Product Description
SALSA® MLPA® Probemix P225-E1 PTEN

To be used with the MLPA General Protocol.

Version E1
For complete product history see page 11.

Catalogue numbers:
- **P225-025R:** SALSA MLPA Probemix P225 PTEN, 25 reactions.
- **P225-050R:** SALSA MLPA Probemix P225 PTEN, 50 reactions.
- **P225-100R:** SALSA MLPA Probemix P225 PTEN, 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.

Intended purpose
The SALSA MLPA Probemix P225 PTEN is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplications in *PTEN* in genomic DNA isolated from human peripheral whole blood specimens. P225 PTEN is intended to confirm a potential cause for and clinical diagnosis of PTEN Hamartoma Tumour Syndrome (PHTS) and for molecular genetic testing of at-risk family members. PHTS includes Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome (BRRS), *PTEN*-related Proteus syndrome (PS), and Proteus-like syndrome (PLS). This probemix can also be used for the detection of deletions or duplications in the *PTEN* pseudogene (*PTENP1*) in a research setting.

Copy number variations (CNVs) detected with P225 PTEN should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Most defects in *PTEN* are point mutations, none of which will be detected by MLPA. It is therefore recommended to use this assay in combination with sequence analysis.

Assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, parental evaluation, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

This device is not intended to be used for standalone diagnostic purposes, pre-implantation or prenatal testing, population screening, or for the detection of, or screening for, acquired or somatic genetic aberrations. Only in a research setting can this device be used for the detection of, or screening for, acquired or somatic genetic aberrations, e.g. from DNA extracted from formalin-fixed paraffin embedded (FFPE) or fresh tumour materials.

[1] Please note that this probemix is for In Vitro Diagnostic use (IVD) in the countries specified at the end of this product description. In all other countries, the product is for Research Use Only (RUO).

[2] To be used in combination with a SALSA MLPA Reagent Kit and Coffalyser.Net analysis software.

Clinical background
Phosphatase and tensin homolog (*PTEN*) is a tumour suppressor gene that is mutated in a large number of cancers at high frequency. Defects in the *PTEN* gene are the main cause of PTEN Hamartoma Tumour Syndrome (PHTS), which is a dominantly inherited cancer predisposition syndrome, characterized by multiple hamartomas in several areas of the body. PHTS includes Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome (BRRS), *PTEN*-related Proteus syndrome (PS), and Proteus-like syndrome (PLS) (GeneReviews: https://www.ncbi.nlm.nih.gov/books/NBK1488/).

CS (OMIM #158350) is inherited in an autosomal dominant manner and comprises 85% of PHTS cases. The incidence of CS is estimated to be 1:200,000. Affected individuals usually have macrocephaly, trichilemmomas, and papillomatous papules, and present by their late 20s. The lifetime risk of developing breast cancer is 85%, with an average age of diagnosis between 38 and 46 years. The lifetime risk for thyroid cancer (usually follicular, rarely papillary, but never medullary thyroid cancer) is approximately 35%. The risk for endometrial cancer may approach 28%.

BRRS is inherited in an autosomal dominant manner. It is present at birth and is characterized by macrocephaly, intestinal hamartomatous polyposis, lipomas, pigmented macules of the glans penis, intellectual disability (50% of the cases) and development delay. The risk of developing cancer in BRRS patients with a *PTEN* pathogenic variant is similar to patients with CS.

PS is a highly variable, severe disorder characterized by progressive segmental or patchy overgrowth of diverse tissues of all germ layers, affecting the skeleton, skin, adipose tissue and central nervous systems. PS is a rare condition with an incidence of less than 1 in 1 million people worldwide and is associated with tumours, pulmonary complications, and deep vein thrombosis.

PLS describes individuals that do not meet the diagnostic criteria of Proteus syndrome, but share many of the characteristic signs and symptoms associated with this condition. Inheritance is autosomal dominant in those with a *PTEN* mutation.

PTENP1 can regulate cellular levels of PTEN (via binding to mRNAs that target *PTEN*) and thereby suppresses cell growth. It has been shown that *PTENP1* is selectively lost in sporadic colon cancer (Poliseno et al. 2010). A specific *PTENP1* deletion (not *PTEN*) was also demonstrated in human melanoma (Poliseno et al. 2011). Furthermore, the importance of *PTENP1* as a tumour suppressor has been recently shown in head and neck squamous cell carcinoma (Liu et al. 2017). *PTENP1* copy number detection is of great importance in cancer research, however, the clinical validity of this gene is not yet fully established.

Gene structure
The *PTEN* gene spans 108 kilobases (kb) on chromosome 10q23.31 and contains 9 exons. The *PTEN* LRG_311 is available at www.lrg-sequence.org and is identical to GenBank NG_007466.2.

The *PTENP1* pseudogene spans 3.9 kb on chromosome 9p13.3 and contains 1 exon. The *PTEN* NCBI sequence is NR_023917.1. No LRG sequence is available for this pseudogene.

Transcript variants
For *PTEN,* multiple transcript variants have been described. Transcript variant 1 encodes multiple isoforms due to the use of alternative translation initiation codons and is a reference standard in the RefSeqGene project. The *PTEN* transcript variant 1 (NM_000314.8; 8515 nt; coding sequence 846-2057; https://www.ncbi.nlm.nih.gov/gene/5728) encodes three isoforms: PTEN-L (or PTENalpha), the longest isoform resulting from an upstream non-AUG (CUG) start codon; and two shorter isoforms resulting from downstream AUG start codons. PTEN, the most abundant isoform, is derived from the 5'-most AUG start codon.

The *PTENP1* gene is transcribed as a long noncoding RNA. Information about the *PTENP1* sequence was obtained from NR_023917.1 (3932 nt; https://www.ncbi.nlm.nih.gov/gene/11191).

Exon numbering

The *PTEN* exon numbering used in this P225-E1 PTEN product description is the exon numbering from the LRG_311 sequence. The *PTENP1* exon numbering used is the exon numbering from the RefSeq NR_023917.1. 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 sequences. As changes to the databases can occur after release of this product description, the NM_sequence or NR_sequence and exon numbering may not be up-to-date.

Probemix content

The SALSA MLPA Probemix P225-E1 PTEN contains 49 MLPA probes with amplification products between 130 and 496 nucleotides (nt). This includes 22 probes for the *PTEN* gene, at least one probe per exon, 10 flanking probes, and two probes for the pseudogene *PTENP1*. In addition, 15 reference probes are included that detect autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes are available 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 from peripheral whole blood specimens or, in a research setting, DNA derived from fresh or FFPE tumour tissue, 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 unrelated individuals who are from families without a history of PHTS. 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 number NA20125 from the Coriell Institute has been tested with this P225-E1 probemix at MRC Holland and can be used as a positive control sample to detect a heterozygous duplication of the complete *PTEN* gene. Several flanking probes (from *BMPR1A* until *HTRA1)* are also affected. Sample ID number ACC-50 (OPM-2) from the Leibniz Institute DSMZ has been tested at MRC Holland and can be used as a positive control sample to detect a homozygous *PTEN* exon 3-7 deletion. The quality of cell lines can change; therefore samples should be validated before use.

Performance characteristics

PTEN copy number changes explain 3% of CS and 11% of BRRS cases (GeneReviews https://www.ncbi.nlm.nih.gov/books/NBK1488/). It is unknown how many PLS and PS cases can be explained by copy number changes in *PTEN*, however, the association between *PTEN* mutations and PLS and PS is well established (Eng 2003, Loffeld et al. 2006, Orloff and Eng 2008, Smith et al. 2002, Zhou et al. 2001). Analytical performance for the detection of deletions/duplications in the *PTEN* gene is very high and can be considered >99% (based on a 2009-2022 literature review).

Analytical performance can be compromised by: SNVs or other polymorphisms in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

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 expected results of *PTEN* and *PTENP1* specific MLPA probes are allele copy numbers of 2 (normal), 1 (heterozygous deletion), 3 (heterozygous duplication), 0 (homozygous deletion) or 4 (homozygous duplication).

The standard deviation of each individual probe over all the reference samples should be ≤0.10 and the final ratio (FR) of each individual reference probe in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the cut-off values for the FR of the probes in the table below can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions. In a research setting, when using DNA samples isolated from tumour tissues, the criterium for the FR of each individual reference probe in patient samples is not applicable.

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 mosaic cases. Analysis of parental samples may be necessary for the correct interpretation of complex results.
- 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 *ITIH5, CELF2, ZNF25, PCDH15, ANXA7, LGI1, SUFU, HTRA1* 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.

P225 specific note
- In a research setting, when using DNA samples isolated 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.
- Probemix P225 does not cover the additional exon located in *PTEN* intron 5, part of transcript variant 2 (NM_001304718.2).

Limitations of the procedure
- In most populations, the major cause of genetic defects in the *PTEN* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P225 PTEN.
- 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.

- When used on tumour DNA (for research use only): 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.

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.

***PTEN* mutation database**

Cosmic *PTEN* mutation database https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=PTEN and the LOVD *PTEN* mutation database https://databases.lovd.nl/shared/genes/PTEN. We strongly encourage users to deposit positive results in the Cosmic Database and/or in the LOVD Database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report copy number changes detected by the reference probes, false positive results due to SNVs and unusual results (e.g., a duplication of *PTEN* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P225-E1 PTEN

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]			
		Reference	*PTEN*	*PTENP1*	Flanking
64-105	Control fragments – see table in probemix content section for more information				
130 *	Reference probe 19616-L26704	4p13			
137 *	Reference probe 03797-L04594	21q22			
142 *	**PTEN probe** 21999-L30837		**Exon 4**		
148 *	Reference probe 14199-L23450	2q13			
155	**PTEN probe** 13690-L15159		**Exon 6**		
162 ¥ Δ	**PTEN probe** 07685-L31034		**Exon 8**		
168 * « ¬	**SUFU probe** 21051-L31033				10q24.32
173 *	**PTEN probe** 22000-L30838		**Exon 2**		
178	**PTEN probe** 17314-L20922		**Exon 3**		
184 * Δ ¬	**ZNF25 probe** reverse-L06666				10**p**11.21
190 *	Reference probe 10710-L31035	6p12			
195 ¥ ±	**PTEN probe** 06729-L31036		**Exon 2**		
201 ¥	**PTEN probe** 18254-L31140		**Exon 1**		
208 *	**PTEN probe** 22001-L30839		**Exon 4**		
214	**PTEN probe** 07686-L15591		**Exon 9**		
222 ¥	**PTEN probe** 17387-L30897		**Exon 1**		
229 *	Reference probe 00967-L31037	3q12			
239 ¥ ¬	**PCDH15 probe** 08751-L22240				10q21.1
246 *	Reference probe 08715-L30393	9q21			
252 ¬	**CELF2 probe** 17393-L22030				10**p**14
259 ¬	**BMPR1A probe** 19351-L28591				10q23.2
266 *	Reference probe 07391-L30898	12q1			
274 ¥ ¬	**LGI1 probe** 19294-L30901				10q23.33
280 *	**PTENP1 probe** 22040-L30965			**Exon 1**	
286	**PTEN probe** 17390-L14811		**Exon 2**		
292 *	Reference probe 16435-L30904	18q21			
299 ¥ ¬	**RET probe** 18081-L30902				10q11.21
305 ¬	**ANXA7 probe** 18380-L25185				10q22.2
312 *	Reference probe 13396-L30900	6q12			
319	**PTEN probe** 03639-L21321		**Exon 6**		
328	**PTEN probe** 19293-L25664		**Exon 3**		
337 ¥	**PTEN probe** 17396-L31245		**Exon 9**		
344	**PTEN probe** 18694-L24032		**Exon 3**		
352	Reference probe 05273-L25208	2p22			
359 ±	**PTEN probe** 17397-L25715		**Exon 8**		
369 ¥ ¬	**HTRA1 probe** 08602-L30903				10q26.13
379	**PTEN probe** 03638-L24933		**Exon 5**		
391 *	Reference probe 08872-L30905	1p31			
400 *	**PTENP1 probe** 22042-L31246			**Exon 1**	
409	**PTEN probe** 13032-L22244		**Exon 5**		
418 *	Reference probe 11562-L12309	5q31			
427	Reference probe 08839-L22026	2p13			
436	**PTEN probe** 13692-L21061		**Exon 7**		
444	**PTEN probe** 17395-L21062		**Exon 9**		
454 ¬	**ITIH5 probe** 17392-L21057				10**p**14
465	**PTEN probe** 17394-L21385		**Exon 1**		
475	**PTEN probe** 17386-L22174		**Exon 7**		
486	Reference probe 13594-L22376	19p13			
496 *	Reference probe 14894-L31209	15q15			

[a] See section Exon numbering on page 3 for more information.

* New in version E1.

¥ Changed in version E1. Minor alteration, no change in sequence detected.

± SNP rs146326040 and rs562164491 (probe 195 nt and 359 nt, respectively) could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

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

« 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.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the *ITIH5, CELF2, ZNF25, PCDH15, ANXA7, LGI1, SUFU, HTRA1* flanking probes or reference probes are unlikely to be related to the condition tested.

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. P225-E1 probes arranged according to chromosomal location

Table 2a. *PTEN*

Length (nt)	SALSA MLPA probe	Gene/exon[a]	Ligation site / location	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
Centromeric flanking probes					
454 ¬	17392-L21057	*ITIH5*	10**p**14	CGAGCAGAGTCA-TCTTGGATGGTG	3.6 Mb
252 ¬	17393-L22030	*CELF2*	10**p**14	TCCCCCGGTCAT-GGTCGGAAAAGG	27.0 Mb
184 Δ ¬	05760-L06666	*ZNF25*	10**p**11.21	ATGTTATTGTGG-AATTCACCAAGG	4.6 Mb
299 ¬	18081-L30902	*RET*	10q11.21	TCCTCTACCTTA-ACCGGAGCCTGG	12.9 Mb
239 ¬	08751-L22240	*PCDH15*	10q21.1	ACAACATGCTGA-TCAAAGGGACTG	19.0 Mb
305 ¬	18380-L25185	*ANXA7*	10q22.2	AGTCCCACCAGG-TGGAGCAGGCTT	13.8 Mb
259 ¬	19351-L28591	*BMPR1A*	10q23.2	TACTTACATGCA-TGTGTTATTAAT	0.9 Mb
PTEN on 10q23.31. Ligation sites are according to NM_000314.8.					
		start codon	*846-848 (Exon 1)*		
201	18254-L31140	Exon 1	14-13 reverse	GCTGCAGCTTCC-GAGAGGAGAGAA	0.8 kb
222	17387-L30897	Exon 1	781-782	CCTGCAGAAGAA-GCCCCGCCACCA	0.2 kb
465	17394-L21385	Exon 1	5 nt after Exon 1	TTGACCTGTATC-CATTTCTGCGGC	29.5 kb
286	17390-L14811	Exon 2	Intron 1 - 925	TAAAGTACTCAG-ATATTTATCCAA	0.1 kb
173	22000-L30838	Exon 2	998-997 reverse	CTTACTACATCA-TCAATATTGTTC	0.2 kb
195 ±	06729-L31036	Exon 2	217 nt after Exon 2 reverse	TATCACATAAGT-ACCTGATTATGT	31.0 kb
344	18694-L24032	Exon 3	145 nt before Exon 3	GGGGTATTTGTT-GGATTATTTATT	0.2 kb
328	19293-L25664	Exon 3	28 nt after Exon 3	ATTTGTATGCTT-GCAAATATCTTC	0.2 kb
178	17314-L20922	Exon 3	226 nt after Exon 3	TTGATCTGCTTT-AAATGACTTGGC	5.0 kb
142	21999-L30837	Exon 4	282 nt before Exon 4	AGCACCTGAATT-TACAGTACTCTG	2.3 kb
208	22001-L30839	Exon 4	1093-1094	CAAATTTAATTG-CAGAGGTAGGTA	0.1 kb
379 #	03638-L24933	Exon 5	1250-1251	GGTGTAATGATA-TGTGCATATTTA	0.1 kb
409	13032-L22244	Exon 5	7 nt after Exon 5	AAAAGGTAAGTT-ATTTTTTGATGT	18.9 kb
155 #	13690-L15159	Exon 6	1389-1390	ATAGCTACCTGT-TAAAGAATCATC	0.1 kb
319	03639-L21321	Exon 6	1473-1472 reverse	CTTACTGCAAGT-TCCGCCACTGAA	5.7 kb
475 #	17386-L22174	Exon 7	1550-1551	ACACGACGGGAA-GACAAGTTCATG	0.1 kb
436	13692-L21061	Exon 7	4 nt after Exon 7	TAAAAAAGGTTT-GTACTTTACTTT	3.0 kb
359 # ±	17397-L25715	Exon 8	1787-1788	AATGACAAGGAA-TATCTAGTACTT	0.1 kb
162 # Δ	07685-L31034	Exon 8	45 nt after Exon 8	GACTTGTATGTA-TGTGATGTGTGT	4.1 kb
444	17395-L21062	Exon 9	6 nt before Exon 9	TAAATTTTCTTT-CTCTAGGTGAAG	0.3 kb
337 #	17396-L31245	Exon 9	2171-2170 reverse	AGAGAATTGTTC-CTATAACTGGTA	0.8 kb
214 #	07686-L15591	Exon 9	3003-3002 reverse	ACAGCATCTGAA-TTTTAGCACTGG	5.8 Mb
		stop codon	*2055-2057 (Exon 9)*		

Length (nt)	SALSA MLPA probe	Gene/exon[a]	Ligation site / location	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
Telomeric flanking probes					
274 ¬	19294-L30901	*LGI1*	10q23.33	CTGAAATGGCTA-GTGGAATGGCTT	8.8 Mb
168 « ¬	21051-L31033	*SUFU*	10q24.32	GAGGCCTGGTGA-GAAATGTGTGAT	19.9 Mb
369 ¬	08602-L30903	*HTRA1*	10q26.13	AATTGTTTCGCA-AGTAAAGAGAGC	

Table 2b. *PTENP*

Length (nt)	SALSA MLPA probe	*PTENP1* exon[a]	Ligation site NR_023917.1	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
400 #	22042-L31246	Exon 1	2109-2110	TTTCTCTTTTCG-TGACCAATCTTG	0.3 kb
280 #	22040-L30965	Exon 1	2423-2424	AGTTTGCAGTTA-GCTAAGAGAAGT	

[a] See section Exon numbering on page 3 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 rs146326040 and rs562164491 (probe 195 nt and 359 nt, respectively) could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

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

« 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.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the *ITIH5, CELF2, ZNF25, PCDH15, ANXA7, LGI1, SUFU, HTRA1* flanking probes 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.

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)
391	08872-L30905	*LEPR*	1p31	AGCACATACTGT-TACGGTTCTGGC
352	05273-L25208	*SPAST*	2p22	CGAGCCACAGCA-AAAAGAGCCCTC
427	08839-L22026	*DYSF*	2p13	TGCCATGAAGCT-GGTGAAGCCCTT
148	14199-L23450	*EDAR*	2q13	GAGAGTTCTGTG-GGTGGAGAGAAG
229	00967-L31037	*SENP7*	3q12	CAGTCATTTGTT-TTCCATGGTTAG
130	19616-L26704	*ATP8A1*	4p13	CAGATTCTTCTT-CGAGGAGCTCAG
418	11562-L12309	*MYOT*	5q31	TCAGGATCTCAA-CAAGGAAGAGCA
190	10710-L31035	*PKHD1*	6p12	GGTTCCTGCTCT-TTCCAGTACCTC
312	13396-L30900	*EYS*	6q12	AAGGTTTGATGT-ACTCACCTACAA
246	08715-L30393	*PCSK5*	9q21	AAGCTGAGACCT-AGTTCCAGAGGG
266	07391-L30898	*COL2A1*	12q13	TGAACCTGGTGA-ACCTGGTGTCTC
496	14894-L31209	*SPG11*	15q15	GGACAATTCGCT-TTGGCCAGGAGG
292	16435-L30904	*MYO5B*	18q21	GAACAGCTCAAC-AACCAAATCCTG
486	13594-L22376	*CACNA1A*	19p13	ACTGGAGGAATG-GCAGCCCCTGGT
137	03797-L04594	*KCNJ6*	21q22	CTCGAAGCTCCT-ACATCACCAGTG

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

Related SALSA MLPA probemixes

ME001 Tumour suppressor mix 1	Tumour suppressor genes; including *KLLN/PTEN* shared promoter region.
P067 PTCH1	Gorlin syndrome; gene included: *PTCH1*.
P081/P082 NF1 mix 1 & mix 2	Neurofibromatosis type 1; gene included: *NF1*.
P101 STK11	Peutz-Jeghers syndrome; gene included: *STK11*.
P158 JPS	Juvenile Polyposis; genes included: *BMPR1A*, *SMAD4* and *PTEN*.
P256 FLCN	Birt-Hogg-Dube syndrome; gene included: *FLCN*.
P472 SUFU	Familial Medulloblastoma and Meningioma; gene included: *SUFU*.

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P225 product history	
Version	*Modification*
E1	All *KLLN* and HhaI digestion control probes have been removed, thus methylation detection is no longer possible in the shared promoter region of *PTEN/KLLN* genes by MS-MLPA with this probemix. In addition, *PTEN* exon 4 probes have been replaced and one probe has been added for exon 2; two *PTEN*-flanking and two *PTENP1* probes have been replaced; ten reference probes have been replaced and one has been added. Nine probes have a modification in length, not in the targeted sequence.
D2	One probe has a small change in length but no change in the sequence detected.
D1	One probe for *PTEN* exon 3 and one flanking probe for *PTEN* have been added and four flanking probes have been replaced. An HhaI digestion control probe has been included and several reference probes have been replaced.
C1	Several new target and reference probes have been added. P225 may also be used to detect methylation of *PTEN* and *KLLN*.
B3	The 88 and 96 nt control fragments have been replaced (QDX2).
B2	The number of PTEN probes has been increased to 25.
B1	Eight new PTEN probes have been added and three variable PTEN probes have been removed. In order to facilitate analysis of tumour DNA, several chromosome 10 probes have been included.
A1	Extra control fragments at 88, 96, 100 and 105 nt have been added.
A0	First release.

Implemented changes in the product description
Version E1-06 – 19 January 2024 (04P) - Length (nt) updated for PTEN probe 06729-L31036 in Table 1 and Table 2a.
Version E1-05 – 07 June 2022 (04P) - For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36). - Changes to the intended purpose. The sentence ‘Only in a research setting can this device be used for the detection of, or screening for, acquired or somatic genetic aberrations, e.g. from DNA extracted from formalin-fixed paraffin embedded (FFPE) or fresh tumour materials’, has been added. - Changes to the required specimens section. Information is added how to use FFPE material for MLPA. - Various minor changes to the performance characteristics. The percentage of PTEN copy number changes in CS has been changed. - The remark: Probemix P225 is not suitable for methylation analysis has been removed. - Minor change to the interpretation of results. The criterium for the final ratio of each individual reference probe in patient samples, is not applicable when using SALSA MLPA Probemix P225 PTEN on tumour material, in a research setting.

- Updated the References and Selected publication Section.
- Various minor textual or layout changes.

Version E1-04 – 16 June 2021 (04P)
- Product description rewritten and adapted to a new template.
- Various minor textual or layout changes.
- Various minor textual changes in the Performance characteristics.
- One point from the Interpretation of results section updated to state that copy number changes detected by reference probes or *ITIH5, CELF2, ZNF25, PCDH15, ANXA7, LGI1, SUFU, HTRA1* flanking probes are unlikely to have any relation to the condition tested for.
- Ligation sites of the probes targeting the *PTEN* gene updated according to new version of the NM_ reference sequence.
- Warning added to Table 1 and Table 2a for probes at 162 nt (07685-L31034) and 184 nt (05760-L06666) being more variable.
- Warning added to Table 1 and Table 2a for probe at 168 nt (21051-L31033) located in or near a GC-rich region.
- Warning for the flanking probes in Table 1 and Table 2a updated: Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the *ITIH5, CELF2, ZNF25, PCDH15, ANXA7, LGI1, SUFU, HTRA1* flanking probes or reference probes are unlikely to be related to the condition tested.
- Updated all references in the Selected Reference section.
- UK has been added to the list of countries in Europe that accept the CE mark.

Version E1-03 – 29 April 2020 (04)
- Product is now registered for IVD use in Israel.
- Version E1-02 – 10 April 2019 (04)
- Corrected *PTEN* exon number in Table 1 for PTEN probe 18254-L31140.
- Changed the number of PTEN/KLLN probes in ME001 probemix to three in “Probemix P225 not suitable for methylation analysis” on page 3 and “Confirmation of results paragraph” on page 5.

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