Terragene®

Viable Spore Count

A brief comparison between USP and Terragene validated methods

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For detailed guidance on how to perform viable spore counting of Terragene's biological indicators, we recommend consulting our validated procedure described in the Technical Note: Terragene Spore Count Protocol. You can also refer to our step-by-step spore count video tutorial available on the Terragene YouTube channel.

Introduction

Biological indicator (BI) manufacturers must report specific product quality parameters, among which is the nominal spore population. For each type of sterilization process, international standards define the minimum nominal population that a BI must contain. As a result, viable spore counting is a key technique for assessing BI quality: it determines the number of microorganisms inoculated in a BI lot and, in some cases, supports the quantitative evaluation of microbial reduction achieved during a sterilization process.

Standards such as ISO 11138-1:2017 and the United States Pharmacopeia (USP) provide spore-counting guidelines, and manufacturers may develop and validate their own based on them. These custom protocols often incorporate additional methodological considerations tailored to the characteristics of each product and the challenges associated with the chosen spore carrier material. This protocol, once validated, is used to demonstrate product compliance, and must be available to any party seeking to verify the population stated in the quality certificates provided with each BI lot.

The aforementioned standards differ in several recommendations regarding the extraction, cultivation, and enumeration steps, as well as the precautions that must be taken throughout the process.

The recommendations in ISO 11138-1:2017 are broad in scope. Manufacturers are free to choose the materials and techniques used for the elution of microorganisms from the test samples. However, the selected method must be validated in advance, and a minimum of four test samples per lot must be evaluated. ISO also recommends preparing a series of serial dilutions before plating the samples onto the appropriate agar media.

In contrast, the USP protocol is more prescriptive. It requires a minimum of three test samples and provides step-by-step instructions for sample elution, plating, and colony enumeration. The procedure also varies depending on the type of the spore carrier—paper carriers, non-paper carriers, or suspensions. Although the USP is the preferred reference for most pharmaceutical laboratories, its protocol remains general and does not fully address the variability in materials and manufacturing processes that distinguish the many BI brands available on the market.

Both protocols agree that colony counts between 30 and 300 CFU provide the most reliable range for accurate enumeration.

Comparison between Terragene and USP protocols

Terragene has developed and validated spore-counting protocols specifically optimized for each type of BI it manufactures. These protocols are designed in accordance with the recommendations and guidelines established in ISO 11138-1:2017.

The pharmaceutical sector is the one that most frequently performs viable count testing as part of its internal quality assurance procedures, aiming to verify the nominal population stated in the batch certificates of analysis. However, the results they obtain often may not match their expectations. The reason is straightforward: as mentioned above, these assessments are usually performed following the USP protocol.

Terragene BIs are designed and validated in accordance with ISO standards, and anyone wishing to perform a spore count or verify the population must comply with the manufacturer's protocol. These standards specify that population verification must fall within 50% and 300% of the population declared by the manufacturer for the batch, **provided that the manufacturer's protocol is followed strictly.**

To better understand the differences between both protocols, the following comparison summarizes the key points:

Number of test samples
The USP recommends using **three test samples**, whereas Terragene recommends using a **minimum of four test samples** per batch/exposure, in line with ISO guidelines. Increasing the number of samples enhances count accuracy by providing a more reliable average.

Elution
For elution, the USP distinguishes between paper and non-paper carriers. For paper carriers, lacing the **three carriers together** in a 250 mL blender cup with 100 mL of chilled, sterile Purified Water and blending until a homogeneous suspension is obtained. For non-paper carriers, USP suggests sonication or shaking on a reciprocal shaker for an appropriate period of time.
Terragene's validated procedure does not differentiate between paper and non-paper carriers for this step. Elution is performed by placing **each individual carrier in a 2 mL tube** compatible with a homogenizer, along with **2–3 mm glass beads**. Each tube should contain **600 µL of deionized distilled water and 400 µL of ethanol**.
Eluting each carrier helps to minimize the error that could generate a single separation process. In addition, using glass beads for spore recovery, specially from metallic carriers, provides superior abrasion, efficiency, and reproducibility compared to vortexing alone. The beads facilitate consistent detachment of firmly adhered spores, yielding more accurate and quantitative recovery. Vortexing, while simpler and faster, relies only on liquid turbulence, often resulting in variable and incomplete recovery from metallic surfaces. The use of glass beads and a homogenizer makes the separation process more effective, avoiding the resistance that could be generated by certain materials in the separation process in a blender.
Finally, the use of a 40:60 ethanol:water solution instead of 100% water. Ethanol helps to slightly decrease the polarity of the medium to facilitate the elution of spores from the carriers.

Heat shock condition
While USP recommends two different temperature and time ranges for doing the thermal shock, between 95-100°C / 15 minutes for thermophiles and 80-85°C / 10 minutes for mesophiles, Terragene has validated highly consistent spore recovery for both *Geobacillus stearothermophilus* and *Bacillus atrophaeus* under the same standardized heat shock temperature: 85 °C for 15 minutes for *G. stearothermophillus* and for 5 minutes for *B. atrophaeus, B. subtilis and B. pumilus*. This harmonized approach simplifies laboratory workflows, since the only variable to take into account is the timelapse.

Cooling after heat shock
For cooling the spores after the heat shock, USP recommends cooling in an ice-water bath at 0-4 °C. Instead, Terragene has validated cooling spores at 0 °C for 15 minutes, which provides a more controlled and reproducible stabilization step compared to holding them at 0–4 °C for only 5–10 minutes. The fixed temperature of 0 °C eliminates variability associated with refrigeration ranges, while the extended time ensures uniform metabolic inactivation across the spore population. This stricter and standardized approach reduces inter-laboratory variability.

Serial dilution
Deionized water: Its chemically inert nature keeps spores suspended without contact with salts or nutrients that might stabilize, activate, or otherwise influence them. This avoids protective or growth-enhancing effects that could distort the results. Because it prevents osmotic protection, minimizes background interference, and offers a standardized medium across laboratories, deionized water supports more consistent and reproducible counts.

Plating method
As for the plating method, USP describes the pour plate method, which consists of pouring molten agar over the inoculum in a Petri dish, allowing the sample to mix with the medium, and letting both solidify together.
In contrast, Terragene uses the spread plate method, where the inoculum is distributed across the surface of a pre-solidified agar medium using a sterile spreader.
The spread plate method offers several advantages over the pour plate technique. Since *G. stearotermophilus* and *Bacillus* species are aerobic organisms, surface spreading ensures that all colonies develop under oxygen exposure, leading to better recovery. In pour plates, spores may become trapped within the agar and either not germinate properly or form poorly developed colonies. The spread plate method also eliminates the heat stress associated with molten agar,

resulting in a clearer and more uniform colony morphology.
Overall, spreading improves reproducibility and reliability of colony counting compared with the inherent variability and potential underestimation associated with pour plate methods.

Culture medium
When it comes to culture media, the two protocols differ significantly in their level of specificity. The USP simply recommends using Soybean–Casein Digest Agar Medium. Terragene, on the other hand, provides a more precise and validated approach by specifying a culture medium that has been tested to ensure optimal spore recovery and accurate counting. Terragene's recommended medium is LBA supplemented with calcium chloride and glucose, formulated to enhance germination and promote robust colony development. Its composition is detailed below:
- Acid casein peptone 1.0% p/v
- Yeast extract 0.5% p/v
- Sodium chloride 0.5% p/v
- Calcium chloride 0.05% p/v
- Glucose 0.03% p/v
- Agar 1.5% p/v

Incubation temperature
The USP specifies incubation at 55–60 °C for BIs used in steam sterilization, and at 30–35 °C for those used in ethylene oxide or dry-heat sterilization.
Based on its optimized culture medium and validated performance data, Terragene provides more precise incubation conditions:
G. stearothermophilus: incubate at (60 ± 2) °C for 48 hours.

B. atrophaeus, B. subtilis, or *B. pumilus*: incubate at (37 ± 2) °C for 24 hours.

These conditions ensure optimal germination and consistent, reliable colony development for each species.

Conclusion

While the USP offers a general framework for viable spore counting, its procedures do not fully reflect the diversity of carrier materials, conditioning processes, and manufacturing characteristics that distinguish the BIs available on the market. Terragene's validated methods bridge this gap by providing a more precise, product-specific, and robust approach that optimizes every stage of the procedure, from elution and heat shock to plating, incubation, and culture media selection.

Terragene BIs are conditioned to respond efficiently to the different sterilization processes used in the industry. As a result, their behavior during viable count testing may differ from that expected under protocols validated by other manufacturers. For this reason, any user wishing to perform spore counting on Terragene indicators must strictly follow Terragene's validated protocol. This method is based on the recommendations of ISO 11138-1:2017 and has been optimized to deliver accurate, consistent results within the error range required by the standard.

By standardizing critical variables, Terragene's protocol ensures superior accuracy and reproducibility in population verification. These improvements reduce inter-laboratory variability and minimize the risk of underestimating true spore loads.

References

- International Organization for Standardization. (2017). *ISO 11138-1:2017 Sterilization of health care products—Biological indicators—Part 1: General requirements.* ISO.
- Terragene. (s. f.). *TN 04: Determination of viable microorganism count in biological indicators.*
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