Verification of an RT-qPCR Assay System for Liquid Biopsy Surveillance of Treatment-Resistant *ESR1* Mutations

Poster G095

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

Asuragen, a Bio-Techne Brand, Austin, TX

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. However, this is challenging due to the necessary sensitivity and specificity required to monitor low-prevalence variants.
- The ExoLution™ Plus cfDNA + exoRNA Isolation Kit* and QuantideX® qPCR *ESR1* exoMutation Kit* bundle includes reagents, consumables, and automated analysis software to reliably detect 11 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

The most common subtype of breast cancer is hormone receptor-positive (HR+)/human epidermal growth factor receptor 2-negative (HER2-). In cases of HR+/HER2- metastatic breast cancer (mBC), resistance to aromatase inhibitors—core components of endocrine therapy (ET)—frequently emerges through mutations located within the ligand-binding domain of *ESR1*. Current NCCN Practice Guidelines in Oncology emphasize that the detection of *ESR1* resistance mutations at the time of disease progression plays a critical role in informing the choice of second-line treatments, such as elacestrant. Furthermore, recent evidence from the PADA-1 clinical trial highlights the advantage of switching therapies upon the detection of circulating cell-free resistance mutations, even before radiologic signs of disease progression appear. This report presents the development and evaluation of a multiplex RT-qPCR assay and software platform, designed for reliable and efficient detection of 11 critical ET-resistance-associated *ESR1* mutations from plasma liquid biopsy samples.

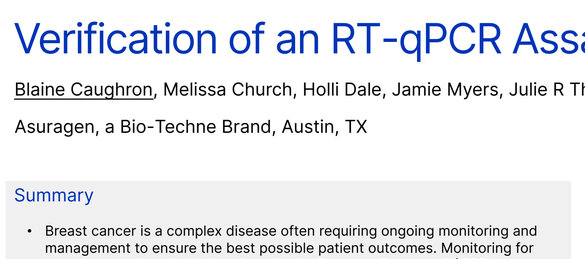

Figure 1. ExoLution Plus cfDNA + exoRNA Isolation Kit and QuantideX qPCR *ESR1* exoMutation Kit Workflow. A minimum of 2 mL of plasma is processed using a novel extraction method optimized to co-enrich cfDNA and exoRNA (included with the QuantideX qPCR *ESR1* exoMutation Kit*). RT product of co-isolated 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 multiple types, cfDNA & 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 plasma from mBC 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-micron filter. cfDNA and exoRNA were then co-isolated using the ExoLution Plus cfDNA + exoRNA Isolation Kit.

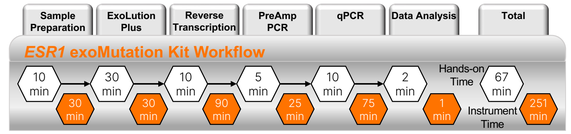

Figure 2. Combined Workflow of ExoLution Plus cfDNA + exoRNA Isolation Kit and QuantideX® qPCR *ESR1* exoMutation Kit Can Be Performed in <6 hrs. Hands-on time, including plasma preprocessing steps, was measured to take approximately 1 hour for a batch size of 6 samples. Instrument time was main contributor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

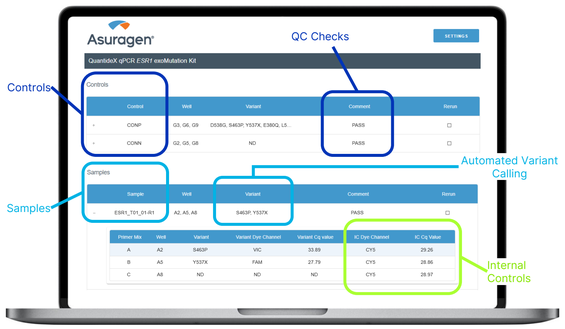

Figure 3. Example Output of QuantideX qPCR *ESR1* exoMutation Analysis Module. Custom software provides fully automated quality controls and variant calling. Analysis results can be exported in LIMS-compatible format (.csv).

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

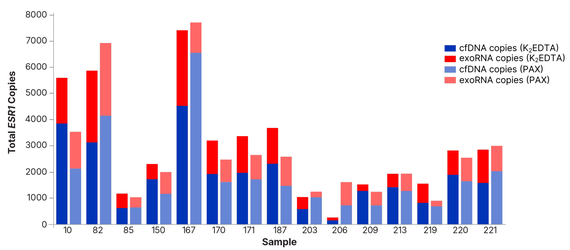

Figure 4. cfDNA and exoRNA Distribution Across 15 Presumed Normal Samples Shows an Increase in *ESR1* copies due to exoRNA. Plasma samples (2 mL) collected from 15 presumed normal subjects underwent cfDNA and exoRNA co-isolation from K_2EDTA and PAXgene Blood ccfDNA Tubes (PAX). Eluates underwent RT with (RT) and without enzyme (NRT), then *ESR1* copies were determined by ddPCR. *ESR1* copies calculated from the RT reaction includes product from both cfDNA and exoRNA, whereas the *ESR1* copies calculated from the NRT reaction would include 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%.

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%

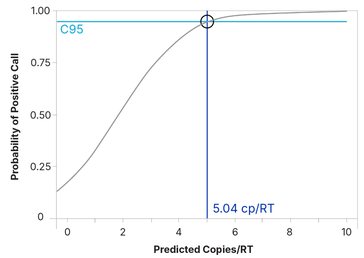

Figure 5. Example Resultant Curve of Probit Analysis Used to Determine LoD. S463P depicted here at 5.04 copies per RT reaction in a background of 10,000 total copies equated to 0.05% VAF. For each variant, a probit-linked inverse prediction fit model was generated to determine the relationship between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

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.

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. NPA ≥ 97% Across Multiple Blood Collection Tube Types. Sample panel consisted of 21 negative plasma samples per collection type, 42 total. Each co-isolation used 2 mL plasma, and samples were analyzed on the QS5 platform.

Platform	Input (mL)	OPA
QS5	2	92%
	4	93%
7500	2	92%
	4	90%
QS7	2	96%
	4	95%

Table 4. Variant OPA ≥ 90% Across Plasma Input Range on Each Supported qPCR Platform. mBC plasma sample aliquots as well as cell line conditioned plasma were co-isolated at 2 mL and 4 mL input. Amplified products were analyzed on all 3 qPCR platforms.

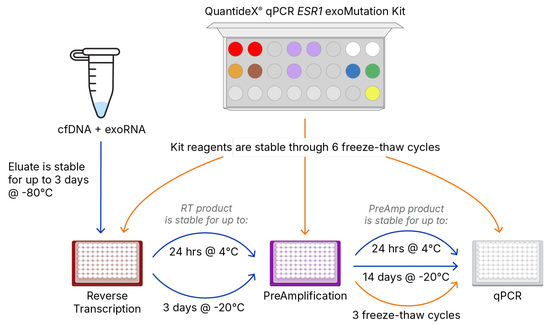

Figure 6. ExoLution Plus cfDNA + exoRNA Isolation Kit Eluates, QuantideX qPCR *ESR1* exoMutation Kit Reagents, and Assay Intermediate Products are Stable Across Multiple Optional Stopping Points and Storage Parameters. OPA, PPA, and NPA were ≥ 90% for all indicated conditions, including confirmatory 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.

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 variant-negative 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 a push-button automated software within a single laboratory shift.

Scan code to learn more

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 AMP, November 2024