Product Description
SALSA® MLPA® Probemix P109-B2 ABCB4

To be used with the MLPA General Protocol.

Version B2
For complete product history see page 7.

Catalogue numbers:
- **P109-025R:** SALSA MLPA Probemix P109 ABCB4, 25 reactions.
- **P109-050R:** SALSA MLPA Probemix P109 ABCB4, 50 reactions.
- **P109-100R:** SALSA MLPA Probemix P109 ABCB4, 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 P109 ABCB4 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *ABCB4* gene, which is associated with several syndromes, including progressive familial intrahepatic cholestasis type 3 (PFIC3), low phospholipid-associated cholelithiasis (LPAC; also known as gallbladder disease 1, GBD1), and familial intrahepatic cholestasis of pregnancy (ICP).

ABCB4 (ATP-binding cassette subfamily B member 4, also known as *MDR3*) is a member of the superfamily of ATP-binding cassette *(ABC)* transporters. These proteins transport various molecules across extra- and intracellular membranes. *ABCB4* is involved in transporting phospholipids across the hepatocyte membrane and releasing them in the bile.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK3/.

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 *ABCB4* exon numbering used in this P109-B2 ABCB4 product description is the exon numbering from the NG_007118.2 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 P109-B2 ABCB4 contains 37 MLPA probes with amplification products between 136 and 454 nucleotides (nt). This includes 25 probes for the *ABCB4* gene, of which three probes are located upstream of the *ABCB4* gene and 22 probes target 22 of the 28 exons. Furthermore, it also contains two probes for the *ABCB1* gene which is located near the *ABCB4* gene. In addition, ten reference probes are included and detect ten different 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 *ABCB4* deficiency syndromes. 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 *ABCB4* gene are small (point) mutations, none of which will not be detected by using SALSA MLPA Probemix P109-B2 ABCB4.

- 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.

***ABCB4* mutation database**

https://databases.lovd.nl/shared/genes/ABCB4. 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 *ABCB4* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P109-B2 ABCB4

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*ABCB4*	*ABCB1*
64-105	Control fragments – see table in probemix content section for more information			
136	Reference probe 00662-L00158	6p		
142 ¬	**ABCB4** probe 04712-L04130		**Upstream**	
148	**ABCB4** probe 04730-L04147		**Exon 20**	
154	**ABCB4** probe 04721-L04139		**Exon 9**	
160	Reference probe 13336-L14762	18q		
166 ¬	**ABCB4** probe 04713-L04131		**Upstream**	
172	**ABCB4** probe 04731-L04148		**Exon 21**	
178	**ABCB4** probe 04722-L04140		**Exon 10**	
186	Reference probe 11148-L16373	8p		
193 ¬	**ABCB1** probe 01115-L00005			**Exon 17**
203	**ABCB4** probe 04732-L04149		**Exon 23**	
210	**ABCB4** probe 04723-L13718		**Exon 11**	
220	Reference probe 13568-L15025	1q		
229	**ABCB4** probe 04715-L04133		**Exon 2**	
238	**ABCB4** probe 04733-L04150		**Exon 24**	
247	**ABCB4 probe** 04724-L04158		**Exon 13**	
256	Reference probe 02469-L01913	15q		
274 ¬	**ABCB4** probe 05081-L04132		**Upstream**	
283	**ABCB4** probe 04734-L04151		**Exon 25**	
293	**ABCB4** probe 04725-L04142		**Exon 15**	
301	Reference probe 11202-L11885	17q		
310	**ABCB4** probe 04717-L04135		**Exon 5**	
319	**ABCB4** probe 04735-L04152		**Exon 26**	
328	**ABCB4** probe 04726-L04143		**Exon 16**	
346	Reference probe 16277-L18569	20q		
355	**ABCB4** probe 04718-L04136		**Exon 6**	
364	**ABCB4** probe 04736-L04153		**Exon 27**	
373	**ABCB4** probe 04727-L04144		**Exon 17**	
382	Reference probe 04278-L23577	12q		
391	**ABCB4** probe 04719-L04137		**Exon 7**	
400	**ABCB4** probe 04737-L04154		**Exon 28**	
409	Reference probe 12280-L13223	22q		
418	**ABCB4** probe 21007-L29225		**Exon 4**	
427 ¬	**ABCB1** probe 02214-L13719			**Exon 3**
436	**ABCB4** probe 04720-L04138		**Exon 8**	
445	**ABCB4** probe 04729-L04146		**Exon 19**	
454	Reference probe 11144-L11828	10q		

[a] See section Exon numbering on page 1 and 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. *ABCB4* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*ABCB4* exon[a]	Ligation site NM_018849.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
427 ¬	02214-L13719	*ABCB1* Exon 3	NM_000927.4; 163 nt before exon 3	ACCCCGCGCACA-GGAAAGCCCCTG	55.5 kb
193 ¬	01115-L00005	*ABCB1* Exon 17	NM_000927.4; 2524-2525	GGATCACAAGCC-CAAGACAGAAAG	65.4 kb
		Start Codon	*54-56 (Exon 2)*		
142 ¬	04712-L04130	Upstream	3784 nt before exon 1	AGGAGGGATCCA-GCAGGCATGGGG	2.2 kb
166 ¬	04713-L04131	Upstream	1563 nt before exon 1	TTCAAGTCAAGT-TCCACCTTAGCT	1.0 kb
274 ¬	05081-L04132	Upstream	549 nt before exon 1	CACCAACAGCGT-TCCTCCTCCCCA	0.8 kb
229	04715-L04133	Exon 2	72-73	TTGAGGCGGCAA-AGAACGGAACAG	12.6 kb
418	21007-L29225	Exon 4	243-244	GTACCATCATGG-CCATAGCTCACG	8.3 kb
310	04717-L04135	Exon 5	365-366	TCGCTGCTAAAT-CCAGGCAAAATT	1.5 kb
355	04718-L04136	Exon 6	451-452	TGCTGCCTATAT-ACAAGTTTCATT	1.4 kb
391	04719-L04137	Exon 7	719-720	GTGATAATGGCC-ATCAGCCCTATT	1.6 kb
436	04720-L04138	Exon 8	798-799	AACTAGCTGCTT-ATGCAAAAGCAG	3.0 kb
154	04721-L04139	Exon 9	984-985	TAATATATGCAT-CATATGCACTGG	2.2 kb
178	04722-L04140	Exon 10	1098-1099	TCAGTGTTGGCC-AGGCTGCCCCAT	1.2 kb
210	04723-L13718	Exon 11	1214-1215	GGACACAAACCA-GACAGCATCAAA	3.4 kb
247	04724-L04158	Exon 13	1500-1501	CGGTGCTGTTTT-CCACCACAATTG	8.8 kb
293	04725-L04142	Exon 15	1841-1842	ACGGTCCGAAAT-GCAGATGTCATC	4.6 kb
328	04726-L04143	Exon 16	1970-1971	CAGATCCAGTCA-GAAGAATTTGAA	2.9 kb
373	04727-L04144	Exon 17	2143-2144	ACCAGTGTCCTT-TCTGAAGGTCCT	4.0 kb
445	04729-L04146	Exon 19	2395-2396	GAAAGCTGGCGA-GATCCTCACCAG	1.5 kb
148	04730-L04147	Exon 20	2513-2514	CTTGCCACAGAT-GCTGCCCAAGTC	1.1 kb
172	04731-L04148	Exon 21	2536-2537	CTCCCAGGCCAC-AGGAACCAGGTT	5.6 kb
203	04732-L04149	Exon 23	2930-2931	TTTCGATTTGGT-GCATATCTCATT	2.6 kb
238	04733-L04150	Exon 24	3050-3051	GACTATGCTAAA-GCTAAGCTGTCT	1.2 kb
283 #	04734-L04151	Exon 25	3218-3219	CTTCAGGGGCTG-AGCCTGGAGGTG	1.8 kb
319 #	04735-L04152	Exon 26	3447-3448	TATTTGACTGCA-GCATTGCCGAGA	3.2 kb
364	04736-L04153	Exon 27	3653-3654	ATCAGACAACCT-CAAATCCTCCTG	1.0 kb
400 #	04737-L04154	Exon 28	3818-3817 reverse	TCCTTGACTCTC-CCATTCTGAAAC	
		Stop Codon	*3912-3914 (Exon 28)*		

[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.

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 P109 ABCB4

- Degiorgio D et al. (2016). ABCB4 mutations in adult patients with cholestatic liver disease: impact and phenotypic expression. *J Gastroenterol*. 51(3): 271-280.
- Gordo-Gilart R et al. (2016). Heterozygous ABCB4 mutations in children with cholestatic liver disease. *Liver Int*. 36(2): 258-267.
- Gordo-Gilart R et al. (2015). Functional analysis of ABCB4 mutations relates clinical outcomes of progressive familial intrahepatic cholestasis type 3 to the degree of MDR3 floppase activity. *Gut.* 64(1): 147-155.
- Jirsa M et al. (2014). ABCB4 mutations underlie hormonal cholestasis but not pediatric idiopathic gallstones. *World J Gastroenterol*. 20(19): 5867-5874.
- Condat B et al. (2013). Prevalence of low phospholipid-associated cholelithiasis in young female patients. *Dig Liver Dis*. 45(11): 915-919.
- Pasmant E et al. (2012). First description of ABCB4 gene deletions in familial low phospholipid-associated cholelithiasis and oral contraceptives-induced cholestasis. *Eur J Hum Genet*. 20(3): 277-282.

P109 product history	
Version	*Modification*
B2	Three reference probes have been replaced and one has been removed. In addition, one probe length has been adjusted.
B1	Six reference probes have been replaced, one ABCB1 probe has been removed and new control fragments have been added (QDX2).
A2	Small change in length and peak height of three probes but no change in sequence detected. In addition, four extra control fragments at 88-96-100 and 105 nt have been added.
A1	First release.

Implemented changes in the product description
Version B2-02 –03 February 2022 (04P) - Product description rewritten and adapted to a new template. Ligation sites of the probes targeting the *ABCB4* gene updated according to new version of the NM_ reference sequence. *Version B2-01 – 02 May 2018 (01P)* - Product description restructured and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. - Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. *Version 13 – 24 August 2016 (55)* - Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included). - Various minor textual changes. *Version 12 – 22 July 2015 (54)* - Figure based on the use of old MLPA buffer (replaced in December 2012) removed. - Various minor textual changes throughout the document.

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