Xpert™ MTT Cell Assay Teaching Kit

Product Code: CCK020

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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.

CCK020, Xpert™ MTT Cell Assay Teaching Kit has been developed for teaching quantitative cell proliferation and cytotoxicity using MTT cell assay. The kit is sufficient for 100 tests (one 96-well microplate).

Significance of reagents provided in the kit
a. MTT reagent (powder)
   MTT (3 - [4, 5- dimethylthiazol - 2 - yl] - 2, 5- diphenyl tetrazolium bromide) is a yellow coloured water soluble tetrazolium dye. Mitochondrial enzyme lactate dehydrogenase, produced by metabolically active cells reduces MTT to water-insoluble formazan crystals. When dissolved in appropriate solvent, these formazan crystals exhibit purple colour. The intensity of the purple colour is directly proportional to the number of viable cells and can be measured spectrophotometrically at 570nm.

b. Cell based assay buffer
   It is a physiological buffer for reconstitution of MTT powder.

c. Solubilization solution
   It is an organic solvent for dissolving formazan crystals.

2. 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

### 3. Kit contents

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Code TC247 MTT reagent</td>
<td>1 x 6mg</td>
<td>4°C</td>
</tr>
<tr>
<td>Description (powder)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Code TL1111 Cell based</td>
<td>1 x 2ml*</td>
<td>RT*</td>
</tr>
<tr>
<td>Description assay buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Code TCL094 Solubilization</td>
<td>1 x 10ml*</td>
<td>RT*</td>
</tr>
<tr>
<td>solution</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*RT* Room Temperature  
*Quantities supplied in excess to compensate operational losses

### 4. 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

### 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 Xpert™ MTT Cell Assay Teaching Kit. Procedure for optimizing cell density is outlined in section 6.3.

**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.

- Read the plates within 1 hour of addition of solubilization solution.

**Culture Medium**

- Phenol red interferes with the measurement of formazan; therefore the cell culture media used for this assay should not contain phenol red.
- 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 650nm.

6. Directions for use

Users are advised to review entire procedure before starting the assay

6.1 Preparation of MTT reagent
Aseptically add 1.2ml of cell based assay buffer in MTT vial and completely dissolve the powder. MTT powder dissolves slowly in the buffer. Vigorous vortexing is needed to dissolve the powder completely. Concentration of the resulting solution is 5mg/ml. 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, 0.22µm 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 -20°C till further use.)

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% CO₂ 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.)

6.3 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 6.2.
2. Adjust the cell density to 1 x 10⁶ cells/ml.
3. Serially dilute the cell suspension from 1 x 10⁶ to 1 x 10⁴ 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 needle-shaped crystals (Refer fig. 1). Slow growing cell lines require longer time to develop formazan crystals.

11. After incubation period, add 100μl of solubilization solution to each well.
12. Stir gently on a gyratory shaker to enhance dissolution of the crystals.
13. Read the absorbance on a spectrophotometer or an ELISA reader at 570nm with a reference wavelength higher than 650nm.
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 (>650nm) (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.

6.4 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 37°C in a 5% CO₂ 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.
   \textbf{(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 and add 100µl solubilization solution to each well.
7. Gentle stirring on a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals especially in dense cultures.
8. Read the absorbance on a spectrophotometer or an ELISA reader at 570nm with a reference wavelength of higher than 650nm.
9. Subtract the average 570nm absorbance values of the control wells from the average 570nm absorbance values of corresponding experimental wells.
10. Measure the absorbance of all the assay wells again at a wavelength higher than 650nm. Subtract these values from the values obtained at 570nm. This reading will help you eliminate non-specific readings from your assay result.
11. Plot the absorbance values on the Y-axis and your experimental parameters on the X-axis.

6.5 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.

7. Storage and shelf life

- 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.

8. 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.

BHK-suspension cells were serially diluted and treated with MTT reagent provided in Xpert™ MTT Cell Assay Teaching Kit, in a 96-well microtiter plate. After incubation for 2, 3 and 4 hours in a humidified incubator at 37°C, 5% CO₂, absorbance was read at 570nm using an ELISA plate reader. The absorbance data was processed as given in point (9). As indicated in the graph, there is linear correlation between cell number and absorbance (R² = 0.993)

9. Advantages

- \textbf{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
- \textbf{Easy reagent preparation}: Ready to mix and pre-weighed reagents offer ease of reagent preparation
- \textbf{Reproducibility}: Entire assay can be performed in a single plate. Cells and reagents need not be transferred. This facilitates reproducibility of the results
- \textbf{Sensitivity and accuracy}: Tetrazolium salt reduction strongly correlates with the metabolic activity of the cells. This allows use of very low cell densities
- \textbf{Safety}: No radioisotopes are involved
- \textbf{Fast}: Use of multi-well ELISA plates allows the processing of large number of samples
### 10. Troubleshooting points

Use the following troubleshooting guidelines for technical assistance.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour change in MTT reagent</td>
<td>Microbial contamination or contamination with a reducing agent</td>
<td>Discard the contaminated vial of MTT and prepare fresh reagent aseptically</td>
</tr>
<tr>
<td></td>
<td>Exposure of the reagent to light</td>
<td>Wrap the reagent bottles and culture plates with aluminium foil</td>
</tr>
<tr>
<td>Very high absorbance values</td>
<td>Improper selection of the filter for reading the absorbance</td>
<td>Choose appropriate filters</td>
</tr>
<tr>
<td></td>
<td>Too much reduction of MTT due to high cell densities</td>
<td>Repeat the assay with reduced cell densities</td>
</tr>
<tr>
<td></td>
<td>Too much reduction of MTT due to long incubation period</td>
<td>Repeat the assay with reduced incubation period</td>
</tr>
<tr>
<td></td>
<td>Microbial contamination</td>
<td>Discard. Repeat the assay with new media and reagents</td>
</tr>
<tr>
<td>Very low absorbance values</td>
<td>Very low cell density</td>
<td>Repeat the assay with high cell densities</td>
</tr>
<tr>
<td></td>
<td>Short incubation period</td>
<td>Repeat the assay with longer incubation period. Certain cell types require longer incubation period of up to 24 hours</td>
</tr>
<tr>
<td></td>
<td>Improper selection of filter for reading the absorbance</td>
<td>Choose appropriate filters within the range of 550-600nm</td>
</tr>
<tr>
<td></td>
<td>Incomplete solubilization of formazan crystals</td>
<td>Allow the formazan crystals to dissolve completely, by mixing with a pipette or gyratory shaker</td>
</tr>
<tr>
<td>Random absorbance values/ poor consistency of replicates</td>
<td>Inaccurate pipetting technique or inaccurate equipment</td>
<td>Perform the assay using automated electronic pipettes for seeding the cell suspension and adding the reagents</td>
</tr>
<tr>
<td></td>
<td>Test compound under study is responsible for improper response of the cells to MTT</td>
<td>Refer to the pharmacological properties of the compound</td>
</tr>
<tr>
<td>Blank/ medium control (i.e. medium without cells) give high absorbance readings</td>
<td>MTT reagent not dissolved completely in cell based assay buffer</td>
<td>Ensure MTT reagent is fully dissolved in cell based assay buffer</td>
</tr>
<tr>
<td></td>
<td>Microbial contamination</td>
<td>Discard. Repeat the assay with new media and reagents</td>
</tr>
</tbody>
</table>

**Flexibility:** MTT works on adherent as well as suspension cell lines

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