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A Standard Reference Material to determine the sensitivity of techniques for detecting low-frequency mutations, SNPs, and heteroplasmies in mitochondrial DNA

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Abstract

Human mitochondrial DNA (mtDNA) mutations are important for forensic identifications and mitochondrial disease diagnostics. Low-frequency mutations, heteroplasmies, or SNPs scattered throughout the DNA in the presence of a majority of mtDNA with the Cambridge Reference Sequence (CRS) are almost impossible to detect. Therefore, the National Institute of Science and Technology has developed heteroplasmic human mtDNA Standard Reference Material (SRM) 2394 to allow scientists to determine their sensitivity in detecting such differences. SRM 2394 is composed of mixtures ranging from 1/99 to 50/50 of two 285-bp PCR products from two cell lines that differ at one nucleotide position. Twelve laboratories using various mutation detection methods participated in a blind interlaboratory evaluation of a prototype of SRM 2394. Most of these procedures were unable to detect the mutation when present below 20%, an indication that, in many real-life cases, low-frequency mutations remain undetected and that more sensitive mutation detection techniques are urgently needed.
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Now that the sequencing of the human genome has essentially been completed, one of the current major and daunting tasks is to detect all the single-nucleotide polymorphisms (SNPs) that are present in the human genome and determine whether they cause silent changes, contribute to the diversity of the population, or cause mutations leading to disease. Venter et al. estimated that 2.1 million SNPs are present in the human genome [1], while the International Human Genome Sequencing Consortium headed by Francis Collins and the International SNP Map Working Group estimated that there are 1.42 million SNPs or 1 every 1900 bp [2,3]. The SNP Consortium, a collaboration between the public and the private sectors, has discovered and characterized nearly 1.8 million SNPs (http://snp.cshl.org/) (accessed 11/01/2004). In addition, the International HapMap Project is undertaking the development of a haplotype map (the HapMap) of the human genome to depict the common patterns of human DNA sequence variation. The HapMap is expected to be a key resource to help researchers in finding mutations affecting health, disease, and an individual's responses to drugs and environmental factors and in distinguishing these mutations from those that are inherited and can serve as biomarkers of one's ethnicity (see http://www.hapmap.org/abouthapmap.html for more information).

Most complex diseases, such as diabetes, cancer, stroke, heart disease, depression, and asthma, are believed to result from the interaction of multiple genes and environmental factors. Since 99.9% of the human DNA sequence is commonly shared by most individuals, the genetic variation in the remaining 0.1% forms the basis of human diversity, inheritable diseases, and drug responses. Discovering and

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understanding the effects of these variants will provide investigators with new insights into the complex causes and possible cures of human diseases. However, to compare all the SNPs in individuals with a disease to those without the disease would be an enormously daunting task as well as extremely expensive and time consuming. A better approach is to examine pooled samples from each group. However, with this approach, one needs to be able to detect the relevant low-frequency mutations that may be missed with current mutation detection techniques.

The detection of SNPs, mutations, and heteroplasmies in human mitochondrial DNA (mtDNA) presents similar problems experienced by those trying to find low-frequency mutations in pooled samples. Since there can be hundreds to thousands of mitochondria in cells and the cellular mtDNA can harbor heteroplasmies (i.e., mtDNA with sequences that agree with the Cambridge Reference Sequence (CRS) and mtDNA that contains mutant nucleotides), which may be present in different proportions depending on the tissue [4], the problem can be equated to finding a needle in a haystack. Although a number of mutation detection techniques are currently available, there is no standard or positive control that allows an investigator to determine the sensitivity of their techniques.

The mutations and SNPs that occur in human mtDNA are important tools in forensic studies and mtDNA disease diagnostics as well as anthropological and evolutionary research [5–10]. The advantages of using mtDNA for human identification are: (1) the multiple polymorphisms in the control (noncoding) region can be used to distinguish between non-maternally related individuals [11,12], (2) the mtDNA of maternally related individuals can be used to verify the identification of the individual of interest especially when reference samples from the victims or suspects are not available [13,14], and (3) the large numbers of mitochondria/cell [15] permit mtDNA extraction from minute or degraded samples when there is insufficient nuclear DNA to make the identification [8,16,17]. Another advantage is that mtDNA can be obtained from body parts such as human hair [4,18], bones [8], fingernails [4], body fluids, or other tissues [19] that usually do not provide nuclear DNA.

One disadvantage of using mtDNA for human identification is the possibility that the existence of a heteroplasmy may produce an ambiguous result. Such heteroplasmies in the HV1 and HV2 regions (the noncoding regions primarily used for human identification) have been shown to be more common than originally suspected [14,20–22]. The failure to detect heteroplasmies in earlier studies [23] may be due to the less sensitive detection techniques used at that time. Even today, using the polymerase chain reaction (PCR) and automated sequencing to detect mutations, it is difficult to detect a heteroplasmy that is present in less than 20% of the mtDNA; and even at the 20% level, it still can be missed if one is not specifically looking for it (see Interlaboratory evaluation of the prototype SRM under Results).

In human identification cases, the detection of one of the heteroplasmic bases in one tissue sample and the other heteroplasmic base in a second sample has caused difficulties in the interpretation. The possibility of this occurring was exemplified in a recent study that found various proportions (ranging from 10 to 100%) of an HV1 heteroplasmy in 24 different hairs from a single individual [4]. In this study, the ratio of the heteroplasmy even varied between the root and the shaft in some of the hairs. Thus, two hair samples (one from the crime scene and one from the suspect) with an unknown heteroplasmy could lead to a false negative and the determination of a mismatch, when, in fact, the samples are from the same individual.

On the other hand, when the same heteroplasmy is detected in two samples, the presence of that heteroplasmy may be used to provide further support that the sample came from the suspected individual or that the two individuals providing the samples are maternally related [24]. At this time, it is not clear whether these heteroplasmies exist in the mtDNA in an individual mitochondrion, in different mitochondria in the same cell, or in mitochondria from different cells within the same tissue.

MtDNA SNPs, heteroplasmies, insertions, and deletions have also been implicated in many human diseases primarily involving the neuromuscular system, but deafness, diabetes, epilepsy, progressive dementia, hypoventilation, cardiac insufficiency, renal dysfunction, and sudden onset blindness have also been correlated with mtDNA heteroplasmic mutations. Most of these diseases cause no symptoms until the percentage of the mtDNA carrying the mutation exceeds some threshold. Thus, many mtDNA diseases appear to be age-dependent (i.e., the patient shows the symptoms of the disease only on aging, a possible indication that the mutant mtDNA molecules may have a selective advantage) [25]. Recent results found that about 1/8000 individuals either have or are at risk of developing a mitochondrial disease [26]. Therefore, the ability to detect a low-frequency heteroplasmic mutation in the presence of a majority of CRS mtDNA is extremely important for correctly diagnosing diseases, predicting the risk of developing mitochondrial diseases, and providing pertinent genetic counseling to families at risk.

In this paper, we describe the development of National Institute of Science and Technology (NIST) SRM 2394, a Standard Reference Material (SRM) designed to allow investigators to determine the sensitivity of their low-frequency mutation, SNP, and heteroplasmic detection techniques and, it is hoped, to encourage the development of even more sensitive methods. The results of an interlaboratory evaluation of a prototype of this SRM by 12 laboratories indicated that more sensitive mutation detection techniques would be extremely valuable and that some laboratories are currently engaged in this effort. The final SRM 2394 is currently available from the Office of Standard Reference Materials at NIST; more information on SRM 2394 can be found at www.nist.gov/srm.

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