Ulrike Kircheis[1], Markus Wehrl[2]

1 Charité – Universitätsmedizin Berlin, Technische Hygiene, Berlin, Deutschland
2 wfk – Cleaning Technology Institute e.V., Krefeld, Deutschland

Method for Testing the Cleaning and Disinfecting Efficacy of Washer-Disinfectors for Flexible Endoscopes

The method described in this article is attached as annex 9 to the «Guideline of DGKH, DGSV, DGVS, DEGEA and AKI for the Validation of Cleaning and Disinfecting Processes for Thermolabile Endoscopes by Washer-Disinfectors», that was already published [1].

The test procedure described in the technical specification DIN ISO/TS 15883-5 «Washer-disinfectors – Part 5: Test soils and methods for demonstrating cleaning efficacy», annex I, applies reactivated sheep blood as test soil in combination with *Enterococcus faecium* in order to test the cleaning and disinfecting efficacy of automatic reprocessing [2].

The determination of the cleaning efficacy is based on the quantification of protein content as the main parameter and uses the modified OPA-method. it was published recently as annex 8 «Method for Testing the Cleaning efficacy of Washer-disinfectors for Flexible Endoscopes» to the above mentioned guideline [3, 4]. The here presented annex 9 will describe a detailed method for the determination of the cleaning and disinfecting efficacy (overall process). For the establishment of this method the working group of the guideline initiated the formation of a team named «Method group».

Coordinating persons of the «Method group» were: Priv. Doz. Dr. Holger Biering representing the instrument Preparation Working group (Arbeitskreis Instrumentenaufbereitung, AKi), Dr. Birgit Kampf, representing manufacturers of flexible endoscopes and Prof. Dr. Heike Martiny, representing the German Society for Hospital Hygiene (Deutsche Gesellschaft für Krankenhaushygiene e. V., DGKH). The «Method group» was supported by Verona Schmidt, member of the coordinators of the working group of the guideline and representing AKI.

Members of the «Method group» were the following institutions and companies: Biotec GmbH, represented by Dr. Olaf Kaup; Charité – Universitätsmedizin Berlin, represented by Dr. Ulrike Kircheis; HS System- und Prozesstechnik, represented by Ingo Hannemann; Hybeta GmbH, represented by Christiaan Meijer and Christoph Keller; HygCen Centrum für Hygiene und medizinische Produktsicherheit GmbH, represented by Johanna Köhnlein; Simicon GmbH, represented by Paul Gerhard Simon and Dr. Nicole Büchl; SMP GmbH, represented by Klaus Roth and Dr. habil. Ludger Schnieder; Verbund für Angewandte Hygiene (VAH) and Universität Bonn, represented by Dr. Jürgen Gebel; wfk – Cleaning Technology Institute e. V., represented by Dr. Markus Wehrl.

1 Introduction

The cleaning and disinfecting efficacy of the overall process of a washer-disinfector (Wd) for flexible endoscopes has to be checked both in type testing and in performance qualification testing within validation.

The test procedure as per DIN ISO/TS 15883-5, annex I is specified and described in detail in the following article. For the preparation of the test pieces polytetrafluorethylene (PTFE-) tubes with a length of 200 cm and an inner diameter of 2 mm are used. The test soil consists of reactivated sheep blood and *Enterococcus faecium* as test organism. The evaluation of the overall process efficacy is based on the quantification of the number of surviving test organisms after a visual inspection of the test pieces.

Departing from DIN ISO/TS 15883-5 this is done by so called «end point determination» (see figure 1): test pieces are soiled, processed and filled with liquid selective

German Society of Hospital Hygiene

Joachimstaler Straße 10
10719 Berlin, Germany
Tel: +49 30 8855 1615
Fax: +49 30 8851 029
E-Mail: info@krankenhaushygiene.de
Internet:
www.krankenhaushygiene.de.

Key Words

Flexible endoscopes
Reprocessing
Testing
Disinfection
Enterococcus faecium
Annex 9
Guideline

Corresponding Authors

Dr. rer. nat. Ulrike Kircheis

Charité – Universitätsmedizin Berlin
Technische Hygiene
Hindenburgdamm 27
12203 Berlin
E-Mail: ulrike.kircheis@charite.de

Dr. rer. nat. Markus Wehrl

wfk - Cleaning Technology Institute e.V.
Campus Fichtenhain 11
47807 Krefeld
E-Mail: m.wehrl@wfk.de

agar afterwards in order to detect all surviving test organisms.

If more information on the failure or insufficiency of a process is needed, additional tests for the quantification of surviving test organisms can be performed. For this purpose test organisms are eluted with a solution containing neutralizer, see figure 1. The eluate is filtered and the membrane filter is incubated on agar media. in the case of high cell numbers dilution series can be plated additionally. After this the eluted test pieces are filled with liquid selective agar in order to detect test organisms which were not eluted.

For the evaluation of cleaning-disinfection processes reduction factors (RF-values) are established. The RF-values are calculated from the number of surviving test organisms retrieved from the end point determination or alternatively from membrane filters and dilution series, each in proportion to the initial challenge of test organisms.

The following article specifies the test method including growing of test organisms, preparation of the test soil and test pieces, determination of the end point, elution of residing test organisms, and evaluation of results. An overview of the workflow is given in figure 1.

2 Material

2.1 Test soil

– sheep blood heparinised with 10 IE heparin ml^{-1}. The usage of pooled blood, i. e. a mixture of blood from several animals, is recommended (e. g. from Acila GmbH, Fiebig Nährstofftechnik). The quality of the blood should be in accordance with the criteria described in annex 8 [3, 4]
– protamine hydrochloride or protamine sulfate, applied to a final concentration of 15 IE ml^{-1} blood (e. g. Protamin Valeant 1000 i.e. ml^{-1} of Valeant Pharmaceuticals Germany GmbH)
– test organism: *Enterococcus faecium* (*E. faecium*) (ATCC 6057, DSM 2146)

2.2 Materials for test pieces

– PTFE-tubes, length 200 cm, inner diameter 2 mm, wall thickness 0.5 mm (e. g. from VWR international GmbH, Order-No: 228-4134). The influence of a pre-cleaning process of these PTFE-tubes was assessed and turned out not to be essential. The PTFE-tubes are applied as single-use products
– sections of silicone-tube, length approximately 2 cm, inner diameter 2 mm (e. g. from Carl Roth, Order-No: 9559.1); if necessary other sizes
– if applicable red locking cones (e.g. from Angiokard Medizinische Spritzguss & Entwicklungstechnik GmbH, Order-No: AK 64900)

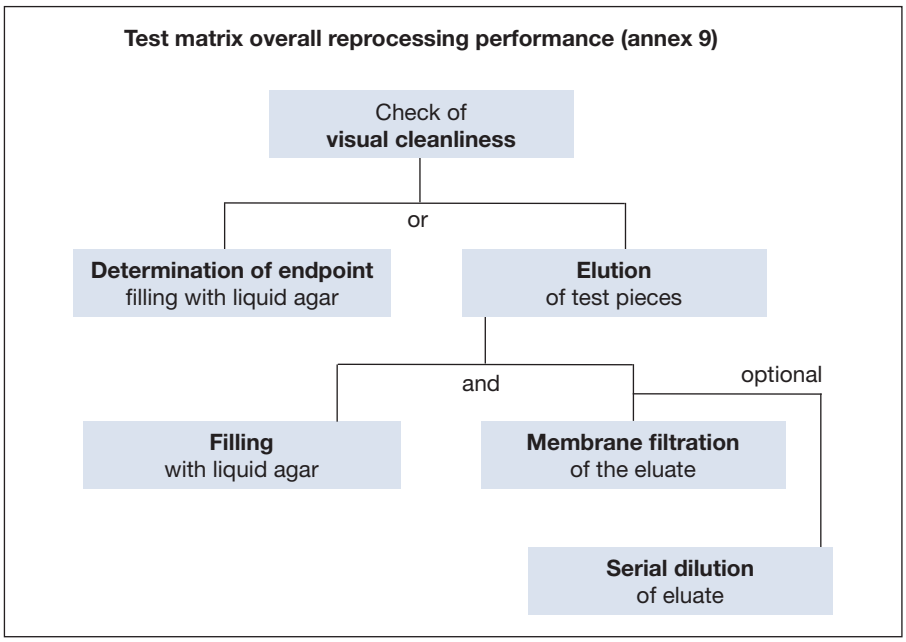

Figure 1: Test matrix for the evaluation of the overall reprocessing performance (annex 9).

2.3 Equipment and consumables

– centrifuge
– balance with a resolution of ≤ 1 mg, calibrated
– micro litre pipettes for diverse volumes, calibrated
– water bath with a temperature range of up to 60 °C
– membrane filtration apparatus for filters with Ø 50 mm
– shaker
– vortex mixer
– 50 ml centrifuge tubes (e. g. Falcon™ tube)
– glass beads, Ø 3–4 mm
– glass beakers, diverse sizes
– 10 ml syringes (single use)
– 20 ml syringes (single use)
– 60 ml syringes (single use) (bladder syringe) or 50 ml syringe for perfusors (e. g. from B. Braun)
– membrane filters, pore size 0.45 µm, Ø 50 mm
– pipette tips, diverse sizes
– drigalski spatula
–cable straps
– scalpels

2.4 Media and solutions

– liquid kanamycin esculin azide agar (55–60 °C)
– kanamycin esculin azide agar (KAA-agar)
– test tubes with 10 ml casein soy bean peptone broth (CSl)
– casein soy bean peptone agar (CSA)
– 0.9 % physiological sodium chloride solution (NaCl-solution)
–if applicable neutralization medium

All materials, solutions and media used for the culturing and preparation of microorganisms have to be sterile. Microbial media have to be stored cool until use.

3 Methods

3.1 Preparation of the test organism suspension

At day 1 *E. faecium* cells of a densely grown surface culture on CSA-agar (at maximum second passage of the surface culture) are transferred into a test tube containing CSl medium. After incubation for 24 hours at 36 ± 1 °C the cells are vortexed (day 2). An aliquot of 100 µl is transferred into another test tube with fresh CSl medium and incubated for 24 hours at 36 ± 1 °C. Experience

has shown, that two subsequent culturing steps in liquid media result in a better bacterial growth. On day 3 the cells are suspended and 100 µl aliquots are used for inoculation on CSA petri dishes that are incubated for 72 hours at 36 ± 1 °C. Approximately 10 to 15 densely grown agar dishes have to be used for the soiling of one test piece.

At day 6 the cells are harvested from the petri dishes by washing of with 5 ml NaCl-solution for each dish using a drigalski spatula. The suspension of all dishes is collected in a glass beaker whose bottom is completely covered with glass beads.

The suspension is homogenized by shaking for 10 minutes at 250 rpm, then transferred to centrifuge tubes and centrifuged for 10 minutes at 3000 g. The supernatant is discarded and the cell pellet is suspended in a small volume of NaCl-solution. glass beads are added (approximately one quarter of the suspension volume) and the suspension is vortexed for 1 minute at approximately 500 rpm. An aliquot of 100 µl is taken for the subsequent quantification of colony forming units (CFu_S) of the bacteria suspension (see 3.7).

3.2 Preparation of the test soil

The heparinised sheep blood, the protamine solution and the bacteria suspension are warmed up to room temperature and each component is mixed thoroughly. The test soil for one test piece is made up of the following constituents:

- 11.4 ml heparinised sheep blood
- 0.42 ml bacteria suspension
- 0.18 ml protamine solution containing 180 IE

One by one, the components are transferred to a beaker, mixed thoroughly yet very carefully to prevent shear forces and a possible bubbling of the blood. To determine the coagulation period of the sheep blood, a stop watch is started right after the addition of protamine. After mixing the test soil an aliquot of 1.0 ml is taken and diluted in 9.0 ml NaCl-solution for the subsequent quantification of colony forming units (CFu_T) (see 3.7).

3.3 Preparation of test pieces

The PTFE-tubes are marked at one end (e. g. using cable straps) and a piece of silicone-tube is fixed to allow the connection of a syringe to load the test soil. The weight of the prepared tubes is measured using a balance.

PTFE-tubes are soiled in a horizontal position. 10 ml of the test soil are taken up with a syringe and are injected into the tubes. After an incubation of 30 seconds, 2 × 10 ml air is injected into the test pieces to purge excessive soil which is collected in a beaker.

In order to determine the time period required for blood coagulation, the beaker is swayed slightly. The onset of coagulation is characterized by a gel-like hardening of the surface, the time is assessed using a stop watch. Required time and room temperature are documented. The complete coagulation has to set in less than 30 minutes and is characterized by a solid and no longer gel-like consistence. If these requirements are not met, the test soil and the respective batch of sheep blood have to be discarded

The soiled test pieces are incubated for one hour at room temperature in a horizontal position, to allow complete coagulation of the soil. Contrary to DIN ISO/TS 15883-5, annex I, the patency is checked. For this 20 ml of air is taken up with a syringe and injected slowly into the test pieces. Test pieces which turn out to be blocked are discarded. Afterwards the weight of the test pieces is measured using a balance in order to calculate the weight of the contained test soil.

3.4 Application of test pieces in a WD

For the determination of the overall cleaning-disinfection performance test pieces are mounted to the respective connections in the Wd in such a way that they will be flushed in the same direction as during soiling. The mounting has to be leak-proof and to withstand applied flushing pressures.

After the process in the Wd the test pieces are removed. Approximately 2 cm of the inlet-side which served as connection to the Wd are cut with a sterile scalpel and discarded. New sterile silicone adapters are put on the test pieces. Test pieces that serve as positive control can be analysed without cutting the inlet-side.

3.5 Determination of end point

After removal the test pieces are inspected visually for cleanliness and the results are documented. The endpoint evaluation quantifies all surviving test organisms that remain in the test pieces. For the evaluation it has to be assured that no disinfectant residues are present in the test pieces. By using a 10 ml syringe the test pieces are completely filled with warm (55–60 °C) liquid KAA-agar avoiding air bubbles and any discharge at the outlet-port. The ends of the test piece are connected to each other in ring shape by silicone adapters; alternatively, the ends may be closed off by cones. Test pieces filled with agar are incubated at 36 ± 1 °C. growth of surviving test organisms is indicated by formation of a black colour. After 24 hours the number of colony forming units (CFU_E) is counted, after 48 hours the test pieces are checked again, all results are documented.

3.6 Elution of test organisms

An elution of test pieces is conducted for: i) analysis of reprocessing procedures with low performance, ii) to exclude any interference of disinfectant residues and iii) to quantify the microbial recovery rate of positive controls.

Processed test pieces are at first eluted with a suitable neutralization medium and subsequently filled with liquid KAA-agar. By this all test organisms are detected both in the test pieces and in the eluate. The selection of suitable neutralization media is based on information and recommendations provided by the disinfectant manufacturer or can be chosen using the standard methods of the DGHM [5].

For the elution of test organisms 50 ml neutralization medium is taken up with a syringe and injected into the test piece. The medium is injected in the same direction as the test soil and the preceding flushing in the Wd. Medium residues in the tubes are expelled by subsequent injection of 2 × 50 ml air. The eluate is collected in a sterile beaker (e. g. 200 ml volume), whose bottom is completely covered with glass beads, and homogenized by shaking for 3 minutes at 250 rpm. Afterwards the test piece is filled with liquid KAA-agar (see 3.5).

The eluate is membrane filtered. To transfer the eluate quantitatively from the beaker to the filter, 150 ml of NaCl-solution is filled into the beaker, briefly shaken and filtered as well. Then the membrane filter is transferred onto KAA-agar medium and incubated at 36 ± 1 °C. The total number of colonies (CFU_M) is counted after 24 hours, a check-up follows after 48 hours, all results are documented. The quantification of high numbers of test organisms is essential for the evaluation of non-processed positive controls (CFU_C) or for the optimization of processes with low RF-values. Therefore an aliquot of 1 ml is taken from the eluate, diluted serially and plated on KAA-agar medium (CFU_D) (see 3.7).

Table 1: Recommended serial dilutions of the different samples and required cell concentrations.

Sample (chapter)		Recommended serial dilution	Required CFU ml^{-1}
Suspension of test organisms (3.1)	KBE_S	10^{-7} to 10^{-10}	≥ 1.0×10^{11}
Test soil (3.2)	KBE_P	10^{-5} to 10^{-8}	≥ 3.5×10^{9}
Eluate of reprocessed test pieces (3.6)	KBE_V	10^{0} to 10^{-2}	–
Eluate of positive controls (3.6)	KBE_K	10^{-1} to 10^{-6}	–

3.7 Quantification of test organisms

The quantification of test organisms by plating is done in duplicate. For the quantification of colony forming units (CFU) samples are diluted serially – according to table 1 – and aliquots of 100 µl are plated on KAA-agar medium. The dishes are incubated at 36 ± 1 °C, a growth of test organisms is indicated by formation of a black colour.

4 Quality control

For the quality control of test pieces regarding the used type of PTFE material it is referred to annex 8 of the guideline [3, 4]. The number of test organisms of the bacteria suspension (CFU_S) and of the test soil (CFU_T) has to be quantified for each batch of test pieces; the required values are given in table 1.

For each batch of prepared test pieces at minimum one positive control (non-processed test piece) has to be analyzed. For this purpose the bacterial recovery rate is calculated, that serves exclusively for internal quality control. For the determination test organisms are eluted and quantified by plating serial dilutions (CFU_C) (see 3.6, 3.7). The bacterial recovery rate (RRB) is calculated as follows:

$$RR_B = 100 * \frac{CFU_C}{WTS * CFU_T}$$

CFU_C: number of test organisms (test piece – positive control)$^{-1}$ retrieved by elution and serial dilution. Counted CFU are corrected for the respective dilution and entire volume of the eluate

WTS: gravimetrically determined weight of test soil in a test piece given in [g], that equals the volume in [ml] for an assumed density of 1.0 g ml^{-1}

CFU_T: number of test organisms in the test soil ml^{-1}

Previous experiences suggest the recovery rate to exceed 0.1 % significantly but to range below 2.0 %. Results of respective round robin tests will be published soon.

Previous experiences suggest the recovery rate to exceed 0.1 % significantly but to range below 2.0 %. Results of respective round robin tests will be published soon.

– room temperature
– period until onset of coagulation of test soil
– amount of test soil in the test pieces after patency check
– calculated CFU number (test piece)$^{-1}$

5 Analysis of test pieces

Test pieces applied for evaluation of the overall reprocessing performance have to contain ≥ 1×10^9 CFU (test piece)$^{-1}$. The initial amount of test organisms in a test piece (CFU_{TP}) is calculated as follows:

$$CFU_{TP} = WTS * CFU_T$$

WTS: gravimetrically determined weight of test soil in a test piece given in [g], that equals the volume in [ml] for an assumed density of 1.0 g ml^{-1}

CFU_T: number of test organisms in the test soil ml^{-1}

Test pieces that do not meet this requirement have to be excluded from the evaluation.

Subsequently the microbial reduction factor (RF-value) is calculated for every test piece. The RF-value is the logarithmic ratio of the initial number of test organisms and the remaining number of test organisms after the process.

The determination of surviving test organisms in processed test pieces can be carried out by various methods (see figure 1) resulting in:

CFU_E:
number of test organisms (test piece)$^{-1}$ resulting of the end point determination (3.5) or of the filling of test pieces after elution (3.6). For the evaluation 10 to 100 CFU (test piece)$^{-1}$ are considered. Cell numbers between 0 and 10 CFU (test piece)$^{-1}$ are set to 1. Numbers exceeding the counting range are set to 100 and the RF-value is expressed as «<».

CFU_M:
number of eluted and membrane filtered test organisms filter^{-1} (3.6). For the evaluation between 6 and 150 CFU filter^{-1} are considered. Cell numbers between 0 and 6 CFU filter^{-1} are set to 1. Numbers exceeding the counting range are set to 150 and the RF-value is expressed as «<».

CFU_D:
number of eluted and diluted test organisms (test piece)$^{-1}$. For the evaluation counted CFU numbers are corrected for the respective dilutions and the entire volume of the eluate.

5.1 Determination of end point (3.5)

The RF-value is calculated as follows:

$$RF = \log\left(\frac{CFU_{TP}}{CFU_E}\right)$$

5.2 Elution and membrane filtration (3.6)

The RF-value is calculated as follows:

$$RF = \log\left(\frac{CFU_{TP}}{CFU_E + CFU_M}\right)$$

5.3 Elution and membrane filtration and serial dilution (3.6)

The RF-value is calculated as follows:

$$RF = \log\left(\frac{KBE_{PK}}{KBE_E + KBE_M + KBE_V}\right)$$

5.4 Documentation

Additionally to the information of quality management (see 4) the following information has to be recorded for every single test piece for the evaluation and calculation of the RF values:
– amount of containing test soil
– number of test organisms (CFU_{TP})
– reduction factor (RF value)
– used method

6 Application of the method

6.1 Visual cleanliness

After removal of the test pieces from the Wd the cleanliness is assessed visually. If any residue is detected the method of annex 9 does not apply as the reprocessing procedure failed.

6.2 Evaluation of cleaning performance

The method of annex 9 can be applied additionally to obtain information about the cleaning efficacy of the reprocessing procedure. The assessment of the cleaning effect using microbial reduction does not replace the obligation to quantify the cleaning efficacy using annex 8 of the guideline.

6.3 Storage and shipping

The described method applies to test pieces which are prepared directly at the site of testing and examined immediately after processing in the Wd according to the test matrix given in figure 1. If test pieces are intended to be used more than 1 hour after preparation or shipped, it has to be demonstrated and documented, that the measures taken are sufficient to assure equivalent and comparable results. This applies to storage and shipping of processed test pieces as well.

6.4 Equivalency to method

Any deviation or modification of the method shall only be acceptable, if comparative investigations had demonstrated equivalency and consistency with the method given here.

6.5 Reproducibility

The reproducibility of the method had already been investigated, proved and published previously [6, 7].

6.6 Simultaneous reprocessing

During application of test pieces no simultaneous reprocessing of flexible endoscopes intended for application on humans shall take place.

6.7 Requirements on the test organism

Requirements on the heat resistance of the used test organism *Enterococcus faecium* (ATCC 6057, DSM 2146) are given in DIN ISO/TS 15883-5, annex I, Chapter I.7.4, which have to be met.

7 References

1. DGKH, DGSV, DGVS, DEGEA, AKI: Leitlinie zur Validierung maschineller Reinigungs-Desinfektionsprozesse zur Aufbereitung thermolabiler Endoskope. ZentralSteril 2011;Suppl. 3.
2. DIN ISO/TS 15883-5. Reinigungs-Desinfektionsgeräte – Teil 5: Prüfanschmutzungen und -verfahren zum Nachweis der Reinigungswirkung (ISO/TS 15883-5); Deutsche Fassung CEN ISO/TS 15883-5:2005. Berlin: Beuth Verlag, 2006.
3. Wehrl M, Kircheis U. Methode zur Überprüfung der Reinigungsleistung von Reinigungs-Desinfektionsgeräten für flexible Endoskope. HygMed 2011;36:402–406.
4. Wehrl M, Kircheis U. Methode zur Überprüfung der Reinigungsleistung von Reinigungs-Desinfektionsgeräten für flexible Endoskope. ZentralSteril 2011;5:352–356.
5. Gebel J, Werner HP, Kirsch-Altena A, Bansemir K. Standardmethoden der DGHM zur Prüfung chemischer Desinfektionsverfahren. mhp Verlag GmbH, Wiesbaden, 2002.
6. Zühlsdorf B, Martiny H. Intralaboratory reproducibility of the German test method of prEN ISO 15883-5 for determination of the cleaning efficacy of washer-disinfectors for flexible endoscopes. J Hosp Infect 2005;59:286–291.
7. Zühlsdorf B, Kampf G, Floss H., Martiny H. Suitability of the German test method for cleaning efficacy in washer-disinfectors for flexible endoscopes according to prEN ISO 15883. J Hosp Infect 2005;61:46–52.