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Technical Datasheet

EZcount™ G6PD Cell Assay Kit

Product Code: CCK069

1. Introduction:

Glucose-6-Phosphate Dehydrogenase (G6PD) is a cytosolic enzyme present in all cells including RBCs. This enzyme participates in Pentose Phosphate Pathway, a metabolic pathway that supplies reducing energy to cells by maintaining the level of NADPH. Plasma membrane of live cells is impermeable to G6PD. However, it is rapidly released into the culture medium when plasma membrane is damaged. The quantity of the released G6PD is used as a measure of cell cytotoxicity.

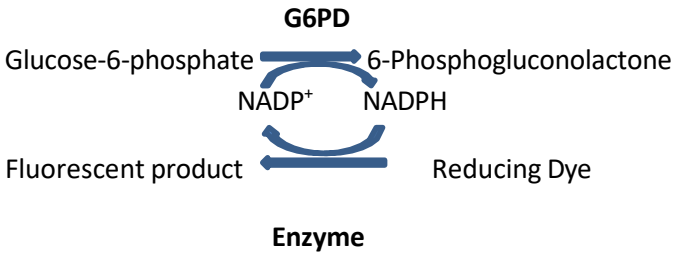
CCK069, EZCount™ G6PD Cell Assay Kit has been designed for rapid determination of cell death. The dye used in the assay is non-radioactive, water soluble and stable in most of the culture media. The assay has many advantages as it is more sensitive, faster, easily reduced and has low background signals.
This kit can be used to determine cytotoxic effect of any drug/ chemical/ metabolite using cells as model.

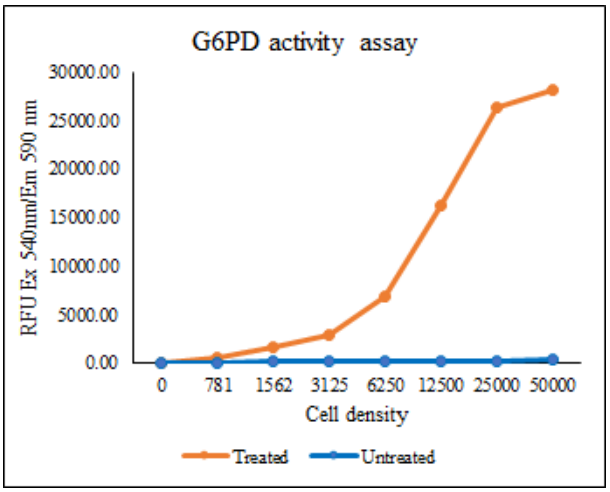
2. About the Assay:

EZcount™ G6PD Cell Assay Kit is based on an enzyme coupled reaction in which the G6PD released from damaged cells hydrolyses G6P in presence of $NADP^+$ to generate 6- Phosphogluconate, H^+ and NADPH. This NADPH in presence of Enzyme reduces dye to produce highly fluorescent product and $NADP^+$. The resulting fluorescence signal is proportional to the amount of G6PD released and this release is proportional to the number of dead cells in the sample. The reaction takes place in two steps

Step 1: G6PD catalyzes the conversion of G6P to 6-Phosphogluconolactone via reduction of $NADP^+$ to NADPH.

Step 2: Enzyme in the Reaction mix reduce the dye in the presence of NADPH to produce highly fluorescent product and $NADP^+$ which can be measured fluorometrically.





Standard curve obtained for treated and untreated cells.

3. Kit contents:

Code	Contents	Quantity	Storage
CCK069(A)	G6PD Reaction Mix	5X10mL	-20°C
CCK069((B)	G6PD Activator	5X100µL	-20°C
CCK069 (C)	G6PD Lysis Buffer	10mL	-20°C

The kit is sufficient for 500 assays.

4. Materials required but not provided in the kit:

- Cells in appropriate medium
- Adjustable pipettes and a repeat pipette
- 96-well plate (Transparent well)/ 96-well solid plate (Black well for Fluorescence).
- 96-well plate reader capable of measuring the fluorescence at 540nm excitation and 590nm emission

5. 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™ G6PD Cell Assay Kit.

5.1 Assay controls

Untreated control

Untreated control well contains cells at different densities same as treated wells but should not be lysed with lysis buffer. Untreated control should be included in assay to ensure that the dye is not interacting with live cells.

Background control

Background control, also known as vehicle control, should be included in the assay to obtain G6PD values generated due to the solvent or culture media in which test compound is dissolved. Background control wells contain the solvent or culture media at a concentration used for dissolving the test compound without cells which already liberated G6PD in the media.

5.2 Incubation period and temperature

Significant fluorescence signal is produced under recommended assay conditions i.e. 30 to 60 minutes incubation at 37°C depending on the cell type. If higher fluorescence signal is required, incubation time can be increased.

5.3 Accuracy

1. To obtain statistically significant data, perform the assay in duplicates or more.
2. 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.
3. Use of a repeating pipettor is recommended to deliver the reagents to the wells. This saves time and helps maintain more precise incubation times.
4. 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.

5.4 Culture Medium

Phenol red interferes with the measurement of fluorescence/ absorbance; therefore, the culture media used for the assay should not contain phenol red.

5.5 Lysis Buffer

Lysis Buffer should be added properly to each well. It is difficult to observe the lysis of cells under microscope in black well plate so it is required to perform the assay in duplicates with one transparent well plate.

5.6 Light sensitivity of G6PD reagent

G6PD reagents are extremely sensitive to light. Prolonged exposure of the reagent to light results in increased background and decreased sensitivity.

6. Directions for use:

Users are advised to review entire procedure before starting the assay

6.1 Preparation of Reagents:

Reaction mix:

Prepare reaction mix by adding 100µl (Part B) in Part A which is 10mL Reaction mix should be prepared freshly according to requirement. A 10ml of Activated reaction mix is sufficient for approximate one 96 well plate.

Note:

Thaw all the other reagents of kit (CCK069) and equilibrate it at room temperature before use. Components of Part A and Part B can be thawed once only.)

6.2 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 overnight or till confluence is reached in phenol red free media.

Harvest the cells by trypsinization in a phenol red free medium and use for the assay. Refer section 5.4 for specifications of the medium required for the assay.

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

6.3 Assay Procedure

1. Seed cells in 90µl medium in triplicates in a 96-well plate (black, clear bottom) at the required cell density in treated and untreated wells. Incubate the plate for 12 – 14 hours or overnight for attachment of cells. Add 90µl culture media in background/blank control well. Refer table 1 for G6PD assay scheme

Note:

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

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 of cell lysis buffer to treated wells and add 10µl of DPBS to untreated wells.
3. Observe the plate under microscope for the lysis of cells, generally it takes 5-10 minutes at 37°C for up to 20,000 cells/well.
4. After cell lysis, add 10µl of reaction mix to each well in both treated and untreated wells.
5. Incubate the plate at 37°C for 30 to 60 minutes.

   Important Note: Wrap the plate in aluminum foil to avoid exposure to light.
6. Read fluorescence using excitation at 540nm and emission at 590nm.

	Treated Wells	Untreated Control	Background Control
Cells	90µl	90µl	-
Culture Medium	-	-	90µl
Lysis solution	10µl	-	-
DPBS	-	10µl	10µl
Reaction mix	100µl	100µl	100µl
Total volume	200µl	200µl	200µl

Table 1: G6PD Cell Assay Scheme

Advantage of G6PD assay over LDH assay:

1. In LDH assay, reaction occurs by Lactate to Pyruvate conversion by LDH. Hence, the presence of pyruvate in medium affects the assay performance. But in case of G6PD assay, Pyruvate in the media does not hamper the reaction. Therefore, culture medium with pyruvate can be used.
2. In LDH assay, there is high background signals in serum supplemented culture media, because LDH activity of cell culture sera is higher. Hence, low serum or serum- free media can only be used for LDH assay. But in case of G6PD, serum supplemented culture media can be used as it does not have G6PD activity.

7. Analysis of Results:

Calculate the average fluorescence values from duplicate/triplicate values and calculate the percentage cytotoxicity using the formula given below –

$$\text{Percentage Cytotoxicity} = 100X \frac{(\text{Treated} - \text{Blank})}{\text{Blank}}$$

8. Storage and Stability:

G6PD reagents are photosensitive. Store it away from bright light.

G6PD reagent is a mixture of enzymes and substrates involved in conversion of G6P to 6-Phosphogluconolactone. Prolonged exposure of the reagent may cause loss in activity of enzymes.

For long term use, store the reagent at -20°C.

Use before expiry date given on the product label.

9. Troubleshooting points:

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
Absorbance / fluorescence values higher than expected	Colored / fluorescent test compound being tested	Determine the inherent absorbance/ fluorescence of compound without adding G6PD reagent mixture
	Production of excess G6PD by dead cells	Repeat the assay with reduced cell densities
Very low absorbance values	Production of very low amount of G6PD	Repeat the assay with higher cell densities
	Inadequate incubation with lysis buffer	Expose the cells to lysis buffer for slight longer duration and observe the plate for proper ell lysis.
Random absorbance values/ poor consistency of replicates	Pipetting error	Perform the assay using automated electronic pipettes for seeding the cell suspension and adding the reagents