# 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.

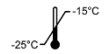
Visit the SALSA® MLPA® Probemix P021 SMA product page on our website to find Certificates of Analysis and a list of related products.

| **Product Name** | **SALSA® MLPA® Probemix P021 SMA** |
|---|---|
| **Version** | **B1** |
| **Catalogue numbers** | **P021-025R (25 reactions)**<br>**P021-050R (50 reactions)**<br>**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<br>EK1-CY5<br>EK5-FAM<br>EK5-CY5<br>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<br>COLOMBIA<br>ISRAEL |
| 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<br>1057 DL, Amsterdam, the Netherlands |
| E-mail | info@mrcholland.com (information & technical questions);<br>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 A2 version*
Thirteen SMN probes were added and four probes were replaced, nine reference probes were replaced, eleven reference probes and six flanking probes were removed, and several 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 determining the presence of 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<br>• 50-250 ng purified human genomic DNA, dissolved in 5 µl $TE_{0.1}$ buffer, pH 8.0-8.5<br>DBS card<br>• 3.2 mm punch |
| Collection method | Human peripheral whole blood<br>• Standard methods<br>DBS card<br>• Whatman 903 paper |
| Extraction method | Methods tested by MRC Holland:<br>Human peripheral whole blood<br>• QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual)<br>• Promega Wizard Genomic DNA Purification Kit (manual)<br>• salting out (manual)<br>DBS card<br>• NaOH based extraction (manual, for more details see Appendix 2) |

| Sample types | | |
|---|---|---|
| Test sample | • provided by user | |
| Reference samples (required) | • provided by user<br>• selection of reference samples is important for correct determination of *SMN1* and *SMN2* copy numbers<br>• extraction method, tissue type, DNA concentration (and) treatment as similar as possible in all test and reference samples<br>• have an *SMN1*, *SMN2* and *NAIP* copy number of two, no *SMN1/2Δ7-8* copies and ≤0.10 standard deviation for all probes<br>• have a final ratio of ~1.0 for all probes<br>• 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<br>• use SD082 to facilitate the selection of suitable reference samples<br>• **SD082 should never be used as a reference sample** | |
| No-DNA control (preferably) | • provided by user<br>• $TE_{0.1}$ buffer instead of DNA<br>• 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.

Please refer to Appendix I for notes on data analysis when using DBS card specimens.

## 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[#] |

* Due to the nature of the exon 7 and 8 mismatch between *SMN1* and *SMN2*, a small background signal can be visible. This background signal can be caused by the *SMN2* exon 7 or 8 probes when no *SMN1* exon 7 or 8 is present, or vice versa. This background signal might be displayed as an intra ratio percentage instead of a final ratio (more details: https://www.mrcholland.com/r/intra-ratio-percentage).

[#] 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 |

* This median final ratio value mentioned here is the 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 final ratios (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%.<br><br>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.<br><br>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.<br><br>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 FeCl3 (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.<br><br>In total, 1/252 probe measurements across all replicates tested showed an ambiguous result, caused by either EDTA, NaCl, FeCl3 or Heparin. A total of 12/252 probe measurements across all replicates tested showed an ambiguous result, caused by hemoglobin.<br><br>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.<br><br>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%.<br><br>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* | From literature, 95-100% of the disease is caused by a homozygous deletion of the *SMN1* exon 7 (Wadman et al, 2020, Kekou et al, 2020, Veldhoen et al. 2021) and for 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).<br><br>* 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 | | SMN Non-specific (7-8) |
| 5q13.2 | *SMN2* | Exon 7 | 0.8 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 | | SMN Non-specific (7-8) |
| 5q13.2 | *SMN2*(+*SMN1*) | Intron 7 | 0.1 kb | 265 | 22124-L31136 | | 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 | | 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 | | SMN Non-specific (7-8) |
| 5q13.2 | *SMN1*(+*SMN2*) | Intron 7 | 0.1 kb | 265 | 22124-L31136 | | 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 | Exon 16 | | 310 | 20763-L28665 | ß | |
| 1q | Reference | Exon 23 | | 445 | 20431-L27913 | | |
| 2q | Reference | Exon 9 | | 211 | 18964-L24756 | | |
| 4q | Reference | Exon 14 | | 247 | 19086-L24973 | | |
| 8p | Reference | Exon 10 | | 373 | 18296-L25750 | | |
| 9q | Reference | Exon 4 | | 337 | 19746-L26529 | | |
| 10p | Reference | Exon 15 | | 256 | 19625-L26284 | | |
| 11p | Reference | Exon 8 | | 355 | 19127-L25074 | | |
| 17p | Reference | Exon 7 | | 436 | 19646-L26317 | | |
| 18q | Reference | Exon 2 | | 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). 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. Annotations of several probes with targets at the edge of or slightly outside the coding region, were altered. The exon numbering from the previous version of this Product Description is disclosed between brackets.
The *NAIP* exon number is derived from MANE project and based on the MANE Select transcript.
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. |
| ב | This probe is insensitive to sample DNA depurination. Use of depurinated DNA will lead to a **higher** signal of this probe. |
| Ø | This probe targets a sequence outside of the known coding region. Copy number alterations of only this probe 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. |

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.

## 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 Probemix P060 SMA Carrier and SALSA MLPA Probemix P460 SMA (Silent) Carrier cannot be used for confirmation of results.

Technique-specific limitations
See the MLPA General Protocol.

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| **Implemented changes in the product description** |
|---|
| *Version B1-07 – 29 January 2025 (03S)*<br>- Product description adapted to new template.<br>- Intended purpose updated, specifying the assay is manual. Prenatal samples removed and function of device on DBS cards further clarified.<br>- The probe targeting *NAIP* is no longer intended for diagnostic use.<br>- Description of probe targets at the edge of or slightly outside the coding region has been adjusted. No change in actual target sites.<br>- Probemix is now IVDR certified.<br>- Probemix is now registered for IVD use in Colombia.<br>- Probemix is no longer registered for IVD use in Morocco. |

## 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 as stated in sections 7 and 8 are important:

1. The exon 7 difference between the *SMN1* and *SMN2* gene, 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*, the P021-B1 probemix 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<br>*SMN1* exon 8: 0 copies<br>*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<br>*SMN1* exon 8: > 0 copies<br>*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<br>*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<br>*SMN1* exon 8: 1 copy<br>*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<br>*SMN1* exon 8: ≠1 copy<br>*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<br>*SMN1* exon 8: 2 copies<br>*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<br>*SMN1* exon 8: ≠2 copies<br>*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 calculated 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.

| **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)*<br>*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;*<br>*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,*<br>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 washed 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 2°C to 6°C for potential follow-up assays. Prolonged storage is possible at -25°C to -15°C.
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, the P021 SMA probemix 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 |