Product Description SALSA® MLPA® Probemix P021-B1 SMA

To be used with the MLPA General Protocol.

Version B1. As compared to version A2, thirteen SMN probes detecting sequences in both *SMN1* and *SMN2* have been added. The probes detecting exon 7 or 8 of either *SMN1* or *SMN2* have been changed. Almost all flanking probes have been removed; most reference probes have been replaced. For complete product history, see page 17.

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, available for various number of reactions. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman capillary sequencers, respectively (see www.mlpa.com).

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

Precautions and warnings: For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mlpa.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.

Important note on exon numbering: The exon numbering for the SMN genes that is used throughout this P021 SMA product description is based on the classic exon numbering as used in most scientific literature: exons 1, 2a, 2b, 3, 4, 5, 6, 7 and 8. In contrast, the exon numbering currently adopted by NCBI and mentioned in the LRG_676 and LRG_677 sequences is 1-9. The exon numbering for the *NAIP* gene is based on RefSeq transcript NM_004536.2. The NM sequences used in this product description date from May 2018 and may be subject to changes made by NCBI.

Intended use: This SALSA MLPA probemix P021 SMA is an in vitro diagnostic (IVD)[1] or research use only (RUO) assay for the detection of copy number changes in the *SMN1* gene for a) patient diagnosis, b) confirmation of a potential cause and clinical diagnosis, and c) carrier testing of spinal muscular atrophy (SMA). Secondly, the assay can be used for *SMN2* copy number determination in (presymptomatic) SMA patients as an aid in prognosis and for treatment eligibility.
This assay is for use with human DNA extracted 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.** foetal 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.

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. Point mutations, which cause SMA in a small number of cases, will not be detected by MLPA. This assay is not intended to be used as a standalone assay for clinical decisions. The results of this test must be interpreted by a clinical molecular geneticist or equivalent.

[1]Please note that this probemix is for In Vitro Diagnostic use (IVD) in the countries specified at the end of this product description. In all other countries, the product is for Research Use Only (RUO).

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 differences in exon 7. *SMN2* is translated much less efficient 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. 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 only 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 0 copies on the other (2+0) (Alias et al. 2004, 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 - 3 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 is important for determining a patient's eligibility for treatment with Spinraza (nusinersen), a drug that can be used to treat SMA by increasing the amount of full-length SMN protein produced from *SMN2* (Otessen 2017).

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.

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

Gene structure: 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 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 *SMN2Δ7-8* in this product description. The *NAIP* gene (17 exons) spans ~27 kb of genomic DNA and is located on chromosome 5q13.2, about 60 kb from the *SMN1* gene.

Transcript variants:
Three ***SMN1*** **transcript variants** are described in https://www.ncbi.nlm.nih.gov/gene/6606. Transcript variant d (NM_000344.3, 1641 nt, coding sequence 164-1048) is the predominant variant and is a reference standard in the NCBI RefSeqGene project. 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 page 1).

Four ***SMN2*** **transcript variants** have been described, see https://www.ncbi.nlm.nih.gov/gene/6607. Transcript variant d (NM_017411.3, 1643 nt, coding sequence 164-1048) 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_075013.1) 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 sites of the *SMN2*-specific MLPA probes are indicated according to the NM_017411.3 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 page 1).

Three ***NAIP*** **transcript variants** have been described, see https://www.ncbi.nlm.nih.gov/gene/4671. The sequence of *NAIP* transcript 1 (NM_004536.2, 6551 nt, coding sequence 719-4930) is a reference standard in the NCBI RefSeqGene project. 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.

Probemix content: SALSA MLPA probemix P021-B1 SMA contains 32 probes with amplification products between 175 and 445 nt:

- 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*).
- One probe for the *NAIP* gene.
- Ten reference probes. The identity of the genes detected by the reference probes is available upon request.

- 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 respectively *SMN1* (important in determining carrier status) and *SMN2* (important for disease prognosis).
- 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 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 exon 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 (*SMN2Δ7-8*) 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.mlpa.com.

Length (nt)	Name
64-70-76-82	Q-fragments (Only visible with <100 ng sample DNA)
88-96	D-fragments (Low signal of 88 or 96 fragment indicates incomplete denaturation)
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.mlpa.com).

MLPA technique validation: Internal validation of the MLPA technique using a minimum of 20 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. This validation experiment can also identify suitable reference samples having two copies each of *SMN1* and *SMN2* and no copies of *SMN2Δ7-8*. An extensive list of Coriell cell lines that can be useful for internal validation is present in Table A of this P021 product description.

Required specimens:
This assay is for use with human DNA extracted 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. foetal 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 read the section on DNA sample treatment found in the MLPA General Protocol.

Reference samples: 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. Analyse the samples with the Coffalyser.Net data analysis software (www.mlpa.com; free of charge)
c. Select samples with two *SMN1*, two *SMN2* and two *NAIP* copies and without *SMN2Δ7-8* copies. Suitable reference samples should have
    - A probe ratio of ~1.0 for the four specific probes: *SMN1* exon 7 & exon 8; *SMN2* exon 7 & 8 **AND**
    - A probe ratio of ~1.0 for all other probes (*SMN1*, *SMN2*, *NAIP,* reference probes).

In most populations, more than 50% of samples from healthy individuals that are tested will be suitable as reference sample. In certain African populations, only 20% of samples tested are suitable as reference sample.

As an extra aid, include MRC-Holland's *SALSA Reference Selection DNA SD082* or *Coriell sample HG01701* in your experiment to help identify suitable references. Both samples contain two copies of the *SMN1*, *SMN2* and *NAIP* genes. Note that *SD082* and *HG01701* should only be used to *identify* reference samples and not as reference samples for data normalisation purposes in routine use. A vial of *SALSA Reference Selection DNA SD082* is included with each P021 SMA order.

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 as much as possible. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol.

Other DNA samples: Inclusion of a positive sample in each experiment is recommended. Coriell Biobank (https://catalog.coriell.org) and DSMZ (https://www.dsmz.de/home.html) have a diverse collection of DNA samples which may be included as a positive control in your MLPA experiments. Table A shows Coriell samples that have been tested by MRC-Holland. Note that the quality of cell lines can change, therefore all samples should be validated before use.

SALSA Reference selection DNA SD082: The SD082 Reference selection DNA provided with this probemix can be used to find suitable reference DNA samples from your own sample collection for further use in MLPA experiments. It is strongly advised to use DNA sample and reference DNA samples extracted with the same method and derived from the same source of tissue. For certain applications, the selection of suitable reference DNA samples is complicated. Inclusion of one reaction with 5 µl SD082 Reference selection DNA facilitates the identification of suitable reference DNA samples. We recommend the use of this SD082 Reference selection DNA only for initial experiments on DNA samples from healthy individuals with the intention to identify suitable reference DNA samples. We do not recommend it for use in all experiments. For further instructions, consult the SD082 Reference selection DNA product description provided. **This product is for research use only (RUO), except when used in combination with a probemix for in vitro diagnostic (IVD) purpose, as specified at the end of this product description.**

Performance characteristics: In the vast majority of 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. 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% (manuscript in preparation). 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 2005-2018 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: SNPs or other polymorphisms (e.g. indels) 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 must 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.mlpa.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.

Table A. Samples from Coriell biobank tested by MRC-Holland for *SMN1*, *SMN2* and *SMN2Δ7-8* copy number (CN)

Coriell biobank Sample ID	*SMN1* CN exon 7	*SMN2* CN exon 7	*SMN1* CN exon 8	*SMN2* CN exon 8	*SMN2Δ7-8* CN
NA00232; NA10684	0	2	0	2	-
NA22592; NA09677; NA03813	0	3	0	3	-
NA03815; NA20760	1	1	1	1	-
NA20787	1	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	2	0	2	0	-
NA19794; HG03940	2	0	2	0	1
NA20767	2	0	2	0	2
HG02514; HG03663; HG03636	2	1	2	1	-
HG02648; HG01365	2	1	2	1	1
HG01631	2	1	2	1	2
HG01701; HG01942; HG01935	2	2	2	2	-
Reference Selection DNA SD082	2	2	2	2	-
HG01617	2	2	2	2	1
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	1
HG01755; HG03650	3	1	3	1	-
NA20775; HG01137	3	1	3	1	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	-
Cell lines 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	-

Interpretation of results:

General points

The SMA 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. 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 completely 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 respectively the 274 and 281 nt probes, 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.

Normal variation in the general population

5. 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.
6. The presence of more than two *SMN1* copies in healthy individuals is a relatively frequent phenomenon, especially in those of African descent (Hendrickson et al. 2009; Sangaré et al. 2014).
7. Complete absence of *SMN2*, as determined by the *SMN2*-specific exon 7 probe (281 nt), is a relatively common phenomenon in healthy individuals and has no known clinical consequences.
8. One or two extra copies of SMN exons 1-6 (*SMN2Δ7-8*) are often present, in particular in samples with no, or only one, *SMN2* copy. A frequency of 8% has been reported in Swedish carriers and non-carriers and a frequency of 23% in Spanish carriers and non-carriers. *SMN2Δ7-8* copies are very rare in patients (Arkblad et al, 2006; Calucho et al, 2018; our unpublished results). The clinical significance of *SMN2Δ7-8* is not yet clear.

SMA Patients

9. 95% of the SMA patients have no *SMN1* copies, as shown by a complete absence of the *SMN1*-specific exon 7 (274 nt) probe amplicon.
10. 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 this. 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), most commonly 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.
11. Besides the four probes that are specific for exon 7 or 8 of either *SMN1* or *SMN2*, the P021-B1 probemix contains an additional 7 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*. See below.
12. A homozygous deletion of *NAIP* exon 5 is frequently observed in SMA patients, but is very rare in healthy individuals. Although some publications indicate a worse prognosis for SMA patients with a homozygous *NAIP* exon 5 deletion, MRC-Holland is of the opinion that the evidence that this probe is useful for patient prognosis is inconclusive.

SMA Carriers

13. 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.
14. 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.

15. Although rare, individuals with two *SMN1* copies may still be a carrier. 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.

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

Table B. Interpretation Table

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 copies > 0 *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 5 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 10 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 cases.
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.
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 15 above.
SMN1 exon 7: 2 copies *SMN1* exon 8 ≠2 *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 15 above.

Table C. Relationship between Probe Ratio and Copy Number for P021 SMA

Most probes in P021 detect a sequence that is present in *both* the *SMN1 and SMN2* gene. 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** (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	**Probe Ratio**	1	1	1	1	1	1
	Copy Number	2	2	2	2	**4**	**4**
SMA patient *deletion of SMN1 (both alleles)*	**Probe 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*	**Probe 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*	**Probe Ratio**	0.5	1.5	1	1	0.75	1
	Copy Number	1	3	2	2	3	4
SMA patient *3 copies SMN2*	**Probe 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* One *SMN2Δ7-8* copy present	**Probe 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*	**Probe 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 SMN2Δ7-8* copy	**Probe 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 probe ratio value of the *SMN2*-specific exon 7 probe (281 nt), converted to copy number; see Table D.
2. The median probe ratio value of the seven probes detecting exons 7 or 8 of both *SMN1* plus *SMN2* (193, 230, 265, 364, 391, 400, 427 nt), converted to copy number; see Table D.
3. The median probe ratio value of the probes detecting exons 1 to 6 of both *SMN1* plus *SMN2*, (382, 184, 221, 328, 319, 288, 346, 409, 199, 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 probe ratio value is within the specified range and one probe 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, involving the exon 1-6 probes, should only be taken into consideration when this 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 somewhere 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 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.

Table D. Relationship between Probe Ratio and Copy Number for *SMN2* in *SMN1*-negative patients.

This table assumes that all reference samples meet the criteria described in section "Reference samples".
This table is intended only for samples that do not have any copies for *SMN1* exon 7.

Probe ratio of the 281 nt SMN2 specific probe for exon 7 (range)	***SMN2* copy number**
0 - 0.10	0 (never observed in patients)
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
***Median* probe ratio of non-specific SMN exon 7-8 probes* or exon 1-6 probes** (range)**	***SMN2* copy number**
0.15 - 0.35	1
0.40 - 0.60	2
0.65 - 0.85	3
0.90 - 1.10	4
1.15 - 1.35	5
1.40 - 1.60	6
1.65 - 1.85	7
All other values	Ambiguous

* This median probe ratio value mentioned here is the median value of the 7 probes detecting exons 7 or 8 of both *SMN1* plus *SMN2* (193, 230, 265, 364, 391, 400, 427 nt).

** Median value of the 10 probes detecting exon 1-6 of *SMN1* plus *SMN2* (184, 199, 221, 288, 319, 328, 346, 382, 409, 418 nt).

General Point 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 probe ratio (or Dosage Quotient (DQ) as it is referred to in the MLPA General Protocol) 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 a correct interpretation of complex results.
- False positive results: please note that abnormalities detected by a single probe or multiple consecutive probes still may be a false positive result. Incomplete DNA denaturation (e.g. due to salt contamination) can 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.
- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (Schwartz et al. 2007 described this for *DMD)*. 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 in some cases not result in inactivation of that gene copy.
- Copy number changes detected by reference probes are unlikely to have any relation to the condition tested for.

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. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (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 1 chromosome and 0 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 only a single probe always require confirmation by another method. 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. Sequence analysis can establish whether mutations or polymorphisms are present in the target sequence of an MLPA probe.

Most problems observed with the MLPA technique are due to a bad sample DNA quality. In case of doubt on results, we recommend to repeat the P021 reaction using an independently obtained DNA sample.

Absence of *SMN1* exon 7 can be confirmed with the Salsa Melt assay MC002 SMA Newborn Screen. MC002 reactions use a simple PCR reaction followed by melting curve analysis. The MC002 SMA assay is available from MRC-Holland.

SMN mutation databases:
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 SMN databases. 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@mlpa.com.

Table 1. SALSA MLPA Probemix P021-B1 SMA

Length (nt)	SALSA MLPA probe [a]	Chromosomal position (hg18) [b]			
		Reference	*SMN1*	*SMN2*	*NAIP*
64-105	Control fragments – see table in probemix content section for more information				
175	Reference probe 00808-L00638	18q21			
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	2q36			
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	4q35			
256 *	Reference probe 19625-L26284	10p11			
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	1q24			
319 *	**SMN1/SMN2 probe** 14132-L15557		**Exon 2b**		
328 *	**SMN1/SMN2 probe** 21514-L30019		**Exon 2b**		
337 *	Reference probe 19746-L26529	9q34			
346 *	**SMN1/SMN2 probe** 21513-L30018		**Exon 3**		
355 *	Reference probe 19127-L25074	11p15			
364	**SMN1/SMN2 probe** 01814-L00807		**Exon 8**		
373 *	Reference probe 18296-L25750	8p11			
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	17p13			
445 *	Reference probe 20431-L27913	1q32			

* New in version B1 (from lot B1-1018 onwards).

¥ 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.

(a) Probes designated as *SMN1*/*SMN2* are not specific for either gene and will indicate a combined copy number of both genes. For exons 1-6, these probes will indicate the combined copy number of the *SMN1*, *SMN2* and *SMN2Δ7-8* genes.

(b) The exon numbering for SMN genes used in this product description is based on the classic exon SMN numbering used in most literature (1, 2a, 2b, 3, 4, 5, 6, 7 and 8). The exon numbering for *NAIP* is based on RefSeq transcript NM_004536.2. NM sequences used are from May 2018 and may be subject to change by NCBI at a later date.

Table 2. P021 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	exon[a]	Ligation site	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
SMN2 [b] *stop codon*			*1046-1048 in NM_017411.3 (Exon 7)*		
301	21491-L29984	Exon 8	1287-1288	GTAAAAGACTGA-GGTGGGGGTGGG	0.1 kb
364 @	01814-L00807	Exon 8			0.1 kb
400 @	22126-L31138	Exon 8			0.1 kb
427 @	22125-L31137	Intron 7			0.1 kb
265 @	22124-L31136	Intron 7			0.1 kb
391 @	22123-L31135	Intron 7			0.1 kb
229 @	22122-L31134	Intron 7			0.1 kb
281†	**21489-L30892**	**Exon 7**	**1003-1004**	TTACAGGGTTTT-AGACAAAATCAA	0.2 kb
193 @	22121-L31133	Intron 6			5.7 kb
418 >	22194-L31251	Exon 6			1.4 kb
199 >	21518-L30023	Exon 5			2.0 kb
409 >	01816-L30922	Exon 4			0.2 kb
346 >	21513-L30018	Exon 3			0.1 kb
288 >	21516-L30893	Exon 3			0.9 kb
319 >	14132-L15557	Exon 2b			0.1 kb
328 >	21514-L30019	Exon 2b			2.5 kb
221 >	21517-L30022	Exon 2a			13.7 kb
184 >	21519-L30024	Exon 1			0.2 kb
382 > Δ	22196-L31253	Exon 1			875.4 kb
SMN2 *start codon*			*164-166 in NM_017411.3 (Exon 1)*		
SMN1 *start codon*			*164-166 in NM_000344.3 (Exon 1)*		
382 > Δ	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 >	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**	**1003-1004**	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	1287-1288	GTAAAAGACTGG-GGTGGGGGTGGG	58.7 kb
SMN1 *stop codon*			*1046-1048 in NM_000344.3 (Exon 7)*		
NAIP *stop codon*			*4928-4930 in NM_004536.2 (Exon 17)*		
238	01259-L00811	Exon 5	1287-1288	TTCATATATAGG-TAAACAGGACAC	
NAIP *start codon*			*719-721 in NM_004536.2 (Exon 4)*		

> These probes detect *SMN1*, *SMN2* and *SMN2Δ7-8*.

@ 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.

(a) This product description uses the classic *SMN1*/*SMN2* exon numbering used in most literature of exons 1, 2a, 2b and 3-8. NM sequences used are from May 2018 and may be changed by NCBI at a later date.
(b) Note that the orientation of the *SMN2* gene is yet not established with certainty. Table 2 is based on Figure 1 in the review of Butchbach (2016).

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*.

Related SALSA Melt Assay products

- MC002 SMA Newborn Screen: Spinal Muscular Atrophy (SMA), to identify newborns with homozygous deletion of *SMN1* exon 7.

References

- Alías L et al. (2014). Improving detection and genetic counseling in carriers of spinal muscular atrophy with two copies of the SMN1 gene. *Clin Genet.* 85:470-475.
- Alías L et al. (2018). Utility of two *SMN1* variants to improve spinal muscular atrophy carrier diagnosis and genetic counselling. *Eur J Hum Genet. 2018 Jun 14*. [Epub ahead of print]
- Arkblad EL. et al. (2006). Multiplex ligation-dependent probe amplification improves diagnostics in spinal muscular atrophy. *Neuromuscul Disord.* 16:830-8.
- Ben Shachar S et al. (2011). Large-scale population screening for spinal muscular atrophy: clinical implications. *Genet Med.* 13:110-114.
- Butchbach (2016). Copy Number Variations in the Survival Motor Neuron Genes: Implications for Spinal Muscular Atrophy and Other Neurodegenerative Diseases. *Front Mol Biosci* 3:7. doi: 10.3389/fmolb.2016.00007.
- Calucho M. et al. (2018). Correlation between SMA type and *SMN2* copy number revisited: an analysis of 625 unrelated Spanish patients and a compilation of 2,834 reported cases. *Neuromuscul Disord* 28:208-15.
- Feldkötter et al. (2002). Quantitative analyses of *SMN1* and *SMN2* based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am J of Hum Genetics* 70:358-68.
- Helmken C. et al. (2003). Evidence for a modifying pathway in SMA discordant families. *Hum Genet* 114:11-21.
- Hendrickson BC. et al. (2009). Differences in *SMN1* allele frequencies among ethnic groups within North America. *J Med Genet.* 46:641-644.
- Luo M. et al. (2014). An Ashkenazi Jewish *SMN1* haplotype specific to duplication alleles improves pan-ethnic carrier screening for spinal muscular atrophy. *Genet Med*. 16:149-56.
- Ottesen EW (2017). ISS-N1 makes the First FDA-approved Drug for Spinal Muscular Atrophy. *Transl Neurosci.* 8:1-6.
- Prior T.W. et al. 2009. A positive modifier of Spinal Muscular Atrophy in the SMN2 gene. *Am J Hum Genet* 85:408-413.
- Sangaré et al. (2014). Genetics of low spinal muscular atrophy carrier frequency in sub-Saharan Africa. *Ann Neurol.* 75(4):525-32
- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
- Thauvin-Robinet C et al. 2012. Homozygous SMN1 exons 1-6 deletion: Pitfalls in genetic counseling and general recommendations for spinal muscular atrophy molecular diagnosis. *Am J Med Genet* 158A:1735-1741.
- Varga RE et al. (2012). MLPA-based evidence for sequence gain: pitfalls in confirmation and necessity for exclusion of false positives. *Anal Biochem.* 421:799-801.

Selected publications about SALSA® MLPA® Probemix P021 SMA

- Bai J-L et al. (2014). Subtle mutation detection of *SMN1* gene in Chinese spinal muscular atrophy patients: implication of molecular diagnostic procedure for *SMN1* gene mutations. *Genetic testing and molecular biomarkers*. 18:546-551.
- Bai J et al. (2017). The *SMN1* common variant c. 22 dupA in Chinese patients causes spinal muscular atrophy by nonsense-mediated mRNA decay in humans. *Gene*.
- Brkusanin M et al. (2015). Joint effect of the *SMN2* and *SERF1A* genes on childhood-onset types of spinal muscular atrophy in Serbian patients. *J Hum Genet*. 60:723-728.
- Fang P et al. (2015). Molecular characterization and copy number of *SMN1*, *SMN2* and *NAIP* in Chinese patients with spinal muscular atrophy and unrelated healthy controls. *BMC Musculoskelet Disord*. 16:11.
- Fernández RM, Peciña A, Muñoz-Cabello B, Antiñolo G & Borrego S (2016). Co-segregation of a homozygous *SMN1* deletion and a heterozygous *PMP22* duplication in a patient. *Clinical case reports*. 4:879-884.
- Ganji H et al. (2015). Detection of intragenic *SMN1* mutations in spinal muscular atrophy patients with a single copy of *SMN1*. *Journal of child neurology*. 30:558-562.
- Kaneko K, Arakawa R, Urano M, Aoki R & Saito K (2017). Relationships between long-term observations of motor milestones and genotype analysis results in childhood-onset Japanese spinal muscular atrophy patients. *Brain and Development*. 39:763-773.
- Kubo Y, Nishio H & Saito K (2015). A new method for *SMN1* and hybrid SMN gene analysis in spinal muscular atrophy using long-range PCR followed by sequencing. *J Hum Genet*. 60:233.
- Li L et al. (2017). Evaluation and comparison of three assays for molecular detection of spinal muscular atrophy. *Clin Chem Lab Med*. 55:358-367.
- Lin X et al. (2017). Modeling the differential phenotypes of spinal muscular atrophy with high-yield generation of motor neurons from human induced pluripotent stem cells. *Oncotarget*. 8:42030.
- Liu Z et al. (2016). New multiplex real-time PCR approach to detect gene mutations for spinal muscular atrophy. *BMC Neurol*. 16:141.
- Medrano S et al. (2016). Genotype-phenotype correlation of SMN locus genes in spinal muscular atrophy children from Argentina. *Eur J Paediatr Neurol*. 20:910-917.
- Noguchi Y et al. (2016). Telomeric Region of the Spinal Muscular Atrophy Locus Is Susceptible to Structural Variations. *Pediatr Neurol*. 58:83-89.
- Qu YJ et al. (2015). Association of copy numbers of survival motor neuron gene 2 and neuronal apoptosis inhibitory protein gene with the natural history in a Chinese spinal muscular atrophy cohort. *J of child neurology*. 30:429-436.
- Qu YJ et al. (2016). Mutation Spectrum of the Survival of Motor Neuron 1 and Functional Analysis of Variants in Chinese Spinal Muscular Atrophy. *J Mol Diagn*. 18:741-752.
- Qu YJ et al. (2016). A rare variant (c. 863G> T) in exon 7 of *SMN1* disrupts mRNA splicing and is responsible for spinal muscular atrophy. *European Journal of Human Genetics*. 24:864.
- Rudnik-Schöneborn S et al. (2016). Distally pronounced infantile spinal muscular atrophy with severe axonal and demyelinating neuropathy associated with the S230L mutation of *SMN1*. *Neuromuscular Disorders*. 26:132-135.
- Steinkellner H, Etzler J, Gmeiner BM & Laccone F (2015). Detection of survival motor neuron protein in buccal cells through electrochemiluminescence-based assay. *Assay and drug development technologies*. 13:167-173.
- Theodorou L et al. (2015). Genetic findings of Cypriot spinal muscular atrophy patients. *Neurol Sci*. 36:1829-1834.
- Yamada H et al. (2015). Two Japanese patients with SMA Type 1 suggest that axonal-SMN may not modify the disease severity. *Pediatric neurology*. 52:638-641.
- Wadman RI et al. (2016). A Comparative Study of SMN Protein and mRNA in Blood and Fibroblasts in Patients with Spinal Muscular Atrophy and Healthy Controls. *PloS one*. 11:e0167087.
- Wan Y & Zhang J (2016). Pyramidal signs in a Caucasian patient with spinal muscular atrophy: a case report. *Folia neuropathologica*. 54:418-421.

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 B1-01 – 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.

Version 37 – 12 April 2017 (55)
- Reference sample section created and information concerning the possibility of SD019 usage included.

Version 36 – 17 March 2017 (55)
- Product description adapted to a new lot (lot number added, new picture included).
- Various minor textual changes throughout the document.

Version 35 – 10 January 2017 (55)
- Warning added in Table 1, 445 nt probe 00802-L00320.

Version 34 – 13 April 2016 (55)
- Product description adapted to a new lot (lot number added, new picture included).

More information: www.mlpa.com; www.mlpa.eu	
	MRC-Holland bv; Willem Schoutenstraat 1 1057 DL, Amsterdam, the Netherlands
E-mail	info@mlpa.com (information & technical questions); order@mlpa.com (orders)
Phone	+31 888 657 200

IVD	EUROPE* CE
RUO	ALL OTHER COUNTRIES

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

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

SMA newborn screening

P021-B1 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

When using P021-B1 for newborn screening, use only 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 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 in the liquid.
5. Seal the plate.
6. Heat the samples for 15 minutes at 99°C in a thermocycler with heated lid.
7. Spin down using a short spin 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 - 6°C for potential follow-up assays. Prolonged storage is possible at -25°C – -15°C.
9. Continue with the MLPA General protocol (www.mlpa.com)

- Prepare 10 mM NaOH: Mix 1 ml 1 N NaOH (Sigma S2770) + 99 ml 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 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 SALSA P021 assay is influenced by contamination of DNA samples with DNA of other newborns. Cleaning punchers between their 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.

Notes:

- It is essential to use Coffalyser.NET for results interpretation.
- When analyzing 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 to verify that that the MLPA P021 SMA assay has been performed with sufficient DNA.
- The standard deviation over the reference probes should be below 0.10.