Product Description SALSA® MS-MLPA® Probemix ME011-D1 Mismatch Repair Genes

To be used with the MS-MLPA General Protocol.

Version D1. As compared to version C1, one HhaI digestion control probe 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. 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.mlpa.com).

Certificate of Analysis: Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mlpa.com.

Precautions and warnings: For professional use only. Always consult the most recent product description AND the MS-MLPA General Protocol before use: www.mlpa.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 use: This SALSA MLPA probemix ME011 Mismatch Repair Genes is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] to determine methylation status of selected GCGC sites in the promoter regions of *MLH1*, *MSH2*, *PMS2* and *MSH6* genes and to detect the *BRAF* p.V600E (=c.1799T>A) point mutation in colorectal and endometrial cancer, in order to stratify the risk of having Lynch syndrome and identify cases which might benefit from subsequent germline genetic testing. In addition, this assay can be used to detect deletions or duplications in the 3' region of the *EPCAM* gene and the promoter regions of *MLH1*, *MSH2*, *PMS2* and *MSH6* genes in order to aid diagnosis of Lynch syndrome.

This assay is for use with peripheral blood derived genomic DNA and also for DNA derived from fresh, frozen and FFPE tissue of colorectal and endometrial cancer, given that tumour cell content of the patient sample is higher than 50%. 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. Methylation status, deletions and duplications and the mutation status of *BRAF* p.V600E should be verified by another technique. Copy number changes detected by only a single probe always require validation 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, parental evaluation, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

[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, SALSA HhaI, and SD086 Binning DNA.

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 (life time risk 52-82%), endometrial cancer (life time risk 25-60%), gastric cancer (life-time risk 6%-13%), ovarian cancer (life time risk 4-12%) 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. 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 (Herman et al. 1998, Cunningham et al. 1998, Kane et al. 1998, Esteller et al. 1998, 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 (Pinto et al. 2018, Goel et al. 2011, Morak 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, are 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* mutational 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 has 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 has 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 has 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 has 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 has 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 has 18 exons. The *BRAF* LRG_299 is available at www.lrg-sequence.org and is identical to GenBank NG_007873.3.

Transcript variants:

- *MLH1* (https://www.ncbi.nlm.nih.gov/gene/4292) has multiple transcript variants of which transcript variant 1 (NM_000249.4, 2494 nt, coding sequence 31-2301) represents the longest transcript. The ATG translation start site is located in exon 1 and the stop codon is located in exon 19.
- *MSH2* (https://www.ncbi.nlm.nih.gov/gene/4436) has two transcript variants of which transcript variant 1 (NM_000251.3, 3115 nt, coding sequence 37-2481) represents the longest transcript. The ATG translation start site is located in exon 1 and the stop codon is located in exon 16.
- *MSH6* (https://www.ncbi.nlm.nih.gov/gene/2956) has multiple transcript variants of which transcript variant 1 (NM_000179.3, 4265 nt, coding sequence 90-4172) represents the longest transcript. The ATG translation start site is located in exon 1 and the stop codon is located in exon 10.

- *PMS2* (https://www.ncbi.nlm.nih.gov/gene/5395) has multiple transcript variants of which transcript variant 1 (NM_000535.7, 5093 nt, coding sequence 31-2619) represents the longest transcript. The ATG translation start site is located in exon 1 and the stop codon is located in exon 15.
- *EPCAM* (https://www.ncbi.nlm.nih.gov/gene/4072) has one transcript variant (NM_002354.3, 1547 nt, coding sequence 196-1140). The ATG translation start site is located in exon 1 and the stop codon is located in exon 9.
- *BRAF* (https://www.ncbi.nlm.nih.gov/gene/673) has multiple transcript variants of which transcript variant 1 is the oldest and the most used in literature (NM_004333.6, 6459 nt, coding sequence 227-2527). The ATG translation start site is located in exon 1 and the stop codon is located in exon 18.

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)
MLH1	NM_000249.4	LRG_216 (NM_000249.3)
MSH2	NM_000251.3	LRG_218 (NM_000251.2)
MSH6	NM_000179.3	LRG_219 (NM_000179.2)
PMS2	NM_000535.7	LRG_161 (NM_000535.5)
EPCAM	NM_002354.3	LRG_215 (NM_002354.2)
BRAF	NM_004333.6	LRG_299 (NM_004333.4)

The exon numbering and NM_sequence used have been retrieved on 01/2020. As changes to the NCBI database can occur after release of this product description, 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, including three EPCAM probes, 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 rs104894994 single nucleotide polymorphism (SNP), which will only generate a signal when respectively the mutation or the SNP are present. In addition, 13 reference probes are included that are not affected by HhaI digestion and target relatively copy number stable regions in 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. The identity of the genes detected by the reference and digestion control probes are available in Table 2b and online (www.mlpa.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.mlpa.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal of 88 nt and 96 nt fragment 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.mlpa.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. SALSA HhaI enzyme (SMR50; MRC Holland) must be used in combination with this probemix as this restriction enzyme has been validated for use with MS-MLPA by MRC-Holland. 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.

Required specimens: Genomic DNA derived from peripheral blood and from fresh, frozen or formalin-fixed paraffin-embedded (FFPE) tumour tissue of colorectal and endometrial cancer, free from impurities known to affect MLPA reactions. Note that 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. 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 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.

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/home.html) have a diverse collection of biological resources which may be used as a positive control DNA sample 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 to detect and methylation status and copy number alterations (CNAs) of target sequences of *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM* genes, as well as the presence of *BRAF* p.V600E mutation. 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	*EPCAM, MSH2* and *MSH6*	Heterozygous deletion	All MMR genes not methylated	-
NA10401[1]	2p21-p16.3	*EPCAM, MSH2* and *MSH6*	Heterozygous duplication	All MMR genes not methylated	-
NA04127	3p22.2	*MLH1*	Heterozygous duplication	All MMR genes not methylated	-
NA07081	7p22.1	*PMS2*	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 MLH1 probe targets; all other genes – not methylated	present
DK-MG-UN[2]	7p22.1	*PMS2*	Heterozygous duplication	All MMR genes not methylated	-
SK-N-MC[3]	3p22.2	*MLH1*	Heterozygous deletion	Low methylation (10-15%) for all MLH1 probe targets; all other genes – not methylated	-

[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 238 and 398 nt are detected in this sample.
[3] Heterozygous loss for the digestion control probe at 132 nt and for reference probes at 178 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 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 is 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, SD086 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 or SNP signal. 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.mlpa.com.

Performance characteristics: 15-20% sporadic colorectal and endometrial cancer cases are explained by *MLH1* promoter methylation. *BRAF* p.V600E mutation, is identified in ~15% of sporadic colorectal cancers and is strongly associated with *MLH1* methylation. Analytical sensitivity and specificity are above 95% for *MLH1* promoter methylation detection by this assay based on literature review for the period of 2008-2019. In up to 90% of hereditary 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, hypermethylation has been detected in less than 1% for *MLH1*, 5% for *MSH2* (1-3% due to *EPCAM* deletion), 0% for *PMS2* and *MSH6* (only somatic hypermethylation described in advanced colorectal cancer).

Analytical performance can be compromised by: SNPs or other polymorphisms (e.g. indels) 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.mlpa.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 DQ of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Heterozygous deletion	0.40 < DQ < 0.65
Heterozygous duplication	1.30 < DQ < 1.65
Heterozygous triplication/Homozygous duplication	1.75 < DQ < 2.15
Ambiguous copy number	All other values

Please note that these above mentioned dosage quotients are only valid for germline testing. Dosage quotients are affected both by percentage of tumour cells and by possible subclonality.

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in 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. Incomplete DNA denaturation (e.g. due to salt contamination) can 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.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- When running MLPA products, the capillary electrophoresis protocol may need optimization. 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: lower injection voltage / injection time settings, 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 in the HhaI digested reaction, 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 digested 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.

To determine if a test sample has a significantly increased methylation level for a particular probe, compare the final ratios of the probe in the digested reaction with the baseline level.

Probe	**Reference sample 1**	**Reference sample 2**	**Reference sample 3**	**Average**	**Standard deviation**	**Baseline level** (average+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

- Final ratio of a probe in a digested test sample > baseline: methylation is increased.
- Final ratio of a probe in a digested test sample ≤ baseline: methylation is *not* increased.

- Interpretation of methylation positive samples is dependent on the application used.
- 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 absent upon complete digestion by HhaI.
- We have no data showing that methylation detected by a particular probe indeed influences the corresponding mRNA levels.

NOTE: In case digestion control probes are not fully digested (methylation percentage > 5%), please contact info@mlpa.com for more information.

ME011 probemix 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 (G**C**GC to G**T**GC), 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.
- 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.

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. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (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 of 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 false positive results due to SNPs and unusual results to MRC-Holland: info@mlpa.com.

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

Length (nt)	SALSA MLPA probe	HhaI 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 * п ‡	Digestion control probe 22576-L31899	+	100%			10p22	
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 ¥ Đ ‡ «	**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 Đ	**MSH2 probe** 02735-L02162	+	100%		2p21		
369 п	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.

п 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).
∧ 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.

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

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 *MSH2*
MSH2 at 2p21. Indicated ligation sites are in NM_000251.3.					
184	06227-L07711	**MSH2** upstream	233 nt before exon 1 *reverse*	GAAACCCGCAGACGCGCATCCT-TAGTAGAGCTCCTT TCTGTGTTTACTCAGCTGCAAGGCTTG	0.1 kb
352 Ð	02735-L02162	**MSH2** upstream	156 nt before exon 1	CAGTAGCTAAAGTCACCAGCGTGCGCGGGA-AGCTGGGCCGCGTCTGCTTATGATTGGTTGCCGC	0.2 kb
254	06226-L31127	**MSH2** exon 1	5-6	GGAAACAGCTTAGTGGGTGTGGGGTCGCGCATT-TTC TTCAACCAGGAGGTGAGGAGGTTTCGACATGGCGGTG	379.8 kb to *MSH6*
MSH6 at 2p16.3. Indicated ligation sites are in NM_000179.3.					
148 ∧ «	21588-L30979	**MSH6** upstream	228 nt before exon 1	CGGCCAGCCCCGCGGCGTGAGGGA-AGGGGAGCTCAGCAGTTCCCCGCGCGGGGCC	0.2 kb
213 Ð ‡ «	06230-L29780	**MSH6** upstream	37 nt before exon 1	CGGCGAGGCGCCTGTTGATTGGCCACT-GGGGCCCGGGTTCCTCCGGCGGAGCGCGCCT	0.1 kb
167 «	06228-L30148	**MSH6** exon 1	41-40 *reverse*	CAACCGTTCTGTCGGACGGAGCTCCTAAAA-GCACC GCATCTACCGCGCGGCTCCTGCTGGCGGGAAATCTG	–
MLH1 on 3p22.2. Indicated ligation sites are in NM_000249.4. The most important methylation region for *MLH1* expression, the Deng **C**-region, is from -248 nt to -178 nt before the transcription start site (Deng et al. 1999, Capel et al. 2007), 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 *MLH1* mRNA expression. It is not possible for us to design extra MLPA probes in these regions as there are no other HhaI sites. Methylation of the Deng A- and B-regions is not specifically correlated with loss of *MLH1* expression.					
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 GGAGGTGCTTGGCGCTTCTCAGGCTCCTCCTCT	0.1 kb
201 ‡	06222-L26305	**MLH1** upstream	215 nt before exon 1 (Deng **C**)	CAATAGGAAGAGCGGACAGCGATCTCTAACGCGCAA-GC GCATATCCTTCTAGGTAGCGGGCAGTAGCCGCTTCAGG	0.2 kb
289 ∫	22572-L31773	**MLH1** exon 1	25-24 *reverse*; **SNP-specific**	GGCTCTTCTGG***T***-GCCAAAATGTCG	0 kb
172 ±	01686-L28585	**MLH1** exon 1	18-19 (Deng **D**)	CTTCCGTTGAGCATCTAGACGTTTCCTTGGCTCT-TC TGG***C***GCCAAAATGTCGTTCGTGGCAGGGGTTATTC	0.2 kb
297	02258-L30977	**MLH1** intron 1	93 nt after exon 1	CGGACACGCCTCTTTGCCCGGGCAGA-GGCATGTACAGCGCATGCCCACAACGGCGGAGGCC	–

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
PMS2 at 7p22.1. Indicated ligation sites are in NM_000535.7.					
154	11966-L13112	**PMS2** intron 1	20 nt after exon 1	GCTCGAGGTGAGCGGGGCTCGCAGTCT-TCCGGTGTCCCCTCTCGCGCGCCCTCTTTGAGAC	0.1 kb
142 #	07935-L16571	**PMS2** upstream	32 nt before exon 1	GCCAATGGGAGTTCAGGAGGCGGA-GCGCCTGTGGGAGCCCTGGAGGGAACTTTCCCAGT	0.2 kb
338 Λ #	07934-L16147	**PMS2** upstream	276 nt before exon 1	GGCAGAACCAAAGCAAAAGGGGGTAGCGCGTGCCAAAG-GCCAACGCTCAGAAACCGTCAGAGGTCACGACGGAGAC	–
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 TTGGTCTAGCTACAG**A**-GAAATCTCGATG	–

The HhaI sites are marked with grey. Ligation sites are marked with -. Complete probe sequences are available at www.mlpa.com.

[a)] See above section on exon numbering 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.

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: 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. ME011-D1 Reference and digestion control probes ordered by chromosomal location

Length (nt)	SALSA MLPA probe	Gene	Chromosomal position	Location (hg18) in kb
127	19551-L30430	DYSF	2p13	02-071,750
369 ¤	21589-L27783	SLC9A2	2q12	02-102,641
190	12780-L28100	EDAR	2q13	02-108,894
178	18842-L30565	FLNB	3p14	03-058,129
232	05709-L30439	CASR	3q21	03-123,484
123	18709-L13645	IL4	5q31	05-132,038
160	10694-L26069	PKHD1	6p12	06-051,717
386	13404-L17499	EYS	6q12	06-064,556
238	08070-L07851	DNAI1	9p13	09-034,491
208	10644-L30437	PCSK5	9q21	09-077,962
398	00973-L30443	TSPAN15	10q21	10-070,937
132 ¤	22576-L31899	ANXA7	10p22	10-074,844
137	03896-L21555	CTTN	11q13	11-069,957
323	09065-L30441	CACNA1A	19p13	19-013,289
263	15809-L30975	ATP1A3	19q13	19-047,175

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

Notes:

- The digestion control probes at 132 nt and 369 nt should provide no, or a very low (<5%) 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 might occur 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.
- In FFPE tissue-derived DNA samples from healthy individuals the digestion control probe at 132 nt showed a little more than 5% signal in digested samples; and accordingly all the MS-MLPA target probes had inefficient HhaI digestion with up to 24% residual signal. Therefore, samples with residual signal for digestion control probe at 132 nt should be treated with caution.

Colorectal cancer (CRC) related SALSA MLPA probemixes

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

References

- Atanesyan L et al. (2017). Optimal Fixation Conditions and DNA Extraction Methods for MLPA Analysis on FFPE Tissue-Derived DNA. *Am J Clin Pathol.* 147:60-8.
- Bouzourene H et al. (2010). Selection of patients with germline MLH1 mutated Lynch syndrome by determination of MLH1 methylation and BRAF mutation. *Fam Cancer.* 9:167-72.
- Capel E et al. (2007). Assessment of MLH1 promoter methylation in relation to gene expression requires specific analysis. *Oncogene.* 26:7596-600.
- Cunningham JM et al. (1998). Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Res.* 58:3455-60.
- Deng G et al. (1999). Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression. *Cancer Res.* 59:2029-33.
- Esteller M et al. (1998). MLH1 promoter hypermethylation is associated with the microsatellite instability phenotype in sporadic endometrial carcinomas. *Oncogene.* 17:2413-7.
- Goel A et al. (2011). De novo constitutional MLH1 epimutations confer early-onset colorectal cancer in two new sporadic Lynch syndrome cases, with derivation of the epimutation on the paternal allele in one. *Int J Cancer.* 128:869-78.
- Herman JG et al. (1998). Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci U S A.* 95:6870-5.
- Hömig-Hölzel C and Savola S (2012). Multiplex ligation-dependent probe amplification (MLPA) in tumor diagnostics and prognostics. *Diagn Mol Pathol.* 21:189-206.
- Kane MF et al. (1997). Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res.* 57:808-11.
- Morak M et al. (2018). Comprehensive analysis of the MLH1 promoter region in 480 patients with colorectal cancer and 1150 controls reveals new variants including one with a heritable constitutional MLH1 epimutation. *J Med Genet.* 55:240-8.
- Nygren AO et al. (2005). Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Res.* 33:e128.
- Perez-Carbonell L et al. (2010). Methylation analysis of MLH1 improves the selection of patients for genetic testing in Lynch syndrome. *J Mol Diagn.* 12:498-504.
- Pinto D et al. (2018). Contribution of MLH1 constitutional methylation for Lynch syndrome diagnosis in patients with tumor MLH1 downregulation. *Cancer Med.* 7:433-44.
- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
- Simpkins SB et al. (1999). MLH1 promoter methylation and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancers. *Hum Mol Genet.* 8:661-6.
- Varga RE et al. (2012). MLPA-based evidence for sequence gain: pitfalls in confirmation and necessity for exclusion of false positives. *Anal Biochem.* 421:799-801.

Selected publications using SALSA MS-MLPA Probemix ME011 Mismatch Repair Genes

- Carnevali I et al. (2017). Somatic Testing on Gynecological Cancers Improve the Identification of Lynch Syndrome. *Int J Gynecol Cancer.* 27:1543-9.
- Castillejo A et al. (2015). Prevalence of MLH1 constitutional epimutations as a cause of Lynch syndrome in unselected versus selected consecutive series of patients with colorectal cancer. *J Med Genet.* 52:498-502.
- Cini G et al. (2019). Toward a better definition of EPCAM deletions in Lynch Syndrome: Report of new variants in Italy and the associated molecular phenotype. *Mol Genet Genomic Med.*e587.
- Crucianelli F et al. (2014). MLH1 constitutional and somatic methylation in patients with MLH1 negative tumors fulfilling the revised Bethesda criteria. *Epigenetics.* 9:1431-8.
- Damaso E et al. (2018). Primary constitutional MLH1 epimutations: a focal epigenetic event. *Br J Cancer.* 119:978-87.
- Dasgupta S et al. (2020). Granular dot-like staining with MLH1 immunohistochemistry is a clone-dependent artefact. *Pathol Res Pract.* 216:152581.
- Egoavil C et al. (2013). Prevalence of Lynch syndrome among patients with newly diagnosed endometrial cancers. *PLoS One.* 8:e79737.

- Gausachs M et al. (2012). MLH1 promoter hypermethylation in the analytical algorithm of Lynch syndrome: a cost-effectiveness study. *Eur J Hum Genet.* 20:762-8.
- Geurts-Giele WR et al. (2014). Somatic aberrations of mismatch repair genes as a cause of microsatellite-unstable cancers. *J Pathol.* 234:548-59.
- Gylling A et al. (2009). Large genomic rearrangements and germline epimutations in Lynch syndrome. *Int J Cancer.* 124:2333-40.
- Hitchins MP (2016). Finding the needle in a haystack: identification of cases of Lynch syndrome with MLH1 epimutation. *Fam Cancer.* 15:413-22.
- Jensen LH et al. (2013). Regulation of MLH1 mRNA and protein expression by promoter methylation in primary colorectal cancer: a descriptive and prognostic cancer marker study. *Cell Oncol (Dordr).* 36:411-9.
- Kamińska K et al. (2019). Prognostic and Predictive Epigenetic Biomarkers in Oncology. *Molecular Diagnosis & Therapy.* 23:83-95.
- Kato A et al. (2016). Isolated Loss of PMS2 Immunohistochemical Expression is Frequently Caused by Heterogenous MLH1 Promoter Hypermethylation in Lynch Syndrome Screening for Endometrial Cancer Patients. *Am J Surg Pathol.* 40:770-6.
- Kuiper RP et al. (2011). Recurrence and variability of germline EPCAM deletions in Lynch syndrome. *Hum Mutat.* 32:407-14.
- Ligtenberg MJ et al. (2009). Heritable somatic methylation and inactivation of MSH2 in families with Lynch syndrome due to deletion of the 3' exons of TACSTD1. *Nat Genet.* 41:112-7.
- Lynch HT et al. (2015). Milestones of Lynch syndrome: 1895-2015. *Nat Rev Cancer.* 15:181-94.
- McCarthy AJ et al. (2018). Heterogenous loss of mismatch repair (MMR) protein expression: a challenge for immunohistochemical interpretation and microsatellite instability (MSI) evaluation. *J Pathol Clin Res.*
- Morak M et al. (2018). Comprehensive analysis of the MLH1 promoter region in 480 patients with colorectal cancer and 1150 controls reveals new variants including one with a heritable constitutional MLH1 epimutation. *J Med Genet.* 55:240-8.
- Najdawi F et al. (2017). Lessons learnt from implementation of a Lynch syndrome screening program for patients with gynaecological malignancy. *Pathology.* 49:457-64.
- Niessen RC et al. (2009). Germline hypermethylation of MLH1 and EPCAM deletions are a frequent cause of Lynch syndrome. *Genes Chromosomes Cancer.* 48:737-44.
- Ozer O et al. (2013). Methylation profile analysis of DNA repair genes in hepatocellular carcinoma with MS-MLPA. *Diagn Mol Pathol.* 22:222-7.
- Peltomaki P (2014). Epigenetic mechanisms in the pathogenesis of Lynch syndrome. *Clin Genet.* 85:403-12.
- Perez-Carbonell L et al. (2010). Methylation analysis of MLH1 improves the selection of patients for genetic testing in Lynch syndrome. *J Mol Diagn.* 12:498-504.
- Pico MD et al. (2020). Clinical and Pathological Characterization of Lynch-Like Syndrome. *Clin Gastroenterol Hepatol.* 18:368-74 e1.
- Pineda M et al. (2012). MLH1 methylation screening is effective in identifying epimutation carriers. *Eur J Hum Genet.* 20:1256-64.
- Pinto D et al. (2018). Contribution of MLH1 constitutional methylation for Lynch syndrome diagnosis in patients with tumor MLH1 downregulation. *Cancer Med.* 7:433-44.
- Sahnane N et al. (2015). Microsatellite unstable gastrointestinal neuroendocrine carcinomas: a new clinicopathologic entity. *Endocr Relat Cancer.* 22:35-45.
- Spaepen M et al. (2013). EPCAM germline and somatic rearrangements in Lynch syndrome: identification of a novel 3'EPCAM deletion. *Genes Chromosomes Cancer.* 52:845-54.
- Sugawara T et al. (2015). Efficient screening strategy for lynch syndrome in Japanese endometrial cancer. *Tohoku J Exp Med.* 235:117-25.
- Takahashi K et al. (2017). Clinical characteristics of Lynch-like cases collaterally classified by Lynch syndrome identification strategy using universal screening in endometrial cancer. *Gynecol Oncol.* 147:388-95.
- van Lier MG et al. (2010). A review on the molecular diagnostics of Lynch syndrome: a central role for the pathology laboratory. *J Cell Mol Med.* 14:181-97.
- Zauber NP et al. (2010). Microsatellite instability and DNA methylation of endometrial tumors and clinical features in young women compared with older women. *Int J Gynecol Cancer.* 20:1549-56.

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 *MGMT*-specific probes have been replaced and three extra *MGMT*-specific probes have been added. One *MSH3*-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 D1-01 — 28 April 2020 (02M)
- Product description rewritten and adapted to a new template and to a new product version.
- 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.

Version C1-03 – 23 January 2019 (01M)
- Reference added on page 2 about the use of FFPE samples for MLPA.
- Interpretation of methylation results on tissue derived DNA samples is modified on page 4 and 5.
- *Colorectal cancer (CRC) related SALSA MLPA probemixes* - table updated on page 9.

Version C1-02 – 03 December 2018 (01M)
- Note about the interpretation of results on digestion probes has been added below Table 1.

Version C1-01 – 12 September 2018 (01M)
- Product description restructured and adapted to a new template.
- Product description adapted to a new product version (version number changed, lot number added, changes in Table 1 and Table 2).
- Various minor textual or layout changes.
- More information on *EPCAM* and constitutional *MLH1* methylation was added in clinical background.

- Interpretation of methylation results on tissue derived DNA samples was added.
- Warning added to Table 1 and 2 for probe sensitivity to overdigestion.
- Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.
- Related probemixes updated.
- New references were added for ME011.

More information: www.mlpa.com; www.mlpa.eu	
	MRC-Holland bv; Willem Schoutenstraat 1 1057 DL, Amsterdam, The Netherlands
E-mail	info@mlpa.com (information & technical questions); order@mlpa.com (orders)
Phone	+31 888 657 200

	EUROPE* CE
	ALL OTHER COUNTRIES

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