

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Rabbit anti-Human Ku70 Polyclonal, IgG

Cat ID# - PA-RP 000006

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 Donkey Anti-Mouse, F(ab')₂ (H + L)

Cat ID# - SA 000042

Ku70 is a crucial protein involved in the Non-Homologous End Joining (NHEJ) pathway, one of DNA's central mechanisms for repairing double-strand breaks (DSBs).

Recognition and Binding to DNA Breaks: Ku70 and Ku80 form the Ku heterodimer. This heterodimer has a high affinity for DNA ends and is responsible for recognizing and binding to DSBs. Its structure allows it to slide onto the DNA strand and form a ring around the break, stabilizing it.

Recruitment of Additional NHEJ Proteins: After binding to the DNA break, the Ku heterodimer acts as a scaffold, recruiting and assembling other proteins essential for the NHEJ pathway. This includes DNA-PKcs (DNA-dependent protein kinase catalytic subunit), which are crucial in further processing the break.

Protection of DNA Ends: By binding to the ends of the DNA at the site of the break, Ku70/80 helps protect the DNA from further degradation or unwanted processing, ensuring that the ends are preserved for the repair process.

Alignment of DNA Ends: Ku70/80 helps align the broken DNA ends, facilitating the ligation step in NHEJ. This alignment is crucial for accurate repair, even though NHEJ does not require extensive sequence homology.

Participation in End Processing and Ligation: Once other NHEJ factors have been recruited, Ku70/80 can assist in processing DNA ends if necessary. Ultimately, it helps bring the DNA ends together for ligation by the ligase complex (such as DNA Ligase IV, XRCC4, and XLF), completing the repair process.

In summary, Ku70, as part of the Ku heterodimer, plays a central role in the NHEJ pathway by recognizing and binding to DNA breaks, recruiting and organizing other repair proteins, protecting DNA ends, and facilitating the alignment and ligation necessary to repair double-strand 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 Ku70 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 Donkey Anti-Mouse, 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 Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:

Single Plane (2D) - Z-plane 81 of 160 total - Panel A

Z Stack - (3D)

Z-Stack = 160 Slices (4.77 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 3560 X 4889
Image Size (Scaled) = 46.98 µm X 64.51 µm
Bit Depth = 16 Bit
Image Center Position = X: 55.92 mm, Y: 39.25 mm
Stage Position = X: 55.92 mm, Y: 39.25 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Z Stack - (3D) - Panel B

Z-Stack = 160 Slices (4.77 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 1825 X 2228
Image Size (Scaled) = 24.08 µm X 29.40 µm
Bit Depth = 16 Bit
Image Center Position = X: 55.92 mm, Y: 39.25 mm
Stage Position = X: 55.92 mm, Y: 39.25 mm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

Single Plane (2D) - Z-plane 81 of 160 total - Panel C

Z Stack - (3D)

Z-Stack = 160 Slices (4.77 µm)

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 933 X 960
Image Size (Scaled) = 12.31 µm X 12.61 µm
Bit Depth = 16 Bit

Image Center Position = X: 55.92 mm, Y: 39.25 mm
Stage Position = X: 55.92 mm, Y: 39.25 mm
ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

Z Stack - (3D) - Panel D**Z-Stack = 78 Slices (2.31 μ m)**

Channels = 3
Scaling (Per Pixel) = 13.20 nm X 13.20 nm X 30.00 nm
Image Size (Pixels) = 361 X 249
Image Size (Scaled) = 4.76 μ m X 3.29 μ m
Bit Depth = 16 Bit
Image Center Position = X: 55.92 mm, Y: 39.25 mm
Stage Position = X: 55.92 mm, Y: 39.25 mm
ROI Center Offset = X: 0.00 μ m, Y: 0.00 μ m

647 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 640
Channel Name = T1-Axiocam 820-SR
Excitation Wavelength = 640
Emission Wavelength = 729
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820
Camera Adapter = 1X
Exposure Time = 100 ms
Depth of Focus = 1.13 μ m
Binning Mode = 2,2
Laser Wavelength = 640 nm @1.00%
Frame Time = 1.33 s
Scan Direction = Unidirectional
Grating = 27.5 μ m grating

488 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 488
Channel Name = T2-Axiocam 820-SR
Excitation Wavelength = 488
Emission Wavelength = 525
Effective NA = 1.4

Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820
Camera Adapter = 1X
Exposure Time = 120 ms
Depth of Focus = 0.81 μm
Binning Mode = 2,2
Laser Wavelength = 488 nm @1.50%
Frame Time = 1.59 s
Scan Direction = Unidirectional
Grating = 36.5 μm grating

DAPI -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 405
Channel Name = T3-Axiocam 820 #2-SR
Excitation Wavelength = 405
Emission Wavelength = 445
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 100 ms
Depth of Focus = 0.69 μm
Binning Mode = 2,2
Laser Wavelength = 405 nm @3.00%
Frame Time = 676.00 ms
Scan Direction = Unidirectional
Grating = 27.5 μm grating

Post Processing - SIM Processing

Processing Dimension - 3D
Result Sampling = 4
Process Sampling = N/A
Channels = 3
Algorithm = SIM
SIM Settings = Standard
Auto Sharpness = On
Sharpness = 9.4422 (647), 10.1352 (488), 8.6429 (DAPI)
Fitting = Fast Fit
Normalization = Auto

Split View - Panel A

The first panel on the left features Rabbit anti-Human BRCA1 Recombinant Monoclonal, IgG visualized with Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(a'')₂ (H + L), the second panel features alpha tubulin visualized with Identifyn® SRM

Alexa Fluor™ 555 Affinity Purified Donkey Anti-Mouse, F(ab')₂ (H + L), the third panel features DAPI nuclear staining, and the last panel on the right features the merged image of all channels. This image was taken at Z-Position 81 of 160.

3D Image Processing - Panel B

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)

3D Image Processing - Panel D

3D Volume Projection = Precise

Appearance = Threshold 4% all channels, Ramp 96% all channels, Maximum 100% all channels

Background = Black

Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping

Projection = View Angle 15°, Scale Z 15%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Panel A - This panel presents a split view from the image acquisition at z-plane 81 out of 160, illustrating the signal and localization of Rabbit anti-Human Ku70 Polyclonal IgG. The left-hand panel highlights the primary antibody, Rabbit anti-Human Ku70 Polyclonal IgG, labeled with Identifyn® SRM Alexa Fluor™ 647 (red). The next panel to the right displays an Alpha Tubulin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 488 (green). The following panel shows DAPI nuclear staining (blue), while the rightmost panel features a merged image of all three channels. A white box indicates a region of interest (ROI) for further investigation in Panel B.

Panel B - This panel presents a three-dimensional maximum projection of Rabbit anti-Human Ku70 Polyclonal IgG from the ROI identified in Panel A. It is counterstained with Identifyn® SRM Alexa Fluor™ 647 secondary antibody (white; the default red color has been changed in Zeiss Zen software to enhance visual contrast), along with the Alpha Tubulin primary antibody labeled with Identifyn® SRM Alexa Fluor™ 488 (green) and DAPI (blue), utilizing super-resolution Structured Illumination Microscopy (SIM) imaging. A white box indicates a region of interest (ROI) for further investigation in Panel C.

Panel C - This panel displays a two-dimensional image from the ROI in Panel B at z-plane 81 out of 160, illustrating the signal and localization of Rabbit anti-Human Ku70 Polyclonal IgG as seen in Panel A. It is counterstained with Identifyn® SRM Alexa Fluor™ 647 secondary antibody (Teal; the default red color has been changed in Zeiss Zen software to enhance visual contrast). A white box indicates a region of interest (ROI) for further investigation in Panel D.

Panel D- This panel provides a three-dimensional volume projection of Rabbit anti-Human Ku70 Polyclonal IgG from the ROI identified in Panel C. It is counterstained with Identifyn® SRM Alexa Fluor™ 647 secondary antibody (Teal, the default red color has been changed in Zeiss Zen software to enhance visual contrast). No adjustments to thresholds or manipulations of any signal were made; however, the View Angle was reduced from 35% (default) to 15%, and the Scale Z was adjusted from 100% (default) to 15%, collapsing the Z aberration and improving two-dimensional visualization for the viewer.

Note - Using super-resolution Structured Illumination Microscopy (SIM) with the Elyra 5, we observe enhanced structural details of Ku70 that were previously seen as "pan-nuclear" or diffuse in diffraction-limited techniques like widefield imaging and confocal microscopy. Although super-resolution Airyscan imaging offers more defined images, it still lacks the detail observable with SIM, which achieves 60-80 nm resolution in the X and Y axes.

In Panels A and B, Ku70 shows some organization, localizing to the nucleus. Panel C highlights a region of interest from Panel B, revealing structural details of Ku70 at SIM resolution. The organization appears similar but demonstrates a less cohesive pattern compared to other nuclear proteins, particularly those that are more ubiquitous, such as H2AX.

Based on thousands of images taken by Identifyn® across various nuclear targets, we face a challenge: does this represent the limit of resolution and possibly not reflect reality, or does it provide a crude visualization of Ku70's localization, organization, or structure?

Image Corrections

Identifyn® SRM Alexa Fluor™ 647 Affinity Purified Goat Anti-Rabbit, F(a'')₂ (H + L) was pseudo-colored in Zen Software from red, the default color, to teal to provide visual contrast.

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