EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
I.5 Systems Toxicology Unit
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

Report on the Full Test Submission Assessment
Test Method: TM2015-03
The SkinEthic™ Human Corneal Epithelium (HCE)
Eye Irritation Test (EIT)

Report on the Full Test Submission Assessment

Test Method:

SkinEthic™ Human Corneal Epithelium Eye Irritation Test (SkinEthic™ HCE EIT)

SUMMARY OF THE ASSESSMENT

The test method has been developed for prediction of eye irritation potential of chemicals, able to distinguish substances requiring official classification for eye irritation or serious eye damage (UN GHS) from 'non-irritants' (no classification category).

The method is based on tissue culture topical exposure with cell viability assay response.

The test submitter requests peer-review based on a ring trial among 3 laboratories.

Modules 1 – 6, i.e. Test Definition (TD), Within-Laboratory Reproducibility (WLR), Transferability (TF), Between-Laboratory Reproducibility (BLR), Predictive Capacity (PC), and Applicability Domain (AD), have been assessed.

Validation of WLR, TF, BLR, and PC is comprehensive and quality assured.

As an additional element, the test method development has overcome a general limitation of related protocols susceptible to interference from coloured substances (MTT-reduction assay) implementing HPLC as an alternative for endpoint quantitative analysis.

1. MODULE 1: TEST DEFINITION

1.1 Human health/environmental/biological effect addressed by the test method and biological/ mechanistic relevance

The SkinEthic™ HCE test method addresses eye irritation caused by topical exposure to chemicals, manifested *in vivo* as local inflammation and/or opacity, resulting mechanistically from cell damage (cytotoxicity).

The *in vitro* test system uses immortalized human corneal epithelial cells, cultured to form a Reconstructed human Cornea-like Epithelium (RhCE), i.e. a three-dimensional tissue similar to the human corneal epithelium. The test method was developed to model *in vivo* topical exposure, with prediction of positive or negative irritation response from cell viability assay. Tissue viability is determined quantitatively as a percentage, relative to a negative control (100% viable) by standardised MTT assay (photometric measurement of purple formazan production from enzymatic reduction of the vital dye MTT). Tissues treated with eye irritants show a decrease in viability relative to the negative control, with discrimination of positive or negative GHS classification defined by an optimised viability threshold percentage (prediction model).

The method is supported comprehensively by references to technical literature, and is mechanistically relevant to effective assessment of topical human eye irritation resulting from local cytotoxicity.

1.2 Purpose and need of the test method

The method is intended for inclusion in a comprehensive testing and assessment strategy, (so-called Top-Down/Bottom-Up approach) particularly relevant for industrial chemicals or chemicals used in human exposure products, such as cosmetics ingredients which are banned from animal testing. The method is therefore required as an alternative, also effectively reducing the need for animal studies by their partial replacement. The SkinEthic™ HCE is the second RhCE test method that is validated following EpiOcular™ EIT. It is however important to have at least two of these methods validated and accepted by regulatory authorities in order to guarantee the widespread availability of this technology and avoid potential market monopolies.

In a tiered assessment strategy, the test is applicable as a first step in Bottom-Up discrimination of 'non-irritants' (GHS no category) or as a confirmatory last step in Top-Down identification of 'irritants' (GHS categories 1 and 2). However, the method is not intended to differentiate category 1 from 2.

1.3 Technical specifications

1.3.1 Protocol of the test method

Comprehensive protocols (SOPs) for eye irritation testing of liquids (EITL) and solids (EITS) as used in the validation study are available (Attachments 1a & 1b, respectively). Final protocols intended for test method users were also submitted in DB-ALM format (Attachments 1c & 1d, respectively).

Critical procedural elements from the SOPs are clear and complete in the TST summary, including:

- test system description (Human Cornea Epithelium tissue model, with quality control).

- TT: test treatment (application, exposure, incubation, MTT-formazan extraction).

- viability determination (MTT formazan assay: OD measurement, HPLC).

Acceptance criteria (for qualified test, qualified run, and complete test):

- NgC: negative control (PBS): $1.4 \leq OD \leq 2.5$ (mean of 2 replicate tissues).

- PC: positive control (methyl acetate): viability $\leq$ 30% (mean of 2 tissue replicates).

- viability difference between run replicates ≤ 20 (NgC, PC, TT).

The acceptance criteria were applied consistently to the 200 chemicals tested (120 in 3 laboratories and 80 additional by the lead laboratory, each in triplicate) where occurrence of non-qualified (NQ) runs was exceptional (Attachment 3a) with rare necessity for repeat runs.

The SOPs are clear and complete, demonstrated in the ring trial by their routine and successful implementation by both the lead and naïve laboratories.

1.3.2 Data analysis and prediction model

The procedure for experimental data processing and analysis is clear in the TST, including:

- Calculation of the mean OD for each individual tissue.

- Calculation of the percentage viability relative to the mean OD of negative control.

- Interpretation of the viability results according to the prediction models respective of EITL (60% threshold) and EITS (50% threshold).

The processing and analysis of all data from the three laboratories in the ring trial was contracted to an independent consultant statistician who has compiled 2 comprehensive reports, respective of the liquid and solid protocols (Attachments 8a and 8b). The reports are clear and concise, uniformly applying the acceptance criteria and prediction model to determine within laboratory reproducibility (WLR) between laboratory reproducibility (BLR) and predictive capacity (PC).

1.3.3 Test items used for developing and optimising the prediction model

The test method was developed within L'Oreal's laboratory using 125 chemicals, including 71 liquids and 54 solids (Attachment 2).

A principal criterion for selection of test chemicals was availability of supporting complete and quality assured *in vivo* Draize eye irritation data. The selection was limited to commercially available chemicals.

The chemicals, incorporating 44/125 (35%) previously selected for an original ring trial eye irritation validation study (EIVS) provided a range of properties, including:

- Chemical class (functional group): soap/surfactant, organics (neutral, acid and base) and inorganic base.

- Several colour interfering chemicals, MTT reducers and MTT reducing coloured chemicals.

- GHS classification: 49% not classified (NC) and 51% of classified (C) (divided as 53% Cat 1 and 47% of Cat 2.

As distribution of physical state and GHS classification category, the 125 chemicals covered: 34 Cat 1 (19 liquids and 15 solids), 21 Cat 2A (16 liquids and 5 solids), 9 Cat 2B (4 liquids and 5 solids) and 61 No Cat (32 liquids and 29 solids).

The complement of chemicals used for development and optimization represents a significant and balanced set.

The full history of the method development and optimization, including the original EIVS ring trial, is clearly summarized in section 1.1.11 of the TST, complete with literature references.

1.3.4 Occurrence of non-qualified test

Acceptance criteria (for qualified test, qualified run, and complete test) are:

- NgC: negative control (PBS): $1.4 \leq OD \leq 2.5$ (mean of 2 replicate tissues).

- PC: positive control (methyl acetate): viability $\leq$ 30% (mean of 2 tissue replicates).

- viability difference between test treatment (TT) run replicates ≤ 20 (NgC, PC, TT).

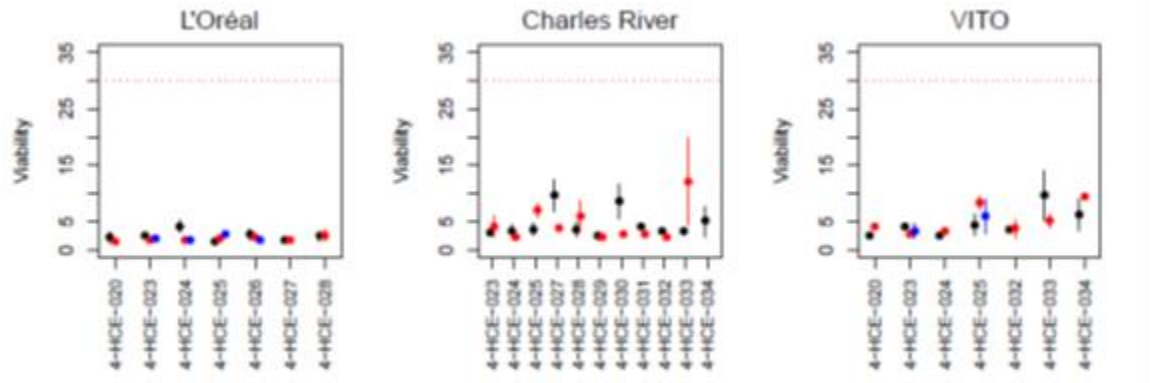
The TST indicates frequency of non-qualified runs is ≤ 1%. The TST also states that a single run composed of at least two tissue replicates should be sufficient for a test chemical when the result is unequivocal. However, in cases of borderline results, such as non-concordant replicate measurements, a second test should be considered, as well as a third one in case of discordant results between the first two tests (frequency in occurrence < 1%).

The statistics reports (Attachments 8a and 8b) summarise within and between laboratory variability of the viability for the positive control (PC) respective of the liquids (EITL) and solids (EITS) protocols:

Liquids (EITL):

	L'Oréal	CRL	VITO
Mean	2.12	4.49	4.89
SD	0.67	2.69	2.35
Min	1.40	2.09	2.35
Max	4.11	12.13	9.54

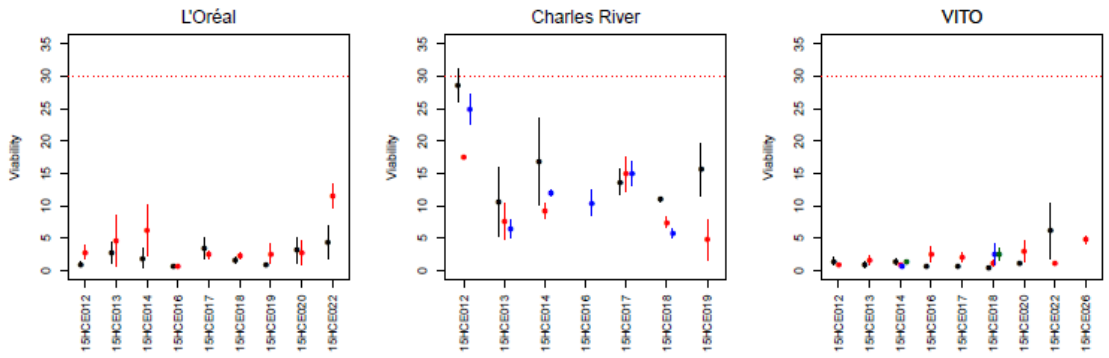
These values are illustrated graphically in the EITL statistics report *(Attachment 8a, Figure 3: Viability of the positive control for different batches (up to 3 series: black, red, blue) determined for duplicate tissues (mean value: dots, single values: bars)* copied here for clarification:



Solids (EITS):

	L'Oréal	CRL	VITO
Mean	3.1	12.9	1.9
SD	2.6	6.3	1.4
Min	0.7	4.8	0.6
Max	11.6	28.6	6.2

These values are illustrated graphically in the EITS statistics report *(Attachment 8b, Figure 3: Viability of the positive control for different batches (up to 4 series: black, red, blue, green) determined for duplicate tissues (mean value: dots, single values: bars)* copied here for clarification:



The PC mean viabilities (generally < 5%) are significantly lower than the 30% acceptance threshold, allowing scope for permitted variability within a qualified run/test. Nevertheless, the PC is also generally reproducible with maximum viabilities unequivocally acceptable.

However, the PC results for EITS from CRL are an exception, with greater variability and maximum viability near the threshold. Independent expert opinion has noted a probable cause for this variability as due to improper spreading of the PC methyl acetate when applied to the tissues, which would explain the larger variability observed for EITS than EITL due to the considerably longer exposure time for solids (4 hours) than for liquids (30 min). In fact, the longer exposure time for solids versus liquids would rather be expected to diminish variability

It is suggested the positive control acceptance criterion threshold could be reduced, perhaps to 15%. The results indicate this would be ample, assuming the tissues are accurately dosed with methyl acetate, while allowing detection of any variability in case of the contrary.

Application of the 30µL methyl acetate to the tissue evidently requires care to ensure even spreading, with higher importance for the solids protocol (EITS) due to the significantly longer exposure time used with this protocol.

1.3.5 Known limitations and drawbacks of the test method

The TST acknowledges that the test method is not intended to differentiate between UN GHS/EU CLP Category 1 (serious eye damage) and UN GHS/EU CLP Category 2 (eye irritation). Gases and aerosols have not been assessed. While it is conceivable that these can be tested using the technology, the current SkinEthic™ HCE EIT does not allow testing of gases and aerosols. No other limitations were identified by the submitter.

The SkinEthic™ HCE technology, associated to production of model and media are proprietary to Episkin SA, France (www.episkin.com) which is covered by IPR. The SkinEthic™ HCE EIT protocols are however publicly available and its principles could be applicable to other similar Reconstructed human Tissue models.

1.3.6 Overall conclusions on Module 1

Module 1: Test Definition: The TST provides clear and comprehensive information relevant to Module 1.

2. MODULE 2: WITHIN-LABORATORY REPRODUCIBILITY (WLR)

2.1 Rationale for the selection of test items used for assessing WLR

The TST confirms chemical selection for WLR is significant, with relevant scope, supported by complete and quality assured *in vivo* Draize eye irritation data, for comparative evaluation of *in vitro* method predictive capacity.

The selection covers:

- solid and liquid physical states.

- the full range of *in vivo* eye irritation GHS Categories (1, 2A, 2B, or No Category).

- the *in vivo* determinants of classification (cornea opacity, iritis, conjunctiva redness, chemosis, reversibility/persistence).

- wide representation of organic functional groups.

- known chemical structures.

- coloured and/or direct MTT reducers.

- availability through laboratory retail supply, at reasonable cost.

This information is compiled in a Table including over 200 chemicals (Attachment 2) indicating the 120 chemicals (60 solids, 60 liquids) used for ring trial assessment of WLR.

2.2 Assessment of within-laboratory reproducibility of experimental data

WLR from the ring trial has been fully assessed by an independent consultant statistician, with comprehensive reports available respective of the two protocols, EITL (Attachment 8a) and EITS (Attachment 8b). The reports also provide all raw data in appendices. Summary data, including additional chemicals tested by the lead laboratory, have also been compiled for overview:

- Attachment 3b: EITL: 60 ring trial chemicals, 3 labs (L'Oreal, VITO, CRL).

- Attachment 3c: EITL: 45 additional chemicals, 1 lab (L'Oreal).

- Attachment 3d: EITS: 60 ring trial chemicals, 3 labs (L'Oreal, VITO, CRL).

- Attachment 3e: EITS: 35 additional chemicals, 1 lab (L'Oreal).

WLR (concordance of predicted classification) based on the set of 120 chemicals, was reported as follows:

- CRL: 91.7% (EITL 88.3% and EITS 95.0%).

- VITO: 94.2% (EITL 93.3% and EITS 95.0%).

- L'Oreal: 95.8% (EITL 95.0% and EITS 96.7%).

WLR for the extended set of 200 chemicals (tested by L'Oreal only) was:

- 95.0% (EITL 93.3% and EITS 96.8%).

The TST concludes that the SkinEthic™ HCE EIT method (liquids/solids) has been shown to exceed the minimum requirement for WLR of 85% set by the validation management group (VMG) of EIVS. The WLR is also comparable to that obtained previously for a

similar method, EpiOcular™ EIT (as described in OECD Test Guideline 492): 96.3%, 98.1% and 98.1% for the liquids protocol in three laboratories and 96.6% for the solids protocol in one laboratory.

2.3 Identification and discussion of outlying values

Evaluation of WLR was based on concordance of viabilities (with respect to prediction model) with descriptive statistics presented as two-by-two scatter plots for the three independent runs. Non-concordant results were identified chemical by chemical, including indication of non-qualified (NQ) runs in exceptional cases.

2.4 Overall conclusions on Module 2

Module 2: Within-Laboratory Reproducibility (WLR): The TST provides clear and comprehensive information relevant to Module 2.

3. MODULE 3: TRANSFERABILITY (TF)

3.1 Test items used for assessing the TF

Transferability of the method was demonstrated using 18 chemicals (9 solids / 9 liquids) including strong colorants and MTT reducers known to cause interference, aiming to cover all experimental eventualities.

3.2 Assessment of the test method TF

The TST indicates two training days are required for a naïve laboratory, including practical application and data evaluation.

Actual transfer of the method was arranged over two weeks, testing the 18 chemicals in replicate independent series to allow evaluation of:

- adherence to acceptance criteria,

- single and dual operator comparison,

- predictive concordance.

Results demonstrated accurate and reproducible implementation.

The training exercise is described in full, with detailed method SOPs (Attachments 4a and 4b) and assessment reports (Attachments 5a, 5b, 5c, and 5d).

SOP implementation (transfer) by the naïve laboratories is also reported in full (Attachments 6a and 6b) indicating the method is both robust and transferable.

3.3 Overall conclusions on Module 3

Module 3: Transferability: The TST provides clear and comprehensive information in support of Module 3.

4. MODULE 4: BETWEEN-LABORATORY REPRODUCIBILITY (BLR)

4.1 Rationale for the selection of test items used for assessing BLR

The TST confirms chemical selection for BLR is significant, with relevant scope, supported by complete and quality assured *in vivo* Draize eye irritation data, for comparative evaluation of *in vitro* method predictive capacity.

The selection covers:

- solid and liquid physical states.
- the full range of *in vivo* eye irritation GHS Categories (1, 2A, 2B, or No Category).
- the *in vivo* determinants of classification (cornea opacity, iritis, conjunctiva redness, chemosis, reversibility/persistence).
- wide representation of organic functional groups.
- known chemical structures.
- coloured and/or direct MTT reducers.
- availability through laboratory retail supply, at reasonable cost.

This information is compiled in a Table including over 200 chemicals (Attachment 2) indicating the 120 chemicals (60 solids, 60 liquids) used for ring trial assessment of BLR.

4.2 Assessment of between-laboratory reproducibility of experimental data

BLR from the ring trial has been fully assessed by an independent consultant statistician, with comprehensive reports available respective of the two protocols, EITL (Attachment 8a) and EITS (Attachment 8b). The reports also provide all raw data in appendices. Summary data have also been compiled for overview:

- Attachment 7a: EITL: 60 ring trial chemicals, 3 labs (L'Oreal, VITO, CRL).
- Attachment 7b: EITS: 60 ring trial chemicals, 3 labs (L'Oreal, VITO, CRL).

BLR (concordance of predicted classification) for liquids, based on pair-wise comparison, was reported as follows:

- L'Oreal versus CRL: 93.3% (56/60 chemicals).
- L'Oreal versus VITO: 95.0% (57/60 chemicals).
- CRL versus VITO: 98.3% (59/60 chemicals).

Fifty six of the 60 chemicals were consistently classified (NC/C) by the three laboratories resulting in a BLR of 93.3% (95% CI: 84.1% - 97.4%).

BLR (concordance of predicted classification) for solids, based on pair-wise comparison, was reported as follows:

- L'Oreal versus CRL and L'Oreal versus VITO: 96.7% (58/60 chemicals).
- CRL versus VITO: 100%.

Fifty eight of the 60 chemicals were consistently classified (NC/C) by the three laboratories resulting in a BLR of 96.7% (95% CI: 88.6% - 99.1%).

The TST concludes overall BLR for the SkinEthic™ HCE EIT method, based on the set of 120 chemicals, was 95.0% (EITL 93.3% and EITS 96.7%) exceeding the defined minimum requirement of 80% set by the VMG of EIVS.

For comparison, the TST reports BLR from a previous ring trial validation of the similar test method EpiOcular™ EIT (as described in OECD Test Guideline 492) was 94.4% for liquids and 92.0% for solids.

4.3 Identification and discussion of outlying values

Evaluation of BLR was based on concordance of viabilities (with respect to prediction model) with descriptive statistics presented as two-by-two scatter plots for the three laboratories. Non-concordant results were identified chemical by chemical, including indication of non-qualified (NQ) runs in exceptional cases.

4.4 Overall conclusions on Module 4

Between-Laboratory Reproducibility (BLR): The TST provides clear and comprehensive information relevant to Module 4.

5. MODULE 5: PREDICTIVE CAPACITY (PC)

5.1 Rationale for the selection of test items used for assessing PC

The TST confirms chemical selection for assessment of PC is comprehensive, supported by complete and quality assured *in vivo* Draize eye irritation data, for comparative evaluation of *in vitro* method relevance.

The selection covers:

- solid and liquid physical states.
- the full range of *in vivo* eye irritation GHS Categories (1, 2A, 2B, or No Category).
- the *in vivo* determinants of classification (cornea opacity, iritis, conjunctiva redness, chemosis, reversibility/persistence).
- wide representation of organic functional groups.
- known chemical structures.
- coloured and/or direct MTT reducers.

This information is compiled in a Table including over 200 chemicals (Attachment 2) indicating the 120 chemicals (60 solids, 60 liquids) used in the ring trial, with an additional 80 chemicals tested by the lead laboratory, for assessment of PC.

5.2 Assessment of the predictive capacity of the test method

PC from the ring trial has been fully assessed by an independent consultant statistician, with comprehensive reports available respective of the two protocols, EITL (Attachment 8a) and EITS (Attachment 8b). The reports also provide all raw data in appendices. Summary data, including additional chemicals tested by the lead laboratory, have also been compiled for overview (Attachment 9).

PC (ring trial) was evaluated by comparing *in vitro* viability with respect to prediction model (all runs, per laboratory and cumulatively) with documented *in vivo* classifications according to GHS.

The statistics report summarises the frequency distribution of true versus false predictions, respective of irritant classification (C) and non-irritant classification (NC). From these frequencies are calculated the sensitivity (rate of correct prediction for C, with false negatives), the specificity (rate of correct prediction for NC, with false positives) and overall accuracy (rate of correct prediction, C or NC) expressed as percentages.

Liquids protocol (EITL) predictive capacity (ring trial):

in vivo	**Cumulative**		**L'Oréal**		**CRL**		**VITO**	
	C	**NC**	**C**	**NC**	**C**	**NC**	**C**	**NC**
Classified	283	5	96	0	94	2	93	3
No Category	77	175	29	55	23	61	25	59
Total	**540**		**180**		**180**		**180**	
Sensitivity (%)	98.3		100		97.9		96.9	
False Negatives (%)	1.7		0		2.1		3.1	
Specificity (%)	69.4		65.5		72.6		70.2	
False Positives (%)	30.6		34.5		27.4		29.8	
Accuracy (%)	84.8		83.9		86.1		84.4	

From statistical bootstrap resampling (which estimates uncertainty in predictive capacity, as 95% CI) (10,000 re-samples at n=1 for the 60 chemicals) the statistics report indicates overall predictive capacity for the liquids protocol (EITL):

Parameter	**Estimate**	**95% CI**
Sensitivity (%)	98.2	93.8; 100
Specificity (%)	69.4	60.7; 75.0
Accuracy (%)	84.8	80.0; 88.3

Solids protocol (EITS) predictive capacity (ring trial):

in vivo	**Cumulative**		**L'Oréal**		**CRL**		**VITO**	
	C	**NC**	**C**	**NC**	**C**	**NC**	**C**	**NC**
Classified	249	21	83	7	83	7	83	7
No Category	63	206	22	68	19	71	22	67
Total	**539**		**180**		**180**		**179**	
Sensitivity (%)	92.2		92.2		92.2		92.2	
False Negatives (%)	7.8		7.8		7.8		7.8	

Specificity (%)	76.6	75.6	78.9	75.3
False Positives (%)	23.4	24.4	21.1	24.7
Accuracy (%)	84.4	83.9	85.6	83.3

From statistical bootstrap resampling (which estimates uncertainty in predictive capacity, as 95% CI) (10,000 re-samples at n=1 for the 60 chemicals) the statistics report indicates predictive capacity for the solids protocol (EITS):

Parameter	**Estimate**	**95% CI**
Sensitivity (%)	91.9	90.0; 93.3
Specificity (%)	76.6	73.3; 80.0
Accuracy (%)	84.3	81.7; 86.7

The TST also reports sensitivity, specificity and accuracy for the extended set of chemicals (including 45 additional liquids and 35 additional solids tested by the lead laboratory only) quoting similar figures.

5.3 Identification and discussion of false predictions

The TST presents a detailed review of misclassifications, including correlation with *in vivo* determinants of GHS classification.

The statistics report indicates the misclassified chemicals were frequently the same between the three laboratories.

5.4 Overall conclusions on Module 5

Predictive Capacity (PC): The TST provides comprehensive information relevant to Module 5.

6. MODULE 6: APPLICABILITY DOMAIN (AD)

6.1 Applicability and limitations of the test method identified through testing and suggested applicability domain

The TST demonstrates the test method has been successfully applied to a range of physical properties and chemical classes, confirming a broad applicability domain. No further limitations were identified from the data acquired in the validation study.

6.2 Overall conclusions on Module 6

Applicability Domain (AD): The TST provides comprehensive information relevant to Module 6.

7. MODULE 7: PERFORMANCE STANDARDS (PS)

7.1 Suggestions for essential test method components, possible reference items, accuracy and reliability values

Performance Standards (PS) not addressed by TST but already available at OECD based on the similar method EpiOcular™ EIT (OECD Series on Testing and Assessment No. 216).

7.2 Overall conclusions on Module 7

Performance Standards (PS) not addressed by TST but already available at OECD based on the similar method EpiOcular™ EIT (OECD Series on Testing and Assessment No. 216).

8. ESSENTIAL INFORMATION FOR A SPECIFIC VALIDATION PROCESS

External Validation Studies (not coordinated by EURL-ECVAM)

This part of the TST aims to report the Test Submitter's conclusions regarding the outcome of an external (pre)validation study and his/her recommendations on future activities. In EURL ECVAM led-studies these two elements are developed and proposed by the validation management group and are integral part of the information provided in the study report for peer-review.

8.1.1 Study organisation and management

The TST confirms compliance with standards for validation study organisation, management, and conduct, including:

- thorough scientific basis for method R&D, with technical competence in tissue model production.
- comprehensive SOPs (protocols) for method implementation.
- comprehensive training for transfer to naïve laboratories.
- comprehensive chemicals (test items) selection.
- clear coordination and responsibilities for the ring trial (project management, chemicals management, data management).
- independence of ring trial testing and data statistics analysis.
- complete documentation.

8.1.2 Study objective

The study objectives (to validate WLR, TF, BLR, and PC for use of the method as an initial step in a Bottom-Up approach) have been clearly defined, and are appropriate.

8.1.3 Summary of the study results

The study results are clearly summarised and consistent with the reports.

8.1.4 Study conclusions

The study conclusions are clear and justified.

8.1.5 Study recommendations

The study scope is clear.

8.1.6 Overall conclusions

Validation of WLR, TF, BLR, and PC is comprehensive and quality assured.

8.2 Essential information for a Retrospective Validation Study

8.2.1 Information/data sources and selection criteria applied/information and data collected

Not applicable.

8.2.2 Protocols identified

Not applicable.

8.2.3 Data analysis and Prediction Models

Not applicable.

8.2.4 Overall conclusions

Not applicable.

8.3 Essential information for a Validation Study based on Performance Standards (for similar or updated test method(s)

8.3.1 Performance standards based on the validated reference test method(s)/essential test method components (used for similarity assessment)

Not applicable.

8.3.2 Overall conclusions

Not applicable.

9. OTHER

The MTT-reduction assay for tissue viability, relevant to all *in vitro* test methods based on Reconstructed human Tissues (RhT) is limited by interference with coloured chemicals.

The test method R&D has overcome this limitation using High/Ultra High Performance Liquid Chromatography Performance (HPLC-UPLC)-spectrophotometry for endpoint detection of formazan.

The HPLC-UPLC method has been shown to be highly reproducible (BLR) between different laboratories.

Based on this, the TST concludes that HPLC/UPLC is relevant to all *in vitro* RhT test methods irrespective of the test system and test method and can be applied to any of the other RhT test systems within the relevant OECD Test Guidelines. Indeed, the HPLC/UPLC-spectrophotometry technique has already been implemented in OECD TGs 431 (*in vitro* skin corrosion based on RhE), 439 (*in vitro* skin irritation based on RhE) and 492 (*in vitro* serious eye damage/eye irritation based on RhCE).

10. OVERALL CONCLUSIONS ON THE OUTCOME OF THE ASSESSMENT

Modules M1 – M6 are complete. M7 is already available OECD Series on Testing and Assessment No. 216.

11. CLARIFICATIONS/ADDITIONAL INFORMATION TO BE REQUESTED TO THE TEST SUBMITTER

List for each Module the additional clarifications/information to be requested to the Test Submitter

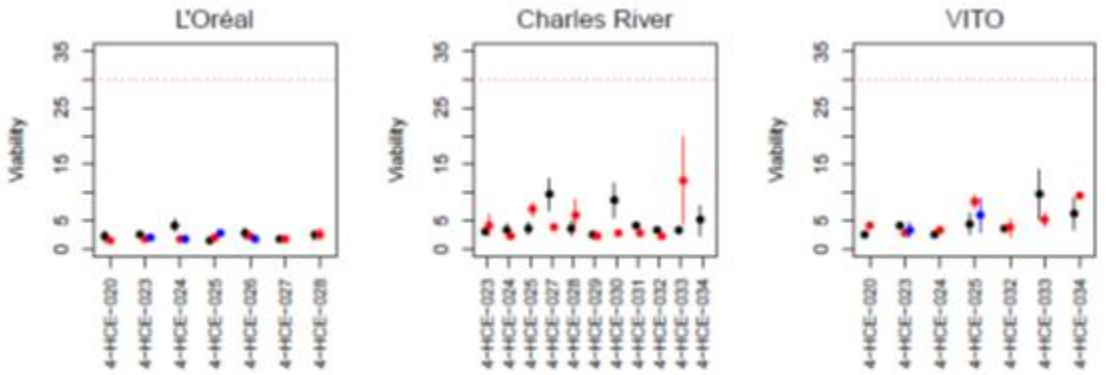
In section 1.3.4 (Occurrence of non-qualified test) the following observations were noted regarding positive control viability and variation with respect to the acceptance criterion (viability $\leq$ 30%, mean of 2 tissue replicates):

The statistics reports (Attachments 8a and 8b) summarise within and between laboratory variability of the viability for the positive control (PC) respective of the liquids (EITL) and solids (EITS) protocols:

Liquids (EITL):

	L'Oréal	CRL	VITO
Mean	2.12	4.49	4.89
SD	0.67	2.69	2.35
Min	1.40	2.09	2.35
Max	4.11	12.13	9.54

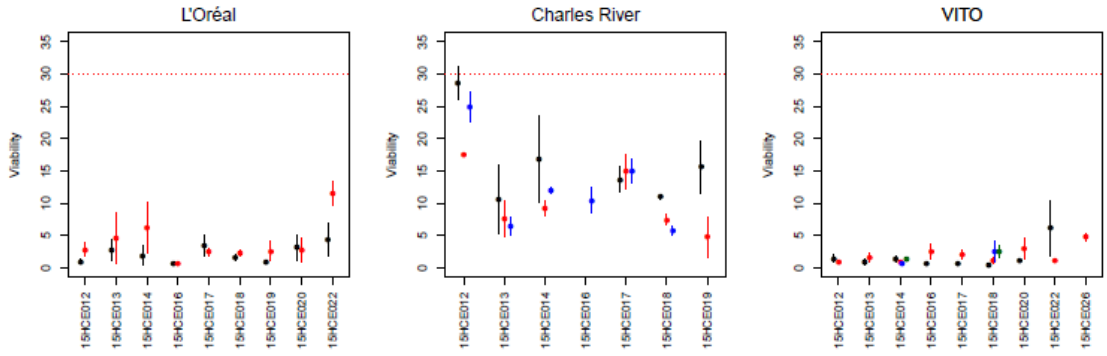
These values are illustrated graphically in the EITL statistics report *(Attachment 8a, Figure 3: Viability of the positive control for different batches (up to 3 series: black, red, blue) determined for duplicate tissues (mean value: dots, single values: bars)* copied here for clarification:



Solids (EITS):

	L'Oréal	CRL	VITO
Mean	3.1	12.9	1.9
SD	2.6	6.3	1.4
Min	0.7	4.8	0.6
Max	11.6	28.6	6.2

These values are illustrated graphically in the EITS statistics report *(Attachment 8b, Figure 3: Viability of the positive control for different batches (up to 4 series: black, red, blue, green) determined for duplicate tissues (mean value: dots, single values: bars)* copied here for clarification:



The PC mean viabilities (generally < 5%) are significantly lower than the 30% acceptance threshold, allowing scope for permitted variability within a qualified run/test. Nevertheless, the PC is also generally reproducible with maximum viabilities unequivocally acceptable.

However, the PC results for EITS from CRL are an exception, with greater variability and maximum viability near the threshold. Independent expert opinion has noted a probable cause for this variability as due to improper spreading of the PC methyl acetate when applied to the tissues, which would explain the larger variability observed for EITS than EITL due to the considerably longer exposure time for solids (4 hours) than for liquids (30 min). In fact, the longer exposure time for solids versus liquids would rather be expected to diminish variability.

It is suggested the positive control acceptance criterion threshold could be reduced, perhaps to 15%. The results indicate this would be ample, assuming the tissues are

accurately dosed with methyl acetate, while allowing detection of any variability in case of the contrary.

Application of the 30µL methyl acetate to the tissue evidently requires care to ensure even spreading, with higher importance for the solids protocol (EITS) due to the significantly longer exposure time used with this protocol.