Product Description
SALSA® MLPA® Probemix P017-D1 MEN1

To be used with the MLPA General Protocol.

Version D1
For complete product history see page 9.

Catalogue numbers:
- **P017-025R:** SALSA MLPA Probemix P017 MEN1, 25 reactions.
- **P017-050R:** SALSA MLPA Probemix P017 MEN1, 50 reactions.
- **P017-100R:** SALSA MLPA Probemix P017 MEN1, 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 P017 MEN1 is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplications in the *MEN1* gene in genomic DNA isolated from human peripheral whole blood specimens. P017 MEN1 is intended to confirm a potential cause for and clinical diagnosis of multiple endocrine neoplasia type 1 (MEN1) and for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with P017 MEN1 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 *MEN1* gene 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, pre-implantation or prenatal testing, population screening, or 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 (MEN) is an autosomal dominant cancer predisposition syndrome characterized by tumours found in two or more endocrine glands. MEN type 1 (MEN1) is primarily 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. Additionally, adrenal tumours, duodenal, thymic and lung neuroendocrine tumours, lipomas, facial angiofibroma, and collagenoma can be found. Most tumours are non-metastasizing, but many can cause striking and serious clinical effects due to 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, 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 of age.

The single gene associated with MEN1 syndrome is *MEN1*. Heterozygous *MEN1* pathogenic variants are found in ~90% of familial MEN1 syndrome patients and in ~65% of sporadic cases. Loss of heterozygosity (LOH) is frequently observed in MEN1 tumours suggesting that *MEN1* acts as a tumour suppressor gene, as postulated by the Knudson 2-hit hypothesis. 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).

Please note that this probemix is not suited to detect deletions or duplications in DNA extracted from fresh tumour tissue or from formalin-fixed paraffin embedded (FFPE) tumour materials. Probemix P244 (only version D1) can be used in a research setting to detect CNVs in the *MEN1*-region in DNA from tumour material.

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

Transcript variants
For *MEN1,* multiple variants have been described. Transcript variant 1 is the most predominantly used 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.

Exon numbering
The exon numbering used in this P017-D1 MEN1 product description is the exon numbering from the LRG_509 sequence. 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 and exon numbering may not be up-to-date.

Probemix content
The SALSA MLPA Probemix P017-D1 MEN1 contains 25 MLPA probes with amplification products between 142 and 373 nucleotides (nt). This includes 15 probes for the *MEN1*-region. In addition, 10 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).

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, 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.

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 MEN1. 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. The quality of cell lines can change; therefore samples should be validated before use.

Performance characteristics
The expected percentage of deletions/duplications is 1-4% of all *MEN1* mutations (Concolino et al. 2016, Lemos and Thakker 2008, Romanet et al. 2019 and the following databases: *www.umd.be/MEN1*, *databases.lovd.nl/shared/genes/MEN1* and *www.ncbi.nlm.nih.gov/clinvar/*). Analytical performance for the detection of deletions/duplications in *MEN1* is very high and can be considered >99% (based on a literature review covering 2007 to 2020).

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*-region specific MLPA probes are allele copy numbers of 2 (normal), 1 (heterozygous deletion), 3 (heterozygous duplication), 0 (homozygous deletion) or 4 (homozygous duplication / 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.

- 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 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* gene. 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* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P017 MEN1.
- 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.

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.

***MEN1* mutation database**

The UMD-MEN1 mutation database (www.umd.be/MEN1/) and the LOVD for the *MEN1* gene (databases.lovd.nl/shared/genes/MEN1). We strongly encourage users to deposit positive results in one of these databases. 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 *MEN1* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P017-D1 MEN1

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*MEN1*
64-105	Control fragments – see table in probemix content section for more information		
142	Reference probe 15097-L16868	9q33	
148	Reference probe 09970-L10429	17q12	
154 «	**MEN1 probe** 17113-L20265		Exon 9
161 «	**MEN1 probe** 03404-L20714		Exon 9
167 «	**MEN1 probe** 13158-L14680		Exon 4
175	Reference probe 11571-L20271	16q21	
185	Reference probe 03200-L03342	1q41	
195 «	**MEN1 probe** 01663-L20715		Exon 1
202 «	**MEN1 probe** 13159-L14681		Exon 5
211	Reference probe 16649-L19182	10q23	
228 «	**MEN1 probe** 01664-L20716		2b
234 «	**MEN1 probe** 17112-L20814		Exon 6
241 «	**MEN1 probe** 13157-L20717		Exon 1
247 *	Reference probe 19086-L24973	4q35	
256 «	**MEN1 probe** 01164-L20713		Exon 10
274	Reference probe 13393-L14850	6q12	
283 «	**MEN1 probe** 01665-L14816		Exon 3
292 *	Reference probe 13325-L14751	18q21	
301 «	**MEN1 probe** 01666-L01245		Exon 7
310 *	Reference probe 12442-L13443	14q24	
328 ¬	**SNX15 probe** 01667-L14817		Upstream
337 «	**MEN1 probe** 03403-L09561		Exon 8
355 « +	**MEN1 probe** 13161-L14683		Upstream
362 « ¬	**SF1 probe** 17117-L20815		Downstream
373	Reference probe 04278-L03682	12q12	

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

* New in version D1.
« 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 264-265. 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. *MEN1* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*MEN1* exon[a]	Ligation site NM_000244.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
328 ¬	01667-L14817	*SNX15* gene		CGAAGGATGACT-TCCTGCGGCACT	216.6 kb
355 « +	13161-L14683	Upstream	315 nt before exon 1	GCACACAGAGTA-GGCATCTTTATA	0.3 kb
195 « »	01663-L20715	Exon 1	34-35	GAGATCCCAGAA-GCCACAGCGCAG	0.2 kb
241 «	13157-L20717	Exon 1	107 nt after exon 1	GTGTGGGATGTA-AGCGCGGAGGTG	0.6 kb
		start codon	*111-113 (Exon 2b)*		
228 « »	01664-L20716	Exon 2b	250-251	CGTGGAGCATTT-TCTGGCTGTCAA	2.0 kb
283 « »	01665-L14816	Exon 3	672-673	AGGATCATGCCT-GGGTAGTGTTTG	0.4 kb
167 « »	13158-L14680	Exon 4	825-826	GTGACCGCAAGA-TGGAGGTGGCGT	0.2 kb
202 « »	13159-L14681	Exon 5	931-932	GCTGCTCTATGA-CCTGGGACATCT	0.7 kb
234 « »	17112-L20814	Exon 6	1036-1037	CCTCTACCACAA-GGTGGGGGCATC	0.4 kb
301 « »	01666-L01245	Exon 7	1092-1093	ACCCCTACATGT-ACCTGGCTGGCT	0.6 kb
337 «	03403-L09561	Exon 8	1218-1219	ACAAGGAGTTCT-TTGAAGTAGCCA	0.6 kb
161 « »	03404-L20714	Exon 9	1390-1391	CGGCATCTGCAA-ATGGGAGGAGGG	0.1 kb
154 «	17113-L20265	Exon 9	1464-1463 reverse	CTGTCCCTCAAA-ACGGCCTAGGGA	0.6 kb
256 « »	01164-L20713	Exon 10	1855-1856	CGCCATCAAGCT-GCAACTCACGGC	27.9 kb
		stop codon	*1956-1958 (Exon 10)*		
362 « ¬	17117-L20815	*SF1* gene		GATTCCAGGAAT-GCCTACAGTTAT	-

[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.

« 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 P244-D1 AIP-MEN1-CDKN1B.
¬ 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 264-265. 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

- P169 Hirschprung-1: Contains probes for the *RET* gene, associated with MEN2A and MEN2B.
- P177 CASR: Contains probes for the *CASR* gene, associated with familial hypocalciuric hypercalcaemia, a disease with certain similar symptoms as PHPT.
- P244 AIP-MEN1-CDKN1B: Version D1 of P244 contains mostly the same *MEN1* probes and *MEN1* flanking probes as this P017-D1 probemix (Table 2b). The *MEN1* upstream and exon 8 probes have different ligation sites. P244-D1 is complemented with probes for the *AIP* gene, other genes in the 11q13 region and probe for the *CDKN1B* gene. Note that P244 (only version D1) is suited for use on tumour DNA in a research setting.
- 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

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Selected publications using SALSA MLPA Probemix P017 MEN1

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Note: *MEN1* copy number variations have also been detected with the P244 AIP-MEN1-CDKN1B probemix. See the selected publications listed in the P244 product description for relevant publications thereof.

P017 product history	
Version	*Modification*
D1	One target probe has been removed and three reference probes have been replaced.
C1	Two probes for *MEN1* exon 7 and one probe for *MEN1* exon 10 have been included. The downstream flanking probe and several reference probes have been replaced. In addition, the 88 and 96nt control fragments have been replaced (QDX2).
B1	Four extra probes for the *MEN1* gene have been included. In addition, four extra control fragments at 88-96-100-105 nt have been added.
A1	First release.

Implemented changes in the product description

Version D1-04 – 03 February 2021 (04P)
- Product description rewritten and adapted to a new template.
- Intended Purpose was rewritten.
- *References* and *Selected Publications* were curated and new literature was included.
- UK has been added to the list of countries in Europe that accept the CE mark.

Version D1-03 – 15 June 2020 (02P)
- Product is now registered for IVD use in Israel.

Version D1-02 – 28 August 2019 (02P)
- Product description restructured and adapted to a new template.
- Added note regarding the use of P244-D1 on tumour DNA in a research setting.
- Notes are added to Table 2 for the probes that are the same in P017 and P244.
- Various minor textual changes.

Version D1-01 – 31 October 2018 (04)
- Product description restructured and adapted to a new template.
- P017-D1 is now CE-marked.
- Various minor changes were made to Table 1 and Table 2.

Version 19 – 09 August 2016 (55)
- Product description adapted to a new version (lot number changed, changes in table 1 and 2, new picture included).
- P466 CDC73 included as related probemix.
- Various minor textual changes

Version 18 – 09 June 2016 (55)
- Warning added in Table 1 and 2, 265 nt probe 13160-L20738.
- Manufacturer's address adjusted.

Version 17 – 14 April 2016 (53)
- Exon numbering of the MEN1 gene has been changed on page 3 and 4.
Various minor textual changes on page 1.

More information: www.mrcholland.com; www.mrcholland.eu	
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IVD	EUROPE* CE ISRAEL
RUO	ALL OTHER COUNTRIES

*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.