Proteometer-CV Kit
For mAb Charge Variant Analysis
Quaternary Pump Configuration

Instructions for Use

NOVILYTIC

NOTE: Please Refer to Instructions for Instrument Setup Prior to Proceeding with these Instructions

A. General Purpose of Method

This method describes the determination of mAb charge variants and titer of therapeutic mAbs and other proteins that contain the Fc domain of human IgG. It is intended primarily to monitor mAb quality directly in clarified fermentation broth without Protein A purification during product development. The method utilizes HPLC systems equipped with two pumps and a fluorescence detector.

The mobile phase gradient for optimal separation of charge variants is mAb dependent. Additionally, the sample matrix may affect the chromatography of some mAbs, for example, those with isoelectric points at or below pH 7.0. The recommended workaround for analysis of such mAbs is provided at the end of the general use instructions.

B. Proteometer-CV Kit Components

1. Proteometer-CV Fluorescent Reagent: Lyophilized material (Mobile Phase CV-MP Component F); 5 vials (store at -20°C until use) (Part no. RGN-PCV-001)
2. Proteometer-CV MD Buffer: Solid material, 20 packets (Part no. BUF-PCV-001)
3. Proteometer-CV Mobile Phase A Salts: Solid material, 25 packets (Part no. BUF-PCV-002)
4. Proteometer-CV Mobile Phase B Salts: Solid material, 5 containers (Part no. BUF-PCV-003)
5. Proteometer-CV Reconstitution Reagent (DMSO): 5 mL; (Part no. REC-PCV-001)
6. Proteometer-CV Reactor (Part no. RCT-PCV-001)
7. Connector tubing (Part no. TUB-PCV-001)
8. Proteometer-CV Tee (Part no. TEE-PFV-001)
9. Proteometer-CV Union (Part no. ZDV-PFV-001
10. Proteometer-CV MD Unit (Part no. MD-PCV-001)

C. Required User Supplied Equipment & Reagents

1. Dual pump HPLC system equipped with refrigerated autosampler, fluorescence detector, and software capable of electronic data collection and processing. **The entire system must be flushed thoroughly with ultra-pure water prior to installation of Proteometer-CV Kit (see Instructions for Instrument Setup for details)**
   Note: The dual pump HPLC system has one quaternary pump (primary) pump connected to the autosampler on the HPLC
2. Client-provided mAb reference standard
3. Clarified Fermentation Broth (CFB) for mAb dilution (mAb-free)
4. Vacuum source for mobile phase filtration
5. Pipet capable of dispensing 20 – 200 µL
6. Pipet capable of dispensing 100 – 1000 µL
7. 1 L beaker, 250 mL beaker
8. Ultra-pure water, 18.2 MΩ cm^{-1} @ 25 °C, or equivalent
9. Microcentrifuge tubes, low binding, 1.5 mL (Eppendorf Cat. No. 0030108442, or equivalent)
10. Media bottles, three
11. Low actinic media bottle (1 L), one
12. Acetonitrile, HPLC grade or equivalent
13. HPLC autosampler vials
14. Filtration apparatus equipped with 0.22 µm polyethersulfone (PES) membrane, or equivalent

D. Quaternary Pump Configuration – Preparation of Primary Pump Mobile Phases

1. **CV-MPAq**

a. Reconstitute five packets of Proteometer-CV Mobile Phase A salts (Section B.3) in approximately 200 mL ultra-pure water contained in a 250 mL beaker. Ensure quantitative transfer by rinsing out the contents of the packets into the beaker. Stir well until dissolved.

b. Check the pH and adjust to 6.0 ± 0.03 with NaOH or HCL if necessary. Bring to a final volume of 250 mL with ultra-pure water and filter through a 0.22 µm filter (Section C.14) into a media bottle (Section C.10). This is CV-MPAq. Mobile phase preparation may be scaled up, if necessary.

2. **CV-MPB**

a. Add one container of Proteometer-CV Mobile Phase B salts (Section B.4) to approximately 200 mL ultra-pure water contained in a 250 mL beaker. Ensure quantitative transfer by rinsing out the contents of the container into the beaker. Stir well until dissolved.

b. Bring to a final volume of 250 mL with ultra-pure water and filter through a 0.22 µm filter (Section C.14) into a media bottle (Section C.10). This is Mobile Phase CVS-MPB. Mobile phase preparation may be scaled, if necessary.

3. **CV-MPC**

   a. Prepare CV-MPC by filling a 1 L media bottle with ultrapure water, 18.2 MΩ cm^{-1} @ 25 °C.

E. Preparation of Secondary Pump Mobile Phase CV-MDf

1. Reconstitute one vial of Proteometer-CV Fluorescent Reagent (Section B.1) by adding 0.5 mL of DMSO from ampoule (Section B.5). Gently mix contents to dissolve and

incubate at room temperature in the dark for at least 30 minutes (this stock solution is stable up to 4 weeks at 2-8 °C).

2. Reconstitute four packets of Proteometer-CV MD Buffer (Section B.2) to approximately 800 mL 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 a 0.22 µm filter (Section C.14) into the low actinic media bottle (Section C.10). This is Proteometer-CV Buffer 1.
4. Measure 50 mL of HPLC grade acetonitrile (Section C.12) and combine with filtered Proteometer CV Buffer 1 prepared in section E.3.
5. Quantitatively transfer the solution of reconstituted Proteometer Reagent Component A (Step E.1) to Proteometer CV Buffer1/acetonitrile mixture prepared in Section E.4. Mix by swirling. This is CV-MDf (recommended for use within 48 hours). Mobile phase preparation may be scaled, if necessary.

F. HPLC Configuration and Proteometer-CV Setup

Please wash the system thoroughly with water before executing the following steps. For optimal resolution of peaks on the chromatogram, it is important to keep the dead volume of the system to a minimum by using zero dead volume connector provided and short connection tubing.

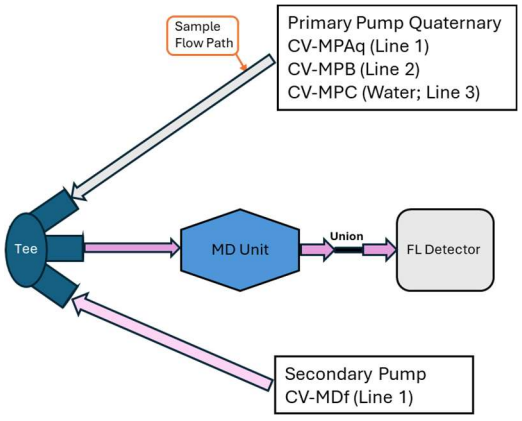
Please follow the instructions applicable to your HPLC.

1. Quaternary Pump Configuration

a. Install Mobile Phase CV-MPAq (Section D.1), CV-MPB (Section D.2), and CV-MP C (Section D.3) on the HPLC system primary pump, lines 1, 2, & 3, respectively and prime thoroughly.

b. Install Mobile Phase CV-MDf (Section E) on the HPLC system secondary/auxiliary pump and prime thoroughly.

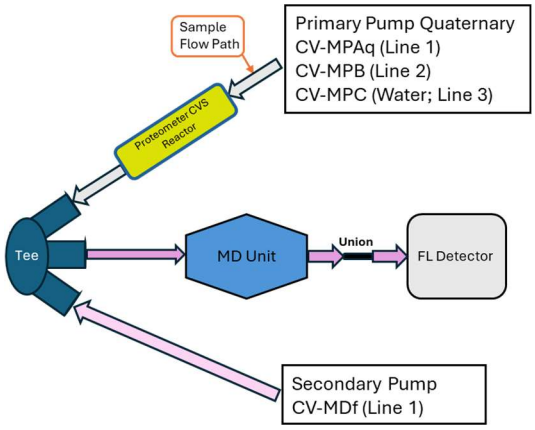
c. With the flow rate set to 0 mL/min, install the Tee, MD unit, and union connector as shown in the diagram.



d. Set the mobile phase contribution on the primary pump to 33% of each mobile phase and set the mobile phase of the secondary pump to 100% contribution for Mobile Phase CV-MDf.

e. Set both pumps to 0.1 mL/min and equilibrate system at these conditions for approximately 30 minutes.

f. With the flow rate set to 0 mL/min, install the Proteometer-CV reactor with the outlet of the column plumbed to the inlet of the Tee using the connector tubing as shown in the diagram.



g. Condition the Proteometer-CV reactor and system components for 20 minutes at the conditions as set in section F.1.d (above).

h. Ramp both the primary and secondary pumps to 0.6 mL/min in 0.1 mL/min increments every 15 seconds.

To avoid damage to the Proteometer-CV reactor, the flow rate of the primary pump must always be greater than or equal to the flow rate of the secondary pump

i. Allow the system to equilibrate until baseline is stable with fluorescence monitoring at Excitation 488 nm / Emission 520 nm. Inspect plumbing for leaks and tighten or re-seat leaky fittings where necessary.

j. The quaternary gradient profile is dependent on the mAb of interest. To optimize charge variant separations, it is recommended to screen gradients beginning with a 0 to 50% CV-MPB over 15 minutes followed by a 2-minute wash at 80% CV-MPB Re-equilibrate system at initial conditions for approximately four minutes prior to initiating subsequent injections. Percent CV-MPA is maintained at 20% throughout the entire run. Flow rates over both pumps are set to 0.6 mL/min.

k. The following gradient profile performed well for most mAbs:

Quaternary (Primary) Pump					Secondary Pump (0.6 mL/min)
Time (min)	Flow mL/min	CV-MPAq %	CV-MPB %	CV-MPC %	CV-MDf %
0.00	0.6	20	0	80	100
2.00	0.6	20	0	80	100
2.01	0.6	20	7	73	100
4.00	0.6	20	7	73	100
19.00	0.6	20	14	66	100
19.01	0.6	20	80	0	100
21.01	0.6	20	80	0	100
21.02	0.6	20	0	80	100
25.00	0.6	20	0	80	100

l. The complete list of instrument parameters is as follows:

- Primary Pump (Quaternary):
  - Line 1: CV-MPAq
  - Line 2: CV-MPB
  - Line 3: CV-MPC

ii. Secondary Pump:

- Line 1: CV-MDf

- Flow Rate: 0.6 mL/min on both Primary and Secondary pumps
- Maximum Pressure of Reactor: 6000 psi (414 bar)
- Mode: Gradient
- Column Oven Temperature: Ambient
- Auto-Sampler Temperature: 4°C
- Injection Volume: Variable
- Run Time: 25 minutes
- Fluorescence Wavelength Settings: Excitation 488 nm / Emission 520 nm
- Detector Settings: Factory Default

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 factory default parameters, perform repeated injections of standard mAb in CFB (Section H.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. Preparation of mAb Samples, Standards, and Controls

The system should be free of potential contaminants, as indicated by the absence of peaks in the chromatograms from Blank A (or Blank C, if applicable). If peaks are present in Blank A (or Blank C), appropriate measures should be taken to clear the system of potential contaminants.

Peaks present in Blank B at the retention time of the mAb must be subtracted from the corresponding sample peak area. If no significant interference is observed, this control may be omitted in future analysis in these matrices.

mAb samples: Fermentation broth 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 the broth. You may store each day's CFBs in aliquots as you typically would until the day of analysis. Before transferring to an autosampler vial, centrifuge at 2000 x *g* for 30 seconds to remove any particulates.

mAb-free clarified fermentation broth (CFB): Please note that you will also require an aliquot of mAb-free CFB as a control to monitor interference from non-mAb broth components. mAb-free CFB may be obtained from a culture transformed with empty vector or by depleting mAb from an aliquot of the test CFB using immobilized Protein A in batch mode.

For every mAb, there is a linear dynamic range in which the relative ratio of acidic:main:basic charge variants is insensitive to the amount of mAb injected. It is recommended that comparative studies of charge variant profile be performed on sample injections containing mAb amounts within this linear dynamic range or on a constant amount of total mAb.

CFB components can compete with and affect binding of certain mAbs to the Proteometer-CV Reactor. This has been observed for mAbs with low pI and for IgG2 mAbs. Adjustment of the sample pH to approximately 6.0 or less improves binding to the Proteometer-CVS Reactor. Reducing sample volume (less CFB proteins) might also help.

CFB may be viscous. Use care when pipetting CFB.

Samples may be injected from autosampler vials or multiwell plates

Prepare controls, standards and samples as follows:

1. Controls:

Blank A: For the Quaternary Pump configuration, Blank A is a 1:4 (v/v) mixture of CV-MPAq with CV-MPC. (Section H.2)

Blank B: mAb-free CFB

Blank C: If the standard is prepared in buffer, an aliquot of this buffer is used as Blank C

2. Standard: Prepare sufficient volume of a 1 mg/mL solution of the test mAb (Section C.2) by dilution with mAb-free CFB to perform desired injections. Prior to use, centrifuge at 2000 x *g* for 30 seconds to remove any particulates.

3. Samples: Transfer sufficient volume of particulate-free CFB containing the test mAb to perform desired injections for charge variant analysis into autosampler vial(s)

I. Data Acquisition

1. For the establishment of a linear dynamic range for analysis, inject variable amounts of mAb (Section H.2) to generate a standard curve using the sum of the acidic, main, and basic peak areas; this should be repeated daily.

2. Inject samples (Section H.3) from desired time points and adjust sample load if necessary to fall within established linear dynamic range.

3. Inject samples at consistent concentrations for optimal method reproducibility.

J. Processing and Reporting

1. Create a processing method to consistently integrate acidic, main, basic, and total peak areas in all injections of your mAb. Retention times are unique to the mAb and HPLC system in use.

2. Use the corresponding total peak areas determined in the standard curve (Section I.1) to calculate the concentration of the unknown mAb in the fermentation samples.

3. Calculate the percent acidic content as follows:

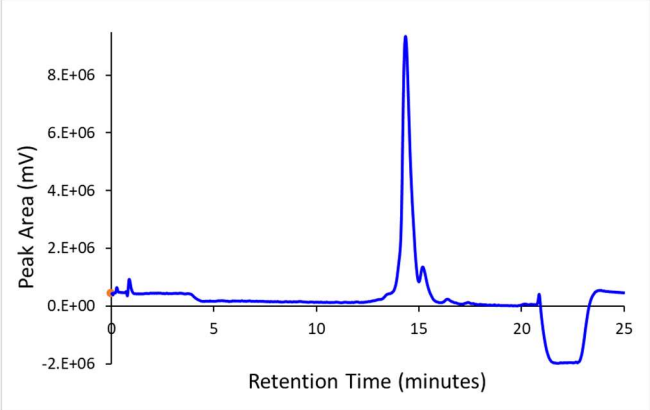
$$\% \text{ Acidic content} = \frac{100 * \text{area of acidic mAb peaks}}{\text{total mAb peak area}}$$

4. Calculate the percent main content as follows:

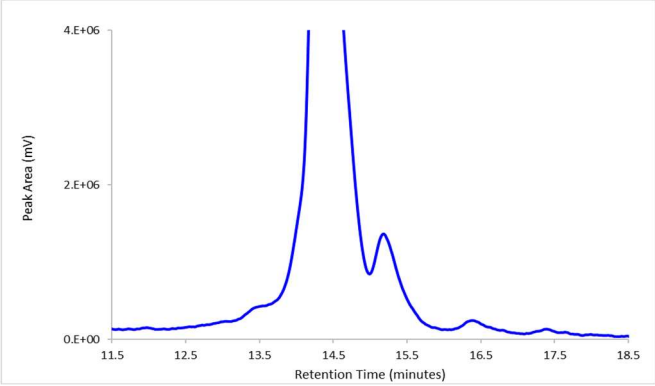
$$\% \text{ Main content} = \frac{100 * \text{area of main mAb peak}}{\text{total mAb peak area}}$$

5. Calculate the percent basic content as follows:

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



Example Chromatogram (Full Scale): NIST mAb, 16µg



Example Chromatogram (Expanded View): NIST mAb, 16µg

K. Proteometer-CV Reactor Extended Storage Conditions

Flush the reactor with at least 4 mL of ammonium sulfate containing 0.02% sodium azide at a flow rate of 0.25 mL/min (~16 min.). Cap ends and store at room temperature.

Ordering Information

Description	Part Number
300 inj. Proteometer-CV Kit (1-Reactor, 1-MD unit, 5 reagent vials, 20 MD buffer packets, 25 mobile phase A salts, 5 vials mobile phase B salts, 1 vial reconstitution reagent (DMSO))	KIT-PCV-003-01
Proteometer-CV Reactor	RCT-PCV-001
Proteometer-CV MD Unit	MD-PCV-001
Proteometer-CV Reagent (5 vials)	RGN-PCV-001
Proteometer-CV Mobile Phase A Salts (25 buffer packets)	BUF-PCV-001
Proteometer-CV Mobile Phase B Salts (5 buffer packets)	BUF-PCV-002
Proteometer-CV MD Buffer (20 buffer packets)	BUF-PCV-003
Proteometer-CV Mixing Tee	TEE-PFV-001
Proteometer-CV Tubing Union	ZDV-PFV-001

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