in the plasma. Plasma deoxyribonucleases are known to exist, but their activity in vitro at room temperature is not well characterized. The increasing concentrations of total DNA with concurrent stable concentrations of cell-free fetal DNA argue against this theory, although what happens after 24 h remains unknown.

Intact fetal cells in the cellular fraction of the maternal blood do not measurably contribute to the amount of cell-free DNA after blood sampling. Cell-free fetal DNA concentrations are stable up to 24 h after phlebotomy and therefore are useful as an additional analyte for the noninvasive screening for complications of pregnancy.

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References

Quantification of Mitochondrial DNA Heteroplasmy by Temporal Temperature Gradient Gel Electrophoresis, Richard G. Boles,1,2 Divya Chaudhari,3 Jan Söderkvist,3 Mark Podberesny,1 and Masamichi Ita1‡ (1 Division of Medical Genetics, Childrens Hospital Los Angeles, Los Angeles, CA 90027; 2 Department of Pediatrics, Keck School of Medicine at the University of Southern California, Los Angeles, CA 90033; 3 Colibri Pro Development, S-187 76 Täby, Sweden; ‡ address correspondence to this author at: Division of Medical Genetics, Box 9, Childrens Hospital Los Angeles, Los Angeles, CA 90027; fax 323-665-5937, e-mail rboles@chla.usc.edu; † current address: University of Toronto, Ontario, MSS1A1 Canada; ‡ current address: Boston University School of Medicine, Boston, MA 02118)

Mutations of mitochondrial DNA are increasingly becoming recognized as causes of a wide variety of disorders, most notably neuromuscular and multisystem. In the vast majority of disease-causing mitochondrial DNA (mtDNA) mutations reported to date, mutated and wild-type DNA coexist in the same cell, a condition referred to as “heteroplasmy” (1). Different tissues can contain various proportions of mutant and wild-type species, and although far from a perfect correlation, determination of these proportions in accessible tissues has some clinical relevance (1) and is often performed by molecular diagnostic laboratories.

Because >100 disease-associated sequence changes have been reported in virtually every region of the mtDNA (2), rapid heteroplasmy screening assays have been used by some laboratories to detect both rare and novel mutations. One such assay, temporal temperature gradient gel electrophoresis (TTGE), is sensitive and specific for mtDNA heteroplasmy. In addition, each heteroplasmy variant produces a distinctive band pattern (3). In TTGE, whole cellular DNA is isolated from tissue and the segment of interest is PCR-amplified. Gel electrophoresis of amplified products occurs with the gel immersed in buffer solution, and the entire gel slowly and methodically warms through the melting temperature range particular to the specific DNA segment, allowing the separation of DNA fragments based on minute sequence differences. At the conclusion of the run, gels are banded in ethidium bromide and photographed by a charge-coupled device digital camera system (3, 4).

In the presence of a single mtDNA species (homo-plasmy), a single gel band results. If heteroplasmy is present, melting and reannealing after the last PCR cycle produces two homoduplex and two heteroduplex double-stranded species with different mobilities at approximately their melting temperature, corresponding to the presence of multiple gel bands (3, 4). Although in theory four bands should occur in the presence of heteroplasmy, in practice the homoduplex and/or heteroduplex bands may not be well separated, producing two, three, or four bands (4). Because the heteroduplexes harbor a physical bulge at the site of the sequence mismatch, their mobility in the gel is retarded relative to the homoduplexes, an intuitive assumption that has been repetitively demon-
To investigate the possibility of using TTGE as a quantitative method, we constructed “artificial heteroplasmies” by mixing DNA samples obtained from blood from two individuals known to differ in sequence (among the amplified segment) by a single nucleotide, T3197C, in the IRNA\text{16S(LIR)} gene, or by a 9-bp insertion in the small noncoding area between COX2 and IRNA\text{18S} (corresponding to triplication of the sequence from nt 8272 to nt 8280, whereas duplication of the 9-bp sequence is the norm) (4). This research was determined to be exempt by the Children's Hospital of Los Angeles Institutional Review Board because it used blood samples left over after clinical testing. Before mixing, the total DNA concentration in both samples was determined at 260 and 280 nm by use of a GeneQuant II RNA/DNA Calculator (Amersham Biosciences), and the appropriate quantities of the two samples were mixed together to create various proportions. PCR and TTGE were performed as reported previously (in the “L” or “K” fragment) (3, 4). The quantity of DNA added per lane was −500 ng. The relative intensities of the various bands were measured with an Alpha Imager 2000 (Alpha Innotech Corporation). The percentage of heteroplasmies was calculated as shown below, whereas band intensities are listed in arbitrary units.

Let:

\[ p = \text{the proportion of wild-type mtDNA} \]
\[ q = \text{the proportion of polymorphic mtDNA} \]
\[ W = \text{the measured intensity of the wild-type homoduplex band} \]
\[ M = \text{the measured intensity of the polymorphic homoduplex band} \]
\[ H_{\text{om}} = \text{the measured intensity of the combined homoduplex bands; } H_{\text{om}} = W + M \]
\[ H_{\text{ct}} = \text{the measured intensity of the combined heteroduplex bands} \]

It follows that:

\[ p^2 = \frac{W}{W + H_{\text{ct}} + M} \]  
\[ 2pq = \frac{H_{\text{ct}}}{W + H_{\text{ct}} + M} \]  
\[ q^2 = \frac{M}{W + H_{\text{ct}} + M} \]  
\[ p^2 + 2pq + q^2 = 1 \]

Solving for \(p\) and \(q\) produces:

\[ p = \frac{1}{2} \left( 1 + \frac{H_{\text{om}} - H_{\text{ct}}}{H_{\text{om}} + H_{\text{ct}}} \right) \]  
\[ q = \frac{1}{2} \left( 1 + \frac{H_{\text{om}} - H_{\text{ct}}}{H_{\text{om}} + H_{\text{ct}}} \right) \]

In these two-solution equations, \(p\) and \(q\) have the opposite values of plus and minus of the term inside the square root, but the equation cannot determine which one is which. However, in reality this potential problem is easily solved when the unknown sample is mixed with a near-equal quantity (as estimated on agarose gel) of wild-type DNA and analyzed by TTGE in a separate lane. Heteroduplex band intensity is always highest at \(p = q = 0.5\), and falls off as \(p\) and \(q\) move in either direction from equal proportions. If \(q\) is <0.5, then mixing the unknown with

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**Fig. 1.** TTGE gel demonstrating various degrees of heteroplasmy for the T3197C polymorphism (A), and percentage of mtDNA polymorphism calculated from TTGE band intensities vs the amounts known to be present in the constructed mixture (B). (A), the numbers at the top of the gel represent the actual percentages of the 3197C polymorphic species in the artificially constructed mixture. The bottom two, somewhat separated bands are the two homoduplex species and the top two, closely spaced bands are the two heteroduplex species. (B), ● T3197C polymorphism (\(y = 0.60x + 23; r = 0.999\)); □ 9-bp insertion (\(y = 0.60x + 22; r = 0.992\)). Points shown for T3197C are the means of four experiments; the error bars indicate the highest and lowest values obtained. The percentage of heteroplasmy for the insertion does not exceed 50% because the initial sample diluted was not homoplasmic, with initial proportions determined by the PCR-restriction fragment length polymorphism method.

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**Flow Diagram:**

1. TTGE gel demonstrating various degrees of heteroplasmy for the T3197C polymorphism.
2. Percentage of mtDNA polymorphism calculated from TTGE band intensities vs the amounts known to be present in the artificially constructed mixture.
3. Points shown for T3197C are the means of four experiments.
4. Error bars indicate the highest and lowest values obtained.
5. The percentage of heteroplasmy for the insertion does not exceed 50% because the initial sample diluted was not homoplasmic, with initial proportions determined by the PCR-restriction fragment length polymorphism method.
wild-type dilutes the proportion further from 0.5, and the heteroduplex bands become less intense. Conversely, if \( q \) is \( >0.5 \), then this mixing brings the proportion closer to 0.5, and the heteroduplex bands become more intense.

Shown in Fig. 1B are our results using TTGE to quantify constructed heteroplasmonic samples for the two different mtDNA polymorphisms: T3197C and the 9-bp insertion. Similar results have been obtained with heteroplasmy of the A3243G pathogenic mutation in \( tRNA^{\text{Leu(UUR)}} \), data not shown. For each heteroplasmic sequence variation, the calculated vs actual percentage of mtDNA heteroplasmy demonstrates a highly linear relationship. Interassay precision is high, as shown in Fig. 1B, in which the error bars denote the range of values obtained in four separate measurements for calculated vs actual percentage of heteroplasmy for the T3197C polymorphism. Thus, TTGE allows for accurate quantification of the heteroplasmic proportions when a calibration curve can be run alongside a patient sample of unknown heteroplastic proportions.

However, as Fig. 1B also demonstrates, the lines do not intercept the origin because of background fluorescence, as is readily apparent on inspection of the gels. With careful attention to factors such as the timing of ethidium bromide staining, background can be reduced but not eliminated, probably owing much to scattered fluorescence from nearby lanes (6). In our experience, this minimal background factor varies little from run to run, and our calibration curves appear to be independent of the specific sequence substitution (Fig. 1B). Thus, with our instrumentation, even in cases in which a calibration curve is not possible or practical, we can correct for background fluorescence and estimate the percentage of heteroplasmy for \( p \) and \( q \), using the linear equation

\[
y = 0.60x + 22
\]

obtained from Fig. 1B. As the background fluorescence captured is likely dependent on the specific instrumentation used in imaging and quantification, the linear coefficients in another laboratory probably will vary from those obtained in ours. However, the same principle should apply, and each laboratory could develop an equation to correct for background fluorescence based on data obtained with its own equipment.

The literature contains several methods of mtDNA quantification, including Southern blotting (5), PCR-SSCP (5, 7), PCR-restriction fragment length polymorphism analysis (8), last one cycle hot PCR (9), real-time fluorescence PCR (10), primer extension (11), and solid-phase minisequencing using PCR with allele-specific oligonucleotides (12). Like PCR-SSCP, but unlike many of the other methods listed, TTGE enables a relatively direct measurement of heteroplasmy. Although quantification by TTGE offers no real advantage in terms of accuracy or convenience compared with other methods, the value of TTGE occurs when TTGE is used as the mutation detection method; in that situation, it provides an immediate estimate of the proportion of heteroplasmy on first viewing the gel. If the sequence variation has been encountered previously, putative identification can be made based on the distinctive gel pattern. Strong support for the sequence variant’s identity can be provided by a second TTGE run in which the unknown sample is run alongside a “standard” sample of its suspected identity in various known heteroplasmic proportions; accurate quantification of the heteroplasmic proportions can be achieved in the same run by mixing the unknown sample with a sample of a known, “normal” sequence (the latter to distinguish \( p \) from \( q \)).

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