Verification of a Highly Sensitive, Circulating Cell-Free DNA and Exosomal RNA RT-qPCR Assay System to Monitor *ESR1* Mutations from a Liquid Biopsy

Poster
P4-07-25

Kevin Kelnar, Blaine Caughron, Holli Dale, Melissa Church, Julie R Thibert, Jamie Myers, Sarah Statt, and Brian C Haynes

Asuragen, a Bio-Techne Brand, Austin, TX

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, ongoing 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 automated analysis software to reliably detect 11 *ESR1* resistance mutations across 6 target codons 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.
- The Kit design was verified on cfDNA and exoRNA isolated from multiple collection tube types, linearized plasmids, and cell line conditioned media samples across multiple operators, storage conditions, qPCR platforms, plasma inputs, and kit lots, resulting in robust and precise assay performance.

Introduction

At a rate of 90 new cases per 100,000 women per year, hormone receptor-positive/human epidermal growth factor receptor 2-negative (HR+/HER2-) is the most prevalent subtype of breast cancer. Mutations in the ligand binding domain of the estrogen receptor gene (*ESR1*) are the leading cause of resistance to aromatase inhibitor therapy. With repeat biopsy collection rarely performed after starting therapeutic treatment, early identification of *ESR1* mutations in metastatic breast cancer in plasma is critical after progression on aromatase inhibitor therapy and is in alignment with recent NCCN guideline updates. Making the decision to switch to elacestrant, a second line therapeutic, as early as possible, hinges upon the presence of *ESR1* resistance mutations, highlighting the importance of early detection in an easily accessible sample type. We report the performance of an assay that detects 11 *ESR1* acquired resistance mutations associated with HR+/HER2- mBC from plasma liquid biopsies that utilizes RT-qPCR technology combined with a novel isolation method that captures exosomal RNA in addition to cfDNA to improve mutation detection sensitivity.

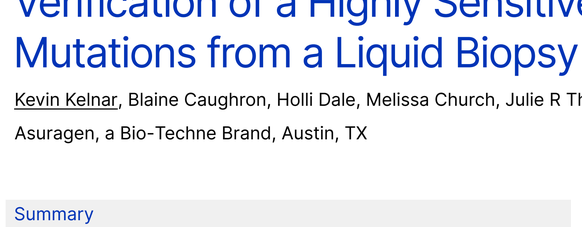

Figure 1. ExoLution Plus cfDNA + exoRNA Isolation Kit and QuantideX qPCR *ESR1* exoMutation Kit Workflow. A minimum of 2 mL of plasma is processed using a novel extraction method optimized to co-enrich cfDNA and exoRNA (included with the QuantideX qPCR *ESR1* exoMutation Kit*). RT product of co-isolated cfDNA and exoRNA is followed by PreAmp PCR. Diluted PreAmp PCR product is interrogated by qPCR for the 11 *ESR1* mutations in three multiplexed reactions. A PCR internal control (IC1, IC2, and IC3) is included in each multiplex reaction for sample QC purposes.

Materials and Methods

Multiple study-specific sample panels consisting, in total, of 90 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. A novel extraction method that co-isolates cell-free DNA (cfDNA) and exosomal RNA (exoRNA) within the ExoLution Plus cfDNA + exoRNA Isolation Kit was used to process purchased mBC plasma samples.

Studies assessed single-site precision, specificity, plasma input, in-use storage conditions, kit freeze-thaws, blood collection tube type, qPCR platform equivalency, and established limit of detection (LoD) per variant. Variant status was determined by an in-house single-plex qPCR-based method.

Whole blood collected in K_2EDTA (BD) and PAXgene® Blood ccfDNA (Qiagen) tubes was centrifuged to separate the plasma prior to extraction. The plasma was subsequently filtered using a 0.8 µm filter. cfDNA and exoRNA were then co-isolated using the ExoLution Plus cfDNA + exoRNA Isolation Kit.

	Sample Preparation	ExoLution™ Plus	Reverse Transcription	PreAmp PCR	qPCR	Data Analysis	Total
Hands-On Time	10 min	30 min	10 min	5 min	10 min	2 min	67 min
Instrument Time	30 min	30 min	90 min	25 min	75 min	1 min	251 min

Note: Hands-on time will vary depending on the number of samples being run. Above is based on 6 samples.

Figure 2. Combined Workflow of ExoLution Plus cfDNA + exoRNA Isolation Kit and QuantideX qPCR *ESR1* exoMutation Kit Can Be Performed in <6 hrs. Hands-on time, including plasma preprocessing steps, was measured to take approximately 1 hour for a batch size of 6 samples. Instrument time was the main contributor to assay time, measured at just over 4 hours.

Samples were reverse transcribed and then amplified using the QuantideX qPCR *ESR1* exoMutation Kit on the Applied Biosystems (ABI) Veriti, and PCR products were detected on the ABI 7500 Fast Dx (7500), QuantStudio 5 Dx (QS5), and QuantStudio 7 Pro Dx (QS7). The .sds and .eds files were analyzed using the QuantideX qPCR *ESR1* exoMutation Analysis Module.

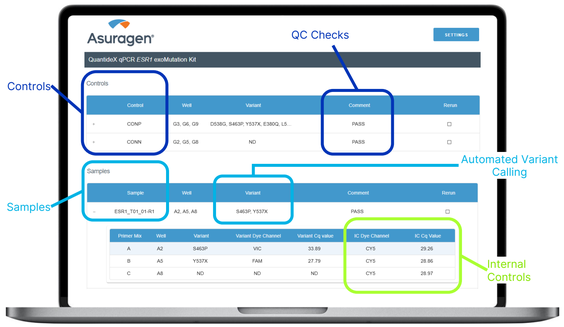

Figure 3. Example Output of QuantideX qPCR *ESR1* exoMutation Analysis Module. Custom software provides fully automated quality controls and variant calling. Analysis results can be exported in LIMS-compatible format (.csv).

Variant-level positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) were calculated by comparison of assay result to reference variant status, with 'positive' defined as qPCR detection at any Cq, and 'negative' defined as not detected (ND).

Results

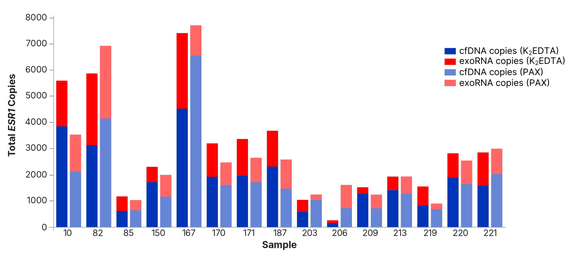

Figure 4. cfDNA and exoRNA Distribution Across 15 Presumed Normal Samples Shows an Increase in *ESR1* copies due to exoRNA. Plasma samples (2 mL) collected from 15 presumed normal subjects underwent cfDNA and exoRNA co-isolation from K_2EDTA and PAXgene Blood ccfDNA Tubes (PAX). Eluates underwent RT with (RT) and without enzyme (NRT), then *ESR1* copies were determined by ddPCR. *ESR1* copies calculated from the RT reaction included product from both cfDNA and exoRNA, whereas the *ESR1* copies calculated from the NRT reaction included product only from cfDNA, shown in blue. Calculated RNA fraction is shown in red. Percentage of exoRNA contribution did vary by sample, with a median value of 38.1%, representing a >60% increase in total *ESR1* copies over cfDNA alone.

Table 1. 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%

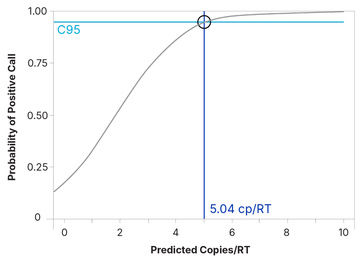

Figure 5. Example Resultant Curve of Probit Analysis Used to Determine LoD. S463P depicted here at 5.04 copies per RT reaction in a background of 10,000 total copies equated to 0.05% VAF. For each variant, a probit-linked inverse prediction fit model was generated to determine the relationship between 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 detect.

Table 2. 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 three alternative *ESR1* mutation detection kits on market.

Variant	Asuragen (qPCR)	Company A (qPCR)	Company B (qPCR)	Company C (dPCR)
D538G	0.08%	0.40%	0.33%	0.01%
S463P	0.05%	0.08%	0.33%	0.03%
Y537S	0.03%	0.10%	0.20%	0.03%
Y537C	0.03%	0.40%	0.33%	0.03%
Y537N	0.03%	0.20%	0.07%	0.03%
Y537D	0.03%	-	0.13%	-
E380Q	0.03%	1.00%	0.13%	0.03%
L536R	0.03%	0.70%	0.26%	0.03%
L536H	0.04%	0.80%	0.13%	-
L536P	0.03%	0.90%	-	-
V422del	0.03%	-	-	-

Tube Type	Variant NPA
K_2EDTA	97% (166/171)
PAXgene Blood ccfDNA	98% (167/171)

Table 3. Variant-Level 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.

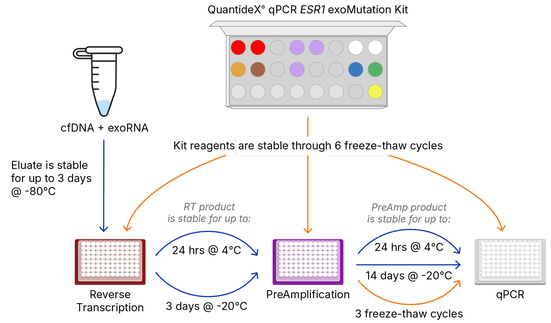

Figure 6. ExoLution Plus cfDNA + exoRNA Isolation Kit Eluates, QuantideX qPCR *ESR1* exoMutation Kit Reagents, and Assay Intermediate Products are Stable Across Multiple Optional Stopping Points and Storage Parameters. OPA, PPA, and NPA were ≥ 90% for all indicated conditions, including confirmatory testing beyond claimed stability. Experiments used surrogate samples of variant-positive plasmid DNA in a background of fragmented wild type DNA, covering all 11 variants, cell line conditioned plasma samples, and presumed normal plasma.

Table 4. *ESR1* resistance mutations detected and confirmed in mBC setting consistent with expected prevalence. A cohort of 21 mBC samples were evaluated, revealing 9/21 *ESR1* positives (43%).

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

Conclusions

- 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.
- Following CLSI guidelines, probit analysis was used to determine LoD for each variant across three qPCR platforms, resulting in %VAF values ranging from 0.03% to 0.08%.
- Analytical specificity of plasma samples exhibited a variant-level NPA ≥ 97%.
- The data showcased here supports routine testing of plasma samples from PAXgene® Blood ccfDNA tubes or K_2EDTA tubes for 11 of the most common and actionable *ESR1* mutations, with results via push-button automated software within a single laboratory shift.

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
5. Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures; Approved Guideline—Second Edition. CLSI document EP17-A2. Wayne, PA: Clinical and Laboratory Standards Institute; 2012.

*For Research Use Only. Not for use in diagnostic procedures.

Presented at SABCS, 2024