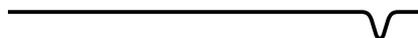# Assessing AST released in a cell culture model of liver toxicity using AlphaLISA.

Author

Jeanine Hinterneder, Ph.D.

Revvity, Inc.

Introduction

Aspartate transaminase (AST) or aspartate aminotransferase, also known as AspAT/ASAT/AAT or serum glutamic oxaloacetic transaminase (SGOT), is an important enzyme in amino acid metabolism. AST catalyzes the reversible transfer of an α-amino group between aspartate and glutamate. The enzyme is found in metabolically active organs and tissues, such as the liver, heart, skeletal muscle, kidneys, brain, and red blood cells. Inflamed or injured liver cells leak higher than normal amounts of AST, and other enzymes, resulting in elevated levels found in the bloodstream. A blood test for AST is commonly used as one measure in the detection of liver damage.[1]

AlphaLISA™ technology allows for the detection of molecules of interest in a homogeneous, no-wash format. In the AlphaLISA assay illustrated in Figure 1A, a biotinylated anti-AST antibody binds to streptavidin-coated Alpha Donor beads while another anti-AST antibody is conjugated directly to AlphaLISA Acceptor beads. Both antibodies bind to human AST and bring the Donor and Acceptor beads into close proximity. Excitation of the Donor beads with light at 680 nm provokes the release of singlet oxygen molecules to activate the Acceptor beads so that they emit a sharp peak of light at 615 nm. This light emission is proportional to the concentration of AST protein in the sample.

Assessing AST released in a cell culture model of liver toxicity using AlphaLISA.

Liver toxicity is a serious concern when developing any new drugs and should be measured early on in the drug development pipeline. Human hepatoma cell lines such as HepG2 cells are widely used for *in vitro* studies of liver function, metabolism, and drug toxicity.[2] We demonstrate here the utility and benefits of using AlphaLISA assays for identifying and quantifying levels of AST protein present in cellular lysate and released into the supernatant from a human hepatoma cell line. We induced toxicity by titrating cell cultures with toxic compounds,

and demonstrate how sensitive and simple it is to rapidly assay for changes in AST expression in both supernatant and cellular lysate from HepG2 cell cultures. The data illustrate the benefits of using AlphaLISA assays as a fast, powerful, homogeneous platform for screening modulators of AST expression and release in cultured human cells. The technology has a number of distinct advantages including high signal to background, wide dynamic range, and an extremely simple, straight-forward, no-wash protocol.
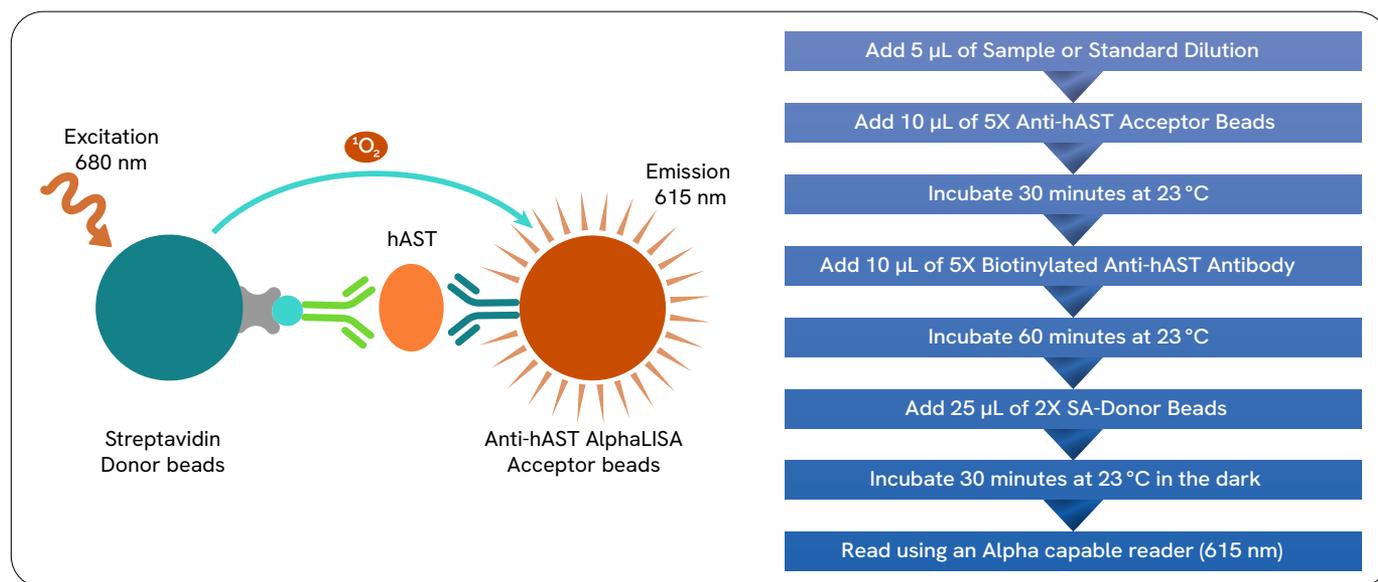

Figure 1: A) Human Aspartate Transaminase (hAST) AlphaLISA detection assay schematic. B) AlphaLISA AST Assay detection workflow.

Reagents

- HepG2 cell line (Hep G2; ATCC® HB-8065™)
- Culture media: E-MEM (ATCC #30-2003) + 10% FBS (ThermoFisher #11875-093)
- Doxorubicin hydrochloride (TOCRIS #2252)
- DMSO (Sigma #D2650)
- Cisplatin (Sigma #479306)
- Hoechst 33342 (Life Technologies #H3570)
- CellTox™ Green Cytotoxicity Assay (Promega #G8742)
- AlphaLISA human AST detection kit (Revvity #AL3078)

Protocol

HepG2 cells were seeded (100 µL/well) into 96-well black ViewPlates™ (Revvity #6005225) and grown for 3 - 5 days in culture media before treatment for 1 or 2 days with toxic compounds. For toxicity analysis, cultures were treated for two days with a titration of toxic compound diluted in culture media (complete media replacement). For AST detection assays, culture media (supernatants) was collected and, in some wells, cells were then lysed with 100 µL of Immunoassay Buffer (provided in the kit; Revvity #AL000) for 15 - 30 minutes on a rotational shaker (DELFIA™ PlateShaker set at ~600 - 700 RPM). Supernatants and lysates were either used immediately or transferred to polypropylene StorPlates (Revvity, #6008290) and frozen at -20 °C to be thawed within one week for measurement with AlphaLISA human AST detection assays (#AL3078).

AlphaLISA assays were performed as indicated in the kit manual in 384-well white OptiPlates™ (Revvity #6007299) and the assay workflow is described in Figure 1B, where only a 5 µL sample is needed per well and with a total incubation time of only two hours.

As a secondary measure of cell death, after supernatants were collected, some wells were labeled with the Hoechst 33342 and CellTox Green dyes by diluting in media (1:1000 final dilution) and adding to wells. CellTox Green dye measures changes in membrane integrity by binding to the DNA of compromised cells. Upon binding DNA, the dye's fluorescence increases and toxicity is measured as a function of the green fluorescence intensity in the nuclei.

Data collection and analysis

The AlphaLISA assays were measured using either a Revvity EnVision™ multilabel plate reader or an EnSight™ multimode plate reader using default values for standard Alpha detection. Standard curves for the AlphaLISA AST assay were performed in sample diluents (kit buffer or culture media) using recombinant standards provided in each kit. Curves were plotted in GraphPad Prism according to a nonlinear regression using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) and $1/Y^2$ data weighting. The lower detection limit (LDL) of the assay was calculated by taking three times the standard deviation of the average background values and interpolating concentration from the standard curve. Interpolated concentrations represent the amount of protein in 5 µL of sample. Overall amounts of AST protein levels were calculated by multiplying by the volume of supernatant or lysate in each well.

Automated cell imaging and analysis of Hoechst and CellTox Green labeled cells were done using the cell imaging module and Kaleido™ software on the EnSight multimode plate reader. Images of each well were acquired using 385 nm and 465 nm LED excitation. Cell nuclei were identified from the Hoechst stain and CellTox Green intensity was measured from the nuclear region of each cell automatically using the Object Counting and Object Intensity Analysis functions.[3]

AlphaLISA human AST detection in HepG2 cells

Aspartate transaminase (AST) has a rather ubiquitous expression profile and expression levels are known to be high in hepatocellular models. To determine if AST is expressed and released at detectable levels in HepG2 cultures, cells were plated in 96-well microplates at six different densities ranging from 32,000 cells titrated down to 1,000 cells and grown for four days before media was replaced entirely and the toxic compound doxorubicin was added to half the wells. After 24 hours, the supernatant was collected from these wells and assayed for AST concentration. The data, presented in Figure 2, show that HepG2 cells release significant levels of AST protein that are detectable with the AlphaLISA assay. Additionally, increasing cell numbers potentiate the AST concentration and treatment with a toxic compound enhances the release of AST into culture media. A clear effect of doxorubicin treatment on AST release is apparent in wells seeded with as few as 2,000 cells. At the two highest concentrations seeded, the cultures were completely confluent (visual observation) and this is illustrated in the data as a leveling off of AST. 25,000 cells per well were chosen for the subsequent experiment.

AST concentrations as a measure of cellular toxicity

The next step was to show that AlphaLISA measurement of AST is a good measure of cellular toxicity. To do this, HepG2 cells were plated at 25,000 cells per well and cultured for three days before media was replaced with media containing 10-point titrations of two test compounds known for their toxic effects at higher concentrations. After a two day treatment, cell supernatants were collected and half the cells were lysed. Supernatants and lysates were frozen and assayed the next day using AlphaLISA and data are shown in Figure 3. The data from this experiment illustrate how increasing concentrations of toxicity-causing compounds result in cellular release of AST into the supernatant. As AST concentration in supernatant increased, the amount of AST left inside the cells decreased, as shown by the opposite profile observed in cell lysate samples.
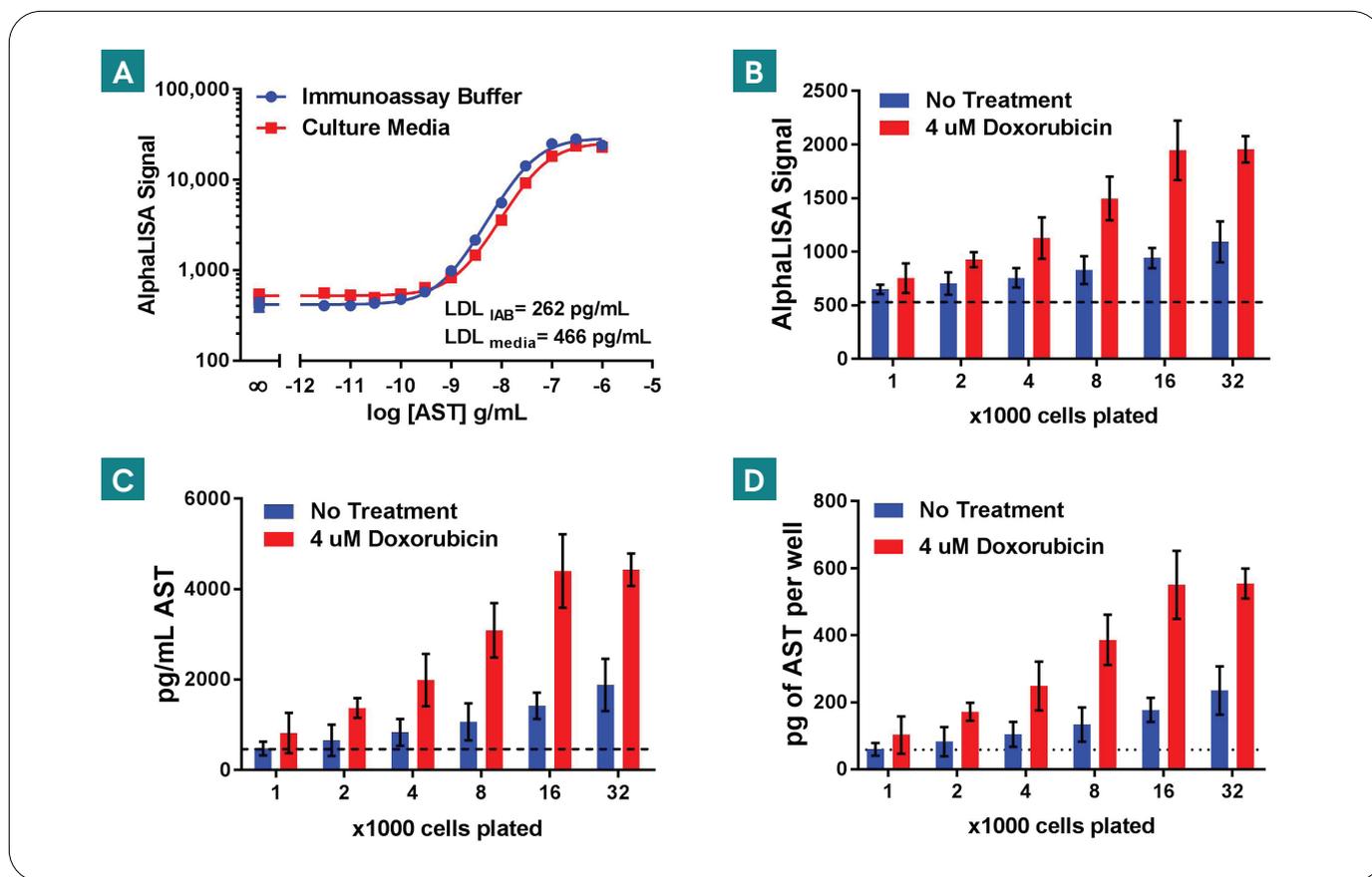
Figure 2: AlphaLISA assay measures AST amounts in HepG2 supernatant. AST levels increase with cell number and treatment with a toxic compound. A) Standard curves generated from a 12-point half-log titration of human AST were run in both assay buffer and culture media. The lower limits of detection (LDLs) are indicated. (B) Cells were seeded at six different densities, cultured four days, and media was replaced with or without 4 µM doxorubicin. The next day, supernatants were collected and assessed for AST concentration with AlphaLISA. AST concentrations in pg/mL (C) are interpolated using the standard curve and converted to overall amount(pg) of AST per well (D) by multiplying by volume. Dotted lines indicate the LDL in media. All data points are an average of three wells.
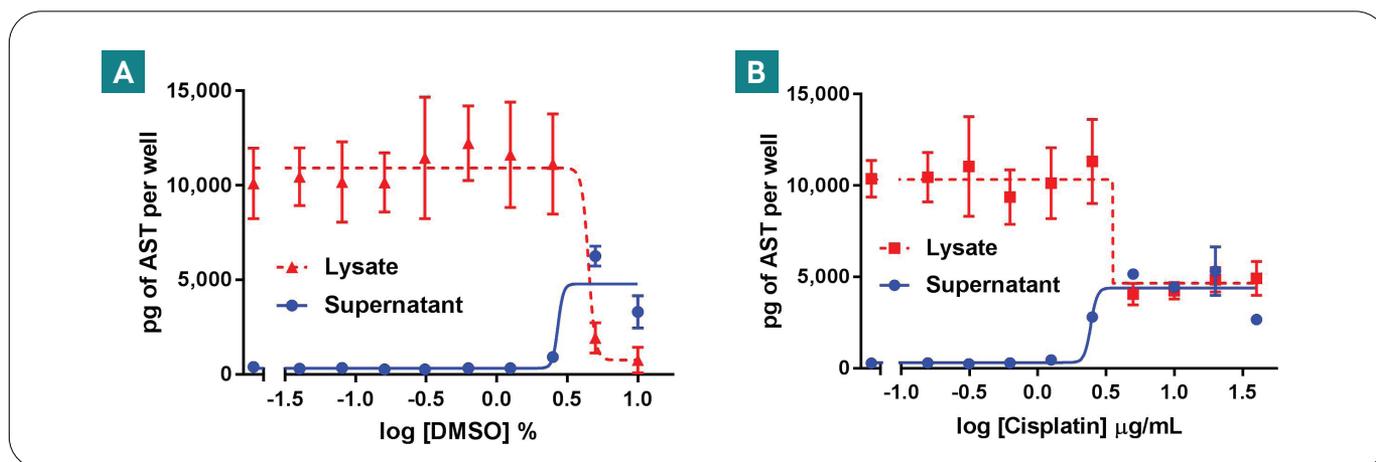

Figure 3: Treatment with two toxic compounds modulate AST concentrations in HepG2 supernatant and cell lysate in opposing directions. HepG2 cells were plated at 25,000 cells per well and cultured for three days before media was replaced with media containing 10-point titrations of DMSO (A) and Cisplatin (B). After a two day treatment, cell supernatants were collected, half the cells were lysed and the samples were assayed for AST concentrations with AlphaLISA. Data points were an average of six wells for supernatants and three wells for lysates. Toxicity curves were generated using a four-parameter (variable slope) dose-response curve fit.

To determine if the changes in AST concentrations are consistent with a measure of cell death, an independent measure of cellular toxicity was assessed using a dye that fluorescently labels dead and dying cells. As cells die, the CellTox Green dye passes through compromised membranes and binds to DNA causing the fluorescent signal to increase. AST toxicity profiles of both compounds measured with the fluorescence-based toxicity assay were consistent with concentrations of AST measured in cellular supernatant using the AlphaLISA technology.
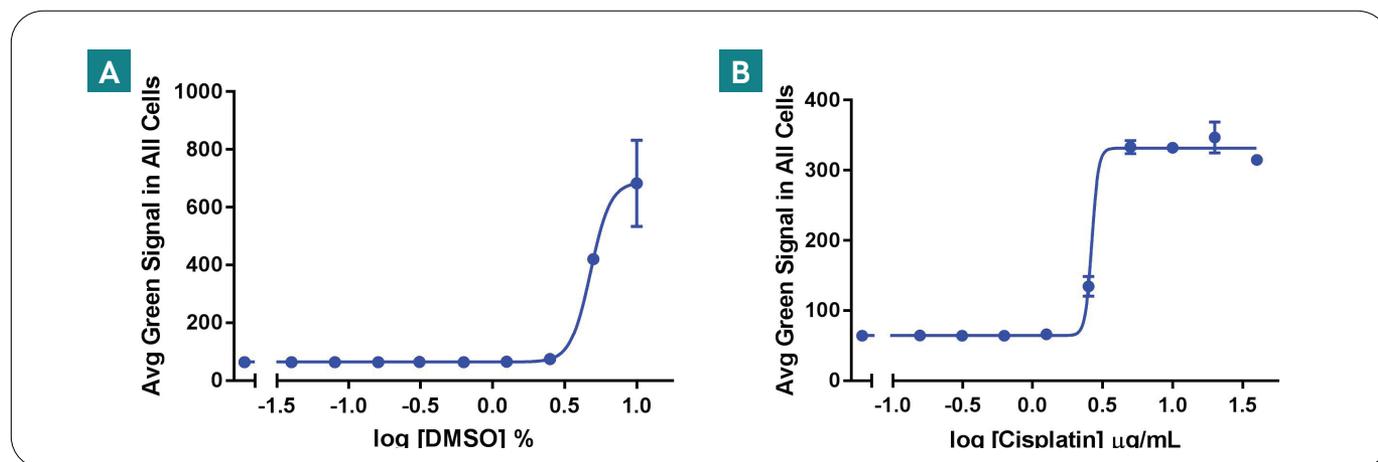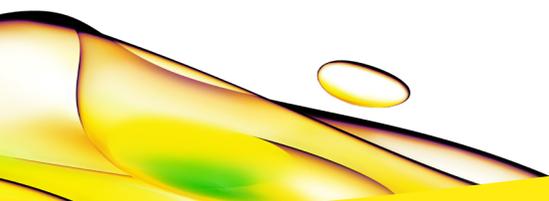

Figure 4: Toxic profiles of two compounds measured using cell imaging and analyzed based on CellTox Green fluorescence. HepG2 cells cultured for three days, then treated for two days with titrations of toxic compounds, were labeled with nuclear dye Hoechst and CellTox Green dye to label all cells and dead cells (respectively). Images were captured and fluorescence of nuclei analyzed automatically with the EnSight multimode plate reader and graphed to show the resulting toxicity profile for DMSO (A) and Cisplatin (B). Data shown are an average of three wells.

References

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