The use of K48 linked diUbiquitin containing an internally quenched fluorescent (IQF) dye pair as a substrate for deubiquitylases.

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June 2010

BACKGROUND

Ubiquitin and polyubiquitylation

Ubiquitin is a small polypeptide that can be conjugated via its C-terminus to the ε-amino groups of lysine residues in target proteins. Formation of this isopeptide bond between a single ubiquitin and the target substrate is termed monoubiquitylation. Conjugation of additional ubiquitin moieties to this initial ubiquitin via isopeptide bond through one of the seven lysine residues present in ubiquitin leads to the formation of polyubiquitin chains. The best characterized polyubiquitin chains result from the conjugation of ubiquitin moeities through Lys48 (K48-linked chains).The most well-characterized consequence of K48 polyubiquitylation is proteasome-mediated degradation of the target protein.

Deubiquitylating enzymes

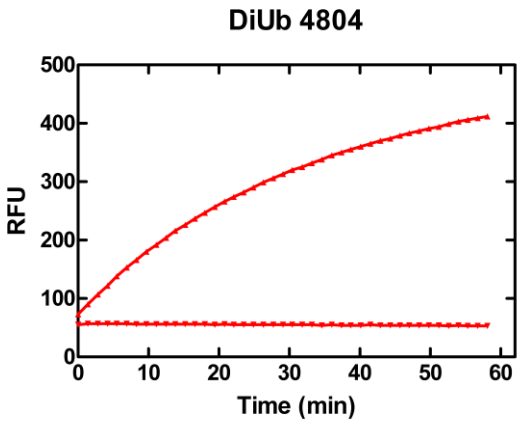
Protein ubiquitylation is reversible through the action of deubiquitylating enzymes (DUBs). These enzymes are capable of recognizing and cleaving the isopeptide bond between ubiquitin moeities or between ubiquitin and the target protein. These isopeptidases have been divided into five families based on sequence homology. These families include the ubiquitin-C terminal hydrolases (UCH), the ubiquitin specific processing proteases (USPs), the Machado-Joseph Disease domain proteases, the Otubain proteases (Otu), and JAMM domain proteases. Although a number of DUBs can cleave ubiquitin molecules with small adducts at the C-terminus, the true substrate for most of these enzymes is the isopeptide bond. The mechanistic basis for recognition and cleavage of an isopeptide bond is widely considered fundamentally different from normal amide bonds. Removal of ubiquitin or polyubiquitin can affect cellular physiology in a number of ways, and several isopeptidases have been linked to pathologies such as cancer, cardiovascular disease, and neurodegeneration.

About the DiUbiquitin (DiUb) IQF substrates

The assay is based on the gain in fluorescent signal that is produced following DUB cleavage of the isopeptide bond present between two ubiquitin moieties. In this instance, the two ubiquitins are linked via an isopeptide bond between the C-terminal glycine of one ubiquitin and specific lysine of the second ubiquitin. Each of the two ubiquitins is singly labeled with a different dye molecule, one of which is a reporter fluorophore (TAMRA) and the other is an efficient fluorescence quencher. As the diubiquitin is cleaved at the isopeptide bond, the proximity of the quencher to the reporting fluorophore is reduced, resulting in an increased fluorescence signal. Currently, LifeSensors provides IQF diUbiquitin substrates linked via either K48 or K63. In addition, LifeSensors is expanding this product line to include K11 and K29 linkages, with the goal of having a comprehensive panel of IQF substrates for all linkage types in Q4 of 2010.

METHODOLOGY FOR ASSAYING USP2 core WITH DiUb K48

200nM DiUb K48-4 (DUK4804) was used to monitor USP2core (10nM) activity over 30min at RT. Both DiUb K48-4 and USP2core were diluted to final concentrations in 50mM Tris, pH 7.5, 0.15M NaCl, 1mM DTT. The addition of low concentrations of detergents or protein additives (e.g. 0.05% CHAPS, 0.1% Tween-20, 0.1% BSA) did not affect the assay. After mixing reactants, TAMRA emission was monitored (Ex = 540/35, Em = 580/20, 570nm dichroic mirror) on a Synergy2 plate reader (BIOTek).



Progression of DiUb cleavage by USP2core: 200nM DiUb K48-4 was incubated with (closed symbols) or without (open symbols) 10nM USP2core. The Increase in TAMRA fluorescence was monitored as described above.

REAGENT DESCRIPTION

Buffer: 50mM Sodium MES, pH 5.5
Storage: Reagent is stable up to 6 months at 4ºC. Long term storage at -80ºC is recommended. Avoid repeated freeze/thaw cycles.

ADDITIONAL MATERIALS REQUIRED

USP2core (LifeSensors cat. no. DB501)

96-well Assay Plates, black (Greiner BioONE 655076)

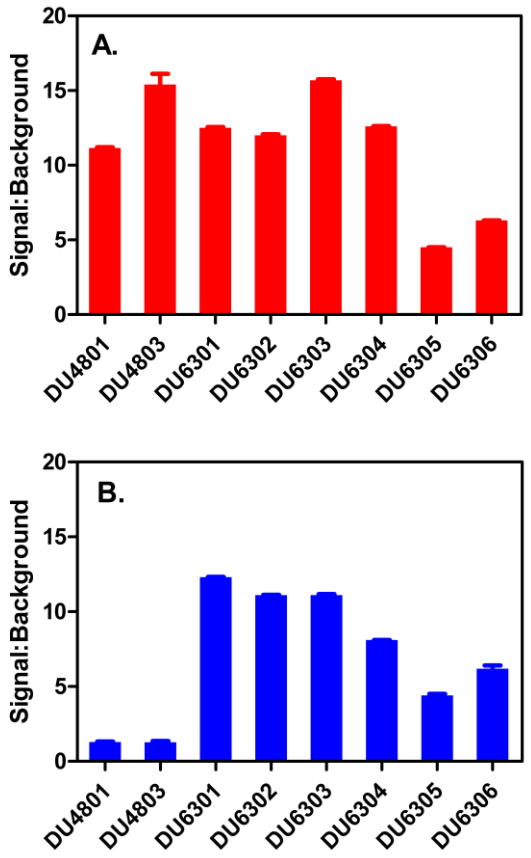
The use of these reagents has been validated on the BIOTek Synergy2 plate reader. Filters or monochromators compatible with monitoring the fluorescence of TAMRA (Exc. 540 nm/Emm. 580 nm) are required. **In addition, the use of a dichroic mirror with a cutoff in the range of 550-570 is highly recommended to ensure maximum signal-to-background.** Further optimization of the plate reader optics (e.g. signal gain, height reads, etc.) is also recommended. Any fluorescence or multimode plate reader capable of the configuration described above is suitable for this assay.

SUGGESTED PROTOCOL (96 well plate)

1. Dilute DiUb K48 substrate to 200nM, or 2x the desired final concentration, in assay buffer of choice (e.g. 50mM Tris, pH 8.0, 0.05% CHAPS, 10mM DTT).
2. Dilute the USP2core to 2X desired final concentration in buffer of choice. A range of enzyme concentrations, spanning at least three orders of magnitude, is recommended.
3. Dispense 50μL of DiUb K48 substrate (or assay buffer as no enzyme control) into black assay plate wells.
4. Add 50μL of USP2core and read immediately in a pre-configured fluorescence plate reader (see above) for 30min to 1hour.

Representative Data for DiUb panel

Beyond differential linkages (e.g. K48, K63, K11), LifeSensors has created subpanels of IQF diubiquitin substrates within each linkage. Because each DUB is likely to recognize and cleave substrates with unique steric considerations, these subpanels vary in location of reporter fluorophore and quencher. It is recommended that each DUB be empirically evaluated against a panel to select the the optimal fluorophore/quencher pairing.



100nM USP2core (Panel A.) or AMSHcore (Panel B.) was incubated for 1 hour with 100nM substrate. Fluorescence was measured and compared with no enzyme control to determine signal:background. While USP2c does not exibit linkage specificity, it nonetheless prefers certain fluor/quencher location combinations. AMSHc displays both linkage and location specificity.

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. Juang, Y-C. (**2012**) "OTUB1 co-opts Lys48-linked ubiquitin recognition to suppress E2 enzyme function." *Mol Cell* 45:384-97.

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).

Our proprietary substrates and technologies, including IQF DiUb substrates, are in part licensed from Progenra, Inc., a leader in ubiquitin proteasome pathway (UPP) drug discovery. *www.progenra.com*

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