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**
	-25˚C – -15˚C
	Keep away from heat or direct sunlight
	Read instructions before use
CE IVD	**EU (candidate) member states, members of European Free Trade Association (EFTA) and the UK.** **Israel** **Thailand**
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

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

+ Cy5-labelled probe is light sensitive.

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 kit packaging. When stored under the recommended conditions, a shelf life of until the expiry date as printed on the box is guaranteed, also after opening. The SALSA Melt Assay MC002 kit should not be exposed to more than 10 freeze-thaw cycles. If the product is received in damaged packaging, please contact MRC Holland or the distributor.

3. Product Documentation

- MC002 Instructions for Use (IFU; current document).
- MC002 Certificate of Analysis (CoA): document specifying storage conditions and quality test data; 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 assay for the detection of homozygous deletions of exon 7 in the *SMN1* gene in genomic DNA isolated from human peripheral whole blood specimens or dry blood spot (DBS) cards. MC002 SMA Newborn Screen is intended to confirm a potential cause for and clinical diagnosis of spinal muscular atrophy (SMA) and for molecular genetic screening of newborns. The MC002 assay cannot determine absolute *SMN1* or *SMN2* copy numbers with the exception of 0 copies.

In most populations, 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 should be confirmed with SALSA MLPA Probemix P021 SMA or SALSA MLPA Probemix P060 SMA Carrier using either DNA purified from peripheral blood or a crude extract from washed DBS cards, prepared as described in protocol C of section 7.2

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

This device is not intended to be used for standalone diagnostic purposes, pre-implantation 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.

4.1. **Comparison of MRC Holland SMA products**

MRC Holland offers four different assays for SMA that fit the complete range of genetic testing needs. The table below indicates which product is most suitable for each application.

		MC002	P021	P060	P460
	CE-marked	yes	yes	yes	yes
	Technique	Melt Assay	MLPA	MLPA	MLPA
Used for	Neonatal patient screening	●	○	○	-
	Patient detection	-	●	○	○
	Carrier detection	-	○	●	●
	Silent Carrier‣ detection	-	-	-	●
	Patient detection confirmation	-	√	√	√
Coverage	*SMN1* exon 7 specific	√◊	√	√	√
	SMN1 exon 8 specific	-	√	√	√
	SMN2 exon 7 specific	√◊	√	√	√
	SMN2 exon 8 specific	-	√	√	-
	SMN1+SMN2 exon 1-8	-	√	-	-
	Silent Carrier polymorphism probes	-	-	-	√

● Primary test
○ Secondary test
‣ Increased detection of Silent Carriers.
- Not possible to detect
◊ MC002: no absolute copy numbers aside from 0 determined.
√ Suitable to detect

5. Summary

5.1. **Disease**

Spinal muscular atrophy (SMA) is a severe, recessive, neuromuscular disease for which treatment options are 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, Strunk et al. 2019). 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 can be performed on 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.

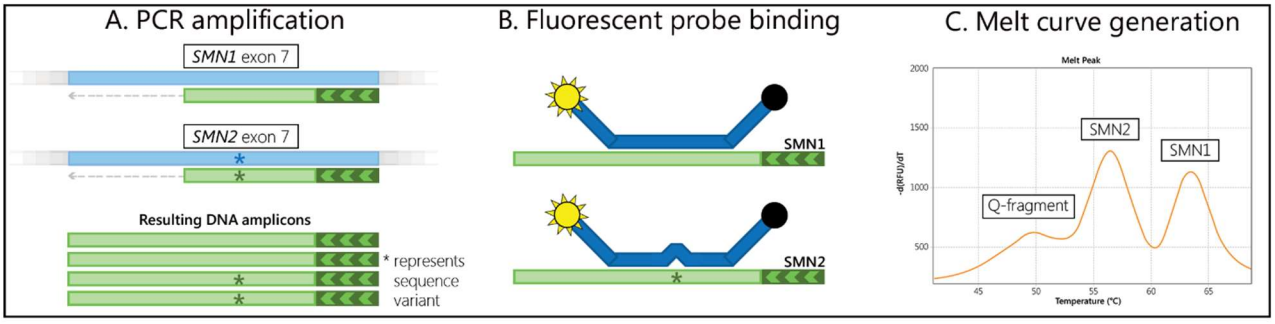

Figure 1. Summary of assay steps. (A) The exon 7 regions of *SMN1* and *SMN2* are amplified with a single set of primers, with one primer in excess. (B) A fluorescently-labelled probe binds to the amplicons. (C) The resulting melt curve indicates *SNM1* and *SMN2* sequence presence and if there was sufficient 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. See CoA for safety statement.
- Internal validation of the MC002 SMA assay is essential.
- 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.
- SNPs can influence the peak pattern of MC002-A SMA Newborn Screen. Please refer to chapter 12 for an overview of SNPs confirmed to influence the MC002-A peak pattern.

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[1])
- 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.
- 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 or SALSA MLPA Probemix P060 SMA Carrier.

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.

[1] 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.

- Store the remaining extracts in a refrigerator between 2°C and 6°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.
   - 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 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 on the SALSA SD074 Threshold DNA need follow-up testing by SALSA MLPA Probemix P021 SMA or P060 SMA Carrier.
- 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. 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 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**

- 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).
- SALSA 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).
- SALSA SD075 Positive DNA should generate a high *SMN2*-specific melt peak (56°C) and have a 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; (Strunk et al. 2019)). 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 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.

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 of preparing the 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 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 the detection of Cy5. For the example instruments presented in Chapter 10.1, 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.

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*

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, the reactions should be heated again for 120 seconds at 99°C prior to continuing with steps 4 and 5.

** For example instruments (10.1) QuantStudio 5 and Bio-Rad CFX96 Touch, step and hold steps (+0.4°C / 5 seconds) are recommended.

10.5. **Experimental Set-up**

Each MC002 experiment/plate should include the following:

- At least two SALSA SD074 Threshold DNA reactions (high *SMN2*:*SMN1* ratio).
- At least one SALSA 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, blood derived 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 a master mix by mixing 19 µl MC002 probemix + 1 µl MC polymerase for each reaction.
   - When preparing master mix, include a 5-10% 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.
   - Master mix can be prepared and dispensed at room temperature.
   - Start the PCR reaction within 3 hrs of preparing the 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 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.

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 High Resolution Melting (HRM) program is required. The following table shows the settings for the example instruments presented in Chapter 10.1.

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

11. Interpretation of Results

Visual confirmation of SALSA Melt Assay results is mandatory. Do not rely on the automatic calling by the 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, Appendix 2)? 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 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**

The following table shows the settings for the example instruments presented in Chapter 10.1. It should be noted that the melting temperatures (Tm) between example machines differs. Determining the relevant Tm should be part of the internal validation of the SALSA MC002 kit.

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. It is important to note, that 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 or SALSA MLPA Probemix P060 SMA Carrier 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:

- Samples where the *SMN1*:*SMN2* peak height ratio is higher than the SALSA SD074 Threshold DNA samples are considered to have at least one *SMN1* copy. These samples do not need follow-up testing. To acquire height ratio, divide the -dRFU/dT value of the *SMN1* peak at maximum height by the -dRFU/dT value of *SMN2* at maximum height.
- Samples that have an *SMN1*:*SMN2* peak height ratio equal to, or lower than, the SALSA SD074 Threshold DNA samples, need follow-up testing by SALSA MLPA Probemix P021 SMA or SALSA MLPA Probemix P060 SMA Carrier.
- 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.

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[2] 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. For confirmation of *SMN1* exon 7 absence, SALSA MLPA Probemix P060 SMA Carrier can also be used. This MLPA probemix allows for easier interpretation of results. *However*, the P060 probemix is *not* suitable for reliable *SMN2* copy number determination. If *SMN2* copy number determination is also included in the purpose of your newborn screening, it is essential to use P021 SMA.

12. 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

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

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, 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	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	MLPA	Carrier testing for spinal muscular atrophy. Quantification of exons 7 and 8 of *SMN1*.
P460 SMA (Silent) Carrier Probemix	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.
- Strunk 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 09 – 17 November 2022 - Thailand was added as a country where MC002 SMA is registered as an IVD. *Version 08 – 22 September 2022* - Role of visual examination during interpretation of results clarified in Chapter 11.2. - *SMN1*:*SMN2* ratio calculation clarified in Chapter 11.2. *Version 07 – 02 August 2022* - Adjusted Table in Chapter 1: added "purified from non-hazardous micro-organisms". - Section on eligible thermocyclers in Chapter 10.1 rewritten. Validated instruments are mentioned as examples throughout the IFU. *Version 06 – 29 June 2021* - IFU rebranded. - The Intended Purpose has been updated. - Information on related MRC Holland SMA products added. - Minor textual changes. - References to 2000R volume removed. - UK added to the list of European countries that accept the CE-mark. - Guideline on use of unvalidated PCR platforms added to chapter 10.3. - Information on SNPs and population dependent homozygous *SMN1* exon 7 deletions underlying SMA added to Chapter 12. - Probemix P060 SMA Carrier added as confirmation test for homozygous *SMN1* exon 7 deletions. - Clarification added on use of SALSA SD074. - Note added to chapter 11.2, on Tm shifts having to be uniform across peaks. - SNP warning added to chapter 6. - SNP remark moved from chapter 11.2 to chapter 12. - Section 2, 3 updated. - Removed: "In addition, the remaining DNA may be suitable for other tests such as a TREC circle qPCR." From section 7.2 - Removed "Can be used as alternative Threshold DNA" from the table in section 18. *Version 05 – 02 October 2020* - LightCycler 480 version specified. - SMN2:SMN1 ratio description of SALSA SD074 changed throughout document. - Ramping speed removed from table in chapter 10.3, additional information added on LightCycler 480 I filter settings. - Clarification of "1 data point / 0.4°C" added in chapter 10.4. - Outdated information removed from chapter 11.2. *Version 04 – 09 April 2020* - Israel was added as a country where MC002 SMA is registered as an IVD. *Version 03 – 15 August 2019* - Table paragraph 10.3 updated. - Information under table 10.4 updated. - Paragraph 10.6 updated. - Minor textual changes. *Version 02 – 13 June 2019* - A heating step was added to the thermocycler protocol (paragraph 10.4). The corresponding table was updated. - Table paragraph 11.2 updated. - Reference added. - Certificate of Analysis (COA) description updated. - Requirement for thermocyclers added (paragraph 10.1) *Version 01 – 12 October 2018* - 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 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.

In the assay 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 reaction 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).

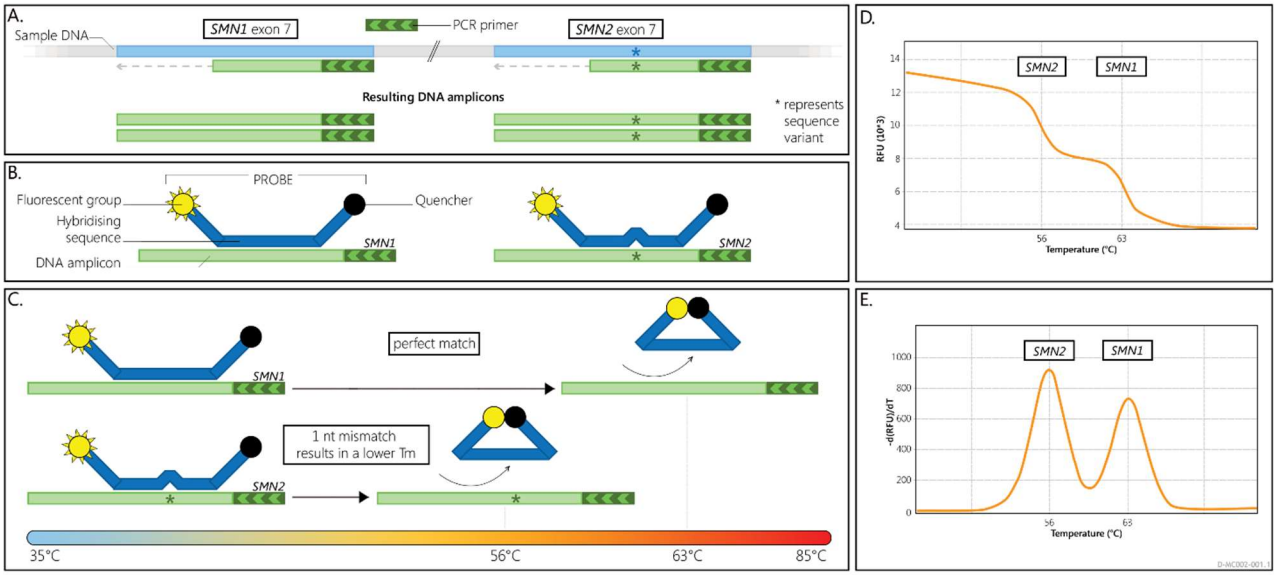

Figure 2. **SALSA Melt assay technical explanation.** (A) The exon 7 sequence of *SMN1* and *SMN2* is amplified using a single PCR primer pair, with one primer in excess (asymmetric PCR). (B) A fluorescent probe binds to one strand of the *SMN1* and *SMN2* amplicons. The 5' fluorescent group is now separated 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 low amount of sample DNA.

Melt curve profiles obtained are compared to the melt curves obtained on the SALSA 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 SALSA SD074 Threshold DNA, must be further tested by an independent technique, such as SALSA MLPA Probemix P021 SMA or SALSA MLPA probemix P060 SMA Carrier. SALSA 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.

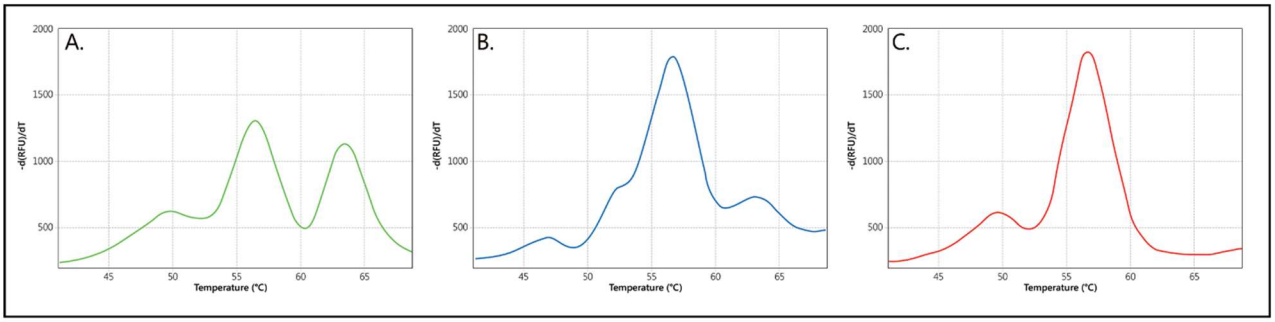

Figure 3. **Typical MC002 results.** Expected melt peak profiles for standard samples. (A) Melt peak profile of an unaffected individual with specific *SMN1* (63°C) and *SMN2* (56°C) peaks showing an *SMN1*:*SMN2* ratio of 1:1. (B) SALSA SD074 Threshold DNA melt peak profile with specific *SMN1* (63°C) 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.

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).

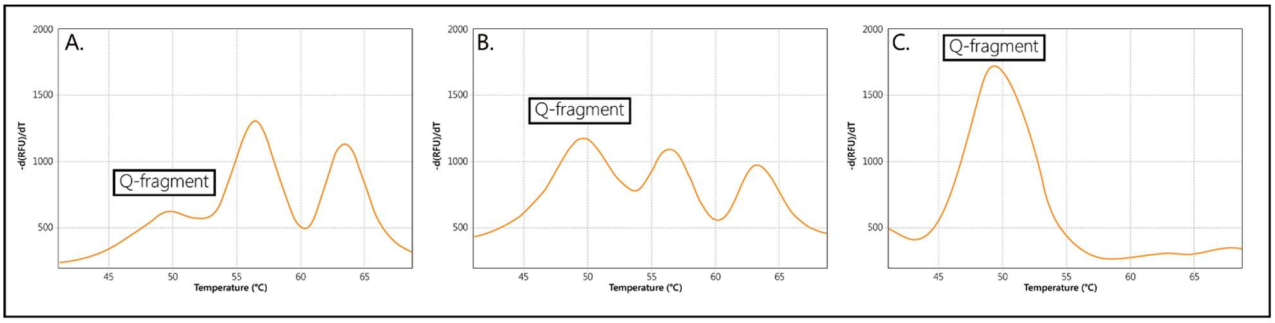

Figure 4. Example MC002 melt peak patterns. (A) A sample that contains 1 copy of both the *SMN1* and *SMN2* genes; the amount of sample DNA is rather low but sufficient, resulting in a visible, but low, Q-fragment specific peak. (B) A sample that has insufficient sample DNA, resulting in the Q-fragment 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 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 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 SALSA 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 Institute 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	
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.