

ab140361- Lactate Dehydrogenase B / LDH- B Activity Assay Kit

Instructions for Use

For the quantitative measurement of lactate dehydrogenase B / LDH-B activity in human, bovine or goat samples. This product is for research use only and is not intended for diagnostic use.

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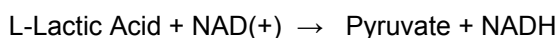
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1. Introduction

Abcam immunocapture enzyme activity assays apply a novel approach, whereby target enzymes are first isolated by immunocapture from tissue or cell samples before subsequent functional analysis. All of our activity kits utilize highly validated monoclonal antibodies and proprietary buffers, which are able to capture enzymes in their fully-intact, functionally-active states.

A mouse monoclonal antibody specific for the B isoform of lactate dehydrogenase LDH-B is pre-coated onto the wells of a microplate. Detergent extracted sample is added to those wells. After the target enzyme has been immunocaptured in the well, substrate is added, and enzyme activity is analyzed by measuring the change in absorbance of the product of the reaction. By analyzing the enzyme's activity in an isolated context, outside of the cell and free from any other variables such as competing enzymes or enzyme isoforms, an accurate measurement of the enzyme's functional state can be made.

Lactate dehydrogenase catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD⁺. The enzyme activity is determined by following the production of NADH in the following LDH-B catalyzed reaction:



The generation of NADH is coupled to the 1:1 reduction of a reporter dye to yield a colored (yellow) reaction product whose concentration can be monitored by measuring the increase in absorbance at 450 nm (dye molar extinction coefficient - $37000 \text{ M}^{-1}\text{cm}^{-1}$).

Background: Functional lactate dehydrogenase are homo or hetero tetramers composed of M and H protein subunits encoded by the *LDH-A* and *LDH-B* genes, respectively. The major isozymes of skeletal muscle and liver has four muscle (M) subunits; while the main isozymes for heart muscle contains four (H) subunits. The other variants contain both types of subunits.

Elevated levels of LDH indicate tissue breakdown in hemolysis. LDH can be used as a marker of myocardial infarction, peaking at days 3 and lasting through day 10. Other disorders indicated by elevated LDH include cancer, meningitis, encephalitis, acute pancreatitis, and HIV. Defects in LDH-B are a cause of hereditary LDH-B deficiency; however LDH-B deficiency is usually asymptomatic.

Limitations:

- FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC PROCEDURES.
- Use this kit before expiration date.
- Do not mix or substitute reagents from other lots or sources.
- If experimental samples generate values outside of the range of the standard sample curve, further dilute the

experimental samples with 1X Incubation Buffer and repeat the assay.

- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

Technical Hints:

- To avoid cross contamination, change pipette tips between additions of each standard, sample and between reagent additions. Also use separate clean, dry reservoirs for each reagent.
- Cover plate during incubation steps.
- Thorough and consistent wash technique is essential for proper assay performance. Wash buffer must be forcefully dispensed and completely removed from the wells by aspiration or decanting. Remove remaining wash buffer by inverting the plate and blotting on paper towels.

2. Assay Summary

Prepare samples as instructed.

Determine the protein concentration of extracts.



Equilibrate all reagents to room temperature.



Create standard protein dilution series.

Dilute sample to desired protein concentration in Incubation buffer.

Add 100 μ L sample to each well used. Incubate 2 hours at room temperature.



Aspirate and wash each well four times.



Freshly prepare 1X Activity Solution then add 100 μ L to each well.

Pop bubbles and immediately record the color development at 450nm for 15 to 45 minutes.

3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

Item	Quantity
10X Buffer	25 mL
10X Blocking Buffer	6mL
Extraction Buffer	15 mL
Reaction Base Buffer	10 mL
100X NAD ⁺	Lyophilized, rehydrate in 0.25 mL H ₂ O
100X Reagent Dye	Lyophilized, rehydrate in 0.25 mL H ₂ O
100X Coupler	Lyophilized, rehydrate in 0.25 mL H ₂ O
1X LDH-B Control	1 µg (Lyophilized)
LDH-B Microplate (12 x 8 antibody coated well strips)	96 Wells

4. Storage and Handling

All components are shipped +4°C. Reagent dye, coupler, LDH-B and NAD⁺ are shipped lyophilized. Before use, rehydrate according to the instructions in Section 6 - Reagent Preparation and vortex each tube thoroughly to dissolve. After hydration, unused amounts of these four materials should be aliquoted and stored at -20°C, avoiding additional freeze/thaw cycles. Store all other components at 4°C. This kit is stable for 6 months from receipt.

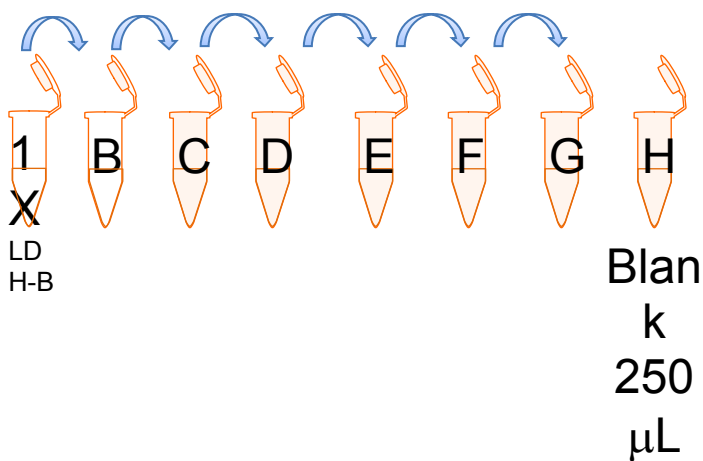
5. Additional Materials Required

- Standard absorbance microplate reader capable of kinetic reading at 450nm
- Multichannel pipette (50 - 300 µL) and tips
- PBS (1.4 mM KH₂PO₄, 8 mM Na₂HPO₄, 140 mM NaCl, 2.7 mM KCl, pH 7.3)
- 1.5 mL microtubes
- Paper towels
- Deionized water
- Optional plate shaker for all incubation steps

6. Reagent Preparation

- 6.1. Prepare 1X Wash Buffer by adding 25 mL 10X Buffer to 225 mL nanopure water.
- 6.2. Prepare 1X Incubation Buffer by adding 6 mL 10X Blocking Buffer to 54 mL 1X Wash Buffer.
- 6.3. Immediately prior to use, prepare the 100X NAD⁺ by resuspending with 250 μ L of nanopure water and vortexing thoroughly until dissolved.
- 6.4. Immediately prior to use, prepare the 100X Coupler, and 100X Reagent Dye by resuspending each with 250 μ L of nanopure water and vortexing thoroughly until dissolved.
- 6.5. Immediately prior to use, prepare 1X Activity Solution. For an entire plate add 100 μ L 100X NAD⁺, 100 μ L 100X Coupler, 100 μ L 100X Reagent Dye to 9.7 mL Reaction Base Buffer provided.
- 6.6. Immediately prior to use, prepare 1X LDH-B Control by resuspending the lyophilized protein in 1 mL 1X Incubation Buffer and vortexing thoroughly until fully dissolved. The final concentration of LDH-B protein is 1 μ g/mL. This stock of standard material is then used to generate a standard curve in labeled tubes; an example dilution series is described below. A different dilution series could be used if more precision is needed in a certain protein range. It is highly recommended to aliquot the remaining stock material and store the aliquots at 80°C.

6.7. To prepare control dilution series, label seven tubes B, C, D, E, F, G and H. Add 250 μL of 1X Incubation Buffer to each of the seven tubes. Transfer 250 μL from the stock 1X LDH-B (from step 6.6) tube to tube B, mix well. Using a fresh pipette tip, transfer 250 μL from tube B to tube C, mix well. Repeat for tubes C through G. Do not add anything to tube 8, it is the zero standard.



7. Test Sample Preparation

Note: Extraction buffer does not contain protease inhibitors. It can be supplemented with a premade protease inhibitor cocktail or PMSF/leupeptin/pepstatin, additionally a phosphatase inhibitor cocktail can be added if desired. Supplements should be used according to manufacturer's instructions.

7.1 Cell Lysates

- 7.1.1. Collect non adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 10 min at 4°C.
- 7.1.2. Rinse cells twice with PBS.
- 7.1.3. Solubilize cell pellet at 2×10^7 /mL in Extraction Buffer.
- 7.1.4. Incubate on ice for 20 minutes. Centrifuge at 16,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C for 6 months. The sample protein concentration in the extract may be quantified using a protein assay.

7.2 Tissue lysates:

- 7.2.1. Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (Dounce homogenizer recommended).
- 7.2.2. Suspend the homogenate to 25 mg/mL in PBS.
- 7.2.3. Solubilize the homogenate by adding 4 volumes of Extraction Buffer to a sample protein concentration of 5 mg/mL (final concentration of 1 mg/mL).
- 7.2.4. Incubate on ice for 20 minutes. Centrifuge at 16,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C for 6 months. The sample protein concentration in the extract may be quantified using a protein assay.

8. Assay Procedure

Equilibrate all reagents and samples to room temperature before use. It is recommended all samples and standards be assayed in duplicate.

- 8.1. Prepare all reagents, and samples as directed in the previous sections.
- 8.2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
- 8.3. Add 100 μL of each diluted sample per well, it is recommended to include a dilution series of the standard protein as a reference. Also include 1X Incubation Buffer as a zero standard.
- 8.4. Cover/seal the plate and incubate for 2 hours at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
- 8.5. Aspirate each well and wash, repeat this three more times for a total of four washes. Wash by aspirating or decanting from wells then dispensing 300 μL 1X Wash Buffer into each well as described above. Complete removal of liquid at each step is essential to good performance. After the last wash, remove the remaining buffer by aspiration or decanting. Invert the plate and blot it against clean paper towels to remove excess liquid.

8.6. Gently add 100 μ L fresh prepared 1X Activity Solution, described in section 6.5, to each well minimizing the production of bubbles.

8.7. Pop any bubbles immediately and record absorbance in the microplate reader prepared as follows:

Mode:	Kinetic
Wavelength:	450 nm
Time:	30-45 min.
Interval:	12 sec
Shaking:	Shake before and between readings

8.8. *Alternative*— In place of a kinetic reading, at a ***user defined***, time record the endpoint OD data at 450 nm in all wells. Better sensitivity is likely with the kinetic reading.

8.9. Analyze the data as described below.

9. Data Analysis

Average the control sample dilution series readings and plot against their concentrations after subtracting the zero reading. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A five parameter algorithm (5PL) was used for the example data, though other equations can be examined to see which provides the most accurate results (e.g. linear, semi-log, log/log, 4 parameter logistic). Unknown samples' activity should be interpolated from the standard curve. This determined relative activity is the amount of reference sample required to generate the same amount of activity as the unknown sample and is usually expressed as a per cent value. Samples producing signals greater than that of the highest standard should be further diluted in 1X Incubation Buffer and reanalyzed.

Lactic-acid conversion to pyruvate and production of NADH by LDH-B in each well is 1:1 proportional with dye reduction and increase in absorbance at 450 nm (dye $\epsilon_{450\text{nm}} = 37 \text{ mM}^{-1} \text{ cm}^{-1}$). For simplicity the activity can be expressed as the change in absorbance per minute per amount of sample loaded into the well. Activity was collected as described in this protocol using a Molecular Devices microplate reader. Activity is clearly measurable in the 0.33-125 $\mu\text{g/mL}$ range when such a fit is applied for HeLa cell lysate. For the LDH-B standard protein, the working range is 8-500 ng/mL .

9.1 TYPICAL STANDARD CURVE – *For demonstration only.*

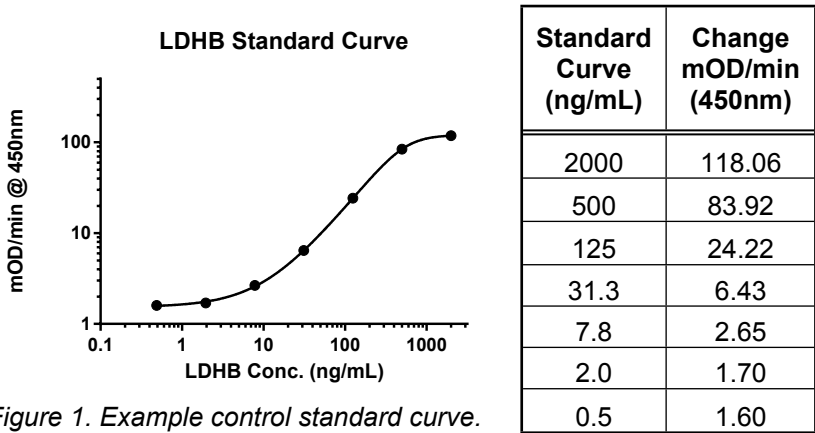


Figure 1. Example control standard curve.

9.2 EXPERIMENTAL RESULTS – *For demonstration only.*

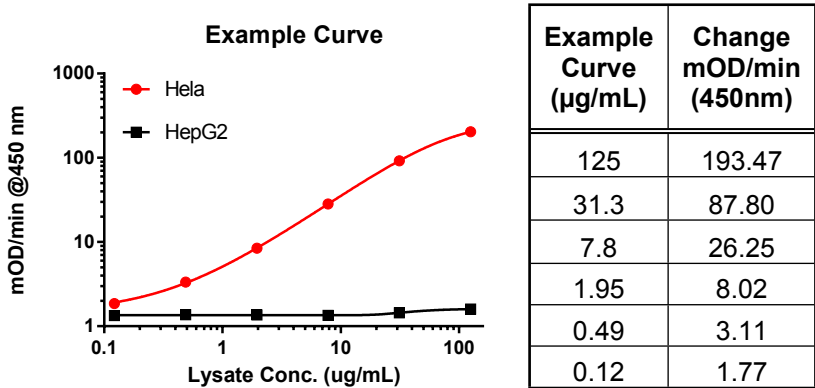


Figure 2. Lysate example curve. LDH-B activity was measured from Hela cell lysate, which contains hetero-tetramer isozyme composed of both (M) LDHA and (H) LDH-B subunits. However, the liver derived cell line, HepG2, which contains mainly homo-tetramer of subunit M (LDHA), shows no enzymatic activity.

9.3 REPRODUCIBILITY

Parameter	CV%
Intra (n=3, 4 assays)	1.5-5.0
Inter (n=4 days)	5.0

9.4 SENSITIVITY

	Theoretical measurable concentration	Lowest measured concentration	Tested Working Range
LDH-B	3.42 ng/mL	8.0 ng/mL	8-500 ng/mL
Hela Lysate	0.17 µg/mL	0.33 µg/mL	0.33-125 µg/mL

9.5 RECOVERY

The standard protein was diluted in four different mixtures to measure the interference of the solutions.

LDH-B Diluted In:	Average Recovery (%)	Range (%)
Control	100%	-
50% Culture Media	94%	91-101%
50% Extraction Buffer	112%	101-130%
10% Mouse Serum	95%	86-111%

9.6 SPECIFICITY

9.6.1 SPECIES CROSS REACTIVITY

The species cross reactivity of this assay was demonstrated by testing the sera from multiple species. The kit shows a good reaction with human, goat and bovine samples, but shows no reaction with mouse or rat sera which may also contain LDH-B. This species cross reaction specificity is due to the species specificity of the capture antibody.

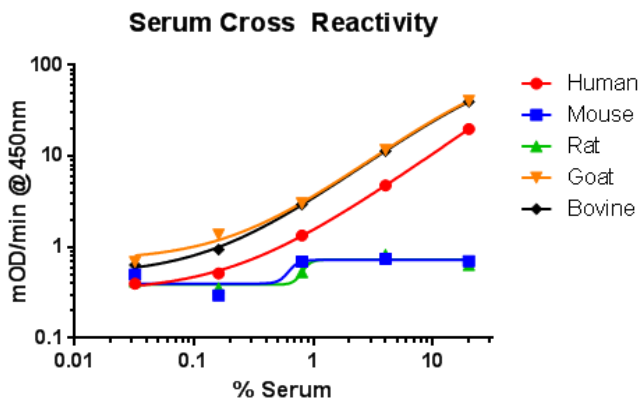


Figure 3. Species cross reactivity shown with sera.

9.6.2 ISOFORM SPECIFICITY

LDH-B isoform specificity was validated by mass spectrometry, activity assay and western blot. For western blot validation, the capture antibody was used to isolate LDH-B from a variety of materials and the protein was eluted from the well and transferred to a membrane

after SDS-PAGE. The membrane was blotted with anti-LDH-B antibody as well as anti-LDH antibody, shown below in Figure 4.

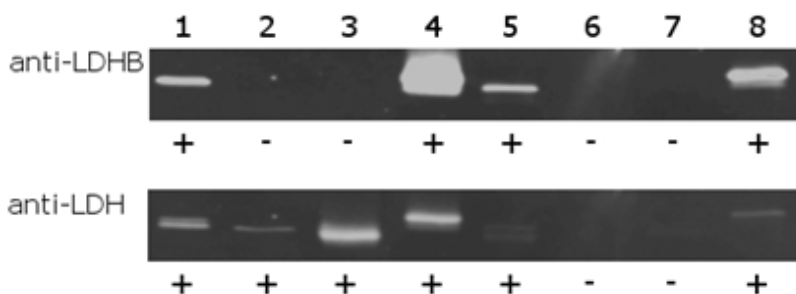


Figure 4. Isoform specificity validated by western blot.

Lane 1 - HeLa cell Lysate

Lane 2 - HepG2 Cell Lysate

Lane 3 - LDHA (purified from rabbit skeletal muscle)

Lane 4 - LDH-B (recombinant)

Lane 5 - HeLa - In-well extracted with capture mAb

Lane 6 - HepG2 - In-well extracted with capture mAb

Lane 7 - LDHA - In-well extracted with a capture mAb

Lane 8 - LDH-B - In-well extracted with capture mAb

The western blot results indicate that the capture antibody isolates active LDH-B, but does not isolate LDHA (which would be detected by the anti-LDH antibody). This result is also supported by Figure 2 which shows that the liver derived cell line, HepG2, shows no enzymatic activity.

10. Troubleshooting

Problem	Cause	Solution
Poor standard curve	Inaccurate Pipetting	Check pipits
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Bubble in well	Ensure all bubbles are popped before reading plate

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