

**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')<sub>2</sub> (H + L)***Cat ID# - SA 000063***Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Donkey Anti-Mouse, F(ab')<sub>2</sub> (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  $\mu$ 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')<sub>2</sub> (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')<sub>2</sub> (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 Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1X Tubelens, was used with the following parameters for each dye:

**Z Stack - (3D) - Panel A, Z-Plane 48 of 95**

**Z-Stack = 95 Slices (2.82  $\mu$ m)**

Channels = 2

Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm

Image Size (Pixels) = 3155 X 2512  
Image Size (Scaled) = 66.61  $\mu\text{m}$  X 53.03  $\mu\text{m}$   
Bit Depth = 16 Bit  
Image Center Position = X: 57.77 mm, Y: 35.27 mm  
Stage Position = X: 57.77 mm, Y: 35.27 mm  
ROI Center Offset = X: 0.00  $\mu\text{m}$ , Y: 0.00  $\mu\text{m}$

**Z Stack - (3D) - Panels B and C, Colocalization Measurement, same Z-Plane.**  
**Z-Stack = 95 Slices (2.82  $\mu\text{m}$ )**

Channels = 2  
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm  
Image Size (Pixels) = 365 X 360  
Image Size (Scaled) = 7.71  $\mu\text{m}$  X 7.60  $\mu\text{m}$   
Bit Depth = 16 Bit  
Image Center Position = X: 57.77 mm, Y: 35.27 mm  
Stage Position = X: 57.77 mm, Y: 35.27 mm  
ROI Center Offset = X: 0.00  $\mu\text{m}$ , Y: 0.00  $\mu\text{m}$

**647 -**

Reflectors = SR HE LBF 405/488/561/642  
Beam Splitter = 405, 488, 561, 642  
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642  
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800  
Contrast Method = Fluorescence  
Illumination Wavelength = 640  
Channel Name = T1-Axiocam 820  
Excitation Wavelength = 640  
Emission Wavelength = 729  
Effective NA = 1.4  
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800  
Imaging Device = Axiocam 820  
Camera Adapter = 1X  
Exposure Time = 100 ms  
Depth of Focus = 1.13  $\mu\text{m}$   
Binning Mode = 2,2  
Laser Wavelength = 640 nm @ 1.0%  
Frame Time = 1.33 s  
Scan Direction = Unidirectional  
Grating = 36.5  $\mu\text{m}$  grating

**488 -**

Reflectors = SR HE LBF 405/488/561/642  
Beam Splitter = 405, 488, 561, 642  
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642  
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800  
Contrast Method = Fluorescence  
Illumination Wavelength = 488  
Channel Name = T2-Axiocam 820  
Excitation Wavelength = 488  
Emission Wavelength = 525

Effective NA = 1.4  
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800  
Imaging Device = AxioCam 820  
Camera Adapter = 1X  
Exposure Time = 120 ms  
Depth of Focus = 0.81  $\mu\text{m}$   
Binning Mode = 2,2  
Laser Wavelength = 488 nm @ 1.00%  
Frame Time = 1.59 s  
Scan Direction = Unidirectional  
Grating = 27.5  $\mu\text{m}$  grating

### **Post Processing - SIM Processing**

Processing Dimension = 3D  
Result Sampling = 4  
Process Sampling = N/A  
Channels = 2  
Algorithm = SIM  
SIM Settings = Standard  
Auto Sharpness = On  
Sharpness = 11.0018 (647), 11.2478 (488)  
Fitting = Fast Fit  
Normalization = Auto

### **2-Dimensional View - Panel A**

Lattice SIM optical section acquired at Z-plane 48 of 95 showing 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')<sub>2</sub> (H + L) (red), and Mouse anti-Human H2Ax Monoclonal, IgG1 – (1H1D9/B7), detected using Identifyn® SRM Alexa Fluor™ 488 affinity-purified Donkey Anti-Mouse F(ab')<sub>2</sub> (H + L) (green). A white box denotes the region of interest (ROI) selected for colocalization analysis in Panels B and C.

### **Colocalization View - Panels B and C**

Horizontal Axis - AF647, Threshold 5707, Range Auto  
Vertical Axis - AF488, Threshold 15509, Range Auto

Image Measurement = Entire Field of Panel B

Colocalization analysis of phospho-ATM (X-axis) and H2Ax (Y-axis) was performed across the entire image acquisition.

Phospho-ATM shows a lack of colocalization with H2Ax, consistent with the known biology of phospho-ATM signaling and total H2Ax distribution.

### **Summary**

Panels B and C – Enlarged regions of interest (ROIs) from the same Lattice SIM acquisition shown in Panel A, demonstrating the spatial relationship between phospho-ATM and

H2Ax following etoposide-induced DNA damage. 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')<sub>2</sub> (H + L) (red), and Mouse anti-Human H2Ax Monoclonal, IgG1 – (1H1D9/B7), detected using Identifyn® SRM Alexa Fluor™ 488 affinity-purified Donkey Anti-Mouse F(ab')<sub>2</sub> (H + L) (green), demonstrate minimal direct signal overlap throughout the analyzed nuclear volume despite robust phospho-ATM aggregate formation and strong DNA damage-associated response.

At Lattice SIM resolution (~60–80 nm XY), the apparent broad colocalization observed in lower-resolution widefield datasets becomes substantially resolved into spatially distinct signaling territories. This finding is biologically consistent with the established role of activated phospho-ATM as an upstream DNA damage response kinase that phosphorylates H2Ax into γH2AX following DNA double-strand break induction. Because the present dataset measures total H2Ax rather than γH2AX specifically, strong molecular colocalization with phospho-ATM is not expected. Instead, phospho-ATM localizes to active checkpoint-associated signaling domains and DNA damage-responsive chromatin territories, while total H2Ax remains broadly distributed throughout the nucleosomal landscape of the nucleus.

Importantly, despite the relative absence of direct overlap, phospho-ATM exhibits a pronounced intranuclear response to etoposide treatment, forming concentrated punctate aggregates consistent with localized ATM activation and recruitment to DNA-damage-associated signaling microenvironments. The progressive loss of apparent colocalization with increasing spatial resolution strongly supports the specificity of the Identifyn® phospho-ATM antibody, as the observed localization pattern closely mirrors the known biology of ATM activation rather than nonspecific chromatin-wide staining.

Collectively, these findings demonstrate that while diffraction-limited imaging modalities can suggest strong colocalization between phospho-ATM and total H2Ax, super-resolution Lattice SIM imaging reveals that these signals largely occupy adjacent or overlapping nuclear response territories rather than identical molecular structures. This distinction underscores the importance of increasing spatial resolution within the Stepdown Methodology™ when interpreting DNA damage-associated signaling relationships and assessing the true molecular organization of repair-associated chromatin domains.

### Image Correction

NONE

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Image Analysis and Data Presentation by B.T. Bennett

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