The flash-small-pool PCR: how to transform blotting and numerous hybridization steps into a simple denatured PCR

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Numerous human diseases are associated with abnormal expansion of unstable trinucleotide repeats (TNRs). TNR instability mechanisms are complex, and remain only partially understood. Small-pool-PCR (SP-PCR) is the reference method to assess TNR instability. SP-PCR amplifies a low number of DNA molecules and is followed by Southern blot. However, SP-PCR remains expensive and time consuming. Here, we describe an optimized SP-PCR that can be done in a day, which reduces cost and experimental biases: the flash-small-pool PCR (FSP-PCR). This method consists of a fluorescent PCR on a few DNA molecules, followed by an alkaline gel electrophoresis revealed with a near infra-red detector system. With reduced experimental steps, cost, and time consumption, microsatellite analysis will become more accessible due to FSP-PCR.

Introduction

An increasing number of human neuromuscular and neurodegenerative diseases are due to unstable trinucleotide repeats (TNRs), including Huntington's disease (with CAG repeats) [1], myotonic dystrophy type 1 (with CTG repeats) [2–4], Fragile X Syndrome (FXS, with CGG repeats) [5] or Friedreich's ataxia (with GAA repeats) [6]. In the general population, repeats remain stable in tissues and through parental transmissions. In patients, the abnormally expanded repeats are unstable and generally biased towards expansions in both germline and somatic cells. In some TNR-associated diseases, such as myotonic dystrophy, larger repeats are associated with more severe symptoms and with an earlier age of onset (anticipation phenomenon) [7]. In somatic cells, TNR instability is age-dependent, tissue-specific and may be associated with symptom progression [8,9]. Therefore, evaluation of TNR instability is critical for prognosis and to better understand these diseases.

Triplets instability appears as a complex result of different mechanisms [7,10]. Development of different models such as mouse, bacterial, yeast, or cell models has given insight about the processes underlying TNR instability but, to date, the dynamic of repeats changes remains only partially understood [10–13]. Further analysis on TNR instability will provide clues for new therapeutic approaches to avoid repeat expansion or to induce TNR contraction down to a non-pathogenic length. Furthermore, accurate TNR instability analysis is necessary to characterize new models and new experimental tools [14–16].

SP-PCR is a powerful method to accurately analyze TNR instability [17–20]. The serial dilutions involved in this method resolve the heterogeneous smear detected by standard PCR experiments. However, current protocols are time consuming, with many steps that can affect the experiment quality such as blotting quality, and hybridization. Moreover, current protocols require many reagents and remain expensive. To eliminate this disadvantage, and to facilitate the spread of this high-sensitive method, we have developed an easier and faster method: the flash-small-pool PCR (FSP-PCR) to detect microsatellite repeat-containing PCR products. This protocol comes without biohazard, and does not require a blotting step or hybridization. Moreover, this protocol is faster, feasible in 24 h only, while current procedures take at least 3 days.

In short, this protocol consists of a classical small-pool-PCR performed with one of the primers labeled with near infrared dye (IRD), loaded on a denaturing agarose gel, and analyzed with fluorescent analyzer, here the Odyssey® CLx Infrared Imaging System (LI-COR Biosciences – GmbH, NE, USA). In this study, we focused on CTG repeat expansion in DM1 patients.

Material & methods

This protocol has been adapted from previously described procedures [16–18]. The primers used are listed in Table 1. See

METHOD SUMMARY

To analyze more quickly and easily triplet nucleotides repeats, we optimized the commonly used small-pool PCR protocol using a near infrared-labeled primer and alkaline gel electrophoresis. Our protocol gives robust results, and avoids the many experimental reagents and biases of the classic small-pool PCR.

Table 1. List of primers used in flash-small-pool PCR.

Primer name	Primer sequence
Forward primer DMC	5′-AACGGGGCTCGAAGGGTCCT-3′
Reverse labeled primer DMBR	5′-IRD700-CGTGGAGGATGGAACACGGGAC-3′

supplementary material for a more detailed protocol.

Restriction endonuclease digestion

All DNA quantifications were done with Qubit® Fluorometer (ThermoFisher Scientific, MA, USA) following manufacturer recommendations. To facilitate DNA accessibility, genomic DNA was digested with 50 U of Hind III endonuclease (Fermentas, MA, USA).

FSP-PCR

Genomic digested DNA was serially diluted (1.5 ng.µl^{-1}, 300 pg.µl^{-1}, 60 pg.µl^{-1}, 12 pg.µl^{-1}) in deionized milli Rho water with 0.1 M of carrier oligonucleotide (Table 1). PCR mix was prepared as follows (final volume = 7 µl per well): 0.2 µM of unlabeled-forward primer, 0.2 µM of IRD-Reverse primer (Metabion, Planegg, Bavaria, Germany), 1X PCR buffer (ThermoFisher Scientific, ref. SM-0005; MA, USA), 5 U.µl^{-1} of Thermo Perfect Taq (Integro, Zaandam, The Netherlands), Deionized milli Rho water up to 6.5 µl. Each PCR reaction was done with 0.5 µl of each serial DNA dilution. PCR program was launched in a Thermal Thermocycler (Applied Biosystems, CA, USA): one 5-min hold at 96°C, followed by 28 cycles: 96°C (45 s), 68°C (45 s), 70°C (3 min), and a chase at 68°C (1 min) and 70°C (10 min). No PCR optimization was needed because of infra-red labeling (IRD).

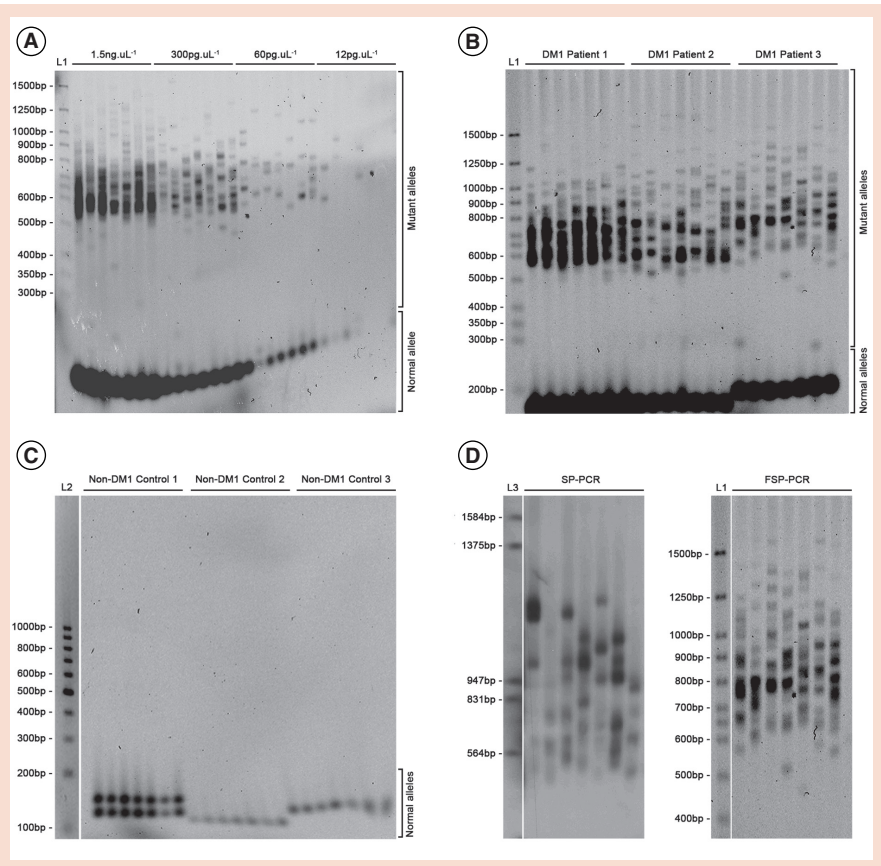

Figure 1. FSP-PCR using blood DNA from patients carrying 150 CTG and non-DM1 controls. (A) The FSP-PCR was performed with successive dilutions of a DM1 patient blood DNA (1.5 ng.µl^{-1}, 300 pg.µl^{-1}, 60 pg.µl^{-1} and 12 pg.µl^{-1}). For each dilution, the seven lanes represent seven PCR replicates. PCR products corresponding to normal and mutant alleles are indicated in brackets. Gel agarose percentage: 1.5%. **(B)** FSP-PCR using blood DNA from three DM1 patients. For each patient, seven PCR replicates were performed using the 300 pg.µl^{-1} dilution. Gel agarose percentage: 1.5%. **(C)** FSP-PCR using blood DNA from three non-DM1 control individuals. For each patient, seven PCR replicates were performed using the 300 pg.µl^{-1} dilution. Gel agarose percentage: 3%. **(D)** Left panel: SP-PCR (seven replicates using the DNA dilution 300 pg.µl^{1}) revealed with a $(CAG)_7$ probe labeled with digoxygenin after Southern blot. Right panel: FSP-PCR (seven replicates using the DNA dilution 300 pg.µl^{1}) revealed directly with Odyssey® CLx Infrared Imaging. L1: 50–1500 bp DNA sizing standard (LI-COR Biosciences – GmbH). L2: Thermo Scientific GeneRuler 100 bp DNA Ladder (ThermoFisher Scientific). L3: DNA Molecular Weight Marker III, DIG-labeled (Roche). DM-1: Myotonic dystrophy type 1; FSP-PCR: Flash-small-pool PCR.

Denaturing agarose gel electrophoresis of PCR products

A 20 × 40 cm 2.5% agarose gel [19] (50 nM of NaCl and 4 mM of EDTA) was pre-soaked in a cold room for at least 1 h in running buffer (NaOH 30 mM, EDTA 2 mM, volume adjusted with milli Rho water). 2 µl of the PCR reactions were used in each well. The ladder used was the 50–1500 bp DNA sizing standard (LI-COR Biosciences – GmbH, USA). The gel electrophoresis was set up as follows 30 min at 140 V, then 2 V/cm (80 V for a 20 × 40 cm gel) overnight. Gels were directly observed with the Odyssey® CLx Infrared Imaging System and analyzed with Image Studio™ software (LI-COR Biosciences – GmbH).

Human DNA samples

DNA from DM1 patients were obtained from the Genethon DNA and Cell Bank in collaboration with the DM scope registry. Consent was obtained for the use of DNA samples in this study.

Results & discussion

We provide here an optimized and robust protocol to assess microsatellite instability. While the current protocols with radioactivity or digoxygenin labeling require expensive reagents and days of experimental procedures, this new protocol provides results in a much simpler way (the experimental steps are resumed to one PCR and one agarose gel migration only), and within a decreased procedure time. This protocol has been tested using serial dilution of blood DNA from a DM1

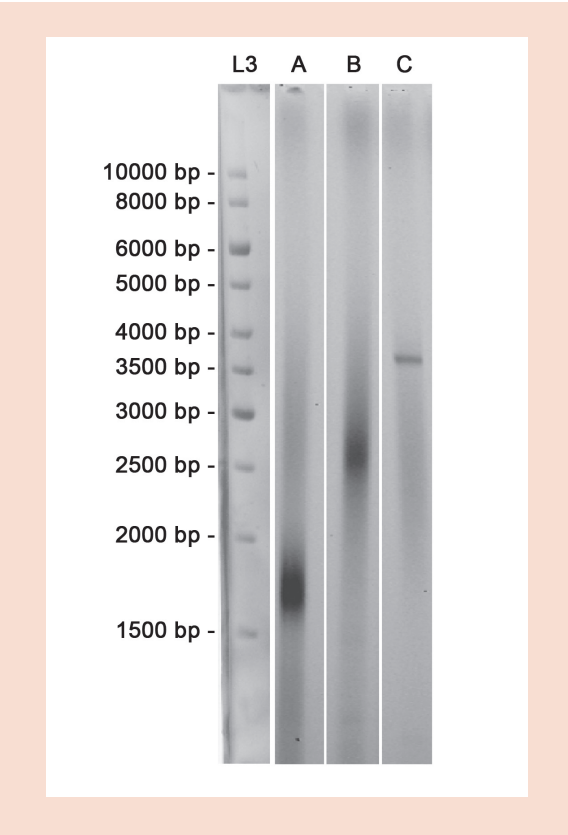

Figure 2. CTG repeat length determination using IRD-labeled leading primer and alkaline gel electrophoresis. PCR with DMC/DMBR primers on 2 ng of DNA extracted from human cerebellum samples with **(A)** 500 CTG repeats (1645 bp expected) or **(B)** 900 CTG (2845 bp expected), and **(C)** mice tail with 1200 CTG (3745 bp expected). L3: Thermo Scientific GeneRuler 1 kb DNA Ladder (ThermoFisher Scientific). The ladder lane was revealed with ethidium bromide while the rest of the gel with the PCR products was revealed with Odyssey® CLx Infrared Imaging.

patient carrying 150 CTG repeats. Figure 1A shows clearly the CTG repeat somatic mosaicism in the different dilutions as observed in classical small-pool PCR [16]. FSP-PCR was repeated with blood DNA from three different DM1 patients carrying between 150 and 200 CTG repeats. PCR amplification using 0.5 µl of the 300 pg. μl^{-1} dilution shows again clearly the CTG repeat mosaicisms resulting from somatic instability (Figure 1B and Supplementary Figure S1). As expected, no somatic mosaicism was detected in non-DM1 individuals (Figure 1C and Supplementary Figure S1). We can note different repeat sizes for normal alleles between individuals, reflecting *DMPK* normal allele polymorphisms [21]. In order to verify the efficiency of FSP-PCR, we compared the CTG repeat expansion mosaicism using FSP-PCR and classical SP-PCR (Figure 1D) [18]. The magnitude of the CTG repeat instability was comparable using both methods showing normal sensitivity with FSP-PCR. We also used various DNA samples carrying different CTG repeat expansions including DNA extracted from DM1 cerebellum samples (with 500 and 900 CTG repeats) and tail DNA from the DMSXL mouse model carrying 1200 CTG repeats. Using 2 ng of DNA, we were able to detect up to 1200 CTG repeats (Figure 2). The results obtained demonstrate that the protocol is useful for detecting a wide range of CTG repeat lengths both in human (blood and tissues) and in mice. Since no optimization was necessary in the original PCR program used with classical unlabeled primers, we assume that this protocol can be easily adapted to any usual PCR protocol [17–20].

Recently, it has been reported that ethidium bromide modifies the mobility of CAG•CTG alternative DNA structures generated by PCR during electrophoresis [22]. Using a denaturing gel and fluorescent primers, this protocol overcomes the possible migration bias resulting from secondary DNA structures formed during PCR. Moreover, we used here IRD700 labeling, as it is empirically known to provide sharper detection than the IRD800 labeling [23]. To our knowledge, there are no IRD-labeled ladders with bands higher than 1500 bp. This problem can be easily overcome by using a usual standard and performing a TBE 1X/ethidium bromide bath after analysis with the Odyssey CLx Infrared Imaging System: fluorescent and UV pictures can be overlaid in computational post-treatment (Figure 2). Another solution can be to create your own labeled ladder as already described elsewhere [24].

In conclusion, the FSP-PCR protocol using fluorescent PCR, an alkaline gel electrophoresis, and a near Infra-red detector system is a highly sensitive, easy, reliable, and faster method for assessing microsatellite instability.

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Author contributions

E.D. designed and performed the experiments and prepared the manuscript. G.G. supervised the study and corrected the manuscript.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/btn-2018-0035

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