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

Sarah Statt[1], Julie R. Thibert[1], Liangjing Chen[1], J. Aquiles Sanchez[2], Kurt Franzen[2], Megan Yociss[1], Elliot Hallmark[1], Melissa Church[1], Stela Filipovic-Sadic[1], Gary J. Latham[1] and Johan Skog[2]

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

SUMMARY

- Breast cancer is a complex disease that often requires ongoing monitoring and management to ensure the best possible patient outcomes. Monitoring for genetic mutations, including those in the *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 interrogates both exosomal nucleic acids (RNA and DNA) and cfDNA, reports multiple mutations, provides a streamlined workflow, and accommodates a range of mutant allele fractions.
- We demonstrate consistent and specific RT-qPCR down to 3 *ESR1* mutants (in a background of 5,000 wild-type copies, or 0.06% mutant allele fraction), congruent with ultra-sensitive mutation detection from 1-2 mL plasma.

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 the 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.

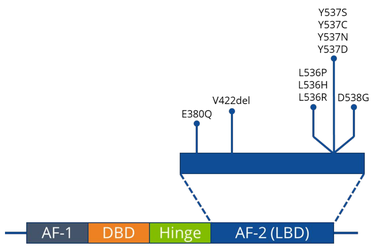
METHODS

To measure the range of ESR1 exosomal RNA copies among individual plasma samples, exosomal nucleic acids (RNA and DNA) and circulating cell-free DNA (cfDNA) were co-isolated using the ExoLution Plus Isolation Kit (Exosome Diagnostics) on eight presumed normal female plasma samples. Reverse Transcription (RT) was completed on the entire exosomal RNA/DNA and cfDNA eluate using SuperScript VILO Master Mix (Thermo Fisher) and 5 µL of RT product was added to a 40 µL QIAcuity digital PCR (dPCR) reaction.

Due to the scarcity of samples containing *ESR1* mutations, plasmids containing the 10 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 dPCR (BioRad QX200). IVT product was created by linearizing plasmids with BamHI (NEB), followed by MEGAshortscript T7 kit (Thermo Fisher) and MEGAclear Transcription Clean-Up Kit (Thermo Fisher).

Contrived samples were created by adding 5,000 copies of *ESR1* cDNA into 8 mL of pooled presumed normal female plasma prepared from K2EDTA blood collection tubes and processed through a 0.8 µm filter. Exosomal nucleic acids and cfDNA were co-isolated using the ExoLution Plus Isolation Kit (Exosome Diagnostics).

RT-qPCR and qPCR-based target enrichment was performed using modified QuantideX® reagents (Asuragen) and mutations were confirmed on either the QuantStudio 5 or 7500 Fast qPCR Platform (Thermo Fisher).



Common Name	COSMIC ID	Coordinate GRCh38	RefBase	AltBase
E380Q	COSV52782264	Chr6:152011697	G	C
V422del	COSV52789441	Chr6:152061020..152061022	GTGG	G
L536P	COSV52782930	Chr6:152419920	T	C
L536H	COSV52795259	Chr6:152098785	T	A
L536R	COSV52787207	Chr6:152419920	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

Figure 1. *ESR1* Acquired Resistance Mutations. In patients with HR+/HER2- metastatic breast cancer, *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.

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

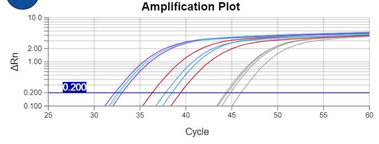
Table 1. Measurement of *ESR1* copy number in eight presumed normal female plasma samples. Copy number of wild-type *ESR1* exosomal RNA/DNA and cfDNA recovered from 2mL plasma from eight individual presumed normal female plasma samples was measured using dPCR. *ESR1* copies ranged from 1,133 to 8,256 per 2mL plasma, with an average of 3,584 copies. Assuming a limit of detection of 5 mutant copies, the minimal minor allele frequency (MAF) ranged from 0.06% to 0.44% MAF, with an average of 0.14%. PC = Positive Control (synthetic DNA)

Sample	Positive Partitions	Negative Partitions	Conc. (copies per µL)	Est. Copies per 2 mL Plasma	Avg. Copies per 2 mL Plasma	Minimal MAF Assuming 5 Mutant Copies and 2 mL Plasma
1	251	25,195	13.3	2,554	2,717	0.18%
1	276	25,151	15	2,880		
2	274	25,176	14.7	2,822	2,909	0.17%
2	282	25,153	15.6	2,995		
3	641	24,825	34.7	6,662	6,768	0.07%
3	637	24.796	35.8	6,874		
4	235	25,215	12.6	2,419	2,448	0.20%
4	231	25,046	12.9	2,477		
5	153	25,313	8.2	1,574	1,642	0.30%
5	162	25,261	8.9	1,709		
6	800	24,674	43	8,256	8,016	0.06%
6	738	24,706	40.5	7,776		
7	114	25,338	5.9	1,133	1,191	0.42%
7	125	25,347	6.5	1,248		
8	290	25,148	15.3	2,938	2,986	0.17%
8	293	25,119	15.8	3,034		
NTC	0	25,477	0	N/A	N/A	N/A
NTC	0	25,445	0	N/A		
PC	2340	23,066	129.9	N/A	N/A	N/A
PC	2264	23,192	127.3	N/A		
Average across all 8 samples				3,584	3,584	0.14%

2A

Mutant Copies	50	50	5	3	0		
WT Copies	0	5000	5000	5000	5000		
Target	100% MAF Avg. Ct	1% MAF Avg. Ct	0.1% MAF Avg. Ct	0.06% MAF Avg. Ct	0% MAF WT Avg. Ct	WT - Mutant 0.1% Avg. Ct	WT - Mutant 0.06% Avg. Ct
D538G	32.98	33.16	37.2	38.07	47.08	9.88	9.01
Y537S	30.58	30.52	36.56	35.70	42.85	6.29	7.15
Y537C	32.47	32.42	37.89	37.67	44.70	6.81	7.03
Y537N	30.92	30.35	35.36	33.80	40.25	4.89	6.46
Y537D	30.98	30.52	34.20	36.22	44.43	10.23	8.21
L536R	30.84	30.78	35.49	35.11	39.80	4.31	4.69
L536H	30.64	31.04	34.15	34.73	42.38	8.23	7.65
L536P	32.52	33.05	36.04	36.22	38.10	2.06	1.89
E380Q	30.09	30.02	35.96	35.04	42.05	6.10	7.02
V422Del	31.75	31.84	35.96	36.26	43.40	7.45	7.14

2B



2C

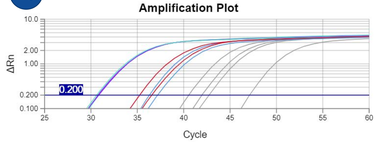
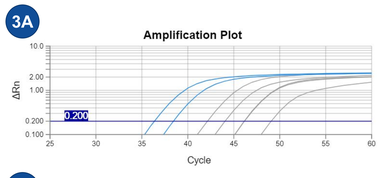
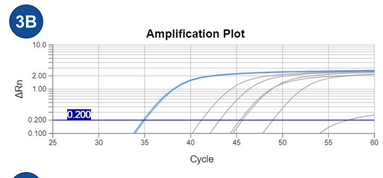


Figure 2. Prototype *ESR1* qPCR assays can detect Minor Allele Fractions (MAFs) at 0.06% or 3 mutant copies for 9 out of 10 mutations. 4 replicates of linearized wild-type (WT) plasmid and 2 replicates of varying proportions amounts of mixed mutant and WT plasmid were run in qPCR for 60 cycles to investigate the sensitivity of each qPCR assay. As seen in the overview table in **2A.)** and more specifically in traces shown in **2B.)** for Y537C and **2C.)** for Y537S, a > 3 Ct difference between the WT target (grey) and the 0.06% mutant target (red) was maintained across 4 replicates of WT and 2 replicates of 0.06% mutant target (3 mutant copies in the background of 5,000 WT copies) for 9 of the 10 *ESR1* targets (L536P did not meet this acceptance criteria). Key: 50 mutant copies (100% MAF) = teal; 1% MAF (also 50 mutant copies) = purple; 0.1% MAF = blue; 0.06% MAF = red; 0% MAF = grey

3A



3B



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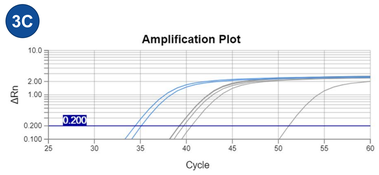


3D

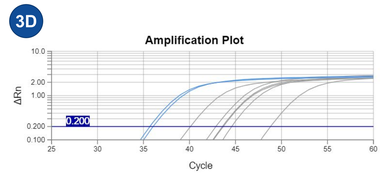
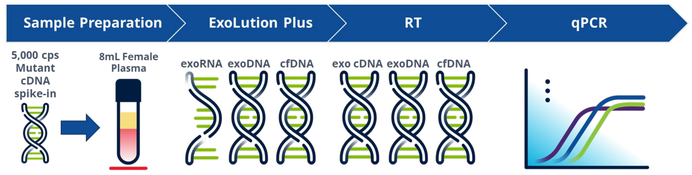


Figure 3. Consistent and specific amplification of *ESR1* qPCR mutation signal was maintained in the presence of WT template. Using the qPCR assays for the 4 mutations present in *ESR1* codon 537, 6 replicates of linearized WT plasmid were run for 60 cycles to investigate the occurrence of false positive signals when only WT targets were present. As seen in **3A.)** Y537C, **3B.)** Y537S, **3C.)** Y537N, and **3D.)** Y537D, > 3 Ct difference between the lowest Ct for the WT target and the highest Ct for 0.1% mutant target was maintained across 6 replicates of WT and 2 replicates of 0.1% mutant target (5 mutant copies in the background of 5,000 WT copies). Key: 0% MAF = grey; 0.1% MAF (5 mutant copies in 5,000 WT copies) = blue

4A



4B

cDNA Spike-in Sample	Positive Partitions	Negative Partitions	Conc. (cps/µL)	Est. Copies per qPCR reaction
D538G	174	15004	13.6	68
Y537S	262	14741	20.7	103.5
Y537C	289	15047	22.4	112
Y537N	232	15420	17.6	88
Y537D	225	14733	17.8	89
L536R	222	14872	17.4	87
L536H	201	12705	18.5	92.5
L536P	247	12456	23.1	115.5
E380Q	210	12616	19.4	97
V422Del	246	13982	20.5	102.5

Figure 4. Mutant *ESR1* IVT cDNA spiked into pooled plasma is effectively recovered using ExoLution Plus. 4A.) Complete assay workflow reflecting input of 5,000 copies of *ESR1* cDNA (created by RT of IVT product) for each of the 10 mutations added individually to 8mLs of pooled plasma from presumed normal females and immediately processed using the ExoLution Plus Isolation Kit. Reverse Transcription (RT) was completed on the entire exosomal RNA/DNA (exoRNA/exoDNA) and cfDNA (data table 4B), followed by qPCR (Table 2). **4B.)** dPCR was utilized to estimate copies of *ESR1* mutant utilized in experiment shown in Table 2.

Table 2. RT-qPCR of *ESR1* mutant cDNA spike-in demonstrates differentiation between mutant and WT background in pooled plasma samples. Across 2 replicates, RT-qPCR *ESR1* assays were able to differentiation between mutant (originating from cDNA spike-in) and background WT present in female plasma samples. ~100 copies of each spiked-in *ESR1* mutant IVT cDNA shown in Figure 4 was carried into the qPCR step. Undetermined values were excluded from calculations.

	Wild-Type (WT) Plasma			Mutant cDNA spike-in			WT - Mutant Avg. Ct
Target	Rep1	Rep2	Average	Rep1	Rep2	Average	
D538G	45.59	Undetermined	45.59	34.12	33.83	33.97	11.61
Y537S	Undetermined	Undetermined	Undetermined	29.54	29.62	29.58	Undetermined
Y537C	Undetermined	Undetermined	Undetermined	32.30	32.05	32.18	Undetermined
Y537N	41.35	Undetermined	41.35	29.84	29.75	29.80	11.55
Y537D	Undetermined	59.53	59.53	30.05	29.97	30.01	29.52
L536R	55.59	47.80	51.70	29.41	29.66	29.54	22.16
L536H	Undetermined	40.28	40.28	29.56	29.63	29.59	10.69
L536P	42.63	44.61	43.62	34.10	33.37	33.73	9.88
E380Q	Undetermined	51.49	51.49	30.99	30.57	30.78	20.71
V422Del	Undetermined	Undetermined	60.00	30.18	30.72	30.45	Undetermined

CONCLUSIONS

- 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 utilizing the ExoLution Plus Isolation Kit.
- Prototype *ESR1* qPCR mutation assays detected mutant allele fractions as low as 0.06% or 3 mutant copies in the background of 5,000 wild-type copies for 9 out of 10 mutations investigated, maintaining high specificity when only wild-type template is present.
- This prototype technology has the potential to address several challenges associated with mutation monitoring in liquid biopsies by expanding detection of mutant analytes (exosomal RNA and DNA), improving analytical sensitivity (novel reagents and analysis software), and increasing accessibility (qPCR instrument install base).

REFERENCES

1. Hartkopf, AD et al. Breast Care 2020;15:347–354; DOI: 10.1159/000508675
2. Dustin, D. et al. Cancer 2019; 125(21): 3714-3728; DOI: 10.1002/cncr.32345
3. (n.d.). FDA approves elacestrant for ER-positive, HER2-negative, ESR1-mutated advanced or metastatic breast cancer. FDA. https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-elacestrant-er-positive-her2-negative-esr1-mutated-advanced-or-metastatic-breast-cancer
4. (n.d.). NCCN Guidelines Version 4.2023. NCCN. https://www.nccn.org/professionals/physician_gls/pdf/breast.pdf

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