Product Description SALSA® MS-MLPA® Probemix ME011-C1 Mismatch Repair Genes

To be used with the MS-MLPA General Protocol.

Version C1. As compared to the previous version (lot B3-1017), this probemix has been extensively revised: digestion control probes, BRAF V600E mutation-specific probe and EPCAM probes added; all MGMT, MLH3, MSH3 probes removed; majority of reference probes replaced. For complete product history see page 11.

Catalogue numbers:

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

To be used in combination with a SALSA MLPA reagent kit, available for various number of reactions. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman 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. MRC-Holland cannot provide assistance with data interpretation.

General Information: The SALSA MS-MLPA Probemix ME011 MMR is a **research use only (RUO)** assay for the detection of aberrant methylation of one or more sequences of the *MLH1, MSH2, PMS2* and *MSH6* genes, and the presence of the *BRAF* c.1799T>A; p.Val600Glu point mutation to determine a sporadic nature of Lynch syndrome-related cancer in tumour tissue. This probemix can also be used to detect deletions/duplications in the promoter regions of the aforementioned genes and the *EPCAM* 3' region to determine whether follow-up germline Lynch syndrome testing should be considered.

CpG-islands are located in or near the promoter region or other regulatory regions of approximately 50% of human genes. Aberrant methylation of CpG-islands has been shown to be associated with transcriptional inactivation of genes in a wide spectrum of human cancers. The genes mentioned above are frequently silenced by methylation in tumours, but are unmethylated in blood-derived DNA, with the exception of constitutional epimutations.

The Mismatch Repair (MMR) system is critical for the maintenance of genomic stability. Defects in the cell's MMR system may lead to the accumulation of mutations resulting in the initiation of cancer. Several MMR genes are involved in Lynch syndrome (formerly known as hereditary nonpolyposis colon cancer (HNPCC)). Genetic alterations in the *MLH1* and *MSH2* genes have been found in up to 90% of Lynch syndrome cases. Genetic alterations in *MSH6* and *PMS2* genes are less frequently detected in Lynch syndrome patients. Around 1-3% of Lynch syndrome 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.

In sporadic colon cancer, hypermethylation is a more frequent mechanism than point mutations or copy number alterations for transcriptional silencing of the *MLH1* gene. The probes targeting GCGC sites in the C and D "Deng" region of the *MLH1* gene are of main interest (Deng et al. 1999). In addition, the *BRAF* p.Val600Glu mutation is a frequently found pathogenic variant in sporadic colorectal cancers. Hypermethylation of the *MLH1* promoter or the *BRAF* p.Val600Glu mutation are rare in tumours of Lynch syndrome patients.
Of note, constitutional inactivation of *MLH1* by methylation, together with a somatic mutation in the functional allele, has been reported as a rare cause of Lynch syndrome and may in rare cases be a heritable disease mechanism (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 Lynch syndrome patients or described as somatic cause in colorectal or endometrial tumours.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1211/

This SALSA MS-MLPA Probemix is not CE/FDA registered for use in diagnostic procedures. Purchase of this product includes a limited license for research purposes.

Gene structure and Transcript variants:
Entrez Gene shows transcript variants of each gene: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene
For NM_ mRNA reference sequences: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide
Locus Reference Genomic (LRG) database: http://www.lrg-sequence.org/

Probemix content: The SALSA MS-MLPA Probemix ME011-C1 MMR contains 37 (MS-)MLPA probes with amplification products between 123 and 454 nt. Fourteen MS-MLPA probes contain a *HhaI* recognition site and provide information on the methylation status of *MLH1, MSH2, MSH6* and *PMS2*. 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.Val600Glu mutation, which will only generate a signal when the mutation is present. In addition, 17 reference probes are included which are not affected by *HhaI* digestion and target relatively quiet regions in colorectal tumours. 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 probes is available in table 2d 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 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 or 96 nt fragment indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

No DNA controls results in only five major peaks shorter than 121 nucleotides (nt): four Q-fragments at 64, 70, 76 and 82 nt, and one 19 nt peak corresponding to the unused portion of the fluorescent PCR primer. Non-specific peaks longer than 121 nt AND with a height >25% of the median of the four Q-fragments should not be observed. Note: peaks below this 25% threshold are not expected to affect MS-MLPA reactions when sufficient amount of sample DNA (50-250 ng) is used.

MS-MLPA technique: The principles of the MS-MLPA technique (Schouten et al. 2002, Nygren et al. 2005) are described in the MS-MLPA General Protocol (www.mlpa.com). The MS-MLPA technique should always be internally validated before use in your laboratory. 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*. We recommend using Promega's *HhaI* enzyme (R6441) as this is the only restriction enzyme that has been validated for use with MS-MLPA by MRC-Holland. More information on the use of MLPA in tumour applications can be found in Hömig-Hölzel and Savola, 2012.

Required specimens: Extracted DNA, which includes DNA derived from paraffin-embedded tissues, free from impurities known to affect MLPA reactions. 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 base-line methylation level for each methylation specific probe when possible. 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 selecting reference samples, please note that methylation patterns may vary between tissues and even age groups! Reference samples should be derived from healthy individuals without a history of Lynch syndrome-related cancers. 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 NA10401 from the Coriell Institute and DU-4475 (ACC-427) from the Leibniz Institute DSMZ have been tested at MRC-Holland and can be used as positive control samples to detect a partial trisomy of chromosome 2 (including all probes for *EPCAM*, *MSH2* and *MSH6*, the digestion control probe for *SLC9A2* and the reference probes for *DYSF* and *EDAR*), and the *BRAF* p.Val600Glu mutation, respectively. The quality of cell lines can change, therefore samples should be validated before use.

SALSA Binning DNA SD029: The SD029 Binning DNA provided with this probemix can be used as Binning DNA sample for binning of one mutation-specific probe (226 nt probe 08780-SP0039-L08904 *BRAF* p.Val600Glu mutation). SD029 Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequence detected by the above mentioned probe. Inclusion of one reaction with 5 µl SD029 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 signal(s), as for this purpose true mutation/SNP positive patient samples or cell lines should be used. It is strongly advised to use DNA sample and reference DNA samples extracted with the same method and derived from the same source of tissue. For further details, please consult the SD029 Binning DNA product description provided. **This product is for research use only (RUO).**

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 base-line methylation levels for each methylation specific probe.

Interpretation of copy number results: The standard deviation of all probes in the reference samples should be <0.10. When this criterion is fulfilled, the following cut-off values for the dosage quotient (DQ) of the probes can be used to interpret MLPA results for autosomal or pseudo-autosomal chromosomes:

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 DQs 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. Analysis of parental samples may be necessary for correct interpretation of complex results.

- False positive results copy number probes: 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. 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.
- False positive results MS-MLPA probes: Rare cases are known in which an apparent methylation of a methylation sensitive probe proved to be due to a sequence change (i.ei SNP) in or very nearby an *HhaI* site.
- 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 MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (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*.
- 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 are unlikely to have any relation to the condition tested for.

Interpretation of methylation results on blood and tissue derived DNA samples:
The amount of baseline methylation is probe-, tissue- and in certain cases also age-specific. Depending on the gene, the location of the MS-MLPA probe in the CpG island, and the sequence detected by the probe, there can be differences in baseline methylation in a subset of cells. Moreover, certain tissue types and DNA extraction methods can result in residual compounds in the DNA sample possibly altering *HhaI* enzyme activity. Consequently, the amount of methylation of certain sequences can vary between DNA samples.

To prevent overestimation of the methylation signal of a target sequence in a test sample, the baseline signal in healthy tissue derived DNA samples after *HhaI* digestion for MS-MLPA probes should be taken into account in the data-analysis. This baseline methylation signal for each individual target probe can be determined by analysing a sufficient number (>3) of samples from healthy individuals, extracted from the same or similar source of tissue, that has been treated the same way as the samples of interest (e.g. FFPE vs. fresh frozen), and processed with the same DNA extraction protocol. To correct for baseline methylation, the following steps have to be performed:

Step 1
First the baseline of each MS-MLPA probe should be determined by calculating the mean of the ratios of healthy reference samples and add 2x the standard deviation to each mean probe ratio. For example:

	Reference sample 1	Reference sample 2	Reference sample 3	Mean Reference samples	Standard deviation	2x standard deviation	Baseline =Mean+2x standard deviation
MS-MLPA Probe 1	0.08	0.00	0.06	0.047	0.034	0.068	**0.11**
MS-MLPA Probe 2	0.05	0.07	0.03	0.05	0.016	0.033	**0.083**
MS-MLPA Probe 3	0.02	0.02	0.02	0.02	0	0	**0.02**

Step 2
To determine the methylation percentage per probe, the baseline value per probe should be subtracted from the ratio of the tumour sample of interest. For example:

	Sample 1	Sample 1 - baseline	Above baseline?	Sample 2	Sample 2 -baseline	Above baseline?
MS-MLPA Probe 1	0.05	0.05-0.11= **-0.065**	no	0.16	0.16-0.11= **0.045**	yes
MS-MLPA Probe 2	0.18	0.18-0.083= **0.097**	yes	0.36	0.36-0.083= **0.28**	yes
MS-MLPA Probe 3	0.02	0.02-0.02**= 0**	no	0.20	0.20-0.02= **0.18**	yes

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

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

ME011 MMR specific notes:
- Please note that several probes have multiple *HhaI* restriction sites. All of these sites need to be methylated in order to not be digested!
- In tumour tissues, reference probes are more prone to show deviating copy number results. To get the correct interpretation, it can help to turn the slope correction off in Coffalyser.Net analysis, when regions targeted by reference probes are affected by copy number alterations.

Limitations of the procedure:
- In most populations, the major cause of genetic defects in *MLH1, MSH2, MSH6* and *PMS2* genes are small (point) mutations, most of which will not be detected by using SALSA MS-MLPA Probemix ME011 MMR.
- 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 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 an apparent methylation of a methylation sensitive probe proved to be due to a sequence change in or very nearby an *HhaI* site.
- MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. 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.

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 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/COSMIC mutation database: 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 copy number changes detected by the reference probes, false positive results due to SNPs and unusual results to MRC-Holland: info@mlpa.com.

Table 1. SALSA MS-MLPA Probemix ME011-C1 MMR

Length (nt)	SALSA MLPA probe	HhaI site	% expected signal reduction[a]	Chromosomal position (hg18)	Mutation specific probe
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 «	**PRICKLE1 probe** 16042-L30431	+	100%	12q12	
137 *	Reference probe 03896-L21555	-	-	11q13	
142	**PMS2 probe** 07935-L16571	+	100%	7p22.1	
148 ¥	**MSH6 probe** 21588-L30979	+	95%	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	
202 ¥	**MLH1 probe** 06222-L26305	+	100%	3p22.2	
208 *	Reference probe 10644-L30437	-	-	9q21	
214 Đ ¥	**MSH6 probe** 06230-L30974	+	100%	2p16.3	
220 *	Reference probe 05939-L05368	-	-	1q22	
226 * Ж §	**BRAF probe** 08780-SP0039-L08904	-	-	7q34	**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	
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	+	95%	7p22.1	
352 Đ ¥	**MSH2 probe** 02735-L02162	+	100%	2p21	
369 * п	**SLC9A2 probe** 21589-L27783	+	100%	2q12.1	
386 *	Reference probe 13404-L17499	-	-	6q12	
398 *	Reference probe 00973-L30443	-	-	10q21	
410 *	Reference probe 20130-L16852	-	-	10p11	
427 *	**EPCAM probe** 13215-L14404	-	-	2p21	
440 *	Reference probe 20247-L30445	-	-	12q13	
454 *	Reference probe 17129-L25056	-	-	11p11	

* New in version C1 (from lot C1-0518 onwards).
¥ Changed in version C1 (from lot C1-0518 onwards). (Small) change in length, no change in sequence detected.
п Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.
« 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.
± Target sequence of this probe contains SNP rs104894994 (C/T) in the GCGC site, +6 nt from the ligation site. When the T-allele of this validated SNP (with an allele frequency of 0.068%) is present, *HhaI* digestion will not occur, resulting in a false methylation positive signal.
Đ This probe is sensitive to overdigestion, which can occur in case of lower ligation-digestion temperature (<48°C).
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.
§ Mutation-specific probe. This probe will only generate a signal when the *BRAF* p.Val600Glu mutation is present.

a) Expected signal reduction on blood DNA derived samples. On tumour samples these percentages can be different.

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 396 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 Hha1 digestion, or by a rare SNP in the digestion site. In these cases information obtained with 132 nt digestion control probe should be ignored.

Table 2. ME011-C1 probes arranged according to chromosomal location

Table 2a. Chromosome 2p

Length (nt)	SALSA MLPA probe	Gene	Exon	Ligation site	**Partial sequence**, for copy number probes (24 nt adjacent to ligation site)/ **Complete sequence**, for MS-MLPA probes (HhaI site is highlighted in grey)	Distance to next probe
197	11983-L30436	***EPCAM***	Exon 8	NM_002354.2; 1228-1229	GTTATTTCCAGA-AAGAAGAGAATG	1.5 kb
310 #	13131-L03603	***EPCAM***	Exon 9	NM_002354.2; 1352-1353	AAATGGACACAA-ATTACAAATGTG	0.1 kb
427	13215-L14404	***EPCAM***	Exon 9	NM_002354.2; 1483-1482 reverse	GGTCAAATTTCA-AGATTGGTAAAG	16.1 kb
184	06227-L07711	***MSH2***	Upstream	NM_000251.2; 144 nt before exon 1 reverse	CGAAACCCGCAGACGCGCATCCT-TAGTAGAGCTCCTTTCTGTGTTTACTCAGCTGCAAGGCTTG	0.1 kb
352 Đ	02735-L02162	***MSH2***	Upstream	NM_000251.2; 67 nt before exon 1	CAGTAGCTAAAGTCACCAGCGTGCGCGGGA-AGCTGGGCCGCGTCTGCTTATGATTGGTTGCCGC	0.2 kb
254	06226-L31127	***MSH2***	Exon 1	NM_000251.2; 94-95	CCAGGAAACAGCTTAGTGGGTGTGGGGTCGCGCATT-TTCTTCAACCAGGAGGTGAGGAGGTTTCGACATGGCGGTG	379.8 kb
148	21588-L30979	***MSH6***	Upstream	NM_000179.2; 165 nt before exon 1	CCTACGTCGGCCAGCCCCGCGGCGTGAGGGA-AGGGGAGCTCAGCAGTTCCCCGCGCGGGGCC	0.2 kb
214 Đ	06230-L30974	***MSH6***	Exon 1	NM_000179.2; 26-27	CCAATCGCGAGGCGCCTGTTGATTGGCCACT-GGGGCCCGGGTTCCTCCGGCGGAGCGCGCCT	0.1 kb
167	06228-L30148	***MSH6***	Exon 1	NM_000179.2; 104-103 reverse	GTCTCAACCGTTCTGTCGGACGGAGCTCCTAAAA-GCACCGCATCTACCGCGCGGCTCCTGCTGGCGGGAAATCTG	

Đ This probe is sensitive to overdigestion, which can occur in case of lower ligation-digestion temperature (<48°C).
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 exon numbering used in this ME011-C1 MMR product description is identical to the LRG_215 for *EPCAM*, LRG_218 for *MSH2* and LRG_219 for *MSH6*. The exon numbering and NM sequences used are from 07/2018, but can be changed by NCBI after the release of the product description. Please notify us of any mistakes: info@mlpa.com.

Table 2b. Chromosome 3p

Length (nt)	SALSA MLPA probe	Gene	Exon	Ligation site NM_000249.3	**Complete sequence**, for MS-MLPA probes (HhaI site is highlighted in grey)	Distance to next probe
247	07187-L26307	***MLH1***	Upstream	460 nt before exon 1 (Deng, **A**-region)	TCAGGTACGTCCGCCACATACCGCTCGTAGTAT-TCGTGCTCAGCCTCGTAGTGGCGCCTGACGTCGCGTT	0.3 kb
278	06221-L31128	***MLH1***	Upstream	184 nt before exon 1 reverse (Deng, **B**-region)	CTTGATATGTGCCTCTGCTGAGGTGATCTGGCGCAGA-GCGGAGGAGGTGCTTGGCGCTTCTCAGGCTCCTCCTCT	0.1 kb
202	06222-L26305	***MLH1***	Upstream	47 nt before exon 1 (Deng, **C**-region)	CAATAGGAAGAGCGGACAGCGATCTCTAACGCGCAA-GCGCATATCCTTCTAGGTAGCGGGCAGTAGCCGCTTCAGG	0.2 kb
172 ±	01686-L28585	***MLH1***	Exon 1	186-187 (Deng, **D**-region)	GCTTCCGTTGAGCATCTAGACGTTTCCTTGGCTCT-TCTGGCGCCAAAATGTCGTTCGTGGCAGGGGTTATTC	0.2 kb
297	02258-L30977	***MLH1***	Intron 1	93 nt after exon 1	CCTAACGGACACGCCTCTTTGCCCGGGCAGA-GGCATGTACAGCGCATGCCCACAACGGCGGAGGCC	

The most important methylation region for *MLH1* expression, the Deng **C**-region, is from -248 nt to -178 nt before the transcription start site. The transcription start site that Deng et al. (1999) used for reference lies 21 nt before the ATG start codon.
The second most important region, the Deng **D**-region, is from -9 nt to +15 nt (Deng et al. 1999, Capel et al. 2007). For this reason, methylation of the 202 nt and 172 nt probes will be the most important determinant for 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.

± Target sequence of this probe contains SNP rs104894994 (C/T) in the GCGC site, 6 nt right from the ligation site. When the T-allele of this validated SNP (with an allele frequency of 0.068%) is present, *HhaI* digestion will not occur, resulting in a false methylation positive signal.

Note: The exon numbering used in this ME011-C1 MMR product description for *MLH1* is the exon numbering identical to the LRG_216 sequence. The exon numbering and NM sequence used is from 07/2018, but can be changed by NCBI after the release of the product description. Please notify us of any mistakes: info@mlpa.com.

Table 2c. Chromosome 7

Length (nt)	SALSA MLPA probe	Gene	Exon	Ligation site	**Partial sequence**, for mutation specific probe (24 nt adjacent to ligation site)/ **Complete sequence**, for MS-MLPA probes (HhaI site is highlighted in grey)	Distance to next probe
154	11966-L13112	***PMS2***	Intron 1	NM_000535.6; 20 nt after exon 1	GCTCGAGGTGAGCGGGGCTCGCAGTCT-TCCGGTGTCCCCTCTCGCGCGCCCTCTTTGAGAC	0.1 kb
142 #	07935-L16571	***PMS2***	Exon 1	NM_000535.6; 25-26	GGCCAATGGGAGTTCAGGAGGCGGA-GCGCCTGTGGGAGCCCTGGAGGGAACTTTCCCAGT	0.2 kb
338 #	07934-L16147	***PMS2***	Upstream	NM_000535.6; 219 nt before exon 1	GGCAGAACCAAAGCAAAAGGGGGTAGCGCGTGCCAAAG-GCCAACGCTCAGAAACCGTCAGAGGTCACGACGGAGAC	134.1 **M**b
226 § Ж	08780-SP0039-L08904	***BRAF***	Exon 15	NM_004333.4; 1820-1821, 1860-1861 **p.V600E** (c.1799T>**A**) **mutation**	TTCTTCATGAAG-ACCTCACAGTAAAAATAGGT GATTTTGGTCTAGCTACAG**A**-GAAATCTCGATG	

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.
§ Mutation-specific probe. This probe will only generate a signal when the *BRAF* p.Val600Glu mutation is present.
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.

Note 1: The exon numbering used in this ME011-C1 MMR product description is identical to the LRG_161 for *PMS2* and LRG_299 for *BRAF*. The exon numbering and NM sequences used are from 07/2018, but can be changed by NCBI after the release of the product description. Please notify us of any mistakes: info@mlpa.com.

Note 2: 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 2d. Reference probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene	Location (hg18)	**Partial sequence** (24 nt adjacent to ligation site)
220	05939-L05368	*LMNA*	1q22	CTCCTCTGTTTT-CTCTCTTAGAGC
127	19551-L30430	*DYSF*	2p13	CCATTGCCAAGA-AGGTCAGTGTCC
190	12780-L28100	*EDAR*	2q13	TGGACATTGCAA-TGATCAGGGCAG
178	18842-L30565	*FLNB*	3p14	CCATCCAAGGTT-AAAATGGATTGC
232	05709-L30439	*CASR*	3q21	AGTGTGTGGAGT-GTCCTGATGGGG
123	18709-L13645	*IL4*	5q31	ATCGACACCTAT-TAATGGGTCTCA
160	10694-L26069	*PKHD1*	6p12	CAGTTTCTGTAT-TTCCTAAAACAG
386	13404-L17499	*EYS*	6q12	TGGCTACAGTGA-ATACACTCCAGA
238	08070-L07851	*DNAI1*	9p13	AGGGATCTGTTT-GCAGTGGGATAT
208	10644-L30437	*PCSK5*	9q21	ACGCAGAAGCCA-TGGTGATGGAGG
410	20130-L16852	*ZNF25*	10p11	CAGGTGATTCCT-GGGGCTGCCAGC
398	00973-L30443	*TSPAN15*	10q21	CATCATCATGGA-GCACTCTGTCAC
454	17129-L25056	*MYBPC3*	11p11	CACCCAACTATA-AGGCCCTGGACT
137	03896-L21555	*CTTN*	11q13	AGGCAGAGCTGA-GCTACAGAGGCC
440	20247-L30445	*COL2A1*	12q13	GTCTGGCTGGTC-AGAGAGGCATCG
323	09065-L30441	*CACNA1A*	19p13	CTCAGGCCTTCT-ACTGGACTGTAC
263	15809-L30975	*ATP1A3*	19q13	CCCAACGACAAC-CGATACCTGCTG

Colorectal cancer (CRC) related SALSA MLPA probemixes

	Condition		Gene	Probemix
COLORECTAL CANCER (CRC) PROBEMIXES	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

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ME011 Product history	
Version	*Modification*
C1	This probemix has been extensively revised: digestion control probes, BRAF 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 probes have been replaced and three extra MGMT probes have been added. One MSH3 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 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.

Version 28 – 4 January 2018 (16)
- New references added for ME011 on page 2.
- Lengths of several probes have been modified to reflect the true amplification length in Table 1 and Table 2.
- Added "MV location (HG18)" column in Table 1 and removed from Table 2a and 2b.
- Removed two columns from Table 1 indicating whether the probes are used as references for copy number and/or methylation analysis.
- Added footnotes about variable probes and non-significant MGMT MS-MLPA probes in Table 1 and Table 2a.
- Added a footnote about the MGMT probes in ME012 probemix in Table 2a.
- Added "Distance to the next probe" column in Table 2a.
- Modified NM-sequence and ligation site information for MGMT probes in Table 2a.
- Updated the NM-sequence version for PMS2 probes in Table 2a.
- Split Table 2 into Table 2a (target probes) and Table 2b (reference probes).
- Added a note about multiple GCGC sites in one MS-MLPA probe on page 7.
- Modified formatting of MGMT probe sequences on page 8 for easier visualisation on black and white print.
- Removed paragraph on page 8 about MGMT probed in the previous ME011-A1 version.

Version 27 – 28 September 2017 (16)
- Three probes (01685-L01265, 11634-L12398, 11947-L00795) adjusted in Table 1 as not being reference probes for methylation in the "Reference Probe for methylation" column.

Version 26 – 15 February 2017 (16)
- Probe length adjusted for reference probe 03797-L04594 in Table 1 and 2.
- Probe length adjusted for MGMT probe 14135-L16573 in Table 1.
- Minor textual and layout changes.
- List of related SALSA MLPA probemixes updated.

Version 25 – 06 December 2016 (16)
- Warning regarding HhaI enzymes that are resistant to heat inactivation added under Methylation-specific MLPA section.

Version 24 – 26 October 2015 (15)
- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new pictures included).
- Footnotes added for Table 1 and 2.
- Expected signal reduction percentages added to Table 1.
- References modified on page 2.

Version 23 – 06 May 2015 (14)
- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new pictures included).

Version 22 (13)
- New references added on page 2.

Version 21 (13)
- Product description adapted to a new product version (product version number and lot number changed, small changes in Table 1 and Table 2, new pictures included).

- New references added on page 1.

Version 20 (12)

- Product description adapted to a new product version (version number and lot number changed, small changes in Table 1 and Table 2, new picture included).
- New references added on page 1.

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