



Best Practice Guidelines for Molecular Analysis in Spinal Muscular Atrophy

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Disclaimer

These Guidelines are based, in most cases, on the reports drawn up by the chairs of the disease-based workshops run by EMQN and the CMGS. These workshops are generally convened to address specific technical or interpretative problems identified by the QA scheme. In many cases, the authors have gone to considerable trouble to collate useful data and references to supplement their reports. However, the Guidelines are not, and were never intended to be, a complete primer or "how-to" guide for molecular genetic diagnosis of these disorders. The information provided on these pages is intended for chapter authors, QA committee members and other interested persons. Neither the Editor, the European Molecular Genetics Quality Network, the Clinical Molecular Genetics Society, the UK Molecular Genetics EQA Steering Committee nor the British Society for Human Genetics assumes any responsibility for the accuracy of, or for errors or omissions in, these Guidelines.

Description of the disease

The spinal muscular atrophies are a clinically and genetically heterogeneous group of neuromuscular disorders caused by degeneration of anterior horn cells. In proximal spinal muscular atrophy (SMA), the muscles of the extremities closest to the trunk are more severely and earlier affected. SMA is clinically classified into three types: acute, intermediate, mild, and adult (SMA types I, II, III, and IV, respectively)^{1,2}. Used nomenclature is summarised in Table 1. Criteria for clinical classification of SMA I, II, and III patients are summarised in Table 2. With an estimated birth prevalence of ~1/10,000^{3,4}, and a carrier frequency of 1/40-1/60^{5,6} the SMAs are among the most frequent autosomal recessive hereditary disorders. The SMA 5q13 region shows a complex structure at the genomic level, including a 500-kilobase pair duplication and inversion⁷. The Survival of Motor Neuron (SMN) gene exists as two homologues, at least one telomeric gene (SMN1) and in the vast majority of chromosomes at least one centromeric gene (SMN2) per chromosome in normal (non-carrier) individuals, although also chromosomes with an intact SMN1 gene exist that lack SMN2⁷. The two SMN genes (SMN1 and SMN2) are highly homologous and contain only five base-pair differences that occur within their 3' regions⁸. These

differences in exons 7 and 8 are used to distinguish SMN1 and SMN2 in DNA analysis. Deletions of the SMN1 gene appear to be directly involved in SMA⁷, since exon(s) 7 (and 8) of SMN1 are undetectable in over 95% of patients, irrespective of their clinical type^{7,9-12,14}, either as a result of homozygous deletions, or because of conversion of sequences of SMN1 into those of the SMN2 gene^{7,11-16}. The identification of several small mutations in the SMN1 gene in patients without a deleted or sequence-converted SMN1 allele^{7,17-25,29} and the existence of an *Smn1*-/*-SMN2* mouse model^{38,39} provide strong evidence that the SMN1 gene is the primary SMA-determining gene. The clinical phenotype of SMA appears to be partly determined by the underlying mutational mechanism. Most cases of SMA type I result from homozygous gene-deletion events, whereas the milder forms of the disease, SMA types II and III, are more likely to result from gene conversion of SMN1 into SMN2; SMA type II from a gene conversion event in one allele in addition to a hemizygous deletion event in the other allele; in SMA type III from gene conversions in both alleles^{13,15,16,26,27}. As a result, patients suffering from the milder forms of SMA carry more copies of the SMN2 gene. In the mouse model, multiple copies of human SMN2 seem to prevent SMA development in *Smn1*-deleted animals (almost) completely. The absence of SMN1 exon(s) 7 (and 8) in SMA patients can be used as a diagnostic tool for SMA²⁸, with a sensitivity of approximately 95% in clinically strictly defined SMA patients in most populations. Most available assays are not quantitative, however. Based on Hardy-Weinberg equilibrium, the remaining patients (SMA patients without a homozygous SMN1 deletion) are virtually all assumed to have a hemizygous SMN1 absence of at least exon 7, either by deletion or by conversion. Several papers including those by McAndrew *et al.*, Wirth *et al.*, Gerard *et al.*, and Scheffer *et al.*^{21,29,30,41} have described quantitative assays based on competitive amplification of SMN1 and SMN2 exons 7 to determine their gene-copy number.



Common reasons for referral

Confirmation of the clinical diagnosis or suspicion of SMA by the detection of homozygous SMN1 exon 7 (and exon 8) deletions is fast, inexpensive, and reliable. The clinical sensitivity is around 95%, the clinical specificity is over 99%. Therefore, no restricting clinical criteria are warranted before undertaking DNA analysis. This is certainly so in neonatal cases in which a rapid molecular analysis and diagnosis may render more invasive procedures (i.e. a muscle biopsy) unnecessary. A different situation exists when hemizygous SMN1 deletion analysis or point mutation analysis is asked for. Our guidelines are summarised in Table 3.

Other reasons for referral include carrier detection by direct hemizygous SMN1 exon 7 deletion analysis and/or indirect linkage analysis. Carrier detection by direct analysis is indicated in siblings and close relatives of SMA patients with a homozygous SMN1 exon 7 (and 8) deletion, in parents of SMA patients who died, and in partners of ascertained carriers without positive family history for SMA. Indirect analysis can only be applied in family members of a patient, assuming that sufficient family members are available for analysis to establish linkage phase. Direct analysis is strongly recommended in these cases.

A third indication for DNA analysis is prenatal analysis. This is often requested by parents of an SMA patient, and may also be indicated for couples who have been shown to be carriers of a mutant SMN1 allele.

Approaches

General Strategy

1. Appropriate patients selected for molecular analysis
2. Confirmation of clinical diagnosis by analysis of homozygous SMN1 exon 7 (and 8) deletion
3. Before embarking on hemizygous SMN1 deletion analysis in cases without homozygous deletion, additional clinical information is requested according to criteria summarised in Table 3
4. Heterozygous deletion analysis by dosimetric SMN1 exon 7 test. If a patient, fulfilling the clinical criteria of SMA, is found heterozygous, there is a 95% chance that (s)he will be compound heterozygous for a deletion and a (point) mutation (see below)
5. Point mutation analysis (optional; see below)
6. Carrier detection by dosimetric SMN1 exon 7 test and/or linkage analysis in relatives. Prior to dosimetric analysis in relatives testing of parents of the homozygously SMN1 deleted index case is strongly recommended (cf.

section SMN1 Exon 7 Dosage Analysis and Carrier Testing)

7. Carrier testing in partners of ascertained carriers by dosimetric SMN1 exon 7 test
8. Prenatal analysis by homozygous SMN1 exon 7 (and 8) deletion analysis or by a combination of (point) mutation analysis and linkage analysis. Some labs confirm the results by linkage analysis using markers flanking SMN1. Exclusion by microsatellite analysis of an interfering degree of admixture of maternal cells in the foetal tissue is obligatory

Confirmation of Clinical Diagnosis

The ability to detect deletions in SMN1 means that testing to confirm a diagnosis of SMA in affected individuals is possible. The presence of a homozygous deletion of exons 7 (and 8) is consistent with a diagnosis of SMA given that there are clinical grounds for suspecting SMA. The absence of a homozygous SMN1 deletion does not rule out a diagnosis of SMA since approximately 6% of cases are known not to have a homozygous SMN1 deletion, a proportion of those SMA patients will have a point mutation in one or extremely rarely in both SMN1 alleles instead⁴⁵. In routine SMN1 testing, a DNA-laboratory often has no or only scarce clinical data on a given patient. In that case, whenever a homozygous SMN1 deletion is not found in a patient, one should doubt the diagnosis of SMA. In analyses performed to confirm the clinical diagnosis of SMA, the presence of a hemizygous SMN1 exon 7 (and 8) deletion identified by a quantitative determination of the number of SMN1 exon 7 copies may provide further support for the diagnosis of SMA (appr. 95-96% of SMA patients have at least a heterozygous SMN1 deletion). Nevertheless, formal confirmation of the clinical diagnosis in those cases can only be obtained after identification of subtle SMN1 mutation(s).

The clinical subtype of SMA appears to be determined (in part) by the nature of the SMN1 mutations. In general, SMA type 1 is associated with deletions in both alleles, SMA type 2 with a deletion in one, and a gene conversion in the other allele, and SMA type 3 with gene conversions in both alleles. However, these data, when available, can at present not be used to predict the clinical course of the disease.

Carrier Testing

Several papers including those by McAndrew *et al.*, Wirth *et al.*, Gerard *et al.*, and Scheffer *et al.*^{21,29,30, 41} have described quantitative assays based on competitive amplification of SMN1 and SMN2 exons 7 to determine their gene-copy number. These techniques have now been implemented in a number of service labs for carrier detection.



SMN1 *de novo* deletions occur at a frequency of approximately 2% of SMA patients, the majority being of paternal origin³². Testing of parents of homozygously deleted SMA patients may give additional information on *de novo* deletions. It has been shown in the majority of cases that these *de novo* deletions are associated with a meiotic recombination or meiotic gene conversion. Hence the recurrence risk in such particular families would theoretically not be elevated.

A complication/pitfall in the dosimetric analysis of SMN1 exon 7 is the occurrence of two (or more) SMN1 genes on a single chromosome 5 in about 4%, as observed by McAndrew *et al.*²¹, Wirth *et al.*³⁵, and others. This would bear on the sensitivity of this technique for carrier identification, since individuals with an SMN1 [1,1] genotype (a single SMN1 gene on each chromosome; non-carriers) cannot be distinguished from individuals with an SMN1 [0,2] genotype (both SMN1 genes on a single chromosome; carriers). Also, in carrier testing of individuals without a positive family history for SMA, e.g. testing of partners of known carriers, it should be taken into account that mutant non-deletion SMN1 alleles (approximately 2.5% of mutant SMN1 alleles) will not be detected. The consequences for the sensitivity and specificity of carrier testing by a dosimetric method have been discussed^{29,30}.

Since the frequency of carriers with two SMN1 genes per chromosome is 4%, compared to only 1% of parents being non-carrier due to a *de novo* mutation, no direct conclusions could be drawn from carrier testing in parents of affected individuals. This problem can be resolved by the identification of a recombination event associated with the *de novo* mutation by indirect analysis, e.g. by using the multicopy markers Ag1-CA and C212³² or other flanking markers. Alternatively, the occurrence of a *de novo* deletion can be confirmed by normal SMN1 dosage in the parents of the apparent non-carrier parent, i.e. two of the grandparents of the affected child. When two SMN1 genes on a single chromosome (opposite to a chromosome lacking an intact SMN1 gene) cause the normal SMN1 dosage in the carrier parent, one grandparent will have an SMN1 dosage compatible with a single SMN1 gene. The other grandparent will have an SMN1 dosage compatible with three SMN1 genes. When the normal dosage in the parent is caused by a *de novo* mutational event in the affected child, both grandparents will have normal SMN1 dosage.

Carrier testing in relatives of SMA patients, e.g. siblings and/or uncles and aunts could also be performed by indirect analysis using flanking markers. A prerequisite is the availability of sufficient DNA samples from family members for establishing linkage phase and informativity of the markers.

Prenatal Testing

Prenatal analysis is carried out preferably by direct mutation detection, e.g. by testing for a homozygous SMN1 exon 7 (and 8) deletion. A prerequisite is the identification of a homozygous deletion in the index patient, followed by the identification of a hemizygous deletion in both parents.

Homozygous deletions of SMN1 exons 7 and 8 in unaffected haplo-identical sibs of SMA patients have been described^{10, 11}. Parents of a SMA patient may actually have a homozygous SMN1 deletion themselves. Although it is hard to predict whether the parental SMN1 deletion allele that had not been transmitted to the affected child may have a pathogenic effect at all, it is our opinion that it cannot be considered simply as a non-risk SMN1 allele. Such an allele should be dealt with as an allele with a potential pathogenic effect.

Prenatal testing based on linkage analysis is useful in families with a clinically ascertained patient with proximal SMA without a homozygous SMN1 deletion. It is recommended that this approach is only used when a hemizygous SMN1 deletion has been identified in the index case. An exception could be made in special cases with a clinically quite clear diagnosis of SMA and consanguinity of the parents, making homozygosity for a rare nondeletion mutant SMN1 allele in the index case more likely. Prenatal analysis in families with non-deleted patients should generally not be performed. Flanking markers may also be used to confirm the results of deletion analysis in prenatal testing, and to exclude interfering admixture of maternal cells in the foetal sample. Some laboratories prefer the use of markers from other chromosomes for exclusion of maternal admixture from foetal tissue to circumvent unwanted information about the foetal carrier status.

Linkage analysis

Linkage analysis is useful for carrier testing if quantitative deletion analysis is not feasible.

In postnatal applications, linkage-based testing of unaffected siblings, or preferably the grand parents of the index case, may be useful to identify a possible meiotic recombination associated with a *de novo* deletion or rearrangement involving SMN1. Also, uniparental isodisomy may be identified by the use of flanking markers.

Some laboratories have the policy to include unaffected siblings in a linkage analysis only when necessary, in order to avoid problems with the identification of carriers in young children. However, this depends on the local situation.



Technical protocols

Analysis of Homozygous SMN1 Deletions

Homozygous deletions of exons 7 and 8 of SMN1 can be detected by several methods including

- (1) SSCP - either on polyacrylamide (8% on 12cm gels works well) or MDE gels. Primer sequences and PCR conditions are summarised in the section "Primer Sequences". The deletion can be visualised by silver staining of the gels as well as by radioactivity or fluorescence⁴². SSCP has the advantage that point mutations in the gene may be detected as well as deletions. However, the method will also identify neutral polymorphisms. A study from Wang *et al.*³¹ have shown that 4% of asymptomatic family members carry a polymorphic variant of exon 7 of SMN1 (telomeric) which looks like a homozygous deletion on SSCP gels.
- (2) Restriction enzyme digestion^{28,29}. For exon 7 the enzymes *DraI* or *HinfI* could be used to separate SMN1 and SMN2 in combination with specific primers, for exon 8 *DdeI* can be used. Primer sequences and PCR conditions are summarised in the section "Primer Sequences". These are rapid assays, and have the additional advantage that the pathogenic absence of SMN1 exon 7 and/or 8 is very specifically tested for.

Gene conversion can be detected by SSCP analysis, dependent on local laboratory conditions used. The restriction digest method does not differentiate between a true deletion and a gene conversion, but correctly determines absence of SMN1 exons 7 and 8 from their normal genomic position.

Some labs prefer to confirm exon 7 only deletions by a second, independent test (in the majority of cases absence of both exons 7 and 8 is detected; these test results confirm each other).

It is recommended that samples from patients known to have a homozygous SMN1 deletion (positive reference samples) as well as samples known to contain intact SMN1 allele(s) (negative reference samples) are always run on gels, in addition to a no template control reaction to exclude carry-over contamination.

Dosage Analysis of Hemizygous SMN1 Deletions

A number of quantitative assays based on competitive amplification of SMN1 and SMN2 exons 7 to determine their gene-copy number have been described^{21,29,30,42}. Primer sequences and PCR conditions are summarised in the section "Primer Sequences". Technical details and pitfalls can be found in the original papers by McAndrew *et al.*, Wirth *et al.*, Gerard *et al.*, and Scheffer *et al.*. The latter system makes use of fluorescently labelled primers, enabling the analysis to be performed on an automated sequencer. Reference samples with

known SMN1 exon 7 copy number should be included on the gels. These assays make use of internal standard plasmids that are added to the reaction mixture for monitoring of the efficiency of the amplification. For availability of these standards and internal controls, the corresponding authors of the respective papers should be contacted.

New PCR technology, e.g. based on real time PCR (e.g. Taqman or FRET technologies), allows the quantification of the PCR during the course of the reaction. Applications for SMA are currently under development.

Since in clinical DNA service practice the majority of the tests used are currently based on qualitative analyses, it is strongly recommended that implementation of a quantitative SMN1 assay should be preceded by a thorough validation of sensitivity and specificity by the laboratory wishing to undertake this testing.

Linkage analysis

A number of informative microsatellite markers flanking the SMA gene are summarised in Table 4³³⁻³⁷. It should be possible to find an informative proximal and distal marker in the majority of cases so that the overall error based on linkage with flanking markers is <1%.

There are also multi-copy markers including C212 and C272 (Ag1-CA), which lie near the 5' end of SMN1^{4,43,44}. These can also be used although the interpretation of alleles requires considerable experience.

SMN1 Point Mutation Analysis

SMN1 point mutation analysis is beyond the scope of current routine DNA service for SMA. In case where analysis of subtle mutations is necessary, laboratories offering this facility (e.g. Bonn, Groningen) should be contacted. In these cases, the clinical diagnosis should meet the criteria formulated in Table 3.

Primer sequences

The primer sequences are taken from the references indicated. Individuals should consult the appropriate reference for full instructions and information on primer use. While every effort has been made to ensure the accuracy of the primer sequences, individual users should confirm these sequences before use.

SMN1/2 exon 7 and exon 8 amplification for SSCP

Lefebvre *et al.*⁷:

Exon 7:

R111: 5' - AGACTATCAACTTAATTCTGATCA - 3'

541C770: 5' - TAAGGAATGTGAGCACCTTCCTTC - 3'



(modified by Matthijs *et al.*⁴² into SMA7B: 5' - TCCTTAATTTAAGGAATGTGAGCA - 3')

Exon 8:

541C960: 5' - GTAATAACCAAATGCAATGTGAA - 3'

541C1120: 5' - CTACAACACCCTTCTCACAG - 3'

SMN1/2 exon 7 homozygous deletion tests using restriction enzymes *DraI* or *HinfI*

Van der Steege *et al.*²⁸:

R111 is used as forward primer.

X7-Dra: 5' - CCTTCCTTCTTTTGGATTTGTTT - 3'

DraI will cleave SMN2 exon 7 specifically.

Wirth *et al.*²⁹:

SMN7F: 5' - CTTCTTTTATTTTCTTACAGGGATT - 3'

SMN7R: 5' - TCCACAAACCATAAAGTTTTAC - 3'

The SMN1 exon 7 product will contain two *HinfI* sites, the SMN2 exon 7 product will contain one *HinfI* site.

SMN1 exon 8 homozygous deletion test using *DdeI*

541C960 and 541C1120 are used as forward and reverse primers, respectively.

DdeI will cleave SMN2 exon 8 specifically.

SMN1 exon 7 dosage analysis

McAndrew *et al.*²¹:

R111 and X7-Dra are used as forward and reverse primers, respectively.

The reference PCR product from the CFTR gene is amplified using

CF621F: 5' - AGTCACCAAAGCAGTACAGC - 3' as forward primer, and

CF621R: 5' - GGGCTGTGCAAGGAAGTGTTA - 3' as reverse primer.

For monitoring the efficiency of the PCR, cloned DNA of an SMN and a CFTR internal standard are added to the multiplex reaction.

Wirth *et al.*²⁹:

SMN7F and SMN7R are used as forward and reverse primers, respectively.

The reference PCR product is amplified using CF621F and CF621R.

For monitoring the efficiency of the PCR, cloned DNA of an SMN and a CFTR internal standard are added to the multiplex reaction.

Scheffer *et al.*³⁰:

R111 (fluorescently labelled) and X7-Dra are used as forward and reverse primers, respectively.

The reference product from the RB1 gene is amplified using

Rbex13F: 5'-ATTACACAGTATCCTCGACA-3'

RBex13R (fluorescently labelled): 5'-TATACGAAGTGGAAAGATGC - 3'

For monitoring the efficiency of the PCR, cloned DNA of an SMN and an RB1 internal standard are added to the multiplex reaction.

Internet resources

<http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispim?600354> (OMIM entry for SMN1)
<http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispim?253300> (OMIM entry for SMA1)
<http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispim?253550> (OMIM entry for SMA2)
<http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispim?253400> (OMIM entry for SMA3)
<http://bioinfo.weizmann.ac.il/cards-bin/cardsearch.pl?search=SMA> (Genecards for SMA and SMN1 and SMN2)
<http://gdbwww.gdb.org/gdb-bin/genera/genera/hgd/Gene?key=GDB%3A120378&!sub=0> (SMA entry in Genome Database)
ftp://ftp.genethon.fr/pub/Gmap/Nature-1995/data/data_chrom5 (Genethon genetic map of chromosome 5)
<http://www.ncbi.nlm.nih.gov/genemap/map.cgi?MAP=GB4&BIN=180&MARK=2660> (Genemap 99 section including SMN1)
<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=PubMed> (PubMed search page)
<http://archive.uwcm.ac.uk/uwcm/mg/search/5215173.html> (Human Gene Mutation Database entry for SMN1)
<http://www.neuro.wustl.edu/neuromuscular/synmot.html#sma5q>

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Table 1 Nomenclature and Gene ID in SMA

OMIM #	OMIM Name	Gene Name	GDB ID
#253300	Spinal muscular atrophy I; (SMA 1, Werdnig-Hoffmann disease)		
#253550			
#253400			
*600354	Spinal muscular atrophy II; (SMA 2)	Survival of Motor Neuron 1, telomeric (SMN1)	GDB:120378 (Gene) GDB:4405727 (Syndromic region)
	Spinal muscular atrophy III; (SMA 3; Kugelberg-Welander disease)		

The gene nomenclature that has been accepted by the nomenclature committee is SMN1 for the telomeric (pathogenic) SMN gene and SMN2 for the centromeric (copy) gene.

Table 2 Criteria for Clinical Classification of SMA Patients

Clinical Subtype	Age of Onset (in months)	Motor Milestones	Death (in years)
SMA type I	< 6	Never sit unaided	< 2
SMA type II	< 18	Never walk unaided	> 2
SMA type III	> 18	Stand unaided	adult

An SMA type IV has been described by Pearn³. In this subtype the clinical symptoms start after the age of 30 years in the majority of cases.

Table 3 Guidelines for the Clinical Indications for DNA Analysis

Clinical Presentation	Test
Any reasonable suspicion of SMA	Homozygous SMN1 exon 7 and 8 deletion test
Clinical criteria supporting the diagnosis of SMA types I, II, or III should be in agreement with those described ⁴⁰ , including characteristic abnormalities in a muscle biopsy	Hemizygous SMN1 exon 7 deletion test/SMN1 point mutation screening

Table 4 Genetic Markers Flanking the SMN1 Gene at 5q11.2-q13.3

Marker	Location	Recombination	PIC	Size
D5S679	Proximal	3%	0.63	180-222
D5S680	Proximal	3%	0.57	141-173
D5S125	Proximal	2%	0.38	143 and 147
D5S681	Proximal	2%	0.68	142-156
D5S435	Proximal	1%	0.68	128-144
D5S629	Proximal	1 %	0.81	233-253
D5S823	Proximal	1 %	0.54	128-150
D5S1556/D5F150	Intragenic/SMN1 promoter region	0 %	0.98	90-122
D5S149	Intragenic/SMN1 promoter region	0 %	0.99	166-203
D5S557	Distal	1%	0.46	148-172
D5S610	Distal	1%	0.80	106-124
D5S351	Distal	1%	0.74	196-234
5'-MAP1B	Distal	1%	0.76	~100
3'-MAP1B	Distal	1 %	0.72	212-226
D5S112	Distal	3%	0.74	94-120
D5S127	Distal	5%	0.84	96-114
D5S39	Distal	7%	0.74	212-220

Primer sequences, PCR conditions, etc. can be found in the Genome Database (<http://gdbwww.gdb.org>).