A kit targeting detection of *ESR1* mutations from circulating exosomal RNA and cell-free DNA supports longitudinal studies into endocrine therapy resistance in a broadly accessible RT-qPCR format

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Sarah Statt[1], Julie R Thibert[1], Konrad Mueller[1], Melissa Church[1], Jamie Myers[1], Holli Dale[1], Liangjing Chen[1], Elliot Hallmark[1], Megan Yociss[1], Kevin Kelnar[1], Kurt Franzen[2], Johan Skog[2], and Brian Haynes[1]

[1]Asuragen, a Bio-Techne Brand, Austin, TX; [2]Exosome Diagnostics, a Bio-Techne Brand, Waltham, MA

Summary

Breast cancer is a complex disease often requiring ongoing monitoring and management to ensure the best possible patient outcomes. Monitoring for genetic mutations, including those in the Estrogen Receptor 1 (*ESR1*) gene, provides valuable information about tumor progression and response to treatment for select cases.

We describe a comprehensive methodology for targeted clinical RT-qPCR monitoring of *ESR1* mutations in plasma that utilizes both exosomal RNA (exoRNA) and circulating cell-free DNA (cfDNA), interrogates 11 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 across 2 users at our expected Limit of Detection of 0.1% MAF, congruent with ultra-sensitive mutation detection from plasma.

Residual clinical sample testing supports the feasibility of the QuantideX® qPCR *ESR1* exoMutation Kit*, focusing on subjects with stage IV metastatic breast cancer (mBC) (HR+/HER2-) on active aromatase inhibitor therapy, +/- CDK 4/6 inhibitor for at least one year.

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.

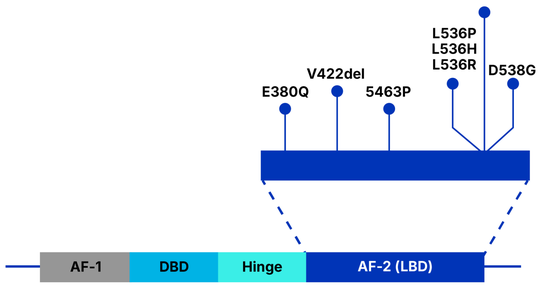

Figure 1. *ESR1* acquired resistance mutations. In patients with HR+/HER2- mBC, *ESR1* mutations are a common cause of acquired resistance to aromatase inhibitors. A key mechanism of endocrine resistance is mutation of the ligand-binding domain (LBD); mutations investigated in our preliminary studies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

To measure the range of *ESR1* copies among individual plasma samples, circulating exosomal RNA (exoRNA) and cell-free DNA (cfDNA) were subjected to an in-house method optimized to co-enrich these two analytes (5,6) (ExoLution Plus, which is included with the QuantideX qPCR *ESR1* exoMutation Kit*) on 15 presumed normal female plasma samples. The exoRNA and cfDNA eluate was split into two reactions, with one reverse transcription (RT) reaction using RT enzyme and one RT reaction without enzyme (no RT) to determine the RNA/DNA fraction contributing to overall *ESR1* signal, as quantified by ddPCR via in-house designed assays (Bio-Rad QX200).

Due to the scarcity of samples containing *ESR1* mutations, plasmids containing the 11 clinically-relevant mutations shown in Figure 1, in conjunction with corresponding wild-type plasmids for the regions of interest, 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 either the QuantStudio 5 or 7500 Fast qPCR Platform (Thermo Fisher).

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) and processed to separate plasma. 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. In-house designed singleplex ddPCR assays of the 11 *ESR1* mutations were used for mutation status confirmation (Bio-Rad QX200).

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

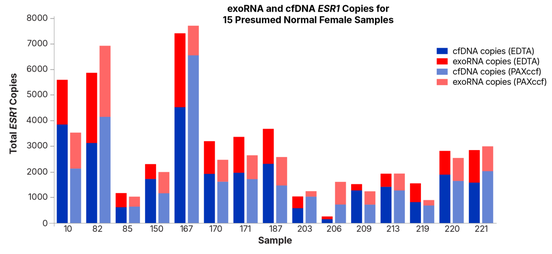

Figure 3. exoRNA and cfDNA Distribution Across 15 Presumed Normal Female Samples Shows an Increase in *ESR1* copies due to exoRNA. Plasma samples (2 mL) collected from 15 presumed normal female subjects underwent exoRNA and cfDNA co-isolation from EDTA tubes processed within 4 hours and PAXgene Blood ccfDNA Tubes (PAXccf) processed within 3 days. RT with enzyme was completed on half the elute and RT without enzyme (no RT) was completed on the other half. ddPCR was utilized to determine *ESR1* copies. *ESR1* copies calculated from the RT with enzyme reaction would include product from both exoRNA and cfDNA (labeled as total *ESR1* copies) whereas the *ESR1* copies calculated from the no RT reaction would include product only from cfDNA portion, shown in blue. Calculated RNA fraction is shown in red. PAXccf had no marked impact on yields, showing the stability tube can preserve material up to 3 days post blood draw. Percentage of exoRNA contribution did vary by sample, with a median value of 38.1%.

Table 2. QuantideX qPCR *ESR1* exoMutation Kit Controls 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). CONP results in a positive signal in all mutant and internal control channels across all 3 tubes whereas CONN results in a positive signal only in our internal control (IC1, IC2, and IC3) channel across all 3 tubes. A single user tested CONP and CONN in duplicate. RT was completed on CONP and CONN, 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. As expected, CONP was positive across all 3 channels for all 3 tubes and CONN was only positive in the internal control channel.

ND = signal not detected within 45 cycles

Tube #	Target	CONP		CONN	
		Rep 1	Rep 2	Rep 1	Rep 2
1	D538G	33.74	33.75	ND	ND
	S463P	35.03	35.11	ND	ND
	IC1	32.93	32.90	32.33	32.41
2	Y537X	30.65	30.67	ND	ND
	E380Q	26.11	26.29	ND	ND
	IC2	32.57	32.78	32.11	31.62
3	L536X	28.35	28.09	ND	ND
	V422del	36.55	36.63	ND	ND
	IC3	32.49	31.79	31.74	31.13

Table 3. Two User Study Shows Concordant Results Utilizing 0.1% MAF (Minor Allele Frequency) and Wild-Type (WT) Samples for all 11 Mutations. Two independent users performed *ESR1* RT-qPCR target enrichment on 11 0.1% MAF mutant contrived samples. Internal controls (IC1, IC2, and IC3) served as a sample QC, noting sufficient *ESR1* copies were present in all samples except those noted by #. As expected, CONP was positive across all channels (mutant or MUT and internal control) for all 3 tubes and CONN was only positive in the internal control channel (data not shown). All 0.1% MAF samples were robustly detected across both users and multiple replicates, noting ND# cases for User 1 were excluded due to lack of adequate IC signal, indicating user error for sample loading. All known WT samples were negative for mutation calls across both users and multiple replicates.

ND = signal not detected within 45 cycles

Tube #	Target	User 1 - 0.1% MAF				User 2 - 0.1% MAF				User 1 - WT	User 2 - WT
		MUT Channel				MUT Channel				MUT Channel	MUT Channel
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 1	Rep 2	Rep 3	Rep 4	Rep 1 -8	Rep 1 -8
1	D538G	34.72	35.87	35.11	35.26	34.24	34.43	34.82	35.30	ND	ND
	S463P	30.29	30.34	29.90	30.00	30.54	30.28	31.50	31.47	ND	ND
2	Y537S	33.01	33.00	32.51	32.79	31.31	31.15	31.14	31.13	ND	ND
	Y537C	34.05	33.77	33.20	33.61	32.00	31.95	30.85	30.91	ND	ND
	Y537N	32.79	32.76	33.24	33.15	33.09	32.99	31.85	31.77	ND	ND
	Y537D	31.94	31.87	31.53	31.52	31.90	31.76	31.11	31.17	ND	ND
	E380Q	29.13	29.29	29.57	29.61	27.34	27.45	27.73	27.67	ND	ND
3	L536R	32.18	31.65	32.18	31.60	30.57	30.24	31.73	31.32	ND	ND
	L536H	32.44	32.25	31.41	31.07	31.68	31.20	30.58	30.64	ND	ND
	L536P	ND#	32.88	31.59	31.92	32.15	31.84	32.56	31.94	ND	ND
	V422del	ND#	38.74	38.77	38.97	34.14	34.05	34.14	34.48	ND	ND

Table 4. Evaluation of QuantideX qPCR *ESR1* exoMutation Kit* with Stage IV mBC Plasma Samples. Plasma samples (2 mL) 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 and cfDNA co-isolation from PAXgene Blood ccfDNA Tubes. Assay workflow (Fig. 2) followed RT, Pre-Amp PCR, and multiplex qPCR reactions targeting 11 *ESR1* mutations run in duplicate. The internal control (IC1, IC2, and IC3) amplified when in the presence of minimum required sample input, serving as a sample QC. All 6 patient samples had sufficient *ESR1* copies within IC1, IC2, and IC3 (data not shown). As expected, CONP was positive across all 3 channels for all 3 tubes (Fig. 4A) and CONN was only positive in the internal control channel (Fig. 4B). Sample 6 was shown to be positive for Y537X (Fig. 4C) and further verified via singleplex ddPCR (data not shown) and qPCR (Fig. 4D) to be positive for Y537C.

ND = signal not detected within 45 cycles

Tube #	Target	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
		MUT Channel		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	Rep 2	Rep 1	Rep 2
1	D538G	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	S463P	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	Y537X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	38.02	37.11
	E380Q	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3	L536X	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	V422del	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

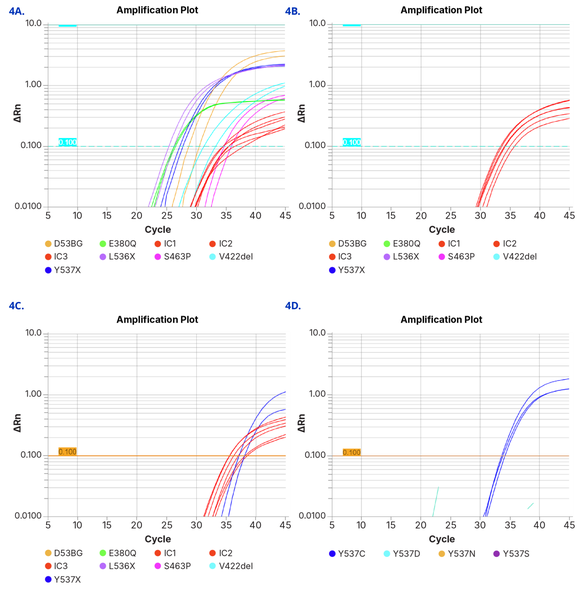

Figure 4. *ESR1* Assay qPCR Amplification Curves for Kit Controls and *ESR1* Mutant Positive Clinical Sample (Sample 6). 4A.) qPCR amplification curves for CONP (run in duplicate) with the 6 clinical samples tested; as expected, CONP was positive across all 3 channels for all 3 tubes, indicating a successful qPCR reaction was completed. 4B.) qPCR amplification curves for CONN (run in duplicate) with the 6 clinical samples tested; as expected, CONN was only positive in the internal control channel for all three tubes (IC1, IC2, and IC3, shown in red), indicating no contamination was present on the plate and RT was successful. 4C.) qPCR amplification curve for Sample 6 (run in duplicate), showing the internal control was successful across all three tubes (IC1, IC2, and IC3, shown in red) as well as a mutant positive signal for Y537X (shown in blue). 4D.) Allele specific primers for Y537X codon (S, C, D, or N) were used in singleplex to determine specific mutation in Sample 6. Each reaction was performed in triplicate and resulted in positive Y537C amplification (shown in blue), as verified by ddPCR (data not shown).

Conclusion

A fast, efficient, and sensitive exosome-based *ESR1* RT-qPCR mutation assay panel was developed and evaluated, demonstrating the reliable and specific detection of rare variants in contrived liquid biopsy specimens using an in-house method (ExoLution Plus) optimized to co-enrich exoRNA and cfDNA (included with the QuantideX qPCR *ESR1* exoMutation Kit*).

QuantideX qPCR *ESR1* exoMutation Kit* was shown to be robust and consistent across multiple users. 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 has the potential to address several challenges associated with mutation monitoring in liquid biopsies by expanding detection of mutant analytes (exosomal RNA and cfDNA), improving analytical sensitivity (novel reagents and analysis software), and increasing accessibility by enabling analysis on widely accessible qPCR instruments.

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

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