

PlentiPlex™ MSI

Made by PentaBase

INSTRUCTIONS FOR USE

PlentiPlex™ MSI for Detection of Microsatellite Instability in Cancer Patients



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Δ IMPORTANT:

Please read these instructions carefully before using PlentiPlex™ MSI assays. It is recommended to save the “Instructions for use” for future use. Purchasers of PlentiPlex™ MSI assays are only granted the right of use, but no general licensing or patent rights.

1. INTENDED USE

PlentiPlex™ MSI assays are intended for in vitro diagnosis of impaired mismatch repair (MMR) affecting prognosis and selection of treatment in cancer patients. MMR deficiency is normally manifested as replicative errors affecting the length (number of nucleotides) of repeated sequences in the genome commonly referred to as micro-satellites. The PlentiPlex™ MSI assays offer length analysis of 5 micro-satellite loci, recommended by the Bethesda and Hamelin guidelines, enabling evaluation of MMR status on a ThermoFisher scientific DNA sequencer instrument.

PlentiPlex™ MSI assays should be used on human DNA samples from fresh, frozen or formalin-fixed paraffin-embedded (FFPE) tissue. PlentiPlex™ MSI assays **are not intended for diagnosing of cancer**, but only as an aid to assist the oncologist’s treatment planning. The tests are provided in one or more boxes, containing the necessary components for the multiplexed thermal amplification of five micro-satellite loci recommend for MSI diagnosis by the Bethesda and Hamelin guidelines. The generated amplicons should be analyzed on a compatible Sanger sequencing instrument like the Applied Biosystems 3130 Series Genetic Analyzer. Consumables for the fragment analysis on the sequencer are not included in the PentiPlex MSI assay.

1.1 INDICATIONS FOR USE

Micro-satellites 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 DNA MMR system. Uncorrected micro-satellite errors are commonly referred to as micro-satellite instability (MSI) and used to assist clinicians and genetic counsellors for the diagnosis of a type of neoplastic inherited syndrome (Vaksman and Garner, 2015).

Inherited deregulation by epigenetic and genetic changes in genes (like PMS2, MLH1, MSH2, or MSH6) and their gene products leading to compromised MMR response is known as Lynch syndrome (LS) or hereditary nonpolyposis colorectal cancer. Apart from high risk of developing colorectal cancer, 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 tumors (Richman S, 2015). In patients with colorectal cancer, MSI seems to be associated with a slightly better prognosis and low benefit of 5-flourouracil-based therapeutics. Uniform evaluation of MSI status is in the “Bethesda Guidelines” (Boland et al., 1998) suggested to include two mononucleotide micro-satellites (BAT25 and BAT26) and three dinucleotide micro-satellites (D2S123, D5S346 and D17S250). With five micro-satellites analyzed patients can be categorized as Micro-satellite stable, MSS (0 markers exhibiting instability), Micro-satellite instable-low, MSI-L (1 marker being unstable) or Microsatellite instable-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 tumors 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 analysis of the “Hamelin Panel” namely the five mononucleotide loci; BAT25, BAT26, NR-21, NR-22 and NR-24 (Suraweera et al, 2002). Apart from increasing sensitivity the monomorphic nature of the Hamelin Panel loci has been suggested to eliminate the need for parallel investigation of paired samples from patients comparing un-involved (germline) tissue to tumor genomic DNA, (gDNA).

2. SUMMARY AND EXPLANATION OF THE ASSAYS

PlentiPlex™ MSI assays are CE-IVD marked in accordance with EU Directive 98/79/EC on Medical Equipment for in vitro diagnostics and used for the indirect evaluation of the efficiency of a human patient’s DNA mismatch repair system.

3. TECHNOLOGY AND REAGENTS

PlentiPlex™ MSI assays are based on PentaBase’s sensitive DNA technology. The MSI analysis system comprises 5 primer pairs (Table 1 and 3) with forward primers being fluorescently labeled as indicated in Table 2 and 4:

Table 1: MSI Bethesda Panel primers

Marker	Forward primer	Reverse primer
BAT25	5'-TCGCCTCCAAGAATGTAAGT-3'	5'-TCTGCATTTAACTATGGCTC-3'
BAT26	5'-TGACTACTTTTGACTTCAGCC-3'	5'-AACCATTCAACATTTTAAACCC-3'
D2S123	5'-AAACAGGATGCCTGCCTTA-3'	5'-GGACTTCCACCTATGGGAC-3'
D5S346	5'-ACTCACTCTAGTGATAAATCGGG-3'	5'-AGCAGATAAGACAGTATTACTAGTT-3'
D17S250	5'-GGAAGAATCAAATAGACAAT-3'	5'-GCTGGCCATATATATTTAAACC-3'

Table 2: MSI Bethesda Panel loci information

MSI loci detected with PlentiPlex™ MSI Bethesda panel				
Marker	Repeated sequence	Size range * (bp)	Fluorophore	Excitation/emission (nm)
BAT25	(A) ₂₅	121-127	PentaGreen	495/516
BAT26	(A) ₂₆	122-128	PentAltRed	564/590
D2S123	(CA) ₁₃ TA(CA) ₁₅ (T/G A) ₇	197-227	PentaGreen	495/516
D5S346	(CA) ₂₆	96-122	PentAltOrange	554/576
D17S250	(CA) ₂₄	147-169	PentAltYellow	532/552

(* Size range determined using the ABI PRISM 3130 Genetic Analyzer. Size range may vary when using alternative instruments and/or configurations)

Table 3: MSI Hamelin Panel primers

Marker	Forward primer	Reverse primer
BAT25	5'-TCGCCTCCAAGAATGTAAGT-3'	5'-TCTGCATTTAACTATGGCTC-3'
BAT26	5'-TGACTACTTTTGACTTCAGCC-3'	5'-AACCATTCAACATTTTAAACCC-3'
NR-21	5'-TAAATGTATGTCTCCCTGG-3'	5'-ATTCCTACTCCGATTACACA-3'
NR-22	5'-GAGGCTTGTAAGGACATAA-3'	5'AATTCGGATGCCATCCAGTT3'
NR-24	5'-CCATTGCTGAATTTACCTC-3'	5'-ATTGTGCCATTGCATTCCAA-3'

Table 4: MSI Hamelin Panel loci information

MSI loci detected with PlentiPlex™				
Marker	Repeated sequence	Average size (bp)	Fluorophore	Excitation/emission (nm)
BAT25	(T) ₂₅	120	PentaGreen	495/516
BAT26	(A) ₂₆	124	PentAltRed	564/590
NR-21	(T) ₂₁	103	PentAltYellow	532/552
NR-22	(T) ₂₂	142	PentaGreen	495/516
NR-24	(T) ₂₄	132	PentAltYellow	532/552

4. ASSAY FORMAT

4.1 FORMAT

PlentiPlex™ MSI assays are supplied as ready-to-use assays in either 1, 12 or 60 reactions in pre-aliquoted 0.2 ml PCR strips or tubes suitable for most standard 96-well plate format PCR instruments. All reagents needed for the PCR amplification of extracted genomic DNA are pre-loaded into the strips/tubes (see Table 1-5 for details).

PlentiPlex™ MSI assays contain the following reagents:

Reference assay

- Reference assay primer/probe mix (fluorescently labeled as indicated in Table 2 and 4)
- Master mix

Mutation assays

- Mutation assay primer/probe mix (fluorescently labeled as indicated in Table 2 and 4)
- Master mix

Table 5: Contents of PlentiPlex™ ready-to-use assays. Each tube contains 7 µL in total

Strip #	Tube #	Content	Template to be added
M1	A	Reference 1	Germline gDNA
	B	MSI Bethesda Panel Multiplex	Tumor gDNA
M2	A	MSI Hamelin Panel Multiplex	Germline or Tumor gDNA

4.2 REFERENCE ANALYSIS

Thermal amplification of Bethesda panel reference DNA takes place simultaneously with the MSI analysis, but in its own tube/well. The reference assay contains an identical primer/probe mix as the mutation analysis assay and gives information about the length of the investigated microsatellites in the germline DNA of the specific patient. The reference assay requires addition of genomic DNA (gDNA) from normal tissue (non-tumor tissue) from the patient. When using PlentiPlex™ MSI Hamelin Panel assays, there is no designated reference tube. The PlentiPlex Hamelin Panel can be used with or without a patient specific, germline DNA reference (see section 8.2: PLENTIplex™ MSI HAMELIN PANEL ANALYSIS, for further information).

4.3 MUTATION ANALYSIS

The mutation assay is used to detect variations in the micro-satellite length in the tumorigenic tissue compared to normal tissue. It requires addition of gDNA from the tumor tissue investigated in the patient. MSI is recognized as varying length (retention times) of one or more amplicons between the normal and the tumorigenic templates when compared after analysis on an appropriate DNA sequencer. If there is no difference between normal and tumorigenic tissue, the patient is characterized as microsatellite stable, MSS. See Section 7 for more details about analysis.

4.4 EQUIPMENT AND REAGENTS NOT SUPPLIED WITH PLENTIplex™

PlentiPlex™ MSI ready-to-use assays are shipped in regular profile 0.2 mL PCR strips. The use of PlentiPlex™ will require the following additional equipment and consumables:

- Template DNA (normal and tumor-derived)
- PCR instrument
- DNA sequence analyzer compatible with the fluorophores in the PLENTIplex™ MSI ASSAYS
- Plastic-ware (tubes/plates) that are compatible with the instruments^
- Formamide (use highly deionized formamide)
- DNA ladder for sequence analyzer (Genescan™ 500 LIZ™ dye or equivalent for detection in the orange channel)
- Heating block (for sequencer plastic ware) 95°C

- Ice bath
- Dedicated pipettes and tips for preparing PCR mixes
- Dedicated pipettes and tips for addition of DNA sample
- Centrifuge for spinning tubes/plates

5. SAFETY, SHIPMENT AND STORAGE

General laboratory precautions should be taken. PlentiPlex™ MSI assays should only be used by personnel who has been trained in the appropriate techniques. All chemicals and biological material should be considered as potentially hazardous. When working with the assays, suitable personal protective equipment (lab-coat, disposable gloves and safety glasses) should be used. It is recommended that all work is carried out in appropriate facilities. All waste should be disposed as clinical waste.

5.1 PRECAUTIONS

The following precautions should be taken when working with PlentiPlex™ MSI assays:

- The assays are only for *in vitro* diagnostic use
- PlentiPlex™ MSI assays are 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)
- PlentiPlex™ MSI assays should not stand alone in the decision about starting specific treatment
- Avoid several freeze/thaw cycles of the reagents as this might impair the performance of PlentiPlex™ MSI assays. Use the reagents when thawed.
- Verify eligibility of the DNA samples as samples of poor quality might affect the analysis
- The delivered reagents should not be diluted further. Further dilution can cause loss of performance
- Use the specified volumes. It is not recommended to reduce the specified volumes as the results can be affected
- No reagents should be substituted by others if the optimal performance should be maintained
- To protect the probes the assays should be protected from light. For more information, see section 5.3 "Storage".
- Use caution not to contaminate reagents and samples. It is recommended to separate preparation of PCR mixes and DNA addition. 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 to their original specifications. DNA Sequencers used in the validation studies have been calibrated with ThermoFisher Dye SET G5 (6-FAM, VIC, NED, PET, LIZ).

5.2 SHIPMENT

PlentiPlex™ ready-to-use assays are shipped on dry ice or super cooled ice packs and should be frozen on arrival. If the PlentiPlex™ packaging has been opened during transport or if the products are not cold upon arrival, please contact PentaBase ApS. (see section 13 "Additional Information"). Please also contact PentaBase ApS if the shipment is missing a certificate of analysis, reagents or a "Quick Guide".

5.3 STORAGE

PlentiPlex™ assays should after arrival immediately be stored at maximum -15°C. Repeated freeze/thaw cycles should be avoided. If the assays are stored under the recommended conditions, it should be stable until the expiry date stated on the box.

6. SPECIMENS

Specimens should be human gDNA extracted from formalin fixed paraffin-embedded (FFPE), fresh or fresh-frozen tumor sections and comparable germline gDNA. The samples should be collected and stored after standard pathology methodology to ensure optimal quality. Extracted gDNA should be stored at maximum -18°C until use.

6.1 RECOMMENDED PROCEDURE FOR EXTRACTION OF GENOMIC DNA

Several methods to extract gDNA from FFPE, fresh or fresh-frozen material can be used. Different methods have been validated under the development and validation of PlentiPlex™ including the Maxwell® 16 FFPE Plus LEV DNA Purification Kit (Promega, Cat. #AS2000 and AS3000). Regardless of method, it is recommended to follow the manufacturers protocol for gDNA extraction.

7. PLENTIPLX™ MSI PROTOCOL

PlentiPlex™ MSI Bethesda and Hamelin panels have different requirements to the reference sample as described below.

7.1 PLENTIPLX™ MSI BETHESDA PANEL PROTOCOL

When using PlentiPlex™ MSI Bethesda panel assays, a patient-specific reference (normal gDNA of non-tumor origin) analysis must be included for MSI analysis of the corresponding tumor biopsy. The reference and MSI analysis tubes should be processed and analyzed in the same PCR- and sequencing run to ensure minimal handling and run variation.

- Thaw the reaction mixtures and spin down
- Add 5 µL extracted gDNA (WT and tumor DNA (app. 1-40 ng/µL)) to the reference and MSI analysis respectively
- Close lids and spin down. Check for air bobbles
- Place the strips in a PCR instrument and run the protocol described in Table 6

7.2 PLENTIPLX™ MSI HAMELIN PANEL PROTOCOL

Analysis with PlentiPlex™ MSI Hamelin Panel assays can be done without a patient specific reference (normal gDNA of non-tumor origin) sample, as long as at least one germ-line (non-tumor) gDNA sample is included in each run on the fragment analysis (see 8.2 PLENTIPLX™ MSI HAMELIN PANEL ANALYSIS below for further details) to ensure minimal handling and run variation.

- Thaw the reaction mixtures and spin down
- Add 5 µL extracted tumor gDNA (app. 1-40 ng/µL) to the MSI assay
- Prepare at least one reference sample (non-tumor gDNA) for each PCR- and fragment analysis run
- Close lids and spin down. Check for air bobbles
- Place the strips into the PCR instrument and run the protocol described in Table 6

Table 6: PlentiPlex™ MSI PCR protocol

Protocol	Temperature	Time	Number of cycles
Hold	94°C	600 sec.	1
Cycling	94°C	50 sec.	10
	55°C	50 sec.	
	72 °C	50 sec.	
Cycling	89°C	30 sec.	25
	55°C	30 sec.	
	72 °C	30 sec.	
Hold	72 °C	10 sec.	1
Hold	10 °C	∞	1

7.3 PLENTIplex™ MSI POST PCR PROTOCOL

After PCR, dilute PCR amplicons 1:10 with DNase free water

- Mix 1 µL of diluted DNA template with 9 µL of formamide and 0.25 µL of size standard DNA ladder in plastic ware compatible with gene analyzer configurations
- Place in heating block at 95°C for 120 secs
- Place immediately on ice bath at 4°C for at least 10 min
- Evaluate amplicon size applying standard protocols on DNA sequence analyzer
- Analyze the samples in accordance with the analysis rules. For more information, see section 8 “Data analysis”

8. DATA ANALYSIS

Micro-satellite loci are evaluated individually by comparing the length of amplicons obtained with the patient’s reference gDNA to those observed when applying the patient’s tumor-derived gDNA. Both shorter and longer micro-satellite amplicons are indicative of instability.

8.1 PLENTIplex™ MSI BETHESDA PANEL ANALYSIS

With five micro-satellites analyzed, patients can be categorized as MSS (0 markers exhibiting instability), MSI-L (1 marker being unstable) or MSI-H (2 or more loci being unstable, see Table 7). Typical size ranges are described in Table 10 and examples of fragment analyses are shown in Figures 1-8.

Table 7: PlentiPlex™ MSI Bethesda Panel Analysis

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

8.2 PLENTIplex™ MSI HAMELIN PANEL ANALYSIS

It has been shown that Hamelin’s panel can be analysed using non-related germline DNA as reference, requiring only tumor DNA from the patient to be analysed (Surrawera et al. 2002). The germline DNA should be included in each run on the instrument, to account for inter run variations. Using a non-related germline DNA as reference you are only able to differentiate between MSS and MSI-H, whereas inclusion of the patient’s germline DNA as reference, allows for discrimination between MSS, MSI-L and MSI-H.

8.2.1 PLENTIplex™ MSI HAMELIN PANEL ANALYSIS WITH NON-MATCHED WT GDNA AS REFERENCE

When using non-matched WT gDNA as reference, with five micro-satellites analyzed, patients are categorized as MSS (0 markers exhibiting instability) or MSI-H (3 or more loci being unstable, see Table 8). When only 1 or 2 of the 5 loci exhibit micro-satellite length alteration(s) we recommend to re-run the sample preferably with inclusion of patient matched germline (non-tumor) gDNA as reference.

Table 8: PlentiPlex™ MSI Hamelin Panel Analysis (non-matched reference)

Unstable loci (#)	Categori
0	MSS
1-2	MSS/ Not valid (re-run sample)
3-5	MSI-H

Typical size ranges are described in Table 11 and examples of fragment analyses are shown in Figures 4-8.

8.2.2 PLENTIplex™ MSI HAMELIN PANEL ANALYSIS WITH TUMOR-MATCHED NON-INVOLVED WT gDNA AS REFERENCE

When using tumor-matched non-involved patient DNA as reference, with five micro-satellites analyzed, patients can be categorized as MSS (0 markers exhibiting instability), MSI-L (1 marker being unstable) or MSI-H (2 or more loci being unstable, see Table 9).

Table 9: PlentiPlex™ MSI Hamelin Panel Analysis (matched reference)

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

Typical size ranges are described in Table 11 and examples of fragment analyses are shown in Figures 4-8.

Table 10: MSI Bethesda Panel loci identification

Marker	Detected in channel	Label	Typical size range (bp)
BAT25	Blue	PentaGreen	121-127
BAT26	Red	PentAltRed	122-128
D2S123	Blue	PentaGreen	197-227
D5S346	Yellow	PentAltOrange	96-132
D17S250	Green*	PentAltYellow	147-169

*Risk of crosstalk between green and yellow channel, so that green signal can be observed in yellow channel.

Table 11: MSI Hamelin Panel loci identification

Marker	Detected in channel	Label	Average size (bp)
BAT25	Blue	PentaGreen	120
BAT26	Red	PentAltRed	124
NR-21	Green*	PentAltYellow	103
NR-22	Blue	PentaGreen	142
NR-24	Green*	PentAltYellow	132

*Risk of crosstalk between green and yellow channel, so that green signal can be observed in yellow channel.

Data examples

The Figures below illustrate both MSS and MSI cases. MSI cases with differences between the reference tissue and the tumor tissue are highlighted by red brackets.

Figure 1: Di-nucleotide locus – D2S123/D5S346/D17S250 – stability

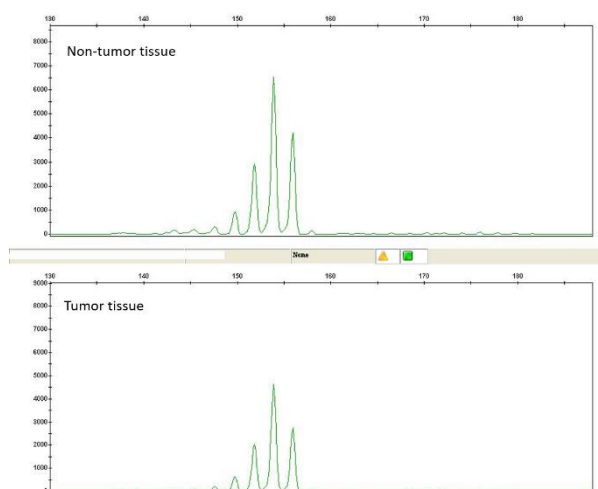


Figure 2: Di-nucleotide locus – D2S123/D5S346/D17S250 – instability

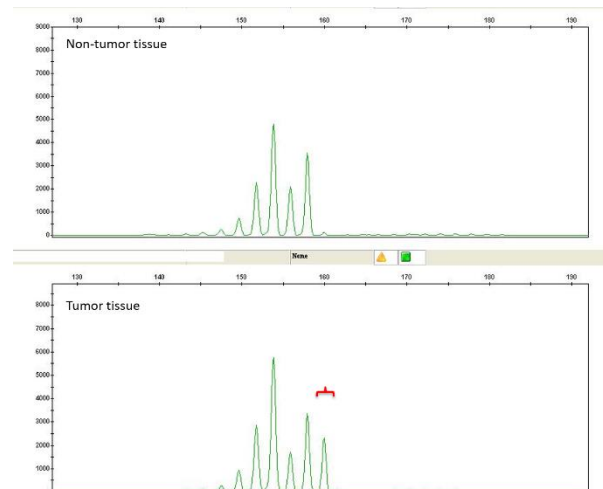


Figure 3: Di-nucleotide locus – D2S123/D5S346/D17S250 – instability

Figure 4: Di-nucleotide locus – D2S123/D5S346/D17S250 – instability

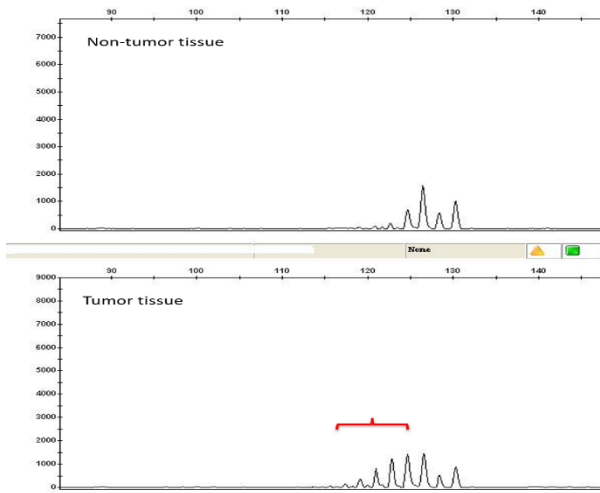


Figure 5: Mono-nucleotide locus – BAT25/26 – stability

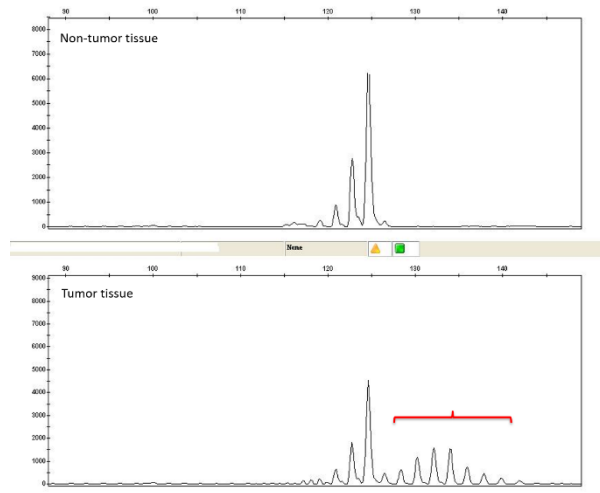


Figure 6: Mono-nucleotide locus – BAT25/26 – instability

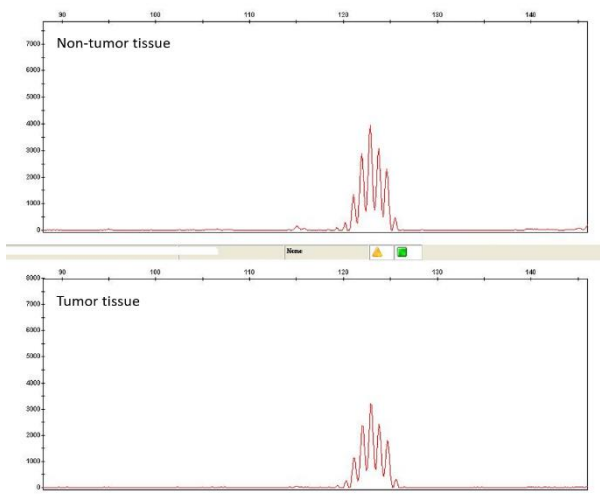


Figure 7: Mono-nucleotide locus – BAT25/26 – instability

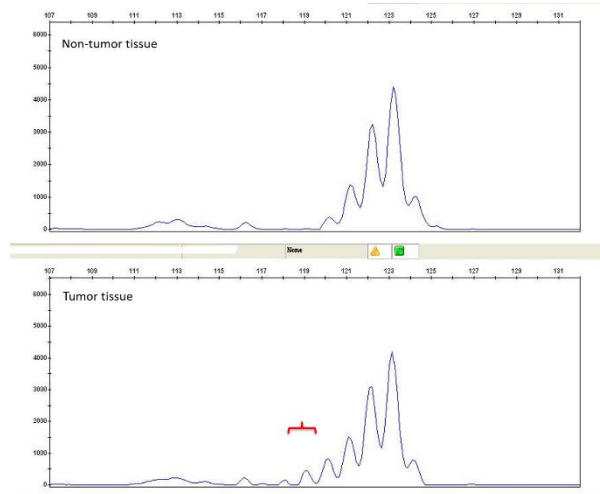


Figure 8: Mono-nucleotide locus – BAT25/26 – instability

9. TROUBLESHOOTING

The troubleshooting guide shown in Table 12 covers some of the most frequent questions and problems that can occur with the use of PlentiPlex™ and how they might be solved.

Table 12: Troubleshooting

Problem	Solution
Low or no signal	Amplification has not taken place efficiently. Try and re-run the PCR. If no signal is observed again, please try to re-extract gDNA.
Extra signals in Yellow channel	The primers labelled with PentaAltYellow can yield cross signal into the yellow channel. Please disregard signals in the yellow channel above 140 base pairs.

10. REFERENCES

Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, and Srivastava S. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res.* 1998 58(22):5248-57.


Richman S. Deficient mismatch repair: Read all about it (Review). *International Journal of Oncology* 2015 47: 1189-1202

Suraweera N, Duval A, Reperant M, Vaury C, Furlan D, Leroy K, Seruca R, Iacopetta B, and Hamelin R. *Gastroenterology.* 2002 123(6):1804-11.

Vaksman Z. and Garner HR. Somatic microsatellite variability as a predictive marker for colorectal cancer and liver progression. *Oncotarget* 2015 6(8): 5760-71.

11. MANUFACTURER AND DISTRIBUTORS

11.1 MANUFACTURER

	PentaBase ApS Lumbyvej 19G 5000 Odense C, Denmark
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11.2 TECHNICAL ASSISTANCE

For technical assistance in Denmark, contact PentaBase ApS:

www.pentabase.com

support@pentabase.com

Phone: +45 3696 9496

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

12. PLENTIplex™ PRODUCT OVERVIEW

Gene	PlentiPlex™ Ready-to-use	Strip #	Catalogue # 1; 12; 60 reactions
MSI	MSI Bethesda Panel Ready-to-use	M1	7000-7002
MSI	MSI Hamelin Panel Ready-to-use	M2	7010-7012

13. ADDITIONAL INFORMATION

The CE IVD labeled PlentiPlex™ assays are medical equipment intended for *in vitro* diagnostic in compliance with EU's Directive 98/79/EC. Products or parts of it must not be resold or transferred without PentaBase's acceptance. PentaBase ApS takes certain reservation for changes. PentaBase ApS disclaim all responsibility for any errors that may appear in this Instructions for use. Furthermore, PentaBase ApS disclaim all responsibility for misinterpretation that can occur by using this product.

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13.1 DATE OF REVISION

SensiScreen® protocol was revised April 2018.

Changes from previous version

PROCEDURE No.	Effective Date	Significant Changes	Previous PROCEDURE No.
1	June 2017	New procedure	N/A
1.1	February 2018	Revised section 11 and 13	1
1.2	March 2018	Added the option to use non-matched WT DNA in the Hamelin panel and revised analysis guidelines accordingly (section 4.1, 7.2 and 8)	1.1
1.3	April 2018	The PCR protocol for the Bethesda panel is now the same as the one used for the Hamelin panel	1.2

