Product Description
SALSA® MLPA® Probemix P060-B2 SMA Carrier

To be used with the MLPA General Protocol.

Version B2
For complete product history see page 15.

Catalogue numbers:
- **P060-025R:** SALSA MLPA Probemix P060 SMA Carrier, 25 reactions.
- **P060-050R:** SALSA MLPA Probemix P060 SMA Carrier, 50 reactions.
- **P060-100R:** SALSA MLPA Probemix P060 SMA Carrier, 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 4).

Please note: This product should not be used to determine *SMN2* copy numbers for the purpose of determining disease prognosis or eligibility for SMA therapeutics. For the most reliable *SMN2* copy number detection, use SALSA MLPA Probemix P021 SMA (from version B1 onwards).

Intended purpose
The SALSA MLPA Probemix P060 SMA Carrier is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplication in exons 7 and 8 of the *SMN1* gene 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. P060 SMA Carrier 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. This probemix can also be used for the detection of copy number changes of exons 7 and 8 of the *SMN2* gene, as an interpretation aid for *SMN1* copy number determination.

Copy number variations (CNVs) detected with P060 SMA Carrier should be confirmed with a different technique. In particular, CNVs detected by the SMN1 exon 7 probe always require confirmation by another method. Point mutations, which cause SMA in a small number of cases, will not be detected by MLPA. It is therefore recommended to use this assay in combination with sequence analysis.

Assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, parental evaluation, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

This device is not intended to be used for standalone diagnostic purposes, pre-implantation screening, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1]Please note that this probemix is for in 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 and 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.

		P060	P460	P021	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

Clinical background

Spinal muscular atrophy (SMA) is a neuromuscular disorder characterised by degeneration of the anterior horn cells of the spinal cord, leading to symmetrical muscle weakness and atrophy. The estimated incidence of SMA is 1:6,000-1:10,000: the second most common lethal autosomal recessive disorder in Caucasians, after cystic fibrosis (Ben-Shachar et al. 2011, Smith et al. 2007). SMA is usually divided into four clinical groups based on age of onset and maximum function obtained.

Two (highly similar) genes play a pivotal role in SMA: *SMN1* and *SMN2*. Most individuals* have two copies of each gene. The SMA region on 5q13.2, containing the telomeric *SMN1* and the centromeric *SMN2*, is a complicated inverted repeat area displaying high instability, leading to frequent deletions and gene conversions. *SMN1* and *SMN2* can only be distinguished by two single nucleotide differences: one in exon 7 and one in exon 8. The single nucleotide difference between *SMN1* and *SMN2* in exon 7 affects mRNA splicing in *SMN2* resulting in an altered SMN protein with a limited half-life and function.

A total of 95-98% of SMA patients (this percentage is lower in SMA patients from African descent) show homozygous deletion of at least exon 7 of the telomeric *SMN1* gene (Labrum et al. 2007). The remaining 3-5% present compound heterozygosity with a point mutation on one chromosome and a deletion/gene conversion on the other. Such a point mutation will not be detected by this P060 SMA Carrier MLPA assay and should be identified by sequencing. In a small number of patients, the *SMN1* defect is a copy number change of *SMN1* exons 1-6 which can be detected with the SALSA MLPA Probemix P021 SMA (Arkblad et al. 2006).

The great majority of SMA carriers can be identified by the presence of a single *SMN1* exon 7 copy. The one copy frequency in the US is 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. Approximately 3-8% of SMA carriers (27% of African Americans) have one functional and one defective *SMN1* copy, or have two *SMN1* copies on one chromosome and 0 copies on the other (2+0) (Alias et al. 2014, Ben-Shachar et al. 2011, Hendrickson et al. 2009, Miskovic

et al. 2011, Smith et al. 2007). Dosage analysis cannot determine the difference between '1+1' and '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 in parents of SMA patients who have 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 (Silent) Carrier, will, depending on ethnicity, increase the chance of identifying silent carriers.

The *SMN2* copy number is very variable with only 60-70% of individuals having two copies. Provided that at least one functional *SMN1* copy is present, complete absence of the centromeric *SMN2* gene has no known clinical consequences. More information on spinal muscular atrophy can be found in http://www.ncbi.nlm.nih.gov/books/NBK1352/.

*In people of African descent, the percentage of SMA patients with a homozygous exon 7 deletion may be lower (Labrum et al. 2007). This assay does not detect other causes of SMA such as pathogenic point mutations.

Gene structure
SMN1 and *SMN2* are part of a 500 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.

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 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).

Exon numbering
The exon numbering for the *SMN* genes that is used throughout this P060-B2 SMA Carrier 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.

Probemix content
This SALSA MLPA Probemix P060 SMA Carrier contains 21 MLPA probes with amplification products between 154 and 342 nt (Table 1) including two probes each for *SMN1* and *SMN2* (Table 2) and 17 reference probes that detect sequences outside this region. The identity of the genes detected by the reference probes is available online (www.mrcholland.com).

- The **SMN1 Exon 7 probe 14919-L17081** (183 nt) is the most important probe as it can be used to determine *SMN1* copy number, which is important for deducing SMA diagnosis and carrier status. This probe is specific

for *SMN1* and will give no significant signal on *SMN2*. The probe has its ligation site at the C-to-T transition in exon 7, which is the site that affects RNA splicing in *SMN2*.

- The **SMN1 Exon 8 probe 14881-L17082** (218 nt) is able to distinguish between *SMN1* and *SMN2* at exon 8 (G-to-A transition). The signal of this probe indicates the copy number of *SMN1* exon 8. In approximately 95% of the samples, the copy number detected by the *SMN1* exon 7 and exon 8 probes is identical. This *SMN1* exon 8 probe cannot be used to quantify the number of *SMN1* copies, as an exon 8 mutation will still result in a functional protein. Only the *SMN1* exon 7 probe should be used to determine the *SMN1* copy number. In the majority of the remaining 5% of samples, gene conversion between *SMN1* and *SMN2* has resulted in a chimeric gene containing the *SMN1* exon 7 sequence and the *SMN2* exon 8 sequence. Such a hybrid gene results in a functionally identical protein to the SMN1 protein.

- The **SMN2 Exon 7 probe 14921-L17083** (282 nt) identifies the *SMN2* exon 7 copy number, which aids in the detection of gene-conversion events. The *SMN2* copy number has no influence on SMA carrier status.

- The **SMN2 Exon 8 probe 14878-L17084** (301 nt) identifies the *SMN2* exon 8 copy number, which aids in the detection of gene-conversion events. The *SMN2* copy number has no influence on SMA carrier status.

The summary of these findings and what they mean for carrier/patient status can be found in Table B (page 8). Figures depicting variation as can be detected by P060 SMA Carrier can be found on page 12 (Figures 1-4).

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)
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).

The choice of reference samples is important for the correct determination of the *SMN1* copy numbers. MRC Holland is not able to provide reference DNA samples. One reason is that MLPA reactions on all samples, including reference samples, should be done on DNA extracted by the same method, as stated above. It is strongly advised to first make a selection of suitable reference samples with known copy numbers before SMA testing is started. One method of doing this is to test a number (e.g. 16) of healthy individuals. Identification of samples having two copies of both *SMN1* and *SMN2* genes should usually be simple as in most populations these will constitute the majority of the samples. The SALSA Reference Selection DNA SD082 can help in identifying suitable reference DNA samples from your own collection. Please note that in some populations, such as African-Americans, the number of individuals with a total of three *SMN1* copies may be almost identical to those with two copies (Hendrickson et al. 2009).

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 P060-B2 probemix at MRC Holland and can be used as a positive control samples to detect copy number variations 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 P060-B2 SMA Carrier for *SMN1* and *SMN2* copy numbers (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*
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; HG00255; NA19437; HG01377	3	0	3	0
HG01755; HG03650; 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
HG02697	4	1	3	2

* *SMN2* probes in this probemix cannot reliably distinguish between 4 or more copies. The indicated copy numbers have been validated using SALSA MLPA Probemix P021 SMA.

SALSA Reference Selection DNA SD082

The selection of suitable reference DNA samples that can be used with P060 SMA Carrier is very important. 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 or can be ordered separately. 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. 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. **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

The expected number of Caucasian SMA carriers that can be detected with this MLPA probemix is approximately 95%, which is higher than in other populations (>90% in Ashkenazi Jewish, Hispanic and Asian populations, ~71% in African Americans) (Hendrickson et al. 2009). The expected number of Caucasian SMA patients that can be detected with this MLPA probemix is between 95-98%, which is also higher than in other populations. The remaining ~5% is caused by small mutations or a deletion of other *SMN1* exons, usually in combination with a heterozygous *SMN1* deletion (compound heterozygosity) (Feldkötter et al. 2002). The small mutations can be found using sequence analysis techniques.

The analytical sensitivity and specificity for the detection of deletions/duplications in the *SMN1* and *SMN2* genes (based on a 2006-2021 literature review) 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* and *SMN2* specific MLPA probes are allele copy numbers of 2 (normal), 0 (homozygous deletion), 1 (heterozygous deletion), 3 (heterozygous duplication) and 4 (heterozygous triplication or homozygous duplication).

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
Copy numbers ≥4	FR > 1.75[#]
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).

[#]*SMN2* probes in this probemix cannot reliably distinguish between 4 or more copies. A final ratio of > 1.75 for *SMN2* probes should be interpreted as 4 or more copies. SALSA MLPA Probemix P021 SMA can be used for a more accurate SMN2 copy number determination.

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.

In case of a gene conversion event whereby a different copy number is found for *SMN1* exon 7 compared to *SMN1* exon 8, *SMN2* exon 7 and 8 copy numbers serve only as an interpretation aid of the *SMN1* copy numbers. Table A shows multiple examples of samples where gain or loss of *SMN1* copy numbers corresponds with loss or gain of *SMN2* copy numbers, respectively, thereby confirming *SMN1* copy number results. Gene conversions in samples with copy number of 4 or more cannot be confirmed by SALSA MLPA Probemix P060 SMA Carrier.

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. In this product description, the classic 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 P060 SMA Carrier 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 the 183 and 282 nt probes respectively, is the only clinically relevant difference between these two genes.
4. Determining *SMN2* copy number is not relevant for SMA carrier testing.
5. Analysis of parental samples may be necessary for correct interpretation of complex results.

6. 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

7. In 5-10% of all cases, the *SMN1* (218 nt) and *SMN2* (301 nt) **exon 8** probes will show a different copy number compared to the *SMN1* (183 nt) and *SMN2* (282 nt) **exon 7** probes. In these cases, the copy number of *SMN1* is only determined by the exon 7 probe. The nucleotide difference targeted by these exon 8 probes is not clinically relevant.
8. 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).
9. Complete absence of *SMN2*, as determined by the *SMN2*-specific exon 7 probe (282 nt), is a relatively common phenomenon in healthy individuals and has no known clinical consequences.

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 (183 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. A small number of patients have a deletion of other exons, in particular exons 1-6. These can be detected with the SALSA MLPA Probemix P021-B1 SMA.

SMA Carriers

12. An individual with a single *SMN1* exon 7 copy (as determined by the *SMN1*-specific exon 7 probe at 183 nt) is a SMA carrier.
13. 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.
14. 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). SALSA MLPA Probemix P460 SMA (Silent) Carrier 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. Detection of these carriers is compromised, as MLPA and other techniques are not able to identify carriers who have one chromosome lacking *SMN1* with the other chromosome carrying two copies of *SMN1*. See also the P460 SMA (Silent) Carrier product description (www.mrcholland.com).

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 determining *SMN1* exon 7 sequence are found. Due to gene conversion, one or more copies of the characteristic *SMN1* exon 8 sequence appear to have become incorporated in the *SMN2* gene.

Finding	Conclusion	Explanation
- *SMN1* exon 7: 1 copy. *SMA symptoms*	SMA patient	If the patient has SMA symptoms, but one copy of *SMN1* exon 7 is present, the patient may belong to the group presenting compound heterozygosity. Sequencing might reveal a defect in the remaining *SMN1* copy.
- *SMN1* exon 7: 1 copy. - *SMN1* exon 8: 1 copy. *No SMA symptoms*	SMA carrier	One copy of *SMN1* is absent, making the person a carrier. The absence of one copy of the *SMN1* exon 8 sequence confirms this.
- *SMN1* exon 7: 1 copy. - A: *SMN1* exon 8: > 1 copies. - B: *SMN1* exon 8: 0 copies. *No SMA symptoms*	SMA carrier	One copy of *SMN1* is absent, making the person a carrier. A: due to gene conversion, one (or more) copies of *SMN1* exon 8 have become incorporated in the *SMN2* gene. B: an *SMN2* exon 8 copy has replaced the *SMN1* exon 8 copy, resulting in a higher *SMN2* exon 8 copy number compared to *SMN2* exon 7.
- *SMN1* exon 7: 2 copies. *No SMA symptoms*	Most likely not a SMA carrier	Most likely this person is not a carrier. However, there is a possibility that both *SMN1* copies lie on one chromosome. If there is a reason to believe that the person is a carrier (i.e. child is SMA patient), he/she may belong to the 3-8% of carriers where this is indeed the case.

- 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. The P060 SMA Carrier probemix 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.
- *SMN2* probes in this probemix cannot reliably distinguish between 4 or more copies.

Confirmation of results

Copy number changes detected by P060-B2 SMA Carrier 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 P021 SMA and P460 SMA (Silent) Carrier cannot be used for confirmation of results.

SMA mutation database

http://grenada.lumc.nl/LSDB_list/lsdbs/SMN1. 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 P060-B2 SMA Carrier

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*SMN1*	*SMN2*
64-105	Control fragments – see table in probemix content section for more information			
154	Reference probe 02595-L17085	5q		
163	Reference probe 02291-L17086	3p		
172	Reference probe 02978-L17087	4q		
183	**SMN1 probe** 14919-L17081		**Exon 7**	
191	Reference probe 00559-L17088	11q		
200	Reference probe 00976-L17298	11p		
208	Reference probe 12490-L17096	1q		
218	**SMN1 probe** 14881-L17082		**Exon 8**	
228	Reference probe 14498-L17101	20p		
237	Reference probe 02334-L17301	12q		
245	Reference probe 14293-L17100	15q		
255	Reference probe 13128-L17099	9q		
264	Reference probe 07630-L17091	10q		
272	Reference probe 14361-L17098	4q		
282 «	**SMN2 probe** 14921-L17083			**Exon 7**
292	Reference probe 00824-L17097	3q		
301 «	**SMN2 probe** 14878-L17084			**Exon 8**
311	Reference probe 06425-L17092	6p		
321	Reference probe 01042-L17093	8q		
331	Reference probe 01043-L17094	8q		
342	Reference probe 13399-L17297	6q		

[a] See section Exon numbering on page 3 for more information.

Note: The exon numbering used in this P060-B2 SMA Carrier product description and in the P060-B2 SMA Carrier 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.

« A low signal can be caused by Salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

Table 2. SMN probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site NM_017411.4 (*SMN2*), NM_000344.4 (*SMN1*)	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
282 «	14921-L17083	*SMN2* exon 7	857-858	TTACAGGGTTTT-AGACAAAATCAA	0.8 kb
301 «	14878-L17084	*SMN2* exon 8	1141-1142	GTAAAAGACTGA-GGTGGGGGTGGG	> 100 kb
183	14919-L17081	*SMN1* exon 7	857-858	TTACAGGGTTTC-AGACAAAATCAA	0.7 kb
218	14881-L17082	*SMN1* exon 8	1141-1142	GTAAAAGACTGG-GGTGGGGGTGGG	

[a] See section Exon numbering on page 3 for more information.

Note: The exon numbering used in this P060-B2 SMA product description and in the P060-B2 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.

« A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

SALSA MLPA Probemix P060 SMA Carrier sample pictures

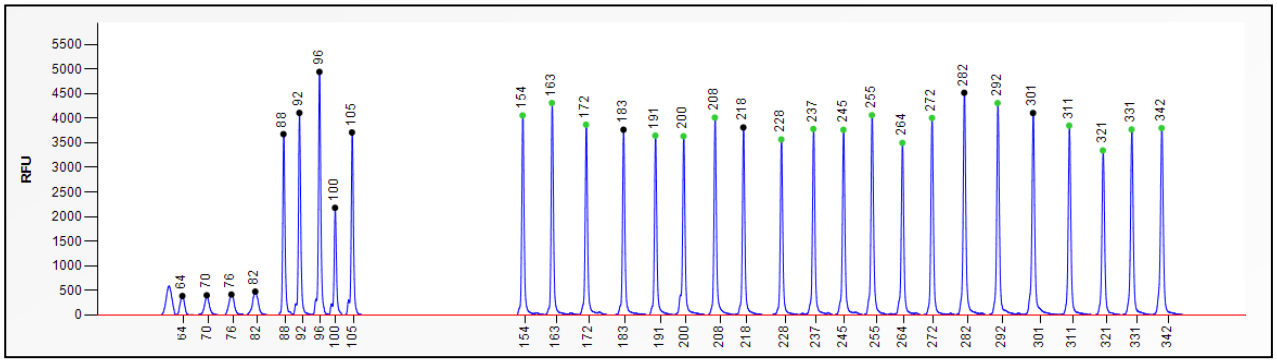

Figure 1. Capillary electrophoresis pattern of a sample of approximately 50 ng human male control DNA (2 copies each of *SMN1* and *SMN2*) analysed with SALSA MLPA probemix P060 SMA Carrier (version B2).

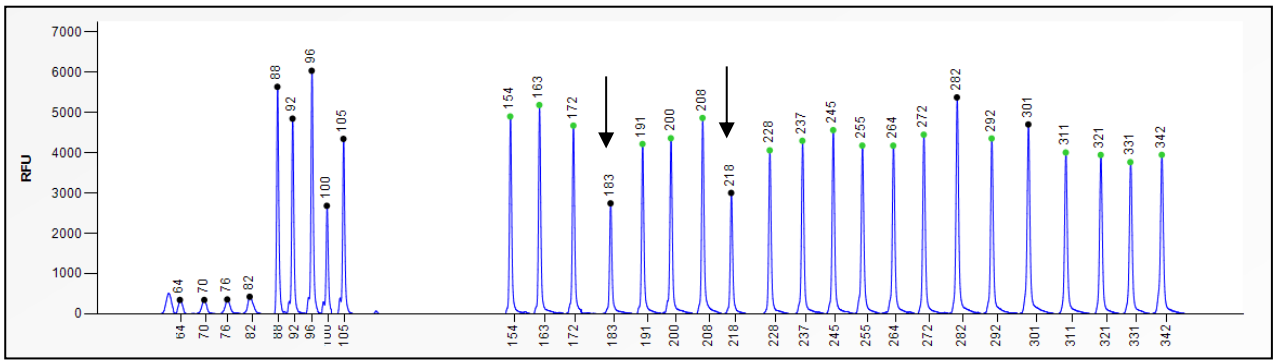

Figure 2. Capillary electrophoresis pattern of a sample of approximately 50 ng human male SMA-carrier DNA (1x *SMN1*, 2x *SMN2*) analysed with SALSA MLPA probemix P060 SMA Carrier (version B2).

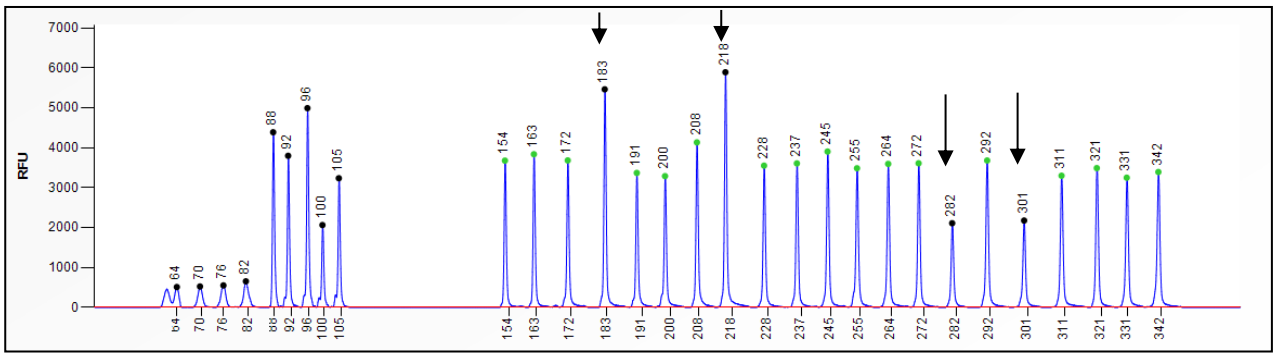

Figure 3. Capillary electrophoresis pattern of a sample of approximately 50 ng human male DNA (3x *SMN1*, 1x *SMN2*) analysed with SALSA MLPA probemix P060 SMA Carrier (version B2).

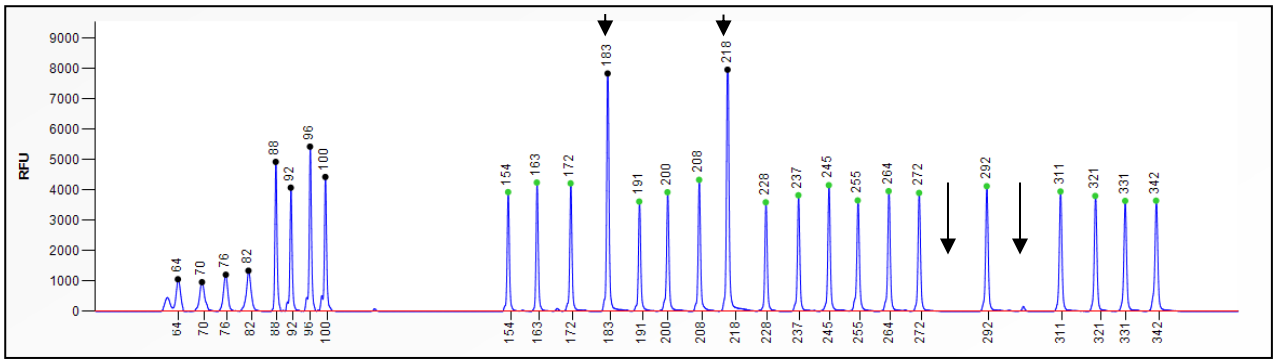

Figure 4. Capillary electrophoresis pattern of a sample of approximately 50 ng human female DNA (4x *SMN1*; 0x *SMN2*) analysed with SALSA MLPA probemix P060 SMA Carrier (version B2).

Related SALSA MLPA probemixes

P021 SMA	Spinal Muscular Atrophy (SMA), to determine *SMN1* and *SMN2* copy number changes (patients). P021 contains probes for all *SMN1*/*SMN2* exons as well as additional probes for exons 7 and 8 for a precise *SMN1* and *SMN2* copy number detection.
P460 SMA (Silent) Carrier	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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P060 product history	
Version	*Modification*
B2	The 88 and 96 nt DNA denaturation control fragments have been replaced (QDX2).
B1	Completely redesigned product. SMN2 exon 7 and 8 probes are now included.
A2	Two extra control fragments at 100 and 105 nt, specific for chromosome X and Y, have been added.
A1	First release.

Implemented changes in the product description

Version B2-11 – 04 June 2024 (04P)
- Added that the *SMN2* probes in this probemix cannot distinguish between 4 and >4 copies of *SMN2* throughout this Product Description. Updated table in the Interpretation of results section to reflect this.
- Name of SALSA MLPA Probemix P460 updated to (Silent) Carrier.

Version B2-10 – 11 December 2023 (04P)
- Removed samples with 5 copies from Positive samples section.
- Removed OMIM numbers from Clinical background section.
- Adjusted footnotes under Table 2.
- Product no longer registered for IVD use in Morocco.
- Various textual changes.

Version B2-09 – 11 July 2022 (04P)
- Table on Final ratios adjusted.
- Information about background signal in Interpretation of results section adjusted.

Version B2-08 – 26 April 2022 (04P)
- Appendix I updated: clarification added to protocol and notes, note added.
- Added note to section Data analysis on DBS cards.
- Corrected cross-reference to section exon numbering under Tables 1 and 2.
- Remark on probe orientation added under Table 2.
- Minor textual changes.

Version B2-07 – 31 May 2021 (04P)
- Product description rewritten and adapted to new template.
- Intended purpose updated.
- Ligation sites of the probes targeting the *SMN1* and *SMN2* genes updated according to new version of the NM_ reference sequence.
- Added a comparison table between MRC Holland SMA products.
- Warning added to Table 1 and 2 on the salt sensitivity of the *SMN2* Exon 7 and 8 probe.

- Appendix 1 updated. - UK added to the list of European countries that accept the CE-mark. Version B2-06 – 25 June 2020 (04) - Costa Rica was added to the list of countries where this product is registered as IVD.

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	EUROPE*  COLOMBIA ISRAEL COSTA RICA
	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. P060-B2 SMA Carrier for use on dry blood spot (DBS) cards

P060-B2 SMA Carrier can be used to determine *SMN1* exon 7 copy number on DBS material when the extraction method as described in this appendix is used.

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, 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 P060 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 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 P060 SMA Carrier Probemix is influenced by contamination of DNA samples with DNA of other samples. 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.
- Instead of the extraction protocol provided here, commercially available extraction kits validated for extraction of DNA from DBS cards can be used.
- It is *NOT* recommended to add multiple punches from the same DBS card to the extraction volume as this will increase the amount of contaminants that interfere with the MLPA reaction.

Notes on data analysis:

- It is essential to use Coffalyser.Net software for data analysis.
- When analysing the data, ensure that the four DNA Quantity Fragments (Q-fragments; at 64, 70, 76, 82 nt) are not higher than 50% of the Benchmark fragment (92 nt). This to verify that that the P060 SMA Carrier 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 P060 SMA Carrier 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