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		Co., Ltd.

LabCyte CORNEA-MODEL24 EYE IRRITATION TEST OPERATION PROTOCOL Ver. 2.7

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83 1. RATIONALE AND BACKGROUND

84 1.1 The LabCyte CORNEA-MODEL24 EYE IRRITATION TEST

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

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- 92

1.2 BACKGROUND OF LabCyte24 EIT

93 Assessment of ocular irritation is an essential part of early testing procedures for the evaluation and hazard classification of substances. Therefore, it plays an important role in the safety evaluation of 94 95 general consumer products and materials. Novel substances used in consumer products must undergo comprehensive toxicological evaluation for eye irritation and a variety of other adverse outcomes. To 96 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 concerns related to animal welfare, because it is painful to the rabbits. Thus, alternative strategies and 100 101 tests are urgently required in order to evaluate the eve 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 studied for links to the biological role of tissue and gene regulation. Three-dimensional RhCE models are 103 104 useful as a multilayered, standardized tissue that mimics the human corneal epithelium.

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

110

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111 **1.3 BASIS OF THE TEST METHOD**

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

The relative viability of the tissue exposed to a test chemical is measured using the WST-8 assay immediately after exposure and again after a post-exposure period. A viability of 40% of the negative control value was used as the cutoff in identifying test chemicals as an irritant (GHS Category 1 or 2) or an non-irritant (GHS No Category). Some culture environments might allow the detection of very small quantities of cytokines secreted by the corneal epithelial tissue in response to topical application of test 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 activity of the cellular mitochondria) could interfere with the WST-8 endpoint. This is only a problem, 129 130 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 described in Section 3.2 "TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH 133 134 WST-8 ENDPOINT".

135

136**1.5BRIEF BASIC PROCEDURE**

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

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For liquid test chemicals, tissues are topically exposed for 1 minute. Preferably, three tissues are used for each test chemical as well as for the positive and negative controls. After exposure, tissues are thoroughly rinsed and blotted to remove the test chemical, then transferred to fresh medium and post-incubated for 24 hours. For solid test chemicals, tissues are exposed for 24 hours but are not 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 dilution with Earle balanced salt solution (EBSS). After a four-hour WST-8 incubation, the orange 146 147 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 chemicals that produce a relative cell viability below 40% of the negative control are predicted to be 150 151 irritants.

152

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 maintaining their phenotype (Rheinwald and Green, 1975; Green, 1978). Reconstruction of the human 157 158 cultured corneal epithelial tissue is achieved by cultivating and proliferating the corneal epithelial cells on an inert filter substrate with a surface area of 0.3 cm^2 at an air-liquid interface for 13 days using an 159 160 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.

164

165

166

1.5.1.1 QUALITY CONTROL OF THE TEST SYSTEMS

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

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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	$(0.1 \le IC_{50} \le 0.4).$
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	$0.5 \le$ Mean OD (A450/650) measured value ≤ 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 $\leq 40\%$

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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.

Standard Deviation (SD) of tissue viability of three identically treated replicates for the negative
 control, positive control, and test chemicals ≤ 18%

201

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

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

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

According to GHS classifications, a substance is an irritant (Category 1 or 2) if the mean relative

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

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

211

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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 <u>Table 2</u> 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)		
		0.5 cm).		
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

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

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

227

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229 2.1.3 INSTRUCTIONS FOR USE OF LabCyte CORNEA-MODEL24

Always incubate all of the culture inserts after opening the package. Do not store the culture inserts 230 231 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. • Assay Medium, 100 mL (J-TEC: 402250) 1 240 4 • Cell Counting Kit-8, 500 test (Dojindo: CK04) 241 24-well assay plate (Becton, Dickinson and Company: 353047) 242 • 7-8 243 • 96-well plate (Becton, Dickinson and Company: 353072 or equivalents) 1 PBS, 500 mL (Invitrogen: 14190-144 or equivalents) 2 or 3 bottle 244 • 245 Earle balanced salt solution (EBSS), 500 mL (SIGMA-ALDRICH: E3024) 1 bottle • Sterile cotton buds (JAPAN COTTON BUDS: 10A754D or equivalents) 246 • 1 box • Micro-pipette tips (sterile: 10~200µL, 200-1000µL) 247 248 • 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 (10-100µL) 255 256

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257	2.3 OTHERS
258	2.3.1 EQUIPMENT/INSTRUMENTS
259	• Safety cabinet (or clean bench)
260	• Water bath (37°C)
261	• CO ₂ incubator (37°C, 5% CO ₂ , capable of maintaining high humidity)
262	• Autoclave
263	• 96-well multi-plate reader (required filters: 450 nm, 650 nm)
264	• Precision balance (0.1 mg)
265	Aspirator
266	• Stop-watches
267	• Adjustable micro-pipette (10–200 μL, 200–1000 μL)
268	Sharp-edged forceps (sterile)
269	Micro spatula (sterile)
270	• Beaker (1–2 L: sterile)
271	• Sterilizable poly wash bottle (500–1000 mL: sterile) with wide mouth (mouth $>$ 3-mm dia.)
272	Mortar with pestle
273	
274	Also, it would be convenient to have the following.
275	• Positive-displacement-type pipette (10–100 μ L)
276	

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3. TEST METHOD

- *Procedures described in Sections 3.1.1 to 3.1.3 and Sections 3.3.1 to 3.3.3 are to be performed
 aseptically in a safety cabinet or clean bench. Procedures other than those mentions in the previous
 sentence need not be performed aseptically. Refer to Section 2.1.3 "INSTRUCTIONS FOR USE
- 281 OF LabCyte CORNEA-MODEL24".
- 282

3.1 PREPARATIONS

284 3.1.1 POSITIVE CONTROL

- 285 (1) Ethanol is used as a positive control for liquid test chemicals.
- 286 (2) Lauric acid is used as a positive control for solid test chemicals.
- 287

288 3.1.2 NEGATIVE CONTROL

- 289 (1) PBS is used as a negative control for liquid test chemicals.
- 290 (2) Non-treatment is a negative control for solid test chemicals.

291

292 3.1.3 POLY WASH BOTTLE FOR PBS

- 293 (1) Sterilize poly wash bottle by autoclave.
- 294 (2) Fill the sterilized poly wash bottle with sterile PBS.

295

296

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TEST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOINT

299 There are two kinds of test chemicals that interfere with the WST-8 assay. 300 (a) Test chemicals that stain corneal epithelial tissues directly. 301 (b) Test chemicals that react directly with WST-8. 302 The test chemicals that can dye the corneal epithelial tissues will be extracted from the colored tissue 303 during WST-8 reaction, affecting the OD measurement. Also, the test chemicals that can directly 304 reduce the WST-8 medium will affect the OD measurement due to unexpected reduction reaction by 305 residues of test chemicals in the culture inserts. These test chemicals are needed to perform additional experiments. The procedure is described below. 306 307 308 3.2.1 DETECTION OF THE CHEMICALS THAT STAIN THE TISSUE 309 **STEP 1 (PRELIMINARY TEST)** 310 3.2.1.1 311 (1) Add 50μ L (Liquid) or 10mg (Solid) of the test chemical into wells of 24-well assay plate preliminarily filled with 0.5mL of distilled water. Untreated distilled water is used as control. 312 313 (2) Close the lid of 24-well assay plate and incubate the mixture in CO_2 incubator for 15 minutes.

- 314 (3) After incubation, mix gently and evaluate the color change of the distilled water by visual check.
- (4) When the color of the solution changes significantly, the test chemical is presumed to have the
 potential to stain the tissue. A functional check on viable tissues (Step2) should be performed. If the
 color of the solution does not change significantly, the test chemical is determined not to have a
 potential to stain the tissue.
- 319 (5) Record the details of steps 1 to 4 above in **MDS 1-2**.
- 320

298

3.2

321 3.2.1.2 STEP 2 (FUNCTIONAL CHECK ON VIABLE TISSUE)

- 322 (1) Add 50 μL (liquid) or 10 mg (solid) of the test chemical, which could clearly change the color of the
 323 distilled water in step 1, onto the surface of the epidermis tissues. PBS are used as negative control.
- 324 (2) Go to the Section <u>3.3 EXECUTION OF THE TEST and perform the experiments according to the</u>
 325 procedures, expect for the WST-8 reaction. In the section of WST-8 assay, use the EBSS that does

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326		not contain WST stock solution, instead of diluted WST-8 medium to evaluate the affected OD
327		values by colored diluted water. (correcting tissue).
328	(3)	The corrected OD is calculated using the following formula.
329		Corrected OD = A - (B - C)
330		
331		A : the OD of viable tissue exposed to a test chemical.
332		B : the mean OD of correcting tissue exposed to a test chemical.
333		C : the mean OD of correcting tissue exposed to the negative control.
334		
335	(4)	If a corrected OD is below 0, the OD is considered to be 0.
336	(5)	When a cell viability that is calculated according to the procedures described in Section 3.3.4.4 is
337		\leq 40%, the test chemical is predicted to be an irritant (GHS Category 1 or 2) and there is no need
338		to calculate a corrected value.
339		
340		
341	3.2	2 DETECTION OF CHEMICALS THAT DIRECTLY REDUCE WST-8
342	3.2	.2.1 STEP 3 (PRELIMINARY TEST)
343	(1)	Dilute the cell counting kit-8 (WST-8 stock solution) with EBSS (Cell Counting Kit-8:EBSS = 1:10),
344		and then prepare the diluted WST-8 medium.
345		*Prepare WST-8 medium before use.
346		Dispense 0.3 mL of diluted WST-8 medium into each well of the 24-well assay plate.
347	(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.
348		The diluted WST-8 medium is used as control.
349	(3)	Put on the lid of 24-well assay plate and incubate in a CO ₂ incubator for about 4 hours.
350	(4)	After incubation, shake the mixture gently and evaluate the color change of the diluted WST-8
351		medium by visual check.

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	Ethanol	5% Triton X100	Glycolic acid	m-Phenylene diamine	Monoe	ethanolamine	Calcium thioglycollate
Ph	oto 1	Example of test	chemicals to inter	tere the WS	I-8 assa	ay directly	(STEP 1).
	When test cl	nemicals like m-Pl	henylene diamine, N	Ionoethanolar	nine, or	Calcium th	ioglycollate have
	colored the c	liluted WST-8 med	dium, Step 4 must be	e performed.		 .	
(5)	Significant o	coloring of the di	luted WSI-8 mediu	m by the test	chemic	al indicates	the interference
	between test	chemicals and the	e w S I-8 assay medi	um. The addit	ional fur	ictional che	ck is required for
(c)	these interference chemicals. Go to step 4 described below.						
(6)	Record the d	letans of steps 1 to	5 above in IVIDS 1	<u>-3</u> .			
3.2	2.2.2 STEP	4 (FUNCTIONA	L CHECK ON FR	EEZE-KILLE	ED TISS	UE)	
(1)	Add 50 uL o	of a liquid test che	mical or 10 mg of s	olid a test che	emical th	at clearly c	hanged the color
. ,	of the dilute	d WST-8 medium	(3.2.2.1. STEP 3)	to the surface	of the co	orneal epithe	elial tissues.
(2)	Go to <u>Sec</u>	tion 3.3 "EXE	CUTION OF TH	E TEST" ar	nd perfo	rm the ex	periments using
	freeze-killed	corneal epithelia	al tissues, instead	of using viab	ole corne	al epithelia	al tissues The
	freeze-killed	tissues are prepa	red by placing untro	eated Labcyte	CRNEA	-MODEL	in-80°C or lower
	for more tha	n 30 min.					
	Record the d	letails of freeze-ki	lling the tissue in <u>M</u>	DS 1-3.			
(3)	The correcte	d OD is calculated	l using the following	g formula.			
	Corrected ($\mathbf{D}\mathbf{D} = \mathbf{A} - (\mathbf{B} - \mathbf{C})$					
	A : the OD c	of viable tissue exp	bosed to a test chemi	cal.			
	B : the mean	OD of freeze-kill	ed tissue (correcting	; tissue) expos	ed to a te	est chemical	1.
	C : the mean	OD of freeze-kill	ed tissue (correcting	; tissue) expos	ed to the	negative co	ontrol.
(4)	If a corrected	d OD is below 0, t	he OD is considered	to be 0.			

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- 376 (5) Calculate a cell viability according to the procedures described in **Section 3.3.4.4.** In case that the
- 377 cell viability is $\leq 40\%$, the test chemical is predicted to be an irritant (GHS Category 1 or 2) and
- 378 there is no need to calculate a corrected value.

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381 3.3 EXECUTION OF THE TEST

382

383 3.3.1 PREPARATION OF LabCyte CORNEA-MODEL24 (DAY -1)

- 384 (1) Warm the assay medium to 37°C for 30 minutes
- in a water bath.
- 386 (2) Dispense 0.5 mL/well of the warm assay
 387 medium into the six wells of the 1st row of each
 388 assay plate for LIQUID/SOLID.
- 389 See Fig. 1.
- 390 (3) Open the LabCyte CORNEA-MODEL24391 aluminum package.



- 392 (4) Open the LabCyte CORNEA-MODEL24 plate lid and pick up the culture inserts using sterile393 forceps.
- *Do not touch the surface of the corneal epithelial tissue in the culture inserts.
- 395 *Use forceps to remove any agar medium396 sticking to the outside of the culture inserts.
- 397 (5) Transfer the culture inserts to the assay medium
 398 in the wells of the 1st row using sterile forceps.
 399 See Fig. 2.
- 400 (6) * Avoid the formation of air bubbles under the
 401 tissue inserts. Close the lid on the plate and
 402 place it in a CO₂ incubator.



- 403 (7) Incubate overnight (15–30 hours) until ready to perform Section 3.3.2 "APPLICATION OF
 404 LIQUID TEST CHEMICALS, RINSING AND POST-INCUBATION" or Section 3.3.3
 405 "APPLICATION OF SOLID TEST CHEMICALS AND RINSING".
- 406 (8) Record the details of steps 1 to 7 above in **MDS 2**.
- 407
- 408
- 409

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410	3.3.2	APPLICATION OF <u>LIQUID</u> TEST CHEMIC	ALS AND R	INSING (DAY 0~1)
411				
412	3.3.2.1	PREPARATION OF WELLS FOR POST-IN	CUBATION	(3 RD ROW)
413	(1) War	rm the assay medium to 37°C for 30 minutes		
414	usin	g a water bath.	Fig. 3	Assay Plate for LIQUID
415	(2) Take	e out the assay plate for <u>LIQUID</u> from the	l st row (pre-incubation)	0
416	CO_2	incubator.	2 nd row	
417	(3) Ope	in the lid of the assay plate for LIQUID, and	3 rd row (post-incubation)	ŎŎŎŎŎŎ
418	use	a micropipette to fill the six wells of the 3 rd	4 th row	
419	row	with 0.5 mL/well of the warm assay medium.		
420	See	Fig. 3.		
421	(4) Clos	se the lid of the assay plate and perform Section	3.3.2.2 "AP	PLICATION OF LIQUID TEST
422	CHI	EMICALS AND RINSING" continuously.		
423	(5) If th	ne test chemicals are not applied immediately,	store the Ass	say Plate for LIQUID in a CO_2
424	incu	bator until ready apply but for no more than 12 ho	ours.	
425	(6) Reco	ord the details of steps 1 to 5 above in MDS 3-1 .		
426				
427				

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428 3.3.2.2 APPLICATION OF <u>LIQUID</u> TEST CHEMICALS AND RINSING

429 (1) Take out the assay plate for <u>LIQUID</u> from the CO_2 incubator.

Using a micropipette, apply 50 μ L of a liquid test 430 (2)chemical to the surface of the corneal epithelial 431 tissues in the 1st row of the assay plate. Each test 432 chemical is to be tested in three wells (N=3). 433 Carefully apply the test chemical to the central 434 part of each corneal epithelial tissue. After 435 application, close the lid of the assay plate and tap 436 the sides of the plate to spread the liquid test 437 chemicals to spread out over the entire corneal 438



- epithelial surface. If necessary, use a micro spatula to spread the liquid test chemical over the entiresurface. Take care not to press down on the surface of the corneal epithelial with the spatula.
- 441 *For viscous <u>LIQUID</u> test chemicals, use a wide orifice cell saver tip (See Photo 2.) or positive
 442 displacement type pipette.
- *Use a pipette or other equipment to familiarize
 yourself beforehand with the characteristics
 the test chemicals.
- 446 *Assay no more than two test chemicals on one447 24-well assay plate.
- 448 See Fig. 4.

Each chemical is tested in three wells, using

Fig. 4 Assay Plate for LIQUID Test chemical 1 Test chamical 2

450 three tissues (N = 3).

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

- 452 (4) Close the lid and incubate each well for 60 ± 10 seconds in the cabinet at room temperature.
- *Keep the lid of the assay plate closed at all times except when applying test chemicals. Leaving the
 lid open could affect the quantity of the test chemical in the well due to air circulation in the
 cabinet.

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456	(5)	Wait for 60 ± 10 seconds after applying a test chemical,		
457		then open the assay plate for $\underline{\text{LIQUID}}$ and pick up a	Photo 3	Rinse I
458		culture insert with sterile forceps.		
459	(6)	Discard the test chemical on the tissue by decantation		
460		and tapping it on a beaker. Fill the culture insert to		
461		overflowing with PBS from a poly wash bottle.		NT?
462		* Keep PBS flush during rinsing to wash away the test		
463		chemical from the tissue surface	AV.	
464		See Photo 3.	Photo 4	Rinse 2
465		*To avoid damaging the tissue with too forceful a		
466		stream, use a wide-mouth nozzle on the poly wash	9	
467		bottle.		
468	(7)	Decant the PBS into the beaker. Remove as much of the		0
469		PBS inside the culture insert as possible by tapping it on	20	
470		the beaker.		
471		See Photo 4.		
472	(8)	Repeat steps 6 and 7 at least 10 times to remove as much	as possible of	the residual test chemical or
473		the tissue surface.		
474		*Depending upon the physical properties of the test		
475		chemical, bubbles might form in an insert during	Photo 5 F	Rinse 3
476		washing. Continue washing until all bubbles		
477		disappear.	Carl	
478	(9)	Using a sterile cotton bud, gently remove as much as		
479		possible of the leftover PBS both inside and outside the	2	
480		culture insert.	INA	1
481		See Photo 5.		
482		*Take care not to press down on the surface of the tissue		
483		with the sterile cotton bud.		

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484		
485	(10) Wipe the culture insert and then place it in the well	
486	of the same column in the 3rd row.	Fig.5 Assay Plate for LIQUID Test chemical 1 Test chemical 2
487	See Fig. 5.	
488	*Take care to prevent air bubbles from forming	(pre-incubation) 2 rd row (observed a solution)
489	under the culture inserts.	$\begin{array}{c} (\text{chemical approximation}) \\ 3^{d'} \text{row} \\ (\text{ringe}) \end{array} \qquad $
490	(11) Repeat steps 1 to 11 for all culture inserts at one- to	
491	three-minute intervals.	
492	(12) Record the details of steps 1 to 12 above in MDS 3-1 .	
493		
494	3.3.2.3 POST-EXPOSURE INCUBATION	
495	(1) After performing Section 3.3.2.2 "APPLICATION	OF LIQUID TEST CHEMICALS AND
496	<u>RINSING</u> , close the lid of the assay plate for <u>LIQUII</u>	<u>D</u> and place it in a CO_2 incubator as soon as
497	possible.	
498	(2) Incubate for 24 ± 1 hours.	
499	(3) Record the details of steps 1 and 2 above in MDS 3-1 .	
500		
501		
502		

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503 3.3.3 APPLICATION OF SOLID TEST CHEMICALS AND RINSING (DAY 0-1) 504 (2ND PREPARATION OF WELLS FOR SOLID TEST CHEMICAL APPLICATION 505 3.3.3.1 ROW) AND FOR POST-INCUBATION (3RD ROW) 506 507 (1) Warm the assay medium to 37°C for 30 minutes using a water bath. 508 (2) Take out the assay plate for SOLID from the CO_2 Fig.6 Assay Plate for SOLID 509 incubator. 1st row 510 (3) Open the lid of the assay plate for SOLID and use (pre-incubation 2nd row a micropipette to fill the 12 wells in the 2^{nd} and 3^{rd} 511 (chemical app 3rd row rows with 0.5 mL/well of warm assay medium . (rinse) 512 4th row 513 See Fig. 6. (4) Close the lid of the assay plate and go to **Section** 514 3.3.3.2 "APPLICATION OF SOLID TEST CHEMICALS" continuously. 515 516 (5) If the test chemicals are not applied immediately, store the Assay Plate for LIQUID in a CO2 517 incubator until ready apply but for no more than 12 hours. 518 (6) Record the details of steps 1 to 5 above in **MDS 3-2**. 519 3.3.3.2 APPLICATION OF SOLID TEST CHEMICALS 520 521 (1) Take out the assay plate for SOLID from the CO_2 Photo 6 Application of SOLID 522 incubator. Using a precision balance, weigh out 10 ± 2 mg of 523 524 the solid test chemicals. If necessary, crush and grind the solid test chemicals in a mortar with 525 pestle. Apply the solid test chemical to the surface 526 527 of the corneal epithelial tissue. See Photo 6. 528 If necessary, use a micro spatula to spread the test chemical gently over the entire surface. Use three 529 530 wells per test chemical (N = 3).

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Assay Plate for SOLID Fig.7 531 See Fig. 7. Test chemical 1 Test chemical 2 532 1st row (pre-incubation) (2) Place the exposed culture insert in the well of 533 2nd row (chemical application the same column in the 2nd row (chemical 534 3rd row (rinse) 535 application). 4th row 536 See Fig. 8. 537 *Take care to prevent air bubbles from forming Assav Plate for SOLID Fig.8 under the culture inserts. 538 Test chemical 1 Test chemical 2 539 (3) Close the lid of the assay plate for SOLID and lst row (pre-incubation) 540 place it in a CO₂ incubator. Incubate for 2nd row (chemical application 24 ± 1 hours. 541 3rd row (rinse) (4) Record the details of steps 1 to 3above in 542 4th row 543 MDS 3-2. 544 **RINSING OF SOLID TEST CHEMICALS** 545 3.3.3.3 546 (1) After incubation, take out the assay plate for SOLID from the CO_2 incubator. 547 (2) Open the assay plate for SOLID and pick up a culture insert with sterile forceps. (3) Discard test chemicals on the tissue by tilting the insert and tapping if on a beaker. Fill the culture 548 insert to overflowing with PBS from a poly wash bottle. 549 550 * Keep PBS flush during rinsing to wash away the test Photo 7 Rinse 1 chemical from the tissue surface. 551 552 See Photo 7. *To avoid damaging the tissue with too forceful a 553 554 stream, use a wide-mouth nozzle on the poly wash 555 bottle. 556 (4) Tilt the insert to discard the PBS into the beaker. Remove as much of the PBS inside the culture insert as 557 558 possible by tapping it on the beaker.

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559		See Photo 8.		Photo	8	Rinse 2
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.		Y		
563	(6)	Using a sterile cotton bud, gently remove as much	n as			
564		possible of the leftover PBS both inside and outs	side	21		
565		the culture insert.		-		
566		See Photo 9.		Photo	9	Rinse 3
567	(7)	If it proves difficult to remove completely all	the	1 martin		States
568		residual test chemical from the corneal epithelial tis	sue	-		
569		surface, remove as much as possible and continue	e to			
570		step 8.				- da
571	(8)	Place the rinsed culture insert in the well of the sa	ime	1	(
572		column in the 3rd row (rinse).			11	
573		See Fig. 9.				
574		*Take care to prevent air bubbles from forming	Fi	ig.9	Assa 'est ch	y Plate for SOLID micial 1 Test chemical 2
575		under the culture inserts.	l st row (pre-inc	cubation)		
576	(9)	Record the details of steps 1 to 8 above in MDS	2 nd row (chemic	cal application)		
577		<u>3-2</u> .	3 rd row (rinse)			
578		After rinsing step, perform Section 3.3.4	4 th row			
579		"WST-8 assay" continuously.				
580						
581						

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582 3.3.4 WST-8 ASSAY (DAY 1)

583

584**3.3.4.1PREPARATION OFWELLS 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),
- and then warm the diluted WST-8 medium. Prepare the additional dilute WST-8 medium for blanksin the WST-8 assay.
- 589 *Prepare WST-8 medium before performing the590 WST-8 assay.
- 591 (3) Take out the assay plate for <u>LIQUID</u> from the CO₂
 592 incubator or prepare the assay plate for <u>SOLID</u>.
- 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 <u>LIQUID or SOLID</u>) and pick up a culture insert with sterile forceps.

603 (3) Remove residual culture medium on a cluture

insert by washing in PBS dish 1(first time) and the

- 605 in PBS dish 2 (second time). After washing, wipe606 the bottom with a paper towel.
- 607 (4) After washing and wiping, place the culture insert608 in the well of the same column in the 4th row.
- 609 See Fig. 11.



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- 610 * Avoid the formation of air bubbles under the culture inserts.
- 611 (4) Close the lid of the assay plate and place it in the CO_2 incubator.
- 612 Incubate for 4 hours \pm 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, take out the assay plate from the CO_2 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

620 (freeze-killed tissue or tissue reacted with only EBSS that does not contain WST-8 stock solution).

Fig.12A Allocation on a 96-well plate for living tissue												
	1	2	3	4	5	6	7	8	9	10	11	12
А	Blank											
В	NC for	NC for	NC for	PC for	PC for	PC for	NC for	NC for	NC for	PC for	PC for	PC for
	LIQUID-1	LIQUID-2	LIQUID-3	LIQUID-1	LIQUID-2	LIQUID-3	SOLID-1	SOLID-2	SOLID-3	SOLID-1	SOLID-2	SOLID-3
С	LIQUID	LIQUID	LIQUID	LIQUID	LIQUID	LIQUID	SOLID	SOLID	SOLID	SOLID	SOLID	SOLID
	1-1	1-2	1-3	2-1	2-2	2-3	1-1	1-2	1-3	2-1	2-2	2-3
D	LIQUID	LIQUID	LIQUID	LIQUID	LIQUID	LIQUID	SOLID	SOLID	SOLID	SOLID	SOLID	SOLID
	3-1	3-2	3-3	4-1	4-2	4-3	3-1	3-2	3-3	4-1	4-2	4-3
Е	LIQUID	LIQUID	LIQUID	LIQUID	LIQUID	LIQUID	SOLID	SOLID	SOLID	SOLID	SOLID	SOLID
	5-1	5-2	5-3	6-1	6-2	6-3	5-1	5-2	5-3	6-1	6-2	6-3
F	LIQUID	LIQUID	LIQUID	LIQUID	LIQUID	LIQUID	SOLID	SOLID	SOLID	SOLID	SOLID	SOLID
	7-1	7-2	7-3	8-1	8-2	8-3	7-1	7-2	7-3	8-1	8-2	8-3
G	LIQUID	LIQUID	LIQUID	LIQUID	LIQUID	LIQUID	SOLID	SOLID	SOLID	SOLID	SOLID	SOLID
	9-1	9-2	9-3	10-1	10-2	10-3	9-1	9-2	9-3	10-1	10-2	10-3
Н												
		•	•	•	•							

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Fig.1	Fig.12B Allocation on a 96-well plate for correcting tissue.											
	1	2	3	4	5	6	7	8	9	10	11	12
А	Blank											
В	NC for LIQUID-1	NC for LIQUID-2	NC for LIQUID-3				NC for SOLID-1	NC for SOLID-2	NC for SOLID-3			
С	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
Е	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
Н												
Add	reacted	WST-8	mediur	n to the	same l	ocations	s as livi	ng tissu	es (Fig.	12A).		

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

625

626 **3.3.4.4 OPTICAL DENSITY MEASUREMENTS OF THE REACTION MEDIUM**

- (1) Using a 96-well plate reader, measure OD at 450 nm and 650 nm and then used the following
 equation to determine a composite OD for each well.
- 629

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

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

- 633
- (2) Calculate the mean OD for the negative control, a cell viability for each individual tissue, and a
 mean cell viability (including SD) for each test chemical using the following equations.
- 636
- 27

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		Mean OD _{NC} = $\frac{\text{Sum of}}{1}$	the OD _{NC} for three repl	icate tissues
			3	
637			Fach ticque OD	
	Т	issue cell viability (%) $=$		x 100
638				
		Sum tota	l of cell viability (%) for	three replicate tissues
	Mean cell vi	ability (%) =	3	
639				
640	(0) D 1 1	letails of steps 1 and 2 above i	1 MDS 4-2.	
040	(3) Record the c	letuns of steps 1 and 2 above h		
640 641	(3) Record the c			

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643 **4. ASSESSMENT**

644

6454.1CONDITIONS FOR A VALID TEST

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

647

646

- Tissue viability: $0.5 \le \text{mean OD}$ (A450/650) measured value for negative control ≤ 1.6
- Positive control: mean tissue viability for positive control $\leq 40\%$
- SD: SD (negative control, positive control and each test chemicals) of tissue viability of 3
 identically treated replicates ≤ 18 %
- 652

653 4.2 ASSAY CRITERIA

- 654 The criteria for in vitro prediction are shown below.
- After exposure to a chemical, if cell viability is 40% or less, the chemical is predicted to be an
- 656 irritant (GHS Category 1 or 2), otherwise it is predicted to be a non-irritant (GHS No Category).
- 657 See Table 3.
- 658

659 <u>Table 3</u> Prediction model of LabCyte24 EIT

Tissue Viability	Prediction
≤ 40%	Category 1 or 2
> 40%	No Category

660

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5. References

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667	

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aboratory name:	Т	est name:	Test no. :
. LabCyte CORNE	A-MODEL24		
Date received:			
Lot no.:			
Expiration date:			
Accessories: Assay a 24 well	medium, 30mL □ (assay plate □	Lot no.:	Expiration date:
Note			
. Assay medium			
. Assay medium Date received:			
. Assay medium Date received: Lot no.: Expiration date:			
. Assay medium Date received: Lot no.: Expiration date: <u>Note</u>			
. Assay medium Date received: Lot no.: Expiration date: <u>Note</u>			
Assay medium Date received:	Operator:	Check date:	Study director:

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TE: ST	ST FOR DETECTING CHEMICALS THAT INTERFERE WITH WST-8 ENDPOIN [®] EP-1 (3.2.1.1)
Lat	poratory name: Test name: Test no. :
1.	Add distilled water (0.5 mL) to the wells of the 24-well assay plate.
Т	o add distilled water (0.5 mL) Execution date/time:
2.	Apply test chemicals to the wells of the 24-well assay plate.
3.	Culture the 24-well assay plate in CO ₂ incubator for 4 hours.
Т	ime of WST-8 reaction started:
Т	ime of WST-8 reaction completed:
4.	Check the color of water.
5.	Test chemical information and check list of coloring potential.

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	

____ Operator:_____ Check date:_____ Study director:___ Date: Secretariat Check date: _ Name:_

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Note

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0.4.1	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE	J-TEC
October, 2018	MODEL: LabCyte CORNEA-MODEL24	Japan Tissue Engineering Co., Ltd.

741 742 743	MDS TES STE	5 1-3: T FOR DETECTING C P-3 (3.2.2.1)	HEMICALS TH	IAT INTERF	ERE WITH WST-8 END	POINT
744 745	Labo	pratory name:_	Test name:		Test no. :	
743 746	1.	Preparation of WST-8	dilution medium			
748	Wa	arm EBSS for 30 minutes.		Time/date:		
750 751 752 753	CC EB Vol	CK-8: (Lot no.: SS: (Lot no.: lumeL			Expiration date: Expiration date: Time/date completed:))
754 755	2.	Add WST-8 dilution me	edium (0.3mL) to	o the wells of	the 24-well assay plate.	
756 757 758	То	add WST-8 dilution mediu	m (0.3mL) □	Time/o	date executed:	
759 760 761	3.	Apply test chemicals to	o the wells of the	e 24-well ass	ay plate.	
762 763	4.	Culture the 24-well as	say plate in CO ₂	incubator fo	r 4 hours.	
764	Tin	ne of WST-8 reaction start	ed:			
766 767 768	Tin	ne of WST-8 reaction com	pleted:			
769 770 771	5.	Check the color of WS	T-8 medium.			
772	6.	Test chemical informat	ion and checked	d list of WST	-8 assay interfere.	

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

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	
	LIQUID	50 µL			SOLID	mg	

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Note			
Date:	Operator:	Check date:	_ Study director:
Secretariat	Check date:	Name:	

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October, 2018	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Japan Tissue Engineering Co., Ltd.
MDS 1-4:		

Laboratory name:	Test name:	Test no :
1. Transfer LabCyte CORNI	EA-MODEL24 tissues to 50 m	L tube or appropriate sterile con
2. Freeze tissues in the -80°	C deep-freezer for 30 minutes (1	l st freezing).
Store for 30 minutes.	□ Time/date:	
3. Thaw tissues in the 37°C i	ncubator for 15 minutes.	
Store for 15 minutes.	Time/date:	
4. Freeze tissues in the -80°	C deep-freezer for more than 30	minutes (2 nd freezing).
Store for more 30minutes.	□ Time/date:	
5. Just before using, thaw tis	sues in the 37°C incubator for 1	5 minutes.
Store for 15 minutes.	Time/date:	
Noto		
Note		
Date: Operator:	Check date:	Study director:

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October, 2018	IN VITRO EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE	J-TEC
	MODEL: LabCyle CORNEA-MODEL24	Co., Ltd.

Laborat	ory name:	Te	est name:		Test no. :
l. Wai 24-\	m up the assay me vell assay plate.	dium and add	0.5mL of the a	ssay medium t	to the wells of the 1st ro
Assay m	edium: (Lot no.:			Expiration da	te:
Warm fo Add 0.5i	r 30 minutes. nL of assay medium	□ to each well □	Time/date: Time/date:		Number of plate:
2. Trar	nsfer culture inserts	to wells in the	e 1st row on the	e 24-well assa	y plate.
LabCyte	CORNEA-MODEL2	4(Lot no.:		Expiration da	te:
	Time/date execu	ted:			
Confirm	that there are no bub	bles under the o	cell culture inser	t.	
3. Lab	Cyte CORNEA-MC	DEL24 is cult	ured in CO ₂ inc	cubator overnig	ght.
T '					
i ime/da	te of culture started:				
<u>Note</u>					
Date:	Opera	tor:	Check da	ite:	Study director:

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October, 2018	IN VITRO EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Japan Tissue Engineering Co., Ltd.

863 MDS 3-1(LIQUID):

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864 APPLICATION OF LIQUID TEST CHEMICA	LS, RINSING AND POST-INCUBATION (3.3.2)
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Laboratory name: _____ Test name: _____ Test no. : _____ 865 866

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

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.

Time/date execution started:

879 LIQUID test chemical information 3.

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 2 3	:		
		LIQUID, viscous	50 µL	1 2 3	:		
		LIQUID, viscous	50 µL	1 2 3	:	:	
		LIQUID, viscous	50 µL	1 2 3	:	:	
		LIQUID, viscous	50 µL	1 2 3	-	:	
		LIQUID, viscous	50 µL	1 2 3			
		LIQUID, viscous	50 µL	1 2 3			
		LIQUID, viscous	50 µL	1 2 3			
		LIQUID, viscous	50 µL	1 2 3			
		LIQUID, viscous	50 µL	1 2 3			

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4. After exposure to test chemical for 1 minute, wash out the LabCyte CORNEA-MODEL24 and transfer the culture inserts to the 3rd row on the 24-well assay plate. (Lot no.: PBS:

Expiration date:)	
	/	

Hit PBS stream on the tissue surface directly.												
_								 		 		

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

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

Time/date post-incubation started:

Time/date post-incubation completed:

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	IN VITRO EYE IRRITATION TEST USING	I LTEC
October, 2018	HUMAN CORNEAL TISSUE	JIEC
	MODEL: LabCyte CORNEA-MODEL24	Japan Tissue Engineering
		Co., Ltd.

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902 203	Secretariat	Check date:	Name:		
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October, 2018	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Japan Tissue Engineering Co., Ltd.
MDS 3-2(<u>SOLII</u> APPLICATION	D): OF <u>SOLID</u> TEST CHEMICALS AND RINSING (3.3.3	
Laboratory name:	Test name: Test no	
1. Warm up the the 24-well a	assay medium and add 0.5mL of the assay medium to ssay plate for <u>SOLID</u> .	the wells of 2 nd and 3 rd row on
Assay medium:	(Lot no.: Expiration date:)

> Warm for 30 minutes. Time/date:

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

2. Apply test chemicals to the LabCyte CORNEA-MODEL24 and transfer the culture inserts to the 2nd row on the 24-well assay plate. <u>920</u> 921

Time/date completed:_____ 922 923 Time/date started:

924 3. SOLID test chemical information

Test chemical	Lot no.	Physical state	Crush and grind		Test che	mical amo	ount.	Time of application	Exposure period (24hours)
Non treatment (Negative control)								:	
Lauric acid (Positive control)		SOLID		(mg,	mg,	mg)	:	
		SOLID		(mg,	mg,	mg)	:	
		SOLID		(mg,	mg,	mg)	:	
		SOLID		(mg,	mg,	mg)	:	
		SOLID		(mg,	mg,	mg)	:	
		SOLID		(mg,	mg,	mg)	:	
		SOLID		(mg,	mg,	mg)	:	
		SOLID		(mg,	mg,	mg)	:	
		SOLID		(mg,	mg,	mg)	:	
		SOLID		(mg,	mg,	mg)	:	
		SOLID		(mg,	mg,	mg)	:	

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

Time/date exposure started: Time/date exposure completed:

After exposure to test chemical, wash out the LabCyte CORNEA-MODEL24 and transfer the 5. 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:

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October, 2018	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Japan Tissue Engineering Co., Ltd.

<u>Note</u>			
Date:	Operator:	_ Check date:	_Study director:
Secretariat	Check date:	_ Name:	

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October, 2018	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE MODEL: LabCyte CORNEA-MODEL24	Japan Tissue Engineering Co., Ltd.

·		163(110
1. Preparation of VVS1-8		
Warm EBSS for 30 minutes.	□ Time/date:	
CCK-8: (Lot no.: EBSS: (Lot no.:	<u></u>	Expiration date:
Volume <u>mL</u>		Time/date completed:
2. Add WST-8 dilution m	edium (0.3mL) to the wells in	the 4th row on the 24-well assay plate
To add WST-8 dilution mediu	ım (0.3mL) □ Time/c	date executed:
3. After the operation, the	e blotted tissue transfer to we	ells of 4th row of 24-well assay plate.
Time/date started:	Time/da	ate completed:
Confirm that there are no	hubbles under the cell culture	a insort
Commit that there are no		e insert.
4. Culture LabCyte COR	NEA-MODEL24 in CO2 incub	pator for 4 hours.
Time of WST-8 reaction star	ed:	
Time of W/ST 8 reaction com	nlotod:	
	pieteu.	
Note		
Date: Oper	ator: Check da	ate:Study director:

MDS 4-2: SAMPLING OF REACTING WST-8 SOLUTION (3.3.4.3) AND MEASUREMENT (3.3.4.4) aboratory name:	Ve	rsion 2.7	7	<i>IN VIT</i> HU MC	RO EY MAN C DEL: L	E IRRI ORNEA abCyte C	TATION L TISSU CORNEA	TEST E -MODE	USIN EL24	NG J	apan Tis	•TE ssue Eng Ltd.	C gineerin
aboratory name: Test name: Test no. : A Reacting WST-8 solution (200 µL) is transferred to each well on the 96-well plate. Transfer to the 96-well plate. Time/date executed: Sample location on 96-well plate.	MD Sa) s 4-2: Mplin(<u>G OF RI</u>	EACTIN	<u>G WST</u>	-8 SOLI	JTION (<u>3.3.4.3)</u>	AND M	IEASUR		Г (3.3.4.	4)
1. Reacting WST-8 solution (200 µL) is transferred to each well on the 96-well plate. Transfer to the 96-well plate. Time/date executed: Sample location on 96-well plate. LQUID SQLID A 5 6 7 8 9 10 11 A Diank Nemeral Nem	Labor	ratory na	ame:			Test	name: _			Test	no. :		
Transfer to the 96-well plate. Time/date executed: Supple location on 96-well plate. LIQUD SOLD A 5 6 7 8 9 10 11 A D 6 7 8 9 10 11 A D A 5 6 7 8 9 10 11 A D D SOLD A 5 6 7 8 9 10 11 A D D D D D D 1 <t< td=""><td>1.</td><td>Reactin</td><td>ig WST-</td><td>8 solutio</td><td>n (200 µ</td><td>ıL) is tra</td><td>nsferred</td><td>to each</td><td>well on</td><td>the 96-\</td><td>vell plate</td><td>e.</td><td></td></t<>	1.	Reactin	ig WST-	8 solutio	n (200 µ	ıL) is tra	nsferred	to each	well on	the 96-\	vell plate	e.	
Time/date executed:	Trai	nsfer to t	he 96-we	ell plate.									
Sample location on 96-well plate. LIQUID SOLID A 1 3 4 5 6 7 8 9 10 11 B PBS-1 PBS-2 PBS-3 Emarch-1 Emarch-2 Emarch-3 New ternt	Tim	e/date e>	xecuted:				_						
LIQUID SOLID 1 2 3 4 5 6 7 8 9 10 11 A blank Image: A Image: A 5 6 7 8 9 10 11 A blank Image: A	San	nole loca	ition on 9	6-well pla	ate.								
1 2 3 4 5 6 7 8 9 10 11 A blank		LIQU	<u>JID</u>	oo p.e				<u>SOLI</u>	<u>D</u>				
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c 10001 10002 100	B	PBS-1	PBS-2	PBS-3	Ethanol-1	Ethanol-2	Ethanol-3	Non-treat	Non-treat	Non-treat	Lauric	Lauric	Laurio
D I	С							inent-i	ment-2	ment-3	aciu-i	aciu -z	aciu -
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2. Analyze extract OD at 450nm and 650nm, and calculate the OD(450nm-650nm). Analyze OD at 450nm and 650nm. Calculate the OD (450nm-650nm). Calculate cell viability and SD. Cell viability and SD are recorded on a separate data sheet. The data sheet is attached to the back of this sheet. Check for input errors. Time/date executed: Note Note Secretariat Check date: Name:	н												
Note	2. Ana Calo Calo Cell The Che Tim	Analyze lyze OD culate the culate ce viability data she cck for in e/date ex	e extract at 450nn e OD (45 ill viability and SD a eet is atta put errors xecuted:_	OD at 4 n and 650 0nm-650 v and SD. are record ached to t s.	50nm a)nm. nm). ded on a he back	nd 650n separate of this sh	m, and o data she eet. —	calculate et.	the OD	(450nm	-650nm)		
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0.1.1.0010	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE	J-TEC
October, 2018	MODEL: LabCyte CORNEA-MODEL24	Japan Tissue Engineering
		Co., Ltd.

REVISION HISTORY

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

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Ver.2.2	 Revised clerical error. 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. In the section 3.3.2 "APPLICATION OF LIQUID TEST CHEMICALS AND RINSING (DAY 0~1)", explained temperature condition (room temperature) at chemical 	Sep., 2013
	application.	
Ver.2.3	 Revised clerical error. In the section 3.3. "TEST METHOD", explained about a blank preparation at WST-8 assay. In the section 3.3. "TEST METHOD", changed the condition of WST-8 reaction from standing to shaking. In the section 3.3. "TEST METHOD", explained about a blank preparation at WST-8 assay. 	Feb., 2014
Ver.2.3.1	1) Revised clerical error.	Mar., 2014
	2) About the prediction result of eye irritation, changed the classification from irritation/no irritation to GHS classification.	
Ver.2.3.2MTT	1) At the analysis of cell viability, changed the assay method from WST-8 assay to MTT assay.	Jul., 2014
Ver.2.4.1	 At the analysis of cell viability, changed the assay method from MTT assay to WST-8 assay. In the section 3.3. "TEST METHOD", changed the condition of WST-8 reaction from shaking to standing. In the section 3.3. "TEST METHOD", changed the dilution solution of WST-8 reaction from PBS to EBSS. In the section 3.3. "TEST METHOD", changed the reaction period of WST-8 from 5 hours to 4 hours. The standard of the additional testing was mentioned about a 	Jan., 2015

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

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October, 2018	<i>IN VITRO</i> EYE IRRITATION TEST USING HUMAN CORNEAL TISSUE	J-TEC
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		Co., Ltd.

	test chemicals.	
Ver. 2.5.4	1) In the section 3.3. "TEST METHOD", added attention point of	June, 2016
	the washing procedure.	
	2) In the section 4.3 "ASSESSMENT FLOWCHART", Change	
	numbers of test run from three independent run to single run.	
Ver. 2.5.5	1) Revised clerical error.	October, 2016
	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 %.	
Ver.2.5.6	1) Revised clerical error.	February, 2017
	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.	
Ver.2.6	1) Changed the assay acceptance criterion of negative control	June, 2018
	from 0.5 ≤ and ≤1.3 to 0.5 ≤ and ≤ 1.6.	
Ver.2.7	1) Revised clerical errors. There is no change of protocol.	