UbiQ

targeting the ubiquitin system

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Ub-FANCD2(557-565)-FP *(human, synthetic)*

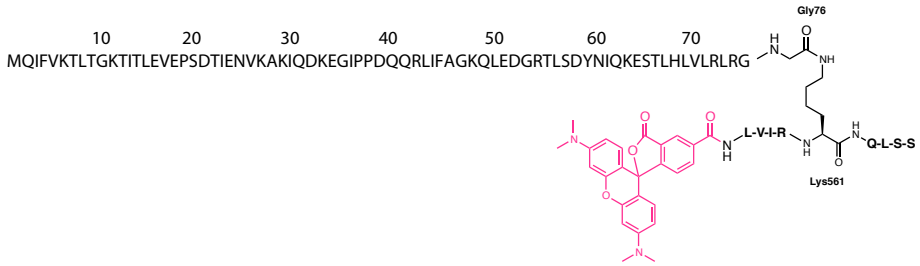
UbiQ code : UbiQ-029
Batch # : B01082013-001 Protocol # : P01082013-001

Product Information

Amount : 0.16 mg, lyophilized powder (purple)
Purity : ≥95% by RP-HPLC and SDS-PAGE
Mol. Weight : 10002 Da by MS (calc Mw 10002 Da)
Storage : −20°C; buffered solution at −80°C. Please avoid multiple freeze/thaw cycles.

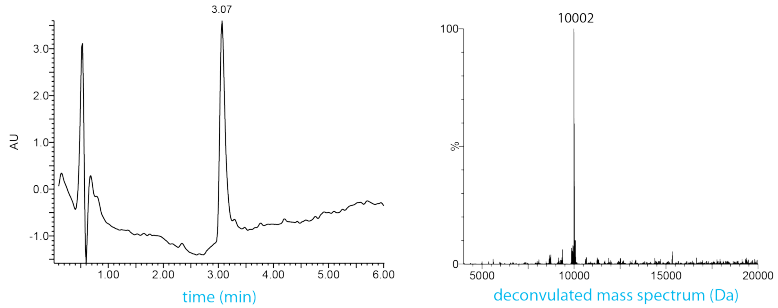
Background. Class II fluorescence polarization HTS reagent based on the peptide sequence 557 – 565 of FANCD2, a DNA repair protein which is monoubiquitinated on Lys561. The peptide is modified on the N-terminus with a 5-carboxytetramethylrhodamine (5-TAMRA) and conjugated at Lys561 to ubiquitin via a native isopeptide bond. Typical substrate concentrations range from 10–100 nM. DUB concentrations can range from 0.01-10 nM but depend on specific assay conditions and method of detection.

Structure (5-TAMRA)-LVIRK^{561}(Ub)QLSS



Important: sample preparation. Dissolve the powder in as little DMSO as possible (*e.g.* 10 mg/mL = 1000 µM, if not clear then heat slightly) and add this DMSO stock slowly to the required buffer (please note the order of addition). *If possible, it is prefered to dilute the DMSO stock into milliQ and then buffer the solution.* For experimental details see page 2 and the open-access reference 4. The concentration of 5-TAMRA-Lys(Ub)-Gly-OH can be verified by comparing the fluorescence intensity with that of a known concentration of TAMRA.

A



B

A: LC-MS analysis. Mobile phase A = 1% CH_3CN, 0.1% formic acid in water (milliQ) and B = 1% water (milliQ) and 0.1% formic acid in CH_3CN. Phenomenex Kinetex C18, (2.1×50 mm, 2.6 µM); flow rate = 0.6 mL/min, runtime = 6 min, column T = 40°C. Gradient: 5% ⇨ 95% over 3.5 min.
B: SDS-PAGE analysis. Fluorescence scan (exc 550 nm, emi 590 nm), 12% SDS-PAGE gel.

Experimental Details Fluorescence polarization assay

FP assays were performed on a PerkinElmer Wallac EnVision 2010 Multilabel Reader with a 531 nm excitation filter and two 579 nm emission filters. Fluorescence intensities were measured in the S (parallel) and P (perpendicular) direction. FP values are given in mP (millipolarization) and calculated using the following formula:

$$Polarization\ (mP) = \frac{S - (G \cdot P)}{S + (G \cdot P)} \cdot 1000$$

The confocal optics were adjusted with the average P and S values for TAMRA and the grating factor (G) was determined using a polarization value (L) for TAMRA of 50 mP using the following formula:

$$G = \frac{average\ S}{average\ P} \cdot \frac{1 - (\frac{L}{1000})}{1 + (\frac{L}{1000})}$$

The assays were performed in "non binding surface flat bottom low flange" black 384-well plates (Corning) at room temperature in a buffer containing 20 mM Tris·HCl, pH 7.5, 5 mM DTT, 100 mM NaCl, 1 mg/mL 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) and 0.5 mg/mL bovine gamma globulin (BGG). Each well had a volume of 20 µL. Buffer and enzyme were predispensed and the reaction was started by the addition of substrate. Kinetic data was collected in intervals of 2.5 or 3 min. From the obtained polarization values (P_t) the amount of processed substrate (S_t) was calculated with to the following equation:

$$S_t = S_0 - \left[S_0 \times \frac{P_t - P_{min}}{P_{max} - P_{min}} \right]$$

Where P_t is the polarization measured (in mP); P_{max} is the polarization of 100% unprocessed substrate (determined for every reagent at all used substrate concentrations); P_{min} is the polarization of 100% processed substrate; S_0 is the amount of substrate added to the reaction.

From the obtained P_t values the values for initial velocities (v_i) were calculated, which were used to determine the Michaelis-Menten constants (K_m, V_{max} and k_{cat}) by fitting the data according to the formula below (where $k_{cat} = V_{max}/[E]$). All experimental data was processed using Ms Excel and Prism 4.03 (GraphPad Software, Inc.).

$$v_i = \frac{V_{max} \times S_0}{K_m + S_0}$$

Literature. (1) Tirat, A. et al. *Anal. Biochem.* **2005**, *343*, 244-255. (2) Huang et al. *Methods Mol. Biol.* **2009**, *565*, 127. (3) Levine *et al. Anal. Biochem.* **1997**, *247*, 83. (4) Geurink and El Oualid et al. *ChemBiochem*, **2012**, *13*, 293. (5) Sheng et al. *Nat. Struct. Mol. Biol.* **2006**, *13*, 285.