APPENDIX H

EpiOcular assay protocol

1. Tissue

EpiOcular[TM] OCL-200 tissues were purchased from MatTek Corporation (Ashland, MA, USA) with medium and other reagents as part of the EpiOcular assay package.

2. EpiOcular assay protocol

EpiOcular assay was examined based on the method of Kaluzhny et al. (2011) with minor modification. Briefly, each EpiOcular tissue was transferred to a 6-well plate well containing 1.0 ml of assay medium. The tissues were incubated for overnight at 37±1°C, under 5% CO_2 and 95% air atmosphere. After this incubation, tissues were pre-treated for 30 ± 2 minutes with 20 μL of DPBS (-) (Sigma-Aldrich Co.). Exposure condition was separated by the form of test substances. For the liquid substances, the 50μL of test substances were applied topically onto each tissue and tissues were incubated for 30 ± 2 minutes. Each test substance and control were tested with duplicate tissues. After exposure, the tissues were rinsed by dipping, swirling, and decanting three times using three different beakers filled with DPBS (-). The tissue was then transferred to a 12-well plate well containing 5.0 ml of assay medium and immersed for 12 ± 2 minute at room temperature. After discarding the medium on the tissues by decantation, the tissues were transferred to a 6-well plate containing 1 ml of assay medium. Then the tissues were incubated for 120 ± 5 minutes (post incubation). For solid substances, 50 mg of test substances were applied to the each tissue. The treated tissues were incubated for 90 ± 5 minutes in the same way. The rinsing and immersing were the same procedure described for the liquids. However, solids-treated tissues were incubated for 18 ± 0.25 hours as post incubation. Each tissue was transferred to a 24-well plate well containing 0.3 ml of assay medium containing 1.0 mg/ml MTT and incubated for 180 ± 10 minutes. Then, the tissues were transferred to a 24-well plate containing 2 ml of isopropanol. After leaving the tissues for two hours at room temperature, 200 μl aliquots from each well were transferred to a 96-well plate in duplicate. The absorbance was measured at 550 nm with a plate reader. The ratio of absorbance (%) on each test substance to that of control was represented as relative viability. The mean viability values for two tissues were obtained.

3. Category classification of eye irritation by the EpiOcular assay

Category classification of eye irritation by the EpiOcular assay ("NI" or "I") was determined based on the relative viability. A test chemical that had a relative viability of 60% or less was categorized as an "I" and a concentration of test material that had a relative viability greater than 60 % was categorized as a "NI".

4. Direct reduction of MTT by test substance

It should be noted that there are some chemicals which have a ability to directly reduce MTT. These abilities may cause the false negative results or under-prediction. To avoid these false results, it is necessary to know whether the test substance can reduce MTT directly. A 1 mg/mL MTT solution is prepared and 100 μL or 100 mg of test substance was added to 1 mL of MTT solution. After incubation in the dark at room temperature for 60 min, the colors of mixed solution were checked. If the solution color turned blue or purple, the test substance has reduced the MTT; the absence of darkening indicates that the test substance did not directly reduce MTT.

If the test substance reduced MTT directly, the false negative result may be obtained depending on the binding capacity of test substance to the tissue. In the case, a functional check by using freeze-killed tissue controls must be performed. Freeze-killed tissues were treated according to the standard testing procedure provided by the manufacturer. If the test materials bind to the tissue and MTT reduction occurs, corrective measure was used.