Identifyn Standards for Optical Microscopy Methods™

Identifyn Primary Antibody

Rabbit anti-Human γH2Ax (Serine 139) Recombinant Monoclonal, Mouse Fc, IgG (BLR014M)
Cat ID# - PA-RR 000005

Identifyn® Secondary Antibodies

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

Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Donkey Anti-Rabbit, F(ab')$_2$ (H + L)
Cat ID# - SA 000043

Gamma H2AX (γH2AX) is a variant of the histone H2A protein that plays a crucial role in the cellular response to DNA double-strand breaks (DSBs). Its primary function is as a marker of DNA damage, particularly in the context of double-strand breaks (DSBs). When DSBs occur in chromatin, γH2AX is rapidly phosphorylated specifically at the serine 139 residue (referred to as Ser139 phosphorylation or γ-phosphorylation) near the site of the break. This phosphorylation event forms γH2AX foci, visualized via immunofluorescence microscopy techniques as an aggregate of fluorescently labeled protein at the site of breaks.

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, Rabbit anti-Human γH2Ax Recombinant Monoclonal, Mouse Fc, IgG (BLR014M), 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), Fcγ Fragment Specific was incubated at room temperature for 1 hour at a dilution of 1:1000.

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™ 555 Affinity Purified Donkey Anti-Rabbit, F('b')$_2$ (H + L) 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 72 of 147 total - *Panel A*

Z Stack - (3D) - *Panels A, B, & C*
Z-Stack = 147 Slices (4.38 μm)

Channels = 3

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Scaling (Per Pixel) = 0.030 µm X 0.030 µm X 0.030 µm
Image Size (Pixels) = 1476 X 1476
Image Size (Scaled) = 44.18 µm X 44.18 µm
Bit Depth = 16 Bit
Image Center Position = X: 71.24 mm, Y: 48.14 mm
Stage Position = X: 71.24 mm, Y: 48.14 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

647 -
Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 64 µm
LSM MBS = MBS 488/561/639, 405/730
LSM SBS = SBS SP 615
Laser Wavelength = 639 nm @0.02%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 3.0
Rotation = 0°
Sampling = 2.36
Pixel Time = 0.23 µs
Frame Time = 4.84 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 = 645-700
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 650 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = 7.9 (3D, Auto)

555 -
Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 57 µm
LSM MBS = MBS 488/561/639, 405/730
LSM SBS = Mirror
Laser Wavelength = 561 nm @0.007%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 3.0
Rotation = 0°
Sampling = 2.36
Pixel Time = 0.23 µs
Frame Time = 4.84 s
LSM Scan Speed = 12

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Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 553
Emission Wavelength = 568
Effective NA = 1.4
Detection Wavelength = 565-625
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 650 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = 7.4 (3D, Auto)

DAPI -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00 AU / 41 µm
LSM MBS = MBS 488/561/639, 405/730
LSM SBS = Mirror
Laser Wavelength = 405 nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 3.0
Rotation = 0°
Sampling = 2.36
Pixel Time = 0.23 µs
Frame Time = 4.84 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 = 430-460
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)

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

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Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

3D Image Processing - *Panel C*

3D Maximum Projection = Precise
Appearance = Threshold 1% 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)

Clipping Image Process = A clipping plane was introduced to pronounce regions of special interest.
X-Z = Activate was selected, textured opaque was chosen, and a green outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Position of Clip = -14%
Clip X = 30°
Clip Z = 0°
Y-Z = Activate was selected, textured opaque was chosen, and a dark red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Position of Clip = -24%
Clip Y = -30°
Clip Z = 0°

Summary

Panel A - This panel displays a 2-dimensional view from z-plane 72 of 147, illustrating the signal and localization of Rabbit anti-Human γH2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), Actin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 555 (orange), and DAPI nuclear staining (blue). Panel demonstrates the localization of γH2Ax within the nuclear compartment using DAPI.

Panel B – This panel presents a 3-dimensional Maximum Projection of Rabbit anti-Human γH2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), Actin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 555 (light grey, changed from default orange to provide better visual contrast), and DAPI nuclear staining (blue).

Panel C - This panel presents a 3-dimensional Volume Projection to demonstrate further the nuclear localization of Rabbit anti-Human γH2Ax Recombinant Monoclonal, Mouse Fc, IgG2a. The projection shows Rabbit anti-Human γH2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647 (red), Actin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 555 (light grey, changed from default orange to provide better visual contrast), and DAPI nuclear staining (blue) at confocal resolution The image employs a double "wedge" or a double cut plane on the X-Z (green outline) and Y-Z planes (dark red outline. In the X-Z cut plane, the position is -14%, the angle X is at 30 degrees, and the angle Z is 0 degrees. In the Y-Z cut plane, the position is -24%, the angle X is at -30 degrees, and the angle Z is 0 degrees. The wedge

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allows us to define clearly the γH2Ax localization from multiple angles within the nuclear compartment.

Panel D - A clipped image from Zeiss Zen image acquisition settings for this z-stack comprising 147 planes. **We note that the 639nm laser power is set at 0.02% to obtain maximal imaging of the Rabbit anti-Human γH2Ax Recombinant Monoclonal, Mouse Fc, IgG2a primary antibody labeled with Identifyn® SRM Alexa Fluor™ 647.**

Panels A, B, and C all demonstrate the Rabbit anti-Human γH2Ax Recombinant Monoclonal, Mouse Fc, IgG2a labeled with Identifyn® SRM Alexa Fluor™ 647 localized to the nucleus Additionally, we observe that as we move to confocal resolution, the diffuse pan-nuclear staining seen in the widefield and widefield SIM images now becomes more punctate, providing somewhat better visualization of γH2Ax localizatio. Furthermore, not only do we now see localized protein aggregates, but diffuse structural details are beginning to emerge, which will be shown in more detail as we move into super resolution.

Image Corrections

Panel A - Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, $F(ab')_2$ (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast.
Panel B - Identifyn® SRM Alexa Fluor™ 555 Affinity Purified Donkey Anti-Mouse, $F(ab')_2$ (H + L) was pseudo-colored in Zen Software from orange, the default color, to white to provide visual contrast.

Panel C - Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, $F(ab')_2$ (H + L) was pseudo-colored in Zen Software from red, the default color, to white to provide visual contrast.

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

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