Technical Data Sheet

PE Mouse Anti-Underphosphorylated Retinoblastoma (Rb) Set

Product Information

Material Number:	**550502**
Reactivity:	QC Testing: Human
Component:	**51-14445X**
Description:	PE Mouse Anti-Underphosphorylated Retinoblastoma (Rb)
Size:	100 tests (1 ea)
Vol. per Test:	20 µl
Clone Name:	G99-549
Immunogen:	Recombinant full-length human Rb
Isotype:	Mouse IgG1
Storage Buffer:	Aqueous buffered solution containing BSA and ≤0.09% sodium azide.
Component:	**51-13855X-8**
Description:	PE Mouse IgG1 Isotype Control
Size:	100 tests (1 ea)
Vol. per Test:	20 µl
Clone Name:	MOPC-21
Isotype:	Mouse IgG1, κ
Storage Buffer:	Aqueous buffered solution containing BSA and ≤0.09% sodium azide.

Description

The retinoblastoma gene encodes a nuclear phosphoprotein (Rb or p110Rb) which is expressed in most normal cells of vertebrates and acts as a tumor suppressor. The underphosphorylated form of Rb is mainly found in resting or fully differentiated cells, whereas the more highly phosphorylated forms are present in proliferating cells. Thus, the underphosphorylated form of Rb can act as a marker of cells in G0/G1 phase of the cell cycle. Growth factors act to promote cells through the G1 and S phase of the cell cycle and during this time Rb undergoes sequential phosphorylation. Rb can act as a gatekeeper to regulate the cell cycle because in the underphosphorylated form it binds to critical regulatory proteins including E2F which acts to repress transcription. When Rb becomes phosphorylated, E2F is released and transcription can occur. Disregulation of the cell cycle can be caused by mutations in Rb as well as in p53, which can lead to unregulated growth or oncogenesis. The expression of underphosphorylated Rb has been found to be unregulated following induction of cell cycle arrest. There are numerous methods to induce cell cycle arrest and a typical model system is the use of TPA to induce cell cycle arrest in MOLT-4 cells. Clone G99-549 has been reported to recognize only the underphosphorylated form of the human Rb protein. The epitope for clone G99-549 has been reported to be mapped to amino acids 514-610 of human Rb.

Preparation and Storage

Store undiluted at 4°C and protected from prolonged exposure to light. Do not freeze.
The monoclonal antibody was purified from tissue culture supernatant or ascites by affinity chromatography.
The antibody was conjugated with R-PE under optimum conditions, and unconjugated antibody and free PE were removed.

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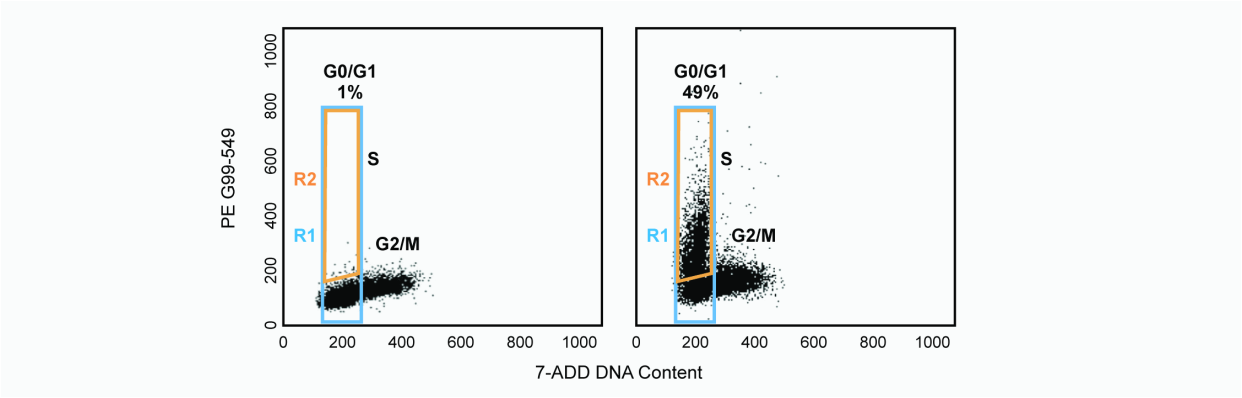

Profile of underphosphorylated Rb in MOLT-4 cells analyzed on a BD FACSCalibur™ flow cytometry system. *Cells were untreated (left panel) or were treated with TPA (200-1000 ng/ml, 24 hr) (right panel) to induce cell cycle arrest. Cells were then fixed, permeabilized and stained with PE mouse anti-underphosphorylated Rb antibody (Clone G99-549, Component No. 51-14445X) or with the PE mouse IgG1 isotype (negative) control antibody (Clone MOPC-21, Component No. 51-13855X-8). Cells were counterstained with 7-AAD (Cat. No. 559925) for DNA content. TPA treatment increased the expression of underphosphorylated Rb in the G0/G1 phase of the cell cycle. Cell cycle phase is denoted as G0/G1, S and G2/M.*

Application Notes

Application

Intracellular staining (flow cytometry)	Routinely Tested

Recommended Assay Procedure:

Methods for staining and analyzing underphosphorylated RB

Induction of Cell Cycle Arrest

The expression of underphosphorylated Rb has been reported to be upregulated following the induction of cell cycle arrest. The expression of underphosphorylated Rb in MOLT-4 cells, for example, has been shown to be upregulated following the induction of cell cycle arrest by TPA treatment. TPA (12-O-TetradecanoylPhorbol-13-Acetate), also known as Phorbol 12-Myristate-13-Acetate (PMA), may be obtained from Sigma-Aldrich (catalog number P8139). Numerous other methods to induce cell cycle arrest have been reported for various cell types and each investigator is encouraged to optimize protocols for their own experimental system. In a typical model system, TPA may be used to induce cell cycle arrest in MOLT-4 cells (Human T-lymphoblasts; ATCC CRL-1582). TPA has been shown to induce growth arrest in certain leukemia and cancer cells.

Induction of Cell Cycle Arrest in MOLT-4 Cells by TPA

1. Make a TPA stock solution at 1 mg/ml in ethanol.
2. Cells should be in log-phase growth. 1 x 10^6 cells per test is recommended.
3. Culture MOLT-4 cells in the absence (as a negative control) or presence of TPA (200-1000 ng per 1 ml of cell culture media) for 24 hr to induce cell-cycle arrest. TPA may be pipetted directly into the flask.
4. Following a 24 hr incubation, harvest cells and follow the fixation and staining protocol.

Fixation and Staining Protocol

Reagents

1. 1X PBS.
2. 10% formaldehyde methanol-free solution, EM grade (EM Sciences, Cat. No. 15712-10). Dilute to 1% solution in 1X PBS before use.
3. 80% ethanol in deionized water.
4. 0.25% Triton X-100 made up in 1X PBS (Sigma-Aldrich, Cat. No. X-100)
5. 7-AAD Staining Solution (Cat. No. 559925). Use 5 µl per test. 7-AAD (7-Amino-actinomycin D) is a convenient, ready-to-use nucleic acid dye solution that is used for DNA staining. 7-AAD fluorescence is detected in the far-red range of the spectrum (650 nm longpass filter). Cell cycle phase can be estimated by DNA content.
6. PE Mouse Anti-Underphosphorylated Retinoblastoma (Rb) Antibody (Cat. No. 550502; Component No. 51-14445X).
7. PE Mouse IgG1 Isotype Control (Cat. No. 550502; Component No. 51-13855X-8).

Procedure

1. Harvest, count and pellet cells following standard procedures.
2. Wash cells by resuspending the pellet in 20 ml of 1X PBS; centrifuge at 1000 rpm for 10 min and aspirate supernatant. Repeat wash once.
3. Resuspend cells in 1% methanol-free, formaldehyde/PBS solution while vortexing. Use 500 µl fixative for every 1x10^6 cells and incubate for 15 min on ice.
4. Wash cells as in Step 2.
5. While vortexing, add 10 ml ice-cold 80% ethanol drop by drop, to the cell pellet. Incubate cells at -20°C for at least 24 hr before staining. For long term storage, keep cells at -20°C in 80% ethanol.
6. Just prior to staining, remove ethanol by centrifugation at 1000 rpm for 10 min and wash cells as in Step 2.

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7. Resuspend pellet from Step 6 to a concentration of 1 x 10^7 cells/ml in 0.25% Triton X-100 made up in 1X PBS. Let sit on ice for 5 min then wash with 1X PBS twice as in Step 2. Resuspend the final pellet in 1X PBS to a concentration of 1 x 10^7 cells/ml.
8. For each test, take 100 µl of the cell suspension (1x10^6 cells), add 20 µl of the PE Mouse IgG1 Isotype Control (component no. 51-13855X-8), or 20 µl of the PE Mouse Anti-Underphosphorylated Retinoblastoma (Rb) Antibody (component no. 51-14445X) and incubate at room temperature for 30 min in the dark.
9. Wash cells by adding 2 ml of 1X PBS to each tube and gently resuspending cells; centrifuge at 1000 rpm for 5 min and aspirate supernatant. Repeat wash once.
10. Resuspend each pellet from Step 9 in 500 µl 1X PBS and add 5 µl of 7-AAD to stain the DNA. Incubate at room temperature for 15 min in the dark. Analyze stained cells by flow cytometry within 4 hr. Store stained cells at 4°C prior to analysis.

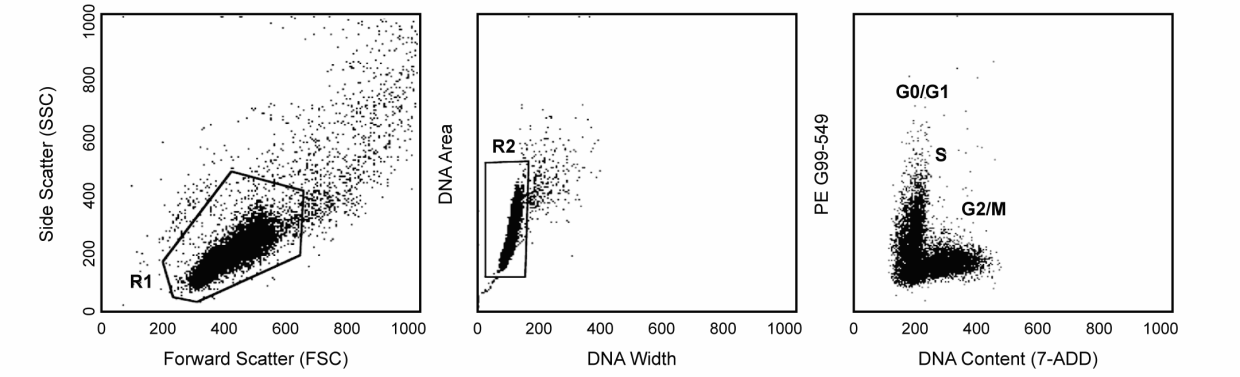
Data Acquisition and Analysis (using a BD FACSCalibur™ flow cytometry system)

Data Acquisition

1. In this example, all fluorescence channels are set to a linear scale to provide the necessary sensitivity to measure underphosphorylated Rb. The forward scatter (FSC) and side scatter (SSC) channels are typically set to linear. PE is acquired in FL2 and 7-AAD in FL3. The 7-AAD display channel (FL3) must be set to have both FL3 area and FL3 width. The FL3 area provides the cell cycle distribution; the FL3 width provides the data for doublet discrimination. Set the DDM parameter in the Detectors/Amps window to DDM parameter FL3.
2. Prepare a FSC versus SSC dot plot to visualize cells and make sure the cells are on scale. Make a region (R1) around the cells of interest (left panel).
3. Prepare a FL3 width versus FL3 area dot plot to visualize single cells versus doublets or triplets, and make a region (R2) around the single cells to exclude doublets (middle panel).
4. Prepare a FL3 area (x-axis) versus FL2 (PE, y-axis) to analyze the underphosphorylated Rb fluorescent signal versus DNA content (right panel). For FL2 use the voltage and/or gain to place the isotype control between 0 and 200 (right panel), while at the same time ensuring a positively stained sample is not off the upper end of the scale or above 1000 (right panel). Underphosphorylated Rb is a relatively low abundance molecule; thereby careful adjustment of the voltage/gain is required. For FL3 area, it is traditional to set the voltage so that the cells in the G0/G1 phase of the cell cycle are centered on 200. The FL3 voltage setting might have to be adjusted slightly for each test to ensure that G0/G1 cells are centered on 200.

Data Analysis

1. Open the data file that is the FSC versus SSC dot plot. Draw a region (R1) around the cells of interest (left panel, from Data Acquisition).
2. Open the data file that is the FL3 width versus FL3 area dot plot and plot the cells from R1. Draw a region (R2) around the single cell population (middle panel, from Data Acquisition).
3. Open data file that is FL3 area versus FL2 dot plot showing the cells from R2. Draw a region (R3) around the G0/G1 area of the cell cycle. The region should be centered on 200. Draw a new region (R4) that has the same vertical boundaries as R3 but has the horizontal cutoff directly above and skirting the isotype control cells. Region 4 will be the amount (%) of cells that are positive for underphosphorylated Rb.



Data Acquisition. *Left panel, Cells of interest are selected by drawing a gate (R1) to exclude cell debris and clumps with high forward and high side scatter. Middle panel, To visualize single cells versus doublets or triplets, a second gate (R2) is drawn on the DNA area versus DNA width fluorescence dot plot, gated on R1. DNA area is an estimation of DNA content. Right panel, Data is visualized on DNA content (7-AAD versus PE G99-549 fluorescence dot plot). MOLT-4 cells were treated with TPA (200-1000 ng/ml, 24 hr), fixed, permeabilized and stained with PE mouse anti-underphosphorylated Rb antibody (clone G99-549, Component No. 51-14445X). Cell cycle phase is denoted as G0/G1, S or G2/M.*

Suggested Companion Products

Catalog Number	Name	Size	Clone
559925	7-AAD	2.0 ml	(none)

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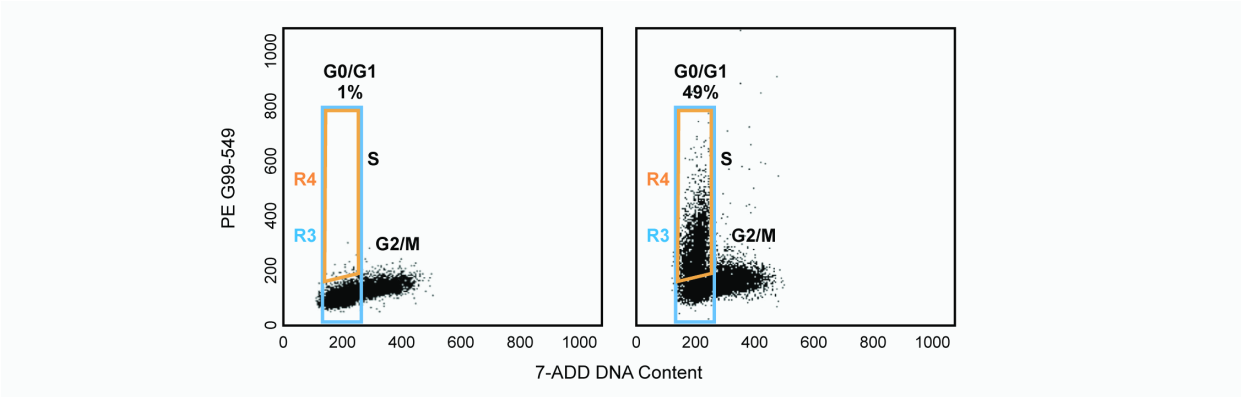

Data Analysis. *Comparison of underphosphorylated Rb expression in proliferating (Left panel; untreated) and cell cycle arrested (Right panel; TPA treated) MOLT-4 cells. The R3 gate (blue) is centered on 200 encompassing the G0/G1 phase of the cell cycle. The frequencies (%) of underphosphorylated Rb positive cells are shown in gate R4 (orange), which has a horizontal cutoff directly above and skirting the isotype control background levels. TPA treatment (200-1000 ng/ml, 24 hr) increased the expression of underphosphorylated Rb in the G0/G1 phase of the cell cycle from 1% to 49% (TPA).*

Product Notices

1. This reagent has been pre-diluted for use at the recommended Volume per Test. We typically use 1 × 10^6 cells in a 100-µl experimental sample (a test).
2. An isotype control should be used at the same concentration as the antibody of interest.
3. Caution: Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
4. Source of all serum proteins is from USDA inspected abattoirs located in the United States.
5. For fluorochrome spectra and suitable instrument settings, please refer to our Multicolor Flow Cytometry web page at www.bdbiosciences.com/colors.
6. Please refer to www.bdbiosciences.com/pharmingen/protocols for technical protocols.

References

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