QuantideX®
qPCR *ESR1* exoMutation Kit

Protocol Guide

Research Use Only. Not for use in diagnostic procedures.

REF A01031

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Purpose

The QuantideX® qPCR *ESR1* exoMutation Kit is an *in vitro* nucleic acid amplification kit for the qualitative detection of variants located in the ligand binding domain of the Estrogen Receptor 1 (*ESR1*) gene. The Kit is designed for reverse transcription and preamplification on DNA and RNA extracted from the plasma fraction of human whole blood performed on a thermal cycler, followed by qPCR. Data analysis is performed using the QuantideX qPCR *ESR1* exoMutation Analysis Module which is designed to report the presence or absence of the targeted variants.

The QuantideX qPCR *ESR1* exoMutation Kit is for research use only (RUO) and not for use in diagnostic procedures.

Limitations

- The QuantideX® qPCR *ESR1* exoMutation Kit is a qualitative assay and does not provide quantitative outputs.
- This Kit has been evaluated for use with total nucleic acid isolated using the ExoLution Plus cfDNA + exoRNA Isolation Kit from 2 - 4 mL of the plasma fraction of whole blood.
- Whole blood samples must be collected using either K_2EDTA-treated blood collection tubes and processed into plasma ≤ 4 hours, or PAXgene Blood ccfDNA Tubes and processed into plasma within 3 days.
- All variants were verified on plasma, surrogate plasma, or plasmid mixes as described in the **Analytical Assessment** section. Not all variants were verified with procured plasma samples due to lack of sample availability.
- Cross-reactivity of allele-specific primers may occur between mutations in codons 536-538, resulting in a positive result with more than one target mutation reported.

Warnings and Precautions

- Use appropriate personal protective equipment when working with these materials.
- Follow Universal Precautions in compliance with OSHA 1910.1030, CLSI M29, or other applicable guidance when handling human samples.
- RNase/DNase contamination can cause degradation of the RNA samples and cDNA products. Use nuclease-free filter pipette tips and nuclease-free tubes. Clean bench surfaces before beginning work.
- RNA, DNA, and PCR carryover contamination can result in false positive signals not detected by the analysis tool. Use appropriate precautions in sample handling, workflow, and pipetting.
- Prior to use, ensure that all centrifuges, pipettes, thermal cyclers, and qPCR instruments are maintained, calibrated, and validated according to the manufacturer's instructions.
- Use of alternative plastics and instruments other than those listed in **Required but not Provided** section may increase risk of QC failures.
- Do not open qPCR plates after amplification has started. PCR carryover contamination can result in false positive signals. Use appropriate precautions in sample handling, workflow, and pipetting.
- Maximum number of samples tested in a single batch (n=30) is due to available plate space.
- The Kit-included controls (CONN and CONP) must be included in each batch run.

- The Kit generates waste potentially containing hazardous components and therefore requires handling and disposal according to local safety regulations.

Principle of the Procedure

The QuantideX® qPCR *ESR1* exoMutation Kit is a targeted, multiplexed RT-qPCR nucleic acid amplification test for the qualitative detection of 11 of the most prevalent pathogenic variants (**Table 1**) located in the ligand binding domain of *ESR1* and an internal control. The variant panel is associated with drug resistance in Hormone Receptor-positive (HR+) metastatic breast cancer (mBC) (Hartkopf, et al., 2020). The panel coverage and assay design accommodate NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) that prefer assessment of *ESR1* ligand-binding domain mutations at progression following prior lines of endocrine therapy. The co-extraction of cell free DNA (cfDNA) and exosomal RNA by the ExoLution Plus cfDNA + exoRNA Isolation Kit increases available analyte for the QuantideX qPCR *ESR1* exoMutation Kit to detect low, emerging fractions of nucleic acid from a tumor.

The QuantideX qPCR *ESR1* exoMutation Analysis Module (the Software) is an all-in-one data analysis and reporting tool. The Software combines variant detection and sample-level QCs and is able to report multiple variants per sample by assigning a positive signal per dye channel with the appropriate variant. The resultant batch analysis can be exported for downstream reporting.

Table 1. *ESR1* variants detected by the QuantideX qPCR *ESR1* exoMutation Kit.

Common Target Name	Cosmic ID	Coordinate GRCh38	Reference Base	Alternate Base
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

Workflow

The assay workflow includes sample preparation, isolation, reverse transcription, preamplification PCR, qPCR (3 wells per sample), and data summarization in the provided Software. For this workflow, whole blood is processed into plasma following a double centrifugation and pre-filter step, followed by total nucleic acid (TNA) isolation of cfDNA and exosomal RNA from 2 – 4 mL of plasma using the ExoLution Plus cfDNA + exoRNA Isolation Kit. 10 µL of the resultant TNA is used in a 15 µL reverse transcription (RT) step to convert exosomal RNA into cDNA, followed by mutant target enrichment in a 22.5 µL preamplification (PreAmp) step. After a 1:40 dilution, 2 µL of diluted PreAmp PCR products are added to 8 µL qPCR master mixes containing Primer Mix A, B, or C. Following the qPCR step, the resultant file

must be processed by the QuantideX qPCR *ESR1* exoMutation Analysis Module. Qualitative results are generated for all targeted variants and internal controls. See **Figure 1** for a schematic of the workflow.

The Kit includes reagents for multiplexed, qualitative qPCR to produce variant-positive amplicons labeled with FAM or VIC dyes. The presence of a variant will result in a signal (i.e., a Ct or Cq value) in these fluorescent channels, whereas no signal means absence of the variant. The internal control amplicon is labeled with CY5 and will exhibit a signal in all three qPCR primer mixes in a valid sample test.

The reagents are adapted for RT and PreAmp use on the ABI Veriti Thermal Cycler, and the qPCR reagents utilize hydrolysis probe technology for analysis on the ABI QuantStudio 5 Dx, ABI QuantStudio 7 Pro Dx, or the ABI 7500 Fast Dx Real-Time PCR systems. The test includes reagents sufficient for 50 reactions performed in up to 6 independent batches. The included CONN is not expected to show any variant results. The included CONP generates positive results for two variants in each qPCR primer mix. All samples and controls are run in singleton.

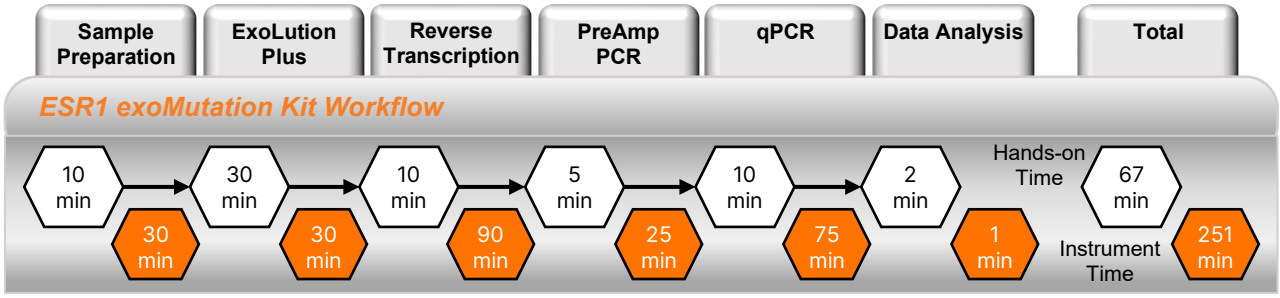


Figure 1. The workflow is streamlined from sample-to-answer and can be performed in less than 6 hours with ~1 hour of total hands-on time for 6 samples.

Supplied with the Kit

Item #	Description	Cap Color
145388	Diluent (6 tubes)	Clear
A01034	RT Buffer	Red
A01035	RT Enzyme	Red
A01014	ESR1 CONN	Orange
A01015	ESR1 CONP	Amber
A01023	PreAmp Buffer	Purple
A01024	PreAmp Enzyme Mix	Purple
A01025	PreAmp Primer Mix	Purple
A01026	qPCR Buffer	White
A01027	qPCR Enzyme Mix	White
A01028	qPCR Primer Mix A	Blue
A01029	qPCR Primer Mix B	Green
A01030	qPCR Primer Mix C	Yellow

- The QuantideX qPCR *ESR1* exoMutation Analysis Module, REF A01043, is required for analysis. The Analysis Module Software User Guide, document number 0004887, is available for download on the myAsuragen customer portal https://asuragen.com/myasuragen.

Use of Batch Run Controls

The assay requires use of the Kit-included positive (CONP) and negative (CONN) controls, which must be tested in singleton in every batch run for data analysis and quality control. The positive control provides a positive signal in all dye channels utilized (FAM, VIC, and CY5) across all qPCR primer mixes. The negative control provides an internal quality control to ensure the RT step is functioning properly in the batch run. For details on the positive and negative control, refer to the **Quality Control Procedures** section of this protocol guide.

Required but not Provided

Item numbers, where provided, may vary by region. Please contact Asuragen Technical Support for assistance.

- Reagents for nucleic acid isolation are not included in this Kit. The ExoLution Plus cfDNA + exoRNA Isolation Kit is required for co-isolation of cfDNA and exosomal RNA from plasma. The ExoLution Plus Kit protocol guide, document number 00004771, is available for download on the myAsuragen customer portal https://asuragen.com/myasuragen.
- General laboratory equipment and workspace to perform RT and PCR procedures
- Centrifuge capable of spinning 96-well plates
- Micro-centrifuge or benchtop centrifuge capable of spinning reagent tubes
- Vortex mixer
- Pipettes with an accuracy range between 0.2–2 µL, 1–10 µL, 2–20 µL, 20–200 µL, and 100–1000 µL
- Multi-channel pipette unit capable of pipetting 1–10 µL
- 96-well thermal cycler: Applied Biosystems™ Veriti™ 96-well Thermal Cycler
- 96-well PCR semi-skirted plates for use with the Applied Biosystems Veriti 96-well Thermal Cycler, or equivalent
- One of the following Applied Biosystems instruments:
    - Applied Biosystems QuantStudio™ 5 Dx Real-Time PCR System
    - Applied Biosystems QuantStudio™ 7 Pro Dx Real-Time PCR System
    - Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument
- Thermo Fisher Scientific Design and Analysis 2 Software

  Note: If not utilizing Applied Biosystems Diomni Enterprise Software, QuantStudio 5 Dx and QuantStudio 7 Pro Dx runs require analysis in Design & Analysis 2 Software prior to use in the QuantideX qPCR *ESR1* Analysis Module.
- Adhesive foil seals (Beckman Coulter 538619), or equivalent for use with Applied Biosystems thermal cyclers

  CRITICAL: It is not recommended to use alternate plate seal types (e.g., adhesive clear seals) or strip caps to seal plates and tubes, as this can lead to sample cross-contamination.
- MicroAmp™ EnduraPlate™ Optical 96-Well Clear Reaction Plates for use with QuantStudio 5 Dx and QuantStudio 7 Pro Dx (ABI A36924, or equivalent)
- MicroAmp™ Fast Optical 96-Well Reaction Plate for use with ABI 7500 Fast Dx, ABI P/N 4346907, or equivalent

- Compression Pad for use with Applied Biosystems thermal cyclers: Applied Biosystems P/N 4312639, or equivalent
- MicroAmp™ Optical Adhesive Film for sealing qPCR plates, ABI P/N 4311971, or equivalent

Number of Reactions

- The provided reagents are sufficient for up to 50 reactions, including master mix overage.

Reagent Stability

- The reagents are stable through the labelled date when stored under the specified conditions.
- The kitted reagents support 6 freeze-thaw cycles. Additional uses are not recommended.

Storage, Handling, & Disposal

- Store frozen reagents in a non-frost-free freezer protected from light at -15 to -30°C.
- Use appropriate personal protective equipment when working with these materials.
- Do not pool components from different reagent lots or other kits.
- Do not use reagents after the labeled expiration date.
- Ensure all qPCR primer mixes are handled separately with care to minimize the risk of cross-contamination. Never have multiple qPCR primer mix tubes or master mix tubes open simultaneously.
- Do not interchange the reagent tube caps; this may cause cross-contamination or degradation of reagents.
- Prior to reagent use:
    - Keep enzyme mixes frozen or on ice until needed. Flick to mix, spin down to collect contents.
    - Completely thaw all non-enzyme reagents at room temperature (approximately 30 minutes). Vortex after thawing, spin down to collect contents.
- Ensure all controls are handled separately to minimize the risk of cross-contamination.
- Prior to opening, briefly centrifuge each component to collect the solution at the bottom of each vial.
- Use assembled master mixes immediately after formulation.
- Dispose of contents/containers in accordance with local, regional, national, territorial, provincial, and international regulations. Review the Safety Data Sheets (SDS) to determine the safe disposal of materials for your region. SDSs are available for download at https://asuragen.com/myasuragen. Avoid release to the environment.
- Contact Asuragen Technical Support immediately if the reagent packaging arrives damaged or opened.

Procedural Steps

Sample Handling

Total nucleic acid extracted from 2 - 4 mL of the plasma fraction of whole blood collected in K_2EDTA collection tubes (processed into plasma ≤ 4 hours) or PAXgene Blood ccfDNA Stability Tubes (processed into plasma ≤ 3 days) must be isolated using the ExoLution Plus cfDNA + exoRNA Isolation Kit. Only the cfDNA and exoRNA fraction are isolated and nearly the entire eluate (10 µL of the 12 µL elution) is utilized in the RT step of the QuantideX qPCR *ESR1* exoMutation Kit. Therefore, it is impossible to confirm sample quantity or purity via a spectrophotometer.

The ExoLution Plus cfDNA + exoRNA Isolation Kit references use of a syringe filter (0.8 µm Leur Lock filter: Millipore P/N SLAAR33SB, or equivalent) during the plasma pre-processing step. The syringe filter step is not a requirement during plasma pre-processing steps but should be incorporated if typical workflow leads to clogged Column Units during the ExoLution Plus cfDNA + exoRNA Isolation Kit workflow. Certain collection procedures or using frozen plasma may require the pre-filter step.

It is essential to use the upstream ExoLution Plus cfDNA + exoRNA Isolation Kit; other commercially available isolation kits have not been verified with this assay. ExoLution Plus cfDNA + exoRNA Isolation Kit isolates should proceed directly to the **RT Master Mix Setup and Incubation** section, or store the isolated material at -80°C for up to 72 hours before use.

Note: Although not verified, similar results are expected when isolated material is stored at -20°C.

Pre-Analytical Steps

qPCR Instrument Filter Settings

Prior to the qPCR step, all targets (QuantStudio 5 Dx, QuantStudio 7 Pro Dx) and detectors (ABI 7500 Fast Dx) must be programmed on the qPCR instruments prior to first use. Future uses can utilize these settings. The target names with the associated reporters in **Table 2** must be used exactly as shown (target names are case-sensitive) for the QuantideX qPCR *ESR1* exoMutation Analysis Module to recognize if correct detectors were utilized during run setup. Reference the appropriate instrument manufacturer's user guide for target assignment and run setup.

Table 2. qPCR instrument target naming and associated reporter. Ensure "none" is selected as the quencher for each target.

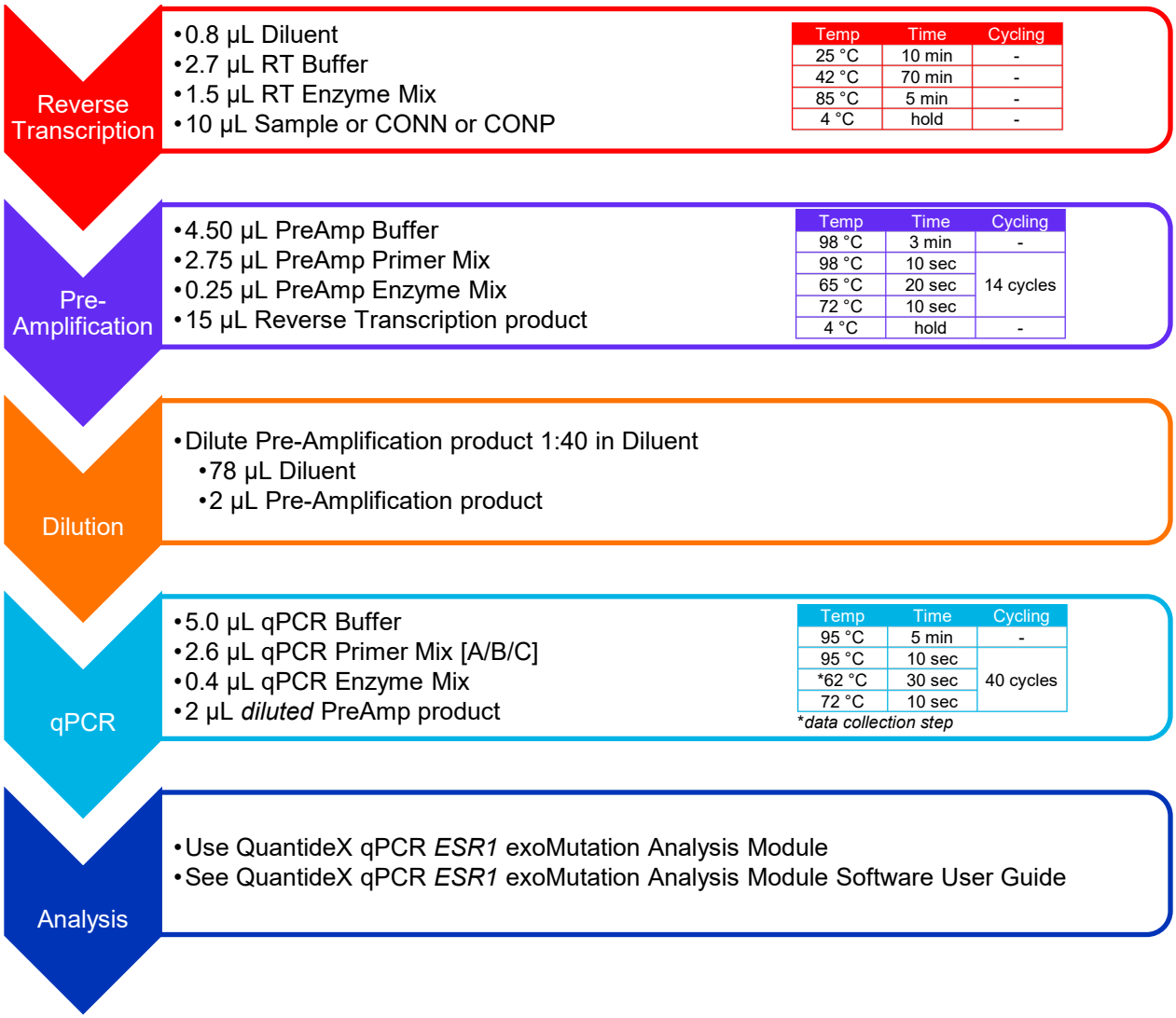
Primer Mix	Target Name	Reporter
A	D538G	FAM
	S463P	VIC
	IC1	CY5
B	Y537X	FAM
	E380Q	VIC
	IC2	CY5
C	L536X	FAM
	V422del	VIC
	IC3	CY5

Control and Sample Naming

In the qPCR step, review the manufacturer restrictions of the qPCR instrument for naming guidance related to character use and overall number of characters. In addition, the following naming rules must be observed:

- The Kit-included positive control must be named CONP but is not case sensitive. Within a batch run, only one well per primer mix can be named CONP. The Software will provide a single CONP result in the analysis module.
- The Kit-included negative control must be named CONN but is not case sensitive. Within a batch run, only one well per primer mix can be named CONN. The analysis module will provide a single CONN result in the Software.
- Each sample must be assigned the same unique name across all 3 qPCR Primer Mixes. The analysis module will provide a single result for each sample in the Software.
- These characters \ / : * ? ‘ “ < > | are not permitted.

Protocol Overview



The protocol is written for a single reaction; master mixes should be prepared for the appropriate number of reactions at each protocol step. The provided reagents are sufficient for up to 50 reactions each of qPCR Primer Mix A, B, and C, performed in up to 6 independent batches, including 15% overage for all master mix formulations.

The workflow should proceed in a unidirectional manner starting with a dedicated pre-amplification area and moving to a segregated post-amplification area. Amplified product should remain in the post-amplification area to minimize the risk of amplicon contamination.

RT Master Mix Setup and Incubation

Note: Create master mix and setup plate on ice or cold block in a pre-amplification area.

1. Thaw the **RT Buffer**, **Diluent**, **ESR1 CONN**, and **ESR1 CONP** at room temperature. Thaw samples on ice.
2. If the **RT Buffer** is cloudy, allow to thaw 5 more minutes and briefly vortex. Repeat as needed.
3. Briefly vortex and centrifuge the thawed reagents and samples.
4. Leave the **RT Enzyme** in the freezer or on ice until needed. When ready to add to master mix, gently flick the tube (do not vortex) and pulse spin.
5. Transfer the controls and samples to empty wells of a 96-well plate in the following order.
   a. 10 µL of **ESR1 CONN**
   b. 10 µL of each test sample
   c. 10 µL of **ESR1 CONP**

   Note: Avoid using corner wells. A suggested plate layout for a 6-sample (8-reaction) batch is below. Alternatively, if continual cross-contamination is observed, move CONP and CONN away from other reactions, giving at least one blank column as space between samples and controls.

	1	2	3	4	5	6	7	8	9	10	11	12
A		CONN										
B		Sample1										
C		Sample2										
D		Sample3										
E		Sample4										
F		Sample5										
G		Sample6										
H		CONP										

   Note: A single reaction of both the CONN and CONP must be included in each batch run and is required for analysis by the QuantideX qPCR *ESR1* exoMutation Analysis Module.
6. In a 1.5 mL microcentrifuge tube, assemble the RT master mix for the appropriate number of reactions (*n* samples + 2 controls) in the order listed below, adding 15% overage.

Item	Reagent	Vol/rxn (µL)
145388	Diluent	0.8
A01034	RT Buffer	2.7
A01035	RT Enzyme Mix	1.5
	Total	**5.0**

7. Gently vortex the RT master mix and briefly centrifuge the tube to collect contents.
8. Add 5 µL of RT master mix to each well containing sample or control. Place the pipet tip just below the surface of the master mix for each aspiration, using a new tip for each well.
9. Seal the plate with an adhesive foil seal or equivalent.
10. Briefly vortex and centrifuge the plate, ensuring all volume is collected at the bottom of the wells.

11. Transfer plate to an ABI Veriti thermal cycler, using the conditions below.
    - Cycling:

Temp	Time
25°C	10 min
42°C	70 min
85°C	5 min
4°C	hold

    - Ramp rate: 3.9°C/sec (standard ramp rate for ABI Veriti)
    - Reaction volume: 15 µL
12. After the RT reaction is complete, proceed directly to the **Pre-Amplification Master Mix Setup and Thermal Cycling** section, or place RT product plate in storage (4°C up to 24 hours or -20°C up to 72 hours).

Pre-Amplification Master Mix Setup and Thermal Cycling

Note: PreAmp master mix is added into the existing RT product plate after RT reaction is complete.

1. If needed, thaw plate containing RT product at room temperature.
2. Briefly vortex and centrifuge the plate containing RT product.
3. In a pre-amplification area, thaw the **PreAmp Buffer** and **PreAmp Primer Mix** at room temperature.
4. Briefly vortex and centrifuge the thawed reagents.
5. If **PreAmp Buffer** is cloudy, allow to thaw 5 more minutes and briefly vortex. Repeat as needed.
6. Leave the **PreAmp Enzyme Mix** in the freezer or on ice until needed. When ready to add to PreAmp master mix, gently flick the tube (do not vortex) and centrifuge to collect contents.
7. In a 1.5 mL microcentrifuge tube, assemble the PreAmp master mix in the order listed below, adding 15% overage.

Item	Reagent	Vol/rxn (µL)
A01023	PreAmp Buffer	4.50
A01025	PreAmp Primer Mix	2.75
A01024	PreAmp Enzyme Mix	0.25
	Total	**7.5**

8. Gently vortex the PreAmp master mix and centrifuge to collect contents.
9. Carefully remove adhesive seal from the RT product plate, making sure not to disturb any wells.
10. Add 7.5 µL of PreAmp master mix into each well already containing RT product, pipetting up and down 3 times to ensure adequate liquid transfer. Place the pipet tip just below the surface of the master mix for each aspiration, using a new tip each time.
11. Seal the plate with a new adhesive foil seal or equivalent.
12. Briefly vortex and centrifuge the plate, ensuring all volume is collected at the bottom of the wells.

13. Transfer plate to an ABI Veriti thermal cycler, using the conditions below.
    - Cycling:

Temp	Time	Cycling
98°C	3 min	-
98°C	10 sec	14 cycles
65°C	20 sec	
72°C	10 sec	
4°C	hold	-

    - Ramp rate: 3.9°C/sec (standard ramp rate for ABI Veriti)
    - Reaction volume: 23 µL *(rounded up from 22.5 µL)*
14. When complete, move sealed plate to a post-amplification area. Proceed directly to the **PreAmp Product Dilution** section or place plate in storage (4°C up to 24 hours or -20°C up to 14 days).

PreAmp Product Dilution

1. If needed, thaw plate containing PreAmp product at room temperature.
2. Briefly vortex and centrifuge the plate containing PreAmp product.
3. Thaw the **Diluent** at room temperature.
4. In a new 96-well plate, add 78 µL of **Diluent** to each well matching the layout of the PreAmp product plate.
5. Add 2 µL of PreAmp product to the respective well in the plate containing Diluent. Pipette up and down at least three times to ensure adequate transfer.
6. Seal the plate with a new adhesive foil seal or equivalent.
7. Thoroughly vortex the plate.
8. Centrifuge the plate, ensuring all volume is collected at the bottom of the wells.
9. Keep diluted PreAmp product at 2 to 8°C until needed but must be used the same day as dilution.
10. Proceed immediately to the **qPCR Master Mix Setup and Thermal Cycling** section.

qPCR Master Mix Setup and Thermal Cycling

Note: The **qPCR Enzyme Mix** is very viscous. Avoid formation of bubbles when assembling the qPCR master mixes, transferring qPCR master mixes to the plate, and when adding controls and samples.

1. In a pre-amplification area, thaw the **qPCR Buffer** and **qPCR Primer Mix A**, **B**, and **C** at room temperature.
2. Briefly vortex and centrifuge the thawed reagents to collect contents.
3. Leave the **qPCR Enzyme Mix** in the freezer or on ice until needed. When ready to add to master mix, gently flick the tube (do not vortex) and briefly centrifuge.

4. In three separate 1.5 mL microcentrifuge tubes, assemble three qPCR master mixes (one for each qPCR Primer Mix) in the order listed below, adding 15% overage.

Item	Reagent	Vol/rxn (µL)
A01026	qPCR Buffer	5.0
A01028 or A01029 or A01030	qPCR Primer Mix A **or** qPCR Primer Mix B **or** qPCR Primer Mix C	2.6
A01027	qPCR Enzyme Mix	0.4
	Total	**8.0**

5. Gently vortex the three qPCR master mixes and briefly centrifuge to collect contents.

6. Assuming the suggested plate layout from the **RT Master Mix Setup and Incubation** section was used, a suggested qPCR layout is below. Each master mix is plated, then the diluted PreAmp product is added across all three mixes.
   Note: If continual cross-contamination is observed, move CONP away from other reactions, giving at least one blank column as space between samples and controls

	1	Master Mix A 2	3	Master Mix B 4	5	Master Mix C 6	7	8	9	10	11	12
A		CONN		CONN		CONN						
B		Sample1		Sample1		Sample1						
C		Sample2		Sample2		Sample2						
D		Sample3		Sample3		Sample3						
E		Sample4		Sample4		Sample4						
F		Sample5		Sample5		Sample5						
G		Sample6		Sample6		Sample6						
H		CONP		CONP		CONP						
		Master Mix A		Master Mix B		Master Mix C						

7. Transfer 8 µL of Master Mix A to corresponding wells of a qPCR optical plate.

8. Transfer 8 µL of Master Mix B to corresponding wells.

9. Transfer 8 µL of Master Mix C to corresponding wells.

10. Move the qPCR plate containing Master Mixes A, B, and C to the same post-amplification area containing the diluted PreAmp product.

11. Briefly vortex and centrifuge the plate containing diluted PreAmp product.

12. Carefully remove adhesive seal from the diluted PreAmp product plate, making sure not to disturb any wells.

13. Add 2 µL of diluted PreAmp product into 3 wells: one each for qPCR Master Mix A, B, and C. Pipette up and down at least 3 times to ensure adequate transfer. Use a new tip for each well.

14. Thoroughly seal the plate with an optical adhesive sealing film.

15. Briefly vortex the plate, ensure bubbles are not introduced into the well.

16. Centrifuge the plate, ensuring all volume is collected at the bottom of the wells.

Note: If there are excessive bubbles in any well after centrifugation, gently tap the sealed qPCR plate on the benchtop. Re-centrifuge the plate at 1,500 g for 1 minute. See the**Importance of Avoiding Bubbles in qPCR Setup** section.

17. Immediately transfer plate to the pre-programmed qPCR instrument, using the cycling conditions below.
    - Cycling

Temp	Time	Cycling
95°C	5 min	-
95°C	10 sec	40 cycles
62°C*	30 sec	
72°C	10 sec	

*data collection step

- Standard Run Mode
- Reaction volume: 10 µL
- Channels: FAM, VIC, CY5
- Targets: see **Table 2**; targets are specific to each primer mix
- Passive Reference: ROX
- Run Analysis:
  - Baseline
    - Manual, Start: 3, End: 15 for ABI 7500 Fast Dx
    - Manual, Baseline Start: 3, Baseline End: 15 for ABI QuantStudio 5 Dx and QuantStudio 7 Pro Dx
  - Threshold
    - Manual, FAM, VIC, CY5 at 0.2 for ABI 7500 Fast Dx
    - Manual, FAM, VIC, CY5 at 0.1 for ABI QuantStudio 5 Dx and QuantStudio 7 Pro Dx

Data Analysis

ABI 7500 Fast Dx (SDS v1.x)

1. When the run has completed, click on the Analyze button.
2. Click Save to save the analyzed run.
3. Import the SDS file into the QuantideX qPCR *ESR1* exoMutation Analysis Module and submit for analysis.

ABI QuantStudio 5 Dx

Thermo Fisher Design & Analysis 2 Software

1. When the run has completed, save the file.
2. Open the run file (.eds) in the Thermo Fisher Scientific Design and Analysis 2 Software.
3. Ensure analysis settings match baseline and threshold settings state in qPCR Master Mix Setup and Thermal Cycling step.
4. Click Analyze to save the analyzed run.

5. Import the SDS file into the QuantideX qPCR *ESR1* exoMutation Analysis Module and submit for analysis.

Note: Diomni Enterprise Software is compatible with the QS5 Dx Instrument but has not been verified with the QuantideX qPCR *ESR1* exoMutation Analysis Module.

ABI QuantStudio 7 Pro Dx

Thermo Fisher Design & Analysis 2 Software

1. When the run has completed, save the file.
2. Open the run file (.eds) in the Thermo Fisher Scientific Design and Analysis 2 Software.
3. Ensure analysis settings match baseline and threshold settings state in qPCR Master Mix Setup and Thermal Cycling step.
4. Click Analyze to save the analyzed run.
5. Import the SDS file into the QuantideX qPCR *ESR1* exoMutation Analysis Module and submit for analysis.

Diomni Enterprise Software (RUO Workspace)

1. When the run has completed, navigate to the Results tab and click on the run name.
2. Ensure analysis settings match baseline and threshold settings stated in qPCR Master Mix Setup and Thermal Cycling step.
3. Click on the Analyze button and save the analyzed file.
4. Download the EDS file.
5. Import the EDS file into the QuantideX qPCR *ESR1* exoMutation Analysis Module and submit for analysis.

Application of a Cq Cutoff

The QuantideX® qPCR *ESR1* exoMutation Kit is designed to optimize limit of detection (LOD) across all variants. If desired, a maximum Cq value (Cq cutoff) may be applied to balance analytical sensitivity (LOD) and specificity. Verification data is presented in the **Analytical Assessment** section with no Cq cutoff, making the cutoff the total number of PCR cycles (40 cycles). As an example, **Table 3** and **Table 4** show impact to sensitivity and specificity levels at different Cq cutoffs ranging from 34 cycles to 40 cycles. The application of any Cq cutoff is subject to the user lab's discretion.

Table 3. Variant-level limit of detection for the QuantideX qPCR *ESR1* exoMutation Kit with a Cq Cutoff applied across a range of Cq values. Single-value LODs denote that a uniform %VAF was established across all three supported qPCR platforms (ABI 7500 Fast Dx, ABI QuantStudio 5 Dx, and ABI QuantStudio 7 Pro Dx). In contrast, a range of values reflects variant-specific %VAF across all three qPCR platforms.

Variant	LOD %VAF, range across platforms by Cq Cutoff			
	34	36	38	40
D538G	0.11-0.17%	0.08-0.11%	0.07-0.10%	0.06-0.10%
S463P	0.12-0.14%	0.05-0.11%	0.04-0.07%	0.04-0.07%
Y537S	0.03%	0.03%	0.03%	0.03%

Variant	LOD %VAF, range across platforms by Cq Cutoff			
	34	36	38	40
Y537C	0.03%	0.03%	0.03%	0.03%
Y537N	0.03%	0.03%	0.03%	0.02-0.03%
Y537D	0.03%	0.03%	0.03%	0.03%
E380Q	0.03%	0.03%	0.03%	0.03%
L536R	0.03%	0.03%	0.03%	0.03%
L536H	0.04-0.07%	0.04-0.05%	0.04-0.05%	0.04%
L536P	0.03-0.04%	0.03-0.04%	0.03-0.04%	0.03-0.04%
V422del	0.03%	0.03%	0.03%	0.03%

Table 4. Evaluation of sample-level analytical specificity (exclusivity) for the QuantideX qPCR *ESR1* exoMutation Kit on the ABI QuantStudio 5 Dx with a Cq Cutoff applied across a range of Cq values in mutant-negative plasma samples collected in K_2EDTA and PAXgene Blood ccfDNA BCT. NPA=negative percent agreement.

Cq Cutoff	34	36	38	40
NPA (Sample Level)	100%	95%	93%	93%

Importance of Avoiding Bubbles in qPCR Setup

Avoid formation of bubbles when assembling the qPCR master mixes, transferring qPCR master mixes to the qPCR plate, and when adding controls and samples. Bubbles in wells of the qPCR plate are likely to contribute to signals that may cross threshold settings. The signals can be observed as fluorescent drift in amplification plots and as wavy signal in the multicomponent plots. Continued centrifugation typically does not remove all bubbles in the qPCR plate. The best resolution for bubble elimination is to gently tap the sealed qPCR plate on the countertop. **Figure 2** exhibits the impact of bubbles in the multicomponent and amplification plot.

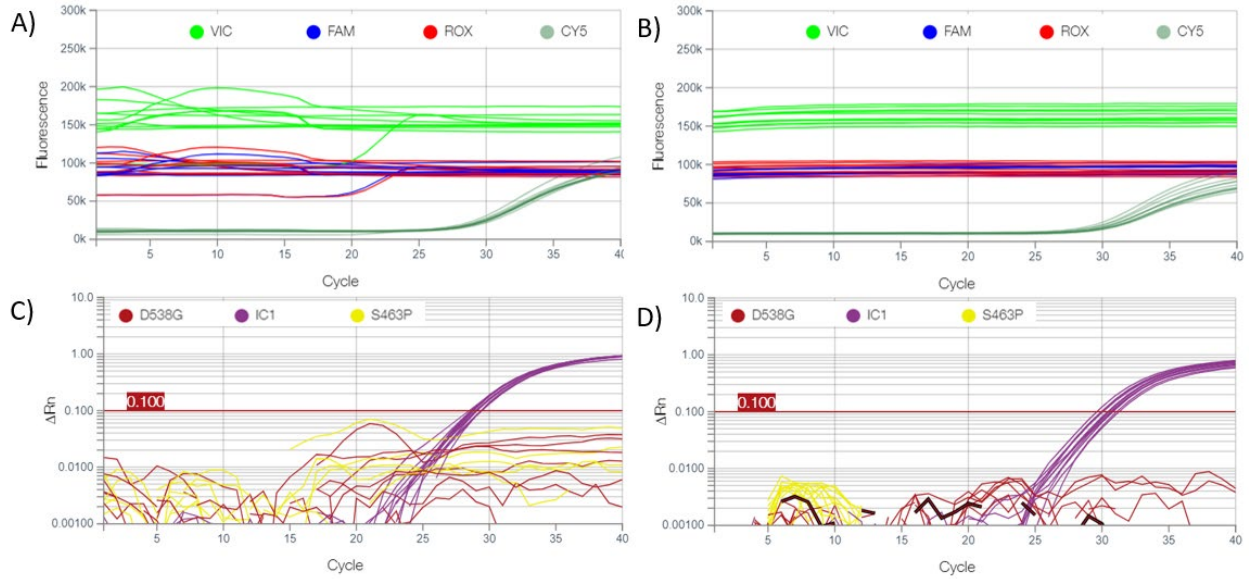


Figure 2. Multicomponent plot view of a qPCR plate containing A) bubbles and B) no bubbles resolved by tapping the sealed qPCR plate on the countertop, resulting in an even signal in all dye channels. Amplification plot view of the same qPCR plate exhibiting C) noisy background with signal nearing the threshold setting and D) clean background signal as a result of no bubbles.

Quality Control Procedures

Positive Control (CONP)

The positive control (CONP) is required in singleton for all 3 qPCR Primer Mixes in every batch run for data analysis and quality control. The CONP generates a positive signal in all dye channels (FAM, VIC, CY5) across all qPCR primer mixes (**Figure 3**). Specifically, per qPCR primer mix, the CONP will generate a positive signal for two variants and the internal control (IC). CONP results are included in the QuantideX qPCR *ESR1* exoMutation Analysis Module generated CSV report.

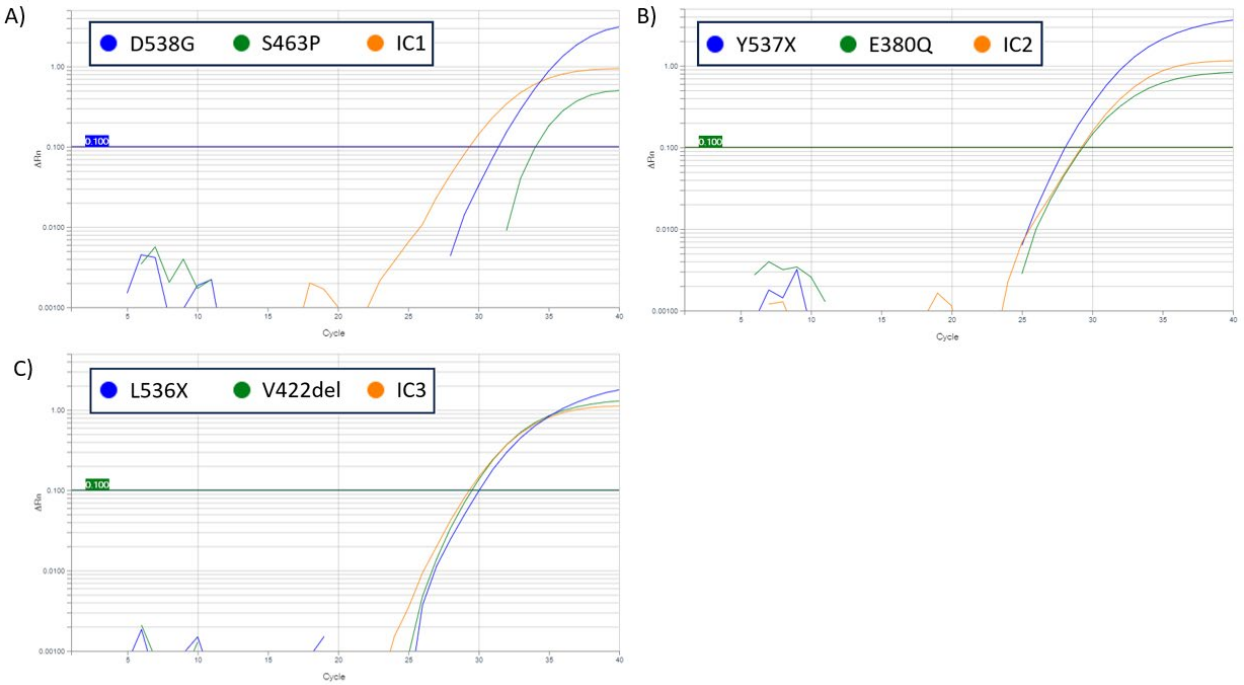


Figure 3. Example of QuantideX qPCR *ESR1* exoMutation Kit Positive Control (CONP) qPCR amplification trace for A) Primer Mix A [D538G, S463P], B) Primer Mix B [Y537X, E380Q], and C) Primer Mix C [L536X, V422del].

If an expected variant is not detected then the comment column for CONP will display “Expected Variant(s) not detected” and the rerun box for CONP and all samples will be checked. If the internal controls (IC1, IC2 and/or IC3) are not detected, then the comment column for CONP will display “Internal Control: not detected” and the rerun box for CONP and all samples will be checked. If the expected targets are not identified then the comment column for CONP will display “Invalid number of target(s)” and the rerun box for CONP and all samples will be checked. These QC failures alert the user that the batch run is invalid. For further details, refer to the **Troubleshooting** section and the QuantideX qPCR *ESR1* exoMutation Analysis Module Software User Guide (00004887).

A valid CONP will display PASS in the comment column in the results view of the QuantideX qPCR *ESR1* exoMutation Analysis Module and in the control section of the CSV report. In the results view, the CONP dropdown table will display the variants detected, the variant dye channel, and associated Cq values. The internal control (IC) dye channel and IC Cq values will be displayed (**Figure 4**).

	Control	Well	Variant	Comment	Rerun
–	CONP	G9, H1, H4	D538G, S463P, Y537X, E380Q, L536X, V422del	PASS	☐

Primer Mix	Well	Variant	Variant Dye Channel	Variant Cq value	IC Dye Channel	IC Cq Value
A	H1	D538G | S463P	FAM | VIC	30.99 | 33.51	CY5	29.24
B	H4	Y537X | E380Q	FAM | VIC	27.33 | 26.31	CY5	29.23
C	G9	L536X | V422del	FAM | VIC	26.80 | 27.65	CY5	29.43

Figure 4. Example of a valid CONP result from the QuantideX qPCR *ESR1* exoMutation Analysis Module.

Negative Control (CONN)

The negative control (CONN) is required in singleton in every batch run for data analysis and quality control. The CONN generates a signal in only the CY5 dye channel for the internal control (IC) across all qPCR primer mixes (**Figure 5**). CONN results are included in the QuantideX qPCR *ESR1* exoMutation Analysis Module generated CSV report.

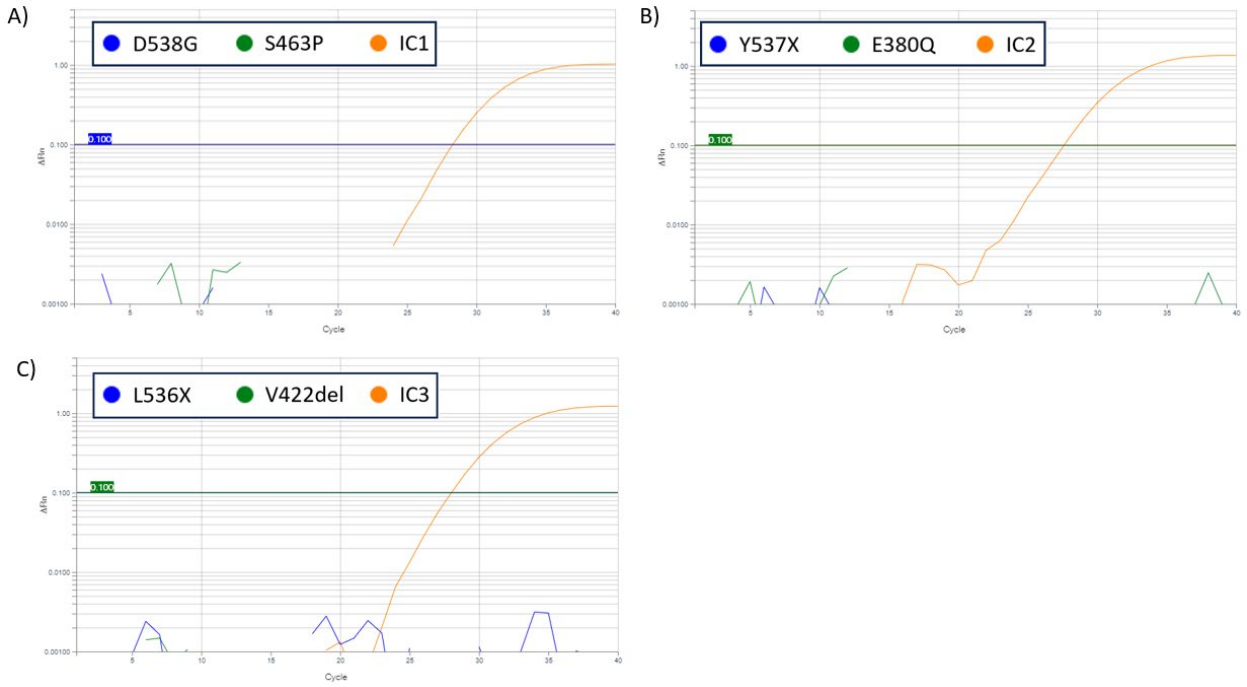
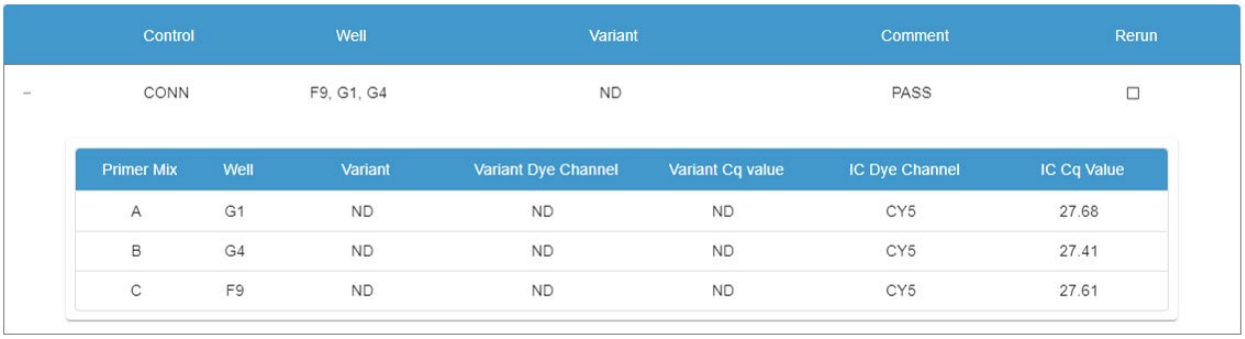


Figure 5. Example of QuantideX qPCR *ESR1* exoMutation Kit Negative Control (CONN) qPCR amplification trace for A) Primer Mix A, B) Primer Mix B, and C) Primer Mix C.

If a variant is detected then the comment column for CONN will display “Variant(s) detected” and the rerun box for CONN and all samples will be checked. If the internal controls (IC1, IC2 and/or IC3) are not detected, then the comment column for CONN will display “Internal Control: not detected” and the rerun box for CONN and all samples will be checked. If the expected targets are not identified then the comment column for CONN will display “Invalid number of target(s)” and the rerun box for CONN and all samples will be checked. These QC failures alert the user that the batch run is invalid. For further details,

refer to the **Troubleshooting** section and the QuantideX qPCR *ESR1* exoMutation Analysis Module Software User Guide (00004887).

A valid CONN will display PASS in the comment column in the results view of the analysis module and in the control section of the CSV report. In the results view, the CONN dropdown table will display ND (Not Detected) in the variant, the variant dye channel, and variant Cq value columns. The internal control (IC) dye channel and IC Cq value will be displayed (**Figure 6**).



Control	Well	Variant	Comment	Rerun
CONN	F9, G1, G4	ND	PASS	☐

Primer Mix	Well	Variant	Variant Dye Channel	Variant Cq value	IC Dye Channel	IC Cq Value
A	G1	ND	ND	ND	CY5	27.68
B	G4	ND	ND	ND	CY5	27.41
C	F9	ND	ND	ND	CY5	27.61

Figure 6. Example of a valid CONN result from the QuantideX qPCR *ESR1* exoMutation Analysis Module. ND = Not Detected.

No Template Control (NTC)

Use of a negative control (i.e., NTC) is optional and not required. If a qPCR NTC is desired, the Kit Diluent may be used in place of the sample volume. In the QuantideX qPCR *ESR1* exoMutation Analysis Module generated CSV report, an NTC sample is displayed in the list of samples tested. A properly functioning NTC exhibits no dye signal in the qPCR run. The comment column for an NTC will display “Internal Control: not detected” and the rerun box will be checked.

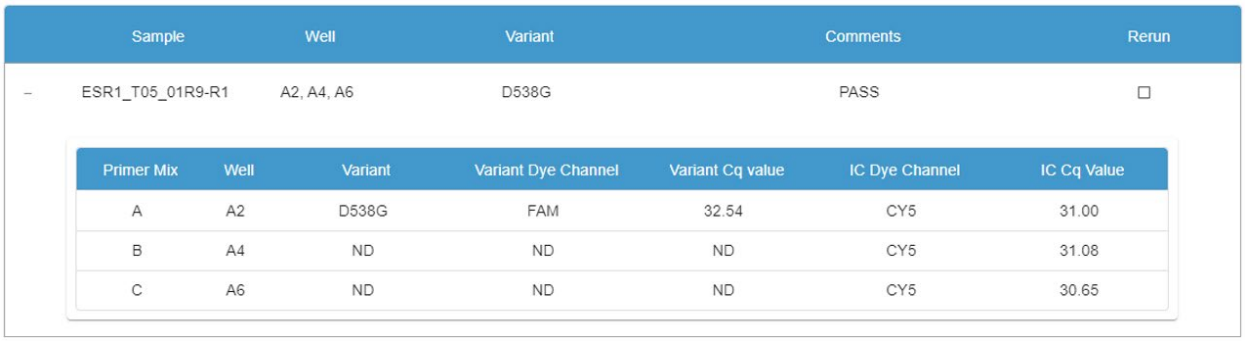
When included, if the NTC produces detectable dye signal, the batch run may be considered invalid. However, since the NTC is not required, the operator or laboratory will need to determine how to use this information for identifying batch run failures.

Samples

Each sample is only required to be tested in singleton. It is expected for each sample to generate a signal in the CY5 dye channel for the internal control (IC) across all qPCR primer mixes. A valid sample will display PASS in the comment column in the results view of the analysis module and in the sample section of the QuantideX qPCR *ESR1* exoMutation Analysis Module generated CSV report.

If no variants are detected, the results view of the QuantideX qPCR *ESR1* exoMutation Analysis Module sample dropdown table will display ND (Not Detected) in the variant, variant dye channel, and variant Cq value columns. The internal control (IC) dye channel and Cq value will be displayed.

If variants are detected, the results view of the QuantideX qPCR *ESR1* exoMutation Analysis Module sample dropdown table will display the variants detected, the variant dye channel, and associated Cq values. ND (Not Detected) will be displayed for variants not present in the sample. The internal control (IC) dye channel and IC Cq values will be displayed (**Figure 7**).



	Sample	Well	Variant	Comments	Rerun
–	ESR1_T05_01R9-R1	A2, A4, A6	D538G	PASS	☐

Primer Mix	Well	Variant	Variant Dye Channel	Variant Cq value	IC Dye Channel	IC Cq Value
A	A2	D538G	FAM	32.54	CY5	31.00
B	A4	ND	ND	ND	CY5	31.08
C	A6	ND	ND	ND	CY5	30.65

Figure 7. Example of a valid sample result containing a D538G positive variant from the QuantideX qPCR *ESR1* exoMutation Analysis Module. ND = Not Detected.

To ensure quality results, the QuantideX qPCR *ESR1* exoMutation Analysis Module contains automated QC checks for all samples. For more details on these QCs, refer to the **Troubleshooting** section and the QuantideX qPCR *ESR1* exoMutation Analysis Module Software User Guide (00004887).

Troubleshooting

Observation	Potential Cause	Action
Incorrect baseline or threshold settings	Analysis settings for the baseline and/or threshold do not match expected settings	Open the .sds or .eds file and review the baseline and threshold settings. See **qPCR Master Mix Setup and Thermal Cycling** for baseline and threshold settings appropriate per qPCR instrument. Reanalyze the run and submit for analysis in QuantideX qPCR *ESR1* exoMutation Analysis Module.
Please analyze a file from a supported platform	Non-supported instrument file submitted for analysis	Confirm the correct file was submitted for analysis. See **Required but not Provided** for a list of supported qPCR instruments. See the QuantideX qPCR *ESR1* exoMutation Analysis Module Software User Guide for supported file types.
Invalid number of target(s)	Incorrect target name setup	See qPCR Instrument Filter Settings in **Pre-Analytical Steps**; confirm target naming according to **Table 2**. Reanalyze the run and submit for analysis in QuantideX qPCR *ESR1* exoMutation Analysis Module.
	Incorrect assignment of target(s) to plate layout	Ensure qPCR setup naming of all controls and samples contain a single assignment of each target. A valid sample or control contains 9 targets; confirm target naming according to **Table 2**.
	Incorrect control and/or sample naming	Ensure CONN and CONP are labeled as identified in **Pre-Analytical Steps**. Incorrectly named controls lead to incorrect assignment in the QuantideX qPCR *ESR1* exoMutation Analysis Module. The affected control(s) will be flagged for rerun. Additionally, all samples in the batch will be marked for rerun, including samples that were named correctly. Misnaming of sample replicates results in the sample flagged for a rerun. Ensure sample name is consistent across all wells for the sample as identified in **Pre-Analytical Steps**.
	Variant observed in CONN	Verify no contamination has occurred. Verify qPCR master mixes were properly formulated. Confirm CONN was added to the appropriate well. If CONN has a variant detected, CONN will be flagged for rerun. Additionally, all samples in the batch will be marked for rerun.
	Unexpected variant(s) observed in	If continual cross-contamination is observed, arrange plate layout with placement of CONP away from CONN and samples, giving at least one blank column as space between samples and controls.

Observation	Potential Cause	Action
	CONN and samples	Ensure recommended foil seals and plates are used.
	Absence of a positive signal in all channels for CONP	Verify qPCR master mixes were properly formulated. Verify CONP was added to the appropriate well. If CONP has a variant detected, CONP will be flagged for rerun. Additionally, all samples in the batch will be marked for rerun.
Invalid reporter(s) for target(s)	Incorrect target name setup	See qPCR Instrument Filter Settings in **Pre-Analytical Steps**; confirm target naming according to **Table 2**. Reanalyze the run and submit for analysis in QuantideX qPCR *ESR1* exoMutation Analysis Module. All affected controls and samples in the batch will be marked for rerun.
CONP not found	Incorrect control naming	Ensure CONP is labeled as identified in **Pre-Analytical Steps**. Incorrectly named CONP leads to incorrect assignment in the QuantideX qPCR *ESR1* exoMutation Analysis Module. CONP will be flagged for rerun. Additionally, all samples in the batch will be marked for rerun, including samples that were named correctly.
CONN not found	Incorrect control naming	Ensure CONN is labeled as identified in **Pre-Analytical Steps**. Incorrectly named CONN leads to incorrect assignment in the QuantideX qPCR *ESR1* exoMutation Analysis Module. CONN will be flagged for rerun. Additionally, all samples in the batch will be marked for rerun, including samples that were named correctly.
PCR Cycle QC	Incorrect number of PCR cycles completed	The recommended number of cycles for the qPCR step is 40. If >40 qPCR cycles were intentionally run, click “OK” on the popup window to accept the results. See **qPCR Master Mix Setup and Thermal Cycling** for recommended cycling conditions.
Internal Control: Not Detected	No signal detected for IC1, IC2, and/or IC3	Ensure correct volume of template was utilized in all steps. Verify qPCR master mixes were properly formulated. If CONN or CONP have internal control not detected (ND) for IC1, IC2, and/or IC3, affected control(s) and all samples will be flagged for rerun.
	Incorrect target name setup	See qPCR Instrument Filter Settings in **Pre-Analytical Steps**; confirm target naming according to **Table 2**. Reanalyze the run and submit for analysis in QuantideX qPCR *ESR1* exoMutation Analysis Module.
	Incorrect assignment of	Ensure qPCR setup naming of all controls and samples contain a single assignment of each target. A valid sample or

Observation	Potential Cause	Action
	target(s) to plate layout	control contains 9 targets; confirm target naming according to **Table 2**.
	Incorrect internal control naming	Ensure IC1, IC2 and IC3 are labeled as identified in **Pre-Analytical Steps**. Incorrectly named IC1, IC2 and IC3 lead to incorrect assignment in the QuantideX qPCR *ESR1* exoMutation Analysis Module. The affected control(s) will be flagged for rerun. Additionally, all samples in the batch will be marked for rerun, including samples that were named correctly.
	Variant observed in CONN	Verify no contamination has occurred. Verify qPCR master mixes were properly formulated. Confirm CONN was added to the appropriate well. If CONN has a variant detected, CONN will be flagged for rerun. Additionally, all samples in the batch will be marked for rerun.
	Absence of a positive signal in all channels for CONP positive control	Verify qPCR master mixes were properly formulated. Verify CONP was added to the appropriate well. If CONP has a variant detected, CONP will be flagged for rerun. Additionally, all samples in the batch will be marked for rerun.
Expected Variant(s) not detected	One or more expected variants are not detected (ND) in CONP	Ensure correct control was used for CONP. Verify qPCR master mixes were properly formulated. CONP and all samples will be flagged for rerun.
	Incorrect target name setup	See qPCR Instrument Filter Settings in **Pre-Analytical Steps**; confirm target naming according to **Table 2**. Reanalyze the run and submit for analysis in QuantideX qPCR *ESR1* exoMutation Analysis Module.
Variant(s) detected	Unexpected variant(s) have been detected in CONN	Ensure correct control was used for CONN. Verify no contamination has occurred. CONN and all samples will be flagged for rerun.
	Incorrect target name setup	See qPCR Instrument Filter Settings in **Pre-Analytical Steps**; confirm target naming according to **Table 2**. Reanalyze the run and submit for analysis in QuantideX qPCR *ESR1* exoMutation Analysis Module.
qPCR instrument does not complete programmed run	Instrument malfunction	Ensure instrument has been installed according to manufacturer recommendations.

Observation	Potential Cause	Action
		Repeat qPCR using freshly diluted pre-amplified product. See **PreAmp Product Dilution**.
Aberrant background signal present in qPCR amplification plot	Bubbles in qPCR plate	Employ pipetting techniques that minimize addition of bubbles to qPCR plate setup. Tap sealed qPCR plate on countertop and visually confirm all bubbles have been removed prior to starting qPCR run. Adjusting the qPCR initial denaturing step to 95°C for 15 min may aid in bubble elimination. **NOTE**: This alteration of the prescribed qPCR cycling conditions has not been verified.

Analytical Assessment

The QuantideX® qPCR *ESR1* exoMutation Kit analytical assessment presented here did not utilize a Cq cutoff, making the cutoff the total number of PCR cycles (40 cycles). To view the impact to the sensitivity (LOD) and specificity data with the implementation of a Cq cutoff, see **Application of a Cq Cutoff**.

Within-lab Precision

To assess precision of the QuantideX qPCR *ESR1* exoMutation Kit, six surrogate samples were prepared that contained 10 mutant copies from two different plasmid DNAs (10 copies Plasmid1 + 10 copies Plasmid2 = 20 copies total) mixed into 9,980 wild type copies of fragmented, cell line DNA. All 11 variants were represented in the sample panel at the targeted 0.1% VAF.

Testing was performed in duplicate using the complete workflow starting from nucleic acid across 12 batch runs with two operators, three kit lots, two Applied Biosystems™ Veriti™ 96-well thermal cycler, and two Applied Biosystems QuantStudio™ 5 Dx Real-Time PCR Systems, generating 72 measurements per sample, or 864 sample measurements in total.

Out of 864 valid measurements generated by the study, the variant-level statistics were >96% in agreement with expected results for each operator and kit lot (**Table 5**). Positive percent agreement (PPA) was ≥99.6% (287/288) across all expected positive calls, with the only missed call being for S463P (95.8%, 23/24). Additionally, variant-level overall percent agreement (OPA) was ≥96%, with %CV of Cq values per target ranging from 2.6 to 6.6% (**Table 6**).

Table 5. Variant-level metrics for the within-lab precision study of the QuantideX qPCR *ESR1* exoMutation Kit between kit lots and operators. OPA=overall percent agreement, PPA=positive percent agreement, NPA=negative percent agreement.

Measure	Lot 1	Lot 2	Lot 3	Operator 1	Operator 2
OPA	99%	98%	99%	97%	99%
PPA	100%	100%	99%	99%	100%
NPA	98%	96%	98%	97%	99%

Table 6. Variant-level metrics for the within-lab precision study of the QuantideX qPCR *ESR1* exoMutation Kit per variant. OPA=overall percent agreement, PPA=positive percent agreement, NPA=negative percent agreement.

Target	PPA (n)	NPA (n)	OPA (n)	Cq Mean	Cq StdDev	%CV
D538G	100% (48)	94% (96)	96% (144)	31.93	2.11	6.6%
S463P	96% (24)	99% (120)	99% (144)	33.95	2.10	6.2%
Y537X	100% (96)	94% (48)	98% (144)	28.88	1.75	6.0%
E380Q	100% (24)	100% (120)	100% (144)	28.99	0.74	2.6%
L536X	100% (72)	94% (72)	97% (144)	28.65	1.51	5.3%
V422del	100% (24)	100% (120)	100% (144)	29.43	1.34	4.6%

Limit of Detection (LoD)

The LoD was estimated for each variant across all supported qPCR platforms using CLSI EP12 and EP17 as guidance. A titration of mutant-positive plasmid DNA in a background of wild type, fragmented cell line DNA was tested at 0%, 0.01%, 0.03%, 0.05%, and 0.1% variant allele frequency (VAF). All samples were tested across 20 replicates (1x RT>1x PreAmp>1x qPCR) per concentration. Resultant PreAmp products underwent qPCR testing on the ABI 7500 Fast Dx, ABI QuantStudio 5 Dx, and ABI QuantStudio 7 Pro Dx.

Per variant and qPCR platform, 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 crosses the 95% probability line (i.e., the C95) was reported as the estimated VAF that the assay can determine a sample to be positive 95% of the time. Copies/RT was converted to %VAF since 10,000 total copies were input into each reaction. Resultant LoDs ranged from 0.025% to 0.083% VAF across all 3 supported qPCR platforms, with individual qPCR platform LoDs reported per variant in **Table 7**.

Table 7. Variant-level limit of detection for the QuantideX qPCR *ESR1* exoMutation Kit across all three supported qPCR platforms: ABI 7500 Fast Dx, ABI QuantStudio 5 Dx, and ABI QuantStudio 7 Pro Dx.

Variant	ABI QuantStudio 5 Dx (n=120)	ABI QuantStudio 7 Pro Dx (n=120)	ABI 7500 Fast Dx (n=120)	Across qPCR Platforms (n=360)
D538G	0.082%	0.100%	0.064%	0.083%
S463P	0.066%	0.039%	0.042%	0.050%
Y537S	0.025%	0.026%	0.026%	0.026%
Y537C	0.029%	0.029%	0.029%	0.029%
Y537N	0.025%	0.026%	0.026%	0.025%
Y537D	0.029%	0.029%	0.029%	0.029%
E380Q	0.028%	0.029%	0.029%	0.028%
L536R	0.028%	0.029%	0.029%	0.029%
L536H	0.041%	0.041%	0.045%	0.043%
L536P	0.028%	0.028%	0.039%	0.031%
V422del	0.030%	0.030%	0.030%	0.030%

Analytical Specificity (Exclusivity)

Analytical specificity (exclusivity) was tested *in vitro* to exhibit a “Not Detected” call on samples determined to be negative (i.e., wild type) for all targets detected by the assay. The study was performed on previously characterized, mutant-negative plasma samples from K_2EDTA and PAXgene Blood ccfDNA collection tubes. Per collection tube, one operator extracted total nucleic acid from 21 mutant-negative samples using 2 mL plasma in the ExoLution Plus cfDNA + exoRNA Isolation Kit. Each sample was tested in singleton in the QuantideX qPCR *ESR1* exoMutation Kit. The overall variant-level NPA for K_2EDTA tubes was 97% and for PAXgene Blood ccfDNA tubes was 98% as shown in **Table 8**.

Additionally, *in silico* analysis was performed using a proprietary, internal method. Primers were screened to avoid potential off-target amplicons.

Table 8. Evaluation of variant-level analytical specificity (exclusivity) in mutant-negative plasma samples. NPA=negative percent agreement.

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

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