Cell-APOPercentage

Apoptosis

Assay

Detection and measurement of cell apoptosis

biocolor

life science assays

internet manual downloaded from www.biocolor-assays.com
Colorimetric Protocol

1. Seed a 24 well tissue culture plate with 5 x 10^4 cells in 500 µl culture medium and incubate the cells at 37°C / 5% CO₂ until confluence is reached (~ 24h).

<table>
<thead>
<tr>
<th>Reagent Blank</th>
<th>Reagent Blank</th>
<th>Negative Control</th>
<th>Negative Control</th>
<th>Positive Control</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>Sample 1</td>
<td>Sample 4</td>
<td>Sample 4</td>
<td>Sample 7</td>
<td>Sample 7</td>
</tr>
<tr>
<td>Sample 2</td>
<td>Sample 2</td>
<td>Sample 5</td>
<td>Sample 5</td>
<td>Sample 8</td>
<td>Sample 8</td>
</tr>
<tr>
<td>Sample 3</td>
<td>Sample 3</td>
<td>Sample 6</td>
<td>Sample 6</td>
<td>Sample 9</td>
<td>Sample 9</td>
</tr>
</tbody>
</table>

2. Prepare dilutions of test apoptotic agent(s) at selected concentrations using the suggested layout above. Controls (-ve & +ve) should be included with each experiment.

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

<table>
<thead>
<tr>
<th></th>
<th>Reagent A (500µl/well)</th>
<th>Reagent B (500µl/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent Blank</td>
<td>Culture medium / serum</td>
<td>Reagent A</td>
</tr>
<tr>
<td>Negative Control (0% apoptosis)</td>
<td>Culture medium / serum</td>
<td>Reagent A + 5% v/v dye</td>
</tr>
<tr>
<td>Positive Control (100% apoptosis)</td>
<td>Culture medium / serum + reference apoptotic agent</td>
<td>Reagent A + 5% v/v dye</td>
</tr>
<tr>
<td>Test Samples (&gt; 0%, &lt;100%)</td>
<td>Culture medium / serum + test apoptotic agent</td>
<td>Reagent A + 5% v/v dye</td>
</tr>
</tbody>
</table>

4. Remove the culture medium from each well of the incubated plate and add 500µl of Reagent A, supplemented with serum (if required by the cells), 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 500µl 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 1000µl/well PBS to remove non-cell bound dye. (NOTE: Careful pipetting is advised as some apoptotic agents can cause detachment and loss of cells).

7. Add trypsin (50µl) 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 200µl Dye Release Reagent to each well and shake plate for 10 minutes.

9. Transfer contents of each well (250µl) to a 96 well flat bottom plate and read absorbance at 550nm, (or blue-green filter), using a microplate reader. Bubbles in wells affect results, burst with clean pin or transfer 200µl instead of 250µl to microplate.

FOR ASSAY CALCULATIONS GO TO PAGE 4
Supplementary Protocol

Analytical Digital Photomicroscopy – The dye reagent used has a purple-red colour, which allows detection of apoptosis with a conventional inverted microscope.

Photomicrograph images obtained may be transferred, as jpeg files, to a computer where the stained area is counted in pixels using Image J software (free from; http://rsbweb.nih.gov/ij/).

The digital analytical photomicroscopy protocol and videos demonstrating the procedure can be found on our website.

Assay Developed and Produced

by

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Photomicrograph of live CHO cells labelled with APOPercentage Dye for 30 minutes at 37°C / 5% CO₂, (Microscope magnification x400).

Prior to photography the dyed cells were washed with DMEM and then covered to a depth of 3 to 4 mm with DMEM without Phenol Red but containing a supplement of 5% serum.

The above soft focus image is from a CHO control well free from any added apoptotic inductors. The incidence of a dye labelled apoptotic cell in control wells is low, about 1 per 20,000 cells. Apoptotic cell numbers do tend to gradually increase with subsequent sub-culturing.
Cell-APOPercentage™
Apoptosis Assay

TECHNICAL INFORMATION

ASSAY PROTOCOL
Colorimetric Protocol

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The assay has been designed for research work only.
Handle the APOPercentage Assay using Good Laboratory Practice.

APOPercentage™ Apoptosis Assay Patent No: GB2356929
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Intended Applications

The Cell-APOPercentage™ Apoptosis Assay is a detection and measurement system to monitor the occurrence of apoptosis in mammalian, anchorage-dependent cells during in vitro culture.

The assay uses a dye that is selectively imported by cells that are undergoing apoptosis. Necrotic cells cannot retain the dye and therefore are not stained.

Protocol and Format

**Colorimetry protocol** - The dye that accumulates in 30 minutes within labeled cells is released into solution and the concentration of released intracellular dye measured using a microplate colorimeter.

This manual describes the colorimetric protocol in a 24-well plate format. Alternative microwell plates, microscope chamber slides and T-flasks are also suitable for use with the assay. If using a 96 well plate with the colorimetric method it is recommended that the contents of 3 wells be pooled before reading results in the microplate reader to provide a detectable absorbance.

**APOPercentage Kit Pack Sizes and Storage Conditions**

**Assay Kit Product Code: A1000** sufficient for 4 x 24 wells or 6 x 96 wells, microwell format.

All components are stable for up to one year unopened when stored at 25°C. Store at 4°C once opened and use within 30 days.
Assay Kit Components

1. APOPercenage Dye (5ml)

The dye is dissolved in a phosphate buffered saline (PBS) solution, pH 7.4, prepared using pyrogen-free water. The dye solution was prepared by filtering the dye solution through a 0.2μm filter into sterile sealed glass ampoules.

For assay use: dye reagent aliquots should be removed from this vial using a sterile needle and syringe (1 ml or 2 ml capacity is suitable). A dye solution that becomes turbid, or where particles appear, should be discarded as sterility has possibly been compromised.

2. APOPercentage Dye Release Reagent (150ml)

An alkali solution that disrupts the cell membrane and releases intracellular accumulated dye.

3. Phosphate Buffered Saline (PBS), sterile solution (100ml) – starter bottle.

The APOPercenage Assay has been designed for use within the ‘Clean Room’ of a Cell Culture Unit.

Other Components required - not supplied

The Colorimetric Protocol Requires

(a) A fresh working solution of trypsin as used for cell release during cell sub-culturing.

(b) A Microplate Colorimeter to measure released dye, (a BMG Labtech Spectrostar Nano Microplate Reader was used in the present work).

(c) 24 well plates for culturing cells during apoptosis testing.

(d) 96 well plates for use when reading cell dye released absorbance values.

The Detection Protocol Requires

An inverted stage microscope with an attached digital camera.
**MODE OF ACTION**

The onset of the execution phase of apoptosis has been linked to translocation of phosphatidylserine from the interior to the exterior surface of the mammalian cell membrane, experimentally supported by annexin-V binding to phosphatidylserine. Phosphatidylserine transmembrane movement results in the uptake of the APOPercantage Dye by apoptotic committed cells. This dye uptake continues until *blebbing* occurs. No dye is then released from the now defunct cell, other than that within isolated ‘blebs’.

Each apoptotic inducer has an optimum time and concentration to be effective. This optimum time and concentration will be affected by the cell line examined.

**ASSAY PREPARATIONS**

**Manual:** This protocol should be considered as a start-up guide only. Seeding cell density for particular cells used and incubation time required for apoptotic agent of choice will need to be optimized.

**Format:** The APOPercantage™ Assay has been designed for use with anchorage dependent mammalian cells seeded in sterile tissue culture containers. A 24 well plate format has been used as a working example of the assay.

**Seeding Density:** In order to compare experiments carried out on different days the cell seeding density and the time required to obtain a confluent layer should be maintained.

**Negative Control:** Occurrence of apoptosis within an uninduced primary cell/cell line should be assayed to examine the background frequency of dye labeled cells.

**Positive Control:** The use of 10mM H2O2 for four hours to induce 100% dye labelled cells is recommended. Test results can be expressed as a percentage of this data to display percentage apoptosis.

**Reagent Blank:** A reagent blank sample should be included in each experiment to take into account the background absorption value due to, for example, Phenol Red and serum if used in culture medium.

**Cell Adhesion:** If unacceptable cell loss is encountered with test agent(s), consider growing the cells on a gelatin gel covered by culture medium (protocol available from our website).
COLORIMETRIC PROTOCOL EXAMPLE CALCULATIONS

1. Follow protocol on inside front cover of manual.

2. Subtract the mean value of reagent blank replicates from test results obtained as shown in table below. (This experiment was carried out with triplicate samples).

<table>
<thead>
<tr>
<th>H$_2$O$_2$ (mM)</th>
<th>Raw Absorbance Data</th>
<th>Mean Reagent Blank Value</th>
<th>Raw Data Minus Reagent Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.062</td>
<td>0.058</td>
<td>0.009</td>
</tr>
<tr>
<td>0.5</td>
<td>0.066</td>
<td>0.068</td>
<td>0.013</td>
</tr>
<tr>
<td>1.0</td>
<td>0.067</td>
<td>0.080</td>
<td>0.014</td>
</tr>
<tr>
<td>2.5</td>
<td>0.179</td>
<td>0.196</td>
<td>0.126</td>
</tr>
<tr>
<td>5.0</td>
<td>0.226</td>
<td>0.217</td>
<td>0.173</td>
</tr>
<tr>
<td>7.5</td>
<td>0.234</td>
<td>0.247</td>
<td>0.181</td>
</tr>
<tr>
<td>10.0</td>
<td>0.245</td>
<td>0.236</td>
<td>0.192</td>
</tr>
</tbody>
</table>

3. Plot mean absorbance values ± 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.**

Effect of Hydrogen Peroxide (0 – 10mM) for 4 hours on CHO Cells.

Results expressed as mean absorbance for triplicate wells ± S.E.M. (n = 3)
APOPERCENTAGE DYE USED TO LABEL CELLS EXHIBITING APOPTOSIS IN ANCHORAGE-DEPENDENT MAMMALIAN CELLS, USING A RANGE OF APOPTOTIC INDUCERS

Hydrogen Peroxide (H$_2$O$_2$) (5mM)

3T3 Cells

1 HOUR  
2 HOURS  
3 HOURS

CHO Cells

1 HOUR  
2 HOURS  
3 HOURS
Cyclohexamide (CHX) (10mM)

3T3 Cells
2 HOURS

4 HOURS

6 HOURS

CHO Cells
2 HOURS

4 HOURS

6 HOURS
Cisplatin (2mM)

3T3 Cells
2 HOURS 4 HOURS 6 HOURS

CHO Cells
2 HOURS 4 HOURS 6 HOURS