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2 **STATEMENT ON THE SCIENTIFIC VALIDITY OF CYTOTOXICITY/CELL-**
3 **FUNCTION BASED IN VITRO ASSAYS FOR EYE IRRITATION TESTING**
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6 At its 31st meeting, held on 7 and 8 July, 2009 at the European Centre for the Validation of
7 Alternative Methods (ECVAM), Ispra, Italy, the non-Commission members of the ECVAM
8 Scientific Advisory Committee (ESAC)¹ unanimously endorsed the following statement:
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10 The replacement of traditional animal-based test methods by alternative ones should ideally
11 be obtained by one-to-one replacements: to keep the testing regime simple and economical
12 one single alternative method should, wherever feasible, be sufficient to generate data of
13 equal or better quality than the traditional test.
14

15 However, in the case of eye irritation it is currently generally accepted that, in the foreseeable
16 future, no single *in vitro* eye irritation test will be able to replace the *in vivo* Draize eye test to
17 predict across the full range of irritation for different chemical classes. However, strategic
18 combinations of several alternative test methods within a (tiered) testing strategy may be able
19 to replace the Draize eye test.
20

21 A possible conceptual framework for such a (tiered) testing strategy has been developed
22 within an ECVAM workshop (Ref. 1). The framework is based on alternative eye irritation
23 methods that vary in their capacity to detect either severe irritant substances (EU R41; GHS
24 'Category 1') or substances considered non-irritant (EU 'Non-Classified'; GHS 'No Category').
25 According to this framework the entire range of irritancy may be resolved by arranging tests
26 in a tiered strategy that may be operated from either end: to detect first severe irritants and
27 resolve absence of irritancy ("Top-Down Approach") or to proceed inversely, starting with the
28 identification of non-irritants first ("Bottom-Up Approach"). Mild irritancy will be resolved in
29 a last tier in both approaches.
30

31 To evaluate the scientific validity of possible building blocks of such a test strategy and to
32 assess their possible placement within a Bottom-Up and Top-Down Approach, ECVAM has
33 undertaken a retrospective validation study of four cell-based *in vitro* methods.
34

35 The test methods evaluated were:

- 36
- 37 a. Cytosensor Microphysiometer (INVITTOX Protocols 97 and 102 modified)²
 - 38 b. Fluorescein Leakage (INVITTOX Protocols 71, 82, 86 and 120);
 - 39 c. Neutral Red Release (INVITTOX Protocol 54 and PREDISAFETM);
 - 40 d. Red Blood Cell haemolysis (INVITTOX Protocols 37 and 99),
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42 The four test methods, including ten protocol variations, were subjected to independent,
43 expert review with respect to their use to either

¹ Details can be found in the PRP report

² Invitox protocols can be downloaded from ECVAM's database service on Alternative Methods to Animal Experimentation, DBALM: <http://ecvam-dbalm.jrc.ec.europa.eu>



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

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

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

60
61 Following independent ESAC peer review of this retrospective validation study and
62 considering the potential test strategies in which the tests may be used, the ESAC concluded
63 the following:

64 1. CYTOSENSOR MICROPHYSIOMETER TEST METHOD

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

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69
70 A. The **Cytosensor Microphysiometer test method (INVITTOX Protocol 102 modified)** is
71 considered to have been scientifically validated and to be ready for consideration for
72 regulatory use as an initial step within a **Top-Down Approach** to identify ocular corrosives
73 and severe irritants (EU R41, GHS Category 1, and EPA Category I) from all other classes for
74 the chemical applicability domain of water-soluble chemicals (substances and mixtures).

75
76 B. Furthermore, the **Cytosensor Microphysiometer test method (INVITTOX Protocol 102**
77 **modified)** is considered to have been scientifically validated and to be ready for consideration
78 for regulatory use as an initial step within a **Bottom-Up Approach** to identify non-irritants
79 (EU:NC; GHS: NC; EPA: cat IV) from all other classes only for water-soluble surfactants and
80 water-soluble surfactant-containing mixtures.

81
82 C. On the basis of a thorough evaluation of the data compiled in the course of the ECVAM
83 validation study, the ESAC concludes that the **Cytosensor Microphysiometer** test method
84 does NOT correctly identify moderate and mild ocular irritants (EU: R36; GHS: Cat 2A/B;
85 EPA: Cat II/III). Therefore, the test method can only be employed to make decisions on two
86 of the three categories of the eye irritation classification scheme (see A and B). Consequently,
87 ESAC does NOT recommend this test method as a full replacement method. It should be
88 noted in this context that the **Top-Down and Bottom-Up Approach** foresees the theoretical
89 possibility of a *default* mild/moderate categorization (e.g. EU R36 or GHS Cat 2) of all those
90 substances neither identified as ocular corrosives and severe irritants (see A) nor as "non-



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

96
97 Although these recommendations are based on the evaluation of data sets obtained using
98 specific hard- and software, it is anticipated that other Cytosensor Microphysiometer
99 equipment and software may become available with either equivalent or better performance
100 and will need to be efficiently validated. Depending on the similarity of new equipment with
101 respect to the validated one, this may be performed as a *Similar Method Validation* ('me-too')
102 or an *Update Validation*. ESAC therefore recommends the development of Performance
103 Standards for the Cytosensor Microphysiometer test method.

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

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110 2. FLUORESCHEIN LEAKAGE TEST METHOD

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

117
118 Additional testing and further refinement, in particular with respect to variability and
119 definition of the applicability domain, by expanding the dataset of tested chemicals and direct
120 comparison with *in vivo* data is recommended and should be kept under review.

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122 With regard to the

- 123 • Neutral Red Release (INVITTOX Protocol 54 and PREDISAFETM);
- 124 • Fluorescein Leakage (INVITTOX Protocols 82, 86 and 120);
- 125 • Red Blood Cell haemolysis (INVITTOX Protocols 37 and 99),

126 ESAC considers that the available evidence is insufficient³ to support a recommendation that
127 they are ready for consideration for regulatory use.

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

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³ Details can be found in the PRP report



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

140

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

146

147 Joachim Kreysa

148 Head of Unit

149 In vitro methods Unit

150 European Centre for the Validation of Alternative Methods

151

152 Ispra, 10th July 2009



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153 **REFERENCECS**

154

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

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

163

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

165

- 166 Ms Argelia Castaño (Spain)
167 Ms Maija Dambrova (Latvia)
168 Ms Alison Gray (ESTIV)
169 Ms Katalin Horvath (Hungary)
170 Ms Dagmar Jírová (Czech Republic)
171 Mr Roman Kolar (Eurogroup for Animals)
172 Ms Elisabeth Knudsen (Denmark - acting as moderator at the meeting)
173 Mr Manfred Liebsch (Germany)
174 Mr Gianni Dal Negro (EFPIA)
175 Mr. Walter Pfaller (Austria)
176 Mr Tõnu Püssa (Estonia)
177 Mr Dariusz Sladowski (Poland)
178 Mr Jon Richmond (UK)
179 Ms Vera Rogiers (ECOPA)
180 Mr Michael Ryan (Ireland)
181 Ms Annalaura Stamatii (Italy)
182 Mr Jan van der Valk (The Netherlands)
183 Mr Carl Westmoreland (COLIPA)
184 Mr Timo Ylikomi (Finland)

185

186 The following Commission Services and Observer Organisations were involved in the
187 consultation process, but not in the endorsement process itself:

188 **Commission services**

- 189 Mr Joachim Kreysa (DG JRC, Head of In vitro methods Unit/ECVAM, chairman)
190 Mr Claudius Griesinger (DG JRC, ESAC secretariat)
191 Ms Susanne Hoke (DG ENTR)
192 Ms Susanna Louhimies (DG ENV)
193 Mr Juan Riego Sintes (DG JRC)

194

195 **The following observers were present**

- 196 Mr Hajime Kojima (JaCVAM)
197 Mr William Stokes (NICEATM)
198 Ms Marilyn Wind (ICCVAM)