Add the following:

▲⟨1788.2⟩ MEMBRANE MICROSCOPE METHOD FOR THE DETERMINATION OF SUBVISIBLE PARTICULATE MATTER

1. INTRODUCTION

The membrane microscope method (MM) may be applied to samples of both large-volume and small-volume parenteral injections, as well as ophthalmic solutions. This test enumerates solid[1] particulate matter that is ≥10 µm after collecting, rinsing, and drying on a microporous membrane filter. Semisolid substances may also be isolated, although all liquid and sub-micrometer material will pass through the membrane. Because a wide range of test aliquots may be utilized, particle counts may be determined on a per-volume or a per-container basis without dilution or extrapolation.

Contrary to the light obscuration (LO) or flow imaging (FI) methods, particles are not measured using the MM method in their in-situ state. Particles are isolated from the formulation and examined on an air-dried substrate, and thus they may have changed from their wetted state in the final product. MM is not a reliable method for analyzing particles <10 µm; however, these particles can be analyzed using LO and FI. Because of their deformable shape and low contrast, protein particles can be difficult to detect or pass through the filter. For these reasons, this technology is best-suited for the analysis of foreign particles, which is useful in root cause analysis.

To perform the MM assay, the analyst estimates the size of retained materials viewed at 100× magnification, tabulating them into two (or more) size categories. In this process, materials on the membrane surface that do not appear solid may be encountered, showing little or no surface relief like a "stain" on the membrane. *Particulate Matter in Injections* ⟨788⟩ advises not to size or enumerate such semisolid particles due to comments in the past from large-volume injectable manufacturers that encountered brown stain-like residues after heat sterilization of dextrose solutions using terminal sterilization. However, if a carbohydrate solution or similar formulation is not being tested, recognizing the presence of such material may help to determine changes that would increase formulation robustness. Consistent evidence of such materials may be an indication that further product development is necessary to understand their content. The nature of these materials and the subsequent decision to count or investigate must be based upon product formulation experience. Interpretation of microscopical enumeration may be aided by testing a sample by the LO method or a validated alternative method and characterizing by Fourier transform infrared spectroscopy (FTIR) or another spectral microscopy method (see *Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections* ⟨1787⟩). Information is provided in this chapter to aid the analyst in using the MM method.

2. TEST APPARATUS

2.1 Principles of Operation

Sample packages are mixed and opened, and product fluid is drained or transferred into a clean filtration barrel. Free particles from the product are captured on a membrane surface. The membrane-retained material is rinsed and dried for subsequent microscopic examination. Sizing and counting of particles is conducted at 100× using two size thresholds: ≥10 µm and ≥25 µm. Particle content is reported as the total count in each category.

[1] Soft particles and semisolid substances may also be retained.

2.2 Basic Configuration

Elements of the filtration and microscopic equipment are described in ⟨788⟩. Additional considerations are discussed here.

FILTRATION APPARATUS

Use a filter funnel suitable for the volume to be tested, such as a 25-mm 50 mL or 47-mm 300 mL barrel. Larger barrels are also available. However, the barrel inner diameter will determine the final counting surface; for example, an inner diameter of about 16 mm for 25-mm membranes or about 37 mm for 47-mm membranes. The funnel may be made of plastic, glass, or stainless steel. Use a filter support made of stainless steel or sintered glass as the filtration base. The goal is a flat, dry, even-colored, and unmarked membrane background.

Cleaning and rinsing the apparatus is facilitated with a pressurized solvent dispenser capable of delivering fluids passed through a terminal membrane filter at a range of pressures from 10 to 80 psi.

MEMBRANES

Chapter ⟨788⟩ recommends a black or dark gray membrane with a pore size of <1 µm; however, finer pore size membranes will have smoother surfaces, enhancing the resolution of particles in the microscope. Smaller pore sizes may impede the filtration of more viscous sample fluids during the assay. The black or dark gray membrane is recommended to provide good contrast for the array of particle colors, shapes, and transparencies. Other colored membrane types may be suitable depending on the product.

Alternative membrane choices may also be used to improve or alleviate incompatibility during product filtration. Product compatibility is an issue for mixed cellulose ester membranes or for the isolated materials used for further compositional analysis. Gold-coated or metallic silver filters may be used for these applications, especially spectroscopic analysis or particle type identification.

The test apparatus is described in ⟨788⟩. Additional comments are the following:

- A compound binocular microscope that corrects for changes in interpupillary distance by maintaining a constant tube length must be used.
- The objective must be of 10× nominal magnification with flatness correction and color correction, such as a planar achromat or better in quality, with a minimum 0.25 numerical aperture (NA).
- The objective must be compatible with an episcopic illuminator attachment.
- The eyepieces must be matched. In addition, one eyepiece must be designed to accept and focus an eyepiece graticule.
- The microscope must have a mechanical stage capable of holding and traversing the entire filtration area of a 25-mm or 47-mm membrane filter in order to completely scan the isolate surface.
- One episcopic and one oblique illuminator are required. Both illuminators must be of sufficient output to provide a bright and even source of illumination and may be equipped with blue daylight filters to decrease operator fatigue during use.
- The *USP* graticule as described in ⟨788⟩ is used.

STAGE MICROMETER

Used for calibrating the microscope configuration and graduated in 10-µm increments, the stage micrometer is preferably utilized each day of use. For initial calibration, utilize a stage micrometer that is traceable to the National Institute of Standards and Technology (NIST) to verify the *USP* graticule dimension features. A commercial stage micrometer graduated in 10-µm increments to verify proper setup may be used for daily calibration/verification.

2.3 Instrument Dynamic Range

The following considerations apply to both human operator and machine vision. Although the instrument has a wide dynamic range and this technique may be used for visible particles at the upper end, the focus in this section is at the lower end, a subvisible range.

SIZE ACCURACY

The microscopical assay uses 100× observation by reflected illumination in an air mount that produces optical diffraction effects, e.g., blurriness. Thus, the practical resolution at the specified size thresholds is about 1 µm. At a nominal 100× magnification, the smallest particles (10 µm) are easily discriminated from the background. Particles <10 µm can be observed via the MM method, but differentiation of particles close to the thresholds is important in the sizing process.

Because there is a diffraction limit of about 1 µm, it may be difficult to discriminate 9 µm from 10 µm or 24 µm from 25 µm due to suboptimal illumination, interfering background, particle features, et cetera. As a best practice, if there is doubt in determining whether an object is a particle, it should be counted. The operator should note the predominant particle morphologies descriptively and may perform further analysis if appropriate.

PARTICLE CONCENTRATION

The filtration operation will capture any solid material that is suspended within the sample fluid. For a blank (e.g., filtered water), there may be several particles ≥10 µm. The particle load is typically higher for samples.

Considering the ⟨788⟩ small-volume injection limit of 3000 particles that are ≥10 µm per container, several thousand particles may be isolated from a 10-container pool. That particle load is atypical, but when encountered, must be processed. Full counting

of a high particle load membrane is possible but time consuming and fatiguing when thousands of particles are present. Partial or statistical counting is recommended and is described later in this chapter in *Partial Count Procedure*. The partial counting approach extrapolates the count of a high particle load membrane from a defined portion of the filtration area. A practical upper limit for counting is around ≥3000 particles per membrane. The ≥3000 particle load typically takes a trained analyst an hour or more to count. Consider reducing the sample volume (e.g., fewer product samples) to reduce the total load of isolated particles. Finally, when a high particle load sample cannot be adequately processed, pursue an alternative analytical strategy.

THRESHOLDING/SENSITIVITY

Training is essential because the assay requires the operator's judgment of particle size by comparison to the calibrated reticle. Calibration of the microscope after optimization of the illumination allows the operator to make the best judgment on whether a particle should or should not be included in the particle count.

For automated systems, the same rules for image analysis apply. Because the 10-µm and 25-µm thresholds are used, the sensitivity at these sizes is essential. The *General Notices* require that accuracy be within 10%; therefore the system must be able to recognize 10 ± 1 µm. Thresholding implies the definition of actual particle boundaries versus background, which can be difficult for the human operator and even more so for the instrumental logic applied for feature recognition.

3. BLANK AND SAMPLE HANDLING

A blank determination is necessary at the beginning of each test sequence to verify minimal contribution from the background, equipment, or personnel. Test sequence can be defined as per shift, product family, series of filtrations (manifold), or sample. The suitability of the selected test sequence is dependent upon the operational needs of the laboratory system; the ability to clean glassware between samples, the number of different products, and the volume of sample tested. However, the blank should be considered as a system suitability check. Failure of the blank would suggest that all samples that were run after the previous blank are questionable.

To determine the blank count, duplicate the sample preparation process regarding the apparatus and membrane types. Assemble a clean filtration apparatus with a fresh membrane, rinse the interior with filtered water to drain, and then deliver 50 mL or more volume of filtered water to the filtration funnel while applying vacuum, drawing the entire volume of water through the membrane filter. Remove the membrane from the filter funnel base and place it onto a holding device, typically, on a strip of double-sided tape on a microscope slide or in a commercial membrane holder or Petri dish. After allowing the membrane to dry (it must be counted dry), examine the entire filtration area microscopically at 100× magnification. If NMT 20 particles ≥10 µm and NMT 5 particles ≥25 µm or larger are present within the filtration area, the background particle level is sufficiently low to meet the criteria in ⟨788⟩, *Method 2 Microscope Particle Count Test*. If particle load exceeds these limits, repeat the procedure.

There is value in further limiting the background counts for both ⟨788⟩ and *Particulate Matter in Ophthalmic Solutions* ⟨789⟩ testing in regard to good laboratory practice, and more specifically in regard to the ≥25-µm and ≥50-µm particle limits in ⟨789⟩, which may be more restrictive than injectable limits considering the total particle content allowed for the (usually) small unit volumes.

4. TEST ENVIRONMENT

The recommended operational detail to enhance the successful performance of the MM assay is given in this section. It is ideal to use two unidirectional airflow hoods (UAFH) or other unidirectional airflow (UAF) enclosures, one for "wet" sample preparations and the other an enclosure for the microscope counting phase. The UAFH should have a capacity sufficient to envelop the area in which the analysis is prepared. The UAFH provides high-efficiency particulate air (HEPA) filtered air that meets the International Organization for Standardization ISO 14644-1 standard (*1*).

Throughout the operational procedure (in the HEPA environment), it is preferable to use powder-free gloves and low-shedding clothing. Before conducting the test, clean the work surfaces of the UAF enclosure with an appropriate filtered solvent. Glassware and equipment should be rinsed successively with a warm, residue-free solution of detergent, hot water, and filtered distilled or deionized water. [NOTE—Prior to use, pass the distilled or deionized water through membrane filters of at least 0.2-µm nominal pore size.] If an alternate solvent must be used, use proper safety precautions. Perform the rinse in the UAF enclosure. Allow the glassware and filtration apparatus to dry in the enclosure, upstream of all other operations. The enclosure is preferably located in a separate room that is supplied with filtered cool, conditioned air and maintained under positive pressure with respect to the surrounding areas.

4.1 Microscope Preparation

The microscope optical alignment and illumination are critical for the success of this method. Although it is not difficult to differentiate a 10-µm particle from a 25-µm particle at 100× with reflected light, the decision regarding the boundary at each size category is difficult with inadequate equipment, maintenance, or optical alignment. Also, operator fatigue is caused by poor microscope alignment. The operator will have to determine if a particle is above or below the limit (e.g., the particle size is 9 µm or 11 µm). Optimized system resolution (i.e., the ability to discern discrete points of minimal separation) relies upon good and well-aligned optical systems. Factors including instrument cleanliness, resolution (e.g., objective NA[2]), and focus of both eyepieces, and the graticule will all play significant roles in attaining the best images. In consideration of optimizing the use of the binocular compound microscope, it is best to utilize operators familiar with the instrument and comfortable with its

[2] NA is an indicator of optical light-gathering capability and, thus, resolution. High NA correlates to high resolution.

alignment. The operator conducting the method should align the optics and illumination for his/her use with supervisory/trainer approval.

It is recommended to start with the alignment of the microscope for a typical transmitted illumination observation using a known sample. Any specimen familiar to the operator will suffice; however, a common particle count reference standard suspension, such as the USP Particle Count Set RS, is a good selection because it is also utilized in method system suitability evaluation. A drop of USP Particle Count Set RS is placed between a glass microscope slide and coverslip and viewed microscopically[3] With appropriate interpupillary distance and a comfortable sitting position at the microscope, the operator examines the fields of suspended spheres. One should observe (with ease) the small standard spheres, crisp on a combined field for both eyes. One attains crisp focus and ease of view after the focal adjustment of each eyepiece on a single point on the specimen.

Rotate the graticule in the right microscope eyepiece so that the linear scale is located at the bottom of the field of view, bringing the graticule into sharp focus by adjusting the right eyepiece diopter ring while viewing an out-of-focus specimen. Focus the microscope on a specimen, looking through the right eyepiece only. Then, looking through the left eyepiece, adjust the left eyepiece diopter to bring the specimen into sharp focus.

When the operator is not comfortable using the microscope or does not attain an equivalent crisp focus for each eye in a merged field of view, counting will become a difficult experience and will result in fatigue and flawed size comparison.

Nothing is better for preparing the operator for counting particles than to examine a test membrane as a positive control. Seasoned microscopists may not require this step, but for new operators or individuals conducting many different types of methods in the modern laboratory, familiarization is a prudent exercise. A filter membrane of the type being used for the method —such as a 25-mm, color-contrast, plain, membrane filter of 0.45-µm nominal pore size, containing particles—is a good choice. This may be a sample from a previous method that contains a variety of particle types or one prepared for familiarization. This positive test control will contain natural particles (e.g., flakes, threads, equant particles, and particles of various colors/opacity in a range of sizes) to effectively refresh the operator's sensitivity and facilitate microscope and illumination alignment for optimal viewing.

To examine the membrane preparation, locate a typical array of particles and first bring the illumination into good alignment:

1. Adjust the external, incident illumination at an oblique angle (10°–20° inclined to the stage) so that an even ellipse of reflected light is visible on the membrane and even illumination is evident through the eyepiece field of view (even across the full field). Shadows will be evident from larger particles, such as those with *z*-axis dimension >5 µm (*z*-axis is the microscope's vertical axis).
2. Now adjust the internal episcopic brightfield illuminator to yield an even illumination at a high setting on the transformer control. More importantly, when dialing down the illumination, one observes the evident shadow from the larger particles. In this manner, the high reflectivity of flat, glassy particles and the distinct shadows of more equant (*x*:*y*:*z* ~ 1:1:1) particles are evident.

4.2 Using the Circular Diameter Graticule

In order to properly focus the ocular lenses and attain a balanced single-field view, each operator must bring the *USP* graticule lines into sharp focus by adjusting the eyepiece diopter ring. Next, focus the microscope on a specimen through this same eyepiece, and then, looking only through the other eyepiece, adjust its diopter ring to bring the specimen into sharp focus. The *USP* graticule and specimen particles are now in focus on a well-balanced illumination field.

The *USP* graticule is specifically fabricated for each microscope. The relative error of the graticule used must be ±2% and is initially measured with an NIST traceable stage micrometer. To accomplish this, align the graticule micrometer scale with the stage micrometer so that they are parallel. Compare the scales, using as large a number of graduations on each as possible. Read the number of graticule scale divisions (*GSD*), compared to stage micrometer divisions (*SMD*). Calculate the relative error:

$$\% \text{ Relative error} = 100[(GSD - SMD)/SMD]$$

A relative error of ±2% is acceptable and verifies good alignment, focus, and proper magnification. Thereafter, a day-of-use verification by the microscope operator with the NIST traceable stage micrometer is sufficient to demonstrate proper setup.

The basic technique of measurement, applied with the use of the circular diameter graticule, is to count all particles 10 µm and larger, further categorizing in sizes ranging from 10 to 24 µm and 25 µm or greater. The circular zone or graticule field of view (GFOV) is a useful zone for active sizing and counting. Particles are compared to the linear scale and/or circles to determine their size against the circular diameter design. This is conducted by mentally transforming the image of each particle onto the 10- and 25-µm graticule reference circles. The sizing process is carried out without having to physically superimpose the particle on the reference circles; particles are not moved from their locations within the GFOV (the large circle) for comparison to the reference circles. Compare the longest chord of the particle being sized to the diameter of the black or transparent circles. The circles allow comparison of the particles regardless of their orientation. The linear scale may also be used for size comparison. Use the clear graticule reference circles to size white or transparent particles. Use the black reference circles to size dark particles.

The intent of comparing particles to an equivalent circular diameter is correlation to the LO particle sizing methodology, for which many manufacturers have extensive databases. In practice, particles with nearly circular areas will correlate well with the graticule circle diameters. For particles with one long axis, such as rods and needles, the conversion to circular area will produce more significant bias to smaller estimated sizes. It is simpler, and most conservative, to count particles in longest chord. To use an extreme example, the total count of monodispersions of fine needle crystals would vary greatly dependent upon the size determination utilized.

[3] The microscope objective requires a defined cover slip thickness, nominally 170 µm, or No. 1½.

4.3 Particle Counting Accuracy (System Suitability)

- It is valuable to use a reference count preparation for verifying illumination and as a means to discuss counting and sizing criteria.
- Calibration of the graticule/linear scale should be done as needed to verify proper setup (ocular, objective, and intermediate lenses are easily removed and changed).
- Particle counting accuracy is an important training operation and a reasonable annual check. A monodisperse sphere standard, such as a membrane deposit of the USP Particle Count Set RS, is useful for comparative counts between the trainer and the operators.
- Blank samples are an effective means of confirming the cleaning preparations for the method.

5. TEST PROCEDURE

5.1 Sample Preparation

Preparation of the filtration apparatus and test preparations are discussed in ⟨788⟩ and *Methods for the Determination of Subvisible Particulate Matter* ⟨1788⟩.

Filter large-volume injection (LVI) units individually. Small-volume injection (SVI) units having a volume of 25 mL or more may be filtered individually.

Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply vacuum. If the volume of solution to be filtered exceeds the volume of the filtration funnel, add a portion of the solution, stepwise, until the entire volume is filtered. It is prudent to maintain more than half of the funnel volume of liquid in the filtration funnel between refills, especially if the partial count procedure is to be used (see *Partial Count Procedure*). [NOTE—This step is necessary in order to ensure even distribution of particles on the analytical membrane.] After the last addition of solution, begin rinsing the walls of the funnel by directing a low-pressure stream of filtered water in a circular pattern along the walls of the funnel, and stop rinsing the funnel before the volume falls below about one-fourth of the fill level. Maintain the vacuum until all the liquid in the funnel is gone.

Remove the filtration funnel from the filtration base while maintaining vacuum, then turn the vacuum off, and remove the filter membrane with nonserrated forceps. Place the filter in the prepared holder and label with sample identification. Allow the filter to air-dry in the UAF enclosure with the cover ajar.

5.2. Particle Enumeration

The MM test described in this section is flexible in that typical artifacts such as air and immiscible liquids do not interfere with the final count. The method has a broad size-detection and counting range if applying the partial count procedure. This method may be used where all particles on an analysis membrane surface are counted or where only those particles on some fractional area of a membrane surface are counted.

TOTAL COUNT PROCEDURE

The microscope method is a slower process than the LO or FI methods, but they all have their value. Because the results rely upon the training and experience of human operators (e.g., visual inspection), insufficient training and lack of diligence can result in imprecision (e.g., poor agreement within and among labs) and inaccuracy (e.g., particle size estimates that are inaccurate for nonspherical particle shapes). Operator fatigue can be a problem and is promoted by poor ergonomic fit (chair height), poor or imbalanced ocular focus, and inattention to detail due to interruptions and lack of mental focus. Restricting the eye movement to a field-defining graticule, such as the *USP* counting graticule, restricts eye movement to the central one-third of the field of view. This significantly limits eye movement and may induce fatigue.

Sample size is an important consideration in counting precision. Care must be taken to sample many containers within a batch for a good representation of the particle distribution. Accordingly, the portion of the individual package sampled is critical. Particles may float or settle. Sampling only the first 25 mL of a large volume parenteral or sampling without adequate and recent mixing will lead to serious undercounting. Sampling whole, well-mixed containers with the particles in suspension is the best approach.

Counting the isolated particles is an important parameter. Counting all of the particles retained on the membrane is certainly the best approach, and then the simple problem is determining the correct size for placement into the threshold bins, 10 µm and 25 µm. This will be increasingly important for methods utilizing additional bins for population determination, such as 5-, 50-, and 100-µm. Note that the particle limits for ⟨788⟩ and ⟨789⟩ must be reported as all particles ≥10 µm and all particles ≥25 µm (and all particles ≥50 µm). If the lab method has been configured to count in several bins, such as 10–24 µm, 25–49 µm, and ≥50 µm, et cetera, all bins ≥10 µm must be added to yield the total ≥10-µm count; all bins ≥25 µm need to be added to yield the total ≥25-µm count. Using a number of narrow size bins may be beneficial in product improvement efforts to separate particle groups.

In performing a total count, the graticule GFOV is defined by the large circle of the graticule, and the vertical crosshair is used as a counting target. Count and size particles as they pass across the full diameter line of the GFOV, keeping mental track of the path over the membrane. Scan the membrane in paths that cover the effective filtration area (EFA) adjoining but not overlapping previous scan paths. Repeat this procedure; tabulate particle counts minimally in the 10–24-µm and ≥25-µm thresholds, moving across the membrane until all particles on the membrane within the EFA are counted. Record the total number of particles that are 10–24 µm and the number that are ≥25 µm.

For large-volume products, calculate the particle count, in particles per milliliter, for each unit tested:

$$P_{\Sigma\geq10}/V$$
$$P_{\Sigma\geq25}/V$$
$$P_{\Sigma\geq50}/V$$

in which $P_{\Sigma\geq10}$ is the total particle count obtained from all portions ≥10 µm analyzed; $P_{\Sigma\geq25}$ is the total particle count obtained from all portions ≥25 µm analyzed; $P_{\Sigma\geq50}$ is the total particle count obtained from all portions ≥50 µm analyzed; and V is the volume, in milliliters, of the solution tested.

For example, the analyst has counted the test samples in four bins: a) 10–24 µm, b) 25–49 µm, c) 50–99 µm, and d) ≥100 µm. This would then be calculated as:

$$P_{\Sigma\geq10} = P_{10–24\ \mu m} + P_{25–49\ \mu m} + P_{50–99\ \mu m} + P_{\geq100\ \mu m}$$

For small-volume products, calculate the particle count, in particles per container:

$$P_{\Sigma\geq10}/\eta$$
$$P_{\Sigma\geq25}/\eta$$
$$P_{\Sigma\geq50}/\eta$$

in which $P_{\Sigma\geq10}$ is the total particle count obtained from all portions ≥10 µm analyzed; $P_{\Sigma\geq25}$ is the total particle count obtained from all portions ≥25 µm analyzed; $P_{\Sigma\geq50}$ is the total particle count obtained from all portions ≥50 µm analyzed; and n is the number of units pooled (1 in the case of an individual unit).

PARTIAL COUNT PROCEDURE

When encountering a membrane full of particles, the task of adequately counting all of them is daunting. Consider that an small volume parenteral with an at-limit content of small particles, sampled in a 10-vial pool would have 30,000 10-µm particles on the membrane. Partial or statistical counting of the membrane's EFA may be the only means to attain consistent results. Partial counting should be used not to reduce count times but to estimate the total load on a high-count isolate. Field-defining devices, such as grids on the membrane surface or an ocular GFOV, have been used reliably. An ocular graticule provides a sharp boundary for area definition. Gridded membrane lines are rather broad and have ink-spatter that may be mistaken for particulate matter.

For 25-mm membranes, the EFA is 16-mm in diameter using typical commercial filtration funnels; therefore the EFA ($\pi \times r^2$) = 201 mm^2. Based upon earlier proposals from the Health Industry Manufacturers Association (HIMA) and discussion by Draftz (*2*), acceptable confidence intervals (Poisson distribution, two standard deviations) dictate that for samples with <1000 particles, the imprecision of statistical counting is objectionable. Full count is recommended for such samples. For samples with >1000 particles on a 25-mm isolate membrane, a reasonable estimate of particle population is attained using 20 GFOVs. If a smaller confidence interval for the result is desired, a larger number of fields and particles may be counted.

For 47-mm membranes the EFA has a 37-mm diameter. These larger-diameter membranes may be selected for formulations needing more membrane surface area (i.e., formulations that have slow flow characteristics through 25-mm membranes); in which case, the EFA ($\pi \times r^2$) is ($\pi \times 18.5$ mm^2) = 1075 mm^2. Thus, for 47-mm membrane EFAs, many more GFOVs must be counted to attain similar confidence levels. Using 100 GFOVs for partial counting of 47-mm membranes provides similar statistical confidence to the 20-GFOV/25-mm approach. Accordingly, when a particle load of 1000 or less is present, a full count is recommended.

When a partial count of particles on a membrane is to be performed, the analyst must first ensure that an even distribution of particles is present on the membrane. This is assessed by rapid scanning at 50× to qualitatively scan for clumps of particles. If heterogeneity is observed, one should perform a full count on the membrane. Next, count the 10-µm or larger particles in one GFOV at the edge of the filtration area as well as one GFOV in the center of the membrane. The number of 10-µm or larger particles in the GFOV with the highest total particle count must not be more than twice that of the GFOV with the lowest total particle count. If this criterion is not met, perform a full count of the membrane.

To perform a partial count of the particles on a membrane, include all particles 10–24 µm and ≥25 µm within the GFOV and those that are in contact with the right side of the GFOV circle. Do not count particles outside of the GFOV. Ignore those that touch the left side of the GFOV circle. The dividing line between right and left sides of the GFOV circle is the vertical crosshair and is a useful counting line.

[NOTE—Make the best possible judgment on particle size without changing the membrane position, microscope magnification, or illumination.]

Start at the center edge of the filtration area and begin counting adjacent GFOVs. When the other edge of the filtration area is reached, move one GFOV toward the top of the filter and continue counting GFOVs by moving in the opposite direction. This can be accomplished by one of two methods: define a landmark (particle or surface irregularity in the filter) and move over one GFOV in relation to the landmark, or use the Vernier scale on the microscope method to move 1 mm between GFOVs. To facilitate the latter, adjust the microscope *x*- and *y*-method positioning controls to a whole number at the starting position at the center right edge of the filtration area so each GFOV will be one whole division of movement of the *x*-method positioning control. If the top of the filtration area is reached before the desired number of GFOVs is reached, begin again at the right center edge of the filtration area one GFOV lower than the first time. This time, move downward on the membrane when the end of a row of GFOVs is reached. Continue as before until the number of GFOVs is complete.

For large-volume products, extrapolate the total count of particles per milliliter:

$$P_{\Sigma\geq10}\, A_T/A_P V$$
$$P_{\Sigma\geq25}\, A_T/A_P V$$

in which $P_{\Sigma \geq 10}$ is the total particle count obtained from all fields of view and all size thresholds; A_T is the filtration area of the membrane in millimeters^2 (inner filtration barrel diameter); A_P is the partial area counted in millimeters^2, based on the number of graticule fields counted (GFOV area × number of GFOV counted); V is the volume of solution filtered in milliliters; and $P_{\Sigma \geq 25}$ is the total particle count obtained from all fields of view and all size thresholds ≥25 µm.

For a solution pool (for small-volume product units containing less than 25 mL) or for a single unit of a small-volume product, extrapolate the total count of particles per unit:

$$P_{\Sigma \geq 10} A_T / A_P n$$
$$P_{\Sigma \geq 25} A_T / A_P n$$

in which $P_{\Sigma \geq 10}$ is the total particle count obtained from all fields of view and all size thresholds; $P_{\Sigma \geq 25}$ is the total particle count obtained from all fields of view and all size thresholds ≥25 µm; and n is the number of units counted (one, in the case of an individual unit). For all types of product, if the tested material has been diluted to decrease viscosity, the dilution factor must be accounted for in the calculation of the final test result.

6. AUTOMATED APPROACHES

6.1 Background

The original MM method was designed as an operator-intensive operation, from preparation to filtration to counting. The judgment of the operator regarding the presence and "countability" of isolates is paramount to effective detection, sizing, and counting of the retained particles. There may be alternative approaches. Static image analysis systems offer automated tracking and detection of particles based on contrast against the background. There is increased potential for objective accuracy and precision versus subjective human decision and tabulation. Primary considerations for the qualification of image systems are: recognition of thin or indistinct particles, "acceptance" of background features, discrimination of particles from background, and counting of artifacts. Use of these systems is supported with proper qualification using typical particle types as challenge sets.

To provide a more comprehensive guidance for human or machine vision, this chapter includes qualification recommendations for operators running the membrane assay for demonstrating the applicability of an automated method.

Qualification is a quality or accomplishment that makes someone suitable for a particular job or activity. Discussion points to address this simple goal are as follows:

- Microscope selection, preparation, and use are critical in detecting, sizing, and counting particles in the collected isolate. See *2. Test Apparatus* for background on the type of microscope and illumination used in daily counting.
- Familiarization of the operator is essential in particle recognition and size/count decision making. The following are elements of a proper training program:
  - It is imperative to fully scan the EFA for countable particles:
    - The membrane is tracked under the 10× objective with an *x-y* stage control
    - The lateral tracks must be parallel and not underpassed (gaps) or overpassed (recounted)
    - The edge of the EFA must be recognized and taken as the limit for tracking
  - Real particles ≥10 µm must be tabulated and discerned from the background
  - Blank samples familiarize the operator with the characteristics of the membrane texture, color, and reflectivity
  - Differentiation of 10–24 µm and ≥25 µm (additionally ≥50 µm for ⟨789⟩) particles is imperative

6.2 Qualification

A bimodal suspension of standard spheres is a reasonable means to verify operator recognition of ≥10 µm and ≥25 µm standards to satisfy the above criteria. Use two sphere standards just above the two size thresholds, such as 12–15 µm and 30–35 µm. Prepare a suspension of equal portions of the standard spheres in particle-free water, limiting the total content to ≤1000 in the volume tested. Training operators are charged with preparing and counting the preparations to verify acceptability.

The trainer counts the preparation, once acceptable, three times within a workday. This is the reference count. To promote stability, the membrane standard preparation may be sprayed with a fixative, such as a clear photoprotectant aerosol spray or a hand-pumped clear hairspray to fix the particles to the membrane surface. These applications may be used for months but should always be verified by trainers.

Trainees count the preparation three times in the same workday. The relative standard deviation (RSD)[4] for each threshold for one operator should be small, such as ≤5%. The RSD for each threshold between operator and trainer must be ≤10%.

In addition to manual microscopy, advances in technology have provided for automated testing of membrane-filtered samples. These techniques replace the human eye with a charge-coupled device (CCD) camera for image collection and the human decision-making process with advanced image processing software. While these techniques may seem to be simple, human-error free substitutes, careful consideration of the experimental variables, much like those for manual microscopy, is required for proper method qualification.

System particle size accuracy and count may be determined using traceable etched slides available from national standards organizations. These suffice to provide a calibrated measure of particle size, typically using chromed circular spots on a rigidly controlled sample. This approach also offers the user a self-contained system check with limited overhead once method qualification is complete (i.e., internal verification of system performance).

[4] The relative standard deviation (RSD), also known as the coefficient of variation (CV), is a standardized measure of dispersion of a probability distribution or frequency distribution. It is often expressed as a percentage and is defined as the ratio of the standard deviation to the mean (or its absolute value).

Beyond the initial system verification, it is necessary to directly compare the results of operator-conducted MM to automated methods. Although the mixed-sphere samples suggested for operator familiarization/training are excellent for comparing data collection methods, they represent optimal samples that may not highlight differences between manual and automated approaches. Instead, it is imperative that manual and automated methods be compared in a vein similar to that expressed for manual operator training.

- Using a minimum of three real samples, have the manual operator conduct the assessment of particulates ≥10, ≥25, and ≥50 µm
- Using the automated method for the same samples, have the system conduct three replicates of each sample for assessment of particulates ≥10 and ≥25 µm
- The RSD for the automated method replicates should be ≤3%
- The RSD between the manual and automated methods should be ≤10%
- The manual operator should review automated results to ensure that the automated system accurately captures particle populations observed manually

In general, repeatability of automated methods should be reasonably high (≤1%), and that of a single human observer slightly lower (≤5%). However, differences in method variables between manual and automated methods can lead to large changes in the apparent count and/or size results when comparing techniques. This variance can be far outside that expected for a human operator versus an automated method, which typically points to a shortcoming of the automated method. Such a situation warrants an understanding of why the method results diverge and a question of whether automation of the method is reasonable.

These considerations are usually related to either the microscope illumination or the thresholding mechanism defined in the automated method. Illumination varies among automated systems and can include diascopic (transmitted), episcopic (axial reflected), or oblique lighting. Manual methods have evolved to utilize oblique lighting exclusively, while automated methods may utilize alternate illumination, depending on the substrate selection. This can lead to automated methods with considerably different contrast quality from corresponding manual methods, so care should be taken to closely match the lighting conditions when comparing size/count results. Single-value thresholding schemes, which are quite common for automated systems, may produce fragmented images or miss particles entirely, whereas a human operator can clearly resolve the particle projection and accurately size/count the particles. It is critical to ensure that the qualification of an automated method includes a review of detected particles.

A final topic of consideration involves the selection of membrane type. Not all product forms are amenable to using cellulosic membranes, so alternatives to the standard cellulose-based membrane substrate may be appropriate. Novel membranes are now available that feature superior homogeneity, compatibility with organic solvents, and spectroscopic compatibility. These membrane choices may be used to develop manual or automated methods that meet ⟨788⟩ requirements, with an appropriate qualification of the method.

REFERENCES

1. International Organization for Standardization. ISO 14644-1:2015. Cleanrooms and associated controlled environments —part 1: classification of air cleanliness by particle concentration. Geneva, Switzerland: International Organization for Standardization; 2015.
2. Draftz RG. Microscopical counting, sizing and statistical strategies for LVP contaminants. *Conference Proceedings*, pp. 458–466. International Conference on Liquid Borne Particle Inspection and Metrology. 1987 May 11–13; Arlington VA.

▲ (USP 1-May-2021)