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Technical Datasheet

EZcount™ LDH Cell Assay Kit, Homogeneous

Product Code: CCK058

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.

CCK058, EZCount™ LDH Homogeneous Cell Assay Kit has been designed for rapid determination of cell cytotoxicity in a one step reaction. Non-toxicity of the LDH reagent allows performing the assay in a single culture vessel i.e. in homogenous format. The reagent can accurately determine cell cytotoxicity in the samples containing mixed population of viable and dead cells. This feature makes the method suitable for high throughput automation wherein minimum handling and manipulations are required.

2. About the Assay:

EZcount™ Lactate Dehydrogenase (LDH) Homogenous Cell Assay Kit is based on an enzyme coupled reaction in which the LDH released from cells with damaged membrane reduces the dye to a coloured 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:
Enzyme in the LDH reagent uses NADH to reduce the dye to a coloured product which can be measured either colorimetrically or fluorometrically.

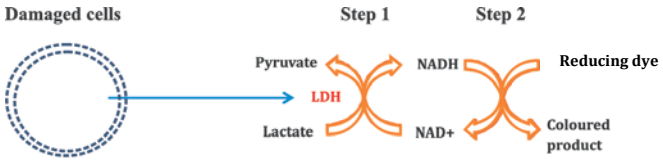

Fig 1: Schematic representation of measurement of LDH released from damaged cells

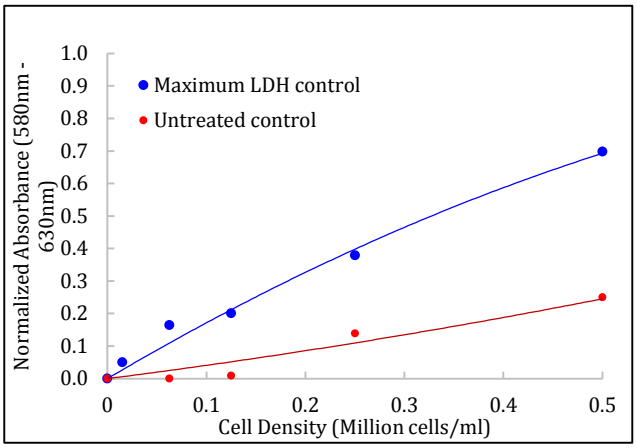

Fig 2: Linear relationship between cell number and absorbance using EZcount™ LDH homogeneous assay
Vero cells were serially diluted and plated in triplicate in 96 well plate. Maximum LDH control cells were treated with lysis solution and PBS was added to untreated control cells to compensate for the volume. LDH reagent was added to all the wells after lysis treatment. As indicated in the graph, high content of LDH released by maximum LDH control resulted in higher absorbance values compared to LDH released from background control cells

3. Kit contents:

The kit is sufficient for 500 assays.

Contents		Quantity	Storage
Code	Description		
CCK058 (A)	LDH reagent	5 x 5ml	-30°C to -10°C
CCK058(B)	Lysis solution	5ml	-30°C to -10°C
CCK058(C)	Stop solution	25ml	-30°C to -10°C

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 580nm and >600nm or fluorescence at 560nm excitation and 590nm emission

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

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 37°C) 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 37°C) 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 37C 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 Assay Procedure

1. Seed 90µl cell suspension in a 96-well plate at the required cell density.
2. In case of adherent cells, incubate the plate in an incubator at 37ºC and 5% CO_2 for 12-14 hours or overnight to allow attachment of the cells to wells. In case of suspension cells, perform the assay immediately after plating the cells.
3. Set up appropriate assay control as mentioned in section 5.1. (Refer Table 1 for complete assay scheme).
4. Thaw lysis solution CCK058(B) and equilibrate it at room temperature.
5. Add 10µl lysis solution CCK058(B) to maximum LDH control wells, 10µl test compound to experimental wells, 10µl solvent to untreated control wells and 10µl DPBS to background control wells.
6. Incubate the plate in an incubator at 37ºC and 5% CO_2 for required period of time (10 mins).
   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.
7. Thaw a bottle of LDH reagent CCK058(A) and equilibrate it at room temperature.
8. Add 50µl of LDH reagent to each well and incubate the plate at room temperature for 30 minutes.
   Note: Wrap the plate in aluminium foil to avoid exposure to light.
9. Thaw stop solution CCK058(C) and equilibrate it at room temperature.
10. For end point assay, add 50µl of Stop solution CCK058(C) to each well and read the absorbance at 580nm as a main wavelength and >600nm as a reference wavelength OR read the fluorescence at 560nm excitation and 590nm emission.
11. For kinetic assay do not add the stop solution. Read the absorbance / fluorescence at required time intervals.

	Maximum LDH Control	Experimental Wells	Untreated Control	Background Control
Cells	90µl	90µl	90µl	-
Culture Medium	-	-	-	90µl
Lysis solution	10µl	-	-	-
Test compound	-	10µl	-	-
Solvent / vehicle	-	-	10µl	-
DPBS	-	-	-	10µl
LDH Reagent Mix	50µl	50µl	50µl	50µl
Stop solution	50µl	50µl	50µl	50µl

Table 1: LDH Cell Cytotoxicity Assay Scheme

7. Analysis of Results:

Calculate the average absorbance / fluorescence values from triplicate values and calculate the percentage cytotoxicity using the formula given below –

$$\text{Percentage Cytotoxicity} = 100 \times \frac{(\text{Experimental} - \text{Background control})}{(\text{Max. LDH control} - \text{Background})}$$

8. 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 -30°C to -10°C .

Use before expiry date given on the product label.

9. Troubleshooting points:

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
High background absorbance / fluorescence	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
Absorbance / fluorescence values higher than expected	Coloured / fluorescent test compound being tested	Determine the inherent absorbance / fluorescence 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