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 67 of 131, Panel A*
Z-Stack = 131 Slices (3.9 µm)

Channels = 3
Scaling (Per Pixel) = 0.035 nm X 0.035 nm X 0.030 nm
Image Size (Pixels) = 731 X 676
Image Size (Scaled) = 25.80 µm X 23.86 µm
Bit Depth = 16 Bit
Image Center Position = X: 95.01 mm, Y: 46.20 mm
Stage Position = X: 95.01 mm, Y: 46.20 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

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Z Stack - (3D) - *3D Volume Projection, Panel B*
Z-Stack = 131 Slices (3.9 µm)

Channels = 3
Scaling (Per Pixel) = 0.035 nm X 0.035 nm X 0.030 nm
Image Size (Pixels) = 191 X 186
Image Size (Scaled) = 6.74 µm X 6.56 µm
Bit Depth = 16 Bit
Image Center Position = X: 95.01 mm, Y: 46.20 mm
Stage Position = X: 95.01 mm, Y: 46.20 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

647 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 328 µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS LP 615
Laser Wavelength = **639 nm @ 2.60%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.0
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.10 µs
Frame Time = 18.77 s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 643 - 735
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
Airyscan Mode = SuperResolution 10.0 (3D, Auto)

488 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 250 µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 505
Laser Wavelength = **488 nm @ 0.3%**
Laser Blanking = Enabled

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Scan Mode = Frame
Scan Zoom = 2.0
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.10 µs
Frame Time = 18.77 s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Detection Wavelength = 499 - 548
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
Airyscan Mode = SuperResolution 10.0 (3D, Auto)

DAPI - *Not Shown in Data Analysis, but Collected in Image Acquisition*
Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00 AU / 214 µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 505
Laser Wavelength = **405 nm @ 0.3%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.0
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.10 µs
Frame Time = 18.77 s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 422 - 477
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 750 V
Detector Offset = 0
Detector Digital Gain = 1.0
Airyscan Mode = SuperResolution 8.5 (3D, Auto)

2-Dimensional View- *Panel A*

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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 and Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG - (BLR014M). A Region of Interest is denoted by a white box to be further examined in the 3-Dimensional Volume Projection in Panel B.

3D Image Processing - *Panel B*

3D Volume Projection = Precise
Appearance = Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

STORM - *Panel C*

STORM imaging from the same product dataset is shown in Panel C to demonstrate the remaining resolution limitations of Airyscan microscopy. While Airyscan provides improved sub-diffraction localization and structural discrimination relative to conventional confocal imaging, STORM imaging further resolves nanoscale organizational features and underlying damage-associated signaling architectures that are not fully distinguishable in the Airyscan dataset.

Summary

Panel A shows a single Airyscan optical section (Z-plane 67 of 131) of 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), following etoposide-induced DNA damage. Relative to conventional widefield, WF-SIM, and confocal microscopy, Airyscan imaging provides improved sub-diffraction structural discrimination, enhanced signal localization, and increased separation of damage-associated signaling territories within the nuclear compartment. ATR.p demonstrated punctate intranuclear redistribution and spatial overlap with γH2Ax-enriched DNA damage regions, consistent with coordinated replication-stress and DNA damage checkpoint activation.

Panel B presents a three-dimensional volume reconstruction of the region of interest identified in Panel A, further illustrating the spatial organization and volumetric distribution of ATR.p and γH2Ax-associated signaling structures throughout the nuclear environment. Compared to preceding diffraction-limited modalities, Airyscan imaging reveals improved structural separation of resolution-limited aggregates and increased visualization of sub-diffraction organizational features; however, definitive nanoscale molecular architecture and precise repair-complex organization remain incompletely resolved at this imaging stage. Accordingly, the observed overlap should continue to be interpreted as coordinated recruitment and overlapping DNA damage-associated signaling territories rather than definitive molecular colocalization.

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Importantly, this dataset represents the next stage of super-resolution refinement within the Identifyn® Stepdown Methodology™, demonstrating the progressive improvement in structural interpretation that accompanies increasing spatial resolution. The transition from widefield to WF-SIM, confocal, and now Airyscan imaging illustrates how incremental improvements in optical sectioning and sub-diffraction signal discrimination progressively refine ATR.p and γH2Ax-associated damage architectures prior to higher-order interrogation by Lattice SIM, SIM^2, and STORM imaging workflows.

Image Correction

NONE

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

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