ab102526
Lactate Dehydrogenase (LDH) Assay kit (Colorimetric)

Instructions for use:

For the rapid, sensitive and accurate measurement of Lactate Dehydrogenase (LDH) in various samples.

This product is for research use only and is not intended for diagnostic use.
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1. BACKGROUND

Lactate Dehydrogenase (LDH) Assay Kit (Colorimetric) (ab102526) quantifies LDH activity in a variety of biological samples such as serum or plasma, cells, culture medium and fermentation. In this assay LDH reduces NAD to NADH, which then interacts with a specific probe to produce a color ($\lambda_{\text{max}} = 450$ nm). The kit can detect 1-100 mU/mL of LDH directly in samples. The assay is quick, convenient, and sensitive.

Lactate dehydrogenase (LDH) is an oxidoreductase (EC 1.1.1.27) present in a wide variety of organisms. It catalyses the inter-conversion of pyruvate and lactate with concomitant inter-conversion of NADH and NAD+. It converts pyruvate, the final product of glycolysis, to lactate in hypoxic conditions, and it performs the reverse reaction during the Cori cycle in the liver. LDH quantification is of clinical interest as serum levels of certain LDH isozymes reflect pathological conditions in particular tissues.

When disease or injury or toxic material damages tissues, the cells LDH is released into the bloodstream. Since LDH is a fairly stable enzyme, LDH has been widely used to evaluate the presence of damage and toxicity of tissue and cells.
2. ASSAY SUMMARY

Sample preparation

↓

Standard curve preparation

↓

Add reaction mix

↓

Measure optical density (OD450 nm) in a kinetic mode* at 25°C for 30 – 60 minutes protected from light

*For kinetic mode detection, incubation time given in this summary is for guidance only.
3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. STORAGE AND STABILITY

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**
5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH Assay Buffer</td>
<td>50 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>LDH Substrate Mix (Lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>NADH Standard (0.5 μmol; Lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>LDH Positive Control (Lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at OD 450 nm
- MilliQ water or other type of double distilled water (ddH₂O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom
- Dounce homogenizer (if using tissue)
- PBS
8. TECHNICAL HINTS

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Ensure plates are properly sealed or covered during incubation steps.

- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.

- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.

- Make sure you have the right type of plate for your detection method of choice.

- Make sure all necessary equipment is switched on and set at the appropriate temperature.
9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening

9.1. **NADH Standard:**
Reconstitute with 0.4 mL ddH$_2$O to generate a 1.25 mM NADH Standard Solution. Aliquot standard so that you have enough volume to perform the desired number of tests. Store aliquots at -20°C. Keep on ice while in use. Use within 1 month.

9.2. **LDH Assay Buffer:**
Ready to use as supplied. Warm to room temperature before use. Store at -20°C.

9.3. **LDH Positive Control:**
Reconstitute the whole vial with 200 μl LDH Assay Buffer. Aliquot the control so that you have enough volume to perform the desired number of tests. Store aliquots at -20°C. Keep on ice while in use. Use within 1 month.

9.4. **LDH Substrate Mix:**
Reconstitute in 1.1 mL ddH$_2$O by mixing solution for 10 minutes. Aliquot substrate so that you have enough volume to perform the desired number of tests. Store aliquots at -20°C. Use within 1 month.
10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be prepared immediately prior use. Do not store for future use.

10.1. Using 1.25mM NADH standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Volume of Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End Conc NADH in well (nmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>125</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>120</td>
<td>50</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>115</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>110</td>
<td>50</td>
<td>7.5</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>105</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>100</td>
<td>50</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL)
11. SAMPLE PREPARATION

General Sample Information

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze samples in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1. Cell (adherent or suspension) samples:

11.1.1. Harvest the amount of cells necessary for each assay (initial recommendation = 1 – 2 x 10⁶ cells).

11.1.2. Wash cells with cold PBS.

11.1.3. Homogenize cells on ice in 2 – 4 volumes of cold Assay Buffer;

11.1.4. Centrifuge cells at 4°C at 10,000 x g for 15 minutes in a cold microcentrifuge to remove any insoluble material.

11.1.5. Collect the supernatant and transfer to a new tube.

11.1.6. Keep on ice.

11.2. Tissue samples

11.2.1. Harvest the amount of tissue necessary for each assay (initial recommendation = 100 g tissue).

11.2.2. Wash tissue with cold PBS.

11.2.3. Homogenize tissue in 2 – 4 volumes of cold Assay Buffer using a Dounce homogenizer (10 – 50 passes) on ice.

11.2.4. Centrifuge samples at 4°C at 10,000 x g for 15 minutes in a cold microcentrifuge to remove any insoluble material.
11.2.5. Collect the supernatant and transfer to a clean tube.
11.2.6. Keep on ice.

11.3. **Erythrocytes:**

11.3.1. Harvest the amount of erythrocytes necessary for each assay (initial recommendation = 0.2 mL).
11.3.2. Wash tissue with cold PBS.
11.3.3. Homogenize cells on ice in 2 – 4 volumes of cold Assay Buffer.
11.3.4. Centrifuge cells at 4°C at 10,000 x g for 15 minutes in a cold microcentrifuge to remove any insoluble material.
11.3.5. Collect the supernatant a transfer to a new tube.
11.3.6. Keep on ice.

11.4. **Serum, Urine and other fluid samples:**

Serum and urine samples can be tested directly by adding sample to the microplate wells.

However, to find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample.

**NOTE:** *We suggest using different volumes of all sample to ensure readings are within the Standard Curve range.*
12. ASSAY PROCEDURE

- Equilibrate all materials and prepared reagents to correct temperature prior to use.
- We recommended to assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

12.1. Set up Reaction Wells

Standard wells = 50 µL standard dilutions
Sample wells = 2 – 50 µL samples (adjust volume to 50 µL/well with LDH Assay Buffer).
Positive control = 2 – 5 µL Positive control (see step 9.3) (adjust volume to 50 µL/well with LDH Assay Buffer).

12.2. Reaction Mix

12.2.1. Prepare 50 µL of Reaction Mix for each reaction. Mix enough reagents for the number of assays (samples and controls) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation: X µL component x (Number reactions +1).

<table>
<thead>
<tr>
<th>Components</th>
<th>Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH Assay Buffer</td>
<td>48</td>
</tr>
<tr>
<td>LDH Substrate Mix</td>
<td>2</td>
</tr>
</tbody>
</table>

12.3. Add 50 µL of Reaction Mix into each standard, sample and positive control sample wells. Mix well.
12.3.1. Measure output immediately at OD 450 nm (T1) on a microplate reader in a kinetic mode, every 2 – 3 minutes, for at least 30 – 60 minutes at 37°C protected from light.

Incubate reaction for up to 4 hours if LDH activity is low.

**NOTE:** *Incubation time depends on the LDH Activity in the samples. We recommend measuring OD in a kinetic mode, and choosing two time points (T1 and T2) in the linear range (OD values A1 and A2 respectively) to calculate the LDH activity of the samples.*

For standard curve, do not subtract A2 from A1. Standard curve can also be read in end point mode (i.e. at the end of incubation time).
13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

13.1. Average the duplicate reading for each standard and sample.

13.2. Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.3. Plot the corrected absorbance values for each standard as a function of LDH activity.

13.4. Draw the best smooth curve through these points to construct the standard curve. Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.5. Activity of LDH in the test samples is calculated as:

\[ \Delta A_{450\text{nm}} = (A_2 - A_1) \]

Where:

A1 is the sample reading at time T1.
A2 is the sample reading at time T2.

Use the \( \Delta A_{450\text{nm}} \) to obtain B nmol of NADH generated by LDH during the reaction time \( \Delta T = T_2 - T_1 \).
13.6. Activity of LDH in the test samples is calculated as:

\[ \text{LDH Activity} = \left( \frac{B}{\Delta T \times V} \right) \times D = \text{nmol/min/mL} = \text{mU/mL} \]

Where:
- \( B \) = Amount of NADH in sample well calculated from standard curve (nmol).
- \( \Delta T \) = Reaction time (minutes).
- \( V \) = Original sample volume added into the reaction well (mL).
- \( D \) = Sample dilution factor.

NADH molecular weight 763 g/mol

**Unit Definition:**

1 Unit LDH = amount of enzyme that catalyzes the conversion of lactate to pyruvate to generate 1.0 \( \mu \text{mol} \) of NADH per minute at pH 8.8 at 37°C.
14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Figure 1: Typical NADH calibration standard curve generated following assay kit protocol.
**Figure 2:** Continuous reading through time of positive control (red), background well (blue) and a human serum sample.
15. QUICK ASSAY PROCEDURE

**NOTE:** This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare NADH standard, LDH substrate mix and positive control (aliquot if necessary); get equipment ready.
- Prepare LDH standard dilution [2.5 – 12.5 nmol/well].
- Prepare samples in optimal dilutions so that they fit standard curve readings.
- Set up plate in duplicate for standard (50μL), samples (50μL) and positive control (50 μL).
- Prepare a master mix for LDH Assay Reaction Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH Assay Buffer</td>
<td>48</td>
</tr>
<tr>
<td>LDH Substrate mix</td>
<td>2</td>
</tr>
</tbody>
</table>

- Add 50 μL Reaction Mix to standard, samples and positive control wells.
- Measure absorbance at OD=450 nm on a microplate reader in a kinetic mode at 37°C for 30 – 60 minutes protected from light.
# 16. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate</td>
</tr>
<tr>
<td>Sample with erratic readings</td>
<td>Samples not deproteinized (if indicated on protocol)</td>
<td>Use provided protocol for deproteinization</td>
</tr>
<tr>
<td></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer, increase number of strokes</td>
</tr>
<tr>
<td></td>
<td>Samples used after multiple free/thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use</td>
</tr>
<tr>
<td></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td>Lower/Higher readings in samples and Standards</td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
<tr>
<td>Problem</td>
<td>Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>---------------------------------------------------------</td>
<td>----------------------------------------------------</td>
</tr>
<tr>
<td>Standard readings do not follow a linear pattern</td>
<td>Pipetting errors in standard or reaction mix</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect concentration</td>
<td>Always refer to dilutions described in the protocol</td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
</tbody>
</table>
17. INTERFERENCES
These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

- FBS – if measuring LDH in cell culture medium

18. FAQs
What is the detection range for this kit?
The detection range is 1 -100 mU/mL of LDH.

Can this kit measure both intra- and extracellular LDH activity?
This kit can measure both intra and extracellular Pyruvate.
For intracellular measurement from cell samples follow the sample preparation step described in the protocol.
For extracellular media, remove any cell debris by spinning down and optimize the volume needed to get values within the linear range of the standard curve.

How specific is this kit? It seems that this kit is only measuring the change between NAD+ and NADP, which can be catalyzed by enzymes other than LDH. Is there any way to be sure that the kit is measuring only LDH activity?
The principle of the assay is the following:
LDH converts Lactate to pyruvate, generating NADH from NAD. The NADH interacts with the probe to generate color at 450 nm. The amount of colored product formed is directly proportional to the LDH activity in the sample. The buffer conditions (pH, presence of an electron transfer agent) are optimized for LDH activity and since the NADH generation is coupled to lactate conversion to pyruvate, this assay specifically detects LDH and no other NAD+ to NADH converting enzymes in the cell. Any nonspecific endogenous NADH can be accounted for by omitting the substrate in the reaction mix. This background value can be subtracted from the sample LDH activity value.
I want to measure LDH alpha (LDH5) activity, which converts pyruvate to lactate. Will this kit work for that purpose?

Lactate dehydrogenase catalyzes the inter-conversion of pyruvate and lactate with concomitant inter-conversion of NADH and NAD+. It converts pyruvate, the final product of glycolysis, to lactate when oxygen is absent or in short supply and it performs the reverse reaction during the Cori cycle in the liver. So LDH5 converts pyruvate to lactate and vice versa. In our assay, NAD+ (produced during pyruvate to lactate conversion by LDH5) is reduced to NADH, which interacts with a probe to produce a color ($\lambda_{\text{max}} = 450 \text{ nm}$).

In essence, this assay can work for this objective. The reaction conditions and the relative amount of pyruvate and lactate decide which direction the reaction will go.

How can adherent cells be used for this assay?

Trypsinization can be used to detach cells. Then collect the cells, spin down, remove medium + trypsin, wash cells with PBS and then proceed with homogenization using the assay buffer in the kit. It is important to be careful since over-trypsinization might damage the plasma membrane and leak LDH into the medium.

Does phenol red in the media affect this assay?

Since only 2 – 50 µL sample are added per well, the color from phenol red is diluted by the assay buffer/reaction mix. Typically this does not affect the assay. The OD450 nm readings are in the yellow-brown range.
19. NOTES
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