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Directional Insert: AK/PAK2 Assay Kit GLO111-01

Kit Components

X **Buffer I:** 1 vial of 1 ml of 50 mM Tris-HCl pH 7.0 buffer containing 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 14 mM β-mercaptoethanol, 0.03% Brij-35 and 2 mg/ml bovine serum albumin.

X **Buffer II:** 1 vial of 1 ml of 50 mM Tris-HCl pH 7.0 buffer containing 10% glycerol, 1 mM benzamidine, 0.1 mM PMSF, 14 mM β-mercaptoethanol and 0.03% Bij-35.

X **Buffer III:** 1 vial of 1 ml of 50 mM Tris-HCl pH 7.0 buffer containing 65% ethyleneglycol, 1 mM benzamidine, 0.1 mM PMSF, 14 mM β-mercaptoethanol and 0.03% Bij-35.

X **AK/PAK2 Catalytic Domain:** 1 vial of 200 ng of purified AK/PAK2 in 40 µl 50 mM Tris-HCl, pH 7.0 buffer containing 14 mM β-mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF, 1 mM EDTA, 65% ethyleneglycol and 0.03% Brij-35.

X **MBP:** 0.5 ml of 10 mg/ml myelin basic protein substrate in 50 mM Tris-HCl, pH 7.0 buffer containing 10% glycerol, 1 mM benzamidine and 0.03% Brij-35.

X **Mg/ATP Solution 1:** One vial containing 1 ml of MgATP cocktail 10 mM magnesium chloride and 1 mM ATP in 50 mM Tris-HCl pH 7.0 buffer containing 10% glycerol, 1 mM benzamidine, 0.1 mM PMSF, 14 mM β-mercaptoethanol and 0.03% Bij-35.

X **Mg/ATP Solution 2:** One vial containing 1 ml of MgATP cocktail 100 mM magnesium chloride and 1 mM ATP in 50 mM Tris-HCl pH 7.0 buffer containing 10% glycerol, 1 mM benzamidine, 0.1 mM PMSF, 14 mM β-mercaptoethanol and 0.03% Bij-35.

Solution required to measure activity with MBP not included with kit:

X [γ-^{32}P]ATP (~ 1500 Ci/mmol)
X Trichloroacetic acid
X Scintillation fluid

Required equipment not included:

X Variable volume pipettes (0-10 µl, 0-20 µl and 0-100 µl) + tips
X Vortex mixer
X Timer
X Water bath
X Plexiglass shielding
X Scintillation vials and counter

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Kit Description

Quantity: The kit provides for greater than fifty AK/PAK2 assays according to the protocol described below.

Storage and Stability: Store in aliquots at -70E C. Stable for at least one year under these conditions.

Use: The kit may be used to determine:

1. AK/PAK2 activity
2. The effect of a test substance
3. Identify new physiological targets of AK/PAK2
4. Identify a related protein kinase
5. Determine if preparations of protein kinases may be free of AK/PAK2 activity.

The kit provides for rapid, convenient and highly reproducible AK/PAK2 activity measurements. The assay is linear for up to 20% incorporation of total ATP. The assay is based on the activation and reaction with MBP and [γ-^{32}P]ATP of AK/PAK2 or related protein kinase. The ^{32}P-labeled MBP is then separated from the residual [γ-^{32}P]ATP by precipitation and washing with trichloroacetic acid using rapid centrifugation in a microcentrifuge. The precipitate is then quantitated by liquid scintillation counting.*****

Auto-Activated AK/PAK2, Mg^{2+}

MBP + [γ-^{32}P]ATP ----------> ^{32}P-labeled MBP + ADP

Safety Warnings and Precautions: This assay kit is designed for research use only and is not recommended for internal use in humans and animals. Please follow your institutional protocols and instructions for handling, use storage and disposal of radioactive materials. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

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Assay Protocol**

1. Rapidly thaw the components of the kit, mix each solution with a vortex and place on ice.

2. Add 45 µl of MgATP Solution 2 to 5 µl (~ 100 µCi) of [γ-^{32}P]ATP (~ 3000 Ci/mmol). This mixture is referred to as "^{32}P-labeled ATPMg solution" below.

Activation mixture:

3. Add 8 µl of Buffer I† to four micro-centrifuge tubes.

4. Add 1 µl of MgATP Solution 1 to one tube and 1 µl of Buffer II to the other tube.

5. Add 1 µl of AK/PAK2 (or test sample) to each tube.

6. For reaction controls, omit AK/PAK2 from the incubations and replace with 1 µl of Buffer III in two additional micro-centrifuge tubes.

7. Upon addition of AK/PAK2, incubate for 15 min at 30EC to activate the kinase.

Reaction with MBP

8. To four new microcentrifuge tubes add 7 µl of Buffer I and 1 µl of the MBP solution,

9. To each of these tubes add a 1 µl aliquot of each activation and control mixture described above.

10. Add 1 µl of the ^{32}P-labeled ATPMg solution, vortex and incubate the mixtures at 30EC.

11. After 10 min at 30EC, add 1 ml of 10% trichloroacetic acid to each tube and vortex gently.

12. Centrifuge the mixtures at 10,000 rpm for 2 minutes in a microcentrifuge and remove the supernatant with a pasteur pipete in accordance with your local radioisotope regulations.

13. Add another 1 ml of 10% trichloroacetic acid to each tube, centrifuge at 10,000 rpm for 2 minutes in a microcentrifuge and decant the supernatant.

13. Repeat this washing procedure twice more‡.

14. After the last wash, add 1 ml of scintillation cocktail to each microcentrifuge tube.

15. Insert each tube into a glass or plastic scintillation vial.

16. Read in scintillation counter.

17. Compare CPM of enzyme samples and CPM of control samples that contain no enzyme (background).

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Notes

*****Buffer II is identical to Buffer I except that no bovine serum albumin is included. Buffer II may be used in the incubations instead of Buffer I, for example, if the phosphorylation of test samples were to be analyzed by autoradiography following SDS-PAGE. To terminate reactions under these conditions, use SDS sample buffer as described (Laemmli UK (1970) Nature 227, 680-685) instead of 10% trichloroacetic acid.

****** We recommend reading the entire protocol before starting.

† Volume of assay buffer may be readjusted when the effect of a compound, on kinase activity is being tested. Appropriate controls should be performed at all times.

‡ Due to some variability in [γ-^{32}P]ATP preparations among suppliers, it may sometimes be necessary to increase or reduce the number of times the washing procedure is performed in order to reduce background readings.