

MLPA[®] DNA DETECTION / QUANTIFICATION PROTOCOL

The use of a thermocycler with heated lid (105°C) is essential. We recommend 0.2 ml PCR-tubes. The MLPA step-by-step protocol for DNA detection/quantification is available at www.mlpa.com.

All quantities mentioned in this protocol are per reaction. Please read all notes carefully before you begin. ^{A1}

1. DNA denaturation and hybridisation of the SALSA MLPA probes

1. Dilute the DNA-sample (20-500 ng DNA, preferred range 50-200 ng DNA) with TE to 5 µl. ^{A2}
2. Heat 5 minutes at 98 °C; cool to 25 °C before opening the thermocycler.
3. Add a mixture of 1.5 µl SALSA probemix (black cap) + 1.5 µl MLPA buffer (yellow cap) to each tube. ^{A3}
4. Mix with care. Incubate 1 minute 95 °C, followed by 16 hrs at 60 °C. ^{A4}

2. Ligation reaction

5. Reduce temperature of the thermocycler to 54 °C. While at 54 °C, add 32 µl Ligase-65 mix to each sample and mix well. ^{B1}
6. Incubate 15 minutes at 54 °C, then heat for 5 minutes at 98 °C. ^{B2}

Ligase-65 mix (made less than 1 hr before use and stored on ice):

- Mix: 3 µl Ligase-65 buffer A (transparent cap) + 3 µl Ligase-65 buffer B (white cap) + 25 µl Water. Add 1 µl Ligase-65 (green* cap) and mix again.

3. PCR reaction

7. Mix in new tubes: 4 µl SALSA PCR buffer (red cap) + 26 µl water + 10 µl MLPA ligation reaction.
8. While these tubes are in a thermocycler at 60 °C, add 10 µl Polymerase mix to each tube and immediately start the PCR reaction. ^{C1-9}

Polymerase mix (made less than 1 hr. before use and stored on ice):

- Mix: 2 µl SALSA PCR-primers (brown* cap) + 2 µl SALSA Enzyme Dilution buffer (blue cap) + 5.5 µl Water. Add 0.5 µl SALSA Polymerase (orange cap). MIX WELL.

PCR Conditions

- 35 cycles: 30 seconds 95 °C; 30 seconds 60 °C; 60 seconds 72 °C. End with 20 minutes incubation at 72 °C.

4. Separation of amplification products by electrophoresis

9. Please find the settings for your sequencer on our website www.mlpa.com/coffalyser/support.html

5. MLPA data analysis

10. Information about data analysis can be found on page 4 and on our website: www.mlpa.com. For troubleshooting, see page 6.

* Please note that on April 16 2008, 2 reagents have changed cap colour. PCR Primer mix now has a brown cap (before purple); Ligase-65 now has a green cap (before brown). This reason this has been done is that the PCR primer mix now comes in a completely brown vial to protect it from light. Please be careful and check the label on the tube to make sure you are using the correct reagents, especially if you also work with older and newer kits.

A. Notes on part 1 - The denaturation / hybridisation reaction

A1. Protect the PCR-primers from light by wrapping the vial in alufoil. It is recommended to centrifuge the contents of the tubes for a couple of seconds before first use, as drops may have adhered to the lid during transport. Furthermore, it is recommended to flip all non-enzyme tubes before pipetting.

A2. We recommend using approximately 100 ng (20-500 ng) of human chromosomal DNA.

- Normalization can only be performed on samples run within the same experiment.
- Reference samples: for conditions in which copy number variations are very rare, it is possible to normalize MLPA data by simple cross-sample comparison. However, for conditions where copy number variations are relatively common (e.g. DMD-Becker) including reference samples is essential. Negative reference samples should be run simultaneously with the other samples.
- As there may be small changes between different lots of a probemix, even when the probe content of a mix is unchanged, it is recommended to compare samples run with the same lot only.
- Although the MLPA reaction is robust and reproducible, the treatment of DNA (storage, extraction method) influences the MLPA peak pattern. This does not affect the analysis as long as one compares samples that have been extracted by the same method and are derived from the same source: e.g. blood vs blood DNA, tumour breast tissue vs. healthy breast tissue (does not need to be from same individual). When working with samples that have been extracted differently is unavoidable, an extra purification step helps.
- DNA should be dissolved in TE (10 mM Tris-HCl pH 8.2; 1 mM EDTA).
- The EDTA concentration in the DNA sample should not exceed 1 mM and the sample volume should not exceed 5 µl. The volume of the reaction is important for the hybridisation speed, which is probe- and salt concentration dependent. The T_m of the probe-target binding also depends on the salt concentration.

A3. The MLPA buffer is viscous and does not mix easily. SALSA probemix and MLPA buffer can be mixed by repeated pipetting at room temperature just before use. Do not centrifuge this mixture as the longer probes might precipitate!! Please note that the MLPA buffer contains a high concentration of salts and other constituents. Usually it will freeze at -20 °C, sometimes it remains liquid.

A4. The hybridisation time can be anywhere between 12 and 24 hrs (we recommend 16-18 hrs, but hybridisation of probes to their targets should be nearly complete after 12 hrs). MRC-Holland uses Biometra Uno II thermocyclers with a heated lid at 105 °C.

The following program covers the complete MLPA reaction:

1) Hybridization reaction

1.	98 °C	<i>5 minutes</i>
2.	25 °C	<i>hold</i>
3.	95 °C	<i>1 minute</i>
4.	60 °C	<i>hold</i>

2) Ligation reaction

5.	54 °C	<i>hold</i>
6.	54 °C	<i>15 minutes</i>
7.	98 °C	<i>5 minutes</i>
8.	4 °C	<i>hold</i>

3) PCR reaction

9.	60 °C	<i>hold</i>
10.	35 cycles:	
	▪ 95 °C	<i>30 seconds</i>
	▪ 60 °C	<i>30 seconds</i>
	▪ 72 °C	<i>60 seconds</i>
11.	72 °C	<i>20 minutes</i>
12.	4 °C	<i>hold</i>

B. Note on part 2 - The ligation reaction

B1. When running a large number of samples simultaneously, the ligation incubation period will of course be longer for the first tubes. This is no problem; just raise the temperature 15 min after you have finished with the last sample.

B2. Following the 98 °C ligase inactivation treatment, samples can be stored at 4°C for up to one week. For longer periods, storage at -20 °C is recommended.

C. Notes on part 3 - The PCR reaction

C1. To avoid contamination, never handle MLPA PCR products with micro-pipettes that have been used for MLPA reactions! Following PCR, the tubes should not be opened in the vicinity of the thermocycler.

C2. Start the PCR as soon as possible after addition of the polymerase mix! The manual hot start as used in this protocol is recommended, but not essential. Preparation of the complete PCR reaction at room temperature or on ice will usually result in almost identical results. In case of a large number of samples we recommend to prepare the samples on ice: after adding the polymerase mix transfer the tubes to a preheated thermocycler (72 °C) and start the PCR reaction immediately. Some users prepare a mixture of water, PCR buffer and polymerase mix, distribute this among all vials and start the PCR reaction by adding the ligation reaction.

C3. Denaturation time of the PCR reaction can be reduced to 20 seconds in most thermocyclers.

C4. All volumes of the PCR reaction can be reduced in order to save reagents.

C5. We recommend running 35 PCR cycles. However, for most users 33 cycles will be more than sufficient and many users could reduce the number of cycles to 30. A lower number of PCR cycles will result in a very minor improvement in the linearity of relative probe signal with target sequence copy number. In contrast, DNA samples containing only 10-20 ng of human DNA may require up to 37 PCR cycles.

C6. PCR products can be stored at 4 °C for at least one week. As the fluorescent labels used are light-sensitive, the PCR products should be stored in a dark box or wrapped in aluminium-foil.

C7. Contamination: MLPA is less prone to PCR-contamination problems than normal PCR reactions. In a normal PCR reaction, contamination with ~5 copies of a previous PCR product carrying the same primers could already result in a false-positive signal. In an MLPA-based PCR reaction, however, such contaminating PCR products are outcompeted by the large numbers of oligos present in the new MLPA reaction. In a typical MLPA reaction, 50 ng human DNA is used, containing 17.000 haploid genomes. The number of ligated probes will be close to this. At the start of the MLPA-based PCR reaction, there will therefore be ~15.000 copies of each of the 40 different ligation products, meaning any residual PCR products will be outcompeted. At MRC-Holland, thermocyclers are in the set up PCR lab. Our strict rule is that following the PCR, the tubes should not be opened in this same room.

C8. Incomplete mixing of the viscous 50% glycerol enzyme solutions with dilution buffers are a major source of errors!

C9. Sequence of the PCR primers (5'-3')

SALSA PCR Forward primer (Labelled): *GGGTTCCCTAAGGGTTGGA

SALSA PCR Reverse primer (Unlabelled): GTGCCAGCAAGATCCAATCTAGA

Alternative one-tube PCR protocol

(Results are more variable as compared to the recommended protocol)

While the tubes containing the 40 µl Ligation reaction are in a thermocycler at 60 °C, add 10 µl Polymerase mix to each tube and start the PCR.

4. Separation of amplification products by electrophoresis

Equipment and reagents

1. Capillary Sequence System with fragment analysis software
2. Hi-Di formamide
3. Labelled size standard (ROX, TAMRA, LIZ-500 or D1-500)

Method

The amount of the MLPA PCR reaction required for analysis by capillary electrophoresis/sequence gel electrophoresis depends on the instrument and fluorescent label used. You can find your settings at www.mlpa.com in the support section under technical MLPA protocols.

5. MLPA Data analysis

Since normalisation of electrophoresis results is essential for obtaining useful MLPA data, data analysis forms a crucial component of the MLPA procedure. Please read these notes carefully. Further information on other options and software can also be found on our website www.mlpa.com.

A) Coffalyser MLPA software

To analyze MLPA data, we recommend using Coffalyser software developed at MRC-Holland, especially for the analysis of large numbers of samples. Coffalyser is an Excel-based program which runs on a Microsoft Office version 2003+. All data normalization steps are built-in functions of Coffalyser. Furthermore, Coffalyser corrects for an effect characteristic of most MLPA probemixes: the probe length-dependent decrease in peak area/height. Coffalyser can be downloaded freely from our website and is updated regularly. An Excel-independent version will be available at the beginning of 2009.

B) Visual examination

Analysis of a limited number of samples can be done by simple visual examination of the capillary electrophoresis peak profiles. A two colour on-screen overlay of sample and reference reaction can be accomplished by Genotyper™ software.

C) Manual spreadsheet-based MLPA analysis

Information on manual spreadsheet-based MLPA analysis can be found on our website: www.mlpa.com.

Notes on data analysis

Important facts about normalization

- Normalize only samples that have been
 - a) extracted by the same method
 - b) run within the same experiment
 - c) run with the same probemix lot
- Make sure you reset the bin settings on your sequencer software when using a different probemix lot, sequencer or run settings

Robustness of the block normalization

MRC-Holland generally defines relative probe signals by dividing [the peak area of each amplification product] by [the total peak area of only the reference probes in the probemix]. The robustness of this normalization method depends on the number of reference runs, reference probes, chromosomal location of reference probes and the origin of the reference samples.

The recommended type of data normalization per product can be found in the accompanying product description.

Probe ratios

MRC-Holland usually defines probe ratios between 0.7-1.3 as normal.

Peak area versus peak height

Both peak area and peak height can be used to calculate probe signals. Generally, using peak areas is the most accurate method. However, using peak heights has advantages when (some) peaks in a sample are very broad. In this case, calculating peak areas often lead to inaccuracies and it is better to use peak height.

Correction for signal sloping

It is possible that in some runs the peaks of the longer MLPA probes are more than 3 times lower than those of shorter probes. This is called *signal sloping* or the *ski-slope effect*. Signal sloping should be corrected for when the signal sloping in a patient sample run differs from the signal sloping in a reference run. Coffalyser automatically corrects for this effect. Alternatively, you can generate series of dosage quotients for each peak by using individual neighbouring peaks as reference peaks, assuming that these are silent.

SALSA MLPA Q- & D-fragments (former DQ- & DD-fragments)

All SALSA MLPA probemixes contain four DNA Quantity control fragments (Q-fragments) and most also contain three DNA Denaturation control fragments (D-fragments). The 4 Q-fragments (64, 70, 76, 82 nt) are not ligation-dependent and are present in small quantities. When the MLPA reaction has been successful, the Q-fragments are dwarfed by the MLPA probes. In contrast, when the peaks of all four Q-fragments are larger than half the size of the peaks of the D-fragments and MLPA probes (Fig. 1A, 1B), either the ligation reaction failed or the amount of sample DNA was less than 20 ng. In either case, the MLPA results are not reliable.

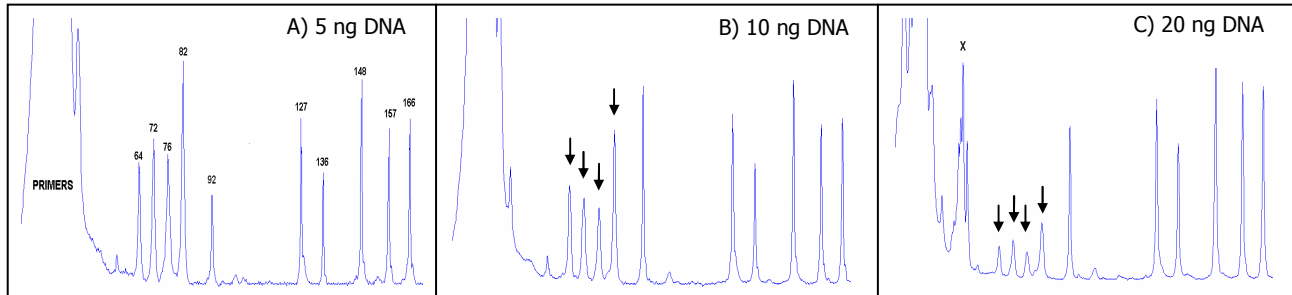


Figure 1. Effect of DNA quantity on Q-fragments. MLPA results with 5 ng (A), 10 ng (B), 20 ng (C). Peaks of the Q-fragments decrease when the amount of sample DNA added is increased. Primer-dimer peaks (x) are often seen at ~55 nt.

In contrast, the three D-fragments (88, 92 and 96 nt) *are* DNA- and ligation-dependent, just like normal MLPA probes. Their peak size should be comparable to that of the MLPA probes; they will be visible when ligation was successful and DNA was sufficient and properly denatured. The 92 nt D-fragment recognizes a 2q14 DNA sequence. The 88 nt D-fragment recognizes a sequence on 6p21.3 in the CpG island preceding the FANCE gene. The 96 nt D-fragment a sequence on 1p36 in the CpG island preceding the TP73 gene. These two CpG islands have a very high percentage of C/G nucleotides, making them extremely difficult to denature. In case the 88 or the 96 nt fragment peak are much lower (<40%) than the 92 nt D-fragment and the other MLPA probes, denaturation of the sample DNA may have been incomplete, resulting in unreliable results for probes detecting sequences in or near (<5 Kb) a CpG island (Figure 2C). Often, only probes for exon 1 will be affected, as exon 1 is generally more CG-rich.

A poor denaturation can be caused by small amounts of ionic impurities (e.g. Mg) in the DNA sample (Fig. 2). In this case, DNA denaturation can be accomplished by prolonging the first 5 min. 98 °C denaturation step (prior to hybridization) to 40 min, or by inclusion of 5% glycerol in the 5 µl DNA sample. Even the apparent deletion of two probes next to each other can be a false positive result due to incomplete denaturation! Results of probes close to CpG islands should always be treated with caution and the signals of the D-fragments should be included in the analysis. More information can be found in 'probes close to a CpG island', available on our website: www.mlpa.com (support section – technical MLPA protocols).

As less is added of the 92 nt control fragment, it becomes a measure for hybridization. If the peak size of the 92 nt D-fragment is much lower than that of the 88 and 96 nt D-fragments, this may indicate that probe-to-target hybridization was incomplete. Incomplete hybridization can be caused by 1) insufficiently long hybridization times, 2) insufficient amounts of probemix and/or 3) MLPA buffer, 4) the use of larger volumes of DNA solution (> 5 µl), 5) a lower than 60 °C hybridization temperature or 6) a lower than 100 °C temperature of the thermocycler lid.

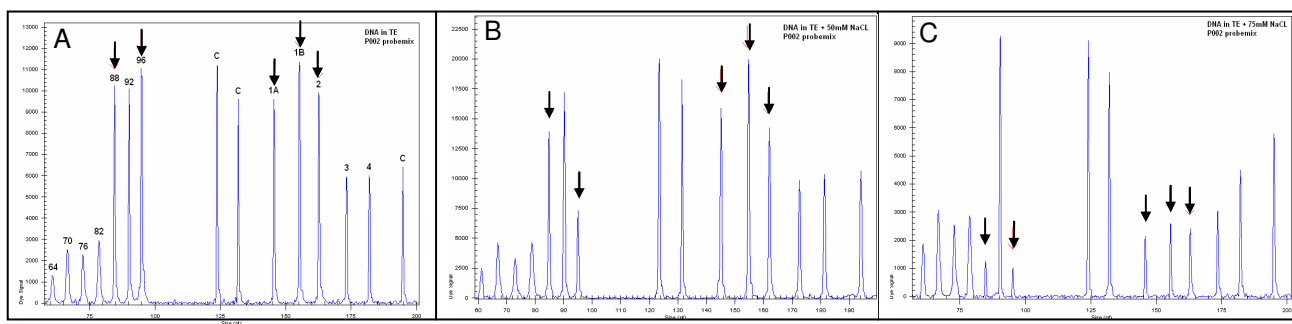
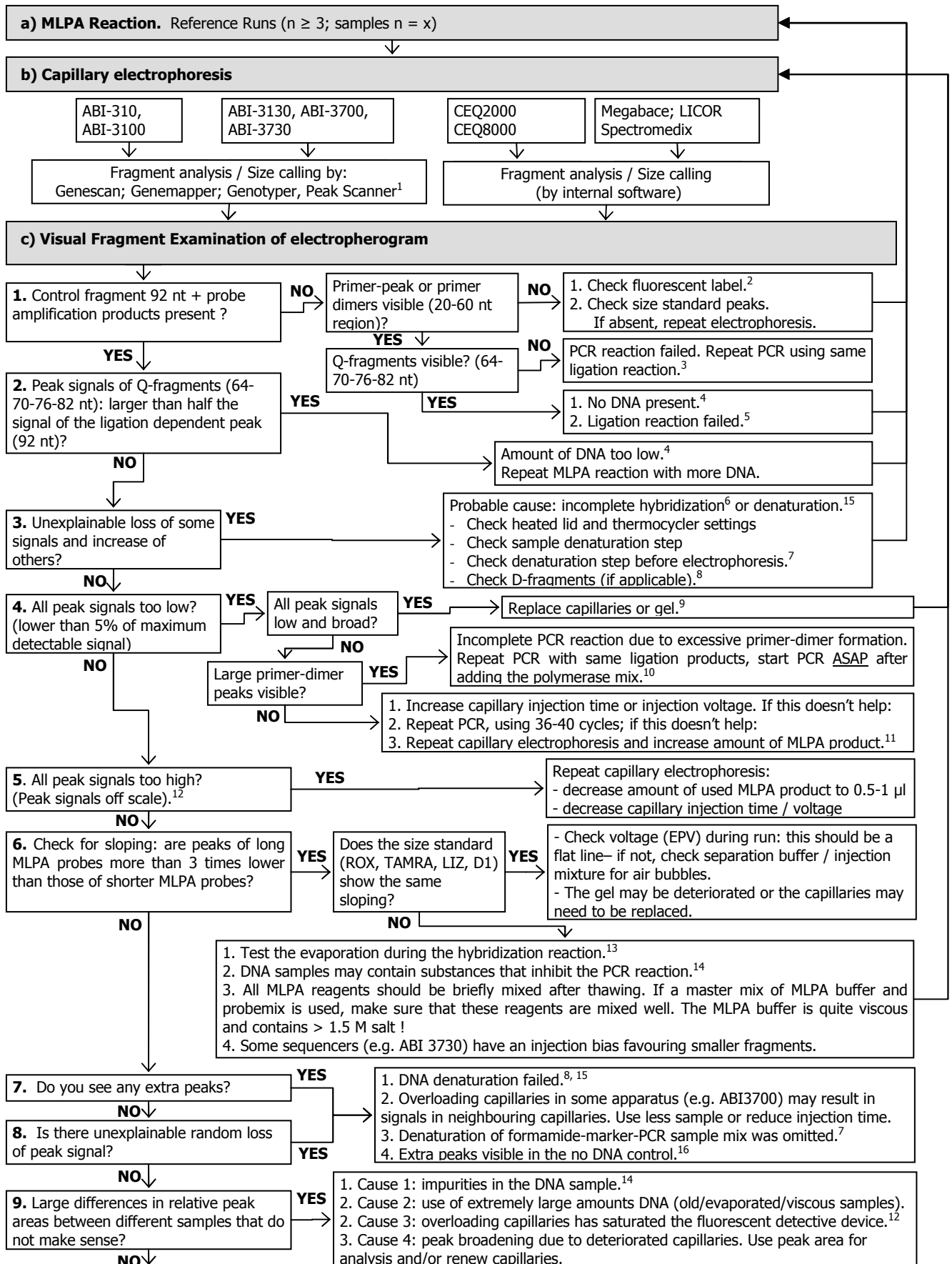


Figure 2. Effect of a poor denaturation (due to a high salt concentration) on D-fragments. MLPA P002 probes signals from DNA in TE (A), with 50mM NaCl (B), 75 mM NaCl (C). Peak size of control fragments 88, 96 and probes for exon 1A, 1B and 2 are reduced in the presence of a high salt concentration due to incomplete denaturation. 64-96 nt: quality control fragments, c: control probe, 1-5: BRCA1 probes for exons 1-5.

MLPA Evaluation & Troubleshooting Flow Scheme



d) Your runs are good enough to continue with analysis.

To proceed, go to your fragment analysis software and deselect all dyes except for the fluorescent dye used to detect the MLPA probe signals (FAM, Cy5.0, IRD800)

10. Export fragment results data of good runs. See <http://www.mlpa.com/coffalyser/support.html> for all run & extraction manuals.

ABI GeneScan (v3.1, v3.7)

ABI GeneMapper (v3.7, v4.0) / Peak scanner software v1.

Beckman CEQ2000 CEQ8000

Megabace software mail:info@mlpa.com

Following the instructions in the [run & extraction manuals](#) (see above), all runs have been transported to either a *.CSV or *.TXT project file. The MLPA product fragment lists should have the following plot settings (column order):

GeneScan (ABI): dye; time; pk length; pk height; pk area; data point

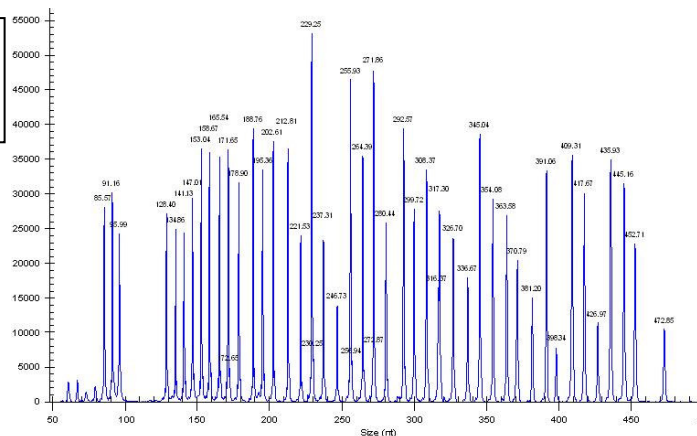
Genemapper (ABI): pk dye; sample file name; pk height, pk area; data point

CEQ2000/8000 (Beckman): sample name; dye; std frag size (nt); est frag size (nt); pk area (rfuxmm); pk height (rfu).

Analyze your runs allowing a minimal peak signal (1% of total area or height), ensuring all data will be stored. Transfer your files to Coffalyser MLPA DAT (see Coffalyser MLPA DAT manual).

All ABI size called files have a tab delimitations, whereas CEQ project files (created using the export fragments/genotypes options) are expected to be comma delimited. Problems with the import of these files are often caused by export of all dyes or differently set plot settings. **Note:** We distinguish two kinds of Genemapper files: separate sample files and combined files containing multiple runs. Both files are exported in the same manner. For the CEQ, we distinguish the CEQ8000 project files containing multiple samples (Export fragments genotypes) and CEQ8000 single files (export sample runs).

11. Make a print of the electropherogram displaying the reference runs. You can use this print for setting the detected lengths in the Coffalyser MLPA-DAT software for MLPA analysis.



Notes on troubleshooting

1. Peak Scanner Software is available free of charge from Applied Biosystems: <https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=603604&tab=Overview>.
2. For Beckman CEQ sequencers, CY5.0 labelled primers are used; for LICOR sequencers IR800 labelled primers. For all other instruments, FAM-labelled primers are used.
3. Each SALSA MLPA probemix contains the Q-fragments at 64-70-76-82 nt. These fragments do not require ligation or presence of sample DNA (see p.5). In case no Q-fragments AND no probe amplification products are present, the PCR reaction has failed. The ligation reaction is very stable and can be stored at 4 °C for more than one week. The amount of reagents in each kit is sufficient for ~120 PCR reactions, making it possible to repeat some PCR reactions. In order to save reagents, it is also possible reduce the volume of the PCR reactions two fold (5 µl ligation reaction + 2 µl 10xPCR buffer + 13 µl water + 5 µl polymerase mix) .
4. Note that optical density (260 nm) measurements often overestimate the DNA concentration. In case of doubt, agarose electrophoresis can be used to check the presence of DNA. Usually, 20 ng DNA will be visible on ethidium bromide/agarose gels and will give a smear in the 10.000-40.000 bp range. Please note that in samples with very low DNA concentrations, the peak size of all four Q-fragments will be increased. In case only one increased peak is seen in the 64-83 nt range, it will not be a Q-fragment. This peak will be due to an aspecific amplification product or long primer dimer, that has the same mobility as one of the Q-fragments.
5. The ligation reaction is very robust. More than 90% of the ligation is complete within 2 minutes incubation at 54 °C. Problems with the hybridization or ligation reaction will usually result in a variable peak pattern that contains most of the probe amplification products.
6. Some probes hybridize faster to their targets than others. The concentration of probes in the probemix has been adjusted to give >90% complete hybridization of each probe in the 16 hrs incubation. Some causes of incomplete hybridization that we have encountered are: 1) No denaturation of sample DNA at 98 °C before addition of MLPA buffer and probemix; 2) Incubation at 60 °C for only 16 minutes instead of 16 hrs; 3) The use of lower than

recommended amounts of probemix and MLPA buffer; 4) Defect heated lid, resulting in evaporation of all fluid during o/n incubation.¹³ Other causes of an unexplainable loss of some signals and increase of others are incomplete sample denaturation^{8,15} and an incomplete denaturation of the formamide/PCR reaction/size standard mix before injection in the capillaries⁷.

7. Brief heating of the formamide/PCR reaction/size standard mix to 80 °C before injection in the capillaries is usually essential. If this heating step is omitted, fragments with a high % GC will remain double stranded, while other fragments might denature at room temperature by the high formamide concentration and give a normal peak. The double stranded fragments will give a very small peak at the expected location.
8. Most MLPA probemixes contain the D-fragments at 88 and 96 nt, which give a warning when the sample DNA was not completely denatured. In case the 88 and 96 nt amplification products are much lower than the 92 and 127-454 nt amplification products, denaturation of the sample DNA might have been incomplete, resulting in unreliable results for probes detecting sequences in or close (<5 Kb) to a CpG island. This can be due to the presence of small amounts of ionic impurities such as Mg in the sample¹⁵. See also p.5.
9. The gel used for capillary electrophoresis can easily deteriorate, especially during a hot summer. Remove the gel from the instrument after use and store at 4 °C. In case the peaks of the size standard are low and broad, is almost certain that the problem lies in the capillaries and/or gel.
10. The standard protocol in which the polymerase mix is added at 60 °C requires that the PCR starts as soon as possible after addition of the polymerase mix to the sample. In case of large numbers of samples, it might be more convenient to prepare the PCR reactions on ice. After addition of the polymerase mix to the last sample, the PCR reactions should be transferred to a preheated thermocycler (72 °C) and the PCR should be started immediately.
11. Peak sizes do not increase linearly with the use of increasing amounts of MLPA PCR products in the formamide/PCR reaction/size standard mix. Addition of a larger volume of the MLPA PCR products, also increases the salt concentration in this formamide-size standard-PCR reaction standard mix. The extra salt competes with the DNA fragments for injection in the capillary. Increasing the injection time / voltage might be more effective.
12. For each type of instrument the maximum level peak height that is quantified correctly is different. The signal of some types of instruments is saturated at a fluorescence level of 10.000 while other brands use arbitrary fluorescence levels that range up to 180.000. Preferably the highest peaks should be less than 75% of the instruments maximum.
13. Excessive evaporation can be checked by the incubation of 8 µl water overnight at 60 °C. The next morning, at least 5 µl water should remain at the bottom of the tube. In case of excessive evaporation, please try a different brand of tubes, check whether the heated lid works or try using mineral oil on top of the PCR mix.
14. The PCR reaction in MLPA is more sensitive to contaminants than normal PCR reactions. Contaminants include phenol, ethanol and salts. We strongly recommend comparing only samples extracted/purified by the same method. Samples derived from paraffin-embedded formalin-fixed tissues should be compared to each other or to DNA from healthy tissue that has undergone similar treatment. We have received several emails indicating problems with DNA purified by automated sample processing. In case of doubts about DNA quality, it is useful to try a DNA sample from another lab. At MRC-Holland, commercially-obtained DNA is also frequently used: Promega cat. nr. G1471 (male) and G1521 (female). It is often possible to clean contaminated samples by ethanol precipitation or silica columns (Qiagen). DNA losses can be reduced by inclusion of 10 µg glycogen (Roche Diagnostics) in ethanol precipitations. In case the Q-fragments are very low or absent, please try using less sample DNA.
15. Incomplete sample denaturation can be the result of ionic impurities such as Fe, Mg or Mn in the DNA sample. The source of these can be e.g. the use of old magnetic particles in automated DNA extraction devices. Complete DNA denaturation can be accomplished by prolonging the 5 minutes 98 °C denaturation step to 40 minutes and / or inclusion of 5% glycerol in the 5 µl DNA sample.
16. The no DNA-control of some probemixes contains an aspecific fragment. With increasing amounts of sample DNA, the size of both the small Q-control fragments and this aspecific fragment will decrease. When 20 ng or more DNA is used, the four Q-control fragments at 64-70-76-82 nt and any aspecific fragments will decrease and cannot influence the results obtained. Aspecific fragments are usually due to sequence similarity between two of the approximately 80 oligonucleotides in a MLPA probemix and not due to contamination. The main problem is that in the PCR reaction of the no DNA-control, there are *150.000.000 copies x number of probes* of long MLPA probe oligonucleotides containing a perfect copy of one of the PCR primers.

Please note

It is important to study printed peak profiles, including the 64-70-76-82 nt Q-fragments region, for troubleshooting. In case you would like to ask our opinion, please email us peak profiles including the Q-fragment region, preferably in a PowerPoint file. It is impossible for us to comment on Excel sheets with analysed data.

SALSA® MLPA® kits are sold by MRC-Holland for research purposes and to demonstrate the possibilities of the MLPA technique. This kit is not CE/FDA certified for use in diagnostic procedures. SALSA MLPA kits are supplied with all necessary buffers and enzymes. Purchase of the SALSA MLPA test kits includes a limited license to use these products for research purposes.

The MRC-Holland team