

**Identifyn Standards for Optical Microscopy Methods™**Identifyn® Primary Antibody**Rabbit anti-Human ATR.p (Threonine 1989) Recombinant Monoclonal, IgG - (BLR032P)***Cat ID# - 000013*Identifyn® Secondary Antibody**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L)***Cat ID# - SA 000060*

ATR (Ataxia Telangiectasia and Rad3-related) is a critical serine/threonine kinase that plays a central role in the cellular response to DNA damage, particularly in response to replication stress and single-strand breaks (SSBs). Unlike ATM (Ataxia Telangiectasia Mutated), which is primarily activated by double-strand breaks, ATR is activated by persistent single-stranded DNA (ssDNA) regions that arise during DNA replication or repair processes.

**Overview of ATR Activation**

ATR is activated when extensive ssDNA accumulates, often resulting from stalled replication forks or other forms of replication stress. This activation is usually mediated by RPA (Replication Protein A), which binds to ssDNA and recruits ATR and its key activator, ATRIP (ATR Interacting Protein).

**Recruitment to Sites of Damage:** RPA-coated ssDNA signals DNA damage or replication stress. ATR, with ATRIP, is recruited to these sites, activating ATR's kinase activity.

**Interaction with the 9-1-1 Complex:** ATR activation is further facilitated by the 9-1-1 complex (comprising RAD9, RAD1, and HUS1), which is loaded onto DNA by RAD17-RFC at sites of DNA damage. This complex interacts with TOPBP1 (Topoisomerase II Binding Protein 1), a key activator of ATR, thereby fully activating ATR kinase activity.

**ATR's Role in Single-Strand Break Repair**

ATR plays a vital role in facilitating and coordinating the repair of single-strand breaks through various mechanisms:

**Checkpoint Activation:** ATR is critical for activating checkpoint signaling pathways, notably by phosphorylating CHK1. This leads to cell cycle arrest or delay, allowing time for DNA repair before cell division continues. This checkpoint activation is crucial in preventing cells from progressing with unrepaired single-strand breaks.

**Coordination of Repair Pathways:** ATR activation helps coordinate DNA repair processes by recruiting repair proteins and integrating signals across various pathways. This coordination ensures that repair proteins involved in base excision repair (BER), homologous recombination, and other SSB-related pathways are recruited and activated as needed.

**Stabilization of Replication Forks:** ATR contributes to the stabilization of stalled replication forks, preventing the collapse into double-strand breaks. This is particularly important in preventing the progression of single-strand breaks into more severe DNA damage.

**ATR in DNA Damage Response**

Beyond its direct role in facilitating single-strand break repair, ATR has broader implications in the DNA damage response:

**Genomic Stability:** By ensuring proper checkpoint activation and coordinating DNA repair, ATR helps maintain genomic stability, reducing the risk of mutations and chromosomal abnormalities.

**Response to Replication Stress:** ATR is particularly critical in responding to replication stress, which can result in single-strand breaks due to replication fork stalling or collapse. ATR's activation in these contexts ensures that DNA repair processes are promptly initiated.

ATR is a central player in responding to single-strand breaks and replication stress, activating checkpoint pathways, coordinating DNA repair, and stabilizing replication forks. Through these functions, ATR significantly contributes to maintaining genomic stability and ensuring that cells do not progress with unrepaired DNA damage.

## Method Plus Damage -

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2  $\mu$ M etoposide-spiked complete growth media for 24 hours, followed by -20 °C methanol fixation and blocked with [Thermo Fisher Fish Serum Blocker cat#37527X3](#). The primary antibody, Rabbit anti-Human ATR.p (Threonine 1989) Recombinant Monoclonal, IgG - (BLR032P), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5 times in 1X PBS and mounted using [ProLong™ Diamond Antifade Mountant with DAPI \(cat# P36962\)](#).

## 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 ATR.p (Threonine 1989) Recombinant Monoclonal, IgG - (BLR032P), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3 times in 1X PBS. Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 5 times in 1X 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) - 2D at Z-Plane 77 of 151, Panel A, from Confocal Data Set**  
**Z-Stack = 151 Slices (4.5  $\mu$ m)**

Channels = 1  
Scaling (Per Pixel) = 0.059  $\mu$ m X 0.059  $\mu$ m X 0.030  $\mu$ m  
Image Size (Pixels) = 1407 X 1407  
Image Size (Scaled) = 82.77  $\mu$ m X 82.77  $\mu$ m  
Bit Depth = 16 Bit  
Image Center Position = X: 91.51 mm, Y: 49.99 mm

Stage Position = X: 91.51 mm, Y: 49.99 mm  
ROI Center Offset = X: 0.00  $\mu$ m, Y: 0.00  $\mu$ m

### **Single Plane (2D) - Control**

Channels = 1  
Scaling (Per Pixel) = 0.059  $\mu$ m X 0.059  $\mu$ m  
Image Size (Pixels) = 2266 X 2266  
Image Size (Scaled) = 133.28  $\mu$ m X 133.28  $\mu$ m  
Bit Depth = 16 Bit  
Image Center Position = X: 91.24 mm, Y: 48.00 mm  
Stage Position = X: 91.24 mm, Y: 48.00 mm  
ROI Center Offset = X: 0.00  $\mu$ m, Y: 0.00  $\mu$ m

### **647 - Plus Damage, from Confocal Data Set**

Reflector = None  
Contrast Method = Fluorescence  
Pinhole = 1.00 AU / 64  $\mu$ m  
LMS MBS = MBS 488/639 & 405/730  
LMS SBS = SBS SP 615  
Laser Wavelength = **639 nm @ 1.8%**  
Laser Blanking = Enabled  
Scan Mode = Frame  
Scan Zoom = 1.6  
Rotation = 0°  
Sampling = 1.20  
Pixel Time = 0.28  $\mu$ s  
Frame Time = 5.28 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 = 647 - 700  
Imaging Device = GaAsP-Pmt3  
Detector Type = GaAsP-PMT  
Detector Gain = 550 V  
Detector Offset = 0  
Detector Digital Gain = 1.0  
LSM Plus Mode = Filter: 8.6 (3D, Auto)

### **647 - Minus Damage - noting the same laser input power and conditions**

Reflector = None  
Contrast Method = Fluorescence  
Pinhole = 1.00 AU / 64  $\mu$ m  
LMS MBS = MBS 488/639 & 405/730  
LMS SBS = SBS SP 615  
Laser Wavelength = **639 nm @ 1.8%**

Laser Blanking = Enabled  
 Scan Mode = Frame  
 Scan Zoom = 1.0  
 Rotation = 0°  
 Sampling = 1.20  
 Pixel Time = 0.23  $\mu$ s  
 Frame Time = 11.26 s  
 LSM Scan Speed = 9  
 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 = 550 V  
 Detector Offset = 0  
 Detector Digital Gain = 1.0  
 LSM Plus Mode = Filter: 8.2 (3D, Auto)

### **Profile View Display** - *Plus DNA Damage, Top*

Profile Definition = Stroke Thickness 1.5, Normal View, Grid Distance 1

Profile View = X and Y axes were set to Auto - **X (Min 0.0 and Max 32.80), Y (Min 0.0 and Max 6383)**

This panel measures phospho-ATR DNA damage-induced foci/aggregates across an arbitrary line, noting that the foci/aggregate signal reached approximately ~7K A.U. at its maximum.

Measured at the Z-plane 77 of 151.

### **Profile View Display** - *Minus DNA Damage, Bottom*

Profile Definition = Stroke Thickness 1.5, Normal View, Grid Distance 1

Profile View = X and Y axes were set to Auto - **X (Min 0.0 and Max 106.7), Y (Min 0.0 and Max 4653)**

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

### **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 0.0 and Max 100000)

### **Arithmetic Mean intensity = 702.066**

The histogram was generated from the yellow-boxed region to quantify the overall phospho-ATR protein response within a defined nuclear area 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 0.0 and Max 200000)

### **Arithmetic Mean intensity = 471.197**

The histogram was generated from the yellow-boxed region to quantify the overall phospho-ATR protein within a defined nuclear area 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.

### **Control Conclusion –**

In the absence of DNA damage, ATR.p (647) exhibited a predominantly low-intensity, diffuse nuclear distribution with minimal focal organization, consistent with basal ATR signaling activity under unstressed cellular conditions. Following etoposide-induced DNA damage, ATR.p exhibited a pronounced increase in fluorescence intensity, along with extensive reorganization into discrete intranuclear foci and higher-order, aggregate-like signaling territories. Quantitative profile-view analysis revealed peak signal intensities approaching ~7K A.U. in the DNA-damaged condition compared to ~4K A.U. in the untreated control. In contrast, histogram analysis showed a substantial increase in the arithmetic mean fluorescence intensity after DNA damage induction.

The observed transition from diffuse low-intensity nuclear staining to highly organized punctate ATR.p-associated signaling architectures is strongly consistent with canonical ATR activation in response to replication stress and DNA damage. These findings support recruitment of activated ATR signaling complexes to RPA-coated single-stranded DNA regions and engagement of downstream DNA damage response (DDR) checkpoint pathways. Importantly, the marked increase in signal intensity, focal organization, and aggregate complexity observed under DNA-damaged conditions remained highly consistent across the sequential workflows of widefield, WF-SIM, confocal, Airyscan, Lattice SIM, SIM<sup>2</sup>, and STORM imaging, further supporting the biological specificity, reproducibility, and structural coherence of the ATR.p-associated DNA damage response observed throughout the Identifyn® Stepdown Methodology™ progression.

Collectively, these findings support the performance and biological specificity of Rabbit anti-Human ATR.p (Threonine 1989) Recombinant Monoclonal, IgG - (BLR032P) across multiple orthogonal imaging modalities and resolution scales. The antibody demonstrated robust signal generation, biologically coherent DNA damage-dependent redistribution, strong preservation of subcellular localization characteristics, and reproducible structural behavior throughout progressively higher-resolution imaging workflows. The combined control, quantitative, and multimodal imaging datasets support the utility of BLR032P as a high-performance research reagent for investigating ATR-associated replication stress signaling and DNA damage response biology under both conventional fluorescence and advanced super-resolution imaging conditions.

**Image Corrections**

NONE

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Image by J.K. Bennett

Image Analysis and Data Presentation by B.T. Bennett

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