Implementation of digital PCR TaqMan™ multiplex assay enables high sensitivity for detection of *ESR1* mutations in liquid biopsy material

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Introduction

ESR1 mutations are drivers of therapy resistance and an established biomarker for selective estrogen receptor (ER) degraders therapy in ER+, HER2- advanced or metastatic breast cancer. While *ESR1* mutations can be detected by sequencing methods, digital PCR offers a cost-effective alternative which is more resistant to inhibitors and may prove especially suitable for liquid biopsy analysis for cancer research. In this study, we evaluated a research use multiplex digital PCR-based approach for identification and differentiation of 8 most common *ESR1* mutations in liquid biopsy specimens in comparison to next-generation sequencing (NGS).

Materials and methods

Assays for research use only were designed to detect 8 most common *ESR1* mutations, which should cover a vast majority of resistance mechanisms: E380Q, L536H, L536P, L536R, Y537C Y537N, Y537S, and D538G in a total of 4 multiplex reactions (Table 1). These selected variants account for activating *ESR1* mutations in more than 91% of all recorded cases of ESR1 mutations in breast cancer.[1] Each reaction contained 2 primer/probe pairs for mutant and 2 for wild-type alleles, labeled with FAM™, VIC™, ABY™, or CY5™ fluorophores (Table 1). Digital PCR was performed on the Absolute Q instrument (Thermo Fisher Scientific). To assess the analytical performance of the assays, commercially available reference material with varying mutational allele frequencies (AF) and varying DNA input was tested. In addition, analytical performance was tested on liquid biopsy research samples previously characterized by NGS. Cell-free DNA was isolated from whole blood using the MagMAX™ Cell-Free DNA Isolation Kit (Thermo Fisher Scientific).

Table 1. Design of multiplex assays for dPCR analysis of ESR1 mutations

dPCR *ESR 1* multiplexes															
Multiplex 1				*Multiplex 2*				*Multiplex 3*				Multiplex 4			
Mutation	COSM-ID	Mutant Probe Dye	Wild type Probe Dye	Mutation	COSM-ID	Mutant Probe Dye	Wild type Probe Dye	Mutation	COSM-ID	Mutant Probe Dye	Wild type Probe Dye	Mutation	COSM-ID	Mutant Probe Dye	Wild type Probe Dye
E380Q	COSM3829320	FAM	VIC	L536H	COSM6843697	FAM	VIC	L536P	COSM6906109	FAM	VIC	L536R	COSM4774826	FAM	VIC
D538G	COSM94250	ABY	Cy5	Y537C	COSM1074637	ABY	Cy5	Y537N	COSM1074635	ABY	Cy5	Y537S	COSM4774826	ABY	Cy5

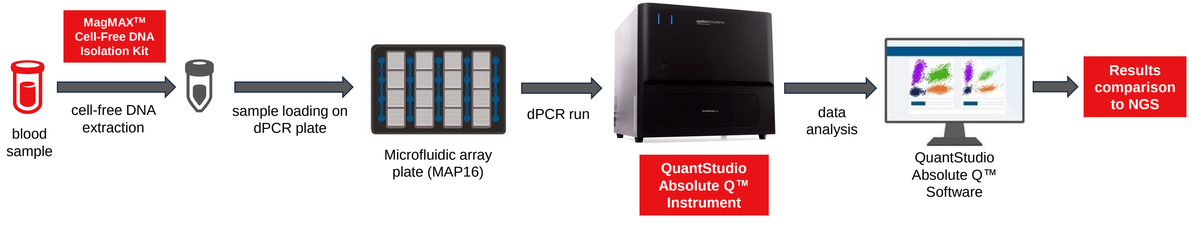

Figure 1. Digital PCR workflow for liquid biopsy specimen and comparison to next generation sequencing

Results

The tested assays demonstrated 100% sensitivity in reference material for the respective mutations. *ESR1* variants could be detected at AF of 0.1% with 20 and 10ng DNA input (Figure 2A-B), while for 2ng DNA input AF down to 0.5% were tested successfully (Figure 2C-D). In total, 5 liquid biopsy samples were analyzed and obtained cell-free DNA concentrations ranged from 0.75 to 23.8ng/µl. The detected AF in liquid biopsy samples corresponded to AF detected with NGS (Table 2).

Figure 2. Allelic frequency detected by ESR1 multiplex dPCR testing of reference material with varying DNA input and expected mutation allele frequencies

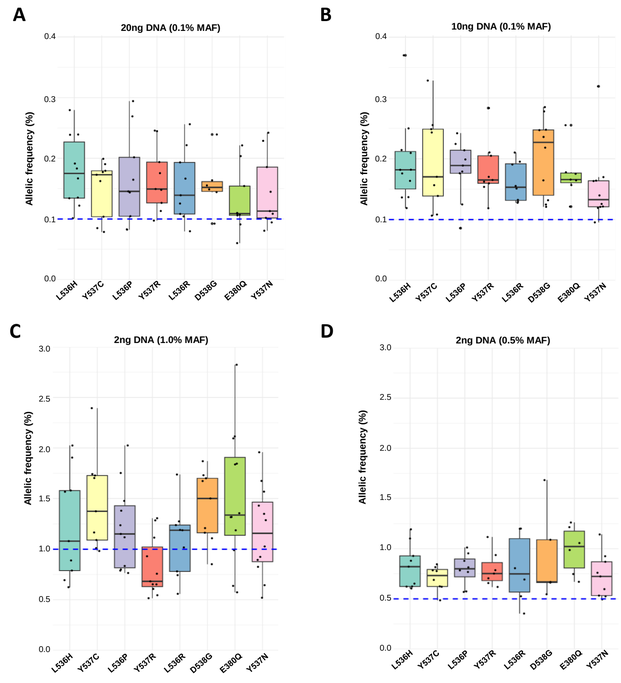

Figure 3. Examples of dPCR result analysis plot for ESR1 mutations detected in A) reference material and B) liquid biopsy sample

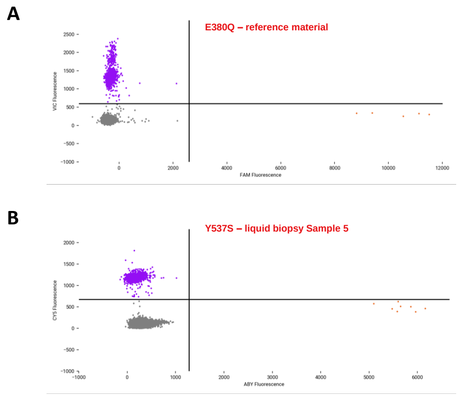

Table 2. ESR1 mutations detected in liquid biopsy material

Sample	*ESR1* mutation	dPCR MAF	NGS MAF
Sample 1	Y537S	2.24%	10.00%
Sample 2	Y537N	3.30%	5.00%
	Y537S	4.50%	4.30%
	D538G	53.57%	51.90%
Sample 3	-	0.0%	0.0%
Sample 4	-	0.0%	0.0%
Sample 5	Y537S	0.84%	0.30%

Conclusions

- **Digital PCR on the Absolute Q instrument offers a highly accurate, rapid and cost-effective technique for *ESR1* mutations detection for cancer research.**
- **This method is particularly suitable for analyzing liquid biopsy material, as it demonstrates high analytical sensitivity and specificity in identifying mutational variants with allele frequencies as low as 0.1%.**

References

1. Grinshpun A, Chen V, Sandusky ZM, et al. ESR1 activating mutations: From structure to clinical application. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, Volume 1878, Issue 1, 2023, 188830. doi:10.1016/j.bbcan.2022.188830.

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