EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
In vitro methods Unit
European Centre for the Validation of Alternative Methods (ECVAM)

STATEMENT ON THE SCIENTIFIC VALIDITY OF CYTOTOXICITY/CELL-FUNCTION BASED IN VITRO ASSAYS FOR EYE IRRITATION TESTING

At its 31st meeting, held on 7 and 8 July, 2009 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC)[1] unanimously endorsed the following statement:

The replacement of traditional animal-based test methods by alternative ones should ideally be obtained by one-to-one replacements: to keep the testing regime simple and economical one single alternative method should, wherever feasible, be sufficient to generate data of equal or better quality than the traditional test.

However, in the case of eye irritation it is currently generally accepted that, in the foreseeable future, no single *in vitro* eye irritation test will be able to replace the *in vivo* Draize eye test to predict across the full range of irritation for different chemical classes. However, strategic combinations of several alternative test methods within a (tiered) testing strategy may be able to replace the Draize eye test.

A possible conceptual framework for such a (tiered) testing strategy has been developed within an ECVAM workshop (Ref. 1). The framework is based on alternative eye irritation methods that vary in their capacity to detect either severe irritant substances (EU R41; GHS 'Category 1') or substances considered non-irritant (EU 'Non-Classified'; GHS 'No Category'). According to this framework the entire range of irritancy may be resolved by arranging tests in a tiered strategy that may be operated from either end: to detect first severe irritants and resolve absence of irritancy ("Top-Down Approach") or to proceed inversely, starting with the identification of non-irritants first ("Bottom-Up Approach"). Mild irritancy will be resolved in a last tier in both approaches.

To evaluate the scientific validity of possible building blocks of such a test strategy and to assess their possible placement within a Bottom-Up and Top-Down Approach, ECVAM has undertaken a retrospective validation study of four cell-based *in vitro* methods.

The test methods evaluated were:

a. Cytosensor Microphysiometer (INVITTOX Protocols 97 and 102 modified)[2]
b. Fluorescein Leakage (INVITTOX Protocols 71, 82, 86 and120);
c. Neutral Red Release (INVITTOX Protocol 54 and PREDISAFE™);
d. Red Blood Cell haemolysis (INVITTOX Protocols 37 and 99),

The four test methods, including ten protocol variations, were subjected to independent, expert review with respect to their use to either

[1] Details can be found in the PRP report
[2] Invittox protocols can be downloaded from ECVAM's database service on Alternative Methods to Animal Experimentation, DBALM: http://ecvam-dbalm.jrc.ec.europa.eu

a) initiate a Bottom-Up Approach, for consideration for regulatory use to identify non-irritants (EU: 'Non Classified'; GSH: 'No Category'; EPA: 'Category IV') from all other classes as part of a tiered testing strategy, or
b) to initiate a Top-Down Approach, to identify ocular corrosives and severe irritants (EU R41, GHS 'Category 1', and EPA 'Category I') from all other classes as part of a tiered testing strategy.

In the absence of internationally agreed performance criteria for either approach, the PRP of the ESAC applied the following criteria:

- any test used to initiate a Top-Down Approach must balance specificity and sensitivity to correctly identify a substantial proportion of severe irritants, with a false positive rate that would not lead to the over-classification of an unreasonable number of materials of lower ocular irritancy potential – an over-classification rate (false positives) of <10% was considered acceptable
- any test used to initiate a Bottom-Up Approach should ideally give no false negatives with respect to human safety, and no false negative should be produced by high-moderate or severe irritants.

Following independent ESAC peer review of this retrospective validation study and considering the potential test strategies in which the tests may be used, the ESAC concluded the following:

1. CYTOSENSOR MICROPHYSIOMETER TEST METHOD

The Cytosensor Microphysiometer test method can be used for two of the three EU and GHS classification categories used for the endpoint of ocular irritation:

A. The **Cytosensor Microphysiometer test method (INVITTOX Protocol 102 modified)** is considered to have been scientifically validated and to be ready for consideration for regulatory use as an initial step within a **Top-Down Approach** to identify ocular corrosives and severe irritants (EU R41, GHS Category 1, and EPA Category I) from all other classes for the chemical applicability domain of water-soluble chemicals (substances and mixtures).

B. Furthermore, the **Cytosensor Microphysiometer test method (INVITTOX Protocol 102 modified)** is considered to have been scientifically validated and to be ready for consideration for regulatory use as an initial step within a **Bottom-Up Approach** to identify non-irritants (EU:NC; GHS: NC; EPA: cat IV) from all other classes only for water-soluble surfactants and water-soluble surfactant-containing mixtures.

C. On the basis of a thorough evaluation of the data compiled in the course of the ECVAM validation study, the ESAC concludes that the **Cytosensor Microphysiometer** test method does NOT correctly identify moderate and mild ocular irritants (EU: R36; GHS: Cat 2A/B; EPA: Cat II/III). Therefore, the test method can only be employed to make decisions on two of the three categories of the eye irritation classification scheme (see A and B). Consequently, ESAC does NOT recommend this test method as a full replacement method. It should be noted in this context that the **Top-Down and Bottom-Up Approach** foresees the theoretical possibility of a *default* mild/moderate categorization (e.g. EU R36 or GHS Cat 2) of all those substances neither identified as ocular corrosives and severe irritants (see A) nor as "non-

classified" substances (see B) in the first two tiers of the strategy. However, the test method's high false negative rate (9-55%) when initiating a top-down approach and high false positive rate (50-69%) when initiating a bottom-up approach exclude the possibility to use the method for default categorization. The test methods can thus not be considered a full-replacement method on its own using the Top-Down and Bottom-Up approach.

Although these recommendations are based on the evaluation of data sets obtained using specific hard- and software, it is anticipated that other Cytosensor Microphysiometer equipment and software may become available with either equivalent or better performance and will need to be efficiently validated. Depending on the similarity of new equipment with respect to the validated one, this may be performed as a *Similar Method Validation* ('me-too') or an *Update Validation*. ESAC therefore recommends the development of Performance Standards for the Cytosensor Microphysiometer test method.

The current chemical applicability domain is limited: whilst in some cases this might be increased by expanding the data set of studied compounds, the test method is not amenable to testing non-water soluble solids, suspensions, or viscous materials.

2. FLUORESCEIN LEAKAGE TEST METHOD

The **Fluorescein Leakage test method (INVITTOX Protocol 71)** is considered to have been scientifically validated and to be ready for consideration for regulatory use as an initial step within a **Top-Down Approach** to identify ocular corrosives and severe irritants (EU R41, GSH Category 1, and EPA Category I) from all other classes for water-soluble chemicals (substances and mixtures).

Additional testing and further refinement, in particular with respect to variability and definition of the applicability domain, by expanding the dataset of tested chemicals and direct comparison with *in vivo* data is recommended and should be kept under review.

With regard to the

- Neutral Red Release (INVITTOX Protocol 54 and PREDISAFE™);
- Fluorescein Leakage (INVITTOX Protocols 82, 86 and120);
- Red Blood Cell haemolysis (INVITTOX Protocols 37 and 99),

ESAC considers that the available evidence is insufficient[3] to support a recommendation that they are ready for consideration for regulatory use.

Similarly, the available evidence for Fluorescein Leakage INVITTOX Protocol 71 does not support a recommendation for its use to initiate a Bottom-Up Approach for regulatory use.

[3] Details can be found in the PRP report

This statement takes account of the dossiers prepared for peer review; the views of independent experts of the ESAC Peer Review Panel (PRP) who evaluated the dossiers against defined validation criteria as well as supplementary submissions made by the Validation Management Group.

In agreement with common practice upon completion of a validation study, ESAC recommends the development of Performance Standards for the Cytosensor Microphysiometer and the Fluorescein Leakage assays to allow the validation of *similar test methods* or *modifications of the validated test methods* based on pre-defined evaluation and acceptance criteria.

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Ispra, 10th July 2009

REFERENCECS

1. Scott, L. et al. (2009) A proposed eye irritation testing strategy to reduce and replace in vivo studies using Bottom-Up and Top-Down approaches. Toxicol In Vitro. May 31. *[Epub ahead of print]*

The ESAC was established by the European Commission, and is composed of nominees from the EU Member States, industry, academia and animal welfare organisations, together with representatives of the relevant Commission services.

This statement was endorsed by the following members of the ESAC:

Ms Argelia Castaño(Spain)
Ms Maija Dambrova (Latvia)
Ms Alison Gray (ESTIV)
Ms Katalin Horvath (Hungary)
Ms Dagmar Jírová (Czech Republic)
Mr Roman Kolar (Eurogroup for Animals)
Ms Elisabeth Knudsen (Denmark - acting as moderator at the meeting)
Mr Manfred Liebsch (Germany)
Mr Gianni Dal Negro (EFPIA)
Mr. Walter Pfaller (Austria)
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The following Commission Services and Observer Organisations were involved in the consultation process, but not in the endorsement process itself:

Commission services
Mr Joachim Kreysa (DG JRC, Head of In vitro methods Unit/ECVAM, chairman)
Mr Claudius Griesinger (DG JRC, ESAC secretariat)
Ms Susanne Hoke (DG ENTR)
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The following observers were present
Mr Hajime Kojima (JaCVAM)
Mr William Stokes (NICEATM)
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