GSI LDH Cytotoxicity Assay Kit DataSheet

Lactate dehydrogenase (LDH or LD) is an enzyme found in nearly all living cells. Lactate dehydrogenase catalyzes the interconversion of pyruvate and lactate with concomitant interconversion 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. At high concentrations of lactate, the enzyme exhibits feedback inhibition, and the rate of conversion of pyruvate to lactate is decreased. Within the heart, lactate dehydrogenase plays the role of converting lactate back into pyruvate so that the pyruvate can be used again to create more energy. Lactate dehydrogenase is composed of four subunits.[1] The two most common subunits are the LDH-M and LDH-H protein, encoded by the LDHA and LDHB genes, respectively. These two subunits can form five possible tetramers (isoenzymes): 4H, 4M, and the three mixed tetramers (3H1M, 2H2M, 1H3M). These five isoforms are enzymatically similar but show different tissue distribution: The major isoenzymes of skeletal muscle and liver, 4M, has four muscle (M) subunits, while 4H is the main isoenzymes for heart muscle in most species, containing four heart (H) subunits. LDH works to prevent muscular failure and fatigue in multiple ways.[2] The lactate-forming reaction generates cytosolic NAD+, which feeds into the glyceraldehyde 3-phosphate dehydrogenase reaction to help maintain cytosolic redox potential and promote substrate flux through the second phase of glycolysis to promote ATP generation. LDH normally appears throughout the body in small amounts and the gene expression is down-regulated by PGC-1α.[3] LDH is expressed extensively in body tissues, such as blood cells and heart muscle. High lactate dehydrogenase 5 expression correlates with high tumoral and stromal vascular endothelial growth factor expression in gastric cancer.[4] Because it is released during tissue damage, it is a marker of common injuries and disease such as heart failure. LDH is often used as a marker of tissue breakdown as LDH is abundant in red blood cells and can function as a marker for hemolysis. A blood sample that has been handled incorrectly can show false-positively high levels of LDH due to erythrocyte damage. It can also be used as a marker of myocardial infarction.

References

1. Millar DB, Frattali V, Willick GE (1969) Biochemistry. 8 (6): 2416–21.
2. Tesch P, et al. (1978). Acta Physiol Scand. 103 (4): 413–20.
3. Summermatter S, et al. (2013). Proc Natl Acad Sci U S A. 110 (21): 8738–43.
4. Kim HS, et al. (2014). Pathobiology. 81 (2): 78–85.

PRINCIPLE OF THE ASSAY

Cytotoxicity is evaluated by the quantification of plasma membrane damage. Lactate dehydrogenase (LDH) is the most widely used marker in cytotoxicity evaluation since it is present in all cell types, and rapidly released into the cell culture medium upon damage of the plasma membrane. The extracellular LDH in the medium can be quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD+ reduction to NADH. Oxidation of NADH by diaphorase leads to the reduction of a tetrazolium salt (INT) to a red formazan product that can be measured spectrophotometrically at 490 nm (Figure 1). The level of formazan formation is directly proportional to the amount of LDH released into the medium, which is indicative of cytotoxicity.

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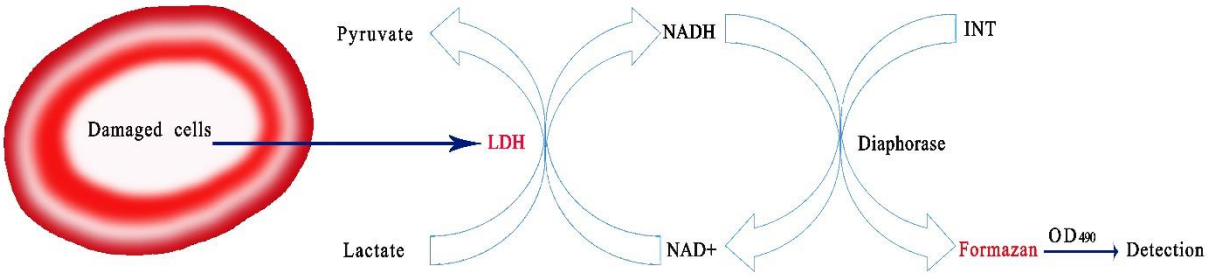

Figure 1 LDH Cytotoxicity Assay Principle

This package insert must be read in its entirety before using this product.

Storage

Store at 4°C as supplied for 6 months. Store at -20°C after reconstitution for up to 3 months.

MATERIALS PROVIDED

Description	Quantity	Description	Quantity	Description	Quantity
INT Substrate Mix	1	Assay Buffer	1	Lysis Solution	1
Stop Solution	1	LDH Positive Control	1	DataSheet/Manual	1

Reagents provided in the kit are sufficient for 1000 tests. When stored as directed, the kit is stable for 6 months from the date of receipt. Bring all reagents to room temperature before use.

MATERIALS NOT PROVIDED BUT REQUIRED

Cultured cell lines, tissue culture 96-well plate, flat-bottom, clear 96-well plate compatible with spectrophotometry, 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), spectrophotometer microplate reader capable of reading 490nm and 680nm absorbance. All materials are available at www.fishersci.com.

Procedural guidelines

1. LDH concentration and activity vary across sera types (such as horse, fetal bovine, or calf serum) commonly used to maintain mammalian cell lines. Therefore, it is important to measure LDH activity in culture media with serum. The endogenous LDH activity present in serum causes background signal in the assay. To reduce background signal, use the minimum serum percentage appropriate for each cell line without compromising cell viability.
2. The protocols provided are for 96-well plates. For 384-well plates, divide the volumes by four.

Reagent Preparations

On the day of the experiment, prepare the following reagents.

1. Warm the Lysis Buffer and Stop Solution to room temperature (takes ~20 minutes).
2. Prepare Assay Buffer Stock Solution: Reconstitute the Assay Buffer with 11 ml PBS to make 10 x Assay Buffer and protect from light.
3. LDH Reaction Mix: Reconstitute the INT Substrate Mix in 1.1 ml ddH2O for 10 min and mix thoroughly. The solution is stable for two months at 4°C. For 100 assays, mix 100 µl of INT Substrate Mix with 10.0 ml of 1 x Assay Buffer. Unused LDH Reaction Mix can be stored at −20°C protected from light for 3 to 4 weeks with tolerance for three freeze/thaw cycles without affecting the activity during the storage period.
4. Reconstitute the LDH Positive Control in 0.5 mL of 1 x Assay Buffer. Store the unused portion at −20°C.

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Assay Procedure

1. Sample Preparation: Collect cells (adherent or suspension) and wash once with fresh regular culture medium, then seed 100 µl cells (with 2-10 x 10^4 cells[2]) in a 96-well plate as the following:

	Background Control	Low Control	High Control	Test Sample
Cell suspension	-	100 µl	100 µl	100 µl
Culture medium	100 µl	-	-	-
Lysis Solution	-	-	10 µl	-
Test Substance	-	-	-	10 µl

   a. Background Control: 100 µl culture medium per well in triplicates with no cells. The Background Control will measure reagents and LDH background from culture medium serum. The background value has to be subtracted from all other values.
   b. Low Control: 100 µl cells in triplicate wells
   c. High Control: 100 µl cells in triplicates, add 10 µl of Cell Lysis Solution each well, mix. To adjust the increase of medium volume, 11 µl of the medium may be used in LDH activity assay at step 5.
   d. Test Sample: 100 µl cells in triplicates, add 10 µl of test substances to each well, mix.

3. Sample Incubation: Incubate cells in an incubator (5% CO2, 90% humidity, 37°C) for the appropriate time of treatment determined for test substance. Gently shake the plate at end of the incubation to ensure LDH is evenly distributed in the culture medium.
4. Centrifuge cells at 600 x g for 10 min to precipitate the cells.
5. Transfer the clear medium solution (10 µl/well) into an optically clear 96-well plate.
6. (optional) to perform an LDH Positive Control assay, add 10 µl of LDH Positive Control into triplicate wells.
7. Add 100 µl of LDH Reaction Mix to each well, mix and incubate for 30 min[6] at room temperature.
8. Add 50 µl of Stop Solution to each well and mix by gentle tapping to dissolve the precipitates. Note: Break any bubbles present in wells with a syringe needle and/or by centrifugation before reading.
9. Measure the absorbance of all controls and samples with a plate reader equipped with 490 nm filter. The reference wavelength should be 650 nm.
10. Calculate a percentage of cytotoxicity with the following equation:
    Cytotoxicity (%) = [(Test Sample OD_{490}- Low Control OD_{490})/(High Control OD_{490} – Low Control OD_{490})] X 100

Precaution and Technical Notes

1. It is critical to follow the procedure step by step otherwise appropriate color development may not occur as expected.
2. Trypsin may be used to remove adherent cells from a culture surface before seeding in a 96-well plate.
3. The number of cells to be used per well depends on the cell types. To optimize the assay, you can do a quick testing by using 2, 4, 8 x 10^4 cells per well, and then follow the assay protocol to determine the cell number you should use. The high control should be OD450nm ~2.0 after 30 min treatment with 10% Cell Lysis Solution, while the low control should be OD450nm < 0.8. The reaction time should be set at ~ 30 min.
4. Positive control (10 µl LDH) can be used to test whether all reagents are working properly to response to active LDH enzyme.
5. If the test substances are not dissolved in PBS, a solvent control may be performed by addition of the same amount of solvent in triplicates without testing substances.
6. The reaction time can be decreased or increased depending on the color development. The plate can be read at multiple time points until the desired reading is observed. The high control should be OD450nm ~2.0, while the low control should be OD450nm < 0.8.
7. LDH contained in the serum may cause a high background value. Adjust serum content to 1% or less as a final concentration.
8. This kit should not be used beyond the expiration date on the label.
9. Use a fresh reagent reservoir and pipette tips for each step.
10. It is recommended that all controls and samples be assayed in triplicate.
11. Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay.

For Research Use Only

Related products

GR107033 GSI Resazurin Cell Viability Assay Kit
GR107046 GSI MTT Assay Kit
GR107047 GSI MTS Assay Kit
GR107048 GSI TTC Assay Kit
GR107049 GSI WST-1 Cell Viability & Proliferation Assay Kit
GR107050 GSI ADCC Assay Kit
GR107051 GSI SOD Assay Kit
GR107052 GSI CDC Assay Kit
GR107053 GSI Lysine Specific Demethylase-1 (CLSD-1) Assay Kit

Troubleshooting Guide

Problem	Possible causes	Solution
Assay not working	• Assay Buffer at wrong temperature • Protocol step missed • Plate read at incorrect wavelength • Unsuitable microplate plate for assay	• Assay buffer must not be chilled-need to be at RT. • Re-read and follow the protocol exactly • Ensure you are using appropriate reader and filter settings. • For colorimetry, clear plate should be used.
Large CV (high variation in replicate value)	• Pipetting error or insufficient mixing of working solution and cell-sample. • Unstable sample type • Samples prepared in the wrong buffer • Samples not deproteinized (if indicated on the datasheet) • Cell/tissue samples not sufficiently homogenized • Too many freeze-thaw cycles • Samples contain impeding substance • Samples are too old or incorrectly stored. • Bubbles present in wells	• Use a positive displacement pipette or mix the plate more thoroughly. • Refer to datasheet for details about incompatible samples. • Use the assay buffer provided or refer to datasheet for instructions. • Use the 10kDa spin column. • Increase sonication time/number of strokes with the Dounce homogenizer. • Aliquot samples to reduce the number of freeze-thaw cycles. • Troubleshoot and also consider deproteinizing samples. • Use freshly made samples and store at recommended temperature until use. • Centrifuge the plate for a longer time or at a higher speed. • Break the bubbles with a syringe needle. • When plating the samples, use a positive displacement or electronic pipette.
Unexpected results	• Measured at wrong wavelength • Samples contain impeding substances • Unsuitable sample type. • Sample reading are outside linear range	• Use appropriate reader and filter settings described in datasheet. • Troubleshoot and also consider deproteinizing samples • Use recommended samples types as listed in the datasheet. • Concentrate/dilute samples to be in the linear range
Lower/higher readings in samples	• Cell density was too low. • Bubble present in wells.	• Repeat determination of optimum cell number. • Avoid bubble formation when pipetting and mixing.

	• Not fully thawed kit components. • Expired kit or incorrectly stored reagents • Reagents sitting for extended periods on ice • Incorrect incubation time/temperature • Incorrect amount used	• Wait for components to thaw completely and gently mix prior use. • Always check expiry date and store kit components as recommended on the datasheet. • Try to prepare a fresh reaction mix prior to each use. • Refer to datasheet for recommended incubation time and/ or temperature. • Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)
High background	• High inherent LDH activity animal sera in cell culture media.	• Reduce serum concentration to 0-5%. • Alternatively, before lysis or treatment of cells, exchange the complete media with media without serum.
High Low Control absorbance	• High cell density • Vigorous pipetting during cell plating	• Repeat determination of optimum cell number for assay • Gently handle cell suspension during plate set-up.