MRC-Holland MLPA®

MLPA essential points

1. Strictly follow the Instructions for Use

NEVER deviate from the instructions for use (MLPA product description and the MLPA General Protocol / MS-MLPA General Protocol), the latest versions of which are available on www.mlpa.com. The general protocols have been carefully optimised to minimise the effects of experimental variation. In the great majority of problems reported to MRC-Holland, customers are found to have deviated from the protocol in some way.

2. Sample treatment

- **DNA samples should contain at least 5 mM Tris buffer pH 8-8.5.** Use TE - never H_2O! - to dilute and dissolve DNA samples. Unbuffered samples degrade rapidly by depurination during the initial 5 min 98°C denaturation step, resulting in suboptimum MLPA results. If unsure how the sample was dissolved, add Tris-HCl (pH 8.0-8.5) to a final concentration of 10 mM.
- **Various other contaminants may affect MLPA**, including heparin and melanin. Not all purification methods remove these contaminants.
- **A260/280 ratio is not the best indicator** of a sample's suitability for MLPA. E.g. the presence of salt or heparin in the DNA sample can influence MLPA results but does not affect the A260/280 ratio. Conversely, contamination of sample DNA with proteins does affect the A260/280 ratio but typically does not influence MLPA results. Use the Q and D control fragments (see below) to verify the quality of your experiment instead.
- **Use 50-100 ng sample DNA**, ideally. Using more sample DNA also introduces more contaminants in the reaction, whereas using <50 ng sample DNA increases the variation of the results. The Q control fragments (64, 70, 76 & 82 nt) indicate whether sufficient sample DNA was used.
- **The presence of salt in DNA samples can cause false positive results** as it hinders the denaturation of CG-rich areas. The D control fragments (88 & 96 nt) indicate whether sample DNA denaturation was successful.
- To minimise the effect of contaminants, compare only DNA **samples extracted by the same method, from the same tissue type** and use **a similar DNA quantity** for all samples within one experiment.
- **Do not use SpeedVac** to concentrate diluted DNA preparations: it may result in very high EDTA and salt concentrations which influence the MLPA reaction.
- **Do not use WGA DNA:** Whole genome amplification (WGA) does not result in equal amplification of CG- and AT-rich genomic regions, thereby introducing a copy number bias.

3. Critical steps

- **Make master mixes** in order to simplify pipetting and reduce variation between samples.
- **Accurate pipetting** of the **hybridisation master mix** is absolutely essential to obtain reliable results.
- **Never vortex enzyme solutions, including the Ligase and Polymerase master mixes**. Vortexing reduces the enzyme activity. Ensure the Ligase master mix is at room temperature before adding it to the samples. This reduces non-specific peak formation.
- Add the **ligase master mix** while the samples are in the thermocycler **at 54°C**. Warm the polymerase enzyme briefly in your hand before adding it to the master mix. This reduces viscosity. **Add the polymerase master mix at room temperature** to the samples.

4. Experimental robustness

- **Use at least 3 reference samples** in each experiment for reliable data normalisation.
- **Check the reproducibility** of probes in your MLPA experiment (with Coffalyser.Net):
    - in the test samples: stdev of reference probes should be < 0.10;
    - in the reference samples: stdev of all probes should be < 0.10.

5. Capillary electrophoresis

- **Do not use old capillary array or gel**, as this gives unreliable results.
- **Ensure MLPA peaks are within the optimal range** of the capillary electrophoresis device. Overloading makes reliable peak quantification impossible.
- Check the size standard to verify the quality of the electrophoresis.

6. Interpretation of results

- A proper understanding of the limitations of MLPA and of the application tested for is essential for a correct interpretation of results.
- Always have probe results (ratios) sorted on genomic location to facilitate interpretation (Coffalyser.Net).
- False negative results: MLPA cannot detect any changes that lie *outside* the target sequence of the probes; it will not detect point mutations unless specifically stated otherwise, nor any balanced changes, such as inversions and translocations. Even when MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region exist.
- False deletions can be due to e.g. 1. SNPs in the sequence detected by the probe (especially in case of single exon deletions); 2. Peak detection problems (peaks below the detection threshold of the capillary electrophoresis instrument; peak binning problems); 3. Sloping; 4. Sample DNA denaturation problems (Coffalyser.Net checks for the last 3 aspects; SNPs need to be examined by sequencing).
- False duplications can be due to e.g. 1. Contamination of the sample DNA with a PCR amplification product; 2. The use of unsuitable reference samples containing aberrant copy numbers; 3. Pseudogenes acquiring the same sequence as the proper gene; 4. The existence of very rare pseudogenes (e.g. SMAD4 pseudogene is present in < 1% of individuals).
- Confirm results if possible by using another method or a suitable MLPA probemix. Analysis of parental samples may be necessary for a correct interpretation of complex results.