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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 antibody. The labeling medium is removed, and cells are fixed and DNA is denatured using fixation/denaturation solution to make BrdU accessible to the Anti-BrdU-HRP antibody. 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)

Code	Description	Quantity	Storage
CCK031(A)	BrdU labeling reagent	1ml	-20°C
CCK031(B)	Fixation-denaturation solution	2 x100ml	2-8°C
CCK031(C)	Anti- BrdU-HRP	0.2ml	2-8°C
CCK031(D)	Antibody dilution solution	100ml	2-8°C
CCK031(E)	Washing buffer 10X	100ml	RT
CCK031(F)	TMB Substrate solution 10X	10ml	2-8°C
CCK031(G)	BrdU Blocking solution 1X	100ml	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
- Phosphate buffered saline
- Stop solution: 1M H_2SO_4

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

- Include appropriate assay controls i.e.
  1. Blank: Medium with BrdU and Anti- BrdU-HRP
  2. Background control: Medium with cells and Anti-BrdU-HRP

Contents	Blank	Background control
Culture medium	100µl	-
Cells	-	100µl
BrdU working solution	10µl	-
Anti-BrdU-HRP	100µl	100µl

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, several 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 reading 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 450nm (primary wavelength).
- Reference wavelength (for non-specific readings) should be >600nm.

7. Directions for use:

Users are advised to review entire procedure before starting the assay

7.1 Preparation of BrdU working solution

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 – 8ºC.
3. Mix 10µl of BrdU labeling reagent with 990µl of media containing 10% FBS.
4. This amount will be sufficient for one 96 well plate (10µl/well).

7.2 Preparation of antibody working solution:

1. Add 20µl of Anti-BrdU-HRP CCK031(C) to 9980µl of antibody dilution solution CCK031(D).
2. This amount will be sufficient for one 96 well plate (100µl/well).

7.3 Preparation of working washing solution 1X:

1. Add 10ml of washing buffer 10X CCK031(E) to 90ml of water.
2. This amount will be sufficient for one 96 well plate (100µl/well).

7.4 Preparation of working TMB substrate 1X:

1. Add 1ml TMB substrate solution 10X CCK031(F) in 9ml of water.
2. This amount will be sufficient for one 96 well plate (100µl/well).

7.5 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 confluence 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).*

7.6 Assay procedure:

1. Seed 100µl 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.

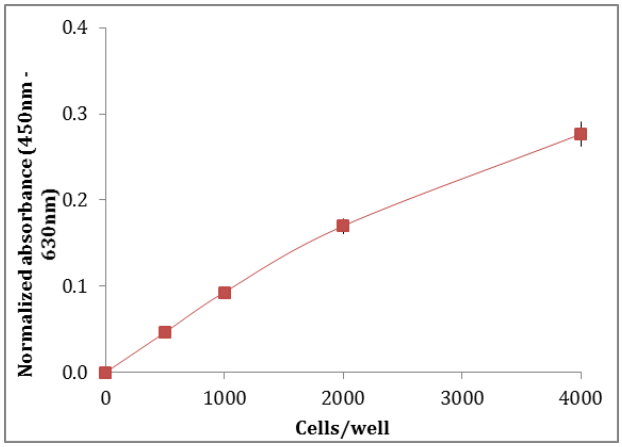
b) If the cell growth modifying agent is any kind of radiation or waves, treat the cells with them for required period of time.

2. Add 10µl/well BrdU working solution and incubate the plate at 37ºC in a 5% CO_2 atmosphere.
3. Allow the cells to grow up to 24 hours or till confluence is reached.
   Note: Incubation time varies depending on cell line and action of test molecule. It should be optimized accordingly.
4. After incubation period, remove the media by flicking off the plate and rinse with DPBS.
5. Add 200µl Fixation-denaturation solution CCK031(B) in each well carefully along the side of the well and incubate for 30 minutes at room temperature.
6. Remove the solution by flicking off the plate and add 100µl working washing buffer in each well carefully along the side of the wells.
7. Wash the wells for 1 minute on the shaker at 200rpm. Repeat the washing step 3 - 5 times.
   Note: Number of washes can be increased or decreased as required. It should be optimized depending on the experimental requirements.
8. OPTIONAL – Blocking by blocking solution
   Add 100µl Blocking solution CCK031(G) in each well carefully along the side of the well and incubate for 30 minutes at room temperature.
9. OPTIONAL - Wash the wells for 1 minute on the shaker at 200rpm. Repeat the washing step 3 - 5 times.
10. Add 100µl working antibody solution to each well and incubate for 1 hour at room temperature.
11. Remove the antibody solution by flicking off the plate and rinse the wells 3 times as mentioned in step 6 and 7.
12. Add 100µl 1X TMB substrate and incubate at room temperature for 5-30 minutes.
    Note: Incubation time with TMB substrate can be optimized till the colour development is sufficient.
13. To optimize incubation time with substrate, measure the absorbance of plate at 370nm (main wavelength) and 492nm (reference wavelength) at various time points such as 10, 20, 30 minutes.
14. Add 25µl stop solution in each well.
15. Read the absorbance on spectrophotometer or an ELISA reader by using 450nm (main wavelength) and >600nm (reference wavelength) as differential filter.
16. Calculate normalized absorbance values by subtracting average 450nm absorbance values from average >600nm absorbance values of corresponding wells.
17. Plot the absorbance values on the Y-axis and your experimental parameters on the X-axis.

8. Storage and Shelf life:

- Store BrdU labeling reagent and working BrdU solution at -20ºC. DO NOT FREEZE-THAW REPEATEDLY.
- Store Anti-BrdU-HRP, Antibody dilution solution, Blocking solution and 10X TMB substrate solution at 2-8ºC.
- Store 10X washing buffer, working washing buffer and fixation-denaturation solution at room temperature.
- Store working antibody solution and substrate solution 1X at 2-8ºC.
- Use before expiry date given on the label.

9. Performance characteristics:



BHK21 cells were serially diluted and treated with BrdU reagent provided in EZcount™ BrdU Cell Assay Kit, in a 96-well plate. After incubation for48 -72 in a humidified incubator at 37ºC, 5% CO_2, BrdU assay procedure was performed.

10. Procedure at a Glance

Step	Details	Volume per well	Duration	Repeats	Temperature
1	Seeding	100ul	-	-	-
2	Addition of test molecule	Assay specific			
3	Addition of BrdU working solution	10ul	Depending upon the experimental requirement	-	37°C, 5% CO_2
4	Removal of medium - flick off	-	-	-	-
5	Wash with DPBS	200ul	~ 1 minute	-	-
6	Fixation and denaturation	200ul	30 minutes	-	Room temperature
7	Washing with working washing solution	100ul	1 minute / wash at 200rpm	3 - 5	Room temperature
8	Blocking – OPTIONAL	100ul	30 minutes	-	Room temperature
9	Washing with working washing solution	100ul	1 minute / wash at 200rpm	3 - 5	Room temperature
10	Anti-BrdU-HRP addition	100ul	60 minutes	-	Room temperature
11	Washing with working washing solution	100ul	1 minute / wash at 200rpm	3 - 5	Room temperature
12	Addition of 1X TMB	100ul	5 - 30 minutes	-	Room temperature
13	Stop solution	25ul	-	-	Room temperature
14	Absorbance measurement (450nm / 630nm)	-	-	-	-

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 minute
	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	Add blocking buffer (100µl/well) and incubate for 30 minutes at room temperature.
		Repeat the experiment with low seeding density.