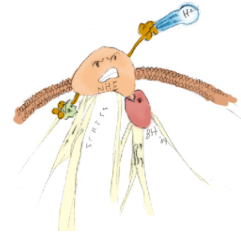


Protocol for Protein Assay with Cell Cultures – 96 well plate version



Protein assay protocol for cell cultures using a non-reducing, native lysate method

Introduction

It is important to realize that the protein kinases, phosphatases and proteases are all active once cells are lysed in a non-reducing sample buffer (without the Lamelli SDS PAGE sample buffer). If you do not stop the reactions, you will not be able to study the effects of the study you are working on. Therefore time and temperature are important keys to achieving repeatable and reliable results. Move quickly and keep the temp of ALL buffers and the cells at ice temp! On the ice bucket is not the same as IN the ice bucket. You can expect an 80% confluent, 35 mm dish of fibroblasts to have a 0.25-0.5 mg/ml protein concentration.

CELL LYSATE PREPARATION

1. Stimulate cells as normal
2. Wash the cells with ice cold PBS two times. Keep the plate of cells ON ice.
 - *If using suspension cells, pellet (1000 x g for 5 min at 4°C) the cells and wash in 1-5 ml of ice cold PBS. Repeat once.*
3. It is important to remove all of the PBS by canting the dish and draining the sheeting liquid. If you don't you will certainly get lots of variation in the protein concentration.
4. Add 1.0 ml of Lysis Buffer to 150 mm dish or 150 µl to 35 mM dishes.
5. Lyse cells with sonication in the cup sonicator. Use 4-10 second bursts. Place Ice in the cup to keep the temp down. Use enough water to cover the depth of the samples. If necessary, use a 26 or 27 gage needle to shear DNA.
6. Immediately pipet 50 µl of each sample into a new microfuge tube.
7. To the remaining sample (in the original tube), add 20µl of 5X SDS PAGE sample buffer sample and boil.

PROTEIN ASSAY (96 well plate method)

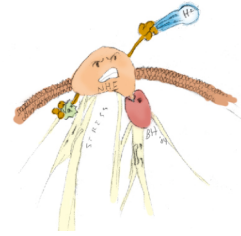
1. Prepare the following standards using the 1.0 mg/ml BSA in the freezer - 0.0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml BSA. Use lysis buffer for your dilutions. Make 1 ml of each BSA standard. These can be refrozen and used again.
2. Add 300 µl of 1X Bradford Protein Assay. (stock Biorad protein assay is 5X – dilute with water Use 10 ml of 5X BioRad Protein Assay to 40 ml of water in a falcon tube. Store at room temp).
3. Add 5 µl of each standard to a 96 well. Do each point in triplicate!
4. Add 5 µl of each unknown lysate sample to a separate well, again do in triplicate.
5. Rock to mix and read with the plate reader at 595 nm.
6. Use the standard protein concentrations to prepare a standard curve of abs vs. µg/µl. Convert mg/ml to µg/µl for ease of use. 1 mg/ml = 1 µg/µl
7. Create a standard curve with Abs on the Y axis and µg/µl on the X axis. Use this curve to calculate the protein concentration in your samples.
8. Adjust for the SDS PAGE buffer you added to the lysates in step 5 in lysates preparation by multiplying the concentration calculated with undiluted sample by 0.833.

WESTERN BLOT CALCULATION Remember that it is important to load equal amounts (mass) of protein in each lane. To do best do this, first we assume that you cannot load more than 30 µl safely on a gel. Then find the sample with the lowest protein concentration. This is the limiting step for maximum protein. *Ideally, it is good to add 1 – 20 µg of a lysate per lane. Too much and the lanes become fuzzy, too little protein and you won't see anything.* Determine the total amount of protein using 30 µl of this sample. Then use that number to calculate the volume to load of each sample.

Calc 1: 30µl x concentration of most dilute sample µg/µl = max protein µg

Calc 2: max protein µg / concentration µg/µl of each sample = volume to load in µl

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ASSAY COMPONENTS

LYSIS BUFFER – use to scrape cells when concerned about phosphorylation levels

Components	Stock Conc.	Final Conc.	mls in 20mls
Tris Buffer, pH 7.5	100 mM	50 mM	10
EDTA	500 mM	1 mM	0.04
EGTA	200 mM	1 mM	0.1
Activated Na ₃ VO ₄	200 mM	0.25 mM	0.1
Na β-glycerophosphate	1 mM	10 mM	0.2
Na fluoride	500 mM	50 mM	2
Triton X-100	10%	0.5%	1
Protease Inhibitors	Use one tablet per 20 ml cocktail		
QS w/ mQ water to 20 ml			

Freeze in 1.0 ml aliquots and do not re-use.

STOCK SOLUTIONS USED TO MAKE LYSIS BUFFER

Sodium Orthovanadate Activation (Na₃VO₄) *Sodium orthovanadate should be activated for maximal inhibition of protein phosphotyrosyl-phosphatases.* This procedure depolymerizes the vanadate, converting it into a more potent inhibitor of protein tyrosine phosphatases.

1. Prepare a 200 mM solution of sodium orthovanadate. (Prepare 100 ml)
2. Adjust the pH to 10.0 using either 1N NaOH or 1N HCl. The starting pH of the sodium orthovanadate solution may vary with lots of the chemical. At pH 10.0 the solution will be yellow.
3. Boil the solution until it turns colorless (approximately 10 minutes).
4. Cool to room temperature.
5. Readjust the pH to 10.0 and repeat steps 3 and 4 until the solution remains colorless and the pH stabilizes at 10.0.
6. Readjust the final volume to 100 ml with water.
7. Store the activated sodium orthovanadate as aliquots at -20°C.

Sodium Fluoride (Sodium NaF) – a phosphatase inhibitor

- Prepare a 500 mM stock solution: store at room temperature

Sodium β-glycerophosphate (BGP) – A general phosphatase inhibitor store at room temp.

Protease Inhibitors – If no tablets of protease inhibitor cocktail are available, a single inhibitor, Phenylmethylsulfonyl fluoride (PMSF) (200 mM stock solution in isopropanol; store at room temperature) can be use.

Note: *Ideally, the remaining protease and phosphatase inhibitors should be added to the solution on the same day you are running the assay (100 µl of aprotinin, leupeptin, and pepstatin; 500 µl of PMSF, Na₃VO₄, and NaF), but with the exception of PMSF the diluted inhibitors are stable in aqueous solution for up to 5 days. PMSF is extremely unstable in aqueous solutions, with a half-life of approximately 30 minutes, and it should be added immediately before use.*