

Identifyn Standards for Optical Microscopy Methods™Identifyn® Primary Antibody**Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG - (BLR031P)***Cat ID# PA-RR 000012*Identifyn® Secondary Antibodies**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(ab')₂ (H + L)***Cat ID# - SA 000063*

ATM (ataxia-telangiectasia mutated) is a crucial serine/threonine kinase that plays a central role in the DNA damage response (DDR), particularly in response to DNA double-strand breaks (DSBs). ATM's activation and subsequent phosphorylation cascades are essential for detecting DNA damage and initiating repair processes. Here's a detailed overview of how ATM is activated, its targets, and its role in the DDR:

Activation of ATM

In response to DNA damage, particularly DSBs, ATM is activated through a series of events:

Detection of DNA Breaks: ATM can sense DNA breaks by associating with the MRN complex (MRE11, RAD50, and NBS1). This complex recognizes DSBs, leading to ATM's recruitment to the damaged site.

Autophosphorylation: Upon detection of DNA damage, ATM autophosphorylates at serine 1981 (S1981), thereby activating it. This phosphorylation event is critical for ATM's kinase activity and its ability to phosphorylate downstream targets.

Dimer-to-Monomer Transition: In its inactive form, ATM is a dimer. It transitions to an active monomeric state upon activation, allowing it to interact with and phosphorylate various downstream proteins.

Phosphorylation Targets of ATM

Once activated, ATM phosphorylates many proteins involved in the DNA damage response, cell cycle regulation, and apoptosis. Here are some critical targets of ATM:

CHK2: ATM phosphorylates CHK2, a cell cycle checkpoint control kinase. This phosphorylation helps to halt the cell cycle, allowing time for DNA repair.

p53: ATM phosphorylates p53, activating this tumor suppressor, leading to cell cycle arrest or apoptosis if the damage is irreparable.

H2AX: ATM phosphorylates histone H2AX, forming γ-H2AX, a marker of DNA damage. This modification helps signal the presence of DSBs and recruit DNA repair machinery.

BRCA1: ATM's phosphorylation of BRCA1 is critical for homologous recombination, a key DNA repair pathway for DSBs.

NBS1: As part of the MRN complex, NBS1 phosphorylation by ATM enhances the complex's DNA repair functions.

53BP1: ATM phosphorylation of 53BP1 helps recruit this protein to sites of DSBs, thereby guiding repair pathways such as non-homologous end joining (NHEJ).

Role of ATM Phosphorylation in DNA Repair

ATM-mediated phosphorylation plays a central role in coordinating the DNA damage response:

Signal Propagation: Phosphorylation cascades initiated by ATM amplify the signal in response to DNA damage, ensuring an efficient response.

Recruitment of Repair Machinery: Phosphorylation of specific proteins helps recruit DNA repair complexes to the sites of DNA damage.

Cell Cycle Regulation: ATM activation can lead to cell cycle checkpoints, preventing cells with damaged DNA from dividing.

Apoptosis: If the DNA damage is too severe to repair, ATM's activation can lead to apoptosis, preventing the propagation of damaged cells

ATM's phosphorylation in response to DNA damage is crucial for detecting DNA double-strand breaks and coordinating the DNA damage response. By phosphorylating a range of critical proteins, ATM orchestrates a complex signaling network that facilitates DNA repair, cell cycle regulation, and apoptosis, thereby maintaining genomic stability.

Method

Plus Damage -

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, DNA damage was induced by treatment with 2 μ M Etoposide-spiked complete growth media for 12 hours, followed by -20 °C methanol fixation and blocking with [Thermo Fisher Fish Serum Blocker \(cat#37527X3\)](#). The primary antibody, Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG - (BLR031P), was incubated at room temperature for 1 hour at a 1:250 dilution. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(ab')₂ (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5X in PBS.

Minus Damage -

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, then fixed in -20 °C methanol and blocked with [Thermo Fisher Fish Serum Blocker \(cat#37527X3\)](#). The primary antibody, Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG - (BLR031P), was incubated at room temperature for 1 hour at a 1:250 dilution. Cells were washed 3X in PBS.

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(ab')₂ (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using [ProLong™ Diamond Antifade Mountant with DAPI \(cat# P36962\)](#).

Z Stack - (3D) - Panel A, Showing Z-Plane 33 of 73

Z-Stack = 73 Slices (2.16 μ m)

Channels = 1

Scaling (Per Pixel) = 0.059 μ m X 0.059 μ m X 0.030 μ m

Image size (Pixels) = 1341 X 1115

Image Size (Scaled) = 78.85 μ m X 65.56 μ m

Bit Depth = 16 Bit

Image Center Position = X: 97.20 mm, Y: 46.14 mm

Stage Position = X: 97.20 mm, Y: 46.14 mm

ROI Center Offset = X: 0.00 μm , Y: 0.00 μm

Single Plane (2D)

Channels = 1
Scaling (Per Pixel) = 0.059 μm X 0.059 μm
Image size (Pixels) = 1066 X 1066
Image Size (Scaled) = 62.72 μm X 62.72 μm
Bit Depth = 16 Bit
Image Center Position = X: 70.58 mm, Y: 54.20 mm
Stage Position = X: 70.58 mm, Y: 54.20 mm
ROI Center Offset = X: 0.00 μm , Y: 0.00 μm

647 - Plus Etoposide, Upper Panel

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 65 μm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 615
Laser Wavelength = **639 nm @ 0.3%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.5
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.26 μs
Frame Time = 5.63 s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 640 - 732
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 8.1 (3D, Auto)

647 - No Etoposide, Lower Panel - Control

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 65 μm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 615
Laser Wavelength = **639 nm @ 0.3%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.1

Rotation = 0°
Sampling = 1.20
Pixel Time = 0.32 μ s
Frame Time = 3.52 s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 640 - 732
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 6.2 (3D, Auto)

Profile View Display - *Plus DNA Damage, Top*

Profile Definition = Stroke Thickness 1.00, Normal View, Grid Distance 1

Profile View = X and Y axes were set to Auto - **X (Min 0.0 and Max 93.6), Y (Min 0.0 and Max 36113)**

This panel measures Phospho-ATM DNA damage-induced foci/aggregates across an arbitrary line, noting that the foci/aggregate signal reached approximately ~30K A.U. at its maximum.

Measured at the Z-plane, 33 of 73.

Profile View Display - *Minus DNA Damage, Bottom*

Profile Definition = Stroke Thickness 1.00, Normal View, Grid Distance 1

Profile View = X and Y axes were set to Auto - **X (Min 0.0 and Max 67.6), Y (Min 0.0 and Max 42444)**

This panel measures Phospho-ATM in the absence of DNA damage across an arbitrary line, noting that the foci/aggregate signal reached approximately ~4K A.U. at its maximum.

Two-dimensional single plane acquisition.

Histogram Display - *Plus DNA Damage, Top*

Histogram Definition = Normal, Histo Table set to Frequency, Bin Count 256, Bin Size was 256, Lower Threshold 0, Upper Threshold 65535

Histogram View = X and Y axes were set to Auto - X (Min 0.0 and Max 65535), Y (Min 0 and Max 2000000)

Arithmetic Mean intensity = 1,176.434

The histogram was generated from the entire image field to quantify the overall Phospho-ATM response following DNA-damage induction. This measurement provides additional support for the observed increase in signal relative to the control condition, complementing the line-profile analysis presented alongside it.

Histogram Display - Minus DNA Damage, Bottom

Histogram Definition = Normal, Histo Table set to Frequency, Bin Count 256, Bin Size was 256, Lower Threshold 0, Upper Threshold 65535

Histogram View = X and Y axes were set to Auto - X (Min 0.0 and Max 65535), Y (Min 0 and Max 1000000)

Arithmetic Mean intensity = 167.861

The histogram was generated from the entire image field to quantify the overall Phospho-ATM protein in the absence of DNA-damage induction. This measurement provides additional support for the observed increase in signal relative to the control condition, complementing the line-profile analysis presented alongside it.

Summary

Following etoposide-induced DNA damage, Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG - (BLR031P) exhibited substantial enrichment of nuclear signal and reorganization into prominent DNA damage-associated semi-resolved aggregate structures relative to the untreated control. Quantitative fluorescence intensity analysis of the damaged sample demonstrated a maximum foci/aggregate line-profile intensity of 34,393.000 A.U. with an arithmetic mean intensity of 1,176.434 A.U. In contrast, the undamaged control sample demonstrated a substantially lower maximum peak intensity of 4,022.000 A.U. and an arithmetic mean intensity of 167.861 A.U. Collectively, these measurements correspond to an approximate 8.55-fold increase in maximum phospho-ATM-associated fluorescence intensity and an approximate 7.01-fold increase in mean nuclear phospho-ATM-associated signal following etoposide-induced DNA damage, supporting significant phospho-ATM-associated signal enrichment following DNA damage induction.

In undamaged cells, the phospho-ATM signal remained comparatively diffuse and low intensity throughout the nucleus, consistent with basal ATM phosphorylation levels under non-stressed cellular conditions. Due to the minimal fluorescence signal in the untreated control sample, the range indicator was activated during line-profile acquisition to identify and visually delineate nuclear positions within the field of view. Without this positional reference, the nuclei would have been difficult to visually distinguish within the line-profile representation due to the substantially reduced phospho-ATM-associated fluorescence signal.

Importantly, the dramatic contrast observed between untreated and DNA-damaged conditions further reinforces the specificity, sensitivity, and imaging robustness of the Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal antibody across the complete IdentifyN® Stepdown Methodology™ workflow. Consistent localization

behavior, low-background nuclear specificity, strong fluorescence intensity preservation, and reproducible foci-associated signal enrichment were maintained across widefield fluorescence microscopy, widefield structured illumination microscopy (WF-SIM), confocal microscopy, Airyscan super-resolution microscopy, lattice SIM, lattice SIM², and STORM single-molecule localization microscopy datasets. Collectively, the ability to preserve biologically coherent signal architecture and maintain compatibility across progressively demanding imaging modalities strongly supports both the performance of the phospho-ATM antibody itself and the broader validity of the Identifyn® multi-modal validation framework.

Image Correction

The phospho-ATM channel (647) was displayed in grayscale to enhance visual contrast.

Image by J.K. Bennett
Image Analysis and Data Presentation by B.T. Bennett
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