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Technical Datasheet

EZcount™ Lactate Dehydrogenase Cell Assay Kit

Product Code: CCK036

1. Introduction:

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme present in all mammalian cells. Plasma membrane of live cells is impermeable to LDH. However it is rapidly released into the culture medium when plasma membrane is damaged. The quantity of the leaked LDH is used as a measure of cell cytotoxicity. Due to low molecular weight (35kDa) LDH is easily released from the cells even with very minor damage or perturbations in the membrane. This makes the LDH release assay very sensitive.

CCK036, EZCount™ LDH Cell Assay Kit has been designed for rapid determination of cell cytotoxicity using an enzyme coupled two step reaction

2. About the Assay:

EZcount™ Lactate Dehydrogenase (LDH) is based on an enzyme coupled reaction in which the LDH released from cells with damaged membrane reduces the tetrazolium dye to a coloured formazan product. The reaction takes place in two steps -

Step 1:
LDH catalyzes the conversion of lactate to pyruvate via reduction of NAD+ to NADH.

Step 2:
NADH thus generated reduces the tetrazolium dye to a coloured formazan product which can be measured spectrophotometrically.

Fig 1: Schematic representation of measurement of LDH released from damaged cells

Applications of LDH cell assay

- Determination of substance mediated cytotoxicity
- Determination of cell mediated cytotoxicity
- Estimation of total cell number

3. Kit contents:

The kit is sufficient for 500 assays.

Contents		Quantity	Storage
Code	Description		
CCK036(A)	LDH reagent	5 x 5ml	-20°C in dark
CCK036(B)	Lysis solution	5ml	-20°C in dark
CCK036(C)	Stop solution	25ml	-20°C in dark

4. Materials required but not provided in the kit:

- Cells in appropriate medium
- Adjustable pipettes and a repeat pipettor
- 96-well plate for culturing the cells
- 96-well plate reader capable of measuring the absorbance at 450nm and more than 600nm.

5. General guidelines:

It is important to optimize experimental factors like cell density, incubation time, media composition and concentration of the agents under investigation prior to use of EZcount™ LDH Cell Assay Kit.

5.1 Assay controls

It is necessary to include different types of controls depending on application of LDH assay. The controls specific for the assay types are given in section 6.3 and 6.4. Given below are the general assay controls to be included irrespective of the type of assay.

1. Maximum LDH control:
   It is recommended to perform maximum LDH estimation to determine total amount of LDH present in the cells. A critical factor that affects the value of maximum LDH control is the time of

addition of lysis solution. Approximate half-life of LDH in the culture medium is 9 – 10 hours. If lysis solution is added at the beginning of exposure period and if the exposure period is greater than 9 hours, the quantity of active LDH remaining at the end of experiment may underestimate the quantity of LDH present in untreated cells. On the other hand, if lysis solution is added at the end of experimental exposure period, cell in the untreated control wells will proliferate and increase in number and may have more LDH present at the end of the exposure period. This will give higher LDH values compared to the LDH values that would be present in the beginning of the exposure period. Considering these possibilities, time of addition of lysis solution should be empirically determined.

2. Background control
   Significant amount of LDH is present in the serum used for supplementing the culture medium. Amount of LDH present in the serum varies batch wise. This contributes to the background absorbance / fluorescence. Hence it is recommended to set a background control that contains medium without cells. Absorbance /fluorescence of background control wells should be subtracted from absorbance / fluorescence values of experimental wells to get corrected absorbance readings. Alternatively, reduced serum media or serum free media can be used to eliminate or reduce the background due to LDH in the serum.

3. Untreated control
   Untreated control, also known as vehicle control should be included in the assay to obtain LDH values generated due to the solvent in which test compound is dissolved. Untreated control wells contain the solvent at a concentration used for dissolving the test compound.

5.2 Incubation period and temperature

Significant fluorescence signal / absorbance is produced under recommended assay conditions i.e. 10 minutes incubation at room temperature.

If higher fluorescence signal / absorbance is required, incubation time or incubation temperature (up to 37ºC) or both can be increased.

5.3 Accuracy

- To obtain statistically significant data, perform the assay in triplicates or more.
- Accuracy of the assay depends on pipetting skills of the personnel. Inappropriate addition and mixing practices may result in erroneous and false-positive or false-negative results.
- Use of a repeating pipettor is recommended to deliver the reagents to the wells. This saves time and helps maintain more precise incubation times.
- Pipette tip should be equilibrated with the reagent before use. This is carried out by slowly filling up the tip with reagent and gently expelling the contents, several times.

5.4 Culture Medium

- Phenol red interferes with the measurement of fluorescence / absorbance; therefore the culture media used for the assay should not contain phenol red.
- Culture medium supplemented with pyruvate reduces the rate of reaction as it reduces the rate of conversion of lactate to pyruvate by LDH. When use of pyruvate containing medium is obligatory, it is recommended to increase either incubation time or incubation temperature (up to 37ºC) or both.

5.5 Stop solution

Stop solution should not be added while performing kinetic studies.

5.6 Light sensitivity of LDH reagent

LDH reagent is extremely sensitive to light. Prolonged exposure of the reagent results in increased background and decreased sensitivity.

6. Directions for use:

Users are advised to review entire procedure before starting the assay

6.1 Preparation of cells:

Always use freshly harvested cells for assay. Seed the cells in a cell culture flask or dish in an amount appropriate for the assay and incubate at 37ºC in a 5% CO_2 environment. Allow the cells to grow up to 24 hours or till confluence is reached.

Harvest the cells in a phenol red free medium and use for the assay. Refer section 5.4 for specifications of the medium required for the assay.

*(**Note**: The quantity of the cell suspension to be seeded in the medium depends upon doubling time of individual cell lines and seeding density to be used in the assay.)*

6.2 Optimization of cell density

It is recommended to determine the optimum cell density for every cell type as different cell types may contain variable amount of LDH. Optimum cell density is that cell density in which the difference between maximum LDH control and untreated control is maximum.

1. Harvest the cells as mentioned in section 6.1.
2. Prepare different cell densities by serial dilution.
3. Seed 90µl of each cell density in triplicates for maximum LDH control and in triplicates for untreated control.
4. Include background control (100µl of culture medium) in triplicates.
5. In case of adherent cells incubate the plate at 37ºC for 12 – 16 hours to allow the cells to attach bottom of plate.
   Suspension cells can be used immediately after seeding.
6. Thaw lysis solution CCK036(B) and equilibrate it at room temperature
7. After incubation, add 10µl lysis solution CCK036(B) to each well of maximum LDH control and add 10µl DPBS to each well of untreated control to compensate for the volume.
8. Incubate the plate at 37ºC and 5% CO2 for 30 – 45 minutes.
9. Observe cell lysis in maximum LDH control cells microscopically.
10. Centrifuge the plate at 1500rpm for 5 minutes to settle the cell debris at the bottom of wells.
11. Aseptically transfer 50µl supernatant from each well to a new 96 well plate.
12. Add 50µl LDH reagent to each well and incubate the plate at room temperature for 15 – 30 minutes (or till the colour development is sufficient.) *Note: Cover the plate with aluminium foil to avoid exposure to light.*
13. Thaw stop solution CCK036(C) and equilibrate it at room temperature.
14. Add 50µl of Stop solution CCK036(C) to each well and read the absorbance at 490nm as a main wavelength and a reference wavelength greater than 600nm.
15. Subtract the average absorbance values of background control wells from average absorbance values of maximum LDH control wells and untreated control wells.
16. Plot a curve of maximum LDH control and untreated control with cell density on X-axis and absorbance on Y-axis.
    ***Note**: Refer figure 2 for more details*

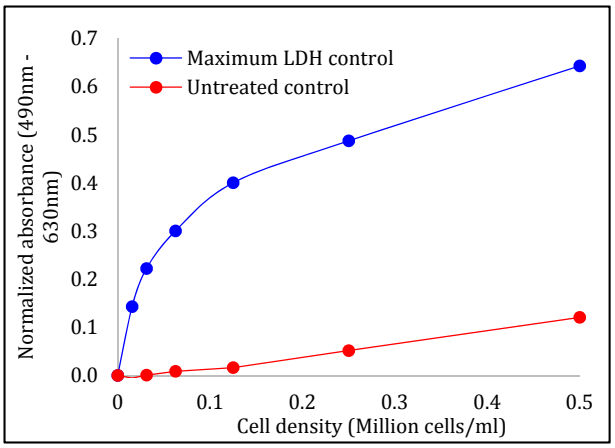

Fig 2: Determination of optimum density of CHO cells
CHO cells were serially diluted and plated in triplicate in 96 well plate. Maximum LDH control cells were treated with lysis solution and PBS was added to untreated control cells to compensate for the volume. LDH reagent was added to all wells after lysis treatment. As indicated in the graph, optimum cell density for CHO cells is 0.5 x 10^6 cells/ml.

6.3 Substance mediated cytotoxicity assay

In addition to the controls mentioned in section 5.1, it is necessary to include vehicle control and volume correction control when performing LDH assay for determination of substance mediated cytotoxicity.

Vehicle control: Corrects the LDH generated due to the solvent used for dissolving the test substance

Volume correction control: Corrects the volume change caused by addition of lysis solution / test substance.

1. Seed 90µl cell suspension in a 96-well plate at the optimized cell density.
2. In case of adherent cells, incubate the plate in an incubator at 37ºC and 5% CO_2 for 12 - 16 hours to allow attachment of the cells to wells.
3. In case of suspension cells, perform the assay immediately after plating the cells.
4. Set up appropriate assay control as mentioned in section 5.1. (Refer Table 1 for complete assay scheme).
5. Thaw lysis solution CCK036(B) and equilibrate it at room temperature.
6. Add 10µl lysis solution CCK036 (B) to maximum LDH control wells, 10µl test compound to experimental wells, 10µl DPBS to untreated control wells and 10µl solvent to vehicle control* wells. Include background control consisting of culture medium without cells.
   Note: The time at which lysis solution is added to the maximum LDH control wells should be determined empirically. Refer section 5.1 for more details.
   **If test compound is dissolved in DPBS, untreated control serves as a vehicle control also.*

7. Incubate the plate at 37ºC and 5% CO_2 for 30 – 45 minutes.
8. Observe cell lysis in maximum LDH control cells microscopically.
9. Centrifuge the plate at 1500rpm for 5 minutes to settle the cell debris at the bottom of wells.
10. Aseptically transfer 50µl supernatant from each well to a new 96 well plate.
11. Thaw a bottle of LDH reagent CCK036(A) and equilibrate it at room temperature.
12. Add 50µl of LDH reagent to each well and incubate the plate at room temperature for 15 – 30 minutes (or till the colour development is sufficient).
    Note: Wrap the plate in aluminium foil to avoid exposure to light.
13. Thaw stop solution CCK036(C) and equilibrate it at room temperature.
14. For end point assay, add 50µl of Stop solution CCK036(C) to each well and read the absorbance at 490nm as a main wavelength and reference wavelength greater than 600nm.
    Note: Refer Fig 3 for substance mediated cytotoxicity assay.
15. For kinetic assay do not add the stop solution. Dire ctly read the absorbance at specified wavelength.
16. Calculate the average absorbance values from triplicate values.

LDH generated due to effect of test compound on the cells

A = Avg absorbance of test – Avg absorbance of background control (or vehicle control if applicable)

Total LDH released from the cells due to complete lysis

B = Avg absorbance of maximum LDH control – Avg absorbance of volume correction control

Baseline LDH released from the cells

C = Avg absorbance of untreated control – Avg absorbance of background control

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

	Maximum LDH Control	Untreated Control	Volume correction control	Background Control	Test	Vehicle control (if applicable)
Cells	90µl	90µl	-	-	90µl	90µl
Culture medium	-	-	90µl	100µl	-	-
Incubate the plate for 12 – 16 hours at 37ºC 5% CO_2						
Test compound	-	-	-	-	10µl	-
Solvent / vehicle	-	-	-	-	-	10µl
Gently swirl to mix the contents and incubate the plate for required period of time at appropriate temperature and humidity conditions						
Lysis solution	10µl	-	10µl	-	-	-
DPBS	-	10µl	-	-	-	-
Gently swirl to mix the contents and incubate the plate at 37ºC, 5% CO_2 for 30 – 45 minutes						
Centrifuge the plate at 1500rpm for 5- 10 minutes						
Transfer the 50µl supernatant from each well to a separate plate						
LDH Reagent Mix	50µl	50µl	50µl	50µl	50µl	50µl
Gently swirl to mix the contents and incubate the plate at room temperature for 15 – 30 minutes or till the colour development is sufficient						
Stop solution	50µl	50µl	50µl	50µl	50µl	50µl
Gently swirl to mix the contents and read absorbance at 490nm (main wavelength) and >600nm (reference wavelength)						

Table 1: Substance mediated cytotoxicity assay scheme

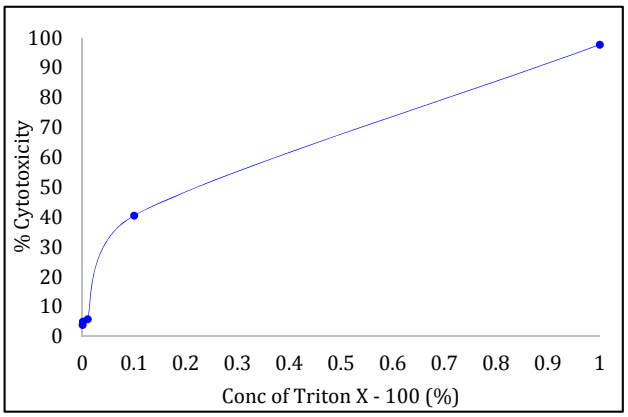

Fig 2: Triton X 100 mediated cytotoxicity in CHO cells

CHO cells were plated in triplicate in 96 well plate at the density of 0.5 x 10^6cells/ml. Maximum LDH control cells were treated with lysis solution and PBS was added to untreated control cells to compensate for the volume. Experimental cells were treated with various concentrations of Triton X-100. LDH reagent was added to all wells after the treatment. Percentage cytotoxicity in each Triton-X-100 concentration was calculated and the graph of % cytotoxicity was plotted against Triton X-100 concentration.

6.4 Cell mediated cytotoxicity assay

Cell mediated cytotoxicity is characterized by cytolysis of target cells by effector cells in a multi-step process. EZcount™ LDH cell assay kit can be used for determination target cell viability in an effector cell mediated cytotoxicity assay.

Most commonly used effector cells are antigen specific cytotoxic T lymphocytes and non-specific natural killer (NK) cells and macrophages.

The target cells to which these effector cells are directed include allogenic cells, malignant cells, virus-infected cells and chemically conjugated cells.

In addition to the controls mentioned in section 5.1, it is necessary to include certain other controls when performing LDH assay for determination of cell mediated cytotoxicity.

Effector cell untreated control: Corrects the spontaneous release of LDH from effector cells

Target cell untreated control: Corrects the spontaneous release of LDH from target cells

Target cell maximum LDH control: Required in calculations to determine 100% release of LDH

Volume correction control: Corrects the volume change caused by addition of lysis solution

1. Prepare serial dilutions of effector cells with culture medium.
2. Add 50µl of effector cell suspension in -
   - Test wells
   - Effector cell untreated control
3. Adjust the density of target cells to twice the optimized cell density.
4. Add 50µl target cell suspension in –
   - Test wells
   - Target cell untreated control
   - Target cell maximum LDH control
5. Add 100µl culture medium in –
   - Volume correction control and
   - Background control
6. Incubate the plate at 37ºC and 5% CO_2 for required period of time.
   Note: refer table 2 for detailed assay scheme.
7. Thaw lysis solution CCK036(B) and equilibrate it at room temperature.
8. Add 10µl lysis solution CCK036(B) to
   - Volume correction control and
   - Target cell maximum LDH control
9. Incubate the plate at 37ºC for 30 minutes.
10. Centrifuge the plate at 1500rpm for 5 – 10 minutes.
11. Transfer 50µl supernatant from each well to a separate 96 well plate.
12. Thaw LDH reagent CCK036(B) and equilibrate it at room temperature.
13. Add 50µl LDH reagent CCK036(A) to each well and wrap the plate with aluminium foil to protect from light.
14. Incubate the plate at room temperature for 15 – 20 minutes or until the colour development is sufficient.
15. Add 50µl stop solution CCK036(C) to each well to stop the colour development.
16. Read the absorbance at 450nm as a main wavelength and reference wavelength greater than 600nm.
17. Calculate average absorbance values from triplicate values.
18. Calculate percentage cytotoxicity using the formula mentioned on next page.

$$\% \textbf{ Cytotoxicity} = 100 \times \frac{\text{Test} - \text{Effector cell untreated control} - \text{Target cell untreated control} - \text{background control}}{(\text{Target cell max. LDH control} - \text{Volume correction control}) - (\text{Target cell untreated control} - \text{background control})}$$

	Test	Target Cell Untreated Control	Effector Cell Untreated Control	Target Cell Max. LDH Control	Volume correction control	Background Control
Effector Cells	50µl	-	50µl	-	-	-
Target Cells	50µl	50µl	-	50µl	-	-
Culture medium	-	-	-	-	100µl	100µl
Incubate the plate for required period of time at 37ºC 5% CO_2						
Lysis solution	-	-	-	10µl	10µl	-
Gently swirl to mix the contents and incubate the plate at 37ºC, 5% CO_2 for 30 – 45 minutes						
Centrifuge the plate at 1500rpm for 5- 10 minutes						
Transfer the 50µl supernatant from each well to a separate plate						
LDH Reagent Mix	50µl	50µl	50µl	50µl	50µl	50µl
Gently swirl to mix the contents and incubate the plate at room temperature for 15 – 30 minutes or till the colour development is sufficient						
Stop solution	50µl	50µl	50µl	50µl	50µl	50µl
Gently swirl to mix the contents and read absorbance at 450nm (main wavelength) and >600nm (reference wavelength)						

Table 2: Cell mediated cytotoxicity assay scheme

7. Storage and Stability:

LDH reagent is photosensitive. Store it away from bright light.

LDH reagent is a mixture of enzymes and substrates involved in conversion of lactate to pyruvate. Prolonged exposure of the reagent may cause loss in activity of enzymes.

For long term use, store the reagent at -20ºC.

Use before expiry date given on the product label.

8. Troubleshooting points:

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
High background absorbance	High LDH content in the serum used for culturing	Decrease the serum concentration to 5%
		Change the lot of serum or source of serum. Increasing levels of LDH activity has been observed in human AB serum, horse serum, fetal bovine serum and calf serum
High absorbance values of untreated control	Higher cell density	Repeat the assay with lower cell densities
	Cells used for the assay not healthy	Check viability and morphology of the cells before using them for the assay. Use healthy cells with more than 90% viability
Absorbance / fluorescence values higher than expected	Coloured test compound being tested	Determine the inherent absorbance of compound without adding LDH reagent mixture
	Production of excess LDH by dead cells	Repeat the assay with reduced cell densities
		Reduce the exposure period of cells with test compound and repeat the assay
Very low absorbance values	Production of very low amount of LDH	Repeat the assay with higher cell densities
	Inadequate incubation with test compound / LDH reagent	Expose the cells to test compound for longer duration and repeat the assay
		Expose the cells to LDH reagent for longer duration and measure the absorbance / fluorescence
	Prolonged exposure of cells to the test compound	Expose the cells to test compound for shorter duration and repeat the assay. (Half-life of LDH in culture medium is 9 – 10 hours. If cells are exposed to test compound for more than 9 – 10 hours, LDH released from the cells may lose the activity, resulting in low readings.
Random absorbance values/ poor consistency of replicates	Pipetting error	Perform the assay using automated electronic pipettes for seeding the cell suspension and adding the reagents
	Test compound under study which is responsible for improper response of the cells to LDH reagent	Refer to the pharmacological properties of the compound