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1 **REVIEW**

2  
3 **An Overview of Current Alternative Models in the Context of Ocular Surface Toxicity**

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16  
17 **Abstract**

18 The 21<sup>st</sup> century has seen a steadily increasing social awareness of animal suffering, with  
19 increased attention to ethical considerations. Developing new integrated approaches to testing  
20 and assessment (IATA) strategies is an Organisation for Economic Co-operation and  
21 Development (OECD) goal to reduce animal testing. Currently, there is a lack of alternative  
22 models to test for ocular surface toxicity (aside from irritation) in lieu of the Draize eye  
23 irritation test (OECD guideline No. 405) performed in rabbits. Five alternative *in vitro* or *ex*  
24 *vivo* methods have been validated to replace this reference test, but only in combination.  
25 However, pathologies like Toxicity-Induced Dry Eye (TIDE), cataract, glaucoma and  
26 neuropathic pain can occur after exposure to a pharmaceutical product or chemical and  
27 therefore need to be anticipated. To do so, new models of lacrimal glands, lens, neurons  
28 innervating epithelia are required. These models must take into account real life exposure  
29 (dose, time, and tear film clearance). The scientific community is working hard to develop  
30 new, robust, alternative, *in silico* and *in vitro* models, while attempting to balance ethics and  
31 availability of biological materials. This review provides a broad overview of the validated  
32 methods for analysing ocular irritation and those still used by some industries, as well as  
33 promising models that need to be optimized according to the OECD. Finally, we give an

34 overview of recently developed innovative models which could become new tools in the  
35 evaluation of ocular surface toxicity within the scope of IATAs.

36

### 37 **Short abstract**

38 Until now, the Draize test in rabbits has been the only test performed to anticipate ocular  
39 toxicity of pharmaceutical compounds, mainly irritation. However, in the field of alternative  
40 approaches, new models must be developed and validated. This review aims to give an  
41 overview of the OECD validated methods and of innovative models, which could become  
42 new tools in the evaluation of ocular surface toxicity.

43

44

45 **Key words:** Draize Eye Test; OECD guidelines; Ocular Surface; In Silico; 3D Multicellular;  
46 Cornea-on-a-chip; Organoids

47

48

### 49 **Introduction**

50 Since the beginning of the 21<sup>st</sup> century, modern toxicology has been focusing on the 3R  
51 principle, “Reduce, Refine, Replace”, established in 1959 by Russell and Burch, stipulating  
52 that the use of laboratory animals should be only a last resort. Since 2013, in Europe, the  
53 cosmetic industry has been confronted with strict prohibition of evaluating its products on  
54 animals. Integrated approaches of testing and assessment (IATA), promoted by the OECD  
55 (Organisation for Economic Cooperation and Development) might enable validation of new  
56 compounds in this sector (Canavez *et al.* 2021).

57 To date, validated alternative models have been available only for the evaluation of potential  
58 ocular surface irritation. Models to predict Toxicity-Induced Dry Eye (TIDE), anterior  
59 segment neuropathies or other ocular surface changes are still in the stage of basic science  
60 research. Furthermore, classification of ocular irritants is based on the United Nations  
61 Organization (UNO) system, *i.e.* the GHS “Globally Harmonized System of Classification  
62 and Labelling of Chemicals” (Luechtefeld *et al.* 2016). This international system distinguishes  
63 severe irritants (Category 1), moderate irritants (Category 2A), mild irritants (Category 2B)  
64 and non-irritants (No Category). However, unlike the Draize test, the *in vivo* reference model  
65 in rabbits, current alternative models for ocular irritation cannot distinguish Category 2A from  
66 2B irritants. These irritants are usually differentiated based on the kinetics of the reversibility

67 of damage. Of note, the lack of reproducibility of the *in vivo* test of reference, the Draize test,  
68 complicates the validation of alternative models by the ICCVAM (Interagency Coordinating  
69 Committee on the Validation of Alternative Methods)(OECD Webinar 2019a).

70 In its first section, this review presents updates in the latest methodology for evaluation of  
71 ocular irritation, first presenting the five *in vitro* or *ex vivo* models validated by the OECD, in  
72 combination, to replace the Draize test (Guideline (GL) 405)(OECD, 2020a): Reconstructed  
73 human Cornea-like Epithelium (RhCE) viability tests (GL 492)(OECD, 2019a), Bovine  
74 Corneal Opacity and Permeability (BCOP) test (GL 437)(OECD, 2020b), Isolated Chicken  
75 Eye (ICE) test (GL 438)(OECD, 2018a), Fluorescein Leakage (GL 460)(OECD, 2017), and  
76 Short Time Exposure assay (STE, GL 491)(OECD, 2020c). The ocular irritation IATA  
77 indicates the combination of tests that should be considered depending on whether the product  
78 is suspected to be an irritant ('top-down' approach) or is thought to be in the non-irritant  
79 category ('bottom-up' approach). This review also presents models used by some cosmetic  
80 companies that either were or still are under evaluation by the OECD, such as the Isolated  
81 Rabbit Eye (IRE) test, Hen's Egg Test on the Chorio-Allantoic Membrane (HET-CAM).  
82 Characteristics and protocol details of the models for ocular irritation are summarized in  
83 Table I.

84 Next, promising models mentioned in the OECD Guidance Document No. 263 (OECD,  
85 2019b) are presented. These models, if optimized and validated, might represent a major asset  
86 in classifying new compounds into Categories 2A and 2B: PorCORA (Porcine Ocular Cornea  
87 Opacity/Reversibility Assay), EVEIT (Ex Vivo Eye Irritation Test), 3D Hemi-Cornea and  
88 SMI (Slug Mucosal Irritation assay).

89 Finally, in order to prevent complex toxicities as TIDE, glaucoma, cataract, some of which  
90 are rare topical side effects, new models presented in the literature could be validated and  
91 incorporated into new IATAs, taking into account real-life exposure, pharmacokinetics and  
92 knowledge already reported in the literature. Therefore, this final section provides an  
93 overview of *in silico* and *in vitro* models which could, in combination, enable complete  
94 evaluation of ocular surface toxicities within the framework of IATAs.

95

## 96 **Alternative models to the Draize test according to OECD GL 405**

97

98 *Reconstructed human Cornea-like Epithelium (RhCE)*

99 Since the last update of GL 492 in 2019 (OECD, 2019a), four models of RhCE are now  
100 available to evaluate ocular surface irritation, two of which are considered Validated  
101 Reference Methods (VRM): EpiOcular™ (VRM1), SkinEthic™ HCE (VRM2), LabCyte  
102 CORNEA-MODEL24 and MCTT HCE™. These RhCE models mimic human corneal  
103 epithelium morphologically, histologically, biochemically and physiologically and can be  
104 used first in a ‘bottom-up’ approach to identify non-irritant substances. Even though cellular  
105 damage can occur through several mechanisms, only cytotoxicity measurements are carried  
106 out. Indeed, cell viability is considered to be proportional to the severity of damage and  
107 representative of the global response of the ocular surface: mild irritants with low transcorneal  
108 penetration alter only the superficial corneal epithelium, whereas moderate or severe irritants  
109 can penetrate more deeply, reaching the corneal stroma and sometimes endothelium. This  
110 global response would be a correct representation of the damage that could occur in humans  
111 after toxic exposure, no matter the cellular mechanisms involved, ranging from slight  
112 conjunctival erythema or edema to severe changes such as corneal opacification.

113 While there may be differences between RhCE models (Table II), mainly concerning the cell  
114 types used and duration of epithelium culture, the testing method is similar: direct application  
115 of the tested compounds on the 3D epithelium and viability cytotoxicity assays reflecting the  
116 mitochondrial metabolic ability of viable cells. If corneal viability diminishes to below the  
117 fixed threshold (specific to each RhCE, see Table II), this will suggest classification of the  
118 compound as an ocular irritant. Above the threshold, the compound will not be classified and  
119 must be combined with another validated GL (437, 438, 460, 491 or in last option, if the test  
120 compound is not a cosmetic, GL 405 *i.e.* Draize test). Nevertheless, the OECD Guidance  
121 Document No. 263 (OECD, 2019b) reports the ongoing OECD evaluation of the EpiOcular™  
122 time-to-toxicity (ET<sub>50</sub>) assay, a test that could enable the differentiation of category 2A from  
123 2B irritants (Kandarova *et al.* 2018). This new protocol is based on multiple time and  
124 concentration exposures. It could represent a major asset in the scope of IATA decision trees,  
125 since as of yet, no validated alternative model alone can distinguish between all the categories  
126 of irritants.

127 Another limitation is that GL 492 can only be used for solids, semi-solids, liquids and waxes,  
128 since gases and aerosols have not undergone validation procedures. Nonetheless, this aspect  
129 should be investigated, since many accidental ocular exposures are caused by volatile  
130 compounds (OECD 2019a).

131

132 *Bovine Corneal Opacity and Permeability assay (BCOP)*

133 Recommended as the first step of a ‘top-down’ strategy, the organotypic BCOP model  
134 described in GL 437 (OECD, 2020b) enables differentiation between severe irritants applied  
135 to isolated bovine cornea from slaughterhouses. It can also identify non-irritants in a ‘bottom-  
136 up’ approach. The eyeballs are kept *ex vivo* for a brief period, during which physiological and  
137 biochemical functions remain unaltered. After excision, corneas are anchored on a corneal  
138 holder composed of two chambers, both filled with preservation medium. Briefly, the  
139 endothelial surface of the cornea is placed on the *o-ring* in the posterior chamber, while the  
140 epithelial surface is positioned in the anterior chamber.

141 Two application methods, adapted to the type of compound being tested, are described in the  
142 GL, but an important parameter is verification that the product covers the entire epithelial  
143 surface and that the washing step is sufficient to retrieve all of the compound. Irritancy  
144 potential is then measured through the *In Vitro* Irritancy Score (IVIS), which combines the  
145 diminution of light transmission capacity (corneal opacity, measured with an opacimeter) and  
146 the increase in fluorescein sodium passage (permeability, *i.e.* the amount of dye dropped in  
147 the anterior chamber and that crosses the corneal thickness). Of note, fluorescein sodium is an  
148 anionic compound, not retained by a healthy, negatively charged epithelium. A substance will  
149 be categorized as a severe irritant if the IVIS is greater than 55 and as a non-irritant if the  
150 IVIS is less than 3. However, if the IVIS is between 3 and 55, additional tests will be required  
151 to distinguish category 2 irritants. It is also possible to complement these results with a  
152 histologic analysis of the cornea, which procedure is described in the Guidance Document No.  
153 160 (OECD, 2018b).

154 Of note, since the last guideline update in June 2020, a second opacimeter can be used  
155 (LLBO), requiring adaptation of the IVIS equation and decision criteria, but the performance  
156 is comparable to OP-KIT, the first opacimeter validated.

157

158 *Isolated Chicken Eye (ICE)*

159 Like the BCOP model, the ICE aims to discriminate Category 1 GHS substances in a ‘top-  
160 down’ strategy but can also be included in a ‘bottom-up’ approach to identify non-irritants.  
161 The ICE is regulated by OECD GL 438, last updated in 2018 (OECD, 2018a). This test uses  
162 enucleated eyes of chickens for human consumption. In this assay, corneas are not excised.  
163 The whole eye is placed in a stainless-steel clamp with the cornea positioned vertically. The  
164 clamp is placed in a superfusion chamber to nourish the cornea. At the start of the test, the

165 clamp is retrieved from the chamber and the cornea positioned horizontally in order to apply  
166 the tested compounds. A qualitative and quantitative evaluation of the cornea is conducted to  
167 establish potential opacities, epithelial morphological alterations (detected by fluorescein  
168 sodium retention) and edema. As for BCOP, corneal opacity and fluorescein retention are  
169 scored, and are associated to a morphological evaluation which is “subjective according to the  
170 interpretation of the investigator” (GL 438). The combination of these scores enables the GHS  
171 classification of test compounds. For instance, with three scores of I, the substance is  
172 considered a non-irritant.

173 Furthermore, since the last GL update, histological features after paraffin embedding can be  
174 analyzed notably for detergents and surfactant irritants (OECD, 2019b). Indeed, there should  
175 be a correlation between erosion, vacuole formation in the inferior area of the epithelium,  
176 presence of pycnotic nuclei and irreversibility of the damage. GL 438 proposes another table  
177 to score those parameters.

178

#### 179 *Fluorescein Leakage (FL)*

180 The FL test follows GL 460 (OECD, 2017). The FL method is performed *in vitro* on a semi-  
181 permeable membrane (insert) leading to a single-layer culture of renal tubular cells of Madin-  
182 Darby Canin (MDCK CB997). This is a well described cell line, known to form tight  
183 junctions and desmosomes. Its organization is similar to the non-proliferative apical corneal  
184 epithelium. Furthermore, permeabilization of corneal epithelium is known to be one of the  
185 first phenomena occurring in toxicity-induced ocular irritation.

186 Changes in tight junctions and desmosomes are proportional to the quantity of fluorescein  
187 sodium that diffuses into the basal chamber, evaluated through FL<sub>20%</sub>, that is to say the  
188 concentration of the tested compound that leads to an FL of 20% compared to negative  
189 controls (single layer of cells not exposed and insert without cells). The substance tested is  
190 categorized as a severe irritant on the GHS ocular irritation classification if the FL<sub>20%</sub> ≤ 100  
191 mg/mL.

192 Integrated into a ‘top-down’ strategy, this simple method enables distinguishing Category 1  
193 chemicals without additional data. However, unlike the previous methods presented, this  
194 method can only be used with water-soluble compounds or mixtures. Indeed, solids in  
195 suspension will precipitate. In addition, it is not applicable to strong bases or acids, volatile  
196 compounds or cellular fixatives, because the toxic mechanisms for these types of compounds  
197 (such as protein coagulation or saponification) cannot be evaluated by FL. Finally, colored or

198 viscous compounds should be tested with other methods, since their complete washout  
199 required before the fluorescent measurement is complicated. Of note, a compound with a  
200 strong affinity for the insert membrane can lead to the same problem. Therefore, this affinity  
201 must be tested, as described in the GL, before beginning the assay. While reversibility of the  
202 epithelial changes cannot yet be evaluated, this will be considered in the next update of the  
203 GL, with the possibility of using FL to separate Categories 2A and 2B of the GHS  
204 classification.

205

#### 206 *Short Time Exposure Assay (STE)*

207 The STE, GL 491 (OECD, 2020c), can be considered in ‘bottom-up’ and ‘top-down’  
208 strategies. STE enables the evaluation of all types of chemicals except volatile compounds  
209 with a vapor pressure above 6 kPa<sup>1</sup> and solid non-surfactants (not water-soluble after at least 5  
210 min in NaCl). This *in vitro* model consists of a confluent monolayer culture of rabbit corneal  
211 fibroblasts (several cell lines are possible, such as CCL60 or SIRC). If cell viability (MTT  
212 assay, see Table 2) is less than 70% with both concentrations, the substance is placed in  
213 Category 1 without any additional assay. If cell viability is above 70% with at least one  
214 concentration, additional tests are required.

215

#### 216 **Other ocular irritation models evaluated by the OECD**

217

##### 218 *Vitrigel-Eye Irritancy Test (EIT) method*

219 While not mentioned in GL 405 as an alternative method, the OECD introduces this method  
220 in its Guidance Document n°263 (OECD, 2019b) and in the 2019 GL 494 (OECD, 2019c),  
221 establishing the protocol for the Vitrigel EIT method. This method can be used only in a  
222 ‘bottom-up’ approach to identify non-irritants. It evaluates the barrier function of a human  
223 corneal epithelium reconstructed on a Vitrigel matrix (ECh-T immortalized cell line ; collagen  
224 gel obtained by rehydration of a hydrogel that has undergone a vitrification  
225 process)(Takezawa *et al.* 2004; Yamaguchi *et al.* 2016) with a Transepithelial Electrical  
226 Resistance (TEER) measurement. Of note, this ohmmeter analysis is sensitive to the number  
227 of cell passages and to room temperature (Srinivasan *et al.* 2015). This measurement is  
228 characterized by three parameters: time lag, intensity and plateau level. A non-irritant product  
229 is identified by a time lag > 180 seconds, an intensity < 0.05% and a plateau level ≤ 5.0%. If  
230 one of these criteria differs, additional studies are required to classify the product. A



231 limitation of this method is its small range of application, being limited to liquids or semi-  
232 liquids with a pH > 5. However, unlike previous methods described, it can be used for volatile  
233 compounds and products that interfere with the detection of formazan in the MTT assay.

234

#### 235 *Ocular Irritection*®

236 Ocular Irritection® is an *in vitro* macromolecular test which is the subject of a GL drafted in  
237 2019 (OECD, 2019d). It is suitable either in a ‘bottom-up’ or ‘top-down’ strategy for solids  
238 and liquids with a pH between 4 and 9. It is an acellular system composed of proteins,  
239 glycoproteins, carbohydrates, lipids and low molecular weight compounds. Ocular  
240 Irritection® test aims to mimic the organized and transparent structure of the cornea after  
241 rehydration (Eskes *et al.* 2014). This enables specific detection of protein coagulation or lipid  
242 saponification mechanisms. Nevertheless, since the system is devoid of cells, cytotoxicity  
243 cannot be evaluated. The matrix and the testing principle are presented in figure 1. Any  
244 change in the matrix organization leads to a modification of the turbidity and reflects the  
245 irritative capacity of the test compound. However, like many alternative models, this testing  
246 method alone is unable to distinguish mild irritants.

247

#### 248 *Cytosensor Microphysiometer (CM)*

249 Because of the lack of commercial availability of the Cytosensor Microphysiometer  
250 technology, the preliminary GL version released in 2012 (OECD, 2012) for the evaluation of  
251 water-soluble compounds, solids, viscous substances or homogenous suspensions has seen its  
252 development discontinued in 2016 (European Commission 2020a). Nevertheless, it could be  
253 integrated into a ‘bottom-up’ or ‘top-down’ approach if similar instruments were to come to  
254 market. It consists of an adherent, confluent, single layer of mice fibroblasts (cell line L929)  
255 cultured on a polycarbonate insert. These cells are designed to represent conjunctival and  
256 corneal epithelia. The test endpoint is the Metabolic Rate Decrement of 50% (MRD<sub>50</sub>), that is  
257 to say the concentration that reduces the acidification rate by 50%. This measurement reveals  
258 irritation potential, since damaged cells will produce less acidic metabolites in the culture  
259 medium. On the one hand, if the MRD<sub>50</sub> ≤ 2 mg/mL, the product is considered to be a severe  
260 irritant in a ‘top-down’ approach. On the other hand, if the MRD<sub>50</sub> > 10 mg/mL, the test  
261 compound is classified as a non-irritant in a ‘bottom-up’ strategy. Of note, the GL mentions  
262 that this testing method could evaluate reversibility if optimized.

263

#### 264 *Neutral Red Release (NRR)*

265 The NRR test evaluates cytotoxicity on a single layer fibroblast or keratinocyte culture loaded  
266 with neutral red 3 hours before the exposure to test compounds (OECD, 2019b). This vital  
267 dye incorporates itself into lysosomes of viable cells. Several protocols have been proposed in  
268 the literature (Zuang 2001), such as the FRAME protocol based on mice embryonic  
269 fibroblasts (3T3-L1 cell line) or the *Clonetics Corporation* protocol using human  
270 keratinocytes. In both cases, the endpoint is the NRR<sub>50</sub>, that is to say the test compound  
271 concentration that releases, in the culture medium, 50% of the neutral red incorporated by  
272 lysosomes. The more toxic a substance is, the more cellular membranes, including lysosomal  
273 membranes, are altered, leading to leakage of intracellular compounds such as neutral red.  
274 Validated by internal procedures in many industries, the ICCVAM is requesting  
275 supplementary data on inter-laboratory reproducibility before publishing a GL on the Neutral  
276 Red Release assay (OECD, 2019b). It is also being considered for use in combination with the  
277 EpiOcular time-to-toxicity assay on RhCE.

278

#### 279 *Red Blood Cell test (RBC)*

280 The RBC test evaluates the ability of test compounds to disrupt red cell membranes (relation  
281 between hemolysis and oxyhemoglobin denaturation) and in this way, to classify products  
282 into GHS Categories 1 or non-classified (OECD, 2019b). RBC test can be conducted on red  
283 blood cells from various species (pig, sheep, rabbit) (Lewis *et al.* 1993; Mehling *et al.* 2007;  
284 Pape *et al.* 1987; Pape 1990). The irritant potential score corresponds to the ratio between the  
285 leakage of red blood cell hemoglobin in the supernatant (H<sub>50</sub> concentration inducing a red cell  
286 hemolysis of 50%) and oxyhemoglobin (denaturation index, DI). If H<sub>50</sub>/DI > 100, the  
287 substance is considered a non-irritant, between 10 and 100 the substance is categorized as a  
288 mild irritant (Category 2), between 1-10 as a moderate irritant, and if the H<sub>50</sub>/DI < 1, the  
289 compound is classified in Category 1 (severe irritant).

290 An application of this method on 12 shampoos and 7 conditioners was proposed by Alves *et*  
291 *al.* (2008), attesting to a 91.6% sensitivity and 100% specificity of the method. However, in  
292 the Guidance Document n°263 (OECD, 2019b), the OECD underscores the necessity for  
293 more data on the types of compound that can be tested, in other words, the method's range of  
294 application. Indeed, while the literature reports other studies on surfactants, mixtures  
295 (Mehling *et al.* 2007) and eyedrops (Martins *et al.* 2012), the chemical and physical properties  
296 of test compounds must be further investigated.

297

298 *Isolated Rabbit Eye (IRE)*

299 Although the IRE test is similar to the ICE (compound exposure time, endpoints ; see Table  
300 1), this organotypic model on the enucleated rabbit eye has not been validated by the  
301 ICCVAM since its 2010 evaluation, due to the lack of a standardized protocol, the lack of  
302 data on decision criteria, and the fact that rabbit eyes come from experimental animals and not  
303 from slaughterhouses as with BCOP or ICE (Lee *et al.* 2017; Prinsen and Koëter 1993).  
304 Nevertheless, the IRE is accepted in the European Union for distinguishing severe irritants  
305 (except alcohols, solids and surfactants, for which there are too many false negatives)  
306 (ICCVAM 2010).

307

308 *Hen's Egg Test on Chorioallantoic Membrane (HET-CAM)*

309 The HET-CAM is an alternative model developed by Luepke in 1985 and modified to classify  
310 irritant compounds. Indeed, the chorioallantoic membrane of the egg is considered to be a  
311 reasonable facsimile of the conjunctiva and its vasculature. Of note, from this model was  
312 derived another model, the Chorioallantoic Membrane Vascular Assay (CAMVA). The main  
313 nuance between the two (see figure 2) could enable differentiation of non-irritants from mild  
314 or moderate irritants.

315 The main advantages of using embryonated eggs are their accessibility, low cost and rapid  
316 growth. These eggs can be kept in an incubator for up to 13 days of maturation. After 14 days  
317 of growth, the development of the embryo is advanced, and the model is then considered an *in*  
318 *vivo* model (Kue *et al.* 2015).

319 While the ICCVAM did not validate this testing method for distinguishing severe irritants  
320 (ICCVAM 2010), this method is still used by some industrials in their internal weight of  
321 evidence WoE, these methods being recognized in the European Union. The procedure for  
322 opening the eggs without breaking the vascular membrane is described in figure 2.

323 However, one should bear in mind that this testing method has been increasingly criticized,  
324 being considered an *in vivo* model even in the first days of embryonic development.

325

326 **Models requiring optimization according to the OECD**

327

328 The models introduced in the following section are models mentioned in the OECD Guidance  
329 Document n°263 as interesting models, if optimized, for evaluation of reversibility of ocular

330 irritation/corrosion, which may thus be able to distinguish between all GHS categories,  
331 including category 2 compounds.

332

### 333 *Porcine Ocular Cornea Opacity/Reversibility Assay (PorCORA)*

334 PorCORA is an organotypic model similar to BCOP, since it is based on the maintenance *ex*  
335 *vivo* of porcine cornea obtained from slaughterhouses. Its added value lies in the air interface  
336 preservation allowing maintenance for 21 days (same as in the Draize reference test, the  
337 amount of time needed to evaluate reversibility of damage). Several steps are required to  
338 prepare the excised corneas (Vij *et al.* 2017). First, the corneas are placed in a 24-well plate,  
339 with the epithelium facing the bottom of the well. A mixture of agar/gelatin/medium is poured  
340 onto the corneas, which are then placed in Petri dishes after gelification. The test compounds  
341 are applied directly to the corneal surface (10  $\mu$ L for liquids, 20 mg for solids) for 5 minutes.  
342 The corneas are then washed with PBS (Piehl *et al.* 2011). Corneal alterations and their  
343 reversibility are then estimated and scored based on the area of staining with fluorescein  
344 sodium over the course of 1, 2, 3, 7, 10, 14 and 21 days after compound exposure (European  
345 Commission 2020b).

346 In this way, Piehl *et al.* demonstrated in 2011 that this method gave similar results to the  
347 Draize reference test (correlation coefficient of 0.98) with reproducible results for the five  
348 control test substances: phosphate buffered saline (PBS), absolute ethanol (EtOH), 3% sodium  
349 dodecyl sulfate (SDS), 1% benzalkonium chloride (BAK), and 10% sodium hydroxide  
350 (NaOH). Furthermore, in this study, PorCORA identified reversible and irreversible effects.  
351 By establishing a PorCORA score, it could be possible to distinguish GHS category 1  
352 products (irreversible alterations before 21 days) from category 2 products (reversible  
353 damages before 21 days, with a score returning to 0).

354 Nevertheless, additional data is needed. Indeed, in this study, Piehl *et al.* found that the  
355 method was too sensitive for surfactants. Similarly, in another study conducted on shampoos  
356 and hair dyes, PorCORA overestimated the irritant potential (Donahue *et al.* 2011). Finally, a  
357 drawback of this model is the progressive opacification of the cornea due to the gel that  
358 prevents the endothelium from correctly regulating corneal stromal fluids (Spöler *et al.* 2015).

359

### 360 *Ex Vivo Eye Irritation Test (EVEIT)*

361 The EVEIT is an air-liquid interface culture system, enabling maintenance of excised rabbit  
362 corneas (from slaughterhouses) for 72 hours following compound application. Briefly,  
363 corneas with a scleral ring are removed and anchored in a chamber filled with a minimal

364 volume of medium to maintain hydrostatic pressure. This *ex vivo* model reflects the  
365 biochemical activity of corneal epithelium and endothelium. Its advantage compared to the  
366 PorCORA system presented above is that the EVEIT does not lead to corneal opacification  
367 during culture (Spöler *et al.* 2015). Decision criteria are evaluated four times over 72 hours  
368 enabling differentiation of non-irritants from category 2A irritants (OECD, 2019b; Spöler *et*  
369 *al.* 2015): macroscopic observation of corneal opacity, fluorescein sodium diffusion, corneal  
370 thickness and structural changes measured by optical coherence tomography. Each  
371 measurement results in a score, similar to those used in the ICE or Draize tests, which were  
372 described by Spöler *et al.* in 2015. If preservation time of the corneas *ex vivo* could be  
373 improved, this testing method could enable differentiation of all category 2 products. Of note,  
374 this method was used by Schrage *et al.* in 2012 to evaluate the effect of artificial tears on  
375 corneal epithelial repair after mechanical damage. This study highlights the fact that the  
376 models presented in this section could serve equally well for toxicity studies as for  
377 pharmacological studies for the development of ophthalmic treatments.

378

### 379 *3D Hemi-Cornea*

380 The first *in vitro* system that may potentially discriminate GHS categories 1 and 2 alone, the  
381 3D Hemi-Cornea combines, in an insert, a corneal human epithelium reconstituted from an  
382 immortalized cell line with human corneal immortalized keratinocytes which represent  
383 stromal cells (Bartok *et al.* 2015; Engelke *et al.* 2013; Zorn-Kruppa *et al.* 2014). The two cell  
384 types are separated by a collagen membrane allowing evaluation of the two cell lines  
385 independently after a 60 min-exposure of the chemical (Zorn-Kruppa *et al.* 2014). This model  
386 is adapted for liquids as well as solids but is constraining since it has to be cultured during 7  
387 days with a daily change of medium. The endpoint measured is metabolic activity and the  
388 cytotoxicity MTT test. The distinction between GHS categories non-irritant, 1 and 2 could be  
389 observed though the extension and/or localisation of corneal changes (Tandon *et al.* 2015).  
390 Moderate irritants lead to a loss of viability of the corneal epithelium and can affect the  
391 stroma, whereas severe irritants lead to severe corneal epithelial and stromal alterations. As a  
392 result, this system properly classifies category 1 compounds and 80% of category 2  
393 compounds, but only 50% of non-irritant substances, with an overestimation of their irritant  
394 potential. A hypothesis to explain these last, disappointing results is that the compounds in  
395 this category were frequently viscous and difficult to remove during the washing steps,  
396 leading to the deterioration of some epithelial layers (Bartok *et al.* 2015). Furthermore, in

397 another study, the irritation potential of compounds with extreme pH were again  
398 overestimated, as in other *in vitro* tests, possibly because of the absence of the mucinous layer  
399 of the tear film, which has a buffer effect *in vivo* (Zorn-Kruppa *et al.* 2014).

400 Nonetheless, this test quoted in the Guidance Document n°263 of OECD seems to be an  
401 option for the evaluation of surfactants, alcohols, ketones, and volatile compounds, in other  
402 words, compounds that, in many other alternative models, lead to false positives results. This  
403 3D hemi-cornea could at the same time allow the evaluation of compound diffusion, since the  
404 test substances need to cross an aqueous collagen membrane.

405

#### 406 *Slug Mucosal Irritation (SMI) assay*

407 Described in the literature for the evaluation of reversible or irreversible ocular (Lenoir *et al.*  
408 2011a) and nasal (Lenoir *et al.* 2013) stinging, itching and/or burning (SIB), the SMI test  
409 measures the liberation of mucus proteins from *Arion lusitanicus* slugs. This method can  
410 screen for ocular discomfort generated by isolated ingredients or final products. As presented  
411 in the schematic protocol Figure 3, the slug's weight is compared before and after every  
412 contact period (CP)(Lenoir *et al.* 2009, 2011a, 2013; Cutuli *et al.* 2021).

413 Developed by Lenoir *et al.*, this test was used to evaluate shampoos and artificial tears. The  
414 results were correlated with a clinical study (Spearman's Rank correlation of 0.986,  $p <$   
415 0.001)(Lenoir *et al.* 2011b). Similarly, Petit *et al.* 2017 was able to reproduce this alternative  
416 model in 2017 to evaluate veterinary products. Recently, a new SMI alternative model, using  
417 a "Yellow slug", was reported to evaluate surface disinfectants used against SARS-CoV-2  
418 (Cutuli *et al.* 2021).

419 Since it can distinguish category 2 irritant products, optimization and validation of this test is  
420 mentioned to be of interest in the OECD Guidance Document n°263. However, depending on  
421 national regulations, this test might be considered animal experimentation (OECD, 2019b).

422

#### 423 **New innovative models for ocular surface toxicity evaluation**

424

425 Mimicking ocular structures *in vitro* is challenging (lacrimal glands, conjunctiva, innervation,  
426 lens, ...). New models are being developed in basic science research, notably using fluidic  
427 and three-dimensional approaches. These technologies of organ-on-a-chip originate from the  
428 area of pharmaceutical research and development (Wilson *et al.* 2015). In addition, this  
429 review will focus on *in silico* approaches, which are required to understand real-life exposure

430 and thus aid in design of the *in vitro* strategy, reducing time and costs of development.  
431 Organoid models will be described in the final part of this section, even though these new  
432 cellular structures are mainly studied for the purpose of replacing deficient patient structures.  
433 Table III proposes an overview of the selected models.

434

#### 435 *In silico models*

436 *In silico* approaches, using computer and mathematical tools, aim to simulate *in vivo*  
437 biological processes, mimicking a multicellular organ crossed by biological flows and  
438 connected to other structures of the organism. Inspired by the “PB-PK”, *Physiologically*  
439 *Based Pharmacokinetics*, approach (predicting absorption, distribution, metabolism and  
440 elimination), these *in silico* methods try to improve toxicological evaluation, taking into  
441 account local metabolism, barriers, ..., and to estimate a toxic dose (Knudsen *et al.* 2015).  
442 Once the organ is modelled, multiple scenarios can be tested by changing dose, time, method  
443 of exposure and other parameters that could influence the risk of toxicity (for instance,  
444 enzyme polymorphisms, pregnant women or pediatric differences in metabolism)(Jones *et al.*  
445 2015).

446 For each product tested, an exhaustive knowledge of its physicochemical properties must  
447 come through computerized channels (Brochot *et al.* 2014). To this end, other *in silico* tools  
448 can contribute to the information in the literature: *Qualitative and Quantitative Structure*  
449 *Activity Relationship* (QSAR) models that can predict biological properties such as affinity,  
450 protein binding, based on chemical structure. These models are available as free access or  
451 commercial software (ECHA 2019). To encourage regulatory acceptance of these QSAR  
452 models, the OECD released Guidance Document n°69 and created a free access toolbox with  
453 some QSAR models (OECD 2020d). First developed for conception of possible  
454 pharmaceuticals, some QSAR models aim to predict ocular irritation and damage based on the  
455 compound’s toxicodynamic properties: acidity, electrophilicity, chemical reactivity, surfactant  
456 effect (OECD, 2019b). For instance, Kulkarni *et al.* (2001) examined membrane interactions  
457 of compounds with the stratified lipophilic corneal epithelium to determine the irritant  
458 potential of substances already classified by the Draize test *in vivo*.

459 For local ocular toxicity, it is essential to mimic three main factors that influence ocular  
460 surface penetration and distribution: static barriers with different transport systems (claudins,  
461 zonula occludens), dynamic clearance (lacrimal fluids, Schlemm’s canal drainage) and  
462 metabolic factors (enzymes, efflux pumps, receptors). In 2018, Pak *et al.* applied these

463 principles to develop an *in silico* rabbit cornea model (epithelium, stroma, endothelium), the  
464 Quasi-3D CoBi (Computational Biology) model which includes passive transport  
465 (paracellular, transcellular) through the corneal epithelium (barrier to the passage of  
466 hydrophilic compounds), transport through the stroma (barrier to the passage of lipophilic  
467 compounds) and protein binding (such as glycosaminoglycans which can retain hydrophilic  
468 compounds). To do so, the research team created a precise geometric representation of the  
469 multilamellar corneal structure, applying complex mathematic equations to reflect the various  
470 flows. Nevertheless, this *in silico* model should be elaborated by adding all of the ocular  
471 structures (such as conjunctiva, tear film, neurons, retina) and should be based on human data  
472 to improve the predictions made through these models. The lack of human data on barriers,  
473 thickness and porosity of layers, local metabolism, physical constants, ..., remains to this day  
474 a barrier to the development of *in silico* models and use as a high throughput tool.  
475 Implementation of this work would be extremely time-consuming and would require a great  
476 deal of computing power but would improve extrapolations.

477 By enabling the identification of target structures, these models could guide the first steps of  
478 the AOP (adverse outcome pathway), which are currently the subject of toxicological  
479 development as supports for implantation of IATA, limiting unnecessary *in vitro* studies.  
480 However, one should bear in mind that if an important metabolic pathway used by the  
481 compound tested is missed in the model, the predictions will not be accurate.

482

### 483 *3D multicellular models*

484 Numerous 3D models are described in the literature, improving the phenotype of the  
485 epithelium formed. Nevertheless, many of them neglect the tear film, which covers the  
486 epithelia of the ocular surface, as well as the innervation of the ocular surface, which are,  
487 however, two central structures in understanding and anticipating TIDE and anterior segment  
488 neuropathies. Thus, this section describes three models that could become assets in the  
489 development of IATAs for ocular surface toxicity: the first model presented would allow  
490 evaluation of tear film thickness and composition, while the following ones would permit  
491 analysis of the toxic impact on neurons interacting with corneal cells.

492

#### 493 - Conjunctiva and lacrimal gland coculture

494 The literature is rich in alternative corneal models but delves less into conjunctival and  
495 lacrimal gland toxicity, although these structures which are essential for production of the tear



496 film, a fundamental structure to be evaluated for the anticipation of TIDE. Nevertheless, in  
497 2017, Lu *et al.* proposed a coculture between rabbit primary epithelial conjunctival cells and  
498 spheroids of rabbit primary lacrimal acinar cells. To our knowledge, this is the first *in vitro*  
499 3D model capable of producing aqueous and mucinous layers of the tear film.

500 After testing several configurations, direct contact between the two cell types, as presented  
501 figure 4, was found to present the best configuration, with optimal epithelial morphology,  
502 permeability, phenotype and lacrimal fluid production, even though direct contact is not the  
503 most physiological configuration (no direct contact in humans between these types of cells).  
504 To highlight the usefulness of their model, they demonstrated the protective effect of  
505 dexamethasone, a corticosteroid known to reduce inflammation of the ocular surface in TIDE,  
506 after exposure to pro-inflammatory IL-1 $\beta$ . This effect could not be seen on a simple  
507 monoculture of conjunctival cells. While this model does not allow the formation of a  
508 complete tear film with a lipid layer, it remains an interesting advance for the *in vitro*  
509 anticipation of TIDE. Further studies could be conducted by adding meibocytes in the culture,  
510 to obtain a complete tear film.

511

512 - 3D model of nerve-stroma interactions

513 To date, only a few models consider corneal innervation in a toxic response. Sharif *et al.*  
514 (2018) explored the corneal stroma-neuron interaction in depth by proposing a 3D coculture  
515 on an insert between HCF (*human primary corneal fibroblasts*) and SH-SY5Y neurons, a  
516 well-characterized human neuroblastoma cell line derived from bone marrow. This model is  
517 based on the *de novo* production of extracellular matrix by fibroblast cells and tries to mimic  
518 the *in vivo* nerve-stroma interaction in the cornea, improving the comprehension and  
519 anticipation of corneal cell damage as well as pathways of neuronal regeneration.

520 However, further studies are needed to characterize the neuronal phenotype of this model and  
521 therefore the ability of this model to mimic toxicity affecting the ocular surface. Indeed, SH-  
522 SY5Y neurons do not have the same phenotype as primary sensory neurons from trigeminal  
523 nerves, the main innervation of the ocular surface (Mélik-Parsadaniantz *et al.* 2018), since  
524 they can develop two distinct phenotypes (neuroblastic or epithelial-like). SH-SY5Y includes  
525 adherent cells but also floating viable cells whose biological significance is not yet  
526 understood. Also, neuroblastic SH-SY5Y cells express tyrosine hydroxylase and dopamine- $\beta$ -  
527 hydroxylase, two catecholaminergic markers, which are not characteristic of trigeminal  
528 neurons, which are primarily sensory neurons (Kovalevich and Langford 2013).

529 Nevertheless, transfected SH-SY5Y could be considered to study certain ocular surface  
530 symptoms such as stinging or itching. This was the objective of the NociOcular test based on  
531 a 2D model of SH-SY5Y expressing the transient receptor potential cation channel subfamily  
532 V member TRPV1, known to be implicated in these ocular surface phenomena (Dua *et al.*  
533 2018). Using this test, Forsby *et al.* (2012) completed an ocular tolerability study of 19  
534 shampoos, resulting in only one false negative and two false positives compared to a clinical  
535 evaluation. NociOcular measures, by fluorescence, the intracellular calcium flux mediated by  
536 the activation of TRPV1 and correlated to ocular discomfort. A similar study was conducted  
537 by Narda *et al.* in 2019) on the ocular tolerance of sunscreens, confirming the need to evaluate  
538 disturbances in neuronal transmission and not just damage to the ocular surface epithelial cell  
539 in a comprehensive study.

540

541 - Triculture of neuronal, epithelial and stromal cells

542 Wang *et al.* 2017 proposed an air liquid interface (ALI) triculture between human primary  
543 corneal cells, human corneal stromal stem cells and Chicken Dorsal Root Ganglion (DRG)  
544 neurons, supported by silk proteins. The use of silk proteins aims to mimic the mechanical  
545 properties of the cornea, so as to favour neuronal development. Figure 5 explains the cellular  
546 organization of the model. Through this set up, Wang *et al.* obtained optimized axonal  
547 development as well as a better epithelium / stromal phenotype and viability. At the moment,  
548 corneal tissue models are limited to one or two weeks of culture and do not include the  
549 nervous component. This ALI culture, integrating corneal interactions with neurons while  
550 conserving its integrity for 28 days, enables to evaluate toxic induced alterations of phenotype  
551 and viability. This model represents a progress in tissue engineering, promoting the  
552 importance of cell types interactions for better differentiation and maturation.

553

554 *Cornea-On-a-Chip models*

555 The focus of much attention in recent decades, organs-on-a-chip seek to miniaturize an organ,  
556 facilitate the assembly of cell types and recreate the dynamics of an organ (Mandenius 2018).  
557 These chips are mainly based on microfluidic technics, using biocompatible polymers such as  
558 polydimethylsiloxane (PDMS), a transparent, flexible and gas impermeable organomineral  
559 material. The advantage of these systems lies in the small amount of biological material  
560 needed, while improving the representation of dynamic *in vivo* parameters compared to a  
561 classic 2D cell culture. Nevertheless, protocols have not yet been standardized, scale-up

562 remains unfeasible for routine experimentation, and the analytical challenge (because of the  
563 small quantity of cells) remains to be solved (Sosa-Hernández *et al.* 2018).

564 Because of the complexity of multicompartmental and multi-layered ocular structures,  
565 establishing an eye-on-a-chip is a hard task. If we focus on the anterior segment, some  
566 corneas-on-a-chip are described in the literature and attempt to include ocular surface flow  
567 (blinking of the eyelids, tear secretion, shear stress). Furthermore, microfluidics and  
568 compartmentalization on a chip are also being considered to improve the mimicry of ocular  
569 surface innervation, taking into account the fact that only nerve endings can be directly  
570 exposed to a topically applied toxicant.

571

572 - Cornea-on-a-chip, ocular flows and shear stress

573 A current limitation of corneal barrier models is the lack of flow to mimic the shear stress  
574 caused on the epithelium by eyelid blinking, which is responsible for tear film movement, and  
575 as a result of drug or toxicant distribution and its effects on the ocular surface. Of note, this is  
576 also a limitation of the Draize test when attempting to most closely approximate human  
577 physiology, since rabbits blink less frequently than humans, resulting in a longer exposure  
578 time (Maurice 1995).

579 In 2018, to study passage through the corneal barrier, Bennet *et al.* 2018 proposed a cornea-  
580 on-a-chip with a pulsatile flow to represent blinking or a continuous flow for tear secretion. A  
581 confluent epithelium of 5 to 7 layers with a stable phenotype and permeability was obtained  
582 on a PDMS chip with a fibronectin coated membrane (mimicking Bowman's layer) and  
583 immortalized human corneal epithelial cells. In this system, eyedrop pharmacokinetics and  
584 toxicity can be evaluated by applying either the continuous or pulsatile flow for 5 hours. After  
585 this experimentation time, 98% of the compounds were found to be eliminated; compared to a  
586 static model, it improves the evaluation of absorption, bioavailability and toxicity.  
587 Nevertheless, additional studies are required to understand the impact of the two types of  
588 flow, since compound penetration appeared more significant with the pulsatile flow.

589 Similarly in 2020, Abdalkader and Kamei published a four chamber microfluidic model with  
590 uni- and bi-directional flow to study the impact of shear stress on corneal epithelium barrier  
591 phenotype. This PDMS system, composed of human corneal epithelial cells on a porous  
592 membrane, aims to simulate human cornea, with an apical side in contact with lacrimal fluid  
593 (bidirectional flow for eye blinking) and a proximal side with the aqueous humor  
594 (unidirectional flow mimicking drainage through Schlemm's canal). After having obtained a

595 stratified (2-3 layers), permeable (evaluation by fluorescein diffusion), phenotyped  
596 (expression of tight junction proteins such as the zonula occludens proteins), they applied  
597 both flows for 24 hours and observed that shear stress did not alter cellular adhesion and  
598 improved the expression of cytokeratins, which are important proteins for flexibility, cellular  
599 elasticity and maintaining corneal barrier integrity. In addition, this model could take into  
600 account the compound real time of remanence in the tissue.

601 Nevertheless, these two models are limited in their representation of the cornea, since they  
602 lack formation of the stromal and endothelial layers, corneal elements that are notably  
603 essential for aqueous humor flow. This limitation is addressed by Bai *et al.* (2020) with their  
604 cornea-on-a-chip, a PDMS compartmentalized chip using primary murine corneas; they  
605 simultaneously isolate both epithelial and endothelial corneal cells and plant them into two  
606 separate compartments with a collagen membrane to mimic Bowman's layer.

607

608 - Cornea and conjunctiva-on-a-chip

609 Another approach to the 3D ocular model on-a-chip was designed in 2019 by Seo *et al.*,  
610 combining human primary corneal epithelial cells and immortalized conjunctival cells  
611 (epithelial and glandular cells), cultured in an ALI system. The primary corneal cells are  
612 incorporated into a collagen matrix which mimics the stromal layer. A perfusion system  
613 mimics tear flow, while a biomimetic system recreates blinking of the eyelids. Their  
614 complementary data gives a better representation of this complex model. Seo *et al.* obtained a  
615 pluristratified epithelium with 7 to 8 layers like human cornea, expressing specific markers  
616 (*ex.* cytokeratins 3, 19) and producing a "tear film" of 6  $\mu\text{m}$  comparable to the *in vivo*  
617 thickness. Like the previous models, they proved that shear stress induced cellular  
618 differentiation and limited pro-inflammatory cytokine production. To attest to the utility of  
619 their model, they demonstrated the anti-inflammatory action of lubricin, a protein-like mucin.  
620 While this model does not include the vasculature or immune cells normally present in the  
621 conjunctiva nor the nerve endings of the ocular surface which participate in tear secretion, this  
622 chip represents a major improvement for pharmacological and toxicological compound  
623 evaluation, especially for a TIDE IATA.

624

625 - Corneal innervation compartmentalization

626 Currently, most ocular surface models, like the flow systems just discussed, neglect toxic  
627 effects on ocular surface innervation, whereas during a toxic exposure, trigeminal nerve

628 endings can be altered, with an indirect impact on neuronal cell bodies. Therefore, stimulating  
629 primary cell cultures of neurons directly does not mimic real life exposure, and, as a result,  
630 mechanisms of toxicity are impossible to analyze properly. In order to improve anatomical  
631 representation of the ocular surface innervation, Sarkar *et al.* (2012) used a Campenot device  
632 to evaluate morphological alterations (neurite fragmentation, axon breaks, lack of  
633 regeneration) of mice primary trigeminal neurons after exposition to BAK, preservative  
634 contained in many eyedrops. With this model, they highlighted a dose-dependent toxicity of  
635 BAK on neurites. Campenot devices were the first systems to allow neuronal  
636 compartmentalization but new microfluidic organ-on-a-chip devices could be considered.  
637 Indeed, these microchips can be precisely designed to optimized axonal guidance of  
638 trigeminal neurons (Courte *et al.* 2018). This innovative system also allows to analyze  
639 separately nerve ending and cell body responses. Finally, this model could be improved by  
640 adding corneal epithelial cells in the distal compartment to allow interaction between these  
641 cells and the nerve endings, coming even closer to corneal physiology. It could provide a  
642 better understanding of toxic mechanisms and facilitate establishment of TIDE AOPs and  
643 screening of new therapeutic agents (anti-inflammatory, axonal regeneration,  
644 neuroprotection). Nevertheless, a limitation of this model is the use of primary murine cells,  
645 which does not entirely respect the 3R rule to “Reduce, Replace, Refine,” central in IATA  
646 development. Even if primary cells are a better representation of a peripheral neuronal  
647 phenotype, in the framework of alternative methods, induced Pluripotent Stem Cells should  
648 be considered, as in the organoid models described below.

649

#### 650 *Organoid models of the anterior segment of the eye*

651 While the definition can vary between authors, organoids are 3D structures, derived from  
652 embryonic stems cells or induced Pluripotent Stem Cells (iPs), capable of self-organization on  
653 their framework (such as porous membrane and hydrogel) (Duboule 2019). A Pubmed search  
654 with “eye organoid” as keywords reports mostly retinal organoids or organoids destined to be  
655 transplanted in humans to replace deficient structures. Few articles address anterior segment  
656 organoids for *in vitro* evaluation of pathologic or toxic pathways. However, some of the  
657 organoids described could be adapted for toxicological studies.

658

659 - Corneal organoids

660 In 2017, Foster *et al.* presented a corneal organoid derived from an IMR90.4 iPSC cell line  
661 (Foster *et al.* 2017) and published their precise methodology in 2020 (Foster *et al.* 2020).  
662 Mature transparent organoids are obtained after 120 days of cellular sequential selection,  
663 forced aggregation and differentiation. Their lamellar structure is composed of epithelial,  
664 stromal and endothelial layers and expresses specific corneal markers (cytokeratins 3, 14,  
665 collagen of type I, V, VII). Even if any toxicological study has already been conducted, this  
666 model could be further optimized to evaluate the impact of toxic compounds on the  
667 interactions between the three main corneal layers (epithelium, stroma, and endothelium).  
668 Nevertheless, cell differentiation sometimes appears incomplete, leading to the presence of  
669 some retinal cells within the corneal organoid. Other protocols presented to obtain corneal  
670 organoids for transplantation seem to result in pure corneal organoids, such as that of  
671 Susaimanickam *et al.* (2017), but additional studies are needed to evaluate the reproducibility  
672 of these models.

673

#### 674 - Lens organoids

675 In 2018, Murphy *et al.* addressed the unsolved problem of obtaining pure lens cells from  
676 human embryonic pluripotent stem cells (CA-1 cell line). Their objective was to elaborate a  
677 simple, reproducible method to study lens pathologies and anticipate toxicity-induced  
678 cataracts. To this end, they put in place a complex, semi-automated selection protocol based  
679 on knowledge of embryonic development, with successive inhibition and activation of the  
680 FGF, TGF- $\beta$  and Wnt pathways (Yang *et al.* 2010) and magnetic selection of ROR1+  
681 expressing cells (orphan receptor expressed on epithelial lens cells). These organoids remain  
682 viable for 42 days, expressing, among others,  $\alpha$  and  $\beta$  crystallins, present *in vivo* in lens fibers  
683 and necessary for focusing of light. In this study, they proved the ability of these microlenses  
684 to evaluate the toxic potential of a drug candidate, Vx-770, tested in 2016 for cystic fibrosis.  
685 This compound, which has induced toxic cataracts in rats, also altered the lens organoids'  
686 ability to focus light. To summarize, after reproducibility and intra-laboratory transferability  
687 is addressed, this innovative model could be used routinely for the evaluation of mechanisms  
688 of toxicity-induced cataract, which still remain poorly understood, as well as the efficacy of  
689 new treatments.

690

#### 691 **Conclusion**

692 The 21<sup>st</sup> century has seen an increase in the movement toward alternative methods to animal  
693 testing, especially since the complete ban of animal experimentation in cosmetics. Ocular  
694 toxicity studies are no exception, and studies still need to be conducted for new compounds.  
695 Indeed, the alternative models to the Draize reference test present similar disadvantages,  
696 among which figure the absence of detection of conjunctival or iris damage, the absence of  
697 evaluation of systemic toxicity that can occur after ocular exposure and the possibility of false  
698 negatives or false positives. Furthermore, none of them alone is able to identify all of the GHS  
699 ocular irritant categories, and reversibility of damage is still difficult to evaluate, explaining  
700 the impetus of the OECD to optimize some other models. In recent decades, toxicology  
701 procedures have aimed to develop IATAs to circumvent these limitations of the alternative  
702 methods. Putting aside Draize reference test, known for its lack of reproducibility which  
703 complexifies the validation of alternative models by the ICCVAM (OECD Webinar 2019a),  
704 and constructing new models, from scratch, based on established AOPs, might be necessary to  
705 improve the robustness of the toxicology approaches and results for human use. Indeed, we  
706 need to break free from Draize eye irritation test and its poor quality of result to improve  
707 inter-laboratory validation of new models (Spielmann 2014) that could enable the  
708 identification of a new category of compounds, very low irritants, which requires finer  
709 sensitivity methods. This validation step is essential to develop robust alternative approaches  
710 to animal testing in the ocular surface field, as it has been done for skin sensitization. Indeed,  
711 in June 2021, OECD released GL 497 on “Defined Approaches for Skin Sensitisation”,  
712 describing the integrated testing strategy and combination of tests that can be used in  
713 toxicology studies in replacement of the reference test on rabbits, the Local Lymph Node  
714 Assay (OECD 2021).

715 In the field of ophthalmology, IATAs should extend the assessment of toxicity to pathologies  
716 other than irritation, especially Toxicity-Induced Dry Eye (TIDE), that can occur after chronic  
717 exposure to very low concentrations (Bonneau *et al.* in press). While much less frequent, a  
718 toxic compound can also lead to, cataract, glaucoma or ocular surface neuropathies after local  
719 exposure. These effects should be considered, taking into account real-life exposure to the  
720 compound, determined through literature searches and *in silico* models. As a result, new  
721 drugs, cosmetic compounds, or other chemicals, should be investigated for acute irritation  
722 and/or for chronic adverse events, depending on real-life use, requiring the development and  
723 validation of models and tests with short and/or repeated exposures.

724 Establishing integrated decision trees for these newly considered adverse events will require a  
725 precise understanding of toxic mechanisms, with the development of Adverse Outcome  
726 Pathways (AOP), a concept also promoted by the OECD with the establishment of new  
727 collaborative tools such as AOP wiki, Effectopedia and the e.AOP.Portal (OECD Webinar  
728 2019b). The innovative models presented in the last section of this review could, after  
729 assessment of robustness and regulatory validation, be included in IATAs. They could be a  
730 key asset to understanding molecular mechanisms and establishing AOPs. Validation of new  
731 models will be a lengthy process, since they should be developed in such a way as to be as  
732 cost-effective and least constraining as possible (ethics and supply logistics).

733

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740

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1020 **Tables**

1021 **Table I. Summary of models validated or under evaluation by the OECD.** \*Values given  
1022 in OECD GL to identify Category 1 or not-classified substances (depending on assay  
1023 applicability) in comparison to the Draize eye irritation test.

1024 **Table II. Summary of validated RhCE models for ocular irritation according to OECD**  
1025 **GL 492.**

1026 **Table III – Summary of innovative models with potential for evaluation of ocular**  
1027 **surface toxicity.**

1028

## 1029 **Figures**

1030 **Figure 1. Schematic presentation of the matrix created in the Red Blood Cell test and**  
1031 **the principle of denaturation** (modified from OECD Webinar 2019a)

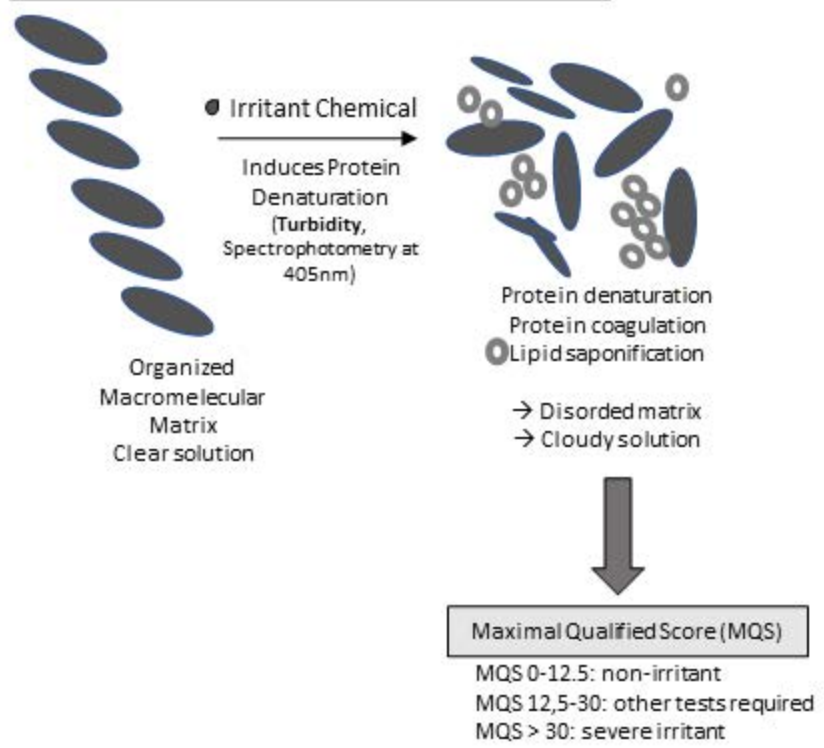
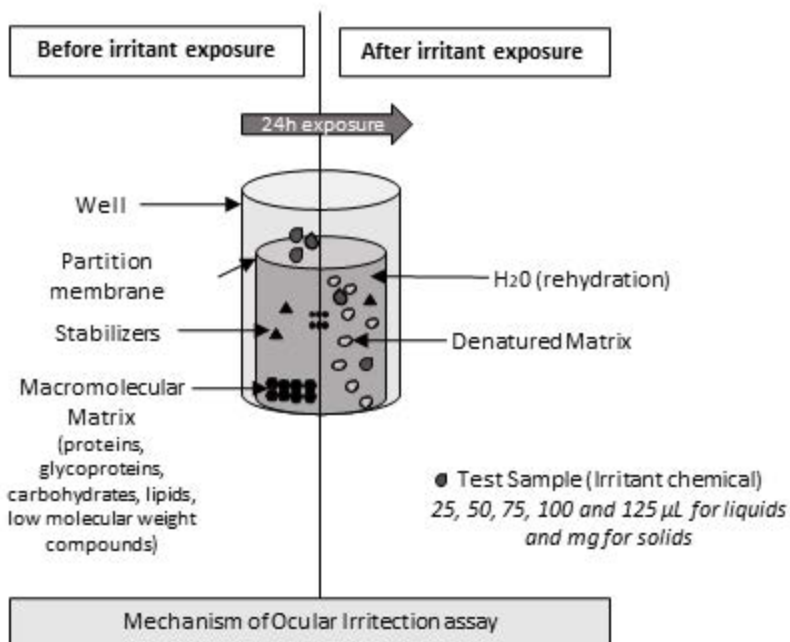
1032 **Figure 2. Procedure to prepare the embryonated egg in the HET-CAM testing method**  
1033 (personal images, not published)

1034 **Figure 3. Schematic evaluation protocol for ocular discomfort in the slug irritation**  
1035 **model** (modified from Lenoir *et al.* 2011). *CP: Contact Period; SIB: Stinging, Itching and*  
1036 *Burning*

1037 **Figure 4. Schematic representation of the coculture established** between conjunctival  
1038 epithelial cells and lacrimal spheroids (modified from Lu *et al.* 2017).

1039 **Figure 5. Schematic comparison of human corneal structure with 3D triculture model**  
1040 **structure** (modified from Wang *et al.* 2017).

1041





Egg shell opening with the tip of pliers after spotting the air compartment by transparency

Enlargement of the opening with scissors

Access to the membranes

Addition of NaCl 0.9% to separate the protective membrane from the vascularized membrane

NaCl 0.9% removal

Protective membrane removal

Access to the vascularized membrane, beginning of eye irritation test



**Egg opening procedure**

**Exposure to test compound**

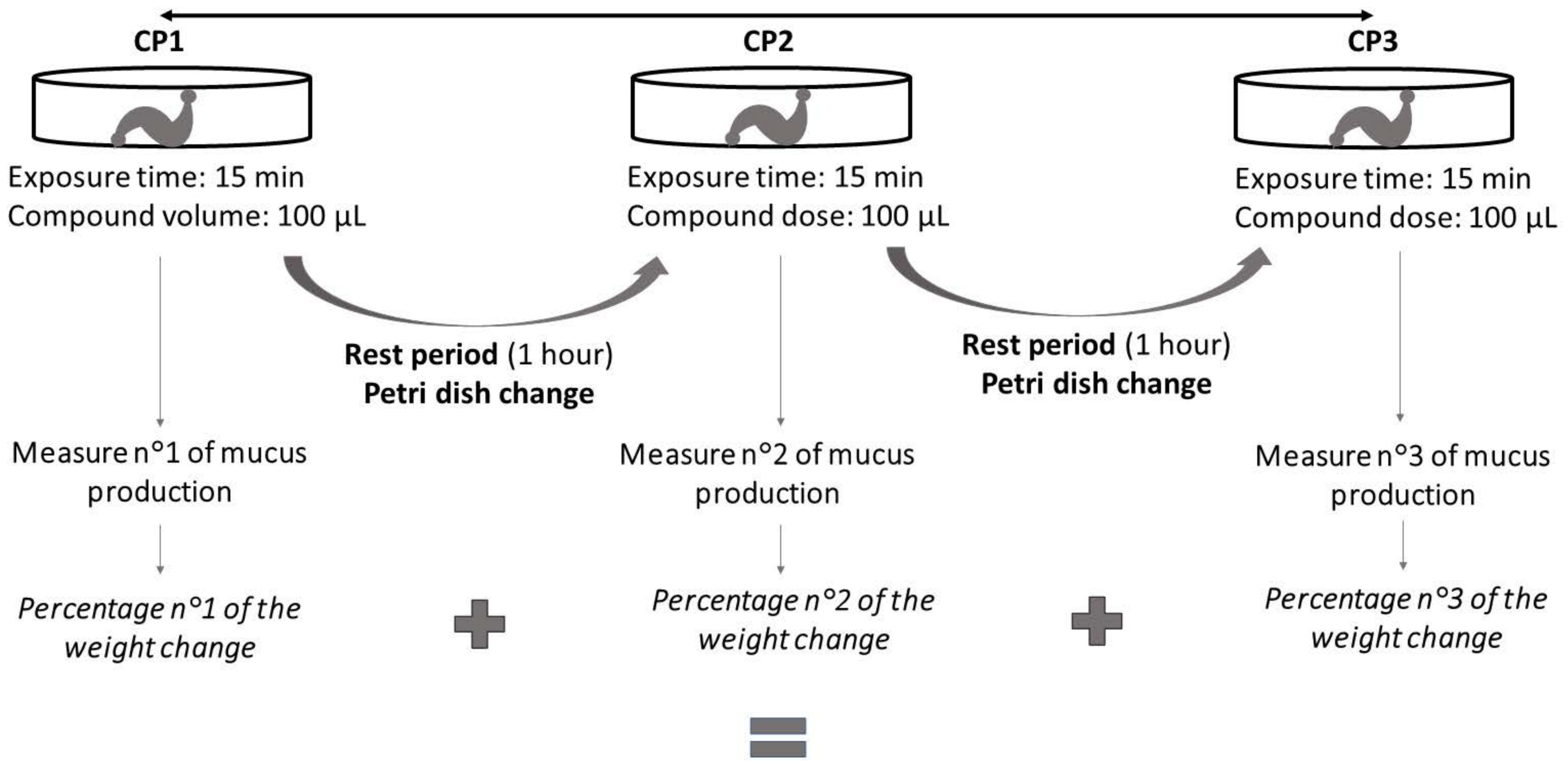
HET-CAM: 30 sec, 2, 5 min

CAMVA: 30 min

**Irritation Score, IS**

- hemorrhage appearance time,
- vessel lysis appearance time,
- protein coagulation apparition time

Conducted over a 24-hour period



Exposure time: 15 min  
Compound volume: 100 µL

Exposure time: 15 min  
Compound dose: 100 µL

Exposure time: 15 min  
Compound dose: 100 µL

Rest period (1 hour)  
Petri dish change

Rest period (1 hour)  
Petri dish change

Measure n°1 of mucus production

Measure n°2 of mucus production

Measure n°3 of mucus production

Percentage n°1 of the weight change

+

Percentage n°2 of the weight change

+

Percentage n°3 of the weight change

=

Total mucus production

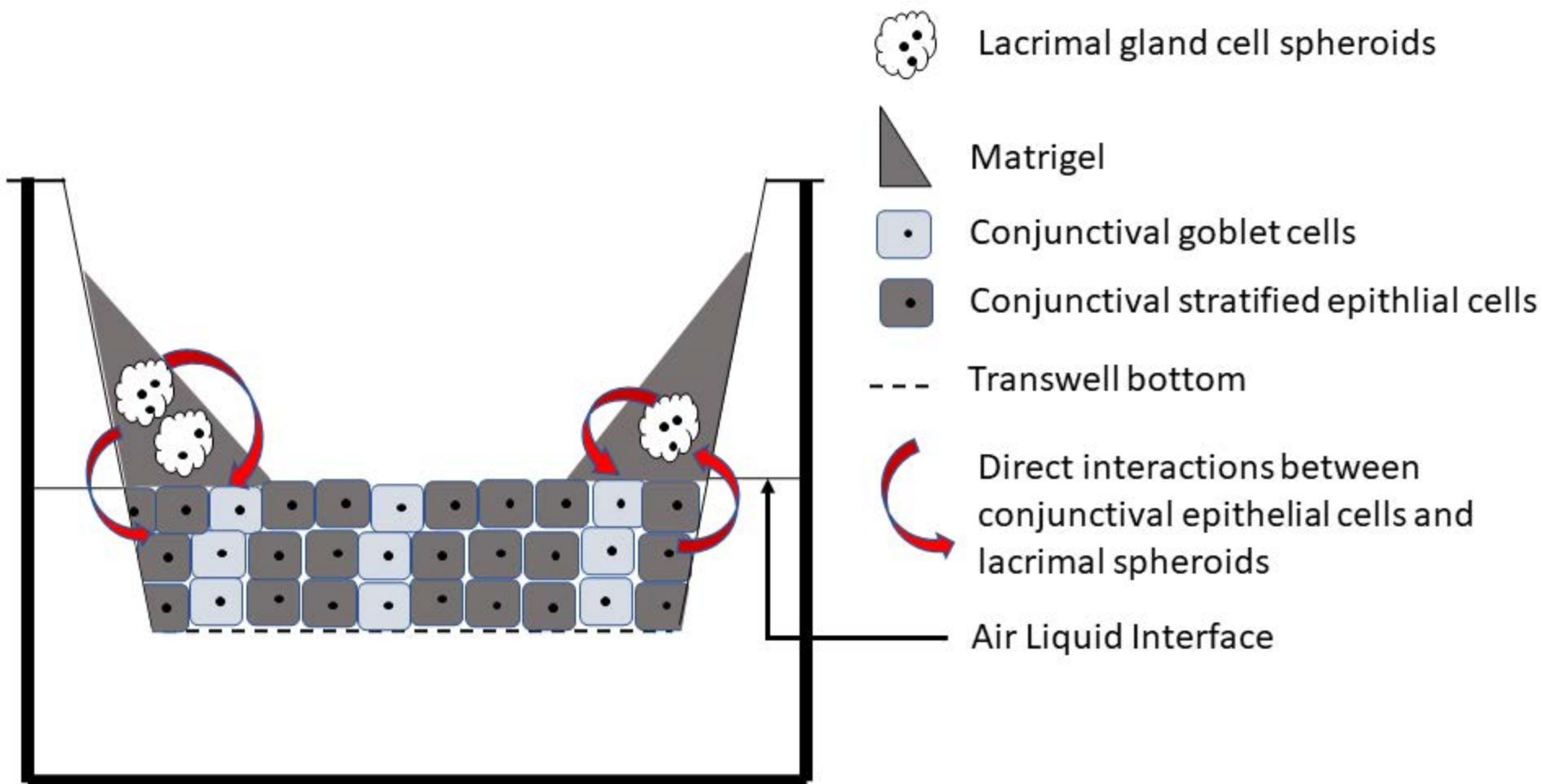
Legends:

 Petri dish

 Slug

\*SIB : stinging, itching and/or burning

**Light SIB\* = 3 to 8%**  
**Moderate SIB\* = 8 to 15%**  
**Severe SIB\* > 15%**



Ocular irritation tests	Draize test	RhCE	BCOP	ICE	Fluorescein Leakage	STE	Vitrigel EIT	Ocular Irritection®	IRE	HET-CAM / CAMVA	CM	NRR	RBC
OECD guideline (last update)	OECD GL 405 (2020)	OECD GL 492 (2019)	OECD GL 437 (2020)	OECD GL 438 (2018)	OECD GL 460 (2017)	OECD GL 491 (2020)	OECD GL 494 (2019)	OECD GL 496 (2019)	Not validated	Not validated	Temporary version released in 2012 (development discontinued in 2016)	Not validated	Not validated
Model	<i>In vivo</i> , albino rabbit	<i>In vitro</i> , 3D human reconstructed epithelium	<i>Ex Vivo</i> , isolated bovine cornea	<i>Ex Vivo</i> , enucleated chicken eye	<i>In vitro</i> , tubular kidney MDCK CB997 cell line, monolayer, semi-permeable membrane	<i>In vitro</i> , monolayer confluent rabbit corneal fibroblasts (ex. CCL60 cell line)	<i>In vitro</i> , human reconstructed epithelium (Vitrigel matrix)	<i>In vitro</i> , acellular system, macro-molecular matrix (proteins, lipids, carbohydrates,...)	<i>Ex vivo</i> , enucleated rabbit eye	Chorioallantoic Membrane of chicken embryo egg	<i>In vitro</i> , mono-layer mice fibroblasts from L929 cell line cultivated on a polycarbonate insert	<i>In vitro</i> , mono-layer of 3T3-L1 fibroblasts or NHEK human keratinocytes (FRAME/ Clonetic protocol)	Isolated red blood cells
Recommended strategy	Last resort (forbidden for cosmetics)	Bottom-Up	Bottom-Up, Top-Down	Bottom-Up, Top-Down	Top-Down	Bottom-Up, Top-Down	Bottom-Up	Bottom-Up, Top-Down	Not recommended	Not recommended	(If validation of GL: Bottom-Up, Top-Down)	Not recommended (supplementary data required)	Not recommended (supplementary data required)
Field of applicability	Liquids, solids, aerosols	Liquids, semi-solids, solids, waxes	Liquids, semi-solids, creams, waxes (including surfactants)	Substances and mixtures	Water-soluble substances and mixtures	All types of products (except volatile substances, non surfactant products)	Chemical products with pH > 5, including volatile or coloured compounds (excluding solids)	Solids and liquids with 4 ≤ pH ≤ 9	Substances and mixtures	Substances and mixtures	Water-soluble compounds (including mixtures), solids/viscous substances / uniform suspensions	Water-soluble substances	Substances and mixtures
GSH categories	1, 2A, 2B, not-classified	Not-classified (in process of validation to distinguish 1, 2A et 2B with EpiOcular® time-to-toxicity assay)	1, not-classified	1, not-classified	1	1, not-classified	not-classified	1, not-classified	(accepted in European Union for category 1)	HET-CAM accepted in European Union for category 1	1, not-classified	not-classified	1, not-classified
Compound exposure time	21 days	See Table 2	10 min (other exposure times if scientific rationale)	10 sec (rinsing removal)	1 min (followed by a 30min incubation of fluorescein)	5 min (two concentrations, 0.5% and 0.05%)	3 min	24h (5 concentrations, 25, 50, 75, 100, 125 µL or µg)	10 sec (rinsing removal)	30 sec, 2, 5 min (HET-CAM) / 30 min (CAMVA)	810 sec (= 13, 5min, followed by a 6 min wash out cycle)	1 or 5 min (FRAME or Clonetic protocol)	10 min to 1 hour at room temperature (under continuous stirring)
Endpoints	<b>Conjunctiva</b> (chemosis, redness, tearing), <b>Corneal</b> opacification, <b>Iris</b> (swelling, light reactivity)	Mitochondrial metabolic capacity	Corneal opacity; Fluorescein retention	Corneal opacity; Fluorescein retention; Morphological alteration (evaluated after 30 min, 1, 2, 3, and 4 hours of product retrieval)	Fluorescein diffusion (spectrophotometry at 530 nm)	Mitochondrial metabolic capacity	TEER (measured every 10 s during 3 min)	Turbidity variations (spectrophotometry at 405 nm)	Corneal opacity, edema; Fluorescein penetration; Epithelial changes (evaluated after 30 min, 1, 2, 3, and 4 hours of product retrieval)	Hemorrhage / vessel lysis / protein coagulation apparition times	Dose-response study, pH changes evaluation over time	Dose-response study, Release of preloaded neutral red, 3 hours before exposure (spectrophotometry at 546-550 nm)	Hemoglobin leakage (photometry at 540 nm); Oxyhemoglobin denaturation (spectrophotometry at 575 nm)
Threshold or Score	Maximal ocular irritation ( <b>Max.O.I</b> )	MTT or WST threshold (see table 2)	<i>In Vitro</i> Irritancy Score ( <b>IVIS</b> )	Addition of scores for each endpoint graded from I to IV	Fluorescein Leakage of 20% ( <b>FL<sub>20%</sub></b> )	MTT threshold	Score that combines time lag, intensity and plateau level	Maximal Qualified Score ( <b>MQS</b> )	Addition of scores for each endpoint	Irritation Score ( <b>IS</b> )	Metabolic Rate Decrement of 50% ( <b>MRD<sub>50</sub></b> )	Neutral Red Release of 50% ( <b>NRR<sub>50</sub></b> )	ratio concentration inducing a red cell hemolysis of 50% / Denaturation index ( <b>H<sub>50</sub>/DI</b> )

Ocular irritation tests	Draize test	RhCE	BCOP	ICE	Fluoresceine Leakage	STE	Vitrigel EIT	Ocular Irritection®	IRE	HET-CAM / CAMVA	CM	NRR	RBC
<b>Accuracy *</b>	Reference	EpiOcular™, 80% (96/112) SkinEthic™ HCE, 84% (168/200)	79% (150/191)	83% (142/172)	77% (117/151)	83% (120/140)	78% (73/93)	74% (65/88)	78% (110/141)	69% (41/59)	Data not found	Variable, protocol dependent	96.7% (Alves et al. 2008)
<b>Specificity *</b>	Reference	EpiOcular™, 37% (21/55) SkinEthic™ HCE, 28% (29/103)	25% (32/126)	7% (9/127)	7% (7/103)	1% (1/86)	70% (23/33)	81% (55/68)	6% (4/62)	64% (18/28)	2% (1/48)	Variable, protocol dependent	100% (Alves et al. 2008)
<b>Sensitivity *</b>	Reference	EpiOcular™, 4% (3/57) SkinEthic™ HCE, 5% (5/97)	14% (9/65)	47% (21/45)	56% (27/48)	51% (20/39)	83% (50/60)	50% (10/20)	34% (27/79)	0% (0/31)	20.5% (7/34)	Variable, protocol dependent	91.6% (Alves et al. 2008)
<b>Main limits</b>	<ul style="list-style-type: none"> <li>- 3R rule ethical problem</li> <li>- Forbidden for cosmetics</li> <li>- Inter/Intra laboratory variability</li> <li>- Over-estimation of toxicities occurring in humans</li> </ul>	<ul style="list-style-type: none"> <li>- No toxicity evaluation of eyelid, iris, and other ocular structures</li> <li>- No evaluation of gas and aerosols</li> </ul>	<ul style="list-style-type: none"> <li>- No toxicity evaluation of eyelid, iris, etc</li> <li>- No evaluation of gas and aerosols</li> <li>- Over-estimation for pour alcohols, ketones</li> </ul>	<ul style="list-style-type: none"> <li>- No toxicity evaluation of eyelid, iris, and other ocular structures</li> <li>- No evaluation of gas and aerosols</li> </ul>	<ul style="list-style-type: none"> <li>- No toxicity evaluation of eyelid, iris, and other ocular structures</li> <li>- No evaluation of gas and aerosols</li> </ul>	<ul style="list-style-type: none"> <li>- No toxicity evaluation of eyelid, iris, and other ocular structures</li> <li>- No evaluation of gas and aerosols</li> </ul>	<ul style="list-style-type: none"> <li>- No toxicity evaluation of eyelid, iris, and other ocular structures</li> <li>- No evaluation of gas and aerosols</li> </ul>	<ul style="list-style-type: none"> <li>- No cytotoxicity evaluation</li> <li>- Reduced field of applicability</li> </ul>	<ul style="list-style-type: none"> <li>- No standardized protocol,</li> <li>- No sufficient data on decision criteria and on inter-laboratory reproducibility</li> </ul>	<ul style="list-style-type: none"> <li>- No standardized protocol</li> <li>- Embryo egg can be considered as animal experimentation depending on countries</li> </ul>	<ul style="list-style-type: none"> <li>- No evaluation of gas and aerosols</li> </ul>	<ul style="list-style-type: none"> <li>- No sufficient data on inter-laboratory reproducibility</li> <li>- Reduced field of applicability (supplementary data needed)</li> </ul>	<ul style="list-style-type: none"> <li>- No sufficient data on field of applicability</li> </ul>

OECD GL 492		EpiOcular™	SkinEthic™ HCE	LabCyte CORNEA-MODEL24	MCTT HCE™
Cell type		Primary human keratinocytes from human epiderma	Immortalized human corneal epithelial cells	Primary human corneal epithelial cells	Primary human corneal epithelial cells
Field of applicability		Solids, liquids, semi-solids and waxes			
Validated Reference Methods (VRM)		MRV1	MRV2	/	/
3D development*		At least three viable cell layers and of a non keratinized surface	At least four viable cell layers that include basal columnar cells, transitory amplifier cells and squamous superficial cells	At least three viable cell layers and of a non keratinized surface	At least three viable cell layers and of a non keratinized surface
Compound exposure time	Liquids	30 min	30 min	1 min	10 min
	Solids <i>(or liquids non applicable with a pipette)</i>	6 hours	4 hours	24 hours	3 hours
Cytotoxicity test** (Non irritant threshold)		MTT (> 60 %)	MTT (>50%)	WST-8 (>40%)	WST-8 (> 35% for liquids; > 60% for solids)

\* The barrier function of the 3D reconstructed-cornea epithelia must be validated based on their ability to resist penetration by cytotoxic compounds such as Triton X-100 and sodium dodecylsulfate.

\*\* The two colorimetric tests, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and WST (water-soluble tetrazolium salts)-8 tests are similar. In the first one, formazan is formed intracellularly, requiring the step of cell lysis with isopropanol before the absorbance measurement, while in the second one, formazan is present directly in the cell culture medium. This colorimetric measure is proportional to the number of live cells (Pauly et al. 2009).

	Multicellular 3D models			Models on-a-chip				Organoids	
	Coculture conjunctiva / lacrimal glands	Coculture SH-SY5Y neurons / stromal corneal cells on an insert	Tri-culture neurons / epithelial cells / stromal cells	Cornea-on-a-chip, lacrimal flows	Cornea-on-a-chip and shear stress	Coculture on-a-chip cornea – conjunctiva	Compartmentalized corneal neurones	Cornea	Lens
Cell types	<ul style="list-style-type: none"> <li>Primary epithelial conjunctival rabbit cells</li> <li>Primary rabbit acinous lacrimal glands spheroids</li> </ul>	<ul style="list-style-type: none"> <li>Primary human corneal fibroblasts</li> <li>Human neuroblastoma SH-SY5Y cell line</li> </ul>	<ul style="list-style-type: none"> <li>HCE human corneal epithelial cell line</li> <li>Stromal human stem cells (hCSCCs)</li> <li>Neuronal cells (DRG)</li> </ul>	<ul style="list-style-type: none"> <li>HCE human corneal epithelial cell line</li> </ul>	<ul style="list-style-type: none"> <li>HCE human corneal epithelial cell line</li> <li><b>Or</b></li> <li>Primary mice epithelial and endothelial corneal cells</li> </ul>	<ul style="list-style-type: none"> <li>Primary human corneal epithelial cells</li> <li>Human immortalized conjunctival cells</li> </ul>	<ul style="list-style-type: none"> <li>Primary trigeminal ganglion mice cells (<i>model improvement possible by adding epithelial corneal cell to form a coculture</i>)</li> </ul>	<ul style="list-style-type: none"> <li>IMR90.4 iPS cell line</li> </ul>	<ul style="list-style-type: none"> <li>human pluripotent embryonic stem cells (hESC line CA1)</li> </ul>
Advantages	<ul style="list-style-type: none"> <li>Production of aqueous and mucinic lacrimal layers</li> </ul>	<ul style="list-style-type: none"> <li>Production <i>de novo</i> of extracellular matrix by fibroblasts</li> <li>Mimic the interactions of nerves with the stroma</li> </ul>	<ul style="list-style-type: none"> <li>Air liquid interface culture on silk protein to better mimic mechanical corneal properties and improve neuronal development</li> </ul>	<ul style="list-style-type: none"> <li>Mimic pulsatile flow generated by eyelid blinking</li> <li>Mimic continuous flow generated by lacrimal secretion</li> </ul>	<ul style="list-style-type: none"> <li>Mimic lacrimal flow generated by eye blinking (bidirectional flow)</li> <li>Mimic aqueous humour evacuation through Schlemm’s canal (unidirectional flow)</li> </ul>	<ul style="list-style-type: none"> <li>Mimic stroma through collagen matrix</li> <li>Mimic lacrimal flow through a perfusion system</li> <li>Mimic eyelid blinking through biomimetic system</li> </ul>	<ul style="list-style-type: none"> <li>Separate nerve endings (distal compartment) from neuronal cell bodies (proximal compartment) to better mimic physiology and independently evaluate the impact of a toxic on nerve endings</li> </ul>	<ul style="list-style-type: none"> <li>Lamellar structure of the cornea (epithelium, stroma, endothelium) identifiable at 30 days of culture</li> </ul>	<ul style="list-style-type: none"> <li>Formation of a fibrillary structure characteristic of the lens</li> </ul>
Evaluated parameters	<ul style="list-style-type: none"> <li>Permeability of tight junctions (conjunctival epithelium) to dextran</li> <li>Lacrimal fluid thickness</li> <li>Epithelial gene marker (KRT4)</li> <li>Mucin gene marker / production (MUC5AC)</li> <li>Inflammatory gene marker (IL-1<math>\beta</math>, MMPs)</li> </ul>	<ul style="list-style-type: none"> <li>Collagen and fibrosis gene markers (alpha-SMA)</li> <li>Structural changes (transmission electron microscopy)</li> <li>Neuronal activation markers (cFOS, TRPV1, TRPM8, <i>etc</i>)</li> </ul>	<ul style="list-style-type: none"> <li>Cell viability (LIVE/DEAD Viability/ Cytotoxicity Kit)</li> <li>Corneal epithelium and stromal phenotype (involucrin, KRT3, connexin 37, ALDH3A1)</li> </ul>	<ul style="list-style-type: none"> <li>Epithelium thickness</li> <li>Corneal epithelium phenotype (ZO-1)</li> <li>Membrane permeability (TEER)</li> </ul>	<ul style="list-style-type: none"> <li>Epithelium thickness</li> <li>Epithelial permeability (fluorescein, dextran)</li> <li>Corneal epithelium phenotype (ZO-1, KRT19, KRT12)</li> </ul>	<ul style="list-style-type: none"> <li>Epithelium thickness</li> <li>Corneal epithelium phenotype (p63, KRT19, KRT3)</li> <li>Lacrimal film thickness</li> <li>Inflammatory cytokines production (IL-<math>\beta</math>, TNF-<math>\alpha</math>) and metalloproteinases (MMP-9)</li> </ul>	<ul style="list-style-type: none"> <li>Inflammatory markers</li> <li>Cell death markers</li> <li>Morphological alterations of axons (CFSE coloration)</li> </ul>	<ul style="list-style-type: none"> <li>Lamellar structure thickness</li> <li>Corneal epithelium phenotype (KRT3, KRT14, p63<math>\alpha</math>, KERA, type I / V / VIII collagen, LUM)</li> <li>Organization of collagen fibrils (transmission electron microscopy)</li> </ul>	<ul style="list-style-type: none"> <li>Lens phenotype (ROR, crystallines <math>\alpha</math> et <math>\beta</math>, integrins, laminins et collagens)</li> <li>Light focusing ability</li> </ul>
References	Lu <i>et al.</i> 2017	Sharif <i>et al.</i> 2018	Wang <i>et al.</i> 2015 Wang <i>et al.</i> 2017	Bennet <i>et al.</i> 2018	Abdalkader <i>and</i> Kamei 2018 Bai <i>et al.</i> 2020	Seo <i>et al.</i> 2019	Vitoux <i>et al.</i> 2020	Foster <i>et al.</i> 2017 Susaimanickam <i>et al.</i> 2017 Foster <i>et al.</i> 2020	Murphy <i>et al.</i> 2018