Product Description
SALSA® MLPA® Probemix ME028-D1 Prader-Willi/Angelman

To be used with the MS-MLPA General Protocol.

Version D1
As compared to version C1, two methylation-specific SNRPN probes have been added. One digestion control and one reference probe have been replaced. Two probes have been adjusted in length, not in sequence detected. For complete product history see page 13.

Catalogue numbers:
- **ME028-025R:** SALSA MLPA Probemix ME028 Prader-Willi/Angelman, 25 reactions.
- **ME028-050R:** SALSA MLPA Probemix ME028 Prader-Willi/Angelman, 50 reactions.
- **ME028-100R:** SALSA MLPA Probemix ME028 Prader-Willi/Angelman, 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.

In several No DNA reactions performed on this probemix, MRC Holland has observed a series of non-specific peaks. These peaks appeared very sporadically and we have not yet been able to establish the cause. However, we found that the amount and height of these peaks is greatly reduced by not spinning down your MLPA reactions in between the ligation and PCR reaction. The non-specific peaks are not expected to influence results. Please notify us if you still regularly observe these peaks: info@mrcholland.com.

Intended purpose
The SALSA MLPA Probemix ME028 Prader-Willi/Angelman is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of copy number variations and methylation status of the 15q11 region in genomic DNA isolated from human peripheral whole blood specimens, or in case of extracted DNA from prenatal samples, from (1) (un)cultured amniotic fluid obtained in week 16 of the pregnancy or later and free from blood contamination, (2) (un)cultured chorionic villi free from maternal contamination (copy number only), (3) fetal blood. ME028 Prader-Willi/Angelman is intended to confirm a potential cause for and clinical diagnosis of Prader-Willi syndrome (PWS), Angelman syndrome (AS), and 15q11 duplication syndrome. In rare cases, this product can also be used for carrier testing of at-risk family members.

Methylation changes and copy number variations (CNVs) found with multiple consecutive probes detected with ME028 Prader-Willi/Angelman should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. In the majority of patients, defects in the 15q11 region are copy number changes, but point mutations can occur which will not be detected by MLPA. For AS, it is therefore recommended to use this SALSA MLPA probemix in combination with sequence analysis of the *UBE3A* gene.

Assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, parental evaluation,

clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

This device is not intended to be used for standalone diagnostic purposes, pre-implantation testing, population screening, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1] Please note that this probemix is for in vitro diagnostic (IVD) use in the countries specified at the end of this product description. In all other countries, the product is for research use only (RUO).

[2] To be used in combination with a SALSA MLPA Reagent Kit, Coffalyser.Net analysis software and SALSA HhaI.

Clinical background

Genomic imprinting is the monoallelic expression of genes, dependent on the parental origin of the chromosome. It plays a role in growth and development. Differentially methylated regions (DMRs) act as imprinting control regions to regulate the expression of imprinted genes. Imprinting disorders like Prader-Willi syndrome (PWS) and Angelman syndrome (AS) originate from a disturbance in this monoallelic expression by disruption or epimutation of imprinted genes (Ishida et al. 2013).

PWS and AS are distinct neurogenetic disorders, both usually caused by chromosomal deletions on chromosome 15q11 or by uniparental disomy (UPD). In UPD, both copies of a chromosome are inherited from a single parent. These 15q11 chromosomal alterations result in an aberrant expression profile of gene loci that are subject to imprinting. Absence of a paternal allele of chromosome 15q11, due to a chromosomal deletion of (part of) the paternal allele or the presence of two imprinted copies due to maternal UPD, results in PWS. The absence of the maternal copy of the same region or paternal UPD causes AS. Table 4 contains an overview of the expected copy number changes and methylation profiles in PWS/AS patients with deletions or aberrant methylation. Rare disorders with similar clinical features as PWS but different genetic etiology have been identified and are often referred to as PWS-like disorders. One of these PWS-like syndromes is caused by *MAGEL2* truncating mutations and was renamed from PWS-like syndrome to Schaaf-Yang syndrome (OMIM #615547).

Paternally expressed genes in the 15q11 PWS/AS region are *MKRN3*, *MAGEL2*, *NDN*, *SNRPN* and the snoRNA cluster; while *UBE3A* is maternally expressed. The PWS-AS Imprinting Centre (IC) (located upstream of the *SNURF-SNRPN* gene) contains both the PWS-SRO (smallest region of deletion overlap) and the AS-SRO. The AS-SRO is required for the PWS/AS region to have the maternal pattern of epigenetic modification and gene expression only if the chromosome has an intact PWS-SRO. The PWS-SRO is a 4.1 kb region that includes the *SNRPN* promoter. The PWS-SRO is unconditionally required for the PWS/AS region to have the paternal pattern of epigenetic modification and gene expression.

Finally, a rare cause of PWS is a small deletion within the SNORD116 cluster, downstream of *SNRPN* (Sahoo et al. 2008). However, this deletion is only relevant when it is absent in parental samples. Additional probes for the 15q11 region are present in the SALSA MLPA Probemix P343 Autism-1 and the SALSA MLPA Probemix P336 UBE3A, please note that these are research use only (RUO) assays.

Additionally, maternal duplications of the PWS/AS critical region on 15q11.2-q13.1 cause the 15q11 duplication syndrome characterized by developmental delay, intellectual disability, hypotonia, and seizures. The extra copy is most commonly a maternal isodicentric 15q11.2-q13.1 supernumerary chromosome (80% of cases) or a maternal interstitial 15q11.2-q13.1 duplication (20% of cases).

The database of genomic variants mentions that copy number changes in the breakpoint region BP1-BP2 (*NIPA1* and *TUBGCP5*) and u1B-u1B* (*SNRPN* exons 1 and 2) region have been found in healthy individuals (see http://dgv.tcag.ca/dgv/app/home). According to Stefansson et al. (2008), a deletion of the BP1-BP2 region is present in 0.19% of normal individuals and in 0.55% of schizophrenia patients. More probes for this BP1-BP2 region can be found in the SALSA MLPA Probemix P211 HSP region (RUO).

More information is available at:
Update of the EMQN/ACGS best practice guidelines for molecular analysis of Prader-Willi and Angelman syndromes | European Journal of Human Genetics (nature.com)

https://www.ncbi.nlm.nih.gov/books/NBK1330/ (PWS),
https://www.ncbi.nlm.nih.gov/books/NBK1144/ (AS), and
https://www.ncbi.nlm.nih.gov/books/NBK367946/ (15q11 Duplication Syndrome).

Gene structure and exon numbering
The exon numbering used in this ME028-D1 Prader-Willi/Angelman product description is the exon numbering from the LRG sequences referred to below, which are available at www.lrg-sequence.org. When no LRG sequence is available for a gene the exon numbering from the GenBank sequences referred to below is used, which are available at www.ncbi.nlm.nih.gov.

The *MAGEL2* gene spans ~2.5 kilobases (kb) on chromosome 15q11.2 and contains 1 exon. The *MAGEL2* LRG_1046 is identical to GenBank NG_016776.1. *MAGEL2* is a paternally expressed imprinted gene.

The *SNRPN* gene (NG_012958.1) spans ~155 kb on chromosome 15q11.2 and contains 13 exons. *SNRPN* is a paternally expressed imprinted gene.

The *UBE3A* gene spans ~109 kb on chromosome 15q11.2 and contains 10 exons. The *UBE3A* LRG_15 is identical to GenBank NG_009268.1. *UBE3A* is a maternally expressed imprinted gene.

The *ATP10A* gene (NG_009282.1) spans ~191 kb on chromosome 15q12 and contains 21 exons. *ATP10A* is a maternally expressed imprinted gene.

The *GABRB3* gene (NG_012836.1) spans ~237 kb on chromosome 15q12 and contains 9 exons.

The *OCA2* gene (NG_009846.1) spans ~351 kb on chromosome 15q12-q13.1 and contains 24 exons.

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 LRG/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 ME028-D1 Prader-Willi/Angelman contains 49 (MS-)MLPA probes with amplification products between 129 and 481 nucleotides (nt). 36 probes are target probes of which eight contain a HhaI recognition site and provide information on the methylation status of the 15q11 chromosomal region. All probes present will also give information on copy number changes in the analysed sample. In addition, 11 reference probes are included that are not affected by HhaI digestion and detect genes located outside the 15q11 region. 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. SALSA HhaI enzyme (SMR50) must be used in combination with this probemix as this restriction enzyme has been validated for use with MS-MLPA by MRC Holland.

Required specimens
Extracted DNA from human peripheral whole blood specimens, or in case of extracted DNA from prenatal samples, from (1) (un)cultured amniotic fluid obtained in week 16 of the pregnancy or later and free from blood contamination, (2) (un)cultured chorionic villi free from maternal contamination (copy number only), (3) fetal blood, 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 fetus. 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. Therefore, only copy number can be determined with ME028 when used on DNA extracted from CVS.

Reference samples
A sufficient number (≥3) of reference samples should be included in each MS-MLPA experiment for data normalisation and to identify the baseline methylation level for each methylation-specific probe. 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! Reference samples should be derived from different unrelated individuals who are from families without a history of PWS, AS, or 15q11 duplication syndrome. 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 ID Coriell biobank	Genotype	Probes affected by CNV	Expected FR	Expected Methylation (MAGEL2, SNRPN probes)
NA13556	Female sample: Heterozygous deletion of a small *SNRPN* region affecting 6 probes; Prader-Willi syndrome.	20694-L28518 to 20687-L31784	0.5	≥ 0.85
NA21887	Female sample: Large heterozygous deletion that includes the *OCA2* gene and the *TUBGCP5*/*NIPA1* region; Angelman syndrome.	20702-L29063 to 20698-L28526	0.5	< 0.05
NA20375	Male sample: Large heterozygous deletion that includes the *OCA2* gene but not the *TUBGCP5*/*NIPA1* region; Angelman syndrome.	20688-L28510 to 20698-L28526	0.5	< 0.05
NA20408	Female sample: Uniparental disomy (no copy number changes); Prader-Willi syndrome.	-	1.0	≥ 0.85
NA13554	Male sample: Heterozygous maternally inherited deletion of *SNRPN* exon alpha (also named exon u5, or exon 4 in NM_022807.3) affecting 6 probes; asymptomatic, healthy individual.	20694-L28518 to 20687-L31784	0.5	< 0.05
NA22397	Male sample: Maternal duplication of 15q11.	20688-L28510 to 20698-L28526 to	>1.3	~ 0.70

The NIBSC Institute in the U.K. provides a panel of WHO certified genomic DNA samples for PWS/AS (Prader Willi & Angelman Syndromes, Human gDNA (1st International Genetic Reference Panel) (nibsc.org)). This panel has been described by Boyle et al. (2011).

Performance characteristics

For PWS, MS-MLPA can detect deletions, UPD, and ID (imprinting defect) and will detect >99% of PWS patients (GeneReviews). For AS, MS-MLPA can detect deletions, UPD, and ID and will detect more than 75% of AS patients. For the remaining AS patients, whose methylation profiles are normal, approximately half carry a point mutation in *UBE3A*, while the molecular basis for the remaining patients is undetermined (Clinical utility gene card for Angelman Syndrome). MS-MLPA can distinguish deletions from disomy, but cannot distinguish between UPD and imprinting defects. Further testing by microsatellite analysis is therefore needed for those cases. For 15q11 duplication syndrome, MS-MLPA can detect >99% of cases. The analytical sensitivity and specificity for the use of ME028 is very high and can be considered >99% (based on a 2005-2022 literature review and external validation).

Analytical performance can be compromised by: SNVs or other polymorphisms in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MS-MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

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 expected results for MLPA probes in the PWS/AS region are allele copy numbers of 2 (normal), 1 (heterozygous deletion), 3 (heterozygous duplication), and 4 (heterozygous triplication). Allele copy numbers of 0 (homozygous deletion) and 4 (homozygous duplication) are very rare. Please see Table 4 for examples of potential 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.

The DNA sequences that are detected by the seven MAGEL2/SNRPN methylation-specific probes are imprinted and expected to be maternally methylated in healthy individuals; fully methylated in Prader-Willi patients and unmethylated in Angelman syndrome patients.

Methylation status	Final ratio (FR)
Fully methylated	FR ≥ 0.85
Unmethylated	FR < 0.05
50% methylated / imprinted	0.40 ≤ FR ≤ 0.65
Ambiguous	All other values

Aberrant methylation of the 15q11 locus: Aberrant methylation of the 15q11 locus can be detected by the seven methylation-specific MLPA probes detecting sequences in the *SNRPN* and *MAGEL2* genes, when compared to results obtained on DNA samples from healthy individuals.

***SNRPN* locus:** The six methylation-specific probes targeting the *SNRPN* gene are located very close to each other. It is expected that all six probes provide similar results. We recommend using the median methylation status of these *SNRPN* probes to determine the methylation status of the *SNRPN* locus and to disregard aberrant methylation detected by a single *SNRPN* MS-MLPA probe.

***MAGEL2* locus:** Imprinting in the 15q11 region outside the *SNRPN* CpG island appears to be not completely established in CVS and fetal cells obtained from amniocytes. Although the MAGEL2 probe at 232 nt behaves more similar to the *SNRPN* CpG island as compared to the NDN probe that was present in ME028 version B, abnormal methylation values for *MAGEL2* are regularly obtained on fetal DNA. **In case of use on DNA derived from fetal tissue it is therefore recommended to disregard the methylation status of the *MAGEL2* locus.**

Please note that these above mentioned final ratios are affected in mosaic samples and fetal samples of dizygotic twin pregnancies. For 15q11 duplication syndrome patients, a duplication in copy number should first be verified before methylation status is assessed. The methylation status in 15q11 duplication patients will not follow the above table. If a duplication is maternally inherited the ratios of imprinted methylation probes are expected to be ~0.7, and if the duplication is paternally inherited the ratios of imprinted methylation probes are expected to be ~0.3.

- 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 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. HhaI-digestion can be considered sufficient when <5% of the signal remains in the digested reaction compared to the undigested reaction.
- mRNA levels: We have no data showing that methylation detected by a particular probe influences the corresponding mRNA levels.
- Copy number changes detected by reference 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.

ME028 specific note:

- This probemix contains one methylation-specific probe in *UBE3A* at 184 nt that is located in an CpG island which is always unmethylated.

Please note that the great majority of Prader-Willi and Angelman samples will show a deletion of 29-35 probes, AND/OR a methylation change of six SNRPN and one MAGEL2 probe. Abnormal copy numbers or methylation changes detected by only one or two probes should be treated with caution.

Limitations of the procedure

- 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.

- For use on (un)cultured amniocytes, contamination of the sample with maternal DNA may lead to wrong conclusions.
- For use on (un)cultured chorionic villi, discrepancies in chromosomal patterns between DNA from chorionic villi and fetus have been described due to maternal contamination, postzygotic nondisjunction, postzygotic isochromosome formation, mosaic situations, and complications in DNA sampling in twin pregnancies (Van den Berg et al. 2006).

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.

LOVD mutation databases

https://www.lovd.nl/. We strongly encourage users to deposit positive results in the LOVD Database. 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 *UBE3A* exons 1 and 3 but not exon 2) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix ME028-D1 Prader-Willi/Angelman

Length (nt)	SALSA MLPA probe	HhaI site	% methylated in normal blood-derived DNA	% expected signal reduction[a]	Chromosomal position (hg18)			
					Reference	*UBE3A*	*SNRPN* ◊	Other 15q
64-105	Control fragments – see table in probemix content section for more information							
129	Reference 18709-L26847				5q			
137	**OCA2** 20700-L28528							15q13.1
142 ¥ Ð	**SNRPN** 20687-L31784	+	50%	50%			SNURF:TSS-DMR	
148	Reference 08372-L08226				17q			
154	**TUBGCP5** 02018-L00865						BP1-BP2 region	
160	**UBE3A** 04620-L00863					Exon 4		
166	Reference 08020-L07801				11q			
172	**MKRN3** 20688-L28510							15q11.2
178 ±	**SNRPN** 04106-L13905	+	50%	50%			SNURF:TSS-DMR	
184 «	**UBE3A** 19804-L28512	+	0%	100%		Exon 1		
190 ±	**SNRPN** 04104-L04294	+	50%	50%			SNURF:TSS-DMR	
195	**UBE3A** 20689-L28513					Exon 3		
202 ¬ ๑	**APBA2** 01314-L00867							15q13.1
208	Reference 07404-L07051				12q			
214	**SNRPN** 12719-L28514					SNORD116 snoRNA cluster		
220	**GABRB3** 01315-L00868							15q12
226 «	**ATP10A** 20691-L28515							15q12
232 «	**MAGEL2** 20701-L28529	+	50%	50%				15q11.2 MAGEL2:TSS-DMR
239	**SNRPN** 20692-L15415						Exon u1B*	
244	Reference 08051-L07832				5p			
250	**SNRPN** 11181-L13997	+	50%	50%			SNURF:TSS-DMR	
256	**SNRPN** 20694-L28518						Exon u5	
264 *	Reference 07630-L17091				10q			
270	**SNRPN** 12182-L28519						Intron u2	
278	**SNRPN** 12179-L13382						Intron u2	
288	**SNRPN** 15261-L16736						Exon u1B	
294	**SNRPN** 01318-L13088						SNURF-SNRPN	
301	**UBE3A** 12082-L28520					Exon 5		
310	Reference 14480-L16200				4q			
317	**OCA2** 20698-L28526							15q13.1
326	**SNRPN** 20697-L28525					SNORD116 snoRNA cluster		
333 *	**SNRPN** 22586-L31906	+	50%	50%			SNURF:TSS-DMR	
342 ¥ π	Digestion control probe 20703-L31907	+	0%	100%	2q			
347	Reference 18474-L29176				6q			
355	**UBE3A** 02034-L12925					Exon 10		
364	**ATP10A** 20695-L28523							
373	**UBE3A** 10878-L11548					Exon 2		
382 ๑	**GABRB3** 10874-L11544							15q12
391	**SNRPN** 12477-L13519						Exon u5	
400	Reference 13588-L24039				1q			
409	**SNRPN** 11177-L28521						SNURF-SNRPN	
418 «	**MAGEL2** 11155-L29062							15q11.2
427 «	**NDN** 04026-L29645							15q11.2
436	**NIPA1** 20702-L29063						BP1-BP2 region	
443 *	**SNRPN** 22587-L31786	+	50%	50%			SNURF:TSS-DMR	
452	Reference 19636-L26295				10p			
461 * π	Digestion control probe 22386-L09311	+	0%	100%	8p			
472	**SNRPN** 12721-L13796					SNORD116 snoRNA cluster		
481	Reference 19033-L24843				8p			

[a] Expected signal reduction on blood DNA derived samples. On other tissue samples these percentages can be different.

* New in version D1.
¥ Changed in version D1. Minor alteration, no change in sequence detected.

◊ Nomenclature according to Beygo et al. (2019) EMQN/ACGS best practice guidelines and Monk et al. (2018) Recommendations for a nomenclature system for reporting methylation aberrations in imprinted domains.
± SNP rs541877352 could influence the 178 nt probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe. The target sequence of the 190 nt probe contains SNP rs189040948 in the GCGC site. When the SNP is present on the unmethylated allele, HhaI digestion will not occur, causing a false fully methylated result.
Π Digestion control: warns for insufficient digestion. Upon digestion, <5% of the signal should remain in the digested reaction compared to the undigested reaction.
ɷ Be cautious when interpreting results if the probe signal of these two probes in the digested reaction is more than 15% lower than expected. The lower signal in digested reactions of these two probes indicates the use of an excess of HhaI enzyme or the use of an enzyme preparation that is unsuitable for MS-MLPA such as HhaI enzymes that are resistant to heat inactivation.
« 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.
¬ This probe is outside the common PWS/AS region. The size of the region showing an aberrant copy number will differ between different PWS/AS patients.
Ð This probe is sensitive to overdigestion, which can occur for example in case of lower ligation-digestion temperature (<48°C).

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Table 2. ME028-D1 target probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene/ Exon[a]	HhaI site	GenBank Ligation site	MV location (hg18)	Imprinted allele	Distance to next probe
Common breakpoint cluster region BP1							
436 ‡	20702-L29063	*NIPA1*		NM_144599.5; 260-261			214.0 kb
154 ‡	02018-L00865	*TUBGCP5*		NM_052903.6; 793-794			964.5 kb
Common breakpoint cluster region BP2							
172	20688-L28510	*MKRN3*		NM_005664.4; 929-930	15-021,362818		77.5 kb
418 «	11155-L29062	*MAGEL2* – Exon 1		NM_019066.5; 3713-3714	15-021,440355		4.1 kb
232 «	20701-L28529	MAGEL2:TSS-DMR	+	NM_019066.5; 348 nt before exon 1, reverse	15-021,444420	Maternal	38.0 kb
427 «	04026-L29645	*NDN*		NM_002487.3; 1030-1029, reverse	15-021,482490		1.1 **M**b
		***SNRPN* ◊**		**NM_022807.5**			
288	15261-L16736	u1B		175-176 in exon 1	15-022,620016		6.1 kb
239	20692-L15415	u1B*		271-272 in exon 2	15-022,626072		64.9 kb
278	12179-L13382	Intron u2		8.2 kb after exon 3	15-022,690980		12.3 kb
270	12182-L28519	Intron u2		12.9 kb before exon 4	15-022,703328		13.4 kb
256	20694-L28518	Exon u5 (AS-SRO)		376 nt after exon 4	15-022,716714		0.6 kb
391	12477-L13519	Exon u5 (AS-SRO)		986 nt after exon 4	15-022,717321		33.8 kb
250	11181-L13997	Intron 2 SNURF:TSS-DMR (PWS-SRO)	+	7.2 kb before exon 5	15-022,751105	Maternal	0.1 kb
178 ±	04106-L13905	Intron 2 SNURF:TSS-DMR (PWS-SRO)	+	7.1 kb before exon 5	15-022,751214	Maternal	0.3 kb
190 ±	04104-L04294	Intron 2 SNURF:TSS-DMR (PWS-SRO)	+	6.8 kb before exon 5	15-022,751480	Maternal	0.3 kb
142 Ð	20687- L31784	Intron 2 SNURF:TSS-DMR (PWS-SRO)	+	6.6 kb before exon 5	15-022,751773	Maternal	0.5 kb
333	22586-L31906	Intron 2 SNURF:TSS-DMR (PWS-SRO)	+	6.1 kb before exon 5	15-022,752234	Maternal	0.7 kb
443	22587-L31786	Intron 2 SNURF:TSS-DMR (PWS-SRO)	+	5.4 kb before exon 5	15-022,752900	Maternal	11.3 kb
294	01318-L13088	Exon 3		726-725 in exon 6, reverse	15-022,764248		8.3 kb
409	11177-L28521	Exon 7		1089-1090 in exon 9	15-022,772555		75.7 kb
214	12719-L28514	SNORD116-1		NR_003316.1; 474 nt after transcript, reverse	15-022,848250		24.4 kb
472	12721-L13796	SNORD116-11		NR_003326.2;	15-022,872658		15.9 kb

Length (nt)	SALSA MLPA probe	Gene/ Exon[a]	HhaI site	GenBank Ligation site	MV location (hg18)	Imprinted allele	Distance to next probe
				426 nt after transcript			
326	20697-L28525	SNORD116-23		NR_003337.2; 464 nt after transcript, reverse	15-022,888546		247.8 kb
		UBE3A		**NM_130838.4**			
355	02034-L12925	Exon 10		2900-2901	15-023,136395		20.3 kb
301	12082-L28520	Exon 5		2172-2173	15-023,156677		11.1 kb
160	04620-L00863	Exon 4		1146-1147	15-023,167740		4.2 kb
195	20689-L28513	Exon 3		626-627	15-023,171919		29.8 kb
373	10878-L11548	Exon 2		565-566	15-023,201674		33.5 kb
184 «	19804-L28512	Exon 1	+	49-50	15-023,235184	N/A	252.8 kb
364	20695-L28523	*ATP10A* – Exon 15		NM_024490.4; 3360-3361	15-023,487957		171.9 kb
226 «	20691-L28515	*ATP10A* – Exon 1		NM_024490.4; 494 nt before exon 1	15-023,659906		684.3 kb
220	01315-L00868	*GABRB3* – Exon 9		NM_021912.5; 1208-1209	15-024,344242		19.6 kb
382 ๑	10874-L11544	*GABRB3* – Exon 7		NM_021912.5; 771-772	15-024,363881		1.4 **M**b
137	20700-L28528	*OCA2* – Exon 23		NM_000275.3; 2501-2502	15-025,763706		187.1 kb
317	20698-L28526	*OCA2* – Exon 3		NM_000275.3; 6 nt after exon 3, reverse	15-025,950765		1.2 **M**b
202 ¬ ๑	01314-L00867	*APBA2*		NM_005503.3; 2605-2606	15-027,196749		

[a] See section Exon numbering on page 3 for more information.

◊ Nomenclature according to Beygo et al. (2019) EMQN/ACGS best practice guidelines and Monk et al. (2018) Recommendations for a nomenclature system for reporting methylation aberrations in imprinted domains.
± SNP rs541877352 could influence the 178 nt probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe. The target sequence of the 190 nt probe contains SNP rs189040948 in the GCGC site. When the SNP is present, HhaI digestion will not occur, resulting in a false methylation positive signal.
๑ Be cautious when interpreting results if the probe signal of these two probes in the digested reaction is more than 15% lower than expected. The lower signal in digested reactions of these two probes indicates the use of an excess of HhaI enzyme or the use of an enzyme preparation that is unsuitable for MS-MLPA such as HhaI enzymes that are resistant to heat inactivation.
« 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.
¬ This probe is outside the common PWS/AS region. The size of the region showing an aberrant copy number will differ between different PWS/AS patients.
‡ The chromosomal order of these probes in the NCBI and BLAT databases (hg18) is different. We used the order that is mentioned by Jiang et al. (2008).
Ð This probe is sensitive to overdigestion, which can occur for example in case of lower ligation-digestion temperature (<48°C).

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Table 3. Methylation-specific target probe sequences detected by ME028-D1

Length (nt)	SALSA MS-MLPA probe	Partial sequence with HhaI site
142	20687-L28509	AGGGGGTGTTGA**GCGC**AGGT-AGGTGTATAATAGTGACCAC
178	04106-L13905	CAGCGAGTCTG**GCGC**AGAGT-GGAGCGGCCGCCGGAGATGC
184	19804-L28512	GATCCGTGTGTCTCCCAAGA-TGGTG**GCGC**TGGGCTCGGGG
190	04104-L04294	GGTATCCTGTCCGCTCGCAT-TGGG**GCGC**GTCCCCCATCCG
232	20701-L28529	GATGTGAGGCGGAGAATGAA-AAAA**GCGC**ATTTACATAAGA
250	11181-L13997	GGAGGGAGCTGGGACCCCTGCA-CTGCGGCAAACAAGCACGCCT**GCGC**GGCCGC
333	22586-L31906	GTGGTGGTGGTGCTTTTTTA-TTAAAACT**GCGC**AATGCCTA
443	22587-L31786	CTGTTCCTTTCCGGTTGTG**G**-**CGC**AGTAGAGGGGGGAGGAG

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.

Table 4. Interpretation of copy number and methylation ratio results

	PWS Deletion	PWS Disomy ¥	Reference	AS Disomy ¥	AS Deletion	Duplication	
Genomic situation of the 15q11 region *	M_	MM	PM	PP	P_	PMM	PPM
Copy number	1	2	2	2	1	3	3
Copy number ratio	0.5	1	1	1	0.5	1.5	1.5
% Methylated	100%	100%	50%	0%	0%	70%	30%
Ratio after digestion	1	1	0.5	0	0	0.7	0.3

* In this row, the paternal and maternal copies of the 15q11 region are indicated with a P or M, respectively.

¥ Next to uniparental disomy, PWS/AS can also be caused by aberrant methylation due to imprinting defects. With the ME028 probemix no discrimination between uniparental disomy and imprinting defects can be made.

Related SALSA MLPA probemixes

ME030 BWS/RSS: Contains probes for Beckwith-Wiedemann syndrome.
ME031 GNAS: Contains probes for the complex *GNAS* region on chromosome 20.
ME032 UPD7-UPD14: Contains probes for uniparental disomy of chromosomes 7 and 14.
ME034 Multi-locus Imprinting: Contains probes for eleven different imprinted locations.
P211 HSP region: Contains additional probes for the BP1-BP2 region.
P325 OCA2: Contains probes for each exon of *OCA2* with the exception of exon 8.
P336 UBE3A: Contains probes for each *UBE3A* exon.
P343 Autism-1: Contains additional probes for *UBE3A* and the 15q13 region.

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ME028 product history	
Version	*Modification*
D1	Two methylation-specific SNRPN probes added. One digestion control and one reference probe replaced. Two probes adjusted in length, not in sequence detected.
C1	The methylation-specific NDN probe has been replaced by a MAGEL2 probe. Two probes for the *OCA2* gene have been included. Four other 15q11 probes, the two digestion control probes and several reference probes have been replaced.
B2	The 88 and 96 nt control fragments have been replaced (QDX2).
B1	A large number of new 15q11 probes have been included in version B1.
A1	First release.

Implemented changes in the product description

Version D1-03 – 21 December 2022 (04M)
- Genotype of positive sample NA22397 is corrected to maternal duplication of 15q11.
- Gene structure and exon numbering section is updated: *SNRPN* gene contains 13 exons and *MAGEL2* gene length spans 2,5 kb in hg18.
- Various minor textual or layout changes.

Version D1-02 – 16 June 2022 (04M)
- Clinical background section is updated.
- Gene structure and transcript variants section and exon numbering section are replaced with the Gene structure and exon numbering section.
- Methylation status table is updated: criteria for unmethylated status is changed from =0% to <5% and methylation percentage is replaced by final ratio (FR).
- LOVD mutation databases section is updated.
- DMR nomenclature updated in Table 1 and Table 2.
- Exon numbering in Table 2 column “GenBank Ligation site” according to NG_012958.
- Warning for SNPs (178 nt probe and 190 nt probe) added under Table 1 and 2.

- Chromosomal position (264 nt probe and 461 nt probe) corrected in Table 1.
- Reference section is updated.
- Various minor textual or layout changes.
- MS from 'SALSA MS-MLPA Probemix ME028' throughout document removed.

Version D1-01 – 20 July 2021 (04M)
- Product description rewritten and adapted to a new template.
- ME028-D1 is now CE marked.
- Product description adapted to a new product version (version number changed, changes in Table 1, Table 2, and Table 3).
- Various minor textual or layout changes.
- Added 15q11 duplication sample to positive DNA sample table.
- Methylation percentages table added to interpretation of results section.
- Added remarks concerning prenatal samples to limitations of the procedure section.
- Updated NM_ reference sequence numbers and ligation sites for all genes except *SNORD116* and *APBA2*. Updated NM_ reference sequence, ligation sites, and exon numbers for *UBE3A* probes.
- Updated table 4 with PPM example.
- Updated references with publication from Beygo et al. 2019.
- Added additional ME028 specific note about UBE3A methylation.

Version C1-04 – 06 September 2019 (01M)
- Advice on not spinning down your MLPA reactions in between the ligation and PCR reaction was added to the warning about non-specific peaks on page 1.
- Catalogue number SALSA HhaI adjusted.

Version C1-03 – 24 January 2019 (01M)
- Exon number changed for positive sample NA13554.

Version C1-02 – 17 December 2018 (01M)
- Use of SALSA HhaI (SMR51) with ME028 added.
- Information regarding 15q11 duplication syndrome added.
- Additional positive control samples included.
- Warning added under copy number status table.
- Digestion control warning updated: removed signal should be gone upon complete digestion and added HhaI-digestion can be considered sufficient when <5% of the signal remains in the digested reaction compared to the undigested reaction.
- Order of TUBGCP5 and NIPA1 switched in Table 2.
- Old exon numbering for UBE3A removed from Table 2.
- Warning added regarding overdigestion of the 142 nt probe.
- Example of duplication added to Table 4.
- Figure 4 removed.
- ME034 added as a related probemix.
- References updated.

Version C1-01 – 14 June 2018 (01M)
- Product description restructured and adapted to a new template.
- Various minor textual and layout changes.
- Table 3 with methylation-specific target probe sequences detected by ME028-C1 added.
- P211 HSP region added to the related probemixes.

Version 59 – 5 January 2018 (16)
- Warning added in Table 1 and Table 2, 226 nt probe 20691-L28515.

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

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