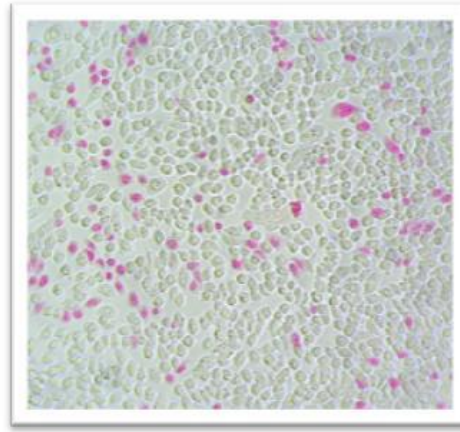
Cell-APOPercentage

Apoptosis

Assay

Detection and measurement of cell apoptosis



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Cell-APOPercentage™

Apoptosis Assay

TECHNICAL INFORMATION

The assay has been designed for research work only.

Handle the APOPercentage Assay using Good Laboratory Practice.

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ASSAY MANUAL

Intended Applications

The Cell-APOP*ercentage*™ 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.

Assay Formats

Direct Analysis
Dye uptake results in individual apoptotic cells staining an intense pink colour. This can be visually assessed using brightfield microscopy. Apoptosis in substrate-adherent cell populations can be readily quantified using image analysis techniques. This technique is the most sensitive. *See page 9 for further detail.*

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/spectrophotometer.
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. *See page 4 for further detail.*

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. APOPercentage Dye (5ml)

The dye is supplied in phosphate buffered saline (PBS), solution of pH 7.4. The sealed glass vial is sterile until opened.

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 may have 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 APOPercentage 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 / Spectrophotometer** to measure released dye.

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

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

The Direct 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 APOPercentage 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 APOP*ercentage*™ 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 H_2O_2 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).

APOPercentage Assay - Colorimetric Protocol

1. Seed a 24 well tissue culture plate with 5 x 10^4 cells in 500 ul culture medium and incubate the cells at 37^0C / 5% CO_2 until confluence is reached (~ 24h).

Reagent Blank	Reagent Blank	Negative Control	Negative Control	Positive Control	Positive 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

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.

	Reagent A (500µl/well)	**Reagent B (500µl/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 500µl of Reagent A, supplemented $^v/_v$ 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% CO_2.

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% CO_2. 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 10minutes.

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.

COLORIMETRIC PROTOCOL - EXAMPLE CALCULATIONS

1. Follow protocol provided

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).

H_2O_2 (mM)	Raw Absorbance Data			Mean Reagent Blank Value	Raw Data Minus Reagent Blank		
0	0.062	0.061	0.058	0.053	0.009	0.008	0.005
0.5	0.066	0.072	0.068		0.013	0.019	0.015
1.0	0.067	0.080	0.071		0.014	0.027	0.018
2.5	0.179	0.187	0.196		0.126	0.134	0.143
5.0	0.226	0.228	0.217		0.173	0.175	0.164
7.5	0.234	0.239	0.247		0.181	0.186	0.194
10.0	0.245	0.255	0.236		0.192	0.202	0.183

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.

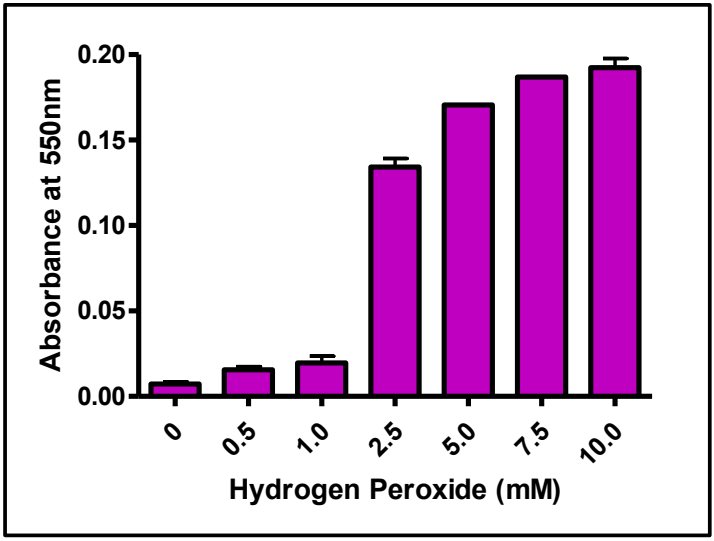


Fig. 1 ***Colorimetric Quantification.***

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

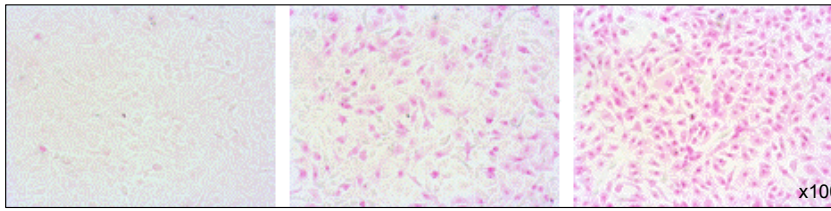
Results expressed as mean absorbance for triplicate wells ± S.E.M. (n = 3)

APOPERCENTAGE DYE USED TO LABEL CELLS EXHIBITING APOPTOSIS IN ANCHORAGE-DEPENDENT MAMMALIAN CELLS, USING A RANGE OF APOPTOTIC INDUCERS

Hydrogen Peroxide (H_2O_2) (5mM)

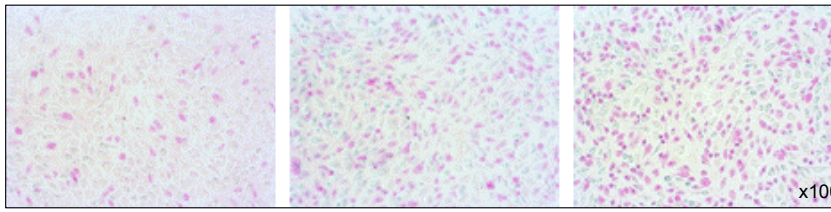
3T3 Cells

1 HOUR **2 HOURS** **3 HOURS**



CHO Cells

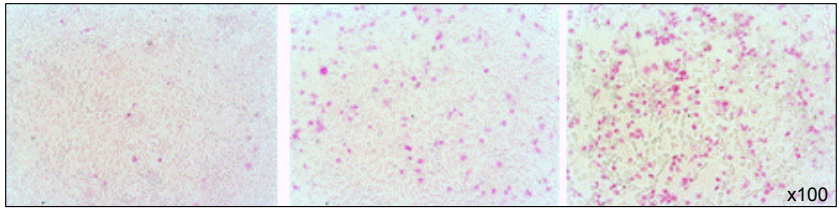
1 HOUR **2 HOURS** **3 HOURS**



Cyclohexamide (CHX) (10mM)

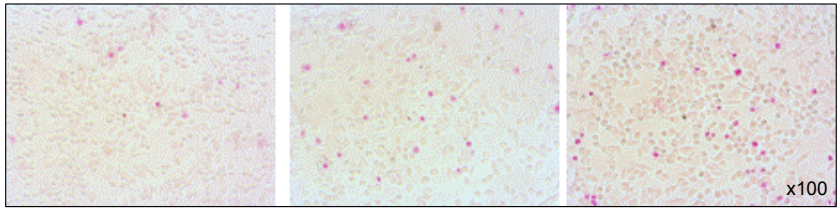
3T3 Cells

2 HOURS **4 HOURS** **6 HOURS**



CHO Cells

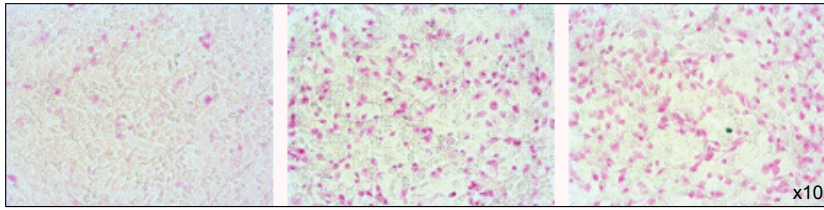
2 HOURS **4 HOURS** **6 HOURS**



Cisplatin (2mM)

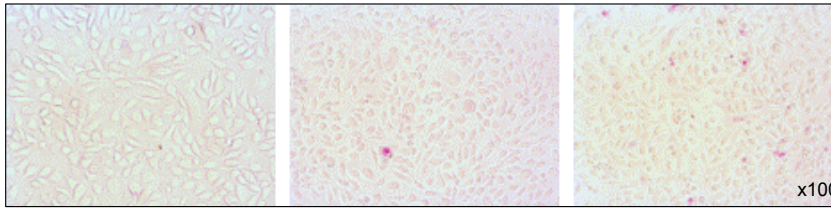
3T3 Cells

2 HOURS **4 HOURS** **6 HOURS**



CHO Cells

2 HOURS **4 HOURS** **6 HOURS**



Supplementary Direct detection Protocol

The dye reagent used has a purple-red colour, which allows visual direct detection of apoptosis in single cells using a conventional inverted microscope.

Photomicrograph images obtained may transferred to a computer where the dye-stained area can be 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 the APOPercentage page of our website.

Photomicrograph of live CHO cells labelled with APOPercentage Dye for 30 minutes at 37^0C / 5% CO_2, (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 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.