MS-MLPA General Protocol

Required components

Name	Cat. numbers	Ingredients
SALSA® MLPA® probemix	see probemix product description	synthetic oligonucleotides, oligonucleotides synthesised using non-pathogenic bacterial strain, Tris-HCl and EDTA

To be used with:

- SALSA® MLPA® Reagent Kit (Cat. No: EK1-FAM, EK1-CY5, EK5-FAM, EK5-CY5, EK20-FAM)
- SALSA® HhaI (Cat. No: SMR50)
- Data analysis software Coffalyser.Net™ (Cat. No: n.a.)

For certain applications, can be used with:

Name	Cat. numbers	Ingredients
SALSA® Binning/Reference Selection DNA	SDXXX	Tris-HCl, EDTA, synthetic/control plasmid DNA, human genomic female DNA, cell line DNA

Storage and shelf life of components

Recommended conditions		

A shelf life until the expiry date is guaranteed, also after opening, when stored in the original packaging under recommended conditions. For the exact expiry date, see vial labels. Products should not be exposed to more than 25 freeze-thaw cycles. Do not use the products if the packaging is damaged or opened upon arrival. Leave products in original containers. Waste material must be disposed of in accordance with the national and local regulations.

Safety of components

None of the ingredients are derived from humans, animals, or pathogenic bacteria or pathogenic viruses. 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 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.

Assay principle (MS-MLPA)

Methylation-specific MLPA (MS-MLPA) is a semi-quantitative technique based on the amplification of up to 60 probes, each detecting a specific DNA sequence. The technique starts with denaturation of the sample DNA (see Figure 1 below). Next, a mixture of MLPA probes is added, each probe consisting of two or three oligonucleotides. When hybridisation of all oligos to the sample DNA is complete, the mixture is split over two tubes.

Probes in both tubes are ligated, but the second mixture is also treated with HhaI, which results in digestion of probes which hybridised to an unmethylated target. Next, all ligated probes are amplified simultaneously using a universal PCR primer pair, of which one is fluorescently labelled. This results in a set of PCR amplicons unique to each probe, each with a unique length. The amplicons are then separated by length on a capillary electrophoresis instrument. The resulting sample-specific electropherograms are analysed using Coffalyser.Net. Comparing data from digested and undigested samples to similarly treated reference samples reveals changes in methylation status of the target sequences.

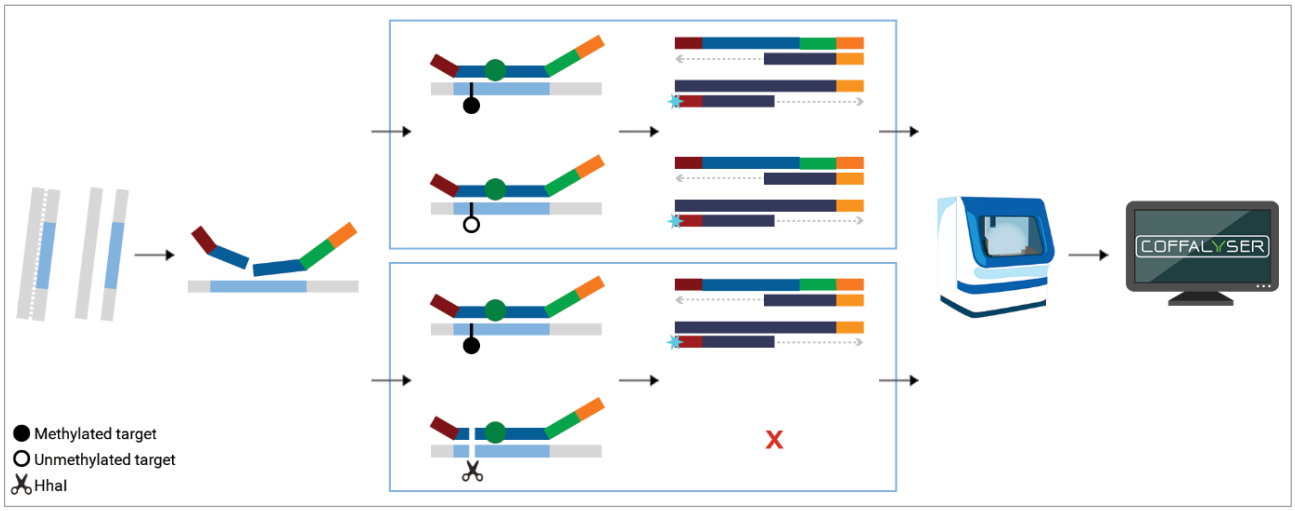


Figure 1. MS-MLPA workflow

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/plates
- Capillary electrophoresis instrument that operates with denaturing conditions and has fragment analysis software; see further details in this Help Centre article
- High quality formamide
- Labelled size standard: Applied Biosystems GeneScan™ 500 LIZ®/ROX™; SCIEX CEQ™ DNA Size Standard kit - 600
- Gel polymer: POP-1, POP-4 or POP-7 (Applied Biosystems); GenomeLab™ Linear Polyacrylamide denaturing gel (SCIEX); Spectrum Compact Polymer4 (Promega); Hitachi DS3000 Polymer4 (Hitachi)

Sample requirements

50-250 ng human DNA (unless stated otherwise) extracted from the tissue indicated in the probemix product description. DNA samples should contain 5-10 mM Tris-HCl buffer pH 8.0-8.5.
Recommended extraction methods:

- QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual)
- Promega Wizard Genomic DNA Purification Kit (manual)
- Salting out (manual)

Precautions and warnings

General precautions

1. Do not use the product if it is damaged or expired.
2. For professional use only. The assay should be performed by professionals trained in molecular techniques.
3. Internal validation of each assay is required, in particular when using it for the first time, or when changing the sample handling procedure, DNA extraction method or instruments used. Use ≥16 different DNA samples from healthy individuals. Validation should show a standard deviation ≤0.10 for every probe, unless described differently in the probemix product description.
4. The person responsible for result interpretation should be aware of the latest scientific knowledge of the application and of any limitations of the MLPA technique that could lead to incorrect results.
5. Coffalyser.Net should be used for data analysis. Use of other software may lead to false results.
6. Always check quality control scores before result interpretation. Only results of samples with good quality scores can be reliably interpreted.
7. Apparent homozygous deletions should be confirmed by visual examination of the electropherogram to exclude false results caused by binning problems or low signals.
8. MLPA 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 the tests should be interpreted by a clinical molecular geneticist or equivalent.

Sample quality precautions

9. DNA depurination caused by insufficient buffering capacity of the sample DNA can result in false results. If it is unknown whether sufficient buffer is present, add Tris-HCl: 4 µl sample DNA + 1 µl 50 mM Tris-HCl pH 8.5.
10. Contaminants remaining after DNA extraction, including salts, heparin, EDTA (>1.5 mM) and iron, may influence assay performance.
11. Salt in DNA samples can cause poor denaturation. This can result in apparent deletions, even of several probes recognising adjacent genomic targets. Do not use QIAGEN M6, M48 and M96 systems; these leave too much salt. For QIAGEN EZ1, use the QIAGEN Supplementary Protocol for MLPA.
12. Do not concentrate DNA; this leads to high EDTA and salt concentrations.

Precautions during execution

13. Never use more than 5 µl DNA solution per reaction. The required DNA quantity is specified in the probemix product description.
14. Do not mix different lots of MLPA probemix.
15. Evaporation that increases concentrations of contaminants and salt may occur during overnight hybridisation or when pipetting the ligation master mix.
    To prevent/reduce evaporation:
    a. Use a multi-channel pipette to reduce handling time;
    b. Ensure that the heated lid works correctly;
    c. Increase or decrease the pressure of the heated lid;
    d. Try to use different reaction tubes;
    e. Put a small drop of mineral oil on top of the DNA sample to cover the liquid surface.
16. Replace capillaries and polymer regularly. 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.
17. Formamide can become acidic, causing depurination and fragmentation of PCR products upon heating. Use high quality formamide and store it in aliquots at -20°C.
18. The volume of PCR product should never be >10% of the total injection mixture. When peaks are low, increase injection time and/or voltage – do not add more PCR product.
19. False results can be obtained if one or more peaks are off-scale. The risk of off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. To reduce the signal, rerun the PCR products using:
    a. Lower injection voltage / shorter injection time;
    b. A reduced amount of PCR products.
20. Contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal. Analysis of a second independently collected and isolated DNA sample can exclude these contamination artefacts.
21. SALSA® HhaI enzyme should be used in all methylation-specific MLPA (MS-MLPA) experiments. Several enzymes sold as "HhaI" are resistant to heat inactivation and are NOT compatible with MS-MLPA. These include, but may not be limited to, Thermo Fisher Scientific enzymes HhaI, ANZA 59 HhaI, and FastDigest HhaI.

Application-specific precautions

See probemix product description.

Test procedure part I – MS-MLPA reaction

Instructions	Thermocycler program
1. DNA denaturation	
1.1 Label 0.2 ml tubes/strips/plates. 1.2 Add 5 µl DNA sample or TE (no-DNA control) to each tube. 1.3 Place tubes in thermocycler, heat at 98°C for 5 minutes, cool to 25°C.	98°C for 5 min 25°C pause
2. Hybridisation	
2.1 Thaw MLPA Buffer and MLPA Probemix, vortex and centrifuge briefly. Pipette when at room temperature 2.2 Prepare HYBRIDISATION MASTER MIX. For one reaction*: MLPA Buffer: 1.5 µl MLPA probemix: 1.5 µl Mix well by vortexing or pipetting. 2.3 Add 3 µl HYBRIDISATION MASTER MIX to each tube. Accurate pipetting is essential at this step! Mix by pipetting. 2.4 Incubate at 95°C for 1 minute, and hybridise at 60°C for 16-20 hours.	95°C for 1 min 60°C pause (16-20 h)
3. Ligation and ligation/digestion	
3.1 Thaw Ligase Buffer A and Ligase Buffer B, vortex and centrifuge briefly. Pipette when at room temperature. Warm Ligase-65 and HhaI in hands for 10 seconds. Do not vortex, just centrifuge briefly. 3.2 Prepare the master mixes. For one reaction*: LIGASE BUFFER A MASTER MIX: Ultrapure water: 10 µl; Ligase Buffer A: 3 µl LIGASE-65 MASTER MIX**: Ultrapure water: 8.25 µl; Ligase Buffer B: 1.5 µl; Ligase-65: 0.25 µl, added last. LIGASE-DIGESTION MASTER MIX**: Ultrapure water: 7.75 µl; Ligase Buffer B: 1.5 µl; Ligase-65: 0.25 µl; SALSA HhaI: 0.5 µl, added last. Mix well by pipetting gently up and down, do not vortex.	20°C pause 48°C pause 48°C for 30 min 98°C for 5 min 20°C pause
3.3 Continue thermocycler program and cool tubes to 20°C. 3.4 Add 13 µl LIGASE BUFFER A MASTER MIX to each tube. Mix well by pipetting gently up and down. 3.5 Place a second set of tubes in the thermocycler, transfer 10 µl of the mixture from each tube to a second tube, and continue program with heating to 48°C. 3.6 At 48°C open tubes **in thermocycler**, add 10 µl LIGASE-65 MASTER MIX to each original tube, mix well by pipetting, close tubes. 3.7 Add 10 µl LIGASE-DIGESTION MASTER MIX to each second tube, mix well by pipetting. 3.8 Close tubes and continue to incubate at 48°C for 30 minutes. 3.9 Heat to 98°C and incubate for 5 minutes to inactivate ligase, cool to 20°C.***	
4. PCR	
4.1 Thaw PCR Primer Mix, vortex and centrifuge briefly. Warm Polymerase in hands for 10 seconds, do not vortex, just centrifuge briefly. 4.2 Prepare the POLYMERASE MASTER MIX. For one pair of reactions*: Ultrapure water: 7.5 µl PCR Primer Mix: 2 µl Polymerase: 0.5 µl Mix well by pipetting up and down, do not vortex. 4.3 At 20°C, add 5 µl POLYMERASE MASTER MIX to each tube, mix well by pipetting but do not spin down the tubes, and continue with PCR program immediately. 4.4 After the PCR, to avoid contamination, do not open tubes in the same room and use a different micropipette to handle the PCR products. 4.5 Store PCR products shielded from light at 4°C for up to 1 week, or between -25°C and -15°C for a longer time.	35 PCR cycles: • 95°C for 30 s • 60°C for 30 s • 72°C for 60 s 72°C for 20 min 15°C pause

*To minimise sample variation, prepare sufficiently large volumes of master mix solutions: 5-10% volume surplus.
**When prepared >1 hour before use, store master mixes on ice or at 4°C, and warm them to room temperature before addition to tubes.
***When the tubes are taken to a separate lab for the PCR, pre-heat the thermocycler for the PCR, e.g. set at 95°C for 1 sec followed by 20°C paused. Minimise the transfer time (e.g. <5 min), place the tubes in the thermocycler and continue with step 4.1. This minimises nonspecific peak formation.

Test procedure part II – Fragment separation

1. Injection settings for commonly used capillary electrophoresis devices supported in Coffalyser.Net	
PCR primer label: FAM	
ABI SeqStudio	Capillaries: 28 cm Injection mixture: • PCR product 0.8 µl • GS500 size standard 0.3 µl (ROX/LIZ) • HiDi formamide 12 µl Seal injection plate, heat at 86°C for 3 minutes, cool at 4°C for 2 minutes.
ABI Prism 3100 ABI 3130 (xL) ABI 3500 (xL) ABI 3730 (xL) ABI SeqStudio Flex	Capillaries: 36 or 50 cm Injection mixture: • PCR product 0.7 µl • GS500 size standard 0.3 µl (ROX) or 0.2 µl (LIZ) • HiDi formamide 9 µl Seal injection plate, heat at 86°C for 3 minutes, cool at 4°C for 2 minutes. *Only 36 cm capillaries.
Hitachi DS3000*	
Promega Spectrum Compact*	
PCR primer label: Cy5	
SCIEX CEQ 2000 SCIEX CEQ 8000 SCIEX CEQ 8800 SCIEX GenomeLab GeXP	Capillaries: 33 cm Injection mixture: • PCR product 1 µl • SS600 size standard 0.5 µl • HiDi formamide / Beckman SLS 28.5 µl Add 1 drop of high quality mineral oil.
2. Electrophoresis settings	
Use default fragment analysis settings appropriate for the application, instrument, polymer and capillary length. Instrument settings may require optimisation to ensure that signals fall within the optimal detection range and that the run is long enough to detect all fragments. The optimal signal ranges (in RFU) and the minimum/maximum signals per instrument can be found in the Coffalyser.Net Reference Manual.	

Quality control and data analysis

The latest version of Coffalyser.Net™ (downloadable from the MRC Holland website) should be used for quality control and data analysis. For detailed instructions, see the Coffalyser.Net Reference Manual.

See also these articles in our Help Centre:

- Explanation about Quantity and Denaturation control fragments
- Information on no-DNA controls

For further help with troubleshooting, visit the troubleshooting pages on the MRC Holland Help Centre.

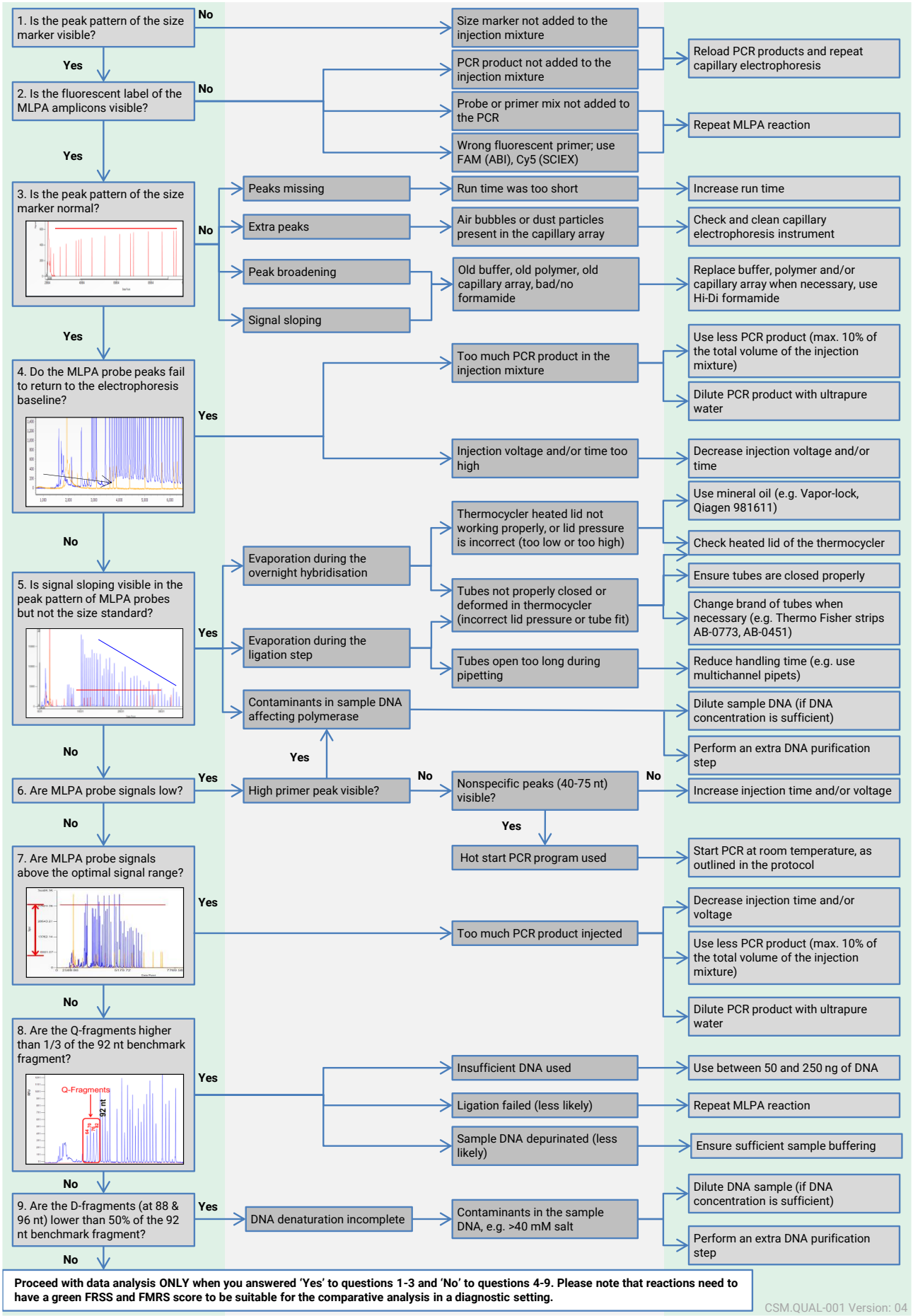
Interpretation and confirmation of results & performance characteristics

Application-dependent; see probemix product description.

Limitations

1. In most populations and for most MLPA applications, the major cause of genetic defects are small (point) mutations, most of which will not be detected by MLPA.
2. MLPA will not detect most inversions, balanced translocations, or copy number changes that lie (partially) outside the sequence detected by an MLPA probe.
3. Analytical performance can be compromised by impurities in the DNA sample, incomplete DNA denaturation (e.g. due to salt contamination), the use of insufficient or too much sample DNA, insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors.
4. Minor differences in experimental execution may affect the MLPA peak pattern. Only include samples in an analysis that were a) included in the same MLPA experiment and b) tested with the same probemix lot.
5. In certain cases, analysis of parental samples might be necessary for correct result interpretation.
6. Certain copy number aberrations can be due to somatic alterations, including large deletions and duplications of entire chromosomes.
7. Small changes (e.g. SNVs, small indels) in the sequence targeted by a probe can cause false positive results, even when >20 nt from the probe ligation site. Sequence changes can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of the probe oligonucleotide to the sample DNA. Sequence changes within a HhaI site can interfere with HhaI digestion and may result in a false positive methylation signal. Deviations detected by MLPA should be confirmed, and single-probe deviations always require confirmation. Sequencing of the target region is recommended.
8. DNA from whole genome amplification reactions is not suitable for MS-MLPA due to amplification bias and the removal of methylation signature.
9. 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. Subtle changes, such as those observed in mosaic cases, may only be distinguished when probes are arranged according to chromosomal location.
10. Not all copy number and methylation changes detected by MLPA are pathogenic. MRC Holland cannot provide information whether a specific deletion or duplication or aberrant methylation will result in disease.
11. Bisulfite-treated DNA samples are not suitable for MS-MLPA reactions.
12. Most MS-MLPA probes detect the methylation of a single HhaI site (GC^{me}GC) found within the sequence detected by the probe. 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.

Troubleshooting flowchart



More details
Online instruction video *How to perform an MLPA reaction*.

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

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.

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CE

Implemented changes in the protocol
Version-013 – 20 June 2024 - Protocol received a new structure and a new design. - Tables in SALSA MLPA assay components replaced with references to product descriptions of components. - Section on additional PCR reagent kit removed. - Section on standard packaging labels removed. - Section on MS-MLPA principle rewritten and figure of the workflow improved. Figure about calculations removed. - Section Materials required but not provided updated. - Information in section Sample treatment and storage reorganised. Section is made shorter and renamed to Sample requirements. Some information was moved to Precautions and warnings. - Section Selecting reference & other control samples removed as this information is also present in product descriptions. - Information in chapter 3 Notes to read before you start moved to Test procedure part I. - Information in chapter 5 and 6 (MS-MLPA protocol in brief and MS-MLPA protocol) combined in Test procedure part I in a new table format. - Instruction to remove tubes from thermocycler after denaturation and place them back after addition of Hybridisation master mix removed. - Instruction to remove tubes from thermocycler after hybridisation and place them back after addition of Ligase-65 and Ligase-Digestion master mix removed. - Instruction to place tubes in the thermocycler before adding Polymerase master mix removed because there is no previous instruction to remove them. - Instruction added not to spin down tubes after addition of Polymerase master mix. - Foot note added to Test procedure part I, giving instructions for the situation when tubes are taken to a separate lab for the PCR. - Information in section 7.1. Notes to read before you start moved to Precautions and warnings. - Table about signal ranges in capillary electrophoresis instruments in section 7.2. replaced with a reference to the corresponding table in the Coffalyser.Net Reference Manual. - Information in chapter 8 (quality control and troubleshooting) replaced with references to the Coffalyser.Net Reference Manual and to knowledge base articles about control fragments and no-DNA controls. - Chapter 9 (Data analysis) replaced with the instruction to download Coffalyser.Net and to read the Coffalyser.Net Reference Manual. - Information in chapter 10 (Interpretation and confirmation) either moved to Precautions and warnings, to Limitations or removed because it is specific for certain applications.