Product Description
SALSA® MLPA® Probemix P305-B3 AGXT

To be used with the MLPA General Protocol.

Version B3
As compared to version B2, three reference probes have been removed and four reference probes have been replaced, in addition six probe lengths have been adjusted. For complete product history see page 7.

Catalogue numbers:
- **P305-025R:** SALSA MLPA Probemix P305 AGXT, 25 reactions.
- **P305-050R:** SALSA MLPA Probemix P305 AGXT, 50 reactions.
- **P305-100R:** SALSA MLPA Probemix P305 AGXT, 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 P305 AGXT is a **research use only (RUO)** assay for the detection of deletions or duplications in the *AGXT* and *GRHPR* genes, which are associated with primary hyperoxaluria type 1 and 2. This probemix can also be used to detect the presence of the *AGXT* c.731T>C (p.I244T), c.33_34insC (c.33dupC; p.Lys12GlnfsX156) and c.508G>A (p.G170R) point mutations.

Primary hyperoxaluria type 1 and 2 are each caused by a deficiency of an enzyme that catalyses glyoxylate conversion (alanine-glyoxylate aminotransferase encoded by *AGXT* and glyoxylate reductase/hydroxypyruvate reductase encoded by *GRHPR*, respectively). This deficiency results in the production of oxalate, which accumulates in the form of calcium oxalate crystals. Persistent hyperoxaluria causes nephrolithiasis and nephrocalcinosis, leading to renal failure, followed by tissue oxalosis with life-threatening complications. There are three types of primary hyperoxaluria, of which type 1 is the most severe, followed by type 2. Both type 1 and type 2 are inherited in an autosomal recessive manner (Howles and Thakker. 2020).

The *AGXT* gene (11 exons) spans ~11.7 kb of genomic DNA and is located on 2q37.3, 241.5 Mb from the p-telomere (only 1.5 Mb from the q-telomere). The *GRHPR* gene (9 exons) spans ~14.3 kb of genomic DNA and is located on 9p13.2, 37.4 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1283/ and https://www.ncbi.nlm.nih.gov/books/NBK2692/.

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 *AGXT* exon numbering used in this P305-B3 AGXT product description is the exon numbering from the NG_008005.1 sequence. The *GRHPR* exon numbering used in this P305-B3 AGXT product description is the exon numbering from the NG_008135.1 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 P305-B3 AGXT contains 31 MLPA probes with amplification products between 132 and 382 nucleotides (nt). This includes 12 probes for the *AGXT* gene (one probe for each exon and two probes for exon 1) and eight probes for the *GRHPR* gene (one for each exon except exon 5). Furthermore, this probemix also contains three probes specific for the *AGXT* c.731T>C (in exon 7), c.33_34insC (in exon 1) and c.508G>A (in exon 4) point mutations which will only generate a signal when the mutation is present. In addition, eight 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 hereditary kidney diseases. 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 NA01229, NA14943 and NA05067 from the Coriell Institute have been tested with this P305-B3 probemix at MRC-Holland and can be used as positive control samples to detect a heterozygous duplication of the *GRHPR* gene (NA05067) and a heterozygous duplication (NA01229) and a heterozygous deletion (NA14943) of the *AGXT* gene. The quality of cell lines can change; therefore samples should be validated before use.

SALSA Binning DNA SD030

The SD030 Binning DNA provided with this probemix can be used for binning of all probes including the *AGXT* c.731T>C, c.33_34insC and c.508G>A mutation-specific probes (*AGXT* probe 09743-L32674, probe 21829-L32675 and probe 09740-L10150, respectively). SD030 Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequence detected by the above mentioned probe. Inclusion of one reaction with 5 µl SD030 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). 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 SD030 Binning DNA product description, available online: www.mrcholland.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.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 *AGXT* and *GRHPR* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P305 AGXT.
- 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.

***AGXT* and *GRHPR* mutation databases**

https://databases.lovd.nl/shared/genes/AGXT and https://databases.lovd.nl/shared/genes/GRHPR. 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 *AGXT* exons 8 and 10 but not exon 9) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P305-B3 AGXT

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*AGXT*	*GRHPR*
64-105	Control fragments – see table in probemix content section for more information			
132	Reference probe 09173-L09347	11q		
137	**GRHPR probe** 11508-L25680			**Exon 8**
143	**GRHPR probe** 10442-L10994			**Exon 3**
148	**AGXT probe** 09735-L10141		**Exon 1**	
154	**AGXT probe** 09745-L10153		**Exon 8**	
160	Reference probe 09992-L10451	7q		
166	**GRHPR probe** 11509-L11002			**Exon 9**
173	**AGXT probe** 09737-L12708		**Exon 2**	
184 *	Reference probe 06748-L06352	8q		
190	**AGXT probe** 09741-L10148		**Exon 5**	
199	**GRHPR probe** 10446-L10999			**Exon 6**
207	**AGXT probe** 09739-L10146		**Exon 4**	
214	**AGXT probe** 09749-L10158		**Exon 11**	
231 ¥ §	**AGXT probe** 09743-L32674		**c.731T>C mutation**	
240 ¥	**AGXT probe** 13371-L32803		**Exon 3**	
247	Reference probe 10808-L11455	4q		
264 ¥ §	**AGXT probe** 21829-L32675		**c.33_34insC mutation**	
274	**GRHPR probe** 10441-L10993			**Exon 2**
283 §	**AGXT probe** 09740-L10150		**c.508G>A mutation**	
288 *	Reference probe 17604-L21601	18q		
299 ¥	**AGXT probe** 22998-L14829		**Exon 1**	
311	**AGXT probe** 09747-L12945		**Exon 10**	
319 ¥	**GRHPR probe** 22933-L32628			**Exon 7**
328	**AGXT probe** 09746-L25678		**Exon 9**	
336	Reference probe 09027-L09281	1q		
346 *	Reference probe 11360-L12085	12p		
355	**AGXT probe** 11902-L12711		**Exon 6**	
364 ¥	**GRHPR probe** 22829-L10997			**Exon 4**
369	**AGXT probe** 12377-L13386		**Exon 7**	
373	**GRHPR probe** 10440-L14001			**Exon 1**
382 *	Reference probe 02459-L01903	15q		

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

* New in version B3.

¥ Changed in version B3. Minor alteration, no change in sequence detected.

§ Mutation-specific probe. This probe will only generate a signal when the mutation is present (c.731T>C 231 nt; c.33_34insC 264 nt; c.508G>A 283 nt), small signals are visible in negative samples at 231 nt and 264 nt. It has been tested on artificial DNA **but not on positive human samples!**

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. P305-B3 probes arranged according to chromosomal location

Table 2a. *AGXT* gene

Length (nt)	SALSA MLPA probe	*AGXT* exon[a]	Ligation site NM_000030.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*43-45 (Exon 1)*		
299	22998-L14829	Exon 1	62 nt before exon 1	GCCCATCCACCA-ATCCTCACCTCT	0.1 kb
264 §	21829-L32675	Exon 1	76-75 *reverse*	AGCAGGGCCTTG-GGGGGGGGTCAC	0.1 kb
148	09735-L10141	Exon 1	198-199	ATGAGCAAGGAT-ATGTACCAGGTA	0.3 kb
173	09737-L12708	Exon 2	362-363	GGCCAATGGCAT-TTGGGGGCAGCG	1.3 kb
240	13371-L32803	Exon 3	426-427	CCGATGACCAAG-GACCCTGGAGGC	0.7 kb
207	09739-L10146	Exon 4	500-501	GCTGCTGTTCTT-AACCCACGGGGA	0.1 kb
283 §	09740-L10150	Exon 4	550-551	TTGATGGCTTCA-GGGAACTCTGCC	1.6 kb
190	09741-L10148	Exon 5	591-592	CTCCTGGTGGAT-TCGGTGGCATCC	1.1 kb
355	11902-L12711	Exon 6	722-intron 6	TGACAAGGCCAA-GTGAGTGACCCA	1.0 kb
369	12377-L13386	Exon 7	66 nt before exon 7	ACAGGACAGCCA-GCGAGACTGCCC	0.1 kb
231 §	09743-L32674	Exon 7	773-772 *reverse*	CCAGCCACTTGG-TGTCCAGGTAGA	0.8 kb
154	09745-L10153	Exon 8	831-832	TACCATCACACA-ATCCCCGTCATC	1.7 kb
328	09746-L25678	Exon 9	969-970	GGGCTGCAGCTC-TTCGTGAAGGAC	0.4 kb
311	09747-L12945	Exon 10	1002-1003	CGGCTTCCCACA-GTCACCACTGTG	1.0 kb
214	09749-L10158	Exon 11	1450-1449 *reverse*	CTGCAGCTCATT-TGGAAGGCACTG	
		stop codon	*1219-1221 (Exon 11)*		

Table 2b. *GRHPR* gene

Length (nt)	SALSA MLPA probe	*GRHPR* exon[a]	Ligation site NM_012203.2	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*56-58 (Exon 1)*		
373	10440-L14001	Exon 1	75-76	GGTGCGACTCAT-GAAGGTGTTCGT	2.1 kb
274	10441-L10993	Exon 2	166-167	TGGGACTCGGAT-GAGCCCATCCCT	1.1 kb
143	10442-L10994	Exon 3	331-332	GCTTTGGATGAA-ATCAAGAAGCGG	0.6 kb
364	22829-L10997	Exon 4	395-396	CCACCGCCGAAC-TCGCAGTCTCCC	3.2 kb
	No probe	Exon 5			
199	10446-L10999	Exon 6	593-594	GTGTCCAGAGAT-TTCTGTACACAG	0.8 kb
319	22933-L32628	Exon 7	679-680	CTGGCTGCCCAA-TCTGATTTCATC	1.6 kb
137	11508-L25680	Exon 8	887-888	AACCACTGCCTA-CAAACCACCCTC	4.7 kb
166	11509-L11002	Exon 9	1027-1028	CCGATGCCTAGT-GAACTCAAGCTG	
		stop codon	*1040-1042 (Exon 9)*		

[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.

§ Mutation-specific probe. This probe will only generate a signal when the mutation is present (c.731T>C 231 nt; c.33_34insC 264 nt; c.508G>A 283 nt), small signals are visible in negative samples at 231 nt and 264 nt. It has been tested on artificial DNA **but not on positive human samples!**

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

- Howles SA and Thakker RV. (2020). Genetics of kidney stone disease. *Nat Rev Urol*. 17:407-421.
- 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 P305 AGXT

- Beck BB et al. (2013). Novel findings in patients with primary hyperoxaluria type III and implications for advanced molecular testing strategies. *Eur J Hum Genet*. 21:162-172.

P305 product history	
Version	*Modification*
B3	Three reference probes have been removed and four reference probes have been replaced, in addition six probe lengths have been adjusted.
B2	Three reference probes have been removed and control fragments have been adjusted (QDX2).
B1	First commercial release.

Implemented changes in the product description
Version B3-01 – 24 May 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). - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. Version B2-01 — 20 October 2020 (02P) - Product description rewritten and adapted to a new template. - Various minor textual or layout changes. - Ligation sites of the probes targeting the AGXT and GRHPR genes updated according to new versions of the NM_ reference sequences. Version 12 – 15 August 2019 (55) - Comment on the mutation specific probes has been adjusted below both Table 1 and 2. The text: , small signals (<5%) are visible in negative samples at 229 nt and 264 nt. has been added.

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