Ziva™ Tox Ultrasensitive Cytotoxicity Assay

Ultrasensitive chemiluminescent immunoassay for the assessment of cell cytotoxicity caused by a wide variety of cytotoxic agents and/or processes, including chemical agents, antibody directed cell cytotoxicity (ADCC), complement lysis, and cytotoxic cells such as cell-mediated cytotoxicity caused by cytotoxic T-lymphocytes (CTL) , natural killer (NK), lymphokine activated killer (LAK) cells, macrophages, or any other effector cells or materials displaying cytotoxic properties.

Cat. No. CM011, 100 Test Kit, Ziva-Tox Ultrasensitive Cytotoxicity Assay

Cat. No. CM012, 500 Test Kit, Ziva-Tox Ultrasensitive Cytotoxicity Assay

Store Kits at +2 to +8°C **For Research Use Only**

Intended Use

The Ziva™ Tox Ultrasensitive Cytotoxicity Assay (Ziva-Tox) is intended for use in the ultrasensitive detection of cytotoxic effects in mammalian cells and cell lines in culture (adherent and suspension cells) in an easy to use and rapid assay format. Ziva-Tox measures the toxic effect of the cytotoxic agent upon cellular DNA synthesis of the target cell. Ziva-Tox is intended for research use only and is not intended for use in medical diagnostic procedures.

Introduction

Cytotoxicity assays are used in a variety of important medical research and industrial applications. In its simplest form, cytotoxicity refers to the ability of an agent (cellular, non-cellular) to cause a toxic effect (e.g., death or damage) to a target cell. Methods used to assess cell death or cytotoxicity have generally focused on three main biological aspects: 1) Uptake of dyes such as trypan blue. Cells that are dead are unable to perform the active transport of exogenous dyes; 2) Release of artificial labels (e.g., ^{51}Cr, ^{111}In). 3) Release of endogenous molecules (e.g., LDH, GAPDH, DNA fragments). Each of these methods depends on perturbations of the cell membrane or disintegration of the treated cell as the result of cytotoxic effects. The kinetics of the events that lead to cell membrane disruption are dependent upon the cellular pathway of cell death. Thus, the user should be aware that the mechanism of cell death (e.g., apoptosis versus necrosis) as the cellular pathway will dictate whether the readout reflects early or late events in cell death.

Historically, the gold standard for measuring cell cytotoxicity has been the ^{51}Cr-release method that detects the release of ^{51}Cr from the cytoplasm of pre-labeled target cells after exposure to effector cells or other cytotoxic agents. This method relies on the agent's or effector cell's ability to initiate cell death, either by permeabilization

of the target cell membrane or activating a biochemical pathway that results in cell death and disintegration. Cytotoxic T lymphocytes (CTL), natural killer (NK) cells, and lymphokine-activated killer (LAK) cells are all common examples of effector cells in cell-mediated cytotoxic models. However, "leaky" cells contribute to high spontaneous release of ^{51}Cr from control cells, which diminish the signal-to-noise ratio. Other confounding factors include target cell damage caused by the radioisotope. This damage increases the assay's background noise and limits the experiment to the observation of cytotoxic events that must take place within 2-6 hours.

Current use of non-radioactive assay kits has reflected a trend in biomedical laboratories to avoid radioisotopes. Non-isotopic cytotoxicity assays include the LDH, G6PDH, or Adenylate Kinase (AK) enzyme release detection assays, and the ATP release detection assay, which monitor the release of cellular proteins or ATP from dying cells upon cell membrane disruption. While these methods have been useful in the past, they may severely underestimate the appearance of the cytotoxicity, because significant and relevant cell toxicity often occurs before cellular membrane permeabilization. That is, to cause cell membrane permeability, crucial intracellular pathways must be committed first, and then permeablization of the membrane occurs over time.

When studying cell-mediated or non-cell mediated (e.g., chemical, ADCC) cytotoxic effects, these types of enzyme or ATP based assays also often suffer from large non-specific contributions from effector cells and non-treated control target cells (i.e., the spontaneous release of the endogenous protein or ATP from the effector cells, and the target cells). This non-specific signal results in a relatively low dynamic range for the assay and causes these types of assays to be generally less sensitive in detecting the appearance of cytotoxic effects associated with exposure of the cytotoxic agent to the target cells. In the case of LDH, it is known that serum-derived LDH adds to the background and the LDH test may have to be performed at a lower serum concentration than is optimal for the cells in the assay. In addition, the quality and viability of the assayed cells must be very high in order to reduce the spontaneous release of the LDH, G6PDH, ADH enzyme or ATP from the control target cells as much as possible.

Other non-isoptopic cytotoxicity assays measure cell proliferation and death indirectly by detecting and quantitating the increase or decrease of the cellular metabolic activity after exposure to cytotoxic agents. These types of assays include ones such as MTT, XTT or WST-1 colorimetric assays that detect formazan products from mitochondrial metabolism. A major shortcoming to these indirect methods has been high background signals that are due to the spontaneous release of metabolic markers from all viable cells. This non-specific signal results in a relatively low dynamic range for the assay and causes the assay to be significantly less sensitive in detecting the appearance of cytotoxic agent-induced cytotoxic effects associated with the treated target cells. Further, non-mitochondrial reductase enzymes which metabolize MTT have been reported to be present in non-mitochondrial sub-cellular components. In addition, these assays are dependent on metabolic processes that may not detect early events of cell death. It is also critical for the background signal to be as low as possible in order to detect the effect of cytotoxicity on target cells. Again the quality and viability of the assayed cells must be very high in order to minimize the spontaneous assay signal from the target cells as much as possible.

The assays discussed above are not optimally suitable for detecting the death of small numbers of target cells in a population of many cells because these assays indirectly measure the activity of all viable cells present.

In contrast, the Ziva-Tox assay directly measures cytotoxicity based on the measurement of a) the extent of BrdU label incorporation into cytotoxic agent treated target cells during or after cytotoxic agent treatment, relative to the extent of DNA synthesis in non-treated control target cells (i.e., measures the extent of cellular DNA synthesis

inhibition caused by the treatment with the cytotoxic agent) or b) the extent of loss of BrdU labeled target cell DNA from cytotoxic agent treated target cells (i.e., target cells which are pre-labeled with BrdU and then treated with the cytotoxic agent), relative to the extent of loss of pre-labeled BrdU DNA from the nontreated target cell control.

The Ziva-Tox assay has the following important advantages.

1) **SIGNIFICANTLY INCREASED DYNAMIC RANGE AND SENSITIVITY:** The Ziva-Tox assay does not have the problem of high spontaneous release of non-specific signal because it directly detects BrdU incorporation into the DNA of surviving cells. Precise biological effects can be measured by comparing the signal of treated target cells to the control wells containing untreated target cells. This results in a much higher dynamic range for the Ziva-Tox assay and causes the assay to be significantly more sensitive in detecting the appearance of agent-induced cytotoxic effects associated with the treated target cells.

2) **MORE FLEXIBILITY IN THE QUALITY OF THE TARGET CELLS:** The quality of the target cells have much less of an effect on the assay results and the cell quality can vary greatly because there is no "spontaneous release" or its equivalent associated with in the treated, and non-treated control, target cells. In addition the serum concentration has little effect on the ability to detect cytotoxic effects associated with the treated target cells.

3) **GREAT FLEXIBILITY IN TIME OF CYTOTOXIC AGENT EXPOSURE TO TARGET CELLS:** Cytotoxic agent plus target cell sample exposure times using the Ziva-Tox assay method are very flexible and the cells can be exposed to the cytotoxic agent for 2-4 hours, or days,, if necessary. This flexibility allows the operator to more fully interrogate the biological response of the target cell to cell-mediated toxicity or potentially cytotoxic agents. Longer incubations, in effect, serve to amplify the interaction between target cells and the cytotoxic cells or reagents. In addition longer exposure times may more closely mimic natural conditions in biological systems.

4) **GREATER FLEXIBILITY IN TIMING AND LENGTH OF BrdU LABELING OF TARGET CELLS:** The Ziva-Tox assay BrdU labeling method is also very flexible. The labeling of the control and target cells can be performed simultaneously with the cytotoxic agent exposure, or in the final 2-24 hours of the exposure to the cytotoxic agent. Precise biological effects can be measured by comparing the Ziva-Tox assay signals of treated target cells to the control wells containing untreated target cells.

5) **GREATER SAMPLE SIZE FLEXIBILITY:** With Ziva-Tox it is possible to interrogate very small numbers of cells (< 1,000 cells per well). Biological effects have been detected using between 500 to 40,000 cells per sample.

6) **EASY AND RAPID ASSAY PROTOCOL**: After the cytotoxic agent treatment and BrdU labeling period, which is dependent on the experimental system, the actual Ziva-Tox assay procedure time can be performed in less than 1 hour.

Principle of the Ziva-Tox Assay

Ziva-Tox is an ELISA-based ultrasensitive cell toxicity assay that uses a chemiluminescent substrate to detect the presence of BrdU incorporated in actively proliferating cells. The Ziva-Tox assay determines a Percent Cytotoxic Effect (see Calculation section below) for the treated target cells, by determining the extent of cellular BrdU DNA synthesis inhibition caused by the treatment of the target cells with the cytotoxic agent. This is performed by measuring the extent of BrdU label incorporated into cytotoxic agent treated target cells during or after cytotoxic agent treatment, relative to the extent of BrdU DNA synthesis in non-treated control target cells. BrdU DNA synthesis is very sensitive to perturbations to a large number of healthy cell functions, revealing if cell DNA synthesis is inhibited or impaired in cells undergoing damage or death from a cytotoxic agent. Therefore, the magnitude of the difference in the amount of BrdU cell DNA detected in cytotoxic agent treated cells relative to the amount of BrdU cell DNA detected in untreated control cells is a measure of the magnitude of the cytotoxic effect on the treated cells. By comparing the signal of treated cells with untreated cells, one can derive a measure of the percent of cell cytotoxicity.

Kit Contents

Abbreviations: µL = microliter: mL = milliliter

Kit Contents, Cat #CM-011, 100 Tests, Ziva-Tox Ultrasensitive Cytotoxicity Assay		
Cat. #	**Amount**	**Reagent Description**
AB0102	1 vial x 50 µL	**Anti-BrdU Antibody- Alkaline Phosphatase Conjugate**
SL0222	1 vial x 200µL	**BrdU Labeling Solution**
SL0232	1 bottle x 5 mL	**Antibody Conjugate Diluent**
SL0342	1 bottle x 5 mL	**Fix Solution**
SL0202	1 bottle x 20 mL	**Stringency Solution**
SL0112	1 bottle x 70 mL	**Preparation Solution, 3X**
BU0072	1 bottles x 160 mL	**Wash Buffer**
SU0052	1 bottle x 5 mL	**Tropix®CDP Star®Ready-to-Use with Sapphire II™** (Chemiluminescent Substrate)
Kit Contents, Cat #CM-012, 500 Tests, Ziva-Tox Ultrasensitive Cytotoxicity Assay		
Cat. #	**Amount**	**Reagent Description**
AB-0106	1 vial x 250 µL	**Anti-BrdU Antibody- Alkaline Phosphatase Conjugate**
SL-0222	1 vial x 200µL	**BrdU Labeling Solution**
SL-0236	1 bottle x 25 mL	**Antibody Conjugate Diluent**
SL-0346	1 bottle x 25 mL	**Fix Solution**
SL-0206	1 bottle x 100 mL	**Stringency Solution**
SL-0116	1 bottle x 340 mL	**Preparation Solution, 3X**
BU-0076	1 bottles x 500 mL	**Wash Buffer**
BU-00762	1 bottles x 300 mL	**Wash Buffer**
SU-0066	1 bottle x 25 mL	**Tropix®CDP Star®Ready-to-Use with Sapphire II™**

		(Chemiluminescent Substrate)

Materials Required But Not Provided With The Kit		
Cat. No.	**Amount**	**Description**
EQ401	1	96-well Microplate Luminometer, Insight-Mi™, Jaden BioScience Inc. or equivalent
CM006	12 vials x 2 mL/vial	Ziva Positive and Negative Control Kit, for Ziva Assays
-	1	37°C, 5% CO_2 Incubator
	-	Sodium Azide or DNA synthesis inhibitor
-	-	Distilled water
136102	1 case	96-well microtiter plates, White NUNC™, flat bottom, Sterile Nunclon Delta Surface, Tissue Culture plates, polystyrene with lids
-	-	96-well microtiter plates, U- or V-bottom, Sterile Tissue Culture plates with lids.
-	3	Pipets capable of delivering 10 µL, 200 µL and 1000 µL Repeat Pipetors capable of delivering 50 µL, 200 µL and 400 µL
-	1	Dedicated Pipetman capable of delivering 50µL for the anti-BrdU mAb-AP conjugate
-	-	Pipet tips: 10 µL, 200 µL and 1000 µL and filtered and unfiltered pipet tips, sterile and non-sterile
-	-	Absorbent paper or paper towels
-	1	Centrifuge for use with microtiter plates, that can reach 1700 RPM

WARNINGS AND PRECAUTIONS

Ziva-Tox Ultrasensitive Cytotoxicity Assay is intended for research use only.

STANDARD FORMAT

The basic STANDARD format is used in different cytotoxicity model systems. A schematic of the STANDARD ASSAY PROTOCOL is outlined below.

ZIVA-TOX STANDARD ASSAY PROTOCOL

Treatment and incubation of cell culture dependent on experimental objectives

BrdU labeling (blue) incubation 2-24 hours

Centrifugation for suspension cells (no need to centrifuge adherent cells)

Optional methods to fix and transfer to assay plate (see detailed protocols)

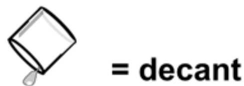
Fix 10 minutes RT

Stringency Solution 10 minutes, RT
Preparation Solution (1 rinse)

Anti-BrdU antibody 20 minutes;
then add 300μL Wash Buffer to antibody

Wash Buffer (4 washes)
Preparation Solution (2 rinses)

Chemiluminescent Substrate
Read luminescence


= decant

= decant & blot

ZIVA STANDARD ASSAY PROTOCOL

Review the Technical Support Section before beginning the assay. This section contains directions for diluting the Anti-BrdU Antibody Conjugate and the 3X Preparation Solution dilutions before use. Warm to room temperature and swirl the Stringency Solution, Preparation Solution, Antibody Diluent, Wash Buffer, and Ziva Controls before use. The Fix Solution, Positive and Negative Controls will form crystals at ≤25 °C; care should be taken to warm and swirl liquid until crystals are dissolved before use.

In general, the cells of interest are incubated under growth conditions with or without the cytotoxic agent. The BrdU labeling reagent is added to the cells either at the same time the cytotoxic agent is added or after the cells have been exposed to cytotoxic agent. Actively proliferating cells incorporate the BrdU label into their DNA. Cell-mediated incubations are typically performed in U- or V-bottom sterile tissue culture plates and, after the centrifugation step and the addition of Fix Solution, the well contents are transferred to a white flat-bottom 96-well-microtiter plate for assay processing. Non-cell mediated studies such as ADCC and chemical cytotoxicity studies use the same white flat bottom sterile 96-well microtiter plate for all of the cytotoxic agent/target cell and BrdU incubation and assay processing steps (e.g., contents of the wells are not transferred to another plate).

After BrdU labeling, suspension cells are centrifuged to the assay surface (adherent cells do not need centrifugation) and the liquid content of the wells is decanted into a sink. During this step unincorporated BrdU is removed. The addition of the Fix Solution causes the cellular DNA in the cell samples to be converted to a single strand state and immobilized to the well surface. The assay plate surface and the immobilized cell DNA is then i) conditioned by a stringency step, ii) rinsed with Preparation Solution and then iii) exposed to the anti-BrdU antibody-alkaline phosphatase conjugate in order to bind the antibody conjugate to the immobilized cellular BrdU DNA. Subsequent wash steps separate and remove any unbound antibody-alkaline phosphatase. Chemiluminescent substrate is then added to the assay surface. The chemiluminescence signal is then measured after an appropriate incubation period and the signal measured should be proportional to the number of BrdU-labeled cells present in the assay well.

1.0 Cell Growth and Sample Preparation

Each operator should determine the optimal experimental model system and strategy for treating the target cell sample with the cytotoxic agent. Such strategies should include consideration of the cell type, and effector model system, incubation conditions and duration required to observe the desired biological effect, BrdU labeling times, and cell densities that are optimal for the type of cytotoxicity model system being studied. The operator should use paired biological controls that are incubated under the same culture conditions applied to the treated cell sample.

In general, each well in a 96-well microtiter plate receives up to 100 µL of the desired target cell concentration in the chosen tissue culture medium. Add up to 100 µL of the desired dilution of cytotoxic agent per well, for a total maximum volume of 200 µL per well. **Note**: Ziva-Tox typically uses at most 100,000 cells per well. Ziva is an ultrasensitive assay, therefore, significantly less cells can be used in typical operations. Again the operators should determine the cell density that is optimal for their own model system.

Cell-mediated Toxicity Studies: Incubate target and effector cells, BrdU, and controls in V- or U-bottom 96-well, sterile cell culture microtiter plates. Ziva-Tox is a very flexible cytotoxicity assay. The operator

can either label the cells with BrdU at the same time the cytotoxic agent is added to the cells, or alternatively the operator can incubate the target cells with the cytotoxic agent first, for the amount of time the operator determines a biological reaction will occur, before adding the BrdU labeling reagent to the test sample. In both cases the target cells should be capable of actively synthesizing DNA, and the incubation times for BrdU labeling should be long enough to allow BrdU incorporation into the cellular DNA (e.g., 1-24 hours) (see Section 2.0 below, **BrdU Labeling Step)**. The use of paired biological control samples are recommended and should be processed under the same conditions as the test sample (see Section below, **Biological Control Samples**).

Non-cell-mediated Toxicity Studies (e.g., chemical and ADCC toxicity studies), use a white flat bottom, 96-well, sterile cell culture microtiter plate. The cells remain in these wells (are not transferred to another plate) for the entire Ziva-Tox assay.

2.0 BrdU Labeling Step

The **BrdU Labeling Solution** is provided at a concentration of 10 mM. To label wells, dilute the stock 1:100 with complete tissue culture media (diluted BrdU concentration equals 10^{-4}M) and pipette 20 µL per well. The recommended final BrdU concentration in the well should be 0.01 mM (1 x 10^{-5}M). Incubate for between 2 to 24 hours or longer in 5-7% CO_2 at 37°C or the operator's required cell culture incubation conditions.

3.0 Cell Centrifugation Step

Remove plate from incubator. Cover plates with lid and centrifuge plates containing suspension cells at ~1600 to 1650 rpm, @ +4°C, 5 minutes. Centrifugation is not required for adherent cells. Decant and blot.

4.0 Fix and Stringency Steps

4.1. Add 50 µL **Fix Solution** to each well containing cell samples. For cell-mediated assays performed in U- or V-bottom microtiter plates, transfer the contents of each well to a white flat bottom 96-well microtiter plate for incubation and the remainder of the assay. For efficient mixing and transfer of the contents, gently pipet the mixture in the well up and down before transferring. . From this point on (for both cell-mediated and non-cell mediated) the assay should be performed in a white flat bottom 96-well microtiter plate. In addition, the operator can add 50 µL to each **Positive or Negative Ziva Controls well on the plate**. Cover plate and incubate @ Room Temperature (RT) for 10 minutes. Decant and blot. **Do Not Add** Fix Solution to the Ziva Positive and Negative Control wells.

4.2 Add 200 µL **Stringency Solution** to each well. Cover plate and incubate @ RT for 10 minutes. Decant and blot.

4.3. Add 400 µL **Preparation Solution** to each well. Cover plate and incubate @ RT for 2 minutes. Decant and blot. Note: If wells hold less than 400 µL, fill reagent to the well rim.

5.0 Antibody- Conjugate Reaction and Wash Steps

5.1 Add 50 µL of the prepared "diluted" **anti-BrdU Antibody Conjugate Solution** to each well. Cover plate and incubate @ RT for 20 minutes. Thereafter, add 300 µL **Wash Buffer** to each well and immediately decant and blot**.** **Note:** Read the Reagent Handling Section. If plate wells hold less than 400 µL, fill the well to the rim with Wash Buffer.

5.2 Wipe the top of the plate with a clean damp absorbent paper.

6.0 Wash Steps.

6.1. Add 400 µL **Wash Buffer** to each well. Decant and blot. **Perform 3*X***

6.2. Add 400 µL **Preparation Solution** to each well. Decant and blot. **Perform 2*X***

6.3 Wipe the top of the plate with a clean damp absorbent paper.

Note: If the plate well volume is less than 400 µL per well, decrease the reagent volume to 300 µL per well and add one extra Wash Step with Wash Buffer (6.1) and one extra Rinse Step with Preparation Solution (6.2). Decant and blot.

7.0 Chemiluminescent Signal Generation and Detection

7.1 Add 50 µL **CDP* Star®Chemiluminescent Substrate** to each well.

7.2 **Chemiluminescent Signal Detection Measurements:** The chemiluminescent assay signal develops over time. The halftime of signal generation is roughly 5-10 minutes at room temperature. The room temperature signal is close to fully developed at 30 minutes after the addition of the substrate and is essentially completely developed at 60 minutes after substrate addition. After 60 minutes the signal changes little over hours. In order to compare the chemiluminescent signals from different assays, the compared signals should be obtained at the same time after the addition of the substrate to the well. Generally the luminometer is programmed to report the chemiluminescent signal for each assay well in terms of Relative Light Units/second (RLU/sec). It is recommended that the plate be placed in the luminometer or in the dark after addition of the substrate until the set read time. Typical read-times are set at 1 sec per well.

ASSAY CONTROLS

General Biological Control Samples

The cytotoxic effect for the Ziva-Tox assay is signaled by the partial or complete inhibition of the treated cell sample DNA synthesis relative to the extent of DNA synthesis obtained for untreated control cells. Therefore in order to calculate the extent of DNA synthesis inhibition in the treated target cells by the cytotoxic agent or effector cell of interest, it is necessary to experimentally determine the maximum extent to which cell DNA synthesis can be inhibited by a cytotoxic agent which inhibits essentially all cellular DNA synthesis in the target cells. Treatment of target cells with 0.1% w/v Sodium Azide (final concentration) is usually sufficient to

completely inhibit DNA synthesis in the target cell. Alternatively, other known DNA inhibitory molecules can be used, at concentrations that inhibit 100% of DNA synthesis.

Therefore, two biological control samples are recommended for each Ziva-Tox assay using paired cell samples: 1) a Maximum Cytotoxic Control, using the same target cell sample dilution (paired sample) incubated with 0.1% w/v sodium azide final concentration and BrdU. The 100% DNA synthesis inhibition control for the Ziva-Tox assay is analogous to the complete lysis control used for many current cytotoxicity assays. In the Calculation Section below, the assay chemiluminescent signal from the Maximum Cytotoxic Control sample is refered to as "**RLUspont.**" In general the "Complete DNA Synthesis Inhibition Controls" should not exceed 10% of the maximal BrdU incorporation; 2) a Minimal Cytotoxic Control consisting of the paired target cell sample incubated with BrdU but not exposed to the cytotoxic agent (e.g., either chemical or biological effector cells). In the Calculation Section below, the assay chemiluminescent signal from the Minimal Cytotoxic Control sample is refered to as "**RLUmax.**" This control provides quantitative information concerning the maximal non-cytotoxic signal associated with the target cell incorporation of BrdU, which is necessary in order to determine the degree of cytotoxicity of the cytotoxic agent.

Cell-Mediated Cytotoxicity Control

Ideally, for a cell-mediated cytotoxicity assay, the effector cells mixed with the target cells should incorporate little or no BrdU into their DNA during the BrdU labeling step. However this may not be the case in the chosen effector cell and there may be an additional signal due to effector cell-related BrdU incorporation during DNA synthesis. This signal must be determined in order to calculate the extent of cell DNA synthesis inhibition caused by the effector cells. The magnitude of this signal will depend upon the nature of the particular effector cell type used. Because of this, it is recommended that an additional control be used in the assay, consisting of effector cells alone (not mixed with target cells) labeled with BrdU. In the Calculation Section below, the assay chemiluminescent signal from this sample is referred to as "**RLUeff.**" In cases where the effector cell extent of DNA synthesis may diminish over time, the user should determine the optimal timing when effector cell DNA synthesis is diminished, and the effector cell cytotoxicity is still efficacious, and use these low DNA synthesis effector cells in the assay. Taking this into account will help to minimize the contribution of the BrdU labeling of the effector cells in the assay. Similar controls are utilized for other cytotoxiciy assay kits, such as the LDH-based kits. See the Calculations section below for a detailed discussion of the use of this data in the "% Specific Cytotoxicity" equation.

Ziva Positive and Negative Control Samples

Ziva Positive and Negative Control samples are used as a general internal and between-assay control. The Ziva Positive Control contains a known amount of BrdU-labeled single strand cell DNA and the Ziva Negative Control contains a known amount of cell single strand DNA not labeled with BrdU. When using the Ziva Positive and Negative Control samples, be aware that a precipitate can form when refrigerated. Care should be taken to re-dissolve the precipitate heat the Control samples in hot tap water for several minutes, or incubate for 10-15 min in a 37˚C bath, and then vortex the controls. Allow the controls to come to roughly room temperature before use. It is recommended that triplicate Positive control and triplicate Negative control wells be used in an assay.

Control Assay Procedure: At the same time that the Fix Solution is being added to cell samples in the assay plate (Section 4.1 of the Ziva STANDARD ASSAY PROTOCOL) or cells samples containing Fix Solution are

transferred to the white 96-well microtiter assay plate, aliquot 50 µL/well of the Positive or Negative Ziva Controls into separate designated control wells. **Do not add the Fix Solution to the Control samples.** Incubate each Control well for 10 minutes at room temperature at the same time that the other assay wells are incubated with Fix Solution. The assay plate is then processed according to the STANDARD ASSAY PROTOCOL starting with Section 4.2 Stringency Step forward.

CDP Star® Substrate Control.

It is useful to include in each assay several substrate-only wells. Such wells are not exposed to the Ab-AP conjugate but do undergo the Step 6 Wash and Rinse Steps outlined in the STANDARD ASSAY PROTOCOL before adding the substrate. Ideally one substrate-only control well should be the first well in the assay that substrate is added to, and one substrate-only control well should be the last well in the assay that the substrate is added to as a control of pipeting sample carryover.

CALCULATIONS:

Typically, ≥ 3 replicate wells of each dilution, sample set or controls are performed. Calculate the mean, standard deviation and coefficient of variation for the data from each dilution or sample set. In addition, it is useful to calculate the following parameters for each well or each set of replicate wells in an assay.

Relative Light Unit/Cell: The replicate mean Relative Light Units (RLU/sec) divided by the known number of cells present in the assay well.

Antibody Directed and Chemical Cytotoxicity Calculations

The **"percent specific cytotoxity"** refers to the percent inhibition of BrdU incorporation that is specifically due to the cytoxic agent (e.g., ADCC or chemical, cytotoxic drug).

$$\%\ \text{Specific Cytotoxicity} = 1 - \left(\frac{RLU\exp - RLUspont}{RLU\max - RLUspont} \right) \times 100\%$$

Where:

RLUexp = the assay Relative Light Unit (RLU) signal from the (Cytotoxic Agent + Target cell + BrdU) sample.

RLUspont = the assay RLU signal from the (Target cell + Azide + BrdU i.e. the complete DNA synthesis inhibition control) cell sample. This represents the *least* amount of BrdU that can be incorporated in to the system.

RLUmax = the assay RLU signal from the (Target cell + BrdU) cell sample. This represents the maximum amount of BrdU that can be incorporated into the system.

Cell-Mediated Cytotoxicity Calculations

The "percent specific cytotoxicity" refers to the percent inhibition of BrdU incorporation that is specifically due to the effect of the Cytotoxic Effector Cells on the Target cells in the experiment. This is determined by the following equation:

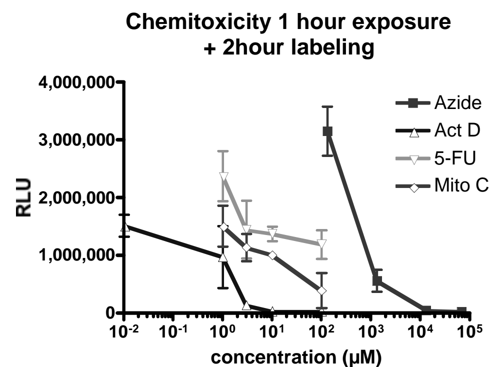
$$\%\ \text{Specific Cytotoxicity} = 1 - \left(\frac{RLU\exp - RLUspont - RLUeff}{RLU\max - RLUspont - RLUeff} \right) \times 100\%$$

Where

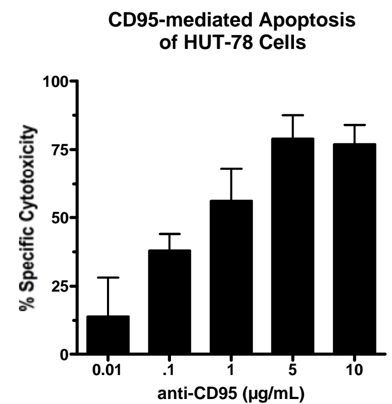
RLUeff = the assay signal from the (Effector cells alone + BrdU) cell sample. Keep in mind that if the experiment requires several different effector to target (E:T) ratios, a set of "effector cells alone" controls must be created for each E:T ratio. Thus, separate calculations for each E:T set will use the corresponding RLUs from each effector cell group.

Assay Performance Characterization:

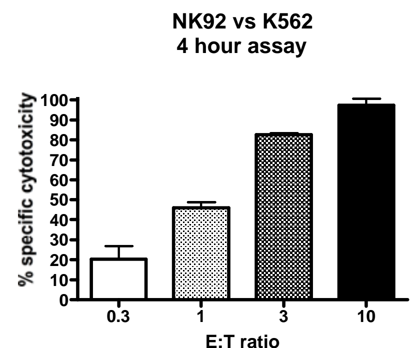
The performance characteristics of the Ziva-Tox Assay were evaluated for Chemical Cytotoxicity, Antibody Directed Cell Cytotoxicity (ADCC), and Cytotoxic T Lymphocyte (CTL) Toxicity using mammalian target cells. In principle, Ziva-Tox measures the incorporation of BrdU in living cells. The difference between the non-treated controls ("100% incorporation capacity") and the experimental wells represents the amount of cytotoxicity present in the experimental well. In all performance tests, the "Complete DNA Synthesis Inhibition Controls" did not exceed 10% of the maximal BrdU incorporation



Example 1 **Detection of chemotoxicity on mouse mastocytoma cell line P815.** P815 mouse mastocytoma cells ($5x10^3$ cells per well; triplicates) were incubated with either Sodium Azide, Actinomycin D, 5-FluoroUracil, or Mitomycin C in 96-well white NUNC Tissue culture microtiter plates for 1 hour in a 37°C, CO_2 incubator. The cytotoxicity of each chemical was evaluated at four different concentrations. After incubation for 1 hour, BrdU was added to label cells for an additional 3 hours. The cells were then assayed with Ziva-Tox in the same white 96-well microtiter plate.

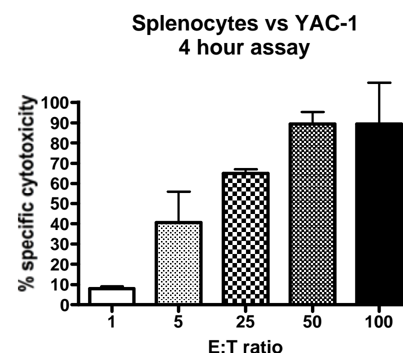


Example 2 **Detection of apoptosis mediated by anti-CD95 (Fas) antibodies.** Human T-cell lymphoma HUT-78 cells ($5x10^3$ cells per well; triplicates) were incubated in the presence of 5 different concentrations of anti-CD95 monoclonal antibodies in a 96-well white NUNC Tissue culture microtiter plate at 37°C, 5% CO_2, overnight. During the last 4 hours of incubation, BrdU was added to label cells and the cells were assayed with Ziva-Tox in the same white 96-well microtiter plate.



Example 3 **Detection of cell cytotoxicity using NK92 natural killer cells (Effector (E)) and K562 target cells (Target (T)).** Human K562 cells ($5x10^3$ cells/well; triplicates) were incubated in the presence of NK92 cells in V-bottom cell culture plates, at the indicated E:T ratios for 4 hours at 37°C, 5% CO_2, in the presence of BrdU. The cells were treated with Fix

Solution and transferred to the white 96-well Nunc microtiter plates and assayed with Ziva-Tox.



<u>Example 4</u> **Detection of cell cytotoxicity using naïve mouse splenocytes (Effector (E)) on YAC-1 cells (Target (T)).** Mouse YAC-1 cells ($5x10^3$ cells/well; triplicates) were incubated in the presence of naïve splenocytes at the indicated E:T ratios indicated for 4 hours of at 37°C, 5% CO_2 in V-bottom cell culture plates with BrdU. The cells were treated with FIX and transferred to white 96-well Nunc microtiter plates and then assayed with Ziva-Tox.

Patents and Trademarks

Jaden has patent applications pending for Ziva Ultrasensitive technology.
The use of CDPStar® is a registered trademark of Tropix, Inc./Applied Biosystems and covered by one or more of the following US Patent Nos. licensed from Applied Biosystems.
4,931,569
5,145,772
5,326,88
5,538,847

Technical Support

ASSAY REAGENT PREPARTION FOR THE ZIVA STANDARD ASSAY

Prior to beginning the assay prepare the following reagents:

1. 1X Working Preparation Solution from the Kit 3X Preparation Solution

<u>Calculate the volume of Kit 1X Preparation Solution needed for the assay.</u>

Determine the number of microtiter plate wells to be used in the assay.

[The total volume of 1X Kit Preparation Solution needed to do the assay for the desired number of assay wells] = [number of microtiter assay wells to be used] x [(0.4 mL/well for each wash) x (3 rinses used per well per assay)] = [__ ml]

Calculate the volume of 3X Preparation Solution needed for the assay.

[volume of 3X Preparation Solution needed for the assay] = (__ mL) ÷ (3).

To prepare the 1X Preparation Solution for the assay make a dilution of: [(__ mL ÷ 3) of 3X Kit Preparation Solution) + [2 x (__ mL ÷ 3) of clean distilled water].

<u>As an example if the number of processed wells is 12, then</u>:

<u>12</u> wells x 0.4 mL/well x 5 (Preparation Solution rinses used per assay well) = <u> 24 </u>mL total volume of 1x Preparation Solution needed.

[(24 mL ÷ 3) of 3X Kit Preparation Solution) + [2 x (24 mL ÷ 3) of clean distilled water].

[(8 mL) of 3X Kit Preparation Solution) + [2 x (8 mL) of clean distilled water]. 8 mL of 3X Kit Preparation Solution + 16 mL of clean distilled water = 24 mL 1X Preparation Solution.

2. Preparation of Anti-BrdU mAb-Alkaline Phosphatase Conjugate Working Solution (Ab Working Soln)

Note: Droplets may form in cap of the Anti-BrdU mAb-AP Conjugate vial during shipment. Centrifuge at low speed (1,000 to 10,000 RPM, 30 seconds) to locate all of the antibody conjugate at the bottom of the vial. Prepare Antibody dilutions before beginning the assay.

Calculate the volume of Ab Working Soln that is needed for the assay:
#___ wells used in the assay x 50 µL Ab Working Soln/well = ____ µL total volume of Ab Working Soln. Add this volume of Antibody Conjugate Diluent to a clean test tube.Calculate the amount of the Anti-BrdU mAb-AP Conjugate to add to the Antibody Conjugate Diluent by using this formula: 10 µL Anti-BrudU mAb-AP Conjugate/1 mL Antibody Conjugate Diluent.

Example: If the total number of wells used is 12, then:

12 wells x 50 µL/well = 600 uL Antibody Conjugate Diluent needed.
Add 10 uL/mL x 0.6 mL = 6 µL Anti-BrdU Ab-AP conjugate to the specified volume of Antibody Conjugate Diluent and then mix.

REAGENT HANDLING AND ASSAY PROCEDURE CONSIDERATIONS FOR THE ZIVA STANDARD ULTRASENSITIVE ASSAY

The Ziva assay is simple to perform. However, it is an ultrasensitive assay and the operator must use greater care than for a standard ELISA assay when performing the assay steps in order to reduce the chance of inadvertent contamination and cross contamination. To achieve ultrasensitivity, past ELISA practices have to be replaced with care in the following areas:

1. **General:** Keep all reagent caps tightly closed to avoid evaporation and store the reagents at the temperatures indicated on the reagent labels. Make sure **all reagents are warmed to room temperature before use** . None of the kit reagents should be frozen.
2. **Pipeting Technique:** To avoid inadvertent contamination or cross-contamination, care should be taken when pipeting biological materials or reagents to:
   - never allow pipet tips to touch one sample and be carried over to the next well which contains a different sample;
   - never allow pipet tips to physically touch the side or bottom of the well into which the reagent is being dispensed, and this is especially important for the addition of antibody-alkaline phosphatase conjugate to a well;
   - avoid creating an aerosol by dispensing the reagent too rapidly from the pipette tip into the microtiter plate well, and this is especially important for the addition of antibody-alkaline phosphatase conjugate to a well.

3. **Spill over:** To avoid cross-contamination, care should be taken to avoid letting a reagent or sample from one well to spill-over or splash to the next during the steps of reagent addition, centrifugation, washing, or blotting. If your plate wells hold less than 400 µL, fill the specified reagent to the well rim.
4. **Anti-BrdU mAb-AP Conjugate.** The anti-BrdU mAb is labeled with alkaline phosphatase (AP). AP can spread easily on laboratory equipment, gloves and surfaces and cause an inadvertent contamination. Careful attention to handling AP is recommended to avoid contamination. Precautions for minimizing the inadvertent spread of AP are the following:
   - 4.1. Use a dedicated pipetman for dispensing the anti-BrdU mAb-AP conjugate. Pipet tips with filters can also be used. Never let used pipet tips contaminate other surfaces.
   - 4.2 Wear gloves and change gloves frequently, especially when preparing and dispensing the anti-BrdU mAb-AP conjugate.
5. **Decanting Technique:** To minimize cross contamination from biological samples and AP, use a clean and previously unused absorbent material such as disposable paper towels, to soak up residual liquid during the decanting steps. Paper towels are acceptable to use. The Technique:
   - 5.1 First, decant well liquid in a sink, by turning the plate over and jarring the liquid out of the plate using a quick **"vertical"** up and down **sharp jerk** (not slanted) dispensing technique.
   - 5.2 Two quick up and down vertical **sharp jerks** are sufficient to remove most of the unwanted liquid. Some operators allow the plate to remain inverted for several seconds between jerks to allow the liquid to fully drain from the well. Be careful to keep the plate away from the sink bottom during the decanting as the liquid may splash back into the wells and cause inadvertent contamination.
   - 5.3 After the decanting process while the plate is still face down, move to the blotting station that has fresh absorbent paper. Quickly blot 1x to first remove the majority of liquid and then more slowly blot 3-5 x more on an unused portion of the paper. After the Fix step, allowing most of the liquid to drain from the wells before proceeding to the next step, reduces within sample well-well variability.
6. **Plate Reuse:** Unused wells on a plate should be covered with a plate seal or parafilm during the assay. To determine whether a well or row of wells has been used or contaminated the plate can be scanned in the luminometer before use to insure that the wells of interest were not inadvertently contaminated from prior use.
7. **Fix Solution and Flex System Reagents**: If stored in the refrigerator, a precipitate will form in these reagents. If this occurs, to redissolve the precipitated components heat the reagent in hot tap water or heat for ~10-15 min in a 37˚C water bath. Then let the reagent equilibrate to room temperature and shake to mix before use.
8. **CDP Star Chemiluminescent Substrate**: Store at 4 to 8 ˚C until use. Allow to warm to room temperature before use. Keep reagent away from heat and light. In order to protect the reagent from inadvertent contamination of the stock bottle with biological or AP contamination, the operator can pour the required volume of reagent into a baked glass tube (~270°C, 1 hr) or a clean glass tube which has been rinsed with distilled autoclaved water, and then dispense the room temperature equilibrated reagent from this tube into the assay wells for their immediate assay.

9. **The luminometer used** to read the Ziva assay microtiter plate chemiluminescent signals should have a known linear dynamic range of signal measurement. It is preferable that the luminometers linear dynamic range of signal measurement be 10^8 or more for optimal effectiveness of the Ziva STANDARD and Ziva FLEX assays. Some standard microtiter plate luminometers have a small linear dynamic range of signal measurement of 10^4 to 10^5. Using a luminometer with a small dynamic range can be useful depending on the user's application, however irrespective of the luminometer used when comparing assay sample signals, each sample experimental or control assay signal measurement compared must be within the linear dynamic signal range of the instrument used to obtain the signal measurements, in order for the comparison to be valid. Further, in order to obtain an accurate S/N ratio for an assay, both the experimental and control assay signal must reflect measurements obtained within the luminometers linear dynamic signal detection range. The Jaden Bioscience 96-well Microplate Luminometer, Insight-Mi™ has a linear dynamic range of signal measurement of about 10^9.

10. **White microtiter plates are known to autoluminesce** when exposed to light, and different white plates autoluminesce to different degrees. For example, it is not wise to locate the luminometer next to an outside window where the microtiter plate to be read can be exposed to direct sunlight as direct sunlight can cause empty and assay wells to give signals of tens of thousands of Relative Light Units/sec (RLU/sec). The autoluminescent signal decays away with time, and the time required depends on the plate type. The time for complete decay of the autoluminescent signal can be tens of minutes to hours. The Jaden Bioscience 96-well Microplate Luminometer Insight-Mi™ design greatly minimizes the magnitude of the detected light generated autoluminescent signal. Black microtiter plates also minimize this autoluminescent effect at the cost of reducing the assay signal by roughly 10 fold or more.

11. **Preparation of primary cell samples for the Ziva-Tox Assay:** If high background numbers are obtained from primary cell samples (e.g., as from freshly obtained splenocytes), this may be due to small numbers of actively dividing adherent cells such as fibroblasts in a heterogeneous mixture of cells. If this cellular population is irrelavent to the experiment, the operator can eliminate their contribution to the assay by performing the following procedure during the cell preparation step: The heterogenous mixture of cells, cytotoxic agent and BrdU are incubated in U- or V-Bottom 96-well culture plates as described earlier. After incubation, the cell sample is gently mixed (aspirated up and down) and transferred to a white flat bottom, 96-well microtiter plate (Do Not Add Fix Solution). After transfer, the cells are centrifuged and rinsed as described in Section 3.0 Cell Centrifugation and Preparation Steps, and then 50 μL Fix Solution is added and processed as described in the Standard Protocol. The unwanted adherent cells such as fibroblasts will remain attached to the U- or V-bottom microtiter plate used for incubation, thereby eliminating their contribution in the experiment.

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