

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 6.

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, 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 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 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://ghr.nlm.nih.gov/gene/GLI3>, <https://ghr.nlm.nih.gov/gene/HOXD13> and <https://ghr.nlm.nih.gov/gene/ROR2>.

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 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, two for the *HOXD13* gene and ten for the *ROR2* gene. In addition, ten reference probes are included detecting different autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes is 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 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 105 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 105 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, 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 the disorder it is used for. 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 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 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 in or near the *GLI3*, *HOXD13* or *ROR2* gene. 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 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 *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. 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.

GLI3, HOXD13 and ROR2 mutation databases: http://grenada.lumc.nl/LSDB_list/lsdbs/gli3; http://grenada.lumc.nl/LSDB_list/lsdbs/HOXD13; http://grenada.lumc.nl/LSDB_list/lsdbs/ror2. We strongly encourage users to deposit positive results in relevant databases. 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 *GLI3* exons 6 and 8 but not exon 7) to MRC-Holland: info@mlpa.com.

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

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)			
		Reference	GLI3	HOXD13	ROR2
64-105	Control fragments – see table in probemix content section for more information				
130	Reference probe 00797-L00463	5q31			
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	16q22			
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	18q21			
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	17q11			
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	3q24			
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	15q21			
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	19p13			
355	GLI3 probe 05571-L05003		Exon 14		
364	ROR2 probe 05583-L05015			Exon 8	
373	GLI3 probe 05572-L05004		Exon 15		
382	Reference probe 14642-L16292	1q23			
391 «	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	4p16			
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	22q11			

« 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 P179-B1 Limb malformations-1 product description is the exon numbering from the RefSeq transcripts NM_000168.5, NM_000523.3, and NM_004560.3. The exon numbering and NM sequences used are from 08/2018, 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 2. P179-B1 probes arranged according to chromosomal location

Table 2a. *GLI3* gene

Length (nt)	SALSA MLPA probe	<i>GLI3</i> exon	Ligation site NM_000168.5	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
		<i>start codon</i>	<i>92-94 (exon 2)</i>		
453 «	16922-L20249	Exon 1	4 nt after exon 1, reverse	TTCGAGCGGGAC-GTACCTGCGGCG	0.2 kb
445 «	16923-L19867	Exon 1	142 nt after exon 1	GCCCAGATTTAG-AGAGCCGCCGTT	13.6 kb
391 «	05556-L04988	Exon 2	77-78	GAGAGCTGAAGT-AATGAGAAGACA	0.1 kb
137 «	05557-L05451	Exon 2	172-173	TCCA CTGAACA-GATGTGAGCGAG	74.7 kb
409	05559-L04991	Exon 3	238-239	CCTGGACAGACT-TATCAGAGAG	0.1 kb
160	05558-L05453	Exon 3	300-301	GGGGCTCAGCAA-AGTCAGTGAGGA	71.5 kb
427	05561-L04993	Exon 4	459-458 reverse	GATGAGGAGGGT-CTGAAAAGAAGA	0.1 kb
190	06148-L04992	Exon 4	500-501	CTCCTGTACCAA-TTGATGCCAGAC	28.2 kb
202	05562-L20251	Exon 5	612-613	CCTGCCCTTCAT-TAGGATCTCCCC	0.1 kb
196	16916-L19860	Exon 5	701-702	ACATGGACTATA-TCCGCTCCTTGC	3.1 kb
209	05563-L04995	Exon 6	799-800	AGCCCAGCAGAA-TACTATCATCAG	5.4 kb
229	16918-L19862	Exon 7	1099-1098 reverse	CTTGACGATAAG-TGACCATAGGAG	13.8 kb
247	05565-L04997	Exon 8	1250-1251	CTGCCCCAAGTT-TTCCAACACAGA	1.0 kb
265	05566-L04998	Exon 9	1400-1401	AGAGGTCCAAGA-TCAAACCCGATG	1.7 kb
288	05567-L20252	Exon 10	1475-1476	CAACCCTTGTC-AGGAGGAAGGGG	0.1 kb
281	16919-L19863	Exon 10	1582-1581 reverse	CTTACGTGCACA-AGCTGCTCTTGG	44.8 kb
301	05568-L05000	Exon 11	1654-1655	CTGGACTGCTCA-AGAGAGCAGAAA	1.1 kb
317	05569-L05001	Exon 12	1855-1856	AAGGCTTTCTCA-AATGCCTCTGAT	5.1 kb
337	05570-L05002	Exon 13	2072-2073	GCAGCCATTAC-AGTCCAGTTCGC	4.6 kb
355	05571-L05003	Exon 14	2227-2228	GGTCAGTCTTCA-TGCAGCAGCCAA	2.1 kb
373	05572-L05004	Exon 15	3392-3393	TGCAGTATTTAA-ATTCCAGAACCC	
		<i>stop codon</i>	<i>4832-4834 (exon 15)</i>		

« 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 2b. *HOXD13* gene

Length (nt)	SALSA MLPA probe	<i>HOXD13</i> exon	Ligation site NM_000523.3	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
		<i>start codon</i>	<i>88-90 (exon 1)</i>		
148 «	05573-L05005	Exon 1	664-665	AGGTATCCTTCT-ACCAGGGCTATA	1.1 kb
172 «	05574-L05454	Exon 2	978-979	GAGTATGCCATT-AACAAATTCATT	
		<i>stop codon</i>	<i>1117-1119 (exon 2)</i>		

« 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 2c. *ROR2* gene

Length (nt)	SALSA MLPA probe	<i>ROR2</i> exon	Ligation site NM_004560.3	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
		<i>start codon</i>	200-202 (exon 1)		
142 «	05575-L05452	Exon 1	74-75	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	330-331	GAACGACCCTTT-AGGACCCCTTGA	18.5 kb
184	16915-L19859	Exon 3	629-630	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	772-773	AACCGGACCATT-TATGTGGACTCG	4.1 kb
294	05581-L20253	Exon 6	872-873	ACCAGTGCTCAC-AGTTCGCCATCC	2.3 kb
325	05582-L05014	Exon 7	1169-1170	CAGGCATGGATT-ACAGAGGAACGG	4.4 kb
364	05583-L05015	Exon 8	1415-1416	TGGGGATTCTGT-ACATCTTGGTCC	2.1 kb
400	16920-L19864	Exon 9	2069-2070	ATGTGCTAGTGT-ACGACAAGCTGA	
		<i>stop codon</i>	3029-3031 (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.

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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.

P179 Product history	
Version	Modification
B1	One <i>GLI3</i> and three <i>ROR2</i> probes have been replaced. Four extra <i>GLI3</i> probes and one extra <i>ROR2</i> 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 B1-01 – 12 September 2018 (01P)

- Product description restructured and adapted to a new template.

Version 15 – 27 November 2015 (55)

- Product description adapted to a new lot (lot number added, small changes in Table 1 and Table 2, new picture included).
- Various textual and layout changes.
- References updated.
- Manufacturer's address adjusted.

Version 14 – 15 July 2015 (54)

- Figure based on the use of old MLPA buffer (replaced in December 2012) removed.

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

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