The members of data analysis group are elected by recommendation of the IL-2 Luc assay VMT, and check and analyze the data obtained in this validation trial from a third-party standpoint. They also take charge of statistical processing in this validation trial.

3) Quality assurance group

The members of record management group are elected by recommendation of the IL-2 Luc assay VMT. They prepares protocol, test chemical preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation trial. They also collects 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.

4) Lead laboratory

The lead laboratory representing the test method is responsible for providing the test method protocol and the eventually necessary data recording or calculation templates. The Trial Coordinator has to ensure that such data recording or calculation templates have been validated before distribution to the test facilities involved in the validation trial. The lead laboratory is also responsible for providing, if necessary, new versions of the protocols during the entire validation trial. The lead lab and the other participating test facilities might be contacted by the VMT for technical issues.

3. 3Sponsor

The validation trial for assessing the validity of IL-2 Luc assay will be financed by the Ministry of Health, Labour and Welfare (MHLW), Japan.

The lead laboratory will support the IL-2 Luc assay validation trial by assuring that reliability is assessed. At the same time, preliminary results of the test method can be evaluated. For this purpose Lead laboratory will support:

- the financial aspects related to the coordination of a validation trial (e.g. organization of VMT meetings where also the involved test facilities can be invited for technical clarifications to the VMT, the publication of the validation trial results)
- the test, reference and control item purchase, coding and distribution to the test facility
- the availability of the test systems to the participating laboratories by supporting the Lead laboratory with the logistics for delivering the test system to the facility
- the independent data analysis and statistical support (biostatistician) based on the study reports generated
- the other costs for participating laboratories

3.4Trial coordination

Dr. Hajime Kojima was appointed as the Trial Coordinator with well-defined roles and responsibilities to coordinate the trial and to establishment of a VMT by supporting of JaCVAM.

The name and location of the Trial Coordinator should be identified in each individual study plan. For the IL-2 Luc assay validation trial, the Trial Coordinator has direct access to the test item coding.

The Trial Coordinator's responsibilities include:

a) Establishment of/support to lead laboratory, including meeting organization

b) Trial communication and coordination with test facilities

c) Recording of document and data flow between test facilities

d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial

e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis

f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratories and other test facilities involved in the validation trial, and should reflect the overall trial

g) Approval with date and signature of all protocols, Study Plans and Study Reports

h) The communication of the results of the trial into the public domain

The role of Trial Coordinator (as the formal representative of the VMT and the single contact point with the SDs) is of fundamental importance. The Trial Coordinator is the single critical point of trial control and must ensure clear lines of communication between the involved test facilities in the trial. The communication line of the Trial Coordinator is with the SDs of the different test facilities. The SDs are the single point of contact with the Trial coordinator (unless otherwise communicated by the participating Test Facilities) to assure a transparent and recorded documentation flow during the trial. The Trial Coordinator should also ensure that appropriate arrangements have been made for the supply of the test systems, and test, control and reference items, which meet the requirements of the trial, and that there are appropriate test method protocols (dated signature by the trial coordinator and the Lead Laboratories) and, if appropriate, validated data recording, data analysis, data reporting sheets for the test method.

It is the responsibility of the Trial Coordinator to approve the study plans send for approval by the test facilities, and any amendments to the study plan, by dated signature.

3.5Training

The lead laboratory will be responsible for issuing a training agenda to the Trial Coordinator for further distribution to the all test facility giving details what training aspects will be covered during the training of the other SDs and Study Personnel at the lead laboratory. Furthermore, after the training, the lead laboratory will issue to the Trial Coordinator a training report and indicating if critical observations are made by the other test facilities regarding the IL-2 Luc assay protocols. In case any critical observations are made a new version of the IL-2 Luc assay protocols might necessary be issued to the other test facilities before initiating the between-laboratory transferability.

3.6 [Module 2] Within-laboratory reproducibility

The within-laboratory reproducibility of the all test facility has been done by an independent biostatistical analysis, under the VMT. The proportion of concordance should be equal or more than 80% as tentative acceptance criteria for phase I validation.

3.7[Module 3] Between-laboratory transferability

This between-laboratory transferability (Module 3, identical to ICCVAM proficiency testing phase) is performed in order to assess the successful transfer of the assay to a test facility unexperienced with that particular test method but having knowledge of similar test systems and endpoint detection methods.

For the transfer of IL-2 Luc assay to the all test facility, the Phase 0 study using non-coded five chemicals was performed. A few concentrations of each test item will be tested in triplicate in 3 independent runs according to the IL-2 Luc assay protocol describing the details of the experimental design.

The five test items selected for the phase I study are coded as follows: A, B, C, D, and E. The all facility will prepare a study according to internal GLP principle. This plan will be submitted to the Trial Coordinator and lead laboratory for approval.

The results of the between-laboratory transferability will be reviewed before progressing with module 4 on the between laboratory reproducibility. If the transferability data do not meet test acceptance criteria, the Trial Coordinator representing the VMT will try to identify the problems and make corrections where needed. At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the Trial Coordinator (study plan in GLP principle, raw data, records and data analysis, study report in GLP principle).

3.8 [Module 4] Between-laboratory reproducibility

Ten coded test items have been selected to confirm the between-laboratory reproducibility in the phase I study. A few concentrations of each test item will be tested in triplicate according to the IL-2 Luc assay method protocol describing the details of the experimental design.

At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the trial coordinator (study plan in GLP principle, raw data, records and data analysis, study report in GLP principle). The proportion of concordance between-laboratory reproducibility should be equal or more than 80% as acceptance criteria,

3.9[Module 5] Predictive capacity

The necessity for further chemical analysis will be subject to a VMT decision once the data of the between laboratory reproducibility has been assessed. Depending on the statistical analysis the lean design for validation as well as the automatisation of the test leading to an increased dataset will be considered.

4. Protocol

In this validation trial, the protocol (ver. 1E) will be used (attached Document #2). This protocol will make up a draft by the lead laboratory and be finalized by VMT.

A measurement of bioluminescence intensity induced with chemical treatment will be measured by luminometer (Phelios: ATTO, Cat #:AB-2350) calibrated using stabilized SLG, SLO and SLR enzymes in this validation trial.

5. Chemicals

5.1 Chemicals Selection

Test chemicals have been selected by chemical repository based on published papers on in vivo immunotoxicity

The applied selection criteria were:

- information on mode/site of action
- coverage of range of relevant chemical classes and product classes quality and quantity of reference data (*in vivo* and *in vitro*)
- high quality data derived from animals and (if available) also humans
- knowledge on interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)
- coverage of range of toxic effects/potencies

- chemicals that do not need metabolic activation
- appropriate negative and positive controls
- physical and chemical properties (feasibility of use in the experimental set-up as defined by the CAS No.)
- single chemical entities or formulations of known high purity
- availability
- costs

In the first phase of the selection procedure, the Chemical Selection Committee identified and collected several existing lists of potential chemical sensitizing in order to establish a primary database. These chemicals had originally been compiled by international experts for various purposes e.g. as reference compounds for validation studies. An extensive literature research was performed by the Chemical Selection Committee in order to insure that the preselected chemical fulfilled the selection criteria described above.

Emphasis was laid on the fact that different potencies (strong, weak and no activity) have been chosen. In addition, it was decided that at least 20% of the total substances to be tested should be negative in order to increase the statistical power of the data analysis.

In the first phase IL-2 Luc assay validation trial with data generation at the test facilities, five chemicals will be tested three times in each test chemical for between-laboratory reproducibility and to confirm transferability. After discussion of Phase I results, detailed test planning of the Phase II will be determined. At this moment, twenty chemicals will be planned in the phase II trial for predictive capacity (Table 2).

Table 2. Outline of test planning at each study in the validation trial.

Study		Chemicals	Test Number	Information obtained
Phase (planning)	0	5 non- coded	1	Between-lab transferability
Phase (planning)	I	5 coded	3	Within & between-lab reproducibility
Phase (planning)	II	20 coded	1	Between-lab reproducibility & predictability

(Planning of Phase II will be determined after discussion of the results of Phase I)

5.2 Chemicals Acquisition, Coding and Distribution

The assessment of within-laboratory reproducibility (Module 2), between laboratory transferability (Module 3) in the all test facilities have been performed with coded chemicals. This IL-2 Luc validation trial plan describes the generation of the missing data sets under coded test item. If the results obtained are not very similar to the previous obtained sets, the VMT has to assess if coded chemicals need to be tested in the all test facilities.

The coding will be supervised by the Trial Coordinator, in collaboration with the chemical repository responsible of coding and distribution of test, reference and control items for the validation trial.

5.3 Handling

Each test facility shall receive through the Trial Coordinator essential information about the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions). Moreover, the SD should receive the safety information concerning the hazards identification and exposure controls/personal protection.

6. Records and archiving

At the end of the trial, the IL-2 Luc assay validation trial report is prepared by the Trial Coordinator or the VMT personnel who appointed by the Trial Coordinator. The trial report summarizes the trial goals, procedures, results and conclusions of the validation trial. This represents the whole validation trial, including archiving and, as such, will cover several study reports, as well as reports for test item supply, data management and statistics. The Trial Coordinator oversees the preparation of the trial report. The Trial Coordinator will be representing the VMT discussions responsible for preparation of the scientific conclusions. Signatories to the trial report include the Trial Coordinator, the statistician, and the SDs of the involved test facilities. Although the SDs may not be involved with the preparation of the trial report, their signatures confirm that the trial report is an accurate reflection of the management and study events. The trial report should contain a statement, signed by the Trial Coordinator, commenting on the accuracy and completeness of the trial report and identifying any significant issues which could have affected the integrity of the trial, including matters of GLP compliance. A QC statement will be included in the trial report, in order to identify what QC monitoring was done and to confirm whether or not the trial report is an accurate reflection of the validation trial data.

7. Study timeline

An approximate schedule for IL-2 Luc assay validation trial is shown in Table 3.

Duration of this validation trial is around twenty -month from May 2016 to Nov 2017.

Table 3. Schedule of IL-2 Luc assay validation trial

Month	Activity
January 2016	Establish the VMT
	Selection of participating research laboratories
	Deliberation, decision and read-through of draft study plan
	Deliberation and decision of protocol
	Preparation of a tentative list of test chemicals
	Distribution of test chemicals, standard chemicals and positive control chemicals
February,2016	Technical transfer using five known chemicals (non-coded) Start of technical transfer **to know between laboratory transferability**
	Data collection of technical transfer (**Phase 0 study**)
Phase I study	
May 2016	Coding and distribution of five coded test chemicals
June, 2016	Start of Phase I study
September, 2016	End of Phase I study
January, 2017	**2nd VMT Meeting** / Phase I results and planning of Phase II study
Phase II study to know between- and within-laboratory reproducibility	
2017	Coding and distribution of coded test chemicals and positive chemicals
2017	Start of Phase II study using 20 coded test chemicals
2017	End of Phase II study
January, 2018	**3rd VMT Meeting** /reviewing of Phase II study results
2018	Completed validation report

Abbreviations

CAS: Chemical Abstracts Service
GLP: Good Laboratory Practice
HRI: Hatano Research Institute
FDSC: Food and Drug Safety Center
JaCVAM: Japanese Centre for the Validation of Alternative Methods
NIHS: National Institute of Health Sciences
OECD: Organization for Economic Co-operation and Development
QC: Quality Control
TG: Test Guideline
VMT: Validation Management Team

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ENV/JM/TG/M(2015)6

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

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ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

OECD

Test Guidelines Programme

Draft Summary Record: Expert Group Meeting on Skin Sensitisation

14-15 October 2015 Paris,
France

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Draft Summary Record: Expert Group Meeting on Skin Sensitisation

14-15 October 2015

Paris, France

1. Opening of the meeting

1. The meeting was chaired by the OECD Secretariat. Participants from Denmark, France, Japan, Korea, Netherlands, Spain, Switzerland, United Kingdom, European Commission (European Chemicals Agency and Joint Research Center), BIAC and ICAPO attended the meeting. The list of participants is available in Annex 1. The Chair introduced the draft agenda, indicating that the main objective was to address comments received from the WNT in July 2015 on the draft Test Guidelines for human Cell Line Activation test (h-CLAT). The second objective was to discuss the status and issues related to other assays on skin sensitisation, either on-going projects included in the workplan or potential new projects.

Part 1: human Cell Line Activation test (h-CLAT)

2. Discussion of the main issues raised by the WNT comments

2. Joao Barroso (EC) presented the main issues with h-CLAT, raised by WNT comments, mainly h-CLAT reproducibility, applicability and limitations. This was followed by a presentation from Roman Liška (EC) of the re- analysis of the within and between laboratory reproducibility of the h-CLAT. He explained how new approaches were used to assess the within and between laboratory reproducibility of the assay in the validation study, i.e. the permutation of runs' predictions that takes into account all possible sequences of events and the bootstrap probabilistic approach.

3. It was clarified that these new approaches can be used since the runs conducted in the test are fully independent: the runs are conducted on different days and if done on the same day, all preparations have to be re-done. How they happen in time is thus random and the runs are interchangeable. The group agreed on the methodology for calculation of the WLR. Based on the new approaches, the value of the WLR in the validation study was found to be between 82.4 and 84.8% (while the WLR in the h-CLAT validation report was of 80.0% and the target WLR was of 85%). Despite the fact that the level of WLR is slightly below the target, it was agreed that the level of WLR is transparently reflected in the TG, which specifies that "The level of reproducibility in predictions that can be expected from the test method is in the order of 80% within and between laboratories". It was noted that the 'Report on re-analysis of the within and between laboratory reproducibility of the h-CLAT' would be made available together with the validation report (after approval process) in the Series on Testing and Assessment on the OECD public website.

4. To better control certain parameters that could be a source of variability and thus to reduce the variability of the assay, some revisions to the draft Test Guideline had been proposed before the meeting. The optimisation work conducted throughout the development of the test method to minimise sources of variability were presented by Masaki Miyazawa and Takao Ashikaga (BIAC). It was in particular proposed to better control cell density (relevant changes brought to paragraph 19 of the draft TG) and the exposure time (paragraphs 24, 25 and 29 revised). In addition, as the reactivity check using controls is regularly performed, the group also agreed to emphasise in the draft TG, on the need to use the data from the reactivity check as a mean of verification of laboratory proficiency. For this purpose, a new paragraph was added in the section on laboratory proficiency and the test report was also revised accordingly.

5. It was agreed that since the result of a test is based on 2 concordant runs, when the first two runs are concordant, it is not necessary to run a third one. The group agreed on the wording of the draft TG that says: "each test chemical is tested in at least two independent runs to derive a single prediction". "If however, the first two runs are not concordant for at least one of the markers (CD54 or CD86), a third run is needed and the final prediction will be

based on the majority result of the three individual runs (i.e., 2 out of 3)" (paragraphs 29 and 33). A figure of the prediction model used in the h-CLAT test method (figure 1 of draft TG) was considered to be a very useful addition to the draft TG.

6. The group also discussed the number of replicates that should be used in the TG and it was agreed that because a prediction is obtained from at least two independent runs, one replicate could be enough. Paragraph 29 was revised to provide this rationale.

7. Regarding the limitation of the assay and description of the applicability domain, it was recognised that the assay presents limitations for the detection of three types of chemicals, which were described in the TG: (i) pro/pre- haptens, (ii) substances of low water solubility and (iii) strong fluorescent chemicals. The group agreed with the proposed text (paragraph 12), after further harmonised with the wording used in the Keratinosens TG. It was acknowledged that the detection of pro/pre-haptens is an area where progress needs to be made (an upcoming workshop dedicated to discussing this topic was mentioned), but the current state of knowledge doesn't allow to provide more details in the TG.

3. Other issues

8. A few other topics were discussed such as the use of the word 'solvent' vs 'vehicle' in the TG. It was agreed to replace 'solvent' by 'solvent/vehicle' throughout the text of the TG to acknowledge the fact that some test chemicals can be dispersed (and 'solvent' would not be appropriate in this case).

9. There was some discussion and request for clarification about the sentence in paragraph 11 relative to the testing of mixture: "However, before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for the testing of the mixture." This sentence was developed and agreed by the WNT at the meeting in April 2014. It was clarified that it should be understood in a context of Classification and Labelling, to express that when a mixture is tested and the result is negative, the substances that are part of the mixture can't be declassified on the basis of the results obtained from the mixture.

10. It was suggested to provide the historical relative fluorescence activity (RFI) value and the CV75 value (75% of cell viability) obtained for each proficiency substance included in Table 1 of Annex 2 of the TG. Having the ranges for these values, derived from the 4 laboratories which participated in the validation study, was considered particularly useful to facilitate comparison of data in case other antibodies and cytotoxicity markers are used. Japan/ EURL ECVAM will go back to the data obtained from the laboratories involved in the validation study and investigate the relevance of providing this information. If relevant, these data will be included in the revised version of the draft TG.

11. In terms of next steps, the Secretariat indicated that Japan/EURL ECVAM will now prepare the next version of the draft TG, based on the outcome of the meeting, and update the responses to comments received from the previous WNT commenting round, as appropriate. The revised draft TG will be circulated to the WNT for written comments before the end of the year. It is expected that the draft TG is then submitted for approval at the next meeting of the WNT, in April 2016.

Part 2: Other assays on the OECD Test Guideline workplan

4. Myeloid U937 Skin Sensitization Test (U-SENS) for identifying skin sensitization potential of chemicals

12. Nathalie Alépée (France) presented the history of the U-SENS™ skin sensitisation test, the outcome of the validation study and the statistical analysis that has been conducted. Like the h-CLAT, this assay addresses the 3rd key event in the skin sensitisation pathway, i.e. activation of dendritic cells. Following pre-validation of the assay, the validation phase started in 2013 and the validation test report was submitted to EURL ECVAM in July 2014. Following EURL ECVAM initial review, responses from France and the revised validation report are expected to be available in November 2015. In view of its subsequent peer review by ESAC, a new ESAC group for skin sensitisation is being established.

13. It was noted that the BLR and WLR values (respectively 84.2% and 91.7%) were above the h-CLAT values. It was suggested that this could come from a few differences between the 2 assays, such as the difference in cell line or the fact that this assay only looks at the expression of one type of cell surface markers (CD86). Some participants considered that the prediction model in case of inconclusive result is complex and might be difficult to use.

14. The possibility to develop a Performance-Based Test Guideline (PBTG) including h-CLAT and the U-SENS test methods was mentioned. It was noted however, that for the time being, considering the respective stages of development of the 2 draft TGs, the h-CLAT would be developed as a stand-alone TG and when the U-SENS is ready, the TG would be adapted and turned into a PBTG that would include both test methods. At that time, Performance Standards (PS) would also need to be developed. It was clarified that PS are a document that accompany a PBTGs and are intended to be a guide for the developers of new or modified test methods, similar to the validated reference methods. They communicate the basis by which new test methods can be determined to have sufficient accuracy and reliability for a specific testing purpose.

5. IL-8 Luc assay: An In Vitro Method for Identifying the Skin Sensitisation Potential of Chemicals

15. Setsuya Aiba (Japan) presented the validation report of the IL-8 Luc assay. This assay is based on IL-8 production, which is also a marker of dendritic cell activation in allergic contact dermatitis. The assay was optimised over the various phases of the validation, leading to significant improvement of the performance of the system.

16. Its peer review started in March 2015 and the final peer review meeting was expected to be held in Japan, on 23/24 October. Initial comments from the peer review panel (PRP) were presented by the chair of the PRP, David Basketter (UK). The validation report was well received by the panel. Regarding the BLR and WLR, the PRP concluded that the "data on BLR, with a sufficient number of test chemicals, exceeded the success criterion". However, although the "average WLR met the success criterion of 80%", "the data on WLR was more limited than comparable validation studies, particularly in respect to the number of chemicals tested". "Consequently, the PRP recommends additional assessment of within laboratory reproducibility with more and different test chemicals using the final protocol and prediction model". It is expected that the PRP report is available early 2016.

Part 3: New projects, not yet on the OECD workplan

6. LLNA: BrdU-FCM

17. Korea plans to submit a Standard Project Submission Form (SPSF) in November for inclusion in the TG workplan of a new project for the development of a TG on non-radioactive Mouse Local Lymph Node Assay using Flow-Cytometry Method (LLNA: BrdU-FCM). The method and outcome of the validation study were presented by Ilyoung Ahn (Korea). The method is a modified method of the LLNA: BrdU-ELISA (TG 442B) which enables to reduce the number of animals tested in pre-screen tests, compared with the existing LLNA test methods. The validation study has been conducted to evaluate its reliability and relevance based on the performance standards available in Annex I of TG 429 and Guidance Document 34. The validation study is almost finished and is planned to be completed in December.

18. The presentation was well received and the pre-screen step of the protocol considered an interesting development. However, as this assay is an in vivo assay it was uncertain if the WNT would consider it as a priority for inclusion in the workplan. As an alternative option, it was suggested that the project could consist in an update TG 442B to include also the BrdU-FCM method, rather than to create another LLNA TG.

7. SENS-IS assay

19. France plans to submit an SPSF in November for inclusion in the TG workplan of a new project for the development of the SENS-IS™ assay. The method and outcome of the validation study were

presented by Hervé Groux (France). The assay is a new approach for the identification of skin sensitisers where genes specifically modulated in sensitised skins allow the detection of sensitisers in a reconstructed human skin model. The results of the validation study, conducted in 3 laboratories were very promising and although it is a patented method the group showed interest and supported further development.

8. Task Force on Hazard Assessment (TFHA) and skin sensitisation activities

20. Joop De Knecht (Secretariat) updated the Expert Group on the activities of the Task Force for Hazard Assessment (TFHA) related to Integrated Approaches to Testing and Assessment (IATA) and their application. He presented the scope of 2 draft documents currently under development and discussion by the TFHA: a Guidance Document on the reporting of IATA and a Guidance Document on the reporting of structured approaches to data integration and individual information sources used within IATA for skin sensitisation. The objective of developing these documents is to provide a consistent approach to the documentation of IATA, which is a preliminary step towards harmonisation. It was noted that a lot of strategies are currently proposed and questioned if the objective is to tend towards a final one with time. It was indicated that the objective is to provide tools for assessment but today it is hardly possible to say if one strategy is better than the other.

平成27年度厚生労働科学研究補助金（化学物質リスク事業）

分担研究報告書

データシートでの毒性判定結果提示のための平均値の比の95%信頼区間の計算の検討

分担研究者：大森崇

研究要旨

[背景と目的] 化学物質免疫毒性評価系としてmodified MITAが構築されつつある。modified MITAのバリデーション研究を行うにあたり、プロトコルに適したデータシートを作成する必要がある。試験施設の実験者がデータシートにデータを入力した段階で判定結果を知る必要が望ましい。毒性判定結果を平均値の比の95%信頼区間を用いる場合、Excelの関数であるt.inv関数ではうまく結果を返すことができないことがわかっている。本研究では、t.inv関数を用いずに山内の近似式として知られる近似式によって適切に平均値の比の95%信頼区間を得ることができるかどうかを検討した。

[方法] これまでに実施された計4168の実データを用い、山内の近似式による97.5%点と比の95%信頼区間の下限の値を統計解析ソフトRで算出したこれらの値と比較することを行った。

[結果] 検討に用いた実データの小数自由度が3~6の範囲であった。この範囲においてパーセント点も95%区間の下限もRと山内の近似のどちらもほぼ同様な値を取っていることがわかった。

[結論] Excelによるデータシートで平均値の比の95%信頼区間を算出する際には、Excelのt分布のパーセント点を計算する関数であるt.inv関数を用いるのではなく、山内の近似式である(1)式によってパーセント点を計算し、平均値の比の95%信頼区間を得ればよい。

A. 研究目的

Multi-ImmunoToxicity assay (MITA)は化学物質免疫毒性評価系として開発された試験法である。現在この試験法の改良がなされIL-8 Luc assayを加えたmodified MITAが構築されている。今後国内外から免疫毒性の専門家を招き、MITAの科学的意義、作成したadverse outcome pathwayならびに試験法プロトコルの妥当性などについて議論する予定である。本研究班では、modified MITAを用いたIL-2転写活性抑制を指標としたT細胞の分化異常誘導化学物質評価系と、IL-8転写活性増強を指標とした気道刺激性物質評価系による試験法ガイドラインをめざしており、その目的のために多施設のバリデーション研究を計画する必要がある。

多施設バリデーション研究では、施設内/施設間再現性の評価ならびに関連性の評価が必要となる。その際、各試験実施施設に被験物質が送付され、これらの物質を用いて提案されたプロトコルに基づき実施された試験の結果が試験法のプロトコルに沿って作成されたデータシートに入力される。最終的な施設内/施設間再現性や関連性の評価は、

入力されたデータは解析施設に集められ、作成されたデータセットの下で行われる。

ところで提案される試験法は複数の繰り返し実験の結果で判定されることが多い。modified MITA においてもこの試験法を構成する一つである IL-8 Luc assay は複数の実験結果によって毒性の判定が行われる。IL-8 Luc assay は 4 回の実験結果の中で 2 回の陽性結果が得られれば毒性ありと判定することとなっている。このことはすべての被験物質について 4 回の実験を行うことをしなくても最低 2 回の実験結果で毒性を判定できる場合があることを意味する。必要最小限の実験でバリデーション研究を行うことができるならばコスト削減や期間短縮が期待できる。しかし、そのためには試験実施施設における実験実施者が、各実験の実験データをデータシートに入力した段階で実験結果が陽性であるのか陰性であるのかを知る必要がある。つまり、入力したデータシートは実験結果が提示されるように設計する必要がある。

データシートは多施設で試験を行うどのような施設でも簡便に扱うことができ、後の試験法の普及を考えると特別なソフトウエアを必要としないことが望ましいであろう。我々は Microsoft 社の Excel が広く普及している現状を考慮し、Excel を用いたデータシートを作成してきた。modified MITA の場合もその予定である。

毒性試験法はある被験物質について複数の濃度とその反応の関係で毒性を評価することが多い。ある 1 回の実験において、被験物質がの濃度が 0 である反応の測定値の平均値と特定の濃度の反応の測定値の平均値の比の大きさで陽性/陰性の判断を行うことが多く、modified MITA の場合も例外ではない。2 つの平均値の比の値に基づく判定では、陽性か陰性かの判定を行うための基準値の大きさは試験系が開発される過程で決められることが多い。一方で、比を算出する際に用いた個々の測定値のばらつきを考慮して、統計的な差によって判定することも可能であり、これは 95%信頼区間を用いることで行うことができる。

平均値の比の 95%信頼区間には、t 分布の 97.5%点が必要となる。[1] t 分布の 97.5%値は自由度と呼ばれるパラメータの関数であるため、自由度を与える必要がある。平均値の比の場合、比を構成するそれぞれの平均値を得るために用いたデータで得られるそれぞれの分散が等しいと仮定を置く場合には、自由度は整数となるが、そのような仮定ができない場合は、小数自由度を求めて使うことなる。[2] 毒性試験の場合、被験物質の濃度が 0 の測定値のばらつきは、特定での測定値のばらつきより小さくなることが観察されることが多いため、分散が等しいという仮定を置くことは避けることが望ましいであろう。つまり、小数自由度を用いた t 分布の 97.5%点が必要となる。ところが、Excel に組み込まれている t 分のパーセント点を計算する関数である t.inv 関数は、小数自由度を適切に反映しておらず、小数の値を入力しても整数に切り上げた自由度でのパーセント点を返すものになっている。先に記述したよう、データシートは Excel を用いることが望ましいとなると、小数自由度に対応した平均値の比の 95%信頼区間をシート上で計算できる必要がある。通常の統計ソフトに導入されているパーセント点の計算方法は公開されていないため知ることができない。一方、t 分布のパーセント点の近似式として山内の近似式として知られる方法がある。[3] 山内の近似式は Excel に組み込むことができる程度に簡便な近似式である。

そこで、本研究ではすでに研究結果が得られている modified MITA を構成する一つの試験法である IL-8 Luc assay のデータを用いて、山内の近似式により算出される 97.5%点と 95%信頼区間の下限について、統計解析ソフトである R で計算され

る結果の比較を行い、山内の近似式のデータシートへの適用可能性を検討することを目的とした。

B. 研究方法

B.1. 検討に用いたデータ

これまでに IL-8 Luc assay は、バリデーション研究を通して得られた 379 実験分データを用いた。この試験は 0 濃度と 11 の濃度の試験からなるため、計 4168 のパーセントが得られることになる。

B.2. 山内の近似式

自由度 ν の t 分布の 97.5%点を $t_{0.975}(\nu)$ とすると、山内の近似式は $t_{0.975}(\nu)$ を

$$t_{0.975}(\nu) \approx u + \frac{y_1(u)}{\nu} + \frac{y_2(u)}{\nu^2} + \cdots + \frac{y_5(u)}{\nu^5} \qquad (1)$$

で近似する。ただし $y_1(u) = (u^3 + u)/4$,

$y_2(u) = (5u^5 + 16u^3 + 3u)/96$,

$y_3(u) = (3u^7 + 19u^5 + 17u^3 - 15u)/384$,

$y_4(u) = (79u^9 + 776u^7 + 1482u^5 - 1920u^3 - 945u)/92160$,

$y_5(u) = (27u^{11} + 339u^9 + 930u^7 - 1782u^5 - 756u^3 + 17955u)/368640$,

$u = 1.96$ である。[3)]

B.3. パーセント点と 95%信頼区間の下限の比較

1468 の比のデータについて、上記の式で得られる山内の近似式により算出される 97.5%点の値と 95%信頼区間の下限の値を、統計解析ソフトである R で計算されるそれぞれの値と比較した。

C. 研究結果

C.1. 小数自由度の要約

検討に用いた 1468 の比のデータについての要約統計量を表 1 に示す。

表 1　小数自由度の要約統計量

サイズ(n)	平均値	標準偏差	最小値	中央値	最大値
4169	4.49	1.12	3.00	4.58	6.00

自由度の範囲は 3～6 の範囲にあることがわかる。

C.2. 97.5%点の比較結果

検討に用いた 1468 の比のデータについて R により得られる 97.5%を横軸に、山内の近似式により得られる 97.5%を縦軸にとった散布図を図 1 に示す。

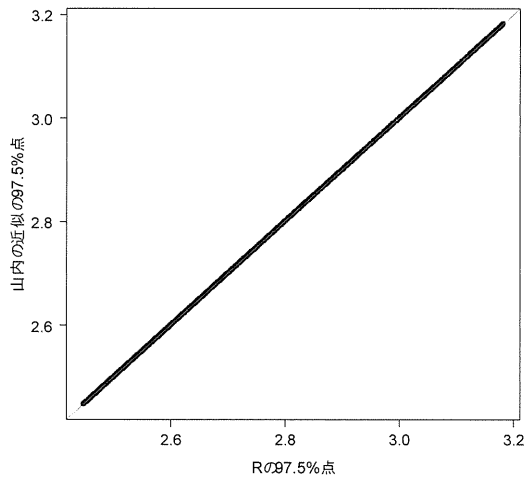

図 1　R と山内の近似の 97.5%点

C.3. 95%信頼区間の下限の比較結果

検討に用いた 1468 の比のデータについて R により得られる 95%信頼区間の下限を横軸に、山内の近似式により得られる 95%信頼区間の下限を縦軸にとった散布図を図 2 に示す。図において点線で示した参照線は、それぞれ 1 の値のところであり、これは 95%信頼区間の下限を用いたときの統計的有意差の基準となる値である。

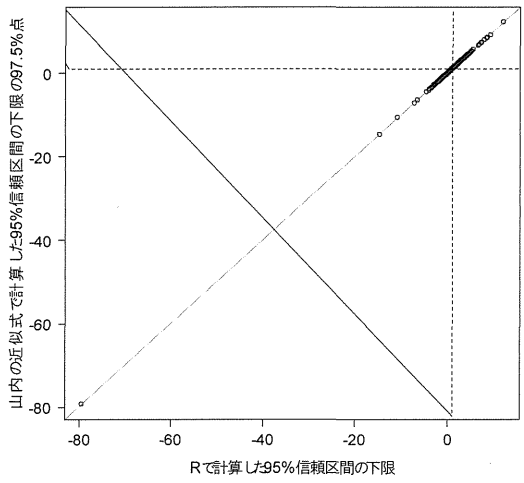

図2　Rと山内の近似の95%信頼区間の下限

D．考察

表1では1468の比のデータの自由度の中央値は4.58である。この値を用いてExcel2010のt.inv関数で、97.5%点を計算するとエラーもなく2.776445という値が返される。しかし、この値は自由度を4としてt.inv関数で計算しても同じ値である2.776445が返されてしまう。一方、Rのパーセント点を計算する関数qtを用いた場合、自由度が4.58の場合には2.643129が、自由度が4の場合には2.776445が返され、小数自由度に対して適切な値を返していることがわかる。

図1よりパーセント点はRと山内の近似のどちらもほぼ同様な値を取っていることがわかる。また、図2より95%区間の下限もRと山内の近似のどちらもほぼ同様な値を取っていることがわかる。よって、山内の近似式は統計解析ソフトRを用いて解析するのと同様の結果を得ることができることがわかった。

よって、Excelでデータシートを構築する場合には、Excelのt分布のパーセント点を計算関数であるt.inv関数を用いるのではなく、山内の近似式である(1)式によって計算し、比の95%信頼区間を得ればよい。この結果を今後modified MITAのバリデーション研究で行う際に作成するデータシートを反映させることにする。

Excelは広く普及しているソフトウエアである。Rはフリーのソフトウエアであるという利点があるものの、実験実施者にとって統計ソフトは馴染みがあるソフトウエアではないため、Rでの解析を実験実施者に求めるべきではないであろう。また、Excelのt.inv関数の問題はExcelのバージョンが更新されることで問題は解消されるかもしれない。しかしながら、どのような実験施設も常に最新のExcelを有し、利用しているとは考えにくい。

E．結論

以上より、Excelによるデータシートで平均値の比の95%信頼区間を算出する際には、Excelのt分布のパーセント点を計算する関数であるt.inv関数を用いるのではなく、山内の近似式である(1)式によってパーセント点を計算し、平均値の比の95%信頼区間を得ればよい。

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F．健康危険情報

なし。

G．研究発表

なし。

H．知的財産権の出願・登録状況

なし。

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厚生労働科学研究費補助金（化学リスク研究事業）
免疫毒性評価試験法Multi-ImmunoTox assayの国際validationへ向けての検討
分担研究報告書

化学物質のMITAによる解析、validation、プロトコール作成

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研究要旨
厚生労働科学研究費補助金事業「多色発光細胞を用いたhigh-throughput免疫毒性評価試験法の開発」にて開発した新たな*in vitro*免疫毒性評価試験法（Multi-ImmunoTox assay：MITA）について現時点で得られたdata setおよび相場によるIL-2転写活性抑制を中心とした免疫毒性AOPをもとに国際バリデーション用の試験法プロトコール、データシート、記録用紙を作成した。施設間試験の実施者に対し試験法の説明会を実施し技術移転を図った。国際バリデーションに先立ち技術移転性を確認するため5物質でのトレーニングを行い、プロトコールの問題点を改良しPhase I試験に臨む予定である。

キーワード：試験法プロトコール、技術移転性、バリデーション

A．研究目的

厚生労働科学研究費補助金事業「多色発光細胞を用いた high-throughput 免疫毒性評価試験法の開発」にて開発した新たな *in vitro* 免疫毒性評価試験法（Multi-ImmunoTox assay：MITA）のOECD（Organisation for Economic Co-operation and Development）における試験法ガイドライン（Test Guideline：TG）化を目的とし、試験法プロトコールを作成し国際バリデーションの準備を行う。

B．研究方法

以下の方法によりIL-2およびIFN-γプロモーター活性の測定を行った。ヒトTリンパ芽球性白血病由来細胞株JurkatにIL-2プロモーターに制御されたSLGルシフェラーゼ遺伝子（緑色に発色）、IFN-γプロモーターに制御されたSLOルシフェラーゼ遺伝子（橙色に発色）、GAPDHプロモーターに制御されたSLRルシフェラーゼ遺伝子（赤色に発色）を導入した#2H4細胞を1ウェル当たり2×10^5個、黒色の96-well プレート(Greiner bio-one)に播種し化学物質を加え、37℃、5%CO_2下で1時間培養した。つづいて25nM PMAと1μM Ioの混合物(PMA/Io)で刺激し37℃、5%CO_2下で6時間培養した。その後、細胞溶解剤とルシフェラーゼ反応の基質であるルシフェリンの混合剤であるTripluc luciferase assay reagent (TOYOBO)を混合し、室温で10分振盪させたのちマルチプレート対応型ルミノメーターにてルシフェラーゼ活性を測定した。SLG、SLO、 SLRルシフェラーゼは共通の基質の存在により同時に発光するが、2枚の光学的フィルターにより分離し、各

ルシフェラーゼの発光量 (SLG-luciferase activity (SLG-LA) 、 SLO-luciferase activity (SLO-LA)、SLR-luciferase activity (SLR-LA)) を検出した。また細胞数の違いや各種刺激後の生存率の違いを勘案しSLG-LA、SLO-LAをSLR-LAで除することによりそれぞれnormalized SLG-luciferase activity(nSLG-LA), normalized SLO-luciferase activity(nSLO-LA)を算出した。さらに以下の式に%suppression抑制率を計算した。

% suppression = (1-薬物存在下でのnSLG-LAまたはnSLO-LA/薬物非存在下でのnSLG-LAまたはnSLO-LA) X 100

各実験において得られた結果は、一元配置分散分析を行い、その後Dunnett検定により有意な抑制効果，増強効果があるか否かを検討した。しかし、この実験を3回繰り返し検討すると、3回の実験結果が必ずしも一致していない薬剤が存在した。そこで、一致が見られなかった薬剤に関しては、3回の繰り返し実験の結果のなかから%suppressionの絶対値（免疫抑制物質に関しては正の値、増強物質に関しては負の値となる）が最も大きい値を選びStudent's t-testを行い、そこで統計的有意差の得られた場合、その結果を薬剤の最終的判定結果とした[1]。

C. 結果

C-1. 試験法プロトコール、データシート、記録用紙の作成

現時点で得られたdata setおよび相場によるIL-2転写活性抑制を中心とした免疫毒性AOPを参考とし、IL-2, IFN-γレポーター細胞である#2H4細胞を用いた試験法プロトコール、Multi-Immuno Tox Assay protocol ver. 008.1Eを作成し、国際バリデーションに向け英訳した（添付文書１）。データ入力、結果表示用にエクセルファイルをベースとしたdata sheet、Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006を作成した（添付文書２）。さらに参加施設用の記録用紙を作成し各施設に配布した（添付文書３）。

C-2. 試験法の説明会を実施

初めてMITAを行う参加施設の実施者を対象とし当研究室にて説明会を2015年8月と2016年2月の計２回開催した。当研究室において参加者の手技によりPMA/Ioに対する#2H4細胞の反応および陽性コントロール化学物質による抑制が認められることを確認した（図１）。

C-3. Phase 0 試験の実施

MITAの国際バリデーションPhase I試験に先立ち技術移転性を確認するためPhase 0試験用に以下の化学物質を参加3施設に送付した。(2-Aminoanthracene, CH_3HgCl, Chloroquine diphosphate salt, Citral, Dexamethasone) 現在、これらの化学物質を３回ずつアッセイするPhase 0試験を施行中である。

D. 考察

現時点でのクライテリアでは、低濃度で亢進し、高濃度で抑制が見られる化学物質については最終的な結果がばらつくことが予測される。今後Phase 0の結果を参照としPhase Iへ向けたクライテリアの改変を検討する。

E. 結論

国際バリデーション用の試験法プロトコール、データシート、記録用紙を作成した。施設間試験の実施者に対し試験法の説明会を実施し技術移転を図った。国際バリデーションに先立ち技術移転性を確認するため5物質でのトレーニングを行い、プロトコールの問題点を改良しPhase I試験に臨む予定である。

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