

APIS PIK3CA qPCR Kit Handbook

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Handbook



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Applicable

to:

03101

03102



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 PIK3CA qPCR 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.

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1. Intended Use

The APIS PIK3CA qPCR Kit is a Research Use Only (RUO) real time polymerase chain reaction (qPCR) assay for the detection of *PIK3CA* mutations in deoxyribonucleic acid (DNA).

The APIS PIK3CA qPCR Kit is a product used for basic laboratory research only. Not to be used in diagnostic procedures.

2. Product description

The APIS PIK3CA qPCR 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 PIK3CA 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.

An enzyme mix is provided with the kit. 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 PIK3CA qPCR 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. 10 µL of sample is required to assess all mutations detected by the kit.

Table 1: Targets detected by the APIS PIK3CA qPCR Kit.

Reaction Mix	Mutation	Nucleic acid change	COSMIC ID
1	H1047L	3140 A>T	COSV55873401
	H1047R	3140 A>G	COSV55873195
	Reference	N/A	N/A
2	C420R	1258 T>C	COSV55874020
	E542K	1624 G>A	COSV55873227
	E545K	1633 G>A	COSV55873239

3. Material Provided

3.1 Kit Contents

APIS PIK3CA qPCR Kit (24 samples in singlicate + controls)

Component	Colour	Volume
Primer Probe mix 1	Clear	1x 252 μ L
Primer Probe mix 2	Clear	1x 252 μ L
Enzyme Mix	Yellow	2x 514 μ L
Positive Control (PC)	Black	1x 126 μ L
No Template Control (NTC)	White	1x 126 μ L

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™, HEX™ and Cy® 5 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 10 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 PIK3CA qPCR Kit have been diluted optimally. Do not dilute reagents further.
- 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.
- Repeated thawing and freezing should be avoided.
- 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 24 samples can be analysed simultaneously in one qPCR run.

Thaw template DNA and all kit components. It is important to mix the solutions completely before use to avoid localised differences in concentration.

9.1.1 Master Mix Preparation

Prepare enough volume of master mix for one technical replicate per DNA sample, one PC, one NTC, and one additional replicate (n+1) per mix to allow sufficient overage volume for PCR setup.

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 one reaction.

Master Mix ID	Volume of Enzyme Mix per reaction (μL)	Primer Probe Mix tube	Volume of Primer Probe Mix per reaction (μL)
Mix 1	10	PPmix 1	5
Mix 2	10	PPmix 2	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.
- 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	1x
Master Mix Total	15.0	-
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.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Mix 1 Sample 1	Mix 1 Sample 2	Mix 1 Sample 3	Mix 1 Sample 4	Mix 1 Sample 5	Mix 1 Sample 6	Mix 1 Sample 7	Mix 1 Sample 8		Mix 1 PC		Mix 1 NTC
B	Mix 2 Sample 1	Mix 2 Sample 2	Mix 2 Sample 3	Mix 2 Sample 4	Mix 2 Sample 5	Mix 2 Sample 6	Mix 2 Sample 7	Mix 2 Sample 8		Mix 2 PC		Mix 2 NTC
C												
D	Mix 1 Sample 9	Mix 1 Sample 10	Mix 1 Sample 11	Mix 1 Sample 12	Mix 1 Sample 13	Mix 1 Sample 14	Mix 1 Sample 15	Mix 1 Sample 16				
E	Mix 2 Sample 9	Mix 2 Sample 10	Mix 2 Sample 11	Mix 2 Sample 12	Mix 2 Sample 13	Mix 2 Sample 14	Mix 2 Sample 15	Mix 2 Sample 16				
F												
G	Mix 1 Sample 17	Mix 1 Sample 18	Mix 1 Sample 19	Mix 1 Sample 20	Mix 1 Sample 21	Mix 1 Sample 22	Mix 1 Sample 23	Mix 1 Sample 24				
H	Mix 2 Sample 17	Mix 2 Sample 18	Mix 2 Sample 19	Mix 2 Sample 20	Mix 2 Sample 21	Mix 2 Sample 22	Mix 2 Sample 23	Mix 2 Sample 24				

Figure 1: Suggested 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 PIK3CA qPCR 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	45
	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 PIK3CA qPCR Kit.

Mix	Target	Dye	Quencher	ΔRn Estimated Threshold (QS5 Dx)*
Mix 1	H1047L	HEX™	BHQ-1®	75,000
	H1047R	FAM™	BHQ-1®	100,000
	Reference	Cy®5	BHQ-2®	Auto
Mix 2	C420R	HEX™	BHQ-1®	12,000
	E542K	Cy®5	BHQ-2®	52,000
	E545K	FAM™	BHQ-1®	44,000

*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 PIK3CA qPCR Kit.

Mix	Target	Detection Channel (max emission nm)	Acceptable Negative Control Ct Range	Acceptable Positive Control Ct Range
Mix 1	H1047L	HEX (555)	Undetermined	$\geq 28.06 - \leq 33.06$
	H1047R	FAM (520)	Undetermined	$\geq 26.61 - \leq 31.61$
	Reference	Cy5 (668)	Undetermined	$\geq 24.26 - \leq 29.26$
Mix 2	C420R	HEX (555)	Undetermined	$\geq 26.35 - \leq 33.09$
	E542K	Cy5 (668)	Undetermined	$\geq 28.32 - \leq 34.10$
	E545K	FAM (520)	Undetermined	$\geq 28.30 - \leq 33.72$

*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.

10. Kit Capabilities

10.1 Analytical Sensitivity

10.1.1 Limit of Blank

The limit of blank (LoB) was determined by testing mutation-negative cfDNA samples derived from cell-line, mutation-negative human plasma, and contrived samples consisting of WT DNA fragments.

Each sample was tested using two kit lots and one QS5 Dx instrument, across multiple days, yielding from 96 to 108 replicates per target. Delta Rn thresholds as per the QS5 Dx were applied. The study design was based on the CLSI guideline EP17-A2. For each target the overall rate of correct sample interpretation was $\geq 99\%$. 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. Blank samples were contrived with WT DNA copies.

Target	Cell Line cfDNA (n=12)	Human Plasma cfDNA (n=12)	WT DNA fragments (blank) (n=72 or 84)
	Detection Rate	Detection Rate	Detection Rate
H1047L	0/12	0/12	0/72
H1047R	0/12	0/12	0/72
C420R	0/12	0/12	0/84
E542K	0/12	0/12	1/84
E545K	0/12	0/12	0/84

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 QS5 Dx instruments and two kit lots. Delta Rn thresholds as per the QS5 Dx were applied. The study design was based on the CLSI guideline EP17-A2. The LoD was defined as the lowest mutant allele frequency with $\geq 95\%$ correct calls. The calculated LoD for each target is summarised in Table 8.

Table 8: LoD for the APIS PIK3CA qPCR 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.

Target	LoD (%MAF)	Mutant DNA copies at LoD	WT DNA copies at LoD
H1047L	0.50%	25	4975
H1047R	0.50%	25	4975
C420R	0.30%	15	4985
E542K	0.36%	18	4982
E545K	0.50%	25	4975

10.2 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. No cross reactivity was noted between the mutations within the APIS PIK3CA qPCR kit (Table 9).
















Table 9: Specificity and cross-reactivity observed between the mutations in the APIS PIK3CA qPCR Kit. Green denotes specificity.

Target	DNA fragment				
	H1047L	H1047R	C420R	E542K	E545K
H1047L					
H1047R					
C420R					
E542K					
E545K					

11. Troubleshooting

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

12. 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
	Distributor		

13. Contact Information



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

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14. Ordering Information

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