Product Description SALSA® MLPA® Probemix P289-A3 LMX1B

To be used with the MLPA General Protocol.

Version A3. As compared to version A2, one probe has been adjusted in length. For complete product history see page 5.

Catalogue numbers:
- **P289-025R:** SALSA MLPA Probemix P289 LMX1B, 25 reactions.
- **P289-050R:** SALSA MLPA Probemix P289 LMX1B, 50 reactions.
- **P289-100R:** SALSA MLPA Probemix P289 LMX1B, 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 P289 LMX1B is a **research use only (RUO)** assay for the detection of deletions or duplications in the *LMX1B* gene, which is associated with Nail-patella syndrome.

The *LMX1B* gene (8 exons) spans ~87 kb of genomic DNA and is located on chromosome 9q33, ~128 Mb from the p-telomere.

Nail patella syndrome (NPS) is an autosomal dominant disorder characterised by nail and skeletal malformations, nephropathy and glaucoma. The renal change resembles glomerulonephritis. Anomalies in a single gene, *LMX1B*, underlie this disease. Genomic alterations affecting the function of the LMX1B gene are found in up to 91% of families with NPS (Ghoumid et al. 2016). *LMX1B* encodes a transcription factor containing two zinc-binding LIM domains (exons 2&3) and a homeodomain (exons 4-6). This transcription factor is involved in multiple developmental processes, including those of the limbs and kidneys, which explains the pleiotropic effect of variation in this gene. The LIM domains are responsible for the interaction with other proteins, whereas the homeodomain binds to DNA. Absence or inactivation of the LIM domain inhibits the interaction with other transcription factors involved in DNA binding. Although clear genotype-phenotype associations have not been found for extrarenal manifestations, mutations in the homeobox are frequently associated with nephropathy (Harita et al. 2016). Along exhaustive information on *LMX1B* sequence variation, MLPA revealed new LMX1B gene deletions (Bongers et al. 2008; Ghoumid et al. 2016). Please note that the MLPA probemix used by Bongers et al. is different from this P289 probemix.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1132.

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 *LMX1B* exon numbering used in this P289-A3 LMX1B product description is the exon numbering from the RefSeq transcript NM_002316.4, which is identical to LRG_1014 for *LMX1B*. The exon

numbering and NM_ sequence used have been retrieved on 11/2019. 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 P289-A3 LMX1B contains 18 MLPA probes with amplification products between 165 and 319 nucleotides (nt). This includes eight probes for the *LMX1B* gene, covering all exons. Furthermore, this probemix also contains a flanking probe for the 9q34 region. In addition, nine 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, 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 nephropathy. 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 or in or near the *LMX1B* 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 *LMX1B* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P289 LMX1B.
- 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.

***LMX1B* mutation database:** https://databases.lovd.nl/shared/genes/LMX1B. 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 *LMX1B* exons 3 and 5 but not exon 4) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P289-A3 LMX1B

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a] Reference	*LMX1B*
64-105	Control fragments – see table in probemix content section for more information		
165	Reference probe 09632-L09917	17q11	
172	**LMX1B probe** 09238-L09566		**Exon 5**
178	Reference probe 05458-L04861	22q11	
184	Reference probe 09822-L10232	10q26	
190	**LMX1B probe** 09236-L09564		**Exon 3**
195	Reference probe 09256-L09448	7q22	
211	Reference probe 15521-L17376	16q13	
229	**LMX1B probe** 13166-L14470		**Exon 8**
235 ¥	**LMX1B probe** 21265-L12575		**Exon 4**
247	Reference probe 06747-L06351	8q12	
256	**LMX1B probe** 10657-L09567		**Exon 7a**
265 «	**LMX1B probe** 09235-L09563		**Exon 2**
274	Reference probe 05284-L04665	14q22	
283 «	**LMX1B probe** 13167-L14471		**Exon 1**
292	**LMX1B probe** 13168-L14472		**Exon 6a**
301	Reference probe 12783-L13918	2q13	
310 ¬	**ENG probe** 03011-L02451		9q34
319	Reference probe 07332-L06969	1q25	

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

¥ Changed in version A3. 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.

Table 2. LMX1B probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*LMX1B* Exon[a]	Ligation site NM_002316.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*523-525 (Exon 1)*		
283 «	13167-L14471	Exon 1	236 nt after exon 1	CCAGGCTTAGGT-TCTAGAGCTGCC	0.7 kb
265 «	09235-L09563	Exon 2	799-800	CCCTCACCACCA-GCTGCTACTTCC	75.5 kb
190	09236-L09564	Exon 3	1015-1016	GCCAGCTGCTGT-GCAAGGGTGACT	2.2 kb
235 ¥	21265-L12575	Exon 4	1097-1098	GAGCGAGGATGA-AGATGGGGACAT	0.4 kb
172	09238-L09566	Exon 5	1 nt before exon 5	CTCTCTGAGCCA-GGTCCGAGAGAC	0.2 kb
292	13168-L14472	Exon 6a	3 nt before exon 6a	GCGCTCTCCCTG-CAGATGAAGAAG	2.2 kb
256	10657-L09567	Exon 7a	1487-1488	GATCGTGGCCAT-GGAACAGAGCCC	0.9 kb
229	13166-L14470	Exon 8	2122-2123	GGTGACCTGAGA-AGCGTGTGTACC	1123.1 kb
		stop codon	*1708-1710 (Exon 8)*		
310 ¬	03011-L02451	*ENG* gene		CTTACTCCAGCT-GTGGCATGCAGG	

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.

¥ Changed in version A3. 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.

References

- Bongers EM et al. (2008). Identification of entire LMX1B gene deletions in nail patella syndrome: evidence for haploinsufficiency as the main pathogenic mechanism underlying dominant inheritance in man. *Eur J Hum Genet.* 16:1240-1244.
- Ghoumid J et al. (2016). Nail-Patella Syndrome: clinical and molecular data in 55 families raising the hypothesis of a genetic heterogeneity. *Eur J Hum Genet*. 24:44-50.
- Harita Y et al. (2017). Spectrum of LMX1B mutations: from nail-patella syndrome to isolated nephropathy. *Pediatr Nephrol*. 32:1845-1850.
- 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 P289 LMX1B

- Ghoumid J et al. (2016). Nail-Patella Syndrome: clinical and molecular data in 55 families raising the hypothesis of a genetic heterogeneity. *Eur J Hum Genet*. 24:44-50.
- Jiang S et al. (2014). A microdeletion of chromosome 9q33.3 encompasses the entire LMX1B gene in a Chinese family with nail patella syndrome. *Int J Mol Sci*. 15:20158-20168.

P289 Product history	
Version	*Modification*
A3	One probe has been adjusted in length.
A2	Three reference probes have been replaced and one removed. Control fragments have been adjusted (QDX2).
A1	First release.

Implemented changes in the product description
Version A3-01 - 06 January 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 *LMX1B* gene updated according to new version of the NM_ reference sequence. - For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36). *Version 07 - 10 October 2016 (55)* - Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included). - Warning for incomplete denaturation added for *LMX1B* probe 09236-L09564 in table 1 and 2. - New references added on page 2. - Various minor textual changes on page 1. *Version 06 - 03 August 2015 (49)* - Electropherogram picture(s) using the old MLPA buffer (replaced in December 2012) removed.

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