Mitotic Index Assay Kit (Fluorescent)

(version B)

Catalog No. 18020

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Introduction

Active Motif's Mitotic Index Assay Kit enables you to determine the percentage of cells that are undergoing mitosis within a specified population. The Mitotic Index Assay Kit includes a phospho-histone H3 (Ser 28) monoclonal antibody (clone HTA28) and a Chromeo™ 488 fluorescently labeled secondary for detection. Propidium iodide is provided to stain DNA in all cells to give a total cell count for analysis. Paclitaxel is included to use as a positive control treatment.

Cell division is a complex and tightly regulated process that is marked by mitosis. During the progression of interphase in stage G2 to M phase, chromatin is packed into condensed chromosomes for nuclear division to continue. During this event, histones may be phosphorylated in a specific and temporally regulated manner. Recently, studies have shown that histone H3 is specifically phosphorylated at serines 10 and 28 during mitotic chromatin condensation[1,2]. Therefore, the phosphorylated histone H3 serine 28 residue is a useful marker for mitosis and this specific phosphorylation site can be used to determine the mitotic index of a population of cells.

The mitotic index, or proportion of cells undergoing mitosis within a specified cell population, is easily determined by calculating the percentage of cells positive for the histone H3 (Ser 28) signal relative to the total number of cells (Figure 1). The total number of cells is determined by the red emission signal from nuclei as a result of the propidium iodide stain. After reading the propidium iodide signal, the phospho-histone H3 (Ser 28) is detected by the Chromeo 488 Goat anti-Rat IgG secondary antibody which emits a bright green signal. This bright green fluorescence reveals cells undergoing mitosis (Figure 2). The drug paclitaxel prevents mitotic spindle assembly and results in cells that are arrested during mitosis in late G2/M phase to provide a reading for high-mitotic index within a treatment population. This treatment provides a reference to compare the effect of test compounds on mitosis.

product	format	catalog no.
Mitotic Index Assay Kit	5 x 96 reactions	18020

Kit Performance and Benefits

The Mitotic Index Assay Kit is for research use only. Not for use in diagnostic procedures.

Antibody Specificity

The anti-phospho-Histone H3 (Ser 28) clone HTA28 is raised in rat and generated against a synthetic peptide corresponding to residues 23-35 with a phosphorylated serine 28 residue of human histone H3. The antibody cross reacts with human, mouse, hamster and bovine samples and is expected to cross react with other mammalian species based on sequence homology. The Chromeo™ 488 Goat anti-Rat IgG detects both heavy and light chains of rat immunoglobulins.

Chromeo™ 488 Dye Fluorescent Properties

Chromeo 488 has an excitation peak at 488 nm and peak emission at 517 nm.

Propidium Iodide Fluorescent Properties

Propidium iodide has an excitation peak at 530 nm and peak emission at 625 nm.

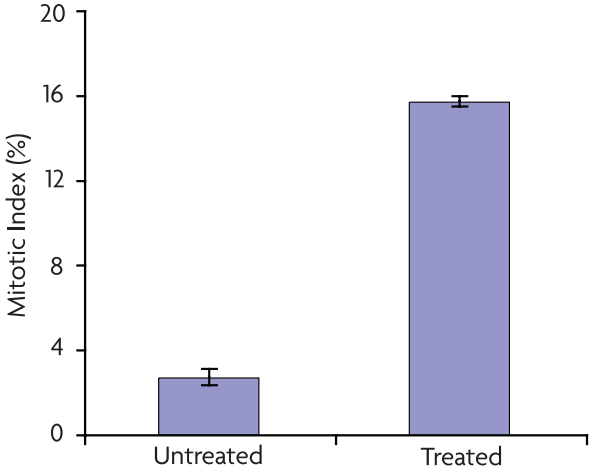

Figure 1: Fluorescence analysis was performed on 18 wells of untreated and paclitaxel-treated cells to obtain the average percent mitotic index value. HeLa cells were treated with 1 µM paclitaxel diluted in complete medium for 6 hours.

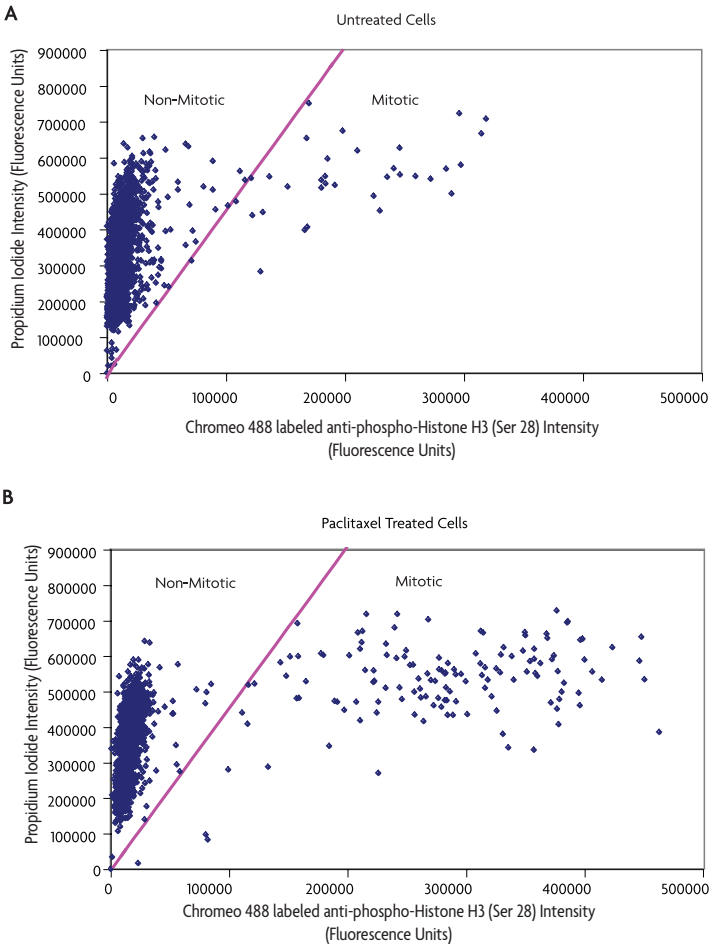

Figure 2: Single cell analysis of an untreated versus a paclitaxel treated population quantified using the IsoCyte™. Panel A shows few cells undergoing mitosis in an untreated cell population. Panel B shows the shift in cell population detected by the 488 signal to the right of the dividing line following paclitaxel treatment.

Kit Components and Storage

Store components at the temperatures indicated below. Paclitaxel and Propidium Iodide are in DMSO and will be frozen when stored at 4°C. Aliquot Paclitaxel and Propidium Iodide to avoid subjecting to multiple freeze-thaws. The antibodies may also be aliquotted and stored at -20°C for long-term storage.

This kit has been optimized for use in high-throughput screening assays with object-based fluorescent imaging systems. This assay does not perform well on standard fluorescent plate readers, and we do not recommend using this assay with standard fluorescent plate readers.

Reagent	Quantity	Storage / Stability
anti-phospho-Histone H3 (Ser 28)	12.5 µl	4°C for 6 months
Chromeo™ 488 Goat anti-Rat IgG	25 µl	4°C for 6 months
Propidium Iodide (1 mg/ml)	100 µl	4°C for 6 months
Paclitaxel (1 mM)	10 µl	4°C for 6 months
RNase A (lyophilized)	1.1 mg	4°C for 6 months

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Additional materials required

- 100% ice cold methanol
- 5% BSA in PBS
- PBS
- 96 well plates (Corning, catalog number 3904)
- multi-channel pipette
- Object-based fluorescent imaging system with 488 nm excitation and 510-540 nm and 600-650 nm emission filters

Alternative treatment agents

- Colchicine (Calbiochem, catalog number 234115)
- Etoposide (Calbiochem, catalog number 341205)

Reagent Preparation

RNase A

Rehydrate the lyophilized RNase A prior to use by adding 110 µl H_2O to make a 10 mg/ml stock solution.

Propidium iodide/RNase A solution

Dilute propidium iodide and RNase A 1:1000 in PBS together for a final concentration of 1 µg/ml propidium iodide and 10 µg/ml RNase A.

For example: the volume required to treat one 96 well plate is 19.2 ml (200 µl x 96 wells = 19200 µl). Add 20 µl of propidium iodide and 20 µl of RNase A to 20 ml of pre-warmed PBS, then add 200 µl to each well. .

Paclitaxel

Dilute the 1 mM Paclitaxel stock solution 1:1000 in complete cell medium to make a 1 µM final concentration. Remove existing medium and add 100 µl of the paclitaxel solution to each well.

5% BSA Solution

Prepare a 5% BSA solution in PBS. This solution is used in the blocking step and to dilute the primary and secondary antibodies. If the solution is prepared in advance, it should be stored at 4°C. For 500 reactions in 96 well plates, 80 ml of 5% BSA is sufficient.

Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

This protocol has been used with HeLa cells and macrophages. Due to variability between wells, we recommend treating one full column of each plate with paclitaxel for reference.

1. Day 0: seed cells at 2500/well in 200 µl/well in black walled, clear plastic bottomed 96 well plates. Plates can be left untouched in the tissue culture hood for 20 minutes after seeding to aid cell settling and to avoid cells being pushed to the edge of the wells.

   Note: The number of cells may need to be optimized depending on the cell type used.

2. Day 1: Remove media from positive-control wells and add 100 µl/well of 1 µM paclitaxel for desired length of treatment.

   Note: Six hours was the amount of time required to see an adequate degree of mitotic arrest in HeLa and macrophage cells.

3. Remove medium from all wells and apply 50 µl of 100% ice cold methanol for 10 minutes to fix and permeabilize the cells.
4. Remove methanol and wash cells once with 200 µl PBS. PBS may be cold or warmed to room temperature.
5. Remove PBS and apply 50 µl of 5% BSA in PBS for one hour at room temperature to block cells.
6. Remove BSA solution and add 50 µl per well of primary antibody as 1:2000 dilution in 5% BSA solution overnight at 4°C.

   Note: Primary incubation may be performed for one hour at room temperature.

7. Wash cells twice with 200 µl of room temperature PBS.
8. Add 50 µl/well of the Chromeo™ 488 Goat anti-Rat IgG secondary antibody as 1:1000 dilution in 5% BSA Solution for one hour at room temperature.
9. Wash as in step 7.
10. Pre-warm propidium iodide/RNase A solution to 37°C and add 200 µl/well.
11. Incubate for 20 min at 37°C. There is no need to wash the cells before imaging.

    Note: Turn on imaging equipment to allow lasers to warm up.

12. Image plate with fluorescent imaging system.

References

1. Goto, H. *et al.* (1999) *J. Biol. Chem.* 274(36): 25543-25549.
2. Hirata, A. *et al.* (2004) *J. Histochemistry & Cytochemistry* 52(11): 1503-1509.

Appendix

Section A. Troubleshooting Guide

PROBLEM	POSSIBLE CAUSE	RECOMMENDATION
Little or no signal is observed from detecting anti-phospho Histone H3 (Ser 28)	Cells are dividing slowly. This could be due to recent thaw, ill-health or cell type.	Do not use cells that have been recently thawed.
		Make sure cells have adequate nutrients and CO_2 and are maintained at 37°C.
		Change to a cell line that divides more rapidly.
No increase in mitotic index is observed after paclitaxel treatment.	Cells are dividing too slowly to be arrested in mitosis.	Increase the length of time that cells are incubated with paclitaxel. If treating overnight, decrease paclitaxel concentration to 250 to 500 nM to avoid cyto-toxicity.
		Change cell model.

Section B. IsoCyte Imaging and Data Analysis

IsoCyte scanning conditions

Use Ch1 (510-540 nm emission filter) to image the Chromeo™ 488 Goat anti-Rat IgG-labeled phospho Histone H3 (Ser 28) and Ch3 (600 LP or 650 LP filter) to image the propidium iodide.

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 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.

Section C. Related Products

Fluorescent Dyes	Format	Catalog No.
Chromeo™ 488 NHS-Ester	1 mg	15511
	5 mg	16511
Chromeo™ 494 NHS-Ester	1 mg	15111
	5 mg	16111
Chromeo™ 546 NHS-Ester	1 mg	15211
	5 mg	16211
Chromeo™ 642 NHS-Ester	1 mg	15311
	5 mg	16311
CE Dye 503	1 kit	15101
CE Dye 540	1 kit	15102

Fluorescent Secondary Antibodies	Format	Catalog No.
Chromeo™ 488 Goat anti-Mouse IgG	1 mg	15031
Chromeo™ 488 Goat anti-Rabbit IgG	1 mg	15041
Chromeo™ 494 Goat anti-Rabbit IgG	1 mg	15042
Chromeo™ 546 Goat anti-Mouse IgG	1 mg	15033
Chromeo™ 546 Goat anti-Rabbit IgG	1 mg	15043
Chromeo™ 642 Goat anti-Mouse IgG	1 mg	15034
Chromeo™ 642 Goat anti-Rabbit IgG	1 mg	15044

Fluorescent Cell Stain	Format	Catalog No
LavaCell™	200 µg	15004

Protein Labeling	Format	Catalog No.
LigandLink™ pLL-1 Kit	1 kit	34001
LigandLink™ Fluorescein Label	300 rxns	34101
LigandLink™ Hexachlorofluorescein Label	300 rxns	34104
LigandLink™ pLL-1-NFκB p65 Kit	1 kit	34004
LigandLink™ pLL-1-p53 Kit	1 kit	34005
LigandLink™ pLL-1-STAT1 Kit	1 kit	34006

Luciferase Assays	Format	Catalog No.
RapidReporter® Gaussia Luciferase Assay	100 rxns	33001
	1000 rxns	33002
RapidReporter® pRR-High vector	10 µg	33003
RapidReporter® pRR-High Assay	100 rxns	33004
RapidReporter® pRR-Low vector	10 µg	33005
RapidReporter® pRR-Low Assay	100 rxns	33006
RapidReporter® pRR-High-CRE vector	10 µg	33007
RapidReporter® pRR-High-CRE Assay	100 rxns	33008
RapidReporter® pRR-High-NFκB vector	10 µg	33009
RapidReporter® pRR-High-NFκB Assay	100 rxns	33010
RapidReporter® pRR-High-GR vector	10 µg	33011
RapidReporter® pRR-High-GR Assay	100 rxns	33012
RapidReporter® pRR-High-STAT3 vector	10 µg	33013
RapidReporter® pRR-High-STAT3 Assay	100 rxns	33014
RapidReporter® pRR-High-STAT1 vector	10 µg	33015
RapidReporter® pRR-High-STAT1 Assay	100 rxns	33016

Technical Services

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

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