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Assay Technologies Ltd.

APIS Breast Cancer Subtyping Kit

Instructions for Use

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

APIS Breast Cancer Subtyping Kit is a product for *in-vitro*-diagnostic use.

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.

UK CA IVD

Revision History:

Revision	Date	Description
01	11AUG23	New Document
02	01DEC23	Updated NTC volume to 500 µL and amendment to background color for Enzyme mix in Section 3.1

Contents

APIS Assay Technologies

APIS Assay Technologies are committed to realise the clinical 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 Breast Cancer (BC) Subtyping Kit is a real-time nucleic acid-based in vitro diagnostic test. The kit detects and enables relative gene expression quantification of nine human mRNA target genes extracted from formalin-fixed, paraffin embedded (FFPE) pre-operative core needle biopsies (CNB) or FFPE resected breast tumour tissue from patients with invasive breast cancer. Specimens are processed using the RNeasy® DSP FFPE Tissue Kit for manual sample preparation and the QuantStudio™ 5 Dx instrument for automated amplification and detection. Automated result interpretation is performed using the APIS BC Subtyping Analysis Software.

The test will detect the relative expression of seven mRNA target genes – *ESR1*, *PGR*, *ERBB2*, *MKI67*, *CCNA2*, *PCNA*, and *KIF23* and two reference genes *IPO8* and *PUM1*.

The test will report the individual biomarker status (Positive or Negative) for *ESR1*, *PGR*, *ERBB2*, and High or Low status for *MKI67* and the proliferation measure from the combined expression of *MKI67*, *CCNA2*, *PCNA*, and *KIF23*. Marker status will be used to report the molecular classification: Luminal A-like, Luminal B-like (HER2 negative), Luminal B-like (HER2 positive), HER2 positive (non-luminal), and Triple Negative subtypes.

The Breast Cancer Subtyping Kit is intended for use in assessing the mRNA levels in invasive breast cancer tissue obtained in from patients and prepared as FFPE specimens. The assay is intended as an aid in diagnosis of patients in conjunction with other laboratory and clinical data and is not intended to be used as the sole basis for diagnosis, treatment, or other patient management decisions.

The BC Subtyping kit is intended for use by qualified and trained clinical laboratory personnel.

2. Summary and Explanation of the Test

Breast cancer is one of the most common cancers in women, with approximately two million new breast cancer cases per year (Harbeck 2019). Breast cancer is the most common cause of female cancer mortality in developing countries (Coughlin 2019) and the second most common cause of cancer (after lung cancer) in developed countries (Breast Cancer Research Foundation; Jemal et al. 2011).

Breast cancer (BC) is a heterogenous disease on the molecular level (Sachs et al. 2018). Treatment has evolved to take this heterogeneity into account with emphasis on biologically directed therapies to reduce the adverse effects of chemotherapy-based treatment regimens (Waks and Winer 2019). The intrinsic classifications of Perou et al. (2000) distinguished four subtypes of breast cancer, luminal A and luminal B (expressing the oestrogen receptor (ER)), human epidermal growth factor receptor 2 (HER2) enriched (without ER expression) and basal-like tumours that express no ER or HER2.

Currently, clinical practice typically uses a surrogate classification of five subtypes based on histological and molecular characteristics. Tumours expressing ER and/or progesterone receptor (PR) are considered hormone receptor- positive breast cancers, whereas tumours that do not express ER, PR or HER2 are triple- negative breast cancer (TNBC) (Harbeck 2019). Adjuvant therapy choice is primarily based on the molecular subtype of the patient's tumour sample.

The gold standard for molecular subtyping is reconstruction of the subtype using IHC to measure protein expression as a stand in for the markers. Although many studies of IHC based subtyping have shown clinical similarity to gene expression profiling, the methodology does have some limitations. Despite efforts to standardise staining and analysis protocols the inter-and intra-variability of IHC is of concern (van Bockstal et al. 2020; van Dooijeweert et al. 2020). The quality of the determination of these markers is essential for effective therapy.

The Breast Cancer Subtyping Kit aims to reduce this intra and inter variability as well as reducing laboratory and pathologist analysis time, thereby improving patient outcomes and costs.

The Breast Cancer 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 theamplicon is spanning an exon-exon junction. The probe binds to the target sequence between the primers.

The Breast Cancer 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 Breast Cancer Subtyping Kit are listed in Table 1.

Table 1 Targets detected by the APIS Breast Cancer Subtyping Kit

Reaction Mix	Protein Name	Gene Symbol	Full name
1	ER	*ESR1*	Oestrogen receptor 1 (Gene ID: 2099)
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

Reaction Mix	Protein Name	Gene Symbol	Full name
			(Gene ID: 9493)
	PUM1	*PUM1*	Pumilio homolog 1 (Gene ID: 9698)

3. Material Provided

3.1 Kit Contents

APIS Breast Cancer Subtyping Kit
(24 clinical samples in duplicate + controls)

Component	Colour	Volume
Primer Probe mix 1	Red	1x 68µL
Primer Probe mix 2	Yellow	1x 68µL
Primer Probe mix 3	Green	1x 68µL
Primer Probe mix 4	Blue	1x 68µL
Enzyme mix	White	2x 1500µL
Buffer mix	Purple	2x 550µL
Positive Control (PC)	Black	1x 71µL
Water for No Template Control (NTC)	Clear	1x 500µL

4. Materials Required but Not Provided

Reagents

- QIAGEN RNeasy® DSP FFPE Kit (QIAGEN Cat. No. 73604)
- 100% ethanol (do not use denatured alcohol, which contains other substances such as methanol or methylethylketone), molecular biology grade
- RNA Quantification Reagents- see RNA Quantification and Normalisation on page 9
- Nuclease free water for sample dilutions

Consumables

- Sterile pipette tips with filters
- Sterile 1.5 mL microcentrifuge tubes
- MicroAmp™ EnduraPlate™ Optical 96-Well Clear GPLE Reaction Plates with Barcode (Thermo Fisher Scientific Cat. No. 4483348 or 4483351)
- MicroAmp™ Optical Adhesive Film (Thermo Fisher Scientific Cat. No. 4311971)

Equipment

- Adjustable volume pipettes
- Digital shaking dry bath fitted with 1.5 mL tube block and shaker
- Microcentrifuge
- Vortex
- PCR plate and reagent cool rack
- Adhesive Film Applicator
- Plate vortex
- Plate centrifuge
- QuantStudio™ 5 Dx + computer with QuantStudio™ Dx IVD software (version 1.0.2 or above) (Thermo Fisher Scientific Cat. No. A32005C)
- RNA quantification equipment (e.g., Qubit™ Fluorometer (Thermo Fisher Scientific Cat. No. Q33238))
- Cool block or ice

5. Platform and Software

The Breast Cancer Subtyping Kit is specifically designed for use with the RNeasy DSP FFPE Kit for manual sample preparation and the QuantStudio 5 Dx instrument operating with a personal computer installed with:

- QuantStudio 5 Dx IVD software (version 1.0.2 or above)
- QuantStudio 5 Dx IVD run template (BC_Subtyping_Template)
- Breast Cancer Subtyping Software (V1.2 or above)

Refer to QuantStudio 5 Dx System user guide for information concerning the QuantStudio 5 Dx instrument. The QuantStudio 5 Dx instrument must be maintained according to the requirements in the user guide.

6. 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 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 four 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.

7. Warnings and Precautions

The APIS BC Subtyping Kit is to be used by trained personnel in a professional molecular biology laboratory environment.

For use only with the QuantStudio 5 Dx Instrument. See user manual of instrument for safety information.

For use only with the RNeasy DSP FFPE Kit. See RNeasy DSP FFPE Kit (QIAGEN Cat. No. 73604) for safety information.

Repeated thawing and freezing should be avoided. Do not exceed four freeze-thaw cycles.

The use of FFPE material should be limited to samples no older than 20 months.

8. 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 Breast Cancer Subtyping Kit have been diluted optimally. Do not dilute reagents further.
- Do not use expired or incorrectly stored components.
- 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.

9. 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 (SDSs).

10. Activities Performed Prior to PCR Set Up

- Set up the instrument as detailed in the QuantStudio 5 Dx Real-Time PCR Instrument user guide (Publication Number 100042186).

- Ensure the instrument is calibrated for FAM™, HEX™, Texas Red® and Cy5® dyes. In order to use HEX™ and Texas Red® dyes custom calibration is required; follow the procedure for custom dye calibration as detailed in the QuantStudio™ 5 Dx Real-Time PCR Instrument user guide (Publication Number: 100042186). Note: Instrument calibration requires admin user access.
- Install the Breast Cancer Subtyping Kit PCR template for (BC_Subtyping_Template) on the computer connected to the QuantStudio 5 Dx instrument.

To install the published template in the QuantStudio 5 Dx IVD software, log in to the software with an administrator account or any other account with sufficient permissions and in the menu bar select **Tools→Template Menu**. Click **Install**, then click **Yes** to confirm the **Template Installation Agreement**. Navigate to the desired published template (.edt file), then click **Open**. The template is now installed and accessible in the **Template Menu**.

Contact APIS Assay Technologies to obtain the published template.

11. 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.
- Macro-dissection of DCIS should be performed to prevent any false results from the DCIS tumour.
- 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 and -100°C.

12. Procedure

12.1 RNA Isolation

It is recommended to 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 12.2) 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.

Follow the RNeasy® DSP FFPE Kit Instructions for Use (follow protocol as though you are extracting from four sections). Ensure the digital shaking dry bath used in the procedure has been calibrated and the displayed temperature is correct before starting incubation. Purified RNA should be eluted in 30µL of RNase free water provided in the extraction kit.

12.2 RNA Quantification & Normalisation

Quantify RNA using a fluorometric quantification method that uses nucleic acid binding dyes.

RNA stock concentration must be ≥4 ng/µL to have sufficient volume to proceed to testing.

Dilute RNA stock concentration to 2.5 ng/µL using nuclease free water (not provided with the kit). At least 40µL of the normalised RNA sample should be prepared for testing with the kit.

For each specimen, calculate the volume (µL) of RNA stock needed:

$$RNA\ volume\ (\mu L) = \frac{Final\ diluted\ solution\ concentration\ (ng/\mu L)}{Stock\ solution\ concentration\ (ng/\mu L)} \times Final\ desired\ volume\ (\mu L)$$

For each specimen, calculate the volume (µL) of nuclease free water diluent needed:

$$Water\ volume\ (\mu L) = Final\ desired\ volume(\mu L) - RNA\ volume(\mu L)$$

12.3 Gene Expression 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 2–8°C, template RNA, Enzyme Mix, Buffer Mix, PPmixes, Positive and Negative Control, immediately before plate set up. It is important to mix the solutions completely before use to avoid localised differences in concentration. Ensure no precipitate is present within the Buffer Mix. If precipitate can be observed, mix by vortexing until fully dissolved. 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.

12.3.1 Master Mix Preparation

Prepare a volume of master mix for two technical replicates per RNA sample, and one technical replicate for both positive and negative controls. Prepare enough master mix for two additional replicates (n+2) to allow sufficient overage volume for PCR setup. Ensure set up is performed on ice or at 2–8°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 according to Table 3. 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 1 sample run in duplicate, 1 positive control and 1 negative control (n=4). Master Mix should be prepared for a total of 6 reactions (n+2)

Master Mix ID	Volume of Enzyme Mix (µL)	Volume of Buffer Mix (µL)	Primer Probe mix tube	Volume of Primer Probe mix (µL)
Mix 1	66	24	**PPmix 1**	6
Mix 2	66	24	**PPmix 2**	6
Mix 3	66	24	**PPmix 3**	6
Mix 4	66	24	**PPmix 4**	6

Table 3: Example master mix manufacture volumes for N=1 to 10 samples

Component	Number of Samples To Be Tested (N)									
	1	2	3	4	5	6	7	8	9	10
Enzyme Mix volume (µL)	66	88	110	132	154	176	198	220	242	264
Buffer Mix volume (µL)	24	32	40	48	56	64	72	80	88	96
PPmix volume (µL)	6	8	10	12	14	16	18	20	22	24

Do not exceed N=10, since this is the maximum number of samples that can fit on one plate.

12.3.2 Plate Set up

- Position a 96-well PCR plate on a cooling block.
- Pipette into each well 16 µL of each corresponding Master Mix and 4 µL of RNA sample/Positive Control or Negative Control as shown in the plate layout (Figure 1), colour coded for each of the four mixes 1-4.
- To reduce a risk of cross contamination, negative and positive controls should always be positioned on the right side of the plate.
- The final concentration of components per reaction are detailed in Table 4.

Table 4: 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	2.5 ng/µL (total 10 ng)
Total Reaction Volume	20.0	-

- Seal the plate using a PCR plate seal and the sealing tool.
- Add plate to plate vortex and vortex to mix.
- Introduce the plate in the plate centrifuge and centrifuge for >1 minute to spin contents to bottom of the plate.
- Visually assess the wells for bubbles, if any are present, flick the plate and centrifuge for additional 30 seconds. Repeat until no bubbles are present.
- Place the plate in the QuantStudio 5 Dx instrument.


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

12.4 Run Method

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

Select "New Experiment" in the home page of the QuantStudio 5 Dx IVD software (Figure 2). From the pop-up template menu select the previously installed BC_Subtyping_Template.edt template and click "Create New Experiment".

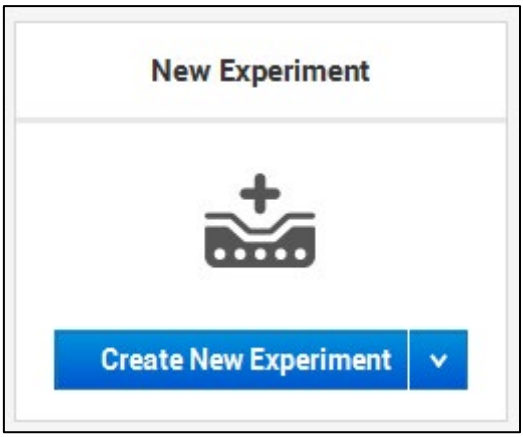

Figure 2: QuantStudio 5 Dx IVD Software New Experiment

The published template includes all the experiment properties and the run method optimised for use with the BC Subtyping Kit and are shown in Figure 3 and Table 5 .

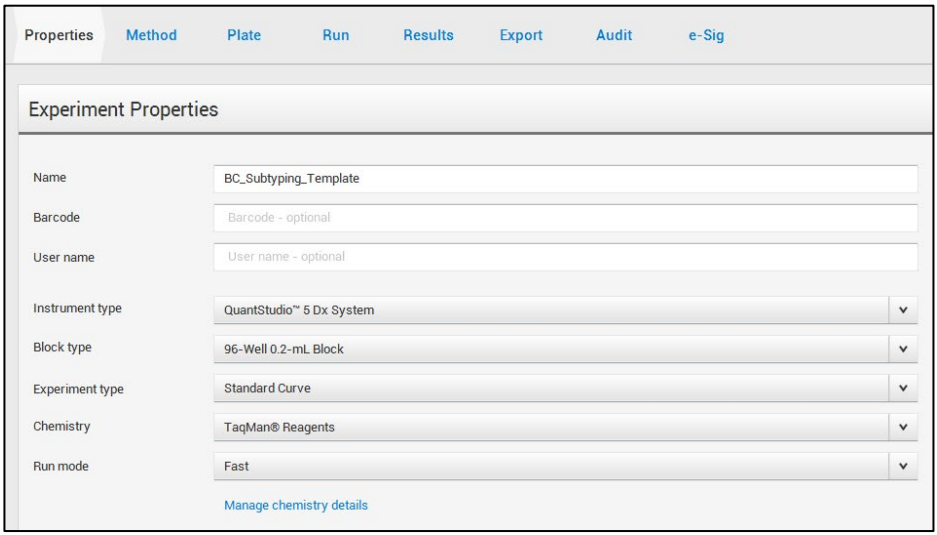

Figure 3: Run properties to be used

Table 5: Cycling parameters

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 set in BC_Subtyping_Template.edt template are shown in Table 6.

Table 6: Target Data Acquisition and Analysis Settings

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

**KRT5* target will not be analysed in the assay.

The "Advanced Setup" in the plate tab should be used to assign sample IDs in place of (Sample 1 to Sample 10). If not all samples are being run on a plate, deselect the samples not being used. Figure 4 shows the plate set up and sample set up view.

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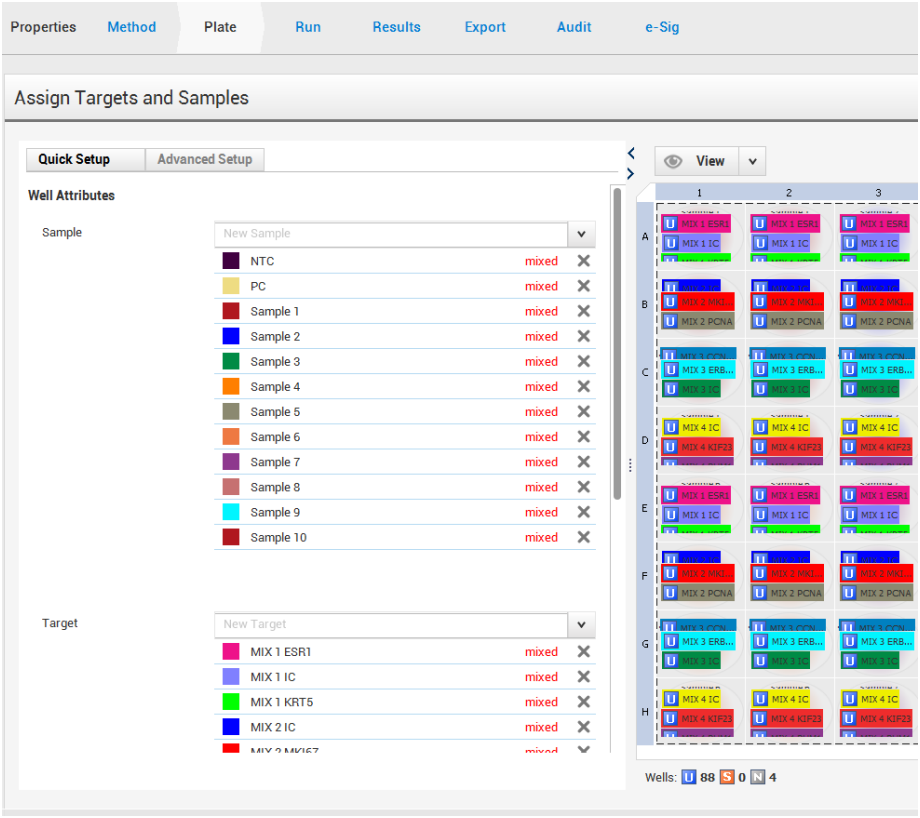
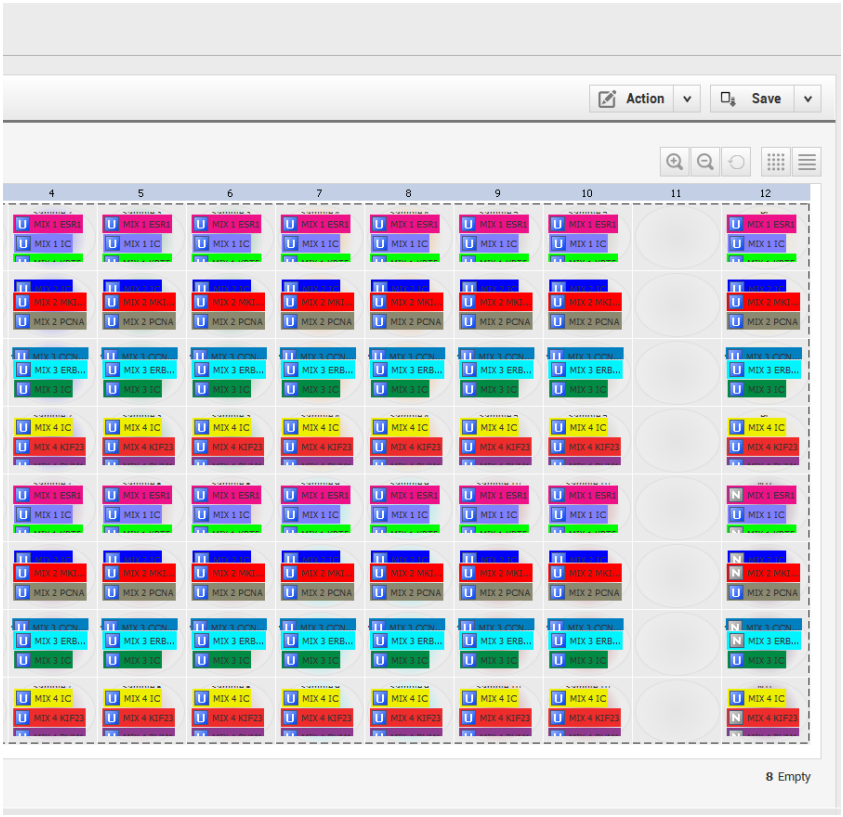


Figure 4: Plate setup and sample ID assignment


Action
Save
4
5
6
7
8
9
10
11
12
8 Empty

Ensure all settings are correct (as detailed in Table 5 and Table 6) and start the run.

12.5 Data Analysis

BC_Subtyping_Template template includes the QuantStudio 5 Dx analysis parameters, automatic baselines and thresholds defined in Table 6 are used for analysis.

12.6 QuantStudio 5 Dx Run Export

Once the run is completed export the Ct values as an .xls file, using the Export Tab as shown in Figure 5. Do not make any changes to the file. This file should be used as input into the BC Subtyping Kit Analysis Software.

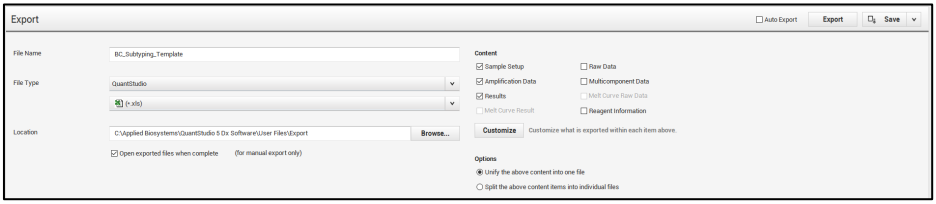


Figure 5: Export tab and export settings

12.7 Results Interpretation

Analysis is carried out using the BC Subtyping software – accessed via a web browser at the following address:
https://www.APISbcsubtypinganalysis.com/

Note: Software functionality has only been tested in Chrome Version 97.0 or higher or Firefox Browser version 95.0.1 or higher with Windows 10 pro.

Import the run file into the software by navigating to the .xls file via the "choose file" button.
Begin analysis by clicking on the "Start Analysis" button (Figure 6).

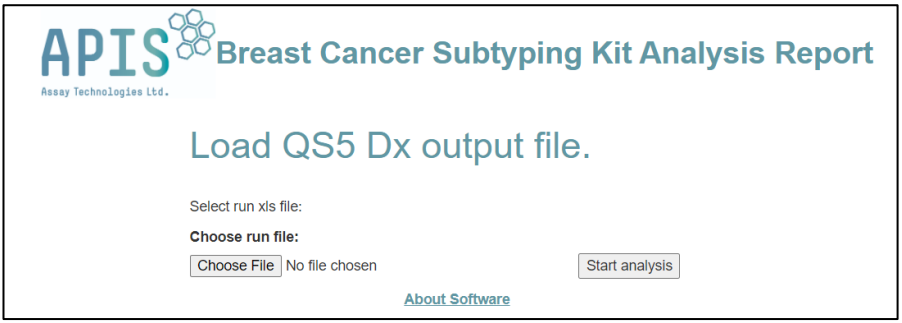
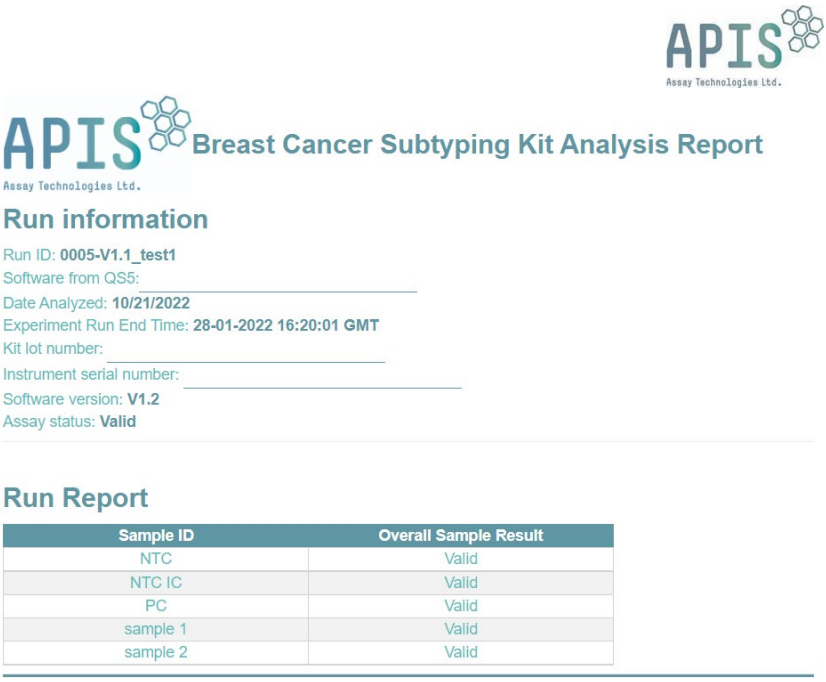


Figure 6: Homepage for the Breast Cancer Subtyping Software

The software will output a summary of run information, with run validity and sample validity, as defined.
Kit lot and instrument serial number should be entered manually by the operator as shown in Figure 7.



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Breast Cancer Subtyping Kit Analysis Report

Run information

Run ID: **0005-V1.1_test1**
Software from QS5:
Date Analyzed: **10/21/2022**
Experiment Run End Time: **28-01-2022 16:20:01 GMT**
Kit lot number:
Instrument serial number:
Software version: **V1.2**
Assay status: **Valid**

Run Report

Sample ID	Overall Sample Result
NTC	Valid
NTC IC	Valid
PC	Valid
sample 1	Valid
sample 2	Valid

Figure 7: Run and Sample Validity Report

12.7.1 Run Validity

The run is deemed valid if the criteria in Table 7 are met. Negative Control results for the targets should be "Undetermined", while the Internal Control (IC) should amplify within each master mix.

Table 7: Run Validity Criteria

Mix	Target	Acceptable Negative Control Ct Range	Acceptable Positive Control Ct Range
MIX1	*ESR1*	Undetermined	26.760-33.082
	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

12.7.2 Sample Validity

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 four mixes should fall within the range specified in Table 8. If the reference genes or IC are out of specification the sample is invalid and should be repeated.

Table 8: Sample Validity Criteria for IC

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

12.7.3 Sample Status Interpretation

The software will provide a result for target and subtype calling for each individual sample (Figure 8). The software allows for both an operator and checker to review and sign off on the results.

A PDF containing all run and sample information can also be printed by selecting the "Print Report" button.

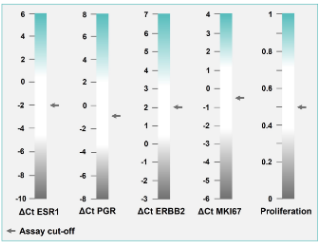
Results

Sample: sample 1

Run Name: 0005-V1.1_test1

Validity: Valid

Marker	Status	dCt
ESR1	Positive	1.31
PGR	Positive	2.69
ERBB2	Negative	1.9
MKI67	Low	-2.53
Proliferation	Low	0.05
Subtype:		**Luminal A**



Comments

Operator:

Operator name | Operator signature | Date

Checker:

Checker name | Checker signature | Date

Figure 8: Sample results

Delta Ct (ΔCt) for each target is automatically calculated by the software as the mean of both reference gene duplicates, subtracted by the mean of the target biomarker (Figure9).

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

Figure 9: 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

Expression of each target is considered as positive/high if the ΔCt of the sample target is above the cut-off values listed in Table 9.

Table 9: ΔCt cut-off values for each target

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

If a ΔCt returned is greater than 12 the sample should be repeated.

A logistic model using the ΔCt of *MKI67*, *CCNA2*, *PCNA* and *KIF23* calculates a proliferation score.

The output of the proliferative signature is a value between 0 and 1, values above 0.5 recorded as high, below as low.

The subtype is determined based on the individual target calls following the logic in Table 10.

Table 10: 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

A list of flags given by the software is detailed in Table 11.

Table 11: Software Flags

Software Flag Example	Description
Run validity failed. Mix 3 CCNA2 Fail - PC out of specified range	Positive Control out of specification. Run is invalid. Run to be repeated.
Run Invalid. Unexpected amplification detected in NTC MIX 1 ESR1 well, Ct.	Unexpected amplification in negative control. Run is invalid. Run to be repeated.
Run Invalid. NTC MIX3 IC well CT value out of the specified range Ct."	Internal Control out of specification in NTC well. Run is invalid. Run to be repeated.
Sample 1 – Sample invalid – IC Mix 1 out of specification	Internal Control Ct out of specification. Sample is invalid. Sample to be repeated.
Sample 1 Sample invalid. Reference gene IPO8 Ct out of specification	Internal Control Ct out of specification. Sample is invalid. Sample to be repeated.

12.8 Repeat Testing of Invalid Runs and Samples

If the run report indicates 'Run Invalid' the whole PCR run should be repeated.

If the run report indicates 'Sample Invalid' the sample should be repeated. Repeat dilution of the invalid specimen RNA stock and repeat testing as described starting in section 2.2.

If the specimen remains invalid after retesting, or insufficient RNA stock remains to prepare another dilution, repeat the entire test procedure for that specimen, starting with RNA Isolation using new FFPE sections.

13. Performance Characteristics

13.1 Analytical Sensitivity

13.1.1 Limit of Blank (LoB)

The LoB was established by testing RNA samples from eight individual clinical FFPE samples negative for each target. RNA from samples was normalised to 2.5 ng/µL. A total of 32 replicates of each sample were tested with four BC subtyping kit lots. The LoB was defined as the highest measured ΔCt that corresponds to the upper 95th percentile in the negative sample. The LoB was estimated for each kit lot and the highest value across all the kit lots was reported as the LoB value. The study design was based on the CSLI guideline EP17-A2.

The LoB values for each target (in terms of ΔCt) detected by the BC subtyping kit are reported in Table 12.

Table 12 Summary of results for each target, including the mean ΔCt, SD and the LoB calculation.

Target	Total no of replicates	Mean ΔCt	SD	LoB
ESR1	342	-8.061	1.362	-5.820
PGR	342	-6.748	2.166	-3.185
ERBB2	342	-2.288	1.508	0.192
MKI67	332	-3.692	1.093	-1.894

13.1.2 Limit of Detection (LoD)

The LoD was established by testing a pooled RNA sample, expressing all target genes detected by the kit, extracted from clinical FFPE specimens. The sample was serially diluted to at least five titration levels per target. Each set of eight replicates was tested with three BC subtyping kit lots and three operators using three instruments over five days for a total of 48 replicates per target following EP17-A2 guidelines. Probit analysis was used to determine the LoD (the lowest concentration of an analyte that can be consistently detected (with ≥95% of probability). Samples that returned a result at LoD were then quantified using digital PCR (dPCR) with an alternative assay to the BC Subtyping Kit, to obtain an absolute quantification in copy number for that sample. The recommended RNA input for the kit is 10ng, thus LoD results are presented both as the predicted Ct from probit analysis and the copy number for each target at LoD for a 10ng RNA aliquot. The claimed LoD for the targets in Ct and target copy number are noted in Table 113.

Table 13 LoD for the BC subtyping targets reported at Ct and RNA copies per 10 ng RNA input.

Target	Ct at predicted LoD	LoD (RNA copies per 10ng input)
ESR1	37.267	124.6
PGR	36.318	0.633
MKI67	38.145	0.018
ERBB2	35.668	185.8
CCNA2	36.244	0.231
PCNA	35.855	0.055
KIF23	36.594	0.121
IPO8	37.211	0.343
PUM1	35.287	0.198

13.2 Measuring Range

13.2.1 Linearity

Linearity was estimated by testing two clinical RNA samples for each biomarker representing high and low positive samples for all targets. Each dilution series had seven levels and two replicates at each level to evaluate the assay linearity across the target range from 4 ng to 120 ng total RNA input. The linear regression analyses were performed for first, second and third order polynomials as per CLSI EP06-A guidelines. The data was considered linear if the non-linear coefficients from the second and third order polynomials were insignificant ($p > 0.05$). When significant non-linear coefficients were observed, the degree of nonlinearity was calculated per CLSI EP06-A guideline.

The estimated regression, the intercepts and slopes from the linear model are shown in Table 14. For all targets to be linear, the RNA input range was between 7.5–80 ng total RNA input.

Table 14 Linear fit for the BC subtyping targets

Target	R^2	Linear Range (ng input)	Slope	Efficiency
High positive *ESR1*	0.997	4–120	-3.441	95.26%
Low positive *ESR1*	0.992	7.5–120	-3.352	98.78%
High positive *PGR*	0.999	7.5–120	-3.31	100.49%
Low positive *PGR*	0.995	4–120	-3.315	100.30%
High positive *ERBB2*	0.999	4–80	-3.423	95.96%
Low positive *ERBB2*	0.993	7.5–120	-3.312	100.43%
High positive *MKI67*	0.998	4–80	-3.174	106.55%
Mid positive *MKI67*	0.989	7.5–120	-3.393	97.12%
High positive *PCNA*	0.998	4–80	-2.990	115.97%
Mid positive *PCNA*	0.997	4–80	-3.350	98.83%
High positive *CCNA2*	0.998	4–80	-3.408	96.53%
Mid positive *CCNA2*	0.982	4–120	-3.379	97.66%
High positive *KIF23*	0.997	4–80	-3.104	109.96%
Mid positive *KIF23*	0.987	7.5–120	-3.577	90.37%
IPO8	0.997	7.5–120	-3.278	101.86%
PUM1	0.992	4–120	-3.326	99.83%

The data support assay linearity across the indicated RNA test concentration of 10 ng.

13.2.2 Minimum Tumour Content

The minimum tumour content on an FFPE section required to result in a correct call for the BC Subtyping Kit was determined. RNA was extracted from FFPE breast cancer samples with low and high expression levels of targets of interest (*ESR1*, *PGR*, *ERBB2* and *MKI67*). Samples with known tumour content were normalised to 2.5 ng/µL and then diluted to tumour concentrations between 1% and 40% using RNA extracted from healthy normal breast tissue. For *MKI67* and *ESR1*, RNA was also extracted from an equal area (cm^2) of healthy and tumour FFPE tissue with 100% tumour content, mixed to contrive samples with a varying degree of tumour content between 10 and 40% and then quantified and normalised to 2.5 ng/µL. All samples were tested with two replicates per level using a single kit lot of the BC Subtyping Kit. The kit's minimum tumour content value was determined as the lowest tumour content concentration which resulted in a positive call (ΔCt) for the assessed targets (Table 15).

Table 15 Minimum tumour content defined for each target.

Target	Minimum Tumour Content
ESR1	10%*
PGR	1%
ERBB2	1%
MKI67	10%
Proliferation signature	10%
Assay Minimum Tumour Content	10%

*ESR1 minimum tumour content was calculated as 10% following both methods. 20% should be used for sufficient RNA to be extracted from the slide.

13.3 Analytical Specificity and Cross-reactivity

13.3.1 Cross-reactivity

ThermoBLAST™ software was used to scan oligonucleotide sequences against large genome databases to detect 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, viruses, bacteria, and soil microbes. When screening the multiplex mixes against the sequence databases, there were no potentially problematic non-target amplicons.

13.3.2 Interference (exogenous and endogenous)

The impact of potential endogenous (necrosis) and exogenous (paraffin wax and RNeasy® DSP FFPE Kit DSP solution, RNeasy® DSP FFPE Kit RBC Buffer, RNeasy® DSP FFPE Kit Wash Buffer/Ethanol) interferants on the BC subtyping kit performance was determined.

For assessment of interference from necrotic tissue, RNA extracted from necrotic tissue was spiked into RNA extracted from FFPE breast cancer samples at 10% and 30% v/v per eluate and normalised to 2.5 ng/µL, a blank sample was also tested. Three samples were tested in the study representing either high, low or negative expression of the BC subtyping targets. Each sample was tested across six replicates, with RT-qPCR runs performed using one kit lot. Correct call was observed for all samples tested. Where a significant difference in ΔCt was observed between the spiked and control samples, this was within acceptable intermediate precision of the assay. Presence of necrotic tissue up to 30% did not have an effect on the performance of the kit.

For assessment of interference of the paraffin wax and components of the RNeasy® DSP FFPE (DSP solution, RBC Buffer, Wash Buffer/Ethanol), three FFPE samples were tested in the study representing either high, low or negative expression of the BC subtyping targets. RNA from FFPE samples were spiked with 1x and 10x concentrations of each interferant, a blank was also tested. The test

concentrations of each interferant were estimated using the CLSI guidelines (EP07-A2).

In total, six replicates of each sample/interferent combination were tested with one BC subtyping lot. Where a significant difference was observed between the spiked and control samples, this was within acceptable intermediate precision of the assay. None of the interferents tested had any impact on kit performance.

13.4 ΔCt Cut-offs

Cut-offs for the individual BC Subtyping Kit assay targets were determined using RNA extracted from FFPE CNB breast tissue samples obtained from unique patient donors. Samples were positive and negative for ER, PR and HER2 expression (or high and low for Ki67) as assessed by IHC. A total of 157 clinical FFPE samples were chosen to determine the cut-offs for *ESR1* and *PGR*. For *ERBB2*, 195 clinical FFPE samples were chosen to determine the cut-off. For *MKI67* and proliferation 591 clinical FFPE samples were chosen to determine the cut-off. RNA samples were tested at 2.5ng/µL (10 ng of RNA into each reaction). All samples were tested with the BC Subtyping Kit with two technical replicates per data point.

If no fluorescent signal was detected after cycle 40 – values were inferred as having a value of 40. ΔCt for each target was calculated as the mean of both reference gene duplicates, subtracted by the mean of the target biomarker. For each target a binary classifier logistic regression model was trained using the ΔCt expression data as input and the IHC status as the binary classifier (positive/negative or high/low). Assay cut-offs were calculated taking into account positive and negative agreement equally, the cut-offs are shown in Table 16.

A proliferative model was trained using a binary classifier logistic regression model. For each target, a regression model was trained using the ΔCt expression data as input and Ki67 IHC status input as the binary classifier (high/low). *MKI67*, *CCNA2*, *KIF23* and *PCNA* markers were input into the model.

Table 16 Assay ΔCt cut-off values calculated for the BC Subtyping Kit targets.

Target	Assay cut-off
ESR1	-1.98
PGR	-0.63
ERBB2	2.00
MKI67	-0.64
Proliferation	0.50

13.5 Repeatability and Reproducibility

The within-site repeatability, intermediate precision and reproducibility of the BC subtyping kit were evaluated by testing six contrived RNA samples. These samples represented a negative, low positive and mid positive sample for each of the targets: *ESR1*, *PGR*, *ERBB2*, *MKI67*/proliferation score. Samples were divided into single use aliquots for testing at three independent laboratories. The study design was based on the CLSI guidelines EP05-A3.

Repeatability and intermediate precision were generated using replicate measurements of the same samples using an identical PCR layout, the same protocol, two operators, two instruments, two kit lots and one site over 10 days to generate a total of 50 replicates per sample.

The proportion of correct calls for each sample tested at: Site 1 (repeatability) along with the corresponding two-sided exact 95% Confidence Intervals (Cis) are reported in Table 17.

Table 17 Kit Repeatability - number of correct calls and two-sided 95% confidence limits for the BC Subtyping Kit targets and proliferation score.

Target	Expression Level	N	Correct Call	% Detection Rate	Lower Confidence Interval (95% CI)	Upper Confidence Interval (95% CI)
ESR1	Mid	50	50	100%	92.89%	100%
	Low	50	50	100%	92.89%	100%
	Negative	50	50	100%	92.89%	100%
PGR	Mid	50	50	100%	92.89%	100%
	Low	50	50	100%	92.89%	100%
	Negative	50	50	100%	92.89%	100%
ERBB2	Mid	50	50	100%	92.89%	100%
	Low	50	50	100%	92.89%	100%
	Negative	50	50	100%	92.89%	100%
MKI67	Mid	50	50	100%	92.89%	100%
	Low	50	50	100%	92.89%	100%
	Negative	50	49	98%	89.35%	99.95%
Proliferation Score	Mid	50	50	100%	92.89%	100%
	Low	50	50	100%	92.89%	100%
	Negative	50	50	100%	92.89%	100%H

Reproducibility was estimated by generating replicate measurements of the same samples using an identical PCR layout, protocol, six operators, three sites, four instruments and three kit lots over 10 days to generate a total of 100 replicates per sample (for *ESR1*/*PGR* Mid and *MKI67*/Proliferation Negative 99 replicates are available).

Reproducibility along with 95% confidence limits are repeated in Table 18.

Table 18 Kit reproducibility – number of correct calls and two-sided 95% confidence limits for the BC Subtyping Kit targets and proliferation score for samples tested across all sites.

Target	Expression Level	N	Correct Call	% Detection Rate	Lower Confidence Interval (95% CI)	Upper Confidence Interval (95% CI)
ESR1	Mid	99	99	100%	96.34%	100%
	Low	100	100	100%	96.38%	100%
	Negative	100	100	100%	96.38%	100%
PGR	Mid	99	99	100%	96.38%	100%
	Low	100	100	100%	96.38%	100%
	Negative	100	100	100%	96.38%	100%
ERBB2	Mid	100	100	100%	96.38%	100%
	Low	100	100	100%	96.38%	100%
	Negative	100	100	100%	96.38%	100%
MKI67	Mid	100	100	100%	96.38%	100%
	Low	100	98	98%	96.38%	100%
	Negative	99	86	87%	78.59%	92.82%
Proliferation Score	Mid	100	100	100%	96.38%	100%
	Low	100	100	100%	96.38%	100%
	Negative	99	99	98.99%	94.50%	99.97%

Variance component analysis (using main effects model) was calculated with the factors nested in a following order: site, operator, instrument, lot and run/day. With one run for each sample performed per day, between-day variability is accounted for by the between-run variability. The variability associated with each factor was reported in terms of standard deviation, % coefficient of variance (for Ct values only) and percent of total variance, in which the variability corresponding to each component was calculated as a percentage of the sum of all variance components. The variance components analysis as measured for Repeatability is shown in Table 19. The variance components analysis as measured for Reproducibility is shown in Table 20.

Table 19 Repeatability Standard Deviation (SD) for ΔCt – Between Lot, Between-run, Between-Operator, Between-Day, Between-Instrument and Total Variance.

Target	Expression Level	Between Operator (SD)	Between- instrument (SD)	Between-lot (SD)	Between-Run (Between Day) (SD)	Within Run (SD)	Total (SD)
ESR1	Mid	0.344	0.031	0.000	0.232	0.266	0.494
	Low	0.136	0.141	0.166	0.195	0.297	0.438
	Negative	0.000	0.000	0.000	0.000	0.803	0.803
PGR	Mid	0.118	0.113	0.263	0.041	0.233	0.390
	Low	0.000	0.000	0.208	0.098	0.24	0.332
	Negative	0.154	0.000	0.000	0.000	0.635	0.654
ERBB2	Mid	0.191	0.000	0.07	0.128	0.18	0.301
	Low	0.000	0.063	0.016	0.139	0.119	0.194
	Negative	0.066	0.000	0.105	0.118	0.134	0.218
MKI67	Mid	0.000	0.096	0.165	0.000	0.220	0.291
	Low	0.000	0.091	0.129	0.058	0.207	0.267
	Negative	0.078	0.117	0.113	0.000	0.215	0.281
Proliferation Score	Mid	0.000	0.010	0.021	0.018	0.033	0.044
	Low	0.000	0.005	0.021	0.013	0.039	0.047
	Negative	0.035	0.018	0.011	0.007	0.039	0.057

Table 20 Reproducibility - Overall Mean, Standard Deviation (SD) for ΔCt –

Between-run, Within – Site, Between Site, Between Lot, Between-Operator, Between-Day, Between-Instrument and Total Variance.

Target	Expression Level	Between Operator (SD)	Between- instrument (SD)	Between-lot (SD)	Between-Run (Between Day) (SD)	Within Run (SD)	Total (SD)
ESR1	Mid	0.339	0.270	0.113	0.342	0.295	0.674
	Low	0.338	0.000	0.495	0.229	0.331	0.722
	Negative	0.149	0.000	0.000	0.682	0.768	1.120
PGR	Mid	0.050	0.122	0.417	0.098	0.269	0.538
	Low	0.092	0.000	0.693	0.189	0.240	0.763
	Negative	0.065	0.000	0.000	0.000	0.626	0.653
ERBB2	Mid	0.127	0.095	0.497	0.218	0.166	0.589
	Low	0.018	0.052	0.441	0.088	0.185	0.514
	Negative	0.067	0.152	0.450	0.229	0.143	0.550
MKI67	Mid	0.057	0.059	0.476	0.072	0.299	0.573
	Low	0.099	0.070	0.405	0.098	0.279	0.520
	Negative	0.043	0.108	0.098	0.066	0.306	0.362
Proliferation Score	Mid	0.008	0.005	0.020	0.011	0.015	0.030
	Low	0.014	0.000	0.040	0.009	0.045	0.065
	Negative	0.037	0.021	0.000	0.025	0.049	0.081

13.6 Accuracy

The accuracy of the BC Subtyping Kit was demonstrated relative to IHC using CNB FFPE clinical specimens. Study design was per CLSI EP12-A2. For each specimen ER, PR, HER2 and Ki67 status was assessed by IHC (and/or ISH for *ERBB2*) before testing with the Breast Cancer Subtyping Kit. RNA was extracted from two to four FFPE sections (5–10 µm) using the RNeasy® DSP FFPE Kit (QIAGEN) following the kit handbook and the Breast Cancer Subtyping Kit instructions for use. Samples with sufficient RNA were diluted to 2.5 ng/µL for testing. A total of 215 samples were used for analysis (195 samples were used for *ERBB2* as samples unresolved for HER2 were removed from this analysis. A total of 214 samples were used for *MKI67* as no Ki67 IHC data was available for one sample). Agreement between the IHC/ISH results and the BC Subtyping Kit was calculated for each marker. Agreement for the BC Subtyping Kit proliferation score is calculated against Ki67 IHC. The overall percent agreement (OPA), positive percent agreement (PPA) and negative percent agreement (NPA) along with the corresponding two-sided exact 95% CIs results are shown in Table 21. Correlation of RNA expression as defined by ΔCt and IHC score % are shown for *ESR1*, *PGR* and *MKI67* in Figure 10, 11 and 12 respectively.

Table 21: Breast Cancer Subtyping Kit Agreement to IHC.

	ESR1 (ER)	*PGR* (PR)	*ERBB2* (HER2)	*MKI67* (Ki67)	Proliferation Signature
Positive Percent Agreement (PPA)	95.3% (90.6-97.7) 142/149	93.6% (87.9-96.7) 117/125	93.8% (79.9-98.3) 30/32	93.8% (87.0-97.1) 90/96	89.6% (81.9-94.2) 86/96
Negative Percent Agreement (NPA)	86.4% (76.1-92.7) 57/66	66.7% (56.4-75.5) 60/90	90.8% (85.4-94.3) 148/163	58.5% (49.5-77.0) 69/118	71.2% (62.4-78.6) 84/118
Overall Percent Agreement (OPA)	92.6% (88.3-95.4) 199/215	82.3% (76.7-86.8) 177/215	91.2% (86.4-94.8) 178/195	74.3% (68.1-79.7) 159/214	79.4% (73.4-84.6) 170/214

Resolution testing of discordant samples was conducted using an independent validated dPCR test. The OPA, PPA and NPA, along with the corresponding two-sided exact 95% CI following composite resolution are shown in Table 22.

Table 22 Breast Cancer Subtyping Kit Agreement to IHC following dPCR resolution testing.

	ESR1 (ER)	*PGR* (PR)	*ERBB2* (HER2)	*MKI67* (Ki67)
Positive Predictive Agreement (PPA)	99.3% (96.2-99.9) 145/146	97.7% (93.5-99.2) 128/131	100% (89.6-100) 33/33	99.1% (95.0-99.8) 109/110
Negative Predictive Agreement (NPA)	91.3% (82.3-96.0) 63/69	77.4% (67.4-85) 65/84	92.6% (87.5-95.7) 150/162	71.2% (61.8-79.0) 74/104
Overall Percent Agreement (OPA)	96.7% (93.4-98.4) 208/215	89.8% (85.0-93.1) 193/215	93.8% (89.6-96.4) 183/195	85.5% (81.9-89.1) 183/214

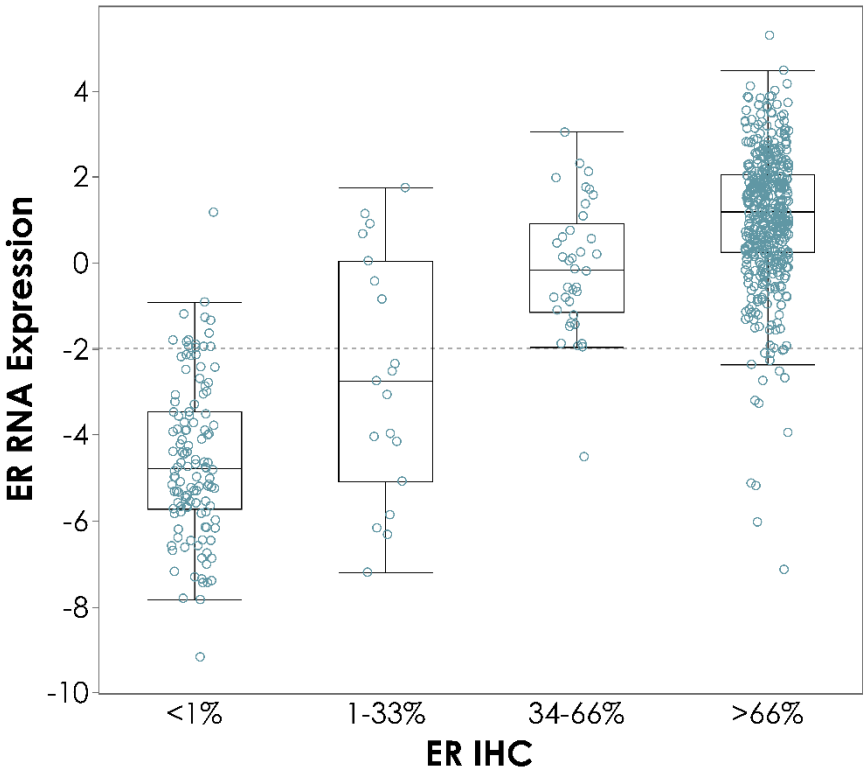


Figure 10 Correlation to ER IHC Score for ESR1

Concordance scatter plot between APIS BC Subtyping Kit and IHC % based biomarker assessment. Analysis of 652 breast cancer CNB specimens tested across four kit lots and five qPCR instruments. Y axis; APIS mRNA gene expression (ΔCt value) with assay cut-off (dashed line). X axis; Semi-quantitative IHC positive nuclei. ER negative when <1%

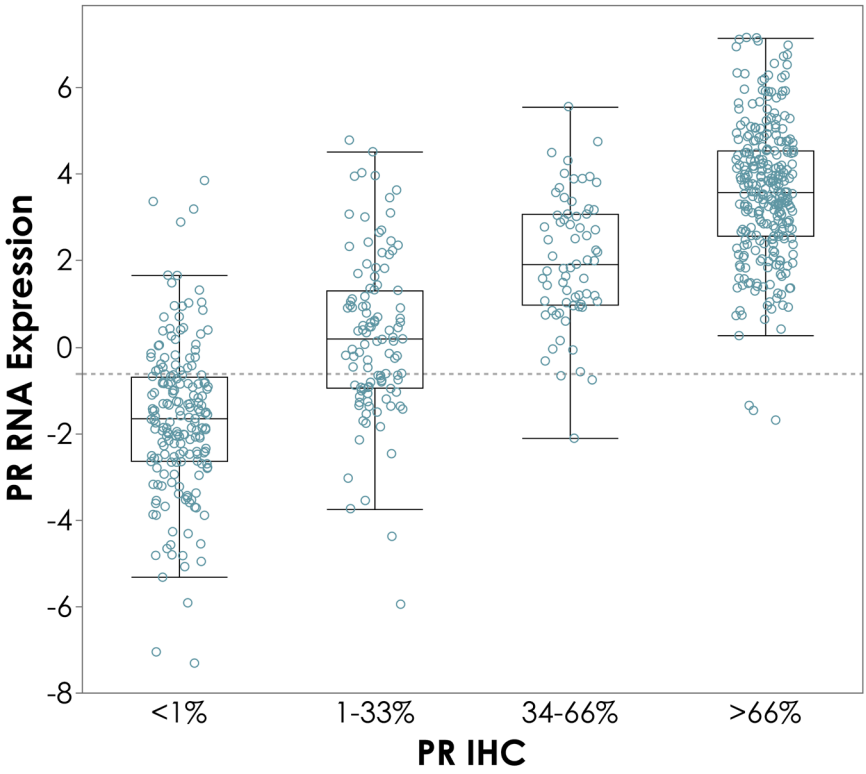


Figure 11 Correlation to PR IHC Score for PGR

Concordance scatter plot between APIS BC Subtyping Kit and IHC % based biomarker assessment. Analysis of 652 breast cancer CNB specimens tested across four kit lots and five qPCR instruments. Y axis; APIS mRNA gene expression (ΔCt value) with assay cut-off (dashed line) for positive/negative calling. X axis; Semi-quantitative IHC positive nuclei %. PR negative when <1%

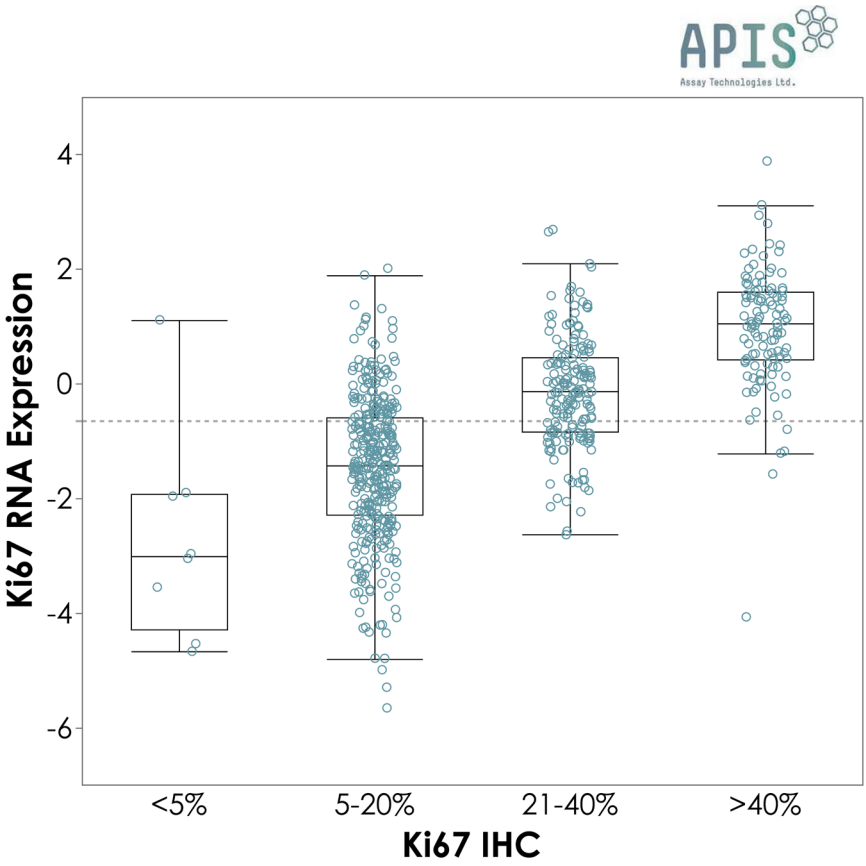


Figure 12 Correlation to Ki67 IHC Score for MKI67

Concordance scatter plot between APIS BC Subtyping Kit and IHC-based biomarker assessment. Analysis of 650 breast cancer CNB specimens tested across four kit lots and five qPCR instruments. Y axis; APIS mRNA gene expression (ΔCt value) with assay cut-off (dashed line) for positive/negative calling. X axis; Semi-quantitative IHC positive nuclei %.

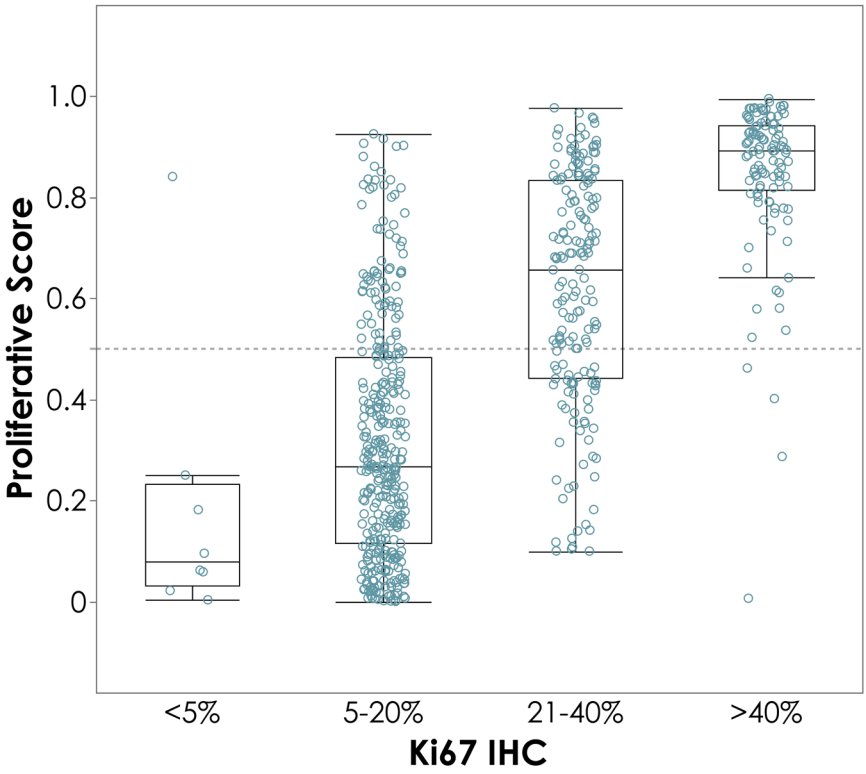


Figure 13 Correlation to KI67 IHC Score for proliferative signature

Concordance scatter plot between APIS BC Subtyping Kit and IHC-based biomarker assessment. Analysis of 650 breast cancer CNB specimens tested across four kit lots and five qPCR instruments. Y axis; APIS proliferative score with a cut-off (dashed line) for high/low calling. X axis; Semi-quantitative IHC positive nuclei %

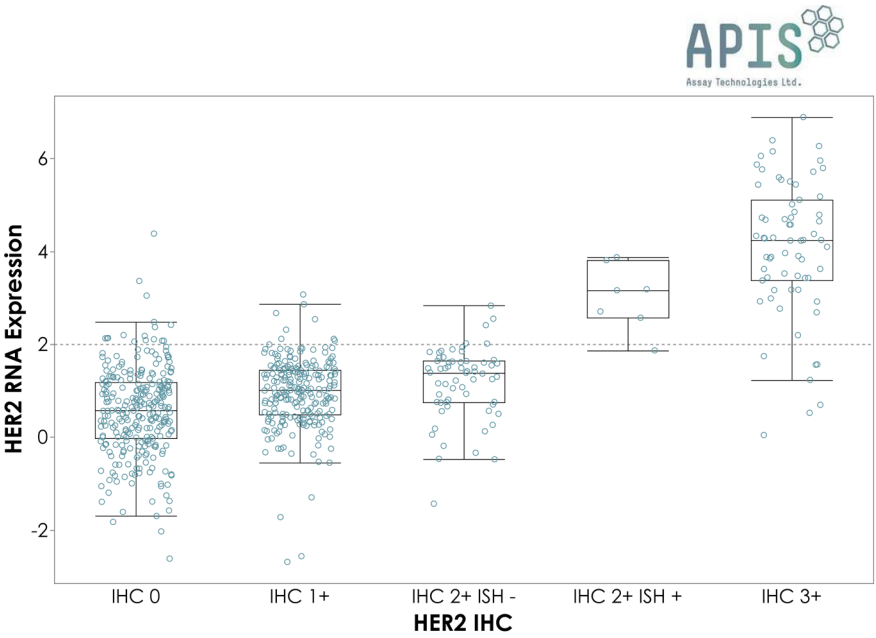


Figure 14 Correlation to HER2 IHC Score for ERBB2

Concordance scatter plot between APIS BC Subtyping Kit (HER2/ERBB2) and IHC-based biomarker assessment. Analysis of 652 breast cancer CNB and resected specimens. HER2 scoring as per ASCO/CAP guidelines; negative (0, 1+), positive (3+), unresolved (2+) sample status assigned positive or negative upon reflex testing. Y axis; APIS mRNA gene expression (ΔCt value) with assay cut-off (dashed line) for positive/negative calling. X axis; IHC score. High correlation observed between APIS BC Subtyping Kit and IHC score. Overlap between "1+" and "0" HER2 IHC status and ERBB2 RNA expression

13.7 Guardbanding

Guardbanding was performed to determine the robustness of the BC Subtyping Kit.

13.7.1 Thermal Cycling Guardband

The tolerance of the BC subtyping Kit to temperature variations of the reverse transcriptase, denaturing and annealing step during PCR that could be introduced by the QuantStudio 5 Dx instrument. The standard cycling conditions for the BC Subtyping Kit are a reverse transcription (RT) step at 50°C for 10 min, an initial Taq activation at 95°C for 30 s, followed by 40 cycles of denaturation at 94°C for 10 s and annealing at 60°C for 30 s. Temperature control on the QuantStudio 5 Dx 0.2mL block is to within 0.25°C; RT, annealing and denaturation temperatures were tested at ±1°C for each step. Four FFPE clinical RNA samples were selected, two high relative expression of *ESR1* and low *MKI67*, and two high relative expression of *MKI67* and low *ESR1*. Two replicates per sample per condition were assessed. RT temperature at -0.3°C was also tested.

No effect on kit performance was observed by varying temperatures of the annealing and denaturation steps. The kit performance was affected when the RT step was tested at 1°C below the set condition, however this was not observed when tested at -0.3°C. The BC Subtyping Kit performance is not affected when the PCR temperature varies within the accuracy of the QuantStudio 5 Dx instrument.

13.7.2 Volumetric Guardband

The acceptable tolerance for operator pipetting variation that still produces reliable results were determined. The standard volumes as stated in the instructions for use are 15 µL master mix (manufactured by mixing 11 µL of enzyme mix and 4 µL of buffer mix), and 1 µL of PPmix. The volumetric tolerance was tested by varying the volume of each individual component at set up by keeping the volume of the other components constant. Each component volume was varied by ±20%. The sample used for this study comprised a single lot of positive control contrived from synthetic RNA for each target. Each combination was plated out as 16 µL total master mix with 4 µL of template. Two

replicates per sample per condition were assessed across two PCR runs.

Volumetric errors in setup between -20% to +5% did not affect the performance of the targets or IC, with all data falling within precision of the assay. The minimum reaction number for kit's set up is six thus the minimum pipetting volume will be 6µL, with a deviation in volume from that set on the pipette acceptable -20% to +5%. Pipettes meeting calibration with a suitable volume range all meet this precision. Thus, volumetric changes within the precision of pipets do not affect performance of the BC Subtyping Kit.

13.7.3 PCR Setup Guardband

The effect of thawing and incubation time before PCR set up on BC Subtyping kit performance was determined. Two FFPE clinical RNA samples were selected, one sample with high relative expression of *ESR1* and low *MKI67*, and one sample with high relative expression of *MKI67* and low *ESR1*. Four replicates per sample per condition were assessed across two PCR runs.

Kit component handling
A single kit was thawed at 4°C and used for immediate PCR set-up for two identical PCR runs (nominal condition). A separate kit of the same lot was thawed at room temperature for one, two, and four hours, with PCR set-up at the end of each incubation point.

Master mix handling
A single kit lot was thawed at 4°C and used for immediate PCR set-up for two identical PCR runs (nominal condition). Additional master mix was incubated at room temperature for one, two, and four hours, with PCR set-up at the end of each incubation point.

Performance was lost at one hour by incorrect target calls for both master mix set-up and kit components incubated at room temperature. Thus, users are recommended to always thaw reagents at 2-8°C and to set up and run PCR plates immediately.

13.8 Cross-contamination

Assessment of cross-contamination and carry-over within the extraction and PCR system was performed by testing six ESR1 positive and twenty-five ESR1 Negative samples. Seven extraction runs were performed: negative samples only, mixed samples checkerboard, negative samples only, mixed samples checkerboard and finally negative samples only. All samples were diluted to 2.5 ng/µL for PCR testing. PCRs were performed in duplicate with two replicates per sample being tested. The study was design as per CLSI EP10-A3-AMD.

The correct call for negative samples was calculated. The proportion of negative samples which reported the correct call was 85/86, the percentage of correct calls was 98.8% (95% CI 93.7-99.8).

The data shows that potential carry-over and cross contamination within the extraction and PCR do not cause false positive results when using the RNeasy DSP FFPE Kit and the BC Subtyping Kit.

13.9 Specimen Stability

13.9.1 FFPE and RNA Stability

A literature search was conducted to determine the effect of formalin fixation and RNA storage stability. The literature showed that RNA extracted from freshly fixed, and paraffin embedded material has been shown to be almost intact with high integrity. Degradation of nucleic acids is noted in FFPE tissue stored at room temperature, this being slowed by storage at 2-8°C, and prevented by storage at -20°C or -80°C.

While it is noted that FFPE tissues stored for longer yield a reduced quantity of nucleic acid, these tissues could be used for the analysis of some small amplicons <150bp in samples stored up to 10 years being suitable for use.
However, FFPE material should not be used older than 20 months from embedding with the BC subtyping kit.

RNA is stable at -80°C for up to a year without degradation.

13.9.2 RNA Freeze Thaw

The effect of freeze-thaw of RNA on the performance of the BC Subtyping Kit was assessed. RNA extracted from two representative clinical breast cancer FFPE samples (*ESR1* positive or *MKI67* high) were used for testing. The two RNA stock samples were thawed up to five times at 2-8°C for 30 min, diluted to a concentration of 2.5ng/µL, returned to the freezer and the dilutions then tested by RT-qPCR. ΔCt values for both samples were within the precision of the kit across all five freeze thaw cycles tested, therefore freeze-thaw had no effect on kit performance.

13.10 Kit Stability

Storage (real time), transport (simulated) and in-use (freeze-thaw) stability were assessed. RNA from two contrived FFPE breast cancer samples was used for testing (one positive for *ESR1* and one positive for *MKI67*).

The study design is based on the CSLI guidelines (EP25A).

The kits lots were stored at '-20°C' (-10 to -30°C') in a temperature controlled and monitored freezer.

For the storage stability study 10 time points were assessed, starting with time point 0 and then following days 30, 58, 86, 124, 154, 182, 246, 301 and 366. A further time point at 459 days is planned. Three kit lots were assessed during the storage stability study. For each of the time points to be tested, sufficient RNA aliquots for each of the two samples were prepared and stored at -50 to -100°C. At each of the time points three kit lots were used to test three replicates of each RNA sample. For time point 0 the kits were tested twice in order to obtain more results for the baseline performance assessment. At each subsequent time point kits and RNA will be withdrawn from storage and used to repeat the initial testing. Monthly sampling showed stability for the *ESR1*, MKI67, *IPO8* and *PUM1* targets across a period of 12 months.

One kit lot was assessed for in-use stability. 5 times, during the testing period, the components of the kit were defrosted for 30 min on frozen armor beads and then tested by RT-qPCR. After being thawed for 30 min, the two kits were returned to the freezer.

Three kit lots were assessed for transport stability. The kit was placed on dry ice for five days ±2 hours and subsequently stored in a '-20°C' freezer for two days ±2 hours. This process was repeated twice to simulate the transport from the manufacture to the distributor and from the distributor to the customer. Taking into consideration that any of the stages could face a delay, a third simulation took place repeating the storage on dry ice for five days ±2 hours and subsequently in the freezer.

100% correct call was observed for all time points tested. The BC Subtyping Kit is stable when stored at -20°C ±10°C and when freeze thawed four times. The real time stability data supports shelf life for 12 months. The kit is stable when transported on dry ice for three times for five days with two days at -30°C to -15°C between transportation.

13.11 Clinical Performance

Clinical accuracy of the BC Subtyping Kit was demonstrated relative to IHC using CNB and resected FFPE clinical specimens. 421 samples were collected prospectively. Testing was performed at one external site. For each specimen ER, PR, HER2 and Ki67 status was assessed by IHC (and/or ISH for *ERBB2*) before testing with the APIS Breast Cancer Subtyping Kit. RNA was extracted from 1–3 FFPE sections (2 µm) using the RNeasy DSP FFPE Kit (QIAGEN) following the kit handbook and the Breast Cancer Subtyping Kit instructions for use. Samples with sufficient RNA were diluted to 2.5 ng/µL for testing. A total of 378 samples were used for analysis. Agreement between the IHC/ISH results and the BC Subtyping Kit was calculated for each marker. Agreement for the BC Subtyping Kit proliferation score is calculated against Ki67 IHC. The overall percent agreement (OPA), positive percent agreement (PPA) and negative percent agreement (NPA) along with the corresponding two-sided exact 95% CIs results are shown in Table 23.

Table 23 Breast Cancer Subtyping Kit Agreement to IHC

	ESR1 (ER)	*PGR* (PR)	*ERBB2* (HER2)	*MKI67* (Ki67)	Proliferation Signature
Positive Percent Agreement (PPA)	94.2% (91.0-96.3) 293/311	89.3% (85.2-92.4) 251/281	88.9% (74.6-95.6) 32/36	69.2% (61.9-75.6) 119/172	73.8% (66.8-79.8) 127/172
Negative Percent Agreement (NPA)	89.6% (80.0-94.8) 60/67	88.7% (80.8-93.5) 86/97	97.4% (95.1-98.6) 333/342	88.8% (83.7-92.4) 182/205	86.3% (81.0-90.4) 177/205
Overall Percent Agreement (OPA)	93.4% (90.4-95.5) 353/378	89.2% (85.6-91.9) 337/378	96.6% (94.2-98.0) 365/378	79.8% (75.5-83.6) 301/377	80.6% (76.2-84.5) 304/377

Resolution testing of discordant samples was conducted using an independently validated dPCR test. The accuracy, sensitivity, and specificity, along with the corresponding two-sided exact 95% CIs following composite resolution are shown in Table 24.

Table 24: Breast Cancer Subtyping Kit Agreement to IHC following dPCR resolution testing.

	ESR1 (ER)	*PGR* (PR)	*ERBB2* (HER2)	*MKI67* (Ki67)
Clinical Sensitivity	98.3% (96.2-99.3) 296/301	93.5% (89.9-95.8) 257/275	97.1% (85.1-99.5) 33/34	89.5% (83.4-93.5) 128/143
Clinical Specificity	94.8% (87.4-98.0) 73/77	95.1% (89.1-97.9) 98/103	97.7% (95.5-98.8) 336/344	94.0% (90.2-96.4) 220/234
Clinical Accuracy	97.6% (95.5-98.7) 369/378	93.9% (91.0-95.9) 355/378	97.6% (95.5-98.7) 369/378	92.3% (89.2-94.6) 348/377

High clinical accuracy of the APIS Breast Cancer Subtyping Kit is observed.

14. Troubleshooting

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

15. Limitations

The product is intended for use with the QIAGEN RNeasy® DSP FFPE Kit.

The product is intended for use only on a QuantStudio 5 Dx real-time PCR cycler. Strict compliance with the Kit Handbook is required for optimal results.

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 analysisusing 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.

16. Associated Documents

Document No.	Document Title
100049556	QuantStudio™ 5 Dx IVD Software v1.0 USER GUIDE
100042186	QuantStudio™ 5 Dx Real-Time PCR Instrument USER GUIDE
1106945EN	RNeasy® DSP FFPE Kit Instructions for Use (Handbook)

17. 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

Symbol	Definition
	In-vitro Diagnostic (IVD)
	Keep away from sunlight
	Manufacturer
	Negative control
	Positive control
	Serial number
	Temperature limits, Upper and Lower
	Use by date

18. References

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