Virus Specimen Preparation in Biosafety Level 3-4 Laboratories

mPrep™ System

Applications Note #506

Introduction

Viruses are examined with transmission electron microscopy (TEM) to screen for unknown disease-causing agents, evaluate structure, elucidate mechanisms, verify molecular biology assays, and enable diagnosis [1–3].

Viruses are commonly prepared for TEM by applying droplets of virus suspensions onto Formvar®-filmed TEM grids [4]. After virus attachment to the film surface, grids are rinsed, negatively stained with uranyl acetate [2, 3, 5], sometimes rinsed again, blotted, and then stored in a grid box until placed into the TEM. Each of these preparative steps requires the use of fine-tipped forceps to pick up delicate grids and move them between successive reagents and storage containers. While it can be challenging to handle grids without dropping or damaging them in standard TEM labs, the difficulty increases considerably when highly pathogenic viruses are involved because preparation must take place in a level 3 or 4 Biosafety Laboratory (BSL3/4). In a BSL3/4 lab, positive pressure suits with restrictive facemasks make it difficult to accurately see grids, while double layer gloves reduce the tactile sensitivity and dexterity needed for grid handling. The sharp-tipped forceps used for grid handling also pose a puncture risk for the protective suits and gloves. Finally, since TEMs are usually outside BSL3/4 suites, the grids must also be treated with decontamination agents such as fixatives before analysis.

This application note demonstrates an easy method for preparing pathogenic viruses for TEM within BSL suites and standard EM labs. With this method the individual TEM grids are not directly handled inside the BSL suite. Instead, a single-or multi-channel pipettor is used to deliver reagents to one or simultaneously to dozens of grids held inside mPrep/g™ capsules. This eliminates the puncture risk from forceps in the BSL3/4 suite, reduces the amount of effort required, and improves the reproducibility. This also makes this methodology equally advantageous for preparation of non-pathogenic viruses.

Please note that the mPrep/g capsule processing method can be modified for studies with different types of viruses and different preparations, as described in the Protocol Variations section below.

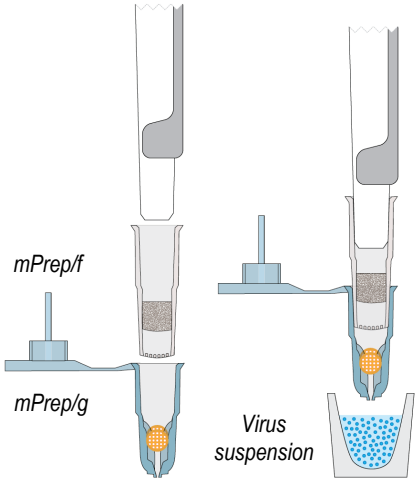

Figure 1: Set up mPrep/g capsules with grids and apply viruses. A single mPrep/g capsule containing two grids is attached to a single-channel pipettor via an mPrep/f filter coupler. In practice, identification labels are attached to capsules, and multichannel pipettors can be used to process several grids.

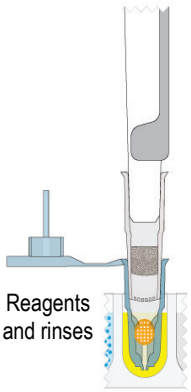

Figure 2: Transfer viruses and reagents into mPrep/g capsules by pipetting from reagent vessel. Rest the ends of the capsule(s) in the bottom of microplate wells or reagent reservoirs. Aspirate virus suspensions and reagents into mPrep/g capsules. Use a lab stand (not shown) to support pipettor vertically.

Sample Preparation

Advance Preparation in the EM Lab

1. Formvar-filmed 400 mesh Cu grids were prepared using standard methods [4].
2. Using forceps, two Formvar-filmed grids were inserted into each labeled mPrep/g capsule.

MICROSCOPY INNOVATIONS LLC
213 Air Park Rd Suite 101
Marshfield WI 54449-8626
888.302.3925 or 715.384.3292
info@microscopyinnovations.com

3. Reagents, including glutaraldehyde fixative, osmium tetroxide (OsO_4), and uranyl acetate stain, were prepared in the EM lab.
4. These following items were transferred to the BSL3/4 suite:
    a. mPrep/g capsules loaded with Formvar-filmed grids
    b. reagents
    c. Pipetman Neo® 8-channel P200 pipettor (Gilson)
    d. mPrep/f™13 Extreme filter couplers
    e. 96-well low adhesion polypropylene microplates
    f. reagent reservoirs
    g. 50 ml centrifuge tubes or other suitable sealable containers.

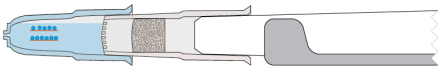

Figure 3: Sediment viruses onto filmed grids. Setting mPrep/g capsules horizontally can enhance sedimentation of certain viruses onto filmed grids. Viruses can also be adsorbed onto filmed grids with the pipettor and grids held vertically.

Virus Preparation in the BSL Suite

1. The mPrep/g capsules loaded with Formvar-filmed grids were attached to the 8-channel pipettor using mPrep/f filter couplers on each pipettor channel (Figure 1).
2. Virus suspensions were prepared in the BSL suite and then transferred into individual microplate wells or reagent reservoirs.
3. In the study shown here, the volume of the pipettor was set to 50 µl. The virus suspension was then aspirated into the mPrep/g capsules and the pipettor was laid on its side for 10 minutes with grids oriented so viruses could sediment onto films (Figure 3). Alternately, this step can be done with the pipettor in a vertical position (Figure 2).
   Note: This step can be done various ways depending on the virus and its properties as discussed below in Protocol Variations.
4. The pipettor was picked up and the plunger was pressed to dispense the virus to waste (Figure 2).
5. Glutaraldehyde fixative was aspirated into capsules, incubated for 10 minutes, and then dispensed to waste (Figure 2).
6. The deionized (dI) water wash was then aspirated into capsules and dispensed to waste in 3 rinse cycles (Figure 2).
7. The mPrep/g capsules were removed from the pipettor and placed into a 50 ml centrifuge tube containing filter paper soaked in 1% OsO_4 solution (Figure 4). The tube was sealed for over 1 hour to decontaminate virus with OsO_4 vapor. The sealed tube was then transferred to the EM lab for final preparation.

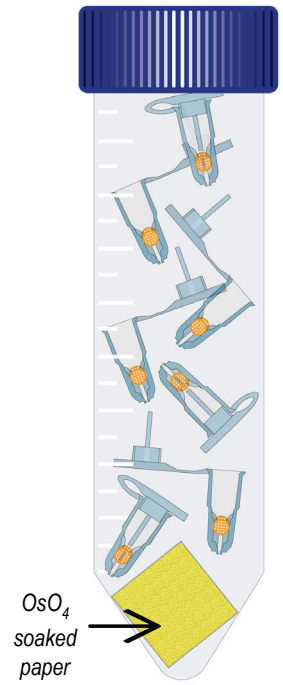

Figure 4: Decontaminate and transfer to vessel. Placing virus-loaded grid-containing capsules into a vessel filled with osmium tetroxide vapor can be used to decontaminate viruses. Capping the vessel allows safe transport of the grid-containing capsules into the EM lab.

Final Preparation in the EM Laboratory

1. The container with the mPrep/g capsules was opened in the fume hood.
2. The mPrep/g capsules with viral grid specimens were attached to the 8-channel pipettor, using new mPrep/f filter couplers on each pipettor channel (Figure 5).
3. The volume of the pipettor was set to 40 µl, and the grids were washed 3x with dI water (Figure 5).
4. Grids were negatively stained with uranyl acetate by aspirating 40 µl stain, holding for 10 seconds (or up to 1 minute, if needed), and then dispensing to waste.

5. The mPrep/g capsules were removed from the pipettor and then the grids inside the mPrep/g capsules were blotted dry by touching a wedge of filter paper to where the grids contact the slots inside mPrep/g capsules (Figure 6).
6. Grids were removed from the mPrep/g capsule and examined with TEM.

Results and Discussion

Virus preparation with mPrep/g capsules produced excellent TEM images. Figure 7 shows an example of Ebola virus prepared in a BSL4 containment facility and Figure 8 shows an example of a quantitative study with virus-like particles (VLPs) prepared in BSL2 conditions. Compared against previous droplet methods for sample preparation, preparation of both the Ebola and VLP specimens required much less effort and provided greater consistency:

- Preparation throughput increased dramatically. Droplet methods for grid preparation in BSL4 conditions typically produced about 20% usable grids. By contrast, mPrep/g processing achieved well-prepared grids nearly 100% of the time.
- Grid preparation was much easier and safer. Forceps handling of grids was eliminated in the BSL suite.
- Multiple grids were prepared identically. Use of the 8-channel pipettor allowed viruses and reagents to be processed simultaneously in multiple mPrep/g capsules.
- Handling time to prepare grids was much less. During adsorption and reagent treatments, lab personnel were free to perform other tasks.

Protocol Variations

The sample preparation protocol can be readily adapted for various types of viruses, for different concentrations of viruses, or to perform other TEM virus studies such as immuno-labeling.

Sedimentation of viruses: Virus suspensions may sediment at different rates. If the viruses are not in suspension near the grid, they will not adsorb or attach to the filmed grid. Several methods can be used to control the surface concentration of adsorbed viruses on the grids:

- For viruses that readily stay in suspension, it is easy to keep the pipettor in a vertical position for the desired adsorption time (Figure 2). This also reduces the volume needed for all processing steps for each capsule to 35–40 µl. Note that it may be necessary to vary the adsorption time from the 10 minutes used above.
- For viruses that sediment rapidly, the pipettor can be laid on its side after aspiration of the suspension with grids oriented horizontally so the viruses will deposit and adsorb on the Formvar film (Figure 3). For this method, the pipettor volume needs to be about 50 µl to ensure that both grids in the capsule are covered with reagent when the pipettor is laid on its side. This method was used to generate the results shown here.

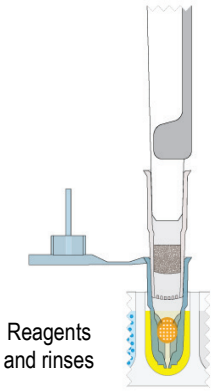

Figure 5: Deliver stains and labels to the grids in the EM lab. Rest the capsule end in the microwells or reagent reservoirs to the rinses, uranyl acetate, and any other stains or labels into the mPrep/g capsules.

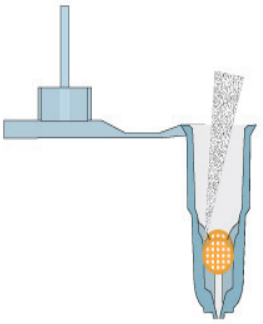

Figure 6: Blotting grids. Grids are blotted to remove negative stain and to accelerate drying. Insert a filter paper wedge or other absorbent material into the mPrep/g capsules and touch where the grids are held in the capsule slots.

- For viruses where the extent of sedimentation is unknown, it is recommended to keep the pipettor vertical in the microplate well and aspirate and dispense the suspension in and out of the mPrep/g capsule(s) every 10–30 seconds to agitate and keep all viruses suspended. The volume setting for the pipettor should be 35–40 µl for all steps. This method helps provide a representative sample of the virus preparation when there are mixtures of viruses or aggregates that sediment at different rates.

Enhance virus concentration on the grids: Filmed grids may be treated to enhance nanoparticle adsorption by aspirating appropriate reagents into the mPrep/g capsule to treat the film. For example, agents (such as poly-l-lysine or specific binding ligands or antibodies) can be coated onto the filmed grid by aspirating the agents into the capsule and rinsing prior to introducing the virus suspension.

Staining and labeling: Negative stains other than uranyl acetate may be used [6]. Immunolabeling reagents, positive stains, and other fluid reagents can be easily delivered to the grids and viruses by aspiration into the mPrep/g capsules.

Pre-fixing viruses: It is possible to pre-fix the viruses in glutaraldehyde prior to applying them to the grids. This can enable grid preparation in the EM lab and thus outside the BSL3/4 containment facility.

Increasing the number of grids prepared: The method is easily extended to simultaneously prepare many grids, by inserting 1 or 2 grids per capsule, stacking up to 4 capsules per channel, and/or using multichannel pipettors (Table 1 and Figure 9). When using a multi-channel pipettor, different reagents can be arrayed into different microplate wells to simultaneously prepare different grids with different protocols. This can also be used for procedures such as immuno-labeling where it desirable to prepare titrations and controls.

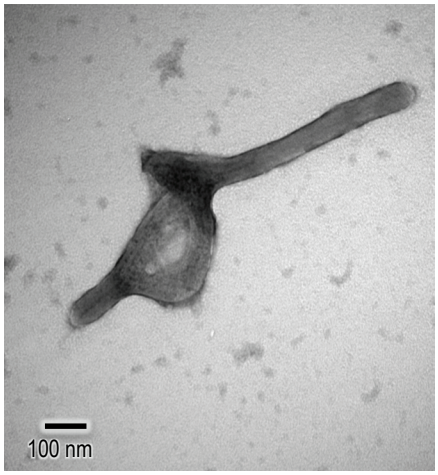

Figure 7: Ebola virus. Prepared in BSL4 conditions.

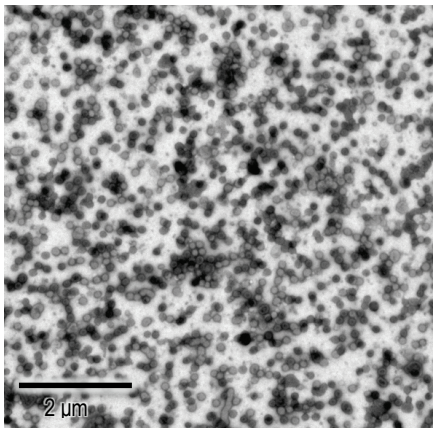

Figure 8: Virus-like particles. Prepared in BSL2 conditions.

Table 1: Preparing multiple grids with mPrep/g capsules

Use	To Prepare	Maximum Capacity per Pipettor
single channel pipettor	1–2 grids in 1 capsule	up to 8 grids in one 4-capsule stack
8-channel pipettor	8–16 grids in 8 capsules	up to 64 grids in eight 4-capsule stacks
12-channel pipettor	12–24 grids in 12 capsules	up to 96 grids in twelve 4-capsule stacks

Conclusions

The methods described here can simplify the preparation of pathogenic virus specimens within BSL3/4 labs. By reducing grid handling and enabling simultaneous identical preparation of multiple samples, mPrep/g processing provides greater reproducibility to improve research quality. These methods can be extended to the preparation of non-pathogenic and inactivated viruses, providing the same advantages even when bio-containment is not required.

References

1. Curry A, Appleton H, Dowsett B (2006) Application of transmission electron microscopy to the clinical study of viral and bacterial infections: present and future. *Micron*: 37(2): 91-106. Epub 2005 Nov 2.
2. Goldsmith CS, Miller SE (2009) Modern Uses of Electron Microscopy for Detection of Viruses. *Clinical Microbiology Reviews*, Oct. 2009, p. 552–563.
3. Laue M (2010) Electron microscopy of viruses. *Methods Cell Biol*: 96:1-20. doi: 10.1016/S0091-679X(10)96001-9.
4. Bozzola JJ, Russell LD (1999) *Electron Microscopy*, 2nd Ed, Jones and Bartlett. Boston.
5. Brenner S, Horne RW (1959) Negative staining method for high resolution electron microscopy of viruses. *Biochim Biophys Acta*: 34:103–10.
6. Benmeradi N, Payre B, Goodman SL (2015) Easier and Safer Biological Staining: High Contrast UranyLess Staining of TEM Grids using mPrep/g Capsules. *Microsc Microanal:* 21 (suppl 3) 2015, 721.

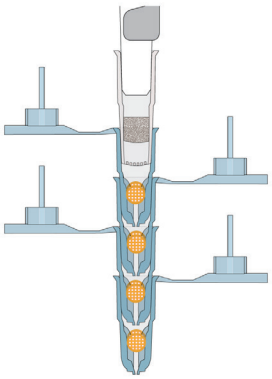

Figure 10: Stacking capsules to simultaneously process multiple grids. Inserting 1 or 2 grids per capsule and stacking up to 4 capsules per channel of the pipettor enables processing of many grids.

Acknowledgements

We gratefully acknowledge Mr. Mitchell Monninger, Ms. Candace Blancett and SPC Chrystal Nguessan from Dr. Mei Sun's laboratory at the US Army Medical Research Institute of Infectious Diseases (US AMRIID, Pathology Division, Ft. Detrick, MD) for performing this study and for providing permission to share this work.

Safety Disclaimer

MICROSCOPY INNOVATIONS DOES NOT GUARANTEE THAT THIS PROTOCOL WILL RESULT IN THE COMPLETE DECONTAMINATION OF ALL VIRUSES. Follow all appropriate and necessary safety protocols for handling pathogenic viruses, including the United States Occupational Safety and Health Administration (OSHA) Bloodborne Pathogens Standard (29 CFR § 1910.1030). Users should also follow all appropriate safety guidelines for handling toxic reagents such as glutaraldehyde, uranyl acetate and osmium tetroxide.

Ordering Information

Product #	Item Description/Catalog Information
G1600	16 mPrep/g capsules & 16 label sets in capsule/grid storage box
F1602	16 mPrep/f13 Extreme filter couplers with 13 µm filter in capsule/grid storage box
PL96PP500	Non-sterile, PP, 96-well microwell plates, 500 µl, 10/sleeve
R15-50HDPE	Q300-1550 - 15ml Reagent Reservoir, non-sterile, HDPE, 50/PK
KIT_xxx	Starter kits with mPrep capsules and accessories including Gilson Pipetman Neo® pipettor (various custom kits available — please inquire)

Microscopy Innovations LLC 213 Air Park Rd Suite 101, Marshfield WI 54449 | **T** 715-384-3292 **F** 888-302-3925 | www.microscopyinnovations.com