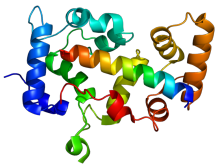
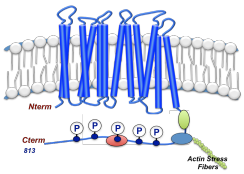


Phospho-westernblot Assay Protocol



Important considerations for kinase sample preparation success

Successful preparation of tissue or cells for the subsequent assay of kinase activity depends largely on the three following factors

- ***Maintain the sample at low temperature*** during the preparation process i.e. washing, scraping and centrifuging the cells after agonist addition. Everything is done with cold buffers and is kept in the ice, NOT on top of the ice.
- ***Minimizing actual elapsed sample preparation time***, i.e. complete the sample preparation protocol from agonist to centrifuging and separation of the two components as soon as possible. No time to wander off and surf the web. Time from harvesting the cells to homogenization of the sample can be critical to keep the protein active and in its respective intracellular location.
- ***Inclusion of protease inhibitors and phosphatase inhibitors*** in the preparation scheme to retain the structural and functional integrity of the kinase. Active kinases are subject to proteolytic activity and phosphatases are kinetically much faster than most kinases
- ***Cells must be "starved"*** synchronizing (into same cell cycle state) and making cells quiescent (resting or Go) by culturing cells 12-16 hours in "low serum" (0.5% FBS) medium. The length of time required depends on cell type. We find with fibroblasts this time works well. Longer incubation without FBS may induce apoptosis and that is another problem… It is best to carefully rinse cells with room temp PBS and replace with media that does NOT contain any FBS for one hour prior to agonist stimulation. During this time you can add inhibitors.

Cell Culture

1. Cells must be "starved" see note above and 70-80% confluent at time of agonist stimulation. There are two typical ways to approach such an experiment.

    a. Seed Day One early in the morning starve that night harvest the next day. Seed at a density such that in the morning of day two the cells will be at 70-80%. Seeding in the morning allows cells to adhere (~6 hours around 5pm) and followed by a gentle removal of media, washing with room temp PBS and replace with medium with 0.5% FBS. The cells must then be processed the next morning

    b. Seed day one, starve day two (3-4 pm) and harvest cells morning day 3. The cells must be seeded at a much lower density for this approach to work. Ensure to triterate well to avoid cells growing in clumps or islands. As for the two-day approach, remove old media after overnight starving, rinse with room temp PBS and replace with FBS free medium for one hour prior to adding agonist.

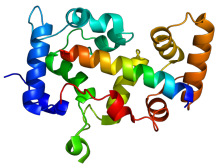
2. Most experiments will work using a 6 well or 35 mm dish. If needed use multiple dishes or a larger dish if the protein concentrations are too load.

3. Volumes. Use 1.0 ml of serum free medium with needed inhibitors or other treatments. Mix the inhibitors with the media prior to adding medium to the cells.

4. When adding agonist it is easy to a 1000X starting concentration of the agonist. If the vehicle (solvent) is DMSO (dimethylsulfoxide) must be less than 1% or preferably 0.5-0.1%. Add agonist while mixing (tipping the dish side to side) to ensure the agonist/solvent doesn't concentrate on an area directly underneath the pipettes.

5. Adding agonist does NOT need to be done sterilely. All prior work should be done aseptically. IF the agonist is sterile maintain the state of the agonist as others may need to use the agonist for longer incubations.

6. Timing. The amount of time of agonist incubation depends on agonist, cell type and cell function being measured. We find 5 – 30 min work best for most. Read literature to find appropriate time.



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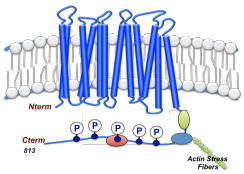

Harvesting – All work from here out MUST be done quickly and ON ICE with ICE COLD Solutions!

Solutions-Equipment: 1X SDS PAGE diluted with water and include phosphatase inhibitors (see below), cell scrapers – re-usable, PBS on ice, ice pan and aspirator system – ensure the second trap is in place, and 27 gage needle for sheering cells.

1. Use the large ice pan filled with ice. Set up an aspirator with glass pippett or a 200 ul pipette tip for aspirations.

2. Remove media and wash 3 times with ice cold PBS. Drip 200-500 ul of PBS. The exact volume is not critical. BUT do NOT wash off cells. From this point on the cells must be on ice. Be certain all of the PBS has been removed after the last wash. This is critical, residual PBS will dilute the protein. Tilt the dish ON ICE and let the residual solution pool at the bottom of the dish. Do this quickly and aspirate the media.

3. Once the last of the PBS has been removed in a timely fashion, add 0.1 ml of **1X** SDS-PAGE sample buffer containing sodium orthovanadate and ß glycerol phosphate. (phosphatase inhibitors should be made and frozen ahead of time as a 100X solution see info below)
   10 mM ß glycerol phosphate
   1 mM Na_3VO_4

4. Add 100 µl of SDS PAGE Sample Buffer (Lamelli SB)

5. Scrape the cells, being careful to scrape all of the cells towards the bottom of the dish

6. Lyse cells with 5 passes through a 27 gauge needle.

7. Boil the sample for 5 min and freeze if not continuing for the day. Spin down the samples for 5 min to avoid using ppt or aggregated sample.

8. Determine the protein assay using the BCA micro assay – follow the BCA micro assay protocol. Use 1X SDS PAGE sample buffer as a blank

9. Adjust samples to either (the same of the most dilute protein concentration) or (2 mg/ml). Load each sample with 10-50 µg protein. Higher mass of protein will likely result in a smearing or the bands, too little protein loaded may result in a difficult to detect westernblot. Start high and go less each time using a new antibody.

Prepare 10 ml of 100X phosphatase inhibitor solution (1.0 M ß glycerol phosphate and 100 µM orthovandadate)
Na Orthovanadate (use at final conc of 1.0 µM)

- Add 0.367 g of sodium orthovanadate to 10 ml miliQ water
- Adjust pH to 10.0. At this pH the solution will be pail yellow
- Boil the solution until it turns colourless (~10 min)
- Allow to cool to room temp and re-adjust to pH 10.0. If the solution turns yellow, boil and repeat until the pH stabilizes at pH10 AND remains colorless. Store in 0.5 ml aliquots at -20oC.

ß glycerol phosphate (use at 10 mM)

- Dissolve 2.16 g of disodium salt (mw=216) in 10 ml water. Freese in 0.5 ml aliquots at -20oC.

Prepare 1.0 ml SDS PAGE Buffer with phosphatase inhibitors.
250 µl 4X or 200 µl 5X SDS PAGE sample buffer with ß ME.
10 µl 100 X orthovanadate
10 µl 100 X ß glycerol phosphate
QS to 1.0 ml with water.