Product Description
SALSA® MLPA® Probemix P280-B4 SLC26A4

To be used with the MLPA General Protocol.

Version B4
As compared to version B3, eight reference probes have been replaced and one has been removed. In addition, one probe length has been adjusted. For complete product history see page 8.

Catalogue numbers:
- **P280-025R:** SALSA MLPA Probemix P280 SLC26A4, 25 reactions.
- **P280-050R:** SALSA MLPA Probemix P280 SLC26A4, 50 reactions.
- **P280-100R:** SALSA MLPA Probemix P280 SLC26A4, 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 P280 SLC26A4 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *SLC26A4* gene, which is associated with Pendred syndrome and autosomal recessive non-syndromic deafness (DFNB4) (Cirello et al. 2018). This probemix can also be used to detect the presence of three point mutations: the L236P (c.707T>C; Exon 6) amino acid substitution mutation, the IVS8+1G>A (Exon 8) donor splice mutation, and the T416P (c.1246A>C; Exon 10) amino acid substitution mutation. These three mutations have been found in several cases of Pendred syndrome (Tsukamoto et al. 2003).

Pendred syndrome is an autosomal recessive disorder that accounts for 4-10% of the cases for hereditary deafness (Tesolin et al. 2021). Besides sensorineural hearing impairment, this syndrome is characterized by the presence of goiter, and a partial defect in iodide organification, which may be associated with insufficient thyroid hormone synthesis (Royaux et al. 2000). Goiter development (diffuse thyroid enlargement) and development of hypothyroidism are variable and depend on nutritional iodide intake. Pendred syndrome is caused by biallelic mutations in the *SLC26A4* gene, which encodes pendrin, a transporter of chloride, bicarbonate and iodide. Mutations in this gene can also cause DFNB4 (Royaux et al. 2000). The *SLC26A4* gene (21 exons) spans ~57.2 kb of genomic DNA and is located on chromosome 7q22.3, ~107 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1467/.

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 *SLC26A4* exon numbering used in this P280-B4 SLC26A4 product description is the exon numbering from the NG_008489.1 sequence. The exon numbering of the NM_000441.2 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 P280-B4 SLC26A4 contains 38 MLPA probes with amplification products between 131 and 436 nucleotides (nt). This includes 24 probes for the *SLC26A4* gene, with at least one probe for each exon. Among these, three probes are specific for the L236P (707T>C; Exon 6; 382 nt) mutation, the IVS8+1G>A (Exon 8; 321 nt) mutation, and the T416P (1246A>C; Exon 10; 227 nt) mutation, which will only generate a signal when the mutation is present. In addition, 14 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 Pendred 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.

SALSA Binning DNA SD071

The SD071 Binning DNA provided with this probemix can be used for binning of all probes including the three mutation specific probes (227 nt probe 20624-L025811 for the T416P mutation, 321 nt probe 09249-L30464 for the IVS8+1G>A mutation, and 382 nt probe 09246-L13230 for the L236P mutation). SD071 Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequence detected by the above mentioned probes. Inclusion of one reaction with 5 µl SD071 Binning DNA in initial MLPA experiments is essential as it can be used to aid in data binning of the peak pattern using Coffalyser.Net software. Furthermore, Binning DNA should be included in the experiment whenever changes have been applied to the set-up of the capillary electrophoresis device (e.g. when capillaries have been renewed). Binning DNA should never be used as a reference sample in the MLPA data analysis, neither should it be used in quantification of mutation signal(s). It is strongly advised that all samples tested are extracted with the same method and derived from the same source of tissue. For further details, please consult the SD071 Binning DNA product description, available online: www.mrcholland.com. **This product is for research use only (RUO).**

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 *SLC26A4* gene are small (point) mutations, of which only three will be detected by using SALSA MLPA Probemix P280 SLC26A4.
- 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.

***SLC26A4* mutation database**

https://databases.lovd.nl/shared/genes/SLC26A4. 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 deletion of *SLC26A4* exons 4 and 6 but not exon 5) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P280-B4 SLC26A4

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*SLC26A4*
64-105	Control fragments – see table in probemix content section for more information		
131 ¥	Reference probe 00797-L25925	5q	
137	**SLC26A4 probe** 09253-L09445		**Exon 11**
142	**SLC26A4 probe** 09258-L11422		**Exon 16**
148	Reference probe 00798-L00316	13q	
154 *	Reference probe 20337-L27719	1p	
160	**SLC26A4 probe** 09248-L09438		**Exon 8**
168	**SLC26A4 probe** 09255-L09447		**Exon 13**
178	**SLC26A4 probe** 09257-L09449		**Exon 15**
184	**SLC26A4 probe** 09251-L09442		**Exon 10**
190	Reference probe 06743-L06347	8q	
195	**SLC26A4 probe** 09256-L09448		**Exon 14**
202	**SLC26A4 probe** 09241-L09431		**Exon 1**
207	Reference probe 06059-L06041	4p	
214	**SLC26A4 probe** 09259-L09451		**Exon 17**
220	**SLC26A4 probe** 09262-L11424		**Exon 20**
227 §	**SLC26A4 probe** 20624-L28511		**Exon 10**
233 *	Reference probe 19624-L26861	10p	
240	**SLC26A4 probe** 09254-L11425		**Exon 12**
247	**SLC26A4 probe** 09261-L09453		**Exon 19**
256 * §	Reference probe 14758-L16455	9q	
265	**SLC26A4 probe** 09242-L09432		**Exon 2**
288 *	Reference probe 17604-L21601	18q	
301 *	Reference probe 10783-L28289	2p	
310	**SLC26A4 probe** 09245-L09435		**Exon 5**
321 §	**SLC26A4 probe** 09249-L30464		**Exon 8**
328 *	Reference probe 16275-L22420	19p	
338	**SLC26A4 probe** 09260-L11427		**Exon 18**
347	**SLC26A4 probe** 19667-L11428		**Exon 7**
355	**SLC26A4 probe** 09243-L09433		**Exon 3**
364 *	Reference probe 10675-L11257	6p	
373	**SLC26A4 probe** 19902-L11426		**Exon 21**
382 §	**SLC26A4 probe** 09246-L13230		**Exon 6**
391	Reference probe 16566-L19057	11q	
400	**SLC26A4 probe** 09250-L09441		**Exon 9**
409	**SLC26A4 probe** 09244-L09434		**Exon 4**
419 *	Reference probe 03854-L23156	17q	
427	**SLC26A4 probe** 12286-L13231		**Exon 6**
436	Reference probe 14085-L15684	15q	

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

* New in version B4.
¥ Changed in version B4. Minor alteration, no change in sequence detected.
§ Mutation-specific probe. This probe will only generate a signal when the L236P (c.707T>C; 382 nt), IVS8+1G>A (Exon 8; 321 nt) or T416P (c.1246A>C; Exon 10; 227 nt) mutation is present. It has been tested on artificial DNA **but not on positive human samples!**

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. *SLC26A4* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*SLC26A4* exon[a]	Ligation site NM_000441.2	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*32-34 (Exon 2)*		
202	09241-L09431	Exon 1	142 nt before exon 1	GGGGTAGGATTT-CTTTCCTGATAG	1.0 kb
265	09242-L09432	Exon 2	89-90	ACAGCTGCAGCT-ACATGGTGTCGC	1.7 kb
355	09243-L09433	Exon 3	297-298	TAGTGACGTCAT-TTCGGGAGTTAG	8.8 kb
409	09244-L09434	Exon 4	376-377	CCTGTCGGATAT-GGTCTCTACTCT	2.1 kb
310	09245-L09435	Exon 5	544-545	GGAACTGTATTA-AATACTACTATG	0.7 kb
427	12286-L13231	Exon 6	679-680	AGGTACTTGGCA-GATCCTTTGGTT	0.1 kb
382 §	09246-L13230	Exon 6	738-739	GGTCTCACAGCC-AAAGATTGTCCT	8.2 kb
347	19667-L11428	Exon 7	894-895	AGTTAAGGAATT-AAATGATCGGTT	0.2 kb
160	09248-L09438	Exon 8	969-970	TGCTACTGCCAT-TTCATATGGAGC	0.1 kb
321 §	09249-L30464	Exon 8	1 nt after exon 8	ATCCCAAGGGGA-TGAGTGTGGTGT	5.6 kb
400	09250-L09441	Exon 9	1077-1078	GTTCTCGGAGAT-GCTGGCTGCATC	1.1 kb
184	09251-L09442	Exon 10	1223-1224	TCTCAGGATTCT-TCTCTTGTTTTG	0.1 kb
227 §	20624-L28511	Exon 10	1277-1278	TCCAGGAGAGCC-CTGGAGGAAAGA	4.2 kb
137	09253-L09445	Exon 11	1326-1327	TGCGATTGTGAT-GATCGCCATTCT	0.3 kb
240	09254-L11425	Exon 12	1434-1435	GCTGTGTGACAT-TCCTCGTCTGTG	1.3 kb
168	09255-L09447	Exon 13	1516-1517	CTGGGGCTGGAT-CTCGGTTTACTA	2.1 kb
195	09256-L09448	Exon 14	1586-1585 reverse	TCCAAGGCCATT-CCAAGAAGGACT	2.1 kb
178	09257-L09449	Exon 15	1665-1666	ACCTCAAGGAGT-GAAGATTCTTAG	1.1 kb
142	09258-L11422	Exon 16	1794-1795	GCTGAGGAAAAT-ACAGAAACTAAT	0.8 kb
214	09259-L09451	Exon 17	1963-1964	AACTCTGAGCTT-CCAGTCAAAGTG	2.5 kb
338	09260-L11427	Exon 18	52 nt after exon 18	CGTAAGCCCTTT-CTCCTATCTGGG	5.7 kb
247	09261-L09453	Exon 19	2200-2201	TTGACGGTCCAT-GATGCTATACTC	2.5 kb
220	09262-L11424	Exon 20	2338-2339	GAAGAACTTGAT-GTCCAGGATGAG	2.9 kb
373	19902-L11426	Exon 21	2408-2409	TGAGCAAGGAAT-ACAAGACAAAAC	
		stop codon	*2372-2374 (Exon 21)*		

[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.

§ Mutation-specific probe. This probe will only generate a signal when the L236P (c.707T>C; 382 nt), IVS8+1G>A (Exon 8; 321 nt) or T416P (c.1246A>C; Exon 10; 227 nt) mutation is present. It has been tested on artificial DNA **but not on positive human samples!**

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

- P153 EYA1 — Branchio-oto-renal dysplasia (BOR) syndrome. Contains probes for the *EYA1* gene.
- P163 GJB-WFS1-POU3F4 — Hereditary hearing loss and Wolfram Syndrome type 1. Contains probes for the *GJB2*, *GJB3*, *GJB6*, *WFS1* and *POU3F4* genes.
- P186 PAX3-MITF-SOX10 — Waardenburg Syndrome (WS). Contains probes for the *PAX3*, *MITF* and *SOX10* genes.
- P191 & P192 Alport-mix 1 & 2 — X-linked Alport syndrome (XLAS). Contains probes for the *COL4A5* gene.
- P461 DIS — Deafness-infertility syndrome (DIS), Autosomal recessive deafness 16 and 22. Contains probes for the *STRC*, *CATSPER2* and *OTOA* genes.

References

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- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
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- 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 P280 SLC26A4

- Nonose RW et al. (2018). Mutation analysis of *SLC26A4* (Pendrin) gene in a Brazilian sample of hearing-impaired subjects. *BMC Med Genet*. 19:73.
- Pique LM et al. (2014). Mutation analysis of the *SLC26A4*, *FOXI1* and *KCNJ10* genes in individuals with congenital hearing loss. *PeerJ*. 2:e384.
- Tesolin P et al. (2021). Pendred syndrome, or not Pendred syndrome? That is the question. *Genes (Basal)*. 12:1569.
- Zhao J et al. (2012). *SLC26A4* gene copy number variations in Chinese patients with non-syndromic enlarged vestibular aqueduct. *J Transl Med*. 10:82.

P280 product history	
Version	*Modification*
B4	Eight reference probes have been replaced and one has been removed. In addition, one probe length has been adjusted.
B3	Two reference probes were replaced and one probe length was adjusted.
B2	Three reference probes were replaced and one was added. Additionally, the control fragments were adjusted.
B1	One extra probe targeting the *SLC26A4* gene was included. 6 reference probes were replaced and one removed. Moreover, one SLC26A4 probe was changed from a wild-type specific probe to a mutation specific probe.
A1	First release.

Implemented changes in the product description
Version B4-01 – 30 November 2021 (04P) - 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). - Ligation sites of the probes targeting the *SLC26A4* gene 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. Version 11 – 14 December 2017 (55) - Product description adapted to a new product version (version number changed, lot number added, new pictures included). Version 10 – 06 July 2017 (55) - Sample DNA SD031 updated to SD071. - Minor textual changes

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