

## **Scanning Transmission Electron Microscopy (STEM) for Accurate Virus Quantification**

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### **Abstract**

Accurate virus quantification is sought, but a perfect method still eludes the scientific community. Electron Microscopy (EM) quantification is a valuable technique because it

provides morphology data and counts all viral particles, including partial or noninfectious particles; however, EM methods have been criticized as inaccurate, non-reproducible, and having detection limits too high to be useful. To address these issues, we developed a consistent, reproducible virus quantification method called Scanning Transmission Electron Microscopy – Virus Quantification (STEM-VQ) which simplifies sample preparation and utilizes large throughput STEM imaging and commercially available software to count particles. In this paper, we present data evaluating alphavirus viral stock preparations that supports this method's reproducibility and accuracy including a comparison of this quantification method to viral plaque assay and the ViroCyt Virus Counter.

## **Introduction**

Quantification is an important factor when studying viral impact on the environment or a host<sup>1-3</sup>. An accurate method of viral particle quantification is desired, but a universally accepted method still eludes the scientific community<sup>1-4</sup>. Routinely, multiple methods of quantification are used to substantiate each other. Commonly used methods for quantification of viruses include EM count, plaque assay, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), epifluorescent microscopy, and analytical flow cytometry<sup>1-3,5,6</sup>. EM quantification is a valuable technique because it provides morphology data and counts all viral particles, including partial or noninfectious particles<sup>2,3,7</sup>; however, EM methods have been criticized as inaccurate, inconsistent, and lacking sensitivity with a detection limit too high to be useful<sup>2,3,6</sup>. To address these shortcomings while preserving the valuable aspects, we developed the STEM-VQ method. This method is an efficient, reproducible EM quantification method which uses the mPrep/g capsules method<sup>8</sup> to improve and simplify sample preparation, and takes advantage of STEM for automatic image acquisition.

EM particle counting was first attempted in the early 1940s<sup>9</sup> using spray or centrifugation techniques to completely deposit the sample on supporting media, followed by negative staining with 2% Uranyl Acetate<sup>2,10-13</sup>. Viral particles within representative fields were imaged with a transmission electron microscope (TEM) and counted. The final concentration was calculated based on the area imaged and the volume of sample applied<sup>9-11,13-16</sup>. Scientists continued adapting application techniques to improve sample distribution by including various sample collection mediums<sup>14,15</sup> and different purification steps to decrease sedimentation that interfered with imaging and counting<sup>9,13,15</sup>. In 1950, scientists began using latex beads of a known concentration as a reference within their sample, allowing for calculations based on the bead concentration instead of the area imaged<sup>9,11-13</sup>. Inclusion of reference beads led to development of the droplet method for sample application<sup>13</sup>. Several variations have been developed, but the most commonly used droplet method involves placing a drop of sample onto a horizontally oriented grid<sup>13,16</sup>. While other methods of counting have been used, typically, the two main approaches for counting TEM images involve either counting a set number of grid squares or a set number of beads. Thresholds for each of these methods have been as few as 3 grid squares or 200 beads, respectively<sup>2,16</sup>. There have also been many different approaches to calculating the final concentration of virus<sup>2,3,5,6,9,14-16</sup>. Automatic processing for image acquisition, analysis, and enumeration has emerged in more recent years with the development of image analysis software and more automated microscopes<sup>1</sup>. Scanning Electron Microscope (SEM) imaging has been used in quantification of particles in the field of material sciences<sup>17</sup> and STEM imaging has been used for particle counting in cell monolayers<sup>1,18-20</sup>, but STEM imaging has not gained widespread use for particle counting material in suspension in biology or virology fields.

We developed a consistent, reproducible virus quantification method called STEM-VQ which simplifies sample preparation and utilizes large throughput STEM imaging and commercially available image analysis software. Briefly, our method continues to use a known concentration of gold beads as a reference, but it involves an altered droplet application method<sup>13</sup> with three major steps (Figure 1A): First, an equal volume of gold beads is mixed with an unknown concentration of virus particles and the mixture is applied to the EM grid using mPrep/g capsules<sup>8,21,22</sup> (Supplementary Figure 1). Use of the mPrep/g capsules reduced direct handling of grids and allowed easy application of sample and buffers when capsules were attached to a pipette. Second, the grids are imaged with STEM using automated ATLAS software. Compared to traditional TEM imaging, STEM imaging eliminates the need for negative staining and allows easy imaging of a much larger area of the grid. Third, ImageJ image analysis software is used to count virus and gold particles. These counts are used to determine the virus to gold ratio and calculate the original virus concentration (Figure 1C). We present a comparison of this quantification method to agarose-based viral plaque assay<sup>23</sup> and the ViroCyt Virus Counter 2100 (VC) (ViroCyt, Boulder, CO, USA)<sup>3</sup>.

## **Methods**

### ***Virus suspension preparation***

Venezuelan equine encephalitis virus (VEEV), INH-9813 strain and eastern equine encephalitis virus (EEEV), V105-00210 strain were received from the University of Texas Medical Branch repository (Dr. Robert Tesh). VEEV INH-9813 was isolated from the serum of an infected individual who presented with clinical disease in Columbia, South America in 1995<sup>24</sup>. The virus was isolated following a single passage on Vero cells (Vero-1). EEEV V105-00210 was isolated

from a human case of EEEV in Massachusetts in 2005, with no passage in animals and a single passage in cell culture<sup>25</sup>. Western equine encephalitis virus (WEEV) McMillan strain was received from the Centers for Disease Control and Prevention, Fort Collins. This virus was originally isolated from a human case of WEEV in Ontario, Canada in 1941<sup>26</sup>. This virus has a passage history including both animals and cell culture. Biosafety level (BSL-)-3 laboratory prepared master (MVS), working (WVS), and sucrose purified (SpVS) virus stocks were prepared using ATCC Vero 76 cells. For MVS and WVS, Vero cells were infected at a multiplicity of infection (MOI) of 1 in EMEM (Cellgro) containing 2% fetal bovine serum (FBS)(HyClone), 1% non-essential amino acids (NEAA)(Gibco), 1% Penicillin-Streptomycin (Pen-Strep; 10,000 U/ml-10 mg/ml, respectively; Sigma-Aldrich), 1% HEPES (1M; Sigma-Aldrich), and 1% L-glutamine (200 mM; HyClone). At 24-32 hours post-infection, supernatants were harvested and clarified by centrifugation (10,000 x g, Sorvall GSA rotor, 30 minutes). Stocks were aliquoted and stored at -70°C for future use. For SpVS, supernatant was collected from Vero cells were infected at a MOI=1 in EMEM containing 5% FBS, 0.5% Pen-Strep, 1% HEPES, 1% L-Glutamine, and 0.1% Gentamicin (50 mg/ml; Sigma-Aldrich) and clarified by centrifugation. Virus was precipitated with 2.3% NaCl and 7% Polyethylene glycol (MW8000, Sigma-Aldrich), with stirring, overnight at 4°C. Virus was pelleted by centrifugation (10,000 x g; Sorvall GSA rotor, 30 minutes). Virus pellets were suspended in 1x TNE Buffer (10 mM Tris, 0.2 M NaCl, 1 mM EDTA, pH 7.4) and layered onto gradients. Virus was banded in 20-60% continuous sucrose density gradients (100,000 x g, 4°C, for 4 hours; SW-28Ti rotor, Beckman). The virus band was collected, aliquoted and stored at -70°C for future use.

For all stocks, deep sequence analysis determined that the sequence of these isolates were consistent with the reported strain of VEEV, EEEV, and WEEV. Additionally, this analysis demonstrated that the virus stocks did not contain contaminating agents.

***STEM-VQ sample preparation by mPrep/g capsules***

Viral stocks were serially diluted beginning at 1:10 in phosphate buffered saline (PBS) with dilution volumes of at least 5 ml to achieve the final dilution for application to EM grids. Two formvar-carbon coated 200 mesh copper EM grids (SPI, Cat#3420C-MB) were inserted into a capsule based-microscopy processing system called mPrep/g<sup>8</sup> (Figure 1A left, Microscopy Innovation, LLC, WI, Cat#G1600 and F1602). Gold beads (40nm, Ted Pella Cat#15707-5, 1.461E10 particles/ml concentration certified by Particle Technology Labs) were sonicated for 10 minutes and transported into biocontainment with grid-loaded mPrep/g capsules with filters, fixatives, and 1% osmium tetroxide. Step 1, in the biocontainment suite, equal volumes of virus (unknown concentration) and gold beads (known concentration) were well mixed (Figure 1B) and 40µl of the resulting suspension aspirated into the mPrep/g capsule while attached to a pipette (Figure 1A left). Step 2, the pipette with mPrep/g attached was laid on its side for 10 minutes with grids oriented horizontally for even sample distribution onto grid formvar. Step 3, the pipette was picked up and the plunger pressed to dispense the virus bead mixture into a waste container. An aliquot (40µl) of fixative (2% glutaraldehyde in water) was then aspirated into capsule, incubated with grids oriented horizontally for 20 minutes, and then dispensed into a waste container. Three rinse cycles were performed by aspirating and immediately dispensing 40µl of deionized (dI) water. An extra ten rinse cycles were needed with samples that were dense such as the spVS conditions. Step 4, the mPrep/g capsule was removed from the pipette and placed, with the lid open, into a 50ml centrifuge tube containing filter paper soaked in 1%

Osmium Tetroxide ( $\text{OsO}_4$ ) suspended in water. The tube was sealed for 1 hour to ensure complete inactivation of the virus by  $\text{OsO}_4$  vapor and transferred to the BSL-2 electron microscopy facility. Step 5, in the BSL-2 EM facility the mPrep/g capsule was removed from the tube and placed onto a pipette. Three rinse cycles were repeated by aspirating 40 $\mu\text{l}$  of dI water followed by dispensing into a waste container. The capsule was removed from the pipette, lid opened, and allowed to air dry. Once dry, the grids were ready to be imaged under STEM. (Supplementary Figure 1) Note, Step 4, inactivation with osmium tetroxide vapor, can be eliminated for samples that do not require BSL-3 or -4 biocontainment.

### ***STEM imaging***

TEM grids were loaded into a Zeiss Sigma Field Emission SEM and imaged with a STEM detector at 30kV. Images were auto-acquired using Zeiss FIBICS ATLAS software: using 35X35  $\mu\text{m}$  frame size, 4nm/pixel spacing, and 2000ns dwell time.

### ***Data analysis by ImageJ***

ImageJ software was used to individually count alphaviruses (70nm in diameter) and nano-gold particles (40nm in diameter) according to their respective particle sizes. Suggested ImageJ macro codes are recorded in supplementary figure 3.

### ***STEM-VQ Statistical Analysis***

#### **Virus to beads ration:**

For each grid, the virus to beads' ratio was estimated as the slope of the linear regression of the virus to bead counts per sampled grid area, forced through the origin. The sampling variance of

the estimated virus to bead ratio obtained from a single grid was taken from the large sample delta-method approximation, as discussed by Kempen and Vilet <sup>27</sup>.

$$\text{var}(r_1) \approx \frac{1}{n} \left( \frac{\text{var}(x)}{\mu_y^4} + \frac{\mu_x^2 \text{var}(y)}{\mu_y^4} - \frac{2\mu_x \text{cov}(x, y)}{\mu_y^3} \right)$$

Where x and y are the bead count and the bead weighted virus count and n is the number of sampled areas. Where multiple grids were run per virus sample, an average of the log virus to bead ratio is formed by linear mixed model. This procedure reweights the average to adjust for the correlation observed between certain sets of grids, as well as the differing sampling variances within grid. Analysis is implemented in SAS Proc Mixed.

#### **Calculation of the concentration of particles:**

The following formula is used to calculate the concentration of particles:

*Unknown Virus Concentration*

*= Virus to beads'ration × Known beads'Concentration*

#### ***Agarose-Based Plaque Assay***

Each virus stock was quantitated by standard agarose-based plaque assay<sup>23</sup>. Virus stocks were serially log-diluted starting at a dilution of 1:10 in Hank's Balanced Saline Solution (HBSS). ATCC Vero 76 cells seeded on 6-well plates were grown to 90-100% confluence. Duplicate wells were infected with 100µL/well of each serial dilution. Plates were incubated at 37°C for 1 hour, with rocking every 15 minutes for even distribution and to keep monolayer from drying. Following the incubation period, wells were overlaid with 0.5% agarose in 2X Basal Medium Eagle with Earle's Salts (EBME) (USAMRIID, Fort Detrick, MD) containing HEPES and 10%

FBS, 1% L-glutamine, 1% NEAA, 1% Pen-Strep, and 0.1% gentamycin, and plates were incubated at 37°C at 5% CO<sub>2</sub>. Twenty four hours later cells were stained by the addition of a second agarose overlay prepared as above and containing 5% neutral red stain (Gibco). The plates were incubated at 37°C at 5% CO<sub>2</sub> for another 24 hours. Residual infectivity was quantitated by counting defined plaques (neutral red exclusion areas). Titer was calculated factoring in the volume of inoculum used per well and the dilution(s) with plaque counts between 10 and 150.

### ***ViroCyt Quantification***

Samples were tested on VC using the ViroCyt reagent kit and following manufacturer's instructions. The strategy was similar to that described for filoviruses in Rossi *et al.*, 2015<sup>3</sup>. Virus preparations were diluted beginning at 1:10 into ViroCyt sample buffer. Serial ¼ log dilutions were prepared from the 1:10 in order to provide samples with values within the linear range of the VC. Briefly, 300µl of each dilution was stained using 150µl of Combo Dye solution, incubated in the dark at room temperature for 30 minutes, and analyzed in the VC. Each dilution was tested in triplicate with inter-sample washes and a cleanliness control run between each sample to verify the flow path was clean. Results were automatically analyzed by the instrument software and reported as virus particles per ml (VP/ml). The sample quantification limit (SQL) for unpurified virus stocks were similar to that previously reported for filoviruses (2.0E+06 VP/ml) while purified virus SQL was lower and equivalent to the lower limit of the linear range of the instrument (5.5E+05 VP/ml). All VC results greater than this value were considered statistically distinguishable from background and therefore reportable. Final virus particle concentrations were established using all samples whose VP/ml counts were

above background and within the linear range of the instrument. Microsoft Excel 2007 (Redmond, WA, USA) was used for linear-regression analysis and determining coefficient of variation between replicates. Instrument performance was validated prior to testing samples by running a manufacturer's control of known concentration.

## **Results**

### **Sample preparation quality correlated with accuracy.**

Uniform particle distribution and minimal background on the EM grid was critical for achieving accurate results<sup>5,6,16</sup> (Figure 2A, 2D). Proper sample preparation, including bead agitation, extensive mixing of the gold beads with the virus, and at least 3 washes with reagent grade water was required. Particles aggregation was always a sample preparation problem (Figure 2B).

Sonication of the bead stock prior to mixing with the virus helped to suspend the bead solution and eliminate clumping that formed when the solution was stored for a lengthy period between uses. Thorough mixing of the virus and gold beads by pipetting the mixture up and down several times evenly distributed sample throughout the solution and helped remove any viral aggregation. Nutrient rich media was required for virus growth, but this media resulted in crystal salt and sugar deposits on the grid which made imaging and counting difficult (Figure 2C, 2E).

Extra rinsing at least 10 times with water helped eliminate these deposits.

Upon data analysis, we found that correlation strength between the gold beads count and virus count was an indicator of good sample preparation quality; and therefore, result accuracy (Figure 2D, 2E). All of the samples used here were well prepared and the standard macro was used. As we have developed this method we have observed that while inferior preparations should be immediately identifiable during imaging, areas within the grid that contain particle clumping or

dirty background may go undetected with our automated imaging process. Samples that contained particle aggregation or dirty background are usually identified by the technician when the data set is poorly correlated or contains extreme outliers (Figure 2E). In this event, the image analysis can be adjusted in a manner appropriate according to the severity of the issue.

Adjustments to the analysis macro code (Supplementary Figure 3) such as tailored thresholding or background extraction often solves the issue. If the particle aggregation or dirty background is severe enough a new grid preparation for imaging is required.

### **Computing bootstrapped standard error to statistically determine the number of images for an accurate STEM-VQ calculation.**

One-hundred areas were imaged from a single grid to determine the number of imaged areas that would be required for an accurate representation of the entire grid (Figure 3A). Two possible estimators of the virus to bead ratio were compared: (1) the ratio of mean virus count to mean bead count (ratio of means) and (2) the slope of the linear regression of virus to bead counts, forced through the origin (regression through the origin). The 100 areas were resampled 50 times with replacement to form a boot-strap<sup>28</sup> estimate of the standard error (Figure 3B). As shown in Figure 3B, the standard error decreased with increasing numbers of sampled areas. Considering a compromise between the costs of increased sampling versus the reduction in error, we determined that 30 imaged areas per grid were needed for accurate quantification. Most of the gains in reliability were realized by  $n=30$ , with further increases in the number of sampled areas yielding only small reductions in variance.

### **Individual sample preparation resulted in some variation and limited analytical errors.**

We found a major source of error came from variability between sample preparations. We analyzed 4 different preparations of 3 different individual VEEV stock samples. Each preparation consisted of two duplicate grids (Figure 4A). The results from the simultaneously prepared duplicate grids in each preparation were very similar to each other, but there were variations between different preparations. Figure 4B shows the standard error calculated from the counts in Figure 4A. The range varied between one standard error above and one standard error below demonstrating that the variability was less than a log, which is commonly considered acceptable for EM particle counts<sup>2,3,29</sup>.

### **Detection limit for accurate counting**

The detection limit for any EM procedure is typically considered  $1\text{E}+07$  particles/ml (P/ml).<sup>2,6</sup> In order to determine the range for accurate counting for STEM-VQ method, we examined serial dilutions using alphavirus samples (Figure 5A). We found that samples containing greater than  $1\text{E}+12$  P/ml typically had too much viral aggregation for an accurate quantification (data not shown). At the lower end, samples below  $1\text{E}+07$  P/ml had too few viral particles in the field of view for an accurate count (data not shown). Particle counts within the range of  $1\text{E}+09$  to  $1\text{E}+12$  P/ml provided accurate detection in 10-fold dilutions for three different sucrose-purified virus stocks (EEEV, WEEV and VEEV) (Figure 5B). All data in serial dilutions were linear, indicating the accuracy of the data set.

### **STEM-VQ method results were comparable to agarose-based plaque assay and ViroCyt Virus Counter results.**

There are many ways to quantify virus, all of which use very different methods to identify particles. Among all methods, plaque assay and the VC are well developed and widely used.

Plaque assay is the most common approach to virus quantification and is typically considered the “gold standard”<sup>2,3</sup>. It measures infectious virus particles by counting the number of plaques formed when virus is applied to a monolayer of cells, giving a count expressed as plaque forming units per ml (PFU/ml)<sup>2-4</sup>. The VC is a flow-based counter which quantifies virus particles in solution<sup>3</sup>. It requires the sample to be stained for protein and nucleic acid and counts particles containing both stains as intact virus particles, resulting in a count expressed as virus particles per ml VP/ml<sup>3</sup>. With STEM-VQ particle images are captured and particles counted electronically, then visually confirmed. Counts are expressed as particles per ml P/ml<sup>2</sup>.

We compared STEM-VQ, plaque assay and VC results for different alphavirus stocks (Figure 6). The linear range of the VC was verified to be between 5.5E+05 and 1E+09 VP/ml. Testing of each virus prep resulted in a linear curve with  $R^2 \geq 0.972$ , slopes between 0.916 and 1.396 and coefficient of variation (%CV)  $\leq 29\%$  using at least 4 concentrations and n between 11 and 18. Since the plaque assay measures infectious particles and the VC counts essentially intact virus particles, we expected VC and plaque assay results to be similar for each virus stocks. Our data agreed with this theory. We also expected the STEM-VQ results to be higher than both plaque assay and the VC since STEM-VQ counts the presence of all particles within a size range and cannot determine if they are infectious. We consistently found STEM-VQ results approximately 1.5 logs higher than the plaque assay and VC results (Figure 6). We do not propose that all types of viruses or variable conditions would result in a 1.5 log difference in plaque assay and EM counting, but we would always expect the EM count to be higher than plaque assay.

**Discussion:**

Virus quantification using TEM has been criticized as difficult and time consuming; issues we wanted to improve with the development of this method. In supplementary Figure 2, we compared and summarized the improvements made to the STEM-VQ method compared to the conventional TEM method. We simplified sample preparation with better distribution by using mPrep/g capsules in the process. The mPrep/g is a small capsule that functions as a pipette tip capable of holding two EM grids<sup>30</sup> (Figure 1A left). Once the grids have been inserted into the capsule, the person preparing the sample simply attaches the mPrep/g capsule to a pipette and no further grid manipulation is needed. Using mPrep/g resulted in much less damage to the grid during sample preparation, providing more data available to collect for more accurate results. It also made sample preparation in biocontainment labs (BSL3 and BSL4) much easier and safer. Each capsule holds 2 grids; therefore, duplicate grids can be made with no further effort. The capsules can also easily be loaded onto a multi-channel pipette, or stacked, so many samples or several replicates of the same sample can easily be prepared<sup>30</sup>. Additionally, uniform particle distribution on the grid is critical for achieving accurate data<sup>5,6,16</sup>. We found samples prepared using the capsule consistently showed more uniform distribution than samples prepared using the traditional droplet method<sup>8</sup>.

Our new automated imaging and analysis procedure saved valuable technician time and allowed for much larger data collection in a shorter period of time. ATLAS automated imaging software enabled the user to select multiple areas to image, optimize the image acquisition settings for quality imaging such as focus, brightness, and contrast, and then the software automatically acquired images from large areas of the sample while unattended. We were able to acquire images of thirty 35x35 $\mu$ m square areas from a 200 mesh grid in less than 3 hours, and a technician needed to be present for only 45 minutes of those 3 hours. This was significantly less

time when compared to traditional methods in which a technician must continually sit at the microscope manipulating the controls and taking individual images. This new method not only saved overall imaging time for faster results, it largely decreased the amount of hands-on time required by a technician. Similarly, ImageJ analysis decreased the time needed to count the particles. Manually counting a well populated grid square requires hours of counting, whereas, using ImageJ software the same images were completed in less than 5 minutes. When counting or imaging is manually performed, accidental overlap or skipping an area frequently occurs. Utilization of software for analysis and automated image acquisition eliminated this error.

Virus quantification is an important step when characterizing challenge material for use in animal models of infectious disease. There are many methods for viral quantification including plaque assay, the VC, and EM<sup>1-3,5,6</sup>. The desired information and practicality of each method should be considered when determining which method to use. The plaque assay can be time consuming, typically requiring many days to complete, and must be performed at the level of containment appropriate for the virus being handled<sup>2,3</sup>. Choosing a cell line, media, and other variables are essential to a successful plaque assay<sup>3</sup>. Plaque assay has the lowest limit of detection<sup>31</sup>. It can only detect infectious particles, which a majority consider more applicable for dosing quantification because infectious particles are responsible for disease; however, there is evidence that noninfectious particles can also effect the host response<sup>32</sup>. Therefore, it is important to evaluate noninfectious as well as infectious particles present in virus challenge stock preparations.

For alphaviruses, the VC results were comparable to plaque assay, but VC has a higher limit of detection with an optimal range of  $5.5E+5 - 1.0E+9$  VP/ml<sup>3</sup>. It must also be operated in the level of containment required for the sample, but it was quick, taking less than an hour to stain

and count a sample. It was also the most affordable option, costing about \$5.00 per run.

However, the VC may provide poor results in samples with high levels of protein in the media <sup>3</sup>.

A major limitation of EM counting methods despite improvements seen with the STEM-VQ method is the relatively high detection limit, a concentration of 1E+07 P/ml remains necessary for accurate results <sup>2,6</sup>. Media containing high levels of salt, protein, or sucrose may lead to poor imaging if not properly rinsed, and poor fixation can lead to loss of sample from the grid or unidentifiable particles <sup>2,5</sup>. After BSL-3/-4 sample application to the grid, which takes about an hour, exposure to osmium tetroxide vapor quickly deactivates any virus and allows the rest of the work to be performed outside biocontainment <sup>8</sup>.

Despite its challenges, EM quantification is valuable due to its ability to count total particles and provide gross morphology data. It should be noted that although this method allowed for gross morphological evaluation; more detailed observations such as protein coat on virus particles requires additional EM procedures such as negative staining with TEM imaging. These EM methods can also be applied to other noninfectious nano-particles such as virus-like-particles (VLPs), whereas plaque assays and VC are unable to quantify VLPs. EM may also be able to provide insight into VLPs development or changes due to manipulations through morphologic evaluation <sup>33,34</sup>. STEM-VQ and VC particle counts can be used in conjunction with other quantification methods, typically plaque assay, to create ratios that provide insight into both infectivity of a virus stock and the quality of the virus preparation. These ratios (P:PFU and VP:PFU) are important comparators when examining alterations in quality of virus stocks which can arise due to mutation, poor handling techniques, or sequential passaging. <sup>19,35,36</sup>

The STEM-VQ method simplifies sample preparation, imaging, and data analysis for particle concentration analysis using electron microscopy. These changes have increased the accuracy and reproducibility of this assay.

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**Author Contribution:**

C.D.B. and M.G.S. designed and developed the method. Ke.A.K. and M.G.S set up the STEM imaging system. D.P.F and S.L.N provided statistical guidance and analysis. E.M.M., A.E.P. and P.J.G. performed sample preparation and plague assay experiment. B.J.K. and C.A.R. performed ViroCyt (VC) experiment. C.D.B., M.K.M, and Ka.A.K. Performed STEM-VQ experiment. C.D.B. and M.G.S. wrote the manuscript with the help of all coauthors.

**Additional Information:**

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### Figure Captions:

**Figure 1: STEM-VQ Method overview.** (A) The three major phases that are needed for determining particle concentration are illustrated: (left) sample preparation using mPrep/g system, (middle) STEM imaging, (right) Particle counting using imageJ. (B) A mixture of a

known concentration of gold beads with an unknown concentration of virus stock, followed by application of the mixture onto an EM grid for STEM imaging is illustrated. (C) Formula used to calculate the number of unknown viral particles based on the known concentration of gold beads and the virus-gold ratio.

**Figure 2: Good quality sample preparation produces data points that have a strong linear**

**correlation:** A) An example of evenly distributed virus and beads. B) This sample is too highly concentrated, which leads to clumping and inability to determine an accurate count. C) This sample contains large amount of crystal sediments and debris in the background; this background material is difficult to differentiate from viral particles when utilizing image analysis software. D) A strongly correlated data set results from well prepared samples as in A, each data point, 10 total, represents the quantity of virus and beads in a 35 x 35 $\mu$ m area on a single EM grid. E) A poorly correlated data set indicates a poorly prepared sample as depicted in panels B and C, each data point, 10 total, represents the quantity of virus and beads in a 35 x 35 $\mu$ m area on a single EM grid. Scale bars are 100nm.

**Figure 3: Computing bootstrapped standard error to statistically determine the number of**

**images required for an accurate STEM-VQ method.** (A) particle count data representing data from 100 imaged areas of a single grid. (B) Bootstrap estimates of the standard error computed by simulating 500 resamples of the 100 areas. Most of the reduction in error is achieved by 30 images.

**Figure 4: Individual preparation causes small variation among same virus stock sample.**

All counts are calculated from 30 different images per sample. (A) STEM-VQ particle count data

of duplicate grids from 3 different individual virus stock samples prepared 4 different times. (B) Standard error from 4 different preparations for the 3 different individual virus stock samples.

**Figure 5: STEM-VQ data from different sample dilutions indicate accurate counting result.**

All counts are calculated from 30 different images per sample. (A) Results from 3 different alphavirus stocks using 3 different dilutions. (B) Comparison of the data from different dilutions.

**Figure 6: STEM-VQ method results are consistent with plaque assay and ViroCyt counting results.**

(A) Quantification results for 5 different alphavirus stocks using 3 different quantification methods. All EM counts are calculated from 30 different images per sample. (B) Comparison of the results from different methods in graphical format. EM results are higher than ViroCyt and plaque assay because it counts the presence of all particles, including non-infectious.