Product Description

SALSA® MLPA® Probemix ME012-B1 MGMT-IDH-TERT

To be used with the MS-MLPA General Protocol.

Version B1
As compared to version A1, two MS-MLPA probes for *MGMT* promoter, two mutation-specific probes for *TERT* promoter and one digestion control probe have been added; depurination-sensitive and the majority of reference probes have been replaced. For complete product history see page 13.

Catalogue numbers:

- **ME012-025R:** SALSA MLPA Probemix ME012 MGMT-IDH-TERT, 25 reactions.
- **ME012-050R:** SALSA MLPA Probemix ME012 MGMT-IDH-TERT, 50 reactions.
- **ME012-100R:** SALSA MLPA Probemix ME012 MGMT-IDH-TERT, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit, SALSA Binning DNA SD094, 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 MLPA Probemix ME012 MGMT-IDH-TERT 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 *IDH1* (p.R132H=c.395G>A and p.R132C=c.394C>T), *IDH2* (p.R172K=c.515G>A and p.R172M=c.515G>T) and *TERT* promoter (C228T and C250T) point mutations in a DNA sample.

Gliomas, glioneuronal and neuronal tumours are the most common central nervous system (CNS) neoplasms, subdivided into six groups according to the fifth edition of the WHO classification of CNS tumours: adult-type diffuse gliomas (encompassing oligodendroglioma, glioblastoma and astrocytoma), paediatric-type diffuse low-grade gliomas, paediatric-type diffuse high-grade gliomas, circumscribed astrocytic gliomas, glioneuronal and neuronal tumours, ependymomas (Torp et al. 2022; Gritsch et al. 2022). Molecular genetic characteristics play an increasingly major role in classification, diagnostics and prognosis of CNS tumours.

IDH1 and *IDH2* mutation (*IDH*-mutation) status represents an important diagnostic and prognostic marker in gliomas (Riemenschneider et al. 2010, van den Bent et al. 2010). The presence of *IDH*-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 *IDH*-mutant or *IDH*-wildtype (Wesseling and Capper 2018).

Point mutations in *TERT* promoter region generate novel transcription factor binding sites and thus increase the expression of telomerase enzyme encoded by *TERT*. Common *TERT* promoter mutations are known as C228T and C250T, referring to C>T transitions at hg19/GCRh37 chr5:1295228 and chr5:1295250 positions, respectively. These mutations are predominantly present in oligodendroglioma and are associated with poor prognosis and reduced survival in the absence of *IDH*-mutation (Labussière et al. 2014). *TERT* promoter mutation, in combination with *IDH*-mutation and 1p/19q codeletion, is characteristic of oligodendroglioma.

Absence of *TERT* promoter mutation, coupled with the presence of *IDH*-mutation, designates astrocytoma (Cahill et al. 2015; Eckel-Passow et al. 2015).

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 genes 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.

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). Assessment of both the *IDH*-mutation 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 *IDH*-mutation and *MGMT* methylation status is suggested to predict survival in glioblastoma better than either biomarker alone (Molenaar et al. 2014). The presence of *TERT* promoter mutation in combination with unmethylated *MGMT* defines glioblastoma with the poorest prognosis (Arita et al. 2016).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK558954/ and WHO Classification of Central Nervous System Tumours, 5th Edition, Volume 6.

This SALSA 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
Matched Annotation from NCBI and EMBL-EBI (MANE): http://www.ncbi.nlm.nih.gov/refseq/MANE/
Tark – Transcript Archive: http://tark.ensembl.org/

Exon numbering
The *MGMT*, *IDH1*, *IDH2* and *TERT* exon numbering used in this ME012-B1 MGMT-IDH-TERT product description is the exon numbering derived from MANE project (release version 1.0) based on MANE Select transcripts NM_002412.5; NM_005896.4, NM_002168.4 and NM_198253.3, respectively. The abovementioned NM_sequences were also used for determining each probe's ligation site, 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.

Probemix content
The SALSA MLPA Probemix ME012-B1 MGMT-IDH-TERT contains 31 (MS-)MLPA probes with amplification products between 123 and 317 nucleotides (nt). Eight MS-MLPA probes contain at least one HhaI recognition site and provide information on the methylation status of the *MGMT* gene. All probes present will also give information on copy number changes in the analysed sample in the undigested reaction. Moreover, this probemix contains six mutation-specific probes to identify the four most predominant *IDH1* (p.R132H and p.R132C) and *IDH2* (p.R172K and p.R172M) and two *TERT* promoter region point mutations (C228T and C250T) in glioma. In addition, 13 reference probes are included that are not affected by HhaI digestion and target relatively copy number stable regions in various cancer types, including gliomas. Also, three digestion control probes are included in this probemix indicating whether or not restriction endonuclease digestion in the MS-MLPA reaction was complete. Furthermore, this probemix contains one DNA depurination-sensitive probe. Complete/partial probe sequences and the identity of the genes detected by the reference probes are available online and in Tables 2 and 3 (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 without a history of CNS tumours. More information regarding the selection and use of reference samples can be found in the MS-MLPA General Protocol (www.mrcholland.com).

Positive control DNA samples
MRC Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. Coriell Institute (https://catalog.coriell.org) and Leibniz Institute DSMZ (https://www.dsmz.de/) have diverse collections of biological resources which may be used as positive control DNA samples in your MLPA experiments. Sample ID numbers NA00959, NA05299, NA20263 from the Coriell Institute; 42-MG-BA (ACC 431), DK-MG (ACC 277), GMS-10 (ACC 405), HEP-G2 (ACC 180), HL-60 (ACC 3) and MOLP-8 (ACC 569) from the Leibniz Institute DSMZ; Human Methylated & Non-methylated DNA Set (cat. no. D5014, Zymo Research); Methylated Human Control DNA (cat. no. N1301, Promega); Human high methylated genomic DNA (cat. no. 80-8061-HGHM5, EpigenDx) and Methylated Control DNA (cat. no. M8750, Sigma-Aldrich) have been tested with this ME012-B1 probemix at MRC Holland and can be used as positive

control samples to detect *MGMT* copy number alterations (CNAs), methylation status and *TERT* promoter point mutations. The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Source	*MGMT* CNA*	*MGMT* methylation status#	Point mutation detected
NA00959	Coriell Institute	heterozygous duplication	all probe targets *non*-methylated	none
NA05299	Coriell Institute	heterozygous deletion	all probe targets *non*-methylated	none
NA20263	Coriell Institute	heterozygous deletion	all probe targets *non*-methylated	none
42-MG-BA (ACC 431)	DSMZ	none	all probe targets methylated (except 175 nt probe)	*TERT* C228T
DK-MG (ACC 277) ±	DSMZ	heterozygous deletion	all probe targets methylated	*TERT* C250T
GMS-10 (ACC 405)	DSMZ	heterozygous deletion	all probe targets methylated (except 175 and 215 nt probes)	*TERT* C228T
HEP-G2 (ACC 180)	DSMZ	none	all probe targets methylated	*TERT* C228T
HL-60 (ACC 3)	DSMZ	none	all probe targets methylated	none
MOLP-8 (ACC 569)	DSMZ	heterozygous deletion	all probe targets *non*-methylated	none
Methylated Human Control DNA π ±	Promega	none	positive control – all targets methylated	none
Human high methylated genomic DNA π	EpigenDx	none	positive control – all targets methylated	none
Methylated Control DNA π	Sigma-Aldrich	none	positive control – all targets methylated	none

* Indicated CNA applies to genomic sequence targeted by MGMT MS-MLPA probes: *MGMT* promoter region, however, the whole extent of CNA present in this cell line cannot be determined by this ME012-B1 MGMT-IDH-TERT probemix.
# "Methylated" refers to cases where MS-MLPA probes have ratios higher than baseline methylation.
π Digestion control probe at 208 nt is fully digested in this methylated control sample. The HhaI digestion of this probes is NOT dependent on the methylation state of the target DNA, as the GCGC site is located in the probe's stuffer sequence.
± due to SNP rs16906252, 187 nt MGMT probe 22670-L31908 shows reduced signal is in this sample.

SALSA Binning DNA SD094
The SD094 Binning DNA provided with this probemix can be used for binning of all probes including the six mutation-specific probes: *IDH1* probe 19529-L16492 at 203 nt (p.R132H=c.395G>A), *IDH1* probe 19926-L32919 at 232 nt (p.R132C=c.394C>T), *IDH2* probe 20643-L32911 at 151 nt (p.R172K=c.515G>A), *IDH2* probe 20643-L32910 at 154 nt (p.R172M=c.515G>T), *TERT* probe S1295-L32988 at 127 nt (C250T) and *TERT* probe S1309-L32882 (C228T) at 146 nt. SD094 Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequences detected by the above mentioned probes. Inclusion of one reaction with 5 µl SD094 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. 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 SD094 Binning DNA product description, available online: www.mrcholland.com.
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.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 this criterion is 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/gain	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication/gain	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 or in or near the *MGMT* and *TERT* genes. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MS-MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Digestion Control Probes. The target sequences of the digestion control probes are unmethylated in most blood-derived DNA samples. The signals of the digestion control probes should be gone upon complete digestion by HhaI.
- mRNA levels. We have no data showing that methylation detected by a particular probe indeed influences the corresponding mRNA levels.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.

- False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: a lower injection voltage or a shorter injection time, or a reduced amount of sample by diluting PCR products.

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

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

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

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

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

To determine if a test sample has a significantly increased methylation level for a particular probe, compare the methylation ratio of the probe with the baseline level.
- Methylation ratio of a probe in test sample > baseline: methylation is increased.
- Methylation ratio of a probe in test sample ≤ baseline: methylation is *not* increased.

For obtaining final ratios in Coffalyser.Net, please check https://www.mrcholland.com/r/me012/final-ratios in MRC Holland Support Help Centre.

Interpretation of methylation positive samples is dependent on the application used. For example, in glioblastoma, 8% (average of five CpGs (Quillien et al. 2012)) and 12.5% (average of four CpGs (Yuan et al. 2017)) were indicated to be the optimal threshold for defining *MGMT* baseline methylation by using pyrosequencing.

Note: In case digestion control probes are not fully digested (>0.10), please contact info@mrcholland.com for more information.

ME012 specific notes:
- Please note that two MGMT probes have multiple HhaI restriction sites. All of these sites need to be methylated in the DNA template in order for the probes not to be digested.

- *MGMT* probes 20118-L27105 at 139 nt, 14133-L32908 at 175 nt, 19736-L26793 at 190 nt and 23231-L32916 at 264 nt are not completely digested in DNA samples derived from blood and show 5-15% background signal after HhaI digestion. Low methylation ratios obtained with these probes should be treated with caution.
- The CpG site at chr10:130966742 (hg38) is targeted by two *MGMT* probes: 20118-L27105 at 139 nt and 23231-L32916 at 264 nt. However, the 139 nt probe additionally targets two other CpG sites (see Table 2). Thus, for methylation status determination of the CpG at chr10:130966742, the 264 nt probe is more suitable.
- Probes with consecutive lengths might become small (2 nts) when the fragment separation is performed on Beckman instrument, and thus the users are encouraged to ensure that the peak binning is correctly done.

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. 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 database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report false positive results due to SNVs and unusual to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix ME012-B1 MGMT-IDH-TERT

Length (nt)	SALSA MLPA probe	HhaI site	MS-MLPA probes	Mutation-specific probes	Chromosomal position (hg18)	
			expected signal reduction[a]	mutation details	target probes	reference/ other probes
64-105	Control fragments – see table in probemix content section for more information					
123 *	Reference probe 18709-L13645	-				5q31
127 * § ◊	**TERT probe** S1295-L32988	-		C250T	5p15.33	
139 ¥ Δ ‡ ∧	**MGMT probe** 20118-L27105	+	95-100%		10q26.3	
146 * § Δ	**TERT probe** S1309-L32882	-		C228T	5p15.33	
151 ¥ §	**IDH2 probe** 20643-L32911	-		p.R172K=c.515G>A	15q26.1	
154 ¥ §	**IDH2 probe** 20643-L32910	-		p.R172M=c.515G>T	15q26.1	
160 *	Reference probe 19970-L27032	-				4p16
165 ¥	**MGMT probe** 19735-L32914	+	100%		10q26.3	
171 ¥	Reference probe 10904-L32913	-				9q34
175 ¥ ∧	**MGMT probe** 14133-L32908	+	90-95%		10q26.3	
184 ¥	Reference probe 04857-L32200	-				5p13
187 * ±	**MGMT probe** 22670-L31908	+	100%		10q26.3	
190 ∧	**MGMT probe** 19736-L26793	+	95-100%		10q26.3	
197	Reference probe 06937-L29022	-				11q12
203 § +	**IDH1 probe** 19529-L16492	-		p.R132H=c.395G>A	2q33.3	
208 π	Digestion control probe 19490-L25113	+	100%			2q12
215 ๑	**MGMT probe** 12250-L27780	+	100%		10q26.3	
222 *	Reference probe 19781-L28158	-				1q25
232 ¥ §	**IDH1 probe** 19926-L32919	-		p.R132C=c.394C>T	2q33.3	
238	Reference probe 18055-L22445	-				16q23
242 ¥ Δ ‡	**MGMT probe** 23256-L32917	+	100%		10q26.3	
251	Reference probe 06712-L29006	-				15q24
256 * π	Digestion control probe 22932-L32372	+	100%			3p14
264 * ∧	**MGMT probe** 23231-L32916	+	85-90%		10q26.3	
269 ¥	Reference probe 16433-L32907	-				18q21
277	Reference probe 13796-L24815	-				3q25
285	Reference probe 19493-L29004	-				3p12
294	Reference probe 13393-L28788	-				6q12
303 * ∫	Depurination-sensitive probe 05697-L05139	-				12q24
310 π	Digestion control probe 17305-L29021	+	100%			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.

* New in version B1.

¥ Changed in version B1. Minor alteration, no change in sequence detected.

§ Mutation-specific probe. This probe will only generate a signal when the indicated mutation is present. It has been tested on artificial DNA and on cancer cell lines **but not on positive human samples!**

◊ Signal of this probe on cancer cell line samples with reported one mutant allele is approximately third of average reference probe signal.

± Target sequence of this probe contains SNP rs16906252 (C/T) in the GCGC site, 7 nt left from ligation site. When the T-allele of this validated SNP (with an allele frequency of 6.7%) is present, HhaI digestion will not occur, resulting in a false methylation positive signal. In addition, T-allele of this SNP may result in signal reduction in undigested reaction, therefore, 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.

π 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.

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

+ 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 (SD094), which is provided with each vial of ME012 probemix. Instructions to create a manual binset can be found in MRC Holland Support Help Centre: https://support.mrcholland.com/

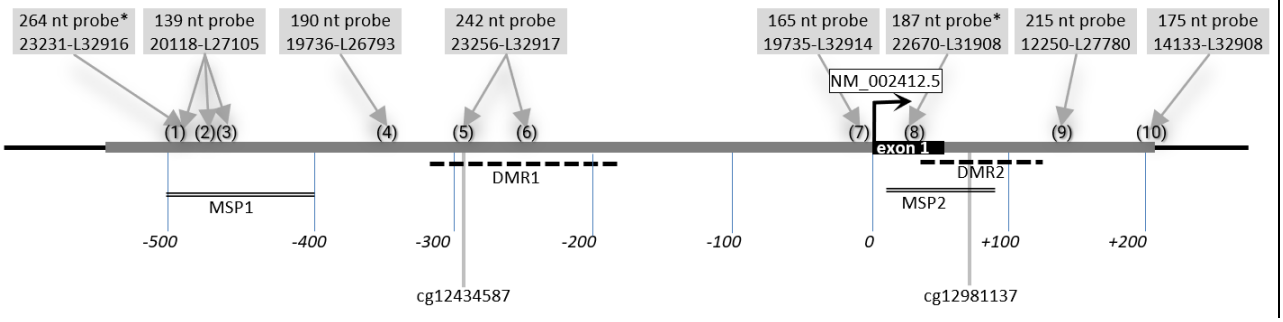
∫ Reduced signal of this probe indicates that sample DNA is possibly depurinated. An extremely low signal of this probe might indicate a very poor sample DNA quality; please critically review your MLPA results in this case.

ɷ Target sequence of this probe contains SNP rs186050433 (C/T) in the GCGC site. When the T-allele of this validated SNP (with an allele frequency of 0.59%) is present, HhaI digestion will not occur, resulting in a false methylation positive signal.

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 table above. Single probe aberration(s) must be confirmed by another method.

Table 2. ME012-B1 MS-MLPA probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Ligation site[a]	Complete sequence	CpG location[b]
MGMT, at 10q26.3. Ligation sites are according to NM_002412.5. Two methylation hot spots in the promoter region of the *MGMT* gene are suggested to denote silencing of the *MGMT* gene (Qian and Brent. 1997); these hot spots overlap with two distinctly methylated regions (DMR1 and DMR2) identified in glioblastoma cells and xenografts (Malley et al, 2011). Two CpG sites within these regions (cg12434587 and cg12981137) were shown to have a prognostic value and a strong association with patient survival (Bady et al. 2012). This ME012-B1 probemix includes eight MS-MLPA probes targeting CpG sites within and surrounding the above mentioned regions. The probe at 242 nt covers the cg12434587; the cg12981137 is not within an HhaI recognition site and thus is not targeted by an MS-MLPA probe in this ME012 probemix. Please see figure below for information on the location of the targeted GCGC-sites of methylation-specific probes in the promoter region of the *MGMT* gene. Note: the CpG site (1) targeted by 264 nt probe is also in the target sequence of 139 nt probe. However, the 139 nt probe additionally targets two other CpG sites, thus for methylation status determination of the CpG site (1) the 264 nt probe is more suitable.				
264 Λ	23231-L32916	520 nt before exon 1	GCTCAGCGTAGCCGCCCCGAGCAGGA-CCGGGATTCTC ACTAAGCGGGCGCCGTCCTACATA	chr10:129,466,742 (1)
139 Δ ‡ Λ	20118-L27105	457 nt before exon 1 *reverse*	GCGTGCAAGCGACCTGCCACGT-GCCCGAGTGGTCCTGA AAGCGCGCGGGGGTCGTAGGACGGCGCCCGCTTAGTGAGA	chr10:129,466,742 (1) chr10:129,466,761 (2) chr10:129,466,763 (3)
190 Λ	19736-L26793	383 nt before exon 1	CCTGTGACAGGAAAAGGTACGGGCCATTTGGCAAACTAAG-GCACAGAGCCTCAGGCGGAAGCTGG GAAGGCGCC GCCCGGCTTGTACCGG	chr10:129,466,888 (4)
242 Δ ‡	23256-L32917	263 nt before exon 1 *reverse*	CTGAGGCAGTCTGCGCATCCTCGCTGGA-CGCCGGCAC GCTGGCCCTGGTCCTCCGGCAGCGCCGCTGCCCTG	chr10:129,466,945 (5) chr10:129,466,991 (6)
165	19735-L32914	7-6 *reverse*	ACCGCGAGGACCTGCGGGCGTCGGGACGCAA-AGCGTTCTAGGGGCGCGGGCTGTCCCAGCATATCCGG	chr10:129,467,232 (7)
187 ±	22670-L31908	47-48	GTCCTCGCGGTGCGCACCGTT-TGCGACTTGGTGAGTG TCTGGGTCGCCTCGCTCC	chr10:129,467,279 (8)
215 ɷ	12250-L27780	73 nt after exon 1	CTCGGGACGGTGGCAGCCTCGAGTGGT-CCTGCAGGCGCCCTCACTTCGCCGTCGGGTGT	chr10:129,467,378 (9)
175 Λ	14133-L32908	154 nt after exon 1 *reverse*	AGAAAGGCTGGGCAACACCTGGGAGGCACTT-GGGGCGCACCTGGAGCTCGCCCGGGATGGGT	chr10:129,467,445 (10)



→	Locations of individual CpG sites within HhaI recognition sites targeted by MGMT probes: (1)-(10).
▬	CpG island in *MGMT* promoter region.
- - - - -	Methylation hotspots/DMR1 and DMR2 (Qian and Brent. 1997; Malley et al. 2011).
═	MSP1 and MSP2 amplicons according to van Nifterik et al. 2010.
*	New GCGC-sites targeted by this probemix from ME012-B1 version onwards.

[a] See section Exon numbering on page 2 for more information.

[b] Genomic coordinates (hg38) indicate the location of the first C of CpG site(s) within HhaI recognition sites targeted by MGMT probes. Numbers in the brackets are used to enumerate the CpG sites targeted by MS-MLPA probes in ME012-B1. The HhaI sites are marked with grey. Ligation sites are marked with –.

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

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

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

± Target sequence of this probe contains SNP rs16906252 (C/T) in the GCGC site, 7 nt left from ligation site. When the T-allele of this validated SNP (with an allele frequency of 6.7%) is present, HhaI digestion will not occur, resulting in a false methylation positive signal. In addition, T-allele of this SNP may result in signal reduction in undigested reaction, therefore, in case of apparent deletions, it is recommended to sequence the region targeted by this probe.

ⓟ Target sequence of this probe contains SNP rs186050433 (C/T) in the GCGC site. When the T-allele of this validated SNP (with an allele frequency of 0.59%) is present, HhaI digestion will not occur, resulting in a false methylation positive signal.

Notes:

- Please be aware that two MGMT probes have multiple HhaI restriction sites. All of these sites need to be methylated in the template in order for the probe not to be digested.
- 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. ME012-B1 Mutation-specific probes

Length (nt)	SALSA MLPA probe	Exon[a]	Ligation site	Mutation details	Partial sequence (24 nt adjacent to ligation site)
IDH1, at 2q33.3. Ligation sites are according to NM_005896.4. The probes at 202 nt and 230 nt will only give a signal when respectively the p.R132H and p.R132C mutation is present in the sample.					
203 +	19529-L16492	6	618-619	p.**R132H**=c.395G>A	CATCATAGGTC**A**-TCATGCTTATGG
232	19926-L32919	6	617-616 *reverse*	p.**R132C**=c.394C>T	ATAAGCATGAC**A**-ACCTATGATGAT
IDH2, at 15q26.1. Ligation sites are according to NM_002168.4. The probes at 151 nt and 154 nt will only give a signal when respectively the p.R172K and p.R172M mutation is present in the sample.					
154	20643-L32910	5	593-594	p.**R172M**=c.515G>T	CACCATTGGCA**T**-GCACGCCCATGG
151	20643-L32911	5	593-594	p.**R172K**=c.515G>A	CACCATTGGCA**A**-GCACGCCCATGG
TERT, at 5p15.33. Ligation sites are according to NM_198253.3. The probes at 146 nt and 127 nt will only give a signal when respectively the C228T and C250T mutation is present in the sample. These mutations are also known with other names in literature and databases, with the most common ones being NM_198253.2: c.1-124C>T or C>T at chr5:1295228 (hg19, reverse strand) for C228T; and NM_198253.2: c.1-146C>T or C>T at chr5:1295250 (hg19, reverse strand) for C250T.					
146 Δ	S1309-L32882	1	45 nt before exon 1	**C228T**	GAGGGCCCGGA**A**-GGGGCTGGGCGG
127 ◊	S1295-L32988	1	67 nt before exon 1	**C250T**	CGGGGACCCGG**A**-AGGGGTCGGGAC

[a] See section Exon numbering on page 2 for more information.

+ This probe might show very low unspecific background signal in wild-type samples. This background signal at 202 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 (SD094), which is

provided with each vial of the ME012 probemix. Instructions about how to create a manual binset can be found in the MRC Holland Support Help Centre: https://support.mrcholland.com/

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

◊ Signal of this probe on cancer cell line samples with reported one mutant allele is approximately third of average reference probe signal.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Table 4. ME012-B1 reference and digestion control probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene	Chromosomal position (hg18)	Location (hg18) in kb
222	19781-L28158	*NCF2*	1q25	01-181,803
208 π	19490-L25113	*SLC9A2*	2q12	02-102,641
256 π	22932-L32372	*FLNB*	3p14	03-057,969
285	19493-L29004	*GBE1*	3p12	03-081,775
277	13796-L24815	*KCNAB1*	3q25	03-157,716
160	19970-L27032	*EVC2*	4p16	04-005,684
184	04857-L32200	*NIPBL*	5p13	05-037,080
123	18709-L13645	*IL4*	5q31	05-132,038
317	11898-L24065	*PKHD1*	6p12	06-052,039
294	13393-L28788	*EYS*	6q12	06-064,546
310 π	17305-L29021	*ESCO2*	8p21	08-027,688
171	10904-L32913	*SETX*	9q34	09-134,200
197	06937-L29022	*BEST1*	11q12	11-061,481
251	06712-L29006	*HEXA*	15q24	15-070,436
238	18055-L22445	*PLCG2*	16q23	16-080,527
269	16433-L32907	*MYO5B*	18q21	18-045,743

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

Note: The digestion control probes at 208, 256 and 310 nt should provide no, or a very low (<10%) signal in digested samples. HhaI digestion of the 256 and 310 nt probes 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. Rare cases have been observed where the 256 and 310 nt probes generate a signal while the probe at 208 nt does not. In such cases, complete digestion might have been hampered by sample DNA methylation at that site, e.g. in tumour derived hypermethylated DNA, by impurities affecting HhaI digestion, or by a rare SNP in the digestion site. In these cases information obtained with 256 and/or 310 nt digestion control probes should be ignored.

Related SALSA MLPA probemixes

P088 Oligodendroglioma 1p-19q Contains probes for copy number detection of 1p and 19q chromosomal regions and for *IDH1* (p.R132C, p.R132H) and *IDH2* (p.R172K, p.R172M) point mutation detection.

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

P370 BRAF-IDH1-IDH2 Contains probes for *BRAF*, *FGFR1*, *MYB* and *MYBL1* genes and the 9p21.3 region, for duplications leading to *SRGAP3-RAF1*, *KIAA1549-BRAF* and *FGFR1-TACC1* fusions, 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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Selected publications using SALSA MLPA Probemix ME012 MGMT-IDH-TERT

- Mäki-Nevala S et al. (2021) Immunoprofiles and DNA Methylation of Inflammatory Marker Genes in Ulcerative Colitis-Associated Colorectal Tumorigenesis. *Biomolecules*. 11:1440.
- Afkhami M et al. (2016) Detection of MGMT promoter methylation in malignant gliomas. *Journal of Clinical Oncology*. 34:e23131-e.

ME012 product history	
Version	*Modification*
B1	Two MS-MLPA probes for *MGMT* promoter, two mutation-specific probes for *TERT* promoter and one digestion control probe have been added; depurination-sensitive and the majority of reference probes have been replaced.
A1	First release.

Implemented changes in the product description

Version B1-02 – 16 May 2023 (04M)
- Minor textual change throughout the document: “MS-MLPA probemix” replaced with “MLPA probemix”.
- Added a footnote regarding 127 nt TERT probe signal in Table 1 and 3: “Signal of this probe on cancer cell line samples with reported one mutant allele is approximately third of average reference probe signal.”
- Adjusted *MGMT* methylation status information for DK-MG cell line in Positive sample table on page 4.
- Added a new reference on page 12.

Version B1-01 – 02 May 2023 (04M)
- Product description adapted to a new template and new product version (version number changed, changes in Table 1 and Table 2).
- Probemix name has changed to “MGMT-IDH-TERT” (was “MGMT-IDH1-IDH2”).
- General information section on page 1 rewritten.
- SALSA Binning DNA paragraph rewritten because a new SD is supplied with ME012-B1 version (SD054 is replaced with SD094).
- Various minor textual or layout changes.
- Table structure rearranged and content is modified (Tables 2a, 2b and 3 modified to Table 2, 3 and 4).
- MGMT probe figure has been moved to Table 2 and was adapted to the new probemix content.
- Small changes of probe lengths in Table 1 and 2, and throughout the document in order to better reflect the true lengths of the amplification products.
- Positive samples section on pages 3 and 4 is completely re-written with inclusion of new samples and with merging the methylation, copy number and point mutation information into one table.
- ME012 specific notes modified.
- Added information about final ratio display in Coffalyser.Net in the Interpretation of methylation results on blood and tissue derived DNA samples section.

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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