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Technical Datasheet

EZcount™ MTS Homogenous Cell Assay Kit

Product Code: CCK070

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™ Homogenous MTS 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 water soluble tetrazolium dye MTS [2-(4-Iodophenyl)-3 - 4 - nitrophenyl) - 5 - (2, 4 - disulfophenyl) - 2H - tetrazolium sodium salt] to water soluble coloured formazan product by metabolically active cells. This reduction is mediated by mitochondrial dehydrogenases. The intensity of coloured formazan is directly proportional to the number of cells and can be measured spectrophotometrically at 490nm.

Unlike MTT assay, MTS assay does not involve solubilization step. This feature makes MTS assay more accurate, sensitive and time-saving. CCK070 makes use of single ready-to-use reagent as an advantage over CCK053 (EZcountTM Homogenous MTS Cell Assay Kit) which involves an additional activator.

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

The reagents supplied in the kit are sufficient for 1000 assays (Ten 96-microwell plates).

Code	Description	Quantity	Storage
CCK070(A)	MTS Homogenous reagent	10 X 2.0ml	-20°C

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 at 490nm and >600nm.

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™ MTS Homogenous Cell Assay Kit. Procedure for optimizing cell density is outlined in section 7.2.

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.
- 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 MTS differently. For this reason, plating density and incubation period for every cell line should be optimized to obtain results in linear range.

Culture Medium

- Phenol red may interfere with the measurement of formazan; 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 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 490nm (primary wavelength) and >600nm (reference wavelength.

7. Directions for use

Users are advised to review entire procedure before starting the assay

7.1 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.)*

7.2 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 7.1.
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. Thaw one bottle of CCK070(A) and equilibrate at Room Temperature before use. *Reagent in one bottle is sufficient for one 96-well plate.*
8. Add 20µl of MTS homogenous solution to each well including controls and swirl to mix the dye uniformly.
9. Wrap the plate with aluminium foil to avoid exposure to light.
10. Return the plate to the incubator for 2 to 4 hours.

11. Observe the plate at periodic intervals for development of colour.
12. Stir gently on a gyratory shaker to enhance uniform mixing of colour.
13. Read the absorbance on a spectrophotometer or an ELISA reader at 490nm (main wavelength) and >600nm (reference wavelength).
14. Determine the average values from triplicate readings and subtract from this value the average value for blank (i.e. medium control). Specific absorbance = Absorbance (test) – Absorbance (blank)
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.

7.3 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 20µl MTS homogenous reagent to each well.
4. Swirl the plate to mix the dye uniformly.
5. Wrap the plate with aluminium foil to avoid exposure to light.
6. 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.)*
7. Remove the plate from incubator after incubation.
8. Read the absorbance on a spectrophotometer or an ELISA reader at 490nm (main wavelength) and >600nm (reference wavelength).
9. Subtract the average absorbance values of the control wells from the average absorbance values of corresponding experimental wells.
10. Plot the absorbance values on the Y-axis and your experimental parameters on the X-axis.

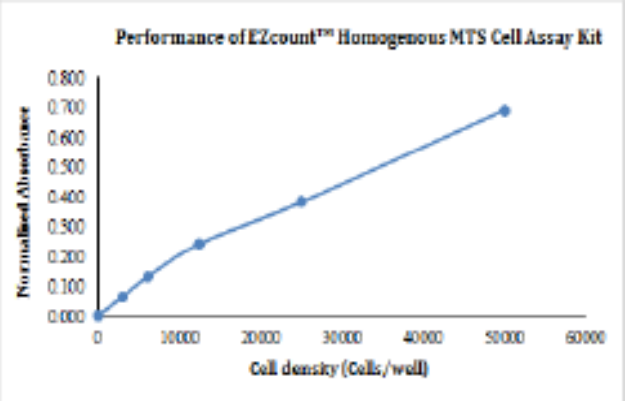
7.4 Interpretation of Data

1. The linear portion of the MTS 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

- Store MTS Homogenous reagent at -20ºC.
- If not consumed in single experiment, we recommend the storage of the MTS Homogenous solution at -20ºC till further use.
- Repeated freezing and thawing may result in loss in activity of the reagent and increased background absorbance.
- Shelf life of the complete kit is 9 months.
- Use before expiry date given on the label.

9. Performance characteristics



The sensitivity of MTS 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 MTS reagent provided in EZcount™ MTS Homogenous Cell Assay Kit, in a 96-well microtiter plate. After incubation for 4 hours in a humidified incubator at 37ºC, 5% CO_2, absorbance was read at 490 nm using an ELISA plate reader. The absorbance data was processed as given in point (7.4). As indicated in the graph, there is linear correlation between cell number and absorbance.

10. Advantages

Time saving: Absorbance can be measured without involving solubilization with organic solvent.
Easy reagent preparation: Ready to mix 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**: MTS works on adherent as well as suspension cell lines. It does not affect viability of cells and allows determination of cell viability even after 24 hours. The plates can be read and returned to incubator several times for further colour development.

11. Troubleshooting points

Use the following troubleshooting guidelines for technical assistance

Problem	Cause	Solution
Colour change in MTS reagent	Microbial contamination or contamination with a reducing agent	Discard the contaminated vial of MTS and prepare fresh reagent aseptically
	Exposure of the reagent to light	Wrap the reagent bottles and culture plates with aluminium foil
	Improper selection of the filter	Choose appropriate filters
Very high absorbance values	Too much reduction of MTS due to high cell densities	Repeat the assay with reduced cell densities
	Too much reduction of MTS 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
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 MTS	Refer to the pharmacological properties of the compound
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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