Peer Review Panel Evaluation of the Hand1-Luc EST (Embryonic Stem Cell Test) as a Non-Animal Test for Evaluating the Developmental Toxicity of Chemicals

Japanese Center for the Validation of Alternative Methods
National Institute of Health Sciences
Tokyo, Japan

Report completed by the Peer Review Panel on October, 2nd, 2017

Table of contents

Executive Summary

The Japanese Center for the Validation of Alternative Methods (JaCVAM) convened an independent scientific Peer Review Panel (PRP) to evaluate the validation status of the Hand1-Luc EST (Embryonic Stem Cell Test) as a non-animal test for evaluating the developmental toxicity of Chemicals in accordance with established international criteria (OECD, 2005) (1). The Hand1-Luc EST is an *in vitro* test for developmental toxicity, which could be used within an "Integrated Approach to Testing and Assessment" (IATA) for developmental toxicity *in vitro*.

JaCVAM provided the members of the PRP with the validation report, all supporting documents of the study and a questionnaire with 14 evaluation criteria were sent to the members of the PRP before the first meeting of the PRP. In conducting its evaluation, the PRP addressed each of the evaluation criteria that correspond to internationally harmonized validation and acceptance criteria (OECD GD 34).

On November 22 -23, 2016, in Osaka, Japan, the PRP met with the Validation Management Team (VMT) of the study and discussed all aspects of the validation study and in particular the evaluations and questions in the questionnaires submitted by the members of the PRP. The recommendations of PRP have been taken into account and the validation report of Hand1-Luc EST validation study was revised by the VMT (2, 3, 4, 5).

Overall conclusion: The documents supporting the assessment of the new method are complete and allow a sufficient evaluation of the validity of the method. The management and conduct of the multi-center validation study by the VMT were excellent and they are exceeding the usual standard of multi-center studies as far as communication among participants. VMT is concerned and also data exchange and analysis and implementing quality assurance.

Compared to *in vivo* testing for developmental toxicity the test does not require to sacrifice animals, considerably reduces cost, time and required expertise. With the high positive predictive value of the validated prediction model the new *in vitro* test is ideally fit to identify chemicals with a high potential of developmental toxicity within an IATA for assessing developmental toxicity.

Regulatory rationale: At present, there is no *in vitro* test guideline available to assess developmental toxicity. Therefore, the Hand1-Luc EST has been developed to meet the requirements, which the original mEST could not satisfy (6). The validation study showed that Hand1-Luc EST test allows to identify positive developmental toxicants with high confidence and can be used as a high-throughput test for screening purposes. However, to predict developmental toxicity more accurately, this test will need to be combined with other assays within an IATA for *in vitro* developmental toxicity testing.

Scientific rationale: In the Hand1-Luc EST mouse Hand1-ES (KOB1) cells are used, which are transfected with a vector containing the luciferase gene monitored by the Hand1 promoter. Test chemicals are applied to the Hand1-ES cells from the start of differentiation, when the three primordial tissues are not yet formed (day 0= day 3.5 *in vivo*) until day 5 (=8.5 *in vivo*), when ESC are differentiating into cardiomyocytes (mesoderm induction) (2, 3, 4, 5). Thus, depending on the mode of action of chemicals, it is possible to cover a large number of organs formed or specific organs only. Indeed, if the genes playing important roles in the three germ layer formation are affected by the chemical, then all the downstream genes governed may affect the ect-, endo- and mesoderm layer formation.The Hand1 gene is involved in the development and differentiation of heart, limbs and facial bones (7, 8, 9, 10). Therefore, chemicals triggering malformations in these organs may be detected with the Hand1-Luc assay.

Limitations: Differentiation is measured by luciferase activity and thus chemicals interfering with luciferase protein should not be tested. Protease inhibitors cannot be detected in the

Hand1-Luc EST, since protease activity is used to assess cell viability. The Hand1-Luc EST can also not evaluate the effect of metabolites due to the inability of ES cells to metabolize compounds.

Validation study reference chemicals: A sufficiently number of representative, coded chemicals (28) was used in the validation study to evaluate the performance of method. Details of the chemical selection procedure are described in detail in the "chemical selection report". The experts of the PRP concluded that the selection of test chemicals to demonstrate performance of the assay was appropriate.

Assay Reproducibility: The experts of the PRP concluded that the validation report provides excellent information on variability (e.g. coefficient of variation, CV) of the data that were measured in the individual laboratories and on the CV of the within- and between-laboratory reproducibility.

Test method predictivity: The prediction model has been established with a high number of embryotoxicants (71) with different toxicological mechanisms (11). The experts of the PRP were satisfied with the determination of accuracy, predictive capacity and the way in which existing data on developmental toxicity of relevant species have been taken into account.

Data quality: During the validation study quality checks were carefully conducted by an independent expert. All of the participating laboratories are GLP certified and the study was conducted in the spirit of Good Laboratory Practice (GLP). All data were available to the experts of the PRP for review.

Test method protocols: As far as the identification of positive and negative results was concerned, detailed information is provided in the revised version of the protocol, including the acceptance criteria and the calculation used to fit curves to obtain IC_{50} and ID_{50} values. Therefore, the experts of the PRP concluded that the protocol is complete and sufficiently detailed for new laboratories to conduct the Hand1-Luc EST assay.

Applicability domain: There is some evidence that the Hand1 gene and its functions are very similar between human and mouse. The applicability of the Hand1-Luc EST is restricted to the examination of all the pathways related to the Hand1 gene (formation of the heart, limbs and craniofacial bones) and the early period of development (from 3.5 to 8.5 days after fertilization in the mouse). The Hand1-Luc EST can also detect embryotoxicity by disruption of genes involved in the development of the three germ layers (Ectoderm, Mesoderm and Endoderm) due to the exposure to chemicals in and after the undifferentiated stage.

Introduction

The Japanese Center for the Validation of Alternative Methods (JaCVAM) convened an independent scientific Peer Review Panel (PRP) to evaluate the validation status of the Hand1-Luc EST (Embryonic Stem Cell Test) as a non-animal test for evaluating the developmental toxicity of chemicals in accordance with established international criteria (OECD, 2005) (1). The Hand1-Luc EST is an *in vitro* test for developmental toxicity, which may be used within an "Integrated Approach to Testing and Assessment" (IATA) for developmental toxicity.

JaCVAM provided the members of the PRP with the validation report and all supporting documents of the validation study and provided the members of the PRP with a questionnaire of 14 evaluation criteria before the first meeting of the PRP. During the evaluation the PRP discussed each of the evaluation criteria, which are addressing acceptance criteria of international validation studies.

On November 22 -23, 2016, in Osaka, Japan, the PRP met with the Validation Management Team (VMT) of the Hand1-Luc EST validation study and discussed all aspects of the validation study and in particular the topics addressed in the questionnaires submitted to the PRP. The comments and recommendations of the PRP were taken into account in the revised version of the validation report and discussed during a teleconference of the PRP and the VMT on May 27, 2017.

This report summarizes the final evaluation and conclusions of the experts of the PRP following the 14 criteria order submitted by JaCVAM.

Evaluation Criterion 1: A rationale for the test method should be available, including description of toxicological mechanisms, a clear statement of scientific need, and regulatory application.

Chemicals that exhibit the potential for developmental toxicity should be identified and eliminated in the early stages of the development of new chemicals to which consumers, patients and workers are exposed. All of the current guidelines for reproductive toxicity (OECD 414, 415, 416, 421, 422, 426, 443) are requiring to sacrifice a high number of pregnant animals. In addition, the cost triggered by all those experiments is high and specific expertise to conduct them is required. With the implementation of Directive 2010/63/EU, the need for validated alternative methods, in particular in vitro replacement methods, for the safety evaluation of cosmetic substances and products became crucial. This is maintained in the EU Cosmetics Directive (EC) No 1223/2009. However, there is presently no *in vitro* test guideline available to assess one of the endpoints, which are covered by *in vivo* reproductive toxicity tests, e.g. developmental toxicity. Therefore, the Hand1-Luc EST *in vitro* test for developmental toxicity has been developed to meet the requirements, which the original mEST could not satisfy (6).

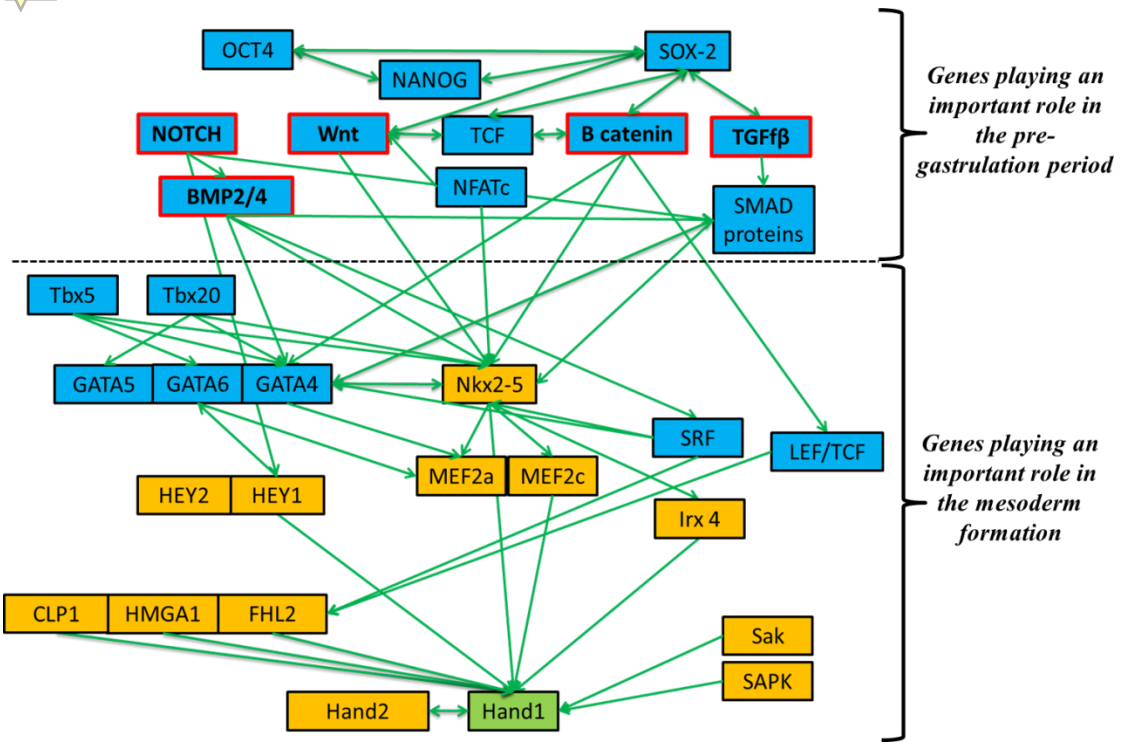
The validated protocol is using Hand1-ES (KOB1) cells transfected with a vector containing the luciferase gene monitored by the Hand1 promoter. Hand1-ES (KOB1) cells are differentiated into cardiomyocytes during 120 hrs of culture (5 days). The proposed protocol includes assays for estimating cytotoxicity followed by measurement of Hand1 promoter activity (differentiation toxicity). This test does not require to sacrifice any pregnant animals, and considerably reduces cost, time and required expertise (2). With the high positive predictive value of the validated prediction model the new *in vitro* test is ideally fit to identify chemicals with a high potential of developmental toxicity within a future IATA for *in vitro* developmental toxicity in vitro.

Compared to the original EST, the Hand1-Luc EST is easier to perform, requires lower quantity of chemicals, has a wider applicability (because the prediction model is based on a higher number of chemicals and Hand1 gene alteration data can be used to create AOP) and

considers concentrations in the culture medium close to the saturated free concentration in fetuses measured in developmental toxicity studies *in vivo*. Therefore, the test allows to identify positive embryotoxicants with high confidence and could become a high-throughput test for eliminating true developmental toxicants and serve as a tool for screening purposes. However, to predict developmental toxicity more accurately, this test will need to be combined with other in vitro assays in an IATA.

A short summary is provided to describe the mechanism of developmental toxicity, which is covered by the Hand1-Luc assay.

Figure 1 shows the possible pathways and genes related to Hand1.



If a chemical affects one of these genes, it may be detectable by measuring Hand1 gene expression. In downstream pathways, Hand1 is related to the development of different organs and the depletion or reduction of its expression is reported to have a negative effect on development. Chemicals have different individual modes of action, since some may affect specific pathways (enzyme inhibition), others may affect a particular physiological function (for example, ion channel inhibition), and others that may affect DNA synthesis (enzyme inhibition or intercalating agent).

In the Hand1-Luc EST, chemicals are applied to the cells from the beginning of differentiation when the three primordial tissues are not yet formed (day 0= day 3.5 *in vivo*) until day 5 (=8.5 *in vivo*) when mESC are differentiated into cardiomyocytes (mesoderm induction). Thus, depending on the mode of action of chemicals, it would be possible to cover a large number of organs formed or specific organs only. Indeed, if the genes playing important roles in the three germ layer formation are affected by the chemical, then all the downstream genes governed may affect the ect-, endo- and mesoderm layer This may be measured by an alteration of the Hand1 gene expression since the mesoderm would be also affected.

In this assay, the measurements are performed 120 hrs after inducing the ES cells into cardiomyocytes. During this time window, the heart, the placenta, the neural tube and the ear are formed. The somite segmentation has begun and the first branchial arch has maxillary

and mandibular components at day 8.5. The limb buds are however not yet visible until day 9. Hand1 gene is involved in the development and differentiation of heart, limbs and facial bones (7, 8, 9, 10). Thus, chemicals triggering malformations in these organs may be detected with the Hand1 assay. However, some chemicals may alter genes expressed 120 hrs. (5 days) after the beginning of differentiation and may not be detected by this assay.

Concerning species differences, the Hand1 gene is well preserved among different mammal species and the amino acid sequence homology between mouse and human is 92% (NCBI homology alignment browser; Basic Local Alignment Search Tool (BLAST)).

As suggested before, a combination with other tests analyzing the development of other organs (bones, neurons, digestive gut) will be crucial to be able to cover prenatal development. For example, three different tests covering the three different germ layers (endo-, meso-, ectoderm) would allow for the understanding of the possible pathways involved in abnormal development and provide information for identifying AOPs.

Evaluation Criterion 2: The relationship between the test method endpoint(s) and the biological effect and to the toxicity of interest should be addressed, describing limitations of the test methods.

Differentiation is measured by luciferase activity and thus chemicals interfering with luciferase protein should not be tested. Protease inhibitors cannot be detected in the Hand1-Luc EST since protease activity is used to assess cell viability.

Another limitation is that the Hand1-Luc EST can not evaluate the effect of metabolites due to the inability of ES cells to metabolize compounds. Metabolism is an important issue of all *in vitro* toxicity tests.

The experts of the PRP concluded that users of the test must be aware of these general limitations of all of the currently available *in vitro* developmental toxicity tests.

Evaluation Criterion 3: A detailed test method protocol should be available

Questions of the experts addressed the appropriateness of the final volume of the vehicles. This point has been detailed in the revised version of the protocol. The highest concentration of PBS (-) that does not alter differentiation is 5% and for DMSO it is 0.1% (lead laboratory data). Moreover, in many *in vitro* tests the highest test concentration is 1000µg/ml. Thus, if the molecular weight of a test chemical is 200, then the final concentration would be equivalent to 5mM, a very high concentration.

As far as the identification of positive and negative results is concerned, detailed information is provided in the revised version of the protocol, including the acceptance criteria and the calculation used to fit curves to obtain the IC_{50} and ID_{50} values.

The experts of the PRP concluded that the protocol appears complete and adequate in detail for a laboratory to conduct the study.

Evaluation Criterion 4: Within- and between-laboratory reproducibility of the test method should be demonstrated

Since in the first draft provided no measure of variability (e.g. coefficient of variation), all CV values have been added in the appropriate appendix to the revised validation report.

Moreover, the report mentioned several times discrepancies between the laboratories regarding the establishment of the maximum dose. Therefore, a paragraph has been added to warn the experimenter to pay particular attention during the precipitation evaluation especially for DMSO dissolved liquids where small bubbles could be hard to distinguish inside the assay medium.

The experts of the PRP concluded that the additional improvements provide excellent information on the within- and between-laboratory reproducibility.

Evaluation Criterion 5: Demonstration of the test method's performance should be based on testing of representative, preferably coded reference chemicals

The PRP concluded that a sufficiently high number of representative, coded chemicals (28) was used in the validation study to evaluate the performance of the method. Details of the chemical selection procedure are described in detail in the "chemical selection report".

Some misclassifications have been noted when comparing *in vitro* and *in vivo* data. The most obvious reason is when the chemical interferes with a pathway not related to Hand-1. Another reason is when the chemical disturbs the development during a period which is not covered by the Hand-1 Luc test. However, this cannot yet be taken into account due to lack of information on AOPs in the field of developmental toxicity both in experimental animals and humans.

The experts of the PRP concluded that the selection of test chemicals to demonstrate performance of the assay was appropriate.

Evaluation Criterion 6: Accuracy or predictive capacity should be demonstrated using representative chemicals. The performance of test methods should have been evaluated in relation to existing relevant toxicity data as well as information from the relevant target species.

The comments of the experts were quite positive, e.g., the prediction model uses only one equation and the solubility of the chemicals is taken into account for the prediction which becomes a way to express the solubility of chemicals in the body fluids. The prediction model has been established with a high number embryotoxicants (71) covering a wide spectrum of mechanisms of toxicity (11).

An evaluation in relation to the target species is quite difficult since the developmental toxicity of chemicals is very variable among different animal species and humans. However, in the current validation study all data banks and publications that are publicly available and covering the endpoint developmental toxicity have been properly taken into account by the managers of the validation study in order to determine the performance of the test method.

Thus, the members of the PRP were satisfied with the determination of accuracy, predictive capacity and the way in which existing data on developmental toxicity data from relevant species were taken into account.

Evaluation Criterion 7: All data supporting the assessment of the validity of the test method should be available for expert review

All data were available to the experts for review. The experts noted that, in several experiments, ID_{50} values were higher than IC_{50} and that this this particular issue was not discussed in the validation study. Therefore, the relevance of such data should be explained and their relevance for determination of the performance of the test.

It was explained that this phenomenon was also observed in the original EST especially for negative chemicals that do not show a gap between the IC_{50} and the ID_{50}. The VMT tried to improve this point by operating measurements in the same plate which was proved to be a good way. During the validation study, they also faced this problem and tried to correct it as much as possible by implementing the curve fitting (2 and 3 parameter curve fittings) according to recommendations by VMT members. As a result, a better ratio IC_{50}/ID_{50} (see the validation report) was obtained. However, in some cases the IC_{50} value was still lower than the ID_{50}. This may be due to the fact that since two different devices were used (luminometer and fluorometer) measuring two different endpoints (cytotoxicity and luciferase activity), the

resulting measurements were not identical for IC_{50} and ID_{50}. Although this may happen, the gap observed when the ID_{50} was higher than the IC_{50} is not dramatically high and does not compromise the validity of the data.

Thus the members of the PRP were satisfied with the availability of the data and the explanations given by the VMT.

Evaluation Criterion 8: Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of Good Laboratory Practice (GLP)

Since there were some doubts that the validation study had been conducted according to principles of GLP, the VMT provided the following explanation:

The validation report has been corrected in part 2.5 (Quality Check monitoring). According to the OECD guidance 34, p21: *"Clear and comprehensive standardized test method protocols, preferably in compliance with GLP Principles, together with standard operating procedures (SOP), as appropriate. This should include a description of the test system, exposure conditions, dose selection procedures, endpoint(s) assessed, measurements taken, specialized equipment or supplies that may be needed, measures of variability, the way in which the results are calculated and expressed, and the use of positive and negative controls and other performance checks."* (1)

During the validation, the quality check was carefully operated by an independent expert. An example of an empty quality check sheet is available for each phase. The QC sheet, the protocol and the calculation sheet contain, what is required by the OECD GD 34:

- Description of the test system: Described in the protocol and in the validation report
- exposure conditions: described in the protocol
- dose selection procedures: described in the protocol
- endpoint(s) assessed: described at the end of the protocol and revealed by the calculation sheet provided
- measurements taken: described in the protocol
- specialized equipment or supplies that may be needed: described in the protocol
- measures of variability: defined and calculated automatically in the calculation sheet
- the way in which the results are calculated and expressed: described at the end of the protocol and the calculations used in the excel sheet were validated
- the use of positive and negative controls and other performance checks: 5-FU was used as a positive control. No negative control was used.

Finally, since all the participating laboratories possess the GLP certification, the following sentence was added at the end of part 2.5:"*Finally, all the participating laboratories possess the GLP certification. The present study was conducted in the spirit of Good Laboratory Practice (GLP).*"

Taking into account these detailed explanations, the experts of the PRP concluded it appears that the answer is "yes."

Evaluation Criterion 9: Applicability domain of the validity of the test method should be defined for expert review

There is some evidence that the Hand1 gene and its function is very similar between human and mouse. The applicability of the Hand1-Luc EST is restricted to the examination of all the pathways related to the Hand1 gene (formation of the heart, limbs and craniofacial bones) and the early period of development (from 3.5 to 8.5 days after fertilization in the mouse). The Hand1-Luc EST can also detect embryotoxicity by disruption of genes involved in the development of the three germ layers (Ectoderm, Mesoderm and Endoderm) due to the

exposure to chemicals in and after the undifferentiated stage.
The PRP experts concluded that the documents provided are demonstrating that the VMT of the validation study have extensively evaluated the applicability domain of the test method and that the applicability domain is sufficiently described in the revised validation report and that, therefore, the answer is “yes”.

Evaluation Criterion 10: Proficiency chemicals should be set up in the proposed protocol

Apparently due to language problems the VMT misunderstood this point. As a consequence, a list of proficiency chemicals has been added to the protocol. Therefore, the PRP experts concluded that the apparent error has been corrected properly in the revised protocol.

Evaluation Criterion 11: Performance standard should be set up with proposed protocol

As described for the proficiency chemicals definition, apparently due to language problems the VMT misunderstood this point. As a consequence, the performance standards have been added in Appendix 44 of the revised validation report.

Therefore, the PRP experts concluded that the apparent error has been corrected properly in the revised protocol.

Evaluation Criterion 12: Advantages in terms of time, cost and animal welfare

Since this information is missing in the validation report, the VMT provided the following information on the Hand1-Luc EST, which the PRP experts accepted as adequate:

The following table shows the approximate cost for one run with one chemical

Material		**Original EST**	**Hand1-Luc EST**
	price		
Cells	D3 (600$/vial)	600$	/
	3T3 (600$/vial)	600$	/
	KOB1-ES (Hand1) (2200$/vial)	/	2200$
Plates	1$/Petri dish	8$	/
	1$/ 60cm plate	8$	
	11$/ 24 well plate	88$	/
	6$/ 96 well plate	100$	/
	15$/96U well white plate	/	15$
Chemical	50$/100mg	~300 mg(=150$)	~30 mg (=15$)
Assay Medium	2$/ml	500 ml (=1000$)	30ml 0$ (contained in the 2200$ kit)
Maintenance medium	3$ +1$(LIF)/ml 2$ + 1$(LIF)/ml	/ 30$	50 ml 0$ (contained in the 2200$ kit) /
MTT assay	70$/100mg	15mg=21$	/
Cell titer fluor	8$/µl	/	80$
Steady glo	13$/ml	/	104$
Total with cells purchase		2605$	2414$
Total without cells purchase		1405$	214$ + ~300$ (medium) = 514$

Furthermore, with the time that can be saved the human resources becomes much cheaper for the Hand1-Luc EST as shown below.

		Original EST	**Hand1-Luc EST**
Time		4 days with 4 hours per day: 16 hours	2 days, 1 hours per day: 2 hours
Human resources ($)	1hr ~ 90$	1440$	180$

In a near future, the kit should be cheaper than it is and an option available is to sell cells only once by conclusion of Material Transfer Agreement that allows the user to create its own stock.

Finally, raising the number of chemicals does not affect very much the time to conduct the Hand1-Luc EST especially during cell seeding.

Evaluation Criterion 13: Completeness of all data and documents supporting the assessment of the test method

The data and documents supporting the assessment of the new method are complete and allow a sufficient evaluation of the validity of the method. The experts of the PRP have therefore concluded that in general the documents are complete.

Evaluation Criterion 14: Validation management and conduct

The management and conduct of the multi-center validation study of the Hand1-Luc EST by the VMT were excellent and they are exceeding the usual standard of multi-center studies as far as communication among participants and VMT is concerned and also data exchange and analysis and implementing quality assurance.

Therefore, the experts of the PRP are concluding that the answer is “yes” based on the summaries of the face-to-face meetings and the conference calls.

Conclusions

The panel concluded that the reproducibility and predictivity of the Hand1-Luc EST assay is sufficient to support its use as an *in vitro* test to identify with high confidence chemicals which are toxic to prenatal development. It could become a high-throughput test for eliminating developmental toxicants early within the screening of chemicals to be used in patents and consumers and to which workers and the environment are exposed. The panel also concluded that compared to *in vivo* testing for developmental toxicity the new *in vitro* test does not require to sacrifice pregnant animals, considerably reduces cost, time and required expertise. The panel proposed that with the high positive predictive value of the validated prediction model the new *in vitro* test ideally fits to identify chemicals with a high potential of developmental toxicity within an IATA for assessing developmental toxicity *in vitro*. In this integrated strategy, negative results in the Hand1-Luc EST assay would require further testing *in vitro* or *in vivo* animals.

Thus, it should not be used as a stand-alone assay.

Appendix 1

Independent Peer Review Panel Members

Horst Spielmann MD, Peer Review Panel Chairman,
Professor, Inst. Pharmacy, FU Berlin, Germany, horst.spielmann@fu-berlin.de
Horst Spielmann is Professor for Regulatory Toxicology in the Institute for Pharmacy, Faculty of Biology, Chemistry, and Pharmacy at the Freie Universität Berlin in Berlin, Germany. He is the former and first head of the National German Center for the Validation of Alternative Methods (ZEBET) at the Federal Institute for Risk Assessment (BfR) in Berlin, Germany, where he directed the standardization and international validation of several *in vitro* methods and also served as the head of the Department of Scientific Services. He served as Germany's first national representative on the Scientific Advisory Committee for the European Centre for the Validation of Alternative Methods at the European Commission's Joint Research Centre in Ispra, Italy, and has served as an expert for many years for the European Commission's Framework Programs on alternatives to animal testing and *in vitro* toxicology. In December 2012, he was appointed as the Animal Welfare Officer for the State of Berlin.

Roland Buesen DVM, BASF SE, Ludwigshafen, Germany, roland.buesen@basf.com,
Roland Buesen, Ph.D., is a veterinary specialist for pharmacology and toxicology with extensive experience in basic and applied health science research areas such as toxicology and cell biology. Since 2007, he works as a study director for several *in vivo* toxicity assays according to OECD and other guidelines including teratogenicity and reproductive toxicology assays. Before 2007, he gained qualified practical experience in all aspects of cell culture techniques, immunology assays, high performance liquid chromatography, as well as development and experimental improvement of *in vitro* assays. He holds several publications in refereed journals dealing with *in vitro* as well as *in vivo* toxicology issues. Dr. Buesen has been speaker at national and international meetings.

Elise Grignard PhD, EURL ECAM, EU JRC, Ispra, Italy, Elise.GRIGNARD@ec.europa.eu
Elise Grignard is a Scientific Officer at the, at the European Union Reference Laboratory for alternatives to animal testing (European Commission, Directorate General Joint Research Centre), contributing to the identification and evaluation of in vitro methods for their potential validation, in the field of endocrine disruption and reproductive toxicity. She is a member of the ICCVAM Developmental and Reproductive Toxicology Working Group, and contributes to the OECD work through the Validation Management Group on Non-Animal Testing and the Advisory Group on Endocrine Disrupters Testing and Assessment.

Pertti Hakkinen PhD, National Library of Medicine, National Institutes of Health, Bethesda, USA, hakkinenp@mail.nlm.nih.gov,
Pertti Hakkinen is the Senior Toxicologist and the Toxicology and Environmental Health Science Advisor (to the Director) in the Division of Specialized Information Services of the National Institutes of Health's National Library of Medicine (NIH NLM). He provides leadership in the development of new resources in exposure science, toxicology, risk assessment, and risk communication, and enhancements to existing resources in these fields. Also, he is NIH NLM's Principal Agency Representative on ICCVAM. In addition, he is the project leader for the Chemical Hazards Emergency Medical Management (CHEMM) tool, an online information resource for alternatives to animal testing (ALTBIB), and the recently updated and enhanced ToxTutor® educational resource. Further, he is an Adjunct Associate Professor in Preventive Medicine & Biostatistics in the F. Edward Hébert School of Medicine

at the Uniformed Services University of the Health Sciences (USU) in Bethesda and a co-leader of the Environmental Health Sciences graduate level course offered by the Foundation for Advanced Education in the Sciences (FAES) at the NIH.

Koichi Imai, Professor, Osaka Dental University, Osaka, Japan, **imai@cc.osaka-dent.ac.jp**

Professional career

2014 Professor of Osaka Dental Univ. (Dept. of Biomaterials), Osaka, Japan
2010 Asso. Prof. of Osaka Dental Univ. (Dept. of Biomaterials)
2005 Head of Analytical Instrument Facility, Inst. Dental Research, Osaka Dental Univ.
Head of Low Temperature Facility, Institute of Dental Research, Osaka Dental Univ.
Head of Laser Facility, Institute of Dental Research, Osaka Dental Univ.
1989 Head of Tissue Culture Facility, Institute of Dental Research, Osaka Dental Univ.

Scientific positions

2017 Board member of ISO/TC106 (Dentistry)
2016 Vice-president of the "Japanese Association of Regenerative Dentistry"
2016 President of the "Nano Biomedical Society"
2014 Director of the "Japanese Society for Dental Materials and Devices"
2014 Director of the "Osaka Odontrogical Society"
2012 Director of the "Bio-Integration Society"
2007 Director of the "Japanese Society for Alternative to Animal Experiments"
2003 Board member of ISO/TC194 (Biological evaluation of medical devices)

Norihito Matsumoto PhD, Ono Pharmaceutical Co., Ltd., Osaka, Japan, **no.matsumoto@ono.co.jp**,

Norihito Matsumoto is the head of Mechanistic Toxicology Group in Safety Research Laboratories of Ono Pharmaceutical Co., Ltd. His areas of expertise are mechanism research in toxicology and exploratory toxicology including hepatotoxicity, gen-toxicity, phototoxicity and embryotoxicity during the early stage of drug development. He is the councilor of the Japanese Society of Toxicology and the Japanese Society for Alternatives to Animal Experiments.

Hyung Sik Kim, Professor, Sungkyunkwan University, Soul, Korea, hkims@skku.edu

Professional career

Since 2013 Professor, School of Pharmacy, SungKyunKwan University
2012 - 2013 Professor, College of Pharmacy, Pusan National University.
2007- 2012 Associate Professor, College of Pharmacy, Pusan National University.
2006 - 2009 Vice Dean, College of Pharmacy, Pusan National University.
2003 - 2006 Assistant Professor, College of Pharmacy, Pusan National University
2002 - 2003 Visiting Fellow in National Institutes of Health (NIH), USA
1997 - 2003 Senior Scientist, National Institute Tox. Research (NITR), Koran FDA, Korea
1994 -1996 Teaching Assistance, College of Pharmacy, SungKyunKwan U., Suwon, Korea

Research areas: Chemical carcinogenesis/mechanism of action, Epigenetic trans-generational study for cancer development, Screening & testing for novel anticancer agents (HDAC inhibitor, Topo-II inhibitors), Development of biomarkers associated with chemical carcinogenesis, Metabolomics profiling associated with aging and inflammation, Action mechanism for toxicities of environmental xenobiotics in molecular and cellular levels

Appendix 2

Acknowledgements

The Peer Review Panel members gratefully acknowledge Florian Le Coz from Sumitomo Chemical Co Ltd. Osaka, for his invaluable assistance with translation and editing during the peer review process. The Panel also acknowledges the members of the Validation Management Team for the completeness of the validation study reports, their cooperation and responsiveness to requests for additional information and analyses, and their responsive consideration of suggestions and updating of the validation study reports and test method protocol. Finally, the Panel expresses its appreciation to Dr. Hajime Kojima and his staff at the National Institute of Health Sciences for their excellent support and arrangements for the peer review panel meetings

Appendix 3

Glossary

5-FU	5-Fluorouracil
3T3	BALB/c 3T3 cells derived from mouse embryonic fibroblast cells (American Type Culture Collection)
BfR	Bundesinstitut für Risokobewertung, Federal Institute for Risk Assessment (G)
BASF	Badische Anilin und Soda Fabrik AG, Ludwigshafen (G)
CellTiter fluor	Cell Viability Assay is a non-lytic, single-reagent-addition fluorescence assay that measures the relative number of viable cells in a population.
D3	Mouse embryonic stem cells clone D3
DMSO	Dimethyl Sulfoxide
EST	Embryonic Stem Cell Test
EURL ECVAM	European Union Reference Laboratory at the JRC, Ispra (I)
Hand1	Heart and neural crest derivatives expressed 1
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IC_{50}	Cytotoxicity was expressed as the concentration of chemical that reduces the viability of cells to 50%
ID_{50}	The inhibition of differentiation was expressed as the concentration of the test chemical that reduces the luminescence by 50%
JaCVAM	Japanese Center for the Validation of Alternative Methods
KoCVAM	Korean Center for the Validation of Alternative Methods
METI	Ministry of Economy Trade and Industry
NEDO	New Energy Development Organization
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD	Organization for Economic Co-Operation and development
PBS	Phosphate Buffer Saline
Steady-Glo	Product designed for a high-throughput quantitation of luciferase expression in mammalian cells is commonly performed by batch processing of 96- and 384-well plates.
VMT	Validation Management Team

Appendix 4

References

1. OECD. Guidance Document No. 34: Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Organization for Economic Cooperation and Development, Paris, France, 2005 http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2005)14&doclanguage=en

2. Le Coz F., Suzuki N., Nagahori H., Omori T., Saito K. (2015): Hand1-Luc embryonic stem cell test (Hand1-Luc EST): a novel rapid and highly reproducible in vitro test for embryotoxicity by measuring cytotoxicity and differentiation toxicity using engineered mouse ES cells. J. Toxicol. Sci. 40(2): 251-261

3. Hand1-Luc EST Validation Management Team. Hand1-Luc Embryonic Stem Cell Test (Hand1-Luc EST), Draft Protocol Ver.10 E, 2017, February 1st

4. Hand1-Luc EST Validation Management Team. Validation Study Report on Hand1-Luc EST (Embryonic Stem Cell Test) As a Non-animal Test Evaluating Developmental Toxicity of Chemicals, March 2017.

5. Hand1-Luc EST Validation Management Team. Validation Study Report on Hand1-Luc EST (Embryonic Stem Cell Test) As a Non-animal Test Evaluating Developmental Toxicity of Chemicals, October 2016.

6. Bremer S., Hartung T. (2004): The use of embryonic stem cells for regulatory developmental toxicity testing in vitro- the current status of test development. Curr. Pharm. Des. 10(2): 2733-2747

7. Firulli BA., Fuchs RK., Vincentz JW., Clouthier DE., Firulli AB. (2014): Hand1 phosphoregulation within the distal arch neural crest is essential for craniofacial morphogenesis. Development 141(15): 3050-3061

8. Laurie LE., Kokubo H., Nakamura M., Saga Y., Funato N. (2016): The transcription actor Hand1 is involved in Runx2-Ihh-regulated endochondral ossification. PLOS ONE doi 10.1371: 1-10

9. McFadden DG., Barbosa AC., Richardson JA., Schneider MD., Srivastava D., Olson EN. (2005): The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. Development 132(1): 189-201

10. Reamon-Buettner, SM., Ciribilli, Y., Traverso, I., Kuhls, B., Inga, A., and Borlak, J. (2009): A functional genetic study identifies HAND1 mutations in septation defects of the human heart. Hum. Mol. Genet. 18(19): 3567-3578

11. Nagahori H., Suzuki N., Le Coz F., Omori T., Saito K. (2016). Prediction of in vivo developmental toxicity by combination of Hand1-Luc embryonic stem cell test and metabolic stability test with clarification of metabolically inapplicable candidates. Toxicol. Lett. 259: 44-51