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RESEARCH

Validation of Milliflex® Quantum for Bioburden Testing of Pharmaceutical Products

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ABSTRACT: This article reports the validation strategy used to demonstrate that the Milliflex® Quantum yielded non-inferior results to the traditional bioburden method. It was validated according to USP <1223>, European Pharmacopoeia 5.1.6, and Parenteral Drug Association Technical Report No. 33 and comprised the validation parameters *robustness, ruggedness, repeatability, specificity, limit of detection and quantification, accuracy, precision, linearity, range,* and *equivalence in routine operation.* For the validation, a combination of pharmacopeial ATCC strains as well as a broad selection of in-house isolates were used. In-house isolates were used in stressed state. Results were statistically evaluated regarding the pharmacopeial acceptance criterion of ≥70% recovery compared to the traditional method. Post-hoc test power calculations verified the appropriateness of the used sample size to detect such a difference. Furthermore, equivalence tests verified non-inferiority of the rapid method as compared to the traditional method. In conclusion, the rapid bioburden on basis of the Milliflex® Quantum was successfully validated as alternative method to the traditional bioburden test.

KEYWORDS: Bioburden testing, Rapid microbiological methods, Validation, Non-inferiority test, Compendial method.

LAY ABSTRACT: Pharmaceutical drug products must fulfill specified quality criteria regarding their microbial content in order to ensure patient safety. Drugs that are delivered into the body via injection, infusion, or implantation must be sterile (i.e., devoid of living microorganisms). Bioburden testing measures the levels of microbes present in the bulk solution of a drug before sterilization, and thus it provides important information for manufacturing a safe product. In general, bioburden testing has to be performed using the methods described in the pharmacopoeias (membrane filtration or plate count). These methods are well established and validated regarding their effectiveness; however, the incubation time required to visually identify microbial colonies is long. Thus, alternative methods that detect microbial contamination faster will improve control over the manufacturing process and speed up product release. Before alternative methods may be used, they must undergo a side-by-side comparison with pharmacopeial methods. In this comparison, referred to as *validation,* it must be shown in a statistically verified manner that the effectiveness of the alternative method is at least equivalent to that of the pharmacopeial methods. Here we describe the successful validation of an alternative bioburden testing method based on fluorescent staining of growing microorganisms applying the Milliflex® Quantum system by MilliporeSigma.

1. Introduction

In the pharmaceutical industry, the term *bioburden* often comprises the enumeration of microbial bioload in the bulk solution (compounding solution) prior to microbial reducing filtration, before sterile filtration, and/or before heat sterilization. Several definitions of bioburden are found in the current regulatory documents (1). For example, the U.S. Food and Drug

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Administration (FDA) Guidance for Industry (2) defines bioburden as "the total number of microorganisms associated with a specific item prior to sterilization".

Traditionally, bioburden is analysed by the membrane filtration method according to European Pharmacopoeia (Ph. Eur.) 2.6.12 (3) or USP <61> (4), but also the plate count or most probable number (MPN) method can be applied. As these methods are described in the compendial chapters of the pharmacopeia, they are regarded as validated. Alternative methods may be applied provided that non-inferiority to the compendial methods has been demonstrated. To this end, validation criteria of the Ph. Eur. 5.1.6., USP <1223>, or PDA Technical Report No. 33 (5–7) may be used to demonstrate non-inferiority. Overviews on these chapters, which were currently updated, are given, for example, by Miller (8, 9).

Health authorities encourage pharmaceutical manufacturers to use rapid or alternative microbiological methods (RMMs), especially for in-process testing (10–13). RMMs have several advantages compared to traditional methods such as faster product release, faster reaction time to non-compliance or deviations, and increased automation, resulting in a better control of the manufacturing process (e.g., 14, 15). The aim of the current work was to find a suitable RMM system for the enumeration of bioburden of parenteral drug product bulk solutions.

Several applications are available on the market that may potentially be used to perform bioburden testing—for example, autofluorescence using Growth Direct™ from RapidBioMicrosystem, adenosine triphosphate (ATP) bioluminescence using Milliflex® Rapid system from MilliporeSigma (a trademark of Merck KGaA, Darmstadt, Germany)—for an overview see reference 16. Another possibility is the Milliflex® Quantum where incubated samples are stained using carboxyfluorescein diacetate (CFDA), which is a non-fluorescent substrate that is cell membrane–permeable. Within the cell the CFDA is cleaved by unspecific intracellular esterases to carboxyfluorescein, which is a green-fluorescent that can be excited with blue light (λ = 488 nm). Because carboxyfluorescein is only poorly membrane-permeable, the fluorochrome is accumulated and retained within viable cells. The fluorochrome is excited within the Milliflex® Quantum Reader, and colonies of metabolically active microorganisms can be counted visually by the analyst, that is, there is no automated enumeration by the system. The read-out is, as for the traditional method, in colony-forming units (CFU). Because of the bright fluorescent signal, colonies can be seen at smaller sizes, resulting in a reduced incubation time. Prestudies indicated that the benefits of the Milliflex® Quantum would be a reduced incubation time from 3–7 days—the traditional method according to Ph. Eur. 2.6.12 or USP <61> (3, 4)—to 72 h and still provide accurate, valid results that would enable investigations to be rapidly initiated when required. The staining with CFDA is a well-described and widely applied technique (see, e.g., references 17, 18) that can be considered non-destructive to the largest extent if the dye is used at the proper concentration (19). There are, however, also reports indicating that CFDA may have an impact on replicating cells (20), especially when used at inadequate concentrations. Furthermore, the fluorescence signal could outshine adjacent smaller colonies, as observed for ATP bioluminescence methods (M. Goverde, personal observation). Therefore, the staining as such, in correlation with the incubation time, must be validated in order to avoid too low enumeration results.

A first evaluation of the Milliflex® Quantum by MilliporeSigma found it to be suitable for bioburden testing of bulk solutions. Although it still enumerates CFUs by human eye and is thereby comparable to the traditional method (i.e., membrane filtration method and same growth media), it was decided to validate the Milliflex® Quantum method with all validation criteria according to the pharmacopeial recommendations because two relevant factors were changed: (1) the incubation time was reduced in our case from 5–7 days to 72 h and (2) samples are stained with a chemical that could influence microbial growth. To our knowledge this is the first publication on the validation of Milliflex® Quantum used for a more rapid enumeration of bioburden according to pharmacopeial validation criteria.

2. Methods

2.1. Equipment and Materials

The equipment and expandable materials used were purchased from MilliporeSigma (Lyon, France) and are listed below (the catalogue number is given in parentheses). For both the traditional bioburden (TBB) test and the rapid bioburden (RBB) test, the

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TABLE I
List of All Isolates Used for Specificity. For In-house Strains the Method of Stress Is Indicated

Species	Strain	Stress	Agar
Pseudomonas aeruginosa	ATCC 9027	No stress	TSA
Staphylococcus aureus	ATCC 6538	No stress	TSA
Bacillus subtilis	ATCC 6633	No stress	TSA
Escherichia coli	ATCC 8739	No stress	TSA
Burkholderia cepacia	ATCC 25416	No stress	TSA
Stenotrophomonas maltophilia	In-house	Heat stress: 60 °C, 2 min	TSA
Bacillus idriensis	In-house	At least 7 days nutrient starvation	TSA
Bacillus clausii	In-house	At least 7 days nutrient starvation	TSA
Staphylococcus epidermidis	In-house	Heat stress: 60 °C, 2 min	TSA
Staphylococcus warneri	In-house	Heat stress: 60 °C, 3 min	TSA
Pseudomonas stutzeri	In-house	Heat stress: 60 °C, 2 min	TSA
Kocuria rhizophila	In-house	Heat stress: 70 °C, 2 min	TSA
Aspergillus brasiliensis	ATCC 16404	No stress	SDA
Candida albicans	ATCC 10231	No stress	SDA
Penicillium sp.	In-house	Heat stress: 50 °C, 3 min	SDA

same nutrient media cassettes and filter funnels were used:

- Milliflex® Quantum Reader (MXQREAD01)
- Milliflex® Quantum membrane transfer tool with seal (MXQTRT001, MXQSEAL01)
- Removal rack with liquid media cassette adapter (REMRACK01, REMSPARE1)
- Milliflex® Plus Pump (MXPPLUS01)
- Milliflex® Plus Pump Head (MXPHEAD01)
- Milliflex® cassettes with tryptic soy agar (TSA) (MXSMCTS48)
- Milliflex® cassettes with Sabouraud dextrose agar (SDA) (MXSMCSD48)
- Milliflex® HA 100 mL funnel unit (0.45 µm cellulose esters membrane) and fluorescent reagents (MXQTV0KT1)
- Milliflex® liquid cassettes (MXLMC0120)

As inoculum and rinsing fluid a solution with 1 g Bacto Peptone (No. 211677 from Becton Dickinson, Franklin Lakes, NJ) in 1000 mL water purified at a pH of 7.1 ± 0.2 was used.

2.2. Inoculum

For the validation of Repeatability BioBall™ Single Shot from *Bacillus subtilis* (catalogue no. 56024, bioMérieux, Marcy-l'Étoile, France), *Pseudomonas aeruginosa* (catalogue no. 56040, bioMérieux) and *Aspergillus brasiliensis* (catalogue no. 56022, bioMérieux) were used. These three strains were used to represent Gram-positive bacteria, Gram-negative bacteria, and a mould.

For other validation parameters in-house prepared strains were used in a stressed state to represent worst-case scenarios. The stress protocols (e.g., by application of heat or by nutrient depletion) are described in Gray et al. (21). As shown by Gray et al., application of heat stress led to the most reproducible delay before start of growth and reduction in microbial numbers for non-spore forming microorganisms, as compared to other stress conditions (e.g., chemical stress). For bacteria that could form endospores, spore formation was triggered by nutrient starvation and cold stress, and the resulting spore suspensions were used for the experiments. Table I gives an overview of strains used and the stress parameters applied.

2.3. Workflow of Milliflex® Quantum and Traditional Bioburden (TBB) Test

Milliflex® Quantum: Filtration of the sample was performed in a laminar air flow cabinet (LAF). All

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materials used and the Milliflex® Plus Pump were disinfected with 70% ethanol, while the head of the Milliflex® Plus Pump was autoclaved. The filtration was performed in the LAF using the "dry-out" function of the Milliflex® Plus Pump. If indicated, the membrane was rinsed according to the adequate rinsing protocol. Then the filter funnel was closed, removed from the Milliflex® Plus Pump, and transferred onto a Milliflex® agar cassette. The cassettes containing SDA were incubated at 20–25 °C, and the ones containing TSA at 30–35 °C, for a maximum of 72 h (if not required differently by the experimental setup, e.g., for *robustness*).

After incubation, the staining was performed using the Milliflex® Quantum Membrane Transfer Tool, a removal rack which assists in opening the Milliflex® cassettes harboring the membranes, and the staining reagent. The stained filters were incubated for 30 min at 30–35 °C (if not required differently by the experimental setup). Then the enumeration of the bright spots, representing CFDA-stained colonies, with the Milliflex® Quantum was performed manually and documented on worksheets [good manufacturing practice (GMP) documents].

If indicated, the membrane was further incubated. This was necessary for verification of non-destructiveness of the staining, and may also be necessary for identification of the contaminants in routine use of the method. To that end, the membrane was separated from the liquid media cassette using the removal rack, transferred onto a fresh agar cassette, and further incubated under the already previously used incubation conditions.

A negative control with the rinsing fluids was carried out per test session. This was done to check whether the sterilization of the fluids and the working technique of the analyst were adequate.

Traditional Bioburden (TBB) Test: The TBB test was performed as described in USP <61> and Ph. Eur. 2.6.12 (3, 4) using the membrane filtration method. The preparation was the same as described above for the Milliflex® Quantum. The same microbial suspension in the rinsing solution was filtered (and rinsed if needed), and the filter funnel was closed and removed from the MX Plus Pump. Subsequently, the filter funnel was transferred onto a Milliflex® agar cassette. Following placement of the membrane on the agar cassette, all removable parts of the filter funnel were removed, and the cassette now harboring the filter membrane was closed with its lid. The cassettes containing SDA were then incubated at 20–25 °C and the ones containing TSA at 30–35 °C. In order to validate the rapid method in a worst-case approach, TSA cassettes were incubated at least 5 days and SDA cassettes at least 7 days during the validation (routine conditions are TSA for 3–5 days and SDA for 5–7 days). After incubation, the colonies were visually counted with the human eye with intermediate readings, if necessary. The count to be reported was the one with the highest amount of colonies.

2.4. Risk–Benefit Analysis

A theoretical comparison of the compendial membrane filtration method according Ph. Eur. 2.6.12 or USP <61> (3, 4) and the Milliflex® Quantum method was performed in order to identify differences. Each single step of the analysis was listed for both methods, and based on this comparison it was decided whether the difference required validation or not.

2.5. Validation Parameters

The TBB test served as reference method against which the RBB test was validated. The aim of the validation was to demonstrate that the RBB test was an appropriate alternative method to the TBB. This was achieved by satisfying the defined validation parameters from USP <1223>, Ph. Eur. 5.1.6, and PDA Technical Report No. 33 (5–7). Validation was deliberately performed without focusing on a particular type of product. Thus, most experiments were performed without using any product (an exception was the validation parameter *equivalence*).

1. ***Robustness:*** *Robustness* describes the reliability of the method in routine use. The application of small but deliberate variations in method parameters must not lead to significantly different results. *Robustness* towards different incubation times and different staining times was evaluated by the supplier. However, validation of these parameters was repeated because different incubation and staining times were assessed in the present validation as compared to the supplier validation. An incubation time of 72 h minus 2 h or plus 48 h, and a staining time of 30 min minus 15 min or plus 30 min, were validated. For validation of both parameters, a low inoculum (10–100 CFU) of the microorganisms of interest was inoculated in 100 mL of the rinsing

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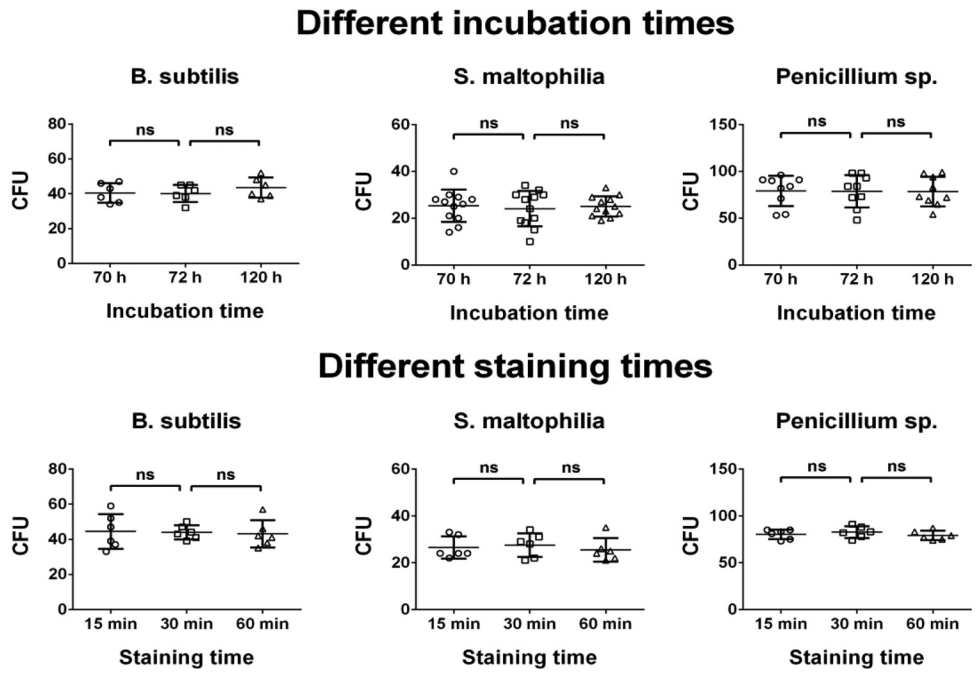

Figure 1

Results for the validation parameter *robustness* for different incubation times (upper graphs) and staining times (lower graphs) for the three test strains. The sample size (N) was in general 6, it had to be increased for validation of different incubation times for *S. maltophilia* (N = 12) and for *Penicillium* sp. (N = 9) in order to reach sufficient test power.

solution. The whole 100 mL volume was then filtered and processed as described above (see paragraph 2.3). Slow- or fast-growing microorganisms, from either from large or small colonies, were used: *Bacillus subtilis* ATCC 6633, *Stenotrophomonas maltophilia* (in-house isolate, heat stress at 60 °C for 2 min), and *Penicillium* sp. (in-house isolate, heat stress: 50 °C for 3 min). For all used microorganisms, the inoculum was 10–100 CFU. Two test runs with three replicates per microorganism and incubation time or staining time were used, respectively. However, the sample size was increased if the statistical test power was below 0.8 (see below paragraph 2.6 and Figure 1). As the obtained results were consistent with the assumption of normally distributed data, results were evaluated using a 2-sample t-test at a confidence level of 95%. The acceptance criteria were no statistically significant difference in CFU count between the lower and the standard incubation or staining time, and no statistically significant difference in CFU count between the higher and the standard incubation or staining time using the 2-sample t-test (see paragraph 2.6 for further details).

2. ***Ruggedness:*** *Ruggedness* describes the reproducibility of test results under different routine circumstances (i.e., alteration of analysis parameters that represent unavoidable changes). *Ruggedness* is normally expressed as the lack of influence of operational and environmental variables on the test results of the microbiological method. Both Ph. Eur. 5.1.6 and USP <1223> (5, 6) state that *robustness/ruggedness* determination is best suited to demonstration by the supplier of the method. *Ruggedness* was shown by the supplier, Merck KGaA, and described in the supplier method validation summary. *Ruggedness* parameters covered by the supplier were different filter funnel lots, media lots, analysts, instruments, and staining reagent lots. In conclusion, results generated by the supplier on *ruggedness* parameters were reviewed and evaluated to be sufficient. No further experiments were dedicated to show *ruggedness.*

3. ***Repeatability:*** *Repeatability* describes the reproducibility of test results under near-identical conditions: analysis of samples under routine circumstances (performed by the same analyst using the

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same equipment and, if possible, the same consumable lots) at different times of day and on different days. The experimental procedure was the same as for *robustness* (see above) but without variation of incubation or staining time. The following microbial strains were used: *Bacillus subtilis* NCTC 104001, *Pseudomonas aeruginosa* NCTC 129241, and *Aspergillus brasiliensis* NCPF 22751 using BioBall™. BioBall™ were used since they have an extremely precise and consistent mean CFU value. In total, four runs (two in the morning and two in the afternoon each on a different day) with five replicates per run and microorganism were performed. Acceptance criterion was that there should be no statistically significant difference between the different test runs for each strain using a one-way analysis of variance (ANOVA) at a confidence level of 95% (for further details on statistics see paragraph 2.6).

4. ***Specificity:*** The *specificity* of a method is defined as the potential to detect a broad range of microorganisms. For the Milliflex® Quantum, *specificity* was demonstrated for 15 different microorganisms, including Gram-negative rods, Gram-positive spore-forming bacteria, Gram-positive cocci, yeasts, and molds (Table I). All in-house isolates were stressed (see Table I). The chosen isolates can be considered representative for any pharmaceutical manufacturing operation site. The procedure of analysis with the Milliflex® Quantum was the same as described under *robustness* but without variation of incubation or staining time (see above). In addition, membranes were transferred onto fresh Milliflex® cassettes after reading and were re-incubated at 20–25 °C or 30–35 °C for another 4 days and colonies were re-counted by unaided eye (qualified analyst). The latter was performed to check if the staining had any negative effect on microbial viability. For the TBB, testing was performed as described in paragraph 2.3 using the same filtration volume and inoculum as for the RBB test. Two test runs with three replicates per microorganism were made. The CFU count of the RBB test should not be statistically different as compared to the TBB test (except the rapid method yields superior results). For each strain, a 2-sample t-test at a confidence level of 95% was performed. Furthermore, the CFU counts obtained for all strains were pooled and a non-inferiority test for log-normal distributed data at a confidence level of 95% with a non-inferiority boundary of 70% was applied. To analyze for the effect of the staining, a paired t-test at a confidence level of 95% was used (for further details on statistics see paragraph 2.6).

5. ***Limit of Detection* and *Quantification:*** The *limit of detection* is defined as the lowest number of microorganisms that can be detected under the stated experimental conditions. The *limit of quantification* is the lowest amount of microorganisms that can be accurately counted with acceptable *accuracy* and *precision* under the stated experimental conditions. The *limit of quantification* was not in the scope for validation of the Milliflex® Quantum because, like for the traditional method, enumeration of colonies is also executed by an operator. Therefore, the *limit of quantification* has to be the same for the RBB test as for the TBB test.

In general, for the bioburden for parenteral products an acceptance criterion of 10 CFU/100 mL is often used (22), but it can also be higher depending on the application (e.g., biotech active pharmaceutical ingredients). Therefore, a detection limit of at least 5 CFU would be appropriate for the intended application and still leave an ample safety margin.

In order to investigate the *limit of detection,* 100 mL aliquots of the rinsing solution were inoculated with approximately 50, 5, 0.5, and 0.05 CFU of the microorganisms of interest. The samples were tested for microbial burden using the TBB test and the RBB test in parallel (see paragraph 2.3 for further details).

The selection of microorganisms used was intended to cover a broad range of possible contaminants: a slow-growing Gram-negative rod, a Gram-positive coccus, a spore-forming Gram-positive rod, a small size Gram-negative rod, a mold, and a yeast (see Figure 3 for species names). All in-house isolates were stressed as indicated in Table I, except *Brevundimonas diminuta* (which was an in-house water isolate).

Two test runs with 10 replicates per concentration and microorganism were performed. For each concentration of each test method, the number of replicates that showed microbial growth was recorded. A 10-replicate MPN table was used to determine the upper and lower confidence limit of the MPN for each method according to USP chapter <1223> (6) using the MPN table from the FDA Bacteriological Analytical Manual, Appendix 2 (23). The three highest dilutions of the microbial solution were used

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for evaluation, unless the highest dilution did not show any microbial growth. In that case, the three lowest dilutions were used for determination of the confidence limits of the MPN. If the confidence limits (95%) obtained for both methods overlapped, it was concluded that no statistical difference regarding the *limit of detection* exists (for further details on statistics see paragraph 2.6).

6. ***Accuracy, Precision, Linearity,*** **and** ***Range:*** The *accuracy* of the microbiological method is the closeness of the actual test results obtained with the RBB test compared to the TBB test. It may also be expressed as the percentage recovery of microorganisms with the alternative method as compared to the traditional method.

The *precision* of a microbiological method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of laboratory microorganisms across the range of the test. The *precision* of a microbiological method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation).

The *linearity* of a microbiological test method is its ability to ensure that results are proportional to the concentration of microorganisms present in the sample within a given range.

The *range* is the maximum level of microorganisms that can be accurately counted and was determined based on the *linearity* data.

As for *limit of detection,* 100 mL aliquots of the rinsing solution were inoculated with approximately 200, 100, 30, 10, and 5 CFU of the microorganisms of interest. The samples were tested for microbial burden using the TBB test and the RBB test in parallel. As microorganisms, fast-growing Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538) and slow-growing Gram-negative bacteria (*Stenotrophomonas maltophilia* in-house isolate, heat-stressed at 60 °C for 2 min) were used in three test runs with five replicates per concentration of microorganism.

For satisfying the validation parameter *accuracy,* the count obtained with the RBB test should not be statistically significantly different from the TBB (except the rapid method yields superior results) using the 2-sample t-test at a 95% confidence level for each dilution of each microorganism. This was possible because the obtained data appeared to be normally distributed. The *precision* of the RBB test as compared to the TBB test was assessed using a test for equal variances (Bartlett's test) at a 95% confidence level for each dilution of each microorganism. The RBB test should not have statistically significantly different variance as compared to the traditional method (except if variance is significantly smaller). For *linearity,* linear regression analysis of the data obtained (mean of the 15 replicates per concentration and microorganism obtained with the RBB test or the TBB test) was performed. A correlation coefficient of $r^2 = 0.95$ or better and a slope ranging from 0.8 to 1.2 was considered satisfactory (for further details on statistics see paragraph 2.6).

7. ***Equivalence:*** The objective of this test was to demonstrate non-inferiority of the alternative method based on the Milliflex® Quantum RBBtest in comparison to the TBB test under routine circumstances. To that end, three different product solutions were tested with both methods in parallel, using the product-specific rinsing protocol previously defined in suitability tests. Because bioburden samples typically do not harbor high numbers of microorganisms, samples were manually inoculated with different microorganisms prior to test performance. Manual inoculation was necessary, as a comparison of mainly zero values does not allow assessment of whether equivalence of the methods is given. Inoculation was performed with mixed cultures of two to four different strains from Table I with 10–100 CFU.

For each of the three product solutions, two test runs with 15 samples each were conducted. The mixed cultures used for inoculation of the samples were varied within each test run; the 15 samples were split in groups of five samples, which were inoculated with a different mixed culture each. Filtration and incubation was performed as described in paragraph 2.3.

Statistically significant equivalence between the RBB test and the TBB test using a non-inferiority test at a confidence level of 95% with a non-inferiority boundary of 70% was used for statistical evaluation. Calculation was performed for each product solution and each incubation condition (20–25 °C on

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SDA and 30–35 °C on TSA). For all but one data set (product no. 3, SDA 20–25 °C), the obtained results were consistent with the assumption of normally distributed data. For product no. 3, SDA 20–25 °C, assumption of a negative-binomial distribution provided a better fit (for further details on statistics see paragraph 2.6).

2.6. Statistical Analysis

For statistical analysis Minitab® Release 16 Statistical Software, GraphPad Prism 5, or R 2.8.1 were used. Any data intended for statistical analysis was subjected to a normality test using the Ryan-Joiner test. If the test for normality failed, the data was subjected to a transformation by using the logarithm of the CFU counts. If the data had to be transformed by using the logarithm of the CFU count, zero-values had to be excluded because the logarithm of zero does not exist. If data remained not normally distributed despite log transformation, non-parametric alternatives for the t-test (Mann-Whitney test), paired t-test (Wilcoxon matched pairs test), or the ANOVA (Kruskal-Wallis test) were used.

For experiments that involved spiking with microbial suspensions, outliers were identified with Grubb's test for outliers and excluded from any calculations (including normality tests).

In order to compare two groups, the 2-sample t-test or the paired 2-sample t-test was used. The latter was used when the individual values of the two data sets were strictly dependent on each other. For example, this test was applied to compare counts from the same plate with the Milliflex® Quantum and by eye after re-incubation. For a comparison of more than two groups of data, the ANOVA test was used instead of a t-test.

In order to assess if two methods have a comparable *precision,* tests for equal variance were applied. For normally distributed data, Bartlett's test was used. For not normally distributed data, its non-parametric counterpart, Levene's test, was used.

In order to statistically assess equivalence between the alternative method and the traditional method, the non-inferiority test was used with a lower boundary of 70% as stated in USP chapter <1227> (24). The rapid method and the traditional method are equivalent if the lower limit of the 95% confidence interval of the mean difference in recovery (rapid method compared to traditional method) is completely within the non-inferiority boundary. The non-inferiority test used in this validation requires the assumption that the data follows particular statistical models. For the present validation, the non-inferiority test assuming normal, log-normal, or negative-binomial distributions was used. The most adequate distribution model for the experimental data was chosen before conducting the non-inferiority test.

Finally, test power calculations were used in order to verify adequate sample size for the validation experiments. In reference to USP chapter <1010> (25), a test power of ≥0.8 was regarded as acceptable.

To calculate sample size, standard deviations from pre-experiments were used to estimate sample size required for the validation experiments with a test power >0.8. Furthermore, post-hoc test power analyses were employed to verify that test power to detect a meaningful difference was indeed ≥0.8. These post-hoc test power analyses were based on the data generated in the validation experiments, applying their actual means, standard deviations, and sample sizes. If for a data set post-hoc test power was <0.8, an additional test run was performed and the post-hoc test power calculation repeated taking into consideration the additional data. If post-hoc test power was still insufficient, this process was repeated until sufficient test power was reached.

The largest acceptable difference in microbial recovery between the rapid method and the traditional method was defined as 30% in reference to USP chapter <1227> (24). This means that the rapid method was required to recover at least 70% of the mean microbial count of the traditional method. For all experiments, the confidence level was defined as 95% because 5% possibility of type I error seemed acceptable.

3. Results

3.1. Risk–Benefit Analysis

Table II summarizes the process of analysis for both methods and the identified differences. Two process steps were found to be relevant and require validation: incubation time and evaluation of the test result (including staining time).

TABLE II

Summary of All Relevant Process Steps, Comparing the Traditional Bioburden (TBB) Test with the Milliflex® Quantum System. The Last Column Votes if There Is Any Difference between the Two Methods

Process Step	Traditional Test	Milliflex® Quantum test	Difference
Sampling procedure of bioburden samples	According to internal SOPs	According to internal SOPs	No difference
	Sample must be representative for the whole batch/portion	Sample must be representative for the whole batch/portion	
Test environment	Aseptic working technique in a laminar air flow cabinet	Aseptic working technique in a laminar air flow cabinet	No difference
Method used	Membrane filtration method (most cases) or pour-plate method	Milliflex® Quantum will only be used for filterable products (i.e., membrane filtration method)	No relevant difference
Membrane filters/pore size and application	Membrane filter/funnels with a nominal pore size of 0.45 μm and about 50 mm in diameter applied onto solid nutrient media	Membrane filters/funnels with a nominal pore size of 0.45 μm and about 50 mm (filtration area) in diameter applied onto solid nutrient media	No difference
Membrane filters/composition	Filter material is in general mixed ester cellulose or other suitable filter membrane material	Filter material is in general mixed ester cellulose (standard filtration kit of the supplier) or other suitable filter membrane material	No difference
Type and volume of rinsing fluid(s)	Type and volume of rinsing fluid(s) is determined during suitability tests	Type and volume of rinsing fluid(s) is determined during suitability tests	No difference
Growth media	Solid nutrient media: tryptic soy agar (TSA), Sabouraud dextrose agar (SDA)	Solid nutrient media: tryptic soy agar (TSA), Sabouraud dextrose agar (SDA)	No difference
Growth promotion of the media	Growth promotion is performed according to USP <61> and Ph. Eur. 2.6.12	Growth promotion is performed according to USP <61> and Ph. Eur. 2.6.12	No difference
Sample volume to be tested	1–200 mL for the determination of bioburden (depends on the product)	1–200 mL for the determination of bioburden (depends on the product)	No difference
Revalidation of the bioburden test	After changes which could have an influence on the test result. Each change is evaluated in a change control process	After changes which could have an influence on the test result. Each change is evaluated in a change control process	No difference
Incubation time	Bioburden test samples are incubated for a total of 5–7 days for incubation on SDA and 3–5 days for incubation on TSA	Reduced time period of incubation of at least 72 h.	**Difference must be validated**
Incubation temperature	Bioburden test samples are incubated 20-25 °C for SDA and 30–35 °C for TSA	Bioburden test samples are incubated 20–25 °C for SDA and 30–35 °C for TSA	No difference
Evaluation of the test result	Membranes are visually enumerated and microbial colonies are enumerated by a qualified operator	Membranes are stained with CFDA, which gets converted into the fluorochrome carboxyfluorescein inside viable cells. The fluorescent colonies are enumerated by a qualified operator using the Milliflex® Quantum. Since the Milliflex® Quantum does not harbor an enumeration algorithm, the operator visually counts the fluorescent colonies	**Difference must be validated**

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TABLE II
(Continued)

Process Step	Traditional Test	Milliflex® Quantum test	Difference
Recording of GxP-relevant data	GxP-relevant data is recorded on paper worksheets	GxP-relevant data is recorded on paper worksheets	No difference
Acceptance criteria for bioburden	Action levels depend on the product and range from 10 CFU/100 mL to 100 CFU/1 mL for total viable count	Action levels depend on the product and range from 10 CFU/100 mL to 100 CFU/1 mL for total viable count	No difference

SOP: standard operating procedure.

3.2. Validation Parameters

3.2.1. Robustness: *Robustness* towards different incubation times as well as staining times was successfully demonstrated (Figure 1). The results obtained after 72 h incubation (representing the reference incubation time) were compared to results obtained after 70 h and 120 h incubation, respectively. For the incubation time, the results are not significantly different with shorter and longer incubation times as compared to the reference incubation time of 72 h. Thus, slightly shorter or longer incubation times do not lead to significantly different results. It should be noted that the maximum incubation time covered in this validation is 5 days (120 h), which corresponds to a suitable incubation time for the TBB test. Therefore, staining would not be required in routine testing in such a case, and all possible incubation times longer than 72 h but shorter than 5 days can be considered validated.

It was assessed whether the length of staining has an impact on the obtained results. For staining times ranging from 15 to 60 min, no significant differences were detectable. Therefore, minor deviations from the standard staining time of 30 min, which still are within the validated range, can be considered acceptable. In conclusion, *robustness* towards different incubation times and different staining times was successfully demonstrated.

3.2.2. Repeatability: The validation parameter *repeatability* aims towards demonstration that an alternative method consistently yields similar results if the same experiment is repeated on different days and times of day. For all three tested microorganisms no significant differences were detected (Figure 2). In conclusion, *repeatability* was successfully demonstrated.

3.2.3. Specificity: The results of *specificity* are summarized in Table III. For some strains the sample size needed to be increased due to insufficient test power. Additionally, in three cases one value each was identified as an outlier with the Grubb's test and was therefore excluded from the calculation and statistical analysis (see footnote in Table III). For all but one strain no significant difference between the RBB test and the TBB test was found. *Bacillus subtilis* showed a significant lower mean in the RBB test compared to the TBB test. Nonetheless, the mean recovery of *B. subtilis* by the RBB test reaches 81% of the mean

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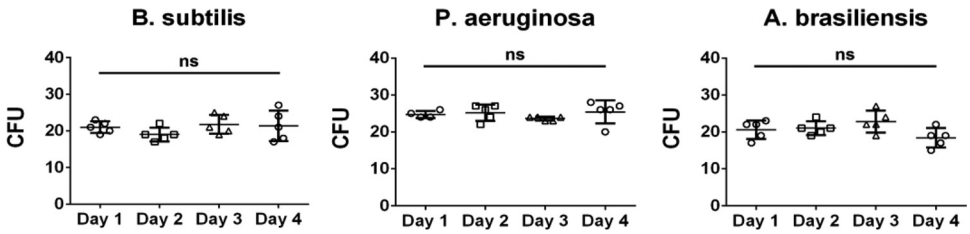

Figure 2

Results for the validation parameter Repeatability. Experiments were performed on four different days for *B. subtilis*, *P. aeruginosa* and *A. brasiliensis*. N = 5 for each day.

recovery of the TBB test, which remains acceptable in microbiological testing where recoveries of 50–200% or 70% are generally acceptable [Ph. Eur. 2.6.12 and USP <1227>, respectively (3, 24)]. In addition, to further investigate this result, a non-inferiority test with the *B. subtilis* data was performed in order to unambiguously demonstrate that recovery by the RBB test is above the specified 70% acceptance criterion. In this test, the mean recovery of the RBB test was 81% while the lower limit of the 95% confidence interval was 74%, that is, the lower limit of the 95% confidence interval was within the 70% boundary (Figure 3). Therefore, statistical proof for non-inferiority is provided. In summary, for each individual strain included in the validation, a sufficient recovery regarding the 70% acceptance criterion was statistically demonstrated.

TABLE III
Summary of the Sample Size, CFU Means (SD = Standard Deviation) for All Strains for the Validation Parameter *Specificity* Including Statistical Calculations. Significant *P*-values Are Printed in Bold. RBB = Rapid Bioburden, TBB = Traditional Bioburden

Species	Sample Size RBB/ TBB	RBB mean ± SD [CFU]	TBB mean ± SD [CFU]	*P*-value	Test Power
Pseudomonas aeruginosa	6/6	33 ± 3	40 ± 7	0.06	0.99
Staphylococcus aureus	5[1]/6	53 ± 4	50 ± 6	0.42	1.00
Bacillus subtilis	8[1]/9	36 ± 4	45 ± 4	**0.00**	1.00
Escherichia coli	9/9	23 ± 5	20 ± 4	0.25	0.93
Burkholderia cepacia	6/6	76 ± 12	72 ± 7	0.53	0.99
Stenotrophomonas maltophilia	6/6	33 ± 7	31 ± 6	0.59	0.82
Bacillus idriensis	6/6	21 ± 2	22 ± 4	0.51	0.97
Bacillus clausii	9/9	54 ± 13	51 ± 14	0.67	0.79[2]
Staphylococcus epidermidis	6/6	33 ± 5	33 ± 6	0.92	0.92
Staphylococcus warneri	9/9	38 ± 8	37 ± 8	0.80	0.87
Pseudomonas stutzeri	6/6	48 ± 7	41 ± 8	0.13	0.95
Kocuria rhizophila	6/6	79 ± 7	77 ± 9	0.64	1.00
Penicillium sp.	6/6	88 ± 12	90 ± 6	0.72	1.00
Aspergillus brasiliensis	6/5[1]	46 ± 7	48 ± 2	0.52	0.99
Candida albicans	9/9	32 ± 7	35 ± 8	0.45	0.90

[1] One value was identified as statistically significant outlier with Grubb's test and therefore excluded from the calculation.

[2] Test power was 0.01 below the specified 0.8 after performing nine replicates. However, the mean CFU recovery of the RBB test was better compared to the TBB test. Thus, no risk that the RBB test could fail the acceptance criterion of 70% recovery compared to the TBB test is present. A test power of 0.79 is therefore regarded as sufficient in that case.

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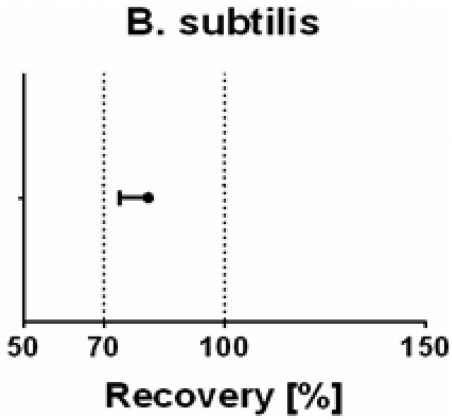

Figure 3

For *B. subtilis* the RBB method was tested against the TBB method by the non-inferiority test at a confidence level of 95% with a non-inferiority boundary of 70%. With this test it could be shown that the difference between the recovery of the RBB lies above the 70% boundary and therefore the recovery of *B. subtilis* is acceptable.

Additionally, the non-destructiveness of the staining procedure for each individual strain included in the validation was verified. No significant differences were detectable when comparing the RBB test count to visual enumeration of the same plates after re-incubation to a total incubation time of 7 days (data not shown).

Taking into account the data generated for all strains, statistical proof of non-inferiority of the RBB test count after less than 72 h of incubation was provided as compared to the traditional method, and as compared to re-incubation of the same plates to a total incubation time of 7 days. This further proves that the reduced incubation time of 72 h for the RBB method on basis of the Milliflex® Quantum is sufficient. In conclusion, *specificity* and non-destructiveness were successfully demonstrated.

3.2.4. Limit of Detection: The results of the *limit of detection* are given in Figure 4. In all cases the 95% confidence intervals of the traditional and the rapid bioburden test did overlap. Thus, no statistically significant difference regarding the *limit of detection* of any of the test strains was observed. In conclusion, an adequate *limit of detection* was successfully demonstrated.

3.2.5. Accuracy, Precision, Linearity, and Range: *Accuracy* was successfully demonstrated over a range from approximately 200 to 5 CFU (Table IV and V). No significant differences in the mean CFU values were detectable between the RBB test and the TBB test. For the lowest concentration of approximately 5 CFU, the test power was below 0.8, because for such low microbial numbers the standard deviation is high in relation to the mean. This represents a typical feature of microbiology since no "half" CFU can exist

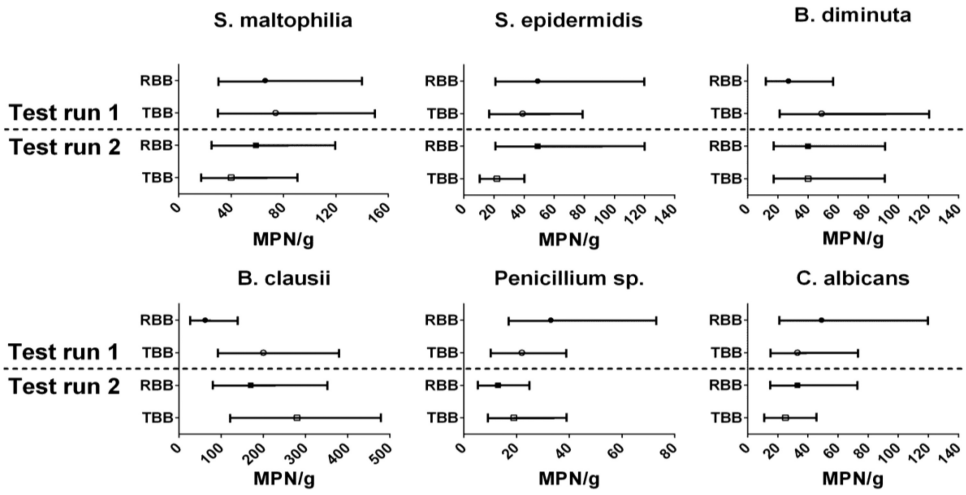

Figure 4

Results for the validation parameter *limit of detection* using the MPN approach. Results for two test runs using six different isolates are shown. MPN = most probable number, RRB = rapid bioburden, TBB = traditional bioburden.

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TABLE IV
Results for the Validation Parameters Accuracy and Precision for *Staphylococcus aureus* with the Statistical Evaluation. For Accuracy the 2-sample t-test and for Precision the Bartelett's Test Was Used. RRB = Rapid Bioburden, TBB = Traditional Bioburden, CFU = Colony Forming Unit, SD = Standard Deviation

S. aureus		Dilution 1		Dilution 2		Dilution 3		Dilution 4		Dilution 5	
		RBB	TBB	RBB	TBB	RBB	TBB	RBB	TBB	RBB	TBB
Sample size		15	15	15	15	15	15	15	15	15	15
Mean ± SD (CFU)		161 ± 27	160 ± 23	85 ± 12	78 ± 15	27 ± 3	28 ± 4	8 ± 3	8 ± 2	4 ± 2	4 ± 2
Accuracy	*P*-value	0.65		0.20		0.53		0.95		0.83	
	Test power	1.00		1.00		1.00		0.81		0.58[1]	
Precision[2]	*P*-value	0.59		0.47		0.33		0.10		0.52	
	Test power	0.81		0.81		0.81		0.81		0.81	

[1] For microbial counts below 10 CFU, the standard deviation becomes extremely high in relation to the mean CFU value. Therefore, test power is by definition very low. Because of the nearly identical mean values, we are of the opinion that *accuracy* was suitably demonstrated and did not further increase sample size.
[2] Statistical data evaluation was not performed for each individual test run, but rather the data from all test runs was pooled. Test power calculations indicated that this was the better approach; otherwise, test power would not have been sufficient in several occasions. By pooling the data, test power and therefore the capability to detect statistical differences was increased.

on an individual agar plate. The coefficient of variation (relation between mean and standard deviation) must by definition become high for low microbial numbers. Because the difference in mean microbial count between the RBB test and the TBB test was very small (only 0.10 and 0.13 CFU, respectively), we are of the opinion that *accuracy* was also demonstrated for low microbial numbers.

Precision was successfully demonstrated, that is, no significant differences regarding the standard deviations of the RBB test and the TBB test were detected (Tables IV and V).

Linearity was successfully shown for both species investigated, that is, both the r^2-values and the slopes of the linear regression lines were within the specified ranges (Figure 5).

In conclusion, *accuracy, precision,* and *linearity* were successfully demonstrated. Furthermore, the obtained data indicated that the operational range of the RBB assay should be the same as for the TBB assay. This is strengthened by the fact that both methods rely on visual enumeration of microbial colonies through a qualified analyst.

3.2.6. Equivalence in Routine Operation: *Equivalence in routine operation* between the RBB test and the TBB test was successfully demonstrated by parallel testing of artificially spiked product solutions with a selection of mixed cultures. Non-inferiority against a 70% boundary was demonstrated through statistics for all product solutions and both growth media (Figure 6). Furthermore, mean microbial recovery was nearly identical between the RBB test and the TBB test in all cases (Figure 6). The mean microbial number was dependent on the mixed cultures used for inoculation, which was particularly visible for SDA, as that nutrient medium does not promote growth of most bacteria. The restrictive growth promotion of SDA was also the reason why for calculation of the non-inferiority test a negative binomial distribution had to be used in one case; because no growth was observed for several mixed cultures consisting only of bacteria, normal distribution in contrast to a negative binomial distribution did not provide a suitable approximation. In conclusion, *equivalence in routine operation* was successfully demonstrated.

TABLE V

Results for the Validation Parameters Accuracy and Precision for *Stenotrophomonas maltophilia* with the Statistical Evaluation. For *Accuracy* the 2-sample t-test and for *Precision* the Bartelett's Test Was Used. RRB = Rapid Bioburden, TBB = Traditional Bioburden, CFU = Colony Forming Unit, SD = Standard Deviation

S. maltophilia		Dilution 1		Dilution 2		Dilution 3		Dilution 4		Dilution 5	
		RBB	TBB	RBB	TBB	RBB	TBB	RBB	TBB	RBB	TBB
Sample size		14[1]	15	15	15	15	14[1]	15	15	14[1]	15
Mean ± SD [CFU]		181 ± 13	188 ± 10	92 ± 11	98 ± 8	27 ± 3	25 ± 4	9 ± 3	8 ± 2	5 ± 2	5 ± 2
Accuracy	*P*-value	0.12		0.14		0.21		0.84		0.89	
	Test power	1.00		1.00		1.00		0.85		0.75[2]	
Precision[3]	*P*-value	0.49		0.29		0.50		0.09		0.70	
	Test power	0.81		0.81		0.81		0.81		0.81	

[1] One value was identified as statistically significant outlier with Grubb's test and therefore excluded from the calculation.

[2] For microbial counts below 10 CFU, the standard deviation becomes extremely high in relation to the mean CFU value. Therefore, test power is by definition very low. Because of the nearly identical mean values, we are of the opinion that *accuracy* was suitably demonstrated and did not further increase sample size.

[3] Statistical data evaluation was not performed for each individual test run, but rather the data from all test runs was pooled. Test power calculations indicated that this was the better approach; otherwise, test power would not have been sufficient in several occasions. By pooling of the data, test power and therefore the capability to detect statistical differences was increased.

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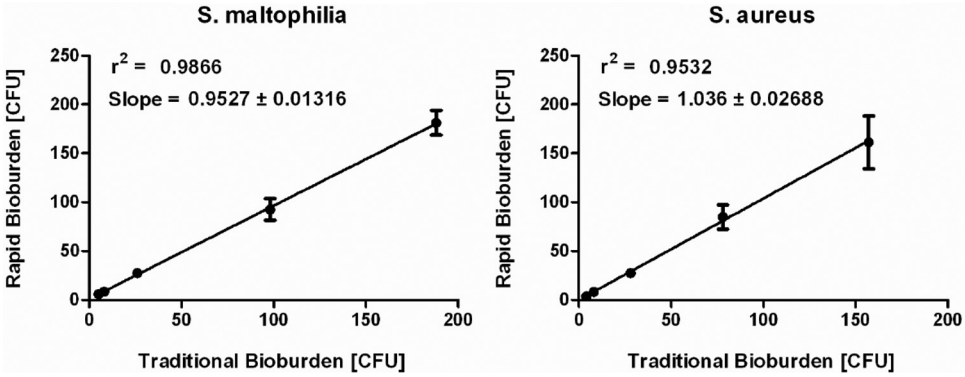

Figure 5

Linear regression analysis for enumeration of different concentrations of *S. maltophilia* and *S. aureus*. For TBB (x-axis), the mean value was used as reference against which the rapid bioburden results were plotted. For rapid bioburden (y-axis), the mean values and standard deviations are indicated. The linear regression line, r^2-value (correlation coefficient), and 95% confidence interval of the slope are shown. CFU = colony-forming units.

4. Discussion

In the present study the Milliflex® Quantum was validated as an alternative microbiological method for bioburden testing of compounding solutions for aseptically produced medical products using the approaches of USP <1223>, Ph. Eur. 5.1.6, and PDA Technical Report No. 33 (5–7). The aim was to reduce the incubation time from 5 to 7 days of the traditional method according USP <61> and Ph. Eur. 2.6.12 (3, 4) to 72 h. Although MilliporeSigma did validate the method and provided a report, the risk–benefit analysis showed that mainly two aspects (incubation time, evaluation of results with staining time) needed further validation. Therefore, a validation with the validation parameters *robustness, ruggedness, repeatability, specificity, limit of detection and quantification, accuracy, precision, linearity, range*, and *equivalence in routine operation* was performed using a combination of pharmacopeial strains as well as a broad selection of in-house isolates. The latter were used in stressed state as indicated by Gray et al. (21). Results obtained with the alternative method were statistically evaluated regarding the pharmacopeial acceptance criterion of ≥70% recovery compared to the traditional method [USP <1227> (24)]. Because statistical non-significance can be due to insufficient sample size, post-hoc test power calculations were used to verify the appropriateness of the used sample size; if indicated, the sample size was increased. The use of test power calculations is highly important in order to not miss significant differences in recovery between the rapid method and the traditional method due to

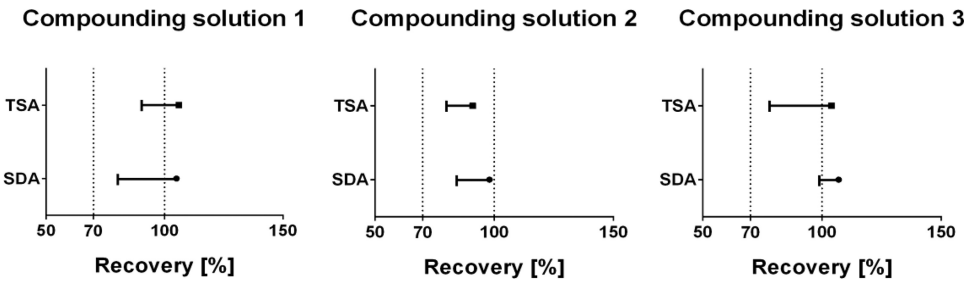

Figure 6

The RBB method was compared to the TBB method for three products that were inoculated with mixed microbial cultures of isolates of Table I for both agar media used (TSA and SDA). The RBB methodnwas tested against the TBB method by the non-inferiority test at a confidence level of 95% with a non-inferiority boundary of 70%. In all cases the RBB method lies above the 70% boundary.

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insufficient sample size. Furthermore, our approach of defining a starting sample size on the basis of preliminary studies and prospective test power calculations, followed by retrospective power analysis and adjustment of the number of replicates if indicated, allows for solid statistical assessment of the study while keeping the manual workload manageable.

For all validated parameters the Milliflex® Quantum showed no statistical lower recovery, thereby showing its suitability to replace the traditional method and to provide the results within 72 h instead of 5 to 7 days. There was one exception that needed further investigation. For *specificity* a significantly lower count of 36 ± 4 CFU compared to 45 ± 4 CFU was found for *B. subtilis* with the Milliflex® Quantum. This difference, however, was smaller than 30% as demonstrated through a non-inferiority test and therefore not relevant in our context. Furthermore, *B. subtilis* is one of the test strains used for the suitability test for a product of interest. Thus, *B. subtilis* would be included in every product-specific suitability study, and capability of the RBB test to detect sufficient *B. subtilis* is verified for each individual product. Therefore, no risk that significant under-recovery of *B. subtilis* would occur through the use of the RBB method is present. For all other Bacilli, no differences were detectable. It is further worth noting that our approach of using a 2-sample t-test for comparing the RBB test and the TBB test regarding *specificity* may not have been ideal. As our validation target regarding the parameter *specificity* was to demonstrate recovery ≥70%, the general use of the non-inferiority test would have been more fitting. However, as the 2-sample t-test may indicate significant differences even if this difference is smaller than 30% (as happened with *B. subtilis*), our validation approach for *specificity* may rather have been overly strict.

Probably one of the most important validation parameters is *equivalence in routine operation.* In the present study three different product compounded solutions were spiked with a mixture of the strains used. We could demonstrate statistically significant non-inferiority of the rapid method as compared to the traditional method. In conclusion, the RBB test on basis of the Milliflex® Quantum was successfully validated as alternative method to the TBB test.

The benefits of Milliflex® Quantum as a rapid method are its ease of use and reliability. It also is a non-destructive method and therefore the isolates can be identified if needed. Furthermore, the initial investments are moderate especially compared to some other RMMs, and the RBB method has a small laboratory footprint. However, the reduction of time is modest, there is more hands-on time and reagents cost than for the TBB method, and finally the read-out is still performed by the analyst. If the Milliflex® Quantum would automatically perform the CFU read-out and offer an interface to a laboratory information management system (LIMS), a much higher benefit would result. These improvements are under evaluation at the supplier to improve the attractiveness of the system for routine use.

Other systems using florescent staining or intrinsic fluorescence were validated or evaluated. Gurramkonda et al. (26) used resazurin to enumerate bacteria. In that case, a hand-held enumeration system was developed for flexibility and speed. However, in their study only *Escherichia coli* was used as test strain and the application is a rapid screen for viable cells followed by the traditional method. Irie et al. (27) show that intrinsic fluorescence can be detected at 1 CFU with the IMD-A from BioVigilant. A comparable system can be used for water testing [IMD-W (Instantaneous Microbial Detection System for Pharmaceutical Waters) from BioVigilant], thus the next step would be to evaluate the ability of IMD-W to enumerate the bioburden in bulk solutions. However, here the challenge is that the IMD-W does not count CFU units, and also viable but non cultivable (VBNC) units are counted as part of the bioburden.

Another application for RBB screening is the use of flow cytometry (e.g., *28, 29*). This is a very rapid method, but there are limitations such as a high *limit of detection* of approximately 100–1000 CFU (29) and also the count of VBNC units. Flow cytometry as well as detection of CO_2 and ATP bioluminescence were evaluated by Hiom et al. (30) for their ability to detect microorganisms in hospital intravenous pharmaceuticals. They found very good correlations between the RMMs and the aerobic microbial count in a time frame of 18 to 72 h. But for all three methods an enrichment phase in broth was needed, and therefore the methods were only qualitative and not quantitative. An interesting system for bioburden testing is the Growth Direct System by Rapid Micro Biosystems. This system is very close to the traditional method, as it also enumerates microbial colonies on a membrane that is incubated on agar. However, the system

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detects colonies consisting of approximately 100 individual cells, as compared to the approximately 10^6 cells the human eye needs to detect a colony. Therefore, CFU are detected much earlier than by eye, and the system is fully automated with a LIMS interface (31).

Finally, microorganisms can be detected by polymerase chain reaction (PCR) within hours using adequate protocols. But depending on the primers used, the detection may be very specific to a narrow microbial spectrum and is so far mostly used for the detection of indicator pathogenic microorganisms (e.g., 32, 33).

Before routine use, product-specific suitability tests are needed in order to demonstrate that the method is suitable for each product of interest. Such a suitability study is comprised of two further points: (1) The *sample effects study* determines whether the product of interest interferes with the RBB assay. To that end, it is demonstrated that the product of interest does not fluoresce within the Milliflex® Quantum Reader to an extent that reliable detection of microbial colonies could be jeopardized. Furthermore, it is demonstrated that the product of interest does not inhibit the staining reaction. (2) Analogous to the TBB test, a suitable rinsing protocol needs to be validated, which prevents that product residues inhibitory for microbial growth remain on the membrane. If these points are fulfilled then the Milliflex® Quantum can be used for routine bioburden testing.

In conclusion, the present work demonstrated that the Milliflex® Quantum is a suitable system to enumerate bioburden in pre-filtration bioburden solutions at a reduced incubation time of 72 h. Validation of the method made use of a broad spectrum of compendial microbial strains as well as stressed in-house isolates. The improved time-to-result is modest but can be of high importance. Attractiveness of the instrument could be further increased with an automated read-out and LIMS interface, thereby reducing hands-on time and increasing data integrity.

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Conflict of Interest Declaration

The authors declare that they have no competing interests. M. Goverde received financial support from Merck KGaA for writing the present publication.

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