

# Sized Right for Particle Analysis

## Introduction

Particle size matters. Whether you're studying a protein formulation, a small molecule formulation or a lipid nanoparticle, characterizing the size of your particles is a key parameter used in assessing the quality of a drug product<sup>1</sup>.

The HORIZON<sup>®</sup> system from Halo Labs employs Backgrounded Membrane Imaging (BMI) technology to measure and characterize subvisible particles in a high-throughput, low volume format. BMI has its roots in membrane microscopy – a USP lot release method that images particles on a membrane surface. The fully automated, fluidics-free HORIZON<sup>®</sup> system utilizes disposable membrane plates and automated image

processing to rapidly generate reliable and accurate particle counts and sizing. It can perform particle characterization in up to 96 samples in a single run using as little as 25  $\mu$ L per sample. BMI is also insensitive to solution refractive index, enabling reliable measurement of translucent protein aggregates.

## The HORIZON<sup>®</sup> system for fast and reliable particle analysis

The HORIZON<sup>®</sup> system uses sophisticated image-processing techniques to analyze images and acquire particle data. The key is to first take a background image of the membrane. After samples are filtered through by vacuum and the particles are captured, the same membrane is re-imaged with sample particles on the

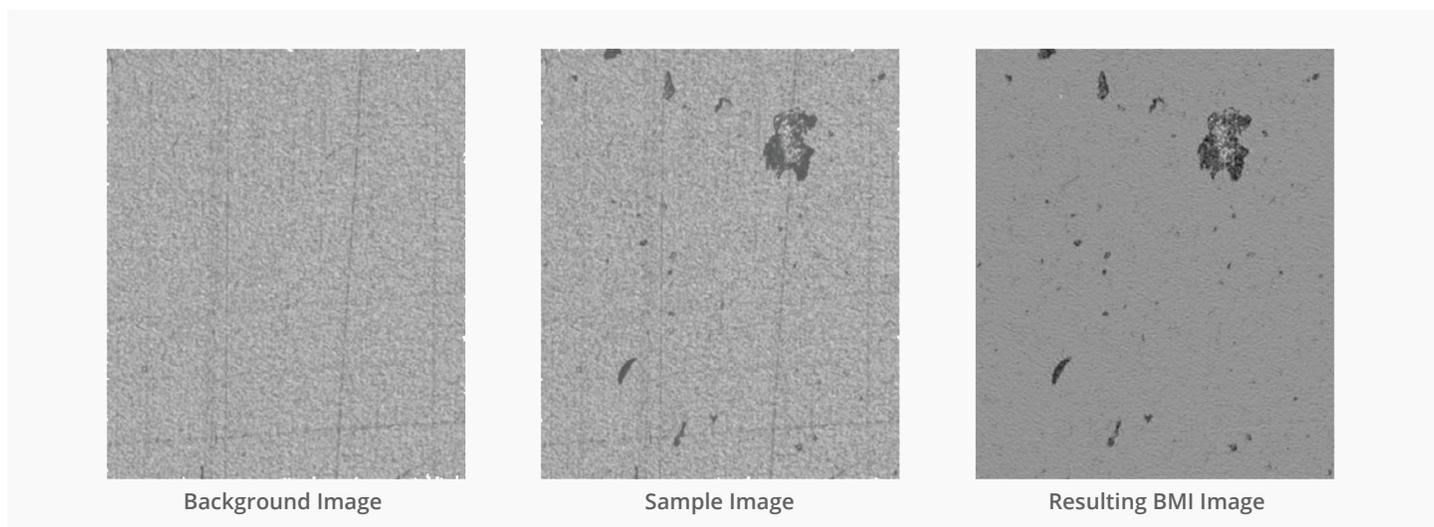
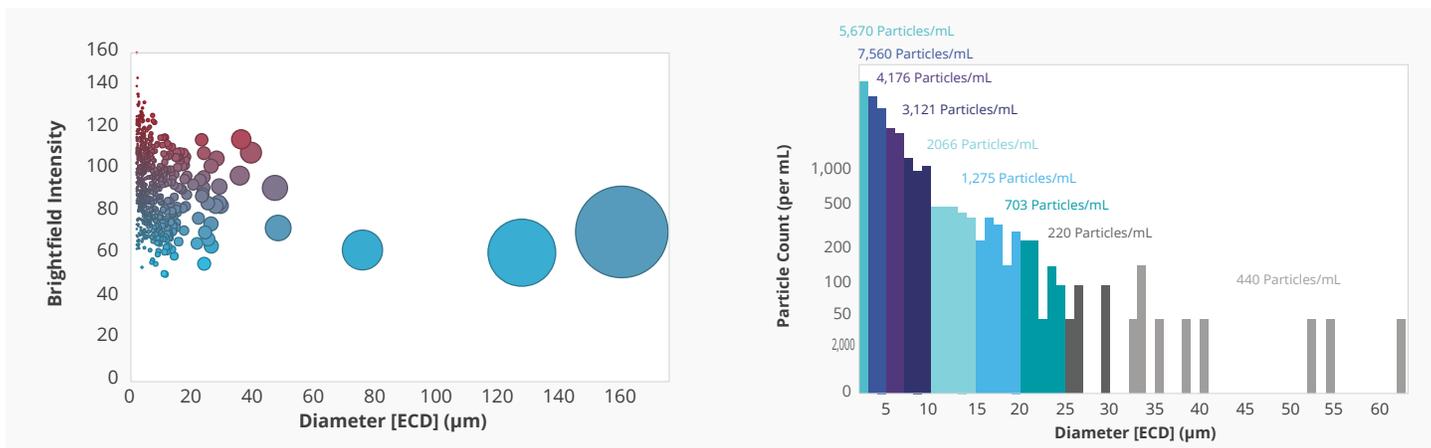


FIGURE 1: Image backgrounded on the HORIZON<sup>®</sup> system for accurate high contrast particle images.



**FIGURE 2:** Interactive charts of individual wells in the VUE software enable visualization of data by multiple characteristics.

surface. The sample image is precisely aligned with the background image and then processed pixel by pixel so that the background texture is eliminated, and particles are viewed in high contrast (Figure 1). Backgrounding the image leads to high reproducibility and minimal interference from membrane texture and imperfections.

### Easy visualization with the VUE software

The HORIZON<sup>®</sup> system comes with the VUE software, an intuitive, streamlined analysis suite designed to quickly provide key insights about your data. The automated data analysis gives quick and easy access to particle characterization parameters like Equivalent Circular Diameter (ECD), aspect ratio, circularity, etc. Individual images can be viewed for every particle. Interactive scatter plots and histograms enable the user to visualize data by multiple characteristics (Figure 2) and easily obtain useful statistics such as size distribution and variability.

### Verification of sizing accuracy using NIST-traceable size standards

Routine periodic instrument performance checks are an important way to ensure integrity and reproducibility of results obtained from any particle analysis platform. Particle size standards may be used to validate systems across their dynamic ranges and for regular performance checks of an instrument.

Certified, ready to use microsphere suspension size standards with traceability to National Institute of

Standards and Technology (NIST) are convenient and cost-effective and can be used with the HORIZON<sup>®</sup> instrument to verify and validate particle sizing accuracy. In this method paper we describe a simple procedure incorporating NIST-traceable bead standards to verify accuracy of measurements on the HORIZON<sup>®</sup> system.

### Method

The key steps for utilizing NIST-traceable bead size standards to verify particle sizing on the HORIZON<sup>®</sup> system are as follows:

- 1 Select and prepare size standard to measure.
- 2 Enter correct settings in the VUE Software.
- 3 Prepare the HORIZON<sup>®</sup> instrument plate and measure on the instrument.
- 4 Analyze data to determine average size value of single particles.

### Select and prepare size standard

The NIST-traceable standards recommended for the HORIZON<sup>®</sup> system verification procedure are COUNT-CAL<sup>™</sup> Count Precision Size Standards polystyrene beads from Thermo Fisher Scientific ([www.thermofisher.com/order/catalog/product/](http://www.thermofisher.com/order/catalog/product/)). COUNT-CAL<sup>™</sup> standards are available in a selection of precise sizes ranging from 5–30 µm (TABLE 1) and have a tight size distribution specification of <3%. This product is preferred over other monosized bead standards as they come pre-diluted to

THERMO FISHER PN	DIAMETER	PARTICLE DIAMETER	CONCENTRATION
CC05-PK	5 µm	5.010 µm ± 0.035 µm	3000 particles/mL
CC10-PK	10 µm	10.00 µm ± 0.08 µm	3000 particles/mL
CC15-PK	15 µm	15.02 µm ± 0.15 µm	3000 particles/mL
CC30-PK	30 µm	29.75 µm ± 0.56 µm	3000 particles/mL

**TABLE 1:** NIST-traceable polystyrene bead sizing standards recommended for use in verification of the HORIZON® system.

a concentration that is suitable for membrane particle analysis.

Vials of COUNT-CAL™ standards should be stored refrigerated at 2–8°C when not in use, and never frozen. Mix the product thoroughly by gentle inversion by hand prior to and during use.

## Enter correct settings in the VUE software

### Select particle detection profile

The VUE software v1.4 offers two selections for particle detection profile. The “HaloLabs Recommended” setting should be used for sizing verification as it is an improved algorithm that will yield the most accurate results. This profile must be selected over the default “HaloLabs Original” profile via the following steps:

- 1 In the VUE software, start a new experiment and lay out the sample plate according to HORIZON® system Operation Manual, Section 3-2.
- 2 From the pull-down menu in the top left corner of the software window, select “Global Settings”.
- 3 In Global Settings pop-up window there is a pull-down menu option for “Particle Detection Profile”. Select “HaloLabs Recommended” as the detection profile (Figure 3a).

## Set up correct size bins

The VUE software v1.4 has a feature that enables user-defined custom size bins. Once the COUNT-CAL™ size standard to measure for this procedure has been selected, it is very important to adjust the bins to match the chosen bead size.

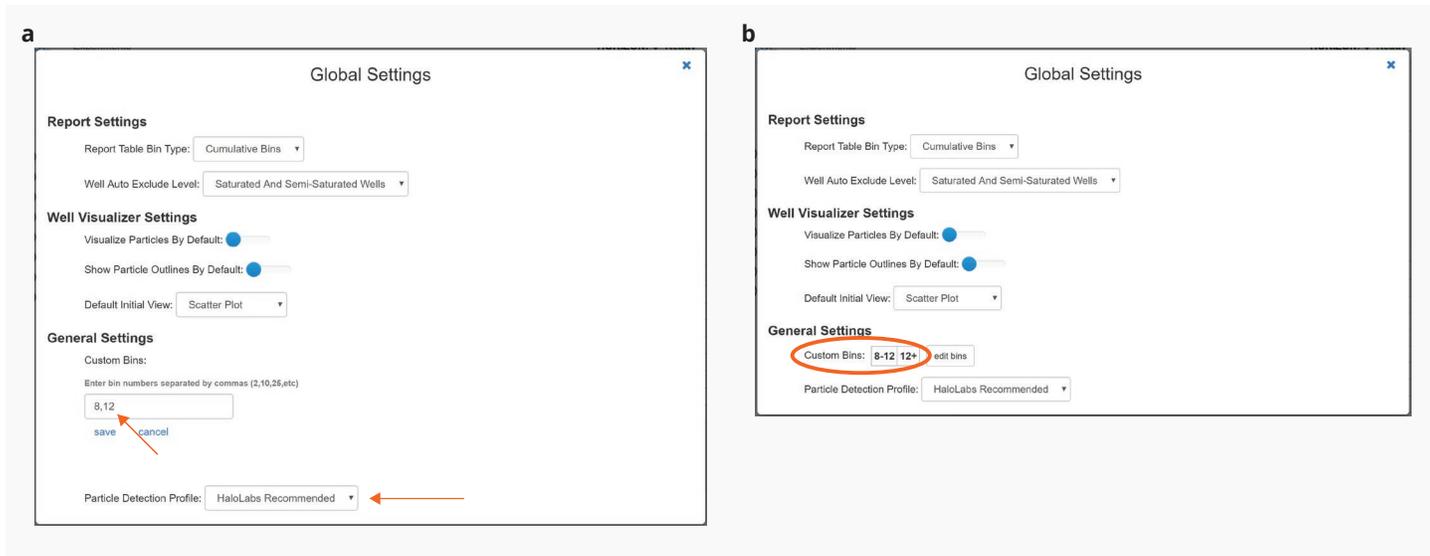
Entering the appropriate bin settings ensures that only singlet beads are analyzed when determining average particle size. In membrane analysis, synthetic spherical bead particles >5 µm can aggregate as they roll on the membrane and gravitate towards the center of the well upon applying the vacuum. However, the VUE software is equipped to distinguish these aggregates and separately analyze singlet beads for the purposes of confirming sizing accuracy.

Bin values to use for this procedure are listed according to bead size in TABLE 2. Use the following steps to change the bin settings:

- 1 In the Global Settings pop-up window, under General Settings, click the “Edit Bins” button.
- 2 Type in the bin values specified in TABLE 2, separated by commas (Figure 3a).
- 3 Under Report Settings, verify that “Report Table Bin Type” selected is “Cumulative Bins”.
- 4 Click the “Save” button. Custom bin settings should now reflect the desired range (Figure 3b).
- 5 Close the Global Settings window.

BEAD SIZE	BIN TO ANALYZE	BIN VALUES TO ENTER IN GLOBAL SETTINGS
5 µm	3 µm – 7 µm	3, 7
10 µm	8 µm – 12 µm	8, 12
15 µm	12 µm – 18 µm	12, 18
30 µm	24 µm – 36 µm	24, 36

**TABLE 2:** Bins to define in the VUE software based on NIST bead standard(s) being analyzed.



**FIGURE 3:** Entering the correct settings in the VUE software v1.4 under Global Settings, shown here for 10  $\mu\text{m}$  bead standard. (a) Selecting Particle Detection Profile and entering bin values from TABLE 2 under the General Settings header. (b) Bin values after clicking “Save” should reflect the desired ranges.

### Prepare the HORIZON<sup>®</sup> system plate

After setting up your experiment in the software and entering correct size bins, a background image of the plate wells must be acquired. Background the wells to be used in the bead validation experiment by loading the HORIZON<sup>®</sup> system plate in the instrument and clicking the “Acquire Background” button in the experiment window. Once backgrounding is completed, eject the plate and use the following procedure to load the bead samples onto the backgrounded wells:

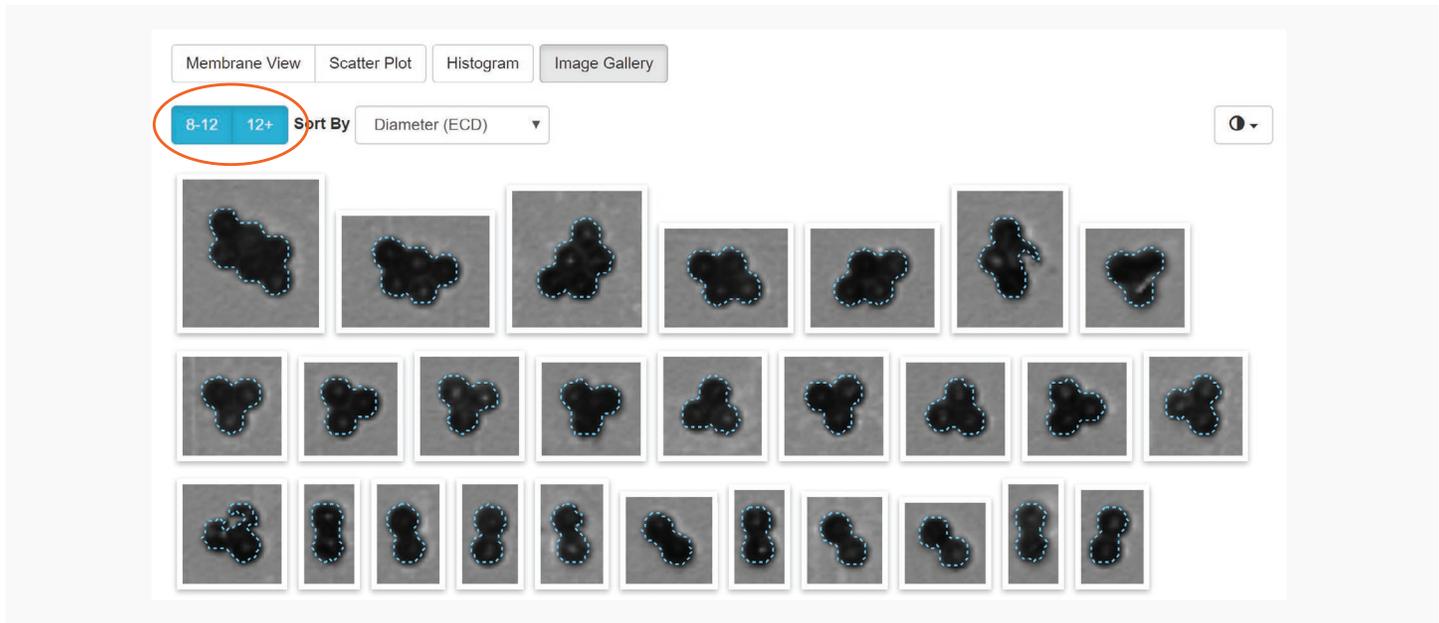
- 1 Place the plate onto the loading manifold.
- 2 Mix the bead standard vial by inverting at least 10 times immediately before pipetting.
- 3 Pipette 50  $\mu\text{L}$  of sample into each well according to plate layout. Pipette sample into a maximum of 8 wells (1 column).
- 4 Once 8 wells are loaded, turn on the vacuum pump, open the manifold vacuum switch and allow the sample to pull through the membrane for 10 seconds.

- 5 If needed, repeat steps 2–4, mixing the vial again each time, until all designated wells have sample.
- 6 Place the plate onto filter paper and pull vacuum for additional 10 seconds to ensure the membrane is fully dried.

Vacuum should be applied after pipetting a maximum of 8 samples (1 plate column) at a time when loading bead suspensions onto the HORIZON<sup>®</sup> system plate. More frequent sample pull-through limits the time for bead particles to settle after pipetting, thus ensuring more even distribution across the membrane. Additional vacuum applications do not impact wells in which particles have already been captured. Also, frequent mixing of the standard vial helps keep beads separate and evenly suspended for more consistent sampling.

### Analyze the data to determine average particle size

Once the plate is prepared, place it in the HORIZON<sup>®</sup> instrument and click “Start Measurement” to begin data acquisition. The light on the front panel will change colors when measurement is complete.



**FIGURE 4:** Image gallery of 10 µm NIST beads run on the HORIZON® system, sorted by diameter, with both bins selected. From this view, the blue “12+” button (the largest bin) can be clicked to de-select and remove aggregated beads from the data set.

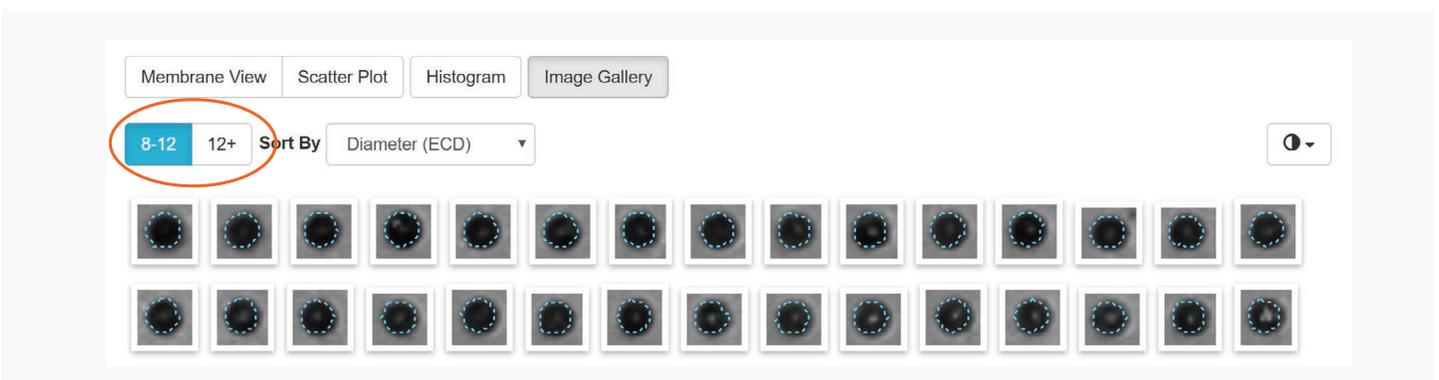
Return to the VUE software and click “View Experiment Report” to see the compiled results. On the plate map, click on the well containing the standard to analyze. Then click “Image Gallery”. Individual images of particles detected in the sample will be displayed (Figure 4).

The custom bins established prior to running the plate will both be highlighted above the image gallery (Figure 4, top of window). Particles from the largest bin primarily contain aggregated beads and need to be

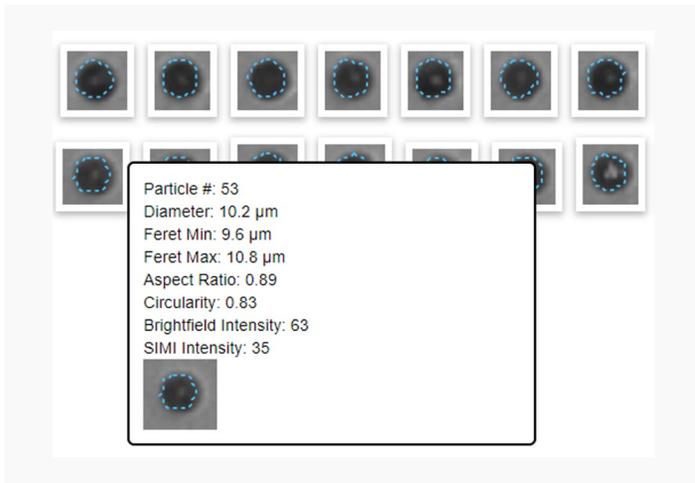
excluded. Click on the largest bin (“X+” bin) to de-select it. The aggregates are now hidden leaving only the singlet particles in the gallery to analyze (Figure 5).

Calculating the average size of single beads captured can be done in two simple steps:

- 1 Identify the median particle in the bin.
- 2 Average the median particle size with that of the two surrounding beads.



**FIGURE 5:** Image gallery of 10 µm NIST beads after de-selecting the largest bin. In this example using 10 µm beads, only particles that are between 8 µm and 12 µm (singlet beads) are now viewable.



**FIGURE 6:** Example particle data seen when hovering over each individual image.

The median particle is identified by using the particle number designation. All particles on the membrane are numbered from largest to smallest. A particle’s number, diameter, and other characteristics can be viewed by hovering the mouse over its image (Figure 6). In the image gallery view, particle images are ordered by default by their number — with the largest at the top left, smallest at the bottom right.

Hover over the largest and the smallest particle number in the bin and record their particle numbers (Figure 7). Then calculate the average of the two numbers. The particle with the number corresponding to this average is the median particle. For example, in Figure 7 the largest particle in the bin is #34 and the smallest is #73. The average of 34 and 73 is 53, therefore particle #53 is the median particle of the bin.

Next, hover over the images of the median particle and then the two particles surrounding it on each side and record the diameters. Calculate the average of these three numbers. This value represents the measured average bead size for the well.

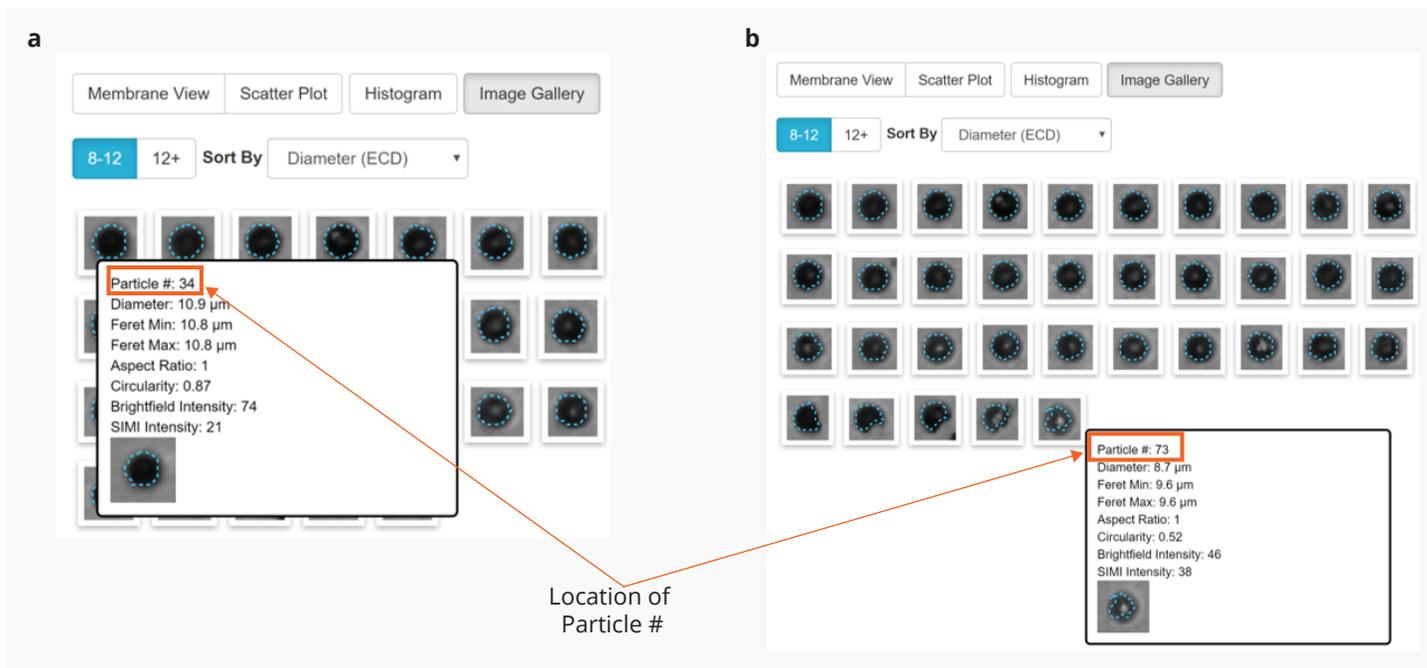
To illustrate using the Figure 7 example, the diameters of particles # 53 (median), #52, and #54 are:

$$\#52 = 10.3 \mu\text{m}$$

$$\#53 = 10.2 \mu\text{m}$$

$$\#54 = 10.2 \mu\text{m}$$

The average of these values rounded to 1 decimal = 10.2  $\mu\text{m}$ .



**FIGURE 7:** Example showing the particle numbers for the smallest and largest beads in the image gallery. To find the median particle number, simply average these two particle numbers.

## Case study

Using the procedure described, the diameter for four of the NIST-traceable COUNT-CAL™ bead standards was measured using the HORIZON® system. Eight wells were analyzed for each bead size and resulting average diameters and standard deviations are shown in TABLE 3. All bead sizes were determined to within 2.2% of the vendor-certified size specification, indicating accurate measurement by the HORIZON® system membrane analysis system.

NIST BEAD STANDARD (µm)	DIAMETER (µm)	DIAMETER STD DEVIATION	HORIZON MEASURED ECD AVERAGE (µm) N=8	HORIZON MEASURED ECD STD DEVIATION N=8
5	5.01	0.04	4.9	0.06
10	10	0.08	10.0	0.15
15	15.02	0.15	15.0	0.12
30	29.75	0.56	29.4	0.28

**TABLE 3:** NIST-traceable bead sizing standards and the measured size using the HORIZON® system. Data represents average measurements from 8 wells.

## Conclusion

Here we have demonstrated a straightforward procedure for utilizing commercially available certified NIST-traceable size standards to verify performance and accuracy of the HORIZON® system. Generating particle characterization data you can trust is vital, and the described method offers an additional tool to give you confidence that the instrument is measuring particle size accurately.

Our expert applications team is ready to support your work on the HORIZON® system. If you have questions or would like to request assistance with implementing this protocol, reach out to us at [support@halolabs.com](mailto:support@halolabs.com). 

## References

1. Shekunov BY, Chattopadhyay P, Tong HHY, Chow AHL. Particle Size Analysis in Pharmaceuticals: Principles, Methods, Applications. *Pharmaceutical Research*. 2007 Feb; 24(2): 203–227.

