MicroSight® MSI

1-Step HRM Analysis

In vitro Diagnostic Assay for Detection of Microsatellite Instability in Cancer Patients - For Analysis of Paired and Unpaired Tumour Samples

INSTRUCTIONS FOR USE

REFERENCE NUMBERS
Ready-to-Use (RTU)
7201 (16 reactions)

Version 3.0
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CE IVD

Table of Contents

1 Intended purpose

MicroSight® MSI 1-step HRM Analysis is based on Real-Time Polymerase Chain Reaction (RT-PCR) followed by high-resolution melt (HRM) study on a BaseTyper™ 48 Real-Time PCR Instrument - 48 well block with 4 channel detection, Gentier 48E/48R Real-time PCR system or a BaseTyper™ 48 Quiet HRM Real-Time PCR System. The assay is intended for the qualitative evaluation of microsatellite stability (MSS)/instability (MSI), in human DNA from solid biopsies such as Formalin-Fixed Paraffin-Embedded (FFPE) tissue. MSI is known as a prognostic marker and a predictive factor of response to certain therapies like check point inhibitors and 5-fluorouracil. MicroSight® MSI 1-step HRM Analysis is an in vitro diagnostic medical device intended as an aid to correct treatment selection.

1.1 Intended user

MicroSight® MSI 1-step HRM Analysis is intended for use by healthcare professionals or qualified laboratory personnel specifically instructed and trained in the techniques of real-time PCR, HRM measurements as well as proficient in handling biological samples. Medical interventions based on results from this product requires medical authorisation.

2 Background

Microsatellites are genetic motifs consisting of short (1-6 bases) nucleotide sequences repeated up to 100 times comprising approximately 3% of the human genome. During replication, these sequences are susceptible to errors, both deletions and insertions, normally corrected by the mismatch repair (MMR) system. Uncorrected microsatellite errors are commonly referred to as MSI, and this is observed with a deficient MMR system where the replication errors result in different lengths of the microsatellites in the MMR system-deficient tissue. MSI is used to assist clinicians and genetic counsellors in the diagnosis of a type of neoplastic inherited syndrome[1].

Inherited deregulation by epigenetic and genetic changes in genes (like PMS2, MLH1, MSH2, or MSH6) and their gene products leading to compromised MMR system response is known as Lynch syndrome (LS) or hereditary nonpolyposis colorectal cancer (CRC). Apart from a high risk of developing CRC, people suffering from LS also have significantly increased risk of developing endometrial cancer, gastric cancer, ovarian cancer, small bowel (small intestinal) cancer, pancreatic cancer, urinary tract cancer, kidney cancer, bile duct cancer, and certain skin and brain tumours[2]. In patients with CRC, MSI is associated with a slightly better prognosis and low benefit of 5-flourouracil-based chemotherapy. More recently, immune checkpoint inhibitors have demonstrated great efficacy in patients with MSI, not only in CRC, leading FDA and EMA to approve the treatment of any MSI solid tumour with immunotherapies[3,4].

Uniform evaluation of MSI status is in the "Bethesda Guidelines"[5] suggested to include two mononucleotide microsatellites (BAT25 and BAT26) and three dinucleotide microsatellites (D2S123, D5S346 and D17S250). With five microsatellites analysed, patients can be categorised as microsatellite stable, MSS (0 markers exhibiting instability), microsatellite unstable-low, MSI-L (1 marker being unstable), or microsatellite unstable-high, MSI-H (2 or more loci being unstable). As individual dinucleotide markers of the Bethesda panel only exhibit instability in 60-80% of MSI-H tumours, it has been suggested to exchange these markers with an expanded set of mononucleotide markers. Thus, Suraweera and co-workers suggested to evaluate MSI status by five mononucleotide loci: BAT25, BAT26, NR21, NR22, and NR24[6]. Apart from increasing sensitivity, the monomorphic nature of this panel has been suggested to eliminate the need for parallel investigation of paired samples from patients comparing un-involved (germline) tissue to tumour genomic DNA (gDNA). Alternative mononucleotide loci such as MONO27 have revealed equally high specificity and sensitivity[7].

3 Test principle

By detecting MSS/MSI, MicroSight® MSI 1-step HRM Analysis offers a status of the patients mismatch repair (MMR) system affecting prognosis and selection of treatment in cancer patients. MicroSight® MSI 1-step HRM Analysis analyses the stability/instability of five quasi-monomorphic mononucleotide microsatellite sequences; BAT25, BAT26, NR22, NR24, and MONO27[5,6,7]. The PCR products obtained from the analysis are analysed based on their melting profile, which varies according to e.g., heterozygosity and length, where especially length plays an important role in MSI analysis.

A tumour biopsy contains a mix of normal cells and tumour cells. If the tumour has MSI, it will contain both the original length of the microsatellite due to the normal cells in the biopsy and microsatellites with alteration in length from the MSI tumour cells. The different lengths of the microsatellites affect the shape of the melting curve, and thus it is possible to differentiate between MSS and MSI tumours by comparing the shape of the melting curve of the tumour tissue to the normal tissue[8]. When applying a universal reference, which is from homozygous DNA, a tumour with heterozygous loci will be analysed as unstable. However, due to the low frequency of heterozygosity in quasi-monomorphic loci, the possibility to have more than one locus with heterozygosity is substantially negligible. However, utilising a universal reference it is not possible to characterise tumours as MSI-L as heterozygosity occurring in a single locus would be

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[1] Vaksman and Garner, 2015; PMID: 25691061
[2] Richman S, 2015; PMID: 26315971
[3] Le et al., 2015; PMID: 26028255
[4] Le et al., 2017; PMID: 28596308
[5] Boland et al., 1998; PMID: 9823339
[6] Suraweera et al., 2002; PMID: 12454837
[7] Bacher et al., 2004; PMID: 15528789
[8] Martín-Núñez et al., 2012; PMID: 21946052

observed as positive. Misclassification of MSI-H due to intrinsic heterozygosity is quite null, keeping in mind that co-occurrence of even two heterozygotic loci is rare[6,7], and the tumour sample must be unstable in at least three loci to be characterised as MSI-H when using a universal reference with MicroSight® MSI 1-step HRM Analysis. However, it is still being debated whether it is of clinical relevance to differentiate patients as being MSS or MSI-L[9,10].

MicroSight® MSI 1-step HRM Analysis is intended for analysis of human genomic DNA (gDNA) from fresh, frozen, or FFPE CRC or endometrial cancer tissue biopsies. MicroSight® MSI 1-step HRM Analysis has high requirements for precise block temperature regulation and performance of HRM module and can only be used with BaseTyper™ Real-Time PCR-instruments.

3.1   Explanation of the assay

The MicroSight® MSI 1-step HRM Analysis assay is based on PentaBase's highly sensitive DNA technology. MicroSight® MSI 1-step HRM Analysis comprises five primer pairs, including (Su)Primer™ and labelled EasyBeacon™ probes covering five microsatellites (BAT25, BAT26, NR22, NR24, and MONO27).

3.1.1   EasyBeacon™ Probes

EasyBeacon™ probes are PentaBase's alternative to molecular beacons labelled with a fluorophore at the 5'end, a quencher at the 3'end, an internal quencher, and with the addition of pentabases, thereby removing the need for adding a stem and giving the probe a significantly improved signal-to-noise ratio, higher specificity and higher sensitivity compared to conventional molecular beacon probes. EasyBeacon™ probes in the MicroSight® MSI 1-step HRM Analysis assay are labelled with PentaGreen™ (detected on the same channel as FAM) in combination with a Green Quencher™.

3.1.2   SuPrimers™

SuPrimers™ are standard DNA primers modified with one or more pentabases. The pentabases provide increased specificity and sensitivity and reduce primer-dimer formation.

3.2   Product variants

MicroSight® MSI 1-step HRM Analysis is one product supplied as Ready-to-Use (RTU), which can be used in Paired or Unpaired analysis. The RTU version is pre-dispensed and only need addition of template before analysis.

3.3   Principle of the procedure

Non-tumour tissue and tumour tissue DNA from the same patient (Paired analysis) or universal reference DNA and tumour tissue DNA (Unpaired analysis) are amplified using the MicroSight® MSI 1-step HRM Analysis assay in separate strips. The melt curves of the tumour tissue DNA are compared to the melt curves of non-tumour tissue DNA or universal reference DNA to detect differences in the melt shape, which reflects the length variation of the microsatellite loci. See section 7 for more details.

4 Reagents and materials

The materials provided with the MicroSight® MSI 1-step HRM Analysis can be found in **Table 1**. Materials and instruments required, but not provided can be found in **Table 2**.

4.1   Storage and stability

Assay performance has been evaluated after storage at -20°C for 6 months. Assay performance was found to be within specifications. Further studies are ongoing to investigate further shelf-life extension.

4.1.1   In-use stability

Assay performance has been evaluated during repeated freeze-thaw cycles in a 72-hour time period. The MicroSight® MSI 1-step HRM Analysis assay was subjected to 8 freeze-thaw cycles. At each cycle, the assay was thawed at room temperature and exposed to light for at least 30 min. 3 reactions were subsequently transferred to new tubes and stored at 4°C until analysis. The stock mix was frozen in 2 h intervals or overnight. Assay performance was found to be unchanged after 8 freeze-thaw cycles. Do not leave thawed assays at room temperature for an extended period (>1 hour).

4.1.2   Shipping stability

Examination of stability after prolonged exposure to high temperature and subsequent long-term storage at correct temperature (-20°C) has not been tested. Therefore, the contents of any shipment received that are no longer frozen should be regarded as damaged and returned to PentaBase.

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[9] Li et al., 2020; PMID: 31956294
[10] Puliga et al., 2021; PMID: 33721595

4.2 Materials provided

MicroSight® MSI 1-step HRM Analysis is supplied as Ready-to-Use assays in pre-aliquoted 0.2 mL PCR strips. All reagents needed for the PCR amplification and microsatellite length variation analysis of extracted genomic DNA are pre-loaded into the strips. Furthermore, a universal reference and a positive (unstable in all five loci) and negative (stable in all five loci) control are included when purchasing MicroSight® MSI 1-step HRM Analysis (**Table 1**). The universal reference and the negative control both consist of 1 ng/µL human gDNA with similar lengths of the investigated five loci, and the positive control consists of a 20% 1 ng/µL human MSI cancer cell-line, with divergent lengths of the five loci, in 80% 1 ng/µL universal reference gDNA.

In addition, software analysis templates for paired and unpaired analysis for the BaseTyper™ 48.4 Quiet HRM Real-Time PCR System will be provided by email.

NOTE: The universal reference and the negative and positive controls should only be used for MicroSight® MSI 1-step HRM Analysis.

Table 1. List of materials provided with MicroSight® MSI 1-step HRM Analysis as Ready-to-Use (RTU).

Description	Tube no.	Kit components	Contents
	RTU		
16x5 strips with assay	A B C D E	BAT25 simplex BAT26 simplex NR22 simplex NR24 simplex MONO27 simplex	Synthetic DNA and PCR Master Mix
Universal reference	-	Universal reference (8 reactions*)	Human gDNA in TE buffer
Positive control	-	Positive control (2 reactions*)	Human MSI cancer cell-line in 80% 1 ng/µL human gDNA in TE buffer
Negative control	-	Negative control (2 reactions*)	Human gDNA in TE buffer

*1 reaction corresponds to one 5 well strip.

4.3 Materials and Instruments required but not provided

Table 2. Materials and instruments required but not provided.

Materials
Pipettes (1-10 µL, 10-100 µL)
Sterile pipette Tips
Centrifuge for spinning PCR tubes, strips or plates
Vortex instrument
DNA extraction method or instrument
DNA extraction method or instrument (E.g., BasePurifier™ 32, PentaBase A/S, Ref. No. 715)
DNA Extraction kit for FFPE samples (E.g., Nucleic Acid Extraction Kit for FFPE DNA Extraction, Xi'an TianLong Science and Technology Co., Ltd., distributed by PentaBase A/S, Ref. No. T165H)
Real-Time PCR instrument
BaseTyper™ 48.4 Quiet HRM Real-Time PCR System (PentaBase A/S ref. no. 754) or Gentier 48E/48R Real-Time PCR system (Xi'an Tianlong Science and Technology Co., Ltd., China)

NOTE: Please be aware that this assay is only compatible with the PCR instruments described in **Table 2**.

5 Warnings and precautions

- For *in vitro* diagnostic use.
- MicroSight® MSI 1-step HRM Analysis is not intended for diagnosing any type of cancer, but only as a supplement for other prognostic factors for the selection of patients who might benefit from a specific treatment (companion diagnostics).
- Good laboratory practices and careful adherence to the procedures specified in these Instructions for Use are necessary. Wear laboratory coats, laboratory gloves, and eye protection when handling biological samples and reagents. Laboratory gloves must be changed between handling different biological samples. Work in clean environments to avoid contaminations.
- Remove gloves and wash hands thoroughly after handling samples and reagents.
- Be aware that Ready-to-Use PCR strips from different LOTs should not be used for the same patient either for paired or unpaired analysis. Only one LOT should be used to analyse a patient.
- Do not use reagents that have expired.
- Do not use damaged MicroSight® MSI 1-step HRM Analysis tubes or strips.
- Do not use a MicroSight® MSI 1-step HRM Analysis pre-dispensed in a Ready-to-Use PCR tube that has been dropped while open.
- Do not open the tubes or unseal wells during or after amplification following completing the PCR program.
- Be aware of the placement and orientation of the PCR tubes in the PCR machine in relation to how the samples are named in the PCR software.
- Consult relevant real-time qPCR Instrument User Guides for additional warnings, precautions, and procedures to reduce the risk of contamination.
- Dispose of used MicroSight® MSI 1-step HRM Analysis tubes, pipette tips, and specimen tubes according to local, state, and federal regulations for biological material.
- The reagents should not be diluted to a lower concentration than stated in the protocol. This may affect the performance of the assay.
- Do not substitute the reagents with others, as it may affect the performance of the assay.
- Inadequate or inappropriate sample collection, storage, and transport may yield incorrect or invalid results.
- Verify eligibility of the DNA samples as DNA samples can be non-homogeneous and of varying quality, which might affect the analysis. In cases of suspected DNA degradation, it is recommended to verify DNA integrity and the amount of amplifiable DNA by a PCR-based method.
- To protect the included fluorescent probes, avoid exposure to light for extended periods.
- Use caution not to contaminate reagents and samples. Dedicated pipettes should be used, and it is recommended to have separate areas for sample preparation and PCR running.
- All used instruments and equipment should be calibrated and perform in accordance with their original specifications.
- Do not reuse any of the reagents included in MicroSight® MSI 1-step HRM Analysis.
- Do not use any of the reagents included in MicroSight® MSI 1-step HRM Analysis for other purposes than MicroSight® MSI 1-step HRM Analysis assay analysis.

6 Sample handling

6.1 Sample collection, transport, and storage

Specimens should be human gDNA extracted from formalin-fixed paraffin-embedded (FFPE), fresh, or fresh-frozen tumour sections and, for paired analysis, comparable germline gDNA (non-tumour tissue DNA). It is recommended that FFPE samples are collected, transported, processed, and stored according to ISO 20166-3:2018.[11] to ensure optimal DNA quality.

6.2 Sample purification

Extraction of genomic DNA from FFPE samples should be performed using genomic DNA extraction kits and/or procedures specially designed for handling of FFPE samples according to the manufacturer´s instructions including steps for deparaffinisation and sample digestion to remove PCR inhibitors embedded in the sample. It is recommended to evaluate DNA integrity and amplifiability by PCR-based methods according to ISO 20166-3:2018.

MicroSight® MSI 1-step HRM Analysis assay is valid using a DNA concentration of 1-10 ng/µL. This concentration range is based on a Real-Time PCR quantification assay, and it is recommended to evaluate DNA integrity and amplifiability by PCR-based methods according to ISO 20166-3:2018. We recommend using **2-5 ng/µL** if the concentration is determined by any other method than Real-Time PCR.

NOTE: It is important that the normal and tumour DNA in paired analysis is diluted to the **same** concentration.

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[11] Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for formalin-fixed and paraffin-embedded (FFPE) tissue — Part 3: Isolated DNA

7 Procedure

7.1 BaseTyper™ instrument program setup

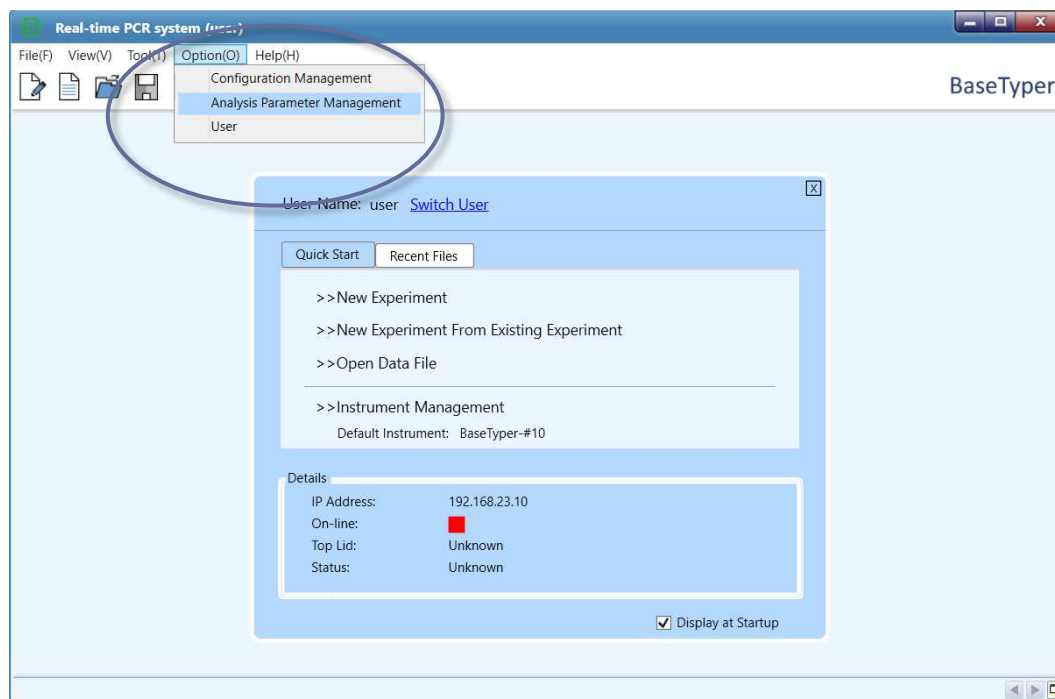1. Click on **Option(O)** and go to **Analysis Parameter Management (Figure 1)**.

Figure 1. BaseTyper™ Instrument Analysis Parameter Management setup.

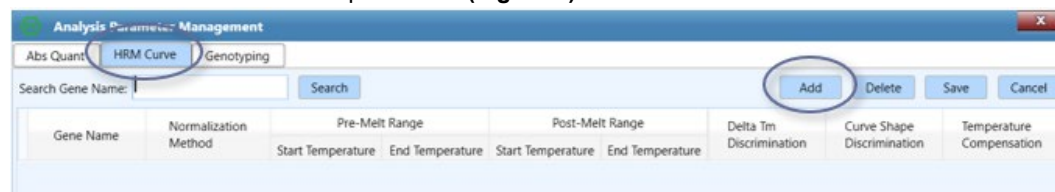2. Click on the fan **HRM Curve** and press **Add (Figure 2)**.

Figure 2. Adding HRM curve.

3. Type "BAT25" under the column **Gene Name** and fill in the columns as shown in **Figure 3**.
4. Repeat this for BAT26, NR22, NR24, and MONO27, by clicking on **Add** and type in the information shown below.
5. Remember to press **Save** after you finished typing in all the information. **NOTE:** It is only necessary to do the set-up once. However, if the software is re-installed, or if another computer is set up for the analysis, this procedure must be repeated.
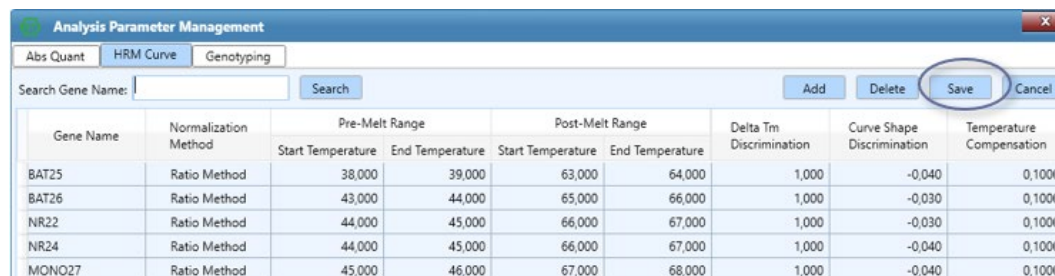

Gene Name	Normalization Method	Pre-Melt Range		Post-Melt Range		Delta Tm Discrimination	Curve Shape Discrimination	Temperature Compensation
		Start Temperature	End Temperature	Start Temperature	End Temperature			
BAT25	Ratio Method	38,000	39,000	63,000	64,000	1,000	-0,040	0,1000
BAT26	Ratio Method	43,000	44,000	65,000	66,000	1,000	-0,030	0,1000
NR22	Ratio Method	44,000	45,000	66,000	67,000	1,000	-0,030	0,1000
NR24	Ratio Method	44,000	45,000	66,000	67,000	1,000	-0,040	0,1000
MONO27	Ratio Method	45,000	46,000	67,000	68,000	1,000	-0,040	0,1000

Figure 3. HRM curve setup.

7.2   Paired samples procedure

IMPORTANT: When using MicroSight® MSI 1-step HRM Analysis for **paired sample analysis**, a patient-specific reference (normal gDNA of non-tumour origin) must be included for MSI analysis of the corresponding tumour biopsy. The reference and MSI analysis strips should be placed in the same PCR heating block to ensure minimal run variation.

7.2.1   Preparation of MicroSight® MSI 1-step HRM Analysis assay

1. Use two Ready-to-Use PCR strips (strip 1 and strip 2) for each patient to be analysed. **NOTE:** Four patients can be analysed in one run. The BaseTyper™ Instrument measures fluorescence from the side of the strips which allows for marking of strips with e.g., patient or sample number on the lids.
2. Spin down the PCR strip (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the tubes. **NOTE:** Do not leave thawed assays at room temperature for an extended period (>1 hour), alternatively keep on ice.

7.2.2   Adding DNA to the tubes

The concentration of the DNA must be in the range of 1-10 ng/µL, but it is recommended to use 2-5 ng/µL. Use the same concentration for the normal and tumour patient DNA.

1. Vortex DNA samples briefly prior to use.
2. Add 5 µL of DNA from the **normal** tissue of patient 1 to each of the 5 tubes of strip 1. Seal all tubes.
3. Add 5 µL of DNA from the **tumour** tissue of patient 1 to each of the 5 tubes of strip 2. Seal all tubes.
4. Repeat for patient 2 to 4.

NOTE: It is recommended to run a strip with each of the included controls (positive and negative control) when implementing the assay and hereafter occasionally including one or more of the controls in the runs. The controls can only be tested against the universal reference. The controls and universal reference are loaded in a similar fashion as the normal/tumour DNA. However, only one strip is required for the control and universal reference.

7.2.3   Placing strips in the PCR instrument

1. Briefly vortex strips (2-5 sec.) to enhance elimination of air bubbles and spin down the PCR strips (1-2 minutes at 4000-5000 rpm).
2. Place the PCR tubes in the BaseTyper™ Instrument. Patient 1 non-tumour sample is placed with the mark in position A1, and patient 1 tumour sample is placed with the mark in position B1. Repeat for patient 2 (C1, D1), 3 (E1, F1), and 4 (H1, G1) (**Figure 4**).
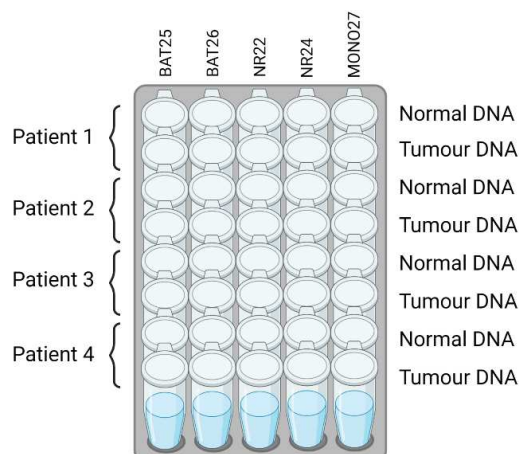

Figure 4. Placement of strips in the BaseTyper™ Instrument.

7.3   Unpaired samples procedure

IMPORTANT: When using MicroSight® MSI 1-step HRM Analysis assay for **unpaired sample analysis**, a no patient-specific reference should be included for MSI analysis. A universal reference is provided with the kit. For each PCR run, one strip containing the universal reference must be included.

7.3.1   Preparation of MicroSight® MSI 1-step HRM Analysis assay

1. Use one Ready-to-Use PCR strip for the reference gDNA and one Ready-to-Use PCR strip for each patient to be analysed. **NOTE:** Seven patients can be analysed in one run. The BaseTyper™ Instrument measures fluorescence from the side of the strips which allows for marking of strips with e.g., patient or sample number on the lids.
2. Spin down the PCR strip (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the tubes. **NOTE:** Do not leave thawed assays at room temperature for an extended period (>1 hour), alternatively keep on ice.

7.3.2 Adding DNA to the tubes

The concentration of the DNA must be in the range of 1-10 ng/μL, but it is recommended to use 2-5 ng/μL.

1. Vortex DNA samples briefly prior to use.
2. Add 5 μL of DNA from the **universal reference** tube to each of the 5 tubes of the first strip. Seal all tubes.
3. Add 5 μL of DNA from the **tumour** tissue of patient 1 to each of the 5 tubes of the second strip. Seal all tubes.
4. Repeat step 3 for patient 2 to 7.

NOTE: It is recommended to run a strip with each of the included controls (positive and negative control) when implementing the assay and hereafter occasionally including one or more of the controls in the runs. The controls can only be tested against the universal reference. The controls are loaded in a similar fashion as the universal reference and the tumour DNA.

7.3.3 Placing strips in the PCR instrument

1. Briefly vortex strips (2-5 sec.) to enhance elimination of air bubbles and spin down the PCR strips (1-2 minutes at 4000-5000 rpm).
2. Place the PCR tubes in the BaseTyper™ Instrument. The universal reference strip is placed with the mark in position A1, and patient 1 tumour sample is placed with the mark in position B1 (**Figure 5**).
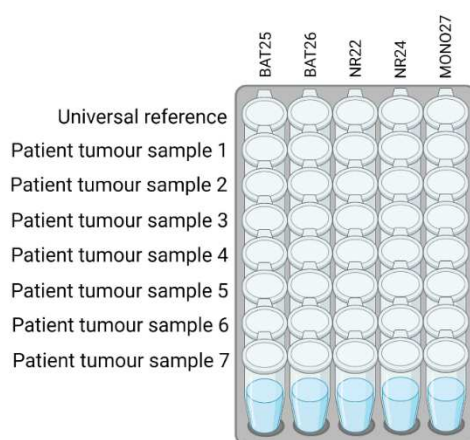

Figure 5. Placement of strips in the BaseTyper™ Instrument.

7.4 BaseTyper™ Instrument experimental setup – Paired Analysis

1. In the BaseTyper™ Instrument software, select **New Experiment From Existing Experiment** (**Figure 6**) and open the template "MSI BASETYPER TEMPLATE – PAIRED SAMPLES v1.0". The template will be provided by PentaBase A/S by email. **NOTE**: The template can be selected in two tabs in the software.
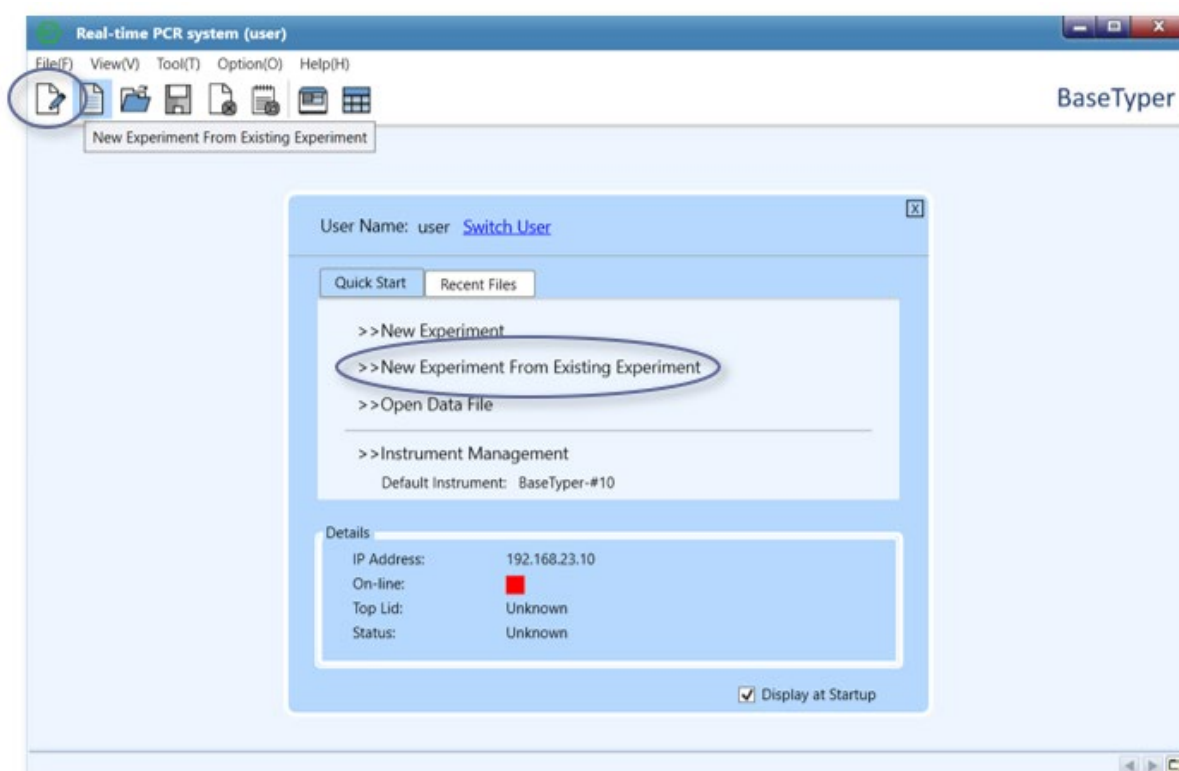
Figure 6. Setting up a new paired sample analysis experiment on the BaseTyper™ Instrument.

2. Give the experiment a name. It is recommended to name the experiment with the tumour sample ID numbers.
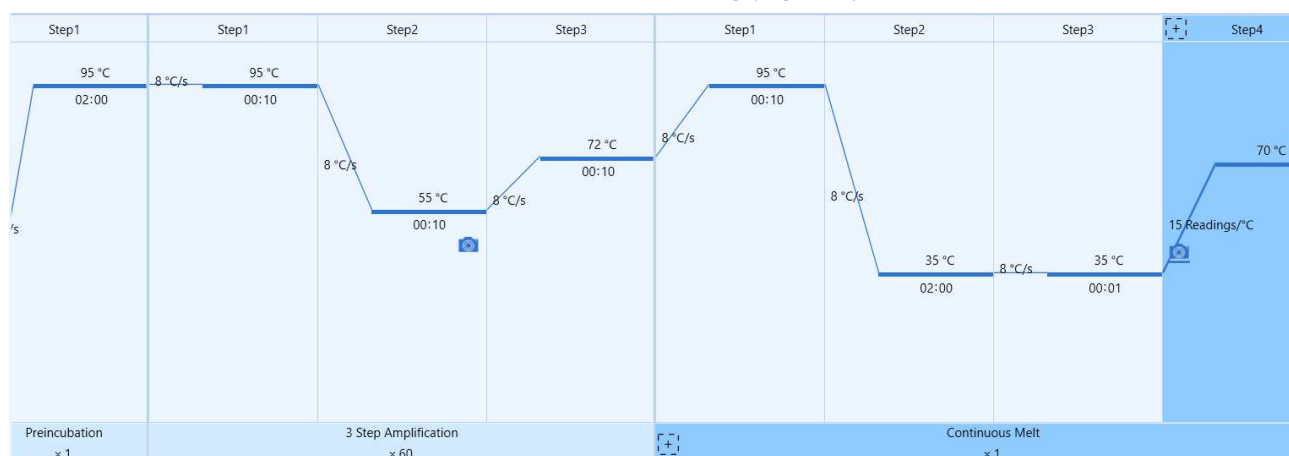3. The temperature profile can be viewed under **Run Setting** (**Figure 7**).

Figure 7. Setting the MicroSight® MSI 1-step HRM Analysis run program on the BaseTyper™ Instrument.

4. Remember to set the **Reaction Volume** to 24 µL under **Experiment** in the **Run Setting** tap.
5. Go to the **Sample Setting** tab (**Figure 8**).
6. Name the samples by marking a row from 1-5.
    a. For row A1-A5: In the **Sample ID** box type the sample ID after "Normal", e.g., the sample ID will be "Normal 324". Press "enter" on the keyboard.
    b. For row B1-B5: In **the Sample ID** box type the sample ID after "Tumour", e.g., the sample ID will be "T-324". Press "enter" on the keyboard.
    c. Repeat for all rows containing samples.
7. The naming of samples can also be set after the run has finished, but **not during the run**!
8. Do not change any other properties than the Sample ID. This can cause failure of analysis.

Figure 8. Sample settings for paired sample analysis.

7.4.1 Starting the run
1. Go to the **Run Monitoring** tab (**Figure 9**).
2. Select the instrument under **Model** in the Run Info window and press **Run**. The run will automatically be saved in the folder you chose when setting up the run. If auto-saving was not selected, save the run manually before closing the program.
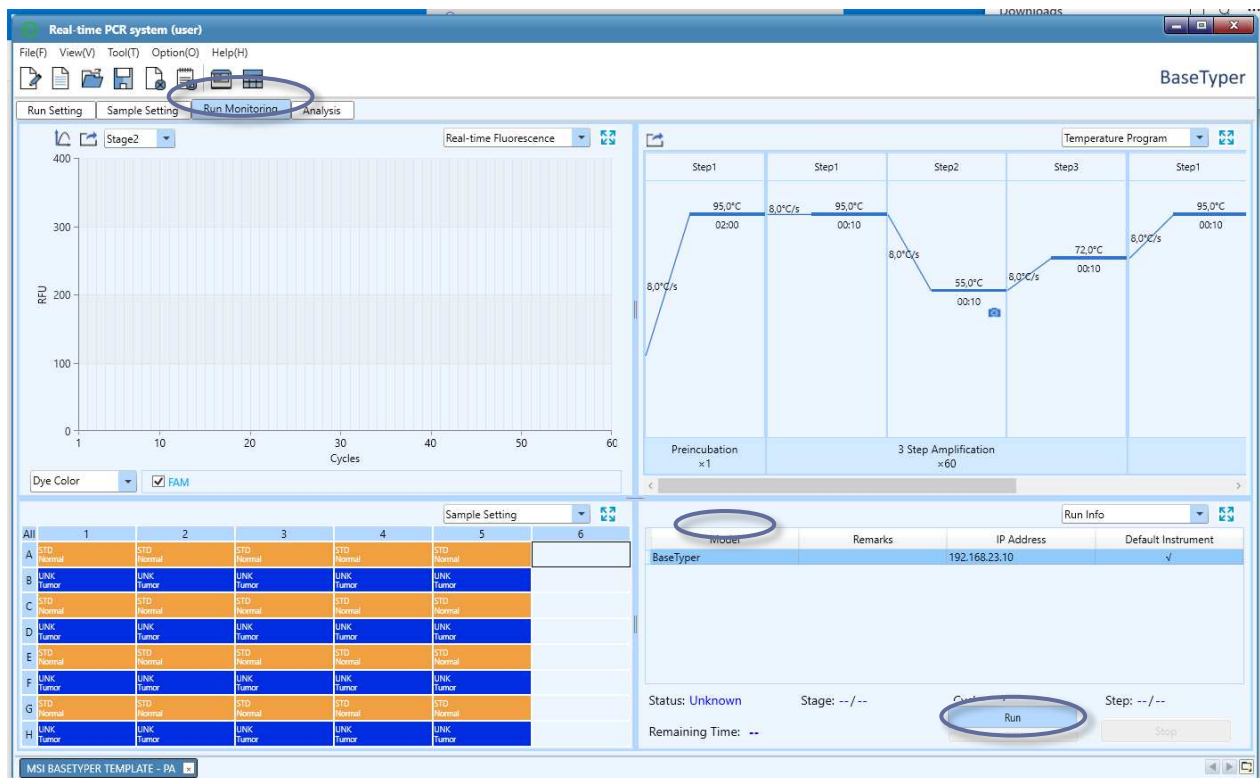

Figure 9. Starting the run at the BaseTyper™ Instrument.

7.5 BaseTyper™ Instrument experimental setup – Unpaired Analysis

1. In the BaseTyper™ Instrument software, select **New Experiment From Existing Experiment** (**Figure 10**) and open the template "MSI BASETYPER TEMPLATE – UNIVERSAL REFERENCE v2.1". The template will be provided by PentaBase A/S by email. **NOTE**: The template can be selected in two tabs in the software.
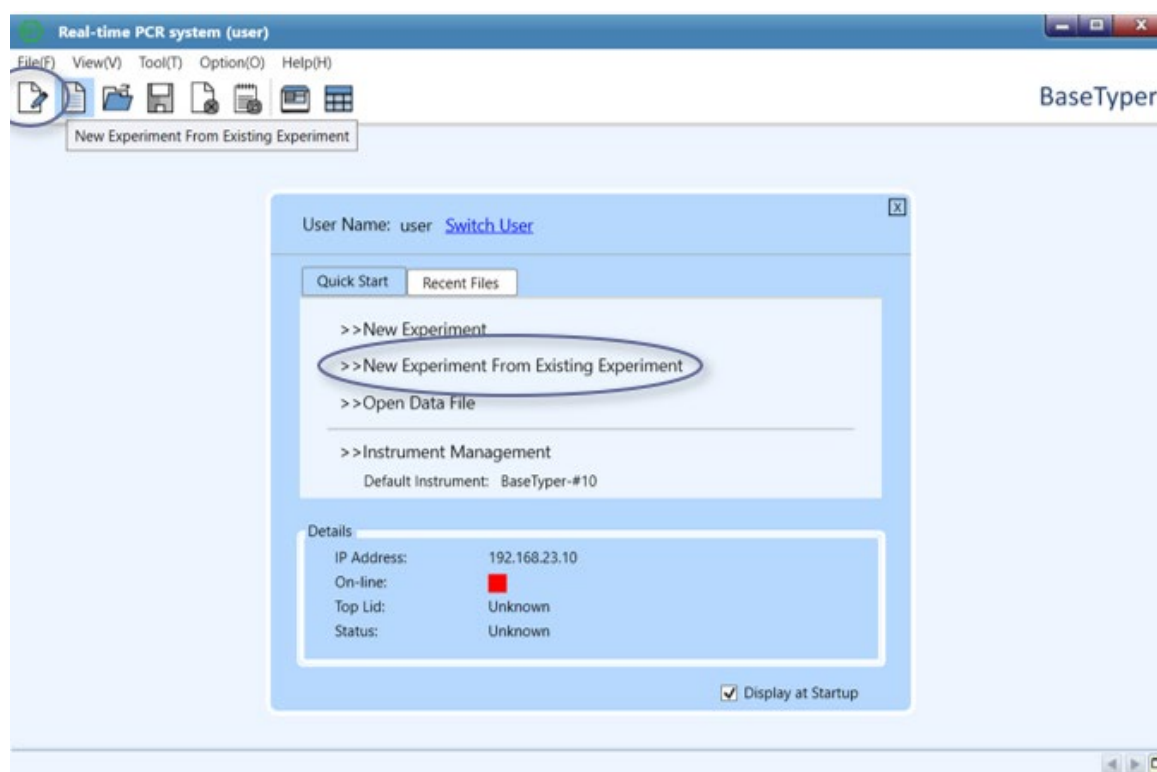

Figure 10. Setting up a new unpaired sample analysis experiment on the BaseTyper™ Instrument.

2. Give the experiment a name. It is recommended to name the experiment with the tumour sample ID numbers.
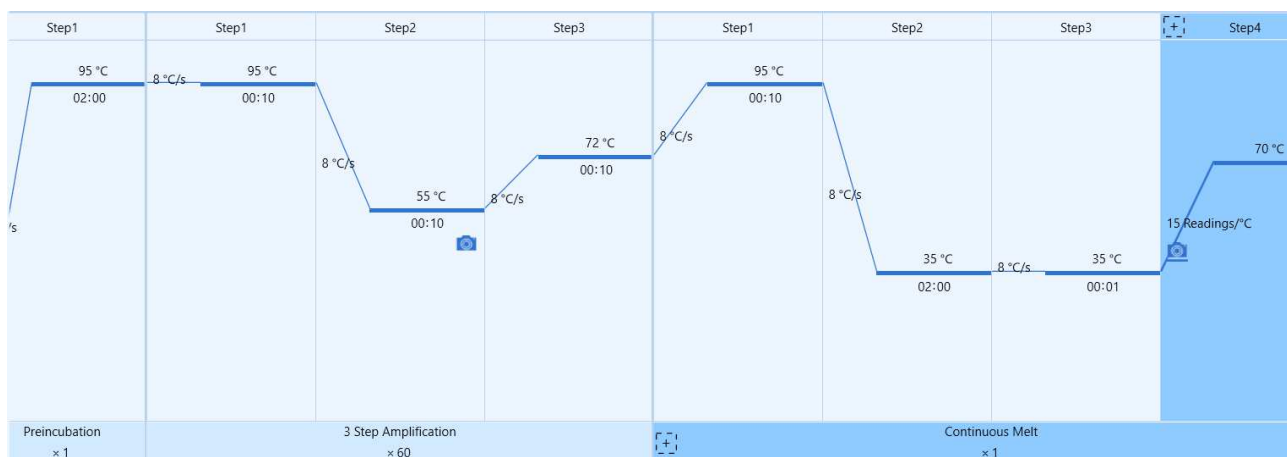3. The temperature profile can be viewed under **Run Setting** (**Figure 11**).

Figure 11. Setting the MicroSight® MSI 1-step HRM Analysis run program on the BaseTyper™ Instrument.

4. Remember to set the **Reaction Volume** to 24 µL under **Experiment** in the **Run Setting** tap.
5. Go to the **Sample Setting** tab (**Figure 12**).
6. Name the samples by marking a row from 1-5.
   a. You should not change anything in the universal reference (position A1-A5).
   b. For row B1-B5: Delete the name "Tumour sample 1" and type the tumour ID in the **Sample ID** box. Press "enter" on the keyboard.
   c. Repeat for all rows containing samples.
7. The naming of samples can also be set after the run has finished, but **not during the run**!
8. Do not change any other properties than the Sample ID. This can cause failure of analysis.
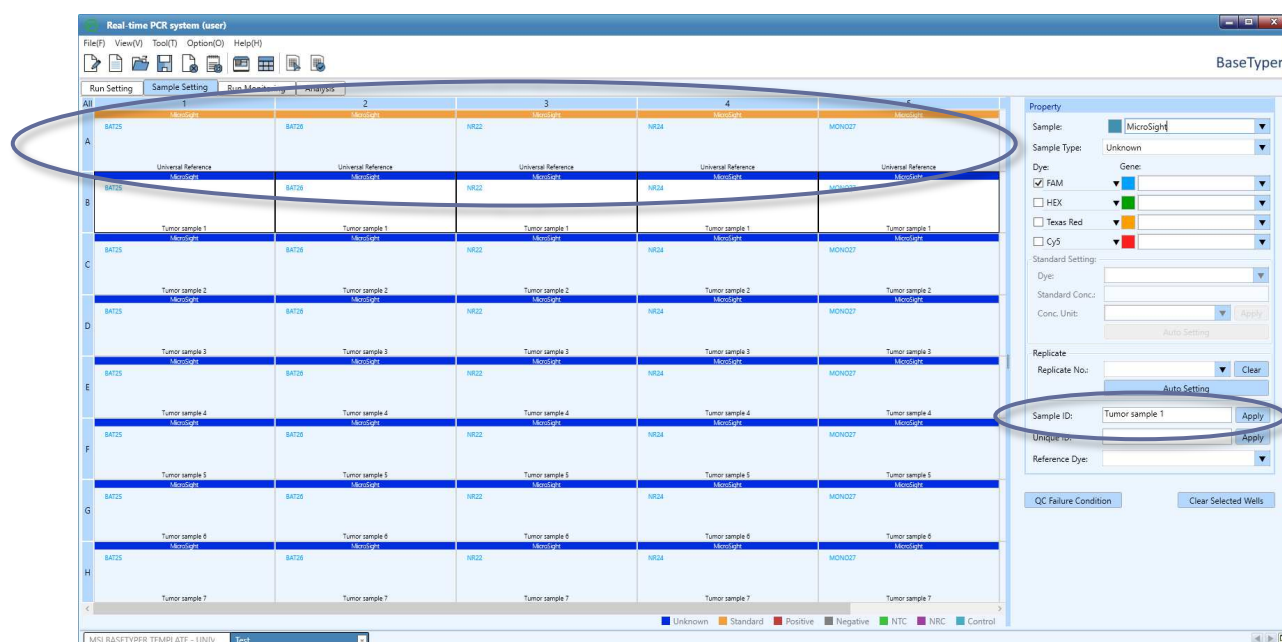
Figure 12. Sample settings for unpaired sample analysis.

7.5.1   Starting the run
1.   Go to the **Run Monitoring** tab (**Figure 13**).
2.   Select the instrument under **Model** in the Run Info window and press **Run**. The run will automatically be saved in the folder you chose when setting up the run. If auto-saving was not selected, save the run manually before closing the program.
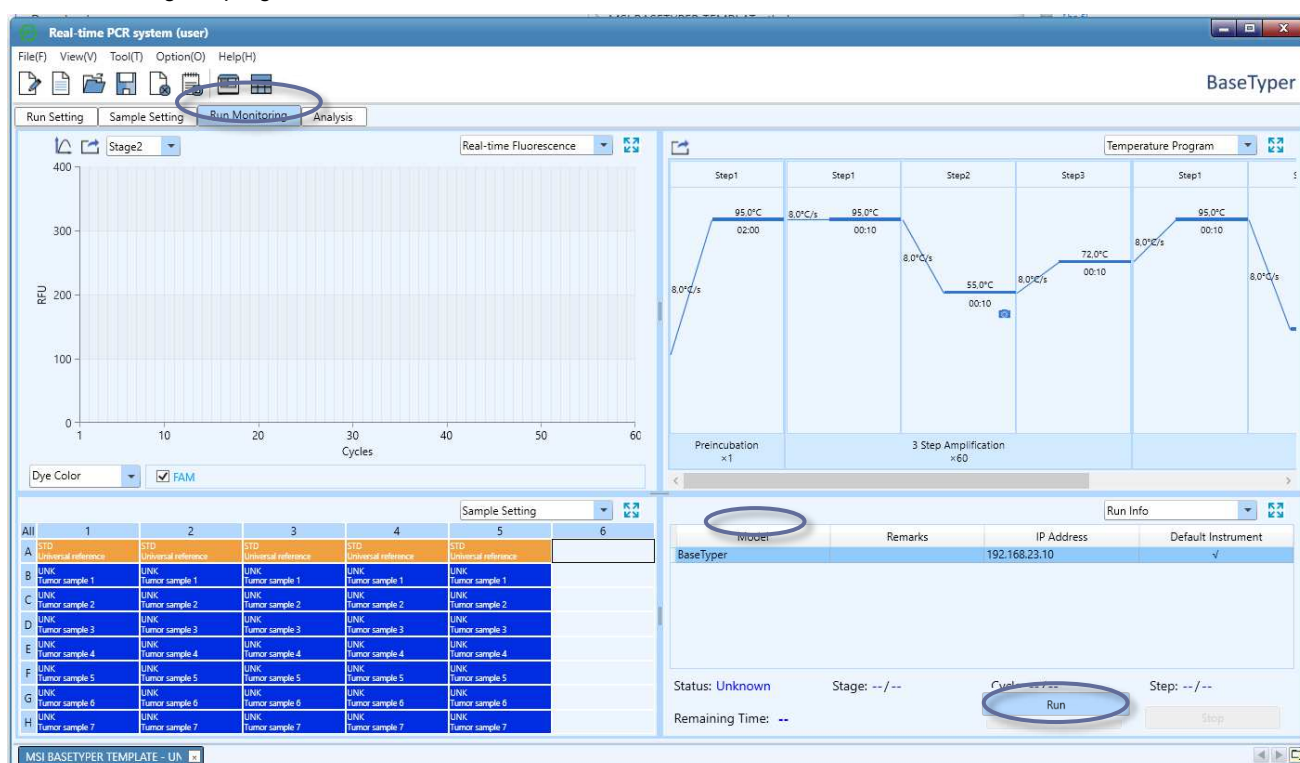

Figure 13. Starting the run at the BaseTyper™ Instrument.

8 Data Analysis

Microsatellite loci are evaluated individually by comparing the melt properties of amplicons obtained with the patient's tumour DNA to those observed when applying the patient's non-tumour DNA (paired analysis) or the universal reference DNA (unpaired analysis). Both shorter and longer microsatellite amplicons are indicative of instability.

8.1 Validation of sample concentration

The Ct value of the MONO27 assay is used to validate that the concentration of amplifiable DNA in the sample is acceptable. This is done by reading the Ct value for MONO27 for each sample (including the universal reference for unpaired analysis) under **Analysis → Abs Quant (Stage2_Step2)** (**Figure 14).**
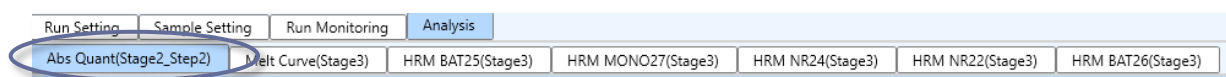

Figure 14. Validation of sample concentration using MONO27 Ct values.

If the Ct value for MONO27 is higher than 35, the sample concentration is too low, and the run is invalid (**Table 3**). Perform a new sample purification and repeat the analysis. **NOTE**: In the specific case where the Ct values for the remaining 4 loci are all below the Ct criteria outlined in **Table 7**, the sample concentration is acceptable, and the high MONO27 Ct value is due to pipetting errors. In this case, rerun the sample and make sure to add sample to all tubes of the strip.

Table 3. Quality control criteria for the Ct value of MONO27.

MONO27	Interpretation	Comments
$Ct_{(MONO27)} \leq 35$	Valid	Sample concentration is acceptable
$Ct_{(MONO27)} > 35$	Invalid	Sample concentration is too low except in cases where the Ct values for the 4 other loci are acceptable.

8.2 Baseline and threshold settings

The automatic baselining and threshold setting performed by the BaseTyper™ software should be used. **NOTE**: manual baselining may be beneficial in cases where air bubbles in the Real-Time PCR reaction mixture are distorting automatic baselining. Press ![icon] **Analysis Settings** and select **Manual Baseline (Figure 15).** To change the baselining for all wells, select one well and press **Ctrl + A**. Change the **Start Cycle** to a cycle after the air has disappeared (e.g., 15) and the **End Cycle** to a cycle number before the exponential phase of the PCR curves (e.g., 20). Press **OK**.
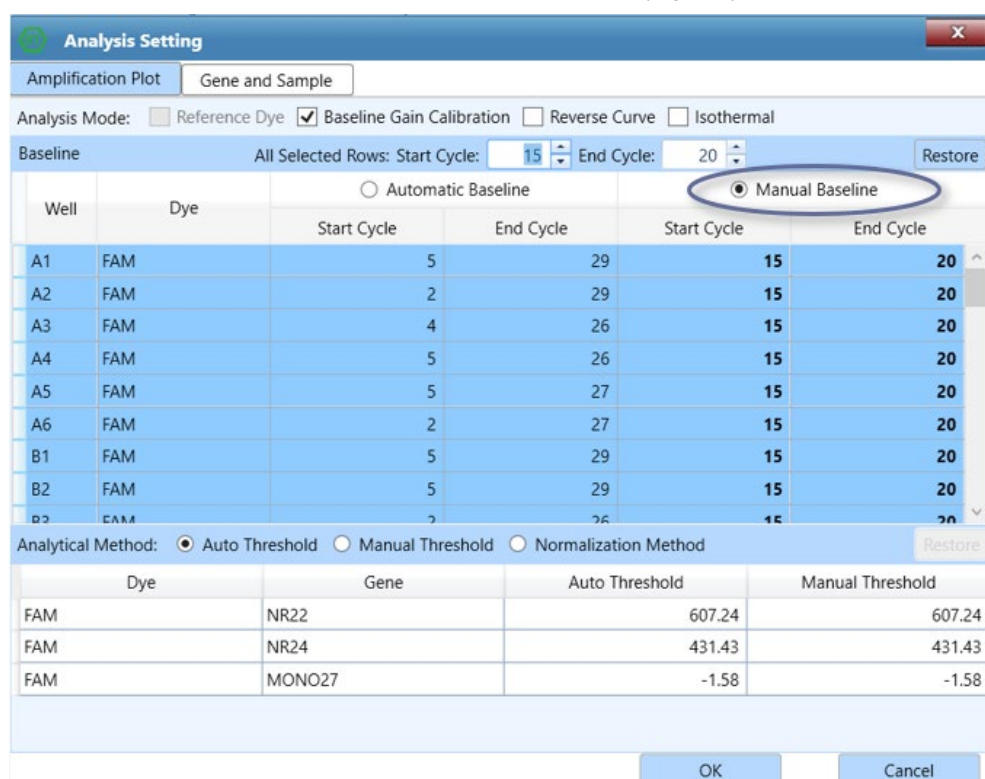

Figure 15. Adjusting the baseline manually.

8.3 Interpretation of results

8.3.1 Evaluation of Microsatellite locus stability for Paired and Unpaired samples

1. Go to the **Analysis tab** and press ![icon] **New Analysis** (**Figure 16**).
2. Select **High Resolution Melt** in the **New Analysis** window.
3. Click the **All Select** checkbox in the **Select HRM Gene** window and press **OK**.
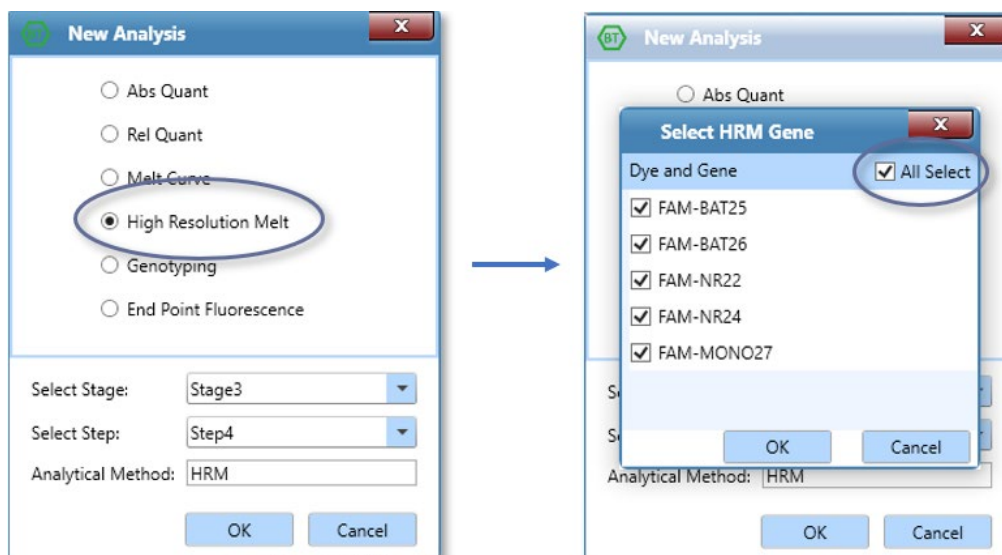

Figure 16. Selecting HRM paired sample analysis.

4. Five HRM tabs will then open, one for each locus (**Figure 17**).

Figure 17. Selecting HRM paired sample analysis

5. Go to **HRM BAT25(Stage3)**. Under **Result Table** you find the stability result for that locus.
    A "**+**" in the "**Calling**" column means that the sample is unstable in that locus, and a "**-**" in the "**Calling**" column means that the sample is stable. Repeat for BAT26, NR22, NR24, and MONO27. An example for paired sample analysis is shown in **Figure 18A**. Tumour sample 1 and 2 are stable in the BAT25 locus and sample 3 and 4 are unstable. **Figure 18B** shows an example for unpaired analysis; tumour sample 1, 3, 5 and 7 are unstable in the BAT25 locus and tumour sample 2, 4, and 6 are stable.

A.

B.

Figure 18. A. Paired sample analysis. **B.** Unpaired sample analysis. A "+" in the "Calling" column means that the sample is unstable in that locus, and a "-" in the "Calling" column means that the sample is stable.

8.3.2 Overall evaluation of Microsatellite stability

8.3.2.1 Paired sample analysis

With five microsatellites analysed by MicroSight® MSI 1-step HRM Analysis using **paired tumour samples,** patient samples are categorised as being either microsatellite stable (MSS, zero loci being unstable), microsatellite unstable-low (MSI-L, one locus being unstable) or microsatellite unstable-high, (MSI-H, two or more loci being unstable, see **Table 4**).

Recommendation: samples with only two to three unstable loci should be rerun until two runs produce the same result.

Table 4. Evaluation of microsatellite stability of paired tumour samples using MicroSight® MSI 1-step HRM Analysis.

Unstable loci (#)	Category
0	MSS
1	MSI-L
2-5	MSI-H

8.3.2.2 Unpaired sample analysis

With five microsatellites analysed by MicroSight® MSI 1-step HRM Analysis using **unpaired tumour samples,** patient samples are categorised as being either microsatellite stable (MSS, zero to two loci being unstable) or microsatellite unstable-high, (MSI-H, three or more loci being unstable, see **Table 5**).

Recommendation: samples with only two unstable loci should be rerun until two runs produce the same result.

Table 5. Evaluation of microsatellite stability of unpaired tumour samples using MicroSight® MSI 1-step HRM Analysis.

Unstable loci (#)	Category
0-2	MSS
3-5	MSI-H

Patients analysed using the unpaired analysis need more unstable microsatellites before being categorised as MSI-H due to the possibility of the patients having heterozygote microsatellites – see section 2 for the explanation of how heterozygosity affects the MSI status calling.

8.3.3 Data example for paired analysis

A case of an unstable and a stable locus from a paired analysis is illustrated in **Figure 19**. Similar plots can be observed for an unpaired analysis (not shown), where the universal reference replaces the normal tissue.
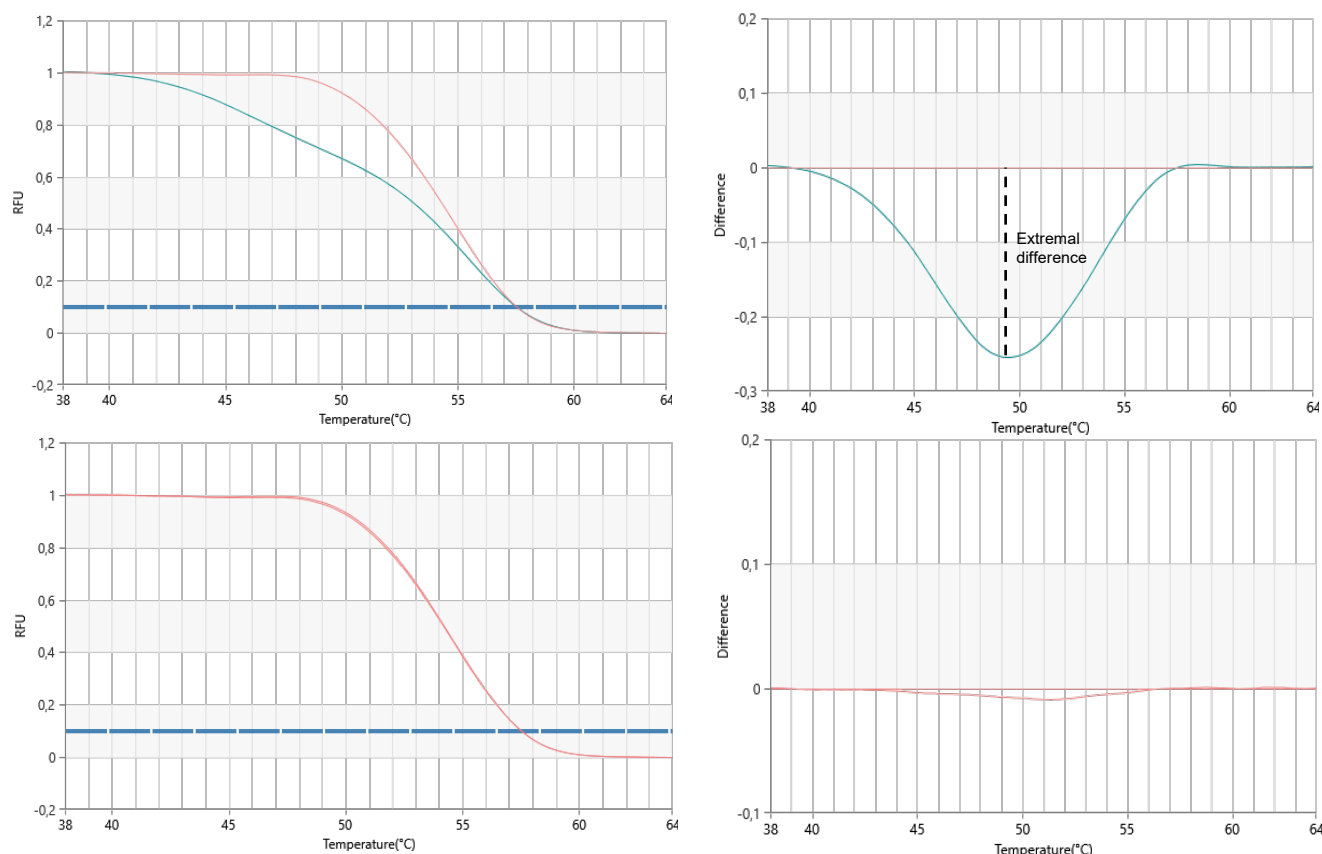
Figure 19. Microsatellite unstable and stable locus. Left: The normalised melting curves. If a difference is observed between the curve of the non-tumour tissue and tumour tissue the locus is unstable (upper, left graph). If no difference is observed between the curves of the tissue samples the locus is stable (lower, left graph). Right: The difference plots.

Normalised HRM curves and difference plots are shown on the left and on the right, respectively, of **Figure 19**. The difference plots are created by setting a reference curve (curve from non-tumour tissue or universal reference) as baseline curve, and the baseline curve is subtracted from the tumour curve. Thereby the difference in Relative Fluorescence Unit (RFU) is plotted as a function of the temperature (the maximum difference in RFU equals the extremal difference value). If the difference curve crosses a threshold set in the PCR software, the tumour is deemed as microsatellite unstable in the given locus. If the difference curve does not cross the threshold, the locus is considered as microsatellite stable. The thresholds for calling a locus stable or unstable were individually set for each locus (see **Table 6**). The thresholds for each locus. based on data from a development cohort to retain the highest sensitivity and specificity.

Table 6. The thresholds for each locus.

Locus	Threshold (RFU)
BAT25	-0.04
BAT26	-0.03
NR22	-0.03
NR24	-0.04
MONO27	-0.04

8.3.4 Invalid results

If the Extremal difference value (found in the Result Table) is below -0.9 or above 0.9 (**Figure 20**) for one or more tubes, no template has been added to the tube(s) and this may cause a false MSI result. Verify that the sample concentration is acceptable by evaluating the MONO27 Ct value as described in section 8.1 "Validation of sample concentration" and that the Extremal difference for MONO27 is above -0.9 and below 0.9. If the MONO27 Ct value is valid (≤35), the sample concentration is acceptable, and the Extremal difference value(s) found in other tube(s) is/are due to sample not being added to these tubes. Rerun the sample analysis and make sure to add sample to all tubes. If in contrast the MONO27 Ct value is above 35, the sample concentration is too low, and it is recommended to reperform DNA extraction and rerun the sample analysis.

8.3.4.1 Control of sample loading

If the Extremal difference value (found in the Result Table) is below -0.9 or above 0.9 (**Figure 20**), the addition of sample to the specific tube can be controlled by reading the Ct value for the particular locus under **Analysis → Abs Quant(Stage2_Step2)**.The Ct control criteria for the different loci are outlined in **Table 7**. In cases where one of the locus

Ct values are invalid, the sample should be rerun unless all locus Ct values are invalid in which case the sample concentration is too low and a new DNA purification should be performed.

Table 7. Ct control criteria for added template for all loci.

Locus	Ct value	Conclusion
BAT25	>38	Invalid
	≤38	Valid
BAT26	>33	Invalid
	≤33	Valid
NR22	>36	Invalid
	≤36	Valid
NR24	>33	Invalid
	≤33	Valid
MONO27	>35	Invalid
	≤35	Valid

8.3.4.2 Signal in NTC

No Ct value should be observed in the case of an added no template control (NTC). If a Ct value is observed for a NTC sample, this could indicate contamination. Find the cause of contamination by checking or replacing all potential sources of the contamination such as pipettes and instruments. If the contamination cannot be located, contact PentaBase A/S or your local distributor.
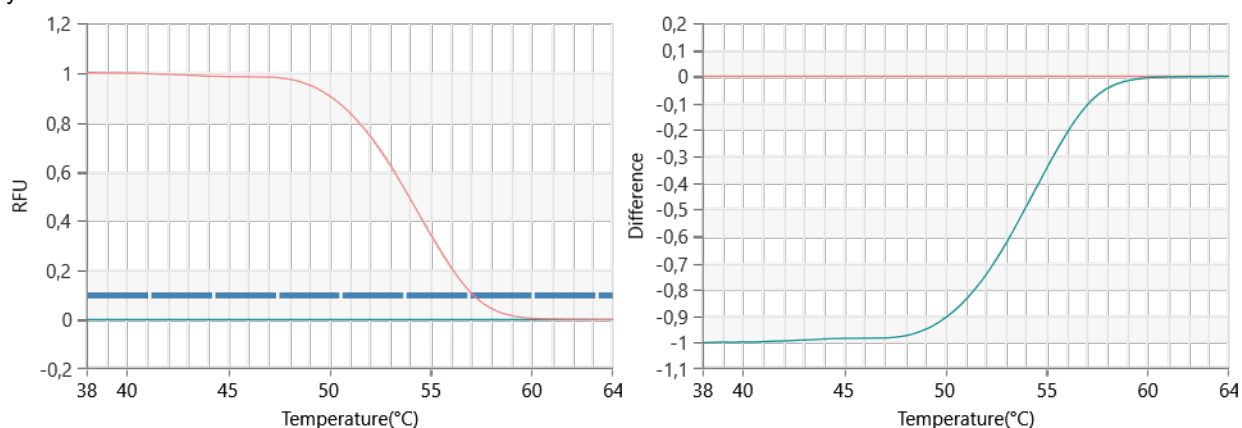

Figure 20. False locus MSI result caused by no added template DNA for the tumour sample (green curve) or by a NTC control. Left: The normalised melting curves. Right: The difference plot.

9 Performance evaluation

9.1 Analytical sensitivity – Limit of Detection

The minimum concentration of DNA required for the analysis, without risking a false positive MSI calling, was tested to be 0.05 ng/µL for all loci. This was also the lowest concentration tested. The evaluation was carried out using 20 replicates of gDNA from a FFPE MSS sample for each locus (data not shown).

The limit of detection (LOD) for MicroSight® MSI 1-step HRM Analysis was determined to be 10% MSI for four out of the five loci (BAT25, BAT26, NR22, and MONO27) both for paired and unpaired analysis (**Table 8**). All five loci were found to be unstable at 25% while four out of five loci were unstable at 10%. For NR24, 14 out of 20 replicates were found to be unstable at 15% (**Table 8**). The LOD for MicroSight® MSI 1-step HRM Analysis is therefore 10% MSI, as only two and three loci should be unstable for a sample to be MSI for paired and unpaired analysis, respectively.

The LOD was evaluated using three or 23 replicates of DNA from a 100% MSI human colorectal carcinoma cell-line (1 ng/µL) which was diluted in a background of gDNA purified from a FFPE MSS sample (1 ng/µL) and compared to both Universal control (1 ng/µL) and the gDNA from the FFPE MSS sample (1 ng/µL).

Table 8. LOD of MicroSight® MSI 1-step HRM Analysis using 100% MSI FFPE human colorectal carcinoma cell-line DNA.

Locus	% of DNA from 100% MSI FFPE human colorectal carcinoma cell-line		
	10%	15%	25%
BAT25	23/23	-	3/3
BAT26	23/23	-	3/3
NR22	23/23	-	3/3
NR24	0/3	14/20	3/3
MONO27	23/23	-	3/3
Conclusion	**MSI**	**NA**	**MSI**

9.2 Analytical precision

Genomic DNA extracted from tumour and non-tumour FFPE tissues from 30 CRC patients (from our development cohort) was analysed in five different laboratories to investigate inter-laboratory variation. Very low variation was observed between samples, and all MSS patients (25/25) and MSI (5/5) patients were correctly classified using both paired sample and universal reference in all five laboratories. All laboratories had 100% agreement for all loci in the analysis of paired samples. Laboratories 1, 3, and 4 had 100% agreement in the analysis of BAT25, NR22, NR24, and MONO27 using universal reference. Laboratory 2 found two MSS samples to be unstable in BAT25 and one MSS sample to be unstable in BAT26 using the universal reference. Laboratories 3 and 4 found one MSS sample to be unstable in BAT26 using the universal reference. Laboratory 5 found one MSS sample to be unstable in MONO27 using the universal reference. These five cases were not found by the other laboratories.
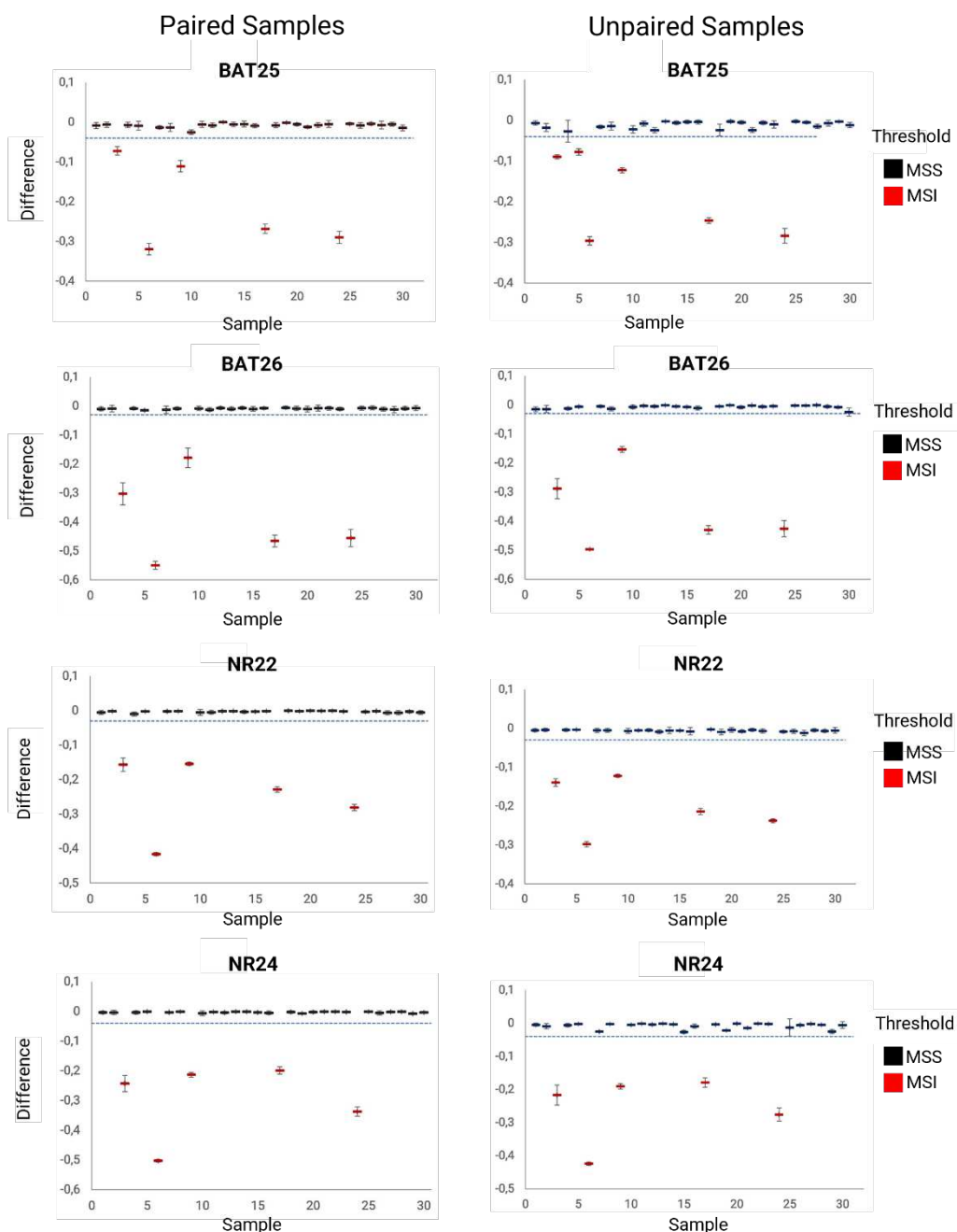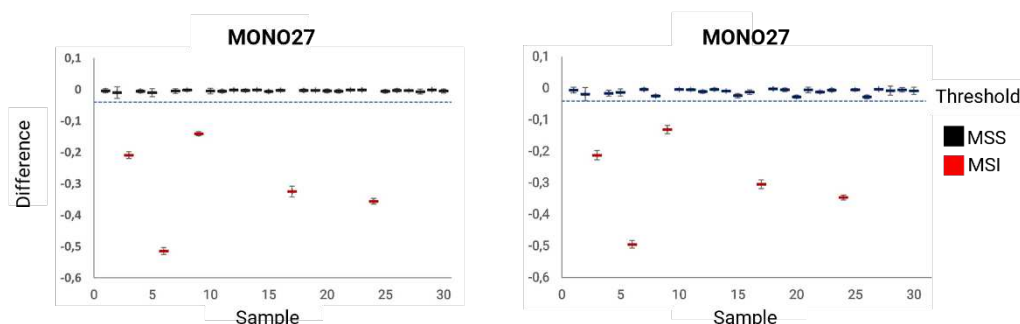
Figure 21. Low inter-laboratory variation. 30 samples from a development cohort were tested using MicroSight® MSI 1-step HRM Analysis in five laboratories. Depicted is the average difference +/- the standard deviation for BAT25, BAT26, NR22, NR24, and MONO27 for each sample.

9.3 Specificity

To evaluate the specificity of MicroSight® MSI 1-step HRM Analysis, an *in-silico* analysis assessing the primers specificity to the human reference genome using Nucleotide-Nucleotide BLAST 2.13.0+ was performed. The human reference genome (sequence: GRCh38) was downloaded from https://www.ncbi.nlm.nih.gov/genome/guide/human/ on 20th of May 2022. The BLAST output pre-processing included:

1. Determining possible primer pairs.
2. Discarding pairs with a product length more than 5000 bp and with coverage less than 85%.

Primer pairs showed 100% alignment with their target regions (NC_00002.12 for BAT26, MONO27, and NR24, NC_00004.12 for BAT25, and NC_000011.10 for NR22) as illustrated in **Figure 22**. Additional high similarity regions were identified for the NR24 primers, but no high similarity regions were identified for the combined primer pair (**Figure 22**).
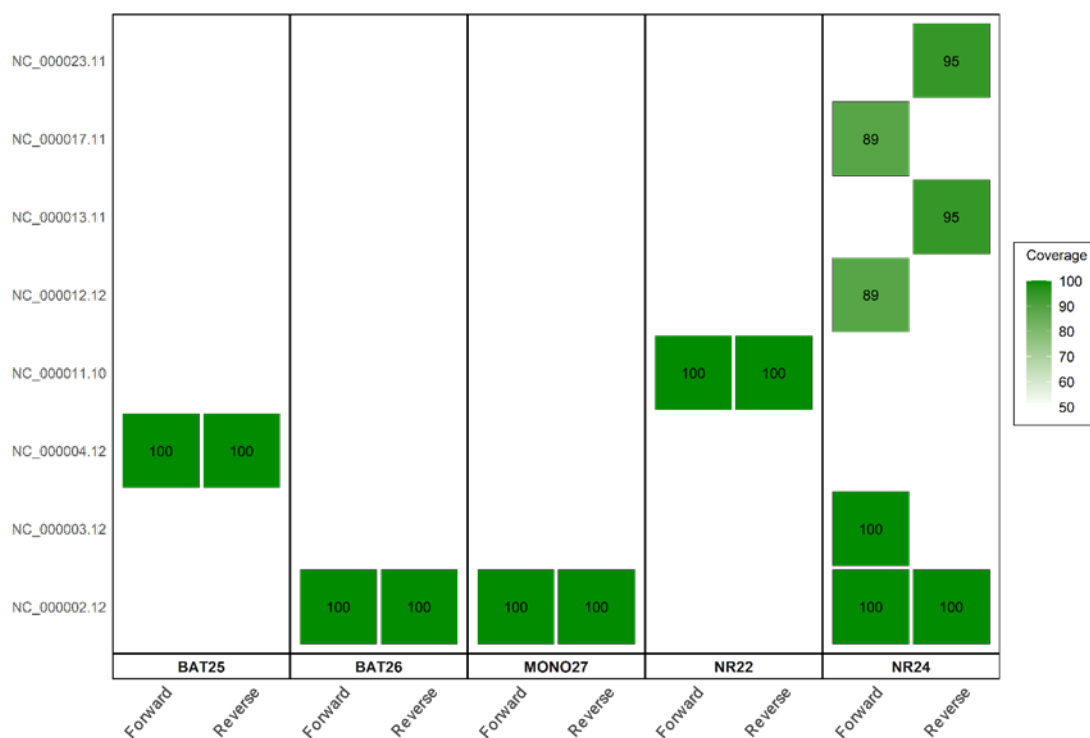
Figure 22. MicroSight® MSI 1-step HRM Analysis primer specificity heatmap to human reference genome (GRCh38). The NCBI accession numbers of different chromosome are shown on the left and the primer targets are listed at the bottom of the heatmap. The coverage on the heatmap is based on High Scoring Pairs (HSP). Specifically, Query Coverage per HSP (QCovHSP). The QCovHSP score takes into consideration whether the sequence is trimmed or contain a mismatch. The value corresponds to the percentage of the whole primer length that has been aligned/matched to the sequence(s). The values in the boxes correspond to the primer coverage (%), which represent an alignment score of the primer and the corresponding chromosome in the human genome.

9.4   Clinical evaluation

9.4.1   CRC patient samples

Validation of MicroSight® MSI 1-step HRM Analysis on CRC patient samples was performed in four different laboratories in Italy. The assay was used for both unpaired and paired analysis in a retrospective analysis of FFPE samples from 127 patients with histologically confirmed CRC previously analysed with traditional capillary electrophoresis (**Table 9 and Table 10**). Each of the four laboratories used different panels for the capillary electrophoresis. The samples were evaluated by MicroSight® MSI 1-step HRM Analysis assay by comparing the melt properties of amplicons obtained when applying the patient's tumour-derived genomic gDNA with those observed when applying the patient's non-tumour DNA (paired samples) or a universal reference (unpaired samples). As MSI-L cannot be called by MicroSight® MSI 1-step HRM Analysis for unpaired samples, the MSI-L samples were excluded from the cohort for this analysis. Refer to section 3 for more information.

Table 9 and **Table 10** show the results of tested samples and the agreement between MicroSight® MSI 1-step HRM Analysis and fragment analysis by capillary electrophoresis in the three categories (MSS/MSI-L/MSI-H) for unpaired and paired samples, respectively. Except one sample that was MSI-H by MicroSight® MSI 1-step HRM Analysis but MSS by capillary electrophoresis, there was 100% agreement between MicroSight® MSI 1-step HRM Analysis and the comparator methods when using unpaired samples analysis (**Table 9**). Similarly, only two discrepant cases were found in the paired samples analysis (**Table 10**). The one and two cases under unpaired and paired samples, respectively, not matching the MSS status and thereby lowering the agreement for this calling was due to:

1. One paired sample being called MSI-L using MicroSight® MSI 1-step HRM Analysis paired samples, because of instability in NR22, but MSS on the fragment analysis, which does not cover NR22.
2. One sample being called MSS by the fragment analysis and MSI-H using MicroSight® MSI 1-step HRM Analysis, both with unpaired and paired samples.

Table 9. Summary of clinical evaluation of MicroSight® MSI 1-step HRM Analysis on CRC patient samples using unpaired sample analysis.

Clinical peformance		MicroSight® MSI 1-Step HRM Analysis			
Unpaired FFPE tumour biopsies from 127 CRC patients		MSS	MSI-L	MSI-H	Total
Capillary electrophoresis comparator methods	MSS	78	NA	1	79
	MSI-L	11	NA	0	11
	MSI-H	0	NA	37	37
	Total	89	NA	38	127
Overall agreement (excluding MSI-L) was 115/116 or 99.1% (CI95: 95.3-99.9%)					
MSS percentage agreement was 78/79 or 98.7% (CI95: 93.2-99.8%)					
MSI-L percentage agreement was NA					
MSI-H percentage agreement was 37/38 or 97.4% (CI95: 86.5-99.5%)					

The agreement in MSI-L was zero percent except for one laboratory having 20% MSI-L agreement. This was in part due to some paired samples being called MSI-L caused by instability in NR21 or in dinucleotide loci in the fragment analysis, while they were called MSS using MicroSight® MSI 1-step HRM Analysis, which does not include NR21 or dinucleotide loci, as dinucleotides have been shown to have a lower specificity and sensitivity compared to mononucleotides[6,7,12].

Table 1010. Summary of clinical evaluation of MicroSight® MSI 1-step HRM Analysis on CRC patient samples using paired sample analysis.

Clinical performance		MicroSight® MSI 1-Step HRM Analysis			
Paired FFPE tumour biopsies from 127 CRC patients		MSS	MSI-L	MSI-H	Total
Capillary electrophoresis comparator methods	MSS	77	1	1	79
	MSI-L	10	1	0	11
	MSI-H	0	0	37	37
	Total	87	2	38	127
Overall agreement was 115/127 or 90.6% (CI95: 84.2-94.5%)					
MSS percentage agreement was 77/79 or 97.5% (CI95: 91.2-99.3%)					
MSI-L percentage agreement was 1/11 or 9.1% (CI95: 1.6-37.7%)					
MSI-H percentage agreement was 37/38 or 97.4% (CI95: 86.5-99.5%)					

9.4.2   Endometrial cancer patient samples

Furthermore, MicroSight® MSI 1-step HRM Analysis was validated on endometrial cancer patient samples in a laboratory in Switzerland. The assay was again used with both unpaired and paired analysis in a retrospective analysis of FFPE samples from 38 patients with confirmed endometrial cancer previously analysed with traditional capillary electrophoresis **(Table 11 and Table 12)**. The samples were evaluated by the MicroSight® MSI 1-step HRM Analysis assay in a similar fashion as for the CRC samples.

Table 11 and Table 12 show the results of tested samples and the agreement between MicroSight® MSI 1-step HRM Analysis and fragment analysis by capillary electrophoresis in the two categories (MSS/MSI-H) for unpaired and paired

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[12] Ferreira et al., 2009; PMID: 19521971

samples, respectively. There was 100% agreement between MicroSight® MSI 1-step HRM Analysis and the comparator methods when using both unpaired (**Table 11**) and paired (**Table 12**) samples analysis.

Table 11. Summary of clinical evaluation of MicroSight® MSI 1-step HRM Analysis on endometrial cancer patient samples using unpaired sample analysis.

Clinical performance Unpaired FFPE tumour biopsies from 38 endometrial cancer patients		MicroSight® MSI 1-Step HRM Analysis		
		MSS	MSI-H	Total
Capillary electrophoresis comparator methods	MSS	19	0	19
	MSI-H	0	19	19
	Total	19	19	38
Overall agreement was 38/38 or 100%				
MSS percentage agreement was 19/19 or 100%				
MSI-H percentage agreement was 19/19 or 100%				

Table 1211. Summary of clinical evaluation of MicroSight® MSI 1-step HRM Analysis on endometrial cancer patient samples using paired sample analysis.

Clinical evaluation: Paired FFPE tumour biopsies from 38 endometrial cancer patients		MicroSight® MSI 1-Step HRM Analysis		
		MSS	MSI-H	Total
Capillary electrophoresis comparator methods	MSS	19	0	19
	MSI-H	0	19	19
	Total	19	19	38
Overall agreement was 38/38 or 100%				
MSS percentage agreement was 19/19 or 100%				
MSI-H percentage agreement was 19/19 or 100%				

10 Limitations

- MicroSight® MSI 1-step HRM Analysis can only be run on a BaseTyper™ 48.4 Quiet HRM Real-Time PCR System.
- MicroSight® MSI 1-step HRM Analysis has only been validated for use with colorectal and endometrial cancer FFPE specimens.
- Incorrect collection, storage, DNA extraction, transportation or handling of the sample could cause false test results due to low amount or poor quality of gDNA or the presence of PCR inhibitors in the sample.
- MicroSight® MSI 1-step HRM Analysis is validated for use with 5-50 ng of DNA per reaction. Using DNA input amounts lower or higher than this may lead to incorrect test results.
- A negative test result (MSS) does not exclude the presence of MSI DNA at levels below the detection limit of the assay.
- The Ct value of the MONO27 assay is only indicative of the amount of template added to the reaction and cannot be used for precise quantification of DNA.
- Variation between BaseTyper™ 48.8 Quiet HRM Real-Time PCR Systems might influence the results and/or the performance of the MicroSight® MSI 1-step HRM Analysis.

11 Symbols

The following symbols are used in the labelling of MicroSight® MSI 1-step HRM Analysis.

	Date/country of manufacture	**IVD**	diagnostic medical device
	Use-by date		Do not reuse
	Contains sufficient for *<n>*		Manufacturer
	Temperature limit	CE	CE marking of conformity; this device is in conformity with the applicable requirements for CR of an *in vitro* diagnostic medical device
	Consult electronically available instructions for use		

12 Manufacturer

PentaBase A/S
Petersmindevej 1A
DK-5000 Odense, Denmark

Telephone: +45 36 96 94 96
Email: info@pentabase.com
Webpage: www.pentabase.com

For technical assistance in all other countries, contact your local distributor. A complete list of distributors is available at www.pentabase.com.

NOTICE TO USERS: any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.