AAPS Interlaboratory Study

Tier 1 Method: Non-reduced and reduced CE-SDS

Introduction

CE-SDS can be used to quantify monomeric purity, heavy and light chain relative abundance, and glycan occupancy of a monoclonal antibody.

Samples can be reduced or nonreduced prior to analysis. For the non-reduced analysis, the native protein is denatured with SDS prior to separation to mask the protein native charges. An alkylating agent such as iodoacetamide (IAM) may be added to the sample to minimize disulfide shuffling. The non-reduced sample can help quantify fragmentation levels (partial mAbs lacking one or both heavy or light chains) and monomeric purity.

For reduced analysis, the sample is treated with SDS, and either dithiothreitol (DDT) or beta-mercaptoethanol (BME) to reduce the native protein structure (disrupt disulfide bonds). A recombinant mAb can be reduced to glycosylated heavy chain (HC), non-glycosylated heavy chain (NGHC), and a light chain (LC). The reduced form can help to quantify heavy and light chains, heavy chain glycan occupancy, and to determine the content of non-reducible size variants. Peak assignments to LMW combination of heavy (H) and light (L) chains are often based on their apparent molecular weight and migration relative to the monomer.

Important Notes: This protocol is not a Standard Operating Protocol and assumes the required instrument is in good working order and the analysis is performed by experienced user(s). Required calibration or check standards should be run according to the manufacturer instructions. This protocol does not contain all the details of the analysis as it is not specific to the needs of each instrument/make/model. The analyst should rely on their best judgment, routine practices, and knowledge of the technique to conduct the study; this protocol should be used as a guideline for the analysis. Samples should be analyzed immediately after thawing and preparation.

Equipment and Materials List (ensure chemicals are molecular biology grade and are suitable for electrophoresis)

1. CE-SDS separation system with a UV detector
2. SDS-MW Analysis Kit or equivalent
3. Reducing agent (i.e. 2-mercaptoethanol (BME))
4. Alkylating agent (i.e. Iodoacetamide (IAM))
5. Protein samples and formulation buffers supplied by sample originators
    a. Buffers, aliquots of unstressed, stir stressed, light stressed from Shipping #1
6. Pipets and pipette tips as recommended by manufacturer.
7. Cleaning solutions or detergents to run between samples, as recommended by the manufacturer
8. Particle free water/deionized ultra-filtered water or equivalent
9. 0.5 mL Micro-Centrifuge capped vials

Reagents and Solutions

Table 1: The following samples and their buffers will be provided by sample originators. Buffers, aliquots of unstressed, stir stressed and light stressed from Shipping #1 should be used for this analysis. All stressed material were generated at nominally 1 mg/mL.

Proteins	Formulations	Samples
Amgen IgG2	10 mM sodium acetate, pH 5.0	Unstressed, 1 mg/mL Stir stressed, 1 mg/mL Light stressed, 1 mg/mL
NISTmAb IgG1	12.5 mM L-histidine, 12.5 mMol L-histidine HCl, pH 6.0	Unstressed, 1 mg/mL Stir stressed, 1 mg/mL Light stressed, 1 mg/mL

Procedures

Instrument Set Up

1. Prepare the instrument and components as described by the manufacturer.
2. Cut the bare fused silica capillary to desired length before installing it use and install it according to the manufacturer's instructions.
3. Set up the instrument with the parameters from Table 1. This table is modified from the paper by Turner et al.(1).
4. Prepare and run an instrument qualification standard and ensure the results are as expected.

Table 2: Initial conditions for CE-SDS methods, adapted from Turner et al. (Ref 1)

Parameter	**Setting**
Auxillary data channels	Current; max = 300.0 µA
Temperature (cartridge & sample storage)	25.0 °C
Peak detect threshold	2
Peak detect peak width	9
Electropherogram scan data rate	2 Hz
Scan range	190 to 400 nm

Filter	Normal; Peak width: 16 to 25 points
Electropherogram channel	Wavelength = 220 nm; Bandwidth = 10 nm

Sample Preparation

1. Thaw the samples from Shipping #1 and aliquot the Tier 1 vials (for the unstressed, stir, and light stressed mAb samples) as described in the instructions into smaller aliquots for the various methods and freeze until ready to use. Be sure to follow the procedure for vigorously mixing the sample prior to aliquoting.
2. For this analysis, thaw the aliquots designated for CE-SDS measurements (prepared and frozen in #1) by bringing them to room temperature for no more than 15 minutes. It is recommended that these samples be analyzed within 2 hours of thawing.
3. A proper mixing of the sample prior to analyzing is important to eliminate particle clumping. Prior to analysis, mix the aliquots in the following manner pipetting the sample with a 200 uL tip near the bottom of the sample tube 10 times in different directions but not touching the bottom or creating bubbles. Remove sample from near the bottom of the vial, from about ¾ of the depth of the tube from the top of the liquid before dispensing the sample into another container containing the buffer.
4. The mixed samples can be used for analysis but it might be necessary to spin down a few samples. If additional sample preparation is needed, detail it in the data sheet.
5. The final concentration of the protein should be optimally between 0.5 mg/mL and 1 mg/mL and the final volume for each sample should be near 100 µL (it may be a little higher after addition of the internal standard protein). Be sure to prepare all stocks on the same day as analysis.
6. Using the reducing and alkylating agent of choice, prepare the reduced and non-reduced samples keeping in mind the above concentration and sample requirement.
7. Be sure to document this information in the **CE-SDS Data** template.

Sample Measurement

1. Review the **CE-SDS Data** template for the requested information before beginning the measurements.
2. Load samples in triplicates (3 injections from 1 sample prep).
3. Run all the non-reduced samples separately from the reduced samples (see sequence of runs on data collection sheet).

Understanding Results

Data analysis

1. Analyze the electropherograms using the software that accompanies the instrument. Migration times and corrected peak areas should be recorded for the non-reduced and reduced sample analysis.
2. See Table 3 below that describes the data that should be recorded and calculated. Additional data can be recorded, as needed.

Table 3: Measured and calculated assay results for CE-SDS analyses (modified from paper by Turner et al.)

Sample	**Measurand Needed**	**Calculated Quality Parameters**
Non-reduced samples	10 kDa internal standard migration time (min) Monomer migration time (min) Corrected area of monomer Corrected area of fragment peaks: L, H, H:L, H:H, H:H:L, clip species	Monomeric purity (%)
Reduced	10 kDa internal standard migration time (min) Migration time (min) of L and H Corrected area of L, NGH, H, and thioether	Heavy chain relative abundance (%) Light chain relative abundance (%) Glycan occupancy (%) *Thioether relative abundance (RA) (%)

L = light chain; H = heavy chain; H:L = heavy chain:light chain fragment; H:H = heavy chain:heavy chain fragment; H:H:L = heavy chain:heavy chain:light chain fragment; NGH = aglycosylated/non-glycosylated heavy chain

Data Reporting

Instrument parameters used, measured, and calculated data should be entered in the **CE-SDS Data** reporting template**.** If any corrections to any data are performed, report the magnitude and basis of the corrections.

Troubleshooting

For any errors associated with failed system suitability tests, low or unsteady current, broad peaks and poor resolution, check the instruction manual of your CE-SDS system.

Appendix

Use the equations below for the calculations:

$$\text{Monomeric purity (\%)} = \frac{CA(monomer)}{CA(monomer) + \Sigma CA(fragments)} \times 100\%$$

$$\text{Glycan Occupancy (\%) of heavy chain} = \frac{CA(Heavy)}{CA(Heavy) + CA(Nonglycosylated\ Heavy)} \times 100\%$$

$$\text{Heavy Chain RA (\%)} = \frac{CA(Heavy)}{CA(Light) + CA(Heavy) + CA(Nonglycosylated\ Heavy) + CA(Thio)} \times 100\%$$

$$\text{Light Chain RA (\%)} = \frac{CA(Light)}{CA(Light) + CA(Heavy) + CA(Nonglycosylated\ Heavy) + CA(Thio)} \times 100\%$$

$$\text{Thioether RA (\%)} = \frac{CA(Thio)}{CA(Light) + CA(Heavy) + CA(Nonglycosylated\ Heavy) + CA(Thio)} \times 100\%$$

Further Information

For any specific questions regarding this method, please contact Katharina Yandrofski at katharina.yandrofski@nist.gov. Please copy aapsinterlab@nist.gov on your email.

References

1. Turner A, Yandrofski K, Telikepalli S, King J, Heckert A, Filliben J, et al. Development of orthogonal NISTmAb size heterogeneity control methods. Anal Bioanal Chem. 2018;410(8):2095-110. doi: 10.1007/s00216-017-0819-3.