



Ziva™ Ultrasensitive BrdU Cell Proliferation Assay

Ultrasensitive chemiluminescent immunoassay for the detection of cell proliferation by detecting BrdU incorporation into cells actively synthesizing cellular DNA: A non-isotopic alternative, with superior sensitivity compared to the “Gold Standard” ³H-thymidine incorporation assay.

Cat. No. CM003, 100 Test Kit, Ziva Ultrasensitive BrdU Cell Proliferation Assay

Cat. No. CM004, 500 Test Kit, Ziva Ultrasensitive BrdU Cell Proliferation Assay

Store Kits at +2 to +8°C

For Research Use Only

Intended Use

The Ziva™ Ultrasensitive BrdU Cell Proliferation Kit (Ziva-CPA) is intended for the ultrasensitive detection of cell proliferation using mammalian cells and cell lines in culture (adherent and suspension cells) in an easy to use, rapid assay format. Ziva is a non-isotopic immunoassay based on the measurement of 5'-bromo-2'-deoxyuridine (BrdU) incorporation into cellular DNA during DNA synthesis. Ziva is not for use in medical diagnostic procedures. Ziva is intended for research use only.

Introduction

Cell proliferation assays are used in a variety of important medical research applications. In its simplest form, cell proliferation can be detected and quantitated by counting labeled cells directly. Various cell staining techniques are used to facilitate cell counting. However, these direct methods are not useful for detecting the proliferation of small numbers of cells within a population of cells. Alternatively, cell proliferation can be indirectly detected and quantitated using assays such as MTT, XTT or WST-1 colorimetric assays which detect the metabolic activity of a cell population as the cell number increases. A major shortcoming to these indirect methods has been high assay backgrounds and relative assay insensitivity. Another widely used indirect method for detecting cell proliferation is the chemiluminescent ATP detection assay. These indirect cell proliferation assays have very limited utility for detecting the proliferation of small numbers of proliferating cells within a population of many cells. This greatly limits the application of these indirect assays. As an example, for a T cell proliferation assay, generally only a small fraction of a large population of viable cells present in the assay may be actively proliferating and of interest. The “Gold Standard” method for detecting cell proliferation has long been the ³H-thymidine method which incorporates tritiated thymidine into the synthesis of the new DNA in proliferating cells. This method provides a method for detecting and

Jaden BioScience Inc.

quantitating a low number of proliferating cells, and is capable of detecting the proliferation of a small fraction of cells in a large population of cells. The Lower Limit of Detection (LLOD) of cells for the ^3H -thymidine method is about 200-500 cells. However, current trends in lab techniques and requirements of smaller clinical laboratories have necessitated the use of non-isotopic labels and smaller numbers of cells. Current commercially available colorimetric, fluorescent, and chemiluminescent cell proliferation assays which detect newly synthesized cell DNA have been developed as an alternative to ^3H -thymidine. These types of assays generally detect the incorporation of BromodeoxyUridine (BrdU) a DNA precursor into cell DNA by using a labeled anti-BrdU antibody and detection substrate, in an ELISA format. These non-isotopic BrdU ELISAs typically take an average of 2.3 hours to perform. However, most assays detection limits are in the 10^3 cell range, with very few of these assays claiming an assay cell LLOD equal to the ^3H -thymidine method. Information is very limited with regard to these assays ability to detect a small fraction of proliferating cells within a large population of non-proliferating cells.

In contrast, Ziva™ can be performed in less than 1 hour and is approximately 200-500 times more sensitive than the ^3H -thymidine method (i.e., has a cell LLOD 200-500 times lower than the ^3H -thymidine method). In a spike-in experiment, Ziva has been used to detect 1-4 BrdU labeled actively proliferating cells in a background of 100,000 non-proliferating viable cells.

Principles of Ziva Assays

Ziva-CPA is an ELISA based ultrasensitive BrdU Cell Proliferation assay which uses a chemiluminescent substrate to detect the presence of an anti-BrdU antibody labeled with alkaline phosphatase. The Ziva-CPA assay has two different formats, the STANDARD and the FLEX formats. These formats differ in how the cells are prepared for the Ziva-CPA assay. The assay procedure for both formats is the same from the stringency step forward.

Kit Contents

Abbreviations: μL = microliter; mL = milliliter

Kit Contents, Cat #CM-003, 100 Tests, Ziva-CPA		
Cat. #	Amount	Reagent Description
AB0101	1 vial x 50 μL	Anti-BrdU Antibody- Alkaline Phosphatase Conjugate
SL0221	1 vial x 200 μL	BrdU Labeling Solution
SL0231	1 bottle x 5 mL	Antibody Conjugate Diluent
SL0061	1 bottle x 5 mL	Fix Solution
SL0201	1 bottle x 20 mL	Stringency Solution
SL0111	1 bottle x 70 mL	Preparation Solution, 3X
BU0071	1 bottles x 160 mL	Wash Buffer
SU0051	1 bottle x 5 mL	Tropix®CDP Star®Ready-to-Use with Sapphire II™ (Chemiluminescent Substrate)
Kit Contents, Cat #CM-004, 500 Tests, Ziva-CPA		
Cat. #	Amount	Reagent Description

Jaden BioScience Inc.

AB-0105	1 vial x 250 µL	Anti-BrdU Antibody- Alkaline Phosphatase Conjugate
SL-0221	1 vial x 200µL	BrdU Labeling Solution
SL-0235	1 bottle x 25 mL	Antibody Conjugate Diluent
SL-0065	1 bottle x 25 mL	Fix Solution
SL-0205	1 bottle x 100 mL	Stringency Solution
SL-0115	1 bottle x 340 mL	Preparation Solution, 3X
BU-0075	1 bottles x 500 mL	Wash Buffer
BU-00752	1 bottles x 300 mL	Wash Buffer
SU-0065	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	Positive and Negative Control Kit, for Ziva Assays
CM007	1	Flex System Kit
CM008	1	500 Test Kit, plus Flex System Reagent
CM009	1	Positive and Negative Control Kit plus Flex System Reagent
-	1	37°C, 5% CO ₂ Incubator
-	-	Distilled water
136102	1 case	Microplates, 96-well Microplates, NUNC™, Sterile Nunclon Delta Surface, Tissue Culture plates, white polystyrene
-	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 Ultrasensitive BrdU Cell Proliferation Assay is intended for research use only.

STANDARD FORMAT

The basic STANDARD format is widely used in different cell proliferation assays. The cells of interest are incubated under growth conditions with BrdU in white 96 well-microtiter plates. Actively proliferating cells incorporate the BrdU label into their DNA. After labeling, suspension cells are centrifuged to the assay surface (adherent cells do not need centrifugation) and rinsed with 1X Preparation Solution and centrifuged again. Residual liquid is decanted into a sink. During this step unincorporated BrdU is washed away. FIX Solution is then added to the well and cellular DNA in the immobilized cells is converted to a single strand state and immobilized to the well surface. The assay surface and the immobilized cell DNA is then conditioned by a stringency step and then exposed to 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. 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. A schematic of the STANDARD ASSAY PROTOCOL is outlined below.

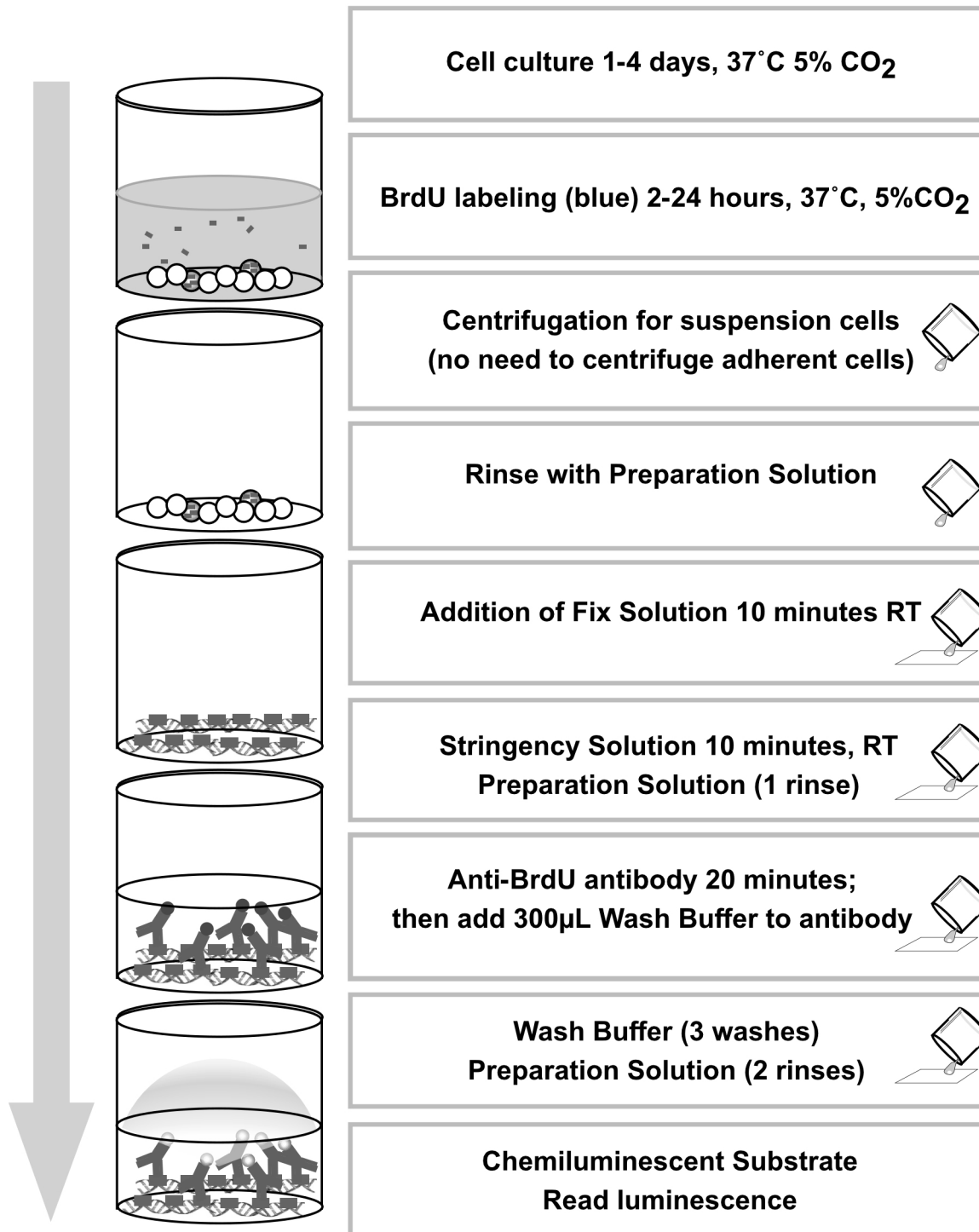
FLEX SYSTEM FORMAT


For the **FLEX System** format, suspension cells or adherent cells are labeled with BrdU and incubated *in vivo* or *in vitro* in a V-Bottom or U-bottom microtiter plate. Actively proliferating cells incorporate the BrdU label into their DNA. After labeling, all or an aliquot of sample cells in suspension or suspended adherent cells are added directly to the FLEX solution where the cellular DNA is converted to a single strand state. Cells added to the FLEX System reagent can be in standard physiological saline or PBS or cell growth media or biological sample. The FLEX cell mixture (biological sample and Flex System) is then added to an assay container for storage or a white 96-well microtiter plate well for processing. The sample single strand cell DNA is immobilized to the well surface. The assay surface is then conditioned through a stringency step and anti-BrdU antibody-alkaline phosphatase conjugate is exposed to the assay surface in order to bind the antibody conjugate to the immobilized cellular BrdU DNA. Wash steps then separate and remove any unbound antibody-enzyme conjugate and then chemiluminescent substrate is 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 container.

Ziva's FLEX System Reagent, performs both a fixation and a storage function. Cell samples (suspension or adherent cells) do not need to be rinsed or centrifuged. Cell samples are added directly into storage containers containing the FLEX System reagent and either stored for later batch testing or tested immediately with the Ziva assay. With the exception of the cell preparation and fixation steps the assay protocols for the FLEX System assay and Ziva STANDARD ASSAY PROTOCOL are identical.

Results from the Ziva STANDARD ASSAY PROTOCOL are dependent on the cell line or sample used and the exact incubation times should be optimized individually for each experimental design. The following outlines the STANDARD ASSAY PROTOCOL that can be used for most actively proliferating cells.

ZIVA™-CPA STANDARD ASSAY PROTOCOL



 = 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 1X Preparation Solution dilutions before use. Warm to room temperature and swirl Fix Solution, Flex System-Reagent, Stringency Solution, Preparation Solution, Antibody Diluent, Wash Buffer, and Controls before use.

1.0 Cell Growth and Sample Preparation

Each user should determine the cell densities, culture conditions and incubation times that are optimal for their systems. Use a white, flat-bottom, cell culture 96-well microtiter plate. Add to each well up to 100 μ L of the desired cell concentration in the chosen Tissue Culture Medium (TCM). Gently add up to 100 μ L of the desired dilution of cell stimulatory factor and/or test reagent per well, for a total maximum volume of 200 μ L per well. **Note:** Ziva-CPA typically uses at most 100,000 cells per well. Ziva is an ultrasensitive assay, therefore, significantly less cells can be used in typical operations. The operator should determine the optimal cell concentration for their application.

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 μ L per well. The recommended final BrdU concentration in the well should be 0.01 mM (1×10^{-5} M). Incubate for between 2 hours to 24 hours or longer in 5-7% CO₂ at 37°C or the operator's required cell culture incubation conditions.

3.0 Cell Centrifugation and Preparation Steps

- 3.1 Remove plate from incubator. Add **Preparation Solution** to each well to bring the volume of liquid up to the top rim of the well. Usually about 200 μ L is added to each well. Do not overflow the wells into the next wells. Cover plates with lid and centrifuge plates containing suspension cells at centrifuge ~1600 to 1650 rpm, @ +4°C, 5 minutes. Centrifugation is not required for adherent cells. Decant and blot.
- 3.2. Add 400 μ L **Preparation Solution** to all wells; for suspension cells, cover plate with lid, centrifuge ~1600 to 1650 rpm, @+4°C, 5 min. Centrifugation is not required for adherent cells. Decant and blot. **Note:** Be careful to avoid splashing. If plate well's hold less than 400 μ L liquid, fill the Preparation Solution to the well rim.

4.0 Cell Fix and Stringency Steps

- 4.1. Add 50 μ L **Fix Solution** to each well containing cell samples. Alternatively, the operator can add 50 μ L to each well of either **Positive or Negative Controls** or 50 μ L of cell-FLEX System Reagent mixture. Cover plate and incubate @ Room Temperature (RT) for 10 minutes. Decant and blot. **Do Not Add** Fix Solution to the Controls or the cell-Flex System mixture.

Jaden BioScience Inc.

- 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 Wash Buffer to the well rim.
- 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 3X**
- 6.2 Add 400 μL **Preparation Solution** to each well. Decant and blot. **Perform 2X**
- 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 **Detection Measurements:** The chemiluminescent assay signal develops over time. The half-time 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 is 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.

Ziva™ FLEX System Cell Preparation Procedure

The basic Ziva FLEX System cell sample preparation procedure is performed in the following order:

1. Add X volumes of the cell sample of interest to $3X$ volumes of the FLEX System Reagent in a suitable container and mix the resulting solution thoroughly. Each well requires 50 μL /well cell-FLEX System mixture for a single well assay. Depending on the size of the cell sample volume a suitable container can be a 0.5 mL screw cap microcentrifuge tube, a 15 mL polypropylene conical screw cap tube that can be centrifuged or a larger polypropylene storage container.

Jaden BioScience Inc.

- 1.1 A convenient small volume of cell sample to use for a single well FLEX System assay is 20 μ L of the cell sample of interest, added to 60 μ L of FLEX System Reagent.
- 1.2 A convenient volume of cell sample to use for 3 replicates well FLEX System assay is 50 μ L of the cell sample of interest. Fifty microliters of cell sample added to 150 μ L of FLEX System Reagent provides enough volume of cell-FLEX System mixture for 3 well replicates of cell-FLEX System mixture.
2. Heat the cell sample-FLEX System mixture in a waterbath for 20 minutes at 75°C, and then cool the tube to room temperature.
3. Centrifuge the mixture to pellet precipitate which may form in the mixture. For example a microfuge tube should be centrifuged for 2 minutes at 8000 to 12000 RPM, and a 15 mL conical propylene culture tube should be centrifuged at top speed for 10 minutes in a simple table top centrifuge. The cell-FLEX System mixture supernatant can be assayed immediately after centrifugation or saved and assayed later. If saved, the cell-FLEX System mixture should again be centrifuged just before use. Prepared samples can typically be stored at refrigerated conditions, however the user should determine their own storage conditions for the specific prepared cell sample (cell-Flex System mixture).
4. Remove 50 μ L from the centrifuged tube being careful not to disturb any pellet, and put it into a tissue culture microtiter plate well to start the Ziva-CPA assay. Unused cell-FLEX System mixture can be saved for later use or archive purposes.
5. Cover the well and incubate for 10 minutes at room temperature along with other samples processed in Step 4.1 Fix Step. DO NOT ADD Fix Solution to the Cell-FLEX System Mixture. Decant and blot and then proceed to Step 4.2 (the Stringency Step) of the Ziva STANDARD ASSAY PROTOCOL.

ASSAY CONTROLS

Negative Control, Non-BrdU Labeled Cells

The Ziva-CPA assay is designed to detect and quantitate the chemiluminescent signal in Relative Light Units (RLU). The RLU signal is specifically associated with the immobilized cell BrdU-labeled DNA in a cell sample. It is necessary to determine how much of the total assay RLU signal is associated with BrdU labeled cell DNA, and how much of the total RLU signal is not associated with BrdU cell DNA. Non-cell associated BrdU RLU signal is commonly caused by the non-specific immobilization of signal generating molecules (in this case antibody- alkaline phosphatase (AP) molecules or Ab-AP molecules) to the microtiter well surface during the assay that is later exposed to the assay substrate. This non-specific signal immobilization can be caused by the non-specific binding of signal generating Ab-AP molecules directly to the well surface itself or to some non-DNA cell or growth media component which becomes immobilized on the well surface. It should be noted that the assay non-specific binding can vary for different cells and different fetal bovine serum lots.

Therefore, a necessary control for each Ziva cell proliferation assay is a paired cell sample which is not BrdU labeled but is essentially identical to the BrdU-labeled cell sample in composition and treatment. This control

Jaden BioScience Inc.

provides quantitative information concerning the non-specific signal associated with the BrdU negative cells which is necessary in order to determine the fraction of the total BrdU-labeled cell RLU signal which is specifically associated with cell BrdU-labeled DNA.

Assay Lower Limit of Detection

The lower limit of detection of the assay can be determined by incubating actively dividing cells with the BrdU labeling solution as described in Step 2 of the Standard Assay Protocol and a paired cell sample without exposure to BrdU. After incubation, count the cell numbers of both the labeled and unlabeled cells and make dilutions. Aliquot 100 μ L of each BrdU labeled and unlabeled cell dilution into separate wells. Generally, 3 replicates of each control dilution are typically assayed. See the Calculations Section below.

Ziva Positive and Negative Control Samples

When using the Ziva Positive and Negative Control samples, warm to room temperature and vortex before use. Aliquot 50 μ L of either the Ziva Positive control (containing a known amount of BrdU-labeled single strand cell DNA), or Ziva Negative control (containing a known amount of cell single strand DNA not labeled with BrdU), to a well at the same time that the Ziva STANDARD ASSAY PROTOCOL Fix reagent is being added to the other cell sample assay wells (Step 4.1 Cell Fix Step of the STANDARD ASSAY PROTOCOL). 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 rest of the assay for each Ziva Control well is identical to the STANDARD ASSAY PROTOCOL starting with Section 4.2 Stringency Step of the STANDARD ASSAY PROTOCOL.

Standard Curve

The operator may create a standard curve by assaying at least 3 different dilutions of a known positive or BrdU labeled sample.

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. 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 or sample set 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.

The Signal-to-noise (SN) ratio: SN ratio = (the assay RLU/sec value for an assay well) \div (the RLU/sec value for the assay negative control well)

Jaden BioScience Inc.

Lower Limit of Detection (LLOD): The LLOD represents the lowest number of BrdU labeled cells which can be detected by the assay in a statistically significant way. When an SN ratio of 2/1 for an assay well is the criterion of statistical significance, the LLOD can be calculated for the well using the following relationship.

LLOD for an assay well = $[(1 / (\text{the assay well SN}-1))] \times (\text{the Number of BrdU labeled cells or cell equivalents in the assay well})$

Assay Performance Characterization:

The performance characteristics of the Ziva™ Ultrasensitive BrdU Cell Proliferation Assay were evaluated with mammalian cells and the results are presented below. The evaluation was conducted primarily in terms of the parameters, RLU/sec, RLU/cell, SN ratio, and LLOD. The LLOD values were calculated on the basis that a SN ratio = 2 as a statistically significant SN ratio for the assay.

Figure 1 and Table 1 present results for the effect of the time on the exposure of P815 cells to 10^{-5} M BrdU in culture in the Ziva Standard assay. Jaden's Insight-Mi luminometer was used to measure RLU/sec signals and the LLOD for a Ziva Standard assay comparing 1000 BrdU labeled P815 cells and 1000 unlabeled P815 cells.

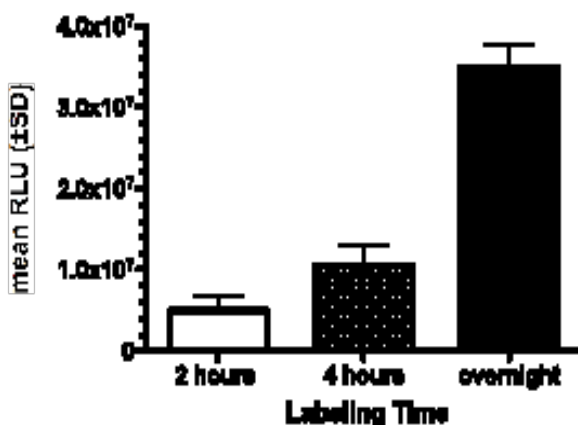


Figure 1. Effect of the time on the exposure of P815 cells to 10^{-5} M BrdU in Culture using the Ziva Standard Ultrasensitive BrdU Cell Proliferation Assay.

Table 1

	2 Hours	4 Hours	Overnight
Mean Positive RLU/sec (n = 4)	4,935,875	10,567,650	34,893,500
Positive RLU/Cell	4,936	10,568	34,894
Mean Negative RLU/sec (n = 4)	26,214	34,397	43,144
Negative RLU/Cell	26	34	43
Signal: Noise (SN)	188	307	809
LLOD	5	3	1

Jaden BioScience Inc.

In this experiment, 1000 P815 cells per well were cultured in RPMI-1640, 10%FBS in the presence or absence of 10^{-5} M BrdU and incubated at 37°C, 5% CO₂. Samples of the BrdU labeled P815 cells and unlabeled P815 cells were sampled at 2 hours, 4 hours, and after overnight incubation. Cell samples were counted and cell dilutions prepared at 1×10^4 cells/mL. 100 μ L/well (1000 cells per well) of each cell dilution was assayed in quadruplicate in a white 96-well Delta Nunc tissue culture microtiter plate and processed using the Ziva Standard ASSAY PROTOCOL. RLU/sec values presented were obtained 60 minutes after the addition of the substrate to the assay well. The typical kinetics of chemiluminescent signal generation in the Jaden Insight Mi Luminometer are presented in terms of % of the maximum signal (which occurs about 60 minutes after the addition of the substrate to the well): 5 min signal- 30% to 40% of maximum signal; 15 min signal- 50% to 70% of maximum signal; 30 min signal- 80% to 90% of maximum signal. These results illustrate the ultrasensitive detection capability of the Ziva standard assay, and further illustrate that, as expected, the RLU/cell, the SN ratio, increase with labeling time while the LLOD decreases. These results also illustrate the broad dynamic range of detection of the Jaden Insight-Mi Luminometer and its utility using the Ziva assay. Most microtiterplate luminometers cannot accurately measure high signals (RLU/sec) over about 10^6 RLU/sec.

Figure 2 and Table 2 present results illustrating the capability of the Ziva assay to detect 1 proliferating cell (a BrdU labeled P815 cell) in the presence of a large number (10^5) of non-proliferating cells (non-BrdU labeled mouse splenocyte cells).

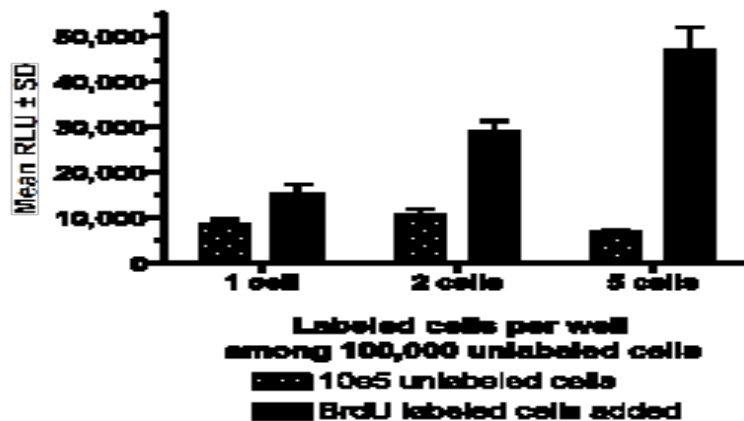


Figure 2. Capability of the Ziva FLEX System assay for detecting low numbers proliferating cells (BrdU labeled P815 cells) in the presence of large numbers of non-proliferating cells (non-BrdU labeled mouse splenocytes). These data illustrate the ultrasensitive performance characteristics of the Ziva FLEX System assay.

Table 2

# BrdU-labeled Cells among 10 ⁵ unlabeled cells:	1 Cell Equivalent	2 Cell Equivalents	5 Cell Equivalents
Mean Positive RLU (n = 3)	15,364	29,238	47,092
RLU/cell	6741	9207	7994
Mean Negative RLU Background (10 ⁵) (n = 3)	8,623	10,824	7,120
Signal-to-Noise (SN)	1.8	2.7	6.6
LLOD	1.3	1.2	0.9

In this experiment, P815 cells were cultured in RPMI-1640, 10%FBS in the presence of 10⁻⁵M BrdU incubated overnight at 37°C, 5% CO₂. The BrdU labeled P815 cells were counted and serial dilutions prepared in Ziva cell-FLEX System mixtures. Each FLEX System dilution contained 100,000 unlabeled mouse splenocytes per 50 µL of cell-FLEX System mixture. Fifty microliters of the cell-FLEX System mixture dilution was pipetted into a well (n = 3) and assayed using the Ziva™ Flex System assay. Calculations in Table 2 demonstrate the raw data with RLU/cell, SN ratio values, and LLOD values.

Figure 3 presents results from a comparison of the ³H-thymidine assay and the Ziva standard assay and illustrates that the non-isotopic Ziva Standard Ultrasensitive BrdU Cell Proliferation Assay has a much lower proliferating cell detection LLOD than does the ³H-thymidine assay.

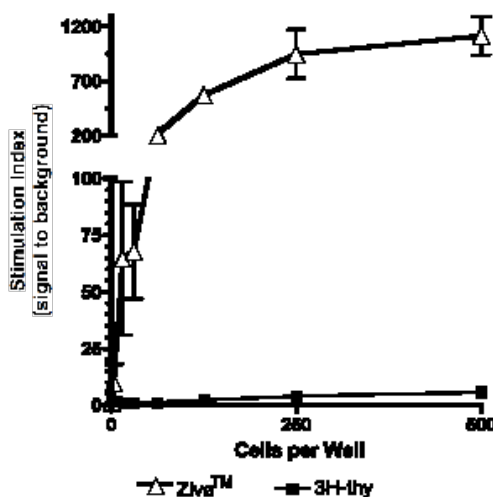


Figure 3. Comparison of the Ziva Standard assay and the ³H-thymidine assay measurement of the Stimulation Index. P815 mouse mastocytoma cells were titrated by serial dilution into a 96-well plate. Duplicate plates set ups were used; one pulsed with ³H-thymidine and the other labeled with BrdU and the respective plates were both assayed according to each method’s protocols.

In this experiment, the Ziva Standard Ultrasensitive BrdU Cell Proliferation Assay and the ³H-thymidine assay performance was compared. Serial dilutions of P815 cells were cultured in RPMI-1640, 10%FBS in the

Jaden BioScience Inc.

presence of 10^{-5} M BrdU or $1\mu\text{Ci}$ ^3H -thymidine and incubated overnight at 37°C , 5% CO_2 . The BrdU-labeled cells were then assayed using Ziva™ Standard Assay Protocol. Cells exposed to tritium were harvested according to the standard methods for ^3H -thymidine labeled cells and counted according to standard procedures. All dilutions for each test method were assayed in triplicate. The measurement comparison is presented as the Stimulation Index, which represents the ratio of the positive signal in experimental wells to the signal generated from cells without label.

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 PREPARATION 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

Calculate the volume of Kit 1X Preparation Solution needed for the assay.

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 (5 washes 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].

As an example if the number of processed wells is 12, then:

12 wells x 0.4 mL/well x 5 (Preparation Solution rinses used per assay well) = 24 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 (8,000 to 10,000 RPM, 30 seconds) to locate all of the antibody 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 μ L Ab Working Soln/well = ___ μ L 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 μ L Anti-BrdU mAb-AP Conjugate/1 mL Antibody Conjugate Diluent.

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

12 wells x 50 μ L/well = 600 μ L Antibody Conjugate Diluent needed.

Add 10 μ L/mL x 0.6 mL = 6 μ L Anti-BrdU Ab-AP conjugate to the specified volume of Antibody Conjugate Diluent. 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:

- 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** (except anti-BrdU mAb, and BrdU labeling reagents). None of the kit reagents should be frozen.
- 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.
- 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,

Jaden BioScience Inc.

- washing, or blotting. If your plate wells hold less than 400 μ L, 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. Pipets 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 to soak up residual liquid during the decanting steps. Paper towels are acceptable to use. The Technique:
 - 5.1 First, dispense 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. 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 all liquid to be drained from the wells before proceeding to the next step, reduces within sample well-well variability. Be careful not to allow the test wells to overlap sections of the absorbent material that has been exposed to previously blotted liquid, as this may cause inadvertent contamination.
 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 heat the reagent in hot tap water to redissolve the precipitated components. Then let the reagent equilibrate to room temperature and shake to mix before use.
 8. **CDP Star Chemiluminescent Substrate:** Store at 4 to 8 $^{\circ}$ 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 (\sim 270 $^{\circ}$ C, 1 hr) or a clean glass tube which has been rinsed with distilled autoclaved water, and then dispense the 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

Jaden BioScience Inc.

STANDARD and Ziva FLEX assays. Some standard microtiter plate luminometers have a linear dynamic range of signal measurement of about 10^6 (1,000,000). Using luminometers with a small dynamic range can be useful depending on the user's application, however the sample signal measurement must be within the linear dynamic signal range of the instrument used to reliably compare to another sample. 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.

Jaden BioScience Inc.
3950 Sorrento Valley Blvd., Suite 400
San Diego, CA 92121

858 320-0097 telephone
858 320-0057 fax

LB8-012 Rev 02-19-10