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MAX DISCOVERY™

Aspartate Transaminase (AST) Cytotoxicity Assay Kit Manual

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MaxDiscovery™ Aspartate Transaminase (AST) Cytotoxicity Assay Kit is intended for laboratory use only, unless otherwise indicated. This product is NOT for clinical diagnostic use. MaxDiscovery is a Trademark of Bioo Scientific Corporation (BIOO).

GENERAL INFORMATION

Product Description

The *MaxDiscovery™ Aspartate Transaminase (AST) Cytotoxicity Kit* is a plate-based colorimetric enzymatic assay for the determination of the aspartate transaminase enzyme in cultured cell lines. Aspartate transaminase (AST) also known as aspartate aminotransferase or (sGOT) is a metabolic enzyme expressed in a host of mammalian cell types. Upon cell damage or lysis the enzyme is released into the cell culture supernatant and is thus a useful tool for the investigation of cytotoxicity effects of experimental drug formulations and cell transfection.

The kit uses a spectrophotometric, kinetic assay to detect changes in aspartate transaminase levels directly from cultured cell media. The unique features of the kit are:

- High sensitivity
- A flexible, versatile assay format that can be modified to meet user needs
- High compatibility across a broad range of cell lines
- A robust enzyme-based assay which does not require expensive instrumentation
- High reproducibility
- Specially formulated BIOO Booster reagent which significantly improves assay signal

Procedure Overview

The *MaxDiscovery™ Aspartate Transaminase (AST) Cytotoxicity Kit* uses a coupled enzymatic reaction scheme: aspartate and α-ketoglutarate are first converted to glutamate and oxaloacetate which is converted by malate dehydrogenase to make malate and NAD^+. The conversion of the NADH chromophore to NAD^+ product, measured at 340 nm, is proportional to the level of AST enzyme in the sample. The absorbance of each well at 340 nm is measured using a plate reader. The concentration of AST in each sample is then directly determined from the change in absorbance at 340 nm over a 30 minute assay length. Dilutions of the AST Control, included in the kit, can be used to construct a standard curve to calibrate the assay and confirm assay linearity. This is described in more detail in Section, “Data Analysis.”

Although AST is present in a host of human tissues, its release varies among cell types. Therefore, the most useful analysis of cytotoxicity in a cultured cell line involves measuring cytotoxicity as a percentage of damaged or lysed cells. In this analysis, the AST level of the TREATED sample is compared to both an UNTREATED culture, and a LYSED control culture.

Example calculation for percent cytotoxicity:

Cytotoxicity (%) = [(TREATED sample – UNTREATED sample) / (LYSED sample – UNTREATED sample)] X 100.

Where TREATED sample, UNTREATED sample, and LYSED sample represent the AST activities of the treated, untreated and lysed samples.

Kit Contents, Storage and Shelf Life

The *MaxDiscovery™ Aspartate Transaminase (AST) Cytotoxicity Kit* has the capacity for 192 determinations or testing of 84 samples in duplicate (using 12 wells for standards). The kit also contains enough material to construct four standard curves. Store the kit at 4°C. The shelf life of the kit is 12 months, after receipt, when the kit is properly stored.

Kit Contents	Amount	Storage
Clear Microtiter Plate	2 x 96-well Plate (8 wells x 12 strips)	4ºC
Opaque Plate	2 x 96-well Plate (500 µL capacity)	4ºC
AST CytoMix Reagent	2 bottles	4ºC
BIOO Booster Reagent	1 tube	4ºC
AST Enzyme Control	2 tubes	4ºC
AST Dilution Buffer	7.5 mL	4ºC

Required Materials Not Provided With the Kit

- Microtiter plate reader (*340 nm*)
- Microcentrifuge tubes
- Deionized or distilled water
- Multichannel pipet or repeating pipettor (*Optional*)

Warnings and Precautions

BIOO strongly recommends that you read the following warnings and precautions to ensure your full awareness of the techniques and other details you should pay close attention to when running the assays. Periodically, optimizations and revisions are made to the kit and manual. Therefore, it is important to follow the protocol included with the kit. If you need further assistance, you may contact your local distributor or BIOO at techsupport2@biooscientific.com.

- Do not use the kit past the expiration date.
- Try to maintain a laboratory temperature of (20–25°C/68–77°F). Avoid running assays under or near air vents, as this may cause excessive cooling, heating and/or evaporation. Also, do not run assays in direct sunlight, as this may cause excessive heat and evaporation. Cold bench tops should be avoided by placing several layers of paper towel or some other insulation material under the assay plates during incubation.
- Make sure you are using only distilled deionized water since water quality is very important.
- When pipetting samples or reagents into an empty microtiter plate, place the pipette tips in the lower corner of the well, making contact with the plastic.

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SAMPLE PREPARATION

The versatility of the *MaxDiscovery™ Aspartate Transaminase (AST) Cytotoxicity Kit* allows users a great degree of freedom in choosing their cell culture parameters. Although BIOO provides a general cell culture outline that may prove useful for most cell lines, it is understood that many users cannot modify their experiments to fit a specific cytotoxicity assay. Therefore, BIOO has provided a means whereby users can modify the parameters of the AST cytotoxicity assay to fit their experiments. For users who do not have rigid cell culture parameters, a general cell culture preparation guide is provided below. For those who cannot alter their cell culture parameters, an AST Optimization Guide is provided in the Assay Protocol section.

Cell Culture Preparation

The suggested seeding concentration for most adherent cell lines is between 5.0 x 10^4 and 2.5 x 10^5 cells per mL. We suggest that users perform each experimental condition in either duplicate or triplicate.

1. Seed cells in either a 96-well plate in 100 µL of culture medium, or in a 24-well plate in 400 µL of culture medium.
2. Culture the cells in a CO_2 incubator for at least 16 hours, and then replace the cell culture media. Allow cells to grow to at or near confluence.
3. Add test substances to wells designated “TREATED”, and incubate for 2 – 24 hours.
4. Upon completion of desired experimental incubation, for 96-well plate samples add 5 µL of BIOO Booster reagent to the wells designated “LYSED” and pipet up and down. Add 5 µL of dH_2O to all other wells. For 24-well plate samples use 20 µL of either BIOO Booster reagent or dH_2O.
5. After a 20 minute incubation, pipet the “LYSED” wells up and down, and transfer cell culture supernatants from all wells to the provided opaque 96-well plate. Discard the cell culture plate.
6. For 96-well plate samples add 5 µL of BIOO Booster reagent to each well. For 24-well plate samples add 20 µL. Gently triturate (pipet up and down) each sample 4 – 5 times to mix.

Note: Rows B – H can be used for other test substances and/or extra dosing concentrations.

Typical Cell Culture Plate Layout (96-well plate)						
	1, 2	3, 4	5, 6	7, 8	9, 10	11, 12
ROW A	UNTREATED culture	LYSED culture	TREATED culture (dose 1)	TREATED culture (dose 2)	TREATED culture (dose 3)	TREATED culture (dose 4)

ASPARTATE TRANSAMINASE (AST) DETECTION PROTOCOL

Reagent Preparation

IMPORTANT: Make sure you read "Warnings and Precautions" section on page 2. ALL REAGENTS AND THE MICROTITER PLATE SHOULD BE BROUGHT UP TO ROOM TEMPERATURE BEFORE USE (30 MIN AT 20–25°C/68–77°F).

Preparation of AST CytoMix Reagent

To reconstitute the AST CytoMix Reagent, add exactly 30 mL of deionized or distilled water to the Reagent Mix powder. Mix by swirling or inverting the bottle 10 times. Allow contents to dissolve for 10 minutes at room temperature.

IMPORTANT: The reconstituted AST CytoMix Reagent can be left at room temperature for short periods (30 min) prior to use. Between uses, the reconstituted Reagent Mix should be stored at 4 °C (for up to 4 months).

Preparation of AST Control Dilutions for Standard Curve (***Optional***)

1. Add 190 µL AST Dilution Buffer to the AST Enzyme Control tube. Invert tube two times to dissolve any contents at the top of tube. Vortex or tap briefly to ensure mixing.

   NOTE: There is enough material to construct 2 Standard Curves. Use a fresh tube of AST Enzyme Control for each Standard Curve. Discard any remaining diluted AST Enzyme Control after using it to make the dilutions, in Step 2, for the Standard Curve.

2. Label six microfuge tubes: 1, 2, 3, 4, 5, 6 (Neg). Then make 6 serial dilutions of the AST Enzyme Control (3 concentration increments per log) using the AST Dilution Buffer as described in the table below.

   NOTE: Make the AST Enzyme Control Dilutions for the Standard Curve fresh each time that the Standard Curve is performed.

Standard Tube #	Preparation	Relative Dilution*
1	Add 5 µL diluted AST Enzyme Control to 1195 µL AST Dilution Buffer. Mix.	1
2	Add 100 µL of Standard Tube #1 +115 µL of AST Dilution Buffer. Mix.	2.15
3	Add 100 µL of Standard Tube #2 +115 µL of AST Dilution Buffer. Mix.	4.63
4	Add 100 µL of Standard Tube #3 +115 µL of AST Dilution Buffer. Mix.	10
5	Add 100 µL of Standard Tube #4 +115 µL of AST Dilution Buffer. Mix.	21.5
6 (Neg)	Add 150 µL of AST Dilution Buffer.	NA

**Only needed for the generation of the Standard Curve.*

Assay Optimization Guide

This guide provides users with a general outline to maximize the versatility of the *MaxDiscovery™ Aspartate Transaminase (AST) Cytotoxicity Kit*. Since AST expression differs across cell types, it is critical to calibrate the assay parameters so that the change of absorbance of the LYSED control sample remains linear across the length of the assay. This allows the LYSED control well to provide an accurate measure of total AST concentration in a cell culture well.

1. Add 20 µL of the LYSED positive control sample to a microplate well
2. Add 280 µL of Reagent Mix to the well
3. Immediately measure the absorbance of each sample at 340 nm (= initial reading). After exactly 30 min, measure the absorbance again (= final reading)

If the absorbance change between the "final reading" and the "initial reading" is greater than 0.8, decrease the sample size used in the assay to 10 µL. If the absorbance change remains above 0.8, continually shorten the assay length to 15 min, 10 min, and 5 min, until the absorbance change is below 0.8.

If the absorbance change between the "final reading" and the "initial reading" is less than 0.1, increase the sample size used in the assay to 50 µL. If the absorbance change remains below 0.1, increase the assay length to 60 minutes. If by using the 60 min assay length, the change of absorbance remains below 0.1, consider altering your cell culture conditions to increase cell density.

Note: In these cases, the parameter changes determined using this optimization procedure should then replace all parameters in the Assay Protocol below.

Assay Protocol

All optimizations decided upon in the Assay Optimization Guide should replace parameters listed below.

1. Add 20 µL of each sample or standard (in duplicate) to the microplate wells.
2. Add 280 µL of AST CytoMix reagent to the well. (☝ Using a multichannel pipet or repeating pipettor is recommended).
3. Immediately measure the absorbance of each sample at 340 nm (= initial reading). After exactly 30 min, measure the absorbance again (= final reading).

DATA ANALYSIS

Cytotoxicity Analysis (%)

Use the formula below to calculate percent cytotoxicity of a treated cell culture supernatant.

1. Calculate the absorbance change for each sample by subtracting the "final reading" from the "initial reading".
2. Calculate the average absorbance change for each condition run in duplicate, including UNTREATED, LYSED, and TREATED conditions.
3. Substitute the values into the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{TREATED sample} - \text{UNTREATED control}}{\text{LYSED control} - \text{UNTREATED control}} \times 100$$

Where TREATED sample, UNTREATED control, and LYSED control represent the average absorbance change of their respective samples.

Standard Curve Construction *(Optional)*

A calibration curve to confirm assay linearity can be constructed using the AST Enzyme Control Dilutions calibration standards as described below:

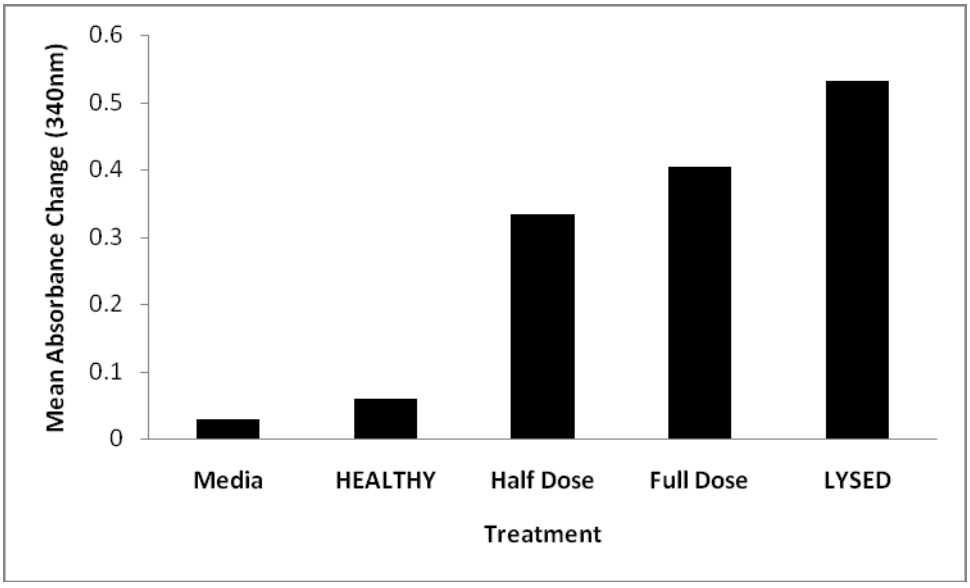
1. For each calibration point, calculate the *average absorbance change.* To do this, subtract the average final absorbance value of each point from its corresponding average initial absorbance value.
2. For each standard, plot the average change in absorbance along the y-axis (from lowest in value to highest in value) and the inverse value of the relative dilution number* (i.e. 0.047, 0.1, 0.22, 0.47 and 1) on the x-axis. For Tube #6 (Neg) use "0".

*Relative dilution numbers can be found in the table on page 4.

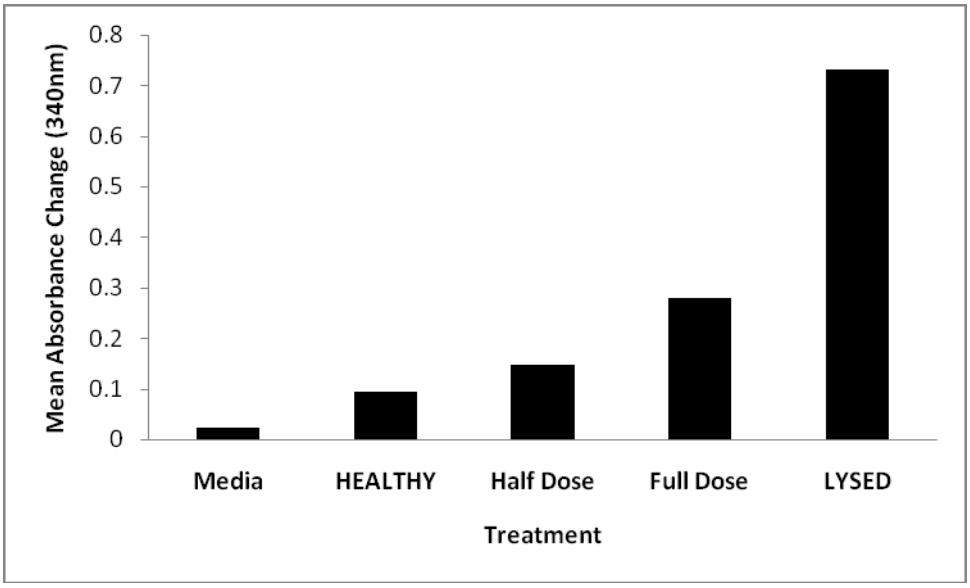
APPENDIX: Results of AST Cytotoxicity Assay Kit

A. Cells were seeded in a 24-well plate at a concentration of 2.5 X 10^5 / mL, and allowed to grow to confluence over 48 hours. Cell culture media was replaced and Cytotoxic Transfection Agent X was added in two increasing doses to the wells. Cells were further incubated 16 hours before harvesting cell culture supernatant.

1. HeLa cells

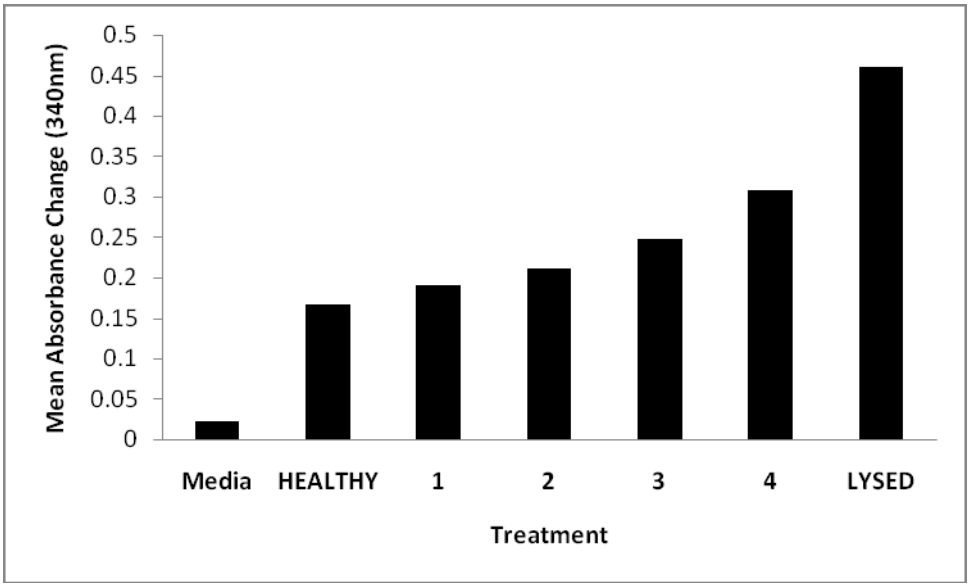


2. A549 cells



B. Cells were seeded in a 96-well plate at a concentration of 10^5 cells / mL, and allowed to grow over 24 hours. Cell culture media was replaced and Cytotoxic Transfection Agent X was added in four increasing doses to the wells. Cells were further incubated 16 hours before harvesting cell culture supernatant.

1. HepG2 Cells



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