Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human BAP1.p Polyclonal, IgG
Cat ID# - PA-RP 000005

Identifyn® Secondary Antibodies

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(ab')$_2$ (H + L)
Cat ID# - SA 000063

Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific
Cat ID# - SA 000012

BAP1 (BRCA1-associated protein 1) is primarily recognized as a nuclear deubiquitinase (DUB) that plays a crucial role in regulating chromatin structure, transcription, DNA repair, and cell cycle progression. While extensively studied in the context of homologous recombination (HR), BAP1 is increasingly acknowledged for its important role in base excision repair (BER).

Phosphorylation of BAP1 (BAP1.p) occurs at several sites, each associated with distinct regulatory functions. The most well-characterized site is Serine 592 (S592), which is primarily phosphorylated by ATM or ATR, with ATR being the more likely kinase, especially in response to DNA damage such as single-strand breaks (SSBs), double-strand breaks (DSBs), and replication stress. Another phosphorylation site, Serine 718 (S718), is also targeted by ATM or ATR. However, its functional role is less well-defined. It is believed to contribute to DNA damage response signaling and may help regulate chromatin remodeling activities. Threonine 599 (T599) has been identified as a potential ATM target based on limited phosphoproteomic data, although its exact function remains unclear. Lastly, Serine 2 (S2) is phosphorylated by casein kinase 2 (CK2) in contexts not related to DNA damage, appearing to play a role in stabilizing the BAP1 protein.

BAP1.p modulates its enzymatic activity, subcellular localization, and interaction networks, thereby influencing DNA repair processes. Although direct evidence linking phosphorylated BAP1 to base excision repair (BER) is limited compared to homologous recombination (HR) and double-strand break (DSB) repair, accumulating data suggest that it plays an indirect but functionally significant role. hosphorylated BAP1 retains deubiquitinase activity, potentially with altered substrate specificity, and facilitates chromatin remodeling by deubiquitinating histone substrates such as H2Aub, H2AK119ub1, and H2AK15ub. This process reduces chromatin compaction and enhances accessibility for BER machinery, including OGG1, APE1, and POLβ. In this context, BAP1.p may help stabilize open chromatin near DNA lesions, promoting the recruitment of BER enzymes and ensuring the efficient excision and repair of oxidative or alkylated bases.

Furthermore, BAP1 phosphorylation may indirectly regulate PARP1 activity through interactions with PARP1 regulators, thus helping to balance PARP1 activation and prevent hyperactivation during the initiation of base excision repair (BER). Additionally, BAP1 phosphorylation is often mediated by upstream DNA damage response kinases, such as ATM and ATR, following DNA damage, including single-strand breaks (SSBs). This positions BAP1.p as a downstream effector that integrates base excision repair (BER) with

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broader double-strand DNA damage response (DDR) signaling and chromatin state regulation.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2µM 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 is Rabbit anti-Human BAP1.p Polyclonal, IgG, 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-Rabbit, $F(ab')_2$ (H + L) was incubated at room temperature for 1 hour at a dilution of 1:1000.

The second primary antibody, Alpha Tubulin Monoclonal Antibody (DM1A), 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 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 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI cat#P36962.

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 93 of 171 total - *Panel A*

Z Stack - (3D) - *Panel A & B*

Z-Stack = 171 Slices (5.1 µm)

Channels = 3
Scaling (Per Pixel) = 0.043 µm X 0.043 µm X 0.030 µm
Image Size (Pixels) = 1276 X 1276
Image Size (Scaled) = 55.08 µm X 55.08 µm
Bit Depth = 16 Bit
Image Center Position = X: 82.48 mm, Y: 48.63 mm
Stage Position = X: 82.48 mm, Y: 48.63 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - *Panel C*

Z-Stack = 171 Slices (5.1 µm)

Channels = 3
Scaling (Per Pixel) = 0.043 µm X 0.043 µm X 0.030 µm
Image Size (Pixels) = 641 X 490
Image Size (Scaled) = 27.67 µm X 21.15 µm
Bit Depth = 16 Bit
Image Center Position = X: 82.48 mm, Y: 48.63 mm

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Stage Position = X: 82.48 mm, Y: 48.63 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

647 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 65 µm
LSM MBS = MBS 488/639, 405/730
LSM SBS = SBS SP 615
Laser Wavelength = 639 nm @0.08%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.4
Rotation = 0°
Sampling = 1.64
Pixel Time = 0.26 µs
Frame Time = 4.20 s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 636-732
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = 7.3 (3D, Auto)

488 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 50 µm
LSM MBS = MBS 488/639, 405/730
LSM SBS = Mirror
Laser Wavelength = 488 nm @0.05%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.4
Rotation = 0°
Sampling = 1.64
Pixel Time = 0.26 µs
Frame Time = 4.20 s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8

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Excitation Wavelength = 493
Emission Wavelength = 517
Effective NA = 1.4
Detection Wavelength = 489-556
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = 8.6 (3D, Auto)

DAPI -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 44 µm
LMS MBS = MBS 488/639, 405/730
LSM SBS = Mirror
Laser Wavelength = 405 nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.4
Rotation = 0°
Sampling = 1.64
Pixel Time = 0.26 µs
Frame Time = 4.20 s
LSM Scan Speed = 12
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 440-475
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = 6.8 (3D, Auto)

Split View - *Panel A*

The top-left panel features Rabbit anti-Human BAP1.p Polyclonal, IgG visualized with Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, $F(ab')_2$ (H + L), the top-right panel shows Alpha Tubulin visualized with Identifyn® SRM Alexa Fluor™ 488 Affinity Purified Goat Anti-Mouse, IgG (H + L), (Subclasses 1+2a+2b+3), Fcy Fragment Specific, the bottom-left panel features DAPI nuclear staining, and the bottom-right panel features the merged image of all channels. This image was taken at Z-Position 93 of 171.

3D Image Processing - *Panel B*

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3D Maximum Projection = Precise
Appearance = Threshold 4% all channels, Ramp 0% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Tone Mapping Enabled
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)
Wedge = X-Z and Y-Z

Clipping Image Process = Clipping planes are introduced to pronounce regions of special interest.

X-Z = Activate was selected, textured opaque was chosen, and a blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = 25%
Clip X = 30°
Clip Z = 15°

Y-Z = Activate was selected, textured opaque was chosen, and a red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -38%
Clip Y = -30°
Clip Z = 0°

3D Image Processing - *Panel C*

3D Maximum Projection = Precise
Appearance = Threshold 4% all channels, Ramp 0% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Tone Mapping Enabled
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)
Wedge = X-Z and Y-Z

Clipping Image Process = Clipping planes are introduced to pronounce regions of special interest.

X-Z = Activate was selected, textured opaque was chosen, and a blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -47%
Clip X = 30°
Clip Z = 0°

Y-Z = Activate was selected, textured opaque was chosen, and a red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -10%
Clip X = -30°

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Clip Y = 0°

Summary

Panel A - This panel showcases a split view. From z-plane 93 of 171, illustrating the signal and localization of Rabbit anti-Human BAP1.p Polyclonal IgG The upper left section features the Rabbit anti-Human BAP1.p Polyclonal IgG primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red). The upper right displays an Alpha Tubulin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 488 (green). The lower left section shows DAPI nuclear staining (blue), while the lower right presents a merged image of the three channels. Panel A notes a Region of Interest (ROI) outlined by a white box, which is explored further in Panel C.

Panel B – This panel presents a 3-dimensional Maximum Projection of Rabbit anti-Human BAP1.p Polyclonal IgG labeled with Identifyn® SRM Alexa Fluor™ 647 (red) and Alpha Tubulin primary antibody with Identifyn® SRM Alexa Fluor™ 488 (green), as well as DAPI (blue) at confocal resolution. The image employs a double "wedge" or a double cut plane on the X-Z (blue outline) and Y-Z planes (red outline). In the X-Z cut plane, the position is 25%, the angle X is 30 degrees, and the angle Z is 0 degrees. In the Y-Z cut plane, the position is -38%, the angle X is -30 degrees, and the angle Z is 0 degrees.

Panel C - This panel presents a 3-dimensional Maximum Projection from the ROI in Panel A, further to demonstrate the nuclear localization of BAP1.p primary antibody. The projection shows Rabbit anti-Human BAP1.p Polyclonal IgG labeled with Identifyn® SRM Alexa Fluor™ 647 (red) and Alpha Tubulin primary antibody with Identifyn® SRM Alexa Fluor™ 488 (green), as well as DAPI (blue) at confocal resolution. The image employs a double "wedge" or a double cut plane on the X-Z (blue outline) and Y-Z planes (red outline). In the X-Z cut plane, the position is -47%, the angle X is at 30 degrees, and the angle Z is 0 degrees. In the Y-Z cut plane, the position is -10%, the angle X is at -30 degrees, and the angle Z is 0 degrees.

Panel D - A clipped image from Zeiss Zen image acquisition settings for this z-stack comprising 171 planes. **We note that the 639 nm laser power is set at 0.08% to obtain maximal imaging of the BAP1.p primary antibody labeled with Identifyn® SRM Alexa Fluor™647.**

Panels A, B, and C all demonstrate the BAP1.p primary antibody localized to the nucleus in response to the Etoposide-induced DNA damage, which is shown using specific z-planes demonstrating the X, Y orientation and cut plane maximum projections demonstrating the X and Y-Z localization within the nucleus.

Image Corrections

None

Image by J.K. Bennett
Image Analysis by B.T. Bennett
Data Presentation by P.D. Amistoso

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