Guideline[1] of "Deutsche Vereinigung zur Bekämpfung der Viruskrankheiten e.V." (DVV; German Association for the Control of Virus Diseases)[2] and Robert Koch Institute (RKI; German Federal Health Authority) for testing the virucidal efficacy of chemical disinfectants in the human medical area

Version of 1 August 2008

1 Introduction

The June 2005 [1] version of the guideline was revised due to the fact that certain sections needed clarification or additions since it had come into effect. Therefore, the implementation of interference control was introduced in accordance with the European standard. Furthermore, it was necessary to develop specific controls for the testing of chemo-thermal disinfection procedures, which had already been found useful in practice.

Questions concerning the replacement of the poliovirus (due to the planned eradication of poliomyelitis [3]) with an adequate alternative test virus have not yet been clarified conclusively. Due to this, the poliovirus (polio-vaccine strain type I, strain LSc-2ab) will remain the current test virus.

As the European standard [2] for the quantitative suspension test for the assessment of virucidal efficacy still does neither allow a biometric evaluation of the tests nor an approval as limited virucidal activity against enveloped viruses, the now amended version of the guideline remains valid for awarding the DVV certificates.

This guideline describes the procedure of suspension experiments. The virucidal efficacy tests shall be conducted both without and with additional protein load. A sufficient titre reduction allows the conclusion that the formula tested has virucidal properties under these test conditions.

Recommendations for the application of the agents in practice can be concluded from the results of the suspension experiments only to a limited degree. Such favourable conditions as during the homogeneous suspension are seldom to be found in practice. Suspension test results should not be regarded as practical application in every case but they allow conclusion of the efficacy of single disinfectants and therefore also a comparison of the efficacy of different disinfectants.

Different parameters of the in vitro test (virus used, cells used, passage number, cytotoxicity, etc.) can influence the results. Furthermore, the titration conditions (dilution factor of samples for titration and number of tested replicates per dilution, etc.) define the test's accuracy and influence, in combination with the parameters mentioned before, the virucidal efficacy of the disinfectant to be tested. Thus, particular emphasis is laid on the biometrical aspects in this guideline. This is to make sure that the ascertained reduction factor (see below) is most likely to reflect the "true efficacy".

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[1] The description "directive" was changed to "guideline" due to legal reasons
[2] Expert Committee "Virus Disinfection": Members in alphabetical order Dr. J. Blümel, PD Dr. D. Glebe, Prof. Dr. D. Neumann-Haefelin, Prof. Dr. H.F. Rabenau*, Dr. I. Rapp, PD Dr. F. von Rheinbaben, Prof. Dr. B. Ruf, Prof. Dr. A. Sauerbrei, Dr. I. Schwebke*, Dr. J. Steinmann, Dr. H. Willkommen, Prof. Dr. M.H. Wolff, Prof. Dr. P. Wutzler
* contributing authors

In this guideline, the terms "limited virucidal activity" (effective against enveloped viruses) and "virucidal activity" (effective against non-enveloped viruses; efficacy against non-enveloped viruses includes efficacy against enveloped viruses) are used in the sense of the position defined by the Virucide Study Group of the RKI [4]. However, it must be taken into account that the term "virucidal activity" does not include all known pathogenic viruses, as some viruses, especially the hepatitis A virus (HAV) or parvoviruses, may have a higher resistance than the test viruses used.

Practical tests for hand, surface and instrument disinfection are not part of the content of this guideline; further guidelines relating to these subjects are scheduled.

2   Test Viruses
The following viruses shall be used:

2.1 Chemical disinfection
2.1.1 Claiming a "limited virucidal activity"
- Vaccinia virus, Elstree strain[3],
- Bovine Viral Diarrhoea Virus (BVDV), NADL strain.
2.1.2 Claiming a "virucidal activity"
- Vaccinia virus, Elstree strain[3],
- Poliovirus vaccine strain type I, strain LSc-2ab,
- Adenovirus type 5, strain adenoid 75,
- Polyomavirus[4] (SV 40), strain 777.

2.2 Chemo-thermal disinfection (temperature > 40°C)
- Bovine Parvovirus, Haden strain

Reference sources for the virus strains and for virus suspensions can be found on the DVV Homepage (www.dvv-ev.de - Fachausschuss "Virusdesinfektion"). An inquiry can also be made to the German Association for the Control of Virus Diseases (DVV), Institute for Virology and Antiviral Therapy, Friedrich-Schiller-University Jena, Hans-Knöll-Str. 2, D - 07745 Jena.

3   Preparation of virus suspensions
Viruses shall be propagated in cell cultures or other adequate systems. The methods which are used to produce virus suspensions may differ, depending on the test virus used. The virus concentration on suspensions used shall not be less than 10^8 $TCID_{50}$/ml (tissue culture infectious dose).

As an example, this goal can be achieved by the following: Cultures infected with virus can be frozen and thawed with the complete medium and cell detritus at least once before harvest. Notwithstanding, if adenovirus is propagated in the cell culture, the growing medium is replaced by 1/10 volume of new medium before freezing and thawing. Virus stocks may be centrifuged for 60 min at 40,000 x g and +4°C, e.g. after previous low-speed centrifugation, in order to concentrate virus and cells at the bottom of the tube. The sediment is resuspended in PBS (phosphate-buffered saline) and centrifuged again at high speed in order to eliminate cell detritus. Alternatively,

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[3] Employees who are conducting experiments with viruses shall be vaccinated accordingly
[4] Former description: Papovavirus SV40

an ultrasound treatment of the infected cells may be conducted to prepare high-titre virus suspensions.

The titre of the virus may also be less than 10^8 $TCID_{50}$/ml, as long as a titre reduction of at least 4 log_{10} steps during examination of the disinfectant can be demonstrated.

4   Preparation of disinfectant dilution

The disinfectant shall be diluted with water of standardised hardness (see Appendix 3). The disinfectant dilution shall be chosen in such a way that the final concentration to be examined shall be achieved in the mixture of virus suspension and disinfectant preparation. This is normally done by a 1.25 fold pre dilution of the concentrated disinfectant (see item 5). This means for preparations that will be used undiluted or are intended for ready-to-use solutions, no concentration exceeding 80% can be tested. With disinfectants that will be used undiluted, no preparations with increased solvent concentrations have to be tested.

Additional testing in a 90% concentration (0.1 parts by volume of virus suspension, 0.9 parts by volume of double-distilled water or FCS, 9 parts by volume of disinfectant) would be acceptable if the mechanism of action justifies this.

5   Conduct of suspension experiments

Virus suspension, fetal calf serum (FCS)[5], double-distilled water and diluted disinfectant are tempered at 20°C. One part virus suspension is mixed with one part FCS or with one part double-distilled water, respectively, then eight parts diluted disinfectant (1.25-fold) is added and stirred. This mixture is kept at 20 ± 0.5°C for the duration of the contact time to be tested.

If disinfectants are intended to be utilised at temperatures below 20°C, the tests shall be conducted at the respective temperature (e.g. 4°C). Chemo-thermal processes at temperatures higher than 40°C are only to be tested with parvovirus at temperatures instructed by the manufacturer.

Four contact times have to be tested usually for the agent according to the range of application:

      either 0.5; 1; 2.5; 5 (if necessary 1.5; 2) min
      or 5; 15; 30 and 60 min.

Agents, which will be used in practice for disinfection within a short contact time only (e.g. hand disinfectants), shall be tested primarily with short contact times. For disinfectants that require a long contact time in practice, the testing of short contact times may be omitted. Concentrations and contact times for the disinfectant are to be chosen in such a way that the result will display the dependency of the virucidal effect of the agent on the concentration and the contact time (kinetics).

The efficacy of the disinfectant shall be tested both without and with FCS exposure (10% final concentration in the test preparation). The virus control preparations shall include the same level of FCS concentration as the test samples.

All experiments must be carried out in at least two independent assay runs on different days.

5.1   Testing of chemo-thermal disinfection

Notwithstanding of contact times mentioned under item 5, testing of chemo-thermal disinfection methods shall be executed according to the requested contact time. The kinetics of the virus inactivation shall become evident from the choice of further

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[5] For experiments with the test viruses BVDV and parvovirus use FCS that neither contains antibodies against BVDV or parvovirus nor the viruses themselves.

contact times and/or concentrations. At least two different concentration-time relations shall be tested.

Those tests shall, if the disinfection procedure consists of several components (e.g. washing and disinfection agent), each be conducted with a) the washing agent or wash increaser only and b) the complete procedure - washing agents and disinfectant - in separate tests at the designated temperature. The tests under a) and b) are also to be conducted at a temperature of 20°C (see table 1).

6   Determination of the infectivity of virus suspensions

Two test principles come into considerations a quantal assay (end point dilution method) or a quantitative assay (plaque assay). At the end of the specified contact time the mixture of virus suspension and disinfectant shall be diluted in a dilution series using ice-cold culture medium (e.g. 0.5 ml mixture in 4.5 ml medium; smaller amounts are not recommended - procedures differing from these requirements need to be justified). Test tubes with the diluted samples shall be placed immediately after preparation in an ice bath (0 - 4°C). The diluted samples are to be inoculated in cultures immediately (stating the time frame in the experiment protocol). It shall be ensured that the disinfectant is neutralised immediately after its contact time to avoid aftereffects of the disinfectant (see 7.3).

If necessary, the effect of the disinfectant can be counteracted by applying gel filtration, micro filtration, or chemical neutralisation. The chosen procedure shall be described in detail and with specific control methods and it shall be proved that the virus detection (virus titre) is not affected.

The infectivity of the test samples can be determined in macro or micro assays. As a parameter for infectivity the cytopathic effect can be identified using the end point dilution method or a plaque assay.

7   Control and comparison experiments
7.1   Virus controls
For virus control, the titre of the virus suspension (not treated with disinfectant) under test conditions without or with FCS load is determined.

7.1.1 Virus control without protein load (FCS)
One part of virus suspension and nine parts of double-distilled water are mixed. After the maximum contact time has elapsed, serial dilutions are prepared and the titre is determined.

7.1.2 Virus control with protein load (FCS)
One part of virus suspension, one part of FCS and eight parts of double-distilled water are mixed. After the maximum contact time has elapsed serial dilutions are prepared and the titre is determined.

7.2   Cytotoxicity control
The cytotoxicity control for the disinfectant described below intends to discriminate virus induced cytopathic changes from cell-toxic effects:

Therefore two parts of double-distilled water are mixed with eight parts disinfectant solution. In the same way as for determining virus infectivity, serial dilutions are prepared for the inoculation of cell cultures.

If the cytotoxicity of the disinfectant is so strong that a decrease of the infectivity titre of 4 $\log_{10}$ cannot be detected, cytotoxicity can be reduced by applying gel filtration, micro filtration, or appropriate chemical neutralisation agents after the contact time

has elapsed. The chosen procedure shall be described in detail and with specific control methods and it shall be demonstrated that the virus detection (titre) is not affected.

7.3 Aftereffect control

Controls concerning aftereffects are to be conducted if, especially after short contact times, an uncontrolled method dependent aftereffect of the disinfectant beyond the contact time cannot be precluded. Generally, the time elapsed between the completion of the contact time and the preparation of the dilution series for titration shall not exceed 15-30 sec.

One part virus suspension is mixed with one part FCS or double-distilled water; then eight parts of a suitable disinfectant solution is added, mixed, and incubated in an ice-cold bath for titration for the time that is needed to complete the dilution series following to the contact time of the disinfectant. Dilution series in order to determine titres are prepared. To determine the disinfectant dilution that no longer exhibits aftereffects, the first two dilutions are usually used. The disinfectant shows a negligible or non-existing aftereffect if the difference of this titre in comparison with the virus control is $\leq 0.5 \log_{10}$.

If other procedures than the dilution procedure described under item 6 are applied to decrease cytotoxicity (see item 7.2) or to neutralise the effect of the disinfectant (e.g. gel filtration, micro filtration or chemical neutralisation), an analogous test is to perform.

7.4 Interference control - control of cell susceptibility

The aim of the interference control is to verify that the susceptibility of the cells for the virus infection is not influenced negatively by the treatment with the disinfectant.

Two parts double-distilled water or one part FCS and one part of double-distilled water are mixed with eight parts diluted disinfectant, which does not exhibit aftereffects (see item 7.3) or cytotoxicity (see item 7.2). These mixtures are to remain in contact with the cell culture for one hour analogous to the determination of infectivity of the virus suspensions (see item 6 and Appendix 1). As a corresponding negative control, the cell cultures are exposed to PBS in the same manner parallel to the disinfectant mixtures and are incubated for one hour under the same conditions. Following to the incubation the disinfectant solution or PBS is removed from the cell cultures. Afterwards, the dilution series of the virus suspension (considering the dilution factor applied for determining the infectivity after exposure to the disinfectant) are prepared and the titres on these cell cultures are calculated. The difference between the titre of the disinfectant treated cells in comparison with the virus control (see item 7.1.1 and item 7.1.2) shall not exceed $0.5 \log_{10}$.

7.5 Cell control

The cells are treated as in the test suspension experiments, although in this case they only get incubated with cell culture medium.

7.6 Formaldehyde control

In addition to every test run, a control test using formaldehyde at pH 7.0 without FCS load needs to be conducted at $20 \pm 0.5°C$. The concentration (during experiment preparation) shall be 0.7 g formaldehyde/100 ml, the contact time shall be 5, 15, 30 and 60 min (for poliovirus also 120 min; a 5 min contact time can be omitted). For this experiment one part virus suspension is mixed with four parts phosphate buffer (0.1 M; pH 7.0) and five parts 1.4% formaldehyde solution (see Appendix 2).

For poliovirus the difference in the titre of the virus control (see item. 7.1.1) and the titre of this formaldehyde control shall be in the range of 0.5 to 2.5 $\log_{10}$ using a contact time of 30 min and in the range of 2.0 to 4.5 $\log_{10}$ for a contact time of 60 min [1].

7.7   Control and comparison tests for chemo-thermal disinfection
For chemo-thermal disinfection tests the use of the formaldehyde control as a reference is not useful, as, on the one hand, aldehyde is not used as an inactivating agent here and, on the other hand, viruses are highly resistant to formaldehyde. In order to verify the adequacy of the parvoviruses intended for use in the test, besides the controls described between items 7.2 and 7.5, additional control experiments are required that are described below (see table 1). These controls shall verify that neither the temperature and the pH-value alone nor these parameters in combination (without exposure to the disinfectant) affect the efficacy against parvoviruses.

7.7.1 Virus control at 20±0.5°C and process temperature
The control described in items 7.1.1 and 7.1.2 shall be conducted at 20±0.5°C and at the designated temperature for this procedure at a pH-value of 7.

7.7.2 Virus control at pH-value of procedure
The controls described under items 7.1.1 and 7.1.2 shall be determined at the designated temperature and at the designated pH-value the process.

Table 1 Control and comparison tests for testing disinfection procedures for chemo-thermal disinfection

Virus titre	20°C	Process temperature
Control as described under items 7.1.1 and 7.1.2	X	X
Control as described under items 7.1.1 and 7.1.2 for the pH-value of the test solution (washing agents + disinfectant)	X	X
Test approach as described under items 5.1 (a and b)	X	

8   Calculation of reduction factor
The assessment of the efficacy of the dilution of the disinfectant is carried out by calculating the reduction factor. The reduction factor is the difference between the infectivity titre obtained without exposure to the disinfectant [($\log_{10}$ TCID$_{50}$/ml or $\log_{10}$ plaque-forming units (PFU)/ml of virus controls (see item 7.1.1 and item 7.1.2)] and the infectivity titre obtained after exposure to the disinfectant (see item 6). The titres shall be calculated with their 95% confidence intervals from which the reduction factor with the 95% confidence interval shall be calculated (see Appendix 6).

9   Test report
The results of the experiment are summarized in tabular form and, if necessary, as graphs including the results of the formaldehyde comparison test. The test report shall include the batch number of the disinfectant to be tested, the active ingredients of the disinfectant as well as detailed information about the testing method, the results (determined reduction factors with 95% confidence interval) and an evaluation of the findings (see Appendices 4 and 5). In the suspension test virucidal activity of a disinfectant concentration is shown by demonstrating a virus titre reduction of at least 4 $\log_{10}$ within the advised contact time.

Literature

1   Leitlinie der Deutschen Vereinigung zur Bekämpfung der Viruskrankheiten (DVV) e.V. und des Robert Koch-Instituts (RKI) zur Prüfung von chemischen Desinfektionsmitteln auf Wirksamkeit gegen Viren in der Humanmedizin.[Guideline of the German Association for the Control of Virus Diseases (DVV) and the Robert Koch Institute (RKI) for testing chemical disinfectants regarding their efficacy in fighting viruses within the field of human medicine.] (2005) Bundesgesundheitsblatt, 48 (2005), 1420-1426

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Author: Fachausschuss „Virusdesinfektion" of the DVV, contributing authors:
Prof. Dr. H.F. Rabenau, Institute for Med. Virology, Paul-Ehrlich-Str. 40, 60596 Frankfurt/M, Germany
Dr. I. Schwebke, Robert Koch-Institute, Nordufer 20, 13353 Berlin, Germany

Appendix 1
Information for conducting infectivity tests
The infectivity of the suspensions and their dilutions can be determined in the $TCID_{50}$ assays, conducted as (microtitre-) plate, tube assay or as a plaque assay.

a) (Microtitre-) plate assay
Example: Pipette 0.05-0.2 ml of the respective sample dilution per well of a microtiterplate. Sample dilutions may either be applied onto adhered cells or added to 0.05-0.2 ml of cell suspension. If adhered cell cultures are used, the culture medium is exchanged after an absorption time of 1-2 h. The cell cultures are incubated at 37°C (usually for 5-15 days) and afterwards assessed under an inverse light microscope for cytopathic effects. The titre of the samples is expressed as $TCID_{50}$/ml.

b) Tube assay
Example: Confluent cell monolayers in culture tubes are inoculated with 0.2 ml of each sample dilution. After an absorption period of 1-2 h at 37°C, the culture medium is removed and replaced by fresh medium. The cell cultures are incubated at 37°C (usually for 5-15 days) and afterwards assessed under an inverse light microscope for cytopathic effects. The titre of the samples is expressed as $TCID_{50}$/ml

c) Plaque assay
Example: Confluent cell monolayers in 24-well plates are inoculated with 0.5-1 ml of the sample dilution. After an absorption period of 1-2 h at 37°C the inoculae are removed and the cell monolayer is rinsed with medium. Afterwards the cell monolayer is covered with 1 ml overlay and incubated in the CO_2 incubator for 3-7 days. (The overlay consists of agar which is solved in double-distilled water to a final concentration of 2%. After cooling, the same volume of double-concentrated culture medium which has been previously warmed to ca. 40°C is added.) Afterwards fixation and staining with 0.3% crystal violet in 3% formalin is carried out. After 2-3 h, the overlay can be removed by rinsing with tap water and the cell layer is rinsed with distilled water. The plaques are enumerated under the microscope. The infectivity of the samples is indicated as PFU/ml.

Appendix 2
**Quantitative determination of formaldehyde**
The formaldehyde concentration of formaldehyde solutions available on the market usually differs and may also depend on storage conditions. Thus it is essential to conduct a quantitative determination of the formaldehyde concentration of the formaldehyde solution or its dilution used in the control test. It needs to be taken into account that formaldehyde solutions which have been prepared from concentrated formaldehyde solutions (e. g. formalin) contain polymers and show the maximum efficacy after a few days at room temperature.

Quantitative determination of formaldehyde for example with hydroxylammonium chloride:
The principle of the method is as follows:
Formaldehyde reacts with hydroxylammonium chloride creating the corresponding oxime. In this reaction, the equivalent amount of hydrogen ions are released, which shifts the pH-value of the reaction batch into the acidic range:
$$H_2C=O + {}^+H_3N\text{-}OH + Cl^- \longrightarrow H_2C=NOH + H^+ + Cl^- + H_2O$$
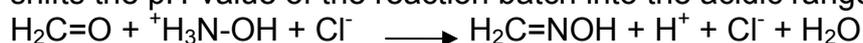
The pH-value is titrated with sodium hydroxide to its former value. The amount of sodium hydroxide used indicates the concentration of the sample.

Execution of the determination:
From the sample to be examined, an amount containing between 100 and 150 mg of formaldehyde is pipetted into a recipient suitable for titration at the pH meter and filled with distilled water to approx. 100 ml. The solution is calibrated at the pH meter to exactly pH 3.0 with approx. 0.5 N hydrochloric acid. 25 ml of an approx. 0.5 N hydroxylammonium chloride solution, which has been calibrated to pH 3.0 before, is added and the mixture incubated at room temperature for 10 min. Then the solution is titrated back to pH 3.0 with 0.5 N sodium hydroxide at the pH meter.
Calculation of formaldehyde concentration in the sample:

$$\frac{\text{Consumed amount of 0.5 N NaOH in ml x 30.03}}{\text{Volume of sample in ml x 20}} = \begin{array}{l}\text{Conc. of formaldehyde}\\\text{in sample in g in 100 ml.}\end{array}$$

Appendix 3
Preparing water of standardised hardness
Two solutions are needed for the preparation:
Solution A:
Dissolve 19.84 g anhydrous magnesium chloride ($MgCl_2$) and 46.24 g anhydrous calcium chloride ($CaCl_2$) in double-distilled water and fill up to 1000 ml (equivalent amounts of aqueous salts may also be used). Sterilise the solution in a steam steriliser. The solution can be stored between 2°C and 8°C for up to one month.
Solution B:
Dissolve 35.02 g of sodium bicarbonate ($NaHCO_3$) in double-distilled water and fill up to 1000 ml. Sterilise the solution applying the method of membrane filtration. The solution can be stored between 2°C and 8°C for up to one week.
For the preparation of 1 litre of water of standardised hardness, at least 600 ml of sterile double-distilled water are filled into a sterilised 1000 ml volumetric flask. 6.0 ml of solution A and 8.0 ml of solution B are added, stirred, and filled up to 1000 ml. The solution shall exhibit a pH-value of 7.0 + 0.2. If necessary, the pH-value is calibrated with 1 N sodium hydroxide (NaOH) or 1 N hydrochloric acid (HCl), respectively. Water of standardised hardness shall be prepared freshly under aseptic conditions and must be used within 24 h.
The hardness covers 300 ppm, based on the calcium carbonate content.

Appendix 4
Tabular and graphical display of test results
a) Table 2 Example of tabular display of test results
   (Information about cytotoxicity of disinfectant and about maximum detectable decrease of infectivity titre)

Test virus	final concentration of disinfectant (%)	Virus titre of control titration ($\log_{10}$ TCID$_{50}$/ml or $\log_{10}$ PFU/ml) including 95% confidence interval	Virus titre of "rest virus" titration ($\log_{10}$ TCID$_{50}$/ml or $\log_{10}$ PFU/ml) including 95% confidence interval				Reduction factor after … (min) including 95% confidence interval			
			1.CT[1]	2 CT	3. CT	4. CT	1. CT	2. CT	3. CT	4. CT

[1] CT = Contact time

b) Examples of graphical depiction of test results

Infectivity titre

($\log_{10}$ TCID$_{50}$/ml or $\log_{10}$ PFU/ml)

Contact time (min)

Infectivity titre

($\log_{10}$ TCID$_{50}$/ml or $\log_{10}$ PFU/ml)

Contact time (min)
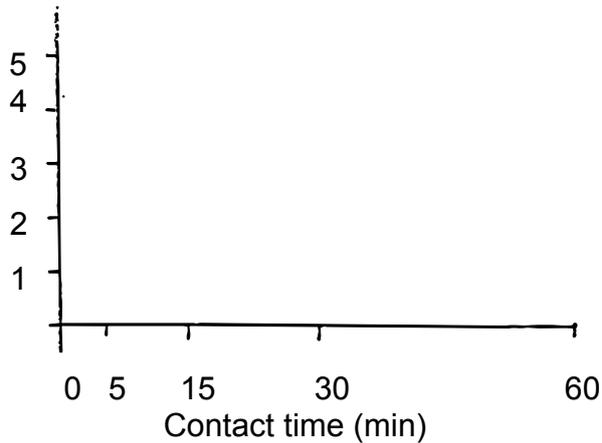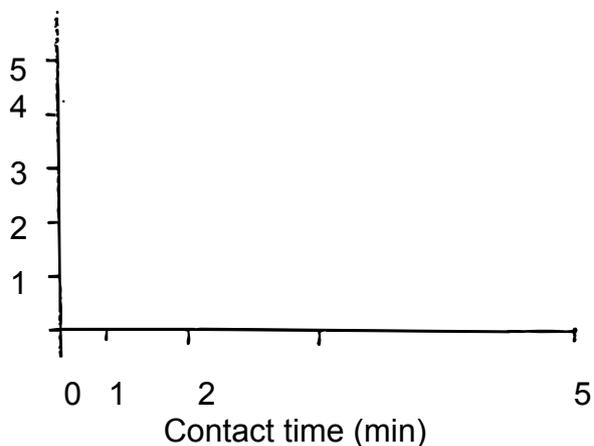
Appendix 5
Information for composing expert reports concerning disinfectant tests

- In the introduction, it shall be described briefly for which application area (e.g. hand, surface, instrument or laundry disinfection) the agent will be used. It is especially important to indicate under which conditions the agent will be used and why the agent is considered effective (e.g. by citing literature references where appropriate).

- All active ingredients, designated working concentrations, and the identity of the test sample shall be named.

- The disinfectant shall be described precisely: Lot number, production date, expiry date, physical properties, colour, pH-value (the pH-value of the test solutions shall be measured in form of the test sample and in form of the working dilution with double-distilled water. This does not apply to alcoholic solutions >60%).

- Origin, preparation, and passage history of the test virus as well as of the cell line shall be described.

- The test method and the controls shall be described in detail. A reference to the guideline is not sufficient. In particular, describe the preparation and, if necessary, the way of concentration of the test virus suspension in detail.

- The results of all tests need to be given in a table as raw data and as calculated $TCID_{50}$ or PFU data, including the 95% confidence intervals. The method for calculating the titre shall also be mentioned. This can be done for example using the Spearman-Kärber method [5, 6] (see item 6). The statistical evaluation and determination of the 95% confidence interval for the reduction factor (RF) shall be conducted according to the guidelines provided in Appendix 6.

Appendix 6

Biometrical evaluation of experiment approaches and assessment of the disinfecting effect on the virus (reduction factor [RF]):

For the evaluation of disinfectant efficacy, the virus titre before and after exposure to the disinfectant is determined and the reduction factor (RF) calculated including its 95% confidence interval. The virus titrations are conducted in such a way that the virus titre exhibits a 95% confidence interval of ≤ 0.5 log_{10}. The number of replicates per dilution (e.g. 8, 12 or 16) and the dilution factor in the dilution series (e.g. 3, 5 or 10) used for the titration shall be determined respectively. All tests need to be conducted in two independent test runs on different days.
A sufficient titre reduction with respect to the disinfectant can be assumed if the average RF (see below) is at least 4 log_{10}. The results shall not be affected by cytotoxicity, interferences or aftereffects of the disinfectant.

The virus titre ($TCID_{50}$/ml or PFU/ml) can be determined using several methods, e.g. the Spearman-Kärber formula [5,6].

A 6.1 Calculating the virus titre with 95% confidence interval

Example:

Using the Spearman-Kärber method the logarithmic infectivity titre in $TCID_{50}$/ml is calculated as follows:

$$m = x_k + d/2 - d \sum p_i$$

Key:

m = negative decadal logarithm of the titre based on the test volume

x_k = logarithm of lowest dose (dilution level) at which all test objects exhibit a positive reaction

d = logarithm of dilution factor

p_i = observed reaction rate

The standard deviation (s) from m is calculated as follows [7]:

$$s_m = \sqrt{d^2 \sum \{(p_i (1 - p_i) / (n - 1)\}}$$

Key:

s_m = standard deviation of logarithmic titre

d = logarithm of dilution factor

p_i = observed reaction rate

n = number of test objects per dilution

95% confidence interval of the titre is approximately 2 s_m. When calculating the titre, take into account the pre-dilution of the sample and calculate the titre to the same volume.

A 6.2 Calculating the reduction factor and its 95% confidence interval

The reduction factor (RF) is calculated as the difference between the logarithmic virus titre before ("control titration", see item 7.1.1 and item 7.1.2, equals titre to a) and after exposure to disinfectant ("rest virus", see item 6, equals titre to b).

The reduction factor (RF) is thus calculated as follows:

$$RF_{T1} = a - b$$

Key:

RF_{T1} = reduction factor from first test run

a = $\log_{10}$ $TCID_{50}$/ml of control titration of the first test run

b = $\log_{10}$ $TCID_{50}$/ml of "rest virus" titration of the first test run

The 95% confidence interval of the RF of the first approach ($K_{RF(T1)}$) is calculated as follows:

$$K_{RF(T1)} = \sqrt{(2 s_a)^2 + (2 s_b)^2}$$

Key:
$K_{RF(T1)}$ = 95% confidence interval of the RF of the first test run
s_a = standard deviation of control titration of the first test run
$2s_a$ = 95% confidence interval of control titration of the first test run
s_b = standard deviation of "rest virus" titration of the first test run
$2s_b$ = 95% confidence interval of "rest virus" titration of the first test run

All tests need to be conducted in two independent test runs. The reduction factor and the 95% confidence interval for each test run is calculated.

If no virus can be detected any more in the test run with disinfectant ("rest virus"), the 95% confidence interval is calculated according to:

$$K_{RF(TkV)} = \sqrt{2\,(s_a)^2}$$

Key:
$K_{RF(TkV)}$ = 95% confidence interval of the RF in the case no virus can be detected in the test run with disinfectant ("rest virus")

A 6.3 Calculating the average reduction factor ($RF_{(mi)}$) and its 95% confidence interval.

The average RF from both approaches and its 95% confidence interval is calculated as follows:

$$RF_{(mi)} = (RF_{T1} + RF_{T2})\,/\,2$$

Key:
$RF_{(mi)}$ = average reduction factor
RF_{T1} = reduction factor from first test run
RF_{T2} = reduction factor from second test run

The 95% confidence interval of the average RF ($K_{RF(mi)}$) is calculated as follows:

$$K_{RF(mi)} = \tfrac{1}{2}\sqrt{(K_{RF(T1)})^2 + (K_{RF(T2)})^2}$$

Key:
$K_{RF(mi)}$ = 95% confidence interval of the average reduction factor
$K_{RF(T1)}$ = 95% confidence interval of the reduction factor of the first test run
$K_{RF(T2)}$ = 95% confidence interval of the reduction factor of the second test run

A 6.4. Calculation example
Titre calculation of control titration of the first test run (T1):

Table 3: Exemplary values of a test result

Dilution	Log_{10}	Number of positive cultures per dilution	p_i
1: 10	1	16/16	1.0
1: 100	2	16/16	1.0
1: 1,000	3	16/16	1.0
1: 10,000	4	16/16	1.0
1: 100,000	5	16/16	1.0
1: 1,000,000	6	16/16	1.0
1:10,000,000	7	12/16	0.75
1:100,000,000	8	8/16	0.50
1:1,000,000,000	9	2/16	0.13
1:10,000,000,000	10	0/16	0

Initial parameter: 100 µl inoculum per well, 16 replicates per dilution and a log_{10} dilution series
A similar accuracy is reached using a 1:3 dilution series and testing 8 replicates per dilution

The titre is calculated according to the numerical values shown in table 2 (see item A 6.1):

$$m = -6 + \tfrac{1}{2} - 1 \times 2{,}38 = 7{,}88$$

Under consideration of the test volume applied, the titre amounts to
8.88 log_{10} $TCID_{50}$/ml

The standard deviation and the 95% confidence interval of the titre is calculated from the values given (cf. item A 6.1) as follows:

$$s_m = \sqrt{1^2 \sum \{(p_i (1 - p_i) / (16 - 1)\}}$$

$p_1 = 1$, $p_2 = 0.75$, $p_3 = 0.5$ and $p_4 = 0.13$ and n is 16 in all dilutions. Therefore

$P_1 (1 - p_1) / (n_1 - 1) = 0$
$p_2 (1 - p_2) / (n_2 - 1) = 0.75 (1 - 0.75) / (16 - 1) = 0.0125$
$p_3 (1 - p_3) / (n_3 - 1) = 0.50 (1 - 0.50) / (16 - 1) = 0.0167$
$p_4 (1 - p_4) / (n_4 - 1) = 0.13 (1 - 0.13) / (16 - 1) = 0.0075$

$s_m = \sqrt{1 (0.0125 + 0.0167 + 0.0075)}$
$s_m = 0.19$, i.e. $2s_m = 0.38$

Therefore the titre of the control titration is 8.88 ± 0.38 log_{10} $TCID_{50}$/ml.

If the "rest virus" of the first test run is calculated analogously, an exemplary value of 3.50 ± 0.32 $\log_{10}$ $TCID_{50}$/ml is calculated.

The reduction factor (RF) is calculated as follows (cf. item A 6.2):

$$RF_{T1} = 8.88 - 3.50$$

The RF of the first test run is 5.38 $\log_{10}$.

The 95% confidence interval of the RF ($K_{RF(T1)}$ is calculated according to (cf. item A 6.2):

$$K_{RF(T1)} = \sqrt{(2 \times 0.19)^2 + (2 \times 0.16)^2} = 0.50$$

Therefore the reduction factor of the first test run is 5.38 ± 0.50 $\log_{10}$.

Assuming that the titres of the control titration and the "rest virus" titration in the second test run under analogous basic conditions are 8.25 ± 0.22 $\log_{10}$ $TCID_{50}$/ml and 3.25 ± 0.34 $\log_{10}$ $TCID_{50}$/ml, a value of 5.0 ± 0.40 for RF_{T2} including 95% confidence interval is calculated.

The average RF from both runs including 95% confidence interval is calculated as follows (cf. item A 6.3):

$$RF_{(mi)} = (5.38 + 5.0) / 2 = 5.19$$

The 95% confidence interval of the RF ($K_{RF(mi)}$ is calculated according to (cf. item A 6.3):

$$K_{RF(mi)} = \tfrac{1}{2} \sqrt{(0.50)^2 + (0.40)^2} = 0.32$$

The total RF from both test runs, (including 95% confidence interval) and with these exemplary values, thus is 5.19 ± 0.32 $\log_{10}$.

The individual titrations comply with the guideline's requirements concerning accuracy, as the 95% confidence intervals of the calculated titres are ≤ 0.5 $\log_{10}$.