Product Description

SALSA® MLPA® Probemix P495-A1 CYP11A1-CYP11B1-CYP11B2

To be used with the MLPA General Protocol.

Version A1
For complete product history see page 7.

Catalogue numbers:

- **P495-025R:** SALSA MLPA Probemix P495 CYP11A1-CYP11B1-CYP11B2, 25 reactions.
- **P495-050R:** SALSA MLPA Probemix P495 CYP11A1-CYP11B1-CYP11B2, 50 reactions.
- **P495-100R:** SALSA MLPA Probemix P495 CYP11A1-CYP11B1-CYP11B2, 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 P495 CYP11A1-CYP11B1-CYP11B2 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *CYP11A1*, *CYP11B1* and *CYP11B2* genes, which are associated with renal insufficiency, congenital adrenal hyperplasia and aldosteronism.

Cytochrome P450 family 11 genes *CYP11A1*, *CYP11B1* and *CYP11B2* encode enzymes which are involved in steroid biosynthesis in the mitochondria of the adrenal cortex. Steroid biosynthesis starts with the conversion of cholesterol into pregnenolone, which is catalysed by the cholesterol side-chain cleavage enzyme, also known as desmolase, encoded by *CYP11A1*. *CYP11A1* disruption causes a block in all aspects of adrenal and gonadal steroid synthesis, leading to congenital adrenal insufficiency (Buonocore F and Achermann JC 2020; OMIM #613743). *CYP11B1* encodes 11β-hydroxylase, which catalyses the conversion of 11-deoxycortisol to cortisol and the conversion of 11-deoxycorticosterone to corticosterone amongst others. A deficiency of 11β-hydroxylase causes autosomal recessive disorder 11β-hydroxylase-deficient congenital adrenal hyperplasia, 11-OHD CAH (OMIM #202010; 5-8% of CAH cases). This is the second most common form of CAH after (*CYP21A2* related) 21-OHD CAH which accounts for ~90% of CAH cases. CAH involves a decreased synthesis of cortisol and corticosterone and an accumulation of their precursors, which leads to androgen excess and hypertension.

CYP11B1 and *CYP11B2* share ~95% sequence homology and are located in close proximity (~31 kb) on chromosome 8q. *CYP11B2* encodes aldosterone synthase, which is involved in the synthesis of aldosterone. Aldosterone synthase deficiency is inherited in an autosomal recessive manner and usually presents with salt wasting, hyperkalaemia and hypotension (Hui et al. 2014). Recombination of *CYP11B1* and *CYP11B2* by unequal crossing over between these highly homologous genes and their regulatory elements have been reported. On the allele lacking the normal copies of *CYP11B1* and *CYP11B2,* the *CYP11B* chimeric gene results in *CYP11B2* being regulated by adrenocorticotropic hormone (ACTH), which normally controls expression of *CYP11B1*. This chimeric variant can lead to autosomal recessive 11β-hydroxylase deficiency or aldosterone synthase deficiency (Portrat et al. 2001). On the duplication allele, a complementary chimeric *CYP11B* gene

results in *CYP11B1* being regulated by angiotensin II and potassium, which normally control expression of *CYP11B2*. This can cause amongst others autosomal dominant glucocorticoid-remediable aldosteronism (GRA) or hyperaldosteronism (Lifton et al. 1992; Menabò et al. 2016; OMIM #103900).

The *CYP11A1* gene (9 exons) spans ~30 kb of genomic DNA and is located on 15q24.1, about 72 Mb from the p-telomere. The *CYP11B1* and *CYP11B2* genes (9 exons each) span ~7.5 kb and ~7.3 kb of genomic DNA, respectively, and are located on 8q24.3, about 144 Mb from the p-telomere (close to the q-telomere).

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 *CYP11A1* exon numbering used in this P495-A1 CYP11A1-CYP11B1-CYP11B2 product description is the exon numbering from the NG_007973.2 sequence. The *CYP11B1* exon numbering is the exon numbering from the NG_007954.1. The *CYP11B2* exon numbering is the exon numbering from the NG_008374.1. 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 P495-A1 CYP11A1-CYP11B1-CYP11B2 contains 38 MLPA probes with amplification products between 129 and 441 nucleotides (nt). This includes ten probes for the *CYP11A1* gene, nine probes for the *CYP11B1* gene and nine probes for the *CYP11B2* gene (see Table 2). 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 renal disease or disorders of sex development. 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 NA03184 and NA20263 from the Coriell Institute have been tested with this P495-A1 probemix at MRC Holland and can be used as positive control samples to test a heterozygous duplication of *CYP11A1* and a heterozygous duplication of *CYP11B1* and *CYP11B2*, respectively. 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 *CYP11A1* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P495 CYP11A1-CYP11B1-CYP11B2.
- 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.

***CYP11A1*, *CYP11B1* and *CYP11B2* mutation databases**

https://databases.lovd.nl/shared/genes/CYP11A1, https://databases.lovd.nl/shared/genes/CYP11B1 and https://databases.lovd.nl/shared/genes/CYP11B2. 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 *CYP11A1* exons 2 and 4 but not exon 3) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P495-A1 CYP11A1-CYP11B1-CYP11B2

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]			
		Reference	*CYP11A1*	*CYP11B1*	*CYP11B2*
64-105	Control fragments – see table in probemix content section for more information				
129	Reference probe 18709-L26847	5q			
136	**CYP11A1 probe** 23183-L32284		**Exon 6**		
142	**CYP11B1 probe** 23206-L32822			**Exon 3**	
149	**CYP11A1 probe** 23184-L32805		**Exon 2**		
155	Reference probe 20337-L27719	1p			
160	**CYP11A1 probe** 23185-L32283		**Exon 4**		
176	**CYP11B1 probe** 23212-L32827			**Exon 9**	
183	Reference probe 09496-L28060	11q			
187	**CYP11A1 probe** 23186-L32806		**Exon 1**		
196	**CYP11B2 probe** 23194-L32811				**Exon 2**
202	**CYP11B1 probe** 23208-L32824			**Exon 5**	
211	Reference probe 05281-L31289	14q			
220	**CYP11B2 probe** 23198-L32814				**Exon 5**
230	**CYP11B1 probe** 23207-L32823			**Exon 4**	
238	**CYP11A1 probe** 23187-L32225		**Exon 9**		
247	Reference probe 11138-L16379	13q			
255	**CYP11A1 probe** 23188-L32807		**Exon 3**		
265	**CYP11B1 probe** 23205-L32821			**Exon 2**	
274	**CYP11B2 probe** 23200-L32816				**Exon 7**
283	Reference probe 13346-L14772	18q			
292	**CYP11B2 probe** 23203-L32819				**Exon 9**
301	**CYP11A1 probe** 23189-L32227		**Exon 5**		
309	**CYP11B1 probe** 23204-L32820			**Exon 1**	
319	Reference probe 15385-L17792	3p			
328	**CYP11B2 probe** 23193-L32810				**Exon 1**
337	**CYP11A1 probe** 23190-L32808		**Exon 1**		
346	**CYP11B1 probe** 23209-L32825			**Exon 6**	
355	Reference probe 11614-L12374	12p			
364	**CYP11A1 probe** 23191-L32809		**Exon 8**		
375	**CYP11B2 probe** 23196-L32813				**Exon 4**
382	**CYP11B2 probe** 23195-L32812				**Exon 3**
391	Reference probe 14984-L16720	6q			
401	**CYP11A1 probe** 23214-L32829		**Exon 7**		
409	**CYP11B1 probe** 23211-L32879			**Exon 8**	
415	**CYP11B2 probe** 23201-L32880				**Exon 8**
422	**CYP11B1 probe** 23213-L32828			**Exon 9**	
429	**CYP11B2 probe** 23202-L32818				**Exon 9**
441	Reference probe 05916-L05361	21q			

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

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. P495-A1 probes arranged according to chromosomal location

Table 2a. *CYP11A1* gene

Length (nt)	SALSA MLPA probe	*CYP11A1* exon[a]	Ligation site NM_000781.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*62-64 (Exon 1)*		
187	23186-L32806	Exon 1	93-94	CCCACGCTCAGT-CCTGGTCAAAGG	0.2 kb
337	23190-L32808	Exon 1	250-251	CTAAACCTGTAC-CATTTCTGGAGG	19.4 kb
149	23184-L32805	Exon 2	373-374	GTCATCGACCCT-GAAGATGTGGCC	3.0 kb
255	23188-L32807	Exon 3	660-661	GGACATCAGTGA-TGACCTGTTCCG	1.2 kb
160	23185-L32283	Exon 4	780-781	CATCTACCAGAT-GTTCCACACCAG	0.8 kb
301	23189-L32227	Exon 5	918-919	GAACTTCTACTG-GGAATTGAGACA	3.4 kb
136	23183-L32284	Exon 6	1110-1109 *reverse*	CCCGCAGCATAT-CCTGCACCTTCA	0.4 kb
401	23214-L32829	Exon 7	1274-1273 *reverse*	CATGTAATCTCG-AAGAACCAAGTC	0.6 kb
364	23191-L32809	Exon 8	1398-1397 *reverse*	AGTAGGTGATGT-TCTTGTCTTTGC	0.6 kb
238	23187-L32225	Exon 9	1512-1513	GGAGAACTTCAG-AGTTGAAATCCA	
		stop codon	*1625-1627 (Exon 9)*		

Table 2b. *CYP11B1/CYP11B2* genes

Length (nt)	SALSA MLPA probe	Exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		CYP11B2 gene	**NM_000498.3**		
		start codon	*4-6 (Exon 1)*		
328	23193-L32810	Exon 1	205-206	TGCACCTGGAGA-TGCACCAGACCT	0.6 kb
196	23194-L32811	Exon 2	13 nt after exon 2	TAAGCGGCGAGT-TGGGAGCTGAGA	2.0 kb
382	23195-L32812	Exon 3	10 nt after exon 3	GACCTTCCCGCA-TGGCCCACACCT	0.1 kb
375 #	23196-L32813	Exon 4	609-610	GCCAGCAACTTA-GCTCTTTTTGAA	0.5 kb
220	23198-L32814	Exon 5	858-859	CGCCCTCAACAC-TACACAGGCATC	1.5 kb
	No probe	Exon 6			
274 #	23200-L32816	Exon 7	1147-1146 reverse	CACTCGCTCCAA-AAACAGACCCAC	0.2 kb
415	23201-L32880	Exon 8	1230-1231	TTCCTCTACTCG-CTGGGTCGCAAT	1.4 kb
429	23202-L32818	Exon 9	2209-2210	CTTGTTCAAGCA-GCGAGTGTTGGG	0.4 kb
292	23203-L32819	Exon 9	2567-2568	AGTGCCCCAGGG-TGAGAACGTGCC	31.2 kb
		stop codon	*1513-1515 (Exon 9)*		
		CYP11B1 gene	**NM_000497.4**		
		start codon	*13-15 (Exon 1)*		
309	23204-L32820	Exon 1	114-115	GTCCCCAGGACA-GTGCTGCCCTTT	0.5 kb
265	23205-L32821	Exon 2	268-267 reverse	CACCATGCCTGC-TCCTCCCAAGTC	2.0 kb
142 #	23206-L32822	Exon 3	453-454	CTGAATCCAGAA-GTGCTGTCGCCC	0.4 kb
230 #	23207-L32823	Exon 4	755-756	GTCTCGCTGGAC-CAGCCCCAAGGT	0.4 kb
202	23208-L32824	Exon 5	918-917 reverse	GCCTTGATGGCA-TCTGGCGACAGT	0.4 kb
346	23209-L32825	Exon 6	10 nt before exon 6	GTCTGCAGGAGA-CACAGCTGCAGG	0.7 kb
	No probe	Exon 7			
409	23211-L32879	Exon 8	1224-1225	ACATTGGTGCGC-GTGTTCCTCTAC	0.8 kb
176	23212-L32827	Exon 9	1527-1528	ATCAACTAATCA-CGTCTCTGCACC	0.6 kb
422	23213-L32828	Exon 9	2102-2103	TGTCCTGACATC-CCCATTTTCAAG	
		stop codon	*1522-1524 (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.

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

- P050 CAH: Contains probes for the *CYP21A2* gene.
- P312 POR: Contains probes for the *POR* gene.

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P495 product history	
Version	*Modification*
A1	First release.

Implemented changes in the product description
Version A1-01 – 25 October 2022 (04P) - Not applicable, new document.

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