

# Use of the CytoTox 96(TM) Assay in Routine Biocompatibility Testing *In Vitro*

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*The complete evaluation of potential biomaterials should include testing for overt toxicity, long-term foreign body responses and mutagenicity. The release of lactate dehydrogenase from cultured cells exposed to a biomaterial provides a sensitive and accurate marker for cellular toxicity. Materials which cause overt toxicity in this screening procedure should be dismissed as candidates for use in vivo. The CytoTox 96(TM) Assay is a fast, accurate and cost-effective test for LDH release that is as applicable to routine biocompatibility testing as it is to immunological studies of cellular cytotoxicity.*

## Introduction

The widespread use of natural and synthetic materials as implantable biological devices ("biomaterials") in human and veterinary medicine has led to great advances in health care. Biomaterials are now used routinely in all major surgical specialties, including orthopedic surgery, cardiothoracic surgery, ophthalmic surgery and plastic/reconstructive surgery. New biomaterials are currently under development and are being tested in centers throughout the world.

There are clear legal, moral and ethical requirements to screen all new biomaterials for adverse effects on cells and tissues. Adverse tissue responses may include overt toxicity leading to cell death around an implant, chronic inflammatory reactions usually in the form of foreign-body reactions, or in the long term, neoplastic changes. In addition, the effects of the implanted material (and its breakdown products, if the material undergoes degradation *in vivo*) must be examined to rule out the possibility of adverse systemic effects, such as autoimmune disease. The composite picture emerging from these tests is a measure of the biological compatibility, or biocompatibility, of the test material.

Although animal studies are ultimately required before a new material can be licensed for clinical use, all potential materials are screened first in tissue culture systems. Current *in vitro* biocompatibility assays are based upon the exposure of cultured cells to test materials, either as a solid sample or as particles. For orthopedic implants, where mechanical wear of the implant (e.g., a hip or knee replacement) may generate particulate debris, both forms of the biomaterial are generally tested (2). The release of a variety of enzymes and chemical mediators may be affected by exposure to a biomaterial. Biochemical analysis of supernatant and cell fractions from tissue culture experiments, using techniques such as bioassays, radio-immunoassays, spectrophotometry and enzyme-linked immunoassays (ELISAs), facilitates the exact quantitation of mediators released from test cells. This article will focus on the use of the enzyme lactate dehydrogenase as a marker for cell toxicity in biocompatibility testing and will describe the use of a 96 well format LDH assay system, the CytoTox 96 Assay.

## Lactate dehydrogenase as a marker of cell damage

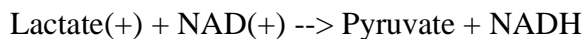
Lactate dehydrogenase (LDH) is a cytosolic enzyme present within all mammalian cells. The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid (3). *In vitro* release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability. As a result, the release of lactate dehydrogenase has proved to be a popular and reliable test for cytotoxicity in both immunological studies, where it has superseded the radioactive chromium release test as an assay for cellular cytotoxicity (4,5), and in biocompatibility studies, where it has now become an important *in vitro* screening test (6,7).

In order to assess the effects of a biomaterial on mammalian cell cultures, cells are exposed to varying concentrations of test material over a period of days. The release of LDH into culture supernatant correlates with the amount of cell death and membrane damage, providing an accurate measure of the cellular toxicity induced by the test substance.

## LDH assay systems

### Direct assays for LDH activity

Until recently, the direct spectrophotometric assay of Wroblewski and La Due (8) was the technique of choice for LDH measurements in our laboratory. This assay is based upon the ability of LDH to catalyze the reaction:

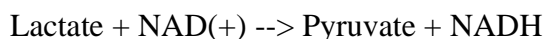


Changes in optical absorbance, measured at 340nm, reflect changes in the concentration of NADH and hence the level of LDH in the test sample. Enzyme activity can be expressed directly in terms of optical absorbance values or converted into international units (IU) by reference to enzyme standards. LDH levels are measured in both the culture supernatant and the cell lysate. The percentage LDH release is calculated by dividing the level of LDH in the medium by the total amount of LDH in the medium and cell lysate.

Although this spectrophotometric assay is easy and cost-effective, it can be extremely time-consuming if a large number of samples are to be tested.

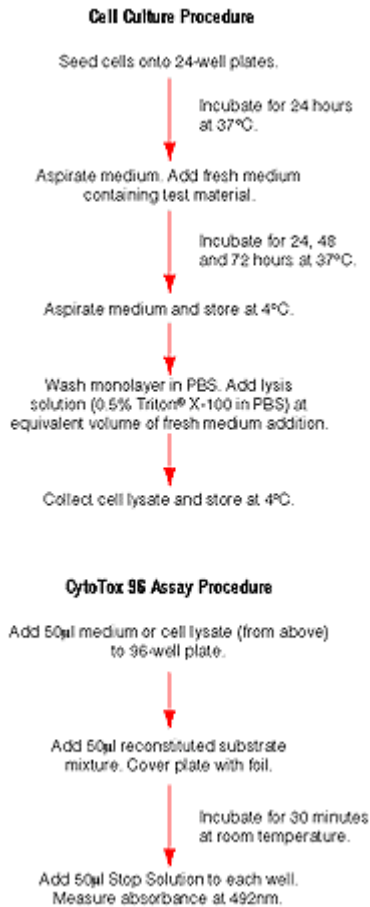
### Coupled assay for LDH activity

Promega's CytoTox 96 Assay is based upon a coupled enzymatic assay involving the conversion of a tetrazolium salt, 2-*p*-(iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT), into a formazan product. The reaction is catalyzed by LDH released from cells and diaphorase present in the assay substrate mixture. [Figure 1](#) summarizes the CytoTox 96 protocol for testing LDH levels in biocompatibility experiments; the chemical reactions are presented below:



In the CytoTox 96 Assay, formazan concentrations are determined by measuring optical absorbance at 492nm (A492) in a 96 well format. To measure the red formazan product accurately, adequate controls should be included to adjust values for the presence of phenol red in the tissue culture medium and for

any endogenous LDH activity arising from animal serum supplements to the tissue culture medium. We currently use phenol red-free medium and include appropriate "culture medium alone" controls in all experiments.



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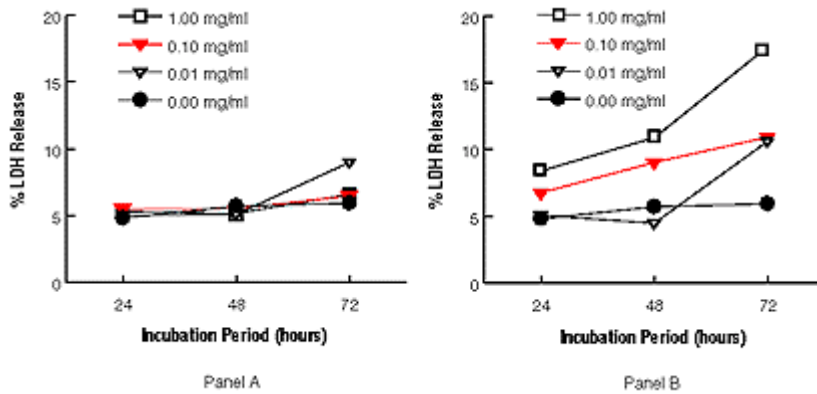
**Figure 1. Protocol for the determination of LDH release from cells exposed to biomaterials.**

## Calculation of results

Results from the CytoTox 96 Assay may be expressed either as optical absorbance values or as international units of enzyme (calculated from a standard curve obtained with the positive LDH control which is included in the system). For biocompatibility testing, we compare the A492 values from test cultures ("Test A492") with those from control cultures. A492 values for samples of both supernatant and cell lysate are determined. In order to correct for any endogenous LDH activity in animal serum, the A492 value of "medium alone" (medium incubated in the absence of cells) is subtracted from all other values. The percentage LDH release is given by:

$$\% \text{ LDH Release} = \frac{\text{Test A492}}{\text{Total A492}} = \frac{\text{Test A492}}{\text{Test A492} + \text{Cell Lysate A492}}$$

Typical results from the assay are shown in [Figure 2](#).

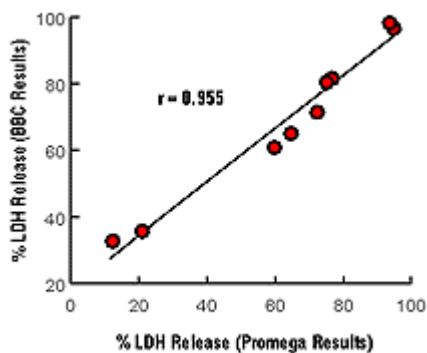


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**Figure 2. Typical results from an experiment to determine the effects of particulate materials on human fibroblasts.** Significantly less LDH is released from cells exposed to the relatively inert chromium (Panel A) than is released from cells exposed to the toxic silica (Panel B).

## Validation of the CytoTox 96 Assay

To validate the accuracy of the CytoTox 96 Assay, LDH levels in samples from a routine biocompatibility experiment were measured using both the CytoTox 96 Assay and the standard spectrophotometric system, consisting of a spectrophotometer linked to a BBC microcomputer. A comparison of the LDH results obtained from the two assays shows that there is excellent correlation between the two assay systems ([Figure 3](#)).



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**Figure 3. Comparison of LDH results from two assay systems.** LDH release from macrophages exposed to a toxic substance (silica) was measured by both the standard spectrophotometric assay (BBC System) and by the CytoTox 96 Assay. There is an excellent correlation between the two sets of data.

## Discussion

Lactate dehydrogenase has proved to be an extremely useful *in vitro* marker for cellular toxicity. LDH assays are of great value both in immunology for the quantification of cytotoxicity, and in the evaluation of biomaterial biocompatibility. We have compared two LDH assay systems: a standard one-stage spectrophotometric assay and the CytoTox 96 coupled-enzyme assay system. Although the two techniques produce similar results, the 96-well format and significantly faster sample throughput make

the CytoTox 96 Assay an ideal choice for routine use in any laboratory performing high-throughput biocompatibility testing.

## References

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## Ordering Information

Product	Size	Cat.#
CytoTox 96(TM) Non-Radioactive Cytotoxicity Assay	10 96 well plates	G1780

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