INSTRUCTIONS

O-GlcNAc Western Blot Detection Kit

24565

1435.4

Number	Description
24565	***O*-GlcNAc Western Blot Detection Kit,** contains sufficient reagents for approximately 10 mini-blots

Kit Contents:

Part A:

M-PER™ Mammalian Protein Extraction Reagent, 25mL, store at room temperature

Dilution Buffer (also functions as Blocking Buffer) (10X), 2 × 50mL, store at 4°C

BupH™ Phosphate Buffered Saline, 17 packs, store at room temperature

Surfact-Amps™ 20 (10% Tween™-20 Detergent), 3 × 10mL, store at room temperature

Goat Anti-Mouse IgM (μ), HRP Conjugate, 75μg, store at 4°C

SuperSignal™ West Dura Luminol/Enhancer Solution, 50mL, store at room temperature

SuperSignal West Dura Stable Peroxide Solution, 50mL, store at room temperature

Part B:

Anti-*O*-GlcNAc Monoclonal Antibody (MAb CTD 110.6), 100μL, store at -20°C

***O*-GlcNAc Western Blot Positive Control** (*O*-GlcNAc-modified BSA), 100ng, store at -20°C

Storage: Upon receipt store components as indicated. The Anti-*O*-GlcNAc Antibody is shipped with dry ice, and all other components are shipped at ambient temperature.

Introduction

The Thermo Scientific™ *O*-GlcNAc Western Blot Detection Kit is a complete kit designed for the detection of *O*-GlcNAc-modified proteins. This kit contains the most specific mouse monoclonal antibody available for on-membrane detection of the *O*-GlcNAc posttranslational modification and an extremely sensitive chemiluminescent substrate.

O-GlcNAc, the modification of serine and threonine residues of nuclear and cytoplasmic proteins with *O*-linked β-N-acetylglucosamine, is one of a growing number of post-translational modifications thought to modulate the function/activity of proteins in cells. This modification appears to be strictly intracellular, in contrast to classical protein glycosylation. Like phosphorylation, *O*-GlcNAc is highly dynamic with rapid cycling in response to cellular signals or cellular stages and occurs at sites on the protein backbone that are similar to those modified by protein kinases. Recently, perturbations in the regulation of *O*-GlcNAc have been implicated in the etiology of type II diabetes, cancer and neurodegenerative diseases. However, studies to understand the functional role of *O*-GlcNAc in cellular regulation have been hampered by the lack of highly specific and sensitive methods to detect *O*-GlcNAc.

The monoclonal antibody included in the Pierce *O*-GlcNAc Western Blot Detection Kit reacts only to the β-*O*-linked serine or threonine GlcNAc modification. There is no cross-reactivity with the α-*O*-GlcNAc linkage, the α/β-*O*- GalNAc modification or the other *N*-linked oligosaccharides. Furthermore, Thermo Scientific™ SuperSignal™ West Dura Extended Duration Substrate allows low picomole sensitivity and *O*-GlcNAc-modified protein detection in less than one minute after exposing the blot to X-ray film.

Additional Materials Required

- Western blot membrane: Use any suitable protocol to separate proteins by electrophoresis and transfer them to a nitrocellulose membrane. Other membrane types may be used; however, optimization may be required.
- Protease inhibitors (e.g., Thermo Scientific™ Halt™ Protease Inhibitor Kit, Product No. 78410)
- PUGNAc (*O*-[2-acetamido-2-deoxy-D-glucopyranosylidene]amino *N*-phenyl carbamate), available from Toronto Research Chemicals (Product No. A157250)
- Glucosamine
- X-ray film (e.g., Thermo Scientific™ CL-XPosure™ Film, Product No. 34090)
- Film cassette, developing and fixing reagents for processing X-ray film
- Rotary platform shaker for agitation of membrane during incubations

Material Preparation

Wash Buffer	Reconstitute one BupH Phosphate Buffered Saline **(**PBS) pack with 500mL of ultrapure water and add 2.5mL of 10% Tween-20 Detergent for a final concentration of ~0.05%.
Blocking Buffer	Dilute the 10X Dilution Buffer (also functions as Blocking Buffer) to 1X in Wash Buffer (e.g., add 1mL of 10X Dilution Buffer to 9mL of Wash Buffer).
Goat Anti-Mouse IgM-HRP Conjugate	Prepare a 1mg/mL stock solution by reconstituting the antibody in 75µL of ultrapure water. Once reconstituted the antibody is stable for approximately one month at 4°C. Alternatively, aliquot the stock solution into single-use volumes, add an equal volume of glycerol and store for up to one year at -20°C. Note that adding glycerol will change the stock concentration to 0.5mg/mL.
O-GlcNAc Western Blot Positive Control	Reconstitute in 100µL of ultrapure water and apply 5µL (5ng) per well. Store at -20°C.

O-GlcNAc Western Blot Procedure

Note: Lysate from Jurkat cells can be prepared according to the following protocol. Alternatively, lysate may be prepared from the cell line of choice according to published protocols.

A. Preparation of Cell Lysates

1. Grow Jurkat cells according to standard protocol.
2. Three hours before collection add PUGNAc and glucosamine to cells at a final concentration of 50µM and 4mM, respectively.
3. Pellet the cells by centrifugation at 400 × *g* for 10 minutes at 4°C.
4. Discard the supernatant and wash the cells once by resuspending the cell pellet in wash buffer. Pellet the cells by centrifugation at 400 × *g* for 10 minutes at 4°C.
5. Add protease inhibitors (e.g., Halt Protease Inhibitor Kit, Product No. 78410) to the Thermo Scientific™ M-PER™ Mammalian Protein Extraction Reagent before adding to the cell pellet.
6. Add M-PER Reagent to the cell pellet. Use at least 10mL of M-PER Reagent for each 1 gram of wet cell pellet.
7. Mix and incubate the cells at room temperature for 10 minutes. Centrifuge cells at 18,000 × *g* for 5 minutes at 4°C to remove cell debris. Transfer supernatant (i.e., cell lysate) to a new tube.

B. Western Blotting and Detection

1. Separate 25µg of the cell extract and optionally, 5ng (5µL) of the O-GlcNAc Positive Control using a 10% or 4-20% Tris-Glycine SDS gel and transfer the proteins to a nitrocellulose membrane.
2. Remove blot from the transfer apparatus and block nonspecific sites on the membrane by completely immersing the membrane with blocking buffer (approximately 50mL) for 1 hour at room temperature (RT) or overnight at 4°C.
3. Prepare a 1:5000 dilution of the Anti-*O*-GlcNAc Monoclonal Antibody in Blocking Buffer. An example of a 1:5000 dilution is to add 6µL of the stock antibody solution to 30mL of Blocking Buffer.

   Note: Aliquot the Anti-*O*-GlcNAc Monoclonal Antibody in single-use volumes and store at -20°C.
4. Remove Blocking Buffer and add ~30mL of the diluted antibody to the membrane. Incubate blot for 1 hour with shaking at RT.
5. Wash membrane six times by completely immersing in wash buffer (approximately 50mL) and agitating for 5 minutes.
6. Prepare a 10µg/ml secondary antibody solution by diluting the 1mg/mL stock solution 1:100 in Blocking Buffer (e.g., add 5µL of stock solution to 495µL of Blocking Buffer). Prepare approximately 30mL of a 1:5000 dilution of the 10µg/mL Goat Anti-Mouse IgM-HRP in Blocking Buffer.

   Note: Discard excess 10µg/mL antibody solution.
7. Remove Wash Buffer, add the diluted secondary antibody and incubate for 1 hour at room temperature with shaking.
8. Wash the membrane 4-6 times by completely immersing the membrane in Wash Buffer (approximately 50mL) and agitating for 5 minutes each.
9. Prepare SuperSignal West Dura Working Solution by mixing 5mL of the Stable Peroxide Solution and 5mL of the Luminol/Enhancer solution. Apply Working Solution to the membrane and incubate for 5 minutes at room temperature. Use 0.12mL substrate solution per cm^2 of membrane. The Working Solution is stable for 24 hours at room temperature.

   Note: Exposure to the sun or any other intense light can harm the working solution. For best results keep the working solution in an amber bottle and avoid prolonged exposure to any intense light. Short-term exposure to typical laboratory lighting will not harm the working solution.
10. Remove blot from Working Solution and place it in a plastic membrane protector. A plastic sheet protector works very well, although plastic wrap may also be used. Use an absorbent tissue to remove excess liquid and to carefully press out any bubbles from between the blot and the membrane protector.
11. Place the protected membrane in a film cassette with the protein side facing up. Turn off all lights except those appropriate for film exposure (e.g., a red safelight).

    Note: Film must remain dry during exposure. Use gloves during the entire film-handling process.
12. Carefully place film on top of the membrane. Expose film for 30 seconds. Exposure times may be varied to achieve optimal results.

    Note: If using a phosphor imaging device (e.g., Bio-Rad's Molecular Imager™ System) or a CCD Camera (e.g., Alpha-Innotech Corporation's ChemiImager™ System), longer exposure times may be necessary.
13. Develop film using appropriate developing solution and fixative.

    Note: After film has been developed, the exposure time may be varied to achieve optimal results. If signal is too intense, reduce the exposure and development incubation times or optimize the system by decreasing the antigen and/or antibody concentrations. Alternatively, use Thermo Scientific™ Pierce™ Background Eliminator (Product No. 21065). Blot may be stripped and re-probed if necessary. For best results, use Thermo Scientific™ Restore™ Western Blot Stripping Buffer (Product No. 21059).

Troubleshooting

Problem	Possible Cause	Solution
Reverse image on film (i.e., white bands with a black background)	Too much HRP in the system	Dilute HRP-conjugate at least 10-fold
Membrane has brown or yellow bands		
Blot glows in the darkroom		
Signal duration is less than 24 hours		
Weak or no signal	Too much HRP in the system caused signal to fade quickly	Dilute HRP-conjugate at least 10-fold
	Insufficient quantities of antigen or antibody	Increase amount of antibody or antigen
	Inefficient protein transfer	Optimize transfer
	Reduction of HRP or substrate activity	*See note below
High background	Too much HRP in the system	Dilute HRP-conjugate at least 10-fold
	Inadequate washing	Increase length, number or volume of washes
	Film has been overexposed	Decrease exposure time or use Erase-It® Background Eliminator
	Antigen or antibody is too concentrated	Decrease amount of antigen or antibody
Spots within the protein bands	Inefficient protein transfer	Optimize transfer procedure
	Unevenly hydrated membrane	Perform manufacturer's suggestions for membrane hydration
	Bubble between the film and the membrane	Remove all bubbles before exposing blot to film
Speckled background on film	Aggregate formation in the HRP-conjugate	Filter conjugate through a 0.2µm filter

*To test the activity of the system in the darkroom, prepare 1-2mL of the SuperSignal Substrate Working Solution in a clear test tube. With the lights turned off, add 1µL of undiluted HRP-conjugate to the Working Solution. The solution should immediately emit a blue light that will fade during the next several minutes.

Related Thermo Scientific Products

21065 **Pierce Background Eliminator Kit,** for eliminating background from film

23225 **BCA Protein Assay Reagent Kit**

78410 **Halt™ Protease Inhibitor Cocktail Kit**

88013 **Nitrocellulose Membrane (7.9 cm × 10.5 cm),** 15/pkg

34090 **CL-XPosure Film (5" × 7" sheets),** 100 sheets/pkg

21059 **Restore Western Blot Stripping Buffer,** 500mL

General References

Comer, F.I., *et al.* (2001). Characterization of a mouse monoclonal antibody specific for *O*-Linked N-acetylglucosamine. *Anal Biochem* **293:**169-77.

Zachara, N.E. and Hart, G.W. (2002). The emerging significance of *O*-GlcNAc in cellular regulation. *Chem Rev* **102:**431-8.

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