Background information

The procedure for the population recovery of biological indicators (BI) on carriers differs for different types of biological indicators, particularly which way the sample is extracted. Important is to extract the sample in such a way that all spores of the BI are ideally separated from each other and undamaged after the separation process. A damage of spores by ultrasound or mixer treatment is not known. Used solvents for dilution, close attention to a heat-shock promoting germination and fresh nutrient agar of good quality are very important variables of a good population recovery. When carrying out the dilution series, glass vessels should be used, since spores can be deposited on the walls of plastic vessels due to the high hydrophobicity of the plastic.

When using suspensions, no solubilization is necessary, but when working with them, any increase in temperature to room temperature may be associated with a loss of population. Once the suspension is dried on carriers, this problem no longer exists. Therefore, the use of a cool box or working on ice is generally recommended when working with suspensions to minimize population loss.

Extraction of the sample depending on the material:

Spore carriers made of materials that can be shredded with a mixer or Turrax: e.g. strips, threads or discs of paper and glass fiber (Tyvek, PET, other material etc. depending on the capacity of the shredder)

Aseptically remove at least 4 untreated **carriers (e.g. spore strips)** from the glassine envelope and place them in a sterile high-speed mixer with 100 ml sterile water and homogenise for 3-5 min until no larger particles are visible in the suspension. If a mixer is not used (e.g. replacement by glass beads), experience shows that smaller populations are obtained. A subsequent use of an ultrasonic bath can have a very positive effect on the recovery, as potentially aggregated spores are separated.

Spore carriers made of materials that cannot be crushed using a mixer or Turrax (if no mixer is available): e.g. strips or discs made of stainless steel, solid glass carriers or plastic materials (Tyvek can also be difficult to extract)

Soak at least 4 untreated carriers (e.g. spore strips) for at least 10 min and if possible, stir in the liquid with a sterile magnetic stirrer. Afterwards, treat in an ultrasonic bath for 10 min and then mix thoroughly using a vortex or treat again for another 10 min with a magnetic stirrer. With all solid carriers, such as metal strips or discs (PET/Tyvek etc.), if the mechanical action during the removal process is too low, there may arise problems of insufficient population recovery. It can be solved very well by adding detergents which do not influence the growth. In internal tests, small amounts of Tween 80 or the reagent Fluid D (0,1 % Peptone, 01 % Tween 80, pH 7,1) have proven to be very good alternative. [Caution! Despite its good suitability, this method conflicts with USP 55, which recommends a recovery in sterile water.]

Spore suspensions and Stearo-Ampoules

Biological indicator suspensions and Stearo-Ampoules with growth medium contain spores already in suspension and can be used directly after vortexing. However, before dilution there should also be a short ultrasound treatment of 5 min to ensure that spore agglomerates are separated.

Special case samples coming from LTSF processes
If spore strips (or other biological indicators [BI]) have been exposed to formaldehyde (e.g. for testing according to a formaldehyde sterilisation procedure), the BIs (e.g. strips) must be post-treated before population determination in order to remove them from growth-inhibiting ("masking") formaldehyde residues. For this purpose, the sterilised spore strips are transferred aseptically to 2% Na_2SO_3 solution for 10 min and then are heat-activated at 90-93°C for one hour. Then the strips are homogenised as described above. A further heat-shock treatment (see below) is no longer necessary. For further information see DIN EN ISO 11138-5 or Gömann et al.: Reaction kinetics of the low temperature steam formaldehyde (NTDF) sterilisation process, Zentr.Steril. 2000, 8 (5): 290-296.

Dilution of the (prepared) suspension

According to USP (United States Pharmacopeia) with sterilised, distilled and cooled (2-8°C) water a dilution series has to be made in such a way that a concentration of approx. 10^2 CFU/ml (colony forming units/ml) is reached.
(Note: Experience has shown that higher population values are obtained when using cooled (4-8°C) water at all dilution steps)
This is best achieved per dilution step by diluting 1 ml of the initial concentration with 9 ml sterile distilled water (1:10 dilution).

Always make sure that the suspension is homogeneously mixed during dilution. For safety reasons it is recommended to prepare and evaluate 2 different dilution series from the initial suspension (double preparation). If the initial concentration of the samples is only approximately known, several dilution series must be prepared and evaluated. The dilution stage containing a germ concentration of about 30 - 300 CFU/ml is then used for the evaluation.
(Note: a good digestion of the samples has to be ensured. If necessary, ultrasonic treatment of the first 1:10 or 1:100 dilution is recommended before the heat shock is performed (ultrasound intensity of a cleaning bath does not harm the spores)).

Execution with and without heat shock

According to USP, the spores should be treated with a heat shock. It is recommended to treat half of the suspension with subsequent temperatures and times and leave the rest untreated. The heat shock represents a germination signal for the spores and should actually lead to a higher recovery. In the case of *B. atrophaeus*, however, a smaller population may also result from the heat shock treatment, as the spores have little resistance to moist heat - this does not represent a deficiency. The population data on the GKE certificate always refer to the recovery after heat shock treatment.

- *Bacillus pumilus* and *Bacillus atrophaeus* at 80 - 85°C for 10 min exposure time
- *Geobacillus stearothermophilus* at 95 – 100°C for 15 min exposure time

Here the warm-up time of the samples must be taken into account, so that effectively longer times than 10 minutes or 15 minutes are required (the exposure time starts when the lower exposure temperature is reached).
(Note: The time of the heat shock definitely starts when the specified temperature values are reached (80°C for *B. atrophaeus* or 95°C for *G. stearothermophilus*). In order to be able to reach the indicated temperature bands within the test vessels at all, a rather higher temperature setting value of the bath is recommended, especially for *G. stearothermophilus* (e.g. 99°C setting value).

After the treatment cool the suspensions quickly in an ice water bath (0-4°C).

Plating of the samples

Place 1 ml of each of the heat-treated and untreated suspensions in different Petri dishes and pour 15-20 ml of liquid TSA (Tryptic Soy Agar) adjusted to approx. 45°C over each one. Mix the solutions as quickly as possible by evenly rotating the agar plate. Each preparation should be determined three times.
(Note: For incubation always use fresh agar, which has only been boiled up once in total. The quality of the agar deteriorates with repeated boiling, which is very likely to result in a low recovery. Different manufacturers offer different culture media for spores (GKE uses TSA from BD)).

As negative control, prepare another Petri dish with used sterile water and as positive control, prepare another Petri dish with a suspension of known population.

Incubate all plates for 48 hours at optimal growth temperatures:

- *Bacillus pumilus* and *Bacillus atrophaeus* at 33 - 37°C
- *Geobacillus stearothermophilus* at 55 - 60°C

(Note: If the wrong temperature is selected, no growth is possible. Insufficient incubation time below 48 h provides worse results).

After incubation, count the bacterial colonies on the plates, which should be between 30 and 300 CFU, and determine the average population of the three treated plates. Plates with less than 10 colonies should not be included in the calculation because of possible surface absorption effects and the associated errors. Care should be taken to ensure that the heat-shock-treated plates are evaluated separately from the untreated plates when calculating the average population.

Finally, the population of the biological indicator can be calculated with the counted CFU per agar plate, taking the dilution factor into consideration.

Compare the obtained data of the untreated and the heat-shock-treated incubation results. It is possible that the heat shock treatment leads to higher recovery rates than the results without heat treatment, because the heat exposure is a germination signal. The population numbers stated on GKE certificates are determined heat shock and incubation according to USP. According to DIN EN ISO 11138-1 the recovery rate of spore strips must be in the range of -50/+300%, based on the values provided in the certificate of the batch.