Product Description SALSA® MLPA® Probemix P359-A3 PLOD1

To be used with the MLPA General Protocol.

Version A3. For complete product history see page 7.

Catalogue numbers:
- **P359-025R:** SALSA MLPA Probemix P359 PLOD1, 25 reactions.
- **P359-050R:** SALSA MLPA Probemix P359 PLOD1, 50 reactions.
- **P359-100R:** SALSA MLPA Probemix P359 PLOD1, 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 P359 PLOD1 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *PLOD1* gene, which is associated with *PLOD1*-related kyphoscoliotic Ehlers-Danlos syndrome.

The Ehlers-Danlos syndromes (EDS) are a group of heritable connective tissue disorders that share the common features of skin hyperextensibility, articular hypermobility, and tissue fragility. Currently, there are six major types of EDS.
PLOD1-related kyphoscoliotic Ehlers-Danlos syndrome (also known as EDS type VI; OMIM # 225400) is an autosomal recessive generalized connective tissue disorder, characterised by progressive curvature of the spine (kyphoscoliosis), fragile eyes, severe muscle weakness (hypotonia), and generalized joint hypermobility in association with skin fragility. It is caused by mutations in the procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 *(PLOD1)* gene, which encodes lysyl hydroxylase 1. This enzyme plays an important role in the formation and stabilization of collagens. The expected number of *PLOD1* deletions/duplications which can be detected with this MLPA probemix is approximately 33% (Giunta et al. 2005, Pousi et al. 1994, Pousi et al. 1998, Yeowell et al. 2000). The *PLOD1* gene contains 19 exons, spans ~41 kb of genomic DNA, and is located on 1p36.22.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1462/.

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 *PLOD1* exon numbering used in this P359-A3 PLOD1 product description is the exon numbering from the RefSeq transcript NM_000302.4. 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 P359-A3 PLOD1 contains 27 MLPA probes with amplification products between 135 and 409 nucleotides (nt). This includes 19 probes for the *PLOD1* gene, two probes for exon 1, and one probe for each other exon with the exception of exon 9. In addition, eight 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 *PLOD1*-related kyphoscoliotic Ehlers-Danlos 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 or in or near the *PLOD1* 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 *PLOD1* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P359 PLOD1.
- 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.

***PLOD1* mutation database:** https://databases.lovd.nl/shared/genes/PLOD1. We strongly encourage users to deposit positive results in the LOVD Database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report copy number changes detected by the reference probes, false positive results due to SNPs and unusual results (e.g., a duplication of *PLOD1* exons 3 and 5 but not exon 4) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P359-A3 PLOD1

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	PLOD1
64-105	Control fragments – see table in probemix content section for more information		
135	Reference probe 15484-L17324	18q12	
148	**PLOD1 probe** 15464-L17304		**Exon 12**
160	**PLOD1 probe** 15465-L17305		**Exon 18**
178	**PLOD1 probe** 15467-L17307		**Exon 7**
186	**PLOD1 probe** 15468-L18084		**Exon 11**
199	**PLOD1 probe** 15469-L17309		**Exon 8**
212	**PLOD1 probe** 15470-L17310		**Exon 19**
220 *	Reference probe 02452-L01896	15q21	
227	**PLOD1 probe** 15471-L17311		**Exon 13**
237	Reference probe 07909-L08509	17q23	
247	Reference probe 08189-L08083	11q12	
265 «	**PLOD1 probe** 15472-L17312		**Exon 1**
274	**PLOD1 probe** 15473-L17313		**Exon 17**
281	Reference probe 14418-L16419	6p12	
293	**PLOD1 probe** 15474-L17314		**Exon 5**
301	**PLOD1 probe** 15475-L17315		**Exon 3**
310	Reference probe 06597-L06155	8q24	
318	**PLOD1 probe** 15476-L17316		**Exon 2**
328	**PLOD1 probe** 15477-L17317		**Exon 6**
337	**PLOD1 probe** 04685-L04063		**Exon 10**
346	**PLOD1 probe** 15478-L17318		**Exon 16**
355 «	**PLOD1 probe** 15479-L17319		**Exon 1**
364 *	Reference probe 14059-L27828	5q33	
373	**PLOD1 probe** 15480-L17320		**Exon 15**
382	**PLOD1 probe** 15481-L17321		**Exon 4**
400 Ж	**PLOD1 probe** 15482-SP0259-L17322		**Exon 14**
409	Reference probe 07208-L06858	7p14	

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

* New in version A3 (from lot A3-0915 onwards).
« 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.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

Table 2. *PLOD1* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	PLOD1 exon[a]	Ligation site NM_000302.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*64-66 (Exon 1)*		
355 «	15479-L17319	Exon 1	333 nt before exon 1	AGGTAACGCCAT-TAACCCGTGCAA	0.7 kb
265 «	15472-L17312	Exon 1	199 nt after exon 1	TTCGGGAAGGAA-AGGGTGACAGCT	13.0 kb
318	15476-L17316	Exon 2	230-231	CTACAAGATCCA-GGTAAGGGGTTT	1.9 kb
301	15475-L17315	Exon 3	26 nt after exon 3	GCTTCCTAGCCT-GGGCCCCTCCGC	0.5 kb
382	15481-L17321	Exon 4	447-448	GTCTTCTCTGCT-GAGGAGCTCATC	2.1 kb
293	15474-L17314	Exon 5	50 nt before exon 5	CCCCTGACTTAG-AGGCTGGAAGCT	2.3 kb
328	15477-L17317	Exon 6	4 nt before exon 6	ACCTTCTTTCCT-GCAGGAGCAGAT	2.1 kb
178	15467-L17307	Exon 7	733-734	AGTTTGAAATGG-GCCATGTGAGAG	1.0 kb
199	15469-L17309	Exon 8	865-866	AAACAGGCTGCA-CCGTGTGTGACG	2.8 kb
337	04685-L04063	Exon 10	1091-1092	GCATGGCAGCGA-GTACCAGTCTGT	2.9 kb
186	15468-L18084	Exon 11	1235-1236	GACCGAGCCCAA-CAGCCTGCGGCT	0.6 kb
148	15464-L17304	Exon 12	1341-1342	AGTGCAGATGGC-TACTATGCCCGT	0.4 kb
227	15471-L17311	Exon 13	1423-1424	ATATTTCAAACA-TCTACTTGATCA	0.8 kb
400 Ж	15482-SP0259-L17322	Exon 14	1556-1557 and 1580-1581	GTTCCTGACCAA-24nt spanning oligo-CTCCCTAGACAG	0.8 kb
373	15480-L17320	Exon 15	1649-1650	CTTCCCACAGGA-CTGGAAGGAGAA	0.8 kb
346	15478-L17318	Exon 16	1747-1748	TCCCCATCTTCA-CGGAGGTGGCCT	3.8 kb
274	15473-L17313	Exon 17	1944-1945	ACGGAGAAGCTC-TACCCCGGCTAC	2.2 kb
160	15465-L17305	Exon 18	2073-2074	GCCCTGAACCGA-GTCGGGGTGGAT	2.0 kb
212	15470-L17310	Exon 19	2445-2446	ATGGCTGGGGCT-CTCCGTGGTGTT	
		stop codon	*2245-2247 (Exon 19)*		

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.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

Related SALSA MLPA probemixes

P143 MFN2-MPZ	Contains probes for the *MFN2* gene located downstream of *PLOD1* gene
P155 EDS	Contains probes for the *COL3A1* and *TNXB* genes (Vascular Ehlers-Danlos syndrome (vEDS) and classic-like Ehlers-Danlos syndrome (clEDS))
P271/P272 COL1A1/ COL1A2	Contains probes for the *COL1A1* and *COL1A2* genes (Osteogenesis imperfecta)
P331/P332 COL5A1	Contains probes for *COL5A1* gene (Classic Ehlers-Danlos syndrome (cEDS))

References

- Giunta C et al. (2005). Mutation analysis of the PLOD1 gene: an efficient multistep approach to the molecular diagnosis of the kyphoscoliotic type of Ehlers-Danlos syndrome (EDS VIA). *Mol Genet Metab.* 86:269-276.
- Pousi B et al. (1994). Alu-Alu recombination results in a duplication of seven exons in the lysyl hydroxylase gene in a patient with the type VI variant of Ehlers-Danlos syndrome. *Am J Hum Genet.* 55:899-906.
- Pousi B et al. (1998). A compound heterozygote patient with Ehlers-Danlos syndrome type VI has a deletion in one allele and a splicing defect in the other allele of the lysyl hydroxylase gene. *Hum Mutat.* 11:55-61.
- 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.
- Yeowell HN et al. (2000). Mutational analysis of the lysyl hydroxylase 1 gene (PLOD) in six unrelated patients with Ehlers-Danlos syndrome type VI: prenatal exclusion of this disorder in one family. *Hum Mutat.* 16:90.

Selected publications using SALSA MLPA Probemix P359 PLOD1

- Ritelli M et al. (2015). Insights in the etiopathology of galactosyltransferase II (GalT-II) deficiency from transcriptome-wide expression profiling of skin fibroblasts of two sisters with compound heterozygosity for two novel B3GALT6 mutations. *Mol Genet Metab Rep.* 2:1-15.

P359 Product history	
Version	*Modification*
A3	Two reference probes have been replaced.
A2	One reference probe has been replaced and the control fragments have been adjusted (QDX2).
A1	First release.

Implemented changes in the product description
Version A3-06 — 12 November 2019 (02P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *PLOD1* gene updated according to new version of the NM_ reference sequence. *Version 05 - 10 February 2016 (55)* - Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included).

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