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Technical Datasheet

EZcount™ BrdU Cell Assay Kit

Product Code: CCK031

1. Introduction:

Cell proliferation and death are essential processes for tissue generation and regeneration, organ development etc. in mammals and are usually under stringent control of extra and intracellular factors. Non-physiological alterations in levels of these factors lead to anomalous cytogenetic behavior of cells which in turn leads to cell transformation, uncontrolled cell growth - the initiating event for cancer development. Pharmaceutical research is hence largely focused on effects of drugs, cytotoxic agents and biologically active compounds which affect cytogenetics.

Multiple procedures are available for determination of cell proliferation and cytotoxicity. Simple and cheap methods for estimating cell viability (or death) are Trypan Blue exclusion and Erythrocin B staining. However, these methods are not sensitive enough and cannot be used for high throughput screening. Measuring the uptake of radioactive substances, usually tritium-labeled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances. Tetrazolium salts have been used to develop a quantitative colorimetric assay to estimate mammalian cell survival and proliferation. The assay detects living, but not dead cells and the signal generated is dependent on the metabolic state of the cells. This method can therefore be used to measure cytotoxicity, proliferation or activation. The results can be read on a multi-well scanning spectrophotometer or a standard ELISA reader and show high degree of precision.

2. About the Assay:

The EZcount™ BrdU Cell Assay kit is designed for determination of cell proliferation and / or effect of cytotoxic agent. When cells are cultured with labeling medium containing Pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU), BrdU gets incorporated in the DNA of proliferating cells in place of thymidine. BrdU labelled proliferating cells can be detected using anti- BrdU (mouse) detection antibody. The labeling medium is removed and cells are fixed and DNA is denatured using fixing/ denaturing solution to make BrdU accessible to the anti-BrdU antibody. Further, HRP conjugated secondary antibody, goat anti-mouse IgG, binds to the primary anti-BrdU antibody. Later, HRP substrate, TMB is added for color development. The magnitude of the absorbance for the developed color directly correlates to the quantity of BrdU incorporated into cells, which is a direct indication of cell proliferation.

3. Applications:

- **Cell proliferation:** Quantification of changes in proliferative activity of cells caused by trophic factors, cytokines and growth promoters.
- **Cell cytotoxicity**: Evaluation of effects of inhibitors or inducers of apoptosis, cytotoxic reagents, carcinogens and toxins.
- **Drug discovery**: High-throughput screening of various anti-cancer drugs.

4. Kit contents:

This kit is sufficient for 1000 assays (Ten 96-well microplates)

Following table enlists the components provided in the kit. Store them at their respective temperature after receiving the kit.

Code	Description	Quan-tity	Shipping tempe-rature	Storage tempe-rature
CCK031(A)	BrdU Solution (1000X)	0.1 mL	-20 °C	-20 °C
CCK031(B)	Fixing/ denaturing solution	100 mL	2-8 °C	RT
CCK031(C)	Anti- BrdU Detection Antibody (Primary)	0.05 mL	-20 °C	-20 °C
CCK031(D)	Goat Anti-Mouse IgG, HRP-Conjugated Antibody (Secondary)	0.015 mL	-20 °C	-20 °C
CCK031(E)	Diluent powder for antibodies	8 x 0.5 g	2-8 °C	2-8 °C
CCK031(F)	Diluent for antibodies	2 x 100 mL	2-8 °C	2-8 °C
CCK031(G)	TMB Substrate	2 x 50 mL	2-8 °C	2-8 °C
CCK031(H)	Stop Solution	100 mL	2-8 °C	2-8 °C
CCK031(I)	Diluent Powder for Wash Buffer	4 x 3 g	2-8 °C	2-8 °C
CCK031(J)	Wash buffer (10X)	100 mL	2-8 °C	2-8 °C

•Quantities supplied in excess to compensate operational losses

5. Materials required but not provided in the kit:

- Cells in appropriate medium
- Adjustable pipettes and a repeat pipettor
- Flat bottom 96-well microtiter plate for culturing the cells
- 96-well plate reader capable of measuring the absorbance

6. General guidelines:

It is important to optimize experimental factors like cell density, incubation time, media composition and concentration of the agents under investigation prior to use of EZcount™ BrdU Cell Assay Kit.

Assay controls

Contents	Blank	Background control
Culture medium	100 µL	-
Cells	-	100 µL
BrdU working Solution	100 µL	-
Anti-BrdU Antibody	100 µL	100 µL
HRP conjugated Antibody	100 µL	100 µL

- Include appropriate assay controls i.e.
  1. Blank: Medium with BrdU and both antibodies
  2. Background control: Medium with cells and both antibodies, no BrdU

Accuracy

- To obtain statistically significant data, perform the assay in triplicates or more.
- Accuracy of the assay depends on pipetting skills of the personnel. Inappropriate addition and mixing practices may result in erroneous and false-positive or false-negative results.
- Use of a repeating pipettor is recommended to deliver the reagents to the wells. This saves time and helps maintain more precise incubation times.
- Pipette tip should be equilibrated with the reagent before use. This is carried out by slowly filling up the tip with reagent and gently expelling the contents, 2-3 times.

Incubation period

- Different cell lines have different properties such as metabolic activity and doubling time. For this reason, plating density and incubation period for every cell line should be optimized to obtain the results in linear range.

Temperature

- Temperature affects the performance of the assay because of its effect on enzymatic rates. It is crucial to run the assay at a uniform temperature to ensure reproducibility across a single plate or among stacks of several plates. Since absorbance or fluorescence readings are measured at room temperature, it is important to ensure adequate equilibration of assay plates after removal from a 37 °C incubator to avoid differential temperature gradients. Stacking large numbers of assay plates in close proximity should be avoided to ensure complete temperature equilibration.

Measurement of absorbance

- Absorbance can be read with a filter in the wavelength range of 450 nm (primary wavelength).
- Reference wavelength (for non-specific readings) should be >600 nm.

7. Directions for use:

Users are advised to review entire procedure before starting the assay

This kit for total 1000 assays is recommended to be used for preparing working solutions for minimum 250 assays at once. These prepared working solutions are suggested to be used within a span of one week of preparation.

7.1 Preparation of BrdU working solution (1X):

1. Working solution should be prepared just prior to adding to the cells.
2. Thaw the vial of BrdU Labeling Reagent CCK031(A) at 2 – 8 ºC.
3. Mix 25 µL of BrdU labeling reagent with 24975 µL of growth media containing (2-10 % FBS).
4. This amount will be sufficient for 250 wells (100 µL/well).

7.2 Preparation of working antibody diluent solution:

1. For primary antibody working solution, empty the contents of 1 bottle of diluent powder for antibodies CCK31(E) in 24987.50 µL of antibody diluent CCK31(F). Vortex this mixture for appropriate mixing of the components. Later, add 12.5 µL anti-BrdU antibody CCK31(C) in this solution and pipette well for proper mixing.
2. For secondary antibody working solution, empty the contents of 1 bottle of diluent powder for antibodies CCK31(E) in 24996.43 µL of antibody diluent CCK31 (F). Vortex this mixture for appropriate mixing of the components. Later, add 3.57 µL HRP conjugated antibody CCK31(D) in this solution and pipette well for proper mixing.
3. This amount of each antibody solution will be sufficient for 250 wells (100 µL/well).

7.3 Preparation of working washing solution 1X:

1. For step 5 of the protocol flow chart (Section 10), prepare 1X Wash buffer by adding 2.5 mL of 10X Wash buffer CCK031(J) in 22.5 mL of purified water and mix well. This amount will be sufficient for 250 assays for 1 wash each (100 µL/well) after BrdU incubation.
2. For steps 9 and 11 of the protocol flow chart, (Section 10) prepare 1X Wash buffer by emptying the contents of 1 bottle of diluent powder for Wash buffer CCK031(I) in a 200 mL bottle. Further, add 15 mL of 10X Wash buffer CCK031(J) in this bottle and vortex for appropriate mixing. Make up the volume to 150 mL with purified water, mix well.
3. This amount will be sufficient for 250 assays for 3 washes each (100 µL/well) after primary and secondary antibody treatment.

7.4 Preparation of cells:

1. Always use freshly harvested cells for assay. Seed the cells in a cell culture flask or dish in an amount appropriate for the assay and incubate at 37 ˚C in a 5 % CO_2 environment. Allow the cells to grow up to 24 hours or till confluency is reached. ***(Note**: Quantity of the cell suspension to be seeded in the medium depends upon doubling time of individual cell line and seeding density to be used in assay).*

7.5 Assay procedure:

1. Seed 100 µL cell suspension in a flat bottom 96- well plate at the pre-optimized cell density, with or without the cell growth modifying agent.

***Note**:*

a) If the cell growth modifying agent is a cytokine, metabolite, growth factor or any other compound, add its required quantity in the culture system.

b) If the cell growth modifying agent is any kind of radiation or waves, treat the cells with them for required period of time.

2. Incubate the cells for 24-72 h based on the growth profile of cells. After incubation, discard the media.
3. Add 100 µL/well BrdU working solution and incubate the plate at 37 ºC in a 5 % CO_2 atmosphere.
4. Allow the cells to grow up from 1 to 24 h or as per cell growth optimization.
   Note: Incubation time varies depending on cell line and action of test molecule. It should be optimized accordingly.
5. After incubation period, remove the media by flicking off the plate and rinse with 100 µL of 1X Wash buffer without diluent powder.
6. Add 100 µL fixation/denaturation solution CCK031(B) in each well carefully along the side of the well and incubate for 30 minutes at room temperature.
7. Remove the solution by flicking off the plate and allow it to air dry for a minute.
8. Add 100 µL working primary antibody solution to each well and incubate for 1 h at room temperature. Remove the antibody solution by flicking off the plate and rinse the wells 3 times with 1X Wash buffer as mentioned in step 9 of Procedure table (Section 10).
9. Add 100 µL working secondary antibody solution to each well and incubate for 30 min at room temperature. Remove the antibody solution by flicking off the plate and rinse the wells 3 times with 1X Wash buffer as mentioned in step 11 of Procedure table (Section 10).
10. Add 100 µL TMB substrate and incubate at room temperature for 5-30 minutes.
    Note: Incubation time with TMB substrate can be optimized till the color development is sufficient.
11. Add 100 µL stop solution in each well.
12. Read the absorbance on spectrophotometer or an ELISA reader by using 450 nm (main wavelength) and >600 nm (reference wavelength) as differential filter.
13. Calculate normalized absorbance values by

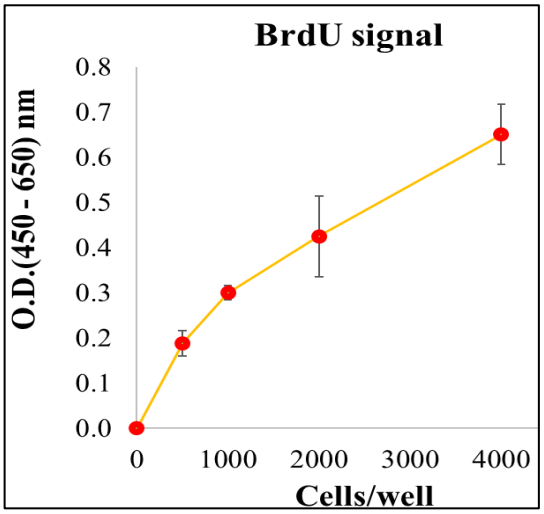
subtracting average >600 nm absorbance values from average 450 nm absorbance values of corresponding wells.

14. Plot the absorbance values on the Y-axis and your experimental parameters on the X-axis.

8. Storage and Shelf life:

- Store BrdU solution at -20 ºC. DO NOT FREEZE-THAW REPEATEDLY.
- Store both antibodies and their working solution at -20 ºC. Working solution can be used till one week after preparation.
- Store both diluent powders, diluent for antibodies, TMB substrate and stop solution at 2-8 ºC.
- Store fixing/denaturing solution at room temperature.
- Store working 1X wash buffers at 2-8 ºC to be used till one week after preparation.
- Use all components before expiry date given on the kit label.

9. Performance Characteristics



CHO-K1 cells were serially diluted and treated with BrdU reagent provided in EZcount™ BrdU Cell Assay Kit, in a 96- well plate. After incubation for 48 h in a humidified incubator at 37ºC, 5% CO_2, cell incubation was done for 2.5 h in BrdU working solution and then assay procedure was performed.

10. Procedure at a Glance

Step	Details	Volume per well	Duration	Repeats	Temperature
1	Seeding	100 µL	-	-	-
2	Addition of test molecule	Assay specific			
3	Addition of BrdU working solution	100 µL	Depending upon the experimental requirement	-	37 ºC, 5% CO_2
4	Removal of medium - flick off	-	-	-	-
5	Rinsing with Wash buffer (1X)	100 µL	~ 1 minute	-	-
6	Fixation and denaturation	100 µL	30 minutes	-	Room temperature
7	Removal of medium - flick off	-	-	-	-
8	Anti-BrdU-antibody addition	100 µL	60 minutes	3 - 5	Room temperature
9	Washing with Wash buffer containing diluent (1X)	100 µL	1 minute / wash	-	Room temperature
10	HRP conjugated-antibody addition	100 µL	30 minutes	3 - 5	Room temperature
11	Washing with Wash buffer containing diluent (1X)		1 minute / wash		
12	Addition of TMB solution	100 µL	5 - 30 minutes	-	Room temperature
13	Stop solution	100 µL	-	-	Room temperature
14	Absorbance measurement (450nm / 650nm)	-	-	-	-

11. Troubleshooting points:

Problem	Cause	Solution
Very low absorbance values	Very low cell density	Repeat the assay with high cell densities
	Short incubation period	Repeat the assay with longer incubation Period.
	Short incubation period with fixation/ denaturation solution	Increase incubation period with fixation / denaturation solution to 60 minutes.
	Short incubation period with antibody	Increase incubation period with antibody.
	Short incubation period with TMB substrate	Increase incubation period with TMB Substrate.
Very high absorbance values	Very high cell density	Repeat the assay with reduced cell densities.
	Long incubation period	Repeat the assay with reduced incubation Period.
	Long incubation period with fixation/ denaturation solution	Decrease incubation period with fixation/ denaturation solution.
	Long incubation period with antibody	Decrease incubation period with antibody
	Long incubation period with TMB substrate	Decrease incubation period with TMB Substrate.
Random absorbance values/ poor consistency of replicates	Inefficient pipetting techniques	Perform the assay using automated electronic pipettes for seeding the cell suspension and adding the reagents.
	Test compound under study which is responsible for improper response of the cells.	Refer to the pharmacological properties of the compound.
Blank/ medium control (i.e. medium without cells) give high absorbance readings	Non-specific binding of Antibody conjugate	Avoid mixing of tips. Repeat the assay for rechecking the performance. Verify appropriate addition of BrdU in the wells to avoid undesirable signals in blank.