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Toward a Fluorescent SSCP Technique that Detects all Mutations: F-DOVAM-S

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Abstract

Although DOVAM-S (detection of virtually all mutations SSCP) in effect detects all mutations and is less costly than direct sequencing, the technique currently requires the use of radioactivity. F-DOVAM-S (fluorescent DOVAM-S) was developed to replace the isotopic label with fluorescence and to increase throughput via dye color multiplexing. As proof-of-principal, two multi-temperature slab gel electrophoresis conditions were evaluated through the blinded analysis of mutations in the FIX genes of 88 Hemophilia B (HB) patients and 7 wild type controls. Using only 2 conditions, it was determined that F-DOVAM-S had a detection sensitivity of 97%. It is anticipated that when three to four optimized conditions are employed, F-DOVAM-S will detect all mutations. Three patient samples were multiplexed per well using three different fluorescent dyes (6FAM, VIC and NED), demonstrating that it is possible to analyze up to 44 kb of diploid, color-coded amplification product per gel lane. This value corresponds to a throughput of about 4 Mb of DNA analyzed per 96-well gel, which is about triple that of conventional radiolabeled DOVAM-S. Throughput is further enhanced by the rapidity at which the fluorescent signal can be captured and the resultant multi-color chromatograms analyzed. Given these data, F-DOVAM-S has the potential to be a particularly powerful technology for clinical diagnosis since it allows the mutation analysis of multiple patients to be performed within 24 hours.

Keywords

fluorescence; SSCP; mutation detection; β -lactamase; multiplexing; F-DOVAM-S; clinical diagnosis

Introduction

Since its inception in 1989, single strand conformation polymorphism (SSCP)¹ analysis remains a popular method of detecting unknown mutations[1-11]. Underlying SSCP analysis is the principle that, when separated by electrophoresis under non-denaturing conditions, a single-strand DNA molecule will adopt one or more secondary/*tertiary* conformations that will

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¹*Abbreviations used:* SSCP, single strand conformation polymorphism; DOVAM-S, detection of virtually all mutations single strand conformation polymorphism; F-DOVAM-S, fluorescent detection of virtually all mutations single strand conformation polymorphism; TBE, Tris-Borate-ethylenediaminetetraacetic acid; TRI/TRI, tricine-ethanolamine; CCD, charge-coupled device; FIX, factor nine; HB, hemophilia B.

cause it to migrate through the gel matrix in a unique manner[12;13]. Thus, a sequence that varies by as little as a single base can be identified on a gel or chromatogram as a band or peak that exhibits altered mobility and/or shape[13]. Typically, a single SSCP assay condition will not detect all mutations in a large sample set that contains a variety of different point mutations. Temperature, pH, salt concentration, glycerol concentration, and DNA fragment length are all variables that can influence the sensitivity of the technique[2;14;15]. Our laboratory has taken two approaches to increase sensitivity. The first has been to couple SSCP with another method such as bidirectional dideoxyfingerprinting or restriction endonuclease digestion so that multiple mutation-containing fragments will be generated[16;17]. The second approach has been to develop a standard set of sensitive and complementary electrophoretic SSCP conditions that together enable the detection of virtually all mutations. We have termed this technique DOVAM-S (detection of virtually all mutations SSCP)[18].

DOVAM-S is routinely performed in our laboratory in a high-throughput format: up to 22 radioisotopically labeled DNA segments are multiplexed, denatured and electrophoretically separated in a single lane of a non-denaturing gel. In this manner, about 500 kb of diploid DNA can be scanned per set of 5 gels, where each gel represents a different generic electrophoresis condition. The technique has been demonstrated to detect all mutations and when it is used in conjunction with Sanger sequencing, it reduces cost three-fold[19].

The application of fluorescent labeling to DOVAM-S eliminates the drawbacks associated with radioactivity, enables multiplexing by dye color, and permits immediate data acquisition since the fluorescent signal is captured by a CCD camera. Using ABI software, the peak patterns associated with the resultant fluorescent chromatograms are comparatively easier to read than the band patterns on radioactive gels. Furthermore, because color-multiplexing makes it possible to include an internal standard in every lane, bands that exhibit only a slightly altered mobility can be more readily identified in a fluorescent format than through conventional DOVAM-S.

For the development of multiplex fluorescent DOVAM-S, our strategy has been to enhance throughput and fidelity by coding individual patient samples in unique colors. Using the fluorescent dyes 6FAM, VIC and NED, three chromatograms can be generated in each gel lane. As well, it has been our goal to reduce the number of generic electrophoresis conditions necessary to detect all mutations. In developing the method, we added a number of features to simplify and enhance the F-DOVAM-S technology. They include: 1) a modified ABI 377 genetic analyzer that can operate at sub-ambient temperatures, 2) a fluorescently labeled non-interacting forward and reverse primer/tail pair consisting of sequence derived from the *E. coli* β -lactamase gene, and 3) two generic conditions that are each electrophoresed at three different temperatures. Herein, we demonstrate in the blinded analysis of 88 hemophilia B patient samples and 7 wild type controls that F-DOVAM-S, when run under 2 different generic conditions, detects mutations in the human FIX gene with a sensitivity of 97%. We also demonstrate that three patient samples labeled with distinct fluorescent dyes can be analyzed in a single gel lane, tripling the throughput of radiolabeled DOVAM-S. These results, along with the enhanced features of F-DOVAM-S, reveal the clinical potential of SSCP for low-cost, rapid mutation identification from the viewpoint of individual patient analysis.

Materials and methods

Patient samples and Genomic Regions Scanned

Genomic DNA from 88 hemophilia B patients and 7 wild type individuals was collected and divided into 3 sets, designated A, B and C. The experimenter had no knowledge of the samples, other than a coded identifier. Specimens belonging to each set were labeled in one of three colors: blue, green or yellow. For every specimen, the complete coding region and flanking

intronic sequence of the human FIX gene was amplified and scanned. The sizes of the ten PCR products, designated as segments A to H3, as well as the corresponding genomic regions that were scanned, are illustrated in Table S1.

Primers and Polymerase chain reaction

Five-prime dye-labeled β -lactamase tail-specific primers were purchased from Applied Biosystems (Foster City, CA, USA). The fluorescent dyes utilized were 6FAMTM (blue), VIC[®] (green) and NEDTM (yellow). Gene specific primers containing the β -lactamase tail were purchased from Integrated DNA Technologies (Coralville, IA, USA). A comprehensive list of the primers is given in Table S1. PCR reactions were performed with four primers multiplexed in 50 μ L volumes. Each reaction consisted of the following components: 1 $\times$ GeneAmp PCR Buffer II (10 mM Tris-HCl pH 8.3, 50 mM KCl), 2 mM MgCl₂, 2 units of AmpliTaq Gold, (Applied Biosystems, Foster City, CA, USA), 150 ng of genomic DNA template, 200 μ M dNTP (Roche Diagnostics Corp., Indianapolis, IN, USA), 70 nM sense and antisense tail-labeled gene-specific primer, and 0.2 μ M sense and antisense dye-labeled tail-specific primer. Thermocycling was performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) according to the following three stage program: hold at 95°C for 5 min./stage 1 (95°C for 30 sec., 40°C for 45 sec., 72°C for 1 min.) for 5 cycles/stage 2 (95°C for 30 sec., 60°C for 45 sec., 72°C for 1 min.) for 15 cycles/stage 3 (95°C for 30 sec., 40°C for 1 min., 72°C for 1 min.) for 30 cycles/hold at 72°C for 1 min./hold at 4°C indefinitely.

Sample Multiplexing

Multiplexed samples were created by combining appropriate amounts of dye-labeled segments A to H3 from one specimen in *Set A*, one in *Set B*, and one in *Set C*. Specifically, segments labeled in 6FAM, VIC, and NED were combined in a ratio of (4:15:25) μ L, so that 30 segments were multiplexed for every sample. In this manner, a total of 33 multiplexed, color-coded samples were generated. Multiplexed samples were subsequently purified on Centricon YM100 columns (Millipore Corp., Bedford, MA, USA), dried, and resuspended in equal volumes of 10 mM Tris, pH 8.0.

Electrophoresis and F-DOVAM-S conditions

Samples were prepared for F-DOVAM-S analysis by combining 0.25 μ L of multiplexed, purified product with 0.75 μ L of ROX400HD size standard (Applied Biosystems, Foster City, CA), 0.5 μ L of H₂O, and 2.5 μ L of loading buffer (50% formamide/7 M urea, 2 mM EDTA/0.01% bromophenol blue/0.01% xylene cyanol FF). Prior to electrophoresis, samples in loading buffer were denatured for 5 min. at 95°C and snap cooled. Appropriate volumes of denatured product were loaded onto a 0.2 mm thick, 36 cm long acrylamide slab gel and electrophoresed under 1) the F-DOVAM-S TBE condition, 2) the F-DOVAM-S TRI/TRI condition and 3) the denaturing condition. The TBE condition consisted of a gel made of 10% HR1000 (Genomys, Foster City, CA, USA)/50 mM TBEⁱ/2.5% Glycerol, and running buffer composed of 50 mM TBE. The TRI/TRI condition consisted of a gel made of 10% PAGE^{plus} (AMRESCO, Solon, OH, USA)/30 mM TRI/TRIⁱⁱ/1% Glycerol, and running buffer composed of 30 mM TRI/TRI. The denaturing condition consisted of a gel made of 5% Long Ranger (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA)/89 mM TBEⁱⁱⁱ/6M urea, and running buffer composed of 89 mM TBE. For the F-DOVAM-S TBE and TRI/TRI conditions, electrophoresis was initially performed at 20 Watts constant power at three sequential temperatures – 4°, 12° and 21°C – for two hours per temperature on a remote apparatus attached to a water bath, as illustrated in Fig. 1C. The gel was then transferred to a

ⁱ50 mM Tris/50 mM Boric Acid/1 mM EDTA, pH 8.3

ⁱⁱ30mM triethanolamine/30 mM tricine, pH 7.9

ⁱⁱⁱ89 mM Tris/89 mM Boric Acid/2 mM EDTA, pH 8.2

regular ABI 377 Genetic Analyzer containing fresh buffer and electrophoresed at 80 Watts constant power for 18 hours at 30°C. For the denaturing condition, electrophoresis was performed directly on an ABI 377 genetic analyzer at 3 kV for 4 hours at 51°C.

Data Analysis

Fluorescent chromatograms were visually analyzed for mutations using Applied Biosystems GeneScan® software, version 3.0. A segment was judged as having a mutation if it possessed at least one of the following criteria: altered peak shape, change in the number of peaks, or altered strand mobility. Upon completion of the study, the number of mutations correctly identified was scored by an independent examiner who had access to the coded patient information.

Results and discussion

Novel Features are incorporated into F-DOVAM-S

In addition to fluorescence, several other features were integrated into DOVAM-S in order to mitigate cost and enhance performance (Figure 1). In order to dramatically lower the cost of fluorescent labeling, a generic β -lactamase tail (Panel A) was introduced to reduce the number of tagged oligonucleotides that need to be synthesized, which in turn dramatically lowers the cost of fluorescent labeling. Using this method, only two fluorescent oligonucleotides are needed per color set, regardless of which gene is analyzed or how many segments it contains. In this study, six fluorescent primers were used to label a total of 30 FIX gene segments for every sample. It has been our experience that for multiplexed samples, this approach is preferable to in-house post-labeling because it results in fewer fluorescent artifacts on non-denaturing gels. In order to further save on time and reagent cost, the gene-specific and tail-specific amplifications were performed as a single PCR (Figure 1B).

Recently, it has been demonstrated for SSCP that altering the temperature over the course of electrophoresis increases the sensitivity of mutation detection[20]. To take advantage of this phenomenon so as to reduce the number of DOVAM-S conditions, a remote gel apparatus was created that allowed the temperature to be changed during electrophoresis (Figure 1C). This system also permitted electrophoresis to be performed at 4°C without the chance of condensate damaging the optics of an ABI 377 Genetic Analyzer. Together, these features constitute a cost-effective, high-throughput F-DOVAM-S platform that generates multicolor fluorescent gels (Figure 1D).

F-DOVAM-S is highly multiplexed

For each patient, the entire coding sequence of the human FIX gene was amplified as 10 segments ranging in size from 189 to 362 base pairs (Figure 2E). The longest exon, Exon H, was divided into 3 amplicons. All segments of an individual's FIX gene were labeled in one of three fluorescent dyes: 6FAM (blue), VIC (green) or NED (yellow). In this manner, amplification product from different specimens was color-coded. To make up a complete sample, color-coded segments from three patients were combined with a ROX400 standard and run in a single gel lane, for a total of 30 segments or 8.3 kb of haploid DNA sequence. Panels 2A to 2C depict the denaturing chromatograms for sample 20, which was composed of labeled segments amplified from patient specimens *A20*, *B20* and *C20*. Note that each chromatogram is characterized by a flat base line and the absence of spectral artifacts. Panel D shows the 3 chromatograms as they appear in a single lane. Under the denaturing condition DNA segments run primarily according to size; nevertheless, a small amount of higher order structure can be observed in the peak patterns, which is consistent with our previous observations. This approach of coding different patient specimens in specific colors has allowed us to actualize a F-DOVAM-S platform that is, to our knowledge, the most multiplexed

form of SSCP[21-23]. In fact, if 5 fluorescent dyes are employed, as is possible with formats like the Applied Biosystems DS-33 dye set, one can multiplex 4 patients per lane while reserving the fifth dye for an internal marker. In this manner, a throughput of 44 kb of diploid sequence per lane, or 4 Mb per 96-well gel, can be achieved if 22 gene segments are multiplexed for each patient. Such throughput makes F-DOVAM-S amenable to clinical diagnosis because multiple patients can have one or more genes analyzed within 24 hours.

Different buffer conditions yield distinct chromatograms

Figure 3 shows representative F-DOVAM-S electropherograms that were generated under the TBE condition (panel A) and under the TRI/TRI condition (panel B). Each pattern is unique in that its segments differ in peak shape, relative height, as well as migration time. For example, under the TBE condition, the two strands comprising segment H1 are broad and adjacent to one another. However, under the TRI/TRI condition, these strands adopt a much more narrow shape and overlap with the strands from segment H2. Although most segments resolve into 2 or more distinct peaks, some only resolve into one. Segment G, electrophoresed under the TBE condition (panel A), and segment H3, electrophoresed under the TRI/TRI condition (panel B), are examples of such amplicons. Differential strand mapping has revealed that these segments produce sense and antisense strands with the same mobility. Overall, DNA migrates faster when electrophoresed under the TRI/TRI condition compared to the TBE condition. Whereas the ten FIX segments migrate past the detector over a range of approx. 700 to 3600 units under the TRI/TRI condition, the same segments migrate over a range of approx. 3000 to 9000 units under the TBE condition.

Mutation detection and sensitivity

Fluorescence was incorporated into DOVAM-S for several reasons. One was to establish a method of generating color coded chromatograms that are comparatively easier to analyze than slab gel autoradiograms. As well, coding different specimens in unique dyes allows several patient samples to be run in a single lane. Figure 4 illustrates the three fluorescent chromatograms that were generated by running sample 20 on a non-denaturing acrylamide gel under the F-DOVAM-S TBE condition. Specifically, panels A to C show the 6FAM, NED and VIC chromatograms for patients A20, B20, and C20 respectively. It is clear that when these specimens are electrophoresed under the non-denaturing F-DOVAM-S TBE condition, DNA migrates as a complex function of both molecular structure and size. In contrast, when the same specimens are electrophoresed under the denaturing condition, DNA migrates primarily according to size (Figure 2, Panels A to D). Furthermore, as indicated by the arrows in figure 4, panels A to C, mutation containing segments that are electrophoresed under a non-denaturing condition clearly display at least one of the following characteristics: altered peak shape, change in the number of peaks, or altered strand mobility. In fact, the specimens coded A20, B20 and C20 actually correspond to hemophilia B cases HB79, HB521 and HB172. Importantly, in the blinded analysis, each patient was scored correctly as having a mutation in the indicated segment.

In addition to incorporating fluorescence into DOVAM-S, another goal of this project was to reduce the number of generic conditions that are needed to detect virtually all mutations. Conventional DOVAM-S requires a set of five electrophoresis conditions in which three different buffers — TBE, TRI/TRI, and CAPSO — are utilized[18]. In this blinded analysis, TBE and TRI/TRI were used as the buffers for two multi-temperature conditions. As illustrated in Table I, 97% of the mutations were detected using only these conditions. Importantly, most of the mutations were heterozygous (Tables S2-S4) and could be classified as micromutes^{iv}.

^{iv}A micromute is defined as a single base substitution; a deletion, insertion or microindel of 50 nucleotides or less, or a tandem, clustered or doublet mutation. Typically, micromutes are detected with high sensitivity through direct sequencing.

All wild type samples were correctly identified. Although a small number of mutations were not detected, it is anticipated that when the number of optimized conditions for either slab gel or capillary electrophoresis is increased to 3 or 4, F-DOVAM-S will detect virtually all mutations.

F-DOVAM-S can be used to characterize polymorphism

The human Factor IX gene contains a polymorphic region in Exon F marked by the substitution G>A which is located at nucleotide position 20422[24;25]. Panels A to C in Figure 5 illustrate that the hemi- and heterozygous patterns associated with this SNP could be clearly differentiated utilizing F-DOVAM-S. Importantly, F-DOVAM-S could also be used to distinguish the presence of other mutations in Exon F, regardless of which 20422 allelic pattern was present (Figure 5D).

Clinical Applications

Now that the human genome sequence is known, many new technologies have arisen to extract genetic information from individuals. For instance, hybridization micro-arrays capable of assessing copy number variation on a genome wide scale have recently been described.[26] In addition, there are a number of technologies under development for whole genome sequencing by companies such as Solexa, Applied Biosystems, 454 Life Sciences, Intelligent Biosystems, Helicos, and NABsys.[27] In all probability, one or more of these 'next-generation' sequencing platforms will supplant the majority of today's existing technology; nevertheless, it will be a number of years before such systems can be demonstrated to possess the reproducibility and accuracy necessary to replace capillary electrophoresis Sanger sequencing for clinical mutation detection.

For molecular epidemiology and clinical molecular diagnosis, it is particularly important to obtain the sequence of a patient's DNA with high accuracy and in a cost-effective, high-throughput manner so that mutations can be correctly identified in a timely fashion. Once a disease associated mutation is found in a family with one or more affected individuals, genetic counseling and treatment options can be provided. Because the genes associated with such diseases are often large and the mutations distributed over multiple exons, all coding segments and flanking regions must be sequenced. As well, to minimize the number of false negatives, the technique used to obtain the nucleotide sequence must have near perfect performance in terms of sensitivity and specificity. Currently, bi-directional Sanger sequencing is commonly used for diagnostic purposes. The drawback to this 'gold standard' of mutation detection is that the operational cost of capillary electrophoresis sequencing is still relatively high. However, the cost of clinical mutation identification can be reduced by at least three-fold by employing DOVAM-S, which has virtually perfect sensitivity, as a filter to exclude wild type amplicons from Sanger sequencing.[19] In our extensive experience, an average 90% of wild type sequence can be excluded from direct sequencing by DOVAM-S for autosomal disease, and up to 95% for X-linked disease. Importantly, the purpose of this study was to show, as proof-of-principal, that DOVAM-S could be adapted to fluorescence in order to further increase throughput, simplify data analysis, and to make the technique more user friendly.

The results of this study suggest that F-DOVAM-S has the potential to be a low cost, high-throughput tool for clinical molecular diagnosis. Because coding specific patients in different colors allows several specimens to be multiplexed in a single gel lane, a large number of individuals can have their entire gene analyzed within 24 hours. Furthermore, the cost of fluorescent labeling is kept low by the addition of a common β -lactamase tail to all amplified segments. In this study, F-DOVAM-S had a detection sensitivity of 97% using only 2 conditions. It is anticipated that the technique will detect virtually all mutations when 3 to 4 optimized conditions are employed for either slab gel or capillary electrophoresis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Evolving from DOVAM-S to F-DOVAM-S: selected features. A) β -lactamase tail All gene-specific primers include one of two 5' terminal sequences derived from the *E. Coli* β -lactamase gene. The two β -lactamase sequences shown in blue in Table S1 constitute a forward and reverse tail pair and give rise to a PCR product that consists of a central gene segment flanked by two labeled β -lactamase moieties. In this manner, sets of labeled amplicons can be generated using only two fluorescent primers. **B) Multiplex PCR.** Labeled segments are generated by performing the two PCR amplifications as one reaction. Gene specific primers containing the 5' β -lactamase sequence give rise to an unlabeled gene segment with flanking β -lactamase moieties (product 1), which in turn serves as the template for the two labeled β -lactamase tail specific primers that generate the final product (product 2). **C) Remote ABI 377.** A stripped down Applied Biosystems 377 Genetic Analyzer allows F-DOVAM-S gels to be run at subambient temperatures without the risk of condensate damaging the optical system. After DNA samples are separated on the remote apparatus, the gel is transferred to a regular ABI 377 and subjected to further electrophoresis during which time the fluorescent signal is captured. **D) Multi-colour fluorescent gels.** Once a spectral correction matrix has been applied, fluorescent gels can be resolved into their component dye colours and analyzed with GeneScan software.

Figure 2. Thirty FIX gene segments are multiplexed in a single lane

Shown are chromatograms generated from a single multiplexed F-DOVAM-S sample, sample 20, which was separated on an acrylamide gel under denaturing conditions. The sample consists of a ROX400 size standard and FIX amplification product from 3 patients. The amplification product for each specimen is labeled in a unique color and is composed of 10 gene segments; thus, a total of thirty FIX segments are analyzed in every lane. Note that under denaturing conditions the DNA segments migrate according to size; nevertheless some higher order structure is evident. **Panels A to C** depict the chromatograms for patients *A20*, *B20* and *C20* labeled in 6FAM, VIC and NED, respectively. **Panel D** shows the three chromatograms in an overlaid format. The identity of each segment (A to H3) is indicated at the bottom of the figure. **Panel E**, far right, depicts the actual appearance of a multiplexed FIX sample on a denaturing slab gel. The name and size of each segment in base pairs (bp) is indicated.

Figure 3. Different F-DOVAM-S conditions produce distinct chromatograms

Shown are representative non-denaturing chromatograms of a FIX patient sample labeled with the fluorescent dye VIC that was run under **A)** the TBE condition and **B)** the TRI/TRI condition. The identity of each strand (A to H3) was determined from a mapping gel. Specifically, the chromatograms differ with respect to peak shape and strand migration time.

Figure 4. Mutations are detected in three patients analyzed in a single gel lane

Illustrated are non-denaturing chromatograms for sample 20, which is the sample depicted in Figure 2. Electropherograms were generated by running the DNA on a non-denaturing acrylamide gel under the F-DOVAM-S TBE buffer condition. **Panels A to C** show the chromatograms for the specimens coded A20, B20 and C20 that were labeled with 6FAM, VIC and NED, respectively. **Panel D** shows the three chromatograms in an overlaid format. Specimens A20, B20 and C20, each scored correctly as possessing a mutation, correspond to patient samples HB79, HB521 and HB172. Visible in red is the ROX400 internal marker.

Figure 5. Mutations can be detected in a highly polymorphic segment

The FIX polymorphism 20422 G>A located in Exon F yields a reproducible pattern when analyzed under the F-DOVAM-S TBE condition. Results were verified by direct sequencing.

Panel A depicts the homozygous SNP pattern for patient HB625, **panel B** the homozygous pattern for a WT sample, and **panel C** the heterozygous pattern for patient HB592. **Panel D** illustrates the pattern associated with patient HB687 who, in addition to being heterozygous for the G>A polymorphism at base position 20422, also has an A inserted at position 20526.

Table 1

Sample Set	A	B	C
Number of Samples in set	32	31	32
Number of WT Samples	3	2	2
Dye Label	6FAM™ (Blue)	VIC® (Green)	NED™ (yellow)
Gel Conditions Used	TBE, TRI/TRI	TBE, TRI/TRI	TBE, TRI/TRI
WT samples correctly identified	YES	YES	YES
Number of Mutations Identified	30/31	30/31	30/31
Per Cent Sensitivity	97	97	97
Per Cent Sensitivity TBE	82		
Per Cent Sensitivity TRI/TRI	77		
Combined Per Cent Sensitivity	97		