MTT cell proliferation assay

PSR

Cell-based assays are often used for screening collections of compounds to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death. Cell-based assays also are widely used for measuring receptor binding and a variety of signal transduction events that may involve the expression of genetic reporters, trafficking of cellular components, or monitoring organelle function.

There are a variety of assay methods that can be used to estimate the number of viable eukaryotic cells.

MTT which is positively charged and readily penetrates viable eukaryotic cells

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Detailed procedure

Equipment and Materials

PBS	Sterile tubes (5 mL)
MTT (2 mg/ml in PBS) – filter and keep dark, prepare freshly	Serological pipettes
DMSO (Dimethyl sulfoxide)	Sterile pipette tips
96-well plate (flat bottom)	
37°C incubator	Laminar flow hood
Multi-channel pipette	Inverted microscope

Reagents and storage conditions

Reagent	storage
MTT Reagent	2-8°C
Detergent Reagent	18-24°C

Procedure

Short 96 well assay:
Each condition should be done in triplicate or more.

1. **Day one**: Trypsinize one T-25 flask and add 5 ml of complete media to trypsinized cells. Centrifuge in a sterile 15 ml falcon tube at 1000 rpm in the swinging bucked rotor (~130 x g) for 10 min.
2. Remove media and resuspend cells to 1.0 mL with complete media.
3. Count and record cells per mL. Remember to remove the cells aseptically when counting.
   - Starting with chamber 1 of the hemacytometer, count all the cells in the 1 mm center square and four 1 mm corner squares. Non-viable cells will stain blue. Keep a separate count of viable and non-viable cells. (See cell culture protocol of RCPN available in nano.tbzmed.ac.ir).

   Note: Cells should be seeded at densities between 5000 and 10,000 cells per well since they will reach optimal population densities within 48 to 72 hours
4. Add 200 µL of cells (7500 total cells) into each well and incubate overnight.
5. **Day two**: Treat cells on day two with agonist, inhibitor or drug.
   - If removing media, do very carefully. This is where most variation in data may occur.
   - Final volume should be 200 µL per well.

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6. **Day three**: Add 50 µL of 2 mg/mL MTT to each well. Include one set of wells with MTT but no cells (blank). All should be done aseptically.
7. Incubate for 4 hours at 37°C in incubator.
8. Carefully remove media. Do not disturb cells and do not rinse with PBS.
9. Add 200µL DMSO and Sorenson's buffer (0.1 M Glycine, 0.1 M sodium chloride, PH=10.5) into each well to dissolve the formazan.
10. Cover with tinfoil and agitate cells on orbital shaker for 15 min.
11. Assays will include:
    a) Blank wells containing medium only
    b) Untreated control cells
    c) Test cells treated with the substance to be assayed
12. Remove plate cover and measure the absorbance in each well, including the blanks, at 570 nm in a microtiter plate reader. [Absorbances can be read with any filter in the wavelength range of 550 - 600 nm. The reference wavelength should be higher than 650 nm. The blanks should give values close to zero (+/- 0.1).]. (Spectrophotometric plate reader, ELx800, Biotek)
13. If the readings are low return the plate to the dark for longer incubation.

Troubleshooting

Problem: MTT Reagent is blue-green.

Cause	Remedy
Contamination with a reducing agent or cell/ bacterial contamination.	Discard. Remove aliquots of new MTT reagent using sterile technique.
Excessive exposure to light.	Store solution in the dark at 4°C.

Problem: Blanks (medium only) give high absorbance readings

Cause	Remedy
The medium is contaminated with cells/ bacteria/yeast (visible under microscope).	Discard. Check medium before plating. Use sterile technique for cell plating in biological hood. Use sterile 96-well plate.
The medium contains ascorbic acid.	Incubate plate in the dark. Find alternative medium if possible.

Problem: Absorbance readings too high.

Cause	Remedy
Cell number per well too high.	Decrease cell density at plating..
Contamination of culture with bacteria or yeast.	Discard. View wells prior to addition of MTT Reagent to check for contamination.

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Problem: Absorbance readings are too low.

Cause	Remedy
Cell number per well is too low.	Increase cell density at plating.
Incubation time for reduction of MTT is too short. No purple color visible in cells when viewed under microscope.	Increase incubation time with MTT Reagent until purple color is evident inside cells when viewed under microscope. Longer incubation of up to 24 hours may be required for some cell types.
Incubation time for solubilization of formazan dye too short (intact cells with intracellular dye visible when viewed under the microscope).	Increase incubation time with detergent reagent or incubate at 37°C. View under microscope to ensure no crystals remain out of solution.
Cells not proliferating due to improper culture conditions or inadequate time of recovery after plating.	Check that culture conditions (medium, temperature, humidity, CO2, etc.) are appropriate. View cells periodically to check condition. Increase time in culture after plating for cell recovery.

Problem: Replicates have different values.

Cause	Remedy
Inaccurate plating or pipetting.	Increase accuracy of cell plating, check accuracy of pipette.

References

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