Date of Shipment : Xxxxxx XX. 20XX 6208a00-210225-230126

National Institute of Advanced Industrial Science and Technology

National Metrology Institute of Japan

Reference Material Report

NMIJ RM 6208-a
No. +++

Monoclonal Antibody Solution, AIST-MAB

This reference material (RM, Monoclonal antibody solution, AIST-MAB) is produced in accordance with the NMIJ's management system and in compliance with ISO 17034 and ISO/IEC 17025. This RM is a recombinant monoclonal antibody (humanized IgG1κ) phosphate buffer solution produced from Chinese hamster ovary-derived cell line. This RM is intended for use in the validation of analytical procedures and analytical instruments for antibody quantification. It is also intended for use in the analytical method development, its evaluation in characterization, and the internal and external quality control of the analyses.

Indicative Value

The indicative value of this RM is given in the table below. It is mass concentration of heterotetrameric-structure monoclonal antibody (humanized IgG1κ) with *N*-linked glycan at 20 °C. The uncertainty of the indicative value is the expanded uncertainty obtained by multiplying the combined standard uncertainty by a coverage factor (k) of 2, and it is the half-width of an interval of confidence estimated to have a level of confidence of approximately 95 %. The amino acid sequence is shown in "Technical Information" 1), the glycan structures are shown in 2), distribution of charge variants is shown in 3), and contents of aggregates and truncated forms are shown in 4).

Substance	Indicative value Mass concentration (g/L)	Expanded uncertainty Mass concentration (g/L)
Monoclonal antibody (humanized IgG1κ)	5.00	0.19

Analysis

The indicative value was determined by applying the amino acid sequence shown in "Technical Information 1)", the molecular weight (148056) obtained from the most abundant glycan structure of glycosylation shown in 2), and the density shown in 5) to the results of amino acid analyses using isotope-dilution mass spectrometry. Amino acid analyses were conducted after spiking stable isotope-labeled amino acids to sample solutions. The following two methods were used for the amino acid analyses:

1) Microwave-assisted liquid-phase hydrolysis followed by reversed-phase chromatography utilizing the pre-column derivatization method/tandem mass spectrometry:

The liquid-phase hydrolysis was performed by using hydrochloric acid for three hours while being heated by microwave to 160 °C. Then the hydrolyzed amino acids, namely aspartic acid, glutamic acid, proline, valine, isoleucine, leucine, phenylalanine, and alanine, were quantified by using *N*-butylnicotinic acid succinimide ester as a pre-column derivatization reagent.

2) Gas-phase hydrolysis followed by hydrophilic interaction chromatography/tandem mass spectrometry:

Gas-phase hydrolysis was performed by using hydrochloric acid at 150 °C for 48 hours. Then, without derivatization, hydrolyzed amino acids, namely aspartic acid, glutamic acid, proline, valine, isoleucine, leucine, phenylalanine, and alanine, were quantified.

Metrological Traceability

The indicative value is determined by amino acid analysis based on isotope-dilution mass spectrometry using the standard solution prepared with the following materials: L-aspartic acid (NMIJ CRM 6027-a), L-glutamic acid (NMIJ CRM 6026-a), L-proline (NMIJ CRM 6016-a), L-valine (NMIJ CRM 6015-a), L-isoleucine (NMIJ CRM 6013-a), L-leucine (NMIJ CRM 6012-a), L-phenylalanine (NMIJ CRM 6014-a), and L-alanine (NMIJ CRM 6011-a). The density was determined by a vibration-type density meter calibrated by the JCSS density standard solution.

Expiration of Report

This report is valid for one year from the date of shipment, provided that this RM is stored in accordance with the instructions given in this report.

Description of the Material

This RM is a monoclonal antibody solution dissolved in 10 mmol/L potassium phosphate buffer (pH 7.0) and is in the form of colorless and clear liquid. Approximately 1 mL of this RM was dispensed in a polypropylene vial, and the vial was sealed in an aluminum-laminated plastic bag.

Homogeneity

Homogeneity of this RM was evaluated by using the relative area percentage of the main peak obtained in cation exchange chromatography. The uncertainty derived from homogeneity has been incorporated in the expanded uncertainty of the indicative value. Homogeneity was also found sufficient in the evaluation using the relative area percentage of the monomer peak in size exclusion chromatography and that using absorbance at 280 nm.

Instructions for Storage

This RM should be kept under –80 °C and protected from light.

Instructions for Use

Prior to use, the bag should be left at room temperature for approximately 30 minutes. The vial should be gently inverted for five times to homogenize the RM. If the RM is stored at room temperature, it should be used up within 24 hours after it is melted. Up to five times of a freeze-thaw cycle does not affect the indicative value, the relative area percentage of the main peak in cation exchange chromatography, the relative area percentage of the monomer peak in size exclusion chromatography, or absorbance at 280 nm.

Precautions for Handling

This RM is for *in vitro* research use only. Refer to the safety data sheet (SDS) on this RM before use.

Preparation

This RM was expressed and purified by using Chinese hamster ovary-derived cell line in Manufacturing Technology Association of Biologics. The culture supernatant of antibody-producing cell lines in serum-free culture medium was purified by chromatographic techniques including protein A affinity, anion-exchange, and cation-exchange. The purified material was treated with virus removal filter, concentration through ultrafiltration, and buffer exchange in a facility compliant with Good Manufacturing Practice (GMP).

Technical Information

The detailed information is given for the analytical techniques used to calculate the indicative value (1-5) and extinction coefficient (6). These represent the measurement results at the time of the commencing the distribution of this RM.

1) This RM has heterotetrameric structure consisting of two light chains and two heavy chains, featuring two-molecule *N*-linked glycan. The amino acid sequences of the light and heavy chains were designed as follows. Peptide mapping of this reference material by trypsin, lysylendopeptidase and Glu-C digestion identified peptides covering the entire sequence of the light and heavy chains shown below.

Light chain

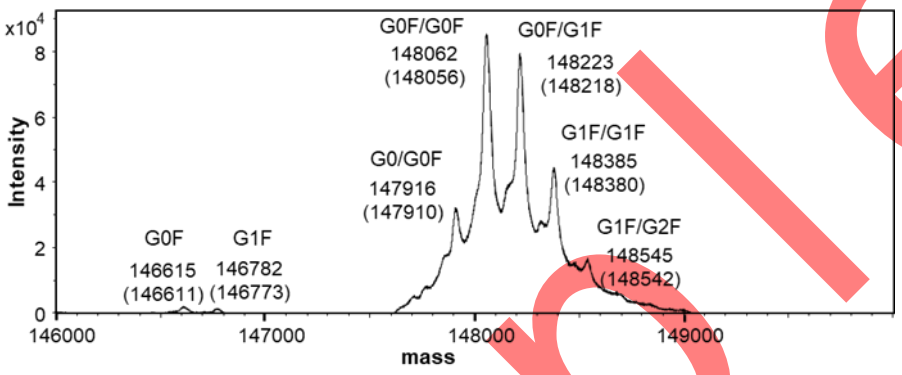
```
1 DIQMTQSPSS LSASVGDRVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYS ASFLYSGVPS RFSGSRSGTD
```

```
 71 FTLTISSLQP EDFATYYCQQ HYTTPPTFGQ GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY
141 PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN
211 RGEC
```

Heavy chain

```
  1 EVQLVESGGG LVQPGGSLRL SCAASGFNIK DTYIHWVRQA PGKGLEWVAR IYPTNGYTRY ADSVKGRFTI
 71 SADTSKNTAY LQMNSLRAED TAVYYCSRWG GDGFYAMDYW GQGTLVTVSS ASTKGPSVFP LAPSSKSTSG
141 GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS
211 NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW
281 YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ
351 VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
421 QQGNVFSCSV MHEALHNHYT QKSLSLSPG
```

2) Mass spectrum of this RM measured by high-resolution mass spectrometer (Intact mass spectrometry) is shown below.



This RM of 3 μL was measured by LC-MS and the deconvoluted mass spectra of multi-charged ions was obtained. This RM is glycoprotein having two molecules of *N*-linked glycan in a molecule. The figure above shows the measured masses and the assignment of the glycan structure with the molecular weights in parentheses. The most common structure was the glycosylated form modified with two G0F glycans (molecular weight: 148056).

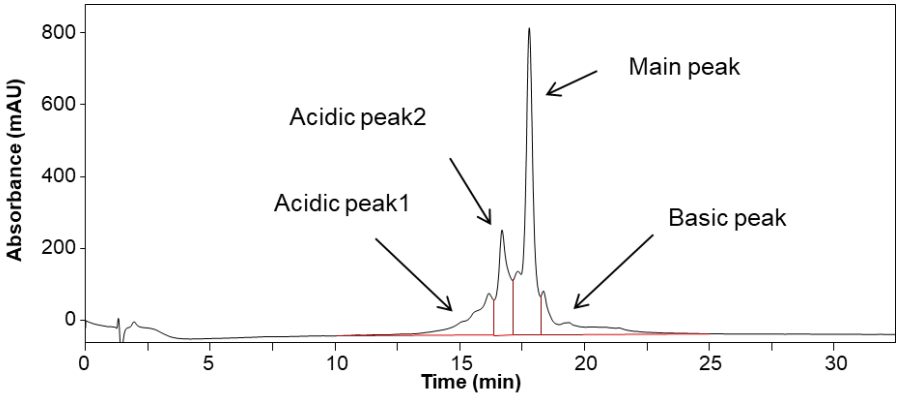
* Structure of G0F: GlcNAcβ(1-2)Manα(1-3)[GlcNAcβ(1-2)Manα(1-6)]Manβ(1-4)GlcNAcβ(1-4)[Fucα(1-6)]GlcNAc

GlcNAc: *N*-Acetylglucosamine, Man: mannose, Fuc: fucose

3) Distribution of charged variants of this RM was evaluated by high-performance liquid chromatograph-ultraviolet detector (LC-UV) using cation exchange chromatography column. The chromatogram was shown below. The chromatogram indicates four primary charged variants, namely a main peak, two associated acidic peaks (1, 2), and a basic peak. The ratio of the changed isomers is expressed as the relative area a percentage of the peaks.

Acidic peak1: (17.5 ± 0.1) %, Acidic peak 2: (18.4 ± 0.1) %, Main peak: (47.8 ± 0.2) %, Basic peak: (16.4 ± 0.1) %

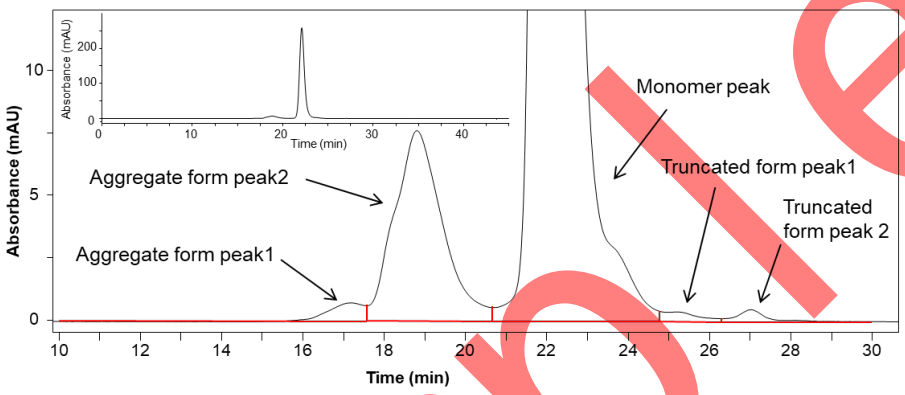
(The value to follow the symbol ± represents the standard deviation of measurement.)



The cation exchange chromatography column used in this analysis was BioPro IEX SF (YMC, 4.6 mm in internal diameter; 100 mm in length; 5 µm in particle size). This RM of 5 µL was injected into the LC-UV system and eluted with a flow rate of 0.5 mL/minute in a linear gradient (10-80 %B) for 30 minutes [Mobile Phase A: 20 mmol/L 2-(*N*-morpholino) ethanesulfonic acid (MES) (pH 6.0), Mobile Phase B: 20 mmol/L MES, 200 mM NaCl (pH 6.0)]. Absorbance was detected at wavelength of 215 nm.

4) Contents of aggregates and truncated forms of this RM were evaluated by LC-UV using size exclusion chromatography. The chromatogram is shown below. The ratio of the aggregates and truncated forms is expressed as the relative area percentage of the peaks.

Aggregate Peak1: (0.42 ± 0.02) %, Aggregate Peak2: (5.72 ± 0.14) %, Monomer Peak: (93.7 ± 0.2) %, Truncated Form Peak1 and Truncated Form Peak2: (0.16 ± 0.01) % (The value following the symbol ± represents the standard deviation of measurement.)



The size exclusion chromatography column used in this analysis was TSK gel G3000SW$_{XL}$ (TOSOH Corp., 7.8 mm in internal diameter; 300 mm in length; 5 µm in particle size). This RM of 10 µL was injected into the LC-UV system and eluted with a flow rate of 0.4 mL/minute by using mobile phase [100 mmol/L sodium phosphate buffer containing 100 mmol/L Na_2SO_4 (pH6.8)]. Absorbance was detected at wavelength of 280 nm.

5) The density of this RM measured by a vibration-type density meter is 1.0027 g/cm^3 at 4 °C and 1.0008 g/cm^3 at 20 °C.

6) Extinction coefficient of this RM at wavelength of 280 nm was obtained based on absorbance measured by UV-2550, spectrophotometer Shimadzu Corp., (band path: 1 nm) using antibody concentration and optical cell whose optical path length was evaluated (Starna Scientific Ltd., 1/Q/1, 1 mm path length, quartz cell). The effects of light scattering were corrected by the method specified in Japanese Pharmacopoeia*.

As a result, the extinction coefficient was determined to be (1.41±0.03) L/(g・cm).

(The value following the symbol ± represents the standard uncertainty of measurement.).

The extinction coefficient without the correction of light scattering was (1.43±0.03) L/(g・cm)

(The value following the symbol ± represents the standard uncertainty of measurement.).

* Japanese Pharmacopoeia 17th Edition (English version), Total Protein Assay (2478)

NMIJ Analysts

The technical manager for this RM is KATO M., the production manager is KINUMI T., and the analysts are KINUMI T., SAIKUSA K., MIZUNO R., and EYAMA S.

Information

If substantive technical changes occur that affect the value assignment before the expiration of this report, NMIJ will notify the registered customers. Customer registration on the NMIJ Website (given below) will facilitate notification. Technical reports regarding this RM can be obtained from the contact details given below.

Reproduction of Report

In reproducing this report, it should be clearly indicated that the document is a copy.

Note

This RM was developed based on the results of the research "Discovering and Manufacturing Pharmaceutical Infrastructure for Next-Generation Treatments and Diagnoses (JP17ae0101003, JP18ae0101056, and JP19ae0101056)" of Japan Agency for Medical Research and Development (AMED) and through cooperation with Manufacturing Technology Association of Biologics.

February 25, 2021

ISHIMURA Kazuhiko
President
National Institute of Advanced Industrial Science and Technology

If you have any questions about this RM, please contact:
National Institute of Advanced Industrial Science and Technology,
National Metrology Institute of Japan,
Center for Quality Management of Metrology, Reference Materials Office,
1-1-1, Umezono, Tsukuba, Ibaraki 305-8563, Japan
Phone: +81-29-861-4059; Fax: +81-29-861-4009, https://unit.aist.go.jp/nmij/english/refmate/

NMIJ RM 6208-a Monoclonal antibody solution (AIST-MAB)
Supplementary information

The measurement results including primary structure of Monoclonal antibody solution (NMIJ RM 6208-a, AIST-MAB) are summarized as the "Technical note (case study) A, B", which can be downloaded from NMIJ Website given below.
https://unit.aist.go.jp/nmij/english/refmate/

Technical note (case study) A summarizes the measurement results for this reference material including the primary structure obtained at AIST/NMIJ, Technical note (case study) B summarizes the measurement results obtained by Manufacturing Technology Association of Biologics. These measurement results are case study for various analyses, are not included in the scope of the Reference Material Report.