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APOPTEST™-Biotin kit for 100 tests

CATALOG NUMBER: MUB-8100B 100 tests
SPECIES: recombinant human Annexin-A5

QUALITY CONTROL:

Annexin A5-conjugated to Biotin (Annexin V-Biotin)
Annexin V-Biotin: 1:8 stoichiometric complex.
Purity: > 99% pure according to Fast Protein Liquid Chromatography.
Quality: > 99% of the protein has full phospholipid binding properties according to ellipsometry
Additives : None

BACKGROUND

The APOPTEST™-BIOTIN is designed to measure Apoptosis, also know as Programmed Cell Death (PCD) in different experimental settings where APOPTEST™-FITC is less suitable. With APOPTEST™-Biotin it is possible to study PCD of adherent cell types in vitro, to perform tricolor analysis of PCD and antigen expression in vitro, and to measure PCD in intact organisms and in tissue samples. APOPTEST™-BIOTIN can be applied to samples derived from mammals, avian and insects (Ref. 1).
During the process of PCD, the plasma membrane of cells start to expose the phospholipod phosphatidylserine at their outer surface before the membrane looses its integrity and allows propidium iodide to enter the cell (Ref 2-4). The APOPTEST™-Biotin employs the property of Annexin V to bind to phosphatidylserine in the presence of Ca^{2+}. In addition, the use of propidium iodide in *in vitro* assays allows to discriminate between viable cells (Annexin V negative and propidium iodide negative), apoptotic cells (Annexin V positive and propidium iodide negative) and (secondary) necrotic cells (Annexin V positive and propidium iodide positive) (Fig. 1).

SPECIFICITY

The conjugation protocol to form Annexin A5 with Biotin to a 1:8 stoichiometric complex has not changed the native phospholipid binding properties of Annexin A5. Binding kinetics show a fast association of Annexin V-Biotin with the phospholipid membrane if phosphatidylserine and Ca^{2+} are available. In the assay, the binding buffer contains Ca^{2+} to allow the interaction between Annexin V-Biotin and phosphatidylserine.

PRODUCT

Each APOPTEST™-Biotin product contains:
1 vial containing 500µl recombinant Annexin V-Biotin solution.
6 vials containing 1.7ml 10x concentrated binding buffer.
1 vial containing 250µg red solid propidium iodide.

APPLICATION

The APOPTEST™-Biotin has several applications:
A) to measure PCD in adherent cells in vitro (Ref. 5)
B) to measure PCD and antigen expression in adherent cells.
C) to measure PCD and antigen expression in cells in suspension where methanol fixation is required.
D) to measure PCD in situ in cells their histological context in whole mounts, paraffin en semithin sections and at the ultrastructural level (Ref 1, 6-8).
The procedures described in this datasheet allow to determine and quantify apoptosis with the flow cytometer or microscope. For measuring PCD in cells in suspension <u>without</u> simultaneous intracellular antigen labeling in the presence of methanol we kindly advise you to use our APOPTEST™-FITC (Ref. 2-3).

PROCEDURES

A. Detection of apoptosis of adherent cell types in culture: Bicolor analysis using Annexin V-Biotin, streptavidin-FITC and propidium iodide.

Materials
APOPTEST™-Biotin
Streptavidin-FITC (not included)
Adherent cells
Ice

Apparatus
Flow cytometer

Method
1. Culture the cells of interest in the wells of a 24 wells plate.
2. Induce apoptosis according to your specific protocols.
3[i]. Add 5 µl of Annexin V-Biotin stock solution to 500µl culture medium at the time point of analysis.
4[ii]. Incubate the cells for at least 5 minutes with Annexin V-Biotin.
5. Remove the supernatant containing the detached cells from the well.
6. Wash the detached cells twice with Ca^{2+} adjusted culture medium or 1x binding buffer to remove unbound Annexin V-Biotin.
7. Rinse the adherent cells in the wells twice with Ca^{2+} adjusted culture medium or 1x binding buffer before harvesting.
8[iii]. Harvest by scraping with a rubber policeman.
9. Dilute the cells with Ca^{2+} adjusted culture medium or 1x binding buffer to 10^5-10^6 cells/ml.
10. Add streptavidin-FITC and propidium iodide (2.5 µg/ml final concentration) to the cell sample.
11. Incubate for 15 minutes on ice before flow cytometric analysis.

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12. The two color dot plot will show three distinct populations. a) the viable cells which were not damaged during collection by scraping. These cells have low FITC and low PI signal, b) the damaged viable cells, which have low FITC and high PI signal and c) the apoptotic cells, which have high FITC and low PI signal. Depending on the cell type and the fraction (adherent or detached) a fourth population may be present. d) the secondary necrotic cells with high FITC and high PI signal.

B. Detection of apoptosis of adherent cell types in culture: Tricolor analysis using Annexin V-Biotin, streptavidin-FITC, a PE-labeled antibody against an intracellular antigen of choice and propidium iodide.

Materials
APOPTEST™-Biotin.
Streptavidin-FITC (not included)
PE-labeled antibody against antigen of interest
Adherent cells
Ice
Apparatus
Flow cytometer
Method
1. Culture the cells of interest in the wells of a 24 wells plate.
2. Induce apoptosis according to your specific protocols.
3[i]. Add 5 µl of Annexin V-Biotin stock solution to 500 µl culture medium at the time point of analysis.
4[ii]. Incubate the cells for at least 5 minutes with Annexin V-Biotin.
5. Remove the supernatant containing the detached cells from the well.
6. Wash the detached cells twice with Ca^{2+} adjusted culture medium or 1x binding buffer to remove unbound Annexin V-Biotin.
7. Rinse the adherent cells in the wells twice with Ca^{2+} adjusted culture medium or 1x binding buffer before harvesting.
8[iii]. Harvest by scraping with a rubber policeman.
9. Fix the cells by resuspending the cell pellet by incubating for 5 minutes in methanol of -20°C.
10. Wash the cells once with PBS and once with PBS, containing 1 mg/ml BSA.
11. Add to 100 µl resuspended cell sample FITC-labeled streptavidin and a PE-labelled antibody against an antigen of choice.
12. Incubate for 1 hour at room temperature.
13. Wash twice with PBS/BSA and finally resuspend the cells in PBS, supplemented with 2.5 µg/ml propidium iodide and 100 µg/ml RNAse.
14. Incubate for 15 minutes on ice before flow cytometric analysis.
15. This protocol enables you to study the expression of an intracellular antigen of choice during the process of apoptosis.
16. If you have FITC-labeled antibodies you can make use of streptavidin-PE for the tricolour analysis to label bound Annexin V-Biotin.

C. Detection of apoptosis of suspended cell types in culture requiring methanol fixation: Tricolor analysis using Annexin V-Biotin, streptavidin-FITC, a PE-labeled antibody against an intracellular antigen of choice and propidium iodide.

Materials
APOPTEST™-Biotin
Streptavidin-FITC (not included)
PE-labeled antibody against antigen of interest
Methanol
Cells in suspension
Ice
Apparatus
Flow cytometer
Remark
Apoptosis of suspended cells can be easily measured using APOPTEST™-FITC (Annexin V FITC). However, if you are performing a tricolor analysis using methanol for fixation, Annexin V-FITC is not recommended because of the loss of FITC signal during fixation.
Method
1. Dilute the 10x concentrated binding buffer 10 fold with distilled water and place the diluted buffer on ice.
2. Resuspend the cells in ice cold diluted binding buffer to 10^5-10^6 cells/ml.
3. Add 5 µl Annexin V-Biotin to 500 µl cell sample and incubate for 5 min on ice.
4. Wash twice with diluted binding buffer to remove unbound Annexin V-Biotin.
5. Fix the cells by resuspending the cell pellet in methanol of -20°C and incubating for 5 minutes.
6. Wash the cells once with PBS and once with PBS, containing 1 mg/ml BSA.
7. Add to 100 µl resuspended cell sample FITC labeled streptavidin and a PE-labeled antibody against an antigen of choice.
8. Incubate for 1 hour at room temperature.
9. Wash twice with PBS/BSA and finally resuspend the cells in PBS, supplemented with 2.5 µg/ml propidium iodide and 100 µg/ml RNAse.
10. Incubate the sample 15 minutes on ice before flow cytometric analysis.
11. This protocol enables you to study the expression of an intracellular antigen of choice during the process of apoptosis.
12. If you have FITC-labeled antibodies you can make use of streptavidin-PE for the tricolor analysis to label bound Annexin V-Biotin.

D. Detection of PCD in cells in their histological context: detection in tissue specimens
The presence of PCD in tissues from human or other species, can be detected using Annexin V-Biotin. Many studies have shown that detection of PCD with Annexin V-Biotin in situ precedes detection of PCD on basis of DNA fragmentation as detected with the TUNEL assay (Figure 2), and labeling of PCD can be achieved throughout the tissue of interest from whole organism to the ultrastructural level (Figure 3). This datasheet provides a single procedure to visualize early and late apoptotic cells in fresh tissue samples. For

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other applications, such as detecting PCD in intact organisms please contact MUbio for specific advise about the appropriate procedures. MUbio Products has extensive hands-on expertise with the Annexin V technology in this context.

Materials

APOPTEST™-Biotin kit
Streptavidin-HRP (not included)
DAB substrate to detect Avidin-HRP (not included)
10xFormalin (not included)
Fresh tissue sample
Eppendorf test tube

Apparatus

Tissue embedding equipment
Microtome
Microscope

Method

1. Dilute the 10x concentrated binding buffer 10 fold with distilled water.
2. Add 100µl of diluted binding buffer to the test tube. Keep the temperature range of the solution between room temperature and 37°C.
2. Add 10 µl Annexin V-Biotin stock solution to the test tube containing 100µl 1x binding buffer.
2. Emerge the fresh tissue sample in the diluted binding buffer with added Annexin V-biotin.
3. Incubate and gently rotate for 30 minutes at room temperature or 37°C.
4. Wash the tissue twice with 1x binding buffer for 5 minutes each time.
5. Prepare fixative by diluting formalin with 1x binding buffer, resulting in a 10% buffered Ca^{2+} containing formalin fixative.
6. Fix the tissue over nigh at 37°C, dehydrate the tissue and embed in paraffin.

7[iv]. Cut up to 5µm sections and visualize cell bound Annexin V-Biotin in the sections by performing standard procedures to stain for HRP in paraffin sections, such as by applying DAB substrate.

This procedure will provide semi-quantitative information as to the extent of apoptosis in a certain section or in serial sections (Ref 8.)

SPECIES REACTIVITY

Annexin V-FITC binds to phosphatidylserine of all cell types (Ref. 7) and species tested (Ref. 1).

STORAGE CONDITIONS

Annexin V-Biotin solution and the 10x concentrated binding buffer

Store in the dark at 2-8°C.

Propidium iodide

Lyophilized propidium iodide should be stored in the dark at a temperature below 25°C. After dissolving in demineralized water, store the propidium iodide solution in the dark at 2-8°C.

Stability

When at 2-8°C, the product is stable at least until the expiry date printed on the vials.

REFERENCES

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8. SM van den Eijnde, J Lips, L Boshart L, C Vermeij-Keers, E Marani, CPM Reutelingsperger, CI De Zeeuw, (1999) Eur J Neurosci 11:712-724.

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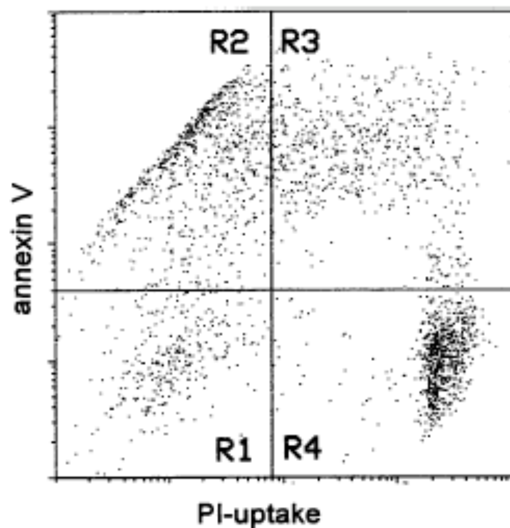

Fig. 1, adapted from reference number 2: Different labeling patterns in this assay identify the different cell populations, i.e., region R1: vital cells (PI-negative/ annexin V-negative), region R2: apoptotic cells (PI-negative/annexin V-positive), region R3: dead cells (PI-positive/annexin V-positive) and region R4: damaged cells (PI-positive/annexin V-negative).

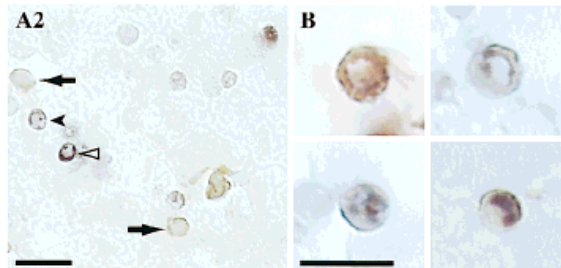

Fig. 2, adapted from reference number 6: Paraffin sections through day 13 limbs of AnxV-biotin injected mouse embryos that have been stained both for PS exposure at the plasma membrane via Annexin V-Biotin and for DNA fragmentation via the TUNEL method; no counterstaining was applied. A2 is showing in one field the three types of labeled cells that were observed: (i) cells that showed Annexin V-biotin labeling at the plasma membrane only (arrows), (ii) pyknotic cells that were double labeled for Annexin -biotin and TUNEL (arrowhead), and (iii) pyknotic cells and cell fragments that appeared only TUNEL-positive (open arrowhead). In B, four Annexin V and TUNEL double-positive cells are depicted in subsequent stages of apoptosis. Such cells were having chromatin margination (upper left and upper right) or nuclear fragmentation (lower left), or were clearly pyknotic (lower right). Scale bars equal 10 µm (A2) or 5 µm (B).

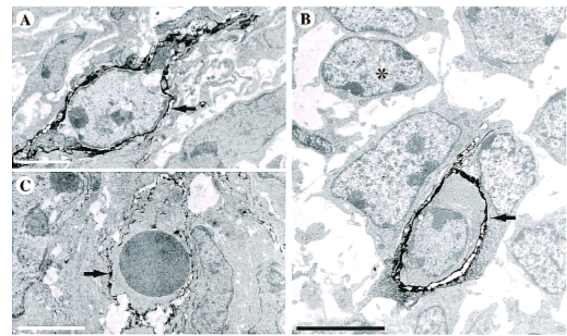

Figure 3, adapted from reference number 6: Electron micrographs, showing Annexin V-biotin labeled cells located in the interdigital region of a day 13 mouse embryo. Cells were found labeled at early stages of apoptosis, when showing first signs of chromatin condensation (A), ingested by a phagocyte (B), and up to the later stages of apoptosis when clearly pyknotic (C). Viable cells were unlabeled (asterix). Scale bars equal 2 µm.

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Datasheet version: MUB8100B 090709

Footnotes

[i] Annexin V-Biotin binds optimally to apoptotic cells at free Ca^{2+} levels of more than 1.5 mM. Should the medium contain less, adjust the level by adding $CaCl_2$ or replace the medium by 1x binding buffer after diluting the 10x binding buffer with distilled water. With regards to the culture medium, our experience is that RPMI1640 is less suitable for the assay. Other media like DMEM are recommended.

[ii] The incubation temperature may vary from 4°C to 37°C. Please be aware that Annexin V-Biotin is unstable at temperatures above 42°C.

[iii] Methods of harvesting using trypsin and EDTA reduces the amount of cellular bound Annexin V-Biotin and may induce viable cells to expose PS. The extent to which this occurs depends on the cell type.

[iv] Please note that the boundaries of the tissue may bind Annexin V-Biotin because of damage to the viable cells at these edges during tissue isolation. It is recommended not to consider these tissue boundaries for PCD analysis.

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