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

CycLex Cellular BrdU ELISA Kit Ver.2

For 200 Assays

Cat# CY-1142V2

Intended Use..... 1
Storage..... 1
Introduction.....2
Principle of the Assay.....2-4
Materials Provided.....5
Materials Required but not Provided..... 5
Precautions and Recommendations..... 6
Detailed Protocol..... 7-9
Troubleshooting..... 10
Reagent Stability.....10
Example of Test Results..... 11
References.....12

Intended Use

The MBL Research Product **CycLex Cellular BrdU ELISA Kit Ver.2** is a non-isotopic immunoassay used for the semi-quantitative measurement of bromodeoxyuridine (BrdU) incorporation in newly synthesized DNA during DNA synthesis in culture cells.

Individual users should determine appropriate conditions when using other types of samples.

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.
- Do not expose reagents to excessive light.

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 Ver.2 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 MBL Research Product **CycLex Cellular BrdU ELISA Kit Ver.2** based on the incorporation of the pyrimidine analogue BrdU instead of [^{3}H]-thymidine into the DNA of proliferating cells that are cultured in 96-well cell culture plates. After its incorporation into DNA, BrdU in cells is detected by anti-BrdU monoclonal antibody.

BrdU is added to wells of 96-well cell culture plate during the final 2 to 24 hours of cell culture. BrdU will be incorporated into the DNA of dividing cells. To enable an antibody binding to the incorporated BrdU, cells are fixed and DNA is denatured. Anti-BrdU monoclonal antibody is pipetted into the wells and allowed to bind to any incorporated BrdU. Unbound antibody is washed away and horseradish peroxidase (HRP)-conjugated anti-mouse IgG is added. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H2O2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance reflects the relative amount of incorporated BrdU in the cells.

Summary of Procedure-1: for adherent cells

Culture adherent cells in 96-well cell culture plate at 10^3-10^5 cells/well
↓ Incubate for O/N in CO_2 incubator at 37°C
Treatment with test compounds
↓ Incubate for an appropriate time in CO_2 incubator at 37°C
Add 10X BrdU Labeling Reagent
↓ Incubate for 2-24 hours in CO_2 incubator at 37°C
Discard the culture medium
↓
Add 200 µL of Fixing/Denaturing Solution
↓ Stand for 30 minutes at room temp.
Discard the Fixing/Denaturing Solution
↓
Add 200 µL of Blocking Solution
↓ Stand for 10 minutes at room temp.
Discard the Blocking Solution
↓
Add 50 µL of anti-BrdU monoclonal antibody
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 50 µL of HRP conjugated anti-mouse IgG
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 50 µL of Substrate Reagent
↓ Incubate for 10-20 minutes at room temp.
Add 50 µL of Stop Solution
↓
Measure absorbance at 450 nm

Summary of Procedure-2: for suspension cells

Culture suspension cells in 96-well cell culture plate at 10^3-10^5 cells/well
↓ Incubate for O/N in CO_2 incubator at 37°C
Treatment with test compounds
↓ Incubate for an appropriate time in CO_2 incubator at 37°C
Add 10X BrdU Labeling Reagent
↓ Incubate for 2-24 hours in CO_2 incubator at 37°C
Centrifuge at 1,000 rpm for 10 minutes
↓
Discard culture media
↓
Air dry the cells by hair-dryer for 15 minutes
↓
Add 200 µL of Fixing/Denaturing Solution
↓ Stand for 30 minutes at room temp.
Discard the Fixing/Denaturing Solution
↓
Add 200 µL of Blocking Solution
↓ Stand for 10 minutes at room temp.
Discard the Blocking Solution
↓
Add 50 µL of anti-BrdU monoclonal antibody
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 50 µL of HRP conjugated anti-mouse IgG
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 50 µL of Substrate Reagent
↓ Incubate for 10-20 minutes at room temp.
Add 50 µL of Stop Solution
↓
Measure absorbance at 450 nm

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. Store protected from light at 4°C for several months or at -20°C for long-term storage.

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

Blocking Solution: One bottle containing 50 mL of 1X Solution. Ready to use.

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

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

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

Stop Solution: One bottle containing 20 mL of 1 N H_2SO_4. Ready to use.

Materials Required but not Provided

- **96-well cell culture plate:** Cell culture grade, flat bottom
- **Centrifuge with a rotor for microplate:** Only for suspension cell.
- **Hair-dryer:** Only for suspension cell.
- **Cell culture media**
- **1X PBS:** pH 7.2
- **Orbital microplate shaker**
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **(Optional) Microplate washer:** Manual washing is possible but not preferable
- **Microplate 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.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- 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.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with Substrate Solution which contains hydrogen peroxide.
- **CAUTION: Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **CAUTION: Stop Solution is a strong acid. Wear disposable gloves and eye protection when handling the solution.**
- **CAUTION: Fixing/Denaturing Solution is a strong alkaline. Wear disposable gloves and eye protection when handling the solution.**

Detailed Protocol

The **CycLex Cellular BrdU ELISA Kit Ver.2** includes all reagents except cell culture plates, for detection of BrdU incorporated into nascent DNA in cultured cells. Since experimental conditions may vary, treatment cells with test compounds 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 Working Solutions

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** and **1000X BrdU Labeling Reagent**.

1. Prepare a working solution of **Wash Buffer** by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water (**ddH_2O**). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Prepare **10X BrdU Labeling Reagent** (110 µM BrdU) by diluting **1000X BrdU Labeling Reagent** 1:100 with a sterile culture medium. 10X BrdU Labeling Reagent can be stored for up to several weeks protected from light at 4°C.

Assay Procedure

1. Culture Cells and Treatment with Compounds

1) Plate cells into a 96-well cell culture plate 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 plate for overnight in CO_2 incubator at 37°C.

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 etc.

4) Incubate the plate for appropriate time in CO_2 incubator at 37°C.

2. Incorporation of BrdU

1) Add **10 µL** of **10X BrdU Labeling Reagent** into 100 µL of culture medium. (A final concentration of BrdU should be 10 µM.)

2) Incubate the plate for ~24 hours* in CO_2 incubator at 37°C.

* For some applications, a labeling for a few hours may be adequate, but prolongation of the incubation time up to 24 hours increases the amount of BrdU incorporated into cellular nascent DNA and thus may lead to increased absorbance values and sensitivity.

3. Fixing and Denaturing

Fixing and denaturing the cells in the 96-well plates should be done as soon as the desired treatment is 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 plate at 400 x g for 10 minutes and then suction using a cannula. 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) Add **200 µL** of **Fixing/Denaturing Solution** slowly to each well to be careful cells are not detached from the plate, and incubate **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 solution still within the wells.

4) Immediately add **200 µL** of **Blocking Solution** slowly to each and incubate **for 10 minutes at room temperature (ca.25°C)**.

5) Remove Blocking Solution from wells with a wrist-flick. While still inverted, tap the plate gently onto absorbent paper to remove any excess solution still within the wells.

4. Detection of Signals

1) Add **50 µL** of **Primary Antibody Solution** to each well and incubate **for 60 minutes* 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 Solution with a wrist flick.

3) Wash wells **5 times** with **300 µL** of **Wash Buffer** per well **for 2 minutes each, shaking at ca. 200 rpm on an orbital microplate shaker**. Remove Wash Buffer in-between each wash with a wrist flick.

4) Add **50 µL** of **Secondary Antibody Solution** to each well and incubate **for 60 minutes at room temperature (ca.25°C)**.

5) Remove Secondary Antibody Solution with a wrist flick.

6) Rinse wells **once** with **200 µL** of **Wash Buffer** per well.

7) Remove Wash Buffer with wrist flick and tap the plate onto absorbent paper.

8) Wash wells **5 times** with **300 µL** of **Wash Buffer** per well **for 2 minutes each, shaking at ca. 200 rpm on an orbital microplate shaker**. Remove Wash Buffer in-between each wash with a wrist flick.

9) After last wash with Wash Buffer, rinse wells **once** with **300 µL** of **1X PBS**.

Remove **1X PBS** with a wrist flick and tap onto absorbent paper. Ensure that that no liquid remains in the well.

10) Add **50 µL** of **Substrate Reagent** to each well. Avoid exposing the plate 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.

11) Incubate the plate **for 10-20 minutes* at room temperature (ca.25°C)**.

* The incubation time may be extended up to 20 minutes if the reaction temperature is below 20°C).

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

13) 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 plate 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.

Troubleshooting

1. **Since the signals are quite influenced by the conditions of cell line, cell number, and incorporation time of BrdU, the optimal experimental conditions must be determined by the individual user.**

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 samples and controls should be assayed 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), may indicate insufficient washing or vigorous washing. **Wash the plate thoroughly and gently**.

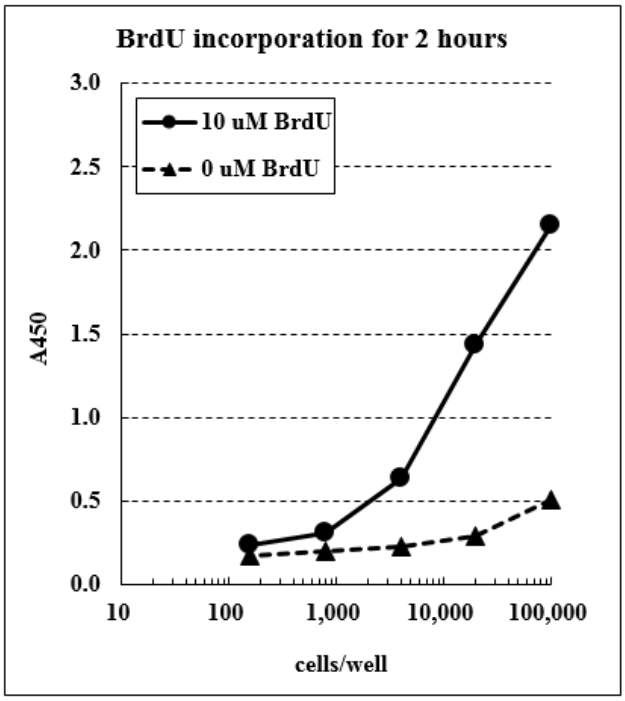
5. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

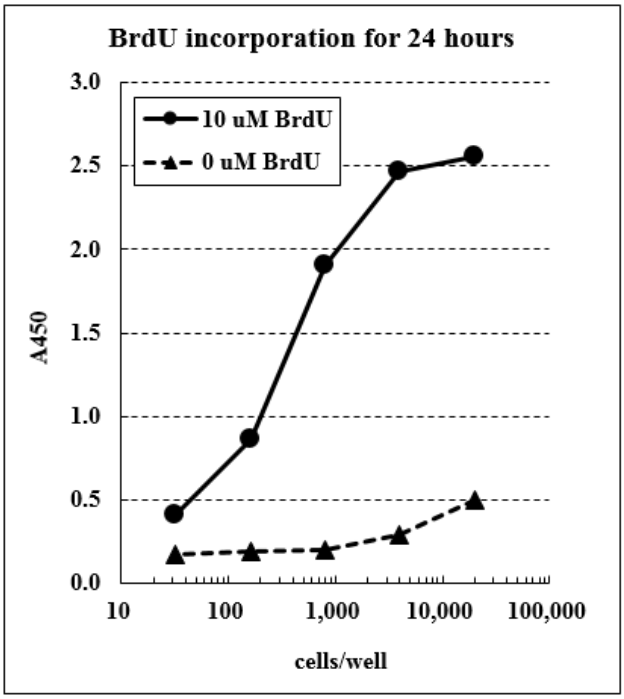
Reagent Stability

All of the reagents included in the **CycLex Cellular BrdU ELISA Kit Ver.2** have been tested for stability. Reagents should not be used beyond the stated expiration date.

Example of Test Results

Fig. Measurement of BrdU incorporation for 2 hours or 24hours on different numbers of HeLa cells. The number of cells per well indicates the one at the time of cell plating. See the step "1. Culture Cells and Treatment with Compounds" of "Assay Procedure" in the section "Detailed Protocol" above.





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