Product Description
SALSA® MLPA® Probemix P465-A1 ACADM

To be used with the MLPA General Protocol.

Version A1
For complete product history see page 7.

Catalogue numbers:
- **P465-025R:** SALSA MLPA Probemix P465 ACADM, 25 reactions.
- **P465-050R:** SALSA MLPA Probemix P465 ACADM, 50 reactions.
- **P465-100R:** SALSA MLPA Probemix P465 ACADM, 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 P465 ACADM is a **research use only (RUO)** assay for the detection of deletions or duplications in the *ACADM* gene, which is associated with Medium-Chain Acyl-Coenzyme A Dehydrogenase Deficiency (MCAD). This probemix can also be used to detect the presence of the wild-type sequence of c.985A>G/p.Lys329Glu (rs77931234) point mutation.

MCAD (OMIM 201450) is a disorder of fatty acid β-oxidation. Fatty acid β-oxidation fuels hepatic ketogenesis, a major source of energy for peripheral tissues once glycogen stores become depleted. This disorder is characterised by intolerance to prolonged fasting, impaired ketogenesis, low plasma and tissue carnitine levels, among others. The disorder may be severe, and even fatal, in young patients. Defects in the *ACADM* gene on chromosome 1 are the main cause of MCAD. Most of the known pathologic defects are point mutations but several articles describe small and large deletions of the *ACADM* gene.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1424/.

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 *ACADM* exon numbering used in this P465-A1 ACADM product description is the exon numbering from the LRG_838 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 P465-A1 ACADM contains 27 MLPA probes with amplification products between 161 and 384 nucleotides (nt). This includes 15 probes for the *ACADM* gene, of which one probe specific for the wild-type sequence of the c.985A>G/p.Lys329Glu (rs77931234) mutation, which will only generate a signal when the wildtype sequence is present and three flanking probes that are located upstream and downstream of the *ACADM* gene. Furthermore, this probemix also contains 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.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 Medium-Chain Acyl-Coenzyme A Dehydrogenase Deficiency (MCAD). 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 *ACADM* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P465 ACADM.
- 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.

***ACADM* mutation database**

https://databases.lovd.nl/shared/genes/ACADM. 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 *ACADM* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P465-A1 ACADM

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*ACADM*
64-105	Control fragments – see table in probemix content section for more information		
161	Reference probe 17517-L21416	2q	
167	**ACADM probe** 20646-L28419		**Exon 12**
174	**ACADM probe** 20647-L28619		**Exon 9**
179	Reference probe 01779-L17334	13q	
185 ¬	**LHX8 probe** 20648-L28421		**Upstream**
191	**ACADM probe** 20649-L28618		**Exon 4**
196	**ACADM probe** 20650-L28423		**Exon 7**
204	Reference probe 18317-L27218	15q	
212 ¬	**BCL10 probe** 17948-L28198		**Downstream**
220	**ACADM probe** 20651-L28424		**Intron 4**
229 ∞	**ACADM probe** 20652-L28620		**Exon 11**
244	Reference probe 18056-L22446	16q	
256	**ACADM probe** 20653-L28426		**Exon 12**
262	Reference probe 12434-L13435	14q	
274	**ACADM probe** 20654-L28427		**Exon 6**
283	**ACADM probe** 20655-L28428		**Exon 3**
292	Reference probe 17265-L20654	6q	
301	**ACADM probe** 20656-L28429		**Exon 2**
310	**ACADM probe** 20657-L28430		**Exon 5**
319	**ACADM probe** 20658-L28431		**Exon 10**
329	Reference probe 14481-L16201	4q	
336	**ACADM probe** 20659-L28432		**Exon 1**
346	Reference probe 03080-L02480	8q	
355	**ACADM probe** 20660-L28433		**Exon 8**
367 ¬	**FUBP1 probe** 18571-L23903		**Downstream**
376	**ACADM probe** 20671-L28613		**Exon 11**
384	Reference probe 18677-L24031	11p	

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

∞ Wild type sequence detected. A lowered probe signal can be due to an *ACADM* exon 11 deletion or the presence of a c.985A>G/ p.Lys329Glu (rs77931234) point mutation. Other variants near the ligation site can also cause a lowered signal. A positive result must be confirmed by another method.
¬ 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.

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. ACADM probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*ACADM* exon[a]	Ligation site NM_000016.6	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
185 ¬	20648-L28421	*LHX8*	Upstream	TCTTCTAACATG-GGTGGAGACAGC	567.5 kb
		start codon	*80-82 (Exon 1)*		
336	20659-L28432	Exon 1	204 nt before exon 1	AAACCAAACCAG-GACTATCAGAGA	3.9 kb
301	20656-L28429	Exon 2	139-138, reverse	TGCTGTGATCTC-CAATGAAAACGA	4.3 kb
283	20655-L28428	Exon 3	251-252	AATTTGCCAGAG-AGGAAATCATCC	0.2 kb
191	20649-L28618	Exon 4	299-300	TCTTCTAGTATC-CAGTCCCCCTAA	0.3 kb
220 +	20651-L28424	Intron 4	213 nt after exon 4	CAACCTTAACTT-GCACCTAAACCT	0.4 kb
310	20657-L28430	Exon 5	419-418, reverse	TGTACATCCATA-AGCCAATTCTTC	1.3 kb
274	20654-L28427	Exon 6	542-543	AGGAGCCATTGA-TGTGTGTGAGTA	5.2 kb
196	20650-L28423	Exon 7	595-596	GTAGCTGGTATA-AAGACCAAAGCA	5.9 kb
355	20660-L28433	Exon 8	770-771	ATACCCCAGGAA-TTCAGATTGGGA	3.6 kb
174	20647-L28619	Exon 9	833-832, reverse	CACATCTTCGAA-GACAATTCCTCT	1.0 kb
319	20658-L28431	Exon 10	992-991, reverse	AGTTTTCCTTTC-CAGGGCATACTT	10.7 kb
229 ∞	20652-L28620	Exon 11	1064-1065	AAATGGCAATGA-AAGTTGAACTAG	0.2 kb
376	20671-L28613	Exon 11	1230-1229, reverse	CTACAGGATATT-CTGTATTAAATC	1.5 kb
256	20653-L28426	Exon 12	1413-1414	GAAAAAAGAAAG-GGCTTTAACGTT	0.6 kb
167	20646-L28419	Exon 12	2001-2002	AGGTAGCCTTTG-GTCTATTGTACA	2201.7 kb
		stop codon	*1343-1345 (Exon 12)*		
367 ¬	18571-L23903	*FUBP1*	Downstream	CCATCATGGCGA-TGGACCGGGAAA	7302.6 kb
212 ¬	17948-L28198	*BCL10*	Downstream	TCTTCAACTACA-CTTCCCAGACCT	

[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.

∞ Wild type sequence detected. A lowered probe signal can be due to an *ACADM* exon 11 deletion or the presence of a c.985A>G/ p.Lys329Glu (rs77931234) point mutation. Other variants near the ligation site can also cause a lowered signal. A positive result must be confirmed by another method.
¬ 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.
+ This probe sequence is located in exon 5 of the NM_001286043.1 sequence (ligation site at 812-813) which represents transcript variant 5.

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

P076 ACADVL-SLC22A5 Contains probes for the *ACADVL* and *SLC22A5* genes, involved in Very Long-Chain Acyl-coenzyme A Dehydrogenase (VLCAD) 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 P465 ACADM

- Kaku N et al. (2018). Diagnostic potential of stored dried blood spots for inborn errors of metabolism: a metabolic autopsy of medium-chain acyl-CoA dehydrogenase deficiency. *J. Clin. Pathol.*, *71*(10), 885-889.

P465 product history	
Version	*Modification*
A1	First release.

Implemented changes in the product description
Version A1-03 – 15 September 2021 (04P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *ACADM* gene updated according to new version of the NM_ reference sequence. *Version A1-02 — 01 November 2019 (02P)* - Text referring to the c.985A>G/p.Lys329Glu point mutation has been altered in sections General Information and Probemix content and below tables 1 and 2. *Version A1-01 — 17 July 2019 (02P)* - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *ACADM* gene updated according to new version of the NM_ reference sequence. *Version 01 – 07 October 2015 (55)* - Not applicable, new document.

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