Comparability Study of Manual and Automated Particle Characterization with MFI

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Introduction

Micro-Flow Imaging (MFI) has become a standard application for particle analysis of protein formulations[1] because of its ability to easily detect particle size and morphology for a diverse range of particle contaminants, including translucent protein fragments and silicone micro-droplets. Recently, the US Pharmacopeial Convention proposed changes to the USP <787> standard, recommending the use of particle morphology detection through technologies such as Micro-Flow Imaging for sub-visible particle characterization[2,3].

Higher throughput and standardization are needed to meet the demand for biopharmaceutical testing of sub-visible particle characterization. The MFI 5000 series instruments can be automated with the addition of the Bot1 autosampler which reduces the hands-on time for sample evaluation. Because the automated mode requires a different sample presentation, the method parameters are often modified in order to provide the same results in automated mode as it does in manual mode. Here we outline the process for transferring a manual protocol to an automated format with the Bot1 autosampler and the MFI 5000 series, with regard to sample volume and mixing parameters.

To demonstrate the comparability of running the MFI system in manual and automated modes, we ran a model protein system comprised of 1% BSA at multiple sites. Each sample was run at least eight times in both the manual and automated modes at two different laboratories. No statistically relevant differences were observed between manual and automated runs at either facility.

Materials and Methods

SAMPLE PREPARATION AND INSTRUMENT OPERATION

- 1% BSA sample was prepared, heated at 60 °C overnight (at least 12 hours) with shaking at 1200 rpm at the ProteinSimple facility. The sample was then aliquotted and stored at –80 °C. Frozen aliquots were shipped to the Takeda facility on dry ice and stored at –80 °C until use.
- Flow cell was washed with 1 mL of 10% Triton-X 100 in distilled, deionized (DDI) water (0.2 micron filtered) and rinsed 4X with 1 mL water before each run.
- A fourth stirring step was added to the protocol to ensure particles did not settle during the sampling process on the Bot1 (see **Table 1**).
- Manual pipetting steps were performed by turning off the Bot1 autosampler and manually pipetting sample into the MFI 5200 system via the sample inlet port. The same analysis method was used for both the automated and manual protocols (**Figure 1**).

METHOD TRANSFER

Translating a manual method to an automated method requires modification of the protocol to ensure comparable results due to differences in sampling handling between the two methods.

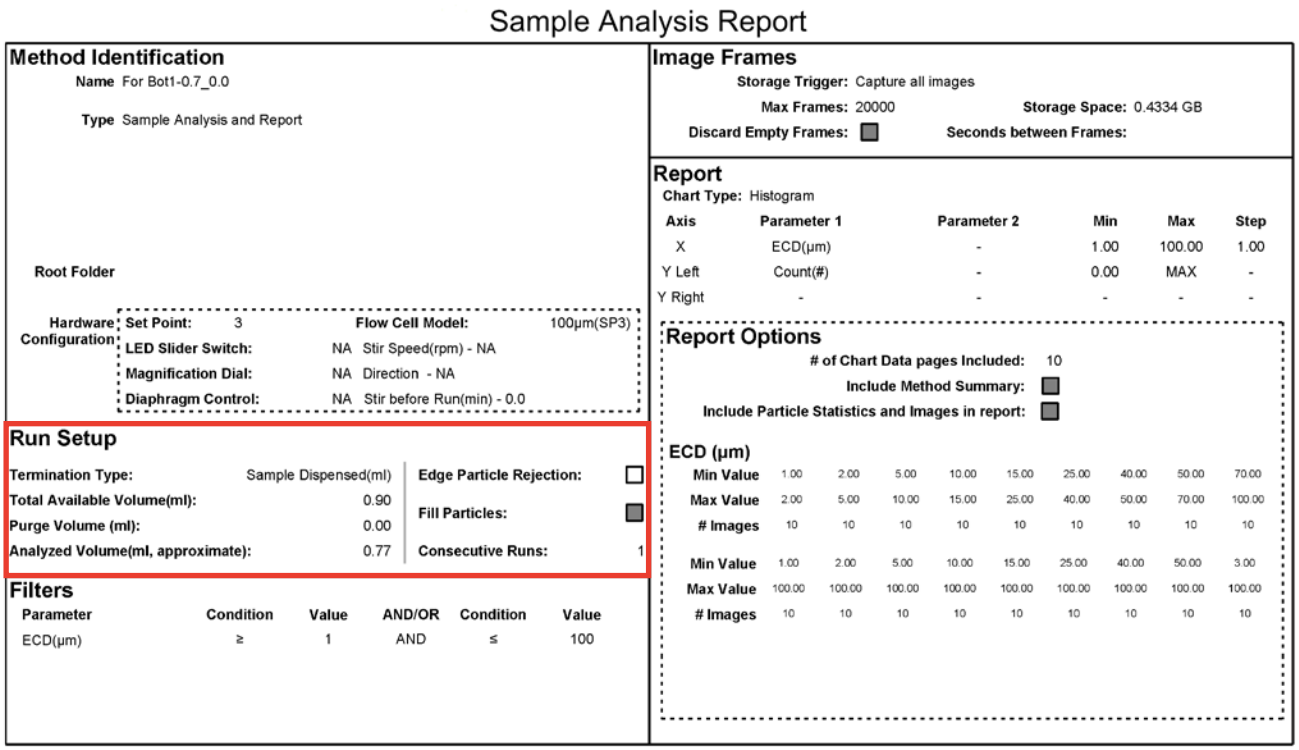
Method transfer considerations include:

- **Changes in sample introduction —** manual pipetting or stirrer with syringe barrel mix differently than an automated pipettor. The user can control the duration and speed of mixing with the automated pipettor.
- **Suitability of sample type —** manual or automated sample requirements are similar; highly viscous or extremely concentrated samples may not be suited for automation.
- **Sample volumes —** sample and purge volumes may change due to new labware and fluid path.
- **Ambient temperature —** samples should be stable at room temperature during an automated run.

OPERATION	LIQUID	VOLUME (ML)
Flush	Filtered water or filtered buffer	0.90
Flush	Filtered water or filtered buffer	0.90
Flush	Filtered water or filtered buffer	0.90
Dry system		
Flush	Filtered water or filtered buffer	0.90
Optimize Illumination	Filtered water or filtered buffer	0.22
Baseline	Filtered water or filtered buffer	0.70
Flush	Filtered water or filtered buffer	0.90
Dry system		
Stir 4 cycles, speed 5	Sample (well 1)	0.90
Flush	Sample (well 1)	0.50
Stir 4 cycles, speed 5	Sample (well 1)	0.90
Optimize Illumination	Sample (well 1)	0.22
Stir 4 cycles, speed 5	Sample (well 1)	0.70
Analysis Run	Sample (well 1)	0.70
Flush	Filtered water or filtered buffer	0.90
Flush	Filtered water or filtered buffer	0.90
Flush	Filtered water or filtered buffer	0.90

TABLE 1. Description of automated Protocol D used for 1% BSA solution. Original version of Protocol D uses 0.90 mL for Flush step. Steps from 'Flush' after Baseline through 'Analysis Run' are repeated for each sample in the run. Stir of four cycles was used prior to each sample analysis to ensure particles remained in suspension.

Sample volumes should be chosen based on particle concentration in the sample, as described in the next section. Sample volumes should also be adjusted further to accommodate the sample delivery format of the instrument configuration. Thus, moving from a manual to automated protocol may require changes to sample volume and flush volume initially to achieve accurate results. This is due to the fixed sample delivery format which includes 1–2 mL deep well plates, the 1 mL pipette tips and the interface to the inlet port (see **Table 2**).


Sample Analysis Report

Method Identification
Name For Bot1-0.7_0.0
Type Sample Analysis and Report
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)
Total Available Volume(ml): 0.90
Purge Volume (ml): 0.00
Analyzed Volume(ml, approximate): 0.77
Edge Particle Rejection:
Fill Particles:
Consecutive Runs: 1
Filters
Parameter Condition Value AND/OR Condition Value
ECD(µm) ≥ 1 AND ≤ 100
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) - 1.00 100.00 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 3.00
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 1. Analysis method for manual and automated samples at both ProteinSimple and Takeda sites. This analysis method was used with the batch protocol for modified Bot1 Protocol D. All samples were analyzed under the same conditions, with an analysis volume of 0.77 µL, and Optimize Illumination volume of 0.22 µL. Particle baseline was established with 0.2 µm-filtered DDI water.

	MANUAL OPERATION	BOT1 PROTOCOL D	MODIFIED BOT1 PROTOCOL D
Bot1 flush with sample	N/A	0.90 mL*	0.50 mL
Sample volume dispensed (defined in Method)	0.90 mL	0.70 mL	0.77 mL
Sample purge volume (defined in Method)	0.20 mL	0.00 mL	0.00 mL
Optimized Illumination volume and liquid type	0.22 mL of buffer (MFI 5200)	0.22 mL of sample (MFI 5200)	0.22 mL of sample (MFI 5200)
Dead volume	0.10 mL	0.03 mL	0.03 mL
Total Sample Volume	**1.20 mL**	**1.85 mL**	**1.52 mL**

*Volume may be reduced by optimizing the batch protocol for a specific sample type. Optimize Illumination step is equivalent to a purge step, so additional purge step is not required.

TABLE 2. Method comparison of manual to modified Bot1 Protocol D. The Bot1 Protocol D was optimized further to reduce flush volume, resulting in a total sample volume of 1.52 mL for Modified Bot1 Protocol D, which was the automated protocol used in this study.

CRITERIA	DESCRIPTION
Volume	Minimum 900 µL*
Throughput	>10 samples/day (or unattended operation)
Labware	1 or 2 mL deepwell plates
Mixing format	Aspirate and dispense steps using automated pipettor
Ambient temperature	Samples can be tested at room temperature (ambient)
Viscosity	Some viscosity, not highly viscous (≤10 cP units)
Concentration	>50–150 mg/mL for protein formulations
MFI 5000 Series	Bot1 is used with MFI 5000 series

*Required sample volume may be reduced following method optimization.

TABLE 3. Recommended criteria for MFI 5000 series with Bot1 autosampler.

Optimization of the automated protocol for the 1% BSA solution was performed as follows. We used a larger volume initially in Protocol D and then modified pipetting parameters as described in the modified Protocol D to reduce the volume requirements (**Table 2**). Additional stirring is required to counteract the effects of settling time during the automated process, so that samples are well mixed at the time of analysis. Sample types used with the MFI system in manual or automated mode should also be evaluated for suitability, as outlined in **Table 3**.

GUIDELINES FOR SAMPLE VOLUME

Particle concentration in the sample can aid in determining required volume for accurate sizing and concentration measurements. The plots in **Figure 2** show the impact of particle concentration on the appropriate volume. Optimal measurements are achieved for values at the 0.00% line of each graph. For example, in samples with particle concentrations of 10,000 P/mL or greater, volumes of at least 500-900 µL should be used. Note that in samples with particle concentrations of 1,000 P/mL, volumes of 1 mL or greater should be used.

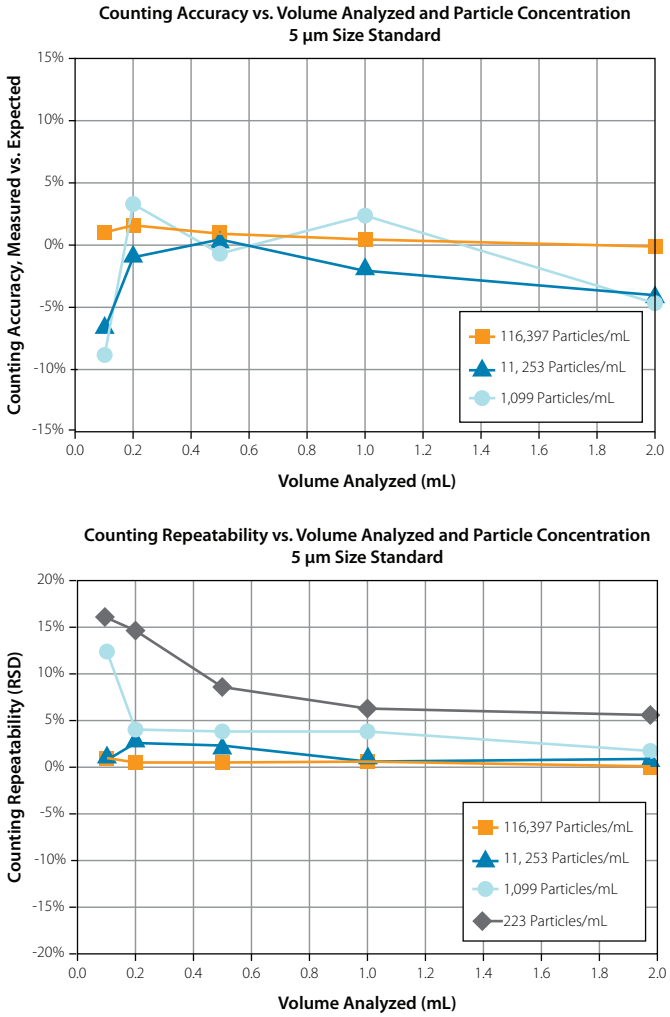


FIGURE 2. Impact of volume on counting accuracy for differing particle concentrations (top). Impact of volume on counting reproducibility for differing particle concentrations (bottom).

ACCOUNTING FOR DIFFERENCES IN SAMPLE HANDLING AND VOLUME REQUIREMENTS

The automated protocol uses 1.52 mL of sample as compared to the manual protocol's 1.20 mL. Volume and mixing steps for the automated protocol were optimized to ensure consistent counts and concentrations between replicates. As shown in **Table 2**, the initial total volume requirements of 1.85 mL for Protocol D were reduced to 1.52 mL in the modified version by adding automated mixing steps and reducing the sample flush volume. To reduce the total volume required, the stir speed was increased slightly from a setting of 3 to 5 (users can control the

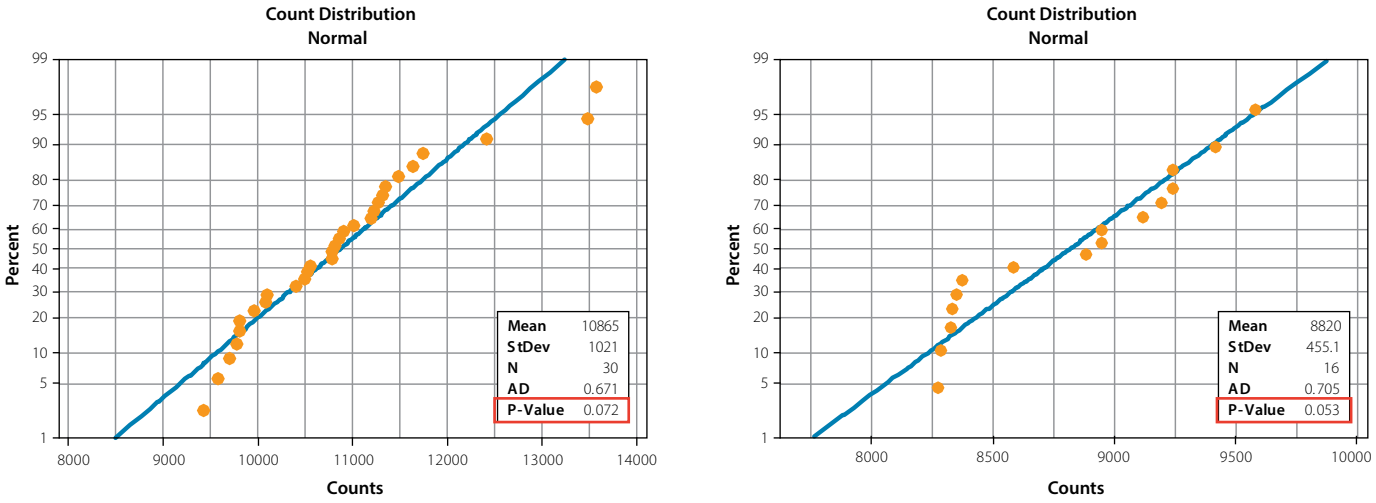


FIGURE 3. Data distribution (counts) at ProteinSimple (left) and Takeda (right). Anderson-Darling normality test was applied to the data distribution of counts. Both data sets were found to have a normal distribution, with a P-value > 0.05.

speed of this setting from 117 to 700 µL/sec). With this change, the actual sample flush volume required was reduced to 500 µL from 900 µL with no additional variation due to precipitation or aggregation. In each case, two runs of four replicates each were averaged and analyzed for count and concentration. Results from each method were analyzed using standard statistical tests as outlined in the next section.

Results

At least eight manual and automated runs were performed at each site, using the same manual and automated protocols for pipetting and analysis (see **Figure 1**).

Data were analyzed to assess normality of data set, and were not found to significantly deviate from normal distribution. P-values were 0.072 and 0.053 for ProteinSimple and Takeda, respectively. Variances between automated and manual methods were not statistically different by f-test (**Figure 3**).

Employing a standard analysis for statistical variance (ANOVA) indicated that only between 0 and 4% of variance in particle counts can be attributed to method type (automated versus manual, **Figure 4**), which is not significant. Regression analysis showed no relationship between other input variables and variance in the data (**Figure 5**).

Comparison of data showed results at Takeda were approximately 2000 counts/mL lower than those at

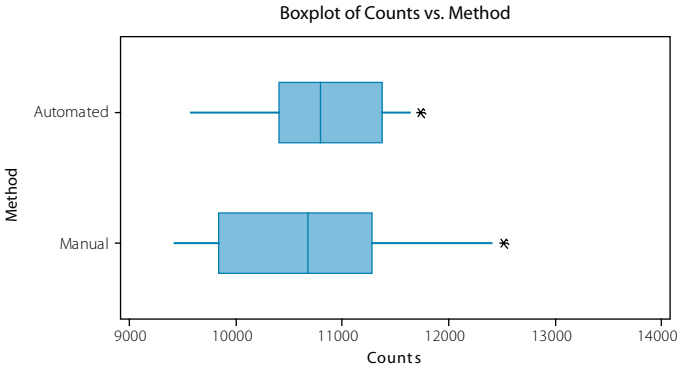
One-way ANOVA: Counts versus Method

```
Source  DF        SS       MS     F      P
Method   1    203214   203214  0.19  0.667
Error   28  30009739  1071776
Total   29  30212953

S = 1035   R-Sq = 0.67%   R-Sq(adj) = 0.00%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level       N   Mean  StDev -------+---------+---------+---------+--
Automated  14  10953    952      (---------------*---------------)
Manual     16  10788   1102  (--------------*--------------)
                          -------+---------+---------+---------+--
                             10500     10850     11200     11550

Pooled StDev = 1035
```



One-way ANOVA: Counts versus Method

```
Source  DF      SS     MS     F      P
Method   1  315900 315900  1.58  0.229
Error   14 2790735 199338
Total   15 3106636

S = 446.5   R-Sq = 10.17%   R-Sq(adj) = 3.75%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level      N    Mean  StDev ------+---------+---------+---------+---
Automated  8  8960.2  492.1            (------------*------------)
Manual     8  8679.1  395.6  (------------*------------)
                          ------+---------+---------+---------+---
                             8500      8750      9000      9250

Pooled StDev = 446.5
```

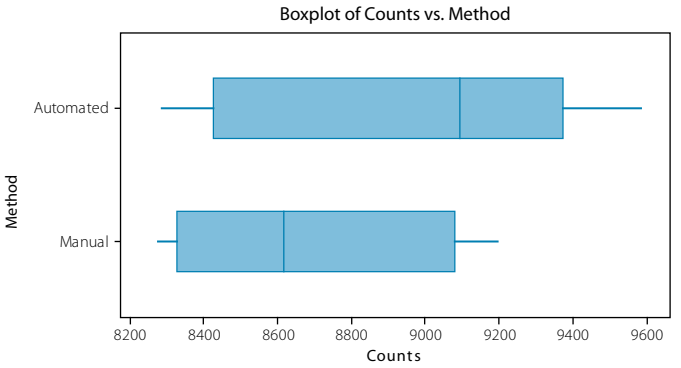


FIGURE 4. Impact of method type on counts at ProteinSimple (top) and Takeda (bottom). Analysis using one-way ANOVA did not show a significant effect for method type on particle counts, with an R-Sq(adj) of 0.0% for ProteinSimple and an R-Sq(adj) of 3.75% for Takeda.

```
Predictor      Coef  SE Coef      T      P
Constant    10534.6    519.9  20.26  0.000
Automated     172.3    410.2   0.42  0.678
Set 2         279.3    404.0   0.69  0.496
Day 2         224.6    410.2   0.55  0.589
Run 2         107.0    553.1   0.19  0.848
Run 3        -266.9    553.1  -0.48  0.634
Run 4         164.7    605.9   0.27  0.788

S = 1106.28   R-Sq = 6.8%   R-Sq(adj) = 0.0%

PRESS = 48120295   R-Sq(pred) = 0.00%

Analysis of Variance

Source          DF        SS       MS     F      P
Regression       6   2064274   344046  0.28  0.940
Residual Error  23  28148679  1223856
Total           29  30212953

Source      DF  Seq SS
Automated    1  203214
Set 2        1  585203
Day 2        1  463322
Run 2        1  153389
Run 3        1  568725
Run 4        1   90420
```

```
Predictor     Coef  SE Coef      T      P
Constant    8885.2    249.4  35.62  0.000
Automated    398.8    245.6   1.62  0.136
Set 2         41.4    229.8   0.18  0.861
Run 2       -162.4    313.1  -0.52  0.615
Run 3       -590.6    319.1  -1.85  0.094
Run 4       -189.8    319.1  -0.59  0.565

S = 442.818   R-Sq = 36.9%   R-Sq(adj) = 5.3%

PRESS = 5405327   R-Sq(pred) = 0.00%

Analysis of Variance

Source          DF       SS      MS     F      P
Regression       5  1145756  229151  1.17  0.388
Residual Error  10  1960880  196088
Total           15  3106636

Source      DF  Seq SS
Automated    1  401933
Set 2        1   15025
Run 2        1   21197
Run 3        1  638197
Run 4        1   69403
```

FIGURE 5. Linear regression analysis was used with dummy coding to evaluate relevant variables (manual method, automated method, day) as a group. Analysis shows no significant impact of system inputs (automation, day to day variance) on particle counts. The various system inputs could account for 0.0% (ProteinSimple, left) and 5.3% (Takeda, right) of the variation in the data. An R-Sq (adj) of >30% would indicate a significant impact.

MANUAL		
RUN	COUNTS	COUNTS/ML
1	8783	11184
2	9745	12409
3	8530	10862
4	8238	10490
Average	8824.00	11236.25
Std Dev	565.63	720.25
%CV	6.41%	6.41%
RUN	COUNTS	COUNTS/ML
5	7918	10083
6	7703	9809
7	7815	9951
8	7403	9427
Average	7709.75	9817.50
Std Dev	192.73	245.40
%CV	2.50%	2.50%
AVERAGE OF ALL 8 MEASUREMENTS		
Average	8227.83	10477.17
Std Dev	875.47	1114.73
%CV	10.64%	10.64%

AUTOMATED		
RUN	COUNTS	COUNTS/ML
1	8463	10780
2	8444	10773
3	8483	10812
4	9003	11479
Average	8598.25	10961.00
Std Dev	234.09	299.43
%CV	2.72%	2.73%
RUN	COUNTS	COUNTS/ML
5	8840	11274
6	9124	11633
7	8905	11343
8	10572	13471
Average	9360.25	11930.25
Std Dev	707.47	899.69
%CV	7.56%	7.54%
AVERAGE OF ALL 8 MEASUREMENTS		
Average	9058.00	11545.67
Std Dev	787.21	1001.27
%CV	8.69%	8.67%

TABLE 4. Count and concentration for automated versus manual format for 1% BSA, ProteinSimple.

ProteinSimple. This difference is likely related to sample quality in terms of sample stress or degradation during shipment. Neither site showed any significant difference in counts or concentration between the manual and automated method, confirming that similar results can be obtained using either format.

COMPARISON OF THE MANUAL VERSUS AUTOMATED METHOD, PROTEINSIMPLE

Data from tests performed at ProteinSimple are listed in Table 4. The automated format of 1% BSA protein solution produced consistent counts and concentration data, compared to the manual format. The modification to Protocol D reduces the sample flush volume without impacting the consistency of results between replicates in the automated method. The standard deviation for both methods was 5% or less for all samples tested.

COMPARISON OF THE MANUAL VERSUS AUTOMATED METHOD, TAKEDA

Data from tests performed at Takeda are listed in Table 5. At the Takeda site, the automated format of 1% BSA protein solution produced consistent counts and concentration data which were also comparable to the manual format. The standard deviation for both methods was 6% or less for all samples tested. Takeda results were similar to ProteinSimple's in terms of successful transfer of the 1% BSA assay to an automated format.

MANUAL			AUTOMATED		
RUN	COUNTS	COUNTS/ML	RUN	COUNTS	COUNTS/ML
1	6570	8947	1	6641	8950
2	6833	9197	2	6477	8586
3	6263	8275	3	6093	8288
4	6344	8333	4	6965	9418
Average	6502.52	8688.06	Average	6544.21	8810.20
Std Dev	221.60	394.50	Std Dev	313.97	421.88
%CV	3.41%	4.54%	%CV	4.80%	4.79%
RUN	COUNTS	COUNTS/ML	RUN	COUNTS	COUNTS/ML
5	6748	8884	5	7043	9241
6	6198	8349	6	7043	9241
7	6670	9123	7	6261	8373
8	6235	8326	8	7373	9585
Average	6462.72	8670.23	Average	6930.14	9110.15
Std Dev	248.28	343.69	Std Dev	409.09	448.12
%CV	3.84%	3.96%	%CV	5.90%	4.92%
AVERAGE OF ALL 8 MEASUREMENTS			AVERAGE OF ALL 8 MEASUREMENTS		
Average	6542.31	8804.16	Average	6806.43	8996.05
Std Dev	267.33	379.17	Std Dev	416.26	452.95
%CV	4.09%	4.31%	%CV	6.12%	5.03%

TABLE 5. Count and concentration for automated versus manual format for 1% BSA, Takeda.

Conclusion

The MFI 5200 produces the same high quality particle characterization in manual or automated mode. This study showed that method optimization could further reduce the sample volume required without impairing concentration accuracy. Statistical analysis confirmed that these protocols are robust and provide an example of standardization of methods across instrument configurations.

In the Takeda development laboratory, MFI was originally implemented due to its advantages over conventional techniques (HIAC-based light obscuration used according to Ph. Eur. 2.9.20/USP <788>) for measurement of sub-visible particles in the µm range (e.g. 2–10 µm).

Those advantages included:

- Lower sample consumption versus light obscuration
- 1–10 µm particle detection
- MFI's ability to distinguish between particle types based on morphology parameters

Initially, a manual MFI 4200 system was obtained for the Takeda development work which included formulation development, primary container selection, stability studies, etc. While the advantages over HIAC-based light obscuration were obvious in terms of volume range, particle size and morphology, manual MFI use became labor-intensive and repetitive with larger development

studies containing up to 50 samples per run. Reduction of hands-on time associated with manual operation was thus a primary goal, and became the main reason for upgrading to the automated MFI 5200 system with Bot1 autosampler at an early stage. In addition, the improved reproducibility associated with the automated format further contributed to the decision to upgrade.

The option to automate provides a key advantage for particle characterization of protein formulations, offering many benefits compared to more common techniques. Automated protocols allow for much greater throughput and less hands-on time, with up to 80 samples per unattended run. Implementation of automation for higher throughput and standardization can help address demand for more rapid and consistent screening methods in particle characterization.

References

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2. In-Process Revision: <787> subvisible particulate matter in therapeutic protein injections (USP36-NF31 IS), February 2013.

3. AAPS update on USP expert committee for subvisible particle analysis, L Narhi, *Newsletter of the AAPS Aggregation and Biological Relevance Focus Group*, 2012; 3(2).

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