Biosolution Co.,Ltd.

Protocol for *in vitro* eye irritation using a new 3D reconstructed human cornea model, MCTT HCE™

CONTENTS

Definitions and Abbreviations

EIT: Eye Irritation Test

HCE: Human Corneal Epithelium

TEER: Trans Epithelial Electric Resistance

ET-50: Effective Time-50

OD: Optical Density

NC: Negative Control

PC: Positive Control

T: Test substance

OD_{blank}: Diluted WST-1 solution

OD_{NCraw}: Raw OD negative control living tissues

OD_{PCraw}: Raw OD positive control living tissues

OD_{Traw}: Raw OD test substance living tissues

OD_{NC}: OD negative control living tissues (OD_{NCraw} - OD_{blank})

OD_{PC}: OD positive control living tissues (OD_{PCraw} - OD_{blank})

OD_{T}: OD test substance living tissues (OD_{Traw} - OD_{blank})

DPBS: Dulbecco's Phosphate-Buffered Saline without Ca^{2+} & Mg^{2+}

SDS: Sodium Dodecyl Sulfate

WST-1: 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, Cell proliferation reagent

$\%NSC_{living}$: Cell viability of living tissue without WST-1 incubation

$\%NSC_{killed}$: Cell viability of killed tissue without WST-1 incubation

$\%NSWST\text{-}1_{killed}$: Cell viability of killed tissue with WST-1 incubation

mg: Milligram

mL: Milliliter

µL: Microliter

mm: Millimeter

nm: Nanometer

℃: Degree Celsius

RT: Room temperature

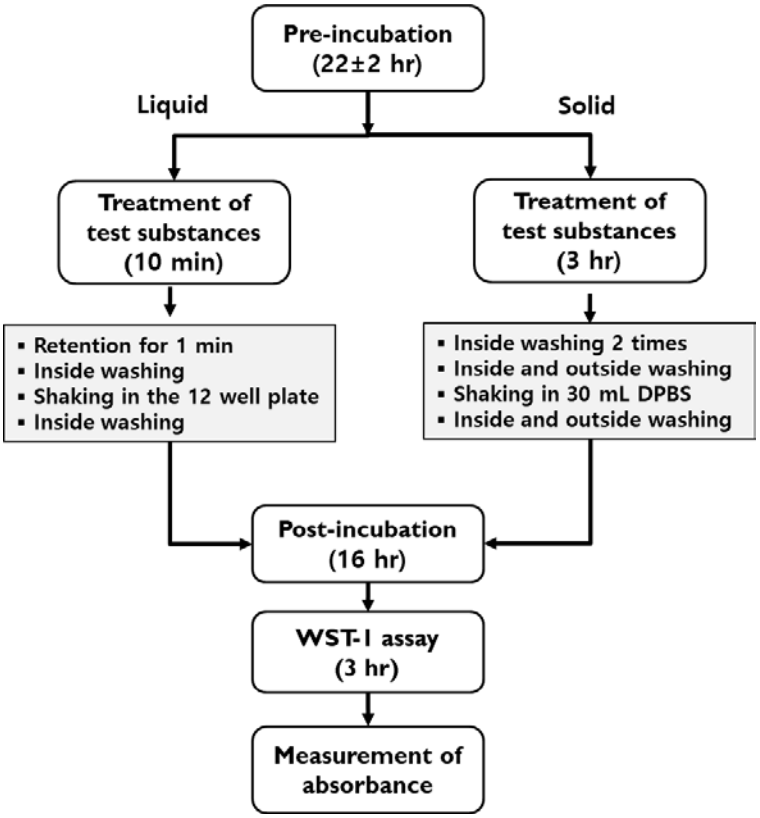
%: Percent

hr/hrs: Hour/hours

min: Minute

Protocol Overview

MCTT HCE™ models, which passed the quality control (Optical Density (OD) values of negative control 0.8 - 1.2 ET-50 (the time of exposure to 0.3% Triton X-100 estimated to decrease the viability by 50%) 17.6 – 41.0 min, and histological examination) of the manufacturer, are delivered to the testing labs in a 24-well format on agarose gel in refrigerated condition. Upon receipt of the shipment, culture medium is warmed in a 37°C thermostat for 30 min. As preparation for the pre-incubation step, 900 μL of the pre-warmed medium is added to each well of a 6-well plate using micropipette and the HCE model insert is carefully transferred to the wells using forceps. Then the well-plate is pre-incubated at 37°C, 5% CO_2 for 22 ± 2 hr. Following pre-incubation, 40 μL of liquid substance or solution, or 40 mg of solid substance is topically applied to the upper epithelial surface of the model insert (0.6 cm^2). Then the tissue is incubated again (37°C, 5% CO_2 condition) for 10 ± 1 min or 3 hr ± 5 min depending on the physical state of the test substance (Fig.1). Next, the tissue is washed to remove the test substance and further incubated for 16 ± 1 hr. Finally, the resulting tissue viability is evaluated by the WST-1 assay.



[Fig. 1] Overview of the optimized eye irritation test method for MCTT HCE™ model

Main Protocol

1. Background

This protocol is for the *in vitro* eye irritation test using reconstructed human corneal epithelium, MCTT HCE™, as a replacement test for the *in vivo* Draize eye irritation test using rabbits.

2. Purpose

This protocol describes *in vitro* eye irritation test using reconstructed human corneal epithelium, MCTT HCE™, as a replacement test for the *in vivo* eye irritation test using rabbits. MCTT HCE™ is designed to closely mimic the biochemical and physiological properties of the human cornea. Eye irritancy of chemicals begins with the penetration of chemicals into the cell layer and is caused by subsequent damages of the corneal epithelium. Cell viability of the MCTT HCE™ model is measured by enzymatic conversion of the vital dye WST-1 [3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide] into a yellow formazan salt that is quantitatively measured in the supernatant naturally released from the tissue following incubation with tetrazolium salt. The purpose of the MCTT HCE™ eye irritation test (MCTT HCE™ EIT) is to predict the ocular irritancy of test substances in compliance with the UN GHS classifications for eye irritation.

3. Materials

3.1 RhCE Model: MCTT HCE™

The MCTT HCE™ consists of normal human-derived corneal epithelium, which has been cultured to form a multilayered, highly differentiated model of the human cornea. The MCTT HCE™ consists of organized basal cells, wing cells and squamous cells arranged in patterns analogous to those found *in vivo*. The MCTT HCE™ tissues are cultured for 14 days on the surface (diameter 12 mm, 0.67 cm^2) of specially prepared cell culture inserts in Millicell™ (Millipore, USA) culture plates and shipped in a 24-well format on shipping agarose gel together with the necessary amount of culture media. In addition, MCTT HCE™ tissue can be frozen during shipment. Therefore, temperature changes during shipment shall be recorded.

The MCTT HCE™ system is manufactured according to defined quality control procedures. All biological components of the epidermis and the culture medium are tested by the manufacturer to confirm the absence of viral, bacterial, fungal and mycoplasma contamination. The Effective Time-50 (ET-50) value following exposure to Triton X-100 for each MCTT HCE™ lot, OD value of the negative control (PBS) by MTT assay, and histology are provided by the manufacturer within a week after shipment.

3.2 Assay quality controls in testing laboratory

The absolute OD of negative control (NC) tissues (treated with sterile DPBS) measured by WST-1 assay is an indicator of tissue viability of the MCTT HCE™ lot delivered to the testing laboratory.

The assay meets acceptance criterion if the mean OD_{450} of the NC (DPBS) tissue is ≥ 1.6 and ≤ 3.0. The reduction in cell viability is the measure for response of the MCTT HCE™ tissue to ocular irritancy. The assay meets another acceptance criterion if the mean viability of PC (SDS 2% or methyl acetate) is reduced to ≤ 35% of that of the negative control tissue.

3.3 Test material and vehicle

Ingredients must be treated 'as is' (not diluted). The most appropriate solvent must be used and the scientific basis for the selection must be provided. If vehicles other than DPBS are used, the effects on the cell viability must be considered.

3.4 Materials and reagent

Process	Instruments and reagents	Manufacture	Purpose of use
Culture	Laminar flow cabinet		Sterile condition
	Cell incubator		Tissue culture at 37°C, 5% CO_2
	Water bath at 37°C		Warming medium and DPBS
	Extra sterile 6-well plates	BD 353046	Tissue culture
	Media	From Biosolution	Tissue culture
	Forceps (Small sterile blunt-edged forceps)		Holding insert
Treatment of test material	DPBS	LONZA 17-512	Negative control, washing test material
	2 (aq) % SDS (Sodium Dodecyl Sulfate) [CAS: 151-21-3]	Sigma L4509	Positive control
	Methyl acetate [CAS: 79-20-9]	Sigma 186325	Positive control
	Balance		Measuring weight of solid material
	Weighing papers		Measuring weight of solid material Treatment of test material
	Spatula		Measuring weight of solid material
	Mortar and pestle		Grinding of coarse solid materials
	Auto pipette and tips		All process
	Extra sterile 6-well plates		Tissue culture & Treatment of test material
	Stop-watches/Timers		Treatment of test material & washing
	Serological pipette and pipette aid		Washing

	500 mL beakers		Washing
	50 mL beakers		Washing
	Sterile 12-well plates	BD 353043	Washing
	General laboratory materials (latex gloves, paper towels, 70% EtOH etc.)		-
WST-1 assay	WST-1 (3-4,5-dimethyl thiazole 2-yl 2,5-diphenyltetrazolium bromide) [CAS: 150849-52-8]	Roche 11 644 807 001 Or 05 015 944 001	Measurement of cell viability
	WST-1 formazan standard (4-[1-(4-Iodophenyl)-5-(4-nitrophenyl)formaz-3-yl]-1,3-benzene Disulfonate, Disodium Salt) [CAS: 150849-53-9]	From Biosolution	Evaluation of linearity
	DPBS	LONZA 17-512	Diluting WST-1
	24-well sterile plates	BD 353047	Treatment of WST-1
	96-well plates	BD 353072	Measuring OD (Optical Density) value
	Centrifuge		Separation of formazan solution from debris
	ELISA Plate reader (96-well)		Measuring OD (Optical Density) value

4. Methods

4.1 Experimental time schedule

Protocol	Day 1	Day 2	Day 3
Delivery	O		
Pre-incubation	O	O	
Treatment		O	
Washing step		O	
Post-incubation		O	O
WST-1 application			O
Reading Optical Density			O

4.2 Preparations of reagents, test chemicals, and instruments

Prepare all reagents just before the commencement of testing. Pre-warmed (37°C) sterile DPBS should be used as a negative control. Weigh 0.02 g Sodium Dodecyl Sulfate (SDS) and dissolve it in 1 mL sterile DPBS (final concentration; 2%). For a liquid test chemical, aliquot 200 µL (sufficient

amount for treating 2 different tissues) into an amber vial. For a solid test chemical, aliquot 40 mg onto weighing paper for each of 2 replicates (in case the chemical might be absorbed into parchment paper, the chemical should be prepared just before treatment). If solid materials are not finely powdered, they must be ground into a fine powder using pestle and mortar for weighing purposes. For materials that are of undefined physicochemical state, such as waxy or crystalline, that deter weighing, pre-warming in a 37°C water bath for 15 min may be helpful. After weighing, store the test substances protected from light. Prepare the diluted WST-1 solution (1:25) in pre-warmed DPBS (e.g. WST-1 400 µL: DPBS 9.6 mL). The WST-1 solution should be prepared just before treatment and covered to protect it from light. Also, the linearity of the photometer shall be checked for each test with the WST-1 formazan standard provided by Biosolution.

Record on [Data sheet MCTT HCE™_EI/Sheet/007-v1.7, MCTT HCE™_EI/Sheet/008-v1.7]

[Fig. 2] Measured solid test material in the weighing paper (left); WST-1 solution, after diluting in DPBS, wrapped with foil to protect it from the light (right).

4.3 Test for interference of chemicals with WST-1 endpoint and correction procedures

In order to prevent the possible interference with the WST-1 endpoint from colorful chemicals or chemicals with direct reducing potential, ‘direct staining by test materials’ or ‘reactivity of test substance with WST-1’ may be checked before the application of test materials. However, when a test substance is determined to be an irritant, the correction is not necessary since corrections always over-predict the classification by subtracting the cell viability.

4.3.1 Direct staining by test material

1) Preliminary test : Add 1 mL of deionized water and 40 µL (liquid) or 40 mg (solid) of the test chemical to each eppendorf tube and incubate the mixture in a cell incubator (37°C, 5% CO_2, 95% RH) for 60 min.

2) Take 200 μL of the reacted solution in duplicate and transfer to a 96-well plate. If an OD value measured at 450 nm is more than 0.1, correction of the cell viability may be necessary (see 4.3.3).

4.3.2 Reactivity of test substance with WST-1

Test substances may reduce WST-1 directly to form formazan. This test may be performed before the eye irritation test to determine interferences.

1) Preliminary test: Add 40 μl (liquid) or 40 mg (solid) of the test substance to 1 mL of diluted working WST-1 solution (1:25). Place the mixture in the incubator (37°C, 5% CO_2, 95% RH) for 3 hr. At the end of the exposure time, shake the mixture and evaluate the presence of formazan.
2) If the solution changes color significantly, the test substance is presumed to have the potential to reduce WST-1. Correction of the cell viability may be necessary (see 4.3.3).

4.3.3 Correction of cell viability for the interference of WST-1 assay

1) When the test substance is only colorant;

The test substance may color-stain the tissue. Conduct the test according to the main protocol but use DPBS instead of WST-1 solution (NSC_{living} [Non-specific color in living tissues control]). This shall be done in duplicate and used as an additional negative control ($\%NSC_{living}$) for the test substance in calculating cell viability based on the following equation,

$$True\ tissue\ viability = [\%Viability_{test}] - [\%NSC_{living}]$$

2) When the test substance is non-colorant, but WST-1 reducer;

Conduct the test according to the main protocol but use a freeze-killed tissue (NSWST-1 [Non-specific WST-1 reduction control]) instead of a viable tissue. The freeze-killed tissue has no metabolic activity, but is capable of absorbing and binding to the test substance. Upon receipt of the tissue, the tissue must be frozen in a 24-well plate at -80°C for 48 hr. Then transfer the tissue into a 6-well plate containing fresh medium and allow 10 min for it to stabilize. The resulting cell viability (%NSWST-1) is used to correct cell viability as follows,

$$True\ tissue\ viability = [\%Viability_{test}] - [\%NSWST\text{-}1]$$

This functional check is not done for every test run, but can be done once, in duplicate, for each test substance of concern.

3) When the test substance is both colorant and WST-1 reducer;;

In this case, the correction needs to be done for both colorant and WST-1 reducer as described in 4.3.3 1) and 2), but the color interference will be subtracted twice. To achieve this, another functional check with freeze-killed tissue must be conducted with DPBS instead of WST-1 solution (NSC_{killed} [Non-specific color in killed tissues control]). Prepare freeze-killed tissue as described above and conduct the test once, in duplicate, for each test substance. Final cell viability is calculated as follows,

$$True\ tissue\ viability = [\%Viability_{test}] - [\%NSWST\text{-}1] - [\%NSC_{living}] + [\%NSC_{killed}]$$

4.4 Receipt of MCTT HCE™

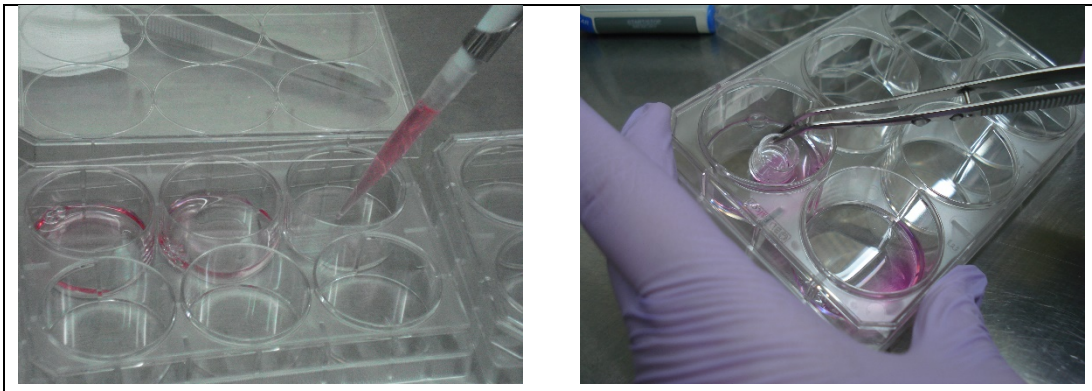
Pre-warm the assay medium to 37℃. Within 5 min of receipt and before beginning incubation, perform a visual inspection of the inserts to check if tissue surfaces are even, without excess moisture, and there are no air bubbles under the insert. Agarose gel has to be flexible to absorb possible shock during shipment and must be intact upon receipt of the tissues. Do not use tissues with defects or fissures, or with excessive moisture on the surface.

Record on [Data sheet MCTT HCE™_EI/Sheet/001-v1.7]

4.5 Pre-incubation

Pipette 0.9 mL of the pre-warmed assay medium into each well of sterile 6-well plates. Remove the shipped multiwell plate from the refrigerator. Under a sterile laminar flow, open the plastic bag containing the 24-well plate with epidermal tissues. Carefully take out each insert containing the epidermal tissue. Place the tissue into a 6-well plate prefilled with 0.9 mL medium. Any air bubbles trapped underneath the insert should be removed. Place the 6-well plates containing the tissues into a humidified (37℃, 5% CO_2) incubator for 22 ± 2 hrs.

Record on [Data sheet MCTT HCE™_EI/Sheet/002-v1.7]

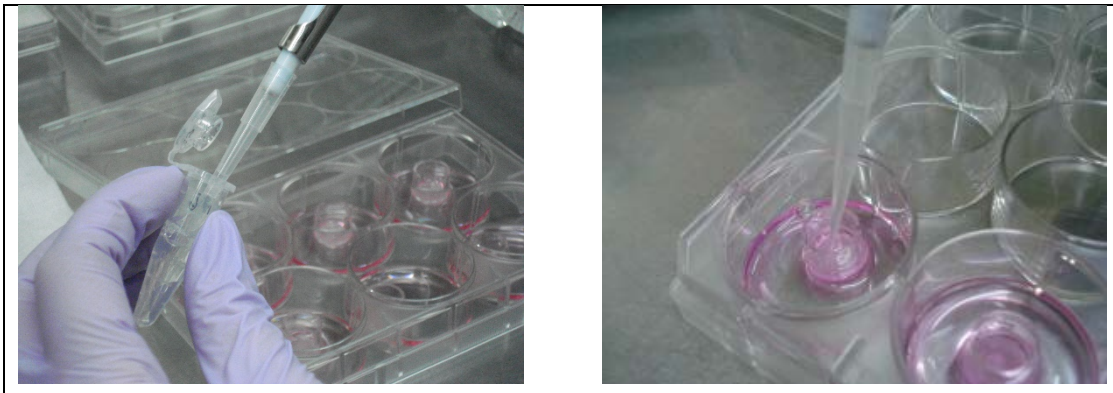


[Fig. 3] Pre-incubation: Pipette 0.9 mL of the pre-warmed assay medium into each well of a sterile 6-well plate (left); then place the tissue into the wells (right)

4.6 Application and washing of liquid chemicals

- Application of the chemical:
  Liquid test substances should be applied in a chemical hood since many of them are volatile and odorous. Take 40 μL of the test material with a micropipette and slowly dispense onto near the center of the cornea model (Fig.4). Rotate the insert to uniformly apply to the whole surface. When application of the test substance onto all 6-well units is complete, put it in a 5% CO_2 cell incubator at 37°C and incubate for 10 ± 1 min.

  Record on [Data sheet MCTT HCE™_EI/Sheet/003-v1.7]



[Fig. 4] Application of liquids: Pipette 40 μL of liquid test material (left); Dispense directly atop the tissue and spread gently (right).

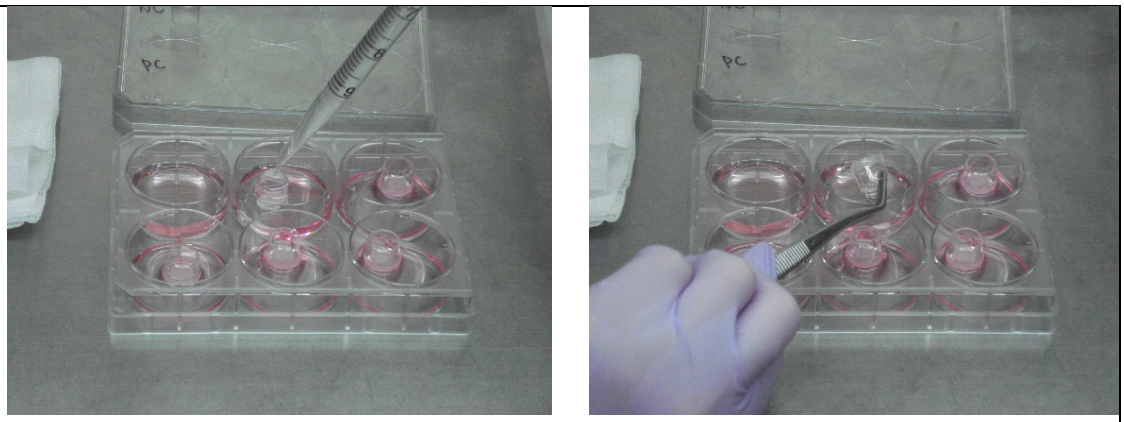
- Washing: Washing procedure for the 6-wells should be completed within 20 min. DPBS for washing should be pre-warmed in a 37°C thermostat before use. During washing, place 2-3 cm of gap between the tip of the pipette aid and the tissue and flow DPBS along the well-wall. Remaining DPBS in-between each washing step is left until the final washing step and shall be removed all at once.
- Detailed procedures are as below.

1) After 10 ± 1 min of incubation, take the plate from the incubator. Add 4 mL of DPBS, using pipette aid, into the insert to overflow the material inside, and leave for about 1 min. Then, flip over the insert using forceps to remove the material inside the insert. The DPBS washing interval between the wells should be the same as the application interval. For example, if the substance is applied at 30 sec intervals, washing should also be performed at 30 sec intervals.
2) Take 10 mL of DPBS using pipette aid to wash the insert. Hold the insert with the forceps and apply the DPBS to the insert to remove all material inside. The 10 mL of DPBS is applied over approximately 10 sec (1 mL/ sec). Washing procedure of this test using DPBS should be maintained at this rate.
3) Place those 6 inserts in each well of a 12-well plate. At 10-sec intervals, add 4 mL of DPBS to the insert in each well to overflow the material inside, and leave for about 1 min. Then

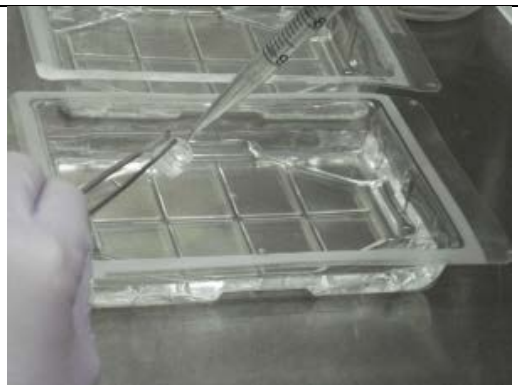
grab the insert with forceps and gently shake it 5 times in DPBS to remove debris and place the inserts on the cover of the 12-well plate.

4) Hold the insert with forceps and rinse inside and outside of the insert thoroughly with 10 mL of DPBS using pipette aid.
5) Finally, remove residual DPBS inside the insert using micropipette and by absorbing with the sterilized gauze, remove the DPBS on the outside the insert as well.

Record on [Data sheet MCTT HCE™_EI/Sheet/003-v1.7]

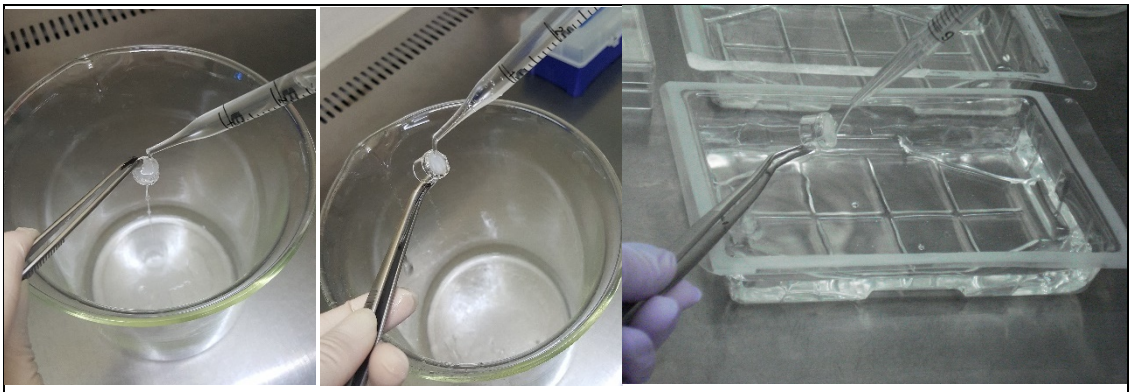


[Fig. 5] 1st washing procedure of liquid material; overflow with sterile DPBS 4 mL staying the tissue insert for 1 min (left), remove excess of DPBS by gently shaking the insert (right).

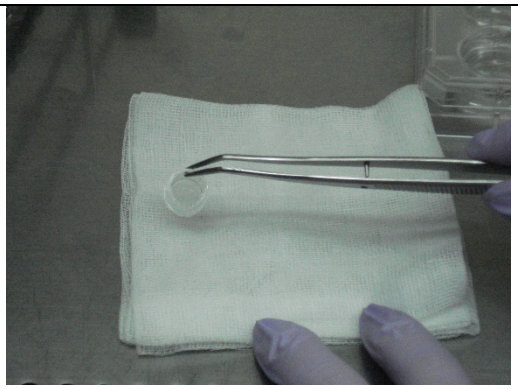


[Fig. 6] 2nd washing procedure of liquid material; with sterile DPBS 10 mL, overflowing the tissue insert.

[Fig. 7] 3rd washing procedure of liquid material; overflowing DPBS 4 mL in 12-well plate and then incubate for 1 minute.



[Fig. 8] 4th washing procedure of liquid material; inside and outside of the tissue once with DPBS 10 mL.



[Fig. 9] Blot insert on gauze and remove any rests inside of insert.

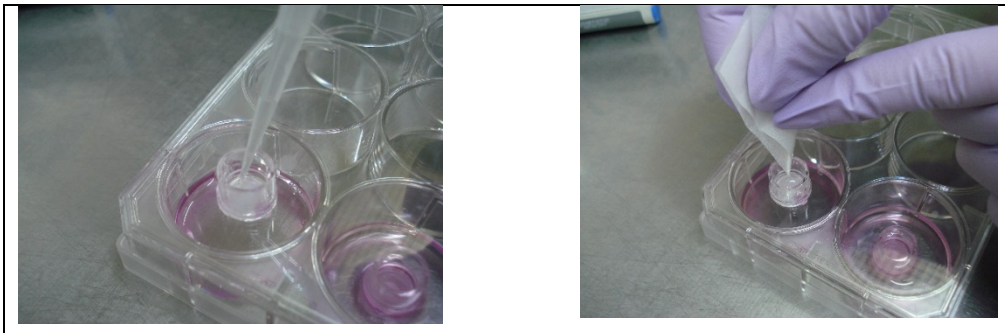
4.7 Application and washing of solid chemicals

- Application of the chemical:

  Start the procedure by taking the cornea model out of the 6-well plate and place it on a cover of the 6-well plate. First of all, wet the surface of the cornea model with 40 μL of DPBS using a micropipette, then apply 40 mg of the test substance atop and in the center of the cornea

model. Then, gently shake the insert to allow the substance to spread evenly over the entire surface (Fig. 10). Once application of test substance is completed in 6 well units, put it in a 5% CO_2 cell incubator at 37℃ for 3 hr ± 5 min.

Record on [Data sheet MCTT HCE™_EI/Sheet/003-v1.7]

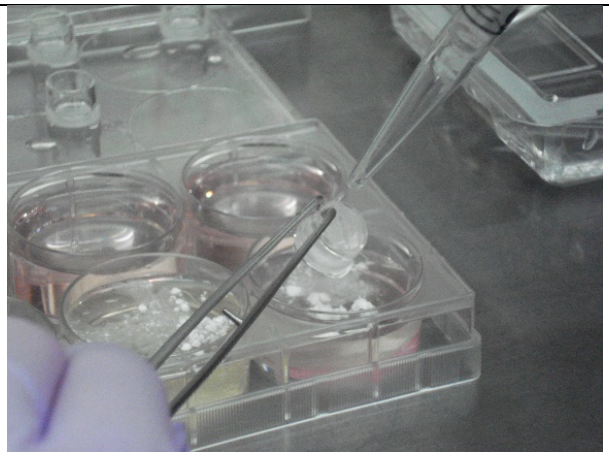


[Fig. 10] Application of solids; First, apply 40 μL of DPBS for wetting the surface of the insert (left), and then apply the weighed test chemical to the insert.

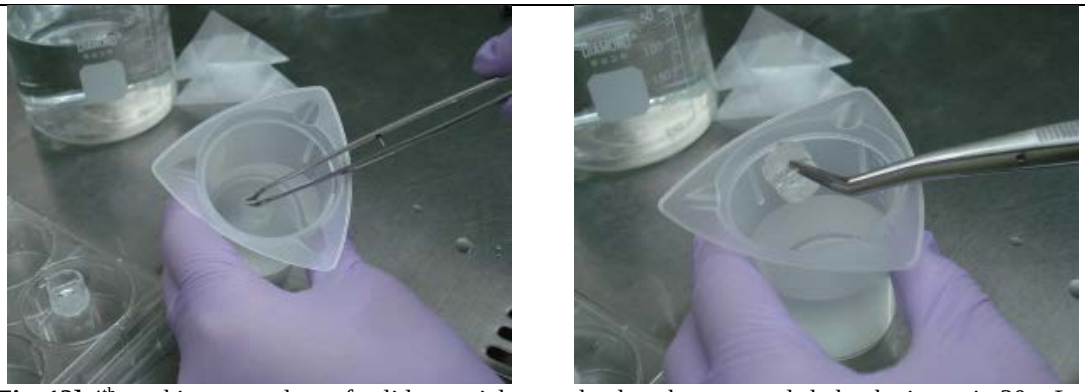
- Washing: The washing process for all 6-wells should be done within 20 min. DPBS for washing should be pre-warmed in a 37°C thermostat before use. During washing, place 2-3 cm of gap between the tip of the pipette aid and the tissue and flow DPBS along the well-wall. Leave remaining DPBS in-between each washing step until the final washing, at which point, remove it all at once.
- Detailed procedures are as follows:

1) After the 3 hr ± 5 min incubation, take the plate out of the incubator. Take 10 mL of DPBS using a pipette aid. Hold the insert with forceps and apply the DPBS to the insert to remove materials inside. The DPBS washing interval between the wells should be the same as the application interval. If the solid material is not removed during this process, gently wipe away only the solid material with a sterile swab. Be careful not to scratch or damage the surface of the cornea model.
2) Repeat step 1).
3) Pick up the insert with the forceps and take 10 mL of DPBS using auto pipette. Turn the insert back and forth when rinsing with DPBS so that the both sides of the insert are rinsed thoroughly.
4) To remove the debris, hold the insert with forceps and gently shake the insert more than 5 times in a 50 mL beaker containing 30 mL of DPBS. Each time the insert gets rinsed in DPBS, take the insert out of the beaker and turn upside down to remove the liquid inside insert. If remaining solid is still noticed by visual inspection, wash it sufficiently at this stage. If the entire solid is not removed even after washing up to 10 times, do not wash it any more so as to prevent damaging the cornea. Unwashed beakers should not be used again for another material.
5) Repeat step 3).
6) Finally, remove residual DPBS inside the insert using a micropipette and by absorbing with sterilized gauze. Remove the DPBS on the outside of the insert as well.

Record on [Data sheet MCTT HCE™_EI/Sheet/003-v1.7]



[Fig. 11] 1st washing procedure of solid material; rinse inside of the insert with 10 mL sterile DPBS.



[Fig. 12] 4th washing procedure of solid material: completely submerge and shake the insert in 30 mL DPBS (left); decant DPBS (right).

4.8 Post-incubation

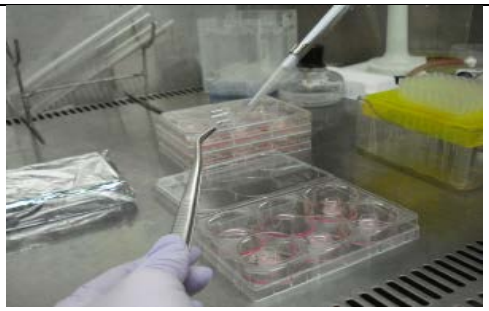
Transfer washed tissue inserts to new 6-well plates pre-filled with 0.9 mL pre-warmed fresh assay medium. Incubate tissue in the incubator for next 16 ± 1 hr.

Record on [Data sheet MCTT HCE™_EI/Sheet/004-v1.7]

4.9 WST-1 Assay

1) After post-incubation, remove medium inside and outside of inserts using pipette.
2) After removing all medium, transfer inserts into 24-well plates, prefilled with 0.2 mL of WST-1 (1:25 diluted). Add 0.1 mL of WST-1 solution inside the insert.
3) Cover the plate with aluminum foil and place in the incubator (37℃, 5% CO_2) for 3 hr ± 5 min.

Record on [Data sheet MCTT HCE™_EI/Sheet/005-v1.7]



[Fig. 13] Remove medium inside and outside of inserts using pipette.

[Fig. 14] Application of WST-1: Fill the 24-well plates with 0.2 mL of WST-1 (1:25 diluted) (left); transfer inserts into each well (center); Add 0.1 mL of WST-1 solution inside inserts (right).

[Fig. 15] Protect the plate from light after application of WST-1 solution.

4.10 Optical density measurements of the extracts

1) After WST-1 incubation is completed, collect all reaction solution (inside and outside of the insert) into the respective well of another plate. Mix the solution to homogeneity by pumping up and down with the micropipette inside the wells of the 24-well plate. Transfer the resulting solutions into microtubes and centrifuge at 200 g for 3 min to remove debris.
2) Transfer 200 μL aliquots of the supernatant (duplicate for each tissue) into a 96-well plate and then check for absence of air bubbles (if air bubbles are present, remove them with a 26 G syringe needle). The recommended plate configuration is given in the spread sheet below.
3) Use working WST-1 solution as blanks. Read OD in a 96-well plate spectrophotometer using wavelength 450 nm. Print measured OD values for records.

Record on [Data sheet MCTT HCE™_EI/Sheet/006-v1.7]

4) 96-well plate design for OD measurement is as follows,

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Blank	Blank	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	
B	NC1	PC1	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Tissue 1
C	NC2	PC2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Tissue 2
D	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	
E	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	
F	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	
G	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	
H	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	

1) Blank: diluted WST-1 solution is used as blank (duplicate)
2) NC, Negative Control: Formazan extract solution of DPBS-treated tissues (1 well/tissue, duplicate/NC)
3) PC, Positive Control: Formazan extract solution of PC-treated tissues (1 well/tissue, duplicate/PC)
4) T, Test substance: Formazan extract solution of each test substance-treated tissues (1 well/tissue, duplicate/T)

5. Results

5.1 Cell viability

Cell viability is calculated with the ratio of OD values of treated tissue over that of NC.

1) Blanks (OD_{blank}): Mean value of OD_{blank} for each plate.
2) Negative control (OD_{NC}): Mean OD value of negative control (OD_{NCraw}) minus mean OD value of blanks (OD_{blank}). Cell viability of negative control should be 100%.

$$'OD_{NC} = OD_{NCraw} - OD_{blank}'$$

3) Positive control (OD_{PC}): Mean OD value of positive control (OD_{PCraw}) minus mean OD value of blanks (OD_{blank}).

$$'OD_{PC} = OD_{PCraw} - OD_{blank}'$$

4) Test substance (OD_T): Mean OD value of test substance (OD_{Traw}) minus mean OD value of blanks (OD_{blank}).

$$'ODT = OD_{Traw} - OD_{blank}'$$

*Cell viability of the test tissue: Calculate the cell viability of a sample using the equation below

$$\text{Cell Viability (\%)} = \frac{\text{Mean Measured } OD_T}{\text{Mean Measured } OD_{NC}} \times 100$$

* OD_{NCraw} : OD value of negative control from spectrophotometer

* OD_{PCraw} : OD value of positive control from spectrophotometer

* OD_{Traw} : OD value of test substance from spectrophotometer

5.2 Criteria for re-test

1) OD value of negative control was below 1.6 or over 3.0.

2) Cell viability of positive control above 35% (CV>35%).

3) Difference of cell viability between duplicate tissues above 20%.

4) Mean value of cell viability was between 30% and 40% for liquid, while 55% and 65% for solid (borderline chemical).

5.3 Data interpretation

Different cut-off values of 35% and 60% indicated the best predictive value for liquid and solid, respectively. According to the EU and the GHS classification (R/38 Category 2 or No Category), an irritant is predicted if the mean relative tissue viability of at least two individual tissues exposed to the test chemical is reduced to 35% for liquid and 60% for solid or below of the mean viability of the negative controls. The test chemical was defined as a non-irritant if the tissue viability was higher than 35% and 60% for liquid and solid, respectively. Otherwise, it was determined to be an irritant.

	Prediction model	Classification
Liquid	Mean tissue viability is ≤ 35%	Irritant (I) R38
	Mean tissue viability is > 35%	Non-Irritant (NI)
Solid	Mean tissue viability is ≤ 60%	Irritant (I) R38
	Mean tissue viability is > 60%	Non-Irritant (NI)

6. Experimental Performance Standards

This Assay should be performed according to Good laboratory practice (GLP).

7. List of Data Sheet

- Delivery
- Pre-incubation
- Treatment
- Post-incubation
- WST-1 assay
- Result of Optical Density
- Preparation of reagents
- Record of test substances
- Record of results for Excel input

Data Sheet – Delivery

Test No.		Test Date	20 . .

Delivery [Day 1 – 20 . .]

Receipt of MCTT HCE™ tissues		
Date	Time	Recipient
20 . .	h min	

Record paper for temperature

Condition of MCTT HCE™ tissues

Lot no.:

Number of tissue: _____tissue (24 tissue/plate)

[Plate A]

	1	2	3	4	5	6
A						
B						
C						
D						

[Plate B]

	1	2	3	4	5	6
A						
B						
C						
D						

Checking point

- air bubbles : (a)
- flat surface : (f)
- extensive moisture on the surface : (e)
- condition of agarose gel : (c)
- no finding: (-)

Draw a diagonal line on the unused plate diagram

KIT Components

Assay medium	㎖ x = ㎖		
Expiration Date	20 . .		

Study Personnel		Signature		Date	20 . .
Study Director		Signature		Date	20 . .

MCTT HCE™_EI/Sheet/001-v1.7

Data Sheet – Pre-incubation

Test No.		Test Date	20 . .

Pre-incubation [Day 1 ~ Day 2 – 20 . . ~ 20 . .]					
Start of pre-incubation time	:	Study Personnel	(sign)	Date	20 . .
End of pre-incubation time	:	Study Personnel	(sign)	Date	20 . .

Study Personnel		Signature		Date	20 . .
Study Director		Signature		Date	20 . .

MCTT HCE™_EI/Sheet/002-v1.7

Data Sheet – Treatment

Test No.		Test Date	20 . .

Treatment [Day 2 – 20 . .]

Test Chemical Application

 # No of chemical: total _____samples with NC and PC

Code	Liquid(L) /Solid(S)	Treatment time		Start of Rinsing		Post-incubation	Remarks
		Well 1	Well 2	Well 1	Well 2		
NC		: :	: :	: :	: :	:	
PC		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	
		: :	: :	: :	: :	:	

Study Personnel		Signature		Date	20 . .
Study Director		Signature		Date	20 . .

Data Sheet – Post-incubation

Test No.		Test Date	20 . .

Post-incubation [Day 2 ~ Day 3 – 20 . . ~ 20 . .]					
Start of post-incubation time	:	Study Personnel	(sign)	Date	20 . .
End of post-incubation time	:	Study Personnel	(sign)	Date	20 . .

Study Personnel		Signature		Date	20 . .
Study Director		Signature		Date	20 . .

MCTT HCE™_EI/Sheet/004-v1.7

Data Sheet – WST-1 assay

Test No.		Test Date	20 . .

WST-1 assay [Day 3 – 20 . .]

Application [# No of chemical: total _____samples with NC and PC]

Code	End of post-incubation	Start of WST-1 incubation	End of WST-1 incubation	OD reading	Remarks
NC	:	:	:		
PC	:	:	:		
	:	:	:		
	:	:	:		
	:	:	:		
	:	:	:		
	:	:	:		
	:	:	:		
	:	:	:		
	:	:	:	:	
	:	:	:		
	:	:	:		
	:	:	:		

Study Personnel		Signature		Date	20 . .
Study Director		Signature		Date	20 . .

MCTT HCE™_EI/Sheet/005-v1.7

Data Sheet – Results of Optical Density

Test No.		Test Date	20 . .

Results of Optical Density [Day 3 – 20 . .]

[Identification]

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Blank	Blank	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	
B	NC1	PC1	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Tissue 1
C	NC2	PC2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Tissue 2
D	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	
E	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	
F	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	
G	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	
H	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	

[96-well plate spectrophotometer OD values]

Study Personnel		Signature		Date	20 . .
Study director		Signature		Date	20 . .

Data Sheet – Preparation of reagents

Test No.		Test date	20 . .

WST-1 [Day 3 - 20 . .]

[WST-1 solution in PBS]

- ✓ Supplier:
- ✓ CAS No.:
- ✓ Lot No.:
- ✓ Expiration date:
- ✓ Volume:
- ✓ PBS volume added:

Study Personnel		Signature		Date	20 . .
Study director		Signature		Date	20 . .

MCTT HCE™_EI/Sheet/007-v1.7

Data Sheet – Record of test substances

Test substance name or code			
Description of Physical consistence (color etc.)			
Total weight test substance and vial(g)			
Storage condition		Storage No	
Receipt date	20 . .	Expiration date	20 . .

Date	Initial quantity	Used quantity	Residual quantity	Study personnel
20 . .				
20 . .				
20 . .				
20 . .				
20 . .				
20 . .				
20 . .				
20 . .				
20 . .				
20 . .				
20 . .				
20 . .				
20 . .				
20 . .				

Study director		Signature		Date	20 . .

MCTT HCE™_EI/Sheet/008-v1.7

Data Sheet – Record of results for Excel input

HCE DATA SHEET_[Day3]_WST-1 assay

Date	YYYY.MM.DD
Lot No.	HCM
Operator	

Red_Box안만 입력 및 변경

TEST MATERIALS

Code	Test Material	Conc. (%)
NC	PBS	100
PC	SDS	2
T1		
T2		
T3		
T4		
T5		
T6		
T7		
T8		
T9		
T10		
T11		
T12		
T13		
T14		
T15		

WST-1 - PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Blank	Blank											
B	NC1	PC1	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Tissue 1
C	NC2	PC2	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Tissue 2
D	T11	T12	T13	T14	T15								Tissue 1
E	T11	T12	T13	T14	T15								Tissue 2
F													
G													
H													

WST-1 - IMPORT (OD)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

RESULTS - OD values (OD-blank)

Blank	0	0	0.000

OD	MCTT HCE			
	1	2	Mean	STD
NC	0.000	0.000	0.000	0.00
PC	0.000	0.000	0.000	0.00
T1	0.000	0.000	0.000	0.00
T2	0.000	0.000	0.000	0.00
T3	0.000	0.000	0.000	0.00
T4	0.000	0.000	0.000	0.00
T5	0.000	0.000	0.000	0.00
T6	0.000	0.000	0.000	0.00
T7	0.000	0.000	0.000	0.00
T8	0.000	0.000	0.000	0.00
T9	0.000	0.000	0.000	0.00
T10	0.000	0.000	0.000	0.00
T11	0.000	0.000	0.000	0.00
T12	0.000	0.000	0.000	0.00
T13	0.000	0.000	0.000	0.00
T14	0.000	0.000	0.000	0.00
T15	0.000	0.000	0.000	0.00

RESULTS - Cell vaibility

Cell viability (%)	MCTT HCE						Re-test			
	1	2	Mean	SD	Difference	Prediction	Diff. > 20	40≤CV<50	NC OD	PC CV
NC	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
PC	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T1	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T2	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T3	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T4	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T5	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T6	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T7	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T8	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T9	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T10	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T11	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T12	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T13	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T14	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!
T15	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Re-test	#DIV/0!

* 4항목 중 하나라도 re-test라고 표기되면 반드시 re-test를 실시해야만 함.