




Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 1 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

1
2
3
4
5
6
7
8
9
10


**LabCyte CORNEA-MODEL24
EYE IRRITATION TEST
OPERATION PROTOCOL
Ver. 2.6**

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 2 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

11	TABLE OF CONTENTS	
12		
13	1. RATIONALE AND BACKGROUND	4
14	1.1 The LabCyte CORNEA-MODEL24 EYE IRRITATION TEST	4
15	1.2 BACKGROUND OF LabCyte24 EIT	4
16	1.3 BASIS OF THE TEST METHOD.....	5
17	1.4 LIMITATION OF THE METHOD.....	5
18	1.5 BRIEF BASIC PROCEDURE.....	5
19	1.5.1 LabCyte CORNEA-MODEL24	6
20	1.5.1.1 QUALITY CONTROL OF THE TEST SYSTEMS	6
21	1.5.1.2 PRECAUTIONS.....	7
22	1.5.2 ASSAY QUALITY CONTROL	7
23	1.5.2.1 ASSAY ACCEPTANCE CRITERION 1: NEGATIVE CONTROL	7
24	1.5.2.2 ASSAY ACCEPTANCE CRITERION 2: POSITIVE CONTROL.....	7
25	1.5.2.3 ASSAY ACCEPTANCE CRITERION 3: STANDARD DEVIATION (SD)	8
26	1.6 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL).....	8
27	2. MATERIALS.....	9
28	2.1 LabCyte CORNEA-MODEL24.....	9
29	2.1.1 LabCyte CORNEA-MODEL24 KIT COMPONENTS	9
30	2.1.2 SHIPMENT OF LabCyte CORNEA-MODEL24	9
31	2.1.3 INSTRUCTIONS FOR USE OF LabCyte CORNEA-MODEL24.....	10
32	2.2 CONSUMABLES.....	10
33	2.3 OTHERS	10
34	2.3.1 EQUIPMENT/INSTRUMENTS	11
35	3. TEST METHOD.....	12
36	3.1 PREPARATIONS.....	12
37	3.1.1 POSITIVE CONTROL.....	12
38	3.1.2 NEGATIVE CONTROL.....	12
39	3.1.3 POLY WASH BOTTLE FOR PBS	12
40	3.2 TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOINT	13
41	3.2.1 DETECTION OF THE CHEMICALS THAT STAIN THE TISSUE	13
42	3.2.1.1 STEP 1 (PRELIMINARY TEST).....	13
43	3.2.1.2 STEP 2 (FUNCTIONAL CHECK ON VIABLE TISSUE).....	13
44	3.2.2 DETECTION OF CHEMICALS THAT DIRECTLY REDUCE WST-8	14
45	3.2.2.1 STEP 3 (PRELIMINARY TEST).....	14
46	3.2.2.2 STEP 2 (FUNCTIONAL CHECK ON FREEZE-KILLED TISSUE)	15
47	3.3 EXECUTION OF THE TEST	16
48	3.3.1 PREPARATION OF LabCyte CORNEA-MODEL24 (DAY -1).....	16
49	3.3.2 APPLICATION OF LIQUID TEST CHEMICALS AND RINSING (DAY 0~1).....	17
50	3.3.2.1 PREPARATION OF WELLS FOR POST-INCUBATION (3 RD ROW)	17
51	3.3.2.2 APPLICATION OF LIQUID TEST CHEMICALS AND RINSING.....	18
52	3.3.2.3 POST-EXPOSURE INCUBATION	20
53	3.3.3 APPLICATION OF SOLID TEST CHEMICALS AND RINSING (DAY 0~1).....	21
54	3.3.3.1 PREPARATION OF WELLS FOR SOLID TEST CHEMICAL APPLICATION (2 ND ROW) AND FOR	
55	POST-INCUBATION (3 RD ROW).....	21
56	3.3.3.2 APPLICATION OF SOLID TEST CHEMICALS.....	21

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 3 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

57	3.3.3.3	RINSING OF SOLID TEST CHEMICALS.....	22
58	3.3.4	WST-8 ASSAY (DAY 1).....	24
59	3.3.4.1	PREPARATION OF WELLS FOR WST-8 ASSAY.....	24
60	3.3.4.2	WST-8 REACTION.....	24
61	3.3.4.3	SAMPLING THE REACTED WST-8 MEDIUM.....	25
62	3.3.4.4	OPTICAL DENSITY MEASUREMENTS OF THE REACTION MEDIUM.....	26
63	4.	ASSESSMENT.....	28
64	4.1	CONDITIONS FOR A VALID TEST.....	28
65	4.2	ASSAY CRITERIA.....	28
66	5.	References.....	29
67	MDS 1-1:	RECEIPT OF LabCyte CORNEA-MODEL24 (2.1.2).....	30
68	MDS 1-2:	TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8	
69	ENDPOINT STEP-1 (3.2.1.1).....		31
70	MDS 1-3:	TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8	
71	ENDPOINT STEP-1 (3.2.2.1).....		32
72	MDS 1-4:	PREPARATION OF FREEZE KILLED TISSUE (3.2.2).....	33
73	MDS 2:	PREPARATION OF LabCyte CORNEA-MODEL24 (3.3.1).....	34
74	MDS 3-1(LIQUID):	APPLICATION OF LIQUID TEST CHEMICALS, RINSING AND	
75	POST-INCUBATION (3.3.2).....		35
76	MDS 3-2(SOLID):	APPLICATION OF SOLID TEST CHEMICALS AND RINSING (3.3.3)	
77		37
78	MDS 4-1:	WST-8 ASSAY (3.3.4.1, 3.3.4.2).....	39
79	MDS 4-2:	SAMPLING OF REACTING WST-8 SOLUTION (3.3.4.3) AND	
80	MEASUREMENT (3.3.4.4).....		40
81	REVISION HISTORY.....		41
82			

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 4 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

83 **1. RATIONALE AND BACKGROUND**

84 **1.1 The LabCyte CORNEA-MODEL24 EYE IRRITATION TEST**

85 The LabCyte CORNEA-MODEL24 eye irritation test (LabCyte24 EIT) is designed to identify test
86 chemicals that cause acute eye irritation by measuring cytotoxic effects using the
87 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
88 (WST-8) assay on the reconstructed human corneal epithelial (RhCE) model. The LabCyte24 EIT is not a
89 kit, but LabCyte CORNEA-MODEL24 tissues are commercially available at a minimum of 24 LabCyte
90 CORNEA-MODEL24 tissues per order.


91

92 **1.2 BACKGROUND OF LabCyte24 EIT**

93 Assessment of ocular irritation is an essential part of early testing procedures for the evaluation and
94 hazard classification of substances. Therefore, it plays an important role in the safety evaluation of
95 general consumer products and materials. Novel substances used in consumer products must undergo
96 comprehensive toxicological evaluation for eye irritation and a variety of other adverse outcomes. To
97 date, the *in vivo* Draize eye test has been the international standard assay for acute ocular toxicity
98 evaluation (irritation and corrosion) and is described (including optimizations and refinements) in OECD
99 Test Guideline 405. However, the use of this test has been questioned and strongly criticized for ethical
100 concerns related to animal welfare, because it is painful to the rabbits. Thus, alternative strategies and
101 tests are urgently required in order to evaluate the eye irritation potential of new chemicals. Corneal
102 epithelial cells on the surface of the eye are to first to be exposed to substances and have been widely
103 studied for links to the biological role of tissue and gene regulation. Three-dimensional RhCE models are
104 useful as a multilayered, standardized tissue that mimics the human corneal epithelium.

105 The LabCyte24 EIT was developed as a replacement for the Draize eye irritation test. The Draize
106 scoring system is heavily weighted towards corneal damage (80 out of 110 total score), because
107 irreversible damage to the cornea can lead to blindness. Since damage to the cornea is so crucial for
108 human health, corneal tissue can be considered a useful tool for the development of *in vitro* eye irritation
109 testing.

110

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 5 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

111 **1.3 BASIS OF THE TEST METHOD**

112 Damage induced by eye irritants generally progresses from the corneal epithelium through the stroma
113 and potentially to the endothelium, and the LabCyte24 EIT is able to provide information on the first
114 stages of this progression. Irritants damage cells while penetrating the corneal epithelium layer, and the
115 cytotoxic progression can be estimated by analyzing cell viability in the LabCyte CORNEA-MODEL24
116 tissue using standardized methods. Although the tissues represent only the corneal epithelium, (very mild
117 responses would also be reflective of some conjunctival irritation), it can be used to estimate deeper
118 damage as far as the stroma by the analyzing cell viability.

119 The relative viability of the tissue exposed to a test chemical is measured using the WST-8 assay
120 immediately after exposure and again after a post-exposure period. A viability of 40% of the negative
121 control value was used as the cutoff in identifying test chemicals as an irritant (GHS Category 1 or 2) or
122 an non-irritant (GHS No Category). Some culture environments might allow the detection of very small
123 quantities of cytokines secreted by the corneal epithelial tissue in response to topical application of test
124 chemicals.

125


126 **1.4 LIMITATION OF THE METHOD**

127 One limitation of this assay method is potential interference of the test chemical with the WST-8
128 endpoint. A colored test chemical or one that directly reduces WST-8 (and thereby mimics dehydrogenase
129 activity of the cellular mitochondria) could interfere with the WST-8 endpoint. This is only a problem,
130 however, if there are significant residual levels of the test chemical on or in the tissue at the time of the
131 WST-8. Although this is considered unlikely, if it did happen, both the actual metabolic WST-8 reduction
132 and the quasi direct WST-8 reduction by a colored test chemical can be quantified using the procedure
133 described in **Section 3.2 “TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH**
134 **WST-8 ENDPOINT”**.

135

136 **1.5 BRIEF BASIC PROCEDURE**

137 On the day of receipt, LabCyte CORNEA-MODEL24 tissues are incubated overnight to release
138 transport–stress-related compounds and debris.

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 6 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

139 For liquid test chemicals, tissues are topically exposed for 1 minute. Preferably, three tissues are used
140 for each test chemical as well as for the positive and negative controls. After exposure, tissues are
141 thoroughly rinsed and blotted to remove the test chemical, then transferred to fresh medium and
142 post-incubated for 24 hours. For solid test chemicals, tissues are exposed for 24 hours but are not
143 post-incubated.


144 After post-incubation of tissue exposed to liquid test chemicals or after exposure of tissue exposed to
145 solid test chemicals, the tissues are each transferred to a well containing the WST-8 medium in a 1:10
146 dilution with Earle balanced salt solution (EBSS). After a four-hour WST-8 incubation, the orange
147 water-soluble formazan salt is formed in the WST-8 medium by cellular mitochondria and optical density
148 (OD) of the WST-8 medium is measured using a spectrophotometer at 450 nm and 650 nm as reference.
149 Relative cell viability is calculated for each tissue as % of the mean of the negative control tissues. Test
150 chemicals that produce a relative cell viability below 40% of the negative control are predicted to be
151 irritants.

153 **1.5.1 LabCyte CORNEA-MODEL24**

154 The LabCyte CORNEA-MODEL24 is a commercially available RhCE model produced by Japan
155 Tissue Engineering Co. Ltd. It comprises normal human corneal epithelial cells that are derived from a
156 human cornea. The cells are cultured with 3T3-J2 cells as a feeder layer in order to expand them while
157 maintaining their phenotype (Rheinwald and Green, 1975; Green, 1978). Reconstruction of the human
158 cultured corneal epithelial tissue is achieved by cultivating and proliferating the corneal epithelial cells
159 on an inert filter substrate with a surface area of 0.3 cm² at an air-liquid interface for 13 days using an
160 optimized medium containing 5% fetal bovine serum. This results in the formation of a multilayer
161 structure comprising a fully differentiated corneal epithelium with features mimicking those of a normal
162 human corneal epithelium. For delivery, LabCyte CORNEA-MODEL24 tissues are embedded in an
163 agarose gel containing a nutrient solution and shipped in 24-well plates.

165 **1.5.1.1 QUALITY CONTROL OF THE TEST SYSTEMS**

166 LabCyte CORNEA-MODEL24 tissue is manufactured in accordance with well-defined quality

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 7 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

167 assurance procedures. Each production batch was comes with quality control documentation that
168 identifies storage conditions, RhCE instructions for use, lot number and origin, histology (demonstration
169 of human multilayered corneal epithelial-like structure), cell viability, and barrier function integrity
170 ($0.1 \leq IC_{50} \leq 0.4$).

171
172

173 **1.5.1.2 PRECAUTIONS**

174 Corneal epithelial cells are taken from healthy donors who are free of HIV or hepatitis. Nevertheless,
175 always adhere to the following procedures for the handling of biological materials:

- 176 (a) Always wear gloves during handling of the eye and kit components.
177 (b) All corneal epithelial tissue as well as all material and media that came in contact with it should be
178 decontaminated prior to disposal using special containers or autoclaving.

179

180 **1.5.2 ASSAY QUALITY CONTROL**

181

182 **1.5.2.1 ASSAY ACCEPTANCE CRITERION 1: NEGATIVE CONTROL**

183 The absolute OD of the negative control for either liquid or solid test chemicals (NC-L or NC-S)
184 tissues (treated with sterile PBS for liquid test chemicals or untreated for solid test chemicals) in the
185 WST-8 assay is an indicator of tissue viability obtained in the testing laboratory after shipping and
186 storing procedures and under specific conditions of use.

187 **$0.5 \leq \text{Mean OD (A450/650) measured value} \leq 1.6$**


188

189 **1.5.2.2 ASSAY ACCEPTANCE CRITERION 2: POSITIVE CONTROL**

190 Ethanol is used as the positive control (PC) for liquid test chemicals (PC-L) and is tested concurrently
191 with the liquid test chemicals. Lauric acid is used as the PC for solid test chemicals (PC-S) and is tested
192 concurrently with the solid test chemicals.

193 Concurrent here means that the PC-L and the PC-S are to be tested for each run.

194 **Mean tissue viability $\leq 40\%$**

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 8 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

195

196 **1.5.2.3 ASSAY ACCEPTANCE CRITERION 3: STANDARD DEVIATION (SD)**

197 Since eye irritation potential is predicted from the mean viability of three individual tissues, the
198 variability of tissue replicates must kept at an acceptably low level.

199 **Standard Deviation (SD) of tissue viability of three identically treated replicates for the negative**
200 **control, positive control, and test chemicals \leq 18%**

201

202 **1.6 DATA INTERPRETATION PROCEDURE (PREDICTION MODEL)**

203 The United Nations Globally Harmonized System (GHS) (United Nations, 2003) was used as a
204 reference for the in vivo eye irritation classification of test chemicals.

205 For the purpose of this EIT, an eye irritant is defined as a substance that induces reversible ocular
206 lesions after administration to rabbits.


207 According to GHS classifications, a substance is an irritant (Category 1 or 2) if the mean relative
208 viability of three individual tissues exposed to the test chemical is falls below 40% of the mean viability
209 of the negative control. **(Refer to Table 1.)**

210 **Table 1** Prediction model of LabCyte24 EIT

<i>In vitro</i> results	Prediction
Tissue viability is \leq 40%	Category 1 or 2
Tissue viability is $>$ 40%	No Category

211

212

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 9 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

213 **2. MATERIALS**

214 **2.1 LabCyte CORNEA-MODEL24**

215 **2.1.1 LabCyte CORNEA-MODEL24 KIT COMPONENTS**

216 LabCyte CORNEA-MODEL24 kit components are shown in **Table 2**.

217 **Table 2** LabCyte CORNEA-MODEL24 Kit Components

Component	Qty	Description
LabCyte CORNEA-MODEL24 plate	1 plate	Contains 24 culture inserts with tissues fixed in nutritive agar medium for transport (usable area: 0.3 cm ²).
Assay Medium	1 bottle	Basic medium for incubation (30 mL). Keep refrigerated.
24-well plate	1 plate	Blank plate for use in assay. Store at room temperature.

218


219 **2.1.2 SHIPMENT OF LabCyte CORNEA-MODEL24**

220 LabCyte CORNEA-MODEL24 is packed in a special Icompo container available from and delivered
221 by NIPPON EXPRESS CO., LTD. After the kit is delivered, examine the contents and make sure that all
222 components (LabCyte CORNEA-MODEL24 plate, assay medium, and 24-well assay plate) are included
223 in the package. Also confirm lot numbers and expiration dates. Record details in Methods Documentation
224 Sheet (MDS) 1. (See **MSD-1**).

225 NIPPON EXPRESS will pick up the Icompo container at a later date (generally, the day after delivery),
226 and it should be returned together with an shipping invoice and the insulating materials.

227

228

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 10 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

229 **2.1.3 INSTRUCTIONS FOR USE OF LabCyte CORNEA-MODEL24**

230 Always incubate all of the culture inserts after opening the package. Do not store the culture inserts
231 after opening.

232 The human corneal epithelial tissue cells used in the LabCyte CORNEA-MODEL24 originate from a
233 normal human donor and are HIV-, HBV-, HCV-, and HPV-negative. They are to be handled,
234 nevertheless, with due care and in accordance with laboratory biosafety guidelines for handling
235 human-derived materials.

236

237 **2.2 CONSUMABLES**

238 The following consumables are required.

239 *The following quantities are necessary to assay between one and six 6 test chemicals at a time.

- 240 • Assay Medium, 100 mL (J-TEC: 402250) 1
- 241 • Cell Counting Kit-8, 500 test (Dojindo: CK04) 4
- 242 • 24-well assay plate (Becton, Dickinson and Company: 353047) 7-8
- 243 • 96-well plate (Becton, Dickinson and Company: 353072 or equivalents) 1
- 244 • PBS, 500 mL (Invitrogen: 14190-144 or equivalents) 2 or 3 bottle
- 245 • Earle balanced salt solution (EBSS), 500 mL (SIGMA-ALDRICH: E3024) 1 bottle
- 246 • Sterile cotton buds (JAPAN COTTON BUDS: 10A754D or equivalents) 1 box
- 247 • Micro-pipette tips (sterile: 10~200 μ L, 200-1000 μ L)
- 248 • Microtubes (1.5mL)
- 249 • Dish (10cm)
- 250 • Paper towel

251


252 Convenient consumable items are shown followings.

253 Also, it would be convenient to have the following.

- 254 • Capillary & piston for positive-displacement-type pipette (10-100 μ L)

255

256 **2.3 OTHERS**

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 11 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

257 **2.3.1 EQUIPMENT/INSTRUMENTS**


- 258 • Safety cabinet (or clean bench)
- 259 • Water bath (37°C)
- 260 • CO₂ incubator (37°C, 5% CO₂, capable of maintaining high humidity)
- 261 • Autoclave
- 262 • 96-well multi-plate reader (required filters: 450 nm, 650 nm)
- 263 • Precision balance (0.1 mg)
- 264 • Aspirator
- 265 • Stop-watches
- 266 • Adjustable micro-pipette (10–200 µL, 200–1000 µL)
- 267 • Sharp-edged forceps (sterile)
- 268 • Micro spatula (sterile)
- 269 • Beaker (1–2 L: sterile)
- 270 • Sterilizable poly wash bottle (500–1000 mL: sterile) with wide mouth (mouth > 3-mm dia.)
- 271 • Mortar with pestle

272

273 Also, it would be convenient to have the following.

- 274 • Positive-displacement-type pipette (10–100 µL)

275

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 12 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

276 **3. TEST METHOD**

277 *Procedures described in Sections 3.1.1 to 3.1.3 and Sections 3.3.1 to 3.3.3 are to be performed
278 aseptically in a safety cabinet or clean bench. Procedures other than those mentions in the previous
279 sentence need not be performed aseptically. Refer to **Section 2.1.3 “INSTRUCTIONS FOR USE**
280 **OF LabCyte CORNEA-MODEL24”**.

281

282 **3.1 PREPARATIONS**

283 **3.1.1 POSITIVE CONTROL**

- 284 (1) Use Ethanol as a positive control for liquid test chemicals.
285 (2) Use Lauric acid as a positive control for solid test chemicals.

286

287 **3.1.2 NEGATIVE CONTROL**

- 288 (1) Use PBS as a negative control for liquid test chemicals.
289 (2) Use non-treatment as a negative control for solid test chemicals.

290


291 **3.1.3 POLY WASH BOTTLE FOR PBS**

- 292 (1) Sterilize poly wash bottle using an autoclave.
293 (2) Fill the sterilized poly wash bottle with sterile PBS.

294

295

296

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 13 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

297 **3.2 TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOINT**

298 There are two kinds of test chemicals that interfere with the WST-8 assay.

299 (a) Test chemicals that stain corneal epithelial tissues directly.

300 (b) Test chemicals that react directly with WST-8.

301 Test chemical that stain the corneal epithelial tissues could possibility transfer from the corneal
302 epithelial tissues to the WST-8 reaction buffer and affect the OD measurements. Test chemicals that react
303 directly with WST-8 can also affect OD measurements, if the test chemical is present in the corneal
304 epithelial tissues when the WST-8 viability test is performed. The procedure for detecting such test
305 chemicals is described below.

306

307 **3.2.1 DETECTION OF THE CHEMICALS THAT STAIN THE TISSUE**

308 **3.2.1.1 STEP 1 (PRELIMINARY TEST)**

309 (1) Add 50 μ L (Liquid) or 10mg (Solid) of the test chemical into wells of 24-well assay plate
310 preliminarily filled with 0.5mL of distilled water. Untreated distilled water is used as control.

311 (2) Close the lid of 24-well assay plate and incubate the mixture in CO₂ incubator for 15 minutes.

312 (3) After incubation, shake the mixture gently and evaluate the staining of the distilled water
313 macroscopically.

314 (4) When the color of the solution changes significantly, the test chemical is presumed to have the
315 potential to stain the tissue and a functional check on viable tissues (Step2) should be performed.

316 When the color of the solution does not change significantly, the test chemical is determined not to
317 have a potential to stain the tissue.


318 (5) Record the details of steps 1 to 4 above in **MDS 1-2**.

319

320 **3.2.1.2 STEP 2 (FUNCTIONAL CHECK ON VIABLE TISSUE)**

321 (1) dd 50 μ L (liquid) or 10 mg (solid) of the test chemical, which clearly changed the color of the
322 distilled water in step 1, onto the surface of the epidermis tissues. Distilled water is used as negative
323 control.

324 (2) Follow all procedures described in the Section 3.3 EXECUTION OF THE TEST. However,
325 incubate the tissue for 3 hours in medium (EBSS) without WST-8 instead of incubating in the
326 diluted WST-8 medium to evaluate the staining of the WST-8 medium (correcting tissue).

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 14 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

327 (3) The corrected OD is calculated using the following formula.

328 **Corrected OD = A – (B – C),**

329 where

330 A is the OD of viable tissue exposed to a test chemical,

331 B is the mean OD of correcting tissue exposed to a test chemical, and

332 C is the mean OD of correcting tissue exposed to the negative control.

333

334 (4) If a corrected OD is below 0, the OD is considered to be 0.

335 (5) When a cell viability that is calculated according to the procedures described in **Section 3.3.4.4** is
336 $\leq 40\%$, the test chemical is predicted to be an irritant (GHS Category 1 or 2) and there is no need
337 to calculate a corrected value.

338

339

340 **3.2.2 DETECTION OF CHEMICALS THAT DIRECTLY REDUCE WST-8**

341 **3.2.2.1 STEP 3 (PRELIMINARY TEST)**

342 (1) Dilute the cell counting kit-8 (WST-8 stock solution) with EBSS (Cell Counting Kit-8:EBSS = 1:10),
343 and then prepare the diluted WST-8 medium.

344 *Prepare immediately prior to use and use as soon as possible.

345 Fill each well of the 24-well assay plate with 0.3 mL of diluted WST-8 medium.

346 (2) Add 50 μ L of a liquid test chemical or 10 mg of a test chemical to the wells of 24-well assay plate.
347 The diluted WST-8 medium is used as control.

348 (3) Close the lid of 24-well assay plate and incubate the in a CO₂ incubator for about 4 hours.

349 (4) After incubation, shake the mixture gently and evaluate the staining of the diluted WST-8 medium
350 macroscopically.



Ethanol


5% Triton X100

Glycolic acid

m-Phenylene
diamine

Monoethanolamine

Calcium
thioglycollate

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 15 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

351 **Photo 1** Example of test chemicals to interfere the WST-8 assay directly (STEP 1).

352 When test chemicals like m-Phenylene diamine, Monoethanolamine, or Calcium thioglycollate have
353 colored the diluted WST-8 medium, Step 2 must be performed.

354 (5) Significant coloring of the diluted WST-8 medium by the test chemical can interfere with the WST-8
355 assay and therefore requires the additional functional check of Step 2.

356 (6) Record the details of steps 1 to 5 above in **MDS 1-3**.

357

358 **3.2.2.2 STEP 4 (FUNCTIONAL CHECK ON FREEZE-KILLED TISSUE)**

359 (6) Add 50 µL of a liquid test chemical or 10 mg of solid a test chemical that clearly changed the color
360 of the diluted WST-8 medium (**3.2.2.1. STEP 3**) to the surface of the corneal epithelial tissues.

361 (7) Follow all procedures described in **Section 3.3 “EXECUTION OF THE TEST”**. Instead of using
362 viable corneal epithelial tissues, however, use corneal epithelial tissues that were freeze-killed at
363 -80°C or lower for 30 min.

364 Record the details of freeze-killing the tissue in **MDS 1-3**.

365 (8) The corrected OD is calculated using the following formula.

366 **Corrected OD = A – (B – C),**

367 where

368 A is the OD of viable tissue exposed to a test chemical,

369 B is the mean OD of freeze-killed tissue (correcting tissue) exposed to a test chemical, and

370 C is the mean OD of freeze-killed tissue (correcting tissue) exposed to the negative control.


371

372 (9) If a corrected OD is below 0, the OD is considered to be 0.

373 (10) When a cell viability that is calculated according to the procedures described in **Section 3.3.4.4** is
374 ≤ 40%, the test chemical is predicted to be an irritant (GHS Category 1 or 2) and there is no need
375 to calculate a corrected value.

376

377

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 16 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

378 **3.3 EXECUTION OF THE TEST**

379

380 **3.3.1 PREPARATION OF LabCyte CORNEA-MODEL24 (DAY -1)**

381 (1) Warm the assay medium to 37°C for 30 minutes
382 in a water bath.

383 (2) Fill the six wells of the 1st row of each assay
384 plate for LIQUID/SOLID with 0.5 mL/well of
385 the warm assay medium.

386 **See Fig. 1.**

387 (3) Open the LabCyte CORNEA-MODEL24
388 aluminum package.

389 (4) Open the LabCyte CORNEA-MODEL24 plate lid and pick up the culture inserts using sterile
390 forceps.

391 *Do not touch the surface of the corneal epithelial tissue in the culture inserts.

392 *Use forceps to remove any agar medium
393 sticking to the outside of the culture inserts.

394 (5) Transfer the culture inserts to the assay medium
395 in the wells of the 1st row using sterile forceps.

396 **See Fig. 2.**

397 *Do not allow air bubbles to form under the
398 tissue inserts.

399 (6) Close the lid on the plate and place it in a CO₂ incubator.

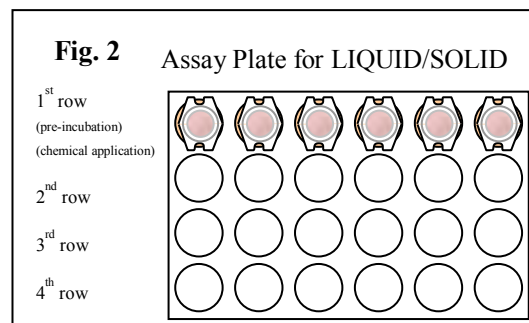
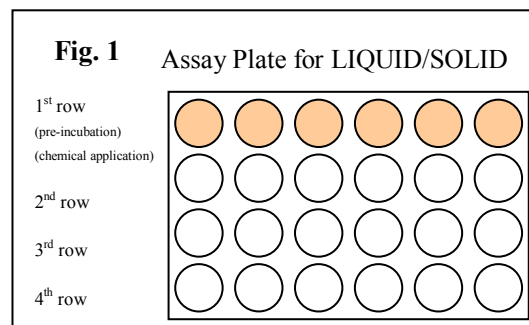
400 (7) Incubate overnight (15–30 hours) until ready to perform **Section 3.3.2 “APPLICATION OF**
401 **LIQUID TEST CHEMICALS, RINSING AND POST-INCUBATION”** or **Section 3.3.3**
402 **“APPLICATION OF SOLID TEST CHEMICALS AND RINSING”**.


403 (8) Record the details of steps 1 to 7 above in MDS 2.

404

405

406



Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 17 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

407 **3.3.2 APPLICATION OF LIQUID TEST CHEMICALS AND RINSING (DAY 0~1)**

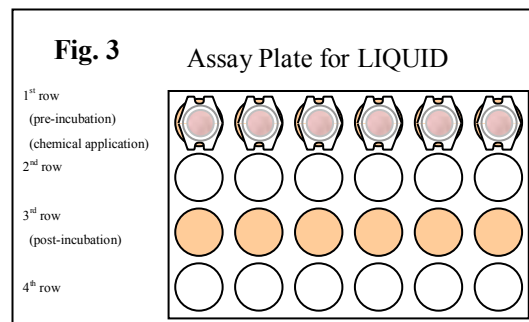
408

409 **3.3.2.1 PREPARATION OF WELLS FOR POST-INCUBATION (3RD ROW)**

410 (1) Warm the assay medium to 37°C for 30 minutes
411 using a water bath.

412 (2) Remove the assay plate for LIQUID from the CO₂
413 incubator.

414 (3) Open the lid of the assay plate for LIQUID, and
415 use a micropipette to fill the six wells of the 3rd
416 row with 0.5 mL/well of the warm assay medium.



417 **See Fig. 3.**


418 (4) Close the lid of the assay plate and perform **Section 3.3.2.2 “APPLICATION OF LIQUID TEST**
419 **CHEMICALS AND RINSING”** continuously.

420 (5) If the test chemicals are not applied immediately, store the Assay Plate for LIQUID in a CO₂
421 incubator until ready apply but for no more than 12 hours.

422 (6) Record the details of steps 1 to 5 above in **MDS 3-1**.

423

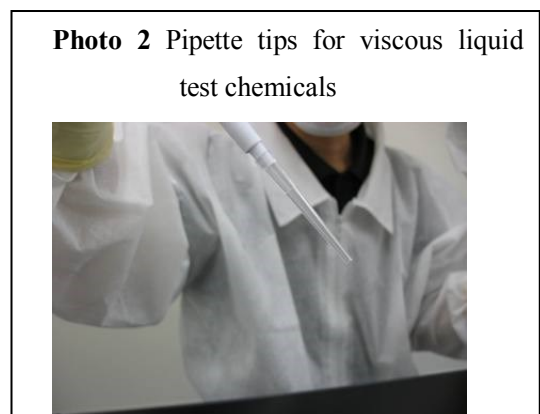
424

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 18 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

425 **3.3.2.2 APPLICATION OF LIQUID TEST CHEMICALS AND RINSING**

426 (1) Remove the assay plate for LIQUID from the CO₂ incubator.

427 (2) Using a micropipette, apply 50 µL of a liquid test
428 chemical to the surface of the corneal epithelial
429 tissues in the 1st row of the assay plate. Each test
430 chemical is to be tested in three wells (N=3).
431 Carefully apply the test chemical to the central
432 part of each corneal epithelial tissue. After
433 application, close the lid of the assay plate and tap
434 the sides of the plate to spread the liquid test
435 chemicals to spread out over the entire corneal

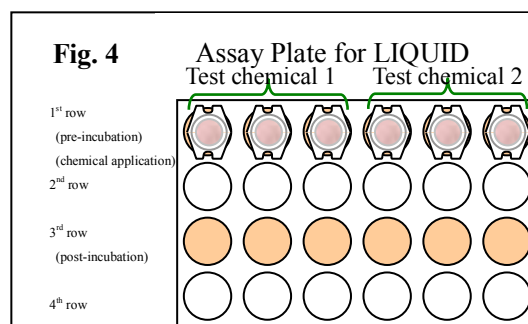


436 epithelial surface. If necessary, use a micro spatula to spread the liquid test chemical over the entire
437 surface. Take care not to press down on the surface of the corneal epithelial with the spatula.

438 *For viscous LIQUID test chemicals, use a wide orifice cell saver tip (See Photo 2.) or positive
439 displacement type pipette.

440 *Use a pipette or other equipment to familiarize
441 yourself beforehand with the characteristics
442 the test chemicals.

443 *Assay no more than two test chemicals on one
444 24-well assay plate.




445 See Fig. 4.

446 Each chemical is tested in three wells, using
447 three tissues (N = 3).

448 (3) Apply test chemicals to each well at an interval of one to three minutes.

449 (4) Close the lid and incubate each well for 60 ±10 seconds in the cabinet at room temperature.

450 *Keep the lid of the assay plate closed at all times except when applying test chemicals. Leaving the
451 lid open could affect the quantity of the test chemical in the well due to air circulation in the
452 cabinet.

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 19 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

453 (5) Wait for 60 ±10 seconds after applying a test chemical,
454 then open the assay plate for LIQUID and pick up a
455 culture insert with sterile forceps.

456 (6) Discard the test chemical on the tissue by tilting the
457 insert and tapping it on a beaker. Fill the culture insert
458 to overflowing with PBS from a poly wash bottle.

459 *Apply a stream of PBS directly to the tissue surface to
460 wash away the test chemical. Maintain a uniformly
461 strong stream of PBS while washing the tissue.

462 **See Photo 3.**

463 *To avoid damaging the tissue with too forceful a
464 stream, use a wide-mouth nozzle on the poly wash
465 bottle.

466 (7) Tilt the insert to discard the PBS into the beaker.
467 Remove as much of the PBS inside the culture insert as
468 possible by tapping it on the beaker.

469 **See Photo 4.**

470 (8) Repeat steps 6 and 7 at least 10 times to remove as much as possible of the residual test chemical on
471 the tissue surface.

472 *Depending upon the physical properties of the test
473 chemical, bubbles might form in an insert during
474 washing. Continue washing until all bubbles
475 disappear.

476 (9) Using a sterile cotton bud, gently remove as much as
477 possible of the leftover PBS both inside and outside the
478 culture insert.

479 **See Photo 5.**

480 *Take care not to press down on the surface of the tissue

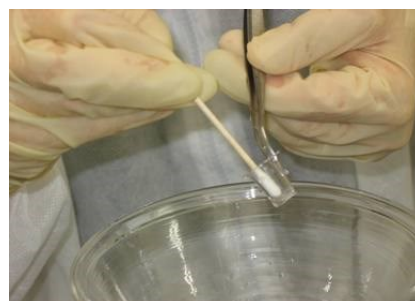
Photo 3 Rinse 1




Photo 4 Rinse 2



Photo 5 Rinse 3



Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 20 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

481 with the sterile cotton bud.

482 (10) Repeat steps 6 to 9 to remove any residual test chemical from the corneal epithelial tissue surface.

483 (11) Blot the culture insert and then place it in the well
484 of the same column in the 3rd row.

485 **See Fig. 5.**

486 *Take care to prevent air bubbles from forming
487 under the culture inserts.

488 (12) Repeat steps 1 to 11 for all culture inserts at one- to
489 three-minute intervals.

490 (13) Record the details of steps 1 to 12 above in **MDS 3-1**.

491

492 3.3.2.3 POST-EXPOSURE INCUBATION

493 (1) After performing **Section 3.3.2.2 “APPLICATION OF LIQUID TEST CHEMICALS AND**
494 **RINSING”**, close the lid of the assay plate for LIQUID and place it in a CO₂ incubator as soon as
495 possible.

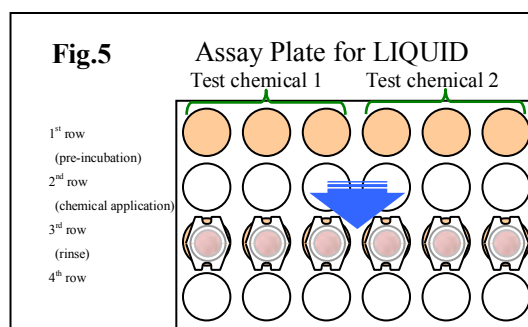
496 (2) Incubate for 24 ±1 hours.


497 (3) Record the details of steps 1 and 2 above in **MDS 3-1**.

498

499

500



Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 21 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

501 **3.3.3 APPLICATION OF SOLID TEST CHEMICALS AND RINSING (DAY 0–1)**

502

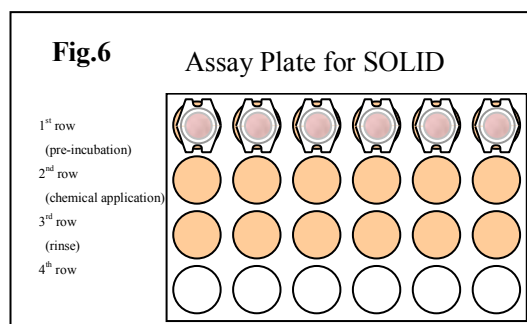
503 **3.3.3.1 PREPARATION OF WELLS FOR SOLID TEST CHEMICAL APPLICATION (2ND**
504 **ROW) AND FOR POST-INCUBATION (3RD ROW)**

505 (1) Warm the assay medium to 37°C for 30 minutes using a water bath.

506 (2) Remove the assay plate for SOLID from the CO₂
507 incubator.

508 (3) Open the lid of the assay plate for SOLID and use
509 a micropipette to fill the 12 wells in the 2nd and 3rd
510 rows with 0.5 mL/well of warm assay medium .

511 **See Fig. 6.**



512 (4) Close the lid of the assay plate and perform

513 **Section 3.3.3.2 “APPLICATION OF SOLID TEST CHEMICALS”** continuously.

514 (5) If the test chemicals are not applied immediately, store the Assay Plate for LIQUID in a CO₂
515 incubator until ready apply but for no more than 12 hours.

516 (6) Record the details of steps 1 to 5 above in **MDS 3-2**.

517

518 **3.3.3.2 APPLICATION OF SOLID TEST CHEMICALS**

519 (1) Remove the assay plate for SOLID from the CO₂
520 incubator.

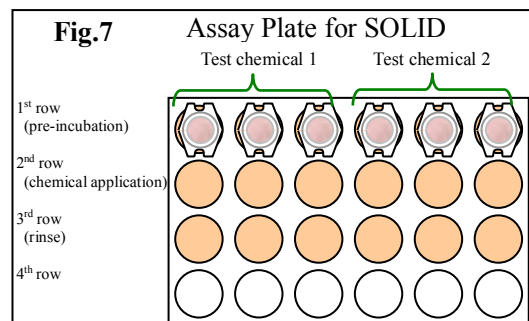
521 Using a precision balance, weigh out 10 ±2 mg of
522 the solid test chemicals. If necessary, crush and
523 grind the solid test chemicals in a mortar with
524 pestle. Apply the solid test chemical to the surface
525 of the corneal epithelial tissue.

526 **See Photo 6.**

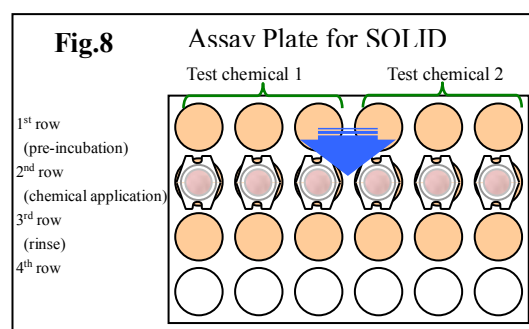


527 If necessary, use a micro spatula to spread the test chemical gently over the entire surface. Use three
528 wells per test chemical (N = 3).

529 See Fig. 7.
530 Each chemical is tested in three wells, using
531 three tissues (N = 3).
532 (2) Place the exposed culture insert in the well of
533 the same column in the 2nd row (chemical
534 application).



535 See Fig. 8.
536 *Take care to prevent air bubbles from forming
537 under the culture inserts.
538 (3) Close the lid of the assay plate for SOLID and
539 place it in a CO₂ incubator. Incubate for
540 24 ±1 hours.
541 (4) Record the details of steps 1 to 4 above in
542 **MDS 3-2**.



544 3.3.3.3 RINSING OF SOLID TEST CHEMICALS

545 (1) After incubation, remove the assay plate for SOLID from the CO₂ incubator.
546 (2) Open the assay plate for SOLID and pick up a culture insert with sterile forceps.
547 (3) Discard test chemicals on the tissue by tilting the insert and tapping it on a beaker. Fill the culture
548 insert to overflowing with PBS from a poly wash bottle.


549 *Apply a stream of PBS directly to the tissue surface to
550 wash away the test chemical. Maintain a uniformly
551 strong stream of PBS while washing the tissue.

552 See Photo 7.

553 *To avoid damaging the tissue with too forceful a
554 stream, use a wide-mouth nozzle on the poly wash
555 bottle.

556 (4) Tilt the insert to discard the PBS into the beaker.



Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 23 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

557 Remove as much of the PBS inside the culture insert as
558 possible by tapping it on the beaker.

559 **See Photo 8.**

560 (5) Repeat steps 3 and 4 at least 10 times to remove as
561 much as possible of the residual test chemical on the
562 tissue surface.

563 (6) Using a sterile cotton bud, gently remove as much as
564 possible of the leftover PBS both inside and outside
565 the culture insert.

566 **See Photo 9.**

567 (7) If it proves difficult to remove completely all the
568 residual test chemical from the corneal epithelial tissue
569 surface, remove as much as possible and continue to
570 step 8.

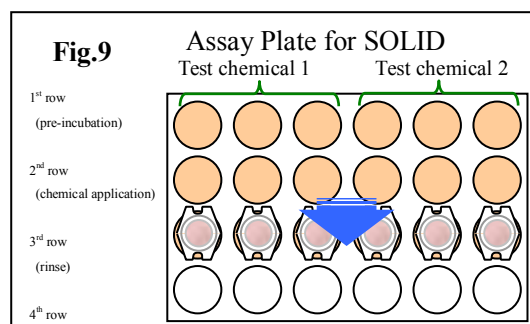
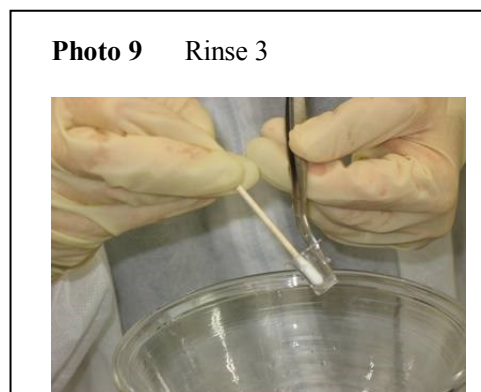
571 (8) Place the rinsed culture insert in the well of the same
572 column in the 3rd row (rinse).

573 **See Fig. 9.**

574 *Take care to prevent air bubbles from forming
575 under the culture inserts.

576 (9) Record the details of steps 1 to 8 above in **MDS**
577 **3-2.**

578 After rinsing step, perform **Section 3.3.4**
579 **“WST-8 assay”** continuously.



582 **3.3.4 WST-8 ASSAY (DAY 1)**

583

584 **3.3.4.1 PREPARATION OF WELLS FOR WST-8 ASSAY**

- 585 (1) Warm the EBSS to 37°C for 30 minutes using a water bath.
- 586 (2) Dilute the cell counting kit-8 (WST-8 stock solution) with EBSS (Cell Counting Kit-8:EBSS = 1:10),
- 587 and then warm the diluted WST-8 medium. Blanks for the WST-8 assay are prepared using the
- 588 diluted WST-8 medium as follows.

589 *Prepare immediately prior to performing the

590 WST-8 assay.

- 591 (3) Remove the assay plate for LIQUID from the CO₂
- 592 incubator or prepare the assay plate for SOLID.

- 593 (4) Open the lid of the assay plate and use a
- 594 micropipette to fill each well of the 4th row with
- 595 0.3 mL/well of the warm diluted WST-8 medium.

596 See Fig. 10.

597 Close the lid of the assay plate and perform **Section 3.3.4.2 “WST-8 REACTION”** continuously.

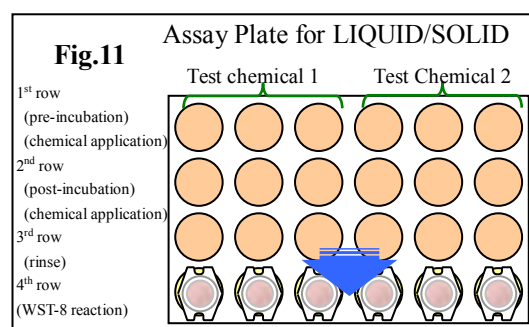
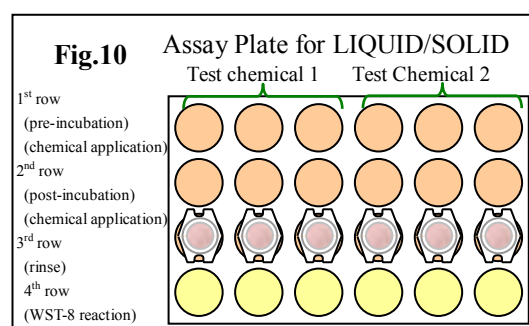
- 598 (5) Record the details of steps 1 to 4 above in **MDS 4-1**.

599

600 **3.3.4.2 WST-8 REACTION**

- 601 (1) Add about 20 mL of PBS each to two dishes: PBS dish 1 and PBS dish 2.
- 602 (2) Open an assay plate (for either LIQUID or SOLID) and pick up a culture insert with sterile forceps.
- 603 (3) Clean the bottom of the culture insert so it is free
- 604 of residual culture medium by washing it first in
- 605 PBS dish 1 and the in PBS dish 2. After washing,
- 606 wipe the bottom with a paper towel.
- 607 (4) After washing and wiping, place the culture insert
- 608 in the well of the same column in the 4th row.

609 See Fig. 11.



610 *Take care to prevent air bubbles from forming under the culture inserts.

611 (4) Close the lid of the assay plate and place it in the CO₂ incubator.

612 Incubate for 4 hours ± 20 minutes.

613 Record the details of steps 1 to 5 above in **MDS 4-1.**

614

615 **3.3.4.3 SAMPLING THE REACTED WST-8 MEDIUM**

616 (1) After incubation, remove the assay plate from the CO₂ incubator.

617 (2) Open the lid of the assay plate and remove the culture inserts from the 4th row with forceps.

618 Transfer 200 µL of the reacted WST-8 dilution medium into the wells of a 96-well plate.

619 *Figs. 12A and 12B show typical allocations on a 96-well plate for both living and correcting tissue.

Fig.12A Allocation on a 96-well plate for living tissue

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank											
B	NC for LIQUID-1	NC for LIQUID-2	NC for LIQUID-3	PC for LIQUID-1	PC for LIQUID-2	PC for LIQUID-3	NC for SOLID-1	NC for SOLID-2	NC for SOLID-3	PC for SOLID-1	PC for SOLID-2	PC for SOLID-3
C	LIQUID 1-1	LIQUID 1-2	LIQUID 1-3	LIQUID 2-1	LIQUID 2-2	LIQUID 2-3	SOLID 1-1	SOLID 1-2	SOLID 1-3	SOLID 2-1	SOLID 2-2	SOLID 2-3
D	LIQUID 3-1	LIQUID 3-2	LIQUID 3-3	LIQUID 4-1	LIQUID 4-2	LIQUID 4-3	SOLID 3-1	SOLID 3-2	SOLID 3-3	SOLID 4-1	SOLID 4-2	SOLID 4-3
E	LIQUID 5-1	LIQUID 5-2	LIQUID 5-3	LIQUID 6-1	LIQUID 6-2	LIQUID 6-3	SOLID 5-1	SOLID 5-2	SOLID 5-3	SOLID 6-1	SOLID 6-2	SOLID 6-3
F	LIQUID 7-1	LIQUID 7-2	LIQUID 7-3	LIQUID 8-1	LIQUID 8-2	LIQUID 8-3	SOLID 7-1	SOLID 7-2	SOLID 7-3	SOLID 8-1	SOLID 8-2	SOLID 8-3
G	LIQUID 9-1	LIQUID 9-2	LIQUID 9-3	LIQUID 10-1	LIQUID 10-2	LIQUID 10-3	SOLID 9-1	SOLID 9-2	SOLID 9-3	SOLID 10-1	SOLID 10-2	SOLID 10-3
H												

620

621

Fig.12B Allocation on a 96-well plate **for correcting tissue**

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank											
B	NC for LIQUID-1	NC for LIQUID-2	NC for LIQUID-3				NC for SOLID-1	NC for SOLID-2	NC for SOLID-3			
C	LIQUID 1-1	LIQUID 1-2	LIQUID 1-3	LIQUID 2-1	LIQUID 2-2	LIQUID 2-3	SOLID 1-1	SOLID 1-2	SOLID 1-3	SOLID 2-1	SOLID 2-2	SOLID 2-3
D	LIQUID 3-1	LIQUID 3-2	LIQUID 3-3	LIQUID 4-1	LIQUID 4-2	LIQUID 4-3	SOLID 3-1	SOLID 3-2	SOLID 3-3	SOLID 4-1	SOLID 4-2	SOLID 4-3
E	LIQUID 5-1	LIQUID 5-2	LIQUID 5-3	LIQUID 6-1	LIQUID 6-2	LIQUID 6-3	SOLID 5-1	SOLID 5-2	SOLID 5-3	SOLID 6-1	SOLID 6-2	SOLID 6-3
F	LIQUID 7-1	LIQUID 7-2	LIQUID 7-3	LIQUID 8-1	LIQUID 8-2	LIQUID 8-3	SOLID 7-1	SOLID 7-2	SOLID 7-3	SOLID 8-1	SOLID 8-2	SOLID 8-3
G	LIQUID 9-1	LIQUID 9-2	LIQUID 9-3	LIQUID 10-1	LIQUID 10-2	LIQUID 10-3	SOLID 9-1	SOLID 9-2	SOLID 9-3	SOLID 10-1	SOLID 10-2	SOLID 10-3
H												

Add reacted WST-8 medium to the same locations as living tissues (Fig.12A).

622

623 (3) Record the details of steps 1 to 2 above in **MDS 4-2**.

624

625 3.3.4.4 OPTICAL DENSITY MEASUREMENTS OF THE REACTION MEDIUM

626 (1) Using a 96-well plate reader, measure OD at 450 nm and 650 nm and then used the following
627 equation to determine a composite OD for each well.

628


629
$$\text{Composite OD} = (\text{OD}_{\text{TC}} \text{ at } 450 \text{ nm} - \text{OD}_{\text{blank}} \text{ at } 450 \text{ nm}) - (\text{OD}_{\text{TC}} \text{ at } 650 \text{ nm} - \text{OD}_{\text{blank}} \text{ at } 650 \text{ nm})$$

630 *If the plate reader can be programmed to perform this calculation automatically, then only the
631 composite OD value need be recorded.

632

633 (2) Calculate the Mean OD for the negative control, a cell viability for each individual tissue, and a
634 mean cell viability (including SD) for each test chemical using the following equations.

635

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 27 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

$$\text{Mean OD}_{\text{NC}} = \frac{\text{Sum of the OD}_{\text{NC}} \text{ for three replicate tissues}}{3}$$

636

$$\text{Tissue cell viability (\%)} = \frac{\text{Each tissue OD}_{\text{TC}}}{\text{Mean OD}_{\text{NC}}} \times 100$$

637


$$\text{Mean cell viability (\%)} = \frac{\text{Sum total of cell viability (\%)} \text{ for three replicate tissues}}{3}$$

638

639 (3) Record the details of steps 1 and 2 above in **MDS 4-2**.

640

641

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 28 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

642 **4. ASSESSMENT**

643

644 **4.1 CONDITIONS FOR A VALID TEST**

645 An eye irritation test is considered valid if all three of the following criteria have been met.

646

- 647 • Tissue viability: $0.5 \leq \text{mean OD (A450/650) measured value for negative control} \leq 1.6$
- 648 • Positive control: mean tissue viability for positive control $\leq 40\%$
- 649 • SD: SD (negative control, positive control and each test chemicals) of tissue viability of 3
650 identically treated replicates $\leq 18\%$

651

652 **4.2 ASSAY CRITERIA**

653 The criteria for in vitro prediction are shown below.

654 After exposure to a chemical, if cell viability is 40% or less, the chemical is predicted to be an
655 irritant (GHS Category 1 or 2), otherwise it is predicted to be a non-irritant (GHS No Category).

656 See Table 3.


657

658 **Table 3** Prediction model of LabCyte24 EIT

Tissue Viability	Prediction
$\leq 40\%$	Category 1 or 2
$> 40\%$	No Category

659

660


Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 29 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

661 **5. References**

662 Green H. (1978) Cyclic AMP in relation to proliferation of the epidermal cell: New view. Cell, 15,
663 801-811.

664 Rheinwald J.G. and Green H. (1975) Serial cultivation of strains of human epidermal keratinocytes:
665 The formation of keratinizing colonies from single cells. Cell, 6, 331-343.

666

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 30 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

667 **MDS 1-1:**
668 **RECEIPT OF LabCyte CORNEA-MODEL24 (2.1.2)**

669
670 Laboratory name: _____ Test name: _____ Test no. : _____

671
672 1. LabCyte CORNEA-MODEL24
673

674 Date received: _____
675

676 Lot no.: _____
677

678 Expiration date: _____
679

680
681 Accessories: Assay medium, 30mL (Lot no.: _____ Expiration date: _____)
682

683 24 well assay plate
684
685

<u>Note</u>

686
687
688 2. Assay medium
689

691 Date received: _____
692

693 Lot no.: _____
694

695 Expiration date: _____
696

<u>Note</u>

698
699
700 Date: _____ Operator: _____ Check date: _____ Study director: _____
701

702
703 Secretariat Check date: _____ Name: _____
704
705

707 **MDS 1-2:**
708 TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOINT
709 STEP-1 (3.2.1.1)

710 Laboratory name:_____ Test name: _____ Test no. : _____
711

- 712 1. Add distilled water (0.5 mL) to the wells of the 24-well assay plate.
713
714 To add distilled water (0.5 mL) Execution date/time: _____
715
716 2. Apply test chemicals to the wells of the 24-well assay plate.
717
718 3. Culture the 24-well assay plate in CO₂ incubator for 4 hours.
719
720 Time of WST-8 reaction started: _____
721
722 Time of WST-8 reaction completed: _____
723
724
725 4. Check the color of water.
726
727
728 5. Test chemical information and check list of coloring potential.
729

Test chemical	Physical state	Amount	Coloring	Test chemical.	Physical state	Amount	Coloring
PBS (NC)	LIQUID			Non treatment (NC)			
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	

730
731 Note

732 Date:_____ Operator:_____ Check date:_____ Study director:_____

733
734 Secretariat Check date:_____ Name:_____

735
736
737
738
739

740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771

MDS 1-3:
TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOINT
STEP-3 (3.2.2.1)

Laboratory name:_____ Test name: _____ Test no. : _____

1. Preparation of WST-8 dilution medium

Warm EBSS for 30 minutes. Time/date: _____
CCK-8: (Lot no.: _____ Expiration date: _____)
EBSS: (Lot no.: _____ Expiration date: _____)
Volume _____ mL Time/date completed: _____

2. Add WST-8 dilution medium (0.3mL) to the wells of the 24-well assay plate.

To add WST-8 dilution medium (0.3mL) Time/date executed: _____

3. Apply test chemicals to the wells of the 24-well assay plate.

4. Culture the 24-well assay plate in CO₂ incubator for 4 hours.

Time of WST-8 reaction started: _____
Time of WST-8 reaction completed: _____

5. Check the color of WST-8 medium.

6. Test chemical information and checked list of WST-8 assay interfere.

Test chemical	Physical state	Amount	WST-8 assay interfere	Test chemical.	Physical state	Amount	WST-8 assay interfere
PBS (NC)	LIQUID			Non treatment (NC)			
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	
	LIQUID	50 µL			SOLID	mg	


772
773

Note

774
775
776
777
778
779
780
781

Date:_____ Operator:_____ Check date:_____ Study director:_____

Secretariat Check date:_____ Name:_____

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 33 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

782 **MDS 1-4:**
783 **PREPARATION OF FREEZE KILLED TISSUE (3.2.2)**


784
785 Laboratory name: _____ Test name: _____ Test no. : _____

- 786
787 1. Transfer LabCyte CORNEA-MODEL24 tissues to 50 mL tube or appropriate sterile container.
788
789
790 2. Freeze tissues in the -80°C deep-freezer for 30 minutes (1st freezing).
791
792 Store for 30 minutes. Time/date: _____
793
794
795 3. Thaw tissues in the 37°C incubator for 15 minutes.
796
797 Store for 15 minutes. Time/date: _____
798
799
800 4. Freeze tissues in the -80°C deep-freezer for more than 30 minutes (2nd freezing).
801
802 Store for more 30minutes. Time/date: _____
803
804
805 5. Just before using, thaw tissues in the 37°C incubator for 15 minutes.
806
807 Store for 15 minutes. Time/date: _____
808
809
810
811
812
813
814
815

Note

816
817 Date: _____ Operator: _____ Check date: _____ Study director: _____
818
819

820 Secretariat Check date: _____ Name: _____
821
822
823

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 34 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850

MDS 2:
PREPARATION OF LabCyte CORNEA-MODEL24 (3.3.1)

Laboratory name: _____ Test name: _____ Test no. : _____

1. Warm up the assay medium and add 0.5mL of the assay medium to the wells of the 1st row on the 24-well assay plate.

Assay medium: (Lot no.: _____ Expiration date: _____)

Warm for 30 minutes. Time/date: _____
Add 0.5mL of assay medium to each well Time/date: _____ Number of plate: _____

2. Transfer culture inserts to wells in the 1st row on the 24-well assay plate.

LabCyte CORNEA-MODEL24(Lot no.: _____ Expiration date: _____)

Time/date executed: _____

Confirm that there are no bubbles under the cell culture insert.

3. LabCyte CORNEA-MODEL24 is cultured in CO₂ incubator overnight.

Time/date of culture started: _____

Note

851
852
853
854
855
856
857
858
859
860
861

Date: _____ Operator: _____ Check date: _____ Study director: _____

Secretariat Check date: _____ Name: _____

862 **MDS 3-1(LIQUID):**

863 **APPLICATION OF LIQUID TEST CHEMICALS, RINSING AND POST-INCUBATION (3.3.2)**

864 Laboratory name: _____ Test name: _____ Test no. : _____

- 865
866 1. Warm up the assay medium and add 0.5mL of the assay medium to the wells of the 3rd row on the
867 24-well assay plate for LIQUID.

868 Assay medium: (Lot no.: _____ Expiration date: _____)

869 Warm for 30 minutes. Time/date: _____

870 Add 0.5mL of assay medium to each well Time/date: _____ Number of plate: _____

- 871
872
873 2. Apply test chemicals to the LabCyte CORNEA-MODEL24.

874 Time/date execution started: _____

- 875
876 3. LIQUID test chemical information

Test chemical.	Lot no.	Physical state	Test chemical vol.	Time of application	Time of rinsing	Exposure period (1 minute)
PBS (Negative control)		LIQUID	50 µL	1	:	□
				2	:	□
				3	:	□
Ethanol (Positive control)		LIQUID	50 µL	1	:	□
				2	:	□
				3	:	□
		LIQUID, viscous	50 µL	1	:	□
		LIQUID, viscous	50 µL	2	:	□
		LIQUID, viscous	50 µL	3	:	□
		LIQUID, viscous	50 µL	1	:	□
		LIQUID, viscous	50 µL	2	:	□
		LIQUID, viscous	50 µL	3	:	□
		LIQUID, viscous	50 µL	1	:	□
		LIQUID, viscous	50 µL	2	:	□
		LIQUID, viscous	50 µL	3	:	□
		LIQUID, viscous	50 µL	1	:	□
		LIQUID, viscous	50 µL	2	:	□
		LIQUID, viscous	50 µL	3	:	□
		LIQUID, viscous	50 µL	1	:	□
		LIQUID, viscous	50 µL	2	:	□
		LIQUID, viscous	50 µL	3	:	□

- 879
880 4. After exposure to test chemical for 1 minute, wash out the LabCyte CORNEA-MODEL24 and
881 transfer the culture inserts to the 3rd row on the 24-well assay plate.

882 PBS: (Lot no.: _____ Expiration date: _____)


883 Hit PBS stream on the tissue surface directly.

884 Confirm that there are no bubbles under the cell culture insert. Time/date completed: _____

- 885
886 5. Culture LabCyte CORNEA-MODEL24 in CO₂ incubator for 24 hours.

887 Time/date post-incubation started: _____

888 Time/date post-incubation completed: _____

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 36 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.


892

<u>Note</u>

893
894
895
896
897
898
899
900
901
902
903
904
905

Date: _____ Operator: _____ Check date: _____ Study director: _____

Secretariat Check date: _____ Name: _____

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 37 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

MDS 3-2(SOLID):

APPLICATION OF SOLID TEST CHEMICALS AND RINSING (3.3.3)

Laboratory name: _____ Test name: _____ Test no. _____

1. Warm up the assay medium and add 0.5mL of the assay medium to the wells of 2nd and 3rd row on the 24-well assay plate for SOLID.

Assay medium: (Lot no.: _____ Expiration date: _____)

Warm for 30 minutes. Time/date: _____

Add 0.5mL of assay medium to each well Time/date: _____ Number of plate: _____

2. Apply test chemicals to the LabCyte CORNEA-MODEL24 and transfer the culture inserts to the 2nd row on the 24-well assay plate.

Time/date started: _____ Time/date completed: _____

3. SOLID test chemical information

Test chemical	Lot no.	Physical state	Crush and grind	Test chemical amount.	Time of application	Exposure period (24hours)
Non treatment (Negative control)					:	<input type="checkbox"/>
Lauric acid (Positive control)		SOLID	<input type="checkbox"/>	(mg, mg, mg)	:	<input type="checkbox"/>
		SOLID	<input type="checkbox"/>	(mg, mg, mg)	:	<input type="checkbox"/>
		SOLID	<input type="checkbox"/>	(mg, mg, mg)	:	<input type="checkbox"/>
		SOLID	<input type="checkbox"/>	(mg, mg, mg)	:	<input type="checkbox"/>
		SOLID	<input type="checkbox"/>	(mg, mg, mg)	:	<input type="checkbox"/>
		SOLID	<input type="checkbox"/>	(mg, mg, mg)	:	<input type="checkbox"/>
		SOLID	<input type="checkbox"/>	(mg, mg, mg)	:	<input type="checkbox"/>
		SOLID	<input type="checkbox"/>	(mg, mg, mg)	:	<input type="checkbox"/>
		SOLID	<input type="checkbox"/>	(mg, mg, mg)	:	<input type="checkbox"/>
		SOLID	<input type="checkbox"/>	(mg, mg, mg)	:	<input type="checkbox"/>

4. Culture LabCyte CORNEA-MODEL24 in CO₂ incubator for 24 hours.

Time/date exposure started: _____


Time/date exposure completed: _____

5. After exposure to test chemical, wash out the LabCyte CORNEA-MODEL24 and transfer the culture inserts to the 3rd row on the 24-well assay plate.

PBS: (Lot no.: _____ Expiration date: _____)

Hit PBS stream on the tissue surface directly.

Time/date started: _____ Time/date completed: _____


Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 38 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

Note

942
943
944
945
946
947

Date: _____ Operator: _____ Check date: _____ Study director: _____

Secretariat Check date: _____ Name: _____

Version 2.6	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Page 39 of 44
June, 2018		 Japan Tissue Engineering Co., Ltd.

948 **MDS 4-1:**
949 **WST-8 ASSAY (3.3.4.1, 3.3.4.2)**

950
951 Laboratory name: _____ Test name: _____ Test no. : _____

952 1. Preparation of WST-8 dilution medium

953 Warm EBSS for 30 minutes. Time/date: _____
954
955 CCK-8: (Lot no.: _____ Expiration date: _____)
956
957 EBSS: (Lot no.: _____ Expiration date: _____)
958
959 Volume _____ mL Time/date completed: _____

960 2. Add WST-8 dilution medium (0.3mL) to the wells in the 4th row on the 24-well assay plate.

961 To add WST-8 dilution medium (0.3mL) Time/date executed: _____
962

963 3. After the operation, the blotted tissue transfer to wells of 4th row of 24-well assay plate.

964 Time/date started: _____ Time/date completed: _____
965

966 Confirm that there are no bubbles under the cell culture insert.
967

968 4. Culture LabCyte CORNEA-MODEL24 in CO₂ incubator for 4 hours.

969 Time of WST-8 reaction started: _____
970
971 Time of WST-8 reaction completed: _____
972

973
974
975
976
977
978
979
980
981
982
983
984
985
986

Note

987
988
989
990 Date: _____ Operator: _____ Check date: _____ Study director: _____

991 Secretariat Check date: _____ Name: _____
992
993
994
995
996
997

998 **MDS 4-2:**
999 **SAMPLING OF REACTING WST-8 SOLUTION (3.3.4.3) AND MEASUREMENT (3.3.4.4)**
1000

1001 Laboratory name: _____ Test name: _____ Test no. : _____
1002

1003 1. Reacting WST-8 solution (200 µL) is transferred to each well on the 96-well plate.
1004

1005 Transfer to the 96-well plate.
1006

1007 Time/date executed: _____
1008
1009

1010 Sample location on 96-well plate.

	<u>LIQUID</u>						<u>SOLID</u>					
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank											
B	PBS-1	PBS-2	PBS-3	Ethanol-1	Ethanol-2	Ethanol-3	Non-treatment-1	Non-treatment-2	Non-treatment-3	Lauric acid-1	Lauric acid-2	Lauric acid-3
C												
D												
E												
F												
G												
H												

1011
1012
1013
1014 2. Analyze extract OD at 450nm and 650nm, and calculate the OD(450nm-650nm).
1015

1016 Analyze OD at 450nm and 650nm.

1017 Calculate the OD (450nm-650nm).

1018 Calculate cell viability and SD.

1019 Cell viability and SD are recorded on a separate data sheet.

1020 The data sheet is attached to the back of this sheet.


1021 Check for input errors.

1022 Time/date executed: _____
1023
1024

Note

1025
1026 Date: _____ Operator: _____ Check date: _____ Study director: _____
1027
1028


1029 Secretariat Check date: _____ Name: _____
1030

Version 2.6	<p style="text-align: center;"><i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24</p>	Page 41 of 44
June, 2018		 <p style="text-align: center;">Japan Tissue Engineering Co., Ltd.</p>


1031 **REVISION HISTORY**

1032


Rev.	CONTENT	Date Revised
Ver.1.1	1) First version	Aug., 2012
Ver.1.2	1) Revised clerical error.	Aug., 2012
Ver.1.3	1) Revised clerical error.	Sep., 2012
Ver.1.3PS	<p>Added the following supplementary explanation.</p> <p>1) Added more detail explanation about the conditions of WST-8 reaction.</p> <p>2) Added the formula of SD.</p>	Sep., 2012
Ver.2.1	<p>1) Revised clerical error.</p> <p>2) In the section 1.2 “BACKGROUND”, changed statement about animal testing (Draize test).</p> <p>3) In the section 3.3. “TEST METHOD”, explained the washing protocol of the <u>LIQUID</u> and <u>SOLID</u> test chemical more briefly.</p> <p>4) In the section 3.3. “TEST METHOD”, changed the WST-8 dilution rate with PBS from 1:10 to 1:5.</p> <p>5) In the section 3.3. “TEST METHOD”, changed the reaction period of WST-8 from 5 hours to 4 hours.</p> <p>6) In the section 3.3. “TEST METHOD”, changed the condition of WST-8 reaction from shaking to standing.</p> <p>7) In the section 3.3. “TEST METHOD”, changed the application-amount of <u>SOLID</u> chemicals from 50mg to 10mg.</p> <p>8) As assay acceptance criteria, added that SD (test chemicals) of tissue viability of 3 identically treated replicates ≤ 20 %.</p> <p>9) In the prediction model of this EIT, changed the cut-off value of the mean viability from 50% to 40%.</p>	May, 2013

Version 2.6	<p style="text-align: center;"><i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24</p>	Page 42 of 44
June, 2018		 <p style="text-align: center;">Japan Tissue Engineering Co., Ltd.</p>

Ver.2.2	<ol style="list-style-type: none"> 1) Revised clerical error. 2) In the section 3.3. "TEST METHOD", additionally explained that the solid test chemical is crush and grind in a mortar with pestle if necessary. 3) In the section 3.3.2 "APPLICATION OF LIQUID TEST CHEMICALS AND RINSING (DAY 0~1)", explained temperature condition (room temperature) at chemical application. 	Sep., 2013
Ver.2.3	<ol style="list-style-type: none"> 1) Revised clerical error. 2) In the section 3.3. "TEST METHOD", explained about a blank preparation at WST-8 assay. 3) In the section 3.3. "TEST METHOD", changed the condition of WST-8 reaction from standing to shaking. 5) In the section 3.3. "TEST METHOD", explained about a blank preparation at WST-8 assay. 	Feb., 2014
Ver.2.3.1	<ol style="list-style-type: none"> 1) Revised clerical error. 2) About the prediction result of eye irritation, changed the classification from irritation/no irritation to GHS classification. 	Mar., 2014
Ver.2.3.2MTT	<ol style="list-style-type: none"> 1) At the analysis of cell viability, changed the assay method from WST-8 assay to MTT assay. 	Jul., 2014
Ver.2.4.1	<ol style="list-style-type: none"> 1) At the analysis of cell viability, changed the assay method from MTT assay to WST-8 assay. 2) In the section 3.3. "TEST METHOD", changed the condition of WST-8 reaction from shaking to standing. 3) In the section 3.3. "TEST METHOD", changed the dilution solution of WST-8 reaction from PBS to EBSS. 4) In the section 3.3. "TEST METHOD", changed the reaction period of WST-8 from 5 hours to 4 hours. 5) The standard of the additional testing was mentioned about a 	Jan., 2015

Version 2.6	<p style="text-align: center;"><i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24</p>	Page 43 of 44
June, 2018		 <p style="text-align: center;">Japan Tissue Engineering Co., Ltd.</p>

	<p>borderline result.</p> <p>9) In the prediction model of this EIT, changed the cut-off value of the mean viability from 40% to 50%.</p>	
Ver.2.4.2	1) Changed the assay acceptance criterion of negative control from 0.5 < and <2.0 to 0.6 < and <1.5.	Mar., 2015
Ver.2.4.2m	1) The judgement of the chemical which is a result of the borderline was added.	Jul., 2015
Ver.2.5.1	<p>1) Revised clerical error.</p> <p>2) In the prediction model of this EIT, changed the cut-off value of the mean viability from 50% to 40%.</p> <p>3) Changed the judgement of equivocal results.</p> <p>4) Changed the assay acceptance criterion of negative control from 0.6 < and <1.5 to 0.5 < and <1.3.</p> <p>5) In the section 3.2. "TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOINT", revised description has clearly.</p> <p>6) Added MDS1-2 and MDS 1-3.</p>	Aug., 2015
Ver.2.5.1m	<p>1) Revised clerical error.</p> <p>2) Changed allocation for a 96-well plate of pattern which consists of freeze killed tissue (Fig.12B).</p>	Sep., 2015
Ver. 2.5.1mr	1) Revised Fig.2B	Sep.,2015
Ver. 2.5.2	<p>1) In the section 3.3. "TEST METHOD", explained the washing protocol of the <u>LIQUID</u> and <u>SOLID</u> test chemical more briefly.</p> <p>2) In the MDS 3-1 and the MDS 3-2, added the check box about the washing procedure.</p> <p>3) In the MDS 3-2, added the check box about crush and grind of</p>	Sep.,2015

Version 2.6	<p><i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24</p>	Page 44 of 44
June, 2018		 <p>Japan Tissue Engineering Co., Ltd.</p>

	test chemicals.	
Ver. 2.5.4	<p>1) In the section 3.3. "TEST METHOD", added attention point of the washing procedure.</p> <p>2) In the section 4.3 "ASSESSMENT FLOWCHART", Change numbers of test run from three independent run to single run.</p>	June, 2016
Ver. 2.5.5	<p>1) Revised clerical error.</p> <p>2) As assay acceptance criteria, changed that SD (negative control, positive control and test chemicals) of tissue viability of 3 identically treated replicates from $\leq 20\%$ to $\leq 18\%$.</p>	October, 2016
Ver.2.5.6	<p>1) Revised clerical error.</p> <p>2) Detection protocol of coloring interference is changed from using WST-8 medium to distilled water and correction of coloring interference is changed from using freeze-killed tissue to using living tissue without WST-8 reaction.</p>	February, 2017
Ver.2.6	<p>1) Changed the assay acceptance criterion of negative control from $0.5 \leq$ and ≤ 1.3 to $0.5 \leq$ and ≤ 1.6.</p>	June, 2018

1033

1034