

MLPA®

COST-EFFECTIVE AND SENSITIVE GENOMIC and METHYLATION PROFILING

Multiplex Ligation-dependent Probe Amplification (MLPA®) is a method that can detect copy number changes of up to 50 nucleic acid sequences in one simple reaction. MLPA® quickly identifies amplifications and deletions of a large number of genes, and can be used for DNA and mRNA profiling of both blood and tumour-derived samples. Furthermore, the MLPA® method can also be used to analyse CpG-methylation (MS-MLPA®). MLPA® has rapidly established its reputation as a trustworthy and efficient method. Hundreds of publications about the technique have appeared since its introduction in 2002, and MLPA® is now used in more than 900 laboratories worldwide.

Current applications of MLPA®

- Detection of small rearrangements. BRCA1, BRCA2, MSH2, MLH1, DMD, APC, SMA, NF1, NF2, VHL, TSC1/2, MECP2, NSD1, LDLR, FBN1, CFTR, DPYD, COL5A1, CACNA1A, PKHD1, BRIP1, SLC26A4, LMN1B, PRSS1, FRMD7, TPMT, FLCN, DNAI1, EP300, DNAH5, UBE3A, PCCA, PCDH15 and many more.
- Detection of large chromosomal rearrangements. Williams syndrome, Prader-Willi/Angelman syndrome, DiGeorge syndrome, Cri du Chat, Pelizaeus-Merzbacher, CMT1, HNPP, etc.
- Detection of copy number changes of subtelomeric regions.
- Detection of copy number changes of complete chromosomes (also in amniotic fluid samples): chromosomes 13, 18, 21, X, Y.
- Tumour diagnostics. DNA copy number changes in ALL, CLL, oligodendrogliomas, melanomas, neuroblastomas, etc.
- Methylation quantification. Prader-Willi/Angelman, Beckwith Wiedemann, MGMT, MLH1, Fragile X and inactivation of tumour suppressor genes.
- mRNA analysis for genes involved in apoptosis and inflammation.
- Over 300 different SALSA® MLPA® kits are available from MRC-Holland, Amsterdam. New products are constantly being developed, usually in cooperation with research labs worldwide.

Multiplex Ligation-dependent Probe Amplification (MLPA®)

- Detection of copy number of to 50 genomic DNA sequences in one simple, PCR-based reaction.
- Requires only 20 ng human DNA (3000 cells / ~ 0.5 ml amniotic fluid).
- Possible to use cell lysates rather than purified DNA.
- Can discriminate sequences differing in a single nucleotide.
- Identical protocol for many different applications.
- High throughput: results available within 24 hrs.
- Requires only a thermocycler and a sequence-type electrophoresis system.
- All reagents are included in the kit.

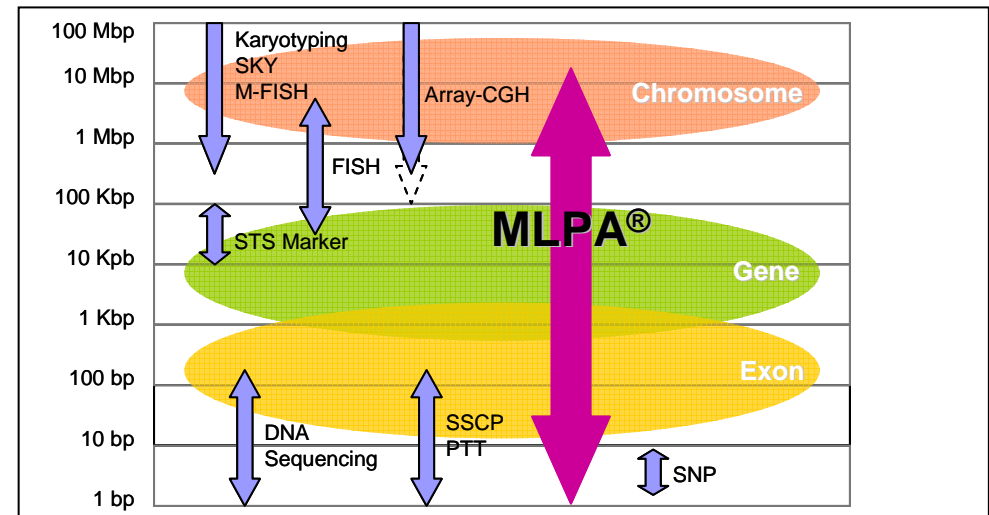


Figure 1 – MLPA® compared to other techniques. MLPA® can detect a wide range of genomic alterations, from single point mutations to large chromosomal deletions/duplications.

How does MLPA® work?

Basically, MLPA® is a method which makes a nucleic acid sample suitable for a multiplex PCR reaction, in which up to 50 specific nucleic acid sequences are amplified simultaneously, using a single PCR primer-pair. The resulting amplification products are separated by sequence type electrophoresis. MLPA® tests are designed so that the length of each amplification product is unique. The length increases in a stepwise-fashion by 6 or 9 nucleotides, with a total size range between 120-500 nucleotides. This size range provides an optimal fragment separation and a low background on sequence type gels.

An ordinary multiplex PCR requires one pair of primers for each fragment to be amplified. These primers are present in large amounts during the reaction, resulting in various problems. Firstly, since the efficiency of different primer pairs usually varies, it is difficult to use a normal multiplex PCR amplification for the relative quantification of target sequences. Secondly, small differences in reaction conditions often result in large differences in the results obtained.

The reason MLPA® is more robust is that all fragments are amplified by using a single PCR primer pair. But how can one amplify and quantify 50 different sequences with just one pair of primers? With a traditional multiplex PCR, this would be impossible. The trick of MLPA® is that it is not the sample DNA that is amplified, but the MLPA® probes that are added to the sample. Furthermore, MLPA® probes consist of two separate oligonucleotides, each containing one of the PCR primer sequences. It is only when these two hemi probes are both hybridised to their adjacent targets that they can be ligated. Only ligated probes will be amplified exponentially in a PCR reaction. The number of probe ligation products therefore depends on the number of target sequences in the sample.

Let's explain this in more detail. In order to amplify the 50 sequences that we want to detect and quantify, we add a mixture of 50 unique MLPA® probes to the nucleic acid sample. Each one of these is complementary to a given target sequence. As said, the MLPA® probes actually consist of two parts: two oligonucleotides that hybridise immediately adjacent to each other, each containing one of the sequences recognised by the PCR primer pair. After hybridisation to their adjacent targets, the two hemi probes are ligated by a specific ligase enzyme. Note that the ligation reaction is so specific that it is able to distinguish sequences differing in a single nucleotide.

The resulting ligation product contains both PCR primer sequences in one fragment and hence will be amplified exponentially during the PCR reaction. In contrast, probe oligonucleotides that are not ligated will contain only a single primer sequence. As a consequence, non-ligated hemi probes will not be amplified exponentially and will not generate a signal. The removal of unbound probes is therefore not necessary in MLPA®.

There will always be some sequences whose amplification rate per cycle will be 1-2% lower than others, thus resulting in a lower final peak area. Hence, a single MLPA® amplification profile is not sufficient to determine whether there are any copy number changes: any peak profile should always be compared to that of a reference sample. Compared to this reference sample, the relative peak area of each amplification product then reflects the relative copy number of the probe's target sequence in the analysed patient sample. A deletion of one or more exons in a patient thus becomes apparent as a decrease in relative peak area of the amplification products of the probes corresponding with these exons (see Figure 2).

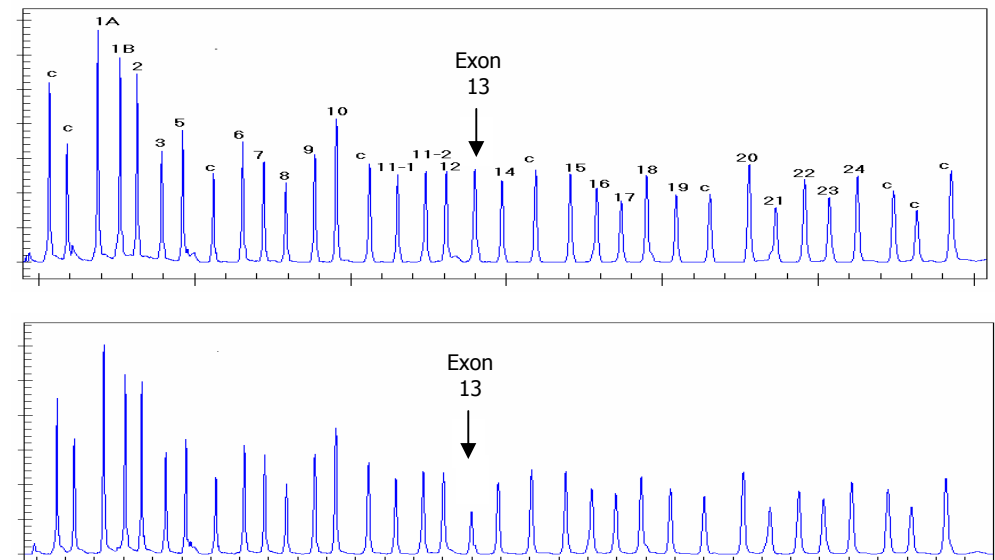


Figure 2 – MLPA® peak pattern. Reference DNA (top) and DNA from a BRCA1 del-exon13 individual (bottom) analysed by SALSA® MLPA® probemix P002 BRCA1. C= reference probe; 1A–24 designate the BRCA1 exons detected by the corresponding probe. When comparing the peak pattern of the patient (bottom) to the reference sample, the exon 13 peak is markedly lower in the patient.

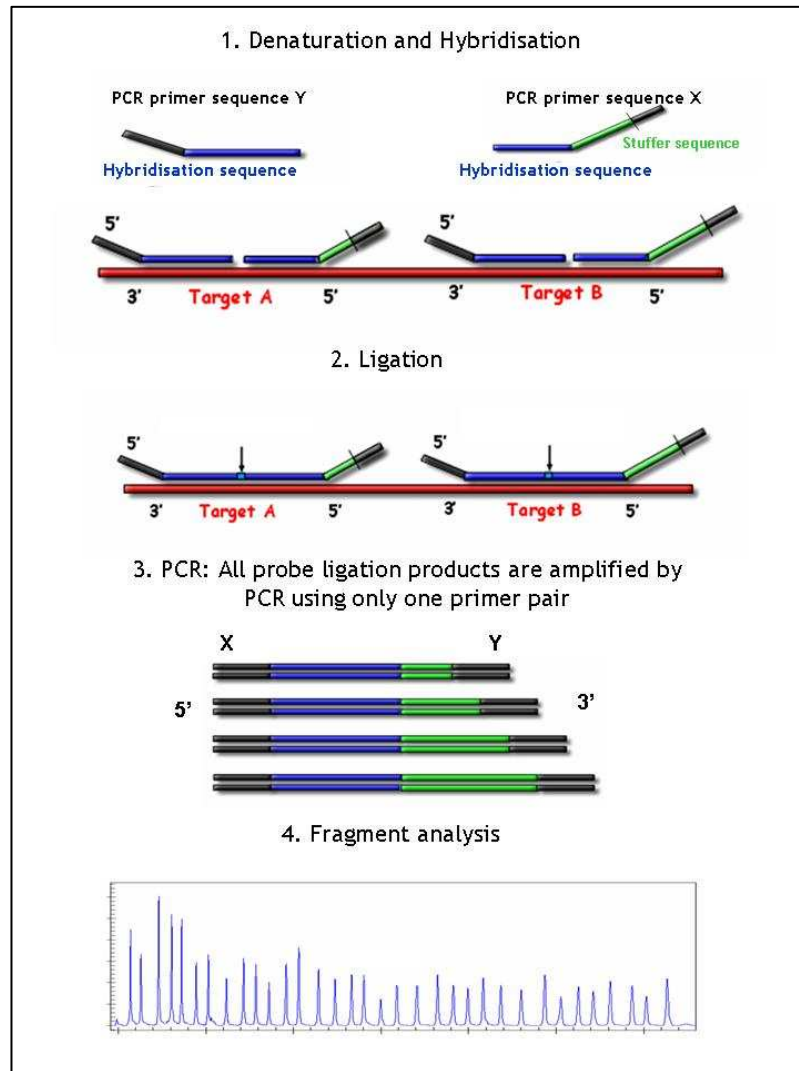


Figure 3 – Outline of the MLPA® technique. After hybridisation to their target sequence in the sample DNA, the probe oligonucleotides are enzymatically ligated. One probe oligonucleotide contains a non-hybridising stuffer sequence of variable length. Ligation products can be amplified using PCR primer sequences X and Y amplification product of each probe has a unique length (130-500 nt). Amplification products are separated by electrophoresis. Relative amounts of probe amplification products, as compared to a reference DNA sample, reflect the relative copy number of target sequences.

The concentration of probes present in an MLPA® reaction and the duration of the hybridisation reaction are sufficient to allow almost complete hybridisation of target sequences to the corresponding probe oligonucleotides. In a typical MLPA® reaction, approximately 500.000.000 copies of each probe oligonucleotide are present, while there are only 20.000 copies of most target sequences in a 60 ng human DNA sample. Prolonging the hybridisation reaction or addition of more probes will not influence the results obtained, making the reaction extremely robust.

As said, probe molecules that do not find a target sequence will not be ligated or exponentially amplified, and will thus not generate a fluorescent signal. As a result, the relative signal strength of each amplification product is determined primarily by the copy number of the target sequence in the sample. Because excess MLPA® probes do not have to be removed, the protocol for an MLPA® reaction is extremely simple.

MLPA® protocol (new)

1. DNA denaturation: heat 5 minutes at 98 °C.
2. Hybridization: add SALSA probemix and MLPA buffer. Incubate 1 minute at 95 °C, hybridize for 16 hr at 60 °C.
3. Ligation: add ligase mix and incubate 15 minutes at 54 °C. Heat inactivate the ligase for 5 minutes at 98 °C.
4. Add primers, dNTPs and polymerase and start PCR.
5. Capillary electrophoresis: export fragment lengths and peak areas to analysis software or spreadsheet. Analyze results.

In 2011, a significant improvement to the MLPA method was introduced in the form of a new, one-tube MLPA protocol. It includes a new PCR primer mix, which significantly reduces primer-dimer formation and experimental variation. Furthermore, less pipetting and less plastics are needed and PCR setup is now possible at room temperature. The changes in the protocol make the MLPA method more robust and easier to perform.

Advantages of MLPA®

1. The MLPA® technique can be used for many different applications; the only difference between different assays is the probe mix used.
2. MLPA® is multiplex: one reaction provides information on up to 50 targets. For most applications, this is sufficient to answer the specific questions asked by a physician.
3. MLPA® reactions are cost effective: the price for an MLPA® kit for 100 reactions is € 1145. This includes all reagents (probemix, buffers, ligase, polymerase, dNTPs and labelled PCR primers).
4. MLPA® is reproducible and easy to perform, and large numbers of samples can be tested simultaneously.
5. MLPA® is sensitive: only 20 ng of human DNA is required, and results do not depend on the amount of sample DNA used.
6. MLPA® can distinguish sequences that differ in only a single nucleotide, and is able to detect small copy number differences - e.g. 3 vs. 2 copies of a given gene sequence - in the complex mixture of the human genome.
7. MLPA® detects a sequence of only ~60 nucleotides and can thus be used to detect deletions or duplications of a single exon.
8. Investment costs are low for most users: the necessary equipment for MLPA® consists of a thermocycler and DNA sequencing electrophoresis equipment, both of which are present in most molecular biology laboratories. Although capillary electrophoresis is preferred, MLPA® has also been used in combination with LICOR and ABI 373 and 377 slab gels.

Limitations of MLPA®

1. MLPA® can only detect copy number changes of the sequences detected by the MLPA probes.
2. A mutation or polymorphism in the sequence detected by a probe can also cause a reduction in relative peak area.
3. MLPA® is primarily a method which identifies genomic deletions/insertions - it is not a suitable method to detect unknown point mutations. Nevertheless, MLPA® is able to discriminate *known* (point) mutations, as probes can be designed so that the ligation site is located directly at the site of the (point) mutation. Ligation will then only occur on non-mutated sequences, resulting in a decreased fluorescent signal in the case of mutated DNA.
4. MLPA® reactions are more sensitive to contaminants (PCR inhibitors such as small remnants of phenol) than ordinary PCR reactions. However, it will be quickly detected whether this has been the case, as it is predominantly those amplification products with lower average peak areas (e.g. the longer probes)

- that will show reduced signals. Moreover, good results *are* obtained on DNA extracted from FFPE tissues.
5. Developing SALSA® MLPA® probemixes as sold by MRC-Holland is complicated, expensive and time-consuming: each probe requires the design and preparation of a phage M13 clone, the purification of its single stranded DNA and digestion with expensive restriction endonuclease. Nevertheless, more than 300 MLPA® kits are currently available from MRC-Holland, and it is always possible to send in requests for new probe mixes. For research purposes, you can also design your own synthetic MLPA probes. Guidelines and sequences of the PCR primers are available on www.mlpa.com.
 6. In contrast to FISH, MLPA® cannot yet be used to investigate single cells. MLPA® analysis of DNA samples from cell mixtures will give the average copy number per cell. In case of tumour analysis, it will be difficult to detect certain genomic alterations if the DNA sample contained less than 50% cancer cells.

Methylation-specific MLPA® (MS-MLPA®)

MS-MLPA® is a semi-quantitative methylation detection method. The technique is a variant of the standard MLPA® method. A major application of MS-MLPA® is the detection of aberrant methylation of the imprinting regions, such as the Prader-Willi/Angelman region. A second major application lies in the detection of methylation changes of CpG islands that are located near the promoter regions of tumor suppressor genes in DNA derived from tumor cells.

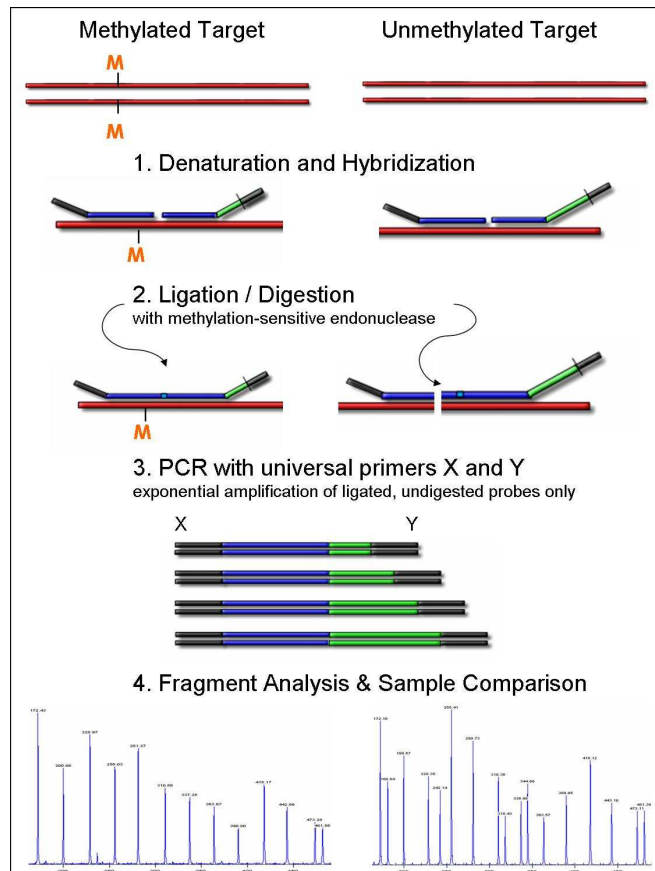


Figure 4 – Outline of the MS-MLPA® technique. 1. Just like a normal MLPA® reaction, the MS-MLPA® protocol starts with sample DNA denaturation and overnight hybridization of probes to their specific DNA targets. MS-MLPA probes contain a recognition site of a methylation-sensitive restriction endonuclease (e.g. HhaI or HpaII); reference probes do

not. 2. The reaction is split into two tubes. One tube is processed as a standard MLPA (only ligation; not shown) and the reaction in the other tube (shown here) is ligated and digested with the methylation-sensitive restriction enzyme HhaI (ligation + digestion). Hybrids of probes with unmethylated sample DNA are digested. 3. Each non-digested probe has a ligation product of a unique length, which is amplified exponentially by PCR. 4. For each sample, two peak patterns are produced: one from the ligation-only reaction (for determining copy number changes – not shown), and one from the ligation and digestion reaction (for methylation profiling - shown). The peak patterns are analysed by comparing them to those obtained on reference samples. Left peak pattern: ligation and digestion reaction of a healthy control showing only the 13 reference probes not containing a HhaI restriction site. The other 26 probes all detect a sequence containing a HhaI restriction site which is unmethylated in normal blood-derived DNA. Right peak pattern: ligation and digestion reaction of a tumour sample. The extra 4 peaks visible here are due to the fact that some of the sequences detected were methylated in this tumour-derived DNA, protecting the probe-DNA hybrid from endonuclease digestion.

The main difference with standard MLPA® is that the MS-MLPA® probes contain a recognition site for the methylation-sensitive restriction enzyme *Hha* I. During ligation, part of the reaction is digested with the HhaI enzyme resulting in digestion of the MS-MLPA® probes targeting unmethylated sequences.

One MS-MLPA® kit can be used for the analysis of 100 DNA samples. Please note that because of the two-step process described in figure 4, each DNA sample will generate two PCR reactions which need to be analysed by sequence type electrophoresis: one ligated-undigested sample, and one ligated-digested sample. More information about MS-MLPA® is available in the MS-MLPA® methylation protocol.

RT-MLPA® for mRNA analysis

Reverse Transcriptase MLPA® (RT-MLPA®) is another adaptation of the MLPA® technique which can be used for mRNA profiling as an alternative to real-time PCR and micro-arrays. Applications include profiling mRNA levels of apoptosis and inflammation genes.

RT-MLPA® does not detect mRNAs directly, as the Ligase-65 enzyme used cannot ligate DNA oligonucleotides when annealed to an RNA target sequence. To solve this, the SALSA RT-MLPA® probes in this problem mix are complementary to cDNA, and the RT-MLPA® kit contains a mixture of Reverse Transcriptase primers to enable cDNA production. This RT primer mix contains one RT primer for each of the probes. Each RT primer is designed to anneal immediately adjacent to the probe recognition site. Since the combined hybridizing sequences of the two

probe oligonucleotides are only 52-73 nt, the RT primers have to be elongated by a mere 50-70 nt to generate a cDNA sequence long enough to be detected. Because short cDNA fragments are already sufficient for the detection of the target sequences, the influence of RNA breakdown on test results is expected to be small. It is even possible to use the mRNA RT-MLPA® kit on RNA extracted from paraffin-embedded, formaldehyde-treated tissues, although success rates will strongly depend on the storage conditions of the tissues (e.g. pH-level, age of the tissue block, etc.). More information about RT-MLPA® is available in the RT-MLPA® protocol.

Articles and quotes

Over 900 articles on MLPA® applications have been published to date.

- *"MLPA analysis represents a simple, rapid and sensitive tool for the detection of CYP21A2/CYP21A1P deletions/duplications in CAH molecular diagnosis. Compared to Southern blot, MLPA may be considered a high throughput analysis."* Concolino, P. et al. (2009). Clin Chim Acta. 402(1-2): 164-70.
- *"This report illustrates that MLPA technique represents an efficient method to screen for large F8 gene deletions in sporadic undiagnosed carriers of haemophilia A."* Lannoy N., et al. (2009). Identification of de novo deletion in the factor VIII gene by MLPA technique in two girls with isolated factor VIII deficiency. Haemophilia. 2009 May;15(3):797-801.
- *"The MS-MLPA assay gave reproducible, accurate methylation and copy number results in the 126 samples assayed"*. Scott, RH. et al. (2008) J Med Genet. 45(2):106-13. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) robustly detects and distinguishes 11p15 abnormalities associated with overgrowth and growth retardation.
- *"This MS-MLPA assay can detect both copy number variations and methylation defects of the 11p15.5 critical region within one single experiment and represents an easy, low cost and reliable system for the molecular diagnostics of BWS and SRS."* Priolo, M. et al. (2008) Eur.J. Human Genetics 16:565-571. MS-MLPA is a specific and sensitive technique for detecting all chromosome 11p15.5 imprinting defects of BWS and SRS in a single-tube experiment.
- *"In summary, MLPA represents an easy, low cost and reliable system in the molecular diagnostics of SRS"*. Eggermann, T. et al (2008) Clin Genet. 73(1): 79-84. Use of multiplex ligation-dependent probe amplification increases the detection rate for 11p15 epigenetic alterations in Silver-Russell syndrome.
- *"MLPA not only was able to precisely detect all present changes in our set of samples, but also was, in some instances, able to identify small mutations"*.
- Djarmati, A. et al (2007) Movement Disorders 22(12):1708-1714. Rapid and Reliable Detection of Exon Rearrangements in Various Movement Disorders Genes by Multiplex Ligation-Dependent Probe Amplification.
- *"We strongly recommend the use of multiplex ligation probe amplification as a first screening method for the detection of copy number aberrations in patients with mental retardation because of its cost-effectiveness"*. Madrigal I. et al (2007) Genet Med. 9(2):117-22. MLPA as first screening method for the detection of microduplications and microdeletions in patients with X-linked mental retardation. "
- Arkblad, EL. et al (2006) Neuromuscul Disord.16(12):830-8. Multiplex ligation-dependent probe amplification improves diagnostics in spinal muscular atrophy.
- Kirchoff, M. et al (2006) Eur J Med Genet. 50(1): 33-42. MLPA analysis for a panel of syndromes with mental retardation reveals imbalances in 5.8% of patients with mental retardation and dysmorphic features, including duplications of the Sotos syndrome and Williams-Beuren syndrome regions.
- *"We conclude that automatic computer assisted MLPA is a rapid, simple and reliable method for detection of aneuploidies in prenatal diagnostics"*. Gerdes, T. et al (2005) Eur. J. Human Genetics 13: 171-175. Computer-assisted prenatal aneuploidy screening for chromosome 13, 18, 21, X and Y based on MLPA. "
- *"Multiplex ligation-dependent probe amplification has rapidly gained acceptance in genetic diagnostic laboratories due to its simplicity, relatively low cost, capacity for reasonable high throughput and robustness"*. Bunyan D.J. et al (2004) British Journal of Cancer 91: 1155-1159. Dosage analysis of cancer predisposition genes by multiplex ligation-dependent probe amplification.
- *"We suggest that the MLPA assay be utilized as an economical primary screen in families calculated to be at an elevated risk of HNPCC"; "MLPA represents a simple and elegant method to detect unusual copy numbers of genomic sequences"*. Ainsworth P.J. et al (2004) Clinical Genetics 66: 183-188. Family cancer histories predictive of a high risk of hereditary non-polyposis colorectal cancer associate significantly with a genomic rearrangement in hMSH2 or hMLH1.
- *"This study shows that MLPA is a fast and reliable screening method, potentially suitable for use in routine diagnostics"*. Rooms, L. et al (2004) Hum. Mutat. 23: 17-21. Subtelomeric deletions detected in patients with idiopathic mental retardation using Multiplex ligation-dependent probe Amplification (MLPA).
- *"Our results show that MLPA is a rapid, reliable, and sensitive technique, which allows high-throughput screening" "the method is reliable and very suitable to be included in the routine molecular analysis of predisposed families"*. Hogervorst, F.B.L. et al (2003) Cancer Research 63, 1449-53. Large genomic

deletions and duplications in the BRCA1 gene identified by a novel quantitative method.

- *"This novel semi-quantitative method is precise, sensitive, rapid, robust, and high throughput"*. Slater H.R. et al (2003) J. Med. Genet. 40: 907-912. Rapid, high-throughput prenatal detection of aneuploidy using a novel quantitative method (MLPA).
- *"In summary, the MLPA technique is a fast and efficient test for the detection of genomic deletions in the MMR genes". "it reveals alterations that might escape detection using conventional diagnostic techniques"*. Gille, J.J. et al (2002) Br. J. Cancer 87: 892-7. Genomic deletions of MSH2 and MLH1 in colorectal cancer families detected by a novel mutation detection approach.
- The MLPA technique has been first described by Schouten, J.P. et al: Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acid Research (2002) 30, e57.*