Product Description
SALSA® MLPA® Probemix P025-A5 Canavan disease

To be used with the MLPA General Protocol.

Version A5
As compared to version A4, two reference probes have been replaced and one reference probe has been removed. For complete product history see page 6.

Catalogue numbers:
- **P025-025R:** SALSA MLPA Probemix P025 Canavan disease, 25 reactions.
- **P025-050R:** SALSA MLPA Probemix P025 Canavan disease, 50 reactions.
- **P025-100R:** SALSA MLPA Probemix P025 Canavan disease, 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 P025 Canavan disease is a **research use only (RUO)** assay for the detection of deletions or duplications in the *ASPA* gene, which is associated with Canavan disease.

Canavan disease is an autosomal recessive disorder, characterised by a spongy degeneration of the central nervous system, resulting in blindness, megalocephaly, severe mental defects, atonia of neck muscles, and hyperextension of legs and flexion of arms. Although average life expectancy of Canavan patients is only 18 months, several congenital, infantile, and late-onset forms of Canavan disease have been reported.

Canavan disease is caused by defects in the *ASPA* gene on chromosome 17p13.3, coding for aspartoacylase. Diagnosis can be performed by measuring aspartoacylase activity in cultured fibroblasts. DNA methods detecting the gene defect are desirable for prenatal diagnosis as aspartoacylase activity is low or undetectable in direct or cultured normal chorionic villi and in normal cultured amniocytes. Although Canavan disease occurs in people from all ethnic backgrounds, it is most common in people from Ashkenazi Jewish heritage. In this population, patients mostly carry one or more of the three common point mutations, E285A, Y231X, and A305E. In other populations, a variety of mutations have been found, including deletions of one or more *ASPA* exons.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1234/.

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/
Matched Annotation from NCBI and EMBL-EBI (MANE): https://www.ncbi.nlm.nih.gov/refseq/MANE/
Tark – Transcript Archive: http://tark.ensembl.org/

Exon numbering
The *ASPA* exon numbering used in this P025-A5 Canavan disease product description is the exon numbering from the MANE project (release version 1.0) based on MANE Select transcripts NM_000049.4 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 MANE sequence. 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 P025-A5 Canavan disease contains 21 MLPA probes with amplification products between 141 and 355 nucleotides (nt). This includes seven probes for the *ASPA* gene and four flanking probes for the 17p12-p13 region: two probes each located in the upstream and downstream region of the *ASPA* gene. 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.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 Canavan disease. 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 *ASPA* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P025 Canavan disease.
- 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 *ASPA* exons 4 and 6 but not exon 5) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P025-A5 Canavan disease

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*ASPA*	Flanking probes
141	Reference probe 03088-L22752	16p		
148 ¬	**TRPV1 probe** 01472-L00946			68 kb downstream
166 *	Reference probe 10398-L20610	9q		
173	Reference probe 01301-L00857	5q		
183	**ASPA probe** 07937-L07718		**Exon 1**	
202	Reference probe 03559-L02925	3p		
220 *	Reference probe 13789-L31486	10q		
229 ¬	**PMP22 probe** 01461-L00926			11.7 Mb downstream
238	Reference probe 21319-L29725	7q		
247	**ASPA probe** 01324-L00870		**Exon 1**	
265	**ASPA probe** 01325-L07456		**Exon 5**	
274	Reference probe 00978-L00565	15q		
283	**ASPA probe** 01326-L00873		**Exon 4**	
292 ¬	**GEMIN4 probe** 01079-L00646			2.7 Mb upstream
301	**ASPA probe** 01327-L00872		**Exon 3**	
310	Reference probe 12442-L13443	14q		
319	**ASPA probe** 01328-L00871		**Exon 2**	
328	Reference probe 01043-L00619	8q		
337	**ASPA probe** 01659-L01241		**Exon 6**	
346 ¬	**RAP1GAP2 probe** 00689-L08392			478 kb upstream
355	Reference probe 07736-L07426	20q		

[a] See section Exon numbering on page 2 for more information.

* New in version A5.

¬ 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. *ASPA* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*ASPA* exon[a]	Ligation site NM_000049.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
				Distance from p-telomere to first probe: 597.0 kb	
292 ¬	01079-L00646	*GEMIN4*		AAACAGTGATAG-ACGTCAGCACAG	2.25 **Mb**
346 ¬	00689-L08392	*RAP1GAP2*		CTTTTTATTTAG-CTCCAGAGAAAG	478 kb
		start codon	*164-166 (Exon 1)*		
183	07937-L07718	Exon 1	163-162, reverse	CAAGAAGTCATT-TCACCAAGTAGT	0.1 kb
247	01324-L00870	Exon 1	244-245	GAGCTAACCGGA-GTATTTCTGGTT	5.5 kb
319	01328-L00871	Exon 2	529-530	ACCTCTAACATG-GGGTGCACTCTT	1.8 kb
301	01327-L00872	Exon 3	623-624	TACCCTGCTACG-TTTATCTGATTG	5.7 kb
283	01326-L00873	Exon 4	705-706	AGAAGTTGGTCC-TCAGCCTCAAGG	5.1 kb
265	01325-L07456	Exon 5	837-838	GGTCTATAAAAT-TATAGAGAAAGT	4.5 kb
337	01659-L01241	Exon 6	936-937	ACTGCATCCTGG-GGATCCCATGTT	68.0 kb
		stop codon	*1103-1105 (Exon 6)*		
148 ¬	01472-L00946	*TRPV1*		CAGCCCGAGGAA-GTTTATCTGCGA	11.7 **Mb**
229 ¬	01461-L00926	*PMP22*		TTAACATCCCTT-GCATTTGGCTGC	

[a] See section Exon numbering on page 2 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.

¬ 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.

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 P025 Canavan disease

- Kaya N et al. (2008). Genome-wide gene expression profiling and mutation analysis of Saudi patients with Canavan disease. *Genet Med.* 10:675-84.

P025 product history	
Version	*Modification*
A5	Two reference probes have been replaced and one reference probe has been removed.
A4	Two reference probes have been removed and one reference probe has been replaced.
A3	Three reference probes have been replaced and the control fragments adjusted (QDX2).
A2	Three reference probes have been replaced and four small control fragments have been included.
A1	First release.

Implemented changes in the product description
Version A5-01 – 21 November 2023 (04P) - Product description rewritten and adapted to a new template. Version A4-01 — 3 February 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 ASPA gene updated according to new version of the NM_ reference sequence. - Exon numbering of the ASPA gene has been changed. - Warning added to Table 1 for variability of reference probe 00976-L00563. Version 14 – 16 March 2018 (55) - Information added on positive control DNA samples on page 2. - Various minor textual changes. Version 13 – 28 September 2017 (55) - Product description adapted to a new product lot (lot number added, new picture included).

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