Vitrigel-Eye Irritation Test (EIT) method

Report of the Peer Review Panel

on

the validation study of the Vitrigel-EIT method to be used in a bottom-up approach for eye hazard identification according to the UN GHS classification

Final version, 19 October 2017

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1. Executive Summary

The present peer review panel (PRP) was established to independently evaluate the scientific validity of the Vitrigel-Eye Irritation Test (EIT) method to distinguish UN GHS No Category (No Cat.) test chemicals from test chemicals requiring classification according to the UN GHS classification scheme as e.g., an initial test in a bottom-up testing strategy approach (OECD GD 263, 2017). Vitrigel-EIT underwent a modular validation study (Anon., 2017a) coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM) and conducted by an international Validation Management Team (VMT) comprised of representatives from the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM), the U.S. National Toxicology Program's Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM-ICCVAM) and the Korean Center for the Validation of Alternative Methods (KoCVAM).

The Vitrigel-EIT method, is based on a three dimensional human corneal epithelium (HCE) cultured on a Collagen Vitrigel Membrane (CVM) scaffold, and assesses the relative change over time of the Transepithelial Electrical Resistance (TEER) of epithelial cells as an endpoint for the integrity or disruption of the tight junction of the corneal epithelium (Anon., 2017a). The within- and betweenlaboratory reproducibility were of 90% (average of three laboratories) and 92% respectively, based on the testing of 10 and 36 coded chemicals (Anon., 2017a). The PRP considered these within- and between-laboratory reproducibility to be sufficient, but would have appreciated having further statistical justification for the number of chemicals used to assess the within-laboratory reproducibility.

Based on the results of the validation and of in-house studies, a reduced applicability domain was proposed by the VMT (Anon., 2017a), based on the following exclusion criteria: i) exclude test chemicals having a pH $\leq$ 5.0 based on 2.5% solution; ii) exclude solid test chemicals having both, log P $\geq$ 2.5 and density < 0.95 g/cm^3 or > 1.10 g/cm^3. The predictive capacity of the reduced applicability domain of Vitrigel-EIT was evaluated based on a total of 76 test chemicals representing 22 UN GHS Cat. 1, 22 UN GHS Cat. 2 and 32 UN GHS No Cat. chemicals, including both liquids and solids and covering a wide range of chemistries. A sensitivity of 93% (41/44), a specificity of 69% (22/32) and an accuracy of 83% (63/76) were found for the reduced applicability domain. Three false negatives (i.e. 7%) of 44 UN GHS classified test chemicals were found, all three being *in vivo* UN GHS Cat. 2 test chemicals (Anon., 2017a). The PRP considered this as an acceptable rate, as it is within the overall probability of about 12% of chemicals identified by the *in vivo* Draize eye test as either UN GHS Cat. 2 or UN GHS No Cat. in a repeated *in vivo* test, due to the *in vivo* method's inherent within-test variability (OECD GD 263, 2017).

Based on the above and the considerations described within this report, the PRP concluded that the Vitrigel-EIT method is valid for use as an initial test in a bottom-up testing strategy approach for identification of test chemicals not requiring classification and labelling for eye irritation or serious eye damage (UN GHS No Cat. chemicals), when used within the limited applicability domain of test chemicals having pH > 5.0 (based on 2.5% solution), and excluding solids having both a log P $\geq$ 2.5 and a density of < 0.95 g/cm^3 or > 1.10 g/cm^3.

2. Peer Review Panel Composition

Chantra Eskes (chair)	SeCAM, Switzerland
Pertti (Bert) Hakkinen	The National Library of Medicine, NIH, USA
Sebastian Hoffmann	seh consulting + services, Germany
Tae Cheon Jeong	Yeungnam University, Korea
Jill Merrill	FDA, USA
Sanae Takeuchi	P&G Innovation Godo Kaisha, Japan

3. Background

The Vitrigel-EIT method underwent a modular validation study coordinated by JaCVAM and conducted by an international Validation Management Team comprising representatives from EURLECVAM, NICEATM-ICCVAM and KoCVAM (Anon., 2017a). The validation study was designed to assess the usefulness of the Vitrigel-EIT test method as an alternative to the *in vivo* Draize test method to identify ocular non-irritants in a bottom-up testing strategy approach according to the UN GHS classification scheme.

The international Peer Review Panel (PRP) first met in July 2016 to discuss the outcome of the validation study with the Validation Management Team (VMT), and to review, in the absence of the VMT, the validation study of the Vitrigel-EIT method. The peer review of the Vitrigel-EIT validation study was conducted based on 14 evaluation criteria as requested by JaCVAM and summarized in Table 1. Upon requests for clarifications by the PRP on a number of elements, the VMT provided replies that were discussed via teleconferences in December 2016 and in June 2017. Based on suggestions from the PRP, the VMT carried out a final revision of the documentation addressing the open issues, which was distributed to the PRP in August 2017 (Anon., 2017a).

Table 1: Evaluation criteria guiding the peer review of the Vitrigel-EIT method.

Peer Review Evaluation criteria	
1	A **rationale for the test method** should be available, including a description of the human health effect, a clear statement of the scientific need, and the regulatory application
2	The **toxicological mechanisms and the relationship** between the test method endpoint(s) **with the biological effect as well as the toxicity of interest** should be addressed, describing limitations of the test method
3	A detailed **test method protocol** should be available
4	The **within- and between-laboratory reproducibility** of the test method should be demonstrated
5	Demonstration of the test method's performance should be based on testing of representative, preferably coded **reference chemicals**
6	**Predictive capacity** should be demonstrated using representative chemicals. The performance of the test method should be evaluated in relation to existing relevant toxicity data as well as information from the relevant target species
7	All **data** should adequately support the assessment of the validity of the test method for peer review
8	All data from the validation study supporting the validity of a test method should be obtained in accordance with the **principles of Good Laboratory Practice** (GLP)
9	**Applicability domain** of the test method should be defined
10	**Proficiency chemicals** should be set up in the proposed protocol
11	**Performance standard** should be set up with proposed protocol
12	**Advantages** in terms of time, cost and animal welfare

13	**Completeness of all data and documents** supporting the assessment of the validity of the test method
14	**Validation study management and conduct**

4. Peer Review Panel Evaluation

4.1. Rationale for the test method

The PRP agreed that a rationale for the test method has been provided, including a description of the human health effect and the regulatory application. Vitrigel-EIT is proposed as an alternative to the *in vivo* Draize eye irritation test method, to be used as an initial test in a bottom-up testing strategy approach (Anon., 2017a). Therefore, the validation study was designed to assess the capacity of Vitrigel-EIT to distinguish UN GHS No Cat. test chemicals from test chemicals requiring classification according to the UN GHS classification scheme.

4.2. Toxicological mechanisms and relationship with the biological effect and toxicity of interest

The PRP considered that the toxicological mechanisms and the relationship between the test method endpoint(s) with the biological effect as well as the toxicity of interest were briefly addressed within the validation report (Anon., 2017a), and were further addressed during a presentation given by the test method developers during the first PRP meeting in July 2016.

The Vitrigel-EIT method is based on a three dimensional air–liquid interface culture system composed of human corneal epithelium (HCE) cultured on a Collagen Vitrigel Membrane (CVM) scaffold comprising high-density collagen fibrils that are equivalent to *in vivo* connective tissues (Takezawa et al., 2011; Yamaguchi et al., 2013, 2016). The HCE model used is based on HCE-T cells, which is a SV40-immortalized cell strain (Araki-Sasaki et al., 1995), that can maintain stable characteristics of the corneal epithelial cells in culture (Kim et al., 2016, Yamasaki et al., 2009).

The Vitrigel-EIT method assesses the relative change over time of the Transepithelial Electrical Resistance (TEER) of epithelial cells as an endpoint for the integrity or disruption of the tight junction of the corneal epithelium. In particular, three parameters are used to correlate the TEER change over time to the prediction of eye hazard requiring classification: the TEER time lag, intensity and plateau level (Anon., 2017a, 2017b).

4.3. Test method protocol

The PRP concurred that a detailed test method protocol is available (Anon., 2017b) and the changes made during the validation study, as well as the rationale for these changes, are clearly described within the validation report and the protocol provided (Anon., 2017a, 2017b). Table 2 summarizes the main protocol changes undertaken during the different phases of the validation study.

Table 2. Overview of the different phases, evaluations and protocols used in the modular validation study of the Vitrigel-EIT method.

Phases	**N. tested chemicals**	**Assessment**	**Protocol used**	**Main protocol updates as compared to previous protocol**
Phase 0	5 non-coded chemicals	Transferability	1.30e	
Phase I	10 coded chemicals	WLR & BLR	1.51e	- Reference control acceptance criteria: TEER plateau level between 10% to 30% inclusive. - Measurements conducted at room temperature (18°C ≤ T ≤ 30°C). - Time from start of exposure to start of measurement: within 2 seconds.
Phase II	10 coded chemicals	BLR	1.61e	- Reference control acceptance criteria: TEER plateau level between **10% to 40%** inclusive. - Measurements conducted at ambient temperature (**22°C ≤ T ≤ 30°C**). - Procedural clarifications (e.g. chemicals solubilisation, clarifying procedures for volatilizing chemicals; clarifying procedures in case of technical issues).
Phase III	36 coded chemicals	BLR & PC	1.71e	- Reference control acceptance criteria: TEER plateau level 10% or more (upper level to be decided at the end of phase III of the validation study). - Measurements conducted at **28 ± 2 °C.**
In-house data	132 noncoded chemicals (including 96 chemicals not tested in phase III)	PC		57 out of the 96 chemicals were tested according to the latest version of the protocol, i.e., with measurements conducted at 28 ± 2 °C.
Final protocol			1.80e	- Inclusion of a section on applicability domain. - Inclusion of pH measurement of 2.5% solutions. - Procedural clarifications for dissolving chemicals. - Time from start of exposure to start of measurement: within 2 to 5 seconds.

BLR: between-laboratory reproducibility; PC: predictive capacity; WLR: within-laboratory reproducibility.

4.4. Within- and between-laboratory reproducibility

Based on results obtained with 10 coded chemicals tested in Phase I, the within-laboratory reproducibility (WLR) was found to meet the success criteria established by the VMT (see Table 3). The 10 chemicals comprised 6 classified (two Cat. 1 and four Cat. 2) and 4 No Cat. test chemicals according to the UN GHS classification scheme (Anon., 2017a). The Validation Study report notes that such a number of chemicals was chosen based on the "*biostatistician's opinion about the statistical validity of the number of test chemicals used for the (EURL-)ECVAM validation study for skin sensitization*" (chapter 3.3.2.2, p. 21 of Anon., 2017a). The PRP would have appreciated having further statistical justification for the number of chemicals (10) used to assess the within-laboratory reproducibility, and whether such number was considered sufficient by the VMT.

The between-laboratory reproducibility (BLR) was also found to meet the success criteria established by the VMT (see Table 3). The BLR was evaluated based on the results obtained with 36 coded chemicals from phase III comprising 24 classified (i.e., 12 Cat. 1 and 12 Cat. 2) and 12 No Cat. test chemicals according to the UN GHS classification scheme. Phase II results were not considered in the evaluation of BLR due to the fact that temperature was found to affect reproducibility, and that during that phase temperatures varied from 22°C to 30°C, whereas it was controlled to be 28±2°C during phase III (Anon., 2017a). Although the number of UN GHS No Cat. chemicals tested to establish BLR was smaller than the number of UN GHS classified chemicals, this is justifiable in the case of Vitrigel EIT which is proposed as an initial test in a bottom-up testing strategy approach, and should therefore should demonstrate appropriate sensitivity (i.e., low rate of false negative predictions).

Table 3. Within- and between-laboratory reproducibility of the Vitrigel-EIT method.

Phases	**N. tested chemicals**	**Assessment**	**WLR**			**BLR**	**VMT success criteria**
			Lab A	**Lab B**	**Lab C**		
Phase I	10 coded chemicals	WLR & BLR	80% (8/10)	100% (10/10)	90% (9/10)	-	≥ 80% WLR
Phase III	36 coded chemicals	BLR & PC	-	-	-	92% (33/36)	≥ 80% BLR

BLR: between-laboratory reproducibility; Lab A: Hatano Research Institute, Food and Drug Safety Center – FDSC (Hatano, Kanagawa); Lab B: Bozo Research Center –BRC (Tokyo); Lab C: Daicel Corporation (Himeji, Hyogo); PC: predictive capacity; VMT: validation Management Team; WLR: within-laboratory reproducibility.

4.5. Selection of test chemicals

The PRP considered that a representative set of chemicals was selected to assess the betweenlaboratory reproducibility and the predictive capacity of the Vitrigel EIT method. It is noted that due to the limited sample size in phase III, additional data obtained in-house for further 57 chemicals tested at 28 ± 2°C (Yamaguchi et al., 2016) were used to assess the predictive capacity of

the assay. Results obtained with the 57 test chemicals were added to the dataset obtained with the 36 chemicals tested in phase III of the validation study, resulting in a total of 93 test chemicals used to assess the predictive capacity of the Vitrigel-EIT assay (Anon., 2017a).

The 93 chemicals used to assess the predictive capacity of the assay comprised 29 Cat. 1, 31 Cat. 2 and 33 No Cat. chemicals according to the UN GHS classification scheme (see appendix 8.13 of Anon., 2017a), having a balanced distribution of the three UN GHS categories of eye hazard. Furthermore, the selected chemicals had representation of both solids and liquids (see Table 4), and covered a wide range of chemistries.

Chemicals from phases I, II and III were tested coded, whereas chemicals tested in house were tested non-coded. It is noted that an overlap of 5 (out of 10) chemicals existed between phases I and II, an overlap of 4 chemicals existed between phases I (out of 10 chemicals) and III (out of 36 chemicals), and an overlap of 9 chemicals existed between phases II (out of 10 chemicals) and III (out of 36 chemicals). The PRP considers this overlap to be helpful as it allowed to demonstrate improvement of reproducibility through the progression of protocol modifications as described in Table 2.

Table 4: Representation of liquids, solids and the various UN GHS categories of the selected chemicals.

		UN GHS Cat. 1	UN GHS Cat. 2	UN GHS No Cat.	**Total**
Phase I	Liquids	1	2	2	**5**
	Solids	1	2	2	**5**
	Total	**2**	**4**	**4**	10
Phase II	Liquids	1	3	1	**5**
	Solids	3	0	2	**5**
	Total	**4**	**3**	**3**	10
Phase III	Liquids	4	9	7	**20**
	Solids	8	3	5	**16**
	Total	**12**	**12**	**12**	36
In-house	Liquids	7	9	20	**36**
	Solids	10	10	1	**21**
	Total	**17**	**19**	**21**	57

4.6. Predictive capacity

The predictive capacity of Vitrigel-EIT was initially assessed based on the 36 chemicals tested coded in phase III of the validation study. In a second step 57 chemicals tested in a non-coded manner

inhouse were added to this analysis, resulting in a total of 93 test materials (Anon., 2017a). Based on the outcome of this analysis, a reduced applicability domain of the assay was proposed by the VMT as described in section 4.9 of this report. When applying the reduced applicability domain, 17 test chemicals (of the 93) were excluded representing eight chemicals from the phase III of the validation study (4 Cat. 1, 3 Cat. 2 and 1 No Cat.) and nine chemicals from the in-house tested dataset (3 Cat. 1 and 6 Cat. 2). As a consequence, the predictive capacity of the reduced applicability domain of the Vitrigel-EIT was based on a total of 76 test chemicals as shown in Table 5. The 76 test chemicals represented 22 Cat. 1, 22 Cat. 2 and 32 No Cat. chemicals according to the UN GHS classification scheme (cf. appendix 8.13 of the validation report). Out of these, 28 were tested coded during phase III of the validation study (8 UN GHS Cat. 1, 9 UN GHS Cat. 2 and 11 UN GHS No Cat. chemicals) and 48 were tested non-coded in-house (14 UN GHS Cat. 1, 13 UN GHS Cat. 2 and 21 UN GHS No Cat. chemicals).

Table 5. Predictive capacity of the Vitrigel-EIT method.

	All tested chemicals				**Reduced Applicability Domain**			
	Phase III 36 coded chemicals		**In-house** 57 non-coded chemicals	**Combined** 93 chemicals	**Phase III** 28 coded chemicals		**In-house** 48 noncoded chemicals	**Combined** 76 chemicals
	Per laboratory	Overall*			Per laboratory	Overall*		
Sensitivity	75.0 – 83.3% (18-20/24)	79.2% (19/24)	86.1% (31/36)	**83.3% (50/60)**	88.2 – 94.1% (15-16/17)	88.2% (15/17)	96.3% (26/27)	**93.2% (41/44)**
Specificity	41.7% (5/12)	41.7% (5/12)	85.7% (18/21)	**69.7% (23/33)**	36.4% (4/11)	36.4% (4/11)	85.7% (18/21)	**68.8% (22/32)**
Accuracy	63.9 – 69.4% (23-25/36)	66.7% (24/36)	86.0% (49/57)	**78.5% (73/93)**	67.9 – 71.4% (19-20/28)	67.9% (19/28)	91.7% (44/48)	**82.9% (63/76)**

* Based on a majority of predictions from the participating laboratories.

Only three false negatives (out of 44 UN GHS classified test chemicals) were found with the reduced applicability domain, all of which were *in vivo* UN GHS Cat. 2 test chemicals. These included the waxy, water insoluble solid camphene (CAS 79-92-5; UN GHS Cat. 2B), α-hexylcinnamaldehyde (CAS 10186-0; UN GHS Cat. 2A) and 6-methylpurine (CAS 2004-03-07; UN GHS Cat. 2B). It is noted that no UN GHS Cat. 1 was under-predicted as No Cat. when applying the reduced applicability domain of Vitrigel EIT method. Furthermore, the false negative rate obtained with the Vitrigel EIT for its proposed reduced applicability domain is within the overall probability of about 12% of chemicals identified by the *in vivo* Draize eye test as either UN GHS Cat. 2 or UN GHS No Cat. in a repeated *in vivo* test, due to the method's inherent within-test variability (OECD GD 263, 2017; OECD TG 492, 2015).

4.7. Data supporting the validity of the assay

The data presented to the PRP did support the conclusions from the validation study report.

4.8. Accordance with the principles of Good Laboratory Practices

Based on the information available to the PRP, the study was conducted in the spirit of GLP.

4.9. Applicability Domain

The test method is proposed to be used for the identification of UN GHS No Cat. test chemicals an initial test in a bottom-up testing strategy approach. Based on the results of the validation and inhouse studies, a reduced applicability domain was proposed by the VMT (Anon., 2017a), in which two exclusion criteria are proposed as follows:

- exclude pH ≤ 5.0 based on 2.5% solution
- exclude solids having both a log P ≥ 2.5[1] and a density of < 0.95 g/cm^3 or > 1.10 g/cm^3.

A possible rationale provided by the VMT for the proposed reduced applicability domain is the fact that acidic substances were reported to influence TEER measurements (Yamaguchi et al., 2016), and the fact that substances having log P ≥ 2.5 and a density of < 0.95 g/cm^3 or > 1.10 g/cm^3 may be water-insoluble.

4.10. Proficiency chemicals

A list of 10 proficiency chemicals divided into 3 UN GHS Cat. 1, 3 UN GHS Cat. 2 and 4 UN GHS No Cat. has been proposed (Appendix 8.9 of Anon., 2017a). The peer-review panel welcomed the proposed list of proficiency chemicals, but was of the opinion that such a list might require further consideration during the process of regulatory acceptance. In particular, the PRP believes that it is important to ensure that the proposed proficiency chemicals have reproducible results between laboratories and between runs, and that it is based on the most up-to-date version of the protocol. In that regard, chemicals that may merit further consideration include camphene (CAS 79-92-5), αhexylcinnamaldehyde (CAS 101-86-0), toluene (CAS 108-88-3) and 3-methoxy-1,2-propanediol (CAS 623-39-2).

[1] It was noted by the VMT that logP values used to define the applicability domain were obtained at 20°C or 25°C. Furthermore it was noted that the logP value at 28°C is rarely different from the logP value at 20°C, as the impact of temperature is generally of only 0.001-0.01 per 1°C of the logKow value.

4.11. Performance Standards

The PRP agreed that performance standards can aid the regulatory acceptance process following the peer-review, but are not needed at the present stage for the PRP evaluation.

4.12. Advantages in terms of time, costs and animal welfare

A comparison between the Vitrigel-EIT and adopted alternative test methods for eye hazard assessment has been provided in the validation report in terms of time, practicality, costs, mechanistic relevance, limitations and predictive capacity (Anon., 2017a). Furthermore, Table 6 shows the predictive capacity of the Vitrigel-EIT method as compared to the alternative test methods currently adopted for the identification of UN GHS No Cat. test chemicals for eye hazard assessment.

In particular, the PRP noted that the Vitrigel-EIT is based on a different mechanism of action compared to the alternative assays currently adopted since it assesses the epithelial barrier function based on a three dimensional culture system composed of human corneal epithelium.

Table 6. Comparison of Vitrigel predictive capacity to the alternative test methods currently adopted for identification of UN GHS No Cat. test chemicals.

	BCOP (OECD TG 437)	**ICE (OECD TG 438)**	**STE* (OECD TG 491)**	**RhCE (OECD TG 492)**	**Vitrigel EIT****
Accuracy	69% (135/196)	82% (125/152)	90% (92/102)	80-84% (n>112)	83% (63/76)
False positive rate (1-specificity)	69% (61/89)	33% (26/79)	19% (9/48)	28-37% (n>55)	31% (10/32)
False negative rate (1-sensitivity)	0% (0/107)	1% (1/73)	2% (1/54)	4-5% (n>57)	7% (3/44)

* Reduced applicability domain as proposed within TG 491;

** Reduced applicability domain as proposed by the VMT (see section 4.9).

4.13. Completeness of data and documents

The PRP appreciated the transparency with which all the Vitrigel-EIT assay material was presented, in particular regarding the protocol modifications during the various phases of the assay validation. The PRP agreed that the data and documents provided were complete and sufficient to evaluate the Vitrigel-EIT validation study.

4.14. Validation study management and conduct

The PRP considered that the Vitrigel-EIT underwent a validation study in accordance with internationally accepted principles (OECD Guidance Document 34, 2005) and following a modular approach to validation (Hartung et al., 2004).

4.15. Other considerations

The PRP agreed that the Vitrigel-EIT method does not seem to pose issues related to intellectual property rights. Indeed, the validation study report states that (Anon., 2017a):

- all components and reagents used in the test method are commercially available;
- the HCE-T cells can be globally distributed from RIKEN BioResource Center;
- the Vitrigel-EIT method is available without any restriction by its intellectual property rights;
- Vitrigel is registered trade mark of National Agriculture and Food Research Organization (Tsukuba, Japan).

5. Conclusions

The PRP concluded that the Vitrigel-EIT method, when based on its proposed applicability domain, can be considered as a valid assay for the identification of test chemicals not requiring classification and labelling for eye irritation or serious eye damage (UN GHS No Cat. chemicals). This is valid for the limited applicability domain of test chemicals having pH > 5.0 based on 2.5% solution; and excluding solids having both a log P ≥ 2.5 and a density of < 0.95 g/cm3 or > 1.10 g/cm3.

6. Acknowledgements

The PRP is grateful to the members of the VMT for their hard work and to JaCVAM for their support in setting up and hosting meetings in Japan, as well as for the setting up of several telephone conferences.

7. References

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