Bundesgesundheitsbl 2020 · 63:645–655
https://doi.org/10.1007/s00103-020-03115-w
Published online: 9 April 2020

Holger F. Rabenau[1] · Ingeborg Schwebke[2] · Johannes Blümel[3] · Maren Eggers[4] · Dieter Glebe[5] · Ingrid Rapp[6] · Andreas Sauerbrei[7] · Eike Steinmann[8] · Jochen Steinmann[9] · Hannelore Willkommen[10] · Peter Wutzler[7]

[1] Institute for med. Virology, University Hospital Frankfurt, Frankfurt/Main, Germany
[2] Robert Koch Institute, Berlin, Germany
[3] Paul-Ehrlich-Institut, Langen, Germany
[4] Labor Prof. Dr. G. Enders MVZ GbR, Stuttgart, Germany
[5] Institute for med. Virology, University Gießen, Gießen, Germany
[6] Laboratory Dr. Merk & Colleagues, Ochsenhausen, Germany
[7] Institute of Virology and Antiviral Therapy, University Jena, Jena, Germany
[8] Department for Molecular & Medical Virology, Ruhr-University Bochum, Bochum, Germany
[9] Dr. Brill + Partner GmbH Institute for Hygiene and Microbiology, Bremen, Germany
[10] RBS Consulting, Erzhausen, Germany

Guideline for testing chemical disinfectants regarding their virucidal activity within the field of human medicine

as of December 1st, 2014
Prepared by the German Association for the Control of Virus Diseases (DVV) and the Robert Koch Institute (RKI)

1 Introduction

This guideline describes the performance of suspension testing of activity of chemical disinfectants against viruses. These tests must be conducted both without and with the addition of fetal calf serum (FCS) as protein load. A titre reduction of at least four logarithmic steps (4 $\log_{10}$) allows the conclusion that the disinfectant tested has virus-inactivating properties under the tested conditions.

Several parameters of the *in vitro* tests can influence the results (e.g. virus, cells, passage number, cytotoxicity). Furthermore, the titration conditions for the determination of the viral concentration (i.e. serial dilution factor of samples and number of tested replicates per dilution) define the test's accuracy and therefore also influence the evaluation of the virucidal activity of the disinfectant to be tested. Thus, this guideline lays particular emphasis on the biometrical aspects of testing.

Recommendations for the practical use of the agents can only be drawn from the results of these suspension tests to a limited degree because the conditions found in homogeneous suspensions are seldom found in practice. However, conclusions on the general activity of the disinfectant tested can be drawn based on the results of suspension tests.

Therefore, for practical tests (e.g. [1]), this guideline serves as preliminary test and as basis for determining practical test conditions.

The terms "limited virucidal activity" (effective against enveloped viruses such as influenza virus, hepatitis B virus, hepatitis C virus, HIV) and "virucidal activity" (active against non-enveloped viruses; activity against non-enveloped viruses includes activity against enveloped viruses) are used as defined in the position paper of the Virucide Working Group of the RKI [2][1].

It should be noted that the term "virucidal activity" does not include all known pathogenic viruses, as certain viruses, such as the hepatitis A virus (HAV) or

This article is a translation of the German announcement *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*: Bundesgesundheitsbl 2015 58:493–504. DOI https://doi.org/10.1007/s00103-015-2131-8

[1] This document was updated in 2017, current version see https://www.rki.de/DE/Content/Infekt/Krankenhaushygiene/Desinfektionsmittel/Downloads/BGBl_03_2017_Schwebke.pdf?__blob=publicationFile.

parvoviruses, may have a higher resistance than the test viruses used.

2 Test viruses

The following viruses shall be used for the disinfectant efficacy test:

2.1 Chemical disinfection

2.1.1 Claiming a "limited virucidal activity"

- vaccinia virus, Elstree strain[2] or Modified Vaccinia Virus Ankara (MVA),
- Bovine Viral Diarrhoea Virus (BVDV), NADL strain.

2.1.2 Claiming a "virucidal activity"

- poliovirus vaccine strain type I, strain LSc-2ab,
- adenovirus type 5, strain adenoid 75 ATCC VR-5,
- SV40, strain 777,
- murine norovirus (MNV), strain S99.

2.2 Chemo-thermal disinfection (temperature >30 °C)

- bovine parvovirus, Haden strain or Minute virus of Mice (MVM) ATCC VR-1346.

Reference sources for the virus strains and for virus suspensions can be found on the DVV Homepage (www.dvv-ev.de—Fachausschuss "Virusdesinfektion"). In addition, the following test viruses are available from the Friedrich-Loeffler-Institute: BVDV, MNV (S99), poliovirus, SV40.

3 Preparation of virus suspensions

Viruses shall be propagated in cell cultures. The methods used to produce virus suspensions may differ, depending on the test virus used. The suspension should have a titre of not less than 10^8 $TCID_{50}$/ml.

The virus titre can be below 10^8 $TCID_{50}$/ml—but must not be below 10^6 $TCID_{50}$/ml. The use of titres lower than 10^8 $TCID_{50}$/ml must to be noted and justified in the test report. The titre of the virus used in the test must allow a determination of a titre reduction of at least 4 log_{10} steps during the disinfectant testing procedure[3].

4 Preparation of the disinfectant dilution

The disinfectant tested shall be diluted with water of standardized hardness (WSH, see Appendix D). The dilution shall be chosen in such a way that the final concentration to be examined is achieved in the mixture of virus suspension and disinfectant preparation. Therefore, the disinfectant dilutions used for these tests shall be 1.25 fold higher concentrated than the concentration which will be tested (see item 5).

Products intended for undiluted use or ready-to-use solutions are diluted with aqua bidest.[4] to determine the kinetics. The test conditions described below can therefore only test concentrations of 80% or lower for these products.

Additional testing at a 90% concentration (0.1 parts by volume of virus suspension, 0.9 parts by volume of bidest. water or FCS, 9 parts by volume of disinfectant) is acceptable, if justified by the mechanism of action.

For disinfectants that will be used undiluted, no preparations with increased ingredient concentrations are allowed to be tested.

5 Conduct of suspension tests

Fetal calf serum (FCS)[5], bidest. water and the disinfectant dilution are adjusted to 20 °C. One part of the virus suspension is mixed with one part of FCS or bidest. water; then eight parts of the diluted disinfectant (1.25-fold) are added and mixed. This mixture is kept at 20 ± 2 °C for the duration of the contact time to be tested.

If disinfectants are intended for use at temperatures below 20 °C, the tests shall be conducted at respective temperatures (e.g. 4 °C). Usually, four contact times must be tested depending on the field of application, including the intended contact time for the application:

- either 0.5; 1; 2.5 and 5 (if necessary 1.5; 2) min[6]
- or 5; 15; 30 and 60 min.

Disinfectants, which will only be used for disinfection within short contact times (e.g. hand disinfectants), shall be tested primarily with short contact times. For disinfectants that require long contact times in practice, the testing of short contact times may be omitted if necessary. Concentrations and contact times for the disinfectant shall be chosen in such manner that the result will show the dependency of the virucidal effect of the product on the concentration and the contact time (kinetics).

The activity of the disinfectant shall be tested both without and with FCS load (10% final concentration when disinfectant tested as 80% concentration or 9% when disinfectant tested as 90%; see item 4). The virus control samples shall include the same level of FCS concentration as the test samples.

All tests shall be carried out in at least two independent test runs on different test days.

[2] Employees conducting tests with this virus must be vaccinated.

[3] Should this not be possible (in exceptional cases), large-volume-plating (LVP) s. item 6 shall be applied.

[4] European standards define water as bidest. water free of cell-toxic substances and prepared in a glass distillation device. As an alternative, the use of Aqua ad iniectabilia according to European Pharmacopoeia is recommended. As a consequence, in case no sterile glass distilled water is used, distilled or de-mineralized water, whose relevant parameters such as conductivity and TOC correspond to those mentioned in the European Pharmacopoeia for Aqua ad iniectabilia, shall be used.

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

[6] To duly conduct hand disinfection, contact times determined in practical tests for bacterial efficacy are required. For the approval of virucidal efficacy based on a suspension test, contact times must not be shorter than indicated, even if proof of virucidal efficacy within shorter time periods does exist.

Table 1 Control tests for testing chemo-thermal disinfection methods

Virus titre	20 °C	Process temperature	60 °C/10 min
Control according to items 7.1.1 and 7.1.2	X	X	–
Test run according to item 5.1 (a and b)	X	X	–
Reference control	–	–	X

5.1 Testing of chemo-thermal disinfection procedures

Chemo-thermal processes at temperatures higher than 30 °C are to be tested with parvoviruses at the temperatures specified by the manufacturer. Deviating from the contact times mentioned under item 5 the contact time claimed for the procedure shall be tested primarily. The kinetics of the virus inactivation should become evident from the choice of further contact times and/or concentrations. At least two different concentration-time relations must be tested.

Those tests must, as long as the disinfection procedure consists of several components (e.g. washing and disinfection agent), be conducted with both a) the washing agent or detergent boosters only and b) the complete procedure – washing agents and disinfectant – in separate test runs at the designated temperature and if necessary, under appropriate application conditions (e.g. time at which the disinfectant is added). The tests under a) and b) must also be conducted at 20 °C (see **Table 1**). The controls according to 7.1 shall be conducted at process temperature.

Fetal calf serum (FCS) and bidest. water are each adjusted to 20 ± 2 °C or to the process temperature. The disinfectant solution or all process components are prepared according to the user instructions for the respective process and are applied once at 20 °C and then at process temperature. One part of virus suspension (20 ± 2 °C) and one part of FCS or of bidest. water (each at the required temperature) are mixed; then eight parts of the diluted disinfectant (1.25-fold; at the process temperature) are added and mixed. This mixture is kept at 20 ± 2 °C or at process temperature for the duration of the contact time to be tested.

6 Determination of the infectivity of samples in the suspension test

Two test principles can be considered as methods for the determination of the virus concentration: quantal assays (end point dilution method) or quantitative assays (plaque assay). They can be performed as macro or micro assays. The cytopathic effect i.e. the transformation of cells as a result of virus propagation, is evaluated as an indicator for virus infection.

At the end of specified contact times the test suspension (see item 5) 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). The test tubes with the diluted samples shall be placed in an ice bath (0–4 °C) immediately after preparation. The diluted samples are to be inoculated in cell cultures immediately (stating the time frame in the experiment protocol). It shall be ensured that the disinfectant does not cause aftereffects (see item 7.3).

If the cytotoxicity of the disinfectant is so strong that it is impossible to detect a decrease of the infectivity titre of 4 $\log_{10}$, an attempt can be made to reduce the cytotoxicity (e.g. by applying gel filtration, micro filtration or appropriate chemical neutralization agents). These procedures may be carried out even if the effects of the disinfectant cannot be sufficiently stopped by dilution at the end of the contact time. The chosen procedure shall be described in detail with specific control methods and it shall be proved that the virus detection (virus titre) is not affected. In case of gel filtration or micro filtration, all relevant test conditions shall be tested with and without filtration (the sole testing of the virus control with and without filtration is not sufficient). The test samples with disinfectants must also be tested with and without filtration (see also memorandum of the Virus Disinfection Expert Committee, appendix G).

So-called "large-volume-plating" (LVP) is another method that can be used to improve or refine detection of a decrease in infectivity at the end of the contact time with the disinfectant. This method must only be applied if it is impossible to reach a titre reduction of ≥4 $\log_{10}$ using the previously described method (end point dilution method without cytotoxicity reduction measures) due to reasons described in the previous paragraph. LVP improves the detection limit and can only be used if no or very little infectivity can be demonstrated in the infectivity tests (end point dilution method or plaque assay). The test mixture is diluted with just enough culture medium that no cytotoxicity can be demonstrated for this test. The diluted sample is inoculated immediately (stating the time frame) in microtitre plates on as many cell cultures as possible. The number of plates and thus the test volume applied determines the detection limit.[7]

After incubation under specific virus relevant titration conditions, the cell cultures are examined microscopically for cytopathic effects. The virus concentration or rather the detection limit is calculated from the number of infected cell cultures or from the total sample volume used showing no infectivity. That calculated value is then used for the calculation of the reduction factor (see item 8). The chosen procedure shall be described in detail.

7 Control and comparison tests

7.1 Virus controls

For the virus control, the titre of the virus suspension (not treated with disinfectant) is determined under test conditions without or with FCS load.

[7] Therefore, volumina of at least 62.5 ml should be spread onto (not less than six) 96-well plates (as mentioned in appendix 1). The use of smaller amounts requires a justification.

7.1.1 Virus control without protein load (FCS)

One part of virus suspension and nine parts of water of standardized hardness (for products to be applied undiluted: bidest. water) are mixed. After the maximum contact time has elapsed, serial dilutions (see item 6) shall be prepared and the titre shall be determined.

7.1.2 Virus control with protein load (FCS)

One part of virus suspension, one part of FCS and eight parts of water of standardized hardness (for products to be applied undiluted: bidest. water) are mixed. After the maximum contact time has elapsed, serial dilutions (see item 6) are prepared and the titre is determined.

7.2 Cytotoxicity control

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

Two parts of water of standardized hardness (for products to be applied undiluted: bidest. water) or one part of water of standardized hardness (for products to be applied undiluted: bidest. water) and one part of FCS respectively are mixed with eight parts of disinfectant solution. From the resulting solution, serial dilutions for inoculation of cell cultures are prepared in the same way as described for determination of virus infectivity (see item 6).

7.3 Aftereffect control

Aftereffect controls should be conducted if, especially after short contact times, an uncontrolled aftereffect of the disinfectant due to methodology beyond contact time cannot be excluded. 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 s.

One part of test mixture (consisting of the test preparation (see item 5) with a suitable disinfectant solution) is mixed with nine parts ice-cold culture medium and incubated in an ice-cold bath for titration for the time period that is needed to start the dilution series (see item 6) following the contact time of the disinfectant. After this period of time, a dilution series is prepared from the suspension in order to determine the titre. The first two dilutions are usually used to determine the disinfectant dilution that no longer exhibits aftereffects. The disinfectant shows a negligible or non-existing aftereffect if the difference of this titre in comparison to the virus control is ≤0.5 $\log_{10}$.

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 treatment with the disinfectant.

Two parts of bidest. 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 A). 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. Then, 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 titres in the PBS or disinfectant treated cells shall not exceed 0.5 $\log_{10}$.

7.5 Cell control

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

7.6 Reference control

In addition to each replicate of test and control, a comparative test using a suitable reference substance must be conducted.

Testing of a reference substance serves to demonstrate the suitability of the test viruses, i.e. to prove that their tenacity towards a certain active substance is consistent. Choice of reference substance should be determined by the active ingredient of the product to be tested, as different properties of the test viruses are to be expected depending on the mechanism of action. Formaldehyde should be used for aldehydic products, peracetic acid (PAA) for oxidative products and ethanol for alcoholic products. For disinfectants relying on a mixture of active ingredients, the reference substance should be chosen according to the predominant active ingredient. The reference control test is conducted according to item 5 but without use of a protein load, requiring the product test solution to be replaced by the reference test solution.

Test laboratories are requested to determine laboratory internal reference values. Based on these data, binding values for the assessment of antiviral studies will be set at a later date.[8] Indicative data on reduction factors for selected viruses and reference substances are listed in appendix B.

7.6.1 Reference control for aldehyde containing products

The test must be conducted at pH 7.0, without serum load and at 20 ± 0.5 °C. The concentration of formaldehyde in the applied solution is 0.7 g/100 ml, the contact times of 5, 15, 30 and 60 min (for poliovirus: 30, 60 and 120 min) are evaluated. For this test, one part of virus suspension is mixed with four parts of phosphate buffer (0.1 M; pH 7.0) and five parts of a 1.4% formaldehyde solution (see appendix C).

7.6.2 Reference control for chemothermal disinfection procedures using peracetic acid

These tests are performed with parvoviruses. 0.005% peracetic acid is tested at 60 °C with a contact time of 10 min. Determination of concentra-

[8] Such data can be transferred to the DVV expert comitee (see http://www.dvv-ev.de).

tion and manufacturing conditions for peracetic acid solution are described in Appendix C.

8 Calculation of the reduction factor

For the evaluation of disinfectant activity, the virus titre (including its 95% confidence interval) shall be determined without (titre a) and with exposure to the disinfectant (titre b) and the reduction factor (RF) calculated including its 95% confidence interval shall be calculated. The virus titre ($TCID_{50}$/ml or PFU/ml) can be determined using several methods, e.g. the Spearman-Kärber formula [3–5] for endpoint titrations, or the Taylor or Poisson formula [5, 6] for LVP (item 6). An exemplary calculation is included in appendix H.

8.1 Calculation of the virus titre and its 95% confidence interval according to Spearman and Kärber [3–5]

The logarithmic infectivity titre according to Spearman and Kärber ($\log_{10}$ $TCID_{50}$/ml) is calculated with the following formula:

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

Key:

m = negative decadal logarithm of the titre based on the test volume
x_k = logarithm of the lowest dose (dilution level) at which all cell cultures exhibit a positive reaction
d = logarithm of the dilution factor
p_i = observed reaction rate

The standard deviation (s) of m is calculated as follows [5]:

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

Key:

s_m = standard deviation of the logarithmic titre
d = logarithm of the dilution factor
p_i = observed reaction rate
n = number of test objects per dilution

The 95% confidence interval (k) of the titre is equivalent to approximately 2 s_m.

The pre-dilution of the sample has to be taken into account when calculating the titre.

8.2 Calculation of the reduction factor (RF) and its 95% confidence interval

The RF is calculated as the difference between the logarithmic virus titre of the respective virus control ("control titration", see item 7.1.1 and 7.1.2, titre a) and after exposure to the disinfectant ("residual virus", titre b).

The RF is thus calculated as follows:

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

Key:

RF_{T1} = reduction factor from the first test run
a = $\log_{10}$ $TCID_{50}$/ml or $\log_{10}$ PFU/ml of virus control titration of the first test run
b = $\log_{10}$ $TCID_{50}$/ml or $\log_{10}$ PFU/ml of residual virus titration of the first test run

The 95% confidence interval of the RF of the first test run ($K_{RF(T1)}$) is calculated from the confidence intervals (k) of the virus titres, with k_a being the 95% confidence interval of the virus control titre (a) and k_b being the 95% confidence interval of the residual virus titre (b). The 95% confidence interval (k) of the titre is equivalent to approximately 2 s_m. The 95% confidence interval of the RF is calculated as follows [5]:

$$K_{RF(T1)} = \sqrt{(k_a)^2 + (k_b)^2}$$

Key:

$K_{RF(T1)}$ = 95% confidence interval of the RF of the first test run
k_a = 95% confidence interval of virus control titration of the first test run
k_b = 95% confidence interval of residual virus titration of the first test run

The reduction factor and the 95% confidence interval must be calculated for each test run.

8.3. Calculating the titre and reduction factor when using the large-volume-plating (LVP) method

The detection of residual virus can be improved by the testing of a large sample volume (LVP).

If no or only very little virus ("residual virus") is detected in the test run with disinfectant, the Spearman-Kärber method would show an overstated titre b.[9] If some viral infectivity is still found using the LVP method, the virus concentration can be calculated according to the following formula. The formula is derived from the Taylor series representing an approach to exponential functions (Taylor formula). The result, converted into a logarithmic value, is titre b and is used for the calculation of the reduction factor. One $TCID_{50}$ is equivalent to 0.69 infectious virus particles.

$$c = \frac{D}{V_w} * \left(-\ln\frac{n - n_p}{n}\right)$$

Key:

c = concentration of infectious virus particles
D = dilution
V_w = volume per well
n number of inoculated wells
n_p = number of virus-positive wells

If no virus is detected using the LVP method, the Taylor formula is invalid

[9] Using the Spearman-Kärber method for calculating the titer implies testing dilutions of a sample whereas the lowest dilution should have a reaction rate of 100% and the highest dilution a reaction rate of 0. The more dilutions are tested in range < 100% but > 0% the more precise is the determination of the titer ($TCID_{50}$/ml). If the reaction rate of the lowest dilution tested is < 100%, it is assumed when calculating that the next lower dilution would have shown a 100% reaction rate. In case the reaction rate is low, maybe ≤ 25% (2 of 8 wells) or even 0, the assumption of a 100% reaction rate of the next lower dilution may cause an overstated titer. This is especially relevant for low dilution factors (3 or 5), as the reaction rate decreases in case of multiple dilution steps according to experience.

and therefore the Poisson formula must be applied. It includes the statistical distribution of few virus particles in a large volume [5]. It is the calculation of the virus concentration necessary to receive a positive result for a given sample volume with a probability of 95%. The number of virus particles is calculated according to the following formula, which, in consideration of the dilution factor and converted into a logarithmic value, represents titre b and is used for the calculation of the reduction factor:

$p = e^{-cv}$ and derived according to c

$c = \ln p / -V$

Key:

p is the probability of not detecting a virus; the probability of not detecting a virus must not be higher than 5% ($p = 0.05$) so that the number of virus particles is calculated which can be detected with a probability of 95%

c = concentration of the infectious virus particles

V = test volume

The reduction factor is calculated as described in item 8.2. For practical reasons, titres calculated according to the Taylor formula or the Poisson formula are quoted without 95% confidence intervals ($k_b = 0$). Therefore, in terms of figures, the 95% confidence interval of the reduction factor correlates with the 95% confidence interval of the control titration titre (a); i.e. the formula translates to:

$$K_{RF(T1)} = \sqrt{(k_a)^2}$$

Key:

$K_{RF(T1)}$ = 95% confidence interval of the reduction factor of the first run

k_a = 95% confidence interval of the virus control titration

8.4 Calculating the average reduction factor ($RF_{(av)}$) and its 95% confidence interval

The average RF from both test runs and its 95% confidence interval are calculated as follows:

$$RF_{(av)} = (RF_{T1} + RF_{T2})/2$$

Key:

$RF_{(av)}$ = average reduction factor

RF_{T1} = reduction factor from the first test run

RF_{T2} = reduction factor from the second test run

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

$$K_{RF(av)} = \sqrt{\frac{(K_{RF(T1)})^2 + (K_{RF(T2)})^2}{2}}$$

Key:

$K_{RF(av)}$ = 95% confidence interval of the average reduction factor

$K_{RF(T1)}$ = 95% confidence interval of the RF of the first test run

$K_{RF(T2)}$ = 95% confidence interval of the RF of the second test run

9. Biometrical evaluation of the test runs and assessment of the virus-inactivating properties (reduction factor [RF])

The disinfectant is assumed to have caused a sufficient titre reduction if the average RF is at least 4 $\log_{10}$. The results shall not be affected by cytotoxicity, interferences or aftereffects of the disinfectant.

The virus titrations must be conducted such 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.

10 Test report

The test results are summarized in tabular form and, if necessary, as graphs including the results of the reference substance. 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 including raw data) and an evaluation of the findings (see item 9).

Corresponding address

Ingeborg Schwebke
Robert Koch Institute
Nordufer 20, 13353 Berlin, Germany
Schwebkei@rki.de

Maren Eggers
Labor Prof. Dr. G. Enders MVZ GbR
Rosenbergstraße 85, 70193 Stuttgart, Germany
eggers@labor-enders.de

Conflict of interest. H.F. Rabenau, I. Schwebke, J. Blümel, M. Eggers, D. Glebe, I. Rapp, A. Sauerbrei, E. Steinmann, J. Steinmann, H. Willkommen and P. Wutzler declare that they have no competing interests.

Appendix A

Information for conducting infectivity tests

The infectivity of the suspensions and their dilutions can be determined in plates as micro assays. The end point dilution method ($TCID_{50}$) (micro and macro assay) or the plaque assay (micro assay), may be applied to determine the titre.

Micro assay in plates

Example: Pipette 0.05–0.15 ml of the respective sample dilution per well of a 96 well cell culture plate (6 to 8 wells per dilution level). Sample dilutions may either be applied onto adhered cells or added to 0.05–0.15 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 in-

Table B.1 Reference values for reduction factors using several reference substances and viruses

Test virus/agent	Alcoholic products: Ethanol		Aldehydic products: Formaldehyde		Oxidative products: PAA	
	Conc./ct	RF	Conc./ct	RF	Conc./ct	RF
vaccinia virus	n.y.d.	n.y.d.	0.7%/n.y.d.	n.y.d.	n.y.d.	n.y.d.
poliovirus	n.y.d.	n.y.d.	0.7%/30 min	0.5–2.5	n.y.d.	n.y.d.
			0.7%/60 min	2–4.5		
adenovirus	n.y.d.	n.y.d.	0.7%/n.y.d.	n.y.d.	n.y.d.	n.y.d.
SV40	n.y.d.	n.a.	0.7%/n.y.d.	n.y.d.	n.y.d.	n.y.d.
MNV	n.y.d.	n.y.d.	0.7%/n.y.d.	n.y.d.	n.y.d.	n.y.d.
MVM	n.a.	n.a.	n.a.	n.a	0.005%/10 min/60 °C (50 ppm)	1.5–2.5

n.y.d. not yet determined, *conc.* concentration, *ct* contact time, *n.a.* not applicable

fectivity of the samples is calculated as $TCID_{50}$/ml.

Appendix B

Reference values for reduction factors using several reference substances and viruses (table B.1).

Appendix C

Determination of chemicals

a) Quantitative determination of formaldehyde

The formaldehyde concentrations of formaldehyde solutions available on the market usually differ and may also depend on storage conditions. Thus, it is essential to conduct a quantitative determination of the formaldehyde concentration or its dilution used in the control test. It needs to be considered that formaldehyde solutions which have been prepared from concentrated formaldehyde solutions (e.g. formalin) contain polymers and develop 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 into the acidic range:

$$H_2C = O + {}^{+}H_3N - OH + Cl^{-} \longrightarrow$$
$$H_2C = NOH + H^{+} + Cl^{-} + H_2O$$

The pH-value is titrated with sodium hydroxide to its former value. The amount of sodium hydroxide used indicates the concentration of the sample.

Carrying out the determination:

An aliquot of the sample to be examined containing 100 to 150 mg of formaldehyde is pipetted into a container suitable for titration at the pH meter and distilled water is added 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 previously calibrated to pH 3.0, is added and the mixture incubated at room temperature for 10 min. The solution is then titrated back to pH 3.0 with 0.5 N sodium hydroxide at the pH meter.

Calculation of the formaldehyde concentration in the sample:

$$\frac{\text{Consumed amount of 0.5 N NaOH in ml} \times 30.03}{\text{Volume of sample in ml} \times 20}$$

= concentration of formaldehyde in sample per g in 100 ml.

b) Titration of PAA

Peracetic acids are equilibrium systems consisting of the following components:

Peracetic acid, hydrogen peroxide, acetic acid and water.

Peracetic acid is stored at 4 °C.

A stock solution of peracetic acid is prepared using a peracetic acid solution with a concentration of approximately 40%. The shelf life of the stock solution is maximum one day at 4 °C.

The determination of the PAA concentration by titration is therefore performed a maximum one day before the test.

Substances:

- Peracetic acid
- ortho-phosphoric acid 85% p. A. grade
- Potassium iodide p. A. grade.
- 1% starch solution—starch p. A. grade
- Ammonium heptamolybdate-tetrahydrate p. A. grade
- 0.1 N sodium thiosulfate solution
- Bidest. water, ice flakes

Preparation of the starch solution:

1 g starch is diluted in 100 ml water, brought to boil and allowed to cool.

(shelf life 4 weeks max.).

For the titration, make sure to dilute with at least 100 ml ice water. If insufficiently diluted, hydrogen peroxide already reacts with iodine under cold conditions.

Procedure:

1. Determination of the PAA concentration

10 ml of 5% ortho-phosphoric acid and approx. 0.5 g potassium iodide are added to 100 ml ice water and mixed well, then 3–4 drops of starch solution are added.

Final concentration of disinfectant in the test run (%)	Virus titre of control titration (log_{10} $TCID_{50}$/ml or log_{10} PFU/ml) including 95% confidence interval	Cytotoxicity	Virus titre of "residual 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[a]	2 ct	3. ct	4. ct	1. ct	2. ct	3. ct	4. ct

Fig E.1 ▲ Example of tabular display of test results (Information on cytotoxicity of disinfectant and on maximum detectable decrease of infectivity titre). *c.t.* contact time

After adding of the stock solution[10] the sample is immediately and quickly titrated with sodium thiosulfate solution while stirring until the blue color disappears. The initial disappearance of the color is crucial; after a short time the solution turns blue again as H_2O_2 starts reacting with the iodide.

Calculation:

$$\frac{\text{ml}\,0.1\,\text{N}\,Na_2S_2O_3 \times 3.803}{10 \times \text{ml sample}} = \%\text{peracetic acid}$$

Consumed amount of sodium thiosulfate solution ≙ concentration of peracetic acid 1 ml 0.1 N sodium thiosulfate solution ≙ 3.803 mg peracetic acid.

2. Determination of the hydrogen peroxide concentration

A spatula-tip of ammonium heptamolybdate is added and the solution is warmed to room temperature, then titrated with sodium thiosulfate solution until discoloration is visible.

Calculation:

$$\frac{\text{ml}\,0.1\,\text{N}\,Na_2S_2O_3 \times 1.701}{10 \times \text{ml sample}} = \%H_2O_2$$

Consumed amount of sodium thiosulfate solution ≙ concentration of H_2O_2.

1 ml 0.1 N sodium thiosulfate solution ≙ 1.701 mg H_2O_2.

Appendix D

Preparing water of standardized hardness (WSH)

Two solutions are needed:

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). Sterilize the solution in a steam sterilizer. The solution may be stored at 2–8 °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. Sterilize the solution by membrane filtration. The solution can be stored at 2–8 °C for up to one week.

For the preparation of 1 l of water of standardized hardness, at least 600 ml of sterile bidest. water are added to a sterilized 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 must have a pH of 7.0 + 0.2. If necessary, the pH is calibrated with 1 N sodium hydroxide (NaOH) or 1 N hydrochloric acid (HCl). Water of standardized hardness shall be prepared freshly under aseptic conditions and must be used within 12 h.

The hardness is 376 ppm, based on the calcium carbonate content.

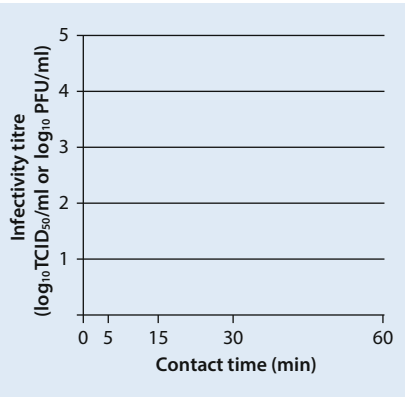
Appendix E

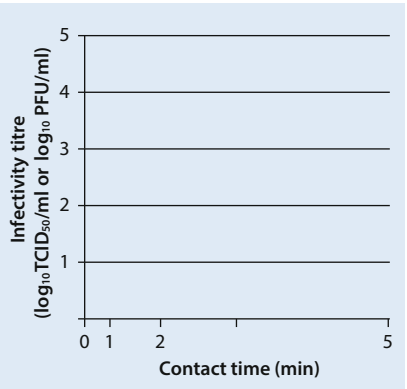
Tabular and graphical display of test results

a) Example of tabular display of test results

(■ Fig. E.1).

b) Examples of graphical depiction of test results





[10] e.g. 1 ml for a 1% peracetic acid solution
10 ml for a 0.1% peracetic acid solution

Table G.1 Example for test results with and without columns

Agent	Concentration	Test virus	Protein load	Cytotoxicity	Column	Virus titre control	Virus titre (log_{10} $TCID_{50}$/ml) after			
							5 min	*15 min*	*30 min*	*60 min*
QAC 1	1%	rotavirus	Bidest. water	3.50	No	7.13 ± 0.37	6.00 ± 0.38	5.63 ± 0.41	5.63 ± 0.41	n. d.
				2.50	Yes	7.00 ± 0.38	≤2.50 ± 00	≤2.50 ± 00	≤2.50 ± 00	n. d.
QAC 2	0.25%	rotavirus	Bidest. water	3.50	No	7.13 ± 0.37	6.75 ± 0.33	6.75 ± 0.44	5.88 ± 0.37	n. d.
				2.50	Yes	7.00 ± 0.38	6.63 ± 0.25	6.50 ± 0.35	6.75 ± 0.44	n. d.
QAC 3	1%	adenovirus	FCS	3.50	No	7.64 ± 0.29	n. d.	n. d.	n. d.	5.79 ± 0.37
				3.50	Yes	7.50 ± 0.40	n. d.	n. d.	n. d.	≤3.50 ± 00
		SV40		3.50	No	7.79 ± 0.37	n. d.	n. d.	n. d.	5.21 ± 0.55
				3.50	Yes	7.79 ± 0.37	n. d.	n. d.	n. d.	≤3.50 ± 00
QAC 4	1%	adenovirus	FCS	3.50	No	7.64 ± 0.29	n. d.	n. d.	n. d.	6.07 ± 0.49
				2.50	Yes	7.50 ± 0.40	n. d.	n. d.	n. d.	≤2.50 ± 00
	0.5%	SV40		4.50	No	7.79 ± 0.37	n. d.	n. d.	n. d.	6.36 ± 0.29
				3.50	Yes	7.79 ± 0.37	n. d.	n. d.	n. d.	≤3.50 ± 00
							1 min	*2 min*	*3 min*	*5 min*
Alcohol + CHG	80%	adenovirus	Bidest. water	4.50	No	8.00 ± 0.46	n. d.	6.75 ± 0.33	n. d.	n. d.
				2.50	Yes	7.25 ± 0.44	n. d.	≤2.63 ± 0.25	n. d.	n. d.

Appendix F

Information on composing expert reports concerning disinfectant activity tests

- Briefly describe what application area (e.g. hand, surface, instrument or laundry disinfection) the agent will be used for in the introduction. It is especially important to indicate which conditions the agent will be used under 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, color, pH-value (the pH-value of the test solutions in the test sample and in the working dilution with WSH shall be documented. This does not apply to alcoholic solutions at concentrations >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 test runs shall be described in detail. A reference to the guideline is not sufficient. In particular, describe the preparation and, if necessary, the method for concentration of the test virus suspension as well as the test method and calculation of the virus concentration (titre) in detail. Any deviation from this guideline shall be described and justified in detail.
- The results of all tests need to be documented 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 [3, 4] or for the LVP the formulas using the Taylor or Poisson method [5, 6]. The statistical evaluation and determination of the 95% confidence interval for the reduction factor (RF) shall be calculated according to the guidelines provided in item 8.

Appendix G

Memorandum on the use of molecular sieve filtration columns in virucidal activity tests

(as of 18 May 2013).

For proof of antiviral activity of disinfectants, a titre reduction of ≥4 log_{10} steps after the specified contact time must be demonstrated during disinfectant tests. However, since some disinfectants exhibit a strong cytotoxicity, this titre reduction cannot always be achieved. For such cases, EN 14476 and the guideline of the DVV/RKI recommend "detoxification" of the tested solutions by molecular filtration, e.g. using Sephadex ™ LH 20 columns. With this method, the cytotoxicity can generally be reduced by one log_{10} step depending on the active substances. In some laboratories, molecular filtration columns are also used for stopping the virus inactivating effect of non-cytotoxic disinfectants. Experiences from several laboratories have shown that infectious viruses are retained in an unpredictable manner in these molecular filtration processes, potentially leading to incorrect results. An antiviral effect is simulated which is not actually present.

This effect has been observed for different viral species and with disinfectants relying on different active ingredients. Table G.1 shows various examples with and without use of molecular filtration columns: The QAV-based formulations 1, 3 and 4 showed a significant retention of infectious viruses in the columns, whereas no such effect was observed in the QAV-based product 2. In direct comparative studies of test solutions – filtered and unfiltered – differ-

Table H.1 Exemplary values for 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
1:1000	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

ences in titres of up to 3 $\log_{10}$ steps were measured. However, this retention effect does not occur during parallel titration of the virus control. These observed effects result in the hypothesis that the critical factor lies within the composition of the test solutions, consisting of disinfectant, virus suspension and, where applicable, protein load.

Several expert reports and publications contain disinfectant efficacy tests relying solely on protocols using molecular sieve filtration. In individual cases, it is difficult to assess the accuracy of this data reliably. This is the case if control measurements are missing that would prove that no residual virus was retained in the columns. We therefore point out that for disinfectant efficacy tests in which the required titre reduction cannot be achieved without "detoxification", all contact times have to be measured both with and without molecular sieve filtration. In doing so, any retention of viruses in the columns can be determined. If necessary, other modifications to the test protocol (e.g. higher virus titre) need to be performed. Molecular sieve filtration is unsuitable for stopping antiviral effects in non-cytotoxic solutions as the necessary timing of this procedure – especially with short exposure times – makes the results less precise than dilution series.

Appendix H

1. Exemplary calculation of the virus titre and the reduction factor according to Spearman and Kärber

Titre calculation of the control titration of the first test run (T1):

The titre is calculated according to the numerical values shown in Table H.1 (see item 8.1):

$$m = -6 + 1/2 - 1 \times 2.38 = 7.88$$

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 8.1) as follows:

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

As $p_1 = 1$, $p_2 = 0.75$, $p_3 = 0.5$ und $p_4 = 0.13$ and n is 16 in all dilutions, therefore

$$P_1(1 - p_1)/(n_1 - 1) = 0$$

$$p_2(1 - p_2)/(n_2 - 1) =$$

$$0.75(1 - 0.75)/(16 - 1) = 0.0125$$

$$\frac{p_3\,(1 - p_3)}{n_3 - 1} = \frac{0.50\,(1 - 0.50)}{16 - 1} = 0.0167$$

$$\frac{p_4\,(1 - p_4)}{n_4 - 1} = \frac{0.13\,(1 - 0.13)}{16 - 1} = 0.0075$$

$$s_m = \sqrt{1\,(0.0125 + 0.0167 + 0.0075)}$$

$$s_m = 0.19,$$

i.e. $2\,s_m = 0.38$

The result is $k_a = 0.38$

Therefore, the titre of the control titration amounts to 8.88 ± 0.38 $\log_{10}$ $TCID_{50}$/ml.

If the residual virus titre of the first test run (figures not shown) is calculated analogously, the result for this example is 3.50 ± 0.32 $\log_{10}$ $TCID_{50}$/ml.

The reduction factor (RF) is calculated as follows:

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

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

The 95% confidence interval of the RF ($K_{RF(T1)}$) is calculated according to (cf. item 8.2):

$$K_{RF(T1)} = \sqrt{(0.38)^2 + (0.32)^2} = 0.50$$

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

Assuming that the titres of the virus control titration and the residual 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, the RF_{T2}, including 95% confidence interval, is calculated as 5.0 ± 0.40.

The average RF from both test runs including 95% confidence interval is calculated according to (cf. item 8.4):

$$RF_{(av)} = (5.38 + 5.00)/2 = 5.19$$

The 95% confidence interval of the average RF ($K_{RF(av)}$) is calculated as follows (cf. item 8.4):

$$K_{RF(av)} = \sqrt{\left(0.50^2 + 0.40^2\right)/2} = 0.45$$

Hence, the total RF from both test runs (95% confidence interval included) for the given example amounts to 5.19 ± 0.45 $\log_{10}$.

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

2. Example calculation for calculating the titre and the reduction factor using the large-volume-plating (LVP) method according to item 8.3

Assuming that only a low amount of infectivity is detected after exposure to the disinfectant resulting in the use of LVP, there are two options for calculating titre b, which can be used to calculate the reduction factor, as described in item 8.2.

a) Calculation according to the Taylor formula:

If some infected cell cultures are found using the LVP method, the titre can be calculated according to the following formula:

$$c = \frac{D}{V_W} * \left(-\ln\frac{n - n_p}{n}\right)$$

c = concentration of the infectious virus particles
D = dilution
V_w = volume per well
n = number of inoculated wells
n_p = number of virus-positive wells

For the example calculation the following values are used:

- $V_w = 0.1$ ml
- $n = 96$
- $n_p = 9$
- $D = 1000$

If these values are inserted into the formula above, the result is 984 infectious particles per ml. One $TCID_{50}$ is equivalent to 0.69 infectious virus particles. The result is therefore a titre of 984 : 0.69 = 1426 $TCID_{50}$ per ml. The value used to calculate the reduction factor is the decadal logarithm of this titre, i.e. 3.15 $\log_{10}$ $TCID_{50}$/ml.

b) Calculation according to the Poisson formula:

If no infected cell cultures are found using the LVP method, the number of infectious virus particles (95% detection limit) is determined as follows:

c = lnp/ – v

Key:

p = is the probability of not detecting a virus; the probability of not detecting a virus must not be higher than 5% ($p = 0.05$). Thus, the virus concentration in a given sample volume that can be detected with a probability of 95% is calculated
V = test volume [ml]

In this example, a sample diluted to 1:1000, due to cytotoxicity, is inoculated into a micro test plate (96 wells, 0.1 ml/well).

This results in the following calculation:

- $p = 0.05$, $\ln p = -2.99$
- $V = 96 \times 0.1$ ml; the tested volume amounts to 9.6 ml

Inserted into the formula the result is a value of ≤0.31 virus particles/ml. Considering the dilution (0.31×1000), the result is a value of 310 infectious virus particles per ml. Since one $TCID_{50}$ is equivalent to 0.69 infectious virus particles, the result is a titre of 310 : 0.69 = 445 $TCID_{50}$/ml whose logarithm is ≤2.65 $\log_{10}$ $TCID_{50}$/ml. This value is the titre used to calculate the reduction factor.

For the calculation of the reduction factor and its 95% confidence interval we assume that the titre of the virus control, as demonstrated by the example above is 8.88 ± 0.38 $\log_{10}$ $TCID_{50}$/ml. 3.15 $\log_{10}$ $TCID_{50}$/ml was determined to be the residual virus titre. Consequently, the reduction factor is (8.88–3.15) 5.73 $\log_{10}$.

Since, for pragmatic reasons, the residual virus titre does not have a 95% confidence interval, the result is a reduction factor of 5.73 ± 0.38 $\log_{10}$.

If no infectivity is found when determining the residual virus titre and if the titre is calculated according to the Poisson formula, the result is a reduction factor of (8.88–2.65) 6.23 $\log_{10}$.

Since, for pragmatic reasons, the residual virus titre does not have a 95% confidence interval, the result is a reduction factor of ≥6.23 ± 0.38 $\log_{10}$. Here the reduction factor is prefixed by the symbol ≥ in order to demonstrate that no residual infectivity was detected, i.e. the reduction factor amounts to at least this value but may also be even higher.

References

1. Rabenau HF, Schwebke I, Steinmann J, Eggers M, Rapp I, Neumann-Haefelin D (2012) Evaluation of a virucidal quantitative carrier test for surface disinfectants (application in the field of human medicine). Hyg Med 37:78–85
2. RKI (2004) Testing and declaration of the virucidal efficacy of chemical disinfectants. Position of the Virucide Study Group of the Robert Koch Institute (RKI). Bundesgesundheitsblatt 47:62–66 (This document was updated in 2017 see https://edoc.rki.de/handle/176904/183, Bundesgesundheitsbl 2017 · 60:353–363 DOI https://doi.org/10.1007/s00103-016-2509-2)
3. Spearman C (1908) The method of "right and wrong cases" ("constant stimuli") without Gauss's formulae. Br J Psychol 2:227–242
4. Kärber G (1931) Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche [A contribution to the collective treatment of a pharmacological experimental series]. Arch Exp Pathol Pharmakol 162:480–483
5. Bekanntmachung über die Zulassung von Arzneimitteln, Anforderungen an Validierungsstudien zum Nachweis der Virussicherheit von Arzneimitteln aus menschlichem Blut oder Plasma vom 20. Dezember 1993/21. Januar 1994. Bundesanzeiger Nr. 84: 4740–4744 bzw. CPMP/BWP/268/95: Note for Guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses. http://www.ema.europa.eu
6. Taylor JR (1997) An introduction to error analysis: the study of uncertainties in physical measurements, 2nd edn. University Science Books, Mill Valley, p 327