Product Description
SALSA® MLPA® Probemix P439-C1 COL4A3

To be used with the MLPA General Protocol.

Version C1
Five target and five reference probes were replaced, one target probe was removed and one target probe was added. Five target and one reference probe were changed in length, not in sequence detected. For complete product history see page 7.

Catalogue numbers:
- **P439-025R:** SALSA MLPA Probemix P439 COL4A3, 25 reactions.
- **P439-050R:** SALSA MLPA Probemix P439 COL4A3, 50 reactions.
- **P439-100R:** SALSA MLPA Probemix P439 COL4A3, 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 P439 COL4A3 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *COL4A3* gene, which is associated with Alport syndrome.

Alport syndrome (AS) is an inherited disorder of the basement membrane, resulting in progressive renal failure due to glomerulonephropathy, variable sensorineural hearing loss, and variable ocular anomalies. It is a genetically heterogeneous disorder, with all forms resulting from mutations in the genes encoding the alpha-3 (*COL4A3*; 2q36.3), alpha-4 (*COL4A4*; 2q36.3), and alpha-5 (*COL4A5*; Xq22.3) chains of type IV collagen, which is a major structural component of the basement membrane (Nozu et al. 2019). AS can be divided into X-Linked AS (XLAS), autosomal recessive AS (ARAS), and autosomal dominant AS (ADAS). XLAS is caused by mutations in the *COL4A5* gene and accounts for approximately 80 % of AS, whereas ARAS and ADAS are caused by mutations in the *COL4A3* and *COL4A4* genes and account for approximately 15 % and 5 % of AS, respectively (Nozu et al. 2019).

Although point mutations in *COL4A3* may be a more common cause of disease, intragenic deletions of *COL4A3* have been reported (Morinière et al. 2014).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1207/

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 *COL4A3* exon numbering used in this P439-C1 COL4A3 product description is the exon numbering from the LRG_230 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 LRG 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 P439-C1 COL4A3 contains 41 MLPA probes with amplification products between 129 and 481 nucleotides (nt). This includes 32 probes for the *COL4A3* gene. In addition, 9 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 Alport syndrome. 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 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.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 *COL4A3* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P439 COL4A3.
- 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.

***COL4A3* mutation database**

https://databases.lovd.nl/shared/genes/COL4A3. 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 SNVs and unusual results (e.g., a duplication of *COL4A3* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P439-C1 COL4A3

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*COL4A3*
64-105	Control fragments – see table in probemix content section for more information		
129	Reference probe 20879-L25093	12q	
135 *	Reference probe 20901-L28955	1p	
145 ¥	**COL4A3 probe** 22966-L32404		**Exon 33**
150 ¥	**COL4A3 probe** 18712-L32518		**Exon 44**
154	**COL4A3 probe** 18714-L24080		**Exon 25**
160	**COL4A3 probe** 18716-L24893		**Exon 11**
166 *	**COL4A3 probe** 22982-L32421		**Exon 23**
172	Reference probe 07915-L07628	8q	
178	**COL4A3 probe** 18717-L24083		**Exon 26**
184	**COL4A3 probe** 18718-L24895		**Exon 30**
190	**COL4A3 probe** 18719-L24085		**Exon 27**
195	**COL4A3 probe** 18720-L24086		**Exon 18**
202	**COL4A3 probe** 18721-L24087		**Exon 51**
211	**COL4A3 probe** 18724-L24090		**Exon 2**
220	**COL4A3 probe** 18723-L24089		**Exon 13**
230 *	Reference probe 10721-L29208	6p	
238	**COL4A3 probe** 18725-L24091		**Exon 48**
253 * Ø	**COL4A3 probe** 22984-L32423		**Intr.1**
265 *	**COL4A3 probe** 22985-L32424		**Exon 35**
270	**COL4A3 probe** 18729-L24095		**Exon 42**
279 *	Reference probe 17450-L32456	16p	
289 *	**COL4A3 probe** 22986-L32425		**Exon 19**
303 ¥	**COL4A3 probe** 18734-L32405		**Exon 41**
310	**COL4A3 probe** 18735-L24101		**Exon 20**
319	**COL4A3 probe** 18736-L24102		**Exon 8**
328	**COL4A3 probe** 18737-L24103		**Exon 49**
335 *	Reference probe 15117-L29502	9q	
346	**COL4A3 probe** 18738-L24104		**Exon 38**
364	**COL4A3 probe** 18740-L24106		**Exon 3**
372	**COL4A3 probe** 18741-L24107		**Exon 9**
382 *	**COL4A3 probe** 22987-L32426		**Exon 6**
392 ¥	**COL4A3 probe** 22967-L24109		**Exon 17**
409	Reference probe 17224-L20551	11p	
418	**COL4A3 probe** 18746-L24112		**Exon 5**
429 *	**COL4A3 probe** 22988-L32427		**Exon 7**
436 ¥	**COL4A3 probe** 18747-L32519		**Exon 47**
445	**COL4A3 probe** 18748-L24897		**Exon 37**
454	**COL4A3 probe** 18749-L25182		**Exon 32**
463	**COL4A3 probe** 18751-L24117		**Exon 28**
472 ¥	Reference probe 12761-L32406	4q	
481 *	Reference probe 19198-L25224	3q	

* New in version C1.
¥ Changed in version C1. Minor alteration, no change in sequence detected.
Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.

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. *COL4A3* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*COL4A3* exon[a]	Ligation site NM_000091.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*53-55 (exon 1)*		
253 Ø	22984-L32423	Intr.1	344 nt after exon 1	AACACCGGATGT-GGAGATGCACGG	72.8 kb
211	18724-L24090	Exon 2	200-199 reverse	TTTGTCTTTACA-GACACAACCCTA	2.2 kb
364	18740-L24106	Exon 3	255-256	ACAGGGGGAGAA-GGGCTTTCCTGG	4.8 kb
418	18746-L24112	Exon 5	404-405	GATTGCCAGGAT-TTTCTGGTTCTC	1.0 kb
382	22987-L32426	Exon 6	460-461	CCTTACGGACTT-GTCGGTGTACCA	0.8 kb
429	22988-L32427	Exon 7	540-539 reverse	AACCTACCGGGA-TCCCTGGGTAGC	0.8 kb
319	18736-L24102	Exon 8	551-552	CCTAGGGTGCTG-CTGGTTTGAAAG	1.0 kb
372	18741-L24107	Exon 9	647-646 reverse	GTGCTGTACCTG-GGGTCCTGGAGC	2.8 kb
160	18716-L24893	Exon 11	743-744	GACCTAGAGGAC-CTAAGGTAGACT	2.2 kb
220	18723-L24089	Exon 13	821-822	GACCCCCGGGAC-CACCAGGAACAG	2.8 kb
392	22967-L24109	Exon 17	1067-1068	GTTTCCCTGGGT-TAATGGGTGAAG	1.2 kb
195	18720-L24086	Exon 18	1095-1094 reverse	TGTCCCCTTTCT-GTCCCTACAGTG	2.3 kb
289	22986-L32425	Exon 19	6 nt after exon 19	CACAAGGTAAGA-ATAAATTTCTTC	1.2 kb
310	18735-L24101	Exon 20	1239-1238 reverse	GACTTCCAGGAA-CTCCGGGGGGAC	6.0 kb
166	22982-L32421	Exon 23	1606-1607	AAAGGAATCCCA-GGTACAAACAAT	3.8 kb
154	18714-L24080	Exon 25	1764-1765	GGGTGTCCCAGG-TGACCCGGGGCT	2.1 kb
178	18717-L24083	Exon 26	1867-1868	TCACAGGCTCTG-AGTGGTGAGAAA	3.4 kb
190	18719-L24085	Exon 27	2058-2057 reverse	CTGGAACTGGTG-TTGAAACACTGA	1.0 kb
463	18751-L24117	Exon 28	2125-2124 reverse	GATCCAGGGATA-CCTTAGAAACAA	3.1 kb
184	18718-L24895	Exon 30	2470-2471	GGGCTTGATGGA-CCACGAGGTACA	1.9 kb
454	18749-L25182	Exon 32	2727-2728	TCAAAGAGGATA-TCCAGGAAATCC	1.3 kb
145	22966-L32404	Exon 33	2777-2778	ATGGAGTGATTG-GGATGATGGGCT	5.4 kb
265	22985-L32424	Exon 35	3004-3003 reverse	TTTCCTTTCTCA-CCTGGATTTCCT	1.7 kb
445	18748-L24897	Exon 37	3247-3246 reverse	TCTCCCTGGAGA-CCATGAATACCT	2.5 kb
346	18738-L24104	Exon 38	3397-3396 reverse	GCTCCCTCAGGT-CCAGCAGGCCCC	2.1 kb
303	18734-L32405	Exon 41	13 nt after exon 41	TAAGCAGTTCTT-CTTCCCTGTCCT	2.4 kb
270	18729-L24095	Exon 42	3762-3763	ACCCACAGGCAT-AGAAGGATTCCC	5.3 kb
150	18712-L32518	Exon 44	3989-3990	AAATATAGGGAG-CACCAGGTACTC	2.0 kb
436	18747-L32519	Exon 47	4333-4334	GAAAAAGGCAAC-AAAGGTTCTAAA	2.7 kb
238	18725-L24091	Exon 48	4436-4437	GGACAACGAGAG-GCTTTGTCTTCA	1.2 kb
328	18737-L24103	Exon 49	4706-4707	TGAACATGGCTC-CCATTACTGGCA	1.9 kb
202	18721-L24087	Exon 51	4979-4980	GCAACTACTATT-CAAATTCCTACA	
		stop codon	*5114-5116 (exon 52)*		

[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.

Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.

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Related SALSA MLPA probemixes

P191 Alport-mix 1	Contains probes for the *COL4A5* gene involved in XLAS.
P192 Alport-mix 2	Contains probes for the *COL4A5* gene involved in XLAS.
P444 COL4A4	Contains probes for the *COL4A4* gene involved in ARAS and ADAS.

References

- Morinière et al. (2014). Improving Mutation Screening in Familial Hematuric Nephropathies through Next Generation Sequencing. *J Am Soc Nephrol*. 25:2740-2751.
- Nozu et al. (2019). A review of clinical characteristics and genetic backgrounds in Alport syndrome. *Clin Exp Nephrol*. 23:158–168.
- 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 P439 COL4A3

- Gillion V et al. (2018). Genotype and outcome after kidney transplantation in Alport syndrome. *Kidney Int Rep.* 3:652-660.
- Weber S et al. (2016). Identification of 47 novel mutations in patients with Alport syndrome and thin basement membrane nephropathy. *Pediatr Nephrol*. 31:941-955.
- Yamamura T et al. (2019). Comparison between conventional and comprehensive sequencing approaches for genetic diagnosis of Alport syndrome. *Mol Genet Genomic med*. 7:e883.

P439 product history	
Version	*Modification*
C1	Five target and five reference probes were replaced, one target probe was removed and one target probe was added. Five target and one reference probe were changed in length, not in sequence detected.
B1	One target probe has been removed and two probe lengths have been adjusted.
A1	First release.

Implemented changes in the product description
Version C1-01 – 04 February 2021 (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 *COL4A3* gene updated according to new version of the NM_ reference sequence. Version 04 – 23 February 2017 (55) - Product description adapted to a new product version (version number changed, lot number added, new pictures included).

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