Chapter 11

Multiplex Ligation-Dependent Probe Amplification (MLPA) for Prenatal Diagnosis of Common Aneuploidies

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Abstract

Multiplex Ligation-dependent Probe Amplification (MLPA) is a method to determine the copy number of up to 60 genomic DNA sequences in a single multiplex PCR based reaction.

MLPA probes consist of two oligonucleotides that can hybridize next to each other on a certain DNA sequence of interest, where they are ligated. All ligated probes are subsequently amplified by PCR using a single set of primers. Each amplified MLPA probe has a unique length and can be visualized and quantified by capillary electrophoresis. As the primers are almost 100% consumed in the PCR reaction, the quantity of each PCR amplicon is proportional to the number of copies of each probe target sequence in the DNA sample. A trisomy 21 can therefore be detected by an approximately 50% increased signal of each chromosome 21 specific probe relative to reference samples.

MLPA with the P095 Aneuploidy probemix for chromosomes 13, 18, 21, X and Y has been used as a rapid detection method on large numbers of samples from uncultured amniotic fluid or from chorionic villi. As compared to FISH and karyotyping, MLPA is more rapid, has a higher throughput, and is less expensive. MLPA however cannot detect low grade mosaicism, female triploidies, and copy number neutral chromosome abnormalities such as inversions and translocations.

Key words Aneuploidy, Multiplex ligation-dependent probe amplification (MLPA), Trisomy, Multiplex polymerase chain reaction (PCR), Amniotic fluid, Chorionic villi, Gene dosage

1 Introduction

Multiplex Ligation-dependent Probe Amplification (MLPA) is a multiplex method to detect abnormal copy numbers of up to 60 different genomic DNA sequences [1]. MLPA reactions are easy to perform, require little hands-on time and results can be obtained within 24 h. Furthermore, it requires only standard equipment that is present in most DNA diagnostic laboratories.

In contrast to normal multiplex PCR, in MLPA not the sample DNA is amplified, but the probes that are hybridized to the target DNA (Fig. 1). Each single probe initially consists of two oligonucleotides (left probe oligo [LPO] and right probe oligo [RPO]), which are designed to hybridize immediate adjacent to each other

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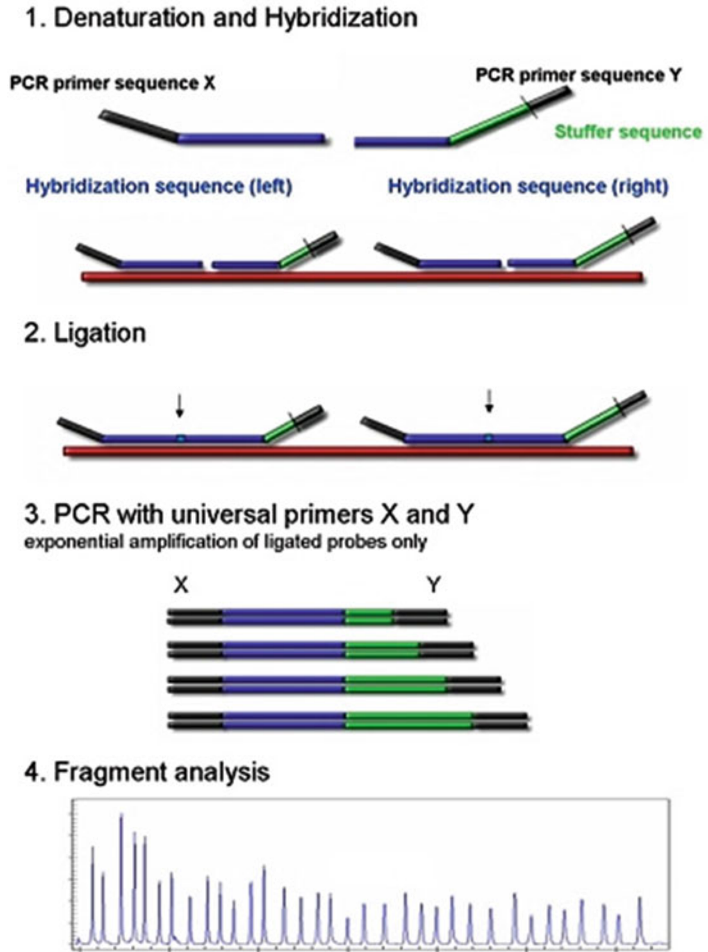

Fig. 1 Outline of the MLPA technique. After hybridization to their target sequence in the sample DNA, the probe oligonucleotides are enzymatically ligated. One probe oligonucleotide contains a non-hybridizing stuffer sequence of variable length. Ligation products can be amplified using PCR primer sequences X and Y, amplification product of each probe has a unique length (90–500 nt). Amplification products are separated by capillary electrophoresis. Relative amounts of probe amplification products, as compared to a reference DNA sample, reflect the relative copy number of target sequences. Adapted from www.mlpa.com

on the target DNA. After hybridization, the two oligonucleotides can be ligated by a specific ligase enzyme, thereby creating a single molecule, but only when both probe oligonucleotides are perfectly hybridized to adjacent sites of the sample DNA. The ends of this probe molecule contain two sequences recognized by a PCR primer pair. After ligation, all probes are amplified by a single PCR primer pair, of which one is fluorescently labeled. Since all probes have a unique length, the resulting MLPA amplified products have a size that ranges from 90 to up to 500 nucleotides, and can be visualized by capillary electrophoresis.

MLPA probes that do not find a target sequence cannot be amplified by PCR, and do not have to be removed. The protocol for an MLPA reaction is therefore very simple (Fig. 1):

1. Denature 20–500 ng DNA by heating to 98 °C in a thermocycler, followed by addition of the MLPA probes and buffer. Leave overnight at 60 °C for hybridization.
2. Add the Ligase and ligase buffers and ligate at 54 °C for 15 min.
3. Inactivate the Ligase by heating to 98 °C. Add PCR primers, dNTPs and polymerase and start the PCR.
4. Analyze the products by capillary electrophoresis.

In MLPA, it is important that all fragments are amplified with the use of only one pair of PCR primers that is present in limited amounts. This abolishes differences in PCR efficiencies, which is normally observed in multiplex PCRs, as well as the difference in DNA input among multiple reactions. As a result, the relative signal strength of each amplification product is determined primarily by the copy number of the target sequence in a DNA sample. By comparing these probe signals with those obtained from reference samples, a decrease or increase in signal intensity can be observed, reflecting a deletion or duplication, respectively (Fig. 2).

The MLPA technique allows discrimination of sequences that differ only in a single nucleotide. MLPA can therefore also be used for the detection of known mutations. A variation on the MLPA technique can be used to determine the methylation status of DNA sequences [2]. MLPA products for more than 400 different

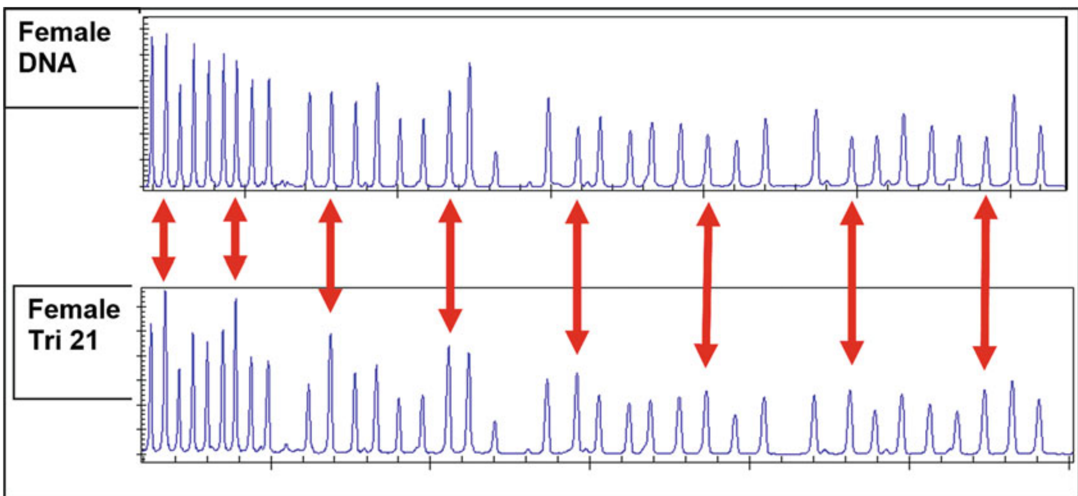

Fig. 2 Detection of trisomy 21 with MLPA. Arrows indicate alterations. An increase of the signal from the chromosome 21 specific probes is seen

applications are commercially available from the MRC-Holland company (www.mlpa.com).These include MLPA probemixes for the detection of:

1. Aneuploidy of chromosomes 13, 18, 21, X, and Y (P095 probemix).
2. Deletions or duplications of specific chromosomal areas, e.g., the P250 probemix for the 22q11 region involved in DiGeorge syndrome.
3. Copy number changes of all 41 unique subtelomeric regions (P036 and P070 probemixes), all centromeric regions (P181, P182 probemix) or 23 different microdeletion syndromes, in a single reaction (P245 probemix).
4. Deletions or duplications involving only one or more exons of a gene (e.g., DMD, BRCA1 or BRCA2). Small chromosomal rearrangements can be detected as the sequences analyzed by MLPA probes are only 60 nucleotides in length. Probemixes for more than 100 different genes are available.

For aneuploidy testing on capillary electrophoresis systems, the SALSA P095 probe mix is available. This probemix contains eight independent probes for each of the chromosomes 13, 18, 21 and X, as well as four Y-specific probes, and is used as a rapid test for aneuploidies of these chromosomes. Extensive tests, several of which included more than 4000 samples, indicated a diagnostic accuracy of MLPA for the detection of common trisomies that is statistically similar (non-inferior) to that of karyotyping or FISH [3–10].

MLPA is not able to detect all chromosomal abnormalities seen with karyotyping. It is designed for detection of specific copy number changes of chromosomes 13, 18, 21 and the sex chromosomes. MLPA analysis is expected to detect high level of chromosomal mosaicism since it will give the average copy number per cell. The P095 probemix is able to detect male triploidies but will not detect cases of 69,XXX [3–6]. Cases of 69,XXY may be difficult to discriminate from maternal DNA contamination as was shown for a case of 69,XXY which was assigned as a sample contaminated by maternal DNA [4]. However we have correctly diagnosed two cases [6]. It should be noted, though, that many triploidies result in fetal ultrasound abnormalities.

The MLPA aneuploidy kit is not designed for detection of balanced chromosomal rearrangements like translocations and inversions. MLPA however provides several opportunities that are not possible with other techniques such as karyotyping. For example, when a quick diagnostic result is warranted in case of fetal abnormalities visualized by advanced ultrasound examination, amniotic fluid samples can first be rapidly tested for the copy number of the most common occurring aneuploidies. If normal,

further selective testing based on clinical preselection could be done for using MLPA probemixes for all subtelomeric regions and the most common microdeletion syndromes.

2 Materials

2.1 Contents of SALSA MLPA Probemix and Reagents

1. SALSA Probemix: Mixture of up to 60 pairs of probe oligonucleotides.
2. SALSA MLPA buffer: Contains 1.5 M salt + additives. Does not always freeze at −20 °C.
3. Ligase-65 enzyme solution. Does not freeze at −20 °C.
4. Ligase-65 Buffer A: Contains cofactor NAD required for the Ligase-65 enzyme.
5. Ligase-65 Buffer B: Contains the salts required by the Ligase-65 enzyme.
6. SALSA PCR Primer mix: Contains one fluorescently labeled and one unlabeled PCR primer + dNTPs.
7. SALSA Polymerase enzyme solution. Does not freeze at −20 °C.

SALSA MLPA kits are stable for at least 1 year when stored in the dark at −20 °C.

All enzymes, nucleic acids, and buffer constituents are non-hazardous.

In addition to standard lab equipment, such as pipettors and water, a thermocycler with heated lid and capillary electrophoresis instrument is required.

2.2 DNA Extraction from Amniotic Fluid

1. PBS .
2. QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany).

2.3 Separation and Quantification of the MLPA Amplification Products by Capillary Electrophoresis Using the Beckman CEQ Apparatus

1. Beckman D1-labeled 60–600 size standard (Beckman Coulter, Brea, CA, USA).
2. Deionized formamide.

3 Methods

3.1 Amniotic Fluid Sample Preparation Protocol: DNA-Isolation

1. Mix the amniotic fluid gently, just before a sample is removed. Most cells might be at the bottom of the tube.
2. Centrifuge a 2 mL sample of amniotic fluid for 5 min at 10,000 × *g*. Remove the supernatant carefully. Wash the pellet

with 1 mL of PBS and centrifuge for 5 min at 10,000 × *g*. Remove the supernatant carefully and resuspend the pellet in 200 μL of PBS. DNA-isolation is done according to the Qiagen Blood and Body Fluid Spin Protocol. DNA is eluted with 50 μL buffer AE instead of 200 μL to increase the DNA concentration. Transfer 5 μL DNA to a 0.2 mL vial tube for the MLPA reaction. Store the remainder at −20 °C.

3.2 DNA Denaturation and Hybridization of the SALSA Probes

1. Heat 5 μL DNA-sample (20–500 ng DNA) (*see* **Notes 1** and **2**) for 5 min at 98 °C in a 0.2 mL vial in a thermocycler with heated (105 °C) lid.
2. Cool to 25 °C before opening the thermocycler.
3. Prepare a mixture of equal volumes SALSA Probemix and MLPA buffer at room temperature. Mix well.
4. Add 3 μL of this mixture to each sample. Mix with care by repeated pipetting.
5. Incubate for 1 min at 95 °C, followed by a 16 h incubation (*see* **Note 3**) at 60 °C (*see* **Note 4**).

3.3 Ligation Reaction

1. Prepare a Ligation master mix containing 3 μL Ligase-65 buffer A, 3 μL Ligase-65 buffer B, 25 μL water, and 1 μL Ligase-65 enzyme for each reaction (*see* **Note 5**). Mix well by repeated pipetting.
2. Reduce the temperature of the thermocycler to 54 °C.
3. Add 32 μL Ligase master mix to the MLPA reaction, while the samples are in the thermocycler, and mix by repeated pipetting.
4. Incubate for 15 min at 54 °C, than heat 5 min at 98 °C for Ligase inactivation. Remove vials from the thermocycler.

3.4 PCR

1. Prepare a Polymerase master mix for each reaction containing 2 μL of SALSA PCR-primer mix, 7.5 μL of water, and 0.5 μL of SALSA Polymerase. Mix well but do not vortex. Store on ice until used (*see* **Note 5**).
2. While the vials are at room temperature, add 10 μL of Polymerase master mix to each vial. Mix by pipetting up and down, place the vials in the thermocycler, and start the PCR reaction (*see* **Note 6**).

3.4.1 PCR Conditions

- 30 s 95 °C.
- 30 s 60 °C.
- 60 s 72 °C; 35 cycles.

End with 20 min incubation at 72 °C (*see* **Note 7**).

3.5 Separation and Quantification of the MLPA Amplification Products by Capillary Electrophoresis

The amount of the MLPA PCR reaction required for analysis by capillary electrophoresis depends on the apparatus and fluorescent label used. As an example, conditions for the Beckman CEQ apparatus are shown:

1. Following the PCR reaction, mix 0.7 μL of the PCR reaction, 0.2 μL of the Beckman D1-labeled 60–600 size standard, 32 μl deionized formamide.
   - Settings: Capillary temperature 50 °C. Denaturation 90 °C for 120 s. Injection time: 1.6 KV for 30 s. Runtime: 60 min at 4.8 KV. Analysis settings: Include peaks >3%; Size standard-600. Slope threshold 1.

3.6 Thermocycler Program for the Complete MLPA Reaction

- 5 min 98 °C; 25 °C pause.
- 1 min 95 °C; 60 °C pause.
- 54 °C pause; 15 min 54 °C.
- 5 min 98 °C; 20 °C pause.
- 30 s 95 °C; 30 s 60 °C; 60 s 72 °C, 35 cycles.
- 20 min 72 °C; 15 °C pause.

N.B. Heated lid is at 105 °C during all steps.

3.7 Data Analysis

For data analysis of an MLPA experiment, Coffalyser.Net software with the appropriate lot specific MLPA product sheet must be used and is available free of charge on MRC-Holland's website. Although Coffalyser.Net uses a more sophisticated algorithm, this section describes the basic principles.

Analysis of MLPA data consists of roughly three different parts:

First, Coffalyser.Net starts with raw data analysis (baseline correction, peak identification) and extensive quality control (e.g., DNA quantity used; complete DNA denaturation, degree of sloping).

Next, MLPA data is normalized and peak signals are translated to probe ratios. The absolute fluorescence measured by capillary electrophoresis cannot be used directly for copy number calculations as it is affected by many variables. First, each probe's measured fluorescence must be normalized within each sample to get meaningful data (intra-sample normalization).

In the third step, the relative probe signals are then used in the inter-sample normalization; final probe ratios are determined by comparing the relative probe peak in the DNA sample of interest to all reference samples. Reference DNA samples are expected to have a normal copy number for both the reference and target probes.

This final probe ratio is also called Dosage Quotient (DQ). Coffalyser.Net calculates the DQ for each probe in each sample.

The MLPA peak pattern of a DNA sample without genomic abnormalities will be identical to that of reference samples: final

Table 1
P095 Dosage Quotients expected in aneuploidy and normal cases when using 46, XY as reference samples. Note that the P095 MLPA probemix cannot make distinction between 69, XXX and 46, XX samples (*)

Sample type	chr. 13 probes	chr. 18 probes	chr. 21 probes	chr. X probes	chr. Y probes
46, XY	1	1	1	1	1
46, XX *	1	1	1	2	0
47, XY,+13	1.5	1	1	1	1
47, XY,+18	1	1.5	1	1	1
47, XY,+21	1	1	1.5	1	1
45, X0	1	1	1	1	0
47, XXX	1	1	1	3	0
47, XXY	1	1	1	2	1
47, XYY	1	1	1	1	2
69, XXX *	1	1	1	2	0
69, XXY	1	1	1	1.33	0.67
69, XYY	1	1	1	0.67	1.33

probe ratios will be ~1.0, reflecting 2 copies for autosomal regions. For heterozygous deletions, probe ratios of ~0.5 are expected, while heterozygous duplications will have a DQ-value of ~1.5. Probes should be arranged based on chromosomal location for correct interpretation; this will also aid in detecting subtle changes such as mosaicism.

3.8 Interpretation of Results

Once the correct DQ values of each probe are established, the relative copy number of each sample can be determined. In theory, all probes located on a certain chromosome should give approximately the same DQ-value, also in cases of aneuploidy (Table 1). In practice, however, certain variables such as SNPs, sample impurities etc., can affect the DQ-value obtained for a certain probe. Also, partial chromosome gains or losses may occur.

Based on Van Opstal and colleagues [6], the following rules are recommended for determination of copy number status in aneuploidy cases:

1. When the DQ-values of at least 4 of the 8 probes for a certain chromosome are equal to or higher than 1.30, and the values for the remaining four probes are close to 1.30, a trisomy for that chromosome should be considered.

2. A monosomy X should be considered if the relative probe signals of probes on the X chromosome are within the normal ranges of those for normal males, and Y signals are absent.
3. A 47, XXY should be considered if the relative probe signals of probes on the Y chromosome are within the normal ranges of those for normal males, and those of the chromosome X probes are within the normal range for normal females.
4. A 47, XYY should be considered if the relative probe signals of probes on the Y chromosome are ~1.8 times those for normal males, and those of the chromosome X probes are within the normal range for normal males.
5. A 47, XXX should be considered if the relative probe signals of probes on the X chromosome are ~2.5 times those for normal males, and Y signals are absent.
6. We recommend follow-up study of samples in which several probes for a certain chromosome are marked as having a statistically abnormal probe value by Coffalyser.Net analysis. The use of a 1.30 cut-off value to distinguish a normal from a trisomy result does not allow detection of mosaic samples. As an example, all chromosome 21 specific probes may have a ratio between 1.10 and 1.20 in a mosaic sample with 30% trisomy 21 cells. Coffalyser.Net analysis may identify such mosaic samples when the experiment was performed well.

4 Notes

1. If necessary, dilute DNA with TE (10 mM Tris–HCl pH 8.0; 0.1 mM EDTA).
2. The volume of the reaction is important for the hybridization speed, which is probe and salt concentration-dependent. Do not use more than 5 μL sample DNA.
3. Minimum recommended hybridization period 14 h. Maximum 20 h.
4. Evaporation may occur during (A) overnight hybridization or (B) pipetting the ligation reaction at 54 °C. In case you suspect evaporation problems, the following may help: (A): Test evaporation by incubating 8 μL H_2O overnight at 60 °C; >5 μL H_2O should remain, or (B): Reduce handling time by using multi-channel pipettes. To reduce evaporation: (1) ensure heated lid works well; (2) increase/decrease pressure of lid on tubes; (3) try different tubes (e.g., Thermo Fischer ABgene AB-0773, AB-0451); (4) use mineral oil (Vapor-lock, Qiagen 981611): add small drop of oil to DNA sample, just enough to cover it. There is no need to remove oil. After addition of MLPA buffer-probemix mixture or polymerase mix, centrifuge

very briefly. After addition of ligase mix, gently pipet up and down.

5. Ligase and polymerase master mixes can be stored at 4 °C for at least 1 h.
6. 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 thermocycler.
7. PCR products can be stored in the dark at 4 °C for at least 4 days.
8. An extensive trouble shooting section is present on the www.mlpa.com website.

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