Instructions for Use
SALSA® MLPA® Probemix P021 SMA

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

Visit the SALSA® MLPA® Probemix P021 SMA product page on our website to find Certificates of Analysis and a list of related products.

Product Name	**SALSA® MLPA® Probemix P021 SMA**
Version	B1
Catalogue numbers	P021-025R (25 reactions) P021-050R (50 reactions) P021-100R (100 reactions)
Basic UDI-DI:	872021148P0215C
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 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	-25°C – -15°C	

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 MOROCCO
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
As compared to version A2, four SMN probes were replaced, thirteen SMN probes were added, one NAIP probe was removed, nine reference probes were replaced, twelve reference probes were removed, five flanking probes were removed and two probe lengths were adjusted.

1. Intended Purpose

The SALSA MLPA Probemix P021 SMA is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative manual assay[2] for the detection of deletions in the *SMN1* gene and deletions and duplications in the *SMN2* gene[3]. P021 SMA is intended to establish or confirm a potential cause for and clinical diagnosis of Spinal Muscular Atrophy (SMA) in genomic DNA isolated from human peripheral whole blood specimens or neonatal Dried Blood Spot (DBS) cards by identifying a homozygous *SMN1* deletion.

Secondly, carrier testing and molecular genetic testing of at-risk family members can be done by determining *SMN1* copy numbers, and P021 SMA can be used as an aid in prognosis and for treatment eligibility by determining *SMN2* copy numbers in (pre-symptomatic) SMA patients using genomic DNA isolated from human peripheral whole blood specimens.

In the majority of SMA patients (~95%), the disease is caused by a homozygous loss of the *SMN1* gene, usually detected by the absence of exon 7 specific markers. In a small number of SMA cases, the causative defect concerns a loss of other exon(s) of *SMN1*. Both defects can be detected by SALSA MLPA Probemix P021 SMA. Copy number variations (CNVs) detected with P021 SMA should be confirmed with a different technique. In particular, deletions 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, prenatal testing, 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 P021 SMA may only be used in a research setting. The following table summarises which probes are for IVD or exclusively restricted to RUO use:

	IVD targets	**RUO target**
P021	*SMN1*, *SMN2*	*NAIP*

2. Sample Requirements

Specimen	Human peripheral whole blood • 50-250 ng purified human genomic DNA, dissolved in 5 µl $TE_{0.1}$ buffer, pH 8.0-8.5 DBS card • 3.2 mm punch
Collection method	Human peripheral whole blood • Standard methods DBS card • Whatman 903 paper
Extraction method	Methods tested by MRC Holland: Human peripheral whole blood • QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual) • Promega Wizard Genomic DNA Purification Kit (manual) • salting out (manual) DBS card • NaOH based extraction (manual, for more details see Appendix 2)

<table>
<tr><th colspan="3">Sample types</th></tr>
<tr><td>Test sample</td><td colspan="2">• Provided by user</td></tr>
<tr><td>Reference samples (required)</td><td colspan="2">• Provided by user
• Correct determination of SMN1 and SMN2 copy numbers is completely dependent on the selection of suitable reference samples
• Reference samples must have an SMN1, SMN2 and NAIP copy number of two, no SMN1/2Δ7-8 copies, a final ratio of ~1.0 for all probes and ≤0.10 standard deviation for all probes
• Because of the occurrence of SMN1 duplications and SMN2 deletions in the healthy population, identifying suitable reference samples can be challenging
• Extraction method, tissue type, DNA concentration, (and) treatment as similar as possible in all test and reference samples
• 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</td></tr>
<tr><td>Reference Selection DNA</td><td colspan="2">• 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</td></tr>
<tr><td>No-DNA control (preferably)</td><td colspan="2">• Provided by user
• $TE_{0.1}$ buffer instead of DNA
• To check for DNA contamination</td></tr>
<tr><td rowspan="2">Positive control samples (preferably)</td><td colspan="2">• Provided by user, or</td></tr>
<tr><td>Available from third parties</td><td>See the table of positive samples on the probemix product page on our website.</td></tr>
</table>

*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.

For a detailed interpretation guide when using DBS card specimens, see Appendix 2.

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

Most probes in P021 SMA detect a sequence present in both the *SMN1* and *SMN2* genes (called **non-specific probes**). In an individual who is diploid for both genes, each of these MLPA probes will therefore detect four copies in total. In contrast, the four MLPA probes that are specific for exon 7 or exon 8 of either *SMN1* or *SMN2* each detect two copies in a diploid individual (and are referred to as **specific probes**).

The expected results for SMN1 (274 nt and 295 nt) and SMN2 (281 nt and 301 nt) specific probes (as well as the NAIP probe 238 nt) are provided in the table below.

Typical Results of Specific Probes Targeting *SMN1, SMN2* and *NAIP*

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
For *SMN2* only 2.20 – 2.65	5	Most probable simultaneous heterozygous duplication and heterozygous triplication
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 (orange box in the ratio chart) instead of a Final Ratio (see our support portal for more details).

Although ratios for single probes can be ambiguous, often the *SMN2* copy number can be determined using the other probes available.

The expected results for the non-specific SMN probes are provided in the table below.

Results of Non-specific Probes Targeting *SMN1*&*SMN2* for SMA patients and carriers

Median* Final Ratio (FR) of non-specific SMN exon 7-8 probes* or exon 1-6 probes*	***SMN2* copy number**	
	No *SMN1* present	**One *SMN1* copy present**
0.15 – 0.35	1	0
0.40 – 0.60	2	1
0.65 – 0.85	3	2
0.90 – 1.10	4	3
1.15 – 1.35	5	4
1.40 – 1.60	6	5
1.65 – 1.85	7	6
All other values	Ambiguous	Ambiguous

* Median value of the seven non-specific SMN probes detecting exons 7 or 8 of both *SMN1* plus *SMN2*.

** Median value of the ten probes detecting exons 1-6 of both *SMN1* plus *SMN2*.

NOTE: In case of an exon 7-8 deletion (*SMN1/2Δ7-8* Vijzelaar et al. 2019), the non-specific SMN exon 7-8 probes versus the exon 1-6 probes will show different median final ratios.

The tables illustrate the relationship between final probe ratio and corresponding copy number. Test results are expected to centre 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.

For a detailed interpretation guide, see Appendix 1.

6. Performance Characteristics

Study	Description
Expected values for copy numbers in normal and affected populations	For genomic blood derived DNA, a total of 518 samples, 483 patient derived DNA samples and 35 reference samples with varying copy numbers of *SMN1* and *SMN2* were tested to check the cut-off values for the expected FRs per copy number across SMN1/2 specific and non-specific probes. The results showed that when the correct reference samples are used and the standard deviation of the reference probes is <0.10, the proposed cut-off values yielded the expected results and could safely be used to determine the FRs. The percentage of correct calls per probe varied between 98.4%-100%. For DBS derived DNA, a study from 15 SMA patients and 62 control samples confirmed the cut-off values determined on genomic DNA are appropriate for use with this sample type, with 100% of correct calls for *SMN1* and 94% of correct calls for *SMN2*.
Limit of detection	To determine the analytical sensitivity, or Limit of Detection (LOD), a study was conducted to validate the minimum and maximum input amounts. For genomic blood derived DNA, results support the use of 50-250 ng of genomic DNA as the recommend input amount. For DBS derived DNA, one 3.2 mm punch was determined to be the best suited to provide accurate results.
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, $FeCl_3$, heparin, EDTA and hemoglobin) can affect the MLPA reaction. A study was performed to assess the potential for interference of endogenous (hemoglobin (0.02 µg/µL) and exogenous (EDTA (1.5 mM), heparin (0.02 U/mL), salts (NaCl (40 mM) and $FeCl_3$ (1 µM)) substances on genomic DNA derived from blood. Certain interferents lead to ambiguous ratios for certain probes. EDTA and NaCl showed a reduction of the FRs for the SMN2 exon 7 probe in a sample with *SMN1*:*SMN2* 0:2 copies, while heparin and $FeCl_3$ showed an increase in the FRs for the SMN1 exon 7 probe in samples with *SMN1*:*SMN2* 0:3 copies and with *SMN1*:*SMN2* 1:4 copies. Hemoglobin showed a reduction of the FRs for multiple SMN1&2 or SMN2 probes in a sample with *SMN1*:*SMN2* 1:4 copies and of the SMN non-specific exon 7-8 probes in a sample with *SMN1*:*SMN2* 1:5 copies. No effect was seen for this interferent for the SMN1 exon 7 probe. Out of 252 probe measurements across all replicates, 1 ambiguous result was observed for each of the following conditions: EDTA, NaCl, $FeCl_3$ and heparin. A total of 12/252 probe measurements across all replicates tested showed an ambiguous result, caused by hemoglobin. For DBS derived DNA, a study was performed to assess the potential for interference of endogenous (hemoglobin (0.08 µg/µl and Bilirubin 0.14 µg/µl) and exogenous (NaOH (1.5 mM, 20 mM and 100 mM)), substances. Only the addition of high amounts of NaOH (100mM) was shown to interfere with the results by completely inhibiting the MLPA reaction. Other substances tested did not affect the results in any of the replicates. 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.
Cross-reactivity	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.
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. For precision studies, results were not shown to be affected by operator, day, or replicate, with a total of 1763/1764 correct calls made, over three different operators, days and repeats, in samples with varying *SMN* copy numbers, leading to a precision of 99.9%. Further accuracy depiction comes from a published study by Milligan et al., 2021 with a comparator method. Three different assays (including P021-B1 SMA) were tested and results showed that across all valid measurements for the three methods, *SMN1* and *SMN2* copy numbers were 98.3% and 99.2% concordant, respectively. When the methods were compared between each other, concordance was 98.4% (95% CI, 94.5%–99.6%) for *SMN1* and 98.4% (95% CI, 94.5%–99.6%) for *SMN2* between P021 SMA and the comparator method.
Clinical validity*	In 95-100% of the cases, SMA is caused by a homozygous deletion of the *SMN1* exon 7 (Wadman et al, 2020, Kekou et al, 2020, Veldhoen et al. 2021). For SMA carriers, ~94% of the cases are caused by heterozygous deletion of the *SMN1* exon 7 (Wadman et al, 2020). These percentages are for the Caucasian population, they will vary based on ethnicity (Hendrickson et al. 2009). In a clinical performance evaluation study on dried blood spot cards from 47 SMA patients and 375 control samples, the diagnostic specificity and sensitivity of the P021 SMA assay were both 100% (Strunk et al. 2019). * Based on a 2005-2021 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	Probe property (exon(s))
5q13.2	*SMN2(+SMN1)*	Upstream (Exon 1)	0.2 kb	382	22196-L31253	Δ ⍩ Ø	SMN Non-specific (1-6)
5q13.2	*SMN2(+SMN1)*	Exon 1	13.7 kb	184	21519-L30024		SMN Non-specific (1-6)
5q13.2	*SMN2(+SMN1)*	Exon 2a	2.5 kb	221	21517-L30022		SMN Non-specific (1-6)
5q13.2	*SMN2(+SMN1)*	Exon 2b	0.1 kb	328	21514-L30019		SMN Non-specific (1-6)
5q13.2	*SMN2(+SMN1)*	Exon 2b	0.9 kb	319	14132-L15557		SMN Non-specific (1-6)
5q13.2	*SMN2(+SMN1)*	Exon 3	0.1 kb	288	21516-L30893		SMN Non-specific (1-6)
5q13.2	*SMN2(+SMN1)*	Exon 3	0.2 kb	346	21513-L30018		SMN Non-specific (1-6)
5q13.2	*SMN2(+SMN1)*	Exon 4	2.0 kb	409	01816-L30922	⍩	SMN Non-specific (1-6)
5q13.2	*SMN2(+SMN1)*	Exon 5	1.4 kb	199	21518-L30023		SMN Non-specific (1-6)
5q13.2	*SMN2(+SMN1)*	Exon 6	5.7 kb	418	22194-L31251		SMN Non-specific (1-6)
5q13.2	*SMN2(+SMN1)*	Intron 6	0.2 kb	193	22121-L31133	Ø	SMN Non-specific (7-8)
5q13.2	*SMN2*	Exon 7	0.1 kb	281	21489-L30892	ß ∫	*SMN2* Specific (7)
5q13.2	*SMN2(+SMN1)*	Exon 7 (Intron 7)	0.1 kb	229	22122-L31134	ß +	SMN Non-specific (7-8)
5q13.2	*SMN2(+SMN1)*	Intron 7	0.1 kb	391	22123-L31135	Ø	SMN Non-specific (7-8)
5q13.2	*SMN2(+SMN1)*	Intron 7	0.1 kb	265	22124-L31136	Ø	SMN Non-specific (7-8)
5q13.2	*SMN2(+SMN1)*	Exon 8 (Intron 7)	0.1 kb	427	22125-L31137	+	SMN Non-specific (7-8)
5q13.2	*SMN2(+SMN1)*	Exon 8	0.1 kb	400	22126-L31138		SMN Non-specific (7-8)
5q13.2	*SMN2(+SMN1)*	Exon 8	0.1 kb	364	01814-L00807		SMN Non-specific (7-8)
5q13.2	*SMN2*	Exon 8	847.7 kb	301	21491-L29984	∫	*SMN2* Specific (8)
5q13.2	*SMN1(+SMN2)*	Upstream (Exon 1)	0.2 kb	382	22196-L31253	Δ ⍩ Ø	SMN Non-specific (1-6)
5q13.2	*SMN1(+SMN2)*	Exon 1	13.7 kb	184	21519-L30024		SMN Non-specific (1-6)
5q13.2	*SMN1(+SMN2)*	Exon 2a	2.5 kb	221	21517-L30022		SMN Non-specific (1-6)
5q13.2	*SMN1(+SMN2)*	Exon 2b	0.1 kb	328	21514-L30019		SMN Non-specific (1-6)
5q13.2	*SMN1(+SMN2)*	Exon 2b	0.9 kb	319	14132-L15557		SMN Non-specific (1-6)
5q13.2	*SMN1(+SMN2)*	Exon 3	0.1 kb	288	21516-L30893		SMN Non-specific (1-6)
5q13.2	*SMN1(+SMN2)*	Exon 3	0.2 kb	346	21513-L30018		SMN Non-specific (1-6)
5q13.2	*SMN1(+SMN2)*	Exon 4	2.0 kb	409	01816-L30922	⍩	SMN Non-specific (1-6)
5q13.2	*SMN1(+SMN2)*	Exon 5	1.4 kb	199	21518-L30023		SMN Non-specific (1-6)
5q13.2	*SMN1(+SMN2)*	Exon 6	5.7 kb	418	22194-L31251		SMN Non-specific (1-6)
5q13.2	*SMN1(+SMN2)*	Intron 6	0.2 kb	193	22121-L31133	Ø	SMN Non-specific (7-8)
5q13.2	*SMN1*	Exon 7	0.1 kb	274	21488-L30891	ß ∫	*SMN1* Specific (7)
5q13.2	*SMN1(+SMN2)*	Exon 7 (Intron 7)	0.1 kb	229	22122-L31134	ß +	SMN Non-specific (7-8)
5q13.2	*SMN1(+SMN2)*	Intron 7	0.1 kb	391	22123-L31135	Ø	SMN Non-specific (7-8)
5q13.2	*SMN1(+SMN2)*	Intron 7	0.1 kb	265	22124-L31136	Ø	SMN Non-specific (7-8)
5q13.2	*SMN1(+SMN2)*	Exon 8 (Intron 7)	0.1 kb	427	22125-L31137	+	SMN Non-specific (7-8)
5q13.2	*SMN1(+SMN2)*	Exon 8	0.1 kb	400	22126-L31138		SMN Non-specific (7-8)
5q13.2	*SMN1(+SMN2)*	Exon 8	0.1 kb	364	01814-L00807		SMN Non-specific (7-8)
5q13.2	*SMN1*	Exon 8	58.7 kb	295	21490-L29983	ß ∫	*SMN1* Specific (8)
5q13.2	*NAIP*	Exon 5		238	01259-L00811		
1q	Reference			310	20763-L28665	ß	
1q	Reference			445	20431-L27913		
2q	Reference			211	18964-L24756		
4q	Reference			247	19086-L24973		
8p	Reference			373	18296-L25750		
9q	Reference			337	19746-L26529		
10p	Reference			256	19625-L26284		
11p	Reference			355	19127-L25074		
17p	Reference			436	19646-L26317		
18q	Reference			175	00808-L00638		

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), as mentioned in OMIM: *SMN1* and *SMN2*. This exon numbering is different from the MANE

select transcripts: NM_000344.4 (*SMN1)* and NM_017411.4 (*SMN2*). For more information, see the probe sequences document available on the product page at [www.mrcholland.com.](http://www.mrcholland.com) Annotations of several probes with targets at the edge of or slightly outside the coding region were altered. The exon numbering of product description version B1-06 is disclosed between brackets.
The *NAIP* exon number is derived from the MANE project and is based on the MANE Select transcript: NM_004536.3.
Note that the exact location of the *SMN* and *NAIP* genes in relation to each other and the orientation of the *SMN2* gene is yet not established with certainty. The table above is based on Figure 2 in the article of Wadman (2020).
Chromosomal bands are based on: hg18

7. Precautions and Warnings

Probe warnings

Δ This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.

ß These probes are sensitive to sample DNA depurination. Use of depurinated DNA will lead to a **lower** signal of this probe. Depurination can occur in acidic conditions, e.g. due to insufficient buffering capacity during sample DNA denaturation. When this occurs only in reference samples, it will result in an increased ratio in the test samples.

⊓ These probes are insensitive to sample DNA depurination. Use of depurinated DNA will lead to a **higher** signal of these probes.

Ø These probes target sequences outside of the known coding region. Copy number alterations of only one of 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 at www.mrcholland.com.

ʃ 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. Copy number quantification by P021 SMA 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.
2. 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.
3. In 5-10% of all cases, the SMN1-specific (295 nt) and SMN2-specific (301 nt) **exon 8** probes will show a different copy number compared to the SMN1-specific (274 nt) and SMN2-specific **exon 7** (281 nt) probes e.g. due to gene conversion. In this case, the copy number of *SMN1* and *SMN2* is only determined by the exon 7 probes. The nucleotide difference that is targeted by these exon 8 probes is not clinically relevant.
4. 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).
5. Complete absence of *SMN2*, as determined by the SMN2-specific exon 7 probe (281 nt), is a relatively common phenomenon in healthy individuals and has no known clinical consequences.
6. One or two extra copies of *SMN* exons 1-6 (*SMN1/2Δ7-8*) are often present, in particular in samples with no, or only one, *SMN2* copy. The frequency of *SMN1/2Δ7-8* is highly population dependent though the clinical significance is not yet clear (Vijzelaar et al. 2019). A frequency of 8% has been reported in Swedish carriers and non-carriers and a frequency of 23% in Spanish carriers and non-carriers. *SMN1/2Δ7-8* copies are very rare in patients (Arkblad et al, 2006; Calucho et al, 2018). (See also Appendix 1, Table B)
7. A homozygous deletion of *NAIP* exon 5 is frequently observed in SMA patients, but is very rare in healthy individuals.
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. Determining the *SMN2* copy number is relevant for patient prognosis, but not for SMA carrier testing.
10. Analysis of parental samples may be necessary for correct interpretation of complex results.
11. 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).
12. 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.
13. 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.
14. Copy number alterations of reference probes are unlikely to be related to the condition tested.

Technique-specific precautions

See the [MLPA General Protocol](http://www.mrcholland.com).

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 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. In a very small number of cases, one *SMN1* exon 7 copy is still present but the remaining part of this *SMN1* copy is affected by a deletion of other *SMN1* exon(s), for instance a deletion

of exons 1-6. Probes for these exons detect both *SMN1* and *SMN2*. A deletion in exons other than exon 7 should therefore only be considered pathogenic if this follows from the individual's clinical context.

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). SALSA MLPA Probemix P460 SMA (Silent) Carrier detects two polymorphisms (described by Luo et al. 2014; Alías et al. 2014) that are associated with an increased risk of individuals being 2+0 carriers.
3. Target probes for *NAIP* CNVs are included to be used for research purposes only and not for diagnostic use.
4. Prenatal samples are included to be used for research purposes only and not for diagnostic use.
5. SALSA MLPA Probemixes P060 SMA Carrier and 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. Alías et al. (2014). Improving detection and genetic counseling in carriers of spinal muscular atrophy with two copies of the *SMN1* gene. *Clin Genet.* 85:470-475.
2. Arkblad EL. et al. (2006). Multiplex ligation-dependent probe amplification improves diagnostics in spinal muscular atrophy. *Neuromuscul Disord.* 16:830-8.
3. Calucho M. et al. (2018). Correlation between SMA type and *SMN2* copy number revisited: an analysis of 625 unrelated Spanish patients and a compilation of 2,834 reported cases. *Neuromuscul Disord* 28:208-15.
4. Hendrickson BC. et al. (2009). Differences in *SMN1* allele frequencies among ethnic groups within North America. *J Med Genet.* 46:641-644.
5. Kekou K et al. (2020). Evaluation of Genotypes and Epidemiology of Spinal Muscular Atrophy in Greece: A Nationwide Study Spanning 24 Years. *Journal of Neuromuscular Diseases*. 1-10.
6. Luo M. et al. (2014). An Ashkenazi Jewish *SMN1* haplotype specific to duplication alleles improves pan-ethnic carrier screening for spinal muscular atrophy.*Genet Med*. 16:149-56.
7. Milligan JN et al. (2021). Multisite Evaluation and Validation of a Sensitive Diagnostic and Screening System for Spinal Muscular Atrophy that Reports SMN1 and SMN2 Copy Number, along with Disease Modifier and Gene Duplication Variants. *The Journal of Molecular Diagnostics*. 23:753-764
8. Sangaré et al. (2014). Genetics of low spinal muscular atrophy carrier frequency in sub-Saharan Africa. Ann Neurol. 75:525-532
9. Strunk A et al. (2019). Validation of a fast, robust, inexpensive, two-tiered neonatal screening test algorithm on dried blood spots for spinal muscular atrophy. *International Journal of Neonatal Screening.* 5:21.
10. Veldhoen ES et al. (2021). Natural History of Respiratory Muscle Strength in Spinal Muscular Atrophy: A Prospective National Cohort Study. *Orphanet J Rare Dis*. 70.
11. Vijzelaar R et al. (2019). The frequency of SMN gene variants lacking exon 7 and 8 is highly population dependent. *Plos one*. 14:e0220211.
12. Wadman RI et al. (2020). Intragenic and structural variation in the SMN locus and clinical variability in spinal muscular atrophy. *Brain communications*. 2:fcaa075.

Implemented changes in the product description
Version B1-09 – 30 March 2026 (03S) - Probemix is registered for IVD use in Morocco. - Minor textual change in Intended purpose. - Reference sample selection box re-elaborated and re-formatted. The reason and importance for correct sample selection was emphasized. - Specified that background signals in *SMN1* or *SMN2* homozygous deletion samples may be displayed as orange boxes in the ratio chart (intra ratio percentage). - MANE select transcripts for *SMN1*, *SMN2* and *NAIP*, and OMIM links for *SMN1* and *SMN2* added to the Probe Content table footnote. - Distance from probe 21489-L30892 to 22122-L31134 changed to 0.1 kb. - In Appendix 2, the word 'washed' was removed from the title "Protocol for DNA extraction from dried blood spots". - Sentence added before Table B explaining that the scenarios described are examples. - Minor textual adjustments. *Version B1-08 – 04 August 2025 (03S)* - Explanation of background signal corrected in footnote under table in Interpretation of Results section. - Warnings for background signal of probes 21489-L30892, 21491-L29984, 21488-L30891 and 21490-L29983 added. - Warnings for probes targeting a sequence outside the known coding region added for probes 22121-L31133, 22123-L31135 and 22124-L31136. - Content of Changes in this Product Version section adjusted. - Minor textual adjustments.

MRC Holland, SALSA, MLPA, digitalMLPA, Coffalyser.Net, Coffalyser digitalMLPA, and their logos are trademarks or registered trademarks of MRC Holland BV. All other brands and names herein are the property of their respective owners.

10. Appendix 1 Interpretation of results, additional information

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 as well as the precautions and limitations stated in sections 7 and 8 are important:

1. The exon 7 difference between the *SMN1* and *SMN2* genes, as targeted by the 274 and 281 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 (274 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 for both specific and non-specific SMN probes.
4. Besides the four probes that are specific for exon 7 or 8 of either *SMN1* or *SMN2*, P021-B1 SMA contains seven additional probes that show the *combined* copy number of exon 7 or exon 8 of *SMN1* plus *SMN2*. In patients that have zero *SMN1* copies (as determined by the SMN1-specific exon 7 probe; 274 nt), these probes therefore indicate the *SMN2* copy number. The median value obtained by these seven probes can be used for a highly accurate estimation of the *SMN2* copy number in patients with homozygous loss of *SMN1*. Further notes on using the non-specific SMN probes are provided below.

SMA Carriers

5. A healthy individual with a single *SMN1* exon 7 copy (as determined by the SMN1-specific exon 7 probe at 274 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 distinct *SMN1* exon 7 are present. 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 3.
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. Check carefully for the copy number of the other exons. See section 8, limitation 1.
SMN1 exon 7: 1 copy *SMN1* exon 8: 1 copy no SMA symptoms	SMA carrier	One copy of *SMN1* exon 7 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 *SMN1* exon 8: ≠1 copy no SMA symptoms	SMA carrier	One copy of *SMN1* exon 7 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 3.
SMN1 exon 7: 2 copies *SMN1* exon 8: 2 copies no SMA symptoms	Most likely not an 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.
SMN1 exon 7: 2 copies *SMN1* exon 8: ≠2 copies no SMA symptoms	Most likely not an 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.

Calculations: *SMN2* copy number quantification for SMA patients with homozygous deletion of *SMN1* exon 7

For SMA patients, three calculations are used to determine *SMN2* copy number:

1. The final ratio value of the SMN2-specific exon 7 probe (281 nt), converted to copy number; see section 5. Table: Typical Results of Specific Probes Targeting *SMN1*, *SMN2* and *NAIP* .
2. The median final ratio value of the seven probes detecting exons 7 or 8 of both *SMN1* plus *SMN2* (193, 229, 391, 265, 427, 400 and 364 nt), converted to copy number; see section 5. Table: Results of Non-specific Probes Targeting *SMN1*&*SMN2* for SMA patients and carriers.
3. The median final ratio value of the probes detecting exons 1-6 of both *SMN1* plus *SMN2*, (382, 184, 221, 328, 319, 288, 346, 409, 199 and 418 nt), converted to copy number; see section 5. Table: Results of Non-specific Probes Targeting *SMN1*&*SMN2* for SMA patients and carriers.

Coffalyser.Net has the option to export all probe values in an Excel file, which can be used to determine the median values of the exon 1-6 or 7-8 probes.

In most patients, the copy number determined under *calculations 1* and *2* should yield an identical *SMN2* copy number. For high *SMN2* copy numbers, and for suboptimal samples, such as crude extracts from dried blood spots, *calculation 2* results in the most reliable copy number estimate as it is derived from a much larger number of probes. In case of a discrepancy in which one final ratio value is within the specified range and one final ratio value is in the flanking ambiguous range (see section 5. Interpretation of results), the copy number that is within the specified range can be assumed to be correct. When both values are in an ambiguous range, or

when both *calculations 1 and 2* lead to a different copy number, the experiment should be repeated, preferably with a new DNA preparation.

Calculation 3, concerning the exon 1-6 probes, should only be taken into consideration when the resulting value indicates a *lower* copy number than *calculations 1* and *2*. For patient samples with one exon 7 copy, *calculation 3* may indicate inactivation of that copy by deletion of exons 1-6. For patient samples with homozygous loss of *SMN1* exon 7, *calculation 3* may indicate that the actual number of complete *SMN2* gene copies is in fact lower. Note that an *increased* copy number for the exon 1-6 probes is frequently observed in carriers and normal individuals, and in a small number of patients. See *Interpretation of Results*.

***SMN2* copy number quantification for SMA patients with one remaining copy of *SMN1* exon 7**

In patients who show clear SMA symptoms but have been found to still retain one *SMN1* exon 7 copy (as determined by the 274 nt probe), the remaining *SMN1* gene copy may be defect.

For patient samples with one remaining copy of *SMN1* exon 7, the same procedure as described above can be used to determine the *SMN2* copy number, while keeping in mind that the copy number determined by *calculation 2* in this case reflects the *SMN2* copy number + one *SMN1* copy. Hence, one copy should be subtracted to obtain the *SMN2* copy number (see section 5, table: Results of Non-specific Probes Targeting *SMN1*&*SMN2* for SMA patients and carriers).

Table B. Relationship between Final ratio and Copy Number for P021 SMA

Most probes in P021 SMA detect a sequence that is present in *both* the *SMN1 and SMN2* genes. In an individual who is diploid for both genes, each of these MLPA probes therefore detect four copies in total. In contrast, the four MLPA probes that are *specific* for exon 7 or exon 8 of either *SMN1* or *SMN2* each detect two copies in a diploid individual.

In the table below, we show examples of how to interpret *SMN1* and *SMN2* copy numbers based on probe final ratios in P021 SMA in samples from SMA patients, carriers or healthy subjects. This list is not exhaustive and does not include all possible genotypes.

Examples		**exon 7 SMN1-specific probe** (274 nt)	**exon 7 SMN2-specific probe** (281nt)	**exon 8 SMN1-specific probe** (295 nt)	**exon 8 SMN2-specific probe** (301 nt)	**SMN exon 1-6 probes** (detect both *SMN1* and *SMN2*)	**SMN exon 7-8 probes** (detect both *SMN1* and *SMN2*)
Reference sample	**Final ratio**	1	1	1	1	1	1
	Copy Number	2	2	2	2	**4**	**4**
SMA Patient							
deletion of SMN1 (both alleles) *Two copies of SMN2*	**Final ratio**	0	1	0	1	0.5	0.5
	Copy Number	0	2	0	2	2	2
both SMN1 copies converted into SMN2 by gene conversion of exon 7 only	**Final ratio**	0	2	1	1	1	1
	Copy Number	0	4	2	2	4	4
compound heterozygosity: gene conversion SMN1 > SMN2 (exon 7 only) on allele 1; *deletion of SMN1 exon1-6 on allele 2*	**Final ratio**	0.5	1.5	1	1	0.75	1
	Copy Number	1	3	2	2	3	4
deletion of SMN1 (both alleles)three copies of SMN2	**Final ratio**	0	1.5	0	1.5	0.75	0.75
	Copy Number	0	3	0	3	3	3
SMA carrier							
deletion of one SMN1 copy, one *SMN1/2Δ7-8* copy present	**Final ratio**	0.5	1	0.5	1	1	0.75
	Copy Number	1	2	1	2	4	3
gene conversion: one SMN1-exon 7 copy converted into SMN2-exon 7	**Final ratio**	0.5	1.5	1	1	1	1
	Copy Number	1	3	2	2	4	4
Healthy subject							
three copies SMN1; zero copies SMN2; one SMN1/2Δ7-8 copy	**Final ratio**	1.5	0	1.5	0	1	0.75
	Copy Number	3	0	3	0	4	3

11. Appendix 2 P021-B1 SMA for use on newborn (dried blood spot) screening cards

Precautions and warnings: Only use specimens collected on cards that are based on Whatman 903 paper and that are not impregnated with chemicals. Cards that have been impregnated with chemicals, such as FTA cards, will require extensive washing. Impregnated cards have not been tested at MRC Holland.

Protocol for DNA extraction from dried blood spots

1. Start with a single 3.2 mm punch of each sample in a microtiter plate that fits in a thermocycler.
2. Add 100 µl 10mM NaOH to each well, ensuring that each punch is fully submerged. Leave for 15' at room temperature (RT), preferably with very slow shaking. Mix by pipetting up and down twice; then remove as much of the fluid as possible.
3. Repeat this wash procedure with another 100 µl 10mM NaOH. Leave for 15' at RT, preferably with very slow shaking. Mix by pipetting up and down twice; then remove as much of the fluid as possible.
4. Add 50 µl 10 mM NaOH to each well, ensuring each punch is submerged in the liquid.
5. Seal the plate.
6. Heat the samples for 15' at 99°C in a thermocycler with a heated lid.
7. Spin down using a short spin (to pull down all liquid from the seal) before removing the seal.
8. Use 5 µl of the extract for the P021 MLPA reaction. Store the remaining DNA in a refrigerator at 2ºC to 6ºC for potential follow-up assays. Prolonged storage is possible at -25ºC to -15ºC.
9. Continue with the MLPA General protocol (www.mrcholland.com).

Notes:

- Prepare 10 mM NaOH: Mix 1 ml 1 M NaOH + 99 ml ultrapure water. Do not store the diluted NaOH solution for more than 1 week.
- The volumes of NaOH solution in which the punches are heated can be adjusted. When the Q fragment peaks are high, indicating a low amount of sample DNA, the amount of NaOH solution in which the punches are heated should be lowered. When the Q fragment peaks are low or absent, a larger volume can be used.
- For heating, a thermocycler with a heated lid should be used. Be careful when opening tubes or removing seals in order to prevent contamination with other samples.
- Similar to other techniques, P021 SMA is influenced by contamination of DNA samples with DNA of other samples. Cleaning punchers between use on different cards is essential, e.g. by taking two punches from clean cards. Alternatively, if multiple punches from a specific card are routinely taken, one of the last punches taken should be used for DNA extraction.
- Instead of the extraction protocol provided here, commercially available extraction kits validated for extraction of DNA from DBS cards can be used.
- It is *NOT* recommended to add multiple punches from the same DBS card to the extraction volume as this will increase the amount of contaminants that interfere with the MLPA reaction.

Notes on data analysis:

- It is essential to use Coffalyser.Net software for data analysis.
- When analysing the data, ensure that the four DNA Quantity Fragments (Q-fragments; at 64, 70, 76, 82 nt) are not higher than 50% of the Benchmark fragment (92 nt). This verifies that the P021 SMA experiment has been performed with sufficient DNA. Coffalyser.Net software calculates this percentage and displays an indication for it according to the table below. (This can be found in the “DNA” column in the “Fragment analysis” screen.) When using P021 SMA on DBS material a warning notification for DNA concentration does not prohibit further analysis of the results.
- It should be noted that use of lower DNA concentrations also reduces the FMRS score in Coffalyser.Net. However, other factors also influence the FMRS score. A reduced FMRS score should therefore always be investigated for causes other than DNA concentration. (Right mouse button on the sample name; select “Open”; expand the FMRS section in the tab “overview”.)
- The standard deviation of all reference probes over the reference samples should be <0.10.

Symbol	Explanation	Notification
✓	Median signal of the Q-fragments below 33% of the signal of the benchmark fragment at 92 nt - proceed with results analysis	Ok
!	Median signal of the Q-fragments between 33% and 50% of the signal of the benchmark fragment at 92 nt - proceed with results analysis	Warning
✗	Median signal of the Q-fragments above 50% of the signal of the benchmark fragment at 92 nt – do NOT proceed with results analysis	Bad