Cellular BrdU ELISA Kit
User's Manual
For Research Use Only, Not for use in diagnostic procedures

Cell-Based ELISA Kit for Measuring BrdU incorporation *in situ*

CycLex Cellular BrdU ELISA Kit

For 200 Assays

Cat# CY-1142

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Intended Use

The CycLex Research Product CycLex **Cellular BrdU ELISA Kit** is a non-isotopic immunoassay used for the semi-quantitative measurement of BrdU (bromodeoxyuridine) incorporation in newly synthesized DNA during DNA synthesis

Applications for this kit include:
1) Detection and quantification of cell proliferation.
2) Monitoring the effects of pharmacological agents on DNA replication in cells.
3) Screening inhibitors of cell proliferation.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Don't expose reagents to excessive light.

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Introduction

Since Reichard & Estborn (1951) introduced a method to trace DNA synthesis by incorporation of radioactively labelled thymidine, the method has been used successfully to assess the particular population of cells, which have been entering S phase in analytical cell biology. Nevertheless, this method has several disadvantages. Autoradiography involves handling of radioactive material including manipulations, which must be performed in the dark. Long exposure times delay the evaluation of the experiment. These disadvantages can be avoided by using bromodeoxyuridine (BrdU) as a thymidine analogue. BrdU is incorporated into replicating DNA and can be detected immunocytochemically after partial denaturation of double stranded DNA, by a specific anti-BrdU monoclonal antibody (Gratzner, 1982). This method allowed the assessment of the population of cells, which are actively synthesizing DNA.

A rapid and convenient method for estimating S-phase cells in a population was developed which detects bromodeoxyuridine (BrdU) incorporation into DNA by means of monoclonal anti-BrdU antibodies in a cellular ELISA format. It has been shown that a precise evaluation of cell proliferation could be performed by the measurement of BrdU incorporation in newly synthesized cellular DNA. In addition, there is a good correlation between the cellular BrdU ELISA and the [^{3}H]-thymidine incorporation assay as shown for a variety of murine and human cell systems, including mitogen- and antigen-stimulated lymphocytes and cytokine-induced proliferation of different cell lines.

The CycLex Cellular BrdU ELISA Kit can be used in many different in vitro cell systems when cell proliferation has to be determined.

- Detection and quantification of cell proliferation induced by growth factors and cytokines.
- Determination of the inhibitory or stimulatory effects of various compounds on cell proliferation in environmental and biomedical research and in the food, cosmetic and pharmaceutical industries.
- Determination of the immunoreactivity of lymphocytes, stimulated by mitogens or antigens.
- Determination of the chemosensitivity of tumor cells to different cytostatic drugs in medical research.

Principle of the Assay

The CycLex Cellular BrdU ELISA Kit based on the incorporation of the pyrimidine analogue BrdU instead of [^{3}H]-thymidine into the DNA of proliferating cells that are cultured in microtiter plates. After its incorporation into DNA, BrdU in the cell is detected by anti-BrdU monoclonal antibody.

During the final 2 to 18 hours of culture, BrdU is added to wells of the microtiter plate. BrdU will be incorporated into the DNA of dividing cells. To enable antibody binding to the incorporated BrdU cells must be fixed, permeabilized and the DNA denatured. Detector anti-BrdU monoclonal antibody is pipetted into the wells and allowed to incubate for one hour, during which time it binds to any incorporated BrdU. Unbound antibody is washed away and horseradish peroxidase-conjugated goat anti-mouse IgG is added, which binds to the detector antibody. The horseradish peroxidase catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent). The color is quantified by spectrophotometry and reflects the relative amount of incorporated BrdU in the cells.

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The CycLex **Cellular BrdU ELISA Kit** is designed to measure the relative levels of BrdU incorporated into nascent DNA in situ. The summary of the assay is shown in below.

Summary of Procedure-1 for adherent cells

Culture adherent cells in microplate at 10^3-10^5 cells/well.

↓ Incubate O/N at 37°C in CO_2 incubator

Treatment with test compound

↓ Incubate an appropriate time at 37°C in CO_2 incubator

Add BrdU at final concentration of 10 μM for 2-18 hr

↓

Discard the culture medium

↓

Add 200 μL of Fixing/Denaturing solution.

↓ Stand for 30 min at room temp.

Discard the Fixing/Denaturing solution

↓

Add 50 μL of Anti-BrdU Monoclonal Antibody

↓ Incubate 1 hr at room temp.
Wash the wells

Add 50 μL of HRP conjugated Anti-Mouse IgG

↓ Incubate 1 hr at room temp.
Wash the wells

Add 50 μL of Substrate Reagent

↓

Add 50 μL of Stop Solution

↓

Measure absorbance at 450 nm

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Summary of Procedure-2 for suspension cells

Culture adherent cells in microplate at 10^3-10^5 cells/well.

↓ Incubate O/N at 37°C in CO_2 incubator

Treatment with test compound

↓ Incubate an appropriate time at 37°C in CO_2 incubator

Add BrdU at final concentration of 10 µM for 2-18 hr

↓

c.f.g. at 1,000 rpm for 10 min.

↓

Discard the culture medium

↓

Air dry the cells by hair-dryer for 15 min

Add 200 ↓ µL of Fixing/Denaturing solution.

↓ Stand for 30 min at room temp.

Discard the Fixing/Denaturing solution

↓

Add 50 µL of Anti-BrdU Monoclonal Antibody

↓ Incubate 1 hr at room temp.
Wash the wells

Add 50 µL of HRP conjugated Anti-Mouse IgG

↓ Incubate 1 hr at room temp.
Wash the wells

Add 50 µL of Substrate Reagent

↓

Add 50 µL of Stop Solution

↓

Measure absorbance at 450 nm

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Materials Provided

All compounds treatment should be assayed in duplicate. The following components are supplied and are sufficient for 200 assays.

Fixing/Denaturing Solution: One bottle containing 50 mL of Fixing/Denaturing Solution that consists of acidified ethanol. Ready to use. *The solution is a strong alkaline. Wear disposable gloves and eye protection when handling.*

1000X BrdU labeling reagent: One vial containing 0.1 mL of 11 mM 5-bromo-2'-deoxyuridine in PBS, pH 7.4 Store at 4°C for several months protected from light. For long-term storage, it is recommended to store at -20°C.

10X Wash Buffer: One 100 mL bottle of 10X buffer containing 2% Tween®-20

Primary Antibody Solution (anti-BrdU Monoclonal Antibody MI-2B1): One vial containing 12 mL of anti-bromodeoxyuridine monoclonal antibody MI-2B1. Ready to use.

Secondary Antibody Solution (HRP conjugated Anti-Mouse IgG): One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-mouse IgG polyclonal antibody. Ready to use.

Substrate Reagent: 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle supplied ready to use, containing 20 mL of 1 N H_2SO_4. *Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling.*

Materials Required but not Provided

- **96-well microplate (tissue culture grade, flat bottom)**
- **Centrifuge with rotor** for microplate (for suspension cell only).
- **Cell culture flasks** for growing and splitting cells.
- **Cell culture media**
- **1X PBS pH 7.2**
- **Pipettors:** 2-20 μL, 20-200 μL and 200-1000 μL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **Microplate washer:** optional (Manual washing is possible but not preferable)
- **Software package facilitating data generation and analysis :**optional
- **500 or 1000 mL graduated cylinder**.
- **Reagent reservoirs**
- **Deionized water of the highest quality**.
- **Absorbent paper:** disposable paper towels
- **Plate reader** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.

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Precautions and Recommendations

Safety Warnings and Precautions: The CycLex ***Cellular BrdU ELISA Kit*** *is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.*

Technical Notes

1. When performing washes manually, avoid introducing bubbles when dispensing liquids into the wells, and ensure each well is filled with buffer, but not overflowing to avoid cross-contamination between wells. Empty wells with a wrist-flick motion over an appropriate receptacle, and while still inverted, blot any remaining moisture onto clean absorbent paper.
2. Agitation of wells during incubation of Blocking Buffer and Antibody steps is recommended to reduce non-specific background. If microtiter plate agitator is not available, a platform vortex at a low setting can be used (e.g. level 1 of Fisher's Genie II platform vortex). If background problems occur, simply increase the number and/or duration of washes.
3. A brief 1X PBS rinse is recommended prior to the addition of the HRP substrate to remove any traces of the Tween-20™ with can interfere with the HRP activity.
4. Do not allow the wells to dry out during the protocol.
5. Incubation temperatures for Primary Antibody and Detection Antibody can be varied and should be empirically determined.

General Notes

- Allow all the components to come to room temperature before use.
- Do not use kit components beyond the indicated kit expiration date.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents in this kit may contain preservatives or other chemicals. Care should be taken to avoid direct contact with these reagents.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the alkaline Fixing/Denaturing Solution, the acidic Stop Solution and Substrate Solution which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **CAUTION: Sulfuric Acid is a strong acid. Fixing/Denaturing Solution is a strong alkaline. Wear disposable gloves and eye protection when handling Fixing/Denaturing Solution and Stop Solution.**

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Detailed Protocol

The CycLex **Cellular BrdU ELISA Kit** includes all reagents except cell culture microplate, for detection of BrdU incorporated into nascent DNA in cultured cells. Since experimental conditions may vary, treatment cells with test compound should be assayed in duplicate. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Reagents

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of 10X Wash Buffer.

1. Prepare a working solution of Wash Buffer by adding 100 mL of the **10X Wash Buffer** (provided) to 900 mL of deionized (distilled) water. Mix well.

2. Prepare 10X BrdU labeling reagent: Dilute **1000X BrdU labeling reagent** 1:100 with sterile culture medium (resulting concentration: 110 µM BrdU). 10X BrdU labeling reagent can be stored for up to several weeks protected from light at 4°C.

Assay Procedure

A. Culture adherent cells in 96-well microplate and treatment with compounds

1. Plate adherent cells into a 96-well microplate at 10^3-10^5 cells/well* in a final volume of 100 µL/well.

   ******For most experiments, 10^4 cells/well are sufficient and adequate for adherent cells.*

2. Incubate the microplate at 37°C over night in CO_2 incubator.

3. Add appropriate amount of test compounds** to each well. Vehicle control should be run in duplicate as a negative control.

   *******mitogens, growth factors, cytokines, cytostatic drugs and etc.*

4. Incubate the microplate at 37°C for appropriate time.

B. Incorporation of BrdU in nascent DNA in cell culture

1. Add 10 µL /well 10X BrdU labeling reagent in 100 µL culture. (Final BrdU concentration should be 10 µM)

2. Incubate the microplate at 37°C for 2-18 hr*** in CO_2 incubator.

   ********For most applications, a 2 h labeling time is adequate. Prolongation of the incubation time will increase the amount of BrdU incorporated into cellular nascent DNA and thus lead to increased absorbance values and sensitivity*

C. Fixing and denaturing the cells to 96-well plate

Fixing and denaturing the cells in the 96-well plates should be done as soon as the desired treatment has completed.

For adherent cells:
1. Remove media from wells with a wrist-flick. Avoid touching the bottom of the well and removing cells.

For suspension cells:
1'. Remove media from wells by centrifugation the 96 well microplate at 400 x g for 10 min and then suction using a canulla. Avoid touching the bottom of the well and removing cells. Dry the cells using a hair-dryer **for about 15 minutes**.

After this step, the labeled and dried cells can be stored for up to one week at 4°C.

2. Immediately add **200 µL/well** of **Fixing/Denaturing Solution**. Add the Fixing/Denaturing Solution slowly to insure cells are not detached from the plastic. Let stand **for 30 minutes at room temperature (ca.25°C).**

3. Remove Fixing/Denaturing Solution from wells with a wrist-flick. While still inverted, tap the plate gently onto absorbent paper to remove any excess fixing agent still within the wells.

D. Detection of Signals (Addition of Primary and Secondary Antibodies and Substrate Reagent)

1. Add **50 µL/well** of **Primary Antibody Solution** and incubate **for 1 hour at room temperature (ca.25°C)**.

Alternatively, this incubation period can be varied between 30–120 minutes, depending on individual requirements

2. Remove Primary Antibody with a wrist flick.

3. Rinse the wells **once** with **200µl/well** of **Wash Buffer**.

4. Remove Wash Buffer with a wrist flick. While still inverted, tap the plate onto absorbent paper.

5. Wash wells **4 times** with **200 µL/well Wash Buffer** for 2 minutes each with shaking at ca. 200 rpm on an orbital microplate shaker. Remove Wash Buffer in-between each wash with a wrist flick.

6. Add **50 µL/well** of **Secondary Antibody Solution** and incubate **for 1 hour at room temperature (ca.25°C).**

7. Remove Secondary Antibody with a wrist flick.

8. Rinse wells **once** with **200 µL/well Wash Buffer**.

9. Remove Wash Buffer with wrist flick and tap plate onto absorbent paper.

10. Wash wells **4 times** with **200 µL/well Wash Buffer** for 2 minutes each with shaking at ca. 200 rpm on an orbital microplate shaker. Remove Wash Buffer in-between each wash with a wrist flick.

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11. After last wash with Wash Buffer, rinse wells **once** with **300 µl/well 1X PBS**. Remove with a wrist flick and tap onto absorbent paper. Ensure that that no liquid remains in the well.

12. **Add 50 µL/well** of **Substrate Reagent**. (Avoid exposing the microplate to direct sunlight Covering the plate with e.g. aluminum foil is recommended). Return Substrate Reagent to 4°C immediately after the necessary volume is removed.

13. Incubate the plate **for 10-15 minutes at room temperature (ca.25°C).** (The incubation time may be extended up to 20 minutes if the reaction temperature is below than 20°C).

14. Add **50 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

15. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Note-2: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine of histone acetylation level of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

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Troubleshooting

1. **The signals are influenced a great deal by cell line and cell number that you plated, please ensure the appropriate cell number for your experiment. See "Example of Test Results Fig.2 and 3".**

2. With some cell lines, higher cell concentrations (more than 2×10^4 cells/well) may lead to increasing absorbance values in the absence of BrdU.

3. All treatments including vehicle control should be run in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

4. Poor duplicates, accompanied by elevated values for wells containing non-treated cells (vehicle control), indicate insufficient washing or vigorous washing. **Wash the plate thoroughly and gently.**

5. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the CycLex **Cellular BrdU ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C. For long-term storage of 1000X BrdU labeling reagent, it is recommended to store at -20°C.

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Example of Test Results

Fig.1 Sensitivity of the CycLex Cellular BrdU ELISA Kit

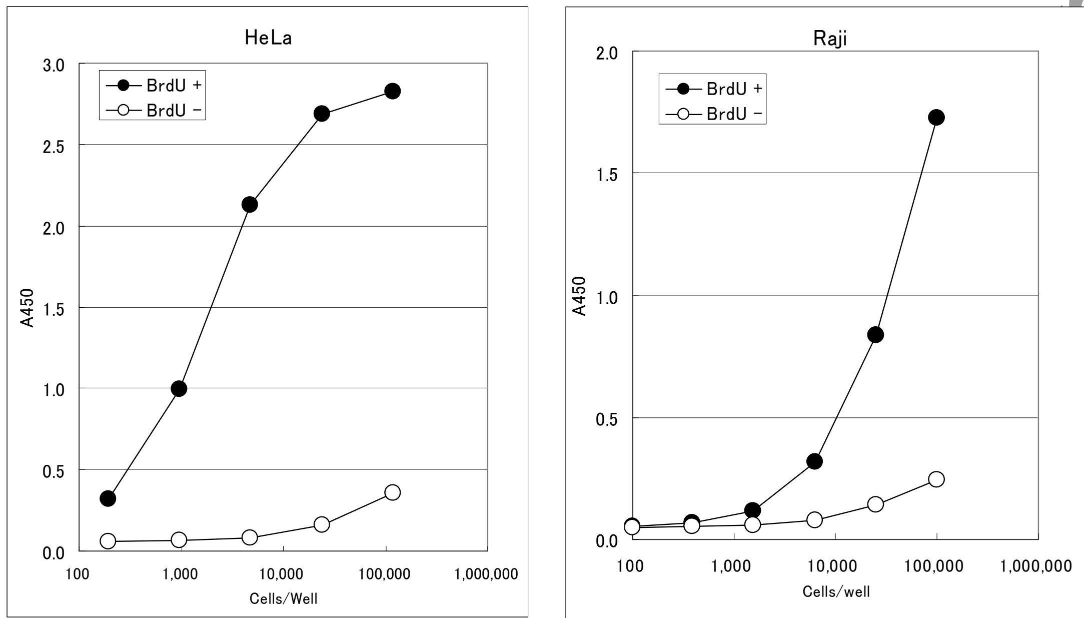

Fig.2 Effect of hydroxyurea on BrdU incorporation in HeLa. The cells (1 x 10^5 /well) were treated with indicated concentration of hyroxyurea for 2 hr then incorporated with 10 uM BrdU for 2hr.

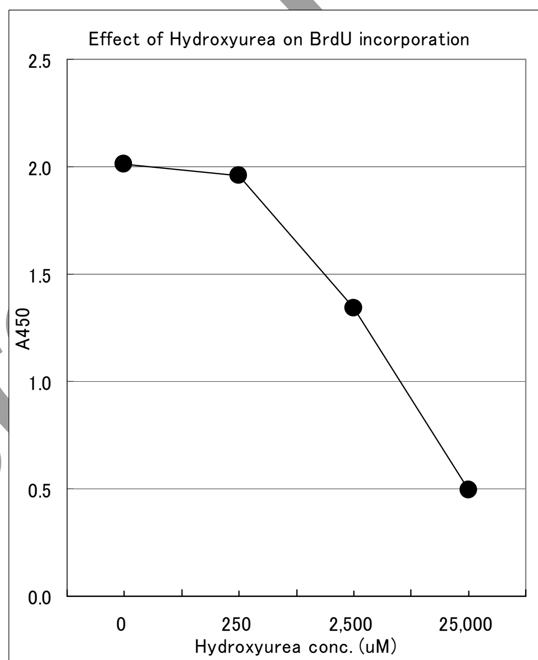

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Fig.3 Effect of hydroxyurea, doxorubicin and SN38 on BrdU incorporation in Raji. The Cells (5 x 10^4 /well) were treated with indicated concentration of drug for 2 hr then incorporated with 10 uM BrdU for 2hr.

Effect of Hydroxyurea on BrdU incorporation
A450
2.5
2.0
1.5
1.0
0.5
0.0
BrdU +
BrdU −
0
250
2,500
25,000
Hydroxyurea (uM)

Effect of Doxorubicin on BrdU incorporation
A450
2.5
2.0
1.5
1.0
0.5
0.0
BrdU +
BrdU −
0
100
1,000
10,000
Doxorubicin (uM)

Effect of SN38 on BrdU incorporation
A450
2.5
2.0
1.5
1.0
0.5
0.0
BrdU +
BrdU −
0
100
1,000
10,000
SN38 (uM)

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Related Products

*** CycLex Cellular Histone Acetylation Assay Kit: Cat# CY-1140**
*** CycLex Cellular UV DNA-Damage Detection Kit: Cat# CY-1141**
*** CycLex BrdU Cellular ELISA Kit: Cat# CY-1142**
*** CycLex Histone H2A.X Phosphorylation Cellular ELISA Kit: Cat# CY-1143**

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