Development of an Ultra-Sensitive Droplet Digital PCR Test for Monitoring ESR1 Variants by Liquid Biopsy

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Introduction

Estrogen receptor 1 (ESR1) mutations are a critical mechanism of acquired resistance to aromatase inhibitor (AI) therapy in hormone receptor-positive (HR+) breast cancer, occurring in approximately 20-40% of patients during treatment[1]. The detection of ESR1 mutations in circulating tumor DNA (ctDNA) through liquid biopsy represents a new approach to real-time monitoring of treatment resistance and therapeutic decision-making. Recent interim results from the landmark SERENA-6 Phase III trial demonstrated the potential clinical utility of ctDNA-guided treatment switching, where median progression-free survival was 16 months in the Camizestrant group versus 9.2 months in the aromatase-inhibitor group when treatment was switched based on ESR1 mutation detection[2]. This study represented the first global Phase 3 trial to demonstrate the potential clinical utility of using ctDNA for treatment guidance and breast cancer management.

We have developed a Droplet Digital™ PCR (ddPCR™) test for the detection of ESR1 variants in plasma-derived cfDNA using the Bio-Rad ddPLEX Mutation Detection Kit. The kitted assay and a supplementary 4-plex assay targets the 11 most prevalent ESR1 variants. Analytical sensitivity studies were performed using synthetic DNA controls diluted into wild-type background DNA at varying input concentrations. Serial dilutions were tested to establish the limit of detection (LOD) across different DNA input amounts, with variant allele frequencies (VAF) ranging from 0.025% to 0.2%.

Materials and Methods

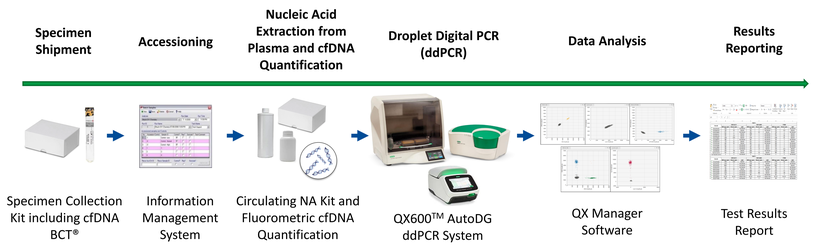


Figure 1. ESR1 Specimen Test Workflow. Samples are shipped to the clinical operations laboratory in Streck cfDNA blood collection tubes for accessioning. After plasma is prepared from whole blood via centrifugation, cfDNA is isolated and quantified. The cfDNA is then input to the ddPCR workflow, which is comprised of reaction mixture preparation, automated droplet generation, PCR, and droplet reading (Bio-Rad QX600 system). Data analysis is performed using QXManager Premium software (Bio-Rad), and a test result report is generated.

Table 1. Two Assay Formats for Detection of 11 ESR1 Variants. The Test is comprised of two different ddPCR assays, the ddPLEX ESR1 Mutation Detection Assay (ddPLEX) and the ESR1 Extended Variant (EXV) Screening Assay. The ddPLEX assay detects and discriminates 7 ESR1 mutations and their corresponding wild-type alleles (in a separate reaction). The EXV assay detects 4 variants in a non-discriminating multiplex as well as wildtype (WT) ESR1. Together, the two assay formats cover the 11 most prevalent endocrine therapy resistance mutations that occur in the ligand binding domain of ESR1[3].

Assay	ESR1 Exon #	Mutant Assay Allele	Mutant Assay Channel	Wild Type Assay Allele	Wild Type Assay Channel
ddPLEX ESR1 Mutation Detection Kit (Bio-Rad, Cat #17009501)	5	E380Q (c.1138G>C)	FAM	380-Wild Type	HEX
	7	S463P (c.1387T>C)	ROX	463-Wild Type	Cy5
	8	L536R (c.1607T>G)	HEX	53X-Wild Type	FAM
		Y537C (c.1610A>G)	Cy5/Cy5.5		
		Y537N (c.1609T>A)	Cy5		
		Y537S (c.1610A>C)	FAM/HEX high		
		D538G (c.1613A>G)	FAM/HEX low		
ESR1 Extended Variant Screening Assay (Bio-Rad, dEXD51651559)	6	V422del (c.1265_1267del)	FAM (non-discriminating multiplex)	ESR1 WT	HEX
	8	L536H (c.1607 T>A)			
		L536P (c.1607 T>C)			
		Y537D (c.1609 T>G)			

Limit of Detection Study Design

Inputs:
- 10 ng and 30 ng nucleic acid
- Targeted Variant Allele Frequency (VAF):
  - 0.2%, 0.1%, 0.05%, 0.025%, 0%

Samples:
- ddPLEX ESR1:
  - Seraseq ctDNA ESR1 Mutation Mix AF1%: Commercial reference material containing pool of all targeted variants
  - Seraseq ctDNA ESR1 Mix Wildtype: Commercial reference material containing only wild type/non-mutated genomic DNA
  - 3 replicates per concentration
- Supplemental ESR1 4-plex:
  - Synthetic dsDNA (gblocks, IDT) diluted in sonicated WT gDNA
  - 2 replicates per sample and concentration

Figure 2. Experiment Design for ESR1 Limit of Detection Study. Analytic materials were diluted in ctDNA-relevant matrices to create contrived positive samples targeting a range of variant allele frequencies from 0.2%-0.025%. Two to three replicate tests were performed using the ddPLEX and EXV assays. A blank sample (matrix diluent only) was also tested to confirm assay specificity. All samples were tested using the ESR1 workflow, and analysis was performed using QX Manager Software (v2.0).

Results

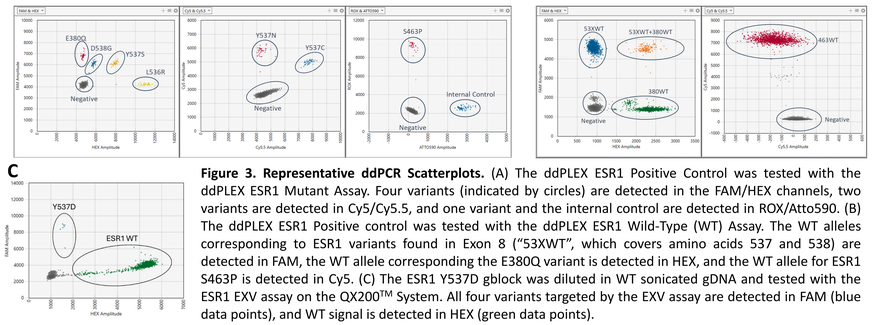
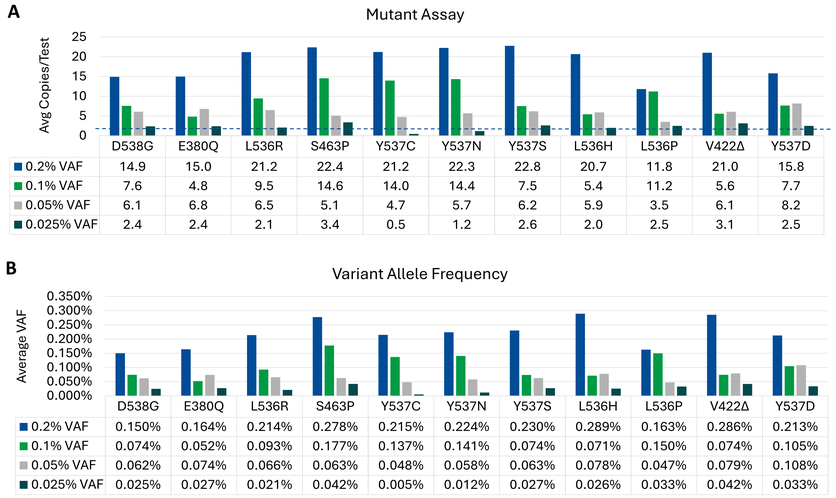


Figure 3. Representative ddPCR Scatterplots. (A) The ddPLEX ESR1 Positive Control was tested with the ddPLEX ESR1 Mutant Assay. Four variants (indicated by circles) are detected in the FAM/HEX channels, two variants are detected in Cy5/Cy5.5, and one variant and the internal control are detected in ROX/Atto590. (B) The ddPLEX ESR1 Positive control was tested with the ddPLEX ESR1 Wild-Type (WT) Assay. The WT alleles corresponding to ESR1 variants found in Exon 8 ("53XWT", which covers amino acids 537 and 538) are detected in FAM, the WT allele corresponding the E380Q variant is detected in HEX, and the WT allele for ESR1 S463P is detected in Cy5. (C) The ESR1 Y537D gblock was diluted in WT sonicated gDNA and tested with the ESR1 EXV assay on the QX200™ System. All four variants targeted by the EXV assay are detected in FAM (blue data points), and WT signal is detected in HEX (green data points).



	D538G	E380Q	L536R	S463P	Y537C	Y537N	Y537S	L536H	L536P	V422Δ	Y537D
0.2% VAF	14.9	15.0	21.2	22.4	21.2	22.3	22.8	20.7	11.8	21.0	15.8
0.1% VAF	7.6	4.8	9.5	14.6	14.0	14.4	7.5	5.4	11.2	5.6	7.7
0.05% VAF	6.1	6.8	6.5	5.1	4.7	5.7	6.2	5.9	3.5	6.1	8.2
0.025% VAF	2.4	2.4	2.1	3.4	0.5	1.2	2.6	2.0	2.5	3.1	2.5

	D538G	E380Q	L536R	S463P	Y537C	Y537N	Y537S	L536H	L536P	V422Δ	Y537D
0.2% VAF	0.150%	0.164%	0.214%	0.278%	0.215%	0.224%	0.230%	0.289%	0.163%	0.286%	0.213%
0.1% VAF	0.074%	0.052%	0.093%	0.177%	0.137%	0.141%	0.074%	0.071%	0.150%	0.074%	0.105%
0.05% VAF	0.062%	0.074%	0.066%	0.063%	0.048%	0.058%	0.063%	0.078%	0.047%	0.079%	0.108%
0.025% VAF	0.025%	0.027%	0.021%	0.042%	0.005%	0.012%	0.027%	0.026%	0.033%	0.042%	0.033%

Figure 4. Limit of Detection Study. (A) Mutant assay results and (B) Variant allele frequency (VAF) for the 30 ng input condition. Bars represent the average mutant copies or VAF, respectively, measured per test for each of the 11 variants and 4 input concentrations. Dotted line: represents the analytic threshold for calling a positive of 2 copies/test.

Table 2. Table of Results for the Limit of Detection (LoD) Study. The variant allele frequency and mutant copies/test observed at the LoD are provided for each of the 11 variants with 30 ng and 10 ng input.

30 ng Input

Target	LoD (VAF)	LoD (Copies/Test)
D538G	0.025%	2.4
E380Q	0.025%	2.4
L536R	0.025%	2.1
S463P	0.025%	3.4
Y537C	0.05%	4.7
Y537N	0.05%	5.7
Y537S	0.025%	2.6
L536H	0.025%	2.0
L536P	0.025%	2.5
V422del	0.025%	3.1
Y537D	0.025%	2.5

10 ng Input

Target	LoD (VAF)	LoD (Copies/Test)
D538G	0.05%	2.1
E380Q	0.1%	3.9
L536R	0.1%	4.5
S463P	0.1%	3.7
Y537C	0.1%	4.3
Y537N	0.1%	3.3
Y537S	0.05%	3.4
L536H	0.025%	2.0
L536P	0.1%	2.6
V422del	0.1%	4.0
Y537D	0.1%	2.9

Acknowledgments

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Results

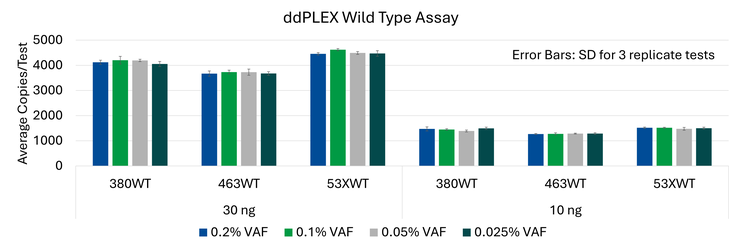


Figure 5. Wild Type Assay Results for ddPLEX Assay. The average copies/test for each of the wild type assay alleles at all 4 input concentrations for both 30 ng and 10 ng inputs are shown. Error bars represent the standard deviation for 3 replicate tests.

Table 3. Table of Results for Wild Type (WT) Measurements. The mutant and wild type copies/test for the WT inputs are provided for each of the 11 assay targets with both 30 ng and 10 ng input.

30 ng Input

Target	MUT (Copies/Test)	WT (Copies/Test)
D538G	0.0	10012
E380Q	0.0	9192
L536R	0.0	10012
S463P	0.0	8053
Y537C	0.0	10012
Y537N	1.7*	10012
Y537S	0.0	10012
L536H/L536P/V422del/ Y537D Multiplex	0.0	7365

10 ng Input

Target	MUT (Copies/Test)	WT (Copies/Test)
D538G	0.0	3109
E380Q	0.0	2998
L536R	0.0	3109
S463P	0.0	2747
Y537C	0.0	3109
Y537N	1.7*	3109
Y537S	0.0	3109
L536H/L536P/V422del/ Y537D Multiplex	0.0	2378

*Negative result. Analytic threshold for positive was set to 2 copies/test.

Conclusions

- The ESR1 ddPLEX and EXV assays combined provide coverage of the 11 most prevalent ESR1 mutations (97% coverage) in breast cancer patients[3] .
- The ESR1 Specimen Test Workflow is run on the QX600™ AutoDG System, for hands-free droplet generation and advanced multiplexing with 6-color detection (Figure 1).
- The Limit of Detection for the 11 ESR1 variants ranged from 0.025% - 0.1% variant allele frequency with at least 2 copies detected per test for inputs ranging from 10 ng to 30 ng nucleic acid (Figure 4 and Table 2).
- Wild type-only controls tested negative for all variants with as much as 30 ng input. (Table 3)
- Assay Development studies are ongoing, including clinical validation and utility (as required).

References and Conflict of Interest Disclosure

[1]Brett, et al. ESR1 mutation as an emerging clinical biomarker in metastatic hormone receptor-positive breast cancer. Breast Cancer Research. 2021; 23(85).

[2]Bidard, et al. First-Line Camizestrant for Emerging ESR1-Mutated Advanced Brest Cancer. NEJM. 2025; 393(6).

[3]Grinshpun, et al. ESR1 activating mutations: From structure to clinical application. BBA – Reviews on Cancer. 2023; 1878(1): 188830.

[3]Cancer Hotspots Database, MSKCC; prevalence of single residue and in-frame indel mutation hotspots identified in 24,529 tumor samples by the algorithm described in [Chang et al. 2017] and [Chang et al. 2016]

Leisa Jackson, Helen Halpin, and Gary A. Pestano, PhD are employees of Biodesix, Inc and hold options or shares therein.

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