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Biochemical and Biophysical Research Communications 339 (2006) 145–150
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# Multiplex real-time PCR for detection of deletions and duplications in dystrophin gene

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Received 27 October 2005
Available online 9 November 2005

**Abstract**

Genetic testing of Duchenne and Becker muscular dystrophies (DMD/BMD) is a difficult task due to the occurrence of deletions or duplications within dystrophin (*DMD*) gene that requires dose sensitive tests. We developed three multiplex quantitative real-time PCR assays for dystrophin exon 5, 45, and 51 within two major hotspots of deletion/duplication. Each exon was co-amplified with a reference X-linked gene and the copy number of the target fragment was calculated by comparative threshold cycle method ($\Delta\Delta C_t$). We compared the performance of this method with previously described end-point PCR fluorescent analysis (EPFA) by studying 24 subjects carrying *DMD* deletions or duplications. We showed that Q-PCR is an accurate and sensitive technique for the identification of deletions and duplications in DMD/BMD. Q-PCR is a valuable tool for independent confirmation of EPFA screening, particularly when deletions/duplications of single exons occur or for rapid identification of known mutations in at risk carriers.


*Keywords:* Duchenne muscular dystrophy; Carrier diagnosis; Real-time PCR; Deletions; Duplications

Duchenne and Becker muscular dystrophies (DMD/BMD) are X-linked neuromuscular disorders affecting 1 in 3500 live-born males caused by mutations in the dystrophin gene (*DMD*). This gene, consisting of 79 exons covering 2.5 Mb of genomic DNA, is characterized by a remarkable mutation diversity [1]. About 65% of DMD/BMD cases show partial gene deletions (~60%) or duplications (~5%) of different sizes that are preferentially clustered in two major hotspots of mutation, spanning exons 3–7 and 44–55 [2,3]. Point mutations and deletion/insertions of few nucleotides account for the remaining cases (35%) [4]. Mutations are either inherited from asymptomatic female carriers (70%) or occur de novo (30%) [1]. Widely used genetic tests for detection of common deletions in affected males rely on amplification of several *DMD* exons by multiplex PCR and evaluation of amplification fragments by agarose or capillary electrophoresis [5,6]. Traditionally identification of female carriers was carried out by different methods including Southern blotting, mRNA, FISH, and haplotype analysis [7–10]. All these approaches are time-consuming and may suffer from a limited sensitivity (e.g., small duplications). To overcome these problems, rapid and low-cost sensitive PCR tests have been developed to analyze simultaneously multiple *DMD* exons, such as multiple exons, agarose or capillary electrophoresis [11,12], multiplex amplifiable probe hybridization (MAPH) [13], and multiple ligation-dependent probe amplification (MLPA) [14]. In these methods, end-point PCRs are accomplished in 20–25 cycles, when amplification is supposed to be in its exponential phase such that a linear

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.006

relationship between quantities of template DNA and PCR products is maintained. These assays should be considered semiquantitative, as the actual amplification profile of the reaction is based on a theoretical assumption. Real-time PCR was specifically developed to quantify specific DNA targets through the monitoring of product formation. This technology has been successfully applied for the detection of hemizygous deletions or duplications in different genetic disorders. Recently a quantitative real-time PCR test based on SYBR Green I dye has been developed for the detection of deletions/duplications of the dystrophin gene [15]. This method however does not allow the amplification of internal reference fragments in the same reaction tube and it may be quantitatively affected by amplification of aspecific PCR products. In the present report, we investigated whether real-time PCR based on TaqMan technology could be successfully applied to the diagnosis of DMD/BMD by studying 11 obligate female carriers of partial *DMD* deletion and 63 additional subjects at risk of *DMD* deletion or duplication, comparing its performance with end-point fragment amplification fluorescent analysis [16].

## Materials and methods

### *Patients*

We selected 11 obligate female carriers from six different families characterized by two DMD/BMD affected male relatives with exon 5, 45 or 51 dystrophin deletions. For confirmation study, we selected 15 possible female carriers with an affected son showing exon 5, 45 or 51 deletions and 48 DMD/BMD males with: (i) immunohistochemically confirmed lack or reduction of dystrophin expression in muscle biopsy and (ii) no *DMD* deletions.

### *Genomic DNA extraction*

Genomic DNA was obtained from peripheral lymphocytes by using the QIAamp DNA blood kit (Qiagen, Chatsworth, CA) and quantified by UV spectroscopy. Purified genomic DNA was resuspended in HPLC pure water to working concentration and stored at 4 °C.

### *Preparation of reference exon plasmid constructs*

DNA was extracted from peripheral blood of a normal subject and amplified by PCR using primers: clEX5/F (5′-TTA TTG CAA CTA GGC ATT TGG TCT C-3′) and clEX5/R (5′-GAT TAA TGT TAC CCA AAA GGA AAC C-3′), and clEX45/F (5′-TGG TAG CAC ACT GTT TAA TCT TTT C-3′) and clEX45/R (5′-CCT TTC ACC CTG CTT ATA ATC TCT C-3′), clEX51/F (5′-TTT TAG CTC CTA CTC AGA CTG TTA C-3′) and clEX51/R (5′-CTG CCA ACT TTT ATC ATT TTT TCT C-3′) for the *DMD* gene and primers clPLP1F (5′-GGC TGA GGG CTT CTA CAC CAC-3′), clPLP1R (5′-ACC TTG TCG GGA TGT CCT AGC-3′) for the PLP1 gene.

In detail, 5 μl of DNA (approximately 0.1 μg/μl) was amplified in a thermal-cycler (Applied Biosystems, Foster City, CA) with a reaction mixture containing 100 pmol each of primers, $MgCl_2$ (1.5 mM), Buffer (1×), 125 μM of each dNTP, and 1 U *Taq* Gold DNA polymerase. After 10-min pre-activation at 95 °C, 35 cycles of amplification with a temperature profile of 94, 58, and 72 °C (30 s each) were performed, followed by a final extension step of 10 min at 72 °C. PCR products were cloned into the pCRII plasmid using the TOPO-TA Cloning kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Plasmids pEx5, pEx45, pEx51, and pPLP1 were purified with the Qiagen plasmid Maxi kit (Qiagen, Chatsworth, CA).

### *End-point fragment amplification fluorescent analysis)*

We modified a previously reported end-point multiplex fluorescent PCR technique [16] by reducing the size of amplified fragments (between 150 and 400 bp) to minimize preferential amplification of short fragments and lowering the number of amplification cycles to 19 to accomplish the reaction within the exponential phase. End-point fragment amplification fluorescent analysis (EPFA) was performed using five different multiplex PCR sets in which 34 dystrophin exons (including exon 5, 45, and 51) were amplified in groups of 6–8 fragments and in combination with three different reference gene fragments (X-linked coagulation factor VIII gene).

Precisely, 150–200 ng of genomic DNA was added to each reaction tube containing 6.5 mM di $MgCl_2$, 0.3 μM of each primer, 200 μM for each dNTP, and 1 U *Taq* DNA polymerase (Q-BIOgene, Basel, CH) in a final reaction volume of 16 μl. Forward primers were labeled with 6-carboxyfluorescein (FAM) or hexachloro-6-carboxyfluorescein (HEX) fluorochromes (sequences of primers available on request). PCR was performed according to the following conditions: initial pre-activation at 94 °C for 7 min; 5 cycles at 94 °C for 30 s, 64 °C for 3.5 min, 5 cycles at 94 °C for 20 s, 64 °C for 5 min, 9 cycles at 94 °C for 20 s, 64 °C for 6.5 min, and final extension at 72 °C for 15 min.

Fragments were then electrophoresed on ABI prism 3100 Genetic analyzer and analyzed by GeneScan software package (Applied Biosystem, Foster City, CA).

The intensity of the fluorescence signal obtained with reference fragments was used to normalize the fluorescence signal obtained with dystrophin exon fragments in controls ($R_x$) and patients ($R_c$) as previously described [16]. For each exon a diagnostic index ($DI_{EPFA}$) was calculated as the mean ratio of normalized signals between the proband and the control ($R_x/R_c$) obtained in two different EPFAs. For normal subjects the expected $DI_{EPFA}$ is about 1. For females with hemizygous *DMD* deletions and males with duplicated exons the theoretical $DI_{EPFA}$ is about 0.5 and 2, respectively.

### *Multiplex quantitative real-time PCR (Q-PCR)*

*Oligonucleotide primers and TaqMan probes.* Primers and probes were designed with Primer Express (Applied Biosystem, Foster City, CA) and Oligo 4.1 primer analysis (National Biosciences, Plymouth, MN) softwares. The selected primers and probes underwent an extensive search in the EMBL and GenBank databases to avoid any significant homology with other known nucleotide sequences.

In detail, a 95-bp fragment of *DMD* exon 5 was amplified using primers EX5F (5′-TAG TGA ATA TTG GAA GTA CTG ACA TCG-3′) and EX5R (5′-GGA TTC TTA CCT GCC AGT GG-3′) and detected using a 24 bp minor groove binder (MGB)-DNA probe (5′-ATC ATA AAC TGA CTC TTG GTT TGA-3′). Similarly, a 149 bp amplicon of *DMD* exon 45 was amplified by primers EX45F (5′-GGT ATC TTA CAG GAA CTC CAG GAT G-3′), EX45R (5′-CAC CGC AGA TTC AGG CTT C-3′), and detected by a 22 bp MGB probe (5′-ATT GGG CAG CGG CAA ACT GTT G-3′). Primers EX51F (5′-GCC ATC TCC AAA CTA GAA ATG C-3′), EX51R (5′-CAA GCA GAG AAA GCC AGT CG-3′), amplified a 106 bp amplicon, which was detected using the 21 bp MGB probe (5′-TCT TCC TTG ATG TTG GAG GTA-3′). All the *DMD* probes were synthesized with the reporter dye 6-carboxyfluorescein (FAM) covalently linked to the 5′end and the dark quencher dabcyl to the 3′end (Applied Biosystems, Warrigton, UK).

Finally, a 135 bp amplicon belonging to proteolipid-1 gene (PLP1) was selected as an X-linked reference and primers PLP-1F (5′-GCG CAG TCA GGC AGA TCT T-3′), PLP-1R (5′-GAC ACA CCC GCT CCA AAG-3′), and a 26 bp probe (5′-CTA CAA GAC CAC CAT CTG CGG CAA GG-3′) were designed. Primers and probe were chosen in order to avoid cross-hybridization with the selected *DMD* sequences. The PLP1 probe was conjugated at the 5′end with the fluorescent dye Yakima Yellow and at the 3′end with the eclipse dark quencher (Eurogentec, Seraing, BE).

*Real-time PCR assays.* The TaqMan reaction was performed using an ABI 7500 instrument (Applied Biosystem, Foster City, CA) in a final volume of 25 μl containing 100 μM dATP, dCTP, dGTP, 200 μM dUTP, 5.5 mM $MgCl_2$, TaqMan reaction buffer 1×, and different amounts of primers and probes (exon 5: 150 nM primers and 200 nM probe; exon 45: 75 nM primers and 200 nM probe; exon 51: 50 nM primers and 200 nM probe; PLP1 exon 3: 600 nM primer and 400 nM probe), 0.625 U AmpliTaq Gold, 0.25 U uracil-*N*-glycosylase, and 100 ng DNA template. The TaqMan PCR cycling conditions were: 15 min pre-activation at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 64 °C for 60 s with an auto-decrement temperature factor of 0.1 °C/cycle.

*Data analysis.* Real-time PCR assay amplifications were carried out in triplicate. For each amplification a computer algorithm calculates the fluorescent signal (ΔRn) generated by the degradation of the hybridized probe and automatically determines the cycle of threshold ($C_t$) at which each PCR amplification reaches a threshold value (usually set at 10 times the standard deviation of the baseline signal) that is inversely proportional to the log number of target copies present in the sample. Relative quantification of *DMD* exons was obtained using a comparative $C_t$ method [17]. The copy number of the unknown samples was normalized to an endogenous reference (PLP1 gene) and expressed relative to a calibrator sample (normal control) using the formula $2^{-(\Delta\Delta C_t \pm SD)}$ where $\Delta\Delta C_t = [mC_{t_{PLP1}}(\text{calibrator sample}) - mC_{t_{DMD}}(\text{calibrator sample})] - [mC_{t_{PLP1}}(\text{test sample}) - mC_{t_{DMD}}(\text{test sample})]$. In each assay, $mC_t$ is the mean obtained from triplicate amplifications and SD represents the standard deviation of the difference calculated from the $mC_t$ SD of *DMD* exons and PLP1. According to this method the expected $2^{-(\Delta\Delta C_t \pm SD)}$ is about 1, 0.5, and 2 for normal, deleted, and duplicated samples, respectively. For each sample a diagnostic index ($DI_{Q\text{-}PCR}$) was calculated as the mean $2^{-(\Delta\Delta C_t \pm SD)}$ obtained from two independent assays.

## Results

### *DMD screening by end-point fragment amplification fluorescent analysis*

To select samples for validation of Q-PCR test, we screened by EPFA 63 subjects at risk of *DMD* exon 5, 45, and 51 deletions or duplications (15 females and 48 males) and identified 10 females with deletions and 3 males with duplications (exon 51 rearrangements are shown as example in Fig. 1).

### *Real-time PCR assays for the detection of deletion/duplication of dystrophin exon 5, 45, and 51*

In order to design a truly quantitative test for confirmation of the female carrier status, we selected a multiplex quantitative real-time PCR approach (Q-PCR). To this end, as internal reference system a fragment of the X-linked proteolipid protein 1 (PLP1) was chosen for single-tube analysis with the 5, 45 or 51 dystrophin exons selected because of being present in the two major deletion/duplication hotspots of the dystrophin gene.

Thus, different amounts of the target sequence cloned in plasmidic vectors were tested in separate tubes or in combination with the selected reference gene PLP1, using four orders of magnitude of DNA input (from 500 to 500,000 copies) to see if the PCR efficiency influenced multiplex PCR. As shown in Fig. 2A, the amplification kinetics of both the reference and the dystrophin exons overlapped, allowing quantitative determination of a diagnostic index ($DI_{Q\text{-}PCR}$) using the $\Delta\Delta C_t$ method (Figs. 2B and C). Moreover, no appreciable differences were detected when the amounts of the two target sequences were measured in the same reaction tube as compared with the measurement obtained by the same reactions performed in separate tubes (see equations of the curves Fig. 2A).

We measured then the $\Delta\Delta C_t$ over a wide range of DNA copy input. As shown in Fig. 2 either using exon fragment cloned in plasmidic vectors (panel B) or full genomic DNA (panel C), the slope obtained in the graphical representation

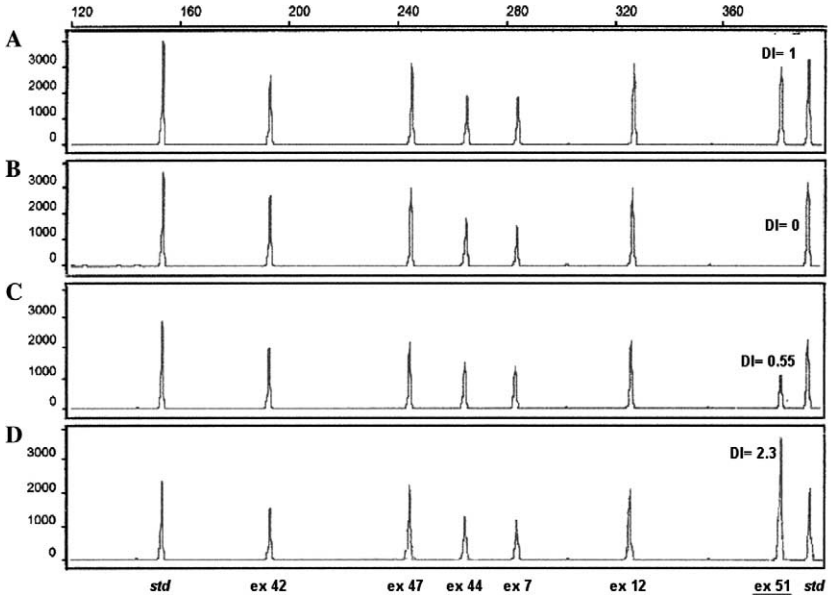


Fig. 1. Electropherograms of dystrophin exons 7, 12, 42, 44, 47, 51, and control fragments (F8 gene, exons 7 and 16) obtained with end-point fragment amplification and capillary electrophoresis. (A) Normal control; (B) male subject with exon 51 deletion; (C) female carrier with exon 51 deletion; (D) male subject with exon 51 duplication. The $DI_{EPFA}$ of exon 51 is shown in all panels.

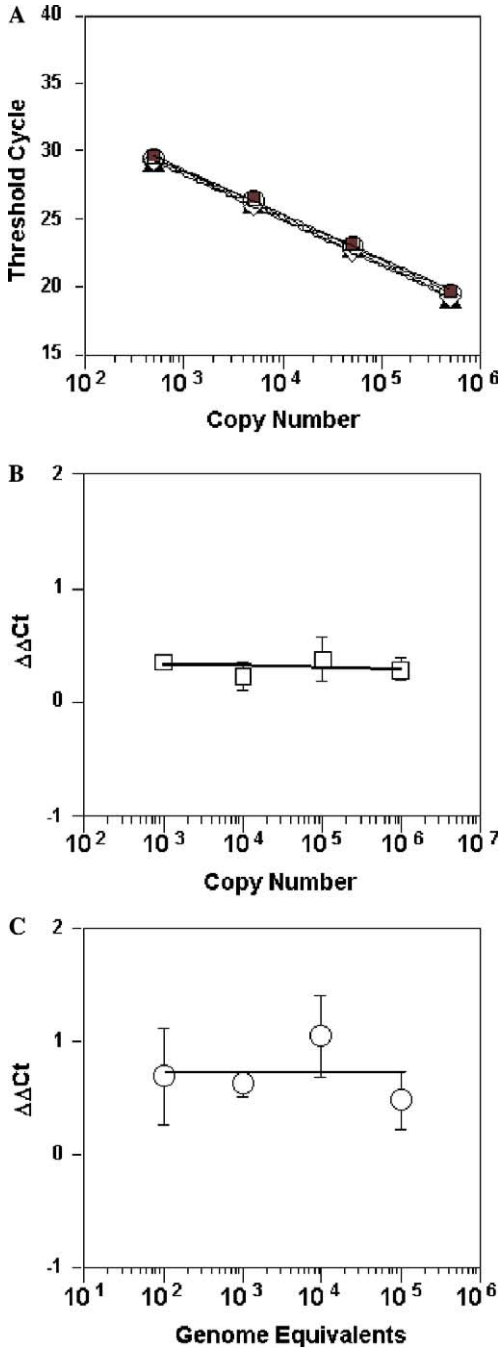


Fig. 2. Amplification kinetics (A) and $\Delta C_t$ quantification measured over a wide range of plasmid and human genomic DNA concentrations featured by the TaqMan assays. (A) Amplification of exon 51 and PLP-1 target sequences performed in a single-tube format (exon 51: equation $y = 3.375x + 15.925$, $R^2 = 0.998$, open diamond; PLP-1: equation $y = 3.315x + 16.283$ $R^2 = 0.999$, dark square) or in two separate tubes (exon 51: equation $y = 3.352x + 15.825$, $R^2 = 1$, open circle; PLP-1: equation $y = 3.281x + 16.53$ $R^2 = 0.999$, filled triangle). (B) Evaluation of exon 45/PLP-1 $\Delta C_t$ intervals measured over a series of plasmid DNA dilutions (from 1,000,000 to 1000 copies). The resulting equation is $y = -0.0032x - 0.298$, $R^2 = 0.004$. (C) Evaluation of exon 5/PLP-1 $\Delta C_t$ interval measured over a series of human genomic DNA dilutions (from 100 to100,000 human genomic equivalents). The resulting equation is $y = -0.021x - 0.655$, $r^2 = 0.012$. Each exon/PLP1 combination was fully validated with a complete set of experiment.

of the DNA copies number vs $\Delta\Delta C_t$ values approximated, as expected, the theoretical value of zero, as indicated (Fig. 2 legend).

### *Combination of EPFA and Q-PCR for diagnosis of dystrophinopathies*

To validate the use of our Q-PCR assays on clinical sample, we selected 11 obligate female carriers, with deletions of *DMD* exon 5 ($n = 2$), 45 ($n = 4$) or 51 ($n = 5$), respectively, and compared its performance with EPFA technique.

The diagnostic index ($DI_{Q\text{-}PCR}$) obtained with the real-time PCR technique ranged, for all the different exon fragments tested, from 0.29 to 0.51 with a mean of 0.38 and a standard deviation of 0.08, Similarly, the $DI_{EPFA}$ ranged from 0.48 to 0.70 with a mean of 0.55 and a standard deviation of 0.08 (Table 1). Overall, both methods unambiguously lead to the identification of female carriers and normal controls.

A second group of normal subjects (9 individuals) and carriers of exon 5, 45, 51 deletions (10 females) or duplications (3 males) was selected on the basis of EPFA screening and analyzed by Q-PCR (Table 2). The $DI_{Q\text{-}PCR}$ calculated in controls ranged from 0.83 to 1.19 with a mean of 0.99 and a standard deviation of 0.13. $DI_{EPFA}$ ranged from 0.94 to 1.00 with a mean of 0.95 and a standard deviation of 0.02. Also in this set of samples Q-PCR and EPFA provided overlapping results.

Altogether, the $DI_{Q\text{-}PCR}$ calculated on female carriers of deletions ranged from 0.29 to 0.60 with a mean of 0.39 and a standard deviation of 0.09, while $DI_{EPFA}$ ranged from 0.41 to 0.70 with a mean of 0.53 and a standard deviation of 0.07. Although the number of duplications tested was limited, the $DI_{Q\text{-}PCR}$ ranged from 1.77 to 2.42 with an average value of 2.11 and a standard deviation of 0.32, the same patients tested with EPFA ranged from 1.9 to 2.3 with a mean of 2.17 and a standard deviation of 0.19.

Table 1
Comparative analysis of EPFA and Q-PCR in female obligate carriers

| Exon | Patient ID | Diagnostic index (SD) | |
|---|---|---|---|
| | | EPFA | Q-PCR |
| 5 | OBL1_FAM1 | 0.52 (±0.01) | 0.51 (±0.07) |
| | OBL2_FAM1 | 0.53 (±0.03) | 0.29 (±0.01) |
| | Control | 0.99 (±0.00) | 1.04 (±0.03) |
| 45 | OBL3_FAM2 | 0.54 (±0.00) | 0.30 (±0.01) |
| | OBL4_FAM2 | 0.70 (±0.01) | 0.31 (±0.04) |
| | OBL5_FAM3 | 0.70 (±0.03) | 0.49 (±0.03) |
| | OBL6_FAM3 | 0.48 (±0.01) | 0.41 (±0.03) |
| | Control | 0.91 (±0.01) | 0.82(±0.01) |
| 51 | OBL7_FAM4 | 0.54 (±0.00) | 0.38 (±0.01) |
| | OBL8_FAM4 | 0.49 (±0.01) | 0.32 (±0.01) |
| | OBL9_FAM5 | 0.52 (±0.03) | 0.31 (±0.08) |
| | OBL10_FAM5 | 0.51 (±0.00) | 0.44 (±0.06) |
| | OBL11_FAM6 | 0.55 (±0.01) | 0.40 (±0.03) |
| | Control | 0.94 (±0.03) | 1.20 (±0.03) |

Table 2
Comparative analysis of EPFA and Q-PCR in subjects with *DMD* deletions and duplications

| Exon | Patient ID | Sex | Diagnostic index (SD) | |
|---|---|---|---|---|
| | | | EPFA | Q-PCR |
| 5 | CAR2 | F | 0.51 (±0.01) | 0.46 (±0.06) |
| | DMD12 | M | 1.90 (±0.01) | 1.89 (±0.03) |
| | DMD41 | M | 2.29 (±0.07) | 1.77 (±0.11) |
| | Control | M | 0.96 (±0.01) | 1.09 (±0.04) |
| | Control | F | 0.95 (±0.03) | 0.90 (±0.03) |
| | Control | F | 0.95 (±0.01) | 0.83 (±0.01) |
| 45 | CAR6 | F | 0.47 (±0.00) | 0.32 (±0.03) |
| | CAR8 | F | 0.53 (±0.01) | 0.29 (±0.01) |
| | CAR11 | F | 0.55 (±0.03) | 0.32 (±0.03) |
| | CAR15 | F | 0.41 (±0.01) | 0.60 (±0.01) |
| | DMD22 | M | 2.20 (±0.04) | 2.34 (±0.07) |
| | Control | M | 0.96 (±0.07) | 0.91 (±0.03) |
| | Control | F | 0.94 (±0.00) | 0.90 (±0.01) |
| | Control | F | 0.94 (±0.01) | 0.93 (±0.01) |
| 51 | CAR3 | F | 0.48 (±0.01) | 0.45 (±0.03) |
| | CAR4 | F | 0.52 (±0.01) | 0.40 (±0.03) |
| | CAR9 | F | 0.56 (±0.03) | 0.51 (±0.03) |
| | CAR10 | F | 0.54 (±0.00) | 0.32 (±0.03) |
| | CAR13 | F | 0.55 (±0.03) | 0.41 (±0.03) |
| | DMD22 | M | 2.30 (±0.04) | 2.42 (±0.28) |
| | Control | M | 0.94 (±0.03) | 0.96 (±0.01) |
| | Control | F | 1.00 (±0.04) | 1.19 (±0.03) |
| | Control | F | 0.94 (±0.00) | 1.05 (±0.03) |

## Discussion

Aim of the study was to assess the ability of quantitative real-time PCR based on TaqMan technology detecting hemizygous duplication or deletions within the dystrophin gene for the diagnosis of DMD/BMD. To this end, we selected exon 5, 45, and 51 within the mutational hotspots of *DMD* gene and developed three multiplex assays in which a target exon was co-amplified with an X-linked reference fragment. To validate the test, we investigated 21 female carriers of *DMD* deletions (11 obligate carriers) and 3 males with *DMD* duplications. We showed that $DI_{Q\text{-}PCR}$ values calculated in controls (0.83–1.19), in female carriers of deletions (0.29–0.60), and males with duplications (1.77–2.42) did not overlap allowing the accurate discrimination of carriers and non-carriers of both deletions and duplications in the dystrophin gene. The investigation of obligate carriers and comparative analysis with previously reported semiquantitative end-point PCR [16] indicate that the method is robust. We also showed that the experimental variability on a given sample in two different runs is low, indicating that the method is highly reproducible. To our knowledge this is the first study implementing the real-time TaqMan technology to the diagnosis of DMD/BMD.

Molecular diagnosis of DMD/BMD is a difficult task due to size of the dystrophin gene coding region and its complex genomic organization, and to a remarkable mutation diversity including dose abnormalities such as deletions and duplications.

Multiplex end-point PCR approaches such as MAPH, MLPA, and EPFA allow semiquantitative analysis of multiple exons and can be effectively used for the rapid screening of the entire *DMD* coding region in both affected males and female carriers [16,15].

MAPH and MLPA analyses are accomplished in two working days and are laborious and time-consuming. EPFA screening may be accomplished in a single day. However, the lack of validated commercial kits requires handling of several primer pairs to set up multiplex PCRs, making the management of this test difficult. Moreover, all these assays require the use of capillary electrophoretic runs on automated sequencers with additional handling and costs.

For carrier testing the mutation is usually known and there is no need to screen the entire gene. Thus, the real-time assay allows the rapid identification of a mutation in a single PCR within few hours and for less than 3 euros. Furthermore, reagents for Q-PCR can be custom-predispensed by producers for an easy handling.

Furthermore, our method could be routinely used to confirm end-point PCR screening data using an independent set of primers and probes, particularly when deletions/duplications are found in a single exon. Indeed, it has been shown that polymorphisms may prevent the correct annealing of probes and primers leading to false-positive [18].

Thus, a combination of these two different assays may be necessary for secure and correct diagnosis of DMD/BMD.

A real-time PCR test based on SYBR green I detection was recently described for DMD/BMD [15]. This method allows the monitoring of amplification reaction but cannot easily discriminate between specific and aspecific PCR products, and does not allow the amplification of control and *DMD* fragments in the same tube. The use of TaqMan fluoresceine-labeled probes ensures a higher level of specificity, allowing the co-amplification of different targets including the reference fragment. In conclusion, we demonstrated that our real-time quantitative PCR-based approach permits the discrimination of normal, deleted, and duplicated *DMD* alleles. The method is highly sensitive, specific, and reproducible, requires minimal quantity of DNA, and is carried out in a single step.

The routine application of this technique to the diagnosis of DMD/BMD will require the development of additional assays for the analysis of exons that are frequently involved in *DMD* rearrangements.

## Acknowledgments

This work was supported by Telethon-Italy (Grant GGP04166) and M.I.U.R.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.11.006.

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