Standard Protocol for the Vitrigel-EIT method

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1. Principle of the method

The Vitrigel-EIT method is a highly sensitive means of testing for eye irritation potency by analyzing a series of transepithelial electrical resistance (TEER) measurements taken over time after exposure to a test chemical. A typical protocol for this test method is as follows.

2. Applicability domain

The following substances are not suitable for testing with this method and are excluded from the applicability domain.

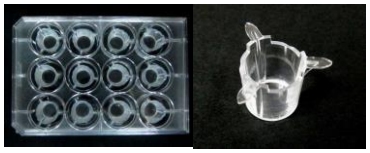
1. Chemicals that have a pH level of 5 or less in solution (see section 5.5.3, step 3).
2. Solids that have both a logP value of at least 2.5 and a density of either less than 0.95 g/cm^3 or more than 1.10 g/cm^3.

3. Materials to be procured by the user

- HCE-T cells (Cell Bank, Riken BioResource Center, RCB2280)
- Clean bench or biological safety cabinet
- CO_2 incubator (37°C, 5% CO_2 in air)
- Phase-contrast microscope
- Water bath
- Centrifuge (150 × g or greater)
- Safety pipette filler
- Micropipettes (100 μL, 1000 μL)
- Tweezers
- 70% Ethanol
- Balance
- Computer (Windows OS with USB port)
- Liquid nitrogen freezer (for cell storage)
- Universal pH test paper (ADVANTEC, 07011030)

4. Vitrigel-EIT kit contents (for 72 tests)

*Other equivalent reagents or materials are acceptable.

Name	Maker	Item code	Quantity	Store at
ad-MED Vitrigel™ comprise 12 collagen vitrigel membrane (CVM) chambers set in a 12-well plate 	Kanto	08360-96	6 plates	r.t.[1]
Culture medium (prepared by Kanto) Composition D-MEM/F-12 5% fetal bovine serum, 5 μg/ml human insulin, 10 ng/ml human epidermal growth factor, 2.5 % dimethyl sulfoxide, 100 U/ml penicillin, and 100 μg/ml streptomycin	 LT[2] Sigma LT LT Sigma LT	 11330-032 F2442 12585-014 PHG0311 D2650 15140-148	1 bottle	2–8°C
0.05% trypsin-0.02% EDTA-2 Na solution	LT	25300-054	10 bottles	−20°C or lower
PBS	Sigma	D8537	1 bottle	2–8°C
0.4% trypan blue solution	LT	15250-061	1 vial	r.t.
Negative control reagent (saline) (prepared by Kanto)			1 vial	2–8°C
Positive control reagent (benzalkonium chloride)	Sigma	B6294	1 vial	r.t.
Reference control reagent (99.5% ethanol)	Kanto	14033-00	1 vial	r.t.
Dimethyl sulfoxide	Sigma	D2650	1 vial	r.t.
TEER recorder (prototype)	Kanto		1 set	r.t.
Tissue culture flask (T-75)	BD Falcon	353024	3 packages	r.t.
5ml Pipette	BD Falcon	357529	1 package	r.t.
15ml Plastic tube	BD Falcon	352096	1 package	r.t.
1.5ml Plastic tube	As one	1-7521-01		
Hemocytometer	TGK	OC-C-S02	1 package	r.t.
Chambers without membrane and 12-well plate for TEER recorder check	Kanto		1 set	r.t.

NB1: room temperature, NB2: Life Technologies

5. Procedure

5.1 Thawing and initial culturing of HCE-T cells

1. Warm culture medium in a water bath at 37°C.
2. Pour 14 ml of the culture medium into a T-75 tissue culture flask and spread it over the bottom surface.
3. Transfer a cryotube of human corneal epithelium (HCE) T cells from the liquid nitrogen freezer to the water bath and agitate gently until completely thawed.
4. Open the cryotube, make sure any sunken HCE-T cells are suspended in the medium, then add the total cell suspension to the culture medium in the T-75 flask, and mix until uniform. Then, transfer the flask to the CO_2 incubator (37°C, 5% CO_2 in air) to start culturing the HCE-T cells.
5. After culturing the cells for at least 2 but no more than 6 hours, observe the morphology of the cells under a phase-contrast microscope. If most of the cells are well attached, as shown in Fig. 1 (a few non-attached cells can be ignored), replace the culture medium with a fresh one and continue the culturing. If not well attached, dispose of the failed cell culture and repeat steps 1 to 4 using a different cryotube of HCE-T cells.

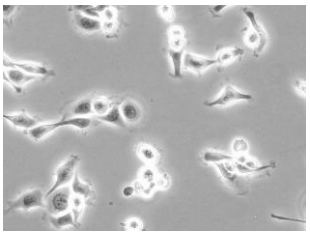


Fig. 1 Phase-contrast microphotograph of HCE-T cells cultured for two hours.

6. After culturing the cells for a few days, observe the morphology of the cells under a phase-contrast microscope. If the cells have proliferated into a monolayer of between 80 and 100% confluency, as shown in Fig. 2, proceed to section 5.2 to passage the cells as described. If not, dispose of the failed culture and repeat steps 1 to 5 using a different cryotube of HCE-T cells.

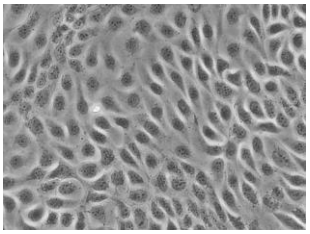


Fig. 2 Phase-contrast microphotograph of HCE-T cells in a min. 80% confluent monolayer appropriate for passaging.

5.2 Passaging HCE-T cells

1. Prepare four new T-75 flasks with 14 ml of fresh culture medium in each flask.
2. Warm the culture medium bottles, PBS, and trypsin solution in a water bath at 37°C.
3. Remove the culture medium from the flask of HCE-T cells that have proliferated into a min. 80% confluent monolayer. Pour 10 ml of PBS into the flask and spread it over the bottom surface to rinse the cells.
4. Remove the PBS from the flask. Pour 5 ml of trypsin solution into the flask, spread it over the bottom surface, and the remove 4.3 ml of the solution. Incubate the flask in a CO_2 incubator for five minutes. Surplus trypsin solution can be stored in a freezer and reused.

5. Tap the bottom and side walls of the flask several times to detach the cells from the bottom surface, as shown in Fig. 3.
6. Pour 4 ml of fresh culture medium into the flask and suspend the cells by pipetting as quickly as possible.
7. Add 1.0 to 1.2 ml of the cell suspension to the culture medium in each of the four new flasks described in step 1 and mix until uniform. Next, place the flasks in a CO_2 incubator to start passaging HCE-T cells in the four new flasks. Thereafter, change the culture medium every other day.

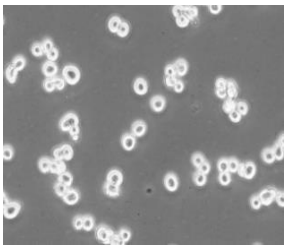


Fig. 3 Phase-contrast microphotograph of HCE-T cells after trypsinization

8. Allow the subculture to proliferate in as many or as few flasks as needed (ordinarily, two to eight).

5.3 Cryopreservation of HCE-T cells

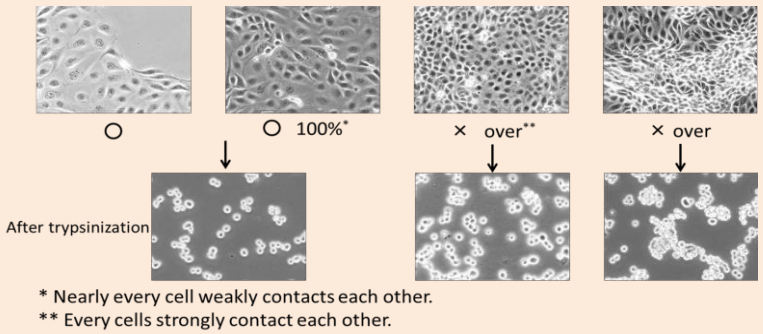
Prepare eight cryotubes of HCE-T cells from the four T-75 flasks prepared in section 5.2 by transferring half of the cells in each flask to a cryotube, as described below.

1. Prepare a culture medium with 20% DMSO by adding 0.8 ml of DMSO to 3.2 ml of culture medium and mixing until uniform.
2. Follow steps 2 to 6 of the procedure described in section 5.2 to create four flasks, in which HCE-T cells have proliferated into a monolayer of between 80 and 100% confluency.
3. Collect the cell suspension in a 50 ml centrifuge tube and spin at 150 × g for three minutes at room temperature.
4. Remove the supernatant and tap the tube several times to loosen the multicellular pellet.
5. Suspend the cells in 4 ml of a fresh culture medium.
6. Add 4 ml of the culture medium with 20% DMSO to the cell suspension and gently mix by pipetting. Quickly dispense 1 ml of the cell suspension to each cryotube.
7. Place the cryotubes in a programmable freezer (or equivalent) and cool at a rate of -1°C/minute to a temperature of -80°C. Preserve the cryotubes in a liquid nitrogen freezer.

5.4 Preparation of Vitrigel-HCE models

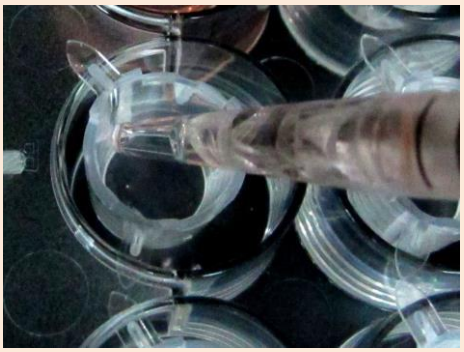
1. Follow the procedure described in section 5.1. to thaw HCE-T cells preserved in the cryotubes.
2. Initiate culturing and allow the HCE-T cells to proliferate into a monolayer of between 50 and 100% confluency. About 80 models can be prepared from a single T-75 flask in which the cells have proliferated into a confluent monolayer.

Caution:
Do not use HCE-T cells in an over-confluent state, because cell-to-cell aggregates are difficult to separate.



3. Follow steps 2 to 6 of the procedure described in section 5.2.
4. Observe the morphology of the cells under a phase-contrast microscope. Proceed to step 5 only if the culture comprises well-separated, individual cells. If cellular aggregates comprising six cells or more are observed over more than 20% of the surface area, dispose of the failed cell culture and return to step 1 of this section.
5. Transfer the cell suspension to a 50-ml tube. Resuspend the cells uniformly and then immediately dispense 100 µL of the cell suspension into a 1.5-ml tube.
6. Add 100 µL of trypan blue solution to the cell suspension and mix well by pipetting. Count the numbers of living and dead cells by using a hemocytometer and calculate the survival rate and density of the cell suspension. If the cell survival rate is lower than 95%, dispose of the failed cell culture and return to step 1 of this section.

Caution:
Do not push a collagen vitrigel membrane with the pipette tip. Use the guide on the side wall of the chamber to fix the pipette tip temporarily.



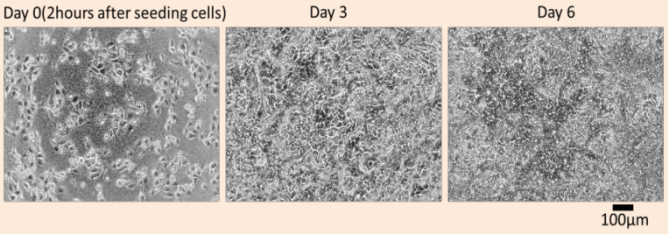
7. For each collagen vitrigel membrane chamber pre-set in a well of a 12-well plate, first pour 1.5 ml of culture medium into the well on the outside the chamber and then pour 0.5 ml of culture medium directly into the chamber itself. Let stand at room temperature for at least 10 minutes but no more than 2 hours.
8. Remove the culture medium from the inside of each collagen vitrigel membrane chamber and pour 0.5 ml of the cell suspension at a density of 1.2×10^5 cells/ml. Transfer the plate to a CO_2 incubator and culture for two days.
9. Change the culture medium in the cell outside the chamber and remove the culture medium

from inside the chamber to start the air–liquid interface culture, then culture for four days in a CO_2 incubator.

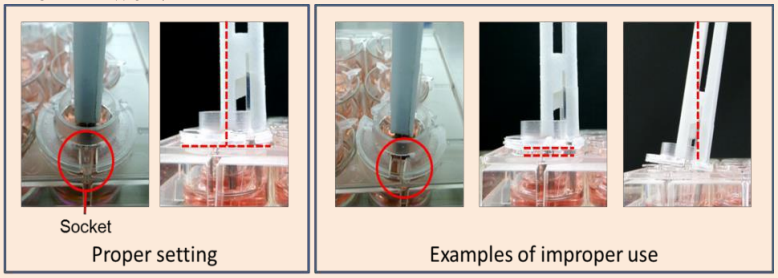
10. Change the culture medium in the cell outside the chamber on the fifth day after seeding.
11. On the sixth day after seeding, pour 0.5 ml of culture medium directly into the inside of the chamber. Adjust the temperature of the model to 28±2°C. Set the electrode to the TEER recorder on the chamber. Measure an initial TEER value for each HCE model before exposure to a test chemical and record the measured values on the data sheet.
12. HCE models with an initial TEER value of 140–220 Ω·cm^2 are acceptable for use in testing. HCE models that pass the above acceptance criterion should be used in a chemical exposure test within the same day.

Caution:
Although no technique for measuring temperature is specified, we recommend pouring culture medium into an empty well of the 12-well plate before adjusting the temperature for all models, then measuring the temperature of the medium inside the wells. If there is no empty well available, use an empty well on a new 12-well plate.

Phase-contrast microphotographs of HCE-T cells cultured in a CVM chamber. It is important to seed cells uniformly. The cells gradually form multilayers.



Set the electrode to the TEER recorder in the chamber by fixing the three chamber arms in the three sockets of the adaptor. The adapter of electrode should stand vertically on the rim of the well.



Caution:
Do not leave a model outside a CO_2 incubator without culture medium in a chamber. Do not leave a model outside a CO_2 incubator over 2 hours.

5.5 Performing tests using the Vitrigel-EIT method

5.5.1 Checking the TEER recorder

1. Pour 3 ml of a 0.45% NaCl aqueous solution at 25±5°C in one well of a 12-well plate and insert a chamber without membrane in the well.
2. Pour 3 ml of a 0.9% NaCl aqueous solution at 25±5°C in one well of 12-well plate and insert a chamber without membrane in the well.
3. Measure the TEER value in both wells. If the TEER recorder is functioning normally, the measured values will satisfy the following conditions:
   (TEER value of 0.45% NaCl aqueous solution) − (TEER value of 0.9% NaCl aqueous solution) ≥ 60 Ω·cm^2

5.5.2 Testing positive, negative, and reference control reagents

1. Equilibrate the culture medium to an ambient temperature of between 22 and 30°C.
2. The Vitrigel-EIT method uses a saline solution as negative control, a benzalkonium-chloride solution as positive control, and an ethanol solution as reference control. Prepare each in a 2.5% (w/v) reagent solution by adding 0.1–0.2 g of sodium chloride, benzalkonium chloride, and ethanol to an appropriate volume of culture medium in a 15-ml tube and mixing until uniform. As long as the proper concentration is maintained, the total quantity of the prepared reagent is unimportant.

   Caution:
   To minimize volatilization, 15-ml tubes should be sealed tightly when not in use.

3. Adjust the temperature of the reagent solution to 28±2°C.
4. Adjust the temperature of a model to 28±2°C.

   Caution:
   Measure temperature by immersing a thermometer in the reagent solution or culture medium.

5. Remove the culture medium from inside the chamber and set the electrode to the TEER recorder on the chamber.

   Caution:
   Add the control reagent within 5 minutes of removing the culture medium from inside the chamber.

6. Bearing in mind the importance of performing the test within the required timeframe, quickly pour 500 µL of the control reagent into the inside of a chamber (within 2-seconds time) and click the Start icon on the measurement software within 2 to 5 seconds of exposure to the control reagent. TEER values are automatically recorded every 10 seconds for 3 minutes thereafter. If the differential between the initial TEER value and the TEER value at 0 seconds is more than ±40 Ω·cm^2, reject the results and retest using a different HCE model.

7. Input the measured TEER values to the data sheet (software), as described in section 5.5.4.
8. Once the measured TEER values are input, the data is automatically analyzed to determine whether or not the success criteria have been satisfied as show below. If the data is marked Pass on the data sheet, proceed to the next test. If the data is marked NG on the data sheet, redo the test starting with preparation of a Vitrigel-HCE model, as described in section 5.4.

   Success criteria

   Negative control: The plateau level is 5% or less of the TEER value at 0 seconds.

   Positive control: The plateau level is 40% or more of the TEER value at 0 seconds.

   Reference control: The plateau level is 10% or more of the TEER value at 0 seconds.
9. Rinse the electrode with pure water, and wipe it with an absorbent paper towel after every test.

5.5.3 Testing test chemicals

1. Equilibrate the culture medium to an ambient temperature of between 22 and 30°C.
2. Prepare a 2.5% (w/v) test chemical solution by adding 0.1–0.2 g of each test chemical to an appropriate volume of culture medium in a 15-ml tube. As long as the proper concentration is maintained, the total quantity of the prepared reagent is unimportant. Mix manually until the test chemical dissolves or for a maximum of one minute. If the test chemical does not dissolve readily, try using the following techniques in the following order to dissolve it: a) mix mechanically for a maximum of one minute using a vortex mixer, b) sonication for a maximum of 20 minutes, or c) heating to a maximum temperature of 70°C. After trying each technique, adjust the temperature of each test chemical solution to 28±2°C and check solubility. Move to the next step of the procedure once the test chemical solution is well dissolved or homogeneously dispersed.

   Caution:
   To minimize volatilization, the 15-ml tubes should be tightly sealed when not in use. The parameters (time, temperature, intensity, etc.) for dissolving test chemicals may be adjusted to accommodate the physiochemical properties of each test chemical.
3. Measure the pH level of the test chemical solution using universal pH test paper.
4. Adjust the temperature of the test chemical solution to 28±2°C.
5. Adjust the temperature of a model to 28±2°C.

   Caution:
   Measure temperature by immersing a thermometer in the test chemical or culture medium.
6. Remove the culture medium from inside the chamber and set the electrode to the TEER recorder on the chamber.
7. Bearing in mind the importance of performing the test within the required timeframe, quickly pour 500 µL of the test chemical solution into the inside of a chamber (within

2-seconds time) and click the Start icon on the measurement software within 2 to 5 seconds of exposure to the test chemical solution. TEER values are automatically recorded every 10 seconds for 3 minutes thereafter. If the differential between the initial TEER value and the TEER value at 0 second is more than 40 $\Omega \cdot cm^2$, reject the results and retest using a different HCE model.

Caution:
For test chemicals that are insoluble, a homogeneous suspension of the test chemical may be prepared immediately before testing.

8. Input the measured TEER values to the data sheet (software), as described in step 5.5.4.
9. Rinse the electrode with pure water or 70% ethanol, and wipe it with an absorbent paper towel after every test.

5.5.4 Automatic analysis system utilizing a data sheet

1. Three individual tests are performed for each test chemical, and the measured TEER values are input to software data sheet running on PC automatically analyze the data. The mean TEER values for the three tests are plotted on a timeline to create a profile of TEER values (dP/dT), which are analyzed for three parameters: time lag (t_1), intensity ($-[P_2 - P_1]/[t_2 - t_1]$), and plateau level ($100 - P_2$). Time lag (t_1) is defined as the maximum time a profile was maintained at $0 \geq dP/dT > -0.03\%$/second. The starting time of plateau level (t_2) after the profile was maintained at ($dP/dT \leq -0.03\%$/second) for a particular period of time was defined as the initial time at which the profile was maintained at $0 \geq dP\ (P_3 - P_2)/dT\ (t_3 - t_2) > -0.03\%$/seconds. The time ($t_3$) is represented in the equation ($t_3 = t_2 + 30$ seconds) because the plateau level was evaluated by the profile for 30 seconds. P_1, P_2, and P_3 are the percentages against the initial TEER value at t_1, t_2, and t_3 after exposure to the test chemical, as shown in Fig. 4.

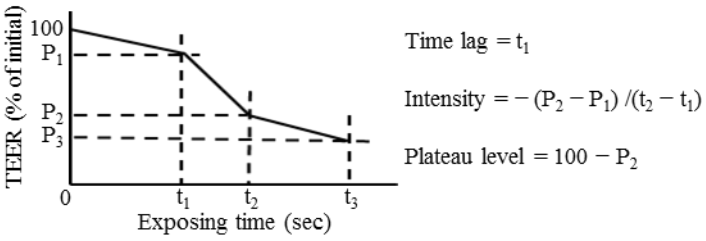


Fig. 4 Schematic analysis of a TEER profile after exposure to a test chemical
Here, t_1 and t_2 represent time lag and starting time of plateau level, respectively. t_3 is defined as $t_3 = t_2 + 30$ seconds. P_1, P_2, and P_3 are the percentages against the initial TEER value at times t_1, t_2, and t_3, respectively.

2. Eye irritation potency was predicted using the following criteria.

Criteria	Result
Time lag ≤ 180 or Intensity ≥ 0.05 or Plateau level > 5.0	Irritant (I)
Time lag > 180 and Intensity < 0.05 and Plateau level ≤ 5.0	Non-irritant (NI)

Revision history

Version 1.60e

Date: June 30, 2014 Description of change

4.411, Procedure 4.5.2., step 1, 3 and Procedure 4.5.3., step1, 3 Replaced the word “room temperature” to “ambient temperature for the experiment” Added an attention about the ambient temperature at procedure 4.4., step 11

Reason

Control of the ambient temperature is important for success the experiment.

Procedure 4.5.2., step 1, 4.5.3., step 1

Change the ambient temperature for a TEER test as follows.

between 18 and 30

↓

between 22 and 30

Reason

Temperature of a HCE model affects values of time dependent TEER change.

Procedure 4.5.2., step 7

Included the reference control as acceptance criteria, and changed the acceptance criterion as follows.

Plateau level is between 10% and 30%, inclusive.

↓

Plateau level is between 10% and 40%, inclusive. Reason

At the 2nd VMT meeting, all members agreed to should be included the reference control as the acceptance criteria for quality preservative of the model.

Expand the acceptance criteria on ground of the test results by the read laboratory.

Procedure 4.5.3., step 2, 5th sentence

Changed the description of the procedure for propagating a test chemical solution as follows. Old:

If the test chemical has not been dissolved, try to dissolve it by the mechanical mixture for a maximum 1-minute period using a voltex, by the sonication for a maximum

20-minute period, or by the heating to 70

New:

If the test chemical has not been dissolved, try to dissolve it by selecting an appropriate technique(s) from the following; mechanical mixture for a maximum 1-minute period

using a vortex mixer, sonication for a maximum 20-minute period, or heating to maximum 70

Reason

At the phase 1 study, some members misunderstood the procedure and they thought that all of three techniques should be done. Replaced the word “voltex” to “vortex mixer” (corrected a typo).

Procedure 4.5.2., step 2 and 4.5.3., step 2

Added an attention “To avoid volatilizing chemicals, the 15ml tube should be tightly lidded after weighing a test chemical to the tube except for adding a culture medium and sampling the 2.5% test chemical solution.”.

Reason

Clarify the attention that the tube should be tightly lidded during the experiment to avoid volatilizing chemicals.

Procedure 4.5.2., step 5 and 4.5.3., step 5

Added a sentence “In case the increasing and decreasing of 40 Ω・cm2 and more is occurred between the initial TEER value and the TEER value at 0 second, reject the experiment and retest using another HCE model.”

Reason

In case the TEER value at 0 second evidently differs from the initial TEER value, it indicates some technical failure in the experiment (e.g., contamination of electrical nose, improper use of electrode).

Version 1.70e

Date: October 15, 2014 Description of change

Procedure 4.4., step 11, Procedure 4.5.2., step 4 and Procedure 4.5.3., step 4

Replaced the word “Let stand for 10 minutes (within 2 hours) at the ambient temperature for the experiment.” to “Adjust the temperature of the model to 282 Added an attention about the way of measuring temperature at procedure 4.4., step 11

Reason

Temperature control of a model is important for success the experiment.

Procedure 4.5.2., step 1 and Procedure 4.5.3., step 1

Replaced the word “ambient temperature for the experiment” to “between 22 to 30

Remove attentions at 4.5.2., step 1 and Procedure 4.5.3., step 1

Reason

Remove the word “ambient temperature”.

Procedure 4.5.2., step 3 and Procedure 4.5.3., step 3

Replaced the word “with the ambient temperature for the experiment” to “to 282 Added an attention about the way of measuring temperature at procedure 4.5.2., step 3 and Procedure 4.5.3., step 3

Reason

Temperature control of a 2.5 w/v% reagent solution is important for success the experiment.

Procedure 4.5.2., step 7

Changed the acceptance criterion of reference control as follows. Plateau level is between 10% and 30%, inclusive.

↓

Plateau level is level is 10% or more

Reason

The upper limit of acceptance criterion will be decided at the end of phase III validation study.

Version 1.80e

March 31, 2015

Description of changes

Section 2. Applicability domain

A new section was added to define an applicability domain based on the test results of 132 chemicals at the lead laboratory.

Section 3. Materials to be procured by the user

The term universal pH test paper (ADVANTEC, 07011030) was added to section 3, because an additional step was added to measure the pH level of the test chemical solution using universal pH test paper.

Section 4. Vitrigel-EIT kit contents

Formerly Section 2, this section was renumbered to accommodate the addition of the new Section 2.

Section 5. Procedure

Formerly Section 5, this section was renumbered to accommodate the addition of the new Section

2.

Section 5.5.3, Step 2

Changed the description as follows.

Old:

If the test chemical has not been dissolved, try to dissolve it by selecting an appropriate technique(s) from the following; mechanical mixture for a maximum 1-minute period using a vortex mixer, sonication for a maximum 20-minute period, or heating to maximum 70°C.

New:

If the test chemical does not dissolve readily, try using the following techniques in the following order to dissolve it: a) mix mechanically for a maximum of one minute using a vortex mixer, b) sonication for a maximum of 20 minutes, or c) heating to a maximum temperature of 70°C. After trying each technique, adjust the temperature of each test chemical solution to 28±2°C and check solubility. Move to the next step of the procedure once the test chemical solution is well dissolved or homogeneously dispersed.

Also, a condition was added to the Caution box at the end of Step 2, which reads:

> The parameters (time, temperature, intensity, etc.) for dissolving test chemicals may be adjusted to accommodate the physiochemical properties of each test chemical.

This was done due to problems with between-laboratory reproducibility during Phase III, when there were non-concordant results for some test chemicals. These discrepancies were attributed to differences in techniques used to dissolve the test chemicals, so we specified procedures for dissolving test chemicals.

Procedure 5.3.3., step 3

Add an extra procedure “Measure pH level of each 2.5% test chemical solution was using Universal pH test paper.”

Reason for revision

Add a procedure in association with setting applicability domains.

Version 1.81e

January 18, 2019

Create a title page