Product Description
SALSA® MLPA® Probemix P498-A1 CSTB

To be used with the MLPA General Protocol.

Version A1
For complete product history see page 6.

Catalogue numbers
- **P498-025R:** SALSA® MLPA® Probemix P498 CSTB, 25 reactions
- **P498-050R:** SALSA® MLPA® Probemix P498 CSTB, 50 reactions
- **P498-100R:** SALSA® MLPA® Probemix P498 CSTB, 100 reactions

SALSA® MLPA® Probemix P498 CSTB (hereafter: P498 CSTB) is to be used in combination with:

1. SALSA® MLPA® Reagent Kit (Cat. No: EK1-FAM, EK1-CY5, EK5-FAM, EK5-CY5, EK20-FAM),
2. Data analysis software Coffalyser.Net™ (Cat. No: n.a.)

Volumes and ingredients

Volumes			Ingredients
P498-025R	**P498-050R**	**P498-100R**	
40 µl	80 µl	160 µl	Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA

The MLPA probemix is not known to contain any harmful agents. Based on the concentrations present, none of the ingredients are hazardous as defined by the Hazard Communication Standard. A Safety Data Sheet (SDS) is not required for this product: none of the ingredients contain dangerous substances at concentrations requiring distribution of an SDS (as per Regulation (EC) No 1272/2008 [EU-GHS/CLP] and 1907/2006 [REACH] and amendments).

Storage and handling

Recommended storage conditions		

A shelf life of until the expiry date is guaranteed, when stored in the original packaging under recommended conditions. For the exact expiry date, see the label on the vial. This product should not be exposed to more than 25 freeze-thaw cycles. Do not use the product if the packaging is damaged or opened. Leave chemicals in original containers. Waste material must be disposed of in accordance with the national and local regulations.

Certificate of Analysis
Information regarding 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
SALSA® MLPA® Probemix P498 CSTB is a **research use only (RUO)** assay for the detection of deletions or duplications in the *CSTB* gene, which is associated with Unverricht-Lundborg disease, also known as Progressive Myoclonic Epilepsy Type 1 (EPM1).

EPM1 is an autosomal recessive neurodegenerative disorder and the most common single cause of progressive myoclonus epilepsy worldwide. Mutations in the *CSTB* gene encoding cystatin B, a cysteine

protease inhibitor, are responsible for the primary defect underlying EPM1. Virtually all affected individuals have an unstable dodecamer repeat expansion in one or both of the *CSTB* alleles (Kälviäinen et al. 2008).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1142/.

This product is not CE/FDA registered for use in diagnostic procedures. The SALSA® MLPA® technique is covered by US patent 6,955,901 and corresponding patents outside the US. The purchase of this product includes a license to use only this amount of product solely for the purchaser's own use.

Gene structure and transcript variants:
Entrez Gene shows transcript variants of each gene: https://www.ncbi.nlm.nih.gov/gene
For NM_ mRNA reference sequences: https://www.ncbi.nlm.nih.gov/nuccore?db=nucleotide
Matched Annotation from NCBI and EMBL-EBI (MANE): https://www.ncbi.nlm.nih.gov/refseq/MANE
Tark – Transcript Archive: https://tark.ensembl.org

Exon numbering
The *CSTB* exon numbering used in this P498-A1 CSTB product description is the exon numbering derived from MANE project (release version 1.0) based on MANE Select transcript NM_000100.4. 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
P498-A1 CSTB contains 15 MLPA probes with amplification products between 130 and 214 nucleotides (nt). This includes five probes for the *CSTB* gene. In addition, 10 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 MLPA (Schouten et al. 2002) are described in the MLPA General Protocol (www.mrcholland.com).

MLPA technique validation
Internal validation using 16 different DNA samples from healthy individuals is required, in particular when using MLPA for the first time, or when changing the sample type or 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. MRC Holland has tested and can recommend the following extraction methods:

- QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual)

- Promega Wizard Genomic DNA Purification Kit (manual)
- Salting out (manual)

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. 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 different reference samples from unrelated individuals should be included in each MLPA experiment for data normalisation. Reference samples should be derived from individuals who are from families without a history of Unverricht-Lundborg 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 deviations to the indicated copy number alteration (CNA) findings might occur.

Data analysis
Coffalyser.Net should be used for data analysis in combination with the appropriate lot-specific Coffalyser sheet. For both, the latest version should be used. Coffalyser.Net 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/gain	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication/gain	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 Coffalyser.Net (Calculations, cut-offs and interpretation remain unchanged.) Please note that Coffalyser.Net 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. single nucleotide variants (SNVs), point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination in the DNA sample) 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 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 *CSTB* gene are dodecamer repeat expansions and small (point) mutations, none of which will be detected by using P498 CSTB.
- 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 in sequence data 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.

***CSTB* mutation database**

https://databases.lovd.nl/shared/genes/CSTB. 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 https://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 *CSTB* exons 1 and 3 but not exon 2) to MRC Holland: info@mrcholland.com.

Table 1. P498-A1 CSTB

Length (nt)	MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*CSTB*
64-105	Control fragments – see table in probemix content section for more information		
130	Reference probe 21303-L29709	3p	
136	Reference probe 22128-L31156	16p	
142	**CSTB probe** 23399-L33139		**Exon 1**
148	Reference probe 17032-L20095	10p	
154	Reference probe 20488-L28069	19p	
160	**CSTB probe** 23400-L33140		**Exon 2**
166	Reference probe 23321-L32967	11q	
172	**CSTB probe** 23401-L28330		**Exon 3**
178	Reference probe 06008-L05433	2q	
184	**CSTB probe** 23402-L33141		**Exon 1**
190	Reference probe 22509-L31658	14q	
196	Reference probe 16322-L18711	7p	
202	**CSTB probe** 23403-L33142		**Exon 3**
208	Reference probe 14966-L16702	6q	
214	Reference probe 11432-L12158	1q	

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

The probe lengths in the table above may vary slightly depending on the capillary electrophoresis machine settings. Please see the most up-to-date Coffalyser sheet for exact probe lengths obtained at MRC Holland.

SNVs located in the target sequence of a probe can influence probe hybridisation and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

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

Length (nt)	MLPA probe	*CSTB* exon[a]	Ligation site[b] NM_000100.4	Partial sequence[c] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*40-42 (Exon 1)*		
142	23399-L33139	Exon 1	31 nt before exon 1	GACCCCAGCGCC-TACTTGGGCTGA	0.1 kb
184	23402-L33141	Exon 1	33-34	CCCTCCGTCGCC-GCCAAGATGATG	1.6 kb
160	23400-L33140	Exon 2	135-134 *reverse*	GGGAACTTCTTG-TTTTCTTTCTCT	0.5 kb
202	23403-L33142	Exon 3	284-283 *reverse*	GGTAGTTAGATA-AGGTCAAGGGCT	0.1 kb
172	23401-L28330	Exon 3	366-367	CCCTTCAGCCAG-AAGACTGACAAA	
		stop codon	*334-336 (Exon 3)*		

[a] See section Exon numbering on page 2 for more information.
[b] Ligation sites are relative to the start of the NM_ sequence, and not relative to the coding sequence.
[c] Complete probe sequences are available at www.mrcholland.com. Please notify us of any mistakes: info@mrcholland.com.

SNVs located in the target sequence of a probe can influence probe hybridisation and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Related products
For related products, see the product page on our website.

References

- Kälviäinen R et al. (2008). Clinical picture of EPM1-Unverricht-Lundborg disease. *Epilepsia*. 49:549-556.
- 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.

P498 product history	
Version	*Modification*
A1	First commercial release.

Implemented changes in the product description
Version A1-01 – 17 December 2024 (05P) - Not applicable, new document.

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