Instructions for Use

SALSA® MLPA® Probemix P165 HSP mix-1

See also the MLPA General Protocol, and the product description of the SALSA® MLPA® Reagent Kit and the Coffalyser.Net Reference Manual.

Visit the SALSA® MLPA® Probemix P165 HSP mix-1 product page on our website to find Certificates of Analysis and a list of related products.

Product Name	**SALSA® MLPA® Probemix P165 HSP mix-1**
Version	C3
Catalogue numbers	P165-025R (25 reactions) P165-050R (50 reactions) P165-100R (100 reactions)
Basic UDI-DI:	872021148P16565
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

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

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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:

C3 version compared to C2 version

One reference probe has been replaced. Five target probes have had a small change in length but no change in sequence detected.

1. Intended Purpose

The SALSA MLPA Probemix P165 HSP mix-1 is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative manual assay[2] for the detection of deletions and duplications in the *SPAST* gene, in order to confirm a potential cause for and clinical diagnosis of spastic paraplegia (SPG) type 4, and for molecular genetic testing of at-risk family members[3]. This assay is for use with genomic DNA isolated from human peripheral whole blood specimens.

Copy number variations (CNVs) detected with P165 HSP mix-1 should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Most defects in *SPAST* are point mutations, none of which will be detected by MLPA. It is therefore recommended to use this assay in combination with sequence analysis.

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, 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, e.g from DNA extracted from formalin-fixed paraffin embedded (FFPE) or fresh tumour materials.

[1] Please note that this probemix is for in vitro diagnostic (IVD) use in the countries specified on page 1 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 and Coffalyser.Net analysis software.

[3] Probes targeting the *ATL1* gene may only be used in a research setting. The following table summarises which probes are for IVD use or exclusively restricted to be used in a research setting:

IVD Target	RUO Target
CNVs: *SPAST*	CNVs: *ATL1*

2. Sample Requirements

Specimen	50-250 ng purified human genomic DNA, free from heparin, 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 and treatment as similar as possible in all test and reference samples. • Have a normal copy number 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 spastic paraplegia.	
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, 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 samples.

3. Test Procedure

See the 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 Results of Reference Probes

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

Typical Results of Probes Targeting Two Copies (*ATL1* and *SPAST*)

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 probe 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.

6. Performance Characteristic

<table>
<tr><th>Study</th><th>Description</th></tr>
<tr><td>Expected values for copy number in normal and affected populations</td><td>To determine the expected values in normal and affected populations a study was conducted on over 1500 MLPA reactions using 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 table in section 5 of this product description can be used. Cut-off values for copy number determination were verified with SALSA MLPA Probemix P165 HSP mix-1 in 21 samples from healthy individuals with normal copy numbers and three samples with known CNVs. The expected FRs for the corresponding copy number were found in all samples tested.</td></tr>
<tr><td>Limit of Detection</td><td>A study using representative probemixes 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. These lower and higher limits of detection were verified using SALSA MLPA Probemix P165 HSP mix-1 on two samples with known CNVs status and on one sample without any aberrations and the expected FRs for the corresponding copy number were obtained using both the lower and upper input amount of DNA.</td></tr>
<tr><td>Interfering substances</td><td>SNPs or other polymorphisms (e.g. indels) in the DNA target sequence and impurities in the DNA sample (e.g. NaCl or KCl, EDTA and hemoglobin) can affect the MLPA reaction.

A study using SALSA MLPA Probemix P165 HSP mix-1 was performed to assess the potential for interference of endogenous and exogenous substances (at the concentrations shown in the table below) on genomic DNA in samples with known CNVs status.
<table>
<tr><th>Interferent</th><th>Source</th><th>Testing Concentration</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 111/120 measurements</td></tr>
<tr><td>NaCl</td><td>Exogenous – DNA extraction</td><td>40 mM</td><td>Copy number: Expected FR for 111/120 measurements</td></tr>
<tr><td>Fe^{3+} ($FeCl_3$)</td><td>Exogenous – DNA extraction</td><td>1 µM</td><td>Copy number: Expected FR for 120/120 measurements</td></tr>
<tr><td>Heparin</td><td>Exogenous – specimen collection tubes</td><td>0.02 U/mL</td><td>Copy number: Expected FR for 116/120 measurements</td></tr>
<tr><td>Hemoglobin</td><td>Endogenous – blood sample</td><td>0.02 µg/µl</td><td>Copy number: Expected FR for 86/120 measurements</td></tr>
</table>
* Results are summarised for all probes across the two samples tested. In addition artificial DNAs have been tested and showed concordant results.

Endogenous (hemoglobin) and exogenous interfering substances (EDTA, heparin, salts (NaCl), and $FeCl_3$) were tested and shown to have mild, severe or no effects on the P165 HSP mix-1 results. At most, these substances led to ambiguous ratios and potential delayed results in the case of EDTA, NaCl and heparin. Hemoglobin led to incorrect ratios and, consequently, to false positive/negative results.

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.

To assess the potential for binding of non-specific targets from human genomic DNA, in silico analysis of the probes used in the assay was performed using Human BLAT Search. All probes in SALSA MLPA Probemix P165 HSP mix-1 were found to be specific.

To support the above in silico data, quality tests on 42 wildtype samples, and two samples with known SPAST CNVs were performed to determine whether probes are specific to their target sequence. 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, three previously genotyped samples were tested using SALSA MLPA Probemix P165 HSP mix-1 and found to have the expected results. Assay precision was tested by repeatedly testing samples with known copy number status over multiple days, and by multiple operators. Results showed a correct call in 599/600 data points, leading to a precision of >99%. In addition artificial DNAs have been tested and showed concordant results (precision >99%).</td></tr>
<tr><td>Clinical validity*</td><td>SPAST: 20-25% of Spastic paraplegia 4 (SPG4) is caused by deletions and duplications in SPAST (Beetz et al. 2006; D'Amore et al. 2018; Depienne et al. 2006; Kadnikova et al. 2019).

*(Based on a 2018-2023 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 – Probe Details Sorted by Chromosomal Position

Chr. position	Target	Exon	Distance to next probe	Length (nt)	Probe number	Warnings
2p22.3	*SPAST*	Upstream (Exon 1)	0.4 kb	472	07279-L21088	Ø
2p22.3	*SPAST*	Exon 1	0.1 kb	142	22526-L31678	
2p22.3	*SPAST*	Exon 1	23.5 kb	235	05265-L04648	
2p22.3	*SPAST*	Exon 2	2.0 kb	161	11061-L21076	
2p22.3	*SPAST*	Exon 3	9.3 kb	184	05949-L05391	
2p22.3	*SPAST*	Exon 4	15.9 kb	196	05268-L04651	
2p22.3	*SPAST*	Exon 5	1.0 kb	214	22527-L31867	
2p22.3	*SPAST*	Exon 6	0.4 kb	463	05950-L05394	
2p22.3	*SPAST*	Exon 7	10.8 kb	269	05270-L21080	
2p22.3	*SPAST*	Exon 8	1.5 kb	296	05271-L21084	
2p22.3	*SPAST*	Exon 9	8.1 kb	319	05272-L04646	
2p22.3	*SPAST*	Exon 10	0.3 kb	346	05273-L04655	
2p22.3	*SPAST*	Exon 11	0.2 kb	208	05951-L05395	
2p22.3	*SPAST*	Exon 12	4.8 kb	400	05274-L04656	
2p22.3	*SPAST*	Exon 13	1.5 kb	137	05952-L05396	
2p22.3	*SPAST*	Exon 14	1.6 kb	364	05953-L05397	
2p22.3	*SPAST*	Exon 15	2.3 kb	427	05275-L04657	
2p22.3	*SPAST*	Exon 16	7.2 kb	445	20720-L28598	
2p22.3	*SPAST*	Exon 17	1.9 kb	241	05658-L05111	
2p22.3	*SPAST*	Exon 17		173	07128-L06737	
14q22.1	*ATL1*	Upstream (Exon 1)	0.1 kb	282	20719-L28597	Ø
14q22.1	*ATL1*	Upstream (Exon 1)	26.7 kb	263	17303-L20798	Ø
14q22.1	*ATL1*	Exon 1 (2b)	0.2 kb	154	05277-L26403	+
14q22.1	*ATL1*	Exon 1 (2b)	27.6 kb	382	05278-L04645	
14q22.1	*ATL1*	Exon 2 (3)	3.1 kb	166	05279-L31680	
14q22.1	*ATL1*	Exon 3 (4)	0.6 kb	178	05280-L04661	
14q22.1	*ATL1*	Exon 4 (5)	2.3 kb	202	05281-L04662	
14q22.1	*ATL1*	Exon 5 (6)	1.7 kb	220	05282-L04663	+
14q22.1	*ATL1*	Exon 6 (7)	17.7 kb	247	05283-L04664	
14q22.1	*ATL1*	Exon 7 (8)	1.1 kb	276	05284-L21081	
14q22.1	*ATL1*	Exon 8 (9)	6.3 kb	302	05285-L04666	
14q22.1	*ATL1*	Exon 9 (10)	1.2 kb	327	05286-L04667	
14q22.1	*ATL1*	Exon 10 (11)	1.3 kb	355	05287-L05110	
14q22.1	*ATL1*	Exon 11 (12)	4.9 kb	373	05288-L04644	
14q22.1	*ATL1*	Exon 12 (13)	4.2 kb	409	05289-L21085	
14q22.1	*ATL1*	Exon 14 (15)		436	05290-L21086	
1p	Reference			310	13275-L14608	
2q	Reference			391	04530-L03919	
3q	Reference			481	03328-L02715	
5q	Reference			130	00797-L00463	
7q	Reference			228	08007-L07788	
8q	Reference			149	10056-L10480	
9q	Reference			254	13128-L14348	
12q	Reference			290	02338-L21083	
17q	Reference			454	03856-L03307	
18q	Reference			190	16424-L18877	
20p	Reference			337	07930-L07660	

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 *SPAST* and *ATL1* exon numbers are derived from 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. Annotation of one probe with a target at the edge of or slightly outside the coding region, is altered. The exon numbering from the previous version of this Product Description is disclosed between brackets.
Chromosomal bands are based on: hg18.

7. Precautions and Warnings

Probe warnings

Ø These probes target sequences outside of the known coding region of the MANE Select transcript. Copy number alterations of only these probes are of unknown clinical significance.

\+ The ligation site of these probes is >20 nt away from the nearest exon. For more information, download the probe sequences document available on the product page on www.mrcholland.com.

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. 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. Sequence changes 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. Deviations detected by this product should be confirmed, and single-probe deviations always require confirmation. Sequencing of the target region is recommended. Please contact MRC Holland for more information: info@mrcholland.com.
4. Copy number alterations of reference probes are unlikely to be related to the condition tested.

Technique-specific precautions
See the MLPA General Protocol.

8. Limitations

Probemix-specific limitations
1. Target probes for *ATL1* CNVs are included to be used for research purposes only and not for diagnostic use.
2. No probe for *ATL1* exon 13 is present.

Technique-specific limitations
See the MLPA General Protocol.

9. References Cited in this IFU

1. Beetz C et al. (2006). High frequency of partial SPAST deletions in autosomal dominant hereditary spastic paraplegia. *Neurology.* 67:1926-1930.
2. D'Amore A et al. (2018). Next Generation Molecular Diagnosis of Hereditary Spastic Paraplegias: An Italian Cross-Sectional Study. *Front Neurol.* 9:981.
3. Depienne C et al. (2006). Exon deletions of SPG4 are a frequent cause of hereditary spastic paraplegia. *Journal of medical genetics.*
4. Kadnikova VA et al. (2019). Mutational Spectrum of Spast (Spg4) and Atl1 (Spg3a) Genes In Russian Patients With Hereditary Spastic Paraplegia. *Sci Rep.* 9:14412.

Implemented changes in the product description

Version C3-04 – 21 March 2025 (03S)
- The product description has been updated to a new template.
- The intended purpose has been updated removing *ATL1* gene as IVD target and specifying assay is manual.
- Percentage of SPG4 cases explained by deletions or duplications in *SPAST* gene updated.
- Exon numbering for all probes targeting the *ATL1* gene updated.
- Description of probe targets at the edge of or slightly outside the coding region has been adjusted. No change in actual target sites.
- SNV(s) rs200452381 and rs145204580 can affect the probe signal. However, the warnings for these SNVs present in previous product description versions have been replaced by a general warning for small sequence changes, included in section Precautions and Warnings.
- Warning for a ligation site >20nt away from the nearest exon added to probes 05277-L26403 and 05282-L04663.
- Warning for probes located outside of coding region added to probes: 07279-L21088, 20719-L28597 and 17303-L20798.
- Probemix is now IVDR-certified