Product Description SALSA® MLPA® Probemix P104-C2 Menkes ATP7A

To be used with the MLPA General Protocol.

Version C2. As compared to version C1, four reference probes have been replaced and three probes have been adjusted in length. For complete product history see page 7.

Catalogue numbers:
- **P104-025R:** SALSA MLPA Probemix P104 Menkes ATP7A, 25 reactions.
- **P104-050R:** SALSA MLPA Probemix P104 Menkes ATP7A, 50 reactions.
- **P104-100R:** SALSA MLPA Probemix P104 Menkes ATP7A, 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 P104 Menkes ATP7A is a **research use only (RUO)** assay for the detection of deletions or duplications in the *ATP7A* gene, which is associated with Menkes disease (MD).

Menkes disease (MD) is a multi-systemic disorder of copper metabolism. Progressive neurodegeneration and connective tissue disturbances, together with the peculiar 'kinky' hair are the main manifestations. MD is an X-linked recessive disorder, and as expected the vast majority of patients are males. MD occurs due to pathogenic variants in the *ATP7A* gene and the vast majority of *ATP7A* pathogenic variants are intragenic mutations or partial gene deletions. ATP7A is an energy dependent transmembrane protein, which is involved in delivery of copper to the copper-dependent enzymes and is therefore critical for copper homeostasis. Severely affected MD patients usually die before the third year of life.

The *ATP7A* gene (23 exons) spans ~140 kb of genomic DNA and is located on chromosome Xq21.1, ~78 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1413/.

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 *ATP7A* exon numbering used in this P104-C2 Menkes ATP7A product description is the exon numbering from the RefSeq transcript NM_000052.7, which is identical to the NG_013224.2 sequence. The exon numbering and NM_ sequence used have been retrieved on 07/2020. 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 P104-C2 Menkes ATP7A contains 34 MLPA probes with amplification products between 130 and 445 nucleotides (nt). This includes one probe for each exon of the

ATP7A gene and two probes for exon 1. In addition, 10 reference probes are included that detect locations on the X-chromosome. 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 of the same sex 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 Menkes disease (MD). It is recommended to use samples of the same sex to facilitate interpretation. 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. 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.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:

Copy number status: Male samples	Dosage quotient
Normal	0.80 < DQ < 1.20
Deletion	DQ = 0
Duplication	1.65 < DQ < 2.25
Ambiguous copy number	All other values

Copy number status: Female samples	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 *ATP7A* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P104 Menkes ATP7A.
- 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.

***ATP7A* mutation database:** https://databases.lovd.nl/shared/genes/ATP7A. 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 SNPs and unusual results (e.g., a duplication of *ATP7A* exons 5 and 7 but not exon 6) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P104-C2 Menkes ATP7A

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*ATP7A*
64-105	Control fragments – see table in probemix content section for more information		
130	Reference probe 13499-L02104	Xp11	
142	**ATP7A probe** 07483-L07140		**Exon 21**
150 ¥	**ATP7A probe** 03109-L32299		**Exon 1**
157	**ATP7A probe** 03117-L02557		**Exon 12**
166 *	Reference probe 14200-L15814	Xq13	
179	Reference probe 05619-L14947	Xq28	
188	**ATP7A probe** 03118-L22384		**Exon 13**
200	**ATP7A probe** 07481-L22385		**Exon 16**
209	**ATP7A probe** 03111-L22386		**Exon 3**
220	**ATP7A probe** 03119-L22387		**Exon 15**
228	**ATP7A probe** 03903-L02554		**Exon 7**
238	**ATP7A probe** 03120-L02560		**Exon 17**
247	Reference probe 03650-L03063	Xp22	
259	**ATP7A probe** 03901-L22388		**Exon 5**
271 ¥	**ATP7A probe** 21527-L22390		**Exon 19**
279	**ATP7A probe** 21087-L02553		**Exon 6**
292	**ATP7A probe** 03122-L02562		**Exon 20**
300	Reference probe 16690-L19263	Xp11	
308 ¥	**ATP7A probe** 21379-L02555		**Exon 9**
320	**ATP7A probe** 03123-L02563		**Exon 22**
328 *	Reference probe 07097-L06677	Xp22	
338	**ATP7A probe** 03116-L14467		**Exon 10**
346	**ATP7A probe** 03149-L14468		**Exon 1**
355 *	Reference probe 03741-L03201	Xq28	
363	**ATP7A probe** 07477-L07134		**Exon 4**
372	**ATP7A probe** 07479-L07136		**Exon 11**
382	Reference probe 02908-L02302	Xq22	
394	**ATP7A probe** 07482-L07139		**Exon 18**
400	**ATP7A probe** 21088-L29507		**Exon 8**
409	**ATP7A probe** 07480-L07137		**Exon 14**
418 *	Reference probe 05124-L04514	Xq26	
427	**ATP7A probe** 12797-L13935		**Exon 23**
436	**ATP7A probe** 12793-L13929		**Exon 2**
445	Reference probe 13119-L14338	Xp11	

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

* New in version C2.

¥ Changed in version C2. Minor alteration, no change in sequence detected.

Table 2. *ATP7A* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*ATP7A* exon[a]	Ligation site NM_000052.7	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*165-167 (Exon 2)*		
150	03109-L32299	Exon 1	40-41	GCAGCTACTGTG-ACTTCTCCGATT	0.1 kb
346	03149-L14468	Exon 1	132-133	TAACCATAGGAT-AGAGAAACCAGG	61.0 kb
436	12793-L13929	Exon 2	95 nt after exon 2	GACACACTGACA-CATTTGGCAATA	16.6 kb
209	03111-L22386	Exon 3	462-463	TGACTTTGCCAT-GGGACCATATCC	0.9 kb
363	07477-L07134	Exon 4	913-914	ATTGGGAGCTAT-TGATGTAGAACG	9.2 kb
259	03901-L22388	Exon 5	1628-1629	AAGAATTCATCT-AAGTGTTACATA	4.5 kb
279	21087-L02553	Exon 6	1761-1762	AAGTAAGGTATA-ATCCTGCTGTTA	6.0 kb
228	03903-L02554	Exon 7	1939-1940	ACACAGAGGGAT-CCTATACTGCTC	2.1 kb
400	21088-L29507	Exon 8	2097-2096 reverse	TCTTATTTCTCG-TTTATGATCTAA	0.4 kb
308	21379-L02555	Exon 9	2269-2270	TATGTTCCTGGA-GCGCCAGATTCT	1.5 kb
338	03116-L14467	Exon 10	2528-2529	CCTATGCTGTTT-GTGTTTATTGCA	1.6 kb
372	07479-L07136	Exon 11	2610-2611	TAATTTCACTAC-AAGCTACAGAAG	1.1 kb
157	03117-L02557	Exon 12	2737-2738	CAAATTTCCAGT-GGATGGTCGTGT	4.4 kb
188	03118-L22384	Exon 13	2812-2813	GCCTGTGGCTAA-GAAACCTGGCAG	0.7 kb
409	07480-L07137	Exon 14	2998-2997 reverse	AAACAAAAACAA-TAAAAGGAACAA	8.3 kb
220	03119-L22387	Exon 15	3129-3130	TACGATTTGCTT-TCCAAGCCTCTA	2.2 kb
200	07481-L22385	Exon 16	3417-3416 reverse	TAGAGGGTGTTC-ACTGTTACTTTC	2.1 kb
238	03120-L02560	Exon 17	3482-3483	GAAACCTTGGGT-ACCTGCATAGAT	5.3 kb
394	07482-L07139	Exon 18	3768-3767 reverse	TTCAGTCATGAA-ATCATTTACATC	1.7 kb
271	21527-L22390	Exon 19	3862-3863	AGACACAGTGAA-GCCTGAAGCAGA	2.0 kb
292	03122-L02562	Exon 20	4012-4013	TTCTCACAAGGT-TGCTAAAGTGAA	0.8 kb
142	07483-L07140	Exon 21	4256-4255 reverse	ACCAGATTATAA-ATTAGAGCAAAG	2.1 kb
320	03123-L02563	Exon 22	4323-4324	TTTTGCAGCCCT-GGATGGGATCTG	0.9 kb
427	12797-L13935	Exon 23	4453-4454	AGGACAGAAGAG-TCCTTCAGAAAT	
		stop codon	*4665-4667 (Exon 23)*		

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.

Related SALSA MLPA probemixes

- P098 Wilson: Contains probes for the *ATP7B* gene involved in Wilson disease.

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 P104 Menkes ATP7A

- Cao B et al. (2017). Identification of novel ATP7A mutations and prenatal diagnosis in Chinese patients with Menkes disease. *Metab Brain Dis.* 32:1123–1131.
- de Gemmis P et al. (2017). 13 novel putative mutations in ATP7A found in a cohort of 25 Italian families. *Metab Brain Dis.* 32:1173–1183.
- Mogensen M et al. (2011). Exon duplications in the ATP7A gene: frequency and transcriptional behaviour. *Orphanet J Rare Dis.* 6:73.
- Moller LB et al. (2015). Partial Gene Duplications in ATP7A Accounts for 5% of the Disease Causing Mutations in Menkes Disease. *Conference paper http://www.researchgate.net/publication/267027743*.
- Moller LB et al. (2012). Clinical expression of Menkes disease in females with normal karyotype. *Orphanet J Rare Dis. 7:6.*
- Moller LB et al. (2011). Diagnosis of copper transport disorders. *Curr Protoc Hum Genet*. Chapter 17:Unit 17.9.
- Schoonveld C et al. (2013). Prenatal diagnostic conundrum involving a novel ATP7A duplication. *Clin Genet.* 84:97-8.

P104 Product history	
Version	*Modification*
C2	Four reference probes have been replaced and three probes have been adjusted in length.
C1	One target probe for *ATP7A* has been removed, one reference probe has been added and two replaced. In addition two probe lengths have been adjusted.
B2	The control fragments have been adjusted (QDX2). Furthermore, three reference probes have been removed and three replaced. Also, a probe targeting the Y-chromosome has been removed.
B1	Two new *ATP7A* specific probes, one reference probe and four control fragments have been added.
A1	First release.

Implemented changes in the product description

Version C2-01 — 28 September 2020 (02P)
- Product description rewritten and adapted to a new template.
- Ligation sites of the probes targeting the *ATP7A* gene updated according to new version of the NM_ reference sequence.

Version 13 – 14 December 2016 (55)
- Information on percentage deletions / duplications removed from page 1.
- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new pictures included).
- Changes of probe lengths in Table 1 and Table 2 in order to better reflect the true lengths of the amplification products.
- Various textual changes on page 1 and throughout the document.

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