

Product Description

SALSA® MS-MLPA® Probemix ME042-C2 CIMP

To be used with the MS-MLPA General Protocol.

Version C2

For complete product history see page 13.

This SALSA MS-MLPA probemix is for basic research and intended for experienced MLPA users only! This probemix enables you to detect aberrant methylation of CpG islands around the transcription start site of eight genes for which an altered methylation status in the CpG Island Methylator Phenotype (CIMP) has been reported in literature. Interpretation of results obtained with this product can be complicated. MRC-Holland cannot provide assistance with data interpretation. Sharing interesting findings as well as suggestions from specialists for improvement of this product or product description are highly appreciated.

Catalogue numbers:

- **ME042-025R:** SALSA MS-MLPA Probemix ME042 CIMP, 25 reactions.
- **ME042-050R:** SALSA MS-MLPA Probemix ME042 CIMP, 50 reactions.
- **ME042-100R:** SALSA MS-MLPA Probemix ME042 CIMP, 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.

MS-MLPA probes targeting promoter regions of the *SOCS1* and *IGF2* genes are sensitive to certain experimental variations, therefore findings of copy number alterations should be treated with caution and confirmed by other methods if possible.

General information

The SALSA MS-MLPA Probemix ME042 CIMP is a **research use only (RUO)** assay for the detection of aberrant methylation of one or more sequences in the promoter regions of the *CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1* genes. This probemix can also be used to detect deletions/duplications in the promoter regions aforementioned *genes*, and the presence of the *BRAF* p.V600E (c.1799T>A) point mutation 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. The *CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1* genes are frequently silenced by methylation in CpG Island Methylator Phenotype (CIMP) tumours, but are unmethylated in blood-derived DNA, with the exception of constitutional epimutations. In addition, DNA methylation analysis can indicate in some cases from which type of tissue the tumour was derived.

CIMP tumours are characterized by the hypermethylation of promoters mainly in tumour suppressor genes and DNA repair genes, which leads to genetic silencing and a loss of protein expression. CIMP is a typical

feature for a subset of colorectal cancers, where also *BRAF* mutations are commonly found (Weisenberger et al. 2006). CIMP status was also identified in glioblastoma, gastric cancer, endometrial and breast cancers (reviewed in Weisenberger (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>
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>

Locus Reference Genomic (LRG) database: <http://www.lrg-sequence.org/>

PhenoGram Plot: <http://visualization.ritchielab.org/phenograms/plot>

Exon numbering

The exon numbering used in this ME042-C2 CIMP product description is the exon numbering from the sequence of NG_032024.1 for *CACNA1G*, of NM_004378.3 for *CRABP1*, of LRG_1031 for *IGF2*, of LRG_216 for *MLH1*, of NM_006161.3 for *NEUROG1*, of NM_001031680.2 for *RUNX3* and of NM_003745.2 for *SOCS1*. **From product description version C2-03 onwards, the exon numbering from the MANE transcripts is used for *CDKN2A*.** Consequently, for *CDKN2A*, the exon numbering has been changed: NM_000077.5 (MANE Select) encoding p16INK4A and NM_058195.4 (MANE Plus Clinical) encoding p14ARF are used. Both NM_000077.5 and NM_058195.4 have distinct first exons (both numbered as exon 1) which contain the translation start codon, and share a common second exon, which is translated in different reading frames (see schematic presentation below). The exon numbering (LRG_11 for *CDKN2A*), used in previous versions of this product description, can be found in between brackets in the Table 2. **Please be aware that the MANE and LRG exon numbering do not always correspond, and MANE exon numbering used here may differ from literature.** The exon numbering of the NG/NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG sequences. As changes to the databases can occur after release of this product description, the NG/NM_ sequence and exon numbering may not be up-to-date.

Probemix content

The SALSA MS-MLPA Probemix ME042-C2 CIMP contains 49 (MS-)MLPA probes with amplification products between 124 and 492 nucleotides (nt). 31 MS-MLPA probes contain an HhaI recognition site and provide information on the methylation status of promoter regions of *CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1* genes. All probes present will also give information on copy number changes in the analysed sample. In addition, 15 reference probes are included that are not affected by HhaI digestion and target relatively copy number stable regions in various cancer types. Also, two digestion control probes are included in this probemix indicating whether or not restriction endonuclease digestion in the MS-MLPA reaction was complete. Complete probe sequences and the identity of the genes detected by the reference probes are available online and in Table 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 cancer. 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 NA06226, NA08039, NA05067, NA02819 and NA13031 from the Coriell Institute and 697 (ACC-042), CADO-ES1 (ACC-255), DU-4475 (ACC-427), HCT-116 (ACC-581), REH (ACC-022) and SU-DHL-8 (ACC-573) from Leibniz Institute DSMZ have been tested with this ME042-C2 probemix at MRC Holland and can be used as a positive control samples to detect copy number and methylation alterations as well as the presence of *BRAF* p.V600E point mutation, as described in the table below. The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Source	Expected copy number alteration*	Expected methylation status of CpGs targeted by MS-MLPA probes in ME042-C2#	<i>BRAF</i> p.V600E mutation
NA02819	Coriell	Gain of <i>CDKN2A</i>	Methylated <i>IGF2</i>	-
NA05067	Coriell	Gain of <i>CDKN2A</i>	none	-
NA06226	Coriell	Gain of <i>SOCS1</i>	none	-
NA08039	Coriell	Gain of <i>SOCS1</i>	Methylated <i>IGF2</i>	
NA04127	Coriell	Gain of <i>MLH1</i>	none	-
NA13031	Coriell	Heterozygous deletion of <i>CACNA1G</i>	Methylated <i>CRABP1</i>	-

697 (ACC-042)	DSMZ	none	Methylated <i>RUNX3</i> , <i>NEUROG1</i> , <i>CDKN2A</i> , <i>IGF2</i> , <i>CRABP1</i> & <i>CACNA1G</i>	-
CADO-ES1 (ACC-255)	DSMZ	Homozygous deletion of <i>CDKN2A</i> and gain of <i>CACNA1G</i>	Methylated <i>NEUROG1</i> , <i>IGF2</i> & <i>CRABP1</i>	-
DU-4475 (ACC-427)	DSMZ	none	Methylated <i>RUNX3</i> , <i>MLH1</i> , <i>NEUROG1</i> , <i>CDKN2A</i> , <i>IGF2</i> , <i>CRABP1</i> & <i>CACNA1G</i>	detected
HCT-116 (ACC-581)	DSMZ	Gain of <i>CACNA1G</i>	Methylated <i>NEUROG1</i> , <i>CDKN2A</i> , <i>IGF2</i> , <i>CRABP1</i> & <i>CACNA1G</i>	-
REH (ACC-022)	DSMZ	Heterozygous deletion of <i>MLH1</i> , homozygous deletion of <i>CDKN2A</i> and gain of <i>SOCS1</i>	Methylated <i>RUNX3</i> , <i>NEUROG1</i> , <i>IGF2</i> & <i>CRABP1</i>	-
SU-DHL-8 (ACC-573)	DSMZ	none	Methylated <i>RUNX3</i> , <i>NEUROG1</i> , <i>CDKN2A</i> , <i>IGF2</i> , <i>CRABP1</i> & <i>CACNA1G</i>	-

*Indicated alteration refers to sequences targeted by (MS-)MLPA probes, however, the whole extent of copy number alteration present in this cell line cannot be determined by this ME042-C2 CIMP probemix.

"Methylated" refers to cases where half or more probes for the specific gene promoter have ratios higher than baseline methylation, the rest is not methylated and is not indicated in this table.

SALSA Binning DNA SD029

The SD029 Binning DNA provided with this probemix can be used for binning of all probes including the BRAF p.V600E (c.1799T>A) mutation-specific probe (226 nt probe 08780-SP0039-L08904). SD029 Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequence detected by the above mentioned probe. Inclusion of one reaction with 5 µl SD029 Binning DNA in initial MLPA experiments is essential as it can be used to aid in data binning of the peak pattern using Coffalyser.Net software. Furthermore, Binning DNA should be included in the experiment whenever changes have been applied to the set-up of the capillary electrophoresis device (e.g. when capillaries have been renewed). Binning DNA should never be used as a reference sample in the MLPA data analysis, neither should it be used in quantification of a mutation signal. It is strongly advised that all samples tested are extracted with the same method and derived from the same source of tissue. For further details, please consult the SD029 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.

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 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.

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.

ME042 specific notes:

- Please note that several probes have multiple HhaI restriction sites. All of these sites need to be methylated in order to not be digested!
- In 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.
- Please note that due to high nucleotide sequence similarity of mutated V600E (GTG to GAG single nucleotide variation) and V600K (GTG to AAG double nucleotide variation) codons, the BRAF V600E probe included in this probemix might give a small signal on a sample with V600K mutation.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *CACNA1G*, *CDKN2A*, *MLH1*, *RUNX3* and *SOCS1* genes are small (point) mutations, most of which will not be detected by using SALSA MS-MLPA Probemix ME042 CIMP.
- 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: <https://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 false positive results due to SNVs and unusual results (e.g., a duplication of two or more non-consecutive *NEUROG1* promoter sequences targeted by ME042 probes) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MS-MLPA Probemix ME042-C2 CIMP

Length (nt)	SALSA MLPA probe	Hhal site	% expected signal reduction ^a	Chromosomal position (hg18)	Mutation-specific probe
64-105	Control fragments – see table in probemix content section for more information				
124	Reference probe S0864-L26216	-		21q22	
132 Δ‡	MLH1 probe 15288-L32721	+	100%	3p22.2	
142 Δ«‡	IGF2 probe 13984-L15560	+	90%	11p15.5	
151	Reference probe 07241-L26836	-		3p11	
156 Δ	SOCS1 probe 15034-L27192	+	100%	16p13.13	
161 Δ	Reference probe 08222-L07936	-		10q26	
166 ^	NEUROG1 probe 13987-L15563	+	90%	5q31.1	
172 «^	IGF2 probe 16653-L19928	+	95%	11p15.5	
178	MLH1 probe 13988-L19727	+	100%	3p22.2	
184	CDKN2A probe 11869-L27058	+	100%	9p21.3	
190	Reference probe 08262-L08125	-		2p22	
195	CDKN2A probe 14001-L00962	+	100%	9p21.3	
201	NEUROG1 probe 10126-L13261	+	100%	5q31.1	
206	CRABP1 probe 13989-L15565	+	100%	15q25.1	
212 ^	NEUROG1 probe 13369-L16567	+	90%	5q31.1	
218	CACNA1G probe 13990-L15566	+	100%	17q21.33	
226 §Ж	BRAF probe 08780-SP0039-L08904	-		7q34	p.V600E (c.1799T>A)
232 Đ	CDKN2A probe 14003-L16397	+	100%	9p21.3	
238 ‡Đ	SOCS1 probe 15035-L27059	+	100%	16p13.13	
244	Reference probe 18664-L24018	-		11p14	
250 Đ	CACNA1G probe 10123-L26834	+	100%	17q21.33	
258 «	RUNX3 probe 13992-L26835	+	100%	1p36.11	
265 ^Đ	CRABP1 probe 13993-L15569	+	95%	15q25.1	
273	CACNA1G probe 10122-L16074	+	100%	17q21.33	
283 ^	NEUROG1 probe 13994-L15570	+	90%	5q31.1	
292	Reference probe 15724-L17704	-		12q12	
300 Δ‡Đ	SOCS1 probe 15037-L16786	+	100%	16p13.13	
310 Đ	CRABP1 probe 13996-L15572	+	100%	15q25.1	
318 ^‡	CRABP1 probe 13997-L15573	+	95%	15q25.1	
328	Reference probe 04007-L03430	-		2q33	
337 *π	Digestion control probe 20703-L27753	+	100%	2q12	
341 ‡‡	CDKN2A probe 14004-L32668	+	100%	9p21.3	
349 ¥«Đ	RUNX3 probe 14005-L32666	+	100%	1p36.11	
355 ¥	MLH1 probe 21553-L32720	+	100%	3p22.2	
364 ^	NEUROG1 probe 14007-L10468	+	90%	5q31.1	
371 Δ«Đ	RUNX3 probe 11131-L16234	+	100%	1p36.11	
382	Reference probe 00973-L00560	-		10q21	
391 ^	NEUROG1 probe 13998-L27056	+	90%	5q31.1	
399 Δ	SOCS1 probe 14393-L16073	+	100%	16p13.13	
407	Reference probe 01237-L24913	-		10p14	
418 Δ«^+	IGF2 probe 14000-L15576	+	95%	11p15.5	
427	Reference probe 15730-L17710	-		2q11	
436 *π«Δ	Digestion control probe 09167-L09460	+	100%	11q13	
445	Reference probe 15733-L17713	-		1p13	
454	Reference probe 10685-L11267	-		6p12	
463 ‡+	MLH1 probe 02260-L01747	+	100%	3p22.2	
475 +	Reference probe 13594-L15051	-		19p13	
484	Reference probe 09682-L10062	-		3p25	
492	Reference probe 14883-L27057	-		14q11	

- ^a Expected signal reduction on blood DNA derived samples. On other tissue or tumour derived samples these percentages can be different.
- * New in version C2.
- ¥ Changed in version C2. Minor alteration, no change in sequence detected.
- § Mutation-specific probe. This probe will only generate a signal when the *BRAF* p.V600E (c.1799T>A) mutation is present. It has been tested on artificial DNA and on cell line DU-4475 (ACC427), **but not on positive human samples!** Please note that this probe might give a small signal on a sample with the *BRAF* p.V600K mutation.
- Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.
- « 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.
- Ð This probe is sensitive to overdigestion, which can occur for example in case of lower ligation-digestion temperature (<48°C).
- Λ This probe is not completely digested in DNA samples derived from blood.
- Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.
- ‡ This probe contains two 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 may show increased copy number ratios if experimental deviations lead to higher reaction volumes during overnight hybridization.

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 2. ME042-C2 target probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene	Ligation site ^a	Location (hg18) in kb	Complete sequence
349 «Ð	14005-L32666	<i>RUNX3</i>	NM_001031680.2; 670-671	01-025,129	CGATGGTGGACGTGCTGGCGGACCACGCA-GGCG AGCTCGTGGCGACCACAGCCCCAACTTCTCT
371 Δ«Ð	11131-L16234	<i>RUNX3</i>	NM_001031680.2; 16 nt before exon 2 reverse	01-025,129	GCTTGGGTCTACGGGAATACGCAT- AACAGCGGCCGTGAGGCGCGCGGGCAGGCGGA
258 «	13992-L26835	<i>RUNX3</i>	NM_001031680.2; 695 nt before exon 2 reverse	01-025,130	TGCTAGAAATTTGCTTAGAACGTCCGGGTC- CCACGGAAGGCGCCTTGCCGCCCTCTCT
355	21553-L32720	<i>MLH1</i>	NM_000249.4; 628 nt before exon 1	03-037,009	TCCGCCACATACCCTCGTAGTAT-TCGTGCTCA GCCTCGTAGTGGCGCCTGACGTGCGGTT
463 +‡	02260-L01747	<i>MLH1</i>	NM_000249.4; 352 nt before exon 1 reverse	03-037,010	CTGCTGAGGTGATCTGGCGCAGA-GCGGAGGAG GTGCTTGGCGCTTCTCAGGCTCCTCTCT
132 Δ‡	15288-L32721	<i>MLH1</i>	NM_000249.4; 215 nt before exon 1	03-037,010	GAGCGGACAGCGATCTTAACGCGCAA-GCGCA TATCCTTCTAGGTAGCGGGCAGTAGCCGCTCAGG
178	13988-L19727	<i>MLH1</i>	NM_000249.4; 93 nt after exon 1	03-037,010	GGACACGCCTCTTTGCCCGGGCAGA-GG CATGTACAGCGCATGCCACAACGGCGGAGGC
166 ^	13987-L15563	<i>NEUROG1</i>	NM_006161.3; 596-595 reverse	05-134,899	TGCGTCCAGGGCCGCGTTCAA-GTTGTGCATG CGTTGGCGCTCGCGATCGTTGGCCTTG
283 ^	13994-L15570	<i>NEUROG1</i>	NM_006161.3; 273-274	05-134,899	TGTCCGTCGGTCTGCACAGCGCAAC-GATGC CAGCCCGCCTTGAGACCTGCATCTCCGACCTC
212 ^	13369-L16567	<i>NEUROG1</i>	NM_006161.3; 183-182 reverse	05-134,899	GCCGCCAGGGCGCACTTACGT-TCCAACA GCCTGGGGTTGTTACTCTGTGCCAGTTGCGGG
391 ^	13998-L27056	<i>NEUROG1</i>	NM_006161.3; 47-48	05-134,899	CTGATCTGATCGCCGGCGACATCA- CTCAGGAGACCGGCCGGCGCGGTGCCCC
364 ^	14007-L10468	<i>NEUROG1</i>	NM_006161.3; 23 nt before exon 1	05-134,900	CCCATTGTTGCGCCGGGTACTTA-AGGGGTC CTGAGGCCAGTCGTGTGCCACACTCGGTGCT
201	10126-L13261	<i>NEUROG1</i>	NM_006161.3; 143 nt before exon 1 reverse	05-134,900	TCATCCCGTGCAAGCCCGGGTATTTGCATAA T-TTATGCTCGCGGGAGGCCCATCGCCCTC

226 § Ж	08780-SP0039-L08904	BRAF p.V600E c.1799T>A	NM_004333.6; 1985-1986 and 2025-2026	07-140,100	CCTTTACTTACTACACCTCAGATATATTTCTTCA TGAAG-ACCTCACAGTAAAAATAGGTGATTTTGG TCTAGCTACAGA- GAAATCTCGATGGAGTGGG TCCCATCAGTTTGAACAGTTGTCTGG
232 Đ	14003-L16397	CDKN2A	NM_000077.5 (p16 ^{INK4A}); 158-157 reverse	09-021,965	CACCTGGATCGGCCTCCGACCGTAAC-TATTCTG GTGCGTTGGGCAGCGCCCCCGCTCCAGCAGC
184	11869-L27058	CDKN2A	NM_000077.5 (p16 ^{INK4a}); 378 nt before exon 1 (2)	09-021,965	TTTTAACAGAGTGAACGCCTCAAACACGCCTT TGCT-GGCAGGCGGGGAGCGCGGCTGGGAG CAGGGAGGC
341 ‡	14004-L32668	CDKN2A	NM_058195.4 (p14 ^{ARF}); 92-93	09-021,984	GCAGGTTCTTGGTGACCCTCCGGA- TTCGGCGCGCTGCGGCCCGCGAGTGAG
195	14001-L00962	CDKN2A	NM_058195.4 (p14 ^{ARF}); 928 nt before exon 1	09-021,985	GGAAGAGGAAAGAGGAAGAAGCGCTCAGAT- GCTCCGCGGCTGTCTGGAAGTTAAAACCGAAA ATAAAAATGG
172 «^	16653-L19928	IGF2	NM_000612.6; 427 nt before exon 5; NM_001127598.3; 318 nt after exon 4	11-002,118	CAGCCCCAAGCCACCTGCATCTGCACTCA- GACGGGGCGCACCCGAGTGCAGCCTCCTGGTG
418 Δ+«^	14000-L15576	IGF2	NM_000612.6; 1517 nt before exon 5; NM_001127598.3; 37-38	11-002,119	CCACCGCCTGCCACAGAGCGTTCGATCGC- TCGCTGCCTGAGCTCCTGGTGCGCCCGCGGAC
142 Δ«^‡	13984-L15560	IGF2	NM_000612.6; 1730 nt before exon 5; NM_001127598.3; 176 nt before exon 3	11-002,119	GAAATTTCTCTCTAGCGTTGCCCAAACACA- CTTGGGTGCGCCGCGCCCTCAGGACGTGG
206	13989-L15565	CRABP1	NM_004378.3; 96-97	15-076,420	GCCACCATGCCAACTTCGCCGGCAC-CTGGA AGATGCGCAGCAGCGAGAATTCGACGAGCTGC
310 Đ	13996-L15572	CRABP1	NM_004378.3; 174 nt after exon 1, reverse	15-076,420	GCTGAACGCGTGGGTTCCGGGATCTCT-ACCA GCTTCTCCGAGACCCGGTGCCTGGGAGACAA
265 ^Đ	13993-L15569	CRABP1	NM_004378.3; 226-227	15-076,420	GTGGAGATCCGCCAGGACGGGGATCAG-TTCTA CATCAAGACATCCACCACGGTGCACCACCTG
318 ^‡	13997-L15573	CRABP1	NM_004378.3; 109 nt after exon 2	15-076,421	CCTTTGCAGCCTGTGGCGCGCCTTCT- TGCAGGGTGTGTACTGGCTGTTTGCAGAGGG GGTTTGTGCATCCTAG
238 ‡ Đ	15035-L27059	SOCS1	NM_003745.2; 438-439	16-011,257	ATTCTACTGGGGGCCCTGAGCGTGCACG- GGGCGCACGAGCGGCTGCGCGCCGAGCCCGT
156 Δ	15034-L27192	SOCS1	NM_003745.2; 102 nt before exon 2	16-011,257	ACTTGGTGTCCGTGCTCGCCCCCT- AGGGCCGGTCCGCCGGGAGCGCCGCCCT
399 Δ	14393-L16073	SOCS1	NM_003745.2; 200 nt after exon 1	16-011,257	CCTTTCTCCGGCCCTAGCCCAAATCGCCCA- GACCAGGCGCGGATCCAGCCTGGCCAGCAGGC GGCG
300 Δ‡Đ	15037-L16786	SOCS1	NM_003745.2; 23 nt before exon 1	16-011,258	CCAGCCCCGCTCCGAGCCGGTTAAA-AGA CTGGCGCAGGGGCGGGCGCGAACAGAGCGA
273	10122-L16074	CACNA1G	NM_018896.5; 455-456	17-045,994	GAGCCTGGGCGCGAAGCGAAGAA- GCCGGAACAAAGTGAGGGGGAGCCGGCCGGC
250 Đ	10123-L26834	CACNA1G	NM_018896.5; 705-704 reverse	17-045,994	CCCGGGCGATCCGGAGAGGGGCA-AGC GGCGCCCTCAGAGGAGGTGTCTCACGCAA
218	13990-L15566	CACNA1G	NM_018896.5; 926-925 reverse	17-045,994	GCGGCTGTCTGGCTCAAGTAGAAGAA- AACCACCGGGCCAGCGCGGGTACGGC

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

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

§ Mutation-specific probe. This probe will only generate a signal when the *BRAF* p.V600E (c.1799T>A) mutation is present. It has been tested on artificial DNA and on cell line DU-4475 (ACC427), **but not on positive human samples!** Please note that this probe might give a small signal on a sample with the *BRAF* p.V600K mutation.

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

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

Ð This probe is sensitive to overdigestion, which can occur for example in case of lower ligation-digestion temperature (<48°C).

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

Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

‡ This probe contains two 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 may show increased copy number ratios if experimental deviations lead to higher reaction volumes during overnight hybridization.

Table 3. ME042-C2 reference and digestion control probes arranged according to chromosomal location

Length (nt)	SALSA MS-MLPA probe	Gene	Chromosomal position (hg18)	Partial sequence (24 nt adjacent to ligation site)	Location (hg18) in kb
445	15733-L17713	<i>TRIM45</i>	1p13	CAGTAGTGGACA-TCCGAGGGGGAG	01-117,465
190	08262-L08125	<i>SPAST</i>	2p22	TCATTAGAATAC-AGGGAGCAGAGA	02-032,141
337 π	20703-L27753	<i>SLC9A2</i>	2q12	GGACACTTTGGA-ATTCCATTGGCA	02-102,641
427	15730-L17710	<i>PROM2</i>	2q11	GAACCTGTCGCA-ACTTCTTGGCCT	02-095,311
328	04007-L03430	<i>BMPR2</i>	2q33	AGATGAGACAAT-AATCATTGCTTT	02-203,087
151	07241-L26836	<i>POU1F1</i>	3p11	AGATCATGAGGA-TGGCTGAAGAAC	03-087,392
484	09682-L10062	<i>CAV3</i>	3p25	GCATCAGCCACA-TCTACTACTCT	03-008,762
454	10685-L11267	<i>PKHD1</i>	6p12	TCTGGCATCTAT-ATCTGCAGTCCC	06-051,876
407	01237-L24913	<i>UPF2</i>	10p14	TGCCATTCTTT-GCATCTCAAAG	10-012,019
382	00973-L00560	<i>TSPAN15</i>	10q21	CATCATCATGGA-GCACTCTGTAC	10-070,937
161 Δ	08222-L07936	<i>PLEKHA1</i>	10q26	ATGCGATTCTGA-CGATCCACATAA	10-124,143
244	18664-L24018	<i>ANO5</i>	11p14	AGAAACACTGGC-CTTGGGCTGTCC	11-022,258
436 Δ«π	09167-L09460	<i>MEN1</i>	11q13	GTGTGGGATGTA-AGCGCGGAGGTG	11-064,335
292	15724-L17704	<i>KIF21A</i>	12q12	TTGTTAGTGATC-AGGCCAACCATG	12-038,037
492	14883-L27057	<i>RPGRIP1</i>	14q11	TCTGAAGTCAGT-GAAGCACAAACT	14-020,873
475 +	13594-L15051	<i>CACNA1A</i>	19p13	ACTGGAGGAATG-GCAGCCCCTGGT	19-013,207
124	S0864-L26216	<i>KCNJ6</i>	21q22	AGCTCCTACATC-ACCAGTGAGATC	21-037,920

« 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.

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

+ This probe may show increased copy number ratios if experimental deviations lead to higher reaction volumes during overnight hybridization.

Complete probe sequences are available at www.mrcholland.com. Please notify us of any mistakes: info@mrcholland.com.

NOTE: The digestion control probes at 337 nt and 436 nt should provide no, or a very low (<10%) signal in digested samples. HhaI digestion of the 436 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 337 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 436 nt probe generates a signal while the probe at 337 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 436 nt digestion control probe should be ignored.

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.

Related SALSA MLPA probemixes

Condition		Gene	Probemix
Lynch syndrome (HNPCC)		<i>MLH1</i>	P003 MLH1/MSH2 P248 MLH1-MSH2 Confirmation ME011 Mismatch Repair Genes
		<i>MSH2</i>	P003 MLH1/MSH2 P248 MLH1-MSH2 Confirmation ME011 Mismatch Repair Genes
		<i>MSH6</i>	P072 MSH6-MUTYH ME011 Mismatch Repair Genes
		<i>PMS2</i>	P008 PMS2 ME011 Mismatch Repair Genes
		<i>EPCAM</i>	P003 MLH1/MSH2 P072 MSH6-MUTYH ME011 Mismatch Repair Genes
Polyposis syndrome	MAP	<i>MUTYH</i>	P378 MUTYH P043 APC P072 MSH6-MUTYH
	AFAP	<i>APC</i>	P043 APC
	FAP	<i>APC</i>	P043 APC
Various cancers types		<i>CDKN2A/CDKN2B</i>	ME024 9p21 CDKN2A/2B region
		tumour suppressor genes	ME001 Tumour suppressor mix 1
			P294 Tumour Loss

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ME042 product history	
<i>Version</i>	<i>Modification</i>
C2	One digestion control probe has been replaced and one added, and several probes have a change in length but not in the sequence detected.
C1	Length change for BRAF V600E mutation-specific probe from 409 nt to 226 nt. In addition, several reference probes have been added or replaced. Seven target probes and one reference probe have a small change in length, but no change in the sequence detected.
B2	The 88 and 96 nt control fragments have been replaced (QDX2).
B1	One MLH1 probe and three SOCS1 probes added. Five reference probes replaced and one added.
A1	First release.

Implemented changes in the product description

Version 05-C2– 22 August 2023 (04M)

- Added probe warning for BRAF mutation-specific probe 08780-SP0039-L08904 in Tables 1 and 2: possible small signal for BRAF V600E mutation probe on a sample with V600K mutation.

Version 04-C2– 1 February 2023 (04M)

- Corrected gene name typo in the table in the ' Positive control DNA samples' section.

Version 03-C2– 19 January 2023 (04M)

- Exon numbering of the *CDKN2A* gene has been changed according to MANE in 2. See also explanation on page 2.

- Modified the table in the ' Positive control DNA samples' section.

Version 02-C2– 17 May 2022 (04M)

- Added ME024, ME001 and P294 probemixes in Related SALSA MLPA probemixes section on page 11.

Version 01-C2– 28 January 2022 (04M)

- Product description rewritten and adapted to a new template.

- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).

- Various minor textual or layout changes.

- Ligation sites of the probes targeting the *CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1* genes updated according to new version of the NM_ reference sequence.

- References updated on page 11.

More information: www.mrcholland.com; www.mrcholland.eu

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