SIGMA

**ProductInformation**

**Apoptosis Detection Kit, Annexin V-CY3**

Product No. **APO-AC**
Store at 2-8 °C

# TECHNICAL BULLETIN

**Product Description**
The annexins are a group of homologous proteins that bind phospholipids in the presence of calcium.[1,2] Apoptosis, or programmed cell death, is an important mechanism of most cells used to negatively select cells that are deleterious to the host. Many cells of the immune system such as thymocytes, self-reactive B and T cells undergo apoptosis as a result of the normal cell selection process. The cellular changes involved in the process include loss of cell membrane phospholipid asymmetry during early stages of apoptosis. In living cells the phosphatidylserine [PS] is transported to the inner plasma membrane leaflet by the enzyme Mg-ATP dependent aminophospho-lipid translocase.[3] However, during the onset of apoptosis, the PS is transported to the external leaflet of the plasma membrane. The PS is then available for binding to annexin V and any of its conjugates in the presence of $Ca^{2+}$ ions.

Apoptotic cells can be differentiated from necrotic cells in several ways. The method employed by this kit involves the use of two labels:

- Annexin-Cy3.18 (AnnCy3) binds to phosphatidylserine that is present in the outer leaflet of the plasma membrane of cells that are starting the apoptotic process. The binding is observed as red fluorescence.

- 6-Carboxyfluorescein diacetate (6-CFDA) is used to measure viability. When this non-fluorescent compound enters living cells, esterases present hydrolyze it, producing the fluorescent compound, 6-carboxyfluorescein (6-CF). This appears as green fluorescence.

Cells can be incubated either with AnnCy3 or 6-CFDA separately or with the two compounds simultaneously. After labeling at room temperature, the cells are immediately observed by fluorescence microscopy. Live cells will be labeled only with 6-CF (green), while necrotic cells will label only with AnnCy3 (red). Cells in the early stage of apoptosis, however, will be labeled with both AnnCy3 (red) and 6-CF (green).

**Components**
The kit includes reagents for 200 tests (which is equivalent to 5-10 x $10^6$ Jurkat cells according to the supplied procedure).

- Annexin V-Cy3.18, 10 µg protein
  Product No. A 4963 , 100 µg/ml solution in 50 mM Tris HCl, pH 7.5, containing 100 mM NaCl

- 6-Carboxyfluorescein diacetate 10 mg
  (6-CFDA), Product No. C 5041

- 10X Binding Buffer, 20 ml
  Product No. B 9796, 100 mM HEPES/NaOH, pH 7.5, containing 1.4 M NaCl and 25 mM $CaCl_2$

Reagents and Equipment Required But Not Provided
(Sigma product numbers have been given where appropriate)
- Cells to undergo apoptosis. An example procedure is given using Jurkat E6-1 cells.[6]
- Apoptosis inducer. Induction may be spontaneous or induced. In the example, staurosporin (Product No. S 4400) dissolved at 100 µg/ml in DMSO is used as inducer.[6]
- Phosphate buffered saline (PBS) Product No. D 8537
- Fluorescence microscope
- Serological centrifuge
- Incubator at 37 °C with 5% $CO_2$ atmosphere
- PolyPrep poly-L-lysine coated slides, Product No. P 0425
- Cover glasses, 24 x 50 mm, Product No. C 8181
- PAP pen for immuno staining, Product No. Z37,782-1

**Preparation Instructions**

Reagents supplied with this kit:

- 1X Binding Buffer
  (10 mM HEPES, pH 7.5, containing 140 mM NaCl and 2.5 mM $CaCl_2$):
  Dilute 10X Binding Buffer (Product No. B 9796) 1:10 with deionized water.

- 50 mM 60CFDA in acetone
  Dissove 2.32 mg 6-Carboxyfluorescein diacetate (6-CFDA, Product No. C 5041) in 0.1ml acetone. Store in an amber vial and protect from light. After opening store the original vial (solid) at –20 °C.

- Double Label Staining Solution
  (1 μg/ml AnnCy3 and 500 μM 6-CFDA in 1X Binding Buffer)
  To prepare 2 ml of Double Label Staining Solution add:

| | |
|---|---|
| 20 μl | AnnCy3, 100 μg/ml solution (Product No. A 4963) |
| 20 μl | 50 mM 6-CFDA in acetone (see above) |
| 200 μl | 10X Binding Buffer (Product No. B 9796) |
| 1.76 ml | Deionized water |

  Store this solution in an amber vial and protect from light.

If single staining is desired:

- Annexin V-Cy3 Conjugate (1 μg/ml AnnCy3 in 1X Binding Buffer):
  Dilute AnnCy3, 100 μg/ml solution (Product No. A 4963) 1:100 with 1X Binding Buffer. Store in an amber vial and protect from light.
- 500 μM cFDA
  Dilute 50 mM cFDA 1:100 in binding buffer. Store in an amber vial and protect from light.

**Storage/Stability**

Store the kit at 2-8 °C. Protect from light

Note: All the solutions supplied in this kit have been filtered with a sterile 0.2 μm filter and the bottles aseptically filled.

For long term stability of the solution when in use, it is recommended to remove an aliquot in a sterile manner in a hood. No preservative is added to these solutions.

**Procedure**

The following procedure is an example using Jurkat cells and Double Label Staining Solution.

**Note:** Staining of Jurkat cells with cFDA can be performed with lower concentrations of cFDA (minimal concentration-100μM.)

1. Induce apoptosis in a cell suspension of Jurkat cells (e.g., by addition of staurosporin to 1 μg/ml). Keep non-induced cells for a zero time control.

2. Incubate for the desired time at 37 °C in a 5% $CO_2$ atmosphere.

3. Wash the cells twice with PBS.

4. Suspend the cells in PBS at a concentration of approximately 0.5-1x$10^6$ cells per ml.

5. Take a PAP pen and draw 2 circles of approximately 1 cm diameter on a PolyPrep poly-L-lysine-coated slide (one for control cells and one for induced sample cells). This will restrict the drop placed on the slide to a specific area.

6. Place 50 μl of the cell suspension (induced or non-induced) in each circle and leave at room temperature for 10 minutes, allowing the cells to be absorbed to the plate.

7. Remove the excess liquid by carefully touching a tissue to the side of the circle. Do not blot directly on top of the sample since this will damage the cells.

8. Wash the cells with three aliquots of 50 μl of 1X Binding Buffer. Blot the excess liquid each time with a tissue as in step 7.

9. Place 50 μl of the Double Label Staining Solution (AnnCy3 + 6-CFDA) on each circle and cover with a petri dish covered with aluminum foil.

10. Incubate for 10 minutes at room temperature. After staining, wash each circle with five aliquots of 50 μl of 1X Binding Buffer as in step 8. This will remove excess label from the cells.

11. Place 35 µl of 1X Binding Buffer on each circle and cover the slide with a 24 x 50 mm cover slip. Observe the results using a fluorescence microscope, then photograph. Use the correct filter and light source depending on the label.

**Note:** When using cells other than Jurkat cells or in the case of non optimal staining, it is recommended to optimized the reagents concentrations required for appropriate staining.

**Results**

By fluorescence microscopy, 6-carboxyfluorescein (6-CF) is observed as green fluorescence and Annexin V Cy3.18 (AnnCy3) is observed as red fluorescence.

There are three possible results:

1) Live cells will only stain with 6-CFDA (green).
2) Necrotic cells will only stain with AnnCy3 (red).
3) Cells starting the apoptotic process will stain both with AnnCy3 (red) and 6-CF (green).

Note: By microscopy, Annexin Cy3 fluoresces more brightly than the Annexin FITC conjugate.

**References**


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5. Breeuwer, P., *et al.*, Appl. Environ. Microbio., **61**, 1614 (1995)
6. Martin, S.J., *et al.*, J. Exp. Med., **182**, 1545 (1995)


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