Product Description
SALSA® MLPA® Probemix P076-B3 ACADVL-SLC22A5

To be used with the MLPA General Protocol.

Version B3
As compared to version B2, four reference probes have been replaced. For complete product history see page 7.

Catalogue numbers:
- **P076-025R:** SALSA MLPA Probemix P076 ACADVL-SLC22A5, 25 reactions.
- **P076-050R:** SALSA MLPA Probemix P076 ACADVL-SLC22A5, 50 reactions.
- **P076-100R:** SALSA MLPA Probemix P076 ACADVL-SLC22A5, 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.

General information
The SALSA MLPA Probemix P076 ACADVL-SLC22A5 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *ACADVL* and *SLC22A5* genes, which are associated with Very Long-Chain Acyl-coenzyme A Dehydrogenase (VLCAD) deficiency and primary carnitine deficiency.

VLCAD deficiency (OMIM 201475) is a fatty acid oxidation disorder that is detected in new-born screening. VLCAD is a metabolic disorder which prevents the converting of certain fats to energy. Defects of the *ACADVL* (acyl-Coenzyme A dehydrogenase, very long chain) gene are the cause of VLCAD deficiency; 80% of the cases of VLCAD deficiency are caused by mutations in the *ACADVL* gene and 20% of the cases are caused by complete or partial deletions of *ACADVL*.

Another fatty acid oxidation disorder is primary carnitine deficiency (OMIM 212140). Most cases of primary carnitine deficiency are caused by mutations in the *SLC22A5* gene (solute carrier family 22 member 5), however, 20% of the cases do not show any mutation and might be caused by (partial) deletions of *SLC22A5*.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK6816/ and https://www.ncbi.nlm.nih.gov/books/NBK84551/.

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 *ACADVL* and *SLC22A5* exon numbering used in this P076-B3 ACADVL-SLC22A5 product description is the exon numbering from the NG_007975.1 and NG_008982.2 sequences. 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 P076-B3 ACADVL-SLC22A5 contains 42 MLPA probes with amplification products between 130 and 503 nucleotides (nt). This includes 20 probes for the *ACADVL* gene, one probe for each exon with the exception of exon 2 and two probes for exon 4, and ten probes for the *SLC22A5* gene, one probe for each exon. In addition, 12 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 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 VLCAD deficiency and primary carnitine deficiency. 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.

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 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. 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 *ACADVL* and *SLC22A5* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P076 ACADVL-SLC22A5.
- 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.

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 SNVs and unusual results (e.g., a duplication of *ACADVL* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P076-B3 ACADVL-SLC22A5

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*ACADVL*	*SLC22A5*
64-105	Control fragments – see table in probemix content section for more information			
130	Reference probe 08640-L08656	3q		
136	**ACADVL probe** 13445-L14900		**Exon 1**	
142	Reference probe 07721-L07431	7p		
148	**SLC22A5 probe** 15914-L18032			**Exon 9**
157	**ACADVL probe** 13447-L19445		**Exon 10**	
166	**SLC22A5 probe** 15915-L18033			**Exon 7**
172	**ACADVL probe** 13443-L14898		**Exon 14**	
178	**SLC22A5 probe** 15916-L18034			**Exon 4**
190	Reference probe 12422-L13423	14q		
196	**ACADVL probe** 13444-L14899		**Exon 9**	
202	**ACADVL probe** 13449-L14904		**Exon 17**	
209 Ж	**SLC22A5 probe** 15917-SP0306-L18035			**Exon 3**
220	Reference probe 12427-L13428	22q		
229	**SLC22A5 probe** 15918-L18036			**Exon 2**
238	Reference probe 01640-L01178	11q		
252	**SLC22A5 probe** 15919-L18037			**Exon 8**
265	**ACADVL probe** 13458-L14913		**Exon 7**	
274 *	Reference probe 21324-L29730	7q		
281	**ACADVL probe** 13452-L14907		**Exon 16**	
292	**ACADVL probe** 13454-L14909		**Exon 6**	
301	**ACADVL probe** 13434-L14889		**Exon 20**	
310	**SLC22A5 probe** 15920-L18038			**Exon 6**
319 Ж	**ACADVL probe** 16854-SP0400-L19648		**Exon 8**	
328 *	Reference probe 19756-L26539	9q		
337	**SLC22A5 probe** 16845-L19635			**Exon 5**
346	**ACADVL probe** 13441-L14896		**Exon 19**	
355	**ACADVL probe** 13442-L15597		**Exon 11**	
364	**ACADVL probe** 21571-L30567		**Exon 12**	
372	**SLC22A5 probe** 15922-L18040			**Exon 1**
383 *	Reference probe 21008-L29226	8q		
391	**ACADVL probe** 13453-L14908		**Exon 4**	
400	**ACADVL probe** 13461-L14916		**Exon 15**	
409	Reference probe 09999-L10331	20q		
419	**ACADVL probe** 13456-L14911		**Exon 5**	
427 *	Reference probe 22582-L31781	5q		
445	**ACADVL probe** 16855-L19649		**Exon 3**	
454	**ACADVL probe** 13437-L14892		**Exon 18**	
463	**ACADVL probe** 13436-L14891		**Exon 13**	
472	Reference probe 12761-L13877	4q		
483	**ACADVL probe** 13439-L14894		**Exon 4**	
492	**SLC22A5 probe** 15923-L18041			**Exon 10**
503	Reference probe 09870-L18172	2p		

[a] See section Exon numbering on page 1 for more information.

* New in version B3.
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.

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. P076-B3 probes arranged according to chromosomal location

Table 2a. *ACADVL*

Length (nt)	SALSA MLPA probe	*ACADVL* exon[a]	Ligation site NM_000018.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*48-50 (Exon 1)*		
136	13445-L14900	Exon 1	41-40, reverse	TGCATCTCCGAA-TCTCTCCGGGCG	0.4 kb
	No probe	Exon 2			
445	16855-L19649	Exon 3	32 nt before exon 3, reverse	CAAGTTCAGGGA-AGGGACTTCCGC	0.1 kb
391	13453-L14908	Exon 4	31 nt before exon 4, reverse	TTTCAGGGCTCT-GGTTGGGTCTGG	0.1 kb
483	13439-L14894	Exon 4	301-302	GCTCACCACAGA-TCAGGTGTTCCC	0.2 kb
419	13456-L14911	Exon 5	381-382	CTGTGTCCCGTT-TCTTCGAGGTAA	0.1 kb
292	13454-L14909	Exon 6	411-412	ATCCCGCCAAGA-ATGACGCTCTGG	0.6 kb
265	13458-L14913	Exon 7	557-558	GTGGGCATGCAT-GACCTTGGCGTG	0.5 kb
319 Ж	16854-SP0400-L19648	Exon 8	4 nt and 31 nt after exon 8	TGGATCAGGCAA-27 nt spanning oligo-CCGCCCAATTCC	0.1 kb
196	13444-L14899	Exon 9	820-819, reverse	AGACCGTGAAGA-TGTCTGCTAGGC	0.5 kb
157	13447-L19445	Exon 10	973-974	AAACACAGCAGA-GGTGTTCTTTGA	0.5 kb
355	13442-L15597	Exon 11	1203-1204	AGCTGGCACGGA-TGGTTATGCTGC	0.4 kb
364	21571-L30567	Exon 12	2 nt before exon 12	CTATGCAACCTC-AGTCCATGGCTT	0.2 kb
463	13436-L14891	Exon 13	13 nt before exon 13	AGTCTCATCTGT-TCTTTGTCCCTA	0.2 kb
172	13443-L14898	Exon 14	1443-1442, reverse	CCGAAGAATGTC-ATTTGTCCCCTC	0.1 kb
400	13461-L14916	Exon 15	1487-1486, reverse	AGCTCCTTTCCT-TTGTCCTATGGG	0.2 kb
281	13452-L14907	Exon 16	19 nt before exon 16	ACTAACCAGTCA-TTCTCCCTCTTC	0.2 kb
202	13449-L14904	Exon 17	1703-1702, reverse	TTCTTGTGTTTT-ATCAGCTTGGCC	0.2 kb
454	13437-L14892	Exon 18	1795-1794, reverse	CTCCTCACCTCG-AGAGAACCACCA	0.1 kb
346	13441-L14896	Exon 19	1848--1847, reverse	GTCACAGAGCAT-TTTCTCATGCTG	0.2 kb
301	13434-L14889	Exon 20	1957-1958	CTTCAAAAGCAT-CTCCAAGGCCTT	
		stop codon	*2013-2015 (Exon 20)*		

Table 2b. *SLC22A5*

Length (nt)	SALSA MLPA probe	*SLC22A5* exon[a]	Ligation site NM_003060.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*264-266 (Exon 1)*		
372	15922-L18040	Exon 1	390-391	CCGTGTTCCTGA-TAGCGACCCCGG	8.3 kb
229	15918-L18036	Exon 2	726-725, reverse	GAAGGAGCCCAA-CAGCACACCCAC	5.7 kb
209 Ж	15917-SP0306-L18035	Exon 3	776-775 and 11 nt before exon 3, reverse	ACGAACAGCACA--26 nt spanning oligo-AGGAGAGTGACA	1.2 kb
178 #	15916-L18034	Exon 4	947-948	TCAGTTCGTATA-ATATTCTCTACG	1.7 kb
337	16845-L19635	Exon 5	1135-1136	GGGACGATTTGA-AGAGGCAGAGGT	1.9 kb
310	15920-L18038	Exon 6	1271-1272	GATCTGCTTCGA-ACCTGGAATATC	1.8 kb
166	15915-L18033	Exon 7	1378-1377, reverse	AGTTCACAAAGA-TGTCCCCATGCA	1.7 kb
252	15919-L18037	Exon 8	1552-1551, reverse	CCATCACCAGGA-CTGTAGCCAAAT	1.3 kb
148	15914-L18032	Exon 9	1751-1752	ATTCTCATGGGA-AGTCTGACCATC	1.2 kb
492	15923-L18041	Exon 10	2549-2550	TGTGAGCTCTTA-AGACCACTCAGC	
		stop codon	*1935-1937 (Exon 10)*		

[a] See section Exon numbering on page 1 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.

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.

This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.

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.

Related SALSA MLPA probemixes

P465 ACADM — Contains probes for the *ACADM* gene, involved in Medium-Chain Acyl-Coenzyme A Dehydrogenase (MCAD) Deficiency.

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 P076 ACADVL-SLC22A5

- Frigeni M et al. (2017). Functional and molecular studies in primary carnitine deficiency. *Hum mutat*, 38(12), 1684-1699.

P076 product history	
Version	*Modification*
B3	Four reference probes have been replaced.
B2	Three reference probes have been replaced and one probe length has been adjusted.
B1	All SLC22A5 probes have been replaced. Two ACADVL probes have been replaced. Five reference probes have been replaced. Il4 probe at chr. 5 has been removed. Control fragments at 88 nt and 96 nt have been replaced (QDX2).
A1	First release.

Implemented changes in the product description
Version B3-01 – 24 September 2021 (04P) - 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 *ACADVL* and *SLC22A5* genes updated according to new version of the NM_ reference sequence. - Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. *Version 08 – 07 March 2018 (55)* - 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). - Related probemix added on page 1. - New reference added on page 2. - Ligation sites of the probes targeting the *SLC22A5* gene updated according to NM_003060.2 sequence which is equal to NG_008982.1. *Version 07 – 26 November 2015 (55)* - 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 throughout the document.

- Ligation sites of the probes targeting the *ACADVL* gene updated according to new version of the NM_ reference sequence.

Version 06 – 12 August 2015 (54)
- Various minor textual changes.
- Figure(s) based on the use of old MLPA buffer (replaced in December 2012) removed.
- “Peak area” replaced with “peak height”.

Version 05 (48)
- Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added.

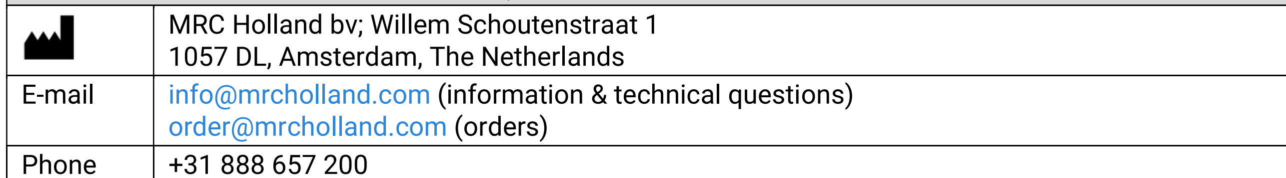
Version 04 (48)
- Remark on RefSeqGene standard and transcript variant added below Table 2.
- Minor textual changes.

Version 03 (46)
- Product description adapted to a new product version (version number changed, lot number added, changes in Table 1 and Table 2, new picture included).
- Minor textual changes.

Version 02 (46)
- Warning added: SLC22A5 probes are more variable.
- Sentence “when only small numbers of samples are tested, visual comparison of peak profiles should be sufficient” removed from data analysis section.

Version 01(46)

Not applicable, new document.



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