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Validation Study on Five Cytotoxicity Assays by JSAAE
IV.
Details of the Colony Formation Assay

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Abbreviations: CF, colony formation; 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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Abstract

The inter-laboratory validation study on 5 cytotoxicity assays conducted by JSAAE has been described in the preceding articles in this issue. Presented here are precise data and the protocols on the colony formation (CF) assay with two cell lines, HeLa S3 (SC) that is common to the other 4 assays and BALB/3T3 that is frequently used in this assay. The CF assay showed high performance rates with both HeLa S3 (SC) and BALB/3T3 A31-1-1 cell lines. Almost all submitted data files were acceptable before ED50 calculation in this assay. Variations on negative controls in the assay revealed technical differences among laboratories and the characteristics of cells applied. The CF assay with BALB/3T3 A31-1-1 cells resulted in the lowest median values of log(ED50) for Tween 20, Tween 80 and cetylpyridinium chloride monohydrate. For propylene glycol assayed with HeLa S3 (SC) cells, the lowest median value of log(ED50) was observed. Thus the CF assay was able to detect with the highest sensitivity the least toxic chemical (#4) and the most severely toxic chemical. CF assay with HeLa S3 (SC) cells gave the largest power for distinction when observing the most and the least toxic chemicals. However, the toxicities of moderately toxic chemicals were not sharply distinguishable from each other. Comparing the mean hinge-spreads, CF assay

with BALB/3T3 A31-1-1 cells showed the largest variation of the log(ED50) values among all assay systems examined in this study. These results suggest that the CF assay is a highly sensitive assay but is influenced by many factors such as culture conditions and techniques of cell handling.

Introduction

As described in the preceding papers (see Validation Article I in this issue), 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 (Watanabe *et al.*, 1989; Ohno *et al.*, 1995).

Colony formation (CF) assay measures reproductive death through clonal cell growth. The CF assay is known to be a highly sensitive method because it involves long-term exposure to chemicals and requires only low cell density (Clemedson *et al.*, 1996a and b; Sasaki and Tanaka, 1991; Sasaki *et al.*, 1991). For these reasons, the CF assay has been adopted in the Japanese guidelines for medical devices (Tsuchiya *et al.*, 1993). On the other hand, it has some disadvantages, e.g., the assay takes about 7-14 days to complete and needs much incubator space. This report supplements Validation Article I (the first article in this issue). We describe here precise results of the CF assay carried out in the validation study, including the protocols, representative hand-plotted dose-response curves, extra ordinal 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), cetylpyridinium chloride monohydrate (#5, severe irritant), sodium lauryl sulphate (#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.

In addition to the common HeLa S3 (SC) cells from human cervix carcinoma, BALB/3T3 A31-1-1 cells from mouse embryo were used in this CF assay. Both cell lines were supplied from RIKEN Cell Bank, Tsukuba Science City.

Data analysis

The submitted data files were first inspected manually to detect simple recording errors. Then hand-plotted dose-response curves drawn from the mean values at each chemical concentration were checked visually. The ED50 values of possibly acceptable candidate data files (see Validation Article I, Tables 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 CF 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 $NaHCO_3$ and pH 7.3 - 7.6) + 10% (v/v) calf serum (CS).
   2) BALB/3T3 A31-1-1 cells: MEM (autoclavable, containing 1.8 mg/ml of $NaHCO_3$ and pH 7.3 - 7.6) + 10% (v/v) fetal bovine serum (FBS).

(2) Subculture for maintenance

HeLa S3 (SC) cells used in the assay were in log phase. Subculture was performed with the dissociation medium consisting of 0.05% trypsin and 0.02% EDTA dissolved in Ca^{++}, Mg^{++}-free Dulbecco's phosphate-buffered saline (PBS(-)). After dissociation of the cells, $2x10^5$ cells were seeded into a 60-mm dish containing 5 ml of culture medium. HeLa S3 (SC) cells were subcultured every 3 - 4 days.

BALB/3T3 A31-1-1 cells were subcultured at 7 day intervals. The cells (100-150 x 10^4 cells/ 60-mm dish) in log phase were treated with 0.25% trypsin in PBS(-), then 10-15 x 10^4 cells were seeded in a 60-mm dish containing 5 ml of culture medium.

1-2. Reagents

(1) PBS(-)

(2) Dissociation medium: 0.05% trypsin and 0.02% EDTA dissolved in PBS(-), 0.25% trypsin in PBS(-).

(3) Methanol

(4) Giemsa solution diluted to approximately 5-10%

2. Test chemical preparation

Test chemicals #1, 2, 4, 5, 6 and 7 were dissolved in PBS(-), sterilized by membrane filtration and then serially diluted with PBS(-). Chemical #3 was dissolved in DMSO. The final concentrations of organic solvents in the culture medium were kept at 0.5% or less.

Since the concentration range employed was different for each chemical, at first the practically attainable maximum concentration was determined and diluted serially, then the assay was carried out with a wide dose range covering approximately 5-6 orders of magnitude. From the results of this preliminary test, the maximum concentration was set at the lowest concentration able to kill 100% of the cells, and this concentration was then diluted serially. The 2 to 3 orders of magnitude of the test concentration range were covered by this serial dilution. Finally the assay was repeated to be able to obtain more than 3 points with a cytotoxic effect occurring between 20-80% of the maximum cytotoxicity.

3. Procedure for the CF assay

3-1. Cell seeding

(1) Cells at approximately 80% confluence were selected, i. e. logarithmically growing cells. After discarding the culture medium and washing the cells with 2 ml of PBS(-), the cells were dissociated by adding 2 ml of the dissociation medium that was immediately aspirated. After remaining for approximately 5 min at room temperature, cells were suspended in 5 ml of PBS(-) and transferred to a 15-ml centrifuge tube containing 5 ml of the culture medium. The cells were collected by centrifugation at 1000 rpm (140 x g) for 3 min.

(2) For HeLa S3 (SC) cells: A cell suspension containing 25 cells/ml in culture medium was prepared. An aliquot (4 ml) of this suspension, was added to each 60-mm dish, i.e., 100 cells/dish. To avoid uneven attachment of the cells, the dish was kept undisturbed for 20 min at room temperature. Then the cells were cultured for 24 hr in a CO2-incubator at 37 °C.

For BALB/3T3 A31-1-1 cells: The cells were plated in 60-mm dishes in the same manner as HeLa S3 (SC) cells.

3-2. Treatment

After the 24-hr incubation, 20 μl of the test chemical solution was added to each dish with very gentle agitation. The dishes were cultured without medium change for a further 13 days with HeLa.S3 (sc) cells or 7 days with BALB/3T3 A31-1-1 cells.

3-3. Fixing and Staining

(1) The culture medium was discarded.

(2) Cells were fixed by adding 3-4 ml of methanol for at least 5 min.

(3) After fixing, cells were stained with the Giemsa solution for at least 15 min.

(4) The solution was discarded and the cells were rinsed with tap water.

(5) Colonies which consisted of more than 50 cells in each dish were counted.

3-4. Worksheet for data collection

A sample of a worksheet (Fig. 1) for data collection was shown to each laboratory. However,

Fig. 1. An example of the worksheet for data collection for the CF assay

Note that 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 softwares. All the worksheets were first transferred to MS-DOS text files and incorporated to the software Excel version 4.0, and then recalculated.

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4. Calculation of cytotoxicity

Plating efficiency (PE) was defined as: (number of colonies/number of cells plated) x 100%. Cytotoxicity was expressed in percentage of relative viability (or survival) which was defined as (mean PE in the treated dishes at a chemical concentration / mean PE in the control dishes) x 100%. This value was submitted for the ED50 calculation in the LAP-JSAAE program.

Results and Discussion

Quality of raw data

To understand the characteristics of raw data and resulting dose-response curves in all submitted data files, hand-plotted dose-response curves* on mean values at each chemical concentration were drawn. At this preliminary step, we found that Lab-12 and Lab-35 had carried out the assay using FBS instead of CS for HeLa S3 (SC) cells; that is a violation of the protocol for this assay. Their data files (7 files for each laboratory) were not accepted for further analyses. No other violation was found in the CF assay (see Validation Article I, Table 3).

Fig. 2 illustrates two examples of the hand-plotted dose-response curves of CF assays. 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. CF assays with HeLa S3 (SC) and BALB/3T3 A31-1-1 cells showed clear S-shape curves as compared with other assays. As shown in Fig. 2a, the dose-response curves drawn from the data file of Lab-36 and Lab-24 were apparently outliers. The ED50 derived from the data file of Lab-36 was an extremely high value. After the meeting on raw data confirmation, Lab-36 was allowed to amend their simple error in recording the data of HeLa S3 (SC) to 1/200 of that which they initially submitted.

The data on chemical #1 submitted from Lab-24 showed an extremely low ED50 value in the assay with HeLa S3 (SC) cells compared with the value for chemical #7 which is the same chemical, Tween 20. As shown in Fig 2b, the hand-plotted dose-response curves of the CF assay with BALB/3T3 A31-1-1 cells varied considerably depending on the laboratory. BALB/3T3 A31-1-1 cells showed a wide range of toxicity responses with chemical #5, cetylpyridinium chloride monohydrate, the most toxic among the 6 chemicals used in the present validation. Extra ordinal dose-response curves were evident from the data files of Lab-24 and Lab-31 (Fig 2b).

We excluded data files containing any abnormality according to the flow chart as described (see Fig. 1 in Validation Article I), then acceptable data files were analyzed by the LAP-JSAAE program. The data files on BALB/3T3 A31-1-1 cells submitted from Lab-36, which were once accepted after amendment as described above for HeLa S3 (SC) cells, were finally rejected by the LAP-JSAAE program.

Characteristics of submitted data files and log(ED50) values

Fig. 3 illustrates the characteristics of accepted data files of CF assays with log(ED50) values. Among the 24 laboratories that participated initially in CF assay, two (Lab-43 and Lab-47) did not submit any data for the assay with either cell line, and three (Lab-4, Lab-17 and Lab-31) did not submit any data with HeLa S3 (SC) cells. The ratio of ED50s of chemical #1 and #7 exceeded 5-fold in Lab-24 and, therefore, all the data files for chemical #1 - #7 as a set was rejected (see Validation Article I, Table 6).

The data of Lab-4, Lab-12 and Lab-36 were incomplete. Many data files on the chemical #4, propylene glycol, in the assay with both cell lines were lacking. This may be due to extremely low toxicity of chemical #4. The protocols for the CF assay required that the maximum concentration of organic solvents should be 0.5% or less. The solvent concentrations used for chemical #4

* Copies of all the hand-plotted dose-response curves are available on request.

Fig. 2. Hand-plotted dose response curves of the CF assays at the 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), CF assays with chemical #1 (Tween 20) on HeLa S3 (SC) cells. b (next page), CF assays with chemical #5 (cetylpyridinium chloride monohydrate) on BALB/3T3 A31-1-1 cells. Points represent the mean of viability for an observed concentration in an assay. The figure in an open symbol indicates the laboratory number.

Fig. 3. Log(ED50) values and visualized characteristics of submitted data files

Open boxes represent 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-wisker plot analysis shown in Fig. 5

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 *vise versa*, was over 5.

A check code was found in data files of chemical #2, #3, #4, #5, or #6.

Data files accepted after amending simple recording errors on reported concentration of chemicals after the discussion meeting held on November 30, 1994.

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 bewteen 20-80% of the maximum effect.

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.

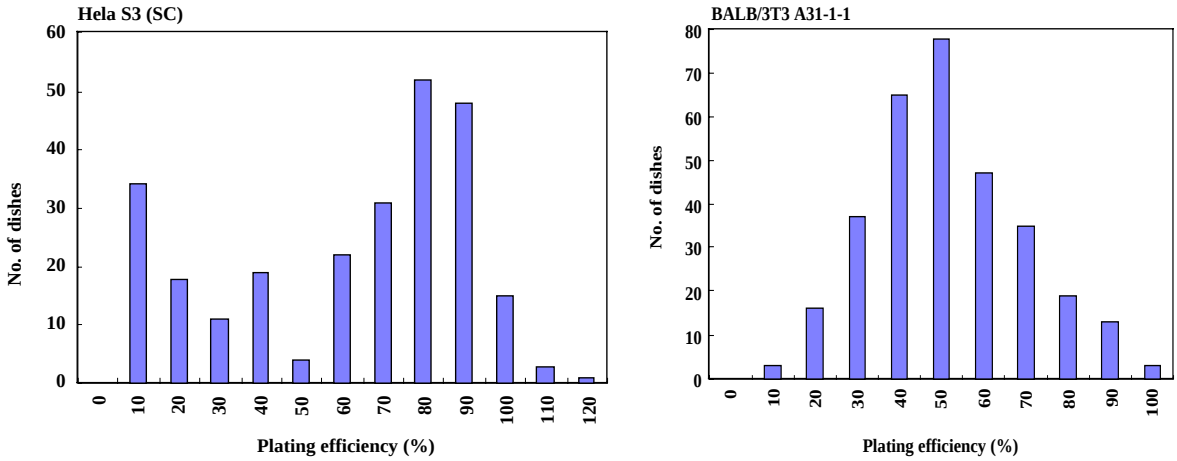

Fig. 4. Distribution of plating efficiencies in negative controls obtained from raw data of the CF assay a, left, HeLa S3 (SC) cells. b,right, BALB/3T3 A31-1-1 cells.

were not enough to observe cytotoxic responses. The data files of chemical #4 were accepted from only 8 laboratories per cell line.

Due to protocol violation, data files from Lab-12 and Lab-35 on HeLa S3 (SC) cells were excluded from analysis. Overall, for assays on HeLa S3 (SC) cells and BALB/3T3 A31-1-1 cells, performance rates were calculated to be 77% and 89%, respectively (see Validation Article I, Table 3).

The capital letters in shaded boxes in Fig. 3 are check codes attached by the LAP-JSAAE program for calculation of ED50 values. Most of the rejected files on chemical #4 were marked with the code C and code A. There was no data file marked with the check code B and D. Thus, a total of 100 files for HeLa S3 (SC) cells and 126 files for BALB/3T3 A31-1-1 cells were finally accepted for further box-whisker-plot analysis (Fig. 4).

Technical stability of the CF assay in participating laboratories

Among the 24 laboratories that participated initially, 15 received the technology transfer (see Table 2 of Validation Article I in this issue). In one of these laboratories, the recipient did not conduct the CF assay but instead a technician did the work. Six laboratories (Lab-5, -12, -17, -24, -28, and -33) did not receive the technology transfer but performed the assay. Lab-43 and -47 did receive the technology transfer but did not submit any data.

To determine technical stability of the CF assay within a laboratory, the variation in the negative controls was calculated on each assay (Table 1). With HeLa S3 (SC) cells, Lab-45 had carried out the assays with the largest variation for negative controls. Their coefficient of variation was 49.6% (see CV-1 in Table 1) in the 4 assays for the 7 chemicals with the average plating efficiency 71.6%; the plating efficiency of their negative controls varied from 18.5 to 91.6% (Table 2, see Mean). Within assay dish lots, the coefficients of variation were between 8.6% and 14.5% with a mean of 11.1% for the 4 assays (see CV-2 in Table 2). This tendency, i.e., the larger variation among assays for different chemicals and the smaller variation among the dishes in an assay, was not necessarily the case for the negative controls observed in other laboratories.

With HeLa S3 (SC) cells, the mean value of plating efficiencies of all participants was 70.1% (Table 1). With BALB/3T3 A31-1-1 cells, the mean value of plating efficiencies of all participants was slightly less at 53.8% (Table 1).

When numbers of dishes with a range of negative control plating efficiency values were plotted from the CF assays with HeLa S3 (SC) and BALB/3T3 A31-1-1 cells, characteristic distributions were observed for the two cell lines (Fig. 4). With HeLa S3 (SC) cells, a bimodal distribution was observed with two peaks at 10% and 80% plating efficiencies, while normal distribu-

Table 1. Variation of the negative controls in CF assays

Lab. No.	HeLa S3 (SC) cells					BALB/3T3 A31-1-1 cells				
	Assays	PE[a] (%)	SD[b]	CV-1[c] (%)	CV-2[d] (%)	Assays	PE (%)	SD	CV-1 (%)	CV-2 (%)
5	1	47.4				2	44.6	1.4	3.1	6.3
10	7	90.6	3.9	4.3	5.8	6	68.3	7.9	11.6	7.7
11	3	87.2	5.7	6.5	10.8	3	46.2	9.6	20.8	14.0
12						5	41.4	9.8	23.7	6.1
13	6	99.0	4.5	4.5	11.1	6	44.5	9.4	21.1	14.1
14	5	19.3	0.7	3.6	5.5	5	39.6	1.9	4.8	4.3
17						2	35.6	0.9	2.5	6.6
20	4	86.2	2.4	2.8	9.2	5	72.4	7.9	10.9	10.2
24						4	81.5	17.2	21.1	6.2
27	4	73.1	6.4	8.8	9.7	4	52.5	0.8	1.5	20.6
28	1	93.5				1	48.5			
30	3	61.6	6.6	10.7	15.2	3	64.7	4.9	7.6	12.2
31						5	85.5	6.6	7.7	8.0
33	4	81.1	13.4	16.5	8.5	3	51.7	15.8	30.6	12.6
35						3	55.8	2.1	3.8	10.8
36	6	20.6	2.8	13.6	18.3					
37	3	74.2	10.5	14.2	7.1	3	51.1	5.7	11.2	5.4
39	6	40.7	1.0	2.5	6.3	6	65.7	9.4	14.3	5.1
40	1	93.8				2	27.4	2.1	7.7	8.6
45	4	71.6	35.5	49.6	11.1	4	39.9	14.7	36.8	15.0
46	3	82.4	4.5	5.5	8.7	4	59.7	7.0	11.7	8.4
	Mean	70.1	25.4	11.0	9.8	Mean	53.8	15.3	13.3	9.6

a Mean plating efficiency of the negative controls in the assays
b Standard deviation
c Coefficient of variation among independent assays calculated from the mean negative control value of each assay
d Coefficient of variation among the negative control dishes within an assay: Mean of the assays

tion with a peak at 50% was observed with BALB/3T3 A31-1-1 cells. In the CF assay, the plating efficiency of BALB/3T3 A31-1-1 cells was greatly affected by serum lot. Therefore, we used the same lot of FBS with BALB/3T3 A31-1-1 cells but did not use the same lot of CS for HeLa S3 (SC) cells. This is one of the possible reasons for the normal distribution profile found with the plating efficiencies of BALB/3T3 A31-1-1 cells, since almost the same participants performed the CF assay using both HeLa S3 (SC) and BALB/3T3 A31-1-1 cells.

Effect of cell lines

As shown in the box-whisker-plots of log(ED50) (Fig. 5), BALB/3T3 A31-1-1 cells exhibited higher sensitivity than HeLa S3 (SC) cells to cytotoxic chemicals except for chemical #4 in the CF assay. Nine and five outliers were observed in the CF assays with HeLa S3 (SC) cells and BALB/3T3 A31-1-1 cells, respectively (Fig. 5). As mentioned in Validation Article I in this issue, the CF assay with HeLa S3 (SC) cells gave the largest "power for distinction" (PFD) of 22, while this assay with BALB/3T3 A31-1-1 cells

Table 2. Intra-laboratory variation of the negative controls in CF assays with HeLa S3 (SC) cells carried out in Lab-45.

Assay for Chemical	Plating efficiency in each dish (%)										
	D-1	D-2	D-3	D-4	D-5	D-6	D-7	D-8	Mean[a]	SD[b]	CV-2[c]
#1, 6, 7	93	95	89	78					88.8	7.6	8.6
# 2	71	89	88	89	101	88			87.7	9.6	10.9
# 4	118	79	82	80	94	88	89	103	91.6	13.3	14.5
# 5	18	19	18	15	21	20	20	17	18.5	1.9	10.4
Mean[d]									71.6		11.1
SD[d]							35.5				
CV-1[e]						49.6					

a Mean plating efficiency of the negative controls in each assay
b Standard deviation
c Coefficient of variation (%) among the negative control dishes within an assay
d Calculated from the four assays
e Coefficient of variation among independent assays calculated from the mean negative control value of each assay

gave the value of 13.4. The value of PFD will be helpful as an indicator to distinguish cytotoxicities of individual chemicals tested in the assay.

Conclusion

As has been described in the first article of this issue, the CF, CV, MTT, and NR assays are recommendable from the view point of performance of the assays. From the view point of inter-laboratory variation, the CF assay with BALB/3T3 A31-1-1 cells was not necessarily the best one. However, the CF assay with HeLa S3 (SC) cells was the best from the view point of the "power for distinction" of toxicities among chemicals.

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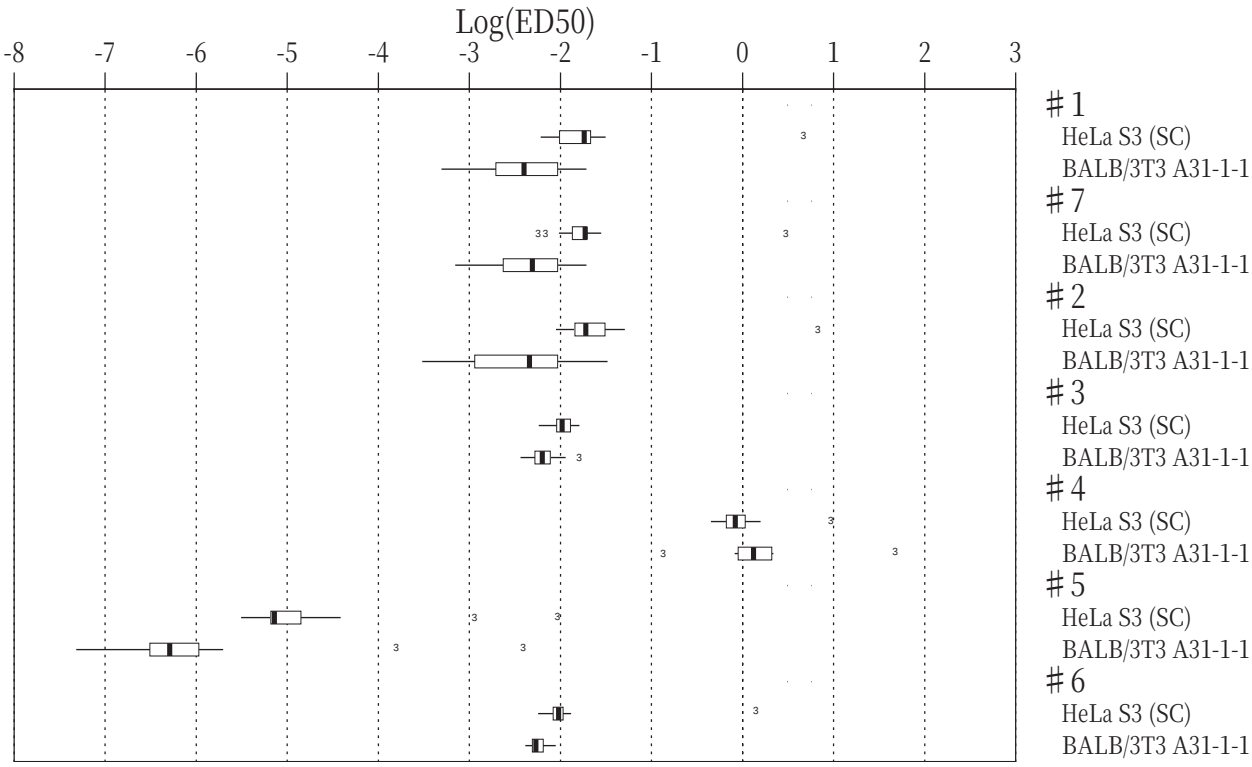

Fig. 5. Box-whisker-plots of results of the CF assay
Asterisks indicate outliers of the corresponding box-whisker-plots.

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