Validation Study on Five Cytotoxicity Assays by JSAAE - VII.

Details of the MTT Assay

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Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; ED50, 50% effective dose; JSAAE, Japanese Society of Alternatives to Animal Experiments; PBS(-), Ca++, Mg++-free Dulbecco's phosphate-buffered saline; PFD, power for distinction.

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The inter-laboratory validation study on 5 cytotoxicity assays conducted by JSAAE has been described in the preceding articles (see Validation Articles I - VI in this issue). Presented here are precise data and the protocols on the MTT assay with two cell lines, HeLa S3 (SC) and SQ-5. Hand-plotted dose-response curves provided unexpected information otherwise difficult to assume from calculated ED50 values and accompanying outputs from the logistic analysis program, LAP-JSAAE. The characteristics of data files from 18 laboratories were visualized together with all the log(ED50) values. Variations on negative controls in the assay revealed technical competence characteristics of each laboratory. Generally low OD590 values have been obtained in the present MTT assay but high inter-laboratory reproducibility was observed with both cell lines. These results will supplement our understanding on the MTT assay carried out in the large-scale inter-laboratory validation.

**Introduction**

As has been described in the preceding articles (see Validation Articles in this issue, Ohno, T., et al., 1997), the Japanese Society of Alternatives to Animal Experiments (JSAAE) organized a first step inter-laboratory validation study on 5 cytotoxicity assays in October 1992, since a battery of appropriate cytotoxicity assays is expected to be the core alternative to the in vivo Draize test in the near future (Ohno, T., et al., 1995).

MTT assay refers to the cytotoxicity test that uses a dye, 3-(4,5-dimethylthiazolyl-2-yl) 2,5 diphenyltetrazolium bromide (MTT). This assay is based on the reduction of the soluble yellow MTT tetrazolium salt to a blue insoluble formazan product by mitochondrial succinic dehydrogenase (Mosmann, 1983). Since this assay showed the advantage of easy handling and high sensitivity, it has been widely used for cytotoxicity determinations. This assay has also been utilized to detect cytotoxic lymphokines produced by human lymphocytes (Green, 1984), and to measure cell activation (Gerlier and Thomasset, 1986). The MTT assay was also vali-
dated as a candidate alternative to the Draize eye irritation test by another program in Japan (Ohno, Y., et al., 1995; Kojima, et al., 1995). This report supplements the Validation Article I in this issue. We describe here the precise results of the MTT assay carried out in the present validation study, including the protocols, representative hand-plotted dose-response curves, extraordinary data files, ED50 values on each accepted data files, and discussions.

**Materials and Methods**

*Chemicals and Cell Lines*

The six chemicals tested and their allocation to 7 coded samples were described in the preceding paper (see Validation Article I). They were Tween 20 (#1, non-irritant), Tween 80 (#2, non-irritant), sucrose fatty acid ester (#3, irritant), propylene glycol (#4, non-irritant), cetlypyridinium chloride monohydrate (#5, severe irritant), sodium lauryl sulfate (#6, moderate irritant) and Tween 20 (#7, same as #1, an intrinsic masked reference chemical). The symbol # and numbers in parentheses are double-mask codes given to these chemicals by the Chemical Bank and the Working Group before their transfer to each laboratory. The two samples (#1 and #7) were the same chemical and were supplied to determine intra-laboratory variation of the final data.

The HeLa S3 (SC) cell line, used in other assays in the present validation study, together with SQ-5 cells from human lung squamous carcinoma, were used in this MTT assay. Both cell lines were supplied from RIKEN Cell Bank.

*Data analysis*

The submitted data files were first inspected manually to detect simple recording errors and then the hand-plotted dose-response curves drawn using mean values at each chemical concentration were checked visually. The ED50 values of possibly acceptable candidate data files (see Validation Article I, Table 3 and 5) were analyzed by computer-assisted LAP-JSAAE program (see Validation Article II) which is based on calculation of dose-response by the non-linear least squares method. Finally, general statistical analyses were applied to the calculated data.

**Protocol of the MTT assay**

1. **Materials**

   1-1. Cultures

   (1) Culture medium

   1) HeLa S3 (SC) cells: Eagle’s minimum essential medium (MEM, autoclavable, containing 1.8 mg/ml of NaHCO₃, pH 7.3 - 7.6) containing 10%(v/v) calf serum (CS)

   2) SQ-5 cells: MEM (autoclavable, containing 1.8 mg/ml of NaHCO₃, pH 7.3 - 7.6) containing 10%(v/v) fetal bovine serum (FBS)

   (2) Subculture

   Both cell lines were used in the assay at the log phase. Subculture was performed with the dissociation medium (see below). After dissociation of the cells, 2x10⁵ cells were seeded into a 60-mm dish containing 5 ml of culture medium. HeLa S3 (SC) cells were subcultured every 3 - 4 days.

   1-2. Reagents

   (1) PBS(-): Ca⁺⁺, Mg⁺⁺-free Dulbecco's phosphate-buffered saline

   (2) Dissociation medium: 0.05% trypsin and 0.02% EDTA dissolved in PBS(-)

   (3) Neutralization medium: Use culture medium for both cell lines.

   (4) MTT solution: MTT (commercial grade) was dissolved in PBS(-) at 5.5 mg/ml, then the MTT solution was sterilized through a 0.45 µ-pore size membrane filter. This stock solution was stored in the dark at 4 Åé. Before use, the stock solution was diluted to one-tenth with the culture medium.

   (5) 4N HCl: HCl was diluted to 4N with distilled water.

   (6) Acid-isopropanol: 4N HCl was mixed with isopropanol in the ratio of 1:100 by volume.

2. **Test chemical preparation**

   Test chemicals were dissolved in PBS(-), ster-
ilized by membrane filtration, and then serially diluted with culture medium. Since the optimal concentration range was different with each chemical, at first the practically attainable maximum concentration was determined. Then several 10-fold serial dilutions were made and the assay was carried out with a wide dose range spanning approximately 5-6 orders of magnitude. From the results of this preliminary test, the maximal concentration was set (the lowest concentration able to kill 100% of the cells) and 2-fold serially diluted. The 2 to 3 orders of magnitude of the test concentration range was covered by this serial dilution. Finally the assay was repeated to be able to obtain more than 3 points of cytotoxic effect between 20-80% of the maximum cytotoxicity.

3. Procedure for the MTT assay

3-1. Cell seeding

(1) The cells at approximately 80% confluence were selected, i.e., logarithmically growing cells. Both cell lines were trypsinized from preculture bottles before adding to the 96-well microplate.

1) The cell suspensions (3x10^4 cells/ml for HeLa S3 (SC) and for SQ-5) were prepared.

2) A 100 µl aliquot of the cell suspension was gently introduced into each well of the test plate.

3) The cells were then cultured in a CO₂ (5% in air) incubator overnight.

3-2. Treatment

(1) After incubation, culture medium was discarded.

(2) One hundred µl of the test chemical dissolved in culture medium was added into each well and then cultured for a further 48 hrs.

3-3. MTT staining

(1) After the 48-hr incubation, 90 ml of culture medium containing the test chemical was discarded and then 90 ml of fresh culture medium was added.

(2) Ninety µl of the medium was discarded and then 90 ml of medium containing MTT at the concentration of 0.55 mg/ml was added into each well. The plate was incubated further for 4 hrs.

(3) The medium containing MTT was discarded by inverting the plates and then each well was washed with 200 ml of PBS(-). The test plate was inverted on paper towels to absorb the remaining drops of water.

(4) Two hundred µl of acid-isopropanol was added to each well.

(5) To extract and solubilize the formazan, the test plate was agitated by microplate shaker for 10 min.

(6) OD590 was measured by an automatic microplate reader.

3-4. Worksheet for data collection

An example of the worksheet (Fig. 1) for data collection was shown to each laboratory. However, each laboratory was allowed to make its own style of the worksheet but using pre-set positions for raw data recording. These worksheets were formed using personal computer software. All the worksheets were first transferred to MS-DOS text files and incorporated into the software Excel version 4.0, then recalculated.

3-5. Calculation of cytotoxicity

\[
\text{Cytotoxicity (\%) = } \frac{(a-b)}{(c-b)} \times 100
\]

where a is OD590 value derived from a well added with a test chemical, b is mean OD590 value derived from blank wells, c is mean OD590 value derived from control wells (i.e., added culture medium as a test chemical).

An observed point in the assay was defined as an average of cytotoxicity derived from replicate determinations for a concentration of the test chemical.

Results and Discussion

Quality of raw data

To understand the characteristics of the raw data and resulting dose response curves in all
submitted data files*, hand-plotted dose response curves were drawn for the mean values at each chemical concentration. At this preliminary step, Lab-23 was found to have carried out the MTT assay without including the positive control, negative control and blank wells in the same plate for both cell lines. Lab-24 carried out the assay with only one plate for each of the two cell lines. Lab-3 carried out the assay with HeLa S3 (SC) cells in the medium containing FBS but not CS, while Lab-42 used SQ-5 cells in the medium containing CS but not FBS. Lab-35 used

These are severe violations of the common rule [3] described in Validation Article I in this issue. Further, Lab-3 carried out the assay with HeLa S3 (SC) cells in the medium containing FBS but not CS, while Lab-42 used SQ-5 cells in the medium containing CS but not FBS. Lab-35 used

* Copies of all the hand-plotted dose-response curves are available on request.
DMEM but not MEM. These are also violations of the protocol. The 56 data files from these laboratories were not accepted for further analyses (see Validation Article I in this issue, Table 3).

Fig. 2 illustrates two examples of the hand-plotted dose-response curves from the MTT assay. Titles indicate the cell line used, and the code number of tested chemical. The plots obtained provided information difficult to assume from calculated ED50 values and accompanying outputs from the logistic analysis program, LAP-JSAAE. As shown in Fig. 2a, Lab-35 did not observe a dose-response curve that crossed over the 50% viability line for chemical #4. The extraordinary results were also observed for chemical #5 by Lab-3 (Fig. 2b). These results suggested that the preparation technique of the test chemical solution was inappropriate in both laboratories. These types of data have been excluded in the program LAP-JSAAE.

Characteristics of submitted data files and log(ED50) values

Fig. 3 illustrates the characteristics of data files for the MTT assay with log(ED50) values calculated from the accepted data files using LAP-JSAAE program. Among the 20 laboratories that participated initially, two (Lab-43 and Lab-47) did not submit any data. Lab-31 did not submit any results on the assay with SQ-5 cells. Lab-4 lacked the data files of chemicals #3, #5, and #6 tested with HeLa S3 (SC) cells and of chemicals #3 and #6 tested with SQ-5 cells. Due to protocol violation, all data files from 3 laboratories (Lab-23, -24 and -35), data files from Lab-3 assayed with HeLa S3 (SC) cells, and data files from Lab-42 with SQ-5 cells, were excluded from the analysis. Thus, the performance rates of runs were 88% and 84%, respectively, for 123 files and 117 files submitted on HeLa S3 (SC) cells and SQ-5 cells, respectively (see Validation Article I in this issue, Table 3).

The capital letters in shaded boxes are check codes in the LAP-JSAAE program. For example, the file submitted from Lab-27 for chemical #4 tested with HeLa S3 (SC) cells was marked with check code A because we failed to calculate ED50 by LAP-JSAAE program due to large variation of the raw data. The check code A was also marked in data submitted from Lab-3, -45 and -46; the Check code C, from Lab-3, -19, -28, -38 and -42; the check code F, from Lab-19 and -42.

Finally, a total of 89 files for HeLa S3 (SC) cells and 83 files for SQ-5 cells was accepted for further box-whisker-plot analysis (Fig. 4). Performance rates based on the finally accepted data files of the assay for HeLa S3 (SC) cells and SQ-5 cells were 64% and 59%, respectively (see Validation Article I in this issue, Table 3).

Technical stability of MTT assay in participating laboratories

Among the 20 laboratories that participated initially, 12 received the technology transfer (see Validation Article I, Table 2). Of these, recipients from 2 laboratories did not conduct the MTT assay but employed a technician. Six laboratories (Lab-13, -19, -27, -28, -35 and -38) did not receive the technology transfer but performed the assay. Lab-43 and Lab-47 did receive the technology transfer but did not submit any data.

To detect technical stability of the MTT assay within a laboratory, the variation of the negative controls on each assay and on each plate in an assay was calculated (Table 1). With HeLa S3 (SC) cells, Lab-14 was shown to have carried out the assays with the largest variation for negative controls. Their coefficient of variation was 42.5% in the 5 assays for 7 chemicals with the average OD590 value 0.160. The actual OD590 values of their negative controls fluctuated from 0.119 to 0.327 (Table 2).

The larger variation among assays for different chemicals and the smaller variation among the plates in an assay was generally observed in OD590 values of the negative controls. The mean value and median value of OD590 values of all participants were 0.549 and 0.457, respectively.

Influence of cell lines

As shown in the box-whisker-plots (Fig. 4), influence of cell lines HeLa S3 (SC) and SQ-5
Fig. 2. Hand-plotted dose-response curves of the MTT assays drawn at the time of submission of data files.

Hand-plotted dose-response curves were drawn to understand the gross characteristics of data files at their submission to The Working Group. a (this page), MTT assays with HeLa S3 (SC) cells on chemical #4 (propylene glycol). b (next page), MTT assays with SQ-5 cells on chemical #5 (cetylpyridinium chloride monohydrate). A point indicates the mean of viability for an observed concentration in the assay. The figure in an open symbol indicates laboratory number.
Fig. 3. Log(ED50) values and visualized characteristics of submitted data files.

- Open boxes are the files finally accepted for comparison of inter-laboratory variation of log(ED50) values without any difficulty in obtaining ED50 values. Asterisks indicate that the log(ED50) value became an outlier in the box-whisker plot analysis as shown in Fig. 4.
- No data file was submitted.
- A data file(s) was submitted but not useful because of severe violation of the protocol.
- A check code was found in data files of chemical #2, #3, #4, #5, or #6.

Definition of error codes are:
Code-A : Calculation of an ED50 value failed because of wide variation of data.
Code-C : No observed point was found in the data file between 20-80% of the maximum effect.
Code-F : RMS is 10 or more. RMS indicates the degree of deviation of the observed dose-response relationship from the logistic model. See details in Validation Article II in this issue.
### Table 1. Variation of the negative controls in MTT assays

<table>
<thead>
<tr>
<th>Lab No.</th>
<th>HeLa S3 (SC) cells</th>
<th>SQ-5 cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assays</td>
<td>OD590&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>0.350</td>
<td>0.027</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>0.856</td>
<td>0.081</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>0.932</td>
<td>0.072</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>0.436</td>
<td>0.170</td>
</tr>
<tr>
<td>13</td>
<td>6</td>
<td>0.358</td>
<td>0.084</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>0.160</td>
<td>0.068</td>
</tr>
<tr>
<td>19</td>
<td>4</td>
<td>0.576</td>
<td>0.114</td>
</tr>
<tr>
<td>27</td>
<td>5</td>
<td>0.692</td>
<td>0.008</td>
</tr>
<tr>
<td>28</td>
<td>7</td>
<td>0.477</td>
<td>0.024</td>
</tr>
<tr>
<td>31</td>
<td>7</td>
<td>0.427</td>
<td>0.140</td>
</tr>
<tr>
<td>38</td>
<td>6</td>
<td>0.372</td>
<td>0.035</td>
</tr>
<tr>
<td>42</td>
<td>1</td>
<td>1.210</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>4</td>
<td>0.244</td>
<td>0.095</td>
</tr>
<tr>
<td>46</td>
<td>4</td>
<td>0.591</td>
<td>0.225</td>
</tr>
<tr>
<td>Mean</td>
<td>0.549</td>
<td>0.289</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean OD590 of the negative controls in the assays  
<sup>b</sup> Standard deviation  
<sup>c</sup> Coefficient of variation among independent assays calculated from the mean negative control value of each assay  
<sup>d</sup> Coefficient of variation among the negative control wells within an assay: Mean of the assays

### Table 2. Intra-laboratory variation of the negative controls in MTT assays with HeLa S3 (SC) cells carried out in Lab-14.

<table>
<thead>
<tr>
<th>Assay for Chemical</th>
<th>OD590</th>
<th></th>
<th></th>
<th>Mean&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CV-2&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td>Plate-1</td>
<td>Plate-2</td>
<td>Plate-3</td>
<td>Mean&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CV-2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>#1</td>
<td>0.135</td>
<td>0.124</td>
<td>0.122</td>
<td>0.127</td>
<td>0.007</td>
<td>5.5 %</td>
</tr>
<tr>
<td>#2, 3</td>
<td>0.327</td>
<td>0.278</td>
<td>0.235</td>
<td>0.280</td>
<td>0.046</td>
<td>16.4</td>
</tr>
<tr>
<td>#4, 5</td>
<td>0.137</td>
<td>0.109</td>
<td>0.110</td>
<td>0.119</td>
<td>0.016</td>
<td>13.4</td>
</tr>
<tr>
<td>#6</td>
<td>0.150</td>
<td>0.146</td>
<td>0.122</td>
<td>0.139</td>
<td>0.015</td>
<td>10.9</td>
</tr>
<tr>
<td>#7</td>
<td>0.152</td>
<td>0.119</td>
<td>0.135</td>
<td>0.135</td>
<td>0.017</td>
<td>12.2</td>
</tr>
<tr>
<td>Mean&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.160</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.7 %</td>
</tr>
<tr>
<td>SD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.068</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>42.5 %</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean OD590 of the negative controls in each assay  
<sup>b</sup> Standard deviation  
<sup>c</sup> Coefficient of variation (%) among the negative control wells (one well/plate) within an assay (3 plates/assay)  
<sup>d</sup> Calculated from the 5 assays  
<sup>e</sup> Coefficient of variation (%) among independent 4 assays calculated from the mean negative control value of each assay
was not observed in the MTT assay. Although we have not standardized the lot of MEM, CS, and FBS, the length of each box that shows hinge-spread is relatively small compared to that obtained from other cytotoxicity assays. The mean hinge-spreads for the chemicals were 0.31 and 0.22 in the MTT assay with HeLa S3 (SC) cells and SQ-5 cells, respectively (see Validation Article I in this issue, Table 7). However, the "power for distinction" (see details in Validation Article II) of the MTT assay with HeLa S3 (SC) cells was smaller than that of both the CF assay and the CV assay with HeLa S3 (SC) cells.

Conclusion

As described in Validation Article I in this issue, the CF, CV, MTT, and NR assays are basically recommendable from the view point of performance of the assays. However, from the view points of inter-laboratory variation and the "power for distinction" of toxicities among chemicals, the MTT assay was revealed not to be the optimal one.

Acknowledgment

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