# WSE-7620 EzCyto LDH Cytotoxicity Kit - Instruction Manual

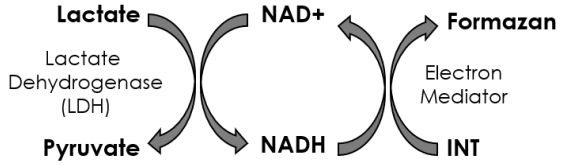
First edition : August 21, 2026

## 1. Safety Precautions

Before using this product, read this instruction manual carefully. Do not operate the product until you fully understand the contents of this manual. This manual describes only the methods for using the product for its intended purpose. Do not use the product for purposes or by methods not specified in this manual. If the product is used for an unspecified purpose or by an unspecified method, the operator is responsible for taking all necessary safety precautions and for any unforeseen circumstances.

## 2. Purpose of use

This kit is designed to quantify cytotoxicity by measuring the activity of lactate dehydrogenase (LDH) released into the culture medium as a result of cell damage. The resazurin reagent included in the kit can also be used to measure the number of viable cells and cellular metabolic activity by fluorescence or colorimetric detection. LDH is released into the extracellular environment when the cell membrane is damaged. Measuring LDH activity in culture supernatant enables evaluation of immune-cell-mediated cytotoxicity, such as that caused by NK cells or CTLs, as well as drug toxicity and cytotoxicity caused by chemical substances. In the culture supernatant, LDH oxidizes lactate to pyruvate while reducing NAD+ to NADH. The generated NADH reduces the tetrazolium salt INT through an electron mediator, producing a water-soluble formazan dye. LDH activity can be quantified by measuring the absorbance of the generated formazan dye at approximately 490 nm. The absorbance is proportional to the amount of LDH released.




Resazurin, on the other hand, is reduced by redox enzymes in the mitochondria and cytoplasm of viable cells and is converted to fluorescent resorufin. The amount of resorufin generated is proportional to the number of viable cells and their metabolic activity.
Reference: Matthew A, et al. Clin Mater. 1994;16(4):189-194.

## 3. Kit Components

| | Volume | Qty. | Storage |
|---|---|---|---|
| Amber vial<br>**LDH Reagent 1**<br>Substrate/dye solution | 5 mL | 2 | Frozen (-20 ℃ or below) |
| Amber tube<br>**LDH Reagent 2**<br>Coenzyme solution | 1 mL | 2 | Frozen (-20 ℃ or below) |
| White bottle<br>**LDH Stop Solusion**<br>Reaction stop solution | 50 mL | 1 | Frozen (-20°C or below); room temperature permitted |
| Clear vial<br>**LDH Lysis Solution**<br>Cell lysis solution (20x conc.) | 4 mL | 1 | Frozen (-20°C or below); room temperature permitted |
| Clear-cap tube<br>**LDH Positive Prevention**<br>Standard enzyme solution (10 kU/mL) | 0.05 mL | 1 | Frozen (-20°C or below) |
| Red-cap<br>**Resazurin Reagent**<br>Cell proliferation measurement | 1.5 mL | 2 | Frozen (-20°C or below); refrigeration permitted |

Prepare LDH Working Solution by mixing one bottle of LDH Reagent 1 with one bottle of LDH Reagent 2. This amount is sufficient for approximately one 96-well plate (about 100 samples).

## 4.Composition

| Name | Main component |
|---|---|
| **LDH Reagent 1**<br>Substrate/dye solution | Lithium lactate |
| **LDH Reagent 2**<br>Coenzyme solution | β-NAD |
| **LDH Stop Solution**<br>Reaction stop solution | Phosphoric acid |
| **LDH Lysis Solution**<br>cell lysate | Polyoxyethylene (10) octylphenyl ether |
| **LDH Positive Control**<br>Standard enzyme solution | Lactate dehydrogenase |
| **Resazurin Reagent**<br>Cell proliferation measurement | 2-Amino-2-hydroxymethyl-1,3-propanediol |

This product contains substances subject to notification under Japan's PRTR Act and Industrial Safety and Health Act in amounts exceeding the applicable exemption thresholds. For details, download and review the SDS for this product from the ATTO website (https://www.atto.co.jp).

## 5. Storage

- This product is shipped refrigerated or frozen. After receipt, protect the reagents from direct sunlight and store each reagent at the appropriate temperature. Unopened reagents are stable through the stated expiration date (approximately one year from the date of manufacture).
- After thawing, LDH Reagent 1 and LDH Reagent 2 can be stored for approximately three months under uncontaminated conditions at 2-10°C.
- LDH Working Solution prepared by mixing LDH Reagent 1 and LDH Reagent 2 is stable for approximately four weeks when frozen at -20°C or below, or approximately two weeks when refrigerated at 2-10°C.

## 6. Disposal

- Dispose of each reagent in accordance with the disposal procedures of your institution.

| Container | Body | Closure / Seal |
|---|---|---|
| Amber / clear vial | Body: polyethylene | Cap: polypropylene |
| White bottle | Body: polypropylene copolymer | Cap: polypropylene |
| Tube | Body: polypropylene | Cap: polypropylene;<br>O-ring: silicone rubber |

## 7. Additional Materials Required

- Measurement vessel: 96-well plate or equivalent
- Measurement instrument: spectrophotometer, plate reader, or equivalent
- Pipettes, tubes, tips, and other standard laboratory supplies

## 8. Precautions for Use

- Allow reagents to return to room temperature before use.
- Protect LDH Working Solution and samples during the reaction (including 96-well plates) from light.
- When dispensing samples or reagents into the measurement

vessel, avoid introducing air bubbles.

- If a phenol-red-containing medium is used, background may increase. If necessary, dilute the sample or use a phenol-red-free medium.
- Depending on the assay system, serum-derived LDH may contribute to background. If necessary, dilute the sample or use low-serum medium.
- Dye may deposit on quartz or glass cuvettes. After use, soak the cuvettes in methanol or diluted chlorine bleach, then wash with a glassware detergent.
- LDH Stop Solution may irritate the skin and eyes. Exercise adequate care during storage and handling.
- When preparing a calibration curve, use the mean values after subtraction of the blank value. Apply the same blank subtraction to measured samples before conversion or calculation.

## 9. Before use

### 9-1. Reagent Preparation

① **LDH Working Solution**

Mix one bottle of LDH Reagent 1 with one tube of LDH Reagent 2 to prepare LDH Working Solution. This prepares the quantity required for approximately one 96-well plate (about 100 samples). LDH Working Solution can be stored for approximately two weeks at 2-10°C or approximately four weeks at -20°C or below.

| LDH Working Solution | LDH Reagent 1 | LDH Reagent 2 |
|---|---|---|
| | 1 bottle (5 mL ) | 1 tube (1 mL ) |

② **Medium Containing LDH Lysis Solution**

Add LDH Lysis Solution to the culture medium at 1/20 of the medium volume. The required volume per well is 100 µL. For example, add 500 µL of LDH Lysis Solution to 10 mL of medium and mix.

＊Medium containing LDH Lysis Solution can be stored for approximately two weeks at 2-10°C.

### 9-2 . Control Setup

**A. Cytotoxicity Caused by Drugs, Chemicals, or Other Substances**

1. **Background Control —** Measures LDH activity derived from the culture medium.
2. **Low Control —** Measures LDH activity derived from cells that have not been treated with the drug or other substance.
3. **High Control —** Add LDH Lysis Solution to measure the maximum LDH activity that can be released from normal cells.
4. **Substance (Drug, etc.) Control 1 —** Measures LDH activity derived from the test substance itself.
5. **Substance (Drug, etc.) Control 2 —** Checks whether the test substance inhibits LDH activity. Use LDH Positive Control.

**B. Cell-Mediated Cytotoxicity**

1. **Effector Cell Control —** Measures LDH activity derived from effector cells.
2. **Low Control (Target) —** Measures LDH activity derived from target cells.
3. **High Control (Target) —** Add LDH Lysis Solution to measure the maximum LDH activity that can be released from target cells.
4. **Low Control (Medium) —** Equivalent to the background control above; measures LDH activity derived from the medium.
5. **High Control (Medium) —** Measures LDH activity derived from medium containing LDH Lysis Solution.

### 9-3 . Method for calculating cytotoxicity

First subtract the medium-derived LDH activity from each measured value. Then calculate cytotoxicity (%) using the equations below.

**1) Cytotoxicity Caused by Drugs, Chemicals, or Other Substances**

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental value - Low control}}{\text{High control - Low control}} \times 100$$

※Before using the equation, subtract the Background Control value from the Experimental value, Low Control, and High Control values.

**2) Cell-Mediated Cytotoxicity**

$$\text{Cytotoxicity (\%)} = 100 \times \frac{[(\text{Effector + Target}) \text{ value} - \text{Effector value}] - \text{Low control (Target)}}{\text{High control (Target) - Low control (Target)}}$$

※Before using the equation, subtract the Low Control (Medium) value from the Effector + Target value, Effector value, and Low Control (Target) value. Subtract the High Control (Medium) value from the High Control (Target) value.

## 10. Procedure

### 10-1. Preliminary Experiment (Determining the Appropriate Cell Number)

Because the amount of intracellular LDH differs among cell types, first determine the optimal cell concentration.

1. Disperse the cells using trypsin or another suitable method and prepare a cell suspension at 4-8 x 10^5 cells/mL.
2. Add 100 µL of medium to columns 1-6 of a 96-well plate.
3. Add 100 µL of the cell suspension to columns 1-6 of row A and mix.
4. Transfer 100 µL of the cell suspension from row A to row B and mix.
5. In the same manner, transfer and mix the cell suspension from B to C, C to D, and so on through F to G to prepare a 1/2 serial dilution of the cells. Do not add cells to row H; use medium only as the background control.

| | High control | | | Low Control | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| A | 40000 cells/well | | | 40000 cells/well | | |
| B | 20000 cells/well | | | 20000 cells/well | | |
| C | 10000 cells/well | | | 10000 cells/well | | |
| D | 5000 cells/well | | | 5000 cells/well | | |
| E | 2500 cells/well | | | 2500 cells/well | | |
| F | 1250 cells/well | | | 1250 cells/well | | |
| G | 625 cells/well | | | 625 cells/well | | |
| H | 0 Background | | | 0 Background | | |

Example well layout

6. Incubate for the required period in a $CO_2$ incubator.

Set the culture period to match the duration of the cytotoxicity test. For adherent cells, incubate overnight to allow the cells to attach; replace the medium as appropriate.

**7.** Add 100 µL of medium containing LDH Lysis Solution to the High Control wells (rows A-H, columns 1-3).

**8.** Add 100 µL of medium to the Low Control wells (rows A-H, columns 4-6).
For the Low Control, sterile water or sterile PBS(-) may be added instead of LDH Lysis Solution, as appropriate.

**9.** Incubate for 30 minutes in a $CO_2$ incubator.

**10.** Collect 100 µL of culture supernatant from all wells (rows A-H, columns 1-6) into a separate 96-well test plate.

**11.** Add 50 µL of LDH Working Solution to each collected supernatant sample.

**12.** React for 10-30 minutes at room temperature, protected from light.

The color-development time is a guideline. It may be adjusted as long as sufficient absorbance is obtained, but use the same reaction time within a given experiment.

**13.** Add 50 µL of LDH Stop Solution to every well used for the reaction.

**14.** Using a microplate reader, measure absorbance at 490 nm. For background correction, also measure absorbance at the reference wavelength of 680 nm and use A490 - A680 as the measured value.

Within the cell-concentration range in which LDH activity is proportional to cell number, select the cell concentration that gives the largest difference between the High Control and Low Control values, and use that concentration for the cytotoxicity test.

## 10-2 . Cytotoxicity Test Using Drugs, Chemicals, or Other Substances .

**1.** Prepare a cell suspension at the cell concentration determined in the preliminary experiment. Wash the cells with the medium to be used.
For 10,000 cells/well, prepare the suspension at 1 x 10^5 cells/mL.

**2.** Seed 100 µL of the cell suspension into each well of a 96-well plate.
For adherent cells, incubate overnight to allow the cells to attach. Replace the medium as appropriate.

**3.** Prepare a new 96-well test plate and dilute the test substance (drug, etc.) with medium or another suitable diluent. Because mixing equal volumes of the substance solution and cell suspension produces a two-fold dilution, prepare the substance at twice the intended final concentration. Prepare 100 µL per well.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | High control | 1× Substance | 1/2× Substance | 1/4× Substance | 1/8× Substance | 1/16× Substance | 1/32× Substance | 1/64× Substance | 1/128× Substance | Low Control | Substance control | Background |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |

Example well layout

When testing with n=3, edge wells may show larger errors. Avoid rows A and H and use the central area of the plate. If reducing the number of dilution steps, similarly avoid columns 1 and 12 and use the central area.

**4.** Incubate for the required period in a CO2 incubator.

**5.** Collect 100 µL of culture supernatant from all wells (rows A-D, columns 1-12) into a separate 96-well test plate.
After collecting the culture supernatant, the remaining cells can be quantified by the resazurin assay described in Section 10-4.

**6.** Add 50 µL of LDH Working Solution to every well containing collected culture supernatant.

**7.** React for 10-30 minutes at room temperature, protected from light.
The color-development time is a guideline. It may be adjusted as long as sufficient absorbance is obtained, but use the same reaction time within a given experiment.

**8.** Add 50 µL of LDH Stop Solution to every well used for the reaction.

**9.** Using a microplate reader, measure absorbance at 490 nm. For background correction, also measure absorbance at 680 nm and use A490 - A680 as the measured value.

### 10-3 . Cell-Mediated Cytotoxicity Test

**1.** Prepare the target-cell suspension at the cell concentration determined in the preliminary experiment. Wash the cells with the medium to be used.
For 10,000 cells/well, prepare the suspension at 1 x 10^5 cells/mL.

**2.** Wash the effector cells with the medium to be used and prepare the effector-cell suspension at twice the cell concentration of the first point in the 1/2 serial dilution.
If starting with an effector-to-target cell ratio of 10:1, 100,000 effector cells/well are required; therefore prepare the effector-cell suspension at twice that concentration, 2 x 10^6 cells/mL.

**3.** Add 100 µL of medium to columns 2-11 of a 96-well plate. Add 100 µL of the effector-cell suspension to column 2 in rows A-H and mix.

**4.** Transfer 100 µL of the cell suspension from column 2 to column 3 and mix.

**5.** Continue in the same manner from column 3 to 4, 4 to 5, and so on through column 10 to 11 to prepare a 1/2 serial dilution of effector cells. Do not add cells to column 12; use medium only as the control.

**6.** Seed 100 µL of target-cell suspension into each well in rows A-D, columns 1-11. Also seed 100 µL of target-cell suspension into rows G-H of column 1.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | High control **Target cell** | Effector Cells (1x) +Target cells | Effector Cells (1/2x) +Target cells | Effector Cells (1/4x) +Target cells | Effector Cells (1/8x) +Target cells | Effector Cells (1/16x) +Target cells | Effector Cells (1/32x) +Target cells | Effector Cells (1/64x) +Target cells | Effector Cells (1/128x) +Target cells | Effector Cells (1/256x) +Target cells | Effector Cells (1/512x) +Target cells | High control **Medium** |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | Low control **Target cell** | Effector Cells (1x) | Effector Cells (1/2x) | Effector Cells (1/4x) | Effector Cells (1/8x) | Effector Cells (1/16x) | Effector Cells (1/32x) | Effector Cells (1/64x) | Effector Cells (1/128x) | Effector Cells (1/256x) | Effector Cells (1/512x) | Low control **Medium** |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

When testing with n=3, edge wells may show larger errors. Avoid rows A and H and use the central area. If reducing the number of dilution steps, similarly avoid columns 1 and 12 and use the central area.

**7.** Add 100 µL of medium to each well in rows E-H, columns 2-12. Also add 100 µL of medium to each well in rows A-D of column 12.

**8.** Incubate for the required period in a CO2 incubator.
Adjust the culture period as appropriate because it depends on the cytotoxicity test.

**9.** Thirty minutes before the end of the culture period, add 10 µL of medium containing LDH Lysis Solution to the High Control wells (rows A-D, columns 1 and 12).

**10.** Incubate for 30 minutes in a CO2 incubator.

**12.** Collect 100 µL of culture supernatant from all wells (rows A-H, columns 1-6) into a separate 96-well test plate.

**13.** Add 50 µL of LDH Working Solution to each collected

supernatant sample.

**14.** React for 10-30 minutes at room temperature, protected from light.

The source manual proceeds from step 10 directly to step 12. The numbering above follows the source document. The color-development time is a guideline; it may be adjusted as long as sufficient absorbance is obtained, but use the same reaction time within a given experiment.

**15.** Add 50 µL of LDH Stop Solution to every well used for the reaction.

**16.** Using a microplate reader, measure absorbance at 490 nm. For background correction, also measure absorbance at 680 nm and use A490 - A680 as the measured value.

Within the cell-concentration range in which LDH activity is proportional to cell number, select the cell concentration that gives the largest difference between the High Control and Low Control values, and use that concentration for the cytotoxicity test.

### 10-4 . Cell Number Measurement by Resazurin Assay

**1.** After collecting the culture supernatant, add 10 µL of Resazurin Reagent to every well containing the remaining cells.

Add 10 µL of Resazurin Reagent per 100 µL of cell culture medium.

**2.** Incubate for 1-4 hours in a CO2 incubator.

The rate and extent of color development vary depending on cell type and cell concentration. Contamination during incubation may affect the measured values; therefore, perform all operations aseptically.

**3.** Using a microplate reader, measure absorbance at 570 nm. For background correction, also measure absorbance at 600 nm and use A570 - A600 as the measured value.

For fluorescence measurement, measure at Ex 540-570 nm and Em 580-610 nm. Fluorescence detection provides higher sensitivity.

## 11. Others

Even when the same protocol is followed, small differences in technique can produce large differences in results. Experimental know-how and related information can be downloaded from the ATTO website: https://www.atto.co.jp/

| Phenomenon | Possible Cause | Corrective Action |
|---|---|---|
| It doesn't produce any color, or its absorbance is too low. | Possible reagent problem. | Check the expiration date and storage conditions and use fresh reagents. Confirm the reagent preparation procedure and the amount added. |
| | Cell number is too low; LDH activity is low. | Optimize conditions by increasing the cell concentration or extending the culture period. |
| | Reaction time is too short. | Extend the color-development time. |
| | Cytotoxicity has not been sufficiently induced. | Review the effector-cell number, drug concentration, and culture period. |
| | Cell solubilization is insufficient. | Check the amount of lysis solution added and the treatment time, and confirm that the cells are completely lysed. |
| High background | High spontaneous LDH release. | Shorten the culture period and check the condition of the cells. |
| | Cells are dying during culture. | Check cell viability, passage number, and culture conditions. |
| | Cells were damaged during collection of the supernatant. | Collect the supernatant gently. If necessary, use supernatant after centrifugation. |
| | Background derived from serum or medium. | Set a medium blank and subtract the background value during analysis. |
| Higher control values | Poor cell condition. | Use healthy cells in the logarithmic growth phase. |
| | Culture period is too long. | Shorten the incubation time. |
| | LDH is released even from effector cells alone. | Set an effector-cell-only control and correct for the background. |
| High control measurement values are low. | Insufficient amount of lysis solution. | Add the specified amount of lysis solution. |
| | Insufficient lysis time. | Incubate for the full recommended period. |
| | Cells are not completely lysed. | Confirm the state of cell lysis under a microscope. |

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