Product Description

SALSA® MLPA® Probemix P179-B1 Limb malformations-1

To be used with the MLPA General Protocol.

Version B1
For complete product history see page 7.

Catalogue numbers:

- **P179-025R:** SALSA MLPA Probemix P179 Limb malformations-1, 25 reactions.
- **P179-050R:** SALSA MLPA Probemix P179 Limb malformations-1, 50 reactions.
- **P179-100R:** SALSA MLPA Probemix P179 Limb malformations-1, 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 P179 Limb malformations-1 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *GLI3*, *HOXD13,* and *ROR2* genes, which are associated with limb malformations, including Greig cephalopolysyndactyly syndrome, Pallister-Hall syndrome and Robinow syndrome.

The *GLI3* gene encodes a protein that belongs to the C2H2-type zinc finger proteins. Defects in the *GLI3* gene cause a wide variety of phenotypes, including Greig cephalopolysyndactyly syndrome (GCPS) and Pallister-Hall syndrome.
The *HOXD13* gene encodes a transcription factor and is involved in distal limb patterning. Four types of *HOXD13* mutations are associated with distinct phenotypes. Expansions in the amino-terminal polyalanine tract cause synpolydactyly (SPD), specific missense mutations cause brachydactyly type E, intragenic deletions or other missense mutations cause SPD with an additional foot phenotype, while a splice site mutation has been reported to cause only foot malformation.
The *ROR2* gene encodes a transmembrane receptor tyrosine kinase, which is particularly important for the chondrocyte lineage. Mutations in *ROR2* have been shown to result in brachydactyly type B, and in autosomal recessive Robinow syndrome.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1446/, https://www.ncbi.nlm.nih.gov/books/NBK1465/, and https://www.ncbi.nlm.nih.gov/books/NBK1240/.

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 *GLI3*, *HOXD13,* and *ROR2* exon numbering used in this P179-B1 Limb malformations-1 product description is the exon numbering from the NG_008434.1, NG_008137.1, and NG_008089.1 sequences, respectively. 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 NG 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 P179-B1 Limb malformations-1 contains 43 MLPA probes with amplification products between 130 and 463 nucleotides (nt). This includes 21 probes for the *GLI3* gene, at least one probe for each exon, two probes for the *HOXD13* gene, and ten probes for the *ROR2* gene, one probe for each exon and two probes for exon 1. In addition, ten 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 *GLI3*, *HOXD13,* and *ROR2* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P179 Limb malformations-1.
- 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.

LOVD 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 *GLI3* exons 7 and 9 but not exon 8) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P179-B1 Limb malformations-1

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]			
		Reference	*GLI3*	*HOXD13*	*ROR2*
64-105	Control fragments – see table in probemix content section for more information				
130	Reference probe 00797-L00463	5q			
137 «	**GLI3 probe** 05557-L05451		**Exon 2**		
142 «	**ROR2 probe** 05575-L05452				**Exon 1**
148 «	**HOXD13 probe** 05573-L05005			**Exon 1**	
154	Reference probe 02409-L03789	16q			
160	**GLI3 probe** 05558-L05453		**Exon 3**		
166	**ROR2 probe** 05577-L05009				**Exon 2**
172 «	**HOXD13 probe** 05574-L05454			**Exon 2**	
178	Reference probe 16888-L19721	18q			
184	**ROR2 probe** 16915-L19859				**Exon 3**
190	**GLI3 probe** 06148-L04992		**Exon 4**		
196	**GLI3 probe** 16916-L19860		**Exon 5**		
202	**GLI3 probe** 05562-L20251		**Exon 5**		
209	**GLI3 probe** 05563-L04995		**Exon 6**		
221	**ROR2 probe** 16917-L19861				**Exon 4**
229	**GLI3 probe** 16918-L19862		**Exon 7**		
238	Reference probe 02519-L01950	17q			
247	**GLI3 probe** 05565-L04997		**Exon 8**		
256	**ROR2 probe** 05580-L05012				**Exon 5**
265	**GLI3 probe** 05566-L04998		**Exon 9**		
274	Reference probe 08545-L08546	3q			
281	**GLI3 probe** 16919-L19863		**Exon 10**		
288	**GLI3 probe** 05567-L20252		**Exon 10**		
294	**ROR2 probe** 05581-L20253				**Exon 6**
301	**GLI3 probe** 05568-L05000		**Exon 11**		
310	Reference probe 03934-L03389	15q			
317	**GLI3 probe** 05569-L05001		**Exon 12**		
325	**ROR2 probe** 05582-L05014				**Exon 7**
337	**GLI3 probe** 05570-L05002		**Exon 13**		
346	Reference probe 02324-L01815	19p			
355	**GLI3 probe** 05571-L05003		**Exon 14**		
364	**ROR2 probe** 05583-L05015				**Exon 8**
374	**GLI3 probe** 05572-L05004		**Exon 15**		
384	Reference probe 14642-L16292	1q			
392 «	**GLI3 probe** 05556-L04988		**Exon 2**		
400	**ROR2 probe** 16920-L19864				**Exon 9**
409	**GLI3 probe** 05559-L04991		**Exon 3**		
418	Reference probe 03065-L02494	4p			
427	**GLI3 probe** 05561-L04993		**Exon 4**		
433 «	**ROR2 probe** 16921-L19865				**Exon 1**
445 «	**GLI3 probe** 16923-L19867		**Exon 1**		
453 «	**GLI3 probe** 16922-L20249		**Exon 1**		
463	Reference probe 08479-L20250	22q			

[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. P179-B1 probes arranged according to chromosomal location

Table 2a. *GLI3*

Length (nt)	SALSA MLPA probe	*GLI3* exon[a]	Ligation site NM_000168.6	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*282-284 (Exon 2)*		
453 «	16922-L20249	Exon 1	5 nt after exon 1, reverse	TTCGAGCGGGAC-GTACCTGCGGCG	0.2 kb
445 «	16923-L19867	Exon 1	142 nt after exon 1	GCCCAGATTTAG-AGAGCCGCCGGT	13.6 kb
392 «	05556-L04988	Exon 2	267-268	GAGAGCTGAAGT-AATGAGAAGACA	0.1 kb
137 «	05557-L05451	Exon 2	362-363	TCCACTCGAACA-GATGTGAGCGAG	74.7 kb
409	05559-L04991	Exon 3	428-429	CCTGGACAGACT-TATCACAGAGAG	0.1 kb
160	05558-L05453	Exon 3	490-491	GGGGCTCAGCAA-AGTCAGTGAGGA	71.5 kb
427	05561-L04993	Exon 4	649-648, reverse	GATGAGGAGGGT-CTGAAAAGAAGA	0.1 kb
190	06148-L04992	Exon 4	690-691	CTCCTGTACCAA-TTGATGCCAGAC	28.2 kb
202	05562-L20251	Exon 5	802-803	CCTGCCCTTCAT-TAGGATCTCCCC	0.1 kb
196	16916-L19860	Exon 5	891-892	ACATGGACTATA-TCCGCTCCTTGC	3.1 kb
209	05563-L04995	Exon 6	989-990	AGCCCAGCAGAA-TACTATCATCAG	5.4 kb
229	16918-L19862	Exon 7	1289-1288, reverse	CTTGCAGATAAG-TGACCATAGGAG	13.8 kb
247	05565-L04997	Exon 8	1440-1441	CTGCCCCAACTT-TTCCAACACAGA	1.0 kb
265	05566-L04998	Exon 9	1590-1591	AGAGGTCCAAGA-TCAAACCCGATG	1.7 kb
288	05567-L20252	Exon 10	1665-1666	CAACCCTTGTCA-AGGAGGAAGGGG	0.1 kb
281	16919-L19863	Exon 10	1772-1771, reverse	CTTACGTGCACA-AGCTGCTCTTGG	44.8 kb
301	05568-L05000	Exon 11	1844-1845	CTGGACTGCTCA-AGAGAGCAGAAA	1.1 kb
317	05569-L05001	Exon 12	2045-2046	AAGGCTTTCTCA-AATGCCTCTGAT	5.1 kb
337	05570-L05002	Exon 13	2262-2263	GCAGCCATTCAC-AGTCCAGGTCGC	4.6 kb
355	05571-L05003	Exon 14	2417-2418	GGTCAGTCTTCA-TGCAGCAGCCAA	2.1 kb
374	05572-L05004	Exon 15	3582-3583	TGCAGTATTTAA-ATTCCCAGAACC	
		stop codon	*5022-5024 (Exon 15)*		

Table 2b. *HOXD13*

Length (nt)	SALSA MLPA probe	*HOXD13* exon[a]	Ligation site NM_000523.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*171-173 (Exon 1)*		
148 «	05573-L05005	Exon 1	747-748	AGGTATCCTTCT-ACCAGGGCTATA	1.1 kb
172 «	05574-L05454	Exon 2	1061-1062	GAGTATGCCATT-AACAAATTCATT	
		stop codon	*1200-1202 (Exon 2)*		

Table 2c. *ROR2*

Length (nt)	SALSA MLPA probe	*ROR2* exon[a]	Ligation site NM_004560.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*266-268 (Exon 1)*		
142 «	05575-L05452	Exon 1	140-141	GAGGTCCTCGAA-GTGGACCCGTTT	0.2 kb
433 «	16921-L19865	Exon 1	2 nt after exon 1	CGGACTTCAGGT-AGGATCTGGCGT	174.1 kb
166	05577-L05009	Exon 2	396-397	GAACGACCCTTT-AGGACCCCTTGA	18.5 kb
184	16915-L19859	Exon 3	695-696	GGATGAAGACCA-TTACCGCCACTG	1.3 kb
221	16917-L19861	Exon 4	59 nt after exon 4	TTGTTATGTAGG-AATCCGGGGTTT	18.6 kb
256	05580-L05012	Exon 5	838-839	AACCGGACCATT-TATGTGGACTCG	4.1 kb
294	05581-L20253	Exon 6	938-939	ACCAGTGCTCAC-AGTTCGCCATCC	2.3 kb
325	05582-L05014	Exon 7	1235-1236	CAGGCATGGATT-ACAGAGGAACGG	4.4 kb
364	05583-L05015	Exon 8	1481-1482	TGGGGATTCTGT-ACATCTTGGTCC	2.1 kb
400	16920-L19864	Exon 9	2135-2136	ATGTGCTAGTGT-ACGACAAGCTGA	
		stop codon	*3095-3097 (Exon 9)*		

[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.

« 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.

Related SALSA MLPA probemixes

P180 Limb malformations-2 Contains probes for the *SALL1*, *SALL4,* and *TBX5* genes.

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 P179 Limb malformations-1

- Demurger F et al. (2015). New insights into genotype–phenotype correlation for GLI3 mutations. *Eur J Hum Genet*. 23:92-102.
- Bednarczyk et al. (2013). Normal exon copy number of the GLI2 and GLI3 genes in patients with esophageal atresia. *Dis Esophagus*. 26:678-81.
- Jamsheer A et al. (2013). Isolated brachydactyly type E caused by a HOXD13 nonsense mutation: a case report. *BMC Med Genet*. 13:4.
- Lima AR et al. (2022). Phenotypic and mutational spectrum of ROR2-related Robinow syndrome. *Hum Mutat.*
- Pereda A et al. (2018). What to consider when pseudohypoparathyroidism is ruled out: iPPSD and differential diagnosis. *BMC Med Genet*, 19(1), 1-10.

P179 product history	
Version	*Modification*
B1	One *GLI3* and three *ROR2* probes have been replaced. Four extra *GLI3* probes and one extra *ROR2* probe have been included.
A2	The 88, 96, 100 and 105 nt control fragments have been included.
A1	First release

Implemented changes in the product description
Version B1-02 – 19 April 2022 (04P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *GLI3*, *HOXD13*, and *ROR2* genes updated according to new version of the NM_ reference sequence. - 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 Tables 1 and 2 for *ROR2* exon 1 probe being located near a GC-rich region. *Version B1-01 – 12 September 2018 (01P)* - Product description restructured and adapted to a new template.

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