MicroSnap™ Equivalence Testing Versus Traditional Methods

Introduction

Traditional methods for microorganism enumeration (i.e., plating) can be highly variable for a number of reasons, including:

1. The assumption that one colony forming unit (CFU) is derived from a single organism. Microbes grow in clusters, chains, or clumps of cells as they reproduce by binary fission. When plating, these clumps are not separated into single cells during sample preparation. Therefore, a colony can be formed from one, two, or more cells.
2. Survivability - bacteria tend to prefer liquid media. They will grow better, resuscitate better and be resuscitated quicker than drier growth products (e.g., Petrifilm™).
3. Stress - if a bacterium that is already stressed is put into an environment that stresses it further, it will suffer and growth will vary depending on the level of stress which is difficult to estimate. For example, if the organism is already desiccated, it might prefer plating over liquid media, etc.
4. Probability - the distribution of bacteria in a sample makes the equivalence angle variable. Two draws from a sample can be at the opposite ends of the specification (a mean CFU/mL of 10 will have a distribution of 1 through 25 CFU per mL), meaning 2 draws can produce results x20 fold apart. Therefore, evaluating Log 0.5 is sometimes the best comparison achievable which is why it is used.

As a result, precision and accuracy in microbiology involve a normal expected variation of 2 – 5-fold (Log 0.3-0.5). This means for a result to be considered significantly different, a variation of > 5-fold (greater than log 0.5) must be observed. This variability is compounded when contamination levels are very low (1-100 CFU) and the distribution of contamination within a sample is uneven. Combined, this reduces the probability of detection and increases the variation and inaccuracy of test results even further. This challenge is compounded by the fact that sample testing typically requires pulling only 25 grams for testing, no matter the size of the batch. Therefore, CFU values are only an estimate of microorganism contamination. This has significant implications when comparing colony count methods with other enumeration methods, especially those that measure every single viable organism. These methods can be expected to be more inclusive and more precise.

Overview

Requirements for Evaluation

When evaluating an alternative method, several factors must be considered during planning and execution. These include:

- Training on both the reference and new method (MicroSnap) to ensure reliable results are generated and correctly interpreted.
- Testing of both positive and negative control samples to ensure typical results are correctly identified and atypical results are not misinterpreted.
- Preparing and testing samples containing a known inoculum at the proper contamination level (separate from control samples).
- Representative sample types from the facility (ones to be tested routinely).
- Statistical data analysis of the results generated.
- Testing sufficient replicate samples (at least 5-10 at each level with more needed at lower contamination levels).

Study Goals

By utilizing the general comparison protocol, users should be able to learn the following skills:

1. Sample preparation and its importance in finding the correct answer
2. How many dilutions to do based on the required limit or historical inoculum size
3. How to run an equivalence study using traditional methods
4. How to run an equivalence study using the rapid method – MicroSnap
5. How to compare a traditional method and compare to MicroSnap tests

MicroSnap Background Details

The MicroSnap detection system consists of an Incubation Device containing a proprietary growth media and a Detection Device containing a bioluminogenic substrate that is measured using a Hygiena™ luminometer, EnSURE™ or EnSURE™ Touch. The two-step test procedure requires a short incubation period facilitating recovery of bacteria followed by a detection step. The incubation time of the sample is determined by the level of sensitivity required. During incubation the bacteria use up available food resources in the media and more of the diagnostic enzymes are created (beta-galactosidase and beta-glucuronidase), which are required for the bioluminogenic reaction. Once a sample is transferred from the Incubation Device to the Detection Device and activated, the specific substrate in the Detection Device reacts with the diagnostic enzymes produced by the bacteria producing light which is read by the Hygiena luminometer.

The light output is directly proportional to the initial starting inoculum. However, there is a potential for harsh samples to interfere with the chemistry kinetics, growth of the bacteria, or even diagnostic enzyme production by the bacteria. Therefore, when using the EnSURE luminometer, it is important to run a standard curve with these sample types to determine the RLU to CFU conversion for the specific material being tested. (More information can be found here). (This is not necessary for the EnSURE Touch as CFUs are directly calculated.)

Equipment, Supplies & Reagents

- Product samples (food, water, surface swab)
- Positive controls (bacterial cultures for comparison)
- MicroSnap Coliform
- MicroSnap Total
- MicroSnap *E. coli*
- MicroSnap EB
- TSA, Petrifilm or selective/chromogenic plates (i.e., HiCrome™, VRBA, Petrifilm CC, Brilliance™)
- Sponge/Sponge Stick for sample collection (i.e., Hygiena Sponge'n Bag™ or StickSponge™)
- Diluent bag and diluent broth (for sponges)
- Stomacher (if testing solid food)
- Plate spreader (for culture plates)
- Pipette tips
- Incubators (30-32°C, 37°C)
- EnSURE (or EnSURE Touch) Luminometer
- SureTrend™ or SureTrend™ Cloud software

Methods

Sample Preparation Overview

Most food samples tested for microbiological contamination are diluted at some stage to make the handling of the sample easier. Modern microbiology relies on the ability of the user to measure small volume accurately. This ability underpins the fact that most limits set by appropriate bodies on the contamination levels in food are fixed at some point and foods are tested for their ability to harbor bacteria that are either higher or lower than these limits. Hence, most solid foods need to be rendered into some form of liquid preparation in order to be diluted down to be able to measure at these levels.

Environmental sampling may or may not require this level of dilution depending on how clean the surface being tested is. Typically, sampling is done after surfaces have been cleaned and before sanitizing; therefore, levels of residual contamination should be much lower.

Food Sample Preparation (10% suspension)

As noted above, most food samples require dilution. To prepare a 10% suspension, add 10 g of food to 90 mL of sterile diluent (commonly used diluents are Maximum Recovery Diluent (MRD), Phosphate Buffered Saline (PBS), water or other commercially available diluent). For small samples, 1 g of food can be added to 9 mL of sterile diluent such as enhanced nutrient broth (HTS-9 - Hygiena offers these prepared vials to simplify workflow).

If the food is a solid, it will need to be homogenized using a stomacher or pulsifier to create a suspension within the diluent. Since the dissolution of nutrients into the diluent can support organism growth, the suspension should be used within a few minutes of preparation to minimize initial growth which could skew the results.

From this initial 10% suspension, further dilutions can be made for analysis, depending on the method being used for detection. This is necessary as different microbiological methods have unique lower and upper limits for statistical accuracy (see table below).

Method of Detection	Abbrev.	Lower Read Level (CFU)	Upper Read Level (CFU)
Membrane Filtration (45 mm)	MF	20	200
Pour Plates (90 mm)	PP	30	300
Petrifilm™ (45 mm)	PF	20	200
Spread plates (90 mm)	SP	20	200
MicroSnap™ 6 hours	MS6	10	10,000
MicroSnap™ 8 hours	MS8	1	1,000

It is recommended that serial dilutions be performed to cover all possible levels of microorganism numbers potentially present. This will result in 4 sample dilution levels of 10%, 1%, 0.1% and 0.01%. It is best to keep these dilutions chilled to minimize the effect of any growth.

Surface Sample Preparation

Environmental sampling can also be performed using similar methods. In this case, sponge sticks can be used to collect surface samples following standard collection procedures. Sponges are then put into a diluent bag to allow the microorganisms to disperse into the diluent.

From this initial suspension, further dilutions can be made for analysis as noted above. The goal is to achieve levels of detection that will be statistically significant based on the method being used for analysis.

Incubation for Equivalence Testing of MicroSnap vs Other Methods

1. Collect sample and place into the MicroSnap Incubation Device. Samples can be as noted above – surface samples, liquids, or diluted food suspensions. Add 1 mL to the Incubation Device.
2. When ready to activate, have plates, media or Petrifilm ready. Ensure enough sample is prepared to run testing in triplicate for each dilution or suspension and for each alternate method being tested.
3. Activate Incubation Device to bring media to bottom of tube (see MicroSnap instructions or video for details)
    a. Make sure to slightly remove the bulb from the tube to release pressure and allow the entire liquid volume to come into contact with the sample.
4. Vortex or shake Incubation Device to mix the sample.
5. At time zero (T=0), perform the following, depending on the sample and methods being tested.
    a. For Petrifilm, place 1 mL of each sample dilution on Petrifilm (recommend 5 replicates for equivalence testing as recommended per BAM, FDA, and AOAC). At the same time, add 1 mL of sample dilution to the MicroSnap Incubation Device and activate as in step 3.
    b. For Pour Plates, mix 1 mL or each sample dilution with 15 mL of melted agar and pour into a standard culture plate. At the same time, add 1 mL of sample dilution to the MicroSnap Incubation Device and activate as in step 3.
    c. For prepared plates (spread plates), two options can be used for testing:
        i. For liquid/suspension samples, equal volumes (typically 100 µl) can be spread on plates or added to the MicroSnap Incubation Device, which is then handled as in Step 3 and 4 above.
        ii. For environmental testing, surface samples can be collected using the swab portion of the MicroSnap Incubation Device. Activate the device as in Step 3. Mix as in Step 4. Remove 100 µl aliquots and plate on agar plates (at least in duplicate). Incubate the remaining media in the Incubation Device according to the MicroSnap instructions or transfer 100 µl to a new MicroSnap Incubation Device and continue from Step 3.
    d. For environmental testing (surface samples), two options can be used for testing:
        i. Surface sample can be collected using the swab portion of the MicroSnap Incubation Device. Activate the device as in Step 3. Mix as in Step 4. Remove 100 µl aliquots and plate on agar plates (at least in duplicate). Incubate the remaining media in the Incubation Device according to the MicroSnap instructions.
        ii. Sponge samples (already in diluent) can be directly plated or added to the MicroSnap Incubation Device. Typically, 1 mL samples can be transferred to Petrifilm and MicroSnap (in triplicate), or 100 µl can be plated directly onto standard plates and MicroSnap Incubation Devices.
6. Incubate the MicroSnap Incubation Devices for 6-8 hours; incubate Petrifilm, poured plates and prepared plates for 24 hours.

Detection

For MicroSnap:

1. After 6-8 hours of incubation, remove 100 µl from the Incubation Device and add it to the MicroSnap Detection Device (run in duplicate or triplicate). The time of incubation (6-8 hours) will determine the level of counts required (at 6 hours, 100 CFU is the lowest detectable level; at 8 hours, 1 CFU is the lowest detectable level). Dilution factors need to be taken into account when these levels are used.
2. Activate the MicroSnap Detection Device. Shake for 3-5 seconds.
3. Insert Detection Device into the EnSURE or EnSURE Touch luminometer; read and record RLU values for EnSURE. (If RLUs are zero, allow the remaining media in the Incubation Device to continue to incubate overnight and test again after 24 hours (T=24) to confirm negativity). If using EnSURE Touch, the device will convert RLU values to CFU values which will be directly displayed.

For pour plates, spread plates or Petrifilm.

1. After the indicated incubation time, count the number of colonies present on each plate/film and record the results. Follow the defined criteria for counting on selective/chromogenic agar.

Results & Discussion

Comparison

Time	Petrifilm Method	MicroSnap	Standard Plates	Selective/Chromogenic Plates
Swab surface to collect sample. Mix well via vortex, shaking or massaging swab				
0 hours	3 x 1 mL onto Petrifilm. Incubate at 30-32°C	2-3 x 1 mL for Incubatio n Device (Step 1). Incubate at 30-32°C	3 x 100 µL onto plates. Incubate at 30-32°C	2 x 100 µL onto plates. Incubate at 37°C
6-8 hours	n/a	Remove 2-3 x 100 µL from Incubation Device and add to Detection Device. Activate and read in EnSURE.	n/a	n/a
Optional 8-12 hours	n/a	Remove 2-3 x 100 µL from Incubation Device. Add to Detection Device. Activate, incubate and read in EnSURE or EnSURE Touch.	n/a	n/a
If at T = 8 hrs RLU<10, continue to incubate remaining media overnight and test again at 24 hours				
24 hours	Count Petrifilm for colony growth	If required, confirm negativity by transferring 2-3 x 100 µL from Incubation Device to Detection Device. Activate, incubate and read in EnSURE or EnSURE Touch.	Count colony growth on plates	Count plates for colonial growth. Count only specifically defined colonies (per selective media instructions).

Calculation of Equivalence

1. In order to verify equivalence, calculate the counts on the Petrifilm, standard plates or pour plates and convert to CFU/g or CFU/mL.

Dilution	Calculations
10%	Multiply colony counts by 10 to obtain CFU/g (CFU/mL)
1%	Multiply colony counts by 100 to obtain CFU/g (CFU/mL)
0.1%	Multiply colony counts by 1,000 to obtain CFU/g (CFU/mL)
0.01%	Multiply colony counts by 10,000 to obtain CFU/g (CFU/mL)

2. For MicroSnap, refer to the look-up table in the kit insert or alternatively, you can manually calculate values as follows:
    a. Take the log of your RLU value
        i. Log^{10} (RLU)
    b. Take the answer from step a. and divide by 0.6 and add 0.5
        i. [Log^{10} (RLU)/0.6] + 0.5
    c. Take 10 to the power of the answer from step b. to get CFU value
        i. $10^{[[Log^{10}(RLU)/0.6] + 0.5]}$

3. Compare values from both methods by running a Log transformation on both CFU/g or CFU/mL and directly comparing. Equivalence is reached if both methods are <0.5 logs apart.

Conclusions

By demonstrating equivalence between MicroSnap and traditional culture methods, users can obtain results more quickly and more accurately. Time to results can be reduced from a minimum of 24 hours to 6-8 hours. In addition, MicroSnap eliminates the normal variability obtained with standard culture methods, giving more confidence in the results obtained.

Furthermore, MicroSnap is suitable for a wide variety of sample types, allowing the lab to test multiple matrices for results the same day. MicroSnap is also easy to use, with a 2-step procedure that requires less materials and labor when compared to traditional plate culture methods. With the added benefit of Hygiena's SureTrend™ software, users can store their testing data in one place and use the built-in reporting features to understand the performance of their sanitation procedures more deeply.

References

1. Petrifilm™ is a trademark of 3M
2. HiCrome™ is a trademark of HiMedia Laboratories
3. Brilliance™ is a trademark of Thermo Fisher Scientific
4. Excel® is a registered trademark of Microsoft