Product Description SALSA® MLPA® Probemix P092-C1 ABCC6

To be used with the MLPA General Protocol.

Version C1. One flanking probe, one reference probe, and one ABCC6 probe have been removed and seven reference probes have been replaced. In addition one probe has been adjusted in length. For complete product history see page 6.

Catalogue numbers:
- **P092-025R:** SALSA MLPA Probemix P092 ABCC6, 25 reactions.
- **P092-050R:** SALSA MLPA Probemix P092 ABCC6, 50 reactions.
- **P092-100R:** SALSA MLPA Probemix P092 ABCC6, 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.mlpa.com).

Certificate of Analysis: Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mlpa.com.

Precautions and warnings: For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mlpa.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 P092 ABCC6 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *ABCC6* gene, which is associated with pseudoxanthoma elasticum (PXE).

PXE is an autosomal recessive multisystem disorder that is associated with accumulation of mineralized and fragmented elastic fibers in the skin, vascular walls, Bruch's membrane in the eye, and the upper gastrointestinal tract. Defects in the *ABCC6* gene on chromosome 16p13.11 cause PXE. The protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. The ABCC6 protein, a member of the multidrug resistance-associated protein (MRP) subfamily, is a putative efflux cellular transporter with unknown physiologic substrate.
The majority of PXE is caused by point mutations in the *ABCC6* gene. A 16.4 kb deletion involving *ABCC6* exons 23-29 is a recurrent pathogenic variant found in multiple populations with varying frequency. It represents approximately 30% of alleles in the US and about 5% of alleles in Europe (Le Saux et al. 2001).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1113/.

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 *ABCC6* exon numbering used in this P092-C1 ABCC6 product description is the exon numbering from the RefSeq transcript NM_001171.5, which is identical to the LRG_1115 sequence. The exon numbering and NM_ sequence used have been retrieved on 04/2020. As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date.

Probemix content: The SALSA MLPA Probemix P092-C1 ABCC6 contains 33 MLPA probes with amplification products between 130 and 454 nucleotides (nt). This includes 22 probes for the *ABCC6* gene

targeting 22 out of 31 exons of the gene. Furthermore, two flanking probes are included to facilitate the determination of the extent of a deletion/duplication. In addition, nine reference probes are included and detect nine different autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mlpa.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.mlpa.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal of 88 nt and 96 nt fragment 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.mlpa.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 unrelated individuals who are from families without a history of pseudoxanthoma elasticum. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol.

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/home.html) have a diverse collection of biological resources which may be used as a positive control DNA sample 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.mlpa.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 dosage quotient (DQ) 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 DQ of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Heterozygous deletion	0.40 < DQ < 0.65
Heterozygous duplication	1.30 < DQ < 1.65
Heterozygous triplication/Homozygous duplication	1.75 < DQ < 2.15
Ambiguous copy number	All other values

- 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. Incomplete DNA denaturation (e.g. due to salt contamination) can 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.
- When running MLPA products, the capillary electrophoresis protocol may need optimization. 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: lower injection voltage / injection time settings, 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 *ABCC6* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P092 ABCC6.
- 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. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (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.

***ABCC6* mutation database:** https://databases.lovd.nl/shared/genes/ABCC6. 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 SNPs and unusual results (e.g., a duplication of *ABCC6* exons 10 and 12 but not exon 11) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P092-C1 ABCC6

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a] Reference	*ABCC6*
64-105	Control fragments – see table in probemix content section for more information		
130 *	Reference probe 14118-L15719	7p22	
139 ¬	**ABCC1 probe** 00557-L09990		Downstream
154	Reference probe 00513-L00093	5q31	
161 ¥	**ABCC6 probe** 07410-L32220		**Exon 5**
168	**ABCC6 probe** 04912-L09992		**Exon 10**
178	**ABCC6 probe** 07415-L07063		**Exon 13**
184 *	Reference probe 19450-L25864	14q31	
196 ¬	**TSC2 probe** 04024-L10855		Downstream
208	**ABCC6 probe** 07417-L07963		**Exon 18**
214	**ABCC6 probe** 07409-L07057		**Exon 4**
220	**ABCC6 probe** 02994-L02433		**Exon 21**
238	**ABCC6 probe** 07419-L07067		**Exon 28**
247	**ABCC6 probe** 02995-L02434		**Exon 22**
265	**ABCC6 probe** 07416-L07064		**Exon 17**
274	**ABCC6 probe** 02996-L02435		**Exon 23**
286 *	Reference probe 02877-L18857	1p33	
301	**ABCC6 probe** 02997-L02436		**Exon 24**
310	**ABCC6 probe** 07408-L07056		**Exon 2**
319	**ABCC6 probe** 07418-L07066		**Exon 27**
328	**ABCC6 probe** 02998-L02437		**Exon 25**
337 *	Reference probe 07930-L07660	20p13	
346	**ABCC6 probe** 02987-L02426		**Exon 11**
355	**ABCC6 probe** 02999-L02438		**Exon 26**
364	Reference probe 01234-L00781	10p14	
372	**ABCC6 probe** 02988-L02427		**Exon 12**
382 ±	**ABCC6 probe** 07414-L07062		**Exon 9**
391 *	Reference probe 21106-L29573	13q32	
400	**ABCC6 probe** 02989-L09994		**Exon 14**
409 Δ	**ABCC6 probe** 07412-L07060		**Exon 7**
419 *	Reference probe 03854-L23156	17q11	
430	**ABCC6 probe** 02990-L09995		**Exon 15**
436	**ABCC6 probe** 03002-L09996		**Exon 30**
454 *	Reference probe 16539-L19029	15q21	

a) See above section on exon numbering for more information.

* New in version C1.
¥ Changed in version C1. Minor alteration, no change in sequence detected.
± SNP rs72664283 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.

¬ 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.

Table 2. *ABCC6* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*ABCC6* exon[a]	Ligation site NM_001171.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*38-40 (Exon 1)*		
	No probe	Exon 1			
310 #	07408-L07056	Exon 2	38 nt after exon 2, reverse	GCCTCCCCCGAA-CATTGCCTGGTT	2.3 kb
	No probe	Exon 3			
214 #	07409-L07057	Exon 4	194 nt after exon 4	CATTTCTTATCA-ACACAATTTCCT	4.9 kb
161 #	07410-L32220	Exon 5	21 nt before exon 5, reverse	GAAAGCAGAAGA-TAAGGAATGGAG	5.8 kb
	No probe	Exon 6			
409 #	07412-L07060	Exon 7	35 nt after exon 7, reverse	CTTTTCTGAAGT-AGCATCAGGTGA	6.6 kb
	No probe	Exon 8			
382 #	07414-L07062	Exon 9	1114-1115	ATGTTCCTCTCA-GCCTGCCTGCAA	4.0 kb
168	04912-L09992	Exon 10	1313-1314	AGAGCGTCCTCT-ACCTCAACGGGC	5.2 kb
346	02987-L02426	Exon 11	1399-1400	TCCGCCCTCACT-GCCATCGCTGTC	2.6 kb
372	02988-L02427	Exon 12	1565-1566	GGGAGGGAGCCT-TTCTGGACAGAG	1.4 kb
178	07415-L07063	Exon 13	1769-1770	TCAACATCCTCA-ACAAGGCCCAGG	1.7 kb
400	02989-L09994	Exon 14	1839-1840	CTTTGACCGTCT-GGTCACCTTCCT	2.1 kb
430	02990-L09995	Exon 15	11 nt before exon 15	AAATGGCCTCTT-GTGCCCTGCAGC	2.6 kb
	No probe	Exon 16			
265	07416-L07064	Exon 17	1 nt after exon 17, reverse	CCCAAACTCTCA-CCTGCTCCCCAA	3.4 kb
208	07417-L07963	Exon 18	32 nt before exon 18	CCTGTAGATGCT-GACTCAGGCCAC	5.6 kb
	No probe	Exon 19			
	No probe	Exon 20			
220	02994-L02433	Exon 21	2729-2730	TCCCTGAGAAGG-ACCGTACCACTT	3.7 kb
247	02995-L02434	Exon 22	2985-2986	GCAGCAGACGCA-GGCAGCCCTGCG	3.8 kb
274	02996-L02435	Exon 23	3102-3103	GTTGCTCTTCCA-GAGGCTCCTGTG	2.7 kb
301	02997-L02436	Exon 24	3388-3389	CGCTTGGAGTCA-GCCAGCTACTCG	1.7 kb
328	02998-L02437	Exon 25	3619-3620	GTGCTGAGCAAA-GCCCACCTCAGT	1.9 kb
355	02999-L02438	Exon 26	3709-3710	CGCAACTGGACA-GACCTAGAGAAC	1.9 kb
319	07418-L07066	Exon 27	2 nt after exon 27	AGGAGAGAAGGT-GAGTGGTTCTCT	2.5 kb
238	07419-L07067	Exon 28	111 nt before exon 28	GAAGGTAGACCT-TTACACAATGAG	4.4 kb
	No probe	Exon 29			
436	03002-L09996	Exon 30	4311-4312	GATCCTCATCCT-GGACGAGGCTAC	142.9 kb
	No probe	Exon 31			
		stop codon	*4547-4549 (Exon 31)*		
139 ¬	00557-L09990	*ABCC1*		CGTGGAATACCA-GCAACCCCGACT	**14 Mb**
196 ¬	04024-L10855	*TSC2*		TGGATGGATGTT-GGCTTGTCCTCG	

a) See above section on exon numbering for more information.

b) Only partial probe sequences are shown. Complete probe sequences are available at www.mlpa.com. Please notify us of any mistakes: info@mlpa.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.

References

- Le Saux O et al. (2001). A spectrum of ABCC6 mutations is responsible for pseudoxanthoma elasticum. *Am J Hum Genet.* 69(4):749-64.
- 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 P092 ABCC6

- Contrò G et al. (2019). A novel ABCC6 variant causative of pseudoxanthoma elasticum. *Hum genome var*, 6(1), 1-4.
- Costrop LM et al. (2010). Novel deletions causing pseudoxanthoma elasticum underscore the genomic instability of the ABCC6 region. *J Hum Genet*. 55;112-117.
- Faria CS et al. (2013). Clinical Phenotypes and ABCC6 Gene Mutations in Brazilian Families with Pseudoxanthoma Elasticum. *Acta Derm Venereol*. 93:739-40.
- Iwanaga A et al. (2017). Analysis of clinical symptoms and *ABCC6* mutations in 76 Japanese patients with pseudoxanthoma elasticum. *J Dermatol.* 44(6):644-50.
- Li Q et al. (2014). Mutations in the ABCC6 Gene as a Cause of Generalized Arterial Calcification of Infancy: Genotypic Overlap with Pseudoxanthoma Elasticum. *J Invest Dermatol*. 134:658-65.
- Nitschke Y et al. (2012). Generalized arterial calcification of infancy and pseudoxanthoma elasticum can be caused by mutations in either ENPP1 or ABCC6. *Am J Hum Genet*. 90;25-39.
- Sanchez-Navarro I et al. (2018). Combining targeted panel-based resequencing and copy-number variation analysis for the diagnosis of inherited syndromic retinopathies and associated ciliopathies. *Sci rep*, 8(1), 1-11.

P092 Product history	
Version	*Modification*
C1	One flanking probe, one reference probe, and one ABCC6 probe have been removed and seven reference probes have been replaced. In addition one probe has been adjusted in length.
B3	The 88 and 96 control fragments have been replaced (QDX2).
B2	Extra control fragments have been added and 11 new probes for the *ABCC6* gene have been added.
A1	First release.

Implemented changes in the product description

Version C1-01 — 19 May 2020 (02P)
- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).
- Various minor textual or layout changes.

Version B3-01 – 20 August 2018 (01P)
- Product description restructured and adapted to a new template.
- Various minor textual changes.
- Reference added.
- Warning added to Table 1 and 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.

Version 13 – 10 January 2017 (55)
- Warning added in Table 1, 418 nt probe 01140-L00610 and 226 nt probe 02699-L02850.

Version 12 – 31 March 2016 (55)
- Various minor textual changes.
- Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included).

Version 11 – 12 August 2015 (54)

- Various minor textual changes.
- Figure(s) based on the use of old MLPA buffer (replaced in December 2012) removed.
- "Peak area" replaced with "peak height".

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