An Exosome-based *ESR1* Monitoring RT-qPCR Kit That Rapidly and Accurately Detects Acquired Resistance Variants at ≤ 0.1% Frequency in Liquid Biopsy Samples

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Summary

Breast cancer is a multifaceted disease that frequently necessitates continuous oversight and management to achieve optimal patient results with improved outcomes. Monitoring for the emergence of resistance mutations, especially those in the Estrogen Receptor 1 (*ESR1*) gene, offers important insights into tumor progression and treatment efficacy.

We have developed a comprehensive methodology for targeted RT-qPCR monitoring of *ESR1* mutations in plasma that utilizes both exosomal RNA (exoRNA) and circulating cell-free DNA (cfDNA), interrogates 11 of the most common and actionable mutations, provides a streamlined workflow, and includes the incorporation of an endogenous/internal control and external batch run control materials (positive, negative) to ensure high-quality results.

We demonstrate consistent and specific RT-qPCR results between 0.1-0.01% Minor Allele Frequency (MAF) depending on the total mutant copies and mutation, congruent with ultrasensitive mutation detection from plasma.

Our data showcased here supports routing testing of clinical samples from PAXgene® Blood ccfDNA tubes or K2-EDTA tubes for 11 of the most common and actionable *ESR1* mutations, with results via a push-button automated software within a single laboratory shift.

Introduction

Hormone receptor-positive/human epidermal growth factor receptor 2-negative (HR+/HER2-) breast cancer is the most common type of breast cancer. Patients with HR+/HER2- metastatic breast cancer (mBC) often become resistant to aromatase inhibitors commonly used in endocrine therapy (ET). Estrogen Receptor 1 (*ESR1*) ligand binding domain mutations are frequently detected in HR+ mBC and have been reported to be associated with ET resistance (1). It is estimated that 20-40% of mBC patients will develop resistance to treatment via mutations in *ESR1* (1). Recent studies have shown that monitoring of *ESR1* mutations in plasma may serve as a predictive biomarker of acquired resistance to ET, showcasing a strong need for sensitive nucleic acid-based assays (2). The recent FDA approval of elacestrant (3) and updated NCCN guidelines for breast cancer calling for *ESR1* mutation testing and recommending elacestrant use when detected (4) further stress the urgency for sensitive detection of *ESR1* ligand-binding domain mutations.

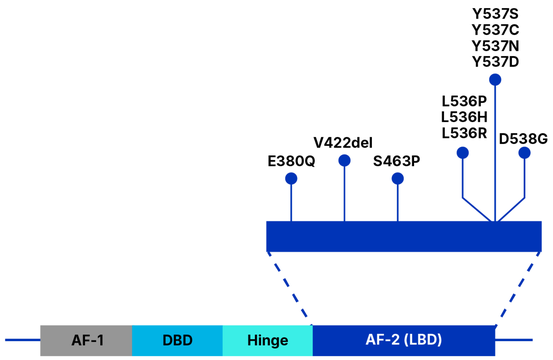

Figure 1. *ESR1* acquired resistance mutations. In patients with HR+/HER2- mBC, *ESR1* mutations are a common cause of acquired resistance to aromatase inhibitors. A key mechanism of endocrine resistance is mutation of the ligand-binding domain (LBD); mutations investigated in our preliminary studies within the LBD are shown.

Table 1. *ESR1* mutations with Hg38 chromosomal locations and COSMIC IDs

Common Name	Cosmic ID	Coordinate GRCh38	RefBase	AltBase
E380Q	COSV52782264	Chr6:152011697	G	C
V422del	COSV52789441	Chr6:152061020..152061022	GTGG	G
S463P	COSV52784970	Chr6:152094402	T	C
L536P	COSV52782930	Chr6:152098785	T	C
L536H	COSV52795259	Chr6:152098785	T	A
L536R	COSV52787207	Chr6:152098785	T	G
Y537S	COSV52783938	Chr6:152098788	A	C
Y537C	COSV52782924	Chr6:152098788	A	G
Y537N	COSV52784978	Chr6:152098787	T	A
Y537D	COSV52804811	Chr6:152098787	T	G
D538G	COSV52781024	Chr6:152098791	A	G

Materials & Methods

Due to the scarcity of samples containing *ESR1* mutations, plasmids containing the 11 clinically-relevant mutations shown in Figure 1, were designed. These constructs contained a T7 promoter to allow for in vitro transcription of mutant and wild-type *ESR1* transcripts. The resulting *ESR1* transcripts also included an additional 84-nucleotide exogenous sequence for precise quantification using ddPCR (Bio-Rad QX200). Linearized plasmids were utilized to create contrived mutant material, spiking ddPCR-verified copies into NA12878 (Coriell) gDNA fragmented to mimic cfDNA utilizing the M220 instrument (Covaris). Multiplex RT-qPCR target enrichment was performed using QuantideX reagents (Asuragen), and mutations were determined utilizing the QuantStudio 5 (QS5), the QuantStudio 7 (QS7), or 7500 Fast qPCR Platform (Thermo Fisher). Primary analysis was completed in Design & Analysis 2 (DA2) software (Thermo Fisher), while secondary analysis and interpretation was performed through a dedicated QuantideX qPCR *ESR1* exoMutation Analysis Module (Asuragen).

QuantideX qPCR *ESR1* exoMutation Kit* controls were formulated to allow for quality control of multi-analyte assay. Two controls, CONP and CONN, were formulated to serve as either a DNA-based positive control (CONP) or an RNA-based negative control (CONN). Briefly, CONP was created by combining multiple ddPCR-verified copies of linearized plasmids to result in a positive signal in all mutant and internal control channels. CONN was created utilizing an IVT product that results in a positive signal only in our internal control (IC1, IC2, and IC3) channel. Briefly, CONN IVT was created by utilizing the MEGAshortscript T7 Transcription Kit (Thermo Fisher) and MEGAclear Transcription Clean-Up Kit (Thermo Fisher) on a linearized plasmid containing the endogenous or internal control sequence.

Plasma samples collected from subjects with stage IV mBC (HR+/HER2-) on active aromatase inhibitor therapy, +/- CDK 4/6 inhibitor for a minimum of 1 year, underwent exoRNA/cfDNA co-isolation from a PAXgene® Blood ccfDNA Tube (Qiagen) or K2-EDTA Tube (BD) and processed to separate plasma. Plasma samples were obtained from a single external source following all regulatory, ethical and informed consent standards. The plasma was filtered using a 0.8 µm filter followed by an in-house method optimized to co-enrich exoRNA and cfDNA (ExoLution Plus, included in the QuantideX qPCR *ESR1* exoMutation Kit*). RT was completed on the entire exoRNA/cfDNA eluate. The RT product was passed through a Pre-Amp PCR, diluted, and added to three multiplex *ESR1* targeted qPCR reactions: Mix A, Mix B, and Mix C. In-house designed singleplex allele-specific qPCR assays of the 11 *ESR1* mutations were used for mutation status confirmation on the QS5 (Thermo Fisher).

Figure 2. QuantideX qPCR *ESR1* exoMutation Kit workflow. A minimum of 2 mL of plasma is processed using an in-house method (ExoLution Plus) optimized to co-enrich exoRNA and cfDNA (included in the QuantideX qPCR *ESR1* exoMutation Kit*). RT was completed on the co-isolated exoRNA and cfDNA, followed by Pre-Amp PCR for sample enrichment. Diluted Pre-Amp PCR product was transferred to 3 tubes for multiplex interrogation of the 11 *ESR1* mutations by qPCR. An internal PCR control (IC1, IC2, and IC3) was included in each multiplex reaction tube for sample QC purposes.

Results

Table 2. Mutant targets within 536, 537, and 538 codons show no cross-reactivity with unintended targets in nearby codons. Contrived 0.1% Minor Allele Frequency (MAF) targets (10 mutant copies in the background of 10,000 Wild-Type copies) were run across all 3 mixes. Amplification and resulting positive calls were as expected: 0.1% D538G only had amplification for Mix A and not Mix B/C, 0.1% Y537S/C/N/D only had amplification for Mix B and not Mix A/C, and 0.1% L536R/H/P only had amplification for Mix C and not Mix A/B.

ND = signal not detected within 40 cycles

0.1% MAF Target	Mix A				Mix B				Mix C			
	MUT Channel D538G		IC Channel IC1		MUT Channel Y537X		IC Channel IC2		MUT Channel L536X		IC Channel IC3	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
D538G	35.84	35.22	30.42	30.68	ND	ND	29.06	29.49	ND	ND	26.36	27.01
Y537S	ND	ND	30.48	30.30	28.74	29.10	29.27	29.29	ND	ND	26.62	26.71
Y537C	ND	ND	30.22	30.49	31.28	31.28	29.09	29.26	ND	ND	26.27	26.66
Y537N	ND	ND	30.23	30.44	29.15	28.87	29.09	29.33	ND	ND	26.53	26.97
Y537D	ND	ND	30.08	30.35	27.57	28.90	29.55	29.53	ND	ND	26.82	26.83
L536R	ND	ND	30.26	30.39	ND	ND	29.49	29.28	26.77	27.14	27.08	27.54
L536H	ND	ND	30.63	30.36	ND	ND	29.82	29.26	28.39	28.54	28.02	28.42
L536P	ND	ND	30.39	31.18	ND	ND	29.45	30.40	30.15	32.21	29.18	30.08

Table 3. 20 replicates of mutation-negative samples show an analytical specificity of ≥90% across two platforms. 10 replicates of 0.1% MAF samples and 20 replicates of known mutation-negative samples were utilized in the workflow shown in Figure 2 starting at the RT step. All 10 replicates of 0.1% MAF samples were identified and shown as min, max and median Cq values across the 10 replicates, showcasing high precision across both the QS5 and QS7 qPCR platforms. Mix A and B had no detection of mutant signal within the negative samples whereas Mix C had 2 false calls within the L536X mutant channel, resulting in an analytical specificity of 90%.

ND = signal not detected within 40 cycles

Mix	Target	QS5 - 0.1% MAF			QS5 - WT	QS7 - 0.1% MAF			QS7 - WT
		MUT Channel			MUT Channel	MUT Channel			MUT Channel
		MIN	MAX	MEDIAN	Rep 1 -20	MIN	MAX	MEDIAN	Rep 1 -20
A	D538G	31.57	35.76	32.89	ND	31.54	38.69	33.01	ND
	S463P	30.17	31.70	30.88	ND	30.01	31.90	30.89	ND
B	Y537S	29.89	32.93	31.58	ND	30.65	35.61	32.84	ND
	Y537C	29.14	30.78	30.16		30.62	32.04	31.58	
	Y537N	28.89	31.68	29.49		30.21	33.05	30.68	
	Y537D	28.11	29.24	28.60		29.10	30.17	29.57	
	E380Q	27.97	29.73	28.24	ND	29.11	30.97	29.61	ND
C	L536R	28.54	29.58	29.03	ND: Rep 1-18 *33.59, 33.54*	29.65	30.99	30.18	ND: Rep 1-18 *36.49, 36.77*
	L536H	28.61	31.27	29.74		30.13	32.31	30.87	
	L536P	29.84	32.07	30.47		30.94	33.48	31.77	
	V422del	29.23	31.32	30.43	ND	33.58	36.43	35.14	ND

Table 4. Sensitivity for all 11 mutations was maintained between 0.1-0.01% at 10 mutant copies with increasing wild-type background . Contrived mutant targets were created maintaining 10 mutant copies in increasing background of wild-type copies of the following amounts: 10,000 (0.1% MAF), 25,000 (0.04% MAF), 50,000 (0.02% MAF), and 100,000 (0.01% MAF). Samples, in duplicate, and kit controls (CONP/CONN), in singleton, were run across all 3 mixes. All 11 *ESR1* mutations were consistently detected down to 0.01%, showcasing the highly specific design of the QuantideX qPCR *ESR1* exoMutation Kit*. At 100,000 wild-type background copies, Mix B Y537X and Mix C L536X had a false signal in the FAM channel, as shown by the light blue text. 1 replicate of S463P resulted in no signal (ND* in red text), which was determined to be due to user error in the formulation of the sample.

ND = signal not detected within 40 cycles; red text indicates unexpected result.

Mix	Target	10,000 WT background 0.1% MAF for MUT				25,000 WT background 0.04% MAF for MUT			
		MUT Channel		IC Channel		MUT Channel		IC Channel	
		Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
A	D538G	32.96	36.04	30.64	31.10	34.55	34.12	29.37	29.41
	S463P	ND*	32.01	30.77	30.19	33.73	33.63	29.34	28.97
	WT	ND | ND	ND | ND	30.33	30.05	ND | ND	ND | ND	29.03	28.36
B	Y537S	31.28	30.33	29.99	29.83	29.78	30.57	28.60	28.39
	Y537C	31.49	30.63	29.81	29.65	32.20	30.88	28.65	28.55
	Y537N	30.18	29.99	29.86	30.07	31.46	29.62	28.81	28.65
	Y537D	29.73	29.75	30.29	29.99	30.79	29.42	29.02	28.68
	E380Q	30.58	29.27	30.82	30.06	29.74	29.56	29.17	28.41
	WT	ND | ND	ND | ND	30.36	29.76	ND | ND	ND | ND	28.82	27.99
C	L536R	32.04	31.36	31.02	30.37	33.10	32.62	29.45	29.30
	L536H	31.75	31.05	30.86	30.37	32.31	30.50	29.60	28.82
	L536P	34.90	33.88	30.50	30.86	33.82	33.03	29.59	28.94
	V422del	34.57	31.77	30.87	30.31	32.39	32.25	29.87	28.84
	WT	ND | ND	ND | ND	30.27	29.65	ND | ND	ND | ND	28.95	28.52

Mix	Target	50,000 WT background 0.02% MAF for MUT				100,000 WT background 0.01% MAF for MUT			
		MUT Channel		IC Channel		MUT Channel		IC Channel	
		Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
A	D538G	35.98	32.23	28.36	28.68	32.86	34.41	27.61	27.65
	S463P	32.67	33.39	28.31	28.26	34.49	36.62	27.08	27.36
	WT	ND | ND	ND | ND	27.83	27.26	ND | ND	ND | ND	26.67	26.36
B	Y537S	29.89	30.11	27.49	27.62	29.49	33.43	26.30	26.74
	Y537C	32.58	31.35	27.87	27.76	31.56	31.65	26.95	26.96
	Y537N	31.55	30.10	27.64	27.55	30.10	31.01	26.36	26.47
	Y537D	28.93	29.55	27.72	27.66	29.57	31.13	26.75	26.93
	E380Q	29.48	29.85	27.88	27.50	30.63	29.76	26.96	26.76
	WT	ND | ND	ND | ND	27.59	27.02	39.01 | ND	ND | ND	26.58	26.09
C	L536R	32.18	31.77	28.47	28.28	31.94	31.68	27.25	27.40
	L536H	29.36	30.72	28.03	28.02	32.25	31.54	27.28	26.88
	L536P	34.10	34.01	29.19	28.35	32.95	34.39	27.56	27.50
	V422del	32.63	32.30	28.41	28.07	35.57	35.12	27.80	27.68
	WT	ND | ND	ND | ND	27.60	27.13	36.35 | ND	ND | ND	26.48	27.46

Table 5. Sensitivity for all 11 mutations was maintained between 0.1-0.01% at varying mutant copies input with consistent wild-type background at 10,000 copies. Contrived mutant targets were created maintaining consistent background of 10,000 wild-type (WT) copies while decreasing mutant copies to achieve the following MAFs: 0.1%, 0.05%, 0.03%, and 0.01%. Samples, in 2 reps for mutants and 4 reps for WT, and kit controls (CONP/CONN), in singleton, were run across all 3 mixes. Y537N, Y537D, E380Q, L536H, and V422del assays were able to detect down to a single mutant copy whereas L536R and L536P were able to detect down to three mutant copies. D538G, S463P, Y537S, and Y537C were able to detect 10 mutant copies. 1 replicate of S463P (same formulation as shown in Table 4) resulted in no signal (ND* in red text), which was determined to be due to user error in the formulation of the sample. Additionally, 1 WT replicate within Mix C did show positive for L536X.

ND = signal not detected within 40 cycles; red text indicates unexpected result.

Mix	Target	10 Mutant Copies in 10,000 WT 0.1% MAF		5 Mutant Copies in 10,000 WT 0.05% MAF		3 Mutant Copies in 10,000 WT 0.03% MAF		1 Mutant Copy in 10,000 WT 0.01% MAF		0 Mutant Copy in 10,000 WT
		MUT Channel		MUT Channel		MUT Channel		MUT Channel		MUT Channel
		Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1-4
A	D538G	30.79	32.99	34.11	ND	34.64	34.51	34.53	ND	ND
	S463P	ND*	32.51	ND	34.88	33.57	ND	ND	36.16	ND
B	Y537S	29.03	29.53	ND	30.77	ND	32.11	34.55	ND	ND
	Y537C	31.30	30.74	ND	31.28	32.00	ND	32.45	36.60	
	Y537N	29.57	30.03	31.12	32.50	31.75	34.21	33.30	31.25	
	Y537D	27.45	28.68	32.11	30.37	30.74	29.43	31.52	31.52	
	E380Q	29.33	29.93	30.69	31.66	31.28	32.18	30.63	32.75	ND
C	L536R	29.58	29.53	28.81	34.46	30.99	30.64	ND	32.63	ND: Rep 1-3 *34.52*
	L536H	29.60	30.38	29.90	29.58	30.82	31.33	33.35	32.59	
	L536P	28.36	30.43	30.18	30.91	32.50	32.02	ND	37.22	
	V422del	32.27	32.09	33.58	31.62	32.22	33.42	33.42	33.81	ND

Table 6. S463P assay can consistently detect 10 mutant copies across multiple instruments. Due to the S463P resulting in no signal as shown in Table 4 and Table 5, we repeated the 0.1-0.01% at varying mutant copies input with consistent wild-type background at 10,000 copies with increased replicates at 0.1% MAF and across 4 7500 fast dx instruments. Across 10 replicates, the S463P assay robustly and consistently detects 10 mutant copies of S463P in the background of 10,000 copies of WT.

ND = signal not detected within 40 cycles; red text indicates unexpected result.

Target	Mutant Copies in 10,000 WT	Rep	Instrument #1	Instrument #2	Instrument #3	Instrument #4
			MUT Channel S463P			
S463P	1	Rep 1	ND	ND	ND	ND
		Rep 2	ND	ND	38.02	ND
	3	Rep 1	34.52	37.30	37.61	36.84
		Rep 2	37.58	ND	38.41	ND
	5	Rep 1	34.86	34.88	34.51	ND
		Rep 2	ND	37.14	37.16	35.52
	10	Rep 1	34.78	34.63	34.94	34.26
		Rep 2	35.02	35.91	35.04	36.47
		Rep 3	34.28	34.37	34.51	34.31
		Rep 4	36.79	35.89	36.22	36.60
		Rep 5	35.88	35.87	34.75	37.76
		Rep 6	36.30	35.23	34.76	35.88
		Rep 7	34.80	34.41	34.26	33.08
		Rep 8	35.86	34.68	34.16	34.44
		Rep 9	34.38	33.82	33.38	33.56
		Rep 10	34.50	34.61	34.14	33.36

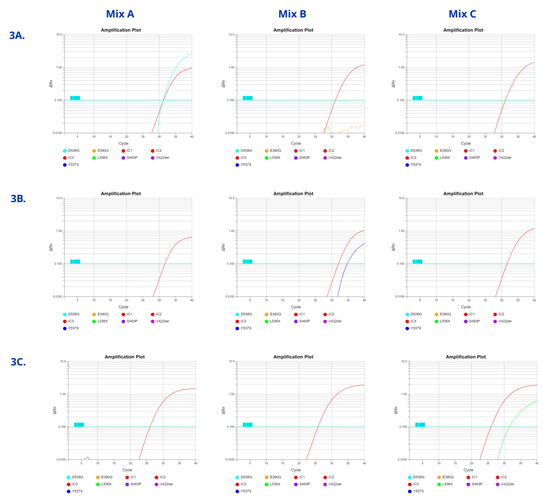

Figure 3. *ESR1* Assay qPCR amplification curves for representative *ESR1* mutant positive clinical samples qPCR amplification curves for four representative clinical samples that were confirmed positive with an orthogonal method are shown. The internal control was successful across all three mixes (IC1, IC2, and IC3) as shown by a red curve in each Mix. Positives are as follows: 3A.) Sample 2 qPCR amplification curve for a positive D538G mutation shown in the Mix A trace 3B.) Sample 4 qPCR amplification curve for a positive Y537X mutation shown in the Mix B trace 3C.) Sample 3 qPCR amplification curve for a positive L536X mutation shown in the Mix C trace.

	Sample	Well	Variant	Comments	Rerun
+	Sample 1	B1, B3, B5	ND	PASS	☐
−	Sample 2	C1, C3, C5	D538G	PASS	☐

Primer Mix	Well	Variant	Variant Dye Channel	Variant Cq value	IC Dye Channel	IC Cq Value
A	C1	D538G	FAM	31.22	CY5	31.27
B	C3	ND	ND	ND	CY5	31.10
C	C5	ND	ND	ND	CY5	31.12

+	Sample 3	D1, D3, D5	L536X	PASS	☐
+	Sample 4	E1, E3, E5	Y537X	PASS	☐

Figure 4. QuantideX qPCR *ESR1* exoMutation Analysis Module graphical user interface. Clinical samples from Figure 3 are shown as the reporting created in a dedicated QuantideX qPCR *ESR1* exoMutation Analysis Module with data for individual wells for Sample 2 shown in an optional sub-table.

Conclusion

A rapid, effective, and highly sensitive exosome-based RT-qPCR mutation assay panel for *ESR1* was created and tested. This panel reliably and specifically identifies rare variants in both contrived and clinical liquid biopsy samples using an in-house method (ExoLution Plus) designed to simultaneously enrich exoRNA and cfDNA, included with the QuantideX qPCR *ESR1* exoMutation Kit*.

QuantideX qPCR *ESR1* exoMutation Kit* was shown to be sensitive down to 0.01% MAF, depending on the mutation, while maintaining a high level of specificity. Inclusion of internal controls (IC1, IC2, and IC3) ensures adequate sample is present to test *ESR1* mutations whereas kit provided external batch run control materials represented by CONP, a DNA-based positive control, and CONN, an RNA-based negative control, act as a quality check for mutant positive clinical samples.

This technology could overcome various obstacles in mutation monitoring for liquid biopsies by enhancing the detection of mutant analytes (such as exosomal RNA and cfDNA), boosting analytical sensitivity through innovative reagents and analysis software, and improving accessibility by allowing analysis on commonly available qPCR instruments.

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*This product is under development. Future availability and performance to be determined.

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