Product Description
SALSA® MS-MLPA® Probemix ME001-D1
Tumour suppressor mix

To be used with the MS-MLPA General Protocol.

Version D1
For complete product history see page 11.

This SALSA MS-MLPA probemix is for basic research and intended for experienced MLPA users only! This probemix enables you to quantify genes or chromosomal regions in which the occurrence of copy number changes is not yet well-established and the relationship between genotype and phenotype is not yet clear. Since it will not provide you with clear cut answers, interpretation of results can be complicated. MRC Holland recommends thoroughly screening any available literature. Suggestions from specialists for improvement of this product or product description are highly appreciated.

Catalogue numbers:
- **ME001-025R:** SALSA MS-MLPA Probemix ME001 Tumour suppressor mix, 25 reactions.
- **ME001-050R:** SALSA MS-MLPA Probemix ME001 Tumour suppressor mix, 50 reactions.
- **ME001-100R:** SALSA MS-MLPA Probemix ME001 Tumour suppressor mix, 100 reactions.

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

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

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

General information
The SALSA MS-MLPA Probemix ME001 Tumour suppressor mix is a **research use only (RUO)** assay for the detection of aberrant methylation of one or more sequences of 25 tumour suppressor genes (TSG). This probemix can also be used to detect deletions/duplications in these TSGs in a DNA sample.

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. These TSGs are frequently silenced by methylation in tumours, but are unmethylated in blood-derived DNA. In addition, DNA methylation analysis can indicate in some cases from which type of tissue the tumour was derived.

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
Matched Annotation from NCBI and EMBL-EBI (MANE): https://www.ncbi.nlm.nih.gov/refseq/MANE/ and http://tark.ensembl.org/
For NM_ mRNA reference sequences: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide

Transcript numbers and exon numbering
The transcript numbers used for all genes in this ME001-D1 Tumour suppressor mix product description are derived from the MANE project (release version 1.0). The MANE Select NM_sequences were also used for determining each probe's ligation site and exon numbering, indicated in Table 2. For *CDKN2A* in particular, both the NM_000077.5 (MANE Select) and NM_058195.4 (MANE Plus Clinical) are used in Table 2. As changes to the databases can occur after release of this product description, the NM_ sequence and exon numbering may not be up-to-date. Please note, that note that in other MRC Holland product descriptions the exon numbering for the genes included in this ME001-D1 might differ in case other resources such as LRG or NG sequences are indicated to be used for exon numbering.

Probemix content
The SALSA MS-MLPA Probemix ME001-D1 Tumour suppressor mix contains 45 (MS-)MLPA probes with amplification products between 121 and 495 nucleotides (nt). 28 MS-MLPA probes contain an HhaI recognition site and provide information on the methylation status of 25 TSGs. All probes present will also give information on copy number changes in the analysed sample. In addition, 16 reference probes are included that are not affected by HhaI digestion and target relatively copy number stable regions in various cancer types. Also, one digestion control probe is included in this probemix indicating whether or not restriction endonuclease digestion in the MS-MLPA reaction was complete. Complete/partial probe sequences and the identity of the genes detected by the reference probes are available in Tables 2, 3 and online (www.mrcholland.com).

This probemix contains nine quality control fragments generating amplification products between 64 and 105 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and one chromosome Y-specific fragment (see table below). More information on how to interpret observations on these control fragments can be found in the MS-MLPA General Protocol and online at www.mrcholland.com.

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

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

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

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

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. More information on the use of FFPE tissue samples for MLPA can be found in Atanesyan et al. (2017).

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 different healthy individuals. More information regarding the selection and use of reference samples can be found in the MS-MLPA General Protocol (www.mrcholland.com).

Positive control DNA samples
MRC Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. Coriell Institute (https://catalog.coriell.org) and Leibniz Institute DSMZ (https://www.dsmz.de/) have diverse collections of biological resources which may be used as positive control DNA samples in your MLPA experiments. The quality of cell lines can change; therefore samples should be validated before use.

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

Interpretation of copy number results
The standard deviation of each individual probe over all the reference samples should be ≤0.10. When these criteria are fulfilled, the following cut-off values for the FR of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

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

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

Please note that these above mentioned final ratios are only valid for germline testing. Final ratios are affected both by percentage of tumour cells and by possible subclonality.

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in subclonal cases.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or

PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.

- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MS-MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Digestion Control Probe. The target sequence of the digestion control probe is unmethylated in most blood-derived DNA samples. The signal of the digestion control probe should be gone upon complete digestion by HhaI.
- mRNA levels. We have no data showing that methylation detected by a particular probe indeed influences the corresponding mRNA levels.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: a lower injection voltage or a shorter injection time, or a reduced amount of sample by diluting PCR products.

Interpretation of methylation results on blood and tissue derived DNA samples:
This probemix is intended for determining if the DNA sequences targeted by the methylation-specific probes show differential methylation as compared to the reference samples. This requires the determination of a "baseline" level of methylation, which can be used to determine if the methylation level in a test sample is significantly different from the reference samples.

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

To determine the baseline methylation level, it is required to test a sufficient number (≥3) of reference samples from healthy individuals. These samples should be derived from the same tissue type, handled using the same procedure (e.g. FFPE vs. fresh frozen), and prepared using the same DNA extraction method.
The baseline methylation level is then calculated by taking the average value of final ratios of the reference samples per probe and adding two times the standard deviation. The table below contains an example. Note that each individual methylation-specific probe should have a separate baseline methylation level and those values should not be averaged between the probes.

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

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

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

Interpretation of methylation positive samples is dependent on the application used.
NOTE: In case digestion control probes are not fully digested (>0.05), please contact info@mrcholland.com for more information.

ME001 specific notes:

- Please note that several MS-MLPA probes have multiple HhaI restriction sites. All of these sites need to be methylated in the template DNA in order for the probe not to be digested.
- In samples from tumour tissues, reference probes are more prone to have deviating copy number results as compared to blood derived germline samples. When regions targeted by reference probes are affected by copy number alterations, it can help to turn the slope correction off in Coffalyser.Net analysis to get the correct copy number interpretation on the target region.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the majority of target genes in this probemix are small (point) mutations, most of which will not be detected by using SALSA MS-MLPA Probemix ME001 Tumour suppressor mix.
- MS-MLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations. Even when MS-MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.
- Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can cause false positive results. Mutations/SNVs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA.
- An MS-MLPA probe targets a single specific HhaI site in a CpG island; if methylation is absent for a particular CpG-site, this does not necessarily mean that the whole CpG island is unmethylated!
- Rare cases are known in which apparent methylation as detected by an MS-MLPA probe proved to be due to a sequence change in or very near the HhaI site.
- MS-MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. Changes in methylation status, gains or losses of genomic regions or genes may not be detected if the percentage of tumour cells is low. In addition, subclonality of the aberration affects the final ratio of the corresponding probe. Furthermore, there is always a possibility that one or more reference probes *do* show a copy number alteration in a patient sample, especially in solid tumours with more chaotic karyotypes.

Confirmation of results

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

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

COSMIC mutation database

http://cancer.sanger.ac.uk/cosmic. We strongly encourage users to deposit positive results in the COSMIC mutation database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

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

Table 1. SALSA MS-MLPA Probemix ME001-D1 Tumour suppressor mix

Length (nt)	SALSA MLPA probe	HhaI site	% expected signal reduction[a]	Chromosomal position (hg18)	
				target	reference/control
64-105	Control fragments – see table in probemix content section for more information				
121	Reference probe 19616-L27455	-	-		4p13
126 π	Digestion control probe S0750-L21493	+	100%		2q12
136 *	**KLLN probe** 10345-L11988	+	100%	10q23.31	
141	**APC probe** 21787-L01968	+	100%	5q22.2	
148 Δ ‡ ๑	**TIMP3 probe** 02255-L31273	+	100%	22q12.3	
156	Reference probe 14199-L27634	-	-		2q13
161	**CDKN2A probe** 01524-L01744	+	100%	9p21.3	
168 ±	**MLH1 probe** 01686-L01266	+	100%	3p22.2	
175	Reference probe 00554-L13516	-	-		12p13
184	**ATM probe** 04044-L03849	+	100%	11q22.3	
190	Reference probe 06237-L20171	-	-		10p11
198	**RARB probe** 04040-L18151	+	100%	3p24.2	
204	Reference probe 18317-L27398	-	-		15q24
210	**CDKN2B probe** 15673-L17639	+	100%	9p21.3	
223 «	**HIC1 probe** 21184-L31271	+	100%	17p13.3	
238 « ‡ ๑	**CHFR probe** 03813-L03753	+	100%	12q24.33	
246 ∞	**BRCA1 probe** 05162-L04543	+	100%	17q21.31	
256	Reference probe 20831-L29007	-	-		8q13
265	**CASP8 probe** 02761-L02210	+	100%	2q33.1	
274 ‡ ๑	**CDKN1B probe** 07949-L07730	+	100%	12p13.1	
280	Reference probe 13350-L26120	-	-		9q21
292	**KLLN probe** 02203-L24130	+	100%	10q23.31	
301	**BRCA2 probe** 04042-L03755	+	100%	13q13.1	
313	Reference probe 06580-L24038	-	-		2q24
319 ∞	**CD44 probe** 03817-L01731	+	100%	11p13	
328	**RASSF1 probe** 18373-L29973	+	100%	3p21	
338	Reference probe 16335-L19822	-	-		3q21
346	**DAPK1 probe** 01677-L01257	+	100%	9q21.33	
353 ∫	**VHL probe** 03810-L01211	+	100%	3p25.3	
364	Reference probe 01234-L00781	-	-		10p14
373	**ESR1 probe** 21467-L11994	+	100%	6q25.1	
382	**RASSF1 probe** 21468-L31467	+	100%	3p21.31	
391	Reference probe 00713-L00108	-	-		19q13
398 «	**TP73 probe** 21387-L01263	+	100%	1p36.32	
409	**FHIT probe** 02201-L01699	+	100%	3p14.2	
416	Reference probe 20960-L29094	-	-		6p12
424	**CADM1 probe** 21385-L03848	+	100%	11q23.2	
429	**PTEN probe** 21788-L31287	+	100%	10q23.31	
436 ¥ ∧ ‡	**CDH13 probe** 07946-L31292	+	95-100%	16q23.3	
445	Reference probe 15733-L17713	-	-		1p13
454 ∞	**GSTP1 probe** 01638-L01176	+	100%	11q13.2	
463 ‡	**MLH1 probe** 21388-L30083	+	100%	3p22.2	
469	Reference probe 20128-L27639	-	-		18p11
483	Reference probe 11677-L12448	-	-		16p11
495	Reference probe 06676-L15676	-	-		11p15

[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 (from lot D1-0219 onwards).
¥ Changed in version in version D1 (from lot D1-0219 onwards). Minor alteration, no change in sequence detected.
± 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 0.14%) is present, HhaI digestion will not occur, resulting in a false positive methylation signal.
∫ Target sequence of this probe contains SNP rs3087462 (C/T) in the GCGC site, 3 nt right from the ligation site. When the T-allele of this validated SNP (with an allele frequency of 2.93%) is present, HhaI digestion on the first GCGC site will not occur, but assessment of the methylation status will be possible based on the second intact GCGC site.
∞ SNPs rs529629382, rs55707108 and rs572419862 in target sequence of probes at 246, 319 and 454 nt respectively could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.
ɷ This probe shows 20-30% reduced signal in digested reactions on methylated control DNA sample.
« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.
π Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.
Λ This probe is not completely digested in DNA samples derived from blood.
‡ This probe contains two 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.

NOTE: The digestion control probe at 126 nt should provide no, or a very low (<10%) signal in digested samples. The signal of the 126 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.

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

Table 2. ME001-D1 MS-MLPA probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene	Ligation site	Location (hg18) in kb	Complete sequence
398 «	21387-L01263	***TP73***	NM_005427.4; 61-62	01-003,559	CGCCCGCGAAGGGGACGCAGC-GAAACCGGGGCCCGCGCCAGGCCAGCCGGGA
265	02761-L02210	***CASP8***	NM_001372051.1; 2.6 kb before exon 1	02-201,831	CTTTCCAATAAAGCATGTCCAGCGCTC-GGG CTTTAGTTTGCACGTCCATGAATTGTCTGCCACA
353 ∫ ‡	03810-L01211	***VHL***	NM_000551.4; 9 nt before exon 1	03-010,158	GCGAAGACTACGGAGGTCGACTCGGG-AGCGCGCACGCAGCTCCGCCCCGCGTCCGACC
198	04040-L18151	***RARB***	NM_000965.5; 173 nt before exon 1	03-025,445	AGAAAACGCCGGCTTGTGCGCTCGCT-GCC TGCCTCTCTGGCTGTCTGCTTTTGCAGGGCTGCT
463 ‡	21388-L30083	***MLH1***	NM_000249.4; 352 nt before exon 1 *reverse*	03-037,010	CTGCTGAGGTGATCTGGCGCAGA-GCGG AGGAGGTGCTTGGCGCTTCTCAGGCTCCTCCTCT
168 ±	01686-L01266	***MLH1***	NM_000249.4; 18-19	03-037,010	CGTTGAGCATCTAGACGTTTCCTTGGCTCT-TCT GGCGCCAAAATGTCGTTCGTGGCAGGGGTTATTC
382	21468-L31467	***RASSF1***	NM_007182.5; 48 nt before exon 1 *reverse*	03-050,353	GGCCACAGGGCGGGCCCCGAC-TTCAGCGCCTCCCCCAGGATCCAGACTG
328	18373-L29973	***RASSF1***	NM_007182.5; 109 nt before exon 1	03-050,353	CCCACAGTCCCTGCACCCAGGTTTCCA-TTGCGCGGCTCTCCTCAGCTCCTTCCCGCCGC
409	02201-L01699	***FHIT***	NM_002012.4; 65 nt after exon 1	03-061,212	CGCGGGTCTGGGTTTCCACGC-GCGTCA GGTCATCACCCCGGAGCCCAGTGGG
141	21787-L01968	***APC***	NM_000038.6; 97 nt before exon 1	05-112,101	CAGCTGTGTAATCCGCTGGATGCGGACC-AGGGCGCTCCCCATTCCCGTCGGGAGCCCGC
373	21467-L11994	***ESR1***	NM_000125.4; 397-398	06-152,171	AGCCCGCCGTGTACAACTACCCCG-AGGGCGCCGCCTACGAGTTCAACGCCGCGGC
161	01524-L01744	***CDKN2A***	NM_058195.4; 928 nt before exon 1; NM_000077.5; 20.6 kb before exon 1	09-021,985	GGGGAAGAGGAAAGAGGAAGAAGCGCTCAGAT-GCTCCGCGGCTGTCGTGAAGGTTAAAACCGAA AATAAAAATGG
210	15673-L17639	***CDKN2B***	NM_004936.4;	09-021,999	GACTAGTGGAGAAGGTGCGACAGCTC-CTGGAAGC

Length (nt)	SALSA MLPA probe	Gene	Ligation site	Location (hg18) in kb	Complete sequence
			451-452		CGGCGCGGATCCCAACGGAGTCAACCGTTTCGG
346	01677-L01257	***DAPK1***	NM_004938.4; 257 nt after exon 1	09-089,303	CGCGAGGATCTGGAGCGAACTGCT-GCGCCTCGGTGGGCCGCTCCCTTCCCTCCCT
292	02203-L24130	***KLLN***	NM_001126049.2; 902-901 *reverse*	10-089,612	CACCGGAGCGGGCGCAGGAGA-GGCCTGCGGGGTGCGTCCCACTCACAGGGAT
136	10345-L11988	***KLLN***	NM_001126049.2; 756-755 *reverse*	10-089,612	CTTTCATTTTTAGGGCAAACGAGCCGAGT-TACCGGGGAAGCGAGAGGTGGGGCGCTGCAAG
429	21788-L31287	***PTEN***	NM_000314.8; 14-13 *reverse*	10-089,613	CTCTCAAACTTCCATCATGGCTGCAGCTTCC-GAGAGGAGAGAACTGAGCGCAGTCGCGTCCC
319 ∞	03817-L01731	***CD44***	NM_000610.4; 117-118	11-035,117	CTCCTTTCGCCCGCGCCCTCC-GTTCGCTCCGGACACCATGGACAAGTTTTGGTGG
454 ∞	01638-L01176	***GSTP1***	NM_000852.4; 64 nt before exon 1	11-067,108	GAAGAGCGGCCGGCGCCGTG-ACTCAGCACTGGGGCGGAGCGGGGCGGGACC
184	04044-L03849	***ATM***	NM_000051.4; 65-66	11-107,599	GGGAGGAGGCGAGAGGAGTCGGGA-TCTGCGCTGCAGCCACCGCCGCGGTTGATACTACTTT
424	21385-L03848	***CADM1***	NM_001301043.2; 283 nt before exon 1	11-114,881	CCTGGAGCCCGAGTCCTTGCACGCCA-GGCGCCCGGGAGAACACTTTTTCCTTGATCCGGGGAAAGCA
274 ‡ ๑	07949-L07730	***CDKN1B***	NM_004064.5; 316-317	12-012,762	CAGCCCCTGCGCGCTCCTAGA-GCTCGGGCCGTGGCTCGTCGGGGTCTGTGTCTTT
238 « ‡ ๑	03813-L03753	***CHFR***	NM_001161346.2; 130 nt before exon 1 *reverse*	12-131,974	CGCGAGAGTAGGCGCGTGGAGG-GCGCTCGGCCATCTTTGATCCTGACCAGGCGACTTCGT
301	04042-L03755	***BRCA2***	NM_000059.4; 102-103	13-031,788	CGGGAGAAGCGTGAGGGGACAGATTTGTG-ACCGGCGCGGTTTTTGTCAGCTTACTCCGGCCAAAAAAGA
436 ‡ Λ	07946-L31292	***CDH13***	NM_001257.5; 169-170	16-081,218	GTTCTGTGCGTTCTCCTGTCCCAG-GTAGGGAAGAGGGGCTGCCGGGCGCGCTCTG
223 «	21184-L31271	***HIC1***	NM_006497.4; 15 nt before exon 1	17-001,905	CGCTCCAGATAAGAGTGTGCGGA-AAGCGCGGCGGGGCTGAGACGCGACCAGGAC
246 ∞ #	05162-L04543	***BRCA1***	NM_007294.4; 59-60	17-038,531	TTCTCAGATAACTGGGCCCCTGC-GCTCAGGAGGCCTTCACCCTCTGCTCTGGGTAAAGGT
148 Δ ‡ ๑	02255-L31273	***TIMP3***	NM_000362.5; 126-127	22-031,528	CCAGCGCCGAGGCAGCCTCGC-TGCGCCCCATCCCGTCCCGCCGGGCACTCGG

The HhaI sites are marked with grey. Ligation sites are marked with –.

± 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 0.14%) is present, HhaI digestion will not occur, resulting in a false positive methylation signal.

∫ Target sequence of this probe contains SNP rs3087462 (C/T) in the GCGC site, 3 nt right from the ligation site. When the T-allele of this validated SNP (with an allele frequency of 2.93%) is present, HhaI digestion will not occur, resulting in a false positive methylation signal.

∞ SNPs rs529629382, rs55707108 and rs572419862 in target sequence of probes at 246 nt, 319 nt and 454 nt respectively could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.

๑ This probe shows 20-30% reduced signal in digested reactions on methylated control DNA sample.

« 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.

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

‡ This probe contains two 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.

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

Table 3. Reference probes arranged according to chromosomal location.

Length (nt)	SALSA MLPA probe	Gene	Chromosomal band (hg18)	Partial sequence (24 nt adjacent to ligation site)	Location (hg18) in kb
445	15733-L17713	*TRIM45*	1p13	CAGTAGTGGACA-TCCGAGGGGGAG	01-117,465
156	14199-L27634	*EDAR*	2q13	GAGAGTTCTGTG-GGTGGAGAGAAG	02-108,894
313	06580-L24038	*SCN2A*	2q24	AACTTGGTTTGG-CAAATGTGGAAG	02-165,907
338	16335-L19822	*RAB7A*	3q21	AGGCCTTCAACA-CAATTCCCCTCT	03-130,015
121	19616-L27455	*ATP8A1*	4p13	CAGATTCTTCTT-CGAGGAGCTCAG	04-042,278
416	20960-L29094	*PKHD1*	6p12	TTTATCCACCAA-GTGGTGTTCCAG	06-052,049
256 #	20831-L29007	*EYA1*	8q13	GTGTATGTTGTT-ATAGGAGATGGT	08-072,286
280	13350-L26120	*PCSK5*	9q21	CATTAGCAAGCA-TTAGAACATCTC	09-077,989
364	01234-L00781	CELF2-region	10p14	GACATTCACTGT-GGAAATTTGGTG	10-011,017
190	06237-L20171	*CREM*	10p11	AGGTGCTACAAT-TGTACAGTACGC	10-035,517
495	06676-L15676	*SMPD1*	11p15	CTGCTGAAGATA-GCACCACCTGCC	11-006,369
175	00554-L13516	*TNFRSF1A*	12p13	CCCTGAGCCCAA-ATGGGGGAGTGA	12-006,321
204	18317-L27398	*SEMA7A*	15q24	CGAGCCACACAA-GGAGTGTCCCAA	15-072,491
483	11677-L12448	*LAT*	16p11	ACCAGTTTGTAT-CCAAGGGGCATC	16-028,905
469	20128-L27639	*RNMT*	18p11	TACAATGAACTT-CAGGAAGTTGGT	18-013,724
391	00713-L00108	*KLK3*	19q13	ATGTGGGTCCCG-GTTGTCTTCCTC	19-056,050

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.

Complete probe sequences are available at www.mrcholland.com.

Related SALSA MLPA probemixes

ME011 Mismatch Repair Genes Contains (MS)-MLPA probes for promoter regions of *MLH1*, *MSH2*, *MSH6, PMS2 and EPCAM* genes, and one *BRAF* V600E mutation-specific probe.

ME012 MGMT-IDH-TERT Contains MS-MLPA probes for the *MGMT* promoter region as well as mutation-specific probes to detect the presence of point mutations in the *IDH1*, *IDH2* genes and the *TERT* promoter.

ME024 9p21 CDNK2A/2B region Contains (MS)-MLPA probes for CDKN2A/2B gene region, *MIR31*, *MTAP* and *PAX5* genes.

ME042 CIMP Contains MS-MLPA probes targeting the promoter regions of the *CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1* genes.

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Note: Above is a selection of references for this probemix; PubMed and Google Scholar provide more references and information on the use of the ME001 probemix.

ME001 product history	
Version	*Modification*
D1	Majority of reference probes and CDKN2B target probe have been replaced. Moreover, two PTEN/KLLN probes and one HhaI digestion control probe have been added, and several probes have a minor change in length but not in the sequence detected.
C2	New control fragments have been added (QDX2).
C1	The 148 nt APC probe has been replaced and extra control fragments have been added at 100 and 105 nt.
B1	The 274 nt CDKN1B & 436 nt CDH13 probes have been replaced.
A1	First release.

Implemented changes in the product description

Version D1-03 – 23 February 2023 (04M)
- Product description rewritten and adapted to a new template.
- Various minor textual or layout changes.
- Ligation sites of the probes targeting all genes, except for *VHL*, *MLH1*, *FHIT* and *PTEN*, were updated according to new version of the NM_ reference sequence or the MANE Select transcript.
- Warning added in Table 3 for 20831-L29007 probe's specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.
- Recent references added in section " Selected publications using SALSA MS-MLPA Probemix ME001 Tumour suppressor mix" on page 10.
- Updated information in the Related SALSA MLPA probemixes section on page 9.
- Small modification of the probemix name from "Tumour suppressor mix 1" to "Tumour suppressor mix".

Version A1-03 – 02 August 2021 (02M)
- In section 'SALSA Binning DNA SD054' on page 3 updated the information about SD054 content replacing 'synthetic DNA' with 'plasmid DNA'.

Version A1-02 – 11 June 2021 (02M)
- ME012-specific note added regarding probes with incomplete HhaI digestion on page 5.

Version A1-01 — 05 August 2019(02M)
- Product description rewritten and adapted to a new template.
- Various minor textual or layout changes.
- Information on findings with positive DNA samples added on page 3.
- Figure 1 completely modified.
- Ligation sites of the probes targeting the MGMT gene updated according to new version of the NM_ reference sequence.
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.
- Table 2c (control probes) replaced with a note on page 7 containing the relevant information.
- Three ME012 probemix specific notes added on page 5.
- Added note about background signal of 203 nt IDH1 mutation-specific probe 19529-L16492 below Table 1 and Table 2a.
- Interpretation of methylation results on tissue derived DNA samples was added. - For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36).

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