APIS

Assay Technologies Ltd.

APIS Breast Cancer Subtyping kit

RUO Handbook (Other Instrumentation)

APIS Breast Cancer Subtyping Kit

Handbook

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REF

Applicable to:
00402 (EU)
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APIS Breast Cancer Subtyping Kit is a product for research use only.

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

Revision History

Revision	Date	Description
ART0050(01)	17FEB2023	N/A
ART0050(02)	11OCT2023	Applicable product codes and distributor added to title page
ART0050(03)	26OCT2023	Updated applicable product code on the title page

Contents

APIS Assay Technologies

Apis Assay Technologies are committed to realise the potential of systems biology and medicine in the diagnosis and personalisation of treatment. We seek to develop biomarkers that deliver significant improvements in the prediction and prevention of disease.

1. Intended Use

The BC subtyping kit is intended for research use only. Not intended for medical purpose or objective.

The BC Subtyping Kit (RUO) is a gene expression assay based on a real-time reverse transcription polymerase chain reaction (RT-qPCR). The kit detects and enables relative gene expression quantification of ten human mRNA target genes extracted from formalin-fixed, paraffin embedded (FFPE) pre-operative core needle biopsies (CNB) or FFPE resected breast tumour tissue.

Product description

The BC Subtyping 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 BC subtyping kit also contains an internal control (IC), a synthetic RNA sequence, which monitors for assay set up, reagent performance and interfering substances. 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 BC Subtyping Kit are listed in Table 1.

Table 1 Targets detected by BC Subtyping Kit

Reaction Mix	Protein Name	Gene Symbol	Full name
1	ER	ESR1	Oestrogen receptor 1 (Gene ID: 2099)
	CK5	KRT5	Keratin 5 (Gene ID: 3852)
2	PR	PGR	Progesterone receptor (Gene ID: 5241)
	Ki67	MKI67	Marker of proliferation Ki-67 (Gene ID: 4288)
	PCNA	PCNA	Proliferating cell nuclear Antigen (Gene ID: 5111)
3	HER2	ERBB2	Erb-b2 receptor tyrosine kinase 2 (Gene ID: 2064)
	CCNA2	CCNA2	Cyclin A2 (Gene ID: 890)
	IPO8	IPO8	Importin 8 (Gene ID: 10526)
4	KIF23	KIF23	Kinesin Family member 23 (Gene ID: 9493)
	PUM1	PUM1	Pumilio homolog 1 (Gene ID: 9698)

2. Material Provided

Kit Contents

Apis Breast Cancer Subtyping Kit (RUO)
(24 samples in duplicate + controls)

Component	Colour	Volume
Enzyme mix	White	2x 1.5mL
Buffer mix	Purple	2x 550µL
Primer Probe mix 1	Red	1x 75µL
Primer Probe mix 2	Yellow	1x 75µL
Primer Probe mix 3	Green	1x 75µL
Primer Probe mix 4	Blue	1x 75µL
Positive Control (PC)	Black	1x 100µL
Water for No Template Control (NTC)	Clear	1x 500µL

3. Materials Required but Not Provided

Reagents

Note: Refer to kit handbooks to ensure all supplementary material is available.

- RNA isolation kit suitable for formalin-fixed, paraffin embedded (FFPE) tissue. (e.g., QIAGEN RNeasy® DSP FFPE Kit (QIAGEN Cat. No. 73604))
- DNase I (If not already included as part of the selected RNA isolation kit)
- 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 qPCR instrument

Equipment

- Real-Time PCR instrument (calibrated for FAM™, HEX™, Texas Red® and Cy5® dyes). Refer to the equipment user guide for further information on instrument calibration.
- RNA quantification equipment (e.g., Qubit™ Fluorometer (Thermo Fisher Scientific Cat. No. Q33238))
- Adjustable volume pipettes
- Centrifuge (for spinning down plates and microcentrifuge tubes)
- Vortex
- Adhesive Film Applicator
- Cool block or ice

4. 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.
- When stored under the recommended storage conditions in the original packaging, the kit is stable until the expiration date stated on the label.
- Prior to use the reagents should be kept cool at 4°C until fully thawed.
- 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.

5. Warnings and Precautions

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

6. General Precautions

- The test is for use with resected or CNB FFPE breast cancer tissue specimens.
- All chemicals and biological materials are potentially hazardous. FFPE 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 BC Subtyping 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.
- Thoroughly decontaminate work area before setting up.
- Do not remove the plate seal after the run has finished.

7. 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).

8. Specimen Storage and Handling

To prepare tissue specimens for RNA extraction:

- Using standard materials and methods, fix the tissue specimen in 10% neutral buffered formalin (NBF) and embed the tissue specimen in paraffin. Using a microtome, cut 5 µm sections from the paraffin block and mount them on glass slides.
- Scrape excess paraffin away from the tissue using a fresh, sterile scalpel.
- Scrape the tumour tissue from the slides into labelled microcentrifuge tubes using a fresh scalpel for each specimen.
- Label, handle and store tumour specimens in a controlled fashion according to local procedures.
- Once extracted, store RNA eluate between -50°C and -100°C.

9. Procedure

9.1 RNA Isolation

Apis recommend extraction of RNA from FFPE sections using the RNeasy DSP FFPE Kit (QIAGEN Cat. No. 73604), or equivalent, according to the manufacturer's recommendations. A DNase step should be either included as part of the RNA isolation procedure or included as a separate step pre-PCR set up.

It is recommended to initially use 2x 5 µm FFPE sections per extraction and to remove any excess paraffin using a fresh sterile scalpel before the extraction procedure. Use of additional thinner sections that total 10µm is acceptable. If upon RNA quantification (as per procedure described in Section 9.2) the RNA yield obtained is insufficient (<4 ng/µL) it is recommended to repeat the procedure using up to 20µm of FFPE sections per extraction.

9.2 RNA Quantification & Normalisation

Quantify the RNA using an appropriate quantification method (e.g., Fluorometer or UV spectrometer).

The RNA input range for the assay is between 7.5–80 ng total RNA input. It is recommended to use a total RNA input of 10ng in a volume of 4µL. A single aliquot of 40µL at 2.5ng/µL is sufficient to run the assay.

9.3 Plate Set Up & Cycling

The master mix contains all the components required for RT-qPCR except the template RNA. We recommend a negative control (without template RNA) and positive control should be included on every run. Up to 10 samples can be analysed simultaneously in one RT-qPCR run.

Thaw, on ice or at 2°C to 8°C, template RNA and all kit components. 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.

9.3.1 Master Mix Preparation

Prepare a volume of master mix for two technical replicates per RNA sample and one technical replicate for the PC and NTC respectively. Prepare enough master mix for two additional replicates (n+2) per mix to allow sufficient overage volume for PCR setup. Ensure set up is performed on ice or at 2°C–8°C.

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 required. 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	11	4	**PPmix 1**	1
Mix 2	11	4	**PPmix 2**	1
Mix 3	11	4	**PPmix 3**	1
Mix 4	11	4	**PPmix 4**	1

9.3.2 Reaction Set up

- Position a PCR plate/tubes on a cooling block.
- Pipette into each well/tube 16 µL of each corresponding Master Mix and 4 µL of RNA sample/Positive Control or Negative Control. An example of set-up in a 96-well PCR plate is shown in the example plate layout (Figure 1), colour coded for each of the four mixes 1-4.
- 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	11.0	1x
Buffer Mix	4.0	1x
Primer Probe mix	1.0	Variable
Master Mix Total	16	-
Sample (Template RNA/PC/NTC)	4.0	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 manufacture instructions.


1
2
3
4
5
6
A
Sample 1 Rep 1
Sample 1 Rep 2
Sample 2 Rep 1
Sample 2 Rep 2
Sample 3 Rep 1
Sample 3 Rep 2
B
Sample 1 Rep 1
Sample 1 Rep 2
Sample 2 Rep 1
Sample 2 Rep 2
Sample 3 Rep 1
Sample 3 Rep 2
C
Sample 1 Rep 1
Sample 1 Rep 2
Sample 2 Rep 1
Sample 2 Rep 2
Sample 3 Rep 1
Sample 3 Rep 2
D
Sample 1 Rep 1
Sample 1 Rep 2
Sample 2 Rep 1
Sample 2 Rep 2
Sample 3 Rep 1
Sample 3 Rep 2
E
Sample 6 Rep 1
Sample 6 Rep 2
Sample 7 Rep 1
Sample 7 Rep 2
Sample 8 Rep 1
Sample 8 Rep 2
F
Sample 6 Rep 1
Sample 6 Rep 2
Sample 7 Rep 1
Sample 7 Rep 2
Sample 8 Rep 1
Sample 8 Rep 2
G
Sample 6 Rep 1
Sample 6 Rep 2
Sample 7 Rep 1
Sample 7 Rep 2
Sample 8 Rep 1
Sample 8 Rep 2
H
Sample 6 Rep 1
Sample 6 Rep 2
Sample 7 Rep 1
Sample 7 Rep 2
Sample 8 Rep 1
Sample 8 Rep 2

7	8	9	10	11	12
Sample 4 Rep 1	Sample 4 Rep 2	Sample 5 Rep 1	Sample 5 Rep 2		PC
Sample 4 Rep 1	Sample 4 Rep 2	Sample 5 Rep 1	Sample 5 Rep 2		PC
Sample 4 Rep 1	Sample 4 Rep 2	Sample 5 Rep 1	Sample 5 Rep 2		PC
Sample 4 Rep 1	Sample 4 Rep 2	Sample 5 Rep 1	Sample 5 Rep 2		PC
Sample 9 Rep 1	Sample 9 Rep 2	Sample 10 Rep 1	Sample 10 Rep 2		NTC
Sample 9 Rep 1	Sample 9 Rep 2	Sample 10 Rep 1	Sample 10 Rep 2		NTC
Sample 9 Rep 1	Sample 9 Rep 2	Sample 10 Rep 1	Sample 10 Rep 2		NTC
Sample 9 Rep 1	Sample 9 Rep 2	Sample 10 Rep 1	Sample 10 Rep 2		NTC

Figure 1: Mix and Sample Layout, rows A and E contain master mix 1, rows B and F contain master mix 2, rows C and G contain master mix 3 and rows D and H contain master mix 4.

9.4 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 RT-qPCR cycling parameters are outlined 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 BC Subtyping 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	

Targets, reporter dyes and quenchers for each PPmix are shown in Table 5. Automatic baseline and threshold settings should be used. Users are recommended to empirically determine the suitability of thresholds for the qPCR instrument under use.

Table 5: Target Data Acquisition and Analysis Settings for the BC Subtyping Kit

Mix	Target	Dye	Quencher	Ct Threshold	Baseline
MIX1	ESR1	FAM™	BHQ-1 ®	Auto	Auto
	KRT5	HEX™	BHQ-1 ®	Auto	Auto
	IC	Cy5®	BHQ-2 ®	Auto	Auto
MIX2	PGR	FAM™	BHQ-1 ®	Auto	Auto
	MKI67	HEX™	BHQ-1 ®	Auto	Auto
	PCNA	Texas Red®	BHQ-2®	Auto	Auto
	IC	Cy5®	BHQ-2 ®	Auto	Auto
MIX3	ERBB2	FAM™	BHQ-1 ®	Auto	Auto
	CCNA2	HEX™	BHQ-1 ®	Auto	Auto
	IPO8	Texas Red®	BHQ-2 ®	Auto	Auto
	IC	Cy5®	BHQ-2 ®	Auto	Auto
MIX4	KIF23	FAM™	BHQ-1 ®	Auto	Auto
	PUM1	Texas Red®	BHQ-2 ®	Auto	Auto
	IC	Cy5®	BHQ-2 ®	Auto	Auto

9.5 Run Export

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.

9.5.1 Recommended Run Validity Criteria

The run is deemed valid when the results for the negative control for the targets produce no amplification.

All reactions contain an Internal control in the Cy5 channel that should amplify within each master mix within the negative control well. Each target should also amplify within the positive control. The recommended run validity criteria are outlined in Table 6.

Table 6: Recommended Run Validity Criteria for the BCSubtyping kit.

Mix	Target	Acceptable Negative Control Ct Range	Acceptable Positive Control Ct Range
MIX1	ESR1	Undetermined	≤33.082
	Internal Control (IC)	≤31.925	N/A
MIX2	PGR	Undetermined	≤33.682
	MKI67	Undetermined	≤35.067
	PCNA	Undetermined	≤34.428
	Internal Control (IC)	≤31.925	N/A
MIX3	ERBB2	Undetermined	≤34.959
	CCNA2	Undetermined	≤33.180
	IPO8	Undetermined	≤33.721
	Internal Control (IC)	≤31.925	N/A
MIX4	KIF23	Undetermined	≤35.216
	PUM1	Undetermined	≤33.395
	Internal Control (IC)	≤31.925	N/A

9.5.2 Recommended Sample Validity Criteria

Recommended sample validity criteria are provided. Their suitably for the PCR instrument should be defined by the user.

It is recommended that for each sample the Ct values of both reference genes, IPO8 and PUM1, should be less than or equal to a Ct of 37.5 in both replicates. For each sample the IC Ct in each of the 4 mixes should fall within a specified range.

The recommended sample validity criteria are outlined in Table 7. If the reference genes or IC are out of specification the sample is invalid and should be repeated.

Table 7: Recommended Sample Validity Criteria for the IC

Mix	Target	Acceptable Ct Range
MIX1	Internal Control (IC)	≤31.925
MIX2	Internal Control (IC)	≤31.925
MIX3	Internal Control (IC)	≤31.925
MIX4	Internal Control (IC)	≤31.925

9.6 Results Interpretation

Target relative expression can be reported as Delta Ct (ΔCt) value. Delta Ct (ΔCt) for each target should be calculated by taking the mean Ct value of both reference gene duplicates (IPO8 and PUM1), subtracted by the mean of the target biomarker (**Error! Reference s ource not found.** 3).

$$\Delta Ct = \frac{\sum_{i=1}^{n} reference\ genes}{n} - \frac{\sum_{i=1}^{j} marker_m}{j}$$

Figure 3: Delta Ct (ΔCt) calculation performed for all targets. n=the total number of reference gene PCR replicates, j the total number of target PCR replicates

ΔCt values should be used to guide the biomarker expression status (positive/high or negative/low).

Users should determine the ΔCt cut-off for the assay empirically through testing known positive and negative samples for each marker.

10. Troubleshooting

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

11. 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.

It is important that the amount and quality of RNA in the sample is assessed prior to performing sample analysis using the PCR Kit.

Attention should be paid to expiration dates and storage conditions printed on the box and labels of all components. Do not use expired or incorrectly stored components.

12. Symbols

Symbol	Definition
	Batch code
	Catalogue number
	Caution
	Consult instructions for use or consult electronic instructions for use
	Contains sufficient for <24> tests
	Do not use if package is damaged and consult instructions for use
	Keep away from sunlight
	Manufacturer
	Negative control

Symbol	Definition
	Positive control
	Serial number
	Temperature limit
	Use by date
	Research Use Only

13. Contact Information

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14. Ordering Information

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