Instructions For Use
SALSA® MC002 SMA Newborn Screen

Version A

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**
Σ	**100, 1000**
Basic UDI-DI:	**872021148MC002YS**
Shipping conditions	**Dry ice or cooling elements**
	-25˚C to -15˚C
	Keep away from heat or direct sunlight
	Read instructions before use
CE 2797 IVD	**Europe Israel Thailand**
RUO	**All other countries**

Any serious incident that has occurred in relation to this product should be reported to MRC Holland and the competent authority of the Member State in which the user and/or the patient is located.

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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1. Kit Components

Available volumes:

- **MC002-100R:** 100 reactions
- **MC002-1000R:** 1000 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, $MgCl_2$, Glycerol, $(NH_4)_2SO_4$
SALSA MC Polymerase	red	Polymerase enzyme	1× 115 µl	5× 230 µl	Glycerol, non-ionic detergents, EDTA, DTT, KCl, Tris-HCl, MC Polymerase enzyme (purified from non-hazardous micro-organisms)
SALSA SD074	blue	Threshold DNA	1× 110 µl	1× 110 µl	Synthetic oligonucleotides, Tris-HCl, EDTA
SALSA SD075	blue	Positive DNA	1× 110 µl	1× 110 µl	Synthetic oligonucleotides, Tris-HCl, EDTA

+ Light sensitive.

2. Storage and Shelf Life

A shelf life of until the expiry date is guaranteed, also after opening, when stored in the original packaging under recommended conditions. For the exact expiry date, see the outer container. This product should not be exposed to more than 10 freeze-thaw cycles. Do not use the product if the packaging is damaged or opened. Leave chemicals in original containers. Waste material must be disposed of in accordance with the national and local regulations.

3. Product Documentation

- MC002 Instructions for Use (IFU; current document).
- MC002 Certificate of Analysis (CoA): quality control specifications; see www.mrcholland.com.

4. Intended Purpose

The Melt Assay SALSA MC002 SMA Newborn Screen is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative manual assay for the detection of homozygous deletions of exon 7 in the *SMN1* gene in DNA isolated from dry blood spot (DBS) cards. MC002 SMA Newborn Screen is intended to determine a potential cause for spinal muscular atrophy (SMA) through molecular genetic screening of newborns. The MC002 assay cannot determine absolute *SMN1* or *SMN2* copy numbers with the exception of 0 copies.

A homozygous loss of the *SMN1* gene, usually detected by the absence of exon 7 specific markers, is the cause of disease in the majority of SMA patients (>95%)[2]. Homozygous *SMN1* exon 7 deletions detected with MC002 SMA Newborn Screen must be confirmed with SALSA MLPA Probemix P021 SMA or an equivalent method using either DNA purified from peripheral blood or a crude extract from washed DBS cards.

Assay results are intended to be used in conjunction with other diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, 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 or prenatal testing, carrier screening, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1]Please note that this kit is for in vitro diagnostic (IVD) use in the countries specified on the first page of this instructions for use. In all other countries, the 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. Assay Summary

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, Strunk et al. 2019). Fluorescence is only measured during melt curve generation.
Absence of the *SMN1*-specific melt peak at 63°C indicates 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 a successful reaction and the use of sufficient sample DNA. The assay can be used with a crude DNA extract prepared from a 1.5 mm or 3.2 mm punch of a DBS card. For more information on the assay see www.mrcholland.com.

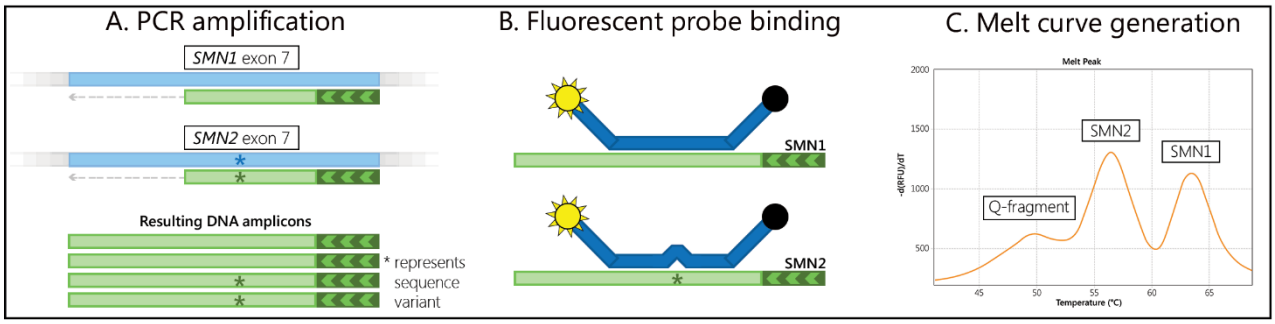

Figure 1. Summary of assay steps. (A) The exon 7 regions of *SMN1* and *SMN2* are amplified with a single set of primers, with one primer in excess. (B) A fluorescently labelled probe binds to the amplicons. (C) The resulting melt curve indicates *SMN1* and *SMN2* sequence presence and if there was sufficient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

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.
- 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.
- SNPs can influence the peak pattern of MC002-A SMA Newborn Screen. Please refer to chapter 10 for an overview of SNPs confirmed to influence the MC002-A peak pattern.
- Never vortex MC Polymerase or MC002 master mix containing MC Polymerase, as this may cause enzyme inactivation.
- Do not combine reagents from different lots.
- 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.
- None of the ingredients are derived from humans, animals, or pathogenic bacteria. This product is not known to contain any harmful agents. Based on the concentrations present, none of the ingredients are hazardous as defined by the Hazard Communication Standard. A Safety Data Sheet (SDS) is not required for this product: none of the ingredients contain dangerous substances at concentrations requiring distribution of an SDS (as per Regulation (EC) No 1272/2008 [EU-GHS/CLP] and 1907/2006 [REACH] and amendments). If spills occur, clean with water and follow appropriate site procedures.

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.
- Crude extracts should be prepared as described in section 7.2 from DBS cards that are based on untreated Whatman 903 paper. Cards impregnated with chemicals, e.g. FTA cards, cannot be used. For validation of other cards, contact MRC Holland.
- 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.

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, and carefully remove the seal/lids after the heating step to prevent contamination with other samples.
- Store the remaining extracts in a refrigerator between 2°C and 8°C for potential follow-up assays. Prolonged storage (longer than one week) is possible when stored between -25°C and -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 (RT).
- 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 RT.
- 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 sample for 15 minutes at 99°C.
- Use 2 µl of the extract for each MC002 reaction.

8. Samples to Include in Each Experiment

8.1. **SALSA SD074 Threshold DNA Sample**

- SALSA SD074 Threshold DNA has one copy of *SMN1* and a high copy number of *SMN2* (high *SMN2:SMN1* ratio) and is expected to 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 SALSA SD074 Threshold DNA in each MC002 experiment.
- Samples that have an *SMN1*:*SMN2* peak ratio that is equal to, or lower than, the reactions with the SALSA SD074 Threshold DNA need follow-up testing by SALSA MLPA Probemix P021 SMA.
- SALSA SD074 Threshold DNA CANNOT be used in MLPA experiments.

8.2. **SALSA SD075 Positive DNA Sample**

- SALSA 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 SALSA SD075 Positive DNA in each MC002 experiment.
- SALSA SD075 Positive DNA CANNOT be used in MLPA experiments.

9. SALSA Melt Assay Technical Validation

9.1. **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 SALSA SD074 Threshold DNA reactions (high *SMN2*:*SMN1* ratio).
- Two SALSA SD075 Positive DNA reactions (homozygous absence of *SMN1*).
- One no DNA control reaction.

9.2. **Expected Results Technical Validation**

	SMN1-specific melt peak (~63°C)	*SMN2*-specific melt peak (~56°C)	Q-fragment (~49°C)
Samples from healthy individuals	Present in all samples	Present in most samples	Lowest peak or absent
SD074 Threshold DNA	Low but distinct peak	High(est) peak	Lowest peak or absent
SD075 Positive DNA	Absent	Present	Lowest peak or absent
NoDNA	Absent	Absent	Only peak present

10. Performance Characteristics

Study	**Description**
Expected values for copy number in normal and affected populations	MC002 is designed to determine if a DNA sample contains 0 or more copies of exon 7 of *SMN1* and *SMN2*. To do this, one cut-off value is used to distinguish between positive (0 *SMN1* exon 7 copies) and negative (1 or more *SMN1* exon 7 copies) results by using SD074 in each experiment. The cut-off applied is detailed in the table below. Peak height ratio — SMN1 copies *SMN1*:*SMN2* peak height ratio > SALSA SD074 Threshold DNA — ≥1 *SMN1*:*SMN2* peak height ratio ≤ SALSA SD074 Threshold DNA — <1 No *SMN1* specific melt peak — <1
Limit of Detection	The analytical sensitivity of MC002 was assessed by conducting two experiments with varying amounts of input DNA; one experiment using cell-line derived purified DNA and one using dry blood spot (DBS) screening card (Whatman 903) derived DNA, both from an adult sample. In the first experiment on the cell-line derived DNA, a positive sample containing 0 copies *SMN1* and 2 copies *SMN2* was serially diluted to 10, 5, 2.5, 1, 0.5, 0.25 and 0.125 ng/reaction and tested. The assay was able to accurately distinguish the *SNM2* peak down to 0.25 ng of input DNA in all replicates tested. The second experiment used DNA isolated from DBS cards. The blood was derived from a healthy patient with normal *SMN1* (>1 copies) and *SMN2* copy numbers. For this, varying input amounts of blood (equivalent to the upper and lower amounts used in practice) were pipetted on the DBS cards: 6 µL (oversaturated), 3 µL (saturated), 4x diluted, 6x diluted and 8x diluted. Four replicates were tested for each amount of input blood sample. The assay was able to accurately distinguish the *SMN1* and *SMN2* peaks in all replicates across all concentrations tested. MC002 was able to accurately detect the expected copy numbers down to 0.25 ng input DNA and in over and under-saturation conditions for DBS cards.
Interfering substances	SNPs or other polymorphisms (e.g. indels) in the DNA target sequence and impurities in the DNA sample can affect the reaction.

Study	Description
	For MC002, DNA is extracted from DBS cards using 10 mM NaOH. The influence of using a higher concentration was therefore tested by using 15-100 mM NaOH. Haemoglobin has a quenching effect on the fluorescence signal, diminishing the height of the melting curve peaks. Therefore, concentrations of 0-16 mg/mL of haemoglobin were spiked into blood derived DNA and tested to assess the effect on the melt peaks. Further, 15, 20 and 100 mM of NaOH, 0.08 µg/µl of hemoglobin and 0.14 µg/µl of bilirubin were spiked in DBS samples and tested alongside un-spiked controls. Results showed that up to 50 mM NaOH can be added before results are affected and that the concentrations of haemoglobin and bilirubin tested do not have an effect on the results obtained with MC002-A. To minimise variability across samples, all samples tested should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method. Additionally, SNPs that could interfere with results were tested: -rs56299889: This SNP causes the *SMN1* peak to be lower, which could lead to a false positive -rs200146682: This SNP causes the *SMN1* peak to shift ~5.5°C lower, making the *SMN1* peak for the SNP carrying allele to coincide with the *SMN2* peak, which could lead to a false positive -rs537638918 polymorphism in *SMN2* results in an extra melt peak 5°C before the *SMN2* peak, that might be misinterpreted as a high Q-fragment melt peak (sample with insufficient DNA).
Cross-reactions	Cross-reactivity is the potential for probes to bind to homologous regions (e.g. pseudogenes) or other cross-reactive sequences. MC002 has been tested for cross-reactivity by using *SMN1*:*SMN2* 0:2 and 2:0 copy samples. For both samples, no non-specific binding signals have been observed for the 0 copies. Additionally 47 confirmed SMA positive samples (*SMN1*=0 copies) and 375 negative controls were tested in-house and none of the samples showed any non-specific signals.
Accuracy	Results of accuracy are derived from trueness and precision studies. For trueness, 3 different samples carrying different possible genotypes (*SMN1*:*SMN2* ratios of 0:2, 1:1 and 2:0) were tested using MC002 as well as SALSA MLPA Probemix P060 SMA Carrier and found to have the expected results. Assay precision was tested by introducing sources of variation in the assay, such as samples, probemix lots, operators and runs and analysing the results obtained. Results showed that the sample genotype and the different probemix lots can cause variation in the melting temperature of the *SMN1* peak. However, none that would cause false results. Variation from lot to lot is to be expected. Furthermore, some variation can be introduced by operators in the fluorescent yield of the *SMN1* peak. Overall even though some variability can be seen in the meting temperature of the *SMN1* peak when testing different variables, none of them could lead to false results, as the temperature range did not exceed a coefficient of variation of 10%.
Clinical validity	From the general literature on SMA, for the Caucasian population, the diagnostic sensitivity for a homozygous exon 7 deletion is expected to be >95%, as up to 5% of the cases are due to other causes. These percentages can vary based on ethnicity (Hendrickson et al. 2009). The above percentages are confirmed by the results from an internal study which showed that MC002 will reliably detect SMA in all of patients that present with a homozygous *SMN1* exon 7 deletion. SMA caused by rare single nucleotide mutations or deletions of exons 1-6 will not be detected by this assay. The diagnostic sensitivity, for the homozygous deletion in exon 7 of the *SMN1* gene is therefore 100% based on this internal study. This is further supported by Kozon et al. 2023 and Mikhalchuck et al. 2023, which both showed a diagnostic sensitivity of 100%.

Summary of Safety and Performance (SSP)
The SSP is available in the European database on medical devices (Eudamed), https://ec.europa.eu/tools/eudamed, or upon request.

11. SALSA Melt Assay Procedure

11.1. **Materials Required but not Provided**

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

- Centrifuge with swingout buckets for multiwell plates.
- Calibrated thermocycler meeting the following specifications:
  - melt curve option available;
  - Cy5 fluorophore detection capability present;
  - heated lid (99-105°C) present;
  - capable of recording at least one datapoint every 0.4°C during melt curve (or 2.5 datapoints every 1°C);
  - compatible with 96-wells plates;
  - a temperature range of 35°C – 99°C.

  MC002 is compatible with machines that meet the above mentioned specifications, but please note that the implementation of instruments always requires in-house validation.

  MC002 was developed and validated with:
    - Bio-Rad CFX96 Touch - With Bio-Rad HSP9655 white/white plates and Bio-Rad MSB1001 MicroSeal Plate Sealing film.
    - Roche LightCycler 480 I - 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.

  These three instruments are used throughout the document to illustrate important settings.

11.2. **Procedure Notes**

- MC Polymerase solution contains 50% glycerol and remains liquid at the recommended storage temperature.
- Start the PCR reaction within 3 hrs of preparing the master mix; during this time, it is not necessary to shield the MC002 master mix from light.
- PCR products can be stored at 4°C for 1 week in case the melt curve generation needs to be performed later, or repeated. As fluorescent dyes are light-sensitive, store PCR products in the dark.
- After use, store all remaining reagents between -25°C and -15°C.

11.3. **Experimental Set-up and Protocol**

Each MC002 experiment/plate should include the following:
- Samples as described in section 8.
- 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 crude extracts from DBS cards, use 2 µl 10 mM NaOH, or an extract from a blank DBS card.

1. Thaw the MC002 probemix tube completely (essential!), vortex and centrifuge 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 opening, as drops may have adhered to the lid.
3. Prepare a master mix:
   - Can be prepared and dispensed at RT.
   - Mix 19 µl MC002 probemix + 1 µl MC Polymerase for each reaction.
   - Include a 5-10% volume surplus to allow for pipetting errors.
   - For 50 reactions can be prepared by adding 55 µl MC Polymerase to a complete tube of MC002 probemix.
   - 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.**
   - Start the PCR reaction within 3 hrs of preparing the master mix.
4. Dispense 20 µl of the master mix in each well.
5. Add 2 µl DNA sample to 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. When the transfer is not completed within 2 hours and/or the samples have not been kept at RT during the transfer period, incubate for 120 seconds at 99°C, before starting the melt curve generation.

11.4. **Instrument Filter Settings**

The excitation peak of the Cy5 fluorophore is at ~650 nm and the emission peak is at ~670 nm. Use the instrument manufacturer's recommendations for Cy5 detection. For the example instruments presented in Chapter 11.1, the following table shows the recommended settings.

Instrument	Acquiring channel	Excitation filter	Detection filter
Bio-Rad CFX96 Touch	4	620 - 650 nm	675 - 690 nm
QuantStudio 5	X5-M5	640 ± 10 nm	682 ±10 nm
LightCycler 480 I	Cy5*	615 nm	670 nm

* Found under Multi Color Hydrolysis Probe filter settings.
When using a PCR platform not mentioned here, please make sure it is calibrated for Cy5 detection and validate the machine before use.
There is no passive reference included in MC002-A SMA Newborn Screen (select "none" in the template).

11.5. **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*

MC Polymerase heat inactivation

3. 99°C *120 seconds*

Melt curve generation, fluorescence detection*

4. Cool rapidly to 35°C and hold at this temperature for 120 seconds.
5. Slowly increase temperature to 85°C with at least 2.5 datapoints / 1°C (1 data point / 0.4°C).**

* When the melt curve generation (step 4+5) is not performed within 2 hours after the last PCR cycle and/or the samples have not been kept at RT during the transfer period, repeat step 3 prior to continuing with steps 4 and 5.

** For example, for instruments QuantStudio 5 and Bio-Rad CFX96 Touch, step and hold steps (+0.4°C / 5 seconds) are recommended. Continuous mode can be used as long as the amount of data points per degree (2.5 datapoints / 1°C) is maintained and this mode is validated before use.

11.6. **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 High Resolution Melting (HRM) program is required. The following table shows the settings for the example instruments presented in Chapter 11.1.

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 I	"Tm calling" option

12. Interpretation of Results

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

12.1. **Quality Control**

Figure 2 shows examples of typical MC002 melt profiles.

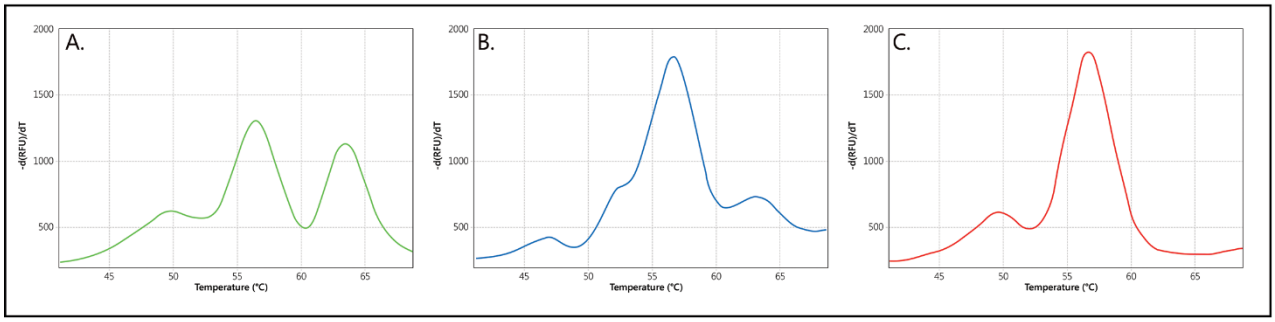

Figure 2. Typical MC002 results. Expected melt peak profiles for standard samples. (A) Melt peak profile of an unaffected individual with specific *SMN1* (63°C) and *SMN2* (56°C) peaks showing an *SMN1*:*SMN2* ratio of 1:1. (B) SALSA SD074 Threshold DNA melt peak profile with specific *SMN1* (63°C) and *SMN2* (56°C) peaks showing a high *SMN2*:*SMN1* ratio. (C) SALSA 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.

Examine the following:

1. The no DNA control reactions: is the Q-fragment specific melt peak (at 49˚C) the only peak present (Figure 3C). If not, contamination of reactions with amplicons of previous experiments may have occurred.
2. The SALSA SD075 reactions: the *SMN1*-specific melt peak at 63°C should be absent (Figure 2C).
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 3B).

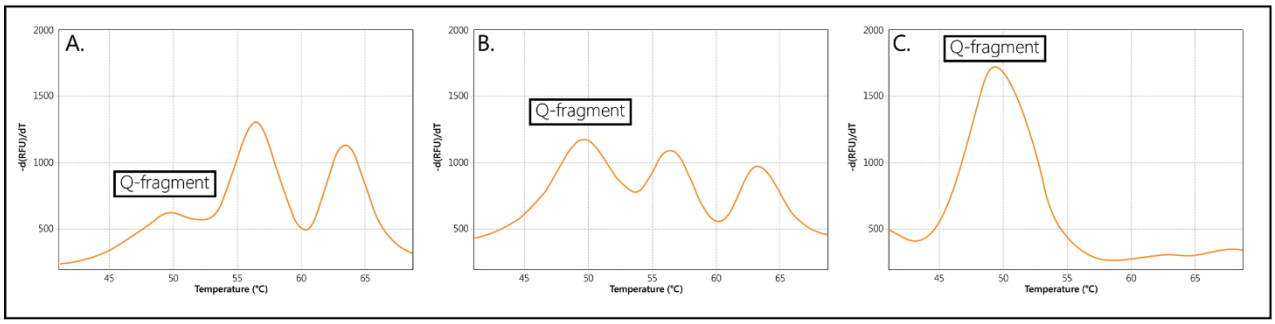

Figure 3. Example MC002 melt peak patterns. (A) A sample that contains 1 copy of both the *SMN1* and *SMN2* genes; the amount of sample DNA is rather low but sufficient, resulting in a visible, but low, Q-fragment specific peak. (B) A sample that has insufficient sample DNA, resulting in the Q-fragment 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.

12.2. **Interpretation of Results**

The following table shows the settings for the example instruments presented in Chapter 11. The melting temperatures (Tm) between example machines differs. Determining the relevant Tm should be part of the internal validation of MC002.

Amplicon	**Melting Temperature (Tm)***			**Comments**
	Bio-Rad CFX96 Touch	**LightCycler 480 I**	**QuantStudio 5**	
SMN1	63°C	66°C	64°C	-
SMN2	56°C	59°C	57°C	-
Q-fragment	49°C	52°C	50°C	HIGH PEAK: INSUFFICIENT SAMPLE DNA USED!

*Tm's are indications and are expected to vary slightly with the instrument and the DNA extraction method used. Tm variation may increase with evaporation. A shift in Tm should always be uniform across peaks.

First, through visual examination of the results determine which samples do not show an *SMN1* peak. These samples need follow-up testing by SALSA MLPA Probemix P021 SMA and should be excluded from the ratio analysis described below.

Then for the remaining samples, compare the melt profile of each sample with that of the SALSA SD074 Threshold DNA reactions:

***SMN1*:*SMN2* peak hight ratio* of samples**	**Result**
Higher than SD074 ratio	At least one *SMN1* copy present
Equal or lower than SD074 ratio (ambiguous samples)	Follow-up testing by SALSA MLPA Probemix P021 SMA needed.

* To acquire peak height ratio, divide the -dRFU/dT value of the *SMN1* peak at maximum height by the -dRFU/dT value of *SMN2* at maximum height.

- 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.

12.3. **Confirmation Testing**

All positive and ambiguous samples (as specified above) should be retested using SALSA MLPA Probemix P021 SMA. SALSA MLPA Probemix P021 SMA[1] 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.

13. Limitations of the Procedure

- In most populations, diagnostic sensitivity is expected to be ~95-98% due to MC002's inability to detect mutations other than homozygous *SMN1* exon 7 deletions. Please note that diagnostic sensitivity is expected to vary between populations (Hendrickson et al. 2009).
- 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 or probe:
  - **rs56299889**: This SNP causes the SMN1 peak to be lower, which could lead to a false positive (no *SMN1*-specific signal detected although at least one *SMN1* exon 7 copy is present). In case a second tier test does not confirm the absence of *SMN1* exon 7, this SNP could be the cause. (Minor allele frequency 0.03, dbSNP version 154.)
  - **rs200146682**: This SNP causes the SMN1 peak to shift ~5.5°C lower, making the *SMN1* peak for the SNP carrying allele to coincide with the *SMN2* peak, which could lead to a false positive (no *SMN1*-specific signal detected although at least one *SMN1* exon 7 copy is present). In case a second tier test does not confirm the absence of *SMN1* exon 7, this SNP could be the cause. (Minor allele frequency 0.00017, dbSNP version 154.)
  - **rs537638918**: This polymorphism in *SMN2* (frequency: ~1:5000 samples) results in an extra melt peak at 5°C before the *SMN2* peak, that might be misinterpreted as a high Q-fragment melt peak (sample with insufficient DNA). 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.
- This assay may be sensitive to yet unconfirmed very rare polymorphisms in the MC002 primer/probe target sequence → possibility of false positives. Please report false results due to SNPs and unusual results to MRC Holland: info@mrcholland.com.

False positive or negative results can also 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.
- Improper mixing of the master mix, e.g. by mixing insufficiently or too vigorously.

[1] From SALSA MLPA Probemix P021 SMA version B1 onwards.

- Excessive evaporation (>48% volume loss) during the MC002 PCR reaction.

14. References

- Helmken C et al. (2003). Evidence for a modifying pathway in SMA discordant families. *Hum Genet* 114:11-21.
- Hendrickson BC et al. (2009). Differences in *SMN1* allele frequencies among ethnic groups within North America. *J Med Genet* 46:641-44.
- Labrum et al. (2007). The molecular basis of spinal muscular atrophy (SMA) in South African black patients. *Neuromuscul Disord* 17:684-92.
- Prior TW 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.
- Strunk A et al. (2019). Validation of a Fast, Robust, Inexpensive, Two-Tiered Neonatal Screening Test algorithm on Dried Blood Spots for Spinal Muscular Atrophy. *Int. J. Neonatal Screen* 5, 21.

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

Implemented changes in the product description and instructions for use
Version 10 – 21 October 2024 - Section 2 rewritten. - Sections 4.1 Comparison of MRC Holland SMA products and 5.1 Disease information moved to the website. - Safety Data Sheet information added as a warning in Section 6. - Clarified interpretation of No DNA reaction (sections 11.1 and 15). - Renamed section 9 and adjusted section 9.2 (information presented in the form of a table). - Section 10 Performance characteristics rewritten. - Section 11 restructured and partially rewritten. - Section 11.2 some procedure notes moved to section 6 Warnings and Precautions. - Section 12.1 sample pictures added. - Section 12.2 partially restructured (information presented in the form of a table). - Section on Related products moved to the website. - Appendix 1: Background Information, Appendix 2: SALSA MC002 SMA Newborn Screen and Appendix 4: DNA Samples for Extensive MC002 Validation moved to the website. - References to peripheral blood as a specimen as well as to SALSA MLPA Probemix P060 SMA Carrier removed throughout the document. - Several minor textual and lay-out changes throughout the document.

15. Appendix 1: 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.
No or only a very low Q-fragment peak is present in the no DNA reaction.	PCR reaction failed, possibly due to PCR inhibitors present in the No DNA reaction.	Check reaction content and repeat MC002 assay.
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 SALSA SD074/SALSA 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 SALSA SD074/SALSA SD075 samples.	DNA samples contain PCR inhibitors.	Test a different DNA purification method.
The Q-fragment melt peak is not the only 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 SALSA SD075 positive DNA sample reaction.		