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

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The method described in this article will be attached as appendix 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 will be published soon.

The test procedure described in EN ISO/TS 15883-5, Annex I, applies reactivated sheep blood as test soil in combination with *Enterococcus faecium* as test organism [1]. This procedure will be supplemented by a cleaning test which relies on the quantitative detection of proteins, as protein content is a suitable parameter for the assessment of soil removal. Protein quantification is performed by using the modified OPA-method [2].

The working group of the guideline initiated the formation of a team named «Method Group» to work out a detailed protocol of the method and to check the method in round robin tests.

Coordinating persons of the «Method Group» and members of the working group of the guideline were: Priv. Doz. Dr. Holger Biering representing the Working Group Instrument Preparation (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 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 GmbH, 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 and Monika Feltgen; Simicon GmbH, represented by Paul Gerhard Simon and Cilli Sedlacek; SMP GmbH, represented by Klaus Roth and Dr. habil. Ludger Schnieder; Verbund für Angewandte Hygiene e. V. (VAH) and Universität Bonn, represented by Dr. Jürgen Gebel; wfk – Cleaning Technology Institute e. V., represented by Dr. Markus Wehrl.

The results of the round robin tests will be published at a later date.

1 Introduction

The cleaning efficacy 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 that could be applied so far (EN ISO/TS 15883-5, Annex I) uses a test soil consisting of reactivated sheep blood and the test organism *Enterococcus faecium*. Here, the cleaning efficacy is determined by the decreasing number of surviving test organisms.

In the procedure established by the «Method Group» a polytetrafluorethylene (PTFE-)tubing with a length of 200 cm and an inner diameter of 2 mm was used as a test piece to assess the cleaning efficacy with regard to the parameter protein content (test piece according to EN ISO/TS 15883-5, Annex I). This test tubing is contaminated with reactivated sheep blood as test soil. For the evaluation of the cleaning efficacy the residual protein content of processed test tubings is assessed. Alternatively the reduction factor (RF) of the protein amount can be calculated.

The modified ortho-phthaldialdehyde (OPA)-method is applied in order to quantify the protein amount. OPA reacts with α- and ϵ-terminal amino groups by forming a fluorescent alkylthio-2-alkylisoindole in the presence of N,N-dimethyl-2-mercaptoethylammoniumchloride. This compound is quantified photometrically at 340 nm. As the OPA-method exclusively detects free primary amino groups of the proteins, the method is being calibrated using the established model protein bovine serum albumin (BSA, fraction V).

The following method describes the preparation of test pieces, the elution of residual test soil, and the quantification of residual proteins using the OPA-method.

KEY WORDS
- flexible endoscopes
- reprocessing
- testing
- cleaning
- modified OPA-method

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2 Material

2.1 Test pieces

2.1.1 Test soiling

- sheep blood heparinized with 10 IE heparin ml^{-1}. The usage of pooled blood, i. e. a mixture of blood from several animals, is preferable. The quality of the blood is approved if the recovery rate of blood proteins is beyond 70 % and clearly below 100 %. Sheep blood is commercially available (e. g. from Acila GmbH, Fiebig Nährstofftechnik)
- 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)
- physiological sodium chloride solution 0.9 % (NaCl solution)

2.1.2 Material for test pieces

- PTFE-tubings with a length of 200 cm and an inner diameter of 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 this PTFE-tubings was assessed and turned out not to be essential
- sections of silicone-tubing, length approximately 2 cm, inner diameter 2 mm (e. g. from Carl Roth, Order-No: 9559.1). Alternatively: silicone-tubings with a length of 1 cm and an inner diameter of 2 mm in combination with silicone-tubings with a length of 2 cm and an inner diameter of 4 mm
- if applicable red locking cones (e. g. from Angiokard, Order-No: AK 64900)

2.1.3 2.1.3 Equipment and consumables

- balance with a resolution of ≤ 1 mg, calibrated
- micro liter pipettes for diverse volumes, calibrated
- 10 ml syringes (single use)
- 20 ml syringes (single use)
- pipette tips, diverse sizes

2.2 OPA method

2.2.1 Equipment and consumables

- UV/VIS-photometer suitable for measurements at $\lambda = 340$ nm
- cuvettes: quartz or single use plastic* (see below) suitable for measurements at $\lambda = 340$ nm
- pH-meter, calibrated

Table 1: Composition of the OPA reagent (2-fold concentrated)

Total volume of the OPA reagent	50 ml
Disodium tetraborate	2.01 g
o-phthaldialdehyde	80.0 mg
2-(dimethylamino)ethanethiolhydrochloride	200 mg
Methanol	2.00 ml
SDS solution 20 % in H_2O (w/w)	2.50 ml
H_2O	ad 50.0 ml

Table 2: Composition of the reagent for the measurement of self-extinction of proteins (2-fold concentrated)

Total volume of the reagent for self-extinction	50 ml
Disodium tetraborate	2.01 g
Methanol	2.00 ml
SDS solution 20 % in H_2O (w/w)	2.50 ml
H_2O	ad 50.0 ml

- balance with a resolution ≤ 1 mg, calibrated
- volumetric flasks 100 ml, class A (e.g. from Carl Roth, Order-No: Y281.1)
- volumetric pipettes 5 ml, class A or AS (e. g. from Carl Roth, Order-No: E976.1)
- volumetric pipettes 2 ml, class A or AS (e. g. from Carl Roth, Order-No: E973.1)
- micro liter pipettes for diverse volumes, calibrated
- pipette tips, diverse sizes
- reaction tubes, diverse
- conical tubes, diverse

** When using single use cuvettes, the constancy of their optical properties should be checked by filling at least 8 cuvettes with 1 % SDS-solution and measuring the extinction at $\lambda = 340$ nm. The difference between the determined extinction values should be ≤ ± 0,005. The usage of single use cuvettes is to be preferred in order to avoid spurious interference caused by improper cleaning of quartz cuvettes.*

2.2.2 Chemicals

- ortho-phthaldialdehyde, p.a.-grade (e. g. Merck, Order-No. 1.11452.0005)
- methanol, p.a.-grade (e.g. Merck, Order-No. 1.06009.1011)
- disodium tetraborate, anhydrous, p.a.-grade (e. g. Merck, Order-No. 1.06306.0250)
- 1 % SDS solution (w/w) in H_2O, adjusted with NaOH to pH = 11 (SDS, sodium dodecylsulfat, grade for biochemical applications [e. g. Merck, Best.-Nr.: 1.12533.0050])
- 20 % SDS solution (w/w) in H_2O, pH value not adjusted
- 2-(dimethyl-amino)ethanethiolhydrochloride, 95 % (e. g. Aldrich, Order-No. D141003-25G)
- L-leucine, grade for biochemical applications (e. g. Merck, Order-No. 1.05360.0025)
- bovine serum albumine (BSA), fraction V, purity ≥ 98 % (e. g. Carl Roth, Order-No. T844.2)
- commercial BSA-standard, fraction V, with specified concentration between 100 – 200 µg ml^{-1} (e. g. Thermo Scientific, Order-No. 23208: ready-to-use set of standards including 7 × 3.5 ml with the following concentrations: 125, 250, 500, 750, 1000, 1500, 2000 µg ml^{-1})
- 0.1 M NaOH solution in H_2O
- H_2O, HPLC-grade (e. g. Merck, Order-No. 1.15333.2500)

2.2.3 Solutions

2.2.3.1 OPA reagent

For the preparation of the 2-fold concentrated OPA-reagent the chemicals given in table 1 are used. The table gives the amount of the respective chemicals for an exemplary total volume of 50 ml. The amounts can easily be adapted to other volumes. First of all, the disodium tetraborate is soluted in the respective volume of H_2O (HPLC-grade) while heating it up to 40 °C. Following this, o-phthaldialdehyde and 2-(dimethyl-amino)-ethanethiolhydrochloride are solubilized in the respective volume of methanol. This solution is added to the disodium tetraborate solution, and finally the respective volume of 20 % SDS solution is added as well. The OPA-reagent has to be prepared freshly each day.

2.2.3.2 Reagent for the measurement of protein self-extinction

For the measurement of protein self-extinction the following 2-fold concentrated reagent is used; its composition is given in table 2. The amounts of the chemicals apply to a final volume of 50 ml that can be adjusted.

2.2.3.3 BSA standards

The OPA-method is calibrated using several standards made up of the model protein bovine serum albumine (BSA), fraction V, which cover a relevant range of concentrations. As the first step in the preparation of the standards 100 mg BSA is dissolved in 1 % SDS solution to a final volume of 100 ml using a volumetric flask. This stock solution (standard no. 1) is diluted further by mixing exactly 5 ml of the respective standard with 5 ml 1 % SDS-solution in a clean beaker using volumetric pipettes.
By balancing each added solution the precision of the dilution can be checked gravimetrically. Alternatively the volumes of the added solutions can be checked using a 10 ml volumetric flask. The error should be ≤ ± 0,3 % (5 ml pipettes, class A/AS). The following BSA-standards are prepared using the scheme given in table 3.

2.2.3.4 Leucine standard

For the preparation of the leucine-standard 2 ml of a 0.01 M L-leucine-solution in H_2O are transferred to a 100 ml volumetric flask by using a volumetric pipette; the flask is then filled up with water. The dilution is checked gravimetrically.

Table 3: Scheme for the preparation of BSA-standards used for the calibration of the OPA method

Standard No.	Protein concentration (µg BSA ml^{-1})	Volume of standard	Volume of solute (1 % SDS solution)
1	1000	5.00 ml stock solution	–
2	500	5.00 ml standard 1	5.00 ml
3	250	5.00 ml standard 2	5.00 ml
4	125	5.00 ml standard 3	5.00 ml
5	62.5	5.00 ml standard 4	5.00 ml
6	31.3	5.00 ml standard 5	5.00 ml
7	15.6	5.00 ml standard 6	5.00 ml
8	7.8	5.00 ml standard 7	5.00 ml
9	3.9	5.00 ml standard 8	5.00 ml
10	0	–	5.00 ml

3 Methods

3.1 Test pieces

3.1.1 Preparation of test soil

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

- 11.4 ml heparinized sheep blood
- 0.42 ml 0.9 % NaCl-solution
- 0.18 ml protamine solution containing 180 IE

One after the other, 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 interval of the sheep blood, a stop watch is started right after the addition of the protamine. After mixing the test soil an aliquot of 100 µl is taken and diluted in 9.9 ml of a 1 % SDS-solution, for a subsequent determination of the protein concentration of the test soil using the OPA-method.

3.1.2 Soiling of PTFE tubings

The PTFE-tubings (length = 200 cm) are marked at one end (e. g. using a cable strap) and a piece of silicone-tubing is fixed to allow for the connection of a syringe which loads the test soil. The weight of the prepared tubing is measured using a balance, then the tubings are fixed horizontally on a table and fixed, e. g. by using adhesive tape.
10 ml of the test soil are taken up with a syringe, that is connected to the silicone-tubing adapter. Following the test soil is injected. After an incubation time of 30 seconds, 2 × 10 ml of air is injected into the test piece to blow out excessive soiling. Expelled soil is collected in a beaker at the tubing ending and, in order to determine the moment the coagulation sets in, the beaker is swayed slightly. The start of the coagulation is recognized by a gel-like hardening of the surface, the period is assessed by using a stop watch. The required time and room temperature are documented.
The soiled test piece is incubated for one hour at room temperature, then the patency is checked. In order to do this, 20 ml of air are taken up with a syringe of just this volume and injected slowly[1]. Afterwards the weight of the test piece is measured using a balance and the weight of the contained soiling is calculated.

3.1.3 Application of Test Pieces in a WD

For the measurement of the cleaning performance, test pieces in WD have to be applied in such a way that they are flushed in the same direction as during soiling. It also has to be ensured that the test pieces are fixed tightly and robustly at the respective connections of the WD to withstand the flushing pressure.

3.1.4 Elution of Test Pieces

For the quantification of protein content in reprocessed test pieces as well as in untreated positive and negative controls, residues have to be eluted. Therefore, 5 ml 1 % SDS-solution are taken up with a 10 ml syringe and injected into the respective test pieces. The injection is conducted in the same direction as the soiling of the test piece and the preceding flushing during reprocessing in WD. The SDS-solution is collected in a beaker and then pulled back into the test piece. After that the syringe is removed and 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. The test piece is incubated for 30 minutes at room temperature. Afterwards, the SDS-solution is expelled into a beaker, then pulled up into the test piece again. This rinsing process is repeated once more. Finally, the test piece is expelled completely and drained by 3 x 20 ml of air to remove residual SDS-solution. The protein content of this eluate is quantified by the modified OPA-method.

3.2 OPA method

The following descriptions refer to a sample volume of 1 ml which might have to be modified with respect to the available equipment. Before usage all solutions have to be warmed up to room temperature. The measuring wavelength is $\lambda = 340$ nm. The measurement of each sample is performed in triplicate. For subsequent calculations the average values are used.

3.2.1 Calibration of OPA method

For the quantification of unknown protein samples the protein concentration is determined as equivalents referring to a special «model» protein. The most common «model» protein is fraction V of bovine serum albumine (BSA). The calibration of the OPA-method is performed using several BSA-standards as given in table 3. For the measurement, 500 µl of the respective standard and 500 µl OPA-reagent (table 1) are pipetted into a cuvette, mixed thoroughly and incubated for 3 minutes. Subsequently the extinction is determined by using a suitable photometer. The measurement starts using standard no. 10 that serves as blank sample for zeroing the photometer (auto zero function, or – for two beam photometers – insertion into the reference beam). Subsequently, the extinctions (E) of the remaining standards are measured. Standards that result in $E > 1.000$ are not being considered for the calibration.

The measured extinction values have to be corrected for the self-extinction of the BSA-protein itself. In order to do this, 500 µl of the respective standard and 500 µl of the reagent for the determination of self-extinction (table 2) are pipetted into a cuvette and mixed thoroughly. The self-extinction is determined by starting with standard no. 10 which serves as blank sample for zeroing the photometer. The values for the self-extinction are subtracted from the extinction values by employing the OPA-reagent. The corrected extinction values that have been thus identified are now used to calculate a regression curve ($y = mx + b$) which is applied to determine the concentration of unknown protein samples.

3.2.2 Checking of OPA method

In order to check the used chemicals and prepared reagents a leucine standard according to 2.2.3.4 is analysed. Therefore the photometer is zeroed using the blank sample. Then, 500 µl of the leucine standard are mixed in a cuvette with 500 µl of the OPA reagent and incubated for 3 minutes, after which the extinction is measured. The self-extinction of the leucine standard is determined as well, and both extinction values are subtracted. The corrected extinction is determined in triplicate, the average has to match $E = 0.641 \pm 0.032$.

An additional check of the OPA method is performed by measuring an external, commercially available BSA standard (fraction V) with a precisely given concentration in the range of 100 – 200 µg ml^{-1}, as stated by the manufacturer. This external standard must not be diluted or modified. After zeroing the photometer, 500 µl of the commercially available standard are added to 500 µl of the OPA reagent and mixed thoroughly. After 3 minutes incubation time the extinction (E) is determined. Finally, the self-extinction is measured and subtracted.

3.2.3 Quantification of protein samples

Before starting measurements the photometer is zeroed using the blank sample (auto-zero function or insertion into reference-beam). After this, the unknown protein samples are measured by mixing 500 µl of the protein sample and 500 µl of the OPA-reagent thoroughly. The mixture is checked visually to make sure that it does not exhibit any precipitation or turbidity. After 3 minutes incubation time the extinction is measured. Samples that give extinction values beyond 1.000 have to be diluted using 1 % of SDS-solution.

To correct for self-extinction of proteins, another 500 µl of the protein sample is mixed thoroughly with 500 µl of the reagent for measurement of self-extinction. The concentration of the used protein sample for this measurement has to be the same as for the measurement with the OPA-reagent. The average value for the self-extinction is subtracted from the average value of the extinction measured with the OPA-reagent. The corrected extinction values are transferred to protein concentration using the established calibration curve.

4 4 Characterisation of test pieces

4.1 Batch characterisation

Each batch of prepared test pieces has to be checked regarding the appropriateness of its quality by:

- Negative controls: For each batch of used PTFE-tubings an adequate number of negative controls (unsoiled tubings) has to be analyzed by elution and subsequent protein quantification to check for adequate quality and to exclude unwanted effects caused by incorrect manufacturing. Depending on the ordered batch size of the PTFE-tubings 1 – 3 negative controls should be analyzed.

- Positive controls: For each batch of prepared test soil at least 1 test piece (untreated positive control) has to be analyzed by elution and subsequent protein quantification to check for adequate quality of the test soil. The recovery rate of proteins has to exceed 70 % but has to range clearly below 100 %.

1 In deviation from standard DIN ISO/TS 15883-5, a patency check is conducted after soiling in this manner.

The recovery rate (RR) of proteins for test pieces has the following correlation:

$$RR = 100 \cdot \frac{RPC\ (\mu g)}{WTS\ (ml) \cdot PC\ (\mu g \cdot ml^{-1})}$$

RPC: eluted residual protein content of the test piece in µg

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}

PC: protein concentration of the used test soil in µg ml^{-1}

4.2 Characterisation of individual test pieces

All information regarding the preparation of test pieces has to be documented carefully, particularly information about the used sheep blood and the PTFE-tubings (e. g. manufacturer, distributor, distribution date, product number and batch number). Moreover, the following information should be documented during the preparation of the test pieces:

- room temperature during preparation
- coagulation time of test soil
- protein concentration of test soil
- weight of test soil in test pieces after patency check

5 Evaluation of test pieces

The analysis of protein residues in WD-processed test pieces provides the following information:

- the level of total residual protein content in processed test pieces
- the reduction factor (RF) for the removal of the test soil. The RF-value is the logarithmized quotient of the protein content of the test piece before and after treatment in a WD.

6 References

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2. Frister H, Meisel H, Schlimme E. Einsatzmöglichkeiten der modifizierten OPA-Methode in der Proteinanalytik. Ernährungs-Umschau 1990; 37(11): 442–445.