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Technical Datasheet

EZcount™ MTT Cell Assay Kit

Product Code: CCK003

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 a high degree of precision.

2. About the Assay

The EZcount™ MTT Cell Assay kit is designed for determination of cell viability and cell proliferation and/or effect of cytotoxic agent. This kit is based on the quantitative measurement of extracellular reduction of the yellow colored water soluble Tetrazolium dye 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) to insoluble formazan crystals by metabolically active cells. This reduction is mediated by mitochondrial enzyme lactate dehydrogenase. When dissolved in a appropriate solvent, these formazan crystals exhibit purple color, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570nm.

The assay procedure involves reconstitution of the pre-measured MTT reagent in the assay buffer, followed by its addition to the culture system. After dissolving the formazan crystals in the solubilization solution, results can be directly read on a suitable reader.

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**	**CCK003-1000***	**CCK003-2500****	
CCK003(A)	MTT reagent (powder)	2 vials	5 vials	2 - 8°C
CCK003(B)	Cell based assay buffer	1 x 20ml•	2 x 20ml•	15-30°C
CCK003(C)	Solubilization solution	1 x 125ml•	2 x 125ml•	15-30°C

* Sufficient for 10 microplates (1000 assays)
** Sufficient for 25 microplates (2500 assays)

• Quantities supplied in excess to compensate operational losses

5. Materials required but not provided in the kit

- Cells in appropriate medium with or 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™ MTT Cell Assay Kit. Procedure for optimizing cell density is outlined in section 7.3.

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)
- Extracellular reducing components such as ascorbic acid, cholesterol, alpha-tocopherol, dithiothreitol present in the culture media may reduce the MTT to formazan. 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 pipette reagents. 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.
- Care should be taken so that no bubbles are introduced into the wells during pipetting or mixing of the reagents.

Incubation period

- Different cell lines may have different properties such as metabolic activity and doubling time and hence respond to MTT differently. For this reason, plating density and incubation period for every cell line should be optimized to obtain results in linear range.
- Formation of formazan crystals can be checked by observing the cells under inverted microscope periodically during incubation. The crystals appear as needle-shaped and dark purple coloured intracellular precipitates. Longer incubation period may be required if adequate amount of crystals are not formed.
- Plates can be read after overnight incubation after addition of solubilization solution.

Culture Medium

- It is advisable to use phenol red free medium. However, if phenol red containing medium is used, appropriate controls should be applied.
- High protein content in the culture medium may lead to precipitation on addition of solubilization solution. Serum is the major factor contributing to high protein content of culture medium. Maximum acceptable concentration of fetal bovine serum is 10%. However, sera with higher protein content than FBS should be used at lower concentrations.

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 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 550-600nm (primary wavelength).
- Reference wavelength (for non-specific readings) should be higher than 600nm.

7. Directions for use

Users are advised to review entire procedure before starting the assay

7.1 Preparation of MTT reagent

Aseptically add 6ml of cell based assay buffer in one MTT vial and completely dissolve the powder. MTT powder dissolves slowly in the buffer. Vigorous vortexing is needed to dissolve the powder completely. MTT solution should appear bright yellow in color.

Note: For long term storage of the MTT reagent, it is recommended to filter sterilize using a 13mm, 0.22µ syringe filter.

MTT reagent is light sensitive. Store the reconstituted reagent in amber colored bottle. If not consumed in single experiment, we recommend the storage of the reconstituted vial at -20ºC till further 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 37ºC 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 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 the cell line under study.
7. Add 10µl of MTT to each well, including controls.
8. Wrap the plate with aluminium foil to avoid exposure to light.
9. Return the plate to the incubator for 2 to 4 hours.
10. Observe the cells at periodic intervals under an inverted microscope for presence of intracellular needle-shaped, dark purple coloured precipitate. Slow growing cell lines require longer time to develop formazan crystals.
11. When the purple precipitate is clearly visible under the microscope, add 100µl of solubilization solution to the wells.
12. Stir gently on a gyratory shaker for 30 minutes to enhance dissolution of the crystals. Mix the solution with the help of micropipette to allow crystals to dissolve.
    Note: Plate can be incubated overnight after adding the solubilization solution followed by dissolution of crystals and reading.
13. Read the absorbance on a spectrophotometer or an ELISA reader at 570nm with a reference wavelength higher than 600nm.
14. Determine the average values from triplicate readings at 570nm and subtract from this value the average value for blank (i.e. medium control) and average value at the reference wavelength.
    Specific absorbance = Absorbance $_{(570nm)}$ (test) – Absorbance$_{(570nm)}$ (blank) – Absorbance$_{(>600nm)}$ (test)
15. Plot absorbance against cell density.
16. Number of cells to be used in the cell proliferation assay should lie within linear portion of the plot.

7.4 Assay procedures

1. Seed 100µl of cell suspension in a 96-well microtiter plate at the required 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 37ºC in a 5% CO_2 atmosphere for the required period of time.
3. After the incubation period, remove the plates from incubator and add MTT reagent to a final concentration of 10% of total volume. This volume should be same as the volume used while determining optimum cell density.
4. Wrap the plate with aluminium foil to avoid exposure to light.
5. 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 for up to 24 hours.
6. Remove the plate from incubator after incubation.
7. After incubation period, add 100µl of solubilization solution to each well.
8. Stir gently on a gyratory shaker for 30 minutes to enhance dissolution of the crystals. Mix the solution with the help of micropipette to allow crystals to dissolve.
   Note: Plate can be incubated overnight after adding the solubilization solution followed by dissolution of crystals and reading.
9. Read the absorbance on a spectrophotometer or an ELISA reader at 570nm with a reference wavelength of 630nm.
10. Subtract the average 570nm absorbance values of the control wells from the average 570nm absorbance values of corresponding experimental wells.

11. Measure the absorbance of all the assay wells again at a wavelength 630nm. Subtract these values from the values obtained at 570nm. This reading will help you eliminate non-specific readings from your assay result.
12. Plot the absorbance values on the Y-axis and your experimental parameters on the X-axis.

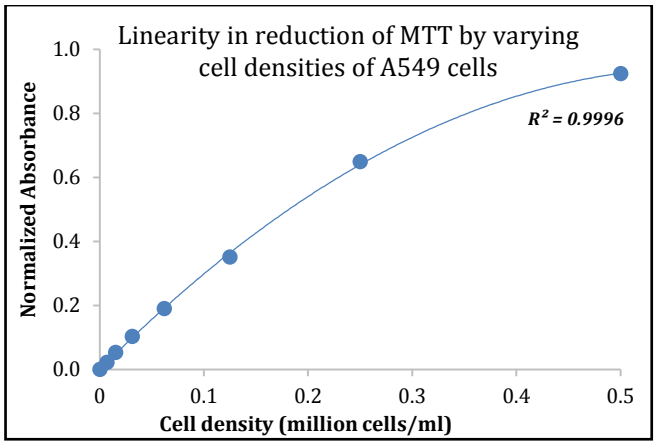
7.5 Interpretation of Data

1. The linear portion of the MTT 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

- MTT reagent is light sensitive. Store the reconstituted reagent in an amber colored bottle. If not consumed in a single experiment, we recommend the storage of the reconstituted vial at -20ºC till further use.
- If stored at 2-8ºC, MTT remains stable for about 4 weeks whereas its stability can be extended for several months if stored at -20ºC.
- Repeated freezing and thawing may result in loss in activity of the reagent, hence aliquoting is preferred.
- Use before expiry date given on the label.

9. Performance characteristics



The sensitivity of MTT to detect changes in cell number has been determined by plotting the graph of normalized absorbance values versus cell number.
A549 cells were serially diluted and incubated with MTT reagent provided in EZcount™ MTT Cell Assay Kit, in a 96-well microtiter plate. After 4hrs of incubation in a humidified incubator at 37ºC, 5% CO_2, absorbance was read at 570nm using an ELISA plate reader.

10. Advantages

- **Time saving**: Absorbance can be measured directly after few minutes of crystal solubilization. Omission of washing and reagent transfer steps saves the time of working
- **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
- **Sensitivity and accuracy:** 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**: MTT works on adherent as well as suspension cell lines

11. Troubleshooting points

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
Colour change in MTT reagent	Microbial contamination or contamination with a reducing agent	Discard the contaminated vial of MTT and prepare fresh reagent aseptically
	Exposure of the reagent to light	Wrap the reagent bottles and culture plates with aluminium foil
Very high absorbance values	Too much reduction of MTT due to high cell densities	Repeat the assay with reduced cell densities
	Improper selection of the filter	Choose appropriate filters
	Too much reduction of MTT 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 550-600nm
	Incomplete solubilization of formazan crystals	Allow the formazan crystals to dissolve completely, by mixing with a pipette or gyratory shaker
Random absorbance values/ poor consistency of replicates	Inaccurate pipetting technique or 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 MTT	Refer to the pharmacological properties of the compound
	MTT reagent not fully dissolved in cell based assay buffer	Ensure MTT reagent is fully dissolved in cell based assay buffer
Blank/ medium control (i.e. medium without cells) give high absorbance readings	Microbial contamination	Discard. Repeat the assay with new media and reagents.

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