Product Description SALSA® MS-MLPA® Probemix ME012-A1 MGMT-IDH1-IDH2

To be used with the MS-MLPA General Protocol.

Version A1. For complete product history see page 12.

Catalogue numbers:
- **ME012-025R:** SALSA MS-MLPA Probemix ME012 MGMT-IDH1-IDH2, 25 reactions.
- **ME012-050R:** SALSA MS-MLPA Probemix ME012 MGMT-IDH1-IDH2, 50 reactions.
- **ME012-100R:** SALSA MS-MLPA Probemix ME012 MGMT-IDH1-IDH2, 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.

General information: The SALSA MS-MLPA Probemix ME012 MGMT-IDH1-IDH2 is a **research use only (RUO)** assay for the detection of aberrant methylation of one or more sequences of the *MGMT* gene. This probemix can also be used to detect the presence of the *IDH1* (p.R132H=c.395G>A and p.R132C=c.394C>T) or *IDH2* (p.R172K=c.515G>A and p.R172M=c.515G>T) point mutations in a DNA sample.

Glioma is the most common neoplasm of central nervous system, comprising three phenotypic groups based on the type of glial cell forming tumour: astrocytomas (including the most aggressive type of brain cancer - glioblastomas), ependymomas, and oligodendrogliomas. Hypermethylation in the promoter region of the *MGMT* gene, encoding for the DNA repair enzyme O^6-methylguanine DNA methyltransferase, is an important prognostic marker and predictor for response to treatment in glioma with alkylating agents such as temozolomide (Weller et al. 2009, Hegi et al. 2005, Pegg 1990). Another important diagnostic and prognostic marker in glioma is the *IDH1* and *IDH2* mutation status (Riemenschneider et al. 2010, van den Bent et al. 2010). The presence of *IDH1* or *IDH2* mutation is suggested to associate with favourable prognosis and a longer survival of glioma patients (Sanson et al. 2009, Zou et al. 2013). The *IDH1/2* mutations described are not activating or inactivating, but probably result in altered enzymatic properties (Hartmann et al. 2009). IDH mutation is a marker for glioma classification since 2016, defining glioblastomas as *IDH1*-mutant or *IDH1*-wildtype (Wesseling and Capper 2018). Assessment of both the *IDH1* mutation status and the *MGMT* methylation status is proposed to be used as a combined predictor for glioblastoma patient survival (Wick et al. 2013). Combined assessment of *IDH1* mutations and *MGMT* methylation status is suggested to predict survival in glioblastoma better than either *IDH1* or *MGMT* alone (Molenaar et al. 2014).

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/

Exon numbering: The exon numbering used in this ME012 MGMT-IDH1-IDH2 product description is the exon numbering from the RefSeq transcript NM_002412.5 for *MGMT*, LRG_610 t1 (identical to NM_005896.3) for *IDH1*, and LRG_611 t2 (identical to NM_002168.3) for *IDH2*. The exon numbering and NM_ sequence used have been retrieved on 07/2019. 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 ME012 MGMT-IDH1-IDH2 contains 31 (MS-)MLPA probes with amplification products between 121 and 317 nucleotides (nt). Six MS-MLPA probes contain an HhaI recognition site and provide information on the methylation status of *MGMT* promoter region. All probes present will also give information on copy number changes in the analysed sample. Moreover, this probemix contains four mutation-specific probes to identify the four most predominant *IDH1* (p.R132H and p.R132C) and *IDH2* (p.R172K and p.R172M) point mutations in glioma. In addition, 18 reference probes are included that are not affected by HhaI digestion and target relatively copy number stable regions in glioma. Also, two digestion control probes and one depurination control probe are included in this probemix, indicating whether or not restriction endonuclease digestion in the MS-MLPA reaction was complete, and whether DNA has been affected by depurination, respectively. Identity of the genes detected by the reference probes are available in Table 2b and complete probe sequences are available 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. 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 healthy individuals without a history of 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 NA00959, NA05299, NA20263 from the Coriell Institute, and EJM, HCT-116, HEP-G2, HL-060, MDA-MB-231, MOLP-8, U-266 from Leibniz Institute DSMZ have been tested with this ME012-A1 probemix at MRC-Holland and can be used as a positive control sample(s) to detect *MGMT* gain/loss and/or methylation. The quality of cell lines can change; therefore samples should be validated before use.

MGMT copy number status of a selection of human fibroblast (Coriell) and cancer cell line (DSMZ) DNA samples.

Sample name	Source	Chromosomal position of CNA	Altered probes in ME012-A1	Expected CNA
NA00959	Coriell Institute	10q26.3	all MGMT probes	Heterozygous duplication
NA05299	Coriell Institute	10q26.3	all MGMT probes	Heterozygous deletion
NA20263	Coriell Institute	10q26.3	all MGMT probes	Heterozygous deletion
MOLP-8	DSMZ	10q26.3	all MGMT probes	Heterozygous deletion

MGMT methylation status of a selection of cancer cell line DNA samples (DSMZ).

Probe length (nt)	HhaI site (GCGC) coordinates (hg18) chr: start-end	Cancer cell line						
		EJM	HCT-116	HEP-G2	HL-060	MDA-MB-231	MOLP-8	U-266
140	chr10:131155014-131155019	-	++++	+++	++++	++++	-	++++
190	chr10:131155141-131155144	-	++++	++	++++	+++	-	++++
124	chr10:131155198-131155201 & chr10:131155244-131155247	-	++++	+++	++++	++	-	+++
160	chr10:131155485-131155488	-	+++	+++	++++	++	-	++++
215	chr10:131155631-131155634	-	++++	++	++++	-	-	-
172	chr10:131155698-131155701	-	+++	++	+++	-	-	-

- 0-10 % after HhaI digestion = no methylation
+ 10-25 % after HhaI digestion = low methylation
++ 25-50 % after HhaI digestion = moderate methylation
+++ 50-75 % after HhaI digestion = high methylation
++++ >75 % after HhaI digestion = (almost) fully methylated

SALSA Binning DNA SD054: The SD054 Binning DNA provided with this probemix can be used for binning of four mutation-specific probes *IDH1* probe 19529-L16492 at 203 nt (p.R132H=c.395G>A), *IDH1* probe 19926-L29107 at 225 nt (p.R132C=c.394C>T), *IDH2* probe 20643-L29002 at 145 nt (p.R172K=c.515G>A) and *IDH2* probe 20643-L29001 at 151 nt (p.R172M=c.515G>T). SD054 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 of SD054 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 signals, as for this purpose true mutation/SNP positive patient samples or cell lines should be used. 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 SD054 Binning DNA product description, available online: www.mlpa.com.

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 standard deviation of each individual probe over all the reference samples should be ≤0.10. When this criterion is 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 *MGMT* gene. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MS-MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Digestion Control Probes: The target sequences of the digestion control probes are unmethylated in most blood-derived DNA samples. The signals of the digestion control probes should be gone upon complete digestion by HhaI.
- 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.
- 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, 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. In glioblastoma, 8% average of five CpGs (Quillien V et al. 2012) and 12.5% mean of four CpGs (Yuan G et al. 2017) were indicated to be the optimal threshold for defining *MGMT* methylation by using pyrosequencing.

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

ME012 probemix specific notes:
- Please note that two probes (S1100-L27104 at 124 nt and 20097-L28960 at 140 nt) have two HhaI restriction sites. Both sites need to be methylated in order to not be digested!
- In samples from tumour tissues, reference probes are more prone to have deviating copy number results than in 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 interpretation of the target region.
- The *IDH1* mutation-specific probe 19529-L16492 at 203 nt might show very low unspecific background signal in wild-type samples. This background signal at 203 nt interferes with Coffalyser data analysis if the assignment of probe identities is not done properly. In order to identify each peak in the peak pattern as a probe, it is recommended to create a manual binset using the SD sample (SD054), which is provided with each vial of ME012 probemix. Instructions to create a manual binset can be found at Coffalyser.Net section in MRC-Holland Support Portal: https://support.mlpa.com/.

Limitations of the procedure:
- 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.

Please report false positive results due to SNPs and unusual results (e.g., a duplication of *MGMT* probes at 140 and 124 nt but not at 190 nt) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MS-MLPA Probemix ME012-A1 MGMT-IDH1-IDH2

Length (nt)	SALSA MLPA probe	HhaI site	% expected signal reduction[a]	Probe details	Chromosomal position (hg18) Target Reference
64-105	Control fragments – see table in probemix content section for more information				
121	Reference probe 19616-L27455	-			4p13
124 ‡	**MGMT probe** S1100-L27104	+	100		10q26.3
135	Reference probe 19551-L26642	-			2p13
140 ∧	**MGMT probe** 20097-L28960	+	90		10q26.3
145 §	**IDH2 probe** 20643-L29002	-		**p.R172K** (c.515G>A)	15q26.1
151 §	**IDH2 probe** 20643-L29001	-		**p.R172M** (c.515G>T)	15q26.1
157	Reference probe 07118-L29026	-			12p13
160	**MGMT probe** 19735-L28999	+	100		10q26.3
166	Reference probe 10904-L11573	-			9q34
172 ∧	**MGMT probe** 14133-L15736	+	95		10q26.3
178	Reference probe 04857-L28909	-			5p13
185 Ж ∫	**Depurination control probe** 20784-SP0891-L28910	-		Depurination control	16p13.2
190	**MGMT probe** 19736-L26793	+	100		10q26.3
197	Reference probe 06937-L29022	-			11q12
203 § +	**IDH1 probe** 19529-L16492	-		**p.R132H** (c.395G>A)	2q33.3
208 п	**HhaI digestion control probe** 19490-L25113	+	100	HhaI digestion control 1	2q12
215 ±	**MGMT probe** 12250-L27780	+	100		10q26.3
225 §	**IDH1 probe** 19926-L29107	-		**p.R132C** (c.394C>T)	2q33.3
229	Reference probe 20525-L29005	-			1q31
238	Reference probe 18055-L22445	-			16q23
244	Reference probe 18664-L24018	-			11p14
250	Reference probe 06712-L29006	-			15q24
256	Reference probe 20831-L29007	-			8q13
265	Reference probe 16433-L29008	-			18q21
270	Reference probe 18257-L29009	-			17p11
277	Reference probe 13796-L24815	-			3q25
285	Reference probe 19493-L29004	-			3p12
294	Reference probe 13393-L28788	-			6q12
302	Reference probe 06548-L28789	-			5q13
310 п	**HhaI digestion control probe** 17305-L29021	+	100	HhaI digestion control 2	8p21
317	Reference probe 11898-L24065	-			6p12

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

§ Mutation-specific probe. This probe will only generate a signal when the indicated mutation is present. It has been tested on artificial DNA **but not on positive human samples!**
± Target sequence of this probe contains SNP rs186050433 (C/T) at HhaI recognition site. When the T-allele of this validated SNP (with an allele frequency of 1.07%) is present, HhaI digestion will not occur, resulting in a false methylation positive signal.
п Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.
∧ 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 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.

∫ Reduced signal of this probe indicates that sample DNA is possibly depurinated. An extremely low signal of this probe might indicate a very poor sample DNA quality; please critically review your MLPA results in this case.
+ This probe might show very low unspecific background signal in wild-type samples. This background signal at 203 nt interferes with Coffalyser data analysis if the assignment of probe identities is not done properly. In order to identify each peak in the peak pattern as a probe, it is recommended to create a manual binset using the SD sample (SD054), which is provided with each vial of ME012 probemix. Instructions to create a manual binset can be found in MRC-Holland Support Portal: https://support.mlpa.com/.

NOTE: The digestion control probes at 208 nt and 310 nt should provide no, or a very low (<10%) signal in digested samples. HhaI digestion of the 310 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 208 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.

Table 2a. ME012-A1 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene/exon	Ligation site	Location (hg18) in kb	Distance to next probe
IDH1, at 2q33.3. Mutations of *IDH1* and *IDH2* (isocitrate dehydrogenase 1 and 2) occur in the majority of lower grade gliomas, and are associated with improved prognosis compared with gliomas with wild-type IDH (Hartmann et al. 2009, Sanson et al. 2009, Cohen AL et al. 2013). IDH mutations are essential biomarkers for molecular classification and prognostication of adult gliomas. The probes at 203 and 225 nt will only give a signal when respectively the p.R132H and p.R132C mutation is present in the sample.					
203 § +	19529-L16492	**IDH1**, exon 6; **p.R132H = c.395G>A**	NM_005896.3; 690-691	02-208.821	0.0 kb
225 §	19926-L29107	**IDH1**, exon 6; **p.R132C = c.394C>T**	NM_005896.3; 689-688 reverse	02-208.821	-
MGMT, at 10q26.3. Two methylation hot spots in the promoter region of the *MGMT* gene, at -249 to -103 nt and +107 to +196 nt in respect to the transcription start site (NM_002412.4), are suggested to denote silencing of the *MGMT* gene (Qian and Brent. 1997). This ME012-A1 probemix includes six MS-MLPA probes targeting CpG sites within and surrounding the above mentioned two methylation hot spots. CpG methylation at specific sites are suggested to provide different prognostic value. The probe at 124 nt covers the cg12434587 site that is suggested to have a strong association with patient survival, according to Bady P et al. 2012. Please see Figure 1 on page 9 for more specific information on the location of methylation-specific probes in the promoter region of the *MGMT* gene.					
140 ∧	20097-L28960	upstream	NM_002412.5; 457 nt before exon 1 reverse	10-131.155	0.1 kb
190	19736-L26793	upstream	NM_002412.5; 333 nt before exon 1	10-131.155	0.1 kb
124 ‡	S1100-L27104	upstream	NM_002412.5; 263 nt before exon 1 reverse	10-131.155	0.3 kb
160	19735-L28999	exon 1	NM_002412.5; 7-6 reverse	10-131.155	0.1 kb
215 ±	12250-L27780	downstream	NM_002412.5; 73 nt after exon 1	10-131.156	0.1 kb
172 ∧	14133-L15736	downstream	NM_002412.5; 154 nt after exon 1 reverse	10-131.156	-
IDH2, at 15q26.1. Mutations of *IDH1* and *IDH2* (isocitrate dehydrogenase 1 and 2) occur in the majority of lower grade gliomas, and are associated with improved prognosis compared with gliomas with wild-type IDH (Hartmann et al. 2009, Sanson et al. 2009, Cohen AL et al. 2013). IDH mutations are essential biomarkers for molecular classification and prognostication of adult gliomas. The probes at 145 nt and 151 nt will only give a signal when respectively the R172K and R172M mutation is present in the sample.					
145 §	20643-L29002	**IDH2**, exon 5; **p.R172K = c.515G>A**	NM_002168.3; 679-680	15-088.433	0.0 kb
151 §	20643-L29001	**IDH2**, exon 5; **p.R172M= c.515G>T**	NM_002168.3; 679-680	15-088.433	-

§ Mutation-specific probe. This probe will only generate a signal when the indicated mutation is present. It has been tested on artificial DNA **but not on positive human samples!**
± Target sequence of this probe contains SNP rs186050433 (C/T) at HhaI recognition site. When the T-allele of this validated SNP (with an allele frequency of 1.07%) is present, HhaI digestion will not occur, resulting in a false methylation positive signal.
∧ This probe is not completely digested in DNA samples derived from blood.
+ This probe might show very low unspecific background signal in wild-type samples. This background signal at 203 nt interferes with Coffalyser data analysis if the assignment of probe identities is not done properly. In order to identify each peak in the peak pattern as a probe, it is recommended to create a manual binset using the SD sample (SD054), which is provided with each vial of ME012 probemix.

Instructions to create a manual binset can be found at Coffalyset.Net section in MRC-Holland Support Portal: https://support.mlpa.com/

Table 2b. Reference probes

Length (nt)	SALSA MLPA probe	Gene	Chr. band (hg18)	MV location (hg18) in kb
229	20525-L29005	CDC73	1q31	01-191.366
135	19551-L26642	DYSF	2p13	02-071.750
285	19493-L29004	GBE1	3p12	03-081.775
277	13796-L24815	KCNAB1	3q25	03-157.716
121	19616-L27455	ATP8A1	4p13	04-042.278
178	04857-L28909	NIPBL	5p13	05-037.080
302	06548-L28789	MCCC2	5q13	05-070.978
317	11898-L24065	PKHD1	6p12	06-052.039
294	13393-L28788	EYS	6q12	06-064.546
256	20831-L29007	EYA1	8q13	08-072.286
166	10904-L11573	SETX	9q34	09-134.200
244	18664-L24018	ANO5	11p14	11-022.258
197	06937-L29022	BEST1	11q12	11-061.481
157	07118-L29026	FGF23	12p13	12-004.352
250	06712-L29006	HEXA	15q24	15-070.436
238	18055-L22445	PLCG2	16q23	16-080.527
270	18257-L29009	AKAP10	17p11	17-019.807
265	16433-L29008	MYO5B	18q21	18-045.743

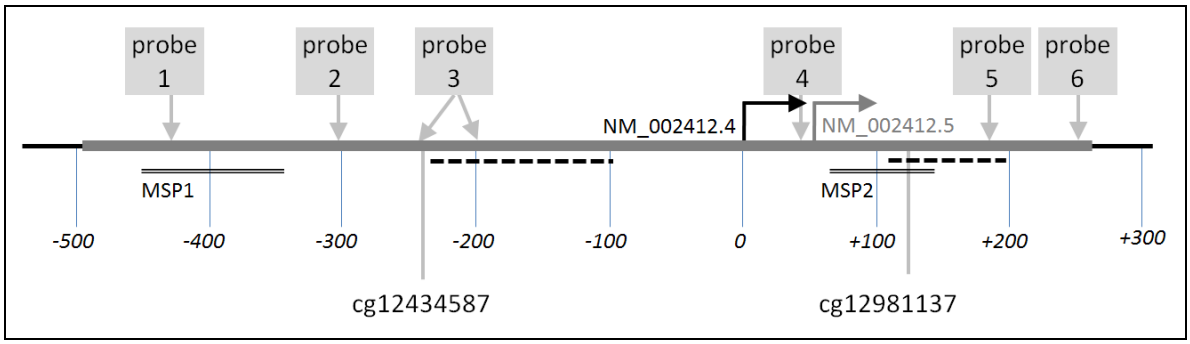
Table 3. Sequences detected by the ME012-A1 probes

Length (nt)	SALSA MLPA probe	Gene	Complete or partial (24 nt adjacent to ligation site) sequences detected by the probes
140	20097-L28960	MGMT	CGGGCGTGCAAGCGACCTGCCACGT-GCCCGAGTGGTCCTGAAAGCGCGCGGGGGTCGTAG
190	19736-L26793	MGMT	CCTGTGACAGGAAAAGGTACGGGCCATTTGGCAAACTAAG-GCACAGAGCCTCAGGCGGAAGCTGGGAAGGCGCCGCCCGGCTTGTACCGG
124	S1100-L27104	MGMT	GGCCTGAGGCAGTCTGCGCATCCTCGCTGGA-CGCCGGCACGCCGGCCCTGGTCCTCCGGCAGCGCCGCTGCCCTGTGC
160	19735-L28999	MGMT	ACCGCGAGGACCTGCGGGCGTCGGGACGCAA-AGCGTTCTAGGGGCGCGGGCTGTCCCAGCATATCCGG
215	12250-L27780	MGMT	CTCGGGACGGTGGCAGCCTCGAGTGGT-CCTGCAGGCGCCCTCACTTCGCCGTCGGGTGT
172	14133-L15736	MGMT	GAAAGGCTGGGCAACACCTGGGAGGCACTT-GGGGCGCACCTGGAGCTCGCCCGGGATGGGT
203	19529-L16492	IDH1	ATAAGTATGAC**A**-ACCTATGATGAT
225	19926-L29107	IDH1	CATCATAGGTC**A**-TCATGCTTATGG
151	20643-L29001	IDH2	TACCATTGGCA**T**-GCACGCCCATGG
145	20643-L29002	IDH2	TACCATTGGCA**A**-GCACGCCCATGG

The HhaI sites are marked with grey. Ligation sites are marked with –. Complete probe sequences are available at www.mlpa.com. Please notify us of any mistakes: info@mlpa.com.

Note: Please be aware that two probes (S1100-L27104 at 124 nt and 20097-L28960 at 140 nt) have two HhaI restriction sites. Both sites need to be methylated in order to not be digested.

Figure 1. Locations of HhaI restriction sites (GCGC) in the target sequence of MGMT MS-MLPA probes (probe 1-6).



CpG island in *MGMT* promoter region

Methylation hotspots: (-249 to -103 nt) and (+107 to +196 nt) from NM_002412.4 transcription start site (Qian XC and Brent TP. 1997).

cg12434587 & cg12981137 CpG sites shown to be important for classification and prognosis (Bady et al. 2012).

MSP1 and MSP2 sites according to van Nifterik KA et al. (2010).

The exact coordinates for HhaI sites (GCGC) of MGMT MS-MLPA probes are shown in the table below.

MS-MLPA probes	Length (nt)	SALSA MLPA probe number	GCGC coordinates (hg18) chr: start-end	GCGC coordinates (hg19) chr: start-end	Distance from/to NM_002412.4 (NM_002412.5) start (bp)
probe 1	140	20097-L28960	chr10:131155014-131155019	chr10:131265024-131265029	- 430 (-487)
probe 2	190	19736-L26793	chr10:131155141-131155144	chr10:131265151-131265154	- 303 (-360)
probe 3	124	S1100-L27104	chr10:131155198-131155201 & chr10:131155244-131155247	chr10:131265208-131265211 & chr10:131265254-131265257	- 246 and - 200 (-303 and -257)
probe 4	160	19735-L28999	chr10:131155485-131155488	chr10:131265495-131265498	+ 38 (-19)
probe 5	215	12250-L27780	chr10:131155631-131155634	chr10:131265641-131265644	+ 184 (+127)
probe 6	172	14133-L15736	chr10:131155698-131155701	chr10:131265708-131265711	+ 251 (+194)

Related SALSA MLPA probemixes

P088 Oligodendroglioma 1p-19q probemix: contains probes for copy number detection of 1p and 19q chromosomal regions and for *IDH1* p.R132C and p.R132H and *IDH2* p.R172K and p.R172M point mutation detection.

P105 Glioma-2 probemix: contains probes for *PDGFRA*, *EGFR*, *CDKN2A*, *PTEN*, *TP53*, *CDK4-MIR26A2-MDM2* and *NFKBIA* genes.

P370 BRAF-IDH1-IDH2 probemix: contains probes for *FGFR1*, *MYB* and *MYBL1* genes and for the 9p21.3 region, as well as probes specific for *BRAF* p.V600E, *IDH1* p.R132H and p.R132C, *IDH2* p.R172M and p.R172K point mutations.

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ME012 Product history	
Version	*Modification*
A1	First release.

Implemented changes in the product description

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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