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Validation of a Rapid Microbiological Method for the Microbiological Examination of Non-sterile and Nonfilterable Drug Products, APIs, and Excipients

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14.1 Introduction

An alternative rapid microbiological test method based on the Celsis Advance system according to USP <1223>, Ph. Eur. 5.1.6, and PDA Technical Report No. 33 was validated for the microbiological examination of non-sterile and nonfilterable drug products, excipients, and APIs. The alternative test method, which for reasons of simplicity will be referred to as Rapid MET throughout this book chapter, replaces both quantitative and qualitative microbiological testing of non-sterile products by combining both these requirements in a single test.

The current test methods for the microbiological examination of non-sterile products have been harmonized and are described in Ph. Eur. 2.6.12, 2.6.13, USP <61>, <62>, and JP <4.05>/I, <4.05>/II. The incubation conditions for the microbial enumeration tests (METs) are 30–35 °C for 3–5 days for total aerobic microbial count (TAMC), and 20–25 °C for 5–7 days for total yeasts and molds count (TYMC). In addition, tests for specified microorganisms composed of enrichment and followed by one or two selection steps have to be performed in order to demonstrate the absence of specified microorganisms in 1 or 10 g of product. In general, these tests last two to three days and in some cases up to six days. Therefore, microbiological examination of non-sterile products may take up to a week before a final result is available. Shortening the incubation time, however, would enable to align microbiological testing with manufacturing concepts such as LEAN manufacturing. Furthermore, the necessity to perform two quantitative (TAMC/TYMC) and several qualitative tests requires a multitude of different nutrient media and substantial hands-on time through the subculturing steps.

One of the major difficulties concerning rapid microbiological method application to non-sterile products is that these in general are nonfilterable. Most RMM systems on the market enabling precise enumeration are, however, based on membrane filtration (Gordon *et al*. 2011; Miller 2012b). Semiquantitative methods or indirect enumeration methods of nonfilterable products via the detection of by-products from microbial metabolism such as CO_2 have recently been developed (Miller 2012a). However, these methods only provide a rough estimation of the microbial count and pharmaceutical products' quality concepts such as trending or expectation or alert levels may not be applicable.

Another approach would be to perform a direct inoculation of the product and instead of providing a microbial count, a presence/absence result for microbial growth may be obtained. Because it is a presence/absence test, the rapid method should only be implemented for products which have an excellent microbiological quality reflected by a record of usually being negative for microbial growth in routine testing with the compendial method. For such products, both enumeration and specified microorganisms tests could be combined in one single test; if the absence of microbial growth is demonstrated for

1 g product, all specifications are met.[1] Thereby, significant reduction of hands-on time could be achieved.

The Celsis Advance System was selected for this purpose, which detects microbial growth based on ATP bioluminescence and may also be used for nonfilterable products. The reagent kit with which the Celsis Advance is operated in this case (called AKuScreen) amplifies the ATP bioluminescence reaction by the addition of ADP, which is converted to ATP by the cellular enzyme adenylate kinase. Adenylate kinase is present in all known microorganisms, and catalyzes conversion of ADP to ATP. Thus, if the enzyme is present in the sample, addition of excess ADP leads to formation of more ATP and hence amplification of the ATP bioluminescence signal. Detection by ATP bioluminescence requires a much smaller amount of microorganisms than visual detection, therefore significantly reducing the necessary incubation times. Intensity of ATP bioluminescence is expressed as relative light units (RLU).

14.1.1 Workflow Rapid MET

Different growth conditions were compared during feasibility studies and the following test setup for routine was defined based on the obtained results:

- 10 g of the product tested is dissolved in 90 ml of dilution buffer. Dilution technique and buffer composition as used for the compendial test methods may be applied.
- 90 ml liquid nutrient medium (TSB supplemented with 4% polysorbate 80 + 0.5% soy lecithin), ~10 g sterilized glass beads of approximately 1 mm diameter, and a sterile magnetic stir bar are added into each of two bottles. 10 ml of the product dilution (corresponding to 1 g drug product/excipient) are transferred into each bottle. These two bottles are the test samples for the Rapid MET with the Celsis Advance. Supplemented TSB was selected because it has been proven superior in recovering spiked microorganisms in a large variety of product-specific method suitability tests (D. Roesti personal observation).
- One bottle is incubated at 20–25 °C, the other one at 30–35 °C. The incubation time is at least 72 hours. Studies including a wide variety of different stressed in-house isolates indicated that this incubation time ensures detection of all relevant strains tested.

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1 In case that a product required the absence of *Salmonella*, either 10 g product had to be used for the Rapid MET or the compendial test for the absence of *Salmonella* had to be performed in parallel to the rapid method. In general, the absence of *Salmonella* is mostly required for products from natural origin. It is of note, however, that most of such products are expected to have a high bioload and therefore be unsuitable for the Celsis Advance System Rapid MET application.

- After the incubation period, the bottles are transferred onto magnetic stirrers and stirred for 20 minutes in order to disperse aggregating microorganisms.[2]
- 50 µl of the samples are measured in duplicate with the Celsis Advance System.

14.1.2 Evaluation of Test Results

Microbiological acceptance criteria are based on Ph. Eur. 5.1.4, USP <1111>, and JP G.4. The compendial method complies, if the microbial limits of the drug product/excipient of interest are not exceeded in the TAMC and TYMC, and if the specified microorganisms are not detected. In the Rapid MET, 1 g of product is evaluated for microbial growth with the Celsis Advance and if no microbial growth is detected, product may be released for MET and absence of specified microorganism in 1 g product (except for *Salmonella*). In case that microbial growth is detected in the Rapid MET with the Celsis Advance, the contaminant(s) will be identified in order to exclude specified or objectionable microorganisms. Furthermore, compendial MET (TAMC/TYMC) will be performed in order to assess whether the level of contamination is above acceptance criteria.

14.2 Method Validation

14.2.1 General Validation Strategy

The aim of the validation was to compare two methodologies (Rapid MET and compendial method) and demonstrate equivalence based on defined validation parameters. The validation parameters consisted of robustness, ruggedness, repeatability, specificity, limit of detection (LOD), accuracy and precision (according to Ph. Eur. 5.1.6 and USP <1123>), and equivalence in routine operations. Demonstration of equivalence between two methodologies 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 in routine operation"). Suitability of a particular product for the Rapid MET is assessed in an additional, product-specific

2 During the feasibility studies, the in-house isolated mold *Penicillium* sp. was not reliably detectable without such pre-treatment; the mold formed such dense aggregates that several cases occurred in which no cell material was by chance pipetted in the assay cuvettes of the Celsis Advance instrument. The glass bead treatment enabled sufficient dispersal of the *Penicillium* sp. and detection with the Celsis Advance System within 72 hours. The combination of small beads and magnetic stirrer had proven most effective when compared to horizontal shaking, overhead shaking, and chemical dispersal through Triton X-100.

study. By demonstrating that the Rapid MET performs at least equivalent to the compendial method and demonstrating that a product in focus for testing with the Rapid MET is suitable for application of that method, it can be ensured that the ability to assess microbiological quality of that product is not compromised by using an alternative to the compendial test method.

Because our application of the method consists of demonstrating the absence of culturable microorganisms in 1 g product, low-level inocula of approximately 1–5 CFU were applied in most validation experiments. Not only pharmacopoeial test microorganisms were used but also in-house isolates. In-house isolates were used in stressed state. Not only does this represent "worst-case" scenarios for detection but it also mimics the actual situation: most contaminants in sterile and non-sterile products are expected to be stressed, e.g. following a treatment with disinfectants, exposition to heat, or dehydration. Therefore, the aim of stress-protocols (e.g. by application of heat or by nutrient depletion) is not to kill all the microorganisms, but rather force surviving microorganisms into a stressed but still viable state with prolonged lag-phase. Upon start of incubation, these microorganisms can, however, recover from their stressed state and return to their normal growth behavior. The used stress protocols were published methods (Gray *et al*. 2010).

14.2.2 Statistical Data Evaluation

14.2.2.1 Fisher's Exact Test and Chi-Square Test

Data were evaluated with several statistical methods. Since the Rapid MET provides qualitative data (presence/absence), Fisher's exact test was used for most evaluations. Fisher's exact test determines whether categorical variables are independent. In our case, the question to be answered was whether successful detection of microorganisms was independent of the test method used (rapid method or compendial method). The null hypothesis was that no relation between the experimental outcome and the used analysis method existed. The null hypothesis was rejected when the p-value was below 0.05 corresponding to a confidence level of 95%. Because Fisher's test belongs to the class of exact tests, it can also be applied for relatively small sample sizes (Fisher 1922). However, because of its methodology, the test is basically limited to comparison of only two data sets.

If more than two sets of data had to be compared (e.g. for the validation parameter "Repeatability," which required evaluation of results from different days and daytimes), the Chi-Square test was used. Similar to Fisher's exact test, the Chi-Square test determines whether categorical variables are independent. The Chi-Square statistic quantifies how much the observed distribution of positive/negative results varies from the theoretical distribution one would expect if no relation between the experimental outcome and the test method exists. This procedure is approximate; it only gets accurate with a certain

sample size (Pearson 1900). Therefore, it was only used if Fisher's exact test could not be applied and also with a higher sample size.

14.2.2.2 Sample Size and Test Power

One of the most important aspects when designing experiments is the choice of an adequate sample size. If sample size is chosen too low, the experimental outcome may be biased through randomness, which in our context mainly means that the ability to detect statistically significant differences is compromised. The ability to detect a statistical difference is often expressed through test power. In reference to USP <1010>, a test power of ≥0.8 can in general be considered acceptable. In our validation, the largest acceptable difference between the rapid method and the compendial method was defined as 30% in reference to USP <1227>.[3] For all experiments, the confidence level was defined as 95%, because 5% possibility of type I error seemed acceptable (Note: Type I errors represented our "risk" of failing a statistical acceptance criterion due to random data fluctuations, although in reality no difference between both methods under examination existed). In general, test power is dependent on the sample size, the largest acceptable difference between the methods under evaluation and the confidence level. Table 14.1 summarizes the impact of these parameters.

Moreover, as can also be inferred from Table 14.1, the sample size dictates which observed effect size leads to a significant difference in the statistical test. With big sample sizes, even minimal differences in recovery lead to statistical

Table 14.1 Variables affecting test power.

Variable factor	Constant factors	Effect on test power
Higher sample size	Largest acceptable difference, confidence level	Higher test power
Lower sample size	Largest acceptable difference, confidence level	Lower test power
Higher largest acceptable difference	Sample size, confidence level	Higher test power
Lower largest acceptable difference	Sample size, confidence level	Lower test power
Higher confidence level	Sample size, largest acceptable difference	Lower test power
Lower confidence level	Sample size, largest acceptable difference	Higher test power

3 Two different methodologies must by definition show differences in the obtained results; particularly if these are (micro-) biological assays. This is also the case for every traditional test method; even such small variables as different operators or nutrient media lots inevitably lead to minor differences. Therefore, a boundary in relation to the reference method should be defined, above which the alternative method operates in a verified and consistent manner.

significance; whereas with small sample sizes also big differences between the data sets of interest may not become statistically significant.

For quantitative data, an additional factor impacting test power is the standard deviation (the higher the standard deviation, the lower the test power). For qualitative data, a factor with a similar impact is the overall success rate. Regarding a microbiological validation, the success rate would represent the overall frequency of microbial detection and is thus dependent on the inoculum used for the validation experiments. The higher the overall success rate, the higher the test power. The reason is that with a higher mean inoculum (e.g. 10 CFU), a negative test result is most probably not due to randomness; whereas with very low mean inocula (e.g. 1 CFU), some samples will by chance not be inoculated. Thus, with low mean inocula and accordingly low overall success rates, it is more difficult to estimate whether a negative test result is due to a weakness of the methodology or due to random absence of microorganisms in the inoculation suspension. Consequently, lower overall success rates result in a lower test power.

In the present study, we applied low-level inocula of 1–5 CFU for 16 different microbial strains. The use of such low inocula inevitably leads to different overall success rates for the different microorganisms represented in the validation. Because the overall success rate for a specific strain remains unknown until the actual validation experiments are performed, it is not possible to give more than an estimate for the required sample size prior to generation of the validation data. This initial sample size subsequently may have to be increased if indicated by the obtained experimental results.

In general, qualitative statistical tests demand for a high number of replicates to reach adequate test power. Such high numbers of replicates can well be accomplished when allowed to pool results obtained for several microbial strains. We have made the experience, however, that evaluations for single microbial strains are often required and sole reliance on pooled data not accepted. Because we envisioned using 16 different microbial strains for demonstration of specificity alone, the overall sample size of the study would have been overwhelming and not justified for our intended application.[4] Therefore, we developed a modified test power procedure, which acted as a tool to decide whether or not sample size should be increased for an individual microbial strain in a particular experiment. To that end, we chose 14 replicates as starting sample size for strain-specific evaluations, which was the lowest reasonable value derived from simulations

4 We believe that the use of a broad microbial spectrum is one of the most important aspects when validating an alternative microbiological method. Concentration on only a few strains may permit to work with higher sample sizes per strain, but we believe that this diminishes the overall weight of the study. Because of the high product volume tested with the Rapid MET as compared to the current method, the risk of diminished ability to evaluate product quality was in general considered low.

and applying our modified test power procedure, which is summarized in the following paragraphs. If indicated by the obtained experimental data, the sample size was increased based on the following definitions:

If the numerical recovery of the rapid method was superior or equivalent to the numerical recovery of the compendial reference method (e.g. both methods detected microbial growth in 12 out of 14 replicates), sample size was not increased.

If the numerical recovery of the rapid method was lower than the numerical recovery of the reference method (e.g. the rapid method detected microbial growth in 12 out of 14 replicates, but the reference method detected growth in 13 out of 14 replicates), our modified test power calculation was performed. If indicated (calculated test power < 0.8), sample size was doubled.

We saw justification to use the lowest value (14 replicates) because of the closeness between the rapid and the compendial method and the fact that the 1 g product required by the Rapid MET often exceeds the product amount tested with the compendial MET. The modified test power calculation took into account the largest acceptable difference of 30% as well as the generated data. To that end, the success rate of the reference method was used as first proportion and 70% of that success rate as second proportion. Furthermore, the confidence level was adjusted according to the generated *p*-value. This, of course, does not represent a formal test power calculation in a strict statistical sense, but rather was used as a tool to decide whether additional data should be generated for providing evidence that the requirement of at least 70% recovery of the rapid method compared to the reference method was fulfilled, while keeping overall sample size at a manageable level.[5] In applications regarded as more critical, the initial sample size could be increased in order to further lower the probability of random sampling error (in case of a sterility test, the authors would, for instance, recommend to use at least 30 replicates per microbial strain as starting sample size).

Table 14.2 shows examples of modified test power calculations with different hypothetical data. In these examples, Method A represents the compendial

5 Our approach based on probability calculations. Briefly, the assumption was made that the compendial reference method has 100% recovery and negative test results are only due to random spiking with sterile inoculation suspension. Probabilistic evaluations for different experimental outcomes were performed, assuming that the alternative method had a recovery of 70% of the compendial reference method. Probabilities for all experimental outcomes which would not trigger an increase in sample size with our definitions outlined above were summed up, resulting in a probability which can also be interpreted as an experimental power. Therefore, our evaluation was rather based on the numerical values. When assuming Poisson-distributed microorganisms, our simulations indicated that the probability to not detect a recovery of less than 70% for an individual strain with a mean microbial count of 1.5 CFU was ~20%, which can be interpreted as a test power of 0.8 as suggested by USP <1010> (for higher microbial numbers, our detection probability increased and was more than 0.9 for mean microbial inocula higher than 2.5 CFU). We considered this approach appropriate for our purpose.

Table 14.2 Examples of our modified test power calculation based on hypothetical data.

Method A +/– (success rate)	Method B +/– (success rate)	*p*-Value Fisher's exact test	Minimal success rate for Method B to fulfill 70% recovery of Method A	Test power	Evaluation
13/1 (0.93)	9/5 (0.64)	0.16	0.65 (0.93 × 0.7)	0.45	Increase sample size
26/2 (0.93)	18/10 (0.64)	0.02	0.65 (0.93 × 0.7)	Not necessary since significant difference	Significant difference
13/1 (0.93)	10/4 (0.71)	0.33	0.65 (0.93 × 0.7)	0.71	Increase sample size
26/2 (0.93)	20/8 (0.71)	0.08	0.65 (0.93 × 0.7)	0.72	Increase sample size
39/2 (0.93)	30/12 (0.71)	0.02	0.65 (0.93 × 0.7)	Not necessary since significant difference	Significant difference
13/1 (0.93)	11/3 (0.79)	0.6	0.65 (0.93 × 0.7)	0.78	Increase sample size
26/2 (0.93)	22/6 (0.79)	0.25	0.65 (0.93 × 0.7)	0.89	Sufficient test power
13/1 (0.93)	12/2 (0.86)	1	0.65 (0.93 × 0.7)	0.88	Sufficient test power
7/7 (0.5)	6/8 (0.43)	1	0.35 (0.5 × 0.7)	0.67	Increase sample size
14/14 (0.5)	12/16 (0.43)	0.79	0.35 (0.5 × 0.7)	0.83	Sufficient test power
5/9 (0.36)	3/11 (0.21)	0.68	0.25 (0.36 × 0.7)	0.54	Increase sample size
10/18 (0.36)	6/22 (0.21)	0.38	0.25 (0.36 × 0.7)	0.39	Increase sample size
20/36 (0.36)	12/44 (0.21)	0.14	0.25 (0.36 × 0.7)	0.34	Increase sample size
35/63 (0.36)	21/77 (0.21)	0.04	0.25 (0.36 × 0.7)	Not necessary since significant difference	Significant difference

For all calculations which took into consideration data of more than only one microbial species, classical *post-hoc* test power calculation was applied.

reference method, whereas Method B represents an alternative method. The largest acceptable difference was defined as 30%. If the test power was not sufficient, sample size was doubled and the ratio of positive and negative results was kept constant (e.g. 26 instead of 13 positive test results) in order to simplify interpretation of the examples.

14.2.2.3 Equivalence Tests

For some validation parameters demonstration equivalence instead of absence of a statistically significant difference had to be provided. While statistical tests like the Chi-Square test evaluate whether a significant difference between different data sets exists, statistical equivalence tests demonstrate the ability of the method of interest to operate within a predefined equivalence boundary. In our case, this equivalence boundary was one-sided (we did not mind if the Rapid MET performed superior to the compendial method), and it was defined that the Rapid MET had to reach at least 70% recovery of the compendial reference method (USP <1227>). We used Fisher's exact test modified for demonstrating one-sided equivalence of success rates (as published by Rasch *et al.* (1998)) for such purposes. The null hypothesis was that the methods did not perform equivalent. The null hypothesis was rejected if the *p*-value of Fisher's exact test modified for one-sided equivalence of success rates was lower than 0.05 (since the test was performed at a confidence level of 95%). Rejection of the null hypothesis meant acceptance of the alternative hypothesis and thus that the methods performed equivalent regarding the boundary of 70%.

14.2.3 Experimental Conditions for Validation Experiments

Unless justified in the text, the general test conditions for the validation were as detailed in Sections 14.2.3.1 and 14.2.3.2.

14.2.3.1 Rapid MET

A low inoculum of the microorganism of interest contained in 10 ml buffer was spiked into glass bottles containing 90 ml liquid nutrient medium (TSB supplemented with 4% polysorbate 80 + 0.5% soy lecithin), ~10 g sterilized glass beads of ~1 mm diameter, and a sterile magnetic stir bar. The bottles were incubated at 30–35 °C (if the microorganism of interest was a bacterium, *Candida albicans* or *Aspergillus brasiliensis*) or at 20–25 °C (if it was a yeast or mold). The incubation time was not more than 72 hours. Following incubation, samples were treated for 20 minutes on a magnetic stirrer. Subsequently, samples were tested for the presence/absence of microorganisms with the Celsis Advance (using the AKuScreen kit).

14.2.3.2 Compendial Method

A low inoculum of the microorganisms of interest contained in 1 ml 0.9% NaCl solution was transferred into a Petri dish and covered with ~20 ml TSA (if the

microorganism of interest was a bacterium, *C. albicans* or *A. brasiliensis*) or SDA (if it was a yeast or mold). SDA plates were incubated at 20–25 °C for at least 7 days, TSA plates at 30–35 °C for at least 5 days. Microbial growth was visually evaluated by a qualified analyst.

14.2.4 Method Validation Results

14.2.4.1 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.

Both Ph. Eur. 5.1.6 and USP <1223> state that Robustness/Ruggedness determination is best suited to be demonstrated by the supplier of the method. Robustness was shown by the supplier of the rapid microbiological method and submitted in a Drug Master File which was accepted by the FDA in May 2010. A selection of robustness parameters covered by the supplier was different reagent reconstitution volumes, reagent reconstitution times, reagent temperatures, sample volumes, reagent injection volumes, and instrument temperatures. Results by the supplier on robustness parameters were reviewed and two additional robustness parameters were identified and therefore included in the validation. These two additional robustness parameters were the incubation time and the length of the glass bead treatment.

Penicillium sp. and *Escherichia coli* were chosen as microbial representatives for this validation aspect. Stressed *Penicillium* sp. represented the worst-case microorganism regarding detection with the Celsis Advance due to its slow growth rate and tendency to form dense aggregates; *E. coli* was chosen due to its exceptionally fast growth rate. One to five CFU of the test strains were inoculated. Results were evaluated using Fisher's exact test at a confidence level of 95%. The results obtained after 72 hours incubation (representing the reference incubation time) were compared to results obtained after 66 and 120 hours incubation, respectively. No significant differences were observed and the test power criterion was passed (Figure 14.1). By demonstrating that also 66 and 120 hours of incubation did not lead to significantly different results, appropriateness of the target incubation time of 72 hours was further supported.

The glass bead treatment which assists in dispersion of aggregating microorganisms (e.g. *Penicillium* sp.) was developed in preliminary studies to the method validation, which indicated that a treatment time of 15 minutes is effective. This duration may, however, also be subject to small variations. The results obtained after 15 minutes glass bead treatment (representing the reference glass bead treatment time) were compared to the results obtained after 10 and 20 minutes glass bead treatment, respectively (Figure 14.1c). Because *E. coli* does not form aggregates, only *Penicillium* sp. was used for that validation aspect. For the comparison of 15 minutes treatment versus 20 minutes treatment, no significant differences were detectable and our test power

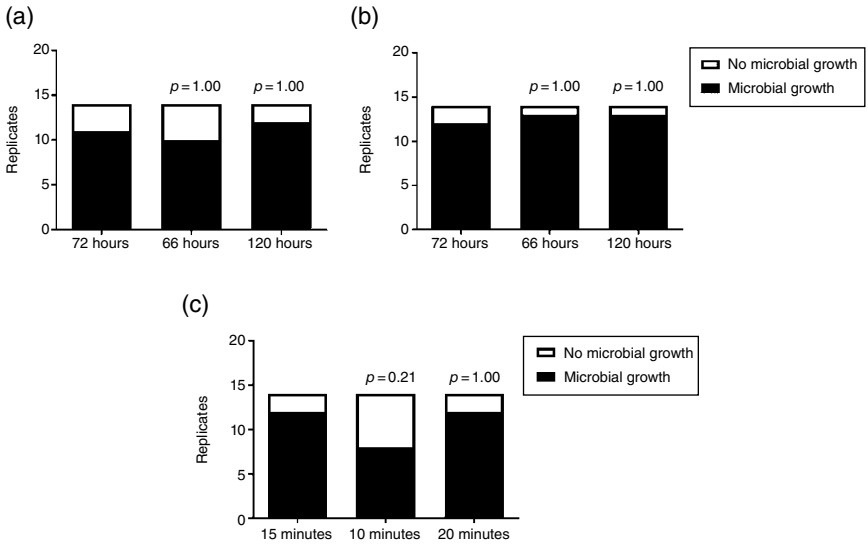

Figure 14.1 Robustness toward different incubation and glass bead treatment times. Robustness toward different incubation times is shown for *Penicillium* sp. (a) and *Escherichia coli* (b). (c) Robustness toward different glass bead treatment times for *Penicillium* sp.

criterion was passed. However, regarding the comparison of 15 minutes treatment versus 10 minutes treatment, the difference was not yet significant, but our test power criterion was not passed, indicating that additional data should be generated. In the light of the obtained data, we refrained from increasing sample size, but concluded that 10 minutes treatment may not be sufficient. Therefore, we decided that duration of the glass bead treatment should be increased from 15 to 20 minutes for routine use as well as for the remaining validation experiments. By performing the glass bead treatment for 20 minutes, it is ensured that minor variations from the target treatment time do not have an impact, since even 15 minutes treatment was shown to be sufficient. Longer treatment times do not have an adverse effect – the treatment is by far not harsh enough to lead to destruction of single microbial cells.

Robustness toward different incubation times was therefore successfully demonstrated for fast-growing and slow-growing aggregate-forming microorganisms. For routine testing, an incubation time of minimum 72 hours will be applied for the Rapid MET on basis of the Celsis Advance. Robustness toward a variation of minus 6 hours and plus 48 hours was demonstrated.

14.2.4.2 Ruggedness

Ruggedness describes the reproducibility of test results through analysis of samples under different routine circumstances (alteration of analysis parameters, which represent unavoidable changes). Ruggedness is normally expressed

as the lack of influence of operational and environmental variables of the microbiological method on the test results. A selection of ruggedness parameters covered by the supplier was different instruments, operators, reagent lots, and cuvette sizes. These parameters were considered adequate and no additional experiments were performed.

14.2.4.3 Repeatability

Repeatability describes the reproducibility of test results through analysis of samples under routine circumstances at different daytimes and on different days (using the same analyst with the same equipment). Due to its slow growth rate and the tendency to form dense aggregates, *Penicillium* sp. represented the worst-case microorganism regarding repeatability. Thus, by demonstrating repeatability for detection of *Penicillium* sp., repeatable detection of fast-growing and/or non-aggregate-forming microorganisms was warranted.

1–5 CFU inocula of *Penicillium* sp. were repeatedly applied on different days and daytimes and several such data sets evaluated for significant differences with Chi-Square test (Figure 14.2). In total, four test runs (two in the morning and two in the afternoon of four different days) each consisting of 20 replicates were performed. Repeatability was acceptable despite a challenging test setup (low numbers of stressed, slow-growing aggregate former). Furthermore, the ability of the assay to deliver acceptable results when repeatedly performed under varying circumstances is shown by each experiment performed during validation.

14.2.4.4 Specificity

The specificity of a method was defined as the potential to detect a broad range of microorganisms, which for a growth-based RMM mainly depends on the

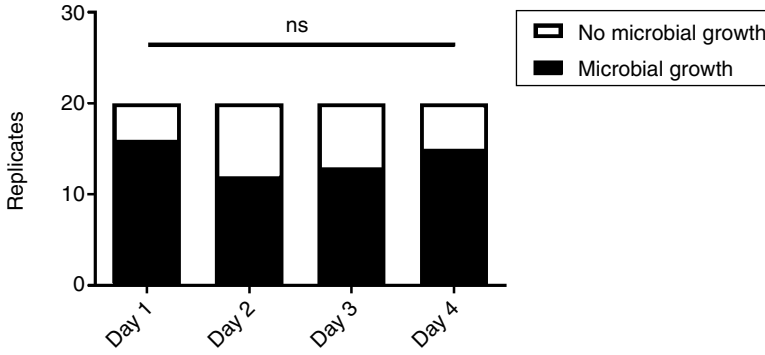

Figure 14.2 Repeatability on different days and daytimes. Results for repeatability on different days and daytimes with *Penicillium* sp. No significant differences were detectable with Chi-Square test (ns, no significant difference).

Table 14.3 Strain selection used for validation of specificity.

Yeast/Mold	Sporulating bacteria	Gram-positive bacteria	Enterobacteria (Gram-negative)	Waterborne Gram-negative bacteria
Candida albicans	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*
Aspergillus brasiliensis	*Bacillus clausii*	*Staphylococcus epidermidis*	*Salmonella abony*	*Burkholderia cepacia*
Penicillium sp.	*Bacillus licheniformis*	*Staphylococcus warneri*		*Pseudomonas stutzeri*
		Kocuria rhizophila		*Stenotrophomonas maltophilia*

fertility of the nutrient medium used. In our validation, specificity was shown by detection of 16 different microorganisms, including Gram-negative rods, Gram-positive sporulating bacteria, Gram-positive cocci, yeasts, and molds. All specified microorganisms mentioned in the harmonized method for Microbiological Examination of Non-sterile Drug Products as described in Ph. Eur. 2.6.13 and USP <62> were included, except from *Clostridium sporogenes* (anaerobic microorganism that would not grow under the defined test conditions). Furthermore, stressed in-house isolates were used. All test microorganisms were spiked with a low inoculum (1–5 CFU) and are summarized in Table 14.3. The 14 replicates per strain were generated in two independent test runs each.

Data for each individual strain was evaluated using Fisher's exact test at a confidence level of 95%. Pooled data from all strains were evaluated for a statistical difference using Chi-Square test at a confidence level of 95%. Furthermore, pooled data for all strains were evaluated for statistical equivalence using Fisher's exact test modified for demonstration of equivalence of one-tailed success rates. If the test power was below 0.8 for an individual strain, additional data were retrieved (Tables 14.4 and 14.5).

14.2.4.5 Limit of Detection

The LOD is defined as the lowest number of microorganisms that can be detected under the stated experimental conditions. Since for the Specificity validation a low inoculum of 1–5 CFU was used, a suitable detection limit as compared to the compendial MET of the Ph. Eur. 2.6.12 and USP <61> was already demonstrated with that series of experiments (see Tables 14.4 and 14.5). Moreover, in the compendial MET, for typical non-sterile and nonfilterable dosage forms such as nonaqueous preparations for oral use, often a diluted product amount may be tested (e.g. 100 mg leading to a maximum detection level of <10 CFU/g).

Table 14.4 Strain-specific specificity results.

Microorganism	Result Rapid MET Growth/No growth	Result Compendial MET Growth/No growth	*p*-Value Fisher's exact test	Test power	Interpretation of Fisher's exact test
Pseudomonas aeruginosa ATCC 9027	12/2	11/3	1	N/A[a]	No significant difference
Staphylococcus aureus ATCC 6538	9/5	12/2	0.38	0.58	No significant difference. Generation of additional data indicated
Additional data for *S. aureus*	19/9	23/5	0.36	0.80	No significant difference
Bacillus subtilis ATCC 6633	13/1	10/4	0.33	N/A[a]	No significant difference
Escherichia coli ATCC 8739	14/0	14/0	No value[b]	N/A[a]	No *p*-value can be calculated. Data suggest sufficient recovery
Burkholderia cepacia ATCC 25416	13/1	12/2	1	N/A[a]	No significant difference
Aspergillus brasiliensis ATCC 16404	14/0	14/0	No value[b]	N/A[a]	No *p*-value can be calculated. Data suggest sufficient recovery
Candida albicans ATCC 10231	14/0	11/3	0.22	N/A[a]	No significant difference
Salmonella abony NCTC 6017	10/4	14/0	0.098	0.43	No significant difference. Generation of additional data indicated
Additional data for *S. abony*	23/5	25/3	0.70	0.95	No significant difference
Staphylococcus epidermidis stressed in-house isolate	10/4	11/3	1	0.81	No significant difference

Microorganism	Result Rapid MET Growth/No growth	Result Compendial MET Growth/No growth	*p*-Value Fisher's exact test	Test power	Interpretation of Fisher's exact test
Kocuria rhizophila stressed in-house isolate	12/2	14/0	0.48	0.84	No significant difference
Bacillus clausii stressed in-house isolate	9/5	11/3	0.68	0.68	No significant difference. Generation of additional data indicated
Additional data for *B. clausii*	19/9	21/7	0.77	0.86	No significant difference
Penicillium sp. stressed in-house isolate	8/6	8/6	1	N/A[a]	No significant difference
Stenotrophomonas maltophilia stressed in-house isolate	11/3	5/9	0.054	N/A[a]	No significant difference
Staphylococcus warneri stressed in-house isolate	14/0	14/0	No value[b]	N/A[a]	No *p*-value can be calculated. Data suggest sufficient recovery
Bacillus licheniformis stressed in-house isolate	11/3	12/2	1	0.85	No significant difference
Pseudomonas stutzeri stressed in-house isolate	5/9	3/11	0.68	N/A[a]	No significant difference

[a] Rapid method numerically equivalent or superior, therefore no test power calculation.
[b] *p*-Value cannot be calculated if both methods recovered microbial growth in all replicates (no 2 × 2 contingency table can be formed).

Table 14.5 Specificity results pooled data from all strains.

Result Rapid MET Growth/No growth	Result Traditional MET Growth/No growth	*p*-Value Chi-Square test	Test power Chi-Square test	*p*-Value Fisher's exact test modified for demonstration of equivalence of one-tailed success rates	Interpretation of results
212/54	212/54	1	1	3.11×10^{-10}	No significant difference. Statistical indication of equivalence.

In conclusion, for each individual strain included in the validation a recovery of >70% was successfully demonstrated. Taking into account the data generated for all strains, no statistically significant difference regarding recovery was detectable with Chi-Square test. Furthermore, through Fisher's exact test modified for demonstration of equivalence of one-tailed success rates, statistical proof of equivalence against a 70% boundary was provided.

Based on the acceptance criteria defined in Ph. Eur. 5.1.4 and USP <1111>, complete absence of specified microorganisms may be required in 1 or 10 g product depending on the product's route of administration (which also is the product amount used for Rapid MET). Therefore, for the LOD study all specified microorganisms mentioned for the growth promotion tests in the Microbiological Examination of Non-sterile Drug Products of the Ph. Eur. 2.6.13, Ph. Eur. 5.1.4, USP <62> and USP <1111> were included except from *C. sporogenes* (anaerobic microorganism that would not grow under the defined test conditions) and *Salmonella* (absence required in 10 g product and therefore most likely out of scope for our Rapid MET application). *Penicillium* sp. was also included in the LOD validation since it represents a worst-case microorganism in terms of detection (low growth rate and strong aggregation). In conclusion, the test strains for LOD validation were *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *E. coli*, *C. albicans*, and stressed *Penicillium* sp. These test strains were serially diluted to extinction (~50 CFU, 5 CFU, 0.5 CFU, and 0.05 CFU per replicate). The Rapid MET was used according to the previously described method. For the compendial method, the test was performed according to the Ph. Eur. 2.6.13 and USP <62> and consisted of enrichment in liquid media followed by selection through selective media. For *Penicillium* sp., 1 ml of test suspension was transferred into a Petri dish and ~20 ml SDA was poured. The SDA plate was then incubated for at least seven days.

Two independent test runs with 10 replicates per dilution and microorganism were performed. From the pattern of replicates, positive or negative for

microbial growth, the 95% confidence interval of the MPN was calculated for each test run with each microorganism using a MPN-table. The 95% confidence interval of the MPN of both methods was evaluated for overlapping. This approach to determine the LOD is referenced in USP <1223>. The ten-replicate MPN-table was obtained from FDA Bacteriological Analytical Manual Appendix 2 (Blodgett 2010).

No significant differences were detectable regarding the LOD (Figure 14.3). For additional evidence, the MPN/g results were plotted and statistically compared using paired *t*-test. No statistically significant difference between the

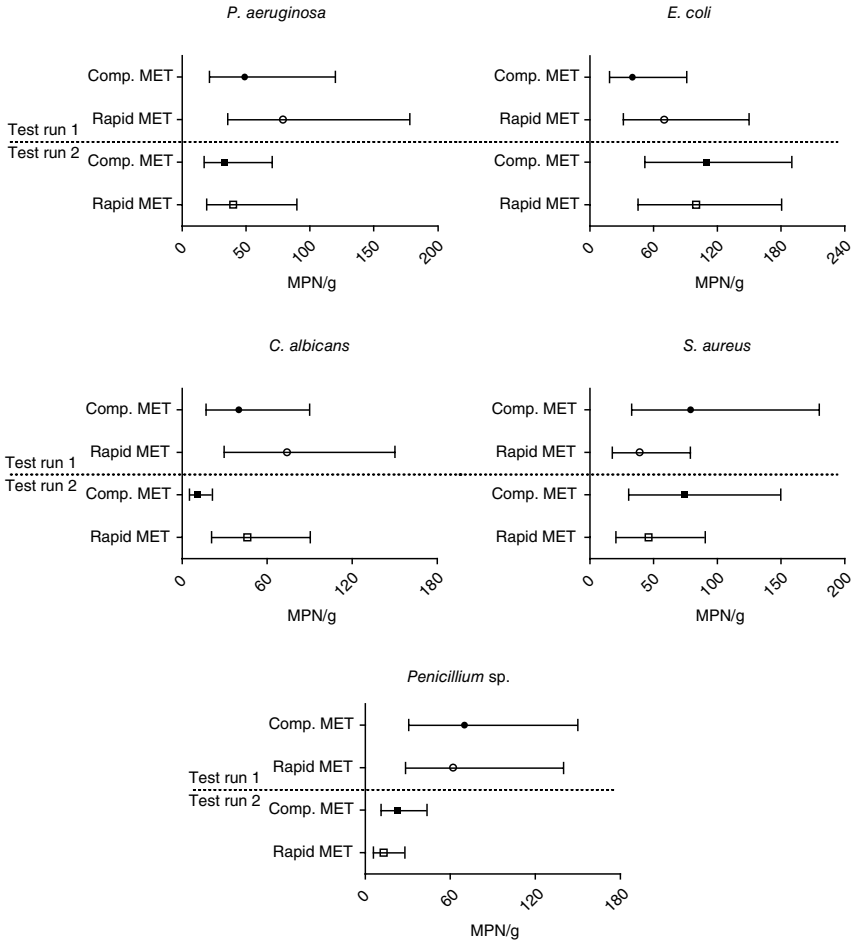

Figure 14.3 Limit of detection. Results of limit of detection compendial method versus Rapid MET. MPN/g as well as the 95% confidence interval of the MPN/g value is indicated. When the 95% confidence intervals overlap there are no significantly different detection limits.

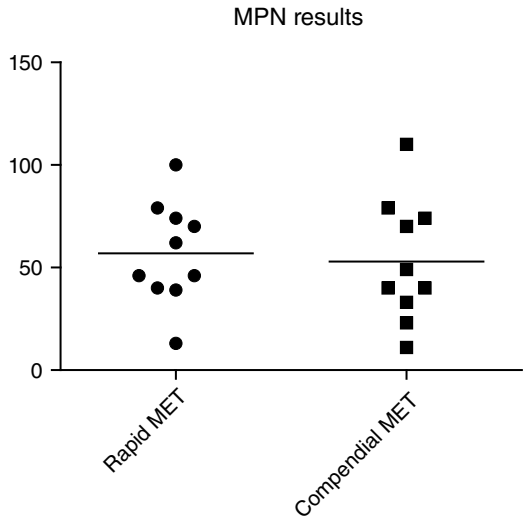

Figure 14.4 Comparison of the obtained MPN/g values. Plotting of the MPN/g results obtained through the detection limit validation. Individual MPN/g values as well as the means are shown.

Rapid MET and the compendial method was detectable and the mean of the obtained MPN/g values was nearly identical for both methods (Figure 14.4).

14.2.4.6 Accuracy and Precision (According to Ph. Eur. 5.1.6)

We decided to insert this validation parameter since the Rapid MET is also planned for registration through European authorities, where according to Ph. Eur. 5.1.6 Accuracy and Precision is also required for qualitative methods. Accuracy and Precision is expressed as the relative rate of false-positive and false-negative results between the rapid method and the compendial method using a standardized, low-level inoculum. The Rapid MET was further challenged for false-negative results by using the microorganisms *Penicillium* sp. and *Bacillus clausii*. *Penicillium* sp. represents the worst-case microorganism regarding detection with the Celsis Advance due to its slow growth rate and tendency to form dense aggregates. *Bacillus clausii* was used as endospore. Thus, in order to multiply and become detectable, *B. clausii* first had to enter into an active state, which strongly depends on the used nutrient medium and incubation conditions. In order to assess the rate of false-positive test results, negative controls were used in which no microorganisms were inoculated.

14.2.4.6.1 Definition of False Negative A false-negative result arises when a test result is negative although the sample has been inoculated. It is of note, however, that some samples would have most probably by chance remained

sterile due to the low inoculum (no microorganisms present in the microbial suspension used for inoculation). In order to challenge for false-negative results, samples were inoculated with 1–5 CFU of the test organisms.

14.2.4.6.2 ***Definition of False Positive*** A false-positive result arises when microbial growth is detected in a negative control. This may either happen because of a microbial contamination or because of an artifact (e.g. ATP-contamination). In order to challenge for false-positive results, samples were inoculated with sterile buffer.

No statistically significant differences between the compendial method and Rapid MET for false-positive and false-negative results were detectable using Fisher's exact test and our test power criterion was passed; thus, comparable accuracy and precision was successfully demonstrated and the rates of false-positive and false-negative results were similar. Exemplary data for *Penicillium* sp. and sterile buffer are shown in Table 14.6. No false-positive results due to ATP contamination were observed.

14.2.4.7 Equivalence in Routine Operation

The objective of this test was to demonstrate the equivalence in routine use of the Rapid MET with the Celsis Advance system as compared to the compendial method. Since the Rapid MET with Celsis Advance system is a qualitative method, only the capacity to detect microbial growth (=presence/absence of microorganisms) was evaluated. Thus, the compendial MET was counted as positive for microbial growth if either a MET (TAMC and TYMC) or a test for specified microorganisms was positive for microbial growth. The experimental setup included samples for which rather few positive test results were expected as well as samples for which several positive test results were expected. Five routine-relevant products were selected, consisting of hard-gelatin capsules, excipients, and film-coated tablets. Thirty random samples from different batches per product were analyzed in parallel according to the compendial

Table 14.6 Example statistical significant tests comparing the rate of false-positive and false-negative results of the compendial versus the rapid MET using *Penicillium* sp. as test strain.

	Rapid MET Growth/No growth	Compendial MET Growth/No growth	*p*-Value Fisher's exact test
False-negative rate	6/14	7/13	1
False-positive rate	0/20	0/20	NA (both methods have no positive results)

Ratios were statistically compared using the Fisher's exact test.

Table 14.7 Equivalence in routine operation.

	Rapid MET Growth/No growth	Compendial MET Growth/No growth
Product 1 (hard-gelatin capsule)	18/12	1/29
Product 2 (excipient)	0/30	1/29
Product 3 (excipient)	30/0	30/0
Product 4 (film-coated tablet)	0/30	2/28
Product 5 (film-coated tablet)	20/10	1/29
Total	68/82	35/125
p-Value Fisher's exact test modified for demonstrating one-tailed equivalence of success rates	$<10^{-15}$	
Interpretation Fisher's exact test modified for demonstrating one-tailed equivalence of success rates	Statistical indication of equivalence	

method described in Ph. Eur. 2.6.12, 2.6.13, USP <61>, <62>, and according to the Rapid MET routine test setup described Section 14.1.

The Rapid MET detected microbial contamination more often than the compendial MET (Table 14.7). This finding is not surprising considering that the rapid method may test a higher amount of product compared to compendial MET since dilution of the product to <1 g is not valid. The *p*-value of Fisher's exact test modified for demonstrating one-tailed equivalence of success rates was clearly below 0.05, indicating statistical equivalence regarding the boundary of 70% recovery.

14.3 Suitability Test (Product-Specific Method Validation)

Additional testing has to be performed for each drug product/API/excipient which is in scope of the Rapid MET in order to demonstrate that the method is also suitable for that product. The suitability study includes the sample effects study and the suitability of the test method.

14.3.1 Sample Effects Study

The sample effects study determines if the drug product/API/excipient itself interferes with the Celsis AKuScreen assay, e.g. by adding turbidity to the sample which impedes the detection of the bioluminescence reaction or by being

bioluminescent itself. The procedure for determining sample effects was based on the supplier's recommendation. Briefly, RLU values generated by the product are measured to assess bioluminescent background, and a standardized amount of ATP is measured with or without product to evaluate whether the presence of product significantly diminishes the signal. We regarded a reduction of the ATP signal through the presence of product by more than 30% as significant.

Furthermore, we used the RLU background of the product to determine a product-specific positive/negative discrimination threshold. The results obtained through measurement with the Celsis Advance are RLU. The RLU values *per se* do not have significance for the intended application, since the system is solely used as a presence/absence method. However, RLU values have to be defined above which a sample is to be considered positive for microbial growth. The approach used by the system supplier is to consider a sample positive for microbial growth if the RLU values are several times higher than the negative control. We applied this approach for validation experiments which did not involve the product – in these cases, a sample was considered positive for microbial growth if RLU values were 10 times higher than the negative control.

However, we did not consider this approach optimal when testing products. First, different products have very different background RLU levels (e.g. capsules in general create stronger background than tablets). Second, the RLU levels of the negative control are prone to a certain extent of variation. In our case, the negative control would be nutrient medium, and throughout the validation we saw that RLU values of nutrient medium were subject to batch-to-batch variations. With maximum 800 RLU, this variation was rather low in our case. However, if a multiple of the RLU value obtained for the negative control is used for discriminating whether a test sample is positive or negative for microbial growth, variability of this discrimination threshold is much higher. Thus, there would be a risk that product background may be above the discrimination threshold if RLU values of the negative control would be low; or below the discrimination threshold if RLU values of the negative control would be high.

For the reasons presented above, we decided to introduce product-specific discrimination thresholds. These are determined in the product-specific method suitability through the sample effects study. With these experiments, luminescent background generation or masking of ATP bioluminescence is assessed. The product-specific discrimination threshold is defined based on the RLU background of the product of interest according to the following workflow.

Three product batches are tested to determine the product RLU background. The highest obtained result is used for the workflow shown in Figure 14.5 in order to mitigate the risk of false-positive results. If further product dilution is indicated, this is achieved through increase of the nutrient medium volume

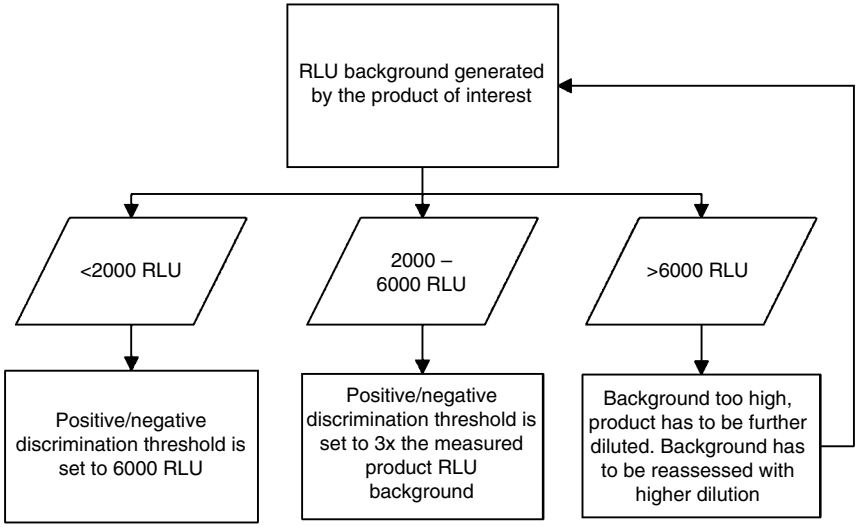

Figure 14.5 Definition of the product-specific positive/negative discrimination threshold.

used for incubation, since the Rapid MET does not allow testing of a smaller product amount than 1 g. The RLU values of even low-level contaminations of slow-growing microorganisms are clearly above the maximum discrimination threshold of 18,000 RLU (3 × 6,000). For example, the average RLU value of positive samples of the LOD 0.5 CFU inoculum of *Penicillium* sp. was 363,031 RLU. Thus, there is no risk that low-level contaminations would lead to false-negative results, due to a too high product-specific discrimination threshold.

The above-presented sample effects concept was already applied for a film-coated tablet final dosage form which is intended as pilot drug product for the registration of the Rapid MET. In that study, we successfully demonstrated that the film-coated tablet did not mask the bioluminescence signal and the average bioluminescent background was 218 RLU. Therefore, samples would be regarded as being positive for microbial growth if more than 6,000 RLU are measured with the Celsis Advance, and the following workflow would then be applied (Figure 14.6).

14.3.2 Suitability of the Test Method

Suitability is shown through detection of a range of microorganisms in the presence of the product of interest, therefore demonstrating acceptable microbial recovery. Ten grams of the product is dissolved in 90 ml of the dilution buffer and stirred. Test microorganisms as the ones requested for the compendial methods (*E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *A. brasiliensis* ATCC 16404, and *C. albicans* ATCC

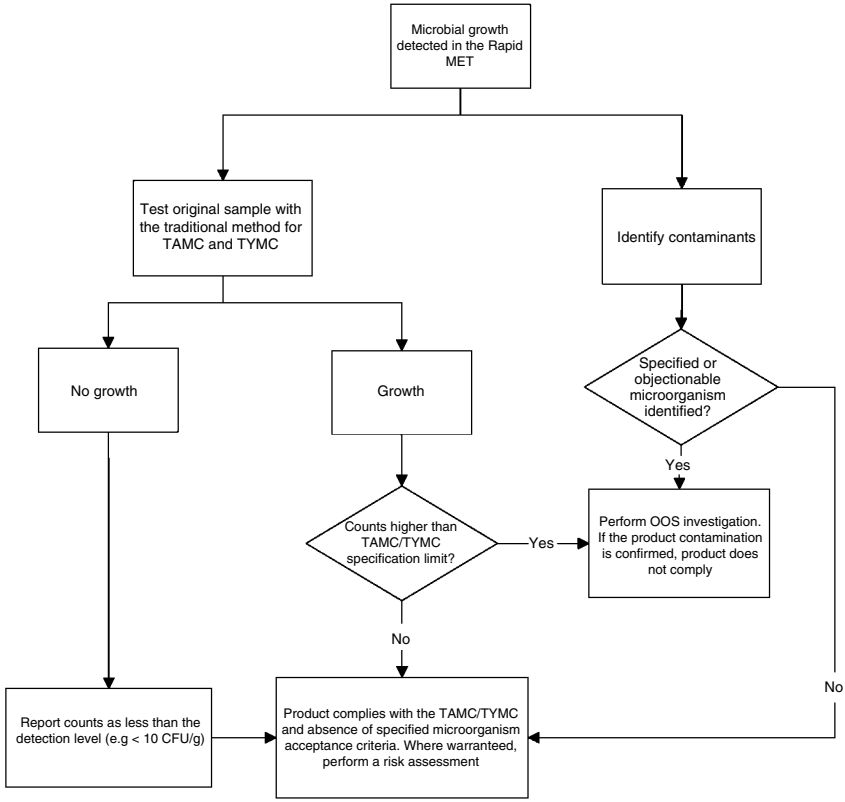

Figure 14.6 Schematic overview of result evaluation with the Rapid MET if growth occurs (OOS, out of specification).

10231) are inoculated individually in the product dilution. Then, 10 ml of the product dilution (corresponding to 1 g product) harboring <100 CFU of the test microorganism are added to glass bottles containing 90 ml TSB + 4% polysorbate 80 + 0.5% soy lecithin, glass beads, and a magnetic stir bar. The bottles are incubated for not more than 72 hours either at 20–25 °C or at 30–35 °C, depending on the type of microorganism. Following incubation, samples are treated for 20 minutes on a magnetic stirrer. Subsequently, samples are tested for the presence/absence of microorganisms with the Celsis Advance (using the AKuScreen kit). Three independent test runs are performed and samples are measured in duplicates. Acceptance criteria are that all test microorganisms are positively detected with the Celsis Advance system and the test microorganism is confirmed with an identification of the recovered microorganism.

14.4 Discussion

The Rapid MET was validated according to USP <1223>, Ph. Eur. 5.1.6, and PDA Technical Report No. 33. Validation was comprised of the validation parameters robustness, ruggedness, repeatability, specificity, LOD, accuracy and precision, and equivalence in routine operation. For the validation, a combination of pharmacopoeial ATCC strains as well as a broad selection of in-house isolates was used. In-house isolates were used in stressed state. Results of the rapid method were statistically compared to the compendial method regarding the USP <1227> acceptance criterion of ≥70% recovery. We developed a modified test power calculation as a tool to confirm the appropriateness of the used sample size to detect such a difference. Furthermore, equivalence of the rapid method as compared to the compendial method was demonstrated in a statistically verified manner. The Rapid MET on basis of the Celsis Advance system (using the AkuScreen reagent kit) was therefore successfully validated as an alternative method to the compendial test for microbiological examination of non-sterile products.

Whereas one can expect that the overall microbial spectrum isolated may slightly differ between two test methods due to factors such as, for instance, the use of different growth media or incubation conditions, the extent of this difference may be estimated through a thorough validation comparing the overall equivalence of the methods based on predefined parameters. Adequate recovery of a range of relevant microorganisms provides solid evidence that the method of interest represents a suitable alternative to the compendial method. Furthermore, it should be verified that the most critical microorganisms (e.g. specified or objectionable microorganisms) that can be isolated with the compendial method should also be recovered in the rapid microbiological method for product release testing. Statistical approaches can be applied for definition of sample sizes and testing of hypotheses with a high probability of correctness. While test power calculations are rather straightforward for quantitative methods, treatment of qualitative data is more difficult. Furthermore, sample sizes for strain-specific qualitative evaluations should still remain at a practical level. We applied a modified test power approach to decide whether sample size should be increased based on our obtained validation data.

Calculation of test power often requires specialized software, which may not be easily accessible to every firm. Furthermore, advanced statistical knowledge may be required. PDA Technical Report No. 33 provides a table which helps microbiologists not familiar to test power calculations by suggesting sample sizes for quantitative experiments; to the knowledge of the authors similar guidance is not available for qualitative experiments. We are of the opinion that our approach to start with a relevant sample size, which may be increased based on the obtained results, represents a reasonable compromise between a still practical sample size and low possibility of experimental bias through random results.

The Rapid MET allowed reduction of the incubation time from 3 to 7 days (TAMC) and 5 to 7 days (TYMC) to 72 hours enabling a faster throughput time of product release testing. The incubation time of 72 hours may seem rather long; however, it confirms our experience that growth-based rapid methods allow to reduce the incubation time approximately by a factor 2–3 compared to the compendial reference method, if a challenging validation approach with stressed isolates is used. Furthermore, our 72 hours also include a generous safety margin. In addition to the reduced incubation time, the main advantage of our use of the Celsis Advance System is that it covers MET and absence of specified microorganisms in one single test, allowing for a significant reduction of the hands-on time and growth media storage. Furthermore, the readout is performed by a validated system and therefore there is reduced challenge on data integrity as for compendial method when the readout is performed by only one person, and there is a possibility for automation and direct data integration into a LIMS system. Internal benchmarking has shown that hands-on time may be reduced by up to 20–30%, depending on the number of tests for specified microorganisms required by the product. With the Rapid MET, 1 g of drug product is always being used by default for testing. Therefore, in case that no microbial growth is detected with the Rapid MET, an additional test for specified microorganisms is not necessary since the absence of microbial growth automatically excludes the presence of any specified microorganisms in 1 g of drug product. The specified microorganisms should, however, be included in the product-specific method suitability study. To provide optimal growth conditions for both bacteria and yeasts/molds, two different incubation temperatures are tested (20–25 and 30–35 °C), although the same medium is used for both tests.

USP <1111> and Ph. Eur. 5.1.4 recommend the absence of bile-tolerant gram-negative bacteria in some products (e.g. inhalants). Bile-tolerant gram-negative bacteria are currently not a well-defined homogenous group but rather defined only on the capacity to grow in bile-salt containing media. If growth occurs in the Rapid MET test, a microbial identification is not sufficient to determine if the contaminant is a representative of that group of specified microorganisms. For this reason, the compendial test for bile-tolerant Gram-negative bacteria has to be performed in parallel. As an alternative, rather than performing the test in parallel by default, a retest with the compendial method for the absence of bile-tolerant Gram-negative bacteria could be performed if growth is observed in the Rapid MET. It is of note that in order to also cover the test for the absence of *Salmonella*, the product-specific suitability test and release testing had to be performed with 10 g product.

Although the Rapid MET with the Celsis Advance will mainly be used for nonfilterable products, testing of filterable products may also be achieved through direct inoculation or filtration of the product on a 0.22 or 0.45 µm filter membrane followed by transfer and incubation of the membrane in the Rapid MET growth medium.

One of the reasons why the Rapid MET with the Celsis Advance was preferred to other existing systems is that the product to be tested can be incubated in a large volume of nutrient medium. Indeed, using a higher volume of nutrient medium can mitigate antimicrobial activity as well as the background of products. The validation work for the Rapid MET was conducted using 90 ml liquid nutrient medium, which now is used as a standard for rapid microbiological examination of non-sterile products. Nevertheless, it was demonstrated that also a nutrient medium volume of 190 ml instead of 90 ml could be used without negative impact on detection limit of the assay (data not shown). If required, even higher volumes may be used, but they would first need to be validated.

One of the main factors affecting microbial recovery and therefore validation of a growth-based alternative method is the nutrient medium. In the Rapid MET with the Celsis Advance, TSB supplemented with 4% polysorbate 80 and 0.5% soy lecithin should be used for routine testing. The use of liquid growth medium is mandatory when performing tests with the Celsis Advance. TSB, as well as its solid equivalent TSA, is a rich nutrient medium offering good growth promotion for a wide variety of microorganisms (Smith *et al.* 1974; MacFaddin 1985). We decided not to use sabouraud dextrose broth for the 20–25 °C incubation temperature, since our method requires the use of products with low bioload; therefore, selection for yeasts/molds from a diverse microbial spectrum is not necessary. Furthermore, TSB can undoubtedly recover a broader microbial spectrum than SDB; thus, we saw it as a more suitable nutrient medium for a presence/absence test. As a matter of fact, TSB incubated at 20–25 °C is the pharmacopoeial incubation condition for detecting yeasts and molds in the sterility test, further indicating that growth-promoting properties for these types of microorganisms should be appropriate. During method validation no evidence for inferior recovery of a slow-growing and stressed mold (*Penicillium* sp.) was observed, and also pharmacopoeial *C. albicans* as well as *A. brasiliensis* strains were adequately recovered. Some mold species, however, have the tendency to form dense aggregates, which may hamper detectability with the Celsis Advance because only a small aliquot of the sample is actually tested for ATP bioluminescence. We have overcome this problem of aggregation by adding the glass bead treatment step, which better homogenizes the microbial cells and therefore ensures that an adequate number is present in the aliquot used for ATP bioluminescence detection.

In case that no microbial growth is detected in the Rapid MET with the Celsis Advance, product may be released for TAMC/TYMC and the absence of specified microorganism in 1 g product. In case that microbial growth is detected in the Rapid MET with the Celsis Advance, the contaminant(s) will be identified. With this approach, it can be assured that also a low-level contamination of specified or objectionable microorganisms would be detected. Furthermore, the original sample of the product would be retested with the compendial MET in order to assess whether the level of contamination is above

acceptance criteria (Figure 14.6). This retest is performed only to provide a count estimate to the detected microbial contamination. If the compendial MET would not recover any microorganisms, the initial microbial finding from the Rapid MET would not be invalidated, but reported as being below the detection limit of the compendial MET (e.g. <10 CFU/g). Furthermore, where warranted, adequate risk assessment for a low-level contamination of that microorganism would have to be performed.

Nevertheless, additionally performing the compendial test for enumeration is time-consuming if microbial growth is detected frequently; thus, products which often exhibit microbial growth may be out of scope for this application. For such products, other RMMs allowing for precise enumeration of microorganisms present in nonfilterable products may be more suitable. For instance, the use of an automated, rapid MPN methodology, which allows for testing of a representative amount of product or enumeration of microcolonies within pour-plated nutrient agar, could represent possible solutions for higher bioload products. Alternatively, product solution could be diluted to the specification limit and a presence/absence test be applied. Consequently, if growth is observed, the limit would be considered exceeded. However, the drawbacks of this approach are the need to perform enumeration tests and absence of specified microorganisms tests in parallel, instead of combining both of these tests in one. Likewise, alert or expectation levels are difficult to define and each finding is a potential out of specification result. Finally, trend analysis of microbial bioload would be challenging and the presence of objectionable microorganisms below the specification level could not be evaluated with such an approach.

14.5 Conclusion

The Rapid MET with the Celsis Advance system was successfully demonstrated to be a possible alternative to the compendial method described in Ph. Eur. 2.6.12, 2.6.13, USP <61>, <62>, and JP 4.05/I and 4.05/II for the microbiological examination of non-sterile products. Our statistical evaluation concept allowed for a robust and scientifically sound validation approach. We defined a process for the product-specific method suitability as well as determination of a product-specific threshold, which if exceeded points toward microbial growth. The Rapid MET using the Celsis Advance can be applied to nonfilterable products of good microbiological quality, allowing for a reduced incubation time to 72 hours and a substantial reduction of hands-on time and improvement in data integrity.

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