Application of FTA[®] Sample Collection and DNA Purification System on the Determination of CTG Trinucleotide Repeat Size by PCR-Based Southern Blotting

Kuang-Ming Hsiao,¹ Her-Maw Lin,² Huichin Pan,¹ Tung-Cheng Li,² Sung-Sheng Chen,⁵ Shuo-Bin Jou,⁶ Ya-Lan Chiu,³ Ming-Fang Wu,¹ Chyi-Chyang Lin,^{1,4*} and Shuan-You Li^{1*}

¹Department of Life Sciences, Chung Shan Medical and Dental College, Taichung, Taiwan

²Institute of Medicine, Chung Shan Medical and Dental College, Taichung, Taiwan

³Department of Medical Technology, Chung Shan Medical and Dental College, Taichung, Taiwan

⁴Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Canada

⁵Department of Neurology, Kaohsiung Medical College, Kaohsiung, Taiwan ⁶Department of Neurology, China Medical College Hospital, Taichung, Taiwan

Myotonic dystrophy (DM) is caused by a CTG trinucleotide expansion mutation at exon 15 of the myotonic dystrophy protein kinase gene. The clinical severity of this disease correlates with the length of the CTG trinucleotide repeats. Determination of the CTG repeat length has been primarily relied on by Southern blot analysis of restriction enzyme-digested genomic DNA. The development of PCR-based Southern blotting methodology provides a much more sensitive and simpler protocol for DM diagnosis. However, the quality of the template and the high (G+C) ratio of the amplified region hamper the use of PCR on the diag-

nosis of DM. A modified PCR protocol to amplify different lengths of CTG repeat region using various concentrations of 7-deaza-dGTP has been reported (1). Here we describe a procedure including sample collection, DNA purification, and PCR analysis of CTG repeat length without using 7-deaza-dGTP. This protocol is very sensitive and convenient because only a small number of nucleate cells are needed for detection of CTG expansion. Therefore, it could be very useful in clinical and prenatal diagnosis as well as in prevalence study of DM. *J. Clin. Lab. Anal.* 13:188–193, 1999.

© 1999 Wiley-Liss, Inc.

Key words: myotonic dystrophy; CTG trinucleotide repeat; DM diagnosis; PCR; FTA[®] Gene Guard System

INTRODUCTION

Myotonic dystrophy (DM) is one of the most frequent neuromuscular genetic diseases in the Caucasian population (incidence of 1 in 8,000) (2). This autosomal dominant disorder is caused by an expansion mutation of the CTG trinucleotide repeat in the 3' untranslated region (3'-UTR) of the myotonin protein kinase gene which was mapped on chromosome region 19q13.3 (3–5). The CTG repeat ranges mostly from 5 to 40 copies in normal individuals and is found to be amplified by up to 2,000 copies in DM patients (6). The size of this trinucleotide repeat is related both to the age of onset and to the severity of the clinical manifestation (7).

Determination of the CTG repeat number can be achieved through polymerase chain reaction (PCR) or Southern blot analysis. PCR technique is routinely used for the detection of small-scale expansion in repeat size. Although the PCR procedure is fast, simple, and inexpensive, size determination of long CTG repeat using this technique is not possible. There-

fore, Southern blot analysis on restriction enzyme-digested genomic DNA (referred to as genomic Southern blot hereafter) remains the primary method for detecting the size of long trinucleotide expansion (3–5). However, this procedure is labor intensive and requires relatively large amounts of genomic DNA. It is thus inconvenient for routine clinical diagnoses, especially for prenatal diagnoses of the disorders.

A combination of PCR and Southern blot analysis proce-

Grant sponsor: National Science Council of the Republic of China; Grant numbers: NSC87-2314-B040-018 and NSC88-2314-B040-021; Grant sponsor: Chung Shan Medical and Dental College Research Fund; Grant numbers: CSMC86-OM-A-011, CSMC86-OM-A-036, CSMC86-OM-B-018, and CSMC86-OM-B-020.

*Correspondence to: Shuan-You Li or Chyi-Chyang Lin, Department of Life Sciences, Chung Shan Medical and Dental College, No.110, Sec.1, Chien-Kuo North Road, Taichung, Taiwan.
e-mail address: syl@mercury.csmc.edu.tw

Received 28 October 1998; Accepted 31 March 1999

dures has been developed for detection of the trinucleotide repeat (5,8,9). In such an assay, PCR-amplified trinucleotide-containing DNA fragments are used instead of restriction enzyme-digested genomic DNA for Southern blot. This gives the advantage of both PCR sensitivity and Southern blotting specificity. Although this assay can distinguish normal DM alleles from expanded ones, signals of the expanded allele are often smears that make the estimation of expansion size difficult (5,9). Recently, Cheng et al. (1) demonstrated that various CTG expansion sizes can be evaluated more precisely by using various concentrations of 7-deaza-dGTP in the PCR mixture. However, the large targets from degraded template DNA remain difficult to amplify by PCR, a common problem often encountered with older samples. To overcome these defects, we developed a sensitive and convenient procedure involving sample collection and storage, DNA purification, and PCR determination of CTG repeat length. Using this procedure, the samples can be stored for a long period of time without affecting the DNA quality for PCR. Furthermore, only a few cells (about 10 lymphoblastoid cells) are needed to determine the size of CTG expansion. The simplicity and sensitivity of this procedure makes it useful for clinical and prenatal diagnosis as well as for prevalence study of DM.

MATERIALS AND METHODS

Sample Collection and DNA Preparation

About 10 ml peripheral blood sample was collected using a heparin- (anticoagulant) rinsed syringe from each tested individual. Seven and half ml of blood specimen was used for transformation of peripheral B lymphocytes by Epstein-Barr virus (EBV) to establish permanent lymphoblastoid cell line. The method used for the transformation was based on the protocol of Neitzel (10) with an additional step of adding 10 µl of phytohemagglutinin (Gibco BRL-Pacific, catalog #10576-015, Tsuen Wan, Hong Kong) to the 10 ml media on day three after culture. For the size determination of CTG repeat by PCR-based Southern blotting (as shown in Fig. 1), the template DNA was directly extracted from blood specimen or from transformed lymphoblastoid cells using Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) following the manufacturer's specifications. For the size determination of CTG repeat by FTA®-PCR system, 1 µl blood specimen (Fig. 2A) or indicated amounts of lymphoblastoid cells (Figs. 2B and 3) were dotted onto an FTA®-treated card (Gibco BRL-Pacific). The FTA® card consists of filter paper impregnated with denaturants that prevent growth of micro-organisms and maintain the integrity of DNA from nucleases, oxidation, and UV damage. At least 1 hr is required for the sample to become dry before processing further. We then used a 1.2 mm-diameter paper puncher (HARRIS MICRO-PUNCH from Gibco BRL) to punch out the sample from the middle of the blood stain on the FTA® card and place it into an amplification tube. The FTA® paper punch was then washed with FTA® Purification Reagent

Fig. 1. Determination of CTG repeat length in DM alleles by improved PCR-based Southern blotting protocol. **A**, Effect of the DNA template amount on the detection of CTG repeat size. Various amounts of DNA samples obtained by serial dilutions from a normal individual (N) and two DM patients (DM1 and DM2) were analyzed as described in Materials and Methods. **B**, Application of the improved PCR protocol in determining the size of CTG expansion. One hundred pg genomic DNA extracted from whole blood was used as a template in all PCR reactions. Lanes 1–4 are normal individuals, lanes 5–7 are asymptomatic individuals, lanes 8–12 are DM patients.

(Gibco BRL) according to the manufacturer's specifications. The complete PCR amplification mix was directly added to the punch containing the purified immobilized DNA. A 20-µl reaction volume was used with all PCR amplification mixes. The FTA® card can be wrapped in plastic wrap and left at room temperature for more than two months or stored in a desiccant for long-term storage.

Determination of CTG Repeat Number of Normal Alleles

For screening study involving normal alleles with lower CTG repeat length, each 20-µl reaction consisted of 1× reaction buffer (containing 1.5 mM MgCl₂, 50mM KCl, 10 mM Tris-HCl (pH9.0), and 0.1% Triton X-100 (Promega, Madison, WI), 0.2 mM dNTP (Promega), 0.5 µM each primer, 4 µCi α-[³²P]dCTP (10 mCi/ml; NEN Life Science Products, Boston, MA), and 1 unit of *Taq* DNA polymerase (Promega). The forward primer, primer H (11), 5'-TCTCCGCC-

Fig. 2. Analysis of the CTG repeat length in the DM locus using FTA[®]-PCR protocol. **A**, Determination of CTG repeat length of normal individuals. One μ l blood specimen was dotted onto an FTA[®] card and DNA was purified and analyzed as described in Materials and Methods. The four tracks to the left contain pGEM sequence, which was used to determine the size of alleles. The corresponding CTG repeat number of signals was shown on the right. **B**, Detection of CTG expansion size using various numbers of cells. Cultured lymphoblastoid cells from a DM patient were counted under microscope and dotted onto the FTA[®] card for CTG-length analysis. The cell number from lanes 1 to lane 4 was 200, 100, 20, and 10, respectively.

AGCTCCAGTCC-3', and reverse primer, primer ER (11), 5'-AAATGGTCTGTGATCCCCC-3', were used for amplification. After an initial template denaturation of 5 min at 96°C, amplification was carried out for 35 cycles of 45 sec at 96°C, 45 sec at 62°C, and 1 min 30 sec at 72°C, then followed by a final extension of 10 min at 72°C in a Perkin Elmer thermal cycler (model 480; PE Applied Biosystems, Foster City, CA). The PCR product was resolved by electrophoresis on 6% urea-polyacrylamide gel (Bio-Rad, Hercules, CA). Dried gels were exposed to Kodak X-AR5 film (Eastman Kodak Company, Rochester, NY) for 1–2 days at –70°C.

Fig. 3. Application of the FTA[®]-PCR system on the diagnosis of a DM family using 10 lymphoblastoid cells. The family pedigree was shown on the top of this figure. Six available samples from this family were also analyzed by Southern blotting on 5 μ g *Eco*RI-digested genomic DNA (shown in the middle of the figure) to confirm the validity of the FTA[®]-PCR protocol using 10 lymphoblastoid cells (shown at the bottom).

PCR-Based Southern Blot Analysis of Expanded CTG Trinucleotide Repeat Length

The various amounts of template DNA used in PCR were obtained by serial dilution from DNA stock (100 nanograms per μ l) extracted from blood specimen or transformed lymphoblastoid cells. The number of cells dotted onto the FTA[®] card was checked by a phase-contrast microscope. PCR reaction was carried out in a total volume of 20 μ l containing 1 μ l DNA template, 1 $\times$ cDNA PCR reaction buffer, 1 M GC-melt, 0.2 mM dNTP, 0.5 μ M primers (H & ER), and 0.8 μ l Advantage-GC cDNA polymerase mix (Clontech, Palo Alto, CA). Cycling conditions were as follows: 1 $\times$ (94°C, 1 min), 35 $\times$ (94°C, 20 sec; 68°C, 5 min), 1 $\times$ (68°C, 5 min), and 15 °C soak using a Perkin Elmer 9600 thermal cycler. Southern blot of the PCR product was performed using a nonisotopic Quick-Light detection system (Life Codes, Stamford, CT) according to the manufacturer's recommendations. PCR products were run on a 1.5 % agarose gel and then transferred to a membrane (GeneScreen Plus[®], NEN[™] Life Science Products). After hybridizing with the alkaline phosphatase-coupled (CTG)₁₀, the membrane was washed according to the manufacturer's instructions. The membrane was then sprayed with Lumi-Phos 480 dioxetane solution, sealed in a development folder, and exposed to X-ray film. This result was con-

firmed by genomic Southern blot analysis using a 1,002-bp DNA probe prepared by PCR using the internal nested primers DM-X (5'-CTCCGGGATCTCCCTAGATAACCTCC-3', from 12320 to 12345 of GeneBank Database accession # L08835) and DM-DR (5'-CTTCCCAGGCCTGCAGTTT-GCCCATC-3') (12). The assay protocol was the same as described previously (3).

RESULTS

Primers flanking the CTG repeat region were designed according the report by Monckton et al. (11) for the determination of both normal and expanded CTG lengths by the PCR. In order to develop a simple PCR protocol that can detect the CTG repeat length without using 7-deaza-dGTP, different DNA polymerases were tested and PCR conditions were optimized (data not shown). Using the described methods, the optimal amount of DNA template to be used for size determination of expanded CTG repeats (DM1, DM2) is 100 picograms (pg) (Figure 1A). Under these conditions, the signals representing normal CTG repeat length in a normal individual (N) and in normal alleles of DM patients (DM1 and DM2) were very faint or even undetectable. If one nanogram (ng) or more DNA template is used, some other products may be detected, possibly reflecting the CTG size heterogeneity. The expanded CTG size determined in Figure 1A was confirmed by the Southern blot analysis on *Eco*RI-digested genomic DNA (data not shown). The application of this improved PCR protocol which uses 100 pg of DNA to determine the various expanded CTG repeat lengths was achieved as shown in Figure 1B. The size of the PCR product is $(132+3n)$ bp; n represents the CTG repeat number. Therefore, the CTG repeat number of the expanded alleles of the DNA samples from lanes 5 to 12, was calculated to be 42, 70, 73, 160, 290, 450, 700, and (1,050/1,180), respectively. The two distinct expanded bands detected in lane 12 could represent the CTG size heterogeneity present in this DM patient. The CTG repeat numbers of the normal alleles of DNA samples from lanes 1 to 12 were (5/13), (5/11), (5/5), (5/11), (5), (5), (5), (13), (5), (14), (5), and (11) as determined by the [³²P]-incorporated PCR method and as described in Materials and Methods. Although the DNA samples of lanes 1, 2, and 4 each contain two normal alleles with different CTG repeat lengths, only one band can be detected that is of similar size to those alleles having 5 CTG repeats. These results indicate that the differences in normal allele sizes, among these samples, are too small to be resolved with the used gel conditions. Hence, although the fragment sizes appear similar, the low repeat numbers of both alleles, such as (5/5) for the DNA sample of lane 3, have resulted in a much weaker signal (Fig. 1B). The observation of the signals representing normal allele bands with 5 CTG repeats, such as lanes 3, 5, 6, 9, and 11, are often faint or undetectable. This suggests the possibility that a shorter CTG repeat

length will result in a higher chance of the probe being washed away under the conditions used.

The CTG region-containing *Eco*RI fragments detected by genomic Southern blotting can be 8.6 kb or 9.6 kb due to the *Alu* polymorphism present in intron 8 of myotonic dystrophy protein kinase (DMPK) gene (13). In all of the DM patients studied to date, the expanded (CTG)_n repeat is always associated with the 1-kb insertion allele of the *Alu* "insertion/deletion" polymorphism. The only exception observed was of a Nigerian kindred (14). This *Alu* insertion polymorphism also shows a total linkage disequilibrium with Taiwanese myotonic dystrophy (unpublished data). Therefore, the size of the detected bands representing the CTG expansion alleles, when using the Southern blot analysis on *Eco*RI-digested genomic DNA, should be $(9,600+3n)$ bp; n represents the CTG repeat number. The calculated CTG repeat numbers for the expanded alleles of the DNA samples of lanes 9–12 in Fig. 1B were 300, 480, 700, and 1,000, respectively, which are similar to those of PCR analysis. Due to the limitation of the resolution, it was almost impossible to precisely estimate the CTG repeat numbers for those alleles with small expansion, such as the DNA samples of lanes 5–8 in Fig. 1B, by genomic Southern blotting (data not shown).

In an attempt to establish a convenient method for sample collection, storage, and subsequent DNA purification, we tested the FTA[®] Gene Guard System (Gibco BRL). In Figure 2A, the results of determining the CTG repeat length within normal range was demonstrated when the immobilized DNA on the FTA[®] card was purified from 1 μ l blood specimen. This purified, immobilized DNA was used as a template for subsequent PCR analysis (referred to as FTA[®]-PCR system hereafter). As has been shown (Figure 2A and data not shown), the major repeat numbers of normal alleles in the Taiwanese population are 5, and 11–13. These numbers are similar to those reported for the European and Japanese populations as well (15). Size determination of CTG expansion in DM alleles by the FTA[®]-PCR system was demonstrated in Figures 2B and 3. In Figure 2B, the DNA purified from the FTA[®] paper was dotted with various numbers of lymphoblastoid cells (10, 20, 100, and 200) from a DM patient with 5 and 190 CTG repeats. After analysis, the results indicated that as few as 10 lymphoblastoid cells were sufficient for the determination of the DM expansion size. When 100 or more cells were used, the detected signal became smeared and revealed that optimal DNA template amounts were critical for precise size determination. The result of the analysis using the FTA[®]-PCR protocol with 10 lymphoblastoid cells and the Southern blot analysis with 5 μ g *Eco*RI-digested genomic DNA from each of the six individuals in a Taiwanese DM family is shown in Figure 3. The expanded CTG repeat numbers of II-4, II-6, II-8, and III-3 determined by FTA[®]-PCR protocol (at the bottom of the figure) was calculated to be 1,020, 1,080, 720, and 950, respectively. The CTG repeat numbers for II-4, II-6, and II-8 obtained by the genomic Southern blotting (in the middle

of the figure) was approximately 1,060, 1,060, and 730, respectively, which were very similar to those of the FTA[®]-PCR analysis. For III-3, the CTG repeat number could be from 800 to 1,300 as calculated from the genomic Southern blotting data. This could also reflect the CTG length mosaicism present in this DNA template, a common phenomenon observed in DM patients (16). The detected CTG repeat size of III-3 by FTA[®]-PCR analysis, 2.9 kb, may represent one of the major sizes present in this patient. In addition, we observed that the quality of DNA purified from the blood specimen and the lymphoblastoid cells stored for two months on FTA[®] paper was just as good as freshly prepared DNA (data not shown). These results indicate that this FTA[®]-PCR protocol is reliable and sensitive.

DISCUSSION

The diagnosis of trinucleotide repeat diseases has become more frequently encountered in the clinical laboratory, especially for those diseases with higher incidence rates such as fragile X syndrome (1 in 850) (17) and DM (1 in 8,000) (2). Thus far, the diagnostic procedures for these diseases involve PCR or Southern blot techniques using DNA extracted from nucleate cells. A basic but unavoidable problem of applying PCR in the clinical laboratory is that the DNA quality of samples may have a significant effect on the testing result. The contamination of microorganisms, nucleases, and UV damage during DNA extraction and storage may cause DNA degradation and thus interfere with the assay process. Due to the fact that old samples with degraded DNA are not suitable for PCR detection of large CTG expansion, DNA preparation from freshly obtained blood samples is often needed. To overcome this problem, we used the FTA[®] Gene Guard System that includes sample collection, storage, and DNA purification. In our experience, the DNA immobilized on FTA[®] cards stored at room temperature for more than two months is still perfect for PCR analysis of DM.

A combination of PCR and Southern blot assay on DM diagnosis was first described by Mahadevan et al. (5). However, the determination of large CTG expansion size by this assay was not possible. An improved PCR protocol was later developed for characterization of large CTG repeat expansion using various percentages of 7-deaza-dGTP in the dG/deaza-dGTP reaction component (1). In that protocol, the optimal percentage of 7-deaza-dGTP in the dG/deaza-dGTP reaction component could vary from 20 to 90%, depending upon the size of the repeat expansion and the method of detection. In this article, we first described a PCR protocol that can determine both the lower CTG repeat number and the large expansion size without using 7-deaza-dGTP. Our protocol used primers that flank the CTG repeat region (primers H and ER) and routinely observed that when the DNA template amount in a 20- μ l PCR reaction mixture was 100 ng or more, the detected signals reflecting the lower CTG expan-

sion were sharp and distinct. However, signals representing large CTG expansion often presented as smearing, as previously reported (5,8,9 and data not shown). This could be due to the heterogeneity of PCR and template DNA. When 100 pg to 1 ng DNA template was used, the size of large CTG expansion could be more precisely determined (Fig. 1 and data not shown). However, we also observed the CTG somatic mosaicism in lymphoblastoid cells. When the DNA from different batches of lymphoblastoid cells of the same DM patients was analyzed, the detected CTG expansion size may vary to some extent (data not shown). This observation was consistent with a recent report by Martorell et al. (16) and may partially explain why when large amounts of DNA (up to 100 ng) was used, the detected signal was often presented as smears.

To investigate whether distinct signal representing expanded CTG length could be detected on trace amounts of blood sample or on only few nucleate cells, the FTA[®] Gene Guard System was used for the preparation of template DNA and then PCR-based Southern blot analysis was performed. As shown in Figures 2 and 3, this procedure can be applied to determine the CTG repeat number of normal as well as DM alleles. We frequently observed that when as few as 10 nucleate cells were used, the detected signal was clear and distinct. Therefore, this procedure could be useful for prenatal diagnosis of DM and population-screening studies of the disease. It is also expected that the protocol can be applied to other trinucleotide expansion diseases, such as Huntington's disease (HD), Machado-Joseph disease (MJD), Spinal and bulbar muscular atrophy (SBMA), Dentatorubral pallidolusian atrophy (DRPLA), and Spinalcerebellar ataxia (SCA), etc.

In conclusion, we established a protocol that has the following improvements. First, a simple and reliable method was used for sample collection and DNA preservation. Second, a simplified and sensitive PCR protocol that did not require 7-deaza-dGTP was introduced for the detection of expanded CTG repeat size. Third, few nucleate cells were needed for determination of the size of trinucleotide repeat.

REFERENCES

1. Cheng S, Barcelo JM, Korneluk RG. Characterization of large CTG repeat expansions in myotonic dystrophy alleles using PCR. *Hum Mutat* 1996;7:304-310.
2. Harper PS. *Myotonic dystrophy*, 2nd edition. London: WB Saunders; 1989.
3. Brook JD, McCurrach ME, Harley HG, et al. Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell* 1992;68:799-808.
4. Fu YH, Pizzuti A, Fenwick RG Jr., et al. An unstable triplet repeat in a gene related to myotonic muscular dystrophy. *Science* 1992;255:1256-1258.
5. Mahadevan M, Tsilfidis C, Sabourin L, et al. Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene. *Science* 1992;255:1253-1255.
6. Harley HG, Rundle SA, Reardon W, et al. Unstable DNA sequence in myotonic dystrophy. *Lancet* 1992;339:1125-1128.

7. Harley HG, Rundle SA, MacMillan JC, et al. Size of the unstable CTG repeat sequence in relation to phenotype and parental transmission in myotonic dystrophy. *Am J Hum Genet* 1993;52:1164–1174.
8. Hecimovic S, Barisic I, Muller A, et al. Expand long PCR for fragile X mutation detection. *Clin Genet* 1997;52:147–154.
9. Petronis A, Heng HHQ, Tatuch Y, et al. Direct detection of expanded trinucleotide repeats using PCR and DNA hybridization techniques. *Am J Med Genet* 1996;67:85–91.
10. Neitzel H. A routine method for the establishment of permanent growing lymphoblastoid cell lines. *Hum Genet* 1986;73:320–326.
11. Monckton DG, Coolbaugh MI, Aashizawa KT, Siciliano MJ, Caskey CT. Hypermutable myotonic dystrophy CTG repeats in transgenic mice. *Nature Genet* 1997;15:193–196.
12. Monckton DG, Wong LJC, Ashizawa T, Caskey CT. Somatic mosaicism, germline expansions, germline reversions and intergenerational reductions in myotonic dystrophy. *Hum Mol Genet* 1995;4:1–8.
13. Mahadevan MS, Foitzik MA, Surh LC, Korneluk RG. Characterization and polymerase chain reaction (PCR) detection of an *Alu* deletion polymorphism in total linkage disequilibrium with myotonic dystrophy. *Genomics* 1993;15:446–448.
14. Krahe R, Eckhart M, Ogunniyi AO, Osuntokun BO, Siciliano MJ, Ashizawa T. De novo myotonic dystrophy mutation in a Nigerian kindred. *Am J Hum Genet* 1995;56:1067–1074.
15. Davies J, Yamagata H, Shelbourne P, et al. Comparison of the myotonic dystrophy associated CTG repeat in European and Japanese populations. *J Med Genet* 1992;29:766–769.
16. Martorell L, Monckton DG, Gamez J, et al. Progression of somatic CTG repeat length heterogeneity in the blood cells of myotonic dystrophy patients. *Hum Mol Genet* 1998;7:307–312.
17. Brown WT. The fragile X: progress toward solving the puzzle. *Am J Hum Genet* 1990;47:175–180.