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NBP1-71669 Protocol

Assay Instruction Manual (NBP1-71669)

Pathway Assay Kit
Akt1 (pS473) & Akt2 (pS474)
Human/Rat/Mouse

Instructions Manual
(For Research Use Only)

Introduction:
Akt (also known as AKT) is a serine/threonine kinase, activated by insulin and various growth and survival factors via a pathway involving PI3 kinase. Activation is mediated through phospholipids binding and phosphorylation by phosphatidylinositol-dependent-kinases at Thr308 in the activation loop and at Ser473 within the carboxy terminus. Three mammalian AKT isoforms of AKT have been identified, AKT1a AKT2 and AKT3 (also known as PKBa, PKBb and PKBg, respectively), which display > 80% sequence identity. Evidence from animal studies suggests that the three AKT isoforms may have some differential, non-redundant physiological functions. Mice lacking AKT2 display insulin resistance and a diabetes-like syndrome, whereas mice lacking AKT1 demonstrate normal glucose homeostasis but are small throughout life. Thus, AKT1 seems to be involved predominantly in control of growth/proliferation, whereas AKT2 regulates cellular metabolism.

Novus has formulated a Denaturing Cell Lysis buffer containing 6M Urea to maximize assay performance.

Materials:
- Antibody-Coated Microwells. 2 x 8-microwell strips (colour coded) - 6 pouches
- Biotin-Conjugated Detection Antibody (color coded caps). Contains 0.05% w/v Sodium Azide* - 6x 1.7ml
- Positive Control (Assay QC) - 1x 1.4ml
- Streptavidin-HRP (SAV-HRP). Contains 3.3mM Thymol - 11ml
- Tetramethylbenzidine (TMB) Substrate - 11ml
- 20 x Wash Buffer Concentrate. Contains 3.3mM Thymol - 25ml
- 5 x Sample Dilution Buffer Concentrate. Contains 0.05% w/v Sodium Azide* - 25ml
- Stop Solution - 5.5ml
- Plate Cover - 3 sheets
- Microwell Strip Holder - 1 holder

Notes:
All components of this kit should be stored at 4C upon receipt.
*Sodium Azide reacts with lead and copper plumbing to form explosive metals azides. Upon disposal, flush drains with copious amount of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water.

Color Coding:
The kit components are color coded, for ease of use. The antibody cap colors match the microwell rim colors, making identification quick and easy.

Akt1 (pS473) - Green
Akt2 (pS474) - Orange

Additional Material Required (Not Supplied):
- Microtitre plate reader capable of measurements at or near 450nm
- Calibrated pipettes with disposable pipette tips. A manifold multichannel pipette is useful for large assays and to minimize pipetting errors.
- Cell Lysis Buffer Preparation for the recommended buffer formulation.
- Wash bottle, manual dispenser or automated plate washer
- Lint-free tissue
- Absorbent paper tissue
- Microcentrifuge Tubes

- Distilled or de-ionised Water

Procedural Notes:
- Ensure all kit components are refrigerated when not in use. All reagents should be allowed to equilibrate to room temperature before use.
- Antibody-Coated microwell strips should be allowed to equilibrate to room temperature before opening the pouch. Any unused strips can be sealed and stored in the provided pouch (containing desiccant) at 4C for 3 months.
- Cell Lysates samples must be prepared using the Denaturing Cell Lysis Buffer containing 6M Urea and protease and phosphatase Inhibitors for optimal assay performance (see Cell Lysis Buffer Preparation).
- Samples should be frozen if not analyzed. Avoid multiple freeze/thaw cycles of frozen samples. Samples must be allowed to thaw on ice prior to analysis.
- Cell lysate samples must be diluted to the desired test concentration with 1x diluted Sample Dilution Buffer (see Reagent Preparation).
- It is recommended that all test samples be run in duplicate.
- When pipetting reagents, maintain a consistent order of addition well to well. This ensures equal incubation times for all wells.
- Cover or cap all reagents when not in use.
- Do not mix reagents from other kits.
- Do not use reagents after the expiry date of the kit.
- Read Absorbances at 450 nm within 30 minutes of assay completion at room temperature.
- All residual wash liquid must be drained from the wells by decanting or aspiration followed by forceful tapping of the plate on absorbent paper tissue. Never insert absorbent paper into the wells. Ensure there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results (see Directions for Washing).
- The TMB substrate is light-sensitive, avoid prolonged exposure to light. Avoid contact of TMB with metal, or colour may develop.

Safety Precautions:
- TMB is toxic if inhaled or swallowed. Avoid contact with skin. Keep container tightly closed when not in use.
- Stop Solution is an acidic solution. Appropriate personal protection equipment must be worn (laboratory coat, gloves, eye protection).

Directions for Washing:
- Incomplete washing will adversely affect the test outcome. All washing must be performed with wash buffer provided.
- Completely aspirate the liquid from all wells by gently lowering an aspiration tip into the bottom of the well. Care must be taken not to scratch the inside of the well.
- After aspiration, fill the wells with 0.3ml of 1x Wash Buffer (see Reagent Preparation). Let soak for 15 to 30 seconds, and then aspirate the liquid. Repeat as directed in the Assay Procedure. After washing, invert the plate and forcibly tap on absorbent paper tissue.
- If a wash bottle is used, flood the plate with wash buffer, completely filling all the wells. After washing, to remove residual solution, invert the plate and forcibly tap on absorbent paper tissue. Do not allow the wells to dry at any time.
- Clean the underside of the wells with lint-free tissue.
- If an automated plate washer is used, follow the operational procedure and programme for 30 second soak cycles.

Reagent Preparation:
- 1x Wash Buffer : Allow 20x Wash Buffer Concentrate to equilibrate to room temperature. Mix to ensure any precipitated salt have re-dissolved. Dilute 25ml 20x Wash Buffer Concentrate into de-ionised or distilled water to a total volume of 500ml. The diluted Wash Buffer is stable at 4C for up to 14 days.
- 1x Sample Dilution Buffer : Allow 5x Sample Dilution Buffer Concentrate to equilibrate to room temperature. Mix to ensure any precipitated salts have re-dissolved. Dilute 25ml 5x Sample Dilution Buffer Concentrate into de-ionised or distilled water to a total volume of 125ml. The diluted Sample Buffer is stable at 4C for up to 14 days.

Cell Lysis Buffer Preparation (denaturing conditions):
Denaturing Cell Lysis Buffer, containing 6M Urea must be used for optimal assay performance. Novus cannot guarantee optimal performance of the assay, if the recommended denaturing cell lysis buffer is not used.

Denaturing Cell Lysis Buffer
10 mM Tris, pH 7.4
150 mM NaCl
1 mM EDTA
1 mM EGTA
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 -80oC. 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 ulof 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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