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EZClick™ TUNEL – *in situ* DNA Fragmentation/Apoptosis Assay Kit

rev 07/20

(Catalog # K191-100; 100 assays; Store at -20°C)

I. Introduction:

Apoptosis is a controlled phenomenon in which apoptotic cells are characterized by changes in nucleus (i.e. chromatin condensation, DNA fragmentation), cytoplasm shrinkage and production of apoptotic bodies. These are subsequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells. DNA strand breaks can be analyzed by TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick End-Labeling) assays. **BioVision's EZClick™ TUNEL-DNA fragmentation/Apoptosis Assay** utilizes modified EdUTP nucleotides, which are incorporated at the 3'-OH ends of fragmented DNA by Terminal deoxynucleotidyl transferase (TdT) enzyme and detected based on a click reaction. This assay enables the easy delivery and incorporation of EdUTP coupled with high selectivity of detection of DNA damage. The assay is performed under mild reaction conditions that preserve the cell morphology, thus enabling the identification of damaged DNA by FACS or fluorescence microscopy. Our assay is fast and capable of detecting a higher percentage of apoptotic cells than the antibody based methods. The kit contains sufficient reagents to detect total/fragmented DNA in apoptotic cells in a 1 X 96-well plate or on 50 cover slips.

II. Applications:

- Detection of DNA fragmentation during apoptosis and programmed cell death
- Screening for genotoxic compounds and effectors of DNA integrity and cellular changes
- Evaluating effects of anti-cancer drugs and genotoxic agents on DNA structure
- In-situ studies of apoptosis and programmed cell death

III. Sample Types:

- Cultured cells, fresh-frozen tissue sections

IV. Kit Contents:

Components	K191-100	Cap Code	Part Number
EZClick™ Wash Buffer (10X)	25 ml	NM	K191-100-1
Fixative Solution	10 ml	WM	K191-100-2
Permeabilization Buffer (10X)	25 ml	NM/Blue	K191-100-3
EZClick™ EdUTP DNA Label (50X)	100 µl	Clear	K191-100-4
Copper Reagent (100X)	100 µl	Blue	K191-100-5
EZClick™ Fluorescent Azide (100X)	100 µl	Red	K191-100-6
Reducing Agent (20X)	500 µl	Yellow	K191-100-7
EZClick™ Total DNA Stain (1000X)	10 µl	Green	K191-100-8
EZClick™ TUNEL Reaction Buffer (10X)	1 ml	Orange	K191-100-9
EZClick™ TUNEL Enzyme	1 vial	Purple	K191-100-10
TUNEL Enzyme Buffer	500 µl	Brown	K191-100-11

V. User Supplied Reagents and Equipment:

- Tissue culture & Flow Cytometry vessels and cell media
- Phosphate Buffered Saline (PBS, pH 7.4)
- Flow cytometer w/lasers capable of excitation at 488 and 530/590 nm emission filters respectively
- Fluorescence microscope w/excitation and emission at 440/490 and 540/580 nm respectively
- **Optional:** Sterile 0.1% Gelatin solution to adhere suspension cells onto surface

VI. Storage Conditions and Reagent Preparation:

Upon arrival, store the entire kit at -20°C protected from light. Briefly centrifuge small vials prior to opening. Read the entire protocol before performing the assay.

- **10X Wash Buffer and 10X Permeabilization Buffers:** Thaw at 37°C to dissolve completely. Dilute the 10X stock buffers by 1:10 in sterile water, mix well. Store at 4°C.
- **Fixative Solution: Divide into aliquots and store at -20°C, protected from light.**
- **EZClick™ TUNEL Enzyme:** Resuspend into 300 µl of TUNEL Enzyme Buffer, mix well. Aliquot and store at -20°C. Use within two months.

VII. Assay Protocol (Notes):

This assay was developed with HeLa (adherent) and Jurkat (suspension) cells and can be modified for any cell line. For this protocol, total assay volume: 100 µl (96 well tissue culture plate). Adjust volumes for other plate formats. Growth conditions, cell number/well etc. should be optimized for your cell type/treatment. We suggest testing several EZClick™ EdUTP DNA Label concentrations to find the best concentration for your experiment. All steps and reagent equilibration should be carried out at room temperature (RT).

1. Preparation of Control and Experimental cells:

a. Obtain cell suspension, and seed directly into tissue culture vessels (HeLa cells (10^5 cells/ ml) and Jurkat (10^6 cells/ ml)), or on coverslips for high resolution microscopy. **To immobilize suspension cells for microscopy**: add 100 µl of 0.1% gelatin solution directly into the wells, tilt the plate to cover the entire well surface, place it in a tissue culture hood for 1 hour. Gently remove the gelatin solution and seed your cells. Allow the cells to recover overnight before the treatment. Prepare wells with cells that will be treated (Experimental Wells) and 4 controls. **A. Unstained Cells:** (FACS Setup Control Cells-- No TUNEL and No EZClick); **B. Positive Control Cells:** (DNase-treated cells after step **1.** *DNAse is not provided*); **C. Background Control Cells:** (EZClick reaction only, no TUNEL); **D. Negative Control Cells** (untreated cells that undergo TUNEL followed by EZClick reaction).

b. Next day, remove the media and induce apoptosis according to your protocol in the wells with **Experimental Cells**. **For immobilized suspension cells**: Centrifuge the plate at 500 x *g* (or the lowest centrifuge setting) for 5 min to deposit the cells onto the surface. Tilt the plate and ***gently*** to remove the media with a pipette tip. *Use the same centrifugation settings throughout the entire protocol*.

c. To terminate the experiment for **adherent cells**, remove culture media, wash the cells once with 100 µl PBS, and then aspirate the PBS. For **suspension cells**, centrifuge the cells at 500 x *g* for 5 min. Discard the supernatant and wash the cells once with 100 µl PBS Centrifuge once again at 500 x *g* for 5 min, then aspirate the PBS. Proceed to the Fixation and Permeabilization.

2. **Fixation and Permeabilization:**

a. For adherent cells: Add 100 µl of Fixative Solution to each well and incubate the cells for 15 min at RT protected from light. Remove the fixative and wash the cells once with 100 µl of 1X Wash Buffer, remove the wash. Add 100 µl of 1X Permeabilization Buffer and incubate the cells for 10 min at RT. Remove the Permeabilization Buffer and wash the cells twice in 100 µl of dH_2O, discard washes.

b. For suspension cells: Re-suspend the cells in 100 µl of Fixative Solution and incubate for 15 min at RT protected from light. Centrifuge and remove the fixative and wash the cells once with 100 µl of 1X Wash Buffer. Centrifuge and remove the supernatant and re-suspend the cells in 100 µl of 1X Permeabilization Buffer. Incubate the cells for 10 min at RT. Centrifuge and remove the Permeabilization Buffer and wash the cells twice in 100 µl of dH_2O, centrifuge and remove washes each time.
Note: for **Positive Control Cells** well(s), treat the cells with 10 U of DNase in 100 µl. Keep the rest of the samples at 4°C in 100 µl of PBS during the DNase treatment of the **Positive Control** wells.

3. **TUNEL Reaction:** This protocol uses 50 µl of the TUNEL reaction cocktail per well (100 µl for cover slips). Cells must be equilibrated to maximize the efficiency of TUNEL reaction. **DO NOT** treat the **Background Control** and **Unstained** cells.

a. Dilute 10X TUNEL Buffer to 1X with dH_2O and add 50 µl in each well and allow the solution to completely cover the surface. Incubate plates for 10 min at RT. Spin down at 500 x g for 5 min, and remove the TUNEL reaction buffer. Add 50 µl of TUNEL buffer to **Background Control** and **Unstained** cells.

b. Prepare 1X TUNEL reaction cocktail according to the table below and add 50 µl to each **Experimental**, **Positive** and **Negative Control** cells well. Incubate the cells for 1 h at 37° C. Alternatively, TUNEL reaction can be carried out overnight at RT. Prepare enough Reaction Cocktail mix for the number of samples that will be analyzed.

	Amount per Reaction
dH_2O	41 µl
TUNEL Reaction Buffer (10X)	5 µl
EdUTP DNA Label (50X)	1 µl
TUNEL Enzyme	3 µl

c. Spin down at 500 x g for 5 min and remove the reaction cocktail. Add 200 µl of 1X Wash Buffer, mix well. Spin down at 500 x *g* for 5 min and remove Wash Buffer. Proceed to EZClick™ reaction.

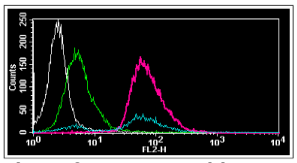
4. **EZClick™ Reaction and total DNA staining: (Background Control, Negative Control, Positive Control & Experimental cell wells)**

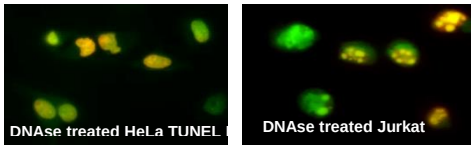
a. Reaction Cocktail: Prepare 1X EZClick™ Reaction Cocktail according to the table below. Reagents should be prepared based on the number of samples to be analyzed. **Add reagents in the exact order as indicated below**. Use the Reaction Cocktail within 15 min of preparation. Cover the plates with foil to *protect from light during, and follow the EZClick™ reaction and DNA staining*.

	Amount per Reaction
PBS	93 µl
Copper Reagent (100X)	1 µl
EZClick™ Fluorescent Azide (100X)	1 µl
Reducing Agent (20X)	5 µl

b. EZClick™ Reaction: Add 100 µl of 1X EZClick™ Reaction Cocktail to each Experimental, Background control, Positive Control and Negative Control cell wells. Add 100 µl PBS to Unstained Control well(s), Incubate the cells for 30 min at room temperature protected from light. Centrifuge and remove supernatant and wash cells three times in 100 µl of 1X Wash Buffer (Centrifuge and remove old buffer after each wash). Re-suspend the cells in 100 µl of PBS. Proceed to DNA staining. If no DNA staining is desired, proceed to Microscopic or FACS analysis. **DNA staining:** Add 2 µl of EZClick™ Total DNA Stain (1000X) to 1998 µl PBS to prepare 1X EZClick™ Total DNA Stain, mix well and add 100 µl per well. Incubate the cells for 20 minutes at room temperature or at 4 °C protected from light. Centrifuge and remove the stain solution; wash the cells once with 100 µl of PBS, centrifuge and remove PBS. **Note**: Cells are compatible with all methods of slide preparation including wet mount or prepared mounting media.

5. **Fluorescence Microscope Imaging**: Analyze samples for red fluorescence generated by TUNEL positive cells and green by total DNA respectively. **FACS analysis:** Harvest the cells by preferred method and wash with 0.5 ml of ice-cold PBS. Transfer the cell suspension into flow cytometry vessels. Analyze samples in FL-2 channel for signal generated by TUNEL positive cells during click reaction. **Note:** Trypsin can be used to collect the adherent cells prior to FACS analysis.

A. 

B. 

Figures: Detection of TUNEL-positive apoptotic strand breaks. Jurkat cells-DNAse treated (10^6 cells/ml) induced strand breaks. **(A) Unstained** cells w/vehicle (white), **Background Control** cells processed for EZClick™ reaction (green), **Negative control** (untreated cells, TUNEL and EZClick™ reaction; blue), DNase-treated cells (pink). **(B) DNase treated** HeLa cells (10^5 cells/ ml) and Jurkat (10^6 cells/ ml). DNA staining and TUNEL and EZClick™ reactions were performed. Green: nuclear stain; Red (TUNEL positive); orange: apoptotic cells.

VIII. RELATED PRODUCTS

Cell Proliferation Assay Kit (Fluorometric) (K307) BrdU Cell Proliferation Assay Kit (K306)

FOR RESEARCH USE ONLY! Not to be used on humans.