PRODUCT DATA SHEET

BrdU Cell Proliferation ELISA

Non-isotopic immunoassay for the quantitation of BrdU incorporation into newly synthesized DNA of actively proliferating cells

Cat. No. KT-076

For Research Use Only. Not for Use in Diagnostic Procedures.

PRODUCT INFORMATION

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PRODUCT
The ***K-ASSAY®*** BrdU Cell Proliferation ELISA is a non-isotopic immunoassay for the quantitation of bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA of actively proliferating cells. This assay is for research use only, not for use in diagnostic procedures.

DESCRIPTION
Evaluation of cell cycle progression is essential for investigations in many scientific fields. Measurement of [^{3}H]-thymidine incorporation as cells enter S phase has long been the traditional method for the detection of cell proliferation. Subsequent quantification of [^{3}H]-thymidine is performed by scintillation counting or autoradiography. This technology is slow, labor intensive and has several limitations including the handling and disposal of radioisotopes and the necessity of expensive equipment.

In an alternative method to [^{3}H]-thymidine uptake, the thymidine analog BrdU is used in place of [^{3}H]-thymidine and is incorporated into newly synthesized DNA strands of actively proliferating cells. Following partial denaturation of double stranded DNA, BrdU is detected immunochemically allowing the assessment of the population of cells, which are actively synthesizing DNA.

The ***K-ASSAY®*** BrdU Cell Proliferation ELISA involves incorporation of BrdU into cells cultured in microtiter plates using the cell layer as the solid phase. The resultant assay is sensitive, rapid, easy to perform and applicable to high sample throughput. In addition to evaluation of cell proliferation, information such as cell number, morphology and analysis of cellular antigens can be obtained from a single culture.

PRINCIPLE
The ***K-ASSAY®*** BrdU Cell Proliferation ELISA involves incorporation of BrdU into cells cultured in microtiter plates using the cell layer as the solid phase. During the final 2 to 24 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. This is all done in one step by treatment with Fixing Solution. BrdU 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 (HRP)-conjugated goat anti-mouse antibody is added, which binds to the BrdU Antibody.

The HRP catalyzes the conversion of the chromogenic substrate tetramethylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent), the intensity of which is proportional to the amount of incorporated BrdU in the cells. The colored reaction product is quantified using a spectrophotometer.

COMPONENTS
- BrdU Reagent: 500X solution of BrdU, 15 µL
- Fixing Solution: 40 mL
- BrdU Antibody, 200X: 120 µL stock solution of the monoclonal antibody to BrdU
- Antibody Diluent: 25 mL solution for dilution of the BrdU Antibody
- Peroxidase Anti-Mouse IgG (2,000X), 15 µL of a peroxidase conjugated goat anti-mouse IgG antibody
- Conjugate Diluent: 25 mL Buffer for dilution of peroxidase conjugated goat anti-mouse IgG antibody
- TMB Substrate: 25 mL, Ready to use TMB solution
- Wash Buffer, 50X: Solution of buffered Tris and Surfactant, 90 mL
- Stop Solution: 25 mL of 2.5 N sulfuric acid

Materials or equipment required but not provided

- 2-20 µL, 20-200 µL, and 200-1,000 µL precision pipettes with disposable tips
- Wash bottle or multichannel dispenser for washing
- 2 L graduated cylinder
- PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4-$7H_2O$, 1.4 mM KH_2PO_4)
- De-ionized or distilled H_2O
- Spectrophotometer capable of measuring absorbance in 96-well plates using dual wavelength of 450-540 or 450-595 nm or a single read at 450 nm.
- Tissue culture microtiter plate (96 well culture dish)
- Sterile reagent troughs
- Micro syringe filter (0.2 µm)
- Syringe

PROTOCOLS

1. Recommended Controls

Two types of controls are recommended to insure validity of the experiment.

1. Blank: Add only tissue culture media (no cells).
2. Background: Cells are present in the wells but do not add the BrdU Reagent.

2. Cell Plating

Seed cells using a sterile 96-well tissue culture plate, cells are plated at 2 x 10^5 cells/mL in 100 µL/well of appropriate cell culture media. Some of the wells on the plate should be set aside for several controls. These should include wells that do not receive cells (media alone), and wells which contain cells but will not receive the BrdU reagent (assay background).

3. Addition of Test Reagent

The test reagent can be a cell proliferation enhancer or alternatively, can be an inducer of growth inhibition or arrest. The test reagent should be made in the cell media at twice the final concentration (2X). 100 µL/well is added on top of the cell wells. The test reagent should be tittered in the assay to determine optimum concentration for inducing cell proliferation or growth arrest. The length of time for test reagent incubation should also be determined for your system (time course study). BrdU addition (see Step 4 below) will occur 2-24 hours prior to the end of the test reagent incubation.

4. Addition of BrdU Reagent

BrdU will be incorporated into proliferating cells and should be added at least 2 hours prior to the end of the test reagent incubation period. Better sensitivity and signal to noise ratios are obtained when longer BrdU labeling times are used. Dilute the 500X concentrated stock 1:500 by adding 8 µL of BrdU stock to 4 mL of cell media. Pipette 20 µL of the diluted BrdU label to the appropriate wells. Reminder: a series of wells should be set aside that do NOT receive the BrdU label (-BrdU control for determining assay background). Incubate the assay 2-24 hours.

5. Fix and Denature Step and Storage of Fixed Plates

For detection of the BrdU label by the anti-BrdU monoclonal antibody, it is necessary to fix the cells and denature the DNA using the solution provided in the kit (Fixing Solution). There is no need to spin the cells prior to addition of the fixing solution. However, if suspension cells are being used, better precision is obtained if the cell plates are spun in a centrifuge prior to the fix/denature step. Plates may be fixed (steps 6-7) and stored at 4°C for assay at a later time. Place dried plates in a sealed dry plastic bag. Zip-lock type bags or heat sealed plastic bags are suitable for this purpose. Plates are stable for at least one month when properly stored.

6. Adherent and Suspension Cells (No-Spin Procedure)

Aspirate the media from the cell wells (this can be done mechanically or the plate can be inverted over appropriate reservoir and blotted on absorbent paper towels). Add 200 µL/well Fixing Solution and incubate at room temperature (RT) for 30 minutes. Aspirate the Fixing Solution and blot the plate dry.

Note: Fixed plates can be stored for up to 1 month at 4°C if stored in a heat sealed or zip-lock bag. If storing your plates for future use, make sure the plates are blotted well and are very dry (NO Fixing Solution should be left in the wells).

7. Suspension Cells (Spin Fix/Denature Procedure)

Spin the plates in the centrifuge (using appropriate centrifuge microtiter plate holders) for 5 minutes at 1,000 rpm. Aspirate the media and add 200 µL/well Fixing Solution. Incubate for 30 minutes at RT. Aspirate the Fixing Solution and blot the plates dry. The assay can be run immediately or plates may be stored for future use (see note above).

8. Wash Step
Dilute the 50X Wash Buffer 1:50 by adding 40 mL to 1.96 liters of distilled water. A microtiter plate washer may be used for all wash steps OR a squirt bottle for manual plate washing may also be used. In either case, the wells should be filled completely with Wash Buffer. **Wash the plate three times with 1X Wash Buffer prior to adding BrdU Antibody.** Aspirate the wash solution after the final wash and blot dry on paper towels.

9. Preparation and Addition of BrdU Antibody
The BrdU Antibody is provided as a 200X concentrated stock solution. Dilute 1:200 by adding 55 µL of the BrdU Antibody to 11 mL of Antibody Diluent. Add 100 µL/well and incubate for 1 hour at RT.

10. Wash Step
Wash as in Step 8 above.

11. Preparation and Addition of the Peroxidase Anti-Mouse IgG conjugate
The Peroxidase Anti-Mouse IgG is provided as a concentrated stock solution. Dilute the Peroxidase Anti-Mouse IgG conjugate 1:2,000 by adding 6 µL to 12 mL of Conjugate Diluent provided. Once diluted, this solution should be filtered using a 0.22 µm syringe filter. This lowers the assay background and improves precision. Pipette 100 µL/well and incubate for 30 minutes at RT.

12. Wash Step and Final Water Wash
Wash as in Step 8 above. **Perform a final water wash by flooding the entire plate with distilled water.** Pat dry on absorbent paper towels.

13. Addition of TMB Substrate
Pipette 100 µL/well TMB Substrate and incubate for 30 minutes at RT **in the dark**. Positive wells will be visible by a blue color, the intensity of which is proportional to the amount of BrdU incorporated in the proliferating cells.

14. Addition of Stop Solution and Reading of the Plate
Stop the reaction by pipetting 100 µL of acid Stop Solution provided to every well. The color of positive wells will change from blue to bright yellow. Read the plate using a spectrophotometric microtiter plate reader set at a dual wavelength of 450/550 nm (alternatively 450/540 nm or 450/595 nm may be used or a single read at 450 nm).

SUMMARY PROTOCOL

1. Cell Plating - no Test Reagent/Drug (skip step 3 below)	Seed cells at 1-2x10^5 cells/mL, 100 µL/well
2. Cell Plating - with Test Reagent/Drug (see step 3 below)	Seed cells at 0.5-4x10^5 cells/mL, 100 µL/well
3. Addition of Test Reagent(s)/Drug	Add 100 µL/well, 2X concentration desired
4. Addition of BrdU Reagent	Dilute 500X stock BrdU, add 20 µL/well (be sure to include a No BrdU control)
5. Incubate	2-24 hours
6. Fix and Denature ***Adherent & Suspension Cells*** No-Spin Procedure ***Suspension Cells*** Spin Procedure	Aspirate (or flick) the media from the cell wells Add 200 µL/well Fixing Solution Incubate 30 minutes at RT Aspirate the Fixing Solution and blot the plates dry Spin the plates for 5 minutes at 1,000 rpm. Aspirate the media, add 200 µL/well Fixing Solution Incubate for 30 minutes, RT Aspirate the Fixing Solution and blot the plates dry
7. Wash Step	Wash X3 with 1X Wash Buffer and blot dry
8. BrdU Antibody	Add 100 µL/well of diluted BrdU Antibody
9. Incubate	1 hour at RT
10. Wash Step	Wash X3 with 1X Wash Buffer and blot dry
11. Conjugate Addition	Add 100 µL/well Peroxidase Anti-Mouse IgG conjugate
12. Incubate	Incubate for 30 minutes at RT

13. Wash Step and Final Water Wash	Wash as above. **Perform a final distilled water wash by flooding the entire plate with distilled water.** Pat dry on absorbent paper towels.
14. Development	Add 100 µL/well TMB Substrate
15. Incubate	30 minutes at RT **in the dark**
16. Stop	Add 100 µL of acid Stop Solution to every well
17. Read	Read at 450/550 nm

STORAGE

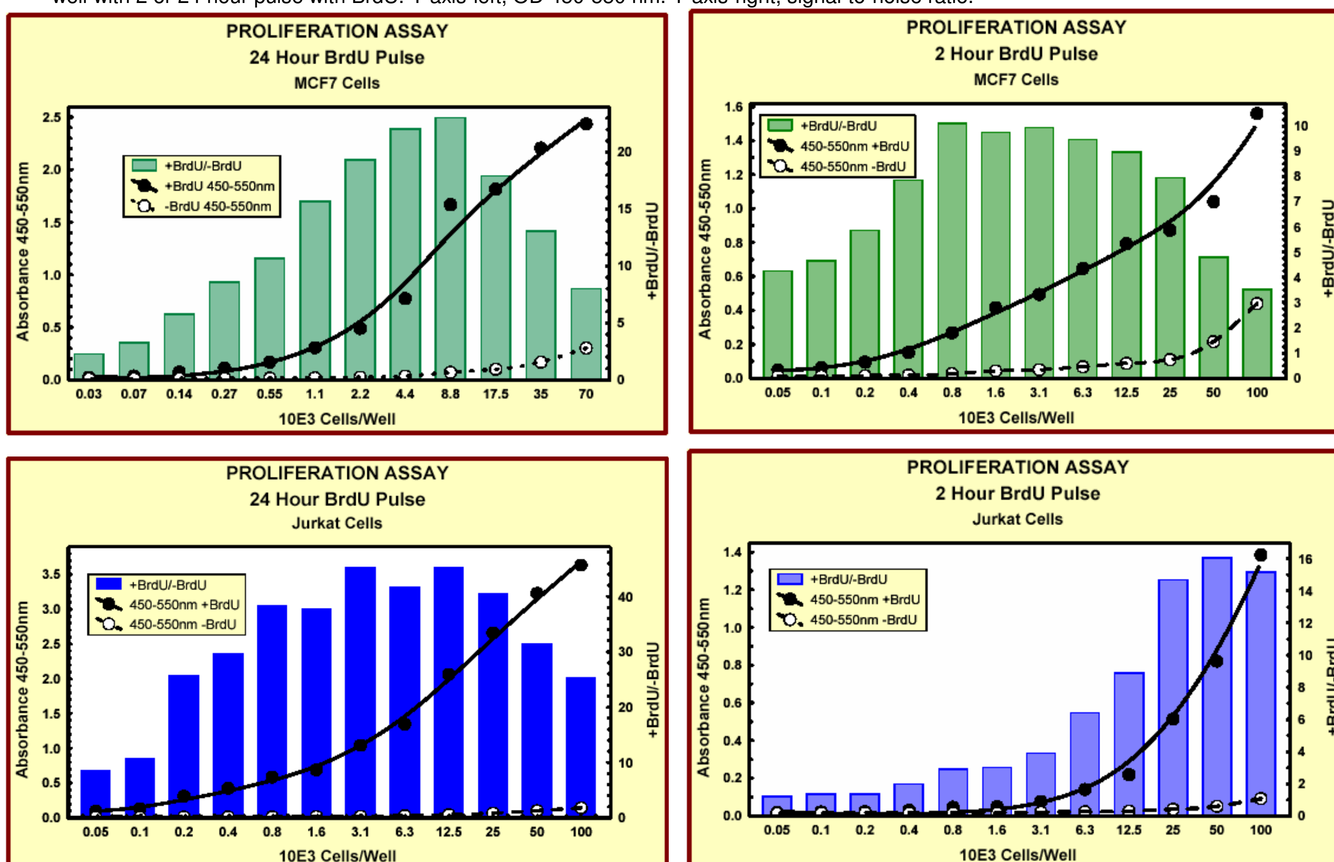
Store all kit components at 4˚C.

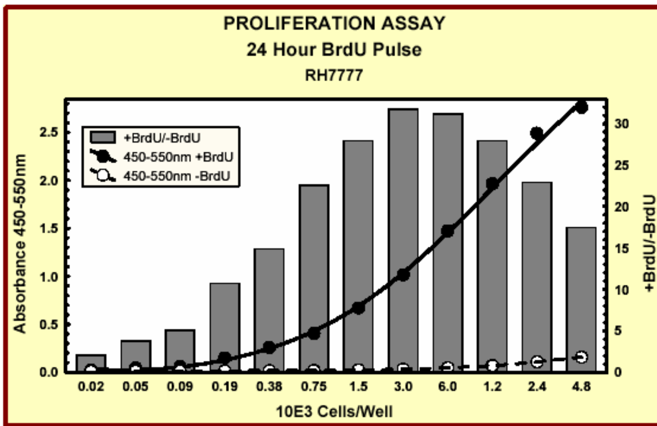
Before first use, remove the Fixing Solution and place at RT for at least 4 hours prior to use. Precipitates that may form should go back into solution.

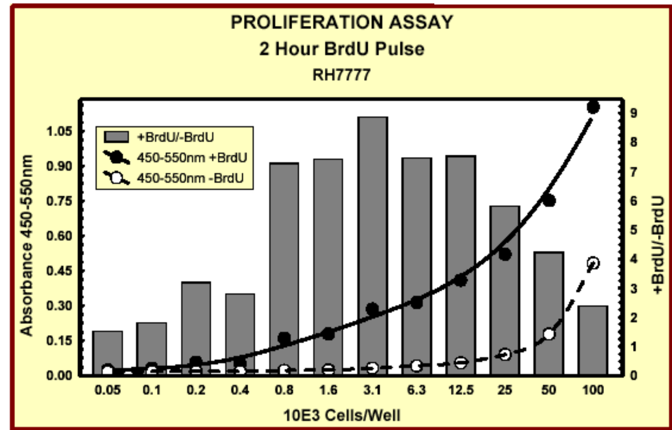
MODEL SYSTEMS

A sensitivity study was performed using the Jurkat (non-adherent) and RH7777 and MCF7 (adherent) cells. Various concentrations of the cells were plated and cultured for 24 hours. The cells were incubated with BrdU Label for 24 hours and incorporated BrdU was detected with the ***K-ASSAY®*** BrdU Cell Proliferation ELISA. There was a direct relationship between the signal and number of proliferating cells at all cell concentrations (Figure 1). The sensitivity of this assay was determined to be 40 cells/well using the mean signal of zero plus two standard deviations; that is, the smallest number of cells that may be distinguished from zero with 95% confidence. Using a two-hour BrdU labeling, 100 cells/well was also significantly higher than the blank control.

FIGURE 1.
K-ASSAY® BrdU Cell Proliferation ELISA, detection of variable numbers of MCF7, Jurkat (non-adherent), or RH7777 (adherent) cells per well with 2 or 24 hour pulse with BrdU. Y axis-left, OD 450-550 nm. Y axis-right, signal-to-noise ratio.







PRECAUTIONS AND RECOMMENDATIONS

1. Do not expose reagent to excessive light.
2. Wear disposable gloves and eye protection.
3. Do not use the kit beyond the expiration date.
4. Do not mix reagents from different kit lots.
5. Do not pipette by mouth or ingest any of the reagents.
6. The buffers and reagents used in this kit contain anti-microbial and anti-fungal reagents. Care should be taken to prevent direct contact with these products.
7. Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
8. Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Dispose of samples properly.

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