CyQUANT™ LDH Cytotoxicity Assay Kit

Catalog Numbers C20300 and C20301

Pub. No. MAN0018500 **Rev.** B.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Invitrogen™ CyQUANT™ LDH Cytotoxicity Assay Kit provides the reagents that are needed for a simple, reliable colorimetric method to quantify cellular cytotoxicity. The kit can be used with different mammalian cell types, including 3D models, for measuring cytotoxicity mediated by chemical compounds and assaying cell-mediated cytotoxicity. Since the LDH concentration in media is an indicator of cellular cytotoxicity the assay can be used to monitor cytotoxicity from the same sample over time. To perform the assay, an aliquot of the cell culture media is transferred to a new plate and the CyQUANT™ LDH Cytotoxicity Assay Kit reaction mixture is added. After a 30-minute incubation at room temperature the assays are stopped by adding the Stop Solution and then absorbance is measured using a microplate reader.

Lactate dehydrogenase (LDH) is a cytosolic enzyme present in many different cell types and is a well-defined and reliable indicator of cytotoxicity. Damage to the plasma membrane releases LDH into the surrounding cell culture media. The extracellular LDH in the media 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.

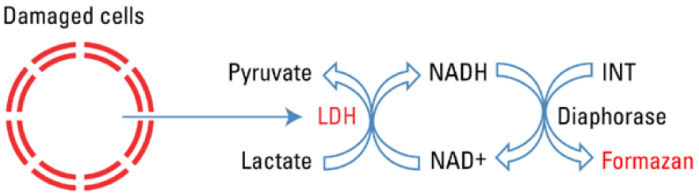

Figure 1 Schematic of CyQuant LDH cytotoxicity assay mechanism.

Contents and storage

Reagents that are provided in the kit are sufficient for 200 reactions in 96-well plates (Cat. No. C20300) or 1,000 reactions in 96-well plates (Cat. No. C20301).

Item	Amount		Storage[1]
	C20300	C20301	
Substrate Mix	1 Vial	5 Vials	Store at –20°C, protect from Light
Assay Buffer	1 x 600 µL	5 x 600 µL	
Lysis Buffer	1 x 2.5 mL	1 x 12 mL	
Stop Solution	1 x 12 mL	1 x 60 mL	
LDH Positive Control	1 x 6 µL	1 x 30 µL	
Absorbance: Monitor the absorbance at 490 nm and 680 nm, using 680 nm as a reference wavelength			

[1] When stored as directed, the kit is stable for 6 months from the date of receipt.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Cultured cell line	MLS
Tissue culture 96-well plate	MLS
Flat-bottom, clear 96-well plate compatible with spectrophotometry	MLS
1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)	MLS
Spectrophotometer microplate reader capable of reading 490nm and 680nm absorbance	MLS

Procedural guidelines

- 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.

- The protocols described in this document are for 96-well plates. For 384-well plates, divide the volumes by four.

Before you begin

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

- Warm the Lysis Buffer and Stop Solution to room temperature (takes ~20 minutes).
- Prepare Substrate Stock Solution: Add 11.4 mL of diH_2O to the entire contents of the Substrate Mix, then mix gently to dissolve.
- Prepare Assay Buffer Stock Solution: Thaw the Assay Buffer to room temperature and protect from light.
- Prepare Reaction Mixture: Combine the 600 µl of Assay Buffer Stock Solution with the 11.4 mL of Substrate Stock Solution, then mix gently and protect from light until use.

  Note: One vial of the Reaction Mixture is sufficient for testing two 96-well plates. Unused Reaction Mixture 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.
- Prepare 1X LDH Positive Control: Dilute 1.5 µl of LDH Positive Control with 1 mL of 1% BSA in PBS. Store the unused portion at −20°C.

Determine the optimum cell number for LDH cytotoxicity assay

1. Prepare a serial dilution of cells (0–10,000 cells/100 µL of media) in two sets of triplicate wells in a 96-well tissue culture plate.

   One set of the serial dilutions is lysed and used to determine the Maximum LDH Release. The second set of serial dilutions is used to determine the Spontaneous LDH release.
2. Incubate the cells overnight in a 37°C incubator with the appropriate level of CO_2.
3. Add 10 µL of sterile water to the Spontaneous LDH Release dilution series of triplicate wells containing cells, then mix by gentle tapping.
4. Add 10 µL of 10X Lysis Buffer to the Maximum LDH Release dilution series, then mix by gentle tapping.

   Note: Do not create bubbles when pipetting because bubbles prevent accurate absorbance readings.
5. Incubate the plate in an incubator at 37°C for 45 minutes with the appropriate level of CO_2.
6. Transfer 50 µL of each sample medium (Spontaneous LDH Activity Controls and Maximum LDH Activity Controls) to a 96-well flat bottom plate in triplicate wells.
7. *(Optional)* To perform an LDH Positive Control assay, aliquot 50 µL of 1X LDH Positive Control into triplicate wells.
8. Transfer 50 µL of Reaction Mixture to each sample well, then mix by gentle tapping.
9. Incubate the plate at room temperature for 30 minutes protected from light.
10. Add 50 µL of Stop Solution to each sample well, then mix by gentle tapping.

    After Stop Solution is added, measure absorbance within 1 to 2 hours.

    Note: Break any bubbles present in wells with a syringe needle and/or centrifugation before reading.
11. Measure the absorbance at 490 nm and 680 nm. To determine LDH activity, subtract the 680-nm absorbance value (background signal from instrument) from the 490-nm absorbance value.
12. Plot the Maximum LDH Release Control absorbance value minus the Spontaneous LDH Release Control absorbance value versus cell number to determine the linear range of the LDH cytotoxicity assay and the optimal number of cells (Figure 2).

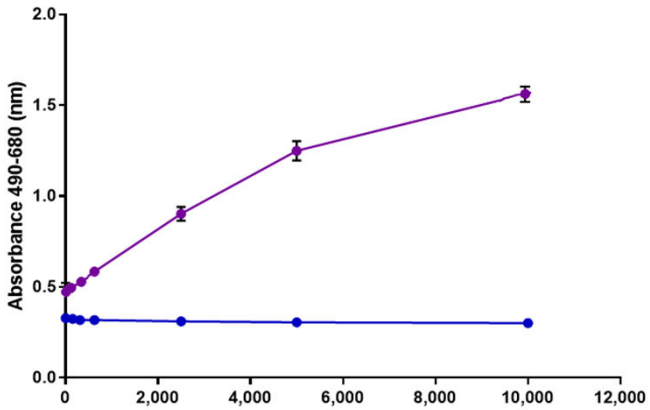

Figure 2 Graph of LDH activity vs. A549 cell number

A549 cells were diluted in a cell culture plate with MEM medium containing 10% fetal bovine serum. The Maximum LDH Release (purple) and the Spontaneous LDH Release (blue) are plotted.

Perform chemical compound-mediated cytotoxicity assay

1. Plate the optimal number of cells/well in 100 µL of medium (as determined in preliminary experiments) in triplicate wells in a 96-well tissue culture plate.

   Note: Include a complete medium control without cells to determine LDH background activity present in sera used for media supplementation. Include a serum-free media control to determine the amount of LDH activity in sera. Plate additional cells in triplicate wells for Spontaneous LDH Activity Controls and Maximum LDH Activity Controls.

2. Incubate cells overnight at 37°C with the appropriate level of CO_2.

3. After overnight incubation, prepare samples according to the following table:

Sample	Action
Spontaneous LDH Activity	Add 10 µL of sterile, ultrapure water to one set of triplicate wells of cells.
Maximum LDH Activity	Add nothing to one set of triplicate wells of cells.
Chemical-treated LDH activity	Add 10 µL of vehicle containing chemical compound to one set of triplicate wells of cells.

4. Incubate the experimental plate in a cell culture incubator at 37°C with the appropriate level of CO_2.

5. To the set of triplicate wells serving as the Maximum LDH Activity Controls, add 10 µL of 10X Lysis Buffer, then mix by gentle tapping.

   Note: Do not create bubbles when pipetting because bubbles prevent accurate absorbance readings.

6. Incubate the plate in a cell culture incubator at 37°C with the appropriate level of CO_2 for 45 minutes.

7. Transfer 50 µL of each sample medium (Spontaneous LDH Activity, Maximum LDH Activity, and Chemical-treated LDH activity) to a 96-well flat-bottom plate in triplicate wells.

8. *(Optional)* To perform an LDH Positive Control assay, aliquot 50 µL of 1X LDH Positive Control into triplicate wells.

9. Aliquot 50 µL of Reaction Mixture to each sample well, then mix well.

10. Incubate the plate at room temperature for 30 minutes protected from light.

11. Add 50 µL of Stop Solution to each sample well, then mix by gentle tapping.

    Note: Break any bubbles present in wells with a syringe needle and/or by centrifugation before reading.

12. Measure the absorbance at 490 nm and 680 nm.

13. To determine LDH activity, subtract the 680-nm absorbance value (background) from the 490-nm absorbance before calculation of % Cytotoxicity.

14. Calculate % Cytotoxicity by using the following formula:

$$\% \text{ Cytotoxicity} = \left[\frac{\text{Compound-treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}}\right] \times 100$$

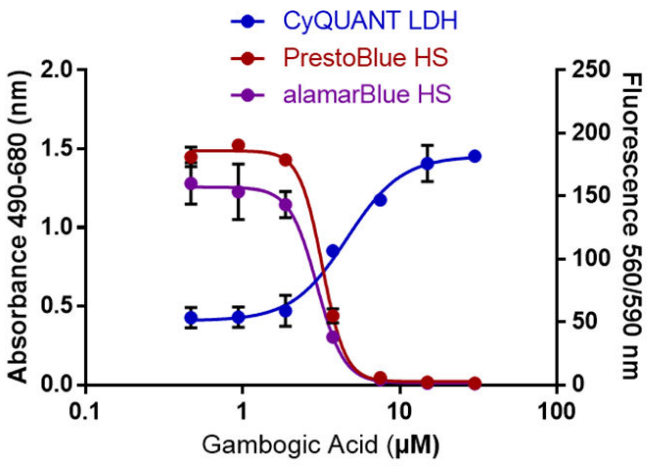

Figure 3 Multiplexing to simultaneously detect changes in cytotoxicity and viability

A549 cells were plated at 5,000 cells per well in a 96-well plate in MEM medium supplemented with 10% FBS and incubated overnight. After the overnight incubation, different concentrations of Gambogic Acid were added to the culture media. Cytotoxicity was measured on aliquots of the drug treated cellular supernatant using the CyQUANT™ LDH Cytotoxicity Assay Kit following the manufacturer instructions. The PrestoBlue™ HS or alamarBlue™ HS was added directly on top of the drug treated cells in media containing FBS. LDH activity was measured using 490-nm absorbance. The fluorescence of PrestoBlue™ HS and alamarBlue™ HS was detected using an excitation of 560 nm and an emission of 590 nm.

Troubleshooting

Observation	Possible cause	Recommended action
High medium control absorbance	High inherent LDH activity in 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 spontaneous control absorbance	High cell density	Repeat determination of optimum cell number for assay.
	Vigorous pipetting during cell plating	Gently handle cell suspension during plate set-up.

Observation	Possible cause	Recommended action
Low absorbance value in experiment	Cell density was too low	Repeat determination of optimum cell number
	Bubble present in wells	Avoid bubble formation when pipetting and mixing.
High variation in replicate values	Pipetting error or insufficient mixing of working solution and cell-sample	Use a positive displacement pipette or mix the plate more thoroughly.
Precipitation of formazan in LDH positive control	1% BSA was not used	Use 1% BSA in the sample.
High variability of absorbance well-to-well	Bubbles present in wells	Centrifuge the plate for a longer time or at a higher speed.
		Break bubbles with a syringe needle.
		When plating the samples, use a positive displacement or electronic pipette.

Limited product warranty

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For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

Revision history: Pub. No. MAN0018500

Revision	Date	Description
B.0	05 March 2019	Changes to before you begin section: Bullet 1 rewritten and moved in to become bullet 2. Original last bullet moved up to become bullet 1 and (takes~20 minutes) was added.
A.0	19 February 2019	Rebranded into a new document.