ELISA PRODUCT INFORMATION & MANUAL

Apoptosis Detection ELISA

NBP2-62139

Enzyme-linked Immunosorbent Assay for quantitative detection of Non-species specific Apoptosis Detection.

For research use only.

Not for diagnostic or therapeutic procedures.

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Apoptosis Detection *ELISA Kit*
NBP2-62139

BACKGROUND

Apoptosis, or programmed cell death, is a highly conserved biochemical mechanism that allows cells to die in a controlled and organized manner. This death process is essential for normal cellular differentiation and tissue homeostasis within multicellular organisms[1].

Principles of the APOPTOSIS DETECTION ELISA

The Apoptosis Detection ELISA is based on the sensitivity of DNA in apoptotic cells to formamide denaturation and the detection of the denatured DNA with a monoclonal antibody to single-stranded DNA (ssDNA). **Formamide is a gentle denaturing reagent, which denatures DNA in apoptotic cells, but not in necrotic cells or in cells with DNA breaks in the absence of apoptosis**[2]. The sensitivity of DNA in apoptotic cells to formamide is not related to DNA breaks, but reflects changes in chromatin associated with apoptosis, such as chromatin condensation and digestion of proteins stabilizing DNA. This assay involves the attachment of cells to 96- well microplates, treatment of the attached cells with formamide and heat, and staining of the ssDNA in apoptotic cells with a mixture of primary antibody and peroxidase-labeled secondary antibody. This protocol, which is based on the one-step detection of ssDNA with the antibody mixture has a higher sensitivity and a lower number of steps than standard two-step ELISAs[3]. This antibody mixture is included in the kit in a ready to use form.

The cells for the Apoptosis Detection ELISA can be prepared in two ways: 1) Cells are grown, treated with apoptosis inducing agents, stained and analyzed in the same microplate. or 2) Cell suspensions obtained from cultures or tissues are transferred into a microplate for staining and analysis. For this assay cells are either fixed with methanol and stored before transfer into plates, or fixed in microplates after transfer to the plate.

Sensitivity

The Apoptosis Detection ELISA can detect 500 apoptotic cells per well and provides a linear relationship between the absorbance and the number of apoptotic cells in the range of 500-5000 cells. A drug inducing 50% apoptosis will produce a signal that is sufficient for drug screening[3]. This assay is suitable for high throughput screening when induction of apoptosis is used as the endpoint of drug activity.

Specificity

The Apoptosis Detection ELISA is highly specific for apoptotic cells. The ssDNA antibody will not react with necrotic cells or with cells with DNA breaks in the absence of apoptosis. In comparison, the TUNEL assay will give positive signals from necrotic cells induced by hyperthermia, detergents or from cells with DNA breaks caused by hydrogen peroxide. These cells will not give a positive signal in the Apoptosis Detection ELISA[2,3](fig.1). *Specificity is a major advantage of the* Apoptosis Detection *ELISA in comparison with the widely used TUNEL assay.*

Universal detection

The detection of apoptotic cells with the Apoptosis Detection ELISA is based on the staining of condensed chromatin in apoptotic nuclei. Since chromatin condensation into compact masses is the most specific and definitive hallmark of apoptosis, this method provides universal detection of apoptosis. Apoptotic events that occur without DNA breaks or without activation of specific caspases will still be detected with the Apoptosis Detection ELISA.

Assay time

This assay can be completed in 3-4 hours, which includes fixation and staining.

Stability and storage

See individual kit components for storage instructions.

References

1. M.J. Arends, *et al*. *Int. Rev Exp. Pathol*. 1991 **32**:223
2. O.S. Frankfurt and A. Krishan. 2001 *J. Histochem. Cytochem.* **49** 369
3. O.S. Frankfurt and A. Krishan. 2001 *J. Immunol. Methods* **253** 133

PLEASE READ THE ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT NOVUS BIOLOGICALS FOR ASSISTANCE IF NECESSARY.

COMPONENTS OF BML-AK120 KIT

100% Formamide
STORAGE: room temperature
QUANTITY: 5 ml

Antibody Mixture
STORAGE: -70°C
Thawed Antibody Mixture can be stored 1-3 days at 4°C or re-frozen once for extended storage.
QUANTITY: 10 ml

Single Stranded DNA (ssDNA)
STORAGE: -70°C
Can be re-frozen repeatedly without any change of activity.
QUANTITY: 2 ml of 0.3 µg/ml in PBS

Blocking Solution
STORAGE: solid can be stored at room temperature.
After reconstitution, store frozen.
QUANTITY: 0.6 g; 20 ml after reconstitution.

Peroxidase Substrate
STORAGE: 2-8 °C, bring to room temperature before use.
QUANTITY: 10 ml

5X Wash Buffer
STORAGE: –70 °C
QUANTITY: 12 ml

Sterile 96-well microplate with lid
STORAGE: room temperature
QUANTITY: one plate and lid

Fixative
STORAGE: room temperature
QUANTITY: 20 ml

OTHER MATERIALS REQUIRED

Equipment

- Microplate reader capable of measuring A_{405}.
- Pipettor or multi-channel pipettor capable of pipetting 50-200 µl accurately
- low speed centrifuge capable of centrifuging microplates.
- 75 °C oven or microplate adapted heating block
- 37 °C incubator

Reagents

- S1 nuclease (optional negative control)
- 1% SDS solution (optional)

PROTOCOL FLOW CHART

Grow cells in 96-well microplate

⇩

Treat with apoptosis inducer (drug)

⇩

Fix cells
(30 minutes)

⇩

Attach cells to surface of wells by drying
(20 minutes)

⇩

Treat with Formamide
(10 minutes)

⇩

Denature DNA

(35 minutes)

⇩

Block with Blocking Solution
(1 hour)

⇩

Incubate with Antibody Mixture
(30 minutes)

⇩

Wash wells 3X with Wash Buffer

⇩

Add Peroxidase Substrate

⇩

Read absorbance at 405 nm

PREPARATION OF REAGENTS

1. Dissolve the 0.6 g of solid in 20 ml distilled H_2O to make blocking solution. Store frozen until ready to use.
2. In new bottle, add 48 ml of distilled H_2O to 5X Wash Buffer. Store frozen until ready to use.

PREPARATION OF CELLS

Grow cells in a 96-well microplate, if possible. The density of the cells is important for the optimal detection of apoptosis by this ELISA. Example: seed 5,000-10,000 cells per well and treat with apoptosis inducing agents 2 days later OR transfer 1,000-10,000 of non-fixed cells / well into 96-well microplate.

EXPERIMENTAL METHODS

I. **Protocol for cells grown in microplate**.

1. Grow and prepare cells as described above.
2. Centrifuge microplate at 200 x g for 5 min, remove medium, add 200 µl of Fixative and incubate at room temperature for 30 min to fix cells.
3. Centrifuge microplate at 200 x g for 5 min, remove fixative and dry plate to attach cells to plate surface. Drying can be performed by incubating at 56°C for 20 min. The microplate with fixed/dried cells can be processed immediately or stored at 4°C in Ziploc bag and analyzed the next day.
4. Add 50 µl/well of 100% formamide, keep plates at room temperature for 10 min and heat at 56°C to denature DNA in apoptotic cells. Heating is performed by incubating plates in preheated oven for 30 min (without lid).
5. Cool plate in refrigerator for 5 min.
6. Remove formamide, add 200 µl/well of Blocking Solution, and place plate in incubator at 37°C for 1 hr to block non- specific binding sites in wells.
7. Remove Blocking Solution, add 100 µl/well of Antibody Mixture and incubate plate at room temperature for 30 min.
8. Rinse wells 3x with 200 µl of 1X Wash Buffer
9. Add 100 µl/well of Peroxidase Substrate, incubate for 30-60 minutes and read absorbance in ELISA plate reader at 405 nm.
   Reaction (color development) can be stopped by the addition of 100 µl of 1% SDS.

II. **Protocol for cells transferred to microplate**.

1. Fix cells in tubes by adding 6 ml of cold methanol to cells suspended in 1 ml of PBS.
   Fixed cells may be stored for 1-3 days at -20 °C.
2. Transfer 1,000-10,000 fixed cells diluted in fixative, as needed, into 96-well microplate.
3. Proceed to step 3 in Protocol I.

CONTROLS

A solution of ssDNA is included in the Apoptosis Detection ELISA to serve as a positive control. An absorbance above 2.4 in wells coated with 30 ng of ssDNA demonstrates good sensitivity of the ELISA for the detection of apoptotic cells. Negative controls can be performed in several ways. First, uninduced cells can be processed in parallel with apoptosis induced cells. Note: these wells may have higher absorbances than other negative control wells because of spontaneous apoptosis. Second, wells coated with medium or fixative without cells can also be used as a negative control. Such wells should have absorbances of 0.05-0.07. Finally, apoptosis induced cells treated with S1 nuclease can be used as a negative control (see below.)

Positive Control

Single stranded DNA (ssDNA) in PBS is provided in the kit to be used as a positive control.

1. Add 100 µl/well of solution containing 0.3 µg/ml ssDNA in PBS (BML-KI167) and dry plate by incubating overnight at 37 °C.
2. Rinse wells once with 1X Wash Buffer.
3. Add 200 µl of Blocking Solution and incubate plates 1 hr at 37°C.

Proceed to step 7 of the protocol. Absorbances of 2.4-2.8 indicate good sensitivity of the assay. Wells treated with PBS instead of ssDNA should have absorbances of 0.05-0.07

S1 Nuclease Negative Control

Treating cells with S1 nuclease after the formamide treatment will eliminate any positive signal.

1. After step 5 of the protocol remove formamide.
2. Add S1 nuclease [100 units/ml in buffer (30 mM sodium acetate (pH 4.6), 4 mM zinc acetate)] or buffer alone.
3. Incubate plates at 37°C for 30 min
4. Rinse 3x with Wash Buffer
5. Add 200 µl of Blocking Solution and incubate plates 1 hour at 37°C

Proceed to step 7 of the protocol. S1 nuclease treatment should decrease absorbance to background levels, while treatment with buffer alone will not affect signal.

QUANTIFICATION

One possible way to quantitate the number of apoptotic cells in the experimental samples is to use, in control wells, a known number of cells and a treatment that will cause a known percentage of these cells to enter apoptosis. A correlation can then be made between absorbance at 405 nm and the number of apoptotic cells.

SAMPLE DATA

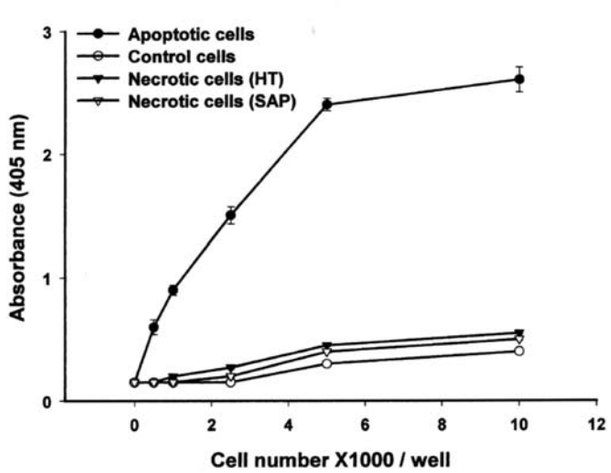

Fig. 1. Specificity and sensitivity of Apoptosis Detection ELISA. Wells were coated with a variable number of methanol fixed MDA-468 cells and stained according to protocol. A pure population of apoptotic cells was obtained by collecting floating cells from flasks with adherent cultures treated with staurosporine. Necrosis was induced by hyperthermia (HT) or saponin (SAP). Data from H_2O_2-treated cells are not shown since their signal was identical to untreated control cells.

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