

Biocolor's APOPercentage Apoptosis Assay™ on BMG LABTECH's microplate reader

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- Monitors occurrence of apoptosis in live, mammalian, anchorage-dependent cells
- Detects and quantifies percentage of apoptosis in cell population

Introduction

Apoptosis is a multistage process during which activity of caspase enzymes fluctuates, DNA becomes fragmented and phosphatidyl serine is transferred to the outside of the cell membrane.

Common methods for analysis include:

1. Caspase activity assay, either colorimetric, fluorescent, luminescent or antibody based.
2. TUNEL assay, based on DNA fragmentation.
3. Annexin-V assay, based on the binding of dye or fluorescently conjugated Annexin-V to phosphatidyl serine which has translocated to the cell membrane exterior during apoptosis.

Necrotic cells must be distinguished using propidium iodide.

APOPercentage Principle

Transfer and exposure of phosphatidyl serine to the exterior surface of the cell membrane has been linked to the onset of apoptosis. Phosphatidyl serine transmembrane movement results in the uptake of APOPercentage Dye by apoptotic committed cells. Dye uptake continues until blebbing of the apoptotic committed cell occurs. No further dye can then enter the defunct cell and the dye that has accumulated within the cell is not released. Necrotic cells do not retain the dye. Dyed cells can be counted using a light microscope or the dye can be released from the cells and measured spectrophotometrically as in the method below.

Materials & Methods

- Minimum cell culture facilities
- BMG LABTECH microplate reader
- 24 and 96 well microplates

Protocol

1. Seed a 24-well tissue culture plate with 5×10^4 cells/well in 500 μ L culture medium and incubate the cells at 37°C/5% CO₂, until confluence is reached (~ 24 h).
2. Prepare dilutions of test apoptotic agent(s) at selected concentrations using the suggested layout below. Controls (-ve & +ve) should be included with each experiment.

Reag. Blank	Reag. Blank	-ve Control	-ve Control	+ve Control	+ve Control
Sample 1	Sample 1	Sample 4	Sample 4	Sample 7	Sample 7
Sample 2	Sample 2	Sample 5	Sample 5	Sample 8	Sample 8
Sample 3	Sample 3	Sample 6	Sample 6	Sample 9	Sample 9

3. Make up double quantity of **Reagent A**. Use half the volume to prepare **Reagent B**.

	Reagent A (500 μ L/well)	Reagent B (500 μ L/well)
Reagent Blank	Culture medium / serum	Reagent A
Negative Control (0% apoptosis)	Culture medium / serum	Reagent A + 5% v/v dye
Positive Control (100% apoptosis)	Culture medium / serum + reference apoptotic agent	Reagent A + 5% v/v dye
Test Samples (>0%, <100%)	Culture medium / serum + test apoptotic agent	Reagent A + 5% v/v dye

4. Remove the culture medium from each well of the incubated plate and add 500 μ L of **Reagent A**, supplemented v/v with serum (if required), to all wells.
5. Incubation time for apoptotic inducer/inhibitor will depend on apoptotic agent used. 30 min before this time period is reached remove **Reagent A**. Immediately replace with 500 μ L **Reagent B** and incubate for the remaining 30 min, at 37°C/5% CO₂.
6. Remove **Reagent B** from each well using a pipette. Gently wash the cells twice with 1000 μ L/well PBS to remove non-cell bound dye. [NOTE: Careful aspiration is advised as some apoptotic agents can cause detachment and loss of cells].
7. Add trypsin (50 μ L) to each well and incubate for 10 minutes at 37°C/5% CO₂. Tap the plate gently by hand after 5 minutes and again after 10 minutes to detach cells from the plastic, cell culture treated surface.
8. Now add 200 μ L Dye Release Reagent to each well and shake plate for 10 minutes.
9. Transfer contents of each well (250 μ L) to a 96 well flat bottom plate and read absorbance at 550 nm. Bubbles in wells affect results, burst with clean pin or transfer 200 μ L instead of 250 μ L to microplate.

This experiment was carried out with triplicate samples.



Instrument Settings

Measurement Type:	Absorbance
No. of Flashes per well:	22
Excitation:	550 nm
Shaking Frequency (rpm):	300
Shaking Mode:	double orbital
Additional Shaking Time:	3s before plate reading
Positioning Delay (s):	0.2

Background

Biocolor is a UK company based in Carrickfergus, N. Ireland, with a network of distributors throughout the world. Biocolor's expertise lies in its unique range of extracellular matrix assays for use with mammalian cells, tissues and fluids: Sircol™- Collagen, Blyscan™- Glycosaminoglycan and Fastin™-Elastin. VolCol is Biocolor's range of bovine and rat collagen products.

More information can be found at www.biocolor.co.uk

Results & Discussion

1. Follow protocol as above to obtain absorption data.
2. Subtract the mean value of reagent blank replicates from test results.
3. Plot mean absorbance values \pm standard error of the mean in a bar chart (Fig. 1) or as a percentage of the Positive Control absorbance value.

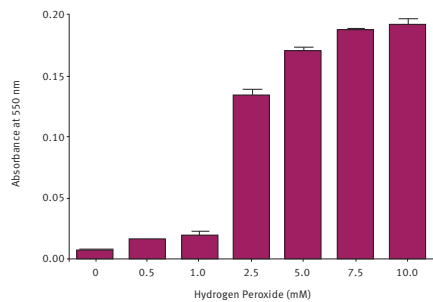


Fig. 1: Colorimetric Quantification. Graph Showing Effect of Hydrogen Peroxide [0 - 10mM] on CHO Cells. Results expressed as mean absorbance for triplicate wells \pm S.E.M. (n = 3). Exposure time to H₂O₂ was 4 hours.

Conclusion

The microplate reader from BMG LABTECH provide a fast, accurate and consistent method for quantifying apoptosis colorimetrically.

