Product Description
SALSA® MLPA® Probemix P180-B4 Limb Malformations-2

To be used with the MLPA General Protocol.

Version B4
For complete product history see page 7.

Catalogue numbers:
- **P180-025R:** SALSA MLPA Probemix P180 Limb Malformations-2, 25 reactions.
- **P180-050R:** SALSA MLPA Probemix P180 Limb Malformations-2, 50 reactions.
- **P180-100R:** SALSA MLPA Probemix P180 Limb Malformations-2, 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 P180 Limb Malformations-2 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *SALL1*, *SALL4,* and *TBX5* genes, which are associated with limb malformations.

The *SALL1* gene product is a C2H2 zinc finger DNA binding protein which is associated with pericentromeric heterochromatin, and is a strong translational repressor. Mutations in *SALL1* cause Townes-Brocks syndrome (TBS), characterised by radial limb malformation, anal abnormalities, abnormal shaped pinnae, and renal anomalies. All cases of TBS described so far are caused by point mutations or small deletions/insertions leading to a truncated protein product.

The *SALL4* gene product is a C2H2 zinc finger DNA binding protein, which is thought to function as a transcription factor. Mutations in *SALL4* cause a variety of phenotypes, including Okihiro syndrome (Duane-radial ray syndrome), characterised by an inability to abduct the eye and radial dysplasia, acro-renal-ocular syndrome, Holt-Oram Syndrome (see below), and malformations previously attributed to thalidomide embryopathy. Most mutations are point mutations or small deletions/insertions leading to a truncated protein product, however recently six large deletions have been associated with Okihiro syndrome.

The *TBX5* gene product is a transcription factor of the T-box family which is particularly important for heart and upper limb development. Point mutations and deletions in *TBX5* have been shown to result in Holt-Oram syndrome (HOS), characterised by radial ray and cardiac (ASD, VSD, Mitral valve prolapse) defects. As the last three exons of *TBX5* are separated by large introns, two probes have been included for these exons.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1373/, https://www.ncbi.nlm.nih.gov/books/NBK1111/ and https://www.ncbi.nlm.nih.gov/books/NBK1445/.

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 *SALL1*, *SALL4,* and *TBX5* exon numbering used in this P180-B4 Limb Malformations-2 product description is the exon numbering from the LRG_674, LRG_675, and LRG_670 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 P180-B4 Limb Malformations-2 contains 29 MLPA probes with amplification products between 128 and 382 nucleotides (nt). This includes 13 probes for the *TBX5* gene, three probes for the *SALL1* gene and four probes for the *SALL4* gene. In addition, nine 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 limb malformations. 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 *TBX5*, *SALL1*, and *SALL4* genes are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P180 Limb Malformations-2.
- 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.

***TBX5*, *SALL1*, and *SALL4* mutation database**

https://databases.lovd.nl/shared/genes. 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 *TBX5* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P180-B4 Limb Malformations-2

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]			
		Reference	*TBX5*	*SALL1*	*SALL4*
64-105	Control fragments – see table in probemix content section for more information				
128	Reference probe 00797-L00093	5q			
134	Reference probe 03571-L02375	7q			
140	**SALL4 probe** 05681-L05123				**Exon 1**
148	**TBX5 probe** 05692-L05134		**Exon 7**		
154	Reference probe 18714-L24080	2q			
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**		
193	**SALL4 probe** 05682-L17147				**Exon 2**
199	**TBX5 probe** 05688-L17144		**Exon 3**		
206	Reference probe 03140-L17145	14q			
213	**TBX5 probe** 05689-L17146		**Exon 4**		
220	**SALL1 probe** 05679-L05121			**Exon 2**	
228	**SALL1 probe** 06208-L05120			**Exon 1**	
238	Reference probe 10085-L10509	8q			
247	**TBX5 probe** 05691-L05133		**Exon 6**		
255	**SALL4 probe** 05683-L05125				**Exon 3**
265	**TBX5 probe** 05693-L05135		**Exon 7**		
274	Reference probe 13479-L14941	1q			
283	**TBX5 probe** 05695-L05137		**Exon 8**		
292	Reference probe 03796-L03237	21q			
303	**TBX5 probe** 05697-L05139		**Exon 9**		
317	**TBX5 probe** 05686-L05128		**Intron 1**		
326 «	**SALL4 probe** 05684-L05126				**Exon 4**
337	Reference probe 03195-L02652	17q			
346	**TBX5 probe** 06209-L05132		**Exon 5**		
373	**SALL1 probe** 15030-L17302			**Exon 3**	
382	Reference probe 06446-L05972	3p			

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

« 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.

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. P315-B4 probes arranged according to chromosomal location

Table 2a. *TBX5*

Length (nt)	SALSA MLPA probe	*TBX5* exon[a]	Ligation site NM_000192.3	Partial sequence[b] (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; 401-402 in NM_181486.4	CTATTCTGGGTA-AGCAGTAAACCC	1.9 kb
184	05687-L05129	Exon 2	726-727	GCCTGACGCAAA-AGACCTGCCCTG	1.9 kb
199	05688-L17144	Exon 3	837-838	AATCAAAGTGTT-TCTCCATGAAAG	2.3 kb
213	05689-L17146	Exon 4	958-959	CTTAATCCCAAA-ACGAAGTACATT	1.0 kb
346 #	06209-L05132	Exon 5	1138-1139	TCCTTCCAGAAA-CTCAAGCTCACC	3.8 kb
247	05691-L05133	Exon 6	1213-1214	TACCAGCCTAGA-TTACACATCGTG	9.3 kb
148	05692-L05134	Exon 7	1340-1341	AGATCACGCAAT-TAAAGATTGAGA	0.1 kb
265	05693-L05135	Exon 7	1421-1422	CAAGAATGCAAA-GGTAGGAAAGTG	19.1 kb
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)*		

Table 2b. *SALL1*

Length (nt)	SALSA MLPA probe	*SALL1* exon[a]	Ligation site NM_002968.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*30-32 (Exon 1)*		
228	06208-L05120	Exon 1	62-63	AAGCCTCAACAT-TTCCAATCCGAC	9.8 kb
220 #	05679-L05121	Exon 2	834-835	CAACATCTTCTA-GTCCTTCTCAAG	5.0 kb
373 #	15030-L17302	Exon 3	4739-4738, reverse	TTACGTCTCAGT-TTCAACTACCAA	
		stop codon	*4002-4004 (Exon 3)*		

Table 2c. *SALL4*

Length (nt)	SALSA MLPA probe	*SALL4* exon[a]	Ligation site NM_020436.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*114-116 (Exon 1)*		
140 #	05681-L05123	Exon 1	93-94	TGCGGCTCTCCC-GGCGCCCCGATG	12.0 kb
193	05682-L17147	Exon 2	2175-2176	ATGATGATGTCA-TCGAAAGCATCG	1.6 kb
255	05683-L05125	Exon 3	2852-2853	AAAGGCAACTTA-AAGGTGGGTTTG	4.3 kb
326	05684-L05126	Exon 4	2969-2970	GTCTCAGAAATC-TTTCCCAAGGAA	
		stop codon	*3273-3275 (Exon 4)*		

[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

P179 Limb Malformations-1 Genes included *GLI3*, *HOXD13*, and *ROR2*.

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 P180 Limb Malformations-2

- Chacón-Camacho ÓF et al. (2017). Clinical and genetic findings in Mexican patients with Duane anomaly and radial ray malformations/Okihiro syndrome. *Rev Invest Clin*, 68(5), 269-274.
- Jourdain AS et al. (2020). Multiplex targeted high-throughput sequencing in a series of 352 patients with congenital limb malformations. *Hum Mutat*, 41(1), 222-239.
- Kimura M et al. (2015). Novel TBX5 Duplication in a Japanese Family with Holt–Oram Syndrome. *Pediatr Cardiol*, 36(1), 244-247.
- Morisada N et al. (2014). 16q12 microdeletion syndrome in two Japanese boys. *Pediatr Int*. 56:e75-8.
- Potuijt JW et al (2022). The pZRS non-coding regulatory mutation resulting in triphalangeal thumb–polysyndactyly syndrome changes the pattern of local interactions. *Mol Genet Genom*, 297(5), 1343-1352.
- Spiridon MR et al. (2018). Holt-Oram syndrome with multiple cardiac abnormalities. *Cardiol Res*, 9(5), 324.
- Unzaki A et al. (2018). Clinically diverse phenotypes and genotypes of patients with branchio-oto-renal syndrome. *J Hum Genet*, 63(5), 647.
- Walencka Z et al. (2016). Clinical expression of Holt-Oram syndrome on the basis of own clinical experience considering prenatal diagnosis. *Ginekol pol*, 87(10), 706-710.

P180 product history	
Version	*Modification*
B4	One reference probe has been replaced.
B3	One reference probe has been replaced.
B2	One reference probe was replaced and the control fragments have been adjusted (QDX2).
B1	One *SALL1* probe and several reference probes have been replaced.
A1	First release.

Implemented changes in the product description

Version B4-02 – 03 February 2023 (04P)
- Product description rewritten and adapted to a new template.
- Ligation sites of the probes targeting the *SALL1* gene updated according to new version of the NM_ reference sequence.

Version B4-01 — 22 August 2019 (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).
- Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.
- Ligation sites of the probes targeting the *SALL4* gene updated according to new version of the NM_ reference sequence.

Version 12 – 17 February 2016 (55)
- 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 layout changes.

Version 11 (53)

- 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).

Version 10 (48)

- Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added.

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