PI (phosphoinositide)-3 Kinase Assay

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[Abstract] PI3-kinases regulate a wide range of cellular responses through the production of phosphatidylinositol 3, 4, 5-trisphosphate [PI (3, 4, 5) P (3)] in cellular membranes. This protocol provides a highly efficient and easy-to-handle method for sensitive detection and quantification of PI 3-kinase activity.

Materials and Reagents

1. Tris
2. NaCl
3. EDTA
4. Triton-X100
5. Glycerol
6. NaF
7. PMSF
8. Aprotinin
9. Leupeptin
10. Sepharose
11. NP-40
12. Tris-LiCl
13. TNE
14. P³² hot ATP (PerkinElmer, catalog number: NEG502A)
15. MgCl₂
16. MnCl₂
17. EGTA
18. Methanol
19. Chloroform
20. Acetic acid
21. Isopropanol
22. Lysis buffer
23. Pre-washed sepharose

24. ATP mix (see Recipes)
25. Phosphoinositol lipid (Avanti Polar Lipid, catalog number: 190082) (see Recipes)

Equipment

1. Centrifuges
2. Sonicator
3. Radiation hood
4. Silica gel TLC plate

Procedure

1. Lyse the cell with cantley lysis buffer plus protease inhibitor (aprotinin 100 µg/ml, Leupeptin 1 µg/ml, PMSF 35 mg/ml), spin at 10,000 x g for 15 min.
2. Take the supernatant after centrifugation. Immunoprecipitate with the antibody against protein of interest at 4 °C on wheel overnight.
3. Add 60 µl pre-washed sepharose (either protein A or protein G) with cut pipet tips to each sample. Put on wheel at 4 °C for 2-3 h.
4. Spin the sample, (you might want to save the supernatant for future analysis). Wash the samples extensively with following:
 - a. 3x 1 ml wash with PBS-1% NP-40
 - b. 3x 1 ml wash with Tris- LiCl
 - c. 2x 1 ml wash with TNE

Make sure the washes will not sit at room temperature (RT) for long. Add 60 µl TNE to each sample.

5. Return the samples on ice while preparing substrates for the assay:
For each sample phosphoinositol lipid, ATP mix and 100 mM MgCl₂ or MnCl₂ is needed.
6. After preparing all the reagents, add 10 µl of PI lipid and 10 µl of 10 mM MgCl₂ to each sample. Now you are ready to perform the assay in the radiation hood.
7. Add hot ATP in the hood. Start the assay by adding 5 µl ATP mix to the sample 10 sec apart and stop each reaction with 20 µl of 8 M HCl when reach 10 min.
Note: make sure to mix the reaction well for each addition.
8. Add 160 µl of 1:1 methanol: chloroform to extract lipid and mix well.
9. Spin the samples in the hot centrifuge for 5 min, remove the upper (water) phase.
10. Wash the organic layer twice with 200 µl 1 M HCl.
11. Spot adequate amount of washed chloroform phase onto a silica gel TLC plate (the sample can be stored at -70 °C if not spotting).

12. Run the plate in the solvent mix (2 M acetic acid/isopropanol 1:2) tank until the solvent front is about 2-3 cm away from the top.
13. Take out the plate and dry in the hood.
14. Wrap the plate and expose on the film at -70 °C overnight.
15. Quantify the phosphatidylinositol3-phosphate spots.

Recipes

For example, if you have 10 samples prepare the reagents as following: Put two extra in: 10+2= 12 samples.

1. PI lipid
 - 2 μ l x 12 samples= 24 μ l
 - Take out 24 μ l PI, dry under the Argon. Bring up to 120 μ l.
 - 10 mM Tris and 1 mM EGTA.
 - Sonicate 10 min.
2. ATP Mix
 - Need total volume 5 μ l x 12 samples= 60 μ l ATP Mix
 - 12 μ l hot ATP (10 uCi/ μ l)
 - 12 μ l Cold 10 mM ATP
 - 36 μ l H₂O
3. MgCl₂
 - 10 μ l x 12 samples= 120 μ l total

References

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