⟨129⟩ ANALYTICAL PROCEDURES FOR RECOMBINANT THERAPEUTIC MONOCLONAL ANTIBODIES

INTRODUCTION

This chapter provides analytical procedures for murine, chimeric, and humanized IgG isotype monoclonal antibodies and subtypes (e.g., IgG1 and IgG2). Subclasses show differences in amino acid sequence and in the number of disulfide bonds, and in some cases they require subclass-specific analysis. This chapter applies to monoclonal antibodies for therapeutic and prophylactic use and for use as in vivo diagnostics. It does not apply to monoclonal antibodies used as reagents in the manufacture of medicinal products, for which applicable requirements are determined by the appropriate regulatory agency.

Naturally occurring polyclonal antibodies in serum or plasma can bind to many different targets as part of the immune system. In contrast, therapeutic monoclonal antibodies for human use are preparations of an immunoglobulin (or a fragment of an immunoglobulin) that have specificity for a target and are derived from a single clone of cells. Therapeutic monoclonal antibodies can bind to and neutralize soluble antigens, and their mechanism of action (MOA) often involves blocking of the ligand from binding to its cognate receptor. Alternatively, therapeutic monoclonal antibodies can recognize and bind to cell-bound antigens and in these cases may also engage the immune system through Fc (crystallizable fragment) mediated effector functions as part of their MOA. Monoclonal antibodies that have a single defined binding specificity are monospecific, whereas recombinantly engineered bispecific monoclonal antibodies can bind to two different targets (e.g., antigens). IgG-type monoclonal antibodies have a molecular weight of approximately 150 kD. Each molecule consists of two heavy and two light polypeptide chains that have a molecular weight of approximately 50 and 25 kD, respectively. Antibodies can be schematically represented as a Y joined by disulfide bridges, where each arm of the Y is called the Fab domain, and the stem of the Y is called the Fc domain. Monoclonal antibodies are glycoproteins that have a glycosylation site in the Fc portion located on each of the heavy chains and have possible additional glycosylation sites in the Fab domain, depending on the molecule.

The specificity of a monoclonal antibody is based on its antigen binding site, which is located in the Fab part of the molecule. The Fc domain contains receptor binding sites that are associated with antibody effector functions that have subclass-specific applications such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Pharmacokinetic effects via binding to the neonatal Fc receptor also are present in this domain.

Monoclonal antibodies are produced in cell culture using a seed lot system with master and working cell banks. After harvest, purification steps ensure that product- and process-related impurities are reduced to an acceptable level.

Currently licensed monoclonal antibody therapeutics include those involved in the activation of effector cells, cell killing, cross-linked induced apoptosis, antagonism against several targets, and agonist antibodies.

This chapter includes purity assessments by size-exclusion chromatography (SE-HPLC), capillary electrophoresis, and analysis of oligosaccharides, and it provides validated procedures for each of these.

Although this chapter is focused on IgG-type monoclonal antibodies, the principles of the tests included can apply to other related molecules, such as IgM or other isotype antibodies, antibody fragments, and Fc-fusion proteins. When the active substance is a conjugated antibody, such tests can be performed on the purified antibody before modification or conjugation.

Alternative methods and/or procedures may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, or in other special circumstances. Such alternative procedures and methods shall be validated as described in *Validation of Compendial Procedures* ⟨1225⟩ and must be shown to give equivalent or better results.

• Size-Exclusion Chromatography

Although SE-HPLC is robust for measuring monomer and high-molecular-weight species (HMWS, aggregates), the quantitation of low-molecular-weight species (LMWS, fragments) can be highly variable depending on the mAb studied. The measurement of LMWS is better quantitated by capillary electrophoresis sodium dodecyl sulfate (CE-SDS).

Mobile phase: Prepare by mixing 10.5 g of dibasic potassium phosphate, 19.1 g of monobasic potassium phosphate, and 18.6 g of potassium chloride per L of water. Verify that the pH is 6.2 ± 0.1. Pass through a membrane filter of ≤ 0.45-µm or smaller pore size.

System suitability solution: Prepare a 10-mg/mL USP Monoclonal IgG System Suitability RS solution in *Mobile phase* by reconstituting the contents of one vial with 200 µL of *Mobile phase*. Reconstituted *System suitability solution* should be used within 24 h after reconstitution, and should be stored at 2°–8° if not used immediately.

System suitability blank: Use *Mobile phase*.

Sample solution: Dilute the sample to 10 mg/mL in *Mobile phase* if dilution is required. Similarly, a blank should be prepared using an equivalent dilution of formulation buffer in *Mobile phase*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 7.8-mm × 30-cm; 5-µm packing L59

Temperatures

Column: Ambient

Autosampler: Maintain at 2°–8°

Flow rate: 0.5 mL/min

Injection volume: 20 µL

Run time: 30 min

System suitability

Sample: *System suitability solution*

[NOTE—Approximate retention times for polymer, dimer, monomer, and LMWS are 12, 15, 18, and 22 min, respectively.]

Suitability requirements

Chromatogram: The chromatogram of the *System suitability solution* is consistent with the typical chromatogram as illustrated in the USP Certificate for USP Monoclonal IgG System Suitability RS.

Chromatogram similarity: The chromatographic profiles of *System suitability solution* injections that bracket injections of *Sample solutions* in a sample set are consistent with each other and with the typical chromatographic profile as illustrated in the USP Certificate for USP Monoclonal IgG System Suitability RS. All named peaks in the typical chromatogram must be present, be well-resolved, and in the same elution order as that in the *System suitability solution* chromatograms.

Quantitative criteria: Bracketing *System suitability solution* injections must meet the following quantitative criteria:
The percent peak area of the HMWS in the *System suitability solution* must be 0.4%–0.67%.
The percent peak area of the main peak in the *System suitability solution* must be 99.1%–99.6%.
The percent peak area of the LMWS in the *System suitability solution* must be NMT 0.2%.

Analysis

Samples: *Blank, System suitability solution*, and *Sample solution*

Injections of the *Sample solution* should be bracketed minimally with single injections of the *System suitability solution*. An injection of a *Blank* should be included as the final injection before the final *System suitability solution* injection.

Calculations: Using a suitable electronic integrator or computer system, analyze the bracketing *System suitability solution* injections. Exclude from integration any peaks that are present in the *Blank*. Protein-related peaks that elute before the main peak are classified as HMWS; those that elute after the main peak are classified as LMWS. Report the percentage of peak areas for the HMWS, main peak, and LMWS.

- **Capillary SDS Electrophoresis (Reduced and Nonreduced)**

Capillary sodium dodecyl sulfate (CE-SDS) electrophoresis is a sensitive, analytical method for quantitative estimation of product-related impurities such as nonglycosylated molecules, half antibodies, and fragments, and thus is also useful as a stability-indicating assay. After denaturation with SDS, the method allows the analysis of the complete antibody under nonreducing conditions, and the analysis of light and heavy chains (LC and HC, respectively) under reducing conditions.

[Note—Slight variations in sample preparation may be necessary depending on the stability of the individual antibody. If 15 min incubation at 70° leads to fragmentation or cleavage of disulfide bonds for a particular antibody sample, adjust the incubation time accordingly.]

SDS sample buffer: Prepare a solution containing 100 mM tris-hydrochloride (tris-HCl), a pH of 8.95 ± 0.05, and 1% (w/w) SDS.

SDS gel buffer: Buffer at a pH of 8.0 containing 0.2% (w/w) SDS and an appropriate hydrophilic polymer acting as a molecular sieve[1]

Alkylation solution: 250 mM iodoacetamide in water prepared immediately before use

System suitability solution: Reconstitute 1 vial of USP Monoclonal IgG System Suitability RS with 0.5 mL of *Water for Injection* (WFI) to a final concentration of 4 mg/mL.

Internal standard solution: 5 mg/mL of 10 kDa internal standard[2] in water containing 0.5% (w/w) SDS and 0.2% (w/v) sodium azide

Nonreduced system suitability solution: Mix 25 µL of the *System suitability solution* with 4 µL of *Internal standard solution*. Mix 66 µL of *SDS sample buffer* with 5 µL of the *Alkylation solution*. Add the *SDS sample buffer* and *Alkylation solution* mixture to the *System suitability solution* and *Internal standard solution* mixture. Mix thoroughly, and heat the mixture at 70° for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

Reduced system suitability solution: Mix 25 µL of the *System suitability solution* with 66 µL of *SDS sample buffer*. Add 4 µL of *Internal standard solution* and 5 µL of neat β-mercaptoethanol. Mix thoroughly, and heat the mixture at 70° for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

Blank solution: Mix 45 µL of water with 50 µL of *SDS sample buffer*. Add 4 µL of *Internal standard solution* and 5 µL of neat β-mercaptoethanol for the reduced conditions or 5 µL of *Alkylation solution* for the nonreducing conditions. Mix thoroughly, and heat the mixture at 70° for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

Nonreduced sample solution: Dilute 100 µg of the sample to be tested with 50–95 µL of *SDS sample buffer* to give a final volume of 95 µL. Add 4 µL of *Internal standard solution* and 5 µL of the *Alkylation solution*. Mix thoroughly, and heat the mixture at 70° for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

Reduced sample solution: Dilute 100 µg of the sample to be tested with 50–95 µL of *SDS sample buffer* to give a final volume of 95 µL. Add 4 µL of *Internal standard solution* and 5 µL of neat β-mercaptoethanol. Mix thoroughly, and heat the mixture at 70° for 15 min. Allow to cool for 3 min at room temperature, centrifuge at 300 × g for 1 min, and transfer 100 µL into autosampler vials.

Instrumental conditions

Mode: CE-SDS

Detector: UV or diode array 220 nm

Capillary: Fused-silica capillary (i.d. 50 µm) cut to a total length of 30 cm with an effective length of 20 cm.[3] The following preconditioning and prefilling steps are required between each run.

Preconditioning of the capillary: Rinse for 3 min at 70 psi with 0.1 N sodium hydroxide followed by 0.1 N hydrochloric acid for 1 min at 70 psi, and water for 1 min at 70 psi.

Prefilling of capillary: Rinse for 10 min at 70 psi with *SDS gel buffer*.

[1] Available from Beckman Coulter as a component of test kit, catalog number A10663, or a suitable equivalent.
[2] Available from Beckman Coulter as a component of test kit, catalog number A10663, or a suitable equivalent.
[3] Beckman Coulter catalog number 338472 or equivalent.

Sample injection: Electrokinetic injection, 5.0 kV reversed polarity for 20 s
Voltage separation
Nonreduced conditions: Run time 40 min at 15 kV, reversed polarity
Reduced conditions: Run time 40 min at 15 kV, reversed polarity
Temperatures
Sample storage: Approximately 25°
Capillary: Approximately 25°

System suitability
Suitability requirements for reducing conditions
Sample: *Reduced system suitability solution*
Electropherogram: The electropherogram of the *Reduced system suitability solution* is consistent with the typical electropherogram illustrated in the USP Certificate for USP Monoclonal IgG System Suitability RS.
Resolution: The main peak of the heavy chain and the peak of the nonglycosylated heavy chain can be clearly identified. The resolution between the nonglycosylated heavy chain and the intact heavy chain is NLT 1.2.
Ratio of nonglycosylated to total heavy chain: Use time-corrected peak areas (see *Capillary Electrophoresis* ⟨1053⟩*, Quantification*, which may be a helpful, but not mandatory resource) for calculation. Calculate the ratio of nonglycosylated to total heavy chain by dividing the relative amount of nonglycosylated heavy chain by the sum of all heavy chains, and multiply by 100. The ratio of nonglycosylated to total heavy chain in the *System suitability solution* should be within the limits of 0.75%–1.34%.
Suitability requirements for nonreducing conditions
Sample: *Nonreduced system suitability solution*
Electropherogram: The electropherogram of the *Nonreduced system suitability solution* is consistent with the typical electropherogram illustrated in the USP Certificate for USP Monoclonal IgG System Suitability RS.
Resolution: The IgG main peak can be clearly identified. The resolution between the IgG main peak and Fragment 1 (F1) is NLT 1.3.
Amount of main peak: Calculate the relative amount of the main peak by dividing the sum of the time-corrected peak area of the main IgG peaks by the sum of all time-corrected peak areas excluding internal standard peaks and all system peaks, and multiply by 100. The relative amount of the main IgG peak of the *System suitability solution* should be within the limits of 61.4%–86.4%.
Relative standard deviation: NMT 2% for the migration of the internal standard in the bracketing *Blank solution*. No other peaks migrating after the internal standard are detectable.

Analysis
Samples: *Blank solution, System suitability solution*, and *Sample solution*
[NOTE—Based on instrument requirements, the field applied across the capillary and the conditions for the sample injection may be adjusted to achieve *System suitability*.] Apply a field strength of 500 V/cm (reversed polarity) for 40 min, using the *SDS gel buffer* as the electrolyte in both buffer reservoirs. Electrophorese the samples, using a 20-s reversed polarity and electrokinetic injection at 5.0 kV, into the anodic end of the capillary, and record the electropherograms at 220 nm. Rinse for 3 min at 70 psi with 0.1 N sodium hydroxide followed by 0.1 N hydrochloric acid for 1 min at 70 psi, and water for 1 min at 70 psi. Inject at least in duplicate.
Calculate the time-corrected peak areas of all peaks migrating after the internal standard peak.
Reduced conditions: Calculate the relative amount of product peak by dividing the sum of the time-corrected peak areas of heavy chain, nonglycosylated heavy chain, and light chain by the sum of all time-corrected peak areas for peaks appearing after the internal standard, and multiply by 100.
Nonreduced conditions: Calculate the relative amount of product peak by dividing the sum of the time-corrected peak area of the main IgG peak by the sum of all time-corrected peak areas for peaks appearing after the internal standard, and multiply by 100.

• **OLIGOSACCHARIDE ANALYSIS—ANALYSIS OF *N*-LINKED OLIGOSACCHARIDES OF MONOCLONAL ANTIBODIES**
The inclusion of oligosaccharide analysis in a specification for a monoclonal antibody drug substance should be assessed using science- and risk-based approaches and should be defined on a product-specific basis. If applicable, one or more of the following analytical approaches can be employed for oligosaccharide profiling or quantitation of individual structures. Other validated methods that are suitable for determination of *N*-linked glycosylation utilizing different modes of separation, labeling, or detection can be used. [NOTE—For monoclonal antibodies with both Fc and Fab *N*-linked oligosaccharides, additional sample preparation procedures (e.g., treatment with the enzyme papain) can be employed to separate the domains before analyses so that information at individual glycosylation sites can be obtained. All additional sample preparation steps for the method require validation.]
[NOTE—Numerical acceptance criteria may be defined for individual glycan structures. If applicable, acceptance criteria must be derived from product-specific information taking into consideration a suitable range of historical batch data.]
Method A: Capillary electrophoresis with laser-induced fluorescence detection
Run buffer: Prepare 100 mM triethanolamine (TEA) and 10% glycerol at pH 7.5 by dissolving 1.492 g of TEA and 10 g of glycerol in 80 mL of water. Adjust with 1 N hydrochloric acid to a pH of 7.5, and dilute with water to a final volume of 100 mL.
Sample buffer: Dilute 1 mL of *Run buffer* with 9 mL of water.
Enzyme reaction buffer: Prepare 50 mM sodium phosphate, pH 7.5.
APTS labeling reagent: Dissolve 5 mg of 8-aminopyrene-1,3,6-trisulfonate (APTS) in 48 µL of 15% acetic acid, and mix.
Sample solution: Add 2 µL of peptide *N*-glycosidase F (PNGase F) (5 units/mL) to 50 µg of sample, and adjust with *Enzyme reaction buffer* to a volume of 50 µL. [NOTE—The unit definition for PNGase F may differ among commercial sources. It may be necessary to experimentally determine the appropriate quantity of PNGase F required for the complete release of *N*-linked oligosaccharides in the sample.] Incubate at 37° for 18 h. Separate released oligosaccharides by centrifugation using a 10,000 molecular-weight-cut-off (MWCO) centrifugal filter. Dry the released

oligosaccharides in a centrifugal vacuum evaporator to dryness. Add 2 µL of *APTS labeling reagent* and 2 µL of 1 M sodium cyanoborohydride to the dried oligosaccharides. Incubate at 55° for 90 min. Add 46 µL of water to quench the reaction and mixture. Add 5 µL of APTS-labeled sample to 95 µL of *Sample buffer*.

System suitability solution: Resuspend each of the suitable glycan standards (10 µg) in 50 µL of water, and mix on a vortex mixer. Add 5 µL of a suitable glycan standard (1 µg) to a microcentrifuge tube. Dry the glycan standard using centrifugal vacuum evaporation at ambient temperature. Add 2 µL of sodium cyanoborohydride to each standard. Add 2 µL of the *APTS labeling reagent* to each standard. Mix the standards on a vortex mixer, and centrifuge briefly to settle the liquid before incubation at 55° for 90 min. Quench the reaction by the addition of 46 µL of water to each standard. Standards can be stored at –20° up to 5 weeks.

Instrumental conditions

Mode: CE

Detector: Laser-induced fluorescence detector (488-nm excitation wavelength; 520-nm emission wavelength)

Capillary: 50-µm inner diameter bare fused-silica capillary with 50-cm total length and 40-cm separation length

Sample injection: 10-s hydrodynamic injection

Separation voltage: 22kV for 50 min

Samples: *Sample solution* and *System suitability solution*

Analysis: Integrate peaks in the resultant electropherogram, and report relative percentage time-corrected peak areas of major glycan structures relevant to the product. Comparison to suitable product-specific reference standard can be performed.

Method B: Liquid chromatography with fluorescence detection

Enzyme reaction buffer: Prepare 100 mM sodium phosphate, a pH of 7.5.

Labeling buffer: Prepare 8% (w/v) sodium acetate trihydrate and 4% (w/v) boric acid solutions in methanol by dissolving 800 mg of sodium acetate trihydrate and 400 mg of boric acid in 10 mL of methanol.

Labeling reagent solution: Prepare a 100-mg/mL stock solution of anthranilic acid (2-AA) in *Labeling buffer.* Mix with an equal volume of 1 M sodium cyanoborohydride in methanol for a final *Labeling reagent solution* containing 50 mg/mL of 2-AA and 0.5 M sodium cyanoborohydride. [NOTE—Keep the labeling reagent protected from light.]

Solution A: Prepare 50 mM ammonium formate, pH 4.4.

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0.0	32.5	67.5
48.0	42.5	57.5
49.0	100	0
53.0	100	0
54.0	32.5	67.5
60.0	32.5	67.5

Sample preparation: Transfer 80 µg of test sample to a microcentrifuge tube. Add 3 µL of PNGase F (2.5 units/mL). [NOTE—The unit definition for PNGase F may differ among commercial sources. It may be necessary to experimentally determine the appropriate quantity of PNGase F required for complete release of *N*-linked oligosaccharides in the sample.] Add 14 µL of *Enzyme reaction buffer.* Add water to 70 µL of total volume, and incubate at 37° for 18 h. Add 70 µL of freshly prepared *Labeling reagent solution*, and incubate at 70° for 2 h in a fume hood. Allow to cool, and add 60 µL of 50% methanol to each sample. Mix, then centrifuge. Remove 140 µL of the supernatant, and transfer to a microcentrifuge tube. Add 500 µL of acetonitrile to the supernatant-containing tube. Load the sample onto a hydrophilic-interaction solid-phase extraction cartridge to remove excess labeling reagent. Wash the cartridge with 95% acetonitrile (approximately 10 mL). Elute the labeled glycans from the cartridge with 0.5 mL of 20% acetonitrile. Dilute the eluate 1:1 with acetonitrile.

Chromatographic system

(See *Chromatography* ⟨621⟩*, System Suitability*.)

Mode: LC

Column: 4.6-mm × 15-cm; 3-µm packing L68

Detector: Fluorescence (360-nm excitation wavelength; 420-nm emission wavelength)

Column temperature: Maintain column at 30°.

Flow rate: 0.8 mL/min

Injection volume: 50 µL

Analysis

Sample: *Sample preparation*

Integrate peaks in the resulting chromatogram, and report relative percentage peak areas of major glycan structures relevant to the product. Comparison to a suitable product-specific reference standard can be performed.

• **OLIGOSACCHARIDE ANALYSIS—SIALIC ACID ANALYSIS**

[NOTE—The range of the standard curve or the mass of test sample may require modification depending on the sialic acid content of the monoclonal antibody.]

Solution A: Prepare 100 mM sodium hydroxide by diluting 10.4 mL of a 50% (w/w) sodium hydroxide solution in 2 L of water. [NOTE—Use high-quality water of high resistivity (18 MΩ-cm or better) that contains as little dissolved oxygen as possible.]

Solution B: Prepare a solution containing 100 mM sodium hydroxide and 1 M sodium acetate by adding 82.0 g of sodium acetate to 800 mL of water. Add 5.2 mL of 50% (w/w) sodium hydroxide, and dilute with water to a final volume of 1000 mL.

Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0.0	93	7
10.0	70	30
11.0	70	30
12.0	93	7
15.0	93	7

Standard stock solution: Prepare 0.2 mM solutions of USP *N*-Acetylneuraminic Acid RS (NeuAc) and USP *N*-Glycolylneuraminic Acid RS (NeuGc) in 20 mM sodium acetate buffer, pH 5.2. Dilute an appropriate volume of this solution with 20 mM sodium acetate buffer, pH 5.2, to obtain a 0.02 mM *Standard stock solution*.

Internal standard stock solution: Prepare a 0.1 mM solution of 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN) in 20 mM sodium acetate buffer, adjusted to a pH of 5.2.

Standard solutions: Prepare as indicated in *Table 3*.

Table 3

Standard	Concentration (µM)	Volume of Standard Stock Solution (µL)	Volume of 20 mM Sodium Acetate Buffer, pH 5.2 (µL)	Volume of Internal Standard Stock Solution (µL)
1	10	250	235	15
2	5	125	360	15
3	4	100	385	15
4	2	50	435	15
5	1	25	460	15
6	0.4	10	475	15

System suitability solution: Prepare 3 µM solutions of NeuAc, NeuGc, and KDN in 20 mM sodium acetate buffer, adjusted to a pH of 5.2. Store the *System suitability solution* at –70°.

Sample solution: Pipet a volume of test sample equivalent to 0.5 mg into a microcentrifuge tube. Dilute with 20 mM sodium acetate buffer, pH 5.2, to a total volume of 475 µL. Confirm the pH with a test strip, and add 10 µL of 10 milliunit/µL of neuraminidase. Incubate for 5 h at 37°. Add 15 µL of *Internal standard stock solution*. Mix on a vortex mixer, and transfer the sample to an autosampler vial. [NOTE—A slight adjustment in sample preparation may be necessary depending on the test sample and the quality of the enzyme. Adjust the incubation time accordingly.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Integrated amperometric detector with gold electrode

Column: 4-mm × 25-cm; 10-µm packing L46

Flow rate: 1 mL/min

Injection volume: 50 µL

Use the following waveform for the electrochemical detector (see *Table 4*).

Table 4

Time (s)	Potential (V)	Integration
0.00	+0.10	—
0.20	+0.10	Begin
0.40	+0.10	End
0.41	–2.00	—
0.42	–2.00	—
0.43	+0.60	—

Table 4 *(continued)*

Time (s)	Potential (V)	Integration
0.44	–0.10	—
0.50	–0.10	—

Analysis

Samples: *Standard solutions*, *System suitability solution*, and *Sample solution*

Integrate the NeuAc, NeuGc, and *Internal standard stock solution* peaks in each chromatogram. For the *Standard solutions*, evaluate the NeuAc and NeuGc peak areas relative to the peak area of the *Internal standard stock solution* as follows.

Area of NeuAc peak/area of *Internal standard stock solution* peak × 100

Area of NeuGc peak/area of *Internal standard stock solution* peak × 100

Generate NeuAc and NeuGc standard curves. Quantify NeuAc and NeuGc in the *Sample solution* by comparison of the relative percent of NeuAc of the *Internal standard stock solution* and the relative percent of NeuGc of the *Internal standard stock solution*, using the standard curves.

Divide the determined NeuAc and NeuGc concentrations by the test sample concentration, and report as molar ratio (i.e., numbers of NeuAc and NeuGc per molecule of test sample).