

Sequential DEXAS: a method for obtaining DNA sequences from genomic DNA and blood in one reaction

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ABSTRACT

Sequential DEXAS (direct exponential amplification and sequencing), a one step amplification and sequencing procedure that allows accurate, inexpensive and rapid DNA sequence determination directly from genomic DNA, is described. This method relies on the simultaneous use of two DNA polymerases that differ both in their ability to incorporate dideoxynucleotides and in the time at which they are activated during the reaction. One enzyme, which incorporates deoxynucleotides and performs amplification of the target DNA sequence, is supplied in an active state whereas the other enzyme, which incorporates dideoxynucleotides and performs the sequencing reaction, is supplied in an inactive state but becomes activated by a temperature step during the thermocycling. Thus, in the initial stage of the reaction, target amplification occurs, while in the second stage the sequencing reaction takes place. We show that Sequential DEXAS yields high quality sequencing results directly from genomic DNA as well as directly from human blood without any prior isolation or purification of DNA.

INTRODUCTION

With the availability of complete genome sequences from many organisms, analysis of nucleotide variation in many individuals is becoming increasingly important. The most common form of genomic variation is single nucleotide polymorphisms (SNPs). A number of methods have been developed to study genomic variation in large numbers of individuals. They include, for example, mass spectrometry (1), mini sequencing (2), pyrosequencing (3), allele-specific PCR (4) and real time PCR (5). However, they all suffer from the disadvantage that they are designed to detect only SNPs that are previously known. In addition, most are as of yet not competitive in terms of cost because they require either expensive instrumentation and/or multiple steps of sample

processing. Here, we describe a new procedure that allows DNA sequences to be determined directly from genomic DNA as well as directly from whole blood. This method requires no special equipment and can easily be automated.

MATERIALS AND METHODS

Samples and primers

Unlabeled or fluorescently labeled oligonucleotides (Table 1) were purchased from Interactiva (Ulm, Germany). Human genomic DNA was purchased from Roche Diagnostics (Mannheim, Germany). Genomic DNA samples from tumor or normal tissues in the mitochondrial DNA (mtDNA) study were kindly provided by M. Fliss (Johns Hopkins University School for Medicine, Baltimore, MD). The genomic DNA samples used for the genotyping study of FXR were kindly provided by S. Grant (Lion Bioscience AG, Heidelberg, Germany).

Modification of AmpliTaq FS DNA polymerase

AmpliTaq FS DNA polymerase was purchased from Applied Biosystems (Foster City, CA) and was used in a Sequential DEXAS reaction after a chemical modification. The modification reaction was carried out according to Palacian *et al.* (6). Briefly, AmpliTaq FS DNA polymerase was incubated at a starting concentration of 1.3 mg/ml in a Tris buffer (50 mM Tris-HCl, 1 mM EDTA, 68 mM HCl, pH 7.5) overnight at 4°C with 4 µl of a 20 mg/ml solution of *cis*-aconitic acid anhydride (Sigma-Aldrich, Milwaukee, WI) in ethanol, resulting in solutions containing molar ratios of *cis*-aconitic acid anhydride to AmpliTaq FS DNA polymerase of 180:1. Control experiments showed that the modified enzyme lacked all activity; however, after heating at 95°C for 10 min almost all activity was regained. During a PCR, it regained ~50% of its activity after 15 cycles and 90% after 20 cycles (Fig. 2).

General Sequential DEXAS parameters

Human genomic DNA was directly amplified and sequenced in a 20 µl Sequential DEXAS reaction, comprising 125 mM Tris-HCl pH 9.3, 5 mM MgCl₂, 5% DMSO, 160 mM betaine, 1 U Tth pyrophosphatase (Roche Diagnostics, Mannheim, Germany), 2 U Platinum Taq DNA polymerase (Life

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Table 1.

Gene/Primer	5'-Position	Label	Sequence (5'→3')	Primer distance
mt-DNAfw-1	16000	IRD 700	GATTCTAATTTAAACTATTCTCTGTTCCTT	469 bp
mt-DNArev-1	16524	IRD 800	TTATGACCCTGAAGTAGGAACCAGATG	
mt-DNAfw-2	15960		AGAAAAAGTCTTTAACTCCACCATT	591 bp
mt-DNArev-2	16601		GAGTGGTTAATAGGGTGATAGACCT	
	Mitochondrial genome			
FXRfw-1	99305	IRD 700	AGGGTGATGAAGTTGTAGCC	376 bp
FXRrev-1	99720	IRD 800	TGGGAAATACAGCTGAGGA	
FXRfw-2	99183		AACATGGTTTCTGCCCTTCAGT	636 bp
FXRrev-2	99863		AGGGACTTGAGCAGCCATTAAC	
	Accession no. AC010200			

Technoloiges, Karlsruhe, Germany), 20 U chemically modified AmpliTaq FS DNA polymerase (Applied Biosystems, Foster City, CA) and primers (Table 1). Unlabeled primers were used at a concentration of 20 pmol and the labeled primers at a concentration of 2 pmol each. The mixtures containing all components except nucleotides were divided into four different tubes containing 2 μ l of the nucleotide mixture. After cycling, reactions (7 μ l) were stopped by the addition of 1 μ l of formamide and dried down to a volume of 1 μ l in a Savant Speed Vac (ThermoQuest, Egelsbach, Germany). An aliquot of 0.2 μ l of the concentrated solution was resolved by 4% PAGE on a Licor DNA-Sequencer Long READIR 4200 (Licor Biotechnology Division, Lincoln, NB) under standard electrophoresis conditions. The sequence data was obtained with the lane tracking software Lanetracker and the base calling software Geneskipper (both EMBL, Heidelberg, Germany).

Sequential DEXAS of mitochondrial DNA

Aliquots of 100 ng of total genomic DNA were used with a 1/200 nucleotide mixture, containing each dNTP at a concentration of 1 mM and one of the four ddNTP at a concentration of 5 μ M. All reactions were run in a GeneAmp PCR system 9700 (Perkin Elmer Cetus, CA). The following cycling parameters were used: 15 cycles of 30 s at 94°C (denaturing), 30 s at 62°C (annealing) and 60 s at 68°C (synthesis), followed by 7 min at 94°C (activation of the sequencing enzyme) and 30 cycles of 30 s at 94°C (denaturing), 30 s at 55°C (annealing) and 60 s at 68°C (synthesis).

Sequential DEXAS of nuclear DNA

For the FXR gene, 200 ng of human genomic DNA was used with a nucleotide solution of 1/400 containing each dNTP at a concentration of 1 mM and one of the four ddNTPs at a concentration of 2.5 μ M. Cycling parameters were identical to the mitochondrial protocol except that annealing during the first 15 cycles was at 61°C and the second cycling process was repeated 35 times.

Sequential DEXAS of human blood

Aliquots of 200 μ l of EDTA-treated blood samples were heated for 15 min at 95°C. After centrifugation at 13 000 r.p.m. for 20 min, an aliquot of 10 μ l was used in a protocol identical to the mitochondrial sequencing protocol from total genomic DNA.

RESULTS

Principle of Sequential DEXAS

In order to determine DNA sequences present in a haploid mammalian genome directly from genomic DNA, it is necessary to amplify the target sequence prior to determining its sequence. During Sequential DEXAS, amplification of the desired target sequence is achieved in the same tube as the sequencing reaction (Fig. 1). However, in contrast to previously developed methods that perform amplification and sequencing in a single tube, such as CAS (7) and DEXAS (8,9), Sequential DEXAS separates the two processes temporally such that the amplification is performed first and the sequencing later. This is achieved by combining two thermostable DNA polymerases in one tube. One enzyme, which discriminates against dideoxynucleotides, performs the amplification of the sequencing target (10) and is supplied as the antibody-modified variant of Taq DNA polymerase (11). The other enzyme, which incorporates dideoxynucleotides (12), performs the sequencing reaction and is supplied as the chemically modified variant of AmpliTaq FS DNA polymerase (6). Whereas the antibody dissociates from the Taq DNA polymerase shortly after the Sequential DEXAS reaction is started, the chemical modification of AmpliTaq FS DNA polymerase remains stable and hence inhibits the polymerization activity for several amplification cycles. However, by an additional heating step after 15 cycles the sequencing enzyme is activated. If one of the primers is fluorescently labeled and four separate reactions each containing one particular dideoxynucleotide are performed, a DNA sequence can be determined directly from genomic DNA as well as from whole blood using this procedure.

Inactivation of AmpliTaq FS DNA polymerase

The activity of unmodified AmpliTaq FS DNA polymerase and the chemically modified AmpliTaq FS DNA polymerase were compared in a PCR in which the amount of PCR product was taken as an indicator of the polymerization activity (Fig. 2). Figure 2 shows that the modified but not activated version of AmpliTaq FS DNA polymerase remains inactive during the first 12 cycles of the thermal cycling process and yielded ~50% of the amplification product after 15 cycles. Thus, the amplification and the sequencing activity provided by the two enzymes can be temporally separated by chemical inhibition of the DNA polymerase used for sequencing.

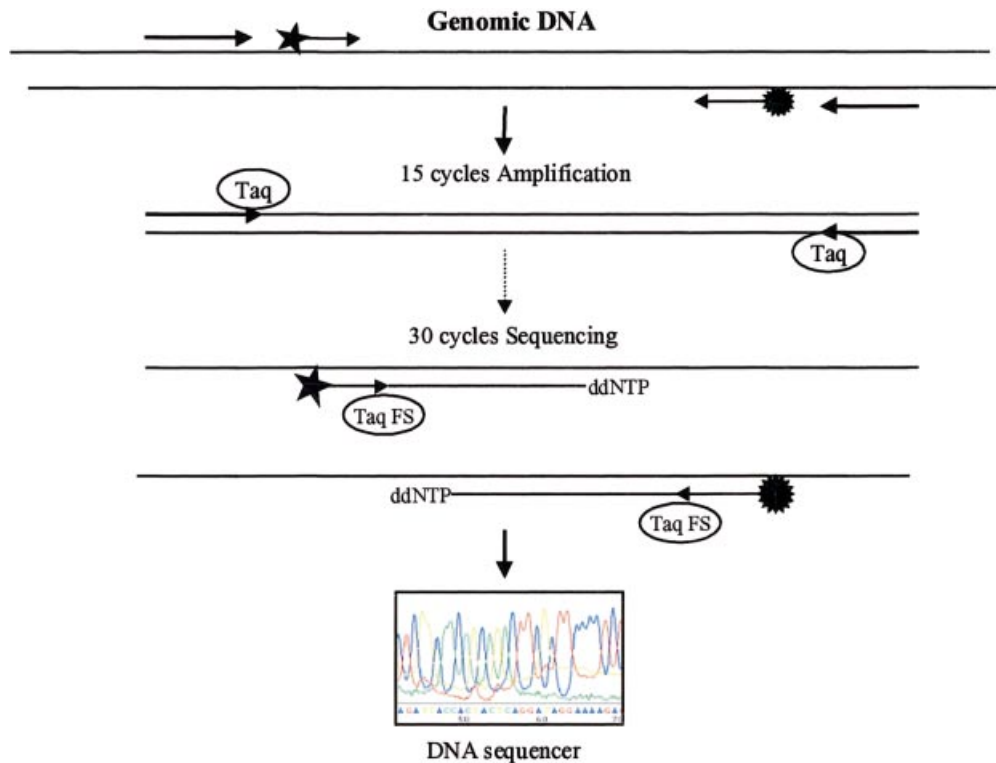


Figure 1. The concept of Sequential DEXAS. The outer pair is unlabeled and provided in a 10-fold higher concentration than the inner primers, which are each labeled with different fluorescent dyes. Two thermostable DNA polymerases are used. The Taq DNA polymerase (Taq) is active from the first cycle of the reaction, whereas the AmpliTaq FS DNA polymerase (Taq FS) is activated by a heating step after the first 15 cycles.

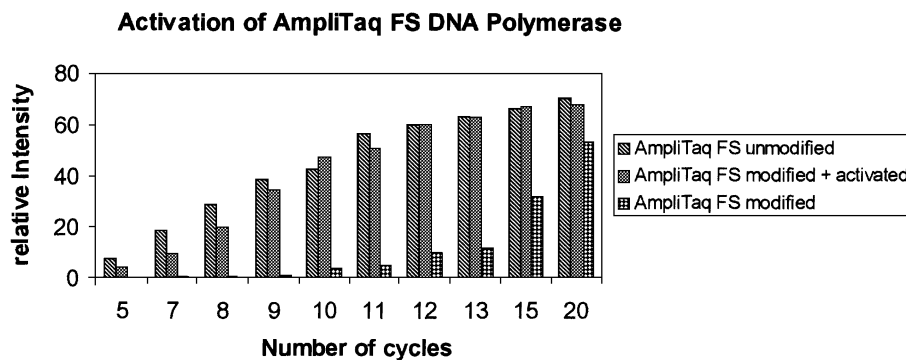


Figure 2. The kinetics of activation of modified AmpliTaq FS. The activity of the chemically modified and not activated AmpliTaq FS DNA polymerase is compared to unmodified AmpliTaq FS DNA polymerase and the modified and activated enzyme.

Nested primers

Figure 3A shows that a Sequential DEXAS reaction performed with one primer pair allows a 469 bp fragment of the mitochondrial genome to be determined from 100 ng of total human DNA. However, the first 30 bases are unreadable due to a large signal likely caused by primer artifacts. Furthermore, peak heights are not uniform, which makes base calling difficult. These problems are also typical of the previously described DEXAS method (8,9), which does not use DNA polymerases that are activated at different times. They can be overcome by using a second primer pair that

carries no label. These so called 'silent primers' are located 50–200 bp outside the region targeted for sequencing by the labeled primers. Figure 3B shows that the addition of external primers substantially improves the sequencing results in terms of the readability of the first 30 bases and the accuracy and total reading length of the sequenced region.

The most accurate results and greatest read lengths (see, for example, Fig. 4) are achieved when the nested unlabeled primers are designed such that they have an 8–10°C higher predicted annealing temperature than the labeled primers. One reason for this could be that the higher annealing temperature of the unlabeled primers causes them to be primarily

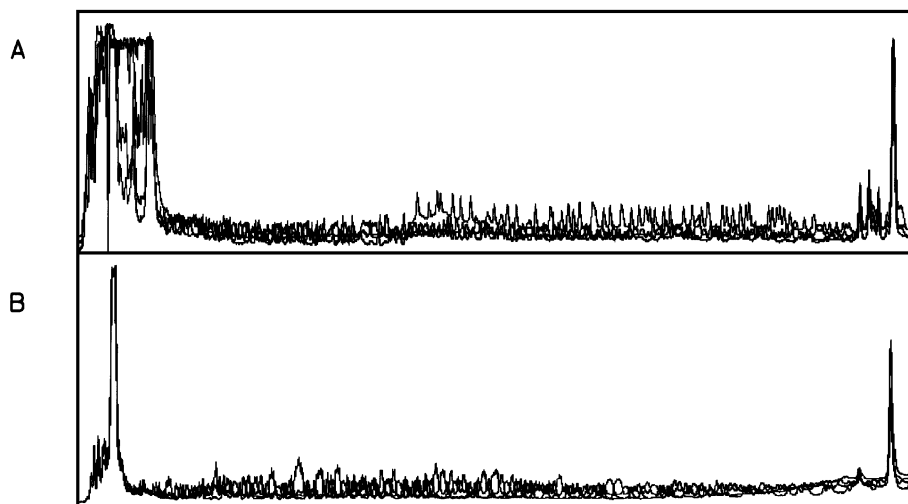


Figure 3. Sequential DEXAS without and with external primers. Sequencing of a 483 bp segment of the mitochondrial control region without external unlabeled primers (A) and with such primers (B).

responsible for the amplification during the first 15 cycles, where a higher annealing temperature is used, whereas the internal labeled primers will principally be responsible for the subsequent sequencing reaction. We have furthermore found that a 10-fold higher concentration of the outer primers than the inner labeled primers results in higher quality results than either a lower or higher relative excess of the outer primers.

Direct sequencing of mitochondrial DNA

Sequential DEXAS was used to determine the DNA sequence of the mitochondrial control region directly from genomic DNA using nested primers. Sequential DEXAS routinely determined the full 483 bases between the inner primers, including the sequence of the reverse primer (Fig. 4). Typically, one incorrect base was called in each reaction. However, the reverse reaction generally resolved such ambiguities. The signal to noise ratio, peak uniformity and spacing is similar to that of a cycle sequencing reaction in most parts of the sequenced region. However, in some areas a higher background is often observed, but this generally has no negative effect on the accuracy of the base calling. For most primer combinations, 100 ng or more results in optimal results, whereas lower amounts of template DNA reduce the read length.

Direct sequencing of nuclear DNA

Sequential DEXAS was used to determine DNA sequences from an intronic region of the human FXR α gene, a member of the nuclear receptor family. Labeled primers were designed flanking a 376 bp segment while the unlabeled primers spanned a region of 636 bp (Table 1). Figure 5 shows a typical reaction where 200 ng of genomic DNA was used as a template. Peak intensities are mostly uniform, allowing 373 bases to be scored from the forward reaction in spite of displacement of the baseline. Four ambiguities are caused by regions with higher background. As for the mitochondrial sequencing, amounts of genomic DNA lower than 100 ng led to significantly shorter reading lengths and lower accuracy. Sequential DEXAS was also applied to the sequencing of a

region comprising exon 8 of the human p53 gene (data not shown), where similar results were obtained.

Variation detection with Sequential DEXAS

In one application, we have used Sequential DEXAS to detect mutations in the control region of the mitochondrial genome from tumors. For this purpose, we screened samples of genomic DNA from tumors and normal tissues in a blind study. DNA samples from cancer patients were provided and previously typed with different methods by Fliss *et al.* (13). Differences in the DNA sequence between normal and tumor material allowed variations to be detected. Figure 6A shows that it is possible to show the presence of three somatic mitochondrial mutations in one sample using Sequential DEXAS and hence to identify the correct patient as indicated in Fliss *et al.* (13).

Sequential DEXAS has also been used to genotype clinical samples with respect to a SNP in the FXR α gene. Figure 6B shows that the Sequential DEXAS reaction allowed both homozygous and heterozygous variants of a SNP to be detected.

Direct sequencing of DNA from human blood

Although procedures have been described that allow PCR to be performed directly on whole blood (14–16), DNA sequence determination from blood requires a time consuming purification of nucleic acids before amplification (17,18) and subsequent DNA sequencing. An aliquot of 200 μ l of frozen-thawed EDTA-treated blood was heated for 15 min at 95°C. Cell debris was pelleted by centrifugation for 20 min and 10 μ l of the supernatant was applied directly to a Sequential DEXAS reaction designed to sequence a 469 bp segment of the mitochondrial genome. Figure 7 shows a typical result from a series of 12 blood samples where 330 bases with four ambiguities were determined; however, the first 130 bases from the primer were unreadable. Further optimizations are on the way to improve both the reading length and quality of Sequential DEXAS performed directly on blood.

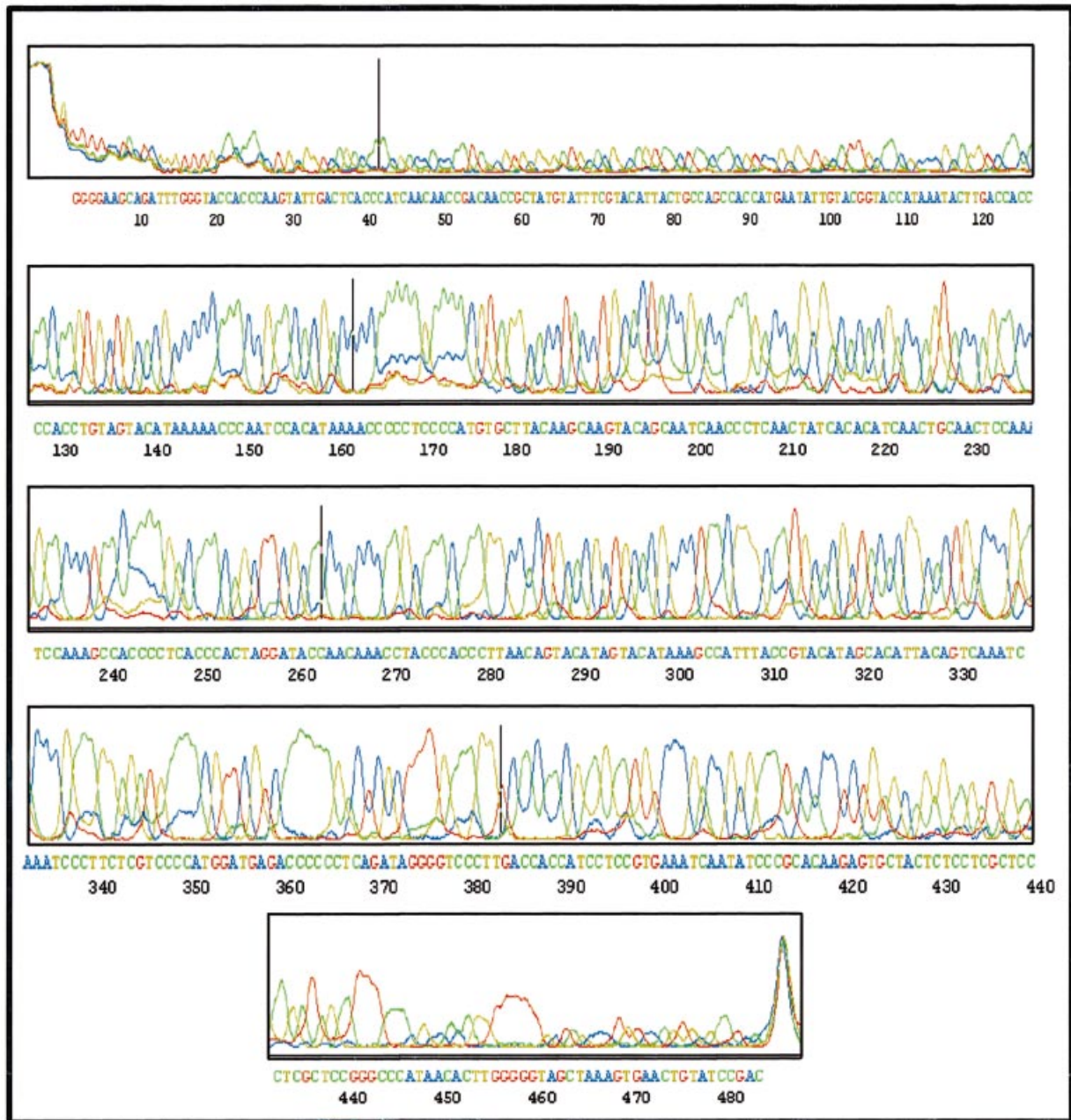


Figure 4. Sequential DEXAS of mtDNA. The entire 483 bases including the reverse primer are called with one ambiguity.

DISCUSSION

Two methods that in principle allow the direct determination of DNA sequences from complex genomes have previously been described. The first such method is CAS (7), which is a two-stage protocol for the coupled amplification and sequencing of DNA. The second method, DEXAS (8,9), achieves amplification of the template DNA as well as the sequencing reaction simultaneously in a single reaction. While CAS requires prior template amplification, DEXAS has the disadvantage of requiring very high amounts of genomic DNA as starting material.

Sequential DEXAS builds on the idea of coupling the two processes of amplification and sequencing in one tube, but separates them in time during the reaction. This allows the amount of template DNA to be reduced because during the first cycles, when the sequencing enzyme is inactive, primer extension will not be terminated before it reaches the other primer binding site due to dideoxynucleotide incorporation. Beyond this, we are the first to demonstrate DNA sequence determination directly on blood samples.

In conclusion, Sequential DEXAS allows DNA sequences to be determined directly from prepared DNA and even directly on blood samples, thus removing the need for

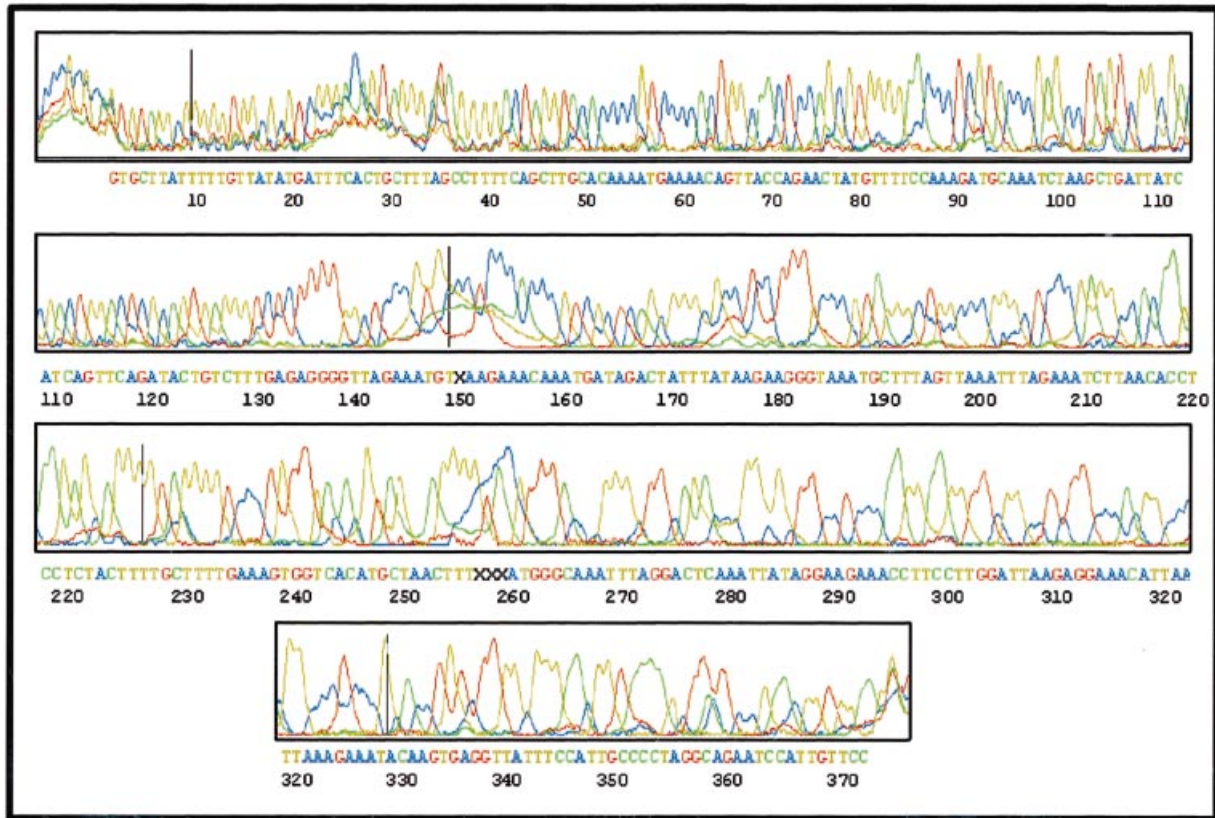


Figure 5. Sequential DEXAS of the FXR α gene. 373 bases were called with four ambiguities.

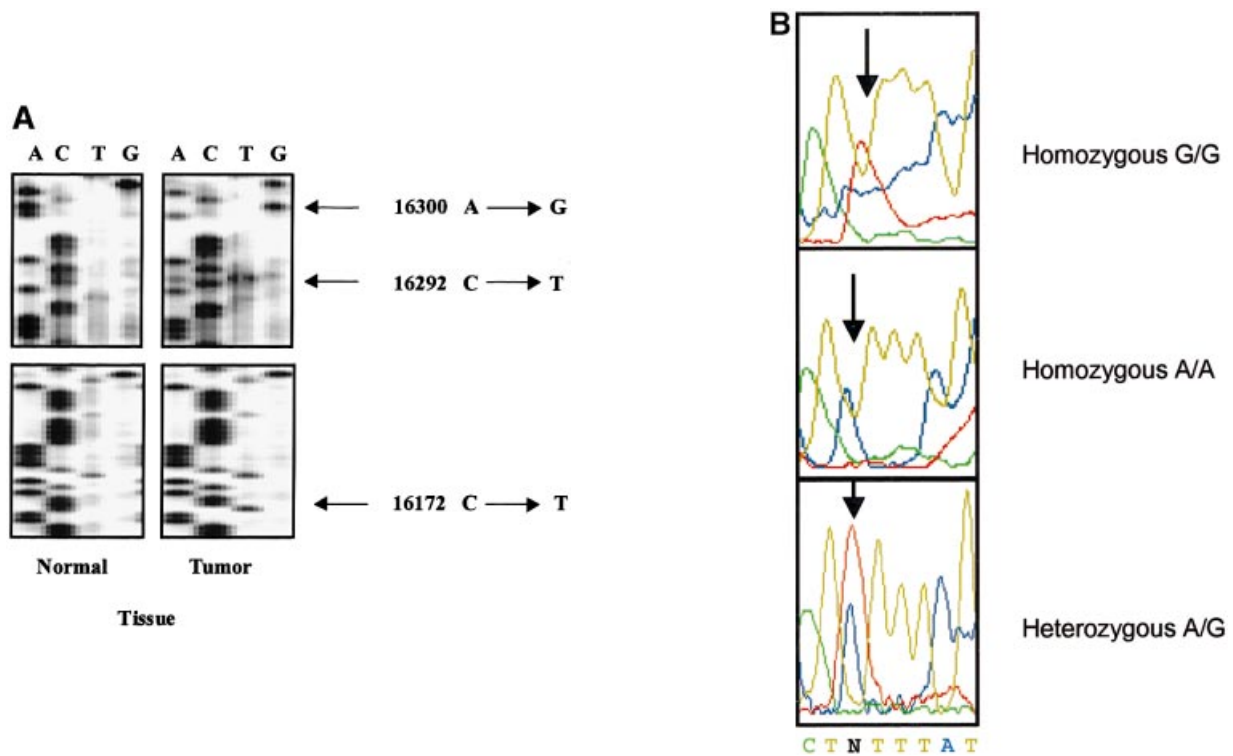


Figure 6. Applications of Sequential DEXAS. (A) Sequential DEXAS was used to compare mtDNA sequences from genomic DNA isolated from tumor and normal tissue from the same individual. Three putative mutations are indicated in the tumor sample. (B) Sequential DEXAS was used to type an intronic SNP in the FXR α gene. Examples of the three possible genotypes are shown.

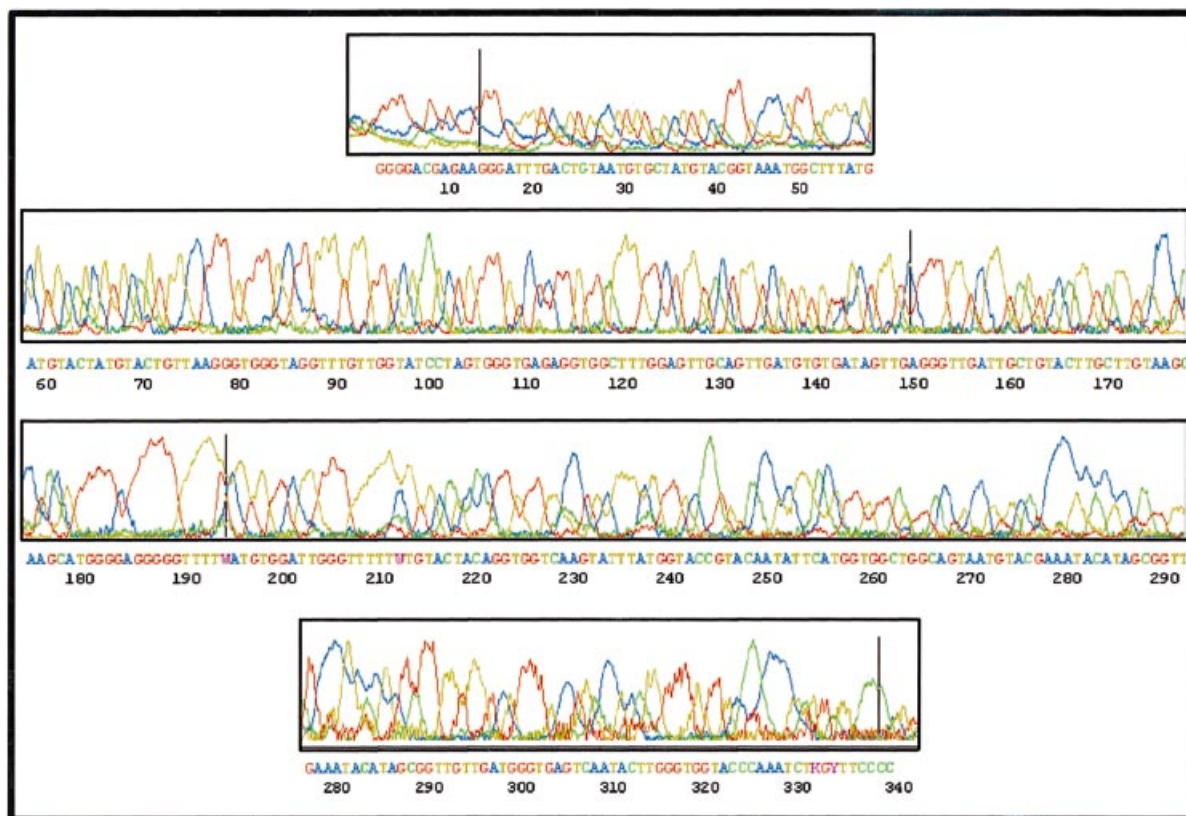


Figure 7. Sequential DEXAS from human blood. An aliquot of 10 μ l of heat-treated human blood yielded a read of 337 bases with four ambiguities.

preparation of DNA from tissues, for cloning or PCR of genomic DNA. A very small number of operations is involved, with the advantage that manual labor and thus the risk of contamination from extraneous DNA is reduced, and, hence, this methodology is eminently suitable for automation.

Potential applications are, for example, the sequencing of all exons from genes known to be involved in certain diseases in large patient groups and the use of mtDNA for large-scale identification after major disasters. To a lesser extent, tag sequencing of complementary DNA clones and identification of bacterial and viral DNA sequences in clinical samples could also be envisioned as potential applications. For several of these applications, Sequential DEXAS will be further developed so that it is also applicable to capillary electrophoresis DNA sequencers.

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