DEVELOPMENT OF A HETEROPLASMIC MITOCHONDRIA DNA STANDARD REFERENCE MATERIAL FOR DETECTION OF HETEROPLASMY AND LOW FREQUENCY MUTATIONS

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The National Institute of Standards and Technology is developing a heteroplasmic human mitochondrial DNA (mtDNA) standard reference material (SRM) to provide quality control to medical, forensic and toxicological scientists who wish to determine their detection limits when examining low frequency mutations or heteroplasmic sites in DNA. The detection of a mutation or polymorphism that is present in every mtDNA molecule is routine. However, in many cases, especially disease-prone individuals, the mutation or polymorphism may exist in only a small proportion of the mtDNA molecules and the disease does not become apparent until a significant number of the mtDNA molecules contain the mutation. The proportion of the DNA with the mutation appears to increase with age (i.e., the mutation becomes more predominant in older individuals, who then exhibit the disease symptoms). Heteroplasmy has also become problematic to the forensic community. It is very difficult to detect a heteroplasmy present at low concentrations. Different hairs from the same individual can have different proportions of the base pairs contributing to the heteroplasmy (see poster of Sekiguchi, Kasai, and Levin). The presence of such heteroplasmic differences may result in exclusion rather than a match. Toxicologists would like to detect mutations that arise in mtDNA from environmental exposures. With the present state-of-the-art techniques, however, low frequency mutations scattered throughout the DNA, are almost impossible to detect.

Technical Strategy: mtDNA mixtures containing a polymorphic/wild type site in different percentages (e.g., 1, 2.5, 5, 10, 20, 30, 40 and 50%) have been constructed from PCR products from two different cell culture lines which differed by one base pair in the amplified region. To construct the mixtures, the concentration of each PCR product was determined by a new chromatographic technique, Denaturing High Performance Liquid Chromatography (DHPLC), which uses an ion-pair reversed-phase column, to separate the DNA fragments based on their size, sequence, and charge density (1). Various sequencing techniques and instrumentation (ABI™ 373, ABI™ PRISM® 310 Genetic Analyzer, Denaturing Gradient Gel Electrophoresis (DGGE)) were used to determine the lowest detectable level of heteroplasmy in our mixtures. We also designed a Peptide Nucleic Acid (PNA) that is complementary to the wild type sequence. Since PNAs bind with higher affinity to DNA than complementary DNA, PCR is inhibited. Thus, the region with the polymorphism is selectively amplified, allowing low concentrations to be enriched to detectable levels.

Results: With automated sequencing techniques using the ABI™ 373 or 310, we were able to unambiguously detect the polymorphism present at the 30% level. Although visible at the 10% and 20% concentrations, it was difficult to distinguish the polymorphism from the background. The use of DGGE increased our resolution to the 5% level. The addition of PNA to the various mixtures, followed by PCR and sequencing also increased the detection resolution to 5%.

Future work: We will conduct an Interlaboratory Evaluation (ILE) on these heteroplasmic mixtures to determine if other laboratories can detect the polymorphism. They will be free to use any technique that they may have developed to detect low frequency mutations or polymorphisms.

Conclusions: The final mtDNA heteroplasmic SRM will include PCR products that contain different concentrations of a heteroplasmic site. Investigators will be able to use this SRM to determine the resolution of their mutation detection techniques and allow them to perfect even more sensitive methods. In-house research will assure that each step of the process is reliable, simple and efficient and that the components of the SRM provide results which are trustworthy and cost effective.

Marino, M.A., Devaney, J.M., Tully, L.A., and Levin, B.C. Mitochondrial DNA Polymorphism Detection and Heteroplasmy Quantitation Using High Performance Liquid Chromatography. Proceedings of the 49th Annual meeting of the American Society of Human Genetics, San Francisco, CA, October 19-23, 1999