

## MLPA<sup>®</sup> DNA METHYLATION QUANTIFICATION PROTOCOL

The use of a thermal cycler with heated lid (105°C) is essential. We recommend 0.2 ml PCR-tubes. HhaI (Promega R6441, 10 units / µl) is not included in the kit.

### **DNA-Denaturation and Hybridisation of the SALSA-probes**

- Dilute the DNA-sample (20-200 ng DNA) with TE to 5 µl.
- Heat 10 minutes at 98°C; Cool to 25°C before opening the thermal cycler.
- Add: 1.5 µl SALSA Probe-mix (black cap) + 1.5 µl MLPA buffer (yellow cap).
- Mix with care. Incubate 1 minute 95°C, then ~16 hrs at 60°C.

### **Ligation + Digestion reaction**

- Add at room temperature a mix of 3 µl Ligase-buffer A + 10 µl water to each sample and mix. Transfer 10 µl to a second tube.
- Incubate 1 minute at 49 °C in the thermal cycler.
- While at 49°C, add 10 µl Lig mix to the first tube and mix (copy number test).
- While at 49°C, add 10 µl Lig-Dig mix to the second tube and mix (methylation test).
- Incubate 30 minutes at 49°C, then heat 5 minutes at 98°C.

#### **Lig mix (made less than 1 hr. before use and stored on ice)**

- Mix 1.5 µl Ligase-65 buffer B (white cap) + 8.25 µl Water. Add 0.25 µl Ligase-65 (brown cap) and mix again.

#### **Lig-Dig mix (made less than 1 hr. before use and stored on ice)**

- Mix 1.5 µl Ligase-65 buffer B (white cap) + 7.75 µl Water. Add 0.25 µl Ligase-65 (brown cap) + 0.5 ul HhaI (Promega R6441, 10 units / µl) and mix again.

### **PCR reaction**

- Mix 5 µl of each MLPA ligation or ligation-digestion reaction with 2 µl 10 X SALSA PCR buffer (red cap) + 13 µl Water.
- While on ice, add 5 µl Polymerase mix to each tube, place them in a preheated thermocycler (e.g. 72°C) and immediately start the PCR reaction.

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

- For each PCR reaction: Mix 1 µl SALSA PCR-primers (purple cap) + 1 µl SALSA Enzyme Dilution buffer (blue cap) + 2,75 µl Water. Add: 0.25 µl SALSA Polymerase (orange cap). MIX WELL.

### **PCR Conditions**

33 Cycles: 30 seconds 95°C; 30 seconds 60°C; 60 seconds 72°C

End with 20 minutes incubation at 72°C.

All reagents included in the methylation kits are identical to those in ordinary MLPA kits. No HhaI enzyme is included. We use Promega HhaI (R6441, 10 units / µl).

## Notes on denaturation / hybridisation reaction

1) We recommend to use between 20 and 200 ng of human chromosomal DNA (In ordinary MLPA reactions, 20-500 ng DNA is used). The DNA should be dissolved in water or 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 volume should not exceed 5 µl. The volume of the reaction is important for the hybridisation speed which is probe- and salt concentration dependent. Besides, the  $T_m$  of the probe-target binding also depends on the salt concentration.

2) SALSA Probe-mix and MLPA buffer can be mixed just in advance at room temperature by repeated pipetting. The MLPA buffer is viscous and does not mix easily. Do not centrifuge this mixture as the longer probes might precipitate !!! Hybridisation periods can be anywhere between 12 and 24 hrs. We use approx. 16 hrs. Hybridisation of probes to their targets is almost complete after 12 hrs. We recommend to spin the contents of the tubes for a few seconds before first use. 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.

3) At MRC-Holland, Biometra Uno II thermal cyclers are used. The heated lid is at 105°C. The following program covers the complete MLPA reaction:

1. 5 minutes 98°C; 25°C pause.
2. 1 minute 95°C; 60°C pause.
3. 49°C pause.
4. 30 minutes 49°C.
5. 5 minutes 98°C; 4°C pause
6. 72°C pause;
7. 30 seconds 95°C; 30 seconds 60°C; 60 seconds 72°C. 35 cycles.
8. 20 minutes 72°C; 4°C pause.

## Note on ligation / digestion reaction

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

## Notes on PCR reaction

1) Never use micro-pipettes for performing MLPA reactions that have been used for handling MLPA PCR products!! Following PCR, the tubes should not be opened in the vicinity of the thermal cycler.

2) In this protocol, preparation of the PCR reaction is performed on ice. This protocol should also work for ordinary MLPA reactions but has not been tested with all probemixes. Start the PCR as soon as possible after addition of the polymerase mix ! Some users prepare a mixture of water, PCR buffer and polymerase mix, distribute this among all vials and start the PCR reaction by addition of the ligation reaction.

3) Denaturation time of the PCR reaction can be reduced to 20 seconds in most thermal cyclers.

4) All volumes of the PCR reaction are reduced two fold as compared to ordinary MLPA reactions.

5) The recommended number of PCR cycles has been increased by us from 33 to 35. For most users however, 33 cycles is sufficient and many users could reduce the number of cycles to 30. A lower number of PCR cycles results in a very minor improvement in the linearity of relative probe signal with target sequence copy number. DNA samples containing only 10-20 ng human DNA may require 37 PCR cycles.

6) THE BLOB. In many PCR reactions an aspecific broad peak is present. Size depends on the apparatus and fluorescent primer used. On our Beckman CEQ2000, the size is 360 bp but we have seen ABI3100 pictures with the blob being clearly visible at 213 bp. This broad "blob" is also visible when no PCR cycles are performed. It might be due to fluorescent primer bound to another molecule or to a detergent micelle and therefore slowed down during electrophoresis. If this blob results in a problem in your MLPA reactions, the

solution might be to increase the number of PCR cycles to e.g. 38, which increases the signal-background ratio.

7) 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.

8) Contamination: MLPA is less prone to PCR contamination problems than ordinary PCR reactions. In an ordinary PCR reaction contamination with 10-100 copies of a previous PCR that used the same primers would result in a false-positive signal. 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 PCR reaction of an MLPA reaction there might therefore be 15.000 copies of each of the 40 different ligation products. At MRC-Holland the PCR apparatus are in the set up PCR lab. We have a strict rule that following the PCR, the tubes should not be opened in this same room. Up to now we do not have contamination problems.

9) INCOMPLETE MIXING OF THE VISCOUS 50 % GLYCEROL ENZYME SOLUTIONS WITH DILUTION BUFFERS ARE A MAJOR SOURCE OF ERRORS.

### **Separation/relative quantification of amplification products electrophoresis**

The amount of the MLPA PCR reaction required for analysis by capillary electrophoresis or by sequence gel electrophoresis depends on the apparatus and fluorescent label used.

**AGAROSE GELS / AGILENT DNA LabChips:** (10-12 probes). *SALSA unlabeled PCR primer-dNTP mix.* Use 10-15 µl of the PCR reaction for ethidium bromide stained 2.5 - 3 % agarose gels.

**ABI-310** (1 capillary): *SALSA 6-FAM PCR primer-dNTP mix.*

Following the PCR reaction, mix: 0.75 µl of the PCR reaction + 0.75 µl water + 0.5 µl internal standard (ROX-500 Genescan, ABI401734) + 12 µl deionised formamide (ABI nr.4311320).

Incubate the mix for 2 minutes at 94°C, and cool on ice.

Capillaries: 5-47 cm 50 µm. ABI402839. POP-4 polymer (But POP-6 will also perform well).

Settings: 5 seconds injection at 15 kV. Run time: 30 minutes at 60°C and 15 kV. Filter C.

**ABI-3100** (16 capillaries) and ABI 3100 Avant (4 capillaries): *SALSA 6-FAM PCR primer-dNTP mix.*

Following the PCR reaction, mix: 1 µl of the PCR reaction + 0.5 µl ROX-labeled internal size standard (ROX-500) + 8,5 µl deionised formamide (ABI nr.4311320).

Capillaries: 36 cm. Polymer: POP-4 or POP-6. Run temperature 60°C. Cap fill volume: 184 steps.

Pre run voltage: 15 kV. Pre run time: 180 sec. Injection voltage: 3.0 kV. Injection time: 10-30 sec.

Run voltage: 15 kV. Data delay time: 1 sec. Run time: 1500 sec.

**ABI-3700** (96 capillaries): *SALSA 6-FAM PCR primer-dNTP mix.*

Following the PCR reaction, mix: 2 µl of the PCR reaction + 0.2 µl ROX-labeled internal size standard (ROX-500 Genescan, ABI 401734) + 10 µl deionised formamide (ABI nr.4311320).

Capillaries : 3700 capillary array, 50 cm (ABI 4305787), POP-4 or POP-6 polymer (ABI 4306733). Settings: 2.5 µl sample volume, 10 seconds injection time at 10 kV. Run voltage 7,5 kV. Run time 4500 sec. Cuvette temperature 48°C. Run temperature 50°C. Filterset D.

**ABI-377** (slab gel): *SALSA 6-FAM PCR primer-dNTP mix.*

Following the PCR reaction, mix: 3 µl of the PCR reaction + 0.5 µl TAMRA-labeled internal size standard (TAMRA-500) + 0.6 µl Dextran-blue (Concentration ?) + 1.9 µl deionised formamide.

Gel used was made with "Long Ranger Singel Pack" type 377-36 cm WTR (company BMA, cat. nr. 50691).

Volume 50 ml, 6 M Ureum, Long Ranger 5%, 1 x TBE, 0.05% APS, 0.7% TEMED, sufficient for two gels. Run conditions: 36 cm gel; 64 slot shark tooth, 3000 Volt; 60 mA; 200 Watt; 51°C. ; Laser Power 40 mW. Run time 3 hr. The signal of the TAMRA sizer is quenched by the FAM label. The size marker is difficult to see and should perhaps be loaded in a separate lane. ROX might be a better choice ?

**LICOR IR2:** *SALSA IR800 PCR primer-dNTP mix.*

Following the PCR reaction, mix: 1 µl of the PCR reaction + 1 µl Formamide + 2 µl loadingmix (LICOR cresol-

red). Incubate the mix for 2 minutes at 65 °C and load 0.8 µl on a 25 cm long; 0.25 mm thick; 48 well; 6 % denaturing sequence gel.

Comment: The LICOR gels give a perfect scan of the complete gel. Results are obtained as a TIFF file and can easily be exported for analysis by software programs. We have not used any software program for quantification of the bands on the TIFF files obtained.

**Beckman CEQ-2000:** *SALSA D4 PCR primer-dNTP mix.*

Following the PCR reaction, mix: 0.7 µl of the PCR reaction + 0.5 µl of the Beckman D1-labeled 60-600 molecular weight marker + 40 µl deionised formamide (ABI nr.4311320) or the more expensive Beckman Sample Loading Solution.

Settings: Capillary temperature: 50 °C. Denaturation: 90 °C for 90 sec. Injection time: 2.0 KV for 60 seconds. Runtime: 60 minutes at 4.8 KV. Analysis settings: Include peaks >3%; Size standard-600 (Beckman nr. 608095); Slope threshold 1.

Comments: Most of our results were obtained on the Beckman CEQ2000, which is much cheaper than the ABI 3100. Compared to all ABI apparatus, it is impressive that you can move the apparatus to another location and start running immediately. No help from ABI specialists for mirror adjustments required. Ease of use is excellent.

**ALF-express** (Amersham-Pharmacia): *SALSA CY5.0 PCR primer-dNTP mix.*

Following the PCR reaction, mix: 2 µl of the PCR reaction + 4.4 µl water + 6.5 µl loading-mix (0.5 mg/ml dextran-blue in formamide / dextran-blue).

Incubate this mix for 2 minutes at 80 °C and load 8 µl on a 6 % denaturing sequence gel.

Comment: The sensitivity of the ALF -Express is very good. Pictures look nice. However the fixed positions of the 40 detectors results in unreliable results, as most often the lanes are not perfectly passing the detectors. Few gels are 100 % perfect. In some cases the signal obtained may even be due to the MLPA reaction loaded in the neighbouring lane. We recommend to compare peak areas of MLPA products with peak areas of the neighbouring MLPA products rather than normalising peak areas to the total peak area of all MLPA peaks.

**Megabace** (Amersham-Pharmacia): *SALSA 6-FAM PCR primer-dNTP mix.*

Following the PCR , mix: 0.6-1.0 µl of the PCR reaction + 0.25 µl ET-550R (sizer) + 7 µl 0.1% Tween-20. Denature 2 minutes at 95 °C and load 1.5-2 minutes at 3 kV. Run 90 minutes at 10 kV.

**Visible Genetics:** *SALSA CY5.0 PCR primer-dNTP mix.*

**SEQUENCE OF THE PCR-PRIMERS (5'-3'):**

SALSA PCR Forward primer (Labeled): \*GGGTTCCCTAAGGGTTGGA

SALSA PCR Reverse primer (Unlabeled): GTGCCAGCAAGATCCAATCTAGA

***MLPA kits are sold by MRC-Holland only for research purposes and to demonstrate the possibilities of the MLPA technique. Our kits are not FDA / CE certified for use in diagnostics.***

**PLEASE NOTE:** Polymerase and PCR primers are included in MLPA kits. However, no license for any patent other than our MLPA patent is conveyed by MRC-Holland to the purchaser of an MLPA kit.

This product is optimized for use in the Polymerase Chain Reaction ("PCR") covered by patents owned by Hoffman-La Roche Inc. and F.Hoffman-La Roche Ltd. No license under these patents is conveyed expressly or by implication to the purchaser by the purchase of this product. A license to use the PCR process is available from the Perkin-Elmer Corporation, 850 Lincoln Center Drive, Foster City, California 94404 U.S.A.

Patent applications on the production and use of thermostable polymerases, such as the SALSA polymerase, have been invalidated in many countries. If the use of heat stable polymerases is still protected by patents in your country, the SALSA polymerase can be disposed of as being non-hazardous waste. Replacement by a licensed preparation of Taq polymerase (5 units/ul) will usually provide you with acceptable MLPA results.

## **Data analysis**

Analysis of a limited number of samples can easily be done by visual examination of the capillary electrophoresis peak profiles. A two color, on screen, overlay of sample and control reaction can be accomplished by genotyper software for instance by changing the values for the length of the size markers by 2 nt for the control sample. For the analysis of large numbers of samples, exportation of peak areas to Excel files is necessary.

A file describing the analysis of results using Genotyper and Excel software (kindly made available by Petra Nederlof of the Netherlands Cancer Institute) can be found on our website [www.mrc-holland.com](http://www.mrc-holland.com). Care should be taken that the template used for recognising the probe peaks is perfect. Wrong settings will result in some probe target sites appearing to be homozygote deleted!

At MRC-Holland we export the size and peak areas obtained to an Excel file. Non specific amplification products, primer dimer peaks and the peaks generated by the MLPA control mix can usually be removed due to their low peak area and/or small lengths. When only the peak areas of the expected MLPA products are left, all peak areas are normalised by dividing each peak area by the combined peak area of all peaks in that lane.

These normalised peak areas are compared to the average results obtained on all samples.

First, the normalised peak areas are divided by the average normalised peak area of that probe amplification product of all samples. All results differing by more than 20 % are highlighted. Raw data of samples that give many aberrations are checked. Very low peaks of both the MLPA products as well as the size standard may indicate failure of the PCR reaction in the capillary. Low MLPA products peaks but high residual primer peak may indicate failure of the PCR reaction. High MLPA control peaks (64, 70, 76, 82 bp) but low MLPA product peaks indicate insufficient sample DNA or failure of the ligation reaction.

Results of samples with non reliable results are removed. Again, for each sample, the normalised peak areas are divided by the average normalised peak area of that probe amplification product of all samples. These results are visualised using Excel graphics.

A deletion of one copy of a probe target sequence will usually be apparent by a reduction in relative peak area for that probe amplification product of 35-55 %. A gain in copy number from two to three copies / diploid genome will usually be apparent by an increase in relative peak area between 30 and 55 %. Standard deviation of peak areas should be below 10 % for all probes.

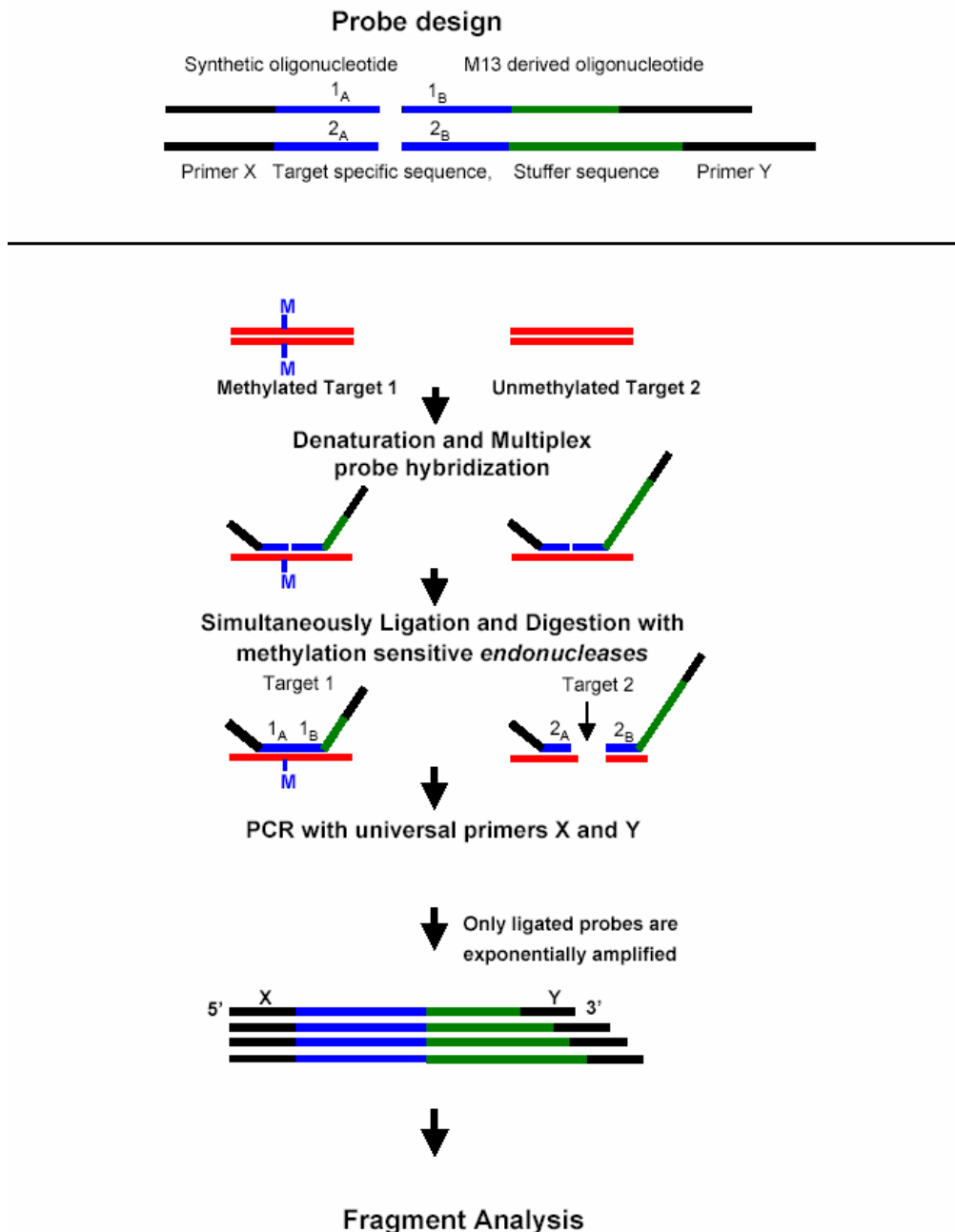
Analysis methods may differ for different applications. Our MSH2/MLH1 exon deletion probe mix P003 contains 19 (out of 42) probes for the MLH1 gene. It is designed to detect samples having a deletion spanning one or more exons, which will usually be apparent by reduction of the relative probe signal of one or a few probes by 40-55%. In samples in which one copy of the complete MLH1 gene was deleted, relative probe signals of the MLH1 exon probes were reduced by less than 40 %. In this case, the MLH1 probe signals should be compared to the probe signals of all non MLH1 probes. Similarly the probe signals of the three ERBB2 (her2-neu) probes in our ERBB2 amplification assay should be compared to the probe signals of the non ERBB2 probes.

More information about MLPA is available at [www.MRC-Holland.com](http://www.MRC-Holland.com)

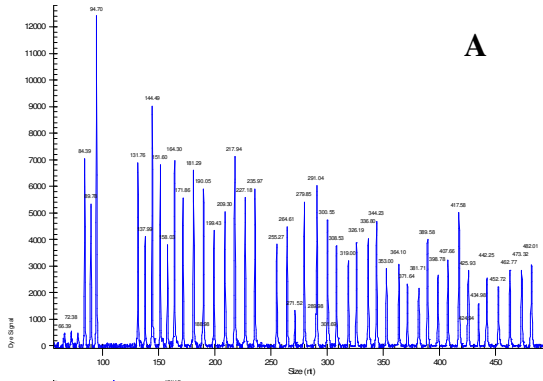
**Contact us at: [info@mrc-holland.com](mailto:info@mrc-holland.com).**

# MS-MLPA®

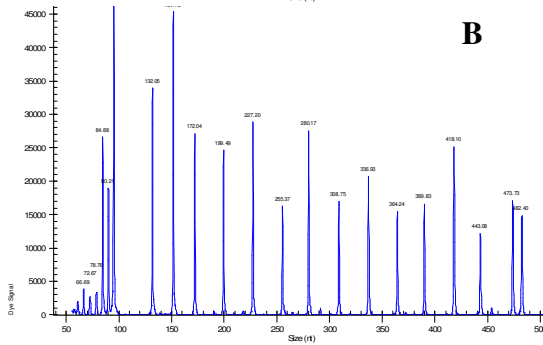
Figure 1 shows an outline of the MS-MLPA procedure. An ordinary MLPA probe harbors two oligonucleotides, one short synthetic and one long M13-derived oligonucleotide and up to 50 probes can be added to each MLPA reaction. Both oligonucleotides contain universal primer sites and the M13 derived oligonucleotide contains a stuffer sequence that varies in length between the different probes. For MS-MLPA, the probe design is similar to an ordinary MLPA probe except that the sequence detected by the MS-MLPA probe contains a recognition sequence for *HpaII* or *HhaI*. Upon digestion of the DNA/MSMLPA probe complex with one of the methylation sensitive enzymes, probes of which the recognition sequence is methylated will generate a signal. If the site is unmethylated the genomic DNA/MS-MLPA probe complex will be digested and prevent exponential amplification and no signal will be detected after fragment analysis. MS-MLPA results of ME001 are viewed in Figure 2.



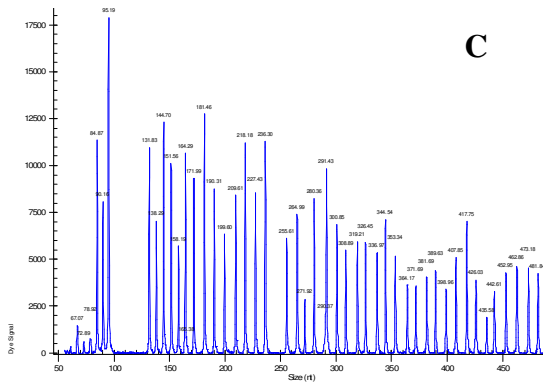
**Figure 1:** MS-MLPA procedure.



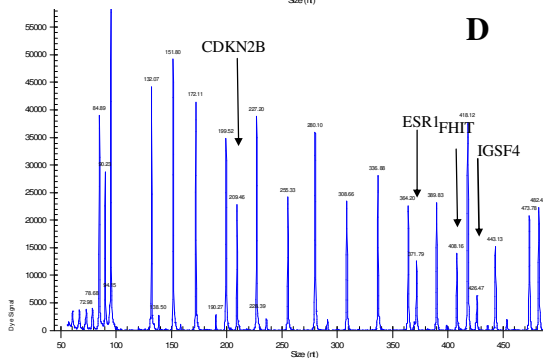
**A** ME001 mix-Control DNA Sample Undigested



**B** ME001 mix-Control DNA Sample Digested with *Hha*I



**C** ME001 mix-Patient DNA Sample Undigested

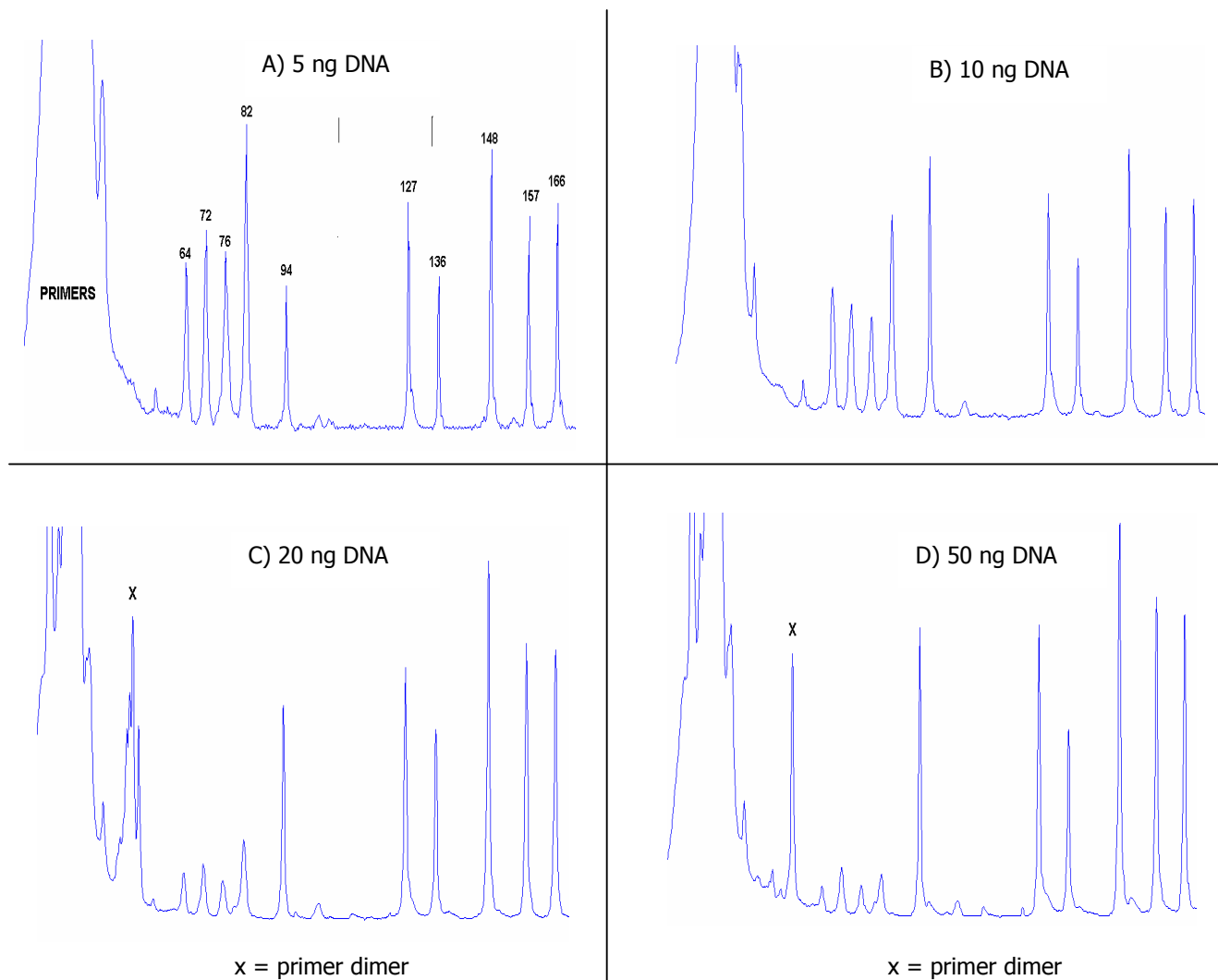


**D** ME001 mix-Patient DNA Sample Digested with *Hha*I

**Figure 2:** MS-MLPA example using ME001. Figure A shows MS-MLPA with undigested control Promega DNA. Figure B shows the same sample but digested with *Hha*I. Using control Promega DNA all the methylation-sensitive probes are digested and thus no amplification product is detected. Only control probes without an *Hha*I site can be detected (B). In Figure C en D a MS-MLPA is depicted of a Patient sample showing methylation of the *CDKN2B*, *ESR1*, *FHIT* and *IGSF4* genes.

## MLPA Control Fragments

In each MS-MLPA kit DQ (DNA Quantity) and DD (DNA Denaturation) control fragments are included. The MLPA DQ fragments will generate amplification products of 64, 70, 76 and 82 bp even when ligation is omitted or when no sample DNA was present. Purpose of these DQ fragments is to give a warning when the amount of sample DNA used was lower than the 20 ng human DNA that is required for reliable MLPA results. The MLPA DD control fragments contain three small control probes with amplification products of 88, 92 and 96 bp. Similar to ordinary MLPA probes, the amplification products of these three control probes are sample DNA- and ligation-dependent. These amplification products are generated by probes that consist of two synthetic probe oligonucleotides and give a warning when the DNA is not completely denatured.



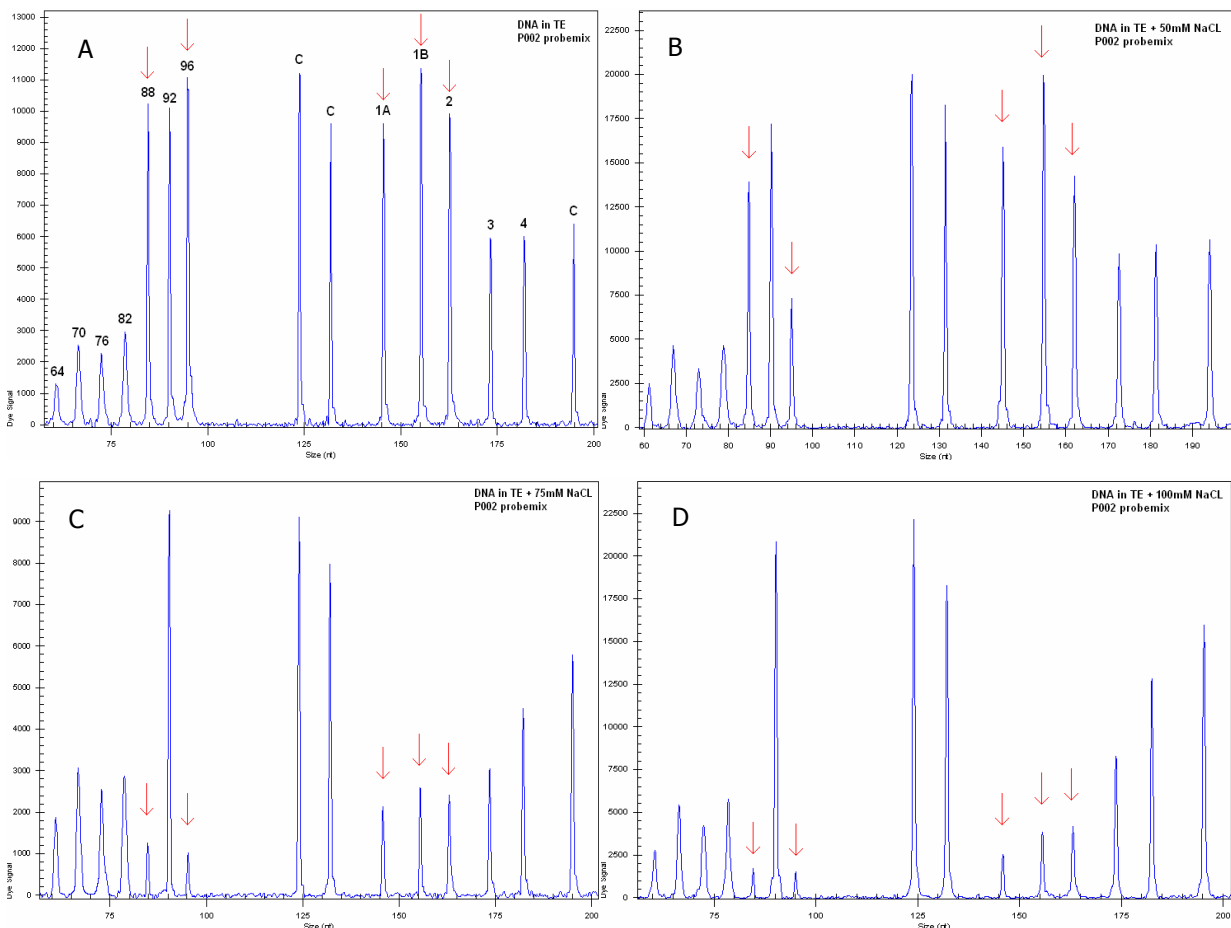
**Figure 3:** MLPA results when 5 ng (A), 10 ng (B), 20 ng (C) or 50 ng DNA is used (D). Primer-dimer peaks are often present at approximately 55 bp.

Amplification products of the MLPA DQ control fragments of 64, 70, 76 and 82 bp will be prominent if the amount of sample DNA is very low (Figure 3). These four fragments do not require a ligation event for amplification. If the 64-70-76-82 bp DQ amplification products have similar or larger peak sizes than the 88-92-96 bp fragments and the 130-472 bp ordinary MLPA probe amplification products, either the ligation reaction failed or the amount of sample DNA was less than 20 ng. In either case, the results obtained may

not be reliable. All signals will probably be low as the 33 cycles of the PCR reaction are not sufficient to generate a good signal (Figure 3).

Besides these 4 small control fragments, the MS-MLPA kits contain three small control probes with amplification products of 88, 92 and 96 bp. Similar to ordinary MLPA probes, the amplification products of these three control probes are sample DNA- and ligation-dependent. These amplification products are generated by probes that consist of two synthetic probe oligonucleotides. The peak size of the 92 bp amplification product reflects the copy number of a 2q14 DNA sequence. The peak area of the 92 fragment should be of similar size as most of the other MLPA amplification products in the 127-454 bp range. In case the 92 bp amplification product is much smaller than the 88 and 96 bp fragments, hybridisation of probes to their targets might not have been complete. We deliberately add less of this 92 bp control probe. Incomplete hybridisation can be the result of the use of insufficient long hybridisation times, the use of insufficient amounts of probemix and/or MLPA buffer, the use of larger volumes of DNA solution (> 5 ul), a lower than 60 °C hybridisation temperature, a lower than 105 °C temperature of the thermocycler lid etc.

The 88 bp fragment is specific for a sequence in the CpG island preceding the FANCE gene on 6p21.3. The 96 bp probe is specific for a sequence within the CpG island preceding the TP73 gene on 1p36. These two CpG islands both have a long sequence with a very high % C/G nucleotides. These chromosomal regions are therefore very difficult to denature. In case the 88 and 96 bp amplification products are much lower than the 92 and 127-454 bp amplification products, denaturation of the sample DNA might have been incomplete, resulting in unreliable results for probes that detect sequences in or close (<5 Kb) to a CpG island (Figure 4) An extensive description of these control probes can be found in 'probes close to a CpG island'.



**Figure 4:** MLPA P002 probes signals from DNA in TE (A), with 50mM NaCl (B), 75 mM NaCl (C) and with 100 mM NaCl (D). 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 bp: quality control fragments, c: control probe, 1-5: BRCA1 probe for exons 1-5.

**Probes close to a CpG island**

Apparent deletions detected by a single MLPA probe always require confirmation by other means. In general, the reduction in probe signal of two or more consecutive MLPA probes by more than 30% is a very strong indication that a true deletion is present. However, apparent deletions of two or more consecutive exons that are all located in close proximity of a strong CpG island have to be treated with caution. An example is the apparent deletion of BRCA1 exon 1A, 1B and 2. In case the sample DNA is incompletely denatured, all seven P002 and P087 probes for BRCA1 exons 1A, 1B and 2 may show reduced relative probe signals.

Complete denaturation of sample DNA is essential for all methods for quantification of specific DNA sequences. Denaturation of DNA is usually accomplished by heating the sample DNA. Before the start of MLPA reactions, the DNA is denatured by heating 5 minutes at 98 °C. Genomic DNA denatures at temperatures around 80 °C when the DNA is dissolved in TE (10 mM Tris-HC : 1 mM EDTA). The presence of higher concentrations of salt increases the denaturation temperature (T<sub>m</sub>) considerably. Besides, this T<sub>m</sub> is dependent on the % C/G of the DNA.

The human genome contains thousands of sequences with a length of several hundreds to thousands of nucleotides, that have a very high % C/G. These CpG islands are often located around the transcription start sites of genes (close before exon 1) and are more difficult to denature than the rest of the genome.

By heating the DNA for several minutes to 98 °C, as well as by pipetting, the size of the genomic DNA is usually reduced to fragments with an average size of 5.000-20.000 nucleotides. Incomplete denaturation of a part of such a long fragment (the CpG island) will keep the two complementary strands together and upon lowering of the temperature the two strands will immediately renature. As a result, a large part of the sequences that are located less than 5-10 Kb from a CpG island will not be available for binding of MLPA probes. These probes will generate a lower signal on incompletely denatured DNA as compared to probes recognising sequences located further away from CpG islands. As a result the apparent copy number of these incompletely denatured sequences seems to be lower compared to samples that are completely denatured. Renaturation of properly denatured, single copy, genomic DNA sequences requires many weeks of hybridisation.

In ordinary MLPA reactions, the size of the amplification products of the three control probes at 88, 92 and 96 bp will be similar (less than 50 % difference in peak height). However, the peak size of the 88 and 96 bp control fragments can be reduced, caused by incomplete denaturation due to CpG islands. As shown in Figure 4 the addition of up to 50 mM NaCl to a DNA sample in TE has no effect on the relative probe signals. At 75 mM NaCl however, the synthetic probes for the CpG islands of FANCE (88 bp) and TP73 (96 bp), as well as the probes for the first BRCA1 exons show a reduced relative probe signal. At 100 mM NaCl, the 88 and 96 bp control probes show a strongly reduced signal due to incomplete denaturation.

In general, most users will use only 1 µl of a DNA preparation and dilute this to 4 µl with TE for MLPA analysis. In order to obtain a salt concentration of 75 mM, the DNA should be 375 mM in salt when only 1 µl is used. Few DNA samples will have a salt concentration of > 375 mM. However, many other ions such as Mg have a much stronger effect on the T<sub>m</sub> of DNA than NaCl at very low concentrations.

DNA preparations isolated by certain lots of commercial kits can give aberrant MLPA results. Impurities present in these DNA preparations may affect the PCR part of MLPA reactions. We expect that samples showing similar peak signals for the 88 and 96 bp control fragments as compared to the 92 bp and the 127-454 bp ordinary MLPA peaks are certainly completely denatured. In case the 88 and 96 bp control fragments apparently are not completely denatured, please try performing an MLPA reaction on lower amounts of sample DNA (lower amounts of ions / impurities) or try addition of 0.5 ul glycerol (resulting in a lower T<sub>m</sub>) to the MLPA reaction.

Please note that our results show that most CpG islands in human genomic DNA that is dissolved in ordinary 1 x PCR buffer (containing 50 mM KCl; 1.5 mM MgCl<sub>2</sub>) are not completely denatured by heating to 95 °C. Amplification products will be obtained by ordinary PCR even for sequences in CpG islands since a small part of the DNA is degraded and will be denatured. Real time PCR for the quantification of sequences located < 5-10 Kb from CpG islands will be unreliable if denaturation is performed in the presence of PCR buffer, as results will depend on the quality of the DNA. Degraded DNA will appear to have a higher copy number of sequences located close to CpG islands as compared to undegraded DNA. For PCR reactions of sequences close to CpG islands, we recommend to denature genomic DNA before the addition of PCR buffer. After denaturation, PCR buffer, dNTPs, primers and polymerase can be added.

## Troubleshooting

### A) No bands visible.

1. Is the molecular weight marker pattern ok ? If not, check capillaries and electrophoresis conditions.
2. Is the PCR primer peak present, and off-scale ? If not, check whether the correct fluorescent label is used. Usually FAM label for ABI apparatus, D4 for Beckman CEQ and IR800 for LICOR.
3. Are the four control fragments visible ? Each SALSA probe mix will generate 4 fragments of 64, 70, 76 and 82 bp even when ligation is omitted. These bands will be small when more than 50 ng human DNA is used for MLPA. If these bands are stronger than the expected MLPA amplification products, either the ligation reaction failed or the amount of sample DNA was much lower than the recommended minimum amount of 20 ng. In either case the results obtained may not be reliable. A fifth band of 94 bp is generated by the control mix, which is ligation-dependent. This is an amplification product of a synthetic MLPA probe specific for the human IL1B gene. This band should be of similar peak area as most of the expected MLPA amplification products. A picture of the control band region is shown on the next page.

**Primer peak ok, but no control fragments and no probe amplification products:** PCR reaction failed. We recommend to repeat the PCR reaction using the same ligation reactions, for instance using a different thermal cycler. Reduce all volumes by 50% to save reagents.

**Primer peak ok, control fragments clearly visible, but no probe amplification products:** No DNA was present in your sample, or the ligation reaction failed. The MLPA reagents are quite stable. After storage of a complete MLPA kit for 12 weeks at room temperature, including probes, ligase and polymerase, good results were obtained. However, the Ligase cofactor NAD, present in Ligase buffer A might be inactivated by more than 15 freeze-thaw cycles. Enzymes may be inactivated by repeated freeze-thaw cycles in freezers operating at -25 / -30°C.

### B) Bands visible, but weak signals.

The tubes of the PCR reaction can be placed in the thermal cycler for another 4 cycles, or a new PCR using the same ligation reactions but with 36-40 cycles might help. Reduce all volumes by 50% to save reagents.

**Increasing the number of PCR cycles to 40, will not influence results!**

### C) Signal strength ok but large differences in relative peak areas between different samples.

This might be due to impurities in the DNA preparations. The use of lower amounts of sample often solves the problem. PCR protocol 2 should be used. Another possibility is overloading of capillaries resulting in saturation of the fluorescence detection device. Broad peaks, especially of longer fragments can be caused by incomplete denaturation during the electrophoresis.

### D) Extra peaks.

Incomplete denaturation during the electrophoresis can result in extra peaks as well as broad peaks especially of longer fragments. An increase in the run temperature might solve this. In some apparatus such as ABI3700, overloading of capillaries may result in signals in neighbouring capillaries.

### E) Low peak area of the longer probes.

This can have many causes. The volume of the DNA sample + probemix +MLPA buffer is 8 µl. Evaporation during the 16 hr. hybridisation reaction is low due to the heated lid. In general, more than 6-6.5 µl fluid remains at the bottom of the tube after this 16 hr incubation. If the volume is lower than 5 µl for instance due to bad closure of the tubes, incorrect heated lid temperature etc. "fading" of the peak heights with increased length of amplification product can occur. Centrifugation of a mixture of MLPA buffer and probemix can cause the larger probes to precipitate. Mix therefore by repeated pipetting. Impurities in DNA samples also affect in particular the longer probes and those probes that already have a lower than average peak area. Finally the electrokinetic injection procedure of some capillary electrophoresis seems to select for injection of smaller fragments. This appears to be more pronounced with ABI sequencers as compared to the Beckman CEQ.

**This methylation MLPA protocol was developed by Anders Nygren, Abdellatif Errami & Jan Schouten at MRC-Holland (submitted for publication).**