Product Description SALSA® MLPA® Probemix P370-C1 BRAF-IDH1-IDH2

To be used with the MLPA General Protocol.

Version C1. As compared to version B1, one *RAF1* target probe and four reference probes have been replaced, and several probes have a change in length but no change in the sequence targeted. SALSA Binning DNA for this probemix has been changed from SD043 to SD054. For complete product history see page 11.

Catalogue numbers:

- **P370-025R:** SALSA MLPA Probemix P370 BRAF-IDH1-IDH2, 25 reactions.
- **P370-050R:** SALSA MLPA Probemix P370 BRAF-IDH1-IDH2, 50 reactions.
- **P370-100R:** SALSA MLPA Probemix P370 BRAF-IDH1-IDH2, 100 reactions.

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

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

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

General information: The SALSA MLPA Probemix P370 BRAF-IDH1-IDH2 is a **research use only (RUO)** assay for detection of the *BRAF* p.V600E & four predominant *IDH1* (p.R132H and p.R132C) and *IDH2* (p.R172M and p.R172K) point mutations, for detection of genomic duplications leading to the *SRGAP3-RAF1, KIAA1549-BRAF* and *FGFR1-TACC1* fusion genes on chromosome arms 3p, 7q and 8p respectively, and for detection of copy number aberrations in the *BRAF, CDKN2A/2B, FGFR1, MYB* and *MYBL1* genes.

The *IDH1* and *IDH2* mutations represent frequent genetic abnormalities in gliomas. Their identification facilitates distinguishing the different glioma entities leading to a more accurate prognosis and treatment (Riemenschneider et al. 2010). *IDH1* and *IDH2* point mutations have been detected with high frequency in diffuse gliomas (Hartmann et al. 2009; Yan et al. 2009), and the presence of these mutations is associated with a longer survival (Sanson et al. 2009; van den Bent et al. 2010). *IDH1/2* mutation is a marker for glioma classification since 2016, defining glioblastomas as *IDH*-mutant or *IDH*-wildtype (Wesseling and Capper 2018). This MLPA probemix contains probes that are specific for the two most frequent *IDH1* (p.R132H and p.R132C) and the two most frequent *IDH2* (p.R172M and p.R172K) mutations.

Activation of the *MAPK* pathway has been detected with high frequency in pilocytic astrocytomas, in particular via a 2 Mb tandem duplication leading to an oncogenic *KIAA1549-BRAF* fusion gene at 7q34 (Jones et al. 2008). Detection of this duplication is of help in differentiating these tumours from diffuse astrocytomas. Alternative *MAPK* pathway activation mechanisms include: 1) the formation of a similar *SRGAP3-RAF1* fusion gene at 3p25, through a 3.6 Mb tandem duplication (Jones et al. 2009), 2) intragenic duplications of *FGFR1* and *FGFR1-TACC1* microamplifications (Zhang et al. 2013; Jones et al. 2013), and 3) certain *BRAF* mutations, in particular the p.V600E mutation (Schiffman et al. 2010; Dougherty et al. 2010). The *BRAF* p.V600E activating mutation in combination with deletion of *CDKN2A* was found to be significantly enriched in cases of low grade glioma that are undergoing transformation to secondary high grade gliomas (Mistry et al. 2015), suggesting to define a clinical distinct subgroup of childhood glioma.

This probemix also includes probes for the *FGFR1, MYB* and *MYBL1* genes and for the 9p21.3 region *(CDKN2A/2B, MIR31).* All these genes and regions are suggested to help in differentiating molecular subtypes of gliomas (see Table 2a for more detailed information). Furthermore, this probemix contains 13 reference probes detecting autosomal chromosomal locations that are regarded as relatively stable in

gliomas and brain tumours. However, it should be noticed that glioma karyotypes can harbour multiple numerical and structural aberrations, which can complicate interpretation of these reference probes.

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
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/

Exon numbering: The exon numbering used in this P370-C1 BRAF-IDH1-IDH2 product description is the exon numbering from the LRG_610 for *IDH1*, NM_014850.4 for *SRGAP3*, LRG_413 for *RAF1*, NG_012330.1 for *MYB*, NG_032965.2 for *KIAA1549*, LRG_299 for *BRAF*, LRG_993 for *FGFR1*, NM_006283.3 for *TACC1*, NM_001080416.4 for *MYBL1* and LRG_611 for *IDH2* gene. The exon numbering and NM_ sequences used for the above mentioned genes have been retrieved on 03/2020. **From product description version C1-04 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 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.** As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date.

Probemix content: The SALSA MLPA Probemix P370-C1 BRAF-IDH1-IDH2 contains 59 MLPA probes with amplification products between 124 and 500 nucleotides (nt). This includes 41 probes for the detection of genomic duplications leading to the *KIAA1549-BRAF, SRGAP3-RAF1* and *FGFR1-TACC1* fusion genes, and for detection of copy number aberrations in the *BRAF, CDKN2A/2B, FGFR1, MYB* and *MYBL1* genes. Furthermore, this probemix also contains five probes specific for the detection of the *BRAF* p.V600E & four predominant *IDH1* p.R132H and p.R132C and *IDH2* p.R172M and p.R172K point mutations, which will only generate a signal when the mutation is present. In addition, 13 reference probes are included that target relatively copy number stable regions in various cancer types including brain tumours. Complete probe sequences are available online (www.mlpa.com) and the identity of the genes detected by the reference probes is available in Table 2b.

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 MLPA General Protocol and online at www.mlpa.com.

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

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

MLPA technique validation: Internal validation of the MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using 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.

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 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 MLPA experiment for data normalisation. 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. Reference samples should be derived from healthy individuals without a history of brain tumours. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol.

Positive control DNA samples: MRC-Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. Coriell Institute (https://catalog.coriell.org) and Leibniz Institute DSMZ (https://www.dsmz.de/home.html) have a diverse collection of biological resources which may be used as a positive control DNA sample in your MLPA experiments. Samples from the Coriell Institute have been tested with this P370-C1 probemix at MRC-Holland and can be used as a positive control samples as described in the table below. The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Chromosomal position of CNA*	Altered target genes in P370-C1	Expected CNA
NA03503	3p25.1-p26.3	*CRBN, SRGAP3, RAF1*	Heterozygous duplication
NA04127	3p25.1-p26.3	*CRBN, SRGAP3, RAF1*	Heterozygous duplication
NA10985	3p25.3-p26.3	*CRBN, SRGAP3*	Heterozygous deletion
NA09367	6q22.33-q23.3	*LAMA2, MYB*	Heterozygous duplication
NA07994	6q23.3-q24.2	*MYB, PLAGL1*	Heterozygous duplication
NA12519	7q34-q35	*KIAA1549, HIPK2, MKRN1, BRAF, CNTNAP2*	Heterozygous triplication/ Homozygous duplication
NA07412	7q34-q35	*KIAA1549, HIPK2, MKRN1, BRAF, CNTNAP2*	Heterozygous deletion
NA08808	7q34-q35	*KIAA1549, HIPK2, MKRN1, BRAF, CNTNAP2*	Heterozygous deletion
NA01220	7q34-q35	*MKRN1, BRAF, CNTNAP2*	Heterozygous duplication
NA02030	8p12-q13.1	*FGFR1, TACC1, MYBL1*	Heterozygous duplication
NA14485	8p11.23-p12	*FGFR1, TACC1*	Heterozygous duplication
NA02819	9p21.3	*MIR31, CDKN2A, CDKN2B*	Heterozygous duplication
NA03226	9p21.3	*MIR31, CDKN2A, CDKN2B*	Heterozygous duplication
NA05067	9p21.3	*MIR31, CDKN2A, CDKN2B*	Heterozygous duplication
NA01750	9p21.3	*MIR31, CDKN2A, CDKN2B*	Heterozygous duplication

* Indicated chromosomal bands accommodate genes targeted by MLPA probes, however, the whole extent of copy number alteration (CNA) present in this cell line cannot be determined by this P370-C1 BRAF-IDH1-IDH2 probemix.

SALSA Binning DNA SD054: The SD054 Binning DNA provided with this probemix can be used for binning of five mutation-specific probes (*BRAF* p.V600E probe M08780-SP0039-L08904 at 226 nt, *IDH1* p.R132H probe M19529-L16492 at 203 nt, *IDH1* p.R132C probe M14787-L16493 at 220 nt, *IDH2* p.R172M probe M20963-L29001 at 244 nt, and *IDH2* p.R172K probe M20963-L29002 at 238 nt). SD054 Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequence detected by the above mentioned probes. Inclusion of one reaction with 5 µl SD054 Binning DNA in initial MLPA experiments is essential as it can be used to aid in data binning of the peak pattern using Coffalyser.Net software. Furthermore, Binning DNA should be included in the experiment whenever changes have been applied to the set-up of the capillary electrophoresis device (e.g. when capillaries have been renewed). Binning DNA should never be used as a reference sample in the MLPA data analysis, neither should it be used in quantification of mutation signals. It is strongly advised that all samples tested are

extracted with the same method and derived from the same source of tissue. For further details, please consult the SD054 Binning DNA product description, available online: www.mlpa.com.

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

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

Copy number status	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Heterozygous deletion	0.40 < DQ < 0.65
Heterozygous duplication	1.30 < DQ < 1.65
Heterozygous triplication/Homozygous duplication	1.75 < DQ < 2.15
Ambiguous copy number	All other values

Please note that these above mentioned dosage quotients are only valid for germline testing. Dosage quotients are affected both by percentage of tumour cells and by possible subclonality.

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic cases.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Incomplete DNA denaturation (e.g. due to salt contamination) can lead to a decreased probe signal, in particular for probes located in or near a GC-rich region. 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 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.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- When running MLPA products, the capillary electrophoresis protocol may need optimization. False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: lower injection voltage / injection time settings, or a reduced amount of sample by diluting PCR products.

P370 specific notes:

- The presence of a clear signal for the 226, 203, 220, 244 and 238 probes (at least 10% of the mean peak height of all reference probes in the sample), indicates the presence of the *BRAF* p.V600E, *IDH1* p.R132H, *IDH1* p.R132C, *IDH2* p.R172M and *IDH2* p.R172K mutation, respectively.This probemix should be used only for detection of the following five point mutations (BRAF p.V600E, IDH1 p.R132H, IDH1 p.R132C, IDH2 p.R172M and IDH2 p.R172K) but not for their quantification.

- 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.
- 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 the V600K mutation.

Limitations of the procedure:
- In most populations the most genetic alterations in *BRAF, IDH1*, *IDH2*, *KIAA1549, SRGAP3, RAF1 FGFR1, TACC1*, *CDKN2A/2B, MYB* and *MYBL1* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P370 BRAF-IDH1-IDH2.
- 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 MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.
- Sequence changes (e.g. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA.
- MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. 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 brain tumours with more chaotic karyotypes.

Confirmation of results: 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 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 SNPs and unusual results (e.g., a duplication of *BRAF* exons 8 and 14 but not exon 12) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P370-C1 BRAF-IDH1-IDH2

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)						
		Reference	*IDH1/2*	3p	6q	Chr. 7	Chr. 8	9p
64-105	Control fragments – see table in probemix content section for more information							
124	Reference probe 18709-L21056	5q31						
130 *	Reference probe 19551-L31871	2p13						
135 ¥	**MYBL1 probe** 22553-L31748						8q13.1	
139 ¥	**SRGAP3 probe** 19365-L31872			3p25.3				
143 ¥	Reference probe 16316-L31755	3q21						
148 «	**MYB probe** 12500-L25742				6q23.3			
155 ¬	**LAMA2 probe** 14924-L25757				6q22.33			
160	**SRGAP3 probe** 19366-L25759			3p25.3				
166	**KIAA1549 probe** 15251-L17567					7q34		
172	**MYBL1 probe** 07915-L07628						8q13.1	
179	**BRAF probe** 10509-L25743					7q34		
184	**FGFR1 probe** 04184-L25753						8**p**12	
190	**TACC1 probe** 19368-L25761						8**p**11.23	
196	Reference probe 18049-L22439	16q23						
203 §	**IDH1 probe** 19529-L16492		**p.R132H**					
208	**KIAA1549 probe** 15252-L17007					7q34		
214 ¥ #	**BRAF probe** 22554-L26031					7q34		
220 §	**IDH1 probe** 14787-L16493		**p.R132C**					
226 § Ж $	**BRAF probe** 08780-SP0039-L08904					**p.V600E**		
232	Reference probe 16428-L25931	18q21						
238 ¥ §	**IDH2 probe** 20963-L29002		**p.R172K**					
244 ¥ §	**IDH2 probe** 20963-L29001		**p.R172M**					
249	**CDKN2A probe** 10333-L17690							9p21.3
254	**RAF1 probe** 15332-L17816			3p25.1				
261	**MYBL1 probe** 07914-L26455						8q13.1	
266	**KIAA1549 probe** 15253-L17561					7q34		
274 ¬	**CNTNAP2 probe** 12947-L25756					7q35		
280	Reference probe 13350-L26120	9q21						
285	**CDKN2A probe** 16533-L26121							9p21.3
292 ¬ «	**IKZF1 probe** 16911-L15654					7**p**12.2		
298	**MYB probe** 17265-L26123				6q23.3			
304 ¬	**SLC26A3 probe** 17066-L26124					7q22.3		
310	Reference probe 16559-L26125	11q13						
319	**KIAA1549 probe** 15255-L17010					7q34		
326	**MYBL1 probe** 19605-L26457						8q13.1	
333 ¬	**PLAGL1 probe** 18472-L26458				6q24.2			
339	**FGFR1 probe** 17635-L26228						8**p**12	
346 *	Reference probe 05273-L04655	2p22						
353	**FGFR1 probe** 04439-L26460						8**p**12	
360	**MKRN1 probe** 15257-L26461					7q34		
366 *	**RAF1 probe** 22758-L32102			3p25.1				
373	**FGFR1 probe** 18296-L25750						8**p**12	
382 *	Reference probe 09717-L31870	12q24						
388	**MIR31 probe** 19508-L26462							9p21.3
395	**HIPK2 probe** 15259-L26463					7q34		
402	**FGFR1 probe** 04440-L26464						8**p**12	
409 #	**BRAF probe** 19324-L25551					7q34		
417	Reference probe 13817-L15311	2q13						
424	**FGFR1 probe** 04441-L21311						8**p**12	
430	**MYB probe** 19369-L26102				6q23.3			
438	**TACC1 probe** 19370-L25763						8**p**11.23	
447	**BRAF probe** 19328-L25555					7q34		
454	**CDKN2B probe** 01531-L13742							9p21.3
463 *	Reference probe 10685-L31869	6p12						
471	**MYB probe** 19371-L26746				6q23.3			
478 ¥	**BRAF probe** 21619-L31757					7q34		
484 ¬	**CRBN probe** 06312-L26467			3p26.3				
494	Reference probe 19137-L26747	21q22						
500 ¥	Reference probe 19675-L27812	4p13						

* New in version C1.

¥ Changed in version C1. Minor alteration, no change in sequence detected.

§ Mutation-specific probe. This probe will only generate a signal when the BRAF p.V600E, IDH1 p.R132H / p.R132C or IDH2 p.R172M / p.R172K mutation is present.

$ Please note that this probe might give a small signal on a sample with the *BRAF* p.V600K mutation.

« 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.

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.

¬ 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.

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.

Table 2a. P370-C1 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene (exon)[a]	Location (hg18) / Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
***IDH1* p.R132H and p.R132C, at 2q33.3** These probes will only give a signal when either the p.R132H or the p.R132C mutation is present in the sample. The p.R132H (c.395G>A) mutation has been detected by sequencing in 664 samples covering 92.7% of *IDH1* mutations, and the p.R132C (c.394C>T) mutation in 29 samples covering 4.2% of the *IDH1* mutations in a cohort of 1010 diffuse gliomas (Hartmann et al. 2009). No probes are present in this probemix for the *IDH1* p.R132S (c.394C>A), p.R132G (c.394C>G) and p.R132L (c.395G>T) mutations which were detected in 11 (1.5%), 10 (1.5%) and two (0.2%) samples, respectively, in the same study by Hartmann et al. 2009.					
203 §	19529-L16492	**IDH1**, ex 6 **c.395G>A=p.R132H**	NM_005896.3; 690-691	CATCATAGGTC**A**-TCATGCTTATGG	0.0 kb
220 §	14787-L16493	**IDH1**, ex 6 **c.394C>T=p.R132C**	NM_005896.3; 689-688 reverse	ATCATCATAGGT-**T**GTCATGCTTAT	-
SRGAP3-RAF1* fusion, at 3p25** Activation of the *RAF1* gene by formation of a *SRGAP3-RAF1* fusion gene in a similar way as the *KIAA1549-BRAF* fusion gene has been described in pilocytic astrocytoma patients (Jones et al. 2009; Forshew et al. 2009; Tatevossian et al. 2010). It is indicated in the 4th column whether the probe is located ***within or *outside* the duplicated region.					
484 ¬	06312-L26467	CRBN	3p26.3; *outside*	CCGATGGCAGCT-TATGTGAATCCT	5.8 **M**b
160	19366-L25759	SRGAP3, ex 20	NM_014850.4; 2861-2862; *outside*	GAAGTGGAGCAG-ATCGAGGCTATT	130.4 kb
139	19365-L31872	SRGAP3, ex 2	NM_014850.4; 812-813; ***within***	GAGCAGCAATCA-GAGTCGCGACTG	3.5 **M**b
254	15332-L17816	RAF1, ex 17	NM_002880.3; 3033-3034; ***within***	TCATGCTGAATT-TTGTCTTCCAGG	34.7 kb
366	22758-L32102	RAF1, ex 2	NM_002880.3; 561-562; *outside*	TGGCAAACTCAC-AGATCCTTCTAA	11.7 kb
***MYB* aberrations, at 6q23.3** MYB is an oncogenic transcription factor that can act both as a transcriptional activator and repressor. Both amplifications of the whole MYB gene and 3' deletions of the MYB gene have been described in paediatric low-grade gliomas (Tatevossian et al. 2010; Zhang et al. 2013).					
155 ¬	14924-L25757	LAMA2	6q22.33	GGGTTTCAAACA-GATGTCAGAGTT	6.0 **M**b
148 «	12500-L25742	MYB, ex 2	NM_001130173.2; 265-266	TGAGGACTTTGA-GATGTGTGACCA	11.2 kb
471	19371-L26746	MYB, ex 10	NM_001130173.2: 1604-1605	CCACCCAAGGTC-TTACCTCCTGCA	6.1 kb
430	19369-L26102	MYB, ex 15	NM_001130173.2; 2285-2286	CTTACAAGCTCC-GTTTTAATGGCA	15.2 kb
298	17265-L26123	MYB, ex 16	NM_001130173.2; 2969-2970	CATTTAATCCAG-ATTGTAAATGCT	8.7 **M**b
333 ¬	18472-L26458	PLAGL1	6q24.2	AAGTTTGTCTGA-AGATTCAAACCT	-
KIAA1549-BRAF* fusion and *BRAF* p.V600E mutation, at 7q34** MAPK pathway activation through a 2 Mb tandem duplication leading to an oncogenic *KIAA1549-BRAF* fusion gene is suggested to be a very frequent event in pilocytic astrocytomas (Jones et al. 2008). The *BRAF* p.V600E mutation, which can be detected by the presence of the 226 nt probe, is also found regularly in this type of tumours. It is indicated in the 4th column whether the probe is located ***within or *outside* the commonly duplicated region. Note that several different fusion variants have been described in the literature. The most common is the *KIAA1549* exon 16-*BRAF* exon 9 fusion. For more information on other fusion variants see e.g. Table 1 in Forshew et al. 2009. When the *BRAF* p.V600E point mutation is detected in combination with deletion of *CDKN2A*, paediatric low grade glioma patients have an increased risk for transformation to secondary high grade glioma; therefore, therapy may need other preventive measures for this defined clinical distinct subgroup (Mistry et al. 2015).					
7p arm					
292 ¬ «	16911-L15654	IKZF1	7p12.2; *outside*	AGACATGTCCCA-AGTTTCAGGTGA	56.9 **M**b

7q arm					
304 ¬	17066-L26124	SLC26A3	7q22.3; *outside*	CCACTTCCTGCA-TGTGGCAGAAAG	31.0 **M**b
166	15251-L17567	KIAA1549, ex 19	NM_001164665.2; 5617-5618; *outside*	TTGCCAGCAGAA-TTGGAGCTCAGC	12.0 kb
266	15253-L17561	KIAA1549, ex 17	NM_001164665.2; 5387-5388; *outside*	AGACTATGGAAT-GACTCCCCCGAC	29.3 kb
208	15252-L17007	KIAA1549, ex 11	NM_001164665.2; 4212-4213; ***within***	GGTCAGCACAAT-AAAGACGACATA	29.7 kb
319	15255-L17010	KIAA1549, ex 4	NM_001164665.2; 3206-3207; ***within***	CCAGACTCCTTT-AATCCTGTCTGT	703.2 kb
395	15259-L26463	HIPK2	7q34; ***within***	ACTACCCATCTA-CACTCTACCAGC	855.8 kb
360	15257-L26461	MKRN1	7q34; ***within***	ACCACCCTCTTA-CACATTTTCAGC	280.6 kb
179	10509-L25743	BRAF, ex 17	NM_004333.6; 8 nt after exon 17; ***within***	CCAAGTAAGTAA-AAGCTTCATGCT	13.5 kb
226 § Ж $	08780-SP0039-L08904	**BRAF**, ex 15 **p.V600E=c.1799T>A**	NM_004333.6; 1985-1986 and 2025-2026	TTCTTCATGAAG-ACCTCACAGTAAA AATAGGTGATTTTGGTCTAGCTACAG**A** -GAAATCTCGATG	0.9 kb
447	19328-L25555	BRAF, ex 14	NM_004333.6; 1964-1965; ***within***	ACCTCAAGAGTA-ATAGTATCCTTC	23.9 kb
409 #	19324-L25551	BRAF, ex 12	NM_004333.6; 1688-1689; ***within***	TGTTGAATGTGA-CAGCACCTACAC	16.4 kb
478	21619-L31757	BRAF, ex 8	NM_004333.6; 1215-1216; *outside*	CAGGCCCCAAAT-TCTCACCAGTCC	14.5 kb
214 #	22554-L26031	BRAF, ex 4	NM_004333.6; 758-759; *outside*	GAGTTACAGTCC-GAGACAGTCTAA	6.2 **M**b
274 ¬	12947-L25756	CNTNAP2	7q35; *outside*	GTGCCTCTGGAT-TGGAATGGAGAA	-
Intragenic duplications of *FGFR1* and *FGFR1-TACC1* microamplifications, at 8p11-p12 Intragenic duplications of exons 10-18 or exons 11-18 of the *FGFR1* gene are detected both in paediatric low grade diffuse gliomas and in pilocytic astrocytomas (Zhang et al. 2013; Jones et al. 2013). These intragenic duplications are suggested to produce autophosphorylation of *FGFR1* and upregulation of the *MAPK/ERK* and *PI3K* pathways. In addition, microamplifications of *FGFR1* and *TACC1* leading to in-frame *FGFR1-TACC1* fusions by joining exon 18 of *FGFR1* with exon 7 of *TACC1*, have been described in in low-grade gliomas (Zhang et al. 2013).					
424	04441-L21311	FGFR1, ex 18	NM_023110.2; 3729-3730	AGCCAATGAACA-GGCATGCAAGTG	1.6 kb
402	04440-L26464	FGFR1, ex 14	NM_023110.2; 2808-2809	TGCATACACCGA-GACCTGGCAGCC	1.1 kb
353	04439-L26460	FGFR1, ex 13	NM_023110.2; 2710-2711	ACCCCAGCCACA-ACCCAGAGGAGC	2.4 kb
373	18296-L25750	FGFR1, ex 10	NM_023110.2; 2259-2260	TCCATGAACTCT-GGGGTTCTTCTG	9.7 kb
184	04184-L25753	FGFR1, ex 5	NM_023110.2; 1481-1482	CAAATGCCCTTC-CAGTGGGACCCC	29.4 kb
339	17635-L26228	FGFR1, ex 2	NM_023110.2; 955-956	TGTGGAGCTGGA-AGTGCCTCCTCT	271.0 kb
190	19368-L25761	TACC1, ex 1	NM_006283.3; 58.8 kb before exon 1	GCTGACTTCGCA-CTTGAGCTCCAG	113.9 kb
438	19370-L25763	TACC1, ex 11	NM_006283.3; 2372-2373	ATGGAGAAGGAA-CAGGCCCTGGCT	28.8 **M**b
Duplications of *MYBL1*, at 8q13.1 *MYBL1* belongs to the Myb family of transcription factors. Gain of 8q13.1 is detected in 28% of paediatric diffuse astrocytomas resulting in partial duplication of *MYBL1* with truncation of its C-terminal negative-regulatory domain (Ramkissoon et al. 2013; Zhang et al. 2013).					
172	07915-L07628	MYBL1, ex 14	NM_001080416.4; 2312-2313	CTGTTGACTGAA-GACATTTCAGAC	25.8 kb
326	19605-L26457	MYBL1, ex 8	NM_001080416.4; 1227-1228	AGAATGAAGTTA-GAAGAAAGCGAA	4.9 kb
135	22553-L31748	MYBL1, ex 5	NM_001080416.4; 836-837	ATCATCTATGAA-GCACATAAGCGG	5.1 kb
261	07914-L26455	MYBL1, ex 2	NM_001080416.4; 443-444	GATCATGATTAT-GAAGTACCACAA	-
***CDKN2A, CDKN2B* and *MIR31* genes, at 9p21.3** Loss of 9p, and especially deletions of the 9p21.3 region including *CDKN2A*, are common in high-grade gliomas. In contrast, *CDKN2A* deletions are rare in anaplastic astrocytomas and glioblastomas with mutated *IDH1* or *IDH2* genes but are more frequent in these tumours without *IDH1/IDH2* mutations (Yan et al. 2009). Additionally, homozygous deletions of *CDKN2A* have been reported to define a subset of malignant astrocytomas in children (Schiffman et al. 2010). When deletion of *CDKN2A* is detected in combination with the *BRAF* p.V600E mutation in paediatric low grade glioma, there is an increased risk for transformation to secondary high grade glioma; therefore, therapy may need other preventive measures for this defined clinical distinct subgroup (Mistry et al. 2015).					
388	19508-L26462	MIR31	9p21.3	AAAGATGGCAAT-ATGTTGGCATAG	456.1 kb
285	16533-L26121	CDKN2A, ex 3 (4)	NM_000077.5; 33 nt before exon 3; NM_058195.4; 33 nt before exon 3	TTGACCTCAGGT-TTCTAACGCCTG	6.7 kb
249	10333-L17690	CDKN2A, intron 1 (2)	NM_000077.5; 138 nt before exon 1; NM_058195.4; 3.8 kb before exon 2	GCCTGGAAAGAT-ACCGCGGTCCCT	25.6 kb
454	01531-L13742	CDKN2B	9p21.3	CCTAGGAAAGGT-GATAGAGCTTAG	-
***IDH2* p.R172M and p.R172K, at 15q26.1** These probes will only give a signal when either the p.R172K or the p.R172M mutation is present in the sample. The p.R172K (c.515G>A) mutation has been detected by sequencing in 20 samples, and the p.R172M (c.515G>T) mutation in six samples, in a cohort of 1010 diffuse gliomas (Hartmann et al. 2009). The same study suggests that *IDH2* mutations occur predominantly in oligodendroglial tumours. No probe is present for the *IDH2* p.R172W (c.514A>T) mutation, which was detected in five samples in this study of 1010 patients.					
244 §	20963-L29001	**IDH2**, ex 5 **c.515G>T=p.R172M**	NM_002168.4; 593-594	TACCATTGGCA**T**-GCACGCCCATGG	0.0 kb
238 §	20963-L29002	**IDH2**, ex 5 **c.515G>A=p.R172K**	NM_002168.4; 593-594	TACCATTGGCA**A**-GCACGCCCATGG	-

a) See above section on exon numbering for more information.

b) Only partial probe sequences are shown. Complete probe sequences are available at www.mlpa.com. Please notify us of any mistakes: info@mlpa.com.

§ Mutation-specific probe. This probe will only generate a signal when the BRAF p.V600E, IDH1 p.R132H / p.R132C or IDH2 p.R172M / p.R172K mutation is present.

$ Please note that this probe might give a small signal on a sample with the *BRAF* p.V600K mutation.

« 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.

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.

¬ 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.

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.

Table 2b. Reference probes arranged according to chromosomal location.

Length (nt)	SALSA MLPA probe	Gene	Location (hg18)	Partial sequence (24 nt adjacent to ligation site)	Location (hg18) in kb
346	05273-L04655	SPAST	2p22	CGAGCCACAGCA-AAAAGAGCCCTC	02-032.215
130	19551-L31871	DYSF	2p13	CCATTGCCAAGA-AGGTCAGTGTCC	02-071.750
417	13817-L15311	EDAR	2q13	TGGCCAGGTGAA-CCAGCGACAGCA	02-108.891
143	16316-L31755	RAB7A	3q21	CACAATAGGAGC-TGACTTTCTGAC	03-130.000
500	19675-L27812	ATP8A1	4p13	CAGATTCTTCTT-CGAGGAGCTCAG	04-042.278
124	18709-L21056	IL4	5q31	ATCGACACCTAT-TAATGGGTCTCA	05-132.038
463	10685-L31869	PKHD1	6p12	TCTGGCATCTAT-ATCTGCAGTCCC	06-051.876
280	13350-L26120	PCSK5	9q21	CATTAGCAAGCA-TTAGAACATCTC	09-077.989
310	16559-L26125	SHANK2	11q13	TGGTGCCAACAA-GGACTCACTCTC	11-070.331
382	09717-L31870	NOS1	12q24	GCTTGCAGATAT-GCATACAGCAGG	12-116.200
196	18049-L22439	PLCG2	16q23	TCCTGTCGCCAG-CTGAGGAGGCGG	16-080.548
232	16428-L25931	MYO5B	18q21	TGGACCCTGATT-GATTTTTATGAT	18-045.743
494	19137-L26747	PSMG1	21q22	TGGAAGCTTTTA-AGCCTATACTTT	21-039.471

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

Related SALSA MLPA probemixes

- **ME012 MGMT-IDH1-IDH2:** Contains probes for detection of *MGMT* methylation status and the four most common *IDH1/2* point mutations.
- **ME024 9p21 CDKN2A/2B region:** Contains probes for detection of methylation status of *CDKN2A/2B* promoter regions and probes for detection of copy number of *MIR31, MTAP, CDKN2A/2B* and *PAX5* genes.
- **P088 Oligodendroglioma:** Contains probes for the 1p arm, the 19q arm, *IDH1/2* and *CDKN2A/2B*.
- **P105 Glioma-2:** Contains probes for *EGFR, TP53, PTEN, CDKN2A/2B, PDGFRA, CDK4, MDM2* and *NFKBIA*.
- **P133 Kallman-2:** Contains 11 probes for 10 of 18 exons of *FGFR1* gene.
- **P298 BRAF-HRAS-KRAS-NRAS:** Contains probes for each exon of *BRAF* gene.
- **P419 CDKN2A/2B-CDK4:** Contains 14 probes for the *CDKN2A* gene, nine probes for the *CDKN2B* gene, nine probes for *CDK4* gene and in total 10 flanking probes for the *CDKN2A* and *CDKN2B* genes.

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P370 Product history	
Version	*Modification*
C1	One *RAF1* target probe and four reference probes have been replaced, and several probes have a change in length but no change in the sequence targeted. SALSA Binning DNA for this probemix has been changed from SD043 to SD054.
B1	Several new probes have been included for *FGFR1, MYB* and *MYBL1*, several probes have been replaced for 3p, 7q and 9p arms and most of the reference probes have been replaced. Also, the control fragments have been replaced (QDX2).
A1	First release.

Implemented changes in the product description

Version C1-04 – 17 January 2023 (02P)
- Exon numbering of the *CDKN2A* gene has been changed according to MANE in Table 2. See also the explanation on page 2.
- Ligation sites of the CDKN2A probes 16533-L26121 and 10333-L17690 have been updated.

Version C1-03 – 10 January 2023 (02P)
- Information about possible small signal for BRAF V600E mutation probe on a sample with the V600K mutation added to the P370 specific notes section and Tables 1 and 2.
- One new reference added for the P370 probemix on page 10.

Version C1-02 – 25 March 2021 (02P)
- SD054 details (plasmid DNA is used instead of synthetic DNA) are updated on page 3.
- New reference added for the P370 probemix on page 10.

Version C1-01 — 23 April 2020 (02P)
- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2a/b).
- Various minor textual or layout changes.
- SALSA Binning DNA for this probemix has been changed from SD043 to SD054 on page 3.
- Ligation sites of the probes targeting the *SRGAP3, MYB, KIAA1549, TACC1* and *MYBL1* genes updated according to new version of the NM_ reference sequence.
- For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36).
- Related SALSA MLPA probemixes list has been updated on page 9.

Version 12 – 18 June 2019 (T08)
- Ligation sites of the probes targeting the *BRAF* gene updated according to new version of the NM_ reference sequence.

Version 11 – 03 April 2019 (T08)
- New references for the P370 probemix added on page 2.
- Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.
- Minor modifications throughout the text.

Version 10 – 20 September 2017 (T08)
- Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included).
- New related MLPA probemix and a reference added on page 2.
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.

- NM sequences and ligations sites for the *MYBL1* and *IDH2* genes have been updated in Table 2.
- Various small textual changes throughout the document.

Version 09 – 03 January 2017 (T08)

- Various textual changes on page 1.
- New references added on page 2.
- Table 2: information on which exon is targeted by the flanking probes removed.

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