# Proteometer-CV Kit
# For mAb Charge Variant Analysis
# Quaternary Pump Configuration
# Instructions for Use

## 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 in section H.

## B. Proteometer-CV Kit Components

1. Proteometer-CV Fluorescent Reagent: Lyophilized material (Mobile Phase CV-MP Component F); **(store at -20°C until use)**, (Part no. RGN-PCV-001)
2. Proteometer-CV MD Buffer: Solid material, (Part no. BUF-PCV-003)
3. Proteometer-CV Mobile Phase A Salts: Solid material, (Part no. BUF-PCV-001)
4. Proteometer-CV Mobile Phase B Salts: Solid material, (Part no. BUF-PCV-002)
5. Proteometer Reconstitution Reagent [dimethyl sulfoxide (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, maximum operating pressure 725 psi or 50 bar, (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 (Section**
   *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. Shake each packet to settle contents to bottom before opening and transfer quantitatively to the beaker by rinsing the empty packets into the beaker. Stir well until dissolved. Check the pH and adjust to 6.0 ± 0.03 with NaOH or HCL if necessary

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 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. Remove one vial of Proteometer-CV Fluorescent Reagent (Section B.1.) from -20 °C freezer and allow to equilibrate to room temperature. Reconstitute vial of Proteometer-CV Fluorescent Reagent by adding 0.5 mL of Proteometer Reconstitution Reagent from container (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).
4. Measure 50 mL of HPLC grade acetonitrile (Section C.12) and combine with filtered buffer prepared in section E.3.
5. Quantitatively transfer the solution of reconstituted Proteometer Reagent Component A (Step E.1) to buffer/acetonitrile mixture prepared in Section E.4 by rinsing the vial into the bottle using the buffer. Mix by swirling. This is CV-MDf (treat as light sensitive, recommended for use within 48 hours). Mobile phase preparation may be scaled, if necessary.

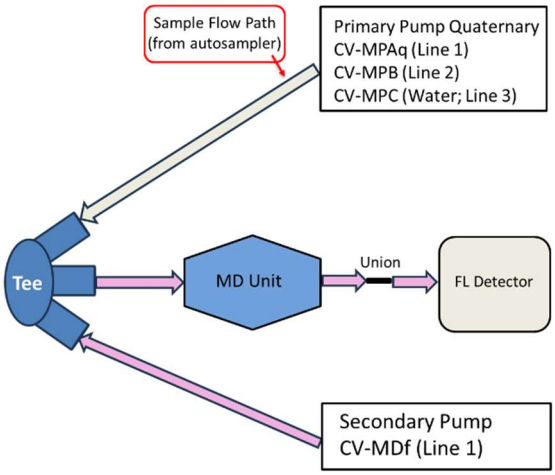
## F. HPLC Configuration and Proteometer-CV Setup

*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.*

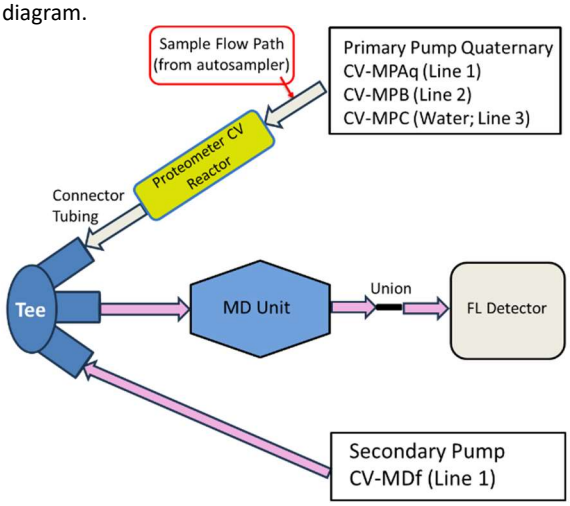
*The following instructions are provided for HPLC system with a quaternary primary pump in line with the autosampler. Please follow the instructions applicable to your HPLC.*

**Quaternary Pump Configuration**

1. Prior to installation of Proteometer-CV Kit:
   Install ultra-pure water vessel to pump lines where mobile phases will flow (three lines on the primary pump in line with the autosampler, one line on the secondary/auxiliary pump). Prime each line for about 3 minutes to remove any residual contaminants and/or organic components from the system. Set the flow rate to approximately 0.3 mL/min and flush for at least 30 minutes.
2. On the primary pump in line with the autosampler, install Mobile Phase CV-MPAq (Section D.1), CV-MPB (Section D.2), and CV-MP C (Section D.3) on lines 1, 2, & 3, respectively and prime thoroughly.
3. Install Mobile Phase CV-MDf (Section E) on the secondary/auxiliary pump and prime thoroughly.
4. With the flow rate set to 0 mL/min, install the Tee, MD unit, and union connector as shown in the diagram.




5. 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.
6. Set both pumps to 0.1 mL/min and equilibrate system at these conditions for approximately 30 minutes.
7. 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.




*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.*

8. Set the mobile phase contribution on the primary pump to 20% CV-MPAq and 80% CV-MPC, and the secondary pump to 100% contribution for Mobile Phase CV-MDf. Set the flow rate of each pump to 0.1 mL/min and condition the Proteometer-CV reactor and system components for 20 minutes.
9. Ramp both the primary and secondary pumps to 0.6 mL/min in 0.1 mL/min increments every 15 seconds.
10. 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.
11. 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. If chromatograms do not show peaks or the detector appears to be saturated, it may be necessary to adjust fluorescence detector settings. Refer to section G.1. to maximize sensitivity without saturating the detector.

**12.** 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 |

**13.** The complete list of instrument parameters using quaternary pump configuration is as follows:

- Primary Pump (Quaternary):
  - Line 1: CV-MPAq
  - Line 2: CV-MPB
  - Line 3: CV-MPC
- 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)
- Maximum Pressure of MD unit: 725 psi (50 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 may be indicated by a breakthrough peak of unretained mAb which is not present in Blank B, the mAb-free CFB injection. 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-CV Reactor. Reducing sample volume (less CFB proteins) may also improve binding. Contact info@novilytic.com for additional assistance if needed.*

*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 of the total mAb peak area, which is 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:

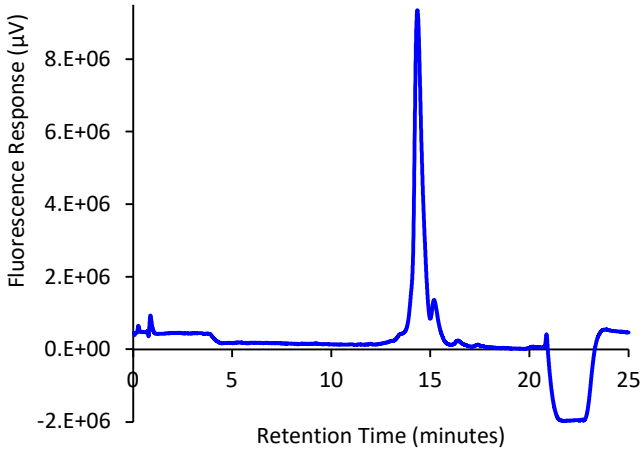
$$\% \text{ Acidic content} = \frac{100 * \text{area of acidic mAb peaks}}{\text{total mAb peak area}}$$

4. Calculate the percent main content as follows:

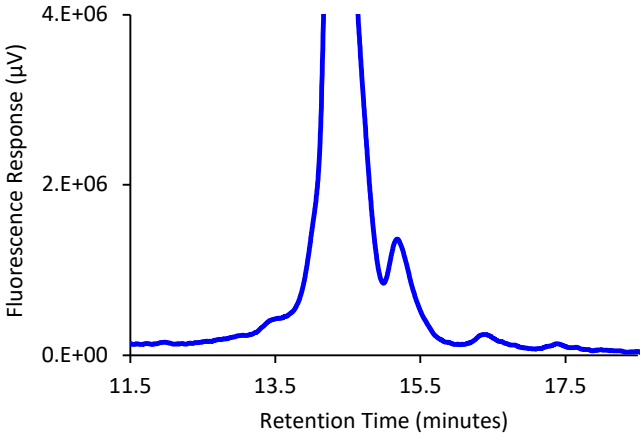
$$\% \text{ Main content} = \frac{100 * \text{area of main mAb peak}}{\text{total mAb peak area}}$$

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



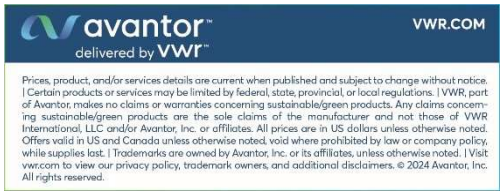

Example Chromatogram (Expanded View): NIST mAb, 16µg

## K. Proteometer-CV Reactor Extended Storage Conditions

Storage solution for the Proteometer-CV Reactor can be prepared with 5 mL of CV-MPAq (Section D.1), 5 mL HPLC grade Ethanol, and 15 mL ultra-pure water for a total volume of 25 mL. This volume may be scaled as necessary. Flush the reactor with at least 16 mL of storage solution at a flow rate of 0.3 mL/min (~55 min.). Cap ends and store at room temperature.

## Ordering Information

| Description | Part Number |
|---|---|
| 60 Injection Proteometer-CV Starter Kit (1-Reactor, 1-MD unit, 4 MD buffer packets, 5 packets mobile phase A salts, 1 container mobile phase B salts, 1 container reconstitution reagent (DMSO)) to be ordered with KIT-PCV-001-01B | KIT-PCV-001-01A |
| 60 Injection Proteometer-CV Starter Kit Reagent (1-Reagent Vial) to be ordered with KIT-PCV-001-01A | KIT-PCV-001-01B |
| 240 Injection Proteometer-CV Consumables Kit (4 reagent vials, 16 MD buffer packets, 20 packets mobile phase A salts, 4 containers mobile phase B salts, 1 container reconstitution reagent (DMSO)) | KIT-PCV-0005-01 |
| 300 Injection Proteometer-CV Full Kit (1-Reactor, 1-MD unit, 20 MD buffer packets, 25 packets mobile phase A salts, 5 containers mobile phase B salts, 1 container reconstitution reagent (DMSO)) to be ordered with KIT-PCV-003-01B | KIT-PCV-003-01A |
| 300 Injection Proteometer-CV Full Kit Reagent (5-reagent vials) to be ordered with KIT-PCV-003-01A | KIT-PCV-003-01B |
| Proteometer-CV Reactor | RCT-PCV-001 |
| Proteometer-CV MD Unit | MD-PCV-001 |
| Proteometer-CV Reagent (4 vials) | RGN-PCV-001 |

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