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Reference Material 8671

NISTmAb, Humanized IgG1κ Monoclonal Antibody

Lot 14HB-D-001

REFERENCE MATERIAL INFORMATION SHEET

Purpose: This reference material (RM) is intended primarily for use in evaluating the performance of methods for determining physicochemical and biophysical attributes of monoclonal antibodies. It also provides a representative test molecule for development of novel technologies for therapeutic protein characterization. This RM can be used for a variety of purposes that may include system suitability tests, establishing method or instrument performance and variability, comparing changing analytical test methods, and assisting in method qualification.

Description: A unit of RM 8671 consists of one internal-threaded polypropylene cryovial containing 800 µL of 10 mg/mL NIST IgG1κ monoclonal antibody (NISTmAb) in 12.5 mmol/L L-histidine, 12.5 mmol/L L-histidine HCl (pH 6.0).

The NIST monoclonal antibody (NISTmAb) is a recombinant humanized IgG1κ expressed in murine suspension culture and has undergone biopharmaceutical industry standard upstream and downstream purification to remove process related impurities. It is an ~150 kDa homodimer of two light chain and two heavy chain subunits linked through both inter- and intra-chain disulfide bonds. The molecule has a high abundance of N terminal pyroglutamination, C terminal lysine clipping, and glycosylation of the heavy chains. The protein also has low abundance post translational modifications including methionine oxidation, deamidation, and glycation [1–4]. These and other product quality attributes were extensively characterized in the ACS Symposium Series for the initial batch of interim material, used as the primary sample (PS) herein [5–7].

Period of Validity: The non-certified values are valid within the measurement uncertainty specified until **29 April 2026**. The value assignments are nullified if the material is stored or used improperly, damaged, contaminated, or otherwise modified.

Maintenance of Non-Certified Values: NIST will monitor this material to the end of its period of validity. If substantive technical changes occur that affect the non-certified values during this period, NIST will update this Reference Material Information Sheet and notify registered users. RM users can register online from a link available on the NIST SRM website or fill out the user registration form that is supplied with the RM. Registration will facilitate notification. Before making use of any of the values delivered by this material, users should verify they have the most recent version of this documentation, available through the NIST SRM website (https://www.nist.gov/srm).

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Non-Certified Values: NIST non-certified values are best estimates based on currently available information and were referred to as "Reference Values" or "Informational Values" from 1987 until July 2020 [8] . Non-certified values are suitable for use in method development, method harmonization, and process control, but do not provide metrological traceability to the International System of Units (SI) or other higher-order reference system. Non-certified values of RM 8671 are provided in the Tables 1 through 5. A thorough expansion on the specific protocols and results described in this RMIS can be found in references [9–13].

Mass Concentration Non-Certified Value: The mass concentration value listed in Table 1 is based on measured decadic attenuance, *D*, at 280 nm. The decadic attenuance, *D*, is computed as the negative logarithm (base 10) of the transmittance, and is analogous to absorbance except for the inclusion of scattering and luminescence effects upon the radiant power exiting the sample [14]. Concentration of the NISTmAb was determined by using a theoretical extinction coefficient (ε) of 1.42 mL · mg^{-1} · cm^{-1} [15,16]. This value was calculated according to the method reported by [16] and further corrected for a glycan mass fraction correction factor of 0.977 [15]. The details of the analytical method and contributions to the uncertainty associated with this mass concentration measurement can be found in reference [9].

Table 1. Mass Concentration for RM 8671 Lot 14HB-D-001 by UV-Vis Spectrophotometry (n = 10 vials).

Average Concentration (mg/mL)	Standard Type A Uncertainty, u_A (mg/mL)	Combined Standard Uncertainty, u_c (mg/mL)	Expanded Uncertainty, U (mg/mL)	Coverage Factor, k
10.004	0.0091	0.0409	0.0818	2

Physicochemical Non-Certified Values: Physicochemical attributes of RM 8671 were measured using qualified size exclusion chromatography (SEC), capillary sodium dodecyl sulfate electrophoresis (CE-SDS), capillary zone electrophoresis (CZE), and dynamic light scattering (DLS) methods [10,11]. These analytical assays were qualified using the primary sample (PS) in order to establish method performance criteria. Qualification exercises were modeled after the ICH Q2(R1) guidelines for method validation and included assessment of linearity, limit of detection (LOD)/limit of quantification (LOQ), range, and precision (repeatability and some intermediate precision). Physicochemical values provided in Tables 2, 3, 4, and 5 were assigned by analyzing Lot 14HB-D-001 with each qualified method (SEC, CE-SDS, DLS, and CZE) while bracketing each analysis with an instrument qualification (IQ) standard and the PS to ensure system suitability. Both the IQ standard and PS were required to pass pre-defined method performance criteria derived from method qualification. The details of the analytical methods and contributions to the uncertainty associated with the measurements can be found in references [9-11]. A thorough expansion on the specific protocols and results for physicochemical measurements can be found in references [9-13].

Table 2. SEC Size Heterogeneity for RM 8671 Lot 14HB-D-001

	Size Heterogeneity (%)	Combined Standard Uncertainty, u_c (%)	Expanded Uncertainty, U (%)	Coverage Factor, k
Monomeric Purity	96.74	0.14	0.41	3
High Molecular Weight	3.06	0.14	0.40	3
Low Molecular Weight	0.20	0.009	0.03	3

Table 3. CE-SDS Size Heterogeneity for RM 8671 Lot 14HB-D-001

	Size Heterogeneity (%)	Combined Standard Uncertainty, u_c (%)	Expanded Uncertainty, U (%)	Coverage Factor, k
Monomeric Purity[(a)]	98.49	0.73	2.19	3
Thioether[(b)]	0.31	0.03	0.09	3
Glycan Occupancy[(b)]	99.38	0.07	0.23	3

(a) Measured using non-reduced CE-SDS (nrCE-SDS)
(b) Measured using reduced CE-SDS (rCE-SDS)

Table 4. DLS Average Hydrodynamic Diameter for RM 8671 Lot 14HB-D-001

Average Hydrodynamic Diameter (nm)	Combined Standard Uncertainty, u_c (nm)	Expanded Uncertainty, U (nm)	Coverage Factor, k
9.92	0.54	1.62	3

Table 5. CZE Charge Heterogeneity for RM 8671 Lot 14HB-D-001

	Charge Heterogeneity (%)	Combined Standard Uncertainty u_c (%)	Expanded Uncertainty, U (%)	Coverage Factor, k
Charge Purity	73.81	0.83	2.50	3
Acidic Variants	16.52	0.48	1.44	3
Basic Variants	9.67	0.63	1.89	3

Additional Information: Additional physicochemical characterization methods including flow imaging analysis and a peptide mapping identity test were also applied to RM 8671 and are reported in Appendix A.

Safety: RM 8671 IS INTENDED FOR RESEARCH USE. RM 8671 IS NOT INTENDED FOR ANIMAL OR HUMAN CONSUMPTION, CLINICAL TESTING, OR THERAPEUTIC USE. As a general rule, personal protective equipment should always be worn during any laboratory procedure. This includes, but is not limited to, safety goggles, gloves, and a laboratory jacket.

Storage: The RM is shipped on dry ice and should remain frozen during shipment. The material should be stored in a frozen state at −80 °C immediately upon receipt. A series of stability samples were evaluated to determine the optimal and most extreme storage conditions under which the sample will likely retain its physicochemical performance for a given method [9]. In all cases, storage of the material at −80 °C is preferred. If aliquot preparation and/or storage at other than −80 °C is necessary, the maximum storage time recommended under given conditions and for certain intended uses is listed in Table 6.

Table 6. Method-Based Alternate Storage Conditions for RM 8671 Values and Associated Control Range[a]

Method	Attribute	Recommended Storage	Max F/T[b] −80 °C (cycles)	Max Storage 4 °C (days)	Control Range
UV	Concentration	−80 °C	5	28	± 2u_c
SEC	Monomeric Purity	−80 °C	5	7	± 3u_c
nrCE-SDS	Monomeric Purity	−80 °C	5	28	± 3u_c
rCE-SDS	Glycan Occupancy, Thioether Content	−80 °C	5	28	± 3u_c
DLS	Hydrodynamic Diameter	−80 °C	5	28	± 3u_c
CZE	Charge Purity	−80 °C	5	28	± 3u_c

[a] Measured values are expected to be within the indicated control range, where u_c is the combined standard uncertainty, based on the alternate storage conditions listed for each individual method.
[b] Freeze/Thaw cycles (F/T)

Instructions for Handling and Use: The vial should be removed from the −80 °C freezer and thawed at room temperature for approximately 30 minutes or until no residual ice crystals remain. Once thawed, the vial should be gently inverted five times to alleviate any concentration gradients that may have formed during the freezing process. The vial should then be quickly spun in a mini-centrifuge to settle any solution that may otherwise remain adhered to the lid or internal threads of the vial. Once opened, the vial contents should be used immediately and/or stored at the pre-defined conditions listed in Table 6 according to the intended use of the material.

Protein solutions of lower concentration may be prepared by transferring an aliquot and diluting to the appropriate volume. Diluents are not furnished with the RM; however, aqueous diluents such as the formulation buffer (12.5 mmol/L L-histidine, 12.5 mmol/L L-histidine HCl, pH 6.0) may be used. Solubility and stability of the material have not been fully tested by NIST under dilute or concentrated conditions and therefore may not conform to expectation in the analytical assays described herein.

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Certain commercial equipment, instruments, or materials may be identified in this Reference Material Information Sheet to adequately specify the experimental procedure. 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.

Users of this RM should ensure that the Reference Material Information Sheet in their possession is current. This can be accomplished by contacting the Office of Reference Materials 100 Bureau Drive, Stop 2300, Gaithersburg, MD 20899-2300; telephone (301) 975-2200; e-mail srminfo@nist.gov; or the Internet at https://www.nist.gov/srm.

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APPENDIX A

Additional Physicochemical Non-Certified Value. Flow imaging analysis was also applied to RM 8671 as an additional physicochemical characterization method and directly compared to data collected for the PS. The details of the analytical method can be found in references [9,11].

Table A1. Subvisible Particle Concentration for RM 8671 Lot 14HB-D-001

Subvisible particle concentration with equivalent diameter greater than 2 μm, $N(d \geq 2\ \mu m)$: 4877 mL^{-1}

Identity: Ultrahigh-performance liquid chromatography coupled with online UV and tandem mass spectrometry detection (UHPLC-UV-MS/MS) was also applied to this RM. A parallel sample preparation of PS was directly compared to the RM with respect to visual appearance and peak retention times of the total ion chromatogram (TIC) and the UV peptide map at 214 nm. Collision induced dissociation MS/MS peak identifications corresponding to peptides derived from the putative NISTmAb sequence were used to confirm identity and assure conformance with the PS. The details of the analytical method can be found in references [9,13]. The NISTmAb primary amino acid sequence is shown Figure A1.

RM 8671 Heavy Chain Amino Acid Sequence

QVTLRESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIRQPPGKALEWLADIWWDDKKHYNPSLKDRLTIS
KDTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSS ASTKGPSVFPLAPSSKSTSGGTA
ALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVD
KRV *EPKSCDKTHTCPPCP* **APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPGK**

RM 8671 Light Chain Amino Acid Sequence

DIQMTQSPSTLSASVGDRVTITCSASSRVGYMHWYQQKPGKAPKLLIYDTSKLASGVPSRFSGSGSGTEFTLT
ISSLQPDDFATYYCFQGSGYPFTFGGGTKVEIK RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Figure A1. Primary amino acid sequence for RM 8671 with variable fragment antigen-binding (Fab) region in normal font, constant Fab region underlined, hinge region in italics, and Fc region in bold.

Storage for Additional Characterization Methods: The RM is shipped on dry ice and should remain frozen during shipment. The material should be stored in a frozen state at −80 °C immediately upon receipt. In all cases, storage of the material at −80 °C is preferred. If aliquot preparation and/or storage at other than −80 °C is necessary, the maximum storage time recommended under given conditions and for certain intended uses is listed in Table A2.

Table A2. Method-Based Alternate Storage Conditions for RM 8671 Additional Characterization Methods.

Method	Attribute	Recommended Storage	Max F/T[(a)] −80 °C (cycles)	Max Storage 4 °C (days)
FI[(b)]	Subvisible Particle Content	−80 °C	0	28
Peptide Map	Identity	−80 °C	ND[(c)]	ND[(c)]

[(a)] Freeze/Thaw cycles (F/T)
[(b)] Flow imaging analysis (FI)
[(c)] Not determined

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APPENDIX B

Source and Preparation: This RM was received as a bulk substance prepared using mammalian cell culture and downstream processing representative of industry state-of-the-art methods. Multiple bulk substance containers were first homogenized to form the 14HB batch. Aliquots of 1 L each were made from the homogenized bulk and designated as individual lots. A single lot (14HB-001) was then diluted 10-fold in USP grade formulation buffer (12.5 mmol/L L-histidine, 12.5 mmol/L L-histidine HCl, pH 6.0) and 800 µL aliquots placed into internally-threaded polypropylene screw top vials. Vials were placed in racks of 96 units each for storage at –80 °C. Sample processing was completed in a sterile environment using pre-sterilized single-use equipment and/or in a class 100 000 cleanroom environment. Material source, preparation, and lifecycle plan is documented in reference [12].

Scientific Contributors: Overall direction and coordination of technical measurements were performed by K. Yandrofski and J.E. Schiel of the NIST Biomolecular Measurement Division. Value assignment measurements were performed at NIST (Gaithersburg, MD) or the Institute of Bioscience and Biotechnology Research (Rockville, MD) by J.E. Schiel, K. Yandrofski, S. Telikepalli, T. Mouchahoir, of the NIST Biomolecular Measurement Division, P. DeRose of the NIST Biosystems and Biomaterials Division and A.H. Turner and J. King, formerly of NIST. Acquisition of the material was performed by J.E. Schiel and M. Tarlov of the NIST Biomolecular Measurement Division and H. Zube. Additional technical guidance was provided by B. Lang and A. Urbas of the NIST Chemical Sciences Division, and J. Travis of the NIST Operations and Strategic Programs Division. Division Management support was provided by M. Tarlov, K. Phinney, J. Marino, and D. Ripple of the NIST Biomolecular Measurement Division, and K. Cole of the NIST Biosystems and Biomaterials Division.

Statistical analysis was provided by A. Heckert of the NIST Statistical Engineering Division.

Support aspects involved with the issuance of this RM were coordinated through the NIST Office of Reference Materials.

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