Simultaneous MLPA-Based Multiplex Point Mutation and Deletion Analysis of the *Dystrophin* Gene

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Abstract

The Multiplex Ligation-dependent Probe Amplification assay (MLPA) is the method of choice for the initial mutation screen in the analysis of a large number of genes where partial or total gene deletion is part of the mutation spectrum. Although MLPA dosage probes are usually designed to bind to normal DNA sequence to identify dosage imbalance, point mutation-specific MLPA probes can also be made. Using the dystrophin gene as a model, we have designed two MLPA probe multiplexes that are specific to a number of commonly listed point mutations in the Leiden dystrophin point mutation database (http://www.dmd.nl). The point mutation probes are designed to work simultaneously with two widely used dystrophin MLPA multiplexes, allowing both full dosage analysis and partial point mutation analysis in a single test. This approach may be adapted for other syndromes with well defined common point mutations or polymorphisms.

Index Entries: MLPA; point mutations; dystrophin; dosage.

1. Introduction

The Multiplex Ligation-dependent Probe Amplification assay (MLPA) ***(1)*** measures DNA sequence copy number and was designed to detect full or partial deletions and duplications in specific genes. It has become the method of choice as the initial mutation screen for many genes where deletions account for a proportion of mutations, for example, the cancer predisposition syndromes where full or partial gene deletions account for 5-10% of cases ***(2)***. However MLPA can be adapted to detect other types of mutation, for example specific probes can be designed for sequences encompassing defined point mutations. By adding common point-mutation-specific (PMS)-MLPA probes to dosage MLPA multiplexes, full dosage analysis and limited point mutation analysis can be performed simultaneously without any significant increase in labor.

In order to investigate the potential of such extended MLPA analysis, we have designed point-mutation-specific probes for a number of mutations in the *dystrophin* gene. This is the largest known human gene, comprising 79 exons that span 2.4 megabases of genomic DNA, and mutations in the gene cause Duchenne or Becker muscular dystrophy (DMD/BMD) ***(3)***. In two-thirds of cases of DMD or BMD, the mutation consists of a

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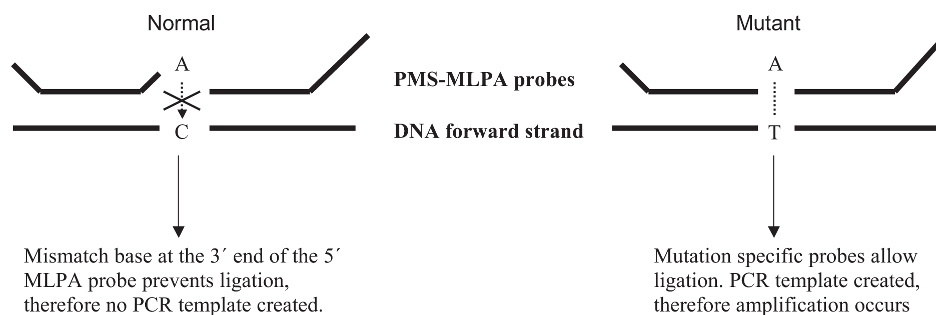

Fig. 1. Hybridization of point-mutation-specific Multiplex Ligation-dependent Probe Amplification assay probes.

deletion including one or more exons ***(3,4)***. For diagnostic purposes, the multiplex PCR protocols generally employed detect over 95% of such deletions by targeting the commonly deleted areas of the gene ***(5–7)***. However, to detect the additional remaining 5% of deletion cases and 7% of cases that have partial gene duplications, most laboratories now use MLPA.

The majority of DMD cases that do not have deletions or duplications have point mutations, the detection of which is difficult and time consuming because of the large size of the *dystrophin* gene, the fact that point mutations can be situated throughout the gene, and the large number of different point mutations that have so far been identified. Comprehensive whole gene point mutation analysis is therefore only performed by specialist laboratories.

The ability to easily identify the more common DMD mutations would be of great help diagnostically. We therefore consulted the Leiden *dystrophin* mutation database (www.dmd.nl), which describes approx 1000 different sequence variants in the *dystrophin* gene. We chose 23 of the most commonly listed point mutations, selecting those that had been seen by multiple research groups. The chosen mutations account for 17% of all the reported point mutation cases on the database.

Appropriate point mutation MLPA probes were designed within the size range of 98–126 base pairs so that they could be combined with the commercially available *dystrophin* dosage MLPA probe mixes produced by MRC Holland (P034 and P035), which range in size from 130 to 490 base pairs.

In order to minimize the possibility of PMS probes interfering with the binding of dosage probes, the PMS-MLPA probes were designed to bind to the forward DNA strand, as the majority of the MRC-Holland MLPA probes bind to the reverse strand. This also reduced the possibility of false-positive results with the PMS-MLPA probes due to G–T mismatches, the commonest type of base mispairing. As most point mutations are C to T transitions, G–T mismatches between a PMS-MLPA probe with a T at the 3' end and the G of a normal sequence are a possibility if probes are designed to bind to the reverse strand. An example of a PMS-MLPA probe which is designed to be specific to a C to T transition and to bind to the forward strand is shown in **Fig. 1**.

The PMS-MLPA probes were directly synthesized with a 5' phosphate added to the second probe of each pair to allow ligation and purified by reversed-phase high-performance liquid chromatography (HPLC) (School of Chemistry, University of Southampton and Thermo Hybaid, Germany).

2. Patients and Controls

Positive controls were available for nine of the mutations in the point mutation probe mixes; c.583C>T (Arg195X), c.2302C>T (Arg768X), c.10171C>T (Arg3391X), c.8608C>T (Arg2870X), c.8944C>T (Arg2982X), c.7657C>T (Arg2553X),

c.8713C>T (Arg2905X), c.10453dupC, and c.10033C>T (Arg3345X). These positive controls were identified as part of the Guy's Hospital *dystrophin* gene mutation diagnostic service and had been previously verified by sequencing. In order to ascertain whether or not the addition of PMS probes adversely affected the function of the MRC-Holland PO34 and PO35 deletion probe multiplex kits, 65 control samples were also analyzed including 50 normal DNA samples, 10 samples each with different dystrophin partial gene deletions, and 5 samples with different partial gene duplications. The partial gene deletion or duplication controls had all been originally referred to the Wessex laboratory for *dystrophin* gene analysis and the mutations were identified using either multiplex PCR or MLPA.

3. Methods

The probe sequences are listed in **Table 1** and are designed to recognize 23 *dystrophin* mutations as listed in **Table 2** (positive control DNA samples were only available for 9 of these). For each multiplex, the probes were diluted to give a final concentration of 1.33 fmol/ml. MLPA analysis was carried out using the standard protocol of MRC-Holland ***(1)***, the only difference being that the amount of Tris-EDTA in the initial denaturation step was reduced to 2.5 ml to allow for the extra 1.5 ml of PMS probe mix. Point mutation mix 1 **(Table 2)** was used in conjunction with MRC-Holland probe mix P034 whereas point mutation mix 2 was used with MRC-Holland probe mix P035. One microliter of the MLPA PCR product was run on an ABI3100 sequencer and the data were analyzed using Genotyper (version 3.7) software (PE Biosystems). The *dystrophin* point mutations appear as additional peaks between 98 and 126 base pairs in addition to the MRC-Holland probe set peaks (*see* **Table 2** for sizes).

4. Results

All nine previously characterized *dystrophin* point mutations were identified by the combined PMS/Dosage MLPA multiplexes **(Fig. 2)**. Furthermore, the 15 known cases with *dystrophin* gene dosage imbalance (10 deletions and 5 duplications) were also identified by the modified multiplex kits with the difference in peak heights being indistinguishable from that seen in the unmodified probe sets (data not shown).

Analysis of normal controls showed there to be no significant difference in peak height measurement ascertained using the modified kits including the PMS probes compared to those using the original MRC Holland dystrophin deletion kits P034 and P035 (data not shown).

A problem was encountered with the accuracy of sizing of amplified fragments using the Genotyper software. All PMS peaks were called by the software as being 2 bp smaller than their actual sizes. This appears to be a software problem rather than an experimental problem, as all of the MRC-Holland dosage probe peaks in the lower size range were also consistently labeled as being 2 bp smaller than published. As the peak sizes for some of the point mutations differ by only 2 bp, it is therefore important to confirm all positive results by sequencing.

5. Discussion

Nine cases with known point mutations were correctly identified by PMS-MLPA analysis, demonstrating proof-of-principle that point mutations can be detected by MLPA. Furthermore, the combined dosage and point-mutation-detection MLPA multiplexes showed no deterioration in ability to detect dosage imbalance mutations. Ten known deletion and five known duplication cases were correctly identified, demonstrating that the efficiency of the MLPA dosage imbalance probes was not compromised by the presence of the additional PMS probes.

The results obtained from the positive control DNAs were very encouraging in terms of the future applications of this technique. PMS-MLPA probes are easy to use and work simultaneously with the commercial MLPA dosage kits, so the amount of additional work that is necessary to screen for both dosage imbalance and selected point mutations is negligible. The adaptation of this methodology to other genes with well defined common point mutations or polymorphisms would be worthwhile.

Table 1
The Sequences of Probes in the Dystrophin Point Mutation Kits (all 5' to 3'). P- = 5' phosphate group.

R145X L	GGGTTCCCTAAGGGTTGGATGGATAATTACGAGTTGATTGTCA
R145X R	P-GACCCAGCTCAGGAGAATCTTTTCGTCACCAATCTAGATTGGATCTTGCTGGCAC
Q1370X L	GGGTTCCCTAAGGGTTGGATTTTTCAGTCTCCTGGGCAGACTA
Q1370X R	P-GATGCTCTGTTCAAGCAACTTTTGAAGAAGGCACTCTAGATTGGATCTTGCTGGCAC
R242X L	GGGTTCCCTAAGGGTTGGACTGGATGGCTTCAATGCTCACTTA
R242X R	P-TTGAGGCAAAACTTGGAAGAGTGACACAGCACGTGCCTTCTAGATTGGATCTTGCTGGCAC
R3381X L	GGGTTCCCTAAGGGTTGGACTTCGCAAAATACCTTTTGGTTCA
R3381X R	P-AAATTTGTTTTTTAGTACCTTGGCCCCTGAGATCCACGAGTCTAGATTGGATCTTGCTGGCAC
R768X L	GGGTTCCCTAAGGGTTGGATTCTGAACTTCTCAGCTTTTTCTC
R768X R	P-ACTCTATGGCCTGCAGCATGAGAGACCCTCGTCTGCCGGACTTCTAGATTGGATCTTGCTGGCAC
R1666X L	GGGTTCCCTAAGGGTTGGAAAGATTTAACCACTCTTCTGCTCA
R1666X R	P-GGAGGTGACAGCTATCCAGTTACTCGGACTCCTCAGTCTGGGCGAATCTAGATTGGATCTTGCTGGCAC
Q60X L	GGGTTCCCTAAGGGTTGGAAAATAAGTCACATACCAGTTTTTA
Q60X R	P-CCCTGTCAGGCCTTCGAGGAGGTCGCTTCTTATAAGAAACGAAGTGCTTCTAGATTGGATCTTGCTGGCAC
R1967X L	GGGTTCCCTAAGGGTTGGAAATTTGTGCAAAGTTGAGTCTTCA
R1967X R	P-AAACTGAGCAAATTTGCTCTCAATGGAAATAACTATAGGTAGTCTAGTTTTCTAGATTGGATCTTGCTGGCAC
R2553X L	GGGTTCCCTAAGGGTTGGACATGCTTGTTAAAAAACTTACTTC
R2553X R	P-AATCCGTAATGATTGTTCTAGCCTAGGTCGGTAGTTGAGGTGGGGGACCCGGGATCTAGATTGGATCTTGCTGGCAC
R2870X L	GGGTTCCCTAAGGGTTGGACTGATACTTAGATCAGGCTGCTCTGTCAGAAATATTCA
R2870X R	P-TACAGTCTCAAGAGTACTCATGATGCACTTGGAGGGCTGGGGTCTAGATTGGATCTTGCTGGCAC
R2905X L	GGGTTCCCTAAGGGTTGGAATTGACCTCCTCAGCCTGCTTTCA
R2905X R	P-TAGAAGCCGAGTGACATTCTGGGCGAGAGAAGACTTCTTACTAGGCTAAGTCTGACTATCTAGATTGGATCTTGCTGGCAC
R3190X L	GGGTTCCCTAAGGGTTGGACAGGACACGGATCCTCCCTGTTCA
R3190X R	P-TCCCCTATTATGAAGAATCAAAGCCTCCCCACCGGCAGTCAAAGGGAAGCTCTTTGCCCATCTAGATTGGATCTTGCTGGCAC
R1314X L	GGGTTCCCTAAGGGTTGGAATTTGGGTTATCCTCTGAATGTCA
R1314X R	P-CATCAAATTTTCAAGTGACTGAAAACACGGGTTCTAGATTGGATCTTGCTGGCAC
R195X L	GGGTTCCCTAAGGGTTGGAGATGTTGAATGCATGTTCCAGTCA
R195X R	P-TTGTGTGGCTGACTGCTGGCAAACTGTGTCGCCTTCTAGATTGGATCTTGCTGGCAC
Q1565X L	GGGTTCCCTAAGGGTTGGACAATTTCAAGCATTTCTCCAACTA
Q1565X R	P-TTGCTTTCTTTCTGTTACCTGAAACTCCGGCGCCGTCCTCTAGATTGGATCTTGCTGGCAC
R2098X L	GGGTTCCCTAAGGGTTGGATTTCTCAACAGATCTGTCAAATCA
R2098X R	P-CCTGCAGGTAAAAGCATATGGATCCCTCCCCCGCTCGTTCTCTAGATTGGATCTTGCTGGCAC
R2982X L	GGGTTCCCTAAGGGTTGGATTTCAGAGGCGCAATTTCTCCTCA
R2982X R	P-AAGTGCCTGTGTGCAATAGTCAAACTACCCGGTTCCTCGTCTCCGCTCTAGATTGGATCTTGCTGGCAC
9204del4 L	GGGTTCCCTAAGGGTTGGAAACTTACTTGATATAGTAGGGCAC
9204del4 R	P-TTTGGCGAGATGGCTCTCTCCCAGTTCAAAGTTCACTCATAGTCCCCTTCTAGATTGGATCTTGCTGGCAC
Q3116X L	GGGTTCCCTAAGGGTTGGAATACTTACAGCAAAGGGCCTTCTA
Q3116X R	P-CAGTCTTCGGAGTTTCATGGCAGTAAGCGCCTAGTCTCCGAGTCACGGCGTCTAGATTGGATCTTGCTGGCAC
S3127X L	GGGTTCCCTAAGGGTTGGAGGTCCAAGGCATCACATGCAGCTC
S3127X R	P-ACAGGCTCAAGAGATCCACTGCAACCCCACCCGCCCCGACACCCGTCAGACCCTTCTAGATTGGATCTTGCTGGCAC
R3345X L	GGGTTCCCTAAGGGTTGGACATTTTATGGCCTTTTGCAACTCA
R3345X R	P-ACCAGAAAAAAAGCAGCTTTGGCAAGCACTGCGGCCGGGACATCCCGAGTCTTCACTCTAGATTGGATCTTGCTGGCAC
R3391X L	GGGTTCCCTAAGGGTTGGACTGCACTGGCAGGTAGCCCATTCA
R3391X R	P-GGGATGCTTCGCAAAATACCTTTTTTGGATCCCGGGTGGGGGGCTGCAAGGTTACCCTTCTAGATTGGATCTTGCTGGCAC
10453dupC L	GGGTTCCCTAAGGGTTGGAGGGCAGGACTACGAGGCTGGCTCA
10453dupC R	P-GGGGGGGAGTCCTGGTTCAAACTTTTTACTGCCGTAGTTCGGCCACCCGTGGACGACGAGTCTAGATTGGATCTTGCTGGCAC

Table 2
The Components of the Dystrophin Point Mutation Probe Mixes

Target	Mutation	MLPA product size
Mix 1		
Dystrophin exon 6	Arg145X (c.433C>T)	98 bp
Dystrophin exon 30	Gln1370X (c.4108C>T)	100 bp
Dystrophin exon 8	Gln242X (c.274C>T)	104 bp
Dystrophin exon 70	Arg3381X (c.10141C>T)	106 bp
Dystrophin exon 19	Arg768X (c.2303C>T)	108 bp
Dystrophin exon 35	Arg1666X (c.4996C>T)	112 bp
Dystrophin exon 3	Gln60X (c.178C>T)	114 bp
Dystrophin exon 41	Arg1967X (c.5899C>T)	116 bp
Dystrophin exon 52	Arg2553X (c.7657C>T)	120 bp
Dystrophin exon 69	Arg3345X (c.10033C>T)	122 bp
Dystrophin exon 59	Arg2905X (c.8713C>T)	124 bp
Dystrophin exon 66	Arg3190X (c.9568C>T)	126 bp
Mix 2		
Dystrophin exon 29	Arg1314X (c.3940C>T)	98 bp
Dystrophin exon 7	Arg195X (c.583C>T)	100 bp
Dystrophin exon 34	Gln1565X (c.4693C{>T)	104 bp
Dystrophin exon 44	Arg2098X (c.6292C>T)	106 bp
Dystrophin exon 60	Arg2982X (c.8944C>T)	112 bp
Dystrophin exon 62	c.9204_9207del	114 bp
Dystrophin exon 64	Gln3116X (c.9337C>T)	116 bp
Dystrophin exon 65	Ser3127X (c.9380C>G)	120 bp
Dystrophin exon 58	Arg2870X (c.8608C>T)	122 bp
Dystrophin exon 70	Arg3391X (c.10171C>T)	124 bp
Dystrophin exon 74	c.10453dupC	126 bp

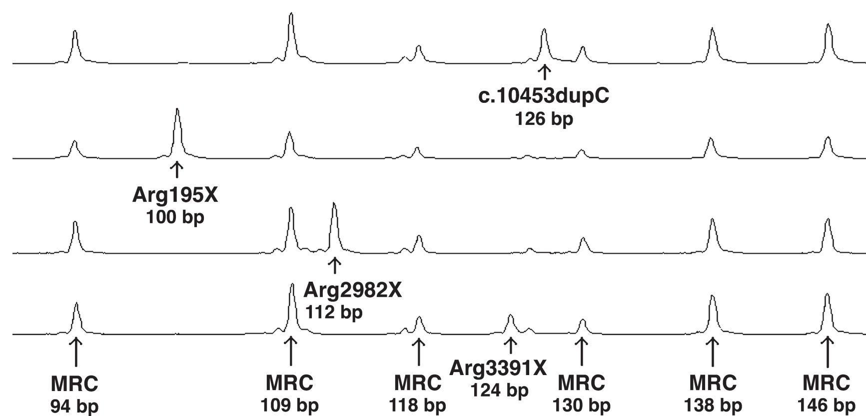

Fig. 2. Examples of the dystrophin point mutation peaks using Dystrophin Point Mutation kit 2 **(Table 2)** in conjunction with the MRC-Holland kit P035. The figure shows a region of the Genotyper trace which includes the MRC-Holland peaks at 94, 109, 118, 130, 138, and 146 bp (labeled MRC). Using the positive control DNAs, additional peaks can be seen at 100 bp (Arg195X), 112 bp (Arg2982X), 124 bp (Arg3391X) and 126 bp (c.10453dupC).

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