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Final Report on Efficacy Assessment of Disinfecting Substances Alternative to Alcohol for Use Against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

The Committee on Efficacy Assessment of Disinfecting Substances Alternative to Alcohol for Use Against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

June, 2020

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Introduction

In March 2020, the Ministry of Economy, Trade and Industry (METI) requested that the National Institute of Technology and Evaluation (NITE) conduct an assessment of the virucidal activity of substances alternative to alcohol-based disinfectants in order to prevent the spread of SARS-CoV-2 infection.
More specifically, the request was to perform the urgent evaluation of virucidal activity of candidate substances against SARS-CoV-2, which were widely available as a daily commodity and expected to be alternatives to alcohol-based disinfectants. This was to be done through a literature survey, given the circumstances of the tight supply and increased demand of alcohol-based disinfectants due to the COVID-19 outbreak.
Toward above-mentioned purpose, NITE established the Committee on Efficacy Assessment of Disinfecting Substances Alternative to Alcohol for Use Against SARS-CoV-2 (Secretariat: METI and NITE). The Committee was responsible for the selection of disinfectant candidates for efficacy evaluation, approval of testing protocols, and other matters necessary for consideration. The Committee was also responsible for judging the validity of the evaluation results, as well as assessing and determining the virucidal activity of the candidate substances against SARS-CoV-2.
As a policy, the Committee tried to keep a good balance among ensuring scientific objectivity, facilitating prompt actions, and providing easy-to-understand information to citizens.
The intended application of the disinfecting substances evaluated by this Committee was the disinfection of environmental surfaces in households and community settings (hereinafter referred to as “surfaces”), which included but were not limited to the surfaces of furniture, sinks, bathtubs, toilets, remote controls for electric appliances, and other fixed items such as counter tops, stairway rails, floors and walls. The Committee assessed the virucidal activity of the candidate substances by using the liquid suspension method of the virus and samples for testing virucidal activity. Evaluations for safety and conditions for the actual use of the effective substances were outside of the scope of the Committee.

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Contents

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1. Outline of the Committee

1-1. Members and Observers of the Committee (in Alphabetical Order)

Members

Names	Titles and Affiliations as of June 25, 2020
Chairperson: MATSUMOTO Tetsuya, M.D., Ph.D.	Chief Professor, Department of Infectious Diseases, International University of Health and Welfare, and Vice President, Japanese Society for Infection Prevention and Control
FUKUZAKI Satoshi, Ph.D.	Professor, Graduate School of Bioresources, Mie University
HANAKI Ken-Ichi, D.V.M., Ph.D.	Director, Management Department of Biosafety and Laboratory Animal, National Institute of Infectious Diseases (NIID)
ISHIWATARI Yukinori	Representative Director, Japan Electrolyzed Water Association
KAGEYAMA Seiji, M.D., Ph.D.	Professor (Division of Virology) and Vice Dean, Faculty of Medicine, Tottori University
KUBOTA Hiroki, Ph.D.	Senior Researcher, Department of Food Additives, National Institute of Health Sciences (NIHS)
MATSUURA Yoshiharu, D.V.M., Ph.D.	Professor, Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University
SAIJO Hiroyuki	Senior Managing Director, Japan Soap and Detergent Association
UEMA Masashi, D.V.M., Ph.D.	Section Chief, 4th Section, Division of Biomedical Food Research, National Institute of Health Sciences (NIHS)

Observers

Names	Titles and Affiliations as of June 25, 2020
IMOTO Yasuo, Ph.D.	Microbial Testing Laboratory Manager, Japan Textile Products Quality and Technology Center (QTEC), Kobe Testing Center

ISHIGURO Hitoshi, Ph.D.	Vice-Leader, Senior Researcher, Kanagawa Institute of Industrial Science and Technology (KISTEC)
KATAYAMA Kazuhiko, Ph.D.	Professor, Laboratory of Viral Infection I, Department of Infection Control and Immunology, Ōmura Satoshi Memorial Institute & Graduate School of Infection Control Sciences, Kitasato University
NAGAI Takeshi, Ph.D.	Researcher, Kanagawa Institute of Industrial Science and Technology (KISTEC)
OGAWA Haruko, D.V.M., Ph.D.	Professor, Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine (OUAVM)
SEJIMA Shunsuke	President, Certified NPO Biomedical Science Association (BMSA)
TAKAGI Hirotaka	Senior Researcher, Management Department of Biosafety and Laboratory Animal, National Institute of Infectious Diseases (NIID)
TAKEDA Yohei, D.V.M., Ph.D.	Specially Appointed Assistant Professor, Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine (OUAVM)

Ministry Officials

Names	Titles and Affiliations as of June 25, 2020
ESAKI Yoshihide	Deputy Director-General, Commerce and Service Industry Policy Group, METI
HINOSHITA Eiji, M.D., Ph.D.	Director, Tuberculosis and Infectious Diseases Control Division, Health Service Bureau, MHLW
KANAI Shinsuke	Director for Policy Planning, Crisis Management Unit, Minister's Secretariat, METI
TANAKA Makoto	Senior Investigator for Surcharge, Representation Division, Consumer Affairs Agency
TANAKA Tetsuya	Director, Bio-Industry Division, Commerce and Service Industry Policy Group, METI
YOSHIMURA Katsumoto	Director, Material Industries Division, Manufacturing Industries Bureau, METI

1-2. Summary of the Activities of the Committee

The First Committee (April 15, 2020)

- Several candidate substances possible to inactivate SARS-CoV-2 were selected based on the literature survey.

The Second Committee (April 30, 2020)

- The results of the evaluation studies of the selected candidates using the Influenza A virus as a surrogate for SARS-CoV-2 were reported.
- The initiation of collaborative studies with the National Institute of Infectious Diseases (NIID) and Kitasato University on the evaluation of the virucidal activity of the candidate substances against SARS-CoV-2 was reported.

The Third Committee (May 21, 2020)

- The first report on the evaluation studies using SARS-CoV-2 was presented. Five surfactants (including quaternary ammonium salts) were judged to be effective.

The Fourth Committee (May 28, 2020)

- The second report on the evaluation studies using SARS-CoV-2 was presented. Two additional surfactants (including quaternary ammonium salts) were judged to be effective.

The Fifth Committee (June 25, 2020)

- The third report of the evaluation studies using SARS-CoV-2 was presented. The results provided by the Obihiro University of Agriculture and Veterinary Medicine (OUAVM), Tottori University and the Japan Textile Products Quality Technology Center (QTEC), in addition to NIID and Kitasato University, revealed that two additional surfactants — hypochlorous acid water (HAW) of 35 ppm or higher of available chlorine concentration (ACC) and sodium dichloroisocyanurate (NaDCC) of 100 ppm or higher of ACC — were judged to be effective against SARS-CoV-2.
- In conclusion, the Committee judged that nine surfactants (including three quaternary ammonium salts) and HAW (including NaDCC) of certain concentration were effective against SARS-CoV-2.

1-3. Schedule of Activities of the Committee

Activities related to the Committee were carried out according to the following schedule.

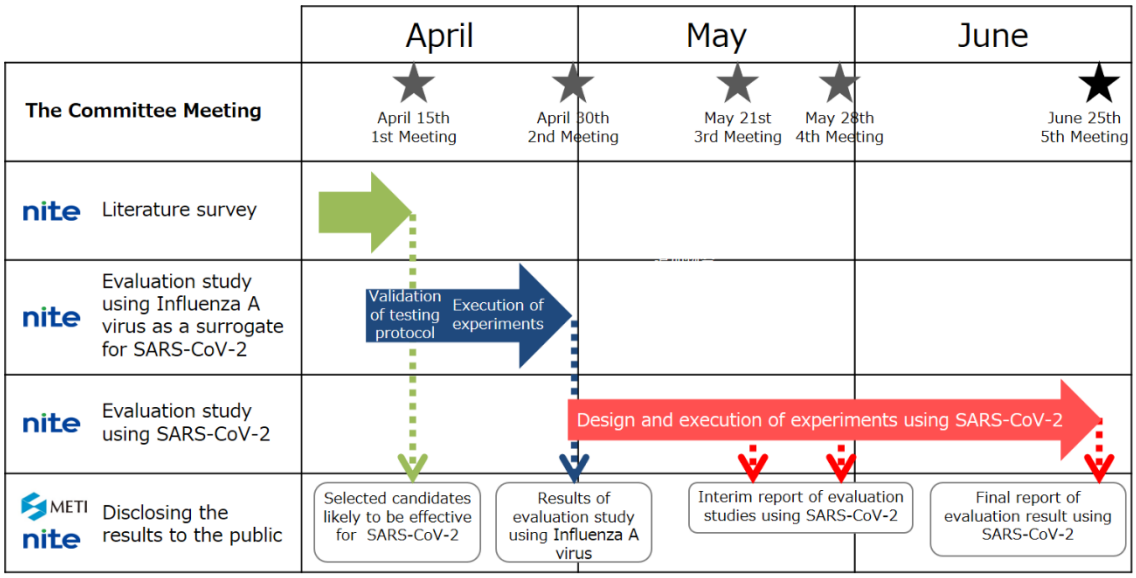


Figure 1. Schedule of Activities of the Committee

Table 1. List of Abbreviation and Acronyms

ACC	Available chlorine concentration
COVID-19	Coronavirus Disease 2019
EW (≤pH 2.7)	Strongly acidic electrolyzed water (≤pH 2.7)
EW (pH 2.7-5.0)	Weakly acidic electrolyzed water (pH 2.7-5.0)
EW (pH 5.0-6.5)	Slightly acidic electrolyzed water (pH 5.0-6.5)
HAW	Hypochlorous acid water
METI	Ministry of Economy, Trade and Industry
NaDCC	Sodium dichloroisocyanurate
NIID	National Institute of Infectious Diseases
NITE	National Institute of Technology and Evaluation
OUAVM	Obihiro University of Agriculture and Veterinary Medicine
QTEC	Japan Textile Products Quality and Technology Center
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
$TCID_{50}$	Median tissue culture infectious dose
min	minutes
s	seconds

2. Candidate Substances for Assessment of their Disinfectant Activity Against SARS-CoV-2

2-1. Selection of Candidate Substances Alternative to Alcohol-Based Disinfectants for Efficacy Assessment

Candidate substance categories for efficacy assessment were selected based upon the following criteria:

- Enough supply of corresponding products was secured in the market.
- Virucidal activity against SARS-CoV-2 was anticipated through the literature survey.

Please note that no information was found indicating the viricidal activity of the candidate substances against SARS-CoV-2 as of May 2020. As such, the virucidal activity of the candidate substances against SARS-CoV-2 was presumed based on scientific evidence regarding their virucidal activities against relevant enveloped RNA viruses. These include other coronaviruses (e.g., SARS coronavirus) and influenza viruses in the literature information (Appendix 5: References 1 to 16).

The preliminary evaluation study of the candidates listed in Table 2 was performed based on the above-mentioned criteria, and the following four substance categories were selected as promising candidates:

- Surfactants (household detergents etc.)
- Hypochlorous acid water (electrolytically-generated and non-electrolytically-generated)
- Quaternary ammonium salts
- Sodium percarbonate (synonymous with sodium carbonate peroxyhydrate)

Table 2. Promising Substance Categories for Disinfection Against SARS-CoV-2

Substance Category	Supply Capacity	Viricidal activity	Targets of use	Necessity for the efficacy assessment against SARS-CoV-2
Hot Water (Heating)	Excellent	Effective	Food Surfaces	Already Recommended by the Government; No Evaluation Required
Alcohol (70% or higher)	Insufficient	Effective	Hand (Food) Surfaces	Already Recommended by the Government; No Evaluation Required
Chlorine Bleach (Sodium Hypochlorite)	Good (Demand Increasing)	Effective	Surfaces	Already Recommended by the Government; No Evaluation Required
Surfactants (Household Detergents etc.)	Excellent	Likely	(Food) Surfaces	Evaluation Study Needed
Hypochlorous Acid Water (HAW) (Electrolytically-Generated and Non-Electrolytically-Generated)	Good	Likely	(Hand) Food Surfaces	Evaluation Study Needed
Quaternary ammonium salts	Good (Demand Increasing)	Likely	(Hand) Food Surfaces	Evaluation Study Needed
Sodium Percarbonate (Synonymous with Sodium Carbonate Peroxyhydrate)	Good	Likely	Surfaces	Evaluation Study Needed

The above table was prepared by the secretariat of the Committee through a literature search.
The status of supply capacity is reflected as of mid-April, 2020.

- While hydrogen peroxide, peracetic acid and iodine-based disinfectants were also listed as promising substance categories, they were not further evaluated because these substances were mainly used in a medical setting.
- Although there were many other promising candidate substances likely to be effective against SARS-CoV-2, they were not evaluated in the Committee mainly due to the limitation of their supply capacity for general use.

2-2. 1) Candidate Substances for Efficacy Assessment Against SARS-CoV-2 – Surfactants, Quaternary Ammonium Salts and Sodium Percarbonate

Twelve candidate substances were chosen for the evaluation study, as listed in Table 3. In addition to the eight surfactants (including one quaternary ammonium salt) that had been tested in the preliminary evaluation study using influenza A virus, three surfactants (including two quaternary ammonium salts) (i.e. sodium alkyl ether sulfates, benzethonium chloride and dialkyldimethylammonium chloride) and sodium percarbonate were added to the evaluation study based on a literature survey.

Table 3. Candidate Substances for the Evaluation Study (Surfactants, Quaternary Ammonium Salts and Sodium Percarbonate)

Subcategory	Name of Substance
Anionic Surfactants	Potassium soap
	Sodium soap
	Sodium alkyl ether sulfates
Nonionic Surfactants	Alkyl glycosides
	Fatty acid alkanol amides
	Polyoxyethylene alkyl ether
Amphoteric Surfactants	Alkyl amidopropyl betaine
	Alkyl dimethyl amine oxide
Cationic Surfactants	Benzalkonium chloride
(Quaternary Ammonium Salts)*	Benzethonium chloride
	Dialkyldimethylammonium chloride
Oxygen Bleach	Sodium percarbonate

* The three substances which were categorized into Cationic Surfactants were also known to be categorized into Quaternary Ammonium Salts.

2-2. 2) Candidates Substances for Efficacy Assessment Against SARS-CoV-2 – Hypochlorous Acid Water (HAW) (Electrolytically-Generated and Non-Electrolytically-Generated)

Several substances from both HAW (electrolytically-generated) and HAW (non-electrolytically-generated) categories were chosen for the evaluation study as listed in Tables 4-1. and 4-2. In this evaluation study, acidic solution containing hypochlorous acid as the main component were considered as HAW.

Please note: There are various types of products manufactured and sold under the name "hypochlorous acid water" in the market, and the majority of these products are categorized into HAW (non-electrolytically-generated); however, there are no widely-accepted product specifications or standards in this category. For instance, the following types of products are sold: a two-liquid premixed type, an ion-exchange type and a powder/tablet type.

Table 4-1. Candidate Substances for the Evaluation Study (Hypochlorous Acid Water (HAW); Electrolytically-Generated)

Subcategory	Electrolytes	pH*	Available Chlorine Concentration* (ppm)
Strongly Acidic Electrolyzed Water (EW (≤pH 2.7))	Sodium chloride	≤2.7	20-60
Weakly Acidic Electrolyzed Water (EW (pH 2.7-5.0))	Sodium chloride	2.7-5.0	10-60
Slightly Acidic Electrolyzed Water (EW (pH 5.0-6.5))	Hydrochloric Acid or Hydrochloric Acid + Sodium chloride	5.0-6.5	10-80

Table 4-2. Candidate Substances for the Evaluation Study (Hypochlorous Acid Water (HAW); Non-Electrolytically-Generated)

Formulation Type	Ingredients	pH*	Available Chlorine Concentration* (ppm)
Two-Liquid Premixed**	Sodium Hypochlorite+Hydrochloric Acid	5.0-6.5	100-300
Two-Liquid Premixed**	Sodium Hypochlorite+Carbonic Acid	5.0-6.5	100-300
Two-Liquid Premixed**	Sodium Hypochlorite+Acetic Acid	5.0-6.5	100-300
Ion-Exchange**	Sodium Hypochlorite	5.0-6.5	100-300
Powder/Tablet	Sodium Dichloroisocyanurate (NaDCC)	5.0-6.5	100-300

*The pH and available chlorine concentration (ACC) indicate the upper and lower limits of the sample. The actual pH and ACC of each tested sample was measured just before the evaluation study.

**Upon the evaluation of HAW of the non-electrolytically-generated type (except for the powder/tablet type), several products corresponding to each formulation type were purchased from the market and the actual evaluation tests were conducted in a blinded fashion. Please note that hydrochloric acid is the most-commonly used as a neutralizing acid among the two-liquid premixed types of HAW.

2-3. Definition of “Hypochlorous Acid Water” Used by This Committee

Various types of products are manufactured and sold under the name "hypochlorous acid water" in the market. Because there have been no widely-accepted product specifications or standards for these products, the following categorization is being tentatively used for this report.

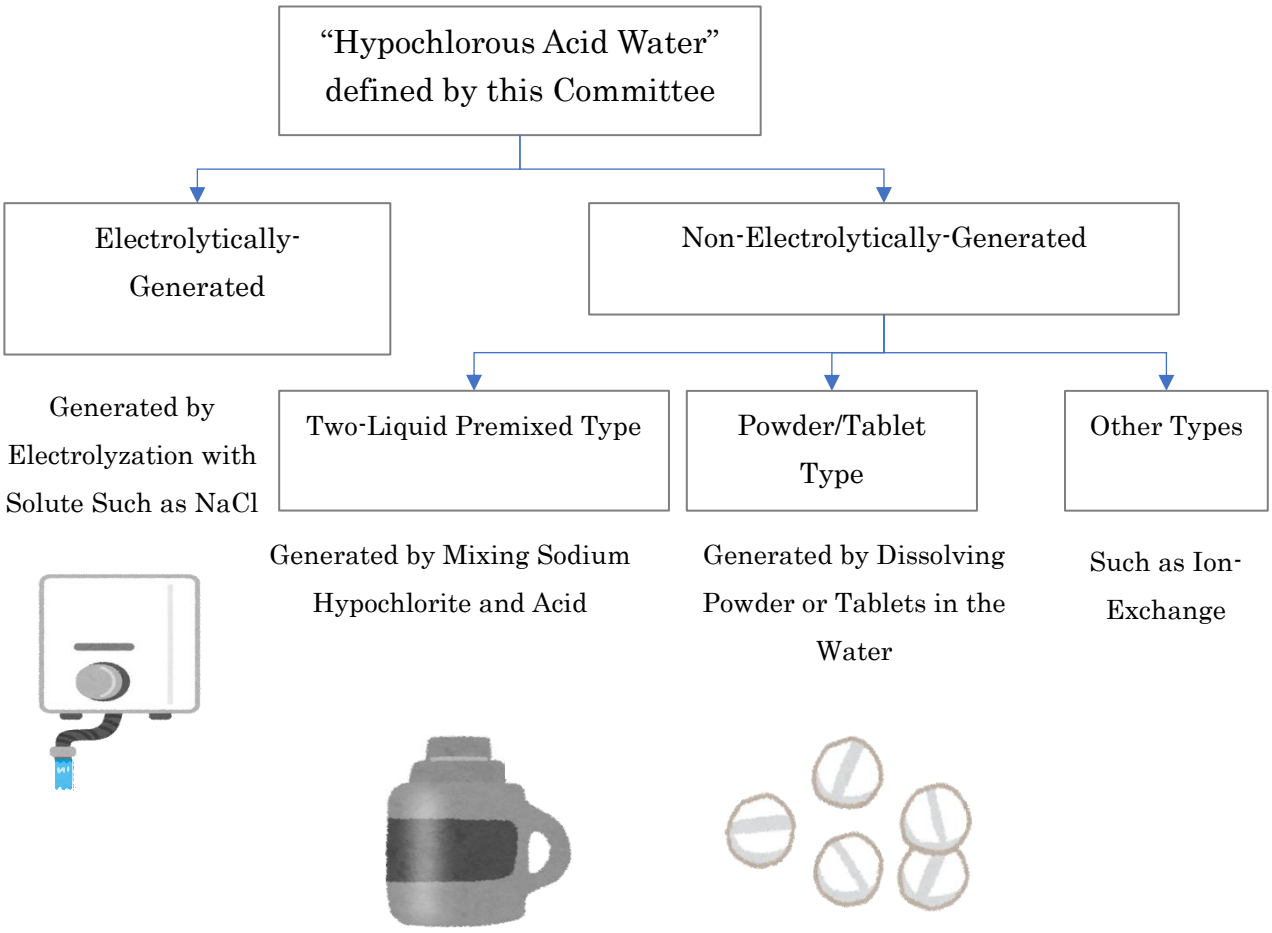


Figure 2. Definition of “Hypochlorous Acid Water” Used by This Committee

3. Outline of Evaluation Studies with SARS-CoV-2 by Participating Organizations

3-1. The evaluation studies were performed by the following organizations in a collaboration manner:

- National Institute of Infectious Disease (NIID)
- Kitasato University
- Obihiro University of Agriculture and Veterinary Medicine (OUAVM)
- Tottori University
- Japan Textile Products Quality and Technology Center (QTEC)

Table 5. Materials and Test Conditions[1] Used in the Evaluation Study of Each Participating Organization

	Organization				
	NIID	Kitasato University	OUAVM	Tottori University	QTEC
Virus Strain	JPN/TY/WK-521	JPN/TY/WK-521 & Kitasato Univ. Isolated strains	JPN/TY/WK-521	JPN/TY/WK-521	JPN/TY/WK-521
Host Cells of Virus Titer Detection	VeroE6/TMPRSS2	VeroE6/TMPRSS2	VeroE6/TMPRSS2	VeroE6/TMPRSS2	VeroE6/TMPRSS2
Medium for Preparation of Virus Stock	DMEM	DMEM	DMEM	DMEM	DMEM
Concentration of FBS for Preparation of Virus Stock	5%	2%	1%	5%	1%
Detection Method of Virus	$TCID_{50}$	CPE Observation and qRT-PCR	$TCID_{50}$	$TCID_{50}$	$TCID_{50}$
Volume Ratio of Virus Stock to Test Substance	1:1[a], 1:9[b], 1:19[c]	1:9[a,c]	1:9[c], 1:19[b,c]	1:9[b], 1:19[b]	1:9[b], 1:19[b]
Initial Virus Concentration	> 4 x 10^6 $TCID_{50}$/50 µl[a] > 7 x 10^6 TCID50/50 µl[b,c]	> 10^4 pfu/µl	> 1 x 10^6 $TCID_{50}$/ml	> 1 x 10^6 $TCID_{50}$/ml	> 1 x 10^7 $TCID_{50}$/ml
Substance Tested	Surfactants Non-Electrolytic HAW Electrolytic HAW	Surfactants Sodium Percarbonate Electrolytic HAW	Non-Electrolytic HAW Electrolytic HAW	Non-Electrolytic HAW	Non-Electrolytic HAW

[1] Test conditions are described in detail in Appendix 2.

FBS, fetal bovine serum; CPE, cytopathic effect; non-electrolytic HAW, HAW (non-electrolytically-generated); electrolytic HAW, HAW (electrolytically-generated)

[a], for surfactants; [b], for HAW (non-electrolytically-generated); [c], for HAW (electrolytically-generated)

4. Results of the Evaluation Studies with SARS-CoV-2

4-1. Results of the Evaluation Studies with SARS-CoV-2 – Surfactants, Quaternary Ammonium Salts and Sodium Percarbonate

- Potassium soap 0.12% (5 min) and sodium soap 0.11% (5 min) showed a less than 99% reduction of viral titer in the evaluation study at NIID. Potassium soap 0.1% (5 min) and sodium soap 0.1% (10 min) did not show virucidal activity in the evaluation study at Kitasato University. On the other hand, potassium soap 0.24% (1 min) and sodium soap 0.22% (1 min) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID.
- Sodium linear alkylbenzene sulfonates 0.1% (20 s) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Virucidal activity was observed at 0.1% (5 min) in the evaluation study at Kitasato University.
- Sodium alkyl ether sulfates 0.5% (5 min) showed a less than 99.9% reduction of viral titer in the evaluation study at NIID. Virucidal activity was not observed at 0.1% (5 min) in the evaluation study at Kitasato University.
- Alkyl glycosides 0.05% (20 s) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Virucidal activity was observed at 0.1% (1 min) in the evaluation study at Kitasato University.
- Fatty acid alkanol amides 0.2% (5 min) showed less than a 99.9% reduction of viral titer in the evaluation study at NIID. Virucidal activity was not observed at 0.1% (5 min) in the evaluation study at Kitasato University.
- Polyoxyethylene alkyl ether 0.1% (5 min) showed a more than 99.99% reduction of viral titer in the evaluation study at NIID. Virucidal activity was not observed at 0.1% (5 min) in the evaluation study at Kitasato University. Polyoxyethylene alkyl ether 0.2% (5 min) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID.
- Alkyl amidopropyl betaine 0.5% (5 min) showed less than a 99.9% reduction of viral titer in the evaluation study at NIID. Virucidal activity was not observed at 0.1% (5 min) in the evaluation study at Kitasato University.
- Alkyldimethylamine oxide 0.05% (20 s) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Virucidal activity was observed at 0.05% (1 min) in the evaluation study at Kitasato University.
- Benzalkonium chloride 0.05% (2 min) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Virucidal activity was observed at 0.05% (1 min) in the evaluation study at Kitasato University.

- Benzethonium chloride 0.05% (1 min) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Virucidal activity was observed at 0.05% (5 min) in the evaluation study at Kitasato University.
- Dialkyldimethylammonium chloride 0.01% (40 s) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Virucidal activity was observed at 0.01% (5 min) in the evaluation study at Kitasato University.
- Sodium percarbonate did not show virucidal activity at 1.0% (5 min) in the evaluation study at Kitasato University. This substance was evaluated only at Kitasato University.
- No cytotoxic effect was observed for each substance at the concentration described above.

Table 6. Summary of Results of the Evaluation Studies with SARS-CoV-2 (Surfactants, Quaternary Ammonium Salts and Sodium Percarbonate)

	Testing organization			
	NIID[1]		Kitasato University[2]	
	Reduction Rate of Virus Titer	Concentration (%)	Virucidal Activity	Concentration (%)
Anionic Surfactants				
Potassium Soap	99.999% or more	0.24	-	0.10
Sodium Soap	99.999% or more	0.22	-	0.10
Sodium Linear Alkylbenzene Sulfonates	99.999% or more	0.10	+	0.10
Sodium Alkyl Ether Sulfates	Less than 99.9%	0.20	-	
Nonionic Surfactants				
Alkyl Glycosides	99.999% or more	0.05	+	0.10
Fatty Acid Alkanol Amides	Less than 99.9%	0.20	-	0.10
Polyoxyethylene Alkyl Ether	99.999% or more	0.20	-	0.10
Amphoteric Surfactants				
Alkyl Amidopropyl Betaine	Less than 99.9%	0.50	-	0.10
Alkyldimethylamine Oxide	99.999% or more	0.05	+	0.05
Cationic Surfactants (Quaternary Ammonium Salts)				
Benzalkonium Chloride	99.999% or more	0.05	+	0.05
Benzethonium Chloride	99.999% or more	0.05	+	0.05
Dialkyldimethylammonium Chloride	99.99% or more	0.01	+	0.01
Oxygen Bleach				
Sodium Percarbonate	NT	NT	-	1.00

[1] The results of the evaluation study at NIID show percentage reduction rates of the virus infectious titer by $TCID_{50}$ calculation method.

[2] In the evaluation study at Kitasato University, when approximately 10,000 virus particles were almost completely inactivated (i.e., less than the detection limit), it was judged as "virucidal activity observed."

NT, Not tested; +, virucidal activity observed; -, virucidal activity not observed.

4-2. Results of the Evaluation Studies with SARS-CoV-2 – Hypochlorous Acid Water (Electrolytically-Generated and Non-Electrolytically-Generated) Except for Sodium Dichloroisocyanurate

- Hypochlorous acid water (HAW) (electrolytically-generated) from ACC 35 to 54 ppm (pH 2.4-5.9) showed a more than 99.9% reduction of viral titer in the reaction times from 20 s to 5 min in the evaluation study at NIID. On the other hand, the same type of HAW from ACC 19 to 26 ppm (pH 2.4-4.2) showed less than a 99.9% reduction of viral titer within the same reaction times.
- HAW (electrolytically-generated) of ACC 50 ppm (pH 5.0 and 6.0) did not show virucidal activity for the reaction times of 1 min and 5 min in the evaluation study at Kitasato University.
- HAW (electrolytically-generated) of ACC 32 ppm (pH 5.3) and 56 ppm (pH 2.5, pH 5.2) showed a more than 99.99% reduction of viral titer for the reaction times of 20 s, 1 min and 5 min in the evaluation study at OUAVM.
- HAW (non-electrolytically-generated) of ACC 50, 100, 150 and 200 ppm (pH 5.2-6.2) showed a more than 99.99% reduction of viral titer for the reaction times of 20 s, 1 min and 5 min in the evaluation study at OUAVM.
- HAW (non-electrolytically-generated) of ACC 100 ppm and 200 ppm (pH 6.0) showed a more than 99.9% reduction of viral titer for the reaction times of 20 s and 1 min in the evaluation study at Tottori University.
- HAW (non-electrolytically-generated) of ACC 51 ppm (pH 6.0) showed a more than 99.99% reduction of viral titer for the reaction times of 20 s and 1 min in the evaluation study at QTEC. On the other hand, the same type of HAW of ACC 27 ppm (pH 6.0) showed less than a 99.9% reduction of viral titer for the reaction times of 20 s and 1 min.
- No cytotoxic effect was observed for each substance at the concentration described above except for ACC 200 ppm of HAW (non-electrolytically-generated) in the evaluation study at OUAVM, at which slight cytotoxicity was observed in periphery of wells.

Table 7. Summary of Test Conditions of HAW in Participating Organizations.

	Organization				
	NIID[1]	Kitasato University[2]	OUAVM[1]	Tottori University[1]	QTEC[1]
Concentration of FBS for Preparation of Virus Stock	5%	2%	1%	5%	1%
Detection Method of Virus Titer	$TCID_{50}$	CPE Observation and qRT-PCR	$TCID_{50}$	$TCID_{50}$	$TCID_{50}$
Volume Ratio of Virus Stock to Test Substance	1:19	1:9	1:19	1:19	1:19

[1] The results of the evaluation studies by these organizations show percentage reduction rates of the virus infectious titer by$TCID_{50}$ calculation method.

[2] In the evaluation study at Kitasato University, when approximately 10,000 virus particles were almost completely inactivated (i.e., less than the detection limit), it was judged as "virucidal activity observed."

4-3. Summary of the Evaluation Studies with SARS-CoV-2 – Hypochlorous Acid Water (Electrolytically-Generated and Non-Electrolytically-Generated) Except for Sodium Dichloroisocyanurate

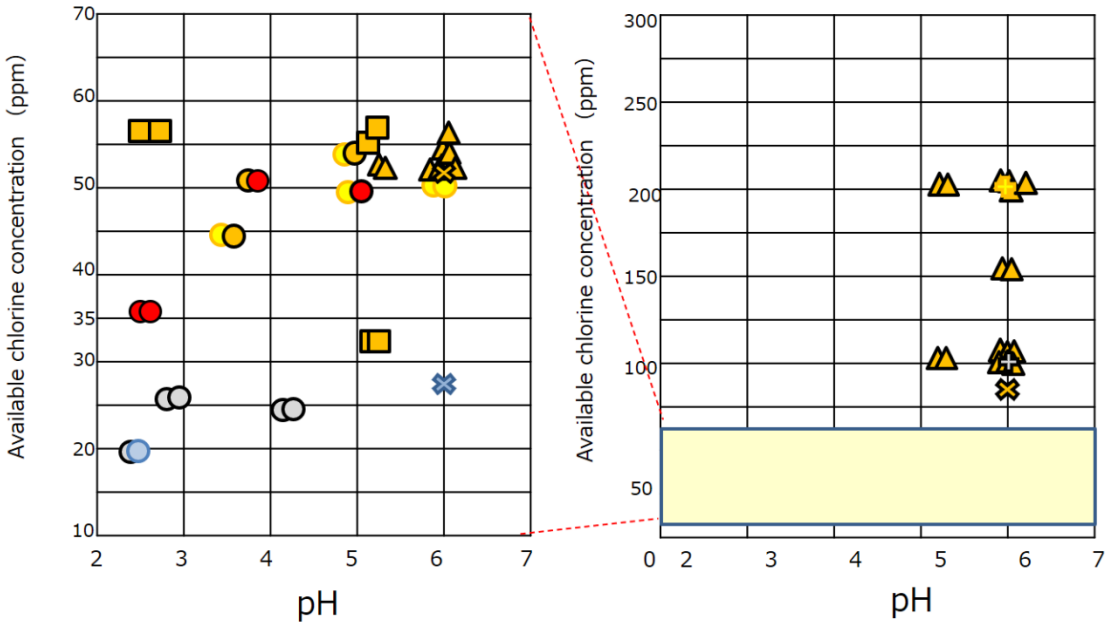


Figure 3. Virucidal Activities of HAW (Electrolytically-Generated and Non-Electrolytically-Generated) Except for Sodium Dichloroisocyanurate
All data for the reaction time of 20 s obtained by NIID, OUAVM, Tottori University and QTEC were plotted in the figure. Please note that the data for Kitasato University are for the reaction times of 1 min and 5 min.

○ Data of HAW (Electrolytically-Generated) at NIID
□ Data of HAW (Electrolytically-Generated) at OUAVM
△ Data of HAW (Non-Electrolytically-Generated) at OUAVM
✚ Data of HAW (Non-Electrolytically-Generated) at Tottori University
✖ Data of HAW (Non-Electrolytically-Generated) at QTEC

99.999% or more
99.99% or more
99.9% or more
99% or more
Less than 99%

Table 8. Summary of Test Conditions of HAW by Participating Organizations (Figure 3)

	Testing Organization				
	NIID	Kitasato University	OUAVM	Tottori University	QTEC
Concentration of FBS for Preparation of Virus Stock	5%	2%	1%	5%	1%
Ratio of Virus Stock to Test Substance	1:19	1:9	1:19	1:19	1:19

4-4. Summary of the Evaluation Studies with SARS-CoV-2 – Hypochlorous Acid Water (Electrolytically-Generated and Non-Electrolytically-Generated) Except for Sodium Dichloroisocyanurate at ACC of ACC 60 ppm or Less

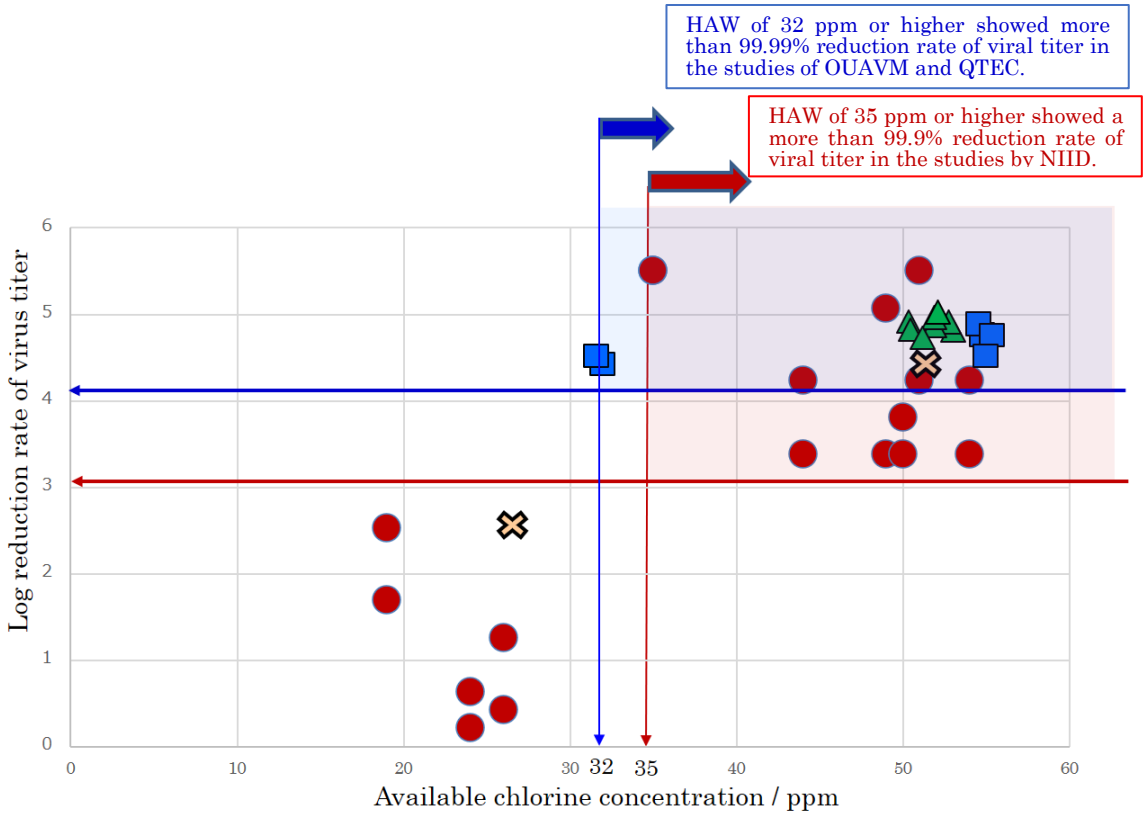


Figure 4. Virucidal Activities of HAW (Electrolytically-Generated and Non-Electrolytically-Generated) Except for Sodium Dichloroisocyanurate at ACC of 60 ppm or less.

● : Data of HAW (Electrolytically-Generated) at NIID
■ : Data of HAW (Electrolytically-Generated) at OUAVM
▲ : Data of HAW (Non-Electrolytically-Generated) at OUAVM
× : Data of HAW (Non-Electrolytically-Generated) at QTEC

4-5. Summary of the Evaluation studies with SARS-CoV-2 – Sodium Dichloroisocyanurate (NaDCC)

- While the NaDCC of ACC 200 ppm or higher showed a more than 99.999% reduction of viral titer at the reaction times of 20 s to 5 min in the evaluation study with a 1:9 volume ratio of virus to sample at NIID, the NaDCC of AC 100 ppm showed a more than 99.9% reduction of viral titer at the reaction times of 40 s to 5 min.
- The NaDCC of ACC 50 ppm or higher showed a more than 99.99% reduction of viral titer for the reaction times of 20 s, 1 min and 5 min in the evaluation study with a 1:19 volume ratio of virus to sample at OUAVM.

Please note: The Committee treated NaDCC as a substance categorized into HAW (non-electrolytically-generated), but the results of the NaDCC solution was herein described separately from HAW for the following reason. While an aqueous solution of NaDCC contains a certain amount of hypochloric acid, free chlorine is released from dichloroisocyanyrate by the dissociation equilibrium reaction. Thus, an aqueous solution of NaDCC has different properties from conventional HAW.

Table 9. Summary of the Test Conditions of Each Organization

	Organization	
	NIID	OUAVM
Concentration of FBS for Preparation of Virus Stock	5%	1%
Detection Method of Virus Titer	$TCID_{50}$	$TCID_{50}$
Volume Ratio of Virus Stock to Test Substance	1:9	1:19

5. Substances Judged to be Effective Against SARS-CoV-2

5-1. Substances Judged to be Effective Against SARS-CoV-2 – Surfactants, Quaternary Ammonium Salts and Sodium Percarbonate

◆ Criteria for Determining Efficacy

Virucidal efficacy of each surfactant, quaternary ammonium salt and sodium percarbonate substance was assessed based on the results of the evaluation studies. A substance was judged to be effective against SARS-CoV-2, when a more than 99.99% reduction of viral titer was shown by NIID and virucidal activity was also shown by Kitasato University. Even when positive results were shown only by either of these organizations, a conclusion was drawn after careful investigation of the results of the evaluation studies obtained by both of these organizations, as the potential for virucidal activity of such substances was still worth considering.

◆ Determination of Efficacy of the Tested Substances

- Potassium soap of 0.12% (5 min) and sodium soap of 0.11% (5 min) showed less than a 99% reduction of viral titer in the evaluation study at NIID. Potassium soap of 0.1% (5 min) and sodium soap of 0.1% (10 min) showed no virucidal activity in the evaluation study at Kitasato University. On the other hand, potassium soap of 0.24% (1 min) and sodium soap of 0.22% (1 min) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Thus, potassium soap of 0.24% or higher and sodium soap of 0.22% or higher were judged to be effective.
- Sodium linear alkylbenzene sulfonates of 0.1% (20 s) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Virucidal activity was also observed at 0.1% (5 min) in the evaluation study at Kitasato University. Thus, sodium linear alkylbenzene sulfonates of 0.1% or higher were judged to be effective.
- Sodium alkyl ether sulfates of 0.5% (5 min showed less than a 99.9% reduction of viral titer in the evaluation study at NIID. Virucidal activity was not observed at 0.1% (5 min) in the evaluation study at Kitasato University. Thus, sodium alkyl ether sulfates were not judged to be effective at a concentration of up to 0.5%.
- Alkyl glycosides of 0.05% (20 s) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Virucidal activity was also observed at 0.1% (1 min) in the evaluation study at Kitasato University. Thus, alkyl glycosides of 0.1% or higher were judged to be effective.
- Fatty acid alkanol amides of 0.2% (5 min) showed less than a 99.9% reduction of

viral titer in the evaluation study at NIID. Virucidal activity was not observed at 0.1% (5 min) in the evaluation study at Kitasato University. Thus, fatty acid alkanol amides were not judged to be effective at a concentration of up to 0.2%.

- While polyoxyethylene alkyl ether of 0.1% (5 min) showed a 99.99% reduction of viral titer in the evaluation study at NIID, virucidal activity was not observed at 0.1% (5 min) in the evaluation study at Kitasato University. On the other hand, the substance of 0.2% (5 min) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Thus, polyoxyethylene alkyl ether of 0.2% or higher was judged to be effective.
- Alkyl amidopropyl betaine of 0.5% (5 min) showed less than a 99.9% reduction of viral titer in the evaluation study at NIID. Virucidal activity was not observed at 0.1% (5 min) in the evaluation study at Kitasato University. Thus, alkyl amidopropyl betaine was not judged to be effective at a concentration of up to 0.5%.
- Alkyldimethylamine oxide of 0.05% (20 s) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Virucidal activity was also observed at 0.05% (1 min) in the evaluation study at Kitasato University. Thus, alkyldimethylamine oxide of 0.05% or higher was judged to be effective.
- Benzalkonium chloride of 0.05% (2 min) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Virucidal activity was also observed at 0.05% (1 min) in the evaluation study at Kitasato University. Thus, benzalkonium chloride of 0.05% or higher was judged to be effective.
- Dialkyldimethylammonium chloride of 0.01% (40 s) showed a more than 99.999% reduction of viral titer in the evaluation study at NIID. Virucidal activity was also observed at 0.01% (5 min) in the evaluation study at Kitasato University. Thus, dialkyldimethylammonium chloride of 0.01% or higher was judged to be effective.
- Sodium percarbonate was tested only at Kitasato University, and virucidal activity was not observed at 1.0% (5 min) in the evaluation study. Thus, sodium percarbonate was not judged to be effective at a concentration of up to 1.0%.

5-2. Summary of Substances Judged to be Effective Against SARS-CoV-2 and Precautions When Eliminating SARS-CoV-2 by Surfactants and Quaternary Ammonium Salts

Substances Judged to be Effective by the Results of the Evaluation Studies

- Sodium linear alkylbenzene sulfonates (0.1% or higher)
- Alkyl glycosides (0.1% or higher)
- Alkyldimethylamine oxide (0.05% or higher)
- Benzalkonium chloride (0.05% or higher)
- Benzethonium chloride (0.05% or higher)
- Dialkyldimethylammonium chloride (0.01% or higher)
- Polyoxyethylene alkyl ether (0.2% or higher)
- Potassium soap (0.24% or higher)
- Sodium soap (0.22% or higher)

  Please note: These results were obtained using the surfactant components themselves. These were not the results of household detergent products that contain these surfactants.

Precautions When Eliminating SARS-CoV-2 from Surfaces with Effective Surfactants

- ✧ Effective elimination of SARS-CoV-2 can be expected by the appropriate use of various types of household detergents containing effective surfactants. It is important to adhere to the instructions for the use of each product.
- ✧ Please note that this assessment verified the efficacy of certain surfactants for disinfection of surfaces against SARS-CoV-2, but was not designed to verify the effectiveness of surfactants for sanitization of the hands or skin.
- ✧ It is also important to pay attention to the safety information on household detergent products. Regarding safety, it is necessary to appropriately use these products in consideration of the safety information and precautions provided by the manufacturer etc.

5-3. Substances Judged to be Effective Against SARS-CoV-2 – Hypochlorous Acid Water (Electrolytically-Generated and Non-Electrolytically-Generated) Except for Sodium Dichloroisocyanurate (NaDCC)

◆ Criteria for Determining Efficacy

Virucidal efficacy of each hypochlorous acid water (HAW) substance was assessed based on the results of the evaluation studies. A HAW substance was judged to be effective against SARS-CoV-2, when a more than 99.99% reduction of viral titer was shown by four of the institutes (NIID, OUAVM, Tottori Univ. and QTEC) and virucidal activity also was shown by Kitasato University. Even when positive results were not obtained by two of the organizations, a conclusion was drawn after careful investigation of the results of the evaluation studies of all of the organizations because the potential for virucidal activity of such samples was still worth considering.

The Committee assumed that the two substances of HAW had the same virucidal activity regardless of their production process, if the available chlorine concentration (ACC) and pH were the same for the substances. Based on this idea, the same criteria for determining efficacy against SARS-CoV-2 were applied to both electrolytically-generated and non-electrolytically-generated HAW.

On the other hand, the Committee judged the efficacy of an NaDCC solution separately from HAW using the following rationale, although it categorized this substance as HAW (non-electrolytically-generated). While an aqueous solution of NaDCC contains a certain amount of hypochloric acid, free chlorine is released from dichloroisocyanyrate by the dissociation equilibrium reaction. Thus, the Committee assumes that an aqueous solution of NaDCC has different properties from conventional HAW.

◆ Determination of Efficacy of the Tested Substances

- HAW of an ACC of 32 ppm or higher demonstrated a more than 99.99% reduction of viral titer in the evaluation study at OUAVM, and HAW of an ACC 51 ppm or higher showed a more than 99.99% reduction of viral titer in the evaluation study at QTEC.
- HAW of an ACC of 35 ppm or higher showed a more than 99.9% reduction of viral titer in the evaluation study at NIID. The level of reduction of viral titer observed at NIID was somewhat different from the levels seen at OUAVM and QTEC. Although it was difficult to clearly identify a cause of this difference, it seemed likely that the composition of the virus stock solution and/or the content of the organic matter in the reaction solution had affected the level reduction of viral titer.

- HAW of an ACC of 50 ppm did not show any virucidal activity in the evaluation study at Kitasato University. The volume ratio of virus stock to the tested sample in the reaction solution was 1:9 in this study. Furthermore, the presence of virucidal activity could be judged in the study at Kitasato University only when approximately 10,000 virus particles were almost completely inactivated (i.e., less than the detection limit) by cytopathic effect and qRT-PCR. Lack of virucidal activity may have been due to these harsh conditions.
- In the study at OUAVM, virucidal activity of HAW of an ACC of 52 ppm was attenuated up to one-tenth, when the volume ratio of virus stock to tested sample in the reaction solution was changed from 1:19 to 1:9. Furthermore, virucidal activity of HAW of an ACC of 51 ppm was drastically attenuated up to one thousandth in the study at QTEC, when it was changed from 1:19 to 1:9. These results indicate that the virucidal effect was greatly influenced by the volume ratio of the virus solution and HAW in the reaction solution. These results offer an important suggestion as to the proper way to use HAW, particularly that at a low ACC concentration, for effective elimination of the virus.
- On the other hand, virucidal activity of HAW of an ACC of 200 ppm was maintained, even when the volume ratio was changed from 1:19 to 1:9 in the study at Tottori University (under the condition of 5% FBS in the virus stock solution). Virucidal activity of HAW of an ACC of 84 ppm was also maintained between these two volume ratios in the study at QTEC (under the condition of 1% FBS in the virus stock solution). From these results, together with the results of HAW at a lower ACC, it was suggested that HAW at a higher ACC would be able to maintain the virucidal effect even under more strict conditions (i.e., at a volume ratio of 1:9).
- It was reported that the activity of hypochlorous acid was attenuated after it bound to an organic matter such as a protein (Appendix 5, Ref. No. 17-21). The same tendency seemed to be observed in these evaluation studies. This observation also offered an important suggestion as to the proper way to use HAW for the effective elimination of the virus.
- The criterion for determining efficacy was basically set at a 99.99% or more reduction of viral titer in this series of evaluation studies. On the other hand, it was revealed though a literature search that the criteria for virucidal effects were set at 99.9% to 99.99% in various guidelines, depending on their purpose or use conditions. Considering these circumstances, it was still worthwhile to take a 99.9% reduction of viral titer into account as a feasible criterion for determining efficacy in the evaluation study at NIID.

- Based on these results, the Committee judged that HAW of an ACC of 35 ppm or higher was effective, which showed a more than 99.9% reduction of viral titer in the evaluation study at NIID as well as a more than 99.99% reduction in the studies at OUAVM and QTEC.

5-4. Substances Judged to be Effective Against SARS-CoV-2 – Sodium Dichloroisocyanurate (NaDCC)

◆ Determination of Efficacy of the Tested Substance

- Basically, the same criteria were applied as those for HAW in determining the efficacy of NaDCC. NaDCC of an ACC of 50 ppm or higher, which showed a more than 99.99% reduction of viral titer in the evaluation study at OUAVM. On the other hand, NaDCC of an ACC of 200 ppm or higher showed a more than 99.999% reduction, but NaDCC of an ACC of 100 ppm showed a more than 99.9% reduction in the evaluation study at NIID. It should be taken into account that NaDCC of an ACC of 100 ppm showed a significant effect in the study conditions at NIID, which were harsher than those at OUAVM.
- Based on these results, the Committee judged that NaDCC of an ACC of 100 ppm or higher was effective, which showed a more than 99.9% reduction of viral titer in the evaluation study at NIID, as well as a more than 99.99% reduction in the study at OUAVM.

5-5. Summary of Substances Judged to be Effective Against SARS-CoV-2 and Precautions When Eliminating SARS-CoV-2 by Hypochlorous Acid Water

Substances Judged to be Effective by the Results of the Evaluation Studies

- The following ACC ranges of HAW and the NaDCC solution were judged to be effective among the samples of hypochlorous acid water (pH 6.5 or less) and the NaDCC solution tested in the evaluation studies.
  - For HAW (electrolytically-generated and non-electrolytically-generated), an ACC of 35 ppm or higher.
  - For an NaDCC solution, an ACC of 100 ppm or higher.

Please note that the Committee judged the efficacy of the NaDCC solution separately from HAW by the following rationale, although NaDCC was initially categorized as a HAW (non-electrolytically-generated). While an aqueous solution of NaDCC contains a certain amount of hypochloric acid, free chlorine is released from dichloroisocyanyrate by the dissociation equilibrium reaction. Thus, the Committee assumes that an aqueous solution of NaDCC has different properties from conventional HAW.

Precautions when Eliminating SARS-CoV-2 from Surfaces with Effective HAW, Including an NaDCC Solution

- Elimination of SARS-CoV-2 can be expected with the appropriate use of an effective HAW.
- Based on the characteristics of HAW, as well as the results of the series of evaluation studies, the recommended use of effective HAW for virus elimination are described as follows:
  - Remove dirt, such as organic matter (hand stains etc.), in advance.
  - Use an enough amount of HAW to eliminate the virus on the surface.
- Please note that this assessment verified the efficiency of HAW within a certain range of ACC for the disinfection of surfaces against SARS-CoV-2, but was not designed to verify the effectiveness of HAW for the sanitization of hands or skin.
- It is also important to pay attention to the safety information on HAW products. Regarding safety, it is necessary to appropriately use these products in consideration of the safety information and precautions provided by the manufacturer etc.

(Reference)

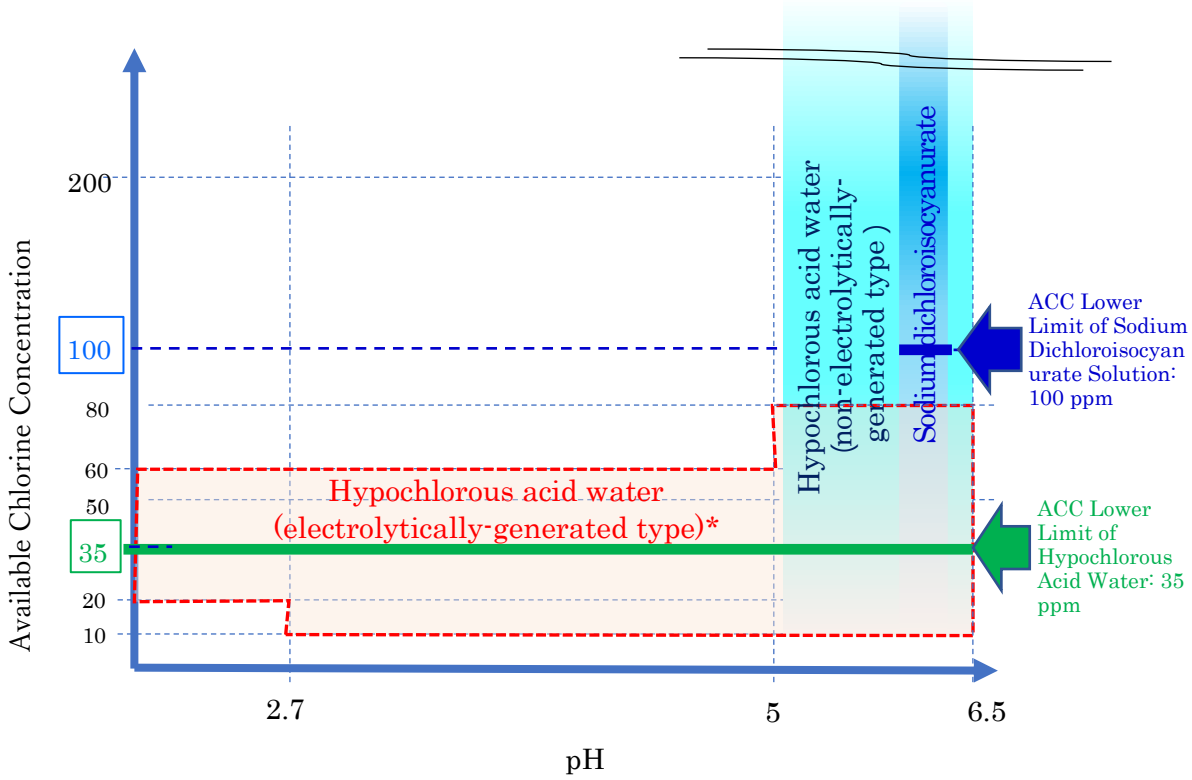


Figure 5. Tested Substances of Hypochlorous acid water (HAW) and Sodium Dichloroisocyanurate (NaDCC) Solution in the Evaluation Studies and their ACC Ranges Judged to be Effective
(See Table 4 for HAW Substances Tested in the Evaluation Studies.)

*Please note that the ranges of pH and ACC plotted for HAW (electrically-generated type) correspond to the ranges set forth by Japan's specifications and standards for food additives.

Important Reminder About the Efficacy Assessment Conducted by the Committee

The Committee judged efficacy of the candidate substances against SARS-CoV-2 based on the criterion set at a 99.99% (i.e., 4 log) or more reduction of viral titer. However, in general criteria for the evaluation of virucidal efficacy set by various guidelines are not necessarily limited to 99.99%, and such criteria seem to have been set based on their purpose or use conditions. Considering these circumstances, one can argue that the efficacy criterion (i.e., a 99.99% or more reduction) set by the Committee in the evaluation studies is somewhat strict. In short, this can be considered as the one with more certainty of virucidal effects. Since the actual virucidal effects are exerted not only by the virucidal potential of these substances but also by their proper use according to their properties, the Committee agrees that the use of the above-mentioned strict criterion leaves a certain margin.

Given the above background, even though a certain substance was not judged to be effective by the Committee against SARS-CoV-2 due to the lack of efficacy in a particular condition (e.g., in a certain concentration) of this series of evaluation studies, this does not necessarily imply denial of virucidal efficacy of the substance. Please note that the efficacy of substances was affected by the study conditions of the evaluation study.

In response to an emergency, the Committee conducted this efficacy assessment for several candidate substances as described above within a limited time frame and with limited resources. In other words, the Committee could not carry out the evaluation of many other substances that could be expected to have a virucidal effect against SARS-CoV-2. Although these substances fell outside of the scope of the evaluation, this does not imply that the Committee has made any determination on an efficacy of these substances against SARS-CoV-2.

The results of the efficacy assessment by the Committee are intended to be used for public relations activities to facilitate public understanding for the presence of alternative substances to alcohol to use for disinfection against SARS-CoV-2 and to encourage a better use of these substances for elimination of SARS-CoV-2 from surfaces in households and community settings.

This assessment was not conducted in accordance with the Act on Securing Quality, Efficacy and Safety of Products Including Pharmaceuticals and Medical Devices (Pharmaceutical Affairs Law), the Food Sanitation Act or any other relevant Japanese laws and regulations.

Closing Remarks

In response to the request by METI under emergency conditions, NITE urgently established and has operated the Committee for the purpose of efficacy assessment of disinfectant substances alternative to alcohol for the use against SARS-CoV-2 as part of the government's countermeasures against the spread of COVID-19. All evaluation studies have been made with the latest information obtained to the extent possible as of June 2020. We greatly expect that further research and evaluations will proceed and that new knowledge will be accumulated in this field.

There were many substances that could not be evaluated due to limited time and resources. We do hope that the results of these evaluation studies can be used for future studies of these substances by other research institutes and the private sector. We would be greatly pleased if the efforts made by the Committee helped prevent the spread of SARS-CoV-2 infection and continued to promote public health.

We would also like to express our deepest gratitude to all the Committee members, institutions and universities that participated in these studies, as well as everyone who contributed to these studies despite the limited time and resources.

Appendix

Testing Protocol of Each Organization

Outline of Testing protocol with SARS-CoV-2

- National Institute of Infectious Disease (NIID) -

- Preparation of virus stock
  - Medium: DMEM + 5% FBS
- Dilution of test substance
  - Surfactant：Diluted with DDW（final concentration: variable)
  - Sodium dichloroisocyanurate: Diluted with DDW（final concentration: 300, 200 and 100 ppm)
  - Hypochlorous acid water; electrolytically-generated type: No dilution required
- Antiviral reaction
  - Surfactant: 1 part of virus + 1 part of test substance solution
  - Sodium dichloroisocyanurate: 1 part of virus + 9 parts of test substance solution
  - Hypochlorous acid water; electrolytically-generated type: 1 part of virus + 19 parts of test substance
  - Reaction Time: 20 s, 40 s, 60 s, 2 min and 5 min.
  - Temperature: room temperature (RT)
- Neutralization of test substance
  - Surfactant: Diluted with equal volume of DMEM + 5% FBS and removed with resin
  - Sodium dichloroisocyanurate: Diluted with 7x volume of DMEM + 5% FBS + 0.01 M $Na_2S_2O_3$ and removed with resin
  - Hypochlorous acid water; electrolytically-generated type: Diluted with 7x volume of DMEM + 5% FBS + 0.01 M $Na_2S_2O_3$.
- Calculation of results
  - $TCID_{50}$ method
  - Positive Control: None
  - Negative Control: Culture medium

Outline of Testing protocol with SARS-CoV-2

- National Institute of Infectious Disease (NIID) - (continued)

Case of surfactant

1 part of virus + 1 part of test substance
(e.g. 80 µl of virus stock (7 x 10^6 $TCID_{50}$/80 µl) + 80 µl of test substance solution in a test tube)

↓

Stand at RT （20 s, 40 s, 60 s, 2 min and 5 min)

↓

Mix with 6x volume of medium (DMEM + 5% FBS) with resin to remove surfactant (= 7^{-1} dilution)
(e.g. Collect 20 µl of the reaction mixture and transfer it to 120 µl of medium with 100 µl bed volume resin (Bio-rad BioBeads SM-2))

↓

Stand at RT for 5 min

↓

Serial 7-fold dilutions with medium (ex, 7^{-2} – 7^{-8})

↓

$TCID_{50}$ is calculated

Case of hypochlorous acid water; electrolytically-generated

1 part of virus + 19 parts of test substance
(e.g. 20 µl of virus stock (3 x 10^6 $TCID_{50}$/20 µl) + 380 µl of test substance in a test tube)

↓

Stand at RT （20 s, 40 s, 60 s, 2 min and 5 min)

↓

Mix with 6x volume of medium (DMEM + 5% FBS + 0.01 M $Na_2S_2O_3$) to neutralize/stop reaction (= 7^{-1} dilution)
(e.g. Add 20 µl of the reaction mixture to 120 µl of medium in a well of titer plate.)

↓

Serial 7-fold dilutions with DMEM + 5% FBS
(ex, 7^{-2} – 7^{-8})

↓

$TCID_{50}$ is calculated

Outline of Testing protocol with SARS-CoV-2

- Kitasato University -

Confirmation of cytotoxicity of test substances and determination of quenching condition

- Prepare 10 times, 100 times, 1,000 times, and 10,000 times dilutions of test substances beforehand and confirm the cytotoxic effect on host cells.
- Determine the dilution ratio (quenching conditions) of the substance to be contacted with the virus stock solution. Avoid the use of dilution ratios indicating cytotoxicity.

Method

- On the day of the test, prepare 96 well plates so that Vero E6/TMPRSS2 cells are 90% confluent. Immediately before the test, wash the plates with serum-free DMEM three times and add 180 µl of DMEM + 2% FBS to each well.
- Add 27 µl of diluted test substance to each well of another empty 96 well plates.
- Put 3 µl of virus stock of SARS-CoV-2 (> 1 x 10^4 pfu/µl) into each well using a multi-pipette and leave for 1 min and 5 min at RT (antiviral reaction step).
- After the reaction, add 270 µl of DMEM + 2% FBS as a stopping solution and pipette 5 times immediately (quenching step).
- Take 20 µl aliquots from each well and transfer to wells (14 wells/one antiviral reaction) of host cell culture plates containing 180 µl medium in advance. Place 1 hour in CO_2 incubator at 37℃.
- Wash cells twice with DMEM + 2% FBS.
- Add 200 µl of DMEM + 2% FBS and place in CO_2 incubator at 37℃.
- Immediately after the addition of the medium (Day 0), and 1 day, 2 days and 3 days after the start of culture, observe the cytopathic effect (CPE).
- On the 3rd day, examine all wells with qRT-PCR (TOYOBO SARS-CoV-2 Detection Kit) and measure SARS-CoV-2 RNA titer.

Outline of Testing protocol with SARS-CoV-2

- Kitasato University –
(continued)

Evaluation and judgment of disinfection effect

- If at least one of the 14 wells has CPE confirmed by the 3rd day and an increase in RNA titer in the well is confirmed, the disinfecting effect of the substance is judged to be null.
- If no increase in RNA titer is observed even if CPE is confirmed, the well is excluded from the evaluation target as this CPE is due to cytotoxicity.
- Only when CPE was not observed in all 14 wells and RNA titer was not increased, it is judged that the disinfection effect is present.
- This judgment means that about 30,000 infectious viruses can be almost completely disinfected (below the detection limit) by the tested substance.

- Please note that the number of infectious virus particles included in one cough droplet is assumed to be 10,000 according to the measured values of patient samples

- Positive control, 70v/v% ethanol
- Negative control, DDW

Outline of Testing protocol with SARS-CoV-2

- Obihiro University of Agriculture and Veterinary Medicine (OUAVM) -

- Preparation of virus stock
  - Medium: DMEM + 1% FBS
- Dilution of test substance (Hypochlorous acid water)
  - Electrolytically-generated type: No dilution required
  - Non-electrolytically-generated type: diluted with DDW (final concentration: 200, 100 and 50 ppm)
- Antiviral reaction
  - 1 part of virus stock + 9 parts of test substance or 1 part of virus stock + 19 parts of test substance
  - Reaction Time: 20 s, 60 s and 5 min
  - Temperature: Room temperature (RT)
- Neutralization of test substance
  - Diluted with dilution buffer (DMEM + 1% FBS + 0.01 M $Na_2S_2O_3$)
- Calculation of results
  - $TCID_{50}$ method
  - Positive Control: 70v/v% ethanol
  - Negative Control: DDW

Outline of Testing protocol with SARS-CoV-2

- Obihiro University of Agriculture and Veterinary Medicine (OUAVM) - (continued)

Case of the ratio 1:9

1 part of virus stock + 9 parts of test substance (e.g. 10 µl of virus stock (5 x 10^6 $TCID_{50}$/ml) + 90 µl of test substance in a test tube)

↓

Incubate at RT (20 s, 60 s and 5 min)

↓

Mix with 9x volume of dilution buffer (DMEM + 1% FBS + 0.01 M $Na_2S_2O_3$) to neutralize/stop reaction (= 10^{-1} dilution)
(e.g. Add 20 µl of the reaction mixture to 180 µl of dilution buffer in cell-seeding 96 well plate)

↓

Serial 10-fold dilutions with dilution buffer (ex, 10^{-2} – 10^{-5})

↓

$TCID_{50}$ is calculated

Case of the ratio 1:19

1 part of virus stock + 9 parts of test substance (e.g. 10 µl of virus stock (5 x 10^6 $TCID_{50}$/ml) + 190 µl of test substance in a test tube)

↓

Incubate at RT (20 s, 60 s and 5 min)

↓

Mix with 9x volume of dilution buffer to neutralize/stop reaction (= 10^{-1} dilution)
(e.g. Add 20 µl of the reaction mixture to 180 µl of dilution buffer in cell-seeding 96 well plate)

↓

Serial 10-fold dilutions with dilution buffer (ex, 10^{-2} – 10^{-5})

↓

$TCID_{50}$ is calculated

Outline of Testing protocol with SARS-CoV-2

- Tottori University -

- Preparation of virus stock
  - Medium: DMEM + 5% FBS
- Dilution of test substance
  - Diluted with DDW（final concentration: 200 and 100 ppm）
- Antiviral reaction
  - 1 part of virus + 9 parts of test substance or 1 part of virus + 19 parts of test substance
  - Reaction Time:　20 s and 60 s
  - Temperature: Room temperature (RT)
- Neutralization of test substance
  - Add 0.02x volume of 0.3 M $Na_2S_2O_3$ solution
- Calculation of results
  - $TCID_{50}$ method
  - Positive Control: None
  - Negative Control: PBS buffer

Outline of Testing protocol with SARS-CoV-2

- Tottori University -

(continued)

Case of the ratio 1:9

1 part of virus + 9 parts of test substance
(e.g. 50 µl of virus stock (6 x 10^6 $TCID_{50}$/ml) + 450 µl of test substance in a test tube)

Incubate at RT (20 s and 60 s)

Mix with 0.02x volume of 0.3 M $Na_2S_2O_3$ solution to neutralize/stop reaction (= 10^0 dilution)
(e.g. Add 10 µl of 0.3 M $Na_2S_2O_3$ solution to the reaction mixture in the tube)

Serial 10-fold dilutions with dilution buffer[a] (ex, 10^{-1} – 10^{-6})

$TCID_{50}$ is calculated

Case of the ratio 1:19

1 part of virus + 9 parts of test substance
(e.g. 25 µl of virus stock (6 x 10^6 $TCID_{50}$/ml) + 475 µl of test substance in a test tube)

Incubate at RT (20 s and 60 s)

Mix with 0.02x volume of 0.3 M $Na_2S_2O_3$ solution to neutralize/stop reaction (= 10^0 dilution)
(e.g. Add 10 µl of 0.1 M $Na_2S_2O_3$ solution to the reaction mixture in the tube)

Serial 10-fold dilutions with dilution buffer[a] (ex, 10^{-1} – 10^{-6})

$TCID_{50}$ is calculated

[a], 1 ml of 5% FBS + 19 ml of PBS + 400 µl of 0.3 M $Na_2S_2O_3$ solution

Outline of Testing protocol with SARS-CoV-2

- Japan Textile Products Quality and Technology Center（QTEC）-

- Preparation of virus stock
  - Medium: DMEM + 1% FBS
- Dilution of test substance
  - Diluted with DDW（final concentration: 80, 50, 25 ppm)
- Antiviral reaction
  - 1 part of virus + 9 parts of test substance or 1 part of virus + 19 parts of test substance
  - Reaction Time:　20 s and 60 s
  - Temperature: Room temperature (RT)
- Neutralization of test substance
  - add 0.1x volume of 0.1 M $Na_2S_2O_3$ solution, 0.1x volume of 10-fold EMEM medium and 0.05x volume of FBS (final concentration: 2% FBS).
- Calculation of results
  - $TCID_{50}$ method
  - Positive Control: 70 w/v% ethanol
  - Negative Control: PBS buffer

Outline of Testing protocol with SARS-CoV-2

- Japan Textile Products Quality and Technology Center（QTEC） - (continued)

Case of the ratio 1:9

1 part of virus + 9 parts of test substance
(e.g. 100 µl of virus stock (1 x 10^7 $TCID_{50}$/ml) + 900 µl of test substance in a test tube)

↓

Contact at RT（20 s and 60 s)

↓

Mix with 0.1x volume of 0.1 M $Na_2S_2O_3$ solution to neutralize/stop reaction,and add 0.1x volume of 10-fold EMEM and 0.02x volume of FBS (final concentration: 2% FBS) (= 10^0 dilution)
(e.g. Add 110 µl of 0.1 M $Na_2S_2O_3$ solution to the reaction mixture in the tube, and add 123 ul of 10-fold EMEM and 25 µl of FBS)

↓

Serial 10-fold dilutions with EMEM + 2% FBS (ex, 10^{-1} – 10^{-6})

↓

$TCID_{50}$ is calculated

Case of the ratio 1:19

1 part of virus + 9 parts of test substance
(e.g. 100 µl of virus stock (1 x 10^7 $TCID_{50}$/ml) + 1,900 µl of test substance in a test tube)

↓

Contact at RT（20 s and 60 s)

↓

Mix with 0.1x volume of 0.1 M $Na_2S_2O_3$ solution to neutralize/stop reaction and add 0.1x volume of 10-fold EMEM and 0.02x volume of FBS (final concentration: 2% FBS) (= 10^0 dilution)
(e.g. Add 220 µl of 0.1 M $Na_2S_2O_3$ solution to the reaction mixture in the tube, and add 246 µl of 10-fold EMEM and 50 µl of FBS)

↓

Serial 10-fold dilutions with EMEM + 2% FBS (ex, 10^{-1} – 10^{-6})

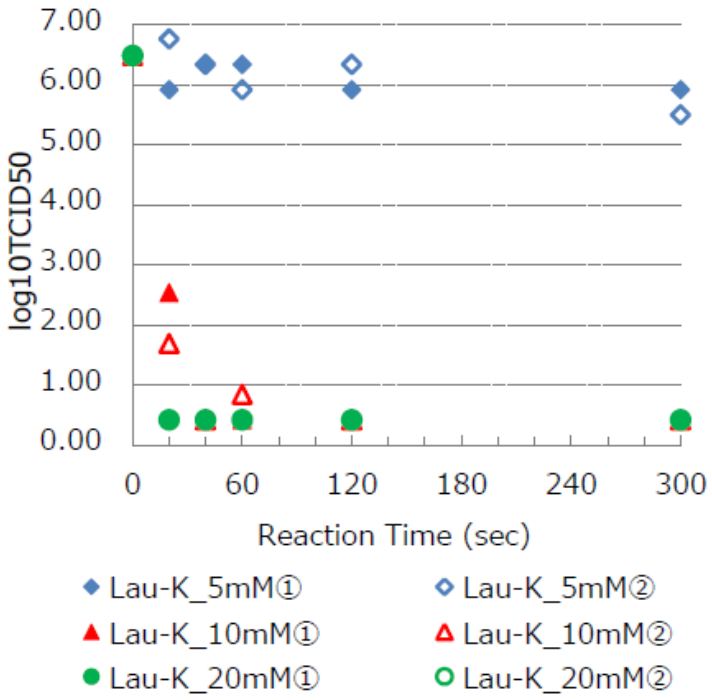
↓

$TCID_{50}$ is calculated

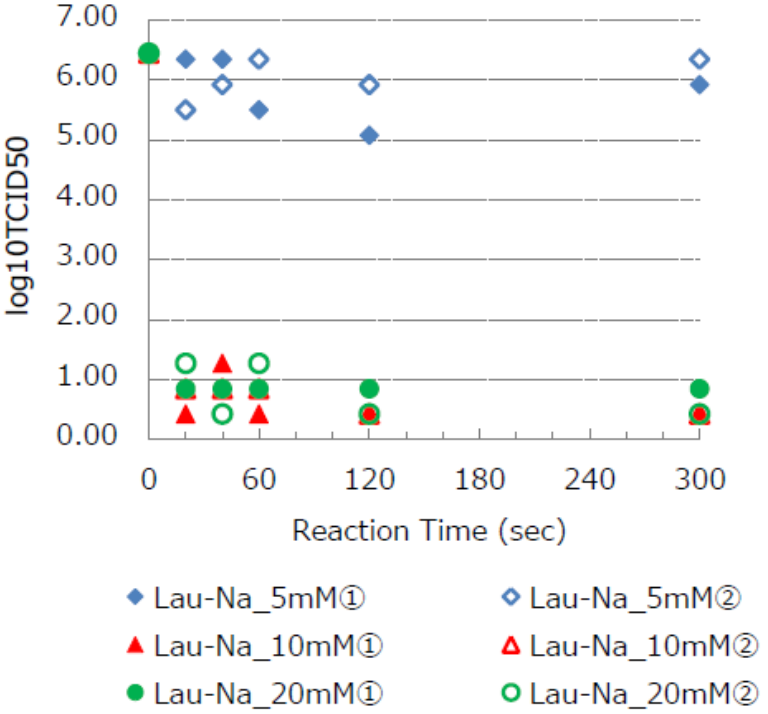
The Results of Surfactants

Anionic Surfactant: Potassium Soap & Sodium Soap

Disinfection of SARS-CoV-2
by Potassium Soap
(Potassium Laurate (Lau-K))



Disinfection of SARS-CoV-2
by Sodium Soap
(Sodium Laurate(Lau-Na))

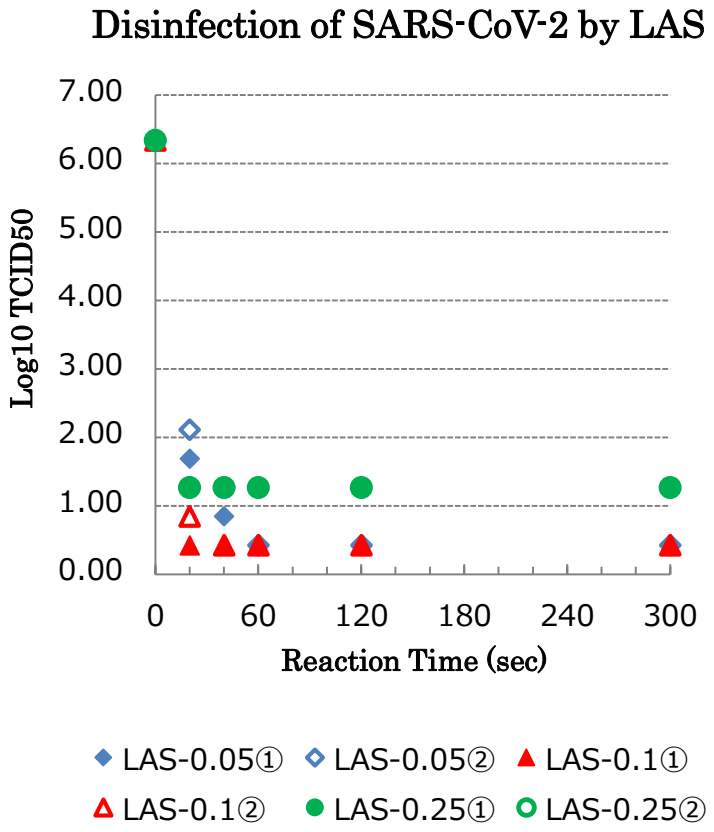


The concentrations of the component (Lau–K) calculated from the purity of the purchased reagent are as follows: 5 mM, 0.12%; 10 mM, 0.24%; and 20 mM, 0.48%.

The volume ratio of virus suspension to anionic surfactant solution is 1:1 (N=2).

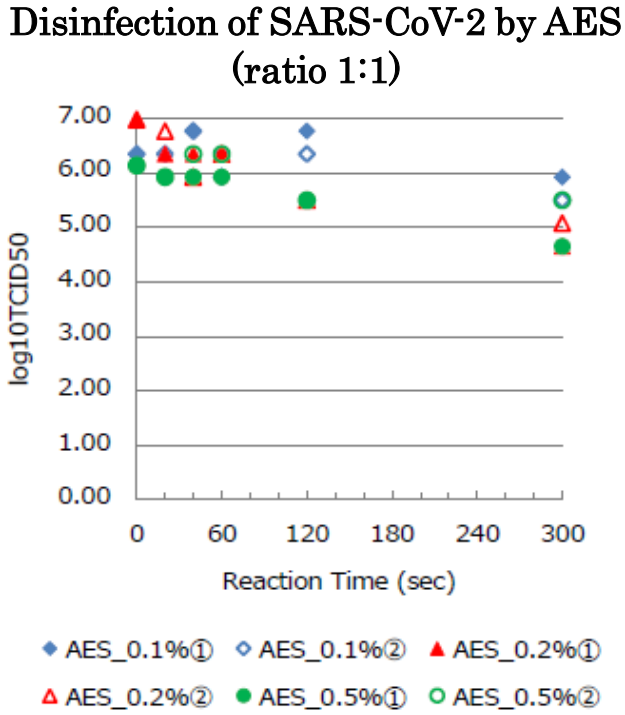
The concentrations of the component (Lau-Na) calculated from the purity of the purchased reagent are as follows: 5 mM, 0.11%; 10 mM, 0.22%; and 20 mM, 0.44%.

Anionic Surfactant: Sodium Linear Alkylbenzenesulfonates (LAS)



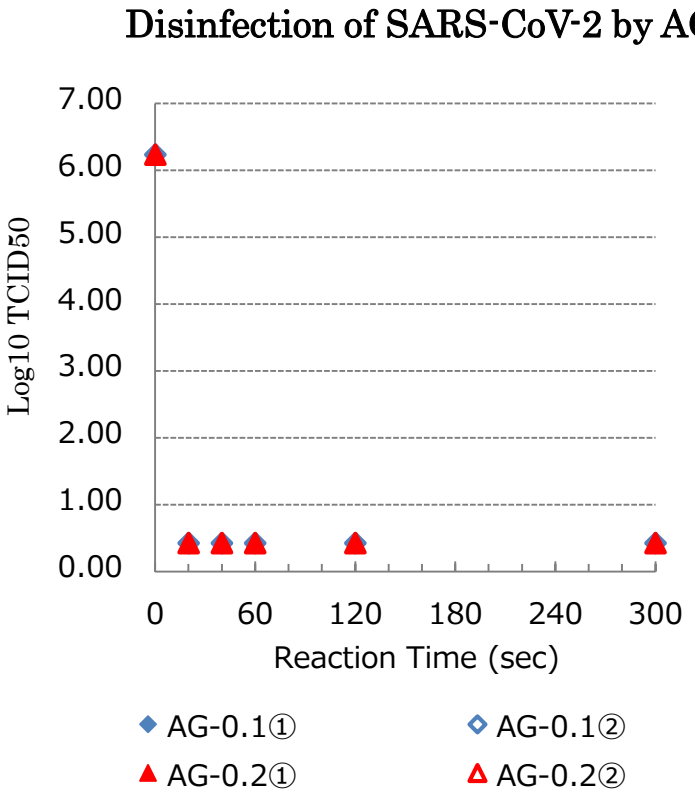
The volume ratio of virus suspension to anionic surfactant solution is 1:1 (N=2).

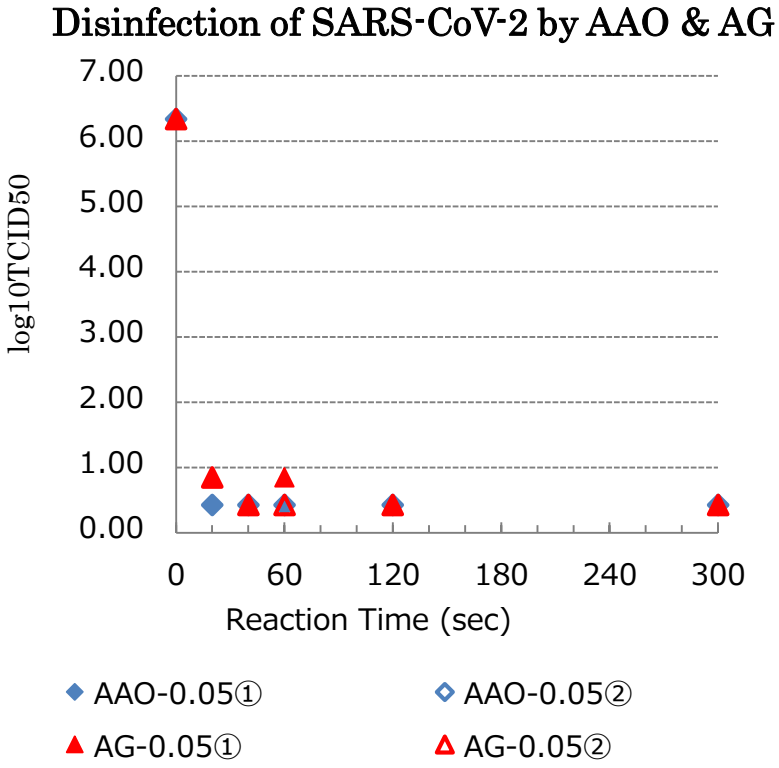
Anionic Surfactant: Sodium Alkyl Ether Sulfates (AES)



The volume ratio of virus suspension to anionic surfactant solution is 1:1 (N=2).

Nonionic Surfactant: Alkyl Glycosides (AG)

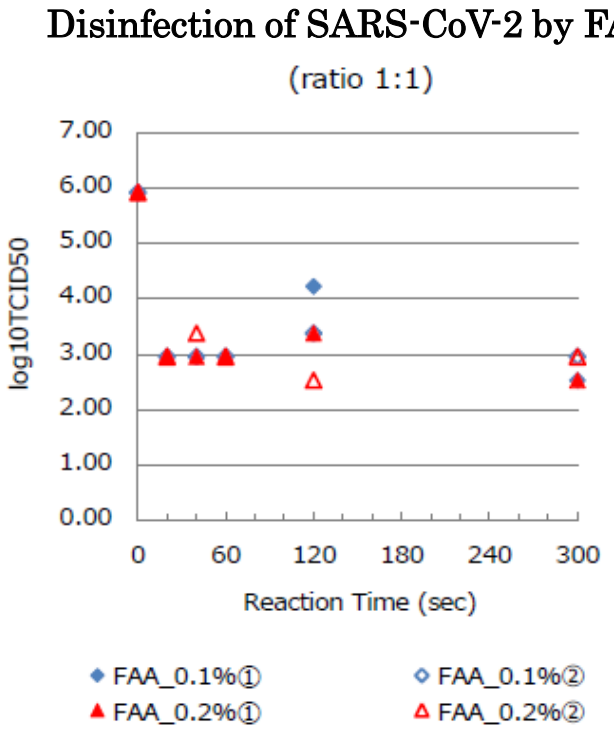




AAO: Alkyldimethylamine Oxide (See page 20)

The volume ratio of virus suspension to nonionic surfactant solution is 1:1 (N=2).

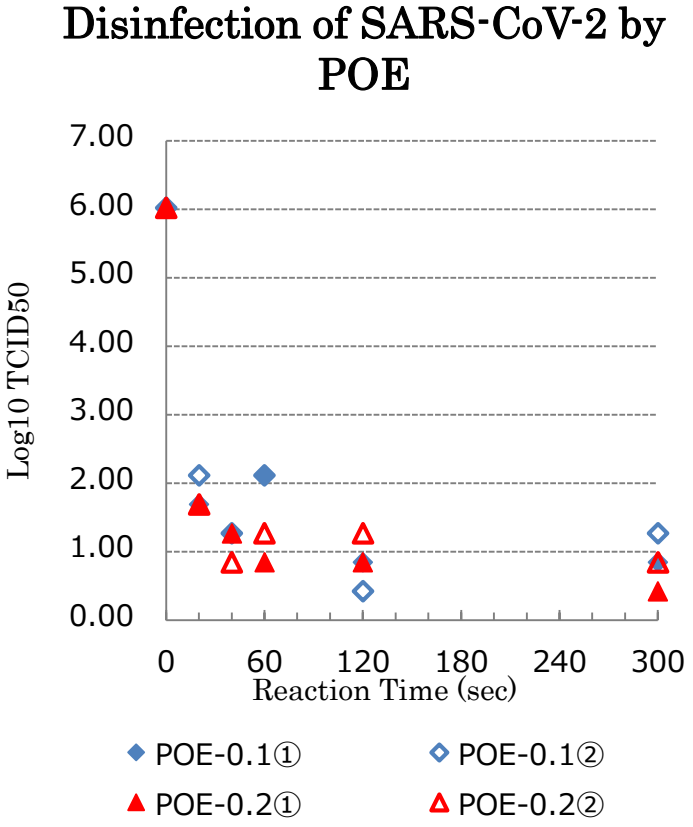
Nonionic Surfactant: Fatty Acid Alkanol Amides (FAA)



The test at 0.5% of FAA failed because of the adverse effect on the test system by the surfactant.

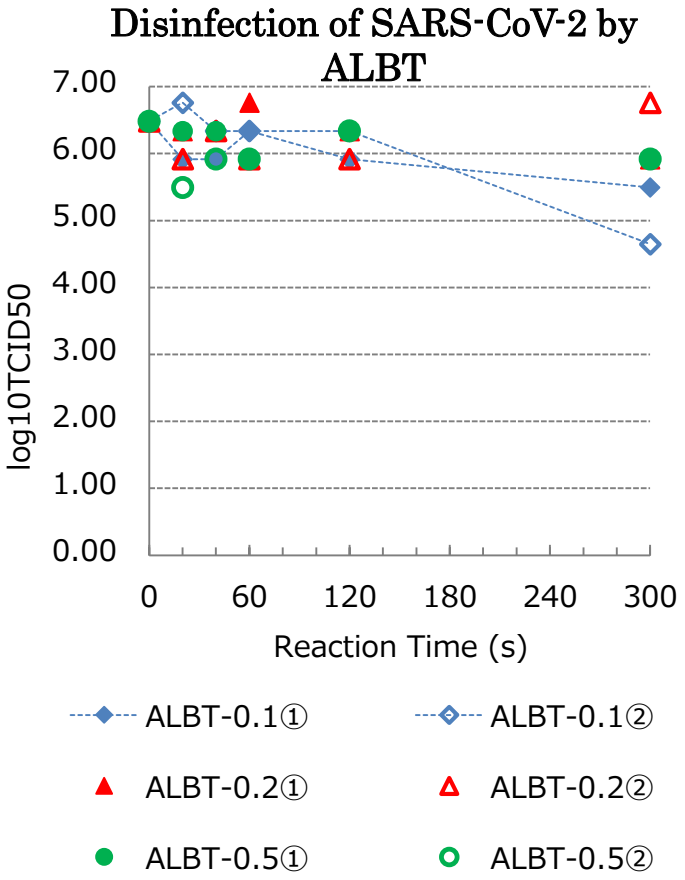
The volume ratio of virus suspension to nonionic surfactant solution is 1:1 (N=2).

Nonionic Surfactant: Polyoxyethylene Alkyl Ether (POE)



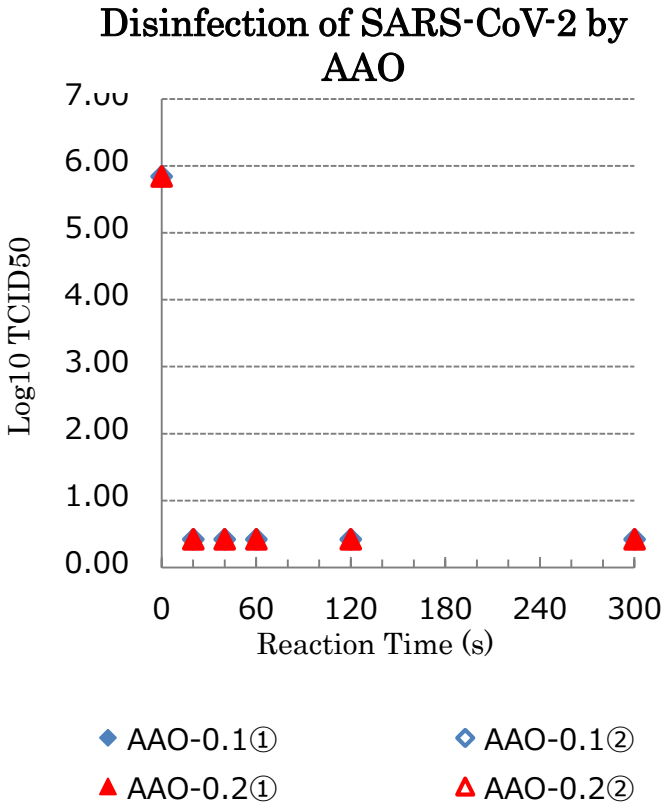
The volume ratio of virus suspension to nonionic surfactant solution is 1:1 (N=2).

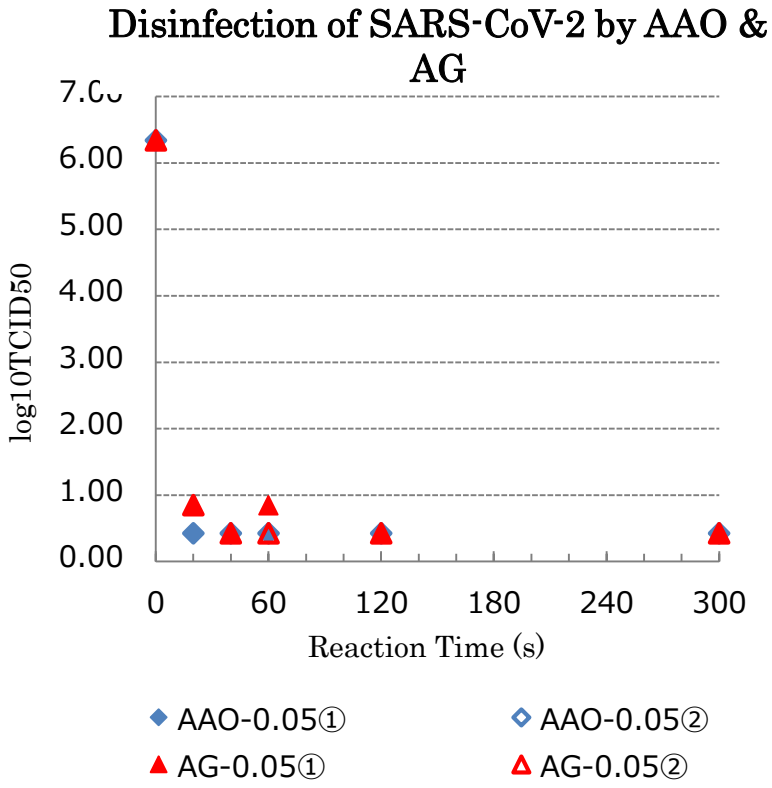
Amphoteric Surfactant: Alkyl amidopropyl betaine (ALBT)



The volume ratio of virus suspension to amphoteric surfactant solution is 1:1 (N=2).

Amphoteric Surfactant: Alkyldimethylamine Oxide (AAO)

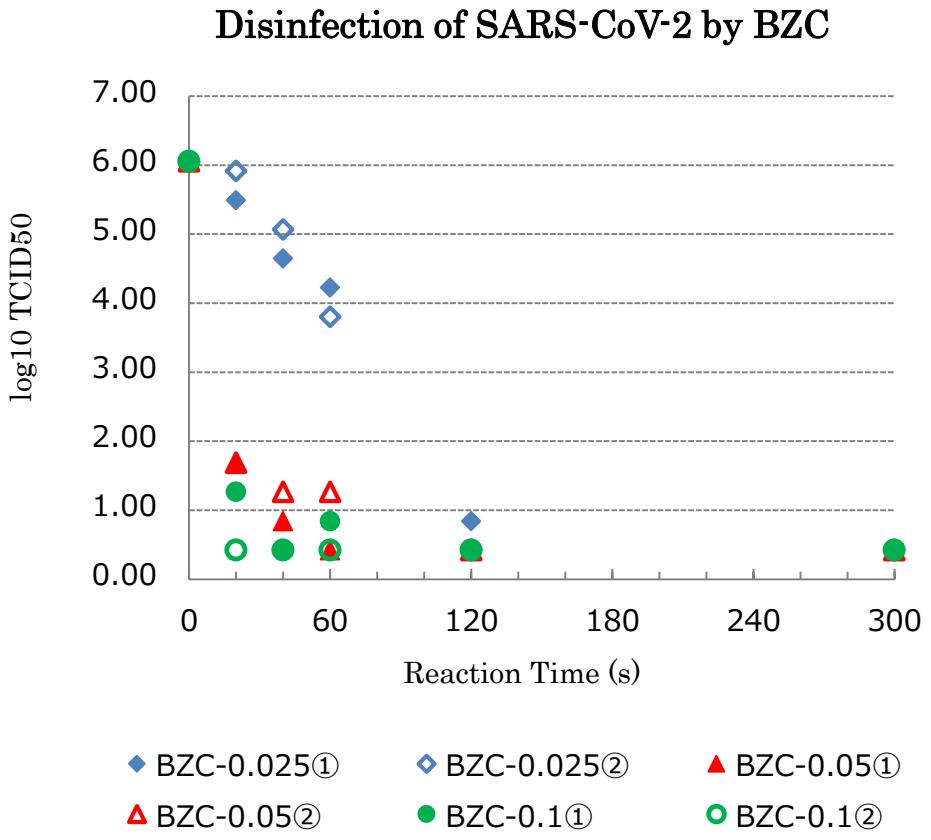




AG: Alkyl Glycosides (See page 16)

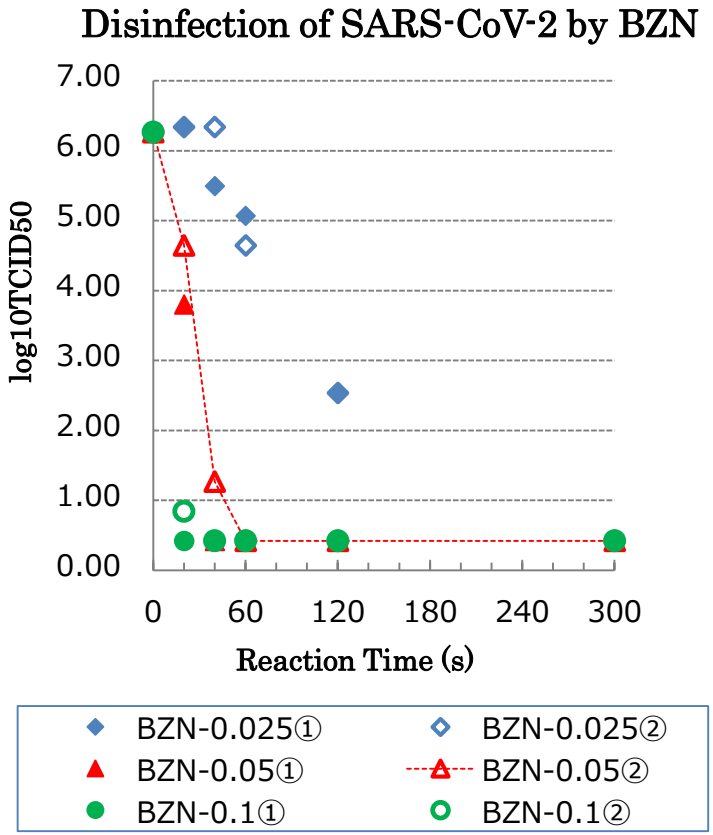
The volume ratio of virus suspension to amphoteric surfactant solution is 1:1 (N=2).

Cationic Surfactant: Benzalkonium Chloride (BZC)



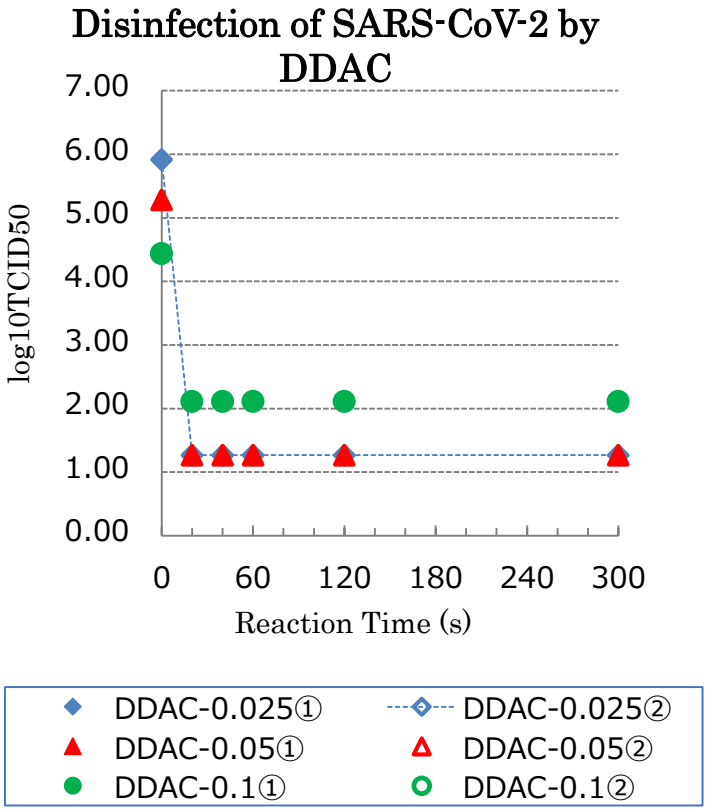
The volume ratio of virus suspension to cationic surfactant solution is 1:1 (N=2).

Cationic Surfactant: Benzethonium Chloride (BZN)

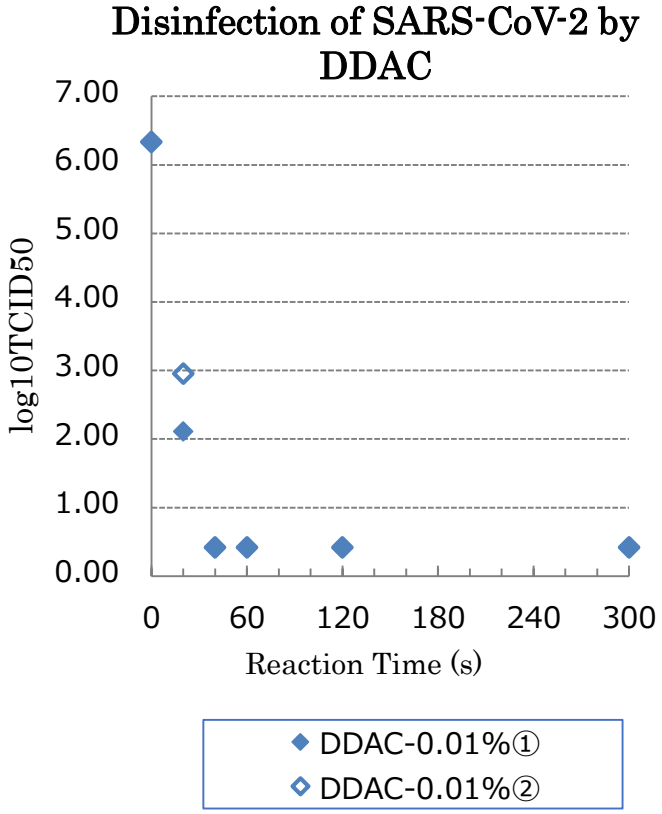


The volume ratio of virus suspension to cationic surfactant solution is 1:1 (N=2).

Cationic Surfactant: Dialkyldimethyldmmonium Chloride (DDAC)



※Since DDAC could not be fully removed by the resin at 0.05% or higher, the initial infectious titer was decreased, and the detection limit was increased due to the cytotoxicity.



The volume ratio of virus suspension to cationic surfactant solution is 1:1 (N=2).

Table1. Effect of Surfactants and Sodium Percarbonate on SARS-CoV-2

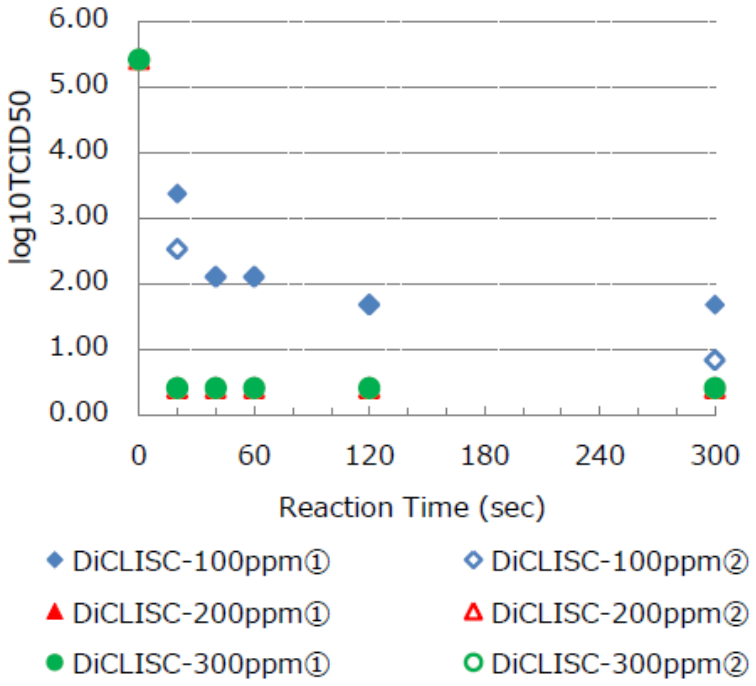
Classification of Disinfectants	Disinfectants	Concentration w/v%					
		0.1		0.05		0.01	
		Reaction Time		Reaction Time		Reaction Time	
		1 min	5 min	1 min	5 min	1 min	5 min
Anionic Surfactants	Potassium Soap	-	-	-	-	-	-
	Sodium Soap	-	-	-	-	-	-
	High Purity Sodium Soap	-	-*	-	-*	-	-*
	Sodium Linear Alkylbenzene Sulfonates	-	+	-	-	-	-
	Sodium Alkyl Ether Sulfates	-	-	-	-	-	-
Nonionic Surfactants	Alkyl Glycosides	+	NT	-	NT	-	NT
	Fatty Acid Alkanol Amides	-	-	-	-	-	-
	Polyoxyethylene Alkyl Ether	-	-	-	-	-	-
Amphoteric Surfactants	Alkyl Amidopropyl Betaine	-	-	-	-	-	-
	Alkyl Dimethyl Amine Oxide	+	NT	+	NT	-	NT
Cationic Surfactants	Benzalkonium Chloride	+	+	+	+	-	+
	Benzethonium Chloride	+	+	-	+	-	-
	Dialkyldimethylammonium Chloride	CT	CT	CT	CT	-	+
Oxygen Bleach	Sodium Percarbonate	-**	-	-	-	-	-

CT, Cell toxicity; NT, Not tested; +, virucidal activity observed; -, virucidal activity not observed.
In this test, when approximately 10,000 viruses were almost completely inactivated (below the detection limit), it was judged as “virucidal activity observed”.
*, Exposure time 10minutes; **, In only one well, RNA titer was not increased and cell death due to the substance was observed.
Viral growth was confirmed by cytopathic effect and amplification of viral RNA by qRT-PCR.

The Results of Hypochlorous Acid Water

Hypochlorous Acid Water; Non-electrolytically-generated Sodium dichloroisocyanurate (NaDCC)

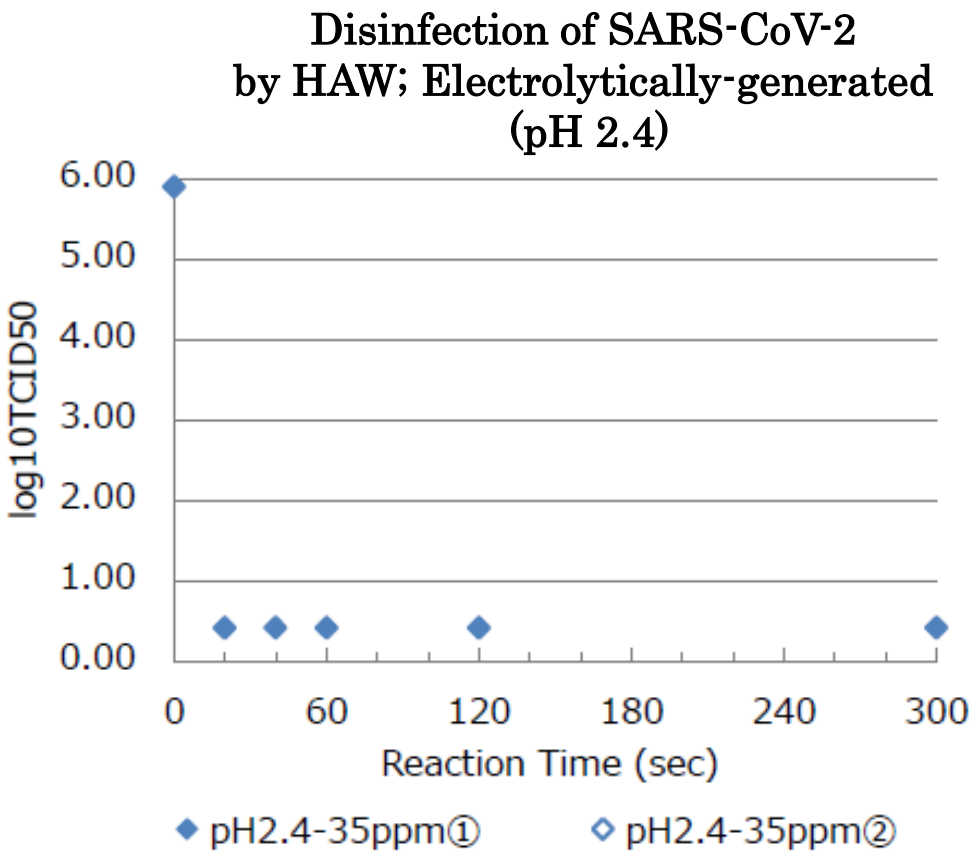
Disinfection of SARS-CoV-2 by NaDCC (DiCLISC)



200 ppm of NaDCC showed more than 99.999% reduction of virus titer after 1 min.
100 ppm NaDCC showed more than 99.9% reduction of virus titer after 1 min.

Test condition
・Virus stock medium: DMEM+5% FBS
・The volume ratio of virus suspension to NaDCC solution solution is 1:9 (N=2).

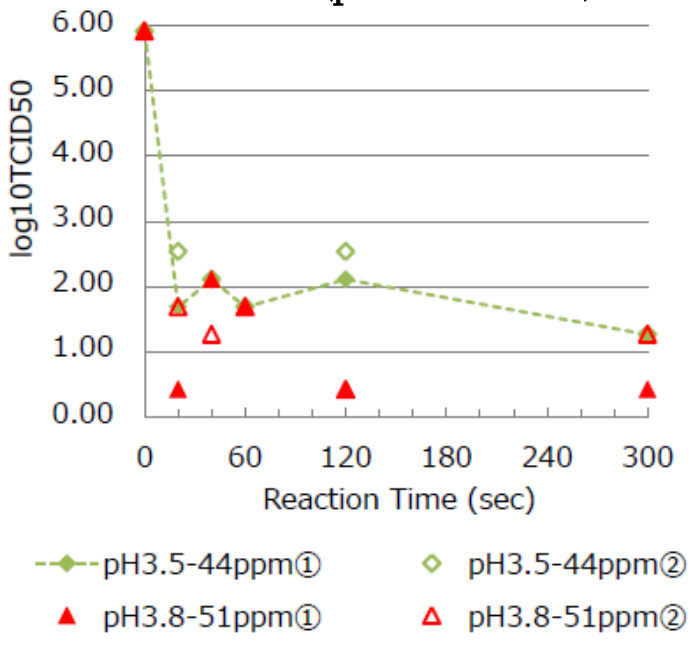
Hypochlorous Acid Water; Electrolytically-generated (strongly acidic electrolyzed water)



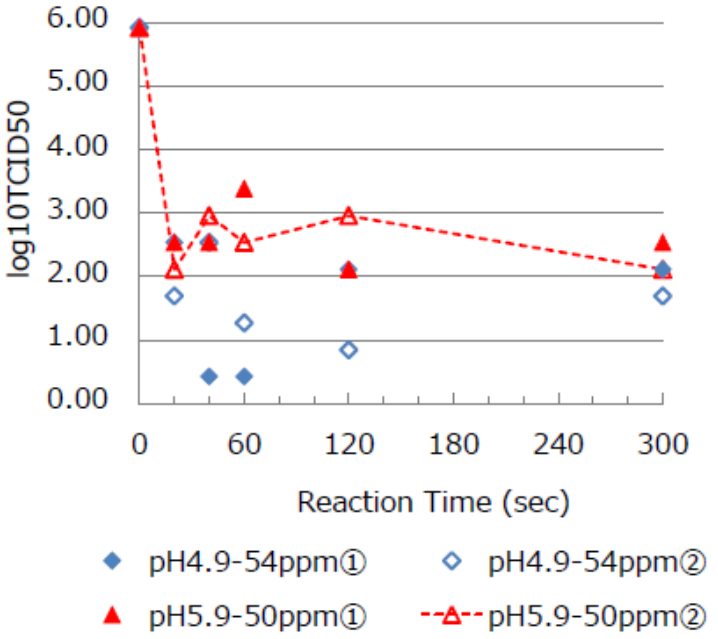
Test condition
・Virus stock medium: DMEM+5% FBS
・The volume ratio of virus suspension to HAW (ACC 35 ppm, pH 2.4) is 1:19 (N=2).
(ACC: Available chlorine concentration)

Hypochlorous Acid Water; Electrolytically-generated (weakly and slightly acidic electrolyzed water)

Disinfection of SARS-CoV-2 by HAW; Electrolytically-generated (pH 3.5 and 3.8)



Disinfection of SARS-CoV-2 by HAW; Electrolytically-generated (pH 4.9 and 5.9)



Test condition

・Virus stock medium: DMEM+5% FBS

・The following four types of HAW were tested: ACC 44ppm, pH 3.5; ACC 51 ppm, pH 3.8; ACC 54 ppm, pH 4.9; and ACC 50 ppm, pH 5.9. (ACC: Available chlorine concentration)

・The volume ratio of virus suspension to HAW is 1:19 (N=2).

Hypochlorous Acid Water; Electrolytically-generated

Table2. Effect of Hypochlorous Acid Water; Electrolytically-generated on SARS-CoV-2

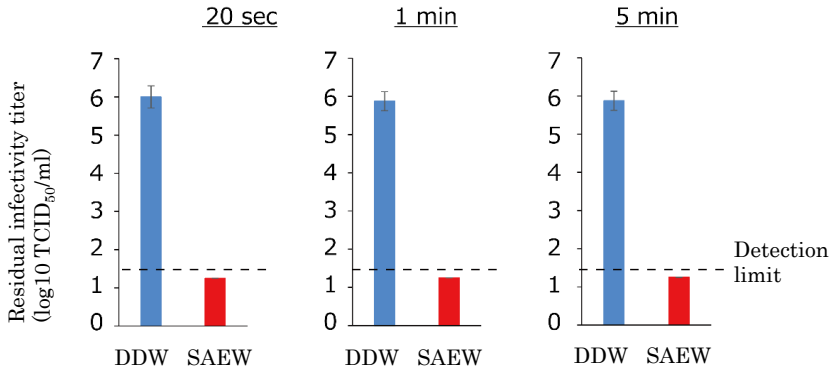
Classification of Hypochlorous Acid Water	Electrolyte	pH	Available Chlorine Concentration (ppm)	Reaction Time	
				1 min	5 min
Slightly Acidic Electrolyzed Water	HCl	5.0	50	-	-
		6.0	50	-	-
	HCl＋NaCl	5.0	50	-	-
		6.0	50	-	-

+, Virucidal activity observed; -, virucidal activity not observed
In this test, when approximately 10,000 viruses were almost completely inactivated (below the detection limit), it was judged as "virucidal activity observed".

Before conducting the test, the available chlorine concentration (ACC) was measured, and it was ensured that there was no discrepancy in the concentration at the time of generation.
Viral growth was confirmed by cytopathic effect (CPE) and amplification of viral RNA by qRT-PCR.
CPE was observed, and the elevated RNA titer reached the upper limit in all wells. In the 70% ethanol-treated group (as a positive control), cells were survived, and the qRT-PCR results were also negative in all wells.

Hypochlorous Acid Water; Electrolytically-generated (strongly acidic electrolyzed water)

Virus suspension (1% FBS):test agent = 1:19



Exposure time		20 sec		1 min		5 min	
Test agents		DDW	SAEW	DDW	SAEW	DDW	SAEW
Infectivity reduction (log_{10} $TCID_{50}$/ml)	Tube 1	6.25	≦1.25	5.75	≦1.25	5.75	≦1.25
	Tube 2	5.75	≦1.25	5.75	≦1.25	5.75	≦1.25
	Tube 3	5.75	≦1.25	5.75	≦1.25	6.25	≦1.25
	Tube 4	6.25	≦1.25	6.25	≦1.25	5.75	≦1.25
	Mean± SD	6.0 ± 0.29	≦1.25 ± 0	5.875 ± 0.25	≦1.25 ± 0	5.875 ± 0.25	≦1.25 ± 0
	Difference value from DDW treatment	-	≧4.75	-	≧4.625	-	≧4.625
Virucidal efficacy (%)		-	≧99.9982	-	≧99.9976	-	≧99.9976

N=4 and two sets of experiment.

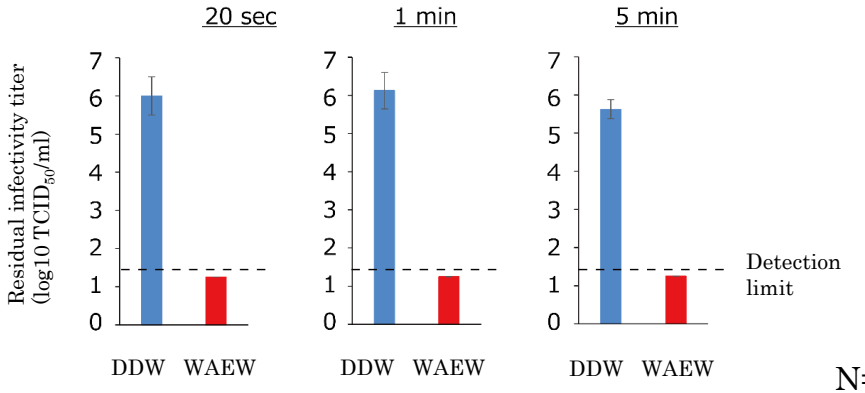
≦1.25:below the detection limit

DDW, double distilled water (negative control).
Strongly acidic electrolyzed water (AAC 56 ppm, pH 2.53).

Virucidal effect of SAEW (56 ppm, pH 2.53) was observed in a short period (20-seconds exposure time) under the condition of test mixture at 1:19 (virus suspension (1% FBS):test agent).

Hypochlorous Acid Water; Electrolytically-generated (slightly acidic electrolyzed water)

Virus suspension (1% FBS):test agent = 1:19



Exposure time		20 sec		1 min		5 min	
Test agents		DDW	WAEW	DDW	WAEW	DDW	WAEW
Infectivity reduction (log_{10} $TCID_{50}$/ml)	Tube 1	6.25	≦1.25	6.25	≦1.25	5.25	≦1.25
	Tube 2	6.25	≦1.25	5.75	≦1.25	5.75	≦1.25
	Tube 3	6.25	≦1.25	5.75	≦1.25	5.75	≦1.25
	Tube 4	5.25	≦1.25	6.75	≦1.25	5.75	≦1.25
	Mean± SD	6.0 ± 0.5	≦1.25 ± 0	6.125 ± 0.48	≦1.25 ± 0	5.625 ± 0.25	≦1.25 ± 0
	Difference value from DDW treatment	-	≧4.75	-	≧4.875	-	≧4.375
Virucidal efficacy (%)		-	≧99.9982	-	≧99.9987	-	≧99.9958

N=4 and two sets of experiment.

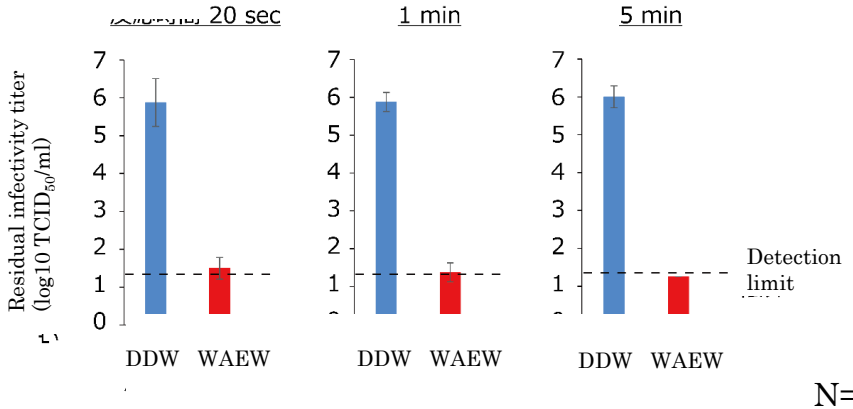
≦1.25:below the detection limit

DDW, double distilled water (negative control).
Slightly acidic electrolyzed water (AAC 54.5 ppm, pH 5.16).

Virucidal effect of WAEW (54.5 ppm, pH 5.16) was observed in a short period (20-seconds reaction time) under the condition of test mixture at 1:19 (virus suspension (1% FBS):test agent).

Hypochlorous Acid Water; Electrolytically-generated (slightly acidic electrolyzed water)

Virus suspension(1% FBS):test agent = 1:19



Exposure time		20 sec		1 min		5 min	
Test agents		DDW	WAEW (32 ppm)	DDW	WAEW (32 ppm)	DDW	WAEW (32 ppm)
Infectivity reduction (log_{10} $TCID_{50}$/ml)	Tube 1	6.75	≦1.75	6.25	≦1.25	6.25	≦1.75
	Tube 2	6.25	2.25	6.25	≦1.25	5.75	≦1.25
	Tube 3	6.25	≦1.75	5.25	≦1.75	6.25	≦1.25
	Tube 4	6.25	≦1.75	6.25	≦1.25	6.25	2.75
	Mean± SD	6.38 ± 0.25	≦1.88 ± 0.25	6 ± 0.5	≦1.38 ± 0.25	6.13 ± 0.25	≦1.75 ± 0.71
	Difference value from DDW treatment	-	≧4.5	-	≧4.625	-	≧4.375
Virucidal efficacy (%)		-	≧99.9968	-	≧99.9976	-	≧99.9958

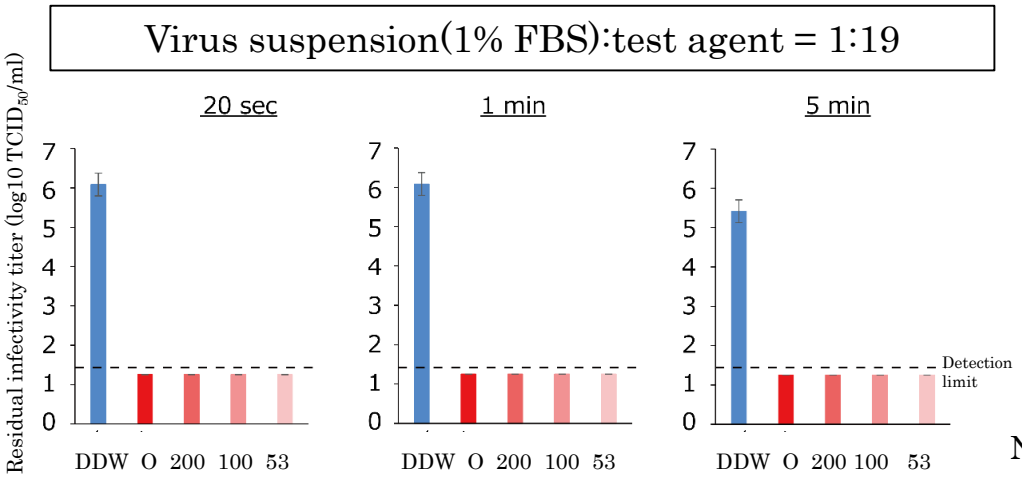
N=4 and two sets of experiment.

≦1.25:below the detection limit

DDW, double distilled water (negative control).
Slightly acidic electrolyzed water (AAC 32 ppm, pH 5.3).

32 ppm WAEW (pH 5.3) showed virucidal effect in a short period (20-seconds exposure time) under the condition of test mixture at 1:19 (virus suspension (1% FBS):test agent).

Hypochlorous Acid Water; Non-electrolytically-generated Anonymous sample A



Virucidal activity at 20 s reaction time

Test agents		DDW	A (original)	A (200 ppm)	A (100 ppm)	A (53 ppm)
Infectivity reduction (log_{10} $TCID_{50}$/ml)	Tube 1	6.25	≦1.25	≦1.25	≦1.25	≦1.25
	Tube 2	5.75	≦1.25	≦1.25	≦1.25	≦1.25
	Tube 3	6.25	≦1.25	≦1.25	≦1.25	≦1.25
	Mean± SD	6.0833 ± 0.29	≦1.25 ± 0	≦1.25 ± 0	≦1.25 ± 0	≦1.25 ± 0
	Difference value from DDW treatment	-	≧4.8333	≧4.8333	≧4.8333	≧4.8333
Virucidal efficacy (%)		-	≧99.9985	≧99.9985	≧99.9985	≧99.9985

N=3 and two sets of experiment.

≦1.25:below the detection limit

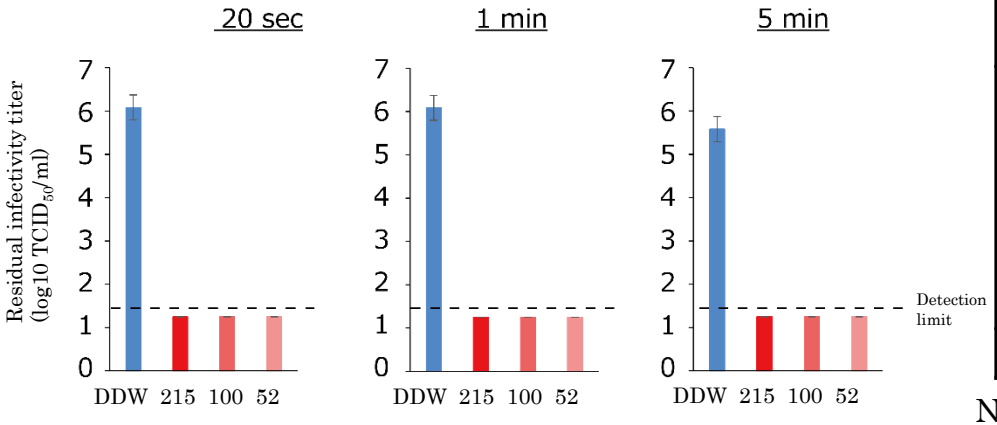
DDW, Double distilled water (negative control).
O, Original concentration (not disclosed) of anonymous sample A (pH 6.0).
The numbers (200, 100 and 53) are available chlorine concentrations (ppm) of diluted anonymous sample A.

Slight cytotoxicity was observed in periphery of wells for the specimen highlighted in light blue.

Anonymous sample A (200, 100 and 53 ppm, pH 6.0) showed virucidal effect against SARS-CoV-2 in a short period (20-seconds exposure time) under the condition of test mixture at 1:19 (virus suspension (1% FBS):test agent).

Hypochlorous Acid Water; Non-electrolytically-generated Anonymous sample B

Virus suspension(1% FBS):test agent = 1:19



DDW, Double distilled water (negative control).
The numbers (215, 100 and 52) are available chlorine concentrations (ppm) of diluted anonymous sample B (pH 6.2).

Virucidal activity at 20 s reaction time

Test agents		DDW	B (215ppm)	B (100 ppm)	B (52 ppm)
Infectivity reduction (log_{10} $TCID_{50}$/ml)	Tube 1	6.25	≦1.25	≦1.25	≦1.25
	Tube 2	6.25	≦1.25	≦1.25	≦1.25
	Tube 3	5.75	≦1.25	≦1.25	≦1.25
	Mean± SD	6.08 ± 0.29	≦1.25 ± 0	≦1.25 ± 0	≦1.25 ± 0
	Difference value from DDW treatment	-	≧4.83	≧4.83	≧4.83
Virucidal efficacy (%)		-	≧99.9985	≧99.9985	≧99.9985

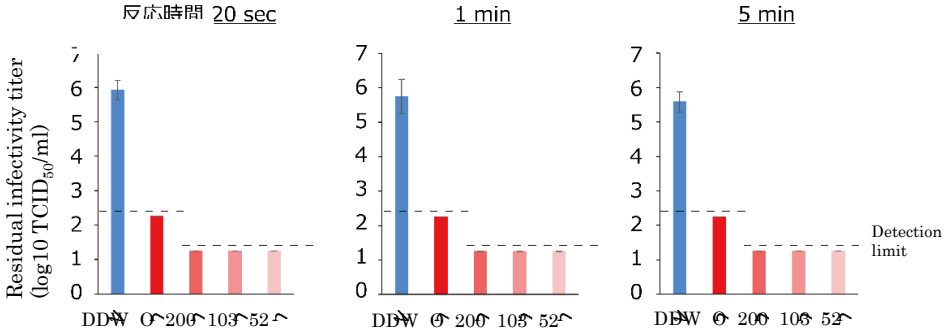
N=3 and two sets of experiment. ≦1.25:below the detection limit

Slight cytotoxicity was observed in periphery of wells for the specimen of 215 ppm at 1 and 5 minutes.

Anonymous sample B (215, 100 and 52 ppm, pH 6.2) showed virucidal effect against SARS-CoV-2 in a short period (20-seconds reaction time) under the condition of test mixture at 1:19 (virus suspension (1% FBS):test agent).

Hypochlorous Acid Water; Non-electrolytically-generated Anonymous sample C

Virus suspension(1% FBS):test agent = 1:19



DDW, Double distilled water (negative control).
O, Original concentration of anonymous sample C (pH 5.2).
The numbers (200, 103 and 52) are available chlorine concentrations of diluted anonymous sample C.

Virucidal activity at 20 s reaction time

Test agents		DDW	C (Original)	C (200 ppm)	C (103 ppm)	C (52 ppm)
Infectivity reduction (log_{10} $TCID_{50}$/ml)	Tube 1	6.25	≦2.25	≦1.25	≦1.25	≦1.25
	Tube 2	5.75	≦2.25	≦1.25	≦1.25	≦1.25
	Tube 3	5.75	≦2.25	≦1.25	≦1.25	≦1.25
	Mean± SD	5.92 ± 0.29	≦2.25 ± 0	≦1.25 ± 0	≦1.25 ± 0	≦1.25 ± 0
	Difference value from DDW treatment	-	≧3.67	≧4.67	≧4.67	≧4.67
Virucidal efficacy (%)		-	≧99.9785	≧99.9979	≧99.9979	≧99.9979

N=3 and two sets of experiment.

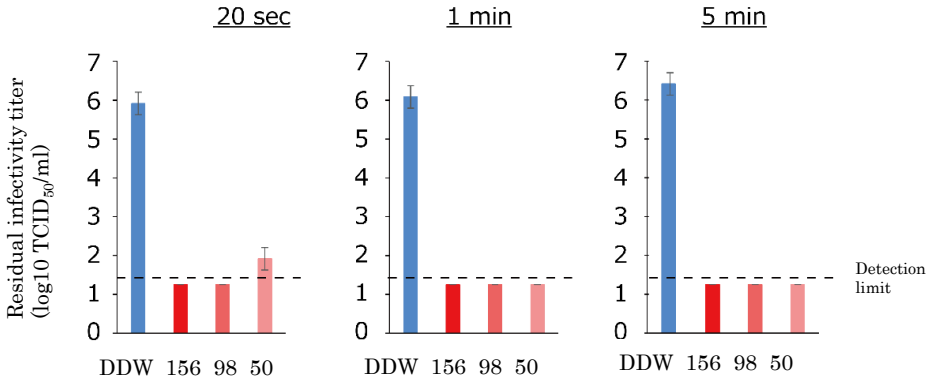
≦1.25:below the detection limit

Global cytotoxicity was observed for the specimens highlighted in gray, and slight cytotoxicity was observed in periphery of wells for the specimen highlighted in light blue.

Anonymous sample C (200, 103 and 52 ppm, pH 5.2) showed virucidal effect against SARS-CoV-2 in a short period (20-seconds rection time) under the condition of test mixture at 1:19 (virus suspension (1% FBS):test agent).

Hypochlorous Acid Water; Non-electrolytically-generated Anonymous sample D

Virus suspension(1% FBS):test agent = 1:19



DDW, Double distilled water (negative control).
The numbers (156, 98 and 50) are available chlorine concentrations (ppm) of undiluted (156 ppm) and diluted anonymous sample D (pH 5.9).

Virucidal activity at 20 s reaction time

Test agents		DDW	D (156ppm)	D (98 ppm)	D (50 ppm)
Infectivity reduction (log_{10} $TCID_{50}$/ml)	Tube 1	5.75	≦1.25	≦1.25	≦1.75
	Tube 2	6.25	≦1.25	≦1.25	≦1.75
	Tube 3	5.75	≦1.25	≦1.25	2.25
	Mean± SD	5.92 ± 0.29	≦1.25 ± 0	≦1.25 ± 0	≦1.92 ± 0.29
	Difference value from DDW treatment	-	≧4.67	≧4.67	≧4
Virucidal efficacy (%)		-	≧99.9979	≧99.9979	≧99.99

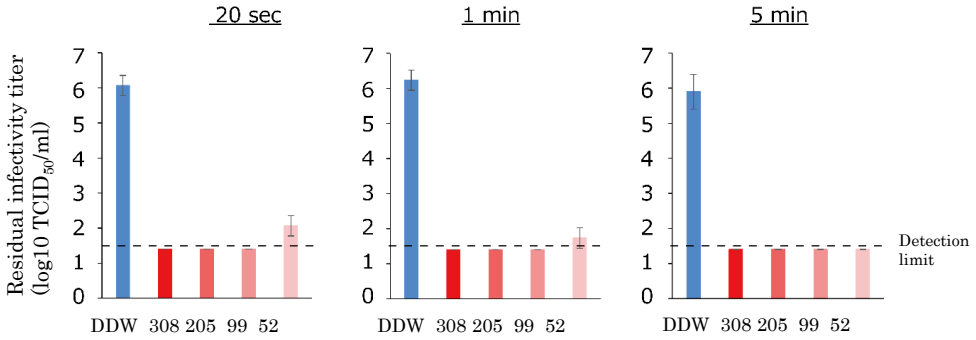
N=3 and two sets of experiment.

≦1.25:below the detection limit

Anonymous sample D (156, 98 and 50 ppm, pH 5.9) showed virucidal effect against SARS-CoV-2 in a short period (20-seconds reaction time) under the condition of test mixture at 1:19 (virus suspension (1% FBS):test agent).

Hypochlorous Acid Water; Non-electrolytically-generated Sodium dichloroisocyanurate (NaDCC)

Virus suspension(1% FBS):test agent = 1:19



DDW, Double distilled water (negative control).
The numbers (308, 205, 99 and 52) are available chlorine concentrations (ppm) of NaDCC solution (pH 6.2).

Virucidal activity at 20 s reaction time

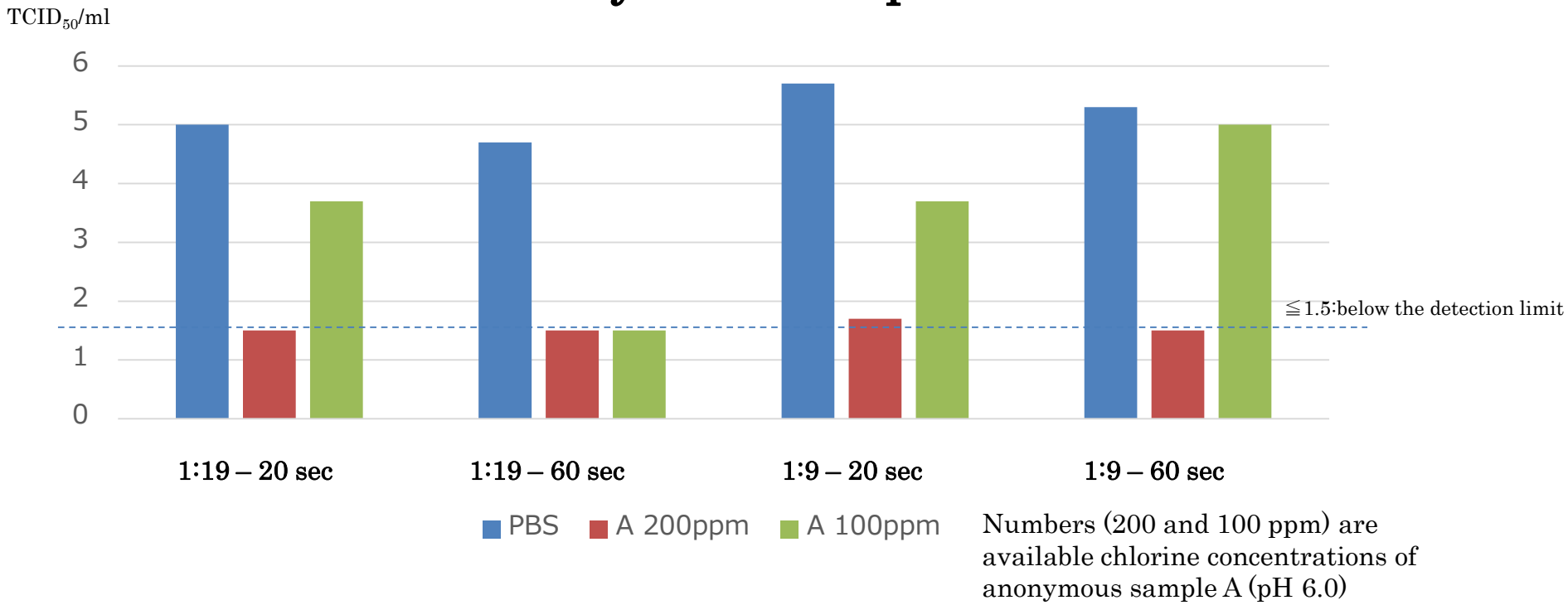
Test agent		DDW	Sodium dichloroisocyanurate			
			308 ppm	205 ppm	99 ppm	52 ppm
Infectivity reduction (log_{10} $TCID_{50}$/ml)	Tube 1	6.4	≦1.4	≦1.4	≦1.4	2.4
	Tube 2	5.9	≦1.4	≦1.4	≦1.4	≦1.9
	Tube 3	5.9	≦1.4	≦1.4	≦1.4	≦1.9
	Mean± SD	6.07 ± 0.29	≦1.4 ± 0	≦1.4 ± 0	≦1.4 ± 0	≦2.07 ± 0.29
	Difference value from DDW treatment	-	≧4.667	≧4.667	≧4.667	≧4
Virucidal efficacy (%)		-	≧99.9979	≧99.9979	≧99.9979	≧99.99

N=3 and two sets of experiment. ≦1.4:below the detection limit

Slight cytotoxicity was observed in periphery of wells for the specimen highlighted in light blue.

NaDCC (308, 205, 99 and 52 ppm, pH 6.2) showed virucidal effect against SARS-CoV-2 under the condition of test mixture at 1:19 (virus suspension (1% FBS):test agent). 200 ppm NaDCC showed more than 99.999% reduction of virus titer after 1 minute.

Hypochlorous Acid Water; Non-electrolytically-generated Anonymous sample A



Numbers (200 and 100 ppm) are available chlorine concentrations of anonymous sample A (pH 6.0)

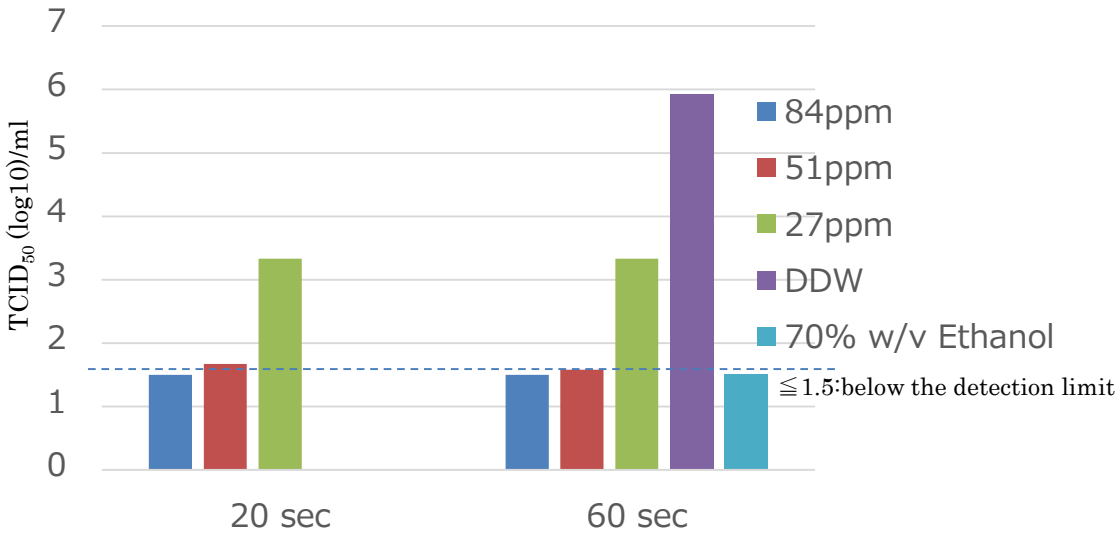
Testing condition：
・virus culture suspension： DMEM medium with 5% FBS
・virus titer：$10^{6.8}TCID_{50}/mL$
・virus suspension：test agent： 1:19 or 1:9
・number of repeats： N=4
・positive control：None
・negative control：PBS（Phosphate Buffered Saline）

At 60-seconds reaction time, 200 ppm of anonymous sample A (pH 6.0) showed reduction of infectious virus titer of 99.9% or more in both test suspension ratios of 1:9 and 1:19 as compared with the negative control group (PBS). On the other hand, at 100 ppm, it was 99.9% or more at 1:19, but under the condition of 1:9, no significant virus titer reduction was observed.

Hypochlorous Acid Water; Non-electrolytically-generated type Anonymous sample A

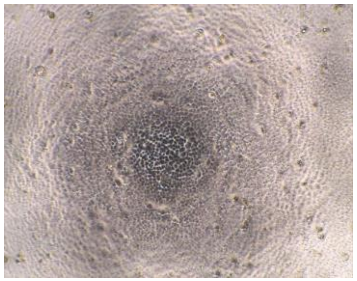
Sample	pH	Available Chlorine Concentration (ppm)	
		Set Value	Measured Value
Anonymous sample A	6.0	80	84
		50	51
		25	27

Testing condition：
・virus culture suspension： DMEM with 1% FBS
・virus titer： 1.8×10^{7} $TCID_{50}$/ml
・virus suspension：test agent： 1:19
・number of repeats： N=3
・positive control：70 w% EtOH
・negative control：MilliQ-water (sterile)

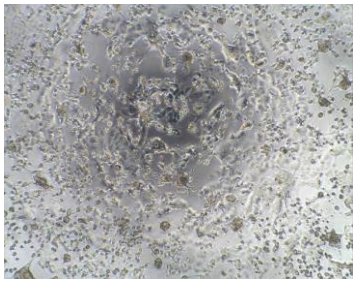


84 ppm and 51 ppm of sample A (pH 6.0) at 60-seconds reaction time showed more than 99.99% reduction of viral titer compared with control treatment (DDW). 27 ppm of sample A showed reduction of viral titer with only 99% or more.

No cytopathic effect



cytopathic effect



The Result of Preliminary Evaluation Tests Using Influenza A Virus as a Surrogate for SARS-CoV-2

Evaluation test using *Influenza A Virus*
Test Agents

Table 1a. List of the Surfactants

Classification of Surfactant	Name of Surfactant	Abbreviation
Anionic Surfactants	Potassium Soap (Potassium Laurate)	Lau-K
	Sodium Linear Alkylbenzene Sulfonates	LAS
Nonionic Surfactants	Alkyl Glycosides	AG
	Fatty Acid Alkanol Amides	FAA
	Polyoxyethylene Alkyl Ather	POE
Amphoteric Surfactants	Alkyl Amidopropyl Betaine	ALBT
	Alkyl Dimethyl Amine Oxide	AAO
Cationic Surfactants	Benzalkonium Chloride[a]	BZC

[a], Also categorized into quaternary ammonium salts

Table 1b. List of Hypochlorous Acid Water; Electrolytically-Generated

Classification of Hypochlorous Acid Water	Electrolyte	pH	Set Value of Available Chlorine Concentration (ppm)
Strongly Acidic Electrolyzed Water	NaCl	≤2.7	40
Weakly Acidic Electrolyzed Water	NaCl	2.7-5.0	30
Slightly Acidic Electrolyzed Water	HCl	5.0-6.5	30
	HCl+NaCl	5.0-6.5	40

Outline of Testing Protocol with *Influenza A Virus*

- Cell culture and virus stock（refer to JIS L 1922/ISO 18184 ）
- Dilution of test substances
  - Surfactants：Diluted with DDW（final concentration 0.1-0.5%）
  - Hypochlorous acid water: No dilution required
- Antiviral reaction（refer to ASTM E1052-20）
  - 1 part of virus + 9 parts of test substance
  - Reaction Time: 1 min and 5 min
  - Temperature: 25 degrees Celsius
- Neutralization of test substances（refer to JIS L 1922/ISO 18184 and ASTM E1052-20）
  - Surfactants: Diluted with 9x volumes of SCDLP medium
  - Hypochlorous acid water: Add one-tenth volume of 0.012 M $Na_2S_2O_3$ solution
- Calculation of results (refer to JIS L 1922/ISO 18184)
  - Plaque assay or $TCID_{50}$ method

Outline of Testing protocol with *Influenza A Virus* (continued)

Case of surfactants

1 part of virus + 9 parts of test substance
(e.g. 100 µl of virus stock (1 x 10^8 $TCID_{50}$/ml) + 900 µl of test substance in a test tube)

↓

Contact at 25℃ (1 min and 5 min)

↓

Mix with 9x volumes of SCDLP medium to neutralize/stop reaction (= 10^{-1} dilution)
(e.g. Add 100 µl of the reaction mixture to 900 µl of SCDLP medium in another test tube)

↓

Serial 10-fold dilutions with cell culture medium (ex, 10^{-2} – 10^{-6})

↓

Plaque assay or $TCID_{50}$ method

Case of hypochlorous acid water

1 part of virus + 9 parts of test substance
(e.g. 100 µl of virus stock (1 x 10^8 $TCID_{50}$/ml) + 900 µl of test substance in a test tube)

↓

Contact at 25℃ (1 min and 5 min)

↓

Mix with 0.1x volume of 0.012 M $Na_2S_2O_3$ solution to neutralize/stop reaction (= 10^0)
(e.g. Add 100 µl of 0.012 M $Na_2S_2O_3$ solution to the reaction mixture in the tube)

↓

Serial 10-fold dilutions with cell culture medium (ex, 10^{-1} – 10^{-6})

↓

Plaque assay or $TCID_{50}$ method

Evaluation test using *Influenza A Virus*
Summary of Results

○ The results obtained from four participating organizations to this study are as follows:
(concentration: 0.1 %, reaction time: 5 min, reduction of virus titer was expressed as $\log_{10}$)

- Surfactants
  - ≥ 4 (99.99%) : Sodium linear alkylbenzene sulfonates, Alkyl glycosides, Polyoxyethylene alkyl ether, Alkyl dimethyl amine oxide
  - ≥ 3 (99.9%) : Potassium soap
  - ≥ 2 (99%) : Fatty acid alkanol amides, Benzalkonium chloride
  - Ineffective : Alkyl amidopropyl betaine

- Hypochlorous acid water; electrolytically-generated
  - ≥ 4 (99.99%) : 4 types of hypochlorous acid water tested in this study (see page 76)

○ The results obtained from participating organizations were closely related each other, so it was judged that the study was successfully performed.

○ These results using influenza A virus indicated that the following substances may have the virucidal activity against SARS-CoV-2 with high probability, and we decided to proceed with the evaluation study of these substances using SARS-CoV-2.

- 5 types of surfactants:
  Sodium linear alkylbenzene sulfonates, Alkyl glycosides, Polyoxyethylene alkyl ether, Alkyl dimethyl amine oxide, Potassium soap

- 4-types of hypochlorous acid water; electrolytically-generated (see page 76)

Virucidal efficacy of tested substances against *Influenza A Virus* Summary of Results

Table 2a. Virucidal Efficacy of Surfactants against Influenza A Virus

Classification of Surfactant	Surfactant	Participating Organization			
		A	B	C	D
		Detection Method of Virus Titer			
		Plaque			$TCID_{50}$
		Strain of Influenzae Virus			
		H3N2 (A/HongKong/8/68) ATCC VR-1679			H1N1 (A/PR/8/34) ATCC VR-1469
Anionic Surfactants	Lau-K	NT	NT	NT	$> 10^3$
	LAS	$> 10^4$	$> 10^4$	$> 10^4$	NT
Nonionic Surfactants	AG	$> 10^4$	NT	$> 10^4$	NT
	FAA	$> 10^2$	$> 10^2$	NT	$> 10^2$
	POE	$> 10^4$	NT	NT	$> 10^4$
Amphoteric Surfactants	ALBT	-	-	NT	-
	AAO	$> 10^4$	$> 10^4$	$> 10^4$	NT
Cationic Surfactants	BZC	NT	$> 10^2$	NT	$> 10^3$

Values show decrease in infectious titer after the reaction time of 5 minutes with each surfactant of 0.1 %.
NT, Not tested; -, ineffective (reduction rate was less than 10^2)

Virucidal efficacy of tested substances against *Influenza A Virus* Summary of Results (continued)

Table 2b. Effect of Hypochlorous Acid Water; Electrolytically-Generated on Influenza A Virus

Classification of Hypochlorous Acid Water	Electrolyte	Participating Organization			
		A	B	C	D
		Detection Method of Virus Titer			
		Plaque			$TCID_{50}$
		Strain of Influenzae Virus			
		H3N2 (A/HongKong/8/68) ATCC VR-1679			H1N1 (A/PR/8/34) ATCC VR-1469
Strongly Acidic Electrolyzed Water	NaCl	NT	NT	NT	$> 10^4$
Weakly Acidic Electrolyzed Water	NaCl	NT	NT	NT	$> 10^4$
Slightly Acidic Electrolyzed Water	HCl	NT	NT	NT	$> 10^4$
	HCl+NaCl	NT	NT	NT	$> 10^4$

Values show decrease in infectious titer after both 1 and 5 min reaction times.
Influenzae A virus used in this study was prepared with ultra-centrifuge.
NT, Not tested

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We would like to thank the organizations below for their participation in the evaluation study using Influenza A virus

BOKEN Quality Evaluation Institute (BOKEN)

Kanagawa Institute of Industrial Science and Technology (KISTEC)

Kitasato Research Center for Environmental Science

Japan Textile Products Quality and Technology Center （QTEC)

(Alphabetical order)

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