

THE USE OF BIOTINYLATED TUBES FOR DETECTION OF POLYUBIQUITINATED PROTEINS BY LIGAND BLOTTING

Steven J. Orcutt and Yilin Yan
Senior Scientists
LifeSensors, Inc.

BACKGROUND

Ubiquitin and Polyubiquitylation in Cellular Processes

Ubiquitin is a small polypeptide that can be conjugated via its C-terminus to lysine residues on target proteins in either monomer form or as polymer chains. The best characterized of this polyubiquitylation is via linkage at either lysine 48 (K48) or 63 (K63) of ubiquitin itself (1). Polyubiquitin modification is, however, a reversible process as these chains are degraded and/or removed by a class of proteases known as deubiquitylases (DUBs). The most prevalent consequence of polyubiquitylation is the proteasome-mediated degradation of target proteins, with the dynamic nature of this signaling greatly impacting both fate and function of many proteins (2). The isolation and identification of these polyubiquitinated proteins, either by western blotting or mass spectroscopy, is critical to illuminating ubiquitin mediated regulation of certain cellular processes and their associated disease states (3).

Unfortunately, traditional techniques for isolation of ubiquitylated proteins suffer from a number of disadvantages. These techniques often rely on the overexpression of fusion-tagged ubiquitin for conjugation to the protein of interest. Tools for the isolation of native ubiquitin do exist, such as ubiquitin binding associated domains (UBAs), based on their natural affinity for ubiquitin (4,5). Similar to ubiquitin antibodies, however, these reagents display a poor affinity for ubiquitin and polyubiquitin chains. For this reason, determination of a protein's ubiquitylation state can often be difficult to detect at best, and subject to experimental artifact at worst.

TUBES: A Revolution in Polyubiquitin Isolation and Characterization

To overcome these obstacles, novel tools have recently been developed by Dr. Manuel Rodriguez and his team at CIC bioGUNE through the generation of Tandem Ubiquitin Binding Entities (TUBEs) (6). TUBEs are essentially tandem UBAs that bind polyubiquitin chains with dissociation constants in the nanomolar range, and have been demonstrated to protect proteins from both deubiquitylation and proteasome-mediated degradation. The nanomolar affinity of TUBEs for polyubiquitylated proteins allows for high efficiency in isolation and characterization of these proteins from cell lines, tissues and organs. TUBEs are rapidly becoming established as one of the most sensitive and versatile research tools for the exploration of the ubiquitin-proteasomal pathway.

PROBING FOR POLYUBIQUITYLATED PROTEINS WITH BIOTIN-TUBES BY LIGAND BLOTTING

When coupled with an appropriate immunoprecipitation (IP) strategy, western blotting for polyubiquitin can be a powerful technique to researchers for the identification or confirmation of polyubiquitinated proteins. However, generating an immune response to a protein integral to a broad range of cellular processes, such as ubiquitin, is expectedly difficult. Accordingly, most polyclonal antibodies currently used for the detection of ubiquitin are less than ideal. Low reactivity and non-specific background can limit detection, while techniques meant to enhance epitope recognition (e.g. treatment with heat or chaotropes) are time consuming and yield results of variable quality.

Biotin-TUBES represent a major leap forward in ubiquitin detection by western blotting. Essentially, ligand blotting (also known as "far western" blotting) for ubiquitin employs biotin-TUBEs substituted for the anti-ubiquitin immunoglobulin as the primary detection reagent. Secondary detection is then accomplished with a streptavidin enzyme conjugate. Ligand blotting with TUBEs requires only minor modifications to existing immunoblot protocols, the most notable of which is the omission of any denaturing steps. Biotin-TUBEs can be used either for the assessment of global polyubiquitylation, or (in combination with an IP strategy) for the identification of discrete polyubiquitinated proteins.

LIGAND BLOTTING METHODOLOGY

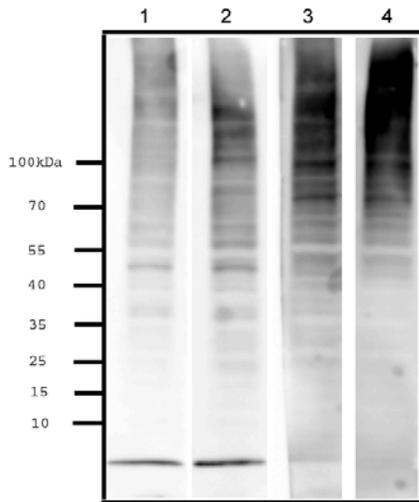
Approximately 40µg of protein extract from Neuro2a cells was subjected to SDS-PAGE, prior to electrophoretic transfer to PVDF. The membrane was sectioned prior to probing with either rabbit polyclonal α-ubiquitin IgG or biotin-TUBE1. For α-ubiquitin IgG, membrane strips were blocked in PBS, 0.1% Tween-20 (PBST) containing 5% milk with and without pre-treatment in boiling water for 10 minutes. Incubation with α-ubiquitin IgG was as per manufacture's instructions. After membrane washing with PBST, membrane strips were incubated with goat anti-rabbit conjugated horse radish peroxidase (HRP) according to the manufacture's instructions. For ligand blotting with biotinylated TUBE1, membrane strips were blocked in PBST containing 3% BSA (Cohn fraction V) **without pre-treatment of strips in boiling water**. Membranes were then incubated with biotin-TUBE1 (either 1.0 or 0.2 µg/ml). After incubation with biotin-TUBE1, membranes were washed prior to incubation with streptavidin conjugated HRP for secondary detection. For all membrane strips, secondary detection was followed by at least 4 washes of PBST, prior to simultaneous development and exposure for detection of proteins by chemiluminescence.

DETECTION OF TOTAL POLYUBIQUITYLATED PROTEIN WITH BIOTIN-TUBES

Probing with α-ubiquitin IgG (Fig.1, Strip 1) yielded the characteristic ladder of bands associated with polyubiquitylation of cellular proteins. In addition, the intensity of immunoreactive bands, particularly of those in the higher molecular weight bands (>100kDa) was increased upon pre-heating of the membrane (Strip 2). When probing

for ubiquitin with biotin-TUBE1, overall band intensity increased dramatically over that seen with α -ubiquitin IgG at both dilutions tested. Working concentrations of biotin-TUBEs were determined to yield approximately 20-100 western blots per product vial.

Detection of Polyubiquitinated Proteins by Far Western



Approximately 40µg of protein from Neuro 2A cells was subjected to SDS-PAGE, prior to electrophoretic transfer. Probing with polyclonal α -ubiquitin was performed without (Strip 1) or with (Strip 2) pre-treatment of the membrane with heat. Strips 3 and 4 were probed with biotinylated-TUBE1 at either 0.2µg/ml or 1µg/ml, respectively, with no prior heating of the membrane.

REFERENCES

1. Pickart, C.M. and Eddins, M.J. (2004). "Ubiquitin: structures, functions, mechanisms." *Biochim biophys Acta*. 1695 (1-3): 55-72.
2. Wilkinson, K. D. (2000). "Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome." *Semin Cell Dev Biol* 11(3): 141-8.
3. Ciechanover, A. (2003) "The ubiquitin proteolytic system and pathogenesis of human diseases: a novel platform for mechanism-based drug targeting." *Biochem Soc Trans* 31(2): 474-81.
4. Shahri, R. (2005) "Diverse polyubiquitin interaction properties of ubiquitin-associated domains." *Nature Structural & Molecular Biology* 12(8): 708-14.
5. Hicke, L. (2005) "Ubiquitin-Binding Domains." *Nature Mol Cell Bio* 6: 610-21.
6. Hjerpe, R., Aillet, F., Lopitz-Otsoa F., Lang, V., England P., and Rodriguez, MS. (2009) "Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities." *EMBO Rep.*, 10(11):1250-8.

About LifeSensors, Inc.

LifeSensors is a biotechnology company located 35 miles west of Philadelphia, Pennsylvania, USA. Founded in 1996, LifeSensors has developed a number of innovative protein expression technologies that enable efficient translation of the genome into proteome.

LifeSensors is well-known for its innovations in an important family of proteins consisting of ubiquitin and ubiquitin-like proteins (UBL) such as SUMO (Small Ubiquitin-like MOdifier).

LifeSensors has been granted several patents to cover the use of SUMO and other UBLs as gene fusion tags to improve the expression and purification of recombinant proteins. Additional patent applications are in various stages of review. Currently, LifeSensors is expanding its protein production capabilities and is developing protein micro array for drug discovery and diagnostics.

TUBEs are licensed by LifeSensors, Inc. from Manuel Rodriquez at CIC bioGUNE. For information on obtaining a license for commercial purposes, contact: Marc Scholtyssek, Sales Manager, LifeSensors, Inc., Malvern PA 19355, Phone: 610.644.8845 ext 305.

all products are for research use only
not intended for human or animal diagnostic or therapeutic uses
LifeSensors, Inc., 271 Great Valley Parkway, Malvern PA 19355
(p) 610.644.8845 (f) 610.644.8616

techsupport@lifesensors.com • www.lifesensors.com • sales@lifesensors.com

Copyright © 2010 LifeSensors, Inc. All Rights Reserved