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COLORIMETRIC CYTOTOXICITY ASSAY KIT

Product # **LK 100**

GUIDE FOR USE

Please read all instructions carefully before beginning the assay

****Store kit at 4°C at all times****

CAUTION

This Oxford Biomedical Research, Inc. product is sold for research and/or in vitro use only. Not for clinical diagnostic use.

STORAGE AND STABILITY

The reagent kit should be stored in the dark at 2-8° C.

INTRODUCTION

This colorimetric cytotoxicity assay measures lactate dehydrogenase (LDH), a relatively stable cytosolic enzyme that is released by cells when they undergo significant membrane damage or cytolysis. The amount of LDH released is proportional to the number of cells damaged/lysed. The amount of enzyme released is monitored by incubation with substrate and a coupled colorimetric reaction. Absorbance data is read using a 96 well plate reader with a 490-492 nm filter.

Two protocols are included with these instructions. One is for cellular cytotoxicity assays in which the lysis of target cells by effector lymphocytes is monitored. The second protocol is for chemotoxicity assays in which the potential toxic effects of chemical agents are are examined by

monitoring their toxicity for indicator cell lines upon challenge with dilutions of the chemical agent.

The LK-100 assay kit is based on the enzymatic reaction catalyzed by lactate dehydrogenase (LDH):

$$NAD^+ + \text{lactate} \longrightarrow \text{pyruvate} + NADH$$

The NADH generated is then reoxidized in a reaction coupled to the reduction of INT, producing a bright red formazan dye:

$$NADH + INT \longrightarrow NAD^+ + \text{formazan (red color)}$$

*PMS serves as an electron carrier in the above reaction

MATERIALS PROVIDED

The kit contains reagents sufficient for eight 96-well plates:

Code No.	Component	Quantity
LK 101	substrate	1 vial
LK 102	assay buffer	1 vial
LK 103	color reagent	4 vials
LK 104	enzyme control	4 vials
LK 105	cell lysing reagent	4 vials
LK 106	color enhancer	1 vial
	Instructions booklet	1

MATERIALS NEEDED BUT NOT PROVIDED

1. Adjustable pipettors to dispense 5-200 µl.
2. Sterile pipette tips.
3. Culture medium as required for the cells to be tested.
4. Assay medium (NOTE: Assay medium should NOT contain serum. Animal sera (including human serum, FBS, etc.) contain measurable amounts of LDH which will significantly increase backbround absorbance readings in this assay. It is recommended that, instead of serum, 1% (w/v) bovine serum albumin be included in the assay medium as a stabilizing agent for the cells and to prevent precipitation of the formazan end product.
5. 96-Well microtiter plates: Use cell culture quality V or round-bottomed for cell mediate cytotoxicity assays. Use flat-bottomed plates for chemotoxicity reactions. Optically clear, flat-bottomed plates are also required for the color development procedure.
6. 37° C Incubator.
7. Centrifuge.
8. 1N HCl.
9. Microtiter plate reader with 490-492 nm filter.

10. Hemacytometer.
11. Microscope.
12. Test systems:
    For cytotoxicity: Effector and target cells.
    For chemotoxicity: Test cell line and test agent.

REAGENT PREPARATION

A. **Enzyme control** (LK-104): Reconstitute one vial with 1.0 ml of assay medium containing 1% (10 mg/ml) bovine serum albumin. Invert the vial several times to dissolve thoroughly.

B. **Lysing reagent** (LK-105): Reconstitute one vial with 1.0 ml of deionized water. Invert the vial several times to dissolve thoroughly.

C. **Substrate mixture** NOTE: The components of the substrate mixture should be reconstituted individually. They should not be mixed together until all of the supernatants and controls have been added to the wells in the assay plate.

1. **Substrate** (LK-101): Reconstitute by adding 10 ml of deionized water. First, slowly add a small amount of water, recap and invert the vial to dissolve part of the contents. Then add the remaining water and mix well. This material is stable for at least three months after reconstitution if stored at 2-8°C.
2. **Assay Buffer** (LK-102): Reconstitute by adding 10 ml of deionized water. Invert the vial to mix. This material is stable for at least 3 months after reconstitution if stored at 2-8° C.
3. **Color Reagent** (LK-103): Reconstitute by addition of 14 ml of deionized water to the amber vial.
   NOTE: This material is packaged under vacuum. Exercise caution when opening the vial. *Protect this reagent from light at all times until it is used.* This material is stable for 24 hrs after reconstitution **provided it is refrigerated, protected from light, and has not been mixed with other components of the substrate mixture**.

ASSAY PROCEDURE

A. Cell mediated cytotoxicity assay:

1. **Preliminary assay**: The level of LDH transcription varies among cell types and cell lines. The maximum release of enzyme from prospective target cells must therefore be determined for to ensure that the intended target cells contain sufficient LDH relative to the effector cells to be used in this assay. The minimum target cell concentration that can be used is also dependent upon the amount of enzyme present. This preliminary assay only needs to be performed for evaluation of a new cell for use in this assay.
   a. Wash the target cells three times with assay medium, then dilute the cells to the desired concentration with assay medium.
   a. Add target cells to the wells of a V-bottomed or round-bottomed microtiter plate to achieve concentrations ranging from 1×10^4 to 1×10^6 per well (triplicate

wells for each concentration are recommended) in a total volume of 145 µl. Cells are then lysed by adding 5 µl of cell lysing reagent (LK-105) to each well and mixing the contents. Plates are centrifuged (10 min @ 200 g), and 100 µl of supernatant is transferred into the corresponding wells of an optically clear, flat-bottomed microtiter plate. Place 100 µl of assay medium into each of three wells to serve as blanks. The amount of enzyme released is determined by the color development procedure described in Part C.

c. Determine the concentration of target cells that yields an absorbance value at least two times the absorbance of the medium background. This is the minimum target cell concentration that should be used to perform assays.

2. **Preparation of cells**: Wash both effector and target cells three times with assay medium. Dilute both cell preparations to the desired concentration with assay medium. Experimental wells should be set up in triplicate or quadruplicate. A sample map of a plate is given in Figure 1, using 8 different effector:target ratios performed in quadruplicate.

Figure 1: Suggested map for cellular cytotoxicity assays

Controls & Blanks			Maximum Release	Spontaneous Release				ExperimentalWells			
blank	blank	blank	max	ESR	ESR	TSR	TSR	E:T ratio 1	E:T ratio 1	E:T ratio 1	E:T ratio 1
ctrl dil 1	ctrl dil 1	ctrl dil 1	max	ESR	ESR	TSR	TSR	E:T ratio 2	E:T ratio 2	E:T ratio 2	E:T ratio 2
ctrl dil 2	ctrl dil 2	ctrl dil 2	max	ESR	ESR	TSR	TSR	E:T ratio 3	E:T ratio 3	E:T ratio 3	E:T ratio 3
ctrl dil 3	ctrl dil 3	ctrl dil 3	max	ESR	ESR	TSR	TSR	E:T ratio 4	E:T ratio 4	E:T ratio 4	E:T ratio 4
ctrl dil 4	ctrl dil 4	ctrl dil 4	max	ESR	ESR	TSR	TSR	E:T ratio 5	E:T ratio 5	E:T ratio 5	E:T ratio 5
ctrl dil 5	ctrl dil 5	ctrl dil 5	max	ESR	ESR	TSR	TSR	E:T ratio 6	E:T ratio 6	E:T ratio 6	E:T ratio 6
ctrl dil 6	ctrl dil 6	ctrl dil 6	max	ESR	ESR	TSR	TSR	E:T ratio 7	E:T ratio 7	E:T ratio 7	E:T ratio 7
ctrl dil 7	ctrl dil 7	ctrl dil 7	max	ESR	ESR	TSR	TSR	E:T ratio 8	E:T ratio 8	E:T ratio 8	E:T ratio 8

ctrl = different dilutions of the LDH control
max = maximal release from target cells.
ESR = spontaneous release from effector cells
TSR = spontaneous release from target cells
E:T = Effector to target cell ratio

3. **Experimental wells**: Add effector and target cells to the wells of round or V-bottomed 96 well microtiter plates so that each well contains a final volume of 150 µl. It is recommended that several ratios of effectors to targets be examined for each concentration of target cells.

4. **Spontaneous release**: Add 100 µl of only effector OR only target cells at each concentration used to separate wells containing 50 µl of assay medium to obtain the spontaneous release of enzyme from these cells.

5. **Maximum release**: Add 100 µl of only target cells at each concentration used to separate wells containing 5 µl of cell lysing solution (LK-105) and 45 µl of assay medium to determine the maximum amount of enzyme released by these cells.

6. **Harvest of supernatants**: Centrifuge cytotoxicity plate(s) for 5 minutes at 1000 rpm, then incubate at 37° C for up to four hours. At the end of the incubation period, remove the microtiter plate(s) from the incubator. Add 50 µl of ice cold assay medium to all wells and centrifuge the plate(s) for 5 minutes @ 200 g. Transfer 100 µl of the supernatants into corresponding wells of an optically clear, flat-bottomed assay plate. In order to obtain a background reading, put 100 µl of assay medium in three blank wells.

7. Proceed to Part C for the control set-up and color development procedure.

B. Chemotoxicity assay procedure (using a test agent):

1. **Preparation of test cells**: Before setting up assay, wash cells to be used 3 times with assay medium. Dilute cells to the desired concentration with assay medium. Place 100 µl of the cell suspension in the wells of a 96 well flat-bottomed tissue culture plate. Cover, place in a humid chamber, and incubate at 37° C for up to 3 days or until at 80% confluency.

   Alternatively, if it is necessary to grow cells in culture medium, which contains serum, rinse the cell monolayer 3 times with serum-free assay medium before using the cells.

2. **Experimental wells**: Experimental wells should be set up in triplicate or quadruplicate. Figure 2 shows a suggested map for the set up of the assay using 2 different test agents at 6 dilutions performed in quadruplicate. Prepare dilutions of the test agent with the assay medium. Water soluble test agents may be diluted and added directly to the cells. Hydrophobic substances need to be dissolved in a suitable, relatively non-toxic solvent (such as DMSO) before being diluted in the assay medium. Insoluble substances may be extracted by an elution process. The test agent is placed in a test tube with assay medium and is rotated end-over-end for 24 hours at 37° C. This allows some toxic elements to leach out into the assay medium, which can then be tested. These dilutions should range from the undiluted test agent to a 1:100,000 dilution, depending on the expected toxicity. Remove the spent media from

the wells and dispense 200 µl of the test agent dilutions into the wells of the 96 well microtiter plate containing the cells to be used for testing.

Figure 2 - Suggested map for chemotoxicity assays

blank	SR	ctrl dil 1	ctrl dil 1	ctrl dil 1	ctrl dil 1	test 1 dil 1	test 1 dil 2	test 1 dil 3	test 1 dil 4	test 1 dil 5	t
blank	SR	ctrl dil 2	ctrl dil 2	ctrl dil 2	ctrl dil 2	test 1 dil 1	test 1 dil 2	test 1 dil 3	test 1 dil 4	test 1 dil 5	t
blank	SR	ctrl dil 3	ctrl dil 3	ctrl dil 3	ctrl dil 3	test 1 dil 1	test 1 dil 2	test 1 dil 3	test 1 dil 4	test 1 dil 5	t
max	SR	ctrl dil 4	ctrl dil 4	ctrl dil 4	ctrl dil 4	test 1 dil 1	test 1 dil 2	test 1 dil 3	test 1 dil 4	test 1 dil 5	t
max	SR	ctrl dil 5	ctrl dil 5	ctrl dil 5	ctrl dil 5	test 2 dil 1	test 2 dil 2	test 2 dil 3	test 2 dil 4	test 2 dil 5	t
max	SR	ctrl dil 6	ctrl dil 6	ctrl dil 6	ctrl dil 6	test 2 dil 1	test 2 dil 2	test 2 dil 3	test 2 dil 4	test 2 dil 5	t
max	SR	ctrl dil 7	ctrl dil 7	ctrl dil 7	ctrl dil 7	test 2 dil 1	test 2 dil 2	test 2 dil 3	test 2 dil 4	test 2 dil 5	t
max	SR	ctrl dil 8	ctrl dil 8	ctrl dil 8	ctrl dil 8	test 2 dil 1	test 2 dil 2	test 2 dil 3	test 2 dil 4	test 2 dil 5	t

max = maximum release
SR = spontaneous release

3. **Spontaneous release**: Remove the spent media from these wells and add 200 µl of fresh assay medium to wells containing cells to be used to determine the spontaneous release of enzyme from the cells.

4. **Maximum release**: Remove the spent media and add 195 µl of fresh assay medium and 5 µl of the lysing reagent (LK-105) to each well containing cells for the determination of the maximum release of the enzyme from the cells.

5. **Harvest of supernatants**: After adding test agent dilutions, lysing reagent, and assay medium, centrifuge the plate(s) for 5 minutes at 1000 rpm and incubate at 37° C for

18-24 hours. At the end of the incubation period, remove the plate(s) from the incubator. Add 50 µl of ice cold assay medium to all wells and centrifuge the plate(s) for 5 minutes at 2000 rpm. Transfer 100 µl of the supernatants into corresponding wells of an optically clear, flat-bottomed microtiter plate(s). In order to obtain a background reading, place 100 µl of assay medium in three blank wells.

6. Proceed to Part C for the control set-up and color development procedure.

C. LDH Controls and color development procedure:

1. **Preparation of enzyme control**: Prepare dilutions from the reconstituted enzyme control (LK-104). For initial experiments, dilutions with the assay medium ranging from 1:20 to 1:2,560 are recommended. These dilutions will allow for a wide range of assay conditions to determine the standard range that is appropriate for a particular assay. To the microtiter plate containing either the cytotoxic or chemotoxic supernatants, place 100 µl of the control dilutions into the control wells. For chemotoxicity assays, it is useful to set up control wells containing both enzyme control dilutions and the test agent to determine if the test agent has any effect on the enzyme.

2. **Preparation of the substrate mixture**: After adding the supernatants and enzyme controls to the wells in the assay plate, prepare the substrate mixture by adding 2.5 ml of reconstituted buffer (LK-102), 2.5 ml of reconstituted substrate (LK-101), and 2.0 ml of color enhancer (LK-106) to the reconstituted color reagent (LK-103) in the amber vial. Recap and mix by inverting several times. The substrate mixture must be used within one half hour of preparation. Discard the remainder after user. The remaining amounts of LK-101, LK-102, and LK-106 should be kept refrigerated at 2-8° C for future use.

3. **Color Development**: Add 100 µl of freshly prepared substrate mixture to each well. Incubate the plate(s) at room temperature and protected from light (the inside of a drawer works well) until purple color develops in assay wells. When sufficient color is developed, usually after ten to sixty minutes, stop the reaction by adding 50 µl of 1N HCl to each well, in the same order in which the substrate mixture was added. Centrifuge the plate(s) for 5 minutes at 2000 rpm to get rid of air bubbles in the wells and then read the absorbance in the wells on a microtiter plate reader at a wavelength of 490-492 nm. The plate can be read at any time up to twenty-four hours after the addition of HCl.

CALCULATION OF RESULTS

A. To determine **percent cytotoxicity** (%C) at a given target to effector cell ratio, calculate the average absorbance readings from the experimental wells (E), the spontaneous release from effector cells (ESR), spontaneous release from target cells (TSR), and the maximum release (M) values from target cells that were lysed, all at the same

concentration as the experimental wells. Substitute these values into the following equation:

$$\%C = \frac{(E - ESR - TSR)}{M - TSR} \times 100$$

B. To determine **percent chemotoxicity** (%T) at each dilution of a test substance, calculate the average absorbance readings from the experimental wells (E). Also calculate the average spontaneous release (S) and maximum release (M) of the cells used. Substitute these values into the following equation:

$$\%T = \frac{E - S}{M - S} \times 100$$

C. Alternatively, to quantitate **the amount of LDH released**, calculate the average absorbance of the controls at each dilution. The reconstituted enzyme control vial (LK-104) contains approximately 71 units LDH/ml. Determine the amount of LDH present in each dilution. Graph the average absorbance of the controls (*y-axis*) against the concentration of LDH in units/ml (*x-axis*) at each dilution to obtain the standard curve. The concentration of LDH in units/ml in the experimental wells can be derived from the curve using the average absorbance at each experimental dilution, finding where that point lies on the standard curve, and following the point down to the corresponding concentration of LDH in units/ml.

D. Another method that can be used is to determine the concentration of the test agent which results in **50% cell destruction or LDH release** (LD-50). This can be determined from the data for the test agent dilutions. Calculate the average maximum release. This represents 100% cell destruction. Also calculate the average absorbances for each test agent dilution. The percentage of cell destruction for each dilution of the test agent is found by the following formula:

$$\text{\% Cell destruction} = \frac{\text{Average absorbance of test agent dilution}}{\text{Average absorbance of test maximum release}} \times 100$$

Using semi-log graph paper, plot the percent of cell destruction for each dilution of the test substances on the *y-axis* and the concentration of the test substance on the *x-axis*. To determine the LD-50 from the graph, find the point where 50% cell destruction intercepts the curve and follow down to the concentration of the test agent. This concentration is the LD-50 value.

TROUBLE SHOOTING

A. No color development after one hour: Check first that the reagents have all been added correctly. If they have been, then it may be necessary to increase the concentration of enzyme control and/or the number of cells used in the assay. If the amount of cells available is limited, the incubation periods may be increased.

B. Color development in less than ten minutes: The concentration of enzyme control and/or the number of cells being assayed needs to be decreased.

C. Inconsistent absorbance readings: Make sure there are no air bubbles in the wells, which will cause a false absorbance reading. Wipe off the bottom of the microtiter plate with a lint-free tissue.

REFERENCES

1. Babson, A.L. and G.E. Phillips. 1965. A Rapid Colorimetric Assay for Serum Lactic Dehydrogenase. *Clin. Chim. Acta.*, 12: 210-215.

2. Korzeniewski, C. and D.M. Callewaert. 1983. An Enzyme-Release Assay for Natural Cytotoxicity. *J. Immunol. Methods*, 64: 313-320.

3. Sherard Braa, S. and D. Triglia. 1991. Predicting Ocular Irritation Using 3-Dimensional Human Fibroblast Cultures. *Cosmetics & Toiletries*, 106(12): 55.

MATERIAL SAFETY DATA SHEET

Gloves and lab coat should be worn at all times while performing this assay. Contents may be harmful if swallowed, inhaled or absorbed through the skin. See *Precautions for Use*.

PRECAUTIONS FOR USE

1. Do not pipette solutions by mouth.
2. Do not eat or smoke in areas where specimens or kit reagents are being handled.

PHYSICAL AND CHEMICAL DATA

Components are stable in closed containers under normal temperatures and pressures.

HANDLING & STORAGE

Safety glasses and gloves should be worn to prevent skin and eye contact. Wear protective clothing such as lab coats. Store kit at 4°C.

HEALTH HAZARDS

Individual components may cause skin irritation or be harmful if swallowed. Avoid contact with skin and eyes.

FIRST AID

Call a physician. If swallowed give water or milk to dilute and induce vomiting. In case of contact with eyes, flush with copious amounts of water for at least 15 minutes, assure adequate flushing by separating eyelids with fingers. In case of skin contact, wash with soap or mild detergent and large amounts of water.

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