Proteometer-UFT Kit Instructions for Use

For mAb Ultra-Fast Titer Analysis
Dual-Pump Method (preferred)

NOVILYTIC

A. General Purpose of Method

This method describes the determination of titer of therapeutic mAbs and other proteins that contain the Fc domain of human IgG. It is intended primarily to monitor mAb concentration in growth media during clone selection and product development utilizing HPLC systems equipped with a fluorescence detector.

The titer result is mAb dependent and the sample matrix may affect the peak response, therefore, it is recommended that a client-provided reference standard be utilized. However, NmAb in a buffer such as 12.5 mM L-Histidine, pH 6.0 may be used as an alternative reference standard for monitoring changes in mAb titer.

B. Proteometer-UFT Kit Components

1. Proteometer-UFT Fluorescent Reagent (Part no RGN-PUF-001): Store at -20 °C until use
2. Proteometer-UFT Buffer (Part no. BUF-PUF-001): Store at room temperature
3. Proteometer-UFT Reconstitution Reagent [dimethylformamide (DMF)] (Part no. REC-PUF-001): Store at room temperature
4. Proteometer-UFT MD Unit (Part no. RCT-PUF-001)
5. Proteometer-UFT Tee (Part no. TEE-PFV-001)
6. Proteometer-UFT Union (Part no. ZDU-PFV-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
   NOTE: System must be flushed thoroughly with ultra-pure water prior to installation of Proteometer-UFT Kit (Section E.1)
2. Client-provided mAb reference material or NIST mAb (NmAb RM 8671)
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. 500 mL beaker
8. Ultra-pure water, 18.2 MΩ cm^{-1} @ 25 °C, or equivalent
9. Acetonitrile, HPLC Grade or equivalent
10. Microcentrifuge tubes, low binding, 1.5 mL (Eppendorf Cat. No. 0030108442, or equivalent)
11. One 500 mL media bottle
12. Two 250 mL media bottles, one low actinic
13. HPLC autosampler vials, or multi-well plate
14. Filtration apparatus equipped with 0.22 µm polyethersulfone (PES) membrane, or equivalent

D. Preparation of Mobile Phases, UFT-MP and UFT-MPf, From Supplied Components

Two mobile phases, UFT-MP and UFT-MPf (UFT-MP supplemented with Proteometer-UFT Fluorescent Reagent) are required for this method. One Proteometer-UFT Buffer packet will make 250 mL of each mobile phase, which is sufficient for approximately 330 injections.

1. *Preparation of Mobile Phase UFT-MPf Component F.* Reconstitute one vial of Proteometer-UFT Fluorescent Reagent (Section B.1) by adding 0.45 mL DMF from ampoule (Section B.3). Gently mix vial contents to dissolve and incubate at room temperature in the dark for at least 30 minutes. This is now Mobile Phase UFT-MPf Component F, and it is usable for up to 4 weeks if stored at -15 to -20 °C.

2. *Preparation of Mobile Phase UFT-MP.* Using scissors, cut open and add one packet of Proteometer-UFT Buffer (Section B.2) to approximately 400 mL ultra-pure water

contained in a 500 mL beaker. Ensure quantitative transfer by rinsing out the contents of the packet into the beaker. Stir well until dissolved. Bring the solution to a final volume of 500 mL with ultra-pure water and filter through the 0.22 µm filter into a 500 mL media bottle. Add 25 mL of acetonitrile and mix well by swirling. This is mobile phase UFT-MP, which is further split into two aliquots.

a. Measure a 250 mL aliquot of UFT-MP and transfer into a 250 mL low actinic media bottle for preparation of mobile phase UFT-MPf.
b. Transfer the remaining mobile phase UFT-MP into a separate 250 mL media bottle for use on the HPLC system.
c. Transfer 250 µL of mobile phase UFT-MP to a maximum recovery autosampler vial (Section C.13) and place in refrigerated autosampler (Blank A).

3. *Preparation of Mobile Phase UFT-MPf*. Prepare UFT-MPf by quantitatively transferring Mobile Phase UFT-MPf Component F (Section D.1) to the low actinic media bottle containing 250 mL UFT-MP (Section D.2.a) Mix by swirling. Mobile phase UFT-MPf volume may be scaled as needed.

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

The HPLC configuration for Proteometer UFT is shown in Figure 1. It is important to keep the dead volume of the system at a minimum by using the zero dead volume union provided (Section B.6) and short connecting tubing. By minimizing the flow-path, it is ensured that the sample peak is captured within a run-time of 1.5 minutes.

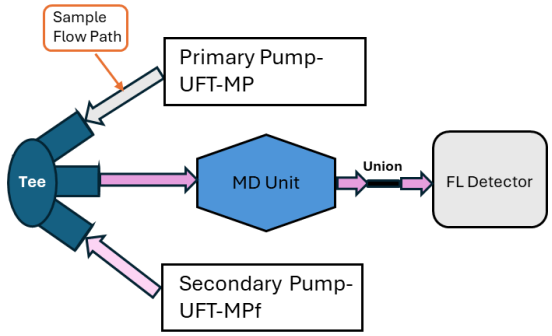

Figure 1. Proteometer-UFT Dual Pump Installation

1. Prior to installation of the Proteometer-UFT Kit:
   a. Install ultra-pure water vessels on the primary and auxiliary pumps where mobile phases will flow.
   b. Prime pump systems 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 MD unit installed:
   a. Install Mobile Phase UFT-MP on the primary pump that is in line with the autosampler system.
   b. Install Mobile Phase UFT-MPf on the secondary/auxiliary pump that is in a separate flow path from the sample flow path.

3. Install the Tee (Section B.5), and the MD Unit (Section B.4) on the HPLC system as shown in the diagram. The lines from the primary and secondary pumps should connect to the outer ports of the Tee and the center port of the Tee should be plumbed to the MD Unit. The MD unit is plumbed directly to the fluorescence detector.

4. Prime the HPLC pumps and equilibrate the HPLC system at initial method conditions of 0.3 mL/min on each pump for approximately 10 minutes.

5. Allow system to equilibrate until baseline is stable with fluorescence monitoring at Ex 552 nm /Em 578 nm. Flow rate may be initially set to 0.1 mL/min until fluorescence signal stabilizes. The complete list of instrument parameters is as follows:
   - Flow Rate: 0.3 mL/min on both primary and secondary/auxiliary pumps
   - Maximum Pressure of MD Unit: 1200 psi (82.7 bar)
   - Mode: Isocratic
   - Column Oven Temperature: Ambient
   - Auto-Sampler Temperature: 4°C
   - Injection Volume: 1-10 µL, constant for all samples in a run (10 µL recommended)
   - Run Time: 1.5 minutes
   - Fluorescence Wavelength Settings: Excitation 552 nm / Emission 578 nm
   - Detector Settings: Factory Default

F. Preparation of mAb Samples, Standards, and Controls

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 3,000 x *g* for 30 seconds to remove any particulates.

mAb-free CFB control: Please note that you will also require an aliquot of mAb-free CFB as a control to monitor interference from non-mAb broth components (Blank B). 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. Prior to use, CFB should be centrifuged at 3000 x *g* for 30 seconds to remove any particulates.

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: UFT-MP (Section D.2.c)
   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. *Standards*: Prepare standards at concentrations that bracket the estimated sample concentration (e.g. 0.1 to 3.75 mg/mL for 10 µL injection volume). Standard solutions should be prepared using client-provided mAb reference material (or NIST mAb) diluted with mAb-free CFB.
3. *Samples*: Place sufficient volume of each CFB/test mAb sample in an autosampler vial or plate well to perform desired injections

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 at set injection volume of mAb in CFB (Section F.2) at the highest µg load you expect to observe in your samples (no greater than 37.5 µg), 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

At constant injection volume (10 µL recommended),

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

2. Generate a standard curve for titer analysis by injecting the prepared mAb standards (Section F.2) such that the amount of mAb injected is between 1 and 37.5 µg. Process the standards following I.1-.2 to obtain peak areas. Plot the peak area versus the mAb standard concentration to determine the linear dynamic range in which all sample concentrations must fall.

3. Inject samples (Section F.3) from desired time points and adjust sample concentration, if necessary, while maintaining volume, to fall within established linear dynamic range. Samples and blanks must be injected at the same volumes as were injected for the standards in H.2.

I. Processing and Reporting

1. Evaluate chromatograms from Blank B (or C, if applicable). The area of peaks present in Blank B at these elution times must be subtracted from the corresponding sample peak area.

2. Create a processing method to consistently integrate peaks in all injections of your mAb. Elution times are dependent on the HPLC system in use.

3. Use the standard curve (Section H.2) to calculate the concentration of the mAb in the unknown fermentation samples by plotting the Peak Area versus the mAb Standard Concentration.

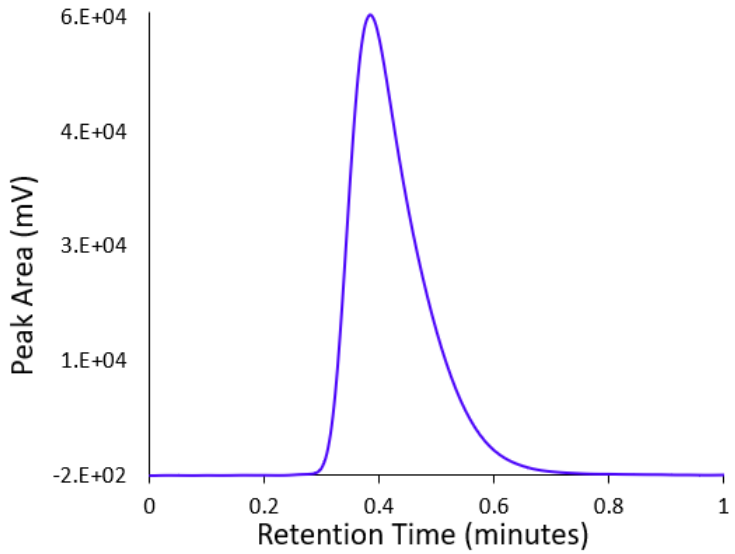

Figure 2. Example Chromatogram: NIST mAb at 25 µg load

J. Proteometer-UFT MD Unit Extended Storage Conditions

Flush with copious amounts of ultrapure water maintained at a low flow rate to avoid excessive backpressure. Force air into the tubing with an empty syringe to displace the water, and store in a closed container.

Ordering Information

Description	Part Number
Proteometer-UFT 300 Inj Kit (1 reagent vial, 1 buffer pack, MD unit, tee, union)	KIT-PUF-002-01
Proteometer-UFT 1000 Inj Kit (4 reagent vials, 4 buffer packs, MD unit, tee,	KIT-PUF-010-01
Proteometer-UFT MD unit	RCT-PUF-001
Proteometer-UFT tee	TEE-PFV-001
Proteometer-UFT union	ZDV-PFV-001
Proteometer-UFT buffer	BUF-PUF-001
Proteometer-UFT reagent	RGN-PUF-001
Proteometer-UFT reconstitution reagent (DMF)	REC-PUF-001

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