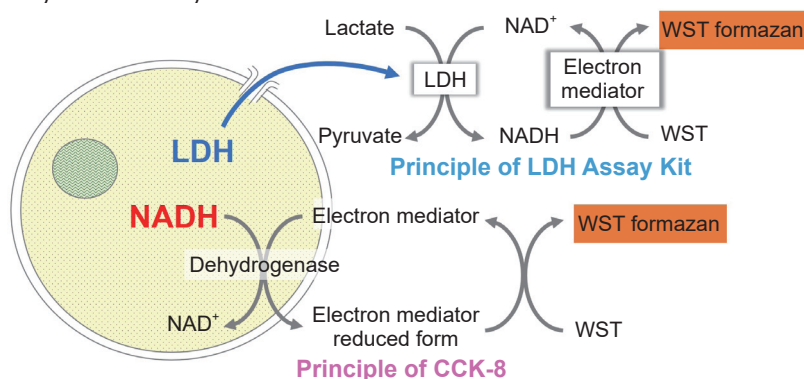


### General information

A method for measuring the number of viable cells or that of dead cells is widely used as a cytotoxicity evaluation. In order to determine the cytotoxicity comprehensively, a toxicity evaluation by combining a plurality of evaluation methods has been increasingly demanded. Viability/Cytotoxicity Multiplex Assay Kit can be performed as a comprehensive cytotoxicity assay. Viability/Cytotoxicity Multiplex Assay Kit includes Cell Counting Kit-8 (CCK-8) and Cytotoxicity LDH Assay Kit-WST (LDH Assay Kit), which measures the number of viable cells and dead cells, respectively.

CCK-8 can determine the number of viable cells by measuring dehydrogenases activity in living cells and LDH Assay Kit can determine the number of dead cells by measuring the amount of released LDH from damaged cell membranes. LDH Assay Kit enables both homogeneous assay by adding the reagent solution in the kit to a cell culture medium directly and non-homogeneous assay by adding the reagent solution to a cell culture supernatant. Two index cytotoxicity evaluations with same cultured cells can be carried out by combining CCK-8 and the non-homogeneous assay in LDH Assay Kit.



### Kit Contents

	Code	Unit size	Components	
Cell Counting Kit-8	CK04	500 tests	Cell Counting Kit-8	5 ml × 1
Cytotoxicity LDH Assay Kit	CK12	500 tests	Dye Mixture	× 1
			Assay Buffer	55 ml × 1
			Lysis Buffer	5.5 ml × 1
			Stop Solution	27.5 ml × 1

### Storage Condition

Store at 0-5°C

### Required Equipment and Materials

- Microplate reader with 450 nm and 490 nm filter
- CO<sub>2</sub> incubator
- 20, 100-200 µl 8-channel pipette
- 96-well tissue culture plate (flat-bottomed)
  - \*Round or V-bottomed plate for suspension cells in non-homogeneous assay.
- 96-well optically clear plate (flat-bottomed)
  - \*For non-homogeneous assay in LDH measurement

### Precaution

- The optimum conditions depend on types of cells. We recommend carrying out a preliminary experiment to optimize the concentration of cells and the incubation time.

### Contents of Technical Manual

#### CCK-8 assay for viable cell detection

- Optimization of cell concentration and incubation time P. 2
- Cell viability assay P. 2

#### LDH Assay for cell death detection

##### Homogeneous assay

- Optimization of cell concentration P. 3
- Cytotoxicity assay P. 4

##### Non-homogeneous assay

- Optimization of cell concentration P. 4
- Cytotoxicity assay P. 5

#### Experimental example for CCK-8 and LDH assay in same cells

- Cell viability and cytotoxicity assay P. 6

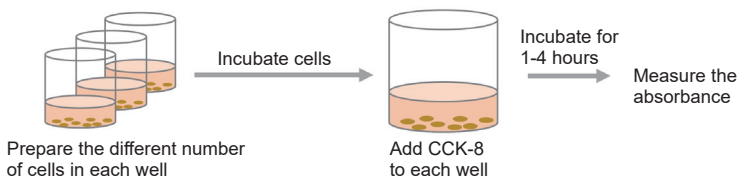
Serial dilution procedure

P. 6

## CCK-8 assay for viable cell detection

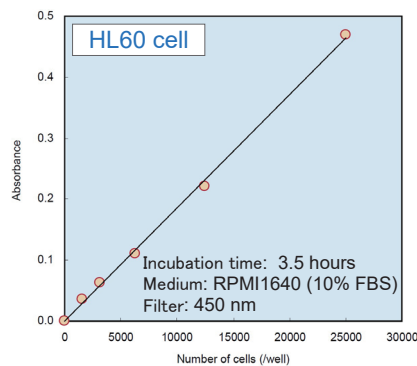
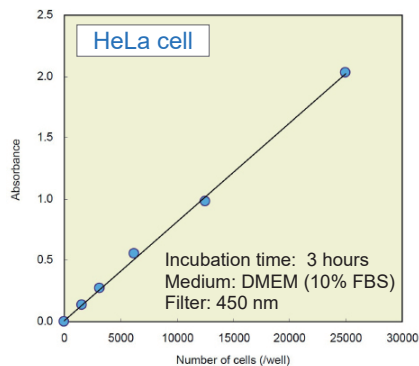
The ideal number of cells and the incubation time for color development should be determined by a preliminary experiment. Prepare cells without addition of test substances in culture medium.

## Optimization of Cell Concentration and Incubation Time

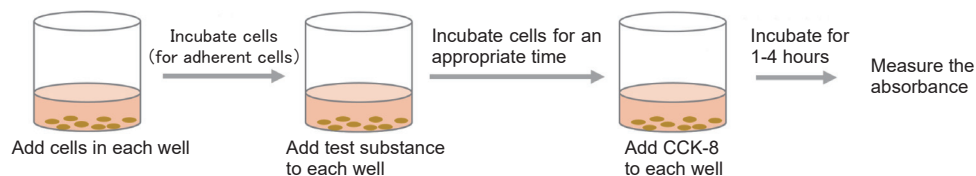


1. Collect cells and wash them with the medium. Prepare  $5 \times 10^5$  cells/ml cell suspension in the medium.
2. Prepare 100  $\mu$ l of 2-fold serially diluted cell suspension in a 96-well tissue culture plate.  
\*Refer the Serial Dilution Procedure in page 6.
3. Incubate cell suspension at 37°C for an appropriate time in a CO<sub>2</sub> incubator.
4. Add 10  $\mu$ l of CCK-8 solution to each well of the plate.  
\*Be careful not to introduce bubbles to the wells, since they interfere the O.D. reading.
5. Incubate the plate at 37°C for 1 - 4 hours in the CO<sub>2</sub> incubator.  
\*Color intensity depends on types of cells. Measure the absorbance every hour and find the optimum incubation time which allows absorbance to be in following range.  
- for cytotoxicity assay : 0.5 - 1.5  
- for proliferation assay : 0.3 - 0.8
6. Measure the absorbance at 450 - 490 nm by using a microplate reader.

< Experimental example : the number of cells vs. absorbance >



## Cell Viability Assay



1. Collect cells and wash them with the medium. Prepare the cell suspension at the optimum concentration which is determined in a preliminary experiment.
2. Add 100  $\mu$ l of the cell suspension to each well of a 96-well tissue culture plate.  
\*For adherent cells, please incubate the culture plate overnight to attach the cells to the plate.
3. Add 10  $\mu$ l of the medium containing test substance that adjusted to the desired concentration. Incubate the plate at 37°C for an appropriate time (e.g. 6, 12, 24, or 48 hours) in a CO<sub>2</sub> incubator.  
\*In case the test substance has color or reducing activity, prepare "Blank" well which includes test substance, CCK-8, and medium without cells.
4. Add 10  $\mu$ l of the CCK-8 solution to each well of the plate.  
\*Be careful not to introduce bubbles to the wells, since they interfere with the O.D. reading.
5. Incubate the plate at 37°C for 1 - 4 hours in the CO<sub>2</sub> incubator.  
\*Please refer to the determined optimum incubation time in preliminary experiment.
6. Measure the absorbance at 450 - 490 nm by using a microplate reader.

## Calculation of Cell Survival Rate

Calculate the average absorbance from each triplicate set of wells and subtract the absorbance of "Blank" from each absorbance of "Sample" and "Control" in the following equation.

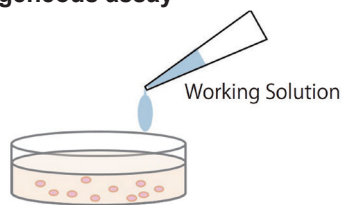
$$\text{Survival rate (\%)} = \frac{(\text{Sample} - \text{Blank})}{(\text{Control} - \text{Blank})} \times 100$$

Control : CCK-8, medium and cells in a well  
Blank : CCK-8 and medium without cells in a well  
Sample : CCK-8, medium, cells and test substances in a well

**LDH Assay for cell death detection**

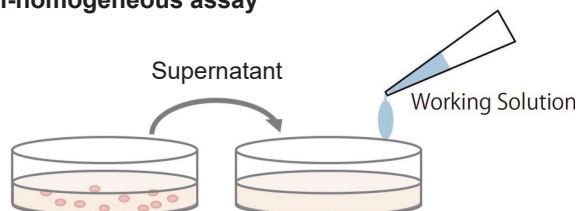
There are two methods: homogeneous assay and non-homogeneous assay, please choose the assay method according to your experiments.

**Homogeneous assay**



Simple procedure: homogeneous assay only requires adding the Working Solution to each well in the presence of live and dead cells, there is no need to transfer cell culture supernatant to a new microplate for measurement. Please refer to page 3 for this procedure.

**Non-homogeneous assay**



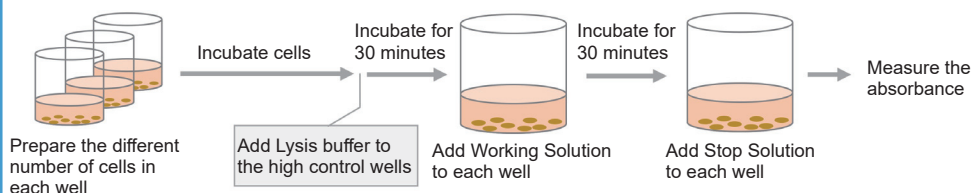
Multi-measuring use: since non-homogeneous assay uses the culture supernatant from each well for the measurement of LDH activity, the sample cells in each well can be applied to some other experiments such as cell viability assay (WST-8 and MTT) and cell staining (nuclear staining, immunostaining). Please refer to page 4 for this procedure.

**Preparation of Reagent**

- 1) To dissolve the powder, add 5 ml of Assay Buffer to the Dye Mixture vial. Close the cap and dissolve the contents completely.
- 2) Add the whole volume of the mixture prepared in 1) to the Assay Buffer bottle.
  - \*Store the Working Solution at 0-5°C and protect it from light. It is stable for 6 months

**Optimization of Cell Concentration**

**Homogeneous assay**



- 1) Collect cells and wash them with the medium. Prepare  $5 \times 10^5$  cells/ml cell suspension in the medium.
- 2) Prepare 100  $\mu$ l of 2-fold serially diluted cell suspension in a 96-well tissue culture plate.
  - \*Refer the Serial Dilution Procedure in page 6.
- 3) Incubate cell suspension at 37°C for an appropriate time in a CO<sub>2</sub> incubator.
- 4) Add 10  $\mu$ l of Lysis Buffer to each well of the high control.
- 5) Incubate the plate at 37°C for 30 minutes in the CO<sub>2</sub> incubator.
- 6) Add 100  $\mu$ l of Working Solution to each well. Protect the plate from light and incubate it at room temperature for 30 minutes.
- 7) Add 50  $\mu$ l of Stop Solution to each well.
- 8) Measure the absorbance at 490 nm by using a microplate reader.
  - \*Plot the data by setting cell concentrations on the x-axis and absorbances on the y-axis. The plot should have two linear lines, one representing High Control and another Low Control. Determine the optimum concentration of cells based on the followings.
    - Eliminate the concentration of cells that has absorbances higher than 2.0.
    - Choose the concentration of cells that has at least 0.2 difference in the absorbance between high control and low control.

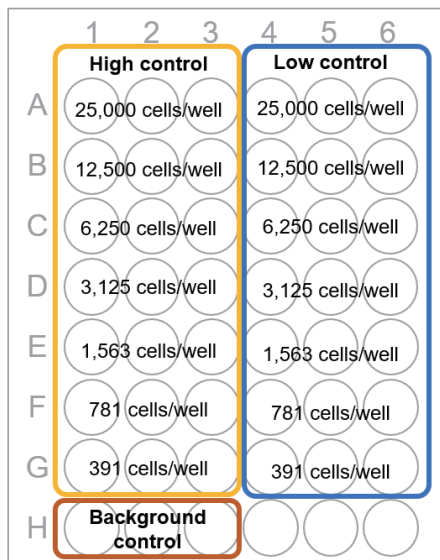


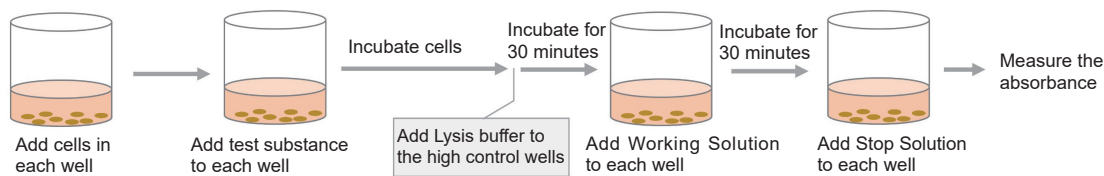
Fig. 1 Plate arrangement

Table 1 Amount of each solution (Homogeneous assay)

	Test substance	High control*	Low control	Background control
Medium	-	50 $\mu$ l	50 $\mu$ l	100 $\mu$ l
Cell suspension	50 $\mu$ l	50 $\mu$ l	50 $\mu$ l	-
Test substance in culture medium	50 $\mu$ l	-	-	-
Lysis Buffer	-	10 $\mu$ l	-	-

\*The difference of total volume of test substance and high control does not affect the result.

- Test substance : The released LDH activity from the cell when the test substance is added to the cell
  - High control : The total LDH activity in the cell (= Maximum LDH release)
  - Low control : The LDH activity released from the untreated cells to the medium (= Spontaneous LDH release)
  - Background control : The LDH activity in the medium
- ※ medium = culture medium



- 1) Add 50 µl of cell suspension to each well of a flat-bottom 96-well tissue culture plate.  
 \*Prepare the double concentration of cell suspension which is confirmed in the preliminary experiment.  
 \*For adherent cells: incubate the plate at 37°C overnight in a CO<sub>2</sub> incubator to allow the cells to adhere and then replace the medium with 50 µl of fresh medium.
- 2) Add 50 µl of medium containing test substance that adjusted to the desired concentration (Refer to Table 1).
- 3) Incubate the plate at 37°C for an appropriate time in a CO<sub>2</sub> incubator.
- 4) Add 10 µl of Lysis Buffer to each well of the high control. Incubate the plate at 37°C for 30 minutes in the CO<sub>2</sub> incubator.
- 5) Add 100 µl of Working Solution to each well. Protect the plate from light and incubate it at room temperature for 30 minutes.
- 6) Add 50 µl of Stop Solution to each well.
- 7) Measure the absorbance at 490 nm by using a microplate reader.

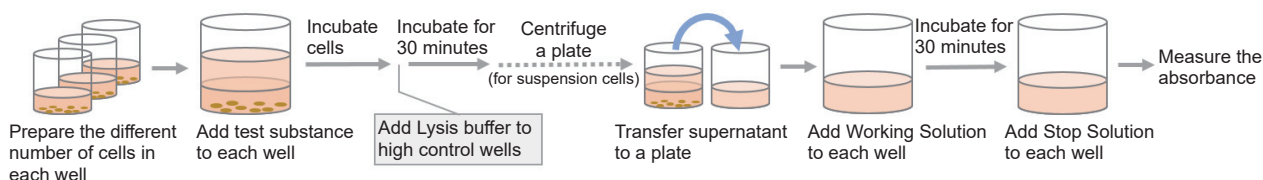
**Calculation of Cytotoxicity**

Calculate the average absorbance from each triplicate set of wells and subtract the absorbance of background from each absorbance of test substance, high control, and low control. Determine the percent cytotoxicity by the following equation.

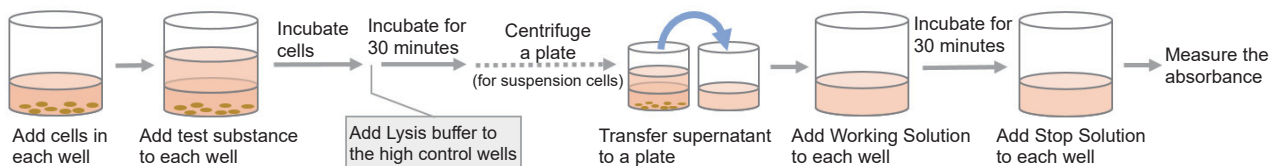
$$\text{Cytotoxicity(\%)} = \frac{(A-C)}{(B-C)} \times 100$$

A: Test substance  
 B: High control  
 C: Low control

**Non-homogeneous assay**



- 1) Collect cells and wash them with the medium. Prepare 5×10<sup>5</sup> cells/ml cell suspension in the medium.
- 2) Prepare 2-fold serially diluted cell suspension in a 96-well tissue culture plate.  
 \*Refer the Serial Dilution Procedure in page 6.
- 3) Add 100 µl of the cell suspension and 100 µl of the medium to each well.
- 4) Incubate the plate at 37°C for an appropriate time in a CO<sub>2</sub> incubator.
- 5) Add 20 µl of Lysis Buffer to each well of the high control.
- 6) Incubate the plate at 37°C for 30 minutes in the CO<sub>2</sub> incubator.
- 7) Centrifuge the plate at 250 × g for 2 minutes to precipitate the cells (for suspension cells).
- 8) Transfer 100 µl of the supernatant from each well to a 96-well clear plate.
- 9) Add 100 µl of Working Solution to each well. Protect the plate from light and incubate it at the room temperature for 30 minutes.
- 10) Add 50 µl of Stop Solution to each well.
- 11) Measure the absorbance at 490 nm by using a microplate reader.  
 \*Plot the data by setting concentration of cells on the x-axis and absorbances on the y-axis. The plot should have two linear lines, one representing High Control and another Low Control. Determine the optimum concentration of cells based on the followings.  
 - Eliminate the concentration of cells that has absorbances higher than 2.0.  
 - Choose the concentrations of cells that has at least 0.2 difference in the absorbance between high control and low control.



- 1) Add 100 µl of the cell suspension to each well of a 96-well tissue culture plate.  
\*For adherent cells: incubate the plate at 37°C overnight in a CO<sub>2</sub> incubator to allow the cells to adhere and then replace the medium with 100 µl of fresh medium.
- 2) Add 100 µl of the medium containing test substance that adjusted to the desired concentration (Refer to Table 2).
- 3) Incubate the plate at 37°C for an appropriate time in a CO<sub>2</sub> incubator.
- 4) Add 20 µl of Lysis Buffer to each well of the high control. Incubate the plate at 37°C for 30 minutes in the CO<sub>2</sub> incubator.
- 5) Centrifuge the plate at 250 × g for 2 minutes to precipitate the cells (for suspension cells).
- 6) Transfer 100 µl of the supernatant from each well to each well of a new 96-well clear plate.
- 7) Add 100 µl of the Working Solution to each well. Protect the plate from light and incubate it at room temperature for 30 minutes.
- 8) Add 50 µl of Stop Solution to each well.
- 9) Measure the absorbance at 490 nm by using a microplate reader.

**Calculation of Cytotoxicity**

Calculate the average absorbance from each triplicate set of wells and subtract the absorbance of background from each absorbance of test substance, high control, and low control. Determine the percent cytotoxicity by the following equation.

$$\text{Cytotoxicity(\%)} = \frac{(A-C)}{(B-C)} \times 100$$

A: Test substance  
B: High control  
C: Low control

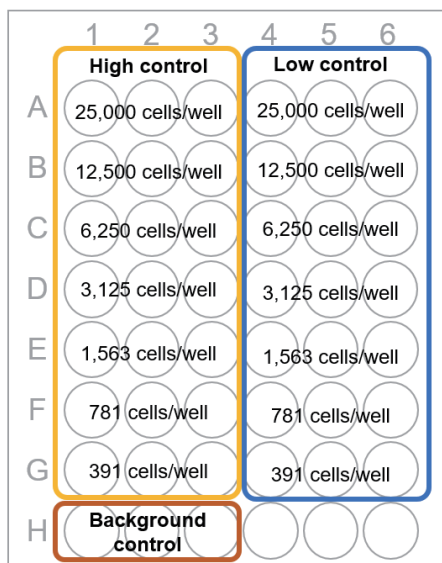


Fig. 2 Plate arrangement

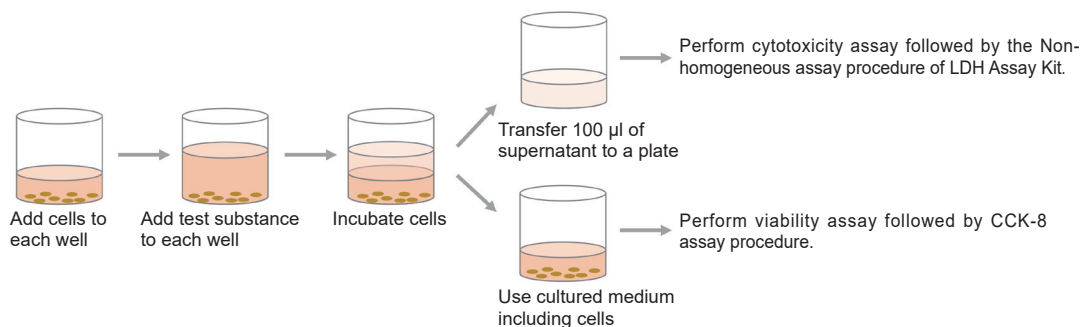
Table 2 Amount of each solution (Non-homogeneous assay)

	Test substance	High control	Low control	Background control
Medium	20 µl	100 µl	120 µl	220 µl
Cell suspension	100 µl	100 µl	100 µl	-
Test substance in culture medium	100 µl	-	-	-
Lysis Buffer	-	20 µl	-	-

- Test substance : The released LDH activity from the cell when the test substance is added to the cell
- High control : The total LDH activity in the cell (= Maximum LDH release)
- Low control : The LDH activity released from the untreated cells to the medium (= Spontaneous LDH release)
- Background control : The LDH activity in the medium  
※ medium = culture medium



## Experimental example for CCK-8 and LDH assay in same cells



- 1) Add 5000 cells/100 µl of HeLa cell to each well of a 96-well tissue culture plate.
- 2) Incubate the plate at 37°C overnight in a CO<sub>2</sub> incubator.
- 3) Add 100 µl of the medium containing test substance that adjusted to the desired concentration.
- 4) Incubate the plate at 37°C for one hour in the CO<sub>2</sub> incubator.  
\*Settle the suitable incubation time for the experiment.
- 5) Add 20 µl of Lysis Buffer to each well of the high control at 30 minutes before the end of incubation time.

### LDH assay using supernatant (under the procedure of Non-homogeneous assay)

- 6) Transfer 100 µl of the supernatant to a new 96-well clear plate.
- 7) Add 100 µl of the Working Solution to each well.
- 8) Protect the plate from light and incubate it at room temperature for 30 minutes.
- 9) Add 50 µl of Stop Solution to each well.
- 10) Measure the absorbance at 490 nm by using a microplate reader.

### CCK-8 assay using cultured medium with cells

- 11) Add 10 µl of CCK-8 to each well where cells have been cultured.
- 12) Incubate the plate at 37°C for 3 hours in the CO<sub>2</sub> incubator.  
\*Settle the suitable colorimetric reaction time for the experiment.
- 13) Measure the absorbance at 450 nm by using a microplate reader.

### < Result of experimental example >

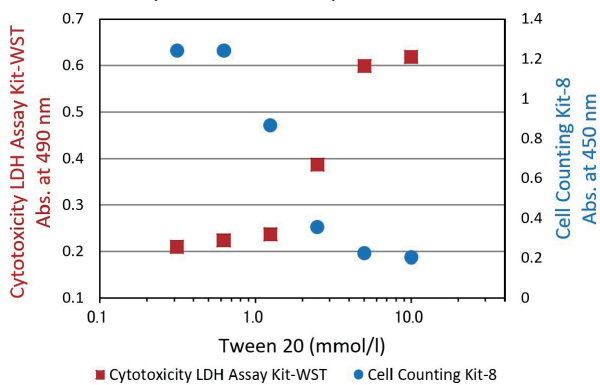


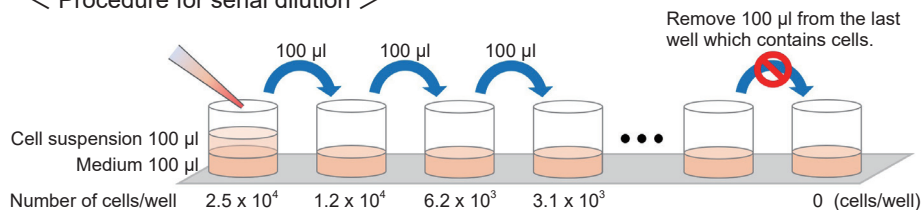
Fig.3 Cytotoxicity assay of Tween 20

Cell : HeLa cell  
Medium : MEM with 10% FBS  
Test substance : Tween 20  
Drug exposure time : 1 hour

## Serial Dilution Procedure

Add 100 µl of medium to each well of a 96-well tissue culture plate. Then, add the cell suspension ( $5 \times 10^5$  cells/ml) to the first well and mix by pipetting. This well contains the maximum number of cells ( $2.5 \times 10^4$  cells/well). Transfer 100 µl from the first well to the next well, and mix by pipetting. Repeat this procedure.

### < Procedure for serial dilution >



If you need more information, please contact Dojindo technical service.

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