APIS

Assay Technologies Ltd.

APIS ESR1 Mutations Kit

Handbook

APIS ESR1 Mutations Kit Σ 24

Handbook

ART0067(04)
November 2023
www.apisassay.com/apis-esr1-mutations-kit

REF

Applicable to:
02201 (UK)
02202 (EU)

APIS Assay Technologies Ltd
Second floor, Citylabs 1.0, Nelson Street,
Manchester, M13 9NQ, UK

APIS Distribution:
APIS Assay Technologies Ltd
Second floor, Citylabs 1.0, Nelson Street, Manchester, M13 9NQ, UK.
+44 (0)161 9388179 – www.apisassay.com

Biocartis Distribution:
BIOCARTIS NV
Generaal De Wittelaan 11 B, 2800 Mechelen, Belgium
+32 15 632 888 - www.biocartis.com

APIS ESR1 Mutations 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 Mutations Kit is a Research Use Only (RUO) real time polymerase chain reaction (qPCR) assay for the detection of a ESR1 mutations in deoxyribonucleic acid (DNA).

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

2. Product description

The APIS ESR1 Mutations Kit employs qPCR with dye-linked oligonucleotides (i.e., 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 specific target sequence located between the primers. To selectively detect ESR1 mutations in high wild-type (WT) background, primer and probe (PP) mixes include blocking oligonucleotides, to prevent elongation, which are specific to the WT target sequence.

Two enzyme mixes are provided with the kit: one is to be combined with PP mixes 1–4 and the second is to be combined with PP mixes 5–6. Positive and Negative Controls (PC and NTC) which monitor for assay set up and reagent performance are also supplied with the kit. A reference control is included, which detects WT DNA to ensure adequate sample is added. All targets detected by the APIS ESR1 Mutations Kit are listed in Table 1.

The assay 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. 30 µL of sample is required to assess all mutations detected by the kit.

Table 1: Targets detected by the APIS ESR1 Mutations Kit (RUO)

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

3. Material Provided

3.1 Kit Contents

APIS ESR1 Mutations Kit
(24 samples in singlicate + controls)

Component	Colour	Volume
Primer Probe mix 1	Clear	1x 189 µL
Primer Probe mix 2	Clear	1x 189 µL
Primer Probe mix 3	Clear	1x 189 µL
Primer Probe mix 4	Clear	1x 189 µL
Primer Probe mix 5	Clear	1x 189 µL
Primer Probe mix 6	Clear	1x 189 µL
Enzyme Mix 1	Yellow	1x 1348 µL
Enzyme Mix 2	Purple	1x 706 µL
Positive Control (PC)	Black	1x 128 µL
No Template Control (NTC)	White	1x 128 µL

4. Materials Required but Not Provided

4.1 Consumables

- Sterile pipette tips with filters
- Sterile 1.5 mL microcentrifuge tubes
- PCR plates/seals or tubes compatible with a qPCR instrument

4.2 Equipment

- Real-Time PCR instrument (calibrated for FAM™ and HEX™ dyes). Refer to the equipment user guide for further information on instrument calibration.
- Adjustable volume pipettes
- Centrifuge (for spinning down plates and microcentrifuge tubes)
- Vortex
- Adhesive Film Applicator
- 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 4 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 DNA, for example, extracted cfDNA.
- Discard any samples or waste according to local safety procedures.
- Reagents in the APIS ESR1 Mutations Kit have been diluted optimally. Do not dilute reagents further.
- Enzyme Mix 1 is for use with PPmix 1 to 4. Enzyme Mix 2 is for use with PPmix 5 and 6. Enzyme Mixes 1 and 2 are not interchangeable due to differences in component concentration and should not be used incorrectly.
- 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.
- Do not remove the plate seal after the run has finished.

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 Plate Set Up & Cycling

The master mix contains all the components required for qPCR, except the template DNA. We recommend including the controls provided with the kit in every run (negative control and positive control). Up to 8 samples can be analysed simultaneously in one qPCR run.

Thaw template DNA and all kit components (thawing on ice or at 2–8°C is recommended). It is important to mix the solutions completely before use to avoid localised differences in concentration.

9.1.1 Master Mix Preparation

Prepare a volume of master mix for one technical replicate per DNA sample, PC and NTC. Prepare enough master mix for two additional replicates (n+2) per mix to allow sufficient overage volume for PCR setup.

⚠ Warning: Ensure the correct Enzyme Mix and Primer Probe Mix is combined to prepare each Master Mix. **Enzyme Mix 1 is for use with PPmix 1 to 4. Enzyme Mix 2 is for use with PPmix 5 and 6.**

For each of the Primer Probe mixes, prepare master mixes in 1.5 mL microcentrifuge tubes immediately before use as per Table 2, adjusting the volumes depending on the required number of reactions. Using a vortex/centrifuge, mix the master mixes for at least 10 seconds and centrifuge to collect the volume at the bottom of the tube.

Table 2: Master mix manufacture for N=1 sample.

Master Mix ID	Enzyme Mix tube	Volume of Enzyme Mix per reaction (µL)	Primer Probe Mix tube	Volume of Primer Probe Mix per reaction (µL)
Mix 1	1	10	**PPmix 1**	5
Mix 2	1	10	**PPmix 2**	5
Mix 3	1	10	**PPmix 3**	5
Mix 4	1	10	**PPmix 4**	5
Mix 5	2	10	**PPmix 5**	5
Mix 6	2	10	**PPmix 6**	5

9.1.2 Reaction Set up

- It is recommended to position the PCR plate/tubes on a cooling block.
- Pipette into each well/tube 15 µL of each corresponding Master Mix and 5 µL of DNA sample/Positive Control or Negative Control (Table 3). An example of set-up in a 96-well PCR plate is shown in Figure 1, colour coded for each of the six mixes, 1–6.
- 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.
- The final concentration of components per reaction are detailed in Table 3.

Table 3: Final concentration of components per reaction.

Component	Volume/reaction (µL)	Final concentration
Enzyme Mix	10.0	1x
Primer Probe mix	5.0	Variable
Master Mix Total	15	-
Sample (Template DNA/PC/NTC)	5.0	Variable
Total Reaction Volume	20.0	-

- Seal the plate using a PCR plate seal and sealing tool.
- Vortex to mix.
- Centrifuge for >1 minute to spin contents to bottom of the plate/tubes.
- Visually assess for bubbles, if any are present, flick the plate/tubes and centrifuge for additional 30 seconds. Repeat until no bubbles are present.
- Place the plate/tubes into the compatible Real-Time PCR instrument following the manufacturer's instructions.

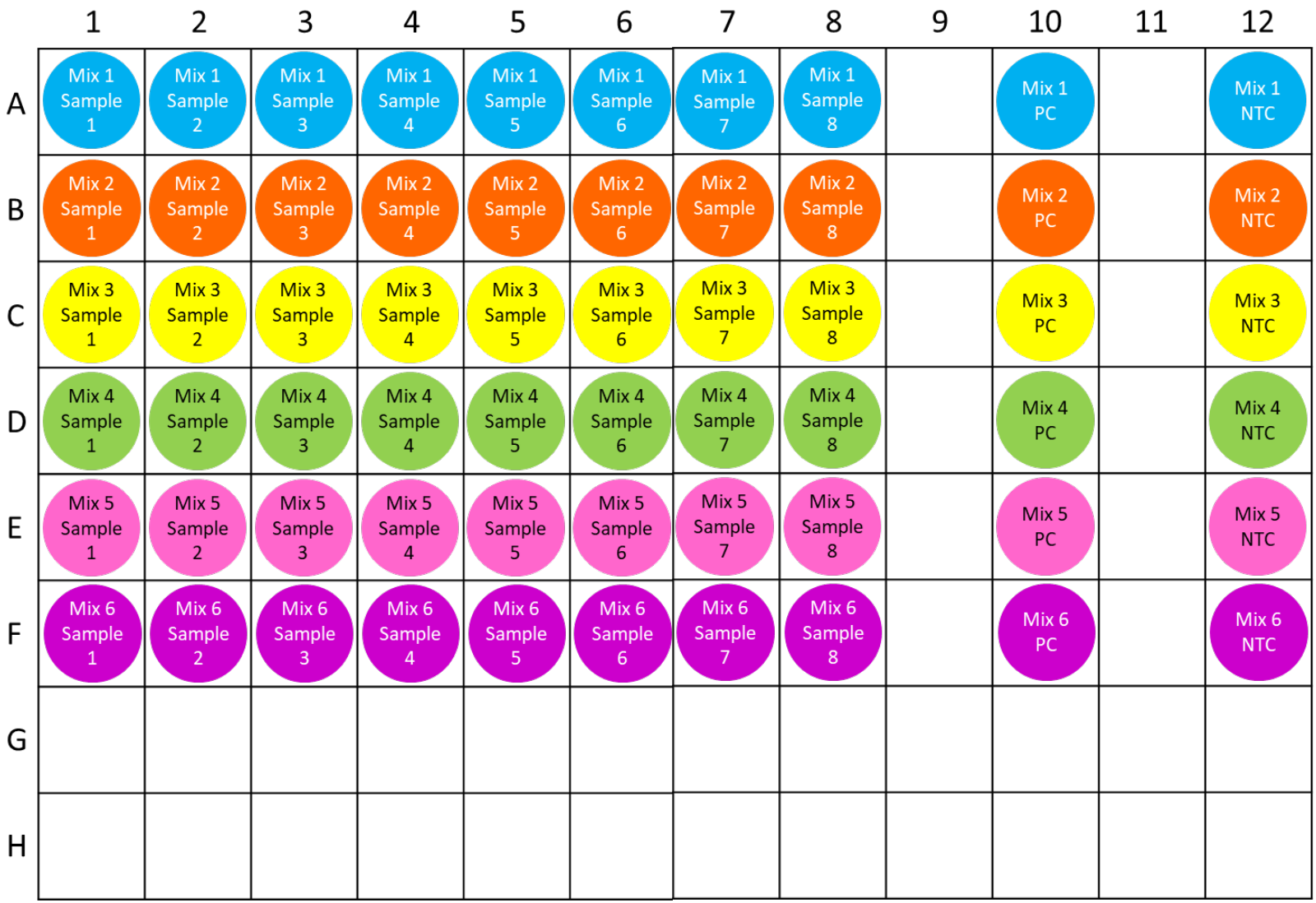

Figure 1: Recommended Mix and Sample Layouts.

9.2 Run Method

Note: prior to set up ensure the instrument is calibrated for the dyes required for this experiment.

Refer to the selected real-time PCR platform user manual to set up the PCR run. The recommended qPCR cycling parameters are outlined in Table 4.

Note: a passive reference dye is not included in the kit – passive reference dye normalisation should not be selected.

Table 4: Recommended cycling parameters for the APIS ESR1 Mutations Kit.

Step	Step Number	Step Name	Temp	Time	Ramp Rate	Cycles
Hold Stage	1	Initial Activation	95°C	10 min	3.29°C/s	1
PCR Stage	1	Denaturation	94°C	10 s	2.53°C/s	40
	2	Annealing Extension Data Acquisition	60°C	30 s	2.53°C/s	

9.3 Threshold Setting

Users are recommended to empirically determine the suitability of thresholds for the qPCR instrument under use. The threshold should be set at the level of detection, or the point at which a reaction reaches a fluorescent intensity above background levels (in this instance WT amplification). Users are recommended to set the thresholds by testing samples positive and negative for selected mutations. For use with the Applied Biosystems™ QuantStudio™ 5 (QS5™) Dx instrument we recommend thresholds provided in Table 5.

Table 5: Target Data Acquisition and Analysis Settings for the APIS ESR1 Mutations Kit.

Mix	Target	Dye	Quencher	ΔRn Estimated Threshold (QS5 Dx)*
Mix 1	D538G	FAM™	BHQ-1®	50,000
	S463P	HEX™	BHQ-1®	36,000
Mix 2	Y537S	FAM™	BHQ-1®	15,000
Mix 3	Y537C	FAM™	BHQ-1®	26,500
Mix 4	Y537N	FAM™	BHQ-1®	17,000
	L536X.1	HEX™	BHQ-1®	28,500
Mix 5	E380Q	FAM™	BHQ-1®	20,000
	P535H	HEX™	BHQ-1®	6,500
Mix 6	L536X.2	FAM™	BHQ-1®	21,500
	Reference	HEX™	BHQ-1®	Auto

*Thresholds to be used only with the QS5 Dx instrument.

9.4 Run Analysis

Once the run is completed, export the Ct (depending on instrument these may also be referred to as Cq or Cp) values. Refer to the selected real-time PCR platform user manual for analysis and export instructions.

9.4.1 Recommended Run Validity Criteria

The run is deemed valid when the results for the negative control for all targets produce no amplification and each target amplifies within the positive control. The recommended run validity criteria for use with the QS5 Dx instrument are outlined in Table 6.

Table 6: Recommended Run Validity Criteria for the APIS ESR1 Mutations Kit.

Mix	Target	Detection Channel (max emission nm)	Acceptable Negative Control Ct Range	Acceptable Positive Control Ct Range
Mix 1	D538G	6-FAM (520)	Undetermined	≥26.5 – ≤30.4
	S463P	HEX (555)	Undetermined	≥29.3 – ≤33.0
Mix 2	Y537S	6-FAM (520)	Undetermined	≥19.0 – ≤33.2
Mix 3	Y537C	6-FAM (520)	Undetermined	≥25.6 – ≤31.9
Mix 4	Y537N	6-FAM (520)	Undetermined	≥26.0 – ≤29.0
	L536X.1	HEX (555)	Undetermined	≥29.6 – ≤32.6
Mix 5	E380Q	6-FAM (520)	Undetermined	≥27.0 – ≤31.2
	P535H	HEX (555)	Undetermined	≥27.3 – ≤30.3
Mix 6	L536X.2	6-FAM (520)	Undetermined	≥28.6 – ≤31.6
	Reference	HEX (555)	Undetermined	≥24.7 – ≤33.8

*Positive control acceptable ranges for use with the QS5 Dx instrument. Users of other instruments are recommended to adjust the specifications as required.

9.4.2 Recommended Sample Validity Criteria

Recommended sample validity criteria for use with the QS5 Dx instrument are provided. Their suitability for other PCR instruments should be determined by the user as required.

It is recommended that for each sample, the Ct value of the reference target should be less than or equal to a Ct of 34.5.

If the reference target is out of specification, the sample is invalid and should be repeated. A repeat extraction with a higher volume of plasma is recommended to increase sample input.

9.5 Results Interpretation

Ct values should be used to guide the mutation status (positive or negative). Samples in which a Ct value is reported, are positive for that target's mutation. If no Ct is reported, the mutation status is negative.

If a positive Ct value is reported for L536X.1 or L536X.2, the ESR1 mutation present in the sample is either L536R, L536P, L536H or L536Q.

10. Performance Characteristics

10.1 Analytical Sensitivity

10.1.1 Limit of Blank

The limit of blank (LoB) was determined by testing negative cfDNA samples derived from cell-line and healthy human plasma, and blank samples contrived with WT DNA fragments. Each sample was tested in two replicates, using two kit lots and one instrument, across multiple days, yielding a total of 144 replicates per target. The study design was based on the CLSI guideline EP17-A2. For each target the overall rate of correct sample interpretation was ≥98%. Results are summarised in Table 7.

Table 7: Detection rates calculated for cell line cfDNA, human plasma cfDNA and blank samples contrived with WT DNA in the limit of blank study. ΔRn thresholds applied for this study are shown.

Target	Cell Line cfDNA (n=48)	Human Plasma cfDNA (n=72)	Blank* (n=24)	ΔRn Threshold
	Detection Rate	Detection Rate	Detection Rate	
D538G	0/48	0/72	0/24	50,000
S463P	0/48	0/72	0/24	36,000
Y537S	1/48	0/72	0/24	15,000
Y537C	0/48	0/72	0/24	26,500
Y537N	0/48	0/72	0/24	17,000
L536X.1	0/48	0/72	0/24	28,500
E380Q	0/48	2/72	0/24	20,000
P535H	0/48	1/72	0/24	6,500
L536X.2	0/48	1/72	0/24	21,500

*Blank sample contrived with 5,000 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), with a total DNA copy number of 5,000 per reaction. A total of 24 replicates were generated per target, across two instruments and two kit lots. The study design was based on the CLSI guideline EP17-A2. The LoD was defined as the highest mutant allele frequency with ≥95% correct calls. The calculated LoD for each target is summarised in Table 8.

Table 8: LoD for the APIS ESR1 Mutations 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 LoD samples. ΔRn thresholds applied for this study are shown.

Target	LoD (%MAF)	Mutant DNA copies at LoD	WT DNA copies at LoD	ΔRn Threshold
D538G	0.40%	20	4980	50,000
S463P	0.08%	4	4996	36,000
Y537S	0.10%	5	4995	15,000
Y537C	0.40%	20	4980	26,500
Y537N	0.20%	10	4990	17,000
L536X.1 (H)	0.80%	40	4960	28,500
L536X.1 (Q)	0.80%	40	4960	28,500
E380Q	1.00%	50	4950	20,000
P535H	0.40%	20	4980	6,500
L536X.2 (R)	0.70%	35	4965	21,500
L536X.2 (P)	0.90%	45	4955	21,500

10.2 Measuring Range

10.2.1 Linear/Dynamic Range

Linearity was determined by testing a dilution series of DNA fragments specific to each mutation. Each dilution series had eight levels with three replicates assessed at each level to determine linearity across the target range from 5 to 10,000 DNA copies. Dynamic range, reaction efficiency and R^2 values for each target are reported in Table 9.

Table 9: Linear/dynamic range for each target in the APIS ESR1 Mutations Kit

Target	Slope	Efficiency (%)	R^2	Dynamic Range (DNA copies)
D538G	-3.43	95.64	1.00	5–10,000
S436P	-3.26	102.49	0.99	5–10,000
Y537S	-3.64	88.22	0.99	5–10,000
Y537C	-3.33	99.67	1.00	5–10,000
Y537N	-3.34	99.10	0.99	5–10,000
L536X.1 (H)	-3.41	96.48	0.99	5–10,000
L536X.1 (Q)	-3.41	96.61	1.00	5–10,000
E380Q	-3.32	99.93	0.99	5–10,000
P535H	-3.35	98.69	1.00	5–10,000
L536X.2 (P)	-3.39	97.43	0.99	5–10,000
L536X.2 (R)	-3.35	98.92	1.00	5–10,000
Reference	-3.33	99.61	0.98	5–10,000

10.3 Analytical Specificity and Cross-reactivity

In silico analysis was performed using ThermoSleuth™ (DNA Software) to scan oligonucleotide sequences against large genome 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 and assessed in triplicate. Cross-reactivity was only found between the mutations located within L536 codon (Table 10), which are reported as L536X for any hits observed (targets labelled as L536X.1 and L536X.2 not differentiated).

Table 10: Specificity and cross-reactivity observed between the mutations in the APIS ESR1 Mutations Kit. Green denotes specificity and red 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
	Batch code		Manufacturer
	Catalogue number		Negative control
	Caution		Positive control
	Consult instructions for use or consult electronic instructions for use		Serial number
	Contains sufficient for <24> tests		Temperature limit
	Do not use if package is damaged and consult instructions for use		Use by date
	Keep away from sunlight		Research Use Only

14. Contact Information

APIS Assay Technologies Ltd.
Second Floor Citylabs 1.0, Nelson Street
Manchester, M13 9NQ, UK
+44 (0)161 9388179
technicalsupport@apisassay.com

This document was last revised:

22 NOV 2023.

15. Ordering Information

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