MANUAL

Internally Quenched Fluorescence – diubiquitin (IQF-DiUb) Substrates

Catalog Numbers:
DiUb48:DU4801, DU4802, DU4803, DU4804, DU4805, DU4806,
DiUb48 panel:DU0101

DiUb63:DUK6301, DUK6302, DUK6303, DUK6304, DUK6305, DUK6306
DiUb63 panel: DU0102

DiUb48, DiUb63 panel: DU0201

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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, K11, 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.

Commercially available assays for measuring isopeptidase activity

Substrate	*Source*	*Biological relevance*
Ubi/Ubl-AMC, -RHO	LifeSensors	Non-physiological
Ubi-AMC	BioMol	
Ubi-AMC/AFC	Boston Biochem	
Ubiquitin LanthaScreen ™ assay	Invitrogen	Non-physiological
CHOP-reporter assay	LifeSensors	Non-physiological, highly sensitive
Internally Quenched Fluorescence-diubiquitin (IQF-DiUb)	**LifeSensors** – **NEW!**	Physiological relevant, highly sensitive, substrate specific

Diubiquitin (DiUb): A Novel Substrate for Robust Fluorescence Readout of DUBs Activity
LifeSensors has developed a line of novel physiological substrates for DUBs – diubiquitin molecules (Diubiquitin, DiUb) that are linked by isopeptide bonds via either K48 or K63, the most abundant forms of poly-ubiquitin linkages. In addition, LifeSensors is currently expanding this product line to include K11 and K29 linkages, with the goal of being able to provide a comprehensive panel of IQF substrates for all linkage types. This assay 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- and K63-linked ubiquitin molecules, potentially providing the basis for their distinct functions. Moreover, the distinct structure of polyubiquitin linkages further illustrates a tremendous diversity among DUBs with regard to their substrate specificity. LifeSensors' IQF-DiUbs offer a variety of K48 and K68-linked substrates 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 or lysine 63 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. In the DiUb assay, each ubiquitin is prepared through site specific labeling at different positions (K63-1-2-3-4-5-6 or K48-1-2-3-4-5-6) in order to allow optimization of a given diubiquitin substrate for an individual DUB. Importantly, the introduction of fluorophore or quencher residue does not affect the native structure of the ubiquitin molecule.
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 compounds
- 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 wide variety of DiUbs allows selection of the best substrate for your enzyme

APPLICATIONS

- Investigation of the linkage specificity and cellular function of DUBs
- Identification of agonist 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 and as panels of six substrates

Buffer: 50mM Sodium MES, pH 6.0.

DiUb K-48, 20 μM each	**DiUb K-63, 20 μM each**	**DiUb K-48 $_{1-6}$, DiUb K-63 $_{1-6}$ panel, 20 μM each**
DiUb K48-1 (DU4801)	**DiUb K63-1 (DU6301)**	**DU0201**
DiUb K48-2 (DU4802)	**DiUb K63-2 (DU6302)**	
DiUb K48-3 (DU4803)	**DiUb K63-3 (DU6303)**	
DiUb K48-4 (DU4804)	**DiUb K63-4 (DU6304)**	
DiUb K48-5 (DU4805)	**DiUb K63-5 (DU6305)**	
DiUb K48-6 (DU4806)	**DiUb K63-6 (DU6306)**	
DiUb K48 $_{1-6}$ panel (DU0101)	**DiUb K63 $_{1-6}$ panel (DU0102)**	

ADDITIONAL ITEMS REQUIRED

1. Control isopeptidase: USP2 core (LifeSensors Cat. no DB501). USP2 core represents the common catalytic core domain of the two isoforms of USP2, USP2a and USP2b. This enzyme is known to cleave polyubiquitin chains formed through both K48 and K63 linkage types. As such, it is active against most of the IQF-DiUb substrates.
2. 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.
3. 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.
4. 384-well black assay plates (Greiner BioONE 781209) or 96- well black assay plates (Greiner BioONE 655076).
5. Fluorescent 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.

SUGGESTED PROTOCOL FOR MONITORING IQF-DiUb CLEAVAGE BY USP2 CORE

To obtain reliable and reproducible results, all samples should be run at least in duplicate.
Allow all reagents to warm to room temperature (20-25°C) before use in the assay.
IQF-DiUb SUBSTRATES ARE LIGHT SENSITIVE AND MUST BE PROTECTED FROM LIGHT AT ALL THE TIMES

1. Dilute individual or combo panel DiUb substrates to 400nM, or 2x the desired final concentration, in assay buffer.
2. Prepare control USP2 core. Dilute USP2 core to 200 nM **(2X)** in assay buffer. A range of enzyme from 10nM to 1µM (final concentration) can be used for USP2 core control to generate a standard curve.
3. For 96-well plate, add 50 µL of USP2 core from step 2 to each control well of black assay plate (row G).
4. Prepare three dilutions of each DUB to be tested. (Suggested: 200nM, 100nM, 10nM).
5. Add 50 µL of assay buffer to "no enzyme" wells (row H).
6. 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).
7. Perform a kinetic read for 30min to 1hour. **Important: The plate should be read immediately after the addition of the enzyme**.

WELL MAP

Typical assay set-up for either the DiUb panel (A) or a single IQF-DiUb (B) To be customized for 384-well plate. The number of DUBs of interest should be determined by the end user. Concentration of USP2 core used: 100 nM. Samples (DUB1, DUB2 and DUB3) are loaded in duplicates (wells 1 and 2) in three dilutions (200nM, 100nM, 10nM).

A.

	DU4801		DU4802		DU4803		DU4804		DU4805		DU4806	
	1	2	3	4	5	6	7	8	9	10	11	12
A	DUB1 200nM	DUB1 200nM										
B	DUB1 100nM	DUB1 100nM										
C	DUB1 10nM	DUB1 10nM										
D	DUB2 200nM	DUB2 200nM										
E	DUB2 100nM	DUB2 100nM										
F	DUB2 10nM	DUB2 10nM										
G	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM
H	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme

B. **DUB screening against a single substrate**. DUBs of interest are loaded in triplicates. DiUb substrates should be prepared in two concentrations (400nM and 200nM, **2X**). Final concentration is shown on the left.

		DUB1			DUB2			DUB3			DUB4		
DiUb subst.	A	DUB1 200nM	DUB1 200nM	DUB1 200nM									
100 nM	B	DUB1 100nM	DUB1 100nM	DUB1 100nM									
100 nM	C	DUB1 10nM	DUB1 10nM	DUB1 10nM									
100 nM	D	DUB1 200nM	DUB1 200nM	DUB1 200nM									
200 nM	E	DUB1 100nM	DUB1 100nM	DUB1 100nM									
200 nM	F	DUB1 10nM	DUB1 10nM	DUB1 10nM									
200 nM	G	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM	USP2 100 nM
	H	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme	No enzyme

STORAGE

Reagent is stable for >4 weeks at 4ºC. Long term storage at -80ºC is recommended. Avoid repeated freeze/thaw cycles.

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.

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