ToxCount™
Cell Viability Assay

(version A)

Catalog No. 18010

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Overview

Active Motif's ToxCount™ Cell Viability Assay provides reagents to perform a simple two-color fluorescence cell viability assay. The ToxCount assay is based on the simultaneous determination of live and dead cells with two probes, calcein AM and ethidium homodimer (EthD-1), that recognize parameters of cell viability including intracellular esterase activity and plasma membrane integrity, respectively.

Calcein AM is a non-fluorescent cell permeable dye that converts to green fluorescent calcein in live cells after acetoxymethyl ester hydrolysis by intracellular esterases. Ethidium homodimer (EthD-1) is a red fluorescent nuclear and chromosome counterstain. This reagent does not permeate live cells and is commonly used to detect dead cells in a population. Because the dyes used in ToxCount are non-fluorescent before interacting with cells, background fluorescence levels are very low. ToxCount data can be acquired using the BlueShift Biotechnologies IsoCyte™ laser scanner or standard fluorescent microscopes.

product	format	catalog no.
ToxCount™	20 x 96 rxns*	18010

* Sufficient components are provided for performing 20 x 96-well assays using the IsoCyte laser scanner. This assay can also be easily adapted for use in smaller or larger formats such as 384-well plates or 6-well plates.

Introduction

ToxCount™ provides a two wavelength cell viability assay based on determination of live and dead cells. The assay uses fluorescent dyes that measure permeability of the plasma membrane of dying cells and the ability of living cells to retain intracellular esterases, respectively. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the non fluorescent cell permeable calcein AM to the intensely fluorescent calcein. In contrast, ethidium homodimer (EthD-1) enters cells with damaged membranes and binds to nucleic acids where it emits a bright red fluorescent signal in dead cells.

ToxCount can be used on multiple fluorescence detection platforms, including multi-well plate scanning, fluorescence microscopy or flow cytometry[1]. The assay is also suitable for use with many adherent cell lines[2], primary cell types[3,4] and small organisms[5]. This method is less expensive, safer and faster than traditional cell viability assays such as 51Cr-release, LDH release and trypan blue exclusion. The calcein AM and EthD-1 fluorescent dyes have been used to study apoptosis[2] and the cytotoxic effects of numerous agents including viral proteins[6], oxalate[7], amyloid proteins[8] and cells such as lymphocytes[1]. The assay is ideally suited for rapid screening of libraries of drugs and small molecules for cytotoxic effects and is particularly applicable to high-content screening using the BlueShift IsoCyte laser scanner.

The dyes provided in ToxCount are easy to use, requiring only 30 minutes loading time. In addition, these dyes can be used in homogeneous 'no-wash' protocols as they are non-fluorescent in solution. Active Motif's ToxCount Kit has been optimized specifically for scanning on the IsoCyte laser scanner. A detailed protocol is available for customers who wish to use the reagents for 96-well, high throughput cytotoxicity assays with the IsoCyte and automated analysis software will rapidly count the number of live and dead cells, providing the user with an easily interpreted readout for each well.

Advantages of the IsoCyte™ laser scanner

The IsoCyte laser scanner from BlueShift Biotechnologies is a bench top laser scanning fluorimeter that measures fluorescence emission signals and their characteristic properties other than intensity and wavelength (*i.e.* fluorescence polarization). The platform has been designed to provide unprecedented flexibility in assay applications, sample formats and fluorophore compatibility. The system scans microplates with industry standard Society of Biomolecular Screening (SBS) allowing measurements to be made from 96, 384 or 1536 well plates.

The scanner's object based technology measures signals associated with objects in the entire well enabling the researcher to:

- Distinguish object signal from background
- Measure object fluorescence intensity
- Provide information regarding cell number, area and location in the well

Kit Components and Storage

Kit reagents are supplied lyophilized and are stable at room temperature. Once the EthD-1 is resuspended in DMSO it must be stored at -20°C. Once the calcein AM has been resuspended in anhydrous DMSO it must be aliquoted, desiccated, protected from light and stored at -20°C as indicated in the Buffer Preparation Section on page 4.

Reagents	Quantity	Storage / Stability
calcein AM	110 µl (1 mM) (lyophilized)	RT for 6 months
EthD-1 (Ethidium homodimer)	110 µl (1 mM) (lyophilized)	RT for 6 months

Additional materials required

- DMSO (anhydrous)
- Serum-free media
- 37°C incubator
- Saponin (Sigma, Cat. No. S4521-25G)
- PBS with calcium and magnesium supplemented with 10 mM glucose
- Light microscope
- Fluorescence microscope/reader or IsoCyte laser based scanner from Blueshift Biotechnologies (Sunnyvale, CA)
- Suitable tissue culture plates such as 96 well, clear bottom, black walled sterile polystyrene plates (i.e. CoStar catalog # 3904)

Reagent Preparation and Protocols

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Preparation of calcein AM Stock Solution

The calcein AM reagent is supplied lyophilized. Prepare a 1 mM calcein AM stock solution by adding 110 µl of anhydrous DMSO to the lyophilized calcein AM in the provided vial. Aliquot in 5 µl fraction and store at -20 ° C desiccated. On the day of the experiment, you will prepare an aqueous working solution containing calcein AM immediately prior to use, and use within one day. **Note:** Reagent must be desiccated once it is resuspended in DMSO as it is susceptible to hydrolysis when exposed to moisture.

Preparation of EthD-1 Stock Solution

The EthD-1 is supplied lyophilized. Prepare a 1 mM EthD-1 stock solution by adding 110 µl of DMSO (does not have to be anhydrous) to the lyophilized EthD-1 in the provided vial. Store at -20 ° C. EthD-1 is stable and insensitive to moisture. Stock solutions of EthD-1 can be stored frozen at -20 ° C for at least one year.

Selection of Optimal Filters

Calcein AM has an excitation/emission spectrum of 496 nm and 516 nm, respectively. A 510-540 nm band pass filter can be used for calcein AM emission. EthD-1 excites at 528 nm and emits at 598 nm. A conventional 600 nm long pass filter can be used to image this dye and to block bleed through from the green channel. If using the IsoCyte, it has a 560 nm dichroic filter to allow transmission of wavelengths longer than 560 nm to the red detection channels.

Preparation of the Cells

Adherent cells may be cultured directly on the microtitre plate. For a 96-well plate used with IsoCyte, we recommend seeding cells at 2.5 x 10^3 cells per well in 200 µl of supplemented medium. Incubate cells at 37 ° C until 60% confluent. **NOTE**: After adding the cells to the plate leave the plate undisturbed in the hood for 20 minutes before placing it in the incubator. Moving the plate immediately after seeding can generate forces that push cells to the outer edges of the well. If using suspension cells, please see Appendix Section C. Troubleshooting on page 11.

Preparation of Saponin Solution

Prepare a 0.1% solution of saponin by dissolving 0.05 g of saponin in 50 ml PBS containing calcium and magnesium or serum free medium. This solution is stable for at least 48 hours.

Preparation of PBS with 10 mM glucose (PBSg)

Prepare PBSg by dissolving 0.09 g of glucose in 50 ml PBS containing calcium and magnesium. We recommend preparing fresh PBSg each day an experiment is to be performed.

Typical ToxCount Experimental Design

The ToxCount protocol volumes are based on a 96-well plate. Sufficient reagents are provided for performing 20 x 96-well plates. However, the assay is also suitable for use with other formats as indicated in Table 1 below.

Plate type	Total vol/well (including test drug/ saponin) (µl)	Vol. dye/well (µl)	Vol. medium/ plate (ml)	calcein AM required/ plate (µl)	EthD-1 required/ plate (µl)	# plates possible
1536	5	2.5	4	4	4	25
384	50	25	10	10	10	10
96	**100**	**50**	**5**	**5**	**5**	**20**
24	500	250	6	6	6	16
12	1000	500	6	6	6	16
6	2000	1000	6	6	6	16

Table 1. A schematic of volumes required in the ToxCount Assay. The protocol is based on a 96-well plate with IsoCyte.

Table 2 below shows a typical experiment which includes individual staining controls for both dyes in treated and untreated wells (to get a readout for background in each channel), and wells that were treated or untreated and stained with both dyes. Once this experiment has been performed for a certain cell type and the background values are known, further experiments can be performed using the Standard Protocol on page 6.

1	2	3	4	5	6	7	8	9	10
Calcein AM Live cells	Calcein AM Dead cells 10 min Saponin	Calcein AM Dead cells 10 min Test Reagent	EthD-1 Live cells	EthD-1 Dead cells 10 min Saponin	EthD-1 Dead cells 10 min Test Reagent	EthD-1 + Calcein AM Live cells	EthD-1 + Calcein AM Dead cells 10 min Saponin	EthD-1 + Calcein AM Dead cells 10 min Test Reagent	No Dyes

Table 2. A typical cytotoxicity/cell viability experiment using ToxCount assay reagents. Cells that have been treated with saponin should not be stained with calcein AM and untreated cells should not stain with EthD-1. The numbers indicate separate columns of a 96 well plate.

IsoCyte Bleed-Through Test

When using the IsoCyte, it is necessary to assess the extent of bleed-through from one channel to another or background levels (according to the Table 2 above). For this test, only 2 ml of calcein AM solution or EthD-1 solution or a 2 ml solution with a mixture of both dyes is prepared. Once this bleed-through experiment has been performed for a certain cell type and the background values are known, further experiments can be performed using the standard protocol on page 6.

NOTE: All working solutions can be prepared with either PBSg or serum free medium. For the IsoCyte, no background interference has been seen from phenol red in the media, however, there may be interference when using a fluorescent microscope.

Step 1: Treatment of cells to induce cell death.

1. Prepare a 0.1% saponin solution by dissolving 0.05 g saponin in 50 ml PBSg or serum-free medium.
2. Remove the media from the wells of the microplate to be tested using a multichannel pipettor.
3. Add 50 µl of the test compounds or saponin solution to the wells designated as "dead" cells for the desired amount of time. For 0.1% saponin we recommend a 10 minute 37°C treatment.
4. Add 50 µl of PBSg or serum free medium to the "live" cells for the desired amount of time.

NOTE: The calcein AM and EthD-1 are supplied lyophilized and instructions for reconstituting are on page 4. If you have already reconstituted the dyes, please ensure the calcein AM and EthD-1 dyes have been thawed for at least 5 minutes and are at room temperature prior to beginning the Step 2 below. This helps avoid the dilution of the solution by ambient moisture condensation. Protect the dyes from light as much as possible.

Step 2: Preparation of 1 µM calcein AM working solution.

1. The calcein AM is supplied lyophilized. Please read the section on page 4 about reconstituting and preparing the calcein AM stock solution. Prepare a 1 µM calcein AM working solution by adding 2 µl of the 1 mM calcein AM stock solution to 2 ml of PBSg or serum free medium.

Step 3: Preparation of 1 µM EthD-1 working solution.

1. The EthD-1 is supplied lyophilized. Please read the section on page 4 about reconstituting and preparing the EthD-1 stock solution. Prepare a 1 µM EthD-1 working solution by adding 2 µl of the 1 mM EthD-1 stock solution to 2 ml of PBSg or serum free medium.

Step 4: Preparation of a mixture of 1 µM calcein AM and 1 µM EthD-1.

1. The calcein AM and EthD-1 are supplied lyophilized. Please read the section on page 4 about reconstituting and preparing the stock solutions. Prepare a mixture of 1 µM calcein AM and 1 µM EthD-1 by adding 2 µl of 1 mM calcein AM stock solution and 2 µl of 1 mM EthD-1 prepared in the previous section to 2 ml PBSg or serum free medium.

Step 5: Perform the Viability Assay.

1. Treat cells with the test compounds or saponin for the desired amount of time.
2. Add 50 µl of 1 µM calcein AM to wells designated "calcein AM only."
3. Add 50 µl of 1 µM EthD-1 to wells designated "EthD-1 only."
4. Add 50 µl of the of 1 µM calcein AM/1 µM EthD-1 mixture to wells designated "EthD-1 + calcein AM"
5. Incubate cells at 37 ° C for 30 minutes.

Standard Protocol

If using the IsoCyte, proceed with the following Standard Protocol after completion of the Bleed-Through Test for a specific cell type. These volumes are based on a 96-well plate. If using a different size plate, please refer to Table 1 on page 5.

NOTE: All working solutions can be prepared with either PBSg or serum free medium. For the IsoCyte, no background interference has been seen from phenol red in the media, however, there may be interference when using a fluorescent microscope.

Step 1: Treatment of cells to induce cell death

1. Prepare a 0.1% saponin solution by dissolving 0.05 g saponin in 50 ml PBSg or serum-free medium.
2. Remove the media from the wells of the microplate to be tested using a multichannel pipettor.
3. Add 50 µl of the test compounds or saponin solution for the desired amount of time. For 0.1% saponin we recommend a 10 minute 37°C treatment.

   NOTE: The calcein AM and EthD-1 are supplied lyophilized and instructions for reconstituting are on page 4. If you have already reconstituted the dyes, please ensure the calcein AM and EthD-1 dyes have been thawed for at least 5 minutes and are at room temperature prior to beginning the Step 2 below. This helps avoid the dilution of the solution by ambient moisture condensation. Protect the dyes from light as much as possible.

Step 2: Perform the Viability Assay

1. The calcein AM and EthD-1 are supplied lyophilized. Please read the section on page 4 about reconstituting and preparing the stock solutions. Prepare a mixture of 1 µM calcein AM and 1 µM EthD-1 by adding 5 µl of 1 mM calcein AM stock solution and 5 µl of 1 mM EthD-1 stock solution to 5 ml PBSg or serum free medium.
2. Add 50 µl of dye mixture to each well.
3. Incubate cells at 37 ° C for 30 minutes.
4. Proceed to cell imaging.

Imaging the cells and Data Analysis using the IsoCyte

The staining of the cells can be checked on a fluorescent microscope before scanning on the IsoCyte. There is no need to remove the dye solution from the well or wash the cells since the dyes are not fluorescent unless they are inside the cell.

Instructions for scanning plates on the IsoCyte

1 Turn on the scanner using the On/Off (I/O) switch located on the left side of the instrument. Wait 5 minutes for the laser to warm up.

2. Start the BBIsoCyte software by double-clicking on the icon on the desktop.
3. Click on the blue arrow shaped icon on the tool bar to eject the plate holder.
4. Insert plate and click icon again to close.
5. Open "ToxCount" method.
6. Select the wells that are to be scanned. The wells will be highlighted in blue if they have been selected.
7. Click on the scan icon.
8. When the scan has finished save the data file.
9. When the analysis is complete, a new folder containing the .csv files will appear in the same directory as the bbx files.

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2. Lucas R. et al. (1998) *Blood* 92(12):4730-41.
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6. Guzman E. and McCrae M.A. (2005) *J Virol Methods* 127(2):119-25.
7. Kohjimoto Y. (1999) *Kidney Int.* 56(4):1432-41.
8. Liu M.L. and Hong S.T. (2005) *Exp Mol Med.* 37(6):559-66.

Appendix

Section A. Optimizing Dye Concentrations

Determination of Optimal Dye Concentrations

Using the IsoCyte, we have determined that 0.5 µM of calcein AM and EthD-1 provide optimal results for staining live or dead cells. However, depending on the cell type assayed or if you are using a fluorescent microscope for detection, optimal dye concentrations may vary. The following method can be used to determine the optimal dye concentration.

1. Remove the ToxCount assay reagents from the freezer and allow them to warm to room temperature.
2. Prepare samples of live cells as well as dead cells on a 96-well plate. Induce cell death using 0.1% saponin for 10 minutes at 37°C.
3. Using samples of dead cells, select an EthD-1 concentration that stains the dead cell nuclei bright red without staining the cytoplasm significantly (recommend 0.1 to 10 µM EthD-1).
4. Using samples of dead cells, select a calcein AM concentration that does not give significant fluorescence in the dead cell cytoplasm (recommend 0.1 to 10 µM calcein AM).
5. Using samples of live cells, check to see that the calcein AM concentration selected above generates sufficient fluorescence in the live cells.
6. The reagent concentrations determined in Step 3 and 5 are optimal for the viability experiments.

Section B. ToxCount Protocol for 8-well chamber slide

If you are using an 8-well chamber slide and evaluating cell viability through use of a fluorescent microscope, please follow the protocol listed below.

1. Seed cells in 250 µl of culture medium at a density of 1 x 10^4 cells per well in an 8 well chamber slide.
2. Incubate cells overnight at 37°C, 5% CO_2.
3. Treat cells with 125 µl 0.1% saponin for 10 minutes or with test compounds for desired amount of time.
4. Prepare a 2X solution of calcein AM and EthD-1 by adding 1 µl from each of the 1 mM stocks to 1 ml of serum free media or PBSg.
5. Add 125 µl of the dye mixture to each well and leave for 30 minutes at 37°C.
6. Image slide on microscope.

Section C. Troubleshooting Guide

PROBLEM/QUESTION	RECOMMENDATION
No staining in sample wells	Use a microscope with a bright field light to check that the cells are still present on the plate and have not lifted off the surface as a result of treatment with toxic reagents.
	Increase the exposure time on the microscope or the gain setting on the IsoCyte.
	Label cells with a higher dye concentration
High background (cell death not induced)	Increase concentration of saponin or try an alternative toxic agent such as methanol to induce cell death
Can I use suspension cells rather than adherent cells?	Yes. Wash suspension cells with 500 -1000 volumes of PBS and sediment by centrifugation. Plate cells in desired plate size in serum free medium to 60% confluency. After cells have settled, treat with 2X solution of test reagent or saponin. Prepare a 3X Dye solution (1.5 µM) and add to wells (final concentration will be 0.5 µM). Incubate for 30 minutes and analyze as desired.

Section D. Related Products

Protein Transfection	Unit	Catalog No.
Chariot™	25 rxns	30025
	100 rxns	30100
Fluorescent Dyes	**Unit**	**Catalog No.**
Chromeo™ 494 Carboxylic Acid	1 mg	15110
	5 mg	16110
Chromeo™ 494 NHS-Ester	1 mg	15111
	5 mg	16111
Chromeo™ 494 Biotin	1 mg	15112
	5 mg	16112
Chromeo™ 494 Streptavidin	1 mg	15113
	5 mg	16113
Chromeo™ 546 Carboxylic Acid	1 mg	15210
	5 mg	16210
Chromeo™ 546 NHS-Ester	1 mg	15211
	5 mg	16211
Chromeo™ 546 Biotin	1 mg	15212
	5 mg	16212
Chromeo™ 546 Streptavidin	1 mg	15213
	5 mg	16213
Chromeo™ 642 Carboxylic Acid	1 mg	15310
	5 mg	16310
Chromeo™ 642 NHS-Ester	1 mg	15311
	5 mg	16311
Chromeo™ 642 Biotin	1 mg	15312
	5 mg	16312
Chromeo™ 642 Streptavidin	1 mg	15313
	5 mg	16313
Protein Labeling		
LigandLink™ pLL-1 Kit	1 kit	34001
LigandLink™ pLL-1-NFkB p65 Kit	1 kit	34004
LigandLink™ pLL-1-p53 Kit	1 kit	34005
LigandLink™ pLL-1-STAT1 Kit	1 kit	34006
LigandLink™ Fluorescein Label	300 rxns	34101
LigandLink™ Hexachlorofluorescein Label	300 rxns	34104

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If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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