New Cleavase® Fragment Length Polymorphism Method Improves the Mutation Detection Assay

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ABSTRACT

Cleavase® Fragment Length Polymorphism (CFLP) analysis is a convenient, accurate and highly sensitive method for the detection and localization of nucleic acid mutations. The assay is well suited for high-throughput screening and can be used to detect mutations in known and unknown nucleic acid samples. A recent improvement in the CFLP assay termed "temperature ramping" or "ramping" is reported here. This procedural improvement eliminates the need for time and temperature optimizations before the actual sample analysis. In this study, we compare the CFLP ramping procedure to the conventional CFLP optimization procedure and demonstrate equal, and in some cases improved, detection of point mutations. With ramping, CFLP reactions are identical for all DNA fragments analyzed, which allows for increased sample throughput, decreased assay time and lower overall cost.

INTRODUCTION

The emerging fields of molecular genetics and pharmacogenomics have increased the demand for technologies capable of high-throughput screening of genetic material for single nucleotide polymorphisms (SNPs). In response to this demand, researchers in academia and the biotechnology industry have developed numerous methods for screening, detecting and identifying single and multiple nucleotide changes in DNA. These methods include single strand conformational polymorphism (SSCP) analysis (10), denaturing gradient gel electrophoresis (DGGE) (25), enzyme mismatch detection (EMD) (31), PCR-restriction fragment length polymorphism (PCR-RFLP) analysis (18), dideoxy fingerprinting

(ddF) (14), base excision sequence scanning (BESS) (9) and DNA sequencing (23).

Third Wave Technologies introduced Cleavase® Fragment Length Polymorphism (CFLP) analysis in 1996 (4,15) as a screening method for the detection of known and unknown mutations in DNA. Each of the previously mentioned screening methods has limitations and varying degrees of sensitivity and specificity for the analysis of mutations (3,28). Of the past in-house studies of CFLP analysis that incorporated at least 70 DNA fragments from various human, bacterial and viral sources, CFLP has been shown to be 100% specific and 96.5%–98% sensitive (3,4,15,17). In other studies, comparable sensitivity rates have been reported for CFLP analysis (1,5,19,21,22,24,26,27,29). In a few studies, low sensitivity rates of 60% and 50% were reported (12,16). CFLP has been successful in detecting numerous mutations in a variety of genes such as: human p53, *CYP21*, *CFTR*, *Col4a5*, *Pkd1*, *FGFR3*, *Brcal*, *MYH7*, *GDNF* and *KVLQT1* (1,5,15,19,22,24,29); bacterial *rpoB*, *KatG* and *mt-rRNr2* (4,15,26); and viral HCV (2,17,27).

Previously, CFLP analysis required the optimization of assay time and temperature for each type of DNA fragment before analysis could begin. This optimization step has been an obstacle in the routine use of the assay. Here, we present a new procedure for performing CFLP analysis, which eliminates the cumbersome optimization step. This new incubation protocol, termed ramping, enables all DNA fragments to be analyzed by the CFLP method using identical reaction conditions. Ramping has not only simplified CFLP analysis by improving execution of the assay, but has in some cases increased its mutation detection ability.

MATERIALS AND METHODS

PCR (Substrate) DNA Fragments

The 701 bp fragment from the UL97 region of human cytomegalovirus was amplified using 2 ng of genomic DNA. The forward and reverse primers were 5' tetrachlorofluorescein (TET)-GGCTGCTGTCTTGCTGCACAA-3' and 5' (TET) GCGAAAGGCGCGTACGCGAC-3', respectively. Each amplification reaction contained 3.5 U of Expand™ High Fidelity *Taq* polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA), 400 nM each primer and 200 µM each dNTP in 100 µL of 1× PCR Buffer [2 mM Tris-HCL (pH 7.5), 10 mM KCl, 100 µM dithiothreitol (DTT), 10 µM EDTA, 0.05% Tween® 20, 0.05% Nonidet™ P-40 (Shell International Petroleum, London, England, UK)] with 1.5 mM MgCl₂, 1 M Betaine and 5% dimethyl sulfoxide (DMSO). After initial denaturation at 94°C for 5 min, the PCRs were amplified for 36 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by a final extension at 72°C for 5 min.

The 479 bp fragment from the *S. aureus* GRLA gene was amplified from approximately 100 ng of genomic DNA. The forward and reverse primers were 5'-Biotin-GATGAGGAG-GAAATCTAGTGAGTG-3' and 5'-CATTGGTTCGAGTGT-CGTATCAT-3', respectively. Each amplification reaction contained 3.5 U of *Taq* DNA polymerase (Sigma, St. Louis, MO, USA), 200 nM each primer and 200 µM each dNTP, 2.3 mM MgCl₂, 9.5% glycerol in 1× reaction buffer (Sigma) in a final volume of 100 µL. After initial denaturation at 94°C for 3 min, the PCRs were amplified for 30 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. PCR products were generated for the 516 bp tyrosinase (human) fragment, 534 bp ITS1 and ITS2 (*Pneumocystis carinii*) fragment and the 193 bp *rpoB* (*Mycobacterium tuberculosis*) fragment as previously reported (2,11,30), except the 5' ends of the primers were labeled with either biotin or TET. Following amplification, the reactions were treated with exonuclease I (Epicentre Technologies, Madison, WI, USA) at 1 U/µL of PCR volume for 30 min at 37°C. The nuclease was deactivated by purification with a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). All DNAs were dissolved in 10 mM Tris-HCL (pH 8.0), 0.1 mM EDTA or sterile distilled water.

CFLP Analysis

Each CFLP reaction contained 100–500 fmoles of substrate DNA in 13 µL of DNA dilution buffer [5 mM MOPS (pH 7.5)]. The reaction was heated to 95°C for 15 s and cooled to either 35°C for ramping or to a predetermined temperature for conventional CFLP digestion. The reaction samples were then mixed with 7 µL of an enzyme solution containing 25 U (1 µL) of Cleavase I enzyme (Third Wave Technologies, Madison, WI, USA), 2 µL of 10× CFLP buffer [100 mM MOPS (pH 7.5), 0.5% Tween 20, 0.5% Nonidet P-40], 2 µL of 2 mM MnCl₂ and 2 µL of DNA dilution buffer. Ramping reactions were incubated at 35°C for 15 s after the enzyme addition, and then the temperature was increased to 85°C at a rate of 0.1°C per s. Non-ramped CFLP reactions were allowed to incubate at the optimal temperature for the time determined in a previous optimization experiment. The reactions were terminated by the addition of 16 µL of stop solution [95% formamide, 10 mM EDTA (pH 8.0), 0.05% xylene cyanol (XC)

and 0.05% bromophenol blue (BPB)] for non-fluorescence detection. For fluorescence imager detection, 0.05% crystal violet was used in place of the XC and BPB.

Gel Electrophoresis and Fluorescence Detection

For analysis using an FMBIO®-100 Fluorescent Method Bio-Image Analyzer (Hitachi Software Engineering, San Bruno, CA, USA), aliquots of 7 µL of CFLP reaction, denatured for 2 min at 85°C, were loaded onto a 20 cm × 20 cm × 0.5 mm 10% denaturing gel [acrylamide:bis (19:1); 7 M urea], with 0.5× TBE buffer [44.5 mM Tris, 44.5 mM borate, 1 mM EDTA (pH 8.0)]. Molecular weight markers of Phi-X174-Hinf 1 (Promega, Madison, WI, USA) were 5' end-labeled with Tamra using a FluoroReporter® Bopdipy® TMR-C5 Oligonucleotide Labeling Kit (Molecular Probes, Eugene, OR, USA). Gel cassettes were transferred to the FMBIO-100 instrument and scanned with a 585 nm filter.

Figure 1. Schematic of the CFLP assay. Wild-type and mutant DNA molecules are heat denatured and cooled to a predetermined temperature. As the DNA strands cool, sequence-dependent secondary structures or “hairpins” unique to each DNA molecule form. The mutation causes a different set of structures to form locally near the sequence change. Cleavase I cuts at the 5' side of the junction of the single- and double-stranded regions of each DNA strand. Enzymatic digestion is performed under conditions of partial cleavage. When a 5' end label is used, a set of labeled fragments is generated that extend out from the labeled end to each structure formed. This results in a nested set of fragments, similar to dideoxynucleotide sequencing. The fragments are separated by rapid electrophoresis on a denaturing acrylamide gel and visualized by means of the labeled end. Mutational changes are visualized as the appearance or disappearance of bands, band mobility shifts or changes in band signal intensity.

Gel Electrophoresis and Chemiluminescence Detection

For chemiluminescence analysis, aliquots of 7 μL of CFLP reaction, denatured for 2 min at 85°C, were loaded onto a 20 cm $\times$ 20 cm $\times$ 0.5 mm 10% denaturing gel [acrylamide:bis (19:1); 7 M urea] with 0.5 $\times$ TBE buffer [44.5 mM Tris, 44.5 mM borate, 1 mM EDTA (pH 8.0)]. Biotinylated molecular weight markers were purchased from Research Genetics (Huntsville, AL, USA).

After electrophoresis, the biotin-labeled CFLP fragments were visualized by chemiluminescence and colorimetric platforms as previously described (11).

RESULTS AND DISCUSSION

Principle of the CFLP Assay

CFLP analysis relies on the formation of individual secondary structures that result when DNA is allowed to cool following a brief heat denaturation (20) (Figure 1). These structures serve as substrates for the structure-specific Cleavase I enzyme, which can be used to produce a set of cleavage products from any given DNA fragment (4). Because formation of these secondary structures is remarkably dependent on nucleotide sequence, cleavage results in the generation of a unique collection of fragments for each sequence analyzed. The similarities and differences between

Figure 2. A conventional CFLP optimization. A matrix of times and temperatures was carried out on 50 fmoles of a 479 bp fragment of the human GRLA gene (7) labeled at the 5' end of the sense strand with biotin. Detection was carried out with the colorimetric substrates NBT and BCIP. The optimal reaction time of 45°C and 7 min can be seen to give the best CFLP pattern and band intensities.

DNA sequences are represented in the CFLP patterns generated. Common sequences result in the generation of similar bands. Sequence differences are evident in the CFLP patterns as one or more of the following: (i) loss or gain of one or more bands, (ii) shifts in the position of bands and (iii) a change in the relative intensities of bands. The structural fingerprints that result from CFLP analysis provide a ready means of identifying the extent to which sequences are related to each other. Figure 1 shows a schematic representation of the CFLP reaction and its underlying principles.

Conventional CFLP Optimization

Conventionally, to perform CFLP analysis on a particular set of DNA samples, an optimization experiment was required to determine the optimal time and temperature conditions for the reaction. Briefly, matrices of time (e.g., 1, 3 and 5 min) and temperature (e.g., 40, 45 and 50°C) were exam-

ined to reveal conditions yielding an optimal CFLP pattern with even distribution of long and short cleavage fragments. A typical CFLP optimization result with a matrix of four temperatures and three time intervals is shown in Figure 2. For this particular DNA fragment being cleaved, the optimal reaction conditions of 45°C for 7 min were chosen since, under these conditions, the CFLP pattern consists of the best representation of the possible fragments. Once the reaction conditions are established, CFLP analysis can be performed on any DNA samples generated using the same PCR primers.

Unfortunately, the optimized conditions determined for a particular DNA fragment can only be used for that type of fragment. DNA of any other origin requires a unique optimization. Since genes generally contain multiple exons and introns, several fragments may be needed for full analysis. Each individual fragment examined could have a unique optima for time and temperature. This new ramping procedure avoids the need for performing the matrix of optimization reactions.

Figure 3. Comparison of the ramping method to the conventional method. Detection for both panels was by chemiluminescence followed by film development. (A) A 479 bp fragment of the GRLA gene labeled on the sense strand with biotin. The ramping method contained 75 fmole per CFLP reaction while the conventional CFLP method contained 50 fmole. Both sets include the same three mutants; Mt1:E84K (GAA:AAA), Mt2:S80Y (TCC:TTC), Mt3:S80Y(TCC:TAC) and one wild-type (7). While the pattern change is very weak between mutant (Mt1) and the wild-type (Wt) sample in the conventional CFLP set (indicated by the asterisk), an intensity pattern change can be seen in the ramping set (indicated by brackets). (B) The method comparison using the kit control DNA: a 516 bp fragment of the human Tyrosinase gene labeled at the 5' end of the antisense strand with biotin. The ramping method contained 400 fmole per CFLP reaction, while the conventional CFLP method contained 200 fmole. Both procedures give very similar patterns, and identify the R422Q (CGG:CAG) mutant (8) (indicated by brackets) by intensity changes in the bands.

Temperature Ramping—An Alternative to Optimization

The ramping method has greatly simplified execution of the CFLP assay. All CFLP kit components and volumes remain unchanged, and it is simply the procedural steps of the assay that have been modified. The conventional optimization experiment is completely eliminated, allowing the user to proceed immediately to CFLP analysis. The procedure involves ramping the reaction temperature from 35°C to 85°C at a rate of approximately 0.1°C per second. The ramping method results in equivalent or improved CFLP banding patterns. Even though the ramping reaction components remain as recommended in the current CFLP analysis protocol, the amount of DNA required in each reaction has been increased 1.5- to two-fold. This is because the ramping method exposes the DNA to the Cleavase I enzyme for 8 or more minutes, whereas in the conventional CFLP reaction, the typical reaction

time is usually between 2 and 5 minutes. The additional DNA minimizes the risk of overdigestion in the ramping reactions.

Figure 3 is an example of two independent fragments that were analyzed using both conventional CFLP and the ramping CFLP procedures. The first example (Figure 3A) is a chemiluminescence film of a 479 bp fragment of the human *GRLA* gene (7) labeled at the 5' end of the sense strand with biotin. Conventional optimization results revealed optimal reaction conditions of 7 min at 45°C (Figure 2). Even though both ramping and conventional CFLP methods display similar CFLP patterns, there are some obvious differences between the two. Surprisingly, the ramping method provides a clear distinction between mutant 1 and the wild-type sample, while the conventional CFLP procedure shows a much more subtle difference between the two. Figure 3B is a chemiluminescence film of a 516 bp fragment of the human Tyrosinase gene (8) labeled at the 5' end of the antisense strand with biotin and

Figure 4. Ramping results generated from four independent DNA targets. CFLP ramping was performed simultaneously on four distinct sets of samples. Even though each DNA set had a different reaction condition, determined from a conventional CFLP optimization experiment, all demonstrated equivalent or better CFLP patterns than seen with the conventional method (data not shown). Set (A) represents four clinical *M. tuberculosis* samples; one wild-type and three rpoB mutants; Mt1: S531L (TCG:TTG), Mt2: H526R (CAC:CGC), Mt3: H526D (CAC:GAC) (26). Set (B) represents two human Tyrosinase gene samples; a wild-type and a mutant: R422Q (CGG:CAG) (8). Set (C) represents the ITS1 and ITS2 region of four distinct *P. carinii* clinical samples with multiple nucleotide differences; Sample 1: Ad, Sample 2: Bg, Sample 3: Ei, and Sample 4: Fg (13). Set (D) represents two sets of CMV strains: the two different parental strains WT1 and WT2, and the two respective ganciclovir resistant mutants; Mt1: M460S (ATG:GTG), Mt2: A594V (GCG:GTG) (6). Concentrations of samples DNA ranged from 200–400 fmoles per CFLP reaction.

determined to have optimal reaction conditions of 2 min at 50°C (data not shown). Again, there are obvious differences between the CFLP patterns generated by each method. The ramping method in Figure 3B reveals a richer and more even distribution of band intensities. Additional amplicons derived from genes such as *rpoB* of *M. tuberculosis*, UL97 of CMV and human Tyrosinase, *GryA*, *GrlA* and *APC*, have been tested comparing these methods (data not shown). In all cases, the CFLP patterns derived from ramping were comparable to or better than the patterns derived using optimized conditions. Ramping has eliminated the need for optimization, and CFLP can be performed using universal conditions no matter what the size or sequence of any given PCR product.

Ramping Universalizes the CFLP Assay

Ramping has universalized the CFLP assay: one reaction condition serves for any PCR product of any size, labeled with any moiety, for any detection platform. Investigators desiring to carry out CFLP on one or several genes, with numerous exons of varying sizes, can now perform CFLP on all these DNAs using identical conditions. Figure 4 is an example of four unrelated DNA fragments cleaved simultaneously and detected by fluorescence scanning on an FMBIO fluorescent method bio-image analyzer. The four DNAs are from bacterial [*Mycobacterium tuberculosis*: *rpoB* gene, 193 bp (26)], human [Tyrosinase gene: Exon 2-4, 516 bp (8)], fun-

gal/protozoan [*Pneumocystis carinii*: ITS1 & ITS2 gene, 534 bp (13)] and viral [Cytomegalovirus: UL97 gene, 701 bp (6)] origin. The four samples were placed together in one thermal cycler, cleaved with the same enzyme master mixture and then temperature ramped at 0.1°C per s from 35°C to 85°C.

CONCLUSION

The demand for cost-effective and sensitive methods for the discovery of nucleic acid polymorphisms has increased dramatically in the fields of molecular genetics and pharmacogenomics. Third Wave Technologies is aware of this need and has modified the CFLP analysis procedure to accommodate the execution of high-throughput screening of known and unknown mutations by generating a universal procedure and eliminating the need for fragment specific optimization. Temperature ramping has eliminated the need for conventional CFLP optimization and allows the use of universal reaction conditions on any PCR product, of any size, with the most common detection platforms. This study demonstrates ramping on five unique PCR products and used three detection platforms: transfer to nylon membrane with both colorimetric detection (Figure 2) and chemiluminescence detection (Figure 3) and fluorescence gel scanning (Figure 4). Ramping simplifies CFLP analysis while retaining, and in some cases enhancing, the versatility and sensitivity it has always had.

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