

Identifyfyn Standards for Optical Microscopy Methods™Identifyfyn® Primary Antibody**Rabbit anti-Human ATR.p (Threonine 1989) Recombinant Monoclonal, IgG - (BLR032P)***Cat ID# - 000013***Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyfyn® SRM Alexa Fluor™ 488, IgG - (BLR014M)***Cat ID# - DC 000003*Identifyfyn® Secondary Antibody**Identifyfyn® 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 through phosphorylation of 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

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 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 γ H2Ax (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 Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, AxioCam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:

Single Plane (2D)

Channels = 3

Scaling (Per Pixel) = 0.110 μ m X 0.110 μ m

Image Size (Pixels) = 834 X 689

Image Size (Scaled) = 91.34 μ m X 75.46 μ m

Bit Depth = 14 Bit

Image Center Position = X: 0.00 μ m, Y: 0.00 μ m

ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

647 -

Reflector = 50 Cy 5

Beam Splitter = 660
 Filter Excitation Wavelength = 625 - 655
 Filter Emission Wavelength = 665 - 715
 Contrast Method = Fluorescence
 Light Source = X-Cite Xylis Lamp Intensity @ 30.00%
 Exposure Time = 300 ms
 Excitation Wavelength = 653
 Emission Wavelength = 668
 Effective NA = 1.4
 Imaging Device = Axiocam 705
 Camera Adapter = 1X
 Depth of Focus = 1.03 μm
 Binning Mode = 2,2

488 -

Reflector = 38 eGFP
 Beam Splitter = 495
 Filter Excitation Wavelength = 450 - 490
 Filter Emission Wavelength = 500 - 550
 Contrast Method = Fluorescence
 Light Source = X-Cite Xylis Lamp Intensity @ 10.00%
 Exposure Time = 100 ms
 Excitation Wavelength = 493
 Emission Wavelength = 517
 Effective NA = 1.4
 Imaging Device = Axiocam 705
 Camera Adapter = 1X
 Depth of Focus = 0.80 μm
 Binning Mode = 2,2

DAPI -

Reflector = 49 DAPI
 Beam Splitter = 395
 Filter Excitation Wavelength = 335 - 383
 Filter Emission Wavelength = 420 - 470
 Contrast Method = Fluorescence
 Light Source = X-Cite Xylis Lamp Intensity @ 5.0%
 Exposure Time = 15 ms
 Excitation Wavelength = 353
 Emission Wavelength = 465
 Effective NA = 1.4
 Imaging Device = Axiocam 705
 Camera Adapter = 1X
 Depth of Focus = 0.72 μm
 Binning Mode = 2,2

Split View - Panel A

The top-left panel 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) 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.

Colocalization View - Panels B and C

Vertical Axis - AF647, Threshold 196, Range Auto
Horizontal Axis - AF488, Threshold 174, Range Auto

Image Measurement = Region of Interest from panel A, measuring the two nuclei shown.

Colocalization analysis between phospho-ATR (Threonine 1989) and γH2AX (Serine 139) (Alexa Fluor 488 direct conjugate) was conducted using a defined ROI containing two nuclei from Panel A. The analysis demonstrates strong spatial colocalization between phospho-ATR and γH2AX foci, supporting ATR activation and localization at DNA damage sites within the nucleus.

Summary

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), alongside Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 488, IgG - (BLR014M), and DAPI nuclear counterstaining. Under widefield fluorescence microscopy conditions, strong nuclear-associated ATR.p signal and discrete γH2Ax-associated DNA damage foci were observed following etoposide-induced replication stress, supporting robust target-specific staining and biologically consistent ATR activation patterns. Apparent colocalization between ATR.p and γH2Ax foci was observed throughout damaged nuclei; however, due to the diffraction-limited resolution of widefield microscopy, the precise nanoscale spatial relationship between ATR.p signaling domains and γH2Ax-associated chromatin structures cannot be definitively resolved at this imaging stage. This dataset therefore serves as the initial step in the Identifyn™ Stepdown Methodology™, establishing biologically consistent damage-associated signal overlap prior to higher-resolution evaluation using confocal, Airyscan, Lattice SIM, SIM², and STORM imaging workflows.

Image Correction

NONE

Image by E.F.Simon
Image Analysis and Data Presentation by B.T. Bennett

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