Product Description
SALSA® MLPA® Probemix P312-B3 POR

To be used with the MLPA General Protocol.

Version B3
For complete product history see page 7.

Catalogue numbers:
- **P312-025R:** SALSA MLPA Probemix P312 POR, 25 reactions.
- **P312-050R:** SALSA MLPA Probemix P312 POR, 50 reactions.
- **P312-100R:** SALSA MLPA Probemix P312 POR, 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 P312 POR is a **research use only (RUO)** assay for the detection of deletions or duplications in the *POR* gene, which is associated with congenital adrenal hyperplasia (CAH) and Antley-Bixler syndrome.

The *POR* gene encodes Cytochrome P450 Oxidoreductase, a membrane-bound enzyme that serves as an electron donor for all microsomal cytochrome P450 enzymes. Specific deficiencies in the *POR* gene lead to clinical manifestations associated with CAH and Antley-Bixler syndrome.

In general, CAH is caused by deficiencies in cytochrome P450 (CYP) enzymes (e.g. CYP21A2) that are downstream in the electron transfer chain from POR (see P050 under 'related products'). Subsequently, POR deficient CAH is caused by the indirect impairment of these CYP enzymes.

Antley-Bixler syndrome is associated with mutations in the *FGFR2* or *POR* gene. *POR* related disease is also thought to be caused by the effect dysfunctional *POR* has on downstream CYP enzymes (Krone et al. 2012).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1419/.

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 *POR* exon numbering used in this P312-B3 POR product description is the exon numbering from the NM_001395413.1 sequence. 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 P312-B3 POR contains 29 MLPA probes with amplification products between 135 and 409 nucleotides (nt). This includes 16 probes for the *POR* gene. Furthermore, this probemix also contains two flanking probes detecting the *HSPB1* gene, which is located approximately 300 kb downstream of *POR*, in order to facilitate the determination of the extent of a deletion/duplication. In addition, eleven 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 congenital adrenal hyperplasia (CAH) and Antley-Bixler 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 *POR* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P312 POR.

- 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.

***POR* mutation database**

https://databases.lovd.nl/shared/genes/POR. 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 *POR* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P312-B3 POR

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*POR*	*HSPB1*
64-105	Control fragments – see table in probemix content section for more information			
135	Reference probe 16316-L21434	3q		
142 ¬	**HSPB1 probe** 09538-L09961			**Exon 1**
148 ¬	**HSPB1 probe** 09540-L09963			**Exon 3**
157	Reference probe 15027-L16778	2q		
166	**POR probe** 13766-L15260		**Exon 1**	
174	**POR probe** 13768-L15262		**Exon 11**	
184	Reference probe 09724-L10074	12q		
196	**POR probe** 12680-L13758		**Exon 3**	
201	**POR probe** 12681-L13759		**Exon 7**	
214	Reference probe 11559-L13505	5q		
220	**POR probe** 12682-L13760		**Exon 4**	
229	**POR probe** 12683-L13761		**Exon 10**	
238	Reference probe 20186-L27463	14q		
247	**POR probe** 12684-L13762		**Exon 6**	
256	**POR probe** 12685-L13763		**Exon 15**	
274	**POR probe** 12686-L13764		**Exon 2**	
283	**POR probe** 12687-L13765		**Exon 5**	
292	Reference probe 03597-L13368	8q		
310	**POR probe** 12689-L13767		**Exon 9**	
319	Reference probe 16623-L19153	17q		
328	**POR probe** 12690-L13768		**Exon 13**	
337	**POR probe** 12691-L13769		**Exon 16**	
346	Reference probe 09073-L22926	19p		
355	**POR probe** 12692-L13770		**Exon 12**	
364	Reference probe 16931-L19874	4q		
373	Reference probe 07788-L07524	20q		
382	**POR probe** 12694-L13772		**Exon 8**	
391	**POR probe** 12695-L13773		**Exon 14**	
409	Reference probe 04683-L04061	1p		

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

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

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. *POR* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*POR* exon[a]	Ligation site NM_001395413.1	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*39-41 (Exon 2)*		
166	13766-L15260	Exon 1	147 nt before exon 1	AAGAGCGCAAAT-TTGCAGGGGGAA	39.1 kb
274	12686-L13764	Exon 2	104-105	GAAGTATCTCTT-TTCAGCATGACG	18.3 kb
196	12680-L13758	Exon 3	20 nt before exon 3	TCCCACGCTCAT-TGCACACTTTTG	7.2 kb
220	12682-L13760	Exon 4	388-387 reverse	TTACCAGGTCAT-ACTCCTCAGGGT	0.8 kb
283	12687-L13765	Exon 5	440-441	GCCCTGGTGGTT-TTCTGCATGGCC	0.8 kb
247	12684-L13762	Exon 6	4 nt after exon 6	GATGGGAAGTGA-GTGCCCACCCTG	0.4 kb
201	12681-L13759	Exon 7	2 nt after exon 7	AGGAGTCCAGGT-GAGCAAGTGCCC	0.6 kb
382	12694-L13772	Exon 8	778-779	CCAGTACGAGCT-TGTGGTCCACAC	1.4 kb
310	12689-L13767	Exon 9	6 nt after exon 9	AATCAGGTACCA-GCTGCCACTGTC	0.2 kb
229	12683-L13761	Exon 10	1094-1095	AACAACCTGGAT-GGTGAGTGCCAC	1.0 kb
174	13768-L15262	Exon 11	1124-1125	CACCCATTCCCG-TGCCCTACGTCC	0.4 kb
355	12692-L13770	Exon 12	1425-1426	CCTCATCCTCCA-AGGTGAGGGCCG	0.5 kb
328	12690-L13768	Exon 13	1572-1573	TGGTGCCCATGT-TCGTGCGCAAGT	0.3 kb
391	12695-L13773	Exon 14	1782-1783	AGCTGGCGCAGT-TCCACAGGGACG	0.2 kb
256	12685-L13763	Exon 15	1894-1895	CCTGTGGAAGTT-GATCGAAGGCGG	0.2 kb
337	12691-L13769	Exon 16	1962-1963	ATGTGCAGAACA-CCTTCTACGACA	316.3 kb
		stop codon	*2070-2072 (Exon 16)*		
		HSPB1 gene			
142 ¬	09538-L09961	Exon 1		CTGACTCTGCTC-TGGACGTCTGCT	1.5 kb
148 ¬ #	09540-L09963	Exon 3		GCGGCAGTCTCA-TCGGATTTTGCA	

[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.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.
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

P029 WBS	Contains probes that detect Williams-Beuren Syndrome causing deletions. These deletions have also been associated with reduced expression levels of several genes, including *POR*.
P050 CAH	Contains probes to detect other CAH related genes including *CYP21A2* gene.
P128 CYP450	Contains probes for several Cytochrome P450 and Glutathione S-transferase genes.

References

- Krone N et al. (2012). Genotype-phenotype analysis in congenital adrenal hyperplasia due to P450 oxidoreductase deficiency. *J Clin Endocrinol Metab*. 97:E257-67.
- 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.

P312 product history	
Version	*Modification*
B3	Five reference probes have been replaced and two reference probes have been removed.
B2	Two reference probes have been replaced and the control fragments have been adjusted (QDX2).
B1	First release.

Implemented changes in the product description

Version B3-02 – 03 February 2023 (04P)
- Product description rewritten and adapted to a new template.
- Ligation sites of the probes targeting the name gene updated according to new version of the NM_ reference sequence.

Version B3-01 — 30 April 2020 (02P)
- 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 *POR* gene updated according to new version of the NM_ reference sequence.
- Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.

Version 08 - 18 October 2016 (55)
- Product description adapted to a new lot (lot number added, small changes in Table 1, new picture included).
- Warning added in Table 1 and 2 for the 274 nt probe 12686-L13764.
- Various textual changes.

Version 07 (50)
- Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included).
- Various textual changes.

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