Western Blot Analysis of Endogenous Gene Expression

Experimental Design Considerations

Western blot analysis of endogenous protein levels can be performed using extracts generated in a variety of ways. To limit possible protein degradation and/or modification during the extraction steps (which is important for accurately determining relative expression levels), we use the simple procedure below which denatures most of the modification and degradation proteins immediately giving the most accurate read out of the true levels of protein at the time of harvest. However, in cases where detection is a problem, a limited purification (e.g. isolation of nuclear extract for the detection of transcription factors) might be required to allow analysis. In such cases, one can carry out the extraction and purification procedures in the presence of protease inhibitors and phosphatase inhibitors (see nuclear extract preparation methods) and hope that such precautions are sufficient to allow for a true reflection of protein levels at time of harvest.

Protocol

For isolation of relatively small numbers of cells (i.e. around one 100mm plate or one T150 flask). For larger amounts of cells, scale up accordingly.

- Harvest cells.
- Add 1ml of 1X PBS, suspend cells, and transfer to an eppendorf tube.
- Spin cells at 4K, 5 minutes in microfuge.
- Take off liquid (with 1ml pipette or with aspirator).
- Assess pellet size (we approximate size by taking another eppendorf tube and adding an amount that we think represents the pellet size. After comparing the accuracy of our guess, we guess again. This is not an exact science. Just get an estimate of pellet size).
- Add equal pellet volume of 1X PBS and suspend cells (very important).
- To lyse cells, add 10 pellet volumes of 1X SDS-PAGE loading buffer and immediately pipette up and down [be quick and vigorous (the soln will be thick and sloppy but pipette up and down as best you can – about 6-8 times)]. (Note: It is very important to pipette up and down immediately after adding SDS-PAGE loading buffer. So don't add loading buffer to several samples and then go back and mix).
- After suspending pellets, poke a hole in the top of the eppendorf tubes with a 20 gauge needle and incubate at 90-95^{o}C for 20-30 minutes (this shears the DNA and gets rid of viscosity).
- Vortex 20 seconds on high (if you have more than 300ul volume, be sure to cover the hole of the tube with your finger while you vortex). If the solution is still viscous, heat for another 5 minutes and vortex again. Store samples at –20^{o}C and they should be stable for years! Samples can be used directly for western blot analysis whenever you desire (however, heat again at 95^{o}C for five minutes before loading gel).

Note:

1) Since we suspend the pellets in a volume that is proportional to the pellet size, the protein concentrations should be nearly the same for different samples (within a single cell line, of course). Therefore, changes in endogenous protein expression can be analyzed by loading the same volume of extract in each lane.
2) The amount of sample that you can load onto an SDS-PAGE gel depends on the particular apparatus. However, with extracts prepared as described, we typically load between 50-100% of the loading capacity of gel apparatus' without any overloading problems.

Solutions

2X SDS-PAGE Loading Buffer**

6.25ml 1M Tris-Cl (pH 6.8)
6.25ml 80% Glycerol
10ml 10% SDS
2.5ml 2-Mercaptoethanol
2.5ml 0.05% (w/v) bromophenol blue
22.5ml H_2O
50ml = Total

**The above recipe is for 2X loading buffer. Make sure you dilute to 1X for the protocol written above.