HiMedia CellCulture
Enabling Breakthroughs

Product Information

EZBlue™ Cell Assay Kit

Product Code: CCK004

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. Quantitative measurement of reduction of Resazurin dyes is an alternate simple, safe and accurate method for determination of cell proliferation. This assay is similar to tetrazolium assay, except that it has the optional advantages of using fluorescent detection methods and omission the of solubilization step.

2. About the Assay

The EZBlue™ Cell Assay Kit is based on reduction of the water soluble, redox dye EZBlue™. The dye itself is dark blue in color and has intrinsic fluorescence. The fluorescence changes when EZBlue™ is reduced by metabolically active cells to the pink resorufin product. The spectral properties of resorufin allows the molecule to be detected using either absorbance or fluorescence. However fluorescence is the preferred method of evaluating the end-point of the reaction as it is more sensitive than absorbance. Dead or non-viable cells are incapable of this reduction and do not contribute to the fluorescence generated by viable cells. The specific mechanism responsible for the reduction of EZBlue™ is unknown, but probably involves the same cellular processes which generate equivalents such as NADH. In comparison to other methods of evaluating cell viability, which require tedious steps like reagent transfer, washing, crystal dissolution, repeated aspiration etc., the use of EZBlue™ is a single step process of addition of the reagent to cells. This ensures a rapid assay with minimal errors and facilitates its use in large scale hi-throughput screenings.

3. Applications

- **Cell proliferation:** Quantification of changes in proliferative activity of cells caused by trophic factors, cytokines, radiations 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

Contents		Kit Code	Storage
Code	**Description**	**CCK004-2500***	
TCL089	EZBlue™ Solution	25ml x 1 bottle	2-8°C

* Sufficient for 25 microplates (2500 assays).

5. Materials required but not provided in the kit

- Cells in appropriate medium
- Adjustable pipettes and a repeat pipettor
- 96-well plate for culturing the cells

- 96-well plate reader or spectrophotometer capable of measuring the absorbance at 570nm or fluorescence at 560nmEX/590nmEM.

6. General guidelines

It is recommended to optimize the experimental factors like cell density, incubation times, media composition and concentration of agents under investigation prior to use of EZBlue™ Cell Assay Kit.

Assay controls

- Include appropriate assay controls i.e.
  1. Medium control (medium without cells)
  2. Cell control (medium with cells but without the experimental drug/ compound)
  3. Vehicle control (medium containing the experimental drug or compound but no cells)

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.

Incubation period

- Different cell lines may respond in different manner to EZBlue™ depending on the properties of the cell lines, such as metabolic activity and doubling time. For this reason plating density and incubation period for every cell line should be optimized to obtain results in a linear range.
- Although EZBlue™ does not affect the cell viability; incubation of cells with EZBlue™ for extended period may lead to reduction of fluorescent resorufin to colorless hydro-resorufin. Therefore determining the exact incubation period is essential.

Sterility

- For slow growing cell lines, longer incubation times are recommended. In such case, care should be taken to maintain sterile environment throughout the incubation period. Contamination leads to incorrect results as microbial contaminants also have capacity to reduce EZBlue™ reagent. This may lead to misinterpretation of the results and failure of the entire assay.

Culture medium

- pH of the medium used for the assay should be between 7.0 to 7.4. Presence of 10% fetal bovine serum has no effect on the results when reduction is measured spectrophotometrically. However, it causes quenching of the fluorescence.

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 the 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.

7. Directions for use

Users are advised to review entire procedure before starting the assay

7.1 Preparation of reagent

Bring the reagent to room temperature before use.

7.2 Preparation of cells

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 for up to 24 hours or till confluence is reached. Harvest the cells and use for assay.

***(Note**: The quantity of the cell suspension to be seeded in the medium depends upon doubling time of individual cell lines and seeding density to be used in the assay.)*

7.3 Procedure for determination of optimum cell density and incubation period to be used in the assay

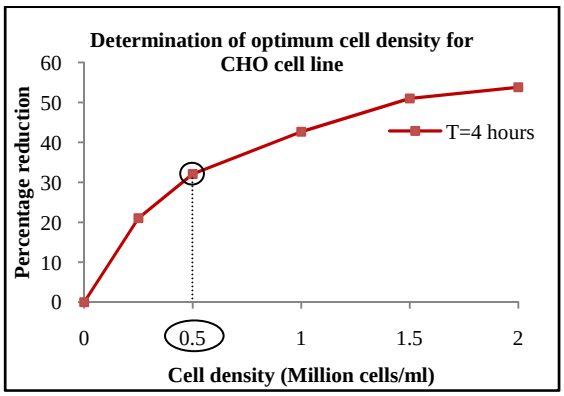
Use the procedure given below to determine optimum plating density and incubation period for cell line. This is a preliminary experiment that should be performed before staring the actual assay.

1. Harvest the cells as explained in section 7.2.
2. Seed 100µl cells in 96-well plate at various cell densities, above and below the cell density expected to be used for the assay.
3. Add 10µl EZBlue™ reagent. (10µl equals to 10% of the total culture volume per well).
4. Wrap the plate in aluminium foil and incubate at 37°C in a 5% CO_2 environment.

5. Read the absorbance at 570nm keeping 600nm as a reference wavelength, or fluorescence at 560nmEX/590nmEM at each hour following plating for first 6 - 8 hours.
6. Process the data and calculate percentage reduction of EZBlue™ by using formulae mentioned in section 9. This data will yield two types of information.

a) Determination of optimum cell density

For each incubation time point, plot the percentage reduction versus seeding cell density. Refer graph 7.3(a).

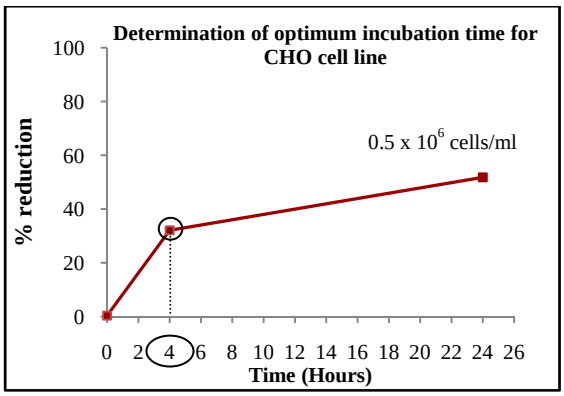


Graph 7.3 (a)

The cell density causing 50% reduction of EZBlue™ when incubation time equals experimental incubation period, represents the optimum cell density for that cell line.

b) Determination of optimum incubation period

For each cell density, plot the percentage reduction versus time period of incubation. Refer graph 7.3 (b).



Graph 7.3 (b)

The period of incubation at which the percentage reduction of EZBlue™ is 50% represents the optimum period of incubation.

***Note**: Usually, the optimum cell density determined will yield the optimum incubation time and vice versa.*

7.4 Assay procedures

7.4.1 Procedure for determining cell proliferation:

1. Seed 100µl cell suspension in a 96-well plate at the required density. Treat them with 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 plate at 37°C in a 5% CO_2 atmosphere for required period of time.
3. After the incubation period, remove the plates from incubator and add EZBlue™ reagent to a final concentration of 10% of total volume. This volume should be same as the volume used while determining optimum cell density. Wrap the plate with aluminium foil to avoid exposure to light.
4. Return the plates to the incubator and incubate for 1 to 8 hours.
   *(**Note**: Incubation time varies for different cell lines. Incubation time should be kept constant while making comparisons.)*
5. Remove plate from the incubator after incubation, followed by mixing on a gyratory shaker.
6. Read the absorbance at 570nm keeping 600nm as a reference wavelength, or fluorescence at 560nmEX/590nmEM.
7. The plate can be reread at a later time in culture period as long as cells remain viable.
   *(**Note**: This time depends on the properties of the cell line as well as nature of the compound under test).*

7.4.2 Procedure for determining cell cytotoxicity

1. a) For adherent cells:

   Seed 100µl cell suspension in a 96-well plate at required cell density, without the test agent. Allow the cells to adhere to the culture plate for about 24 hours. Add the desirable quantity of the test agent

b) For suspension cells:
Seed 100µl cell suspension in a 96-well plate at required cell density. Add the test agent immediately in the desired quantity

2. Incubate the plate for desired period at 37°C in a 5% CO_2 atmosphere.
3. After the incubation period, remove the plates from incubator and add 10µl EZBlue™ reagent. (10µl equals to 10% of the total culture volume per well.)
4. Wrap the plate with aluminium foil to avoid exposure to light.
5. Return the plates to the incubator and incubate for 1 to 8 hours.
*(**Note**: Incubation time varies for different cell lines. Incubation time should be kept constant while making comparisons.)*
6. Remove plate from the incubator after incubation, followed by mixing on a gyratory shaker.
7. Read the absorbance at 570nm keeping 600nm as a reference wavelength, or fluorescence at 560nmEX/590nmEM.
8. The plate can be reread at a later time in culture period as long as cells remain viable.
*(**Note**: This time depends on the properties of the cell line as well as nature of the compound under test).*

8. Storage and Shelf Life

- EZBlue™ reagent is light sensitive. Store the reagent in amber colored bottle.
- If stored at 2-8°C, the reagent remains stable for about 12 months. Stability of the reagent can be extended for several more months by aliquoting and storing at -20°C.
- Use before expiry date given on the label.

9. Processing of the data

A. Measuring proliferation or cytotoxicity using EZBlue™ by spectrophotometry

a) Calculation of percentage difference in reduction between treated and control cells:
Equation for calculation of percentage difference in reduction between treated and control cells:

$$\text{Percentage difference between treated and control cells} = \frac{(Oxi_2 \times T_1) - (Oxi_1 \times T_2)}{(Oxi_2 \times pC_1) - (Oxi_1 \times pC_2)} \times 100$$

---- Equation (I)

Where,

λ_1= 570nm
λ_2= 600nm
Oxi_1= Molar extinction coefficient of oxidized EZBlue™ at 570nm
Oxi_2= Molar extinction coefficient of oxidized EZBlue™ at 600nm
T_1= Absorbance of test wells at 570nm
T_2= Absorbance of test wells at 600nm
pC_1= Absorbance of positive control wells at 570nm (i.e. cells + EZBlue™, without test agent)
pC_2= Absorbance of positive control wells at 600nm (i.e. cells + EZBlue™, without test agent)

Refer the below mentioned table for molar extinction coefficients

Wavelength (nm)	Reduced (Red)	Oxidized (Oxi)
540	104395	47619
570	155677	80586
600	14652	117216
630	5494	34798

b) Calculation of percentage reduction of EZBlue™:
Equation for calculation of percentage reduction of EZBlue™:

$$\text{Percentage reduction of EZBlue}^{TM} = \frac{(Oxi_2 \times T_1) - (Oxi_1 \times T_2)}{(Red_1 \times nC_2) - (Red_2 \times nC_1)} \times 100$$

---- Equation (II)

Where,

Red_1 = Molar extinction coefficient of reduced EZBlue™ at 570nm
Red_2 = Molar extinction coefficient of reduced EZBlue™ at 600nm
nC_1= Absorbance of negative control wells at 570nm (i.e. medium + EZBlue™, without cells)
nC_2= Absorbance of negative control wells at 600nm (i.e. medium + EZBlue™, without cells)

Important Note: *If wavelengths other than those mentioned in the above table are used, calculate correction factor and percentage reduction of EZBlue™ as given in section 9 (C) - Equation V, VI and VII.*

B. Measuring proliferation or cytotoxicity using EZBlue™ by fluorescence

a) Calculation of percentage difference in reduction between treated and control cells:
Equation for calculation of percentage difference in reduction between treated and control cells:

$$\text{Percentage difference between treated and control cells} = \frac{FI_{590} \text{ of test agent}}{FI_{590} \text{ of untreated control}} \times 100$$

---- Equation (III)

Where, FI_{590} = Fluorescence intensity at 590nm emission (560nm excitation)

b) Calculation of percentage reduction of EZBlue™:
Equation for calculation of percentage reduction of EZBlue™:

$$\text{Percentage reduction of EZBlue}^{TM} = \frac{FI_{590} \text{ of test agent} - FI_{590} \text{ of untreated control}}{FI_{590} \text{ of 100\% reduced EZBlue}^{TM} - FI_{590} \text{ of untreated control}} \times 100$$

---- Equation (IV)

C. Measuring reduction in EZBlue™ with different spectrophotometric wavelengths

a) Measure the absorbance of medium control (i.e. medium without cells, with EZBlue™) at higher and lower wavelength.
Similarly, measure the absorbance of only medium (i.e. medium without cells and without EZBlue™) at higher and lower wavelength.
Subtract the absorbance values of medium control from absorbance value of only medium.

$$A(\lambda_H) = \text{Absorbance of EZBlue}^{TM} \text{ in medium} - \text{Absorbance of only medium}$$

$$A(\lambda_L) = \text{Absorbance of EZBlue}^{TM} \text{ in medium} - \text{Absorbance of only medium}$$

----Equation(V)

Where,
$A(\lambda_H)$ = Absorbance of oxidized form at higher wavelength
$A(\lambda_L)$ = Absorbance of oxidized form at lower wavelength

b) Calculation of Correction Factor (CF):

$$\text{Correction Factor (CF)} = \frac{A(\lambda_H)}{A(\lambda_L)}$$

---- Equation (VI)

c) Calculation for percentage reduction of EZBlue™ with different wavelength:

Percentage reduction of EZBlue™ = {A(λ_L) - [A(λ_H) x CF] x 100} **---- Equation (VII)**

Important Note: *The extinction coefficient of EZBlue™ changes with wavelength at every nanometer. Apply correction factors to get correct factors for wavelengths used in the experiment.*

10. Experiment to determine doubling time of CHO cells using EZBlue™

Procedure

1. CHO / Jurkat T cells (0.4 X 10^6) cells were seeded in triplicate wells of a 96-well plate.
2. Medium without cells was used as blank control.
3. EZBlue™ reagent and competitor reagent was added to the cells as per instructions provided.
4. Cells were incubated for varying time intervals and absorbance was measured at 580 and 630 nm respectively and values for percentage reduction were calculated using the formulae provided in section 9(C).
5. Percentage reduction values were then converted to number of cells using linear regression analysis or using the formula: Cell number = (Initial cell number x percentage reduction) + Initial cell number
6. The cell number determined from the experiment was then plotted against the time of incubation.
7. Exponential growth curves were fitted using GraphPad Prism software.

Observations

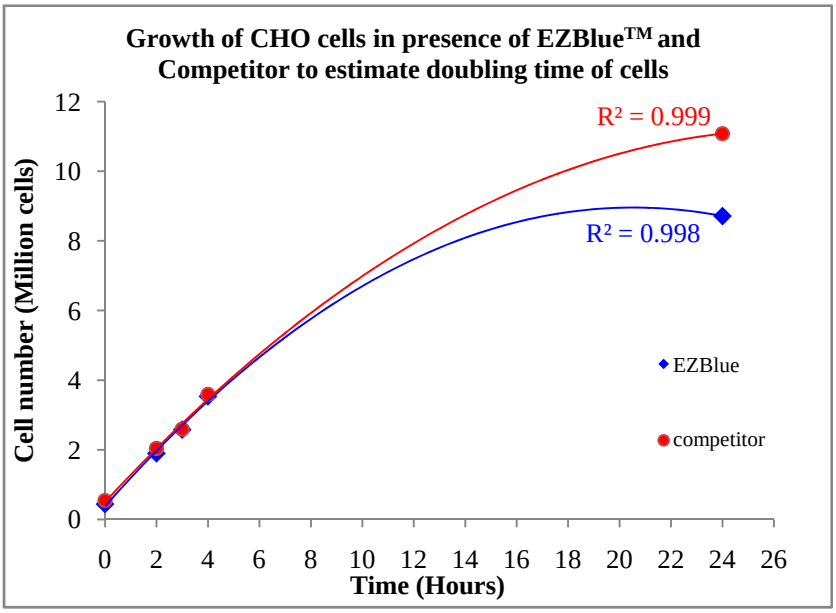
Percentage reduction in EZBlue™ and competitor at different time intervals

Reagent	Cell density (Million cells/ml)	Percentage reduction at different time intervals				
		0 Hour	2 Hours	3 Hours	4 Hours	24 Hours
EZBlue™	0.40	0.099	3.741	5.442	7.834	20.778
Competitor	0.40	0.358	4.098	5.479	7.972	26.696

Analysis

Cell numbers as calculated from values of percentage reduction

Reagent	Initial Cell density (Million cells/ml)	Cell number at different time intervals				
		0 Hour	2 Hours	3 Hours	4 Hours	24 Hours
EZBlue™	0.40	0.44	1.897	2.577	3.534	8.711
Competitor	0.40	0.543	2.039	2.591	3.589	11.078



Results

Doubling times were determined after curve fitting.

	ATCC catalog no. CCL-61	EZBlue™	Competitor
Doubling time (Hours)	12	10.65	9.33

11. Advantages

- **Time saving:** Ready to use EZBlue™ solution saves time of working by eliminating the steps like reagent transfer, washing, crystal dissolution and repeated aspiration
- **Compatibility with multiple instruments:** Reduced EZBlue™ can be detected by spectrophotometer or fluorescence detector depending on availability.
- **Real-time monitoring of cell viability:** Non-toxic nature of EZBlue™ allows real-time monitoring of cell viability
- **Flexibility:** EZBlue™ works on adherent as well as suspension cell lines
- **Cell recovery:** In contrast to MTT assay, EZBlue™ does not lead to cell death. Consequently, cells can be recovered and used in further analysis after incubation with EZBlue™

12. Related products

EZcount™ MTT Cell Assay Kit

Kit Code	
CCK003-1000	**CCK003-2500**
Sufficient for 10 microplates (1000 assays)	Sufficient for 25 microplates (2500 assays)

13. Troubleshooting points

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
High background values	Extensive exposure of reagent to light	Do not expose the reagent to light during experiment. Wrap the assay plates and reagent bottle with aluminium foil to avoid exposure to light
Low fluorescence/ absorbance values	Very low cell density	Increase the incubation time
	Over-incubation of the cell in EZBlue™ may have caused secondary reduction of resorufin to colorless hydro-resorufin	Repeat the assay
	Improper selection of the filter	Choose appropriate filters
High fluorescence/ absorbance values or values above linear range of the reader	Too much reduction of EZBlue™ due to long incubation period	Repeat the assay with reduced incubation time.
	Too much reduction of EZBlue™ due to high cell densities	Repeat the assay with reduced cell densities
Random fluorescence/ absorbance values/ poor consistency of replicates	Inaccurate pipetting technique/ inaccurate equipment	Perform the assay using automated electronic pipettes for seeding the cell suspension and adding the reagents
	Test compound under study is responsible for improper response of the cells to EZBlue™	Refer to the pharmacological properties of the compound

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