Product Description

SALSA® MS-MLPA® Probemix ME030-C3 BWS/RSS

To be used with the MS-MLPA General Protocol.

Version C3
For complete product history see page 11.

Catalogue numbers:

- **ME030-025R:** SALSA MS-MLPA Probemix ME030 BWS/RSS, 25 reactions.
- **ME030-050R:** SALSA MS-MLPA Probemix ME030 BWS/RSS, 50 reactions.
- **ME030-100R:** SALSA MS-MLPA Probemix ME030 BWS/RSS, 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.

Warning: In several No DNA reactions performed on this ME030-C3 probemix, MRC-Holland has recently observed a series of non-specific peaks with predominant lengths at about 100, 118, 172, 209, 249, 260 and 308 nt. 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@mlpa.com.

General information
The SALSA MS-MLPA Probemix ME030 BWS/RSS is a **research use only (RUO)** assay for the detection of aberrant methylation of one or more sequences of the IC2 (KvDMR) and IC1 (H19DMR) domains in the 11p15 chromosomal region associated with Beckwith-Wiedemann syndrome (BWS) and Russell-Silver syndrome (RSS). This probemix can also be used to detect deletions/duplications in the aforementioned chromosomal regions. Additionally, two probes are included for the *NSD1* gene which is associated with Sotos syndrome, a disease that has a similar phenotype. 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).

BWS is a clinically heterogeneous overgrowth syndrome associated with an increased risk for embryonal tumour development. RSS is a genetically heterogeneous disorder involving both intrauterine and postnatal growth retardation. The incidence of both BWS and RSS is estimated to be approximately 1 in 10,000-15,000 newborns and around 85% of the cases are sporadic (Õunap 2016). These conditions are both caused by a genetic or epigenetic alteration within two domains of imprinted growth regulatory genes on chromosome 11p15, leading to deregulated expression of the imprinted genes within this region. Approximately 60-70% of the patients have imprinting abnormalities at one of two imprinted domains IC1 or IC2, and these changes are frequently mosaic (see Figure 1 for a scheme of the imprinted gene cluster). Other known causes of BWS and RSS are uniparental disomy (UPD), trisomy 11p15, mutations in the *CDKN1C* gene, as well as small deletions and translocations. About 10% of RSS cases are caused by maternal UPD for chromosome 7 (Õunap 2016).

This SALSA MS-MLPA Probemix ME030 BWS/RSS is capable of rapidly detecting most causes of BWS and RSS, as both copy numbers and methylation status of the 11p15 region can be determined. This MS-MLPA assay for BWS/RSS can also be useful for screening of childhood cancers, in particular Wilms' tumour. A strong linkage between hypermethylation of the IC1 locus, but not IC2, has been described in these patients resulting in biallelic expression of the *IGF2* gene (Maas et al., 2016). Because of similarities between BWS and Sotos syndrome, two probes for *NSD1* are included.

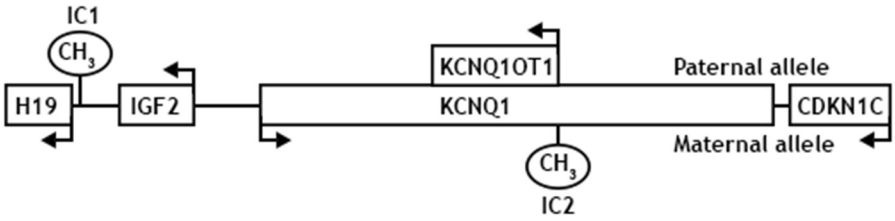

Figure 1. Scheme of the imprinted gene cluster on chromosome 11p15.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1394/ (BWS) and https://www.ncbi.nlm.nih.gov/books/NBK1324/ (RSS).

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 exon numbering used in this ME030-C3 BWS/RSS product description is the exon numbering from the following LRG sequences:

Gene name	LRG sequence
NSD1	LRG_512
H19	LRG_1030
IGF2	LRG_1031
KCNQ1	LRG_287
KCNQ1OT1	LRG_1052
CDKN1C	LRG_533

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 sequences. Furthermore, not all exons may be present in the NM_ sequence that was used for determining a probe's ligation site. Please note that the exon numbers mentioned in Table 2 in the Gene/Exon column and the ligation site column are both based on the LRG exon numbering. 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 ME030-C3 BWS/RSS contains 42 (MS-)MLPA probes with amplification products between 129 and 463 nucleotides (nt). 26 probes are specific for the BWS/RSS 11p15 region. Ten MS-MLPA probes contain an HhaI recognition site and provide information on the methylation status of the BWS/RSS 11p15 region. Two probes are specific for the *NSD1* gene. All probes present will also give information on copy number changes in the analysed sample. In addition, 13 reference probes are included that are not affected by HhaI digestion and detect genes located outside the BWS/RSS 11p15 region. Also, one digestion control probe is 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 BWS/RSS. 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.

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.

***H19* locus and *KCNQ1OT1* locus**

The four MS-MLPA probes targeting the *H19* gene and the four MS-MLPA probes targeting the *KCNQ1OT1* locus are located very close to each other. It is expected that all MS-MLPA probes in each locus provide similar results. We recommend using the average, or the median, methylation status of these probes to determine the methylation status of each locus and to disregard aberrant methylation detected by a single MS-MLPA probe.

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. 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.

ME030 specific notes:

- Due to the high natural variability in methylation levels of the region it is essential to include both test and reference samples in the same experiment. Sample type and DNA extraction method for all samples should be the same.
- Interpretation of the results should only be done by a professional experienced in the field of imprinting disorders, as recommended by Brioude et al (2018).
- The majority of molecular disturbances in RSS and BWS are affected by mosaicism, MS-MLPA is not suitable to detect the low-level mosaicisms present in a subset of these cases (Brioude et al (2018)).
- CDKN1C probe 15054-L18042 at 346 nt is not completely digested in DNA samples derived from blood and shows 10-15% background signal after HhaI digestion. Low methylation ratios obtained with this probe 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.

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.

BWS and RSS mutation database
https://databases.lovd.nl/shared/diseases/00231 and https://databases.lovd.nl/shared/diseases/00232. 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 *KCNQ1* exons 7 and 9 but not exon 8) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MS-MLPA Probemix ME030-C3 BWS/RSS

Length (nt)	SALSA MLPA probe	HhaI site	% methylated in normal blood-derived DNA	% expected signal reduction[a]	Chromosomal position (hg18)	
					Reference	BWS/RSS
64-105	Control fragments – see table in probemix content section for more information					
129	Reference probe 11622-L12379				10q	
135	**H19 probe** 08743-L20532	+	50%	50%		H19DMR/IC1
141	**KCNQ1OT1 probe** 07173-L19191	+	50%	50%		KvDMR/IC2
148	Reference probe 12415-L13416				14q	
154	Reference probe 02944-L02376				7q	
160	**H19 probe** 14731-L01713					Upstream
166	**KCNQ1OT1 probe** 06276-L05782	+	50%	50%		KvDMR/IC2
172 «« Λ	**IGF2 probe** 06269-L20841	+	0%	100%		Exon 4
178	Reference probe 05725-L05164				9q	
184	**H19 probe** 14063-L08764	+	50%	50%		H19DMR/IC1
190	**H19 probe** 16671-L19242					Upstream
196 ««	**CDKN1C probe** 06262-L05768					Exon 1
202 «	Reference probe 05927-L07395				22q	
208	Reference probe 07404-L07051				12q	
214	**H19 probe** 10586-L11141					Upstream
221	**KCNQ1 probe** 14791-L16502					Exon 3
228	**H19 probe** 16670-L19241					Exon 5
238	**H19 probe** 14792-L16503	+	50%	50%		H19DMR/IC1
256	Reference probe 01462-L00927				17p	
266 ~	**KCNQ1 probe** 14733-L18343					Exon 13
274	**KCNQ1OT1 probe** 16654-L19204	+	50%	50%		KvDMR/IC2
284 «	**IGF2 probe** 06272-L05778					Exon 8
292	Reference probe 03262-L20515				3q	
301	**H19 probe** 06266-L05772	+	50%	50%		H19DMR/IC1
310	Reference probe 04528-L03917				2q	
319	**NSD1 probe** 16702-L02529					Exon 23
328	**KCNQ1 probe** 15055-L04802					Exon 7
337	Reference probe 02416-L01862				16q	
346 ««	**CDKN1C probe** 15054-L18042	+	10-15%	90%		Exon 1
355 π	Digestion control probe 09153-L09311	+	0%	100%	8p	
364	**KCNQ1 probe** 16669-L19240					Exon 8
373	**KCNQ1 probe** 14793-L16504					Exon 15
383	Reference probe 00973-L18737				10q	
393 ±	**KCNQ1OT1 probe** 07172-L06781	+	50%	50%		KvDMR/IC2
400	**KCNQ1 probe** 16655-L20510					Exon 9
411 «	**KCNQ1 probe** 03555-L21092					Exon 17
418	**NSD1 probe** 17048-L02071					Exon 21
427	Reference probe 07800-L07555				18q	
436	**KCNQ1 probe** 14732-L02903					Exon 2
445 ««	**CDKN1C probe** 14734-L20842					Exon 3
454	**H19 probe** 10588-L11143					Upstream
463	Reference probe 13471-L11729				2p	

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

± SNP rs568231950 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

« 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 («« extremely sensitive to incomplete denaturation).

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

Λ HhaI-digestion of the 172 nt probe can be considered sufficient when <10% of the signal remains in the digested reaction compared to the undigested reaction.

~ The 266 nt probe 14733-L18343 may show a 20-40% reduced peak height in the digested reactions. The cause of this is not yet known. Please ignore the methylation result of this probe.

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. ME030-C3 target probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene/Exon[a]	HhaI site	Ligation site[a]	MV location (Hg18)	Imprinted allele	Distance to next probe
		NSD1		**NM_022455.5**			
418	17048-L02071	Exon 21		6404-6405	05-176.648452		5.9 kb
319	16702-L02529	Exon 23		7615-7616	05-176.654367		
		H19		**NR_002196.2**			
228	16670-L19241	Exon 5		1879-1880	11-001.973411		2.4 kb
160	14731-L01713	Exon 1		138 nt before exon 1	11-001.975788		0.2 kb
301	06266-L05772	H19DMR/IC1	+	302 nt before exon 1	11-001.975956	Paternal	0.1 kb
238	14792-L16503	H19DMR/IC1	+	447 nt before exon 1, reverse	11-001.976099	Paternal	0.2 kb
184	14063-L08764	H19DMR/IC1	+	617 nt before exon 1	11-001.976269	Paternal	0.3 kb
135	08743-L20532	H19DMR/IC1	+	922 nt before exon 1, reverse	11-001.976564	Paternal	2.3 kb
454	10588-L11143	Upstream		3.2 kb before exon 1	11-001.978896		0.5 kb
214	10586-L11141	Upstream		3.8 kb before exon 1	11-001.979409		3.0 kb
190	16671-L19242	Upstream		6.7 kb before exon 1, reverse	11-001.982389		129.0 kb
		IGF2		**NM_000612.6**			
284 «	06272-L05778	Exon 8		1378-1377, reverse	11-002.111396		6.2 kb
172 «« Λ	06269-L20841	Exon 4	+	427 nt before exon 5 (NM_001127598.2; 318 nt after exon 4)	11-002.117595		321.8 kb
		KCNQ1		**NM_000218.3**			
436	14732-L02903	Exon 2		16 kb after exon 1 (NM_181798.1; 199-200)	11-002.439436		66.1 kb
221	14791-L16502	Exon 3		137 nt before exon 3	11-002.505564		45.1 kb
328	15055-L04802	Exon 7		914-915	11-002.550670		10.6 kb
364	16669-L19240	Exon 8		1034-1035	11-002.561234		1.8 kb
400	16655-L20510	Exon 9		1155-1156	11-002.563022		114.1 kb
		KCNQ1OT1		**NR_002728.3**			
274	16654-L19204	Exon 1 KvDMR/IC2	+	664-663, reverse	11-002.677116	Maternal	0.1 kb
166	06276-L05782	Exon 1 KvDMR/IC2	+	593-592, reverse	11-002.677186	Maternal	0.4 kb
393 ±	07172-L06781	Exon 1 KvDMR/IC2	+	183-184	11-002.677594	Maternal	0.4 kb
141	07173-L19191	Exon 1 KvDMR/IC2	+	191 nt before exon 1, reverse	11-002.677971	Maternal	68.7 kb
		KCNQ1		**NM_000218.3**			
266 ~	14733-L18343	Exon 13	~	1616-1617	11-002.746637		8.1 kb
373	14793-L16504	Exon 15		1791-1792	11-002.754781		71.8 kb
411 «	03555-L21092	Exon 17		2908-2909	11-002.826573		35.0 kb
		CDKN1C		**NM_000076.2**			

Length (nt)	SALSA MLPA probe	Gene/Exon[a]	HhaI site	Ligation site [a]	MV location (Hg18)	Imprinted allele	Distance to next probe
445 ««	14734-L20842	Exon 3		1316-1317	11-002.861603		1.6 kb
196 ««	06262-L05768	Exon 1		311-312	11-002.863233		0.1 kb
346 ««	15054-L18042	Exon 1	+	233-234	11-002.863305		

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

± SNP rs568231950 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

« 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 («« extremely sensitive to incomplete denaturation).

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

Λ HhaI-digestion of the 172 nt probe can be considered sufficient when <10% of the signal remains in the digested reaction compared to the undigested reaction.

~ The 266 nt probe 14733-L18343 may show a 20-40% reduced peak height in the digested reactions. The cause of this is not yet known. Please ignore the methylation result of this probe.

Table 3. Sequences detected by the ME030-C3 probes

Length (nt)	SALSA MS-MLPA probe	Partial sequence with HhaI site
135	08743-L20532	CAGGCCCTCTGGGATGTGGAAGGGCTGGC-CGCGCCTTCGGCAAACCCTCTGTTCCCA
141	07173-L19191	GATCGGTTTTGATGCCACCCGGGCTCAGAT-TGGCCCAGCGGGTCCAGCGCCGCATGAG
166	06276-L05782	GACCGTGTTCAAACCCTCCCAGAGAGA-TGGGGAGGGCCGCGCTGAGGAGAGTCTG
171	06269-L20841	CCAAGCCACCTGCATCTGCACTCA-GACGGGGCGCACCCGCAGTGCAGCCTCC
184	14063-L08764	GTAGAGTGCGCCCGCGAGCCGTA-AGCACAGCCCGGCAACATGCGGTCTTCAGAGT
238	14792-L16503	CTGAGGGGCAGAGGGAAGTGCCGCAA-ACCCCCTGGTGGGCGCGGTGCCAGCCCCCCA
274	16654-L19204	GCGGGGCACACAGCTCACCTCAGCAA-CGCCAGTGATCACCCGTCCCGCGCCGTCCGC
301	06266-L05772	CGGCCCCCAGCCATGTGCAAAGTA-TGTGCAGGGCGCTGGCAGGCAGGGAGCA
346	15054-L18042	CCTCTCCTTTCCCCTTCTTCTCGCT-GTCCTCTCCTCTCTCGCTGCCCGCGTTTGCGCAG
393	07172-L06781	CGTCCTCCTCGGTGCGTCAGTCAT-CGTGGTTCTCCCCGGCGCGCCCCTCGGC

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.

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 4. Interpretation of copy number and methylation ratio results

	BWS IC1 hyper-methylation	BWS pUPD11p15	BWS IC2 hypo-methylation	Reference	RSS IC1 hypo-methylation
Genomic situation of the 11p15 region*	PM	PP	PM	PM	PM
Copy number	2	2	2	2	2
Copy number ratio	1	1	1	1	1
% Methylated IC1	GOM	GOM	50%	50%	LOM
% Methylated IC2	50%	LOM	LOM	50%	50%
Ratio after digestion IC1	GOM	GOM	0.5	0.5	LOM
Ratio after digestion IC2	0.5	LOM	LOM	0.5	0.5
Incidence¥	5%	20%	50%	-	35-50%

* In this row, the paternal and maternal copies of the 11p15 region are indicated with a P or M, respectively.

¥ As reported by GeneReviews (www.ncbi.nlm.nih.gov/books/NBK1394/ & www.ncbi.nlm.nih.gov/books/NBK1324/).
GOM: Gain of methylation; LOM: Loss of methylation

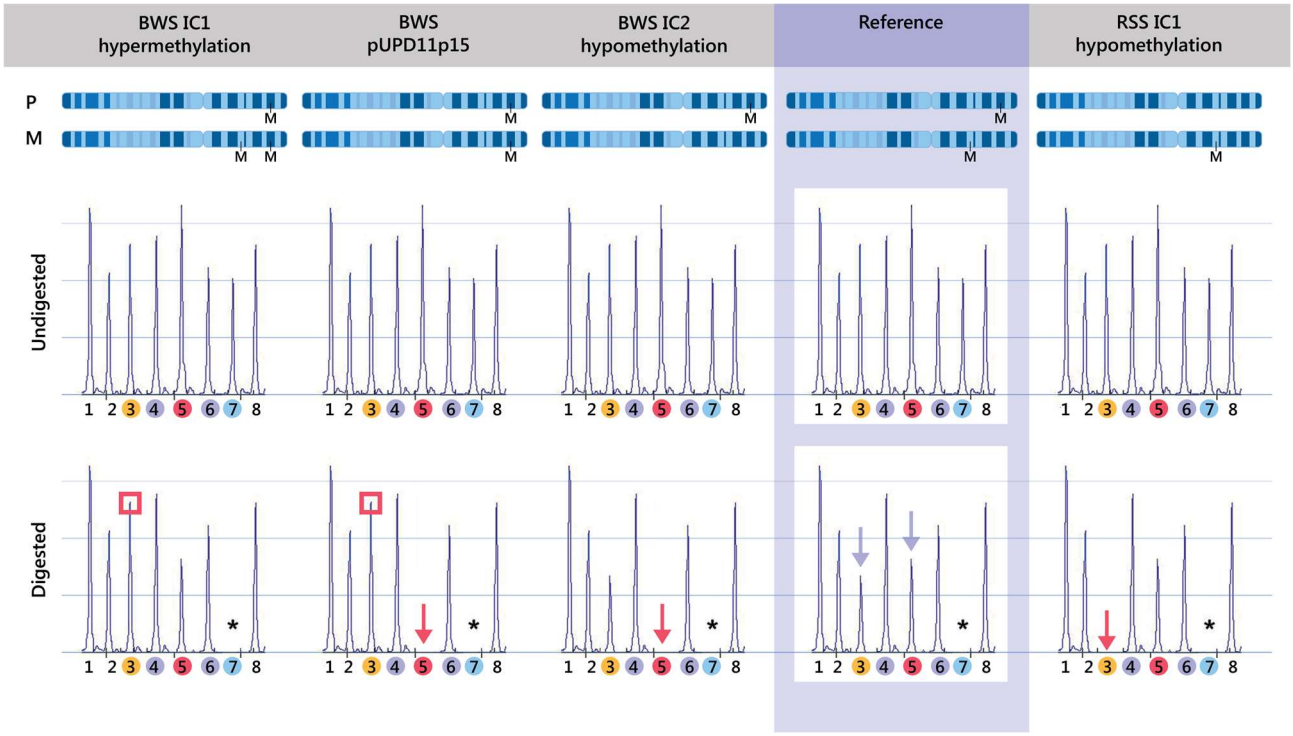

Figure 2. Simplified schematic representation of results that may be obtained with BWS/RSS samples. A cartoon of both copies of chromosome 11 in normal individuals (reference), BWS (left) and RSS (right) is included at the top. Here M indicates methylation of the two imprinted domains IC1 (H19DMR) or IC2 (KvDMR); and P and M indicate the paternal and maternal copy (except in BWS UPD samples), respectively. In case of hypomethylation the affected probes *with* an HhaI site disappear from the electropherogram (see red arrows (↓)). In case of hypermethylation probes *with* an HhaI site are not reduced relative to the undigested samples (see red squares (□)). This is an example, keep in mind that in real samples these changes could be more subtle.

The top row of electropherograms schematically shows *undigested* samples, which are used for copy number analysis. The example shows two reference probes (4, 6), three probes for the 11p15 region *without* an HhaI site (1, 2, 8), two probes for the 11p15 region *with* an HhaI site (3 and 5), of which 3 targets the IC1 region and 5 targets the IC2 region. Furthermore, one digestion control probe with an HhaI site (7) is shown. Copy number ratios are the same in all cases illustrated in this figure.

The bottom row of electropherograms schematically shows the accompanying samples that have been *digested* with HhaI. Compared to the undigested samples, only probes with an HhaI site are affected (3, 5 and 7). The digestion control (7) is unmethylated, and disappears from the electropherograms (indicated with *). The peak height of the **11p15 IC1** probe with an HhaI site (3) and the height of the **11p15 IC2** probe with an HhaI site (5) are reduced by 50% relative to the undigested samples in the reference sample, as indicated with a purple arrow (↓).

The **11p15 IC1** probe's (3) peak height is not reduced relative to the undigested samples (100% methylation) in BWS caused by either IC1 hypermethylation or pUPD11p15 (indicated with a red square (□)), whereas in BWS caused by IC2 hypomethylation this probe is reduced by 50%, similar to the reference samples.

In RSS samples the peak height of this **IC1** probe (3) disappears from the electropherogram completely (indicated with a red arrow (↓)).

The **11p15 IC2** probe's (5) peak height is reduced by 50% relative to the undigested samples in BWS caused by IC1 hypermethylation or RSS caused by IC1 hypomethylation, similar to the reference sample. The **IC2** probe (5) disappears from the electropherogram in cases of BWS caused by either pUPD11p15 or IC2 hypomethylation (indicated with a red arrow (↓)).

Related SALSA MLPA probemixes

P026 Sotos	Contains probes for the *NSD1* gene.
P118 WT1	Contains probes for the *WT1* and *AMER1* regions.
P380 Wilms' tumour	Contains probes for genes implicated in the prognosis of Wilms' tumour.
ME032 UPD7-UPD14	Contains probes for the 7p12, 7q32 and 14q32 imprinted regions.

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- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
- 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 ME030 BWS/RSS

- Eggermann K et al. (2016). EMQN best practice guidelines for the molecular genetic testing and reporting of chromosome 11p15 imprinting disorders: Silver–Russell and Beckwith–Wiedemann syndrome. *Eur J Hum Genet*. 24:1377-87.
- Gede LB et al. (2016). Feasibility study on the use of methylation-specific MLPA for the 11p15 region on prenatal samples. *Prenat Diagn*. 36:100-3.
- Passaretti F et al. (2022). Different mechanisms cause hypomethylation of both *H19* and *KCNQ1OT1* imprinted differentially methylated regions in two cases of Silver-Russell syndrome spectrum. *Genes*. 13:1875.
- Valente FM et al. (2019). Transcription alterations of *KCNQ1* associated with imprinted methylation defects in the Beckwith-Wiedemann locus. *Genet Med*. 21:1808-1820.

ME030 product history	
Version	*Modification*
C3	All probes are identical to lot C1-0711. However, the formation of a secondary structure within the probe oligonucleotides has been reduced.
C2	*KCNQ1OT1* probe at 138 nt has been elongated by 2 nt.
C1	Three probes for *H19* and two for *KCNQ1*, several reference probes, the digestion control probe and the 88 and 96 nt D-fragments have been replaced. One *H19* probe has been removed and one *CDKN1C* probe has been added. For the *NSD1* gene, two probes have been included.
B2	Two extra control fragments at 100 and 105 nt added.
B1	One *H19* probe and one reference probe have been replaced.
A1	First release.

Implemented changes in the product description
Version C3-10 – 22 November 2022 (04M) - Product description rewritten and adapted to a new template. - Various minor textual or layout changes. - Ligation sites of the probes targeting the *NSD1, IGF2 and KCNQ1* genes updated according to new version of the NM_ reference sequence. - Small change of probe length of IGF2 probe at 172 nt in Table 1 and 2 in order to better reflect the true length of the amplification product. - Selected publications using SALSA MS-MLPA Probemix ME030 BWS/RSS section updated. Version C3-09 – 07 July 2022 (02M) - Updated the column "Imprinted allele" in Table 2 for more clarity. Version C3-08 – 15 February 2022 (02M) - Updated Exon numbering section on page 2 for more clarity. - Various minor textual and layout changes throughout the document. Version C3-07 – 01 June 2021 (02M) - ME030 specific note on background digestion of CDKN1C probe at 346 nt added on page 4. Version C3-06 - 22 September 2020 (02M) - ME030 specific notes added on page 4, and 1 reference added. Version C3-05 – 22 July 2019 (02M) - 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. - ME032 was added to the related probemixes section. - Figure 1 was updated for more clarity. - Extra information on *NSD1* added on page 2. Version C3-04 – 14 June 2019 (02M) - Chromosome band in Table 4 legend corrected. - Various minor textual changes. Version C3-03 – 09 May 2019 (02M) - Product description rewritten and adapted to a new template. - Recommended HhaI enzyme changed from Promega's HhaI to SALSA HhaI. - Information added on MS-MLPA technique validation on page 3. - Warning added to Table 1 and 2 about the possible influence of SNP rs568231950 on the probe signal. - Possible techniques for confirmation of methylation status added on page 5. - Warning about off-scale peaks added on page 4. Version C3-02 – 06 June 2018 (01M) - Several small textual changes in Table 4 and Figure 2. - Various minor textual and layout changes throughout the document. Version C3-01 – 12 April 2018 (01M) - Product description restructured and adapted to a new template. - Exon numbering of the *IGF2* gene has been changed. - NM_ reference sequence updated for the *IGF2* gene. - Ligation sites of the probes targeting the *H19* gene updated according to a new version of the NM_ reference sequence. Table and Figure including example results were added on page 8.

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