Product Description
SALSA® MLPA® Probemix P295-B3 SPRED1

To be used with the MLPA General Protocol.

Version B3
For complete product history see page 7.

Catalogue numbers:
- **P295-025R:** SALSA MLPA Probemix P295 SPRED1, 25 reactions.
- **P295-050R:** SALSA MLPA Probemix P295 SPRED1, 50 reactions.
- **P295-100R:** SALSA MLPA Probemix P295 SPRED1, 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 P295 SPRED1 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *SPRED1* gene, which are associated with Legius syndrome.

Legius syndrome (also known as Neurofibromatosis Type 1-like syndrome; NFLS) is characterised by multiple café-au-lait spots. Other features may include an abnormally large head (macrocephaly) and unusual facial characteristics. The syndrome has many symptomatic similarities with Neurofibromatosis Type I (NF1). *SPRED1* (Sprouty-related EVH1 domain containing 1) is a member of the Sprouty family of proteins that regulate growth factor-induced activation of the Ras/MAP kinase cascade. Mutations in the *SPRED1* gene cause Legius syndrome.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK47312/

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 *SPRED1* exon numbering used in this P295-B3 SPRED1 description is the exon numbering from the NG_008980.1 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 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 P295 SPRED1 contains 38 MLPA probes with amplification products between 149 and 454 nucleotides (nt). This includes 23 probes for the *SPRED1* gene. Multiple probes for each of the seven exons (or probes in close proximity of the exon) of the *SPRED1* gene are included. Probes in the upstream region and several probes in intronic regions of the gene have also been included in order to facilitate the detection of breakpoints of intragenic rearrangements. In addition, fifteen 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 Legius syndrome. 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 *SPRED1* gene are small (point) mutations, none of which will not be detected by using SALSA MLPA Probemix P295-B3 SPRED1.
- 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.

***SPRED1* mutation database**

https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php?select_db=SPRED1. We strongly encourage users to deposit positive results in the Leiden Open Variation Database (LOVD) https://databases.lovd.nl/shared/genes/. 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 *SPRED1* exons 3 and 5 but not exon 4) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P295-B3 SPRED1

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*SPRED1*
64-105	Control fragments – see table in probemix content section for more information		
149	Reference probe 10129-L11734	18q	
154	**SPRED1** probe 13044-L14227		Exon 3
160 Ø	**SPRED1** probe 13045-L14228		Intron 4
166	Reference probe 08421-L08304	11q	
172	**SPRED1** probe 13046-L14229		Exon 5
178 Ø	**SPRED1** probe 13047-L14230		Intron 2
185 Ø	**SPRED1** probe 13048-L14231		Intron 1
196	**SPRED1** probe 11017-L11686		Exon 4
202	Reference probe 03709-L25988	9q	
208	**SPRED1** probe 13049-L28437		Exon 4
214	**SPRED1** probe 11015-L28438		Exon 2
220	Reference probe 12427-L13428	22q	
230	**SPRED1** probe 13050-L14233		Exon 1
238	**SPRED1** probe 11019-L11688		Exon 6
247	**SPRED1** probe 11016-L11685		Exon 3
257	Reference probe 17408-L21394	3p	
265	**SPRED1** probe 11013-L13179		Upstream
274 Ø	**SPRED1** probe 13051-L14234		Intron 1
283	Reference probe 08680-L08692	13q	
292	**SPRED1** probe 13052-L14235		Exon 6
301	**SPRED1** probe 11020-L11689		Exon 7
310	Reference probe 12442-L13443	14q	
319	Reference probe 08048-L07829	5p	
328	**SPRED1** probe 13053-L14236		Exon 1
337	Reference probe 09937-L12248	8q	
346	**SPRED1** probe 11018-L11687		Exon 5
355	**SPRED1** probe 13054-L14237		Exon 2
364	Reference probe 12989-L14146	19q	
373	Reference probe 10718-L11300	6p	
384	**SPRED1** probe 13055-L14238		Exon 7
393	Reference probe 14043-L15641	7q	
402	**SPRED1** probe 11021-L11690		Exon 7
409	**SPRED1** probe 13056-L14239		Upstream
420	Reference probe 08839-L13359	2p	
427 Ø	**SPRED1** probe 13057-L14240		Intron 5
436 Ø	**SPRED1** probe 13058-L14241		Intron 2
445	Reference probe 12526-L13576	4q	
454	Reference probe 08579-L08580	17q	

[a] See section Exon numbering on page 1 for more information.

Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.

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. P295-B3 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*SPRED1* exon[a]	Ligation site NM_152594.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		Start Codon	*351-353 (Exon 1)*		
409	13056-L14239	Upstream	824 nt before exon 1	GTGGCTCGACAA-CGATTTGGGAAA	0.3 kb
265	11013-L13179	Upstream	494 nt before exon 1	CGGGCGTGAGGA-GCTGGCCCGCTG	0.5 kb
328	13053-L14236	Exon 1	64-65	GCCCCTCTCTTT-TTCCCTTTCCAC	0.5 kb
230	13050-L14233	Exon 1	138 nt after exon 1	AGATAGCTGATT-TCTTTAGCCATT	8.4 kb
185 Ø	13048-L14231	Intron 1	8515 nt after exon 1	GGAATGTGGAAT-GTAATTCTTTTT	31.7 kb
274 Ø	13051-L14234	Intron 1	5928 nt before exon 2	TGGAACCTAGAT-CTGACTTGTTAT	6.0 kb
214	11015-L28438	Exon 2	431-432	CGAGATGACTCA-AGTGGTGGATGG	0.1 kb
355	13054-L14237	Exon 2	524-525	TGTGCTGACTTT-TTTATCCGTGGA	6.7 kb
436 Ø	13058-L14241	Intron 2	6633 nt after exon 2	CTGTCTGCCTTT-TTTCTCAAAGGT	12.2 kb
178 Ø	13047-L14230	Intron 2	3862 nt before exon 3	GTATTTATGTGA-GTCAAAGAGCTA	3.9 kb
154	13044-L14227	Exon 3	563-564	TGTCAGGTGGTT-TTGGAATGTATG	0.1 kb
247	11016-L11685	Exon 3	678-679	CTGCTGATGCTA-GGGCTTTTGATA	2.3 kb
208	13049-L28437	Exon 4	117 nt before exon 4	TTGCCAGGCAGT-CCAGAAAGATCT	0.2 kb
196	11017-L11686	Exon 4	17 nt after exon 4	AATGGCTTGGAA-GGAATTTGTAAA	10.6 kb
160 Ø	13045-L14228	Intron 4	4307 nt before exon 5	AAGACAGTGAAT-AGTAGGTATTGA	4.3 kb
172	13046-L14229	Exon 5	791-792	GAAGAGGATTCT-TCCAGTTCTCTA	0.1 kb
346	11018-L11687	Exon 5	854-855	AGTGAGCCTTAT-AGAAGCTCAAAT	6.8 kb
427 Ø	13057-L14240	Intron 5	2850 nt before exon 6	TTCATCCTAGAG-GTAACTATTACC	2.9 kb
238	11019-L11688	Exon 6	979-980	CAGAAGTATGGA-ATACGTACAGCG	0.1 kb
292	13052-L14235	Exon 6	84 nt after exon 6	TAATTCTCCATA-TAGTTGAATTGT	1.6 kb
301	11020-L11689	Exon 7	1189-1190	TGATTCCAGTAT-TCAGTTTTCTAA	0.4 kb
402	11021-L11690	Exon 7	1585-1586	AGCTTTGTCTTT-CATTGTACCATG	0.2 kb
384	13055-L14238	Exon 7	1764-1765	AGCTTTTGGCAA-GCAATATGGAAT	
		Stop Codon	*1683-1685 (Exon 7)*		

[a] See section Exon numbering on page 1 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.

Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.

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

- P044 NF2 Contains probes for the *NF2* gene, involved in Neurofibromatosis type 2.
- P081/P082 NF1 Contains probes for the *NF1* gene, involved in Neurofibromatosis type 1.
- P122 NF1 area Contains probes for the 17q11.2 region, involved in Neurofibromatosis type 1.
- P250 DiGeorge Contains probes for the 22q11 region, used for primary screening of this region.
- P258 SMARCB1 Contains probes for the *SMARCB1* gene. Deletions and mutations in *SMARCB1* are associated with malignant rhabdoid tumours and schwannomatosis.
- P455 LZTR1 Contains probes for the *LZTR1* gene, involved in Neurofibromatosis type 3.

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 P295 SPRED1

- Evans DG et al. (2016). Comprehensive RNA analysis of the NF1 gene in classically affected NF1 affected individuals meeting NIH criteria has high sensitivity and mutation negative testing is reassuring in isolated cases with pigmentary features only. EBioMedicine. 7:212-220.
- Palma Milla C et al. (2018). Neurofibromatosis type I: mutation spectrum of NF1 in spanish patients. Ann Hum Genet.
- Pasmant E et al. (2015). Neurofibromatosis type 1 molecular diagnosis: what can NGS do for you when you have a large gene with loss of function mutations? Eur J Hum Genet. 23(5):596-601.
- Pinna V et al. (2015). p. Arg1809Cys substitution in neurofibromin is associated with a distinctive NF1 phenotype without neurofibromas. Eur J Hum Genet. 23(8):1068-1071.
- Van Minkelen R et al. (2014). A clinical and genetic overview of 18 years neurofibromatosis type 1 molecular diagnostics in the Netherlands. Clin Genet. 85(4):318-327.
- Zhang J et al. (2016). Identification of a PTPN11 hot spot mutation in a child with atypical LEOPARD syndrome. Mol Med Rep. 14(3):2639-2643.

P295 product history	
Version	*Modification*
B3	Two reference probes have been replaced.
B2	One reference probe was added.
B1	One reference probe has been removed and six have been replaced, furthermore the control fragments have been adjusted (QDX2).
A1	First release.

Implemented changes in the product description
Version B3-02 – 19 April 2022 (04P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *SPRED1* gene updated according to new version of the NM_ reference sequences. - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. *Version B3-01 – 03 October 2018 (01P)* - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). - Product description restructured and adapted to a new template. - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.

More information: www.mrcholland.com; www.mrcholland.eu	
	MRC Holland bv; Willem Schoutenstraat 1 1057 DL, Amsterdam, The Netherlands
E-mail	info@mrcholland.com (information & technical questions) order@mrcholland.com (orders)
Phone	+31 888 657 200