Miltenyi Biotec

Annexin V MicroBead Kit

Order no. 130-090-201

Contents

Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

1. Description

This product is for research use only.

Components	**2 mL Annexin V MicroBeads**: MicroBeads conjugated to Annexin V.
	2×25 mL 20× Binding Buffer Stock Solution
Capacity	For 10^9 total cells, up to 100 separations.
Product format	Annexin V MicroBeads are supplied in buffer containing stabilizer and 0.05% sodium azide.
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Background information

The Annexin V MicroBead Kit has been developed for positive selection or depletion of cells based on their ability to bind Annexin V–conjugated MicroBeads.

In most normal, viable eukaryotic cells, the negatively charged phospholipid phosphatidylserine (PS) is located in the cytosolic leaflet of the plasma membrane lipid bilayer.[1] PS redistribution from the inner to the outer leaflet is an early and widespread event during apoptosis.[1,2] However, in necrosis, PS becomes accessible due to the disruption of membrane integrity.[2] Apart from necrosis and apoptosis, PS also becomes accessible in activated platelets,[3] in certain cell anomalies like sickle cell anaemia[4], in erythrocyte senescence[5], upon degranulation of mast cells[10], and in certain stages of B cell differentiation.[11] PS exposure also serves as a trigger for the recognition and removal of cells by macrophages.[6,9]

Annexin V is a 35 kDa phospholipid-binding protein and a major cell membrane component of macrophages and other phagocytic cell types. Annexin V has a high affinity to PS in the presence of physiological concentrations of calcium (Ca^{2+})[7] and has already been used to isolate cells with exposed PS using MACS® MicroBeads.[4,8]

1.2 Applications

- Studies on certain cell anomalies (e.g. sickle cell anemia).
- Studies on degranulation of cells (e.g. mast cells).
- Enrichment of dead (apoptotic and / or necrotic) cells.
- Studies on cell activation (e. g. platelets or T cells).
- Studies on cell differentiation (e. g. B cells).

1.3 Reagent and instrument requirements

- Buffer: Prepare 1× Annexin V Binding Buffer from 20× Binding Buffer Stock Solution.
  For 10^7 total cells, dilute 500 µL of 20× Binding Buffer Stock Solution with 9.5 mL of sterile, distilled water. Store at 2–8 °C. Alternatively, prepare 1× Binding Buffer Stock Solution by diluting 25 mL of 20× Binding Buffer with 475 mL of sterile, distilled water.

  ▲ **Note:** Handle under sterile conditions. The use of a different buffer may lead to poor selection results. Binding of Annexin V to PS requires Ca^{2+}. The presence of the ion chelator EDTA will dissolve binding of Annexin V to Phosphatidylserine. 1× Binding Buffer is optimized for best selection results.

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- MACS® Columns and MACS Separators: Annexin V$^+$ cells can be enriched by using MS, LS, or XS Columns or depleted with the use of LD, CS, or D Columns. Cells which strongly express the Annexin V can also be depleted using MS, LS, or XS Columns.

Column	Max. number of labeled cells	Max. number of total cells	Separator
Positive selection			
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS II
LS	10^8	2×10^9	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II
XS	10^9	2×10^{10}	SuperMACS II
Depletion			
LD	10^8	5×10^8	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II
CS	2×10^8		VarioMACS, SuperMACS II
D	10^9		SuperMACS II

▲ **Note:** Column adapters are required to insert certain columns into the VarioMACS™ or SuperMACS™ II Separators. For details refer to the respective MACS Separator data sheet.

- (Optional) Fluorochrome-conjugated Annexin V for flow cytometric analysis, e.g., Annexin V-FITC. For more information about antibodies refer to www.miltenyibiotec.com/antibodies.
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD for flow cytometric exclusion of dead cells.
- (Optional) Pre-Separation Filters (30 µm) (# 130-041-407) to remove cell clumps.

1.4 Isolation principle

PS-exposing cells are enriched by magnetic enrichment using Annexin V MicroBeads. Cells are magnetically labeled with Annexin V MicroBeads and passed through a MACS Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled PS-exposing cells are retained in the column while the unlabeled cells run through. After removal of the column from the magnetic field, the magnetically retained PS-exposing cells can then be eluted as positively selected cell fraction.

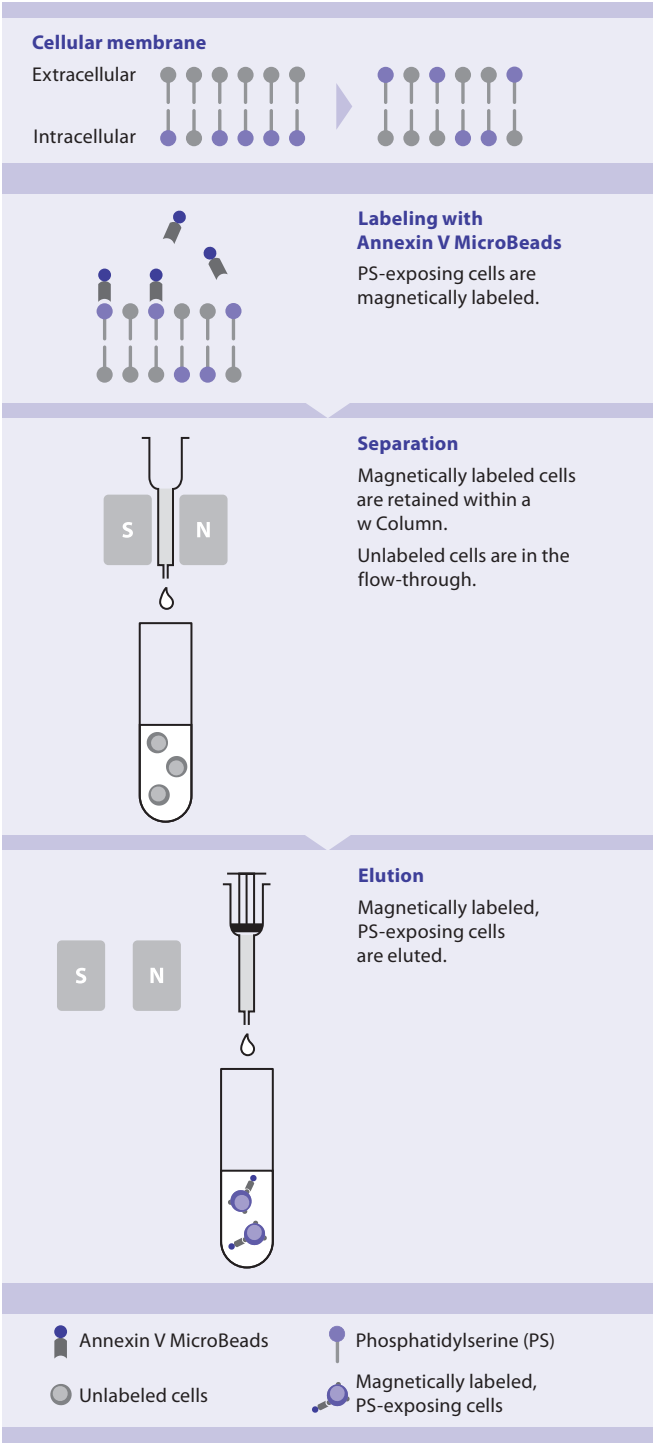

Figure 1: Isolation of PS-exposing cells using the Annexin V MicroBead Kit.

2. Protocol

2.1 Magnetic labeling

▲ When working with cell samples containing platelets (e.g. blood samples), wash samples carefully at a low centrifugation speed (200×g) in order to remove platelets. Use buffer containing the ion chelator EDTA for these washing steps. Activated platelets expose PS and therefore bind Annexin V.[5] Activated platelets also bind to leukocytes, for example, monocytes. In this case, non PS-exposing cells bound to activated platelets may be retained in the magnetic field and contaminate the PS-exposing cell fraction.

▲ The strength of the magnetic labeling using MACS® Annexin V MicroBeads is dependent on the number of binding sites - i.e. the amount of phosphatidylserine exposed - per cell which may differ considerably in different cell types. Thus, magnetic labeling can differ with the type of cells used.

▲ Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.

▲ Volumes for magnetic labeling given below are for up to 10^7 total cells. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^7 total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ For optimal performance it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through 30 µm nylon mesh (Pre-Separation Filters (30 µm), # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.

▲ The recommended incubation temperature is 2–8 °C. Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice may require increased incubation times.

1. Determine cell number.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 80 µL of 1× Binding Buffer per 10^7 total cells.
4. Add 20 µL of Annexin V MicroBeads per 10^7 total cells.
   ▲ **Note:** Increased Annexin V MicroBeads concentrations may impair staining with fluorochrome-conjugated Annexin V.
5. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).
6. (Optional) Add staining reagents, e.g., 10 µL of Annexin V-FITC, and incubate for 5 minutes in the dark in the refrigerator (2–8 °C).
7. Wash cells by adding 1–2 mL of 1× Binding Buffer per 10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
8. Resuspend up to 10^8 cells in 500 µL of 1× Binding Buffer.
   ▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.
   ▲ **Note:** For depletion with LD Columns, resuspend up to 1.25×10^8 cells in 500 µL of buffer.
9. Proceed to magnetic separation (2.2).

2.2 Magnetic separation

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of Annexin V^+ cells. For details refer to the table in section 1.3.

▲ Large numbers of cells in the starting sample require a larger buffer volume when applying cells onto a separation column. Use a maximum cell number of 10^8 cells per 500 µL of 1× Binding Buffer.

▲ Always wait until the column reservoir is empty before proceeding to the next step.

Magnetic separation with MS or LS Columns

1. Place column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.
2. Prepare column by rinsing with the appropriate amount of 1× Binding Buffer:
   MS: 500 µL LS: 3 mL
3. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
4. Wash column with the appropriate amount of 1× Binding Buffer. Collect unlabeled cells that pass through and combine with the flow-through from step 3.
   MS: 4×500 µL LS: 4×3 mL
   ▲ **Note:** Perform washing steps by adding buffer aliquots only when the column reservoir is empty.
5. Remove column from the separator and place it on a suitable collection tube.
6. Pipette the appropriate amount of 1× Binding Buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
   MS: 1 mL LS: 5 mL
7. (Optional) To increase the purity of Annexin V^+ cells, the eluted fraction can be enriched over a second MS or LS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

Magnetic separation with XS Columns

For instructions on the column assembly and the separation refer to the XS Column data sheet.

Depletion with LD Columns

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details refer to the LD Column data sheet.
2. Prepare column by rinsing with 2 mL of buffer.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with 2×1 mL of buffer. Collect total flow-through; this is the unlabeled cell fraction. Perform washing steps by adding buffer two times. Only add new buffer when the column reservoir is empty.

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Depletion with CS Columns

1. Assemble CS Column and place it in the magnetic field of a suitable MACS Separator. For details refer to the CS Column data sheet.
2. Prepare column by filling and rinsing with 60 mL of buffer. Attach a 22G flow resistor to the 3-way stopcock of the assembled column. For details refer to the CS Column data sheet.
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with 30 mL buffer from the top. Collect total flow-through; this is the unlabeled cell fraction.

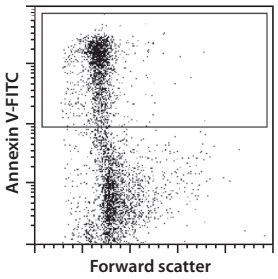
Depletion with D Columns

For instructions on column assembly and separation refer to the D Column data sheet.

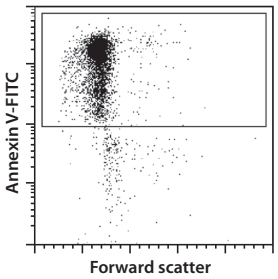
3. Example of an enrichment of dead (apoptotic/ necrotic) cells using the Annexin V MicroBead Kit

Murine thymocytes were treated with anti-Fas monoclonal antibody for 3 hours at 37 °C. PS-exposing cells were enriched by labeling with Annexin V MicroBeads followed by separation on an MS Column in the magnetic field of a MiniMACS™ Separator. Cell fractions were stained with Annexin V-FITC.

Murine thymocytes after treatment with anti-Fas antibody



Positive fraction enriched for PS-exposing cells



4. References

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Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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