

Product Description

SALSA® MS-MLPA® Probemix

ME011-D1 Mismatch Repair Genes

To be used with the MS-MLPA General Protocol.

Version D1

For complete product history see page 15.

Catalogue numbers:

- **ME011-025R:** SALSA MS-MLPA Probemix ME011 Mismatch Repair Genes, 25 reactions.
- **ME011-050R:** SALSA MS-MLPA Probemix ME011 Mismatch Repair Genes, 50 reactions.
- **ME011-100R:** SALSA MS-MLPA Probemix ME011 Mismatch Repair Genes, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit, SALSA HhaI (SMR50) and Coffalyser.Net data analysis software. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman/SCIEX capillary sequencers, respectively (see www.mrcholland.com).

Certificate of Analysis

Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mrcholland.com.

Precautions and warnings

For professional use only. Always consult the most recent product description AND the MS-MLPA General Protocol before use: www.mrcholland.com. It is the responsibility of the user to be aware of the latest scientific knowledge of the application before drawing any conclusions from findings generated with this product.

Intended purpose

The SALSA MS-MLPA probemix ME011 Mismatch Repair Genes is an in vitro diagnostic (IVD)¹ semi-quantitative assay² to determine methylation status of selected GCGC sites in the promoter regions of the human *MLH1*, *MSH2*, *PMS2* and *MSH6* genes and to detect the *BRAF* p.V600E (c.1799T>A) point mutation in order to stratify the risk of having Lynch syndrome. In addition, this assay can be used for the detection of deletions or duplications in the human 3' region of the *EPCAM* gene and the promoter regions of *MLH1*, *MSH2*, *PMS2* and *MSH6* genes in order to confirm a potential cause for and clinical diagnosis of Lynch syndrome and at-risk family members.

This assay is for use with genomic DNA isolated from human peripheral whole blood specimens, fresh, frozen and FFPE tissue of colorectal and endometrial cancer, given that more than 50% of DNA sample is of tumour origin. For analysis of tumour samples, same or similar source of tissue, identical sample treatment, identical DNA extraction method and a minimum of three healthy reference samples should be used.

Copy number variations (CNVs), methylation status and the mutation status of *BRAF* p.V600E detected with ME011 Mismatch Repair Genes should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method.

Assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

This device is not intended to be used for standalone diagnostic purposes, pre-implantation or prenatal testing, and population screening.

¹ Please note that this probemix is for in vitro diagnostic (IVD) use in the countries specified at the end of this product description. In all other countries, the product is for research use only (RUO).

² To be used in combination with a SALSA MLPA Reagent Kit, Coffalyser.Net analysis software and SALSA HhaI.

Clinical background

The main genes in the DNA mismatch repair (MMR) system are *MLH1*, *PMS2*, *MSH2* and *MSH6*. Heterodimers of proteins encoded by these genes (*MLH1/PMS2* and *MSH2/MSH6*) repair and prevent DNA mutations. Defects in the cell's MMR system may lead to the accumulation of mutations resulting in the initiation of cancer. Epigenetic silencing or loss of function mutations in the above-mentioned genes cause MMR deficiency and microsatellite instability (MSI). Heterozygous germline mutations in any of the MMR genes result in Lynch syndrome (LS, also known as hereditary nonpolyposis colorectal cancer, or HNPCC) - an autosomal dominant cancer predisposition condition. LS is characterised by an increased risk of colorectal cancer, endometrial cancer, gastric cancer, ovarian cancer and other cancers such as hepatobiliary tract, urinary tract, brain and skin. Genetic alterations in the *MLH1* and *MSH2* genes have been found in up to 90% of LS cases, whereas *MSH6* and *PMS2* gene alterations are less frequently detected. Around 1-3% of LS cases are explained by *EPCAM* deletions. Elimination of the *EPCAM* transcription termination signal results in transcription continuing into *MSH2* and silencing of the *MSH2* promoter by methylation (Kuiper et al. 2011, Ligtenberg et al. 2009, Niessen et al. 2009). More information on LS is available at <https://www.ncbi.nlm.nih.gov/books/NBK1211/>.

In sporadic MSI-positive colorectal and endometrial carcinomas the *MLH1* promoter is methylated in 10-20% of cases resulting in the loss of *MLH1* expression (Cunningham et al. 1998, Esteller et al. 1998, Herman et al. 1998, Kane et al. 1997, Simpkins et al. 1999). *MLH1* promoter methylation testing is performed in order to differentiate between sporadic MSI and LS. The CpG sites in the C- and D- "Deng" regions of the *MLH1* gene are of main interest (Deng et al. 1999). *MLH1* promoter methylation analysis on tumor tissue can improve the selection of patients for LS genetic testing and thus provide substantial cost reductions (Perez-Carbonell et al. 2010). Of note, in recent years rare cases of constitutional *MLH1* hypermethylation together with a somatic mutation in the functional allele have been reported in LS families (Goel et al. 2011, Morak et al. 2018, Pinto et al. 2018).

Promoter inactivation by methylation of *MSH6* or *PMS2* has not been reported according to our literature review in LS patients or described as a somatic cause in colorectal or endometrial tumours.

BRAF pathogenic variants, most commonly the p.V600E mutation, is another important molecular marker identified in ~15% of sporadic colorectal cancers (Bouzourene et al. 2010). These mutations have a strong association with *MLH1* promoter methylation, and therefore *BRAF* mutation and *MLH1* methylation tests are often performed concurrently. *BRAF* mutation is frequently present in sporadic colorectal cancer with methylated *MLH1*, but not in LS. Given the comparative rarity of *BRAF* mutation and *MLH1* hypermethylation in LS tumours, the testing of those is done in tumour tissue of colon cancer cases to differentiate LS-associated cancer from more common sporadic cancers. *BRAF* pathogenic variants, however, are not common in sporadic endometrial cancers; thus, *BRAF* testing is not helpful in distinguishing endometrial cancers that are sporadic from those that are LS-related.

Gene structure

- The *MLH1* gene spans ~ 57.5 kilobases (kb) on chromosome 3p22.2 and contains 19 exons.
The *MLH1* LRG_216 is available at www.lrg-sequence.org and is identical to GenBank NG_007109.2.
- The *MSH2* gene spans ~ 80.2 kilobases (kb) on chromosome 2p21 and contains 16 exons.
The *MSH2* LRG_218 is available at www.lrg-sequence.org and is identical to GenBank NG_007110.2.
- The *MSH6* gene spans ~ 23.9 kilobases (kb) on chromosome 2p16.3 and contains 10 exons.
The *MSH6* LRG_219 is available at www.lrg-sequence.org and is identical to GenBank NG_007111.1.
- The *PMS2* gene spans ~ 35.9 kilobases (kb) on chromosome 7p22.1 and contains 15 exons.
The *PMS2* LRG_161 is available at www.lrg-sequence.org and is identical to GenBank NG_008466.1.
- The *EPCAM* gene spans ~ 17.9 kilobases (kb) on chromosome 2p21 and contains nine exons.
The *EPCAM* LRG_215 is available at www.lrg-sequence.org and is identical to GenBank NG_012352.2.
- The *BRAF* gene spans ~ 190.8 kilobases (kb) on chromosome 7q34 and contains 18 exons.
The *BRAF* LRG_299 is available at www.lrg-sequence.org and is identical to GenBank NG_007873.3.

Transcript variants

- For *MLH1*, multiple transcript variants have been described. Transcript variant 1 (NM_000249.4, 2494 nt, coding sequence 31-2301; <https://www.ncbi.nlm.nih.gov/gene/4292>) is the longest variant.
- For *MSH2*, two transcript variants have been described. Transcript variant 1 (NM_000251.3, 3115 nt, coding sequence 37-2841; <https://www.ncbi.nlm.nih.gov/gene/4436>) is the longest variant.
- For *MSH6*, multiple transcript variants have been described. Transcript variant 1 (NM_000179.3, 4265 nt, coding sequence 90-4172; <https://www.ncbi.nlm.nih.gov/gene/2956>) is the longest transcript.
- For *PMS2*, multiple transcript variants have been described. Transcript variant 1 is the most predominant and encodes isoform a (NM_000535.7, 5093 nt, coding sequence 31-2619; <https://www.ncbi.nlm.nih.gov/gene/5395>).
- For *EPCAM*, one transcript variant has been described (NM_002354.3, 1547 nt, coding sequence 196-1140; <https://www.ncbi.nlm.nih.gov/gene/4072>).
- For *BRAF*, multiple transcript variants have been described. Transcript variant 1 is the most frequently used one in literature (NM_004333.6, 6459 nt, coding sequence 227-2527; <https://www.ncbi.nlm.nih.gov/gene/673>).

Exon numbering

The exon numbering of target genes used in this ME011-D1 Mismatch Repair Genes product description is the exon numbering from the RefSeq transcripts, which are identical to the corresponding LRG_sequences as noted in the table below. To note, the version of the transcript sequence used in this product description is different from the version used for LRG_sequence.

gene	RefSeq transcript	LRG-sequence (NM_sequence)
<i>MLH1</i>	NM_000249.4	LRG_216 (NM_000249.3)
<i>MSH2</i>	NM_000251.3	LRG_218 (NM_000251.2)
<i>MSH6</i>	NM_000179.3	LRG_219 (NM_000179.2)
<i>PMS2</i>	NM_000535.7	LRG_161 (NM_000535.5)
<i>EPCAM</i>	NM_002354.3	LRG_215 (NM_002354.2)
<i>BRAF</i>	NM_004333.6	LRG_299 (NM_004333.4)

The exon numbering of the NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG sequences. As changes to the databases can occur after release of this product description, the NM_ sequence and exon numbering may not be up-to-date.

Probemix content

The SALSA MS-MLPA Probemix ME011-D1 Mismatch Repair Genes contains 34 (MS-)MLPA probes with amplification products between 123 and 398 nucleotides (nt). 14 MS-MLPA probes contain an HhaI recognition site and provide information on the methylation status of selected GCGC sites in the promoter regions of *MLH1*, *MSH2*, *MSH6* and *PMS2* genes. All probes present will also give information on copy number changes in the analysed sample. Furthermore, the probemix also contains a probe specific for the *BRAF* p.V600E mutation and a probe specific for the alternative T-allele of rs104894994 single nucleotide polymorphism (SNP), which will only generate a signal when respectively the mutation or the SNP is present. In addition, 13 reference probes are included that are not affected by HhaI digestion and target relatively copy number stable regions in various cancer types including colorectal and endometrial cancer. Also, two digestion control probes are included in this probemix indicating whether or not restriction endonuclease digestion in the MS-MLPA reaction was complete. Complete probe sequences and the identity of the genes detected by the reference and digestion control probes are available in Table 2b and online (www.mrcholland.com).

This probemix contains nine quality control fragments generating amplification products between 64 and 105 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and one chromosome Y-specific fragment (see table below).

More information on how to interpret observations on these control fragments can be found in the MS-MLPA General Protocol and online at www.mrcholland.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

MS-MLPA technique

The principles of the MS-MLPA technique (Nygren et al. 2005, Schouten et al. 2002) are described in the MS-MLPA General Protocol (www.mrcholland.com). More information on the use of MLPA in tumour applications can be found in Hömig-Hölzel and Savola (2012).

MS-MLPA technique validation

Internal validation of the MS-MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using MS-MLPA for the first time, or when changing the sample handling procedure, DNA extraction method or instruments used. This validation experiment should result in a standard deviation ≤ 0.10 for all probes over the experiment.

Results of MS-MLPA are highly dependent on the HhaI enzyme used. HhaI enzymes that are resistant to heat inactivation are NOT compatible with the MS-MLPA technique and will give aberrant results. These include, but may not be limited to, Thermo Fisher Scientific enzymes HhaI, ANZA 59 HhaI, and FastDigest HhaI. SALSA HhaI enzyme (SMR50) must be used in combination with this probemix as this restriction enzyme has been validated for use with MS-MLPA by MRC Holland.

Required specimens

Extracted DNA from human peripheral whole blood specimens or fresh, frozen and FFPE tissue of colorectal and endometrial cancer, free from impurities known to affect MLPA reactions. For cancer specimens more than 50% of DNA sample should be of tumour origin. For more information please refer to the section on DNA sample treatment found in the MS-MLPA General Protocol.

Reference samples

A sufficient number (≥ 3) of reference samples should be included in each MS-MLPA experiment for data normalisation and to identify the baseline methylation level for each methylation-specific probe. **Suitable reference samples should be negative for rs104894994 SNP alternative alleles** at the HhaI enzyme recognition site of the 172 nt *MLH1* methylation-specific probe (01686-L28585) to allow complete HhaI digestion and therefore reliable data analysis. The presence of the T alternative allele of this SNP can be detected by the 289 nt SNP-specific probe (22572-L31773). The frequency of the alternative alleles is very low, meaning signal absence for this SNP-specific probe at 289 nt in the majority of reference samples, however a probe signal at 289 nt should be evaluated for each new reference sample. All samples tested, including reference DNA samples, should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method when possible. When selecting reference samples, please note that methylation patterns may vary between tissue types and even age groups! Reference samples should be derived from different healthy individuals without a history of LS-related cancer. More information regarding the selection and use of reference samples can be found in the MS-MLPA General Protocol (www.mrcholland.com).

Positive control DNA samples

MRC Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. Coriell Institute (<https://catalog.coriell.org>) and Leibniz Institute DSMZ (<https://www.dsmz.de/>) have diverse collections of biological resources which may be used as positive

control DNA samples in your MLPA experiments. Sample ID numbers NA13451, NA10401, NA04127 and NA07081 from the Coriell Institute, and DU-4475 (ACC-427), DK-MG-UN (ACC-277) and SK-N-MC (ACC-203) from Leibniz Institute DSMZ have been tested with this ME011-D1 probemix at MRC Holland and can be used as a positive control samples as described in the table below. The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Chromosomal position of CNA*	Copy number altered target genes/ probes in ME011-D1	Expected CNA	Expected methylation status of CpGs targeted by MS-MLPA probes in ME011-D1	BRAF p.V600E
NA13451	2p21-p16.3	<i>EPCAM</i> , <i>MSH2</i> and <i>MSH6</i>	Heterozygous deletion	All MMR genes not methylated	-
NA10401 ¹	2p21-p16.3	<i>EPCAM</i> , <i>MSH2</i> and <i>MSH6</i>	Heterozygous duplication	All MMR genes not methylated	-
NA04127	3p22.2	<i>MLH1</i>	Heterozygous duplication	All MMR genes not methylated	-
NA07081	7p22.1	<i>PMS2</i>	Heterozygous duplication	All MMR genes not methylated	-
DU-4475	3p22.2	normal copy number for all target probes	none	Moderate methylation (~50%) for 247 and 278 nt <i>MLH1</i> probe targets; all other genes – not methylated	present
SK-N-MC ²	3p22.2	<i>MLH1</i>	Heterozygous deletion	Low methylation (10-15%) for all <i>MLH1</i> probe targets; all other genes – not methylated	-
DK-MG-UN ³	7p22.1	<i>PMS2</i>	Heterozygous duplication	All MMR genes not methylated	-

* Indicated chromosomal bands accommodate genes targeted by MLPA probes, however, the whole extent of copy number alteration (CNA) present in this cell line cannot be determined by this ME011-D1 Mismatch Repair Genes probemix.

¹ Heterozygous duplication for the digestion control probe at 132 nt and for reference probes at 127 and 190 nt are detected in this sample.

² Heterozygous loss for the digestion control probe at 132 nt and for reference probes at 178 and 398 nt are detected in this sample.

³ Heterozygous loss for the digestion control probe at 132 nt and for reference probes at 238 and 398 nt are detected in this sample.

SALSA Binning DNA SD086

The SD086 Binning DNA provided with this probemix can be used for binning of all probes including one mutation- and one SNP-specific probe (226 nt probe 08780-SP0039-L08904 *BRAF* p.V600E mutation and 289 nt probe 22572-L31773 *MLH1* rs104894994 (C>T) SNP probe).

Important to know about SD086

i) The rs104894994 SNP detected by 289 nt SNP-specific probe (22572-L31773) is located at the HhaI enzyme recognition site of 172 nt *MLH1* methylation-specific probe (01686-L28585), and therefore, in the digested MS-MLPA reaction 33% residual signal is expected on SD086.

ii) The target sequence of 289 nt rs104894994 SNP-specific probe (22572-L31773) largely overlaps with the target sequence of the 172 nt methylation-specific probe (01686-L28585), resulting in increased signal (one additional copy) of 172 nt probe on SD086 (ratio 1.3-1.65 expected) in the undigested MS-MLPA reaction.

More information on 172 and 289 nt probes can be found in the corresponding footnotes below Table 1 and Table 2a, as well as in the "Interpretation of methylation results on blood and tissue derived DNA samples - ME011 probemix specific notes" section in this product description.

SD086 Binning DNA is a mixture of genomic DNA from healthy individuals and synthetic DNA that contains the target sequence detected by the above mentioned probes. Inclusion of one reaction with 5 µl SD086 Binning DNA in initial MLPA experiments is essential as it can be used to aid in data binning of the peak pattern using

Coffalyser.Net software. Furthermore, Binning DNA should be included in the experiment whenever changes have been applied to the set-up of the capillary electrophoresis device (e.g. when capillaries have been renewed). Binning DNA should never be used as a reference sample in the MLPA data analysis, neither should it be used in quantification of mutation and/or SNP signals. It is strongly advised that all samples tested are extracted with the same method and derived from the same source of tissue. For further details, please consult the SD086 Binning DNA product description, available online: www.mrcholland.com. **This product is for research use only (RUO).**

Performance characteristics

MLH1 promoter methylation, a negative predictive marker for LS, is described in 10-20% sporadic colorectal and endometrial cancer cases. *BRAF* p.V600E mutation is identified in ~15% of sporadic colorectal cancers and is strongly associated with *MLH1* methylation. In up to 90% of hereditary cancer cases (LS), pathogenic variants in the *MLH1* and *MSH2* genes have been found, whereas genetic alterations in *MSH6* and *PMS2* genes are less frequent. Among those variants, promoter hypermethylation has been detected in up to 3% for *MLH1*, 1-3% for *MSH2* (due to *EPCAM* deletion); no studies report promoter hypermethylation for *PMS2* and *MSH6* (only somatic hypermethylation described in advanced colorectal cancer).

The analytical sensitivity and specificity are above 99% for *MLH1* promoter methylation detection by this assay based on literature review for the period of 2008-2020. Analytical performance can be compromised by: SNVs or other polymorphisms in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MS-MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

Data analysis

Coffalyser.Net software should be used for data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet. For both, the latest version should be used. Coffalyser.Net software is freely downloadable at www.mrcholland.com. Use of other non-proprietary software may lead to inconclusive or false results. For more details on MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual. Reference samples should be consulted to identify baseline methylation levels for each methylation-specific probe.

Interpretation of copy number results

The expected results for the *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM* specific MLPA probes are allele copy numbers of 2 (normal), 1 (heterozygous deletion), 0 (homozygous deletion), 3 (heterozygous duplication) or 4 (homozygous duplication). The standard deviation of each individual probe over all the reference samples should be ≤ 0.10 . When these criteria are fulfilled, the following cut-off values for the FR of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Final ratio (FR)
Normal	$0.80 < FR < 1.20$
Homozygous deletion	FR = 0
Heterozygous deletion	$0.40 < FR < 0.65$
Heterozygous duplication	$1.30 < FR < 1.65$
Heterozygous triplication/homozygous duplication	$1.75 < FR < 2.15$
Ambiguous copy number	All other values

Note: The term “dosage quotient”, used in older product description versions, has been replaced by “final ratio” to become consistent with the terminology of the Coffalyser.Net software. (Calculations, cut-offs and interpretation remain unchanged.) Please note that the Coffalyser.Net software also shows arbitrary borders as part of the statistical analysis of results obtained in an experiment. As such, arbitrary borders are different from the final ratio cut-off values shown here above.

Please note that these above mentioned final ratios are only valid for germline testing. Final ratios are affected both by percentage of tumour cells and by possible subclonality. The minimum percentage of cells with a CNA required for reliable copy number analysis by MLPA is 25-30% (Al Zaabi et al. 2010, Coll-Mulet et al. 2008).

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in subclonal/mosaic cases.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region or in or near the *MSH6* gene. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: <http://dgv.tcag.ca/dgv/app/home>. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MS-MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by (Schwartz et al. 2007)). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Digestion Control Probes. The target sequences of the digestion control probes are unmethylated in most blood-derived DNA samples. The signals of the digestion control probes should be gone upon complete digestion by HhaI.
- mRNA levels. We have no data showing that methylation detected by a particular probe indeed influences the corresponding mRNA levels.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: a lower injection voltage or a shorter injection time, or a reduced amount of sample by diluting PCR products.

Interpretation of methylation results on blood and tissue derived DNA samples:

This probemix is intended for determining if the DNA sequences targeted by the methylation-specific probes show differential methylation as compared to the reference samples. This requires the determination of a “baseline” level of methylation, which can be used to determine if the methylation level in a test sample is significantly different from the reference samples.

The baseline methylation level must be determined for every individual methylation-specific probe, and is applicable for one particular experiment. This is important because the level of methylation in samples from healthy individuals depends on the probe’s target sequence and its location in the CpG island, the tissue type and, in certain cases, even on the age of the individual. The detection of methylation can also be influenced by impurities in the DNA sample that alter the activity of the HhaI enzyme. The presence of such impurities may differ between tissue types and DNA extraction methods.

To determine the baseline methylation level, it is required to test a sufficient number (≥ 3) of reference samples from healthy individuals. These samples should be derived from the same tissue type, handled using the same procedure (e.g. FFPE vs. fresh frozen), and prepared using the same DNA extraction method.

The baseline methylation level is then calculated by taking the average value of final ratios of the reference samples per probe and adding two times the standard deviation. The table below contains an example. Note that each individual methylation-specific probe should have a separate baseline methylation level and those values should not be averaged between the probes.

Probe	Reference sample 1	Reference sample 2	Reference sample 3	Average	Standard deviation	Baseline level (mean+2×stdev)
Methylation-specific probe 1	0.08	0.00	0.06	0.047	0.042	0.13
Methylation-specific probe 2	0.05	0.07	0.03	0.050	0.020	0.09
Methylation-specific probe 3	0.02	0.02	0.02	0.020	0	0.02

To determine if a test sample has a significantly increased methylation level for a particular probe, compare the methylation ratio of the probe with the baseline level.

- Methylation ratio of a probe in test sample $>$ baseline: methylation is increased.
- Methylation ratio of a probe in test sample \leq baseline: methylation is *not* increased.

Interpretation of methylation positive samples is dependent on the application used.

NOTE: In case digestion control probes are not fully digested (>0.05), please contact info@mrcholland.com for more information.

- MS-MLPA sensitivity in tumour tissue-derived DNA samples, similar to copy number determination, is affected both by percentage of tumour cells and by possible subclonality, as well as by the baseline level of methylation in reference samples which is determined for each experiment. MS-MLPA sensitivity should be therefore estimated after baseline methylation determination. This baseline or cut-off for ME011 methylation-specific probes used in literature varies between 0.10 and 0.30.

ME011 specific notes:

- The rs104894994 SNP detected by 289 nt SNP-specific probe (22572-L31773) is located at the HhaI enzyme recognition site (GCGC) of 172 nt MLH1 methylation-specific probe (01686-L28585). When the T-allele of this SNP is present resulting in HhaI recognition site disruption (GCGC to GTGC), the digestion will not occur. The presence of this T-allele hampering the HhaI digestion can be detected by the rs104894994 SNP-specific probe at 289 nt included in this ME011-D1 probemix. If this 289 nt probe gives signal, the methylation results obtained with 172 nt MLH1 probe (01686-L28585) are considered as false-positive and should be disregarded.
- Methylation analysis of *MLH1* promoter region: the probes targeting GCGC sites in the so-called Deng C- and Deng D-regions of the *MLH1* promoter are of main interest, as methylation of these regions is strongly associated with *MLH1* silencing. The clinical relevance of methylation only in Deng A and/or B regions is not well established.
- Please note that several probes have multiple HhaI restriction sites. All of these sites need to be methylated in order to not be digested!
- The presence of a clear signal for the 226 nt probe (at least 10% of the mean peak height of all reference probes in the sample), indicates the presence of the *BRAF* p.V600E mutation. However, percentage of tumour cells and possible mutation subclonality affect the assay sensitivity of mutation detection. Reliable detection of point mutation is possible when about 10% of the DNA sample carries a mutation (Homig-Holzel and Savola 2012).
- In case of an unspecific background signal for the *BRAF* mutation-specific (08780-SP0039-L08904) and rs104894994 SNP-specific probes (22572-L31773) in wild-type samples, the assignment of probe identities in Coffalyser.Net (v.140721.1958) data analysis is not done properly. In order to identify each peak in the

peak pattern as a probe, it is recommended to create a manual binset using the SD sample (SD086), which is provided with each vial of ME011 probemix. Instructions to create a manual binset can be found in the Coffalyser.Net Reference Manual at Coffalyser.Net section on the MRC Holland Support Portal: <https://support.mrcholland.com/>.

- MSH6 probe 21588-L30979 at 148 nt and PMS2 probe 07934-L16147 are not completely digested in DNA samples derived from blood and show 5-10% background signal after HhaI digestion. Low methylation ratios obtained with these probes should be treated with caution.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *MLH1*, *MSH2*, *MSH6* and *PMS2* genes are small (point) mutations, most of which will not be detected by using SALSA MS-MLPA Probemix ME011 Mismatch Repair Genes.
- MS-MLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations. Even when MS-MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.
- Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can cause false positive results. Mutations/SNVs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA.
- An MS-MLPA probe targets a single specific HhaI site in a CpG island; if methylation is absent for a particular CpG-site, this does not necessarily mean that the whole CpG island is unmethylated!
- Rare cases are known in which apparent methylation as detected by an MS-MLPA probe proved to be due to a sequence change in or very near the HhaI site.
- MS-MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. Changes in methylation status, gains or losses of genomic regions or genes may not be detected if the percentage of tumour cells is low. In addition, subclonality of the aberration affects the final ratio of the corresponding probe. Furthermore, there is always a possibility that one or more reference probes *do* show a copy number alteration in a patient sample, especially in solid tumours with more chaotic karyotypes.

Confirmation of results

Confirmation of methylation status can be performed with another technique, such as MSP (methylation-specific PCR), pyrosequencing, digestion-based PCR assays, etc. Copy number changes detected by only a single probe always require confirmation by another method. An apparent deletion detected by a single probe can be due to e.g. a mutation/polymorphism that prevents ligation or destabilises the binding of probe oligonucleotides to the DNA sample. Sequence analysis can establish whether mutations or polymorphisms are present in the probe target sequence. The finding of a heterozygous mutation or polymorphism indicates that two different alleles of the sequence are present in the sample DNA and that a false positive MS-MLPA result was obtained.

Copy number changes detected by more than one consecutive probe should be confirmed by another independent technique such as long range PCR, qPCR, array CGH or Southern blotting, whenever possible. Deletions/duplications of more than 50 kb in length can often be confirmed by FISH.

MMR, LOVD and COSMIC mutation databases: <http://www.insight-group.org/>; <http://www.lovd.nl/>; <http://cancer.sanger.ac.uk/cosmic>. We strongly encourage users to deposit germline positive results in the LOVD database or the International Society for Gastrointestinal Hereditary Tumours (INSiGHT) or for somatic mutations in the COSMIC database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on <http://varnomen.hgvs.org/>.

Please report copy number changes detected by the reference probes, false positive results due to SNVs and unusual results to MRC Holland: info@mrcholland.com.

Table 1. SALSA MS-MLPA Probemix ME011-D1 Mismatch Repair Genes

Length (nt)	SALSA MLPA probe	Hhal site	% expected signal reduction ^a	Chromosomal position (hg18)			Mutation-specific
				Reference	MMR and EPCAM	Other	
64-105	Control fragments – see table in probemix content section for more information						
123	Reference probe 18709-L13645	-	-	5q31			
127	Reference probe 19551-L30430	-	-	2p13			
132 * π ‡ †	Digestion control probe 22576-L31899	+	100%		10q22		
137	Reference probe 03896-L21555	-	-	11q13			
142 #	PMS2 probe 07935-L16571	+	100%	7p22.1			
148 ^ «	MSH6 probe 21588-L30979	+	95-100%	2p16.3			
154	PMS2 probe 11966-L13112	+	100%	7p22.1			
160	Reference probe 10694-L26069	-	-	6p12			
167 «	MSH6 probe 06228-L30148	+	100%	2p16.3			
172 ‡	MLH1 probe 01686-L28585	+	100%	3p22.2			
178	Reference probe 18842-L30565	-	-	3p14			
184	MSH2 probe 06227-L07711	+	100%	2p21			
190	Reference probe 12780-L28100	-	-	2q13			
197	EPCAM probe 11983-L30436	-	-	2p21			
201 ‡	MLH1 probe 06222-L26305	+	100%	3p22.2			
208	Reference probe 10644-L30437	-	-	9q21			
213 ¥ † «	MSH6 probe 06230-L29780	+	100%	2p16.3			
220 ¥	EPCAM probe 22424-L31926	-	-	2p21			
226 § Ж	BRAF probe 08780-SP0039-L08904	-	-			p.V600E	
232	Reference probe 05709-L30439	-	-	3q21			
238	Reference probe 08070-L07851	-	-	9p13			
247	MLH1 probe 07187-L26307	+	100%	3p22.2			
254	MSH2 probe 06226-L31127	+	100%	2p21			
263	Reference probe 15809-L30975	-	-	19q13			
278 ‡	MLH1 probe 06221-L31128	+	100%	3p22.2			
289 * †	MLH1 probe 22572-L31773	-	-		rs104894994		
297	MLH1 probe 02258-L30977	+	100%	3p22.2			
310 #	EPCAM probe 13131-L03603	-	-	2p21			
323	Reference probe 09065-L30441	-	-	19p13			
338 ^ #	PMS2 probe 07934-L16147	+	90-95%	7p22.1			
352 †	MSH2 probe 02735-L02162	+	100%	2p21			
369 π	Digestion control probe 21589-L27783	+	100%		2q12		
386	Reference probe 13404-L17499	-	-	6q12			
398	Reference probe 00973-L30443	-	-	10q21			

^a Expected signal reduction on blood DNA derived samples. On other tissue or tumour derived samples these percentages can be different.

* New in version D1.

¥ Changed in version D1. Minor alteration, no change in the sequence detected.

§ Mutation-specific probe. This probe will only generate a signal when the *BRAF* p.V600E mutation is present. It has been tested on artificial DNA and on DU-4475 human cancer cell line derived DNA **but not on positive human samples!**

‡ Target sequence of this probe contains SNP rs104894994 in the GCGC site, +6 nt from the ligation site. When the T-allele of this validated SNP (with an allele frequency of 0.14%) is present, HhaI digestion will not occur, resulting in a false methylation positive signal. The presence of this T-allele hampering the HhaI digestion can be detected by the rs104894994 SNP-specific probe at 289 nt included in this ME011-D1 probemix.

† SNP-specific probe. This probe will only generate a signal when the T-allele of rs104894994 SNP is present. It has been tested on artificial DNA and on selected 1000 genome project samples. If this probe gives signal, the methylation results obtained with 172 nt MLH1 probe (01686-L28585) are considered as false-positive and should be disregarded.

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

- π Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.
- Ð This probe is sensitive to overdigestion, which can occur for example in case of lower ligation-digestion temperature (<48°C).
- Λ This probe is not completely digested in DNA samples derived from blood.
- Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.
- ‡ This probe contains two or more GCGC motifs for HhaI. Loss of probe peak signal (indicative of absence of methylation) will occur not only if both sites are unmethylated but also if one of the sites is unmethylated and the other methylated.
- # This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.

NOTE: The digestion control probes at 132 nt and 369 nt should provide no, or a very low (<10%) signal in digested samples. HhaI digestion of the 132 nt probe depends on the methylation state of the target DNA, as the GCGC site is located in the hybridizing sequence. However, the signal of 369 nt digestion control probe is NOT dependent on the methylation state of the target DNA, as the GCGC site is located in the stuffer sequence of the probe. Rare cases have been observed where the 132 nt probe generates a signal while the probe at 369 nt does not. In such cases, complete digestion might have been hampered by sample DNA methylation at that site, e.g. in tumour derived hypermethylated DNA, by impurities affecting HhaI digestion, or by a rare SNP in the digestion site. In these cases information obtained with 132 nt digestion control probe should be ignored.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation and/or HhaI digestion. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Table 2. ME011-D1 probes arranged according to chromosomal location

Table 2a. ME011-D1 target probes

Length (nt)	SALSA MLPA probe	Gene/exon ^a	Ligation site	Partial sequence, for copy number probes (24 nt adjacent to ligation site)/ Complete sequence, for MS-MLPA probes	Distance to next probe
EPCAM at 2p21. Indicated ligation sites are in NM_002354.3.					
197	11983-L30436	EPCAM exon 8	1065-1066	GTTATTTCCAGA-AAGAAGAGAATG	1.5 kb
310 #	13131-L03603	EPCAM exon 9	1189-1190	AAATGGACACAA-ATTACAAATGTG	0.1 kb
220	22424-L31926	EPCAM exon 9	1320-1319 reverse	GGTCAAATTTCA-AGATTGGTAAAG	16.1 kb to <i>MSH2</i>
MSH2 at 2p21. Indicated ligation sites are in NM_000251.3.					
184	06227-L07711	MSH2 upstream	233 nt before exon 1 reverse	GAAACCCGACAGCGGCATCCT-TAGTAGAGCTCCTTTCTGTGTTACTCAGCTGCAAGGCTTG	0.1 kb
352 Ð	02735-L02162	MSH2 upstream	156 nt before exon 1	CAGTAGCTAAAGTCACCAGCGTGCGGGGA-AGCTGGGCCGCTGCTTATGATTGGTTGCCGC	0.2 kb
254	06226-L31127	MSH2 exon 1	5-6	GGAAACAGCTTAGTGGGTGTGGGTGCGGCATT-TTCTCAACCAGGAGGTGAGGAGGTTTCGACATGGCGGTG	379.8 kb to <i>MSH6</i>
MSH6 at 2p16.3. Indicated ligation sites are in NM_000179.3.					
148 ^ «	21588-L30979	MSH6 upstream	228 nt before exon 1	CGGCCAGCCCCGCGCGTGAGGGA-AGGGGAGCTCAGCAGTTCCCCGCGGGGCC	0.2 kb
213 Ð ‡ «	06230-L29780	MSH6 upstream	37 nt before exon 1	CGGCGAGGCGCCTGTTGATTGGCCACT-GGGGCCCGGGTTCTCCGGCGGAGCGCGCCT	0.1 kb
167 «	06228-L30148	MSH6 exon 1	41-40 reverse	CAACCGTTCTGTCCGACGGAGCTCCTAAAA-GCACC GCATCTACCGCGCGGCTCCTGCTGGCGGGAATCTG	-

MLH1 at 3p22.2. Indicated ligation sites are in NM_000249.4.					
The most important methylation region for <i>MLH1</i> expression, the Deng C -region, is from -248 nt to -178 nt before the transcription start site (Capel et al. 2007, Deng et al. 1999) corresponding to -239 nt to -169 nt from transcription start site of NM_000249.4). The second most important region, the Deng D -region, is from -100 nt to +24 nt in NM_000249.4. For this reason, methylation of the 201 nt and 172 nt probes will be the most important determinant for <i>MLH1</i> mRNA expression. It is not possible for us to design extra MLPA probes in these regions as there are no other HhaI sites (Gausachs et al. 2012). Methylation of the Deng A- and B-regions is not specifically correlated with loss of <i>MLH1</i> expression and the clinical relevance is not well established.					
247	07187-L26307	MLH1 upstream	628 nt before exon 1 (Deng A)	CGTCCGCCACATACCGCTCGTAGTAT-TCGTGCTCA GCCTCGTAGTGGCGCCTGACGTCGCGTT	0.3 kb
278 ‡	06221-L31128	MLH1 upstream	352 nt before exon 1 reverse, (Deng B)	TGTGCCTCTGCTGAGGTGATCTGGCGCAGA-GCGGA GGAGGTGCTTGGCGCTTCTCAGGCTCCTCTCT	0.1 kb
201 ‡	06222-L26305	MLH1 upstream	215 nt before exon 1 (Deng C)	CAATAGGAAGAGCGGACAGCGATCTCTAACGCGCAA- GCGCATATCCTTCTAGGTAGCGGGCAGTAGCCGCTT CAGG	0.2 kb
289 §	22572-L31773	MLH1 exon 1	25-24 reverse; SNP-specific	GGCTCTTCTGGT-GCCAAAATGTCG	0 kb
172 ±	01686-L28585	MLH1 exon 1	18-19 (Deng D)	CTTCCGTTGAGCATCTAGACGTTTCCTTGGCTCT-TC TGGCGCCAAAATGTCGTTCTGTCGAGGGGTTATTC	0.2 kb
297	02258-L30977	MLH1 intron 1	93 nt after exon 1	CGGACACGCCTCTTTCGCCGGGAGCAGA- GGCATGTACAGCGCATGCCACACGGCGGAGGCC	-
PMS2 at 7p22.1. Indicated ligation sites are in NM_000535.7.					
154	11966-L13112	PMS2 intron 1	20 nt after exon 1	GCTCGAGGTGAGCGGGGCTCGCAGTCT- TCCGGTGTCCTCTCGCGCCCTCTTTGAGAC	0.1 kb
142 #	07935-L16571	PMS2 upstream	32 nt before exon 1	GCCAATGGGAGTTCAGGAGGCGGA- GCGCCTGTGGGAGCCCTGGAGGGAACCTTTCCAGT	0.2 kb
338 ^ #	07934-L16147	PMS2 upstream	276 nt before exon 1	GGCAGAACCAAAGCAAAGGGGGTAGCGCGTGCC AAAG-GCCAACGCTCAGAAACCGTCAGAGGTCACGA CGGAGAC	-
BRAF at 7q34. Indicated ligation site is in NM_004333.6.					
226 § Ж	08780- SP0039- L08904	BRAF exon 15	1985-1986 and 2025-2026; p.V600E (c.1799T>A) mutation-specific	TTCTTCATGAAG- ACCTCACAGTAAAAATAGGTGATT TTGGTCTAGCTACAGA-GAAATCTCGATG	-

The HhaI sites are marked with grey. Ligation sites are marked with -. Complete probe sequences are available at <https://www.mrcholland.com/>.

^a See section Exon numbering on page 3 for more information.

§ Mutation-specific probe. This probe will only generate a signal when the *BRAF* p.V600E mutation is present. It has been tested on artificial DNA and on DU-4475 human cancer cell line derived DNA **but not on positive human samples!**

± Target sequence of this probe contains SNP rs104894994 in the GCGC site, +6 nt from the ligation site. When the T-allele of this validated SNP (with an allele frequency of 0.14%) is present, HhaI digestion will not occur, resulting in a false methylation positive signal. The presence of this T-allele hampering the HhaI digestion can be detected by the rs104894994 SNP-specific probe at 289 nt included in this ME011-D1 probemix.

§ SNP-specific probe. This probe will only generate a signal when the T-allele of rs104894994 SNP is present. It has been tested on artificial DNA and on selected 1000 genome project samples. If this probe gives signal, the methylation results obtained with 172 nt MLH1 probe (01686-L28585) are considered as false-positive and should be disregarded.

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

‡ This probe is sensitive to overdigestion, which can occur for example in case of lower ligation-digestion temperature (<48°C).

^ This probe is not completely digested in DNA samples derived from blood.

Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

‡ This probe contains two or more GCGC motifs for HhaI. Loss of probe peak signal (indicative of absence of methylation) will occur not only if both sites are unmethylated but also if one of the sites is unmethylated and the other methylated.

This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.

Note: Please be aware that several probes have multiple HhaI restriction sites. All of these sites need to be methylated in order to not be digested.

Table 2b. Reference and digestion control probes

Length (nt)	SALSA MLPA probe	Gene	Chromosomal position	Location (hg18) in kb
127	19551-L30430	<i>DYSF</i>	2p13	02-071,750
369 π	21589-L27783	<i>SLC9A2</i>	2q12	02-102,641
190	12780-L28100	<i>EDAR</i>	2q13	02-108,894
178	18842-L30565	<i>FLNB</i>	3p14	03-058,129
232	05709-L30439	<i>CASR</i>	3q21	03-123,484
123	18709-L13645	<i>IL4</i>	5q31	05-132,038
160	10694-L26069	<i>PKHD1</i>	6p12	06-051,717
386	13404-L17499	<i>EYS</i>	6q12	06-064,556
238	08070-L07851	<i>DNAI1</i>	9p13	09-034,491
208	10644-L30437	<i>PCSK5</i>	9q21	09-077,962
398	00973-L30443	<i>TSPAN15</i>	10q21	10-070,937
132 π ⌀	22576-L31899	<i>ANXA7</i>	10q22	10-074,844
137	03896-L21555	<i>CTTN</i>	11q13	11-069,957
323	09065-L30441	<i>CACNA1A</i>	19p13	19-013,289
263	15809-L30975	<i>ATP1A3</i>	19q13	19-047,175

π Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.

⌀ This probe is sensitive to overdigestion, which can occur for example in case of lower ligation-digestion temperature (<48°C).

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation and/or HhaI digestion. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Related SALSA MLPA probemixes

Condition	Gene	Probemix
Lynch syndrome (HNPCC)	<i>MLH1</i>	P003, ME011, P248 (confirmation of P003), ME042
	<i>MSH2</i>	P003, ME011, P248 (confirmation of P003)
	<i>MSH6</i>	P072, ME011
	<i>PMS2</i>	P008, ME011
	<i>EPCAM</i>	P003, P072, ME011
Polyposis syndrome	MAP	<i>MUTYH</i> P378, P043, P072
	AFAP	<i>APC</i> P043
	FAP	<i>APC</i> P043

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ME011 product history	
Version	Modification
D1	One of the HhaI digestion control probes has been replaced, four reference probes removed, one SNP-specific probe at GCGC site included and several probes have a change in length but not in the sequence detected.
C1	This probemix has been extensively revised: digestion control probes, BRAF p.V600E mutation-specific probe and EPCAM probes added; all MGMT, MLH3, MSH3 probes removed; majority of reference probes replaced.

B3	One probe has a small change in length but no change in the targeted sequence.
B2	The control fragments have been changed (QDX2).
B1	Two <i>MGMT</i> -specific probes have been replaced and three extra <i>MGMT</i> -specific probes have been added. One <i>MSH3</i> -specific probe is replaced and one removed. The number of reference probes has been increased to 15 and two control fragments at 100 and 105 nt have been added.
A1	First release.

Implemented changes in the product description	
Version D1-05 – 05 March 2024 (04M)	
<ul style="list-style-type: none"> - Reference to SALSA Binning DNA SD086 removed from the intended purpose footnote. - Added “This product is for research use only (RUO)” in the end of ‘SALSA Binning DNA SD086’ section. 	
Version D1-04 – 22 March 2022 (04M)	
<ul style="list-style-type: none"> - Version for internal use only. 	
Version D1-03 – 08 June 2021 (04M)	
<ul style="list-style-type: none"> - Product description rewritten and adapted to a new template. - Intended purpose slightly rephrased. - Minor modifications in sections ‘Clinical background’ and ‘Performance characteristics’ to better reflect the current information from literature. - In section ‘Interpretation of copy number results’ added a sentence about the required tumour cell percentage. - In section ‘Interpretation of methylation results’ added a paragraph regarding MS-MLPA sensitivity. - Added BRAF point mutation sensitivity information to a ME011-specific note on page 8. - Added a ME011-specific note on page 8 about interference of an unspecific background signal for the BRAF mutation-specific (08780-SP0039-L08904) and rs104894994 SNP-specific probes (22572-L31773) in wild-type samples with Coffalyser analysis. - Added a ME011-specific note on page 9 regarding residual signal after HhaI digestion. - Corrected the chromosomal band information for 22576-L31899 probe in Table 1. - Added footnote regarding sensitivity for overdigestion for 22576-L31899 probe in Table 1 and 2b. - In Table 2a added information for MLH1 regarding the clinical relevance of methylation of the Deng A- and B-regions. - Removed several publications and added two using SALSA MS-MLPA Probemix ME011 on page 14 and 15. - For uniformity, the chromosomal locations and bands for target probes in this document are now all based on hg18 (NCBI36). 	
Version D1-02 – 08 March 2021 (02M)	
<ul style="list-style-type: none"> - Added a requirement for selection of reference samples in respect to SNP rs104894994 in the section ‘Reference samples’ on page 4. - UK has been added to the list of countries in Europe that accept the CE mark. 	
Version D1-01 – 28 April 2020 (02M)	
<ul style="list-style-type: none"> - Product description rewritten and adapted to a new template and to a new product version. - ME011-D1 is now CE marked. - Added a table describing genetic alterations detected in positive samples on page 4. - Small changes of probe lengths in Table 1 and Table 2a and 2b in order to better reflect the true lengths of the amplification products. - Table 2a, 2b and 2c combined into one table -Table 2a, and accordingly Table 2d renamed to Table 2b. 	

- Ligation sites of the probes targeting the MLH1, MSH2, MSH6, PMS2 and EPCAM gene updated according to the most recent version (01/2020) of the NM_ reference sequence.
- Modified the selection of publications using SALSA MS-MLPA Probemix ME011 on pages 13-14.
- For reference probes, the column "partial sequence" in Table 2d replaced by "Location (hg18) in kb" information.
- In Table 2a, refined the information regarding Deng region locations in MLH1 promoter.
- Implemented changes in the product description shortened; information is included from C1-01 onwards. Version C1-07 – 20 September 2019 (01M)
- Catalogue number SALSA HhaI enzyme adjusted to SMR50 on page 2.

Version C1-06 – 18 June 2019 (01M)

- Added information about SALSA HhaI enzyme (SMR51) on page 2.
- Ligation site of the BRAF V600E mutation-specific probe updated in Table 2c according to new version of the NM_ reference sequence.
- Minor textual modifications.


Version C1-05 – 13 May 2019 (01M)

- The frequency information of rs104894994 SNP for MLH1 probe 01686-L28585, indicated below Table 1 and 2b, was updated according to NCBI dbSNP.

Version C1-04 – 16 April 2019 (01M)

- Corrected probe length from 396 to 369 in Table 1 note on page 7.
- Information added about the residual signal of 132 nt digestion control probe in Table 1 note on page 7.
- Various minor layout changes.

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	EUROPE* 
	ALL OTHER COUNTRIES

*comprising EU (candidate) member states and members of the European Free Trade Association (EFTA), and the UK. The product is for RUO in all other European countries.