Product Description
SALSA® MS-MLPA® Probemix ME031-C1 GNAS

To be used with the MS-MLPA General Protocol.

Version C1
As compared to version B2, probemix completely revised, details are shown in complete product history see page 11.

Catalogue numbers:
- **ME031-025R:** SALSA MS-MLPA Probemix ME031 GNAS, 25 reactions.
- **ME031-050R:** SALSA MS-MLPA Probemix ME031 GNAS, 50 reactions.
- **ME031-100R:** SALSA MS-MLPA Probemix ME031 GNAS, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit, SALSA HhaI (SMR50) 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 MS-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.

This SALSA MS-MLPA probemix is intended for experienced MLPA users only! The exact link between the *GNAS* complex locus genotype and phenotype is still being investigated. Use of this ME031 GNAS probemix will not always provide you with clear-cut answers and interpretation of results can therefore be complicated. MRC Holland can only provide limited support with interpretation of results obtained with this product, and recommends thoroughly screening any available literature.

General information
The SALSA MS-MLPA Probemix ME031 GNAS is a **research use only (RUO)** assay for the detection of aberrant methylation of one or more sequences of the *GNAS* complex locus. This probemix can also be used to detect deletions/duplications in the *GNAS* complex locus and the *STX16* gene.

Genomic imprinting is the monoallelic expression of genes, dependent on the parental origin of the chromosome. It plays a role in growth and development. Imprinting disorders originate from a disturbance in this monoallelic expression by disruption or epimutation of imprinted genes (Ishida et al. 2013). Loss of methylation in the *GNAS* locus is associated with the genomic imprinting defect inactivating PTH/PTH-related protein signaling disorders subtype 3 (iPPSD3). Mutations in this locus are associated with iPPSD subtype 2 (iPPSD2) (Turan 2017).

iPPSD3, also referred to as pseudohypoparathyroidism Ib, is caused by loss of methylation (LOM) in the *GNAS* complex locus. iPPSD3 is characterised mainly by resistance to parathyroid hormone (PTH) and symptoms that include hypocalcemia, numbness, seizures, tetany, cataracts, and dental problems.

The *GNAS* locus is a complex imprinted locus on chromosome 20 that generates multiple transcripts through the use of several alternative first exons that splice into a common set of downstream exons (see Figure 1). *GNAS* itself encodes the Gsα protein, which is the α-subunit in the heterotrimeric G protein. Due to differential methylation of their promoters, most gene products originate from one parental allele. Transcripts *GNASXL*, which encodes XLαs, *GNAS A/B* (also referred to as 1A) and the antisense transcript *GNAS-AS1* (also referred

to as *NESPAS*) are transcribed from the paternal allele, while *NESP55* (also referred to as *NESP* or *GNAS* transcript variant 4) is transcribed from the maternal allele (Turan and Bastepe 2013). The most downstream promoter (*GNAS* exon 1) is not differentially methylated, which results in *GNAS* expression from both alleles in most tissues but its expression is silenced from the paternal allele in a small number of tissues (Turan and Bastepe 2013).

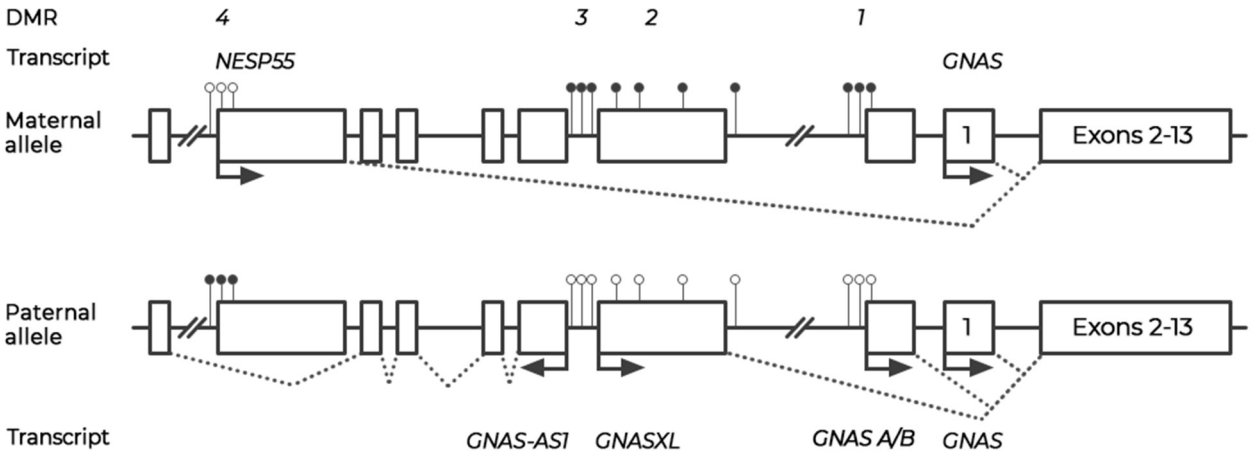

Figure 1. DMR 1, 2, 3 and 4 of *GNAS* locus. Exons are depicted as boxes. Dashed lines depict de exons that are spliced together to form the alternative transcripts. Arrows indicate the direction of transcription. The methylation status of the DMRs is indicated by filled in circles for methylated and empty circles for unmethylated regions. The numbered DMRs are as follows:
1. *GNAS A/B* transcription start site (GNAS A/B:TSS-DMR), covered by *GNAS A/B* exon 1 probes, maternally methylated.
2. *GNASXL* exon 1 (GNAS-XL:Ex1-DMR), covered by *GNASXL* exon 1 and intron 1 probes, maternally methylated.
3. *GNAS-AS1* transcription start site (GNAS-AS1:TSS-DMR), covered by *GNAS-AS1* exon 1 and upstream probes, maternally methylated.
4. *NESP55* transcription start site (GNAS-NESP:TSS-DMR), covered by *NESP55* exon 1 probes, paternally methylated.
Figure adapted from Lemos and Thakker (2015).

The Gsα and XLαs transcripts are involved in downstream signalling from parathyroid hormone (PTH), parathyroid hormone related protein (PTHrP) receptors and other hormone receptors like TSHR and GHRHR. The *GNAS A/B* transcript and the antisense transcript *GNAS-AS1* are not translated into proteins, but are thought to influence Gsα expression via mechanisms that remain to be determined. The *STX16* gene, lastly, is a long range control element of methylation at the *GNAS* locus, located more than 220 kb centromeric of *GNAS* (Turan and Bastepe 2013).

PHPIb is caused by loss-of-methylation (LOM) at *GNAS A/B* located within DMR 1 in the *GNAS* complex locus (Figure 1). LOM can also be observed at *GNAS-AS1 and GNASXL*, which can also be associated with a gain-of-methylation (GOM) at *NESP55*. The autosomal dominant form of PHPIb can be caused by maternal heterozygous deletions in *STX16* (Turan and Bastepe 2013).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK459117/

This SALSA MS-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 *GNAS-AS1* exon numbering used in this ME031-C1 GNAS product description is the exon numbering from the LRG_1051 sequence. The *GNAS* and *STX16* exon numbering used is the exon numbering from the

NG_016194.2 and NG_011831.2 sequences, respectively. The *GNASXL*, *NESP55* and *GNAS A/B* exon numbering used is the exon numbering from the RefSeq transcripts NM_080425.4, NM_016592.5 and NM_001309840.2, 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 MS-MLPA Probemix ME031-C1 GNAS contains 53 (MS-)MLPA probes with amplification products between 126 and 500 nucleotides (nt). This includes 32 probes for the *GNAS* locus and nine probes for the *STX16* gene. 15 MS-MLPA probes contain an HhaI recognition site and provide information on the methylation status of the *GNAS* complex locus. All probes present will also give information on copy number changes in the analysed sample. In addition, ten reference probes are included that are not affected by HhaI digestion and detect genes located outside the *GNAS* complex locus. Also, two digestion control probes are included in this probemix indicating whether or not restriction endonuclease digestion in the MS-MLPA reaction was complete. 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 MS-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)

MS-MLPA technique

The principles of the MS-MLPA technique (Nygren et al. 2005, Schouten et al. 2002) are described in the MS-MLPA General Protocol (www.mrcholland.com).

MS-MLPA technique validation

Internal validation of the MS-MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using MS-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.

Results of MS-MLPA are highly dependent on the HhaI enzyme used. HhaI enzymes that are resistant to heat inactivation are NOT compatible with the MS-MLPA technique and will give aberrant results. These include, but may not be limited to, Thermo Fisher Scientific enzymes HhaI, ANZA 59 HhaI, and FastDigest HhaI. We recommend using SALSA HhaI enzyme (SMR50) as this restriction enzyme has been validated for use with MS-MLPA by MRC Holland.

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 MS-MLPA General Protocol.

The results of methylation-specific MLPA probes tested on chorionic villi samples (CVS) might not reflect the actual epigenetic constitution of the foetus. This is because the locus of interest might not have reached its

final imprinting status in CVS (Paganini et al. 2015). Furthermore, Paganini et al. demonstrated that the rate at which the imprinting is set may differ between, and even within, loci. Consequently, the use of this product on CVS samples should involve examining and validating the methylation status of each individual MS-MLPA probe.

Reference samples

A sufficient number (≥3) of reference samples should be included in each MS-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. When selecting reference samples, please note that methylation patterns may vary between tissue types and even age groups! Reference samples should be derived from different unrelated individuals who are from families without a history of iPPSD. More information regarding the selection and use of reference samples can be found in the MS-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.

Sample name	Source	Chromosomal position of copy number alteration*	Altered target genes in ME031-C1	Expected copy number alteration	Expected methylation ratio
NA08123	Coriell Institute	20q12.32	*STX16, GNAS-AS1, NESP55, GNASXL, GNAS-A/B, GNAS, NELFCD*	Heterozygous duplication of the maternal allele	~0.33 for NESP55 DMR; ~0.67 for GNAS-AS1; GNASXL and GNAS A/B DMRs

* Indicated chromosomal bands accommodate genes targeted by MLPA probes, however, the whole extent of copy number alteration (CNA) present in this cell line cannot be determined by this ME031-C1 GNAS probemix.

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 copy number 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 or in or near the *GNAS* 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 MS-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.
- Digestion Control Probes. The target sequences of the digestion control probes are unmethylated in most blood-derived DNA samples. The signals of the digestion control probes should be gone upon complete digestion by HhaI.
- mRNA levels. We have no data showing that methylation detected by a particular probe indeed influences the corresponding mRNA levels.
- 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.

NOTE: In case digestion control probes are not fully digested (>0.05), please contact info@mrcholland.com for more information.

ME031 specific note(s):

- Please note that several probes have multiple HhaI restriction sites. All of these sites need to be methylated in order to not be digested!

Limitations of the procedure

- iPPSD2 is caused by small (point) mutations in the *GNAS* complex, most of which will not be detected by using SALSA MS-MLPA Probemix ME031 GNAS.
- MS-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 MS-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.
- An MS-MLPA probe targets a single specific HhaI site in a CpG island; if methylation is absent for a particular CpG-site, this does not necessarily mean that the whole CpG island is unmethylated!
- Rare cases are known in which apparent methylation as detected by an MS-MLPA probe proved to be due to a sequence change in or very near the HhaI site.
- No discrimination between uniparental disomy and imprinting defects can be made with this probemix. For this, it is necessary to perform microsatellite analysis in the patient and parents.

Confirmation of results

Confirmation of methylation status can be performed with another technique, such as MSP (methylation-specific PCR), pyrosequencing, digestion-based PCR assays, etc. 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 MS-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.

***GNAS* mutation database:** https://databases.lovd.nl/shared/genes/GNAS. 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 SNPs and unusual results (e.g., a duplication of *GNAS* exons 1 and 3 but not exon 2) to MRC-Holland: info@mlpa.com.

Table 1. SALSA MS-MLPA Probemix ME031-C1 GNAS

Length (nt)	SALSA MLPA probe	HhaI site	% methylated in normal blood-derived DNA	% expected signal reduction[a]	Chromosomal position (hg18)	
					Reference	Target
64-105	Control fragments – see table in probemix content section for more information					
126 ¥	Reference probe 18709-L21698	-			5q	
130 ¥ π	**Digestion control probe** S0750-L25113	+	0%	100%		**2q12**
137	**GNAS-AS1 probe** 18104-L23354	-				**Exon 4**
144 *	**STX16 probe** 22935-L32374	-				**Exon 2**
148 * «	**STX16 probe** 22936-L32375	-				**Exon 1**
157 ¥	Reference probe 05751-L32528	-			5p	
161 *	**GNAS probe** 22937-L32376	-				**Exon 12**
166 * Ð	**GNAS-AS1 probe** 22938-L32377	+	50%	50%		**GNAS-AS1:TSS-DMR**
172	**STX16 probe** 03872-L03320	-				**Exon 3**
177 *	**GNAS-AS1 probe** 22939-L32378	-				**Exon 5**
181 *	**STX16 probe** 22940-L32530	-				**Exon 8**

186 * « ¬	**NELFCD probe** 22941-L32380	-				**Downstream**
197 ‡	**NESP55 probe** 04870-L23097	+	50%	50%		**GNAS-NESP:TSS-DMR**
202	Reference probe 03709-L03163	-			9q	
208 *	**STX16 probe** 22942-L32381	-				**Exon 7**
215	**GNAS probe** 03886-L22604	+	100%	0%		**Exon 9**
221 * «	**GNAS probe** 22943-L32382	-				**Exon 1**
226 *	**GNAS-AS1 probe** 22944-L32531	-				**Exon 2**
232 *	**STX16 probe** 22945-L32384	-				**Exon 4**
236 ¥ Ж	**NESP55 probe** 23056-SP0007-L32537	+	50%	50%		**GNAS-NESP:TSS-DMR**
244	**STX16 probe** 18195-L24186	-				**Exon 6**
250 *	Reference probe 18211-L22195	-			8p	
256 ¥	**GNAS-AS1 probe** 23057-L22606	+	50%	50%		**GNAS-AS1:TSS-DMR**
262 *	**GNAS probe** 22946-L32538	-				**Exon 10**
268 ¥ «	**GNAS A/B probe** 23055-L05694	+	50%	50%		**GNAS A/B:TSS-DMR**
275 * «	**GNAS probe** 22947-L32536	-				**Exon 5**
283	**GNAS probe** 03887-L03335	+	100%	0%		**Exon 13**
292	Reference probe 15724-L17704	-			12q	
300	**GNASXL probe** 03879-L23095	+	50%	50%		**GNASXL:Ex1-DMR**
309 ¥ «	**GNAS probe** 05210-L32533	-				**Exon 4**
315 Ð ‡	**GNASXL probe** 15645-L22676	+	50%	50%		**GNASXL:Ex1-DMR**
320 *	**GNAS-AS1 probe** 22948-L32534	+	50%	50%		**GNAS-AS1:TSS-DMR**
330	**GNAS probe** 15648-L22678	-				**Exon 11**
341 ¥	Reference probe 02664-L32529	-			11q	
346	**STX16 probe** 03874-L03322	-				**Exon 9**
353 *	**GNAS-AS1 probe** 22949-L32592	-				**Exon 1**
364	**GNAS-AS1 probe** 18106-L22533	-				**Exon 3**
373	**GNAS probe** 04465-L01523	-				**Exon 6**
382 π	**Digestion control probe** 18124-L23096	+	0%	100%		**3p14**
391 *	Reference probe 18069-L22459	-			16q	
402 « Ð ‡	**GNAS A/B probe** 03882-L22603	+	50%	50%		**GNAS A/B:TSS-DMR**
411 *	**NESP55 probe** 22950-L32389	+	50%	50%		**GNAS-NESP:TSS-DMR**
418 *	**STX16 probe** 22951-L32390	-				**Exon 5**
427 *	**GNAS probe** 22952-L32391	-				**Exon 8**
432 *	Reference probe 21891-L12107	-			4q	
438 «	**GNAS probe** 06193-L22608	-				**Exon 3**
447 * «	**GNAS A/B probe** 22953-L32535	+	50%	50%		**GNAS A/B:TSS-DMR**
454 *	**GNAS probe** 22954-L32393	-				**Exon 7**
462	**GNASXL probe** 07523-L17516	+	50%	50%		**GNASXL:Ex1-DMR**
468	**GNASXL probe** 21010-L24075	+	50%	50%		**GNASXL:Ex1-DMR**
481 *	Reference probe 08586-L13087	-			17q	
490 « Ж	**GNAS probe** 18168-SP0618-L22702	-				**Exon 2**
500	Reference probe 09682-L22509	-			3p	

* New in version C1.

¥ Changed in version C1. Minor alteration, no change in sequence detected.

« 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.

π Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.

Ð This probe is sensitive to overdigestion, which can occur for example in case of lower ligation-digestion temperature (<48˚C) or when the Hha1 concentration is too high.

Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.

¬ 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 contains two GCGC motifs for HhaI. Loss of probe peak signal (indicative of absence of methylation) will occur not only if both sites are unmethylated but also if one of the sites is unmethylated and the other methylated.

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. ME031-C1 target probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene/Exon/DMR[a]	HhaI site	NM_ sequence/Ligation site	Imprinted allele	Distance to next probe
		STX16		**NM_001001433.3**		
		Start codon		*725-727 (exon 1)*		
148	22936-L32375	Exon 1	-	761-762		7.6 kb
144	22935-L32374	Exon 2	-	6 nt before exon 2		7.9 kb
172	03872-L03320	Exon 3	-	912-913		0.5 kb
232	22945-L32384	Exon 4	-	1011-1012		1.4 kb
418	22951-L32390	Exon 5	-	3 nt after exon 5		1.2 kb
244	18195-L24186	Exon 6	-	19 nt after exon 6		0.6 kb
208	22942-L32381	Exon 7	-	1398-1399		2.5 kb
181	22940-L32530	Exon 8	-	1538-1539		3.8 kb
346	03874-L03322	Exon 9	-	2837-2838		141.6 kb
		Stop codon		*1700-1702 (exon 9)*		
		GNAS-AS1		**NR_002785.2**		
177	22939-L32378	Exon 5	-	1036-1037		20.7 kb
		NESP55		**NM_016592.5**		
		Start codon		*360-362 (exon 1)*		
197 ‡	04870-L23097	Exon 1 / GNAS-NESP:TSS-DMR	+	34 nt before exon 1	Paternal	0.2 kb
236 Ж	23056-SP0007-L32537	Exon 1 / GNAS-NESP:TSS-DMR	+	150-151, 179-180	Paternal	0.2 kb
411	22950-L32389	Exon 1 / GNAS-NESP:TSS-DMR	+	395-396	Paternal	2.0 kb
		Stop codon		*1095-1097 (exon 1)*		
		GNAS-AS1		**NR_002785.2**		
137	18104-L23354	Exon 4	-	571-572		0.3 kb
364	18106-L22533	Exon 3	-	507-508		6.4 kb
226	22944-L32531	Exon 2	-	406-407		1.8 kb
353	22949-L32592	Exon 1	-	230-231		0.3 kb
256	23057-L22606	Exon 1 / GNAS-AS1:TSS-DMR	+	86 nt before exon 1	Maternal	0.4 kb
166 Ð	22938-L32377	Exon 1 / GNAS-AS1:TSS-DMR	+	451 nt before exon 1 reverse	Maternal	1.1 kb
320	22948-L32534	Upstream / GNAS-AS1:TSS-DMR	+	1.5 kb before exon 1	Maternal	1.8 kb
		GNASXL		**NM_080425.4**		
		Start codon		*551-553 (exon 1)*		
300	03879-L23095	Exon 1 / GNASXL:Ex1-DMR	+	1482-1483	Maternal	0.9 kb
462	07523-L17516	Exon 1 / GNASXL:Ex1-DMR	+	2370-2371	Maternal	0.1 kb
315 Ð ‡	15645-L22676	Exon 1 / GNASXL:Ex1-DMR	+	2453-2454	Maternal	0.8 kb
468	21010-L24075	Intr.1 / GNASXL:Ex1-DMR	+	609 nt after exon 1 reverse	Maternal	32.9 kb
		Stop codon		*3662-3664 (exon 13)*		
		GNAS A/B		**NM_001309840.2**		
		Start codon		*213-215 (exon 2)*		
447 «	22953-L32535	Exon 1 / GNAS A/B:TSS-DMR	+	318 nt before exon 1	Maternal	0.2 kb
268 «	23055-L05694	Exon 1 / GNAS A/B:TSS-DMR	+	109 nt before exon 1	Maternal	0.3 kb

402 « Ð ‡	03882-L22603	Exon 1 / GNAS A/B:TSS-DMR	+	155-156	Maternal	2.5 kb
		Stop codon		*1218-1220 (exon 13)*		
		GNAS		**NM_001077488.5**		
		Start codon		*307-309 (exon 1)*		
221 «	22943-L32382	Exon 1	-	408-407 reverse		3.8 kb
490 « Ж	18168-SP0618-L22702	Exon 2	-	501-500; 473-472 reverse		3.4 kb
438 «	06193-L22608	Exon 3	-	547-548		4.6 kb
309 «	05210-L32533	Exon 4	-	584-585		0.2 kb
275 «	22947-L32536	Exon 5	-	732-733		1.6 kb
373	04465-L01523	Exon 6	-	777-778		3.8 kb
454	22954-L32393	Exon 7	-	883-884		0.2 kb
427	22952-L32391	Exon 8	-	940-941		0.2 kb
215	03886-L22604	Exon 9	+	998-999		0.2 kb
262	22946-L32538	Exon 10	-	1057-1058		0.3 kb
330	15648-L22678	Exon 11	-	1231-1232		0.4 kb
161	22937-L32376	Exon 12	-	5 nt after exon 12		0.3 kb
283	03887-L03335	Exon 13	+	1370-1371		75.5 kb
		Stop codon		*1492-1494 (exon 13)*		
186 « ¬	22941-L32380	*NELFCD* gene	-			

[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.
π Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.
Ð This probe is sensitive to overdigestion, which can occur for example in case of lower ligation-digestion temperature (<48˚C) or when the Hha1 concentration is too high.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time. When this occurs in reference samples, it can look like an increased signal in the test samples.
¬ 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 contains two GCGC motifs for HhaI. Loss of probe peak signal (indicative of absence of methylation) will occur not only if both sites are unmethylated but also if one of the sites is unmethylated and the other methylated.

Table 3. Sequences detected by the ME031-C1 probes

Length (nt)	SALSA MS-MLPA probe	Partial sequence with HhaI site
130	S0750-L25113	ATGCTGTGGTAGGGACACTTTGGA-ATTCCATTGGCATTGGGGTGTCTTTGTTAAGTGCCTGGCCAGTAGCGCTGTC
166	22938-L32377	TGGTTCGCCCGCCTGCAGCTCTTA-TACAGCAGCCCGTGCCGTGCGCCCTGCT
197	04870-L23097	CGGCAGAAGTCCGGGCGCGCAACT-TCGCAGAACCTCACTGCCCGTCCC
215	03886-L22604	GACGTGGGTGGCCAGCGCGATGA-ACGCCGCAAGTGGATCCAGTGCTT
236	23056-SP0007-L32537	CCCTTCTTCTTGCTCAGAGAGGCA-AGCAAGGCGCGGAGCTTTAGAAAGTTCTT-AAGTGGTCAGGAAGGTAGGTGCTT
256	23057-L22606	CTCCGACCCCAGCGCCGGGTCAGCCA-TTGGGCAGGGGTCATGCCAATCAAGGCT
268	23055-L05694	GCGTCCCCGGTGGGCCGATTTT-TCGCGCTTCCCCTTCGGTTTATAG
283	03887-L03335	ATCAGCACTGCCAGTGGAGA-TGGGCGTCACTACTGCTACCCTCATTTCACCTGCGCTGTG
300	03879-L23095	CTCCGGACCCCCGTTCGAGAT-TGGCAGCGCCCCGCTGGGGTCGA
315	15645-L22676	AGCCCAAAGCCTCGCGCTCTCTCA-AGGTCAAGAAGGTACCCCTGGCGGAGAAGCGCAGA
320	22948-L32534	AGGGGCGCAGGCACGCGTAGGGATGCGAC-AGGTGGTCGTGAGCCCACCAAAGA
382	18124-L23096	AGGGGCGCGGGTCTGGGTTTCCACGC-GCGTCAGGTCATCACCCCGGAGCC

402	03882-L22603	CCGGCAGGCGCTGCCTTGCGTGT-GAGTGCACCTCACTCACATGTAAGTCGGGGAGCGC
411	22950-L32389	TCCCGGGCTCAGCAGTGGCGCCGA-GCTCGCCATAATTACAACGACCTG
447	22953-L32535	GGGTAAGCGCCGCCGTCGCACACT-TTGCACGCCGCCCAGGGCTACACC
462	07523-L17516	CCGCCGAAAGCCCCAGCGCAACTT-ACTCCGCAACTTTCTCGTGCAAGCCTTC
468	21010-L24075	CACCAGAGTGTGCGAACAGCGAAAGTTT-TTTGCCCGGGCCATGGCGCAAACCACAA

The HhaI sites are marked with grey. Ligation sites are marked with –. Complete probe sequences are available at www.mrcholland.com. Please notify us of any mistakes: info@mrcholland.com.

Note: Please be aware that several probes have multiple HhaI restriction sites. All of these sites need to be methylated in order to not be digested.

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

- ME028 PWS: Probes for the 15q11 imprinted region implicated in Prader-Willi and Angelman syndromes.
- ME030 BWS/RSS: Probes for the 11p15 imprinted region implicated in Beckwith-Wiedemann & Russel-Silver syndromes.
- ME032 UPD7-UPD14: Probes for the 6q24, 7p12.1, 7q32.2 and 14q32.2 imprinted regions.
- ME033 TNDM: Probes for the 6q24 imprinted region implicated in transient neonatal diabetes mellitus.
- ME034 Multi-locus Imprinting: Probes for 11 different imprinted locations in seven different chromosomal regions.

References

- Ishida M et al. (2013). The role of imprinted genes in humans. *Mol Aspects Med*. 34:826-840.
- Lemos MC, Thakker RV. (2015). *GNAS* mutations in Pseudohypoparathyroidism type 1a and related disorders. *Hum Mutat*. 36:11-19.
- Mantovani G et al. (2006). Mutations in the Gs alpha gene causing hormone resistance. *Best Pract Res Clin Endocrinol Metab.* 20:501–513.
- Nygren AO et al. (2005). Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Res*. 33:e128.
- Paganini L et al. (2015). Beckwith-Wiedemann syndrome prenatal diagnosis by methylation analysis in chorionic villi. *Epigenetics*. 10:643-649.
- 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.
- Turan S, Bastepe M. (2013). The *GNAS* complex locus and human diseases associated with loss-of-function mutations or epimutations within this imprinted gene. *Horm Res Paediatr*. 80:229-241.
- Turan S. (2017). Current Nomenclature of Pseudohypoparathyroidism: Inactivating Parathyroid Hormone/Parathyroid Hormone-Related Protein Signaling Disorder. *J Clin Res Pediatr Endocrinol*. 30:58-68.
- 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 MS-MLPA Probemix ME031 GNAS

- Bakker B et al. (2015). A girl with Beckwith-Wiedemann syndrome and pseudohypoparathyroidism Type 1B due to multiple imprinting defects. *J Clin Endocr Metab.* 100:3963-3966.
- Brancatella A et al. (2020). A severe inactivating PTH/PTHrP signaling disorder type 2 in a patient carrying a novel large deletion of the GNAS gene: a case report and review of the literature. *Endocrine*. 67:466-472.

- Colson C et al. (2019). High frequency of paternal iso or heterodisomy at chromosome 20 associated with sporadic pseudohypoparathyroidism 1B. *Bone.* 123:145-152.
- Faias S et al. (2020). Methylation changes at the GNAS imprinted locus in pancreatic cystic neoplasms are important for the diagnosis of malignant cysts. *World J Gastrointest Oncol*. 12:1056-1064.
- Garin I et al. (2015). European guidance for the molecular diagnosis of pseudohypoparathyroidism not caused by point genetic variants at GNAS: an EQA study. *Eur J Hum Genet.* 23:438-444.
- Jin HY et al. (2011). Clinical characterization and identification of two novel mutations of the GNAS gene in patients with pseudohypoparathyroidism and pseudopseudohypoparathyroidism. *Clin Endocrinol (Oxf).* 75:207-13.
- de Lange IM et al. (2016). Macrosomia, obesity, and macrocephaly as first clinical presentation of PHP1b caused by STX16 deletion. *Am J Med Genet Part A*. 170:2431-2435.
- Takatani R et al. (2016). Analysis of Multiple Families With Single Individuals Affected by Pseudohypoparathyroidism Type Ib (PHP1B) Reveals Only One Novel Maternally Inherited GNAS Deletion. *J Bone Miner Res.* 31:796-805
- Yuno A et al. (2013). Genetic and epigenetic states of the GNAS complex in pseudohypoparathyroidism type Ib using methylation-specific multiplex ligation-dependent probe amplification assay. *Eur J Endocrinol*. 168:169-75.

ME031 product history	
Version	*Modification*
C1	Eight target probes were replaced, two target probes were removed and 11 target probes were added; one flanking probe was replaced; four reference probes were replaced and one was removed; four target probes, one digestion control probe and three reference probes were changed in length, not in sequence detected.
B2	One reference probe has been removed and one probe has been adjusted in length, not in sequence detected.
B1	Completely redesigned product.
A2	Eight probes have a small change in length but no change in sequence detected. In addition, the 88 and 96 nt DNA Denaturation control fragments have been replaced and 100 nt/105 nt control fragments have been added (QDX2).
A1	First release.

Implemented changes in the product description
Version C1-01 – 19 March 2021 (04M) - 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). - Change gene names *NESPAS* and *GNAS* exon 1A into *GNAS-AS1* and *GNAS A/B*, respectively. - Updated Figure 1. Version B2-01 — 01 September 2020 (02M) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the *GNAS*, *STX16* and *TH1L* gene and *NESP55* and *GNASXL* transcripts updated according to new version of the NM_ reference sequences. - Warning added to Table 1 and 2 for SNP rs192776054 in the GCGC site of the 166 nt probe. Version 19 – 07 June 2017 (16) - Notification regarding the methylation status of CVS samples added under the Methylation-specific MLPA section on page 3. - Adapted Figure 1 inserted. - Double information about HhaI enzymes removed on page 3. Version 18 – 05 December 2016 (15)

- Warning regarding HhaI enzymes that are resistant to heat inactivation added under Methylation-specific MLPA section.

Version 17 – 17 November 2016 (14)
- Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new pictures included).
- New references added on page 2.
- Exon numbering of the *GNAS* gene has been changed in Table 1 and Table 2.
- Ligation sites of the probes targeting *NESP55* and *GNAS* updated according to new versions 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.

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