



MS-MLPA[®] General Protocol

Instructions For Use

MS-MLPA (Methylation-Specific Multiplex Ligation-dependent Probe Amplification) General Protocol for the detection and quantification of DNA sequences and methylation profiling.

This protocol contains information that is essential for obtaining reliable MS-MLPA results. It must be read in its entirety and used in combination with the appropriate MS-MLPA probemix-specific product description.

SALSA[®] MLPA[®] reagent kits and Coffalyser.Net analysis software are registered for in vitro diagnostic use (IVD) in specific countries (see www.mrcholland.com). In all other countries, these products are for research use only (RUO). When using an IVD-registered probemix for diagnostic purposes, it is essential to combine it with SALSA[®] MLPA[®] reagent kits and Coffalyser.Net analysis software. Country-specific information on the IVD status of probemixes can be found in the appropriate probemix-specific product description and at www.mrcholland.com.

A separate protocol exists for the detection of only DNA copy number (MLPA[®]). This protocol is available at www.mrcholland.com.



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1. INTRODUCTION

Copy number variations (CNVs) and methylation aberrations in human DNA play a role in a large number of disorders. Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) is a semi-quantitative, non-automated technique that is used to determine the relative copy number and methylation status of up to 60 DNA sequences in a single multiplex PCR-based reaction.

1.1. SALSAL MLPA ASSAY COMPONENTS & STORAGE CONDITIONS

NOTE: MS-MLPA and conventional MLPA make use of the same SALSAL MLPA Reagent Kit.

1.1.1. REAGENT KIT ITEM NUMBERS

Cat No	Description	Number of reactions	Fluorescent label PCR primer
EK1-FAM or EK1-Cy5	SALSAL MLPA EK1 reagent kit	100	FAM or Cy5
EK5-FAM or EK5-Cy5	SALSAL MLPA EK5 reagent kit	500	FAM or Cy5
EK20-FAM	SALSAL MLPA EK20 reagent kit	2000	FAM
PCR001-FAM	SALSAL MLPA PCR kit (Optional)	100	FAM
PCR003-FAM	SALSAL MLPA PCR kit (Optional)	300	FAM

1.1.2. REAGENT KIT COMPONENTS

Reagent kit component	Volumes			Ingredients ¹
	EK1	EK5	EK20	
SALSAL MLPA Buffer (yellow cap)	180 µl	5×180 µl	5×700 µl	KCl, Tris-HCl, EDTA, PEG-6000, DTT, oligonucleotides
SALSAL Ligase-65 (green cap)	115 µl	5×115 µl	5×460 µl	Glycerol, EDTA, DTT, KCl, Tris-HCl, non-ionic detergent, Ligase-65 enzyme (bacterial origin)
SALSAL Ligase Buffer A (transparent cap)	360 µl	5×360 µl	5×1420 µl	Coenzyme NAD (bacterial origin)
SALSAL Ligase Buffer B (white cap)	360 µl	5×360 µl	5×1420 µl	Tris-HCl, MgCl ₂ , non-ionic detergent
SALSAL PCR Primer Mix (brown cap)	240 µl	5×240 µl	5×940 µl	Synthetic oligonucleotides with fluorescent dye (FAM or Cy5), dNTPs, Tris-HCl, KCl, EDTA, non-ionic detergent
SALSAL Polymerase (orange cap)	65 µl	5×65 µl	5×240 µl	Glycerol, non-ionic detergents, EDTA, DTT, KCl, Tris-HCl, Polymerase enzyme (bacterial origin)

1.1.3. APPLICATION-SPECIFIC REAGENTS

Application-specific Reagent	Available Volumes (R=number of reactions)	Ingredients ¹
SALSAL MS-MLPA Probemix* (black cap)	40 µl (25R), 80 µl (50R), 160 µl (100R)	Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA
SALSAL HhaI (light blue cap) [‡]	115 µl (200R)	Restriction endonuclease enzyme (bacterial origin), anti-oxidant, glycerol and undisclosed ingredients
Sample DNA [#] (SD) (blue cap)	30 µl or 100 µl	Tris-HCl, EDTA, synthetic/control plasmid DNA, human genomic female DNA, cell line DNA

* Probemixes are designed to be used only in combination with SALSAL MLPA reagent kits.

[‡] SALSAL HhaI can be ordered separately (item code SMR50).

[#] A vial of SD (reference (selection) or binning DNA) is supplied with or can be separately ordered for certain MS-MLPA probemixes. Volumes and ingredients are dependent on SD type.

¹ None of the ingredients are derived from humans, animals, or pathogenic bacteria. 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 these products:** none of the preparations contain dangerous substances (as per Regulation (EC) No 1272/2008 [EU-GHS/CLP] and amendments) 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.









1.1.4. OPTIONAL: ADDITIONAL PCR REAGENT KIT (100 OR 300 REACTIONS)

PCR kit component	Volumes		Ingredients ¹
	PCR001	PCR003	
SALSA PCR Primer Mix (brown cap)	240 µl	3x240 µl	Synthetic oligonucleotides with fluorescent dye (FAM), dNTPs, Tris-HCl, KCl, EDTA, non-ionic detergent
SALSA Polymerase (orange cap)	65 µl	3x65 µl	Glycerol, non-ionic detergents, EDTA, DTT, KCl, Tris-HCl, Polymerase enzyme (bacterial origin)

1.1.5. STORAGE AND SHELF LIFE

All components must be stored directly upon arrival, and after use, 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 is guaranteed, also after opening. For the exact expiry date, see the labels on each vial. Products should not be exposed to more than 25 freeze-thaw cycles.

1.1.6. PACKAGING LABELS

	Manufacturer		Store at
	Lot Number		Keep away from heat or direct sunlight
	Use by		Catalogue Number
	Number of Tests		Read instructions before use
IVD	In Vitro Diagnostic	RUO	Research Use Only

1.2. MS-MLPA ASSAY PRINCIPLE

The principle of MS-MLPA is based on the amplification of up to 60 probes that each detect a specific DNA sequence of approximately 60 nt in length (Figure 1)^{2,3}. The MS-MLPA reaction results in a set of unique PCR amplicons between 64-500 nt in length that are separated by capillary electrophoresis. After initial denaturation of the sample DNA, a mixture of (MS-)MLPA probes is added to the sample. In general, each (MS-)MLPA probe consists of two oligonucleotides that must hybridise to directly adjacent target sequences in order to be ligated into a single probe. During the subsequent PCR reaction, all ligated probes are amplified simultaneously using the same PCR primer pair, resulting in a set of unique PCR amplicons. One PCR primer is fluorescently labelled, enabling the amplification products to be visualised during fragment separation on a capillary electrophoresis instrument. Fragment separation yields a sample-specific electropherogram: the sample peak pattern (Figure 2, top).

To determine both copy number and methylation status of the target DNA, MS-MLPA probemixes contain several methylation-specific probes. These are designed to target DNA sequences which contain a restriction site for the methylation-sensitive restriction enzyme HhaI. After probe hybridisation, the MS-MLPA reaction is split into two parts. One part of the MS-MLPA reaction is processed as a normal MLPA reaction, providing information on copy number status of the target DNA, while the other part is treated with the HhaI enzyme, which provides information on methylation status of the target DNA (Figure 1).

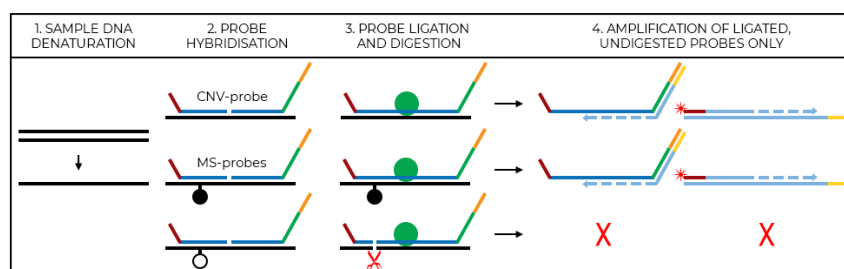


Figure 1. MS-MLPA reaction.

² Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.

³ Nygren AO et al. (2005). Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Res.* 33:e128.

When hybridising to an unmethylated DNA target, the methylation-specific probes will be ligated and simultaneously digested by HhaI (Figure 1, bottom row). A digested MS-MLPA probe will not generate a peak signal because it cannot be amplified. In contrast, when the target sequence of the MS-MLPA probe is methylated, the methyl group will prevent HhaI digestion (Figure 1, middle row). An undigested, ligated probe can be amplified during PCR, resulting in a normal peak signal.

MS-MLPA is a relative technique: only relative differences can be detected by comparing the MS-MLPA peak patterns of DNA samples. The relative height of each individual probe peak, as compared to the relative probe peak heights in various reference DNA samples, reflects the relative copy number of the corresponding target sequence in a sample. Inclusion of reference samples within each experiment is therefore essential. Comparing the electrophoresis patterns of the undigested MS-MLPA reactions allows for the detection of copy number changes (Figure 2 A and B – top row). Comparing the peak patterns of digested reactions may reveal unusual methylation of DNA target sequences (Figure 2 A and B – bottom row).

Figure 2. Profile comparison of MS-MLPA data.

For copy number calculation:

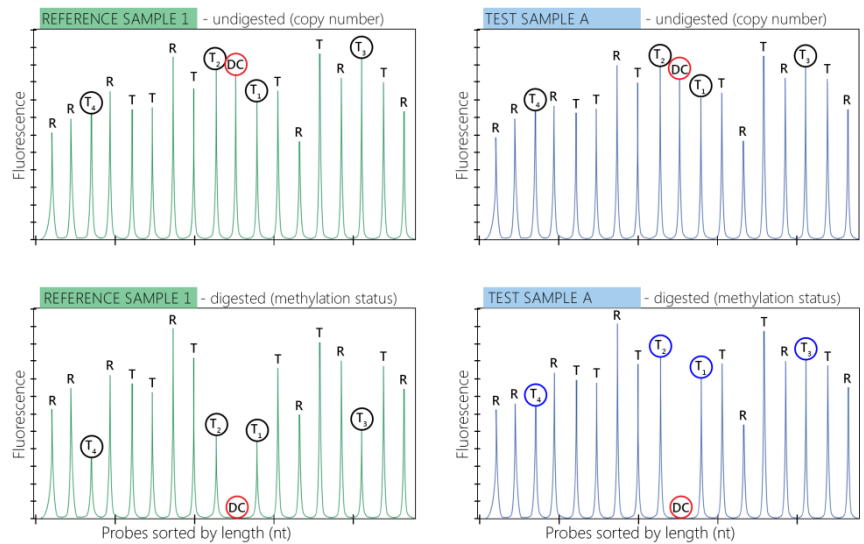
Electropherogram of *Test sample A* – undigested (section A, top right) is compared to the electropherogram of *Reference sample 1* – undigested (section A, top left). *Test sample A* displays the same peak pattern as the reference sample, indicating there are no copy number changes. This is more clearly seen in the probe ratio chart, as displayed by the Coffalyser.Net software, which arranges probes by chromosomal location (section B, ratio charts on top).

For methylation status calculation:

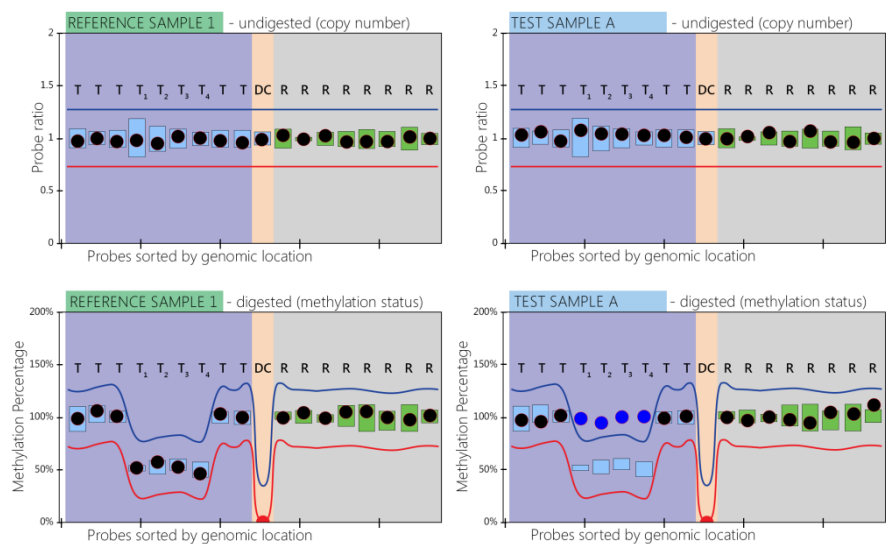
Electropherogram of *Test sample A* – digested (section A, bottom right) is compared to the electropherogram of *Test sample A* – undigested (section A, top right). Similarly, the electropherogram of *Reference sample 1* – digested (section A, bottom left) is compared to the electropherogram of *Reference sample 1* – undigested (section A, top left).

Test sample A – digested shows four target probes with a HhaI site (numbered 1-4 and circled in blue) with a similar peak height to that found in *Test sample A* – undigested. *Reference sample 1* – digested shows the same four target probes with a reduced peak height in comparison to that found in *Reference sample 1* – undigested. When displayed by Coffalyser.Net (arranged by chromosomal location), the results show that these four target probes are 100% methylated in the test sample and 50% methylated in the reference sample (section B, bottom ratio charts). The methylation status of the imprinted region targeted by the four methylation-sensitive probes deviates in *Test sample A* as compared to the reference samples that were derived from healthy individuals. The digestion control probe (DC) detects a sequence that is normally unmethylated in blood-derived DNA, and this probe is used to verify that HhaI digestion was complete. The electropherograms and Coffalyser.Net charts of the digested reference and test samples both show the complete loss of the DC signal.

A. Electropherograms



B. Coffalyser.Net Output



T: target probes, R: reference probes, DC: digestion control probe.

2. ASSAY SETUP INSTRUCTIONS

2.1. MATERIALS REQUIRED BUT NOT PROVIDED

- Ultrapure water
- TE_{0.1} (10 mM Tris-HCl pH 8.0 + 0.1 mM EDTA)
- Calibrated thermocycler with heated lid (99-105°C) and standard laboratory equipment
- 0.2 ml PCR tubes, strips or plates
- Capillary electrophoresis instrument⁴ with fragment analysis software
 - Applied Biosystems: Standard Foundation Data Collection Software
 - SCIEX: GeXP Software Package
- High quality formamide (e.g. Hi-Di Formamide, Applied Biosystems)
- Labelled size standard
 - Applied Biosystems: GeneScan™ 500 LIZ®/ROX™ (preferred; mandatory for use with IVD-registered probemixes), GeneScan™ 600 LIZ®, GeneScan™ 500 TAMRA™
 - SCIEX: CEQ™ DNA Size Standard Kit - 600
- Polymers
 - Applied Biosystems: POP-4 or POP-7 are preferred. POP-6 is not recommended due to its high resolution. SeqStudio: POP-1 is integrated in the cartridge and is suitable.
 - SCIEX: GenomeLab™ Linear Polyacrylamide (LPA) denaturing gel
- Coffalyser.Net analysis software (freely downloadable at www.mrcholland.com)

2.2. SAMPLE TREATMENT AND STORAGE

- Use a total quantity of 50-250 ng of human DNA (50-100 ng is optimal; unless stated otherwise in the probemix-specific product description) in a 5 µl⁵ volume for each MS-MLPA reaction⁶. If necessary, DNA samples can be concentrated by ethanol precipitation, and glycogen (Roche 901393) can be used as a carrier. For more information visit www.mrcholland.com.
- DNA preparations should contain 5-10 mM Tris buffer with a pH of 8.0-8.5 to prevent depurination during the initial denaturation step at 98°C. For example, dissolve and dilute sample DNA in TE_{0.1} (10 mM Tris-HCl pH 8.0 + 0.1 mM EDTA). If it is unknown whether sufficient buffering capacity is present, add Tris-HCl: 4 µl sample DNA + 1 µl 50 mM Tris-HCl pH 8.5.
- Contaminants remaining after DNA extraction, including NaCl or KCl (>40 mM) and other salts, phenol, ethanol, heparin, EDTA (>1.5 mM) and Fe, may influence MS-MLPA performance. MS-MLPA is more sensitive to impurities than multiplex PCR assays. Do not concentrate DNA by evaporation or SpeedVac; this leads to high EDTA and salt concentrations.
- Ensure that the extraction method, tissue type, DNA concentration and treatment are as similar as possible in test and reference samples.
- Extraction methods should not leave a high concentration of contaminants. Do not use QIAGEN M6, M48 and M96 systems, as they leave too much salt. For QIAGEN EZ1, use the *QIAGEN Supplementary Protocol: Purification of genomic DNA from whole blood, optimized for use in MRC-Holland MLPA® assays, using EZ1® DNA Blood Kits* (see www.mrcholland.com). MRC Holland has tested and can recommend the following extraction methods:
 - QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual)
 - Promega Wizard Genomic DNA Purification Kit (manual)
 - Salting out (manual)
- Heparinised blood can only be used when the sample has undergone a purification method to remove the heparin contamination (e.g. Nucleospin gDNA Clean-up XS).
- In certain cases, the SALSA Sample Stabilising Solution (S4; Cat No SMR04, SMR45) (RUO) can improve the quality of the MS-MLPA reaction. See the product description at www.mrcholland.com for more information.
- DNA from whole genome amplification reactions (WGA) is not suitable for MS-MLPA due to amplification bias and because the methylation signature is then removed.
- Bisulfite-modified DNA samples are not suitable for MS-MLPA reactions.
- Aliquot samples and store at -20°C. Contamination with microorganisms can deteriorate samples that are stored at 4°C for an extended period.

⁴ Capillary electrophoresis instruments that do not use denaturing conditions, like QIAGEN QIAxcel or Agilent Fragment Analyzer, cannot be used in combination with MS-MLPA.

⁵ Never use more than 5 µl sample DNA per reaction. Using more than 5 µl DNA reduces the probe and salt concentration. This reduces the hybridisation speed and the stability of the binding of (MS-)MLPA probes to the sample DNA.

⁶ 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-fragments, as is explained in 8.1.

2.3. SELECTING REFERENCE & OTHER CONTROL SAMPLES

- **SELECTING REFERENCE SAMPLES.** Reference samples are DNA samples obtained from healthy individuals with a normal copy number and methylation status for the sequences detected by the target and reference probes. They should be as similar as possible to test samples in all other aspects, including extraction method and sample source. Please note that not all probemixes are suitable for use with DNA from all sources (e.g. formalin-fixed paraffin-embedded (FFPE) tissue). Always check probemix product descriptions for suitable DNA sources. When selecting reference samples, please note that methylation patterns may vary between tissues and even age groups! A sequence that is always methylated in blood-derived DNA may be unmethylated in DNA from other tissues, e.g. amniotic fluid.
- **REFERENCE SAMPLES.** At least three reference samples should be included in each MS-MLPA experiment. When testing >21 samples, include one additional reference sample per seven additional test samples. Distribute reference samples randomly over the experiment to minimise variation. **Multiple reference samples** are needed to estimate the reproducibility of each probe within each MS-MLPA run.
- **COMMERCIAL DNA.** In case of doubts about sample quality, include one or more commercial DNA samples for comparison. We recommend Promega Cat No G1471 male & G1521 female DNA. The commercial DNA should only be used as a control to check sample quality and cannot be used as a reference sample.
- **NO-DNA CONTROL.** It is recommended to include a no-DNA control in every MS-MLPA run. Replace 5 µl DNA by TE_{0.1} (10 mM Tris-HCl pH 8.0 + 0.1 mM EDTA) to check for contamination of TE, MLPA reagents, electrophoresis reagents or capillaries.
- **POSITIVE CONTROL SAMPLES.** Inclusion of positive control samples is recommended when available. MRC Holland does not provide positive samples, but a list of commercially available positive samples is available on www.mrcholland.com. When using cell line DNA, note that cell lines may have acquired additional copy number changes, including gains or losses of complete chromosomes.

3. NOTES TO READ BEFORE YOU START

- Always vortex thawed buffers and probemix, followed by brief centrifugation. All enzyme reagent tubes should be centrifuged briefly. MLPA buffer is typically frozen at -20°C but may remain liquid due to its high salt concentration.
- Before use, warm enzyme vials (Ligase-65, polymerase and HhaI) for 10 sec in your hand to reduce viscosity.
- Enzyme solutions contain 50% glycerol and remain liquid at -20°C. Master mixes containing enzymes should be mixed thoroughly by gently pipetting up and down. Insufficient mixing can result in unreliable results. When preparing master mixes, always add enzymes last. **Never vortex solutions containing enzymes** as enzyme inactivation can occur.
- To minimise sample variation, prepare sufficiently large volumes of master mix solutions (5-10% volume surplus).
- Prepare master mixes (Ligase-65 and polymerase) at room temperature (RT) right before use. If prepared in advance, store master mixes on ice or at 4°C and warm them to RT before addition to MS-MLPA reactions. Non-specific peaks may form in the no-DNA reaction when very cold ligase master mix is added.
- Use multichannel pipettes to avoid excessive evaporation.

4. CRITICAL POINTS FOR OBTAINING GOOD MS-MLPA RESULTS

- Ensure all DNA samples contain 5-10 mM Tris-HCl pH 8-8.5. (Section 2.2)
- Include at least three reference samples, derived from the same tissue type and treated in the same manner as test samples, in every MS-MLPA experiment. (Section 2.3)
- Accurate pipetting of reagents is essential to obtain reliable results. This is especially critical for the 3 µl of hybridisation master mix. (Section 6)
- Use Coffalyser.Net for data analysis. (Section 9)
- Check quality fragments. Complete sample DNA denaturation and HhaI digestion is essential. (Section 8)
- Perform regular CE device maintenance, replacing capillaries and polymer as recommended by the manufacturer. (Section 7)

5. MS-MLPA PROTOCOL - IN BRIEF

1. DNA DENATURATION
 - Heat a 5 µl DNA sample for 5 minutes at 98°C
2. HYBRIDISATION OF PROBES TO SAMPLE DNA
 - Cool down to room temperature, open tubes
 - Add 3 µl hybridisation master mix*
 - Incubate 1 minute at 95°C and hybridise for 16 hours at 60°C
3. LIGATION AND LIGATION-DIGESTION OF HYBRIDISED PROBES
 - Lower thermocycler temperature to 20°C, open tubes

- Add 13 µl of ligase buffer A master mix*
 - Mix well and transfer 10 µl to a second tube
 - Place samples in thermocycler, heat to 48°C and when this temperature is reached:
 - Add 10 µl Ligase-65 master mix* to the first tube (undigested reaction)
 - Add 10 µl Ligase-digestion master mix* to the second tube (digested reaction)
 - Incubate 30 minutes at 48°C
 - Heat inactivate the ligase and HhaI enzymes: 5 minutes 98°C
4. PCR AMPLIFICATION OF LIGATED PROBES
 - Cool down to room temperature, open tubes
 - Add 5 µl polymerase master mix* at room temperature
 - Start PCR (35 x {95°C 30 seconds, 60°C 30 seconds, 72°C 60 seconds}, 72°C 20 minutes, 15°C pause)
 5. FRAGMENT SEPARATION BY CAPILLARY ELECTROPHORESIS
 6. ANALYSE RESULTS WITH COFFALYSER.NET

* Master mixes:

- Hybridisation: 1.5 µl SALSA probemix + 1.5 µl MLPA buffer, per reaction
- Ligase buffer A: 3 µl ligase buffer A + 10 µl ultrapure water, per reaction
- Ligase-65: 1.5 µl ligase buffer B + 8.25 µl ultrapure water + 0.25 µl Ligase-65, per reaction
- Ligase-digestion: 1.5 µl ligase buffer B + 7.75 µl ultrapure water + 0.25 µl Ligase-65 + 0.5 µl HhaI, per reaction
- Polymerase: 3.75 µl ultrapure water + 1 µl PCR primer mix + 0.25 µl SALSA polymerase, per reaction

6. MS-MLPA PROTOCOL

6.1. THERMOCYCLER PROGRAM FOR THE MS-MLPA REACTION

DNA denaturation		
1.	98°C	5 minutes
2.	25°C	pause
Hybridisation reaction		
3.	95°C	1 minute
4.	60°C	16-20 hours
5.	20°C	pause
Ligation & ligation-digestion reactions		
6.	48°C	pause
7.	48°C	30 minutes
8.	98°C	5 minutes
9.	20°C	pause
PCR reaction		
10.	35 cycles:	<ul style="list-style-type: none"> • 95°C 30 seconds • 60°C 30 seconds • 72°C 60 seconds
11.	72°C	20 minutes
12.	15°C	pause

Note: This thermocycler program should be followed unless stated otherwise in the probemix-specific product description.

6.2. DNA DENATURATION (DAY 1)

- Label 0.2 ml tubes, strips or plates.
- Add 5 µl DNA sample (50-250 ng; 50-100 ng is optimal) or TE (no-DNA control) to each tube.
- Place tubes in thermocycler; start MS-MLPA thermocycler program steps 1-2 (see section 6.1).
- Ensure samples are at 25°C before removing tubes from the thermocycler.

6.3. HYBRIDISATION REACTION (DAY 1)

- Prepare hybridisation master mix. For each reaction, mix: 1.5 µl MLPA buffer (yellow cap) + 1.5 µl probemix (black cap). Mix well by pipetting or vortexing.
- After DNA denaturation, add 3 µl hybridisation master mix to each reaction. Accurate pipetting is critical. Mix well by pipetting gently up and down.
- Continue thermocycler program with steps 3-4.

6.4. LIGATION & LIGATION-DIGESTION REACTIONS (DAY 2)

- Prepare a ligase buffer A master mix. For each reaction, mix: 3 µl ligase buffer A (transparent cap) + 10 µl ultrapure water. Mix well by pipetting gently up and down.

- Prepare a Ligase-65 master mix. For each reaction, mix: 8.25 µl ultrapure water + 1.5 µl ligase buffer B (white cap), then add 0.25 µl Ligase-65 enzyme (green cap). Mix well by pipetting gently up and down.
- Prepare a ligase-digestion master mix. For each reaction, mix: 7.75 µl ultrapure water + 1.5 µl ligase buffer B (white cap), then add 0.25 µl Ligase-65 enzyme (green cap) + 0.5 µl SALSA HhaI enzyme (light blue cap). Mix well by pipetting gently up and down.
- Continue thermocycler program with step 5. Remove tubes from the thermocycler.
- Add 13 µl ligase buffer A master mix to each tube. Mix well by pipetting gently up and down. Separate the mixture by transferring 10 µl of the whole mixture to a second tube.
- Place the tubes in the thermocycler. Continue thermocycler program step 6.
- When the thermocycler is at 48°C and **while the samples are IN the thermocycler**, add 10 µl Ligase-65 master mix to the first MLPA reaction (copy number test). Mix well by pipetting gently up and down.
- Add 10 µl of the ligase-digestion master mix to the second MLPA reaction (methylation test). Mix well by pipetting gently up and down.
- Continue thermocycler program with steps 7-9.

6.5. PCR REACTION (DAY 2)

Note: as compared to the PCR reaction in the MLPA General Protocol, the PCR protocol for MS-MLPA described here makes use of half volumes. Some users prefer doing the PCR using double these volumes. The optional SALSA PCR reagents (PCR001 or PCR003) can be ordered for this purpose.

- Prepare polymerase master mix. For each reaction, mix: 3.75 µl ultrapure water + 1 µl SALSA PCR primer mix (brown cap), then add 0.25 µl SALSA polymerase (orange cap). Mix well by pipetting gently up and down.
- **At room temperature**, add 5 µl polymerase master mix to each reaction. Mix well by pipetting gently up and down. **Immediately** place the tubes in the thermocycler and continue the thermocycler program with steps 9-11.
- After the PCR reaction, do not open tubes in the same room as the thermocycler. To avoid contamination, use different micropipettes for performing MS-MLPA reactions and handling MS-MLPA PCR products.
- PCR product can be stored shielded from light at 4°C for 1 week. For longer periods, store between -25°C and -15°C.

7. FRAGMENT SEPARATION BY CAPILLARY ELECTROPHORESIS

7.1. NOTES TO READ BEFORE YOU START

- Size standard, run conditions, polymer, fluorescent dye and volume of MS-MLPA PCR reaction depend on the capillary electrophoresis instrument type. Use the default fragment analysis settings on your capillary electrophoresis instrument applicable for the application, polymer and capillary length. Instrument settings may require optimisation for proper fragment separation.
- Replace capillaries and polymer regularly, by following manufacturer recommendations. Polymer quickly deteriorates after prolonged exposure to >25°C. If size standard peaks are repeatedly low and broad, the capillaries or polymer may have deteriorated.
- Use high quality formamide and store it in aliquots at -20°C. Formamide can become acidic, causing depurination and fragmentation of DNA upon heating.

7.2. ELECTROPHORESIS SPECIFICATIONS

Instrument	Primer Dye	Capillaries	Injection mixture
SCIEX CEQ-2000 CEQ-8000 CEQ-8800 GeXP	Cy5	33 cm	1 µl PCR reaction ^a 0.5 µl CEQ - size standard 600 ^b 28.5 µl HiDi formamide / Beckman SLS Add one drop of high quality mineral oil.
ABI-Prism 3100 (Avant) ABI-3130 (xL) ABI-3500 ^c (xL), Dx ABI-3730 (xL)	FAM	36, 50 cm	0.7 µl PCR reaction ^a 0.3 µl ROX or 0.2 µl LIZ GS 500 size standard 9 µl HiDi formamide Seal the injection plate. Heat 3 min at 86°C, cool for 2 min at 4°C ^d .
ABI-SeqStudio	FAM	28 cm	0.8 µl PCR reaction ^a 0.3 µl ROX/LIZ GS500 size standard 12 µl HiDi formamide Seal the injection plate. Heat 3 min at 86°C, cool for 2 min at 4°C ^d .

^a The volume of PCR product added should never exceed 10% of the total injection mixture.

^b Reduce volume of size standard if needed.

^c For ABI-3500: set run voltage to 15 kV and ensure sufficient run time.

^d Briefly heating the injection mixture before capillary electrophoresis is recommended.

The table below contains the optimal, minimum, and maximum signal ranges for the capillary electrophoresis instruments. If signals fall outside of these values, false results can be obtained. Optimisation of the fragment analysis settings may be required.

Instrument	Optimal signal range (in RFU)	Minimum signal (in RFU)	Maximum signal (in RFU)
SCIEX CEQ/GeXP	9,375 - 136,000	5000	170,000
ABI 310, 3100 & 3130 series	375 - 6,000	200	7,500
ABI 3500, 3730 series & SeqStudio	375 - 24,800	300	31,000

8. MS-MLPA QUALITY CONTROL AND TROUBLESHOOTING

8.1. MS-MLPA QUALITY CONTROL FRAGMENTS

Coffalyser.Net should be used for MS-MLPA data analysis as it automatically performs control fragment checks to ensure minimal quality requirements are met! MS-MLPA probemixes contain quality control fragments that signal if there are problems that may affect MS-MLPA results. Evaluate the quality of the MS-MLPA reaction, including quality control fragments, using the quality control flowchart (section 8.4). Only data that meets the quality requirements is suitable for MS-MLPA result interpretation. To aid in quality assessment, the e-learning modules MLPA quality control fragments and MLPA troubleshooting wizard are available online at www.mrcholland.com.

Almost all SALSA MS-MLPA probemixes contain nine control fragments, as described below:

Name	Length (nt)	Interpretation
Benchmark fragment	92	Benchmark to compare other quality control fragments to.
Quantity fragments (Q-fragments)	64, 70, 76, 82	High when DNA amount is too low or ligation failed. Median of Q-fragment signals $\geq 33\%$ of the 92 nt benchmark fragment \rightarrow DNA quantity insufficient or ligation failed. See Figure 3.
Denaturation fragments (D-fragments)	88, 96	Low in case of poor sample DNA denaturation. Signal $\leq 50\%$ of the 92 nt benchmark fragment \rightarrow DNA denaturation insufficient. See Figure 4.
X & Y fragments	100, 105	Control for sample mix up ⁷ .

Q-FRAGMENTS

The four Q-fragments (64, 70, 76 & 82 nt) provide a control for sufficient DNA addition and successful ligation. The Q-fragments do not need to hybridise to the DNA or be ligated in order to be amplified during PCR. The Q-fragments decrease in height as more sample DNA is included in a reaction (Figure 3).

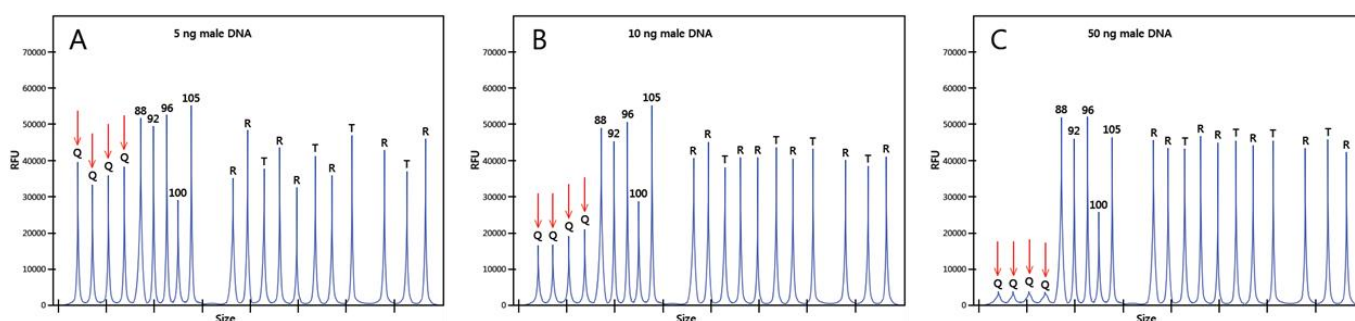


Figure 3. Effect of DNA quantity on Q-fragments. The more sample DNA is used, the lower the Q-fragments. MLPA results with **A.** 5 ng, **B.** 10 ng, **C.** 50 ng DNA. Samples **A.** and **B.** contain insufficient DNA.

D-FRAGMENTS

The two D-fragments (88 & 96 nt) detect sequences in exceptionally strong CpG islands. CpG islands have a high GC-content and are difficult to denature. When the 88 and 96 nt D-fragments are low ($\leq 50\%$ of the 92 nt benchmark fragment) this indicates sample DNA was insufficiently denatured. Poor denaturation can be due to the presence of >40 mM salt in a DNA sample. Incomplete sample DNA denaturation can result in false results!

⁷ Cases are known of males lacking this Y-specific sequence and females carrying this Y-sequence on an X-chromosome.

NOTE: When using ABI POP7 polymer, a non-specific fragment of 80-90 nt is usually present that may coincide with the control fragments!

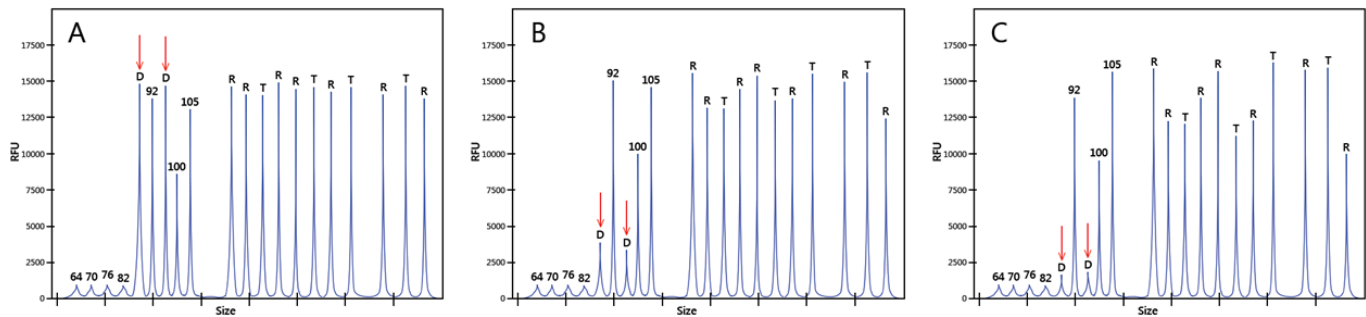


Figure 4. Effect of poor denaturation on D-fragments. D-fragments are low when sample DNA denaturation is incomplete (here induced by adding salt to the sample). MLPA results on DNA sample containing in **A.** TE, **B.** TE + 40 mM NaCl, **C.** TE + 100 mM NaCl. Samples **B.** and **C.** show insufficient denaturation. As MS-MLPA probes more frequently target GC-rich sequences, denaturation problems may be more prevalent in MS-MLPA than in conventional MLPA.

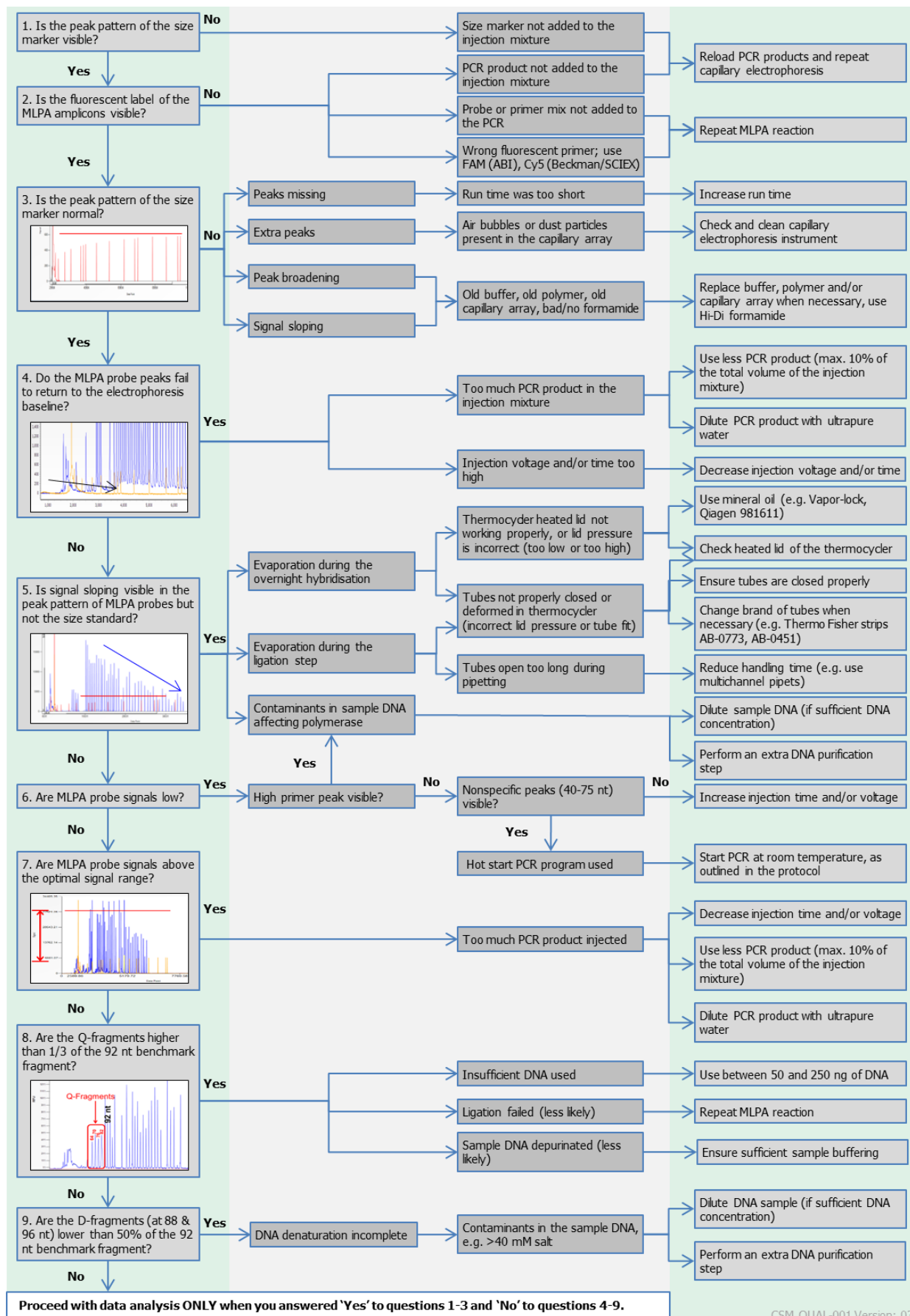
8.2. NO-DNA CONTROL

In a typical no-DNA control, only the four Q-fragments are visible. In some probemixes, a few peaks longer than 100 nt may be visible in no-DNA controls. These non-specific peaks will not influence MS-MLPA results when sufficient sample DNA is used, as they are outcompeted by the exponential amplification of MS-MLPA probes. Notify MRC Holland in case a non-specific peak in the no-DNA control is reproducibly higher than 50% of the median height of the Q-fragments.

8.3. EVAPORATION

Evaporation can occur during (A) pipetting the ligation reaction at 48°C or (B) overnight hybridisation, and causes increased salt concentration. This can result in strong sample DNA secondary structure formation and may inhibit certain probes from binding to their target sequences. In general, plates are more prone to evaporation than strips. In case you suspect evaporation, incubate 8 µl H₂O overnight at 60°C; in the morning, >5 µl H₂O should remain. For suggestions on how to eliminate evaporation, see section 8.4 step 5. When using mineral oil, add just enough to cover the surface. There is no need to remove the oil. After probemix and polymerase master mix addition, centrifuge tubes briefly. After ligase master mix addition, pipet up and down below the oil layer.

8.4. QUALITY CONTROL FLOWCHART



CSM.QUAL-001 Version: 03

9. DATA ANALYSIS

Coffalyser.Net software, in combination with the appropriate lot-specific Coffalyser sheet, should be used for MS-MLPA data analysis. For both, the latest version should be used. The Coffalyser.Net Reference Manual provides step-by-step instructions on MS-MLPA data analysis. Both software and manual are freely downloadable on www.mrcholland.com.

The absolute fluorescence measured by capillary electrophoresis for each probe is affected by many variables and cannot be used directly. The fluorescence of each probe must first be normalised within each reaction (both undigested and digested reactions). For copy number calculation, relative probe signals from the undigested reaction of a test sample are compared with those obtained from the undigested reactions of reference samples. This comparison then allows for the determination of the relative copy number of the target sequences in a sample. For methylation status calculation, the ratio obtained for each probe in the digested reaction is then compared to the ratio obtained in the corresponding undigested reaction. This ratio can be multiplied by 100 to give a methylation percentage. Finally, the methylation percentages in a test sample are compared to the percentages in the reference samples.

Coffalyser.Net selects the best analysis method for each MS-MLPA probemix and offers extensive quality control⁸. For more information on how data analysis is performed, see the Coffalyser.Net Reference Manual. **For IVD-registered MS-MLPA applications, Coffalyser.Net must be used! Using other software may lead to inconclusive or false results!**

10. INTERPRETATION AND CONFIRMATION

- Abnormalities detected by MS-MLPA should be confirmed by an independent technique whenever possible. Copy number and methylation changes detected by a single probe always require confirmation. Sequencing of probe target sequences may show that a lowered probe signal is caused by a mutation/polymorphism. The finding of two heterozygous sequences typically indicates that the sample DNA contains two different alleles. Note that finding a single rare allele by sequencing does not imply that one allele is deleted, as two copies of the rare allele may be present. A homozygous SNP can lead to a partial signal reduction that resembles a heterozygous deletion.
- Not all copy number and methylation changes detected by MS-MLPA are pathogenic. Germline copy number variations reported in healthy individuals can be found at <http://dgv.tcag.ca/dgv/app/home>. MRC Holland cannot provide information whether a deletion or duplication of a specific exon or aberrant methylation will result in disease.
- Certain copy number aberrations can be due to somatic alterations, including large deletions and duplications of entire chromosomes.
- In case of an apparent homozygous deletion, the electropherogram should be visually inspected to identify whether the signal is truly absent. Missing probe signals can be due to binning problems or low signals.
- Copy number changes detected by reference probes or flanking probes are unlikely to be related to the condition tested for. The identity of reference probes is available on request.
- The optimal cut-off values for detecting a significant change in methylation of a sequence is probe dependent and is dependent on sample type and application. In certain cases there might be a significant variation in methylation of specific sequences between different normal individuals. Check the product description of the MS-MLPA probemix for any additional advice on methylation status determination.
- MS-MLPA probes detecting sequences in CpG islands outside imprinting regions, e.g. in promoter regions of genes, often reproducibly yield a low residual signal in the digested reference samples. This might be due to methylation of the sequence in a small percentage of the cells tested. This background signal is often higher in probes detecting sequences near the edge of CpG islands. Similarly, sequences located near the edge of a CpG island more easily lose their protection against methylation and often show a higher frequency of methylation in tumours.
- For many CpG islands located within imprinted chromosomal regions, one parental copy of the CpG island is methylated while the copy inherited from the other parent is unmethylated. The average methylation percentage is therefore 50%. When testing samples for imprinted diseases, the threshold value for abnormal methylation can be determined by testing sufficient DNA samples from healthy individuals.
- Digestion Control Probes: The target sequences of the digestion control probes are unmethylated in most blood-derived DNA samples. The methylation percentage of these probes should be checked to ensure digestion was complete, especially in DNA derived from FFPE tissue. See the probemix-specific product description for more information.

⁸ Coffalyser.Net starts with raw data analysis (baseline correction, peak identification) and provides extensive quality control (e.g. DNA quantity used, complete DNA denaturation, slope correction).

11. PRECAUTIONS AND WARNINGS

- 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. The person responsible for result interpretation should be aware of the latest scientific knowledge of the application in question and of any limitations of the MS-MLPA procedure that could lead to incorrect results.
- Internal validation of each MS-MLPA application is essential, in particular when using MS-MLPA for the first time, or when changing the sample handling procedure, DNA extraction method or instruments used; include at least 16 normal DNA samples. Validation should show a standard deviation ≤ 0.10 for every probe (unless the relevant probemix-specific product description states otherwise). Samples used for validation should be representative of samples used in daily practice.
- Results of MS-MLPA are highly dependent on the HhaI enzyme used. **SALSA HhaI enzyme (SMR50; MRC Holland) should be used** as this restriction enzyme has been validated for use with MS-MLPA by MRC Holland. HhaI enzymes that are resistant to heat inactivation are NOT compatible with the MS-MLPA technique and will give aberrant results. These include, but may not be limited to, Thermo Fisher Scientific enzymes HhaI, ANZA 59 HhaI, and FastDigest HhaI.

12. LIMITATIONS OF THE PROCEDURE

- For most MS-MLPA applications, the major cause of genetic defects are small (point) mutations, most of which will not be detected by MS-MLPA probemixes.
- MS-MLPA cannot detect any deletions or duplications that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations.
- Sequence changes (e.g. SNVs, point mutations, small indels) in or near the target sequence detected by a probe can cause false positive results⁹.
- A point mutation or SNV within the HhaI site may result in a false positive methylation signal as the HhaI enzyme will not be able to digest the probe-sample DNA hybrid.
- Contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal¹⁰. Analysis of a second independently collected and isolated DNA sample can exclude these contamination artefacts.
- In case of poor sample DNA denaturation, apparent deletions, even of several probes recognising adjacent genomic targets, can be a false positive result! Extremely GC-rich chromosomal regions are not denatured at 98°C when more than 40 mM NaCl or KCl is present.
- MS-MLPA tests provide the average copy number and methylation status of the target sequences in the cells from which the DNA sample was extracted. In case several probes targeting adjacent sequences have an unusual value, but do not reach the usual threshold values for a deletion/duplication, mosaicism is a possible cause.
- Minor differences in experimental execution may affect the MS-MLPA peak pattern. Only include samples in an analysis that were a) included in the same MS-MLPA experiment and b) tested with the same probemix lot.
- Subtle changes, such as those observed in mosaic cases, may only be distinguished when probes are arranged according to chromosomal location.
- In certain cases, analysis of parental samples might be necessary for correct result interpretation.
- When running MS-MLPA products, the capillary electrophoresis protocol may need optimization. False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: lower injection voltage / shorter injection time settings, or a reduced amount of sample by diluting PCR products.
- Most MS-MLPA probes detect the methylation of the first cytosine nucleotide in a single HhaI site found within the sequence detected by the probe (G^mCGC). If methylation is absent for this particular CpG-site, it does not necessarily mean that the whole CpG island is unmethylated! We have no data showing that methylation detected by a particular probe indeed influences the mRNA level of that gene.
- The great majority of GC-rich sequences that are located outside of CpG islands are methylated. GC-rich sequences located within CpG islands are often protected against methylation, but the methylation status may differ between tissues and between age groups. Sequences that are methylated in blood-derived DNA might be unmethylated in e.g. amniotic fluid-derived DNA.

⁹ When designing probes, known SNVs are avoided when possible. However, new SNVs are continuously being discovered. Please notify MRC Holland when a polymorphism or a frequent pathogenic mutation influences a probe signal.

¹⁰ Varga RE et al. (2012). MLPA-based evidence for sequence gain: pitfalls in confirmation and necessity for exclusion of false positives. *Anal Biochem.* 421:799-801.

MS-MLPA General Protocol – Document History
Version-010 (30 October 2020)

- There are no changes to the method by which MS-MLPA is performed.
- Text on front page about RUO use of MLPA reagents and MS-MLPA probemixes updated to optional IVD use.
- Section 1.1.4. Additional PCR reagent kits: removed Cy5 from the description of the primer mix, because the PCR001 and PCR003 kits are only sold with FAM primers.
- Figure 1 updated.
- Section 2.1. Added note that the use of GeneScan™ 500 LIZ®/ROX™ is mandatory for use with IVD-registered probemixes.
- Added *"because the methylation signature is then removed"* to the warning about the use of WGA DNA samples.
- Chapter 3. Added HhaI to the instruction to warm enzyme vials. Rephrased instruction about preparing master mixes in advance. Original: *"When prepared >1 hr before use, store master mixes on ice or at 4°C. Master mixes should be warmed to RT before addition to MLPA reactions."* New: *"If prepared in advance, store master mixes on ice or at 4°C and warm them to RT before addition to MS-MLPA reactions."*
- Chapter 4. Added *"and HhaI digestion"* to the warning about the importance of complete DNA denaturation.
- Chapter 5. Added the instruction to mix samples well after the addition of the ligase buffer A master mix. (This is already mentioned in the detailed instructions.) Added the extension *"and when this temperature is reached"* to the step of heating to 48 °C.
- Chapter 9. Added the sentence *"For IVD-registered MS-MLPA applications, Coffalyser.Net must be used!"*
- Chapter 10. Point 2: Added *"or aberrant methylation"* to the sentence *"MRC Holland cannot provide information whether a deletion or duplication of a specific exon will result in disease."* Point 6: Added *"Check the product description of the MS-MLPA probemix for any additional advice on methylation status determination."*
- Chapter 11. Point 3: Added *"SALSA HhaI enzyme (SMR50; MRC Holland) should be used as this restriction enzyme has been validated for use with MS-MLPA by MRC Holland."*
- Chapter 12. Points 3 and 4 and footnote 9: replaced *"SNP"* with *"SNV"*.
- Minor textual changes.

Version-009 (21 October 2019)

- Protocol has been restructured and some sections have been rewritten. There are no changes to the method by which MS-MLPA is performed.
- PCR001-Cy5 and PCR003-Cy5 were discontinued and therefore removed.
- DTT has replaced Beta-Mercaptoethanol in the SALSA MLPA Buffer and SALSA Ligase-65.
- Volume of SALSA HhaI has changed from 65 µl to 115 µl and product code SMR50 has been added.
- Creating a master mix for ligase buffer A (3 µl ligase buffer A (transparent cap)+ 10 µl ultrapure water) for the ligation and ligation-digestion reactions was included.
- ABI 3500 Dx added to section 7.2 ELECTROPHORESIS SPECIFICATIONS.
- Note regarding digestion control probes has been added to the interpretation and confirmation section.
- New limitation of the procedure added regarding off-scale peaks.

Version-008 (18 April 2018)

- Section 6.5: 0.5 µl HhaI enzyme (Promega R6441, 10 units / µl) changed to 0.5 µl Salsa HhaI enzyme (light blue cap).

Version-007 (23 March 2018)

- New Figure 2 added.
- Details on Salsa HhaI added to table in section 4.3. Details regarding Promega HhaI removed.
- Initial settings and ABI-310 removed from electrophoresis specifications table.
- ABI-SeqStudio added to electrophoresis specifications table.
- Table with signal ranges for capillary electrophoresis instruments added.
- Updated Quality control flowchart.
- Critical points for obtaining good results added.
- Information in protocol reorganized and rewritten.

Version-006 (30 November 2016)

- Warning regarding HhaI enzymes that are resistant to heat inactivation added under 2.7 PRECAUTIONS AND WARNINGS.