Product Description
SALSA® MLPA® Probemix P021-B1 SMA

To be used with the MLPA General Protocol.

Version B1
For complete product history see page 18.

Catalogue numbers:
- **P021-025R:** SALSA MLPA Probemix P021 SMA, 25 reactions.
- **P021-050R:** SALSA MLPA Probemix P021 SMA, 50 reactions.
- **P021-100R:** SALSA MLPA Probemix P021 SMA, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit and Coffalyser.Net data analysis software. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman/SCIEX capillary sequencers, respectively (see www.mrcholland.com).

Certificate of Analysis
Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mrcholland.com.

Precautions and warnings
For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mrcholland.com. It is the responsibility of the user to be aware of the latest scientific knowledge of the application before drawing any conclusions from findings generated with this product.

This product requires the identification of suitable reference samples for proper data analysis. For more information, see section Reference samples (page 5).

Intended purpose
The SALSA MLPA Probemix P021 SMA is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplication in the *SMN1*, *SMN2* and exon 5 of the *NAIP* genes in genomic DNA isolated from human peripheral whole blood specimens, prenatal samples, from either (un)cultured amniotic fluid obtained in week 16 of pregnancy or later, free from blood contamination (un)cultured chorionic villi, free from maternal contamination fetal blood or Dried Blood Spot (DBS) cards. P021 SMA is intended to establish or confirm a potential cause for and clinical diagnosis of Spinal Muscular Atrophy (SMA), carrier testing and for molecular genetic testing of at-risk family members. Secondly, the assay can be used for *SMN2* (and *NAIP*) copy number determination in (pre-symptomatic) SMA patients as an aid in prognosis and for treatment eligibility.

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) in *SMN1*. Both defects can be detected by MLPA probemix P021 SMA. Copy number variations (CNVs) detected with P021 SMA 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 vitro diagnostic (IVD) use in the countries specified at the end of this product description. In all other countries, the product is for research use only (RUO).
[2]To be used in combination with a SALSA MLPA Reagent Kit, SALSA Reference Selection DNA SD082, Coffalyser.Net analysis software.

Comparison of MRC Holland SMA products

MRC Holland offers four different assays for SMA that fit the complete range of genetic testing needs. The table below indicates which product can best be used for which purpose.

		P021	P060	P460	MC002
	CE-marked	yes	yes	yes	yes
	Technique	MLPA	MLPA	MLPA	Melt Assay
Used for	Neonatal patient screening	○	○	-	●
	Patient detection	●	○	○	-
	Carrier detection	○	●	●	-
	Silent Carrier‣ detection	-	-	●	-
	Patient detection confirmation	-	-	-	√*
Coverage	*SMN1* exon 7 specific	√	√	√	√◊
	SMN1 exon 8 specific	√	√	√	-
	SMN2 exon 7 specific	√	√	√	√◊
	SMN2 exon 8 specific	√	√	-	-
	SMN1+*SMN2* exon 1-8	√	-	-	-
	Silent Carrier polymorphism probes	-	-	√	-

● Primary test
○ Secondary test
‣ Increased detection of Silent Carriers.
- Not possible to detect
◊ MC002: no absolute copy numbers aside from 0 determined.
√ Suitable to detect
* Exon 1-6 deletions are not detected by MC002

Clinical background

Spinal Muscular Atrophy (SMA) is a group of autosomal recessive neuromuscular disorders characterized by degeneration of the anterior horn cells of the spinal cord, leading to symmetrical muscle weakness and atrophy. SMA is a heterogeneous disease, its phenotype ranging from early onset with a life expectancy of less than 2 years to a late onset with very mild symptoms. SMA disease severity is strongly correlated with *SMN2* copy number. The estimated incidence of SMA is 1:6,000-1:10,000.

Two highly similar genes play a pivotal role in SMA: *SMN1* and *SMN2*. The only clinically relevant difference between the two genes is a single nucleotide difference in exon 7. *SMN2* is translated much less efficiently in a functional SMN protein; therefore, it is the *SMN1* gene which is the determinant factor in SMA. Someone lacking functional copies of *SMN1* is almost always a SMA patient. In most populations, 95-98% of SMA patients show complete absence of at least exon 7 of the *SMN1* gene (this percentage is lower in SMA patients from African descent (Labrum et al. 2007)). Most of the remaining patients have a single copy of the *SMN1* gene which is inactive due to a point mutation or a deletion of exons 1-6. Please note that rare cases have been described of healthy individuals with homozygous loss of *SMN1* exon 7 and only 2 or 3 *SMN2* copies (e.g. Helmken et al. 2003).

SMA carriers are symptom-free. The great majority of SMA carriers can be identified by the presence of a single *SMN1* exon 7 copy. The presence of *more* than two *SMN1* copies is a relatively frequent phenomenon in healthy individuals, especially in individuals of African descent. For more details, see *Interpretation of Results*. About 3-8% of SMA carriers (27% of African Americans) have two *SMN1* copies on one chromosome and zero copies on the other (2+0) (Alias et al. 2014, Hendrickson et al. 2009). MLPA cannot distinguish '1+1' from '2+0' (silent carriers) arrangements. Both situations are simply detected as having two *SMN1* copies leading to false negative results. A thorough molecular analysis should be performed on samples from parents

and blood relatives of SMA patients when initial results indicate two *SMN1* copies. Luo et al. (2014) reported that a haplotype block specific for *SMN1* duplications is present in a large percentage of Ashkenazi Jews and in other ethnic groups. Identifying this haplotype, e.g. with the use of the SALSA MLPA probemix P460 SMA, will help to identify silent carriers.

Most healthy individuals have 0 - 4 copies of *SMN2*. Provided that at least one functional *SMN1* copy is present, complete absence of the centromeric *SMN2* gene seems to have no clinical consequences.

Most patients have 1 - 4 copies of *SMN2*. Establishing the *SMN2* copy number is of importance for SMA patients: the more *SMN2* copies present, the less severe the disease usually is (Feldkötter et al. 2002). Accurate *SMN2* copy number quantification can be important for determining a patient's eligibility for treatment.

Another factor that influences disease severity is the presence of the c.859G>C polymorphism in *SMN2* (Prior et al. 2009). Please note that the *SMN2* copy number and the presence of the c.859G>C variant do not completely explain the differences in disease severity between SMA patients. The c.859G>C polymorphism cannot be detected by P021-B1 SMA.

More information is available at: https://www.ncbi.nlm.nih.gov/books/NBK1352/.

Gene structure

SMN1 and *SMN2* are part of a 500 kilobases (kb) inverted duplication on chromosome 5q13. The *SMN1* gene (9 exons) spans ~28 kb of genomic DNA and is located on chromosome 5q13.2, about 70 Mb from the p-telomere. The *SMN1* LRG_676 is identical to Genbank NG_008691.1 and is available at www.lrg-sequence.org.

The *SMN2* gene (9 exons) also spans ~28 kb of genomic DNA on chromosome 5q13.2. The *SMN2* LRG_677 is identical to Genbank NG_008728.1 and is available at www.lrg-sequence.org.

A third SMN gene variant has been described which contains only exons 1-6 of *SMN1* or *SMN2*. This variant is only present in some individuals (Arkblad et al. 2006; Calucho et al. 2018) and its clinical significance is not yet completely clear. This third variant is referred to as *SMN1/2Δ7-8* in this product description.

The *NAIP* gene (17 exons) spans ~57 kb of genomic DNA and is located on chromosome 5q13.2, about 60 kb from the *SMN1* gene.

Transcript variants

Three ***SMN1* transcript variants** have been described, see https://www.ncbi.nlm.nih.gov/gene/6606. Transcript variant d (NM_000344.4, 1482 nucleotides (nt), coding sequence 18-902) is the predominant and longest variant. In Table 2, the ligation sites of the SMN1-specific MLPA probes are indicated according to this NM sequence. This sequence variant, which contains 9 exons, has the ATG translation start site in exon 1 and the stop codon in exon 7 (known as exon 8 in online databases; see next chapter: Exon numbering).

Four ***SMN2* transcript variants** have been described, see https://www.ncbi.nlm.nih.gov/gene/6607. Transcript variant d (NM_017411.4, 1482 nt, coding sequence 18-902) is the longest transcript and is a reference standard in the NCBI RefSeqGene project. This variant results in exactly the same protein as *SMN1* transcript variant d. However, the predominant transcript variant of *SMN2* is sequence variant a (NM_022875.3) which lacks exon 7 and results in a protein with a different C-terminus that is assumed to be inactive. In Table 2, the ligation site of the SMN2-specific MLPA probe is indicated according to the NM_017411.4 sequence. This sequence, which contains 9 exons, has the ATG translation start site in exon 1 and the stop codon is located in exon 7 (known as exon 8 in online databases; see next chapter: Exon numbering).

Three ***NAIP* transcript variants** have been described, see https://www.ncbi.nlm.nih.gov/gene/4671. The sequence of *NAIP* transcript 1 (NM_004536.3, 7093 nt, coding sequence 944-5155) is the longest variant. In Table 2 the ligation site of the NAIP MLPA probe is indicated according to this NM sequence. The sequence, which contains 17 exons, has the ATG translation start site in exon 4 and the stop codon in exon 17.

Exon numbering

The exon numbering for the *SMN* genes that is used throughout this P021-B1 SMA product description is based on the classic exon numbering as used in most scientific literature: exons 1, 2a, 2b, 3-8. In contrast, the exon numbering currently adopted by NCBI (NG_008691.1 and NG_008728.1 reference sequence) and mentioned in the LRG_676 and LRG_677 sequences is 1-9. As changes to the databases can occur after release of this product description, the NM_sequences may not be up-to-date.

The *NAIP* exon numbering used in this P021-B1 SMA product description is the exon numbering from the RefSeq transcript NM__004536.3 As changes to the databases can occur after release of this product description, the NM_ sequence and exon numbering may not be up-to-date.

Probemix content

The SALSA MLPA Probemix P021-B1 SMA contains 32 MLPA probes with amplification products between 175 and 445 nt. This includes four probes specific for sequences in exon 7 **or** 8 of either *SMN1* **or** *SMN2, 17* probes detecting sequences that are present in both *SMN1* **and** *SMN2* (at least one probe per exon; one additional probe for exon 1, 2b, and 3; seven probes in total for the intron 6-exon 7-intron 7-exon 8 region of both *SMN1* and *SMN2*) and 1 probe for the *NAIP* gene.

In addition, ten reference probes are included that detect autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

- The ***SMN1*-specific exon 7 probe 21488-L30891** (274 nt) **and the *SMN2*-specific exon 7 probe 21489-L30892** (281 nt) are the most important probes in this mix. These two probes distinguish *SMN1* from *SMN2* by having their ligation site at the critical single nucleotide difference between these genes in exon 7, which is a site that affects RNA splicing. These probes can be used to quantify *SMN1* (important in determining carrier status) and *SMN2* (important for disease prognosis) respectively.
- The ***SMN1*-specific exon 8 probe 21490-L29983** (295 nt) **and the *SMN2*-specific exon 8 probe 21491-L29984** (301 nt) distinguish *SMN1* and *SMN2* at the exon 8 G-to-A transition. In approximately 5-10% of cases, the copy number detected by these exon 8 probes does not correspond to that found by the aforementioned exon 7 probes, due to gene conversion. In such cases, only the exon 7 probes should be used to quantify *SMN1* and *SMN2* copy number.
- There are seven **SMN exon 7 & 8 probes** (see Table 2) that detect the *combined* copy number of *SMN1* and *SMN2* genes in the intron 6, exon 7, intron 7 and exon 8 region. In normal individuals carrying two copies of *SMN1* and two copies of *SMN2*, these probes detect four gene copies in total. In case of a homozygous *SMN1* deletion, these probes can be used to more accurately determine the *SMN2* copy number.
- The ten **SMN exon 1-6 probes** (see Table 2) are useful to identify patient samples with a gain or loss of these exons as well as rare carriers who have two *SMN1* exon 7 sequences but one of the *SMN1* genes is non-functional due to a deletion of exons 1-6 (Arkblad et al. 2006; Thauvin-Robinet C et al. 2012). As these probes detect both *SMN1* and *SMN2*, an exon 1-6 deletion detected by these probes should only be considered pathogenic if this is suggested by the individual's clinical context. Please note that many healthy individuals have an extra copy of exons 1-6 (*SMN1/2Δ7-8*) without known clinical significance.

This probemix contains nine quality control fragments generating amplification products between 64 and 105 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and one chromosome Y-specific fragment (see table below). More information on how to interpret observations on these control fragments can be found in the MLPA General Protocol and online at www.mrcholland.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal indicates incomplete denaturation)

Length (nt)	Name
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

MLPA technique
The principles of the MLPA technique (Schouten et al. 2002) are described in the MLPA General Protocol (www.mrcholland.com).

MLPA technique validation
Internal validation of the MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using MLPA for the first time, or when changing the sample handling procedure, DNA extraction method or instruments used. This validation experiment should result in a standard deviation ≤0.10 for all reference probes over the experiment.

Required specimens
Extracted DNA from:

1. Peripheral blood,
2. Prenatal samples, from either
   a. (un)cultured amniotic fluid obtained in week 16 of the pregnancy or later, free from blood contamination
   b. (un)cultured chorionic villi, free from maternal contamination
   c. fetal blood,
3. Dried Blood Spot (DBS) cards, permitted the DNA has been extracted using the method and type of DBS cards described in Appendix I below.

Extracted DNA should be free from impurities known to affect MLPA reactions. For more information please refer to the section on DNA sample treatment found in the MLPA General Protocol.

Reference samples
A sufficient number (≥3) of reference samples should be included in each MLPA experiment for data normalisation. 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. Reference samples should be derived from unrelated individuals who are from families without a history of SMA. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol (www.mrcholland.com).

A careful selection of suitable reference samples, containing two copies of *SMN1*, *SMN2* and *NAIP*, is vital for the correct quantification of *SMN1* and *SMN2* copy numbers with MLPA probemix P021 SMA. As reference samples should be treated identically to patient samples, choose reference samples from your own sample collection. Select suitable reference samples as follows:

a. Test a minimum of 20 healthy individuals (possibly more for populations with high *SMN1/2* variability)
b. Include reactions with Reference Selection DNA SD082 in the experiment
c. Analyse the samples with the Coffalyser.Net data analysis software (www.mrcholland.com)
d. Select samples with two *SMN1*, two *SMN2* and two *NAIP* copies and without *SMN1/2Δ7-8* copies. Suitable reference samples should have:
   - A final ratio of ~1.0 for the four specific probes: *SMN1* exon 7 & exon 8; *SMN2* exon 7 & 8 **AND**
   - A final ratio of ~1.0 for all other probes (*SMN1*, *SMN2*, *NAIP,* reference probes).

As an aid, include MRC Holland's *SALSA Reference Selection DNA SD082* in your initial experiment to help identify suitable reference samples from your own collection. SD082 contains two copies of the *SMN1*, *SMN2* and *NAIP* genes. Note that *SD082* should only be used to *identify* reference samples and ***NOT*** used as reference samples for data normalisation purposes in routine use.

Positive control DNA samples

Like reference samples, MRC Holland cannot provide positive DNA samples either. Inclusion of a positive sample in each experiment is recommended. Coriell Institute (https://catalog.coriell.org) and Leibniz Institute DSMZ (https://www.dsmz.de/) have a diverse collection of biological resources which may be used as a positive control DNA sample in your MLPA experiments. Table A shows Coriell samples that have been tested with this P021-B1 probemix at MRC Holland and can be used as a positive control samples to detect copy number variation in the *SMN1* and *SMN2* genes. The quality of cell lines can change; therefore samples should be validated before use. A large set of positive control DNA samples can also be found in Prior et al. (2021).

Table A. Samples from Coriell biobank tested by MRC Holland with P021-B1 SMA for *SMN1* and *SMN2* copy number (CN)

Coriell biobank Sample ID	Copy number			
	***SMN1* exon 7**	***SMN2* exon 7**	***SMN1* exon 8**	***SMN2* exon 8**
NA00232; NA10684	0	2	0	2
NA22592; NA09677; NA03813	0	3	0	3
NA03815; NA20760; NA20787	1	1	1	1
NA23687; NA23688; NA20764	1	2	1	2
HG00346; HG00281	1	3	1	3
HG01773; HG01774; HG02132	1	4	1	4
NA03814	1	5	1	5
NA19122; HG01941; NA19794	2	0	2	0
HG02514; HG03663; HG03636	2	1	2	1
HG01701; HG01942; HG01935	2	2	2	2
HG01748; HG01971; HG00329	2	3	2	3
HG03625	2	4	2	4
NA19123; HG03258; HG02891	3	0	3	0
HG00255; NA19437; HG01377	3	0	3	0
HG01755; HG03650	3	1	3	1
NA20775; HG01137	3	1	3	1
NA12548; NA20755	3	2	3	2
NA12552; NA20515	3	3	3	3
NA19235; HG03027; HG02769	4	0	4	0
NA19429; HG02836	4	1	4	1
Coriell Sample IDs that have a different copy number for exon 7 and exon 8 due to gene conversion				
NA19177	2	1	3	0
NA21527	2	2	1	3
NA19249	2	2	3	1
NA21526	2	3	1	4
NA19790	3	1	1	3
NA19327	3	1	2	2
NA21513	3	1	4	0
NA19360	4	0	3	1
NA19026	4	1	5	0
HG02697	4	1	3	2
NA19019	4	3	5	2

SALSA Reference Selection DNA SD082
The selection of suitable reference DNA samples that can be used with P021 SMA is crucial. To facilitate the selection of suitable reference DNA samples from your own sample collection, a reference selection DNA sample (catalogue number SD082) is provided with this probemix from MRC Holland and can also be ordered separately. SALSA Reference Selection DNA SD082 should only be used for initial experiments on DNA samples from healthy individuals with the intention to identify suitable reference samples from your own collection. When the SD082 reactions are set as reference samples in the data analysis of an experiment with possible suitable reference samples from your own collection, suitable reference DNA samples will be those samples from healthy individuals that have a final ratio between 0.80 and 1.20 for all probes included in the Probemix. Suitable reference DNA samples selected as described can subsequently be used as reference DNA samples in experiments with patient samples. **SD082 should not be used as a reference sample in subsequent experiments.** For further details, consult the SALSA Reference Selection DNA SD082 product description, available online: www.mrcholland.com.

Performance characteristics
In the vast majority of Caucasian SMA patients (>95%), the disease is caused by a homozygous deletion of the *SMN1* gene, which is readily detected by the *SMN1* specific exon 7 probe in the P021 SMA probemix. This is higher than in other populations (>90% in Ashkenazi Jewish, Hispanic and Asian populations, ~71% in African Americans) (Hendrickson et al. 2009).

The remaining ~5% is caused by small mutations or a deletion of other *SMN1* exons, usually in combination with an heterozygous *SMN1* deletion (compound heterozygosity) (Feldkötter et al. 2002). The small mutations can be found using sequence analysis techniques; deletions of other exons can also detected by this P021 SMA probemix.

In a clinical performance evaluation study on dried blood spot cards from 47 SMA patients and 375 control samples, performed at the Isala clinic in Zwolle, The Netherlands, the diagnostic specificity and sensitivity of the P021 assay were both 100% (Strunk et al. 2019). The diagnostic sensitivity is expected to be approximately 95%-98% in most populations as pathogenic point mutations cannot be detected by P021.

The analytical sensitivity and specificity (based on a 2005-2021 literature review) for the detection of copy number variation and gene conversions is very high and can be considered >99%.

Analytical performance can be compromised by: SNVs or other polymorphisms in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

Data analysis
Coffalyser.Net software should be used for data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet. For both, the latest version should be used. Coffalyser.Net software is freely downloadable at www.mrcholland.com. Use of other non-proprietary software may lead to inconclusive or false results. For more details on MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual. Please refer to Appendix I for notes on data analysis when using DBS card specimens.

Interpretation of results
The expected results for *SMN1* (274 nt and 295 nt) and *SMN2* (281 nt and 301 nt) specific MLPA probes as well as the *NAIP* probe (238 nt) are allele copy numbers of 2 (normal), 0 (homozygous deletion), 1 (heterozygous deletion), 3 (heterozygous duplication) and occasionally 4 (heterozygous triplication or homozygous duplication). For expected results of the remaining probes, please refer to Table C (page 10).

The standard deviation of each individual reference probe over all the reference samples should be ≤0.10 and the final ratio (FR) of each individual reference probe in the patient samples should be between 0.80 and 1.20.

When these criteria are fulfilled, the following cut-off values for the FR of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Final ratio (FR)
Normal	0.80 < FR < 1.20
Homozygous deletion	FR = 0*
Heterozygous deletion	0.40 < FR < 0.65
Heterozygous duplication	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	1.75 < FR < 2.15
Ambiguous copy number	All other values

*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).

Note: The term "dosage quotient", used in older product description versions, has been replaced by "final ratio" to become consistent with the terminology of the Coffalyser.Net software(Calculations, cut-offs and interpretation remain unchanged). Please note that the Coffalyser.Net software also shows arbitrary borders as part of the statistical analysis of results obtained in an experiment. As such, arbitrary borders are different from the final ratio cut-off values shown here above.

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 is important:

1. As stated before, in this product description the traditional exon numbering is used in which the *SMN1* and *SMN2* genes consist of exons 1, 2a, 2b, 3, 4, 5, 6, 7 and 8.
2. Copy number quantification by P021 SMA is highly dependent on a correct selection of reference samples; see section *Reference Samples*.
3. 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.
4. Determining *SMN2* copy number is relevant for patient prognosis, but not for SMA carrier testing.
5. 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.

Normal variation in the general population

6. 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. 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.
7. 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).
8. 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.
9. 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).

SMA Patients

10. 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.
11. 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.
12. Besides the four probes that are specific for exon 7 or 8 of either *SMN1* or *SMN2*, the P021-B1 probemix contains 7 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 7 probes can be used for a highly accurate estimation of the *SMN2* copy number in patients with homozygous loss of *SMN1*.
13. A homozygous deletion of *NAIP* exon 5 is frequently observed in SMA patients, but is very rare in healthy individuals.

SMA Carriers

14. An individual with a single *SMN1* exon 7 copy (as determined by the *SMN1*-specific exon 7 probe at 274 nt) is a SMA carrier.
15. 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.
16. Although rare, individuals with two *SMN1* copies may still be carriers. If the biological parent of a SMA patient is found to have two *SMN1* copies, the following options should be considered:
    a. One *SMN1* copy carries a point mutation or a deletion of other exons than exon 7.
    b. Both *SMN1* copies are located on the same allele. 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. See P460 SMA product description for more information.

The summation of these findings and what they mean for carrier/patient status can be found in Table B.

Table B. 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 point 6 above.
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 point 11 above.
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 in 90-95% of (Caucasian) cases.

Finding	Conclusion	Explanation
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 point 6 above.
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 point 16 above.
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 point 16 above.

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

Most probes in P021 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.

IMPORTANT NOTE: The user of this product should ensure that ALL selected reference samples meet the criteria described in section "Reference samples".

Examples		**exon 7 SMN1-specific probe** (274 nt)	**exon 7 SMN2-specific probe** (281 nt)	**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)*	**Final ratio**	0	1	0	1	0.5	0.5
	Copy Number	0	2	0	2	2	2
SMA patient, *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
SMA patient, *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
SMA patient, *3 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 1 SMN1 copy,* 1 *SMN1/2Δ7-8* copy present	**Final ratio**	0.5	1	0.5	1	1	0.75
	Copy Number	1	2	1	2	4	3
SMA carrier, *gene conversion: 1 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, *3 copies SMN1; 0 copies SMN2; 1 SMN1/2Δ7-8* copy	**Final ratio**	1.5	0	1.5	0	1	0.75
	Copy Number	3	0	3	0	4	3

***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 Table D.
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 Table D.
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 Table D.

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 *points 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 (Table D), 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.

When more samples provide ambiguous results, check if the correct reference samples have been used.

When the standard deviation of the reference probes is > 0.10, the DNA sample or the MLPA reaction, may be of insufficient quality to report results.

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. Such compound heterozygosity has been reported to be present in 2-5% of SMA cases (e.g. Feldkötter et al. 2002). Among the possible causes are:

1. Point mutations in the *SMN1* gene (which will not be detected by the MLPA probemixes for SMA).
2. Copy number changes of other exons in the *SMN1* gene. Most of these copy number changes can be detected with MLPA probemix P021-B1 SMA but, in particular with higher *SMN2* copy numbers, a copy number change detected by a single probe will require independent confirmation, e.g. by long range PCR.

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, once copy should be subtracted to obtain just the *SMN2* copy number (See Table D).

Table D. Relationship between Final ratio and Copy Number for SMN2.

This table assumes that all reference samples meet the criteria described in section "Reference samples".

Final ratio of the 281 nt SMN2-specific probe for exon 7 (range)	***SMN2* copy number**
0 - 0.10	0
0.40 - 0.65	1
0.80 - 1.20	2
1.30 - 1.65	3
1.75 - 2.15	4
2.20 - 2.65	5
All other values	Ambiguous*

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

The following ranges can be used to determine *SMN2* copy numbers when 0 copies or 1 copy (CN values in brackets) of *SMN1* is present.

***Median* final ratio of non-specific SMN exon 7-8 probes* or exon 1-6 probes** (range)**	***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* (193, 229, 391, 265, 427, 400 and 364 nt).

** Median value of the ten probes detecting exons 1-6 of both *SMN1* plus *SMN2* (382, 184, 221, 328, 319, 288, 346, 409, 199 and 418 nt).

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.

General Points for a Correct Interpretation of Results

For a correct interpretation of results, the standard deviation of all probes over the reference samples should be <0.10 and the final ratio of all reference probes in the patient samples should be between 0.80 and 1.20.

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic cases. Analysis of parental samples may be necessary for correct interpretation of complex results.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.

- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: a lower injection voltage or a shorter injection time, or a reduced amount of sample by diluting PCR products.

Limitations of the procedure

- MLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations. Even when MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.
- Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can cause false positive results. Mutations/SNVs (even when >20 nt from the probe ligation site) 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.
- Be aware that 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 the presence of two *SMN1* copies on one chromosome and zero on the other, 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. As mentioned above, the carrier screening in certain populations such as African-Americans and possibly other individuals of African descent may be compromised by a higher frequency of individuals with two or more *SMN1* copies on one chromosome.

Confirmation of results

Copy number changes detected by P021-B1 SMA should be confirmed by another independent technique such as long range PCR and (allele specific) qPCR whenever possible. An apparent deletion detected by a single probe can be due to e.g. a mutation/polymorphism that prevents ligation or destabilises the binding of probe oligonucleotides to the DNA sample. MLPA probemixes P060 SMA Carrier and P460 SMA cannot be used for confirmation of results.

Copy number changes detected by more than one consecutive probe should be confirmed by another independent technique such as long range PCR and (allele specific) qPCR whenever possible.

SMA mutation database

SMN1: http://grenada.lumc.nl/LSDB_list/lsdbs/SMN1.
SMN2: http://grenada.lumc.nl/LSDB_list/lsdbs/SMN2.
We strongly encourage users to deposit positive results in the LOVD *SMN1* database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report copy number changes detected by the reference probes, false positive results due to SNPs and unusual results to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P021-B1 SMA

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]			
		Reference	*SMN1*	*SMN2*	*NAIP*
64-105	Control fragments – see table in probemix content section for more information				
175	Reference probe 00808-L00638	18q			
184 *	**SMN1/SMN2 probe** 21519-L30024		**Exon 1**		
193 *	**SMN1/SMN2 probe** 22121-L31133		**Intron 6**		
199 *	**SMN1/SMN2 probe** 21518-L30023		**Exon 5**		
211 *	Reference probe 18964-L24756	2q			
221 *	**SMN1/SMN2 probe** 21517-L30022		**Exon 2a**		
229 * ‡	**SMN1/SMN2 probe** 22122-L31134		**Intron 7**		
238	**NAIP probe** 01259-L00811				**Exon 5**
247 *	Reference probe 19086-L24973	4q			
256 *	Reference probe 19625-L26284	10p			
265 *	**SMN1/SMN2 probe** 22124-L31136		**Intron 7**		
274 ¥ ‡	**SMN1 probe** 21488-L30891		**Exon 7**		
281 ¥ ‡	**SMN2 probe** 21489-L30892			**Exon 7**	
288 *	**SMN1/SMN2 probe** 21516-L30893		**Exon 3**		
295 ¥ ‡	**SMN1 probe** 21490-L29983		**Exon 8**		
301 ¥	**SMN2 probe** 21491-L29984			**Exon 8**	
310 * ‡	Reference probe 20763-L28665	1q			
319 *	**SMN1/SMN2 probe** 14132-L15557		**Exon 2b**		
328 *	**SMN1/SMN2 probe** 21514-L30019		**Exon 2b**		
337 *	Reference probe 19746-L26529	9q			
346 *	**SMN1/SMN2 probe** 21513-L30018		**Exon 3**		
355 *	Reference probe 19127-L25074	11p			
364	**SMN1/SMN2 probe** 01814-L00807		**Exon 8**		
373 *	Reference probe 18296-L25750	8p			
382 Δ ⊐	**SMN1/SMN2 probe** 22196-L31253		**Exon 1**		
391 *	**SMN1/SMN2 probe** 22123-L31135		**Intron 7**		
400 *	**SMN1/SMN2 probe** 22126-L31138		**Exon 8**		
409 ¥ ⊐	**SMN1/SMN2 probe** 01816-L30922		**Exon 4**		
418 ¥	**SMN1/SMN2 probe** 22194-L31251		**Exon 6**		
427 *	**SMN1/SMN2 probe** 22125-L31137		**Intron 7**		
436 *	Reference probe 19646-L26317	17p			
445 *	Reference probe 20431-L27913	1q			

[a] See section Exon numbering on page 4 for more information.

Note: The exon numbering used in this P021-B1 SMA product description and in the P021-B1 SMA 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 NCBI reference sequences for *SMN1* and *SMN2*. Please notify us of any mistakes: info@mrcholland.com.

¥ Changed in version B1 (from lot B1-1018 onwards). Small change in length, no change in sequence detected.

Δ Warning: The 382 nt exon 1 probe is more variable as compared to other probes.

‡ This probe is 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.

⊐ This probe is unusually insensitive to sample DNA depurination. Use of depurinated DNA will lead to a **higher** signal of this probe.

Table 2. P021 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site NM_000344.4 (*SMN1*), NM_017411.4 (*SMN2*)	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		SMN2 [c] *start codon*	*18-20 in NM_017411.4 (Exon 1)*		
382 >ΔΠ	22196-L31253	Exon 1			0.2 kb
184 >	21519-L30024	Exon 1			13.7 kb
221 >	21517-L30022	Exon 2a			2.5 kb
328 >	21514-L30019	Exon 2b			0.1 kb
319 >	14132-L15557	Exon 2b			0.9 kb
288 >	21516-L30893	Exon 3			0.1 kb
346 >	21513-L30018	Exon 3			0.2 kb
409 >Π	01816-L30922	Exon 4			2.0 kb
199 >	21518-L30023	Exon 5			1.4 kb
418 >	22194-L31251	Exon 6			5.7 kb
193 @	22121-L31133	Intron 6			0.2 kb
281 †‡	**21489-L30892**	**Exon 7**	**857-858**	TTACAGGGTTTT-AGACAAAATCAA	0.2 kb
229 @‡	22122-L31134	Intron 7			0.1 kb
391 @	22123-L31135	Intron 7			0.1 kb
265 @	22124-L31136	Intron 7			0.1 kb
427 @	22125-L31137	Intron 7			0.1 kb
400 @	22126-L31138	Exon 8			0.1 kb
364 @	01814-L00807	Exon 8			0.1 kb
301	21491-L29984	Exon 8	1141-1142	GTAAAAGACTGA-GGTGGGGGTGGG	> 100 kb
		SMN2 *stop codon*	*900-902 in NM_017411.4 (Exon 7)*		
		SMN1 *start codon*	*18-20 in NM_000344.4 (Exon 1)*		
382 >ΔΠ	22196-L31253	Exon 1			0.2 kb
184 >	21519-L30024	Exon 1			13.7 kb
221 >	21517-L30022	Exon 2a			2.5 kb
328 >	21514-L30019	Exon 2b			0.1 kb
319 >	14132-L15557	Exon 2b			0.9 kb
288 >	21516-L30893	Exon 3			0.1 kb
346 >	21513-L30018	Exon 3			0.2 kb
409 >Π	01816-L30922	Exon 4			2.0 kb
199 >	21518-L30023	Exon 5			1.4 kb
418 >	22194-L31251	Exon 6			5.7 kb
193 @	22121-L31133	Intron 6			0.2 kb
274 †‡	**21488-L30891**	**Exon 7**	**857-858**	TTACAGGGTTTC-AGACAAAATCAA	0.1 kb
229 @‡	22122-L31134	Intron 7			0.1 kb
391 @	22123-L31135	Intron 7			0.1 kb
265 @	22124-L31136	Intron 7			0.1 kb
427 @	22125-L31137	Intron 7			0.1 kb
400 @	22126-L31138	Exon 8			0.1 kb
364 @	01814-L00807	Exon 8			0.1 kb
295 ‡	21490-L29983	Exon 8	1141-1142	GTAAAAGACTGG-GGTGGGGGTGGG	58.7 kb
		SMN1 *stop codon*	*900-902 in NM_000344.4 (Exon 7)*		
		NAIP *start codon*	*944-946 in NM_004536.3 (Exon 4)*		
238	01259-L00811	Exon 5	1512-1513	TTCATATATAGG-TAAACAGGACAC	
		NAIP *stop codon*	*5153-5155 in NM_004536.3 (Exon 17)*		

[a] See section Exon numbering on page 4 for more information.

Note: The exon numbering used in this P021-B1 SMA product description and in the P021-B1 SMA 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 NCBI reference sequences for *SMN1* and *SMN2*. Please notify us of any mistakes: info@mrcholland.com.

[b] Only partial probe sequences are shown. Complete probe sequences are available at www.mrcholland.com. Please notify us of any mistakes: info@mrcholland.com.

[c] 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. Table 2 is based on Figure 2 in the article of Wadman (2020).

> These probes detect *SMN1*, *SMN2* and *SMN1/2Δ7-8*.

@ These probes detect *SMN1* and *SMN2*.

† The 274 nt probe is the only probe that is absolutely specific for *SMN1;* the 281 nt probe is the only probe that is absolutely specific for *SMN2*.

Δ Warning: The 382 nt exon 1 probe is more variable as compared to other probes.

‡ This probe is 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.

⊐ This probe is unusually insensitive to sample DNA depurination. Use of depurinated DNA will lead to a **higher** signal of this probe.

Related SALSA MLPA probemixes

P060 SMA Carrier	Spinal Muscular Atrophy (SMA), to determine *SMN1* and *SMN2* copy number (best suited for carrier testing/screening).
P460 SMA	Spinal Muscular Atrophy (SMA), to determine *SMN1* copy number and an increased risk for the 2+0 carrier genotype by detection of two associated polymorphisms (g.27134T>G and g.27706-27707delAT).
P058 IGHMBP2	Autosomal recessive distal spinal muscular atrophy 1 (DSMA1), gene included *IGHMBP2*.

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P021 product history	
Version	*Modification*
B1	As compared to version A2, thirteen SMN probes have been added and four probes have been replaced, nine reference probes have been replaced, eleven reference probes and six flanking probes have been removed, and several probe lengths have been adjusted.
A2	The 88 and 96 nt control fragments have been replaced and 100 + 105 nt X-Y control fragments have been included (QDX2),
A1	First release

Implemented changes in the product description

Version B1-06 – 11 July 2022 (04P)
- Table on Final ratios adjusted.
- Information about background signal in Interpretation of results section adjusted.

Version B1-05 – 26 April 2022 (04P)
- Appendix I updated: clarification added to protocol and notes, note added.
- Information added to Clinical background section.
- Added note to section Data analysis on DBS cards.
- Corrected cross-reference to section exon numbering under Tables 1 and 2.
- Minor textual changes.

Version B1-04 – 28 May 2021 (04P)
- Product description rewritten and adapted to a new template.
- Intended purpose updated.
- Ligation sites of the probes targeting the SMN1, SMN2 and NAIP genes updated according to new version of the NM_ reference sequence.
- Added a comparison table between MRC Holland SMA products.
- Appendix 1 updated.
- Table D updated.
- UK added to the list of European countries that accept the CE-mark.
- Size of the NAIP gene is adjusted.

Version B1-03 – 25 June 2020 (04)
- Israel and Morocco were added to the list of countries where this product is registered as IVD.

Version B1-02 – 05 July 2019 (04)
- Warning added to Table 1 and 2 for depurination risk of several probes.
- Use of unbuffered DNA added to paragraph Limitations of the procedure.

- Obscurification of an exon 1-6 deletion by an *SMN1/2Δ7-8* copy added to paragraph Limitations of the procedure.
- Nomenclature changed from *SMN2Δ7-8* to *SMN1/2Δ7-8*.
- Various minor textual changes.

Version B1-01 – 22 November 2018 (04)
- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2 and throughout the document).
- Product description restructured and adapted to a new template.

Version A2-01 – 24 April 2018 (01P)
- Product description restructured and adapted to a new template.
- Various minor textual or layout changes.

Version 38 – 11 August 2017 (55)
- 3 sequences corrected in table 3.

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IVD	EUROPE* CE ISRAEL MOROCCO
RUO	ALL OTHER COUNTRIES

*comprising EU (candidate) member states and members of the European Free Trade Association (EFTA), and the UK. The product is for RUO in all other European countries.

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

P021-B1 SMA can be used as a 2nd tier confirmation and SMA typing test for Spinal Muscular Atrophy, following a 1st tier screening test using SALSA Melt Assay MC002 SMA Newborn Screen.

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 nt, 70 nt, 76 nt, 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