CyQUANT™ LDH Cytotoxicity Assay – Fluorescence Kit

Catalog Numbers C20302 and C20303

Pub. No. MAN0018656 **Rev.** A.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 – Fluorescence Kit provides the reagents that are needed for a simple, reliable fluorescence-based 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, a sample of the cell culture media is transferred to a new plate and the CyQUANT™ LDH Cytotoxicity Asssay – Fluorescence Reagent Stock Solution is added. After a 10-minute incubation at room temperature the assays are stopped by adding the Stop Solution and then fluorescence 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 resazurin forming the highly-fluorescent resorufin which can be measured using an excitation of 560 nm and an emission of 590 nm (Figure 1). The level of resorufin formation is directly proportional to the amount of LDH released into the medium, which is indicative of cytotoxicity.

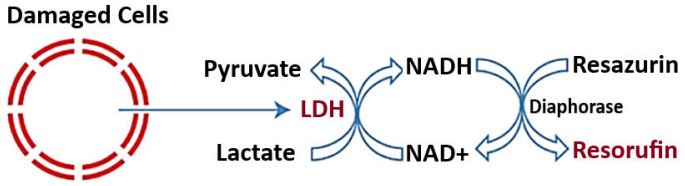

Figure 1 Schematic of CyQUANT™ LDH cytotoxicity assay mechanism.

Contents and storage

Reagents that are provided in the kit are sufficient for 200 reactions in 96-well plates (Cat. No. C20302) or 1,000 reactions in 96-well plates (Cat. No. C20303).

Item	Amount		Storage[1]
	C20302	C20303	
Reagent Mix	1 Vial	5 Vials	Store at –20°C, protect from light
Reporter Mix	1 × 12 mL	5 × 12 mL	
Lysis Buffer	1 × 2.5 mL	1 × 12 mL	
Stop Solution	1 × 12 mL	1 × 60 mL	
LDH Positive Control	1 × 6 µL	1 × 30 µL	
Fluorescence: Use excitation of 560 nm and emission of 590 nm			

[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
Fluorescence microplate reader capable of 560 nm excitation and 590 nm emission	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 Reagent Stock Solution: Add 12 mL of the thawed Reporter Mix to the entire contents of the Reagent Mix (amber glass vial). Mix by vortex to ensure that all materials are fully dissolved. Protect from light.

  Note: One vial of the Reagent Mix is sufficient for testing two 96-well plates. Unused Reagent Stock Solution 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. The third set of wells (a minimum of three wells) contains media only.

   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. The third set containing only media will be used to determine the amount of background fluorescence signal.
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 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, add 50 µL of 1X LDH Positive Control into triplicate wells.
8. Transfer 50 µL of Reagent Stock Solution to each sample well, then mix by gentle tapping. Avoid creating bubbles.
9. Incubate the plate at room temperature for 10 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 fluorescence within 1 to 2 hours.

    Note: Break any bubbles present in wells with a syringe needle and/or centrifugation before reading.
11. Measure the fluorescence by using excitation of 560 nm and emission of 590 nm. To determine LDH activity, subtract the background fluorescence signal (signal resulting from media only wells) from the Spontaneous and Maximum Release samples.
12. Plot the Maximum LDH Release Control fluorescence value and the Spontaneous LDH Release Control fluorescence value versus cell number to determine the linear range of the LDH cytotoxicity assay and the optimal number of cells (Figure 2).

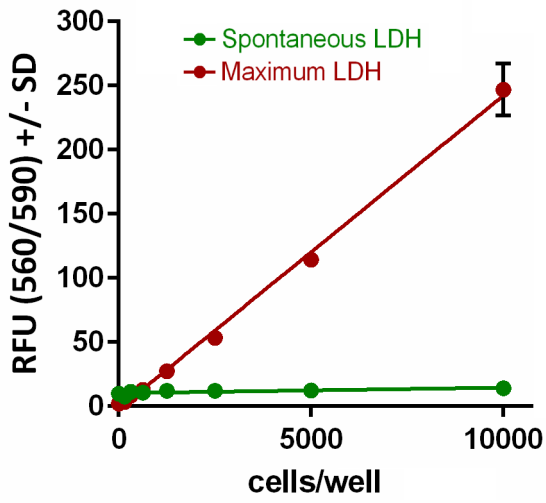

Figure 2 Graph of LDH activity vs. A549 cell number

A549 cells were diluted in a cell culture plate with MEM medium containing 10% fetal bovine serum. The Maximum LDH Release (red) and the Spontaneous LDH Release (green) 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.
Background fluorescence	No treatment or addition is required.

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

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

   Note: Do not create bubbles when pipetting because bubbles prevent accurate 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)* Add 50 µL of 1X LDH Positive Control into triplicate wells to perform an LDH Positive Control assay.

9. Add 50 µL of Reaction Mixture to each sample well, then mix by gently tapping the plate. Avoid creating bubbles.

10. Incubate the plate at room temperature for 10 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 fluorescence by using an excitation of 560 nm and an emission of 590 nm.

13. To determine LDH activity, subtract the background fluorescent signal (media only) from the fluorescence before calculation of % Cytotoxicity.

14. Calculate % Cytotoxicity by using the following formula:

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

Troubleshooting

Observation	Possible cause	Recommended action
High medium control signal	High inherent LDH activity in animal sera in cell culture media	Reduce serum concentration to 1–5%.
		Before lysis or treatment of cells, exchange the complete media with media without serum.
High spontaneous control signal	High cell density	Repeat determination of optimum cell number for assay.
	Vigorous pipetting during cell plating	Gently handle cell suspension during plate set-up.
Low signal 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 variability of signal 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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Life Technologies Corporation | 29851 Willow Creek | Eugene, OR 97402
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Revision history: Pub. No. MAN0018656

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.