



# Certificate of Analysis

## Standard Reference Material<sup>®</sup> 2396

### Oxidative DNA Damage Mass Spectrometry Standards

This Standard Reference Material (SRM) is intended for use in the identification and quantification of oxidatively damaged DNA base components for analysis by isotope-dilution techniques using either gas chromatography/mass spectrometry (GC/MS) [1], or liquid chromatography/mass spectrometry (LC/MS) [2]. A unit of SRM 2396 is a set of 10 components, which includes eight oxidatively-modified, stable isotope-labeled DNA bases (Components 1 through 8), one stable isotope-labeled normal DNA nucleoside (Component 9), and one oxidatively-modified, stable isotope-labeled nucleoside (Component 10). Each vial of SRM 2396 contains 0.2 mL of the designated component described in further detail below.

The certified optical density of Component 10 (see Table 1) is used in the quantification of the DNA. Components 1–8 are included for identification of oxidatively damaged DNA base components by GC/MS. Components 9 and 10 are for identification of nucleosides by LC/MS. In addition, Components 9 and 10 may be used as GC/MS internal standards when modified by acidic hydrolysis. The components are labeled with the stable isotopes <sup>13</sup>C, <sup>15</sup>N, and/or <sup>2</sup>H. The names, abbreviations, structures, and information concentrations of the components are provided in Table 2.

**Certified Value:** The certified value for the optical density of Component 10, 8-OH-dGuo-<sup>15</sup>N<sub>5</sub>, at 294 nm is provided in Table 1 and is based on UV/visible absorption spectroscopy (UV/vis) measurements. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [3]. The expanded uncertainty of the optical density defines an interval within which the unknown value of optical density can be asserted to lie with a level of confidence of approximately 95 % [4].

**Information Values:** Table 2 lists information values for the amount-of-substance concentrations of the SRM 2396 components. An information value is considered a value that will be of use to the SRM user, but insufficient information is available to assess the uncertainty associated with the value. Values are provided in amount-of-substance units (mmol/L) [5].

**Expiration of Certification:** The certification of **SRM 2396** is valid, within the measurement uncertainty specified, until **01 March 2021**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see “Instructions for Storage and Use”). The certification is nullified if the SRM is damaged, contaminated, or otherwise 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.

The overall direction and coordination of the technical measurements leading to the certification were performed by M. Satterfield and M. Dizdar of the NIST Biochemical Science Division.

The analytical determination, technical measurements, and analysis of data for the certification of this SRM were performed by M. Satterfield, D. Leber, P. Jaruga, and M. Dizdar of the NIST Biochemical Science Division.

Statistical consultation for the uncertainty associated with the optical density of Component 10 was performed by S. Leigh and D. Leber of the NIST Statistical Engineering Division.

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Support aspects involved in the issuance of this SRM were coordinated through the NIST Measurement Services Division.

## INSTRUCTIONS FOR STORAGE AND USE

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

**Use:** For the certified and informational values to be applicable, once thawed each SRM component must be processed without delay. Dilutions of these materials may be made as appropriate, but they must be used immediately. Certified and informational values do not apply to any material that has been thawed and subsequently refrozen.

## PREPARATION AND ANALYSIS<sup>(1)</sup>

**Preparation:** Components 1 through 10 were purchased from Cambridge Isotope Laboratories (Andover, MA). Each component was received in solid state and stored at  $-20\text{ }^{\circ}\text{C}$  prior to preparation. Using an analytical microbalance, an appropriate amount of each compound was weighed separately and dissolved in HPLC-grade water in a volumetric flask. After filling to volume and mixing, the individual components were aliquoted (0.2 mL) into sterile microcentrifuge vials and stored at  $-20\text{ }^{\circ}\text{C}$ . Table 2 lists the names of Components 1 through 10, which are used as internal standards for the measurement of the corresponding unlabeled analogs in DNA by GC/MS [1].

**Certified Value:** The certified value for Component 10 provided in Table 1 is based on the measurement of optical density (absorbance) at 294 nm for 10  $\mu\text{L}$  of Component 10 diluted with 90  $\mu\text{L}$  of HPLC-grade water, vortex-mixed, and transferred to a cuvette (Micro Cell 8 mm high, path length 10 mm, with silica windows which transmit from 190 nm to 2500 nm), which was placed in the spectrophotometer. Standard baseline subtraction using the signal at 340 nm was used. Absorbance measurements for the certified value of Component 10, 8-OH-dGuo- $^{15}\text{N}_5$ , by UV/vis were performed on two spectrophotometers. Traceability to the International System of Units (SI) is asserted through the use of SRM 2031 Metal-on-Fused Silica Filters and independent verification using a transfer spectrophotometer.

The uncertainty is expressed as an expanded uncertainty,  $U = ku_c$ , calculated according to the methods in the ISO Guide [4]. The quantity  $u_c$  represents, at the level of one standard deviation, the potential combined effects of the uncertainty due to UV/vis equipment, variability due to Type B uncertainties including components attributable to dilution, purity, and factors influencing the absorbance measurement (temperature of the solution, mass and volumetric). The value of the coverage factor,  $k = 2.02$ , is determined from the Student's  $t$ -distribution with 41 degrees of freedom and a confidence level of 95 %.

Table 1. Certified Optical Density (294 nm) Value for Component 10

ID	Component	Optical Density
10	8-OH-dGuo- $^{15}\text{N}_5$	$0.945 \pm 0.049$

Information values for the amount-of-substance concentrations for the components (mmol/L) are shown in Table 2. The values listed are from averaging the available values determined using the techniques listed in Table 3, which include gravimetric, UV/vis, GC/MS, and LC/MS, as applicable. The wavelength and absorption coefficient used for the UV/vis measurements performed in 2010 are also listed in Table 3 for each of the components, when applicable.

**NIST Analyses:** NIST conducted the UV/vis measurements for Compounds 1 through 6, 9, and 10 in 2003 and 2010. Results were compared to assess the stability and homogeneity of the compounds. The UV/vis results from the 2010 measurements were used in the calculation of the information value for the components and are described below. GC/MS and LC/MS techniques were used to assess the concentration of the components in 2003 and are described below.

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<sup>(1)</sup> Certain commercial equipment, instruments or materials are identified in this certificate to adequately specify the experimental procedures. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Table 2. Nomenclature, Structure, and Information Values of Amount-of-Substance Concentrations of SRM 2396 Components

ID	Component	Concentration (mmol/L)	Structure <sup>(a)</sup>
1	4,6-Diamino-5-formamidopyrimidine- (formyl- <sup>13</sup> C-4,6-diamino- <sup>15</sup> N <sub>2</sub> ) Abbreviation: FapyAde- <sup>13</sup> C, <sup>15</sup> N <sub>2</sub>	0.23	
2	2,6-Diamino-4-hydroxy-5-formamidopyrimidine- (formyl- <sup>13</sup> C-5-amido- <sup>15</sup> N-6-amino- <sup>15</sup> N) Abbreviation: FapyGua- <sup>13</sup> C, <sup>15</sup> N <sub>2</sub>	0.17	
3	8-Hydroxyadenine-8- <sup>13</sup> C-9- <sup>15</sup> N-(6-amino- <sup>15</sup> N) Abbreviation: 8-OH-Ade- <sup>13</sup> C, <sup>15</sup> N <sub>2</sub>	0.15	
4	5-Hydroxycytosine-2- <sup>13</sup> C-1,3- <sup>15</sup> N <sub>2</sub> Abbreviation: 5-OH-Cyt- <sup>13</sup> C, <sup>15</sup> N <sub>2</sub>	0.23	
5	5-Hydroxyuracil-2,4,5,6- <sup>13</sup> C-1,3- <sup>15</sup> N <sub>2</sub> Abbreviation: 5-OH-Ura- <sup>13</sup> C <sub>4</sub> , <sup>15</sup> N <sub>2</sub>	0.14	
6	5-(Hydroxymethyl)uracil-4,5- <sup>13</sup> C-2-α,α- <sup>2</sup> H <sub>2</sub> Abbreviation: 5-(OHMe)Ura- <sup>13</sup> C <sub>2</sub> , d <sub>2</sub>	0.10	
7	<i>cis</i> -Thymine glycol-α,α,α,6- <sup>2</sup> H <sub>4</sub> Abbreviation: ThyGly-d <sub>4</sub>	0.10	
8	5-Hydroxy-5-methylhydantoin-2- <sup>13</sup> C-1,3- <sup>15</sup> N <sub>2</sub> Abbreviation: 5-OH-5-MeHyd- <sup>13</sup> C, <sup>15</sup> N <sub>2</sub>	0.10	
9	2'-Deoxyguanosine- <sup>15</sup> N <sub>5</sub> Abbreviation: dGuo- <sup>15</sup> N <sub>5</sub>	0.13	
10	7,8-Dihydro-8-oxo-2'-deoxyguanosine- <sup>15</sup> N <sub>5</sub> or 8-hydroxy-2'-deoxyguanosine- <sup>15</sup> N <sub>5</sub> Abbreviation: 8-OH-dGuo- <sup>15</sup> N <sub>5</sub>	0.093	

<sup>(a)</sup> Isotope-labeled atoms are indicated by an \* in the structure.

**UV/vis:** For each measurement, an aliquot of 10  $\mu\text{L}$  of the solution was taken and diluted with 90  $\mu\text{L}$  of HPLC-grade water. The tube was vortex-mixed and the solution was transferred into the cuvette (Micro Cell 8 mm high, path length 10 mm, with silica windows, which transmit from 190 nm to 2500 nm), which was placed in the spectrophotometer. After being allowed to equilibrate for 30 seconds the absorbance was recorded at the wavelength maxima shown in Table 3. Measurements were made over three successive days, with four measurements made each day for a total of 12 measurements of each component. Baseline subtraction using the signal at 340 nm for each component was used.

**GC/MS:** The concentrations of Components 1 through 8 were measured by GC/MS with electron-impact (EI) ionization. Non-labeled versions of the components were used as internal standards and were custom synthesized by Program Resources Inc. of Dyncorp, contracted by National Cancer Institute (Frederick, MD) [6], except for Component 1 and Component 6, which were purchased from Sigma Chemical Company (St. Louis, MO). The column was a fused silica capillary column (12.5 m  $\times$  0.2 mm i.d.) coated with cross-linked 5 % phenylmethylsilicone (gum phase, film thickness 0.33  $\mu\text{m}$ ). Aliquots of labeled and non-labeled compounds were mixed and lyophilized in glass vials to dryness for 18 h. For derivatization, 60  $\mu\text{L}$  of a mixture of nitrogen-bubbled bis(trimethylsilyl)trifluoroacetic acid (BSTFA), containing 1 % trimethylchlorosilane and pyridine (1:1, volume fractions) were added to the dried samples. The vials were purged individually with ultra high-purity nitrogen, tightly sealed under nitrogen with Teflon-coated septa, and then vortex-mixed. The derivatization was carried out at 120  $^{\circ}\text{C}$  for 30 minutes. Aliquots of 2  $\mu\text{L}$  of the derivatized samples were injected onto the GC column by means of an automatic sampler (split mode) with a split ratio of 10 to 1. The selected-ion monitoring mode was used to monitor the characteristic ions of the trimethylsilyl (TMS) derivatives of Components 1 through 8 in their EI mass spectra [1,7-10]. Table 4 shows the masses of the characteristic ions of the TMS derivatives of the Components 1 through 10 and their unlabeled analogs that can be used for identification and quantification.

The mass ions of the TMS derivatives for Components 9 and 10 are included for use in quantification. Component 9 quantitatively yields Gua- $^{15}\text{N}_5$  upon acidic hydrolysis and is used as an internal standard for the measurement of guanine in DNA by GC/MS, thus the amount of DNA [11]. Component 10 is used as an internal standard for the measurement of 8-OH-dGuo in DNA by LC/MS after enzymatic hydrolysis of DNA [12], and for the measurement of 8-OH-Gua in DNA by GC/MS after acidic hydrolysis of DNA [13].

**LC/MS:** The concentrations of the Components 9 and 10 were determined by LC/MS with the atmospheric pressure ionization-electrospray (API-ES) process in the positive ionization mode [12] using corresponding non-labeled analogs as internal standards. Separations were performed using a Zorbax Eclipse XDB C18 reversed-phase column (15 cm  $\times$  0.21 cm i.d., 5  $\mu\text{m}$  particle size) with a guard column packed with the same stationary phase (1 cm  $\times$  0.21 cm i.d.). The mobile phase used was a gradient of 0.5 % of solvent B per minute, where solvent A was a mixture of water and acetonitrile (98 %:2 %, volume fractions) and the solvent B was acetonitrile. The flow rate was 0.2 mL/min. The column temperature was held at 30  $^{\circ}\text{C}$ . Selected-ion monitoring mode was used to monitor the characteristic ions of the API-ES mass spectra of Components 9 and 10. These mass spectra consist of a protonated molecular ion ( $\text{MH}^+$ ), a base ion with two H atoms ( $\text{BH}_2^+$ ), and a sodium adduct ion ( $\text{MNa}^+$ ) and are listed in Table 5 [12,14].

Table 3. Molecular Weights, Wavelength, Absorption Coefficients, and Techniques Used to Determine the Concentration of SRM 2396 Components

ID	Component	Molecular Weight	Techniques Used	Wavelength (nm)	Absorption Coefficient ( $\text{L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ )
1	FapyAde- $^{13}\text{C}$ , $^{15}\text{N}_2$	156	Gravimetric, UV/vis, GC/MS	262	4710 [15]
2	FapyGua- $^{13}\text{C}$ , $^{15}\text{N}_2$	172	Gravimetric, UV/vis, GC/MS	266	12882 [15]
3	8-OH-Ade- $^{13}\text{C}$ , $^{15}\text{N}_2$	154	Gravimetric, UV/vis, GC/MS	268	12200 [16]
4	5-OH-Cyt- $^{13}\text{C}$ , $^{15}\text{N}_2$	130	Gravimetric, UV/vis, GC/MS	284	5300 <sup>(a)</sup>
5	5-OH-Ura- $^{13}\text{C}_4$ , $^{15}\text{N}_2$	134	Gravimetric, UV/vis, GC/MS	278	6400 [17]
6	5-(OHMe)Ura- $^{13}\text{C}_2$ , $\text{d}_2$	146	Gravimetric, UV/vis, GC/MS	261	8100 [17]
7	ThyGly- $\text{d}_4$	164	Gravimetric, GC/MS	n/a	n/a
8	5-OH-5-MeHyd- $^{13}\text{C}$ , $^{15}\text{N}_2$	133	Gravimetric, GC/MS	n/a	n/a
9	dGuo- $^{15}\text{N}_5$	272	Gravimetric, UV/vis, LC/MS	254	13000 [17]
10	8-OH-dGuo- $^{15}\text{N}_5$	288	Gravimetric, UV/vis, LC/MS	245 294	12300 [18] <sup>(b)</sup> 10300 [19]

<sup>(a)</sup> For component 4, the average of NIST-measured absorption coefficient values of the labeled component ( $5330 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ ) and unlabeled component ( $5260 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ ) was used to calculate the concentration.

<sup>(b)</sup> There are two absorption maxima for Component 10. The concentration for Component 10 was determined using the absorption coefficient at 294 nm.

Table 4. Ion Masses of GC/MS TMS Derivatives of Components 1 Through 10 and the Unlabeled Analogs (in Parentheses)

ID	Component	$\text{M}^{+\bullet}$ (Da)	$[\text{M}-1]^+$ (Da)	$[\text{M}-15]^+$ (Da)	Fragment Ion (Da)
1	FapyAde- $^{13}\text{C}$ , $^{15}\text{N}_2$	372 (369)	371 (368)	357 (354)	283 (280)
2	FapyGua- $^{13}\text{C}$ , $^{15}\text{N}_2$	460 (457)	-	445 (442)	371 (368)
3	8-OH-Ade- $^{13}\text{C}$ , $^{15}\text{N}_2$	370 (367)	-	355 (352)	-
4	5-OH-Cyt- $^{13}\text{C}$ , $^{15}\text{N}_2$	346 (343)	345 (342)	331 (328)	-
5	5-OH-Ura- $^{13}\text{C}_4$ , $^{15}\text{N}_2$	350 (344)	349 (343)	335 (329)	-
6	5-(OHMe)Ura- $^{13}\text{C}_2$ , $\text{d}_2$	362 (358)	-	347 (343)	-
7	ThyGly- $\text{d}_4$	452 (448)	-	437 (433)	262 (259)
8	5-OH-5-MeHyd- $^{13}\text{C}$ , $^{15}\text{N}_2$	349 (346)	348 (345)	334 (331)	219 (216)
9	dGuo- $^{15}\text{N}_5$ <sup>(a)</sup>	372 (367)	-	357 (352)	-
10	8-OH-dGuo- $^{15}\text{N}_5$ <sup>(b)</sup>	460 (455)	-	445 (440)	-

<sup>(a)</sup> Used for the measurement of Gua- $^{15}\text{N}_5$  after acidic hydrolysis of DNA samples to determine the amount of DNA [11]. The masses of the  $\text{M}^{+\bullet}$  and  $[\text{M}-15]^+$  ions given here are for Gua- $^{15}\text{N}_5$ .

<sup>(b)</sup> Used for the measurement of 8-OH-Gua- $^{15}\text{N}_5$  to determine the amount of 8-OH-Gua in DNA after acidic hydrolysis [12]. The masses of the  $\text{M}^{+\bullet}$  and  $[\text{M}-15]^+$  ions given here are for 8-OH-Gua- $^{15}\text{N}_5$ .

Table 5. LC/MS Ion Masses of Components 9 and 10 and the Unlabeled Analogs (in Parentheses)

ID	Component	$\text{MH}^+$ (Da)	$\text{BH}_2^+$ (Da)	$\text{MNa}^+$ (Da)
9	dGuo- $^{15}\text{N}_5$	273 (268)	157 (152)	295 (290)
10	8-OH-dGuo- $^{15}\text{N}_5$	289 (284)	173 (168)	311 (306)

## REFERENCES

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**Certificate Revision History:** 10 April 2012 (Certified optical density value for Component 10 added, concentration value assignments updated and changed from certified to information values, expiration date updated; editorial changes); 14 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](mailto:srminfo@nist.gov); or via the Internet at <http://www.nist.gov/srm>.*