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 and it must be read in its entirety and be used in combination with the appropriate MS-MLPA probemix-specific product description.

SALSA® MS-MLPA® products are for Research Use Only (RUO).

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

Manufacturer: MRC-Holland B.V. Willem Schoutenstraat 1, 1057 DL Amsterdam, The Netherlands
Website: www.mlpa.com, www.mlpa.eu; Phone: +31 888 657 200
E-mail: info@mlpa.com (information & technical questions), order@mlpa.com (orders)
MS-MLPA General Protocol – Document History

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.

Version-005 (30 May 2016)
- Error in Section 9: 10 µl of polymerase mix changed to 5 µl.
- Adjusted address from manufacturer.

Version-004 (09 August 2013)
- Document COMPLETELY rewritten; New Quality Control Flowchart added; Information presented more concisely.

Version-003 (23 January 2012)
- Sentence removed that the new PCR primer mix has a lot no. of F44 or newer. Old PCR primer mix cannot be recognised by the lot no., but by the absence of the MRC-Holland logo on its label.

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1. EXPLANATION OF SYMBOLS USED
2. INTENDED USE

Copy Number Variations (CNVs) and DNA methylation aberrations in human DNA play a role in a wide range of disorders. Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) is used to determine the copy number and methylation status of up to 60 DNA sequences in a single multiplex PCR-based reaction. MRC-Holland manufactures and sells SALSA MLPA reagent kits and a range of SALSA MS-MLPA probemixes. Together, these can be used to detect deletions/duplications and changes in DNA methylation in a DNA sample. Details on the intended use for each probemix are specified in the SALSA MS-MLPA probemix-specific product description.

3. MS-MLPA ASSAY PRINCIPLE

The principle of MS-MLPA is based on the amplification of up to 60 probes, each of which detect a specific DNA sequence of approximately 60 nt in length (Figure 1).\(^1,2\) The MS-MLPA reaction results in a set of unique PCR amplicons approximately between 64-500 nt in length, which 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 immediately adjacent target DNA 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. One PCR primer is fluorescently labelled, enabling the amplification products to be visualised during fragment separation. This is done on a capillary electrophoresis instrument which yields a specific electropherogram.

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 (Figure 1; 3A). The other part is treated with the HhaI enzyme, which provides information on methylation status of the target DNA (Figure 1; 3B).

![Figure 1. MS-MLPA reaction.](image)

When hybridising to an unmethylated DNA target, the methylation-specific probes will be ligated and simultaneously digested by HhaI (Figure 1; 3B bottom). 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; 3B top). 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. Inclusion of reference samples in the same run is therefore essential (see 9. DATA ANALYSIS). Comparing the electrophoresis patterns of the undigested MS-MLPA reactions allows for the detection of copy number changes (Figure 2). Comparing the peak patterns of digested reactions may reveal unusual methylation of DNA target sequences (Figure 2).

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**Figure 2.** Profile comparison of MS-MLPA data.

**For copy number calculation,** the electropherogram of test sample A – undigested (copy number) is compared to the electropherogram of Reference sample 1 – undigested (only one reference sample is shown). 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 Coffalyser.Net software, which arranges probes by chromosomal location.

**For methylation status calculation,** the electropherogram of test sample A – digested (methylation status) is compared to the electropherogram of test sample A – undigested. Similarly, the electropherogram of reference sample 1 – digested (methylation status) is compared to the electropherogram of reference sample 1 – undigested. Test sample A – digested shows four target probes with a HhaI site (numbered 1-4 and circled in blue) with similar peaks heights to those found in test sample A – undigested. Reference sample 1 – digested shows the same four target probes with a reduced peak height in comparison to those 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. 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.

T: target probes, R: reference probes, DC: digestion control
4. SALSA MS-MLPA ASSAY COMPONENTS & STORAGE CONDITIONS

NOTE: MS-MLPA and standard MLPA make use of the same SALSA MLPA REAGENT KIT.

4.1. REAGENT KIT ITEM NUMBERS

<table>
<thead>
<tr>
<th>Item no.</th>
<th>Description</th>
<th>Number of reactions</th>
<th>Fluorescent label PCR primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EK1-FAM</td>
<td>SALSA MLPA EK1 reagent kit</td>
<td>100</td>
<td>FAM</td>
</tr>
<tr>
<td>EK5-FAM</td>
<td>SALSA MLPA EK5 reagent kit</td>
<td>500</td>
<td>FAM</td>
</tr>
<tr>
<td>EK20-FAM</td>
<td>SALSA MLPA EK20 reagent kit</td>
<td>2000</td>
<td>FAM</td>
</tr>
<tr>
<td>EK1-Cy5</td>
<td>SALSA MLPA EK1 reagent kit</td>
<td>100</td>
<td>Cy5</td>
</tr>
<tr>
<td>EK5-Cy5</td>
<td>SALSA MLPA EK5 reagent kit</td>
<td>500</td>
<td>Cy5</td>
</tr>
<tr>
<td>PCR001-FAM</td>
<td>SALSA MLPA PCR kit (Optional)</td>
<td>100</td>
<td>FAM</td>
</tr>
<tr>
<td>PCR003-FAM</td>
<td>SALSA MLPA PCR kit (Optional)</td>
<td>300</td>
<td>FAM</td>
</tr>
<tr>
<td>PCR001-Cy5</td>
<td>SALSA MLPA PCR kit (Optional)</td>
<td>100</td>
<td>Cy5</td>
</tr>
<tr>
<td>PCR003-Cy5</td>
<td>SALSA MLPA PCR kit (Optional)</td>
<td>300</td>
<td>Cy5</td>
</tr>
</tbody>
</table>

4.2. REAGENT KIT COMPONENTS

<table>
<thead>
<tr>
<th>Reagent kit component</th>
<th>Volumes</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>SALSA MLPA Buffer (yellow cap)</td>
<td>180 µl</td>
<td>KCl, Tris-HCl, EDTA, PEG-6000, oligonucleotides</td>
</tr>
<tr>
<td>SALSA Ligase-65 (green cap)</td>
<td>115 µl</td>
<td>Glycerol, EDTA, Beta-Mercaptoethanol, KCl, Tris-HCl, non-ionic detergent, Ligase-65 enzyme (bacterial origin)</td>
</tr>
<tr>
<td>Ligase Buffer A (transparent cap)</td>
<td>360 µl</td>
<td>Coenzyme NAD (bacterial origin)</td>
</tr>
<tr>
<td>Ligase Buffer B (white cap)</td>
<td>360 µl</td>
<td>Tris-HCl, MgCl₂, non-ionic detergent</td>
</tr>
<tr>
<td>SALSA PCR Primer Mix (brown cap)</td>
<td>240 µl</td>
<td>Synthetic oligonucleotides with fluorescent dye (FAM or Cy5), dNTPs, Tris-HCl, KCl, EDTA, non-ionic detergent</td>
</tr>
<tr>
<td>SALSA Polymerase (orange cap)</td>
<td>65 µl</td>
<td>Glycerol, non-ionic detergents, EDTA, DTT, KCl, Tris-HCl, Polymerase enzyme (bacterial origin)</td>
</tr>
</tbody>
</table>

4.3. APPLICATION-SPECIFIC MS-MLPA PROBEMIX

<table>
<thead>
<tr>
<th>Application-specific MS-MLPA probemix</th>
<th>Available Volumes</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probenmix* (black cap)</td>
<td>40 µl (25R), 80 µl (50R), 160 µl (100R)</td>
<td>Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA</td>
</tr>
<tr>
<td>Salsa HhaI (light blue cap)</td>
<td>65 µl (100R)</td>
<td>Restriction endonuclease enzyme (bacterial origin), anti-oxidant, glycerol and undisclosed ingredients</td>
</tr>
<tr>
<td>Sample DNA* (blue cap)</td>
<td>30 µl or 100 µl*</td>
<td>Tris-HCl, EDTA, synthetic/control plasmid DNA, human genomic female DNA, cell line DNA*</td>
</tr>
</tbody>
</table>

*Probemixes are designed for use only in combination with SALSA MLPA reagent kits.
*SD type (reference, binning, and artificial duplication DNA) and availability is probemix dependent.
*Volumes and ingredients are dependent on SD type.

3 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.

4.4. OPTIONAL: ADDITIONAL PCR REAGENT KIT (100 OR 300 reactions)

<table>
<thead>
<tr>
<th>PCR kit component</th>
<th>Volumes</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR001</td>
<td>PCR003</td>
</tr>
<tr>
<td>SALSA PCR Primer Mix (brown cap)</td>
<td>240 µl</td>
<td>3x240 µl Synthetic oligonucleotides with fluorescent dye (FAM, Cy5 or unlabeled), dNTPs, Tris-HCl, KCl, EDTA, BRIJ, pH 8</td>
</tr>
<tr>
<td>SALSA Polymerase (orange cap)</td>
<td>65 µl</td>
<td>3x65 µl Glycerol, non-ionic detergents, EDTA, DTT (0.1 %), KCl, Tris-HCl, Polymerase enzyme (bacterial origin), pH 7.5</td>
</tr>
</tbody>
</table>

4.5. STORAGE AND SHELF LIFE

All components must be stored directly upon arrival between -25°C and -15°C, shielded from light and in the original packaging. When stored under the recommended conditions, a shelf life of until the expiry date is guaranteed, also after opening. For the exact expiry date, which is at least one year from shipment, see the labels on each vial. Products should not be exposed to more than 25 freeze-thaw cycles.

5. ASSAY SETUP INSTRUCTIONS

5.1. MATERIALS REQUIRED BUT NOT PROVIDED

- Ultrapure water
- TE0.1 (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 equipment (see 7.2 EQUIPMENT AND REAGENTS REQUIRED BUT NOT PROVIDED)

5.2. PRECAUTIONS AND WARNINGS

- SALSA MS-MLPA probemixes are sold by MRC-Holland for Research Use Only (RUO). These probemixes are not CE/FDA or otherwise registered for use in diagnostic procedures.
- Always consult the most recent version of the relevant probemix-specific product description AND the MS-MLPA General Protocol before use.
- 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.
- The MS-MLPA technique should always be internally validated before use in your laboratory. Results of MS-MLPA are highly dependent on the HhaI enzyme used. 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.
- Internal validation of each MS-MLPA application is essential; include at least 16 normal DNA samples. Validation should show a standard deviation <0.10 for each probe (unless the relevant probemix-specific product description states otherwise). Samples used for validation should be representative of samples used in daily practice.
- Bisulfite-modified DNA samples are not suitable for MS-MLPA reactions.
- False positive or negative results can be caused by various factors, including:
  - Sequence changes. See 5.3 LIMITATIONS OF THE PROCEDURE.
  - Impurities in the test or reference DNA samples, including NaCl or KCl (>40 mM) and other salts, phenol, ethanol, heparin, EDTA (>1.5 mM) and Fe.
  - Depurination of sample DNA during the initial 98°C heat treatment. This can occur when the sample has insufficient buffering capacity (sample dissolved in H2O instead of TE). A minimum of 5 mM Tris pH 8.0 in the sample DNA solution is required.
  - Sample DNA denaturation problems causing (part of) the DNA template to be unavailable for the MS-MLPA probes. Certain DNA purification methods, e.g. Qiagen EZ1, result in a high salt concentration in DNA samples. Extremely GC-rich regions are not denatured at 98°C when more than 40 mM NaCl or KCl is present.
  - Use of incorrect DNA quantities (see 5.4 SAMPLE TREATMENT).
5.3. 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 changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations. Even when MS-MLPA did not detect any aberration, the possibility remains that biological changes in that gene or chromosomal region do exist but remain undetected.
- Sequence changes (e.g. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA. A sequence change within the HhaI restriction site of the sample DNA, detected by a methylation sensitive probe, can prevent digestion of that probe.

5.4. SAMPLE TREATMENT

- Use a total quantity of 50-250 ng (50-100 ng is optimal; unless stated otherwise in the probemix-specific product description) of human DNA in a 5 µl volume for each MS-MLPA reaction. If necessary, DNA samples can be concentrated by ethanol precipitation, and glycogen (Roche 901393) can be used as a carrier. More information at www.mlpa.com.
- MS-MLPA is more sensitive to impurities than simple monoplex PCR assays. Contaminants left after DNA extraction (listed in 5.2) may influence MS-MLPA performance.
- To minimise the effect of contaminants or other variables, 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 such as salt. Do not use Qiagen M6, M48 and M96 systems. For Qiagen EZ1, only use the QIAGEN Supplementary Protocol for use in Third Wave Invader® assays (see www.mlpa.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 is not preferred for DNA extraction. Heparin is difficult to remove from DNA preparations and can distort the MS-MLPA PCR reaction. Certain DNA purification methods (e.g. Nucleospin gDNA Clean-up XS) are capable of removing heparin contamination.
- DNA preparations should contain 5-10 mM Tris buffer with a pH of 8.0-8.5 to prevent depurination during initial heat treatment 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 unknown whether sufficient buffer is present, add Tris-HCl: 4 µl sample DNA + 1 µl 50 mM Tris-HCl pH 8.5.

4 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.
5 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.
• EDTA concentration of the samples should not be higher than 1.5 mM. Sample DNA should not be concentrated by evaporation or SpeedVac as this leads to a very high EDTA concentration.
• In case of doubts about DNA quality: a) use only 50 ng of sample DNA; b) clean contaminated samples by ethanol precipitation or clean-up kits (for instance silica based).
• In certain cases, the SALSA Sample Stabilising Solution (RUO) (S4: SMR04, SMR45) can improve the quality of the MLPA reaction. S4 contains several ingredients that reduce the effect of sample DNA impurities. See the S4 product description at www.mlpa.com for more information.
• DNA from whole genome amplification reactions is not suitable for MS-MLPA because of its amplification bias.
• Bisulfite-modified DNA samples are not suitable for MS-MLPA reactions.

5.5. SELECTING REFERENCE & OTHER CONTROL SAMPLES
• REFERENCE SAMPLES. Reference samples should be included in each MS-MLPA experiment. Minor differences in experimental execution may affect the MS-MLPA peak pattern. Only compare samples that are a) included in the same MS-MLPA experiment and b) tested with the same probemix lot.
• MULTIPLE REFERENCE SAMPLES are needed to estimate the reproducibility of each probe within each MS-MLPA run. Use at least 3 different reference samples per MS-MLPA run. When testing >21 samples, add 1 additional reference sample for every 7 additional test samples. Reference samples should be distributed randomly over the experiment to minimise variation.
• SELECTING REFERENCE SAMPLES. Reference samples are DNA samples obtained from healthy individuals in which target and reference probes bind to sequences that are expected to have a normal copy number. They should be as similar as possible to the test samples in all other aspects (see section 5.4). For formalin-fixed paraffin-embedded (FFPE) tissue, use reference samples derived from similarly treated healthy tissue. Not all probemixes are suitable for use with DNA extracted from FFPE material. Please check the probemix-specific product description for suitable tissue types. 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.
• COMMERCIAL DNA. In case of doubts about sample quality, include one or more commercial DNA samples for comparison. We recommend Promega Cat. Nr G1471 male & G1521 female DNA. The commercial DNA should only be used as a control to check sample quality and should not 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 TE0.1 (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. When using cell line DNA, note that cell lines may have acquired additional copy number changes, including gains or losses of complete chromosomes. MRC-Holland does not provide positive samples. A list of commercially available positive samples is available on www.mlpa.com.
• ALIQUOT PRECIOUS REFERENCE/CONTROL SAMPLES and store these samples at -20°C. Contamination with microorganisms can deteriorate samples that are stored at 4°C for an extended period.

6. MS-MLPA REACTION - DNA DETECTION/QUANTIFICATION AND METHYLATION PROFILING

6.1. NOTES TO READ BEFORE YOU START (DAY 1)
• Use a calibrated thermocycler with heated lid (99-105°C).
• After use, store all reagents between -25°C and -15°C.
• Always vortex thawed buffers and probemix before use. MLPA buffer is typically frozen at -20°C but may remain liquid due to its high salt concentration.
• Centrifuge all MLPA reagent tubes for a few seconds before use, as drops may have adhered to the lid.
• Enzyme solutions contain 50% glycerol and remain liquid at the recommended storage temperature. Master mixes containing enzymes should be mixed by gently pipetting up and down. When the viscous enzyme solution is not mixed properly with the buffers, unreliable results will be obtained! However, if the enzyme solution is mixed too vigorously, enzyme inactivation occurs. When preparing master mixes, always add enzymes last, and never vortex solutions containing enzymes.
• To minimise sample variation, prepare sufficiently large volumes of master mix solutions. Include a 5-10% volume surplus to allow for pipetting errors.
• It is recommended to prepare master mixes (Ligase-65 and Polymerase) just before use at room temperature (RT). When prepared more than 1 hr before use, master mixes can be stored on ice or at 4°C. The master mixes
should then be warmed to RT before being added to the MS-MLPA reactions. A large decrease in temperature by addition of very cold ligase master mix can result in increased non-specific peaks in the No DNA reaction.

- When running a large number of samples, use multi-channel pipettes to avoid excessive evaporation.

### 6.2. THERMOCYCLER PROGRAM FOR THE MS-MLPA REACTION

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DNA denaturation</td>
<td>98°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>2.</td>
<td>25°C</td>
<td>pause</td>
</tr>
<tr>
<td>2. Hybridisation reaction</td>
<td>95°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>4.</td>
<td>60°C</td>
<td>pause</td>
</tr>
<tr>
<td>5.</td>
<td>20°C</td>
<td>pause</td>
</tr>
<tr>
<td>3. Ligation &amp; Ligation-Digestion reactions</td>
<td>48°C</td>
<td>pause</td>
</tr>
<tr>
<td>7.</td>
<td>48°C</td>
<td>30 minutes</td>
</tr>
<tr>
<td>8.</td>
<td>98°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>9.</td>
<td>20°C</td>
<td>pause</td>
</tr>
<tr>
<td>4. PCR reaction</td>
<td>35 cycles:</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>11.</td>
<td>60°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>12.</td>
<td>72°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>13.</td>
<td>20°C</td>
<td>pause</td>
</tr>
<tr>
<td>14.</td>
<td>15°C</td>
<td>pause</td>
</tr>
</tbody>
</table>

### 6.3. 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) to each tube. Use TE for a No DNA control.
- Place tubes in thermocycler; start MS-MLPA thermocycler program (see section 6.2). Denature sample DNA for 5 min at 98°C; cool samples to 25°C before removing tubes from thermocycler.

### 6.4. HYBRIDISATION REACTION (DAY 1)

- Vortex MLPA buffer and MS-MLPA probemix vials before use.
- Prepare Hybridisation master mix. For each reaction, mix: 1.5 µl MLPA buffer (yellow cap) + 1.5 µl probemix (black cap). Mix the Hybridisation master mix well by pipetting or vortexing.
- After DNA denaturation, add 3 µl Hybridisation master mix to each sample tube. Mix well by pipetting up and down.
- Continue thermocycler program: incubate for 1 min at 95°C, then 16–20 hours at 60°C.

### 6.5. LIGATION & LIGATION-DIGESTION REACTIONS (DAY 2)

- Vortex the two Ligase Buffer vials before use.
- Warm the Ligase-65 enzyme vial for 10 sec in your hand to reduce viscosity.
- Prepare a Ligase-65 master mix. For each reaction, mix: 8.25 µl Ultrapure Water + 1.5 µl Ligase Buffer B (white cap) + 0.25 µl Ligase-65 enzyme (green cap). Mix well by pipetting gently up and down. Never vortex enzyme solutions.
- 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) and 0.5 µl Salsa HhaI enzyme (light blue cap). Mix well by pipetting gently up and down.
- Continue the thermocycler program: pause at 20°C. Remove the tubes from the thermocycler.
- Add 3 µl Ligase Buffer A and 10 µl Ultrapure water to each tube. Mix by gently pipetting 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 the thermocycler program: pause at 48°C.
• When the thermocycler is at 48°C and while the samples are IN the thermocycler, add 10 µl of the Ligase-65 master mix to the first MLPA reaction (copy number test). Mix by gently pipetting up and down.
• Add 10 µl of the Ligase-Digestion master mix to the second MLPA reaction (methylation test). Mix by gently pipetting up and down.
• Continue the thermocycler program: 30 minutes incubation at 48°C (for ligation and HhaI digestion); 5 minutes at 98°C (for heat inactivation of the enzymes). **Pause at 20°C.** At this point, the tubes can be removed from the thermocycler.

6.6. PCR REACTION (DAY 2)

Note: as compared to the PCR reaction in the standard MLPA 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.

• Vortex SALSA PCR Primer mix vial. Warm Polymerase vial for 10 sec in your hand to reduce viscosity.
• Prepare Polymerase master mix. For each reaction, mix: 3.75 µl Ultrapure water + 1 µl SALSA PCR Primer mix (brown cap) + 0.25 µl SALSA Polymerase (orange cap). Mix well by pipetting up and down; do not vortex.
• **At room temperature,** add 5 µl Polymerase master mix to each MLPA reaction. Mix by pipetting gently up and down. **Directly** place the tubes in the thermocycler and continue the thermocycler program; 35 cycles of 30 seconds 95°C; 30 seconds 60°C; 60 seconds 72°C. End with 20 min incubation at 72°C; pause at 15°C.
• After the PCR reaction, do not open tubes in the room with the thermocycler. To avoid contamination, use different micropipettes for performing MS-MLPA reactions and handling MS-MLPA PCR products.
• PCR product can be stored at 4°C for 1 week. For longer periods, store between -25°C /-15°C. As fluorescent dyes are light-sensitive, store PCR products in a dark box or wrapped in aluminium foil.

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 capillary electrophoresis instrument type. Use the default settings on your capillary electrophoresis instrument that are applicable for the application, polymer and capillary length. Instrument settings may require optimisation for proper fragment separation.
• Using old capillaries or polymer has a detrimental effect on MLPA results. Replace capillaries and polymer regularly; follow instructions of the manufacturer of the capillary electrophoresis instrument. Polymer quickly deteriorates after prolonged exposure to >25°C. In case size standard peaks are repeatedly low and broad, the capillaries or polymer might be deteriorated.
• Formamide can become acidic. This can result in depurination and fragmentation of DNA upon heating. Use high quality formamide and store it in aliquots at -20°C.
• In case all MS-MLPA peaks are low, do not add more MS-MLPA PCR product to the injection mixture! Adding more PCR product increases the salt concentration in the injection mixture which competes with DNA for injection. When an increase in peak heights is desired, increase the injection time or voltage instead. None of the peaks should be off-scale (see section 7.3)!
• Create a new manual bin set in Coffalyser.Net whenever using a different a) MS-MLPA probemix lot, b) size standard, c) capillary electrophoresis instrument, d) run setting e) polymer type, or f) capillary array length.

7.2. EQUIPMENT AND REAGENTS REQUIRED BUT NOT PROVIDED

• Capillary electrophoresis instrument with fragment analysis software. Capillary electrophoresis instruments that do not use denaturing conditions, like QIAxcel, cannot be used in combination with SALSA MS-MLPA.
  o Beckman: GeXP Software Package
  o Applied Biosystems: Standard Foundation Data Collection Software
• High quality formamide (e.g. Hi-Di Formamide, Applied Biosystems)
• Labeled size standard
  o Beckman: CEQ™™ DNA Size Standard Kit - 600
  o Applied Biosystems: GeneScan™ 500 LIZ®/ROX™ (preferred), GeneScan 600 LIZ®, GeneScan 500 TAMRA™
• Polymers
  o Beckman: GenomeLab™ Linear Polyacrylamide (LPA) denaturing gel
7.3. ELECTROPHORESIS SPECIFICATIONS

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Primer Dye</th>
<th>Capillaries</th>
<th>Injection mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beckman/SCIEX CEQ-2000</td>
<td>Cy5</td>
<td>33 cm</td>
<td>1 µl PCR reaction&lt;sup&gt;a&lt;/sup&gt; 0.5 µl CEQ - size standard 600&lt;sup&gt;b&lt;/sup&gt; 28.5 µl HiDi formamide / Beckman SLS Add one drop of high quality mineral oil.</td>
</tr>
<tr>
<td>Beckman/SCIEX CEQ-8000/GeXP single rail</td>
<td>Cy5</td>
<td>33 cm</td>
<td></td>
</tr>
<tr>
<td>Beckman/SCIEX CEQ-8800/GeXP dual rail GeXP</td>
<td>Cy5</td>
<td>33 cm</td>
<td></td>
</tr>
<tr>
<td>ABI-Prism 3100 (Avant) ABI-3130 (xL) ABI-3500&lt;sup&gt;c&lt;/sup&gt; (xL) ABI-3730 (xL)</td>
<td>FAM</td>
<td>36, 50 cm</td>
<td>0.7 µl PCR reaction&lt;sup&gt;a&lt;/sup&gt; 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&lt;sup&gt;d&lt;/sup&gt;.</td>
</tr>
<tr>
<td>ABI-SeqStudio</td>
<td>FAM</td>
<td>28 cm</td>
<td>0.8 µl PCR reaction&lt;sup&gt;a&lt;/sup&gt; 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&lt;sup&gt;d&lt;/sup&gt;.</td>
</tr>
</tbody>
</table>

<sup>a</sup> Never use more than 10% of the PCR product in the injection mixture.
<sup>b</sup> Reduce volume of size standard if needed.
<sup>c</sup> For ABI-3500: from the default settings reduce run voltage to 15 kV and increase run time by 25%.
<sup>d</sup> Briefly heating the injection mixture before capillary electrophoresis is recommended.

The table below presents the optimal, minimum, and maximum signal ranges for the capillary electrophoresis instrument. If the signal falls outside of these values, optimisation of the fragment analysis settings needs to be done. Additionally, if the signal falls outside the optimal range, false results can be obtained.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Optimal signal range (in RFU)</th>
<th>Minimum signal (in RFU)</th>
<th>Maximum signal (in RFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beckman Coulter CEQ/GeXP</td>
<td>9,375 - 136,000</td>
<td>5000</td>
<td>170,000</td>
</tr>
<tr>
<td>ABI 310, 3100 &amp; 3130 series</td>
<td>375 - 6,000</td>
<td>200</td>
<td>7,500</td>
</tr>
<tr>
<td>ABI 3500, 3730 series &amp; SeqStudio</td>
<td>375 - 24,800</td>
<td>300</td>
<td>31,000</td>
</tr>
</tbody>
</table>

8. MS-MLPA QUALITY CONTROL AND TROUBLESHOOTING

For analysis of MS-MLPA data, use Coffalyser.Net software, which can be freely downloaded from www.mlpa.com. The Coffalyser.Net software provides quality scores for each reaction and helps with interpretation of the control fragments that are included in each SALSA MS-MLPA probemix.

8.1. MS-MLPA QUALITY CONTROL FRAGMENTS

**MS-MLPA internal quality control fragments must be checked to ensure minimal quality requirements are met!** MS-MLPA probemixes contain quality control fragments that signal problems that may affect MS-MLPA results. Evaluate the quality of the MS-MLPA reaction, including quality control fragments using the flowchart below (see section 8.4). Only data that meets the quality requirements is suitable for MS-MLPA result calculation. If needed, repeat capillary electrophoresis or MS-MLPA reaction. MLPA Quality Control fragments and MLPA Troubleshooting Wizard E-learning modules are available online at www.mlpa.com.

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Length (nt)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benchmark fragment</td>
<td>92</td>
<td>Benchmark to compare other quality control fragments to.</td>
</tr>
<tr>
<td>Q-fragments</td>
<td>64, 70, 76, 82</td>
<td><strong>High</strong> when DNA amount is too low or ligation failed. Median of four Q-fragment signals ≥33% of the 92 nt benchmark fragment → DNA quantity insufficient or ligation failed. For instance, Figures 4A &amp; 4B.</td>
</tr>
<tr>
<td>D-fragments</td>
<td>88, 96</td>
<td><strong>Low</strong> in case of poor sample DNA denaturation. Signal ≤50% of the 92 nt benchmark fragment → DNA denaturation problems. For instance, Figures 5B &amp; 5C.</td>
</tr>
</tbody>
</table>
**X & Y fragments**

| 100, 105 | Control for sample mix up³. |

**Q-FRAGMENTS**
The four Quantity Fragments (Q-fragments, at 64-70-76-82 nt) measure whether sufficient DNA was added and whether ligation was successful. The Q-fragments do not need to hybridise to the DNA or be ligated to be amplified during PCR. The more sample DNA is added, the lower they get (see Figure 2).

- **ALL FOUR Q-FRAGMENTS HIGH: INSUFFICIENT SAMPLE DNA USED OR LIGATION REACTION FAILED!**

![Figure 2](image)

**Figure 2.** Effect of DNA quantity on Q-fragments. The more sample DNA is used, the lower the Q-fragments. MLPA results with A. 5 ng DNA, B. 10 ng, C. 50 ng.

**D-FRAGMENTS**
The two DNA Denaturation Fragments (D-fragments, at 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 fragments are low (<50% of the 92 nt benchmark fragment) this indicates denaturation problems of the sample DNA. A poor denaturation can be due to presence of >40 mM salt in the DNA sample. Incomplete sample DNA denaturation can result in false results!

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

- **D-FRAGMENTS LOW: SAMPLE DNA DENATURATION INCOMPLETE!**

![Figure 3](image)

**Figure 3.** Effect of poor denaturation on D-fragments. D-fragments are low when sample DNA denaturation is incomplete (here induced by adding salt). MLPA results on DNA sample containing in A. TE, B. TE + 40 mM NaCl, C. TE + 100 mM NaCl. As MS-MLPA probes more frequently target GC-rich sequences, denaturation problems may be more prevalent in MS-MLPA.

**8.2. NO DNA CONTROL**

In a typical No DNA control, only the four Q-fragments are visible. However, MS-MLPA PCR reactions are more prone to non-specific peaks in the No DNA than a normal PCR reaction. In some probemixes, a few peaks may be visible in the No DNA controls. These non-specific peaks should not influence MS-MLPA results: when sufficient sample DNA is used, they are outcompeted by the MS-MLPA probes, similarly to the Q fragments in Figure 4. 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.

³ Rare cases are known of males lacking this Y-specific sequence and females carrying this Y-sequence on an X-chromosome.
8.3. EVAPORATION PROBLEMS
Evaporation can occur during (A) pipetting the ligation reaction at 48°C or (B) overnight hybridisation. In case you suspect evaporation problems (see section 8.4), the following may help: (A) Reduce handling time by using multi-channel pipettes; (B) Test evaporation by incubating 8 µl H₂O overnight at 60°C; >5 µl H₂O should remain. To reduce evaporation: 1. Ensure heated lid works well; 2. Increase/decrease pressure of lid on tubes; 3. Try different tubes (e.g. Thermo Fischer ABgene AB-0773, AB-0451); 4. Use mineral oil (Vapor-lock, Qiagen 981611): add small drop of oil to DNA sample, just enough to cover it. There is no need to remove the oil. After adding MLPA buffer-probemix mixture and polymerase mix: centrifuge very briefly. After addition of ligase mix: gently pipet up and down below the oil layer.
8.4. QUALITY CONTROL FLOWCHART

1. Is the peak pattern of the size marker visible?
   Yes
   No
   Size marker not added to the injection mixture
   PCR product not added to the injection mixture
   Primer mix not added to the PCR
   Reload and rerun sample

2. Is the fluorescent label of the MLPA probe visible?
   Yes
   No
   Primer mix not added to the PCR
   Repeat MLPA reaction

3. Is the peak pattern of the size marker normal?
   Yes
   No
   Peaks missing
   Run time was too short
   Increase run time
   Check and clean capillary electrophoresis instrument

   Extra peaks
   Air bubbles or dust particles present in the capillary array

   Peak broadening
   Old buffer, old polymer, old array
   Replace buffer, polymer, and/or array when necessary

   Signal sloping
   Too much PCR product in the injection mixture
   Use less PCR product (max. 10% of the total volume of the injection mixture)
   Dilute PCR product with ultrapure water

4. Do the MLPA probe peaks return to the electrophoresis baseline?
   Yes
   No
   Injection voltage and/or time too high
   Decrease injection voltage and/or time

   No
   Evaporation during the overnight hybridisation
   Heated lid on the thermal cycler not working properly
   Check heated lid of the thermal cycler
   Use mineral oil

   Tubes not properly closed or deformed in thermocycler
   Ensure tubes are closed properly
   Change brand of tubes when necessary

   Evaporation during the ligation step
   Tubes open too long during pipetting
   Reduce handling time (e.g., use multichannel pipettes)
   Dilute sample DNA
   Perform an extra DNA purification step

5. Is signal sloping visible in the peak pattern of MLPA probes (only)?
   Yes
   No
   Evaporation during the overnight hybridisation
   Heated lid on the thermal cycler not working properly
   Check heated lid of the thermal cycler
   Use mineral oil

   Tubes not properly closed or deformed in thermocycler
   Ensure tubes are closed properly
   Change brand of tubes when necessary

   Evaporation during the ligation step
   Tubes open too long during pipetting
   Reduce handling time (e.g., use multichannel pipettes)
   Dilute sample DNA
   Perform an extra DNA purification step

6. Are MLPA probe signals low?
   Yes
   No
   Primer peak visible?
   No
   Primer dimer visible?
   No
   Increase injection time and/or voltage
   Hot start PCR program used
   Start PCR at room temperature
   Decrease injection time and/or voltage
   Too much PCR product injected
   Use less PCR product (max. 10% of the total volume of the injection mixture)
   Dilute PCR product with ultrapure water

   Insufficient DNA used
   Use between 50 and 250 ng of DNA
   Ligation reaction failed
   Repeat MLPA reaction
   Dilute DNA sample
   Perform an extra DNA purification step

7. Are MLPA probe signals above the optimal detection limit?
   Yes
   No
   Hot start PCR program used
   Start PCR at room temperature
   Decrease injection time and/or voltage
   Too much PCR product injected
   Use less PCR product (max. 10% of the total volume of the injection mixture)
   Dilute PCR product with ultrapure water

   Insufficient DNA used
   Use between 50 and 250 ng of DNA
   Ligation reaction failed
   Repeat MLPA reaction
   Dilute DNA sample
   Perform an extra DNA purification step

8. Are the Q*-fragments higher than 1/3 of the 92 nt benchmark fragment?
   Yes
   No
   DNA Denaturation incomplete
   Contaminants in the sample DNA
   Perform an extra DNA purification step

9. Are the D*-fragments (at 88 & 96 nt) lower than 50% of the 92 nt benchmark fragment?
    Proceed with data analysis ONLY when you answered ‘Yes’ to questions 1-4 and ‘No’ to questions 5-9.
9. DATA ANALYSIS

9.1. COFFALYSER.NET FOR MS-MLPA DATA ANALYSIS

Coffalyser.Net should be used for MS-MLPA data analysis. The Coffalyser.Net Reference Manual provides a step-by-step instruction on MS-MLPA data analysis. Both software and manual are freely downloadable on www.mlpacom.com. This section describes the basic principles of MS-MLPA analysis. Coffalyser.Net analysis is based on these principles but uses a more robust algorithm. In addition, Coffalyser.Net selects the best analysis method for each MS-MLPA probemix and offers extensive quality control. The use of other software may lead to incorrect results!

The analysis of MS-MLPA probemixes consists of two parts:

PART 1: DETERMINING COPY NUMBERS: This is identical to standard MLPA.
PART 2: DETERMINING METHYLATION PROFILE. This is unique for MS-MLPA probemixes.

9.2. PART 1: DETERMINING COPY NUMBERS

The absolute fluorescence measured by capillary electrophoresis cannot be used directly for copy number calculations as it is affected by many variables. First, each probe's measured fluorescence must be normalised within each sample to get meaningful data. Second, various samples need to be compared to establish which sample has aberrant copy number changes. Therefore, MS-MLPA normalisation consists of 2 steps: intrasample normalisation (comparison of probe peaks WITHIN the sample) and intersample normalisation (comparison of relative probe peaks BETWEEN samples.)

1. Intrasample normalisation. Within each sample (undigested), compare each probe peak TO the peaks of the reference probes. Reference probes detect sequences that are expected to have a normal copy number in all samples, and they do not contain a HhaI restriction site. Almost all MS-MLPA probemixes contain 8 or more reference probes located on various chromosomes.

The relative probe peaks determined in step 1 are then used in:

2. Intersample normalisation. Final probe ratios are determined by comparing the relative probe peak in the DNA sample of interest TO all reference samples. Reference DNA samples are expected to have a normal copy number for both the reference and target probes.

The MS-MLPA peak pattern of a DNA sample without genomic abnormalities will be identical to that of reference samples: final probe ratios determined in step 2 will be ~1.0. For heterozygous deletions, probe ratios will be ~0.5. This final probe ratio is also called Dosage Quotient (DQ). Coffalyser.Net calculates the DQ for each probe in each sample. Probes should be arranged based on chromosomal location for correct interpretation; this will also aid in detecting subtle changes such as mosaicism.

How to discriminate normal, deletion and duplication results? Table 1 gives the relationship between copy number status and the typical distribution of DQs.

<table>
<thead>
<tr>
<th>Dosage Quotient Distribution</th>
<th>Copy Number Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQ = 0</td>
<td>0 copies (homozygous deletion)</td>
</tr>
<tr>
<td>0.40 &lt; DQ &lt; 0.65</td>
<td>2≠1 copy (heterozygous deletion)</td>
</tr>
<tr>
<td>0.80 &lt; DQ &lt; 1.20</td>
<td>NORMAL (identical to reference samples)</td>
</tr>
<tr>
<td>1.30 &lt; DQ &lt; 1.65</td>
<td>2≠3 copies (heterozygous duplication)</td>
</tr>
<tr>
<td>1.75 &lt; DQ &lt; 2.15</td>
<td>2≠4 copies (or 1≠2 copies)</td>
</tr>
<tr>
<td>All other values</td>
<td>Ambiguous result</td>
</tr>
</tbody>
</table>

In addition, the standard deviation of each probe should be measured to ensure the result is reliable. Coffalyser.Net software considers a result as statistically significant change if:

7 Coffalyser.Net starts with raw data analysis (baseline correction, peak identification) and provides extensive quality control (e.g. DNA quantity used, complete DNA denaturation, degree of sloping). Slope correction of the peak pattern is one of the many options.

8 The standard deviation differs per probe and depends on many factors, including 1. quality of DNA samples (purity, sample DNA fragmentation, depurination, other modifications); 2. quantity of sample DNA used; 3. number and quality of reference samples; 4.
1. DQ probe 1, sample A > 2 standard deviations higher/lower than average DQ probe 1, ref sample X, Y, Z

Furthermore, to be indicative of a heterozygous deletion or duplication, the following applies:

2. DQ probe 1, sample A below 0.7 or above 1.3 indicates a heterozygous deletion or duplication, respectively\(^9\).

For mosaic and tumour samples, the 0.7/1.3 cut-off lines of Coffalyser.Net are not applicable as the DQs can take on any value: their calculated DQs will depend on a) the magnitude of the copy number change AND b) the percentage of the different cell types in the DNA sample\(^10\). As Coffalyser.Net determines the significance of a probe signal change by evaluating the magnitude of the calculated DQ *in combination with* its statistical significance in the experiment, such a partial copy number change can still be recognised if a) the experiment was performed well, b) a similar DQ was obtained for adjacent probes, and c) there is a sufficient percentage of affected cells.

9.3. PART 2: DETERMINING METHYLATION PROFILE

This part is unique for MS-MLPA probemixes and serves to quantify the percentage of methylation for a distinct probe set within a given sample. Similar to copy number calculation, within each sample, an intrasample normalisation must first take place. The intrasample normalisation is done on both undigested and digested samples. The ratio obtained for a single probe is then compared between the digested and undigested samples. This ratio can be multiplied by 100 to give a methylation percentage. The methylation profile of a test sample is assessed by comparing the methylation percentages obtained on the test sample to the percentages of the reference samples. Reference DNA samples are derived from healthy individuals, expected to have a normal copy number and methylation status for the regions of interest. This way, differences in methylation status between the sample of interest and the reference samples can be identified.
10. INTERPRETATION OF RESULTS

The information provided in the MS-MLPA probemix-specific product description is essential for a correct interpretation of MS-MLPA results.

To judge whether the results obtained are reliable and to interpret MS-MLPA results correctly, a good understanding of the MS-MLPA technique and the application tested for is essential. Keep the following in mind:

- For most applications, the major cause of genetic defects will be small (point) mutations, most of which will not be detected by MS-MLPA.
- 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. Even when MS-MLPA did not detect any aberration, the possibility remains that biological changes in that gene or chromosomal region do exist but remain undetected.
- Sequence changes (e.g. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (even when >20 nt from the probe ligation site) can reduce the probe signal by preventing ligation of the probe oligonucleotides or by destabilising the binding of probe oligonucleotides to the sample DNA.
- Copy number 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 does contain two different alleles. In contrast, note that the finding of only a single rare allele by sequencing does not yet imply that the other, “normal”, allele is deleted: the lower probe signal may be caused by a homozygous SNP, even when the SNP is very rare!
- Long-range PCR and qPCR are often used to confirm (single) exon deletions. It is possible to design your own synthetic MLPA probes for confirmation of results (e.g. Hills A. et al., (2010) Mol Cytogenet. 3:19). Information on MLPA probe design is available on www.mlpa.com (RUO).
- Contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal. Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Not all deletions and duplications detected by MS-MLPA are pathogenic. MRC-Holland cannot provide information whether a deletion or duplication of a specific exon will result in disease. For many genes, exons are described that are only present in certain transcript variants. For some genes, such as DMD, in-frame deletions resulting in mild, or no disease, have been described. A duplication of one or more exons may disrupt that copy of the gene resulting in disease, whereas a complete gene duplication may not be pathogenic. Note that duplications that include the first or the last exon of a gene, may leave one functionally intact copy and may not be pathogenic.
- Germline copy number variations reported in healthy individuals can be found at http://dgv.tcg.ca/dgv/app/home.Certain copy number aberrations can be due to somatic alterations. For instance, a somatic trisomy 12 is an early sign of Chronic Lymphocytic Leukemia (CLL).
- In case of poor sample DNA denaturation, even the apparent deletion of several probes recognising adjacent genomic targets can be a false positive result! The presence of salt in DNA samples (e.g. >40 mM NaCl) prevents DNA denaturation of GC-rich regions. Sequences in the vicinity of such GC-rich regions may denature at 98°C but will possibly reanneal immediately upon cooling as the non-denatured GC-rich sequence holds the two strands together. Binding of probes to their target sequence in these regions will be hindered, resulting in reduced signals for probes located within several kb from such GC-rich regions. This is why the D-fragments should always be examined with great care!
- 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.

11 When designing probes, known SNPs are avoided when possible. However, new SNPs are continuously being discovered. Please notify us when a polymorphism or a frequent pathogenic mutation influences a probe signal.
• In case of an apparent homozygous deletion, the electropherogram should be visually inspected to identify whether the signal is below the detection limit.

• 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\textsuperscript{m}CGC). 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.

• A point mutation 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.

• 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.

• 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.

• 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 quotient is therefore 0.5. When testing samples for imprinted diseases, the threshold value for abnormal methylation can be determined by testing sufficient DNA samples from healthy individuals.

• 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 the CpG island. 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.

• Copy number changes detected by reference probes are unlikely to be related to the condition tested for. The identity of reference probes is available on request.

• In certain cases, analysis of parental samples might be necessary for correct interpretation of results.

MS-MLPA results are more reliable when:

• **Reference samples (undigested):** The standard deviation of all probes is low (<10%) and DQ between 0.8-1.2.

• **Test samples (undigested):** The standard deviation of the reference probes is low (<10%) and DQ between 0.8-1.2.

• **All samples (digested):** The probe signal of the “Digestion Control Probes” should be 4% or less of the corresponding probe signals in the undigested reactions.

• Probes show a decreased or increased signal for adjacent exons (multi-exon deletion or duplication).

• The same result is obtained in a new MS-MLPA run using less DNA (if possible) or using different reference samples. When less DNA is used, possible impurities which may influence the probe signal are diluted.

An MS-MLPA result is unlikely to be reliable when:

• Probes for non-neighbouring exons show a decreased or increased signal, e.g. deletion of exon 3 and 17.

• In the same sample, one or more reference probes show an abnormal copy number.

• Copy number changes are detected with an unusually high frequency in a patient cohort for a certain disease.
11. MS-MLPA PROTOCOL IN A NUTSHELL

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 3 µl Ligase buffer A and 10 µl Ultrapure water to each tube and mix
   • Transfer 10 µl to a second tube
   • Place samples in thermocycler, heat to 48°C
   • 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 to 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

5. CAPILLARY ELECTROPHORESIS OF PCR PRODUCTS

6. ANALYSE RESULTS
   • Determine RELATIVE probe peaks within each sample
   • Compare these results to the results of the reference samples

* Master mixes:
  Hybridisation: 1.5 µl SALSA probemix +1.5 µl MLPA buffer
  Ligase-65: 1.5 µl Ligase buffer B + 8.25 µl Ultrapure water + 0.25 µl Ligase-65
  Ligase-Digestion: 1.5 µl Ligase buffer B + 7.75 µl Ultrapure water + 0.25 µl Ligase-65 + 0.5 µl HhaI
  Polymerase: 3.75 µl Ultrapure water + 1 µl PCR Primer mix + 0.25 µl SALSA Polymerase

PREVENT FALSE POSITIVE OR NEGATIVE RESULTS:

READ THE COMPLETE PROTOCOL AND STRICTLY FOLLOW THIS PROTOCOL and

READ THE MOST RECENT VERSION OF THE PROBEMIX-SPECIFIC PRODUCT DESCRIPTION

THE 7 MOST CRITICAL POINTS FOR OBTAINING GOOD MS-MLPA RESULTS

1. Include at least 3 different reference samples in each MS-MLPA experiment.
2. Use reference samples that were extracted by the same method as the test samples.
4. Check the D-control fragments at 88 and 96 nt for complete DNA denaturation of the samples.
5. Sample DNA elution and dilution solutions should contain 5-10 mM Tris-HCl pH 8-8.5. DNA samples in water will be damaged by depurination during the 5 minute DNA denaturation heating step.
6. Accurate pipetting of the 3 µl Hybridisation master mix is absolutely essential to obtain reliable results.
7. Do not use old capillaries or gel for the electrophoresis.