Comparing ASTM F1671 with a Modified Dot-Blot Method to Evaluate Personal Protective Materials

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Abstract
Effective personal protective equipment (PPE) is critical in preventing the spread of infectious diseases. Appropriate test systems and test soils are needed to adequately evaluate PPE. ASTM test method F903, which specifies the test method setup also used in ASTM F1670 and F1671, has been used for decades to test liquid (ASTM F1670) or viral (ASTM F1671) penetration resistance of PPE fabrics. However, an alteration of the bacteriophage propagation method detailed in the standard was necessary to obtain consistent titers of virus. In this study, modification of the nutrient broth provided consistently higher titers of virus and the use of the top agar in smaller increments prevented premature solidification. This study then compared the standard ASTM F1671 (using bacteriophage \( \phi_{174} \)) with a modified dot-blot method to assess viral penetration of PPE materials. The results indicated that ASTM F1671 and the dot-blot apparatus methods were equivalent. The dot-blot method described here is less labor intensive and faster than the ASTM F1671 method. However, using the dot-blot system, which uses antibodies to detect the bacteriophage and signal amplification, does not indicate if virus viability or infectivity is retained, whereas the ASTM F1671 method indicates both. Nonetheless, the method presented in this investigation is a substantial improvement of a standard method for viral challenge testing of PPE materials.

Although several standards related to PPE testing exist, many were developed years ago. Problems with these standards have surfaced, including the inability for the test soil to maintain the specified surface tension and use of a screen to improve the passing rate of a PPE material.

ANSI/AAMI PB70:2012 categorizes gowns and drapes based on barrier performance and specifies the testing required for each level of performance. Barrier performance is divided into four levels (Level 1, minimal risk situations; Level 2, low risk; Level 3, moderate risk; and Level 4, high risk). Levels 1 through 3 have different water impact and hydrostatic testing requirements, whereas Level 4 requires more stringent fluid and viral penetration testing conditions.

To ensure that healthcare workers and the general public are protected in cases of a deadly viral outbreak, the standard method ASTM F1671 was evaluated for Level 4 gowns using a novel, modified dot-blot method with Level 3 and 4 PPE materials using bacteriophage \( \phi_{174} \) as the biological challenge agent. The bacteriophage \( \phi_{174} \) is a standard viral surrogate used for bloodborne pathogens such as hepatitis B and C viruses and human immunodeficiency virus. As reported previously, in addition to being as effective as the ASTM 1671 method for demonstrating barrier performance of materials, the dot-blot apparatus method has several advantages: It is less costly, uses less sample volume, has no screen effect, and is easier to use. A disadvantage of the smaller dot-blot apparatus is that the surface area of the material tested is smaller.

It is critical that personal protective equipment (PPE) used to protect healthcare workers is safe and effective and meets labeled performance claims for impermeability to deadly viruses such as Ebola and influenza. Preventing healthcare worker exposure is a crucial factor in containing highly contagious infectious diseases.
patients are being transported. The testing pressure specified by many of the PPE standards is 2 psi, whereas maximum pressures exerted on PPE materials for some clinical procedures are reported to range from 10.9 to 60 psi. In addition, clinically relevant artificial test soils (synthetic blood and vomit), in conjunction with microbes, can more accurately simulate stresses on PPE materials. Previous work with the dot-blot method has demonstrated that Level 4 materials fail at higher (10 psi) pressures and with more caustic (vomit) test soils.

With this in mind, a comparison of the dot-blot apparatus and the ASTM apparatus methods was conducted using bacteriophage \( \phi \chi \) in a blood and vomit test soil on a series of Level 3 and 4 PPE materials.

**Materials and Methods**

**Materials**

Bovine serum albumin (BSA); Tween 20; 3,3',5,5'-tetramethylbenzidine (TMB)-enhanced, one-component horse radish peroxidase (HRP) membrane substrate; and secondary antibody goat anti-rabbit immunoglobulin G (IgG) HRP were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Phosphate-buffered saline (PBS) was purchased from Thermo Fisher Scientific (Rockford, IL). Nitrocellulose membranes and blotting paper were purchased from Bio-Rad (Hercules, CA). PBS-T is 0.05% Tween 20 in PBS. *Escherichia coli C* (ATCC 13706) and bacteriophage \( \phi \chi \) (ATCC 13706-B1) were obtained from ATCC (Manassas, VA). Nutrient broth (NB) and nutrient agar (NA) were purchased from Becton Dickenson (Franklin Lakes, NJ). Gowns, drapes and one hospital curtain with different labeled levels of protection were obtained from commercially available sources.

As the purpose of this study was to compare the performance of test apparatus and procedures, rather than to evaluate different PPE materials, the materials and manufacturers are not mentioned. The samples used in this study are identical to those noted in previous works. In addition, the blood test soil and the vomit test soil have also been detailed in those previous studies.

\( \phi \chi \) was propagated on a lawn of permissive *E. coli C* on plates with NA for the bottom layer and agar with NB on top. To produce \( \phi \chi \) for antibody production, *E. coli C* was added to 125 mL NB and grown to an optical density of 0.26 at 600 nm. Next, *E. coli C* bacteria were inoculated with \( \phi \chi \) and incubated for 4 hours. The culture was clarified by spinning at 10,000 \( \times \) g in a Sorval ST 16 centrifuge. The supernatant was retrieved, and the filter sterilized using a 0.22-µm pore size membrane filter (Millipore). \( \phi \chi \) was purified by centrifugation on a 20% sucrose cushion prepared in PBS and filter sterilized with a 0.22-µm filter. A total of 4.5 mL bacteriophages was overlaid on 500 µL sucrose cushion. The \( \phi \chi \) was spun at 118,000 \( \times \) g in an MLS 50 swinging bucket rotor for 2.5 hours at 4°C in Beckman Optima MAX-XP Benchtop Ultracentrifuge. The pellet was resuspended in 200 µL cold PBS. The protein concentration was assessed by using Lowry reagent (Bio-Rad, Richmond, CA) on a 96-well plate format (Molecular Devices, San Jose, CA).

Two rabbits were immunized and boosted with purified \( \phi \chi \) using Freund's complete and incomplete adjuvant, respectively. The rabbits were bled, and the sera was affinity purified on day 70. Reactivity to \( \phi \chi \) was confirmed by enzyme-linked immunosorbent assay. The antibodies were produced by Thermo Fisher.

**Methods**

**ASTM F1671.** *E. coli C* bacteria were grown in bacteriophage NB (containing Polysorbate 80 at a final concentration of 0.01%) at 37°C with shaking (250 rpm in an incubator shaker). The bacterial culture was inoculated with a starter culture of \( \phi \chi \) to generate a fresh stock of the challenge agent postlysis of the bacterial cells. NA plates were used with NB top agar containing 7 g/L Bacto-agar to determine the titer of the generated \( \phi \chi \) bacteriophage stock.

The penetration cell was filled with 60 mL of either blood or vomit artificial test soil with the above generated \( \phi \chi \) bacteriophage challenge suspension (2.5 \( \times \) 10\(^9\) pfu/mL), and the apparatus was observed for any leaks for 5 minutes. Next, the
system was pressurized to 2 psi for 1 minute, then the pressure was turned off and the apparatus was monitored for visual leaks. If liquid penetration was not visible at this point, the specimen was observed again after an additional 54 minutes. At the conclusion of the test, the observed side of the test material was rinsed with assay fluid (5 mL sterile NB) to ensure proper contact of the fluid with the entire viewing surface of the test specimen. The penetration cell was swirled for approximately 1 minute, after which the assay fluid was removed and assayed for the presence of \( \phi\chi_{174} \). The vomit test soil rendered the \( \phi\chi_{174} \) nonviable for the 60 minutes required by the standard test cycle. Some test were run with the bacteriophage in vomit, which survived a 1-minute duration, but this would not be comparable with previous work or represent a worst-case clinically relevant exposure.

A total of 500 µL collected assay fluid was used for the plaque assay. Then, 200 µL of a log-phase culture of E. coli C and 2.5 mL molten top agar was added to the assay fluid and poured it onto bottom agar plates for each experimental setup. The plates were incubated inverted overnight at 37°C and observed for plaque-forming unit (pfu) enumeration the next day. All experiments were repeated in triplicate. Presence of plaques indicated the penetration of the viral surrogate even if liquid penetration was not visible. For a test sample to “pass,” all three plates would be negative for the presence of plaques. The microbiological assays were done inside a biosafety cabinet (BSC) following aseptic techniques. Appropriate negative controls per experiment were included in the study design to rule out contamination during the procedural steps. The procedure is depicted in Figure 1. The test cell was disassembled and disinfected with 1:10 diluted household bleach (final concentration 0.825% sodium hypochlorite) prior to each reuse.

**Dot-blot method.** An acrylic 96-well plate (Minifold I Spot-Blot; Whatman Schleicher & Schuell, now part of MilliporeSigma, St Louis MO; Figure 2), previously used in other studies, was modified. The filtration plate holes, initially around 1 mm diameter, were drilled out to 5 mm, which is slightly smaller than corresponding holes on the 96-well plate (Figure 2). This was done to allow a larger area of the PPE material to be under vacuum. A vacuum gauge (EN 837-1; WIKA Instruments, Lawrenceville, GA), was used to establish and monitor the pressure on the fabric in this test setup. Prewetted nitrocellulose was placed on blotting paper, and the PPE sample was placed on top of the nitrocellulose membrane with the inner-facing material in contact with the nitrocellulose.

![Figure 1. Plaque assay to quantify penetration of viral particles through personal protective equipment](image-url)
The sample plate then was clamped on the PPE/nitrocellulose/blotting paper construct. Approximately 100 µL of 1 × 10⁸ pfu/mL of φX174 bacteriophage was added to the test wells of the sample plate. After resting at ambient pressure for 5 minutes, a vacuum was applied to the dot-blot apparatus (Figure 2) for 1 minute (2 psi = 0.14 bar). The vacuum was discontinued, and the remaining liquid was left in the apparatus at ambient pressure for 54 minutes.

Next, all remaining liquid was removed from sample wells using a multichannel pipette without disassembling the apparatus. The apparatus then was disassembled, and the nitrocellulose membrane was placed in a petri dish. The membrane was washed twice with PBS-T (rocked 5 minutes per wash), blocked with 5% BSA for 60 minutes with rocking, then washed three times with PBS-T (rocked 5 minutes per wash).

The primary antibody (1:2000 in PBS-T) then was added for 60 minutes, followed by washing three times with PBS-T. The goat anti-rabbit IgG HRP secondary antibody (1:5,000 in PBS-T) was added, followed by 60 minutes of incubation with rocking, washing twice with PBS-T, and a final wash with PBS only (rocked 5 minutes per wash). TMB-enhanced, one-component HRP membrane substrate turned blue in the areas in which antibody binding occurred, indicating that bacteriophage had penetrated through the PPE to the nitrocellulose membrane. Figure 3 illustrates a positive (failure), as the bacteriophage had penetrated the material. The blue color indicates antibody binding to the bacteriophage proteins. Cleaning the dot blot with bleach was not possible, as the rubber O-rings in the bottom of the sample well would disintegrate and isopropanol was not effective.

Following use, the apparatus was sprayed with 70% ethanol, washed in soapy warm water, rinsed well with deionized water, and allowed to dry overnight. By itself, 70% ethanol was not sufficient to neutralize the virus, and water temperatures and soap vary; therefore, individual testing of viral neutralization is recommended.

Demonstration and use of both the ASTM F1671 method and the dot blot are available at numerous sites online.

Results and Discussion

The initial challenge in evaluating and comparing the ASTM F1671 and dot-blot methods involved propagating the bacteriophage consistently to a sufficiently high titer. Careful examination of the steps and ingredients used to propagate the bacteriophage indicated some room for improvement. The standard ASTM F1671 requires addition of a surfactant so that the surface tension of the material is comparable with that of blood. If a concentrated stock of bacteriophage is to be diluted in a relevant test soil (e.g., blood, vomit), as performed in this study, adding surfactant may not be necessary. The addition of salts to NB, as required by the standard, was not explained. Therefore, comparison of bacteriophage growth (in pfu/mL) was conducted in (1) NB (phage broth without surfactant or extra salts); (2) phage broth (standard defined); (3) phage broth without salts, with surfactant; and (4) phage broth without surfactant, with salts.

The results of these experiments are shown in Figure 4. NB and phage broth appeared to be equivalent for bacteriophage growth, and making NB is much simpler than making phage broth, especially given
the volume needed for each test (~60 mL). A titer of $10^6$ was sufficiently high to perform the test procedure ($2 \pm 1 \times 10^8$ pfu/mL). If increased titer is desired, propagation in broth with just the added salts (and no surfactant) appears to be ideal.

The standard directs the molten top agar to be held at 45°C until use. The top agar was found to be solidified at this temperature, even when held at 60°C. To address this issue, the (pre aliquoted) top agar was melted in the microwave for 10 seconds, then vortexed for 5 seconds, followed by again microwaving for 5 seconds and vortexing for 5 seconds. This prevented overheating and boiling over of the top agar. After this, the solution was kept in a BSC with intermittent vortexing until the top agar was reached approximately 45°C and then used promptly. Critically, if too much time was taken or too many batches prepared (e.g., eight), gelation would occur in the last couple tubes of top agar.

Table 1 shows a comparison of PPE penetration testing of the ASTM F1671 method with that of the dot-blot method using bacteriophage in blood test soil. Each replicate for the ASTM method represents triplicate samples measured in triplicate, and the data are shown in pfu/mL. For the dot-blot method, triplicate samples were run, and the data indicate the average number of wells (96 per test sample) that tested positive for bacteriophage presence. This is a pass/fail test. Three separate tests are recommended, and any failure of any replicate

<table>
<thead>
<tr>
<th>Broth Type</th>
<th>OD₆₀₀</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0905</td>
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<td>Phage broth -surf, -salt</td>
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<td>Phage broth (+surf, +salt)</td>
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</table>

Figure 3. Example of bacteriophage penetration of a material. The blue color indicates antibody binding to the bacteriophage proteins.

Figure 4. The influence of different broth types on the titer of bacteriophage obtained (in pfu/mL). When the bacteriophage is added to Escherichia Coli C, the optical density (600 nm) will stop increasing when lysis of the bacteria is complete. At that point, the bacteriophage is plated to determine pfu/mL.
means the test material is designated as a failure. However, the ANSI/AAMI PB70 has a rejectable quality level (RQL) of 20% and an acceptable quality level (AQL) of 4%.8 To reliably obtain a statistically sound RQL and AQL, a much larger sample size than three tests for the ASTM F16713 method is needed. The two methods performed comparably; however, larger numbers of samples and participation of other laboratories would be needed to confirm that they are statistically the same.

When the identical series of tests were run at 10 psi only for those samples that had no failures at 2 psi, only one sample passed: gown 1. Given that the pressures, stresses, and strains on gown material in some surgeries exceed 10 psi,12,13 testing at elevated pressures might be worth considering. One concern in using the dot-blot method is that the signal is based on the antibody binding to bacteriophage antigens. This can occur regardless of whether the virus is viable. The ASTM method only generates positive response if the virus is viable and able to infect its host E. coli C. However, the use of an antibody detection system provides penetration results the day of the test, whereas the ASTM method requires at least one additional day.

Next, this methodology was applied using the vomit test soil; however, it rendered φχ174 nonviable during the 60-minute standard testing time. Some experiments were run with a 1-minute exposure period, which the bacteriophage survived. These results would not be comparable with tests runs with other test soils or organisms. A more resilient strain of bacteriophage might be more appropriate for these harsh conditions, or in lieu of a live organism, a tagged bead the size of a small virus could be used. This illustrates the need to examine the clinical and real use of PPE materials to better construct test methods to evaluate them.

Work done at the National Institute for Occupational Safety and Health (NIOSH) may make this a moot comparison.14 The NIOSH study detailed the penetration of different viruses (φχ174 and MS2) in a liquid (NB solution) using a fluorescent dye to enhance visual detection. The results imply that a small difference (0.29 minutes) may exist between the penetration of viruses versus the liquid, suggesting that the segregation of F1670 and F1671 may be unnecessary. Again, this type of system will need further testing prior to concluding with certainty that this method is applicable to many types of viruses and test soils. Also, testing with several more types of PPE materials with a much higher number of samples and the participation of several different laboratories would be needed to incorporate this testing approach into a standard.

### Table 1. Comparison of personal protective equipment penetration testing of the ASTM F1671 method versus the dot-blot method using bacteriophage in blood test soil.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gown</th>
<th>Level</th>
<th>ASTM F1671 Test Cell</th>
<th>Dot Blot</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gown 1</td>
<td>4</td>
<td>0 0 0</td>
<td>0 0</td>
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<tr>
<td>3</td>
<td>Gown 3</td>
<td>4</td>
<td>TNTC TNTC 2.3</td>
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<td>4</td>
<td>TNTC 63 TNTC</td>
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<td>Gown 2</td>
<td>3</td>
<td>TNTC 0 TNTC 52.3</td>
<td>7.7</td>
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<tr>
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<td>Gown 4</td>
<td>3</td>
<td>1 TNTC 0</td>
<td>0 0</td>
</tr>
<tr>
<td>9</td>
<td>Drape 1</td>
<td>NA</td>
<td>TNTC TNTC 0.0</td>
<td>8.7</td>
</tr>
</tbody>
</table>

*Abbreviations used: NA, not available; TNTC, too numerous to count.*
Conclusion
The results from this study indicated that additional optimization is needed for the bacteriophage propagation protocol for ASTM F1671, especially in simplifying the growth medium. No difference was observed in phage titer after propagation in NB, as compared with phage broth; the addition of salts/surfactant can be done prior to use, as needed for testing parameters; and use of NA for bottom agar is simple and adequate.

In addition, alternate methods, such as using a modified dot-blot apparatus, may be worth considering. The modified dot-blot method is less labor intensive and faster, and the results it provides are comparable with those of the ASTM F1671 method. However, the dot-blot system described here detects bacteriophage proteins and may or may not indicate penetration of the whole bacteriophage, whereas in the ASTM F1671 method, only viable phage is detected. A good correlation between colony-forming units (CFUs) and individual bacterial cell counts, via techniques such as flow cytometry, has not, to our knowledge, been reliably demonstrated. Therefore correlation of CFUs to bacterial proteins would also be suspect. A simple pass-fail criterion is currently our best approach in comparing these two methods. Other methods may prove to be superior to both the standard ASTM F1671 method and the dot-blot system used in this study.

Funding
This project was supported in part by an appointment to the Research Participation Program at the Center for Devices and Radiological Health, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the Department of Energy and the Food and Drug Administration (FDA). In addition, this project was funded by the FDA’s Medical Counter Measures (MCMi) program (ECDRHXXXXX404KB).

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References


