Determination of viable count in Bionova® Biological Indicators

The methods described below comply with ISO 11138-1: 2017 Annex A and are applicable to all biological indicators manufactured by Terragene SA. The protocols correspond to the different types of carriers for the different families of biological indicators.

The method used has been validated for the recovery of spores inoculated in the carrier of each biological indicator. Incubation times and temperatures have been validated for each strain considering the effectiveness of the procedure and the morphological characteristics of each of them.

The culture medium designed for optimized counting is the LBA supplemented with calcium chloride and glucose. The 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.

Strains used:

1) *Geobacillus stearothermophilus* ATCC 7953

Two morphologies can be observed, although one usually predominates:

a. *Circular, opaque, smooth and convex colonies.*
b. *Slightly irregular, translucent, umbonal and smooth colonies.*

2) *Bacillus atrophaeus* ATCC 9372

Three morphologies can be observed, although one usually predominates:

a. *Orange, circular, bright colonies with irregular borders.*
b. *Opaque, circular, bright orange colonies with irregular borders.*
c. *White, irregular, wavy and flat colonies.*

3) *Bacillus subtilis* DSM 5230 ATCC 35021

It is usually identified with a single morphology:

a. *Irregular and rough white colonies.*

4) *Bacillus pumilus* ATCC 27142

Two morphologies can be observed, although one usually predominates:

a. *Translucent, elevated and irregular colonies.*
b. *Opaque, circular, smooth, bright and raised colonies.*

Protocol for each type of BI:

A) Indicators with paper filter (discs, strips and self-contained biological indicators), polyethylene (disc and strips) and stainless steel (coupons) carriers.

1. Remove the carrier from the biological indicator and place it in a 2.0 mL tube, adaptable to a sample homogenizer.
2. Add 600 µL of sterile deionized water.
3. Add 400 µL of absolute ethyl alcohol.
4. Mix the solution using a tube mixer.
5. Place six (6) sterile glass beads, 2-3 mm in diameter.
   5.1. In the case of coupons, place the beads on the inoculated side.
6. Let stand at room temperature for 60 minutes.
7. Place the tube in the homogenizer.
8. Select a 60 seconds 3600 rpm program.
9. Start the program.
10. Perform a heat treatment for the activation of the spores.
    10.1. For indicators inoculated with *Geobacillus stearothermophilus*, incubate the samples for 15 minutes in a water bath at 85 °C.
    10.2. For indicators inoculated with *Bacillus atrophaeus*, *Bacillus subtilis* or *Bacillus pumilus*, incubate the samples for 5 minutes in a water bath at 85 °C.
11. Immediately after the heat treatment time has elapsed, place the samples in a water bath at 0 °C for 15 minutes.
12. Prepare 1.5 mL microtubes with 900 µL of sterile deionized water.
13. Perform 1/10 serial dilutions, transferring 100 µL of the spore suspension from tube to tube, after homogenization of the suspension with a mixer. If you do not have a mixer, do it with the micropipette (Figure 1).
14. Sow 100 µL of the corresponding dilution in a Petri dish containing 25 mL of supplemented LBA culture medium. Distribute the sowing content over the entire surface of the plate using Drigalski spatula or sterile glass beads 5-7 mm in diameter.
    Note: for spore populations around $1.0x10^6$ CFU/carrier, sow from the third serial dilution.
15. Incubate the plates in a culture stove.
    15.1. For *Geobacillus stearothermophilus*, incubate at a temperature of (60 ± 2) ºC for 48 hours.
    15.2. For *Bacillus atrophaeus*, *Bacillus subtilis* or *Bacillus pumilus*, incubate at a temperature of (37 ± 2) ºC for 24 hours.
16. Once the incubation is finished, remove the plates from the stove and count the colonies formed on the culture medium. In order to have a reliable count, the number of colonies must be between 30 and 300 CFU.
    Note: In case of observing different morphologies, consider only the colonies with the morphology specified for the corresponding strain.
17. Perform the calculation of the carrier spore population with the following equation:

$$Population\left(\frac{CFU}{Carrier}\right) = Average\ CFU_{plate}\ x\ 10^{(n+1)}$$

Where "Average CFU_{plate}" is the average number of colonies counted on the plates and "n" is the number of serial dilutions made.

B) Self-contained biological indicators with polyethylene carriers.

1. Remove the carrier from the biological indicator and place it in a 2.0 mL tube, adaptable to a sample homogenizer. It is suggested to also transfer the polypropylene microfiber that is on the spore carrier.
2. Add 600 µL of sterile deionized water to the biological indicator tube and rinse the bottom of the tube by repetitively taking and emitting the solution with the micropipette to resuspend the spores. Transfer the liquid to the 2.0 mL tube.
3. Add 400 µL of absolute ethyl alcohol.
4. Mix the solution using a tube mixer.
5. Place six (6) sterile glass beads, 2-3 mm in diameter.
6. Let stand at room temperature for 60 minutes.
7. Place the tube in the homogenizer.
8. Select a 60 seconds 3600 rpm program.
9. Start the program.
10. Perform a heat treatment for the activation of the spores: incubate the samples for 15 minutes in a water bath at 85 °C.
11. Immediately after the heat treatment time has elapsed, place the samples in a water bath at 0 °C for 15 minutes.
12. Prepare 1.5 mL microtubes with 900 µL of sterile deionized water.
13. Perform 1/10 serial dilutions, transferring 100 µL of the spore suspension from tube to tube, after homogenization of the suspension with a mixer. If you do not have a mixer, do it with the micropipette (Figure 1).
14. Sow 100 µL of the corresponding dilution in a Petri dish containing 25 mL of supplemented LBA culture medium. Distribute the sowing content over the entire surface of the plate using Drigalski spatula or sterile glass beads 5-7 mm in diameter.
    Note: for spore populations around $1.0x10^6$ CFU/carrier, sow from the third serial dilution.
15. Incubate the plates in a culture stove at a temperature of (60 ± 2) ºC for 48 hours.
16. Once the incubation is finished, remove the plates from the stove and count the colonies formed on the culture medium. In order to have a reliable count, the number of colonies must be between 30 and 300 CFU.
    Note: In case of observing different morphologies, consider only the colonies with the morphology specified for the corresponding strain.
17. Perform the calculation of the carrier spore population with the following equation:

$$Population\left(\frac{CFU}{Carrier}\right) = Average\ CFU_{plate}\ x\ 10^{(n+1)}$$

Where "Average CFU_{plate}" is the average number of colonies counted on the plates and "n" is the number of serial dilutions made.

C) Indicators without removable carriers (BT91)

1. Remove the ampoule from the biological indicator.
2. Add 600 µL of sterile deionized water into the primary indicator container.
3. Let stand at room temperature for 60 minutes.
4. Add 400 µL of absolute ethyl alcohol.
5. Mix the solution using a tube mixer or by rinsing the bottom of the tube by repetitively taking and emitting the solution with the micropipette.
6. Perform a heat treatment for the activation of the spores: incubate the samples for 15 minutes in a water bath at 85 °C.
7. Immediately after the heat treatment time has elapsed, place the samples in a water bath at 0 °C for 15 minutes.
8. Prepare 1.5 mL microtubes with 900 µL of sterile deionized water.
9. Perform serial dilutions 1/10, transferring 100 µL of the spore suspension from tube to tube, after homogenization of the suspension with a mixer. If you do not have a mixer, do it with the micropipette (Figure 1).
10. Sow 100 µL of the corresponding dilution in a Petri dish containing 25 mL of supplemented LBA culture medium. Distribute the sowing content over the entire surface of the plate using Drigalski spatula or sterile glass beads 5-7 mm in diameter. Note: for spore populations around $1.0x10^6$ CFU / carrier, sow from the third serial dilution.
11. Incubate the plates in a culture stove at a temperature of (60 ± 2) ºC for 48 hours.
12. Once the incubation is finished, remove the plates from the stove and count the colonies formed on the culture medium. For the result of the count to be reliable, the number of colonies must be between 30 and 300 CFU.
    Note: In case of observing different morphologies, consider only the colonies with the morphology specified for the corresponding strain.
13. Perform the calculation of the spores population in the carrier with the following equation:

$$Population\left(\frac{CFU}{Carrier}\right) = Average\ CFU_{plate}\ x\ 10^{(n+1)}$$

Where "Average CFU_{plate}" is the average number of colonies counted on the plates and "n" is the number of serial dilutions made.

D) Self-contained ampoules.

1. Place the self-contained ampoules in sterile 1.5 mL tubes.
   1.1. In the case of the BT23 ampoule it is not necessary, since it has a flat base and can be opened by breaking the ampoule's neck.
2. Break the ampoule using flame sterilized scissors or pliers.
3. Transfer a total volume of 1 mL of the contents of the ampoules to a sterile 1.5 mL tube.
   Note: for BT21, BT22 and BT24 it will be necessary to break more than one ampoule to obtain 1 mL of sample.
4. Perform a heat treatment for the activation of the spores.
   4.1. For indicators inoculated with *Geobacillus stearothermophilus*, incubate the samples for 15 minutes in a water bath at 85 °C.
   4.2. For indicators inoculated with *Bacillus subtilis*, incubate the samples for 5 minutes in a water bath at 85 °C.
5. Immediately after the heat treatment time has elapsed, place the samples in a water bath at 0 °C for 15 minutes.
6. Prepare 1.5 mL microtubes with 900 µL of sterile deionized water.
7. Perform serial dilutions 1/10, transferring 100 µL of the spore suspension from tube to tube, after homogenization of the suspension with a mixer. If you do not have a mixer, do it with the micropipette (Figure 1).
8. Sow 100 µL of the corresponding dilution in a Petri dish containing 25 mL of supplemented LBA culture medium. Distribute the sowing content over the entire surface of the plate using Drigalski spatula or sterile glass beads 5-7 mm in diameter.
   Note: for spore populations around $1.0x10^6$ CFU/carrier, sow from the third serial dilution.
9. Incubate the plates in a culture stove.
   9.1. For *Geobacillus stearothermophilus*, incubate at a temperature of (60 ± 2) ºC for 48 hours.
   9.2. For *Bacillus subtilis*, incubate at a temperature of (37 ± 2) ºC for 24 hours.
10. Once the incubation is finished, remove the plates from the stove and count the colonies formed on the culture medium. For the result of the count to be reliable, the number of colonies must be between 30 and 300 CFU.
    Note: In case of observing different morphologies, consider only the colonies corresponding to the morphology specified for the corresponding strain.
11. Perform the calculation of the population in the carrier with the following equation:

$$Población\left(\frac{CFU}{Carrier}\right) = Average\ CFU_{plate}\ x\ 10^{(n+1)}\ x\ V_{BI}$$

Where "Average UFC_{plate}" is the average number of plate count colonies, "n" is the number of serial dilutions made and "V_{BI}" is the volume of culture medium contained in the ampoule in milliliters (mL).

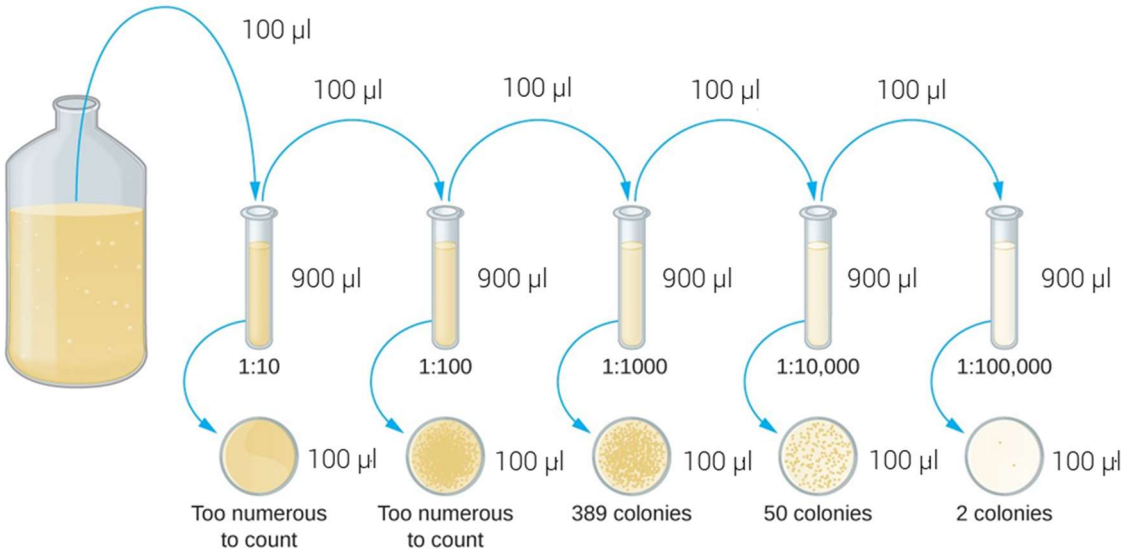

Figure 1: Graphical representation of 1:10 serial dilutions for calculating CFU on plate.

This protocol is the protocol used by Terragene® in order to comply with the ISO 11138 regulation