Validation of a New Highly Sensitive RT-qPCR Test to Monitor *ESR1* Mutations from Liquid Biopsies

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Summary

- The ability to detect *ESR1* mutations early can impact downstream treatment regimens in HR+/HER2- breast cancer patients.
- Molecular methods for detection using multiplex technology provide fast, sensitive, and accurate results particularly from liquid biopsies.
- Having a CLIA-validated test ready for early trial readouts is a key step in filling this critical need for gathering genetic data relevant to treatment options in this patient population.

Introduction

Hormone receptor-positive/human epidermal growth factor receptor 2-negative (HR+/HER2-) is the most common subtype of breast cancer, with an incidence of 90 new cases per 100,000 women annually. In metastatic HR+/HER2- breast cancer, resistance to aromatase inhibitors - a key part of endocrine therapy - often arises from acquired *ESR1* mutations in the estrogen receptor's ligand-binding domain. Because repeat biopsies are rarely performed after treatment begins, detecting *ESR1* mutations in plasma is essential to inform downstream therapy and aligns with updated NCCN guidelines. Early identification of these mutations enables timely transition to second-line therapies like elacestrant with demonstrated efficacy against *ESR1* mutations. Furthermore, recent clinical trials such as SERENA-6 and PADA-1 have demonstrated clinical benefit to changing treatment. This report presents analytical and clinical validation of a multiplex RT-qPCR assay and software platform that detects 11 key *ESR1* resistance mutations in plasma, utilizing a novel method that captures both exosomal RNA and cfDNA to improve sensitivity.

Common Target Name	Coordinate GRCh38	Reference Base	Alternate Base	Primer Mix	Dye
D538G	Chr6:152098791	A	G	Mix A	FAM
S463P	Chr6:152094402	T	C	Mix A	VIC
E380Q	Chr6:152011697	G	C	Mix B	VIC
Y537C	Chr6:152098788	A	G	Mix B	FAM
Y537D	Chr6:152098787	T	G	Mix B	FAM
Y537N	Chr6:152098787	T	A	Mix B	FAM
Y537S	Chr6:152098788	A	C	Mix B	FAM
L536H	Chr6:152098785	T	A	Mix C	FAM
L536P	Chr6:152098785	T	C	Mix C	FAM
L536R	Chr6:152098785	T	G	Mix C	FAM
V422del	Chr6:152061020..152061022	GTGG	G	Mix C	VIC

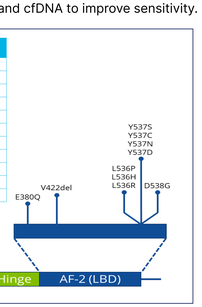

Figure 1. ***ESR1*** **Acquired Resistance Mutations.** In patients with HR+/HER2- metastatic breast cancer, *ESR1* mutations are a common cause of acquired resistance to aromatase inhibitors. A key mechanism of endocrine resistance is mutation of the ligand-binding domain (LBD); mutations investigated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

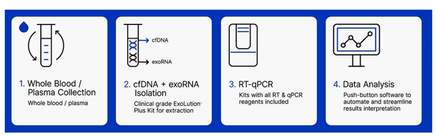

Figure 2. ***ESR1*** **test Workflow, Three Day Turnaround.** Plasma is separated from whole blood drawn in K2-EDTA tubes. Total nucleic acid is isolated using Evolution Plus isolation kit. Reverse transcription, pre-amplification, and qPCR are carried out per manufacturer's instructions. Analysis is exported from Diomni and Quantidex software to look at raw data and positive/negative calls.

Materials and Methods

The QuantideX qPCR *ESR1* exoMutation Kit (Asuragen, Inc.) includes assays and controls for 11 ESR1 mutations (Figure 1). Clinical specimens were procured from patients with stage IV (metastatic) breast cancer (HR+/HER2-) currently on aromatase inhibitor +/- CDK4/6i for ≥12 months with no prior exposure to SERM or SERD therapies. Derivative samples were prepared in wild-type-confirmed plasma spiked with plasmids containing known mutations. These contrived samples were verified for the presence of mutation using droplet digital PCR. The workflow is depicted in Figure 2. Total nucleic acid (free and exosomal nucleic acids) was isolated from 2.0mL of plasma. Samples were subjected to reverse transcription, pre-amplification, and replicated at the qPCR stage, All studies were run on an ABI QuantStudio 7 real-time instrument. Diomni Software (Thermo Fisher) was used to run first-pass QC and QuantideX qPCR *ESR1* exoMutation Analysis Module (Asuragen, Inc) software was used to export Ct values for analysis. Data filtration excluded values >36 and those which amplified in only one replicate. Positive, negative, and overall percent agreement (PPA, NPA, OPA) were calculated based on observed/expected positive and negative calls. The primary objective of the study was to demonstrate analytical validity in a clinical setting with secondary objectives surrounding analytical concordance, sensitivity, and precision. The acceptance criteria for primary analysis for each study were set to ≥85% sensitivity and specificity. The acceptance criteria for secondary analysis for each study were set to ≥90% agreement for each mutation.

Results

- **Analytical Sensitivity** – Limit of Detection (LOD) was verified on the QuantStudio 7 to range from 0.03% to 0.1%.

Table 1. Analytical Sensitivity results. Mutant-positive plasmid DNA was titrated into a background of wild-type fragmented cell line genomic DNA.

Variant	QuantStudio 7 Pro Dx
D538G	0.100%
S463P	0.039%
Y537S	0.026%
Y537C	0.029%
D538G	0.100%
S463P	0.039%
Y537S	0.026%
Y537C	0.029%
Y537N	0.026%
Y537D	0.029%
E380Q	0.029%
L536R	0.029%
L536H	0.041%
L536P	0.028%
V422del	0.030%

- **Analytical Accuracy/Concordance** – Data from 10 positive and 10 negative contrived samples were compared to ddPCR for expected mutations. Samples were randomized and blinded to the operator. 360 datapoints were analyzed with 351 aligning with expected call resulting in 100% PPA and 97.5% OPA (Table 2). Three false positives were detected.

Table 2. Analytical Accuracy Results. Mutations in red were false positive calls.

Row Labels	Expected Call	CLIA ESR1 Call
S0166266	D538G	D538G|L536X|V422del
S0166281	NEG	NEG
S0166282	NEG	NEG
S0166284	NEG	NEG
S0166290	NEG	NEG
S0166291	D538G|E380Q	D538G|E380Q|L536X
S0166292	Y537X	Y537X
S0166293	NEG	NEG
S0166297	V422del	V422del
S0166298	Y537X	Y537X
S0166300	L536X	L536X
S0166301	NEG	NEG
S0166307	E380Q	E380Q
S0166308	NEG	NEG
S0166311	S463P	S463P
S0166314	L536X	L536X
S0166315	NEG	NEG
S0166316	NEG	NEG
S0166319	NEG	NEG
S0166328	D538G	D538G

- **Repeatability** – Three contrived samples were run by one operator replicated 10 times on the run. The three expected variants were D538G, E380Q, and V422del. These had %CVs of 0.7%, 0.3%, and 0.3% respectively (data not shown).
- **Intermediate Precision** – Two operators tested the same three contrived samples in triplicate over five non-consecutive days through the entire workflow. All expected variants were detected by each operator per day run, with no false positives or false negatives resulting in 100% PPA, NPA, and OPA (Table 3).

Table 3. Overall Combined Intermediate Precision Results show %CVs <5% at the Ct level. Values in red only had one replicate out of 30 with a value, so these were excluded from analysis.

Mutation / Expected Mutation	Metric	Samples: CONN None	CONP All	NTC None	S0166267 D538G | E380Q	S0166299 None	S0166313 Y537X | V422del
D538G	Avg	ND	30.71	ND	28.13	ND	ND
	Stdev	ND	0.94	ND	0.62	ND	ND
	%CV	NA	3.1	NA	2.2	NA	NA
S463P	Avg	ND	32.44	ND	ND	ND	ND
	Stdev	ND	0.81	ND	ND	ND	ND
	%CV	NA	2.5	NA	NA	NA	NA
Y537X	Avg	ND	28.09	ND	ND	ND	34.02
	Stdev	ND	0.95	ND	ND	ND	0.45
	%CV	NA	3.4	NA	NA	NA	1.3
E380Q	Avg	ND	29.76	ND	27.75	ND	ND
	Stdev	ND	0.93	ND	0.42	ND	ND
	%CV	NA	3.1	NA	1.5	NA	NA
L536X	Avg	ND	28.71	ND	ND	ND	ND
	Stdev	ND	0.84	ND	ND	ND	ND
	%CV	NA	2.9	NA	NA	NA	NA
V422del	Avg	ND	28.69	ND	ND	ND	26.81
	Stdev	ND	0.85	ND	ND	ND	0.57
	%CV	NA	3.0	NA	NA	NA	2.1

- **Analytical Validity –** Clinical Accuracy sample testing. Procured samples were verified for ESR1 mutations from vendor but confirmed with ddPCR prior to testing. 40 negatives and 5 positives were randomized and blinded to the operator for isolation and testing. Due to the rarity of these mutations, an additional 5 contrived positives were added to the sample set to bring the total to 50. 10/11 positives were correctly identified and there was one false negative resulting in 90.9% PPA and 98.3% OPA.

Table 4A. Clinical Accuracy Results. Values in red were false negative or positive calls.

Sample ID	D538G Avg Ct	D538G StdDev Ct	S463P Avg Ct	S463P StdDev Ct	Y537X Avg Ct	Y537X StdDev Ct	Expected Variant	CLIA ESR1 Call
CONN	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
CONP	30.68	0.80	33.18	0.96	27.91	0.19	ALL	ALL
S0166268	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166269	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166270	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166271	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166272	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166273	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166274	26.70	0.12	NEG	NEG	NEG	NEG	D538G	D538G
S0166275	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166276	NEG	NEG	NEG	NEG	26.77	0.16	Y537X	Y537X
S0166277	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166278	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166279	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166280	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166283	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166285	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166286	32.51	0.68	NEG	NEG	NEG	NEG	D538G	D538G
S0166287	33.81	0.11	NEG	NEG	NEG	NEG	NEG	D538G
S0166288	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166289	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166294	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166295	NEG	NEG	NEG	NEG	30.03	0.31	Y537X	Y537X
S0166296	NEG	NEG	NEG	NEG	NEG	NEG	Y537X	NEG
S0166302	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166303	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166304	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166305	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166306	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166309	35.64	1.16	NEG	NEG	NEG	NEG	D538G	D538G
S0166310	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166312	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166317	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166318	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166321	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166322	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166323	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166324	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166325	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166327	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166328	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166329	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166330	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166331	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166332	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166333	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166334	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166335	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166336	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166337	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166338	27.82	0.10	NEG	NEG	NEG	NEG	D538G	D538G
S0166320	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

Table 4B. Clinical Accuracy Results. Values in red were false negative or positive calls.

Sample ID	E380Q Avg Ct	E380Q StdDev Ct	L536X Avg Ct	L536X StdDev Ct	V422del Avg Ct	V422del StdDev Ct	Expected Variant	CLIA ESR1 Call
CONN	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
CONP	29.42	0.47	29.05	0.69	28.61	0.54	ALL	ALL
S0166268	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166269	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166270	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166271	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166272	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166273	NEG	NEG	32.01	0.11	NEG	NEG	NEG	L536X
S0166274	27.63	0.10	NEG	NEG	NEG	NEG	E380Q	E380Q
S0166275	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166276	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166277	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166278	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166279	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166280	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166283	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166285	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166286	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166287	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166288	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166289	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166294	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166295	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166296	NEG	NEG	31.93	0.40	NEG	NEG	NEG	L536X
S0166302	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166303	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166304	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166305	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166306	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166309	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166310	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166312	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166317	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166318	NEG	NEG	30.62	0.35	NEG	NEG	NEG	L536X
S0166321	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166322	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166323	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166324	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166325	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166327	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166328	NEG	NEG	34.17	1.59	NEG	NEG	L536X	L536X
S0166327	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166328	NEG	NEG	NEG	NEG	27.02	0.05	V422del	V422del
S0166329	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166330	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166331	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166332	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166333	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166335	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166334	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166336	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166336	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166337	NEG	NEG	26.55	0.05	NEG	NEG	L536H	L536X
S0166338	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
S0166320	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

Conclusions

- **Run reliability:** Every plate passed on the first attempt with all controls (CONN/CONP/IC) behaving as expected. No repeats were required.
- **Reproducibility:** Across two operators over five days, call concordance was perfect—positive and negative results matched every time (PPA, NPA, and overall agreement all 100%).
- **Analytical accuracy:** Compared with an orthogonal reference method, results agreed in >97% of comparisons.
- **Clinical accuracy (HR+/HER2- MBC):** The assay correctly identified known mutation-positive cases >90% of the time and showed >98% overall agreement in patient samples.
- **Ongoing work:** Additional stability studies and more clinical specimens are being tested to further strengthen the data set.
- **Bottom line for patient testing:** Performance supports clinical use as a CLIA-validated Laboratory Developed Test*—runs are dependable, results are reproducible across operators and days, and accuracy is high both analytically and in the intended clinical population. Continued data collection should only add confidence.

Scan code to learn more

1. 42CFR493; Code of Federal Regulations Laboratory Requirements, 1990
2. CLSI. *Evaluation of Qualitative, Binary Output Examination Performance*. 3rd ed. CLSI guideline EP12. Clinical and Laboratory Standards Institute; 2023.
3. CLSI. *Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures; Approved Guideline—Second Edition*. CLSI document EP17-A2. Wayne, PA: Clinical and Laboratory Standards Institut
4. (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
5. (n.d.). NCCN Guidelines Version 4.2023. NCCN. https://www.nccn.org/professionals/physician_gls/pdf/breast.pdf

*Research Use Only; not for use in diagnostic procedures. The analytical and clinical performance of this test have not been reviewed by the U.S. Food and Drug Administration.

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