Development and Evaluation of a Highly Sensitive qPCR Assay for *ESR1* Mutation Monitoring

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Summary

- Mutations in the Estrogen Receptor 1 (*ESR1*) gene are the leading cause of treatment resistance in HR+ metastatic breast cancer (mBC). While *ESR1* testing is currently recommended at progression, clinical trials are demonstrating the benefits of switching therapies once mutations are detected in blood, prior to overt progression. Broadly accessible blood-based tests for *ESR1* will be needed to support a paradigm shift to molecular monitoring in the mBC setting.
- The ExoLution™ Plus cfDNA + exoRNA Isolation Kit* and QuantideX® qPCR *ESR1* exoMutation Kit* combination includes reagents, consumables, and analysis software to accurately detect 11 *ESR1* resistance mutations on widely-used qPCR platforms.
- The ExoLution Plus cfDNA + exoRNA Isolation Kit* was developed to co-isolate cell free DNA (cfDNA) and exosomal RNA (exoRNA) from 2-4 mL of filtered plasma in one hour using standard lab equipment.
- The kit performance was evaluated on a cohort comprised of presumed healthy females and females diagnosed with mBC on endocrine therapy (ET). Analytical verification was performed across multiple collection tube types, operators, storage conditions, qPCR platforms, plasma inputs, and kit lots, resulting in robust and precise analytical performance.

Introduction

Hormone receptor-positive/human epidermal growth factor receptor 2-negative (HR+/HER2-) is the most prevalent subtype of breast cancer at a rate of 90 new cases per 100,000 women each year. HR+/HER2- metastatic breast cancer (mBC) is treated through ET in combination with CDK4/6 inhibitors. Mutations in the ligand binding domain of *ESR1* lead to constitutive activation of estrogen receptor signaling and account for up to 40% ET resistance (1). Since the FDA approval of elacestrant (2), the NCCN guidelines have been updated to recommend testing for *ESR1* mutation status (3). A liquid biopsy specimen is recommended because *ESR1* mutations are acquired during treatment and rarely found in primary tumor tissue.

While *ESR1* testing is currently recommended at progression, ongoing clinical trials are establishing the benefits of early therapeutic intervention upon detection of resistance mutations through liquid biopsy monitoring (4). The QuantideX qPCR *ESR1* exoMutation Kit was developed to address the need for more accessible *ESR1* resistance monitoring research. The kit incorporates signal from both cfDNA and exosomal RNA (exoRNA) to detect 11 *ESR1* mutations through an easy-to-implement isolation and qPCR assay workflow.

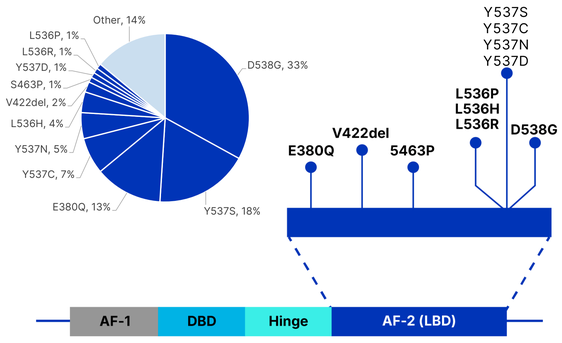

Figure 1. *ESR1* Acquired Resistance Mutations. In patients with HR+/HER2- mBC, *ESR1* mutations are a common cause of acquired resistance to aromatase inhibitors. A key mechanism of endocrine resistance is mutation of the ligand-binding domain (LBD); *ESR1* resistance mutations are further concentrated in 6 codons within the LBD as shown. Overall, these 11 mutations account for 86-90% of all *ESR1* mediated ET resistance (5, 6).

Table 1. The Eleven Most Prevalent *ESR1* Resistance 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

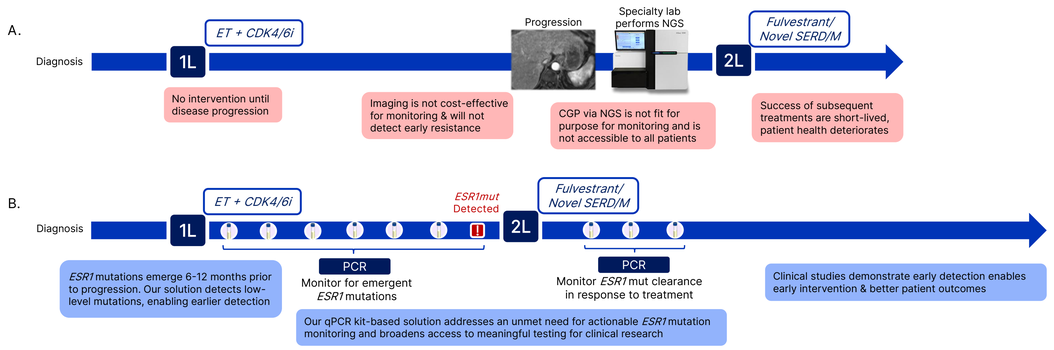

Figure 2. Comparing the Current Standard of Care with Potential Future Standard of Care for HR+/HER2- mBC. Current standard of care for HR+/HER2- mBC (Panel A) recommends ET in combination with CDK4/6i as the frontline therapy (1L), followed by assessing for progression through imaging or other overt measures. Upon radiological progression, liquid biopsy testing for *ESR1* mutation status is performed by NGS comprehensive genomic profiling (CGP) or a targeted PCR assay. Patients positive for *ESR1* mutations are then switched to second line therapy (2L). Ongoing clinical trials (e.g. PADA-1 and SERENA-6) are demonstrating the benefits of switching to 2L upon detection of *ESR1* resistance mutations in blood, prior to overt progression. There is a growing unmet need for accessible testing to address the potential future standard of care (Panel B), wherein patients will be serially monitored for the emergence of *ESR1* mutations and switched to 2L therapy upon detection of molecular resistance.

Materials & Methods

Multiple study-specific sample panels consisting, in total, of 108 samples of differing types, cfDNA and exoRNA from plasma, linearized plasmids, cell line DNA, and cell line conditioned media were used to evaluate the QuantideX qPCR *ESR1* exoMutation Kit performance. cfDNA and exoRNA were co-isolated from 2 mL of plasma collected in K_2EDTA or PAXgene™ Blood ccfDNA tubes from a cohort comprised of presumed healthy females and females diagnosed with mBC on ET. Contrived samples were prepared to assess the limit of detection through a dilution series of synthetic mutant copies in the background of fragmented cell line DNA. RT-qPCR enrichment was performed on widely used thermal cyclers and qPCR instruments (ABI 7500 Fast Dx, QuantStudio™ 5 Dx, QuantStudio™ 7 Pro Dx). Orthogonal methods including dPCR were used to confirm mutant positive results in the mBC subgroup.

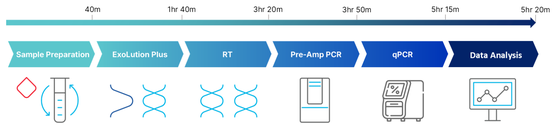

Figure 3. QuantideX qPCR *ESR1* exoMutation Kit Workflow. A minimum of 2 mL of plasma is processed using an in-house method (ExoLution Plus) optimized to co-enrich exoRNA and cfDNA (included in the QuantideX qPCR *ESR1* exoMutation Kit*). RT was completed on the co-isolated exoRNA and cfDNA, followed 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.

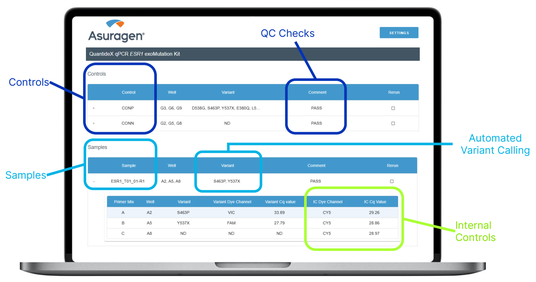

Figure 4. Example Output of QuantideX qPCR *ESR1* exoMutation Analysis Module*. Custom software provides fully automated quality controls and variant calling. Analysis results can be exported in LIMS-compatible format (.csv).

Results

Table 2. Variant-Level OPA, PPA, NPA all ≥ 90% in Single-Site Precision. The precision study incorporated two operators, two qPCR instruments (QS5), three reagent lots, and was performed across six days. Experiments used surrogate samples of variant-positive plasmid DNA in a background of fragmented wild type DNA, covering all 11 variants. Agreement metrics were calculated by reagent lot, by operator, and by variant.

Parameter	OPA	PPA	NPA
Lot 1	99%	100%	98%
Lot 2	98%	100%	96%
Lot 3	99%	99%	98%
Operator 1	97%	99%	97%
Operator 2	99%	100%	99%
D538G	96%	100%	94%
S463P	99%	96%	99%
Y537X	98%	100%	94%
E380Q	100%	100%	100%
L536X	97%	100%	94%
V422del	100%	100%	100%

Table 3. Variant-Level Negative Percent Agreement (NPA) ≥ 97% Across Multiple Blood Collection Tube Types. Sample panel consisted of 30 plasma samples per collection tube type (21 negative, 9 positive), 60 total. Each co-isolation used 2 mL plasma, and samples were analyzed on the QS5 platform.

Tube Type	Variant NPA	Called Neg / Expected Neg
K_2EDTA	97%	166 / 171
PAXgene Blood ccfDNA	98%	167 / 171

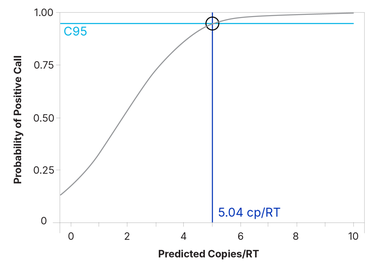

Figure 5. Example Resultant Curve of Probit Analysis Used to Determine Limit of Detection (LoD). S463P depicted here at 5.04 copies per RT reaction in a background of 10,000 total copies equating to 0.05% VAF. For each variant, a probit-linked inverse prediction fit model was generated to determine the relationship between sample hit rate and known analyte concentration. The concentration at which the curve crossed the 95% probability line (i.e., the C95) was reported as the estimated minimum VAF the assay can reliably detect.

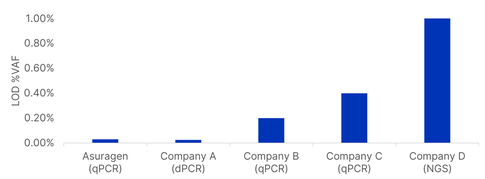

Figure 6. Median LoDs of Commercially Available *ESR1* Mutation Detection Kits. The QuantideX qPCR *ESR1* exoMutation Kit is compared to four other commercially available kits utilizing a range of technologies from dPCR to qPCR and NGS. The QuantideX qPCR *ESR1* exoMutation Kit achieves a median LoD of 0.03%, on par with the best alternative commercial test, which employs dPCR.

Table 4. Established LoDs (%VAF) Ranged from 0.03% to 0.08%. Probit analysis of synthetic DNA (0, 1, 3, 5, 10 copies/RT reaction; 20 replicates each) in a background of fragmented normal DNA (10,000 total copies). Copies/RT was converted to %VAF, which was calculated across all three supported qPCR platforms: 7500, QS5, and QS7. QuantideX qPCR *ESR1* exoMutation Kit displays high sensitivity when compared to claimed LoDs of four alternative *ESR1* mutation detection kits on market.

Variant	Asuragen (qPCR)	Company A (dPCR)	Company B (qPCR)	Company C (qPCR)	Company D (NGS)
D538G	0.08%	0.01%	0.33%	0.4%	1.1%
S463P	0.05%	0.025%	0.33%	0.08%	2.8%
Y537S	0.03%	0.025%	0.20%	0.1%	1.0%
Y537C	0.03%	0.025%	0.33%	0.4%	Unknown
Y537N	0.03%	0.025%	0.07%	0.2%	Unknown
Y537D	0.03%	-	0.13%	-	Unknown
E380Q	0.03%	0.025%	0.13%	1.0%	1.0%
L536R	0.03%	0.025%	0.26%	0.7%	Unknown
L536H	0.04%	-	0.13%	0.8%	Unknown
L536P	0.03%	-	-	0.9%	Unknown
V422del	0.03%	-	-	-	Unknown

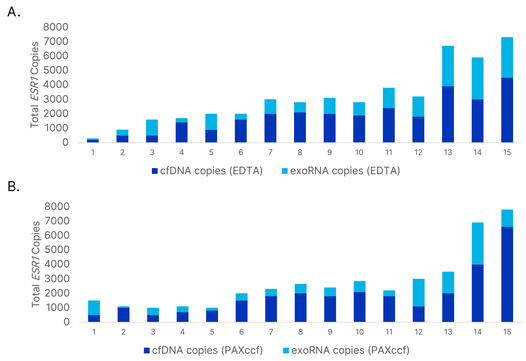

Figure 7. cfDNA and exoRNA Distribution Across 15 Presumed Normal Samples Shows an Increase in *ESR1* Copies Due to exoRNA. Plasma samples (2 mL) collected from 15 presumed normal subjects (x-axis) underwent cfDNA and exoRNA co-isolation from K_2EDTA (Panel A) and PAXgene Blood ccfDNA Tubes (Panel B). Eluates underwent RT with (RT) and without enzyme (NRT), then *ESR1* copies were determined by ddPCR. *ESR1* copies calculated from the RT reaction includes product from both cfDNA and exoRNA, whereas the *ESR1* copies calculated from the NRT reaction would include product only from cfDNA, shown in dark blue. Calculated RNA fraction is shown in light blue. Percentage of exoRNA contribution did vary by sample, with a median value of 38.1%. On average, exoRNA was found to increase the total number of evaluable *ESR1* copies by 60% over cfDNA alone across both tube types.

Table 5. *ESR1* Resistance Mutations Detected and Confirmed in mBC Setting Consistent with Expected Prevalence of *ESR1* Resistance. A cohort of 39 plasma samples from females diagnosed with mBC on ET for at least 1 year were evaluated with the QuantideX qPCR *ESR1* exoMutation Kit, revealing 12/39 *ESR1* positives (31%), consistent with the expected rate of *ESR1* associated resistance in this setting. Mutant positive samples were either determined through upfront screening or confirmatory testing by dPCR. Confirmatory testing is ongoing for P001, P002, and P007.

Sample ID	Detected Variant(s)	Cq Value
202167389	D538G	34.7
17397016	D538G	33.45
17397025	D538G, E380Q, Y537X, L536X	29.31, 33.14, 35.48, 32.43
17397005	D538G, L536X	35.08, 34.29
17397015	E380Q	39.18
17397006	Y537X	36.56
202143632	Y537X	38.7
17397002	Y537X	28.57
17397019	Y537X	26.82
P001	E380Q	38.38
P002	D538G	32.9
P007	Y537X, L536X	26.16, 35.66

Conclusions

- Multiple innovations were leveraged to achieve high sensitivity on qPCR including the incorporation of exoRNA mutation signal enabled through a proprietary isolation kit to co-isolate cfDNA and exoRNA with standard lab equipment.
- An integrated assay and software solution was developed to detect *ESR1* mutations that achieves <0.1% limit of detection in a single day workflow on broadly installed qPCR instruments.
- The QuantideX qPCR *ESR1* exoMutation Kit exhibits precise performance across reagent lots, operators, and variants, with OPA, PPA, and NPA ≥ 94% in a single-site precision study.
- The data supports routine and reliable testing of plasma samples from PAXgene Blood ccfDNA tubes or K_2EDTA tubes for 11 of the most common and actionable *ESR1* mutations.
- The assay technology is readily extensible to treatment response monitoring biomarkers beyond *ESR1*.

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Presenting author, Brian C. Haynes (brian.haynes@bio-techne.com) is a Bio-Techne shareholder and an employee of Asuragen Inc., a Bio-Techne brand.

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