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# ab211153 Anoikis Detection Assay Kit

For the detection of anoikis cell death in live cells by fluorescence microscopy or plate reader.

This product is for research use only and is not intended for diagnostic use.

# Table of Contents

# 1. Overview

Anoikis Detection Assay Kit (ab211153) provides a colorimetric and fluorometric format to measure anchorage-independent growth and monitoring anoikis propelled cell death. Live cells are detected with MTT or Calcein AM. Cell death is detected with Ethidium Homodimer (EthD-1). Background fluorescence levels are very low because the dyes included in the assay are virtually non-fluorescent before interacting with cells.

EthD-1 is an excellent marker for measuring dead cells. EthD-1 is a red fluorescent dye that can only penetrate damaged cell membranes. It will fluorescence with a 40-fold enhancement upon binding ssDNA, dsDNA, RNA, oligonucleotides and triplex DNA.

The kit provides sufficient reagents to evaluated 24 samples on a poly-Hema coated 24-well plate or 96 samples on a hydrogel coated 96-well plate.

Adhesion to the extracellular matrix (ECM) is essential for survival and propagation of many adherent cells. Apoptosis that results from the loss of cell adhesion to the ECM, or inappropriate adhesion is defined as "anoikis". Anoikis, from the Greek word for homelessness, is involved in the physiological processes of tissue renewal and cell homeostasis.

A common feature of carcinoma development and growth is the ability of transformed cells to survive under "anchorage independent" or "spheroid" growth conditions. This resistance to anoikis has been shown to be involved in the loss of cell homeostasis, cancer growth, and metastasis. The inhibition of cell adhesion, spreading, and growth on the ECM is an impediment to the cellular healing process, thus making it a possible therapeutic target. Preventing anoikis and enhancing cell adhesion and spreading is a major goal in the development of cell transplantation techniques, including the therapeutic use of progenitor cells. Further studies aimed at controlling the molecular mechanisms of anoikis resistance will serve to define effective therapies for the treatment of many human malignancies.

## 2. Protocol Summary

Culture cells in anchorage resistant plate
and control plate in parallel

Determine viability (MTT or Calcein AM)

Measure anoikis with EthD-1

## 3. Precautions

**Please read these instructions carefully prior to beginning the assay.**

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

## 4. Storage and Stability

**Store kit in the dark at temperatures described in Materials Supplied section (Calcein AM and EthD-1 should be stored at -20°C, other components should be stored at 4°C). Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

**Δ Note: Avoid multiple freeze/thaw cycles.**

## 5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

## 6. Materials Supplied

| Item | Quantity | | Storage condition (before prep) | Storage condition (after prep) |
|---|---|---|---|---|
| | 24 tests | 96 tests | | |
| Calcein AM (500X) | 50 µL | 50 µL | -20°C | -20°C |
| Ethidium Homodimer (500X) | 50 µL | 50 µL | -20°C | -20°C |
| 24-well Anchorage Resistant plate (poly-Hema coated) | 1 plate | N/A | 4°C | 4°C |
| 96-well Anchorage Resistant plate (hydrogel coated) | N/A | 1 plate | 4°C | 4°C |
| Detergent Solution | 25 mL | 25 mL | 4°C | 4°C |
| MTT Solution | 3 x 1 mL | 3 x 1 mL | 4°C | 4°C |

## 7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Cells
- Cell culture medium
- Inverted fluorescence/light microscope: for monitoring anoikis
- Microplate reader capable of measuring fluorescence at Ex/Em = 485/515 nm (green Calcein AM) and Ex/Em = 525/590 nm (red EthD-1)
- 24-well plate / 96-well plate with clear flat bottom, preferably black
- MilliQ water or other type of double distilled water ($ddH_2O$)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions

## 8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

# 9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

### 9.1 Calcein AM (500X):

Ready to use as supplied. Equilibrate to room temperature prior to use. Store at -20°C.

### 9.2 EthD-1 (500X):

Ready to use as supplied. Equilibrate to room temperature prior to use. Store at -20°C.

### 9.3 Anchorage Resistant Plate:

Ready to use as supplied. Equilibrate to room temperature prior to use. Store at 4°C.

**Δ Note:** Plate can be used over multiple experiment and do not need to be used all at once. If you plan to use the plate over multiple experiments, store plate after use at 4°C. Sterilize unused wells by exposing the plate to UV light under a cell culture hood for 30 minutes before use.

### 9.4 Detergent Solution:

Ready to use as supplied. Equilibrate to room temperature prior to use. Store at 4°C.

### 9.5 MTT Solution:

Ready to use as supplied. Equilibrate to room temperature prior to use. Store at 4°C.

## 10. Assay Procedure – 24 well plate

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- Prepare all reagents as directed in the previous sections.
- We recommend that you assay all controls and samples in duplicate.
- We recommend that you use Calcein AM/EthD-1 detection for this assay is you have access to a fluorescence plate reader, as it more sensitive than the MTT colorimetric detection.

### 10.1 Plate Loading:

10.1.1 Treat your cells of interest with anoikis enhancing or inhibiting agents as required.

10.1.2 Prepare a cell suspension containing 0.1 – 2 x $10^6$ cells/mL in culture media.

10.1.3 Add 0.5 mL cell suspension to each well of the 24-well Anchorage Resistant Plate.

10.1.4 Add 0.5 mL cell suspension to each well of a 24-well control plate.

10.1.5 Culture cells 24 – 72 hours at 37°C and 5% $CO_2$.

**Δ Note:** Time and culture conditions will depend on the cell line and may need to be adjusted.

10.1.6 Proceed to colorimetric (Step 10.2) or fluorometric (Step 10.3) detection as per your instrumentation capabilities.

### 10.2 Colorimetric Detection – MTT:

10.2.1 Add 50 µL of the MTT Reagent to each used well of the 24-well Anchorage Resistant Plate or 24-well control plate.

10.2.2 Incubate plates 2 – 4 hours (overnight incubation might be required) at 37°C. Monitor the cells occasionally with an inverted microscope for the presence of a purple precipitate.

10.2.3 Add 500 µL of Detergent Solution to each well. Gently mix the solution by pipetting.

10.2.4 Cover the plate with foil to protect it from light and incubate in the dark for 2 – 4 hours at room temperature.

10.2.5 Transfer 200 µL to a clear 96-well plate and measure absorbance in each well at OD 570 nm in a microplate reader.

### 10.3 Fluorometric Detection – Calcein AM/EthD-1:

10.3.1 Add 1 µL of Calcein AM (500X) and 1 µL of EthD-1 (500X) to each used well of the 24-well Anchorage Resistant Plate or control plate.

10.3.2 Incubate plates 30 – 60 minutes at 37°C.

10.3.3 Monitor the cells in a fluorescent microscope for the presence of the green Calcein AM (Ex/Em = 485/515 nm) or red EthD-1 (Ex/Em = 525/590 nm).

10.3.4 For quantitative measurement, measure fluorescence in a microplate reader at Ex/Em = 485/515 nm (Calcein) and Ex/Em = 525/590 nm (EthD-1).

**Δ Note:** Cells can be transferred to a black walled plate for measurement in fluorescent plate reader.

## 11. Assay Procedure – 96 well plate

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- Prepare all reagents as directed in the previous sections.
- We recommend that you assay all controls and samples in duplicate.
- We recommend that you use Calcein AM/EthD-1 detection for this assay is you have access to a fluorescence plate reader, as it more sensitive than the MTT colorimetric detection.

### 11.1 Plate Loading:

11.1.1 Treat your cells of interest with anoikis enhancing or inhibiting agents as required.

11.1.2 Prepare a cell suspension containing 0.1 – 2 x $10^6$ cells/mL in culture media.

11.1.3 Add 0.1 mL cell suspension to each well of the 96-well Anchorage Resistant Plate.

11.1.4 Add 0.1 mL cell suspension to each well of a 96-well control plate.

11.1.5 Culture cells 24 – 72 hours at 37°C and 5% $CO_2$.

**Δ Note:** Time and culture conditions will depend on the cell line and may need to be adjusted.

11.1.6 Proceed to colorimetric (Step 11.2) or fluorometric (Step 11.3) detection as per your instrumentation capabilities.

### 11.2 Colorimetric Detection – MTT:

11.2.1 Add 10 µL of the MTT Reagent to each used well of the 96-well Anchorage Resistant Plate or 96-well control plate.

11.2.2 Incubate plates 2 – 4 hours (overnight incubation might be required) at 37°C. Monitor the cells occasionally with an inverted microscope for the presence of a purple precipitate.

11.2.3 Add 100 µL of Detergent Solution to each well. Gently mix the solution by pipetting.

11.2.4 Cover the plate with foil to protect it from light and incubate in the dark for 2 – 4 hours at room temperature.

11.2.5 Transfer 150 µL to a clear 96-well plate and measure absorbance in each well at OD 570 nm in a microplate reader.

## 11.3 Fluorometric Detection – Calcein AM/EthD-1:

11.3.1 Dilute Calcein AM (500X) and EthD-1 (500X) stock solution to 100X with culture medium.

11.3.2 Add 1 µL of 100X Calcein AM/EthD-1 solution to each used well of the 96-well Anchorage Resistant Plate or control plate.

11.3.3 Incubate plates 30 – 60 minutes at 37°C.

11.3.4 Monitor the cells in a fluorescent microscope for the presence of the green Calcein AM (Ex/Em = 485/515 nm) or red EthD-1 (Ex/Em = 525/590 nm).

11.3.5 For quantitative measurement, measure fluorescence in a microplate reader at Ex/Em = 485/515 nm (Calcein) and Ex/Em = 525/590 nm (EthD-1).

**Δ Note:** Cells can be transferred to a black walled plate for measurement in fluorescent plate reader.

# 12. Typical Data

**Typical data** – data provided **for demonstration purposes only.**

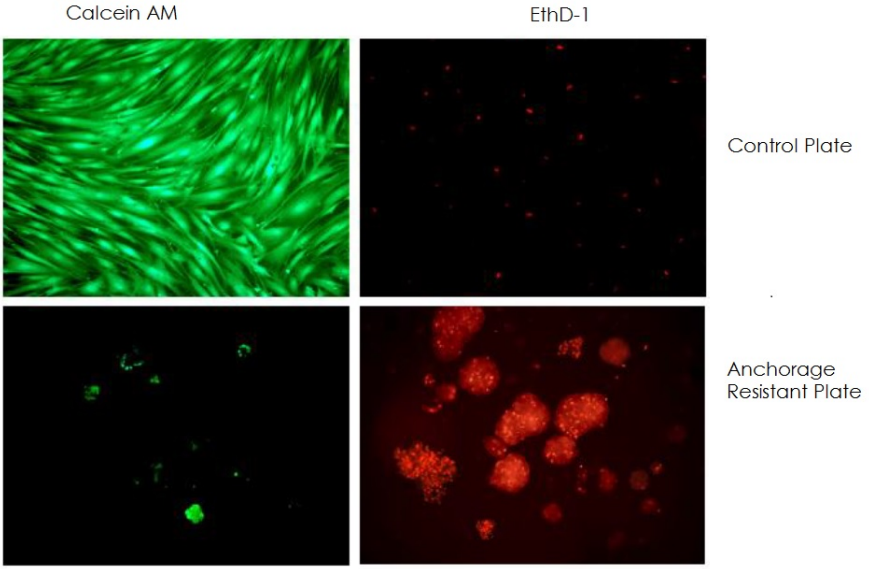


**Figure 1**. Anoikis assay performed on the poly-Hema coated (24-well) plate. Human foreskin fibroblasts BJ-TERT cells were seeded at 40,000 cells/well in a tissue culture control plate (top panel) or an anchorage resistant plate (poly-Hema coated) (bottom panel) and incubated for 24hours. Cells cultured in the anchorage resistant plate show signs of anoikis-like cell death (diminished calcein AM staining and increase EthD-1 staining.

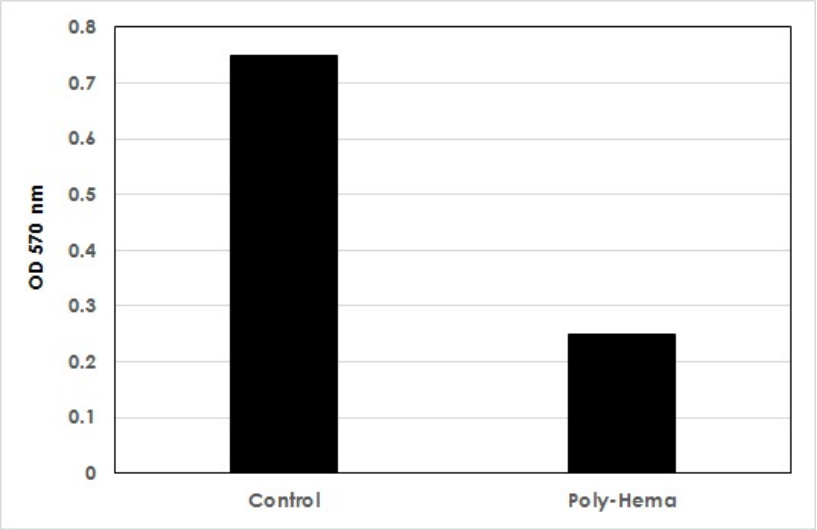


**Figure 2.** Anoikis detection experiment in the 24-well plate. Cell viability determined by MTT in human foreskin fibroblasts BJ-TERT cells seeded at 40,000 cells/well in a tissue culture control plate (control) or an anchorage resistant plate (poly-Hema) and incubated for 24hours.

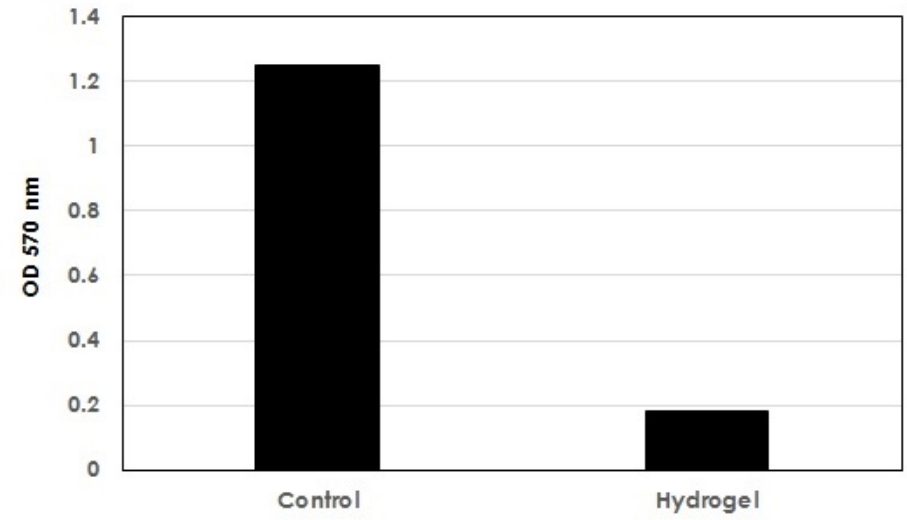


**Figure 3.** Anoikis detection experiment in the 96-well plate. Cell viability determined by MTT in human foreskin fibroblasts BJ-TERT cells seeded at 10,000 cells/well in a tissue culture control plate (control) or an anchorage resistant plate (hydrogel) and incubated for 24hours.

# 13. FAQs

### Q. How does MMT and Calcein AM work?

A. MTT is used for proliferation assays and measures the activity of enzymes in living cells that reduces tetrazolium dye (yellow) to formazan (purple). Calcein AM is also used as a live cell indicator because it is cleaved by intracellular esterases once it enters the cells, producing a green fluorescence. Either MTT or calcein AM can be used to measure cell viability, depending on the type of plate reader available.

### Q. Will this kit differentiate cells that have died by anoikis rather than apoptosis?

A. Anoikis is a form of apoptosis that is induced by the absence of proper cell attachment to the extracellular matrix. Our anoikis assay uses EthD-1 dye to measure cell death, which is not specific to anoikis or apoptosis. However, the assay uses a hydrogel coated plated that cells cannot attach to, which promotes anoikis cell death. Although the assay does not differentiate between apoptosis and anoikis, cell death with this assay can be assumed to be from anoikis based on the assay conditions.

# 14. Notes

## Technical Support



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