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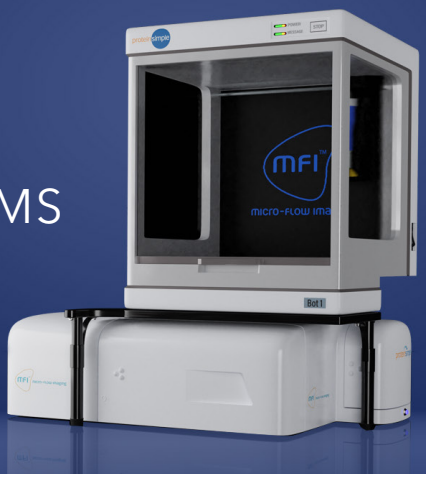
APPLICATION NOTE

# CONFIRMING ACCURATE PARTICLE COUNTING AND SIZING ON MFI SYSTEMS

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## INTRODUCTION

Accurate determination of sub-visible particles and protein aggregates is important to ensure safety and efficacy of biopharmaceutical formulations due to immunogenicity concerns, and biopharmaceutical manufacturers are expected to characterize, monitor and control sub-visible protein particles and protein aggregates in their products. As traditional techniques such as light obscuration lack the sensitivity to distinguish translucent and potentially harmful protein aggregates, other options are needed.

Micro-Flow Imaging (MFI) offers several advantages over traditional techniques in the analysis of sub-visible and visible particles in protein formulations as it's imaging-based and offers direct particle detection. The particle count, size and morphological information provided enable novel and unique insights into particle characterization and quantification with just a single test. As a result, MFI systems can discriminate protein aggregates from the silicone micro-droplet contaminants and air bubbles commonly found in heterogeneous biopharmaceutical samples.

Particle characterization requires highly accurate count and sizing results. To enable this outcome, instrument calibration with NIST-traceable polystyrene beads is used to test instrument operation, re-verify instrument calibration following service, or evaluate method optimization. Polystyrene beads are ideal for particle sizing and sensitivity testing because they easily trigger the MFI imaging detector, yielding consistent and precise sizing and concentration results over a large size range. To ensure correct usage, this application note discusses recommended guidelines on how to use bead calibration for sizing and concentration verification on the MFI 5000 series with or without the Bot1 autosampler.

## WHAT DOES BEAD CALIBRATION TEST?

Polystyrene (PS) beads are useful for evaluating particle detection systems. Their physical characteristics make them ideal tools to qualify:

- Particle Detection — beads are easy to see due to their uniform density and shape.
- Size — beads come in a wide range of sizes that are NIST traceable, offering easy verification of particle sizing.

PS beads cannot address all aspects of particle detection, however. They are unable to duplicate the translucency of protein aggregates in solution or match the range of irregular shapes and specific density differences that can occur with protein fragments. In addition, the circularity and intensity measurements between beads and protein particles can be very different (TABLE 1), even when they are the same size.[1] For these reasons, beads are best suited for sizing accuracy evaluations.

| POLYSTYRENE BEADS | | | | |
|---|---|---|---|---|
| Parameters |  |  |  |  |
| Size (µm) | 10 | 20 | 30 | 50 |
| Intensity mean (0–1023) | 463.47 | 316.62 | 249.22 | 182.54 |
| Circularity (0–1) | 0.93 | 0.92 | 0.94 | 0.94 |
| **PROTEIN PARTICLES** | | | | |
| Parameters |  |  |  |  |
| Size (µm) | 10 | 20 | 30 | 50 |
| Intensity mean (0–1023) | 746.87 | 730.6 | 687.73 | 632.29 |
| Circularity (0–1) | 0.64 | 0.48 | 0.39 | 0.26 |

TABLE 1. Circularity and intensity differences between polystyrene beads and protein particles (Reprinted with permission from publisher).[1]

# MATERIALS

## SELECTING AND HANDLING BEADS

### SIZING STANDARDS

For testing size in the 2–10 µm range, calibration with a 5 µm polystyrene bead is recommended. A 5 µm bead is small enough to test MFI system resolution for sub-visible particles 2–10 µm in size, and ProteinSimple offers NIST Certified Particle Size Standard, 5 µm (P/N 4004-001-001) beads for this purpose.

NIST-traceable polystyrene bead standards are used in the execution of this protocol. Prepare size standards as follows:

- Prepare a sterile 15 mL or 50 mL sample tube with the appropriate dilution volume of sterile, deionized water for the size standard, as described in TABLE 2.
- Perform ten tumbles and ten rolls of the sizing beads as described in the documentation included with the bead standard. Limit the time to transfer beads into an analysis container.

Just prior to use, perform ten tumbles and ten rolls with the diluted sizing standard.

The shelf life of an unopened sizing standard bottle is up to two years, as listed on the bottle label.

### CONCENTRATION STANDARDS

Proper handling of calibration standards for concentration is also essential to accurate results. ProteinSimple offers CountCal™ Particle Concentration Standard, 5 µm (P/N 4004-003-002) beads for this purpose. In order to be sampled correctly from the standards bottle or analysis container the beads must be in suspension, otherwise the values obtained can be lower or higher than the expected concentration of the standard. Moreover, beads will settle over time depending on their size and how long the standards bottle has been sitting. Small, 5 µm beads are mostly neutrally buoyant and therefore require time to settle, but settling time reduces dramatically as bead size increases. To ensure a homogeneous distribution of beads in the sample, proper mixing of bead standards in their bottle is essential before transfer to an analysis container in the MFI system. Without proper mixing, a significant loss of measured sample may occur which can impair data accuracy.

| SIZE | DROPS | DILUTION VOLUME |
|---|---|---|
| **5** µm | 1 | 15 mL |
| **10** µm | 2 | 10 mL |

TABLE 2. Preparing size standards.

Follow these guidelines to correctly transfer beads from the bottle and into an analysis container:

- Perform ten tumbles and ten rolls as described in the documentation included with the bead standard. Once complete, keep the time to transfer beads into an analysis container to a minimum. This becomes more important as the size of the beads increases as larger beads settle faster.
- Use a fresh pipette tip to avoid introducing contaminants into the standards bottle and samples.
- Aspirate from the middle of the fluid in the bottle. If sample is drawn from the top or bottom, a homogenous mixture might not be obtained.
- Once the CountCal bottle is opened, contents must be used within eight hours and then thrown away. The shelf life of an unopened CountCal bottle is six months.

# METHOD DEFINITION

Preparing the bead sample is the first step in ensuring proper evaluation of your system. The next step is to define the method correctly so that only the bead information, and not any other extraneous material, is reported. Extraneous materials are generally outside of the size range for the specific calibration beads, as stated in manufacturer's instructions.

## CONFIGURING THE NUMBER OF RUNS

The documentation included with the concentration standards outlines the analysis of bead standards using a consecutive run approach. For optimal results perform four runs, with the first serving as a purge (these purge results are not included in the analysis), then average the last three runs to calculate the concentration.

If an MFI system with the Bot1 autosampler will be used, the consecutive runs should be configured via a Batch protocol. If a standalone MFI system (without a Bot1 autosampler) will be used, choose the method configuration for Consecutive Runs. The Method Type chosen should be 'Sample Analysis and Report'.

## STIRRING AND VOLUME REQUIREMENTS

For best results with a standalone MFI system, use a stirrer and a syringe barrel for sample introduction. A pipette tip can also be used to introduce sample, but the resulting concentration values may be less accurate due to bead settling effects, and the method will be different.

The next step in defining the method is to establish the volume usage. ***Required volumes will differ for a standalone MFI system versus an MFI system with the Bot1 autosampler.*** In either case, set the Termination Type of the method to 'Sample Dispensed'. TABLE 3 shows the recommended volumes for bead standards by type of sample introduction.

TABLE 4 specifies well dead volume for currently supported labware for the Bot1 autosampler. When using the Bot1 autosampler, note the difference in volume requirements for bead standards (Protocol C) compared to the supported protocol for protein samples (Protocol D). Protocol C should be used to ensure optimal results for evaluation of concentration using 5 µm beads on the Bot1 autosampler.

| | MFI WITH BOT1 | MFI WITH SYRINGE BARREL | MFI WITH PIPETTE TIP |
|---|---|---|---|
| Total Available Volume | 0.9 mL | 1.0 mL | 0.9 mL |
| Purge Volume | 0.0 mL | 0.2 mL | 0.2 mL |

TABLE 3. Recommended volumes for bead standards by sample introduction type.

| BOT1 PARAMETERS | BEAD STANDARDS (PROTOCOL C) | PROTEIN SAMPLES (PROTOCOL D) |
|---|---|---|
| Well dead volume | 0.03 mL | 0.03 mL |
| Flushing (with sample)* | N/A | 0.90 mL |
| Optimize Illumination (with sample) | N/A | 0.22 mL |
| Analysis volume | 0.90 mL | 0.70 mL |
| Volume required per sample (96/1 mL) | 0.95 mL | N/A |
| Volume required per sample (96/2 mL) | 0.95 mL | 1.85 mL** |

* Flushing with sample represents a purge step in Protocol D.
** The actual valuse may be lower, following method optimization.

TABLE 4. Bot 1 autosampler parameters for bead standards versus protein samples. The dead volume specified is for currently supported labware, namely the 96/1 mL and 96/2 mL.

## SETTING UP AN ANALYSIS METHOD FOR SIZING STANDARDS

This section describes how to configure an analysis method for bead sizing verification on the MFI 5000 (or DPA 4000 series) in manual mode. This analysis method can also be modified for use in an automated format on the MFI 5000 with Bot1 Autosampler.

FIGURE 1 shows an example of the steps to create an analysis method for sizing verification of 5 µm beads. To maintain beads in suspension during a standard run, stirring is required. If a Bot1 autosampler will not be used, the Stirring configuration is defined in the Analysis method, as shown in FIGURE 1B. If a Bot1 autosampler will be used, this configuration is done via the Batch protocol, as outlined in the 'Batch Definition with a Bot1 Autosampler' section.

After defining the run set up and stirrer control as shown in FIGURES 1A and 1B, the next step is to define the Particle Statistics, which involves selecting the particle size range described as the Equivalent Circle Diameter (ECD) for a particular bead size in the method for sizing verification. Refer to TABLE 5 for information on the range to choose for size verification beads. If the focus of your study is to detect in the sub-visible particle range from 2–10 µm, choose small bin ranges for bead sizes from 2–10 µm. Since we are testing 5 µm beads, we can select the bin range specifying 3.5 to 6.5, as shown in FIGURE 1D. If testing larger beads, select additional ECD ranges as needed, based on TABLE 5. In that case, configure the step shown in FIGURE 1E to specify additional bin ranges.

TABLE 5 shows the bead standard sizes along with the concentration specification range and the sizing specification range.

| BEAD SIZE (µm) | COUNTCAL ECD RANGE (µm) CONC VERIFICATION | INTERNAL ECD RANGE (µm) SIZE VERIFICATION |
|---|---|---|
| 2 | 1.3 to Max | 1.6 to 2.4 |
| 5 | 3.0 to Max | 3.5 to 6.5 |
| 10 | 7.5 to Max | 8 to 12 |
| 15 | 10 to Max | 12 to 18 |
| 20 | 10 to Max | 16 to 24 |
| 25 | 15 to Max | 20 to 30 |
| 30 | 20 to Max | 24 to 36 |
| 50 | 30 to Max | 40 to 60 |
| 70 | 50 to Max | 56 to 84 |

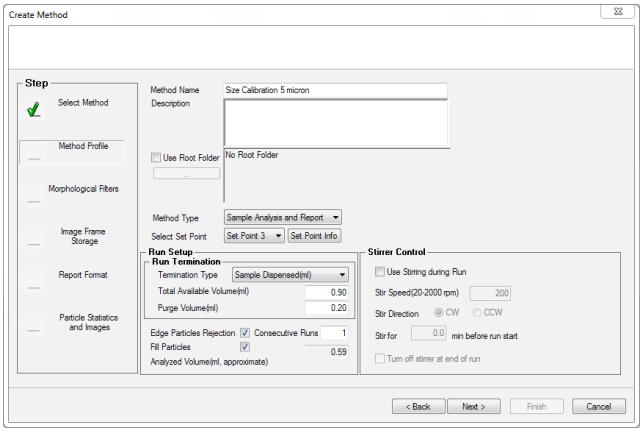
TABLE 5. Bead standards sizes and specification ranges. Beads greater than 25 µm in size are suitable for concentration verification in instruments with setpoint 1 flow cells that are 300 µm in diameter (MFI 4100 and 5100). Beads greater than 25 µm in size are not recommended for instruments using set point 3 flow cells which are 100 µm in diameter (MFI 5200 and 4200).

## SETTING UP AN ANALYSIS METHOD FOR CONCENTRATION STANDARDS

Configuring an analysis method for concentration verification is similar to the format used for sizing. FIGURE 2 shows an example with 5 µm concentration standards.

To maintain beads in suspension during a standard run, stirring is required. If a Bot1 autosampler will not be used, the Stirring configuration is defined in the Analysis method, as shown in FIGURE 2B. For best results, use a stirring speed of 1200 rpm for any of the recommended bead sizes. If a Bot1 autosampler will be used, this configuration is done via the Batch protocol, as outlined in the 'Batch Definition with a Bot1 Autosampler' section.

A

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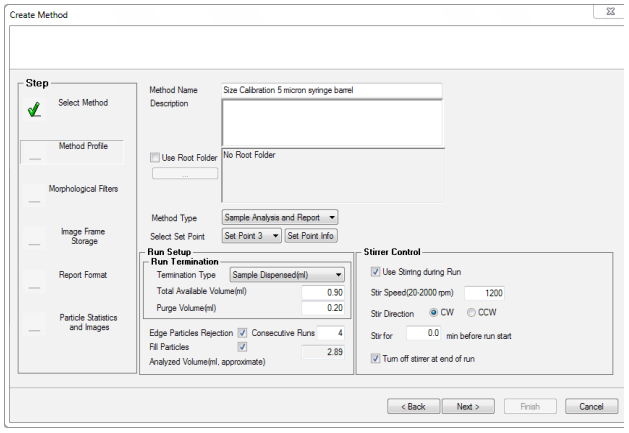


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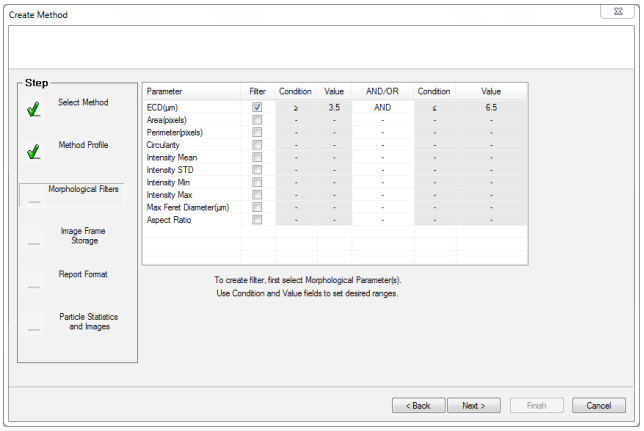


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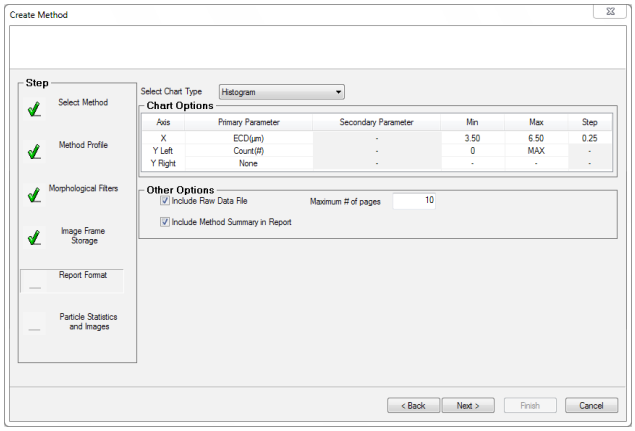


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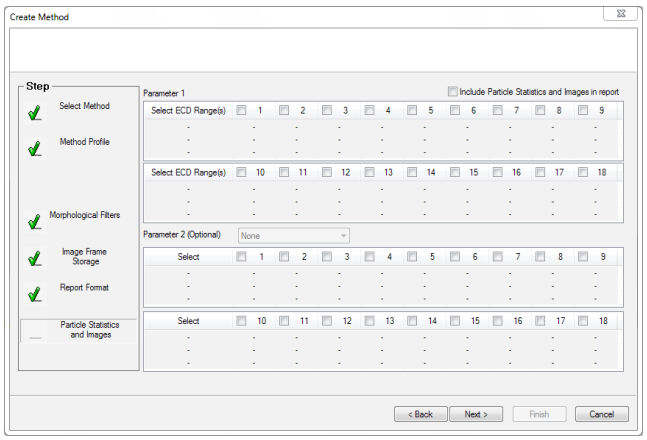
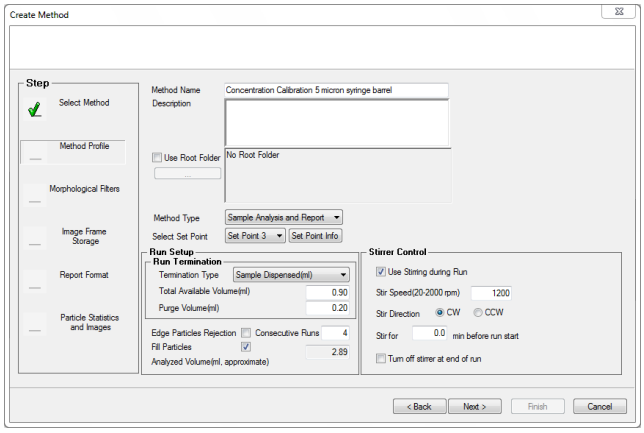



FIGURE 1. Profile of a new method for sizing with 5 µm bead standards. (**A**) Should be used for bead introduction by manual pipette, (**B**) shows bead introduction by syringe barrel, (**C**) describes how to select bead sizing with the ECD filter (pipette or syringe barrel), (**D**) shows the format for the bead sizing histogram, (**E**) shows particle bins unchecked for the 5 µm bead method created in steps **A-D** (deselect the 'Include Particle Statistics and Images in report' checkbox).

A

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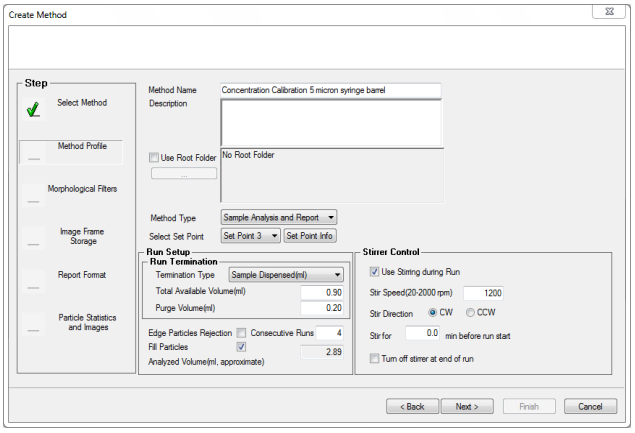


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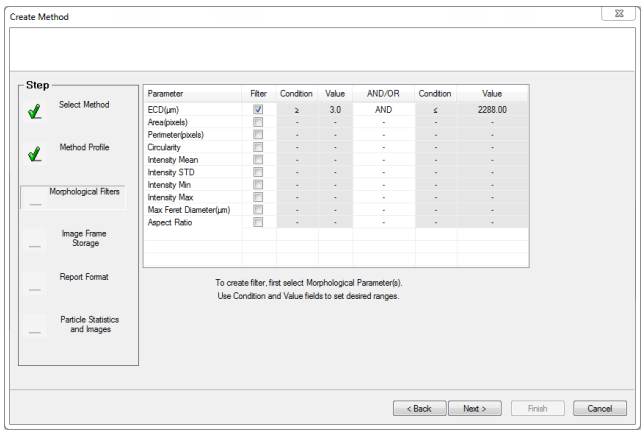


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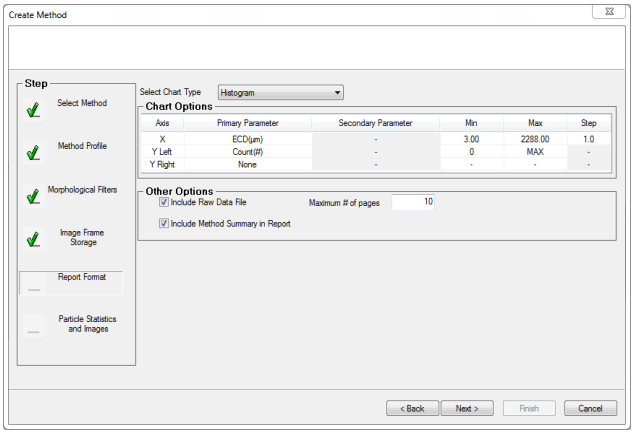


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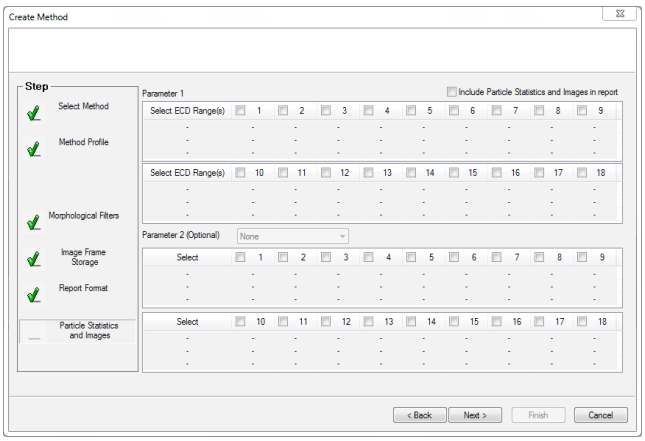



FIGURE 2. Profile of a new method for concentration with 5 µm bead standards. (**A**) Should be used for bead introduction by manual pipette, (**B**) shows bead introduction by syringe barrel, (**C**) describes how to select concentration bead validation with the ECD filter (pipette or syringe barrel), (**D**) shows the format for the bead concentration histogram, (**E**) shows particle bins unchecked for the 5 µm bead method created in steps **A–D** (deselect the 'Include Particle Statistics and Images in report' checkbox).

### BATCH DEFINITION WITH A BOT1 AUTOSAMPLER

With a Bot1 autosampler, the Batch defines how the method will be executed. For optimal results, configure the Batch to run Optimize Illumination in water, and complete the actual Analysis operations immediately after. An example Batch is shown in TABLE 6.

| OPERATION | LIQUID | VOLUME (mL) |
|---|---|---|
| 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 |
| Conditional Baseline | 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 |
| Stir 3 cycles, speed 5 | Sample | 0.90 |
| Analysis Run 1 | Sample | 0.90 |
| Stir 3 cycles, speed 5 | Sample | 0.90 |
| Analysis Run 2 | Sample | 0.90 |
| Stir 3 cycles, speed 5 | Sample | 0.90 |
| Analysis Run 3 | Sample | 0.90 |
| Stir 3 cycles, speed 5 | Sample | 0.90 |
| Analysis Run 4 | Sample | **0.90** |
| Flush | Filtered water or filtered buffer | **0.90** |
| Flush | Filtered water or filtered buffer | **0.90** |

TABLE 6. Example of a Bot1 Batch Protocol for bead standards (Protocol C).

### PARTICLE BASELINE

Control of ambient dust particles in the testing environment avoids high particle backgrounds, which can impact accurate measurement of samples when particle concentrations are low (~1000 P/mL). For a given assay, the sample particle level should be at least 4-fold greater than that of the average particle baseline. For example, if the average of the particle baseline background is about 30–100 P/mL, then samples should have a particle level of at least 400–500 P/mL to demonstrate a significant difference over background. In this example, the calculated ratio of sample level to particle baseline is 400/100 or 4. When the ratio is less than 4, results can be influenced by baseline variability.

## RESULTS

### SIZING ACCURACY

For MFI systems using manual sample introduction, the sizing resolution has been verified for accuracy to within 0.25 µm using NIST-traceable polystyrene beads across a broad range of particle sizes (TABLE 7).

| PS BEAD MEAN SIZE (µm) | ± 5% OF PS BEAD MEAN SIZE (µm) | MFI ECD MEAN SIZE (µm) | % DIFFERENCE OF MFI TO PS MEAN |
|---|---|---|---|
| 300.00 | 315.0 – 285.0 | 305.38 | 1.79% |
| 200.00 | 210.0 – 190.0 | 201.26 | 0.63% |
| 160.00 | 168.0 – 152.0 | 159.19 | -0.51% |
| 99.20 | 104.2 – 94.24 | 98.84 | -0.36% |
| 68.60 | 72.03 – 65.17 | 67.10 | -2.19% |
| 49.80 | 52.29 – 47.31 | 49.41 | -0.78% |
| 40.25 | 42.26 – 38.24 | 39.22 | -2.56% |
| 20.00 | 21.00 – 19.00 | 19.61 | -1.95% |

TABLE 7. MFI system sizing accuracy.

FIGURE 3 shows a plot confirming that a linear relationship exists between measured size and NIST-certified size measurements.

For these reasons, sizing accuracy was well within expected performance parameters listed in TABLE 8.

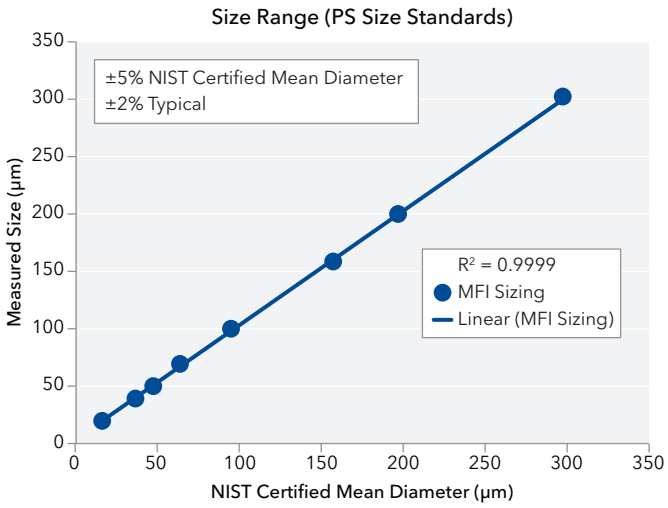



FIGURE 3. DPA-4100 system sizing accuracy across a broad particle size range.

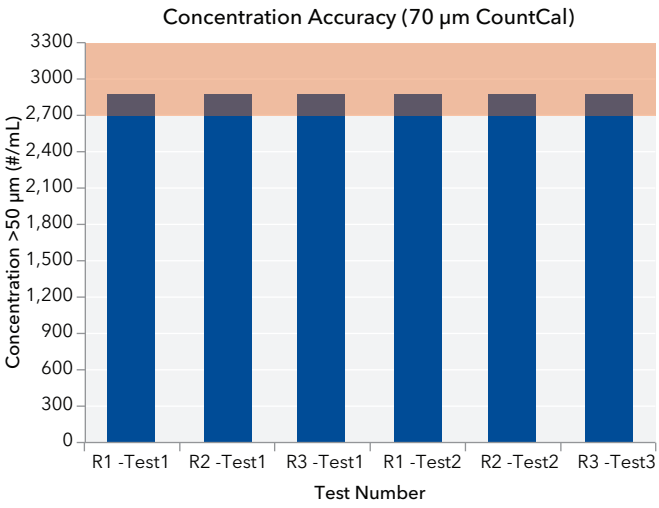



FIGURE 4. DPA-4100 system concentration accuracy. Values are well within the range specified by the manufacturer for concentration measurements (3,000 ± 10%).

| PARAMETERS | 5100 MODEL | 5200 MODEL |
|---|---|---|
| Size Range (µm) | 2 to 300 | 1 to 70 |
| Max Concentration Range without dilution, 2.5 µm object) | 175,000 | 900,000 |
| Analysis Rate (µL/min) | 200 | 150 |
| Sizing Accuracy % (5 µm particles) | ±5 | ±5 |
| Concentration Accuracy % (5 µm particles) | ±10 | ±10 |

TABLE 8. Micro-Flow Imaging performance parameters.

## CONCENTRATION ACCURACY

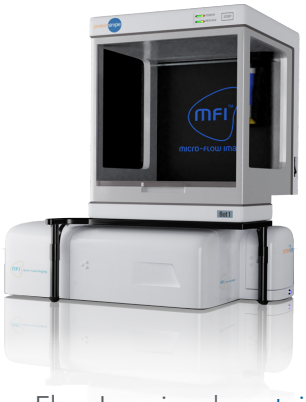
MFI systems accurately measure the concentration of particles across a broad dynamic range. The results in **FIGURE 4** illustrate measurements of 70 µm concentration standards (specified by the manufacturer to be 3000/mL ±10%) on the DPA-4100 instrument, and indicate consistency in sizing accuracy.

# CONCLUSION

Calibration of MFI systems with NIST traceable polystyrene beads is critical for verifying instrument operation and daily performance. Different sample introduction techniques will require different sample handling protocols. Analysis methods for verification of bead sizing and bead calibration are also different and should be tailored to the bead size range. The system parameters used and how standards are handled will impact results, but if the guidelines presented in this application note are followed, precise bead calibration measurements can be achieved.

# REFERENCES

1. Micro-flow imaging: flow microscopy applied to sub-visible particulate analysis in protein formulations. D. Sharma, D. King, P. Oma and C. Merchant, AAPS Journal, 2010; **12(3)**:455–464.



Learn more about Micro-Flow Imaging | proteinsimple.com/mfi_5000.html

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