



Certificate of Analysis

Standard Reference Material[®] 2394

Heteroplasmic Mitochondrial DNA Mutation Detection Standard

This Standard Reference Material (SRM) is composed of human mitochondrial DNA mixtures which simulate different levels of heteroplasmy. SRM 2394 is intended to provide quality control benchmarks for forensic, medical, and DNA scientists to assess the detection sensitivity of low-frequency mutations, single nucleotide polymorphisms (SNPs) in either mitochondrial DNA (mtDNA) or in pooled nuclear DNA samples, or heteroplasmic sites in mtDNA. A unit of SRM 2394 consists of a single protective plastic box containing ten tubes: one tube containing the 100 % (by mass) polymorphic DNA, one tube containing the 100 % (by mass) CRS⁽¹⁾ DNA, and eight tubes containing different mass percentages of the polymorphic/CRS mtDNA mixtures. Each tube contains 25 μ L of DNA at a concentration of 8 ng/ μ L in 10 mM Tris-HCl, pH 8.5.

The DNA mixtures are constructed from the polymerase chain reaction (PCR) products from two different cell culture lines (CHR and GM09947A) that differ by one base pair (bp) at nucleotide position (np) 6371 in the 285 bp amplified region. The cell line (CHR) designated polymorphic has a T at np 6371 and the cell line (GM09947A) containing the CRS sequence has a C at that site.

Certified Values: Table 1 contains the certified mass fraction values of polymorphic/CRS mtDNA mixtures (mass % polymorphic levels are 1 %, 2.5 %, 5 %, 10 %, 20 %, 30 %, 40 %, and 50 %). The uncertainty values calculated on the 50/50 mixture are reported in section, "Uncertainty Analysis". Table 2 contains the certified sequence information of the 285 bp amplicon from the two cell lines (CHR and GM09947A) and shows the one bp difference at np 6371. The entire 16,569 bp of mtDNA from both CHR and GM09947A was sequenced for SRM 2392, and that sequence is given in reference 1, the Certificate of Analysis for SRM 2392 [2], and in NIST SP260-155 [3].

Reference Values: Table 3 contains the reference sequences of the forward and reverse primers used in the PCR amplifications of the 285 bp DNAs that were blended to construct these mixtures.

Expiration of Certification: The certification of **SRM 2394** is valid, within the measurement uncertainties specified, until **31 July 2019** provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Use"). This certification is nullified if the SRM is damaged, contaminated, or modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

Overall direction and coordination of the technical measurements leading to the certification were performed by D.K. Hancock and B.C. Levin formerly of the NIST Biotechnology Division.

The analytical determination, technical measurements, and analysis of data for the certification of this SRM were performed by D.K. Hancock, L.A. Tully, and B.C. Levin formerly of the NIST Biotechnology Division. Complete details can be found in reference 4.

Statistical consultation was provided by S.D. Leigh formerly of the NIST Statistical Engineering Division.

Michael J. Tarlov, Chief
Biomolecular Measurement Division

Gaithersburg, MD 20899
Certificate Issue Date: 09 July 2014
Certificate Revision History on Last Page

Robert L. Watters, Jr., Director
Office of Reference Materials

⁽¹⁾ CRS refers to the Cambridge Reference Sequence [5] and the revised Cambridge Reference Sequence (rCRS) [6].

Support aspects involved in the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

Permissions: The research using CHR and GM09947A DNA was deemed exempt by the NIST Institutional Review Board and the Director of the Chemical Science and Technology Laboratory. This work fits into the exemption category described in 15 CFR 27.101(b)(4) which exempts: "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."

INSTRUCTIONS FOR STORAGE AND USE

Storage: Store frozen at a temperature of $-20\text{ }^{\circ}\text{C}$. Do not store in a self-defrosting freezer because the periodic cycling of temperatures may shorten the shelf-life of this SRM.

Use: Minimize repeated freezing and thawing of these materials as this might shorten the shelf-life of the SRM. If it is necessary to perform many repeated analyses, the SRM may be thawed and the tube contents aliquoted into sterile tubes that can be kept frozen until use. Thawing can be conducted at refrigerator temperatures, room temperature, or at $37\text{ }^{\circ}\text{C}$. After thawing, briefly centrifuge the sample tube to spin down any condensate present in the tube cap, and gently mix to obtain a homogeneous solution. The tube manufacturer cautions that the sample tube caps should be tightened snugly, but not over-tightened. DNA concentrations given are nominal values and are NOT intended for use as concentration standards; only the mass concentration ratios are certified.

SOURCE AND ANALYSIS⁽²⁾

Source of Material: CHR DNA was prepared in the NIST DNA Technologies Group, Biotechnology Division. GM09947A DNA was prepared by Life Technologies, Inc. (Gaithersburg, MD). (Note: The current vendor of GM09947A is Marligen Biosciences Inc., Ijamsville, MD.) The 285 bp amplicons from CHR and GM09947A and their mixtures were prepared by the DNA Technologies Group, formerly of the NIST Biotechnology Division.

NIST Analysis: PCR was used to amplify the 285 bp area of the mtDNA from cell lines CHR and GM09947A using the primer set shown in Table 3. The PCR products were sequenced with an ABI PRISM[®] 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA). Multiple techniques were examined to identify an optimal way to determine the relative mass concentration of each amplicon. The techniques examined included ultraviolet (UV) absorbance at 254 nm, fluorescence analysis using a number of DNA dyes on different platforms, peptide nucleic acid (PNA), Denaturing Gradient Gel Electrophoresis (DGGE), Denaturing High Performance Liquid Chromatography and oligonucleotide hybridization analysis using the Luminex 100 system. Once the relative mass concentrations of the PCR products were determined, the mixtures were prepared.

⁽²⁾ Certain commercial equipment, instruments, materials, or companies are identified in this certificate to specify the experimental procedure. Such identification does not imply recommendation or endorsement by NIST, nor does it imply that the materials or equipment identified are the best available for this purpose.

Table 1. Certified Mass Fraction Values of Polymorphic/CRS mtDNA Mixtures^(a)

Component No.	Color Code	Mass Fraction A (CHR) (%)	Mass Fraction B (GM09947A) (%)
1	Red	100.0	0.0
2	White	0.0	100.0
3	Pink	50.0	50.0
4	Violet	40.0	60.0
5	Blue	30.0	70.0
6	Blue-green	20.0	80.0
7	Green	10.0	90.0
8	Yellow-green	5.0	95.0
9	Yellow	2.5	97.5
10	Orange	1.0	99.0

^(a) Ten color-coded components are included in each kit. These components are labeled with an “A” designating DNA from cell line CHR (polymorphic) and “B” designating DNA from GM09947A (CRS). Components 1 and 2 contain equal mass concentrations of CHR and GM09947A, respectively. The mixtures (components 3 through 10) were prepared by blending appropriate masses of components 1 and 2.

Uncertainty Analysis: The certified values listed in Table 1 correspond to means of the mass concentrations ratios. Each certified DNA ratio is assigned a relative expanded uncertainty about the mean, with coverage factor $k = 2$ (95 % confidence level), of 1.7 %, calculated by combining a between-method variance with a pooled within-method variance [7,8] following the ISO/JCGM Guide [9] and reference 10. Since all mixtures are prepared from the same stock solutions that were adjusted to have equal (1:1) mass concentrations, and no sequential dilutions were performed, the uncertainty reported on the certified DNA ratios is on the actual quotients of the 50/50 mixture of CHR and GM09947A DNA. A consensus mean, across all measured conditions, for such a quotient is 1.002. The 1.7 % relative error ($k = 2$) associated with the number 1.002 is ± 0.017 . The measurand is the total mass fraction for each analyte listed in Table 1. The certified values are metrological traceable to the SI unit of mass.

Table 2. Certified Sequence Information of the Two Amplicons from CHR and GM09947A^(a)

Cell Culture	Amplicon Sequence						
CHR	6242	5' -CGCATCTGC	TATAGTGGAG	GCCGGAGCAG	GAACAGGTTG	AACAGTCTAC	CCTCCCTTAG
	6301	CAGGGAACTA	CTCCCACCCT	GGAGCCTCCG	TAGACCTAAC	CATCTTCTCC	TTACACCTAG
	6361	CAGGTGTCTC	<u>T</u> TCTATCTTA	GGGGCCATCA	ATTTTCATCAC	AACAATTATC	AATATAAAAC
	6421	CCCCTGCCAT	AACCCAATAC	CAAACGCCCC	TCTTCGTCTG	ATCCGTCCTA	ATCACAGCAG
	6481	TCCTACTTCT	CCTATCTCTC	CCAGTCCTAG	CTGCTGGCAT	CACTAT-3'	6526
GM09947A	6242	5' -CGCATCTGC	TATAGTGGAG	GCCGGAGCAG	GAACAGGTTG	AACAGTCTAC	CCTCCCTTAG
	6301	CAGGGAACTA	CTCCCACCCT	GGAGCCTCCG	TAGACCTAAC	CATCTTCTCC	TTACACCTAG
	6361	CAGGTGTCTC	<u>C</u> TCTATCTTA	GGGGCCATCA	ATTTTCATCAC	AACAATTATC	AATATAAAAC
	6421	CCCCTGCCAT	AACCCAATAC	CAAACGCCCC	TCTTCGTCTG	ATCCGTCCTA	ATCACAGCAG
	6481	TCCTACTTCT	CCTATCTCTC	CCAGTCCTAG	CTGCTGGCAT	CACTAT-3'	6526

^(a) The one base pair difference is at nucleotide position 6371 and is shown in bold and underlined. This amplified region of GM09947A agrees with the Cambridge Reference Sequence [5] and the revised Cambridge Reference Sequence [6]. Amplicon sequences are given in the 5' to 3' direction (6242 to 6526).

Table 3. Primers Used to Amplify the 285 bp Amplicon Region of Interest

	Primer	Primer Sequence
Forward	6242	5' CGC ATC TGC TAT AGT GGA GG 3'
Reverse	6526	5' ATA GTG ATG CCA GCA GCT AGG 3'

Note: This amplicon is in the Cytochrome C Oxidase I gene.

REFERENCES

- [1] Levin, B.C.; Cheng, H.; Reeder, D.J.; *A Human Mitochondrial DNA Standard Reference Material for Quality Control in Forensic Identification, Medical Diagnosis and Mutation Detection*; Genomics, Vol. 55, pp. 135–46 (1999).
- [2] SRM 2392; Mitochondrial DNA Sequencing (Human); NIST; U.S. Department of Commerce: Gaithersburg, MD; available at https://www-s.nist.gov/srmors/view_detail.cfm?srm=2392 (accessed Jul 2014).
- [3] Levin, B.C.; Hancock, D.K.; Holland, K.A.; Cheng, H.; Richie, K.L.; *Human Mitochondrial DNA - Amplification and Sequencing - Standard Reference Materials - SRM 2392 and SRM 2392-I*; NIST Special Publication SP260-155; U.S. Government Printing Office: Washington, DC (2003); available at <http://www.nist.gov/srm/publications.cfm> (accessed Jul 2014).
- [4] Hancock, D.K.; Tully, L.A.; Levin, B.C.; *A Standard Reference Material to Determine the Sensitivity of Techniques for Detecting Low-Frequency Mutations, SNPs, and Heteroplasmies in Mitochondrial DNA*; Genomics; Vol. 86, pp. 446–461 (2005).
- [5] Anderson, S.; Bankier, A.T.; Barrell, B.G.; deBruijn, M.H.L.; Coulson, A.R.; Drouin, J.; Eperon, I.C.; Nierlich, D.P.; Roe, B.A.; Sanger, F.; Schreier, P.H.; Smith, A.J.H.; Staden, R.; Young, I.G.; *Sequence and Organization of the Human Mitochondrial Genome*; Nature, Vol. 290, pp. 457–465 (1981).
- [6] Andrews, R.M.; Kubacka, I.; Chinnery, P.F.; Lightowlers, R.N.; Turnbull, D.M.; Howell, N.; *Reanalysis and Revision of the Cambridge Reference Sequence for Human Mitochondrial DNA*; Nature Genetics, Vol. 23, p. 147 (1999).
- [7] Rukhin, A.L.; Vangel, M.G.; *Estimation of a Common Mean and Weighted Means Statistics*; J. Am. Statist. Assoc., Vol. 93, pp. 303–308 (1998).
- [8] Levenson, M.S.; Banks, D.L.; Eberhardt, K.R.; Gill, L.M.; Guthrie, W.F.; Lui, H.k.; Vangel, M.G.; Yen, J.H.; Zhang, N.F.; *An Approach to Combining Results from Multiple Methods Motivated by the ISO GUM*; J. Res. Natl. Inst. Stand. Technol., Vol. 105, pp. 571–579 (2000).
- [9] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement (GUM 1995 with Minor Corrections)*; Joint Committee for Guides in Metrology (JCGM) (2008); available at http://www.bipm.org/utis/common/documents/jcgm/JCGM_1_28_E.pdf (accessed Jul 2014); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at <http://www.nist.gov/pml/pubs/tn1297/index.cfm> (accessed Jul 2014).
- [10] Ellison, S.L.R.; Williams, A.; *EURACHEM/CITAC Guide CG 4: Quantifying Uncertainty in Analytical Measurement*; EURACHEM, 3rd ed.; Eurachem Secretariat: England (UK) (2012); available at <http://www.eurachem.org/> (accessed Jul 2014).

Certificate Revision History: 09 July 2014 (Extension of certification period; editorial changes); 09 June 2009 (Extension of certification period; editorial changes); 09 November 2006 (Editorial changes); 22 December 2004 (Original certificate date).
--

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at <http://www.nist.gov/srm>.