Western Blot for Detecting Phosphorylated STAT3

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[Abstract] The STAT3 transcription factor is an important signaling molecule for many cytokines and growth factor receptors and is constitutively activated in a number of human tumors and possesses oncogenic potential and anti-apoptotic activities. STAT3 is activated by phosphorylation at Tyr705, which induces dimerization, nuclear translocation, and DNA binding. Western blot is most commonly used to detect the activation of STAT3 by using an antibody that is specific for the phosphorylated tyrosine705.

Materials and Reagents

1. Tumor cell lines with constitutive activation of STAT3 (positive control).
   a. DU145 (ATCC, catalog number: HTB-81™)
   b. HepG2 (ATCC, catalog number: HB-8065™)
   c. Hep3B (ATCC, catalog number: HB-8064™)
   d. Huh7
2. Phospho-Stat3 (Tyr705) (D3A7) XP™ Rabbit (Cell Signaling Technology, catalog number: 9145)
3. Stat3 (124H6) Mouse mAb (Cell Signaling Technology, catalog number: 9139)
4. HRP Goat Anti-Rabbit I (BD Biosciences, catalog number: 554021)
5. β-Actin (13E5) Rabbit mAb (Cell Signaling Technology, catalog number: 4970)
6. Anti-mouse IgG, HRP-linked Antibody (Cell Signaling Technology, catalog number: 7076)
   Note: The above antibodies have been tested by the author and may be substituted with the antibodies desired by users.
7. Phosphate buffered saline (PBS)
8. 1x halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, catalog number: 78440)
9. M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, catalog number: 78501)
10. Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, catalog number: 500-0006)
11. 10x Tris/Glycine/SDS (Bio-Rad Laboratories, catalog number: 161-0771)

12. Methanol (Thermo Fisher Scientific, catalog number: A412-20)
13. Tris buffered saline (Bio-Rad Laboratories, catalog number: 170-6435)
14. Tween-20 (Santa Cruz Biotechnology, catalog number: sc-29113)
15. Bovine serum albumin (BSA) (MP Biomedicals, catalog number: 810033)
16. Supersignal west Dura extended duration substrate (Thermo Fisher Scientific, catalog number: 34075)
17. Precision plus protein dual color standards (Bio-Rad Laboratories, catalog number: 161-0374)
18. Restore plus western blot stripping buffer (Thermo Fisher Scientific, catalog number: 46430)
19. Protein lysis buffer (see Recipes)
20. Electrophoresis buffer (see Recipes)
21. 1x Tris buffered saline (TBS) (see Recipes)
22. Transfer buffer (see Recipes)
23. Blocking buffer (see Recipes)
24. Wash buffer (see Recipes)
25. Primary antibody dilution buffer (see Recipes)
26. Blotting membrane (see Recipes)

Equipment

1. Microcentrifuges (Eppendorf, model: 5415 R)
2. Thermolyne Rotomix (BioSurplus, model: 50800)
3. Microcentrifuge tubes
4. Nitrocellulose or PVDF membrane
5. SmartSpec plus spectrophotometer (Bio-Rad Laboratories)

Procedure

A. Protein blotting

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1x PBS; aspirate.
3. Lyse cells by adding 1x protein lysis buffer.
4. Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube.
5. Incubate at room temperature (RT) for 5 min.
6. Microcentrifuge for 10 min at 13,000 rpm.

7. Transfer supernatant to a clean microcentrifuge tube.
8. Measure the protein concentration using the Bio-Rad protein assay dye reagent.
9. Heat a 20 µg sample to 95-100 °C for 10 min; cool on ice.
10. Load 20 µg sample onto SDS-PAGE gel (10 cm x 10 cm) and load 7 µl precision plus protein fual color standards to determine molecular weights.
11. Electrophoresis at constant 80 Volts until the protein dye reaches the bottom of the gel.
12. Electrotransfer to nitrocellulose or PVDF membrane using transfer buffer (constant 30 Volts overnight at 4 °C).

B. Membrane blocking and detection of pSTAT3

1. Incubate membrane in 25 ml of blocking buffer for 1 h at RT.
2. Wash three times for 5 min each with 15 ml of TBS/T.
3. Incubate membrane and primary phospho-Stat3 (Tyr705) (D3A7) XP™ rabbit antibody (1: 1,000) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4 °C.
4. Wash three times for 5 min each with 15 ml of TBS/T.
5. Incubate membrane with HRP-conjugated goat anti-rabbit secondary antibody (1:5,000) and HRP-conjugated anti-biotin antibody (1:1,000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 h at RT.
6. Wash three times for 5 min each with 15 ml of TBS/T.
7. Incubate membrane with 4 ml Supersignal west dura extended duration substrate with gentle agitation for 5 min at RT.
8. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10 sec exposure should indicate the proper exposure time.

C. Detection of STAT3

1. Wash the membrane three times for 5 min each with 15 ml of TBS/T.
2. Incubate membrane with 10 ml restore plus western blot stripping buffer for 15 min to stripe the bonding antibodies.
3. Wash the membrane three times for 5 min each with 15 ml of TBS/T.
4. Repeat steps 11-18 and use Stat3 (124H6) mouse mAb as primary antibody and HRP-conjugated anti-mouse IgG as secondary antibody.

D. Detection of β-actin

1. Repeat steps 19-22 for β-actin detection.

E. Analyzing the level of STAT3 activation

1. Scan the film and measure the intensity of pSTAT3 and STAT3 bands. Calculate the STAT3 activation using the following formula:
   Percentage of STAT3 activation = (intensity of pSTAT3)/ (intensity of STAT3 + intensity of pSTAT3) x 100%.

Recipes

1. Electrophoresis buffer
   To prepare 1 L buffer, add 100 ml 10x Tris/glycine to 900 ml ddH_2O.
2. Protein lysis buffer
   M-PER mammalian protein extraction reagent with 1x halt protease and phosphatase inhibitor cocktail.
3. Transfer buffer
   To prepare 1 L buffer, add 100 ml 10x Tris/Glycine/SDS and 200 ml methanol to 700 ml ddH_2O.
4. 1x TBS
   To prepare 1 L of 1x TBS, add 100 ml 10x TBS to 900 ml ddH_2O.
5. Blocking buffer
   1x TBS, 0.1% tween-20 with 5% (w/v) BSA.
6. Wash buffer (TBS/T)
   1x TBS, 0.1% tween-20
7. Primary antibody dilution buffer
   1x TBS, 0.1% tween-20 with 5% BSA
8. Blotting membrane
   This protocol has been optimized for nitrocellulose membranes, which CST recommends. PVDF membranes may also be used.

References

1. Campbell, C. L., Jiang, Z., Savarese, D. M. and Savarese, T. M. (2001). Increased expression of the interleukin-11 receptor and evidence of STAT3 activation in prostate carcinoma. *Am J Pathol* 158(1): 25-32.
2. Fujimoto, M., Naka, T., Nakagawa, R., Kawazoe, Y., Morita, Y., Tateishi, A., Okumura, K., Narazaki, M. and Kishimoto, T. (2000). Defective thymocyte development and perturbed homeostasis of T cells in STAT-induced STAT inhibitor-1/suppressors of cytokine signaling-1 transgenic mice. *J Immunol* 165(4): 1799-1806.