Product Description SALSA® MLPA® Probemix P473-A2 CTNS

To be used with the MLPA General Protocol.

Version A2. As compared to version A1, five reference probes have been replaced. For complete product history see page 7.

Catalogue numbers:
- **P473-025R:** SALSA MLPA Probemix P473 CTNS, 25 reactions.
- **P473-050R:** SALSA MLPA Probemix P473 CTNS, 50 reactions.
- **P473-100R:** SALSA MLPA Probemix P473 CTNS, 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 P473 CTNS is a **research use only (RUO)** assay for the detection of deletions or duplications in the *CTNS* gene, which is associated with cystinosis. This probemix can also be used to detect the presence of the *CTNS* c.414G>A (p.Trp138Ter; W138X) point mutation.

Cystinosis is classified as a lysosomal storage disorder and is inherited in an autosomal recessive manner. Cystinosis can be divided in three categories: 1) nephropathic cystinosis, 2) intermediate cystinosis/late-onset juvenile or adolescent nephropathic cystinosis, and 3) ocular non-nephropathic cystinosis. Nephropathic cystinosis is characterised by renal tubular Fanconi syndrome, poor growth, impaired glomerular function and accumulation of cystine in almost all cells, leading to cellular dysfunction with tissue and organ impairment. A typical untreated child has short stature, rickets and photophobia. The first signs of the disease are generally noticed after approximately six months of age. Intermediate cystinosis is characterised by all the typical manifestations of nephropathic cystinosis, but onset is at a later age, usually between the age of 15 and 25 years. Ocular non-nephropathic cystinosis is characterised clinically only by photophobia resulting from corneal cystine crystal accumulation.

Defects in the *CTNS* gene on chromosome 17 are the main cause of cystinosis. The *CTNS* gene (13 exons) spans ~26.6 kb of genomic DNA and is located on 17p13.3, 3.6 Mb from the p-telomere. The protein encoded by this gene is cystinosin, an integral lysosomal membrane protein. Several articles have described large deletions in the *CTNS* gene in individuals affected with cystinosis, such as a 9.5 to 16 kb deletion (Forestier et al. 1999) and a 65 kb deletion (Shotelersuk et al. 1998). The latter deletion was later corrected to a 57 kb deletion that includes the *SHPK* (*CARKL*) gene (Touchman et al. 2000; Gahl et al. 2002). This 57 kb deletion is found in almost 50% of affected individuals from Northern European descent. The most common point mutation that is found in affected individuals is the c.414G>A (p.Trp138Ter; W138X) mutation. This mutation was found in 14 out of 108 samples (Shotelersuk et al. 1998).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1400/.

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 *CTNS* exon numbering used in this P473-A2 CTNS product description is the exon numbering from the NG_012489.2 sequence. The exon numbering used for the adjacent *SHPK* gene is the exon numbering from the NG_052852.1 sequence. The exon numbering and NG_ sequences used have been retrieved on 09/2020. 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 P473-A2 CTNS contains 31 MLPA probes with amplification products between 136 and 364 nucleotides (nt). This includes 16 probes for *CTNS* copy number detection, one probe for each exon and two probes for exons 1, 2 and 13, and two flanking probes which detect the adjacent *SHPK* gene. Furthermore, this probemix contains one probe specific for the *CTNS* c.414G>A (p.Trp138Ter; W138X) mutation which will only generate a signal when the mutation is present. 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.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 cystinosis. 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. Sample ID number NA06047 from the Coriell Institute has been tested with this P473-A2 probemix at MRC-Holland and can be used as a

positive control sample to detect a heterozygous deletion of the *CTNS* and *SHPK* genes. The quality of cell lines can change; therefore samples should be validated before use.

SALSA Binning DNA SD064 The SD064 Binning DNA provided with this probemix can be used for binning of the *CTNS* c.414G>A (p.Trp138Ter; W138X) mutation-specific probe (172 nt probe, 21085-L29338). SD064 Binning DNA is a mixture of genomic DNA from healthy individuals and synthetic DNA that contains the target sequence detected by the above mentioned probe. Inclusion of one reaction with 5 µl SD064 Binning DNA in initial MLPA experiments is essential as it can be used to aid in data binning of the peak pattern using Coffalyser.Net software. Furthermore, Binning DNA should be included in the experiment whenever changes have been applied to the set-up of the capillary electrophoresis device (e.g. when capillaries have been renewed). Binning DNA should never be used as a reference sample in the MLPA data analysis, neither should it be used in quantification of mutation signal(s), as for this purpose true mutation/SNP positive patient samples or cell lines should be used. It is strongly advised that all samples tested are extracted with the same method and derived from the same source of tissue. For further details, please consult the SD064 Binning DNA product description, available online: www.mlpa.com.

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. 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 *CTNS* gene are small (point) mutations. Except for the c.414G>A (p.Trp138Ter; W138X) mutation, point mutations will not be detected by using SALSA MLPA Probemix P473 CTNS.
- 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.

***CTNS* mutation database:** https://databases.lovd.nl/shared/genes/CTNS. We strongly encourage users to deposit positive results in the Leiden Open Variation 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 *CTNS* exons 5 and 7 but not exon 6) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P473-A2 CTNS

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	CTNS
64-105	Control fragments – see table in probemix content section for more information		
136 *	Reference probe 21304-L29710	7q31	
142 *	Reference probe 21399-L29876	3q22	
148	**CTNS probe** 21083-L29336		**Exon 7**
154	**CTNS probe** 21023-L29248		**Exon 5**
160	**CTNS probe** 21025-L29508		**Exon 1**
166 *	Reference probe 19517-L26011	19p13	
172 §	**CTNS probe** 21085-L29338		**c.414G>A**
178	**CTNS probe** 21027-L29252		**Exon 13**
185 *	Reference probe 21053-L29282	10q24	
190	**CTNS probe** 21022-L29247		**Exon 2**
197	**CTNS probe** 21035-L29260		**Exon 13**
202	**CTNS probe** 21034-L29514		**Exon 4**
208	Reference probe 12490-L17096	1q32	
214	Reference probe 16426-L18879	18q21	
220	**CTNS probe** 21029-L29254		**Exon 8**
227	**CTNS probe** 21031-L29256		**Exon 1**
239	**CTNS probe** 21026-L29251		**Exon 6**
245	Reference probe 14028-L15626	7q36	
255	Reference probe 06236-L27147	21q11	
265 ¬	SHPK probe 21039-L29264		Telomeric
274	**CTNS probe** 21032-L29257		**Exon 3**
281	Reference probe 15958-L18610	4q25	
292	**CTNS probe** 21030-L29255		**Exon 11**
301	**CTNS probe** 21028-L29253		**Exon 9**
310 ¬	SHPK probe 21038-L29263		Telomeric
319 *	Reference probe 19027-L25662	8p12	
333	**CTNS probe** 21036-L29261		**Exon 12**
340	**CTNS probe** 21037-L29309		**Exon 2**
350	**CTNS probe** 21033-L29258		**Exon 10**
357	Reference probe 18692-L29513	2q23	
364	Reference probe 15131-L16901	14q22	

* New in version A2.

§ Mutation-specific probe. This probe will only generate a signal when the c.414G>A (p.Trp138Ter; W138X) mutation is present. It has been tested on artificial DNA **but not on positive human samples!**

¬ 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.

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

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

Length (nt)	SALSA MLPA probe	CTNS exon[a]	Ligation site NM_001031681.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
265 ¬	21039-L29264	SHPK, exon 2	NM_013276.4; 226-227	GTGAGTAGAATC-CTCCAAGCCCTA	5.9 kb
310 ¬	21038-L29263	SHPK, exon 1	NM_013276.4; 70-71	ATTGACCTGGGC-ACCACATCTGTG	0.3 kb
		start codon	*480-482 (Exon 3)*		
227	21031-L29256	Exon 1	63-64	AGGAGCCGGGAG-GCGCTGGCGGCT	0.2 kb
160	21025-L29508	Exon 1	2 nt after exon 1	CGGAGGTCAGGT-GACAGCGGACCC	0.4 kb
190	21022-L29247	Exon 2	284-285	AACCTTTGCGAG-AGCGCCGGTTGA	0.1 kb
340	21037-L29309	Exon 2	423-424	AGGATTACTGTG-TTTTGTGAGAGC	3.0 kb
274	21032-L29257	Exon 3	7 nt after exon 3 *reverse*	GTGTCATTTCTA-AACTTACCACAT	7.2 kb
202	21034-L29514	Exon 4	2 nt before exon 4	TTGGGTCCTTCC-AGAGTCAAGCGT	1.4 kb
154	21023-L29248	Exon 5	630-631	GGCCACCATTAA-ATGCAACCCTGG	6.1 kb
239	21026-L29251	Exon 6	708-709	CCTAACAGGTTG-TGGTGCCTCCTG	0.3 kb
172 §	21085-L29338	Exon 7	893-894	TTTGTGGCCTGA-TCCATCTCCTTC	0.2 kb
148	21083-L29336	Exon 7	129 nt after exon 7 *reverse*	CGCACTGGGACA-ACTTTCTGGAAA	1.1 kb
220	21029-L29254	Exon 8	1023-1024	TCGGCCTCCTCT-GGGTGCCCTACA	0.1 kb
301	21028-L29253	Exon 9	1052-1053	GAGCAGTTTCTC-CTCAAATACCCC	1.4 kb
350	21033-L29258	Exon 10	1283-1282 *reverse*	ATGTAGGAGAAG-CAGAAGAGAAAC	1.8 kb
292	21030-L29255	Exon 11	1368-1369	GCACTGAGGGCT-GGAGCATTGGCA	0.4 kb
333	21036-L29261	Exon 12	1551-1552	TCTGTTTGTACA-GAAAGAGACCGG	0.5 kb
197	21035-L29260	Exon 13	1766-1767	GTGGCCAGTGAA-CTCAGAGGTGCT	1.8 kb
178	21027-L29252	Exon 13	3529-3530	TCCCAGGTGGGA-GCTGAGGCTAGG	
		stop codon	*1680-1682 (Exon 13)*		

§ Mutation-specific probe. This probe will only generate a signal when the c.414G>A (p.Trp138Ter; W138X) mutation is present. It has been tested on artificial DNA **but not on positive human samples!**
¬ 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.

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.

References

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P473 Product history	
Version	*Modification*
A2	Five reference probes have been replaced.
A1	First release.

Implemented changes in the product description

Version A2-01 — 23 September 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 *CTNS* and *SHPK* genes updated according to new versions of the NM_ reference sequences.
- For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36).

Version 02 – 16 March 2018 (55)
- Information added on positive control DNA samples on page 2.
- Various minor textual changes.

Version 01 – 08 December 2016 (55)
- Not applicable, new document.

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