Determining half-lives of oligomeric proteins by cycloheximide blocking and non-dissociating Western blot

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Abstract

The procedures to determine the half-lives of proteins by pulse-chase analysis and cycloheximide blocking are well established. In the case of oligomeric proteins, these methods do not differentiate whether the stabilities of the monomeric versus oligomeric forms of the protein differ. This method describes how to determine the half-lives of oligomeric proteins that are stable in the presence of low concentrations of the detergent SDS by combining cycloheximide blocking with non-dissociating Western blot (Dutta et al., 2011). The addition of cycloheximide inhibits protein synthesis and non-dissociating Western blot uses low concentrations of SDS to separate stable oligomers from monomers. The amount of the protein of interest detected in samples taken at different times after adding cycloheximide can be used to determine the half-life of the oligomeric protein.

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Protocol

Cells

Step 1.

Seed BSC-1 cells in 6-well plates (5 x 10^5 cells in 3 ml MEM-7.5 FBS per well). Incubate until confluent (12-24 hours) at 37° C and 5% CO2.

Protein expression

Step 2.

Use the VOTE (vaccinia virus/*lac* operon/T7 RNA polymerase/encephalomyocarditis virus IRES [developed by B. Moss, National Institutes of Health, Bethesda, MD]) for expression of species A rotavirus NSP3 or the protein of interest. Infect the cells with recombinant vaccinia virus in one ml of MEM-2.5% FBS using a MOI of 10. Allow the virus to adsorb for two hours swirling gently every 30 min to mix. Aspirate the virus inoculum and add the inducer isopropyl β-D-1-thiogalacopyranoside (0.4 mM) in MEM-2.5% FBS. Incubate 12 hours at 37° C and 5% CO2.

Cycloheximide treatment

Step 3.

Aspirate medium and wash twice with MEM-2.5% FBS. Add two ml MEM-2.5% SFB containing 100 µg/ml cycloheximide and either 0.5 µ MG132 or its diluent dimethylsulfoxide (DMSO). Use as stock 6.25 mM MG132 in DMSO and dilute 1:100 in MEM prior to addition to the cells. Incubate for additional 0, 0.5, 1, 2 and 4 hours.

Harvesting and cells lysis

Step 4.

Scraping cells in 1 mL of cold PBS. Centrifuge at 13.200 g for 10 minutes at 4 ° C. Cell pellet (1.25 x 10^5 cells) is resuspended in 20 ul of lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.4 U / mL DNase I, 0.01 mg / mL RNase A and Complete® protease inhibitor [Roche]). Incubate during 10 minutes at room temperature.

Electrophoresis

Step 5.

Add 20 µl of non-dissociating 2X Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol and 0.8% SDS) to the cell lysate. Incubate 30 minutes on ice. Separate the proteins by running 12% SDS-PAGE in a cold room during 16 hours (4 mA for 0.75 mm gels prepared in 16 x 18 cm plates). Use one lane of the gel for molecular weight markers.

Protein blotting

Step 6.

Transfer proteins to PVDF membranes during 2 hours in semidry system using dissociating transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS and 20% (v/v) methanol).

Antibody staining

Step 7.

Block the PVDF membrane in 5% nonfat dry milk in TBS. Incubate the membrane overnight at 4° C with rat anti-NSP3 (1:20,000) in 1% non-fat dry milk, 0.05% tween-20 in TBS. Incubate the membrane two hours at room temperature with horseradish peroxidase-conjugated anti rat IgG (1:20,000) in 1% non-fat dry milk, 0.05% tween-20 in TBS. Develop the blots with Supersignal West Femto chemilumniescence substrate according to manufacturer's instructions (Pierce, Rockford IL). Perform densitometry analysis with Image J software.