Product Description SALSA® MLPA® probemix P311-B2 CHD

To be used with the MLPA General Protocol.

Version B2. Two reference probes have been replaced, one reference probe has been removed and one probe length has been adjusted. For complete product history see page 7.

Catalogue numbers:
- **P311-025R:** SALSA® MLPA® probemix P311 CHD, 25 reactions.
- **P311-050R:** SALSA® MLPA® probemix P311 CHD, 50 reactions.
- **P311-100R:** SALSA® MLPA® probemix P311 CHD, 100 reactions.

To be used in combination with a SALSA® MLPA® reagent kit, available for various number of reactions. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman 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 P311 CHD is a **research use only (RUO)** assay for the detection of deletions or duplications in the *GATA4*, *NKX2-5*, *TBX5*, *BMP4* and *CRELD1* genes.

Congenital heart disease (CHD) is a common birth defect, of which ventricular septal defects are collectively the most common type. Abnormal cardiac development originates from both environmental and genetic factors. Multiple studies postulate that mutations in several genes could be implicated in CHD.
The transcription factor GATA4 forms a complex with TBX5 which interacts with a heart muscle protein, α-myosin heavy chain. Another factor, the homeobox (developmental) gene, *NKX2-5* also interacts with *MYH6*. Mutations of all these proteins are associated with both atrial and ventricular septal defects. In addition, NKX2-5 is associated with defects in the electrical conduction of the heart and *TBX5* is related to the Holt-Oram syndrome which includes electrical conduction defects and abnormalities of the upper limb. Atrioventricular septal defect (AVSD) can also be caused by mutations in the gene encoding cell adhesion molecule *CRELD1*. Bone morphogenetic protein 4 (*BMP4*) was shown to have a critical role in functional heart formation in model animals; the loss of this protein resulted in various developmental defects.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK500252.

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/

Probemix content: The SALSA MLPA Probemix P311-B2 CHD contains 41 MLPA probes with amplification products between 124 and 475 nt. This includes: eight probes for the *GATA4* gene; four probes for the *NKX2-5* gene; ten probes for the *TBX5* gene; four probes for the *BMP4* gene; two probes for the *CRELD1* gene; and three probes for the 22q11.21 region (DiGeorge region). In addition, nine references probes are included, that detect nine different autosomal chromosomal locations.

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, 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 or 96 fragment indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

No DNA controls results in only five major peaks shorter than 121 nucleotides (nt): four Q-fragments at 64, 70, 76 and 82 nt, and one 19 nt peak corresponding to the unused portion of the fluorescent PCR primer. Non-specific peaks longer than 121 nt AND with a height >25% of the median of the four Q-fragments should not be observed. Note: peaks below this 25% threshold are not expected to affect MLPA reactions when sufficient amount of sample DNA (50-200 ng) is used.

MLPA technique: The principles of the MLPA technique (Schouten et al. 2002) are described in the MLPA General Protocol (www.mlpa.com).

Required specimens: Extracted DNA from peripheral blood, 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: 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 congenital heart abnormalities. 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 Biobank (https://catalog.coriell.org) and 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 must 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 all probes in the reference samples should be <0.10 and the dosage quotient (DQ) of the reference probes 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 or pseudo-autosomal chromosomes:

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 (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 in some cases not result in inactivation of that gene copy.
- Copy number changes detected by reference probes are unlikely to have any relation to the condition tested for.

Limitations of the procedure:

- In most populations, the major cause of genetic defects in the *GATA4*, *NKX2-5*, *TBX5*, *BMP4* and *CRELD1* genes are small (point) mutations, most of which will not be detected by using SALSA® MLPA® Probemix P311 CHD.
- 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 one or more than one consecutive probe (Table 2) 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.

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 *GATA4* exons 5 and 7 but not exon 6) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P311-B2 CHD

Length (nt)	SALSA MLPA probe	Chromosomal position						
		Reference	*GATA4*	*TBX5*	*NKX2-5*	*22q11*	*BMP4*	*CRELD1*
64-105	Control fragments – see table in probemix content section for more information							
124	Reference probe 18709-L21056	5q31						
130	**CTSB probe** 01212-L00766		Down-stream					
136	**CRELD1 probe** 02141-L01620							**Exon 4**
142	**GATA4 probe** 07641-L07326		**Exon 5**					
148 «	**GATA4 probe** 08309-L08282		**Exon 1**					
154 *	Reference probe 21307-L29713	7q31						
160	**TBX5 probe** 05694-L05136			**Exon 8**				
166	**TBX5 probe** 06207-L05127			**Exon 1**				
174	**TBX5 probe** 05696-L05138			**Exon 9**				
184	**TBX5 probe** 05687-L05129			**Exon 2**				
190	**NKX2-5 probe** 12465-L13480				**Exon 1**			
195	**TBX5 probe** 05688-L05130			**Exon 3**				
202	**GATA4 probe** 07643-L07328		**Exon 7**					
208 «	**GP1BB probe** 05464-L10114					**22q11**		
215	Reference probe 08570-L08571	17q23						
229 «	**GATA4 probe** 07697-L07414		Upstream					
238	**GATA4 probe** 07642-L07327		**Exon 6**					
247 «	**TBX5 probe** 05691-L05133			**Exon 6**				
255 «	**BMP4 probe** 12467-L14521						**Exon 1**	
266	**NKX2-5 probe** 11629-L12386				**Exon 2**			
274	**NKX2-5 probe** 12468-L13483				**Exon 1**			
283	**TBX5 probe** 05695-L05137			**Exon 8**				
292	Reference probe 11087-L11770	2p24						
303	**TBX5 probe** 05697-L05139			**Exon 9**				
317	**TBX5 probe** 05686-L05128			**Intron 1**				
328	Reference probe 10682-L11264	6p12						
337	**GATA4 probe** 07639-L07324		**Exon 3**					
346 «	**TBX5 probe** 06209-L05132			**Exon 5**				
355	**GATA4 probe** 07640-L07325		**Exon 4**					
362 «	**GATA4 probe** 07696-L07413		Upstream					
369	Reference probe 12377-L13386	2q37						
382 «	**DGCR8 probe** 08476-L10765					**22q11**		
391 «	**BMP4 probe** 12469-L13484						**Exon 4**	
408 ¥ «	**CRELD1 probe** 12470-L31541							**Exon 11**
418	Reference probe 11008-L11679	4q22						
427	**NKX2-5 probe** 12471-L13486				**Exon 2**			
436 «	**BMP4 probe** 12472-L13487						**Intron 1**	
445	Reference probe 15733-L17713	1p13						
454 «	**BMP4 probe** 12473-L13488						**Exon 3**	
466	**CDC45 probe** 05463-L05808					**22q11**		
475 *	Reference probe 18501-L23726	19q13						

* New in version B2 (from lot B2-1118 onwards).

¥ Changed in version B2 (from lot B2-1118 onwards). Small change in length, no change in sequence detected.

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

Table 2. P311 probes arranged according to chromosomal location

Table 2a. *GATA4*

Length (nt)	SALSA MLPA probe	*GATA4* Exon	Ligation site NM_002052.4	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
362 «	07696-L07413	upstream	8.4 kb upstream of exon 1	CATGCTCAAGAT-AGGCACTGGAGC	1.5 kb
229 «	07697-L07414	upstream	6.9 kb upstream of exon 1	GAGGTTCTTCTT-TAAAATCCATTC	7.0 kb
		Start Codon	*612-614 (exon 2)*		
148 «	08309-L08282	Exon 1	22 nt after exon 1	TTTCTTCCCTTT-CTTTGCTCCTTC	44.6 kb
	No probe	Exon 2			
337	07639-L07324	Exon 3	1231-1232	CTCAGTAGATAT-GTTTGACGACTT	1.3 kb
355	07640-L07325	Exon 4	1513-1514	CTACATGAAGCT-CCACGGGGTACG	4.9 kb
142	07641-L07326	Exon 5	1589-1590	AAGAACCTGAAT-AAATCTAAGACA	1.9 kb
238	07642-L07327	Exon 6	1666-1667	CAACTCCAGCAA-CGCCACCACCAG	1.4 kb
202	07643-L07328	Exon 7	1842-1843	CACAAGGCTATG-CGTCTCCCGTCA	89.3 kb
		Stop Codon	*1938-1940 (exon 7)*		
130	01212-L00766	*CTSB* gene	89.3 kb downstream	AAGTGTAGCAAG-ATCTGTGAGCCT	

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

Note: The exon numbering used in this P311-B2 product description is the exon numbering from the RefSeq transcript NM_002052.4. The exon numbering and NM sequence used is from March 2019, but can be changed (e.g. by NCBI) after the release of the product description. Please notify us of any mistakes: info@mlpa.com.

Table 2b. *NKX2-5*

Length (nt)	SALSA MLPA probe	*NKX2-5* Exon	Ligation site NM_004387.3	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
		Start Codon	*230-232 (exon 1)*		
274	12468-L13483	Exon 1	475-476	CCGGCCAAGTGT-GCGTCTGCCTTT	0.1 kb
190	12465-L13480	Exon 1	10 nt after exon 1	AGGTGAGGAGGA-AACACAGGCCCC	1.6 kb
427	12471-L13486	Exon 2	682-681 reverse	CGCTCCAGCTCA-TAGACCTGCGCC	0.5 kb
266	11629-L12386	Exon 2	1158-1159	CGGGATTCCGCA-GAGCAACTCGGG	
		Stop Codon	*1202-1204 (exon 2)*		

Note: The exon numbering used in this P311-B2 product description is the exon numbering from the RefSeq transcript NM_004387.3. The exon numbering and NM sequence used is from March 2019, but can be changed (e.g. by NCBI) after the release of the product description. Please notify us of any mistakes: info@mlpa.com.

Table 2c. *TBX5*

Length (nt)	SALSA MLPA probe	*TBX5* Exon	Ligation site NM_000192.3	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
		Start Codon	*668-670 (exon 2)*		
166	06207-L05127	Exon 1	509-510	GACGTTGGAAGA-AGACCTGGCCTA	2.2 kb
317	05686-L05128	Intron 1	1.8 kb before exon 2; NM_181486.2; 420-421	CTATTCTGGGTA-AGCAGTAAACCC	1.9 kb
184	05687-L05129	Exon 2	726-727	GCCTGACGCAAA-AGACCTGCCCTG	1.9 kb
195	05688-L05130	Exon 3	837-838	AATCAAAGTGTT-TCTCCATGAAAG	3.3 kb
	No probe	Exon 4			
346 «	06209-L05132	Exon 5	1138-1139	TCCTTCCAGAAA-CTCAAGCTCACC	3.8 kb
247 «	05691-L05133	Exon 6	1213-1214	TACCAGCCTAGA-TTACACATCGTG	28.5 kb
	No probe	Exon 7			
160	05694-L05136	Exon 8	1465-1466	GTGAGGCAAAAA-GTGGCCTCCAAC	0.2 kb
283	05695-L05137	Exon 8	1641-1642	CCATTGTACCAA-GAGGAAAGGTGA	10.1 kb
174	05696-L05138	Exon 9	1724-1725	AAGAAGATTCCT-TCTACCGCTCTA	1.1 kb
303	05697-L05139	Exon 9	2869-2870	TTTGCTTTGGTT-TTGTCCTGCCTT	
		Stop Codon	*2222-2224 (exon 9)*		

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

Note: The exon numbering used in this P311-B2 product description is the exon numbering from the RefSeq transcript NM_000192.3, which identical to LRG_670 sequence. The exon numbering and NM sequence used is from March 2019, but can be changed (e.g. by NCBI) after the release of the product description. Please notify us of any mistakes: info@mlpa.com.

Table 2d. *BMP4*

Length (nt)	SALSA MLPA probe	*BMP4* Exon	Ligation site NM_001202.5	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
		Start Codon	*475-477 (exon 3)*		
255 «	12467-L14521	Exon 1	340-341	TGCAGGGACCTA-TGGTGAGCAAGG	2.1 kb
436 «	12472-L13487	Intron 1	1.1 kb before exon 2	CGCAGGCCGAAA-GCTGTTCACCGT	2.3 kb
	No probe	Exon 2			
454 «	12473-L13488	Exon 3	494-495	TGGTAACCGAAT-GCTGATGGTCGT	1.3 kb
391 «	12469-L13484	Exon 4	856-857	AACATCTGGAGA-ACATCCCAGGGA	
		Stop Codon	*1699-1701 (exon 4)*		

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

Note: The exon numbering used in this P311-B2 product description is the exon numbering from the RefSeq transcript NM_001202.5. The exon numbering and NM sequence used is from March 2019, but can be changed (e.g. by NCBI) after the release of the product description. Please notify us of any mistakes: info@mlpa.com.

Table 2e. *CRELD1*

Length (nt)	SALSA MLPA probe	*CRELD1* Exon	Ligation site NM_001031717.3	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
		Start Codon	*100-102 (exon 2)*		
136	02141-L01620	Exon 4	407-408	CAAGTCAGACTT-CGAGTGCCACCG	6.3 kb
408 ¥ «	12470-L31541	Exon 11	1161-1162	GCATTCCCCATC-TTAACTGATTTA	
		Stop Codon	*1366-1368 (exon 12)*		

¥ Changed in version B2 (from lot B2-1118 onwards). Small change in length, no change in sequence detected.
« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

Note: The exon numbering used in this P311-B2 product description is the exon numbering from the RefSeq transcript NM_001031717.3. The exon numbering and NM sequence used is from March 2019, but can be changed (e.g. by NCBI) after the release of the product description. Please notify us of any mistakes: info@mlpa.com.

Table 2f. *22q11* region (DiGeorge)

Length (nt)	SALSA MLPA probe	*22q11* Gene	Ligation site, region	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
		Telomere			
466	05463-L05808	*CDC45*	NM_001178010.2; 155-156, region A-B	ATGTTCGTGTCC-GATTTCCGCAAA	244.0 kb
208 «	05464-L10114	*GP1BB*	NM_000407.4; 206-207, region A-B	CACAACCGAGCT-GGTGCTGACCGG	386.3 kb
382 «	08476-L10765	*DGCR8*	NM_022720.6; 2993-2994, region A-B	GACTCAGCGACT-GCACCAGTGGCA	

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

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 P311 CHD

- Mutlu ET et al. (2018). Analysis of gene copy number variations in patients with congenital heart disease using multiplex ligation-dependent probe amplification. *Anatol J Cardiol.* 20(01):9-15.
- El Malti R. et al. (2015). A systematic variant screening in familial cases of congenital heart defects demonstrates the usefulness of molecular genetics in this field. *Eur J Hum Genet.* 24:228-236.

P311 Product history	
Version	*Modification*
B2	Two reference probes have been replaced, one reference probe has been removed and one probe length has been adjusted.
B1	One target and one flanking probe have been removed and two reference probes have been replaced.
A2	Control fragments have been adjusted (QDX2). One CRELD1 probe has been removed.
A1	First release.

Implemented changes in the product description
Version B2-01– 24 April 2019 (01P) - Product description restructured and adapted to a new template. - Product description adapted to a new product version. - Small changes in Table 1, and changes in Table 2. *Version 07 (55) – 29 July 2016* - Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included). - Various minor textual changes on page 1. - Exon numbering has changed in table 1 and 2 for genes *NKX2-5*, *TBX5*, *BMP4* and *CRELD1*. - Various minor layout changes *Version 06 (48) – 07 August 2015* - Electropherogram picture(s) using the old MLPA buffer (replaced in December 2012) removed.

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