Easy Particle Analysis for Viscous Samples with MFI

Introduction

Many protein therapeutics in development today are viscous because they contain excipients for stability or have a high concentration of the protein itself. If you use particle analysis techniques like light obscuration, the only way to analyze these samples accurately is to dilute them. But dilution can change the characteristics of the sample, which means more comparability studies to validate the dilution effect. Why make your work harder? MFI lets you skip the whole dilution process.

We studied the MFI 5200 manual system performance across a broad range of sample viscosities and you guessed it — we got precise and quantitative characterization of particle size, concentration, and morphology with even the most viscous solutions. And while the data in this application note is from the MFI 5200 system, we also looked at the MFI 5100 system and saw the same great results: robust particle measurement using neat samples. So if you want out of the dilution boat, read on!

Our Tips for Viscous Sample Introduction

Following these simple guidelines will get you the best data for viscous samples:

Flushing — The most important thing when it comes to viscous samples is to equilibrate your flow cell. Not doing it properly can lead to the formation of Schlieren lines which can cause issues with particle count and morphology. Flush the flow cell either with the sample itself or a buffer with similar optical and physical properties until no Schlieren lines (**Figure 1**) appear along the edge of the flow cell. The volume needed mainly depends on your solution's viscosity, and for very viscous samples you'll need higher flush volumes to equilibrate. We chose 5 mL of flush volume for our experiments here because it worked for all our conditions. But you can often go lower for less viscous samples.

Mixing — Mix your samples really well. A homogenous sample is crucial for accurate sample characterization.

Avoid air bubbles — Air bubbles are more easily trapped in viscous solutions than in aqueous ones. You can avoid introducing them by gently mixing your samples using end-over-end rotation. Avoid the forceful agitation route.

Pump Calibration — For sample viscosities up to 20 cP, standard water calibration is fine, so you don't have to worry about recalibrating your pump.

Morphology — In our study, viscosity didn't have a strong effect on the data. But, if the viscosity of your sample comes from a matrix with a significantly different refractive index, you may see stronger effects.[1] If that's the case, your best bet is to characterize NIST sizing beads in your matrix to see if the sample's refractive index is impacted by solution viscosity.
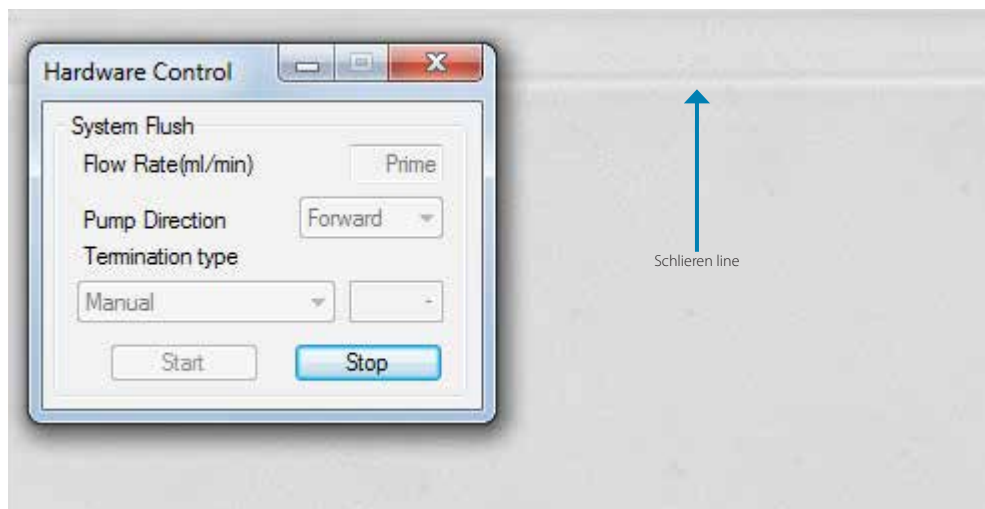
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FIGURE 1. Schlieren line in priming view. Particles coincident with the Schlieren line are interpreted as a single massive particle by the software.

For our study, we compared particle characterization in viscous solutions to water on the MFI 5200 system. The parameters we evaluated included sample volume analyzed, particle count and particle size. We also looked at important morphological parameters to confirm our measurements weren't affected by sample viscosity.

How We Analyzed Samples

For particle count and size, the analysis workflow (**Figure 2**) and the Analysis Method (**Figure 3**) used were the same. The only difference in the Analysis Method was the application of Edge Particle Rejection. For the counting analysis Edge Particle Rejection was turned off, and for the sizing analysis it was turned on. This allowed us to more accurately determine size and count. All samples were introduced to the MFI 5200 system using a syringe barrel and analyzed in triplicate.

SAMPLE VOLUME ANALYSIS

We made samples with viscosities ranging from 1 centipoise (cP) to 20 cP using a series of polyethylene-glycol (PEG) dilutions, then confirmed solution viscosity using a Rheosense Inc. uVisc. Syringe Viscometer.

Viscous samples can be less mobile in the fluid path, so we wanted to find out if this was a potential problem area for the analysis volume. Before each sample was analyzed, the MFI 5200 system was primed with the appropriate PEG solution, and the method was set to analyze 900 µL with 220 µL for optimize illumination and no flush. We then recovered the analyzed volume from the waste line with a pre-weighed Eppendorf tube. As shown in **Figure 4**, the analyzed sample volumes were consistent, regardless of sample viscosity. Because it uses a slow analysis speed, the MFI flow cell precisely analyzed the same amount of sample no matter if it

| Remove syringe barrel from instrument and wash with 20 mL of particle-free water. | Reattach syringe barrel and wash flow cell with 10 mL particle-free water. | Carefully load sample into syringe barrel. Take care to minimize bubbles. | Flush 5 mL of sample through flow cell to get rid of Schlieren lines. | Begin sample analysis. |
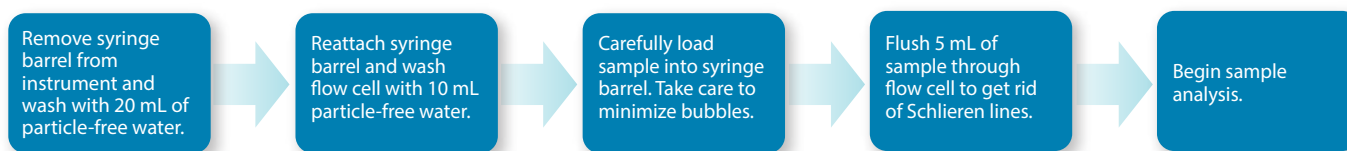
FIGURE 2. Workflow used to analyze viscous samples.

Sample Analysis Report

Method Identification

Name 20150220_Counting_900uL_NoPurge

Type Sample Analysis and Report

Description

Root Folder

Hardware Configuration	Set Point:	3	Flow Cell Model:	100µm(SP3)
	LED Slider Switch:	NA	Stir Speed(rpm) - NA	
	Magnification Dial:	NA	Direction - NA	
	Diaphragm Control:	NA	Stir before Run(min) - 0.0	

Run Setup

Termination Type:	Sample Dispensed(ml)	Edge Particle Rejection:	☐
Total Available Volume(ml):	0.90		
Purge Volume (ml):	0.00	Fill Particles:	☐
Analyzed Volume(ml, approximate):	0.77	Consecutive Runs	1

Filters

| Parameter | Condition | Value | AND/OR | Condition | Value |

Image Frames

Storage Trigger: Capture all images

Max Frames: 20000   Storage Space: 0.4334 GB

Discard Empty Frames: ☐   Seconds between Frames:

Report

Chart Type: Histogram

Axis	Parameter 1	Parameter 2	Min	Max	Step
X	ECD(µm)	-	MIN	MAX	1.00
Y Left	Count(#)	-	0.00	MAX	-
Y Right	-	-	-	-	-

Report Options

of Chart Data pages Included: 10

Include Method Summary: ☐

Include Particle Statistics and Images in report: ☐

ECD (µm)

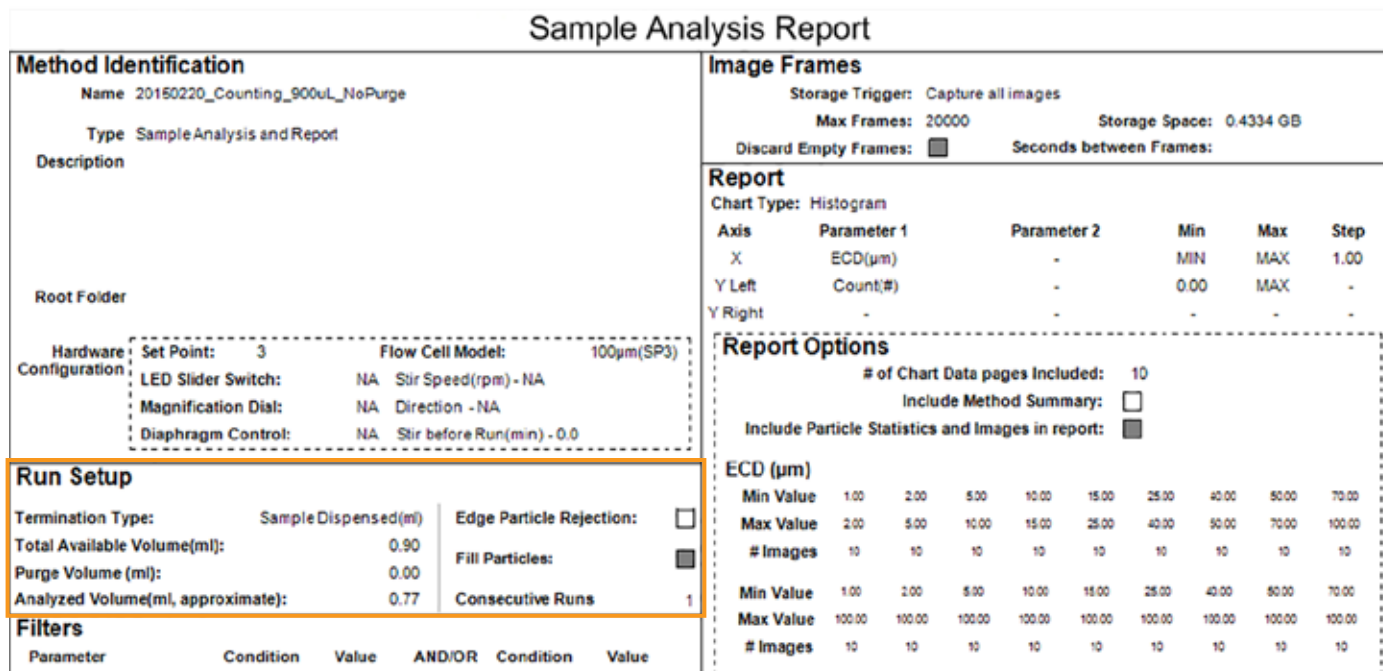| Min Value | 1.00 | 2.00 | 5.00 | 10.00 | 15.00 | 25.00 | 40.00 | 50.00 | 70.00 |
Max Value	2.00	5.00	10.00	15.00	25.00	40.00	50.00	70.00	100.00
# Images	10	10	10	10	10	10	10	10	10
Min Value	1.00	2.00	5.00	10.00	15.00	25.00	40.00	50.00	70.00
Max Value	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
# Images	10	10	10	10	10	10	10	10	10

FIGURE 3. Method used for sample analysis. The Total Available Volume was set to 0.90 mL, which translates to an analyzed volume of 0.77 mL.

was an aqueous solution or a viscous (PEG) one. This also means there isn't any need to recalibrate the pump, which is time saved during setup. How nice is that?

We all know the MFI 5200 system accurately and precisely characterizes particles in the sub-visible range (2 – 25 microns). So the next thing we looked at is how well it stacked up with counting particles in viscous samples.

Same Great Data for Particle Size and Concentration

For our concentration evaluation, NIST-certified 5 µm, 10 µm and 25 µm COUNT-CAL standards (ProteinSimple) were mixed at a 1:1 ratio with the PEG solutions so the final viscosities of the solutions would be at the intended cP range. We confirmed viscosity with the viscometer,
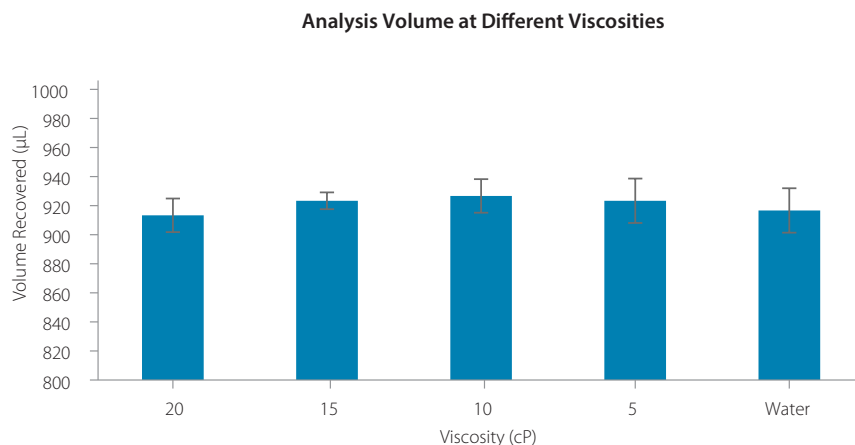
Analysis Volume at Different Viscosities

FIGURE 4. Sample analysis volumes are consistent across a range of viscosities. For all tested samples, values for the analysis were within 5% of the nominal 900 µL, showing that even high viscosity samples can be precisely measured through the flow cell. This precision over a wide range of viscosities is key to accurately determining particle concentration.

and then mixed samples by rotating end over end for 20 minutes before analysis.

Data for the COUNT-CAL bead analysis showed that you can accurately determine particle concentration even with samples as viscous as 20 cP (**Figure 5**). We analyzed data with MVSS 4.0 using standard NIST filters (**Table 1**). COUNT-CAL beads have a published concentration of 3000 particles per mL at their stock concentration, with a variability of +/- 10%. After we diluted the bead stocks 1:1 with a PEG solution, the nominal concentration was 1500 particles per mL. Each condition was measured in triplicate. The recovered concentrations for samples fell within 90 – 110% of the expected 1500 particles/mL, and CVs for the triplicate measurements were below 6%, right within spec.

The Duke Size Standard analysis also showed viscosity had no effect on data quality. Bead standards at all viscosities were measured to within 5% of their expected values (**Figure 6**), confirming that viscosity doesn't impact particle size measurements. So that means you'll get accurate sizing information with samples up to 20 cP. And, replicates for each sample were super tight— variability in the ECD measurements were <1% for the sample in triplicate (**Figure 6**).

So What About Morphology?

Because we know it'll help you identify the morphology of your particles, we also analyzed common parameters on top of the size and count metrics the standards are designed to represent (**Figure 7, 8, 9, 10**). While the data didn't show strong differences in characterization

Concentration of COUNT-CAL Beads in Viscous Matrix

FIGURE 5. Recovered concentrations for samples over a range of viscosities. Error bars represent standard deviations.

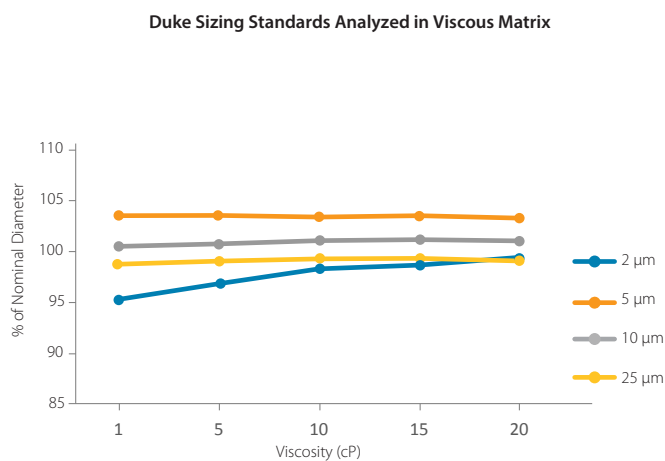
Duke Sizing Standards Analyzed in Viscous Matrix

FIGURE 6. Size measurements from 1 – 20 cP. Bead standards were all within 5% of the expected values. CVs for the triplicate measurements were <1%.

BEAD SIZE	PARAMETER	FILTER
5 µm	ECD	>3.0 µm
10 µm	ECD	>7.5 µm
25 µm	ECD	>15 µm

TABLE 1. Standard NIST filters used to categorize results.

results, statistical analysis did reveal subtle effects that the sample matrix had on particle characterization. We chose the 2 µm bead set for linear regression analysis to get a read on any significant differences between the viscosity conditions. And it turns out that morphologic measures aren't adversely impacted, so no significant changes to intensity max, circularity, and aspect ratio (**Table 2, 3, 4**). We noticed only a very small change in pixel area (**Table 5**).
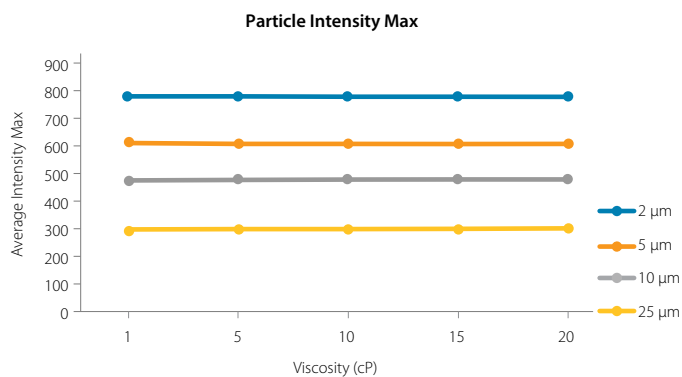

FIGURE 7. Measured particle intensity max. The maximum intensity of the all pixels representing the particle.
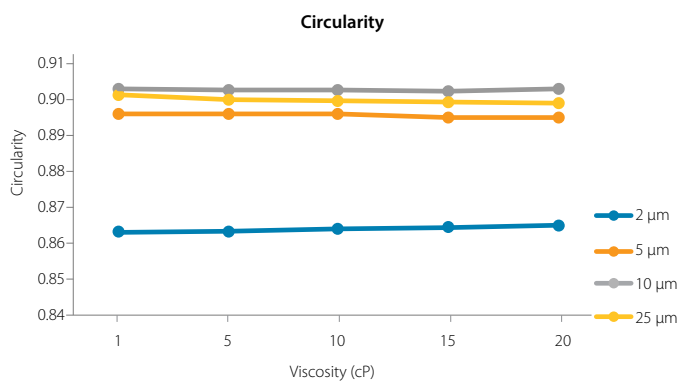

FIGURE 8. Measured circularity. The circumference of an equivalent area circle divided by the actual perimeter of the particle.

INTENSITY MAX OF 2µm BEADS COMPARED TO 1 cP CONDITION			
VISCOSITY	**COEFFICIENTS**	**STANDARD ERROR**	**P-VALUE**
Intercept	778.10	0.57	4.32E-116
5 cP	-0.03	0.57	0.96
10 cP	-0.01	0.57	0.99
15 cP	0.25	0.57	0.66
20 cP	0.69	0.57	0.23

TABLE 2. Particle intensity statistical analysis of 2 µm beads. Linear regression confirmed there was no change in particle intensity max that correlated with viscosity (Model R^2=0.999).

CIRCULARITY OF 2 µm BEADS COMPARED TO 1 cP CONDITION			
VISCOSITY	**COEFFICIENTS**	**STANDARD ERROR**	**P-VALUE**
Intercept	0.86	0.0003	2.35E-132
5 cP	-0.0003	0.0003	0.27
10 cP	-0.0003	0.0003	0.40
15 cP	-0.0006	0.0003	0.06
20 cP	-0.0003	0.0003	0.27

TABLE 3. Circularity statistical analysis of 2 µm beads. Linear regression gave no significant difference in circularity for any of our viscous conditions (Model R^2=0.998).
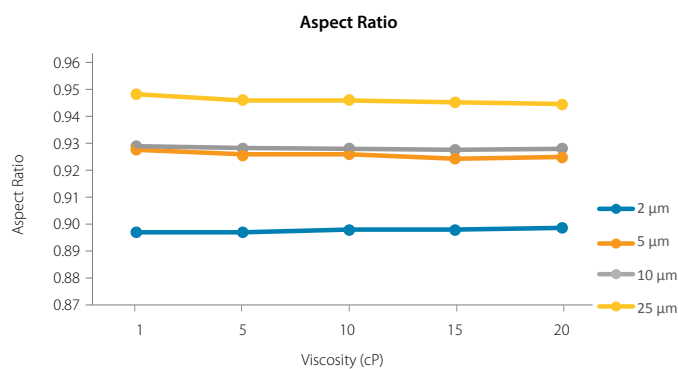
Aspect Ratio

FIGURE 9. Measured aspect ratio. The ratio of the minor axis length of the major axis length of an ellipse that has the same second moments of the particle.
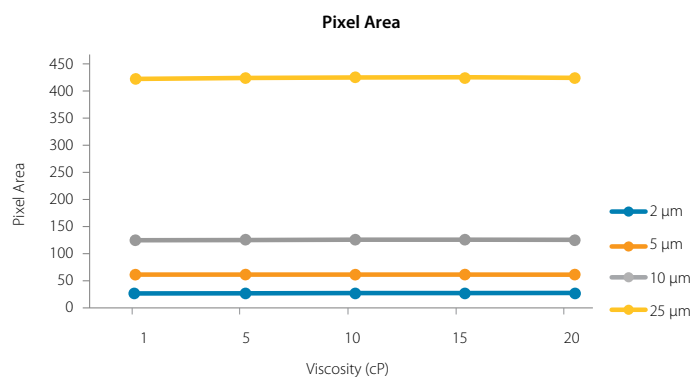
Pixel Area

FIGURE 10. Measured pixel area. The number of pixels representing a particle.

ASPECT RATIO OF 2 μm BEADS COMPARED TO 1 cP CONDITION			
VISCOSITY	COEFFICIENTS	STANDARD ERROR	P-VALUE
Intercept	0.8989	0.00036	3.93E-129
5 cP	-0.0012	0.00036	2.07E-03
10 cP	-0.0010	0.00036	7.53E-03
15 cP	-0.0017	0.00036	2.52E-05
20 cP	-0.0014	0.00036	2.48E-04

TABLE 4. Aspect ratio statistical analysis of 2 μm beads. Linear regression showed a very small change in aspect ratio in viscous conditions. This difference, though consistent, is less than a 1% change in aspect ratio (Model R^2=0.997).

PIXEL AREA OF 2 μm BEADS COMPARED TO 1 cP CONDITION			
VISCOSITY	COEFFICIENTS	STANDARD ERROR	P-VALUE
Intercept	24.28	0.27	2.39E-57
5 cP	0.64	0.27	1.89E-02
10 cP	1.07	0.27	1.83E-04
15 cP	1.22	0.27	2.89E-05
20 cP	0.94	0.27	9.12E-04

TABLE 5. Pixel area statistical analysis of 2 μm beads. Linear regression showed a small but consistent increase in the pixel area under viscous conditions. But we know from the calculated values the change wasn't sufficient enough to impact particle sizing (Model R^2=0.999).

Conclusion

MFI generates absolutely great data, even when sample viscosity is as high as 20 cP. Now you can get the hands-down most consistent measurement of count, size and morphology without having to dilute a single sample! Our analysis of NIST-certified COUNT-CAL concentration standards from 5 to 25 microns in size proved we could accurately determine particle concentrations to within 10% of the nominal value in viscosities ranging from 1 to 20 cP. And sizing data for NIST-certified Duke size standards from 2 to 25 microns was within 5% of the nominal value.

But it gets even better! There were no adverse impacts on morphologic parameters, and no significant changes to aspect ratio, intensity max, or circularity — and only a change of one pixel in particle area. Translation? In a nutshell, you can run your viscous samples straight up with MFI and still get precise and accurate particle characterization.

References

1. How subvisible particles become invisible—relevance of the refractive index for protein particle analysis, S Zolls, M Gregoritza, R Tantipolphan, M Wiggenhorn, G Winter, W Friess and A Hawe, *Journal of Pharmaceutical Sciences*, 2013:102(5):1434-46.

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