Letter

Multiplex ligation-dependent probe amplification is superior for detecting deletions/duplications in Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) (OMIM: 310200) is one of the most commonly inherited neuromuscular diseases, affecting 1 in 3500 males. It is an X-linked disorder caused by mutations in the DMD gene (1) which is composed of 79 exons encoding a 14-kb mRNA (2). Mutations altering the reading frame cause the severe phenotype of DMD, whereas mutations retaining the full-length mRNA cause the more benign phenotype of Becker muscular dystrophy (OMIM: 300376) (3, 4). Knowing the exact mutation in a patient is therefore of prognostic value and a part of the diagnostic tool of these diseases. Structural rearrangements of the gene are found in about two thirds of DMD patients, with approximately 60% carrying deletions and 5–10% carrying duplications, whereas the remaining patients carry point mutations or small insertion/deletions (2, 5, 6). The three multiplex polymerase chain reactions (PCRs), developed by Beggs, Chamberlain and Kunkel, have traditionally been used in a clinical diagnostic set up (7–9) and allow a detection of 90–95% of the deletions in male patients, but duplications are not identified.

Recently, the multiplex ligation-dependent probe amplification (MLPA) method was introduced (10), which allow the relative quantification of up to 40 different nucleic acid sequences in a single reaction tube. The method has proven to be accurate and reliable for identifying deletions and duplications in several genes (11–15). Here, we report our results and experience with the MLPA DMD kits, P034 and P035, commercially available from MRC Holland. These two sets of probes allow a copy number scanning of all 79 DMD exons in just two PCRs.

The PCR products were analysed on a Genetic Analyzer (Applied Biosystems 310) where the individual exons are depicted as a single peak. The relative peak area for each exon was calculated as a fraction of the total sum of peak areas in each sample. Subsequently, the fraction of each peak was divided by the average peak fractions of the corresponding exon in control samples to give the dosage quotient (DQ) (13). Expected DQs in men for single-copy exons are 1, for deleted exons 0, and 2 for duplicated exons. For females, the expected DQs for non-carriers are 1, and for
carriers of a deletion and duplication 0.5 and 1.5, respectively. Initially, we analysed 10 control male samples and calculated the mean DQs and standard deviations (SDs) for each peak. The mean DQ for all peaks was 1.01 (range: 0.94–1.17) and SD was 0.04.

In total, we investigated 196 DNA samples of which the majority, 182, were from male patients, 13 from obligate female carriers and one sample from a young girl with a clinical picture strongly suspicious for DMD.

Deletions in male patients are easily identified by the absence of the respective DQ value, and as the majority of the identified deletions encompass more than one exon no additional analysis is necessary. The absence of only one DMD peak, corresponding to a single exon, was investigated further using PCR primers flanking the exon in question. If the deletion could not be confirmed, the PCR product of that exon was sequenced. Deletion of a single exon in females was confirmed by real-time PCR. Duplications will only be detected if there is a clear difference between DQ of the normal sample and patient sample. In the present series, the DQ found in the samples harbouring duplications did not overlap with the normal DQ range. The DQ for carriers of either deletions or duplications did likewise not overlap with DQ for normal samples making the analysis quite robust (Table 1). Duplication of a single exon was confirmed by real-time PCR.

The MLPA screen of the 196 samples revealed a total of 94 deletions including a complex rearrangement with a non-contiguous deletion, 14 duplications, including a non-contiguous duplication, as well as four point mutations, all expected to result in a truncated protein (Fig. 1). The latter were initially identified as single-exon deletions but were by follow-up analysis shown to be point mutations likely altering hybridization of the MLPA probes.

A total of 112 DMD mutations were identified of which 29 had not previously been discovered using the common multiplex approach. Thus, the MLPA technique has increased our mutation pick-up rate by 33%. Moreover, the technique has enabled us to confidently identify carrier individuals.

The method is easy to handle, highly reproducible on good-quality DNA, and as the assay only requires commonly used technology, it can easily be implemented in a standard diagnostic laboratory. Identified copy number changes involving a single exon must, however, be interpreted with care and verified by alternative techniques.

### References


### Table 1. Dosage quotients from normal controls, males with duplications and female carriers of duplication or deletion

<table>
<thead>
<tr>
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<th>Control</th>
<th>Duplications males</th>
<th>Duplications carriers</th>
<th>Deletions carriers</th>
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<tr>
<td>Mean</td>
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<td>1.46</td>
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<tr>
<td>Range</td>
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<td>1.48–2.38</td>
<td>1.35–1.55</td>
<td>0.47–0.60</td>
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Results from 46 peaks of exons with a duplication (14 patients), from 22 peaks of exons with a duplication (7 carriers) and from 76 peaks of exons with a deletion (7 carriers).

### Fig. 1. Distribution of the new deletions, duplications and point mutations found in this study. Light-grey vertical bars indicate the exons included in our multiplex PCR, used before the introduction of multiplex ligation-dependent probe amplification. The complex deletion encompassing exon 1 and exons 4–11 was only partly detected previously.


