Product Description
SALSA® MLPA® Probemix P444-A3 COL4A4

To be used with the MLPA General Protocol.

Version A3
As compared to version A2, three references probes have been replaced, two reference probes have been removed, and one target probe has been changed in length, but not in the sequence it detects. For complete product history see page 7.

Catalogue numbers:
- **P444-025R:** SALSA MLPA Probemix P444 COL4A4, 25 reactions.
- **P444-050R:** SALSA MLPA Probemix P444 COL4A4, 50 reactions.
- **P444-100R:** SALSA MLPA Probemix P444 COL4A4, 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 P444 COL4A4 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *COL4A4* 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 *COL4A4* may be a more common cause of disease, intragenic deletions of *COL4A4* 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 *COL4A4* exon numbering used in this P444-A3 COL4A4 product description is the exon numbering from the LRG_231 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 P444-A3 COL4A4 contains 43 MLPA probes with amplification products between 130 and 510 nucleotides (nt). This includes 35 probes for the *COL4A4* gene. 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 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 of 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 *COL4A4* gene are small (point) mutations, none of which will not be detected by using SALSA MLPA probemix P444 COL4A4.
- 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.

***COL4A4* mutation database**

https://databases.lovd.nl/shared/genes/COL4A4. 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 *COL4A4* exons 3 and 5 but not exon 4) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P444-A3 COL4A4

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*COL4A4*
64-105	Control fragments – see table in probemix content section for more information		
130	Reference probe 11230-L11913	08q	
136	**COL4A4 probe** 18950-L24741		**Exon 31**
142	**COL4A4 probe** 18951-L24742		**Exon 40**
154 *	Reference probe 03931-L03386	15q	
166	**COL4A4 probe** 18955-L26262		**Exon 42**
172	**COL4A4 probe** 18954-L28346		**Exon 29**
178	**COL4A4 probe** 18956-L24747		**Exon 34**
184	**COL4A4 probe** 18958-L24749		**Exon 22**
190	**COL4A4 probe** 18960-L24751		**Exon 48**
202	**COL4A4 probe** 18961-L25596		**Exon 4**
211	**COL4A4 probe** 18964-L24756		**Exon 9**
220	Reference probe 01782-L01346	13q	
229	**COL4A4 probe** 18965-L24757		**Exon 16**
239	**COL4A4 probe** 18966-L24758		**Exon 6**
247	**COL4A4 probe** 18967-L24759		**Exon 35**
265 Ж	**COL4A4 probe** 18970-SP0730-L24762		**Exon 25**
274 ¥	**COL4A4 probe** 18971-L32672		**Exon 1**
283	Reference probe 10732-L11314	06p	
292	**COL4A4 probe** 18972-L24764		**Exon 19**
301	**COL4A4 probe** 18973-L24765		**Exon 45**
310	**COL4A4 probe** 18974-L24766		**Exon 3**
319	**COL4A4 probe** 18975-L24767		**Exon 7**
328	**COL4A4 probe** 18976-L24768		**Exon 2**
337	**COL4A4 probe** 18977-L24769		**Exon 20**
346	**COL4A4 probe** 18979-L24771		**Exon 44**
355	Reference probe 11614-L12374	12p	
362	**COL4A4 probe** 18980-L24772		**Exon 26**
372	**COL4A4 probe** 18981-L24773		**Exon 33**
382	**COL4A4 probe** 18982-L24774		**Exon 8**
391	**COL4A4 probe** 18983-L24775		**Exon 37**
400	**COL4A4 probe** 18984-L24776		**Exon 46**
409	**COL4A4 probe** 18985-L24777		**Exon 38**
418	**COL4A4 probe** 18986-L24778		**Exon 36**
427 *	Reference probe 20872-L28890	01q	
436	**COL4A4 probe** 18987-L24779		**Exon 13**
445	**COL4A4 probe** 18989-L24781		**Exon 5**
454	**COL4A4 probe** 18990-L27967		**Exon 10**
463	**COL4A4 probe** 18991-L24783		**Exon 41**
472	**COL4A4 probe** 18992-L24784		**Exon 24**
480	**COL4A4 probe** 18994-L24786		**Exon 28**
490	**COL4A4 probe** 18995-L24787		**Exon 32**
500 *	Reference probe 09682-L22509	03p	
510	Reference probe 13438-L22894	05q	

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

* New in version A3.
¥ Changed in version A3. Minor alteration, no change in sequence detected.
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. When this occurs in reference samples, it can look like an increased signal in the test 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. *COL4A4* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*COL4A4* exon[a]	Ligation site NM_000092.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
274	18971-L32672	Exon 1	182 nt before exon 1	CAGGGTAAGGGG-TTAAGGAGGTGA	
328	18976-L24768	Exon 2	246-245 reverse	TCTTCCAGAAGG-TTCTTGTTGACA	16,8 kb
		Start codon	*301-303 (exon 2)*		
310	18974-L24766	Exon 3	389-390	TATACTCATTCT-CTTTTCTGTACA	3,0 kb
202	18961-L25596	Exon 4	464-465	TTGCTCTGTTTG-CCACTGTGTTCC	4,3 kb
445	18989-L24781	Exon 5	624-625	GACAAAGGAGAT-AAGGTAAGCCAC	19,2 kb
239	18966-L24758	Exon 6	658-659	GATTTCCAGGTT-TAGATGGCATAC	1,1 kb
319	18975-L24767	Exon 7	725-726	GAGTGGCCACAA-TGGCTCAAGAGG	1,2 kb
382	18982-L24774	Exon 8	828-829	GGAAATTCAGTG-TTCATTTTAGGT	4,1 kb
211	18964-L24756	Exon 9	10 nt after exon 9 reverse	AGAGCCTGCTCA-GGAGACTTACTG	3,0 kb
454	18990-L27967	Exon 10	903-904	TAGGGATCTTGG-GGTGCAGGAGGA	2,4 kb
436	18987-L24779	Exon 13	1074-1075	GGACCCACCCTG-TTGGTAGAGCCA	5,3 kb
229	18965-L24757	Exon 16	1265-1264 reverse	CCTTTAATCCTG-GAAAACCTGGAG	2,1 kb
292	18972-L24764	Exon 19	15 nt after exon 19	CGGATGCCCTGA-TGTGCCCTGGCT	3,2 kb
337	18977-L24769	Exon 20	1521-1522	ATAGGACCCCCT-GGGCCACAAGGA	4,4 kb
184	18958-L24749	Exon 22	1766-1767	TCTAGGAAATGA-AGGACTCTGTGC	5,5 kb
472	18992-L24784	Exon 24	8 nt after exon 24	TCCAGTATGTAG-AATTCTTCTCTT	8,4 kb
265 Ж	18970-SP0730-L24762	Exon 25	2222-2223; 2252-2253	ACCAGGAGAGCG-30 nt spanning oligo-TGTGAGGGGCCC	2,5 kb
362	18980-L24772	Exon 26	2298-2299	GGTGACACAATT-TCTTGCAACGTA	15,3 kb
480	18994-L24786	Exon 28	2608-2607 reverse	CCTCTTTCCCGG-GGGTCCCAGGTG	3,1 kb
172	18954-L28346	Exon 29	2784-2785	GGCCATTCCTGT-GAAAGAGGTGCT	2,0 kb
136	18950-L24741	Exon 31	3151-3152	GGGGACTGAGAG-GGGCCAAAGGTA	2,9 kb
490	18995-L24787	Exon 32	3253-3252 reverse	TTCTCCTGGGAA-TCCATCATCTCC	2,3 kb
372	18981-L24773	Exon 33	3437-3438	TTTTCCAGGACT-TCCAGGTGACCA	1,3 kb
178	18956-L24747	Exon 34	3513-3512 reverse	CAAACCATACCT-TTAGGTCCTCTT	0,9 kb
247	18967-L24759	Exon 35	3574-3575	GTGAGCCAGGTA-GCCCTGGATGTC	2,6 kb
418	18986-L24778	Exon 36	3696-3697	CCAGGGTGCCCA-GGTACCTTGAAA	4,4 kb
391	18983-L24775	Exon 37	3701-3702	ATCCTCAGGTGA-TCACGGGATGCC	0,8 kb
409	18985-L24777	Exon 38	3863-3864	TCAGAAAGGAAC-TAAAGGTGCTTC	8,8 kb
142	18951-L24742	Exon 40	4073-4074	TCCTGACCCGGG-TCCACCTGGAGA	1,4 kb
463	18991-L24783	Exon 41	4245-4246	AAAGGCTTTCCA-GGATGTGATGGA	1,5 kb
166	18955-L26262	Exon 42	4370-4371	AAAAGGGCCCAC-TGGTCTTCCGGG	2,6 kb
346	18979-L24771	Exon 44	4391-4392	TCCAATTTCAGG-TGAACCGGGGCC	5,7 kb
301	18973-L24765	Exon 45	4597-4598	CCGGACGTAAAG-GTGACACAGGAG	10,0 kb
400	18984-L24776	Exon 46	4791-4792	TTATACCTGGAA-GGGCAAGAGAAA	1,9 kb
		Stop codon	*5371-5373 (exon 48)*		
190	18960-L24751	Exon 48	5431-5430 reverse	GCACAGTCTAGG-AAGTCTTAGCCC	3,1 kb

[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.

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. When this occurs in reference samples, it can look like an increased signal in the test 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.

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.
P439 COL4A3	Contains probes for the *COL4A3* 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 P444 COL4A4

- Gillion V et al. (2018). Genotype and outcome after kidney transplantation in Alport syndrome. *Kidney International Reports*. doi: 10.1016/j.ekir.2018.01.008.
- Yamamura T et al. (2019). Comparison between conventional and comprehensive sequencing approaches for genetic diagnosis of Alport syndrome. *Mol Genet Genomic Med*. 7:e883.

P444 product history	
Version	*Modification*
A3	Three reference probes have been replaced, two reference probes have been removed. One target probe has been changed in length but not in sequence detected.
A2	Two reference probes were replaced and one was added.
A1	First release.

Implemented changes in the product description
Version A3-01–10 September 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 *COL4A4* gene updated according to new version of the NM_ reference sequence. Version A2-01 – 22 February 2018 (01P) - Product description restructured and adapted to a new template. - Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included).

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