT Droplet Digital™ (ddPCR™) System

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Note

This study was originally carried out on the Nio+™ Digital PCR System, which supports seven optical detection channels with the following fluorophores: FAM, Yakima Yellow, ATTO 550, ROX, Cy5, ATTO 700/Cy5.5, and DY-521-XL. The QX700 HT Droplet Digital PCR System (Bio-Rad Laboratories, Inc., catalog #17010628) also supports seven fluorophores, but with a different fluorophore set: FAM, HEX, ROX, ATTO 590, Cy5, Cy5.5, and ATTO 550. Despite the optical difference in two of the fluorophores, the core digital PCR (dPCR) principles of droplet partitioning, endpoint fluorescence detection, and absolute quantification remain equivalent between platforms. Accordingly, the study's findings are applicable to workflows implemented on the QX700 HT ddPCR System.

The *ESR1* 12-plex assay used in this study was replaced by the ddPLEX CrystalTag ESR1 17 mut ddPCR Assay available from Bio-Rad Laboratories (#12026887, #12026888). Mutation detection coverage differs. This study also used Nio Crystal Universal Reporters, formulated to match the fluorescent channel specifications of the Stilla™ Nio Digital PCR System. For equivalent performance when running CrystalTag Assays across all seven channels of the QX700 Droplet Digital PCR System, QX700 CrystalTag Reporters 7 (Bio-Rad, #12027244, #12027265) should be used instead.

This application note describes research-use-only workflows and is not intended to establish clinical performance, diagnostic utility, or applications for patient management.

Key Findings

- The all-in-one QX700 HT Droplet Digital PCR System and associated microfluidic consumable, the RDG16 Cartridge, proved suitable for high-order ddPCR multiplexing approaches, such as color-combination multiplexing, while reducing hands-on time to results
- The *ESR1* 12-plex assay, based on a color-combination approach, was successfully applied for simultaneous monitoring of 10 *ESR1* mutations, one *AKT1* mutation, and a reference target in a single test in research settings
- This hyperplexing approach enabled rapid and robust data analysis using population-based target identification and quantification after setting a single threshold for each fluorescence channel
- This highly multiplexed assay was compatible with plasma and FFPE DNA research samples and demonstrated strong concordance and mutant allele frequency (MAF) correlation with a previous digital PCR study performed using sequential lower multiplex assays

Introduction

Breast cancer is the most diagnosed cancer worldwide representing one in every six cancer-related deaths in women.[1] The majority of breast cancers are characterized by hormone receptor overexpression (HR+ breast cancer), and a strategy of hormone deprivation through endocrine therapy is a commonly used treatment approach. Intrinsic and acquired resistance to endocrine therapy is an inflection point in disease development, resulting in relapse and metastasis.[2]

A key resistance mechanism to endocrine therapy is characterized by the constitutive activity of the estrogen receptor alpha due to mutations in the estrogen receptor 1 (*ESR1*) gene encoding this receptor. *ESR1* mutations have been associated with reduced response to certain endocrine therapies and particularly to aromatase inhibition.[3] *ESR1* mutations are emerging clinical biomarkers in metastatic hormone-positive breast cancer. Recent clinical studies have evaluated *ESR1* mutation monitoring in liquid biopsy samples during treatment and have reported associations between emerging *ESR1* mutations and endocrine therapy resistance.[4]

The QX700 HT Droplet Digital PCR System, ddPLEX CrystalTag ESR1 17 mut ddPCR Assay, and QX700 CrystalTag Reporters 7 mentioned in this app note are for Research Use Only. Not for use in diagnostic procedures.

Due to its high sensitivity and high multiplexing capabilities, ddPCR analysis using the QX700 HT ddPCR System provides detection and quantification of *ESR1* mutations in research liquid biopsy samples and may be useful for biomarker research applications.

To detect and quantify the most frequent *ESR1* mutations in a single test in research settings, a highly multiplexed ddPCR assay was developed: the *ESR1* 12-plex assay. Formalin-fixed, paraffin-embedded (FFPE) DNA and plasma cell-free DNA (cfDNA) samples from breast cancer research samples were analyzed using this assay with the QX700 HT ddPCR System, and the results were compared to previous dPCR results obtained in a sequential strategy at Centre Eugène Marquis.[5]

Methods and Results

Color-Combination Multiplexing Workflow

The QX700 HT ddPCR System is an all-in-one platform for ddPCR analysis capable of simultaneously quantifying multiple biomarkers in a single research sample, simplifying the detection process, minimizing detection variability, and significantly reducing hands-on time and time to results. With seven detection channels, the QX700 HT ddPCR System is suitable for applying high-order multiplexing approaches such as the color-combination strategy.

In the color-combination strategy, each target is encoded by a unique combination of two or more distinct fluorophores, or colors. When two colors are used to detect each target, the maximum multiplexing level is 15-plex with a six-color detection system and 21-plex with a seven-color detection system (Figure 1). This approach has recently been successfully applied using digital PCR systems.[6]

To analyze a color-combination assay, the user is required to adjust only a single threshold for each channel to separate all positive droplets from the negative droplets. Based on this 1D thresholding, QX700 ddPCR System Analysis Software can co-localize fluorescence within the same droplet and thus assign and quantify the presence of a population previously defined with the corresponding color code in the population editing tool. Figure 2 illustrates the population configuration, enabling easy analysis in assays designed using color combination. For a deep dive on how to multiplex with color combination, refer to the note How to Multiplex With Color Combination Using ddPLEX CrystalTag and Droplet Digital PCR (ddPCR) Technologies (bulletin 4000).

Color-combination approach, 21-plex

Target	FAM	HEX	ROX	ATTO 590	Cy5	Cy5.5	ATTO 550
1	✔	✔					
2	✔		✔				
3	✔			✔			
4	✔				✔		
5	✔					✔	
6	✔						✔
7		✔	✔				
8		✔		✔			
9		✔			✔		
10		✔				✔	
11		✔					✔
12			✔	✔			
13			✔		✔		
14			✔			✔	
15			✔				✔
16				✔	✔		
17				✔		✔	
18				✔			✔
19					✔	✔	
20					✔		✔
21						✔	✔

Fig. 1. Multiplexing capability using a color-combination approach in research settings. Each target is assigned a unique combination of fluorescent channels.

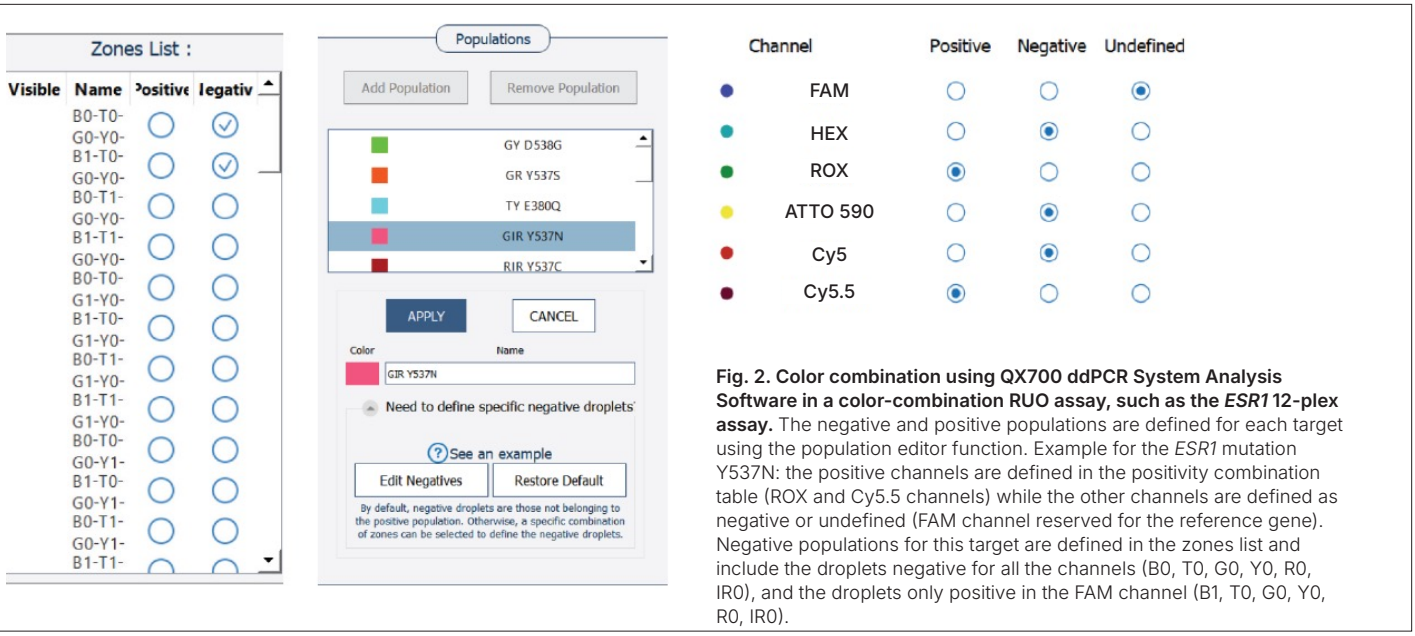


Fig. 2. Color combination using QX700 ddPCR System Analysis Software in a color-combination RUO assay, such as the *ESR1* 12-plex assay. The negative and positive populations are defined for each target using the population editor function. Example for the *ESR1* mutation Y537N: the positive channels are defined in the positivity combination table (ROX and Cy5.5 channels) while the other channels are defined as negative or undefined (FAM channel reserved for the reference gene). Negative populations for this target are defined in the zones list and include the droplets negative for all the channels (B0, T0, G0, Y0, R0, IR0), and the droplets only positive in the FAM channel (B1, T0, G0, Y0, R0, IR0).

ESR1 12-Plex Assay* Design and Analytical Validation

The *ESR1* 12-plex assay is based on the color-combination approach and is designed using CrystalTag technology to enable the simultaneous detection and quantification of 12 targets in the same reaction in research settings. Ten somatic *ESR1* mutations are detected, in color combination, across three exons: exon 5 (E380Q), exon 7 (S463P), and exon 8 (L536H, L536P, L536R, Y537C, Y537D, Y537N, Y537S, D538G).

One additional mutation in exon 2 of the *AKT1* gene (E17K) is detected in a single color (Cy5 channel), while a reference gene (TSN), used for total DNA quantification and MAF calculation, is isolated in a sixth color channel (FAM) to maintain assay sensitivity. The targeted mutations and assay configuration are shown in Table 1.

The ability of the *ESR1* 12-plex assay to detect simultaneously 12 targets in the same research sample was first assessed with synthetic DNA using the QX700 HT ddPCR System and RDG16 Cartridge (Figure 3).

Table 1. Gene and mutations targeted by the *ESR1* 12-plex assay and assay configuration in research settings.

Gene	Mutation	Base Changes	Cosmic	Channels Used
TSN	Ref. gene	N/A	ID N/A	FAM
	E380Q	c.1138G>C	COSM3829320	HEX, ATTO 590
	S463P	c.1387T>C	COSM4771561	HEX, Cy5
	L536H	c.1607T>A	COSM6843697	ATTO 590, Cy5.5
	L536P	c.1607T>C	COSM6906109	ATTO 590, Cy5
	L536R	c.1607T>G	COSM4774826	HEX, ROX
ESR1	Y537C	c.1610A>G	COSM1074637	CY5, Cy5.5
	Y537D	c.1609T>G	COSM6918757	HEX, Cy5.5
	Y537N	c.1609T>A	COSM1074635	ROX, Cy5.5
	Y537S	c.1610A>C	COSM1074639	ROX, Cy5
	D538G	c.1613A>G	COSM94250	ROX, ATTO 590
AKT1	E17K	c.49G>A	COSM33765	Cy5

N/A, not applicable.

*The *ESR1* 12-plex assay was replaced by the ddPLEX CrystalTag ESR1 17 mut ddPCR Assay (#12026887, #12026888). Mutation detection coverage differs.

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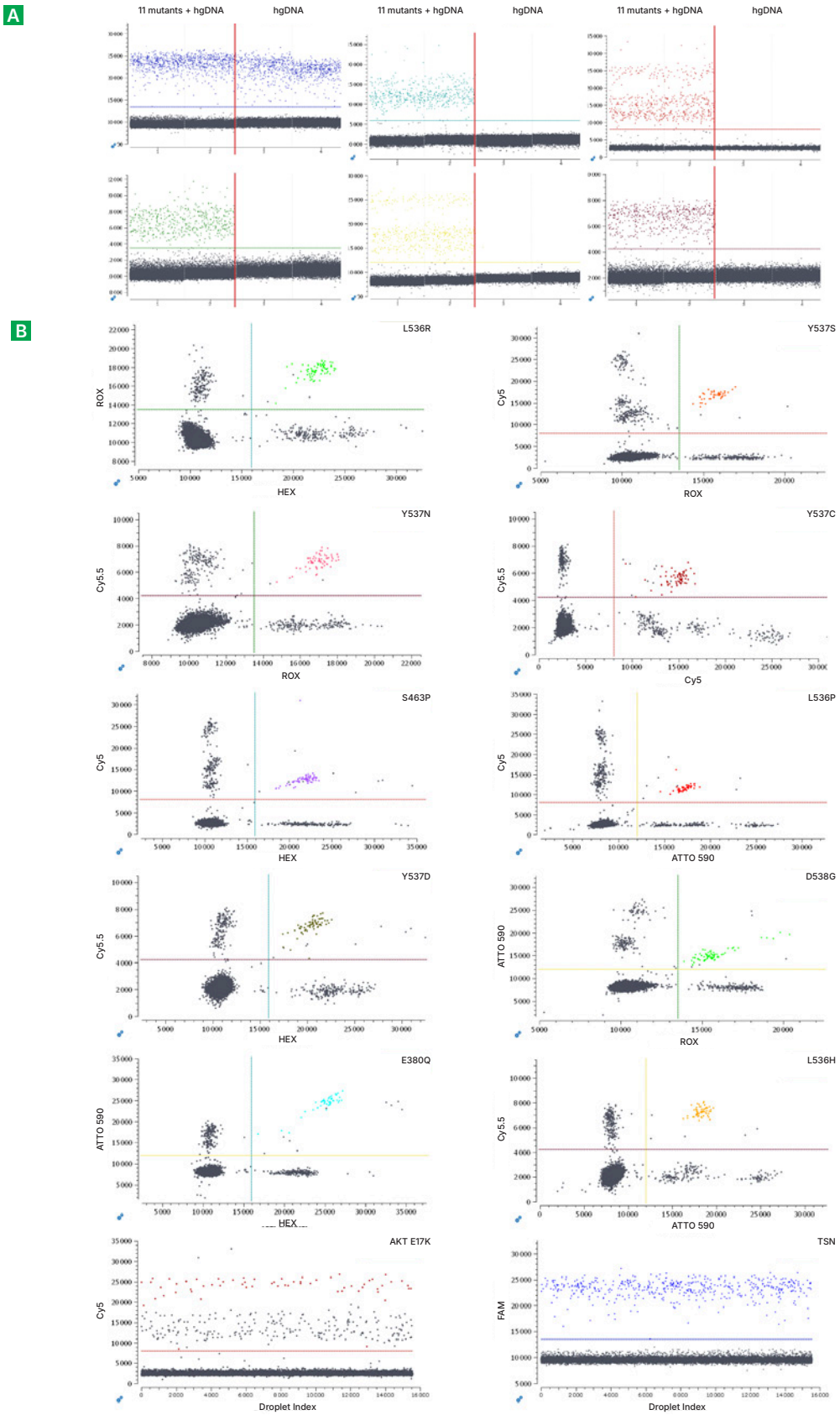


Fig. 3. Analytical validation of the *ESR1* 12-plex assay with synthetic DNA in research settings. A, 1D plots in the six detection channels. Chambers 1–2: positive control = hgDNA (≈ 150 cp/µL) + 11 mutants (≈ 20 cp/µL each) targeted in this assay. Chambers 3–4: negative control = only hgDNA (≈ 150 cp/µL). **B,** visualization of the populations in 1D and 2D plots using QX700 ddPCR System Analysis Software. cp, copies.

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Assay Sensitivity

To determine the analytical sensitivity of each *ESR1* and *AKT1* mutation, the limit of blank (LOB) and limit of detection (LOD) were determined according to the Clinical and Laboratory Standards Institute (CLSI) EP17-A2 standard (Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline).[7] Table 2 shows the theoretical MAF that can be detected with this assay as a function of sample concentration, given that (i) the LOB for the 11 targeted mutations ranges from 0 to 0.27 copies (cp)/µL and the LOD ranges from 0.99 to 1.38 cp/µL (Table 3), and (ii) the RDG16 Cartridge reaction volume is 5 µL per chamber, of which 3.6 µL corresponds to sample volume.

As recommended in the Clinical and Laboratory Standards Institute (CLSI) EP17-A2 standard (Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline), repeat analysis of mutations near the LOD allows confirmation of the result by assessing its repeatability. QX700 ddPCR System Analysis Software supports ultra-high-sensitivity applications (for example, MAF below 0.1%) by pooling two or more replicates to increase the total sample volume analyzed. LOB and LOD values were therefore also determined for two and three pooled RDG16 Cartridge chambers (Table 3). These results showed increased analytical sensitivity when three RDG16 Cartridge chambers were pooled, resulting in LOD values below 0.80 cp/µL for all targets.

Table 2. Theoretical MAF LOD that can be detected with the *ESR1* 12-plex assay in an RDG16 Cartridge for typical sample concentrations.

Sample concentration, ng/µL	Theoretical MAF LOD for One RDG16 Chamber, %
3.5–4	0.12–0.15
3–3.5	0.14–0.18
2.5–3	0.16–0.22
2–2.5	0.20–0.27
1.5–2	0.27–0.36
1–1.5	0.41–0.54
0.5–1	0.82–1.08
0.25–0.5	1.64–2.15

LOD, limit of detection; MAF, mutant allele frequency.

Table 3. LOB and LOD values for the *ESR1* 12-plex assay, determined according to the CLSI EP17-A2 standard (RDG16 Cartridge chamber concentrations in cp/µL).

Number of Chambers LOB/LOD Pooled		Number of Samples	D538G	Y537S	E380Q	Y537N	Y537C	L536H	S463P	L536P	L536R	Y537D	AKT E17K
1	LOB_{95}	95	0	0	0	0	0	0	0	0	0	0	0.27
	LOD_{95}	116	1.25	0.98	1.14	0.99	1.12	1.13	1.11	1.29	1.02	1.12	1.38
2	LOB_{95}	46	0.13	0	0	0	0	0	0	0	0	0	0.16
	LOD_{95}	57	0.94	0.63	0.85	0.70	0.75	0.86	0.84	0.94	0.67	0.76	0.98
3	LOB_{95}	31	0.09	0	0	0	0	0	0	0	0	0	0.18
	LOD_{95}	37	0.80	0.47	0.82	0.57	0.61	0.76	0.73	0.72	0.61	0.72	0.77

cp, copies. LOB, limit of blank; LOD, limit of detection; MAF, mutant allele frequency.

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Detection of *ESR1* Mutations in Plasma and FFPE DNA in Research Settings

The *ESR1* 12-plex assay performance was evaluated using plasma and FFPE DNA from breast cancer research samples on the RDG16 Cartridge and QX700 HT ddPCR System at Centre Eugène Marquis (Rennes, France). DNA extracted from 24 plasma samples (QIAamp Circulating Nucleic Acid Kit, Qiagen) and six FFPE samples (QIAamp DNA FFPE Tissue Kit, Qiagen) was analyzed using the *ESR1* 12-plex assay.

Each plasma sample was run in triplicate. Whenever the result was indeterminate in a single RDG16 Cartridge chamber, replicate chambers were pooled to increase the total sample volume analyzed. A target was considered positive if its concentration was above the LOD and indeterminate if its concentration was between the LOB and LOD.

The results were compared with those obtained previously by Centre Eugène Marquis using the Sapphire Chip and naica™ System in a sequential analysis framework.[5]

In brief, this framework involved an initial screening assay combining a drop-off system covering the 536–540 hotspot with specific detection of the E380Q mutation. If the drop-off result was positive, a targeted test was performed using specific probes to detect three of the main *ESR1* mutations (Y537N, Y537S, and D538G).

If the result was negative for these three mutations, duplex tests were performed to assess less frequent *ESR1* mutations (L536H, L536P, L536R, Y537C, Y537D, Y537H, and L540Q). Except for Y537H and L540Q, the *ESR1* 12-plex assay can identify and quantify each of these *ESR1* mutations, as well as the S463P mutation, in a single reaction.

Out of the 24 cfDNA research samples tested, 14 contained one or more *ESR1* mutations, for a total of 29 detected mutations. Analysis with the *ESR1* 12-plex assay successfully detected and quantified 24 of the 29 mutations, with MAFs ranging from 0.2 to 40.5%. Of the five mutations that were not quantifiable using the *ESR1* 12-plex assay, two had an indeterminate status (concentrations between the LOB and LOD), and two were analyzed using the remaining sample volume, resulting in a sample volume analyzed that was three times lower than in the previous study. The final mutation was present at such a low concentration that two Sapphire Chip chambers had been pooled to detect it in the previous study. More than three RDG16 Cartridge replicates of this sample would have been required for quantification. The differences between the two studies can be explained by the lower DNA sample volume analyzed in this study than in the original study performed by Centre Eugène Marquis. For one sample, two Sapphire Chip chambers were pooled in the original study, while for the other two samples, the remaining DNA volume was insufficient to use the full sample input volume. In all three cases, approximately three times more sample input volume was analyzed in the original study.

Out of the six FFPE DNA research samples tested, three contained mutations, all of which were successfully detected and quantified by the *ESR1* 12-plex assay, with MAFs ranging from 16.0 to 53.4%. The MAFs obtained with the *ESR1* 12-plex assay showed strong correlation with the previous dPCR analysis performed by Centre Eugène Marquis on the same samples (R^2 = 0.9489; Figure 4). Finally, no false-positive events were detected in plasma or FFPE DNA samples, demonstrating the specificity and accuracy of the *ESR1* 12-plex assay in research settings, even when applied to a complex biological matrix.

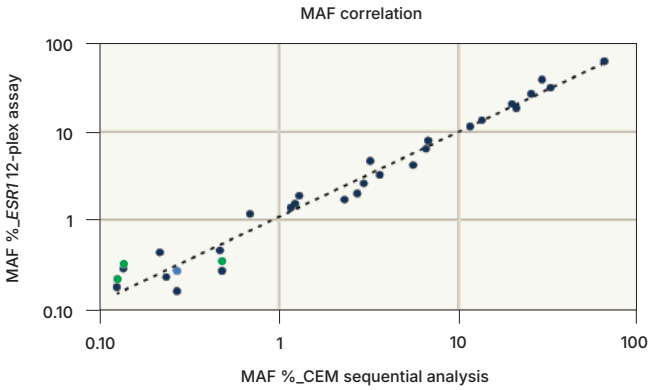


Fig. 4. Correlation of mutant allele frequency (MAF) between results obtained with the *ESR1* 12-plex assay (RDG16 Cartridge, QX700 HT ddPCR System) and a previous study performed by Centre Eugène Marquis (Sapphire Chip, naica System). Green data points indicate mutations for which two RDG16 Cartridge chambers were pooled, whereas the blue data point indicates the mutation for which three Ruby Chip chambers were pooled.

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Mutations detected using two colors can be visualized in 2D using Nio Analyzer Software, enabling the presence of a mutation to be verified, which can be particularly useful for very low MAFs in research settings. Examples of mutations detected during this study are shown in Figure 5.

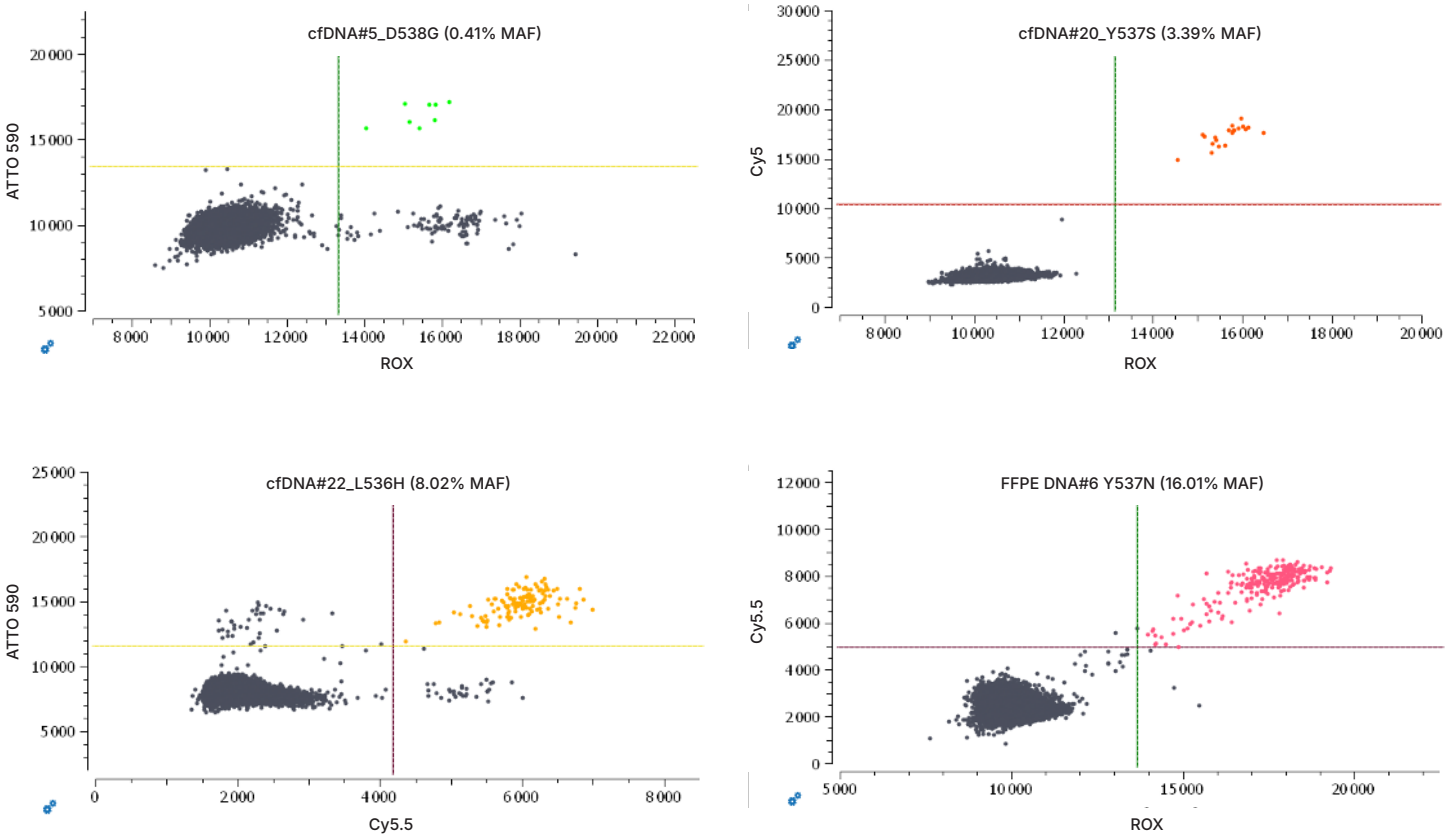


Fig. 5. Examples of mutations detected in cell-free DNA (cfDNA) and formalin-fixed, paraffin-embedded (FFPE) DNA samples using the *ESR1* 12-plex assay in research settings.

Conclusion

The *ESR1* 12-plex assay enabled simultaneous detection and quantification of 10 *ESR1* mutations, one *AKT1* mutation, and a reference target within a single reaction in research settings using color-combination multiplexing. The assay demonstrated analytical sensitivity sufficient to achieve LOD values below 1.38 cp/µL for all targets, with improved sensitivity observed when replicate chambers were pooled.

Analysis of plasma cfDNA and FFPE DNA samples showed strong correlation with previous Crystal Digital PCR studies and successful detection of *ESR1* mutations across a broad range of mutant allele frequencies in research settings. These results demonstrate the utility of highly multiplexed ddPCR assays for sensitive mutation detection and quantification in breast cancer research samples.

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