PO Box 1145, Station CSC, London ON N6A 5K2 Canada
Tel: +1 519 434 5057 Fax: +1 519 434 2639

IDTox™ Aspartate Transaminase (AST) Enzyme Cytotoxicity Kit For Cell Culture Supernatant Samples

SUP6008 192 Wells

Enzyme Immunoassay for the determination of the Aspartate Transaminase enzyme in cell culture supernatant samples.

PRODUCT DESCRIPTION

The ID Labs Aspartate Transaminase (AST) Enzyme 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 serum samples. The 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 IDEnhancer reagent which significantly improves assay signal

PROCEDURE OVERVIEW

The IDTox™ Aspartate Transaminase (AST) Enzyme Toxicity 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 within 5 minutes time. 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.

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 REAGENTS SUPPLIED

The Aspartate Transaminase (AST) Enzyme 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. The shelf life of the kit is noted on the label, when the kit is properly stored, at 2-8°C

Kit Contents	Amount	Storage
Clear Microtitre Plate	2 x 96-well Plate (8 wells x 12 strips)	2-8°C

For *in vitro research use*. CAUTION: Not for human or animal therapeutic use.
Uses other than the labeled intended use may be a violation of local law.

Opaque Microtiter Plate	2 x 96-well Plate (500 µl capacity)	2-8°C
AST Reagent Mix	2 bottles	2-8°C
AST Enzyme Control	2 tubes	2-8°C
AST Dilution Buffer	7.5 ml	2-8°C
IDEnhancer Reagent	1 tube	2-8°C

MATERIALS REQUIRED BUT NOT PROVIDED

Microtiter plate reader (340 nm).
Deionized or distilled water.
1.5 ml microfuge tubes.
Multichannel pipette or repeating pipettor (*recommended but not required*).

WARNINGS AND PRECAUTIONS

It is strongly recommended 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 coming with the kit.

Add standards to plate only in the order from low concentration to high concentration, as this will minimize the risk of compromising the standard curve.

The flexibility of this kit allows users a great degree of freedom in choosing their cell culture parameters. In general, a cell culture outline may prove useful for most cell lines, however it is understood that many users cannot modify their experiments to fit a specific cytotoxicity assay. Users can modify the parameters of the IDTox™ 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, some AST Optimization Tips are provided in the Assay Procedure section (below).

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ASSAY PROCEDURE

PREPARATION OF REAGENTS

Cell Culture Preparation

The suggested seeding concentration for most adherent cell lines is between 5.0×10^4 and 2.5×10^5 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 IDEnhancer 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 IDEnhancer 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 IDEnhancer 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.

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)

ASSAY PROCEDURE

Set up

Make sure you read "Warnings and Precautions" section above. All reagents and the microtiter plate should be brought up to room temperature before use (30 minutes - 1 hour at 20–25°C/68–77°F).

Preparation of Reagent Mix

Preparation of AST Reagent Mix

To reconstitute the AST Reagent Mix, 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.

The reconstituted AST Reagent Mix can be left at room temperature for short periods (30 minutes) prior to use. Between uses, the reconstituted Reagent Mix should be stored at 2-8°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 a few 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 Tips

Because 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 minutes, 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 minutes, 10 minutes, and 5 minutes, 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 minute 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 Tips Section above 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 Reagent Mix 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 minutes, measure the absorbance again (= final reading).

CALCULATION OF RESULTS

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)

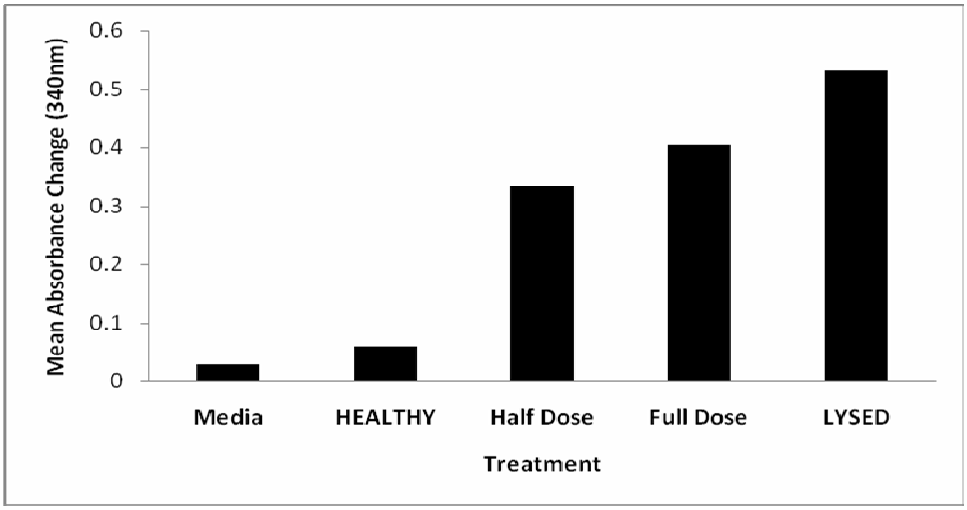
A calibration curve to confirm assay linearity can be constructed using the AST Enzyme Control Dilutions calibration standards as described below:

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 above.

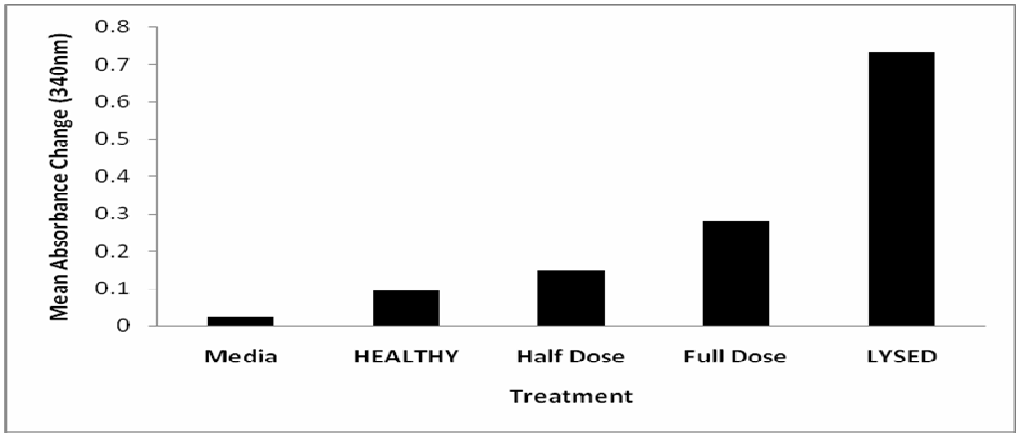
APPENDIX: Results of AST Assay Kit

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