Validation Study on Five Cytotoxicity Assays by JSAAE - VI.

Precise Results of the LDH Release Assay

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Abbreviations: LDH, lactate dehydrogenase; Detergent C, lauryl dimethyl methyl amino betaine acetate; ED50, 50% effective dose; JSAAE, Japanese Society of Alternatives to Animal Experiments; PBS(-), Ca**, Mg** - free Dulbecco’s phosphate-buffered saline.
Abstract

The inter-laboratory validation study on 5 cytotoxicity assays conducted by JSAAE has been described in the preceding articles (see Validation Articles I - V). Presented here are precise data and the protocol of the LDH release assay with two cell lines, HeLa S3 (SC) that is common to the other 4 assays and SQ-5 that is rich in LDH activity. The performance rates in each of the four LDH-release sub-divided assays were extremely low. We concluded that the present LDH release assay is practically immature, although the assay is very informative in principle not only on the cell growth inhibition but also on the cell lysis caused by the tested chemicals.

Introduction

As has been described in the preceding articles (see Validation Articles I - V), 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. LDH release assay measures the activity of lactate dehydrogenase (LDH) released from the cytoplasm of the chemical-treated cells or remaining inside the chemical-treated cells after dissolving the cytoplasmic membrane with a detergent solution. Thus, it could measure both cell killing and cell viability after the chemical treatment in the same culture in an assay if the culture medium and the cell layer were properly separated. Since the activity of cellular LDH corresponds to the number of cells in the culture, it is also a measure of cell growth after a relatively long-term treatment of the cells with chemicals.

This report supplements the preceding articles (see Validation Articles I - V). LDH release assay consists of 4 sub-assays to assess the chemical cytotoxicities from several view points, namely, very acute detergent-like cytolytic activity after 20-min incubation at room temperature (LDH-1 assay, Sasaki et al., 1992), acute cytotoxic activity (i.e., substantial cell killing activity) after 2 hr incubation at 37°C (LDH-2A assay), cell growth inhibition in 48 hr culture (LDH-2B assay), and accumulated cell killing in 48 hr culture (LDH-2B assay) (Wang et al., 1993). We describe here precise results of the four sub-assays carried out in the validation study, including the protocol, representative hand-plotted dose-response curves, extraordinary data files, ED50 values on each accepted data files, and discussions.

Materials and Methods

The six chemicals tested and their allocation to 7 coded samples were described in the preceding paper (see Validation Article I in this issue). They are Tween 20 (chemical #1, non-irritant), Tween 80 (chemical #2, non-irritant), sucrose fatty acid ester (chemical #3, irritant), propylene glycol (chemical #4, non-irritant), cetylpyridinium chloride monohydrate (chemical #5, severe irritant), sodium lauryl sulphate (chemical #6, moderate irritant) and Tween 20 (chemical #7, equal to #1). The symbol # and numbers in parentheses are double-mask-codes given by the Chemical Bank and the Working Group to these chemicals before their transfer.
to each laboratory. The two samples (#1 and #7) were the same chemical and were supplied as an intrinsic masked reference chemical 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 the human squamous carcinoma cell line, SQ-5, were used in the present LDH release assay. Both cell lines were provided as live cultures from RIKEN Cell Bank, Tsukuba. Possibly acceptable candidate data files (see Validation Article I in this issue, Table 3 and 5) were analyzed by computer-assisted LAP-JSAAE program (see Validation Article II in this issue) which is based on calculation of dose-response by the non-linear least squares method.

Protocol of the LDH release assay

1. Materials
1-1. Cultures
(1) Culture medium
   Maintenance 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)
   Experimental medium: MEM (autoclavable, containing 1.8 mg/ml of NaHCO₃, pH 7.3 - 7.6) containing 5%(v/v) fetal bovine serum (FBS)
(2) Subculture for maintenance of the cell lines
   Subculture was performed with the dissociation medium [0.05% trypsin and 0.02% EDTA dissolved in PBS(-)]. After dissociation of the cells, 2x10⁵ cells were seeded into a 60-mm dish containing 5 ml of maintenance medium. The cells were subcultured every 3 - 4 days.
1-2. Reagents
   (1) PBS(-): Ca++, Mg++-free Dulbecco's phosphate-buffered saline
   (2) Detergent C: Lauryl dimethyl methyl amino betaine acetate (Kao Co. Ltd., Tokyo)
   (3) LDH assay kit for toxicology (Kyokuto Pharmaceutical Co. Ltd., Tokyo)

2. Test chemical preparation
   Test chemicals were dissolved in PBS(-) except where indicated, sterilized by membrane filtration, then serially diluted with PBS(-) for LDH-1 assay and with the experimental medium for LDH-2A, -2B, and -2C assays. Since the optimum concentration range was different for each chemical, the practically attainable maximum concentration was first set up followed by several 10-fold serial dilutions; the assay was then carried with a wide dose range spanning approximately 5-6 orders of magnitude. From the results of this preliminary test, the maximum concentration that is the lowest concentration possible to kill 100% of the cells was set and which was 2-fold serially diluted. The span of 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 falling between 20-80% of the maximal cytotoxic effect.
   Test chemicals were prepared at double concentration of the finally expected concentration. One hundred µl samples were added to each well of a 96-well plate.

3. Controls
   (1) Positive control
      Detergent C was dissolved in PBS(-) at 0.4%(w/v); this served as the positive control.
   (2) Negative controls
      For LDH-1 assay:
      Negative control-1: PBS(-) which contained no test sample.
      Negative control-2: Blank well containing no cells.
      For LDH-2A, -2B, and -2C assays:
      Negative control-1 and -2: Experimental medium which contained no test sample.

4. Procedure for LDH-1 assay

4-A. Detection of direct inhibitory activity of
test samples on LDH
(1) Logarithmically growing cells at subconfluence were washed twice with PBS(-).
(2) The cells were frozen at -20 °C after adding 0.1 ml of PBS(-) per 1 cm² culture surface. The dish was kept horizontal during freezing.
(3) The culture dish was thawed at 37°C.
(4) Steps 2 and 3 were repeated twice.
(5) The supernatant of the thawed cells was separated by centrifugation at 3,000 rpm (1,200xg) for 5 min. This supernatant was diluted arbitrarily with PBS(-) and defined as LDH solution-1.
(6) The LDH solution-1 was further diluted with PBS(-) and assayed with the LDH assay kit for toxicology. The supernatant was further diluted to OD560 of 1.5. This solution was defined as LDH solution-2.
(7) Twenty five µl of the LDH solution-2 was added to each well of enzyme immunoassay (EIA) plate. Diluted test samples, 25 µl/well, were added to the LDH solution-2. At this step, both Negative control-1 and Negative control-2 were also prepared.
(8) The well was mixed with a plate mixer for 30 seconds and stood at room temperature.
(9) The dye solution in the LDH assay kit was diluted with the dilution buffer in the kit as indicated by the manufacturer.
(10) This diluted dye solution was poured into a V-bottom reservoir, and then was added at 50µl/well just after 20 min from performing the above step (7).
(11) The plate was incubated for 45 min at room temperature.
(12) The stopping solution provided in the kit was added at 100 µl/well.
(13) Added solutions were mixed with a plate mixer for 30 seconds.
(14) OD560 was measured with a microplate reader.

4-B. Preculture for the test
In the experimental medium, HeLa S3 (SC) and SQ-5 cells were suspended at 4x10⁴ cells/ml and 2x10⁴ cells/ml, respectively. This cell suspension (100µl) was seeded to each well of a 96-well test plate. In the well for the Negative control-2, the experimental medium was added in place of the cell suspension.

4-C. The test of LDH-1 assay
(1) Eighty µl of the experimental medium was removed from each well.
(2) To the well, 200 µl of PBS(-) was added.
(3) From the well, 200 µl of PBS(-) was removed.
(4) To the well, 200 µl of PBS(-) was again added.
(5) From the well, 170 µl of PBS(-) was removed. Finally, 50 µl remained in a well.
(6) From the test sample plate, 50 µl each of test samples, the negative control-1 solution, or the positive control solution was taken and flushed into the same position of the test plate as prepared in the test sample plate by using an 8-channel pipette.
(7) The test plate was incubated at room temperature for 15 min.
(8) Using a microplate mixer, the test plate was shaken for 30 sec.
(9) The plate was then centrifuged at 1000 rpm (140 x g) for 3 min.
(10) The supernatant, 50 µl, was transferred to an EIA plate by using an 8-channel pipette and strictly according to the order of the test sample addition. The time between the addition of the test samples and this transfer was kept at 20 min.
(11) During the incubation, the dye solution was prepared according to the instruction of the LDH assay kit for toxicology (Kyokuto Pharmaceutical Co. Ltd., Tokyo).
(12) The dye solution was pooled in the V-bottom reservoirs; then 50 µl of the dye solution was added to each well of the EIA plate.
(13) The EIA plate was incubated at room temperature for 45 min.
(14) Then 100 µl of the stopping solution in
the LDH assay kit (previously diluted half with distilled water) was added to each well.

(15) The test plate was shaken for 30 sec by using a microplate mixer.
(16) OD560 was measured with a microplate reader. At this step, the absence of abnormal coloring in the Negative control-2 well was confirmed.

5. Calculation of cytotoxicity for LDH-1 assay

For 4-A. Detection of direct inhibitory activity of test samples on LDH

After confirming the existence of a linear relationship between LDH activity and the final concentration of the test chemical, the mean of the OD560 values of the Negative control-1 was set at 1.000 after subtraction of the OD560 of a blank well, and each observed OD560 value was normalized relative to the Negative control-1. The normalized values were defined as the factor for remaining LDH activity (FRLA) at each concentration of the test chemicals. Data from the wells with FRLA of 0.1 or less were not used.

For 4-C. The test of LDH-1 assay

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

where a is the OD560 value derived from a well with added test sample and divided by FRLA at the corresponding chemical concentration,
b is mean OD560 value derived from the negative control-1 wells,
c is mean OD560 value derived from the positive control wells (i.e., 0.2% Detergent C added to the culture medium as a test sample).


6-A. Detection of direct inhibitory activity of test samples on LDH

We have repeated the test described in section 4-A but, instead of PBS(-) in the steps 4-A(5) and 4-A(6), we used the experimental medium. The normalized values were defined as the factor-a for remaining LDH activity (FRLA-a) at each concentration of the test chemicals.

Also, we have repeated this test with, instead of the experimental medium, 1:1 mixture of the positive control solution and the experimental medium. The normalized values were defined as the factor-b for remaining LDH activity (FRLA-b) at each concentration of the test chemicals.

6-B. Preculture for the test of LDH-2A, 2B, and 2C assays

This was the same as described in 4-B.

6-C. The test of LDH-2A, 2B, and 2C assays

[I] LDH-2A, acute cytotoxicity assay under culture condition

(1) Eighty µl of the experimental medium was removed from each well.
(2) To the well, 200 µl of fresh experimental medium was added.
(3) From the well, 200 µl of experimental medium was removed.
(4) To the well, 200 µl of fresh experimental medium was again added.
(5) From the well, 170 µl of experimental medium was removed. Finally, 50 µl was remained in each well.
(6) From the test sample plate, 50 µl each of test samples, the negative control-1 (and -2) solution, or the positive control solution was taken and flushed into the same position of the test plate as prepared in the test sample plate by using an 8-channel pipette.
(7) The test plate were incubated at 37 °C for 2 hr.
(8) To each well, 100 µl of PBS(-) was added except the well of the positive control to which 100 µl of the positive control solution was added.
(9) The test plate was placed for 20 min at room temperature.
(10) The test plate was shaken for 30 sec us-
ing a microplate mixer.
(11) The plate was centrifuged at 1000 rpm (140 x g) for 3 min.
(12) The supernatant, 50 µl, was transferred to an EIA plate by using a 8-channel pipette.
(13) During the incubation, the dye solution was prepared according to the instruction of the LDH assay kit for toxicology (Kyokuto Pharmaceutical Co. Ltd., Tokyo).
(14) Fifty µl of the dye solution which was pooled in the V-bottom reservoirs was added to each well of the EIA plate.
(15) The EIA plate was incubated at room temperature for 45 min.
(16) One hundred µl of 2-fold diluted stopping solution in the LDH assay kit (previously diluted half with distilled water) was added to each well.
(17) The test plate was shaken for 30 sec by using a microplate mixer.
(18) OD560 was measured with a microplate reader. At this step, the absence of abnormal coloring in the Negative control-2 well was confirmed.

[II] LDH-2B (cell growth inhibition) and LDH-2C (cell killing) assays

(1) ~ (6) The same as the corresponding steps for LDH-2A assay.
(7) The test plate was incubated at 37 °C for 48 hr.
(8) The test plate was shaken for 2 min using a microplate mixer.
(9) The plate was centrifuged at 1000 rpm (140 x g) for 3 min.
(10) The supernatant, 50 µl, was transferred to an EIA plate by using an 8-channel pipette.
(11) To each well, 50 µl of the positive control solution was added.
(12) The test plate was placed for 20 min at room temperature
(13) The test plate was shaken for 2 min using a microplate mixer.
(14) The plate was centrifuged at 1000 rpm (140 x g) for 3 min.
(15) The supernatant, 50 µl, was transferred to an another EIA plate by using an 8-channel pipette.
(16) During the incubation, the dye solution was prepared according to the instruction of the LDH assay kit for toxicology (Kyokuto Pharmaceutical Co. Ltd., Tokyo).
(17) Fifty µl of dye solution which was pooled in the V-bottom reservoirs was added to each well of the EIA plate.
(18) The EIA plate was incubated at room temperature for 45 min.
(19) One hundred µl of 2-fold diluted stopping solution provided in the LDH assay kit was added to each well.
(20) The test plate was shaken for 30 sec by using a microplate mixer.
(21) OD560 was measured with a microplate reader. At this step, the absence of abnormal coloring in the Negative control-2 well was confirmed.

6-D. Worksheet for data collection

Samples of worksheets (Fig. 1a - e) for data collection were shown to each laboratory. However, each laboratory was allowed to make their own style of the worksheet with fixed positions of raw data recording. These worksheets were formed on personal computer’s software. All the worksheets were first transferred to MS-DOS text files and incorporated to the software Excel version 4.0, and then recalculated.

Note: Although the responses for LDH-2B and -2C assays are calculated by using OD values of blank, negative- and positive-control obtained in LDH-2A assay, the worksheets of LDH-2B&C assays shown initially to each laboratory did not contain a column where the data from the LDH-2A assay must be recorded. We, therefore, later sent new worksheets to each laboratory with an appended column for the LDH-2A assay data (Fig. 1 d and e).
Fig. 1 Samples of worksheets for data collection for the LDH release assay

For a, Direct inhibition of LDH activity, b, LDH-1, c, LDH-2A, d, LDH-2B&C (cell layer and supernatant), e, LDH-2B&C (supernatant). d and e show new worksheets sent later to each laboratory with an appended column for the LDH-2A assay data, i.e., A) Blank, A) Nega.Cont., and A) Posi.Cont..

Note that each laboratory was allowed to make their own style of the worksheet with fixed positions for raw data recording. These worksheets were formed on personal computer softwares. All the worksheets were first transferred to MS-DOS text files and incorporated to the software Excel version 4.0, and then recalculated.
d
7. Calculation of cytotoxicity for LDH-2A, -2B, and -2C assays*

*For 6A. Detection of direct inhibitory activity of test samples on LDH*

This was the same as described in 5, For 4-A.

*For 6-C, LDH-2A.*

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

where \(a\) is OD560 value derived from a well with added test sample and divided by FRLA at the corresponding chemical concentration, \(b\) is mean OD560 value derived from the negative control-1 wells, \(c\) is mean OD560 value derived from the positive control wells.

*For 6-C, LDH-2B.*

\[
\text{Cytotoxicity-growth inhibition (\%)} = \frac{(m-n)}{m} \times 100
\]

\[
(m-n) = \frac{(e-0.5d)}{2(c-b)} \quad (g-0.5f) = \frac{(g-0.5f)}{2(c-b)}
\]

where \(d\) is OD560 of the negative control-1 transferred to the EIA plate at the step of 6-C, II-(10). \(e\) is OD560 of the negative control-1 transferred to the EIA plate at the step of 6-C, II-(15). \(f\) is OD560 of a test sample transferred to the EIA plate at the step of 6-C, II-(10) and divided by FRLA-a at the corresponding chemical concentration. \(g\) is OD560 of a test sample transferred to the EIA plate at the step of 6-C, II-(15) and divided by FRLA-b at the corresponding chemical concentration.

\[
\text{Cytotoxicity index-cell killing} = \frac{(f-d)}{2(c-b)}
\]

\(\ast\): In this protocol, The Working Group did not describe clearly that, at the calculation of cytotoxicity, one should subtract blank OD value from each raw observed OD value, although we wrote in the Protocols (5. Calculation of cytotoxicity for LDH-1 assay For 4-A. Detection of direct inhibitory activity of test samples on LDH) that the mean of the OD560 value of the negative control-1 was set at 1.000 after subtraction of the OD560 of a blank well. In the calculation of the above-mentioned cytotoxicities, one must subtract each corresponding blank value from each raw OD value. This point was thoroughly examined during the data cleaning process and confirmed by recalculation by The Working Group.

\(\ast\ast\): We used LDH activity in the cells at the beginning of the chemical treatment as the denominator. Therefore, this type of cytotoxicity was not expressed in percentage. In some cases, this index exceeds 1.0, suggesting that the chemical allowed continuation of cell growth during the chemical treatment but finally killed the cells and let them release their LDH into the medium.
Unacceptable data files

In Table 1, we described the reasons for rejecting the data files of each LDH sub-assays. These data files were thought to have severely violated the set protocol. A total of 159 data files was discarded for these reasons.

Quality of raw data

To understand the characteristics of raw data and resulting dose-response curves in all the submitted data files, hand-plotted dose-response curves on mean values at each chemical concentration were drawn.

Fig. 2 illustrates the hand-plotted dose-response curves of chemical #6 (sodium lauryl sulfate) assayed with HeLa S3 (SC) cells. Titles of each figure indicate abbreviation of the LDH release sub-assays (i.e., LDH-1, LDH-2A, -2B, and -2C), the cell line used, and the code number of the tested chemical. Each point indicates the average of viability for an observed concentration in an assay. The number in the small circle indicates the laboratory number.

Since preliminary tests on sodium lauryl sulfate suggested that direct inhibition of LDH activity occurs at concentrations over 0.02%(w/v) (T. Sasaki, personal communication), the same may be possible for this chemical #6 here. Hence, the observed data at over 0.02% of chemical #6 must have been carefully corrected. However, as seen in Fig. 2a, data from Lab-3, -9, and -29 were apparently extraordinal. This tendency was also seen in data from Lab-9 (Fig. 2b and c), Lab-7, and Lab-46 (Fig. 2d).

At this point, we did not care about the possible misunderstanding of the protocol for normalization of the FRLA and subsequent calculation of cytotoxicities by each laboratory.
Fig. 2. Hand-plotted dose-response curves of the LDH release sub-assays prepared at the submission of data files.

Hand-plotted dose-response curves were drawn to understand gross characteristics of data files at their submission to The Working Group. With HeLa S3 (SC) cells and chemical #6 (sodium lauryl sulphate), a, LDH-1; b, LDH-2A; c, LDH-2B; d, LDH-2C. Each point indicates the mean of viability for an observed concentration in an assay. The figure in an open symbol indicates laboratory number.
b
Fig. 3. Log(ED50) values and visualized characteristics of submitted data files.

a, LDH-1. b, LDH-2A. c, LDH-2B. d, LDH-2C.

- 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 (see Validation Article I, Fig. 3, in this issue).
- 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 #1 or #7; then all corresponding data file sets were discarded.
- The ratio of ED50s of chemical #1 and chemical #7, or vice versa, was over 5.
- 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-B**: LDH activity is directly inhibited or stimulated by the test chemical with the correction factor of below 0.1 or above 2.0, respectively. With these factors, correction of the observed LDH activity to the original LDH activity in a sample is essentially meaningless.
- **Code-C**: No observed point between 20-80% of the maximum effect was found in the data file.
- **Code-D**: Data include response of 200% or more where that of negative controls was set at 100%.
- **Code-E**: Of the 95% confidence limits of ED50, the upper limit was over 100 times that of the lower limit.
- **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.
ever, as explained in the foot note * in Materials and Methods, we did not describe clearly in the protocols that, at the calculation of cytotoxicity, one should subtract each corresponding blank value from each raw OD value. Therefore, many of the hand-plotted dose-response curves were not necessarily drawn correctly.

This point was thoroughly examined during the data cleaning process and confirmed by re-calculation by The Working Group. The confirmed data files were utilized for the further statistical analyses.

**Characteristics of submitted data files and log(ED50) values**

As seen in Fig. 3, most of the expected data files were not submitted or were finally rejected by the LAP-JSAAE program for calculation of ED50 values. The capital letters in shaded boxes are the error codes in the LAP-JSAAE program for calculation of ED50 values by the non-linear least squares method. For example, the data file submitted by Lab-3 for chemical #4 tested in the LDH-1 assay with HeLa S3 (SC) cells was marked with check code A because ED50 calculation by the LAP-JSAAE program was not possible. This resulted in a low performance rate as has been described (see Validation Article I). Only 8 data files for chemical #1 were finally accepted for the LDH-1 assay with HeLa S3 (SC) cells. To our surprise, no data file for chemical #6 in the LDH-2C assay with HeLa S3 (SC) cells survived scrutiny (Fig. 2d and Fig. 3d).

The ED50s were examined in each accepted data files. For the LDH-2B assays, none of the chemicals tested were analyzed for their ED50 values with the box-whisker-plot because there were less than 4 remaining acceptable data files.

Technical achievement and its maintenance are evidently the most severe problem in the LDH-release assay. Since such low performance rates were observed in each LDH release sub-assay, the influence of cell lines in each assay could not be statistically analyzed.

**Conclusion**

Regrettably, we concluded that the present LDH release assay, including the four sub-divided assays, is practically immature. The assay, however, is very much informative in principle (Wang et al., 1995) not only on the cell growth inhibition but also on the cell lysis caused by the tested chemicals.

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**References**

