Product Description SALSA® MLPA® Probemix P186-C3 PAX3 MITF SOX10

To be used with the MLPA General Protocol.

Version C3. As compared to version C2, four reference probes have been replaced. For complete product history see page 8.

Catalogue numbers:
- **P186-025R:** SALSA MLPA Probemix P186 PAX3 MITF SOX10, 25 reactions.
- **P186-050R:** SALSA MLPA Probemix P186 PAX3 MITF SOX10, 50 reactions.
- **P186-100R:** SALSA MLPA Probemix P186 PAX3 MITF SOX10, 100 reactions.

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

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

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

General information: The SALSA MLPA Probemix P186 PAX3 MITF SOX10 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *PAX3*, *MITF*, and *SOX10* genes, which are associated with Waardenburg Syndrome (WS).

Several types of WS can be caused by mutations in the *MITF* gene (WS2A), the *PAX3* gene (WS1 / WS3) or the *SOX10* gene (WS2E / WS4C). *MITF* transactivates the gene for tyrosinase, a key enzyme for melanogenesis, and is critically involved in melanocyte differentiation. Absence of melanocytes affects pigmentation in the skin, hair, and eyes, and hearing function in the cochlea. Therefore, hypopigmentation and hearing loss in WS2A are likely to be the results of an anomaly of melanocyte differentiation caused by *MITF* mutations. *PAX3* and *SOX10* regulate *MITF*, and failure of this regulation due to mutations in these genes results in the auditory-pigmentary symptoms in at least some individuals with WS1 / WS3 (*PAX3*) or WS2E / WS4C (*SOX10*).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1531/.

This SALSA MLPA Probemix is not CE/FDA registered for use in diagnostic procedures. Purchase of this product includes a limited license for research purposes.

Gene structure and transcript variants:
Entrez Gene shows transcript variants of each gene: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene
For NM_ mRNA reference sequences: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide
Locus Reference Genomic (LRG) database: http://www.lrg-sequence.org/

Exon numbering: The *PAX3*, *MITF*, and *SOX10* exon numbering used in this P186-C3 PAX3 MITF SOX10 product description is the exon numbering from the RefSeq transcripts NM_181457.4, NM_000248.3, and NM_006941.3, which are identical to the NG_021186.1, LRG_776, and LRG_271 sequences. The exon numbering and NM_ sequence used have been retrieved on 06/2020. As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date.

Probemix content: The SALSA MLPA Probemix P186-C3 PAX3 MITF SOX10 contains 41 MLPA probes with amplification products between 130 and 472 nucleotides (nt). This includes 14 probes for the *PAX3* gene, two probes for all nine exons of the gene except for exon 1 (one probe) and exon 2 (no probe), and 12

probes for the *MITF* gene, one probe for each of the nine exons except for exon 1 and additional four probes for exons 1 and 2 of alternative transcript variant 1 (NM_198159.3). Furthermore five probes for the *SOX10* gene are included, one probe for each of the four exons and two probes for exon 4. In addition, ten 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.mlpa.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.mlpa.com.

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

MLPA technique: The principles of the MLPA technique (Schouten et al. 2002) are described in the MLPA General Protocol (www.mlpa.com).

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 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 unrelated individuals who are from families without a history of Waardenburg syndrome. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol.

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

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

Interpretation of results: The standard deviation of each individual probe over all the reference samples should be ≤0.10 and the dosage quotient (DQ) 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 DQ of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

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

- 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. Incomplete DNA denaturation (e.g. due to salt contamination) can lead to a decreased probe signal, in particular for probes located in or near a GC-rich region of the *SOX10* 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.
- When running MLPA products, the capillary electrophoresis protocol may need optimization. False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: lower injection voltage / injection time settings, or a reduced amount of sample by diluting PCR products.

Limitations of the procedure:

- In most populations, the major cause of genetic defects in the *PAX3*, *MITF*, and *SOX10* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P186 PAX3 MITF SOX10.
- MLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations. Even when MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.
- Sequence changes (e.g. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA.

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.

LOVD mutation database: https://databases.lovd.nl/shared/genes. We strongly encourage users to deposit positive results in the Leiden Open Variation Database (LOVD). 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 SNPs and unusual results (e.g., a duplication of *MITF* exons 5 and 7 but not exon 6) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P186-C3 PAX3 MITF SOX10

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]			
		Reference	*PAX3*	*MITF*	*SOX10*
64-105	Control fragments – see table in probemix content section for more information				
130	Reference probe 00797-L13645	5q31			
136 «	**SOX10 probe** 11174-L11858				**Exon 3**
143	**MITF probe** 10791-L12988			**Upstream**	
160 *	Reference probe 05558-L05453	7p14			
166	**PAX3 probe** 05996-L05421		**Exon 3**		
172	Reference probe 14926-L16659	6q22			
178	**PAX3 probe** 06008-L05433		**Exon 8**		
184	**PAX3 probe** 06000-L05425		**Exon 5**		
190 *	Reference probe 21706-L30730	15q21			
196	**MITF probe** 05431-L04847			**Upstream**	
203	**PAX3 probe** 05999-L29550		**Exon 4**		
211	**MITF probe** 10795-L11435			**Exon 6**	
226 «	**SOX10 probe** 11176-L16376				**Exon 4**
232	**PAX3 probe** 06001-L21404		**Exon 5**		
239	Reference probe 15064-L16822	4q31			
247	**MITF probe** 05432-L04848			**Upstream**	
256 «	**SOX10 probe** 11175-L16380				**Exon 4**
264	**PAX3 probe** 06007-L21163		**Exon 8**		
269	Reference probe 15957-L18109	6q15			
276	**PAX3 probe** 10799-L21168		**Exon 3**		
283	**PAX3 probe** 06005-L05430		**Exon 7**		
291	**MITF probe** 10796-L21164			**Exon 7**	
297	**MITF probe** 10793-L21165			**Exon 4**	
303	**PAX3 probe** 05994-L21166		**Exon 1**		
310 «	**SOX10 probe** 14383-L11857				**Exon 2**
319	Reference probe 10084-L10508	8q22			
326	**MITF probe** 10797-L11437			**Exon 8**	
336	**MITF probe** 05433-L04849			**Exon 2**	
346	**MITF probe** 10794-L11434			**Exon 5**	
358	**PAX3 probe** 05998-L29511		**Exon 4**		
364	**PAX3 probe** 10802-L11442		**Exon 8**		
373	Reference probe 11448-L12178	1q41			
382	**PAX3 probe** 06002-L05427		**Exon 6**		
391	**MITF probe** 20989-L29510			**Exon 3**	
402	**PAX3 probe** 10801-L11441		**Exon 7**		
411	**PAX3 probe** 06003-L05428		**Exon 6**		
422	**MITF probe** 10798-L21167			**Exon 9**	
433 *	Reference probe 16284-L23270	11q13			
442 «	**SOX10 probe** 17728-L16387				**Exon 1**
461	**MITF probe** 17734-L21861			**Upstream**	
472 *	Reference probe 00979-L31258	10p14			

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

* New in version C3.
« 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.

Table 2. P186-C3 probes arranged according to chromosomal location

Table 2a. *PAX3* gene

Length (nt)	SALSA MLPA probe	*PAX3* exon[a]	Ligation site NM_181457.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*384-386 (Exon 1)*		
303	05994-L21166	Exon 1	332-333	CCGCACTCGCCT-TTCCGTTTCGCC	2.7 kb
	No probe	Exon 2			

Length (nt)	SALSA MLPA probe	*PAX3* exon[a]	Ligation site NM_181457.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
166	05996-L05421	Exon 3	300 nt before exon 3	TTCCAGGGATGA-GAAAGTATAACC	0.4 kb
276	10799-L21168	Exon 3	790-791	AATCCGAGACAA-ATTACTCAAGGA	1.3 kb
358	05998-L29511	Exon 4	863-864	ATCCTGAGAAGT-AAATTCGGGAAA	0.4 kb
203	05999-L29550	Exon 4	258 nt after exon 4	CTGGGATTCTAG-AACTGTGAATTG	61.7 kb
184	06000-L05425	Exon 5	1019-1020	TCTGAACCAGAT-TTACCACTAAAG	0.1 kb
232	06001-L21404	Exon 5	1121-1122	TACCCTGACATT-TATACTAGGGAG	10.5 kb
382	06002-L05427	Exon 6	217 nt before exon 6	CTGTTGTTGCAA-GATCATGGTGGG	0.2 kb
411	06003-L05428	Exon 6	1203-1204	GCCGTGCAAGAT-GGAGGAAGCAAG	1.0 kb
402	10801-L11441	Exon 7	1385-1386	CCTCAACCGCTT-CCTCCAAGCACT	0.1 kb
283	06005-L05430	Exon 7	1492-1493	TACAGACAGCTT-TGTGCCTCCGTC	18.1 kb
364	10802-L11442	Exon 8	1680-1681	GCAGTCAGAGAC-TAGACCATATGA	0.1 kb
264	06007-L21163	Exon 8	1760-1761	ACAGGCTACAGT-ATGGACCCTGTC	0.6 kb
178	06008-L05433	Exon 8	282 nt after exon 8 (1815-1816 (NM_181458.4))	GTGCCTTTCATT-ATCTCAAGCCAG	
		stop codon	*1821-1823 (Exon 8)*		

The NM_181457 sequence represents transcript variant PAX3 and is a reference standard in the RefSeq gene project. The 178 nt probe is present in the alternative transcript variant NM_181458 (PAX3D).

Table 2b. *MITF* gene

Length (nt)	SALSA MLPA probe	*MITF* exon[a]	Ligation site NM_000248.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*124-126 (Exon 1)*		
143	10791-L12988	Upstream	197 kb before exon 1 (NM_198159.3; 116-117)	CCAGCAGTGGAA-GGACGGGAAGCG	0.1 kb
196	05431-L04847	Upstream	197 kb before exon 1 (NM_198159.3; 170-171)	CCGGATTTCGAA-GTCGGGGAGGAG	139.5 kb
247	05432-L04848	Upstream	57 kb before exon 1 (NM_198159.3; 288-289)	TAAGCTCCTCCA-GTATGACATCAC	0.1 kb
461	17734-L21861	Upstream	57 kb before exon 1 (NM_198159.3; 389-390)	GCGGCCCAGTTC-ATGCAACAGAGA	58.7 kb
	No probe	Exon 1			
336	05433-L04849	Exon 2	317-318	CATGCCACCGGT-GCCGGGGAGCAG	1.2 kb
391	20989-L29510	Exon 3	436-437	AGTGCCCAGGCA-TGAACACACATT	2.1 kb
297	10793-L21165	Exon 4	538-539	TGGGCTTGATGG-ATCCTGCTTTGC	7.8 kb
346	10794-L11434	Exon 5	602-603	TCTTTATGGAAA-CCAAGGTCTGCC	2.8 kb
211	10795-L11435	Exon 6	756-757	AATCACAACCTG-AGTAAGTTGGTT	4.6 kb
291	10796-L21164	Exon 7	833-834	GTCAAATGATCC-GTGAGTACAATC	2.8 kb
326	10797-L11437	Exon 8	899-900	AAAGTTGCAACG-AGAACAGCAACG	5.6 kb
422	10798-L21167	Exon 9	1101-1102	AACTGCAGCCAA-GACCTCCTTCAG	
		stop codon	*1381-1383 (Exon 9)*		

The NM_000248 sequence represents transcript variant 4 and is a reference standard in the RefSeq gene project. NM_198159 represents transcript variant 1.

Table 2c. *SOX10* gene

Length (nt)	SALSA MLPA probe	*SOX10* exon[a]	Ligation site NM_006941.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*302-304 (Exon 2)*		
442 «	17728-L16387	Exon 1	184-185	CACTTCCTAAGG-ACGAGCCCCAGA	0.8 kb
310 «	14383-L11857	Exon 2	503-504	ACGATGACAAGT-TCCCCGTGTGCA	5.5 kb
136 «	11174-L11858	Exon 3	785-786	AGCGGCTCCGTA-TGCAGCACAAGA	4.0 kb
256 «	11175-L16380	Exon 4	1156-1157	AGCCACGAGGTA-ATGTCCAACATG	1.3 kb
226 «	11176-L16376	Exon 4	2464-2465	AATCAGAGACAA-TTCACAGAGCCT	
		stop codon	*1700-1702 (Exon 4)*		

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

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

« 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.

Related SALSA MLPA probemixes

P153 EYA1	Contains probes for the *EYA1* gene, involved in branchio-oto-renal syndrome.
P163 GJB-WFS1	Contains probes for *GJB2, GJB3, GJB6*, *WFS1* genes, and the *POU3F4* area.
P191/P192 Alport	Contains probes for the *COL4A5* gene, involved in Alport syndrome.
P280 SLC26A4	Contains probes for the *SLC26A4* gene, involved in Pendred syndrome.
P169 Hirschsprung 1	Contains probes for *RET, ZEB2, EDN3,* and *GDNF* genes.
P318 Hirschsprung 2	Contains probes for *SOX10, PSPN, NRTN, EDNRB, GFRA1, GFRA2, GFRA3,* and *PHOX2B* genes.
P354 KIT SNAI2	Contains probes for *KIT* and *SNAI2*, involved in piebaldism.

References

- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
- 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 P186 PAX3 MITF SOX10

- Bocángel MAP et al. (2018). Waardenburg syndrome: Novel mutations in a large Brazilian sample. *Eur J Med Genet*, 61(6), 348-354.
- Jalilian N et al. (2018). A comprehensive genetic and clinical evaluation of Waardenburg syndrome type II in a set of Iranian patients. *Int J Mol Cell Med*, 7(1), 17.
- Kanno A et al. (2017). Frequency and specific characteristics of the incomplete partition type III anomaly in children. *The Laryngoscope*, 127(7), 1663-1669.
- Li H et al. (2017). Identification of a novel De novo heterozygous deletion in the SOX10 gene in Waardenburg syndrome type II using next-generation sequencing. *Genet Test Mol Biomarkers*, 21(11), 681-685.
- Matsunaga T et al. (2013) Genetic analysis of PAX3 for diagnosis of Waardenburg syndrome type I. *Acta Otolaryngol.* 133(4): 345-351.
- Milunsky J et al. (2007) The value of MLPA in Waardenburg Syndrome. *Genet Test.* 11:179-82.
- Trabelsi M et al. (2017). Novel PAX3 mutations causing Waardenburg syndrome type 1 in Tunisian patients. *Int J Pediatr Otorhinolaryngol*, 103, 14-19.
- Wenzhi H et al. (2015). Heterozygous deletion at the SOX10 gene locus in two patients from a Chinese family with Waardenburg syndrome type II. *Int J Pediatr Otorhinolaryngol*, 79(10), 1718-1721.
- Wildhardt G et al. (2013) Spectrum of novel mutations found in Waardenburg syndrome types 1 and 2: implications for molecular genetic diagnostics. *BMJ open* 3(3): e001917.

P186 Product history	
Version	*Modification*
C3	Four reference probes have been replaced.
C2	One reference probe has been replaced and several probe lengths have been adjusted.
C1	Four probes for the *SOX10* gene and two additional *PAX3* probes have been included. Seven reference probes have been replaced.
B1	Several *PAX3* and *MITF* probes have been replaced and extra *MITF* probes have been added.
A1	First release.

Implemented changes in the product description

Version C3-01 — 11 August 2020 (02P)
- Product description rewritten and adapted to a new template.
- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).
- Ligation sites of the probes targeting the *PAX3, MITF*, and *SOX10* genes updated according to new version of the NM_ reference sequences.

Version 11 – 14 October 2016 (55)
- Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included).
- Exon numbering of the PAX3 and MITF genes have been changed.
- Various minor textual changes.

Version 10 (54) – 15 July 2015
- Figure based on the use of old MLPA buffer (replaced in December 2012) removed.

Version 09 (48)
- Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added.

Version 08 (47)
- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included).
- Various minor textual changes.

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