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mAb Size Variant Standard

CONTENTS

I. INTRODUCTION

Size-exclusion chromatography (SEC) is part of the de rigueur analysis of monoclonal antibodies (mAbs) as required by therapeutic regulatory agencies. The inclusion of SEC is generally for aggregate analysis, utilizing the speed and reproducibility of SEC to efficiently separate high molecular weight species (≥300,000 Da), such as dimers and multimers, from monomers (~150,000 Da). SEC also affords the ability to separate low molecular weight species including the Fab/c (~100,000 Da), commonly referred to as the "clip", and the Fab and Fc fragments (~50,000 Da).

The lower molecular weight species are the primary fragments resulting from hydrolytic degradation and are endogenous to a sample; however, they are undesirable in a drug substance/product. The lower molecular weight species, particularly the clip, can be challenging to effectively separate due to their low abundance as well as the need for high resolution chromatography. Unfortunately, the percent composition of the lower molecular weight species can be variable, making standards batch dependent and of lesser utility. As a result, it can often be unclear if a clip species is genuinely absent from a sample or if an assay simply lacks sufficient resolving power.

To address this shortcoming, Waters™ created a mAb Size Variant Standard (p/n: 186009429) which is supplied with a certificate of analysis for each prepared standard lot. The mAb Size Variant Standard comprises NISTmAb (NIST Reference Material [RM] 8671, a humanized monoclonal antibody) and non-reduced IdeS digested NISTmAb fragments $F(ab')_2$ (~100,000 Da) and $(Fc/2)_2$ (~50,000 Da), two mAb fragments with similar molecular weights as the Fab/c and Fab or Fc, respectively. By aliquoting small, standard amounts of IdeS fragments, the mAb size variant helps validate an SEC separation. It should be noted that the clip for the mAb Size Variant Standard will contain both $F(ab')_2$ from the IdeS digest and the Fab/c, and these two products will not resolve as is consistent with them having similar radii of hydration (Figure 1). The $(Fc/2)_2$ fragment is easily resolved from the Fab and Fc fragments.

Each vial comprises 160 µg of stabilized and lyophilized NISTmAb which has been supplemented with 2 µg of purified non-reduced IdeS digested NISTmAb fragments, respectively. NISTmAb was chosen as it is a well characterized benchmark and is used for evaluating the performance of methods for physicochemical and biophysical attributes. After lyophilization of the standard, vials from each lot are reconstituted and tested for uniformity on an ACQUITY™ UPLC™ H-Class Bio System using the NISTmAb RM 8671 as a reference control. Thorough review of the provided certificate of analysis is advised because the mAb Size Variant Standard may have slight differences in properties versus NISTmAb as a result of it being subjected to a buffer change and being lyophilized with trehalose excipient (see Section V. Sample Considerations).

II. RECOMMENDED RECONSTITUTION

For SEC: It is recommended to solubilize the standard to a concentration of 1–2 mg/mL with the addition of 18.2 MΩ water followed by using a standard benchtop vortexer to fully mix the solution. To ensure complete solubilization, it is recommended to vortex for 5 sec each in the upright, inverted, then finally the upright position. For standard analysis with the mAb Size Variant Standard (1–2 µg/µL), it is suggested to use the following injection volumes:

Diameter (mm)	Length (mm)	Injection Volume (µL)
4.6	150	1.8–2.5
	300	3.5–5
7.8	150	5
	300	10

Larger injection volumes can be used but may result in slightly lower resolution between the monomer and the clip. If both monomer and clip relative quantification is desired, it is recommended that a 7.8 x 300 mm column be used. Injections can be made directly from the reconstituted sample from the supplied vial.

Note that because of the low sample volume in the vial it is recommended to set needle depth to 1 mm from the bottom of the vial.

III. STORAGE AND STABILITY

Upon arrival and prior to reconstitution, please store the standard in its original packaging at -20 °C until preparation or its marked expiration date. After reconstitution, it is recommended to use the standard within 24 hours, as longer times can lead to changes in mAb size variant species levels noting that this standard is not intended to be used for mAb component quantitation. If desired, the reconstituted standard can be frozen at 80 °C and thawed for later use noting that the relative amounts of mAb aggregate and fragment might change versus when the standard was freshly prepared.

IV. EXAMPLE CHROMATOGRAPHIC RESULTS

LC system:	ACQUITY UPLC H-Class Bio
Column:	BioResolve™ SEC mAb, 200 Å, 2.5 µm, 7.8 x 300 mm with mAb Size Variant Standard (p/n: 176004595)
Temp.:	Ambient
Mobile phase:	50 mM sodium phosphate pH 7.0, 200 mM KCl
Flow rate:	0.300 mL/min
Sample manager temp.:	8 °C
UV detection:	280 nm, 10 Hz, no filter
Seal wash:	18.2 MΩ water
Needle wash:	18.2 MΩ water
Reconstitution:	160 µL 18.2 MΩ water for the mAb Size Variant Standard
Samples:	1 mg/mL mAb Size Variant Standard 10 mg/mL NISTmAb RM 8671
Injection volume:	10 µL mAb Size Variant Standard and 1 µL NISTmAb

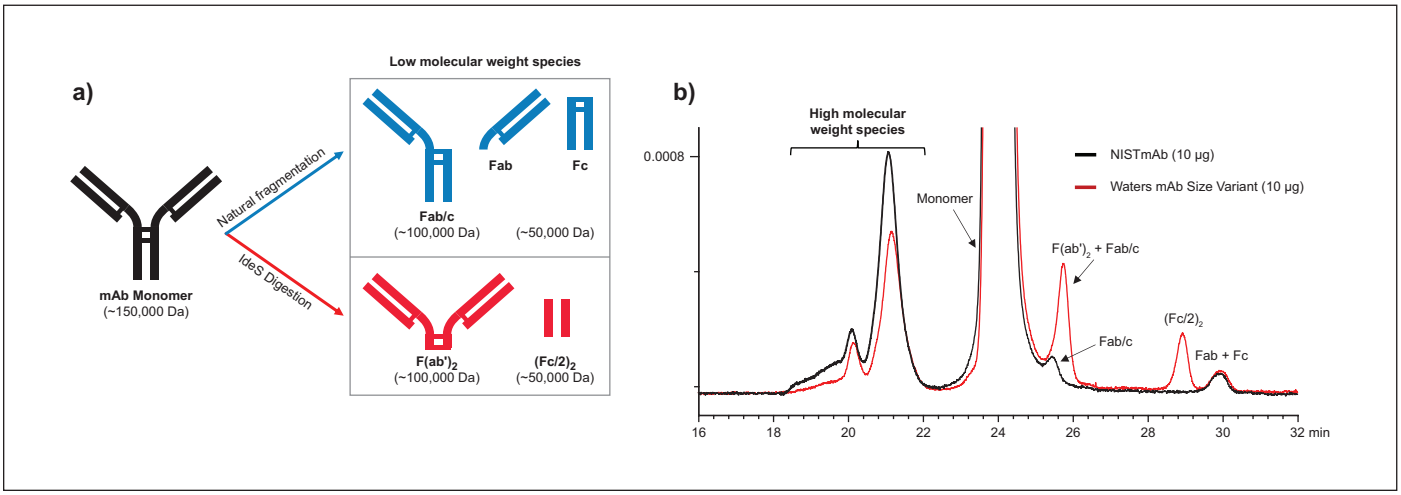

Figure 1. (A) mAb graphic illustrating the difference between the intact monomer and fragments found in NISTmAb and Waters mAb Size Variant Standard. (B) A representative A_{280} SEC chromatogram of NISTmAb (black trace) and modified Waters mAb Size Variant Standard (red trace) cropped to show both high and low molecular weight species, in addition the monomer. Due to similarity in hydrodynamic radii, F(ab')$_2$ and Fab/c are not resolved. Data were collected with a BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 x 300 mm Column with absorbance measured at 280 nm.

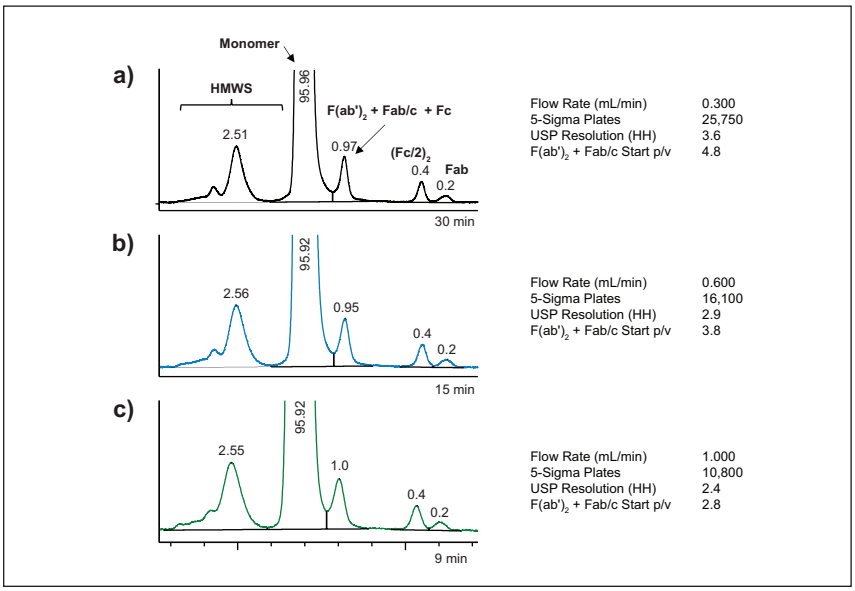

Figure 2. The effect of chromatographic flow rate is shown for a 10 µL injection of the 1.0 mg/mL mAb Size Variant Standard on a BioResolve SEC mAb, 2.5 µm, 7.8 x 300 mm configuration at (A) 0.3 mL/min, (B) 0.6 mL/min, and (C) 1.0 mL/min. Reproducible and accurate percent areas are reported for high molecular weight species (HMWS), monomer peak, F(ab')$_2$ + Fab/c (the "clip"), (Fc/2)$_2$, and Fab. Fab + Fc fragments are clearly detected at a signal-to-noise ratio of 5. The clip is observed at levels of >0.5% area and is best resolved and quantified at the lower flow rate. These representative A280 chromatograms are shown at well below 4.4% of the monomer peak height, making 5-Sigma theoretical plates the most relevant efficiency parameter for SEC.

V. SAMPLE CONSIDERATIONS

It should be noted that the percent of high and low molecular weight species for mAb Size Variant Standard will be different when compared to a standard NISTmAb injection due to the processing and handling associated with the addition of IdeS fragments and lyophilization. NISTmAb generally has between 2.7–3.6% high molecular weight species whereas the mAb Size Variant Standard generally has less, with values varying between 1.9–3.5% from lot to lot. There is a corresponding increase in low molecular weight species with "clip" peak area distribution shifting from an expected value of 0.1–0.2% to 0.8–1.2% for NISTmAb and the mAb Size Variant Standard, respectively.

Additionally, it should be noted that the percent high molecular weight species generally decreases slowly over time, after reconstitution. Presumably, this is due to a shift from soluble aggregates to insoluble aggregates. Accordingly, we recommend testing with a freshly prepared sample.

Flow rate is a critical factor for mAb Size Variant Standard species resolution. As shown in Figure 2, a lower flow rate yields higher 5-Sigma plate counts, USP resolution (Rs), and an improved peak-to-valley (p/v) ratio for the clip ($F(ab')_2$ + Fab/c). This improved chromatography does come at the cost of time, however.

VI. CAUTIONARY NOTE

Depending on user's application, these products may be classified as hazardous following their use, and as such are intended to be used by professional laboratory personnel trained in the competent handling of such materials. Responsibility for the safe use and disposal of products rests entirely with the purchaser and user. The Safety Data Sheet (SDS) for this product is available at www.waters.com/sds.

VII. REFERENCES

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