

SALSA MLPA KIT P021-A1 SMA

Lot 0208, 0807, 0407, 1206, 0706, 0206, 0405

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. The SMA disorder is usually subdivided into three clinical groups. Patients with type I SMA disease (MIM# 253300) show onset at birth or before six months, and usually die of respiratory insufficiency within two years. They are never able to sit or walk. Patients with type II SMA (MIM# 253550) show onset after 6 months. They can sit but are never able to walk unaided, and life expectancy is significantly reduced. Type III SMA (MIM# 253400) patients show first symptoms after 18 months and are able to stand and walk, but often become wheelchair-bound during youth or adulthood.

There are two (highly-similar) genes playing a pivotal role in SMA: SMN1 and SMN2. The two genes can only be distinguished by a single nucleotide difference in exon 7 and 8 (see next page). SMN2 is much less efficient in making the SMN protein, therefore is the SMN1 gene which is the determinant factor. Someone lacking a functioning copy of SMN1 is always a patient, whereas SMA carriers (carrying a single copy of the SMN1 gene) are symptom-free. Establishing the number of SMN2 copy numbers is of importance in SMA patients only: the more SMN2 copies, the better the patient will be able to make up for the loss of SMN1. Approximately 80% of patients with type I SMA carried 1 or 2 SMN2 copies, 82% of patients with type II SMA carried 3 SMN2 copies, and 96% of patients with type III SMA carried 3 or 4 SMN2 copies. Furthermore, among 113 patients with type I SMA, 9 with 1 SMN2 copy lived less than 11 months, 88 of 94 with 2 SMN2 copies lived less than 21 months, and 8 of 10 with 3 SMN2 copies lived 33 to 66 months. In short, the more SMN2 copies a patient has, the less severe the disease is expected to be. More information about the SMA region can be found on the next page.

The SMN1 and SMN2 genes are located in an inverted repeat area on chromosome 5q13. Mutation in other genes in this repeat might influence the SMA phenotype. The BIRC1 gene (also known as NAIP) is telomeric and located close to SMN1. In contrast, the centromeric copy of this gene, called NAIP ψ , is located closer to SMN2. Other genes in the SMA region include GTF2H2 (or BTFp44), SERF1A (or H4F5) and a sequence similar to N-cadherin. This P021-A1 SMA probemix contains probes for several genes in this region. Deletions found in SMA patients usually include SMN1 and SERF1A. In some cases BIRC1 and GTF2H2 are co-deleted.

This SALSA MLPA kit is designed to detect the copy number of the SMN1 and SMN2 genes. The close sequence similarity between these genes complicates the analysis. Heterozygote deletions of probe recognition sequences should give a 35-50% reduced relative peak area of the amplification product of that probe. However, mutations and/or polymorphisms very close to the probe ligation site may also result in a reduced relative peak area. Therefore, apparent deletions detected by a single probe always require confirmation by other methods. Please note that most defects in these genes are expected to be small (point) mutations, most of which will not be detected by this MLPA test.

SALSA® MLPA® kits are sold by MRC-Holland for research purposes and to demonstrate the possibilities of the MLPA technique. This kit is not CE/FDA certified for use in diagnostic procedures. SALSA MLPA kits are supplied with all necessary buffers and enzymes. Purchase of the SALSA MLPA test kits includes a limited license to use these products for research purposes.

The use of this MLPA kit requires a thermocycler with heated lid and sequence type electrophoresis equipment. Different fluorescent PCR primers are available. The MLPA technique has been first described in Nucleic Acid Research 30, e57 (2002).

Related SALSA MLPA kits

- P060 SMA carrier: Spinal Muscular Atrophy (SMA) carrier, to determine only SMN1 copy number changes.
- P058 IGHMBP2: Autosomal recessive distal spinal muscular atrophy 1 (DSMA1), gene included IGHMBP2.

More information

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References of the SALSA MLPA kit P021

- Zapletalova E. (2007). Analysis of point mutations in the SMN1 gene in SMA patients bearing a single SMN1 copy. *Neuromuscul Disord.* 2007 Apr 30.
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SMN region

The SMA region displays high instability, leading to frequent deletions and gene conversions. The copy number of the repeat unit can vary from 0–4 per chromosome. The main cause of this variation is unequal crossing-over between the repeated units during paternal meiosis, leading to deletions of variable sizes. For SMA, the most important of the genes in this repeat region is the Survival Motor Neuron gene, which is designated SMN1 for the telomeric copy and SMN2 for the centromeric copy. The full-length cDNAs of SMN1 and SMN2 are completely identical, except for two nucleotide differences in exons 7 and 8. The exon 7 difference disrupts a putative exonic splicing enhancer in SMN2. As a result, most SMN2 transcripts lack exon 7, whereas SMN1 produces primarily full-length transcripts.

The absence of any functional SMN1 copies results in insufficient amounts of full length transcripts, and is the cause of 95% of all SMA cases. The presence of SMN2 results in a small amount of full length transcripts. Therefore, the more SMN2 copies a patient has, the more full-length SMN2 protein present and the milder the SMA phenotype. The majority of type I SMA patients carry real homozygous deletions of SMN1 and a normal or reduced number of SMN2 copies. The majority of type II and III SMA patients show homozygous absence of SMN1 as a result of gene conversion of SMN1 into SMN2, leading to absence of functional SMN1 and an increase to 3–4 copies of SMN2. Homozygous absence of SMN2 is found in about 5% of controls, but has no clinical phenotype.

Because it is SMN1, not SMN2, which determines both SMA carrier and patient status, distinguishing between the two genes is essential. There are four reasons why establishing carrier or patient status can be problematic, however. First of all, the difference between the two genes is only one nucleotide in exon 7 and one in exon 8, which means that any assay intending to determine carrier status can only do so by focussing on these very small differences. The difference in exon 7 is the crucial one, as it is this distinction which differentiates SMN1 from SMN2 protein efficiency. Second, it has been found that actually only in 95% of the cases in which an absence of the SMN1 exon 7 signal is detected, the SMN1 exon 8 signal is also absent. In the remaining 5%, a normal SMN1 exon 8 signal is found. A likely cause of this finding is the occurrence of a gene conversion between SMN1 and SMN2, resulting in the loss of the distinguishing exon 8 nucleotide. So while the absence of an exon 8 signal alongside an exon 7 absence is an extra confirmation that the SMN1 copy is indeed lacking, an absent exon 7 *without* a corresponding absence of exon 8 should still be considered as a deletion of SMN1. This is because the exon 7 difference is crucial, as this is the one determining the efficiency of the SMN protein. Thirdly, approximately 4% of individuals have a chromosome 5 copy with two SMN1 copies. Detection of some heterozygotes is therefore compromised, as MLPA cannot identify the heterozygotes having one chromosome lacking SMN1, and another chromosome with two copies of SMN1. Therefore, one should keep in mind that a person with two SMN1 copies can STILL be a carrier: if both copies lie on one chromosome arm, there is a 50% chance that not a single SMN1 copy is passed on to the offspring. If a parent of a patient is found to have two copies, this option is a probable explanation. Fourthly and finally, of the 5q13-linked SMA patients, 96.4% show homozygous absence of SMN1 exon 7 and 8, or exon 7 only. The remaining 3.6% present a compound heterozygosity with a subtle mutation on one chromosome and a deletion/gene conversion on the other. Such a subtle mutation is likely not to be detected by the P021 MLPA probemix, and should be established by sequencing. The summation of these findings and what they mean for carrier/patient status can be found in table 1.

Table 1: An overview of the genotype/phenotype relation of SMA patients and carriers.

| Finding | Conclusion | Explanation |
|---|-------------------------------|---|
| 0 copies of SMN1 exon 7 0 copies of SMN1 exon 8 <i>SMA symptoms</i> | 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. |
| 0 copies of SMN1 exon 7 SMN1 exon 8 copies \neq 0 <i>SMA symptoms</i> | SMA patient | SMN1 is absent, as no copies of the determining SMN1 exon 7 sequence are found. Due to gene conversion, 1 or more copies of the characteristic SMN1 exon 8 appear to have become incorporated in the SMN2 gene. |
| 1 copy of the SMN1 exon 7 <i>SMA symptoms</i> | SMA patient | If the patient is clearly a SMA-sufferer, but one copy of SMA still appears to be present, the patient probably belongs to the 3.6% group presenting compound heterozygosity. Sequencing the SMA gene should provide an answer. |
| 1 copies of exon 7 1 copies of exon 8 | 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. |
| 1 copies of SMN1 exon 7 A: SMN1 exon 8 copies > 1 B: SMN1 exon 8 copies = 0 | SMA carrier | One copy of SMN1 is absent, making the person a carrier. A: due to gene conversion, 1 (or more) copies of the characteristic SMN1 exon 8 have become incorporated in the SMN2 gene. B: an SMN2 exon 8 copy has replaced the characteristic SMN1 exon 8 copy. |
| 2 copies of SMN1 exon 7 | Most likely not a SMA carrier | Most likely, the person is not a carrier. However, there is a possibility that both SMN1 copies lie on one chromosome. If there is reason to believe that the person is a carrier (i.e. child is SMA-patient), he/she may belong to the 4% of people where this is indeed the case. |

Description of the 5q probes in P021

- The **SMN Exon 7 probes 1260-L0966 & 1260-L0967** (270 and 276 nt) are the most important probes in this mix. These probes distinguish between SMN1 and SMN2 by having their ligation site at the C-to-T transition in exon 7, which is the site determining RNA splicing. The presence of SMN1 sequences (cytosine at the ligation site) in the sample will result in a peak at 270 nt, whereas the presence of SMN2 (thymidine at ligation site) will result in a peak at 276 nt. These probes can be used both to determine the SMN1 copy number (important in determining carrier status) and to assess the copy number of the SMN2 form relative to normal controls (important to predict severity of the disease in patients).
- The **SMN Exon 8 probes 1812-L1372 & 1812-L1373** (295 and 301 nt) are able to distinguish between SMN1 and SMN2 at the exon 8 G-to-A transition. The presence in the sample of the SMN1 form (cytosine at the ligation site) will result in a peak at 295 nt, whereas SMN2-characteristic sequences (thymidine at the ligation site) will result in a peak at 301 nt. These probes can be used to detect exon 8 deletion of the SMN1 form and assess the copy number of the SMN2 exon 8 form relative to normal controls. In case the copy number detected by these exon 8 probes does not correspond to that found by the exon 7 probes, only the exon 7 probes should be used to determine the SMN1 and SMN2 copy number (see explanation above).
- The **SMN Exon 8 probe 1814-L0807** (364 nt) detects an **exon 8** sequence that is identical in both SMN1 and SMN2 and that can be used to assess the copy number of exon 8 of both forms combined relative to normal controls. A decrease in the 364 nt signal obtained for this probe in comparison to a normal control may indicate a deletion in exon 8 of the SMN1 form and should be interpreted together with the 270 and 295 nt SMN1 specific signals for SMN1 exons 7 and 8, respectively.
- The **SMN probes yielding 382, 400 and 418 nt** peaks are specific for respectively exons 1, 4 and 6 of both SMN1 and SMN2. Deletions of exon 1-6 have been described by *Arkblad EL. Et al. (2006)*.
- The **238 nt BIRC1 probe** detects only the complete BIRC1 gene (also known as NAIP) that co-locates with SMN1. In contrast, the NAIP ψ copy is located close to SMN2 and does not contain the exon 5 sequence detected by the above-mentioned probe. A reduction in signal of 238 nt BIRC1 probe relative to normal controls suggests continuation of the SMN1 deletion into BIRC1 and may indicate a more

severe form of SMA. Note that although BIRC1 deletions are more frequently observed in patients affected by the acute form of SMA, it is not possible to establish an unambiguous correlation between deletion size and clinical severity.

- The **346 nt BIRC1** probe detects a BIRC1 exon 13 sequence. This sequence is present in all copies of BIRC1 (i.e. both NAIP and NAIP ψ forms). There appear to be multiple copies of the incomplete BIRC1 gene on 5q11.
- In some SMA patients, the deletion of SMN1 extends into GTF2H2. Three **GTF2H2 probes** are included to determine the copy number of the two existing GTF2H2 variants. Comparison of the data obtained from these probes can thus be used to determine the extent of this deletion. At present, it is not clear which of these two forms is most closely associated with SMN1. The **328 nt GTF2H2 probe** detects an exon 4 sequence which is present in both variants 1 and 2. Variant 1: the **184 nt GTF2H2 probe** detects the C>G transition in exon 10; it is specific for the variant with a **C** (as opposed to the G) in exon 10. Variant 2: in contrast, the 220 nt probe detects the G>A transition in exon 7, and is specific for the variant with a **G** (as opposed to the A) in exon 7. To be clear: the 220 nt probe usually detects a different variant than the 184 nt probe. However, just as is the case with SMN2 and SMN1 (see page 2), exons changes can occur; as a consequence, you may find both variants vary together (i.e. both deleted or both duplicated).
- The **N-cadherin-like 454 nt probe** detects exon 1 of this sequence (closest to SMN1), which is found on the SERF1A side of SMN1.
- Though a deletion of the **CDH6** (K-cadherin) **148 nt probe** is unlikely, this probe serves as a control for the presence of the entire region.
- **RAD17** with peak size 202 nt is located within 300 Kb from SMN1. The remaining 21 probes are located on other chromosomes and function as reference probes.

False negative results SMA carrier screening

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 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. At this moment we have no possibility to determine if the two SMN1 copies are on the same, or on different chromosomes.

Data analysis

The P021-A1 probe mix contains 37 different MLPA probes with amplification products between 139 and 463 nt. In addition, it contains 7 control fragments generating an amplification product smaller than 120 nt: four DNA Quantity fragments (Q-fragments) at 64-70-76-82 nt and three DNA denaturation control fragments (D-fragments) at 88-92-96 nt. More information on how to interpret observations on these control fragments can be found in the MLPA protocol.

Data generated by this probemix can be intra-normalized by dividing the peak area of each amplification product by the total area of only the reference probes in the probemix (block normalization). Ratios can be obtained by dividing the intra-normalized probe ratio in a sample by the average intra-normalized probe ratio of all reference runs. This type of normalization assumes that no changes occur in the genomic regions targeted by the reference probes.

When only small numbers of samples are tested, visual comparison of peak profiles should be sufficient to easily identify exon deletions. Comparison of results should preferably be performed within one experiment. Only samples purified by the same method should be compared. Confirmation of most exons deletions and amplifications can be done by e.g. Southern blots or long range PCR.

Note that Coffalyser, the MLPA analysis tool developed at MRC-Holland, can be downloaded free of charge from our website www.mlpa.com.

Info/remarks/suggestions for improvement: info@mlpa.com.

SALSA MLPA P021-A1 SMA probemix

| Length (nt) | SALSA MLPA probe | Reference | Chromosomal position | Remarks |
|-------------|---|-----------|----------------------|---|
| 64-70-76-82 | Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA | | | |
| 88-92-96 | D-fragments: Low signal of 88 or 96 nt fragment indicates incomplete denaturation | | | |
| 139 | Reference probe 1061-L0727 | | 12q14 | |
| 148 | CDH6 probe 1254-L0815 | | | |
| 157 | Reference probe 1112-L0549 | | 3q12 | |
| 166 | Reference probe 1448-L0932 | | 17p11.2 | |
| 175 | Reference probe 0808-L0638 | | 18q21 | |
| 184‡ | GTF2H2 probe 1256-L0972 | | | Detects C>G transition |
| 193 | Reference probe 1115-L0005 | | 7q21 | |
| 202 | RAD17 probe 1257-L0184 | | | |
| 211 | Reference probe 1220-L0689 | | 10p15 | |
| 220‡ | GTF2H2 probe 1813-L0818 | | | Detects G>A transition |
| 229 | Reference probe 1120-L0060 | | 11q13 | |
| 238 | BIRC1 probe 1259-L0811 | | | |
| 247 | Reference probe 0816-L0334 | | 21q11 | |
| 256 | Reference probe 0807-L0325 | | 18p11 | |
| 270 | SMN1 probe 1260-L0966 | | | Exon 7 probe - indicates # of SMN1 copies |
| 276 | SMN2 probe 1260-L0967 | | | Exon 7 probe - indicates # of SMN2 copies |
| 286 | Reference probe 0824-L0970 | | 3q21 | |
| 295 | SMN1 probe 1812-L1372 | | | Exon 8 – usually indicates # of SMN1 copies |
| 301 | SMN2 probe 1812-L1373 | | | Exon 8 – usually indicates # of SMN2 copies |
| 310 | Reference probe 0871-L0461 | | 13q34 | |
| 319 | Reference probe 1042-L0791 | | 8q24 | |
| 328‡ | GTF2H2 probe 1262-L0971 | | | Detects both GTF2H2 variants |
| 337 | Reference probe 0812-L0330 | | 21q21 | |
| 346 | BIRC1-D01 probe 1263-L0812 | | | |
| 355 | Reference probe 0965-L0552 | | 2p24 | |
| 364 | SMN1/2 probe 1814-L0807 | | | Detects # of SMN1 + SMN2 copies |
| 373 | Reference probe 1046-L0624 | | 8 | |
| 382 | SMN1/2 probe 1265-L0808 | | | Detects # of SMN1 + SMN2 copies |
| 391 | Reference probe 1160-L0716 | | 3p26 | |
| 400 | SMN1/2 probe 1816-L0809 | | | Detects # of SMN1 + SMN2 copies |
| 409 | Reference probe 0963-L0550 | | 2p14 | |
| 418 | SMN1/2 probe 1815-L0810 | | | Detects # of SMN1 + SMN2 copies |
| 427 | Reference probe 1108-L0679 | | 8q24 | |
| 436 | Reference probe 1057-L0630 | | 17q12 | |
| 445 | Reference probe 0802-L0320 | | 13q34 | |
| 454 | N-Cadherin-like probe 1269-L0813 | | | |
| 463 | Reference probe 0846-L0377 | | 12q24 | |

‡ The signals of the three GTF2H2 probes have been deliberately reduced.

Note: Exon numbering might be different as compared to literature! Please notify us of any mistakes. The identity of the genes detected by the reference probes is available on request: info@mlpa.com.

P021-A1 probes arranged according to chromosomal location

| Length (nt) | SALSA MLPA probe | Distance to next probe |
|-------------|--|------------------------|
| | <i>centromeric site</i> | |
| 202 | RAD17 probe 1257-L0184 | 183.0 Kb |
| 328 | GTF2H2 probe 1262-L0971 | 6.0 Kb |
| 220 | GTF2H2 probe 1813-L0818 | 7.0 Kb |
| 184 | GTF2H2 probe 1256-L0972 | 37.0 Kb |
| 238 | BIRC1 probe 1259-L0811 | 31.0 Kb |
| 346 | BIRC1 probe 1263-L0812 | 27.0 Kb |
| 295 | SMN1 probe 1812A-L1372 (SMN1 specific but a small signal due to SMN2 is present) | 0.2 Kb |
| 364 | SMN1 probe 1814-L0807 (Detects SMN1 + SMN2) | 0.8 Kb |
| 270 | SMN1 probe 1260-L0966 | 6.0 Kb |
| 418 | SMN1 probe 1815-L0810 (Detects SMN1 + SMN2) | 3.0 Kb |
| 400 | SMN1 probe 1816-L0809 (Detects SMN1 + SMN2) | 18.0 Kb |
| 382 | SMN1 probe 1265-L0808 (Detects SMN1 + SMN2) | 44.0 Kb |
| | SERF1B (no probe present) | 10.0 Kb |
| 454 | N-Cadherin-like gene probe 1269-L0813 | |
| | <i>q-telomeric site</i> | |

The order and distances are deduced by analysis of several Genbank files of BAC clones. It is not 100% clear if this is the correct order! The exact location of SMN2, NAIP ψ , CDH6 and the second copy of GTF2H2 is not completely clear.

SALSA MLPA P021-A1 SMA sample picture

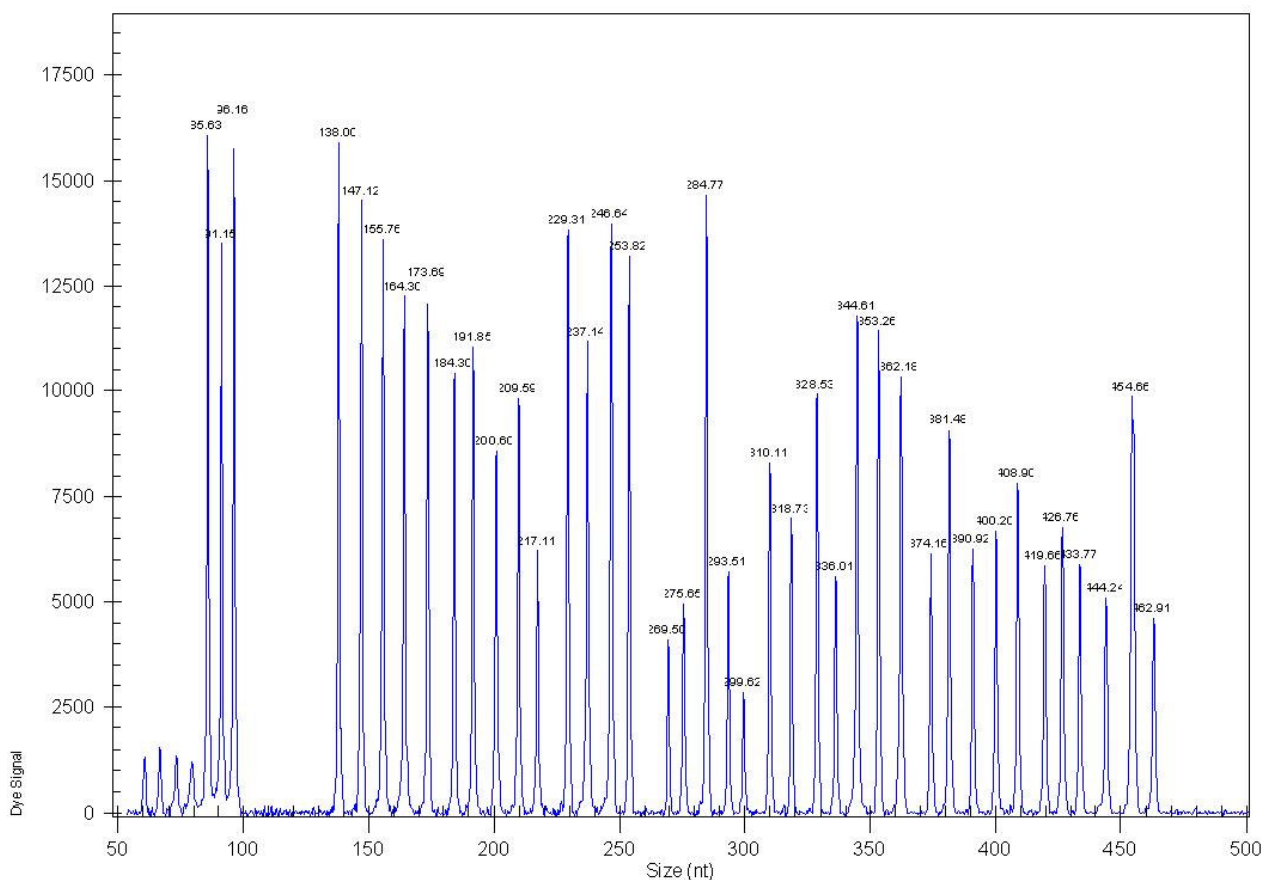


Figure 1. Capillary electrophoresis pattern from a sample of approximately 50 ng human male control DNA analyzed with SALSA MLPA kit P021-A1 SMA (lot 0208).