Product Description
SALSA® MLPA® Probemix
ME024-B3 9p21 CDKN2A/2B region

To be used with the MS-MLPA General Protocol.

Version B3
For complete product history see page 15.

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

Catalogue numbers:
- **ME024-025R:** SALSA MLPA Probemix ME024 9p21 CDKN2A/2B region, 25 reactions.
- **ME024-050R:** SALSA MLPA Probemix ME024 9p21 CDKN2A/2B region, 50 reactions.
- **ME024-100R:** SALSA MLPA Probemix ME024 9p21 CDKN2A/2B region, 100 reactions.

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

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

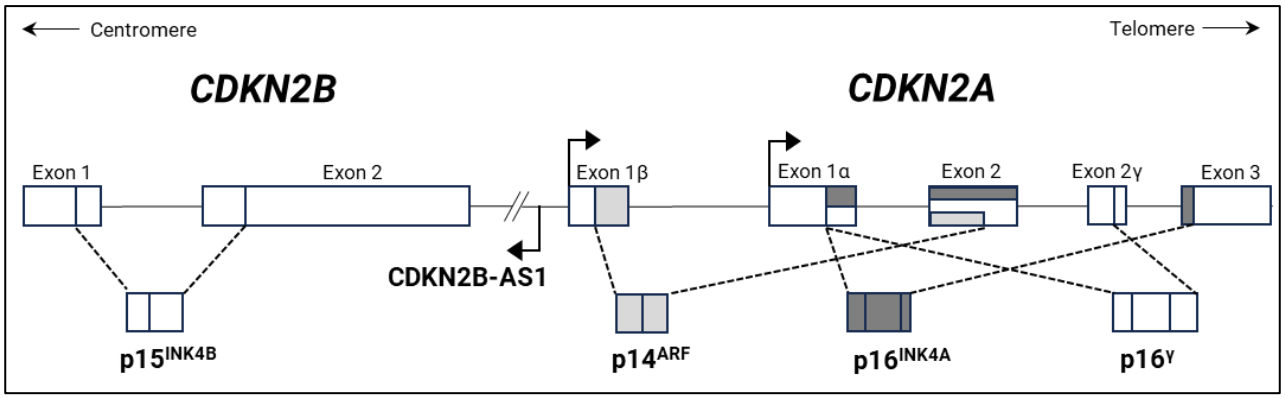
Precautions and warnings
For professional use only. Always consult the most recent product description AND the MS-MLPA General Protocol before use: www.mrcholland.com. It is the responsibility of the user to be aware of the latest scientific knowledge of the application before drawing any conclusions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

General information
The SALSA MLPA Probemix ME024 9p21 CDKN2A/2B region is a **research use only (RUO)** assay for the detection of aberrant methylation of one or more sequences of the *CDKN2A* and *CDKN2B* genes on chromosome band 9p21. This probemix can also be used to detect deletions/duplications in the aforementioned chromosomal region including *MIR31*, *MTAP*, *CDKN2A* and *CDKN2B* genes, and *PAX5* gene on 9p13.

Genomic losses of the 9p21.3 region, encompassing the *CDKN2A/2B* genes, are frequent events in many human cancers. This locus encodes three cyclin-dependent kinase inhibitors $p14^{ARF}$, $p15^{INK4B}$ and $p16^{INK4A}$ (see schematic presentation on page 2). Genomic deletion of one or both copies of these important cell cycle regulatory genes is the main inactivation mechanism in various cancers. *CDKN2A* deletion can extend to the *MTAP* gene, located 110 kb downstream. The *MTAP* gene encodes methylthioadenosine phosphorylase, an important enzyme for the salvage of both adenine and methionine. It is known that many tumour cells require addition of methionine to their growth medium, because their *MTAP* gene is co-deleted with *CDKN2A*. Cells lacking *MTAP* are expected to be sensitive to purine synthesis inhibitors and/or methionine starvation, and therefore homozygous co-deletion of the *CDKN2A* and *MTAP* genes might open possibilities for alternative treatment for cancer patients. Other genes that are frequently co-deleted with *CDKN2A/2B* are *CDKN2B-AS1*, *PAX5*, and microRNA 31 (*MIR31*). Loss of *MIR31* has been shown to have pro-tumorigenic effects on e.g. breast and ovarian cancer (Creighton et al. 2010). *CDKN2B-AS1* (non-protein coding *CDKN2B* antisense RNA 1) is suggested to act as an epigenetic silencer of the *CDKN2B* gene (Yu et al. 2008). The *PAX5* gene, at 9p13,

which is essential for normal B-cell lymphopoiesis, is frequently co-deleted with *CDKN2A* in B-ALL (Kim et al. 2011).
An alternative mechanism of inactivation of the *CDKN2A/2B* genes is hypermethylation of the promoter regions leading to lack of expression of $p14^{ARF}$, $p15^{INK4B}$ and $p16^{INK4A}$ proteins, which further results in uncontrolled cell proliferation and tumour development and progression (Wolter et al. 2001).
Alterations of the *CDKN2A/2B* genes have been also described at the germline level. Germline mutations in the *CDKN2A* gene are frequently associated with predisposition to malignant cutaneous melanoma and pancreatic cancer (Chan et al. 2021). Up to 40% of familial melanomas are associated with *CDKN2A* mutations (Hewitt et al. 2002), including point mutations and various intragenic deletions.

Schematic representation of *CDKN2A* and *CDKN2B* gene structure and encoded proteins



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): https://www.ncbi.nlm.nih.gov/refseq/MANE/
Tark – Transcript Archive: http://tark.ensembl.org/

Exon numbering
From product description version B3-03 onwards, the exon numbering from the MANE transcripts is used for *CDKN2A*, *CDKN2B*, *MTAP* and *PAX5* genes. ME024-B3 9p21 CDKN2A/2B region product description uses the exon numbering from NM_000077.5 for *CDKN2A*, NM_004936.4 for *CDKN2B*, NR_003529.3 for *CDKN2B-AS1*, NR_029505.1 for *MIR31*, NM_002451.4 for *MTAP* and NM_016734.3 for *PAX5*.

For *CDKN2A*, NM_000077.5 (MANE Select transcript) encoding $p16^{INK4A}$ and NM_058195.4 (MANE Plus Clinical transcript) encoding $p14^{ARF}$ are used. Both transcripts have distinct first exons (both numbered as exon 1, also known as 1α and 1β, respectively) which contain the translation start codon, and share a common second exon, which is translated in different reading frames (see schematic representation above). 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.

The exon numbering of the NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG and/or NG_ sequences. 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 ME024-B3 9p21 CDKN2A/2B region contains 48 (MS-)MLPA probes with amplification products between 124 and 500 nucleotides (nt). 10 MS-MLPA probes contain an HhaI recognition site and provide information on the methylation status of *CDKN2A* and *CDKN2B* promoter regions. All probes present will also give information on copy number changes in the analysed sample. In addition, 12 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/partial probe sequences and the identity of the genes detected by the reference probes are available in Table 2 and online (www.mrcholland.com).

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

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

MS-MLPA technique

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

MS-MLPA technique validation

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

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

Required specimens

Extracted DNA, which includes DNA derived from paraffin-embedded tissues, free from impurities known to affect MLPA reactions. For more information please refer to the section on DNA sample treatment found in the MS-MLPA General Protocol. More information on the use of FFPE tissue samples for MLPA can be found in Atanesyan et al. (2017).

Reference samples

A sufficient number (≥3) of reference samples should be included in each MS-MLPA experiment for data normalisation and to identify the baseline methylation level for each methylation-specific probe. All samples tested, including reference DNA samples, should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method when possible. When selecting reference samples, please note that methylation patterns may vary between tissue types and even age groups!

Reference samples should be derived from different healthy individuals 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 NA03226, NA05067, NA02819 and NA01750 from the Coriell Institute, and ACC-200 (COLO-824), ACC-29 (MOLT-16), ACC-255 (CADO-ES1), ACC-437 (MOLT-14), ACC-203 (SK-N-MC), ACC-573 (SU-DHL-8), ACC-42 (697), ACC-581 (HCT-116), ACC-511 (ALL-SIL), ACC-40 (CTV-1), ACC-713 (GRANTA-452), ACC-282 (JURKAT), ACC-742 (BALL-1), ACC-339 (MHH-CALL-3), ACC-22 (REH), ACC-508 (RS4;11) and ACC-578 (TOM-1) from the Leibniz Institute DSMZ, have been tested with this ME024-B3 probemix at MRC Holland and can be used as positive control samples to detect copy number alterations (CNAs) and methylation status of target genes located on 9p (see table below for details). The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Expected CNA◊ ~	Expected methylation status#	
		CDKN2A	***CDKN2B***
Germline samples from Coriell Institute			
NA03226	Heterozygous duplication of all targets on 9p	not methylated	
NA05067			
NA02819	Heterozygous duplication of 9p24.3-p21.3 including telomeric flanking probes, *MIR31*, *MTAP*, *CDKN2A*, *CDKN2B-AS1* and *CDKN2B*	not methylated	
NA01750			
Cancer cell line samples from Leibniz Institute DSMZ			
COLO-824 (ACC-200)	Gain of all targets on 9p	not methylated	
MOLT-16 (ACC-29)	Homozygous deletion of *CDKN2A*, *CDKN2B-AS1* and *CDKN2B*	not applicable	
CADO-ES1 (ACC-255)	Homozygous deletion of *MTAP*, *CDKN2A*, *CDKN2B-AS1* and *CDKN2B*	not applicable*	
MOLT-14 (ACC-437)		not applicable	
SK-N-MC (ACC-203)	none	not methylated	methylated
SU-DHL-8 (ACC-573)		NM_000077.5 methylated NM_058195.4 not methylated	methylated
697 (ACC-42)		NM_000077.5 methylated NM_058195.4 not methylated	methylated
HCT-116 (ACC-581)		NM_000077.5 methylated NM_058195.4 not methylated	not methylated
ALL-SIL (ACC-511)	Heterozygous deletion of *MIR31* and *MTAP* Homozygous deletion of *CDKN2A*, *CDKN2B-AS1* and *CDKN2B*	not applicable	
CTV-1 (ACC-40)	Homozygous deletion of *MTAP*, *CDKN2A and CDKN2B-AS1* Homozygous deletion of *CDKN2B* (partial: only 229 and 458 nt probe targets)	not applicable	methylated
GRANTA-452 (ACC-713)	Heterozygous deletion of flanking genes (*MLLT3* and *KLHL9*), *MIR31* and *PAX5* Homozygous deletion of *MTAP*, *CDKN2A*, *CDKN2B-AS1* and *CDKN2B*	not applicable	
JURKAT (ACC-282)	Homozygous deletion of flanking gene *KLHL9*, and *MIR31*, *MTAP*, *CDKN2A*, *CDKN2B-AS1*, *CDKN2B*	not applicable	
BALL-1 (ACC-742)	Heterozygous deletion of telomeric flanking probes, *MIR31*, *MTAP*, *CDKN2A* (partial: all except 265, 280 and 259 nt probe targets), *CDKN2B-AS1* and *CDKN2B* Homozygous deletion of *CDKN2A* (partial: only 265, 280 and 259 nt probe targets)	not methylated	
MHH-CALL-3 (ACC-339)	Heterozygous deletion of telomeric flanking genes, *MIR31*, *MTAP*, *CDKN2A* (partial: only 136, 157 and 273 nt probe targets), *CDKN2B* and *PAX5* Homozygous deletion of *CDKN2A* (partial: all except 136, 157 and 273 probe targets) and *CDKN2B-AS1*	not applicable	methylated

REH (ACC-22)	Homozygous deletion of flanking gene *KLHL9*, and *MIR31*, *MTAP*, *CDKN2A*, *CDKN2B-AS1*, *CDKN2B*	not applicable	
RS4;11 (ACC-508)	Homozygous deletion of flanking genes (*MLLT3* and *KLHL9*), *MIR31*, *MTAP*, *CDKN2A*, *CDKN2B-AS1* and *CDKN2B*	not applicable	
TOM-1 (ACC-578)	Heterozygous deletion of *MTAP, CDKN2A* (partial: only 357 and 472 nt probe targets), *PAX5* and flanking gene *PCSK5* Homozygous deletion of *CDKN2A* (partial: all except 357 and 472 probe targets)	not methylated	methylated

◊ CNAs detected by reference or control probes are not reported in this table.

~ Indicated chromosomal bands accommodate genes targeted by MLPA probes, however, the whole extent of CNAs present in the samples cannot be determined by this ME024 probemix.

"Methylated" refers to cases where MS-MLPA probes have ratios higher than baseline methylation.

* In this sample, MIR31 probe at 465 nt shows 100% signal loss upon HhaI digestion. Please see specifications for this probe in Table 1.

Data analysis

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

Interpretation of copy number results

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

Copy number status	Final ratio (FR)
Normal	0.80 < FR < 1.20
Homozygous deletion	FR = 0
Heterozygous deletion	0.40 < FR < 0.65
Heterozygous duplication/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. 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.

ME024-B3 9p21 CDKN2A/B/2B region specific notes:

- CDKN2B probe 11867-L12664 at 130 nt, CDKN2A probe 11869-L12666 at 171 nt, CDKN2A probe 01289-L28373 at 238 nt and CDKN2B probe 16064-L18238 at 417 nt are not completely digested in DNA samples derived from blood and show 5-10% background signal after HhaI digestion. Low methylation ratios obtained with these probes should be treated with caution.
- Use of FFPE tissues can result in low quality of the extracted DNA due to sample fixation and storage conditions. This might result in higher probe standard deviations. Warnings during the Fragment Analysis using Coffalyser.Net will indicate that the MLPA experiment was not optimal on the specific sample(s) used. For more information on the use of FFPE tissues with MLPA, please refer to Atanesyan et al. (2017).
- In samples from tumour tissues, reference probes are more prone to have deviating copy number results as compared to blood derived germline samples. When regions targeted by reference probes are affected by copy number alterations, it can help to turn the slope correction off in Coffalyser.Net analysis to get the correct copy number interpretation on the target region.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *CDKN2A* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix ME024 9p21 CDKN2A/2B region.
- MS-MLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations. Even when MS-MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.
- Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can cause false positive results. Mutations/SNVs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA.
- An MS-MLPA probe targets a single specific HhaI site in a CpG island; if methylation is absent for a particular CpG-site, this does not necessarily mean that the whole CpG island is unmethylated!
- Rare cases are known in which apparent methylation as detected by an MS-MLPA probe proved to be due to a sequence change in or very near the HhaI site.
- MS-MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. Changes in methylation status, gains or losses of genomic regions or genes may not be detected if the percentage of tumour cells is low. In addition, subclonality of the aberration affects the final ratio of the corresponding probe. Furthermore, there is always a possibility that one or more reference probes *do* show a copy number alteration in a patient sample, especially in solid tumours with more chaotic karyotypes.

Confirmation of results

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

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

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

Please report false positive results due to SNVs and unusual results (e.g., a duplication of *CDKN2A* exons 2 and 4 but not exon 3) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix ME024-B3 9p21 CDKN2A/2B region

Length (nt)	SALSA MLPA probe	Hhal site	MS-MLPA probes	Chromosomal position (hg18)			
			% expected signal reduction[a]	9p21.3		other	Reference/ Control probes
				CDKN2A	*CDKN2B*		
64-105	Control fragments – see table in probemix content section for more information						
124 ¥	Reference probe 21547-L02274	-					18q11
130 ∧	**CDKN2B probe** 11867-L12664	+	90-95%		exon 1		
136 Ø	**CDKN2A probe** 11868-L13885	+	100%	upstream			
143	Reference probe 14199-L15813	-					2q13
151 Ø	**CDKN2A probe** 08658-L13470	-		intron 1			
157 Ø	**CDKN2A probe** 16065-L18952	+	100%	upstream			
164 ¬	**MLLT3 probe** 16058-L28698	-				9p21.3	
171 ∧	**CDKN2A probe** 11869-L12666	+	95-100%	intron 1			
178	**CDKN2B-AS1 probe** 15671-L17637	-		upstream			
185	Reference probe 18767-L24189	-					10q22
192	**CDKN2B-AS1 probe** 15672-L28771	+	100%	upstream			
199	**CDKN2B probe** 16066-L00960	-			exon 1		
207 *	Reference probe 09718-L31849	-					12q24
214 ¥	**CDKN2B probe** 15673-L31850	+	100%		exon 1		
220 ◊	**CDKN2B probe** 11871-L13741	+	100%		exon 1		
229	**CDKN2B probe** 16059-L18233	-			exon 2		
238 ¥ ∧	**CDKN2A probe** 01289-L28373	+	95-100%	exon 1			
244	**CDKN2A probe** 16060-L19858	-		intron 1			
251	Reference probe 07592-L19744	-					21q21
259	**CDKN2A probe** 15674-L28708	-		exon 2			
265	**CDKN2A probe** 15675-L19896	-		downstream			
273 Ø +	**CDKN2A probe** 02238-L28709	-		upstream			
280	**CDKN2A probe** 16533-L28710	-		exon 3			
287	**PAX5 probe** 16061-L28711	-				9p13.2	
294 #	**MTAP probe** 15677-L28712	-				9p21.3	
301	Reference probe 07127-L28713	-					2p22
312 #	**MTAP probe** 01293-L28714	-				9p21.3	
319 ¬	DOCK8 probe 01130-L00688	-				9p24.3	
328	Reference probe 09065-L28777	-					19p13
339 ¬	GLDC probe 20716-L26930	-				9p24.1	
346 * π	Digestion control probe 20703-L31609	+	100%				2q12
357 Ø	**CDKN2A probe** 01528-L06031	-		intron 1			
364 ¬	KLHL9 probe 16746-L19357	-				9p21.3	
373	Reference probe 05288-L04644	-					14q22
382	**MIR31 probe** 16062-L18236	-				9p21.3	
391	**CDKN2A probe** 08659-L11995	-		exon 1			
400 * ¬	PCSK5 probe 21393-L12855	-				9q21.13	
409 *	Reference probe 02460-L31779	-					15q21
417 ∧	**CDKN2B probe** 16064-L18238	+	90-95%	upstream			
427	**CDKN2A probe** 15680-L19745	+	100%	exon 1			
436 « π	Digestion control probe 09167-L09460	+	100%				11q13
450	Reference probe 09107-L28897	-					4q25
458 Ø	**CDKN2B probe** 20565-L28898	-		downstream			
465 ∫	**MIR31 probe** 13665-L15119	+	0%			9p21.3	
472	**CDKN2A probe** 16536-L19026	-		intron 1			
481	**PAX5 probe** 16063-L18237	-				9p13.2	
490 ¥	Reference probe 06676-L27372	-					11p15
500 *	Reference probe 19675-L27455	-					4p13

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

* New in version B3.

¥ Changed in version B3. Minor alteration, no change in sequence detected.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.
◊ Target sequence of this 220 nt probe contains SNP rs150655569 (C>T/G) in the GCGC site, 10 nt right from the ligation site. When an alternative allele of this SNP (with an allele frequency of 0.18%) is present, HhaI digestion will not occur, resulting in a false methylation positive signal.
+ In several patients from the Netherlands and Belgium, SNP rs551685870 (6-bp deletion GTACGC), in the target sequence of this probe has been reported. Please note that the clinical significance of this deletion is not clear. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
# This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.
∫ This probe is not located on a CpG island. Nevertheless, it has an HhaI site. In our tests it never showed signal reduction upon HhaI digestion on DNA extracted from blood. However, we have observed partial or complete signal reduction of this probe upon HhaI digestion in the DNA of some of the cancer cell lines that we have tested at MRC Holland, e.g. CADO-ES1.
« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.
π Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.
Λ This probe is not completely digested in DNA samples derived from blood.
Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.

NOTE: The digestion control probes at 346 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 346 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 346 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. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Table 2. ME024-B3 9p21 CDKN2A/2B region probes arranged according to chromosomal location

Table 2a. Target and flanking probes

Length (nt)	SALSA MLPA probe	Gene/ exon	Position (hg18)/ Ligation site [a]	**Partial sequence** (copy number probes; 24 nt adjacent to ligation site)/ **Complete sequence** (MS-MLPA probes)	**Distance to next probe**
Telomeric flanking probes					
319 ¬	01130-L00688	*DOCK8*	9p24.3	GAATGTCTTACT-ATTGCTCTGGCA	6.2 **M**b
339 ¬	20716-L26930	*GLDC*	9p24.1	CTCACCTTGATA-TAAGCCCAGGAA	13.8 **M**b
164 ¬	16058-L28698	*MLLT3*	9p21.3	GATAACGACAAT-GACTCTGAAATG	968.4 kb
364 ¬	16746-L19357	*KLHL9*	9p21.3	TGTGCATCCTGA-GCTGTGTGATCT	180.2 kb
MIR31 and ***MTAP*** at 9p21.3. Exon numbering and ligation sites for these probes is according to NR_029505.1 and NM_002451.4, respectively.					
465 ∫	13665-L15119	***MIR31*** exon 1	45 nt after ex 1	CCACCTGCATGCCAGTCCTTCGTGTA-TTGC TGTGTATGTGCGCCCTTCCTTGGATGTGGAT	0.1 kb
382	16062-L18236	***MIR31*** exon 1	58-57 (reverse)	AAAGATGGCAAT-ATGTTGGCATAG	342.7 kb
312 #	01293-L28714	***MTAP*** exon 6	725-726	GAGGTGGTTCTT-GCTAAGGAGGCT	4.5 kb
294 #	15677-L28712	***MTAP*** exon 7	825-826	ACCGGGTCTTAA-AGACCCTGAAAG	108.2 kb
CDKN2A at 9p21.3. *CDKN2A* transcript variants NM_000077.5 (MANE select transcript) and NM_058195.4 (MANE Plus Clinical transcript) encode $p16^{INK4a}$ and $p14^{ARF}$ proteins, respectively (see schematic structure of encoded proteins on page 2 for more information). Exon numbering and ligation sites for *CDKN2A* are based on MANE reference transcripts from the MANE project (release version 1.0) retrieved on 08/2023. Exon numbering used in previous versions is indicated between brackets. ***CDKN2B-AS1*** at 9p21.3. Long non-coding RNA located within the *CDKN2B-CDKN2A* gene cluster. The exon numbering and ligation sites are based on the transcript variant NR_003529.4.					
265	15675-L19896	***CDKN2A*** downstream (4)	NM_000077.5 & NM_058195.4; 182 nt after ex 3	TGAAATGCGGTT-AAAATGATGAAT	0.7 kb
280	16533-L28710	***CDKN2A*** exon 3 (4)	NM_000077.5 & NM_058195.4; 33 nt before ex 3	TTGACCTCAGGT-TTCTAACGCCTG	3.0 kb
259	15674-L28708	***CDKN2A*** exon 2 (3)	NM_000077.5 & NM_058195.4; 45 nt before ex 2	TCCTTTCCGTCA-TGCCGGCCCCCA	3.5 kb
238 ∧	01289-L28373	***CDKN2A*** exon 1 (2)	NM_000077.5; 158-157 (reverse) NM_058195.4; 3.5 kb before ex 2 (reverse)	CCTGGATCGGCCTCCGACCGTAAC-TATTCGG TGCGTTGGGCAGCGCCCCCGCCTCCAGCAGC	0.2 kb
391	08659-L11995	***CDKN2A*** exon 1 (2)	NM_000077.5; 71 nt before ex 1 NM_058195.4; 3.7 kb before ex 2	GCACCGGAGGAA-GAAAGAGGAGGG	0.1 kb
244	16060-L19858	***CDKN2A*** intron 1 (2)	NM_000077.5; 138 nt before ex 1 NM_058195.4; 3.8 kb before ex 2	GCCTGGAAAGAT-ACCGCGGTCCCT	0.2 kb
171 ∧	11869-L12666	***CDKN2A*** intron 1 (2)	NM_000077.5; 378 nt before ex 1 NM_058195.4; 4.0 kb before ex 2	TGAACGCACTCAAACACGCCTTTGCT-GGCA GGCGGGGGAGCGCGGCTGGGAGCAGGGAGGC	6.2 kb
151 Ø	08658-L13470	***CDKN2A*** intron 1 (2)	NM_000077.5; 6.6 kb before ex 1 (reverse) NM_058195.4; 12.7 kb before ex 2 (reverse)	GTTAAGCCTTCA-TAGATGAGTTCT	6.3 kb
357 Ø	01528-L06031	***CDKN2A*** intron 1 (2)	NM_000077.5; 12.9 kb before ex 1 (reverse) NM_058195.4; 6.4 kb after ex 1 (reverse)	TCCAGTAAACTG-ACTCTAAACTTA	1.8 kb
472	16536-L19026	***CDKN2A*** intron 1 (2)	NM_000077.5; 14.7 kb before ex 1 NM_058195.4; 6.4 kb after ex 1	AAGAGTGTTGAA-AGGCCACGACTT	4.9 kb
427	15680-L19745	***CDKN2A*** exon 1 (1)	NM_000077.5; 19.6 kb before ex 1 NM_058195.4; 23 nt before ex 1	GTGCGTGGGTCCCAGTCTGCAGTTA-AGGGGGC AGGAGTGGCGCTGCTCACCTCTGGTGCCAAAGG	0.4 kb
192	15672-L28771	***CDKN2B-AS1*** upstream (1)	NR_003529.4; 32-33	AGCTACATCCGTCACCTGACACGGCCCTACCA- GGAACAGCCGCGCTCCCGCGGATTCTGGTGCTGC	0.2 kb
178	15671-L17637	***CDKN2B-AS1*** upstream (1)	NR_003529.4; 210-211	CGCCAATCAGGA-GGCTGAATGTCA	0.3 kb
136 Ø	11868-L13885	***CDKN2A*** upstream	NM_000077.5; 20.4 kb before ex 1 NM_058195.4; 861 nt before ex 1	ATGGGCTAGACACAAAGGACTCGGTGCT-TGT CCCAGCCAGGCGCCCTCGGCGACGCGGGCAG	0.1 kb

157 Ø	16065-L18952	***CDKN2A*** upstream	NM_000077.5; 20.5 kb before ex 1 NM_058195.4; 928 nt before ex 1	GGGGAAGAGGAAAGAGGAAGAAGCGCTCAGAT-GCTCCGCGGCTGTCGTGAAGGTTAAAACCGAAAATAAA	0.2 kb
273 Ø ± +	02238-L28709	***CDKN2A*** upstream	NM_000077.5; 20.7 kb before ex 1 NM_058195.4; 1.1 kb before ex 1	AGACCGGAGAGA-GAACGTACGCCG	5.1 kb
CDKN2B at 9p21.3. Exon numbering and ligation sites for CDKN2B probes are indicated in NM_004936.4 (MANE select transcript).					
458 Ø	20565-L28898	***CDKN2B*** downstream	2.3 kb after ex 2 (reverse)	CCTAGGAAAGGT-GATAGAGCTTAG	5.3 kb
229	16059-L18233	***CDKN2B*** exon 2	899-900	GCCTGTCTGAGA-CTCACAGGAAGG	3.0 kb
214	15673-L31850	***CDKN2B*** exon 1	451-452	GACTAGTGGAGAAGGTGCGACAGCTC-CTGGAAGCCGGCGCGGATCCCAACGGAGTCAACCGTTTCGG	0.1 kb
130 ∧	11867-L12664	***CDKN2B*** exon 1	319-320	TCGTTAAGTTTACGGCCAACGGTGGAT-TATCCGGGCCGCTGCGCGTCTGGGGGCTGCGGAATGC	0.1 kb
220 ◊	11871-L13741	***CDKN2B*** exon 1	252-253	GAAGCTGAGCCCAGGTCTCCTAGGAAGGA-GAGAGTGCGCCGGAGCAGCGTGGGAAAGAAGGGAA	0.3 kb
199	16066-L00960	***CDKN2B*** exon 1	91 nt before ex 1	CCTCCCGGCGAT-CACAGCGGACAG	0.1 kb
417 ∧	16064-L18238	***CDKN2B*** upstream	163 nt before ex 1	CTCCCTGGCCCAGTCTCTGGCGCA-TGCGTCCTAGCATCTTTGGGCAGGCTTCCCCGCC	15 **M**b
PAX5 at 9p13.2. Exon numbering and ligation sites for PAX5 probes are indicated in NM_016734.3 (MANE select transcript).					
287	16061-L28711	***PAX5*** exon 5	756-757	GTGAGCACGGAT-TCGGCCGGCTCG	18 kb
481	16063-L18237	***PAX5*** exon 2	378-379	CTTGCTCATCAA-GGTGTCAGGCCC	41.1 **M**b
Centromeric flanking probe					
400 ¬	21393-L12855	*PCSK5*	9p21.13	GATGAGCTGGAA-TATGATGACGAG	-

[a] Ligation sites and exon numbering are based on NM sequences from the MANE project. See section Exon numbering on page 2 for more information.

The HhaI sites are marked with grey. Ligation sites are marked with –. Complete probe sequences are available at www.mrcholland.com.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.
◊ Target sequence of this 220 nt probe contains SNP rs150655569 (C>T/G) in the GCGC site, 10 nt right from the ligation site. When an alternative allele of this SNP (with an allele frequency of 0.18%) is present, HhaI digestion will not occur, resulting in a false methylation positive signal.
+ In several patients from the Netherlands and Belgium, SNP rs551685870 (6-bp deletion GTACGC) in the target sequence of this probe has been reported. Please note that the clinical significance of this deletion is not clear. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
# This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.
∫ This MIR31 probe (13665-L15119) is not located on a CpG island. Nevertheless, it has an HhaI site. In our tests it never showed signal reduction upon HhaI digestion on DNA extracted from blood. However, we have observed partial or complete signal reduction of this probe upon HhaI digestion in the DNA of some of the cancer cell lines that we have tested at MRC-Holland, e.g. CADO-ES1.
« 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.
∧ This probe is not completely digested in DNA samples derived from blood.
Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.

Table 2b. Reference and digestion control probes ordered by chromosomal location

Length (nt)	SALSA MLPA probe	Gene	Chromosomal position (hg18)	Location (hg18) in kb
301	07127-L28713	*SPAST*	2p22	02-032,142
346 π	20703-L31609	*SLC9A2*	2q12	02-102,641
143	14199-L15813	*EDAR*	2q13	02-108,894
500	19675-L27455	*ATP8A1*	4p13	04-042,278
450	09107-L28897	*CFI*	4q25	04-110,887
185	18767-L24189	*NODAL*	10q22	10-071,865
490	06676-L27372	*SMPD1*	11p15	11-006,369
436 « π	09167-L09460	*MEN1*	11q13	11-064,335
207	09718-L31849	*NOS1*	12q24	12-116,195
373	05288-L04644	*ATL1*	14q22	14-050,160
409	02460-L31779	*FBN1*	15q21	15-046,567
124	21547-L02274	*NPC1*	18q11	18-019,394
328	09065-L28777	*CACNA1A*	19p13	19-013,289
251	07592-L19744	*ADAMTS5*	21q21	21-027,229

« 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.

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

Related SALSA MLPA probemixes

P419 CDKN2A/2B-CDK4 Contains more probes for *CDKN2A*, *CDKN2B*, *CDK4* and *MTAP* genes, and a mutation-specific probe for *MITF* p.E318K (c.952G>A) point mutation.

P088 Oligodendroglioma 1p-19q Contains more probes for *CDKN2A* and *CDKN2B* genes, 1p and 19q regions, and mutation-specific probes for *IDH1* and *IDH2*.

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

ME042 CIMP Contains MS-MLPA probes for *CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1* genes.

ME001 Tumour suppressor mix Contains MS-MLPA probes for 25 tumour suppressor genes, including *CDKN2A* and *CDKN2B*.

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ME024 product history	
Version	*Modification*
B3	One HhaI digestion control probe and three reference probes have been replaced and one flanking probe has been added. In addition, several probes have a small change in length but not in the sequence targeted.
B2	One flanking probe has been redesigned, two reference probes have been replaced and several probes have a small change in length but no change in the sequence detected
B1	One additional probe for CDKN2A and several probes for genes MIR31, CDKN2B-AS1 and PAX5 on chromosome arm 9p have been included. Several reference probes have been replaced/included. 88 and 96nt control fragments have been replaced (QDX2).
A1	First release.

Implemented changes in the product description

Version B3-04 — 07 September 2023 (04M)
- Product description rewritten and adapted to a new template.
- Schematic figure of CDKN2A and CDKN2B coding regions and proteins updated on page 2.
- Links to gene structure and transcript variants updated.
- Reference transcripts according to MANE project have been updated for *CDKN2B*, *MTAP* and *PAX5* genes in Exon numbering section on page 2 and Table 2.
- NR_sequence version for *CDKN2B-AS1* updated.
- Positive control samples from the DSMZ institute added to the Positive control DNA samples section on pages 4 and 5: ACC-347 (MOLT-14), ACC-42 (697), ACC-511 (ALL-SIL), ACC-40 (CTV-1), ACC-713 (GRANTA-452), ACC-282 (JURKAT), ACC-742 (BALL-1), ACC-339 (MHH-CALL-3), ACC-22 (REH), ACC-508 (RS4;11) and ACC-578 (TOM-1).
- SNP warnings added for probes at 220 nt and 273 nt in Table 1 and Table 2.
- Updated the list of related probemixes on page 13.
- New references added on pages 12 and 13.
- Various minor textual and table layout changes.

Version B3-03 — 19 January 2023 (02M)
- Exon numbering of the *CDKN2A* gene has been changed according to MANE in a Schematic figure on page 2, Table 1 and 2. See also explanation on page 2.
- Ligation sites of the *CDKN2A* and *CDKN2B-AS1* have been updated.
- Exon information of CDKN2B probe 16064-L18238 has been updated to “upstream”.

Version B3-02 — 09 June 2021 (02M)
- ME024-specific note added regarding probes with incomplete HhaI digestion on page 6.

Version B3-01 — 12 December 2019 (02M)
- A warning about non-specific peaks and advice on not spinning down your MLPA reactions in between the ligation and PCR reaction was added on page 1.
- Product description adapted to a new template and to a new product version (version number changed, changes in Table 1 and Table 2a and Table 2b).
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.
- Percentage of expected signal reduction updated in Table 1.
- Warning about the overdigestion in Table 1 for a reference probe at 124 nt has been removed because the probe's performance was improved.
- Ligation site information of CDKN2A 15675-L19896 probe at 265 nt updated according to hg38 information in Table 2.
- NM_sequence version updated for *MTAP* gene in Table 2.
- NM_sequence version and consequently ligation site information updated for *PAX5* probes in Table 2.
- Warning added to Table 2a for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.

Version B2-01 – 19 March 2019 (01M)
- Product description restructured and adapted to a new template.
- Product description completely rewritten.
- For uniformity, the chromosomal positions and bands in this document are now all based on hg18 (NCBI36).
- Small changes of probe lengths in Table 1, Table 2a, Table 2b and Table 3 in order to better reflect the true lengths of the amplification products.

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