Multiplex Ligation-dependent Probe Amplification (MLPA®)

General Protocol

Instructions for use

General MLPA protocol for the detection and quantification of nucleic acid sequences.
To be used only with the appropriate MLPA product description.

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www.mlpa.com
1. INTRODUCTION

Multiplex Ligation-dependent Probe Amplification (MLPA®) is used to determine the copy number of up to 50 DNA sequences in a single multiplex PCR-based reaction. Each MLPA reaction results in a combination of unique PCR amplification fragments ranging between 64 and 500 nt in length, which can be identified and quantified by capillary electrophoresis. In contrast to a standard multiplex PCR, MLPA makes use of only a single PCR primer pair for all its probes, making the method very robust.

Fundamental for the MLPA technique is that it is not sample DNA that is amplified during the PCR, but MLPA probes that have hybridised to the sample DNA. Each MLPA probe consists of two oligonucleotides that have to bind to adjacent DNA target sequences in order to be ligated. Upon ligation, the probe has become a single molecule that will be amplified exponentially during the PCR reaction. Each distinct probe in an MLPA probemix generates a PCR amplicon with a unique length. The up to 60 different PCR amplicons (MLPA probes + quality control fragments), are separated and quantified by capillary electrophoresis. Differences between different DNA samples can be detected by comparing the resulting MLPA peak patterns.

It is important to realise that MLPA is a relative technique, and that only RELATIVE differences can be detected. Within certain limits, the absolute amount of sample DNA used is not important. Similar results are obtained when testing 50 or 250 ng of sample. No conclusions with regard to copy number can be drawn from a single MLPA reaction. Comparison with reference DNA samples analysed in the same run is essential.

For data analysis, a minimum of two calculation steps are used: First, within a sample, one compares the peaks of probes detecting the genes of interest to peaks generated by reference probes (intra-sample normalisation). Reference probes detect sequences which are expected to have a normal copy number in all samples tested. Secondly, the peak pattern of the sample of interest is compared to that obtained on reference DNA samples that were included in the same experiment (inter-sample normalisation). Reference DNA samples are derived from healthy individuals which are expected to have a normal copy number for the reference probes AND the gene(s) of interest. In this way, it is possible to detect abnormal probe signals that indicate deletions or duplications of sequences detected by the MLPA probes.

Please note that results of MLPA® experiments depend on several factors, including experimental setup (selection of test and reference samples), sample purity, instrument settings and the data normalisation method used. For reliable MLPA results, the standard variation for each MLPA probe should be below 10% when tested on normal DNA samples. For this reason, it is essential to follow the procedures described in this protocol accurately. Separate protocols exist for the detection of DNA methylation status (MS-MLPA®) and measuring RNA expression (RT-MLPA®). More information is available on www.mlpa.com.
2. **SALSA® MLPA® KIT CONTENTS & STORAGE CONDITIONS**

2.1. **MATERIAL PROVIDED PER SALSA MLPA KIT (100 REACTIONS)**

<table>
<thead>
<tr>
<th>SALSA MLPA kit component</th>
<th>Volume</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>SALSA MLPA Buffer (yellow cap)</td>
<td>180 µl</td>
<td>KCl, Tris-HCl, EDTA and PEG-6000. pH 8.5</td>
</tr>
<tr>
<td>SALSA Ligase-65 (green cap)</td>
<td>115 µl</td>
<td>Glycerol, BRIJ (0.05 %), EDTA, Beta-Mercaptoethanol (0.1 %), KCl, Tris-HCl. pH 7.5, Ligase-65 enzyme (bacterial origin)</td>
</tr>
<tr>
<td>Ligase Buffer A (transparent cap)</td>
<td>360 µl</td>
<td>NAD (bacterial origin). pH 3.5</td>
</tr>
<tr>
<td>Ligase Buffer B (white cap)</td>
<td>360 µl</td>
<td>Tris-HCl, non-ionic detergents, MgCl₂. pH 8.5</td>
</tr>
<tr>
<td>SALSA PCR Primer Mix (brown cap)</td>
<td>240 µl</td>
<td>Synthetic oligonucleotides, one of which is fluorescently labeled (FAM, Cy5.0 or other dye, dependent on the capillary electrophoresis instrument used), dNTPs, Tris-HCl, KCl, EDTA, BRIJ (0.04 %). pH 8</td>
</tr>
<tr>
<td>SALSA Polymerase (orange cap)</td>
<td>65 µl</td>
<td>Glycerol, BRIJ (0.5 %), EDTA, DTT (0.1 %), KCl, Tris-HCl, Polymerase enzyme (bacterial origin). pH 7.5</td>
</tr>
<tr>
<td>Probemix (black cap)</td>
<td>160 µl</td>
<td>Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA. pH 8.0</td>
</tr>
<tr>
<td><em>SALSA PCR buffer (red cap)</em></td>
<td>480 µl</td>
<td>Tris-HCl, EDTA, KCl, non-ionic detergents. pH 8.5</td>
</tr>
<tr>
<td><strong>Control Plasmid Mix (purple cap)</strong></td>
<td>30 µl</td>
<td>DNA purified from bacteria, Tris-HCl, EDTA. pH 8.0</td>
</tr>
<tr>
<td><strong>Silencer Mix (purple cap)</strong></td>
<td>30 µl</td>
<td>Synthetic oligonucleotides, Tris-HCl, EDTA. pH 8.0</td>
</tr>
<tr>
<td>Product Description</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* Supplied upon request only; not used in the standard protocol
** These components are only available for selected probemixes (see product description)

None of the ingredients are hazardous in the amount present in an MLPA kit as defined in the Hazard Communication Standard (HCS). None of the ingredients are of human or animal origin or are derived from pathogenic bacteria. None of the preparations provided in this kit contain dangerous substances, as defined in European Directive 67/548/EC and its amendments at concentrations requiring distribution of Material Safety Data Sheets as specified in European Directives 1999/45/EC and 2001/58/EC. Therefore, Material Safety Data Sheets for these products are not required.

2.2. **STORAGE AND SHELF LIFE**

All components of the SALSA MLPA kit 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 at least 1 year is guaranteed. See the labels on each vial for the exact expiry date.

2.3. **MATERIALS REQUIRED BUT NOT PROVIDED**

- Thermocycler with heated lid (99-105°C)
- Capillary Electrophoresis Equipment (see p. 3)
- 0.2 ml PCR tubes.
- Standard laboratory equipment

2.4. **PRECAUTIONS AND WARNINGS**

- For professional use only. Always consult the most recent version of the relevant product description AND this general MLPA protocol for DNA detection and quantification before use.
- SALSA® MLPA® kits are sold by MRC-Holland for research purposes and to demonstrate the possibilities of the MLPA technique. These kits are not CE/FDA or otherwise certified for use in diagnostic procedures.
- It is the responsibility of the user to be aware of the latest scientific knowledge of the application in question before drawing any conclusions from findings made with this product.
- Assay performance is dependent on operator proficiency and adherence to procedural directions. Analysis should be performed by properly trained personnel.
- SNPs/mutations within the sequence detected by a probe, as well as differences in purity between sample DNA and reference DNA samples, can result in false positive results. In particular all abnormalities detected by a single MLPA probe should be confirmed by an independent technique.
3. PRE-TEST SETUP INSTRUCTIONS

3.1. SAMPLE TREATMENT

1. Use a total quantity of 50-250 ng of human (genomic) DNA in a 5 µl volume for each MLPA reaction. If necessary, DNA samples can be concentrated by ethanol precipitation. Glycogen (Roche; 901393) can be used as carrier in ethanol precipitations. When using the P125 probemix for human mitochondrial DNA, or when testing DNA from organisms with a genome size smaller than 100 Mb such as bacteria, we recommend using 5-25 ng of sample DNA.

2. Dissolve and dilute sample DNA in TE (10 mM Tris-HCl pH 8.2 + 0.1 mM EDTA). The pH of the DNA preparation should be between 8.0 - 8.5 in order to prevent depurination during the initial heat treatment at 98°C.

3. MLPA does not require a special method or kit for DNA extraction, but the extraction method should not leave a high concentration of contaminants such as salt. For this reason, do not use the Qiagen EZ1, M6, M48 and M96 systems.

4. We have tested and can recommend the following extraction methods:
   - Qiagen Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual)
   - Promega DNA extraction Wizard (manual)
   - Salting out (manual)

5. MLPA is more sensitive to contaminants than simple monoplex PCR assays. Contaminants include (guanidinium) salts, phenol, ethanol, heparin, EDTA, Fe. To minimise the effect of sample DNA contaminants, compare only samples that a) were derived from the same tissue type; b) were extracted using the same extraction method; c) have similar concentrations; d) have undergone similar storage and treatment conditions.

6. EDTA concentration of the samples should not be higher than 2.5 mM. Concentration of sample DNA by evaporation or SpeedVac may result in a very high EDTA concentration which will negatively affect the ligation and PCR reaction.

7. In case of doubts about DNA quality, a) clean contaminated samples by ethanol precipitation or silica based clean-up kits; b) use only 20-50 ng of sample DNA; c) use an alternative PCR protocol in which only part of the ligation reaction is used for the PCR (point 7 under §4.1).

8. Do not use heparinised blood for DNA extraction. Traces of heparin are very difficult to remove from DNA preparations and can distort the MLPA PCR reaction.

9. An RNAse treatment may always be used and is essential when screening for a gene that is very highly expressed in the sample tissue under investigation. Examples are the HBA and HBB genes (blood-derived samples) and (mitochondrial) ribosomal RNA genes (all tissues).

3.2. SELECTING REFERENCE & CONTROL SAMPLES

1. REFERENCE SAMPLES. Small experimental differences between experiments affect the MLPA peak pattern. Therefore, reference samples have to be included in each MLPA experiment! Always compare samples that are a) run within the same experiment; b) tested with the same probemix lot.

2. MULTIPLE REFERENCE SAMPLES are needed to estimate the reproducibility of each probe within each MLPA experiment. Use at least 3 reference samples per MLPA run. When using more than 21 samples, add 1 additional reference sample for each 7 extra samples. Reference samples should be distributed randomly over the sample plate to minimise variation.

3. SELECTING REFERENCE SAMPLES. Reference samples are DNA samples in which the target and reference probe sequences are assumed to have a 'normal' copy number. They are usually obtained from healthy individuals. They should be as similar as possible to the samples to be tested in all other aspects, as specified in point 5 under §3.1 SAMPLE TREATMENT. For samples derived from formalin-fixed paraffin-embedded (FFPE) tissues, we recommend using reference samples derived from similarly treated healthy tissue.

4. COMMERCIAL DNA. In case of doubt about sample quality, it is advised to include one or more commercial DNA samples. We recommend Promega Cat. Nr G1471 male & G1521 female DNA.

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1 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 MLPA probes to the sample DNA.

2 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 §6.3.
4. MLPA REACTION - DNA DETECTION/QUANTIFICATION

4.1. NOTES TO READ BEFORE YOU START (DAY 1)

1. All parts of the MLPA reaction (DNA Denaturation, Probe hybridisation, Ligation of probes and PCR amplification of ligated probes) are performed in a thermocycler with heated lid (99-105°C).
2. Always vortex thawed buffers and probemix briefly before use, as salts and other constituents are concentrated at the bottom of the tube after a freeze-thaw cycle. The MLPA buffer will usually freeze at -20°C but remains fluid in some cases due to its very high salt concentration.
3. Centrifuge the MLPA reagent tubes for a few seconds before use, as drops may have adhered to the lid during transport or vortexing. After use, store all reagents between -25°C and -15°C.
4. Enzyme solutions are supplied in 50% glycerol and remain fluid at the recommended storage temperature. Never vortex solutions that contain enzymes. When preparing master mixes, enzymes should always be added last. Master mixes containing enzymes should be mixed by gently pipetting up and down. When the viscous enzyme solution is not mixed properly with the dilution buffers, unreliable results will be generated! If the enzyme solution is mixed too vigorously though, enzyme inactivation occurs.
5. To minimise sample to sample variation, the volume prepared of each master mix solution should be sufficient for all reactions included in the experiment. We recommend preparing a 5-10% volume surplus to allow for pipetting errors. Ligase-65 master mix and Polymerase master mix should be made less than 1 hour before use and stored on ice.
6. When performing MLPA on a large number of samples, multi-channel pipettes are recommended. In case a large number of samples are run simultaneously, the ligation incubation period of the first tubes will be longer. This has no influence on the results.
7. An alternative MLPA protocol (the previously used “two-tube” protocol) in which only part of the ligation reaction is used for the PCR reaction is available on www.mlpa.com. This alternative protocol may have advantages when using DNA samples that contain impurities such as high EDTA concentrations (>2.5 mM) or PCR inhibitors. To use this protocol, the necessary vial of PCR buffer can be ordered free of charge with each MLPA kit.

4.2. THERMOCYCLER PROGRAM FOR THE 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 pause</td>
</tr>
<tr>
<td>2) Hybridisation reaction</td>
<td>95°C</td>
<td>pause</td>
</tr>
<tr>
<td>3) Ligation reaction</td>
<td>54°C</td>
<td>1 minute pause</td>
</tr>
<tr>
<td>4) PCR reaction</td>
<td>35 cycles: 95°C, 60°C, 72°C</td>
<td>30 seconds 30 seconds 60 seconds</td>
</tr>
<tr>
<td>5)</td>
<td>98°C</td>
<td>20 minutes pause</td>
</tr>
</tbody>
</table>
4.3. DNA DENATURATION (DAY 1)
1. Label 0.2 ml tubes, strips or plates.
2. Add 5 µl of DNA sample (50-250 ng) to each tube (or TE for the 'no DNA control' reaction).
3. Place the tubes in a thermocycler and start the MLPA thermocycler program (see above). Denature sample DNA for 5 minutes at 98°C and cool the samples to 25°C. At this point, the tubes can be removed from the thermocycler.

4.4. HYBRIDISATION REACTION (DAY 1)
1. Vortex the viscous MLPA buffer and the MLPA probemix before use.
2. Prepare a hybridisation master mix containing for each reaction: 1.5 µl MLPA buffer (yellow cap) + 1.5 µl probemix (black cap). Mix the hybridisation master mix well by pipetting or vortexing.
3. After DNA denaturation, add 3 µl of the hybridisation master mix to each sample tube. Mix well by pipetting up and down.
4. Continue the thermocycler program: incubate for 1 minute at 95°C, then for 16 – 20 hours at 60°C.

4.5. LIGATION REACTION (DAY 2)
1. Vortex the two Ligase buffers before use.
3. Continue the thermocycler program: pause at 54°C.
4. When the samples are at 54°C, add 32 µl of the ligase master mix to each reaction tube. Mix by gently pipetting up and down.
5. Continue the thermocycler program: 15 minutes incubation at 54°C (for ligation), followed by 5 minutes at 98°C for heat inactivation of the Ligase-65 enzyme and then pause at 20°C. At this point, the tubes can be removed from the thermocycler.
6. Ligation reaction products can be stored at room temperature for several hours or at 4°C for up to one week.

4.6. PCR REACTION (DAY 2)
Attention! The protocol described here is the one-tube protocol, introduced in June 2011.
The complete ligation reaction is now used for the PCR and the PCR should be started at room temperature. This requires the use of the new MLPA PCR primer mix that can be recognised by the MRC-Holland logo on its label. On the older version of the PCR primer mix, this logo is absent. PCR buffer and Enzyme Dilution buffer are no longer needed in this protocol.

1. Vortex the SALSA PCR primer mix before use. Warm the polymerase for 10 seconds in your hand in order to reduce viscosity.
2. Prepare a polymerase master mix by adding for each reaction: 7.5 µl dH₂O + 2 µl SALSA PCR primer mix (brown cap) + 0.5 µl SALSA Polymerase (orange cap). Mix well by pipetting up and down; do not vortex. Store on ice until use.
3. At room temperature, add 10 µl polymerase mix to each tube. Mix by pipetting gently up and down and continue the thermocycler program: 35 cycles: 30 seconds 95°C; 30 seconds 60°C; 60 seconds 72°C. End with 20 minutes incubation at 72°C and then pause at 15°C.
4. After the PCR reaction, do not open the tubes in the room with the thermocycler. To avoid contamination, use different micropipettes for performing MLPA reactions and for handling MLPA PCR products.
5. PCR reaction products can be stored at 4°C for up to one week. For longer periods, storage between -25°C and -15°C is recommended. As the fluorescent dyes used are light sensitive, the PCR products should be stored in a dark box or be wrapped in aluminium foil.
5. FRAGMENT SEPARATION BY CAPILLARY ELECTROPHORESIS

5.1. NOTES TO READ BEFORE YOU START

- The amount of MLPA PCR reaction, size standard, run conditions, polymer and the fluorescent PCR primer dye required for the capillary electrophoresis, depend on the type of instrument used. The settings given below are standard settings. Instrument settings may require optimisation for proper fragment separation. Please follow the instructions of your capillary electrophoresis supplier on how to optimise your MLPA fragment separation.
- The use of old capillaries or polymer has a detrimental effect on MLPA results. It is important to change the capillaries and polymer regularly. The polymer quickly deteriorates after prolonged exposure to temperatures over 25°C. When the instrument is not used daily, remove the polymer after use and store at 4°C. In case the size standard peaks are low and broad, it is almost certain that the problem lies in the capillaries or polymer.
- Formamide can become acidic when stored for longer periods. This can result in depurination and fragmentation of the DNA upon heating. We recommend storing formamide in aliquots at -20°C.
- In case all MLPA peaks in your electropherograms are low, it is not recommended to add more MLPA PCR product to the injection mixture. Addition of more PCR products also increases the salt concentration in the injection mixture, which competes with the DNA for injection. When an increase in peak heights is desired, increasing the injection time or injection voltage may be more effective.
- Reset the bin settings on your fragment analysis software when using a different MLPA probemix lot, a different size standard, a different capillary electrophoresis instrument or different run settings.

5.2. EQUIPMENT AND REAGENTS REQUIRED BUT NOT PROVIDED

1. Capillary electrophoresis instrument with fragment analysis software.
   - Beckman: GeXP Software Package
   - Applied Biosystems: Standard Foundation Data Collection Software.
2. High quality formamide (e.g. Hi-Di Formamide, Applied Biosystems 4311320).
3. Labelled size standard.
   - Beckman: GenomeLab™ DNA Size Standard Kit - 600
   - Applied Biosystems sequencers: GeneScan™ 500 ROX™, 500 TAMRA™ or 500 LIZ®.
4. Polymers:
   - Beckman: GenomeLab™ Linear Polyacrylamide (LPA) denaturing gel 391438.
   - Applied Biosystems sequencers - preferred: POP-4 or POP-7; POP-6 is not recommended because of its high resolution. For some older sequencers POP-6 may be the only option.

5.3. ELECTROPHORESIS SPECIFICATIONS

Beckman GenomeLab GeXP; CEQ-2000; CEQ-8000; CEQ-8800
- MLA PCR Primer dye: Cy5.
- Recommended capillaries: 33 cm.
- Injection mixture: 0.7 µl PCR reaction + 0.2 µl Beckman D1-labeled CEQ size standard 600 + 32 µl formamide (or Beckman Sample Loading Solution). Add one drop of high quality mineral oil.
- Initial settings: capillary temperature: 50°C; denaturation: 90°C for 120 sec.; injection voltage: 1.6 kV; injection time: 30 sec; runtime: 60 minutes at 4.8 kV.

ABI-Prism 3100, ABI-Prism 3100 Avant, ABI-3130, ABI-3130XL, ABI-3500, ABI-3730, ABI-3730XL
- MLA PCR Primer dye: FAM.
- Recommended capillaries: 36 or 50 cm. **ABI-3500: 50 cm only!**
- Injection mixture: 0.7 µl PCR reaction + ~0.3 µl ROX or 0.2 µl LIZ size standard + 9 µl formamide.
- The chosen filter set should be compatible with the fluorescent dye of the size standard.
- Initial settings 36 cm capillaries: injection voltage: 1.6 kV; injection time: 15 sec.; run voltage: 15 kV; polymer: POP4; run time 1800 sec. For POP7, use run voltage: 10 kV and an increased run time.
- Method: seal the injection plate and **incubate for 2 minutes at 80°C and cool rapidly.**

3 Briefly heating the injection mixture before capillary electrophoresis is essential. If this is omitted, some sequences with a high Guanine and Cytosine content may remain double-stranded, thereby altering their mobility.
**ABI-Prism 310 Genetic Analyzer (1 capillary)**

- MLPA PCR Primer dye: FAM.
- Recommended capillary: 47 cm.
- Injection mixture: 0.75 µl PCR reaction + 0.75 µl dH₂O + 0.5 µl size standard + 13.5 µl formamide.
- Initial settings: injection voltage: 1.6 kV; injection time: 15 sec.; filter set: D; polymer: POP4.
- Method: **Incubate mix for 2 minutes at 80°C and cool rapidly.**

6. **PEAK PATTERN EVALUATION**

Prior to probe ratio calculation, it is essential to first evaluate the quality of your MLPA experiments by inspecting your MLPA peak patterns (electropherograms). This way, potential problems in the MLPA reaction or the fragment separation can be detected before they could skew up analysis. Based on this evaluation, it may be necessary to repeat the capillary electrophoresis or the complete MS-MLPA reaction. First, raw data should be inspected, followed by the size-called data. Only data that has passed the raw data checklist and the peak pattern flowchart is suitable for the actual MLPA data analysis.

6.1. **EVALUATION RAW DATA**

1. Visualise your raw data (.fsa, .scf, .cqf, .esd files) prior to size-calling:
   - a. Beckman: View your raw peak patterns using Coffalyser or Beckman GenomeLab software.
   - b. ABI: view your raw peak patterns using the Foundation Data Collection Software on the instrument itself or by using software like Coffalyser, GeneMapper or Peakscanner.
   - c. Other manufacturers: use the software provided with the capillary electrophoresis instrument.
2. Answer the questions in the RAW DATA CHECKLIST below.
3. When all questions are answered with NO: proceed with evaluation of the sized-called data.

<table>
<thead>
<tr>
<th>Raw Data Checklist</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Does the MLPA peak pattern show severe (unusual) sloping?</td>
<td>☐</td>
</tr>
<tr>
<td>2. Is there a very high baseline?</td>
<td>☐</td>
</tr>
<tr>
<td>3. Are split / shoulder peaks or high background signals present?</td>
<td>☐</td>
</tr>
<tr>
<td>4. Is the size standard pattern unusual, e.g. sloping, broad peaks?</td>
<td>☐</td>
</tr>
<tr>
<td>5. Any signal bleeding in other channels / any spectral pull up/pull down patterns in the dyes?</td>
<td>☐</td>
</tr>
<tr>
<td>6. Are there irregular current patterns?</td>
<td>☐</td>
</tr>
<tr>
<td>7. Are one or more peaks below the recommended minimum signal?</td>
<td>☐</td>
</tr>
<tr>
<td>Beckman CEQ/GeXP: 3000 rfu (or a minimum of 3 x baseline height)</td>
<td>☐</td>
</tr>
<tr>
<td>ABI 3100 series: 300 rfu (or a minimum of 3 x baseline height)</td>
<td>☐</td>
</tr>
<tr>
<td>ABI 3500, 3700-3730: 300 rfu (or a minimum of 3 x baseline height)</td>
<td>☐</td>
</tr>
<tr>
<td>8. Are one or more peaks above the fluorescence maximum of the sequencer?</td>
<td>☐</td>
</tr>
<tr>
<td>Beckman CEQ/GeXP: 170,000 rfu (optimal 6,000 - 140,000)</td>
<td>☐</td>
</tr>
<tr>
<td>ABI 3100 series: 7500 rfu (optimal range 450 - 6,500)</td>
<td>☐</td>
</tr>
<tr>
<td>ABI 3500, 3700-3730: 30,000 rfu (optimal 450 - 25,000)</td>
<td>☐</td>
</tr>
<tr>
<td>9. Are any spike peaks present that could interfere with peak detection?</td>
<td>☐</td>
</tr>
</tbody>
</table>

6.2. **EVALUATION SIZE-CALLED DATA**

1. Size-call your data:
   - a. Beckman: use Coffalyser, or CEQ / GeXP GenomeLab software to size-call and visualise your data.
   - b. ABI: use Coffalyser, Peak Scanner or Genemapper to size-call and then visualise your data.
   - c. Other manufacturers: use the software provided with the capillary electrophoresis instrument.
   - For more information on the size-calling software, please see the website of the manufacturer.
2. Evaluate the MLPA control fragments (64-105 nt). Read §6.3 INTERNAL QUALITY CONTROL
3. Evaluate your peak patterns using the Peak Pattern Evaluation flowchart in Figure 5.
4. When your data has met all criteria, calculate the Dosage Quotient (copy number) using MLPA analysis software. See also §7.
6.3. INTERNAL QUALITY CONTROLS

SALSA MLPA probemixes contain various control fragments with which common errors in the MLPA reaction can be detected.

Table 2 – Internal quality control fragments

<table>
<thead>
<tr>
<th>Name</th>
<th>Length (nt)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>92 nt control</td>
<td>92</td>
<td>Ligation-dependent probe, behaves similarly to other MLPA probes. Forms a benchmark to compare other control fragments to.</td>
</tr>
<tr>
<td>Q-fragments</td>
<td>64, 70, 76, 82</td>
<td>Warn when the amount of DNA was too low. Signals higher than 1/3 of the 92 nt control fragment → DNA quantity insufficient.</td>
</tr>
<tr>
<td>D-fragments</td>
<td>88, 96</td>
<td>Detect denaturation problems. Signal lower than 40 % of the 92 nt control fragments → DNA denaturation problems.</td>
</tr>
<tr>
<td>X &amp; Y fragments</td>
<td>100, 105</td>
<td>Control for sample swapping.</td>
</tr>
</tbody>
</table>

Q-FRAGMENTS

The four Q-fragments (64, 70, 76, 82 nt) are oligonucleotides present in all SALSA MLPA probemixes. They contain both MLPA PCR primer sequences in a single molecule, meaning they do not need to hybridise to the target DNA or be ligated in order to be amplified during the PCR. The Q-fragments are present in such small quantities that their amplicons are completely outcompeted by the amplicons of the MLPA probes when enough sample DNA was used. In other words, when the peaks of the Q-fragments are low or invisible, this signifies that sufficient sample DNA was present and the ligation reaction was successful. In contrast, when the peaks of all four Q-fragments are higher than 1/3 of the height of the 92 nt ligation-dependent control fragment and the MLPA probes, this is a warning that the amount of sample DNA was insufficient or the ligation reaction failed (see Figure 3).

![Figure 3 – Effect of DNA quantity on Q-fragments.](image)

D-FRAGMENTS

Most MLPA probemixes contain two DNA denaturation fragments (D-fragments) at 88 and 96 nt. These are synthetic MLPA probes detecting a sequence within a strong CpG island. CpG islands are chromosomal regions with very high CG content, making them difficult to denature. In case the 88 or the 96 nt fragment peak is much lower (<40 %) than the 92 nt D-fragment and the other MLPA probes, denaturation of sample DNA may have been incomplete, resulting in unreliable results for probes detecting sequences in or near (<5 kb) CpG islands (see Figure 3). This can be due to salt contamination of the DNA sample. The melting temperature of DNA is increased at higher salt concentrations. As CpG islands are often located near mRNA transcription start locations, exon 1 probes are most commonly affected by incomplete DNA denaturation.

Note that there are different control fragment sets used in MLPA probemixes: probemix lots produced between 2008 and December 2010 (lot numbers 0508 to 1210) contain the QDX1 control fragment set, whereas lots produced from January 2011 onwards (lot numbers from 0111 onwards) contain the QDX2 set. The sets only differ in the D-fragments contained; the D-fragments used from 2011 onwards are more sensitive to salt as they detect sequences in the strongest CpG islands of the human genome. In the QDX1 set, it is often only the

4 In case only one or two increased peaks are visible in the 64-82 nt range, this is not a Q-fragment but a non-specific peak.
96 nt fragment that decreases in case of denaturation problems, whereas in the new QDX2 set, both the 88 and 96 nt fragment show a strong signal decrease in case of denaturation problems.

Table 3 – Control Fragments

<table>
<thead>
<tr>
<th>Control Fragment</th>
<th>QDX1 (until lot 1210)</th>
<th>QDX2 (from lot 0111 onwards)</th>
</tr>
</thead>
<tbody>
<tr>
<td>88 nt D-fragment</td>
<td>FANCE (6p21.3)</td>
<td>CARM1 gene (19p13.2)</td>
</tr>
<tr>
<td>92 nt fragment</td>
<td>IL1B (2q13)</td>
<td>IL1B (2q13)</td>
</tr>
<tr>
<td>96 nt D-fragment</td>
<td>TP73 (1p36)</td>
<td>JPH3 gene (16q24.2)</td>
</tr>
<tr>
<td>100 nt X-fragment</td>
<td>AMOT (Xq23)</td>
<td>AMOT (Xq23)</td>
</tr>
<tr>
<td>105 nt Y-fragment</td>
<td>UTY (Yq11.221)</td>
<td>UTY (Yq11.221)</td>
</tr>
</tbody>
</table>

When using ABI POP7 polymer for capillary electrophoresis, a non-specific fragment of ~87 nt is usually present and may coincide with the 88 nt D-fragment. If the analysis of the 88 nt D-fragment is hampered by this non-specific peak, only the 96 nt peak should be used.

Figure 4 – Detection of incomplete sample DNA denaturation by D-fragments (QDX2 control fragment set) MLPA P335 probe signals from male DNA in TE (A), TE + 40 mM NaCl (B), and TE + 100 mM NaCl (C). The peak size of the D-control fragments (88 & 96 nt) is reduced in the presence of 40 mM or more salt due to incomplete sample DNA denaturation. 64-82 nt: Q-fragments, 88-96 nt: D-fragments, r: reference probe, 136-165: target-specific probes.

6.4. No DNA control

In a clean No DNA control reaction, only the Q-fragments at 64-70-76-82 nt generate a probe signal. However, since the MLPA PCR reaction contains billions of long MLPA probe oligonucleotides, each of which containing a perfect copy of one of the PCR primers, MLPA PCR reactions are more prone to having non-specific peaks in the ‘No DNA control’ reaction than a normal PCR. In the great majority of cases, these non-specific peaks do not influence the MLPA results. When sufficient sample DNA is used, they are outcompeted by the ‘real’ MLPA probe amplification products in the same way as the Q-fragments. Please notify MRC-Holland in case a non-specific amplification product in the No DNA reactions is reproducibly higher than the Q-fragments.

6.5. TESTING FOR EVAPORATION PROBLEMS

Excessive evaporation can be tested for by incubating 8 µl water overnight at 60°C. The next morning, at least 5 µl water should still be present at the bottom of the tube.

In case of excessive evaporation, try a different brand of tubes, increase or decrease the pressure of the heated lid on the tubes, check whether the heated lid works properly or try using mineral oil on top of the PCR mix (e.g. Vapor-lock from Qiagen). At high temperatures, the plastic of the tubes is weak. As a result, deformation of tubes can occur when the pressure of the heated lid is too high. This can result in tube leaks and excessive evaporation.
Figure 5. Peak Pattern Evaluation flowchart

1. 92 nt control fragment & probe amplicons present?
   - YES
   - NO

2. Q-fragments: all 4 peaks higher than 1/3 peak of 92 nt control fragment? (see §6.3)
   - YES
   - NO

3. D-fragments (88 & 96 nt): peak(s) lower than 40% of the 92 nt control fragment? (see §6.3)
   - YES
   - NO

4. Excessive primer-dimer formation visible?
   - YES
   - NO

5. Apparently random decrease of some signals and increase of others?
   - YES
   - NO

6. Split peaks visible in several MLPA probe signals?
   - YES
   - NO

7. One or more peak signals too high (off-scale)? (See raw data checklist.)
   - YES
   - NO

8. All peak signals too low? (See raw data checklist.)
   - YES
   - NO

9. Sloping: are peaks of longer MLPA probes more than 3 times lower than those of shorter MLPA probes?
   - YES
   - NO

10. Are there large differences in relative peak heights between samples that don’t make sense?
    - YES
    - NO

11. Proceed with Data Analysis (see §7).

   GO BACK TO §3.1 SAMPLE TREATMENT; §4 MLPA REACTION DNA DETECTION/QUANTIFICATION OR §5 FRAGMENT SEPARATION BY CAPILLARY ELECTROPHORESIS
7. DATA ANALYSIS

Data that has passed raw data and peak pattern evaluation can be used for data normalisation. Since the absolute fluorescence intensities detected by the capillary electrophoresis instrument depend on many factors (degradation of the fluorescent dye, variability between capillaries, pipetting accuracy, etc), this data needs to be normalised to obtain useful MLPA results. In MLPA, normalisation happens in a two-step model. First, the relative fluorescence intensity of each peak within a sample is determined (intra-normalisation); in a second step, this relative peak is compared to that in other samples (inter-normalisation). The final ratio for a given probe in a test sample compared to that same probe in the reference samples is called the Dosage Quotient of that probe.

Various software programs exist that can calculate MLPA dosage quotients like the Coffalyser program from MRC-Holland, but also self-made analysis sheets are frequently used. It is recommended to validate such analysis tools prior to use, e.g. by analysing samples with known copy number changes.

7.1. DOSAGE QUOTIENT INTERPRETATION

For a reliable analysis, the dosage quotient results should meet two criteria:

1) The standard deviation of all probes of the reference runs should be ≤0.1
2) The dosage quotient of the reference probes of the sample runs should be between 0.8 and 1.2

When these criteria have been met, subsequent interpretation of results can be performed. However, when one or more probes in the reference or sample runs do not meet these criteria, it is recommended to re-analyse the raw data to see whether anything in the raw data can explain the deviation of the aberrant probes (see chapter 6). Please note that it is possible to find a copy number alteration in a sequence detected by a reference probe. For many regions of the human genome, rare germline copy number variations have been reported in healthy individuals (http://projects.tcag.ca/variation/). Furthermore, 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 doubts about the quality of the MLPA experiment, repeat the MLPA test / capillary electrophoresis or exclude the sample from the analysis.

8. INTERPRETATION OF RESULTS

To judge whether the results obtained are reliable, sufficient knowledge about the MLPA technique and the application screened for is essential. More info: www.mlpa.com → MLPA procedure → Result interpretation.

Arranging probes according to chromosomal location facilitates the interpretation of results and may reveal more subtle changes such as those observed in mosaic cases. Please keep the following in mind:

- Sequence changes (SNPs, point mutations) in the sample DNA sequence detected by a probe can cause false positive results. Mutations/SNPs (at least up to 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 the probe oligonucleotide to the sample DNA.
- When designing probes, MRC-Holland avoids known SNPs whenever possible. However, new SNPs are continuously being discovered and the number is becoming so large that it is has become impossible to avoid SNPs in all cases. Furthermore, the reported SNPs are not always reliable. Most SNPs in the SNP databases are not validated, some are rare pathogenic variants and many have been detected only once and could be sequencing mistakes. Please notify us when a polymorphism or a frequent pathogenic mutation influences one of the probe signals.
- Most MLPA probes detect a sequence with a length between 55 and 76 nt. Most of our product descriptions contain only a partial probe sequence, usually 12 nt on either side of the ligation site. The complete sequences detected by the probes are available on our website or on request: info@mlpa.com.
- Copy number changes detected by a single probe always require confirmation by other methods. Sequencing is often used to determine whether a mutation or polymorphism affecting the probe signal is present in the probe target sequence. 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 (Hills A. et al., (2010) Mol Cytogenet. 3:19). Information on MLPA probe design is available on www.mlpa.com.
- Frequency of single exon deletions is generally higher in genes with large introns than in small genes.
- Not all deletions and duplications detected by MLPA are pathogenic. For many genes, exons are described that are only present in certain transcript variants. MRC-Holland can not provide information whether or not deletion or duplication of such exons will result in disease. For some genes, 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.

- For most applications, the major cause of genetic defects will be small (point) mutations, most of which
  will not be detected by SALSA MLPA kits. MLPA will not detect most inversions, translocations, nor copy
  number changes that lie outside the sequence detected by the SALSA MLPA probes.

- In case of a poor sample DNA denaturation, even the apparent deletion of several probes
  recognising adjacent genomic sequences can be a false positive result! This is why the D-fragments should always be
  examined with great care. The presence of salt in DNA samples (e.g. 40 mM NaCl or 1 mM MgCl2)
  prevents DNA denaturation of very CG-rich chromosomal regions. Sequences in the vicinity of such CpG
  islands will denature at 98°C but will reanneal immediately upon cooling as the non denatured CpG island
  holds the two strands together. Binding of certain probes to their target sequence will be hindered
  resulting in reduced signals for probes located within several kb from such strong CpG islands.

- MLPA experiments provide the average copy number 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 or duplication, mosaicism is one of the possible
  causes. For example, a 25% mosaic case of trisomy 21 tested with the SALSA MLPA P095 Aneuploidy kit is
  expected to result in ratios of 1.1-1.15 for the eight chromosome 21 probes.

- The majority of MLPA probemixes contain reference probes that are used for data analysis. Copy number
  changes detected by reference probes are unlikely to have any relation to the condition tested for.

- The exact cut-off values for the dosage quotients (DQ) that can be used to determine the allele copy
  numbers are different for each probe and depend on the standard variation of that probe. This standard
  variation depends also on the quality of the DNA samples, the choice of reference DNA samples and the
  quality of the capillary electrophoresis. Internal MLPA technique validation in your laboratory is essential.
  Provided that the standard deviation of all reference probes in the samples tested is below 0.10, and the
  dosage quotients of all probes of the reference sample runs are between 0.85 and 1.15, the following
  cut-off values for the dosage quotients (DQ) of the probes can be used as an indication to identify
  abnormal copy numbers in a typical MLPA probemix targeting human autosomal DNA sequences:

<table>
<thead>
<tr>
<th>Copy Number Status</th>
<th>Dosage Quotient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.85 &lt; DQ &lt; 1.15</td>
</tr>
<tr>
<td>Heterozygous duplication</td>
<td>1.35 &lt; DQ &lt; 1.55</td>
</tr>
<tr>
<td>Homozygous duplication</td>
<td>1.70 &lt; DQ &lt; 2.20</td>
</tr>
<tr>
<td>Heterozygous deletion</td>
<td>0.35 &lt; DQ &lt; 0.65</td>
</tr>
<tr>
<td>Homozygous deletion</td>
<td>0</td>
</tr>
<tr>
<td>Equivocal copy number</td>
<td>All other values</td>
</tr>
</tbody>
</table>

- These values are estimates. In view of the limited availability of positive deletion and duplication samples,
  it is often only possible to determine probe-specific cut-off values for normal samples.

- Analysis of parental samples might be necessary in certain cases for correct interpretation of results.

MLPA results are more plausible when:

- The overall standard variation per probe in the reference samples is low (<10%).
- Probes for adjacent exons show a decreased or increased signal, e.g. exons 2 to 5, indicating a multi-
  exon deletion or duplication.
- The same result is obtained in a new experiment using less (minimum 20 ng) DNA and/or using different
  reference samples. When less DNA is used, possible contaminants which may influence the probe signal
  are diluted. Please note that optimal MLPA results are obtained by using a minimum of 50 ng human
  sample DNA. When less DNA is used, the standard variation of the probes is higher.

A result is unlikely to be reliable when:

- Probes for non-neighbouring exons show a decreased or increased signal, e.g. a duplication of exon 3 and
  17.
- In the same sample, one or more reference probes show an abnormal copy number.
- Many abnormalities are found in a patient cohort of a disease in which copy number changes are known
  to be rare.
9. EXPLANATION OF SYMBOLS USED

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Store at</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOT</td>
<td>Keep away from heat or direct sunlight</td>
</tr>
<tr>
<td>Use by</td>
<td>Cat. No.</td>
</tr>
<tr>
<td>No. of Tests</td>
<td>Read instructions before use</td>
</tr>
</tbody>
</table>

10. MLPA® PROTOCOL FOR DNA ANALYSIS IN A NUTSHELL

1. DNA DENATURATION
   - Heat a 5 µl DNA sample for 5 minutes at 98°C

2. HYBRIDIZATION OF PROBES TO SAMPLE DNA
   - Cool down to room temperature, open the tubes
   - Add a mixture of 1.5 µl SALSA probemix and 1.5 µl MLPA buffer and mix
   - Incubate 1 minute at 95°C + 16 hours hybridisation at 60°C

3. LIGATION OF HYBRIDIZED PROBES
   - Lower thermocycler temperature to 54°C, open tubes
   - Add 32 µl ligase mix, incubate 15 minutes at 54°C
   - Heat to inactivate the ligase enzyme: 5 minutes 98°C

4. PCR AMPLIFICATION OF LIGATED PROBES
   - Cool down to room temperature, open tubes
   - Add 10 µl polymerase mix at room temperature
   - Start PCR

5. CAPILLARY ELECTROPHORESIS OF PCR PRODUCTS

6. ANALYZE RESULTS
   - Determine RELATIVE size of the fluorescent peaks
   - Compare results to reference samples

Ligase mix: 3 µl ligase buffer A + 3 µl Ligase buffer B + 25 µl water + 1 µl Ligase-65
Polymerase mix: 7.5 µl water + 2 µl PCR primer mix + 0.5 µl SALSA polymerase

PREVENT FALSE POSITIVE OR NEGATIVE RESULTS. READ THE COMPLETE PROTOCOL!

This protocol requires the new MLPA PCR primer mix (available from June 2011 onwards).
For more information on this primer mix, see §4.6; for the complete thermocycler program, see §4.2.

Note: Obtaining a detectable signal for each probe is not sufficient. For reliable results, being able to generate MLPA peak patterns on normal samples with a standard variation below 10% for each of the probes is crucial. For this reason, it is essential to follow the procedures described in this protocol accurately.

Thank you for your interest in the MLPA technology. Please help us improve our products and brochures by sending your comments and suggestions to info@mlpa.com. More information on MLPA is available on www.mlpa.com.

MLPA General Protocol – Document Changes:

- 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., on by the absence of the MRC-Holland logo on its label.
- NEW DOCUMENT. Previous MLPA General Protocol completely rewritten for use with the one-tube protocol.