**Identifyn Standards for Optical Microscopy Methods™**

Identifyn® Primary Antibody

**Mouse anti-Human, ATRIP Monoclonal, IgG2b - (4H6H4/G3)**
*Cat ID# - PA-MM 000003*

Identifyn® Secondary Antibodies

**Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific**
*Cat ID# - SA 000056*

ATRIP (ATR Interacting Protein) is a key partner and co-activator of ATR (Ataxia Telangiectasia and Rad3-related), a crucial kinase in the DNA damage response, particularly in response to replication stress and single-strand breaks (SSBs). ATRIP plays a pivotal role in recognizing and binding to single-stranded DNA (ssDNA), facilitating ATR activation at DNA damage sites.

**Overview of ATRIP**

ATRIP is the regulatory subunit that associates with ATR and directs it to sites of DNA damage. It contains a domain that allows it to bind to Replication Protein A (RPA), a complex that binds and stabilizes single-stranded DNA. This interaction with RPA-coated ssDNA is crucial for ATRIP's ability to guide ATR to regions where the DNA damage response is needed.

**Role of ATRIP in Single-Strand Break Repair**

ATRIP plays a central role in the activation of ATR, which is critical to the cellular response to single-strand breaks and replication stress:

**Recognition of Single-Stranded DNA:** ATRIP, through its interaction with RPA, identifies regions of ssDNA. These regions are commonly found at stalled replication forks, resected DNA ends, or sites of single-strand breaks.

**Recruitment of ATR:** Once ATRIP binds to RPA-coated ssDNA, ATR is recruited to the site. This recruitment is crucial for ATR activation, leading to a cascade of events that coordinate the DNA damage response and facilitate DNA repair.

**Activation of Checkpoints:** By facilitating ATR activation, ATRIP indirectly contributes to checkpoint signaling. This leads to CHK1 phosphorylation, which plays a role in cell cycle arrest, providing time for the repair of single-strand breaks before the cell cycle progresses.

**ATRIP's Broader Role in the DNA Damage Response**

Beyond its specific role in single-strand break repair, ATRIP's interaction with ATR has broader implications for the DNA damage response:

**Coordination of DNA Repair:** The ATR/ATRIP complex orchestrates the recruitment of DNA repair proteins. This coordination ensures that repair processes like base excision repair (BER) and homologous recombination can proceed efficiently at sites of single-strand breaks.

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**Response to Replication Stress:** ATRIP is crucial in responding to replication stress, which can lead to single-strand breaks or stalled replication forks. By facilitating ATR activation, ATRIP helps stabilize replication forks and prevent more severe DNA damage, such as double-strand breaks.

ATRIP is a critical regulatory protein that guides ATR to sites of single-strand breaks and other forms of replication stress. By recognizing RPA-coated ssDNA and recruiting ATR, ATRIP plays a pivotal role in activating the DNA damage response, leading to checkpoint activation and coordination of DNA repair processes. Its role in stabilizing replication forks and promoting genomic stability underscores its importance in maintaining genome integrity.

## Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency, followed by -20°C methanol fixation and blocked with Thermo Fisher, Fish Serum Blocker cat#37527X3. The primary antibody, Mouse anti-Human, ATRIP Monoclonal, IgG2b - (4H6H4/G3), 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 Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific was incubated at room temperature for 1 hour at a dilution of 1:1000. Cells were washed 3 times in PBS.

The second primary antibody, Actin Recombinant Rabbit Monoclonal Antibody (JF47-01), 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-Rabbit, IgG (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. *(Not Shown, but in sample)*

## Microscope

Zeiss LSM 980 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:

**Single Plane (2D) - Z-Plane 74 of 141** - *Plus DNA Damage, Top*

Channels = 1 (647)
Scaling (Per Pixel) = 0.059 µm X 0.059 µm
Image Size (Pixels) = 1121 X 1121
Image Size (Scaled) = 65.93 µm X 65.93 µm
Bit Depth = 16 Bit
Image Center Position = X: 83.24 mm, Y: 49.90 mm
Stage Position = X: 83.24 mm, Y: 49.90 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

**Single Plane (2D) - Single Plane**- *Minus DNA Damage, Bottom*

Channels = 1 (647)
Scaling (Per Pixel) = 0.059 µm X 0.059 µm
Image Size (Pixels) = 1612 X 1612
Image Size (Scaled) = 94.79 µm X 94.79 µm
Bit Depth = 16 Bit

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Image Center Position = X: 82.17 mm, Y: 51.27 mm
Stage Position = X: 82.17 mm, Y: 51.27 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

**647 -** Panel A - Etoposide *Induced DNA Damage*

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / 65µm
LMS MBS = MBS 488/561 & 405/730
LMS SBS = SBS SP 615
**Laser Wavelength = 639nm @0.7%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.0
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.30µs
Frame Time = 3.69s
LSM Scan Speed = 12
Scan Direction = Bidirectional
**Line Step = 1**
**Averaging - 8**
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 640 - 668
**Imaging Device = GaAsP-Pmt3**
**Detector Type = GaAsP-PMT**
**Detector Gain = 500 V**
Detector Offset = 0
**Detector Digital Gain = 1.0**
LSM Plus Mode = Filter: 7.8 (3D, Auto)

**647 -** *Panel B - NO Induced DNA Damage*

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / 66µm
LMS MBS = MBS 488/561 & 405/730
LMS SBS = Mirror
**Laser Wavelength = 639nm @0.7%**
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.4
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.24µs
Frame Time = 6.04s
LSM Scan Speed = 11
Scan Direction = Bidirectional
**Line Step = 1**

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**Averaging - 8**
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 640 - 668
**Imaging Device = GaAsP-Pmt3**
**Detector Type = GaAsP-PMT**
**Detector Gain = 500 V**
Detector Offset = 0
**Detector Digital Gain = 1.0**
LSM Plus Mode = Filter: 7.4 (3D, Auto)

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

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 42.3), Y (Min 0 and Max 14933)**

This panel measures ATRIP DNA damage-induced foci/aggregates across an arbitrary line, noting that the foci/aggregate signal reached approximately ~15K A.U. at its maximum.

Measured at Z-plane 71 of 141

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

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 32.2), Y (Min 0 and Max 5879)**

This panel measures ATRIP in the absence of DNA damage across an arbitrary line, noting that the foci/aggregate signal reached approximately ~6K A.U. at its maximum.

Measured at Z-plane 71 of 141

**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 and Max 5000)

The histogram was generated from the yellow-boxed region shown to quantify the overall ATRIP protein response within a defined nuclear area following DNA-damage induction. This measurement provides additional support for the observed signal increase relative to the control condition, complementing the line-profile analysis presented alongside it.

**Histogram Display** - *Minus DNA Damage, Bottom*

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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 and Max 5000)

The histogram was generated from the yellow-boxed region shown to quantify the overall ATRIP protein within a defined nuclear area in the absence of DNA-damage induction. This measurement provides additional support for the observed signal increase relative to the control condition, complementing the line-profile analysis presented alongside it.

**Control Conclusion -**

In undamaged cells, ATRIP exhibits a diffuse nuclear distribution. Upon induction of DNA damage, ATRIP is observed in discrete nuclear foci or aggregates, accompanied by an approximately threefold increase in fluorescence signal intensity, consistent with elevated nuclear ATRIP levels.

**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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