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Product Information

Apo-TRACE™ Apoptotic Cell Staining Kit

Catalog Number **CS1110**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
Apoptosis, a form of programmed cell death, is a biological process important in normal development as well as pathological states. Apo-TRACE™ is a small, non-toxic, organic molecule, which is a member of the *ApoSense*® family of low molecular weight compounds suitable for imaging cell death *in vivo* as well as *in vitro*.[2,3] The *ApoSense* compounds respond to alterations in plasma membrane potential and phospholipid scrambling, which are hallmarks of apoptotic cells. Apo-TRACE has inherent fluorescent properties (excitation at 328 nm and emission at 563 nm) and accumulates in the cytoplasm of apoptotic cells.

This kit is designed to be used for the detection of apoptotic cells after apoptosis induction by observing the accumulation of Apo-TRACE in the cytoplasm of the apoptotic cells. Apo-TRACE staining can be visualized under a fluorescence microscope or alternatively, the molecule can be extracted from the cells for a quantitative evaluation (see Appendix). Apo-TRACE stained apoptotic cells can be separated from the rest of the cells using flow cytometry. A propidium iodide (PI) solution is supplied with the kit to enable staining of non-viable cells. Propidium iodide binds to double-stranded DNA, but it can only cross the plasma membrane of non-viable cells. Thus, a double staining (Apo-TRACE and PI) allows distinguishing between apoptotic and non-viable cells.

The kit was tested on different cell lines (i.e., Jurkat, HeLa, C26, Balb/3T3, CHO, and A-431) using different apoptosis inducers (i.e., anti-Fas antibody, BCNU, and Staurosporine).

Components
The kit is sufficient for up to 350 Apo-TRACE staining assays in 96 well plates

Apo-TRACE solution (15 mg/ml) (Catalog Number V7639)	50 µl
Assay Medium (Catalog Number A5481)	20 ml
Propidium iodide solution (1.0 mg/ml) (Catalog Number P4864)	50 µl

Reagents and Equipment Required but Not Provided

For Apo-TRACE staining

- Fluorescent microscope equipped with appropriate filters. The kit was developed using Olympus IX71 inverted microscope equipped with a Wide UV excitation with long pass emission filters (excitation 350/50 nm, emission 420 long pass). For propidium iodide analysis, a Green/Red filter was used (excitation at 540/25 nm and emission at 605/55 nm).
- Dulbecco's Phosphate Buffered Saline (PBS, Catalog Number D8537)
- Cell culture plates, 96 well, flat bottom (Catalog Number CLS3596) or cell culture plates, 24 well, flat bottom (Catalog Number CLS3524)
- Round, glass cover slips, 12 mm, for cell staining
- Glass slides (Catalog Number CLS29483X1) for observation of cells in suspension.
- Cover glasses (Catalog Number C9802)

For Apo-TRACE quantification

- Fluorimeter equipped with appropriate filters. Apo-TRACE was quantified using Synergy™ HT Fluorimeter equipped with filters for excitation at 360/40 nm, emission at 530/25 nm.
- CelLytic™ M Mammalian Cell Lysis/Extraction Reagent (Catalog Number C2978)
- Cell culture plate, 24 well, flat bottom (Catalog Number CLS3524).
- Nunc® FluoroNunc™ 96 well plates, black (Catalog Number P8741) or equivalent
- Cell scraper (Catalog Number CLS3010)
- Pellet pestle with disposable tip (Catalog Number Z359971 or Z359947) or equivalent, or Dounce tissue grinder set (Catalog Number D8938) or equivalent.

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
Propidium Iodide Working Solution - Just before use, dilute an aliquot of the Propidium iodide solution (Catalog Number P4864) 10-fold in ultrapure sterile water and mix well. The Propidium Iodide Working Solution should be stored protected from light.

Apo-TRACE solution - Warm the Apo-TRACE solution (Catalog number V7639) at 37 °C before each experiment for at least 10 minutes and vortex well.

Working Media - Prepare the appropriate Working Medium by the addition of Apo-TRACE solution (Catalog Number V7639) and Propidium Iodide Working Solution (optional) to the Assay Medium (Catalog Number A5481):

- Working Medium A - for cell staining in 96 well plates or in suspension - Add 2 µl of Apo-TRACE solution to 1 ml of Assay Medium to a final concentration of 30 µg/ml. If counterstaining is desired, also add 5 µl of the Propidium Iodide Working Solution to a final concentration of 0.5 µg/ml.

- Working Medium B - for cell staining on glass slides or in 24 well plates - Add 5 µl of Apo-TRACE solution to 1 ml of Assay Medium to a final concentration of 75 µg/ml. If counterstaining is desired, also add 5 µl of the Propidium Iodide Working Solution to a final concentration of 0.5 µg/ml.

Storage/Stability
The kit is shipped on dry ice and storage at –20 °C is recommended. After first thaw, store the Apo-TRACE solution and the Assay Medium in sterile working aliquots at –20 °C. The Propidium iodide solution can be stored at 2–8 °C.

Procedures
When using a specific *in vitro* model involving apoptosis processes, it is recommended to include a negative control (cells with no apoptosis induction) to account for staining due to non-specific adsorption of Apo-TRACE.

I. Staining adherent cells
The procedure is optimized for HeLa and Balb/3T3 cells. For other cell types, optimize the concentration of Apo-TRACE and the duration of the staining incubation.

A. Staining in 96 or 24 well plates:
1. Seed the cells in a 96 or 24 well plate, and grow them until they reach 80–90% confluence.
2. Induce apoptosis by the method of choice.
3. Monitor the apoptosis process by visualizing the adherent cells under the microscope, in order to make sure that cells have not detached from the growth surface.
4. Carefully remove the medium containing the apoptotic agent from the seeded wells using a pipette.
5. Wash the cells:
   If grown in 96 well plates – carefully add 100 µl/well of PBS or Assay Medium (Catalog Number A5481).
   If grown in 24 well plates - carefully add 250 µl/well PBS or Assay Medium (Catalog Number A5481).
   Remove the wash solution very carefully with the aid of a pipette.
6. Stain the cells:
   For 96 well plates - Add to each well 55 µl of Working Medium A (containing 30 µg/ml of Apo-TRACE and optionally, 0.5 µg/ml of propidium iodide)
   For 24 well plates - Add to each well 250 µl of Working Medium B (containing 75 µg/ml of Apo-TRACE and optionally, 0.5 µg/ml of propidium iodide).
   Incubate the cells at room temperature for ~1 hour protected from light.
   Note: The incubation time is dependent on the apoptosis inducer. Therefore, it can be shortened or lengthened.
7. If required wash the cells as indicated in step 5 (see the Troubleshooting guide).
   Note: The washing step after Apo-TRACE incubation is not necessary if using Working Medium A.
8. Monitor cell staining using a fluorescence microscope equipped with the appropriate filters.

B. Staining on cover slips:
   1. Sterilize round, glass cover slips by heating at 200 °C, by autoclaving, or by γ-irradiation.
   2. Using sterile forceps, place one sterile, round, glass cover slip per well of a 24 well plate.
   3. Seed 5–6 × 10^4 cells on each cover slip and grow them until they reach 80–90% confluence.
   4. Induce apoptosis by the method of choice.
   5. Monitor the apoptosis process by visualizing the cells under the microscope, in order to make sure that cells have not detached from the growth surface.
   6. Carefully remove the medium containing the apoptotic agent from the seeded wells using a pipette.
   7. Wash the cells: Carefully add 250 µl/well of PBS or Assay Medium (Catalog Number A5481). Remove the washing solution very carefully using a pipette.
   8. Stain the cells: Add to each well 250 µl of Working Medium B (containing 75 µg/ml of Apo-TRACE and optionally, 0.5 µg/ml of propidium iodide).
   9. Incubate the cells at room temperature for 15–30 minutes protected from light.
      Note: The incubation time is dependent on the apoptosis inducer. Therefore, it can be shortened or lengthened.
   10. Wash the cells as indicated in step 7.
   11. Put a drop of PBS on top of a glass slide.
   12. Take the cover slip out of the well using forceps and place it up-side-down on the drop of PBS so that the cell surface will touch the PBS.
   13. Monitor cell staining using a fluorescence microscope equipped with the appropriate filters.

II. Staining cells in suspension

The following procedure is for staining cells in tubes. The assay can be adapted to 96 well plates using an inverted microscope, and appropriate centrifuge and plates. In order to minimize cell loss during the washing step(s), it is recommended to use U or V shaped bottom plates for centrifugation (for example, cell culture plates, 96 well, round bottom, Catalog Number CLS3799). For cell visualization, cell culture plates, 96 well, flat bottom, (Catalog Number CLS3596) are recommended**.**

The procedure is optimized for Jurkat cells. For other cell types, optimize the concentration of Apo-TRACE and the duration of the staining incubation.

1. Induce apoptosis in a cell suspension containing 2–5 × 10^6 cells.
2. Centrifuge the cells at 600 × *g* for 5 minutes at room temperature and aspirate the inducer-containing medium.
3. Wash the cells by the addition of 250 µl of PBS or Assay Medium (Catalog Number A5481). Centrifuge the cells as in step 2 and aspirate the wash solution.
4. Add 250 µl of Working Medium A (containing 30 µg/ml of Apo-TRACE and optionally, 0.5 µg/ml of propidium Iodide). Incubate the cells at room temperature for ~1 hour protected from light.
   Note: The incubation time is dependent on the apoptosis inducer. Therefore, it can be shortened or lengthened.
5. An optional step - If required wash the cells. Centrifuge the cells at 600 × *g* for 5 minutes at room temperature and aspirate Working Medium A. Add 250 µl of PBS or Assay Medium (Catalog Number A5481). Centrifuge the cells as indicated previously and aspirate the wash solution.
6. Resuspend the cells in 250 µl of PBS or Assay Medium and transfer 10 µl of cell suspension onto a glass slide and cover the cells with a glass cover slip.
7. Observe the cells under a fluorescent microscope equipped with the appropriate filters.
   Note: If using an inverted microscope, 100 µl per well of cell suspension can be transferred to a 96 well plate and visualized under the microscope.

References

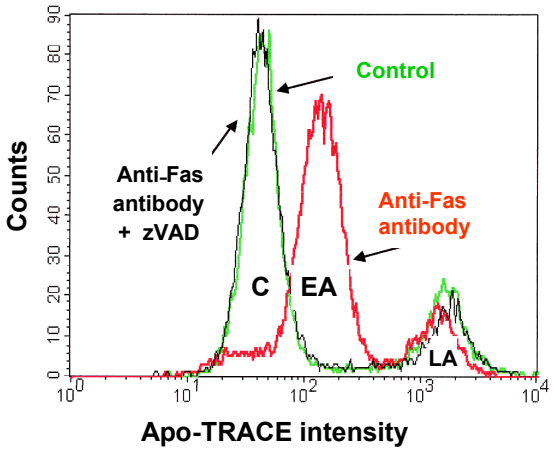
1. Schulze-Osthoff, K., et,al., Apoptosis signaling by death receptors. *Eur. J. Biochem.*, **254**, 439-459 (1998).
2. Damianovich, M., et al., ApoSense: a novel technology for functional molecular imaging of cell death in models of acute renal tubular necrosis. *Eur. J. Nucl. Med. Mol. Imaging*, **33**, 281-291 (2006).
3. Reshef, A., et.al., Novel molecular imaging of cell death in experimental cerebral stroke. *Brain Res.*, **1144**, 156-164 (2007).

Appendix
The following are suggested procedures for detection of Apo-TRACE stained cells by flow cytometry and a quantification method for determining Apo-TRACE accumulation in cells.

I. Analysis by Flow Cytometry (FACS)

1. Grow cells and treat them according to your protocol. Use a negative control of untreated cells.
2. Pellet the cells and wash the pellet with PBS. Adjust the cell density to 10^7 cells/ml using PBS. Note: Adherent cells should be trypsinized prior to centrifugation and washing.
3. Transfer 30 µl of the cell suspension to a tube suitable for FACS analysis.
4. Add 260 µl of PBS and 3.5 µl of Apo-TRACE solution (15 mg/ml, Catalog Number V7639). Incubate at room temperature for 15 minutes protected from light.
5. For double staining add 2.5 µl of Propidium iodide solution (1.0 mg/ml, Catalog Number P4864) and perform a FACS analysis using a FACS analyzer with the appropriate filters. This procedure was tested using FACS Vantage VE (BD Biosciences, San Jose, CA, USA) and CellQuest software, excitation wavelength at 365 nm and emission at 530 nm.
6. The processed population should separate into three groups:
   Live cells that show a low level of UV fluorescence (background)
   Apoptotic cells that show UV fluorescence higher than the background
   Necrotic cells that show a higher UV fluorescence along with red fluorescence.

Figure 1.
FACS analysis of Apo-TRACE accumulation in apoptotic cells and its inhibition by a caspase inhibitor



Apoptosis was induced in Jurkat cells by anti-Fas antibody (0.1 µg/ml) in the absence (red line) or presence (black line) of the pan caspase inhibitor Z-VAD-FMK (50 µM) for 180 minutes. Following incubation, the cells were washed, labeled with Apo-TRACE, and subjected to FACS analysis. The histogram depicts the number of cells (Y axis) vs. Apo-TRACE fluorescence intensity (X axis). There is a marked increase in fluorescence of cells treated with anti-Fas antibody (apoptotic cells, red line). However, the addition of the caspase inhibitor Z-VAD-FMK, causes a marked decrease in cell fluorescence intensity shifting it back to the level of control cells (green line).

C = control
EA = early apoptotic
LA = late apoptotic cells

II. Apo-TRACE extraction from cells

It is highly recommended to include two negative controls:

- Non-induced cells incubated with Apo-TRACE containing Assay Medium.
- Non-induced cells incubated with Assay Medium.

Work in triplicates for each cell treatment.

A. Adherent cells

In order to get a meaningful quantification of the amount of Apo-TRACE in cells, it is better to stain a large amount of cells. Therefore, it is recommended to use 24 well plates.

1. Perform the assay according to Procedures, section IA. Use Working Medium B containing 75 µg/ml Apo-TRACE and wash the cells after incubation with Working Medium B as described in Procedures, section IA, step 5.
2. Detach the cells using a cell scraper.
3. Transfer the detached cells to a 2 ml tube and centrifuge at 600 × *g* for 5 minutes at room temperature.
4. Aspirate the supernatant and break up the cell pellet by finger tapping. Add 500 µl of PBS and mix twice by pipetting.
5. Centrifuge the cells at 600 × *g* for 5 minutes at room temperature.
6. Repeat steps 4 and 5.
7. Aspirate the supernatant and add to each tube 200 µl of CelLytic M Mammalian Cell Lysis/ Extraction Reagent (Catalog Number C2978).
8. Homogenize the cells using a pellet pestle homogenizer (recommended) or equivalent.
9. Centrifuge the homogenate at 14,000 × *g* at 2–8 °C for 20 minutes.
10. Transfer 50 µl aliquots of the supernatant into 2 separate wells of a 96 well black fluorescent plate (Catalog Number P8741). As a blank use 50 µl of CelLytic M Mammalian Cell Lysis/Extraction Reagent.
11. Read the fluorescence intensity with a fluorimeter using the appropriate filters.

B. Cells in suspension

1. Induce apoptosis in a cell suspension containing $1–2 \times 10^7$ cells.
2. Centrifuge the cells at 600 × *g* for 5 minutes at room temperature and aspirate the inducer-containing medium.
3. Wash the cells by the addition of 250 µl of PBS or Assay Medium. Centrifuge the cells as indicated previously and aspirate the washing solution.
4. Add to each well 250 µl of Working Medium B containing 75 µg/ml Apo-TRACE. Incubate the plate for ~1 hour at room temperature in the dark (alternatively wrap in aluminum foil).
5. Centrifuge the cells at 600 × *g* for 5 minutes at room temperature, aspirate the supernatant, and break up the cell pellet by finger tapping. Add 500 µl of PBS and mix twice by pipetting.
6. Repeat step 5.
7. Centrifuge the cells at 600 × *g* for 5 minutes at room temperature and aspirate the supernatant.
8. Add to each tube 200 µl of CelLytic M Mammalian Cell Lysis/Extraction Reagent and continue as described in the procedure for adherent cells (step 8 to the end of the procedure).

Troubleshooting guide

Problem	Reason	Comments and solutions
Apo-TRACE staining is observed in the control cells.	The cells are in bad condition.	1. Start the procedure with fresh cells. 2. Decrease cell staining duration.
Apo-TRACE staining is too weak.	The concentration of Apo-TRACE in the staining mixture is too low.	1. Increase the final concentration of Apo-TRACE in the Assay Medium. 2. Increase the cell staining duration
High background observed with the microscope.	The concentration of Apo-TRACE in the staining mixture is too high.	1. Perform a wash step after the incubation with the Apo-TRACE. 2. Decrease the Apo-TRACE concentration in the Working medium.

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ApoSense technology is covered by the following:
US patent application No. 10/433,668
US patent application No. 11/172,934
European patent application No. 01999555.4
Japanese patent application No. 547886/02
And related patent applications.

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