MANUAL

Internally Quenched Fluorescence – K6-linked Diubiquitin (IQF-K6 DiUb) Substrate
Catalog Numbers: DU 0601

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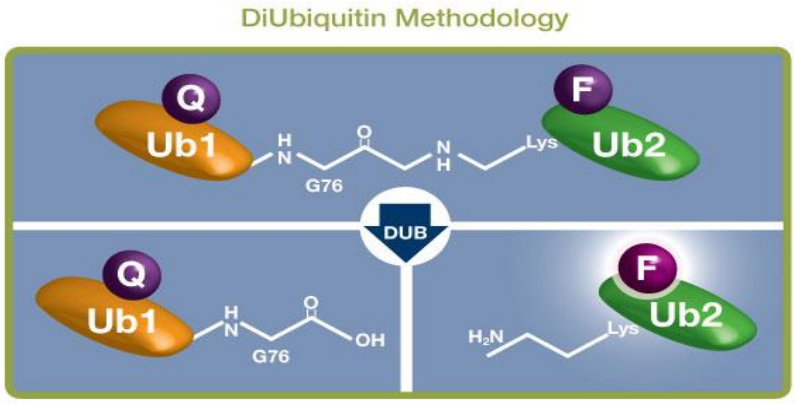
Background

The ubiquitin-proteasome pathway plays a key role in protein modification and degradation. Ubiquitylation is a dynamic and reversible process mediated by the action of deubiquitylating enzymes (DUBs), a family of proteases that specifically cleave ubiquitin-derived substrates. The distinct structures of polyubiquitins determined to date suggest that DUBs act with high specificity towards various Ub linkage types (e.g. K48, K63, K6, etc). DUBs, in general, show remarkable diversity and the members of this family exhibit characteristic developmental and spatial expression patterns, as well as complex biochemical properties. The ability of DUBs to interact with preferred targets, thus modifying cellular functions, further highlights the importance of this pathway in both health and disease. Deregulation of ubiquitylation has been associated with a wide range of pathologies including cancer, muscle atrophy, infectious diseases and neurodegeneration and represents a promising target for therapeutic intervention.
One of the challenges in monitoring DUBs activity is to create physiologically relevant substrate(s) to measure true isopeptidase activity in a format amenable to high-throughput screening (HTS). Currently, commercially available reagents and assays for measuring the activity of Ub/Ubl isopeptidases are based on the cleavage of a linear peptide- or amide-bond that do not reflect the geometry of a true isopeptide bond. In addition, other fluorescence based homogeneous assays for monitoring polyubiquitin chain disassembly suffer from the requirement of multiple enzymatic steps and/or N-terminal modification of ubiquitin molecule, a step known to disrupt conformational integrity The shortcomings of these assay/reagents has resulted in an as yet unmet need for a homogenous, biologically relevant, and HTS amenable assay for DUBs.

K6-linked Diubiquitin (DiUb): A Novel Substrate for Robust Fluorescence Readout of DUBs Activity

K6-linked chains have been implicated in a role in mitophagy. They have been shown to be a selective target for USP30 in the mitochondria. LifeSensors has developed a line of novel physiological substrates for DUBs – diubiquitin molecules (Diubiquitin, DiUb) that are linked by isopeptide bonds via either K48, K63 or K6. These substrates can be used to determine the substrate specificity of numerous DUBs, to monitor the kinetic parameters of DUB mediated isopeptide cleavage, as well as investigate selective de-conjugation of poly-ubiquitylated proteins.



Assay versatility

There is a remarkable difference between the determined structures of K48-, K63- and K6-linked ubiquitin chains, potentially providing the basis for their distinct functions. Moreover, the distinct structure of differently-linked polyubiquitin further illustrates a tremendous diversity among DUBs with regard to their substrate specificity. LifeSensors' IQF-DiUbs offer a variety of substrates that are either K48-, K63- or K6-linked with FRET pair fluorophores uniquely positioned on specific sites of the diubiquitin molecule, thus providing a highly efficient tool to measure selective activity of the DUB of interest.

About the assay

These diubiquitin substrates represent a new class of continuous assay substrates for the cleavage of a true isopeptide bond. The C-terminus of wild type ubiquitin is conjugated via an isopeptide bond to lysine 48, lysine 63 or lysine 6 of a second ubiquitin molecule, with the resultant diubiquitin forming an internally quenched fluorescence FRET pair (IQF) due to a presence of a highly efficient fluorescence quencher on one ubiquitin molecule and a fluorescent reporter (TAMRA) on the second ubiquitin molecule. Cleavage of the diubiquitin molecule by selective DUBs leads to separation of the fluorophore from quencher and subsequent increase in fluorescence signal. The generation of ubiquitin isopeptidase assays using physiological substrates represents a major advancement in the study of this important domain of the eukaryotic proteome.

Benefits

- IQF-DiUbs provide a sensitive, rapid, and robust fluorescent readout of enzymatic activity with minimal interference from screening compound.
- The assay measures cleavage of a physiologically relevant isopeptide bond rather than the linear peptide bond found in other currently available commercial assays for deubiquitylases.
- A variety of DiUbs allows selection of the best substrate for your enzyme.

Applications

- Investigation of the linkage specificity of DUBs
- Identification of agonists or antagonists of specific isopeptidases for HTS and drug discovery
- Determine kinetic parameters mediating substrate/DUB interactions

Components

Reagents are supplied as individual DiUb substrates or as panels of smaller sizes

Buffer: 50mM Sodium MES, pH 6.0.

Additional Items Required

DUB of choice: LifeSensors provides the most comprehensive selection of DUBs available. Please visit www.lifesensors.com or contact a sales representative to learn more.

Assay Buffer: 50mM Tris, pH 8.0, 0.05% CHAPS, 10mM DTT or buffer of choice. The addition of DTT or other reducing agent is required for the assay. Assay condition should be optimized by the end user.

384-well black assay plates (Greiner BioONE 781209) or **96- well black assay plates** (Greiner BioONE 655076).

Fluorescence 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 optimal signal-to-background. Further optimization of the plate reader optics (e.g. signal gain, plate height reads, etc.) is also recommended. Any fluorescence or multimode plate reader capable of the configuration described above should be suitable for this assay.

Ub-TAMRA Reference Standard (Cat.#DU0121). The standard can be used to optimize and calibrate the performance of individual plate readers (or fluorometers) for measurement of the rates of hydrolysis of the IQF-Diubiquitins by DUBs.

Suggested Protocol for Monitoring IQF-K6-linked DiUb cleavage by USP30

- To obtain reliable and reproducible results, all samples should be run at least in duplicate.
- Allow all reagents to warm to room temperature (20-25ºC) before use in the assay.
- IQF-DiUb SUBSTRATES ARE LIGHT SENSITIVE AND MUST BE PROTECTED FROM LIGHT AT ALL THE TIMES
- Dilute K6-linked DiUb substrate to 400nM, or 2x the desired final concentration, in assay buffer.
- Prepare USP30. Dilute USP30 to 200 nM (2X) in assay buffer. A range of enzyme from 10nM to 1µM (final concentration) can be used for USP30 to generate a standard curve.

- For 96-well plate, add 50 µL of USP30 from step 2 to each control well of black assay plate (row G).

- Prepare three dilutions of each DUB to be tested. (Suggested: 200nM, 100nM, 10nM).

- Add 50 µL of assay buffer to "no enzyme" wells (row H).

- Dispense 50µL of each DiUb substrate from step 1 into each well. Note: the concentrations of both DiUb and DUB now is1/2 of the original (Steps 1 and 2).

- Perform a kinetic read for 30min to 1hour. Important: The plate should be read immediately after the addition of the enzyme.

Storage

IQF-DiUbs can be stored at 4ºC for up to 6 months. Long term (> 6 months) storage at -80ºC is recommended. If the products are received frozen on dry-ice please store at -80ºC until ready to use. Avoid repeated freeze/thaw cycles. IQF-DiUb substrates are light sensitive and must be protected from light at all times.

Please note that some physical characteristics and protocols are item specific. Please refer to individual product sheets or application notes now available at www.lifesensors.com for further information.

Limited Use Label License

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transfer or sale of a protein or chemical component made with the product or derivatives of the product,
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