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Product Information

Annexin V-Cy3™ Apoptosis Detection Kit

Catalog Number **APOAC**
Storage Temperature 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 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 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, PS is transported to the external leaflet of the plasma membrane. 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 phosphatidyl-serine present in the outer leaflet of the plasma membrane of cells 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 sufficient reagents for 200 assays (which is equivalent to 5–10 × 10^6 Jurkat cells according to the supplied procedure).

- Annexin V Cy3.18 Conjugate 10 µg protein
  (Catalog Number A4963)
  100 µg/ml solution in 50 mM Tris HCl, pH 7.5, containing 100 mM NaCl

- 6-Carboxyfluorescein diacetate (6-CFDA) 10 mg
  (Catalog Number C5041)

- 10× Binding Buffer 20 ml
  (Catalog Number B9796)
  100 mM HEPES/NaOH, pH 7.5, containing 1.4 M NaCl and 25 mM $CaCl_2$

Reagents and Equipment Required But Not Provided

(Catalog Numbers have been given where appropriate)

- Cells to undergo apoptosis. A procedure is given using Jurkat E6-1 cells.[6]
- Apoptosis inducer - Induction may be spontaneous or induced. In the procedure, staurosporin (Catalog Number S4400) dissolved at 100 µg/ml in DMSO is used as inducer.[6]
- Phosphate buffered saline (PBS, Catalog Number D8537)
- Fluorescence microscope
- Serological centrifuge
- Incubator at 37 °C with 5% CO_2 atmosphere
- Poly-Prep poly-L-lysine coated slides (Catalog Number P0425)
- Cover glasses, 24 × 50 mm (Catalog Number C8181)
- PAP pen for immunostaining (Catalog Number Z377821)

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
1× Binding Buffer (10 mM HEPES, pH 7.5, containing 140 mM NaCl and 2.5 mM $CaCl_2$) - Dilute 10× Binding Buffer (Catalog Number B9796) 10-fold with deionized water.

50 mM 6-CFDA in acetone solution - Dissove 2.32 mg of 6-Carboxyfluorescein diacetate (6-CFDA, Catalog Number C5041) in 0.1 ml acetone. Store solution in an amber vial and protect from light. After opening, store remaining 6-CFDA at –20 °C.

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

20 μl	Annexin V Cy3.18 Conjugate (100 μg/ml solution, Catalog Number A4963)
20 μl	50 mM 6-CFDA in acetone solution
200 μl	10× Binding Buffer (Catalog Number B9796)
1.76 ml	Deionized water

Store the Double Label Staining Solution in an amber vial and protect from light.

If single staining is desired, prepare the following:

- AnnCy3 Solution (1 μg/ml of AnnCy3 in 1× Binding Buffer) - Dilute Annexin V Cy3.18 Conjugate (100 μg/ml solution, Catalog Number A4963) 100-fold with 1× Binding Buffer. Store in an amber vial and protect from light.

- 500 μM CFDA Solution – Dilute 50 mM 6-CFDA in acetone solution 100-fold in 1× 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 solutions 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 a general guideline. It uses Jurkat cells and the Double Label Staining Solution.

Notes: Staining of Jurkat cells with 6-CFDA can be performed with lower concentrations of 6-CFDA (minimal concentration of 100 μM).

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

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 0.5–1 × 10^6 cells per ml.

5. Take a PAP pen and draw 2 circles of ~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 three times with 50 μl of 1× Binding Buffer each. Blot the excess liquid each time with a tissue as in step 7.

9. Place 50 µl of the Double Label Staining Solution (AnnCy3 and 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 five times with 50 µl of 1× Binding Buffer each as in step 8. This will remove excess label from the cells.

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

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-CF (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

1. Pigault C., *et al*., J. Mol. Biol., **236**, 199 (1994).
2. Trotter, P.J., *et al*., Biochem. J., **308**, 591 (1995).
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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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