SALSA® MC002 SMA Newborn Screen

Version A

Instructions For Use

SALSA® Melt Assay for detection of the *SMN1* gene-specific exon 7 DNA sequence in newborns

For neonatal screening of spinal muscular atrophy (SMA)

REF	**MC002-100R, MC002-1000R, MC002-2000R**
Σ	**100, 1000, 2000**
	-25°C – -15°C
	Keep away from heat or direct sunlight
	Read instructions before use
IVD CE	**EU (candidate) member states, members of European Free Trade Association (EFTA).**
RUO	**All other countries**

This product is manufactured by MRC Holland bv in Amsterdam, the Netherlands. The product is sold for use by the end user only and may not be resold, distributed or repackaged without written consent from MRC Holland bv.

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Contents

1. Kit Components

Available volumes:
- **MC002-100R:** 100 reactions
- **MC002-1000R:** 1000 reactions
- **MC002-2000R:** 2000 reactions

Kit component	Cap colour	Contains	MC002-100R	MC002-1000R*	Ingredients
SALSA MC002 probemix	brown	1 Melt curve probe (Cy5-labelled)+ 1 Quantity Fragment (Q-fragment) 2 PCR primers	2× 1000 µl	20× 1000 µl	Synthetic oligonucleotides with and without fluorescent Cy5 dye, dNTPs, Tricine, MgCl2, Glycerol, $(NH_4)_2SO_4$
SALSA MC Polymerase	red	Polymerase enzyme	1× 115 µl	5× 230 µ	Glycerol, non-ionic detergents, EDTA, DTT, KCl, Tris-HCl, MC Polymerase enzyme (bacterial origin)
SD074	blue	Threshold DNA	1× 110 µl	1× 110 µl	Synthetic oligonucleotides, Tris-HCl, EDTA
SD075	blue	Positive DNA	1× 110 µl	1× 110 µl	Synthetic oligonucleotides, Tris-HCl, EDTA

+ Cy5-labelled probe is light sensitive.
* MC002-2000R contains twice the number of vials as MC002-1000R.

2. Storage and Shelf Life

All components must be stored directly upon arrival between -25°C and -15°C, shielded from light and in the original packaging. When stored under the recommended conditions, a shelf life of until the expiry date (see the labels on each vial for the exact expiry date) is guaranteed, also after opening. The SALSA Melt Assay probemix should not be exposed to more than 10 freeze-thaw cycles. If product is received in damaged packaging, contact MRC Holland or distributor.

3. Product Documentation

- MC002 Instructions for Use (IFU; current document).
- MC002 Certificate of Analysis (CoA): lot-specific document specifying storage conditions, quality test data and example melt curves; see www.mrcholland.com.

4. Intended Use

- In vitro diagnostic assay for neonatal screening of spinal muscular atrophy (SMA)[1].
- Melt curve assay for the detection of the *SMN1* gene-specific exon 7 DNA sequence in human DNA extracted from blood, including dried blood spot (DBS) cards.
- This assay allows for the detection of 95-98% of SMA patients in most populations[2].
- Positive results should be confirmed with SALSA MLPA probemix P021 SMA using either DNA purified from peripheral blood or a crude extract from washed DBS cards, prepared as described in this protocol.
- The MC002 assay cannot determine absolute *SMN1* or *SMN2* copy numbers with the exception of 0 copies.

[1] In Vitro Diagnostic use (IVD): only in the countries specified on the title page of this document. In all other countries, this product is for Research Use Only (RUO).
[2] 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.

5. Summary

5.1. Disease

Spinal muscular atrophy (SMA) is a severe, recessive, neuromuscular disease for which treatment options have recently become available. SMA is caused by a complete absence of functional copies of the *SMN1* gene. In most populations, homozygous absence of the exon 7 DNA sequence of the *SMN1* gene is observed in 95-98% of SMA patients. In most remaining cases, point mutations or partial deletions in the *SMN1* gene are the cause of disease. For more information see Appendix 1: Background Information.

5.2. Assay

In the SALSA MC002 SMA Newborn Screen PCR, amplification of exon 7 of the *SMN1* gene and the closely related *SMN2* gene is performed, followed by fluorescent probe binding to the amplicons and generation of a melt curve (Figure 1). Fluorescence is only measured during melt curve generation.
Absence of the *SMN1*-specific melt peak at 63°C is indicative of the absence of the *SMN1* exon 7 DNA sequence. The presence of an *SMN1* (63°C) and/or *SMN2* (56°C) specific melt peak and an absent or low signal for the Q (quantity)-fragment specific melt peak (49°C) indicates successful assay performance and the use of sufficient sample DNA. The assay uses a crude DNA extract prepared from a 1.5 mm or 3.2 mm punch of a DBS card or purified DNA from peripheral blood. More information on the assay can be found in Appendix 2: SALSA MC002 SMA Newborn Screen.

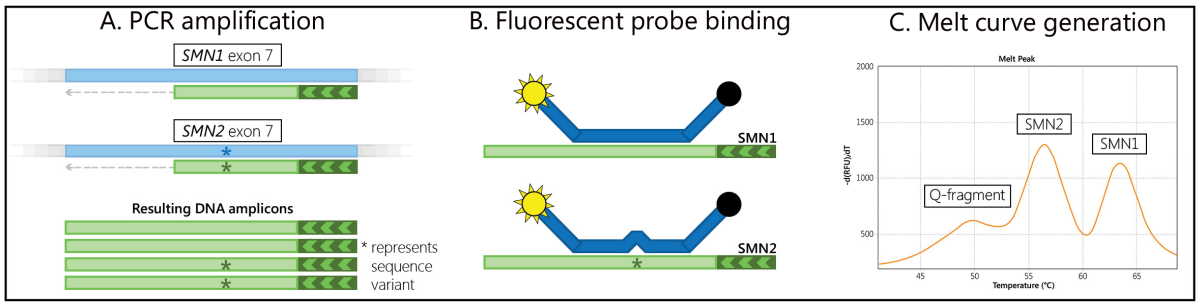

Figure 1. Summary of assay steps. (A) The exon 7 regions of *SMN1* and *SMN2* are amplified with a single set of primers, with one primer in excess. (B) A fluorescently-labelled probe binds to the amplicons. (C) The resulting melt curve indicates *SNM1* and *SMN2* sequence presence and DNA quantity.

6. Warnings and Precautions

- For professional use only. Assay performance is dependent on operator proficiency and adherence to procedural directions. The assay should be performed by professionals trained in molecular techniques.
- Follow good laboratory practice and safety guidelines. See CoA for safety statement.
- Before starting the assay, read the instructions completely and carefully. Always consult the most recent version of the MC002 IFU before use: www.mrcholland.com.
- Internal validation of the MC002 SMA assay is essential.

7. Specimens

7.1. Specimens Required

- The SALSA MC002 SMA Newborn Screen can be used with:
  - Crude DNA extracts from a 1.5 or 3.2 mm punch of a DBS card
  - DNA purified from peripheral blood (concentration 0.5-15 ng/µl; 5-15 ng is optimal[3])
- Crude extracts should be prepared as described in section 7.2 from DBS cards that are based on untreated Whatman 903 paper. For validation of other cards, contact MRC Holland. Cards impregnated with chemicals, e.g. FTA cards, cannot be used.

[3] Optical density (260 nm) measurements often overestimate the DNA concentration, e.g. due to contamination with RNA. Whether the DNA quantity was sufficient can be estimated on the basis of the Q-fragment.

- For purified DNA, the following extraction methods have been validated:
  - Silica-based columns
  - Salting out (manual)
- The extraction method should be similar for all samples used. Heparinised blood may only be used if the sample has been purified by methods that remove heparin (e.g. Nucleospin gDNA Clean-up XS).
- Samples should never be concentrated by evaporation or SpeedVac.

7.2. Preparation of Crude Extracts from Dried Blood Spots

Use one of the following three protocols:
A: Fast, simple and uses the smallest 1.5 mm punch size.
B: Uses the easier-to-handle 3.2 mm punch size but requires sample dilution.
C: A more elaborate method, but the extract prepared can also be used for confirmation reactions with SALSA MLPA probemix P021 SMA. In addition, the remaining DNA may be suitable for other tests such as a TREC circle qPCR.

For all methods:
- Always clean the puncher between different cards (e.g. by taking two punches from an empty card) to avoid sample cross contamination.
- Use a thermocycler with heated lid for heating steps.
- Briefly centrifuge plates/tubes before, and be careful when, removing the seal/lids after the heating step to prevent contamination with other samples.
- Store the remaining extracts in a refrigerator at 2°C – 6°C for potential follow-up assays. Prolonged storage (longer than one week) is possible at -25°C – -15°C.
- Always use a freshly prepared NaOH solution for extraction. Avoid unnecessary exposure of this solution to air and replace weekly.
  - 10 mM NaOH: Mix 1 ml 1 M NaOH (e.g. Sigma S2770) + 99 ml water.

A. Crude extract from an unwashed 1.5 mm punch of a DBS card:
   - Collect a 1.5 mm punch in a well of a 96 well plate or 0.2 ml volume tube.
   - Add 30 µl 10 mM NaOH, ensuring the punch is fully submerged.
   - Seal the plate, or close tubes.
   - Heat the sample for 15 minutes at 99°C.
   - Use 2 µl of this crude extract for each MC002 reaction.

B. Crude extract from an unwashed 3.2 mm punch of a DBS card:
   - Collect a 3.2 mm punch in a well of a 96 well plate or 0.2 ml volume tube.
   - Add 50 µl 10 mM NaOH, ensuring the punch is fully submerged.
   - Seal the plate, or close tubes.
   - Heat the sample for 15 minutes at 99°C.
   - In a new well/tube, dilute 10 µl of this crude extract with 40 µl fresh 10 mM NaOH.
   - Use 2 µl of this diluted crude extract for each MC002 reaction.

C. Crude extract from a washed 3.2 mm punch of a DBS card:
   - Collect a 3.2 mm punch in a well of a 96 well plate or 0.2 ml volume tube.
   - Add 100 µl 10 mM NaOH, ensuring the punch is fully submerged.
   - Incubate for 15 minutes at room temperature.
   - Mix by pipetting the fluid up and down twice; then remove as much of the liquid as possible.
   - Add another 100 µl fresh 10 mM NaOH.
   - Incubate for 15 minutes at room temperature.
   - Mix by pipetting the fluid up and down twice; then remove as much of the liquid as possible.
   - Add 50 µl fresh 10 mM NaOH, ensuring the punch is fully submerged.
   - Seal the plate, or close tubes.
   - Heat the samples for 15 minutes at 99°C.
   - Use 2 µl of the extract for each MC002 reaction.

8. Samples Included in Each Experiment

8.1. SD074 Threshold DNA Sample

- SD074 Threshold DNA has a ratio *SMN1*:*SMN2* = 1:5 and should generate a high *SMN2*-specific melt peak (56°C) and a low, but clearly visible, *SMN1*-specific melt peak (63°C).
- Include two reactions of SD074 Threshold DNA in each MC002 experiment.
- Samples that have an *SMN1*:*SMN2* peak ratio that is equal to, or lower than, the reactions on the SD074 Threshold DNA need follow-up testing by SALSA MLPA probemix P021 SMA.
- SD074 Threshold DNA CANNOT be used in MLPA experiments.

8.2. SD075 Positive DNA Sample

- SD075 Positive DNA should have a complete absence of the *SMN1*-specific melt peak (63°C) and generate a high *SMN2*-specific melt peak (56°C).
- Include at least one reaction of SD075 Positive DNA in each MC002 experiment.
- SD075 Positive DNA CANNOT be used in MLPA experiments.

9. Performance Characteristics

9.1. SALSA Melt Assay Technical Validation

Internal validation of this product is essential before implementation and subsequently when changing DNA extraction method or thermocycler type. Validation testing should include:

- ≥10 samples from unaffected individuals.
- Two SD074 Threshold DNA reactions (ratio *SMN1*:*SMN2* = 1:5).
- Two SD075 Positive DNA reactions (homozygous absence of *SMN1*).
- One no DNA control reaction.

9.2. Expected Results

- All samples from healthy individuals should show the *SMN1*-specific melt peak (63°C).
- Most samples from healthy individuals should show the *SMN2*-specific melt peak (56°C).
- SD074 Threshold DNA should generate a high *SMN2*-specific melt peak (56°C) and a low, but clearly visible, *SMN1*-specific melt peak (63°C).
- SD075 Positive DNA should generate a high *SMN2*-specific melt peak (56°C) and complete absence of the *SMN1*-specific melt peak (63°C).
- No DNA reaction: Q-fragment specific melt peak (49°C) should be the highest peak.

Diagnostic sensitivity*	95%-98%
Diagnostic specificity*	~100%
Analytical sensitivity	100%
Analytical specificity†	>95%

* Diagnostic specificity and sensitivity were both found to be 100% in a clinical performance evaluation study on DBS cards from 47 SMA patients and 375 controls (Isala Clinics, Zwolle, the Netherlands; manuscript in preparation). In most populations, diagnostic sensitivity is expected to be ~95-98% due to MC002's inability to detect mutations other than *SMN1* exon 7 deletions; see Limitations of the Procedure.

† Analytical specificity may be compromised by the factors mentioned in the Limitations of the Procedure section.

10. Protocol

10.1. Materials Required but not Provided

- NaOH for the preparation of crude extracts (e.g. 1 M solution, Sigma S2770).
- Standard laboratory equipment, such as micropipettes and microcentrifuges.
- PCR plates & optical grade adhesive seals, suitable for the instrument used.
- Centrifuge with swingout buckets for multiwell plates.

- Calibrated thermocycler with a melt curve option and a heated lid (99-105°C). The following instruments and materials have been validated[4]:
  - **Bio-Rad CFX96 Touch**
    With Bio-Rad HSP9655 white/white plates and Bio-Rad MSB1001 MicroSeal Plate Sealing film.
  - **Roche LightCycler 480**
    With BIOplastics B17489 plates and BIOplastics 157300 Opti-Seal adhesive seals (www.bioplastics.com).
  - **Thermo Fisher QuantStudio 5**
    With Thermo Fisher N8010560 MicroAmp Optical 96 well plates and Thermo Fisher 4360954 MicroAmp Optical Adhesive Film.

10.2. Procedure Notes

- MC polymerase solution contains 50% glycerol and remains liquid at the recommended storage temperature.
- Never vortex MC polymerase or MC002 master mix, as this may cause enzyme inactivation.
- Do not combine reagents from different lots.
- Start the PCR reaction within 3 hrs after preparing master mix; during this time, it is not necessary to shield the MC002 master mix from light.
- Fluorescence is only measured during the melting curve generation. Never open post-PCR tubes, strips or plates in the room where PCR reactions are prepared.
- PCR products can be stored at 4°C for 1 week. As fluorescent dyes are light-sensitive, store PCR products in the dark. The melt curve generation may be performed, or repeated, up to one week after the PCR reaction.
- After use, store all remaining reagents between -25°C and -15°C.

10.3. Instrument Settings

Instrument	Acquiring channel	Excitation filter	Detection filter	PCR Ramping speed *
Bio-Rad CFX96 Touch	4	620-650 nm	675-690 nm	3.0°C/s
QuantStudio 5	X5-M5	640 ± 10 nm	682 ±10 nm	1.6°C/s
LightCycler 480	Cy5	483 nm	660 nm	2.2°C/s

* Values mentioned are the default machine settings for standard PCR programs.

10.4. Thermocycler Program

DNA denaturation

1. 95°C *60 seconds*

PCR reaction

2. 45 cycles:
   - 95°C *20 seconds*
   - 57°C *30 seconds*
   - 68°C *40 seconds*

Melt curve generation, fluorescence detection*

3. Cool rapidly to 35°C and hold at this temperature for 120 seconds.
4. Slowly increase temperature to 85°C (+0.4°C / 5 seconds)**

* When the melt curve generation (step 3+4) is not performed immediately after the last PCR cycle, the reactions should be heated for 40 seconds at 68°C prior to continuing with steps 3 and 4.
** For QuantStudio 5 and Bio-Rad CFX96 Touch, step and hold steps are recommended.

[4] Other thermocyclers capable of generating a melt curve might be suitable, but have not been tested at MRC Holland; their implementation requires in-house validation.

10.5. Experimental Set-up

Each MC002 experiment/plate should include the following:

- At least two SD074 Threshold DNA reactions (ratio *SMN1*:*SMN2* = 1:5).
- At least one SD075 Positive DNA reaction (homozygous absence of *SMN1*).
- At least one no DNA control reaction: no DNA reactions are intended to check for contamination of e.g. MC002 reagents, pipettes and thermocycler.
  - For experiments using crude extracts from DBS cards, use 2 µl 10 mM NaOH, or an extract from a blank DBS card.
  - For experiments using purified DNA samples: use 2 µl TE (10 mM Tris-HCl pH 8; 1 mM EDTA).

10.6. Experimental Protocol

1. Thaw the MC002 probemix tube and vortex the thawed solution. It is essential that the tube is completely thawed. Centrifuge the probemix tube for a few seconds before opening, as drops may have adhered to the lid.
2. Warm the MC polymerase tube for 10 sec in your hand to reduce viscosity and centrifuge for a few seconds before use, as drops may have adhered to the lid.
3. Prepare an MC002 master mix by mixing 19 µl MC002 probemix + 1 µl MC polymerase for each reaction.
   - When preparing MC002 master mix, include a 5% volume surplus to allow for pipetting errors.
   - A master mix for 50 reactions can be prepared by adding 55 µl MC polymerase to a complete tube of MC002 probemix.
   - MC002 master mix can be prepared and dispensed at room temperature.
   - Start the PCR reaction within 3 hrs of preparing the MC002 master mix.
   - Mix the master mix well by repeatedly pipetting up and down until the viscous MC polymerase and the MC002 Probemix are completely mixed. **Never vortex solutions containing enzymes.**
4. Dispense 20 µl of the MC002 master mix in each well.
5. Add 2 µl DNA sample for each reaction.
6. Seal the plate and centrifuge briefly.
7. Place the plate in the thermocycler; start the MC002 thermocycler program.
   - When the PCR is performed in an instrument with melt curve function, proceed with the melt curve generation immediately after the last PCR cycle.
   - When the PCR is performed in an instrument without melt curve function, transfer the plate to the melt curve instrument and incubate for 40 seconds at 68°C, followed by the melt curve generation.

10.7. Data analysis

Data analysis and quality control are done by visual examination of the melt curve profiles obtained by the standard instrument software. No separate HRM program is required.

Instrument	Melt program data analysed using
Bio-Rad CFX96 Touch	Melt curve tab of the CFX Manager software
QuantStudio 5	Melt curve analysis option of the standard instrument software
LightCycler 480	"Tm calling" option

11. Interpretation of Results

Visual confirmation of SALSA Melt Assay results is mandatory. Do not rely on the automatic calling by PCR platform software.

11.1. Quality Control

See the lot specific MC002 Certificate of Analysis for examples of typical MC002 lot-specific melt profiles (available on www.mrcholland.com).

Examine the following:
1. The no DNA control reactions: is the Q-fragment specific melt peak (at 49°C) at least twice as high as any other peak (Figure 4C)? If not, contamination of reactions with amplicons of previous experiments may have occurred.
2. The SD075 reactions: the *SMN1*-specific melt peak at 63°C should be absent (Figure 3C).
3. The peak profile of each sample: If the Q-fragment peak is higher than any other peak, insufficient sample DNA was present in that reaction and data cannot be interpreted (Figure 4B).

11.2. Interpretation of Results

Amplicon	Tm*	Comments
SMN1	63°C	-
SMN2	56°C	-
Q-fragment	49°C	HIGH PEAK: INSUFFICIENT SAMPLE DNA USED!

*Melting temperatures in this table were obtained on the Bio-Rad CFX96 Touch. Melting temperatures may vary slightly with the instrument and DNA extraction method used and may increase with evaporation.

Compare the melt profile of each sample with that of the SD074 Threshold DNA reactions:
- Samples where the *SMN1*:*SMN2* peak height ratio is higher than the SD074 threshold DNA samples are considered to have at least one *SMN1* copy.
- Samples showing no *SMN1*-specific melt peak, or that have an *SMN1*:*SMN2* peak height ratio equal to, or lower than, the SD074 threshold DNA samples, need follow-up testing by SALSA MLPA probemix P021 SMA.
- Melting temperature differences up to 0.8°C between different reactions are acceptable as these can be due to a small amount of evaporation in a sample.

Please note:
- Complete absence of the *SMN2*-specific melt peak (56°C) is regularly observed. Absence of the *SMN2* gene has no clinical consequences when at least one functional *SMN1* copy is present.
- The rs537638918 polymorphism in *SMN2* (frequency: ~1:5000 samples) results in an extra melt peak at 51°C that might be misinterpreted as a high Q-fragment melt peak (sample with insufficient DNA). An example of a sample with the rs537638918 polymorphism is included in the Certificate of Analysis.
- The number of samples with a similar or lower *SMN1*:*SMN2* ratio as compared to the threshold sample, is expected to be less than 1 in 500 samples tested.

11.3. Confirmation Testing

All positive and ambiguous samples (as specified above) should be retested using SALSA MLPA probemix P021 SMA. SALSA MLPA probemix P021 SMA[5] determines the exon 7 copy number of both the *SMN1* and *SMN2* genes. For SMA patient samples (no *SMN1* exon 7 signal in the P021 SMA test), the *SMN2* copy number is important for prognosis and treatment purposes.

12. Limitations of the Procedure

- This assay is not suitable for SMA carrier testing or copy number determination of the *SMN2* gene.
- This assay considers the homozygous absence of *SMN1* exon 7 as indicative of SMA patient status. However, extremely rare cases exist where such individuals were reported to be symptom-free (Helmken et al. 2003; Prior et al. 2004) → possibility of false positives.
- This assay may be sensitive to rare polymorphisms in the sequences targeted by the MC002 PCR primers → possibility of false positives (no *SMN1*-specific signal detected although at least one *SMN1* exon 7 copy is present). The frequency of such false positive results is expected to be less than 1 in 5000 samples tested (dbSNP version 150 search; January 2018).
- This assay may be sensitive to very rare polymorphisms in the MC002 probe target sequence → possibility of false positives. Please report false results due to SNPs and unusual results to MRC-Holland: info@mrcholland.com.

[5] From SALSA MLPA probemix P021 SMA version B1 onwards.

False positive or negative results also can be caused by experimental factors, including:
- Contamination of reactions with amplicons generated in earlier experiments.
- Contamination of blood extracts with DNA from other newborns. Punchers should be cleaned between use on different DBS cards, e.g. by a blank-card punch.

Assay failure can be caused by:
- Impurities in test samples that strongly affect sample DNA denaturation and/or the PCR reaction or melt curve procedure, including fluorescence quenchers, salts, phenol, ethanol, heparin, EDTA and Fe.
- Improper mixing of the master mix, e.g. by mixing insufficiently or too vigorously.
- Excessive evaporation during the MC002 PCR reaction.

13. Related Products

Product	Technique	Purpose
P021 SMA Probemix CE/IVD	MLPA	Patient testing for spinal muscular atrophy. Quantification of exons 7 and 8 of *SMN1* and *SMN2*. Quantification of the combined *SMN1*+*SMN2* copy number of each other exon.
P060 SMA carrier Probemix CE/IVD	MLPA	Carrier testing for spinal muscular atrophy. Quantification of exons 7 and 8 of *SMN1*.
P460 SMA Probemix RUO	MLPA	Carrier testing for spinal muscular atrophy. Quantification of exons 7 and 8 of *SMN1* with increased chance of detection of silent SMA carriers by inclusion of probes for a specific SNP haplotype.

14. References

- Helmken et al (2003). Evidence for a modifying pathway in SMA discordant families. *Hum Genet* 114:11-21.
- Hendrickson et al. (2009). Differences in *SMN1* allele frequencies among ethnic groups within North America. *J Med Genet* 46:641-44.
- Labrum et al. (2007). The molecular basis of spinal muscular atrophy (SMA) in South African black patients. *Neuromuscul Disord* 17:684-92.
- Prior et al. (2004). Homozygous *SMN1* deletions in unaffected family members and modification of the phenotype by *SMN2*. *Am J Med Genet A* 3:307-10

MC002 Product history	
Version	*Modification*
A	First release (10-2018).

Implemented changes in the product description and instructions for use
Version 1 - First version of the MC002 product description and instructions for use.

15. Appendix 1: Background Information

15.1. Disease Overview

	Incidence		
	Caucasians	**Asians**	**Africans**
SMA Patient	1:6,000-1:10,000	<1:10,000	<1:10,000
SMA Carrier	1:41	1:57	1:92

Sources: Hendrickson et al (2009); NCBI Gene reviews.

Symptoms

- Progressive muscle weakness due to degeneration of lower motor neurons.
- Most common symptoms are difficulty in breathing, swallowing and walking.
- SMA subtypes are distinguished by age of onset and maximum motor function obtained: type I, OMIM# 253300; type II, OMIM# 253550; type III, OMIM# 253400; and type IV, OMIM# 271150.

For more information on SMA: http://www.ncbi.nlm.nih.gov/books/NBK1352/.

15.2. Gene Overview

Gene	# exons	Location	Exon numbering*	Gene copy number unaffected individuals
SMN1	9	5q13.2; telomeric	Traditional: exons 1, 2a, 2b, and 3-8	1-4
SMN2	9	5q13.2; centromeric	Traditional: exons 1, 2a, 2b, and 3-8	0-5

* Note a different exon numbering (exons 1-9) is used by LRG (http://www.lrg-sequence.org/) and NCBI (NG_008691.1; NG_008728.1) .

- 5q13.2 is a complicated, highly variable genomic region, containing a 500 kb inverted duplication.
- The only clinically relevant difference between *SMN1* and *SMN2* is a single nucleotide difference in exon 7 (c.840C>T) affecting mRNA splicing. As a consequence, 90% of *SMN2* pre-mRNAs are alternatively spliced and lack exon 7, while only 10% are full-length transcripts producing a protein that is identical to the protein coded by *SMN1*.
- In unaffected individuals (including carriers), a complete absence of the *SMN2* gene does not have any clinical consequences. SMA patients have at least one *SMN2* copy.

16. Appendix 2: SALSA MC002 SMA Newborn Screen

16.1. SALSA MC002 SMA Newborn Screen Technical Background

SALSA MC002 SMA Newborn Screen provides information on the presence or absence of *SMN1* and *SMN2* exon 7 specific sequences in a sample. The relative ratio of the *SMN1*- and *SMN2*-specific melt peaks reflects the ratio between *SMN1* and *SMN2* copies in the DNA sample tested. This means that a sample with two copies of both *SMN1* and *SMN2* will give the same ratio as a sample with only one copy of both *SMN1* and *SMN2*. No carriers are identified with the MC002 assay as it cannot determine absolute *SMN1* or *SMN2* copy numbers with the exception of 0 copies.

A single PCR primer pair is used to amplify a 180 nt fragment of the *SMN1* and *SMN2* genes that includes exon 7. Formation of at least one of these amplicons is expected in all samples as the complete absence of both the *SMN1* and *SMN2* genes is incompatible with life. In the MC002 PCR reaction, a larger amount of one primer is supplied compared to the other PCR primer. As a result, one strand is formed in excess (asymmetric PCR) (Figure 2A).

A 5' Cy5 fluorescently labelled oligonucleotide probe is also present in each reaction. This fluorescent probe does not affect the PCR reaction. When the probe oligo is free in solution, probe fluorescence is negligible as there is a specific quencher moiety bound to the 3' end of the probe. When the probe oligo is hybridized to a complementary *SMN1* or *SMN2* amplicon strand, the Cy5 dye and the quencher molecule are separated, resulting in maximal fluorescence.

After the PCR reaction, the temperature is lowered and the fluorescent probe hybridizes to the *SMN1* and *SMN2* amplicon strands that were produced in excess, resulting in high fluorescence (Figure 2B). When the reaction mixture is slowly heated, the probe will detach from the amplicon strands at a certain temperature (Figure 2C). This is referred to as the "melting temperature" (Tm) and it is dependent on the sequence of the PCR amplicon.

While slowly heating the reaction mixtures, the fluorescence is measured. The Tm of a probe on a specific sample DNA is identified by a rapid drop in fluorescence during the gradual heating of a sample. These are seen in the so-called melt curve (Figure 2D). A derivative of these melt curves is often used to visualize the Tms as peaks (Figure 2E).

The MC002 probe forms a perfect amplicon-probe hybrid with amplicons that contain the *SMN1* exon 7 wildtype sequence resulting in a Tm of approximately 63°C. When bound to an *SMN2* amplicon, the probe-amplicon hybrid has a 1 nt mismatch, resulting in a Tm that is approximately 7°C lower (~56°C).

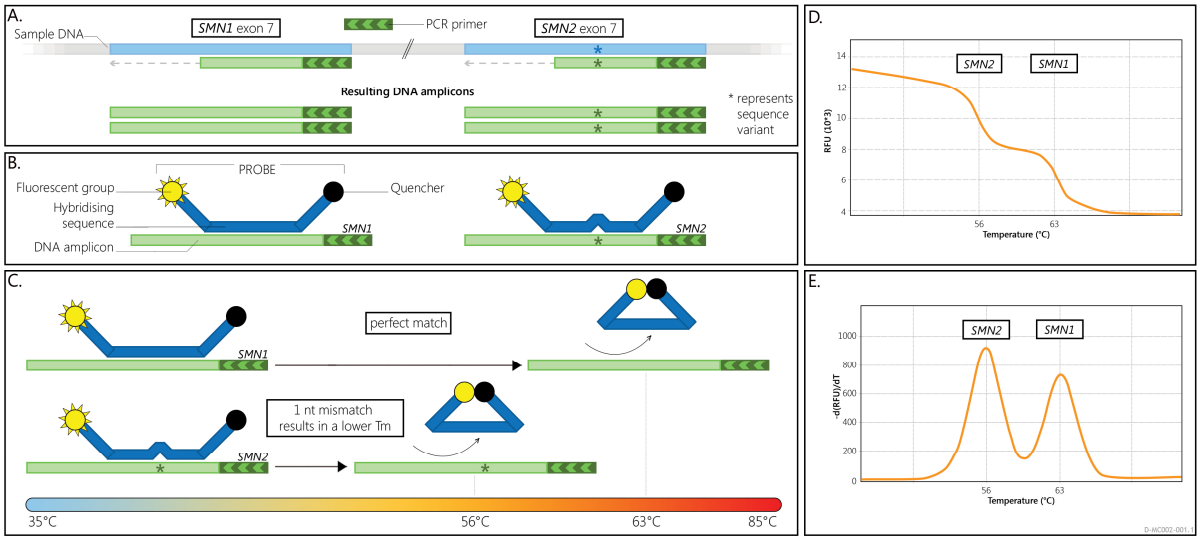

Figure 2. SALSA Melt assay technical explanation. (A) The exon 7 sequence of *SMN1* and *SMN2* is amplified using a single PCR primer pair, with one primer in excess (asymmetric PCR). (B) A fluorescent probe binds to one strand of the *SMN1* and *SMN2* amplicons. The 5' fluorescent group is now separated from its 3' quencher at the other end of the probe oligo, resulting in strong fluorescence emittance. (C) In the upper part of the image, the fluorescent probe binds to the *SMN1* amplicon that has no sequence difference with the probe. In the lower part of the image, the probe binds to the *SMN2* amplicon that has a single nucleotide difference with the probe. When the reaction is slowly heated, the probe-amplicon binding becomes unstable. The probe will dissociate from the *SMN2* amplicons at a lower temperature than from the *SMN1* amplicons, which have no mismatch. When the probe dissociates from its target sequence, fluorescence decreases, as the fluorescent group is quenched by the 3' quencher. (D) A plot showing the fluorescence vs. temperature of a sample with both *SMN1* and *SMN2* amplicons. As the temperature increases, more probe molecules dissociate from the amplicons and the fluorescence decreases. This probe-amplicon dissociation occurs at a lower temperature for the probe-*SMN2* amplicon hybrid that contains a mismatch. (E) The first derivative, -d(RFU)/dT of the curve shown in °C. The graph shows a peak at each temperature with a rapid drop in fluorescence. These temperatures are the Tm for the probe-*SMN2* amplicon hybrid with a 1 nt mismatch and the probe-*SMN1* amplicon hybrid with a perfect match.

When copies of both *SMN1* and *SMN2* are present, two separate Tms will be generated for the probe, visualised as two separate peaks (Figure 3A). If only copies of *SMN1*, or only copies of *SMN2*, are present in a sample, a single Tm/peak will be generated. Please note that an extra, Q-fragment specific, peak with a clearly lower Tm (49°C) may be visible in reactions that contain a very low amount of sample DNA.

Melt curve profiles obtained are compared to the melt curves obtained on the SD074 Threshold DNA known to have a single *SMN1* copy and five *SMN2* copies (Figure 3B). Samples where the *SMN1* peak is absent, or the *SMN1*:*SMN2* peak ratio is lower* as compared to the SD074 Threshold DNA, must be further tested by an independent technique, such as SALSA MLPA probemix P021 SMA. SD075 gives an example of what a

SMA patient profile would look like (Figure 3C). The number of samples requiring further testing in newborn screening programs is expected to be below 0.2%.

* A very low *SMN1*-specific melt peak might be due to contamination of a sample with DNA from another sample. For this reason, not only samples where the *SMN1* peak is absent, but also samples with a very low *SMN1* peak should be further tested.

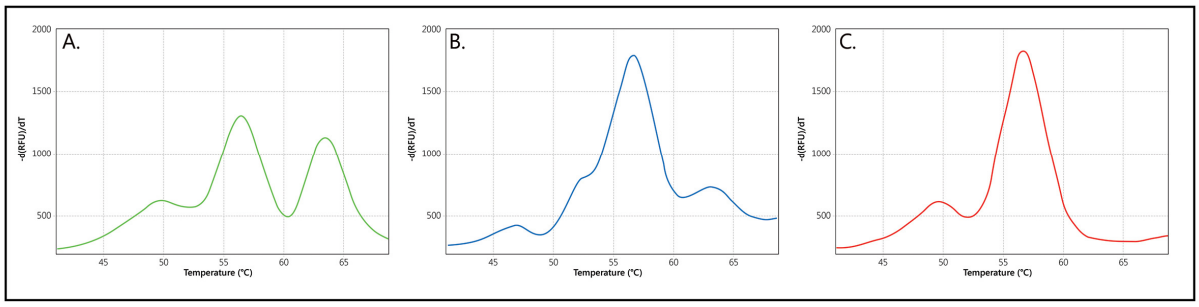

Figure 3. **Typical MC002 results.** Expected melt peak profiles for standard samples. (A) Melt peak profile of an unaffected individual with specific *SMN1* (63°C) and *SMN2* (56°C) peaks showing an *SMN1*:*SMN2* ratio of 1:1. (B) SD074 Threshold DNA melt peak profile with specific *SMN1* (63°C) and *SMN2* (56°C) peaks showing an *SMN1*:*SMN2* ratio of 1:5. (C) SD075 Positive DNA melt peak profile showing an *SMN2* (56°C) peak and absence of the *SMN1* peak with known *SMN1*:*SMN2* ratio of 0:2; indicative of an SMA patient.

16.2. Sample DNA Quantity Determination

The MC002 probemix contains a small amount of a Q-fragment. This is an oligonucleotide that is amplified with the same PCR primer pair as the *SMN1* and *SMN2* amplicons and contains a sequence that is similar to the sequence detected by the Cy5-labelled probe. Binding of the MC002 probe to the Q-fragment amplicon results in a probe-amplicon hybrid with a Tm of 49°C, approximately 7°C lower than the *SMN2* melt peak.

As the Q-fragment is present in very low quantities, the Q-fragment specific melt peak will be absent, or very low, in reactions with sufficient sample DNA as the Q-fragment will be outcompeted by *SMN1* and *SMN2* copies (Figure 4A). When an insufficient amount of sample DNA is present (below 0.25 ng), the Q-fragment specific melt peak will be the highest melt peak present (Figure 4B). When this occurs, reliable conclusions cannot be made. In no DNA reactions, the Q-fragment specific melt peak is the only peak expected (Figure 4C).

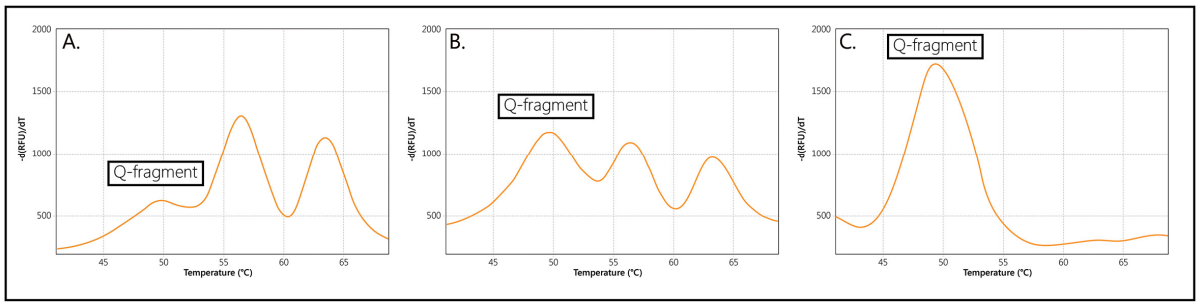

Figure 4. Example MC002 melt peak patterns. (A) A sample that contains 1 copy of both the *SMN1* and *SMN2* genes; the amount of sample DNA is rather low but sufficient, resulting in a visible, but low, Q-fragment specific peak. (B) A sample that has insufficient sample DNA, resulting in the Q-fragment melt peak being the highest of the three peaks. (C) A no DNA reaction where the Q-fragment melt peak is the only peak present.

17. Appendix 3: Troubleshooting

Problem	Cause	Solution
No specific melt curves visible in any reaction.	Programming error(s) in thermocycler / melt program.	Correct errors in the program and repeat the MC002 assay.
	No polymerase enzyme included in the master mix.	Repeat the MC002 assay.
No specific melt curve visible in some reactions.	PCR inhibitors are present in the clinical samples.	Repeat the assay using 5-fold diluted sample, or with a newly prepared sample.
Q-fragment peak is the highest melt peak.	Insufficient sample DNA is present.	Repeat the assay using a more concentrated sample. It is possible to reduce the volume of NaOH solution used for preparation of a crude extract, or to use a lower dilution factor for extracts from 3.2 mm punches. Alternatively, use extracts from washed punches (Protocol C in section 7.2). Do not use more than 2 µl sample DNA / reaction as dilution of the reaction mixture will influence the Tm.
In one or more samples, the Tm observed for *SMN1* and *SMN2* is slightly lower or higher than expected.	Evaporation of reactions results in an increased salt and glycerol concentration. This can result in a slightly altered Tm.	Repeat the MC002 assay when the difference in Tm with the other samples is more than 1°C for both the *SMN1* and *SMN2* melt peak.
An extra melt peak is observed.	An extra melt peak with a Tm below 63°C can be the result of a SNP in either *SMN1* or *SMN2* where the probe binds	No action needed in case a clear *SMN1*-specific melt peak at 63°C is present. When no clear *SMN1* melt peak is visible, perform MLPA follow-up testing.
Melt peaks of all reactions are low.	Wrong type of plate/strips/seal used. Probe signals are higher with correct plate/seals as compared to strips with caps.	Use the recommended type of plates / seals.
	Instrument failure.	Check instrument optics.
Melt peaks of reactions on crude extracts are much lower than the reactions on the SD074/SD075 samples.	Over-quenching of probe signals due to an oversaturated DBS.	When the Q-fragment specific melt peak is low or absent in most samples, the crude extracts can be further diluted by increasing the volume of NaOH solution used for preparation of a crude extract, or by using a higher dilution factor for extracts from 3.2 mm punches. Alternative is to use extracts from washed punches (Protocol C in section 7.2).
Melt peaks of reactions on purified DNA are much lower than the reactions on SD074/SD075 samples.	DNA samples contain PCR inhibitors.	Test a different DNA purification method.
The Q-fragment melt peak is not the highest peak in the no DNA reactions.	Contamination of reactions with amplicons from other samples or from previous experiments.	Never open tubes, strips or plates after the PCR, specifically in the room where reactions are prepared. Do not discard used reaction tubes, strips and plates in the same room where the MC002 reactions are set up or where DNA samples are extracted. Use appropriate plates and seals and visually inspect the rims of the wells to ensure complete sealing. Be very careful with handling the sample plate in which the punch extracts are made.
An *SMN1*-specific melt peak is clearly visible in the SD075 positive DNA sample reaction.		

18. Appendix 4: DNA Samples for Extensive MC002 Validation

In case more extensive MC002 validation is required, DNA samples available from Coriell laboratories can be used (https://www.coriell.org/). The following Coriell cell line-derived DNA samples have been tested at MRC-Holland and verified for the listed *SMN1* and *SMN2* exon 7 copy numbers. Please note that the quality of cell lines can change.

Coriell sample ID	# *SMN1* exon 7	# *SMN2* exon 7	Comments
NA00232	0	2	Can be used as alternative Positive DNA
NA03814	1	5	Can be used as alternative Threshold DNA
HG01773	1	4	
HG00346	1	3	
NA23688	1	2	
HG01748	2	3	
NA03815	1	1	
HG01701	2	2	
NA19019	4	3	
NA12548	3	2	
HG02514	2	1	
HG01755	3	1	
NA19235	4	1	
NA19122	2	0	
HG02051	2	3	Heterozygous for the rare rs537638918 SNP in *SMN2*, resulting in an extra melt peak at 51°C.

Contact info@mrcholland.com for support.