Technical note (case study)

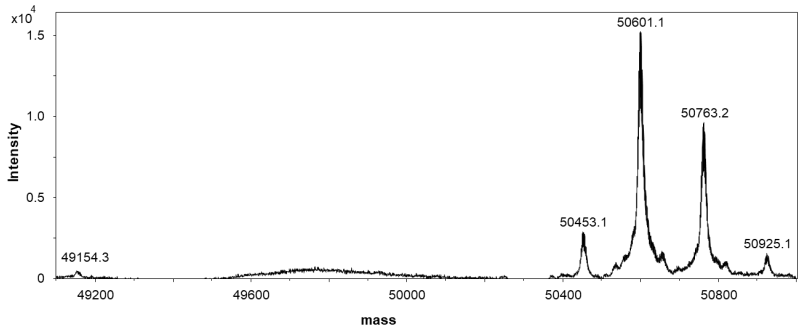
The measurement results of this reference material (AIST-MAB) including the primary structure obtained by AIST/NMIJ are summarized as "Technical note (case study) A". The measurement results obtained by Manufacturing Technology Association of Biologics are summarized as Technical note (case study) B.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

Technical note (case study) A

The measurement results including the primary structure obtained by AIST/NMIJ are shown below.

1. Structural analysis of light and heavy chains by mass spectrometry

The masses of light and heavy chain obtained by reduction with TCEP [tris(2-carboxyethyl)phosphine] of this material were measured by LC-MS. The measurement results and the deconvoluted mass spectrum are shown below. The peaks corresponding glycosylation (G0, G0F, G1F, and G2F) and peak without glycan moiety were identified.

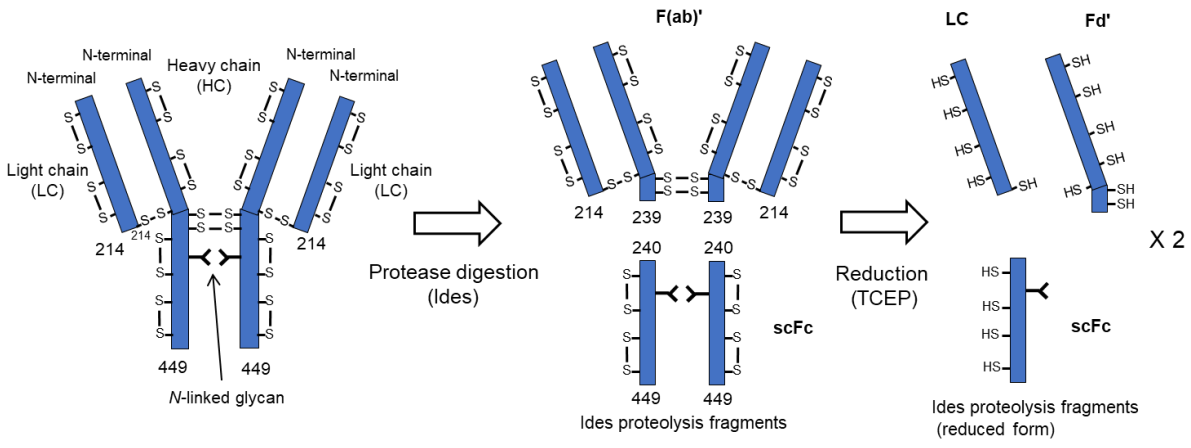


Mass spectrum of heavy chain

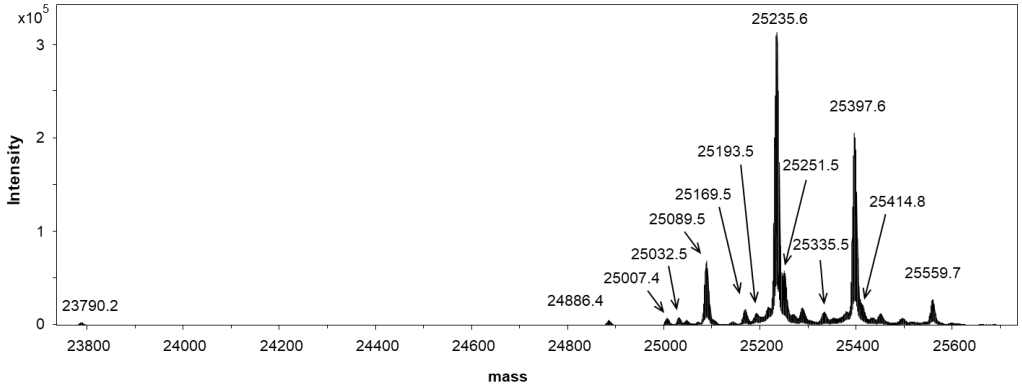
	Glycan structure	Theoretical mass		Measured mass	
		Monoisotopic	Averaged	Monoisotopic	Averaged
Light chain		23428.5	23442.8	23428.5	23442.6
Heavy chain	w/o glycan	49125.4	49155.9		49154.3
	G0	50423.9	50455.1	50423.9	50453.1
	G0F	50570.0	50601.3	50570.0	50601.1
	G1F	50732.0	50763.4	50732.1	50763.2
	G2F	50894.1	50925.5		50925.1

2. Structural analysis of Ides digests by mass spectrometry

The digested products of this material by Ides protease and the reduced material using TCEP were measured by LC-MS. Ides protease possesses papain and pepsin-like substrate specificity, and the digested products shown below were given by hydrolyzed at hinge region.



The measurement results regarding F(ab)', Fd', and scFc, the deconvoluted mass spectrum of scFc fragments were shown in next page.



The mass spectrum of scFc fragment (the averaged mass is shown)

	Glycan structure	Theoretical mass		Measured mass	
		Monoisotopic	Averaged	Monoisotopic	Averaged
F(ab)'		97567.9	97628.0		97629.8
Fd'		25367.5	25383.3	25367.6	25382.6
scFc	w/o glycan	23775.9	23790.7	23775.1	23790.2
	G0-GlcNAc	24871.3	24886.7	24871.4	24886.4
	Man5	24992.4	25007.7	24992.4	25007.4
	G0F-GlcNAc	25017.4	25032.8	25017.4	25032.5
	G0	25074.4	25089.8	25074.5	25089.5
	Man6	25154.4	25169.9	25154.5	25169.5
	G1F-GlcNAc	25179.4	25194.9	25178.4	25193.5
	G0F	25220.5	25236.0	25220.5	25235.6
	G1	25236.5	25252.0	25237.5	25251.5
	Man7	25316.5	25332.0	25319.5	25335.5
	G1F	25382.5	25398.1	25382.6	25397.6
	G2	25398.5	25414.1	25400.5	25414.8
	G2F	25544.6	25560.3	25544.6	25559.7

Twelve of glycosylated peptide chain and peptide chain without glycan were identified.

3. Structural analysis by peptide mapping

The structure shown below was confirmed by peptide mapping of tryptic digest of this material.

1) Partial *N*-terminal pyroglutamylation and addition of lysine residue at the C-terminal were observed.

2) Deamidation of asparagine was observed at the following residues:

Light chain: Asn 30

Heavy chain: Asn 55, Asn 84, Asn 318, Asn 387 or 392 or 393

3) Oxidation of methionine was observed at the following residues:

Heavy chain: Met 107、Met 255

4) Glycosylation was occurred at Asn 300 of heavy chain.

5) The structure of disulfide bond was confirmed by peptide mapping for tryptic digest under non-reducing condition. The disulfide bonds are formed from the following cysteine residues.

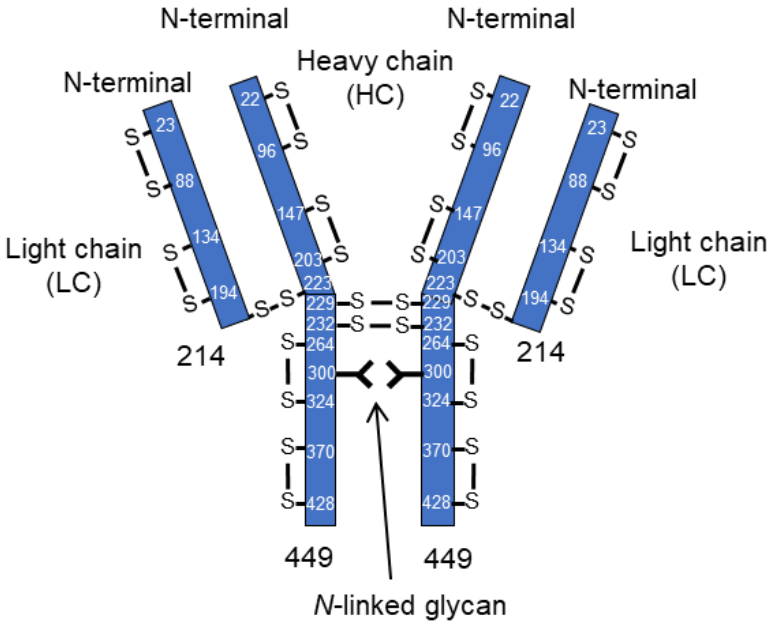
Light chain: Cys 23-Cys 88, Cys 134-Cys 194

Heavy chain: Cys 22-Cys 96, Cys 147-Cys 203, Cys 264-Cys 324, Cys 370-Cys 428

Light chain－Heavy chain: Cys 214 (LC)-Cys 223 (HC)

Heavy chain－Heavy chain: Cys 229 (HC1)-Cys 229 (HC2), Cys 232 (HC1)-Cys 232 (HC2)

The structure of this material including disulfide bonds and glycan was shown below.



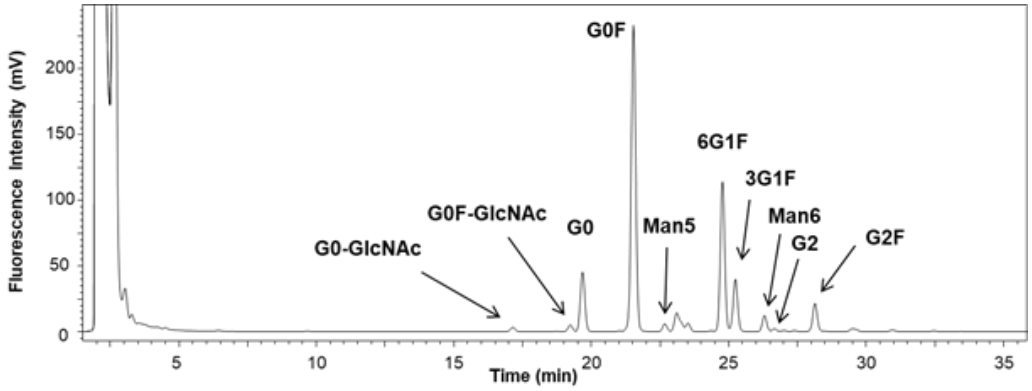
4. Intact mass spectrometry and mass spectometry of light chain and heavy chain for glycanase-treated material

Intact mass spectrometry of the material which was obtained by removing the glycans using *N*-glycanase (PNGase F), and the heavy chain and light chain obtained by reduction using TCEP, were measured by LC-MS. The results are shown below.

	Theoretical mass		Measured mass	
	Monoisotopic	Averaged	Monoisotopic	Averaged
Full body	145076	145165		145172
LC	23428.5	23442.8	23428.6	23442.6
HC	49125.4	49155.9	49125.5	49155.5

5. Glycan mapping for *N*-linked glycan

The glycan structure of this material was analyzed by LC-fluorescence detector after derivatization and reduction with 2-aminobenzamide for a mixture of *N*-linked glycans obtained by PNGase F-treatment of antibody.



Glycan mapping by LC-fluorescence detection

Of the 20 peaks with more than 0.1 % of the total peak area, the structures of 10 of *N*-linked glycans were assigned. The composition of these glycans was determined by area percentage of the peaks and summarized in the table below.

	Area ratio (%)	
	Average	SD
G0-GlcNAc	0.71	0.002
G0F-GlcNAc	1.02	0.004
G0	8.85	0.025
G0F	45.02	0.092
Man5	1.17	0.012
6G1F	21.39	0.051
3G1F	7.78	0.004
Man6	2.38	0.019
G2	0.44	0.006
G2F	4.27	0.017
Other peaks	6.98	0.053

Sample preparation and analytical condition

1. Structural analysis of light and heavy chains by mass spectrometry

To 5 µL of antibody solution, 50 µL water and 5 µL 500 mmol/L TCEP [tris(2-carboxyethyl)phosphine] were added and incubated at 37 °C for 2 h. Two microliter of material was injected into LC-MS.

Liquid chromatograph (LC): Nexera 30A high-performance liquid chromatograph (Shimadzu Corp.)
Column: AQUITY UPLC Protein BEH C4 (Waters Corp., 1.7 µm, 2.1 mm diameter × 100 mm length), Mobile phase A: 0.1 % formic acid/H_2O, B: 0.1 % formic acid/acetonitrile, Flow rate: 0.2 mL/min, Column temperature: 60 °C Gradient condition: 5 %B 2 min, 5-15 %B 1 min, 15-25 %B 3 min, 25-35 %B 15 min
Electrospray ionization high-resolution mass spectrometer (MS): maXis-II time-of-flight mass spectrometer (Bruker Corp.)
Capillary Voltage: 4500 V, nebulizer gas: 1.2 bar, dry gas: 6 L/min, isCID: 30 eV, Quadrupole ion energy 4 eV, Collision energy: 8 eV, Mass range: *m/z* 500-3000, Spectra rate: 3 Hz

2. Structural analysis of Ides digests by mass spectrometry

To 50 µL of antibody solution, 50 µL 50 mmol/L phosphate buffer and 4 µL Ides protease (Ides FabRICATOR, 270 U, Sigma-Aldrich Corp.) were added and at 37 °C for 1 h. Reduced form was obtained by adding an additional 5 µL 500 mmol/L TCEP and incubating at 37 °C for 1 h. Each, 3 µL of material was injected into LC-MS. Measurements were performed as in 1.

3. Structural analysis by peptide mapping

Twenty microliters of the antibody solution was added to 100 µL 8 mol/L guanidine hydrochloride, 1 mmo/L ethylenediaminetetraacetic acid, 250 mmol/L Tris-HCl (pH 7.5), and then 5 µL 500 mmol/L dithiothreitol (DTT) was added and incubated for 1 h at 37 °C. The reaction mixture was then incubated for 1 h in the dark at room temperature with 12 µL 500 mmol/L iodoacetic acid, followed by 5 µL 500 mmol/L DTT. After desalting using a NAP-5 gel filtration column (Cytiva), trypsin (FUJIFILM Wako Pure Chemical, mass spectrometry grade) was added to a 300 µL fraction of desalted NAP-5 elutant at 1:25 (enzyme: substrate) ratio of protein content, and incubated at 37 °C overnight. The reaction was terminated by adding 1 µL trifluoroacetic acid and 5 µL was injected into LC-MS for measurement. For peptide mapping in the non-reduced condition, the same sample preparation as above was performed without DTT and iodoacetic acid.

Liquid chromatograph (LC): Nexera 30A high-performance liquid chromatograph (Shimadzu Corp.)
Column: AQUITY UPLC Peptide BEH C18 (Waters Corp., 3.5 µm, 2.1 mm diameter × 150 mm length), Mobile phase A: 0.1 % formic acid/H_2O, B: 0.1 % formic acid/acetonitrile, Flow rate: 0.2 mL/min, Column temperature: 45 °C Gradient condition: 2 %B 3 min, 2-7 %B 1 min, 7-10 %B 4 min, 10-25 %B 32 min, 25-35 %B 15 min
Electrospray ionization high-resolution mass spectrometer (MS): maXis-II time-of-flight mass spectrometer (Bruker Corp.)
Capillary Voltage: 4500 V, nebulizer gas: 1.2 bar, dry gas: 6 L/min, isCID: 0 eV, Quadrupole ion energy 5 eV, Collision energy: 10 eV, Mass range: *m/z* 200-5000, Spectra rate: 5 Hz
Data dependent MS/MS, Precursor Ions cycle time 1.5 sec, Active exclusion exclude after 1 Spectra, Reconsider Precursor 1.5

4. Intact mass spectrometry and mass analysis of light chain and heavy chain for glycanase-treated material

To 24 μL of antibody solution, 85 μL 50 mmol/L phosphate buffer and 12 μL PNGase F (PNGase F Plus, 1.2 U, Agilent Technologies) were added and at 37 °C for 2 h. Reduced form was obtained by adding an additional 5 μL 500 mmol/L TCEP and incubating at 37 °C for 1 h. Each, 10 μL of material was injected into LC-MS. Measurements were performed as in 1.

5. Glycan mapping for *N*-linked glycan

A series of sample preparation was performed using the EZGlyco mAb-N Kit with 2-AB (Sumitomo Bakelite Co, BS-X4410). Two microliters of the derivatized glycan mixture solution obtained with 8 μL of antibody solution was injected into an LC-fluorescence detection system for analysis.

Liquid chromatograph (LC): Nexera 30A high-performance liquid chromatograph (Shimadzu Corp.)

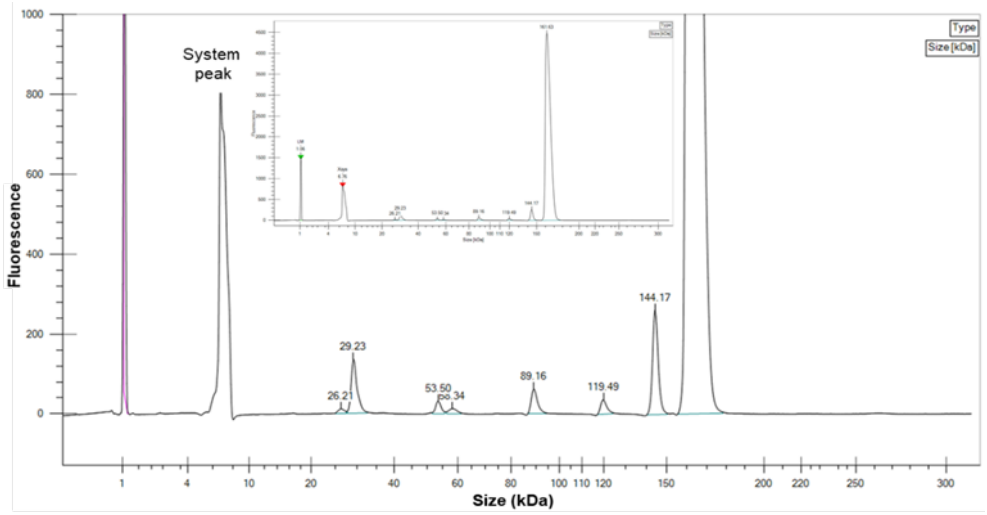
Column: AQUITY UPLC BEH Amide (Waters Corp., 1.7 μm, 2.1 mm diameter × 150 mm length), Mobile phase A: 100 mmol/L ammonium formate, B: acetonitrile, Flow rate: 0.2 mL/min, Column temperature: 45 °C Gradient condition: 75-50 %B 50 min, Fluorescent detection: excitation 330 nm, detection 420 nm.

Technical note (case study) B

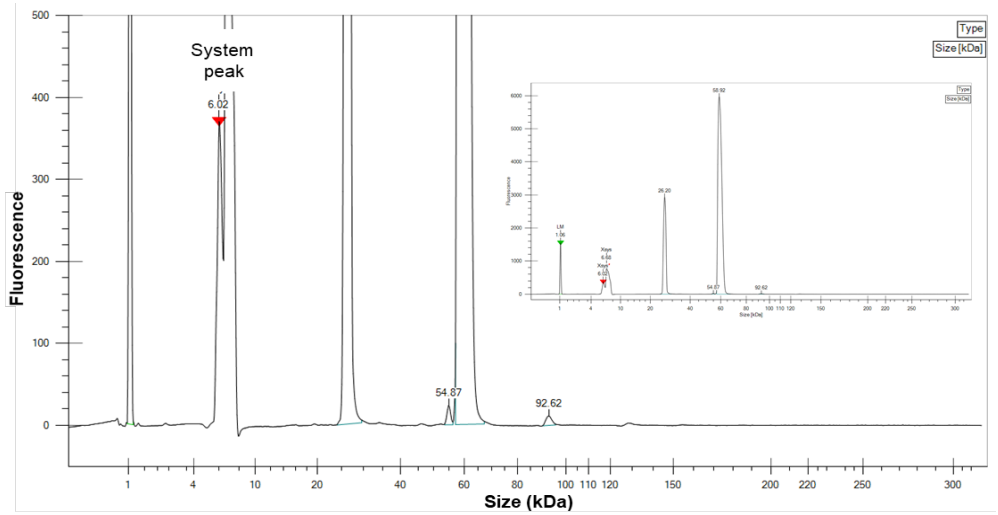
The measurement results obtained by Manufacturing Technology Association of Biologics are shown below.

1. Microchip electrophoresis

The results of SDS (sodium dodecyl sulfate) microchip electrophoresis of this material under non-reducing and reducing conditions using a microchip electrophoresis system are shown below.



Electrophoresis under non-reducing condition



Electrophoresis under reducing condition

	Molecular mass (kDa)		Ratio (%)		Condition of electrophoresis
	Average	SD	Average	SD	
Full body	161.3	0.9	92.9	0.1	non-reduced
Truncated form	143.8	0.8			
	119.1	0.6			
	88.8	0.5			
	58.1	0.3			
	53.2	0.3			
	29.1	0.2			
	26.1	0.1			
	93.0	0.4			reduced
Heavy chain	59.3	0.3	72.6	0	
	55.1	0.2			
Light chain	26.4	0.2	27.2	0.1	

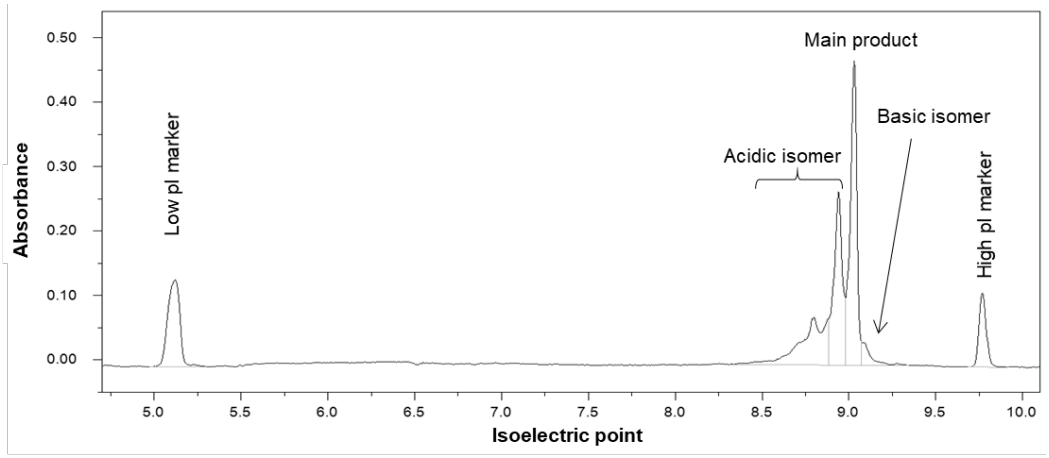
Sample preparation and measurement condition

Two microliters of the sample (2.5 mg/mL antibody solution) was treated in the denaturing/reduction reaction at 70 °C for 10 min using Protein Express Assay Reagent Kit (PerkinElmer), and the results were averaged over three lanes.

Electrophoresis: LabChip GXII electrophoresis system (PerkinElmer)

2. Charge isomer distribution measured by capillary isoelectric focusing

The ratio of charge isomers of this material was measured using a capillary isoelectric focusing system.



Electropherogram obtained by isoelectric focusing

Ratio of the charge isomer

	Average (%)	
	Ratio	SD
Acidic isoform	54.3	0.9
Main product	42.1	0.6
Basic isoform	3.6	0.3

Isoelectric point (9.03 ± 0.003) (after the symbol ± indicates the standard deviation.)

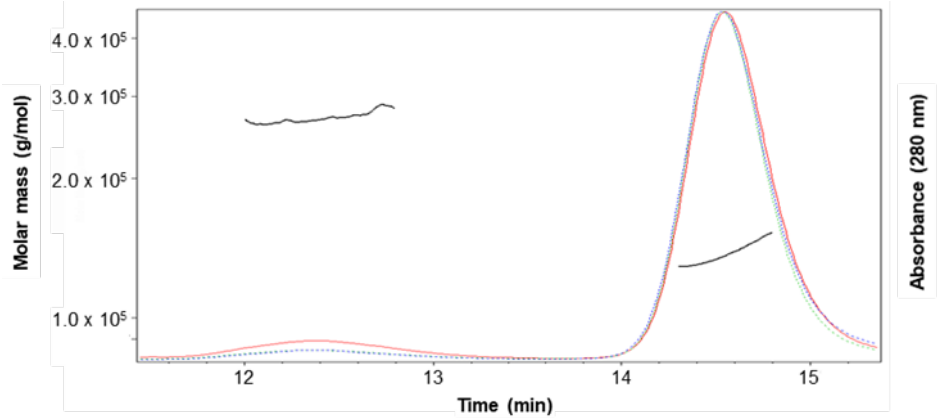
Sample preparation and measurement condition

The sample solution was prepared 0.4 mg/mL as the final concentration of antibody in 4 % Pharmalyte (pH 3-10), 0.35 % methylcellulose, 10 mmol/L arginine solution.

Capillary isoelectric focusing system: iCE3/Alcott720NV (ProteinSimple), Capillary: Fc cartridge (100 μm diameter × 50 mm length), Detection:280 nm, Condition: Prefocusing time: 1 min, 1500 V, Focusing time: 4.5 min, 3000 V. The measurement was repeated three times.

3. Size exclusion chromatography - multi-angle static light scattering (SEC-MALS)

Size exclusion chromatography - Multi-angle static light scattering (SEC-MALS) was used to measure the molecular weight distribution.

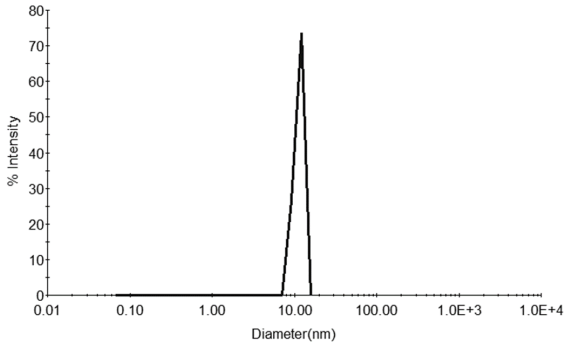


The molecular weight of main peak by this measurement was (1.350 ± 0.004) × 10^5. (after the symbol ± indicates the standard deviation.)

Sample preparation and measurement conditions
Ten microliters of antibody solution (2.5 mg/mL) was injected.
Instrument: High-performance liquid chromatograph: Infinity 1220 LC (Agilent Technologies), Multi-angle light scattering detector: DAWN HELEOS II 8+ (Wyatt Technology), Refractive index detector: Optilab T-rEX (Wyatt Technology)
Column: TSK gel G3000SW_{XL} (TOSOH Corp., 7.8 mm internal diameter × 300 mm length), Mobile phase: 50 mmol/L Phosphate buffer 200 mmol/L NaCl (pH7), Column temperature room temperature. Flow rate: 0.6 mL/min, Detection: UV 280 nm, MALS (dn/dc: 0.185, UV Extinction Coefficient: 1.400).

4. Particle size distribution measured by Dynamic light scattering (DLS)

Dynamic light scattering (DLS) was used to measure the size distribution of nanoscale particles.

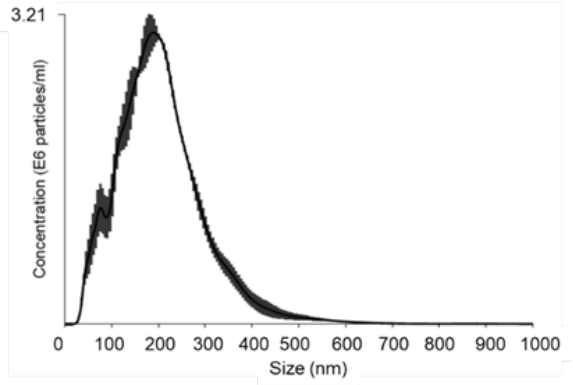


The average particle size was measured to be (11.4 ± 0.2) nm, and the polydispersity index (Pd) obtained by cumulant analysis was (10.6 ± 1.0) %. (after the symbol ± indicates the standard deviation.)

Sample preparation and measurement conditions
Instrument: DynaPro PlateReaderII (Wyatt Technology Corp.), Measurement conditions: measurement time 10 sec, 10 times acquisition, Temperature 25 °C, Viscosity: 0.890 mPa・s (25 °C), reflective index: 1.333 (20 °C, 589nm). The measurement was repeated three times.

5. Particle size distribution measured by nano tracking analysis (NTA)

Nano tracking analysis (NTA) was used to measure the size distribution and particle concentration of subvisible particle.

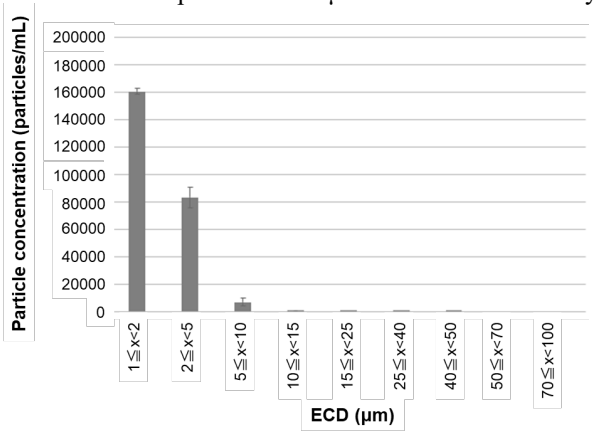


The measurement results showed that average particle size: (206 ± 0.4) nm, mode diameter: (194 ± 7.9) nm, particle concentration: (5.81 ± 0.36) × 10^8 particles/mL. (after the symbol ± indicates the standard deviation.)

Sample preparation and measurement conditions
Instrument: NanoSight NS500 (Quantum Design), Aoftware: NanoSight NTA2.3, Measurement conditions: Camera level: 12, Recording time: 60 sec. The measurement was repeated three times.

6. Aggregated particle (insoluble particulate matter) measured by Micro Flow Imaging (MFI)

Particle concentration of subvisible particles over 1 μm in size was measured by microflow imaging (MFI).



The particle concentration was measured to be (251712 ± 12062) particles/mL. (after the symbol ± indicates the standard deviation.)

Sample preparation and measurement conditions

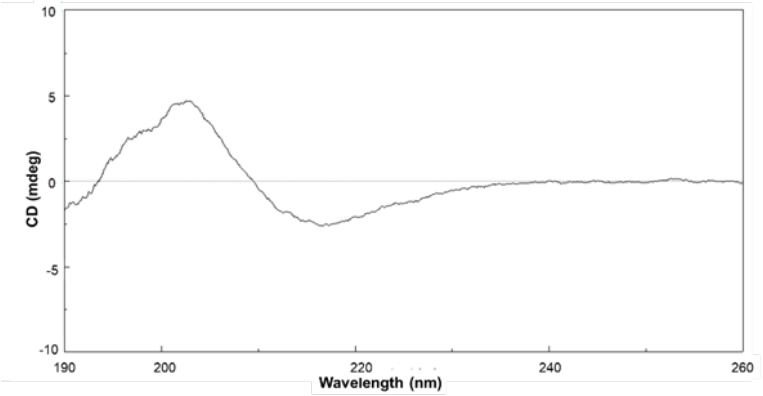
The sample was gently inverted fived time, and subjected to measurement.

Instrument: MFI 5200 (ProteinSimple), Software: MVSS software

Measurement conditions: SetPoint3, 100 μm Depth flow cell, 0.2 mL of sample was used for priming the flow cell, then obtained 226 flames of image by flowing the sample with the flow rate of 0.1 mL/min. The measurement was repeated three times.

7. Circular dichroism spectrum

Circular dichroism (CD) spectrum was measured and ratio of the secondary structure was estimated.



CD spectrum of this material

The estimated ratio of the secondary structure

	Ratio of secondary structure (%)	
	Ratio	SD
Helix	11.3	8.8
Sheet	42.2	5.9
Turn	10.2	2.4
Other	36.3	1.3

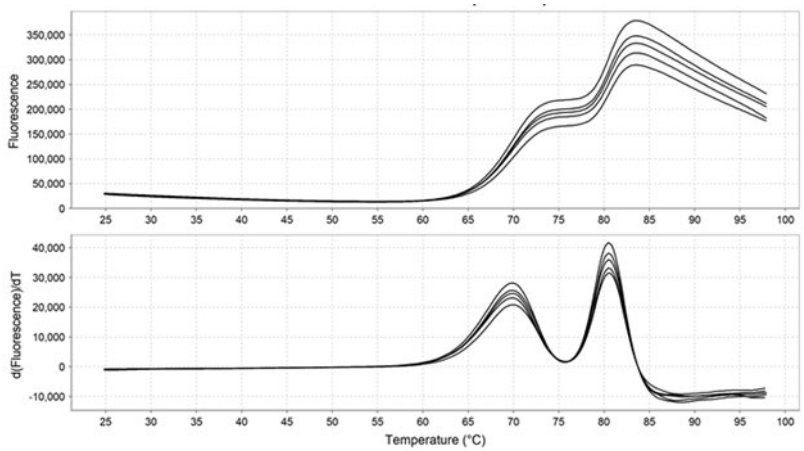
Sample preparation and measurement conditions

Using potassium phosphate buffer as a blank, 0.1 mg/mL of antibody solution was measured using a 1 mm quartz cell with an optical path length.

Instrument: circular dichroism dispersometer J-1500 (JASCO), Estimation of the ratio of the secondary structure was done using the CD multivariate SSE analysis program JWSSE-513(JASCO).

8. Change of the higher-order structure measured by melt curve

The change of the higher-order structure was measured by melt curve using Protein Thermal Shift kit (Applied Biosystems).



*T*m Boltzmann (°C)			*T*m Boltzmann (°C)	
Average	SD		Average	SD
73.64	0.02	Peak 1	69.72	0
		Peak 2	80.36	0

The results are shown in the table for the melting temperatures (*T*m values).

Sample preparation and measurement conditions
Instrument: StepOnePlus Real-Time PCR System (Applied Biosystems), Software: Protein Thermal Shift Software (Applied Biosystems)

9. Analytical results of quantification of impurities

1) Quantification of host cell-derived protein, ELISA
ELISA kit: CHO Host Cell Proteins 3rd Generation F550 (Cygnus Technologies)
Measurement result: 3.2 ng/mg protein (as IgG 5.00 mg/mL)
Limit of quantification: 1.39 ng/mL

2) Quantification of host cell-derived DNA, quantitative PCR
DNA Extraction Kit: DNA Extraction and Amplification Kit D555T (Cygnus Technologies)
Amplification target region: glyceraldehyde dehyde-3-phosphate dehydrogenase
Detection method: SYBR Green I
Measurement results: below the lower limit of quantification
Limit of quantification: 10.0 pg/mg protein (as 5.00 mg/mL of IgG)

3) Quantification of residual protein A, ELISA
ELISA Measurement Kit: Residual Protein A kit AL287 (PerkinElmer)
Results: 0.2 ng/mg protein (as 5.00 mg/mL of IgG)
Limit of quantification: 8.7 pg/mL

4) Quantification of endotoxin, turbidometry
Measurement Kit: Limulus ES-I Single Test (FUJIFILM Wako Pure Chemical)
Results: less than 0.002 EU/mg protein (as 5.00 mg/mL of IgG)
Limit of quantification: 0.01 EU/mL