APIS
Assay Technologies Ltd.

APIS MET Alterations Kit

Handbook

APIS MET Alterations Kit Σ 24

Handbook

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APIS MET Alterations 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 MET Alterations Kit is a Research Use Only (RUO) real time reverse transcription polymerase chain reaction (RT-qPCR) assay for the detection of the MET exon 14 deletion mutation (METΔ14) and the cMET wildtype sequence from ribonucleic acid (RNA).

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

2. Product description

The APIS MET Alterations Kit uses one step RT-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. PCR uses forward and reverse primers to hybridise to a specific RNA sequence to firstly reverse transcribe it into cDNA and then to amplify it. To selectively amplify the mRNA sequence, at least one of the oligonucleotides used to generate the amplicon is spanning an exon-exon junction. The probe binds to the target sequence between the primers.

The assay is designed to detect the relative expression of the wild type cMET transcript, and the RNA product of mutations within the splice junctions that lead to exon-skipping (deletion) of exon 14 (Figure 1).

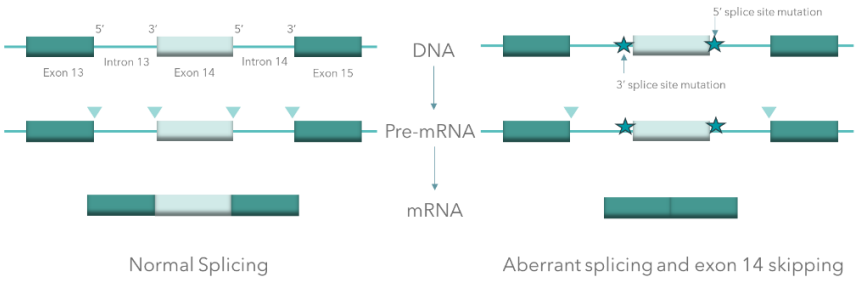

Figure 1. Schematic of normal and aberrant exon 14 splicing as a result of splice site mutations

The APIS MET Alterations Kit contains two reference genes which allow for normalisation of assay input and monitor for assay set up. Positive and Negative Controls (PC and NTC) which monitor for assay set up and reagent performance are also supplied with the kit. Targets detected by the APIS MET Alterations Kit are listed in Table 1.

The assay is designed for use with RNA samples, for example, extracted from FFPE or cell-free RNA (cfRNA).

Table 1. Targets detected by the APIS MET Alterations Kit

Reaction Mix	Gene Symbol	Full name
1	cMET	MET Proto-Oncogene, Receptor Tyrosine Kinase (Gene ID:4233)
	IPO8	Importin 8 (Gene ID: 10526)
2	METΔ14	MET exon 14 deletion splice mutation product
	UBXN4	UBX Domain Protein 4 (Gene ID: 14860)

3. Material Provided

Kit Contents

APIS MET Alterations Kit
(24 samples in duplicate + controls)

Component	Colour	Volume
Enzyme Mix	White	2x 325 µL
Buffer Mix	Purple	1x 647 µL
Primer Probe Mix 1	Red	1x 129 µL
Primer Probe Mix 2	Yellow	1x 129 µL
Positive Control (PC)	Black	1x 148 µL
Water for No Template Control (NTC)	Clear	1x 148 µL

4. Materials Required but Not Provided

Reagents

- RNA isolation kit suitable for the sample type. APIS recommends the use of an RNA isolation kit that includes a DNase step.
- RNA Quantification Reagents (If using a fluorometric quantification method that uses nucleic acid binding dyes).
- Nuclease free water for sample dilutions.

Consumables

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

Equipment

- Real-Time PCR (qPCR) 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

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 upon receipt at –30°C to –15°C in a constant-temperature freezer and protected from light.
- Prior to use, the reagents should be kept cool at 4°C until fully thawed.
- Repeated thawing and freezing should be avoided.
- 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

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

7. General Precautions

- The test is intended for use with RNA.
- Do not assay samples containing more than 3.75 ng/µL of DNA.
- All chemicals and biological materials are potentially hazardous. Specimens and nucleic acids are unlikely to cause any infection hazard, but general Health and Safety procedures should be followed.
- Discard any specimens or waste according to local safety procedures.
- Reagents within the APIS MET Alterations 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.
- Do not exceed 5 freeze-thaw cycles for any single tube of enzyme mix.
- Do not exceed 9 freeze-thaw cycles for the PPmix, Buffer mix or PC components.
- 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 RNA Isolation, Quantification & Normalisation

APIS recommends that the RNA extraction method used includes a DNase step, either as part of the RNA isolation procedure or as a separate step pre-PCR set up.

RNA should be quantified using an appropriate quantification method (e.g., Fluorometer or UV spectrometer).

The RNA input range for the assay is between 0.1-30 ng total RNA. It is recommended to use a total RNA input of 10ng in a volume of 8 µL. A single aliquot of 20 µL at 1.25 ng/µL is sufficient to run the assay. RNA can be diluted and stored at -80°C before use.

For cell free RNA it is recommended to extract samples using a cell-free-specific kit and to use the sample without dilution.

9.2 Plate Set Up & Cycling

The master mix contains all the components required for RT-qPCR except the template RNA. A negative control (without template RNA) and positive control should be included on every run. The kit provides sufficient reagents to run up to 8 plates of 3 samples with the appropriate plate controls. Up to 24 samples can be analysed simultaneously on one RT-qPCR run.

RNA and all kit components should be thawed on ice or at 2°C to 8°C. It is important to mix the solutions completely before use to avoid localised differences in concentration. RNA is known to be fragile and vortexing should be avoided. Mix RNA by gently pipetting up and down three times or flicking the tube multiple times.

9.2.1 Master Mix Preparation

Perform master mix preparation on ice or at 2°C–8°C. Prepare a volume of each 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 each tube of enzyme mix is not thawed more than 5 times.

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	Volume of Enzyme Mix per reaction (µL)	Volume of Buffer Mix per reaction (µL)	Primer Probe mix tube	Volume of Primer Probe mix per reaction (µL)
Mix 1	5	5	**PPmix 1**	2
Mix 2	5	5	**PPmix 2**	2

9.2.2 Plate Set up

- Position a PCR plate on a cool block or ice.
- Following the plate layout in Figure 2 pipette into each well 12 µL of each corresponding Master Mix and 8 µL of RNA/Positive Control or Negative Control.
- To reduce a risk of cross contamination, it is recommended to position the negative and positive controls away from the RNA 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	5.0	1x
Buffer Mix	5.0	1x
Primer Probe mix	2.0	Variable
Master Mix Total	12	-
Sample (Template RNA/PC/NTC)	8.0	Sample 0.1-30ng
Total Reaction Volume	20.0	-

- Seal the plate using a PCR plate seal and sealing tool or cap tubes.
- Vortex to mix.
- Centrifuge for 1 minute to spin contents to bottom of the plate.
- Visually assess for bubbles, if any are present, flick the plate and centrifuge for additional 30 seconds. Repeat until no bubbles are present.
- Place the plate/tubes into the compatible qPCR 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 2. Suggested Master Mix and Sample Layout, rows A, D and G contain Master Mix 1, rows B, E and H Master Mix 2

9.3 Run Method

Note: prior to set up ensure the instrument is calibrated for the dyes required for this experiment (FAM™ and HEX™).

Refer to the selected qPCR 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 MET Alterations Kit.

Step	Step Number	Step Name	Temp	Time	Ramp Rate	Cycles
Hold Stage	1	Reverse Transcription	50°C	10 mins	3.29°C/s	1
	2	Initial Activation	95°C	30 secs	3.29°C/s	1
PCR Stage	1	Denaturation	94°C	10 secs	2.53°C/s	40
	2	Annealing Extension Data Acquisition	60°C	30 secs	2.53°C/s	

9.4 Threshold Setting

Users are recommended to empirically determine the suitability of thresholds for the qPCR instrument used. The threshold should be set at the level of detection, or the point at which a reaction reaches a fluorescent intensity above background levels. For use with the Applied Biosystems™ QuantStudio™ 5 Dx (QS5™ Dx) instrument we recommend thresholds provided in Table 5.

*Table 5. Target Data Acquisition and Analysis Settings (*Recommended for use with the QuantStudio™ 5 DX)*

Mix	Target	Dye	Quencher	ΔRn Threshold	Baseline
MIX1	cMET	FAM™	None	100,000	Auto
	UBXN4	HEX™	None	10,000	Auto
MIX2	METΔ14	FAM™	None	30,000	Auto
	IPO8	HEX™	None	40,000	Auto

For further information of threshold setting on different qPCR platforms refer to Section 13: Appendix A: Threshold Setting.

9.5 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 qPCR platform user manual for analysis and export instructions.

9.5.1 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 acceptance criteria. The recommended run validity criteria for use with the QS5™ Dx instrument are outlined in Table 6. The suitability of the PC acceptance criteria on alternative qPCR platforms should be assessed by the user.

Table 6. Recommended Run Validity Criteria (QS5™Dx

Mix	Target	Acceptable Negative Control Ct Range	Acceptable Positive Control Ct Range
MIX1	cMET	Undetermined	≥26.419-≤31.419
	UBXN4	Undetermined	≥25.108-≤30.108
MIX2	METΔ14	Undetermined	≥25.988-≤30.988
	IPO8	Undetermined	≥26.926-≤31.926

9.5.2 Sample Validity Criteria

Recommended sample validity criteria for use with the QS5™ Dx instrument are provided below. Their suitability on alternative qPCR platforms should be determined by the user, as required.

It is recommended that for each sample, the Ct value of the reference targets (IPO8 and UBXN4) should be less than or equal to a Ct of 38. If the reference target is out of specification, the sample is invalid and should be repeated. A repeat PCR with a higher input of RNA is recommended.

9.6 Results Interpretation

Recommended target cut-off values for use with the QS5™ Dx instrument are provided below, their suitability on alternative qPCR platforms should be determined by the user as required.

9.6.1 METΔ14

Ct values should be used to guide the presence of the METΔ14 transcript (positive or negative for mutations resulting in the splice variant).

Table 7. Recommended Ct cut -off values for METΔ14 when using QS5™ Dx instrument

METΔ14 Status	Ct Value
METΔ14 variant <u>detected</u> (mutation positive)	≤37.15
METΔ14 variant <u>undetected</u> (mutation negative)	>37.15

Figure 2 illustrates METΔ14 Ct values from a range of positive and negative samples.

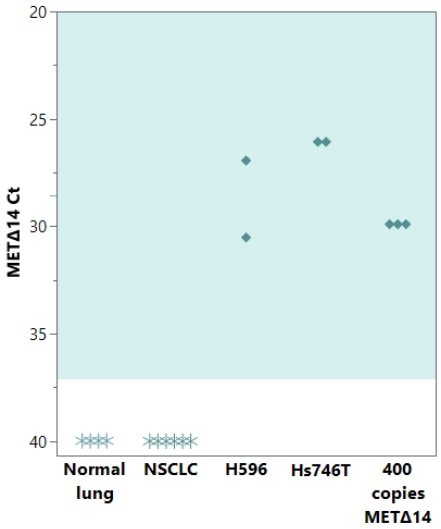

Figure 2. Ct values generated by testing samples positive and negative for METΔ14. Positive samples: RNA derived from cell lines H596 and Hs746T, RNA spiked with synthetic RNA encoding the METΔ14 sequence (400 copies METΔ14 sample). Negative samples: RNA derived from normal human lung and NSCLC specimens. Positive samples are indicated by closed

diamonds. Ct values above which the METΔ14 status is positive are highlighted in blue. Results generated using QS5™ Dx instrument.

9.6.2 cMET

Target relative expression can be reported as Delta Ct (ΔCt) values. The ΔCt for cMET should be calculated by taking the mean Ct value of both reference genes (IPO8 and UBXN4) and subtracting the Ct of cMET, as shown in Figure 3.

$$\Delta Ct = \frac{Ct\ IPO8 + UBXN4}{2} - \text{Ct cMET}$$

Figure 3. ΔCt calculation performed for cMET.

ΔCt values should be used to guide the MET expression status (positive/high or negative/low) (Figure 4). The user should define a ΔCt cut-off for the assay by assessing known positive and negative samples.

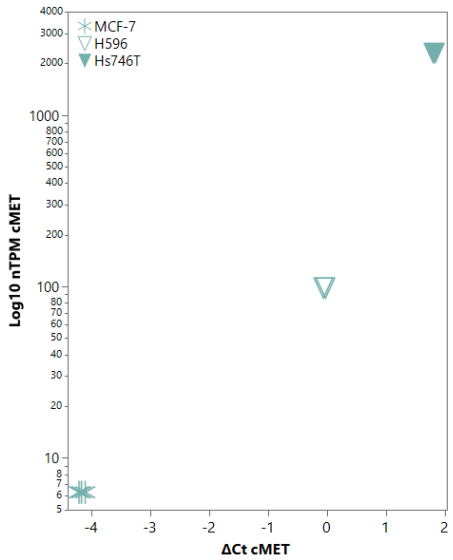

Figure 4. cMET ΔCt values from 3 MET characterised cell lines, MCF-7, H596 and Hs746T plotted against Log10 nTPM (normalised transcripts per million). Hs746T harbours both amplified cMET and the METΔ14 mutation, while HS596 and MCF-7 are negative for MET overexpression. Results generated using QS5™ Dx instrument.

10. Analytical Characteristics

10.1 Analytical Sensitivity

10.1.1 Limit of Blank

The limit of blank (LoB) was determined by testing negative RNA samples derived from cell-line, and total human lung RNA.

For each target the overall rate of correct sample interpretation was ≥98%. Results are summarised in Table 8.

Table 8. Detection rates and LoB calculated for cell line and normal lung RNA.

Target	Cell Line RNA	Normal Lung RNA	ΔCt/Ct LoB
	Detection Rate	Detection Rate	
cMET ΔCt	0/5	0/31	-0.5
METΔ14 Ct	0/3	0/20	38.5 Ct

10.1.2 Limit of Detection

The Limit of Detection (LoD) was estimated for the METΔ14 using copies of a synthetic RNA encoding METΔ14, diluted in a background of 10ng normal total lung RNA (containing approximately 2134 copies of the cMET transcript). The LoD was estimated as the lowest number of METΔ14 copies with a 100% hit rate (8/8 reactions). The LoD for METΔ14 was calculated to be 25 copies, the data is summarised in Table 9.

Table 9. LoD estimation for the METΔ14 target. The estimated minor allele frequency (%MAF) for the deletion for each copy number is indicated. LoD of 25 copies shown in bold

Copies of METΔ14	%MAF	WT DNA copies at LoD	Mean Ct	Hit rate
80	3.75	2134	33.97	8/8
69	3.23	2134	34.90	8/8
58	2.72	2134	35.07	8/8
47	2.20	2134	35.51	8/8
36	1.69	2134	35.64	8/8
25	**1.17**	**2134**	**37.15**	**8/8**
14	0.66	2134	38.26	3/8
3	0.14	2134	40.00	0/8

10.2 Measuring Range

10.2.1 Linear/Dynamic Range

For cMET, IPO8 and UBXN4, linearity was determined by testing a dilution series of normal human lung RNA. To determine linearity for the METΔ14, known copies of a synthetic RNA were diluted in 10ng of normal human lung RNA. Each dilution series had 12 levels with three replicates assessed at each level to determine linearity across a range of 38.28 to 0.04 ng RNA and 2750 to 13 METΔ14 copies. Dynamic range, reaction efficiency and R^2 values for each target are reported in Table 10.

Table 10. Linear/dynamic range for each target in the APIS MET Alterations Kit

Target	Slope	Efficiency (%)	R^2	Dynamic Range (RNA or copies)
IPO8	-3.33	99.66	0.998	38ng-0.3 ng
UBXN4	-3.26	102.65	0.992	38ng-0.15 ng
cMET	-3.43	95.67	0.991	38ng-0.15 ng
METΔ14	-3.69	87.92	0.990	2750-13 copies

11. Troubleshooting

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

12. Limitations

The product is intended for research use only. Not for use in diagnostic procedures.

Dilution of the reagents, other than as described in this handbook, is not recommended, and will result in a loss of performance.

13. Appendix A: Threshold Setting

The threshold should be set to a level higher than the baseline level of fluorescence but well below the plateau of the amplification plot. It must be positioned within the linear segment of the amplification curve, which indicates the detectable log-linear range of the PCR. For clarity, the threshold value should be established within the logarithmic view of the amplification plot to easily identify the log-linear phase of the PCR.

If multiple targets are used in the real-time experiment, the threshold needs to be determined individually for each target. Serial dilutions of template can be used to assess the correct threshold for your instrument, the threshold should be set across a range of template concentrations to determine the optimum threshold for each target (Figure 5).

IPO8 and UBXN4 targets control for the input of sufficient RNA within the assay for RT-qPCR measurements to be valid, as well as normalise the expression of cMET to determine relative expression. For these targets the threshold should be set so that the assay can detect low levels of IPO8, UBXN4 and cMET expression, without false positive calls in negative control.

For the MET Δ14 target users are recommended to set the threshold by testing samples of known positive and negative status to ensure no false positive Ct values are generated.

Refer to the user guide of the qPCR platform in use for further information on threshold setting.

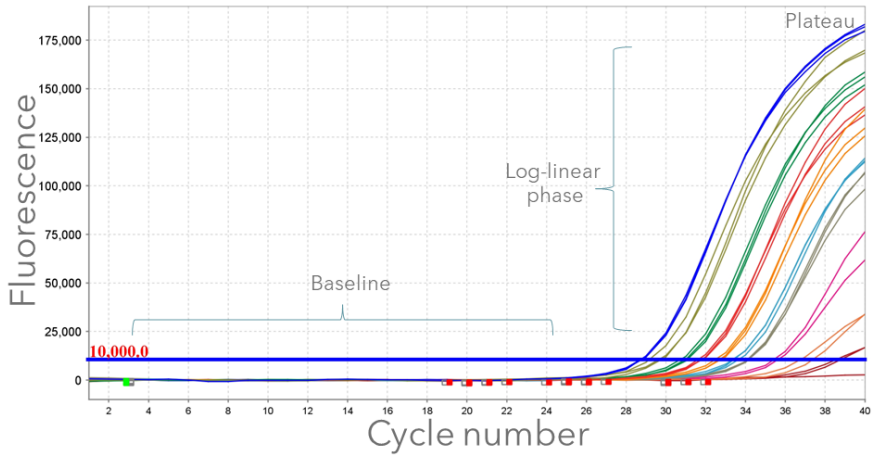

Figure 5. Linear amplification plot for the UBXN4 target across a range of template concentrations (indicated by the coloured lines). The PCR threshold ((blue line) was set close to the inflection point (or the point at which a reaction reaches a level of fluorescence above the background level of the reactions). The PCR threshold was set to ensure that all amplification curves generate a cycle number (Ct value).

14. 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

15. Contact Information

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16. Ordering Information

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