



Short communication

Preparation of viral samples within biocontainment for ultrastructural analysis: Utilization of an innovative processing capsule for negative staining



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Transmission electron microscopy can be used to observe the ultrastructure of viruses and other microbial pathogens with nanometer resolution. In a transmission electron microscope (TEM), the image is created by passing an electron beam through a specimen with contrast generated by electron scattering from dense elements in the specimen. Viruses do not normally contain dense elements, so a negative stain that places dense heavy metal salts around the sample is added to create a dark border. To prepare a virus sample for a negative stain transmission electron microscopy, a virus suspension is applied to a TEM grid specimen support, which is a 3 mm diameter fragile specimen screen coated with a few nanometers of plastic film. Then, deionized (DI) water rinses and a negative stain solution are applied to the grid. All infectious viruses must be handled in a biosafety cabinet (BSC) and many require a biocontainment laboratory environment. Staining viruses in biosafety levels (BSL) 3 and 4 is especially challenging because the support grids are small, fragile, and easily moved by air currents. In this study we evaluated a new device for negative staining viruses called mPrep/g capsule. It is a capsule that holds up to two TEM grids during all processing steps and for storage after staining is complete. This study reports that the mPrep/g capsule method is valid and effective to negative stain virus specimens, especially in high containment laboratory environments.

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1. Introduction

Transmission electron microscopy is an effective tool for viewing the morphology and ultrastructure of small biological specimens that are too small to be seen with a traditional light microscope, such as viruses (Gentile and Gelderblom, 2014; Kruger et al., 2000; Curry et al., 2006; Goldsmith and Miller, 2009). TEMs shoot electrons through a very thin specimen. The electrons interact with the specimen and are focused by an objective lens to form a magnified image on the viewing screen or camera screen. Regions of the sample that bend or block electrons appear dark, while regions

that are electron lucent appear white. Due to the fact that a TEM uses electrons, the resolution is much greater than that of a light microscope, because electrons have a much shorter wavelength than visible light.

Lack of electron dense matter makes viruses difficult to view under a TEM because the electrons are unable to interact with viral material and pass right through the sample. Negative staining is the most common method used to create contrast and view viruses under a TEM. The first negative staining procedure was proposed by Brenner and Horne in 1959, based on an experiment where Hall (1955) and Huxley (1957) observed the appearance of biological structures in reverse contrast when immersed in an electron-dense substance (Kiselev et al., 1990). The process of negative staining has been virtually unchanged over the past half century. Negative staining involves briefly applying a heavy metal salt solution to

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a coated TEM grid, with the virus sample already attached, in an attempt to surround the virus without infiltrating it (Brenner and Horne, 1959). This creates a dark border and maps out the particle's shape. Negative staining can be pictured as the immersion of an object within an electron-dense matrix (Kiselev et al., 1990). The two reagents used as negative stains in our experiments are uranyl acetate (UA) and potassium phosphotungstic acid (PTA). Both of these stains are commonly used to negatively stain small biological samples, such as viruses, protein complexes, and small nanoparticles (Harris, 2007; Bradley, 1967; Suzuki et al., 1987).

The conventional negative staining technique is referred to as the single-droplet negative staining technique (Harris, 2007). It is a manual method that requires precise handling of small and fragile TEM grids with forceps while applying small amounts of virus sample and stain. The typical preparation protocol involves applying a droplet of sample suspension onto the surface of a film-coated TEM grid (Fig. 1A). After attachment of the sample to the film surface, the grid is rinsed to remove non-adherent viruses, and then stained quickly with either UA or PTA for a few seconds to a minute, depending on the sample. Excess liquid is always wicked away from the edge of the grid with filter paper strips and the grid is stored in a grid box until TEM imaging.

The manual method requires the handling of multiple reagents with precise timing to ensure experimental accuracy and reproducibility. It is also time consuming because each TEM grid must be prepared individually. If not handled with care, TEM grids are easily punctured or bent by forceps or contaminated from being in the open air for too long. Processing numerous samples leads to difficulties in tracking the grids and ensuring similar processing for each sample. This sample preparation is more difficult when working in biosafety level (BSL) 3 and 4 biocontainment laboratories, because the required personal protective equipment is cumbersome. Our safety department requires personnel manipulating viruses in BSL-3 biocontainment laboratories to wear 1 to 2 pairs of gloves and work in a biosafety cabinet (BSC). Extra layering of gloves can reduce tactile sensitivity and restrict fine motor movement. The airflow of the BSC protects the user and helps prevent sample contamination; however, the airflow can also cause the samples and stains to dry faster, which can affect the results of a negative stain. The strong airflow in the BSC can also quickly suck up a grid if accidentally dropped. In BSL-4 biocontainment laboratories, personnel are required to wear a positive pressure suit which further restricts physical movement and the window of visibility. The technician wears at least 2 pairs of gloves, with the outer pair is a thick glove which reduces hand motion and tactile sensation, and is required to work in a BSC. Furthermore, the forceps used to handle TEM grids are small and sharp. It poses a risk to the technician due to their ability to puncture gloves. After the completion of staining procedures, the grids are inserted in microfuge tubes filled with Osmium Tetroxide vapor, and transferred out of the biocontainment laboratory. Prior to transfer from the biocontainment laboratory, all surfaces of the microfuge tubes are decontaminated with a disinfectant solution and sealed within double bags with disinfectant solution in the space between the bags. This procedure for removal of specimens from biocontainment can increase the damage to the negative stained EM grids.

In this report we introduce a new method for negative staining grids in biocontainment laboratories that utilizes mPrep/g capsules, a capsule based device for grid handling and staining (Benmeradi et al., 2015; Goodman et al., 2015; Goodman and Kostrna, 2011). The mPrep/g capsule accommodates two TEM grids, minimizes direct handling, and thus reduces the potential for grid damage. The mPrep/g capsule attaches directly to a single or multichannel pipette similar to a pipette tip, allowing for the application of various liquids via pipet aspiration. This enables simultaneous preparation of multiple samples with duplicate grids (Fig. 1B). To

negative stain with mPrep/g capsules the virus sample is first drawn into the capsule and held for 10 min to let the viruses adsorb onto the grid surfaces. The grids with adsorbed virus are subsequently washed with deionized (dI) water and stained with either UA or PTA for a few seconds to 1 min. This process uses the same protocol steps and reagents as the manual droplet method. The difference being that all work occurs inside the mPrep/g capsule with no physical handling of the grids. (Fig. 1C,D).

The purpose of this study was to evaluate mPrep/g capsules as a new method for negative staining of virus samples in biocontainment environments. The mPrep/g capsule method and the manual droplet method were compared, to determine its effectiveness of the mPrep/g negative staining system. This study also examined the EM image quality produced from two different virus inactivation procedures: 1) rapid inactivation, with 1% Osmium Tetroxide (OsO_4) vapor, and 2) a minimum 24 h inactivation with 2% glutaraldehyde, both were conducted using the mPrep/g capsules. Finally, we evaluated two of the most commonly used negative stains, UA and PTA, with regards to EM image quality.

2. Material and methods

2.1. Manual droplet method for negative staining in a biosafety level 2 (BSL-2) lab (Fig. 1A)

Inside the biocontainment laboratory BSC, the virus suspension was mixed well with the same volume of 4% glutaraldehyde to achieve a final concentration of 2% glutaraldehyde. Viruses were inactivated with 2% Glutaraldehyde inside a BSC for 24 h, according to industry standard practice (Moller et al., 2015; Rutala and Weber, 2008), prior to removal and transfer to the BSL-2 TEM facility. In the BSL-2 TEM facility, a drop (8 μl) of the glutaraldehyde treated virus suspension was placed onto a formvar/carbon coated TEM grid for 10 min in a moist chamber to reduce evaporation. It was important to make sure the grid did not dry. Using fine forceps to hold the grid, the liquid was wicked away from the grid surface from the side with filter paper. The grid was then washed three times by touching the grid to the surface of drops of dI water. Remaining water was wicked away by touching filter paper to the side of the grid. A small drop of stain (either 1% UA or 1% PTA) was applied to the grid and allowed to remain from 10 s to 1 min depending on the sample. The stain was wicked away by touching the edge of the grid to a piece of filter paper. The grid was air dried at room temperature and stored for subsequent TEM imaging.

2.2. MPrep/g capsule method for negative staining in biocontainment using aqueous glutaraldehyde and 1% osmium tetroxide vapor inactivation (Fig. 1C)

Step 1. Inside the biocontainment laboratory BSC, 40 μl of virus suspension was aspirated into the mPrep/g capsule attached to a pipette. The pipette remained attached to the mPrep/g capsule until the process was complete. Step 2. The pipette was placed on its side for 10 min with grids oriented horizontally to promote an even distribution of virus particles onto the grids. Step 3. The pipet was picked up and the plunger pressed to dispense the virus solution into a waste container. 40 μl of 2% glutaraldehyde fixative was aspirated into the capsules, covering the grids, and the pipette placed horizontally for 20 min. The fixative was subsequently expelled and 40 μl of dI water was aspirated into the capsules to wash away the fixative. The wash was repeated for a total of 3 rinse cycles. Step 4. 40 μl of stain (either 1% UA or 1% PTA) was aspirated into the capsules for 30 s (time may vary from 10 s to 1 min based on virus sample). Step 5. The mPrep/g capsule was removed from the pipet and the grids blotted dry by touching filter paper to the edge of

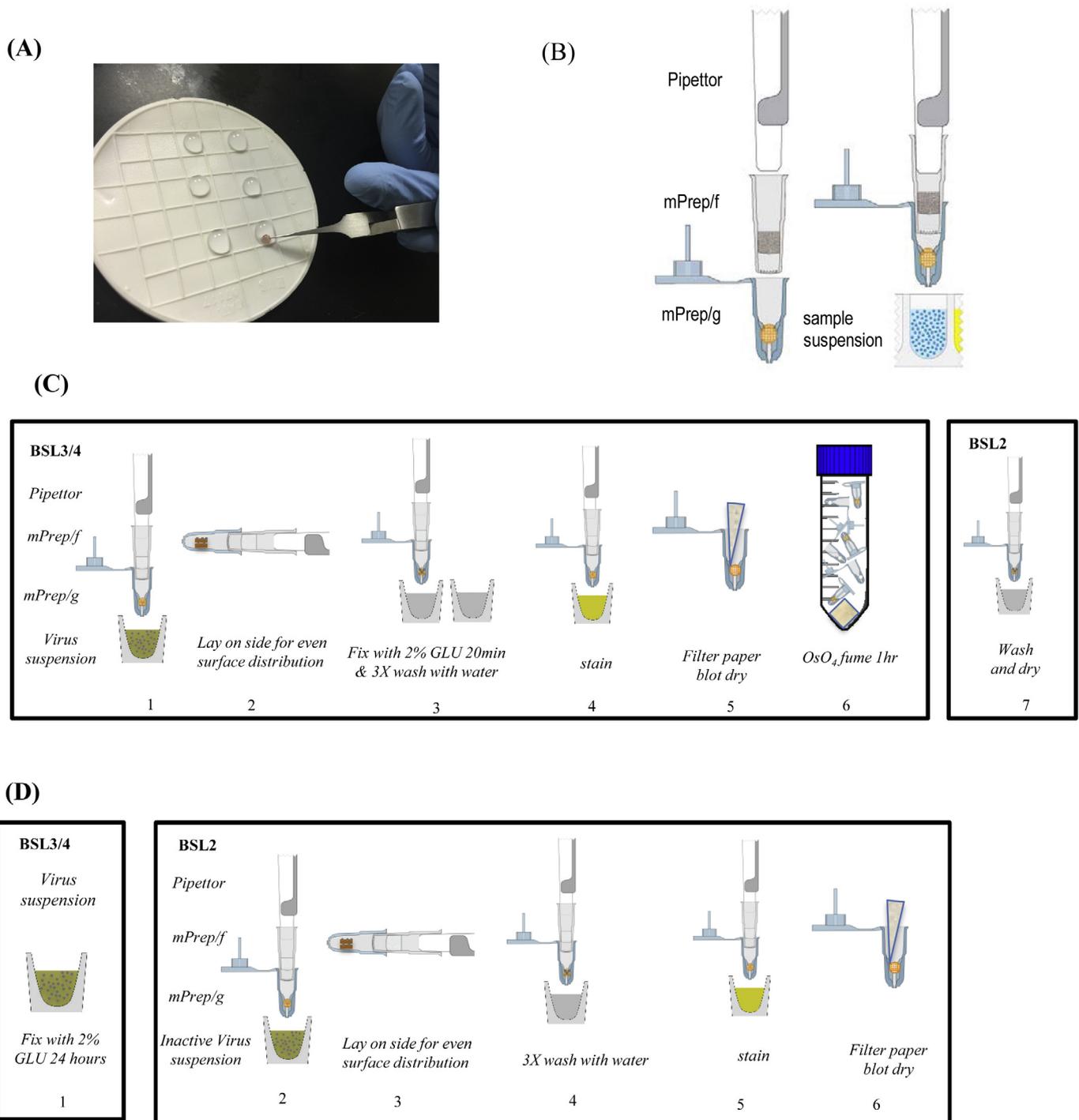


Fig. 1. Negative Staining Methods overview. (A) Manual droplet method of passing EM grids along droplets of reagents and stain. (B) Setting up mPrep/g capsule method with grids and application of sample suspension. (C) Typical procedure using mPrep/g capsule method in biocontainment with short-term inactivation with 1% osmium tetroxide vapor. (D) Recommended procedure using mPrep/g capsule method in biocontainment with long-term inactivation with 2% glutaraldehyde.

the grids. Step 6. The capsule, with the lid open, was then placed into a 50 ml centrifuge tube containing filter paper soaked in a 1% Osmium Tetroxide (OsO₄) solution. The centrifuge tube was sealed for 1 h for complete permeation of the OsO₄ vapor and subsequently decontaminated and transferred to the BSL-2 TEM facility. Step 7. In the BSL-2 TEM facility the mPrep/g capsule was removed from the centrifuge tube and placed onto a pipette. 40 μ l of dl water was aspirated into the capsule and dispensed into a waste container three times. The capsule was removed from the pipette and the grids blotted dry using filter paper touching to the edge of the

grids. After air drying, the grids were stored for subsequent TEM imaging.

2.3. MPrep/g capsule method for inactivation in biocontainment with 2% glutaraldehyde, followed by negative staining in a BSL-2 laboratory. (Fig. 1D)

Step 1, Inside the biocontainment laboratory BSC, the virus suspension was mixed well with the same volume of 4% glutaraldehyde to achieve a final concentration of 2% glutaraldehyde. Viruses

were inactivated with fixative for a minimum of 24 h before packaging, decontamination and transferred to the BSL-2 TEM facility. Step 2. In the BSL-2 TEM facility, the virus and fixative mixture was aspirated into the mPrep/g capsule, containing two TEM grids, attached to a pipette. Step 3. The pipette was placed horizontally for 10 min with the grids oriented in a similar horizontally arrangement to promote an even distribution of virus particles onto the TEM grids. Step 4. The pipette was picked up and the plunger was pressed to expel the virus to a waste container. 40 μ l of dI water was aspirated into the capsules and expelled into the waste container for 3 rinse cycles. Step 5. 40 μ l of stain (either 1% UA or 1% PTA) was aspirated into the capsules for 30 s (time varied from 10 s to 1 min based on virus sample). Step 6. The mPrep/g capsule was removed from the pipettor and the grids blotted dry by touching the edge of the grids to a piece of filter paper. The grids were air dried, and stored for subsequent TEM imaging.

2.4. TEM imaging

All TEM grids were evaluated on a JEOL 1011 transmission electron microscope at 80 kV and all digital images were acquired using an Advanced Microscopy Techniques (AMT) camera system.

3. Results

The goal of this study was to compare the manual droplet method for negative staining to the mPrep/g capsule method, especially the relevance to applications in biocontainment environments. Also, two related aspects of negative staining were also evaluated, 1) UA negative staining and PTA negative staining, and 2) the effect of virus inactivation on TEM image quality for 1 h with 1% osmium tetroxide vapor, compared to 24 h virus inactivation with 2% glutaraldehyde.

3.1. Compare and evaluate EM image quality by manual droplet method versus mPrep/g capsule method

First, we evaluated the imaging quality generated by both manual droplet and mPrep/g capsule methods using Zaire Ebolavirus. Ebolaviruses are members of the *Filoviridae* family, along with Marburg virus. In general, Ebolavirus is 80 nm in diameter and can be over 1000 nm long, making it a large virus. Ebolavirus must be worked with in a BSL-4 biocontainment environment. Fig. 2 shows that the manual droplet methods and the mPrep/g capsule negative staining method both have the ability to produce good quality TEM images. Fig. 2A (manual droplet method) and Fig. 2B (mPrep/g capsule method) show ebolavirus samples that have clearly defined details with nucleocapsid structures in the center of the virion, and visible ebolavirus glycoproteins on the surface.

3.2. Compare and evaluate EM image quality after rapid inactivation with aqueous glutaraldehyde and 1% osmium tetroxide vapor versus 24 h inactivation with 2% aqueous glutaraldehyde, using mPrep/g capsule method

We evaluated EM image quality after two different methods of inactivation using Chikungunya virus. Chikungunya virus is a member of the *Alphavirus* genus in the family *Togaviridae*. It is spherical with a diameter of 60–70 nm. The virion contains an envelope rich in glycoproteins which form trimeric spikes on the viral surface. Chikungunya virus must be handled in BSL-3 biocontainment environment. Rapid inactivation is achieved using 2% glutaraldehyde for 20 min followed by a one hour exposure to 1% osmium tetroxide vapor, with the entire negative staining process occurring in an mPrep/g capsule within a BSC in the biocontainment laboratory (Fig. 1C). However, when using 2% glutaraldehyde for

24 h to inactivate the virus, the inactivation occurs inside a biocontainment environment, but the 1% UA negative stain procedure is carried out using the mPrep/g capsule method in a BSL-2 laboratory (Fig. 1D). Both inactivation procedures don't produce the same quality images (Fig. 3). It is clear from Fig. 3 that fixation in glutaraldehyde without the presence of osmium tetroxide (Fig. 3A,B) show more ultrastructural detail than those of samples prepared with glutaraldehyde and osmium tetroxide (Fig. 3C,D).

3.3. Uranyl acetate (UA) vs phosphotungstic acid (PTA) as a negative stain for aldehyde fixed samples

Examples of UA and PTA negative staining are shown in Fig. 4 on aldehyde fixed virus-like-particles (VLPs). The VLPs are proteins assembled into virus like structures, but do not contain any viral genetic material. They are typically used in vaccine development and for basic viral research. Negative staining is a valuable tool to evaluate VLP assembly and morphology. We used both UA and PTA for negative staining of VLPs with the mPrep/g capsule method. Both stains display high quality results with glycoproteins visible and clearly defined borders of the Ebola nano-VLPs (Carra et al., 2015) (Fig. 4A,B) and Murine Leukemia VLPs (Rein, 2011) (Fig. 4C,D).

4. Discussion

Negative staining is a valuable technique for evaluating viruses, protein complexes and nano-particles. Single-droplet manual staining has been the classic protocol for more than half a century. It is a simple process, but depends largely on sample conditions and requires expertise gained through training for successful completion. There are variations between protocols including inactivation procedures, type of stains used, technician competency, and procedure that impact the negative stain quality. Excellent negative staining is still considered a state-of-the-art skill set and highly desired in many TEM labs.

The mPrep/g capsule method has distinct advantages over the manual droplet method, especially in biocontainment laboratories– the first being technical experience. The manual droplet method requires substantial training and experience before it can be performed successfully, compared to the easy to use mPrep/g capsule method that can be accomplished by entry level technicians with pipetting skills. The biggest challenge with the manual droplet method is the successful handling the TEM grids with forceps, which is made more difficult using BSL-3 and BSL-4 personal protective equipment. The TEM grids are fragile and can easily be damaged or punctured with the forceps. Any damage to the TEM grid is often not revealed until it is viewed with a TEM. The mPrep/g capsule encloses the TEM grids and protects them to eliminate direct handling with forceps in biocontainment. The only time the TEM grids are handled with forceps while using mPrep/g capsules is when they are inserted into and removed from the mPrep/g capsules. Since this is done outside the biocontainment laboratory, grid handling is much easier. One final advantage of the mPrep/g capsule method is that both sides of grid contain virus and stain. This can be useful when the sample concentration is low, but could be detrimental if the sample concentration is high.

Negative staining multiple grids using the manual droplet technique is very time consuming because each sample needs to be individually prepared and stained. This leads to difficulties obtaining consistent and reproducible staining. Keeping track of many individual grids is always a challenge. Each mPrep/g capsule holds two grids and multiple capsules can be attached to a multichannel pipette, streamlining the process. Therefore, mPrep/g capsules ensure all the grids are stained in the same process, same conditions, and same time. The capsules can also be easily labeled to aid

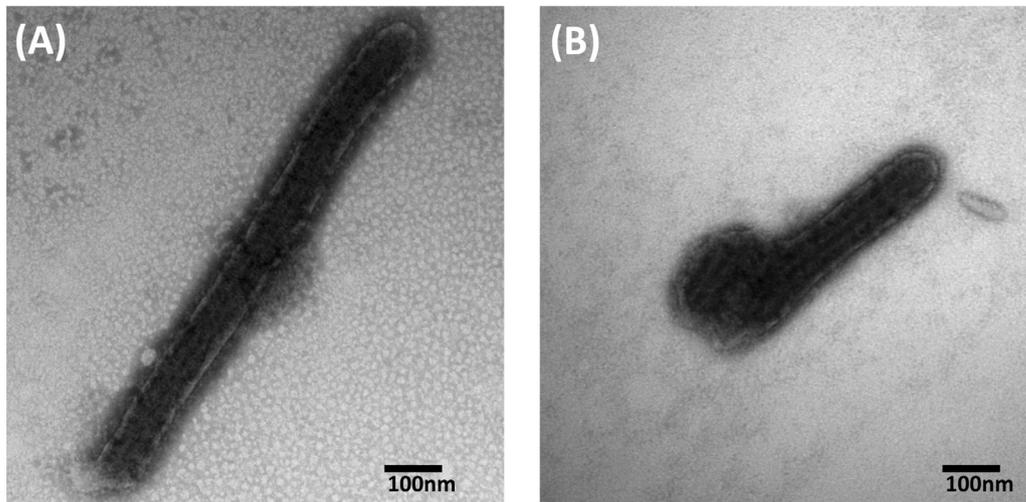


Fig. 2. Comparison of 1% PTA negative stained ebolavirus particles. (A) Negative stained with the manual droplet method. (B) Negative stained with the mPrep/g capsule method.

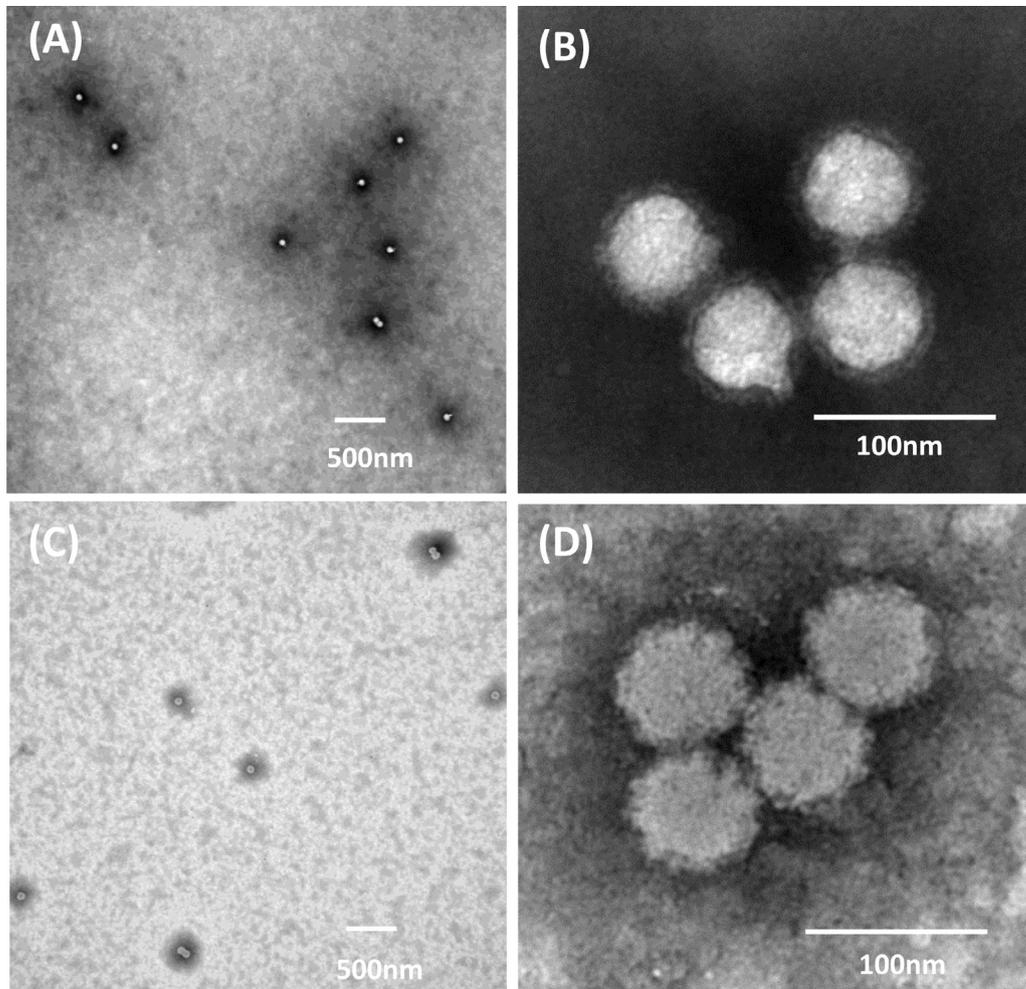


Fig. 3. 1% UA Negative Staining with mPrep/g capsule method of chikungunya virus using different inactivation procedures: (A, B) inactivation for a minimum of 24 h with 2% glutaraldehyde only. (C, D) Rapid inactivation with 2% glutaraldehyde and 1% osmium tetroxide (OsO₄) vapor.

in organization. The other common problem faced when using the manual droplet method is grid contamination. This can occur when the grids are left in the open air for too long or are dropped. The mPrep/g capsules protect the TEM grids from the open air and hold them securely so they are never dropped. mPrep/g capsules provide

greater experimental control and repeatability when preparing and negative staining because the mPrep/g capsule is a more controlled environment.

The biggest problem with using mPrep/g capsules is that TEM grids must be placed into the mPrep capsule manually using for-

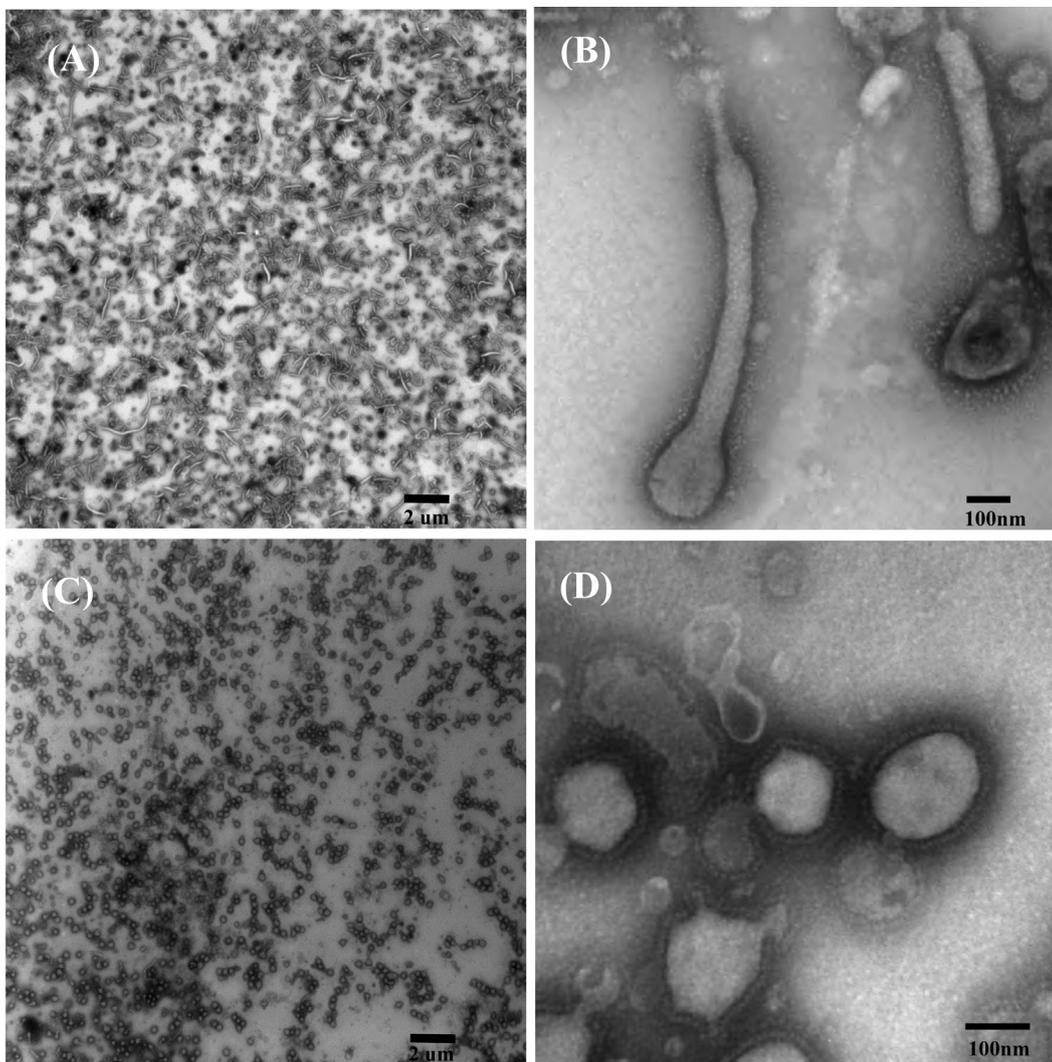


Fig. 4. Examples of Phosphotungstic acid (PTA) and Uranyl Acetate (UA) negatively stained Virus-Like-Particles (VLPs) using mPrep/g capsule method. (A) Low magnification overview of 1% PTA stained ebola nano-VLPs. (B) High magnification TEM image showing structural details of PTA stained ebola nano-VLPs. (C) Low magnification overview of 1% UA stained Murine Leukemia VLPs. (D) High magnification TEM image showing structural details of UA stained Murine Leukemia VLPs with Ebolavirus glycoprotein on their surface.

ceps. Occasionally, grids are bent or damaged. The mPrep/g capsule method also requires slightly more sample and stain than the manual droplet method. The mPrep/g capsule method requires at least 30 μ l of sample and stain per grid, compared to manual droplet method which can be done with as little as 8 μ l.

Virus inactivation is an important part of negative staining, especially when dealing with highly infectious viruses in biocontainment laboratories, as it allows the inactivated virus to be taken out of the biocontainment laboratories. Inactivation using fixatives has the added benefit of fixing the virus to prevent degradation. There are two different ways, used in this study, to inactivate the virus sample. The first involves 20 min in a 2% glutaraldehyde solution, followed by 1 h in a microfuge tube containing 1% osmium tetroxide vapors (Fig. 1C). This inactivation method has the advantage of saving time, as the sample can be taken out of biocontainment laboratory after 1 h and 20 min. However, 1% osmium tetroxide vapor reduces the quality of TEM images (Fig. 3). It was reported that a quick five minute exposure to osmium tetroxide vapor provides great results when negative staining, however longer exposure generated a mixture of positive and negative staining (Barland and Rojkind, 1966). The second inactivation method involves a minimum of 24 h in a 2% glutaraldehyde solu-

tion, with no osmium tetroxide vapor treatment (Fig. 1D). This second method takes longer to complete as the sample is not removed from the biocontainment laboratory for 24 h, but it has the advantage of preparation of negative staining outside of the biocontainment laboratory in a BSL-2 environment, and eliminates the involvement of hazardous osmium tetroxide. Our results demonstrate that 24 h of glutaraldehyde inactivation produces higher quality TEM images than when samples are treated with osmium tetroxide vapor (Fig. 3).

The two negative stains used in this experiment were UA and PTA. Both stains are used as a 1% solution. UA, the acetate salt of uranium, works well as a negative stain because of its dense uranium atoms (Kiselev et al., 1990). PTA, a heteropoly acid, works well as a negative stain because of its tungsten atoms (Kiselev et al., 1990). PTA is occasionally used instead of UA because it is much less toxic and only a mild irritant if inhaled or contacted. UA is highly toxic and mildly radioactive. When negative staining with unfixed samples, the lower pH of UA compared to PTA must be considered. If the pH of the stain doesn't match that of the sample, the sample could be damaged. The viruses used in this experiment require fixation and inactivation, so both UA and PTA can be used to achieve similar results. The results of our experiment show no distinct advantage

to using one stain over the other. Both stains are easy to work with and they both produce good quality TEM images (Fig. 4).

Overall, the mPrep/g capsule method is much easier to use in a biocontainment environment and can be used as an alternative to the manual droplet method. Using mPrep/g capsules saves time and effort while producing consistent high-quality TEM images comparable to what can be produced using the manual droplet method. Short virus inactivation with osmium tetroxide improves speed, but should only be used if reduced image quality is acceptable. Both UA and PTA provided similar results with the fixed virus samples tested in this study; however, results may be different when staining unfixed viruses. Sample quality is the most important factor for achieving high quality negative stained TEM virus images. However, there are other processing factors that can cause poor negative staining results, such as crystals formation in the stains or broken and damaged formvar films on the TEM grids.

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