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Technical Datasheet

EZcount™ XTT Cell Assay Kit

Product Code: CCK015

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 behaviour of cells which in turn leads to cells which in turn leads to cell transformation, uncontrolled cell growth- the initiating event of cancer development . Pharmaceutical research is hence largely focused on effecs 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 a high degree of precision.

2. About the Assay:

The EZcount™ XTT Cell Assay kit is designed for determination of cell viability and cell proliferation and / or effect of cytotoxic agent. This kit is based on quantitative measurement of extracellular reduction of XTT to water soluble orange coloured formazan derivative by metabolically active cells. This reduction is mediated by mitochondrial dehydrogenase enzyme. Reduction of XTT is greatly enhanced by XTT activator. XTT activator mediates reduction of XTT by transferring electron from cell surface/ cell membrane to extracellular XTT.

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:

Contents		Kit Code	Storage
Code	**Description**	**CCK015-1000***	
CCK015 Part A	XTT reagent	10 x 5ml•	-20°C in dark
CCK015 Part B	XTT activator	2 x 0.5ml•	-20°C in dark

•Quantities supplied in excess to compensate operational losses

5. Materials required but not provided in the kit:

- Cells in appropriate medium without phenol red
- 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™ XTT 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 with the cells and solvent in which the experimental drug or compound is dissolved)
- Extracellular reducing components such as ascorbic acid, cholesterol, alpha-tocopherol, dithiothreitol present in the culture media may reduce the XTT to orange coloured formazan derivative. To account for this reduction, it is important to use the same medium in control as well as test wells.

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 may have different properties such as metabolic activity and doubling time and hence respond to XTT differently. For this reason plating density and incubation period for every cell line should be optimized to obtain the results in linear range.

Culture Medium

- Phenol red interferes with the measurement of orange colour; therefore the cell culture media used for this assay should not contain phenol red.

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 XTT reagent:

1. Working solution should be prepared just prior to adding to the cells.
2. Thaw XTT reagent and XTT activator prior to use.
3. Mix 0.1ml of XTT activator with 5ml of XTT properly to form activated XTT solution.
4. This amount will be sufficient for one 96 well plate (50µl/well).
5. Equilibrate the activated XTT reagent at room temperature before use.
6. If precipitation is observed in XTT reagent and XTT activator, warm the reagents in a water bath to 37°C and swirl the bottle and vial until a clear solution is obtained.

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

***(Note**: 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 assay).*

7.3 Pre-assay optimization procedure:

Pre-assay optimization procedure needs to be performed once for each cell line to determine optimum plating density and incubation time.

1. Harvest the cells as explained in section 7.2.
2. Adjust the cell density to 1 x 10^6 cells /ml.
3. Serially dilute the cell suspension from 1 x 10^6 to 1 x 10^3 cells/ml using appropriate culture medium.
4. Seed 100µl of each dilution in 96-well microtiter plate in triplicate.
5. Add medium control in triplicate.
6. Incubate the cells under appropriate conditions depending on specific requirements for that cell line.
7. Add 50µl of freshly prepared activated XTT reagent to each well including control.
8. Return the plate to incubator for 2 to 4 hours.
9. Observe the plate visually at periodic time intervals for appearance of orange color.
10. Once the incubation is over, stir gently on a gyratory shaker to enhance uniform dispersion of reagent.
11. Read the absorbance on spectrophotometer or an ELISA reader at 450nm with a reference wavelength >600nm. If the readings are low, incubate the plate for a longer period.
12. Determine the average values from triplicate readings at 450nm and subtract from this value the average value for blank (i.e. medium control) and average value at >600nm.

    Specific absorbance = Absorbance $_{(450nm)}$ (test) – Absorbance$_{(450nm)}$ (blank) – Absorbance$_{(>600nm}$ (test)

13. Plot absorbance against cell density.
14. Number of cells to be used in the cell proliferation assay should lie within linear portion of the plot.

7.4 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. 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 50µl activated XTT reagent.
4. Return the plates to the incubator and incubate for 2 to 4 hours.
   *(**Note**: Incubation time varies for different cell lines. Incubation time should be kept constant while making comparisons. Some cell lines may require incubation for up to 24 hours.)*
5. Remove the plate from incubator after incubation.
6. Stir gently on the gyratory shaker to enhance uniform dispersion of reagent.
7. Read the absorbance on spectrophotometer or an ELISA reader by using 450nm as primary filter and >600nm as differential filter.
8. Subtract the average 450nm absorbance values of the control wells from the average 450nm absorbance values of corresponding experimental wells.
9. Measure the absorbance of all the assay wells again at a wavelength of >600nm. Subtract these values from the values obtained at 450nm. This reading will help you eliminate non-specific readings from your assay results.
10. Plot the absorbance values on the Y-axis and your experimental parameters on the X-axis.

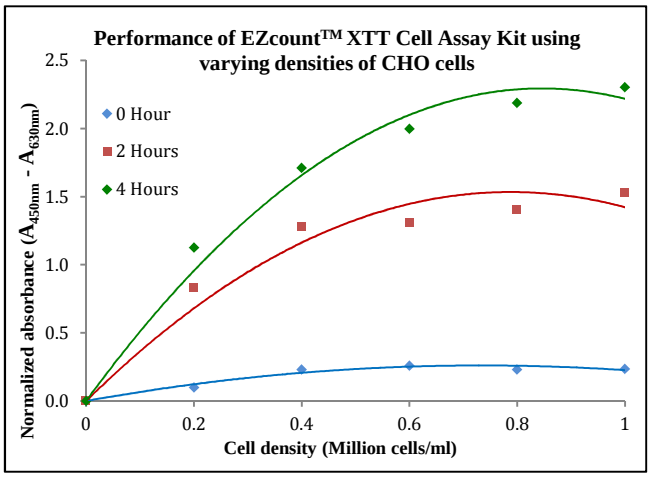
7.5 Interpretation of data:

1. The linear portion of the XTT curve depicts maximum sensitivity to changes induced by experimental parameters.
2. Test values higher than control values indicate increase in cell proliferation and viability and vice versa.

8. Storage and Shelf life:

- XTT reagent and XTT activator are light sensitive. Store them at -20°C in dark.
- Use before expiry date given on the label.
  Note: Repeated freezing and thawing should be avoided. Once thawed, remaining solution should be aseptically dispensed into smaller aliquots for further use.

9. Performance characteristics:



The sensitivity of XTT to detect changes in cell number has been determined by plotting the graph of normalized absorbance values versus cell number.

CHO cells were serially diluted and treated with XTT reagent provided in EZcount™ XTT Cell Assay Kit, in a 96-well plate. After incubation for 0, 2 and 4 hours in a humidified incubator at 37°C, 5% CO_2, absorbance was read at 450nm using an ELISA plate reader. The absorbance data was processed as given in 7.4.

10. Advantages:

- **Time saving**: Absorbance can be measured directly without involvement of solubilization step
- **Easy reagent preparation**: Ready to mix and pre-weighed reagents offer ease of reagent preparation
- **Reproducibility**: Entire assay can be performed in a single plate. Cells and reagents need not be transferred. This facilitates reproducibility of the results
- **Sensitive and accurate:** Tetrazolium salt reduction strongly correlates with the metabolic activity of the cells. This allows use of very low cell densities.
- **Safety**: No radioisotopes are involved.
- **Fast**: Use of multi-well ELISA plates allows the processing of large number of samples.
- **Flexibility**: XTT works on adherent as well as suspension cell lines.

11. Troubleshooting points:

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
Coloration in XTT reagent	Microbial contamination or contamination with reducing agent	Discard the contaminated vial of XTT and prepare fresh reagent aseptically
	Excessive exposure of reagent to light	Store in dark at -20^{o}C
Very high absorbance values	Too much reduction of XTT due to high cell densities	Repeat the assay with reduced cell densities
	Too much reduction of XTT due to long incubation period	Repeat the assay with reduced incubation period
	Microbial contamination	Discard. Repeat the assay with new media and reagents
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. Certain cell types require longer incubation period of up to 24 hours
	Improper selection of filter for reading the absorbance	Choose appropriate filters within the range of 450nm
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 to XTT	Refer to the pharmacological properties of the compound
	XTT reagent and/or activator not fully dissolved in solution	Ensure XTT reagent and/or activator is fully dissolved in solution
Blank/ medium control (i.e. medium without cells) give high absorbance readings	Microbial contamination	Discard. Repeat the assay with new media and reagents.