Product Description

SALSA® MLPA® Probemix P132-A4 Kallmann-1

To be used with the MLPA General Protocol.

Version A4

As compared to version A3, one flanking and two reference probes have been removed. Three reference probes have been replaced. For complete product history see page 7.

Catalogue numbers:

- **P132-025R:** SALSA MLPA Probemix P132 Kallmann-1, 25 reactions.
- **P132-050R:** SALSA MLPA Probemix P132 Kallmann-1, 50 reactions.
- **P132-100R:** SALSA MLPA Probemix P132 Kallmann-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 P132 Kallmann-1 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *ANOS1* (*KAL1*) gene, which is associated with Kallmann syndrome.

Kallmann syndrome is a distinct form of Isolated Gonadotropin-Releasing Hormone (GnRH) deficiency characterised by anosmia or hyposmia. *ANOS1* encodes the extracellular matrix protein, anosmin-1, that plays a key role in the migration of GnRH neurons and olfactory nerves to the hypothalamus.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1334/.

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 *ANOS1* exon numbering used in this P132 Kallmann-1 product description is the exon numbering from the NG_007088.2 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 numbe ring may not be up-to-date.

Probemix content

The SALSA MLPA Probemix P132-A4 Kallmann-1 contains 29 MLPA probes with amplification products between 136 and 400 nucleotides (nt). This includes 16 probes for the *ANOS1* gene and three flanking probes that detect locations on both sides of the *ANOS1* gene. In addition, ten reference probes are included that

detect locations on the X-chromosome. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

This probemix contains ten quality control fragments generating amplification products between 64 and 118 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and two 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)
105-118	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 of the same sex 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 Kallmann syndrome. It is recommended to use samples of the same sex to facilitate interpretation. 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:

Copy Number status: Male samples	Final ratio
Normal	0.80 < FR < 1.20
Deletion	FR = 0
Duplication	1.65 < FR < 2.25
Ambiguous copy number	All other values

Copy Number status: Female samples	Final ratio
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 *ANOS1* gene are small (point) mutations, none of which will not be detected by using SALSA MLPA Probemix P132 Kallmann-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.

***ANOS1* mutation database**

https://databases.lovd.nl/shared/genes/KAL1. 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 *ANOS1* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P132-A4 Kallmann-1

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*ANOS1*
64-118	Control fragments – see table in probemix content section for more information		
136	Reference probe 13085-L14304	Xq21	
142	Reference probe 02928-L03720	Xq27	
148	**ANOS1 probe** 04431-L03817		**Exon 14**
154 *	Reference probe 19375-L25782	Xp11	
159	**ANOS1 probe** 04416-L03802		**Exon 1**
166	**ANOS1 probe** 04423-L05579		**Exon 7**
171	Reference probe 01397-L01608	Xp21	
178	**ANOS1 probe** 04418-L03804		**Exon 2**
184	**ANOS1 probe** 04424-L03810		**Exon 8**
196	Reference probe 20680-L28732	Xq22	
202	**ANOS1 probe** 04419-L03805		**Exon 3**
211	**ANOS1 probe** 05944-L03803		**Exon 1**
220 ¬	**GPR143 probe** 02975-L02406		**Upstream**
228	**ANOS1 probe** 05941-L05940		**Exon 9**
238	**ANOS1 probe** 06402-L09795		**Exon 10**
251	**ANOS1 probe** 05942-L03806		**Exon 4**
263	**ANOS1 probe** 04427-L03813		**Exon 11**
274 *	Reference probe 19689-L26419	Xq23	
283	**ANOS1 probe** 04422-L03808		**Exon 6**
292	**ANOS1 probe** 04428-L03814		**Exon 12**
301	Reference probe 03652-L03065	Xp22	
309	**ANOS1 probe** 05943-L09794		**Exon 5**
318	**ANOS1 probe** 04429-L03815		**Exon 13**
346	**ANOS1 probe** 04430-L03816		**Exon 14**
355 *	Reference probe 12606-L13690	Xq12	
373	Reference probe 01282-L00965	Xq22	
382 ¬	**STS probe** 05192-L04573		**Downstream**
391 ¬	**PUDP probe** 05195-L04576		**Downstream**
400	Reference probe 13114-L14333	Xp11	

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

* New in version A4.
¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

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. *ANOS1* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	*ANOS1* exon[a]	Ligation site NM_000216.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
220 ¬	02975-L02406	*GPR143*		CTGCAACAAAAA-TGAGGGTGACCC	993.6 kb
		start codon	*102-104 (Exon 1)*		
159	04416-L03802	Exon 1	15 nt before exon 1, reverse	CCGGAAAGTTCA-ATCCTTCAACTC	0.3 kb
211	05944-L03803	Exon 1	271-272	CCTGAGCCTGCA-GATCACTCGCAT	32.1 kb
178	04418-L03804	Exon 2	342-341, reverse	AGAACATTGCTT-GTGATTCTGGCA	76.1 kb
202	04419-L03805	Exon 3	380-381	TGCAAGGAATCA-GGGGACCTGAGG	26.6 kb
251	05942-L03806	Exon 4	592-591, reverse	ACCCATTCGAAC-AACATTTCTTCA	9.2 kb
309	05943-L09794	Exon 5	688-689	TACAGAACTGCA-GTCTGGACAGCT	2.6 kb
283 #	04422-L03808	Exon 6	868-867, reverse	GGTACCATCGGC-TGGGTCTTATGT	14.8 kb
166	04423-L05579	Exon 7	1105-1104, reverse	TACTGCTGACCA-TCCAGCTCCAAA	2.2 kb
184	04424-L03810	Exon 8	1231-1230, reverse	CCCAGTACGTTA-TGGCTTGCAATT	14.3 kb
228	05941-L05940	Exon 9	1385-1386	CCCACTCGCCCG-CTGGAAGTCGGA	14.3 kb
238 #	06402-L09795	Exon 10	1498-1499	GTTTCCTGAAGC-GTGTGCCCACAA	2.9 kb
263 #	04427-L03813	Exon 11	1688-1689	CTTAAGGGGAAG-AGCCACAAGCCT	1.1 kb
292 #	04428-L03814	Exon 12	1809-1810	CCGGTCACTTTT-CTTGGAAGATGG	1.3 kb
318 #	04429-L03815	Exon 13	1989-1990	CATCTACTCTTT-ACCGACTGGAAG	1.5 kb
346 #	04430-L03816	Exon 14	2205-2204, reverse	TGTGCATGTCTC-GTGGCCGAAGTT	3.2 kb
148	04431-L03817	Exon 14	5418-5419	ATGATCTTTACT-GAATTTGCCCTT	1195.7 kb
		stop codon	*2142-2144 (Exon 14)*		
382 ¬	05192-L04573	*STS*		AAAAAGCCAACG-CTCCGATCATGA	228.3 kb
391 ¬	05195-L04576	*PUDP*		CTTGCAGCTCCC-GATGTCCAAAGA	

[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.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.
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

P054 FOXL2-TWIST1	Contains probes for the *GPR143* gene.
P133 Kallmann-2	Contains probes for the *FGFR1*, *GNRHR*, *KISSR1*, *GNRH1*, *NELF*, *PROK2* and *PROKR2* genes, which are associated with Kallmann syndrome.
P160 STS	Contains probes for the *STS* gene.

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 P132 Kallmann-1

- Basaran Y et al. (2013). Multiplex ligation dependent probe amplification analysis of KAL1, GNRH1, GNRHR, PROK2 and PROKR2 in male patients with idiopathic hypogonadotropic hypogonadism. *Endokrynol Pol*. 64:285-92.
- Krzymińska A et al. (2011). Large deletion in the KAL1 gene in two related patients with hypogonadotropic hypogonadism: diagnostic usefulness of cytogenetic and molecular methods. *Endokrynol Pol*. 62:224-9.
- Montenegro LR et al. (2013). Combined use of multiplex ligation-dependent probe amplification and automatic sequencing for identification of KAL1 defects in patients with Kallmann syndrome. *Fertil Steril*. 100:854-9.
- Pedersen-White JR et al. (2008). The prevalence of intragenic deletions in patients with idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Mol Hum Reprod*. 14:367-70.
- Stamou MI et al. (2017). Isolated GNRH deficiency: genotypic and phenotypic characteristics of the genetically heterogeneous Greek population. *Eur J Endocrinol*. 176: L1-L5.
- Stamou MI et al. (2019). Unilateral renal agenesis as an early marker for genetic screening in Kallmann syndrome. *Hormones*. 18:103-5.

P132 product history	
Version	*Modification*
A4	One flanking and two reference probes have been removed. Three reference probes have been replaced.
A3	Five reference probes have been replaced, and one flanking probe has been removed. The length of one reference probe has been adjusted, and the 118 nt Y control fragment has been elongated to 121 nt.
A2	The 88, 96, 100 and 105 nt control fragments (QDX2) have been included.
A1	KAL1 exon 10 probe has been replaced. One chromosome Y-specific control fragment at 118 has been added.
A1	First release.

Implemented changes in the product description
Version A4-01 – 10 February 2022 (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). - Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. *Version A3-02 — 13 March 2020 (02P)* - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *ANOS1* gene updated according to new version of the NM_ reference sequence. - Updated gene name *HDHD1* to *PUDP*. - Small changes of probe lengths in Table 1 in order to better reflect the true lengths of the amplification products. - Related probemixes updated. - Selected publications using P132 Kallmann-1 added. *Version A3-01 – 01 March 2018 (01P)* - Product description restructured and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). *Version 14 – 01 February 2017 (55)* - Gene name has been adjusted from KAL1 to ANOS1. - Various minor textual and layout changes. *Version 13 – 19 January 2017 (55)* - Warning added in Table 1, 159 nt probe 04416-L03802, 211 nt probe 05944-L03803, and 400 nt probe 04608-L08387.

Version 12 – 09 December 2015 (55)
- Product description adapted to a new lot (lot number added).
- Various textual changes on page 1, 2, 4 and 5 and minor textual changes throughout the document.
- Warning added on variable probes in Table 1 and 2.

Version 11 – 22 July 2015 (54)
- Figures based on the use of old MLPA buffer (replaced in December 2012) removed.
- Various minor textual changes throughout the document.

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