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Comparison of the complete mtDNA genome sequences of human cell lines – HL-60 and GM10742A – from individuals with pro-myelocytic leukemia and leber hereditary optic neuropathy, respectively, and the inclusion of HL-60 in the NIST human mitochondrial DNA standard reference material – SRM 2392-I☆☆☆★

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☆☆ Orders and requests for information concerning this SRM should be directed to the Standard Reference Materials Program, National Institute of Standards and Technology, 100 Bureau Drive, Stop 3222, Gaithersburg, MD 20899-2322, USA. Tel.: +1-301-975-6776; fax: +1-301-948-3730. E-mail: srminfo@nist.gov, web address http://www.nist.gov/srm.

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Abstract

Forensic and clinical laboratories benefit from DNA standard reference materials (SRMs) that provide the quality control and assurance that their results from sequencing unknown samples are correct. Therefore, the mitochondrial DNA (mtDNA) genome of HL-60, a promyelocytic leukemia cell line, has been completely sequenced by four laboratories and will be available to the forensic and medical communities in the spring of 2003; it will be called National Institute of Standards and Technology (NIST) SRM 2392-I. NIST human mtDNA SRM 2392 will continue to be available and includes the DNA from two apparently healthy individuals. Both SRM 2392 and 2392-I contain all the information (e.g. the sequences of 58 unique primer sets) needed to use these SRMs as positive controls for the amplification and sequencing any DNA. Compared to the templates in SRM 2392, the HL-60 mtDNA in SRM 2392-I has two tRNA differences and more polymorphisms resulting in amino acid changes. Four of these HL-60 mtDNA polymorphisms have been associated with Leber Hereditary Optic Neuropathy (LHON), one as an intermediate mutation and three as secondary mutations. The mtDNA from a cell line (GM10742A) from an individual with LHON was also completely sequenced for comparison and contained some of the same LHON mutations. The combination of these particular LHON associated mutations is also found in phylogenetic haplogroup J and its subset, J_2, and may only be indicative that HL-60 belongs to haplogroup J, one of nine haplogroups that characterize Caucasian individuals of European descent or may mean that haplogroup J is more prone to LHON. Both these mtDNA SRMs will provide enhanced quality control in forensic identification, medical diagnosis, and single nucleotide polymorphism detection.
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Keywords: Forensic identification; GM10742A; Haplogroup J; HL-60; Leber hereditary optic neuropathy (LHON); Medical diagnosis; Mitochondrial DNA sequence; Single nucleotide polymorphism (SNP); Standard reference material (SRM)

1. Introduction

On July 15, 1998, the Federal Bureau of Investigation (FBI) Director signed Standard 9.5, which stated "The laboratory shall check its DNA procedures annually or whenever substantial changes are made to the protocol(s) against an appropriate and available National Institute of Standards and Technology (NIST) standard reference material or standard traceable to a NIST standard". At the present time, there are a number of human DNA standard reference materials (SRMs) available from NIST (Levin et al., 2001). One of these SRMs on mitochondrial DNA (mtDNA SRM 2392) is used by the forensic community for human identification and by the medical community for diagnoses of a number of human diseases now known to be associated with specific mutations, insertions and deletions of mtDNA (Levin et al., 1999). This SRM was prepared by NIST to provide quality control to the scientific community when they amplify and sequence human mtDNA or any DNA and includes DNA from two apparently healthy individuals (CHR and 9947A) plus cloned DNA of an area in CHR containing a C-Stretch beyond which sequencing becomes difficult. All the information necessary to successfully conduct the polymerase chain reaction (PCR) amplification process, cycle sequencing steps, gel separation, and data analysis to obtain the final DNA sequence is provided as well as the information on the sequence of 58 unique primer sets that allow the sequencing of any specific portion or the entire mtDNA (16,569 bp) without any gaps. Following an interlaboratory evaluation, SRM 2392 became available to the public in December 1999.

The FBI acknowledges the utility of DNA SRMs to provide the quality control and quality assurance that the results from forensic laboratories that are sequencing unknown samples are correct. One of the FBI's Combined DNA Index Systems now includes mtDNA from unidentified remains, as well as from relatives of missing persons. In order for authorized laboratories to contribute to these indices, certain quality standards must be met. These include the use of DNA from the human cell line HL-60 as a positive control to be run in conjunction with the unknown samples. HL-60 was chosen as the positive control because of several features present in HL-60 but not in the cell lines currently available in NIST SRM 2392. Some of the advantageous features of HL-60 are well-spaced polymorphisms throughout the non-coding hypervariable regions, HV1 and HV2, of the mtDNA control

region, and no insertions at the HV2 C-stretch area (positions 303–309). One of the current templates in SRM 2392, the CHR DNA has a C-stretch in the HV1 region caused by a T to C change at position 16189 that produces a length heteroplasmy (Bendall and Sykes, 1995; Butler and Levin, 1998). Sequencing through this C-stretch is difficult and time-consuming and results in the need to perform additional sequencing reactions to resolve this region and the area following the C-stretch. The CHR template was chosen by NIST specifically for the C-stretch region since some laboratories wanted the opportunity to address this difficult sequencing problem and try to resolve it. The DNA from the other current SRM DNA template, 9947A, has only two polymorphisms in the HV1 region with respect to the Cambridge Reference Sequence (Anderson et al., 1981; Andrews et al., 1999) and those polymorphisms are at common sites. For the work that the forensic laboratories are doing on human identification, several evenly spaced polymorphisms within the HV1 region are more desirable to differentiate the positive control from the test sample. Thus, the FBI suggested to NIST that the development of a SRM containing the HL-60 DNA template would be of great utility to the forensic community. All of the research and the interlaboratory evaluation of the HL-60 template necessary to produce this new SRM containing HL-60 have been completed and are described in this paper. Both SRM 2392 (containing two DNA templates) and SRM 2392-I (containing one DNA template) should fulfill the needs of forensic laboratories by providing additional quality control when sequencing human mtDNA. Corroboration of the SRM results will provide assurance that the techniques being used for amplification and sequencing unknown DNA are being conducted correctly.

The DNA templates in SRM 2392 came from apparently healthy individuals; however, HL-60 in SRM 2392-I is from a promyelocytic cell line from peripheral blood leukocytes provided by a 36-year-old Caucasian female with acute promyelocytic leukemia. Therefore, it was of interest to determine if the HL-60 DNA contained specific mutations that may be characteristic of this disease. Although more DNA samples from leukemic patients would have to be examined to associate specific mutations with this disease, we did find four polymorphisms that are considered either intermediate or secondary mutations associated with leber hereditary optic neuropathy (LHON) (Wallace et al., 1997). We, therefore, sequenced the mtDNA from cell line GM10742A that was prepared from a patient with LHON for comparison with the HL-60 mtDNA sequence. Two of the four LHON-associated mutations in HL-60 were also found in GM10742A. Two additional mutations associated with LHON were found in GM10742A, one primary and one secondary mutation. Many of the polymorphisms common to both HL-60 and GM10742A (some of which are associated with LHON) characterize the phylogenetic haplogroup J and sub-haplogroup J_2, a subset of J (Finnilä et al., 2001; Helgason et al., 2001; Herrnstadt et al., 2002). Haplogroup J is one of nine haplogroups that characterize Caucasian populations of European decent. A number of papers in the literature indicate that individuals with LHON usually belong to haplogroup J and suggest that those in haplogroup J may therefore be more prone to this mtDNA disease than those in other haplogroups (Brown et al., 2002; Herrnstadt et al., 2002; Torroni et al., 1997).

2. Materials and methods

2.1. HL-60 DNA

DNA from the HL-60 cell culture was extracted, isolated, and quantified by the Professional Services Department of the American Type Culture Collection (ATCC, Manassas, VA).

DNA was isolated from the HL-60 cell culture using the QIAamp DNA Blood Mini Kit (Qiagen, Inc., Valencia, CA). Quantification was determined by the Quantiblot Human DNA Quantification Kit (Applied Biosystems, Foster City, CA). The final concentration, 1.4 ng/μl, is based on 12 replicate tests.

The DNA purity (A260:A280 = 1.9) was determined spectrophotometrically. The integrity of DNA was determined electrophoretically using 0.4% agarose gels.

2.1.1. GM10742A DNA

The lymphoblast cell line GM10742A was purchased from NIGMS Human Genetic Mutant Cell Repository, Coriell Institute of Medical Research,

Camden, NJ. The cells were grown at NIST in RPMI 1640 plus L-glutamine and sodium bicarbonate growth media (Sigma, St. Louis, MO) plus fetal calf serum (20%) (Sigma) plus the antibiotics Streptomycin and Penicillin (final concentration: 100 U/ml) (Sigma). DNA was extracted using the QIAGEN Plasmid Kit following the Plasmid Mini Purification Protocol.

2.1.2. mtDNA primers

Fifty-eight sets of unique primers (19–28 bp) for sequencing any portion or the entire human mtDNA (16,569 bp), including both the HV1 and HV2 regions, were computer-designed using GENE RUNNER FOR WINDOWS (Hastings Software, Inc., Hastings, NY) and were the same as those used for SRM 2392 (Levin et al., 1999) with the exception of the reverse primer of set 51. During the course of this study, this primer was changed since it contained a C at nucleotide position (np) 14,368. Since this is the reverse primer, it would bind to a G at np 14,368. Andrews et al. (1999) in their reevaluation of the placenta originally used to sequence human mtDNA in 1981 (Anderson et al., 1981) found that np 14,368 should have a C at that position. The new reverse primer 51 is 5′-TTAGCGATGGAGGTAGGATT**<u>G</u>**G-3′ (np 14,368 is in bold and underlined) which binds to the C at position 14,368. The 5′ end is np 14,388 and the 3′ end is 14,367. The 58 sets of primers were custom-made by Bio-Synthesis, Inc. (Lewisville, TX); the new reverse primer 51 was obtained from Invitrogen (Carlsbad, CA). Those laboratories using either SRM 2392 or SRM 2392-I should use the new reverse primer 51.

2.1.3. Polymerase chain reaction (PCR) at NIST

The PCR mixture contained: HL-60 DNA (1 μl; 1.4 ng), AmpliTaq Gold® DNA polymerase, (0.5 μl; 2.5 units) (Applied Biosystems), 10 × buffer (5 μl) containing 100 mmol/l Tris–HCl, pH 8.3, 500 mmol/l KCl, 15 mmol/l $MgCl_2$ and 0.01% (w/v) gelatin (Applied Biosystems), 10 mmol/l dNTP mix (1 μl) (Invitrogen), 10 μmol/l forward and reverse primers (1 or 2 μl), plus water to make a final volume of 50 μl.

Thermal cycling was conducted in a PerkinElmer thermocycler Model 9700 and started with 10 min at 95°C, followed by 35 cycles of 20 s at 94°C (denaturation), 10 s at 56°C (annealing), 30 s at 72°C (extension) and ended with a final extension of 7 min at 72°C.

Amplified DNA was purified with a QIAquick PCR Purification Kit (Qiagen) and the purity and size of the PCR product was determined by electrophoresis in 2% agarose gels in 1 × TBE buffer containing 0.5 μg/ml ethidium bromide (Sigma).

2.1.4. Sequencing at NIST

Cycle sequencing using fluorescent dye-labeled terminators was performed with an ABI PRISM® BigDye® Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Applied Biosystems).

Cycle sequencing reactions in both the forward and reverse modes were conducted with a 9700 PerkinElmer thermal cycler and started with 1 min at 96°C. The reaction then underwent 25 cycles of 96°C for 15 s (denaturation), 50°C for 5 s (annealing), and 60°C for 2 min (extension). The DNA products were purified using Edge Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD).

Sequencing and data analysis of the purified DNA were performed using an Applied Biosystems PRISM® Model 310 Genetic Analyzer with POP-6™ polymer system and 47 cm × 50 μm capillaries (Applied Biosystems). Sequence data were analyzed with Sequencing Analysis Software 3.3, and comparisons to the Cambridge Reference Sequence (1981) were performed with Sequence Navigator Software 1.01.

2.2. Interlaboratory evaluation of HL-60

Three laboratories, in addition to NIST, participated in an interlaboratory evaluation of the sequence of HL-60 DNA. These laboratories included the FBI Laboratory, FBI Academy, Quantico, VA 22135; Armed Forces DNA Identification Laboratory (AFDIL), Armed Forces Institute of Pathology, Rockville, MD 20850; and the Georgia Bureau of Investigation (GBI), Decatur, GA 30034. Each laboratory was asked to amplify and sequence the entire mtDNA of HL-60. NIST provided: (1) a tube of DNA containing the extracted DNA ready for PCR amplification; (2) the 58 sets of primers labeled with either F# (forward primer) or R# (reverse primer); (3) a table to record the results; and (4) any other supplies

that were needed and requested by the participants. They were allowed to use any protocol for amplification or sequencing that they wished, but were requested to provide a copy of that protocol to NIST. NIST also requested copies of the electropherograms to enable us to resolve any discrepancies; although as it turned out, there were no discrepancies.

2.2.1. Differences in methodology used by the laboratories in the interlaboratory evaluation

2.2.1.1. Armed forces DNA identification laboratory (AFDIL). AFDIL has developed a high-throughput, automated sequencing procedure using 12 primer sets that produce overlapping PCR products ranging from 825 to 1886 bp. The primers used to amplify the first 11 products were based on those published in Levin et al. (1999) and are: Amp01 – F361/R2216; Amp02 – F1993/R3557; Amp03 – F3441/R4983; Amp04 – F4797/R6526; Amp05 – F6426/R8311; Amp06 – F8164/R9848; Amp07 – F9754/R11600; Amp08 – F11403/R13123; Amp09 – F12793/R14388; Amp10 – F14189/R15396; Amp11 – F15260/R16084. The primers used to amplify the control region were developed at AFDIL and are Amp12 – F15878/R649 (F15878 is TTAACTCCACCATTAGCACC and R649 is TTTGTTTATGGGGTGATGTGA).

2.2.1.1.1. AFDIL PCR amplification. The PCR mixture contained HL-60 DNA (1 μl), AmpliTaq-Gold® DNA polymerase (1 μl) (Applied Biosystems), 10 × PCR buffer (5 μl) (Applied Biosystems), dNTP's (0.2 mmol/l) (Invitrogen), 2 μl of forward and reverse primers (10 μmol/l) (MWG Biotech, High Point, NC) plus dH_2O to a final volume of 50 μl. The 10 × PCR buffer was the same as that used by NIST. Thermal cycling was conducted in a PerkinElmer 9700 thermocycler with the following conditions: 10 min at 96°C (activation of AmpliTaq Gold®), plus 40 cycles of 94°C for 15 s, 56°C for 30 s, and 72°C for 1 min. The purity and size of the PCR products were assessed by electrophoresis in a 0.7% agarose gel containing 0.3 μg/ml of ethidium bromide. The PCR products were purified with Shrimp Alkaline Phosphatase/Exonuclease I (Amersham Pharmacia, Piscataway, NJ). Five μl of exonuclease I (10 U/μl) and 10 μl of Shrimp Alkaline Phosphatase (1 U/μl) was added to each tube containing PCR product. The tubes were heated at 37°C for 15 min followed by 94°C for 15 min in a PerkinElmer 9700 thermocycler.

2.2.1.1.2. AFDIL sequencing. Cycle sequencing was performed with the ABI PRISM® BigDye® terminators (original version) cycle sequencing kit (Applied Biosystems). The sequencing mixture contained 9 μl dH_2O, 6 μl BigDye® dilution buffer (400 mmol/l TRIS, 10 mmol/l $MgCl_2$, pH 9.0), 2 μl BigDye® terminator reaction mixture, 1 μl of forward or reverse primer (10 μmol/l each) and 2 μl of HL-60 PCR product for a total vol of 20 μl. A few of the sequencing primers (e.g. R649) required the use of the ABI PRISM® dGTP BigDye® terminator kit (Applied Biosystems). Thermal cycling was conducted in a PerkinElmer 9700 thermocycler at the following conditions: an initial 1 min denaturation at 96°C, followed by 25 cycles of 94°C for 15 s, 50°C for 5 s, and 60°C for 2 min. The DNA product was purified by filtration through a spin column matrix (Edge BioSystems, Gaithersburg, MD). Electrophoresis and sequencing were performed with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) using POP-6™ polymer (Applied Biosystems) with a 50 cm capillary. Data analysis was executed using Sequencher Plus 4.0.5b11 (Gene Codes, Ann Arbor, MI). The HL-60 sequence differences were identified by comparison to the Cambridge Reference Sequence as revised by Andrews et al. (1999). In most cases, sequence information was acquired for both the forward and reverse directions. In some regions, two separate reactions using the same primer were routinely conducted (indicated by '2 × ' in the following list of primers) to achieve full sequence confirmation. A total of 95 sequencing reactions plus one pGEM reaction were conducted in a 96 well format. If the primer failed the first trial, the reaction was repeated. The finding of a heteroplasmy at nucleotide position (np) 12,071 was also confirmed by an additional PCR and sequencing reaction.

The following primers from Levin et al. (1999) were used to sequence the 12 PCR amplicons:

Amp01 (F361/R2216): F361, R921, F1234, R1425, F873, R2216, F1657, R1769
Amp02 (F1993/R3557): F1993, R2660, R2834, R3557, F2417, R3006, F3234
Amp03 (F3441/R4983): F3441, R3940, F3931 (2 ×), R4982, F4392, R4162

Amp04 (F4797/R6526): F4797 (2 ×), R6526, F5700 (2 ×), F5318, R5882, F6242
Amp05 (F6426/R8311): F6426 (2 ×), R7255, F7645 (2 ×), R8311, F7075, R7792
Amp06 (F8164/R9848): F8164 (2 ×), R9059, F8903, R9848, F8539, R9403, F9309
Amp07 (F9754/R11600): F9754 (2 ×), R10556, F11001 (2 ×), R11600, F10386, R11267
Amp08 (F11403/R13123): F11403 (2 ×), F12357, R13123, F11901 (2 ×), F12601, R12876
Amp09 (F12793/R14388): F12793, R13611, F13518 (2 ×), R14388, F13188, R13343, F13899, R13935
Amp10 (F14189/R15396): F14189 (2 ×), R15396, F14909, R14996, F14470
Amp11 (F15260/R16084): F15260, R16084, F15574, R15774
Amp12 (F15878/R649): F15971, R16175 (2 ×), F16450 (2 ×), R274, F314 (2 ×), R649 (2 ×), F16190, R16400.

In Amp12, F15971 came from Levin et al. (1999). The other primers were designed by AFDIL and were as follows:

R16175: TGGATTGGGTTTTTATGTA
F16450: GCTCCGGGCCCATAACACTTG
R274: TGTGTGGAAAGTGGCTGTGC
F314: CCGCTTCTGGCCACAGCACT
R649: TTTGTTTATGGGGTGATGTGA
F16190: CCCCATGCTTACAAGCAAGT
R16400: GTCAAGGGACCCCTATCTGA.

2.2.1.2. Georgia Bureau of Investigation (GBI). The GBI used the same protocol as that used by NIST with the following exceptions: (1) a PerkinElmer model 9600 was used for thermal cycling; (2) amplified DNA was electrophoresed in 2.75% agarose gels; (3) the cycle sequencing was performed with a Perkin-Elmer model 9600 using the program provided with the BigDye® kit (25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min); (4) some samples that needed to be cycle sequenced again using more amplicon were not purified before precipitation; (5) the samples were precipitated using isopropanol precipitation as per the BigDye® instructions; and (6) comparison of the sequence data was performed with Sequencer 3.1.1 software (Gene Codes, Ann Arbor, MI).

2.2.1.3. Federal Bureau of Investigation (FBI)

2.2.1.3.1. FBI Polymerase chain reaction (PCR). The PCR mixture contained from 0.1 to 1.4 ng HL-60, AmpliTaq Gold® Polymerase (2.5 units) (Applied Biosystems), 10 × PCR buffer (5 μl) (Applied Biosystems), GeneAmp® dNTPs (0.2 mmol/l each) (Applied Biosystems), forward and reverse primers (0.4 μmol/l each), plus dH_2O to a final volume of 50 μl. The 10 × buffer (pH 8.3) was the same as used by NIST. Thermal cycling was conducted in a GeneAmp® PCR System 9700 (PerkinElmer) and consisted of 10 min at 95°C followed by 35 cycles of 94°C for 20 s, annealing temperatures of 50°C (primer set 49), 51°C (primer sets 1, 7, 44, 57), 52°C (primer sets 6, 8, 30, 45), 53°C (primer set 53) and 56°C (all other primer sets) for 10 s, and 72°C for 30 s and ending with a final extension of 7 min at 72°C. Amplified products were purified by treatment with Exo-SAP-IT (5 μl for every 25 μl of PCR product) (USB Corp., Cleveland, OH). Samples of the PCR products were electrophoresed in 1.2% agarose gels containing ethidium bromide to assess the purity, size, and quantity of the PCR products.

2.2.1.3.2. FBI sequencing. Cycle sequencing using fluorescent dye–labeled terminators was performed with an ABI PRISM® dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Applied Biosystems). Thermal cycling was conducted in a GeneAmp® PCR System 9700 (Applied Biosystems) and started with 1 min at 96°C followed by 25 cycles of 96°C for 15 s, 50°C for 1 s and 60°C for 1 min and ended with a final incubation at 15°C for 10 min. These products were purified with Centri-Sep™ spin columns (Princeton Separation, Inc., Adelphia, NJ) and electrophoresed on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) using POP-6™ and either a 47 or 61 cm capillary. Comparison of the sequence data was performed with Sequencer 4.1 software (Gene Codes).

The FBI also discovered the problem with original primer set 51 (see Section 2). The reverse primer incorporated a G at position 14,368 [an error in the original Anderson et al. (1981) sequence that was found and corrected by Andrews et al. (1999)].

However, sequencing with primer set 52 showed a C at position 14,368. They prepared a new set of primers (primer set 51.5) to amplify approximately a 400 bp fragment that encompassed np 14,368. The new primers were: F14217: 5′-CTAATCAACGCCCA-TAATCATAC-3′ and R14620: 5′-GTTTTCTTC-TAAGCCTTCTCC-3′. The new primer set confirmed that the correct base at position 14,368 was a C.

2.3. Permissions

The research to prepare SRM 2392-I containing HL-60 DNA was deemed exempt from the policy of Part 27 of Title 15 of the Code of Federal Regulations by the NIST Institutional Review Board and the Director of the Chemical Science and Technology Laboratory. This work fit into the exemption category described in 15 CFR 27.101(b)(4) which states: "Research, involving the collection or study of existing data, documents, pathological specimens, or diagnostic specimens, if, these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects". ATCC also waived condition 3(c) in their Material Transfer Agreement which states that the "purchaser shall not sell, lend, distribute or otherwise transfer the material or replicates to any others" for the use of HL-60 in the NIST mitochondrial DNA SRM 2392-I. They stated that, in their view, "as a government agency, NIST will not be providing this material as a commercial product despite the collection of fees for the SRM".

3. Results and discussion

HL-60 is a promyelocytic cell line from the peripheral blood leukocytes of a Caucasian female (age 36) with acute promyelocytic leukemia. HL-60 DNA will be available in SRM 2392-I. The two DNA templates available in SRM 2392 are from apparently healthy individuals (both the DNA from CHR and 9947A are from Caucasian females in their twenties and thirties). The entire mtDNA of GM03798 (a 10-year-old apparently healthy Caucasian male) was amplified and sequenced at NIST, discussed in a previous publication (Levin et al., 1999), and is shown in Fig. 1 in this paper; however, that DNA is not included in SRM 2392 or SRM 2392-I. Another mtDNA template (GM10742A) from a 30-year-old Caucasian male patient with LHON was also sequenced at NIST; that sequence is shown in Table 1 and Fig. 1, but the DNA is not included in SRM 2392 or 2392-I. Both GM03798 and GM10742A can be obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden, NJ. At NIST, 58 primer sets (Levin et al., 1999) were used to amplify the entire HL-60 mtDNA and GM10742A at least twice. In the case of HL-60, all the PCR products were sequenced in both the forward and reverse directions for a total of four sequences for each amplicon. During this study, reverse primer 51, which contained np 14,368, was changed (see Section 2) to accommodate the difference found at that position by Andrews et al. (1999) when they resequenced the original placenta used by Anderson et al. (1981). Any sequence ambiguities between experiments at NIST were resolved by additional PCR reactions and sequencing.

The NIST sequencing results from the mtDNA of HL-60, CHR, 9947A and GM10742A are shown in Table 1; all of these sequences plus GM03798 are shown in Fig. 1. Table 1 also compares the CHR, 9947A, HL-60 and GM10742A templates to the original Cambridge Reference Sequence (Anderson et al., 1981) and the revised sequence (Andrews et al., 1999). In Fig. 1, the HL-60 results are shown in black numbers surrounding the circular depiction of human mtDNA; the CHR results are shown in red; 9947A results are shown in green; GM03798 results are shown in blue; and GM10742A are in purple. At the present time, GM03798 and GM10742A are not part of SRM 2392 or SRM 2392-I, and the results are presented for information and comparison only.

The numbering system in Table 1 and Fig. 1 is that of the original Cambridge Reference Sequence (1981). The Cambridge Reference Sequence (1981) was based on a consensus analysis of a placenta, the HeLa cell line and the bovine sequence (the bovine sequence was used in five ambiguous human sites). The 1981 results were reexamined in 1999 by Andrews et al. who resequenced the original placenta. The original Cambridge Reference Sequence was

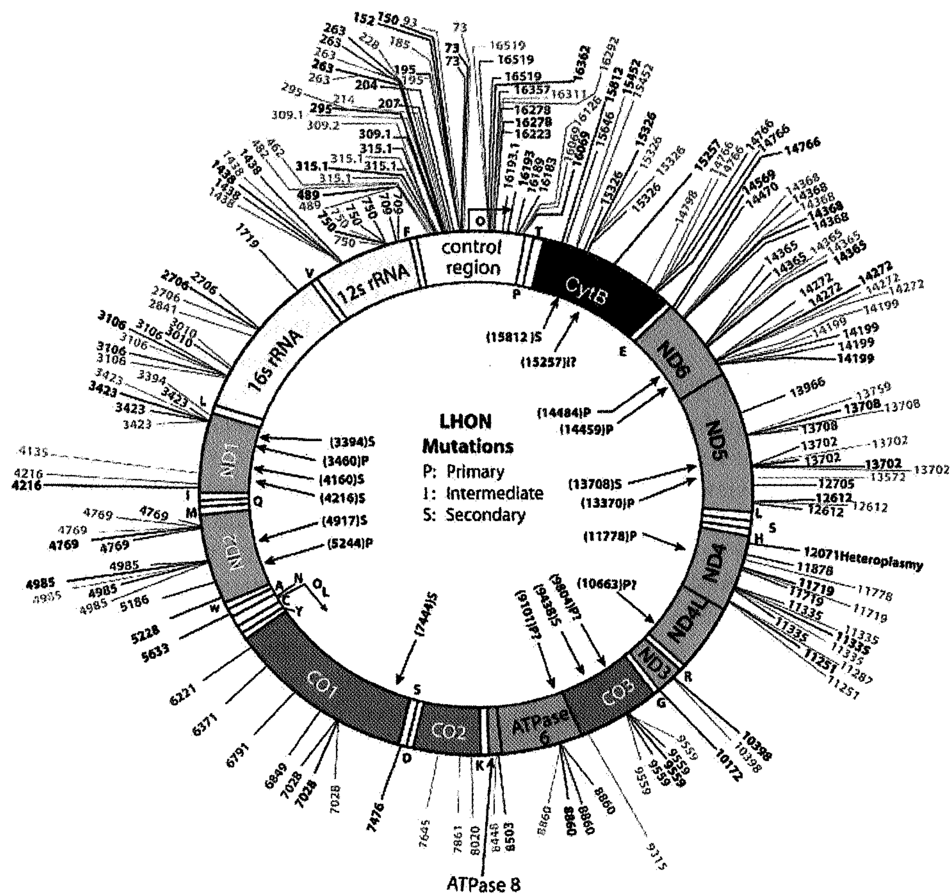

Fig. 1. HL-60 DNA polymorphic differences from the Cambridge Reference Sequence and positions of LHON mutations are added to the figure from Levin et al., 1999. This figure is a schematic of human mtDNA showing its circular double-stranded DNA and all the differences from Cambridge Reference Sequence found in CHR (red), 9947A (green), GM03798 (blue), HL-60 (black), and GM10742A (purple) as numbers along the outside of the color-coded circle. Locations of the control region, rRNAs and genes (see footnote to Table 1 for abbreviations) coded by human mtDNA are shown. The locations of the 22 tRNAs are noted by white areas in the circle and designated by their single letter amino acid code. (Modified from Levin et al., 1999).

found to have a number of rare polymorphisms and errors, which are noted in Table 1 with an asterisk.

Table 1 also shows the unique (U) coding region polymorphisms (either silent with no amino acid change or resulting in an amino acid change) in CHR, 9947A, HL-60 and GM10742A that were not found in the MITOMAP web site database (http://www.mitomap.org). This is a very extensive internet database on the mitochondrial DNA polymorphisms found in the literature. CHR has five polymorphisms (one produces an amino acid change), 9947A has four polymorphisms (two producing amino acid changes), HL-60 has two polymorphisms (one producing an amino acid change and is heteroplasmic), and GM10742A has two (one in the 16sRNA and one that is silent) that were not found in the MITOMAP database.

Table 2 shows the number of differences compared to the Cambridge Reference Sequence found in CHR, 9947A, HL-60 and GM10742A. There are 13, 9, 11 and 12 differences in the non-coding regions of CHR, 9947A, HL-60, and GM10742A, respectively. The non-coding region is the primary area used by the forensic community for human identification. There

are 33, 23, 33 and 31 differences in the coding regions of CHR, 9947A, HL-60, and GM10742A, respectively. These differences would have resulted in 11, 12, 15 and 14 amino acid changes in their respective proteins if one compares the results to the original Cambridge Reference Sequence published by Anderson et al. (1981). If compared to the revised Cambridge Reference Sequence (Andrews et al., 1999), the actual amino acid changes would be reduced to 3 in CHR, 4 in 9947A, and 7 in both HL-60 and GM10472A (the non-normal cell lines sequenced). HL-60 also has a change in the tRNA for alanine and the tRNA for serine. It would be of interest to examine other cell lines from patients with acute promyelocytic leukemia or LHON to see if they have any of these same mutations.

Table 3 shows the HL-60 and GM10742A polymorphisms in the coding regions that (with the exception of one CHR mutation included because of its association with LHON) were not found in the other templates in SRM 2392. This table also indicates if the mutation has been associated with a disease according to the MITOMAP web site database. At least four HL-60 mutations (T4216C in NADH dehydrogenase 1, G13708A in NADH dehydrogenase 5, and G15257A and G15812A in Cytochrome B) were found to be associated with LHON (Wallace et al., 1997). Four mutations associated with LHON were also found in GM10742A; these are T3394C and T4216C in NADH dehydrogenase 1, G11778A in NADH dehydrogenase 4, and G13708A in NADH dehydrogenase 5. Two of these mutations, T4216C and G13708A, were found in both HL-60 and GM10742A. The mutation G13708A was also seen in the CHR template. LHON causes central vision loss in patients in their twenties or thirties although the onset of symptoms can occur both earlier and later. The disease is inherited like other mitochondrial DNA diseases through the maternal lineage. LHON was first associated with a G11778A mtDNA mutation (Wallace et al., 1988) that results in an arginine to histidine change in NADH dehydrogenase 4 at position 340 in a protein that contains 460 amino acids (Lee and Levin, 2002). G11778A is considered a primary mutation causing LHON and was seen in GM10742A, a cell line from a patient who experienced the sudden onset of blindness at the age of 24. Since 1988, 27 missense mutations and one mutation in tRNA for leucine have been associated with LHON (http://www.mitomap.org). Some of these mutations are shown inside the inner circle of Fig. 1. The four mutations considered 'primary' in causing LHON are G14459A, G11778A, G3460A, and T14484C and are presented in order of decreasing severity (Wallace et al., 1997). The mutations associated with LHON found in this study are considered 'primary' (G11778A is in GM10742A), 'intermediate' (G15257A is in HL-60) or 'secondary' (T3394C in GM10742A; T4216C in HL-60 and GM10742A; G13708A in HL-60, CHR and GM10742A; G15812A in HL-60). According to Wallace et al. (1997), the intermediate or secondary mutations may increase the probability of having LHON or may be linked to one of the primary mutations. It is interesting that GM10742A has two of the secondary mutations also found in HL-60, namely T4216C and G13708A. CHR also has the G13708A mutation. Torroni et al. (1997) found that the combination of the np 4216 and 13,708 mutations were more frequent among the LHON patients containing the mutation at 11,778 than among the controls.

Characteristic sets of polymorphisms in human mtDNA are being used to distinguish various groups and to trace their maternal genealogy (Macaulay et al., 1999). Haplogroup J and sub-haplogroup J_2 have specific polymorphisms that distinguish this group of Caucasians with European ancestry from the other eight haplogroups (H, I, K, T, U, V, W and X) that have also been found to characterize those with a European background (Torroni et al., 1997). Both HL-60 and GM10742A have a number of identical polymorphisms that place them in Haplogroup J and Sub-haplogroup J_2 (Table 4).

In the interlaboratory evaluation of the entire sequence of HL-60 that was conducted by three laboratories plus NIST, all four laboratories found the same sequence with the exception of the Georgia Bureau of Investigation who had the problem noted in Section 2 with primer set 51 and was unable to examine that amplicon and therefore, did not find the polymorphism at 14,199.

In conclusion, NIST has sequenced the entire mtDNA (16,569 bp) from the HL-60 cell line multiple times and compared these results with those from AFDIL, FBI, and GBI who participated in the interlaboratory evaluation. All four laboratories

Table 1
Cambridge Reference Sequence nucleotide differences (some unique) found at NIST in the two DNA templates, CHR and 9947A included in SRM 2392; HL-60 included in SRM 2392-I and GM10742A DNA[a]

Comparison with Cambridge Reference Sequence (CRS)

CRS #[d]	Base 1981/1999[e]	Template CHR[b]	Template 9947A[b]	Template HL-60[c]	Template GM10472A	Amino acid change	Region
73	A	G	–[f]	G	G		HV2
93	A	–	G	–	–		HV2
150	C	–	–	T	–		HV2
152	T	–	–	C	–		HV2
185	G	–	–	–	A		HV2
195	T	C	C	–	–		HV2
204	T	C	–	–	–		HV2
207	G	A	–	–	–		HV2
214	A	–	G	–	–		HV2
228	G	–	–	–	A		HV2
263*R[g]	A	G	G	G	G		HV2
295	C	–	–	T	T		HV2
303–309	–	C (ins)	CC (ins)	–	–		HV2
311–315*R	–	C (ins)	C (ins)	C (ins)	C (ins)		HV2
462	C	–	–	–	T		HV2
482	T	–	–	–	C		HV2
489	T	–	–	C	C		HV2
709	G	A	–	–	–		12sRNA
750*R	A	G	G	G	G		12sRNA
1438*R	A	G	G	G	G		12sRNA
1719	G	A	–	–	–		16sRNA
2706	A	G	–	G	G		16sRNA
2841	T	–	–	–	A (U)		16sRNA
3010	G	–	–	–	A		16sRNA
3106–3107*E[h]	C/del	del	del	del	del		16sRNA
3394	T	–	–	–	C	Tyr → His	ND1 LHON
3423*E	G/T	T	T	T	T	Silent	ND1
4135	T	–	C (U)	–	–	Tyr → His	ND1
4216	T	–	–	C	C	Tyr → His	ND1 LHON
4769*R	A	G	G	G	G	Silent	ND2
4985*E	G/A	A	A	A	A	Silent	ND2
5186	A	G (U)	–	–	–	Silent	ND2
5228	C	–	–	G (U)	–	Silent	ND2
5633	C	–	–	T	–		tRNA Ala
6221	T	C	–	–	–	Silent	COI
6371	C	T (U)	–	–	–	Silent	COI
6791	A	G	–	–	–	Silent	COI
6849[i]	A	G(0.3A)[i]	–	–	–	Thr → Ala[i]	COI
7028	C	T	–	T	T	Silent	COI
7476	C	–	–	T	–		tRNA Ser
7645	T	–	C	–	–	Silent	COII
7861	T	–	C (U)	–	–	Silent	COII
8020	G	–	–	–	A	Silent	COII
8448	T	–	C	–	–	Met → Thr	ATPase 8
8503	T	C	–	–	–	Silent	ATPase 8
8860*R	A	G	G	G	nd	Thr → Ala	ATPase 6
9315	T	–	C (U)	–	–	Phe → Leu	COIII
9559*E	G/C	C	C	C	C	Arg → Pro	COIII
10,172	G	–	–	A	–	Silent	ND3
10,398	A	–	–	G	G	Thr → Ala	ND3
11,251	A	–	–	G	G	Silent	ND4
11,287	T	–	–	–	C (U)	Silent	ND4

Table 1 (*continued*)

Comparison with Cambridge Reference Sequence (CRS)							
11,335*E	T/C	C	C	C	C	Silent	ND4
11,719	G	A	–	A	A	Silent	ND4
11,778	G	–	–	–	A	Arg → His	ND4 LHON
11,878	T	C (U)	–	–	–	Silent	ND4
12,071[het]	T	–	–	C/T[het] (U)	–	Phe → Leu[het]	ND4
12,612	A	G	–	G	G	Silent	ND5
12,705	C	T	–	–	–	Silent	ND5
13,572	T	–	C (U)	–	–	Silent	ND5
13,702*E	G/C	C	C	C	C	Gly → Arg	ND5
13,708	G	A	–	A	A	Ala → Thr	ND5 LHON
13,759	G	–	A	–	–	Ala → Thr	ND5
13,966	A	G	–	–	–	Thr → Ala	ND5
14,199*E	G/T	T	T	T	T	Pro → Thr	ND6
14,272*E	G/C	C	C	C	C	Phe → Leu	ND6
14,365*E	G/C	C	C	C	C	Silent	ND6
14,368*E	G/C	C	C	C	C	Phe → Leu	ND6
14,470	T	C	–	–	–	Silent	ND6
14,569	G	–	–	A	–	Silent	ND6
14,766*E	T/C	T	C	T	T	Ile → Thr	ND6
14,798	T	–	–	–	C	Phe → Leu	CYT B
15,257	G	–	–	A	–	Asp → Asn	CYT B LHON
15,326*R	A	G	G	G	G	Thr → Ala	CYT B
15,452	C	–	–	A	A	Leu → Ile	CYT B
15,812	G	–	–	A	–	Val → Met	CYT B LHON
16,069	C	–	–	T	T		HV1
16,126	T	–	–	–	C		HV1
16,183	A	C	–	–	–		HV1
16,184–93	–	C (ins)	–	–	–		HV1
16,189	T	C	–	–	–		HV1
16,193	C	–	–	T	–		HV1
16,223	C	T	–	–	–		HV1
16,278	C	T	–	T	–		HV1
16,292	C	–	–	–	T		HV1
16,311	T	–	C	–	–		HV1
16,362	T	–	–	C	–		HV1
16,519	T	C	C	–	nd		HV1

[a] ATPase 6, ATP synthase 6; ATPase 8, ATP synthase 8; CYTB, Cytochrome B; COI, Cytochrome C Oxidase I; COII, Cytochrome C Oxidase II; COIII, Cytochrome C Oxidase III; del, deletion; het, heteroplasmy found in HL-60 at np 12,071; HV1, non-coding region found from 16,024 and 16,569; HV2, non-coding region found from 1 and 576; ins, insertion; nd, area not amplified or sequenced; ND1, NADH dehydrogenase 1; ND2, NADH dehydrogenase 2; ND3, NADH dehydrogenase 3; ND4, NADH dehydrogenase 4; ND5, NADH dehydrogenase 5; ND6, NADH dehydrogenase 6; (U), Unique polymorphisms found in coding regions of CHR, 9947A, HL-60 and GM10742A determined by comparison with the MITOMAP database (http://www.mitomap.org).

[b] Levin et al. (1999). CHR DNA: Sequence based on two amplifications and cycle sequencing procedures with DNA from the first cell culture line and at least one amplification and cycle sequencing procedure with DNA from the second cell culture line. 9947A DNA: Sequence based on two amplifications and cycle sequencing procedures.

[c] HL-60 DNA: Sequence based on two amplifications and cycle sequencing procedures in both the forward and reverse directions for a total of four sequences.

[d] Numbers correspond to Cambridge Reference Sequence (Anderson et al., 1981).

[e] Base found in 1981 (Anderson et al., 1981)/base found in 1999 (Andrews et al., 1999).

[f] Base pair same as in 1981 Cambridge Reference Sequence.

[g] *R: Rare polymorphisms in Cambridge Reference Sequence discovered by reanalysis of original placenta by Andrews et al. (1999).

[h] *E: Error in Cambridge Reference Sequence discovered by reanalysis of original placenta by Andrews et al. (1999).

[i] Possible heteroplasmic site. This heteroplasmy seen in the mtDNA from the first CHR cell culture line is not seen in the mtDNA from the second CHR cell culture line. It is DNA from the second CHR cell culture line that is supplied in NIST SRM 2392.

Table 2
Number of differences from the Cambridge Reference Sequence

DNA template	Non-coding regions[a]	Coding region	Amino acid changes[b]	Real change[c]
CHR[d]	13	33	11	3
9947A[d]	9	23	12	4
HL-60	11	33	15	7 + 2 tRNA
GM10742A	12	31	14	7

[a] The non-coding regions cover the areas from 16,024 to 16,569 and 1 to 576.
[b] Amino acid changes compared to original 1981 Cambridge Reference Sequence.
[c] Amino acid and tRNA changes compared to revised 1999 Cambridge Reference Sequence and not counting those changes considered a rare polymorphism.
[d] Levin et al. (1999).

found identical results in all amplified regions. SRM 2392-I containing HL-60 will be available in the spring of 2003. SRM 2392 that currently includes the CHR and 9947A templates also will continue to be available. The CHR and 9947A DNAs come from apparently normal individuals, but the HL-60 DNA comes from an individual who has acute promyelocytic leukemia. Since this is the first time that the entire mtDNA from HL-60 has been sequenced, it would be interesting to determine if the differences found in HL-60 are also found in other leukemia patients. Another interesting finding is the four differences in HL-60 that have been associated with LHON disease. For comparison, we also sequenced GM10472A, a cell line from a patient with LHON and found many of the same polymorphisms and mutations. Analysis of these polymorphisms places both HL-60 and GM10742A in Haplogroup J. These results support the premise that individuals in Haplogroup J may be more prone to LHON. At this time,

Table 3
HL-60 and GM10472A Polymorphisms in Coding Regions and not found in original SRM 2392[a]

Nucleotide position	Nucleotide change	Region[b]	Amino acid difference	Associated disease	Reference
2841[GM]	T → A	16sRNA			MA
3010[GM]	G → A	16sRNA			MM, MA
3394[GM]	T → C	ND1	Tyr → His	LHON, NIDDM	MM, MA
4216[HL60;GM]	T → C	ND1	Tyr → His	LHON	MM, MA
5228[HL60]	C → G	ND2	Silent		MA
5633[HL60]	C → T	tRNA ala			MM, MA
7476[HL60]	C → T	tRNA ser			MM, MA
8020[GM]	G → A	COII	Silent		MM, MA
10,172[HL60]	G → A	ND3	Silent		MM, MA
10,398[HL60;GM]	A → G	ND3	Thr → Ala		MM, MA, Mt-1
11,251[HL60;GM]	A → G	ND4	Silent		MM, MA
11,287[GM]	T → C	ND4	Silent		MA
11,778[GM]	G → A	ND4	Arg → His	LHON	MM, MA
12,071[HL60]	T → C/T	ND4	Phe → Leu		MA
13,708[HL60;GMc]	G → A	ND5	Ala → Thr	LHON	MM, MA
14,569[HL60]	G → A	ND6	Silent		MM, MA
14,798[GM]	T → C	CYT B	Phe → Leu		MM, MA
15,257[HL60]	G → A	CYT B	Asp → Asn	LHON	MM, MA
15,452[HL60;GM]	C → A	CYT B	Leu → Ile		MM, MA
15,812[HL60]	G → A	CYT B	Val → Met	LHON	MM, MA

[a] GM, GM10742A; NIDDM, non-insulin dependent diabetes mellitus; MM, http://www.mitomap.org; MA, http://www.cstl.nist.gov/biotech/strbase/mitoanalyzer.html; Mt-1, Levin, BC, Sekiguchi, K, Tully, LA, Chen, JT, and Gropman, A. A patient with chronic progressive external ophthalmoplegia reexamined 30 years later. (Manuscript In preparation, 2003).
[b] See footnote to Table 1.
[c] Also seen in CHR as well as HL-60 and GM10742A.

Table 4
Polymorphisms common to haplogroup J and sub-haplogroup J_2 found in the mtDNA of HL-60 and GM10742A[a]

Polymorphisms characteristic of haplogroup J	Polymorphisms characteristic of sub-haplogroup J_2	Found in HL-60	Found in GM10742A	Also found in	Disease associated
A73G		Yes	Yes	CHR	
C150T		Yes	No		
T152C		Yes	No		
G185A		No	Yes		
T195C		No	No	CHR 9947A	
G228A		No	Yes		
C462T		No	Yes		
T489C		Yes	Yes		
G3010A		No	Yes		
T4216C		Yes	Yes		LHON (secondary)
A10398G		Yes	Yes		
A11251G		Yes	Yes		
G11719A		Yes	Yes	CHR	
A12612G		Yes	Yes	CHR	
G13708A		Yes	Yes	CHR	LHON (secondary)
T14798C		No	Yes		
C15452A		Yes	Yes		
C16069T		Yes	Yes		
T16126C		No	Yes		
T16189C		No	No	CHR	
C16278T		Yes	No	CHR	
T16311C		No	No	9947A	
	C295T	Yes	Yes		
	C5633T	Yes	No		
	C7476T	Yes	No		
	G10172A	Yes	No		
	G15257A	Yes	No		LHON (intermediate)
	G15812A	Yes	No		LHON (secondary)
	C16193T	Yes	No		

[a] References: Torroni et al. (1997); and Finnilä et al. (2001).

since the linkage of HL-60 to the actual donor has been broken, there is no way to determine if this patient actually had LHON.

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LHON Mutations

P: Primary
I: Intermediate
S: Secondary

control region
12s rRNA
16s rRNA
CytB
ND6
ND5
ND4
ND4L
ND3
CO3
ATPase 6
ATPase 8
CO2
CO1
ND2
ND1

O T P E L S H R G K D S W A C Y N O L Q I M L V F

(15812)S
(15257)I?
(14484)P
(14459)P
(13708)S
(13370)P
(11778)P
(10663)P?
(9804)P?
(9438)S
(9101)P?
(7444)S
(3394)S
(3460)P
(4160)S
(4216)S
(4917)S
(5244)P

152 150 93 73 73 73 16519 16519 16519 16362 16292 16357 16311 15812 15452 15452 16278 16278 16223 16126 16069 16069 15646 15326 15326 15326 15326 15326 16193.1 16193 16189 16183

263 263 263 263 263 228 185 195 195 204 295 214 207 309.1 295 309.2 309.1 315.1 315.1 315.1 315.1 315.1 462 482 1438 1438 1438 1438 1438 489 489 750 750 750 750 750 709 709

15257 14766 14766 14766 14766 14766 14798 14569 14470 14368 14368 14368 14368 14368 14365 14365 14365 14365 14365 14272 14272 14272 14272 14272 14199 14199 14199 14199 14199

13966 13759 13708 13708 13708 13702 13702 13702 13702 13702 12705 13572 12612 12612 12612 12071 Heteroplasmy 11878 11778 11719 11719 11719 11335 11335 11335 11335 11335 11287 11251 11251

10398 10398 10172 9559 9559 9559 9559 9559 9315 8860 8860 8860 8860 8503 8448 8020 7861 7645 7476 7028 7028 7028 6849 6791 6371 6221

5633 5228 5186 4985 4985 4985 4985 4985 4769 4769 4769 4769 4769 4216 4216 4135 3423 3423 3423 3423 3423 3394 3106 3106 3106 3106 3106 3010 3010 2841 2706 2706 2706 1719