Appendix 8.1 Study Plan

Version 1.1, 16 December 2011

Draft Study Plan for the validation of Statens Seruminstitut Rabbit Cornea (SIRC) cytotoxicity test as an alternative eye irritation test

Conducted by:

Japanese Center for the Validation of Alternative Methods (JaCVAM)

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1. Aim of the study

This test method is used to measure cytotoxicity of chemicals using Statens Seruminstitut Rabbit Cornea (SIRC) cells and to discriminate between non irritant and irritant in the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Finally the usefulness as alternative method for eye irritation test is examined.

2. Validation Management Team

To make this validation study scientifically pertinent and to assure the smooth conduct of validation, a study organization for validation of SIRC cytotoxicity test as shown in Fig. 1 is established.

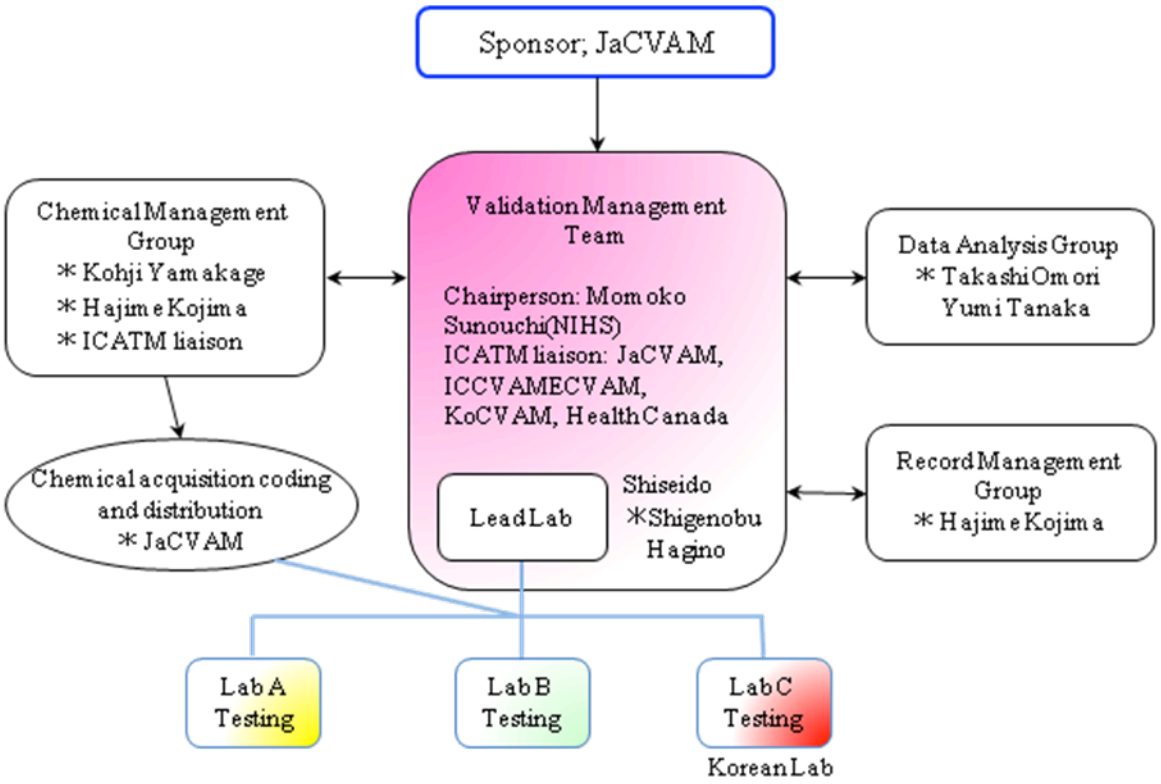

Fig.1 SIRC test Validation Management Team (VMT)

The SIRC cytotoxicity test validation management team (VMT) consisted of the members of the chairperson, chemical management group, data analysis group, record management group, and representative for test development (lead laboratory). The lead laboratory supports to the participating laboratories. The delegates of ICCVAM and ECVAM are liaisons in the VMT and the representatives of the participating laboratories are observers. The VMT will prepare, review, and finalize drafts of study plan and study protocol. In addition, the VMT will also operate and control on the validation study such as checking the progress of study, quality assurance of study records, contact and accommodate with participants and so on.

2-1. Chairperson

The chairperson is elected from among the members of the SIRC cytotoxicity test VMT. He/she prepares drafts of study plan, study protocol and test chemical list, and convenes ad hoc VMT meetings for such reviews and finalizations of study plan, study protocol, and test chemicals list. The chairperson is responsible for operational management of this validation study.

2-2. Chemical management group

The members of chemical management group are elected from among the members of the SIRC cytotoxicity test VMT. They prepare a tentative list of test chemicals and works with the chairperson to make a final decision on the test chemicals to be used in the validation study. The list of coded test chemicals is sent to the chemical distributor.

2-3. Data analysis group

The members of data analysis group are elected from among the members of the SIRC cytotoxicity test VMT, and analyze the data obtained in this validation study from a third-party standpoint. They also take charge of statistical processing in this validation study.

2-4. Record management group

The members of record management group are elected from among the members of the SIRC cytotoxicity test VMT. They prepares protocol, test chemical preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation study. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

2-5. Observers: Researchers responsible for experimental procedures

Each delegate from each laboratory in the validation study is also a observer of the SIRC cytotoxicity test VMT. The delegates or personnel under their supervision carry out experiments according to the study protocol. Upon completion of all experiments, they must submit filled out all record forms, etc. obtained in this validation study to the record management group.

3. Study design

The SIRC cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet. The crystal violet staining method can be used for many cultured cells and can produce the relatively invariable results. Moreover, the operation is simple and easy, and the tested microplate can be stored. No other method can match it.

This SIRC cytotoxicity test validation consists of following three Phases.

1) Phase I for the technical transfer and training
   (within laboratory reproducibility)
2) Phase II for the validation using twenty coded substances
   (within laboratory reproducibility, between laboratory reproducibility)
3) Phase III for the validation using fourty coded substances
   (within laboratory reproducibility, between laboratory reproducibility)

3-1. Research laboratories

This validation study is work out by the participants of previous validation, with selection as necessary. Three laboratories are performed the SIRC cytotoxicity test with 60 chemicals within 80 chemicals selected within a time limit of this validation.

Laboratory Name

1) Bozo Research Center Inc.
2) Nihon Kolmar Co., Ltd
3) Biotoxtech Co. Ltd

3-2. Test chemicals

In this validation study, around 60 test chemicals were selected by the chairperson and chemical management group. All test chemicals are blinded, coded, rotated and distributed by JaCVAM until the end of March, 2012.

3-3. Study duration

Duration of this validation study is a year and a month from September 2011 to August 2012.

3-4. Record collection and analysis

The independent biostatistician of the study will collect the data and organize them in specific data collection software. They will work in close collaboration with the biostatisticians. After decoding they will analyze the data statistically. The data management procedures and statistical tools applied are to be approved by the chairperson and data analysis group.

3-5. Quality assurance

All laboratories will work in the spirit of OECD GLP principle. After completion of experiments, all records will be submitted to the chairperson and record management group. They are checked by record management group.

4. Reporting

(1) The chairperson prepares a report to undergo the international peer review (ICCVAM/ECVAM/JaCVAM/Health Canada) within the framework of ICATM based on the validation data related to the relevance obtained through the SIRC cytotoxicity test validation study.

(2) After obtaining scientifically pertinent validation data related to the relevance through the SIRC cytotoxicity test validation study, the chairperson prepares a research paper for joint publication.

5. Study expense

The total cost for the materials needed to conduct this study, including laboratory supplies such as flasks and plates, cells, sera, culture media and reagents, will be approximately 450,000 yen per each laboratory. A part of study expense will pay JaCVAM out of grants for health science.

6. Study timeline

An approximate schedule for SIRC cytotoxicity test validation study is shown in Table 1.

Table 1. Schedule for SIRC cytotoxicity test validation study

Month	Activity
2011	
September	・Selection of participating research laboratories ・Establish the VMT ・Election and approval of the chairperson and each group
October	・Selection of test substances for Phase I study ・Deliberation of draft study protocol
November	・Deliberation, decision and read-through of draft study plan ・Technical transfer by video-imaging
December 2012 January	・Distribution of non-coded test substances, positive control and relative control substances, medium and fetal calf serum ・Start of Phase I study
February	・End of Phase I study (by early February) ・VMT Meeting /Outline of Phase I study results ・Deliberation and selection of test substances for Phase II study
March	・Preparation, deliberation and decision of Phase I study report ・Distribution of coded test substances for Phase II study
April	・Start of Phase II study ・VMT Meeting /Outline of study results
May	・End of Phase II study ・VMT Meeting /Outline of study results (by late May) ・Deliberation and selection of test substances for phase III
June	・Preparation, deliberation and decision of Phase II study report ・Selection of test substances for phase III ・Distribution of coded test substances for Phase III study
July	・Start of Phase III study
August	・End of Phase III study
September	・VMT Meeting /Outline of study results ・Preparation, deliberation and decision of the reports on Phase III study and the second validation of SIRC cytotoxicity test

Phase I study, Phase II study, Phase III study

Validation Study For The Statens Seruminstitut Rabbit Cornea (SIRC)-CVS Cytotoxicity Test As An Alternative Eye Irritation Test

Draft Study Plan: Phase II-A (Within And Between Laboratory Reproducibilities)

Version 1.51

August 20, 2012

Conducted by:
Japanese Center for the Validation of Alternative Methods (JaCVAM)

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1. Purpose of the study

This test method is used to measure cytotoxicity of chemicals using Statens Seruminstitut Rabbit Cornea (SIRC) cells and to discriminate between non -irritant and irritant. The in vivo standard for the assessment is based on the classification both of the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) and United States Environmental Protection Agency (EPA). Finally the usefulness of SIRC-CVS cytotoxicity test as an alternative method for eye irritation test is examined.

2. Validation Management Team (VMT)

To make this validation study scientifically pertinent and to assure the smooth conduct of validation, a study organization for validation of SIRC-CVS cytotoxicity test as shown in Fig. 1 is established.

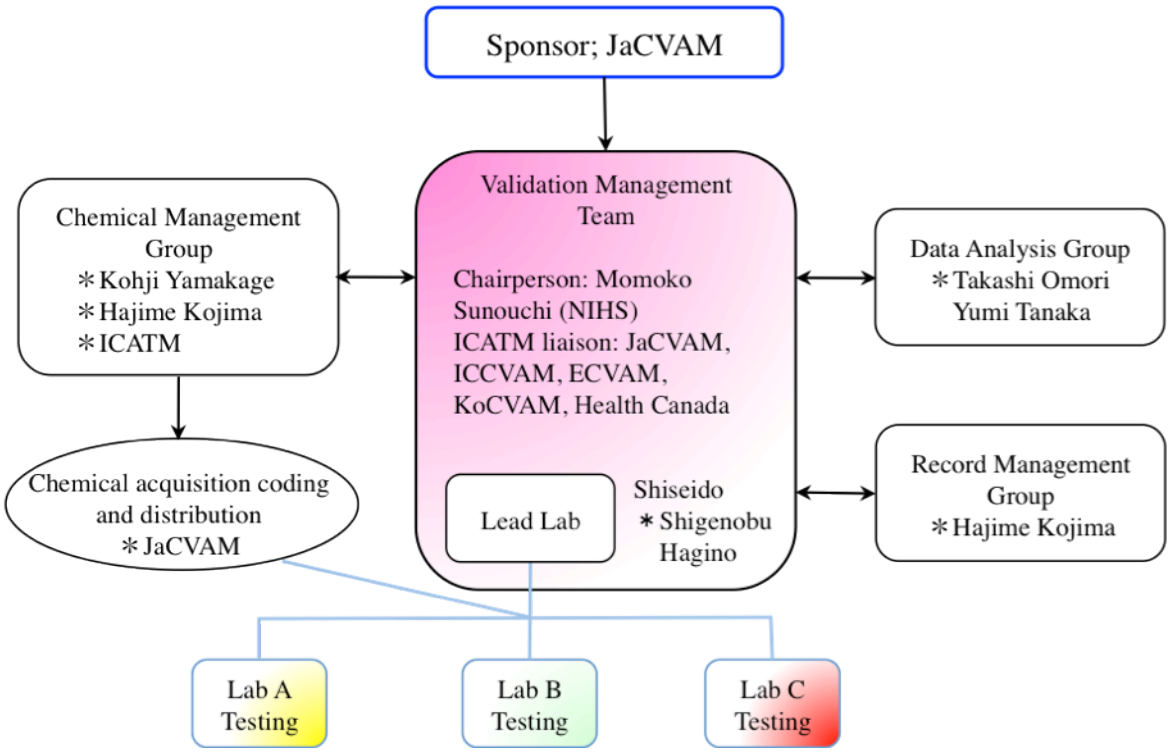

Fig. 1. Study Organization for SIRC-CVS Test Validation

The SIRC-CVS cytotoxicity test validation management team (VMT) consisted of the members of the chairperson, chemical management group, data analysis group, record management group, and representative for test development (lead laboratory). The lead laboratory supports the participating laboratories. The delegates of ICCVAM and ECVAM are liaisons in the VMT and the representatives of the participating laboratories are observers. The VMT will prepare, review, and finalize drafts of study plan and study protocol. In addition, the VMT will also operate and control the validation study such as checking the progress of study, quality assurance of study records, contact and accommodate participants and so on.

2-1. Chairperson

The chairperson is elected from among the VMT members. He/she prepares drafts of study plan, study protocol and test chemical list, and convenes ad hoc VMT meetings for such reviews and finalizations of study plan, study protocol, and test chemicals list. The chairperson is responsible for operational management of this validation study.

2-2. Chemical management group

The members of chemical management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare a tentative list of test chemicals and works with the chairperson to make a final decision on the test substances to be used in the validation study. The list of coded test substances is sent to the chemical distributor.

2-3. Data analysis group

The member of data analysis group are elected from among the members of the SIRC-CVS cytotoxicity test VMT, and analyze the data obtained in this validation study from a third-party standpoint. They also take charge of statistical processing in this validation study.

2-4. Record management group

The members of record management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare protocol, test substance preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation study. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

2-5. Observers: Researchers responsible for experimental procedures

Each delegate from each laboratory in the validation study is also an observer of the SIRC-CVS cytotoxicity test VMT. The delegates or personnel under their supervision carry out experiments according to the study protocol. Upon completion of all experiments, they must submit filled out all record forms, etc. obtained in this validation study to the record management group.

3. Study design

The SIRC-CVS cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet. The crystal violet staining method can be used for many cultured cells and can produce the relatively invariable results. Moreover, the operation is simple and easy, and the results can be confirmed by the measurement of the stored micro plates in any time. No other method can match it.

This SIRC-CVS cytotoxicity test validation consists of following three phases.

1) Phase I for the technical transfer and training
   (Transferability)
2) **Phase II for the validation**
   (within laboratory reproducibility, between laboratory reproducibility**)**
3) Phase III for the validation
   (within laboratory reproducibility, between laboratory reproducibility)

3-1. Research laboratories

Three laboratories are performed the SIRC-CVS cytotoxicity test with sixty substances within eighty chemicals selected within a time limit of this validation.

Laboratory Name

1) Bozo Research Center Inc.
2) Nihon Kolmar Co., Ltd
3) Biotoxtech Co. Ltd

3-2. Selection criteria of test substances

The test substances should be selected in consideration of the various categories such as eye irritant level (GHS and EPA hazard categories), physical form, chemical class and eye lesions produced. The selected substances have high quality in vivo data, especially individual animal data. All of the selected substances are commercially available. This is because they are selected from the substance list of the Eye Irritation Validation Study (EIVS) of ECVAM. All of the selected substances are commercially available. The selected substances have high quality in vivo data, especially individual animal data.

3-3. Test substances

The twenty more substances were selected for the phase II of the validation study at the meeting on February 22 and 23, 2012. The remaining substances will be selected before the next step. All of the test substances for phase II and phase III are used as coded items, so we will provide the list of substances used in the SIRC-CVS test validation after completion of the study. are blinded, coded, rotated and distributed by JaCVAM. Three laboratories will test the same sixty substances. The twenty more substances were selected for the phase II test of SIRC-CVS validation study at the first VMT meeting on February 22 and 23, 2012. Five of them will be used for phase II-A and fifteen for phase II-B. The remaining substances will be selected before phase III test.

Three laboratories will test the same sixty substances.

GHS/EPA category of twenty substances for phase II test are GHS-1/EPA-I; 3 substances, GHS-2A/EPA-II; 3 substances, GHS-2B/EPA-II; 4 substances, GHS-Non/EPA-III; 5 substances and GHS-Non/EPA-IV; 5 substances.

Table 1. Breakdown of substances used for the SIRC-CVS validation study

Phase	The number of the substances	The number of the repetitions	Examination
II-II-A	5	3	Within and between laboratory reproducibilities
II-II-B	15	3	
IIIIII	40	1	Between laboratory reproducibility

3-4. Study duration

Phase II-A validation test will be performed for about ten eleven weeks from the early July late June to the early September, 2012. (See Table 2)

3-5. Record collection and analysis

The independent biostatistician of the study will collect the data and organize them in specific data collection software. They will work in close collaboration with the biostatisticians. After decoding they will analyze the data statistically. The data management procedures and statistical tools applied are to be approved by the chairperson and data analysis group. Any deviations from these principles should be documented along with a discussion of their impact on the study results. The

eye irritation of the test substance is evaluated by using triethanolamine as a relative control in accordance with the protocol, Annex 1. ***Furthermore, in order for SIRC-CVS to have applicability to the EPA classification system, the use of decision criteria based on specific IC50 criteria should be analyzed.***

3-6. Quality assurance

Participating laboratories should conduct all studies according to the principles of Good Laboratory Practices (GLP, OECD 1999). Any deviations from these principles should be documented along with a discussion of their impact on the study results.

4. Reporting

(1) The chairperson prepares a report to undergo the international peer review (ICCVAM/ECVAM/JaCVAM/Health Canada) within the framework of ICATM based on the validation data related to the relevance obtained through the SIRC-CVS cytotoxicity test validation study.

(2) After obtaining scientifically pertinent validation data related to the relevance through the SIRC-CVS cytotoxicity test validation study, the chairperson prepares a research paper for joint publication.

5. Study expense

The total cost for the materials needed to conduct this study, including laboratory supplies such as flasks and plates, cells, sera, culture media and reagents, will be approximately 450,000 yen per each laboratory. A part of study expense will pay JaCVAM out of grants for health science.

6. Study timeline

An approximate schedule for SIRC-CVS cytotoxicity test validation study is shown in Table 2.

Table 2. Schedule for Phase II-A of SIRC-CVS cytotoxicity test validation study

Month	Activity
2012	
June	・ Distribution of five test substances coded for phase II-A study
JulyJune	・ Distribution of the study plan for phase II-A-A and the revised protocol for SIRC-CVS validation phase II study ・ Distribution of five test substances coded for phase II-A study ・ Start of phase II-A study by mid July
July	・ Phase II-A study
August	
September	・ Provision of the data of phase II-A study to the data analysis group by early mid September ・ Data Analysis
October	・ Japanese VMT and the laboratory's meeting /Outline of study results on phase II-A ・ Report to VMT members /Outline of study results on phase II-A ・ End of phase II-A study ・ Distribution of the study plan and fifteen test substances for phase II-B study
November	・ Start of phase II-B study by early November・ Provision of the data of phase II-B test to the data analysis group by late November
December	
2013	
January	・ Provision of the data of phase II-B study to the data analysis group by mid January ・ ・ Data Analysis
February	・ Japanese VMT and the laboratory's meeting /Outline of study results on phase II-B ・ International VMT meeting /Outline of Phase II study results ・ Selection of forty substances for Phase III study ・ Submit the report of phase II in SIRC-CVS cytotoxicity test validation study ・ Preparation and deliberation of Phase II study report

March	・ Distribution of the substances for phase III validation study

7. List of abbriviations and acronyms

ATCC American Type Culture Collection
DMSO Dimethyl Sulfoxide
EPA United States Environmental Protection Agency
FBS Fetal Bovine Serum
GHS Globally Harmonized System of Classification and Labelling of Chemicals
IC_{50} 50% Inhibitory Concentration
JaCVAM Japanese Center for the Validation of Alternative Methods
MEM Minimum Essential Medium
NI Non Irritant
OD Optical density
PBS(-) Phosphate-Buffered Saline (-)
SDS Sodium Dodecyl Sulfate
SIRC cell Statens Seruminstitut Rabbit Corneal Cell
SIRC-CVS Statens Seruminstitut Rabbit Cornea-Crystal Violet Staining

8. About the revision of this study plan

Validation Study For The Statens Seruminstitut Rabbit Cornea (SIRC)-CVS Cytotoxicity Test As An Alternative Eye Irritation Test

Study Plan

Version 1.53

For Phase II-B (Within And Between Laboratory Reproducibility)

October 25, 2012

Conducted by:

Japanese Center for the Validation of Alternative Methods (JaCVAM)

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1. Purpose of the study

This test method is used to measure cytotoxicity of chemicals using Statens Seruminstitut Rabbit Cornea (SIRC) cells and to discriminate between non-irritant and irritant. The in vivo standard for the assessment is based on the classification of the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) and United States Environmental Protection Agency (EPA).

2. Validation Management Team (VMT)

To make this validation study scientifically pertinent and to assure the smooth conduct of validation, a study organization for validation of SIRC-CVS cytotoxicity test as shown in Fig. 1 is established.

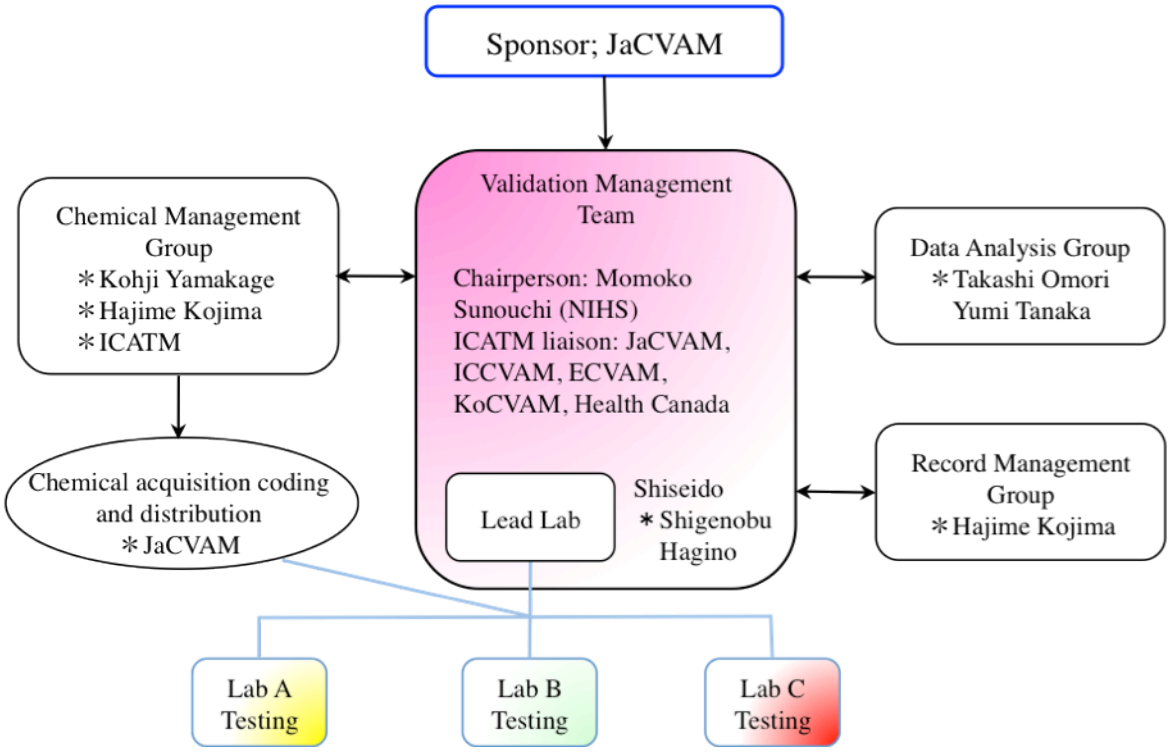

Fig. 1. Study Organization for SIRC-CVS Test Validation

The SIRC-CVS cytotoxicity test validation management team (VMT) consisted of the members of

the chairperson, chemical management group, data analysis group, record management group, and representative for test development (lead laboratory). The lead laboratory supports the participating laboratories. The delegates of ICCVAM and ECVAM are liaisons in the VMT and the representatives of the participating laboratories are observers. The VMT will prepare, review, and finalize drafts of study plan and study protocol. In addition, the VMT will also operate and control the validation study such as checking the progress of study, quality assurance of study records, contact and accommodate participants and so on.

2-1. Chairperson

The chairperson is elected from among the VMT members. He/she prepares drafts of study plan, study protocol and test chemical list, and convenes ad hoc VMT meetings for such reviews and finalizations of study plan, study protocol, and test chemicals list. The chairperson is responsible for operational management of this validation study.

2-2. Chemical management group

The members of chemical management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare a tentative list of test chemicals and works with the chairperson to make a final decision on the test substances to be used in the validation study. The list of coded test substances is sent to the chemical distributor.

2-3. Data analysis group

The member of data analysis group are elected from among the members of the SIRC-CVS cytotoxicity test VMT, and analyze the data obtained in this validation study from a third-party standpoint. They also take charge of statistical processing in this validation study.

2-4. Record management group

The members of record management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare protocol, test substance preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation study. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

2-5. Observers: Researchers responsible for experimental procedures

Each delegate from of the laboratories participating in the validation study is also an

observer of the SIRC-CVS cytotoxicity test VMT. The delegates or personnel under their supervision carry out experiments according to the study protocol (version 2.12) for SIRC-CVS cytotoxicity test. Upon completion of all experiments, they must submit filled out all record forms, etc. obtained in this validation study to the record management group.

3. Study design

The SIRC-CVS cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet. The crystal violet staining method can be used for many cultured cells and can produce the relatively invariable results. Moreover, the operation is simple and easy, and the results can be confirmed by the measurement of the stored microplates in any time. No other method can match it.

This SIRC-CVS cytotoxicity test validation consists of following three phases.

1) Phase I for the technical transfer and training (Transferability)

The study for Phase I was ended.

2) Phase II for the validation

(within laboratory reproducibility, between laboratory reproducibility**)**

The study for Phase II-A was ended.

The study for Phase II-B will be started by late October 2012.

3) Phase III for the validation

(between laboratory reproducibility)

3-1. Research laboratories

Three laboratories perform the SIRC-CVS cytotoxicity tests with sixty substances within eighty chemicals selected within a time limit of this validation.

Laboratory Name

1) Bozo Research Center Inc.
2) Nihon Kolmar Co., Ltd
3) Biotoxtech Co. Ltd

3-2. Selection criteria of test substances

The test substances should be selected in consideration of the various categories such as eye irritant level (GHS and EPA hazard categories), physical form, chemical class and eye lesions produced. The selected substances have high quality in vivo data, especially individual animal data. This is because they are selected from the substance list of the Eye Irritation Validation Study (EIVS)

of ECVAM. All of the selected substances are commercially available.

3-3. Test substances

The twenty more substances were selected for the phase II of the validation study at the meeting on February 22 and 23, 2012. The remaining substances will be selected before the next step. All of the substances for phase II and phase III are used as coded items, so we will provide the list of substances used in the SIRC-CVS test validation after completion of the study.

Three laboratories will test the same sixty substances.

Table 1. Breakdown of substances used for the SIRC-CVS validation study

Phase	The number of the substances	The number of the repetitions	Examination
II-A	5	3	**Within and between laboratory reproducibility**
II-B	**15**	**3**	
III	40	1	Between laboratory reproducibility

3-4. Study duration

Phase II-B validation test will be performed for about eleven weeks from the late October 2012 to the mid January 2013. (See Table 2)

3-5. Record collection and analysis

The independent biostatistician of the study will collect the data and organize them in specific data collection software. They will work in close collaboration with the biostatisticians. After decoding they will analyze the data statistically. The data management procedures and statistical tools applied are to be approved by the chairperson and data analysis group. Any deviations from these principles should be documented along with a discussion of their impact on the study results. The eye irritations of the test substances are evaluated by using triethanolamine as a relative control in accordance with the protocol version 2.12, Annex 1. Furthermore, in order for SIRC-CVS to have applicability to the EPA classification system, the use of decision criteria based on specific IC50 criteria should be analyzed.

3-6. Quality assurance

Participating laboratories should conduct all studies according to the principles of Good Laboratory Practices (GLP, OECD 1999). Any deviations from these principles should be documented along with a discussion of their impact on the study results.

4. Reporting

(1) The chairperson prepares a report to undergo the international peer review (ICCVAM/ECVAM/JaCVAM/Health Canada) within the framework of ICATM based on the validation data related to the relevance obtained through the SIRC-CVS cytotoxicity test validation study.

(2) After obtaining scientifically pertinent validation data related to the relevance through the SIRC-CVS cytotoxicity test validation study, the chairperson prepares a research paper for joint publication.

5. Study expense

The total cost for the materials needed to conduct this study, including laboratory supplies such as flasks and plates, cells, sera, culture media and reagents, will be approximately 450,000 yen per each laboratory. A part of study expense will pay JaCVAM out of grants for health science.

6. Study timeline

An approximate schedule for SIRC-CVS cytotoxicity test validation study is shown in Table 2.

Table 2. Schedule for Phase II-B of SIRC-CVS cytotoxicity test validation study

Month	Activity
2012	
June	・ Distribution of five test substances coded for phase II-A study
July	・ Distribution of the study plan for phase II-A and the revised protocol for SIRC-CVS validation phase II study ・ Start of phase II-A study by mid July
August	
September	・ Provision of the data of phase II-A study to the data analysis group by mid September ・ Data Analysis
October	・ Japanese VMT and the laboratory's meeting on the 16th October Outline of study results on phase II-A ・ End of phase II-A validation study ・ Distribution of the study plan and fifteen test substances for phase II-B study ・ Start of phase II-B study by late October
November	
December	
2013	
January	・ Provision of the data of phase II-B study to the data analysis group by the 15th January ・ Data Analysis
February	・ Japanese VMT and the laboratory's meeting at Kyoto on the 15th February Outline of study results on the phase II-B and on the whole phase II ・ International VMT/he laboratory's meeting at Kyoto on the 16th February Outline of study results on the phase II-B and on the whole phase II Selection of forty test substances for phase III study ・ Submit the report of phase II in SIRC-CVS cytotoxicity test validation study ・ Preparation and deliberation of phase II study report
March	・ Distribution of the test substances for phase III validation study

7. List of abbriviations and acronyms

ECVAM ; European Center for the Alternative Methods
EPA ; United States Environmental Protection Agency
GHS ; Globally Harmonized System of Classification and Labelling of Chemicals
GLP ; Good Laboratory Practice
IC50 ; IC50% Inhibitory Concentration
ICCVAM ; The Interagency Coordinating Committee on the Validation of Alternative Methods
ICATM ; The International Cooperation on Alternative Test Method
JaCVAM ; Japan Center for the Alternative Methods
KoCVAM ; Korean Center for the Alternative Methods
JaCVAM ; Japanese Center for the Validation of Alternative Methods
SIRC cell ; Statens Seruminstitut Rabbit Corneal Cell
SIRC-CVS ; Statens Seruminstitut Rabbit Cornea–Crystal Violet Staining
VMT ; Validation Management Team

Validation Study For The Statens Seruminstitut Rabbit Cornea (SIRC)-CVS Cytotoxicity Test As An Alternative Eye Irritation Test

Study Plan

Version 1.56

Phase III Study (For Predictability)

March 26, 2013

Conducted by:

Japanese Center for the Validation of Alternative Methods (JaCVAM)

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1. Purpose of the study

This test method is used to measure cytotoxicity of chemicals using Statens Seruminstitut Rabbit Cornea (SIRC) cells to discriminate between non-irritant and irritant. The in vivo standard for the assessment is based on the classification of the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) and United States Environmental Protection Agency (EPA).

2. Validation Management Team (VMT)

To make this validation study scientifically pertinent and to assure the smooth conduct of validation, a study organization for validation of SIRC-Crystal Violet Staining (CVS) cytotoxicity test as shown in Fig. 1 is established.

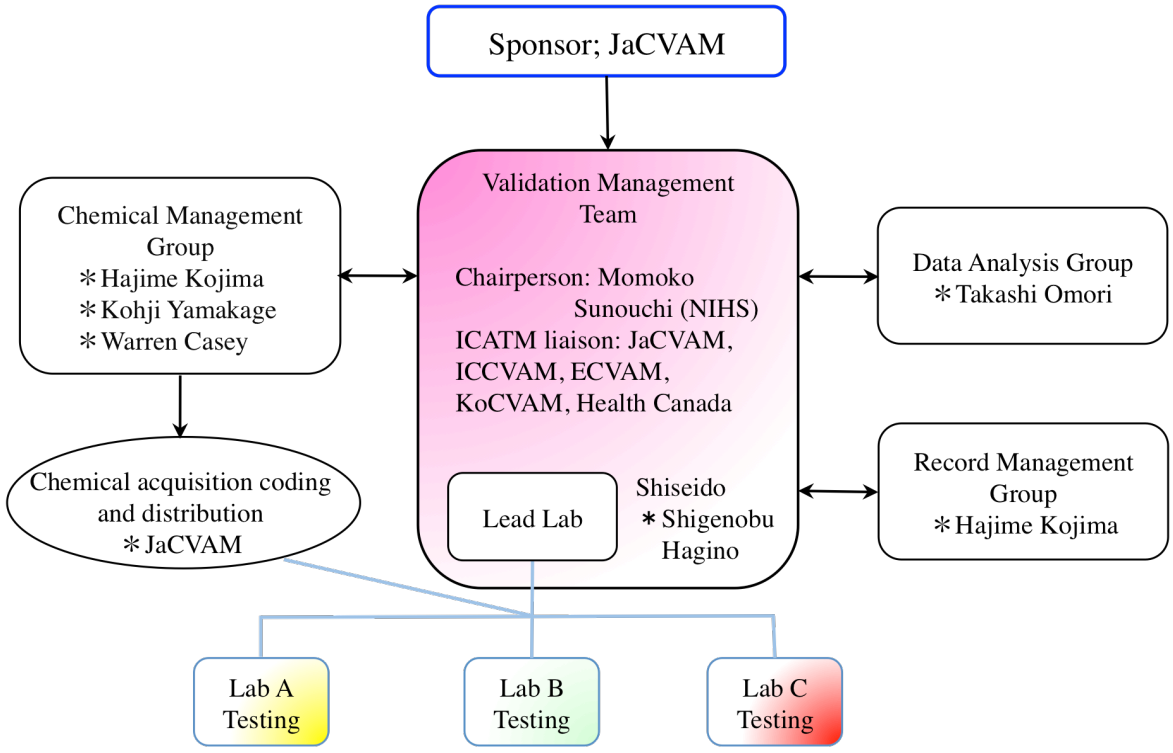

Fig. 1. Study Organization for SIRC-CVS Test Validation

The SIRC-CVS cytotoxicity test validation management team (VMT) consisted of the members of the chairperson, chemical management group, data analysis group, record management group, and representative for test development (lead laboratory). The lead laboratory supports the participating laboratories. The delegates of ICCVAM and ECVAM are liaisons in the VMT and the representatives of the participating laboratories are observers. The VMT will prepare, review, and finalize drafts of study plan and study protocol. In addition, the VMT will also operate and control the validation study such as checking the progress of study, quality assurance of study records, contact and accommodate participants and so on. The VMT members are shown at Table 1.

Table 1. Members of SIRC-CVS Validation Management Team (VMT)

Name	Organization	Action
Momoko Sunouchi	JaCVAM, NIHS Japan	Chairperson
Hajime Kojima	JaCVAM, NIHS Japan	JaCVAM,Chemical Management
Warren Casey	ICCVAM, NIH USA	NICEATM,Chemical Management
Michael Oelgeschlaeger	ECVAM, Federal Institute Risk Assessment, DEU	
Takashi Omori	Doshisha University, Japan	Data Analysis
Kohji Yamakage	FOOD AND DRUG SAFTY CENTER, Hatano Researh Institute,Japan	Chemical Management
Shigenobu Hagino	Shiseido Research Center,Japan	Lead laboratory
KoCVAM		
Health Canada		

2-1. Chairperson

The chairperson is elected from among the VMT members. She prepares drafts of study plan, study protocol and test chemical list, and convenes ad hoc VMT meetings for such reviews and finalizations of study plan, study protocol, and test chemicals list. The chairperson is responsible for operational management of this validation study.

2-2. Chemical management group

The members of chemical management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare a tentative list of test chemicals and works with the chairperson to make a final decision on the test substances to be used in the validation study. The list of coded test substances is sent to the chemical distributor.

2-3. Data analysis group

The member of data analysis group are elected from among the members of the SIRC-CVS cytotoxicity test VMT, and analyze the data obtained in this validation study from a third-party standpoint. They also take charge of statistical processing in this validation study.

2-4. Record management group

The members of record management group are elected from among the members of the SIRC-CVS cytotoxicity test VMT. They prepare protocol, test substance preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation study. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

2-5. Observers: Researchers responsible for experimental procedures

Each delegate from of the laboratories participating in the validation study is also an observer of the SIRC-CVS cytotoxicity test VMT. The delegates or personnel under their supervision carry out

experiments according to the study protocol (version 2.13E) for SIRC-CVS cytotoxicity test. Upon completion of all experiments, they must submit filled out all record forms, etc. obtained in this validation study to the record management group.

3. Study design

The SIRC-CVS cytotoxicity test procedure is based on the measurement of viable cells stained by crystal violet. The crystal violet staining method can be used for many cultured cells and can produce the relatively invariable results. Moreover, the operation is simple and easy, and the results can be confirmed by the measurement of the stored microplates in any time. No other method can match it.

This SIRC-CVS cytotoxicity test validation consists of following three phases.

1) Phase I study (For transferability)

The phase I study by the protocol version 1.6E was ended.

2) Phase II study (For within- and -between laboratory reproducibility)

The phase II-A study by the protocol version 2.09E was ended.

The phase II-B study by the protocol version 2.12E was ended.

3) Phase III study (For predictability)

The phase III study is performed by the protocol version 2.13E.

3-1. Research laboratories

Three laboratories perform the SIRC-CVS cytotoxicity tests with forty substances each.

Laboratory Name

1) Bozo Research Center Inc.

2) Nihon Kolmar Co., Ltd

3) Biotoxtech Co. Ltd

3-2. Selection criteria of test substances

The test substances should be selected in consideration of the various categories such as eye irritant level (GHS and EPA hazard categories), physical form, chemical class and eye lesions produced. The selected substances have high quality in vivo data, especially individual animal data. This is because they are selected based on the substance list of the Eye Irritation Validation Study (EIVS) of ECVAM and others. All of the selected substances are commercially available.

3-3. Test substances

The use of one hundred substances in total was determined for the phase III of the validation study at the VMT meeting on 16th February 2013. These substances were selected by the chemical management group and approved by the VMT members. All of the substances for phase III are used as coded items, so we will provide the list of substances used in the SIRC-CVS test validation after completion of the study.

Each of three laboratories will test the forty substances and ten of the forty will be in common (Table 2).

Table 2. Breakdown of substances used for the SIRC-CVS validation study

Phase	The number of the substances	The number of the repetitions	Examination
III	**100 (coded) in total 40; each laboratory 10; in common 30; different**	**1**	**Predictability**

3-4. Study duration

Phase III validation test will be performed for about twelve weeks from the beginning April 2013 to the end June 2013. (See Table 3)

3-5. Record collection and analysis

The independent biostatistician of the study will collect the data and organize them in specific data collection software. They will work in close collaboration with the biostatisticians. After decoding they will analyze the data statistically. The data management procedures and statistical tools applied are to be approved by the chairperson and data analysis group. Any deviations from these principles should be documented along with a discussion of their impact on the study results. The eye irritations of the test substances are evaluated by using triethanolamine as a relative control in accordance with the protocol version 2.13, Annex 1. Furthermore, in order for SIRC-CVS to have applicability to the EPA classification system, the use of decision criteria based on specific IC50 criteria should be analyzed.

3-6. Quality assurance

Participating laboratories should conduct all studies according to the principles of Good Laboratory Practices (GLP, OECD 1999). Any deviations from these principles should be documented along with a discussion of their impact on the study results.

4. Reporting

(1) The chairperson prepares a report to undergo the international peer review (ICCVAM/ECVAM/JaCVAM/Health Canada) within the framework of ICATM based on the validation data related to the relevance obtained through the SIRC-CVS cytotoxicity test validation study.

(2) After obtaining scientifically pertinent validation data related to the relevance through the SIRC-CVS cytotoxicity test validation study, the chairperson prepares a research paper for joint publication.

5. Study expense

The total cost for the materials needed to conduct this study, including laboratory supplies such as flasks and plates, cells, sera, culture media and reagents, will be approximately 450,000 yen per each laboratory.

6. Study timeline

An approximate schedule for SIRC-CVS cytotoxicity test validation study is shown in Table 3.

Table 3. Schedule for Phase III of SIRC-CVS cytotoxicity test validation study

Month	Activity
2013	
March -	・ Distribution of the medium by early in March ・ Distribution of forty test substances coded from mid-March to early in April
April - June	・ Start of phase III study by mid-April ・ Provision of the data of phase III study to the data analysis group by the end of June
July – September	・ Data Analysis ・ VMT-FTF meetings will be held at the end of September, 2013.
October	
November	
December	・ Submission of the report on the validation study of SIRC-CVS cytotoxicity test as an alternative eye irritation test to JaCVAM steering committee by late December

7. List of abbreviations and acronyms

ECVAM; European Center for the Alternative Methods
EPA; United States Environmental Protection Agency
FTF; Face To-Face
GHS; Globally Harmonized System of Classification and Labelling of Chemicals
GLP; Good Laboratory Practice
IC50; IC50% Inhibitory Concentration
ICATM; The International Cooperation on Alternative Test Method
ICCVAM; Interagency Coordinating Committee on the Validation of Alternative Methods
JaCVAM; Japanese Center for the Validation of Alternative Methods
KoCVAM; Korean Center for the Validation of Alternative Methods
SIRC cell; Statens Seruminstitut Rabbit Corneal cpell
SIRC-CVS; Statens Seruminstitut Rabbit Cornea–Crystal Violet Staining
VMT; Validation Management Team