UbiQ
targeting the ubiquitin system

fluorescence polarization assay

version	V1, 17-9-2015
applicable for	UbiQ assay reagents
reference	Geurink, P.P., et al. A general chemical ligation approach towards isopeptide-linked ubiquitin and ubiquitin-like assay reagents. *ChemBiochem*. **13,** 293-297 (2012).

protocol

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

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

The confocal optics is adjusted with the average P and S values for TAMRA-Lys-Gly and the grating factor (G) can be determined using a polarization value (L) of 50 mP for TAMRA or TAMRA-Lys-Gly (UbiQ-023) using the following formula:

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

The assays is 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 has a volume of 20 µL. Buffer and enzyme are predispensed and the reaction is started by the addition of substrate. Kinetic data is collected in intervals of 2.5 or 3 min. From the obtained polarization values (P_t) the amount of processed substrate (S_t) can be calculated with to the following equation:

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

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) can be calculated, which are 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 is processed using Ms Excel and Prism 4.03 (GraphPad Software, Inc).

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

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