Product Description
SALSA® MLPA® Probemix P098-E1 Wilson disease

To be used with the MLPA General Protocol.

Version E1
As compared to version D1, three target probes, including the wild type probe for N1270S, and two reference probes have been replaced. For complete product history see page 7.

Catalogue numbers:
- **P098-025R:** SALSA MLPA Probemix P098 Wilson disease, 25 reactions.
- **P098-050R:** SALSA MLPA Probemix P098 Wilson disease, 50 reactions.
- **P098-100R:** SALSA MLPA Probemix P098 Wilson 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 P098 Wilson disease is a **research use only (RUO)** assay for the detection of deletions or duplications in the *ATP7B* gene, which is associated with Wilson disease. This probemix can also be used to detect the presence of the R778L (c.2333G>T), A874V (c.2621C>T), H1069Q (c.3207C>A) and N1270S (c.3809A>G) point mutations, and the c.-441_427del15 mutation (15BP-DEL).

Wilson disease is an autosomal recessive disorder characterised by dramatic build-up of intracellular copper. Clinical features include liver disease, neurological problems and psychiatric disturbance. Wilson disease is mainly caused by missense mutations and small deletions and insertions in the *ATP7B* gene (Bandmann et al. 2015). The gene product is a member of the P-type cation transport ATPase family and plays an important role in transporting copper from the liver to other parts of the body (Bandmann et al. 2015). Wilson disease–causing mutations have a reduced penetrance and, although it was believed that the birth genetic prevalence of this disease is about 1:30000, new studies with different methodologies have estimated a significantly higher prevalence, of about 1:7000 (Gao et al. 2019).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1512/

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 *ATP7B* exon numbering used in this P098-E1 Wilson disease product description is the exon numbering from the NG_008806.1 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 NG 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 P098-E1 Wilson disease contains 41 MLPA probes with amplification products between 132 and 445 nucleotides (nt). This includes 28 probes for the *ATP7B* gene, including five probes detecting the wildtype sequence for the R778L (c.2333G>T), A874V (c.2621C>T), H1069Q (c.3207C>A) and N1270S (c.3809A>G) point mutations, as well as the c.-441_427del15 mutation (15BP-DEL). Furthermore, one flanking probe in the *ALG11* gene is included. 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 Wilson 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. Sample ID numbers NA03330, NA05258, NA12606, NA13721 and NA14164 from the Coriell Institute have been tested with this P098-E1 probemix at MRC Holland and can be used as positive control samples to detect the heterozygous duplication of the *ATP7B* gene (NA03330 and NA12606), the heterozygous deletion of the *ATP7B* gene (NA13721 and NA14164) and the monoallelic presence of the H1069Q mutation within the *ATP7B* gene. The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Source	Altered target genes in P098-E1	Expected copy number alteration
NA03330	Coriell Institute	*ATP7B*	Heterozygous duplication
NA05258	Coriell Institute	H1069Q mutation, in exon 14 of the *ATP7B* gene	Heterozygous H1069Q mutation
NA12606	Coriell Institute	*ATP7B*	Heterozygous duplication
NA13721	Coriell Institute	*ATP7B*	Heterozygous deletion
NA14164	Coriell Institute	*ATP7B*	Heterozygous deletion

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 *ATP7B* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P098 Wilson 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.

***ATP7B* mutations database**

https://databases.lovd.nl/shared/genes/ATP7B. 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 SNVs and unusual results (e.g., a duplication of *ATP7B* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P098-E1 Wilson disease

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*ATP7B*
64-105	Control fragments – see table in probemix content section for more information		
132	Reference probe 00797-L21698	5q	
139 ¬	**ALG11 probe** 22059-L31021		***ALG11* Exon 1**
148	**ATP7B probe** 21005-L29223		**Exon 1**
154	Reference probe 14813-L16521	1p	
160	**ATP7B probe** 10737-L19693		**Exon 17**
166 *	Reference probe 09267-L20529	10q	
169 *	**ATP7B probe** 23092-L32589		**Exon 1**
178	**ATP7B probe** 03245-L18515		**Exon 10**
184	**ATP7B probe** 03238-L02675		**Exon 2**
190	Reference probe 11368-L12093	4q	
196	**ATP7B probe** 03246-L18638		**Exon 13**
202	**ATP7B probe** 16668-L19239		**Exon 12**
211	**ATP7B probe** 03239-L02676		**Exon 3**
220 ∞	**ATP7B probe** 16308-L18637		**Exon 14**
229	Reference probe 01828-L01393	16p	
235 ∞	**ATP7B probe** 22065-L31282		**Exon 1**
241	**ATP7B probe** 03240-L19695		**Exon 4**
252	**ATP7B probe** 03248-L18516		**Exon 15**
258 ∞	**ATP7B probe** 04413-L15003		**Exon 8**
265	Reference probe 08575-L08576	17q	
274	**ATP7B probe** 03241-L21643		**Exon 5**
283	**ATP7B probe** 10738-L19698		**Exon 19**
289	Reference probe 14830-L17168	3q	
296	**ATP7B probe** 10736-L19699		**Exon 8**
303	**ATP7B probe** 03242-L19700		**Exon 6**
310	**ATP7B probe** 22061-L31023		**Exon 18**
317 * Ж ∞	**ATP7B probe** 22804-SP1014-L32166		**Exon 18**
328	**ATP7B probe** 03243-L19703		**Exon 7**
336	**ATP7B probe** 22062-L31024		**Exon 20**
346 ∞	**ATP7B probe** 16306-L19705		**Exon 11**
353 *	Reference probe 19475-L26908	18q	
364	Reference probe 01202-L00787	8p	
370 *	**ATP7B probe** 23093-L32590		**Exon 14**
382	**ATP7B probe** 12940-L14808		**Exon 16**
391	**ATP7B probe** 05551-L19706		**Exon 11**
400	Reference probe 12757-L13873	7q	
409	**ATP7B probe** 12866-L19707		**Exon 9**
418	**ATP7B probe** 05555-L19708		**Exon 21**
427	Reference probe 16235-L18488	12p	
436	**ATP7B probe** 22063-L31325		**Exon 21**
445	Reference probe 21728-L30386	15q	

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

* New in version E1.
∞ Wild type sequence detected. A lowered probe signal can be due to one of the following mutations: H1069Q (220 nt probe), 15BP-DEL (235 nt probe), R778L (258 nt probe), N1270S (317nt probe) or A874V (346 nt probe). Other variants near the ligation site can also cause a lowered signal. A positive result must be confirmed by another method.
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.
¬ 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. *ATP7B* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*ATP7B* exon[a]	Ligation site NM_000053.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
139 ¬	22059-L31021	*ALG11* Exon 1		TGCTCACCTCAA-CAACTTGCACAG	0.7 kb
		start codon	*114-116 (exon 1)*		
235 ∞	22065-L31282	Exon 1	328 nt before exon 1 reverse WT at 15BP-DEL mutation	TCGGCCACCTCG-CGCTGGTGCGCT	0.4 kb
148	21005-L29223	Exon 1	74-75	ACCTTTTGCTCT-GAGCCAGATCAG	0.1 kb
169	23092-L32589	Exon 1	2 nt after exon 1	CAGTCGGAAAGT-GAGTTTTGTTCC	36.3 kb
184	03238-L02675	Exon 2	334-335	GTCATGTGTGAA-GTCCATTGAGGA	4.5 kb
211	03239-L02676	Exon 3	1603-1604	GATCAAAGGCAT-GACCTGTGCATC	2.0 kb
241	03240-L19695	Exon 4	1684-1685	GGTTGCCTTGAT-GGCAGGAAAGGC	3.6 kb
274	03241-L21643	Exon 5	1861-1862	CCACAACATAGA-GTCCAAACTCAC	3.1 kb
303	03242-L19700	Exon 6	2011-2012	TTCCCTGGCCCA-GAGAAACCCCAA	1.6 kb
328	03243-L19703	Exon 7	2086-2087	CCTGTGCAGCCT-GGTGTTTGGCAT	1.9 kb
296	10736-L19699	Exon 8	2366-2367	CTGGTGGTTGCT-GTGGCTGAGAAG	0.1 kb
258 ∞	04413-L15003	Exon 8	2446-2445 reverse WT at R778L mutation	GTTCCAGCCACC-GGCCCAGGGCAA	0.8 kb
409	12866-L19707	Exon 9	2499-2500	TGGCTAAACTCA-TGTCTCTCCAAG	7.3 kb
178	03245-L18515	Exon 10	2629-2630	TGGGGGAAAGTT-TCCAGTGGATGG	0.2 kb
346 ∞	16306-L19705	Exon 11	2734-2735 WT at A874V mutation	CACTGTAATTGC-GGGGTCTATAAA	0.1 kb
391	05551-L19706	Exon 11	2793-2794	TGGGCAATGACA-CCACTTTGGCTC	0.3 kb
202	16668-L19239	Exon 12	2870-2869 reverse	TATCCACTAAAC-CGGTCAGCCAGC	3.4 kb
196	03246-L18638	Exon 13	3049-3050	CATCACGGTGCT-GTGCATTGCCTG	2.2 kb
370	23093-L32590	Exon 14	3205-3206	CAAGACTGGCAC-CATTACCCATGG	0.1 kb
220 ∞	16308-L18637	Exon 14	3320-3321 WT at H1069Q mutation	AGCAGTGAACAC-CCCTTGGGCGTG	1.7 kb
252	03248-L18516	Exon 15	3486-3487	GTGCACCGGCCA-GTCACCTGAATG	1.3 kb
382	12940-L14808	Exon 16	3612-3611 reverse	TGTCATAGCGTC-ACTGACATCGCT	2.1 kb
160	10737-L19693	Exon 17	10 nt after exon 17	AGGTACAGCCCT-TTAATGTTGCAA	1.4 kb
310	22061-L31023	Exon 18	3823-3824	GGTTGGCATCAA-CAAAGTCTTTGC	0.1 kb
317 Ж	22804-SP1014-L32166	Exon 18	3880-3881 and 3922-3923 WT at N1270S mutation	CCAGGAGCTCCA-42 nt spanning oligo-TGACTCCCCGGC	0.2 kb
283	10738-L19698	Exon 19	4050-4051	CTAGCATTCACC-TTTCCAAGAGGA	1.8 kb
336	22062-L31024	Exon 20	4225-4226	GCTCTCATCCCT-GCAGCTCAAGTG	0.6 kb
418	05555-L19708	Exon 21	4267-4268	GGAGAGGTATGA-GGCACAGGCGCA	1.8 kb
436	22063-L31325	Exon 21	6094-6095	ATAAACGAGGAA-AAGGTCCTTGCC	
		stop codon	*4509-4511 (exon 21)*		

[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.

∞ Wild type sequence detected. A lowered probe signal can be due to one of the following mutations: H1069Q (220 nt probe), 15BP-DEL (235 nt probe), R778L (258 nt probe), N1270S (317nt probe) or A874V (346 nt probe). Other variants near the ligation site can also cause a lowered signal. A positive result must be confirmed by another method.
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.
¬ 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.

Related SALSA MLPA probemixes

P104 Menkes ATP7A Menkes disease (MD). Contains probes that target the *ATP7A* gene.

References

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P098 product history	
Version	*Modification*
E1	Three target probes, including the wild type probe for N1270S, and two reference probes have been replaced.
D1	Three target probes have been replaced, and three target probes have been added (including a wildtype probe for the c.-441_427del15 mutation and one flanking probe). Also, one reference probe has been replaced and one extra reference probe has been added. Finally, one reference probe has been elongated, no change in sequence detected.
C2	The length of some probes has been adjusted.
C1	Probes for exons 11 and 16 have been removed. Probes for exon 12 and 14 and several reference probes have been replaced. Probes specific for the A874V and H1069Q mutations in *ATP7B* have been added.

B2	The 88 and 96 nt DNA denaturation control probes have been replaced (QDX2) and one probe has a small change in length.
B1	The number of *ATP7B* specific probes has been increased from 16 to 26. All 21 *ATP7B* exons are now covered. In addition, extra control fragments at 100 and 105 nt have been included.

Implemented changes in the product description

Version E1-01 – 4 April 2022 (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 *ATP7B* gene updated according to new version of the NM_ reference sequence.
- Positive samples added.

Version D1-02 – 16 October 2019 (01P)
- Additional information on a second target site for the 320 nt N1270S Wild type probe added to Table 1, Table 2 and Precautions and Warnings.

Version D1-01 – 03 October 2018 (01P)
- Product description restructured and adapted to a new template.
- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).

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

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