

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***Mouse anti-Human H2Ax Monoclonal, IgG1 - (1H1D9/B7)***Cat ID# - PA-MM 000002*Identifyn® Secondary Antibodies**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(ab')₂ (H + L)***Cat ID# - SA 000063***Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, F(ab')₂ (H + L)***Cat ID# - SA 000019*

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

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.

The second primary antibody, Mouse anti-Human H2Ax Monoclonal, IgG1 - (1H1D9/B7), was then incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, F(ab')₂ (H + L) was incubated for 1 hour at room temperature at a dilution of 1:1000. Cells were washed 5X in PBS and mounted using [ProLong™ Diamond Antifade Mountant with DAPI cat#P36962](#).

Microscope

Zeiss LSM980 Confocal with **NIR Detection**, Axio Observer.Z1/7, using a Plan-Apochromat 63x/1.40 Oil DIC M27, was used with the following parameters for each dye:

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

Z-Stack = 73 Slices (2.16 μ m)

Channels = 2

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 μm X 65.56 μm
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

647 -

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)

488 - Acquired but Not Shown in Data Analysis

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 51 μm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 505
Laser Wavelength = **488 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 = 493
 Emission Wavelength = 517
 Effective NA = 1.4
 Detection Wavelength = 511 - 550
 Imaging Device = GaAsP-Pmt3
 Detector Type = GaAsP-PMT
 Detector Gain = 500 V
 Detector Offset = 0
 Detector Digital Gain = 1.0
 LSM Plus Mode = Filter: 7.7 (3D, Auto)

Profile View Display - Panels A & B

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

Profile View = X and Y axes were set to Auto - X (Min 0.0 and Max 30.9), Y (Min 0.0 and Max 43793)

Measured at Z-plane 33 of 73

Zen Acquisition Info Tab - Panel C

Shown are the acquisition parameters for the 647 channel (phospho-ATM) collected using the 639 nm laser at 0.3% power. Scan zoom, scan speed, and line-averaging settings are provided to ensure the transparency and reproducibility of line-profile measurements.

Summary

Panel A – Single Z-plane (Z = 33 of 73) from a confocal acquisition of Rabbit anti-Human ATM.p (Serine 1981) Recombinant Monoclonal, IgG – (BLR031P), detected using Identifyn® SRM Alexa Fluor™ 647 affinity-purified Goat Anti-Rabbit F(ab')₂ (H + L). A line profile traverses a single nucleus to define the region used for quantitative analysis of phospho-ATM aggregate formation and the distribution of fluorescence intensity within the nuclear compartment.

Panel B – Corresponding line-profile analysis demonstrates three prominent phospho-ATM aggregate measurements of 41,708.00, 38,229.00, and 29,400.00 A.U., respectively, indicating substantial local enrichment of phospho-ATM signal following etoposide-induced DNA damage. These intensity peaks are consistent with recruitment and concentration of activated ATM signaling within DNA damage-associated nuclear foci. At confocal resolution, these structures appear as discrete punctate aggregates; however, higher-resolution imaging modalities demonstrate that such apparent foci likely represent clustered signaling assemblies or multi-component repair-associated microenvironments rather than singular molecular structures.

Biologically, ATM phosphorylation represents a central upstream event in the DNA damage response following induction of DNA double-strand breaks. Activated phospho-ATM coordinates checkpoint signaling, chromatin remodeling, and recruitment of

downstream repair factors, including phosphorylation of H2Ax into γ H2AX within damage-associated chromatin territories. The observed focal enrichment is therefore consistent with localized activation of ATM-mediated DNA damage signaling and organization of repair-associated nuclear domains following genotoxic stress.

Panel C – Zeiss ZEN acquisition settings demonstrate that the phospho-ATM signal was acquired with the 639 nm laser at only 0.3% input power, supporting the conclusion that the measured fluorescent intensities reflect true biological enrichment and aggregate formation rather than detector saturation or excessive excitation conditions.

Image Correction

The phospho-ATM (Serine 1981) channel (647) was pseudocolored white to enhance visual contrast.

Image by J.K. Bennett

Image Analysis and Data Presentation by B.T. Bennett

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