Instructions for Use
SALSA® MLPA® Probemix P060 SMA Carrier

See also the MLPA General Protocol, the product descriptions of the SALSA® MLPA® Reagent Kit and SALSA® Reference Selection DNA SD082, and the Coffalyser.Net Reference Manual.

Visit the SALSA® MLPA® Probemix P060 SMA Carrier product page on our website to find Certificates of Analysis and a list of related products.

Product Name	**SALSA® MLPA® Probemix P060 SMA Carrier**
Version	**B2**
Catalogue numbers	**P060-025R (25 reactions)** **P060-050R (50 reactions)** **P060-100R (100 reactions)**
Basic UDI-DI:	**872021148P0605N**
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® Reference Selection DNA SD082	SD082

Comparison of MRC Holland SMA products
MRC Holland offers four different assays for SMA that fit the complete range of genetic testing needs. A comparison of MRC Holland SMA products, indicating which product can best be used for which purpose, can be found at www.mrcholland.com.

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 COLOMBIA ISRAEL COSTA RICA
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
As compared to version B1, the 88 and 96 nt DNA denaturation control fragments have been replaced (QDX2).

1. Intended Purpose

The SALSA MLPA Probemix P060 SMA Carrier is an in vitro diagnostic (IVD)[1] or research use only (RUO) manual semi-quantitative assay[2] for the detection of deletions or duplication in exons 7 and 8 of the *SMN1* gene[3] in genomic DNA isolated from human peripheral whole blood specimens.

P060 SMA Carrier is intended to establish or confirm a potential cause for and clinical diagnosis of Spinal Muscular Atrophy (SMA) (by detecting a homozygous *SMN1* exon 7 deletion) and of SMA carriership (by detecting a heterozygous *SMN1* exon 7 deletion), and for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with P060 SMA Carrier should be confirmed with a different technique. In particular, CNVs detected by the *SMN1* exon 7 probe always require confirmation by another method. Point mutations, which cause SMA in a small number of cases, will not 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, 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 screening, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1] Please note that this probemix is for in 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 and Coffalyser.Net analysis software.

[3] Certain probes targeting additional genes included in P060 SMA Carrier may only be used in a research setting. The following table summarises which probes are for IVD or exclusively restricted to RUO use:

	IVD target	RUO target
P060	*SMN1*	*SMN2*

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 • Selection of reference samples is important for correct determination of *SMN1* copy numbers. • Extraction method, tissue type, DNA concentration (and) treatment as similar as possible in all test and reference samples. • Have an *SMN1* and *SMN2* copy number of two 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 SMA.	
Reference Selection DNA	• SALSA Reference Selection DNA SD082, provided by MRC Holland. • Use SD082 to facilitate the selection of suitable reference samples. • **SD082 should never be used as a reference sample.**	
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 various 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 (*SMN1*/*SMN2*)

Final Ratio (FR)	Copy Number	Description
0*	0	Homozygous deletion (patients)
0.40 – 0.65	1	Heterozygous deletion (carriers)
0.80 – 1.20	**2**	**Normal**
1.30 – 1.65	3	Heterozygous duplication
> 1.75#	4	Copy numbers ≥4
All other values	-	Ambiguous

* There is a high sequence similarity between *SMN1* and *SMN2* exon 7 and 8. In case of a homozygous deletion of one of the probe targets, a small background signal can still be visible. This is caused by nonspecific binding of these probes to the corresponding exon of the homologous gene. This background signal might be displayed as an intra ratio percentage instead of a Final Ratio (see our support portal for more details).

SMN2 probes in this probemix cannot reliably distinguish between four or more copies. A final ratio of > 1.75 for SMN2 probes should be interpreted as four or more copies. SALSA MLPA Probemix P021 SMA can be used for a more accurate *SMN2* copy number determination.

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.

In case of a gene conversion event whereby a different copy number is found for *SMN1* exon 7 compared to *SMN1* exon 8, *SMN2* exon 7 and 8 copy numbers serve only as an interpretation aid of the *SMN1* copy numbers. The table of positive samples on the probemix product page on our website shows multiple examples of samples where gain or loss of *SMN1* copy numbers corresponds with loss or gain of *SMN2* copy numbers, respectively, thereby confirming *SMN1* copy number results. Gene conversions in samples with copy number of four or more cannot be determined by SALSA MLPA Probemix P060 SMA Carrier.

For a detailed interpretation guide, see the Appendix.

6. Performance Characteristics

Study	Description
Expected values for CNVs in normal and affected populations	To determine the expected values 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 reference probe over all the reference samples is ≤0.10, the FRs stated in the copy number table above (page 3) can be used. Standard Cut-off values were verified with SALSA MLPA Probemix P060 SMA Carrier. Five samples were used to cover the range of copy numbers presented in section 5. Table: Typical Results of Probes Targeting Two Copies (*SMN1*/*SMN2*). This resulted in adjustments of the defined cut-off values for 0 copies and 4 or more copies as clarified in this table.
Limit of detection	To determine the analytical sensitivity, or Limit of Detection (LOD), 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.
Interfering substances	SNVs or other polymorphisms (e.g. indels) in the DNA target sequence and impurities in the DNA sample (e.g. NaCl or KCl (>40 mM), EDTA and haemoglobin) can affect the MLPA reaction. 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. Endogenous (haemoglobin (0.02 µg/µL)) and exogenous interfering substances (EDTA (1.5 mM), heparin (0.02 U/mL), salts (NaCl (40 mM))) were tested and shown to have a mild effect on the probemix results, leading to at the most, ambiguous ratios and potential delayed results. Interfering substance $FeCl_3$ (1 µM) was also tested and resulted in a wrong result only for the SMN2 exon 8 probe in one of the tested samples. As this probe is not relevant for patient or carrier detection the adverse effect of $FeCl_3$ contamination is minimal. In general the SMN2 probes were affected by interfering substances, showing an increase in FR as a result of EDTA contamination and reduced FRs as a result of NaCl, $FeCl_3$, heparin and haemoglobin in certain samples. The only exception was a decrease in FR for the SMN1 exon 7 probe as a result of haemoglobin contamination resulting in an ambiguous result, which in turn would not lead to a false result, but could result in a delayed result.
Cross-reactivity	Cross-reactivity is the potential for probes to bind to homologous regions (e.g. pseudogenes) or other cross-reactive sequences. Samples (n=57) with known SMN copy numbers were tested and expected copy numbers were found, attesting to the specificity of the probes in P060 SMA Carrier. 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. The *SMN1* and *SMN2* genes are highly homologous and the only two exon sequence differences are targeted by the two probes (for each gene respectively) in this product.
Accuracy	Results of accuracy are derived from trueness and precision studies. For trueness, previously genotyped samples were tested and found to have the expected results (see also “Cross-reactivity”). For precision studies, five samples were tested in triplicate by three separate operators and over three days by a single operator. The results are not affected by operator or day except for one measurement out of 100 where an ambiguous result was found for an SMN2 probe. This probe is not relevant for patient or carrier detection.
Clinical validity*	SMA patients: 92.5-100% of SMA is caused by a homozygous deletion of *SMN1* exon 7. SMA carriers: 90.5-100%, depending on ethnicity, of SMA is caused by a heterozygous deletion (one copy remaining) of *SMN1* exon 7. (Cao et al. 2020, Sun et al. 2020, Zhao et al. 2022, Davidson et al. 2023) * Based on a 2005-2023 literature review

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	Warning
5q13.2	*SMN2*	Exon 7	0.8 kb	282	14921-L17083	∫
5q13.2	*SMN2*	Exon 8	> 100 kb	301	14878-L17084	∫
5q13.2	*SMN1*	Exon 7	0.7 kb	183	14919-L17081	∫
5q13.2	*SMN1*	Exon 8		218	14881-L17082	∫
1q	Reference			208	12490-L17096	
3p	Reference			163	02291-L17086	
3q	Reference			292	00824-L17097	
4q	Reference			172	02978-L17087	
4q	Reference			272	14361-L17098	
5q	Reference			154	02595-L17085	
6p	Reference			311	06425-L17092	
6q	Reference			342	13399-L17297	
8q	Reference			331	01043-L17094	
8q	Reference			321	01042-L17093	
9q	Reference			255	13128-L17099	
10q	Reference			264	07630-L17091	
11p	Reference			200	00976-L17298	
11q	Reference			191	00559-L17088	
12q	Reference			237	02334-L17301	
15q	Reference			245	14293-L17100	
20p	Reference			228	14498-L17101	

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 *SMN1* and *SMN2* exon numbering used in this product description and lot-specific Coffalyser.Net analysis sheet is the traditional exon numbering (exons 1, 2a, 2b, and 3-8). This exon numbering is different from the MANE select transcripts. For more information, see the probe sequences document available on the product page at www.mrcholland.com.
Chromosomal bands are based on: hg18

7. Precautions and Warnings

Probe warnings

∫ Due to the high sequence similarity between *SMN1* and *SMN2* exon 7 and 8, a small background signal can be visible in homozygous deletion samples (see section **Interpretation of Results** for more information).

Probemix-specific precautions

1. This product should not be used to determine *SMN2* copy numbers for the purpose of determining disease prognosis or eligibility for SMA therapeutics. For the most reliable *SMN2* copy number detection, use SALSA MLPA Probemix P021 SMA (from version B1 onwards).
2. Determining *SMN2* copy number is not relevant for SMA carrier testing.
3. Copy number quantification by P060 SMA Carrier is completely dependent on a correct selection of reference samples. Before testing patient samples, testing of samples from healthy individuals is required to identify suitable reference samples for proper data analysis.
4. Individual MLPA probes can be affected differently by changes in experimental procedures or impurities in samples leading to false positive results. Highly unlikely results such as an unusually high frequency of *SMN1* exon 7 loss (carrier) or *SMN1* exon 7 gain, without loss or gain of the exon 8 probe in most of these samples, should be treated with caution.
5. In 5-10% of all cases, the SMN1 (218 nt) and SMN2 (301 nt) **exon 8** probes will show a different copy number compared to the SMN1 (183 nt) and SMN2 (282 nt) **exon 7** probes e.g. due to gene conversion. In these cases, the copy number of *SMN1* is only determined by the exon 7 probe. The nucleotide difference targeted by these exon 8 probes is not clinically relevant.
6. The presence of more than two *SMN1* copies in healthy individuals is a relatively frequent phenomenon, especially in those of African descent (Hendrickson et al. 2009; Sangaré et al. 2014).
7. Complete absence of *SMN2*, as determined by the SMN2-specific exon 7 probe (282 nt), is a relatively common phenomenon in healthy individuals and has no known clinical consequences.
8. Carrier frequency is strongly population-dependent: in a survey by Hendrickson et al. (2009), the one *SMN1* copy frequency in the US was estimated to be 1:37 for Caucasians, 1:46 for Ashkenazi Jews, 1:56 for Asians, 1:91 for African Americans and 1:125 for Hispanics.
9. Analysis of parental samples may be necessary for correct interpretation of complex results.
10. 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).
11. 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.

12. 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.
13. 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. In ~95% of the Caucasian population, the cause of disease is a homozygous deletion of exon 7. In the remainder of cases, disease is caused by other *SMN1* aberrations.
   In the remaining 5%, the majority of defects will be small sequence changes such as point mutations in the *SMN1* gene. MLPA will not detect these. Detection of small sequence changes is possible by DNA sequencing, but is complicated by the presence of *SMN2* copies. A small number of patients have a deletion of other exons, in particular exons 1-6. These can be detected with the SALSA MLPA Probemix P021-B1 SMA.
2. For carrier screening, false negative results can be obtained. The presence of two *SMN1* copies per cell suggests that the person tested is not a carrier. However, this test result can also be due to:
   a. One *SMN1* copy carries a point mutation or a deletion of other exons than exon 7.
   b. The presence of two *SMN1* copies on one chromosome and zero on the other (2+0 genotype), in which case the person tested is in fact a SMA carrier. MLPA is not able to determine whether the two *SMN1* copies are on the same or on different chromosomes. The frequency of this 2+0 genotype varies per population (Hendrickson et al. 2009). MLPA probemix P460 SMA detects two polymorphisms (described by Luo et al. 2014; Alias et al. 2018) that are associated with an increased risk of individuals being 2+0 carriers.
3. SMN2 probes in this probemix cannot reliably distinguish between 4 or more copies.
4. Prenatal samples are included to be used for research purposes only and not for diagnostic use.
5. SALSA MLPA Probemix P021 SMA and SALSA MLPA Probemix P460 SMA (Silent) Carrier cannot be used for confirmation of results.

Technique-specific limitations
See the MLPA General Protocol.

9. References Cited in this IFU

1. Cao Y et al. (2020). Transmission characteristics of SMN from 227 spinal muscular atrophy core families in China. J *Hum Genet*. 65:469-473.
2. Davidson JE et al. (2023). The Carrier Frequency of Two *SMN1* Genes in Parents of Symptomatic Children with SMA and the Significance of *SMN1* Exon 8 in Carriers. *Genes*. 14:1403.
3. Hendrickson BC et al. (2009). Differences in *SMN1* allele frequencies among ethnic groups within North America. *J Med Genet*. 46:641-644.
4. Sangaré M et al. (2014). Genetics of low spinal muscular atrophy carrier frequency in sub-Saharan Africa. *Ann Neurol*. 75:525-532
5. Sun Y et al (2020). Mutation analysis of 419 family and prenatal diagnosis of 339 cases of spinal muscular atrophy in China. *BMC Med Genet*. 18;21(1):133.
6. Zhao S et al. (2022). Next generation sequencing is a highly reliable method to analyse exon 7 deletion of survival motor neuron 1 (*SMN1*) gene. *Sci Rep*. 12:1-9.

Implemented changes in the product description
Version B2-13 – 04 August 2025 (03S) - Explanation of background signal corrected in footnote under table in Interpretation of Results section. - Warnings for background signal of probes 14921-L17083, 14878-L17084, 14919-L17081 and 14881-L17082 added. - Minor textual adjustments. *Version B2-12 – 29 January 2025 (03S)* - Product description adapted to new template. - Intended purpose updated, specifying the assay is manual. Prenatal samples removed, as well as the function for *SMN2* copy number determination. - The probes targeting *SMN2* are no longer intended for diagnostic use. - Probemix is now IVDR certified.

10. Appendix

General points on interpretation of results

The SMN region on chromosome 5q13 is highly variable, leading to frequent deletions, duplications and gene conversions. For a correct interpretation of results, the following information information as well as the precautions and limitations as stated in sections 7 and 8 are is important:

1. The exon 7 difference between the *SMN1* and *SMN2* genes, as targeted by the 183 and 282 nt probes respectively, is the only clinically relevant difference between these two genes.

SMA Patients

2. 95% of the (Caucasian) SMA patients have no *SMN1* copies, as shown by a complete absence of the SMN1-specific exon 7 (183 nt) probe amplicon.
3. Table A shows examples of various scenarios, including expected results for SMA patients and carriers, as well as the corresponding final ratio and copy numbers.

SMA Carriers

4. A healthy individual with a single *SMN1* exon 7 copy (as determined by the SMN1-specific exon 7 probe at 183 nt) is a SMA carrier.

Table A. Overview of expected results and the corresponding conclusions

Finding	Conclusion	Explanation
- *SMN1* exon 7: 0 copies. - *SMN1* exon 8: 0 copies. SMA symptoms	SMA patient	*SMN1* is absent, as no copies of the distinct *SMN1* exon 7 are present. The absence of both *SMN1* exon 8 copies confirms this.
- *SMN1* exon 7: 0 copies - *SMN1* exon 8: > 0 copies SMA symptoms	SMA patient	*SMN1* is absent, as no copies of the determining *SMN1* exon 7 sequence are found. In 5-10% of cases, the *SMN1* exon 8 copy number does not correspond to the *SMN1* exon 7 copy number, e.g. due to gene conversion. See section 7, precaution 5.
- *SMN1* exon 7: 1 copy. SMA symptoms	SMA patient	Most likely a case of compound heterozygosity caused by either a point mutation or a deletion of other exons in the remaining *SMN1* copy. Sequencing might reveal a defect in the remaining *SMN1* copy.
- *SMN1* exon 7: 1 copy. - *SMN1* exon 8: 1 copy. No SMA symptoms	SMA carrier	One copy of *SMN1* is absent, making the person a carrier. The absence of one copy of the *SMN1* exon 8 sequence confirms this.
- *SMN1* exon 7: 1 copy. - A: *SMN1* exon 8: ≠ 1 copies. - B: *SMN1* exon 8: 0 copies. No SMA symptoms	SMA carrier	One copy of *SMN1* is absent, making the person a carrier. In 5-10% of cases, the *SMN1* exon 8 copy number does not correspond to the *SMN1* exon 7 copy number, e.g due to gene conversion. See section 7, precaution 5.
- *SMN1* exon 7: 2 copies. No SMA symptoms	Most likely not a SMA carrier	Most likely, this individual is not a carrier. However, there is a residual risk that both *SMN1* copies lie on one allele. See section 8, limitation 2.