Identifyn Standards for Optical Microscopy Methods™
Identifyn® Primary Antibody

Rabbit anti-Human ATR.p (Threonine 1989) Recombinant Monoclonal, IgG - (BLR032P)
Cat ID# - 000013

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG - (BLR014M)
Cat ID# - DC 000003

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

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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

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced by treatment with 2 µM etoposide–spiked complete growth medium 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 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.

The second primary antibody, Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG - (BLR014M) was incubated for 1 hour at room temperature at a dilution of 1:250. 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, Panels A, B, and C*
Z-Stack = 151 Slices (4.5 µm)

Channels = 3
Scaling (Per Pixel) = 0.059 µm X 0.059 µm X 0.030 µm
Image Size (Pixels) = 1407 X 1407
Image Size (Scaled) = 82.77 µm X 82.77 µm
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 µm, Y: 0.00 µm

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647 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 64 µm
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 µs
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)

488 -

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 @ 0.2%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.6
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.28 µs
Frame Time = 5.28 s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4

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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: 7.2 (3D, Auto)

DAPI -
Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 43 µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = Mirror
Laser Wavelength = **405 nm @ 0.2%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.6
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.28 µs
Frame Time = 5.28 s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 435 - 470
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 6.8 (3D, Auto)

Split View - *Panel A*

The top-left panel shows Rabbit anti-Human ATR.p (Threonine 1989) Recombinant Monoclonal, IgG - (BLR032P), visualized using **I**dentifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L) secondary antibody. The top-right panel shows Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG - (BLR014M). The lower-left panel shows DAPI staining, and the bottom-right panel shows the three-channel merged image.

Profile View Display - *Panels B & C*

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 32.8), Y (Min 0 and Max 14594)

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This panel quantifies colocalized foci, or resolution-limited aggregates, of phospho-ATR and γH2AX following etoposide-induced DNA damage. Line profile analysis reveals strong spatial concordance and comparable signal intensity distributions, supporting strong coordinated recruitment and overlapping damage-associated signaling territories.

Zen Acquisition Info Tab - *Panel D*

Shown are the acquisition parameters for the 647 channel (phospho-ATR) and 488 channel (γH2AX), collected using the 639 nm laser at 1.8% power and the 488 nm laser at 0.2% power, respectively. Scan zoom, scan speed, and line-averaging settings are provided to ensure the transparency and reproducibility of line-profile measurements.

Summary

Panel A shows Rabbit anti-Human ATR.p (Threonine 1989) Recombinant Monoclonal, IgG - (BLR032P), visualized using Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Donkey Anti-Rabbit, IgG (H + L), together with Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG - (BLR014M), and DAPI nuclear counterstaining following etoposide-induced DNA damage. Relative to both conventional widefield and Apotome SIM imaging, confocal microscopy provides improved optical sectioning, enhanced axial discrimination, and reduced out-of-focus fluorescence contribution, enabling more refined visualization of ATR.p-associated DNA damage response territories throughout the nuclear volume. ATR.p demonstrated strong punctate intranuclear redistribution and damage-associated signaling structures that spatially overlapped with γH2Ax-enriched nuclear regions, consistent with coordinated replication-stress and DNA damage checkpoint activation.

Panels B and C present line-profile analysis across resolution-limited ATR.p and γH2Ax-associated foci, demonstrating strong spatial concordance and comparable fluorescence intensity distributions throughout damaged nuclei. Although confocal microscopy substantially improves volumetric and focal-plane discrimination relative to widefield and WF-SIM imaging, the observed overlap remains constrained by diffraction-limited resolution, preventing definitive nanoscale assessment of individual repair complexes or molecular organization at DNA break sites. Accordingly, the observed signal relationship should be interpreted as coordinated recruitment and overlapping damage-associated signaling territories rather than definitive molecular colocalization.

Importantly, this dataset represents the next refinement stage within the Identifyn® Stepdown Methodology™, progressively improving structural interpretation and signal discrimination prior to higher-resolution Airyscan, Lattice SIM, SIM^2, and STORM interrogation workflows. The transition from widefield to WF-SIM and now to confocal imaging demonstrates how incremental improvements in optical sectioning and spatial resolution progressively refine the apparent overlap between ATR.p and γH2Ax while preserving biologically coherent DNA damage response behavior across modalities.

Image Correction

For Panels B and C, which represent the line-profile analysis and the accompanying image identifying the analyzed region, the ATR.p (647) signal pair was pseudocolored white to enhance visual contrast and improve assessment of fluorescence intensity distribution, focal organization, and spatial relationships among signals across the analyzed nuclear region.

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

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