Lactate Dehydrogenase Assay Kit

Product No.: ENK 13-001

For Research Use Only
Introduction

The lactate dehydrogenase (LDH) assay is very useful for measuring cell death and determining cell number. It is also an effective measurement of the efficiency of sample homogenization. LDH is an intracellular enzyme, which is released from cells when they die and membrane integrity fails, or is liberated when cells are disrupted.

By adding membrane solubilizing detergents to cultured cells, released LDH can be used as a parameter to count cells in that sample. The concentration of cells from unknown samples can be estimated by creating a standard curve of known cells lysed with a mild detergent such as NP-40.

When developing a homogenization procedure for protein isolation or similar objective, aliquots of homogenate can be removed at different times or stages during processing and analyzed for LDH activity. Insoluble debris is first removed by centrifugation, which is followed by an assay of the supernatant, as described below. The relative amount of LDH activity liberated during the process will provide insight as to the best methods and processing times. Over-processing may also be detected by observing decreases of LDH activity.

NAD (nicotinamide adenine dinucleotide) dependent lactate dehydrogenase is a ubiquitous enzyme found in animal, plant, and microbial cells. The enzyme catalyzes a reversible reaction in which lactate and NAD are converted to pyruvate and NADH (Figure 1).

![Coupled enzymatic reaction scheme for the detection of lactate dehydrogenase.](image_url)

For a colorimetric reaction, the hydrogen atom from NADH is transferred to the tetrazolium salt INT using the catalyst phenazine methosulfate (PMS), causing a color change from yellow to red/purple. The amount of reduced INT generated is directly related to the amount of LDH activity in the supernatant, thus the amount of reduced INT generated is proportional to the number of dead or damaged cells.
The optical density of the reduced INT product can be measured spectrophotometrically at 490 nm. Using the molar extinction coefficient (\(\varepsilon\)) of reduced INT (1.84 x 10^4 M^-1 cm^-1), enzyme activity can be calculated using Beer’s Law, Absorbance = \(\varepsilon\) x b (light path) x c (concentration of solute).

For this type of reading, most spectrophotometers are accurate with absorbance measurements between 0.01 and 1.0. Enzyme activity, as measured in units (U), is defined as the amount of enzyme that will convert 1 µmole of substrate to product/minute. For example, cultured cells which are harvested by centrifugation and then lysed with NP-40 lysis buffer could generate an optical density of 0.8 in 5 minutes as measured by a standard spectrophotometer. Using a wavelength of 490 nm and a standard 1 cm cuvette, the concentration can be determined:

\[
0.8 = (1.84 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}) (1 \text{ cm}) \text{ (concentration of reduced INT)}
\]

Rearranged, this equation reads:

\[
0.8 / (1.84 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}) (1 \text{ cm}) = [\text{reduced INT}] = 4.3 \times 10^{-5} \text{ M} = 4.3 \times 10^{-2} \text{ mM reduced INT}
\]

If this reaction was performed in 1 ml, then there would be 4.3 x 10^{-2} µmoles of product in that volume. For a 5 minute reaction, assuming that the product was produced linearly, then the reaction would contain 8.6 mU of LDH.

**Assay Considerations**

Lactate dehydrogenase assays follow the same general rules for most enzyme assays. The assays can be performed either as an endpoint assay (i.e., timed) or kinetic assay. Endpoint assays are simply measurements of the reaction at a defined time period. In LDH assays, the optical density of the reduced INT is measured spectrophotometrically at a specific time. The Stop Solution provided with the kit can be added to rapidly developing assays in order to inhibit the LDH enzyme and stop color development. This reaction should be measured immediately, as the reduced INT will slowly change color at room temperature.

Kinetic assays measure the optical density of the reactions at multiple fixed time intervals in order to generate a graph (absorbance vs. time), which can be further used to calculate the maximum rate of the reaction. Kinetic assays are more laborious and/or require plate reader software that can take multiple readings on a single assay plate. Kinetic assays are considered more accurate as compared to endpoint assays because the maximum rate of the reaction is based on multiple readings instead of a single final optical density reading during a fixed incubation period. In samples that contain a high concentration of enzyme, the time involved in adding analytes to different wells, mixing, and reading can lead to experimental deviations among samples.

It is important with both kinetic and endpoint assays that the enzyme is allowed to process substrate at maximum velocity (\(V_{\text{max}}\)). As for the OPS Diagnostics LDH Assay Kit, any reaction that generates an optical density under 1.0 is running at \(V_{\text{max}}\).
General Assay

The LDH assay involves mixing supernatant or lysates containing LDH with reconstituted Dye Solution and Substrate Buffer. The actual reaction is optimized so one part sample is to be combined with one part reconstituted Dye Solution and two parts Substrate Buffer. This reaction can be measured using a spectrophotometer at 490 nm to quantify activity.

The following assay is intended for a microplate reader, though it can be scaled up for cuvettes or tubes. Samples should be tested in triplicate, plus three negative controls using water. The LDH Positive Control is provided as a reference.

Protocol

1. Prepare the samples prior to rehydrating the solutions for the assay. LDH can be assayed from culture supernatant, a homogenized sample, and chemically lysed cells. Considerations for preparing these samples should include:

   a. Culture medium: LDH can be used to measure cell death in cultured cells. The supernatant can be removed from the culture and assayed directly. If the supernatant contains suspended cells, first centrifuge the sample to remove any cells and/or debris.

   b. Homogenates: Samples which are disrupted by mechanical homogenization can be assayed for enzyme activity. In such cases, centrifuge the sample to remove debris.

Kit Contents

<table>
<thead>
<tr>
<th>Solution A</th>
<th>Substrate Buffer is filter-sterilized and contains Tris (pH 8), lactic acid and Triton X-100. This solution should be handled aseptically, as lactic acid can support microbial growth.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution B</td>
<td>This solution is used to rehydrate the Solution C (lyophilized Dye Solution) and the Positive Control sample.</td>
</tr>
<tr>
<td>Solution C</td>
<td>Each vial contains lyophilized INT, NAD, and PMS and should be reconstituted with 2.5 ml of Rehydration Buffer prior to use. Reconstituted Dye Solution should be used immediately, or stored at -20°C. This solution can be stored frozen for several weeks. Solution that turns clear or red should be discarded.</td>
</tr>
<tr>
<td>Lysis Solution</td>
<td>NP-40 based lysis buffer for cultured cells.</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>A solution of copper sulfate, which inhibits LDH activity.</td>
</tr>
<tr>
<td>Positive Control</td>
<td>Lyophilized LDH for use as a positive control. This vial contains a partially purified LDH. Rehydrate lyophilized control with 1.0 ml of Solution B.</td>
</tr>
</tbody>
</table>
c. Chemically lysed cells: Tissue culture cells can be lysed by adding Lysis Solution (provided in the kit) directly to the cells. For adherent cells, remove the culture medium, rinse with phosphate buffered saline, decant, and then add Lysis Solution. The cells will lysis within several minutes. For non-adherent cells, pellet the cells by centrifugation, wash with PBS, pellet again, and then resuspend in Lysis Solution. For both types of cells, the volume of Lysis Solution which should be used can be the same as culture broth harvested or decanted. If cells are at a high density, more Lysis Solution can be used, while if the density is low, use a smaller volume.

2. Aseptically remove the foil seal and rubber stopper from the Dye Solution vial (Solution C). Dissolve the lyophilized reagents with 2.5 ml of Rehydration Buffer (Solution B). Swirl the vial to mix the contents to reconstitute the lyophilized dye. The reconstituted Dye Solution in each vial is sufficient to perform 100 reactions, or essentially a full 96 well plate.

3. In a clean tube, mix 2 parts Solution A (Substrate Buffer) to 1 part Solution C (reconstituted Dye Solution). Mix only the quantity needed. The Dye/Buffer Solution can be placed in a clean reservoir for dispensing using a multichannel pipette.

4. The sample should be cleared of debris by centrifugation before assaying. Dispense 25 µl of sample into the microwell plate wells. Add 75 µl of the Dye/Buffer Solution to each well with a multi-channel pipette in order to minimize time differences between wells. Dispense the Dye/Buffer Solution slowly to avoid producing air bubbles in the wells.

5. The assay can be run as an endpoint or kinetic assay using software provided with microplate readers. Most accurate measurements use the maximum slope during a kinetic assay (Figure 5). For endpoint assays, incubate the plate at 37˚C until a dark pink/red color develops (Figure 2). Add 25 µl of Stop Solution to each well to inhibit the LDH activity. Read the plate at 490 nm.

<table>
<thead>
<tr>
<th>Color Scale</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light Yellow</td>
<td>Light Yellow</td>
</tr>
<tr>
<td>Medium Yellow</td>
<td>Medium Yellow</td>
</tr>
<tr>
<td>Dark Yellow</td>
<td>Dark Yellow</td>
</tr>
<tr>
<td>Light Pink</td>
<td>Light Pink</td>
</tr>
<tr>
<td>Medium Pink</td>
<td>Medium Pink</td>
</tr>
<tr>
<td>Dark Pink</td>
<td>Dark Pink</td>
</tr>
<tr>
<td>Dark Pink/Red</td>
<td>Dark Pink/Red</td>
</tr>
<tr>
<td>Red</td>
<td>Red</td>
</tr>
<tr>
<td>Dark Red</td>
<td>Dark Red</td>
</tr>
<tr>
<td>Very Dark Red/Purple</td>
<td>Very Dark Red/Purple</td>
</tr>
</tbody>
</table>

Figure 2. Qualitative color scale of reduced INT development.
Creating a Standard Curve for Cells

Cell death and enumeration require a standard curve generated from living cells. As previously stated, tissue culture cells can be counted, added to wells of a microplate, and lysed using the Lysis Buffer provided. Enzyme activity can then be assessed for each concentration of cells in order to construct a standard curve of Enzyme Activity vs. Cell Number. Figure 3 represents the kinetic curves of LDH activity from different concentration mouse L929 cells.

![Figure 3. LDH activity of different concentrations of mouse L929 cell lysates.](image)

There are two ways in which a standard enzyme activity curve can be generated. The first approach which is an endpoint assay, involves selecting a single time point and plotting the optical density at that point against the cell number (Figure 4).

![Figure 4. LDH standard curve for endpoint assays.](image)
The second approach which is a kinetic assay, involves calculating the slope (rate of activity) of the line and plotting it against cell numbers (Figure 5). Both curves are essentially the same though the kinetic curve is more accurate.

In both endpoint and kinetic standard curves, it is very important to select data points or rates from a region of the graph where the data is linear. A typical reaction may start slow, progress through a linear stage and then plateau as reagents become limited and inhibitors accumulate. Accurate comparisons of cell concentrations must be made while the lactate dehydrogenase is operating at maximum velocity, which is reflected in the linear portion of the graph.

**Storage**

The Lactate Dehydrogenase Assay Kit is best stored at 4°C on a long-term basis but may be stored at ambient temperature for a short period of time. The lyophilized Dye Solution is stable until rehydrated at which it should be used immediately, or can be stored at -20°C for a week. Once rehydrated, the lyophilized Positive Control can be stored at 4°C for several weeks, but the enzyme activity may decrease over time. The Stop Solution and Rehydration Buffer are sterile and remain stable unless contaminated. Care should be taken to avoid contaminating any solution with microorganisms or lactate dehydrogenase enzyme. The kit is stable for a minimum of two years when stored at 4°C.

**Disposal**

The components of the kit are non-hazardous, though care should always be taken when handling the reagents. Dispose of reagents and reactions in a responsible manner. Treat the chemicals as if they are biohazards and in accordance with your institution’s guidelines.