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NB300-268 Protocol

Immunoprecipitation protocol for LRRK2 Antibody (NB300-268)

LRRK2 Antibody: https://www.novusbio.com/products/lrrk2-antibody_nb300-268
Protocol for immunoprecipitation of LRRK2 followed by LRRK2 autophosphorylation kinase assay

Cell lysis
3X15 cm plates of SH-SY5Y cells were grown to 80% confluency. The plates were washed twice with PBS and placed on ice. Remaining PBS was aspirated off after tilting plate to remove all PBS. 1.5 ml of cold lysis buffer (buffer A) was added to each plate. The plates were allowed to incubate on ice 5 min until the cells detached. The lysis buffer and cells for each plate were then vigorously passed 6X through a 30.5 guage needle. Lysis buffer and cells were transferred to 3X1.5 ml eppendorf tubes and spun 5 min. at 5,000 rpm in a 4 degree eppendorf microfuge. Lysates were removed from pelleted debris and transferred to new 1.5 ml eppendorf tubes and recentrifuged 10 min. at 13,000 rpm. Lysate was transferred to three new tubes and 1/2 lysate volume of buffer A (-) NaCl was added to each tube.
Preclear
10 ug of rabbit IgG were added to the lysate for each tube and the lysate was vortexed followed by rotating at 4 degrees for 2 hours. 20 ul of protein A sepharose beads (Amersham cat#: 17-0469-01) were added. The tubes were vortexed and then rotated for 1.5 hours at 4 degrees. Lysates were separated from protein A beads beads by low (200 rpm) spin for 2 min and transferred to new eppendorf tubes. A repeat of the protein A sepharose incubation was carried out to remove residual rabbit IgG followed by removal of the protein A beads.

Immunoprecipitation with LRRK2 Ab

To two of the tubes containing precleared lysate were added 7 ul of LRRK2 Ab (7ug). To the remaining tube was added 7ug of rabbit IgG. The tubes were vortexed and allowed to rotate overnight at 4 degrees. The following morning 30 ul of protein A sepharose was added to each tube, the tubes were vortexed and rotated at 4 degrees for 2 hours. The protein A beads were then isolated by brief, low speed centrifugation and were washed 3X in 500ul buffer A (-) NaCl. This was followed by two washes in kinase buffer (buffer B). Protein A beads were resuspended in 1 volume (30 ul) of buffer B for a total of 60ul of immunoprecipitate.
Autophosphorylation kinase reaction, gel electrophoresis and phosphoimaging
On ice, 40 ul of immunoprecipitate from each tube was transferred to a .5ml kinase reaction tube. Each of the three reactions was supplemented with a 5 ul mixture that gave a final reaction concentration of 15 uM cold ATP and 5uCi ATP. The reaction mixtures were vortexed and transferred to a rotator in a 30 degree incubator. The autophosphorylation incubation was allowed to go for 30 minutes and the reaction tubes were taken off the rotator and vortexed every five minutes. The reactions were then halted by addition of 11ul of 5X SDS gel running sample buffer to each of the three samples. 40ul of each sample was then run on a 7% acrylamide-acetate mini-gel. Once the 200Kd molecular weight marker band had run half way down the gel, the gel was stopped dried and exposed blanked to a phosphoimaging cassette (Molecular Dynamics). Following 24 hour exposure, the cassette was assessed for radioactivity on a Storm analyzer.

Buffers

Buffer A (make 10ml both - and + NaCl solutions) = lysis buffer
50mM Tris pH 7.4
150mM NaCl
0.2% NP40
Protease inhibitor cocktail (stock = 100X, Sigma)
0.5mM vanadate
15mM EDTA
adjust to 10 ml with H2O
Buffer B = kinase buffer
10mM hepes
10mM MgCl2
50mM NaCl
protease inhibitor
vanadate

(1mM NaN3 if storing overnight or longer)