APIS
Assay Technologies Ltd.

APIS ESR1 dPCR Kit Handbook

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Handbook

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ESR1 dPCR Kit – APIS Assay Technologies Ltd

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APIS ESR1 dPCR Kit is a product for research use only. Not to be used in diagnostic procedures.

All information contained in this manual was correct at the time of printing. Nevertheless, APIS continuously improves its product and reserves its rights to change specifications, devices, and maintenance procedures at any time and without notification.

RUO

Contents

1. Intended Use

The APIS ESR1 dPCR Kit is a Research Use Only (RUO) digital polymerase chain reaction (dPCR) assay for the detection of *ESR1* mutations in deoxyribonucleic acid (DNA).

The APIS ESR1 dPCR Kit is a product used for basic laboratory research only. Not to be used in diagnostic procedures.

2. Product Description

The APIS ESR1 dPCR Kit enables the detection of 11 *ESR1* mutations in a multiplexed dPCR setup. The kit employs dPCR with dye-linked oligonucleotides (that is, probes labelled with a 5′ reporter dye and a downstream, 3′ dye-free quencher) to detect sequence amplification. During PCR, forward and reverse primers hybridise to the target DNA sequence for amplification, while the probe binds to the target sequence located between the primers. Both mutation-specific primers and probes are included in the kit. To selectively detect ESR1 mutations in high wild-type (WT) background, primer and probe (PP) mixes include blocking oligonucleotides specific to WT target sequence, to prevent elongation.

The kit is for use with dPCR, which involves the partitioning of a PCR reaction into numerous separate reactions, each subjected to thermocycling so that each reaction independently undergoes PCR amplification. After cycling, the PCR reactions are checked for fluorescence and reactions scored as "1" or "0" for amplification. The partitioning of the sample allows the estimation of the number of starting molecules following a Poisson distribution, which enables the possibility of multiple target molecules being in a single partition. Using Poisson's law, the number of target molecules within the sample can be accurately determined. By combining dye linked technology with dPCR, allelic frequences down to 0.1% Mutant Allele Frequency (MAF) can be accurately detected.

The APIS ESR1 dPCR Kit consists of four PP mixes, and Positive and Negative Controls (PC and NTC) which monitor the assay set up and reagent performance. A reference target (detecting WT *ESR1* sequence) is detected by each PP mix, which acts as an internal control and confirms adequate sample is added to the reaction.

The targets detected by the APIS ESR1 dPCR Kit are listed in Table 1.

The kit has been designed for use with any commercial dPCR master mix or platform, and is designed for use with DNA samples, for example, extracted cell-free DNA (cfDNA). It is recommended to extract samples using a cell-free-specific kit and to use the sample without dilution.

Table 1: Targets detected by the APIS ESR1 dPCR Kit. L536 mutations are not differentiated.

PP Mix	Target	Nucleic Acid Change	COSMIC ID
1	D538G	c.1613A>G	COSM94250
	S463P	c.1387T>C	COSM4771561
	Reference	N/A	N/A
2	Y537C	c.1610A>G	COSM1074637
	Y537S	c.1610A>C	COSM1074639
	L536X (L536P)	c.1607T>C	COSM6906109
	L536X (L536R)	c.1607T>G	COSM4774826
	Reference	N/A	N/A
3	L536X (L536H)	c.1607T>A	COSM6843697
	L536X (L536Q)	c.1607_1608delinsAG (TC>AG)	COSM4766050
	Y537N	c.1609T>A	COSM1074635
	Reference	N/A	N/A
4	E380Q	c.1138G>C	COSM3829320
	P535H	c.1604C>A	COSM4944018
	Reference	N/A	N/A

3. Material Provided

3.1 Kit Contents

APIS ESR1 dPCR Kit

Component	Colour	Volume
Primer Probe Mix 1	Clear	1x 252 µL
Primer Probe Mix 2	Clear	1x 252 µL
Primer Probe Mix 3	Clear	1x 252 µL
Primer Probe Mix 4	Clear	1x 252 µL
Positive Control (PC)	Black	1x 210 µL
No Template Control (NTC)	White	1x 210 µL

4. Materials Required but Not Provided

4.1 Reagents and Consumables

- Sterile pipette tips with filters
- Sterile 1.5 mL microcentrifuge tubes
- Digital PCR plates/seals compatible with a dPCR instrument
- Digital PCR master mix suitable for use with a dPCR instrument and dye-linked oligonucleotide chemistry (for example, QIAcuity® probe mix)

4.2 Equipment

- Digital PCR instrument (detection channels: FAM™, HEX™, ROX and Cy®5). Refer to the equipment user guide for compatibility.
- Adjustable volume pipettes
- Centrifuge (for spinning down microcentrifuge tubes)
- Vortex
- Cool block or ice (optional)

5. Reagent Storage and Handling

- If the kit is not frozen on arrival, the outer packaging is damaged or if any component of the kit is not present, please contact APIS Assay Technologies.
- Store kit immediately on receipt at –30°C to –15°C in a constant-temperature freezer and protected from light.
- **When stored under the recommended storage conditions in the original packaging, the kit is stable for 15 months from the date of manufacture.**
- Repeated thawing and freezing should be avoided. Do not exceed 9 freeze-thaw cycles.
- To ensure optimal activity and performance, primer probe mixes must be protected from light to avoid photo bleaching.
- Do not use expired or incorrectly stored components.

6. Warnings and Precautions

This product is for research use only. Not intended for medical purpose or objective.

7. General Precautions

- The test is for use with fragmented DNA, for example, extracted cfDNA or extracted FFPE.
- For use with non-fragmented DNA, dPCR template requires fragmentation to ensure separation across partitions. High molecular weight DNA should be sonicated or digested before use.
- Discard any samples or waste according to local safety procedures.
- Reagents in the APIS ESR1 dPCR Kit have been diluted optimally. Do not dilute reagents further.
- The kit has been developed using high partition numbers (QIAGEN QIAcuity® Nanoplate 26K (24-well)). High-sensitivity or high-partition plates are suggested to ensure detection of low mutant copy numbers.
- Take extreme care to prevent contamination of the kit components with the Positive Control reagent. Cap the tubes promptly after addition of each reagent.
- Take extreme care to prevent cross-contamination between samples. Cap the tubes promptly after addition of each sample.
- Thoroughly decontaminate work area before setting up.

8. Safety Information

- When working with chemicals, always wear suitable personal protective equipment (lab coat, disposable gloves, and protective eyewear). For more information, please consult the appropriate safety data sheets (SDS).

9. Procedure

9.1 Reaction Set Up

The APIS ESR1 dPCR Kit detects a total of 11 *ESR1* mutations across four reactions, using dPCR. We recommend including the controls provided with the kit (negative control and positive control) in every run.

Note: An appropriate instrument and compatible dPCR enzyme mix suitable for use with probe-based chemistry is required for use.

Template DNA and all reaction components should be thawed before use. It is important to mix the solutions completely before use to avoid localised differences in concentration.

Note: The PP mixes in the APIS ESR1 dPCR Kit are supplied at 4x concentration. Using an appropriate dPCR enzyme mix and DNA sample, prepare reaction mixes as per manufactures recommendations, ensuring the ESR1 dPCR is used at 1x concentration.

If using the QIAcuity® Digital PCR System (QIAGEN), follow the reaction volumes described in Table 2 (reaction volumes include overage for pipetting). For use with other dPCR systems, refer to the manufacturer's handbook for reaction mix set up instructions.

Table 2: Reaction set up for N=1 sample including pipetting overage using the QIAcuity Digital PCR System.

Master Mix ID	PP Mix ID	APIS ESR1 dPCR Kit PP mix (µL)	QIAcuity Probe PCR Kit (µL)	Sample/PC/ NTC (up to 11 µL)	Nuclease Free Water (µL)*	Total Master Mix volume (µL)
1	**Mix 1**	11	11	11	11	44
2	**Mix 2**	11	11	11	11	44
3	**Mix 3**	11	11	11	11	44
4	**Mix 4**	11	11	11	11	44

**Variable to account for sample volume - the reaction should be made up to a total volume of 44 µL (44 µL is required to provide pipetting overage).*

9.2 Plate Set Up

Below instructions refer to a set up using the QIAcuity Digital PCR System. For use with other dPCR systems, refer to the manufacturer's handbook for plate set up instructions.

- Pipette 40 µL of each master mix into each well. Reverse pipette to ensure no bubbles are loaded.
- To reduce the risk of cross contamination, it is recommended to position the negative and positive controls away from the DNA samples or on one side of the plate. An example of set-up in a 24-well dPCR plate is shown in Figure 1, and colour coded for each of the four mixes, 1–4.
- Seal the plate using a dPCR plate seal and sealing tool.
- Place the plate/tubes into the dPCR instrument following the manufacturer's instructions.

	1	2	3
A	Sample **1** Mix 1	Sample **2** Mix 1	Sample **3** Mix 1
B	Sample **1** Mix 2	Sample **2** Mix 2	Sample **3** Mix 2
C	Sample **1** Mix 3	Sample **2** Mix 3	Sample **3** Mix 3
D	Sample **1** Mix 4	Sample **2** Mix 4	Sample **3** Mix 4
E	Sample **4** Mix 1	PC Mix 1	NTC Mix 1
F	Sample **4** Mix 2	PC Mix 2	NTC Mix 2
G	Sample **4** Mix 3	PC Mix 3	NTC Mix 3
H	Sample **4** Mix 4	PC Mix 4	NTC Mix 4

Figure 1: Recommended Mix and Sample layouts for a 24 well dPCR plate. PC = positive control; NTC = no template control.

9.3 Run Method

Select appropriate run methods following manufacturer's instructions.

Cycling parameters recommended for use with the QIAcuity Digital PCR System and APIS ESR1 dPCR Kit are outlined in Table 3.

Table 3: Recommended cycling parameters for the APIS ESR1 dPCR Kit with the QIAcuity Digital PCR System.

Step	Step Number	Step Name	Temperature	Time	Cycles
Hold Stage	1	PCR Initial Heat Activation	95°C	2 min	1
PCR Stage	1	Denaturation	95°C	15 s	40
	2	Annealing Extension	60°C	30 s	

Recommended imaging parameters for the QIAcuity Digital PCR System are shown in Table 4. For other instrumentation users should verify exposure duration.

Table 4: Recommended imaging parameters for the APIS ESR1 dPCR Kit.

Channel	Emission (nm)	Target Detected	Exposure Duration (ms)	Gain
Green	518-548	D538G, Y537C, Y537N, E380Q	500	6
Yellow	550-564	S463P, L536P, L536R, L536H, L536Q, P535H	500	6
Orange	580-606	Y537S	400	6
Red	611-653	Reference	300	4

9.4 Threshold Setting

Users are recommended to empirically determine the suitability of thresholds for the dPCR instrument under use. The threshold should be set above any background WT positive partitions. Users are recommended to set the thresholds by testing samples positive and negative for selected mutations. For use with the QIAcuity Digital PCR System we recommend the thresholds provided in Table 5.

Table 5: Target data acquisition and analysis settings for the APIS ESR1 dPCR Kit.

Reaction Mix	Target	Dye	Emission (nm)	Fluorescence Intensity Threshold (QIAcuity Digital PCR System)
1	D538G	FAM	520	75
	S463P	HEX	555	100
	Reference	Cy5	670	50
2	Y537C	FAM	520	50
	Y537S	ROX	604	35
	L536X (L536P)	HEX	555	80
	L536X (L536R)	HEX	555	80
	Reference	Cy5	670	50
3	L536X (L536H)	HEX	555	115
	L536X (L536Q)	HEX	555	115
	Y537N	FAM	520	45
	Reference	Cy5	670	50
4	E380Q	FAM	520	60
	P535H	HEX	555	80
	Reference	Cy5	670	50

9.5 Run Analysis

Once the run is completed, export the results file. Refer to the selected dPCR platform user manual for analysis and export instructions.

9.5.1 Recommended Run Validity Criteria

NTC wells should be examined to detect potential contamination. The run is deemed valid when the results for the negative controls for all targets don't produce positive partitions. Tolerance to positive partitions should be determined for the instrument. For the QIAcuity Digital PCR System the allowable range is 0–2 positive partitions.

The APIS ESR1 dPCR Kit PC is a mixture of synthetic DNA consisting of all ESR1 and reference targets. The PC verifies the reaction integrity and may serve as a guide for data thresholding on dPCR instrumentation. For run validity on the QIAcuity Digital PCR System > 10 copies per target should be observed for the PC targets.

9.6 Results Interpretation

Positive partitions should be used to guide the mutation status (positive or negative). Samples in which positive partitions are greater than the instrument limit of detection (LoD) are positive for that target's mutation. If the positive partition number is less than the instrument LoD, the mutation status is negative. Users should refer to the dPCR instrument under use for the LoD threshold for that instrument. For the QIAcuity® Digital PCR System, there should be >3 partitions for positive calling.

If a positive result is reported for L536X, the ESR1 mutation present in the sample is either L536R, L536P, L536H or L536Q.

10. Kit Capabilities

10.1 Sensitivity

10.1.1 Limit of Blank

The limit of blank (LoB) was determined by testing mutation-negative samples contrived with WT DNA fragments.

Each sample was tested over two runs using two kit lots, one instrument and across multiple days, yielding a total of 12 replicates per target (for E380Q, n=6 samples were tested). For each target the overall rate of correct sample interpretation was 100% (
Table 6).

Table 6: Detection rates calculated for mutation-negative samples contrived with WT DNA in the limit of blank study. Relative fluorescence units (RFU) thresholds applied for this study are shown.

Mix	Target	Mutation Negative* (n=12) Detection Rate	Fluorescence Intensity (RFU) Threshold
1	D538G	0	75
	S463P	0	100
2	L536X (R&P)	0	80
	Y537S	0	35
	Y537C	0	50
3	Y537N	0	45
	L536X (H&Q)	0	115
4	E380Q•	0	60
	P535H	0	80

*Mutation-negative sample contrived with 10,000 and 20,000 WT DNA copies.
•n=6 of 10,000 WT DNA copies.

10.1.2 Limit of Detection

The limit of detection (LoD) was established for each target using samples contrived with WT and mutant DNA at varying mutant allele frequency (MAF), total copy numbers can be found in Table 7. A total of 6 replicates were generated per target, across one instrument and two kit lots. The LoD was defined as the highest mutant allele frequency with 100% correct calls. The calculated LoD for each target is summarised in Table 7.

Table 7: LoD for the APIS ESR1 dPCR Kit in % MAF and DNA copies. The mutant DNA copies at LoD and WT DNA copies at LoD describe the DNA copies of each fragment in the contrived samples. Relative fluorescence units (RFU) thresholds applied for this study are shown.

Mix	Target	LoD %MAF (n=6)	Mutant DNA Copies at LoD	WT DNA Copies at LoD	Fluorescence Intensity (RFU) Threshold
1	D538G	0.10%	20	19,980	75
	S463P	0.10%	10	9,990	100
2	L536X (R)	0.10%	10	9,990	80
	L536X (P)	0.10%	10	9,990	80
	Y537S	0.10%	10	9,990	35
	Y537C	0.10%	10	9,990	50
3	Y537N	0.10%	10	9,990	45
	L536X (H)	0.10%	10	9,990	115
	L536X (Q)	0.10%	10	9,990	115
4	E380Q	0.10%	10	9,990	60
	P535H	0.10%	10	9,990	80

10.2 Specificity and Cross-Reactivity

In silico analysis was performed using ThermoSleuth™ (DNA Software) to scan oligonucleotide sequences against large genomic databases. ThermoSleuth™ assessed all thermodynamically stable hits to determine any possible mishybridisation sites and potential off target amplicons. All oligonucleotides were scanned against sequence databases for all human DNA and RNA sequences. When screening the multiplex mixes against the sequence databases, there were no potentially problematic non-target amplicons.

In vitro specificity and cross-reactivity were determined by assessing DNA fragments specific to each mutation with all the PP mixes. The fragments were prepared at 1,000 DNA copies per reaction and assessed in triplicate. Cross-reactivity was only found between the mutations located within the L536 codon (Table 8), which are reported as L536X for any hits observed (targets labelled as L536X are not differentiated).

Table 8: Specificity and cross-reactivity observed between the mutations in the APIS ESR1 dPCR Kit. Dark green denotes specificity and light green denotes cross-reactivity.

	DNA fragment										
Target	E380Q	S463P	P535H	L536H	L536P	L536R	L536Q	Y537N	Y537C	Y537S	D538G
E380Q											
S463P											
P535H											
L536H											
L536P											
L536R											
L536Q											
Y537N											
Y537C											
Y537S											
D538G											

11. Troubleshooting

For information on troubleshooting, contact APIS Assay Technologies Technical Team via the website (https://www.apisassay.com/)

12. Limitations

Cross-reactivity occurs between mutations on the L536 codon. Four mutations within codon L536 can be detected (L536H, L536Q, L536R, L536P); however, individual calling is not possible.

13. Symbols

Symbol	Definition	Symbol	Definition
LOT	Batch code		Manufacturer
REF	Catalogue number	CONTROL –	Negative control
	Caution	CONTROL +	Positive control
	Consult instructions for use or consult electronic instructions for use	SN	Serial number
	Do not use if package is damaged and consult instructions for use		Temperature limit
	Keep away from sunlight		Use by date
RUO	Research Use Only		

14. Contact Information

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15. Ordering Information

Visit the APIS website at https://www.apisassay.com/
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