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NBP1-71668 Protocol

Assay Instruction Manual (NBP1-71668)

0.5% (v/v) Triton X(R)-100
6M Urea
2 ug/ml Leupeptin
10 uM Pepstatin
3 ug/ml Aprotonin
1 mM Sodium Orthovanadate (Na3VO4)
2 mM Sodium Pyrophosphate (Na4P2O7)
5mM Sodium fluoride (NaF)

Cell Lysate Preparation (adherent cells):
The researcher is recommended to optimize the cell extraction procedures for their own applications.
1. Aspirate media. Treat cells by adding fresh media containing regulator for the desired time.
2. Rinse once with ice cold PBS on ice.
3. Remove PBS and add 0.5 ml of Denaturing Cell Lysis Buffer (see Cell Lysis Buffer Preparation) containing protease/phosphatase inhibitors and PMSF to each plate (10cm diameter, 70% - 80% confluent). Incubate for 10min on ice.
4. Gently scrape cells off the plate and transfer to chilled tubes/vials. Keep on ice
5. Vortex briefly and Microcentrifuge at 14,000 rpm for 10 minutes at 4C.
6. Aliquot the clear lysate to clean tubes. These samples are ready for testing. Lysates can be stored at -80C. Multiple freeze/thaw cycles must be avoided.

Assay Procedure:
The following procedure is for 12 x 8 microwell antibody coated strips. The researcher is advised to scale down or up appropriately, if processing less or more strips.

1. 2x Antibody-Coated Microwell Strips are provided for each target protein (colour coded, 8 microwell/strip).
2. Gently, place the strips in the microwell strip holder. Unused strips can be resealed and stored at 4C. See Procedural Notes for equilibrating the microwell strips to room temperature.
3. Add 100 ul of each diluted cell lysate (sample) and positive control (Assay QC) into the appropriate well. 100ul of 1x Sample Dilution Buffer alone should be added to negative control/blank wells.
a. Positive Control (Assay QC) allows the researcher to assess the performance of the assay
b. As a guide, a minimum concentration of 20ug/well of cell lysate can be used as a starting concentration. However, the researcher may adjust the test concentration for specific treatments or cell lines if desired.
4. Seal with plate cover and press firmly onto top of microwells. Incubate for 2 hours at room temperature.
5. Gently remove the plate cover and wash wells. Thoroughly decant or aspirate solution from wells and discard the liquid. Wash wells 3 times with 0.3 ml of diluted 1x Wash Buffer (see Directions for Washing).
6. Add 100ul of the appropriate Biotin-Conjugated Detection Antibody to each well. Be sure to match the cap colour of the Detection Antibody with the corresponding colour coded Microwell Strips. Seal with plate cover and press firmly onto top of microwells. Incubate for 2 hours at room temperature.
7. Wash wells 3 times as directed in Step 5.
8. Add 100ul SAV-HRP (equilibrated to room temperature) to each microwell. Seal with plate cover and press firmly onto top of microwells. Incubate for 30 minutes at room temperature.
9. Wash wells 3 times as directed in Step 5.
10. Add 100ul of TMB substrate solution (equilibrated to room temperature). Incubate the plate for 20 minutes or until the strongest cell lysate sample turns dark blue at room temperature in the dark.
a. The rate of colour development is dependant on amount of target protein in each sample and thus varies for each sample preparation.
b. Colour development for each target protein must be individually monitored. Consequently, it may be necessary to stop the reaction for each target protein separately, within the same plate.
11. Add 50ul of Stop Solution to each microwell (see section 10 a, b). Gently tap the microtitre plate for a few seconds. The colour in each well should change from blue to yellow.
12. Wipe the underside of the microwells with lint free tissue and read absorbance at 450 nm within 30 minutes of stopping the reaction.

*Note: Total assay time is approximately 5 hours including washing.

Optional Assay Procedure:
- Step 3 (addition of sample) and Step 6 (addition of detection antibody) incubation times can be shortened to 30 mins each with the use of an orbital shaker. (100 rpm recommended)

- Step 8 (addition of SAV-HRP) should be incubated on the orbital mixer for 30 mins. Step 10, (addition of TMB) should be incubated on a benchtop for 30 mins.

To minimize assay drift, it is essential to add samples and detection antibody in as short a time as is practical. This shortened time procedure may result in lower OD values compared to the standard procedure.

*Note: Total assay time for optional assay is approximately 2.5 hours including washing.

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