Proteometer-L Kit Instructions for Use

NOVILYTIC

For mAb Titer & Aggregate Analysis

A. General Purpose of Method

This method describes the determination of titer and relative aggregate content of therapeutic mAbs and other proteins that contain the Fc domain of human IgG. It is intended primarily to monitor mAb quality during product development and production utilizing HPLC systems equipped with a fluorescence detector.

The titer result is mAb-dependent and the sample matrix may affect the separations. Therefore, it is recommended that a client-provided reference standard be utilized.

B. Proteometer-L Kit Components

1. Proteometer-L Fluorescent Reagent: Lyophilized material (Mobile Phase L); each vial makes 1 L mobile phase, (store at -20°C until use); (Part no. RGN-PL-001)
2. Proteometer-L Buffer: Solid material (for 1 L mobile phase); (Part no. BUF-PL-001)
3. Proteometer Reconstitution Reagent [dimethyl sulfoxide (DMSO)]: 5 mL; (Part no. REC-PL-001)
4. Proteometer-L Reactor (stored with 100 mM sodium phosphate, pH ≤7, containing 0.02% sodium azide at room temperature); (Part no. RCT-PL-001)

C. Required User Supplied Equipment & Reagents

1. HPLC system equipped with refrigerated autosampler, fluorescence detector, and software capable of electronic data collection and processing.
2. Client-provided mAb reference standard
3. Clarified Harvest (CH) for mAb dilution (mAb-free)
4. Vacuum source for mobile phase filtration
5. Pipette capable of dispensing 20 – 200 µL
6. Pipette capable of dispensing 100 – 1000 µL
7. 1 L beaker
8. Ultra-pure water, 18.2 MΩ cm^{-1} @ 25 °C, or equivalent
9. Media bottles, low actinic (1 L)
10. Acetonitrile, HPLC grade
11. HPLC autosampler vials, maximum recovery
12. Filtration apparatus equipped with 0.22 µm polyethersulfone (PES) membrane, or equivalent

D. Preparation of Mobile Phase L From Supplied Components

1. Remove one vial of Proteometer-L Reagent (Section B.1) from -20°C freezer and allow to equilibrate to room temperature. Reconstitute vial of Proteometer-L Reagent by adding 0.320 mL of Proteometer Reconstitution Reagent from container (Section B.3). Gently mix contents to dissolve and incubate at room temperature in the dark for at least 30 minutes. This stock solution is stable for up to 4 weeks when stored at 2-8°C.
2. Add one packet of Proteometer-L Buffer (Section B.2) to approximately 800 mL of ultra-pure water contained in a 1 L beaker. Ensure quantitative transfer by rinsing out the contents of the packet into the beaker. Stir well until dissolved.
3. Bring to a final volume of 1000 mL with ultra-pure water and filter through the 0.22 µm filter (Section C.12) into the low actinic media bottle (Section C.9).
4. Measure 50 mL of HPLC grade Acetonitrile (Section C.10) and add to filtered mobile phase.
5. Transfer 250 µL of the solution from Section D.4 to a maximum recovery autosampler vial (Section C.11) and place in refrigerated autosampler (Blank A).
6. Quantitatively transfer the solution of reconstituted Mobile Phase L (Section D.1) to

the bottle of mobile phase prepared in Step 4 by rinsing by rinsing the vial into the bottle using the buffer. Mix by swirling. This is Mobile Phase L (treat as light sensitive, recommended for use within 48 hours). Mobile phase preparation may be proportioned as needed. Scaling should maintain the same ratio of 0.32 mL stock solution to 1050 mL mobile phase (e.g. 0.032 mL stock solution to 105 mL mobile phase).

NOTE: System must be flushed thoroughly with ultra-pure water prior to installation of Proteometer-L Kit (Section E.1)

E. HPLC System Conversion – Proteometer-L Setup and Equilibration

1. Prior to installation of Proteometer-L Kit:
   a. Install ultra-pure water vessel to pump where Mobile Phase L will flow.
   b. Prime pump system for about 3 minutes to remove any residual contaminants and/or organic components from the system.
   c. Set the flow rate to approximately 0.3 mL/min and flush for at least 30 minutes.
2. With no reactor installed:
   a. Install Mobile Phase L on the HPLC system and prime.
   b. Set flow to 0.2 mL/min for 10 minutes with no reactor installed to clear lines.
3. With the flow set to 0 mL/min, install the Proteometer-L Reactor (Section B.4) on the HPLC system.
4. Set flow to 0.1 mL/min and increase to 1.0 mL/min in 0.1 mL/min increments (15 seconds per increment).
5. Allow the system to equilibrate and condition the reactor until baseline is stable (at least 1 hour) with fluorescence monitoring at Ex 450 nm/Em 520 nm. The complete list of instrument parameters is as follows:
   - Mobile Phase: Mobile Phase L (Section D.6)
   - Flow Rate: 1 mL/min
   - Maximum Pressure of Reactor: 5800 psi (400 bar)
   - Mode: Isocratic
   - Column Oven Temperature: Ambient
   - Auto-Sampler Temperature: 4°C
   - Injection Volume: Variable
   - Run Time: 10 minutes
   - Fluorescence Wavelength Settings: Excitation 450 nm / Emission 520 nm
   - Detector Settings: Factory Default

F. Preparation of mAb Samples, Standards, and Controls

Clarified harvest (CH) may be collected at desired time points from a mAb production run prior to, or in parallel with, the analyses. Please use the method that you would normally use to remove the cells/clarify broth. You may store CHs in aliquots as you typically would until the day of analysis. Before transferring to an autosampler vial, centrifuge at 3000 x *g* for 30 seconds to remove any particulates. Please note that you will also require an aliquot of mAb-free CH as a control to monitor interference from non-mAb broth components (Blank B). mAb-free CH may be obtained from a culture transformed with empty vector or by depleting mAb from an aliquot of the test CH using immobilized Protein A in batch mode.

Every mAb sample will contain aggregates (high molecular weight species, HMW) and monomers. Percent aggregate content may vary as a function of the amount of total mAb injected (HMW plus monomer). It is therefore recommended that differences in aggregate content are monitored by injecting samples containing equivalent amounts of total mAb.

1. Samples: Prepare sufficient volume of CH containing the test mAb to perform desired injections. Transfer each sample to a maximum recovery autosampler vial and place in refrigerated autosampler.

2. Standard: Prepare sufficient volume of a 1 mg/mL solution of client-provided mAb by dilution with mAb-free CH* to perform desired injections. Centrifuge at 3000 x *g* for 30 seconds to remove any particulates.
   Transfer aliquots of the prepared mAb standard in CH to maximum recovery autosampler vials and place in refrigerated autosampler.
   *Use caution while pipetting as CH may be viscous.

3. Controls: Transfer 250 µL of mAb-free CH to a maximum recovery autosampler vial and place in refrigerated autosampler (Blank B). If the standard is prepared in buffer, transfer 250 µL of this buffer to a maximum recovery autosampler vial and place in refrigerated autosampler (Blank C).

G. Verification of Instrumental Parameters (Fluorescence Detector Settings)

Each detector has its own strength of fluorescent signal which is dependent on factors such as instrument make and age of detector and lamp.

1. With the detector set to the factory default parameters, perform repeated injections of mAb in CH (Section F.2) at the highest µg load you expect to observe in your samples, adjusting detector settings after each injection, until the signal is maximized without saturating the detector. This should be your detector setting for the duration of testing.

H. Data Acquisition

1. Perform injections of Blank A (Section D.5) and Blank B (or C, if applicable). Evaluate the chromatograms and if peaks are present in Blank A, take appropriate measures to clear up the system of potential contaminants.

2. For titer analysis, inject variable amounts of mAb (Section F.2) to generate a standard curve, establishing the linear dynamic range, using the sum of the monomer and HMW peak areas.

3. Inject samples (Section F.1) Adjust sample volume, if necessary, to ensure that sample load falls within the established linear dynamic range.

4. For relative aggregate analysis, inject samples (Section F.1) at consistent concentrations.

I. Processing and Reporting

1. Evaluate chromatograms from Blanks A and B (or C, if applicable). The profile should be absent from interfering peaks at the retention times corresponding to HMW and monomer mAb species. Peaks present in Blank B or C at these retention times must be subtracted from the corresponding sample peak area. If no significant interference is observed, this control may be omitted moving forward.

2. Create a processing method to consistently integrate HMW and monomer peaks in all injections of your mAb. Retention times are dependent on the mAb and HPLC system in use. In addition to the mAb monomer and dimer peaks, mAb trimer, tetramer, etc. peaks may also be observed. HMW peak area is reported as the sum of dimer, trimer, tetramer, etc. peak areas.

3. Use the standard curve (Section H.2) to calculate the concentration of the mAb in the unknown CH samples.

4. Calculate the aggregate content as follows:

$$\%\ \text{Aggregate content} = \frac{100 * \text{area of HMW mAb peaks}}{\text{total mAb peak area}}$$

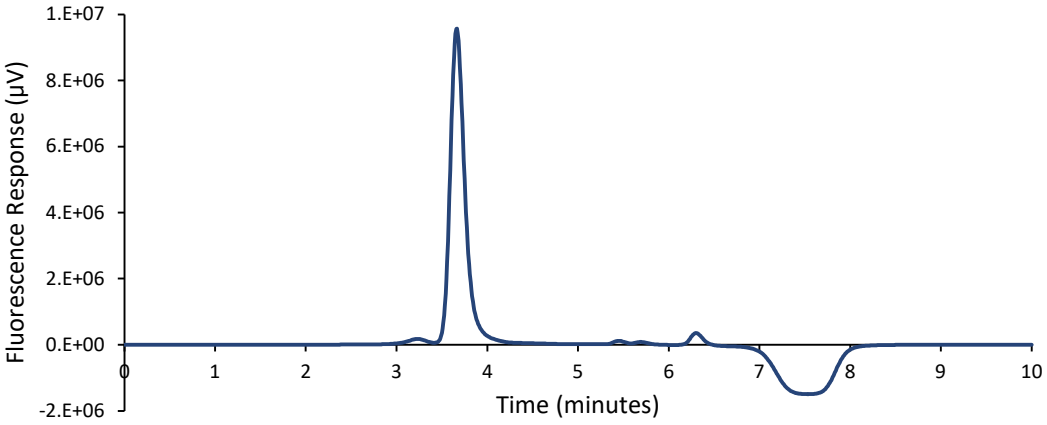


Figure 1. Example Chromatogram: NIST mAb at 16µg load

J. Proteometer-L Reactor Cleaning

In cases of reduced performance, the reactor may be restored using select cleaning solutions after rinsing the reactor with at least five bed volumes of ultra-pure water. The reactor should always be flushed in the direction of the designated flow path, maintaining a low flow rate to avoid excessive backpressure. If suspected contaminants are strongly adsorbed, a solution of 0.5 M guanidine hydrochloride may be used as the cleaning solution. If the suspected contaminants are hydrophobic in nature, solutions containing up to 50% HPLC-grade organics (i.e., EtOH, IPA, or MeOH) may be used instead. Basic contaminants may be flushed out with 0.1% TFA, formic acid, or acetic acid in 15% acetonitrile. A minimum of 20 column volumes of the cleaning solution must be used. Choose the most appropriate cleaning solution for your suspected contaminant, prior to trying others and rinse the reactor with a minimum of five bed volumes of ultra-pure water after flushing with every cleaning solution. If contamination persists, switch cleaning solutions. If all cleaning options have been exhausted, replace the reactor.

K. Proteometer-L Reactor Extended Storage Conditions

For extended reactor storage, equilibrate the reactor with approximately 20 mL of 100 mM sodium phosphate, pH ≤ 7, containing 0.02% sodium azide at room temperature. As an alternative, the reactor may be stored in 20% MeOH/ultra-pure water; however, the reactor MUST be washed thoroughly (approximately 70 mL) with ultra-pure water prior to introduction of the organic component (approximately 20 mL). Please utilize a lower flow rate (e.g. 0.6 mL/min) when running the organic solution to accommodate the higher back pressure observed with MeOH. Note: the same water wash should be performed before re-introducing the reactor to kit operating instructions.

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