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Analysis and Specification of Starch Granule Size Distributions

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Vineeta Bajaj, Ph.D.
Review Editor
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Dear Dr. Vineeta Bajaj,

I, along with coauthors, would like to submit the revised manuscript entitled "**Analysis and Specification of Starch Granule Size Distributions**" for further review for publication in JoVE. We have revised all relevant sections and steps accordingly based on your constructive advices and comments. We have also further revised some protocol steps to make them clearer.

This revised manuscript has not been published or presented elsewhere in part or in entirety, and is not under consideration by another journal. There are no conflicts of interest to declare, and no financial disclosure.

Thank you very much for further consideration. I look forward to hearing from you again.

Sincerely,

Ming Gao, Ph.D.

TITLE:

Analysis and Specification of Starch Granule Size Distributions

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KEYWORDS:

starch granules, granule size distributions, electrical sensing zone, lognormal, two-parameter multiplicative specification

SUMMARY:

Presented here is a procedure for reproducible and statistically valid determinations of starch granule size distributions, and for specifying the determined granule lognormal size distributions using a two-parameter multiplicative form. It is applicable to all granule sizing analyses of gram-scale starch samples for plant and food science research.

ABSTRACT:

Starch from all plant sources are made up of granules in a range of sizes and shapes having different occurrence frequencies, i.e., exhibiting a size and a shape distribution. Starch granule size data determined using several types of particle sizing techniques are often problematic due to poor reproducibility or lack of statistical significance resulting from some insurmountable systematic errors, including sensitivity to granule shapes and limits of granule-sample sizes. We outlined a procedure for reproducible and statistically valid determinations of starch granule size distributions using the electrical sensing zone technique, and for specifying the determined granule lognormal size distributions using an adopted two-parameter multiplicative form with improved accuracy and comparability. It is applicable to all granule sizing analyses of gram-scale starch samples, and, therefore, could facilitate studies on how starch granule sizes are molded by the starch biosynthesis apparatus and mechanisms; and how they impact properties and functionality of starches for food and industrial uses. Representative results are presented from replicate analyses of granule size distributions of sweetpotato starch samples using the outlined procedure. We further discussed several key technical aspects of the procedure, especially, the multiplicative specification of granule lognormal size distributions and some technical means for overcoming frequent aperture blockage by granule aggregates.

INTRODUCTION:

Starch granules are the physical structure in which two main reserve homoglucan polymers in plant photosynthesis and storage tissues, the linear or sparsely branched amylose and the highly branched amylopectin, are orderly packed along with some minor components, including lipids and proteins. Starch granules from various plant species exhibit many three-dimensional (3D) shapes (reviewed in ref.[1,2]), including spheres, ellipsoids, polyhedrons, platelets, cubes, cuboids, and irregular tubules. Even those from the same tissue or different tissues of the same plant species could have a set of shapes with varying occurrence frequencies. In other words, starch granules from a plant species may have a characteristic statistical shape distribution, rather than a specific shape. The non-uniform and non-spherical granule shapes make it difficult to properly measure and define starch granule sizes. Additionally, starch granules from the same tissues of a plant species are of a range of sizes with different proportions, i.e., exhibiting a characteristic size distribution. This size distribution further complicates the analysis and description of starch granule sizes.

Starch granule sizes have been analyzed using several categories of particle sizing techniques (reviewed in ref.[3]), including microscopy, sedimentation/steric field-flow fractionation (Sd/StFFF), laser diffraction and electrical sensing zone (ESZ). However, these techniques are not equally suited for the determination of starch granule sizes in the presence of a granule shape and a size distribution. Microscopy, including light, confocal and scanning electron microscopy, is excellent for the studies of morphology[4-7], structure[8,9] and development[10,11] of starch granules, but hardly suited for defining their size distributions due to some inherent shortcomings. Direct measurements of microscopic granule images or software-assisted image analysis of optical microscopy data (IAOM), which have been used for the determination of granule sizes of starches from several species, including maize[12], wheat[13,14], potato[15] and barley[16], can measure only 1D (usually maximal length) or 2D (surface area) sizes of very limited numbers (tens to a few thousands) of starch granule images. The small granule sampling sizes that are inherently constrained by the techniques could rarely be statistically representative, considering the enormous granule numbers per unit weight of starch (~120 x 10^6 per gram, assuming all 10 µm spheres at 1.5 g/cm³ density), and, therefore, could lead to the poor reproducibility of the results. The Sd/StFFF technique may have high speed and resolution, and narrow size fractions of starch granules[17], but has been rarely used probably because its accuracy could be severely affected by damages, different shapes, and density of starch granules. The laser diffraction technique is the most widely used, and has been applied for starch granule size analyses for all major crop species[3,14,16]. Although the technique has many advantages, it is actually not suited for determinations of starch granule sizes in the presence of a granule shape distribution. Most of the concurrent laser diffraction instruments rely on the Mie light-scattering theory[18] for uniform spherical particles and the modified Mie theory[18] for some other shapes of uniformity. The technique is, therefore, inherently very sensitive to particle shapes, and not entirely suited even for certain shapes of uniformity [19], let alone for starch granules having a set of shapes of varying proportions. The ESZ technique measures the electric field disturbance proportional to the volume of the particle passing through an aperture. It provides granule volume sizes, as well as the number and volume distribution information, etc., at high resolutions. Since the ESZ technique is independent of any optical properties of particles including color, shape,

composition or refractive index, and results are very reproducible, it is particularly suited for determining size distributions of starch granules having a set of shapes.

Starch granule sizes have also been defined by using many parameters. They were often simplistically described by average diameters, which in some cases were the arithmetic means of the microscopically measured maximal lengths of 2D images[12,20], or averages of equivalent sphere diameters[3]. In other cases, the granule size distributions were specified by using size ranges[21,22], the distribution mean volume or mean diameter (sphere equivalent, weighted by number, volume, or surface area) assuming a normal distribution[14, 23-26]. These descriptors of starch granule sizes from various analyses are of a vastly different nature, and not strictly comparable. It could be very misleading if these "sizes" of starch granules from different species or even the same tissues of the same species were directly compared. Furthermore, the spread (or shape) parameter of the assumed normal distributions, i.e., the standard deviation σ (or graphic standard deviation σ_g) measuring the width of the distribution (i.e., the spread of the sizes), has been ignored in most studies.

To resolve the aforementioned critical issues facing starch granule sizing analyses, we outlined a procedure for reproducible and statistically valid determinations of granule size distributions of starch samples using the ESZ technique, and for properly specifying the determined granule lognormal size distributions using an adopted two-parameter multiplicative form[27] with improved accuracy and comparability. For validation and demonstration, we performed replicate granule sizing analyses of sweetpotato starch samples using the procedure, and specified the lognormal differential volume-percentage volume-equivalent-sphere diameter distributions using their graphic geometric means $\bar{x}^*$ and multiplicative standard deviations s^* in a $\bar{x}^*$ ˣ/ (multiply and divide) s^* form.

PROTOCOL:

1. Preparation of starch samples

1.1. Prepare two (or three) gram-scale replicate starch samples from starch-accumulating tissues of various plant species following the established procedures (e.g., potatoes[15], sweetpotatoes[28], wheat grains[13,29], and maize kernels[30], etc.).

1.2. Thoroughly wash starch samples with acetone or toluene 3-4x to minimize granule aggregates and dry them completely.

NOTE: Use extraction procedures that yield more than 1 g of starch per preparation. One or two 0.5-g aliquots from each of the three or two replicate extracts, respectively, are sampled for granule sizing analysis of one starch extract.

2. Electrolyte preparation

2.1. Prepare 500 mL of 50 g/L lithium chloride in methanol for four sizing runs for replication starch samples (100 mL per run plus an extra 100 mL). Preferably, make the electrolyte in large-volume batches, e.g., 4 to 8 L at a time, to minimize the concentration variation.

2.2. Cool the container on ice or in a 4 ˚C cabinet to speed up dissolution of the lithium chloride.

3. Setting up the analyzer

3.1. Choose an aperture tube with a particle diameter range covering the known (in the literature or through trial runs) granule size range of starch samples to be analyzed, e.g., a 100 µm aperture for sweetpotato starches. For starch samples of unknown granule size range, select an appropriate aperture through trial runs using several aperture tubes having overlapping particle diameter ranges.

NOTE: The particle diameter range of an aperture tube is its accurate sizing range between 2 to 60% of, and with an extended sizing range to 80% of its orifice diameter. **Table 1** lists properties of three most useful aperture tubes for sizing granules of major crop starches. If the granule size range of a starch sample is wider than the sizing range of a single aperture tube, perform a multi-tube overlap analysis combining up to five particle size distributions measured with apertures of different sizes. Each aperture is identifiable by its diameter and part number labeled on the tube. Its diameter and serial number contained in a barcode on the tube can be scanned into the analyzer software using the Bar Code Reader on the Control Panel of the analyzer.

3.2. Chose a 100 or 200 mL analytical beaker (over cuvettes) for the determination of starch granule sizes, and set up automatic stirring (below) to maintain a good granule suspension during measurement.

3.3. Create a Standard Operating Method (SOM) to specify run settings, and a Preferences file for analyzing, viewing, and printing the results. Combine SOM and Preferences file into a Standard Operating Procedure (SOP) as needed.

NOTE: For non-standardizable analyses, use SOM to run the analyses, and adjust the SOM settings between runs through the **Edit the SOM** window (see below) as needed. After run completion, analyze, view, and print the run results by changing the Preferences as desired. For standardizable granule sizing analyses, use an SOP to run the analyses.

3.3.1. Start the analyzer software. On the Main Manu, click **SOP | Create SOM Wizard** or **Edit the SOM**, or on the Status Panel, click **Edit SOM**. Use the wizard or the **Edit the SOM** window to select settings for an SOM. Settings typically used for sizing granules of sweetpotato starch samples are summarized in **Table 2**.

3.3.2. Save the created SOM to a File in the SOM Wizard-Summary of Settings window, or in the Edit the SOM window.

3.3.3. On the Main Manu, Click **SOP | Create Preferences Wizard** or **Edit Preferences**. Use the wizard or tabs in the Preferences edit window to select preference settings as those in **Table 3** or others as desired.

3.3.4. Save the selected Preferences to a file in the Create Preferences Wizard-Summary of Settings window or in the **Edit Preferences** window.

3.3.5. On the Main Menu, click **SOP | Create SOP Wizard**. Following the step-by-step guide of the wizard, enter a description, select the SOM and Preferences file to create and save an SOP**.**

4. Granule sizing analyses of the starch samples

4.1. Prepare the Analyzer

4.1.1. Turn on the analyzer, open the software in the computer and verify the Ready status at the top of the Status Panel after its automatic connection to the analyzer.

4.1.2. Fill the electrolyte jar with electrolyte, empty the waste jar if necessary.

4.1.3. Properly install and secure the chosen aperture tube following the guide in the user's manual. For an uncalibrated new aperture tube, calibrate it following the step-by-step guide under **Calibration | Calibrate Aperture** on the Main Menu. For a calibrated aperture tube, verify the calibration following the step-by-step guide of the **Change Aperture Tube Wizard** under the **Run or Calibration | Verify Aperture Calibration** on the Main Menu.

4.1.4. Unlock the assay platform by pushing the lock-release clip (on the middle front of the left sample compartment wall) and manually lower the platform to the bottom. Place an analytical beaker containing 100 mL of electrolyte on the platform, move the stirrer to the stirring position, and manually raise the platform to the self-locking upper position to immerse the aperture tube and stirrer in the electrolyte.

4.1.5. Click **Fill** on the bottom instrument Toolbar to have the analyzer automatically fill the system with the electrolyte and click **Flush** to have the analyzer automatically flush the system.

4.1.6. Load the SOM by clicking **SOP | Load an SOM** on the Main Menu**,** and use the SOM to run an analysis without a Preferences file. Alternatively, load an SOP by clicking **SOP | Load an SOP** on the Main Menu or **Load SOP** on the Status Panel, and use the SOP to run an analysis.

4.1.7. If using an SOP, click **SOP | SOM Info** or **Preference Info** on the Main Menu to verify the SOM and Preference settings. Click **Sample | Enter Sample Info** on the Main Menu or **Edit Info** on the Status Panel to enter the sample information for the run.

4.2. Prepare starch-methanol sample and sizing suspensions

4.2.1. Weigh two or one 0.5 g sample from each of the two or three replicate starch extracts, respectively.

4.2.2. Add each of the 0.5 g starch aliquots to 5 mL methanol in a 50 mL conical centrifuge tube, and fully disperse starch granules using several pulses of low intensity ultrasound (12–24 W/cm^2) from an ultrasonic processor.

4.2.3. Using a disposable transfer pipette, apply one small drop of the starch-methanol suspension (~0.2 mL) to the 100 mL of 50 g/L LiCl methanol electrolyte under constant stirring in the beaker. Close the sample compartment door.

4.3. Perform a sizing run

4.3.1. Click **Preview** in the bottom Instrument Toolbar to start a preview run. On the Status Panel, verify that the dynamically displayed concentration bar is in green, and shows a 5 to 8% nominal concentration range for the suspension.

4.3.2. Click **Stop** on the bottom Toolbar to stop the Preview run. If necessary, dilute the starch-electrolyte suspension by replacing an aliquot of the suspension with the electrolyte, and then repeat a Preview run.

NOTE: The 5% to 8% nominal concentration range of the suspension is critical for completion of a run without stoppage due to aperture blockage by aggregated granules. If needed, adjust the drop-sample size, and/or the concentration of the starch-methanol suspension to make a new starch-electrolyte suspension having the nominal concentration in the optimal range.

4.3.3. After the verification, click **Start** on the bottom Toolbar to start the run. The analyzer automatically completes the run once the total count of sized granules, which is displayed along with the run time on the **Status Panel** in a run, reaches the set Total Count (125,000 or 250,000) by the **Control Mode** of the SOM. Depending on the suspension concentration (within 5-8% range or lower), a single run takes 2 to 5 min or more.

NOTE: When the analyzer automatically detects an aperture blockage per blockage detection settings of the SOM, it will abort the run, flush to unblock the aperture and start a new run. This blockage action is set to maximally repeat for four times before the analyzer cancels the run operation. This run-aborting blockage problem may be overcome by using two technical methods as noted in **Table 2** and detailed in the discussion.

4.3.4. If needed, perform a technical repeat run (see **Table 2** and detailed in Discussion) using the same starch-electrolyte suspension by simply clicking **Start** or **Repeat** on the bottom Toolbar.

4.3.5. After completion of a run or repeat runs, empty the beaker, rinse it with methanol, and refill it with 100 mL fresh electrolyte solution for the next run.

4.3.6. During a run, if an **Extended Size Range** notification dialog appears when the count of granules larger than 60 µm exceeds 0.1% of the total count (per the SOM setting), click **Run 60% to 80%** for running an extended dynamic sizing range to 80% of the aperture diameter.

NOTE: The **Extended Size Range** setting controls actions for granules larger than 60% of the aperture diameter (100 µm, in this case). The setting in the SOM specifies inclusion of starch granules larger than 60 µm when their counts reach over 0.1% of the total count. The completion of the run is still controlled by the total count, and may take slightly less time than otherwise without inclusion of the larger granules totaling less than 0.1% (presumed statically insignificant amount) of the total count.

4.4. Analyze the run results

4.4.1. If an SOM was used to control the runs, select Preferences settings as desired for viewing, printing, and statistical analyses of the results using the **Create Preferences Wizard** or the **Edit Preferences** under the **SOP** on the Main Menu.

4.4.2. Overlay results from multiple runs on a single graph for comparison.

4.4.2.1. Click **Overlay** on the **Main Toolbar** or **File | Overlay** on the Main Menu to access the Overlay window. Navigate to and select multiple desired result files in the Files box, click **Add** to move them to the Selected Files box, and click **OK** to overlay the selected results on a single graph.

4.4.2.2. To add a file to an open overlay, click **RunFile | Open for Overlay** on the Run Menu to access the Overlay window, navigate to the desired file, and click to add.

4.4.3. Average results from replicate analyses (2 extracts x 2 starch-sampling or 3 extracts x 1 starch-sampling), and view or print the average granule size distribution and statistics in a list or graph.

4.4.3.1. On the Main Menu, click **File | FileTool | Average** to open the Average window. Navigate to and select multiple desired result files in the Files box, click **Add** to move them to the Selected Files box, and click **OK** to average the selected results and display the average on a single graph.

4.4.3.2. To include an additional result file in an average distribution, on the Run Menu, click **RunFile | Open and Add to Average** to open the Add to Average window, navigate to and add the file. The new average appears on the graph in the Run (result) window or listing.

5. Specifying the average distribution

5.1. In the Run-Menu window displaying the average distribution, click **Calculate | Averaged Statistics** on the Run Menu to open the statistics summary window, which displays the average statistics in rows, and the graph statistics for the average distribution in the columns.

5.2. Use the graphic geometric mean ($\bar{x}^*$) and S.D. (s^*) in the graph statistics column to specify the average distribution in the $\bar{x}^*$ x/ s^* form. Calculate the CV measuring variations among the averaged replicate distributions by dividing the mean (μ, the same as the $\bar{x}^*$ of the average distribution) of the geometric means of the averaged distributions with the average S.D. (σ) listed in the average statistics row.

NOTE: The average S.D. (for μ) assessing variations among the means of the replicate distributions is different from the graphic geometric S.D. (for $\bar{x}^*$) measuring the spread of the average distribution.

REPRESENTATIVE RESULTS:

To validate the procedure, and demonstrate reproducibility of the determined granule size distribution, we performed replicate sizing analyses of sweetpotato starch samples. We prepared replicate (S1 and S2) starch samples from field-grown sweetpotatoes of a breeding line SC1149-19 at a similar developmental age using a previously described procedure[28]. From each starch extract, two 0.5 g aliquots (a and b) were sampled, suspended in 5 mL of methanol and sonicated with several pulses of low-energy ultrasound to break up aggregates. Each of the two pairs of starch-methanol suspensions was drop-sampled to make a starch-electrolyte suspension, which was then sized twice (technical repeat runs) using the above outlined SOM for a total count of 125,000 granules each. For each single sizing run, once the total count reaches over ~65,000 and ~125,000, the graphic geometric S.D. (s^*) and geometric mean ($\bar{x}^*$) of the displayed differential volume-size distribution no longer significantly change, respectively. Each pair of the repeat runs using one starch-methanol suspension was merged after completion for a total sizing count of 250,000.

Figure 1 shows differential volume-percentage volume-equivalent-sphere-diameter distributions (S1a, S1b, S2a and S2b) for the four replicate sizing analyses of the sweetpotato starch samples, and their average distribution. The CV for the average of geometric means of the four replicate distributions was 3.75 %, demonstrating an excellent reproducibility of the sizing results. Each of the four replicate distributions was determined from a very large sampling size of 250,000 granules, far exceeding the minimal counts (~65,000 and ~125,000) above which the graphic geometric S.D. (s^*) and geometric mean ($\bar{x}^*$) of the displayed differential volume-size distribution in a single sizing run no longer significantly change. Therefore, the determined replicate volume-size distributions were all statistically valid. For better accuracy and comparability (discussed below) of the specification of determined lognormal granule size distributions, all these distributions were specified by using their graphic geometric means ($\bar{x}^*$) and S.D. (s^*) in a $\bar{x}^*$ x/ (multiply and divide) s^* form as listed on the graph. Please note that the granule size distribution of the sweetpotato starch has been rigorously fitted to be lognormal as previously described [28].

[Place **Figure 1** here]

Figure 2 shows the average (or mean) cumulative (<) number- and volume-percentage size distributions of the four replicate sizing analyses, which were transformation views of the

average differential volume-percentage size distribution. The comparison between the cumulative number and volume percentages of starch granules showed that granules having smaller volume-equivalent-sphere diameters accounted for much larger percentages of the total count than the total volume. For example, the numbers of granules having volume-equivalent-sphere diameters smaller or equal to 9.976 µm accounted for 48.53% of the total count, yet only 5.854% of the total volume.

[Place **Figure 2** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Lognormal differential volume-percentage volume-equivalent-sphere size distributions from replicate sizing analyses of sweetpotato starch samples. The sampling scheme for the four replicate sizing analyses were detailed in the result. The four distributions (S1a, S1b, S2a and S2b) from replicate analyses and their average were overlaid and specified using the $\bar{x}^{*\,x}$/ (multiply and divide) s^* form.

Figure 2: Average cumulative (<) number- and volume-percentage size distributions of starch granules from the four replicate sizing analyses of sweetpotato starch samples. The two distributions are transformation views of the average size distribution in Figure 1. The graph compares the cumulative (<) number (left Y-axis) to volume (right Y-axis) percentages of starch granules having volume-equivalent-sphere sizes lower or equal to particular size bins.

Table 1: Three most useful aperture tubes for sizing granules of starches from crop species.

Table 2: Typical SOM settings for controlling sizing runs for sweetpotato starch samples.

Table 3: Typical preference settings for view, analyses and print of results from sizing runs for sweetpotato starch samples.

DISCUSSION:

The outlined procedure has resolved some critical issues in several existing methods for starch granule size analyses, including inappropriate 1D or 2D sizing of 3D granules, distortion of sizing measurements due to none-uniform granule shapes, poor reproducibility and dubious statistical validity due to limited granule-sample sizes, inaccurate or improper specification (especially the use of the average size) of granule sizes in the presence of both granule shape and none-normal size distributions. It uses the ESZ technique that measures 3D sizes (volume) of starch granules and is unresponsive to granule shapes. The design to derive the average granule size distribution from replicate analyses having a very large granule-sample size (4 x 250,000) not only renders the result statistically valid and more reproducible, but also technically mitigates measurement distortions by aggregated and damaged granules to improve sizing accuracy (explained below). As demonstrated in the representative results, the CV for the average of geometric means of replicate distributions determined using the procedure is usually smaller than 5%, indicating a satisfactory reproducibility of the results. Furthermore, the multiplicative specification of both the scale ($\bar{x}^*$) and shape (s^*) of the lognormal granule volume-equivalent-sphere size distribution

more accurately depicts the true nature of distributed granule sizes in a starch sample, and is straightforward to use and universally comparable among granule sizing analyses of starches from same or different sources. Therefore, the procedure enables more accurate, reproducible, and statistically valid determination of starch granule sizes, and proper specification of determined granule lognormal size distributions. It is applicable to all granule sizing analyses of gram-scale starch samples, and could become an essential tool for studies on how starch granule dimensions are molded by the starch biosynthesis apparatus and mechanisms in plant starch-accumulating tissues, and how they impact properties and functionalities of starches for food and industrial uses.

Starch granules are stereo particles having mostly non-spherical shapes so that their sizes must be defined and measured in 3D terms. Thus, the volumes of starch granules best define their sizes, and the volume-equivalent-sphere diameter is the only single 1D size parameter that can be used to properly describe the granule 3D sizes since no stereo objects other than sphere can be defined with a single 1D size parameter. Furthermore, starch granules from all plant species possess a set of shapes with various occurrence frequencies. In the presence of such a shape distribution, any particle sizing techniques that are responsive to particle shapes, e.g., the laser diffraction technique, are not suited for reproducible and statistically valid determinations of starch granule size distributions, as the system error inherent to these techniques cannot be easily corrected with a shape factor. In fact, the error rate (CV) among replicate analyses of granule sizes from the same sweetpotato starch sample using the laser diffraction technique could reach as high as 15-20% [28], indicating very poorly reproducible sizing results. Unfortunately, the impact of granule shapes on sizing starch granules have been mostly overlooked, which resulted in a large body of dubious starch granule size data acquired using shape-responsive particle sizing techniques in the literature.

The two-parameter multiplicative specification defines both the scale ($\bar{x}^*$) and the shape (s^*) of lognormal distributions, and is thus far more precise and meaningful than a single descriptor of mean size or a size range[26]. The multiplicative $\bar{x}^{*\,\times}/\,s^*$, $\bar{x}^{*\,\times}/\,(s^*)^2$, and $\bar{x}^{*\,\times}/(s^*)^3$ intervals, corresponding to $\overline{x} \pm s$, $\overline{x} \pm 2s$ and $\overline{x} \pm 3s$ intervals of a normal distribution, covers approximately 68.3%, 95.5%, and 99.7% confidence intervals of a lognormal distribution, respectively[27]. The geometric mean ($\bar{x}^*$) and S.D. (s^*) of a lognormal granule size distribution correspond to the graphic geometric mean and S.D. of the size distribution curve, which are calculated by the analyzer software and can be selected to display on the on-screen size graph during a sizing run or analyses of results. It is, therefore, rather convenient, and simple to use the multiplicative specification. Additionally, the $\bar{x}^*$ and s^* have been demonstrated to have different physiological implications associated with the starch biosynthesis apparatus[28]. The granule volume-size distributions of starches from various plant species may well be all lognormal since the formation of starch granules in plant starch-accumulating tissues falls into an unconstrained evolving complex system[31] or an intracellular catalytic reaction network[32] characteristic of a lognormal distribution. The bimodal granule size distributions of starches from some plant species, such as those from wheat[13,14], could be regarded as two lognormal distributions. Therefore, the multiplicative specification of granule lognormal volume-equivalent-sphere size distributions may also allow a statistically valid universal comparison of granule sizes

determined from starches of various plant sources and by different measurements, as the $\bar{x}^*$ is in the form of volume-equivalent-sphere diameter and s^* is demensionless.

An appropriate total granule-sizing count for the analysis of a starch (in methanol) sample, which represents the granule sample size, is most critical to successful determination of the granule size distribution of statistical significance for the starch sample. In the case of sweetpotato starch samples, once the total count in a single run reaches over ~65,000 and ~125,000, the graphic geometric S.D. (s^*) and geometric mean ($\bar{x}^*$) of the displayed differential volume-size distribution curve no longer significantly change, respectively, indicating minimal counts for the s^* and $\bar{x}^*$ of statistical significance. The sampling redundancy in sizing 250,000 granules for a starch-methanol sample in the procedure is intended to discount for the aggregated and damaged granules in the sized granule pool. Even assuming that the aggregated and damaged or broken granules accounted for 50% of the total count of 250,000 granules in a completed run or two merged repeat runs, the graphic geometric S.D. and mean of the determined distribution would not have been significantly impacted as they would have been anchored by the intact granules of half of the total count. Furthermore, the more volume-size reduction of the damaged or broken granules, the less impact they have on the distribution. This is because smaller granules take a larger number percentage, but smaller volume percentages of the total sized granules. As demonstrated by the comparison between number and volume cumulative distributions for the same average distribution in **Figure 2**, starch granules with an equivalent-sphere diameter smaller than or equal to 9.967 µm accounted for about 48.53% of the total number, but only 5.854% of the total volume. Thus, any damaged or broken-down granules less than 10 µm would have a very small impact on the differential volume-percentage size distribution. For starch samples of other plant sources, an appropriate total count for their sizing analyses can be the one doubling the minimal count over which the graphic geometric mean ($\bar{x}^*$) of the displayed size distribution in a trial run no longer significantly change.

Technically, the most critical step for a sizing run is to drop a proper amount of the starch-methanol suspension to the electrolyte for an optimal range of 5 to 8% nominal concentration for the starch-electrolyte suspension. To reach the goal, the drop size and the concentration of the starch-methanol suspension may have to be adjusted through trial runs. Concentrations of the starch-electrolyte suspensions higher than the optimal range increase risks of reduced sizing precision, and frequent aperture blockages leading to run abortions, which could make it very difficult to complete a run. But, too low a concentration (e.g. <2%) of the starch-electrolyte suspension may prolong a run too much, and distort frequencies of granules in various size bins due to non-random sampling of granules, which could lead to an unacceptable error rate (the average CV > 5%) for a replicate analysis. The total count for a sizing run also has a major impact on the optimal concentration of a starch-electrolyte suspension, hence on the amount and concentration of the starch-methanol added. The larger the total count for a run, the longer the time for the completion of the run, and thus the more risks for aperture blockages leading to run abortions. The problem of aperture blockage by aggregates worsens when aperture tubes of smaller diameters are used for starch granules of smaller sizes, which makes it very difficult to analyze small starch granules (< 2 µm). This is indeed the major drawback or limitation of the procedure. The aperture blockage problem could be alleviated to a certain extent using some

technical means. One may use more sonication to break up aggregates (inevitably more damaged granules as well) in a starch-methanol suspension, and/or a diluted starch-electrolyte suspension at 2-5% nominal concentrations. Alternatively, one may use technical repeat runs of sizing the minimal total count for stable s^* and $\bar{x}^*$ of the size distributions for a starch type (e.g. about 125,0000 counts for sweetpotato starch) from the same starch-electrolyte suspension, and merge the results of the repeat runs. Each of the four replicate distributions (S1a, S1b, S2a and S2b) shown in **Figure 1** were from two merged technical repeat runs of sizing 125,000 granules each from the same starch-electrolyte suspension. Both methods need to be well tested, as they may increase the replication error rate to an unacceptable level (i.e., the average CV > 5%).

Technical and biological replicate sizing analyses of starch samples from plant sources under similar physiological conditions improve the reproducibility and accuracy of the determined average granule size distribution. Practically, three or four biological replicates of starch samples may be independently extracted from the same tissue under a specific condition. But, we previously found that there was no significant difference in error rates (CV and Standard Errors for the average), and $\bar{x}^*$ and s^* between the average granule size distribution derived from distributions of four biological replicates (i.e., one sizing x one suspension x 4 extracts) and the one from those of two technical sampling each from two biological replicates (i.e., one sizing x 2 starch-methanol suspensions x 2 extracts) [28]. Thus, biological replicate samples could be reduced to two, at least for the sweetpotato starch. Other steps and technical parameters that could be modified or adjusted were specifically noted below each of the steps or the particular parameter in the procedure.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose

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Figure 1

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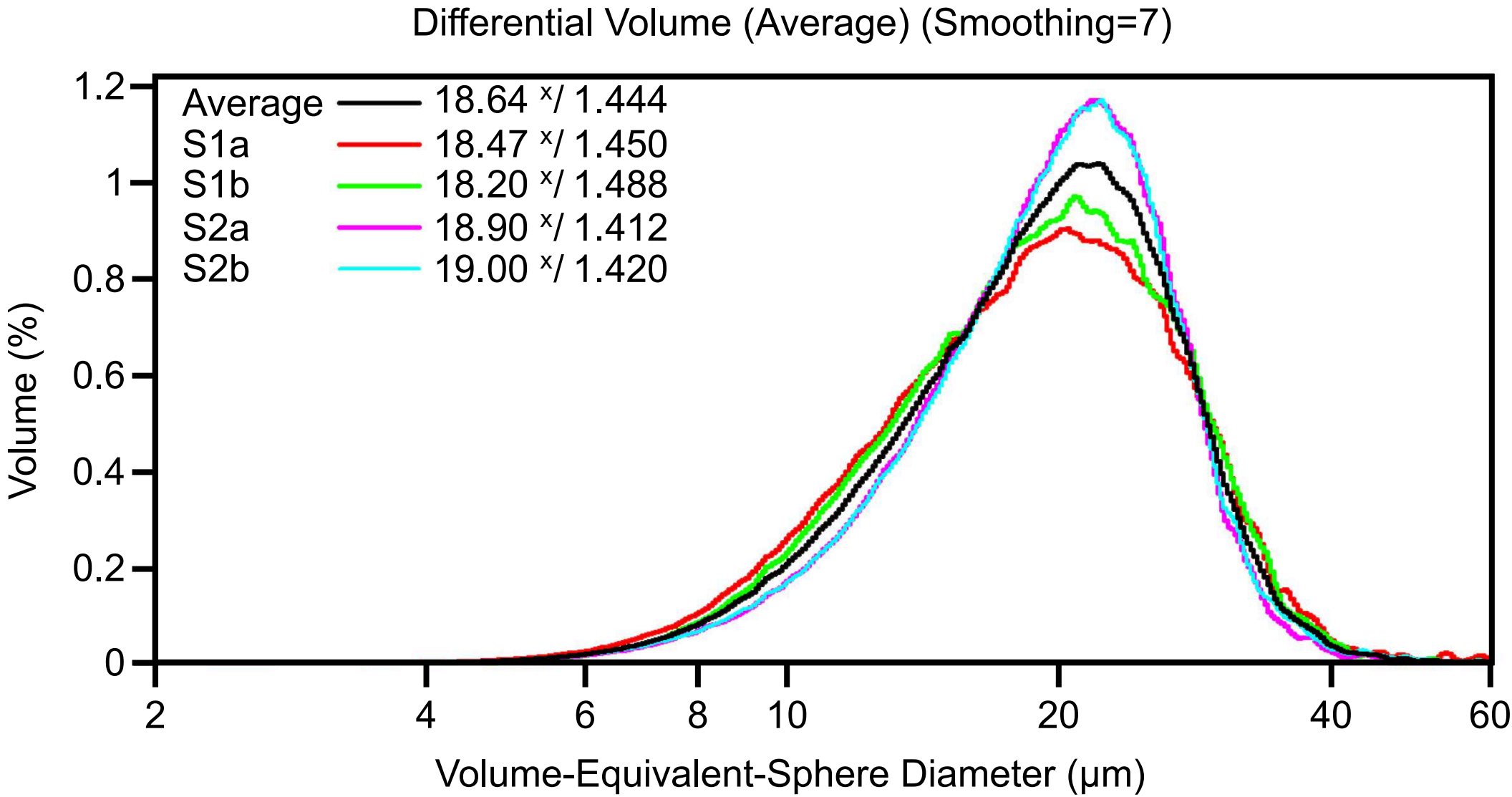


Figure 2

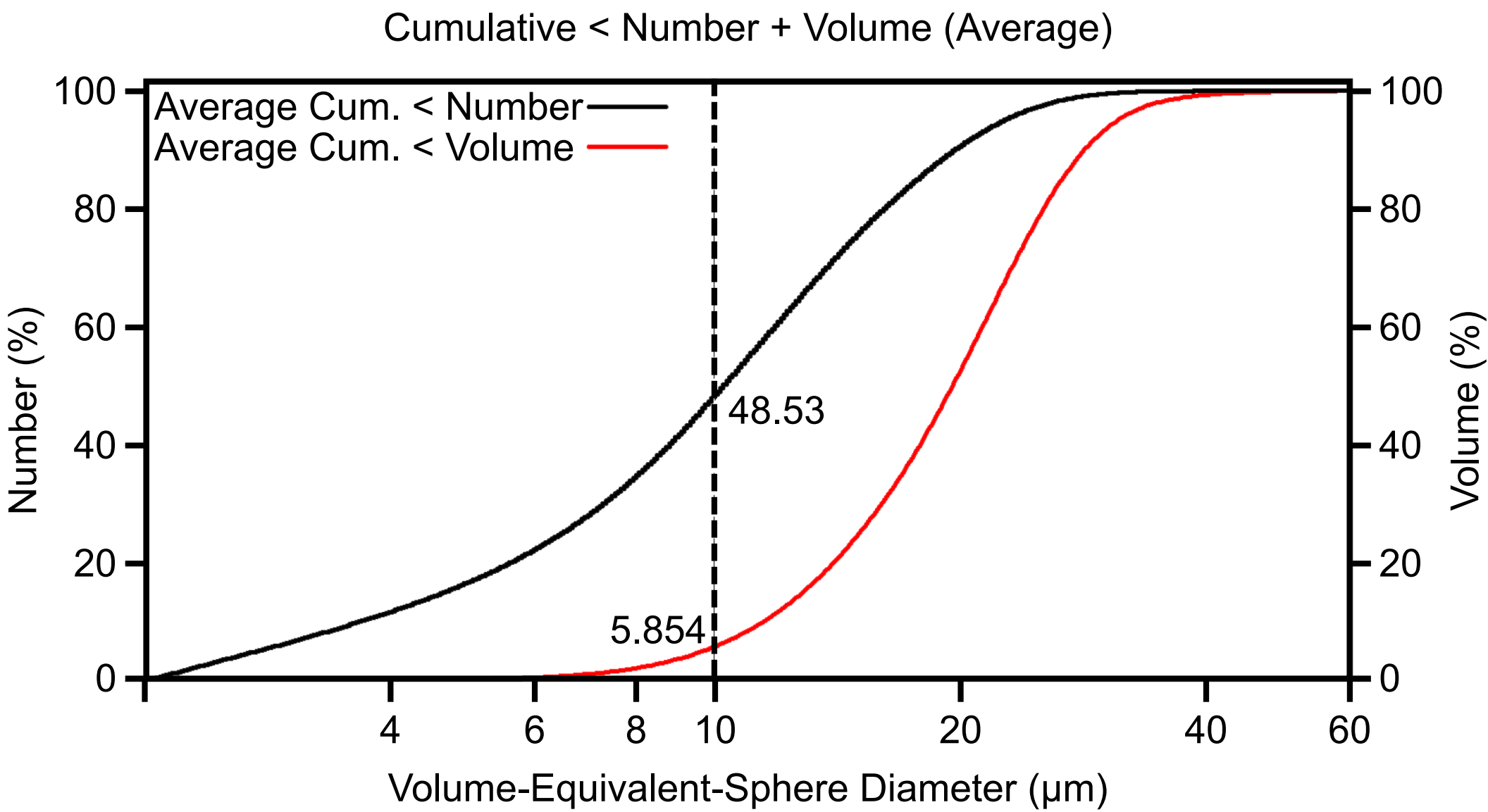


Table 1

Aperture diameter (mm)	Particle Diameter Range (µm)	Particle Volume Range (µm3)
50	1.0 - 40	$0.524 - 33.5 \times 10^3$
70	1.4 - 56	$1.44 - 92.0 \times 10^3$
100	2.0 - 80	$4.19 - 268 \times 10^3$

Table 2 Click here to access/download;Table;Table 2.xlsx

	SOM Settings	Selection
Description	SOM description	Sizing Starch Granules
	SOM author	-
	Sample description	Sweetpotato starch samples
	Electrolyte	50 g L^{-1} Lithium Chloride
	Dispersant	No
	Aperture	100 µm
Control Mode	Control Mode	Total Count [250,000] or [125,000][a]
	Waste Tank	When 80% full
Run Settings	Enter sample info	Yes
	Number of runs	1 (or 2, for repeat runs)
	Flush aperture tube before run	Yes
	Flush aperture tube after run	Yes
	Save file	Yes, including pulse data
	Export data	Yes
	Print report	Yes
	Compare to sample specifications	No
	View	Size
Stirrer Settings	Sample beaker	100 ml Multisizer 4 ST
	Use stirrer	Yes
	Speed	[15], CW (clock wise)
	Stirrer position	Automatic
Threshold, Current and Gain	Sizing threshold	2 µm
	Aperture current	1600 µA
	Preamp gain	2
	Extended size range [b]	When count [> 0.1%] of total count
Pulse to Size Settings	Size bins	400
	Size range	2 to 60 µm
	Bin spacing	Log diameter
	Coincidence correction	Yes
	Pulse Edit	No
Concentration	Sample amount	0.2 ml
	Density	-
	Use pre-dilution factor	-
	Analytic volume	-
	Electrolyte volume	100 ml
	Use dilution factor	No
Blockage	Blockage detection	Automatic (From start of run) Default blockage detection: when count rate <20%, Aperture rate >40%, or concentration spike >40%.
	Blockage action	Cancel, unblock and restart,Up to [4] times
	Show icon	Yes
	Blockage monitor	Count rate

[a]: If repeated unblock and restart could not get the larger-count run completed, make two repeat runs of sizing a lower total count of 125,000 each from the same starch-electrolye suspension, and merge the results of the repeat runs by using [**MergeRuns**] under [**FileTools**] of [**File**] in the **Main Menu**. Alternatively, replace the starch-electrolye suspension with a new one having a lower nominal concentration (2-5%). When preparing a new drop-sample starch-electrolyte suspension, pulse-sonicate the starch-methanol suspension again to break up more aggregates.

[b]: The **Extended Size Range** controls actions for granules larger than 60% of the aperture diameter (100 µm in this SOM). The setting specifies inclusion of starch granules larger than 60 µm when their counts are larger than 0.1% of the total count.

Table 3 Click here to access/download;Table;Table 3.xlsx

	Preference Settings	Selection
Printed Reports	Sample info	Sample, Run Number, Size Bins, Total Counts
	Size graphs	Differential Volume %, Log X Axis, Smooth by Groups of Seven
	Size statistics	Volume, Volume %
	Average statistics	Total Amount, Mean, S.D.
	Overlay statistics	Total Amount, Mean, S.D.
	Listing	Columns: Bin Number, Bin Diameter (center), Diff. Number, Diff. Number %, Diff. Volume %. Bin Grouping: Bin Group Size 7, All Bins, Sum Bins in Group.
Statistics	Type	Geometric[a]
	Range	All
	Results to print	Range, Total Amount, Mean, S.D., 95% Confidence Limits
	Results on graph	Range: All, Total Amount, Mean, S.D.
Averaging and Trend	Average weighting[b]	Volume %
	Distribution[c]	Differential
	Limits	2 S.D.
	Pulse averaging	Use Convert Pulses to Size Range
Export	Data items	Sample Information, Statistics, Average Statistics, Size Listing
	Export extension	.xls
	Number format	123456.78
	Data format	Tab Delimited
	Export folder	Current Folder
	Page setup	Include Custom Title, Print Graphs using Screen Color Include Date
	Graph size:	Half Page
Graph Option	Display:	Screen and Color Printer
	Line color	(Default)
	Line style	(Default)
	Legend	Top Right
	Size	(Default)
	Graph style	Step
	Limit style	Curve
	Fonts and Colors	Default Fonts and Default Colors or as desired.
View Options	Default view	Size, Graph
	Size X axis	Diameter
	Measuring	Particles
	Liter Symbol	L (mL, mL, fL)
	Multisizer pulse data	Graph at most 5010 pulses, List at most 5010 pulses
	Volume units	μm^3
	Numbers	123456.78

[a]: The geometric mean and S.D. statistics specified here are graphic ones that define the scale and shape of the determined differential volume-percentage equivalent-sphere size distribution. They are used to specify the lognormal distribution in the $\bar{x}$* x/ s* form.

[b]: The average weighting refers to how results from multiple runs are averaged by different weighting options. Change these settings in the **Run** Menu for different averaging and view options.

[c]: Select [**Calculate**] to open [**Average Statistics**] in the [**Run Menu**] to see the average statistics in rows, the graph statistics for the average distribution in the "Mean" column.

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_MGao, etal, 2020.xls

Name of Material/ Equipment	Company	Catalog Number
Analytical beaker	Beckman Coulter Life Sciences	A35595
Aperture tube, 100 µm	Beckman Coulter Life Sciences	A36394
Disposable transfer pipettor,	Fisher Scientific (Fishersci.com)	13-711-9AM
Fisherbrand Conical Polypropylene Centrifuge Tubes, 50 ml	Fisher Scientific (Fishersci.com)	05-539-13
Glass beakers, 150 to 250 ml	Fisher Scientific (Fishersci.com)	02-540K
LiCl	Fisher Chemical	L121-100
Methanol	Fisher Chemical	A412-500
Mettler Toledo ML-T Precision Balance	Mettler Toledo	30243412
Multisizer 4e Coulter Counter	Beckman Coulter Life Sciences	B23005
Ultrasonic processor UP50H	Hielscher Ultrasound Technolo	UP50H

Comments/Description

Smart-Technology (ST) beaker

For the MS4E, , 1000 µm

Other disposable transfer pipettors with similar orifice can also be used.

Any other similar types of tubes can be used.

These beakers are used to contain methanol for washing the aperture tube and stirer between runs.

Buy in bulk as the analysis uses a large quantity of methanol.

Any other precision balance with a readablity 0.01 g to 1 mg will work.

The old model, Multisizer 3 can also be used with slight adjustment of parameters. The 4e model comes with a 100 µm aperture tube. Other aperture tubes of different diameter can be purchased separately from the company.

Other laborator sonicator having a low-power (<50 Watt) output can be also used. Both MS1 and MS2 sonotrodes for the particular sonicator can be used to disperse starch granules in 5 ml methanol. Always use the lowest setting first, 20% amplitude and 0.1 or 0.2 cycle, and raise the setting if aggregates persist in suspension.

Response to the Review Editor's comments

We appreciate very much all the constructive comments from the Review Editor. We have revised the sections accordingly based on the comments.

Comments

1. **Citation?**
   Response: Added citation, and update the citations and the reference list.

2. **This section needs more details to make this a standalone manuscript.**
   Response: As discussed with the Review Editor, the highlight of the procedure is taken out. Procedures for starch extractions vary among source plants or tissues. The one used for sweetpotato starch extraction may be of limited application, and is not the emphasis of the article. In addition, the extraction procedure takes two days, and thus not suited for filming. The section is also revised to make it clearer.

3. **If this needs to be filmed, please briefly explain how this is done. Else please remove the highlight and include citations for established procedures.**
   Response: The section is revised with citation for established procedures added, and the highlight removed.

4. **Please include how many starch samples were obtained with how much starting material. (this is for clarifying 2.1).**
   Response: The sample number for replication analysis is stated in the revision. The amount of starch needed for the analysis is stated in the note. As the amounts of starting materials for starch extraction vary widely among procedures, it is noted to use procedures yielding starch sufficient for analysis.

5. **How do you identify this visually?**
   Response: Identification of an aperture tube and import of its diameter and serial number (in the barcode) into the software are noted in the revision.

6. **Only button clicks need to be bolded. Please format accordingly.**
   Response: All the protocol steps were reformatted accordingly to have only the clickable buttons bolded.

7. **How is this (installing an aperture tube) done?**
   Response: The step of installing an aperture tube has been revised to indicate following the User's manual. This is a multi-step process, which is clearly describe and easy to follow in the manual. It is also not the emphasis of the protocol. Thus, it may be better to refer the step to the manual. But, if needed for filming consideration, the process can be revised to include a brief description.

8. **Is this done manually?**

Response: The platform is manually lowered and raised, which has been noted in the revision. The step has been further revised to make it clearer.

9. For how long?
Response: After click the Flush, flushing the system starts and automatically stops when it is complete. The step is revised to note the automatic flushing process.

10. **How?** (To verify SOM and Preferences settings)
Response: How to verify the SOM and Preferences settings in the software has been added.

11. How many grams in 5 mL?
Response: The sampled 0.5-gram starch aliquot is now noted at the step.

12. Significance?
Response: The significance of having a 5 to 8% nominal concentration range of the suspension is noted. The step is further revised to make it clearer.

13. How will you see this?
Response: The step has been revised to clearly state that the analyzer automatically detects an aperture blockage per blockage detection settings of the SOM. When the count rate <20%, Aperture rate >40%, or concentration spike >40% (Blockage detection settings), the analyzer regards the aperture blocked. Although the sudden drop of the count rate and spike of the nominal concentration (the concentration bar turning red) can be seen on the run Status Panel when the blockage happens, the analyzer determines if it is a surmountable transitory blockage or one that has to be unblocked. Also, unblocking of the aperture abort the run. Therefore, it is not meaningful to note the observable sudden drop of the count rate and spike of the nominal concentration.

14. How much time is required?
Response: A single run takes 2 to 5 minutes or more, depending on the total count setting and the suspension concentration, which is noted in the revision. The completion of a run is controlled by the total count setting. The run time vary among various runs using different suspensions. Thus, it may not be that meaningful to note the run time. The step is further revised to make it clearer.

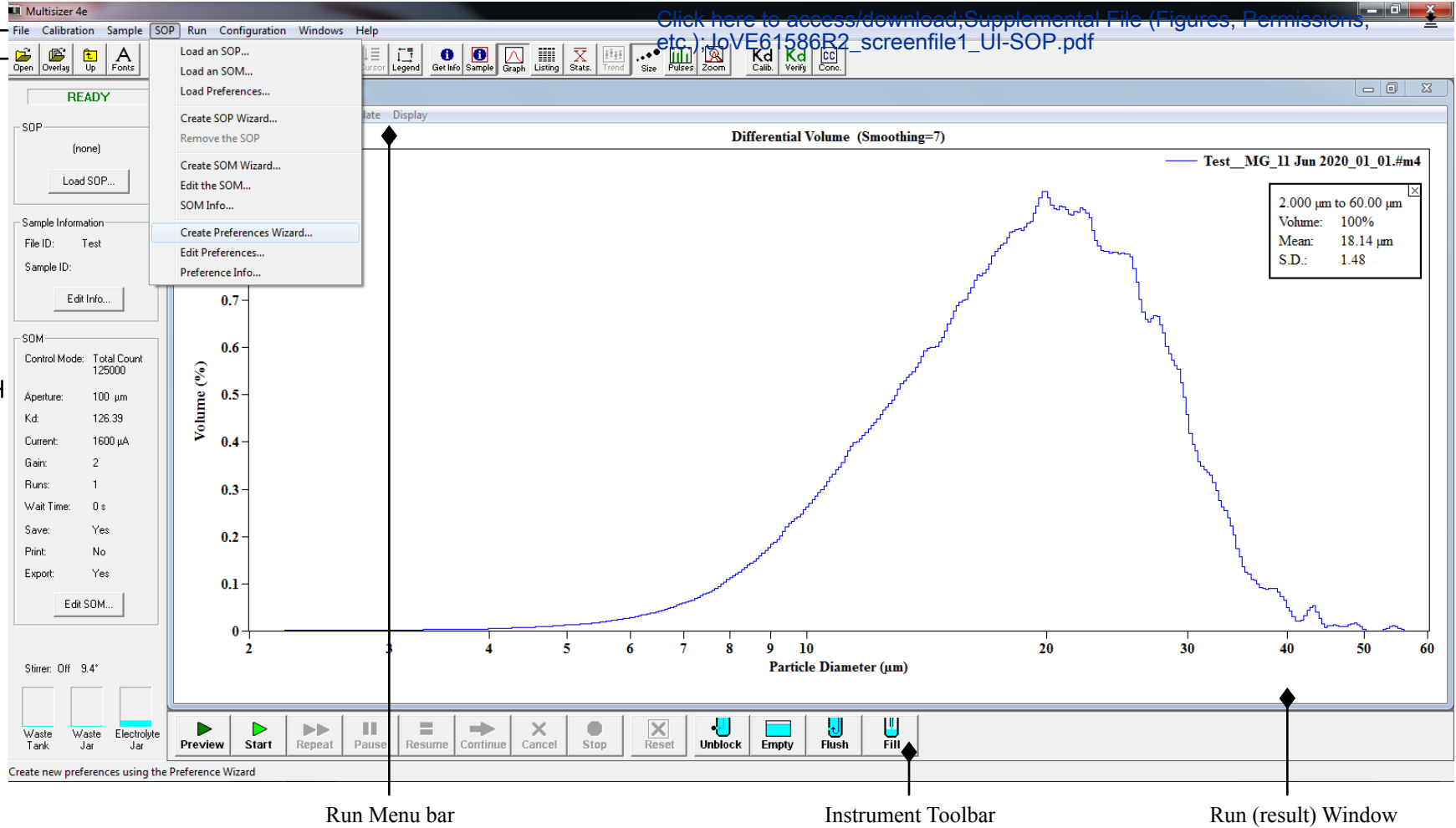
15. How long is this run for?
Response: The technical phrase "for running an extended dynamic sizing range to 80% of the aperture diameter" means inclusion of those granules larger than 6o μm when their counts reach over 0.1% of the total count, but not an extra run. It is controlled automatically, and may not take any extra run time. The step has been revised accordingly.

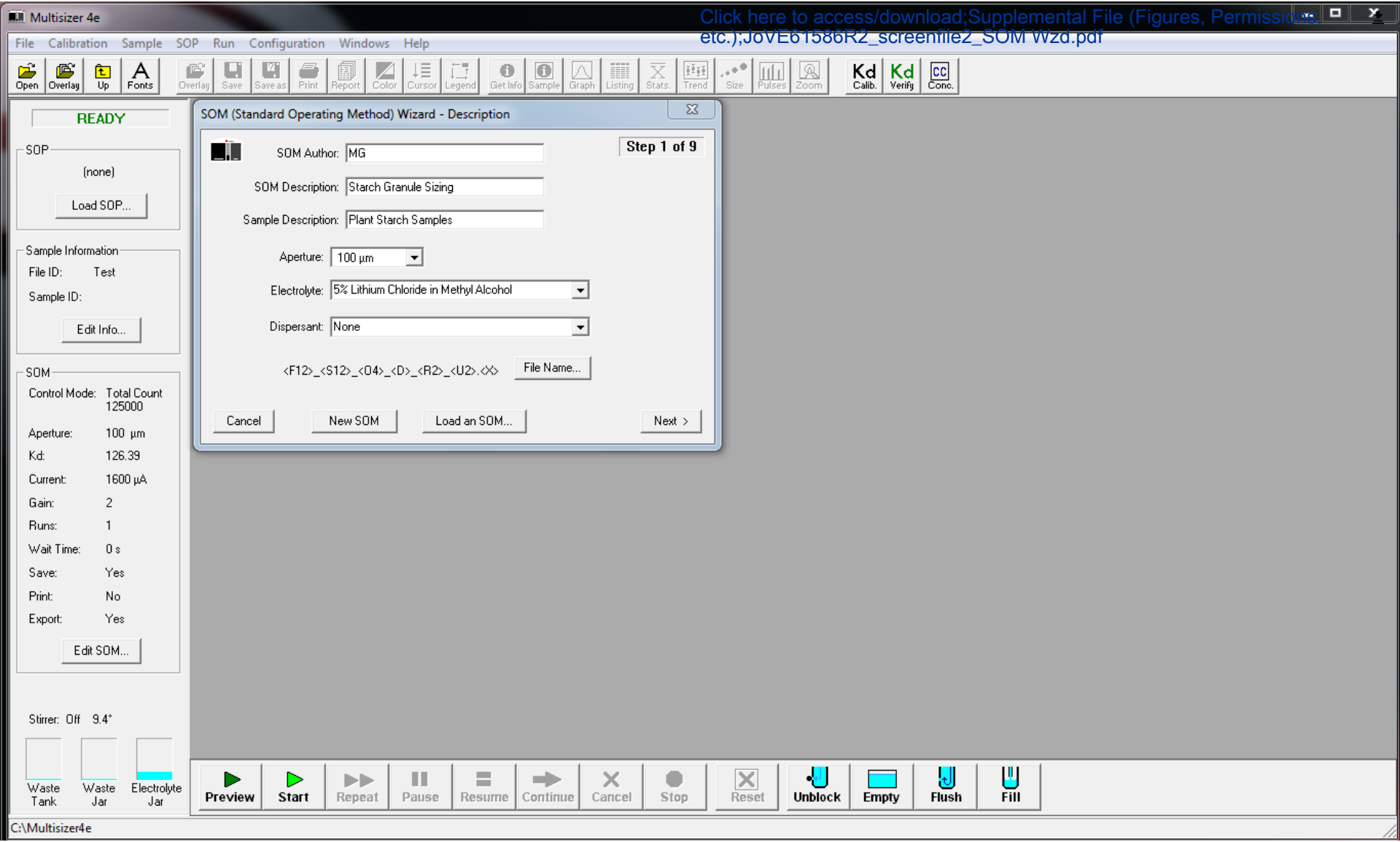
16. Any of these can be used?

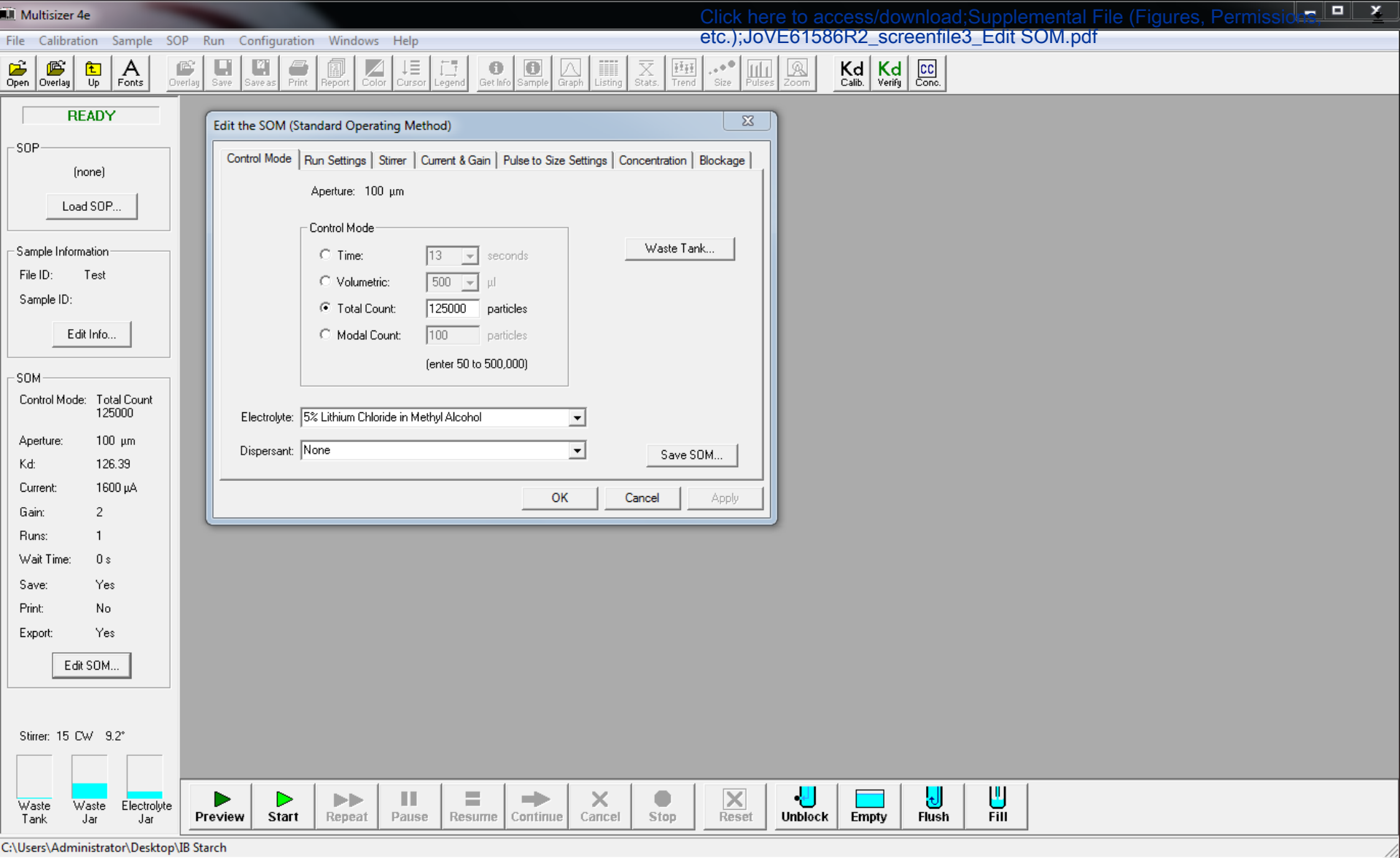
Response: Revised to make it clear that any of these options can be used.

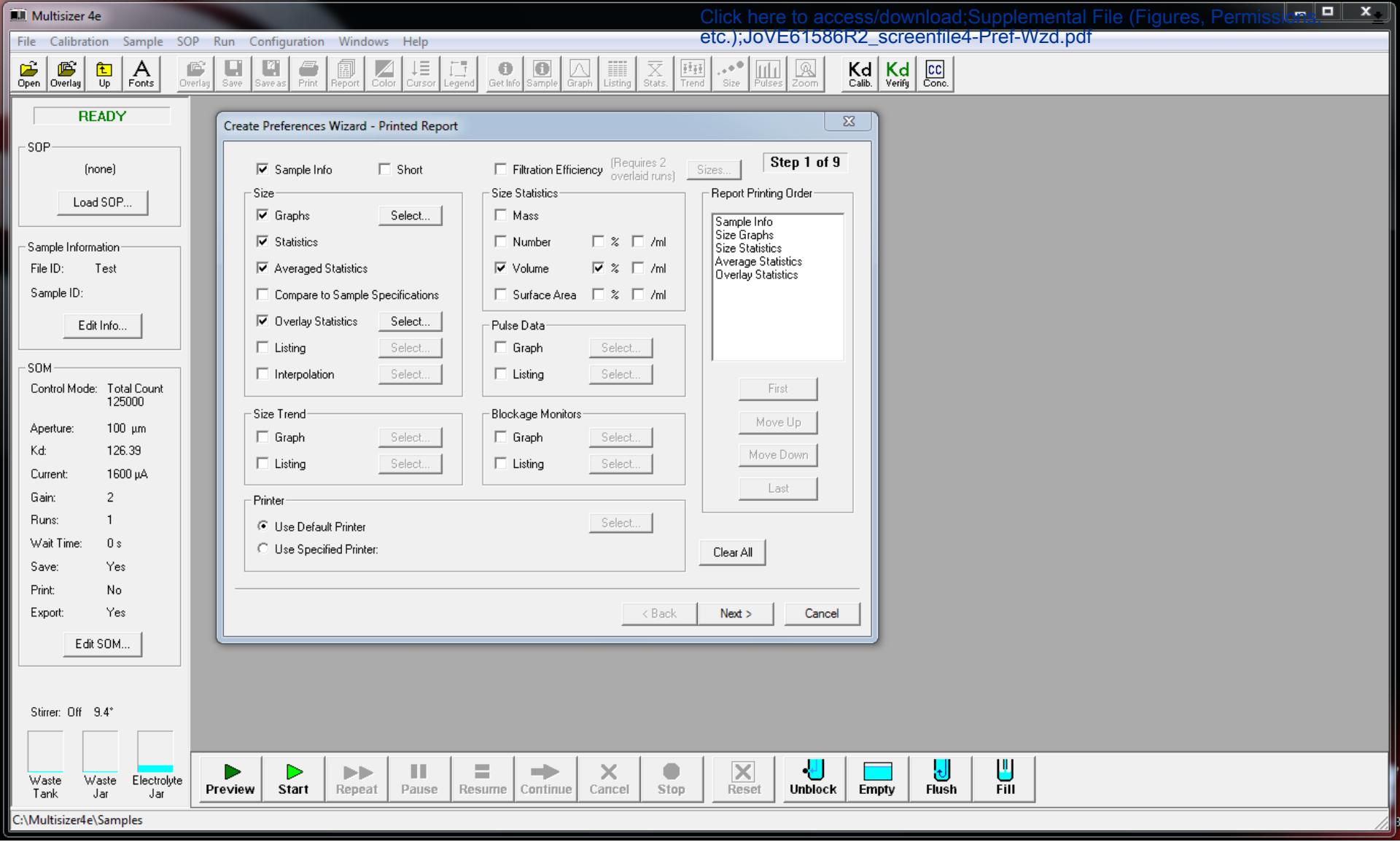
17. Calculations steps cannot be filmed. If this needs filming please include how this is performed.

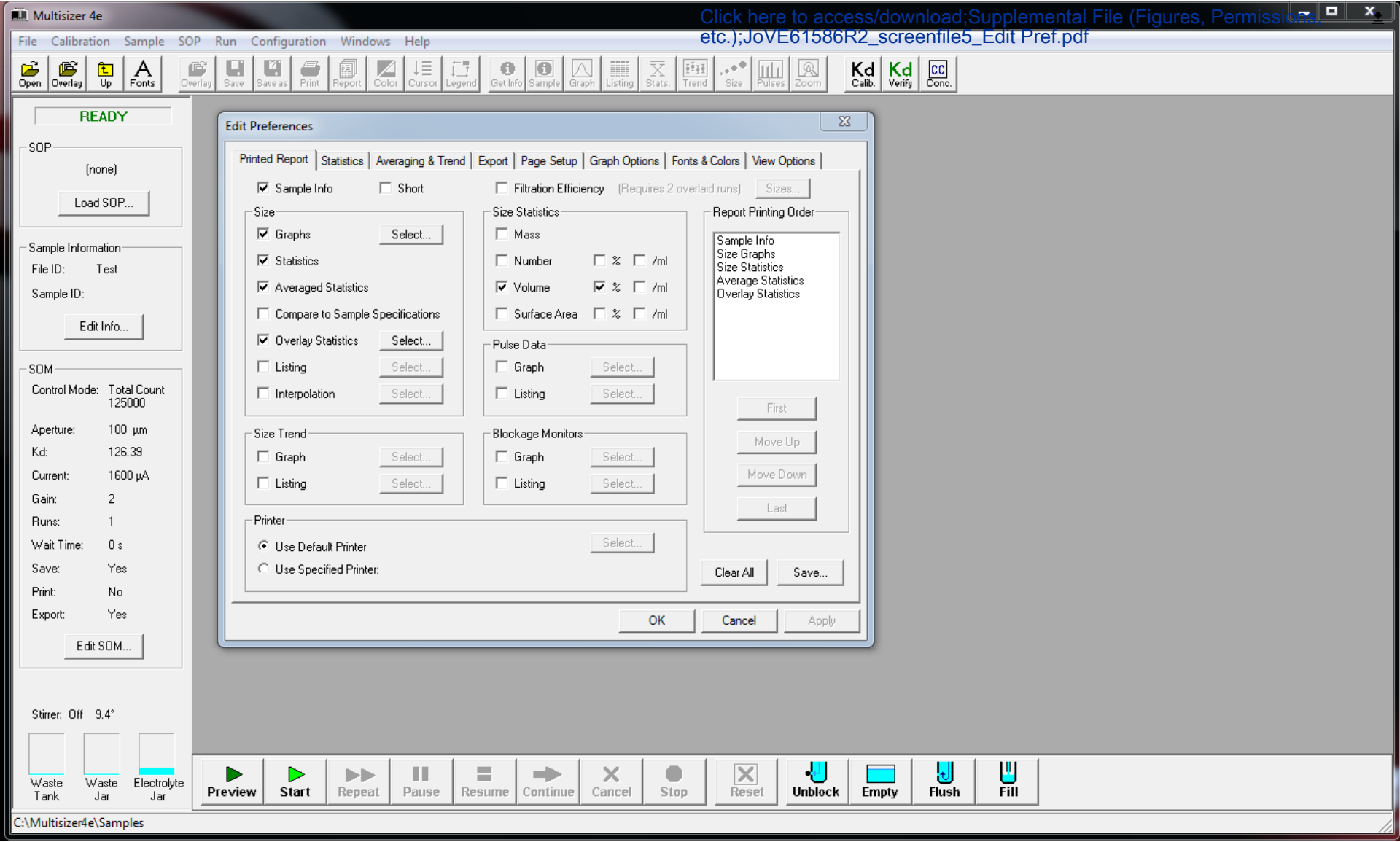
Response: It doesn't need to be filmed as it is a simple calculation. The step has also been revised to make it clearer.

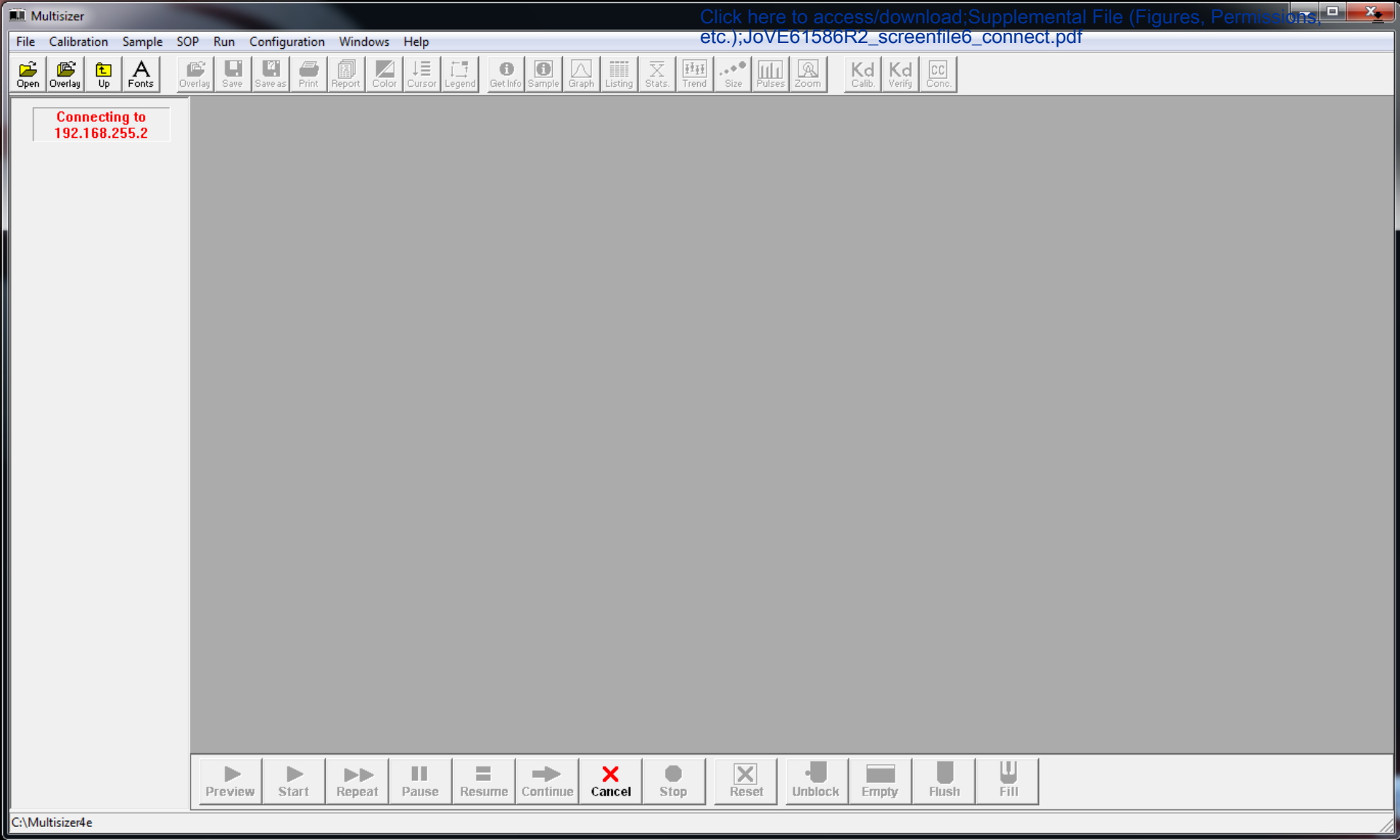


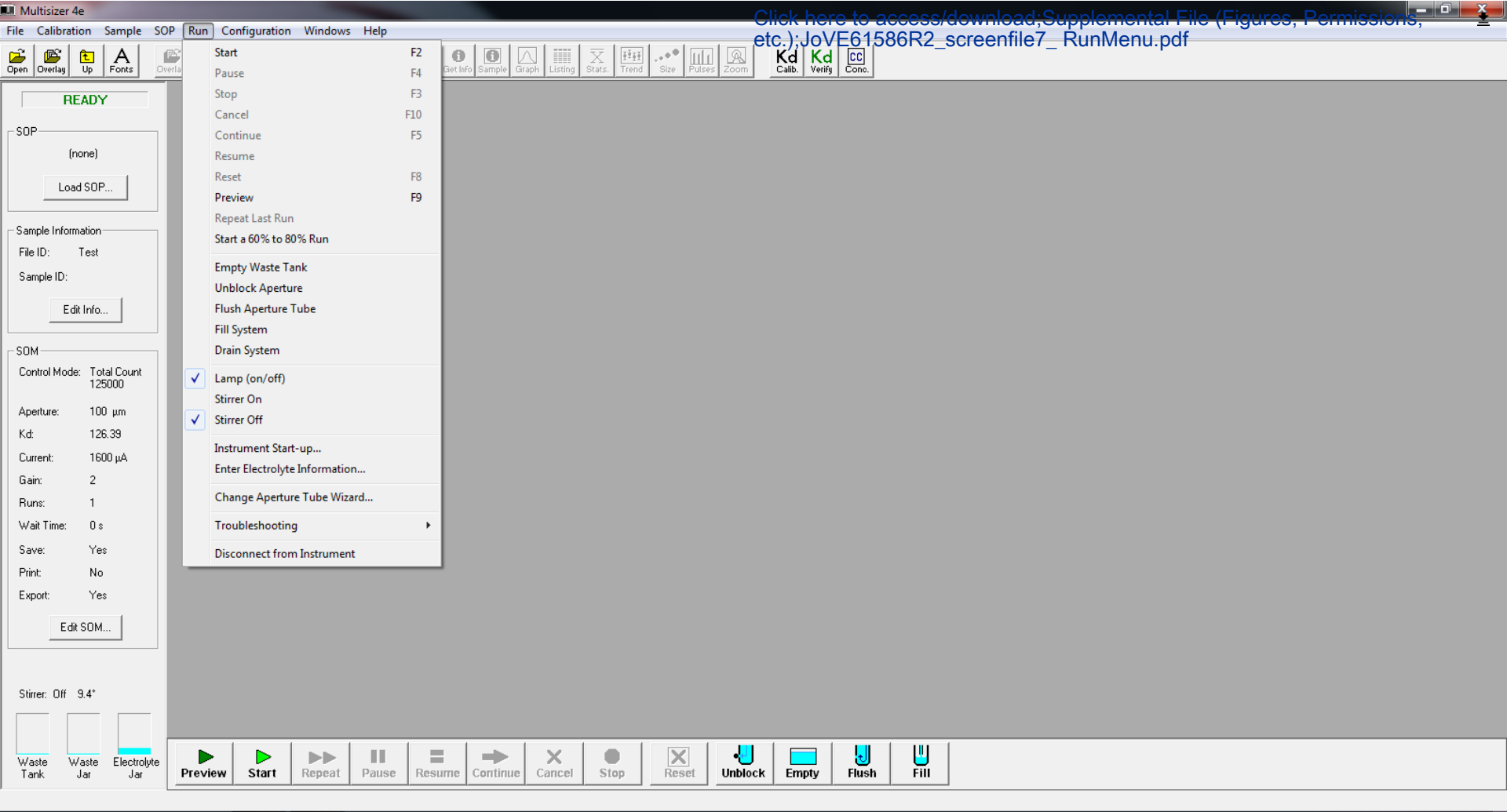


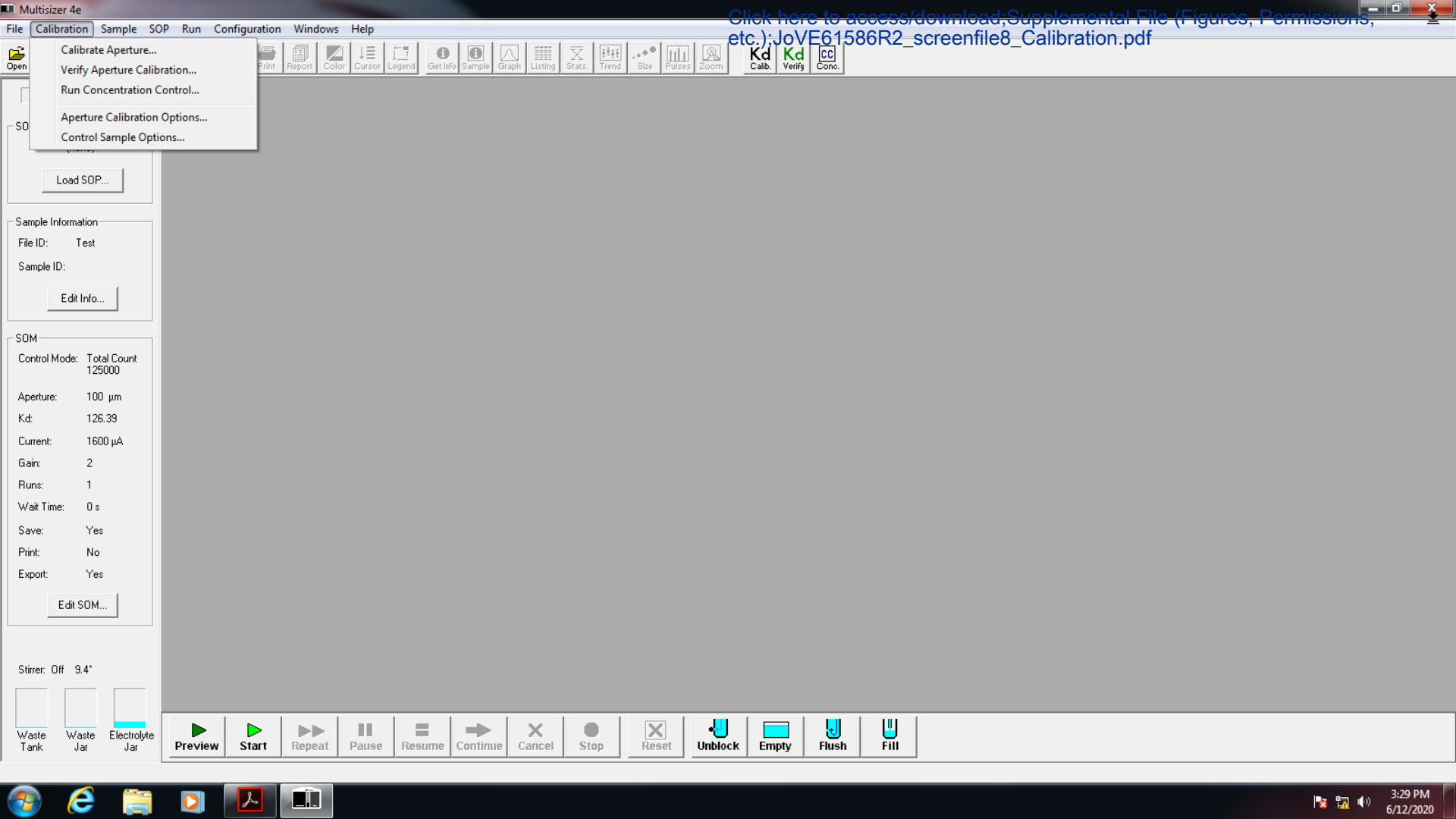


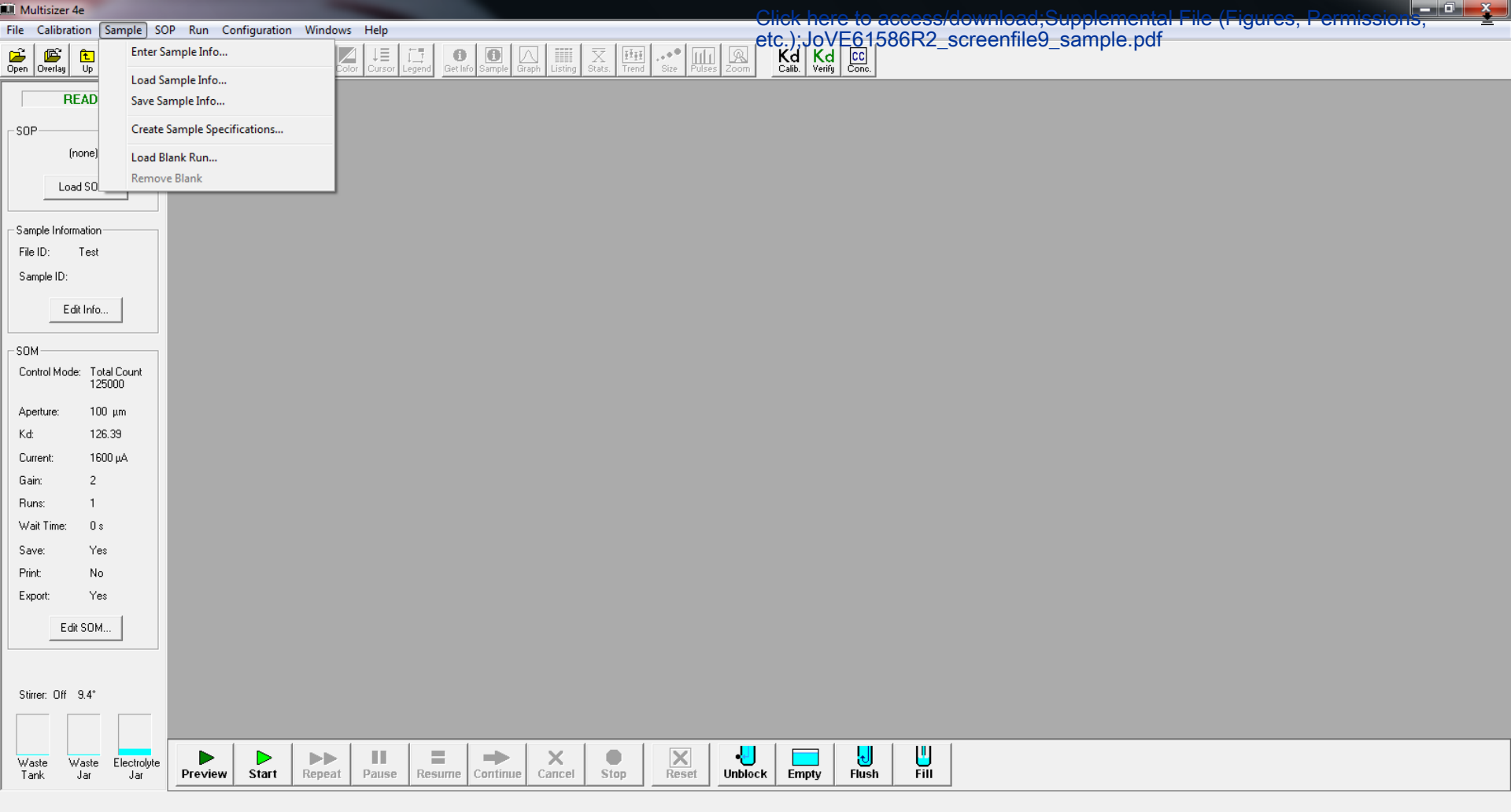


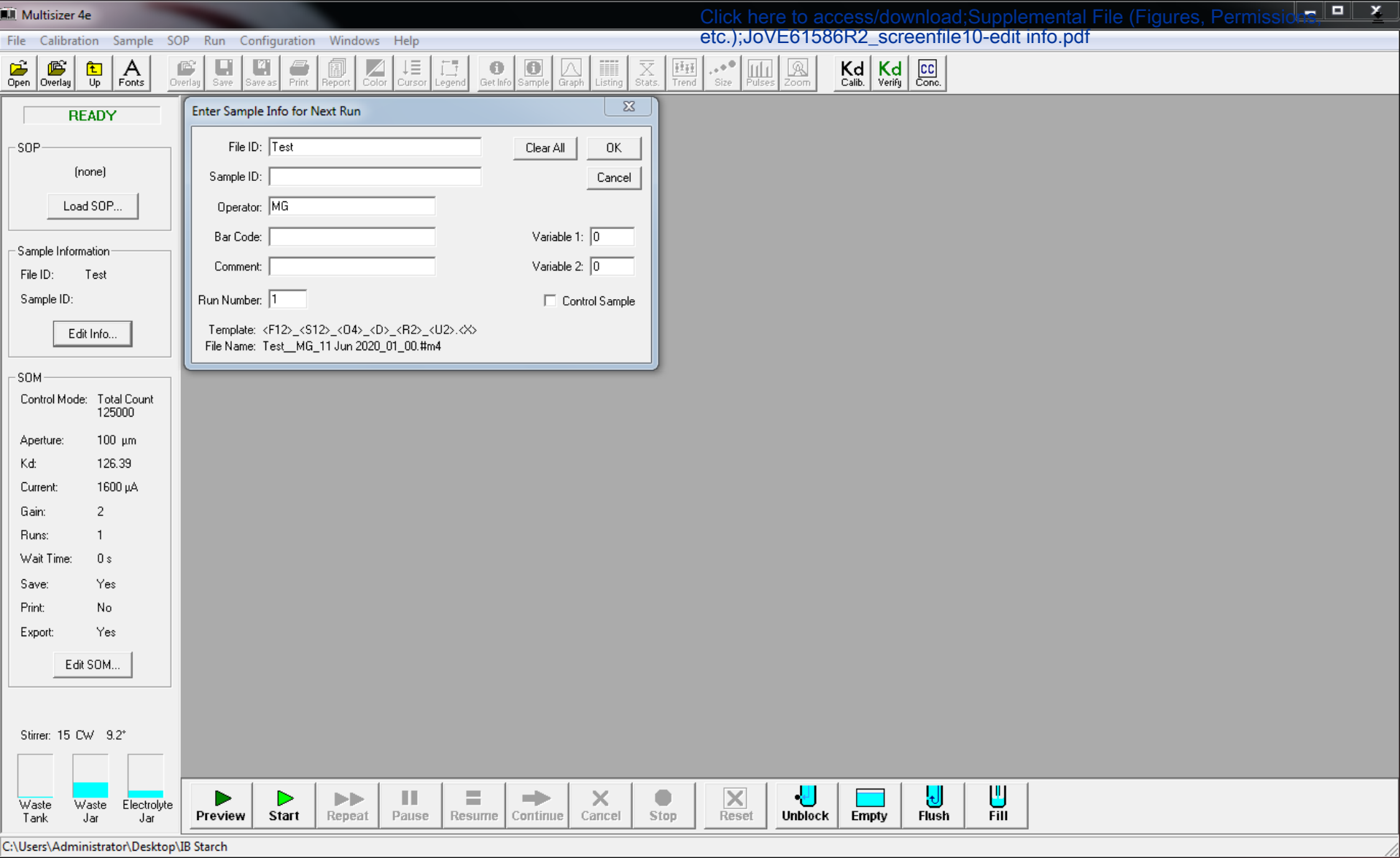


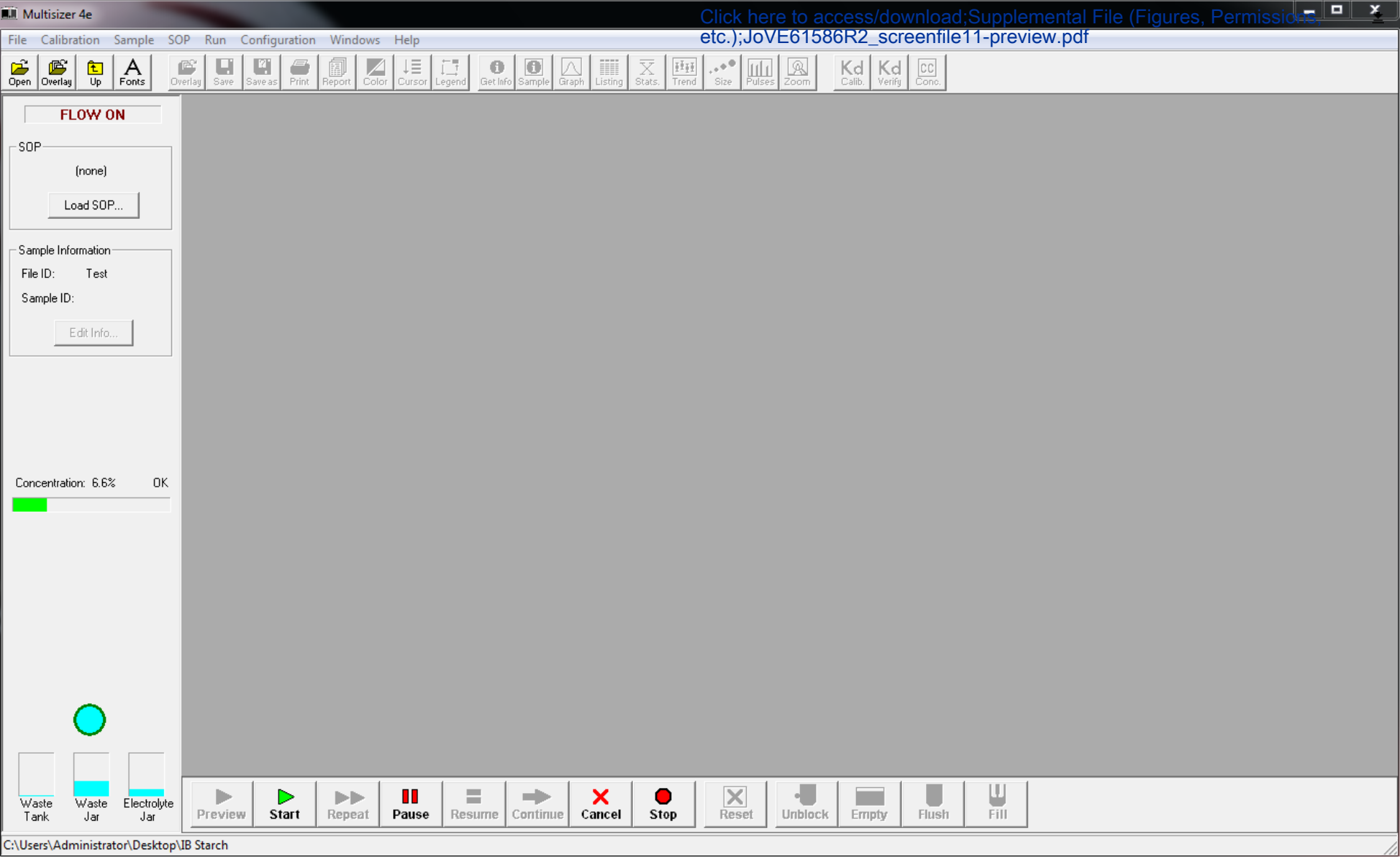












Multisizer 4e

File Calibration Sample SOP Run Configuration Windows Help

Open Overlay Up Fonts | Overlay Save Save as Print Report Color Cursor Legend | Get Info Sample Graph Listing Stats. Trend Size Pulses Zoom | Kd Calib. Kd Verify CC Conc.

RUNNING

Elapsed run time: 122.1 s

Progress

Total Count: 77339

2 85944 60

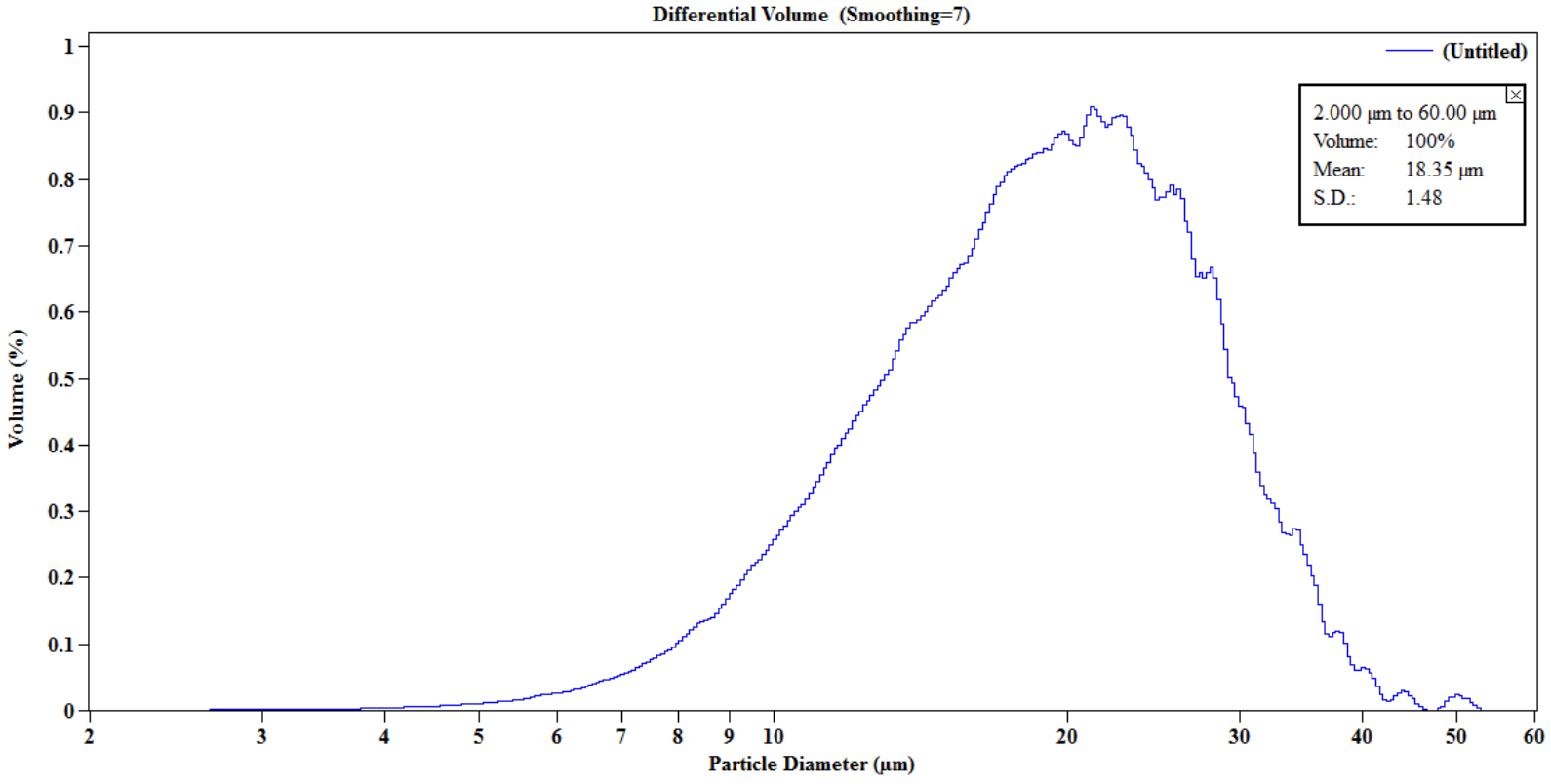
Concentration: 7.9% OK

Count Rate

Waste Tank Waste Jar Electrolyte Jar

Control mode: Total Count 125000 Run 1 of 1

RunFile Edit View Graph Calculate Display



Preview Start Repeat Pause Resume Continue Cancel Stop | Reset Unblock Empty Flush Fill

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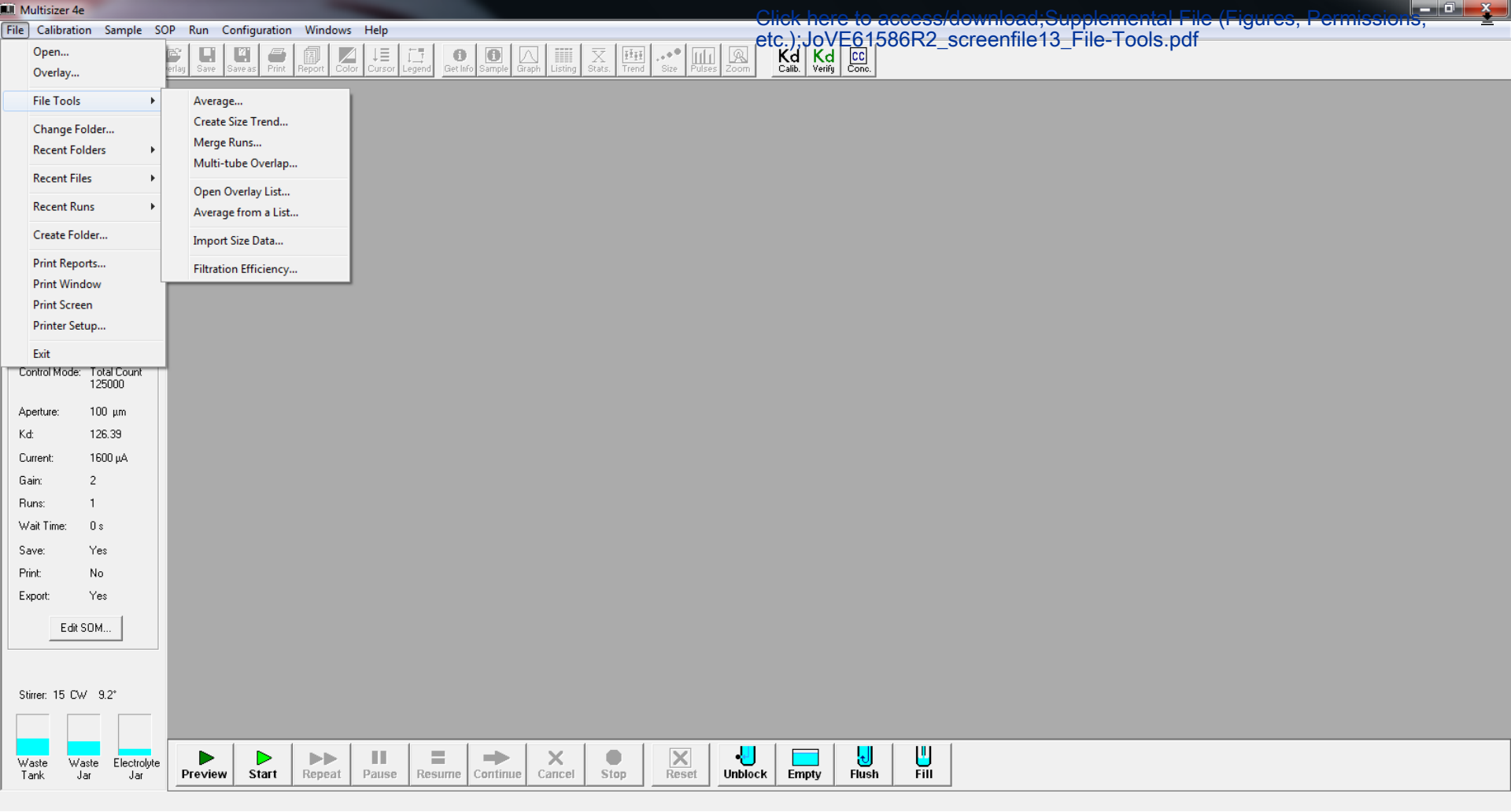


Multisizer 4e

File Calibration Sample SOP Run Configuration Windows Help

Open Overlay Up Fonts | Overlay Save Save as Print Report Color Cursor Legend | Get Info Sample Graph Listing Stats. Trend Size Pulses Zoom | Kd Calib. Kd Verify CC Conc.

READY

SOP
(none)
Load SOP...

Sample Information
File ID: Test
Sample ID:
Edit Info...

SOM
Control Mode: Total Count 125000
Aperture: 100 µm
Kd: 126.39
Current: 1600 µA
Gain: 2
Runs: 1
Wait Time: 0 s
Save: Yes
Print: No
Export: Yes
Edit SOM...

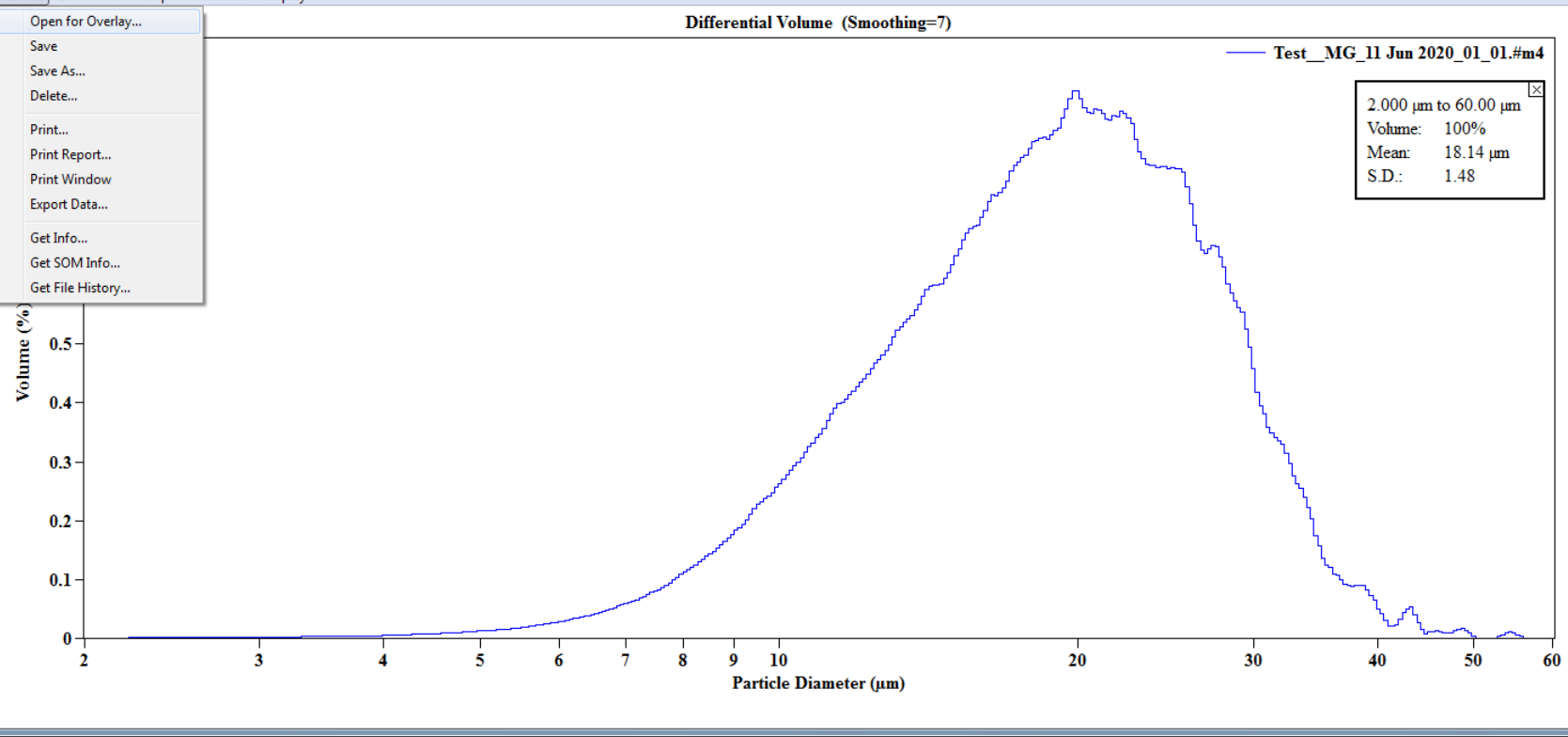
Stirrer: Off 9.4"

Waste Tank | Waste Jar | Electrolyte Jar

Test_MG_11 Jun 2020_01_01.#m4

RunFile Edit View Graph Calculate Display

- Open for Overlay...
- Save
- Save As...
- Delete...
- Print...
- Print Report...
- Print Window
- Export Data...
- Get Info...
- Get SOM Info...
- Get File History...



Preview | Start | Repeat | Pause | Resume | Continue | Cancel | Stop | Reset | Unblock | Empty | Flush | Fill

Open another run in this window

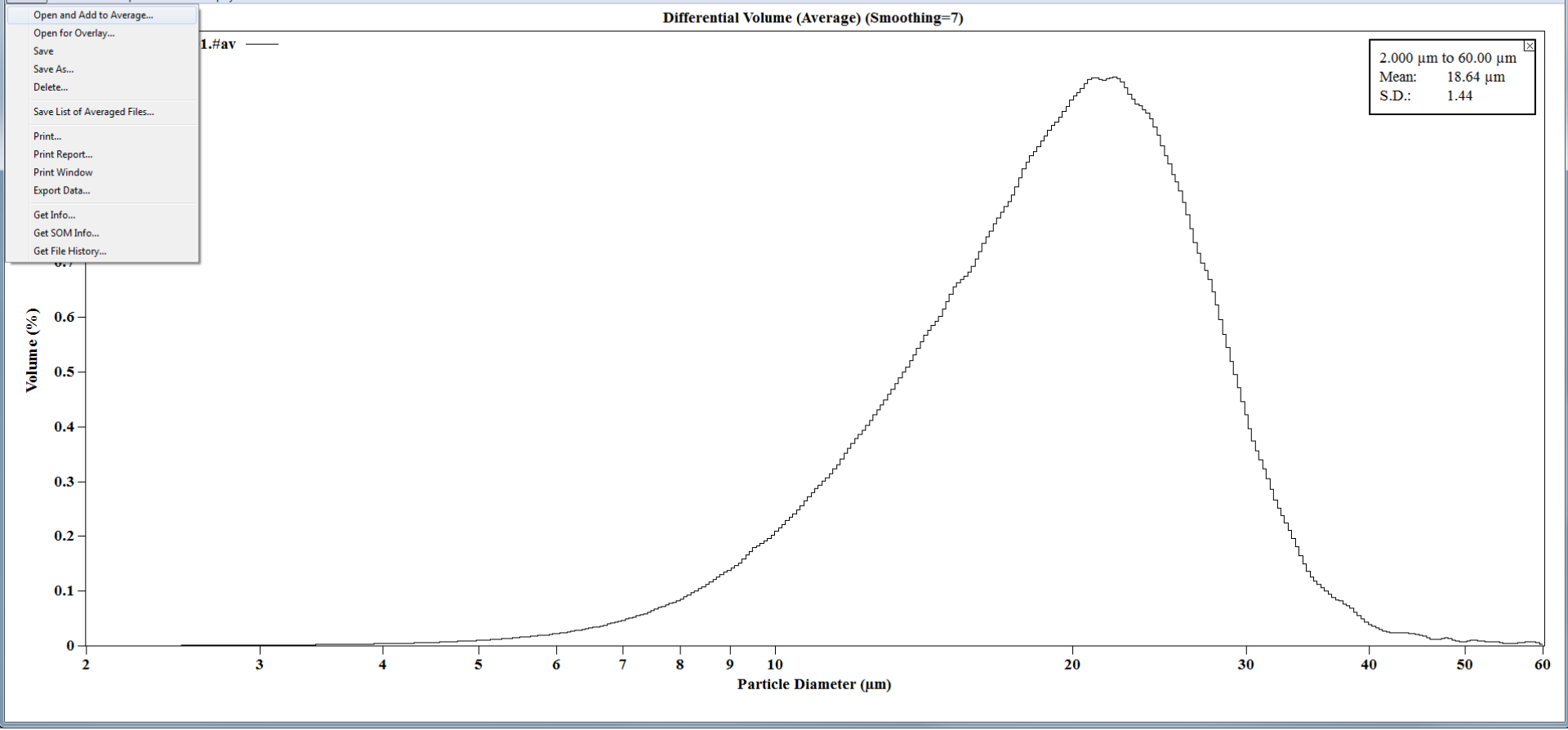
File Preferences Configuration Windows Help

Open Overlay Up Fonts | Overlay Save Save as Print Report Color Cursor Legend | Get Info Sample Graph Listing Stats. Trend Size Pulses Zoom

Q2ab_S2b-F_01.#av

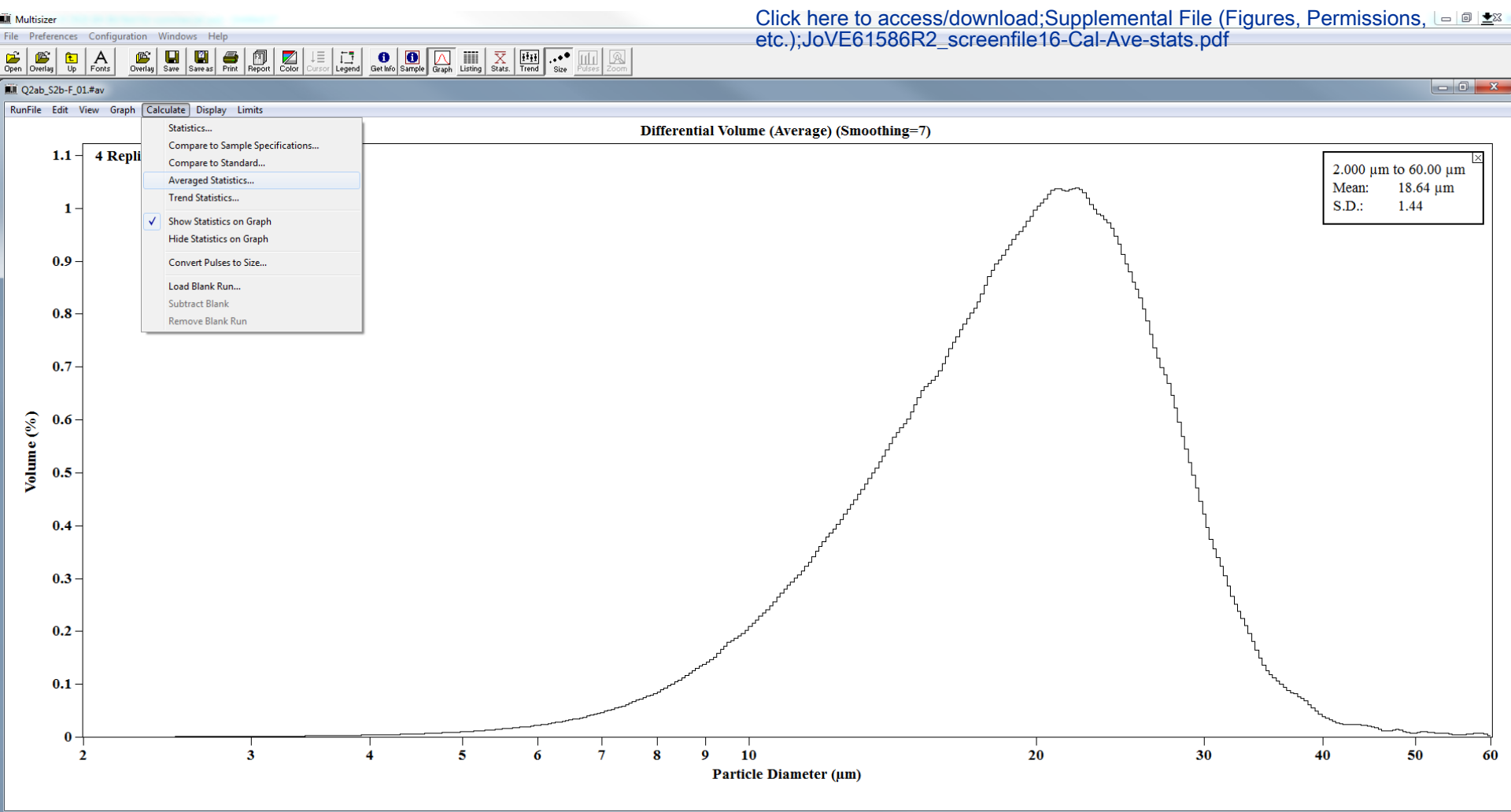
RunFile Edit View Graph Calculate Display Limits

- Open and Add to Average...
- Open for Overlay...
- Save
- Save As...
- Delete...
- Save List of Averaged Files...
- Print...
- Print Report...
- Print Window
- Export Data...
- Get Info...
- Get SOM Info...
- Get File History...



Add a file into the average

Click here to access/download;Supplemental File (Figures, Permissions, etc.);JoVE61586R2_screenfile16-Cal-Ave-stats.pdf



Multisizer

File Preferences Configuration Windows Help

Open Overlay Up Fonts | Overlay Save Save as Print Report Color Cursor Legend | Get Info Sample Graph Listing Stats. Trend Size Pulses Zoom

Q2ab_S2b-F_01.#av

RunFile Edit View Graph Calculate Display Limits

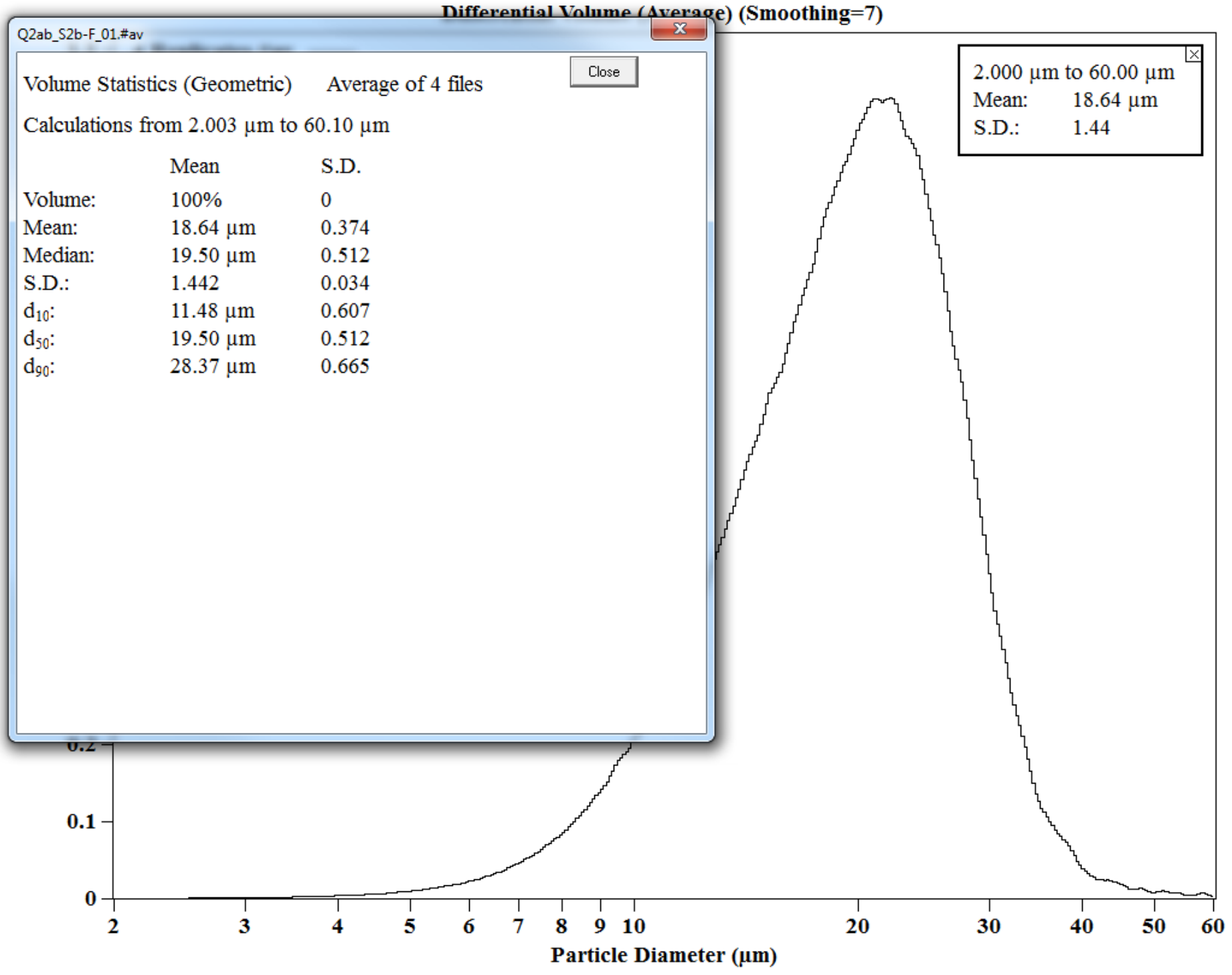
Q2ab_S2b-F_01.#av

Close

Volume Statistics (Geometric) Average of 4 files

Calculations from 2.003 µm to 60.10 µm

	Mean	S.D.
Volume:	100%	0
Mean:	18.64 µm	0.374
Median:	19.50 µm	0.512
S.D.:	1.442	0.034
d_{10}:	11.48 µm	0.607
d_{50}:	19.50 µm	0.512
d_{90}:	28.37 µm	0.665



C:\Users\lab177\Desktop\IB Starch\Sizing Rep Results

Step 3.3.1	JoVE61586R2_screenfile1_UI-SOP JoVE61586R2_screenfile2_SOM Wzd JoVE61586R2_screenfile3_Edit SOM
Step 3.3.3.	JoVE61586R2_screenfile1_UI-SOP JoVE61586R2_screenfile4-Pref-Wzd JoVE61586R2_screenfile5_Edit Pref
Step 3.3.5	JoVE61586R2_screenfile1_UI-SOP
Step 4.1.1.	JoVE61586R2_screenfile6_connect
Step 4.1.3.	JoVE61586R2_screenfile7_ RunMenu JoVE61586R2_screenfile8_Calibration
Step 4.1.7.	JoVE61586R2_screenfile9_sample JoVE61586R2_screenfile10-edit info
Step 4.3.1.	JoVE61586R2_screenfile11-preview
Step 4.3.3.	JoVE61586R2_screenfile11-preview
Step 4.4.2.1	JoVE61586R2_screenfile13_File-Tools
Step 4.4.2.2.	JoVE61586R2_screenfile14_Runfile
Step 4.4.3.1	JoVE61586R2_screenfile13_File-Tools
Step 4.4.3.2	JoVE61586R2_screenfile14_Runfile JoVE61586R2_screenfile15_add-to-Ave
Step 5.1.	JoVE61586R2_screenfile16-Cal-Ave-stats JoVE61586R2_screenfile17-Ave_stat