Determination of the sporicidal activity against *Clostridium-difficile*-spores in the quantitative suspension test*

(Method 18)

18

18.1 Test organism

18.1.1 Testorganism and initial concentration

Clostridium difficile R027	NCTC 13366 (DSM 27147)	1.5 to 5 x 10^7 cfu/ml

18.1.2 Preparation of the C. difficile stock culture

The procedure for *C. difficile* stock culture preparation essentially corresponds to that used for bacteria (➡ *Chapter 6.1.1*). However, instead of TSA and TSB, BHIYT-L agar without taurocholate and lysozyme (➡ *Annex A1.4.12*) and BHI (➡ *Annex A1.4.13*) are used as nutrient media. The stock cultures are incubated at 36 °C ± 1 °C under anaerobic conditions for 72 h and frozen and stored according to ➡ Chapter **6.1.1**.

18.1.3 Preparation of the working culture and C. difficile spore suspension

➡ see diagram **D8.1**, Annex D

Day 1: To prepare the working culture remove single beads from the stock culture in the cryovials using a wire (loop) or forceps and prepare two cultures by streaking them onto BHIYT-L agar (➡ *Annex A1.4.12*) (1st subculture). One is for enrichment, while the other is to verify the purity of the stock by preparing single colonies. Incubate both subcultures at 36 °C ± 1 °C under anaerobic conditions for 48 h in an anaerobic jar until the colonies have reached a diameter of approx. 4 mm. This first subculture serves as working culture. The use of a second or previously stored subculture is not permitted.

Day 2: Prepare Columbia Broth (➡ *Annex A1.4.14*)) in accordance with the manufacturer's instructions. For preliminary reduction of the oxygen and further preparation, transfer 5 ml Columbia broth (➡ *Annex A1.4.14*) to a 15 ml plastic flask with open screw lid and store in an anaerobic jar overnight (at least 12 h).

Day 3: Suspend an isolated colony of the working culture (at least 4 mm) from BHIYT-L agar (➡ *Annex A1.4.12*)) in 5 ml pre-reduced Columbia Broth (➡ *Annex A1.4.14*) and incubate at 36°C ± 1 °C under anaerobic conditions for 24 h. For the following day pre-reduce 20 ml Columbia Broth in 50 ml plastic flasks with open screw lid overnight (at least 12 h).

Alternatively, test procedures based on European standards can be used for testing the sporicidal properties provided that they meet the test design criteria, including cultivation of spores conditions, listed in the requirements.

***Update notification as of 16th January 2019**: Meanwhile the phase 2/step 1 standard EN 17126:2018 has been published.

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Day 4: Inoculate the pre-reduced Columbia Broth (➡ *Annex A1.4.14*) with 50 µl of the 24 h culture and incubate at 36 °C ± 1 °C under anaerobic conditions for 20 h.

When preparing the sporulation medium (➡ *Annex A1.4.15*) the ingredients must be added in the specified order. Fill 500 ml of the sporulation medium in 1 l flasks and autoclave. After cooling, pre-reduce the sporulation medium (➡ *Annex A1.4.15*) in an anaerobic jar for 24 h at 36 °C ± 1 °C. The pH value should rise during this time to 8.2 ± 0.3 (*Note: A total of 2 ml spore suspension is finally derived on Day 17 from the aforementioned 500 ml sporulation medium*).

Day 5: Transfer the entire inoculum of 20 ml Columbia Broth (➡ *Annex A1.4.14*) to 500 ml of the pre-reduced sporulation medium (➡ *Annex A1.4.15)* and incubate this spore culture at 36 °C ± 1 °C under anaerobic conditions for 10 days.

Day 15: Divide the spore culture between two 250 ml centrifuge tubes and centrifuge at 4000 gN for 10 min. [The centrifugation speed (e.g. 10,000 gN) and centrifugation volumes (≤ 250 ml) can be adjusted if good qualitative purification of the spores is assured. The supernatant is discarded as biological waste]. Resuspend the pellet from each 250 ml flask in 50 ml water (➡ *Annex A1.1)* and then combine in one flask. Add water to increase the volume to 250 ml (➡ *Annex A1.1*) and centrifuge at 4000 gN for 10 min. Next, two further wash steps must be performed using 250 ml water in each case (➡ *Annex A1.1)*. After the third centrifugation, resuspend the pellet in 15 ml water (➡ *Annex A1.1)* and transfer to a pre-weighed 50 ml centrifuge tube. Rinse the original centrifuge tube twice with 10 ml water (➡ *Annex A1.1)* and combine the rinsing liquids in the new pre-weighed flask (final volume: 35 ml). After a further centrifugation step at 4000 gN for 10 min, the weight of the pellet is determined and it is stored overnight at 2 °C to 8 °C.

Day 16: For enzymatic digestion of the remaining vegetative cells and cell components resuspend the pellet in 10 ml 0.1 M sodium phosphate buffer (➡ *Annex A1.3.5)*. If the wet weight is more than 700 mg, divide the suspension between two flasks (each 5 ml) and fill the volume in each case to 10 ml with 0.1 M sodium phosphate buffer (➡ *Annex A1.3.5)* and mix.

Add 25 ml of the enzyme buffer (➡ *Annex A1.3.6)* to the resuspended pellet and mix carefully; do not use a rotary mixer (e.g. Vortex).

Process the suspension for 5 min in an ultrasonic bath and incubate for 6 h at 45 °C ± 1 °C in a water bath. For temperature control place a temperature in an identical flask of equal volume to ascertain when the required temperature is reached in the flask. To prevent clump formation repeat the ultrasound step every 2 h ± 0.2 h. After incubation followed by ultrasonic treatment store the suspension overnight at 2 °C to 8 °C.

Day 17: For purification and further storage of the *C. difficile* spore suspension, centrifuge the suspension from the previous day at 4000 gN for 10 min and wash the pellet three times in 10 ml water (➡ *Annex A1.1)*. [The centrifugation speed (e.g. 10,000 gN) and

centrifugation volumes (≤ 250 ml) can be adjusted if good qualitative purification of the spores is assured]. After the wash steps collect the pellet in 30 ml water (➡ *Annex A1.1)* and heat inactivate the spore suspension for 10 min at 70 °C ± 1 °C in a water bath. For temperature control place a thermometer in an identical flask of equal volume to ascertain when the required temperature is reached in the flask and when heat inactivation (10 min at 70 °C ± 1 C°) can start. Immediately after heat inactivation place the flask with the spore suspension for 5 min in an ice bath. Centrifuge again the spore suspension at 4000 gN for 10 min and collect the pellet from 500 ml aliquots of the original culture in 2 ml water (➡ *Annex A1.1)*.

The viable spore count in the spore suspension is determined by performing a sufficient number of decimal dilutions in water (➡ *Annex A1.1)* followed by incubation (➡ *18.4*) in BHIYT-L agar or, when using the spread plate method, on BHIYT-L agar (➡ *Annex A1.4.12*). Record the colony count. Method 18 was validated with the pour plate method. The spread plate method is also a recognized method for *Clostridium difficile*. If the spread plate method is used, the BHIYT-L agar plates must be pre-reduced overnight for at least 12 h at 36 °C in an anaerobic jar.

For purity control phase contrast images of the spore suspension or spore stains should be obtained from the spore suspension. The amount of residual vegetative cells must not exceed 20 % per field of view, otherwise the spore suspension must be washed again or discarded.

Store the spore suspension at 2 °C to 8 °C in a sealable sterile flask (e.g. falcon tube, glass flask) with sterile glass beads. The spores may only be used after a storage time of at least 8 weeks. Before they are used first, the purity and chemical resistance of the spores must be verified according to ➡ 18.1.4 with both validation solutions in all concentrations specified. After storing for 12 months the chemical resistance must be verified again according to ➡ 18.1.4 with both validation solutions in all concentrations specified. Afterwards, repeat every six months.

18.1.4 Determination of viability and chemical resistance of the spore suspension (reference control)

The viability and chemical resistance of the spores of each batch of spores should be verified after storing for at least 8 weeks at 2 °C to 8 °C. The tests are performed using a dilution neutralisation procedure without interfering substance (➡ *18.3.2a*), using as reference precisely determined concentrations of glutaraldehyde[1] (GA) and peracetic acid[2] (PAA). The peracetic acid should be titrated the day before testing the spore suspension in order to determine the actual active ingredient content (➡ *Annex A4)*.

The spore count in the test suspension used for validation of the spore suspension is adjusted to 1.5 – 5.0 x 10^6 cfu per ml using a suitable method.

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The following reductions must be attained in the corresponding concentration-time relations at 20°C ± 1 °C with *Clostridium difficile* spores NCTC 13366:

1.0 % (v/v) – 15 min: GA-solution should a achieve a reduction of < 1.5 lg,

6.0 % (v/v) – 15 min: GA-solution should a achieve a reduction of ≥1.5 lg,

0.01 % (v/v) – 15 min: PAA-solution should a achieve a reduction of < 1.5 lg,

0.04 % (v/v) – 15 min: PAA-solution should a achieve a reduction of ≥1.5 lg.

For each product test conduct in parallel, for internal quality control purposes, a test using the validation solution (GA or PAA) best suited to the product in its lower specified concentration. For example, for a chlorine-cleaving product the inclusion of a PAA validation solution is recommended.

18.1.5 Preparation of the test suspension

Suspend mechanically for 3 min the spore suspension stored with glass beads. While using a suitable method, the viable spore count in the test suspension is adjusted to 1.5 – 5.0 x 10^7 cfu per ml [to 1.5 – 5.0 x 10^8 cfu per ml of ready-to-use (rtu) products which should be tested as 97% solution].

Store this test suspension at 20 °C ± 1 °C and start testing within 2 h.

18.1.6 Determination of the spore count in the test suspension with C. difficile

The adjusted test suspensions are diluted with diluent to obtain test suspensions of 10^1 (10^{-6} dilution) to 10^2 (10^{-5} dilution) cfu/ml. The test suspension is homogenised using a rotary mixer (e.g. Vortex). Take 1 ml aliquots of the diluted test suspension and pour using the pour plate method or, if using the spread plate method, spread onto pre-reduced plates.

Incubate the hardened nutrient media at 36 °C ± 1 °C under anaerobic conditions for 5 d. Count and record the number of cfu on each culture medium. Counter readings which show values between 14 and 330 cfu/plate are given priority.

Before using the test suspension, verify and record the purity using phase contrast microscopy.

18.2 Product test solution

For all products used diluted the concentration of the product test solution must be prepared 1.25-fold higher than the concentration to be tested, due to the method used. For all products used undiluted, the highest concentration that can be tested is the 97% solution (ready-to-use solution).

[1] Glutaraldehyde e. g. from Dow Chemical Company Ltd, product name: BIOBAN™ GA 50 Antimicrobial
[2] Peracetic acid e. g. from Stockmeier Group, product name: Lerasept® spezial

Details on preparation of the product test solution are given in ➡ Chapter **5**.

Products used **undiluted** are referred to as **ready-to-use products** (rtu) below.

18.3 Method

18.3.1 Principle

A test suspension is added to a sample of the product to be tested and this mixture is kept at test temperature. After the chosen and specified contact times, an aliquot quantity of the mixture is immediately neutralised using the validated procedure. In each sample, the colony count is determined and its reduction as compared to the initial colony count is calculated.

➡ see diagram **D8.2**, Annex D

The method of choice is the dilution neutralisation procedure. Membrane filtration procedures are currently not available because of the lack of data.

The tests can be performed under clean or dirty conditions and are tailored to application conditions of the product to be tested.

18.3.2 Dilution neutralisation procedure

Please refer to the respective requirements when selecting the test conditions.

a) without interfering substance (only for validation ➡ *18.1.4*)

Mix thoroughly 8 ml of the product test solution with 1 ml WSH and 1 ml of the test suspension (rtu: mix 9.9 ml of the product test solution with 0.1 ml of the test suspension containing a 10-fold higher spore count). After the required contact times, the test product test suspension mixture is thoroughly mixed once again and 1 ml aliquots are taken and transferred to 9 ml neutraliser (➡ *Annex A1.7*) (= test neutralisation mixture) and mixed. Immediately afterwards, 10^{-1} and 10^{-2} dilutions are prepared in the neutraliser (➡ *Annex A1.7*). After 5 min ± 10 s neutralisation time, 1 ml aliquots from the test neutralisation mixture as well as 1 ml aliquots from the dilutions are poured in duplicate tests onto the BHIYT-L agar or, if using the spread plate method, spread onto pre-reduced plates.

➡ for incubation see **18.4**

➡ for calculation and presentation of the results see **18.5**

b) with 0.03 % albumin (clean conditions)

To simulate clean conditions the quantitative suspension test is performed with product test solutions containing 0.03 % albumin (➡ *Annex A1.8*) which may only be prepared immediately prior to testing.

To that effect, mix thoroughly 8 ml of the product test solution with 1 ml of 0.3% sterile filtered albumin solution and then with 1 ml test suspension (rtu: 9.7 ml of the product test solution is mixed with 0.2 ml of 1.5% albumin solution and 0.1 ml of test suspension containing 10-fold higher spore count).

After the required contact times, the test product test suspension mixture is thoroughly mixed once again and 1 ml aliquots are taken and transferred to 9 ml neutraliser

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➡ for incubation see **18.4**

➡ for calculation and presentation of the results see **18.5**

(➡ *Annex A1.7*) (= test neutralisation mixture) and mixed. Immediately afterwards, 10^{-1} and 10^{-2} dilutions are prepared in the neutraliser (➡ *Annex A1.7*). After 5 min ± 10 s neutralisation time, 1 ml aliquots from the test neutralisation mixture as well as 1 ml aliquots from the dilutions are poured in duplicate tests into the BHIYT-L agar or, if using the spread plate method, spread onto pre-reduced plates.

c) with 0.3 % albumin and 0.3% sheep erythrocytes (dirty conditions)

To simulate dirty conditions the quantitative suspension test is performed with product test solutions containing 0.03 % albumin and 0.3 % sheep erythrocyte (➡ *Annex A1.8*) which may only be prepared immediately prior to testing.

To that effect, mix thoroughly 8 ml of the product test solution with 1 ml solution containing 3 % sterile filtered albumin as well as 3 % sheep erythrocytes and then mix with 1 ml test suspension (rtu: 9.7 ml of the product test solution is mixed with 0.2 ml solution containing 15% albumin as well as 15% sheep erythrocyte solution containing a 10-fold higher spore count).

After the required contact times, the test product test suspension mixture is thoroughly mixed once again and 1 ml aliquots are taken and transferred to 9 ml neutraliser (➡ *Annex A1.7*) (= test neutralisation mix) and mixed. Immediately afterwards, 10^{-1} and 10^{-2} dilutions are prepared in the neutraliser (➡ *Annex A1.7*). After 5 min ± 10 s neutralisation time, 1 ml aliquots from the test neutraliser mixture as well as 1 ml aliquots from the dilutions are poured in duplicate tests into the BHIYT-L agar or, if using the spread plate method, spread onto pre-reduced plates.

➡ for incubation see **18.4**

➡ for calculation and presentation of the results see **18.5**

18.4 Incubation

The viable spore colonies are counted after 5 d incubation time under anaerobic conditions at 36 °C ± 1 °C.

18.5 Evaluation

Culture media with cfu/plate counts between 14 and 330 are given priority for evaluation. The reduction R is calculated according to the following formula:

The test report should list in tabulated form the cfu values per dilution step, associated lg values as well as the resultant R values.

lg R = lg (cfu Co1) – lg (cfu D)

cfu Co1: Number of cfu per ml without product exposure (WSH control)

cfu D: Number of cfu per ml after product exposure

The cfu values per dilution step for controls 2 and 3 (➡ *18.6.1.2 and 3*) should be compared with the cfu values of the dilution steps of the test suspension and checked for plausibility.

18.6 Validation

18.6.1 Validation of the dilution neutralisation procedure

18.6.1.1 WSH control (Co1)

To determine the number of cfu per ml without exposure to the product (Co1), mix with water the respective test suspension, if applicable containing the interfering substance instead of the product test solution. After the required contact times, prepare as described above (➡ *18.3.2*) dilutions (as applicable, dilutions of 10^{-3} to 10^{-4}).

➡ for incubation see **18.4**

➡ for calculation and presentation of the results see **18.5**

Note: The WSH control (Co1) should be between 1.0 x 10^6 and 5,0 x 10^6 cfu/ml.

18.6.1.2 Neutralisation control (Co2)

In order to validate neutralisation (Co2), 1 ml of the highest concentration of the product used in the test is mixed with 9 ml neutraliser and after 5 min ± 10 s neutralisation time* is added to 0.1 ml from a 10^{-2} dilution of the test series run to determine the cfu count (validation suspension) [option for rfu: 0.1 ml of 10^{-3} dilution]. After the longest contact time a 1 ml sample from both a 10^{-1} and a 10^{-2} dilution in neutraliser (➡ *Annex A1.7*) is poured in duplicate onto BHIYT-L agar or, if using the spread plate method, spread onto pre-reduced plates.

➡ see diagram **D8.2**, Annex D

➡ for incubation see **18.4**

➡ for calculation and presentation of the results see **18.5**

Note: If there is insufficient neutralisation in the test [less than 7.5 x 10^2 cfu/ml (= 50 % of the validation suspension in Co2)], a different neutraliser must be chosen.

18.6.1.3 Verification of the non-toxicity of the neutraliser (Co3)

Verification of the non-toxicity of the neutraliser (Co3) is performed in parallel to control 2 but now using WSH instead of the product test solution or, in the case of rfus, water (➡ *Annex A1.1*).

➡ see diagram **D8.3**, Annex D

Note: If a toxic effect is identified in the test [less than 7.5 x 10^2 cfu/ml (= 50% of the validation suspension in Co3)], a different neutraliser must be chosen.

18.6.1.4 Validation of the membrane filtration method

Currently, no membrane filtration method can be performed with *Clostridium difficile* spores due to the lack of data.

*For products with contact times ≤ 10 min after 10 s ± 1 s neutralisation time.

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➡ for incubation see **18.4**

➡ for calculation and presentation of the results see **18.5**

18A Requirements for efficacy testing for VAH certification

For test conditions please refer to Table 18.1 and 18.2

The quantitative suspension test is obligatory for all application methods assessed by VAH. The test conditions (test concentration, test temperature, contact times) are listed in ➡ **Table 18.1**.

Details of the choice of interfering substance (clean or dirty conditions) as well as the required reductions are given in ➡ **Table 18.2**.

From the test results it must be possible to distinguish between the active and inactive range.

➡ see diagram **D8.3**, Annex D

If testing is performed according to EN standards*, the following additional requirements must also be fulfilled:

– Dilutions of the test neutralisation mixture to at least 10^{-2} are indispensable for identification of any neutralisation problems.

Testing with *Clostridium difficile* spores is obligatory for claiming sporicidal activity and requires that testing be conducted in, apart from the quantitative suspension test, also the respective simulated-use tests currently under development.

Table 18.1: Test conditions in the qualitative and quantitative suspension test.

Application procedure	Test concentrations[1]	Test temperature [°C]	Contact times[3]
Surface disinfection	Use dilutions[2]	20 ± 1	1 min[4], 5 min, 15 min, 30 min, 60 min, 240 min
Chemical instrument disinfection (manual immersion method)	Use dilutions[2]	20 ± 1	1 min[4], 5 min, 15 min, 30 min, 60 min
Automated chemical-thermal instrument disinfection	Use dilutions[2]	≥20 to ≤70 ± 1	1 min[4], 5 min, 15 min, 30 min, 60 min
Chemical textile disinfection (manual immersion)	Use dilutions[2]	13 ± 2	4 h, 6 h, 12 h
Automated chemical-thermal textile disinfection	Mixing of detergent and disinfectant in use ratio	30 to 70 ± 1 based on temperatures of wash process	5 min, 10 min, 15 min, 20 min

[1] In addition to the dilutions required for identification of efficacy (activity) range limits. The test concentrations employed should be used in steps not exceeding the factor 10.

[2] Ready-to-use products for products used undiluted.

[3] A minimum of 3 contact times should be tested and the recommended contact time should be the average time.

[4] If 5 min is the claimed contact time, 1 min must also be tested.

* *Update notification*: EN 17126 (phase 2/step 1) has been published in December of 2018.

Table 18.2: Required reduction to demonstrate sporicidal activity in the quantitative suspension test..

Application procedure	**Sporicidal activity:** *Clostridium difficile* **Lg reduction required under clean[1]/or dirty conditions[2]**
Surface disinfection	4 lg
Chemical instrument disinfection (manual immersion method)	4 lg
Automated chemcial-thermal instrument disinfection **20 °C to ≤70 °C**	4 lg
Chemical textile disinfection (manual immersion) **13 °C ± 2**	4 lg
Automated chemical-thermal textile disinfection **30 °C to < 60 °C**	4 lg
Automated chemical-thermal textile disinfection **60 °C to 70 °C**	4 lg

1 In chemical-thermal textile disinfection only for processes with pre-wash.

2 In chemical-thermal textile disinfection only for processes without pre-wash.