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<th>Method/Activity</th>
<th>Aspect</th>
<th>Comments/Recommendations</th>
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| **EPA’s guidance on testing *Clostridium difficile*** | Towelette Testing | • In this session, an overview of the proposed revisions to EPA’s guidance for *C. difficile* claims was provided to participants. See associated ppt presentation for details.  
• Most notable, testing will be conducted using actual towelettes, but will not be based on the expressed liquid.  
• **Action Item:** MLB will consult with Antimicrobials Divisions on use of liquids in QCT-2 for towelettes. |
| **ASTM E2839-11 (Standard Test Method for Production of *Clostridium difficile* Spores) or BEAD/MLB SOP MB-28-01** | Strains | • EPA provided a comprehensive overview of the *C. difficile* strain (in the revised guideline) used in testing.  
• No comments from participants. |
| | Diagnostic characteristics | • Typical characteristics of growth on media and spore/cell morphologies were described by EPA.  
• No comments from participants. |
| | Specialized equipment, media and regents | • The equipment and supplies necessary for testing of *C. difficile* were discussed by EPA. Also, the maintenance and function of the anaerobic chambers were discussed by EPA.  
• A question was raised regarding whether or not EPA has seen any difference in using glass tubes vs. plastic tubes for the RCM step? EPA has not conducted a comparison study.  
• During the session, EPA reiterated the need to maintain fully reduced media for use in recovery assays:  
  • **Action Item:** EPA to clearly identify time frame for reducing liquid and solid media. EPA suggests a minimum of 24 hours under anaerobic conditions. EPA will provide revisions to ASTM E2839-11.  
  • Labs place RCM loosely-capped overnight in anaerobic environment to reduce.  
• EPA demonstrated use of the Coy Anaerobe chamber and the anaerobe jars. |
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<th>Method/Activity</th>
<th>Aspect</th>
<th>Sporulation Protocol</th>
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| ASTM E2839-11   |  - EPA provided a detailed presentation of the spore production protocol.                |  - EPA provided a detailed presentation of the spore production protocol.  
  - When using anaerobe jars, MLB recommended the use of a second jar to remove plate(s) for observation during the 7-10 day incubation.  
  - Recommend use of swinging bucket rotor for centrifugation steps, especially for the purification step. |
| Establishment of frozen stock |  - EPA presented the procedure for generating and storing stock (frozen vegetative stock). |  - EPA presented the procedure for generating and storing stock (frozen vegetative stock).  
  - **Action Item**: EPA to add shelf-life of frozen stock to the standard and SOP – EPA suggested 18 months. |

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- EPA does not use anaerobe jars, but they are considered adequate if used properly.
- Majority of participants use anaerobe jars.
  - Comment: If jar opened, replace indicator packs before closing.
  - Over time, anaerobe jars lids (inner lid) may be compromised and/or crack, thus inspection of the lid’s seal is necessary prior to use.
- Comment: Typical practice for incubation of plates includes sealing the agar plates with parafilm to reduce dehydration; this practice may be employed for incubation of plates in jars for the 5 day period.
- Comment: Some labs buy CABA plates from bioMerieux and reduce them on site. Most labs buy from Anaerobe Systems (pre-reduced).
- Comment: Media performance of in-house media should be conducted to confirm media quality by use of stock spores of known titer to check media. At a minimum, for purchased media, review and maintain the certification documents.
  - Verify performance of purchased media if lot to lot differences in quality are suspected.
  - **Action Item**: EPA will follow-up on the need to include media performance data in the GLP data submissions.
- **Action Item**: Consensus on the use of ST80; everyone using for dispersion.
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<th>Spore Purity and Resistance Testing</th>
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<tr>
<td>ASTM E2839-11</td>
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<td><strong>Action Item:</strong> Revise MLB SOP MB-28-01 to instruct users to bring HistoDenz and spore suspension to room temp prior to purification step, and ensure that liquid in tubes (in heat block or water bath) is at 65°C prior to starting the 10 minute exposure time.</td>
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<th>Other Acceptable Sporulation Protocols</th>
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| ASTM E2895-13   | Mission – One sporulation method | Another acceptable method, ASTM E2895-13, was discussed; some concerns were raised about the enzymatic process of purifying the spores and its impact on spore resistance.  
- A new method, Liver broth method, is currently being balloted by ASTM; and according to one stakeholder, the method is comparatively simple and provides a highly pure spore preparation without a separate purification step.  
- EPA believes it is desirable to have one standard method for spore production.  
  - Users need to consider pros and cons of each method and decide on one method.  
  - **Action Item:** EPA will seek feedback from stakeholders on the use of a single method and will discuss options internally for a long-term resolution.  
- Participants agreed to provide feedback to EPA on any issues associated with qualifying spores for use. |

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<th>Product Efficacy Testing – Quantitative Methodologies</th>
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- Participants expressed concerns that there is only one source of the brushed stainless steel carriers (Pegen).  
- Currently, the standard method allows re-use of carriers.  
  - **Action Item:** Re-use not advisable. Participants to petition ASTM committee to remove option from method. |
| MLB SOP MB-31   | Spore  | Target test spore suspension concentration is 8 to 9 logs/mL. Suspension may be |
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<tr>
<th>Section</th>
<th>Details</th>
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<tbody>
<tr>
<td><strong>Suspension</strong></td>
<td>Diluted on day of use to reach target of 6 to 7 logs/carrier.</td>
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| **Inoculum**     | - Comment: Vortex mix spore suspension every 3 carriers. May use same tip throughout carrier inoculation process.  
                  - **Action Item:** EPA to revise SOP MB-31. |
| **Carrier drying** | - Comment: ASTM standard silent on drying of spore-formers like *C. difficile*.  
               - Currently in MLB's SOP: Inoculate carriers, dry in biological safety cabinet for 30 min with lid off, transfer to desiccator to dry (lids off) 2 hr with vacuum. Thirty minute/no vacuum drying step allows user time to check drying of carriers to ensure inoculum remains on carrier/sufficient levels of inoculum before transferring to desiccator. |
| **PES membranes** | - **Action Item:** EPA to provide source and catalog number for PES filters.  
                   - Pall Corporation #66234 (individual filters)  
                   - Pall Corporation #4806 (microfunnel filter unit, 0.2 µm) |
| **Vials**        | - **Action Item:** EPA to provide source and catalog number for vials.  
                  - Thermo Scientific #2118-9050 (Nalgene, straight-side wide-mouth jar, polypropylene) |
| **Vortexing carriers** | - Comment: Adequate vortexing is essential to remove inoculum from carrier. MLB uses vortex set on highest setting; carrier should be spinning in mixture during the process. Visually inspect carriers to ensure inoculum is completely removed by conducting additional vortexing if necessary.  
                  - Comment: Vacuum should be on during rinsing of vials and dilution tubes. Not specified in MLB's SOP.  
                  - **Action Item:** Revise MLB SOP-MB-31 to keep the vacuum during rinsing. |
| **Filtration**   | - Comment: Some labs have observed that colonies are not obvious on filters early in the incubation time frame (2 days) for treated carriers; a film of growth on filter is observed after 3-5 days. Subculture reveals presence of *C. difficile*. Additional incubation time frame recommended (to 5 days) for treated carriers.  
                  - Comment: Wetness of plates can adversely affect growth of organism on filters.  
                  - Comment: If using microfunnel filter units for filtration after treatment, analysts... |
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| Important time frames | **Antimicrobial Efficacy Test Methods Workshop**
|-----------------------|---------------------------------------------------------
| Important time frames | • EPA reiterated the need to conduct the work as quickly as possible, from end of contact time to completion of filtration. Surviving spores are vulnerable, risk exposure to oxygen.
| | • For example, dilutions should be made with 30 minutes of neutralization and filtration/plating should occur within 50±10 minutes of preparing the dilutions.
| | • Also, if using an anaerobe jar, load plates into jar within 15 minutes following completion of plating – this was a recommendation and should be incorporated in the lab SOPs if using anaerobe jars.
| Questions to consider | **Antimicrobial Efficacy Test Methods Workshop**
| Questions to consider | • How to do neutralization? Not discussed, however, neutralization will be conducted in accordance with BEAD/MLB SOP MB-26-00 using the following revisions specific to *C. difficile*.
| AOAC 2008.05 (Three Step Method) | • The spore suspension will be diluted appropriately in ST-80 to obtain 20-200 CFU/filter.
| Advantages of method | • A suspension test using 10μL of the diluted spore suspension will be employed instead of a carrier-based approach.
| Advantages of method | • Contact time will be 10 minutes and neutralization will be considered adequate if the CFU for all treatments are within 50% of the titer control.
| Disadvantages of method | **Antimicrobial Efficacy Test Methods Workshop**
| Disadvantages of method | • Sensitivity concerns due to sampling (10 μl of 1 mL sample)
| Comparison of QCT-2 to TSM | **Antimicrobial Efficacy Test Methods Workshop**
| Comparison of QCT-2 to TSM | • EPA stated that use of a single test method for efficacy evaluations is desirable in the long term.
| | • EPA has not conducted side by side studies to know if products give same results under both methods.
# Antimicrobial Efficacy Test Methods Workshop

## Highlights from Workshop Sessions

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| ASTM E2896-12 (Standard Test Method for Quantitative Petri Plate Method (QPM)) | **Action Item:** The development of a reference standard (high and low efficacy treatments) for both methods is desirable; sodium hypochlorite is a viable candidate. EPA will initiate studies.  
**MLB provided a full demonstration of the procedure.**  
**Comment:** Humidity may vary during the drying process; however, an environmental monitoring system documents the relative humidity in the incubator. |
| Control of humidity during drying of inoculated plates | **Comment:** Consider pre-dispensing all liquid reagents (dilution blanks) and media for ease of use. |
| Media | **Comment:** Important to use consistent pressure, from test to test, during wiping of carrier during neutralization step.  
**Comment:** Avoid releasing all of active ingredient (i.e., liquid) onto the plate; neutralization is negatively impacted by extreme pressure/high liquid volume – more liquid, more neutralization that has to take place. |
| Uniform wiping procedure | **Comment:** For treated carriers, colonies can be counted on day 3; however, filters with few or no colonies should incubate for up to 5 days.  
**Action Item:** Revise SOPs and ASTM standard for incubation periods for control and treated carriers. |
| Incubation and recording or results | **EPA announced plans to conduct a collaborative study on QPM and *C. difficile.* The goal is launch the study in 2014 to fulfill ASTM data requirements. This study is considered to be the first in a series of evaluations designed to determine the method’s performance. A number of labs expressed interest in participating in the study. The biggest impediment is treatment (antimicrobial wipes) selection – i.e., use of commercially available products.**  
**Action Item:** Develop the study protocol and work with stakeholders for laboratory support. |
| Calculations and interpretation of results | **No comments.**  
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