APIS
Assay Technologies Ltd.

# APIS Breast Cancer Subtyping kit

# RUO Handbook (QS5™ and QS5™ Dx)

# APIS Breast Cancer Subtyping Kit

# Handbook

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**00402 (EU)**
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**APIS Assay Technologies Ltd**
Second floor, Citylabs 1.0, Nelson Street, Manchester, M13 9NQ, UK

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

**US Distribution:**
**Eagle Biosciences, Inc.**
105 NH-RT 101A, STE 12, Amherst, NH, 03031
+1-617-419-2019 - www.eaglebio.com

**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

# 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 for use in diagnostic procedures.**

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/tubes compatible with qPCR instrument (QS5™Dx, MicroAmp™ EnduraPlate™ Optical 96-Well Clear GPLE Reaction Plates with Barcode (Thermo Fisher Scientific Cat. No. 4483348 or 4483351), QS5 MicroAmp™ EnduraPlate™ Optical 96-Well Clear Reaction Plates with Barcode (Thermo Fisher Scientific Cat. No. 4483485)) or compatible equivalent
- Adhesive film (MicroAmp™ Optical Adhesive Film (Thermo Fisher Scientific Cat. No. 4360954 or 4311971) )

## Equipment

- Quantstudio™5 DX or QS5™ with a 0.2ml block (Thermo Fisher Scientific).
- 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. Platform

Apis recommend running the kit on the QuantStudio 5 or QuantStudio™5 Dx (QS5™ or QS5™ Dx) instrument with a 0.2ml block. The instrument should be calibrated for FAM™, HEX™, Texas Red® and Cy5® dyes. Refer to the user guides for further information on instrument calibration.

# 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.
- 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.

## 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 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.

## 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. 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.

# 10. Procedure

## 10.1 RNA Isolation

Apis recommend extraction of RNA from FFPE sections using the RNeasy DSP FFPE Kit (QIAGEN Cat. No. 73604). When using alternate extraction methodology the use of 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 10.2) 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.

## 10.2 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.

## 10.3 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. 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.

### 10.3.1 Master Mix Preparation

Prepare a volume of master mix for two technical replicates per 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 |

### 10.3.2 Plate Set up

- Position a PCR plate on a cooling block or ice.
- Following the plate layout in Figure 1 pipette into each well 16 µL of each corresponding Master Mix and 4 µL of RNA sample/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** | 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 | Sample 2.5 ng/µL (total 10 ng) |
| **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.
- 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 into the PCR instrument following manufacture instructions.


*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.*

## 10.4 Run Method

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

Refer to the QuantStudio™5 DX or QS5™ user manual to set up the PCR run. The recommended experiment properties and RT-qPCR cycling parameters are outlined Figure 2 and Table 4.

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

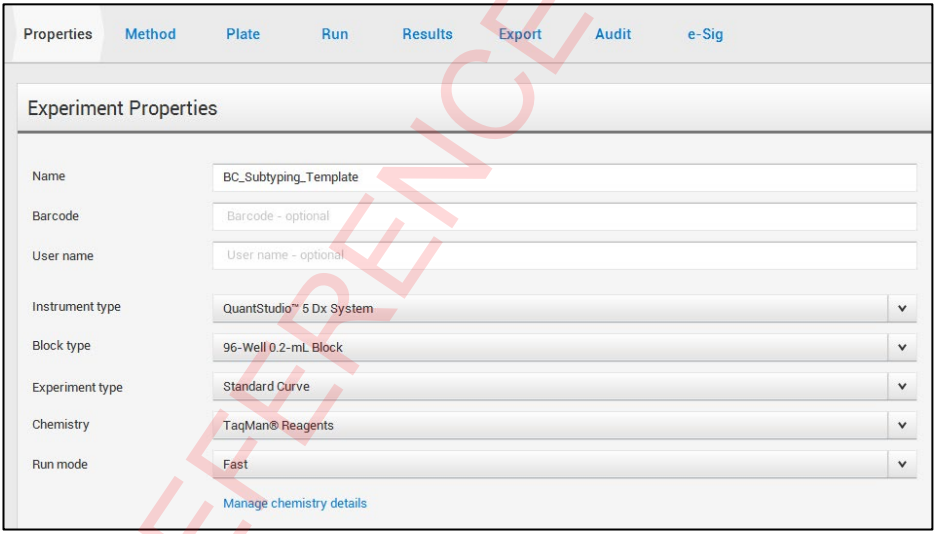


*Figure 2: Run properties to be used*

*Table 4: Recommended Cycling parameters for use with the QuantStudio™ DX and 0.2ml QS5 block.*

| 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, quenchers, and thresholds are shown in Table 5.

*Table 5: Target Data Acquisition and Analysis Settings (*Recommended for use with the QuantStudio™ DX and 0.2ml QS5)*

| Mix | Target | Dye | Quencher | ΔRn Threshold* | Baseline* |
|---|---|---|---|---|---|
| **MIX1** | ESR1 | FAM™ | None | 60,000 | Auto |
| | KRT5 | HEX™ | None | 50,000 | Auto |
| | IC | Cy5® | None | 10,000 | Auto |
| **MIX2** | PGR | FAM™ | None | 50,000 | Auto |
| | MKI67 | HEX™ | None | 27,000 | Auto |
| | PCNA | Texas Red® | None | 85,000 | Auto |
| | IC | Cy5® | None | 10,000 | Auto |
| **MIX3** | ERBB2 | FAM™ | None | 60,000 | Auto |
| | CCNA2 | HEX™ | None | 35,000 | Auto |
| | IPO8 | Texas Red® | None | 150,000 | Auto |
| | IC | Cy5® | None | 10,000 | Auto |
| **MIX4** | KIF23 | FAM™ | None | 160,000 | Auto |
| | PUM1 | Texas Red® | None | 27,000 | Auto |
| | IC | Cy5® | None | 10,000 | Auto |

## 10.5 Run Export

Once the run is completed export the Ct values.

### 10.5.1 Run Validity Criteria

The run is deemed valid when the results for the negative control for the targets are 'Undetermined' (no amplification). Whilst the Internal control (IC) should amplify within each master mix. The run validity criteria for the QS5™Dx and QS5™ are outlined in Table 6.

*Table 6: Recommended Run Validity Criteria (QS5™Dx and QS5 ™)*

| Mix | Target | Acceptable Negative Control Ct Range | Acceptable Positive Control Ct Range |
|---|---|---|---|
| MIX1 | ESR1 | Undetermined | 26.760-33.082 |
| | KRT5 | Undetermined | 28.236–34.004 |
| | Internal Control (IC) | 26.075-31.925 | N/A |
| MIX2 | PGR | Undetermined | 28.292-33.682 |
| | MKI67 | Undetermined | 28.993-35.067 |
| | PCNA | Undetermined | 28.286-34.428 |
| | Internal Control (IC) | 26.075-31.925 | N/A |
| MIX3 | ERBB2 | Undetermined | 26.779-34.959 |
| | CCNA2 | Undetermined | 26.820-33.180 |
| | IPO8 | Undetermined | 27.353-33.721 |
| | Internal Control (IC) | 26.075-31.925 | N/A |
| MIX4 | KIF23 | Undetermined | 28.974-35.216 |
| | PUM1 | Undetermined | 27.281-33.395 |
| | Internal Control (IC) | 26.075-31.925 | N/A |

### 10.5.2 Sample Validity Criteria

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 QS5 ™Dx and QS5 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: Sample Validity Criteria for the IC (QS5™Dx and QS5 ™)*

| Mix | Target | Acceptable Ct Range |
|---|---|---|
| MIX1 | Internal Control (IC) | 26.075-31.925 |
| MIX2 | Internal Control (IC) | 26.075-31.925 |
| MIX3 | Internal Control (IC) | 26.075-31.925 |
| MIX4 | Internal Control (IC) | 26.075-31.925 |

## 10.6 Results Interpretation

Target relative expression can be reported as Delta Ct (ΔCt) values. 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 (Figure 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

Delta Ct values should be used to guide the biomarker expression status (positive/high or negative/low). When using QS5™Dx and QS5 instrument, ΔCt cut-off values presented in Table 8 below can be used. ΔCt values below the cut off can be treated as negative for a given biomarker, and any ΔCt values above the cut off can be treated as positive. ΔCt greater than 12 should be repeated.

*Table 8: ΔCt cut-off values for each target (QS5™Dx)*

| Target | ΔCt Cut off |
|---|---|
| **ESR1** | -1.98 |
| **PGR** | -0.63 |
| **ERBB2** | 2.00 |
| **MKI67** | -0.64 |

A ΔCt cut-off for KRT-5 should be validated by the user.
The subtype can be determined based on the individual target calls following the logic in Table 9.

*Table 9: Subtyping logic table*

| ESR1 | PGR | ERBB2 | MKI67 | Intrinsic subtype |
|---|---|---|---|---|
| + | - | - | - | Luminal A-like |
| + | + | - | - | Luminal A-like |
| - | + | - | - | Luminal A-like |
| + | + | - | + | Luminal B-like (HER2 negative) |
| + | - | - | + | Luminal B-like (HER2 negative) |
| - | + | - | + | Luminal B-like (HER2 negative) |
| + | - | + | + | Luminal B-like (HER2 positive) |
| + | - | + | - | Luminal B-like (HER2 positive) |
| + | + | + | - | Luminal B-like (HER2 positive) |
| + | + | + | + | Luminal B-like (HER2 positive) |
| - | + | + | - | Luminal B-like (HER2 positive) |
| - | + | + | + | Luminal B-like (HER2 positive) |
| - | - | + | + | HER2 enriched (non-luminal) |
| - | - | + | - | HER2 enriched (non-luminal) |
| - | - | - | - | Triple Negative |
| - | - | - | + | Triple Negative |

# 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.

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 allcomponents. Do not use expired or incorrectly stored components.

## 13. 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 |

## 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

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## 15. Ordering Information

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