Cytotoxicity LDH Assay Kit-WST

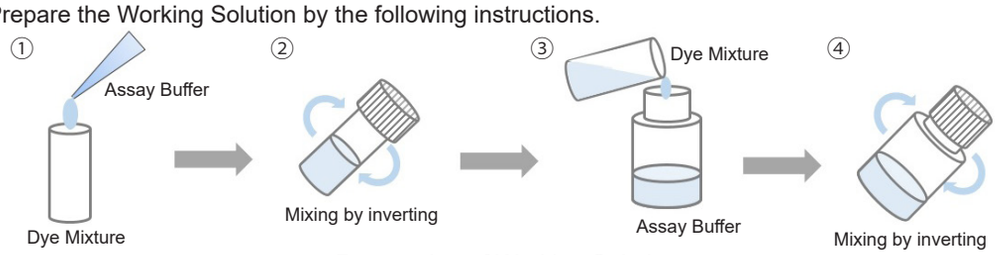
Supplementary Information

Notice to Users

This instruction complements the Technical Manual in the product. Please use this instruction as supplements of the Technical Manual.

Preparation of Reagent

Prepare the Working Solution by the following instructions.



Preparation of Working Solution

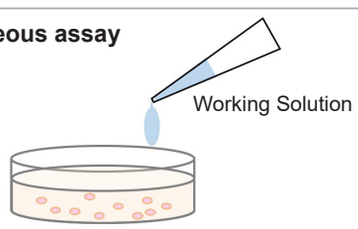
1. Add appropriate volume of Assay Buffer to a Dye Mixture vial and close the cap.
   100 tests: Add 1 ml of Assay Buffer to a Dye Mixture vial.
   500 or 2000 tests: Add 5 ml of Assay Buffer to a Dye Mixture vial.
2. Dissolve the contents completely.
3. Transfer all of the solution of step 2 to Assay Buffer bottle.
4. Close the cap and mix it well.

※ After preparing Working Solution, please store it at 0-5°C and protect from light. Under this condition, it is stable for six months.

Selection of the Assay

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

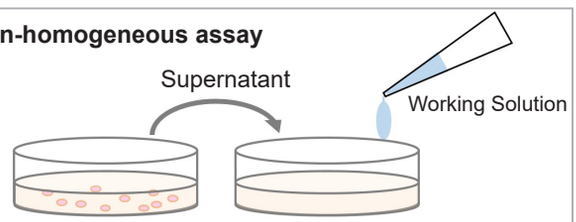
Homogeneous assay



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 2 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 3 for this procedure.

Optimization of Cell Number

The amount of intracellular LDH is different in each type of cells. Therefore, it is required to optimize the number of cells to obtain reliable results before performing your cytotoxicity assay. Measure each absorbance of high control, low control and background control, and then confirm the number of cells for cytotoxicity assay by the instruction below.

High control: LDH activity in the cell (= Maximum LDH release control)
Low control: LDH activity released from the untreated cells
Background control: LDH activity in the medium

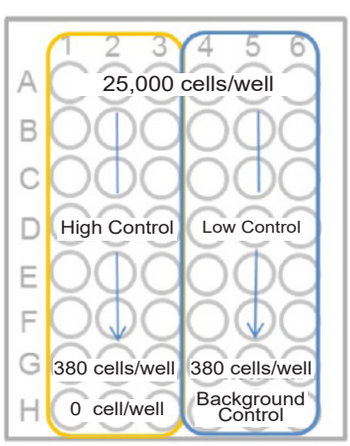


Plate arrangement

<Recommended conditions>
Plot the data by setting cell concentration for the x-axis and absorbance for the y-axis. Then, find the optimum cell number to meet the following condition.

- The difference in the absorbance between high control and low control is at least 0.2.
- The absorbance is lower than 2.0 and positioned on the linear point of plotted curve.

Optimization of Cell Concentration

Preliminary experiment before the cytotoxicity assay

1. Collect cells and wash them with the medium. Prepare cell suspension at 5 x 10^5 cells/ml with the medium.

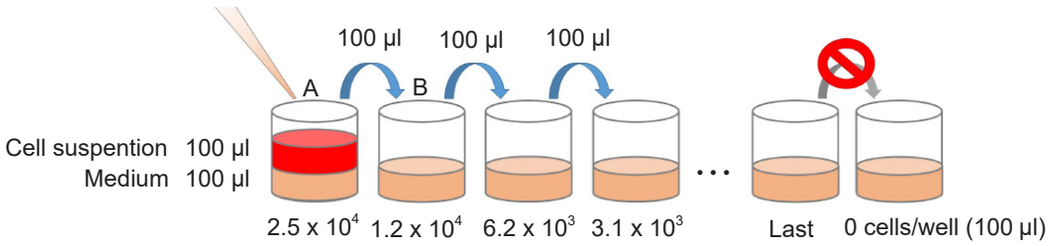
Tips Please prepare the large number of cells such as 10^5-10^6 cells/ml. In case the number of cell is not enough, it may cause experimental errors.

2. Add 100 µl of the medium to each well of a flat-bottom 96-well culture plate.
3. Prepare 2-fold serial dilution of each well in triplicate set of wells for the high control, low-control and background control (medium only).

Tips

〈Serial dilution procedure〉

Add the cell suspension (5 x 10^5 cells/ml) to the first well [A] and mix by pipetting. This well contains the maximum number of cells (2.5 x 10^4 cells/well). Transfer 100 µl from the first well to the next well [B], and mix by pipetting. Repeat this procedure.

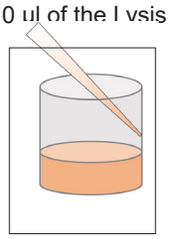


4. Incubate the plate at 37 °C for an appropriate time in a CO_2 incubator.

Tips The incubation time should be determined according to how long cells are exposed to test substance.

5. Add 10 µl of the Lysis Buffer to each well of the high control.

Tips



The amount of Lysis Buffer to be added is small, therefore the tip of the pipette should be touched to the wall. If the reagent remains on the wall, please tap the plate gently and mix with the medium.

6. Incubate the plate at 37 °C for 30 minutes in a CO_2 incubator.
7. Add 100 µl of the Working Solution to each well. Protect the plate from light and incubate it at the room temperature for 30 minutes.
8. Add 50 µl of the Stop Solution to each well.
9. Measure the absorbance at 490 nm by a microplate reader.

* Refer to "Optimization of Cell Number" on page 1 and determine the optimum condition.

Cytotoxicity Assay

1. Add 50 µl of cell suspension to each well of a flat-bottom 96-well culture plate.

Tips
- Please use the doubled concentration of cell suspension, which is confirmed in the preliminary experiment.
- For adherent cells, please incubate the culture plate overnight to attach the cells to the plate. After incubation, replace the medium with 50 µl of the fresh medium and proceed to step 2 .
- For suspension cells, skip this procedure.

2. Add 50 µl of medium containing test substance that adjusted to the desired concentration (Table 1).

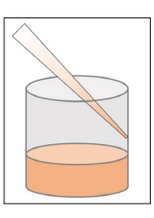
Tips
- Test substance should be prepared to the target concentration with the medium before use it.

3. Incubate the plate at 37°C for an appropriate time in a CO_2 incubator.

Tips
- Please determine the appropriate exposure time of cells to the test substance according to the experimental condition.

4. Add 10 µl of the Lysis Buffer to each well of the high control. Incubate the plate at 37 °C for 30 minutes in a CO_2 incubator.

Tips



The amount of Lysis Buffer to be added is small, therefore the tip of the pipette should be touched to the wall. If the reagent remains on the wall, please tap the plate gently and mix with the medium.

5. Add 100 µl of the Working Solution to each well. Protect the plate from light and incubate it at the room temperature for 30 minutes.
6. Add 50 µl of the Stop Solution to each well.
7. Measure the absorbance at 490 nm by a microplate reader.

Table 1. Overview of the controls (Homogeneous Assay)

	Test substance	High control*	Low control	Background control
Medium	—	50 µl	50 µl	100 µl
Cell suspentions	50 µl	50 µl	50 µl	—
Test substance in culture medium	50 µl	—	—	—
Lysis Buffer	—	10 µl	—	—

*The volume of high control increases by 10% against other controls. However, a volume adjustment is not necessary because this volume difference does not affect the experimental results.

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

Calculation of Cytotoxicity

Calculate the average absorbance from each triplicate set of wells and subtract the background control value from each absorbance. Determine the percent of cytotoxicity by a following equation.

$$\text{Cytotoxicity (\%)} = \frac{\text{Test Substance - Low Control}}{\text{High Control - Low Control}} \times 100$$

Non-homogeneous Assay

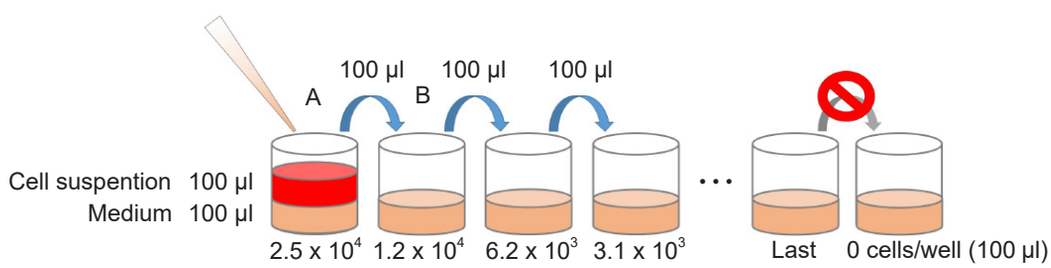
Optimization of Cell Concentration

Preliminary experiment before the cytotoxicity assay

1. Collect cells and wash them with the medium. Prepare cell suspension to 5 x 10^5 cells/ml in the medium.
   Please prerare the large number of cells such as 10^5-10^6 cells/ml.
   Tips In case the number of cell is not enough, it may cause measurement error.
2. Add 100 µl of the medium to each well of a 96-well culture plate.
   Tips Use round or v-bottomed plate for suspension cells, flat-bottomed plate for adherent cells
3. Prepare 2-fold serial dilution of each well in triplicate set of wells for the high-control, low-control and background control (medium only).

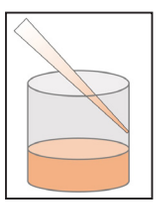
Tips

〈Serial dilution procedure〉
Add the cell suspension (5 x 10^5 cells/ml) to the first well [A] and mix by pipetting. This well contains the maximum number of cells (2.5 x 10^4 cells/well). Transfer 100 µl from the first well to the next well [B], and mix by pipetting. Repeat this procedure.



4. Add 100 µl of the medium to each well.
5. Incubate the plate at 37 °C for an appropriate time in a CO_2 incubator.
   Tips Use the same incubation time in the cytotoxicity assay. Incubation time should be determined according to how long cells are exposed to the reagents after addition of reagents.
6. Add 20 µl of the Lysis Buffer to each well of the high control.

Tips



The amount of Lysis Buffer to be added is small, therefore the tip of the pipette should be touched to the wall. If the reagent remains on the wall, please tap the plate gently and mix with the medium.

7. Incubate the plate at 37 °C for 30 minutes in a CO_2 incubator.
8. Centrifuge the plate at 250 x *g* for 2 minutes to deposit the cells (for suspension cells).
   Tips For adherent cells, you can skip this step.
9. Transfer 100 µl of the supernatant from each well to an optically clear flat-bottom 96-well plate.
10. Add 100 µl of the Working Solution to each well. Protect the plate from light and incubate it at the room temperature for 30 minutes.
11. Add 50 µl of the Stop Solution to each well.
12. Measure the absorbance at 490 nm by a microplate reader.
    * Refer to "Optimization of Cell Number" on page 1 and determine the optimum condition.

Cytotoxicity Assay

1. Add 100 µl of the cell suspension to each well of a 96-well culture plate.

Tips
- For adherent cells: incubate the plate at 37°C overnight in a CO_2 incubator to allow the cells to adhere and then replace the medium with 100 µl of fresh medium.
- Please use the concentration of cell suspension, which is determined in the preliminary experiment.
- For suspension cells, skip this procedure.

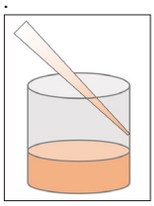
2. Add 100 µl of the medium containing test substance that adjusted to the desired concentration.

3. Incubate the plate at 37 °C for an appropriate time in a CO_2 incubator.

Tips Please determine the exposure time of cells to the test substance according to the experimental condition.

4. Add 20 µl of the Lysis Buffer to each well of the high control. Incubate the plate at 37°C for 30 minutes in a CO_2 incubator.

Tips



The amount of Lysis Buffer to be added is small, therefore the tip of the pipette should be touched to the wall. If the reagent remains on the wall, please tap the plate gently and mix with the medium.

5. Centrifuge the plate at 250 x *g* for 2 minutes to deposit the cells (for suspension cells).

Tips For adherent cells, you can skip this step.

6. Transfer 100 µl of the supernatant from each well to each well of a new optically clear flat-bottom 96-well 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 the Stop Solution to each well.
9. Measure the absorbance at 490 nm by a microplate reader.

Table 2. Overview of the controls (Non-homogenous Assay)

	Test substance	High control	Low control	Background control
Medium	20 µl	100 µl	120 µl	220 µl
Cell suspentions	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

Calculation of Cytotoxicity

Calculate the average absorbance from each triplicate set of wells and subtract the background control value from each absorbance. Determine the percent of cytotoxicity by a following equation.

$$\text{Cytotoxicity (\%)} = \frac{\text{Test Substance - Low Control}}{\text{High Control - Low Control}} \times 100$$

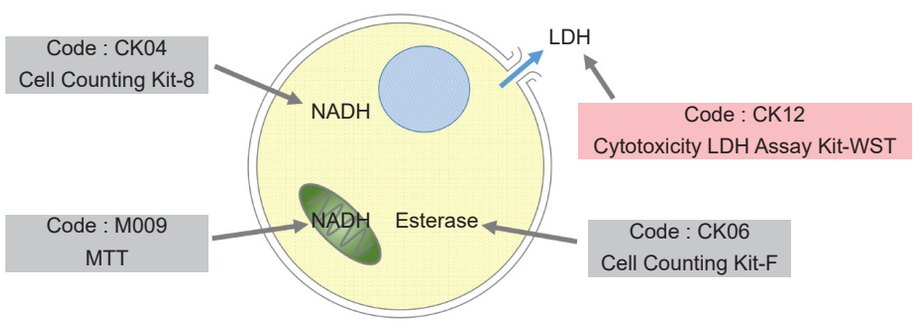
Troubleshooting

Problem	Possible cause	Recommendation
Large variation of absorbance	Bubbles are in the medium.	Please break bubbles by using a needle.If you have a centrifuge for the 96-well plate, please centrifuge it for a minute with 1,000 x *g*.
	Test substance concentration changes by the evaporation of the medium.	Evaporation easily occurs in the outermost wells of the microplate. Therefore, if the plate is incubated for a long time, please add the medium in the outermost wells and do not use them for an assay.
	Reagents are not mixed well.	Please be noted that the amount of the added Lysis Buffer is less than other reagents, and hence it can affect the High Control values. Please mix each reagent and the medium by a plate mixer or tap the surface of the plate with fingers gently when Lysis Buffer, Working Solution, and Stop Solution are added. Also, do not tap the plate too hard so that the medium inside the well would not leak.
	Reagent volume are not accurate.	Calibrate the pipett.
	Reaction time varies among wells by using a single channel pipette.	Multichannel pipettes are recommended to add Working Solution and Stop Solution.
High background values	Medium contains high concentration of LDH.	Use the serum-free medium or the medium contains the serum less than 5%.
	Test substance or the medium has reducing activity.	Some reducing compounds such as ascorbic acid react directly with WST dye. Use medium such as DMEM, RPMI, F-12 do not contain reducing compounds.
Low absorbance values	Test substance or medium inhibit LDH activity or the colorimetric reaction.	Refer to FAQ

FAQ

Q : Can I use this kit with cell viability assay such as Cell Counting Kit -8 and MTT?
A : Yes. Please perform the Non-homogenous Assay, which uses a supernatant culture media. Remained cells can be used for the viability assay by Cell Counting Kit-8 or other reagents.

Q : When I perform cytotoxicity assay with the LDH Kit, can I obtain the correlated data with one performed by Cell Counting Kit-8 ?



The data obtained by the LDH Kit and Cell Counting Kit-8 are not always correlated because each assay measures the different objects.

- The LDH Assay measures the released LDH activity through damaged cell membrans.
- Cell Counting Kit-8 measures the dehydrogenase activity in living cells.

FAQ

Q : Does the Dojindo's LDH Kit includes the standard LDH?
A : No. This kit does not include the standard LDH. If necessary, please purchase it from a reagent manufactures.

Q : Can I measure isozymes of the LDH?
A : No. This product measures the total LDH activity for cytotoxicity assay.

Q. Are there any interfering substances?
A. There are some interfering substances below.

1. Reducing agents

   Since some reducing agents such as ascorbic acid cause a high background, determine if test substance increases the O.D. value of the medium containing the working solution in the absence of cells. This background can be subtracted as the test substance background*.
   *In this case, the O.D. values of the test substance background are required.
   Test substance: test substance + medium + working solution

2. LDH included serum in the medium

   LDH contained in the
   medium increases the O.D. value of each well. The background from the LDH can be subtracted from the O.D. value of each sample. If the background values are too high, use serum-free medium or reduce the concentration of serum less than 5%.

3. Inhibitors of LDH activity

   For example, the medium containing pyruvic acid in high concentration inhibits the colorimetric reaction between LDH and WST. In case the O.D. value of high control is low (close to that of low control in the preliminary experiment), determine if pyruvic acid is contained in the medium.

Dojindo Laboratories
2025-5 Tabaru, Mashiki-machi, Kamimashiki-gun,
Kumamoto 861-2202, Japan Phone::+81-96-286-1515 Fax:+81-96-286-1525
E-mail: info@dojindo.co.jp Web:www.dojindo.co.jp/

Dojindo Molecular Technologies, Inc.
Tel: +1-301-987-2667 Web: http://www.dojindo.com/
Dojindo EU GmbH
Tel: +49-89-3540-4805 Web: http://www.dojindo.eu.com/
Dojindo China Co., Ltd
Tel: +86-21-6427-2302 Web: http://www.dojindo.cn/