

Product Description

SALSA® MLPA® Probemix P244-D1 AIP-MEN1-CDKN1B

To be used with the MLPA General Protocol.

Version D1

As compared to version C1 three new target probes have been included and the length of two target probes has changed. All reference probes are replaced and the total number of reference probes has increased to twelve. For complete product history see page 11.

Catalogue numbers:

- **P244-025R:** SALSA MLPA Probemix P244 AIP-MEN1-CDKN1B, 25 reactions.
- **P244-050R:** SALSA MLPA Probemix P244 AIP-MEN1-CDKN1B, 50 reactions.
- **P244-100R:** SALSA MLPA Probemix P244 AIP-MEN1-CDKN1B, 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 P244-AIP-MEN1-CDKN1B is an in vitro diagnostic (IVD)¹ or research use only (RUO) semi-quantitative assay² for the detection of deletions or duplications in *AIP*, *MEN1* and *CDKN1B* in order to confirm a potential cause and clinical diagnosis of familial isolated pituitary adenoma (FIPA), multiple endocrine neoplasia type 1 (MEN1) or multiple endocrine neoplasia type 4 (MEN4), respectively. This assay is for use with human DNA extracted from peripheral whole blood specimens.

Copy number variations (CNVs) detected with P244 AIP-MEN1-CDKN1B 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 the *AIP*, *MEN1* and *CDKN1B* genes 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, 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, population screening, pre-implantation or prenatal testing. 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 (IVD) use 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

Multiple endocrine neoplasia type 1 (MEN1) is predominantly characterized by the occurrence of primary hyperparathyroidism (PHPT), which occurs in 95-100% of patients; pancreatic neuroendocrine tumours, which occur in 40-75% of patients; and pituitary adenoma, which is found in 30-50% of patients. Most tumours are non-metastasizing, but many can cause striking and serious clinical effects due to the increased secretion of hormones. It is estimated that in the general population 1 to 10 in 100.000 individuals develop MEN1 during their lifetime. Nine out of ten patients diagnosed with MEN1 have the familial form. MEN1 shows dominant autosomal inheritance and the penetrance is >95% by age 40 for confirmed pathogenic mutations. The mean age of death of MEN1 patients is between 50 and 55 years. The single gene associated with MEN1 syndrome is *MEN1*, which encodes the menin protein. Heterozygous *MEN1* pathogenic variants are found in ~90% of familial MEN1 syndrome patients and in ~65% of sporadic cases. Loss of heterozygosity (LOH) of *MEN1* is observed in >90% MEN1 tumours suggesting that *MEN1* acts as a tumour suppressor gene, in line with the Knudson 2-hit hypothesis for tumorigenesis. Besides point mutations, several deletions involving one or more complete exons in the *MEN1* gene have been described (Carroll 2013, Concolino et al. 2016, Lemos and Thakker 2008, Romanet et al. 2019, Thakker 2014), including a pathogenic deletion of just the 5'-UTR (Kooblall et al. 2020).

Pituitary adenomas (PAs) occur with a frequency of ~1 in 1000 in the general population. Most cases are sporadic, but approximately 5% occurs as a familial cancer. The *AIP* gene encodes aryl hydrocarbon receptor-interacting protein (AIP), a tumour suppressor that is involved in the control of cell proliferation and differentiation. *AIP* loss of function mutations are found in 15-25% of familial isolated pituitary adenoma (FIPA) cases, which are subsequently referred to as *AIP*-FIPA. Inheritance is autosomal dominant and the average penetrance is 15-30%, although this may vary greatly. The prevalence of *AIP*-FIPA is estimated at 1:100,000. Similar as for *MEN1*, LOH is frequently observed, suggesting that *AIP* also acts as a tumour suppressor gene (Cai et al. 2013). Although most known germline *AIP* mutations are point mutations, several exon deletions have been reported: exon 1-2, exon 2, exon 1-6 (Georgitsi et al. 2008, Igreja et al. 2010, Marques et al. 2018).

MEN1 and *AIP* are located in close proximity on 11q13, and somatic LOH in *MEN1* and FIPA associated tumours often affects both genes. Apart from tumours in *MEN1* and FIPA patients, LOH of this locus also occurs in sporadic cancers, especially in endocrine tissues. As both genes are considered tumour suppressor genes this double loss may contribute to tumorigenesis. Chromosomal losses of the 11q13 chromosomal band have also been found in other cancers, such as cervical cancer and hibernomas (Newsham 1998; Nord et al. 2010).

MEN4 is a distinct MEN type but the symptoms of MEN4 largely overlap with MEN1 (Pellegata et al. 2006). In a small number (estimated at 1-3%) of *MEN1* mutation-negative patients fulfilling the diagnostic criteria for MEN1, mutations in *CDKN1B* have been detected. Extrapolating from this, the prevalence of MEN4 is very low: <1:300,000. Like MEN1, MEN4 is primarily characterized by PHPT and PA, but the additional tumours show some differences; tumours in the reproductive organs, and adrenal and renal tumours have been found in MEN4 patients. The only way to distinguish MEN4 from MEN1 is by identification of a pathogenic mutation in *CDKN1B*. Somatic mutations in *CDKN1B* have also been identified in sporadic tumours, but LOH of *CDKN1B* in MEN4-related tumours has not been found.

More information on MEN1 can be found on <https://www.ncbi.nlm.nih.gov/books/NBK1538/>
More information on AIP-related FIPA can be found on <https://www.ncbi.nlm.nih.gov/books/NBK97965/>
More information on MEN4 can be found on: <https://omim.org/entry/610755>

Gene structure

The *MEN1* gene spans ~7.2 kilobases (kb) on chromosome 11q13, ~2.6 Mb centromeric from *AIP*, and contains 10 exons. The *MEN1* LRG_509 is available at www.lrg-sequence.org and is identical to GenBank NG_008929.1.

The *AIP* gene spans ~8.1 kb of genomic sequence on chromosome 11q13, ~2.6 Mb telomeric from *MEN1*, and contains 6 exons. The *AIP* LRG_460 is available at www.lrg-sequence.org and is identical to GenBank NG_008969.1.

The *CDKN1B* gene spans 5.0 kb of genomic sequence on chromosome 12p13, and contains 3 exons. No LRG is available for *CDKN1B*, but the GenBank chromosomal sequence is NG_016341.1.

Transcript variants

For *MEN1*, multiple variants have been described. Transcript variant 1 is the most predominant and encodes menin isoform 1 (NM_000244.3; 2785 nt; coding sequence 111-1958). This sequence is the longest of the two *MEN1* reference standards in the NCBI RefSeqGene project. The ATG translation start site is located in exon 2b and the stop codon is located in exon 10. More information about NM_000244.3 and other *MEN1* transcript variants can be found on the NCBI *MEN1* gene page: www.ncbi.nlm.nih.gov/gene/4221.

For *AIP*, multiple variants have been described. Transcript variant 1 is the longest and most abundant transcript and encodes the longest *AIP* isoform (NM_003977.4; 1236 nt; coding sequence 134-1126). The ATG translation start site is located in exon 1 and the stop codon is located in exon 6. More information about NM_003977.4 and other *AIP* transcript variants can be found on the NCBI *AIP* gene page: <https://www.ncbi.nlm.nih.gov/gene/9049>.

For *CDKN1B*, one transcript variant has been described encoding the full length protein (NM_004064.5; 2411 nt; coding sequence 473-1069; <http://www.ncbi.nlm.nih.gov/gene/1027>). The ATG translation start site is located in exon 1 and the stop codon is located in exon 2.

Exon numbering

The *MEN1* exon numbering used in this P244-D1 AIP-MEN1-CDKN1B product description is the exon numbering from the LRG_509 sequence. 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.

The *AIP* exon numbering used in this P244-D1 AIP-MEN1-CDKN1B product description is the exon numbering from the LRG_460 sequence. 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.

The *CDKN1B* exon numbering used in this P244-D1 AIP-MEN1-CDKN1B product description is the exon numbering from the RefSeq transcript NM_004064.5. 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 P244-D1 AIP-MEN1-CDKN1B contains 42 MLPA probes with amplification products between 129 and 463 nucleotides (nt). This includes 25 probes for the *MEN1-AIP* region, five probes for the *CDKN1B* region. In addition, 12 reference probes are included that target relatively copy number stable regions in all cancer types. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

This probemix contains nine/ten 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 human peripheral blood 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 healthy individuals without a history of MEN or FIPA. 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 NA07981 from the Coriell Institute has been tested with this P244-D1 probemix at MRC Holland and can be used as a positive control sample to detect four copies of *CDKN1B* and the flanking regions. The quality of cell lines can change; therefore samples should be validated before use.

Performance characteristics

Deletions and duplications account for 1-4% of all pathogenic *MEN1* mutations and large deletions or duplications account for 2-5% of the pathogenic *AIP* mutations. For *CDKN1B* this percentage is not known: very few pathogenic mutations have been identified and large deletions and duplications appear to be very rare. Analytical performance for the detection of deletions/duplications in *MEN1*, *AIP* and *CDKN1B* is very high and can be considered $>99\%$ (based on a 2007-2020 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 for *MEN1*, *AIP* and *CDKN1B* target probes are allele copy numbers of 2 (normal), 1 (heterozygous deletion), 3 (heterozygous duplication), 0 (homozygous deletion) or 4 (homozygous duplication or heterozygous triplication). 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 following cut-off values for the FR of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Final ratio (FR)
Normal	$0.80 < FR < 1.20$
Homozygous deletion	FR = 0
Heterozygous deletion	$0.40 < FR < 0.65$
Heterozygous duplication	$1.30 < FR < 1.65$
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 or subclonal cases. Analysis of parental samples may be necessary for 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 or in or near the *MEN1-AIP* genes. 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.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *MEN1*, *AIP* and *CDKN1B* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P244 AIP-MEN1-CDKN1B.
- 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 tumours with high chromosomal instability.

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.

Mutation databases

The UMD-MEN1 mutation database (www.umd.be/MEN1/) and the LOVD page for *MEN1*: databases.lovd.nl/shared/genes/MEN1. We strongly encourage users to deposit positive results in any of the *MEN1* databases. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on <http://varnomen.hgvs.org/>.

The FIPA Patients *AIP* Mutations Database: <https://aip.fipapatient.org/menu/main/home> and the LOVD page for *AIP*: <https://databases.lovd.nl/shared/genes/AIP>. We strongly encourage users to deposit positive results in any of the *AIP* mutation databases. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on <http://varnomen.hgvs.org/>.

The LOVD page for *CDKN1B*: <https://databases.lovd.nl/shared/genes/CDKN1B>. We strongly encourage users to deposit positive results in the *CDKN1B* database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on <http://varnomen.hgvs.org/>.

COSMIC mutation database

Somatic mutations in *MEN1*, *AIP* and *CDKN1B* in cancer can be found in the COSMIC database at <https://cancer.sanger.ac.uk/cosmic>.

Please report copy number changes detected by the reference probes, false positive results due to SNVs and unusual results (e.g., a duplication of *AIP* exons 3 and 5 but not exon 4) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P244-D1 AIP-MEN1-CDKN1B

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18) ^a		
		Reference	MEN1-AIP region	CDKN1B
64-105	Control fragments – see table in probemix content section for more information			
129 *	Reference probe 18709-L26847	5q31		
136 ¥ «	MEN1 probe 22342-L02795		Exon 9	
142 * ~	GPRC5A probe 22344-L03556			Downstream
148	CDKN1B probe 18425-L23733			Exon 2
154 *	Reference probe 04540-L28278	2q24		
160 « ~	BRMS1 probe 04155-L03510		11q13	
167 «	MEN1 probe 13158-L14680		Exon 4	
175 «	AIP probe 07380-L09559		Exon 1	
184 *	Reference probe 10904-L27810	9q34		
191 «	MEN1 probe 01663-L01242		Exon 1	
195 «	MEN1 probe 18765-L24187		Exon 6	
202 «	MEN1 probe 13159-L14681		Exon 5	
209 «	AIP probe 07383-L07030		Exon 4	
216 *	Reference probe 09103-L31536	4q25		
220 «	MEN1 probe 01664-L01243		Exon 2b	
229 « ~	RELA probe 01120-L00060		11q13	
238 «	AIP probe 07381-L07028		Exon 2	
247 «	MEN1 probe 01164-L00720		Exon 10	
256 *	Reference probe 08812-L08872	2p13		
266 «	AIP probe 07384-L09556		Exon 5	
277 *	Reference probe 13393-L28135	6q12		
283 «	MEN1 probe 01665-L14816		Exon 3	
292 «	AIP probe 07379-L09558		Exon 1	
301 «	MEN1 probe 01666-L01245		Exon 7	
310 ~	CCND1 probe 05403-L04809		11q13	
319 «	AIP probe 07385-L09557		Exon 6	
326 *	Reference probe 16275-L18567	19p13		
338 ¥ ~	SNX15 probe 01667-L31522		11q13	
346 *	Reference probe 04337-L20895	15q21		
355 « ~	FAM89B probe 04157-L03512		11q13	
364 «	AIP probe 07382-L09069		Exon 3	
373 *	Reference probe 04278-L03682	12q12		
382 ~	SART1 probe 04159-L03514		11q13	
391	CDKN1B probe 18426-L23497			Exon 1
400 « +	MEN1 probe 18427-L23498		Upstream	
409 *	Reference probe 14839-L30627	1p34		
420 * « ~	SF1 probe 22341-L20815		11q13	
427 «	CDKN1B probe 18429-L23500			Exon 3
436 «	MEN1 probe 18430-L23501		Exon 8	
445 *	Reference probe 10709-L11291	6p12		
454 * ~	BCL2L14 probe 22345-L02547			Upstream
463 *	Reference probe 15970-L18122	18p11		

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

* New in version D1.

¥ Changed in version D1. Minor alteration, no change in sequence detected.

« 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 flanking or reference probes are unlikely to be related to the condition tested.

+ The ligation site of this probe is located in an alternative exon 1 present in two alternative transcripts: NM_130803.2 and NM_130804.2. In both transcripts the ligation site is on position 242-243. The significance of deletions/duplications of

only this alternative exon is not clear as this exon is non-coding and alternative transcript variants using other transcription start sites are known.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Table 2. P244-D1 probes arranged according to chromosomal location

Table 2a. *MEN1*-AIP region (11q13)

Length (nt)	SALSA MLPA probe	Gene exon ^a	Ligation site	Partial sequence ^b (24 nt adjacent to ligation site)	Distance to next probe
420 « -	22341-L20815	<i>SF1</i>		GATTCCAGGAAT-GCCTACAGTTAT	27.9 kb
		<i>MEN1</i>	NM_000244.3		
		<i>stop codon</i>	1956-1958 (exon 10)		
247 « »	01164-L00720	Exon 10	1855-1856	CGCCATCAAGCT-GCAACTCACGGC	0.7 kb
136 « »	22342-L02795	Exon 9	1390-1391	CGGCATCTGCAA-ATGGGAGGAGGG	0.6 kb
436 «	18430-L23501	Exon 8	1228-1229	CTTTGAAGTAGC-CAATGATGTCAT	0.6 kb
301 « »	01666-L01245	Exon 7	1092-1093	ACCCCTACATGT-ACCTGGCTGGCT	0.7 kb
195 « »	18765-L24187	Exon 6	1036-1037	CCTCTACCACAA-GGTGGGGGCATC	0.2 kb
202 « »	13159-L14681	Exon 5	931-932	GCTGCTCTATGA-CCTGGGACATCT	0.4 kb
167 « »	13158-L14680	Exon 4	825-826	GTGACCGCAAGA-TGGAGGTGGCGT	0.4 kb
283 « »	01665-L14816	Exon 3	672-673	AGGATCATGCCT-GGGTAGTGTGTTG	2.0 kb
220 « »	01664-L01243	Exon 2b	250-251	CGTGGAGCATTT-TCTGGCTGTCAA	0.7 kb
		<i>start codon</i>	111-113 (exon 2b)		
191 « »	01663-L01242	Exon 1	34-35	GAGATCCCAGAA-GCCACAGCGCAG	0.4 kb
400 « +	18427-L23498	Upstream	337 nt before exon 1	GCGGAAGTGGGA-AACGAGTGCTGC	216.5 kb
338 -	01667-L31522	<i>SNX15</i>		CGAAGGATGACT-TCCTGCGGCACT	545.9 kb
355 « -	04157-L03512	<i>FAM89B</i>		ACAAACACCTGT-GCCAAGACCTGA	88.3 kb
229 « -	01120-L00060	<i>RELA</i>		AAAGGACTGCCG-GGATGGCTTCTA	305.6 kb
382 -	04159-L03514	<i>SART1</i>		CCGCAAGAAGGA-GAAGGAGGTAGT	374.9 kb
160 « -	04155-L03510	<i>BRMS1</i>		CAGAAGAGATGG-AAGCAGAGGGTG	1141.0 kb
		<i>AIP</i>	NM_003977.4		
292 «	07379-L09558	Exon 1	105-106	GAGTCCGGAAGT-TGCCGAAAGGGA	0.1 kb
		<i>start codon</i>	134-136 (exon 1)		
175 «	07380-L09559	Exon 1	186-187	AAAACGTGTGAT-ACAGGAAGGCCG	3.9 kb
238 «	07381-L07028	Exon 2	320-321	CCATGGAGCTCA-TCATTGGCAAGA	2.3 kb
364 «	07382-L09069	Exon 3	519-520	ACAGATGCGTGA-ACACAGCTCCCT	0.7 kb
209 «	07383-L07030	Exon 4	676-677	GCAGTGCCACTT-ATCCACCAGGAG	0.3 kb
266 «	07384-L09556	Exon 5	840-841	GCTGCTGCTCAA-CTACTGCCAGTG	0.5 kb
319 «	07385-L09557	Exon 6	997-998	CAGGCTGACTTT-GCCAAAGTGCTG	2157.1 kb
		<i>stop codon</i>	1124-1126 (exon 6)		
310 -	05403-L04809	<i>CCND1</i>		TCCGCCCTCCAT-GGTGGCAGCGGG	-

Table 2b. *CDKNB1*

Length (nt)	SALSA MLPA probe	<i>CDKN1B</i> exon ^a	Ligation site NM_004064.5	Partial sequence ^b (24 nt adjacent to ligation site)	Distance to next probe
454 ~	22345-L02547	<i>BCL2L14</i>		GTCATGGACAGA-GGTGTCATGGCC	638.3 kb
391	18426-L23497	Exon 1	470-471	GACCCGGGAGAA-AGATGTCAAACG	1.1 kb
		<i>start codon</i>	<i>473-475 (exon 1)</i>		
148	18425-L23733	Exon 2	1022-1023	ATGCCGGTTCTG-TGGAGCAGACGC	2.3 kb
		<i>stop codon</i>	<i>1067-1069 (exon 2)</i>		
427 «	18429-L23500	Exon 3	1199-1200	CCTGTATAAGCA-CTGAAAAACAAC	187.3 kb
142 ~	22344-L03556	<i>GPRC5A</i>		GTCTGCAAGGTG-CAGGACTCCAAC	-

^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.

« 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.

» Detects the same sequence as *MEN1* probes in SALSA MLPA Probemix P017-D1 *MEN1*.

~ 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.

+ The ligation site of this probe is located in an alternative exon 1 present in two alternative transcripts: NM_130803.2 and NM_130804.2. In both transcripts the ligation site is on position 242-243. The significance of deletions/duplications of only this alternative exon is not clear as this exon is non-coding and alternative transcript variants using other transcription start sites are known.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

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

Related SALSA MLPA probemixes

- P017 *MEN1*: Version D1 of P017 contains mostly the same *MEN1* probes and *MEN1* flanking probes as this P244-D1 probemix. P017-D1 is complemented with additional exon 1 and exon 9 probes and the *MEN1* upstream and exon 8 probes have different ligation sites (see also Table 2b). P017 does not contain probes for the *AIP* gene, *CDKN1B* gene and the other genes in the 11q13 region that do not flank *MEN1* directly. Note that P017 is not suited for tumour DNA research.
- P169 Hirschsprung-1: Contains probes for the *RET* gene, associated with MEN2A and MEN2B.
- P177 *CASR*: Contains probes for the *CASR* gene, associated with familial hypocalcaemic hypercalcaemia, a disease with certain similar symptoms as PHPT.
- P466 *CDC73*: Contains probes for the *CDC73* gene that is associated with hyperparathyroidism-jaw tumour syndrome, a disease with certain similar symptoms as PHPT.

References

- 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.
- Cai F et al. (2013). Screening for *AIP* gene mutations in a Han Chinese pituitary adenoma cohort followed by LOH analysis. *Eur J Endocrinol*. 169:867-884.
- Carroll RW (2013). Multiple endocrine neoplasia type 1 (*MEN1*). *Asia Pac J Clin Oncol*. 9:297-309.
- Concolino P et al. (2016). Multiple endocrine neoplasia type 1 (*MEN1*): An update of 208 new germline variants reported in the last nine years. *Cancer Genet*. 209:36-41.

- Georgitsi M et al. (2008). Large genomic deletions in AIP in pituitary adenoma predisposition. *J Clin Endocrinol Metab.* 93:4146-4151.
- Hömig-Hölzel C and Savola S. (2012). Multiplex ligation-dependent probe amplification (MLPA) in tumor diagnostics and prognostics. *Diagn Mol Pathol.* 21:189-206.
- Igreja S et al. (2010). Characterization of aryl hydrocarbon receptor interacting protein (AIP) mutations in familial isolated pituitary adenoma families. *Hum Mutat.* 31:950-960.
- Kooblall KG et al. (2020). Multiple Endocrine Neoplasia Type 1 (MEN1) 5'UTR Deletion, in MEN1 Family, Decreases Menin Expression. *J Bone Miner Res.* 00:1-10.
- Lemos MC et al. (2008). Multiple endocrine neoplasia type 1 (MEN1): analysis of 1336 mutations reported in the first decade following identification of the gene. *Hum Mutat.* 29:22-32.
- Marques P et al. (2018). Emergence of Pituitary Adenoma in a Child during Surveillance: Clinical Challenges and the Family Members' View in an AIP Mutation-Positive Family. *Int J Endocrinol.* 2018:8581626.
- Newsham IF (1998). The long and short of chromosome 11 in breast cancer. *Am J Pathol.* 153:5-9.
- Nord KH et al. (2010). Concomitant deletions of tumor suppressor genes MEN1 and AIP are essential for the pathogenesis of the brown fat tumor hibernoma. *Proc Natl Acad Sci U S A.* 107:21122-21127.
- Pellegata NS et al. (2006). Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans. *Proc Natl Acad Sci U S A.* 103:15558-15563.
- Romanet P et al. (2019). UMD-MEN1 Database: An Overview of the 370 MEN1 Variants Present in 1676 Patients From the French Population. *J Clin Endocrinol Metab.* 104:753-764.
- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Thakker RV (2014). Multiple endocrine neoplasia type 1 (MEN1) and type 4 (MEN4). *Mol Cell Endocrinol.* 386:2-15.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
- 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 P244 AIP-MEN1-CDKN1B

- Belar O et al. (2012). Novel mutations in MEN1, CDKN1B and AIP genes in patients with multiple endocrine neoplasia type 1 syndrome in Spain. *Clin Endocrinol (Oxf).* 76:719-724.
- Cai F et al. (2013). Screening for AIP gene mutations in a Han Chinese pituitary adenoma cohort followed by LOH analysis. *Eur J Endocrinol.* 169:867-884.
- Carvalho RA et al. (2018). Germline mutation landscape of multiple endocrine neoplasia type 1 using full gene next-generation sequencing. *Eur J Endocrinol.* 179:391-407.
- Georgitsi M et al. (2008). Large genomic deletions in AIP in pituitary adenoma predisposition. *J Clin Endocrinol Metab.* 93:4146-4151.
- Igreja S et al. (2010). Characterization of aryl hydrocarbon receptor interacting protein (AIP) mutations in familial isolated pituitary adenoma families. *Hum Mutat.* 31:950-960.
- Iwata T et al. (2014). A novel C-terminal nonsense mutation, Q315X, of the aryl hydrocarbon receptor-interacting protein gene in a Japanese familial isolated pituitary adenoma family. *Endocr Pathol.* 25:273-281.
- Kooblall KG et al. (2020). Multiple Endocrine Neoplasia Type 1 (MEN1) 5'UTR Deletion, in MEN1 Family, Decreases Menin Expression. *J Bone Miner Res.* 00:1-10.
- Marques P et al. (2018). Emergence of Pituitary Adenoma in a Child during Surveillance: Clinical Challenges and the Family Members' View in an AIP Mutation-Positive Family. *Int J Endocrinol.* 2018:8581626.
- Morotti A et al. (2020). The Oncosuppressors MEN1 and CDC73 Are Involved in lncRNA Deregulation in Human Parathyroid Tumors. *J Bone Miner Res.*

- Nord KH et al. (2010). Concomitant deletions of tumor suppressor genes MEN1 and AIP are essential for the pathogenesis of the brown fat tumor hibernoma. *Proc Natl Acad Sci U S A.* 107:21122-21127.
- Pardi E et al. (2017). Mutational and large deletion study of genes implicated in hereditary forms of primary hyperparathyroidism and correlation with clinical features. *PLoS One.* 12:e0186485.
- Pardi E et al. (2013). Aryl hydrocarbon receptor interacting protein (AIP) mutations occur rarely in sporadic parathyroid adenomas. *J Clin Endocrinol Metab.* 98:2800-2810.
- Romanet P et al. (2019). UMD-MEN1 Database: An Overview of the 370 MEN1 Variants Present in 1676 Patients From the French Population. *J Clin Endocrinol Metab.* 104:753-764.
- Tuncer FN et al. (2018). Screening of AIP Gene Variations in a Cohort of Turkish Patients with Young-Onset Sporadic Hormone-Secreting Pituitary Adenomas. *Genet Test Mol Biomarkers.* 22:702-708.

MEN1 copy number variations have also been detected with the P017 *MEN1* probemix. See the selected publications listed in the P017 product description for relevant publications.

P244 product history	
Version	Modification
D1	Three new flanking probes have been included and the length of two probes was changed. All reference probes were replaced and the total number of reference probes was increased to twelve.
C1	Three probes for the missing <i>MEN1</i> exons were included and probes for each exon of the <i>CDKN1B</i> gene are included. Seven reference probes were replaced and one extra reference probe was included.
B1	One <i>MEN1</i> probe was removed and two new <i>MEN1</i> probes were included. Several reference probes were replaced and four extra control fragments at 88-96-100-105 nt were included.
A1	First release.

Implemented changes in the product description
<p>Version D1-02 – 03 February 2021 (04P)</p> <ul style="list-style-type: none"> - Product description rewritten and adapted to a new template. - Intended Purpose was rewritten. - NM sequences for <i>AIP</i> and <i>CDKN1B</i> genes were updated and the ligation sites were also updated according to these new versions of the NM reference sequences in Table 2. - <i>References</i> and <i>Selected Publications</i> were curated and new literature was included. - UK has been added to the list of countries in Europe that accept the CE mark. <p>Version D1-01 - 23 August 2019 (02P)</p> <ul style="list-style-type: none"> - Product description rewritten and adapted to a new template. - P244-D1 is now CE marked. - In a research setting P244-D1 is now suited to be used on tumour DNA. - Product description adapted to a new product version (version number changed, changes in Table 1 & 2). - For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36). <p>Version 09 – 28 April 2016 (55)</p> <ul style="list-style-type: none"> - Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included). - Various minor textual changes on page 1. - Exon numbering of the <i>MEN1</i> gene has been changed in Table 1 and Table 2.

More information: www.mrcholland.com ; www.mrcholland.eu	
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	EUROPE* 
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*comprising EU (candidate) member states and members of the European Free Trade Association (EFTA), and the UK. The product is for RUO in all other European countries.