Instructions for Use

SALSA® MLPA® Probemix ME032 UPD7-UPD14

See also the MS-MLPA General Protocol, the product descriptions of the SALSA® MLPA® Reagent Kit and SALSA® HhaI, and the Coffalyser.Net Reference Manual.

Visit the SALSA® MLPA® Probemix ME032 UPD7-UPD14 product page on our website to find Certificates of Analysis and a list of related products.

Product name	**SALSA® MLPA® Probemix ME032 UPD7-UPD14**
Version	**B1**
Catalogue numbers	**ME032-025R (25 reactions)** **ME032-050R (50 reactions)** **ME032-100R (100 reactions)**
Basic UDI-DI:	**872021148ME032ZH**
Ingredients	**Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA**

Additional Test Components	**Catalogue numbers**
SALSA® MLPA® Reagent Kit	EK1-FAM EK1-CY5 EK5-FAM EK5-CY5 EK20-FAM
SALSA® HhaI	SMR50

Storage and Shelf Life

Recommended conditions		

A shelf life of until the expiry date is guaranteed, also after opening when stored in the original packaging under recommended conditions. For the exact expiry date, see the label on the vial. This product should not be exposed to more than 25 freeze-thaw cycles. Do not use the product if the packaging is damaged or opened. Leave chemicals in original containers. Waste material must be disposed of in accordance with the national and local regulations.

Regulatory Status	
IVD	EUROPE CE 2797
RUO	ALL OTHER COUNTRIES

Label Symbols			
IVD	In Vitro Diagnostic	RUO	Research Use Only

More information: **www.mrcholland.com**	
	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

Any serious incident that has occurred in relation to this product should be reported to MRC Holland and the competent authority of the Member State in which the user and/or the patient is located.

Changes in this Product Version

B1 version compared to A1 version

Six PLAGL1 target probes removed, nine target probes replaced, nine target probes added, two digestion control probes replaced, 11 reference probes replaced, one reference probe removed, and two target probes changed in length, not in sequence detected.

1. Intended Purpose

The SALSA MLPA Probemix ME032 UPD7-UPD14 is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative manual assay[2] to be used with genomic DNA isolated from human peripheral blood. ME032 UPD7-UPD14 is intended for the detection of hypermethylation of the *GRB10* and/or *MEST* differentially methylated regions (DMRs) on 7q12 and 7q32 in combination with one or two copies of the *GRB10* and/or *MEST* genes and their DMRs to confirm a potential cause for Russell-Silver syndrome. Moreover, this assay can detect hypermethylation of the *MEG3*:TSS-DMR on 14q32 in combination with one or two copies of the *DLK1*, *MEG3* and *RTL1* and their DMRs to confirm a potential cause for Kagami-Ogata syndrome (KOS). Lastly, ME032 UPD7-UPD14 allows for the detection of hypomethylation of the *MEG3*:TSS-DMR on 14q32 in combination with one or two copies of the *DLK1*, *MEG3* and *RTL1* genes and their DMRs to confirm a potential cause for and clinical diagnosis of Temple syndrome (TS).

Methylation and copy number (CN) status determined with ME032 UPD7-UPD14 should be confirmed with a different technique. In particular, methylation and CN status determined by only a single probe always require confirmation by another method. Detection of hyper- or hypomethylation in combination with two copies of the respective region requires further investigation to distinguish between uniparental disomy (UPD) and epimutations.

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 or prenatal testing, population screening, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1] Please note that this probemix is for IVD use in the countries specified on page 1 of this product description. In all other countries, this is a RUO product.

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

2. Sample Requirements

Specimen	50-250 ng purified human genomic DNA, dissolved in 5 µl $TE_{0.1}$ buffer, pH 8.0-8.5
Collection method	Standard methods
Extraction method	Methods tested by MRC Holland: • QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual) • Promega Wizard Genomic DNA Purification Kit (manual) • Salting out (manual)

Sample types		
Test sample	• Provided by user	
Reference samples (required)	• Provided by user • Extraction method, tissue type, DNA concentration, treatment, and age group as similar as possible in all test and reference samples. • Have a normal copy number and methylation status, and ≤0.10 standard deviation for all probes. • At least three* independent reference samples required in each experiment for proper data normalisation. Derived from unrelated individuals from families without a history of RSS, KOS or TS.	
No-DNA control (preferably)	• Provided by user • $TE_{0.1}$ buffer instead of DNA • To check for DNA contamination	
Positive control samples (preferably)	• Provided by user if applicable, or	
	Available from third parties	See the table of positive samples on the probemix product page on our website.

* When testing >21 samples, include one extra reference for each 7 test sample.

3. Test Procedure

See the MS-MLPA General Protocol.

4. Quality Control, Data Analysis, and Troubleshooting

Quality Control Fragments in the Probemix	
Length (nt)	**Function**
64-70-76-82	DNA quantity control fragments
88-96	DNA denaturation control fragments
92	Benchmark fragment
100	Chromosome X presence control fragment
105	Chromosome Y presence control fragment

Coffalyser.Net should be used for data analysis in combination with the appropriate product and lot-specific Coffalyser sheet. See the Coffalyser.Net Reference Manual for details on data analysis and quality control.

For troubleshooting help, see the additional resources offered on our support portal.

5. Interpretation of Results

Determining Typical Values in Normal and Affected Populations

The typical final ratio (FR) values stated in the copy number tables were determined in a validation study with samples containing abnormal copy numbers. The standard deviation of each individual probe over all the reference samples was ≤0.10.

Expected Copy Number Results of Reference Probes

Final Ratio (FR)	Copy Number	Description
0.80 – 1.20	2	Normal

Expected Copy Number Results of Target Probes

Final Ratio (FR)	Copy Number	Description
0	0	Homozygous deletion
0.40 – 0.65	1	Heterozygous deletion
0.80 – 1.20	**2**	**Normal**
1.30 – 1.65	3	Heterozygous duplication
1.75 – 2.15	4	Homozygous duplication or Heterozygous triplication
All other values	-	Ambiguous

The tables illustrate the relationship between final ratio and corresponding copy number. Test results are expected to center around these values. Ambiguous values can indicate a technical problem, but may also reflect a biological cause such as mosaicism or a SNV influencing a single probe. It is important to use Coffalyser.Net to determine the significance of values found.

Expected Results of Methylation-Specific Probes in Healthy Individuals

Methylation Ratio (MR)	Methylation status
MR ≥ 0.85 (*MIR380*)	Fully methylated
0.40 ≤ MR ≤ 0.65 (*GRB10*, *MEST*, *MEG3* DMRs)	50% methylated / normally imprinted

It is recommended to use the median MR of the three methylation-specific probes per DMR to determine its methylation status.

Expected Results of Methylation-Specific Probes in Affected Populations

In RSS, KOS, and TS patients, hypomethylation or hypermethylation is expected. A median MR of the three methylation-specific probes in a DMR that is lower than the cut-off values for normal imprinted methylation indicates hypomethylation. A median MR that is higher than the cut-off values indicates hypermethylation.

Possible Results of Digestion Control Probes

Methylation Ratio (MR)*	Digestion status
≤ 0.05	Complete digestion
> 0.05	Incomplete digestion

* Signals ≤ 0.10 are displayed as intra ratio percentage by Coffalyser.Net. For more information see the Coffalyser.Net Reference Manual.

6. Performance Characteristics

Study	Description
Expected values for copy numbers in normal and affected populations	A study was conducted on over 1500 MLPA reactions with samples with and without abnormal copy numbers. When the standard deviation of each individual probe over all the reference samples is ≤0.10, the ranges stated in the copy number tables above can be used. Cut-off values for copy number determination were verified with ME032 UPD7-UPD14.
Expected values for methylation in normal and affected populations	To determine the expected values for methylation in a normal population a study was conducted on samples from healthy individuals. This resulted in more than 750 MRs obtained with the nine methylation-specific probes targeting the three imprinted regions (*GRB10*, *MEST*, *MEG3* DMRs) included in ME032-B1, establishing the range for methylation status in healthy individuals as shown in the table above. Expected values for hypomethylation and hypermethylation could not be determined as clinical patient samples are rare, due to the low prevalence of these syndromes. However, a median MR outside the cut-off values for normal imprinted methylation indicates hypo- or hypermethylation, which was confirmed in six samples with known hypo-/hypermethylation status.

<table>
<tr><td>Limit of Detection</td><td>A study was conducted to evaluate the minimum and maximum amount of DNA acceptable as the assay input. Results support the use of 50-250 ng of human DNA as the recommend input amount. The use of insufficient or too much sample DNA can affect performance.</td></tr>
<tr><td>Interfering substances</td><td>SNVs or other polymorphisms (e.g. indels) in the DNA target sequence and impurities in the DNA sample (e.g. NaCl or KCl, $FeCl_3$, heparin, EDTA and hemoglobin) can affect the methylation-specific MLPA (MS-MLPA) reaction.

A study was performed to assess the potential for interference of endogenous and exogenous substances on genomic DNA. For most probes, expected FRs (FRs within the expected cut-off category) were obtained even in the presence of potential interferents at concentrations shown in the table below.

<table>
<tr><th>Interferent</th><th>Source</th><th>Testing Concen-tration</th><th>Results*</th></tr>
<tr><td>EDTA</td><td>Exogenous – specimen collection tubes</td><td>1.5 mM</td><td>Copy number: Expected FR for 198/200 probes
Methylation: Expected FR for 48/48 probes</td></tr>
<tr><td>NaCl</td><td>Exogenous – DNA extraction</td><td>40 mM</td><td>Copy number: Expected FR for 163/169 probes
Methylation: Expected FR for 38/39 probes</td></tr>
<tr><td>Fe^{3+} ($FeCl_3$)</td><td>Exogenous – DNA extraction</td><td>1 µM</td><td>Copy number: Expected FR for 200/200 probes
Methylation: Expected FR for 48/48 probes</td></tr>
<tr><td>Heparin</td><td>Exogenous – specimen collection tubes</td><td>0.02 U/mL</td><td>Copy number: Expected FR for 200/200 probes
Methylation: Expected FR for 48/48 probes</td></tr>
<tr><td>Hemoglobin</td><td>Endogenous – blood sample</td><td>0.02 µg/µl</td><td>Copy number: Expected FR for 91/200 probes
Methylation: Expected FR for 45/48 probes</td></tr>
</table>

* Results are summarised for all probes across all samples tested.

$FeCl_3$ and heparin did not interfere with copy number and methylation status determination, while an effect was observed for a low number of probes with EDTA and NaCl. Hemoglobin had the largest effect on FRs, in particular for copy number determination.

To minimise variability across samples, 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.</td></tr>
<tr><td>Cross-reactivity</td><td>Cross-reactivity is the potential for probes to bind to homologous regions (e.g. pseudogenes) or other cross-reactive sequences. Quality tests were carried out to determine whether probes are specific to their target sequence and all probes met the quality criteria for specificity.</td></tr>
<tr><td>Accuracy</td><td>Results of accuracy are derived from trueness and precision studies. For trueness, previously genotyped samples and positive and negative control samples for methylation analysis were tested and found to have the expected results. For precision studies, results are not affected by operator, day, or laboratory site.</td></tr>
<tr><td>Clinical validity*</td><td>RSS: According to general literature, RSS is caused by UPD7mat in 5-10% of cases, while microdeletions and epimutations on chromosome 7 have been reported in only a few cases (<1%) (Cerrato et al. 2020, Hoffmann and Heller 2011). Therefore, the molecular cause of RSS can be confirmed by ME032 UPD7-UPD14 in 6-11% of cases. In literature using ME032 UPD7-UPD14, the molecular cause of RSS was identified in 0.9-17.8% of patients referred for RSS molecular testing, depending on the population tested.

KOS: KOS is caused by UPD14pat, microdeletions or epimutations on chromosome 14 (Cerrato et al. 2020). As probes targeting all possible causes of KOS are included in ME032 UPD7-UPD14, the molecular cause of KOS can be confirmed in >99% of KOS cases. In line with this, the molecular cause of KOS was identified in 100% of patients referred for KOS molecular testing in literature using ME032 UPD7-UPD14.

TS: TS is caused by UPD14mat, microdeletions or epimutations on chromosome 14 (Cerrato et al. 2020). Clinical symptoms of TS largely overlap with those of other syndromes, such as RSS and Prader-Willi syndrome (PWS). As a result, TS patients are frequently identified in cohorts initially referred for routine RSS or PWS testing (Mackay et al. 2022). In patients with a TS-like phenotype (including patients suspected of RSS or PWS), the diagnostic sensitivity of ME032 UPD7-UPD14 is estimated to be 1.2-2.3% based on literature using ME032 UPD7-UPD14.

* Based on a 2014-2022 literature review</td></tr>
</table>

Summary of Safety and Performance (SSP)
The SSP is available in the European database on medical devices (Eudamed), https://ec.europa.eu/tools/eudamed, or upon request.

Content – Details Sorted by Chromosomal Position

HhaI site	% methylated in normal blood-derived DNA; imprinted allele	Chr. position	Target	Exon	Distance to next probe	Length (nt)	Probe number	Warnings
-		7p12.2	*GRB10*	Exon 19 (20)	19.8 kb	409	22739-L32033	*
-		7p12.2	*GRB10*	Exon 13 (14)	5.3 kb	256	22732-L32026	*
-		7p12.2	*GRB10*	Exon 10 (11)	56.5 kb	427	18381-L23252	
-		7p12.2	*GRB10*	Exon 6 (7)	107.8 kb	317	22660-L32466	¥
+	50%; tissue-specific	7p12.2	*GRB10*:alt-TSS-DMR		0.4 kb	161	15746-L24414	«
+	50%; tissue-specific	7p12.2	*GRB10*:alt-TSS-DMR		0.1 kb	152	15744-L24405	«
-		7p12.2	*GRB10*:alt-TSS-DMR		0.1 kb	202	22729-L32023	* «
+	50%; tissue-specific	7p12.2	*GRB10*:alt-TSS-DMR		0.1 kb	136	15742-L18941	«
-		7p12.2	*GRB10*:alt-TSS-DMR		79.1 **M**b	226	22731-L32025	* «
-		7q32.2	*MEST*:alt-TSS-DMR		0.5 kb	500	22745-L32467	* «
+	50%; maternal	7q32.2	*MEST*:alt-TSS-DMR		0.1 kb	232	22661-L17775	¥ «
+	50%; maternal	7q32.2	*MEST*:alt-TSS-DMR		0.7 kb	190	15750-L17769	«
+	50%; maternal	7q32.2	*MEST*:alt-TSS-DMR		0.2 kb	184	15749-L17768	«
-		7q32.2	*MEST*:alt-TSS-DMR		0.5 kb	453	23112-L32648	* «
-		7q32.2	*MEST*:alt-TSS-DMR		5.2 kb	172	23111-L32734	* «
-		7q32.2	*MEST*	Exon 5	6.7 kb	418	18343-L23256	«
-		7q32.2	*MEST*	Exon 12		158	22727-L32021	*
-		14q32.2	*DLK1*	Exon 1	1.9 kb	391	22738-L32032	*
-		14q32.2	*DLK1*	Exon 3	3.1 kb	283	22733-L32027	*
-		14q32.2	*DLK1*	Exon 4	77.1 kb	347	15762-L18818	
-		14q32.2	*MEG3/DLK1*:IG-DMR		0.8 kb	373	22736-L32030	*
-		14q32.2	*MEG3/DLK1*:IG-DMR		1.0 kb	445	22740-L32034	*
-		14q32.2	*MEG3/DLK1*:IG-DMR		14.5 kb	148	22726-L32020	*
-		14q32.2	*MEG3*:TSS-DMR		0.1 kb	490	22744-L32038	*
+	50%; paternal	14q32.2	*MEG3*:TSS-DMR		0.2 kb	220	15754-L18942	
-		14q32.2	*MEG3*:TSS-DMR		0.2 kb	124	22725-L32749	*
+	50%; paternal	14q32.2	*MEG3*:TSS-DMR		0.1 kb	292	22668-L32796	* ‡
+	50%; paternal	14q32.2	*MEG3*:TSS-DMR		0.1 kb	118	S0910-L25280	* ±
-		14q32.2	*MEG3*:TSS-DMR		55.7 kb	463	22742-L32036	*
-		14q32.31	*RTL1*	Exon 4	2.4 kb	274	18336-L23249	
-		14q32.31	*RTL1*	Exon 4	140.6 kb	340	15763-L17782	
+	100%; n.a.	14q32.31	*MIR380*	Exon 1		247	18481-L18864	¬
+	0%	2q	Digestion control			132	S0750-L25688	* π
+	0%	8p	Digestion control			355	19043-L29021	* π
-		1p	Reference			400	14839-L16547	*
-		2p	Reference			142	16305-L18830	*
-		3p	Reference			332	17414-L21123	*
-		4p	Reference			115	S0973-L26704	*
-		6p	Reference			472	12029-L12891	*
-		10q	Reference			168	08222-L24899	
-		11p	Reference			364	18676-L24030	*
-		13q	Reference			238	18213-L04552	*
-		16p	Reference			266	22711-L31966	*
-		17q	Reference			511	18539-L32066	*
-		18q	Reference			196	16425-L18878	
-		19q	Reference			300	00348-L00174	*
-		20q	Reference			214	07733-L07423	
-		22q	Reference			436	22240-L31355	*

Probe lengths may vary slightly depending on capillary electrophoresis instrument settings. Please see the most up to date Coffalyser sheet for exact probe lengths obtained at MRC Holland.
The exon numbers are derived from the MANE Project and are based on MANE Select transcripts. For more information, see the probe sequences document available on the product page at www.mrcholland.com. The exon numbering from the previous version of this product description is disclosed between brackets.
The DMR (differentially methylated region) nomenclature used in this product description has been developed by the imprinted loci task force of the European Network for Human Congenital Imprinting Disorders (Monk et al. 2018).
(Alt-)TSS-DMR: (alternative) transcription start site differentially methylated region.
Chromosomal bands are based on: hg18.

7. Precautions and Warnings

Probe changes

*	New probe.
¥	Probe changed in this product version. Minor alteration, no change in sequence detected.

Probe warnings

¬	Flanking probe, included to help determine the extent of a deletion/duplication. Copy number alterations of flanking probes are unlikely to be related to the condition tested.
±	The presence of single nucleotide variants (SNVs) rs577606722 and rs944662889 can affect the probe signal (in digested and/or undigested reactions).
«	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.
π	Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal. The MR of this probe should be checked to ensure digestion was complete.
‡	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.

Probemix-specific precautions

1. This product is not known to contain any harmful agents. Based on the concentrations present, none of the ingredients are hazardous as defined by the Hazard Communication Standard. **A Safety Data Sheet (SDS) is not required for this product**: none of the ingredients contain dangerous substances at concentrations requiring distribution of an SDS (as per Regulation (EC) No 1272/2008 [EU-GHS/CLP] and 1907/2006 [REACH] and amendments).
2. Sample or technical artefacts may appear as a (mosaic) copy number change of the whole/partial gene. Whole/partial gene deletions or duplications should therefore be confirmed by analysis of an independent DNA sample, to exclude false positive results.
3. Methylation and CN status determined by only a single probe always require confirmation by another method.
4. Small changes (e.g. SNVs, small indels) in the sequence targeted by a probe can cause false positive results, even when >20 nt from the probe ligation site. They can reduce the probe signal by preventing ligation of the probe(s) or by destabilising the binding of a probe oligonucleotide to the sample DNA. SNVs rs577606722 and rs944662889 mentioned in this document require additional caution. Please note that any probe can be affected by known or novel SNVs. Sequence changes within a HhaI site can interfere with HhaI digestion and may result in a false positive methylation signal. Sequencing of the target region is recommended. Please contact MRC Holland for more information: info@mrcholland.com.
5. Copy number alterations of reference probes are unlikely to be related to the condition tested.
6. Results of methylation-specific probes tested on chorionic villi samples (CVS) might not reflect the actual epigenetic constitution of the fetus because the locus of interest might not have reached its final imprinting status in CVS. Therefore, only copy numbers can be determined when MS-MLPA is used on DNA extracted from CVS. DNA extracted from prenatal samples can be used **for research use only**.
7. Non-specific peaks were sporadically observed in no-DNA reactions performed on this probemix. Their number and height is greatly reduced by not spinning down the MLPA reactions in between the ligation and PCR reaction. The non-specific peaks are not expected to influence results.

Technique-specific precautions
See the MS-MLPA General Protocol.

8. Limitations

Probemix-specific limitations

1. Molecular causes of RSS other than UPD7mat will not be detected by this probemix.
2. No discrimination between uniparental disomy (UPD) and imprinting defects can be made with this probemix. For this, it is necessary to perform microsatellite analysis in the patient and parents.

Technique-specific limitations
See the MS-MLPA General Protocol.

9. References Cited in this IFU

1. Cerrato F et al. (2020). DNA methylation in the diagnosis of monogenic diseases. *Genes* (Basel). 11:355.
2. Hoffmann K and Heller R et al. (2011). Uniparental disomies 7 and 14. *Best Pract Res Clin Endocrinol Metab*. 25:77-100.
3. Mackay D et al. (2022). First step towards a consensus strategy for multi-locus diagnostic testing of imprinting disorders. *Clin Epigenetics*. 14:143.
4. Monk D et al. (2018). Recommendations for a nomenclature system for reporting methylation aberrations in imprinted domains. *Epigenetics*. 13:117-21.

Implemented changes in the product description
Version B1-05 – 29 January 2025 (03S) - Product description updated to a new template. - Intended purpose was updated to specify that the assay is manual. Removal of references to buccal swab, saliva, testing of at-risk family members, and confirming a clinical diagnosis of RSS and KOS. - Information about prenatal samples added to Probemix-specific precautions. - Performance characteristics updated. Clinical validity updated in line with new intended purpose. - The MIR380 probe at 247 nt annotated as flanking probe. - *GRB10* exon numbering updated according to MANE. - Probemix is now IVDR certified.