Western blot analysis of sub-cellular fractionated samples using the Odyssey Infrared Imaging System

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Method Article

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Abstract

Introduction

This protocol describes nuclear and cytoplasmic fractionation of tissue culture cells and a method for Western blot detection of proteins using the Odyssey Infrared Imaging System. This protocol was used to detect expression of the "small" Tap protein in 293T, HeLa and COS cells. The Odyssey system has several advantages over the more widely used chemiluminescent detection methods: 1) both Alexa Fluor 680 and IRDye800 are stable and the membranes can be stored before scanning 2) in general this system allows better quantification 3) the two-color detection system makes it possible to simultaneously detect two different proteins using secondary antibodies from different species \(see Figure 1) 4) electronic data files allow the intensity of bands to be varied as needed.

Reagents

•Triton X-100 lysis buffer: 50 mM Tris-HCl \(pH 7.5), 0.5% Triton X-100, 137.5 mM NaCl, 10% Glycerol, 1 mM sodium vanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 5 mM EDTA, and Protease Inhibitor Cocktail \(SIGMA; P2714) •2xSDS-PAGE sample buffer: SDS-PAGE sample buffer; 125 mM Tris-HCl \(pH 6.8), 4% SDS, 2% 2-mercaptoethanol, 20% glycerol, and 0.3% BTB •Pre-stained blue protein size maker: Precision Plus Protein Standards \(Bio-Rad) •PVDF membrane: Immobilon-FL membrane \(Millipore) •Transfer buffer: 3.33 g Trizma base and 14.4 g Glycine /1L H_2O with 10% MeOH, prepare 10X buffer without MeOH. •Primary antibody •Secondary antibodies 1) anti-rabbit IgG: Alexa Fluor 680 goat anti-rabbit IgG \(Molecular probes, Invitrogen, http://probes.invitrogen.com, #A21076) use 1:50,000 dilution 2) anti-mouse IgG: IRDye800 conjugated anti-mouse IgG \(Rockland Immunochemicals, http://www.rockland-inc.com/, #610-132-121) use 1:20,000 dilution 3) anti-goat IgG: Alexa Fluor 680 rabbit anti-goat IgG \(Molecular Probes, Invitrogen, http://probes.invitrogen.com, #A21088) use 1: 20,000 dilution •PBS •PBST: 0.1% Tween-20 in PBS •Bovine serum albumin fraction V \(Sigma, A9647) •Blotting buffer: 2% BSA and 0.05% sodium azide in PBST •Sodium azide \(Sigma, S8032)

Equipment

•Sonicator: Sonifier 450 \(Brandson) with microtip \(101-148-0062) •Table top microcentrifuge •SE 260 Mini-Vertical Unit \(Amersham Biosciences, 80-6149-35) •TE22 Mini Tank Transfer Unit \(Amersham Biosciences, 80-6204-26) • Odyssey Infrared Imaging System \(LI-COR Biosciences)

Procedure

A. Quick fractionation of membrane/cytoplasmic and nuclear proteins 1. Lyse cells with 0.5% Triton X-100 lysis buffer. 2. Incubate on ice for 15 min. 3. Separate insoluble nuclei by centrifugation at 13 krpm for 15 min at 4˚C with a tabletop centrifuge \(Eppendorf). 4. Transfer the supernatant \(membrane/cytoplasmic fraction) into a new Eppendorf tube. Take small aliquot for DNA contamination detection. 5.

Rinse the nuclear pellet with the lysis buffer once, then re-suspend in the lysis buffer containing 0.5% SDS and shear released genomic DNA by sonication for 5 seconds with a Brandon Sonifier 450 at output 2. Pre-clear by centrifugation at 13 krpm for 15 min at 4˚C with a tabletop centrifuge. Transfer supernatant to a new tube. 6. Add an equal amount of 2x SDS-PAGE sample buffer to the tubes containing the nuclear and membrane/cytoplasmic fractions and boil both tubes for 10 min. 7. Successful fractionation can be verified by lack of detectable DNA in the membrane/cytoplasmic-fraction using by agarose gel electrophoresis and of beta-tubulin in the nuclear fraction as detected by Western blotting. **B. Western blot analysis using the Odyssey Infrared Imaging System** 1. By using the pre-stained blue protein size markers \(Precision Plus Protein Standards; Bio-Rad) on the SDS-PAGE, the blue marker bands can be visualized later by the Odyssey Infrared Imaging System as orange in the 700 nm window. 2. Using the SE 260 Mini-Vertical Unit, the gel is run at 15 mA while the dyefront is in the stacking gel \(about 90 min). When the dyefront reaches the separation gel increase to 30mA per gel \(about 90 min). 3. Prepare Immobilon-FL membrane by wetting in MeOH for 15 sec, then rinse with ddH_20, and pre-soak in the transfer buffer for more than 30 min. 4. Transfer onto the pre-soaked Immobilon-FL membrane. Use pre-chilled transfer buffer and use a cooling apparatus or keep in the refrigerator with stirring. When using the TE22 Mini Tank Transfer Unit, the guidelines for transfer are: 100 V for 70 min for 50-80 kDa proteins, 90 V for 80 min for 20-50 kDa proteins, but the transfer condition might need to be customized depending on the protein that is to be detected. 5. Rinse the membrane with PBST. 6. Block the membrane with 5% fat-free dried milk in PBST for 30 min at room temperature with gentle agitation. Rince out dried milk with PBST before the next step. 7. Incubate the membrane with primary antibody diluted in the blotting buffer \(1:500-2,000 for most antibodies) for one hour at room temperature with gentle agitation on a rocking plate. These primary antibody solutions can be stored at 4˚C and used several times. 8. Wash the membrane four times with PBST for 15 min each. 9. Incubated the membrane with secondary antibodies diluted in 5% fat-free dried milk in PBST \(see Materials, Reagents for dilutions) for 45 to 60 min at room temperature with gentle agitation on a rocking plate. From this step, keep the membrane away from light. 10. Wash the membrane four times with PBST for 15 min each. 11. Finally, rinse the membrane with PBS before scanning using the Odyssey system. Odyssey system settings: 1) preset: membrane 2) resolution:169 mm 3) quality: medium 4) focus offset: 0.0. Alexa Fluor 680 is detected as orange in the 700 nm window and IRDye 800 is detected as green in the 800nm window.

Timing

About 9.5 hours Sample preparation; 1 hour, SDS-PAGE; 3 hours, Transfer; 1 hour, Western blotting; 4 hours, Scanning and analysis; 30 min

Critical Steps

Do not try to lyse too many cells. It causes contamination of membrane/cytosolic proteins in the nuclear fraction. The appropriate number of cells depend on which cell type you use. For example you can lyse more cells if you use T-cells, since the protein amount per cell is lower.

Troubleshooting

High background: a)Try to soak the membrane in transfer buffer longer. Prior to transfer, we usually keep the membrane in transfer buffer more than 30 min after MeOH activation. b) Too much primary and/or secondary antibodies used. If necessary, perform a titration experiment with the primary antibody to determine the optimal dilution.

Anticipated Results

Proteins will be detected as green or orange bands on the membrane. Pre-stained blue size marker: detected as orange, IRDye800: green, Alexa Fluor 680: orange

Figures

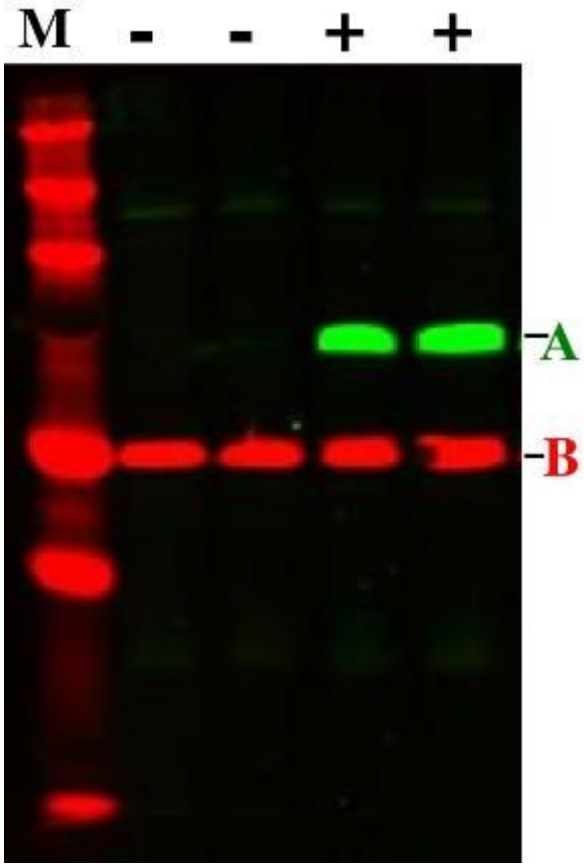

Figure 1

Western blot detection of two different proteins using the Odyssey Infrared Imaging System (2 colour detection) FLAG-tagged proteins (A) in 293T cells transfected with a plasmid expressing a FLAG-tagged version of the mouse Sam68 protein were detected by using an anti-FLAG monoclonal antibody and IRDye800 conjugated anti-mouse IgG as the secondary antibody. As a loading control, beta-tubulin (B) was detected in both transfected and untransfected cells using anti-tubulin rabbit polyclonal antibodies

and Alexa Fluor 680 goat anti-rabbit IgG. M: prestained blue protein size marker, -: untransfected cells +: transfected cells.