Product Description
SALSA® MLPA® Probemix P296-A3 aHUS

To be used with the MLPA General Protocol.

Version A3
For complete product history see page 7.

Catalogue numbers:
- **P296-025R:** SALSA MLPA Probemix P296 aHUS, 25 reactions.
- **P296-050R:** SALSA MLPA Probemix P296 aHUS, 50 reactions.
- **P296-100R:** SALSA MLPA Probemix P296 aHUS, 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 P296 aHUS is a **research use only (RUO)** assay for the detection of deletions or duplications in the *CD46* and *CFI* genes, which are associated with atypical hemolytic uremic syndrome (aHUS).

aHUS is characterised by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia associated with distorted erythrocytes ('burr cells'). The age of onset can range from the neonatal period to adulthood. Generally, aHUS is associated with poor outcome; about 60% of all genetic aHUS progresses to end-stage renal disease (ESRD). Approximately 60% of all aHUS cases have a genetic cause. Mutations in the *CD46* and *CFI* genes account for ~7% and ~6% of all genetic variants leading to aHUS, respectively. Mutations in these genes are typically inherited in an autosomal dominant manner with incomplete penetrance.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1367/.

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 *CD46* and *CFI* exon numbering used in this P296-A3 aHUS product description is the exon numbering from the LRG_155 and LRG_48 sequences, respectively. 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 P296-A3 aHUS contains 35 MLPA probes with amplification products between 142 and 454 nucleotides (nt). This includes 13 probes for the *CD46* gene and 12 probes for the *CFI* gene. In addition, ten 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 atypical hemolytic uremic 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 *CD46* and *CFI* genes are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P296 aHUS.

- 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.

CD46 and CFI mutation database

https://databases.lovd.nl/shared/genes. 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 *CD46* exons 10 and 12 but not exon 11) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P296-A3 aHUS

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*CD46*	*CFI*
64-105	Control fragments – see table in probemix content section for more information			
142	Reference probe 03797-L23466	21q		
153	**CD46 probe** 18762-L11178		**Exon 7**	
166	**CFI probe** 09106-L09165			**Exon 10**
172	**CD46 probe** 09094-L09153		**Exon 10**	
178	**CD46 probe** 20721-L23348		**Exon 6**	
184	**CFI probe** 09099-L09158			**Exon 1**
190	**CD46 probe** 09091-L09150		**Exon 8**	
196	**CFI probe** 09102-L09161			**Exon 4**
202	Reference probe 00992-L00552	2p		
208 Ø	**CD46 probe** 09086-L09145		**Intron 1**	
214	**CFI probe** 09103-L09162			**Exon 5**
221	**CD46 probe** 09088-L09147		**Exon 5**	
226	**CFI probe** 09100-L09159			**Exon 2**
232	**CD46 probe** 09096-L11366		**Exon 12**	
238	Reference probe 14292-L18689	15q		
247 Ø	**CFI probe** 10808-L11455			**Intron 1**
256	**CD46 probe** 09098-L09157		**Exon 14**	
267	Reference probe 14110-L15943	8p		
283	**CFI probe** 09101-L09160			**Exon 3**
292	**CD46 probe** 09085-L24447		**Exon 1**	
301	**CD46 probe** 09097-L09156		**Exon 13**	
310	Reference probe 09245-L09435	7q		
319	**CD46 probe** 09092-L09151		**Exon 9**	
328	Reference probe 02663-L02130	11q		
346	**CFI probe** 09108-L09167			**Exon 12**
355	Reference probe 10134-L10596	18q		
364 Δ	**CFI probe** 09105-L09164			**Exon 9**
377	Reference probe 10693-L19115	6p		
391	**CD46 probe** 09095-L09154		**Exon 11**	
400	**CFI probe** 09109-L09168			**Exon 13**
409	**CD46 probe** 09087-L09146		**Exon 3**	
425	**CFI probe** 09104-L09163			**Exon 6**
433	Reference probe 06948-L06528	3q		
445	**CFI probe** 09107-L09166			**Exon 11**
454	Reference probe 05937-L04835	17q		

[a] See section Exon numbering on page 1 for more information.

Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.
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. P296-A3 probes arranged according to chromosomal location

Table 2a. *CD46*

Length (nt)	SALSA MLPA probe	*CD46* exon[a]	Ligation site NM_002389.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*176-178 (Exon 1)*		
292	09085-L24447	Exon 1	46-47	ACCTGTCCTGCA-GCACTGGATGCT	4.1 kb
208 Ø#	09086-L09145	Intron 1	803 nt before exon 2	GATGTGACACTA-CTGGACTGCCAA	1.4 kb
409	09087-L09146	Exon 3	532-533	ACTTACGAGTTT-GGTTATCAGATG	3.8 kb
221	09088-L09147	Exon 5	3 nt after exon 5	AGTGTAAAGGTA-GTGTTTCAATTT	5.6 kb
178	20721-L23348	Exon 6	865-864, reverse	TCGACTACTGGA-AATCGACATTTG	0.6 kb
153	18762-L11178	Exon 7	1063-1064	AAACCTCCAGCT-TTGAGTCATTCA	0.1 kb
190	09091-L09150	Exon 8	1098-1097, reverse	ACGCTGGAGATT-TTGTAGTGGAAG	2.4 kb
319	09092-L09151	Exon 9	178 nt before exon 9	TTCTTGTACATA-CATCTTACATAC	13.3 kb
172	09094-L09153	Exon 10	79 nt after exon 10	ATGAGGTGCCCA-AACATAGGATCC	1.7 kb
391	09095-L09154	Exon 11	1224-1225	CATTGCTGTGAT-TGTTATTGCCAT	0.6 kb
232	09096-L11366	Exon 12	1278-1279	CCCGTACAGATA-TCTTCAAAGGAG	4.6 kb
301	09097-L09156	Exon 13	1308-1307, reverse	CATCAGTTAGGT-ATGTGCTGAACA	4.1 kb
256	09098-L09157	Exon 14	2203-2204	CTCTGACAGCCA-TAACAGGAGTGC	
		stop codon	*1352-1354 (Exon 13)*		

Table 2b. *CFI*

Length (nt)	SALSA MLPA probe	*CFI* exon[a]	Ligation site NM_000204.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*29-31 (Exon 1)*		
184	09099-L09158	Exon 1	118 nt before exon 1	GATTTCAGCCAA-ATTCTTTCAGAG	1.5 kb
247 Ø	10808-L11455	Intron 1	1318 nt after exon 1	GAAACTAGGGGA-TTCAGAAAAGAT	34.0 kb
226	09100-L09159	Exon 2	260-261	TGTGTGCAACTA-ACAGGAGAAGCT	2.0 kb
283	09101-L09160	Exon 3	444-443, reverse	TTTTGCATATGA-ACATTGTCTTAT	3.0 kb
196	09102-L09161	Exon 4	593-594	ATTGCCGAGGAT-TAGAGACCAGTT	1.0 kb
214	09103-L09162	Exon 5	725-726	GTGTGAATGGGA-AATACATTTCTC	0.2 kb
425	09104-L09163	Exon 6	827-828	GCTTCCATTGCA-AATCGGGTGTTT	10.5 kb
364 Δ	09105-L09164	Exon 9	291 nt before exon 9	GGACATGTGGCA-TCCTTTATCAGC	0.7 kb
166	09106-L09165	Exon 10	1162-1163	ATTCTGACTGCT-GCACATTGTCTC	3.0 kb
445	09107-L09166	Exon 11	1423-1422, reverse	ACGATGCATGTA-TCATTAGGTTGG	3.8 kb
346	09108-L09167	Exon 12	55 nt after exon 12	GGGAGGAGATGT-TTGATAGGGGAA	1.4 kb
400	09109-L09168	Exon 13	1674-1673, reverse	GTTTTCCACAGT-TTTCCCCCCAAC	
		stop codon	*1778-1780 (Exon 13)*		

[a] See section Exon numbering on page 1 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.

Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.
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.
This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.

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

P236 CFH region — Contains probes for the *CFH* and the *CFHRx* genes

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 P296 aHUS

- Alba-Demonínguez et al. (2012). Complement factor I deficiency: a not so rare immune defect. Characterization of new mutations and the first large gene deletion. *Orphanet J Rare Dis* 7:42.
- Gleeson et al. (2016). Chromosomal rearrangement—A rare cause of complement factor I associated atypical haemolytic uraemic syndrome. *Immunobiology*. 221(10):1124-30.
- Szilágyi et al. (2013). The role of complement in Streptococcus pneumoniae-associated haemolytic uraemic syndrome. *Neprhol Dial Transplant* 28:2237-45.
- Szarvas et al. (2014). First-line therapy in atypical haemolytic uremic syndrome: consideration on infants with a poor prognosis. *Ital J Pediatr* 40:101.
- Szarvas et al. (2016). Genetic analysis and functional characterization of novel mutations in a series of patients with atypical hemolytic uremic syndrome. *Mol Immunol*. 71:10-22.

P296 product history	
Version	*Modification*
A3	Three reference probes have been replaced and one removed.
A2	Four reference probes have been replaced.
A1	First release.

Implemented changes in the product description
Version A3-02 – 02 February 2023 (04P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *CFI* gene updated according to new version of the NM_ reference sequence. - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. *Version A3-01 — 15 October 2019 (02P)* - Product description rewritten and adapted to a new template. - Various minor textual or layout changes. - Small changes of probe lengths in Table 1 in order to better reflect the true lengths of the amplification products. *Version 09 (55) - 11 November 2015* - Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included). *Version 08 (49) - 07 August 2015* - Electropherogram picture(s) using the old MLPA buffer (replaced in December 2012) removed.

More information: www.mrcholland.com; www.mrcholland.eu	
	MRC Holland bv; Willem Schoutenstraat 1 1057 DL, Amsterdam, The Netherlands
E-mail	info@mrcholland.com (information & technical questions) order@mrcholland.com (orders)
Phone	+31 888 657 200