

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Draft Proposal for a New Guideline: The Bovine Corneal Opacity and Permeability (BCOP) Test Method for Identifying Ocular Corrosives and Severe Irritants¹

INTRODUCTION

1. The Bovine Corneal Opacity and Permeability (BCOP) test method is an *in vitro* test method that can be used, under certain circumstances and with specific limitations, to identify ocular corrosives and severe irritants^{1,2} (i.e., U.S. Environmental Protection Agency [EPA] Category 1, European Union [EU] R41, the United Nations [UN] Globally Harmonized System of Classification and Labelling of Chemicals [GHS] Category 1). While it is not considered valid as a complete replacement for the rabbit eye test, the BCOP is recommended for use as part of a tiered-testing strategy for regulatory classification and labeling within a specific applicability domain (1)(2). Substances that test positive in this assay and are considered an ocular corrosive or severe irritant after a weight-of-evidence decision will not need to be tested in animals. A substance that tests negative would need to be tested *in vivo* using an accepted test guideline (i.e., OECD Test Guideline 405 (3) or EPA OPPTS 870.1000 (4)) or *in vitro* using an adequately validated test method³.

2. The purpose of this Test Guideline is to describe the procedures used to evaluate the potential ocular corrosivity or severe irritancy of a test substance as measured by its ability to induce opacity and increased permeability in an isolated bovine cornea. Toxic effects to the cornea are measured by: 1) decreased light transmission (opacity), 2) increased passage of sodium fluorescein dye (permeability), and 3) evaluation of fixed and sectioned tissue at the light microscopic level, if applicable. The opacity and permeability assessments of the cornea following exposure to a test substance are considered individually and also combined to derive an *In Vitro* Irritancy Score (IVIS), which is used to classify the irritancy level of the test substance. Histological evaluation of the corneas can be useful for identifying damage in tissue layers that does not produce significant opacity or permeability.

3. The focus of this Test Guideline is on the use of the BCOP test method for the detection of ocular corrosives and severe irritants, as defined by the EPA (5), EU (6), and GHS (7). Ocular irritants that induce lesions that resolve in less than 21 days, as well as

¹ For the purpose of this Test Guideline, severe irritants are defined as those that induce ocular lesions that persist in the rabbit for at least 21 days after administration.

² EPA Category 1 = Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than 21 days (5); EU R41 = Production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application (6); GHS Category 1 = Production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application (7).

³ The *in vitro* test method must be able to correctly identify a BCOP false negative (i.e., a misclassified corrosive or severe irritant), irritants that induce ocular damage that resolves within 21 days, and non-irritants.

32 nonirritants, have been tested using the BCOP test method. However, the accuracy and
33 reliability of the BCOP test method for these categories, as defined by the EPA (5), the
34 EU (6), and the GHS (7), have not been formally evaluated.

35 4. Definitions are provided in **Annex I**.

36 **INITIAL CONSIDERATIONS AND LIMITATIONS**

37 5. This Test Guideline is based on the recommended Interagency Coordinating
38 Committee on the Validation of Alternative Methods (ICCVAM) BCOP test method
39 protocol (**Annex II**), which was developed following an international independent
40 scientific peer review of the validation status and scientific validity of the BCOP (1, 2, 8),
41 with contributions from the European Centre for the Validation of Alternative Methods
42 (ECVAM), the Japanese Center for the Validation of Alternative Methods (JaCVAM),
43 BCOP test method developers, and experienced BCOP users. The ICCVAM BCOP
44 protocol was developed with information obtained from 1) the Institute for In Vitro
45 Sciences (IIVS), a nonprofit foundation that has performed the BCOP assay since 1997 in
46 a Good Laboratory Practice (GLP)-compliant testing facility; and 2) INVITTOX Protocol
47 124 (9), which represents the protocol used for the European Community-sponsored
48 prevalidation study of the BCOP assay conducted in 1997-1998. Both of these protocols
49 are based on the BCOP assay methodology first reported by Gautheron et al. (10).

50 6. The identified limitations for this test method are based on the high false negative
51 and false positive rates observed for certain chemical (*i.e.*, alcohols, ketones) and
52 physical (*i.e.*, solids) classes (see paragraph 49) (2). When substances within these
53 chemical and physical classes are excluded from the database, the accuracy of BCOP
54 across the EU, EPA, and GHS classification systems is substantially improved (2). The
55 current validation database did not allow for an adequate evaluation of some chemical or
56 product classes (*e.g.*, formulations). However, investigators could consider using this test
57 method for testing such substances, whereby a positive result, as part of a weight-of-
58 evidence decision, could be accepted for hazard classification purposes.

59 7. All procedures with bovine eyes and bovine corneas should follow the testing
60 facility's applicable regulations and procedures for handling animal-derived materials,
61 which include, but are not limited to, tissues and tissue fluids. Universal laboratory
62 precautions are recommended.

63 8. Histopathology data have not been formally evaluated for use in this assay.
64 However, a histopathological assessment can be included on a case-by-case basis to
65 identify severe ocular damage that does not produce opacity or permeability changes in
66 the isolated cornea. Histopathology may also be potentially useful when a more complete
67 characterization of damage is needed. Users are encouraged to preserve tissues and
68 prepare histopathology specimens that can be analyzed and used to develop a database
69 and potential decision criteria that may further improve the accuracy of this test method.
70 The OECD Guidance Document on Histopathological Preparation and Evaluation of
71 Tissues from *In Vitro* Ocular Toxicity Test Methods (to be provided) includes detailed
72 procedures on the collection of histopathology data and information on where to submit

73 specimens and resulting data.

74 9. A limitation of the test method is that, although it takes into account some of the
75 ocular effects evaluated in *in vivo* rabbit ocular irritancy tests and to some degree their
76 severity, it does not consider all of the types of ocular damage assessed *in vivo* (i.e.,
77 conjunctival and iridal injuries), nor does it allow for assessing the potential for systemic
78 toxicity associated with this route of exposure. Although the reversibility of corneal
79 lesions cannot be evaluated *per se* in the BCOP assay, it has been proposed, based on
80 rabbit eye studies, that an assessment of the initial depth of corneal injury can be used to
81 predict irreversible or reversible effects (11).

82 10. Efforts are ongoing to expand the applicability domain of the BCOP test method
83 for identifying ocular corrosives and severe irritants, and to characterize its usefulness
84 and limitations for identifying non-severe irritants and nonirritants. Users are encouraged
85 to submit data and histopathology specimens generated according to this Test Guideline
86 to international validation organizations (i.e., the National Toxicology Program
87 Interagency Center for the Evaluation of Alternative Toxicological Methods
88 [NICEATM], the European Centre for the Validation of Alternative Methods [ECVAM],
89 or the Japanese Center for the Validation of Alternative Methods [JaCVAM]). These data
90 may also be used to evaluate possible future use of the BCOP for the identification of
91 nonsevere ocular irritants and nonirritants.

92 **PRINCIPLE OF THE TEST**

93 11. The BCOP test method is an organotypic model that provides short-term
94 maintenance of normal physiological and biochemical function of the bovine cornea in an
95 isolated system. In this test method, damage by the test substance is assessed by
96 quantitative measurements of changes in corneal opacity and permeability with an
97 opacitometer and an ultraviolet/visible (UV/VIS) spectrophotometer, respectively. Both
98 measurements are used to calculate an *IVIS*, which is used to assign an *in vitro* irritancy
99 hazard classification category for prediction of the *in vivo* ocular irritation potential of a
100 test substance.

101 12. The BCOP test method uses isolated corneas from the eyes of freshly slaughtered
102 cattle. Corneas free of defects are dissected with a 2 to 3 mm rim of sclera remaining to
103 assist in subsequent handling, with care taken to avoid damage to the corneal epithelium
104 and endothelium. Isolated corneas are mounted in specially designed corneal holders⁴ that
105 consist of anterior and posterior compartments, which interface with the epithelial and
106 endothelial sides of the cornea, respectively. Both chambers are filled with Eagle's
107 Minimum Essential Medium (EMEM) and the device is then incubated at $32 \pm 1^\circ\text{C}$ for at
108 least one hour to allow the corneas to equilibrate with the media and to resume normal
109 metabolic activity. Following the equilibration period, fresh EMEM is added to both
110 chambers, and a baseline opacity measurement is performed. Corneal opacity is measured

⁴ Corneal holders are available commercially (e.g., Stag Bio [Clermont, France]; Spectro Design [Riom, France]) and detailed drawings of a corneal holder have been published (Northover, A.M. (1995). The Use of the Bovine Isolated Cornea as a Possible *In Vitro* Test for Ocular Irritancy. In: *In Vitro* Toxicity Testing Protocols. Methods in Molecular Biology. Vol. 43. Totowa, NJ: Humana Press, 205-210).

111 quantitatively as the amount of light transmission through the cornea. Permeability is
112 measured quantitatively as the amount of sodium fluorescein dye that passes across the
113 full thickness of the cornea, as detected in the medium in the posterior chamber. Test
114 substances are applied to the epithelial surface of the cornea by addition to the anterior
115 chamber of the corneal holder. Another holder has been designed which may more
116 effectively maintain the normal curvature of the cornea (12), and as such might
117 potentially improve the performance of the BCOP test method⁵.

118 **Source of Bovine Eyes and Selection of Animal Species**

119 13. Cattle sent to slaughterhouses are typically killed either for human consumption
120 (*e.g.*, calves for veal; steers 9 to 30 months old) or for other commercial uses. The cattle
121 in the former category tend to be raised specifically for meat production and thus are of
122 cattle breeds (*e.g.*, Hereford) used to optimize the quality and quantity of beef for human
123 consumption. The cattle in the latter category can include dairy cattle (*e.g.*, Holstein) that
124 are no longer useful for milk production (13)(14). Because cattle have a wide range of
125 weights, depending on breed, age, and sex, there is no recommended weight for the
126 animal at the time of slaughter.

127 **Age of Source Animals**

128 14. Variations in corneal dimensions can result when using eyes from animals of
129 different ages. Corneas with a horizontal diameter >30.5 mm and central corneal
130 thickness (CCT) values $\geq 1100 \mu\text{M}$ are generally obtained from cattle older than eight
131 years, while those with a horizontal diameter <28.5 mm and CCT <900 μM are generally
132 obtained from cattle less than five years old (13). For this reason, eyes from cattle greater
133 than 60 months old are not typically used. Eyes from cattle less than 12 months of age are
134 also not typically used since the eyes are still developing and the corneal thickness and
135 corneal diameter are considerably smaller than that reported for eyes from adult cattle.
136 However, the use of corneas from young animals (*i.e.*, <12 months old) has advantages
137 such as increased availability, a narrow age range, and decreased hazards related to
138 technician exposure to Bovine Spongiform Encephalopathy (15). As further evaluation of
139 the effect of corneal size or thickness on responsiveness to corrosive and irritating
140 substances would be useful, users are encouraged to report as much information as
141 possible on the age, sex, and weight of the animals providing the corneas used in a study
142 and the corresponding corneal diameter and CCT values.

143 **Collection and Transport of Eyes to the Laboratory**

144 15. Eyes are collected by slaughterhouse employees following exsanguination and
145 decapitation of the cattle. To minimize mechanical and other types of damage to the eyes,
146 the eyes should be enucleated as soon as possible after death. To prevent exposure of the
147 eyes to potentially irritating substances, the slaughterhouse employees should not use
148 detergent when rinsing the head of the animal.

⁵ Interested users would need to determine the commercial availability of this holder, potentially by contacting the lead author of reference (12).

149 16. Eyes should be immersed completely in Hanks' Balanced Salt Solution (HBSS) in
150 a suitably sized container. The container should be maintained on ice while taking care to
151 avoid freezing the eyes. Because the eyes are collected during the slaughter process, they
152 might be exposed to blood and other biological substances, including bacteria and other
153 microorganisms. Therefore, it is important to ensure that the risk of contamination is
154 minimized (e.g., by keeping the eyes on wet ice, by using the eyes as soon as possible
155 after slaughter, or by adding antibiotics (e.g., penicillin at 100 IU/mL and streptomycin at
156 100 µg/mL]) to the HBSS used to store the eyes during transport.

157 17. While a formal study to determine the maximal time not to be exceeded during
158 the transport of eyes from the slaughterhouse to the testing facility has not been
159 published, a maximum of five hours has been used in most published BCOP studies. The
160 use of corneas beyond five hours post-collection might be permitted if sufficient evidence
161 demonstrates that a longer time interval does not compromise the results.

162 **Selection Criteria for Eyes Used in the BCOP**

163 18. Once the eyes arrive at the laboratory, they are carefully examined for defects,
164 including opacity, scratches, and neovascularization,. Only corneas from eyes free of
165 such defects are to be used.

166 19. The quality of each cornea is also evaluated at later steps in the assay. Corneas
167 that have an opacity greater than 10 after an initial one-hour equilibration period are to be
168 discarded.

169 20. Each treatment group (test article, concurrent negative control, concurrent
170 positive control) consists of a minimum of three eyes.

171 **PROCEDURE** (see **Annex II** for a detailed protocol for the BCOP test method)

172 **Preparation of the Eyes**

173 21. Corneas free of defects are dissected with a 2 to 3 mm rim of sclera remaining to
174 assist in subsequent handling, with care taken to avoid damage to the corneal epithelium
175 and endothelium. Isolated corneas are mounted in specially designed corneal holders that
176 consist of anterior and posterior compartments, which interface with the epithelial and
177 endothelial sides of the cornea, respectively. Both chambers are filled to excess with
178 EMEM (posterior chamber first), ensuring that no bubbles are formed. The device is then
179 equilibrated at $32 \pm 1^\circ\text{C}$ for at least one hour to allow the corneas to equilibrate with the
180 medium (the approximate temperature of the corneal surface *in vivo* is 32°C).

181 22. Following the equilibration period, fresh EMEM is added to both chambers and
182 baseline opacity readings are taken for each cornea. Any corneas that show tissue damage
183 or an opacity >10 units are discarded. The mean opacity of all equilibrated corneas is
184 calculated. A minimum of three corneas with opacity values close to the median value for
185 all corneas are selected as negative (or solvent) control corneas. The remaining corneas
186 are then distributed into treatment groups and positive/other control groups.

187 23. Because the heat capacity of water is higher than that of air, water would provide
188 the more stable temperature conditions for incubation. Therefore, it is recommended to
189 use a water bath for the incubation at $32 \pm 1^\circ\text{C}$. However, air incubators might also be
190 used, assuming precaution to ensure temperature stability (e.g., by prewarming of holders
191 and media).

192 **Application of the Test Substance**

193 24. Two different treatment protocols are used, one for liquids and surfactants, and
194 one for nonsurfactant solids. For both treatment protocols, the test substance is applied to
195 the epithelial surface of the cornea and incubated horizontally.

196 25. Liquids are tested undiluted, while surfactants are tested at a concentration of
197 10% in 0.9% sodium chloride, distilled water, or other solvent that has been demonstrated
198 to have no adverse effects on the test system. Appropriate justification should be
199 provided for alternative dilution concentrations.

200 26. Corneas are exposed to liquids and surfactants for 10 minutes. Use of other
201 exposure times should be accompanied by adequate scientific rationale.

202 27. Solids are tested as solutions or suspensions at 20% concentration in 0.9% sodium
203 chloride, distilled water, or other solvent that has been demonstrated to have no adverse
204 effects on the test system.

205 28. Corneas are exposed to solids for four hours, but as with liquids and surfactants,
206 alternative exposure times may be used with appropriate scientific rationale.

207 29. Different treatment methods are used depending on the physical nature and
208 chemical characteristics (e.g., solids, liquids, viscous vs. nonviscous liquids) of the test
209 substance. A closed-chamber method is used for nonviscous to slightly viscous liquid test
210 substances, while an open-chamber method is used for semiviscous and viscous liquid
211 test substances. Solid and liquid surfactant test substances and solid test substances are
212 tested using either the closed- or open-chamber method.

213 30. In the closed-chamber method, the control or test substance (0.75 mL) is added to
214 the anterior chamber through dosing holes, which are subsequently sealed with chamber
215 plugs during the exposure period.

216 31. In the open-chamber method, the window-locking ring and glass window from the
217 anterior chamber are removed prior to treatment. The control or test substance (0.75 mL,
218 or enough test substance to completely cover the cornea) is applied directly to the
219 epithelial surface of the cornea using a micropipette or other appropriate device, such as a
220 spatula if solids are being tested.

221 **Post-Exposure Incubation**

222 32. At the end of the 10-minute exposure period, corneas that have been treated with
223 liquids or surfactants are rinsed thoroughly to ensure all materials are removed and they

224 are incubated for an additional two hours at $32 \pm 1^\circ\text{C}$.

225 33. Corneas treated with solids are rinsed thoroughly to ensure all materials are
226 removed, but do not require further incubation beyond the four-hour exposure period.

227 34. At the end of the post-exposure incubation period for liquids and surfactants and
228 at the end of the four-hour exposure period for solids, the opacity of each cornea is
229 recorded. Also, each cornea is observed visually and pertinent observations recorded.
230 Special attention is taken to observe dissimilar opacity patterns, tissue peeling, or residual
231 test substance.

232 **Control Substances**

233 35. Concurrent negative or solvent/vehicle controls and positive controls are included
234 in each experiment.

235 36. When testing a liquid substance at 100%, a concurrent negative control (e.g.,
236 0.9% sodium chloride or distilled water) is included in the BCOP test method so that
237 nonspecific changes in the test system can be detected and to provide a baseline for the
238 assay endpoints. It also ensures that the assay conditions do not inappropriately result in
239 an irritant response.

240 37. When testing a diluted liquid, surfactant, or solid, a concurrent solvent/vehicle
241 control group is included in the BCOP test method so that nonspecific changes in the test
242 system can be detected and to provide a baseline for the assay endpoints. As stated in
243 paragraphs 25 and 27, only a solvent/vehicle that has been demonstrated to have no
244 adverse effects on the test system can be used.

245 38. A known ocular irritant is included as a concurrent positive control in each
246 experiment to verify that an appropriate response is induced. As the BCOP assay is being
247 used in this test guideline to identify corrosive or severe irritants, ideally the positive
248 control should be a reference substance that induces a severe response in this test method.
249 However, to ensure that variability in the positive control response across time can be
250 assessed, the magnitude of irritant response should not be excessive. Based on a large
251 historical database⁶ demonstrating consistent results with a test substance that induces a
252 response near the borderline of the severe irritant response, 100% ethanol is typically
253 used as a positive control in the BCOP. For example, 100% ethanol induces a moderate
254 to severe response (e.g., IVIS = 39.9 - 65.4 [n = 632]; mean = 52.7, standard deviation =
255 6.4).

256 39. The most commonly used positive control for liquid test substances is 100%
257 ethanol⁷, while the most commonly used positive control for solid test substances is 20%
258 (weight to volume) imidazole in 0.9% sodium chloride.

⁶ Sufficient *in vitro* data for the positive control should be generated such that a statistically defined acceptable range for the positive control can be calculated.

⁷ Although alcohols are a limitation of the BCOP test method, 100% ethanol has consistently produced values that fall within the accepted range for opacity, OD₄₉₀, and *In Vitro* Irritancy Score.

259 40. Benchmark substances are useful for evaluating the ocular irritancy potential of
260 unknown chemicals of a specific chemical or product class, or for evaluating the relative
261 irritancy potential of an ocular irritant within a specific range of irritant responses.

262 **Endpoints Measured**

263 41. Opacity is determined by the amount of light transmission through the cornea.
264 Corneal opacity is measured quantitatively with the aid of an opacitometer, resulting in
265 opacity values measured on a continuous scale.

266 42. Permeability is determined by the amount of sodium fluorescein dye that
267 penetrates all corneal cell layers (*i.e.*, the epithelium on the outer cornea surface through
268 the endothelium on the inner cornea surface). Sodium fluorescein solution (4 or 5 mg/mL
269 when testing liquids or solids, respectively) is added to the anterior chamber of the
270 corneal holder, which interfaces with the epithelial side of the cornea. The concentration
271 of sodium fluorescein that crosses into the posterior corneal chamber, which interfaces
272 with the endothelial side of the cornea, is quantitatively measured with the aid of UV/VIS
273 spectrophotometry. Spectrophotometric measurements evaluated at 490 nm are recorded
274 as optical density or absorbance values, which are measured on a continuous scale.

275 43. It is recommended that digital photographs should be taken to document the
276 results obtained in regard to corneal opacity.

277 44. Collection and processing of tissues for histopathological assessment are
278 encouraged to facilitate evaluation of this endpoint for potential inclusion in decision
279 criteria that may improve the accuracy of the test method⁸. A histological evaluation of
280 the type and depth of injury induced by a test substance at the tissue level may be useful
281 when relevant (*e.g.*, when the standard BCOP endpoints of corneal opacity or
282 permeability produce borderline or equivocal results, or to confirm the result of any
283 negative test). This would be particularly relevant to certain classes of chemicals (*e.g.*,
284 peroxides, bleaches), which are known to react with the stromal layer and consequently
285 damage keratocytes, but without effects on permeability and opacity (16).

286 **DATA AND REPORTING**

287 **Data Evaluation**

288 45. Results for opacity and permeability should be combined in an empirically-
289 derived formula that generates an *IVIS* for each test substance.

290 46. Once the opacity and mean permeability (OD₄₉₀) values have been corrected for
291 background opacity and the negative control permeability OD₄₉₀ values, the mean opacity
292 and permeability OD₄₉₀ values for each treatment group are used to calculate an *in vitro*
293 score for each treatment group as follows:

⁸ Individuals interested in collecting and using histopathology data in the BCOP should consult the OECD Guidance Document on Histopathological Preparation and Evaluation of Tissues from *In Vitro* Ocular Toxicity Test Methods.

294 $IVIS = \text{mean opacity value} + (15 \times \text{mean permeability } OD_{490}$
295 $\text{value})$

296 47. The opacity and permeability values should also be evaluated independently to
297 determine whether a test substance induced corrosivity or severe irritation through only
298 one of the two endpoints (see Decision Criteria).

299 48. A standardized scoring scheme and associated decision criteria for histopathology
300 remains to be developed for routine use in the BCOP.

301 **Decision Criteria**

302 49. A substance that induces an $IVIS \geq 55.1$ is defined as a corrosive or severe irritant.
303 As stated in paragraph 1, if the test substance is not identified as an ocular corrosive or
304 severe irritant, additional testing should be conducted for classification and labeling
305 purposes. The BCOP test method has an overall false negative rate of 16% (7/43) to 25%
306 (10/40), when compared to *in vivo* rabbit eye test method data classified according to the
307 EPA (1), EU (2), or GHS (3) classification systems. When substances within these
308 chemical and physical classes are excluded from the database, the accuracy of BCOP
309 across the EU, EPA, and GHS classification systems ranges from 87% (72/83) to 92%
310 (78/85) and the false negative rates range from 0% (0/27) to 12% (3/26). For those
311 substances that produce significant permeability with minimal effects on opacity, it is
312 recommended that the results be critically reviewed in order to understand the mechanism
313 of toxicity. In such cases, and if appropriate, permeability values > 0.600 are used to
314 determine substances as corrosive or severe irritants.

315 50. Even if an ocular corrosive or severe irritant classification is not obtained for a
316 test substance, BCOP data can be useful, in conjunction with *in vivo* data or valid *in vitro*
317 test data, to further evaluate the usefulness and limitations of the BCOP test method for
318 identifying non-severe irritants and nonirritants. Therefore, it is recommended that the
319 complete classification scheme of the BCOP test method (i.e., corrosive/severe irritants,
320 non-severe irritants, or nonirritants) be applied and that these data are reported in parallel
321 with any other data obtained (i.e., from the *in vivo* rabbit eye test or an adequately
322 validated *in vitro* test method). The remaining categories in the BCOP test method
323 classification scheme include the following proposed decision criteria: a substance
324 producing an IVIS from 0 to 3 is considered a nonirritant, from 3.1 to 25 a mild irritant,
325 and from 25.1 to 55 a moderate irritant.

326 51. Benchmark substances are recommended for aiding in the evaluation of responses
327 of test substances of different product or chemical classes. Additionally, histological
328 evaluation of the corneas can be useful to identify severe ocular damage (i.e., corrosivity
329 or severe irritation) that does not produce opacity or permeability changes in the isolated
330 cornea changes (e.g., peroxide-induced stromal damage).

331 **Study Acceptance Criteria**

332 52. A test is acceptable if the positive control gives an IVIS that falls within two

333 standard deviations of the current historical mean, which is to be updated at least every
334 three months. The negative or solvent/vehicle control responses should result in opacity
335 and permeability values that are less than the established upper limits for background
336 opacity and permeability values for bovine corneas treated with the respective negative or
337 solvent/vehicle control.

338 **Test Report**

339 53. The test report should include the following information, if relevant to the
340 conduct of the study:

341 Test and Control Substances:

- 342 • Chemical name(s) such as the structural name used by the Chemical
343 Abstracts Service (CAS), followed by other names, if known;
- 344 • The CAS Registry Number (RN), if known;
- 345 • Purity and composition of the substance or preparation (in percentage(s)
346 by weight), to the extent this information is available;
- 347 • Physicochemical properties such as physical state, volatility, pH, stability,
348 chemical class, water solubility relevant to the conduct of the study;
- 349 • Treatment of the test/control substances prior to testing, if applicable (e.g.,
350 warming, grinding);
- 351 • Stability, if known.

352 Information Concerning the Sponsor and the Test Facility

- 353 • Name and address of the Sponsor
- 354 • Name and address of the test facility
- 355 • Name and address of the Study Director
- 356 • Information on the source of the eyes (e.g., age, sex, weight of the donor
357 animal) and corresponding information on the corneal diameter and CCT
- 358 • Storage and transport conditions of eyes (e.g., date and time of eye
359 collection, time interval prior to initiating testing, transport media, any
360 antibiotics used)

361 Justification of the Test Method and Protocol Used

362 Test Method Integrity

- 363 • The procedure used to ensure the integrity (i.e., accuracy and reliability) of
364 the test method over time (e.g., periodic testing of proficiency substances,
365 use of historical negative and positive control data)

366 Criteria for an Acceptable Test

- 367 • Acceptable concurrent negative control ranges based on historical data
- 368 • Acceptable concurrent positive control ranges based on historical data
- 369 • If applicable, acceptable concurrent benchmark control ranges based on
- 370 historical data

371 Test Conditions

- 372 • Description of test system used
- 373 • Type of corneal holder used
- 374 • Calibration information for devices used for measuring opacity and
- 375 permeability (e.g., opacitometer and spectrophotometer)
- 376 • Information for the bovine corneas used, including statements regarding
- 377 their quality
- 378 • Details of test procedure used
- 379 • Test substance concentration(s) used
- 380 • Description of any modifications of the test procedure
- 381 • Reference to historical data of the model (e.g., negative and positive
- 382 controls, proficiency substances, benchmark substances)
- 383 • Description of evaluation criteria used

384 Results

- 385 • Tabulation of data from individual test samples (e.g., opacity and OD₄₉₀
- 386 values and calculated IVIS for the test substance and the positive,
- 387 negative, and benchmark controls [if included], reported in tabular form,
- 388 including data from replicate repeat experiments as appropriate, and
- 389 means ± the standard deviation for each experiment)
- 390 • Description of other effects observed
- 391 • A digital photograph of each cornea

392 Discussion of the Results

393 Conclusion

394 A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant

395 Studies

- 396 • This statement indicates all inspections made during the study, and the
- 397 dates any results were reported to the Study Director. This statement also
- 398 serves to confirm that the final report reflects the raw data.

399 If GLP-compliant studies are performed, then additional reporting requirements provided
400 in the relevant guidelines (17)(18)(19)(20) should be followed.

401 **REFERENCES**

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- 466

467

ANNEX I

468

DEFINITIONS

469 **Accuracy:** (a) The closeness of agreement between a test method result and an accepted
470 reference value. (b) The proportion of correct outcomes of a test method. It is a measure
471 of test method performance and one aspect of “relevance.” The term is often used
472 interchangeably with “concordance” (see also “two-by-two” table). Accuracy is highly
473 dependent on the prevalence of positives in the population being examined.

474 **Cornea:** The transparent part of the front of the eyeball that covers the iris and pupil and
475 admits light to the interior.

476 **Corneal opacity:** Measurement of the extent of opaqueness of the cornea following
477 exposure to a test substance. Increased corneal opacity is indicative of damage to the
478 cornea. Opacity can be evaluated subjectively as done in the Draize rabbit eye test, or
479 objectively with an instrument such as an “opacitometer.”

480 **Corneal permeability:** Quantitative measurement of damage to the corneal epithelium
481 by a determination of the amount of sodium fluorescein dye that passes through all
482 corneal cell layers.

483 **False negative rate:** The proportion of all positive substances falsely identified by a
484 test method as negative. It is one indicator of test method accuracy.

485 **False positive rate:** The proportion of all negative substances that are falsely identified
486 by a test method as positive. It is one indicator of test method accuracy.

487 **Globally Harmonized System (GHS):** A classification system presented by the United
488 Nations that provides (a) a harmonized criteria for classifying substances and mixtures
489 according to their health, environmental and physical hazards, and (b) harmonized hazard
490 communication elements, including requirements for labeling and safety data sheets.

491 **Good Laboratory Practices (GLP):** Regulations promulgated by the U.S. Food and
492 Drug Administration and the U.S. Environmental Protection Agency, and principles and
493 procedures adopted by the Organization for Economic Cooperation and Development and
494 Japanese authorities, that describe recordkeeping and quality assurance procedures for
495 laboratory records that will be the basis for data submissions to national regulatory
496 agencies.

497 **Hazard:** The potential for an adverse health or ecological effect. A hazard potential
498 results only if an exposure occurs that leads to the possibility of an adverse effect being
499 manifested.

500 **In Vitro Irritancy Score:** An empirically-derived formula used in the BCOP assay
501 whereby the mean opacity and mean permeability values for each treatment group are

502 combined into a single *in vitro* score for each treatment group. The *IVIS* = mean opacity
503 value + (15 x mean permeability value).

504 **Negative control:** An untreated replicate containing all components of a test system.
505 This sample is processed with test substance-treated samples and other control samples to
506 determine whether the solvent interacts with the test system.

507 **Nonirritant:** Substances that are not classified as EPA Category I, II, or III; EU R41 or
508 R36; or GHS Category 1, 2A, or 2B ocular irritants.

509 **Ocular corrosive:** (a) A substance that causes irreversible tissue damage to the eye. (b)
510 Substances that are classified as GHS Category 1, EPA Category I, or EU R41 ocular
511 irritants.

512 **Ocular irritant:** (a) A substance that produces a reversible change in the eye following
513 application to the anterior surface of the eye. (b) Substances that are classified as EPA
514 Category II or III; EU R36; or GHS Category 2A, or 2B ocular irritants.

515 **Ocular severe irritant:** (a) A substance that causes tissue damage in the eye following
516 application to the anterior surface of the eye that does not resolve within 21 days of
517 application or causes serious physical decay of vision. (b) Substances that are classified
518 as GHS Category 1, EPA Category I, or EU R41 ocular irritants.

519 **Opacimeter:** An instrument used to measure “corneal opacity” by quantitatively
520 evaluating light transmission through the cornea. The typical instrument has two
521 compartments, each with its own light source and photocell. One compartment is used for
522 the treated cornea, while the other is used to calibrate and zero the instrument. The
523 difference between photocell signals in the two compartments is measured electronically
524 as a change in voltage, and is displayed digitally, generating numerical opacity values
525 with arbitrary units (but calibrated to opacity standards).

526 **Positive control:** A replicate containing all components of a test system and treated with
527 a substance known to induce a positive response. To ensure that variability in the positive
528 control response across time can be assessed, the magnitude of the severe response
529 should not be excessive.

530 **Reliability:** A measure of the degree to which a test method can be performed
531 reproducibly within and among laboratories over time. It is assessed by calculating intra-
532 and inter-laboratory reproducibility and intralaboratory repeatability.

533 **Solvent/vehicle control:** An untreated sample containing all components of a test
534 system, including the solvent or vehicle that is processed with the test substance-treated
535 and other control samples to establish the baseline response for the samples treated with
536 the test substance dissolved in the same solvent or vehicle. When tested with a concurrent
537 negative control, this sample also demonstrates whether the solvent or vehicle interacts
538 with the test system.

539 **Tiered testing:** A stepwise testing strategy where all existing information on a test
540 substance is reviewed, in a specified order, using a weight of evidence process at each
541 tier to determine if sufficient information is available for a hazard classification decision ,
542 prior to progression to the next tier. If the irritancy potential of a test substance can be
543 assigned based on the existing information, no additional testing is required. If the
544 irritancy potential of a test substance cannot be assigned based on the existing
545 information, a step-wise sequential animal testing procedure is performed until an
546 unequivocal classification can be made.

547 **Validated test method:** An accepted test method for which validation studies have been
548 completed to determine the relevance and reliability of this method for a specific
549 proposed use.

550 **Weight-of-evidence:** The process of considering the strengths and weaknesses of various
551 pieces of information in reaching and supporting a conclusion concerning the hazard
552 potential of a substance.

553

ANNEX II

554

ICCVAM RECOMMENDED PROTOCOL FOR THE

555

BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP) TEST METHOD

556 This proposed protocol for measuring corneal damage was developed following a
557 comprehensive test method evaluation process conducted by ICCVAM, which included
558 an international independent scientific peer review of the validation status and scientific
559 validity of the BCOP (1)(2). It is based primarily on information obtained from 1) the
560 Institute for In Vitro Sciences (IIVS), a nonprofit foundation that has performed the
561 BCOP assay since 1997 in a Good Laboratory Practice (GLP)-compliant testing facility;
562 and 2) INVITTOX Protocol 124 (3), which represents the protocol used for the European
563 Community-sponsored prevalidation study of the BCOP assay conducted in 1997-1998.
564 Both of these protocols are based on the BCOP assay methodology first reported by
565 Gautheron et al. (4). Future studies using the BCOP test method could include further
566 characterization of the usefulness or limitations of the BCOP in a weight-of-evidence
567 approach for regulatory decision-making. Users should be aware that the proposed test
568 method protocol could be revised based on any additional optimization and/or validation
569 studies that are conducted in the future. ICCVAM recommends that test method users
570 consult the NICEATM-ICCVAM website (<http://iccvam.niehs.nih.gov/>) to ensure use of
571 the most current test method protocol.

572

572 **1.0 PURPOSE AND APPLICABILITY**

573 The purpose of this protocol is to describe the procedures used to evaluate the potential
574 ocular irritancy of a test substance as measured by its ability to induce opacity and
575 increase permeability in an isolated bovine cornea. Effects are measured by: 1) decreased
576 light transmission through the cornea (opacity); 2) increased passage of sodium
577 fluorescein dye through the cornea (permeability); and 3) evaluation of fixed and
578 sectioned tissue at the light microscopic level, if applicable. The opacity and permeability
579 assessments of the cornea following exposure to a test substance are considered
580 individually, and also combined to derive an In Vitro Irritation Score (IVIS), which is
581 used to classify the irritancy level of the test substance. Histological evaluation of the
582 corneas can be useful for identifying damage in tissue layers that does not produce
583 significant opacity or permeability.

584 The focus of this protocol is on the use of the BCOP test method for the detection of
585 ocular corrosives and severe irritants, as defined by the U.S. Environmental Protection
586 Agency (EPA)(5), the European Union (EU)(6), and in the United Nations Globally
587 Harmonized System of Classification and Labelling of Chemicals (GHS)(7). Substances
588 other than ocular corrosives and severe irritants (e.g., nonirritants and mild/moderate
589 ocular irritants) have been tested using this protocol; however, the accuracy and
590 reliability of the BCOP test method have not yet been formally evaluated for these other
591 classes of ocular irritancy, as defined by the EPA (5), the EU (6), and the GHS (7).

592 **2.0 SAFETY AND OPERATING PRECAUTIONS**

593 All procedures with bovine eyes and bovine corneas should follow the institution's
594 applicable regulations and procedures for handling human or animal substances, which
595 include, but are not limited to, tissues and tissue fluids. Universal laboratory precautions
596 are recommended, including the use of laboratory coats, eye protection, and gloves. If
597 available, additional precautions required for specific study substances should be
598 identified in the Material Safety Data Sheet for that substance.

599 **3.0 MATERIALS, EQUIPMENT, AND SUPPLIES**

600 **3.1 Source of Bovine Eyes**

601 Eyes from young adult cattle are obtained from a cattle slaughterhouse located within
602 close proximity of the testing facility. The cattle type (breed not specified) can be cows,
603 heifers, steers, or bulls. Because cattle have a wide range of weights, depending on breed,
604 age, and sex, there is no recommended weight for the animal at the time of slaughter.

605 Eyes from very old cattle are not recommended because the corneas tend to have a
606 greater horizontal corneal diameter and vertical corneal thickness that could affect assay
607 performance (8)(Harbell J, personal communication). Additionally, eyes from calves are
608 not recommended since their corneal thickness and corneal diameter are considerably less
609 than that of eyes from adult cattle.

610 **3.2 Equipment and Supplies**

- 611 • Corneal holders (See **Figures 1 and 2**)¹
- 612 • Dissection equipment (scissors, scalpels, forceps)
- 613 • Electric screwdriver
- 614 • Falcon tubes (50 mL)
- 615 • Incubator or water bath
- 616 • Liquinox™ (or equivalent)
- 617 • Microplate reader or UV/VIS spectrophotometer
- 618 • Micropipettors and pipette tips
- 619 • Opacitometer
- 620 • Petri dishes
- 621 • Plastic containers for collection and transport of eyes
- 622 • Sample tubes (5 mL, glass) for permeability determination
- 623 • Spatula
- 624 • Specialized window-locking ring screwdriver
- 625 • Standard tissue culture and laboratory equipment
- 626 • Sterile deionized water
- 627 • Syringes (10 mL) and blunt tip needles (19 Gauge)
- 628 • Vacuum pump
- 629 • Volumetric flasks
- 630 • 96 well plates (polystyrene) or cuvettes of an appropriate size for UV/VIS
- 631 spectrophotometer

632 **3.3 Chemicals**

- 633 • Ethanol (200 proof, absolute, anhydrous, ACS/USP grade)
- 634 • Imidazole
- 635 • Penicillin
- 636 • Sodium chloride
- 637 • Sodium fluorescein
- 638 • Streptomycin

639 **3.4 Solutions**

¹Users should be aware of a newer proposed corneal holder developed by Ubels et al. (9). The ICCVAM Test Method Evaluation Report recommends, “Studies should be conducted to evaluate the impact of using a corneal holder that maintains normal curvature (e.g., the corneal mounting system designed by Ubels et al. [2002]) on accuracy and/or reliability of the BCOP test method.”

640 Follow the manufacturer's recommendations with regard to storage temperature and shelf
641 life of stock solutions. Prepare assay solutions volumetrically.

- 642 • 0.9% (w/v) NaCl in sterile deionized water (saline).
- 643 • 1X Hanks' Balanced Salt Solution with Ca⁺⁺ and Mg⁺⁺ (HBSS) containing
644 100 IU/mL penicillin and 100 µg/mL streptomycin.
- 645 • Dulbecco's Phosphate Buffered Saline (DPBS).
- 646 • Eagle's Minimum Essential Medium without phenol red containing 1%
647 (v/v) Fetal Bovine Serum (complete EMEM), warmed to 32°C.
- 648 • Eagle's Minimum Essential Medium with phenol red containing 1% Fetal
649 Bovine Serum (complete MEM with phenol red, used only for rinsing test
650 substances), warmed to 32°C.
- 651 • Sodium fluorescein (Na-fluorescein) diluted in DPBS to 4 mg/mL for
652 liquid test articles or 5 mg/mL for solid test articles.

653 **4.0 TEST SUBSTANCE PREPARATION**

654 All test substance solutions should be prepared fresh on the day of use.

655 **4.1 Nonsurfactant Liquid Test Substances**

656 Liquid test substances are usually tested undiluted. However, if prescribed, dilutions of
657 aqueous soluble test substances could be prepared in 0.9% sodium chloride.

658 **4.2 Nonsurfactant Solid Test Substances**

659 Nonsurfactant solid test substances should be prepared as 20% (w/v) solutions or
660 suspensions in 0.9% sodium chloride.

661 **4.3 Surfactants**

662 Solid and concentrated liquid surfactants should be prepared and tested as a 10% (w/v,
663 v/v) dilution or suspension in 0.9% sodium chloride.

664 **4.4 Surfactant Preparations**

665 Surfactant-based preparations (e.g., product formulations) are usually tested neat, or can
666 be diluted in 0.9% sodium chloride, with justification of the selected dilution.

667 **5.0 CONTROLS**

668 **5.1 Negative Control**

669 Concurrent negative or solvent/vehicle controls and positive controls are included in each
670 experiment. When testing a liquid substance at 100%, a concurrent negative control (e.g.,
671 0.9% sodium chloride or distilled water) is included in the BCOP test method so that
672 nonspecific changes in the test system can be detected and to provide a baseline for the
673 assay endpoints. It also ensures that the assay conditions do not inappropriately result in
674 an irritant response.

675 **5.2 Solvent/Vehicle Control**

676 When testing a diluted liquid, surfactant, or solid, a concurrent solvent/vehicle control
677 group is included in the BCOP test method so that nonspecific changes in the test system
678 can be detected and to provide a baseline for the assay endpoints. Only a solvent/vehicle
679 that has been demonstrated to have no adverse effects on the test system can be used.

680 **5.3 Positive Control**

681 A known ocular irritant is included as a concurrent positive control in each experiment to
682 verify that an appropriate response is induced. As the BCOP assay is being used in this
683 test guideline to identify corrosive or severe irritants, ideally the positive control should
684 be a reference substance that induces a severe response in this test method. However, to
685 ensure that variability in the positive control response across time can be assessed, the
686 magnitude of irritant response should not be excessive.

687 Based on a large historical database demonstrating consistent results with a test substance
688 that induces a response near the borderline of the severe irritant response, 100% ethanol
689 is typically used as a positive control for liquids in the BCOP. In the *in vivo* rabbit eye
690 test, 100% ethanol is classified as EPA Category III or I (5), GHS Category 2B or 2A (7),
691 and EU nonirritant (6) based on an ECETOC study (10) and a S.C. Johnson & Son study,
692 respectively.

693 Historically, the most commonly used positive control for solid test substances has been
694 20% (w/v) imidazole prepared in saline. In the *in vivo* rabbit eye test, based on an
695 ECETOC study (10), 20% (w/v) imidazole is classified as GHS Category 1 (7) and EU
696 R41 (6).

697 **5.4 Benchmark Substances (if appropriate)**

698 Benchmark substances are useful for evaluating the ocular irritancy potential of unknown
699 chemicals of a specific chemical or product class, or for evaluating the relative irritancy
700 potential of an ocular irritant within a specific range of irritant responses. Appropriate
701 benchmark substances should have the following properties:

- 702 • a consistent and reliable source(s)
- 703 • structural and functional similarity to the class of the substance being
704 tested
- 705 • known physical/chemical characteristics
- 706 • supporting data on known effects in the *in vivo* rabbit eye test
- 707 • known potency in the range of the desired response

708 **6.0 EXPERIMENTAL DESIGN**

709 **6.1 Collection and Transport Conditions of Bovine Eyes**

710 Bovine eyes are typically obtained from a local cattle slaughterhouse, where a
711 slaughterhouse employee excises the eyes as soon as possible after slaughter. Care should

712 be taken to avoid damaging the cornea during the enucleation procedure. Because the
713 eyes are collected during the slaughter process, they might be exposed to blood and other
714 biological substances, including bacteria and other microorganisms. Therefore, it is
715 important to ensure that the risk of contamination is minimized (e.g., by keeping the eyes
716 on wet ice, by using the eyes as soon as possible after slaughter, or by adding antibiotics
717 (e.g., penicillin at 100 IU/mL and streptomycin at 100 µg/mL)) to the HBSS used to store
718 the eyes during transport. The eyes are used within five hours after slaughter.

719 Under conditions where contamination of the bovine eyes with yeast occurs, immersion
720 of the eyes in HBSS containing fungizone should be evaluated.

721 **6.2 Preparation of Corneas**

722 a. Carefully examine all eyes macroscopically. Those exhibiting unacceptable
723 defects, such as opacity, scratches, pigmentation, and neovascularization
724 are rejected.

725 b. Carefully remove the cornea from each selected eye by making an incision
726 with a scalpel 2 to 3 mm outside the cornea, then by cutting around the
727 cornea with dissection scissors, leaving a rim of sclera to facilitate
728 handling. Carefully peel off the iris and lens, ensuring no fragments of
729 these tissues are remaining on the cornea. Take care to avoid damaging the
730 corneal epithelium and endothelium during dissection (see **Figure 3**).

731 c. Store the isolated corneas in a petri dish containing HBSS until they are
732 mounted in holders. Examine the corneas before use, and discard those
733 with defects.

734 d. Mount the corneas in holders (one cornea per holder), by placing the
735 endothelial side of the cornea against the O-ring of the posterior chamber.
736 Place the anterior chamber over the cornea and join the chambers together
737 by tightening the chamber screws. Care should be taken not to shift the two
738 chambers to avoid damaging the cornea.

739 e. Fill both chambers with fresh complete MEM (about 5 mL), always filling
740 the posterior chamber first to return the cornea to its natural curvature. Care
741 should be taken when adding or removing liquid from the posterior
742 chamber to avoid the formation of bubbles and to minimize shear forces on
743 the corneal endothelium.

744 f. Seal each chamber with plugs provided with the holders.

745 g. Incubate the holders in a vertical position at $32 \pm 1^\circ\text{C}$ for at least 60
746 minutes.

747 h. At the end of the initial 1-hour incubation period, examine each cornea for
748 defects, such as tears or wrinkling. Discard corneas with any observed
749 defects.

750 **6.3 Control Cornea Selection and Opacity Reading**

- 751 i. After the 1-hour incubation period, remove the medium from both
752 chambers of each holder (anterior chamber first) and replace with fresh
753 complete MEM.
- 754 j. Take and record an initial opacity reading for each cornea, using an
755 opacitometer or equivalent instrument that has been appropriately
756 calibrated according to the manufacturer's specifications. This initial
757 opacity reading will be used to calculate the final opacity value for each
758 cornea. The testing facility should ensure the opacitometer is functioning
759 properly each day it is used.
- 760 k. Calculate the average opacity value for all corneas.
- 761 l. Select a minimum of three corneas with opacity values close to the average
762 value for all corneas as negative (or solvent/vehicle) control corneas.
- 763 m. Corneas with an opacity reading greater than 10 after an initial one-hour
764 equilibration are discarded.

765 **6.4 Treatment Groups**

766 A minimum of three corneas are treated with each test substance solution or suspension.
767 In addition, three corneas per assay are treated with the concurrent positive control and
768 three corneas per assay are treated with the concurrent negative control. If a benchmark
769 substance is used the day of testing, three corneas should be treated with the benchmark.

770 Different treatment methods are used depending on the physical nature and chemical
771 characteristics (liquid or surfactant versus nonsurfactant solid) of the test substance. The
772 controls used depend on which method is used.

773 **6.5 Treatment of Corneas and Opacity Measurements**

774 6.5.1 Closed-chamber method for nonviscous to slightly viscous liquid test
775 substances

- 776 a. Record the initial opacity readings and label each chamber with the
777 appropriate control or test substance identification. Just prior to treatment,
778 remove the medium from the anterior chamber through the dosing holes
779 using an appropriate aspiration technique (e.g., blunt needle attached to a
780 vacuum pump).
- 781 b. Add 0.75 mL of the control or test substance to the anterior chamber
782 through the dosing holes using a micropipettor. The dosing holes are then
783 resealed with the chamber plugs.
- 784 c. Rotate the holders such that the corneas are in a horizontal position. The
785 holders should be gently tilted back and forth to ensure a uniform
786 application of the control or test substance over the entire cornea.
- 787 d. Incubate the holders in a horizontal position at $32 \pm 1^\circ\text{C}$ for 10 ± 1 minutes.
788 If other exposures times are used, justification must be provided.

- 789 e. Remove the control or test substance from the anterior chamber through the
790 dosing holes and rinse the epithelium at least three times with
791 approximately 2 to 3 mL of fresh complete MEM with phenol red. Perform
792 one last rinse of the epithelium using fresh complete MEM. If it is not
793 possible to remove all visible signs of the test substance, document the
794 observation in the study notebook. Refill the anterior chamber with fresh
795 complete MEM.
- 796 f. Perform a post-treatment opacity reading for each cornea and record the
797 results. Observe each cornea visually and, if applicable, record pertinent
798 observations (e.g., dissimilar opacity patterns, tissue peeling or residual test
799 article).
- 800 g. Incubate the holders in a vertical (anterior chamber facing forward)
801 position at $32 \pm 1^\circ\text{C}$ for 120 ± 10 minutes. If other post-exposure
802 incubation times are used, justification should be provided.
- 803 h. Record a post-incubation opacity reading for each cornea, which will be
804 used to calculate the final corneal opacity value. Observe each cornea
805 visually and record pertinent observations in the study notebook. Special
806 attention is taken to observe dissimilar opacity patterns, tissue peeling or
807 residual test substance, etc.
- 808 6.5.2 Open-chamber method for semiviscous and viscous liquid test substances and
809 surfactant preparations
- 810 a. Record the initial opacity readings and label each chamber with the
811 appropriate control or test article identification. Just prior to treatment,
812 remove the medium from the anterior chamber through the dosing holes.
- 813 b. Remove the window-locking ring and glass window from all appropriate
814 anterior chambers and place the holders into a horizontal position (anterior
815 chamber facing up).
- 816 c. Add test substance to each chamber successively at a constant rate of 15 to
817 30 seconds between each chamber. Apply approximately 0.75 mL of the
818 control or test substance (or enough test substance to completely cover the
819 cornea) directly to the epithelial surface of the cornea using a micropipettor
820 or other appropriate device, such as a spatula. Maintain the holders in a
821 horizontal position (anterior chamber up).
- 822 d. If necessary, to aid in filling the pipette with substances that are viscous,
823 the test article may first be transferred to a syringe. Insert the pipette tip of
824 the positive displacement pipette into the dispensing tip of the syringe, so
825 that the substance can be loaded into the displacement tip under pressure.
826 Simultaneously, depress the syringe plunger as the pipette piston is drawn
827 upwards. If air bubbles appear in the pipette tip, the test article should be
828 expelled and the process repeated until the tip is filled without air bubbles.
829 This method should be used for any substances that cannot be easily drawn
830 into the pipette (e.g., gels, toothpastes, and face creams).

- 831 e. If necessary, immediately upon dosing, slightly tilt the holders to achieve a
832 uniform application of the test article over the entire cornea.
- 833 f. After all of the chambers are dosed, replace the glass windows and
834 window-locking rings.
- 835 g. Incubate the holders in a horizontal position at $32 \pm 1^\circ\text{C}$ for 10 ± 1 minutes.
836 If other exposure incubation times are used, justification should be
837 provided.
- 838 h. Prior to the end of the exposure period, remove the window-locking ring
839 and glass window from each appropriate chamber.
- 840 i. At the completion of the exposure period, successively rinse each cornea in
841 the exposure group according to the intervals that they were dosed. Using a
842 syringe, add fresh complete MEM with phenol red to the inside wall of the
843 anterior chamber creating a “whirlpool or vortex effect”, which causes the
844 test article to be rinsed off the cornea. Take special care not to spray the
845 medium directly onto the cornea. Residual test article that cannot be
846 removed from the cornea by the “whirlpool method” is removed by placing
847 a layer of medium over the cornea (added to the inside wall of the
848 chamber). Spray a gentle stream of medium through the medium layer,
849 directing it towards the residual test article. If after several tries the test
850 article cannot be removed, document this in the study notebook, and
851 proceed to the next step.
- 852 j. Once each cornea is completely rinsed of test article, replace the glass
853 window and window-locking ring. Continue rinsing as stated previously for
854 the “closed chamber method” (see **Section 6.5.1, step e**).
- 855 k. Perform a post-treatment opacity reading for each cornea and record the
856 results. Observe each cornea visually and, if applicable, record pertinent
857 observations (e.g., dissimilar opacity patterns, tissue peeling or residual test
858 article).
- 859 l. Incubate the holders in a vertical (anterior chamber facing forward)
860 position at $32 \pm 1^\circ\text{C}$ for 120 ± 10 minutes. If other post-exposure
861 incubation times are used, justification should be provided.
- 862 m. Record a post-incubation opacity reading for each cornea, which will be
863 used to calculate the final corneal opacity value. Observe each cornea
864 visually and record pertinent observations in the study notebook. Special
865 attention is taken to observe dissimilar opacity patterns, tissue peeling or
866 residual test substance, etc.

867 6.5.3 Solid and liquid surfactant test substances

868 Surfactant test substances are administered following one of the previously described
869 procedures, with one exception, which is noted below:

- 870 • Surfactant test substances are tested on the cornea as a 10% (w/v) solution
871 or suspension prepared in an appropriate solvent/vehicle (e.g., sterile

872 deionized water).

873 6.5.4 Solid nonsurfactant test substances

874 Solid nonsurfactant test substances are administered following one of the previously
875 described procedures, with a few exceptions, which are noted below:

- 876 • Solid test substances are tested on the cornea as a 20% (w/v) solution or
877 suspension prepared in an appropriate solvent/vehicle (e.g., sterile
878 deionized water).
- 879 • Solid test substances are incubated at $32 \pm 1^\circ\text{C}$ for 240 ± 10 minutes.
- 880 • There is no post-treatment incubation period. Thus, immediately following
881 the rinsing process, both chambers are refilled (posterior chamber first)
882 with fresh complete MEM, and the post-treatment opacity readings are
883 taken. During the post-treatment opacity reading, visual observations are
884 performed for each cornea and, if necessary, are recorded in the
885 workbook. Special attention is taken to observe dissimilar opacity
886 patterns, tissue peeling or residual test article, etc. Immediately following
887 these opacity readings and visual observations, the permeability
888 experiment is performed.

889 6.6 Application of Sodium Fluorescein

890 Following the final opacity measurement, permeability of the cornea to Na-fluorescein is
891 evaluated. The Na-fluorescein solution is applied to the cornea by one of two methods,
892 depending on the nature of the test substance:

- 893 a. Liquid and surfactant test substances and surfactant preparations: Remove
894 the medium from both chambers (anterior chamber first). Fill the posterior
895 chamber with fresh complete MEM, and add 1 mL of a 4 mg/mL Na-
896 fluorescein solution to the anterior chamber using a micropipettor. Reseal
897 the dosing holes in the top of both chambers with the chamber plugs.
- 898 b. Solid nonsurfactant test substances: Remove the medium from the anterior
899 chamber only and replace with 1 mL of a 5 mg/mL Na-fluorescein solution.
900 Reseal the dosing holes in the top of both chambers with the chamber
901 plugs.

902 6.7 Permeability Determinations

- 903 a. After adding the Na-fluorescein to the anterior chamber and sealing the
904 chambers, rotate the holders into a horizontal position with the anterior
905 chamber facing up. Tilt the holders slightly, if necessary, to achieve a
906 uniform application of the Na-fluorescein over the entire cornea. Incubate
907 the holders in a horizontal position for 90 ± 5 minutes at $32 \pm 1^\circ\text{C}$.
- 908 b. After the 90-minute incubation period, remove the medium in the
909 posterior chamber of each holder and place into sample tubes pre-labeled
910 according to holder number. It is important to remove most of the medium
911 from the posterior chamber and mix it in the tube so that a representative
912 sample can be obtained for the OD_{490} determination.

- 913 c. After completing the Na-fluorescein penetration steps, the corneas should
914 be fixed in an appropriate fixative (e.g., 10% neutral buffered formalin) at
915 room temperature for at least 24 hours, so that the tissues are available if
916 histology is necessary or requested at a later time. It is important that the
917 corneas not be allowed to dry between transfer from the holders and
918 fixation (submersion in the fixative).
- 919 d. If using a microplate reader to measure optical density, transfer 360 μL of
920 the medium from each sample tube into its designated well on a 96-well
921 plate. The standard plate map provides two wells for each cornea. The first
922 well receives an undiluted sample from each cornea tested. When all of
923 the media samples have been transferred onto the plate, measure and
924 record their OD_{490} . Any OD_{490} value (of a control or test substance
925 sample) that is 1.500 or greater must be diluted to bring the OD_{490} into the
926 acceptable range. A dilution of 1:5 is generally sufficient but higher
927 dilutions may be required. Prepare the dilution from the original sample of
928 medium and transfer 360 μL into the second well designated for that
929 cornea. Reread the plate and record the data from both the undiluted and
930 diluted OD_{490} values. Use the values from this second reading in all
931 calculations. The OD_{490} values of less than 1.500 will be used in the
932 permeability calculation.
- 933 *Note:* The linear range of absorbance of different microplate readers can
934 vary. Thus, each laboratory must determine the upper limit of absorbance
935 (in the linear range) for the microplate reader used in its facility.
- 936 e. If using a UV/VIS spectrophotometer to measure optical density, adjust
937 the spectrophotometer to read at OD_{490} , and zero the spectrophotometer on
938 a sample of complete MEM. Prior to reading samples from the BCOP
939 assay, prepare and read two quality control samples of Na-fluorescein
940 solution to ensure the Na-fluorescein calibration curve (see note below)
941 conducted for the spectrophotometer is still acceptable. If the average of
942 the quality control samples does not fall within the accepted range of the
943 Na-fluorescein calibration curve, then prepare a Na-fluorescein calibration
944 curve prior to running samples from the BCOP assay. If the average of the
945 quality control samples falls within the accepted range of the calibration
946 curve, then proceed to read samples from the BCOP assay. Transfer an
947 aliquot of the mixed medium from the posterior chamber of the BCOP
948 holder into a cuvette, then take and record an absorbance reading using the
949 spectrophotometer. Any solutions giving an OD_{490} beyond the linear range
950 of the spectrophotometer must be diluted in complete MEM, and another
951 reading taken, repeating these steps until the OD_{490} is within the linear
952 range of the spectrophotometer. Repeat these procedures for each sample
953 from the BCOP assay, rinsing the cuvette(s) thoroughly between each
954 sample, until all samples have been read and results recorded.
- 955 *Note:* If conducting this assay for the first time, a calibration curve for the
956 spectrophotometer must be performed, using a series of dilutions of Na-
957 fluorescein solution in complete MEM. A calibration curve should be

958 prepared and used to determine the linear range of the testing facility's
959 spectrophotometer and thus determine the upper limit of absorbance.

960 **6.8 Histopathology**

961 A histopathological evaluation of the corneal tissue might be useful when the standard
962 BCOP endpoints (i.e., corneal opacity and permeability) produce borderline results. A
963 standardized scoring scheme using the formal language of pathology to describe any
964 effects should be used. However, such an evaluation may not be necessary if the test
965 substance belongs to class of materials known to be accurately predicted using only
966 corneal opacity and permeability measurements.²

967 **6.9 Maintenance of the Corneal Holders**

968 Following completion of the assay, clean the disassembled parts of each holder as
969 follows:

- 970 a. Soak the posterior and anterior chambers in a solution of warm tap water
971 and a dime-size or greater amount of Liquinox™ (or equivalent).
- 972 b. Soak the chamber plugs, O-rings, and handle screws in 70% ethanol.
973 Rinse the chamber plugs, O-rings, and handle screws thoroughly in hot tap
974 water and air dry prior to reassembling the chambers.
- 975 c. Clean the interior and exterior surfaces of each pre-soaked posterior and
976 anterior chamber by using a scrubbing sponge. Rinse each posterior and
977 anterior chamber thoroughly in warm tap water and air dry prior to
978 reassembling the chambers.
- 979 d. Match up each numbered posterior chamber with its corresponding
980 anterior chamber, insert an O-ring into the appropriate place, attach a
981 chamber handle screw to the anterior chamber, and finally insert the
982 chamber screws into the anterior chamber.

983 **7.0 EVALUATION OF TEST RESULTS**

984 Results from the two test method endpoints, opacity and permeability, should be
985 combined in an empirically derived formula that generates an *IVIS* for each test
986 substance.

987 **7.1 Opacity**

- 988 a. Calculate the change in opacity for each individual cornea (including the
989 negative control) by subtracting the initial opacity reading from the final
990 post-treatment opacity reading. Then calculate the *average* change in
991 opacity for the negative control corneas.

² The ICCVAM Test Method Evaluation Report recommends “histopathological evaluation of the corneal tissue, using a standardized scoring scheme, should be included when the BCOP test method is conducted. Such data will allow for the development of standardized decision criteria and a more comprehensive evaluation of the usefulness of this endpoint for classifying and labeling substances, especially those that may otherwise produce borderline or false negative results.”

- 992 b. Calculate a corrected opacity value for each treated cornea, positive
 993 control, and solvent/vehicle control (if applicable) by subtracting the
 994 average change in opacity of the negative control corneas from the change
 995 in opacity of each treated, positive control, or solvent/vehicle control
 996 cornea.
- 997 c. Calculate the mean opacity value of each treatment group by averaging the
 998 corrected opacity values of the treated corneas for each treatment group.

999 7.2 Permeability

1000 Microplate Reader Method

- 1001 a. Calculate the mean OD₄₉₀ for the blank wells (plate blanks). Subtract the
 1002 mean blank OD₄₉₀ from the raw OD₄₉₀ of each well (blank corrected
 1003 OD₄₉₀).
- 1004 b. If a dilution has been performed, correct the OD₄₉₀ for the plate blank
 1005 before the dilution factor is applied to the reading. Multiply each blank
 1006 corrected OD₄₉₀ by the dilution factor (e.g., a factor of 5 for a 1:5 dilution).
- 1007 c. Calculate the final corrected OD₄₉₀ value for each cornea by subtracting
 1008 the mean OD₄₉₀ value for the negative control corneas from the OD₄₉₀
 1009 value of each treated cornea.
- 1010 **Final Corrected OD₄₉₀** = (raw OD₄₉₀ – mean blank OD₄₉₀) - mean blank
 1011 corrected negative control OD₄₉₀
- 1012 d. Calculate the mean OD₄₉₀ value for each treatment group by averaging the
 1013 final corrected OD₄₉₀ values of the treated corneas for a particular
 1014 treatment group.

1015 UV/VIS Spectrophotometer Method

- 1016 a. Calculate the corrected OD₄₉₀ value of each treated, positive control, or
 1017 solvent/vehicle control cornea by subtracting the average value of the
 1018 negative control corneas from the original OD₄₉₀ value for each cornea.
- 1019 **Final Corrected OD₄₉₀** = raw OD₄₉₀ - mean blank corrected negative
 1020 control OD₄₉₀
- 1021 b. Calculate the mean OD₄₉₀ value for each treatment group by averaging the
 1022 final corrected OD₄₉₀ values of the treated corneas for a particular
 1023 treatment group.

1024 7.3 In Vitro Irritancy Score

1025 Use the mean opacity and mean permeability values (OD₄₉₀) for each treatment group to
 1026 calculate an *in vitro* score for each treatment group:

$$1027 \quad IVIS = \text{mean opacity value} + (15 \times \text{mean OD}_{490} \text{ value})$$

1028 Additionally, the opacity and permeability values should be evaluated independently to
 1029 determine whether a test substance induced irritation through only one of the two
 1030 endpoints.

1031 8.0 CRITERIA FOR AN ACCEPTABLE TEST

1032 A test is acceptable if the positive control gives an IVIS that falls within two standard
1033 deviations of the current historical mean, which is to be updated at least every three
1034 months. The negative or solvent/vehicle control responses should result in opacity and
1035 permeability values that are less than the established upper limits for background opacity
1036 and permeability values for bovine corneas treated with the respective negative or
1037 solvent/vehicle control.

1038 In the BCOP, 100% ethanol induces a moderate to severe response (*in vitro* score = 39.9 -
1039 65.4 at IIVS [n = 632]; mean = 52.7, standard deviation [SD] = 6.4), while 20% (w/v)
1040 imidazole induces a severe response (*in vitro* score = 69.7 - 136.2 at IIVS [n=125]; mean
1041 = 103, SD = 16.6). The negative/solvent (vehicle) control responses should result in
1042 opacity and permeability values that are less than the established upper limits for
1043 background opacity and permeability values for bovine corneas treated with the
1044 respective negative or solvent/vehicle control.

1045 **9.0 DATA INTERPRETATION**

1046 A substance that induces an *IVIS* ≥ 55.1 is defined as a corrosive or severe irritant. While
1047 this classification system provides a good initial guide to interpretation of these *in vitro*
1048 data, these specific ranges may not be applicable to all classes of substances. For
1049 example, the Sina et al. scoring scale (11) is not appropriate for anionic and nonionic
1050 surfactants since they produce appreciable permeability while inducing little direct
1051 opacity. For these and other substances that produce significant permeability with
1052 minimal opacity, it is recommended that permeability values >0.600 be considered
1053 severe. Benchmark substances are recommended for assaying the responses of test
1054 substances of different product or chemical classes. Additionally, histological evaluation
1055 of the corneas can be instrumental in identifying occult changes (e.g., peroxide-induce
1056 stromal damage).

1057 Even if an ocular corrosive or severe irritant classification is not obtained for a test
1058 substance, BCOP data would be useful in conjunction with *in vivo* data or valid *in vitro*
1059 test data to further evaluate the usefulness and limitations of the BCOP test method for
1060 identifying non-severe irritants and nonirritants. Therefore, it is recommended that the
1061 complete classification scheme of the BCOP test method (i.e., corrosive/severe irritants,
1062 non-severe irritants, or nonirritants) be applied and that these data are reported in parallel
1063 with any other data obtained (i.e., from the *in vivo* rabbit eye test or an adequately
1064 validated *in vitro* test method). The remaining categories in the BCOP test method
1065 classification scheme include the following: a substance producing an IVIS from 0 to 3 is
1066 considered a nonirritant, from 3.1 to 25 a mild irritant, and from 25.1 to 55 a moderate
1067 irritant.

1068 **10.0 STUDY REPORT**

1069 The test report should include the following information, if relevant to the conduct of the
1070 study:

1071 Test and Control Substances:

- 1072 • Chemical name(s) such as the structural name used by the Chemical
1073 Abstracts Service (CAS), followed by other names, if known;

- 1074 • The CAS Registry Number (RN), if known;
- 1075 • Purity and composition of the substance or preparation (in percentage(s)
- 1076 by weight), to the extent this information is available;
- 1077 • Physicochemical properties such as physical state, volatility, pH, stability,
- 1078 chemical class, water solubility relevant to the conduct of the study;
- 1079 • Treatment of the test/control substances prior to testing, if applicable (e.g.,
- 1080 warming, grinding);
- 1081 • Stability, if known.

1082 Information Concerning the Sponsor and the Test Facility

- 1083 • Name and address of the Sponsor
- 1084 • Name and address of the test facility
- 1085 • Name and address of the Study Director
- 1086 • Information on the source of the eyes (e.g., age, sex, weight of the donor
- 1087 animal) and corresponding information on the corneal diameter and CCT
- 1088 • Storage and transport conditions of eyes (e.g., date and time of eye
- 1089 collection, time interval prior to initiating testing, transport media, any
- 1090 antibiotics used)

1091 Justification of the Test Method and Protocol Used

1092 Test Method Integrity

- 1093 • The procedure used to ensure the integrity (i.e., accuracy and reliability) of
- 1094 the test method over time (e.g., periodic testing of proficiency substances,
- 1095 use of historical negative and positive control data)

1096 Criteria for an Acceptable Test

- 1097 • Acceptable concurrent negative control ranges based on historical data
- 1098 • Acceptable concurrent positive control ranges based on historical data
- 1099 • If applicable, acceptable concurrent benchmark control ranges based on
- 1100 historical data

1101 Test Conditions

- 1102 • Description of test system used
- 1103 • Type of corneal holder used
- 1104 • Calibration information for devices used for measuring opacity and
- 1105 permeability (e.g., opacitometer and spectrophotometer)
- 1106 • Information for the bovine corneas used, including statements regarding
- 1107 their quality

- 1108 • Details of test procedure used
- 1109 • Test substance concentration(s) used
- 1110 • Description of any modifications of the test procedure
- 1111 • Reference to historical data of the model (e.g., negative and positive
- 1112 controls, proficiency substances, benchmark substances)
- 1113 • Description of evaluation criteria used
- 1114 Results
- 1115 • Tabulation of data from individual test samples (e.g., opacity and OD490
- 1116 values and calculated IVIS for the test substance and the positive,
- 1117 negative, and benchmark controls [if included], reported in tabular form,
- 1118 including data from replicate repeat experiments as appropriate, and
- 1119 means \pm the standard deviation for each experiment)
- 1120 • Description of other effects observed
- 1121 • A digital photograph of each cornea
- 1122 Discussion of the Results
- 1123 Conclusion
- 1124 A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant
- 1125 Studies
- 1126 • This statement indicates all inspections made during the study, and the
- 1127 dates any results were reported to the Study Director. This statement also
- 1128 serves to confirm that the final report reflects the raw data.
- 1129 If GLP-compliant studies are performed, then additional reporting requirements provided
- 1130 in the relevant guidelines (12)(13)(14)(15) should be followed.

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