GALEAS™ uPCR: ESR1

Ultrasensitive qPCR *ESR1* Mutation Detection Kit

GALEAS™ nonacus

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Revision history

Revision	Date	Revision Description
1.0	August 2024	First iteration
1.1	February 2025	Updated analysis criteria on page 24
1.2	March 2025	Updated Overview on page 4
1.3	June 2025	Updated Table 7 negative control range on page 12
1.4	June 2025	Formatting update

Intended use

GALEAS™ uPCR: ESR1 kit is a qualitative (mutant/wild-type) ultrasensitive qPCR assay that detects *ESR1* clinically relevant mutations in cell-free DNA (cfDNA) known to confer resistance to endocrine therapy in hormone receptor-positive breast cancer patients.

This product is for Research Use Only.

Key features

This kit includes:

- Reagents sufficient for 96 reactions (32 reactions per multiplex)
- Assays covering 11 clinically relevant *ESR1* variants and an internal endogenous control (Table 1)
- Positive and negative controls
- Flexible template concentration
- Streamlined protocol for workflow simplicity

Application	Qualitative PCR test for *ESR1* variant detection
Sample input	Extracted cell-free DNA
Input amount	1-25 ng
Analytical sensitivity	≥90% at mutant allele frequencies of 0.04% to 0.33% *
Analytical specificity	≥98%

*Based on 25 ng input, see Table 9 for full list of targets and associated LOD

Overview

Oestrogen receptor (ER) subtype alpha protein is encoded by the *ESR1* gene. Mutations that occur in the ligand binding domain have been shown to cause resistance to endocrine therapy used for ER-positive, HER2-negative breast cancer treatment. Identification of *ESR1* variants can inform on changes to treatment management.

GALEAS™ uPCR: ESR1 kit can detect 11 clinically relevant variants in the ESR1 gene across three multiplex qPCR reactions targeting the mutations of interest (as shown in Table 1). The kit contains three positive controls at set copy number per target, to confirm assay functionality. A negative control is also included to confirm assay specificity. It contains wild-type genomic DNA (2.5 ng/µl) to be used at an input of 25 ng per reaction.

Table 1. Variants detected by each primer/probe mix and respective controls.

Primer / probe mix	ESR1 Variant (Protein Change)	ESR1 Variant (Nucleic acid change)	Variant prevalence	Detection channel	Controls
1 (32 reactions)	p.E380Q	c.1138G>C	High	HEX	Positive control 1
	p.D538G	c.1613A>G	High	FAM	
	p.Y537S	c.1610A>C	High	FAM	
	p.Y537N	c.1609T>A	High	FAM	
2 (32 reactions)	p.S463P	c.1387T>C	Low	HEX	Positive control 2
	p.Y537C	c.1610A>G	Low	FAM	
	p.L536H	c.1607T>A	Low	FAM	
3 (32 reactions)	p.L536R	c.1607T>G	Low	FAM	Positive control 3
	p.P535H	c.1604C>A	Low	FAM	
	p.L536Q	c.1607_1608delinsAG (TC>AG)	Low	FAM	
	p.Y537D	c.1609T>G	Low	FAM	
	Internal control	Internal control	N/A	HEX	Negative control

Validated instruments and user requirements: The assay has been validated on an AriaMx real-time platform which is controlled by the AriaMx software. The assay is compatible with other equivalent 96-well real-time qPCR cyclers, but validation should be performed prior to use. The kit is required to be operated by trained laboratory staff who are proficient in performing molecular based tests.

Assay performance: The assays have been validated with an input range of 1 to 25 ng. The assays' mutant copy number limit of detection (LOD) for each target is achieved with a >90% positive call rate regardless of total DNA input. The LOD is target dependent and provides an analytical sensitivity as low as 0.04% and as high as 0.3% mutant allele frequency when using 25 ng sample input (see Table 9 for further details). The analytical specificity of each target is ≥98% at an input amount of 25 ng.

Assay analysis: The assay utilises a two-phase cycling protocol. In phase 1, 20 cycles are performed with an annealing temperature of 65°C without fluorescence detection; in phase 2, 35 cycles are performed with an annealing temperature of 60°C with fluorescence detection. All Ct cut-offs and RFU thresholds have been defined utilising the amplification plot generated in phase 2. Therefore, the Ct cut-off of 32 cycles for distinguishing mutant from wild-type is equivalent to 52 cycles in total: 20 dark cycles with high specificity and low PCR efficiency from phase 1 without fluorescence detection and 32 light cycles with high specificity and high PCR efficiency including fluorescence detection from phase 2.

Kit contents

Reagent	Product code	Storage temperature*	Reagent Tube Cap Colour Code
uPCR Buffer	USP_BUF_96	- 20°C	Purple
uPCR Polymerase	USP_POL_96	- 20°C	Red
uPCR ESR1 PP mix 1	USP_EPP1_32	- 20°C	Brown
uPCR ESR1 PP mix 2	USP_EPP2_32	- 20°C	Brown
uPCR ESR1 PP mix 3	USP_EPP3_32	- 20°C	Brown
uPCR Diluent	USP_DIL_96	- 20°C	Green
uPCR ESR1 PC1	USP_EPC1_32	- 20°C	Clear
uPCR ESR1 PC2	USP_EPC2_32	- 20°C	Clear
uPCR ESR1 PC3	USP_EPC3_32	- 20°C	Clear
uPCR NC	USP_ENC_96	- 20°C	Yellow

NOTE: For additional information see the Storage and handling section below

Recommended laboratory reagents and consumables not supplied

Item	Recommended Source
PCR-certified filtered pipette tips (10, 100, 200, 1000 µl capacity)	Various sources available
For Agilent AriaMx qPCR instrument: Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, clear well. For alternative qPCR machines: As machine manufacturer recommends*"	For Agilent AriaMx qPCR instrument: Biorad, Cat# #HSP9601
ABsolute qPCR Plate Seals	Thermofisher, Cat# AB1170
DNA storage - Screw cap micro tube, 2 ml, PCR Performance Tested, Low DNA-binding	Sarstedt, Cat# 72.694.700
1.5-2ml non-LoBind tubes	Various sources available
DNAzap or bleach	Invitrogen, Cat# AM9890
Fluorometer consumables for genomic DNA processing	Invitrogen: Qubit Assay Tubes, Cat # Q32856 Qubit dsDNA BR Assay kit, Cat # Q32853 Qubit dsDNA HS Assay kit, Cat # Q32854)

NOTE: For Bio-Rad CFX96 qPCR instrument we recommend Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white (Bio-Rad, Cat#HSP9655)

Recommended equipment not supplied

Item	Recommended Source
Micro-centrifuge capable of accommodating 1.5-2 ml tubes	Various sources available
Plate centrifuge capable of accommodating 0.2 ml 96 well plates or micro-centrifuge capable of accommodating 8-reaction PCR strip tubes.	Various sources available
Single channel pipettes (10, 100, 200, 1000 µl capacity)	Various sources available
AriaMx Real-time PCR system with AriaMx Software	Agilent, Cat# G8830A.
Biological Safety Cabinet (BCS) class II or PCR hood	Various sources available
Cold block capable of accommodating 1.5-2ml tubes	Various sources available
Fluorometer for genomic DNA fluorometric quantitation	Qubit®3.0 Fluorometer, Invitrogen, Cat # Q33216 Qubit™ 4 Fluorometer, Invitrogen, Cat # Q33238

Plasma processing for optimal cell-free DNA recovery

To achieve optimal results using cell-free cfDNA as input, it is recommended to collect blood in cell-stabilising blood collection tubes, such as Cell3™ Preserver tubes (PRE_C3P_WBS_50) or equivalent, and to extract cfDNA from ≥ 4 ml of plasma. Detailed plasma processing recommendation can be found in Appendix A.

GALEAS™ uPCR is compatible with most cfDNA extraction products or systems which deliver clean nucleic acid isolation (PCR inhibitor free) and should be validated prior to use. We recommend Cell3™ Xtract (PRE_EXT_C3X_#) or Bead Xtract cfDNA kit (PRE_EXT_BXC_#) extraction kits to maximise the sample quality.

To ensure maximum performance, it is recommended to quantify DNA samples prior to use, using a fluorometric assay, such as Qubit. Use of spectrophotometric assays such as Nanodrop is not recommended for DNA quantification due to the inaccuracy in measurement of samples with low concentrations. A minimum of 1 ng and maximum of 25 ng cfDNA can be used (≥5 ng is recommended).

Warning and precautions

This product should be handled only by trained laboratory staff who are proficient in performing molecular based tests and it should be used in accordance with the principles of good laboratory practice. See MSDS for health hazard warnings of individual reagents.

Limitations of use

- This is a qualitative assay and has not been validated for quantification purposes.
- The maximum sample input of 25 ng per reaction should not be exceeded, to ensure specificity.
- The assay has been validated on an AriaMx real-time platform which is controlled by the AriaMx software. The analysis section is based on results obtained with the validated instrument. For any other qPCR instrument the assay requires additional validation from the user.

Storage and handling

The entire kit is stable for 14 days at 4°C and up to 5 freeze-thaw cycles. The polymerase should not freeze, as it is stored in glycerol, and should not be vortexed. It is best practice to store the kit components in the freezer as much as possible when not performing the protocol. The uPCR Polymerase should always be kept on ice or cold blocks when in use. All the remaining reagents can be thawed at room temperature and vortex mixed. Do not store mastermixes for future use.

Optional:
To minimise contamination risks, we recommend to aliquot the entire amount of the positive controls uPCR PC1, uPCR PC2 and uPCR PC3, when thawed for the first time. The aliquots should be then stored at -20°C until used.

To minimise contamination risks, we recommend to aliquot the entire amount of the negative control uPCR NC when thawed for the first time. The aliquots should be then stored at -20°C until used.

GALEAS™ uPCR: ESR1 procedure

qPCR controls, mastermix calculations and plate layouts

Perform mastermix calculations for sufficient volume covering samples and necessary controls. Refer to Table 2 for volume requirements.

A minimum of 1 replicate of positive, negative and No Template control (NTC) must be prepared for each PP mix (refer to Table 3 for guidance).

NOTE: Ensure the mastermix is prepared with 10% overage for consistent dispensing.

Table 2. Description of PCR reaction mix reagent volumes required for each primer/probe mix (total of 3 primer/probe mixes need to be prepared, using uPCR ESR1 PP mixes 1, 2 and 3).

Reagent	Volume	Volume for 32 reactions (Includes 10% overage)
uPCR Buffer	5 µl	176 µl
uPCR Polymerase	0.1 µl	3.5 µl
uPCR ESR1 PP mix	4.9 µl	172.5 µl
Total mastermix volume	**10 µl**	**352 µl**

Table 3. Controls required for each run.

Primer / probe mix	Control required	Minimum number of replicates	Input volume (µl)
1	PC1	1	10
	NTC (uPCR diluent)	1	10
	NC	1	10
2	PC2	1	10
	NTC (uPCR diluent)	1	10
	NC	1	10
3	PC3	1	10
	NTC (uPCR diluent)	1	10
	NC	1	10

NOTE: We recommend running single reactions for patient samples across all 3 multiplexes, along with single reactions of NTC, negative control and corresponding positive control. An exemplary plate layout is displayed in Table 4. Refer to Appendix B for alternative plate layout if using duplicate technical repeats.

Table 4. Recommended plate layout for single reaction per sample per multiplex.
Blue – Mastermix 1, Yellow – Mastermix 2, Pink – Mastermix 3, S = Sample, PC1 = Positive control 1, PC2 = Positive control 2, PC3 = Positive control 3, NC - Negative control, NTC = No Template control.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	S9	S9	S9	S17	S17	S17	S25	S25	S25
B	S2	S2	S2	S10	S10	S10	S18	S18	S18	S26	S26	S26
C	S3	S3	S3	S11	S11	S11	S19	S19	S19	S27	S27	S27
D	S4	S4	S4	S12	S12	S12	S20	S20	S20	S28	S28	S28
E	S5	S5	S5	S13	S13	S13	S21	S21	S21	S29	S29	S29
F	S6	S6	S6	S14	S14	S14	S22	S22	S22	PC1	PC2	PC3
G	S7	S7	S7	S15	S15	S15	S23	S23	S23	NC	NC	NC
H	S8	S8	S8	S16	S16	S16	S24	S24	S24	NTC	NTC	NTC

qPCR set-up protocol

Set-up pre-requisites:

- Perform the following protocol in a pre-PCR environment, preferably within a BCS class II or PCR hood.
- Ensure the UV light in the hood has been utilised for at least 15 minutes prior to performing the experiment.
- If a template spill has occurred previously, use the UV light in the PCR workstation for at least 60 minutes and clean with DNA-zap or bleach.
- The positive controls PC1, PC2 and PC3 (clear cap) and DNA samples should be separated from the rest of the kit components during this procedure by not being placed in the PCR workstation at the same time.

  OPTIONAL: To increase contamination control, a separate UV light capable hood can be used from step 18 onwards which utilises the sample template and positive control.
- Thaw DNA samples to be tested.

1. Remove the uPCR Buffer (**Purple** cap), uPCR PP mix 1, 2 and 3 (**Brown** cap), uPCR NC (**Yellow** cap) and uPCR Diluent (**Green** cap) from the freezer and allow to thaw at room temperature. Once the reagents have thawed, place them in the PCR workstation in a tube rack.
2. Briefly centrifuge, vortex and briefly centrifuge again the uPCR Buffer and uPCR PP mix 1, 2 and 3.
3. Take out the uPCR Polymerase (**Red** cap) from the freezer and place it inside the PCR workstation.

IMPORTANT: Keep the polymerase on a cold block or on ice throughout the procedure.

4. Pipette mix the uPCR polymerase with at least 50% of the total volume in the tube, 10-15 times, to ensure that it is properly mixed.

IMPORTANT: do not vortex mix the uPCR polymerase, as this may damage the enzyme and cause the reagent to froth.

5. Take out and label three 1.5-2 ml tubes for the reaction mastermix preparation described in the following step.
6. Prepare the three reaction mastermixes as per calculations in Table 3. One mastermix should be prepared for each of the three uPCR PP mixes, adding only one uPCR PP per mastermix.

NOTE: Three mastermixes must be created in total, one for each PP mix (1, 2 and 3). All PP mixes utilise the same uPCR Buffer and uPCR Polymerase.

Table 5. Description of uPCR individual reaction volume across all three mastermixes.

Mastermix Name	Mastermix Volume	Template Volume (sample* or control)	Total Reaction Volume
Mastermix (Containing PP mix 1)	10 µl	10 µl	20 µl
Mastermix (Containing PP mix 2)	10 µl	10 µl	20 µl
Mastermix (Containing PP mix 3)	10 µl	10 µl	20 µl

*Minimum of 1 ng, maximum of 25 ng, recommended ≥ 5 ng

NOTE: uPCR Diluent can be used for adjusting the volume of highly concentrated samples.

7. Place the uPCR Buffer, uPCR PP mix 1, 2 and 3 and uPCR polymerase back in the freezer. The uPCR Diluent, uPCR NC, and newly created reaction mastermix 1, 2 and 3 from step 6 should remain in the PCR workstation.

8. Briefly centrifuge, vortex and briefly centrifuge again the prepared reaction mastermix 1, 2 and 3 from step 6.

9. Take out the uPCR-ESR1 positive controls (PC1, PC2, and PC3) from the freezer and place outside of the PCR workstation to thaw at room temperature.

10. Take out a 96-well PCR plate that is recommended for the qPCR instrument being used (unless otherwise stated in the section 'Required laboratory reagents and consumables not supplied'). Place the plate in the PCR workstation.

11. Pipette 10 µl of reaction mastermix into the designated reaction wells.

NOTE: Refer to Table 4 for recommended plate layout

12. Briefly centrifuge, vortex and briefly centrifuge again the uPCR diluent.

13. Add 10 µl of uPCR diluent to the designated NTC reaction wells for reaction mastermixes 1, 2 and 3.

14. Briefly centrifuge, vortex and briefly centrifuge again the uPCR NC.

15. Add 10 µl of the uPCR NC to the designated wells for reaction mastermixes 1, 2 and 3.

16. Remove uPCR diluent, uPCR NC from the PCR workstation and place back in the freezer.

17. Take out DNA samples and briefly centrifuge, vortex and then briefly centrifuge them again.

18. Add 10 µl of DNA sample to the designated wells for reaction mastermixes 1, 2 and 3 to achieve a total reaction volume of 20 µl.

19. Place DNA samples back in the freezer to avoid potential contamination with positive controls in the subsequent steps.

20. Place the uPCR ESR1 PC 1, PC 2 and PC 3 into the PCR workstation.

21. Briefly centrifuge, vortex and then briefly centrifuge again the uPCR ESR1 PC 1, PC 2 and PC 3.

22. Add 10 µl of uPCR ESR1 PC 1 to the designated wells for reaction mastermix 1.

23. Add 10 µl of uPCR ESR1 PC 2 to the designated wells for reaction mastermix 2.

24. Add 10 µl of uPCR ESR1 PC 3 to the designated wells for reaction mastermix 3.

25. Move the uPCR ESR1 PC 1, PC 2 and PC 3 back to the freezer.

26. Place an optically clear pressure activated PCR seal onto the plate and apply pressure with a roller.

27. Spin the 96-well PCR plate in a centrifuge at 1000 rpm briefly. Ensure there are no bubbles in the reaction wells.

28. Load the 96-well PCR plate onto the qPCR thermocycler immediately after setting up the reaction. Refer to cycling conditions for qPCR instrument setup (Table 6).

qPCR thermocycler setup and cycling conditions

Set up the qPCR instrument using the manufacturer's guidelines and input the cycling conditions described below in Table 6. For qPCR instruments with "FAST" blocks, select the fast ramp speed. Select the SYBR/FAM and HEX/VIC filter for all wells to be tested. Select BHQ-1 as the quencher, if required. Set reaction volume to 20 µl.

Table 6. PCR Program cycling conditions.

Step	Cycles	Temperature	Time	Fluorescence capture (data capture)
1	1	95 °C	15 minutes	No
2	20 (phase one)	95 °C	5 seconds	No
3		65 °C	45 seconds	No
4	35 (phase two)	95 °C	5 seconds	No
5		60 °C	45 seconds	No
6		72 °C	20 seconds	Yes

qPCR data analysis and interpretation

The assay utilises a two-phase (Table 6) cycling protocol, in phase one, 20 cycles are performed with an annealing temperature of 65°C without fluorescence capture; and in phase two 35 cycles are performed with an annealing temperature of 60°C with fluorescence capture.

All Ct cut-offs and RFU thresholds have been defined utilising the amplification plot generated in phase two. Therefore, the Ct cut-off of 32 cycles for distinguishing mutant from wild-type is at 52 cycles total: 20 dark cycles with high specificity and low PCR efficiency from phase one without fluorescence capture; and 32 light cycles with high specificity and high PCR efficiency including fluorescence capture from phase two.

Please note data analysis may vary between qPCR instruments and Ct thresholds and cut-offs must be determined empirically by the end user or laboratory if not utilising the Agilent AriaMx instruments. In these instances, Table 7 can be used as a guide to determine appropriate Ct cut-offs using the positive, negative and no-template controls provided in the kit to enable internal validation. If support is required with validating other instruments, please contact Nonacus directly for advice at support@nonacus.com.

The recommended analysis settings to be used on the Agilent AriaMX software are as follows:

- Ct threshold set to 100 ΔR (Fluorescence) or 100 RFU (or equivalent if using alternative instruments) for both SYBR/FAM and HEX/VIC channels.

29. Baseline start and end cycles should be left to auto detection.

Expected results for controls are detailed in Table 7.

Table 7. Interpretation of results for controls relevant to the Agilent AriaMX instrument.

Sample / Control	Expected Ct values					
	Mastermix 1		Mastermix 2		Mastermix 3	
	SYBR/FAM	HEX/VIC	SYBR/FAM	HEX/VIC	SYBR/FAM	HEX/VIC
Positive Control 1 (PC1)	< 18	< 18				
Positive Control 2 (PC2)			< 18	< 18		
Positive Control 3 (PC3)					< 15	
Negative Control (NC)	No amp	No amp	No amp	No amp	No amp	16-20
No Template Control (NTC)	No amp	No amp	No amp	No amp	No amp	No amp

DNA sample data interpretation:

- A Ct value < 32 detected in the SYBR/FAM channel of Mastermix 1, 2 or 3; or in the HEX/VIC channel of Mastermix 1 or 2 is indicative of a positive result for a mutation covered in the respective target assay (see Table 1).
  A Ct value > 32 or no amplification is indicative of a negative result.
- A Ct value < 24 is expected in the HEX/VIC channel of Mastermix 3 to confirm that DNA is present at ≥ 1 ng input. Please be advised if the positive signal is above Ct 24 this might result in decreased sensitivity due to insufficient DNA input.

NOTE: a positive result in more than one target mutation assay may occur, due to limited cross-reactivity across specific mutations (see Table 11 for further information).

Support/troubleshooting

For further guidance, please contact us at **support@nonacus.com.**

Supporting data

All supporting data has been generated using the Agilent AriaMx qPCR instrument and utilising the AriaMx software for data analysis. To achieve the same performance, it is recommended to utilise the same instrument, as performance may slightly differ between qPCR instruments and will require additional validation.

Analytical specificity

The analytical specificity was determined for extracted cfDNA on 25 ng of SensID cfDNA wild type reference standard (cat# SID-000003) per reaction, testing 75 technical replicates for each multiplex. The same testing was also conducted on 25 ng per reaction of SensID wild type genomic DNA reference standard (cat# SID-000004) with 90 replicates for each multiplex. Analytical specificity was also tested on genomic DNA extracted from 25 individual formalin-fixed-paraffin-embedded (FFPE) human tissue specimens at 25 ng input per reaction for each multiplex. Overall ≥98% specificity was obtained for all multiplexes across cfDNA, gDNA and FFPE tissue gDNA.

Table 8. Analytical specificity result comparison for reference standards.

Primer / probe mix	ESR1 Mutation (Protein Change)	Detection channel	SENS-ID cfDNA wt standard (25ng)	SENS-ID gDNA wt standard (25ng)	FFPE tissue gDNA (25ng)
1	E380Q	HEX	0/75 (100%)	0/90 (100%)	0/25 (100%)
	D538G	FAM	1/75 (>98%)	0/90 (100%)	0/25 (100%)
	Y537S	FAM			
	Y537N	FAM			
2	S463P	HEX	1/75 (>98%)	0/90 (100%)	0/25 (100%)
	Y537C	FAM	0/75 (100%)	0/90 (100%)	0/25 (100%)
	L536H	FAM			
3	L536R	FAM	1/75 (>98%)	1/90 (>98%)	0/25 (100%)
	P535H	FAM			
	L536Q	FAM			
	Y537D	FAM			
	Internal control	HEX	Ct 16.15 - 17.86	Ct 14.16 - 15.65	Ct 15.14 – 17.41

Analytical sensitivity and robustness

The analytical sensitivity was determined using contrived samples quantified by digital PCR in a background of 25 ng of wild type gDNA from SensID (cat# SID-000111). Each mutation was assessed individually. The limit of detection was defined as the lowest amount of mutant copies which could be detected in at least 90% of reactions. Three separate runs with 20 replicates per run (60 total replicates) were performed to demonstrate robustness. Nine targets (E380Q / D538G / Y537S / Y537N / S463P / Y537C / L536H / L536R / P535H) were able to achieve 95% detection across three runs. Two targets (L536Q and Y537D) achieved ≥ 90% detection across three runs. The limit of detection is described in Table 9 below.

Table 9. Analytical sensitivity of PP mix 1, 2 and 3.

Primer / probe mix	ESR1 Mutation (Protein Change)	Mutant allele frequency (25 ng input)	Mutant copies at LOD	Run 1	Run 2	Run 3	Overall	Percentage detection overall
1	E380Q	0.13%	10	20/20	20/20	20/20	60/60	100%
	D538G	0.33%	25	20/20	20/20	18/20	58/60	96.66%
	Y537S	0. 20%	15	20/20	20/20	20/20	60/60	100%
	Y537N	0.07%	5	20/20	20/20	20/20	60/60	100%
2	S463P	0.33%	25	19/20	20/20	19/20	58/60	96.66%
	Y537C	0.33%	25	20/20	20/20	20/20	60/60	100%
	L536H	0.13%	10	20/20	20/20	20/20	60/60	100%
3	L536R	0.26%	20	20/20	20/20	20/20	60/60	100%
	P535H	0.07%	5	20/20	20/20	20/20	60/60	100%
	L536Q	0.04%	3	19/20	18/20	19/20	56/60	93.33%
	Y537D	0.13%	10	19/20	16/20	19/20	54/60	90%

Cell-free DNA standards

The sensitivity achieved on cfDNA reference standards was determined by using the SensID ESR1 cfDNA reference standard set (cat# SID-000144). 15ng of SensID reference standard at 1%, 0.3% and 0.1% mutant allele frequency was tested in a background of SensID wild-type cfDNA. Results are shown in Table 10 below.

Table 10. Detection of ESR1 variants in SensID ESR1 reference standard kit.

Primer / probe mix	ESR1 Mutation (Protein Change)	SENS-ID ESR1 standard tube	Lowest mutant allele frequency	Detected?
1	E380Q	4	0.1% in 15ng total	Yes
	D538G	3	0.1% in 15ng total	Yes
	Y537S	2	0.1% in 15ng total	Yes
	Y537N	4	0.1% in 15ng total	Yes
2	S463P	4	0.1% in 15ng total	Yes
	Y537C	1	0.1% in 15ng total	Yes
	L536H	1	0.1% in 15ng total	Yes
3	L536R	3	0.1% in 15ng total	Yes
	P535H	Not included in set	Not applicable	Not applicable
	L536Q	Not included in set	Not applicable	Not applicable
	Y537D	Not included in set	Not applicable	Not applicable

Cross reactivity

The cross reactivity of target assays with other targeted mutations was determined by utilising contrived samples quantified by digital PCR with 2000 mutant copies per reaction in triplicate. Very minor cross reactivity (Ct > 25) was observed between Primer/probe mix 1 with Y537C and Y537D on FAM, likely due to cross-reactivity with Y537S/Y537N (FAM). Very minor cross-reactivity (Ct > 25) was observed between Primer/probe mix 2 and L536R on FAM likely due to cross-reactivity with L536H (FAM). Stronger cross-reactivity was observed between Primer/probe mix 2 and L536Q (c.1607_1608delinsAG (TC>AG)) on FAM, likely due to cross reactivity with L536H (c.1607T>A) on FAM as both produce a T>A change on base 1607 meaning that the L536Q control contains the L536H mutation. However, the L536Q assay does not cross react with the L536H control due to the presence of a C>G change on base 1608. No cross-reactivity occurred for Primer/probe mix 3. Cross-reactivity may result in individual calling not being possible for mutations on the FAM channels in Primer/probe mix 1 and 2.

Table 11. Assay cross reactivity. Green – amplification of expected mutation; Yellow – minimal cross reactivity (Ct > 25); Red – cross reactivity present where individual calling is not possible.

ESR1 Mutation (Protein Change)	Primer / probe mix	E380Q (HEX)	D538G (FAM)	Y537S (FAM)	Y537N (FAM)	S463P (HEX)	Y537C (FAM)	L536H (FAM)	L536R (FAM)	P535H (FAM)	L536Q (FAM)	Y537D (FAM)
Control used @ 2000 copies per reaction		1				2			3			
E380Q												
D538G												
Y537S												
Y537N												
S463P												
Y537C												
L536H												
L536R												
P535H												
L536Q												
Y537D												

Appendix A: Sample processing

Sample quality is crucial for downstream processing; therefore, we recommend use of two independent centrifugation steps for plasma recovery. This ensures removal of any cellular debris which may contain genomic DNA.
The following steps should be followed when processing blood tubes to ensure that high purity cfDNA is recovered:

1) Centrifuge the blood sample at the Blood Collection Tube manufacturer's recommended speed and time.

2) Collect the separated plasma using a 1,000 µl pipette ensuring not to disturb the buffy coat.

3) Aliquot into 1.5–2 ml sterile 'PCR clean' microcentrifuge tubes.

4) Centrifuge the tubes at maximum speed (minimum 10,000 g) for 10 minutes in a microcentrifuge.

5) Taking care not to disturb the cell pellet at the bottom of the tube, retrieve the supernatant and aliquot it into fresh 'PCR Clean' microcentrifuge 1.5–2 ml tubes.

6) Plasma can be stored at -20°C for long term storage. The sample is now ready for cfDNA extraction.

In addition to cfDNA processing, GALEAS™ uPCR: ESR1 can be used with genomic DNA and is compatible with most genomic DNA (including FFPE tissue gDNA) extraction kits or systems which deliver clean nucleic acid isolation (PCR inhibitor free), but this should be validated prior to use.

Fluorometric quantification of extracted DNA must be performed to ensure correct dispensing of the recommended DNA input. To ensure maximum performance, it is recommended to quantify DNA samples using a fluorometric assay, such as Qubit. Use of spectrophotometric assays such as NanoDrop is not recommended for DNA quantification due to the inaccuracy in measurement of samples with low concentrations. A minimum of 1 ng and maximum of 25 ng gDNA can be used (≥5ng is recommended).

The protocol is suitable for gDNA samples with same maximum sample input of 25 ng total.

Appendix B: Alternative plate layouts

The main protocol recommendation is running single reactions per multiplex, however this can be increased to duplicate technical repeats. Alternative recommended plate layout for duplicate replicate sample reaction is shown in Table 12.

Table 12. Recommended plate layout for single reaction per sample per multiplex.
Blue – Mastermix 1, Yellow – Mastermix 2, Pink – Mastermix 3, S = Sample, PC1 = Positive control 1,
PC2 = Positive control 2, PC3 = Positive control 3, NC = Negative control, NTC = No Template control.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	S1	S1	S1	S9	S9	S9	S9	S9	S9
B	S2	S2	S2	S2	S2	S2	S10	S10	S10	S10	S10	S10
C	S3	S3	S3	S3	S3	S3	S11	S11	S11	S11	S11	S11
D	S4	S4	S4	S4	S4	S4	S12	S12	S12	S12	S12	S12
E	S5	S5	S5	S5	S5	S5	S13	S13	S13	S13	S13	S13
F	S6	S6	S6	S6	S6	S6	PC1	PC1	PC2	PC2	PC3	PC3
G	S7	S7	S7	S7	S7	S7	NC	NC	NC	NC	NC	NC
H	S8	S8	S8	S8	S8	S8	NTC	NTC	NTC	NTC	NTC	NTC

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