Product Description
SALSA® MLPA® Probemix P105-D3 Glioma-2

To be used with the MLPA General Protocol.

Version D3
As compared to version D2, one reference probe has been replaced and one reference probe has been added, and several probes have a change in length but no change in the sequence targeted. For complete product history see page 11.

Catalogue numbers:
- **P105-025R:** SALSA MLPA Probemix P105 Glioma-2, 25 reactions.
- **P105-050R:** SALSA MLPA Probemix P105 Glioma-2, 50 reactions.
- **P105-100R:** SALSA MLPA Probemix P105 Glioma-2, 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.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 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 P105 Glioma-2 is a **research use only (RUO)** assay for the detection of deletions or duplications in the following genes *PDGFRA* (4q12), *EGFR* (7p11.2), *CDKN2A* (9p21.3), *PTEN* (10q23.31), *CDK4, MIR26A2, MDM2* (12q14-q15) and *NFKBIA* (14q13.2) and *TP53* (17p13.1).

Gliomas are the most common primary brain tumours and account for one third of central nervous system (CNS) tumours. Gliomas comprise a very heterogeneous group of CNS neoplasms derived from glial cells. There are several oncogenes and tumour suppressor genes, which have been shown to undergo copy number changes in gliomas. Somatic mutations, disruptions, or copy number aberrations in three critical signalling pathways, a) the RTK/PI3K pathway (involving e.g. *EGFR, PDGFRA* and *PTEN* genes), b) the p53 pathway (involving e.g. *CDKN2A, MDM2* and *TP53* genes) and c) the RB pathway (involving e.g. *CDKN2A* and *CDK4* genes), are suggested to contribute to the development of gliomas (Cancer Genome Atlas Research Network 2008). Please see Table 2 for more details.

Epidermal growth factor receptor (EGFR) and its ligands are cell signalling molecules involved in diverse cellular functions. These include cell proliferation, differentiation, motility and survival, and tissue development. Glioblastomas often express EGFR variant III (EGFRvIII), a constitutively active genomic deletion variant of *EGFR* which is characterised by deletions of exons 2-7 of the *EGFR* gene (Sugawa et al. 1990). This probemix allows detection of deletions of *EGFR* that result in EGFRvIII. Please see Table 2 for more details.

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 P105-D3 Glioma-2 product description is the exon numbering from the LRG_309 for *PDGFRA*, LRG_304 for *EGFR,* LRG_311 for *PTEN*, LRG_490 for *CDK4*, NR_029847.1 for *MIR26A2*, NG_016708.1 for *MDM2*, LRG_89 for *NFKBIA*, and LRG_321 for *TP53*. **From product description version D3-03 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 NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG, NG and NR 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 P105-D3 Glioma-2 contains 56 MLPA probes with amplification products between 126 and 500 nucleotides (nt). This includes in total 43 probes for the *PDGFRA*, *EGFR*, *CDKN2A*, *PTEN*, *CDK4, MIR26A2, MDM2*, *NFKBIA* and *TP53* genes. In addition, 13 reference probes are included that detect target relatively copy number stable regions in various cancer types including gliomas. Complete probe sequences and the identity of the genes detected by the reference probes are available in Table 3 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 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)

MLPA technique

The principles of the MLPA technique (Schouten et al. 2002) are described in the 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).

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 different healthy individuals without a history of glioma. More information regarding the selection and use of reference samples can be found in the 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 indicated in the table below from the Coriell Institute and Leibniz Institute DSMZ have been tested with this P105-D3 probemix at MRC Holland and can be used as positive control samples. The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Source	Chromosomal position (hg18) of copy number alteration*	Altered target genes in P105-D3	Expected copy number alteration
NA07081	Coriell	7p11.2	*EGFR*	Heterozygous duplication
NA02819	Coriell	9p21.3	*CDKN2A*	Heterozygous duplication
NA03226	Coriell	9p21.3	*CDKN2A*	Heterozygous duplication
NA05067	Coriell	9p21.3	*CDKN2A*	Heterozygous duplication
NA01750	Coriell	9p21.3	*CDKN2A*	Heterozygous duplication
NA20125	Coriell	10q23.31	*PTEN*	Heterozygous duplication
NA06801	Coriell	14q13.2	*NFKBIA*	Heterozygous duplication
SK-N-MC (ACC-203)◊	DSMZ	10q23.31	*PTEN*	Heterozygous deletion
		17p13.1	*TP53*	Heterozygous deletion, except homozygous deletion of exon 2a
IGR-37 (ACC-237)	DSMZ	4q12	*PDGFRA*	Heterozygous deletion
		9p21.3	*CDKN2A*	Heterozygous deletion, except homozygous deletion of exons 3-4
		10q23.31	*PTEN*	Heterozygous deletion
		12q14-q15	*CDK4, MIR26A2, MDM2*	Heterozygous deletion
		14q13.2	*NFKBIA*	Heterozygous deletion
		17p13.1	*TP53*	Heterozygous deletion
SAOS-2 (ACC-243)◊	DSMZ	4q12	*PDGFRA*	Heterozygous duplication
		12q14-q15	*CDK4, MIR26A2, MDM2*	Homozygous duplication
		14q13.2	*NFKBIA*	Homozygous duplication
		17p13.1	*TP53*	Homozygous deletion of exons 2a-11

* 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 P105-D3 Glioma-2 probemix.

◊ In this indicated cell line sample some of the reference probes are affected by CNAs.

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.

Interpretation of results

The standard deviation of each individual probe over all the reference samples should be ≤0.10. When this criterion is fulfilled, the following cut-off values for the final ratio (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	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 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.
- 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.

P105 specific note

- 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 many tumour samples, genetic alterations in *PDGFRA, EGFR, CDKN2A, PTEN, CDK4, MIR26A2, MDM2, NFKBIA* and *TP53* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P105 Glioma-2.
- 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. 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.
- 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 solid 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 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 *EGFR* exons 2 and 4 but not exon 3) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P105-D3 Glioma-2

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]				Location (hg18) in kb
		Reference	*EGFR*	*PTEN*	Other targets	
64-105	Control fragments – see table in probemix content section for more information					
126	Reference probe 18709-L21698	5q31				05-132,038
131	Reference probe 16316-L22397	3q21				03-130,000
137	**EGFR probe** 06121-L20393		Exon 6			07-055,188
142	**CDKN2A probe** 18753-L24594				Upstream	09-021,985
148	Reference probe 14279-L15949	15q13				15-025,951
157	**NFKBIA probe** 18758-L24126				Exon 3	14-034,942
161	**EGFR probe** 05438-L24607		Exon 5			07-055,186
167	**TP53 probe** 01588-L06028				Exon 1	17-007,531
172	**EGFR probe** 06405-L24605		Exon 1			07-055,054
178	**PTEN probe** 17314-L20922			Exon 3		10-089,675
184	**EGFR probe** 05440-L04856		Exon 7			07-055,189
190	**PTEN probe** 06729-L06339			Exon 2		10-089,644
196	**TP53 probe** 01996-L09268				Exon 2a	17-007,521
202	Reference probe 04542-L03931	2q24				02-166,567
208 ±	**PTEN probe** 17391-L21278			Exon 4		10-089,681
214 #	**PTEN probe** 07686-L15591			Exon 9		10-089,716
220 ¥	**EGFR probe** 17208-L23696		Exon 4			07-055,182
226 ¥	**PTEN probe** 17387-L24930			Exon 1		10-089,614
231	**NFKBIA probe** 18757-L24608				Exon 5	14-034,942
238	Reference probe 15174-L16949	3p13				03-072,510
244	Reference probe 12431-L13432	22q12				22-032,003
250	**CDKN2A probe** 16060-L22417				Intron 1	09-021,965
256	**EGFR probe** 05959-L24612		Exon 13			07-055,197
263	**TP53 probe** 02376-L21409				Exon 4b	17-007,519
269	**EGFR probe** 05969-L24610		Exon 23			07-055,234
277	**NFKBIA probe** 18759-L24127				Exon 4	14-034,942
283 ± ‡	**TP53 probe** 01999-L21074				Exon 7	17-007,518
292	Reference probe 11900-L12706	6p12				06-052,028
299	**TP53 probe** 17420-L29693				Exon 3	17-007,520
309	**CDKN2A probe** 17814-L22631				Exon 3	09-021,958
316	**TP53 probe** 17421-L24613				Exon 5	17-007,519
324	**PTEN probe** 03639-L24076			Exon 6		10-089,702
331	Reference probe 08905-L24614	11p11				11-047,316
339	**CDK4 probe** 17815-L22422				Exon 4	12-056,431
346	**EGFR probe** 17210-L24618		Exon 16			07-055,206
353	**TP53 probe** 17422-L24617				Exon 10	17-007,515
360 #	**PTEN probe** 17397-L24616			Exon 8		10-089,711
366	Reference probe 06760-L24615	8q12				08-061,928
373 #	**PTEN probe** 03638-L22839			Exon 5		10-089,683
385	**CDKN2A probe** 17817-L23295				Exon 2	09-021,961
391	**PDGFRA probe** 03107-L02038				Exon 3	04-054,822
399	**EGFR probe** 05436-L24070		Exon 3			07-055,178
407	**PDGFRA probe** 18755-L24123				Exon 22	04-054,851
413 ¥	**MDM2 probe** 18255-L32191				Exon 10	12-067,517
421	**EGFR probe** 21280-L27637		Exon 2			07-055,178
427 *	Reference probe 08839-L32428	2p13				02-071,767
436 ¥	**EGFR probe** 02063-L32279		Exon 8			07-055,191
443 ¥	**PDGFRA probe** 18756-L32278				Exon 5	04-054,826
450 ¥	**TP53 probe** 17424-L27139				Exon 11	17-007,514
454 ¥	**CDK4 probe** 18752-L32277				Exon 8	12-056,428

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]				Location (hg18) in kb
		Reference	*EGFR*	*PTEN*	Other targets	
463 *	Reference probe 15970-L32276	18p11				18-012,784
472 ¥	**MIR26A2 probe** 18710-L32275				Exon 1	12-056,505
479 ¥ #	**PTEN probe** 17386-L32274			Exon 7		10-089,708
486 ¥	**MDM2 probe** 07178-L32273				Exon 2	12-067,489
493	Reference probe 16456-L24172	18q21				18-045,630
500	Reference probe 17001-L22947	20q11				20-034,954

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

* New in version D3.

¥ Changed in version D3. Minor alteration, no change in sequence detected.

± SNP rs587780544 could influence the probe signal at 208 nt and SNP rs121912660 could influence the signal of the probe at 283 nt. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

‡ The ligation site of this probe is located on a common mutational hotspot both in germline and somatic samples as reported by the IARC TP53 Database (http://p53.iarc.fr/). 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.

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. P105-D3 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene / exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
PDGFRA gene, at 4q12, indicated ligation sites are according to NM_006206.6. *PDGFRA* amplification or gain is detected in 2-18% of gliomas (Alentorn et al. 2012; Bleeker et al. 2012; Cancer Genome Atlas Research Network, 2008). The frequency of *PDGFRA* amplification is shown to increase with tumour grade, and the amplification of *PDGFRA* is suggested to be a poor prognostic factor in anaplastic gliomas and in *IDH1*-mutant de novo glioblastoma multiforme (GBMs) (Alentorn et al. 2012; Phillips et al. 2013).					
391	03107-L02038	*PDGFRA*, ex 3	299-300	GGAGAGTGAAGT-GAGCTGGCAGTA	3,8 kb
443	18756-L32278	*PDGFRA*, ex 5	846-847	ACCTGTGCTGTT-TTTAACAATGAG	25,4 kb
407	18755-L24123	*PDGFRA*, ex 22	3067-3068	ATCCTGCTGTGG-CACGCATGCGTG	-
EGFR gene, at 7p11.2, indicated ligation sites are according to NM_005228.5. *EGFR* amplification is frequently detected in primary glioblastomas and is associated with poor prognosis (Hurtt et al. 1992). Glioma patients with amplification of wt-*EFGR* have been suggested to respond well to EGFR kinase inhibitors, especially in combination therapy (Joshi et al. 2012). **EGFR variant III** (EGFRvIII) is an oncogenic, constitutively active mutant form of *EGFR* that is commonly expressed in glioblastoma. EGFRvIII is formed by an in-frame genomic deletion of exons 2 to 7 of *EGFR*, producing a truncated receptor lacking a portion of the extracellular ligand binding domain. The expression of EGFRvIII is suggested to confer worse prognosis than wt-*EGFR* expression alone (Shinojima et al. 2003).					
172	06405-L24605	*EGFR*, ex 1	223-224	CCGTCCAGTATT-GATCGGGAGAGC	123,1 kb
421	21280-L27637	*EGFR*, ex 2	440-441	TAACTGTGAGGT-GGTCCTTGGGAA	1,0 kb
399	05436-L24070	*EGFR*, ex 3	533-534	TTATGTCCTCAT-TGCCCTCAACAC	3,4 kb
220	17208-L23696	*EGFR*, ex 4	817-818	ACCACCTGGGCA-GCTGTAAGTGTC	4,6 kb
161	05438-L24607	*EGFR*, ex 5	852-853	TGTCCCAATGGG-AGCTGCTGGGGT	1,2 kb
137	06121-L20393	*EGFR*, ex 6	914-915	CTGTGCCCAGCA-GTGCTCCGGGCG	1,5 kb
184	05440-L04856	*EGFR*, ex 7	1117-1118	AGGGCAAATACA-GCTTTGGTGCCA	1,8 kb
436	02063-L32279	*EGFR*, ex 8	1215-1216	AGCTATGAGATG-GAGGAAGACGGC	5,7 kb
256	05959-L24612	*EGFR*, ex 13	1862-1863	CCGAGGCAGGGA-ATGCGTGGACAA	9,6 kb
346	17210-L24618	*EGFR*, ex 16	2169-2170	CTTGAAGGCTGT-CCAACGAATGGG	27,6 kb
269	05969-L24610	*EGFR*, ex 23	3037-3038	AGATCTCCTCCA-TCCTGGAGAAAG	-

Length (nt)	SALSA MLPA probe	Gene / exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
CDKN2A gene, at 9p21.3. Deletion or mutation of *CDKN2A* is detected in ~50% of glioma samples (Beroukhim et al. 2007; Cancer Genome Atlas Research Network, 2008) and a homozygous loss of *CDKN2A* is suggested to be a progression-associated genetic marker in glioma (Appay et al. 2019, Lu et al. 2020).					
309	17814-L22631	*CDKN2A*, ex 3 (4)	NM_000077.5; 830-831; NM_058195.4; 904-905	TTGCGAGCCTCG-CAGCCTCCGGAA	3,0 kb
385	17817-L23295	*CDKN2A*, ex 2 (3)	NM_000077.5; 430-431; NM_058195.4; 504-505	TGCGCGCGGCTG-CGGGGGGCACCA	4,1 kb
250	16060-L22417	*CDKN2A*, intron 1 (2)	NM_000077.5; 138 nt before ex 1; NM_058195.4; 3.8 kb before ex 2	GCCTGGAAAGAT-ACCGCGGTCCCT	19,6 kb
142	18753-L24594	*CDKN2A*, up (ex 1)	NM_000077.5; 19.7 kb before ex 1; NM_058195.4; 175 nt before ex 1	CGCAGGGCTCAG-AGCCGTTCCGAG	-
PTEN gene, at 10q23.31, indicated ligation sites are according to NM_000314.8. Deletion or LOH of 10q is the most common genomic alteration found in primary and secondary glioblastomas (Ohgaki et al. 2004; Beroukhim et al. 2007). Co-expression of *EGFRvIII* and *PTEN* has been suggested to associate with favourable clinical response to EGFR kinase inhibitors (Mellinghoff et al. 2005), while loss of *PTEN* expression seems to correlate with resistance to gefitinib (Guillamo et al. 2009).					
226	17387-L24930	*PTEN*, ex 1	781-782	CCTGCAGAAGAA-GCCCCGCCACCA	29,9 kb
190	06729-L06339	*PTEN*, ex 2	217 nt after ex 2 reverse	TATCACATAAGT-ACCTGATTATGT	31,5 kb
178	17314-L20922	*PTEN*, ex 3	226 nt after ex 3	TTGATCTGCTTT-AAATGACTTGGC	5,2 kb
208 ±	17391-L21278	*PTEN*, ex 4	14 nt before ex 4 reverse	AAAAGAAAAGTT-TAAAAGTGATAT	2,1 kb
373 #	03638-L22839	*PTEN*, ex 5	1250-1251	GGTGTAATGATA-TGTGCATATTTA	19,1 kb
324	03639-L24076	*PTEN*, ex 6	1473-1472 reverse	CTTACTGCAAGT-TCCGCCACTGAA	5,7 kb
479 #	17386-L32274	*PTEN*, ex 7	1550-1551	ACACGACGGGAA-GACAAGTTCATG	3,1 kb
360 #	17397-L24616	*PTEN*, ex 8	1787-1788	AATGACAAGGAA-TATCTAGTACTT	5,4 kb
214 #	07686-L15591	*PTEN*, ex 9	3003-3002 reverse	ACAGCATCTGAA-TTTTAGCACTGG	-
CDK4, ***MIR26A2*** and ***MDM2*** at 12q14.1-q15 Amplification of 12q14-q15, which harbours the *CDK4* and *MDM2* genes, is detected in 14-18% of newly diagnosed glioblastoma patients (Cancer Genome Atlas Research Network, 2008). The *MIR26A2* gene (at 12q14.1) is also shown to be amplified in high-grade glioma, and this amplification is correlated with monoallelic *PTEN* deletion (Huse et al. 2009). In addition, *MIR26A2* is shown to regulate *PTEN* expression and, thereby, *MIR26A2* amplification provides a significant growth advantage for tumour cells (Kim et al. 2010).					
454	18752-L32277	*CDK4*, ex 8	NM_000075.4; 1157-1158	TCTCTGAGGCTA-TGGAGGGTCCTC	2,5 kb
339	17815-L22422	*CDK4*, ex 4	NM_000075.4; 644-645	GGCCTGGCCAGA-ATCTACAGCTAC	73,7 kb
472	18710-L32275	*MIR26A2*, ex 1	NR_029847.1; 43-42 reverse	AGGCCTCACAGA-TGGAAACAGCCT	11,0 **M**b
486	07178-L32273	*MDM2*, ex 2	NM_002392.6; 355-356	CCTACTGATGGT-GCTGTAACCACC	27,5 kb
413	18255-L32191	*MDM2*, ex 10	NM_002392.6; 1195-1194 reverse	TCAGGATCTTCT-TCAAATGAATCT	-
NFKBIA gene, at 14q13.2, indicated ligation sites are according to NM_020529.3. Deletion of NF-κB inhibitor (*NFKBIA*) is detected in about 25% of glioblastomas (Bredel et al. 2011). It is suggested that deletion of *NFKBIA* and amplification of *EGFR* show a pattern of mutual exclusivity and that *NFKBIA* deletion could substitute for *EGFR* amplification (Bredel et al. 2011).					
231	18757-L24608	*NFKBIA*, ex 5	808-807 reverse	CTTCAACAGGAG-TGACACCAGGTC	0,2 kb
277	18759-L24127	*NFKBIA*, ex 4	706-707	GCATCGTGGAGC-TTTTGGTGTCCT	0,6 kb
157	18758-L24126	*NFKBIA*, ex 3	445-446	ATTCGTAGACTC-CACTCCACTTGG	-
TP53 gene, at 17p13.1, indicated ligation sites are according to NM_000546.6. Mutations or homozygous deletions of *TP53* are detected in 35% of newly diagnosed diffuse glioblastomas (Cancer Genome Atlas Research Network, 2008). Clinical and prognostic significance of *TP53* deletions is still under evaluation.					
450	17424-L27139	*TP53*, ex 11	1300-1301	CTCATGTTCAAG-ACAGAAGGGCCT	1,0 kb
353	17422-L24617	*TP53*, ex 10	1188-1189	TGAGGCCTTGGA-ACTCAAGGATGC	3,1 kb
283 ± ‡	01999-L21074	*TP53*, ex 7	981-982	CTGTCCTGGGAG-AGACCGGCGCAC	1,2 kb
316	17421-L24613	*TP53*, ex 5	735-736	TATCCGAGTGGA-AGGAAATTTGCG	0,3 kb
263	02376-L21409	*TP53*, ex 4b	546-547	CAAGATGTTTTG-CCAACTGGCCAA	0,8 kb
299	17420-L29693	*TP53*, ex 3	451-450 reverse	TAGCTGCCCTGG-TAGGTTTTCTGG	0,6 kb
196	01996-L09268	*TP53*, ex 2a	117-118	CTCTTGCAGCAG-CCAGACTGCCTT	10,8 kb
167	01588-L06028	*TP53*, ex 1	58-59	TCCGGGGACACT-TTGCGTTCGGGC	-

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

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

± SNP rs587780544 could influence the probe signal at 208 nt and SNP rs121912660 could influence the signal of the probe at 283 nt. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

‡ The ligation site of this probe is located on a common mutational hotspot both in germline and somatic samples as reported by the IARC TP53 Database (http://p53.iarc.fr/). 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.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Table 3. Reference probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene	Chromosomal band (hg18)	Partial sequence (24 nt adjacent to ligation site)	Location (hg18) in kb
427	08839-L32428	*DYSF*	2p13	TGCCATGAAGCT-GGTGAAGCCCTT	02-071,767
202	04542-L03931	*SCN1A*	2q24	AACACCACAACT-GGTGACAGGTTT	02-166,567
238	15174-L16949	*RYBP*	3p13	GAATCTTTCTGA-AATTGCACATGG	03-072,510
131	16316-L22397	*RAB7A*	3q21	CACAATAGGAGC-TGACTTTCTGAC	03-130,000
126	18709-L21698	*IL4*	5q31	ATCGACACCTAT-TAATGGGTCTCA	05-132,038
292	11900-L12706	*PKHD1*	6p12	TGCTCTCTGGAT-TCAAGACTGAAA	06-052,028
366	06760-L24615	*CHD7*	8q12	GATTTTTACCGT-GTGGTATCCACC	08-061,928
331	08905-L24614	*MYBPC3*	11p11	CGTGGGAGAGGA-CTCCTGCACAGT	11-047,316
148	14279-L15949	*OCA2*	15q13	AGGGGGAAAATA-TCTCACCCTTTC	15-025,951
463	15970-L32276	*PTPN2*	18p11	TCTGCACTAGTA-ACTGACAGTGCT	18-012,784
493	16456-L24172	*MYO5B*	18q21	TCTGACTCATCA-TCTCCCACTTCC	18-045,630
500	17001-L22947	*SAMHD1*	20q11	CCCTGTCACCTC-AAGTTTGAGGAT	20-034,954
244	12431-L13432	*LARGE1*	22q12	ATCCACCTGGTA-TGGTCGACGGGG	22-032,003

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

Related SALSA MLPA probemixes

- **P088 Oligodendroglioma 1p-19q**: Contains probes for chromosomes 1p and 19q, *CDKN2A/2B* and *IDH1* R132H/C and *IDH2* R172M/K point mutations.
- **P370 BRAF-IDH1-IDH2**: Contains probes for chromosomes 3p, 6q, 7q, 8p, 8q, *CDKN2A/2B* and *BRAF* V600E, *IDH1* R132H/C and *IDH2* R172M/K point mutations.
- **P315 EGFR:** Contains more probes for the *EGFR* gene and *EGFR* T790M and L858R point mutations.
- **P225 PTEN**: Contains more probes for the *PTEN* gene.
- **P056 TP53:** Contains more probes for the *TP53* gene.
- **ME012 MGMT-IDH1-IDH2:** Contains probes for detection of methylation status of *MGMT*, and for *IDH1* R132H/C and *IDH2* R172M/K point mutations.
- **ME024 9p21 CDKN2A/2B region:** Contains more probes for the *CDKN2A/2B* genes and 9p21.3 region and also allows detection of methylation status of *CDKN2A* and *CDKN2B*.
- **P419 CDKN2A/2B-CDK4:** Contains more probes for the *CDKN2A, CDKN2B* and *CDK4* genes.
- **P323 CDK4-HMGA2-MDM2:** Contains more probes for the *CDK4* and *MDM2* genes.

References

- Alentorn A et al. (2012). Prevalence, clinico-pathological value, and co-occurrence of PDGFRA abnormalities in diffuse gliomas. *Neuro Oncol*. 14:1393-403.

- Appay R et al (2019). CDKN2A homozygous deletion is a strong adverse prognosis factor in diffuse malignant IDH-mutant gliomas. *Neuro Oncol*. 21:1519-28.
- Atanesyan L et al. (2017). Optimal fixation conditions and DNA extraction methods for MLPA analysis on FFPE tissue-derived DNA. *Am J Clin Pathol*. 147:60-8.
- Beroukhim R et al. (2007). Assessing the significance of chromosomal aberrations in cancer: methodology and application to glioma. *Proc Natl Acad Sci U S A*. 104:20007-12.
- Bleeker FE et al. (2012). Recent advances in the molecular understanding of glioblastoma. *J Neurooncol*. 108:11-27.
- Bredel M et al. (2011). NFKBIA deletion in glioblastomas. *N Engl J Med*. 364:627-37.
- Cancer Genome Atlas Research Network (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 455:1061-8.
- Guillamo JS et al. (2009). Molecular mechanisms underlying effects of epidermal growth factor receptor inhibition on invasion, proliferation, and angiogenesis in experimental glioma. *Clin Cancer Res*. 15:3697-704.
- Hömig-Hölzel C and Savola S. (2012). Multiplex ligation-dependent probe amplification (MLPA) in tumor diagnostics and prognostics. *Diagn Mol Pathol*. 21:189-206.
- Hurtt MR et al. (1992). Amplification of epidermal growth factor receptor gene in gliomas: histopathology and prognosis. *J Neuropathol Exp Neurol*. 51:84-90.
- Huse JT et al. (2009). The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. *Genes Dev*. 23:1327-37.
- Joshi AD et al. (2012). Evaluation of tyrosine kinase inhibitor combinations for glioblastoma therapy. *PLoS ONE.* 7(10):e44372.
- Kim H et al. (2010). Integrative genome analysis reveals an oncomir/oncogene cluster regulating glioblastoma survivorship. *Proc Natl Acad Sci U S A*. 107:2183-8.
- Lu VM et al. (2020). The prognostic significance of CDKN2A homozygous deletion in IDH-mutant lower-grade glioma and glioblastoma: a systematic review of the contemporary literature. *J Neurooncol.* 148:221-9.
- Mellinghoff IK et al. (2005). Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N Engl J Med*. 353:2012-24.
- Ohgaki H et al. (2004). Genetic pathways to glioblastoma: a population-based study. *Cancer Res*. 64:6892-9.
- Phillips JJ et al. (2013). PDGFRA amplification is common in pediatric and adult high-grade astrocytomas and identifies a poor prognostic group in IDH1 mutant glioblastoma. *Brain Pathol*. 23:565-73.
- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
- Shinojima N et al. (2003). Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme. *Cancer Res*. 63:6962-70.
- Sugawa et al. (1990). Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. *Proc Natl Acad Sci U S A*. 87:8602-6.
- Varga RE et al. (2012). MLPA-based evidence for sequence gain: pitfalls in confirmation and necessity for exclusion of false positives. *Anal Biochem.* 421:799-801.

Selected publications using SALSA MLPA Probemix P105 Glioma-2

- Boots-Sprenger S et al. (2013). Significance of complete 1p/19q co-deletion, IDH1 mutation and MGMT promoter methylation in gliomas: use with caution. *Mod Pathol.* 26:922-9.
- Franceschi S et al. (2015). Investigating molecular alterations to profile short- and long-term recurrence-free survival in patients with primary glioblastoma. *Oncol Lett*. 10:2599-3606.
- Fukai J et al. (2020). Molecular characteristics and clinical outcomes of elderly patients with IDH-wildtype glioblastomas: comparative study of older and younger cases in Kansai Network cohort. *Brain Tumor Pathol.* 37:50-9.

- García-Claver A et al. (2013). Gene expression changes associated with erlotinib response in glioma cell lines. *Eur J Cancer.* 49:1641-53.
- Gessi M et al. (2013). H3.3 G34R mutations in pediatric primitive neuroectodermal tumors of central nervous system (CNS-PNET) and pediatric glioblastomas: possible diagnostic and therapeutic implications? *J Neurooncol*. 112:67-72.
- Guillaudeau A et al. (2012). EGFR soluble isoforms and their transcripts are expressed in meningiomas. *PLoS One*. 7(5):e37204.
- Kanamori M et al. (2016). Malignant transformation of diffuse astrocytoma to glioblastoma associated with newly developed BRAFV600E mutation. *Brain Tumor Pathol*. 33:50-6.
- Navarro L et al. (2015). Alteration of major vault protein in human glioblastoma and its relation with EGFR and PTEN status. *Neuroscience.* 297:243-51.
- Molenaar RJ et al. (2014). Rhabdoid glioblastoma in a child: case report and literature review. *Neuro Oncol.* 16:1263-73.
- Motomura K et al. (2012). Immunohistochemical analysis-based proteomic subclassification of newly diagnosed glioblastomas. *Cancer Science.* 103:1871–9.
- Muñoz-Hidalgo L et al. (2020). Somatic copy number alterations are associated with EGFR amplification and shortened survival in patients with primary glioblastoma. *Neoplasia*. 22:10-21.
- Serna E et al. (2014). Correlation between EGFR amplification and the expression of microRNA-200c in primary glioblastoma multiforme. *PLoS One*. 9(7):e102927.
- Shibahara I et al. (2013). The expression status of CD133 is associated with the pattern and timing of primary glioblastoma recurrence. *Neuro-Oncology*. 15:1151-9.
- Shibahara I et al. (2018). Glioblastoma in neurofibromatosis 1 patients without IDH1, BRAF V600E, and TERT promoter mutations. *Brain Tumor Pathol*. 35:10-8.
- Trabelsi S et al. (2015). Adult recurrent pilocytic astrocytoma: Clinical, histopathological and molecular study. *Neurochirurgie*. 61:392-7.
- Weller M et al. (2014). Assessment and prognostic significance of the epidermal growth factor receptor vIII mutation in glioblastoma patients treated with concurrent and adjuvant temozolomide radiochemotherapy. *Int J Cancer*. 134:2437-47.
- Westphal M et al. (2015). A randomised, open label phase III trial with nimotuzumab, an anti-epidermal growth factor monoclonal antibody in the treatment of newly diagnosed adult glioblastoma. *Eur J Cancer*. 51:522-32.

P105 product history	
Version	*Modification*
D3	One reference probe has been replaced and one reference probe has been added, and several probes have a change in length but no change in the sequence targeted.
D2	Two probes have a small change in length, but no change in the sequence targeted.
D1	Several target probes have been replaced, probes for new target genes/regions have been added (*PDGFRA, NFKBIA*, 12q14-q15), and most of the reference probes have been replaced.
C2	The 88 and 96 nt control fragments have been replaced (QDX2).
C1	One *EGFR* probe is replaced; four extra reference probes have been included, four probes have a slightly different length and two extra control fragments at 100-105 have been added.
B1	One *PTEN* probe has been replaced by two new probes.
A1	First release.

Implemented changes in the product description
Version D3-03 – 17 January 2023 (04P) - Exon numbering of the *CDKN2A* gene has been changed according to MANE in Table 1 and 2. See also explanation on page 2. - Ligation site of the CDKN2A probe 18753-L24594 has been updated. Version D3-02 – 04 May 2021 (04P) - Remark added, in section Positive control DNA samples, that some reference probes are affected by CNAs in the indicated cell line samples. Version D3-01 – 21 April 2021 (04P) - Product description adapted to a new product version (version number changed, changes in Table 1, Table 2 and Table 3) and adapted to a new template. - Positive samples added on page 3. - Ligation sites of the probes targeting the PDGFRA, CDKN2A, PTEN, CDK4, MDM2, NFKBIA and TP53 genes updated according to newest version of the NM_ reference sequence. - Warning added to Table 1 and 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. - For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36). - New references added on pages 10-11. - Various minor textual and layout changes. Version 29 – 11 April 2019 (T08) - Ligation sites of the probes targeting the EGFR gene updated according to new version of the NM_reference sequence. - Ligation sites of the probes targeting the CDKN2A gene are added also for the NM_058195.3 reference sequence. Version 28 – 12 December 2018 (T08) - Related probemixes updated - ME012-MGMT-IDH1-IDH2 probemix added on page 2. - New reference added to 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 sequence and ligation sites for CDK4 probes are updated in Table 2. - Minor layout changes. Version 27 – 28 April 2017 (T08) - Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included). - Exon numbering of the EGFR and MDM2 genes has been changed in page Table 1 and Table 2a. - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. - New references added on page 1. - Various minor textual and layout changes.

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