UbiQ
targeting the ubiquitin system

UbiQ protocol P002_DUB fluorescence polarization assay reagents

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UbiQ
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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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A General Chemical Ligation Approach Towards Isopeptide-Linked Ubiquitin and Ubiquitin-Like Assay Reagents

Paul P. Geurink, Farid El Oualid, Anika Jonker, Dharjath S. Hameed, and Huib Ovaa*[a]

Ubiquitin (Ub) and ubiquitin-like proteins (Ubls) form a family of small and highly conserved post-translational modifiers that become linked to target proteins and thus modulate their function (such as degradation, trafficking and signalling).[1] The linkage between a Ub(l) and a target protein most frequently consists of an isopeptide bond between the C-terminal carboxylate of Ub(l) and the ε-amine of a lysine residue. Ub(l) ligation requires the concerted action of enzymes E1, E2 and E3, defined combinations of which provide specificity for the protein target.[2] Next to human Ub, 17 Ubls from nine phylogenetic classes have been reported.[3] Each has its own discrete conjugation and deconjugation enzymes and has a distinct effect on its cellular target. The best-studied Ubls are Nedd8 and SUMO. For example, neddylation of cullin–RING E3 ligases is required for their enzymatic activity.[4] The three human SUMO proteins (SUMO-1, SUMO-2 and SUMO-3) are conjugated to diverse target proteins, thereby often altering their interaction with other proteins through interactions between SUMO and SUMO-binding motifs.[5]

Specific deconjugating enzymes remove Ub and Ubls from target proteins. By doing so, they achieve three major functions.[6] First, as Ub and Ubls are often translated as pro-proteins, they cleave the C termini of Ub and Ubls to generate the mature forms. Secondly, these proteases can reverse Ub(l) signalling functions and recycle free Ub and Ubls. Thirdly, in those cases where chains exist, such as for Ub and SUMO-2 and -3, proteases can perform a chain-editing function. As deregulation of Ub(l) deconjugating activity is linked to the occurrence of a variety of diseases, these are of interest as potential drug targets,[7] and consequently, good assay reagents are required to report enzymatic activity and inhibition. Current assay reagents are mainly based on a Ub(l) part connected by a linear peptide bond to a reporter module—either a fluorogenic or latent enzyme that becomes active upon Ub(l) processing.[7c] In addition, besides lacking the native isopeptide linkage, such reagents cannot be functionalised (beyond the reporter module) to resemble a more physiologically relevant substrate.

A previously reported fluorescence anisotropy/fluorescence polarisation (FP) assay reagent for Ub(l) deconjugating enzymes is based on a fluorophore-labelled lysine, or a peptide linked to Ub by an isopeptide bond (Figure 1).[8] This reagent has two characteristics that make it well-suited for high-throughput investigations of catalytic action.[9] First, it is the only reported assay reagent that incorporates an isopeptide linkage;[8] secondly, its physiological relevance (and potentially its affinity for a deconjugating enzyme) can be enhanced by functionalising the assay reagent with substrate-derived elements around the isopeptide linkage.[10]

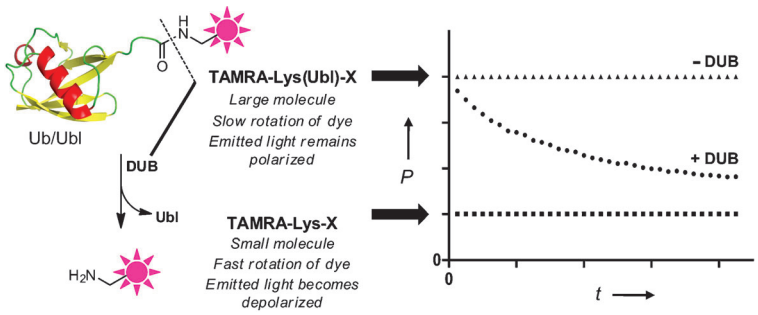

Figure 1. FP assay. When a fluorophore, covalently attached to a small molecule (e.g. a small peptide) is excited by polarised light, it will emit predominantly depolarised light. When it is bound to a high molecular weight molecule (e.g. Ub or a Ubl) the emitted light is much less depolarised. By following the change in fluorescence polarisation, the activity can be monitored. P, polarisation.

Because of the cumbersome enzymatic preparation required for this type of reagent, it has not become the standard in this field. To overcome the limitations set by enzymatic reactions, we and others recently reported methods for the site- and chemoselective Ub modification of peptides.[11] In this approach, isopeptide-linked Ub-conjugates are prepared by native chemical ligation between a 5- or 4-thiolysine-containing peptide (**1**, Figure 2B) and a Ub thioester. Desulfurisation of the intermediate thiolysine side-chain then affords the product with a native isopeptide linkage. The Ub E1 enzyme can be used to generate the required Ub thioester in situ.[11c,12] As E1 enzymes for most Ubls are commercially available, we wondered if the same strategy could also be used for the construction of Ubl-based conjugates. We started investigating the conjugation of the Ubl Nedd8 to a series of ten 5-thiolysine-containing peptides by using this method. The corresponding Nedd8–peptide conjugates were formed rapidly, with full conversion, as judged by SDS-PAGE analysis of the crude ligation mixtures (Figure 2A). Treatment of the peptides with four other Ubls (SUMO-1, -2, -3 and ISG15) and their E1 enzymes under the same ligation conditions gave similar results (Figure S2 in the Supporting Infor-

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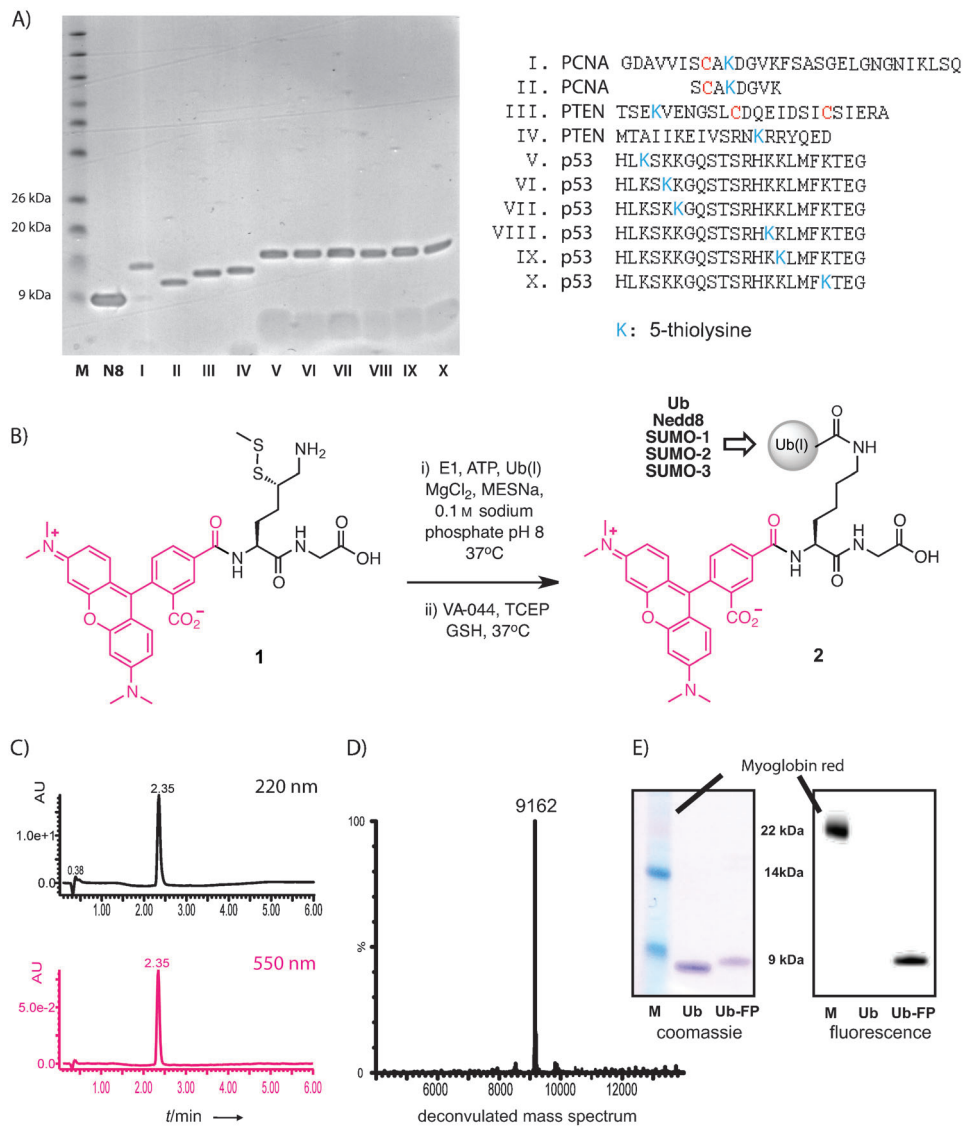

Figure 2. Ligations of Ub(l) with 5-thiolysine-modified peptides by E1-mediated Ub(l) ligation. A) Gel analysis of the crude ligation reactions in which Nedd8 (N8) was ligated to ten different peptides derived from PCNA, PTEN and p53. B) Construction of the Ubl-based FP reagents **2** from TAMRA-labelled dipeptide **1**. C) HPLC, D) MS, and E) gel analysis of FP reagent **2**-Ub after purification.

mation). Next, we tested whether our E1-mediated Ubl ligation could be used for the practical synthesis of various isopeptide-linked Ub(l)-based FP assay reagents.

We started with the synthesis of Ub and Ubl (Nedd8, SUMO-1, SUMO-2 and SUMO-3) conjugates, which were natively linked through an isopeptide lysine bond to 5-carboxytetramethylrhodamine (TAMRA)-labelled 5-thioLys-Gly dipeptide (**2**, Figure 2B). TAMRA-conjugate **1** (1 mM) and Ub(l) proteins (100 μM) were incubated with the appropriate E1 enzyme (150 nM) at 37 °C. In general, LC-MS and SDS-PAGE analysis showed full consumption of Ub(l) protein and formation of the desired ligation product after six hours. Next, the crude ligation product was desulfurised by addition of the radical initiator VA-044 (20 mM), tris-(2-carboxyethyl)phosphine (TCEP, 150 mM) and glutathione (40 mM) with overnight incubation at 37 °C.[13] After HPLC purification and lyophilisation, the desired products were obtained in overall yields of 20–50%. All purified products were analysed by LC-MS and SDS-PAGE (see Figure 2C and the Supporting Information). As all three SUMO proteins contain a native cysteine residue, we anticipated that this might be desulfurised to an alanine residue. However, in all cases LC-MS analysis showed desulfurisation of the thiolysine moiety only, even after prolonged treatment with the desulfurisation cocktail.[11a,b,14]

Next, we tested the FP reagents in deconjugation assays by treating them with three human deubiquitylating enzymes (DUBs; UCH-L3, USP7/HAUSP and USP21), two viral ovarian tumour domain (OTU) DUBs and three SUMO-specific proteases (SENP1, SENP6 and SENP7). These proteases were incubated at six different concentrations with all five Ub(l) FP reagents (**2**, Figure 2B) at room temperature (Figure 3 and the Supporting Information). The completely hydrolysed product (TAMRA-Lys-Gly) was used as a control, and the spectroscopic optics were calibrated by applying an FP value of 50 mP (millipolarisation units) for this tracer. As expected, the Ub FP reagent was efficiently cleaved by all tested deubiquitinases in a concentration-dependent manner, and was unaffected by all three SUMO-specific proteases. The most active DUB in this series appeared to be UCH-L3, which almost completely processed the Ub FP reagent within 80 min at 15.6 pM (Figure 3A). The K_m and k_{cat} values were determined by measuring fluorescence polarisation over time at different substrate concentrations (Figure 3B, Table 1). Michaelis–Menten plots revealed k_{cat} and K_m values that are comparable with those reported for the fluorogenic ubiquitinamidomethyl coumarin Ub-AMC (k_{cat} = 9.1 s^{-1}, K_m = 51 nM).[15]

UCH-L3 is known to exhibit deneddylating activity.[16] The Nedd8-based FP reagent was indeed processed by UCH-L3, although with a lower efficiency than Ub (Figure 3C and D); this is in line with an earlier report.[15] It must be noted that the catalytic breakdown of the Nedd8-based FP reagent stopped at around 60% conversion (Figure 3C). This was also observed for other substrates (vide infra). Currently, the reason for this

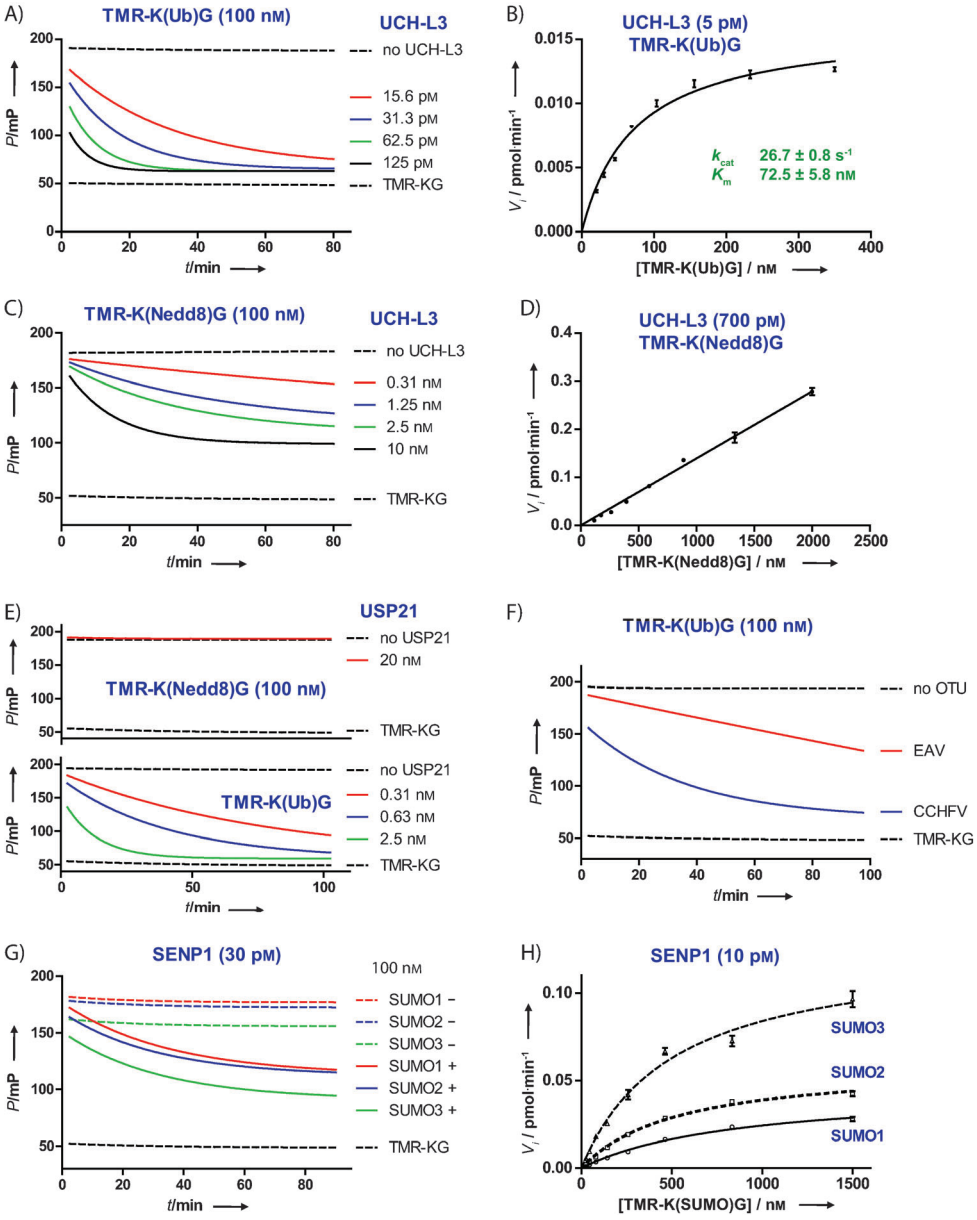

Figure 3. FP assays with Ub(l) FP reagents and different Ub(l) deconjugating enzymes. A) Reaction time-course and B) substrate titration for UCH-L3 and the Ub-derived FP reagent. C) Reaction time course and D) substrate titration for UCH-L3 and the Nedd8-derived FP reagent. E) Reaction time course for USP21 and the Nedd8- (top) and Ub-derived (bottom) FP reagents. F) Reaction time course for the EAV- and CCHFV-derived OTUs and the Ub-derived FP reagent. G) Reaction time course for the SUMO1- ,2- and 3-derived FP reagents in the presence (+) or absence (−) of SENP1. H) Substrate titration for SENP1 and the SUMO1-, 2- and 3-derived FP reagents. Additional results for all tested Ub(l) deconjugating enzymes with different Ub(l) FP reagents at different concentrations are presented in the Supporting Information. *P*: polarisation.

Table 1. Kinetic analysis of Ub(l) proteases for Ub(l) FP substrates.

Enzyme	FP substrate	k_{cat} [s^{-1}]	K_m [nM]	k_{cat}/K_m [$M^{-1}s^{-1}$]
UCH-L3	Ub	27 ± 0.8	73.0 ± 5.8	3.7×10^8
SENP1	SUMO-1	38 ± 2.1	876 ± 93	4.3×10^7
SENP1	SUMO-2	50 ± 1.7	540 ± 41	9.2×10^7
SENP1	SUMO-3	106 ± 5.2	508 ± 59	2.1×10^8
USP7	Ub	3.3 ± 0.22	34600 ± 3400	9.5×10^4
USP7	Ub-PTEN[5–21]	5.0 ± 0.21	22900 ± 1600	2.2×10^5

remains unclear. However, an activity assay using excess Ub showed that this effect cannot be explained by product inhibition (Figure S23). Loss of enzymatic activity over time also does not account for this, as the final mP was independent of enzyme concentration (Figure 3A). Nonetheless, both the Ub and Nedd8 FP reagents are well suited for monitoring UCH-L3 activity. USP21 has also been reported to deconjugate both Ub and Nedd8,[17] although a more recent report shows that USP21 exhibits no deneddylating activity.[18] Our results show that USP21 processes the Ub FP reagent at sub-nanomolar concentrations (Figure 3E, bottom) but not the Nedd8 FP reagent, even at 20 nM (Figure 3E, top), which supports the recent report.

We investigated two viral deubiquitinases that belong to the OTU class, one from equine arteritis virus (EAV) and one from the Crimean–Congo haemorrhagic fever virus (CCHFV). CCHFV is a lethal human pathogen, and it is believed that its inherent DUB activity has a major role in its pathogenic function.[19] Both OTU DUBs were found to efficiently cleave the Ub FP substrate in a concentration-dependent manner (Figure 3F), but they lacked reactivity towards Nedd8[20] and SUMO FP reagents (Figures S14 and S15).

As shown, all tested deubiquitinases efficiently processed the Ub FP reagent, however they proved to be unreactive towards the three SUMO-derived reagents. In contrast, the SUMO-specific protease, SENP1, was unreactive towards the Ub FP reagent but efficiently processed the three SUMO FP reagents at 30 pM and with comparable efficiency (Figures 3G and S16), although there was a slight preference for SUMO-3 (Figure 3H and Table 1). We also tested SENP6 and SENP7 for their ability to process the SUMO-derived FP reagents. It is known that SENP6 and SENP7 exhibit specificity for SUMO-2 and SUMO-3, whereas SENP1 lacks a clear preference for any particular SUMO isoform.[21] Indeed, SENP6 and SENP7 properly processed both the SUMO-2 and SUMO-3 FP

reagents, albeit with a clear preference for SUMO-3 (Figures S17 and S18) in both cases. The SUMO-1 FP reagent was not processed by SENP6 or SENP7 at up to 20 nM.

We further functionalised our FP reagents by introducing a peptide sequence derived from a known ubiquitylated substrate. These context-specific reagents resemble the native environment that a Ub(l) protease encounters better than a single lysine residue. As a test case, our attention was drawn to the tumour suppressor phosphatase PTEN, which contains two major monoubiquitylation sites (Lys13 and Lys289). Monoubiquitylation of these sites is important for regulation of PTEN-mediated tumour suppression and its nuclear import.[22] The major DUB responsible for PTEN deubiquitylation is USP7/HAUSP.[23] Based on the peptide sequences surrounding Lys13 and Lys289, we designed two FP reagents that comprised a TAMRA-labelled 17-amino-acid PTEN peptide (i.e., PTEN[5–-21] and PTEN[281-–297], respectively). The lysine residues were linked by an isopeptide bond to Ub. The ability of full-length USP7 to hydrolyse these FP reagents was assessed in an FP assay at different concentrations of USP7 (Figure 4 and Figure S20). For comparison, all other Ub(l)-derived reagents were also tested. As expected, USP7 could not process the Ubl-derived FP reagents (e.g., Nedd8 and SUMO, Figure S11) but efficiently hydrolysed the TAMRA-Lys(Ub)-Gly reagent. Whereas USP7 showed high activity against the PTEN[5–21]-based FP reagent, it was much less active on the PTEN[281–297]-based FP reagent (Figures 4 and S20). This result was also apparent from a gel-based assay (Figure S21), thereby confirming that the observed difference in reactivity depends on the nature of the FP substrate. It was previously reported that both Lys13 and Lys289 are deubiquitylated by USP7 in vivo.[23] However, to the best of our knowledge, the relative USP7 deubiquitylation rates for these sites are not known. The observed differences in USP7 reactivity here might be explained by an intrinsic preference of USP7 for monoubiquitylated Lys13, although further experiments are needed to substantiate this. The kinetic data for the PTEN[5–21]-derived FP substrate revealed that introduction of the PTEN[5–21]-peptide resulted in a higher k_{cat}/K_m value compared with the unfunctionalised TAMRA-Lys(Ub)-Gly reagent (Table 1 and Figure S22). The kinetic parameters for the PTEN[281–297] reagent were not determined as it was only minimally processed by USP7. It must be noted that introduction of the larger peptides around the isopeptide linkage decreases the dynamic range of the FP assay reported here. However, with a calculated *Z*-score of 0.88 for the PTEN[5–21]-based FP reagent, we believe that the system is well suited for monitoring Ub(l) proteolysis activities in a context-specific manner.[24]

In conclusion, we have shown that the 5-thiolysine-mediated ligation can be used to generate a wide range of isopeptide-linked Ub(l)-based FP assay reagents, something that was not possible with conventional enzyme-based strategies. We and others have demonstrated that these are very powerful tools for measuring Ub(l) deconjugating activity.[8] In principle, our methodology can be adapted to any Ubl for which the E1 enzyme is available. In contrast to any other reagent, it is possible to incorporate substrate-based peptide sequences. This is a major advantage, as it offers the possibility of more physiologically relevant assay reagents.[7c] Overall, the practical generation of FP assay reagents described here will likely "catalyse" both fundamental research in the Ubl field and drug discovery efforts.

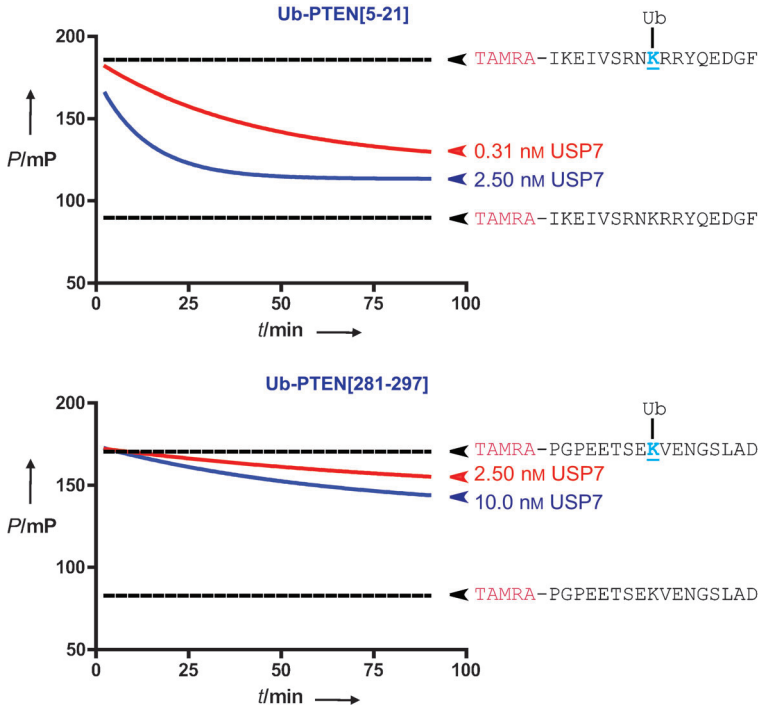

Figure 4. FP assays with full-length USP7 and ubiquitylated PTEN[5–21] (top) and PTEN[281–297] (bottom) derived peptide FP substrates (100 nM). P: polarisation.

Acknowledgements

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Keywords: activity assays · chemical ligation · fluorescent probes · proteases · protein–protein interactions

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Supporting Information

A General Chemical Ligation Approach Towards Isopeptide-Linked Ubiquitin and Ubiquitin-Like Assay Reagents

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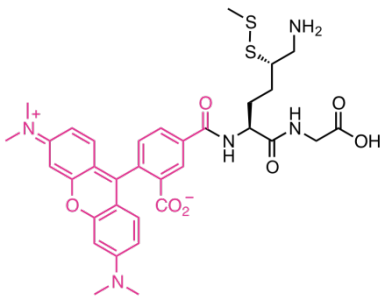
Materials and Methods

Solvents were purchased from BIOSOLVE and general chemicals from Sigma Aldrich. Peptide synthesis reagents were purchased from Novabiochem. VA-044 (2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride) was purchased from WAKO Pure Chemical Industries Ltd. Ubiquitin, SUMO-1, SUMO-2, SUMO-3, Nedd8 and corresponding E1s were obtained from Boston Biochem Inc (USA).

DUB	Origin/organism	Fragment
USP7	Human	full length (1-1102)
UCH-L3	Human	full length (1-230)
OTU from EAV	Equine arteritis virus	cat. domain 261-427 (MBP tagged)
OTU from CCHFV	Crimean-Congo hemorrhagic fever virus	aa 1-169
USP21	Human	cat. domain (221-565)
SENP1	Human	cat. domain (635-1112)
SENP6	Human	cat. domain (635-1112)
SENP7	Human	cat. domain (733-1050)

Table S1. Background of used Ub(l) deconjugating enzymes.

***N*$^{\alpha}$-(5-carboxytetramethylrhodamine)-5*S*-(methyldisulfanyl)-L-lysine-glycine.** To Fmoc-Gly-PEG-PS resin (1.0 g, 0.18 mmol/g) was added piperidine:NMP (20:80 v/v, 5 mL). After shaking for 45 min, the resin was washed with CH_2Cl_2. This procedure was repeated, followed by washing with CH_2Cl_2/NMP (1:1 v/v) and NMP. To the resin was added Fmoc-5-thiolysine-OH (196.8 mg, 0.36 mmol), PyBOP (206 mg, 0.40 mmol), D*i*PEA (138 µL, 0.79 mmol) and NMP (5 mL). After overnight shaking, the resin was thoroughly washed with CH_2Cl_2/NMP (1:1 v/v) and after a final wash diethylether dried under high vacuum. A portion of the resin (344 mg, 62 µmol) was treated with piperidine/NMP (1:4 v/v, 5 mL) for 5 min and washed with NMP. This procedure was repeated twice, after which the resin was washed properly with CH_2Cl_2/NMP (1:1 v/v). To the resulting resin was added 5-carboxytetramethylrhodamine (TAMRA, 107 mg, 250 µmol, 4 eq), PyBOP (129 mg, 250 µmol, 4 eq), D*i*PEA (65 µL, 370 µmol, 6 eq) and NMP/CH_2Cl_2 (5/1 mL). After shaking overnight, the resin was washed with CH_2Cl_2/NMP (1:1 v/v) until the flow-through was colorless. The resin was then treated with TFA/H_2O/Pr_3SiH (90:5:5 v/v/v, 4 mL) for 1 h. Elution of the product was performed with TFA/CH_2Cl_2 (1/1 v/v) followed by MeOH. Concentration and drying under high vacuum gave the title compound as a dark purple oil (45 mg, >99%). LC-MS: R_t = 2.3 min; ES^+ *m/z* $[M + H]^+$= 694.3 Da; *m/z* $[M + 2H]^+$= 347.6 Da. A 10 mM stock solution was prepared in 0.1 M sodium phosphate pH 8.



Fmoc solid phase peptide synthesis

Peptides were synthesized on a Syro II MultiSyntech Automated Peptide synthesizer by standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptides chemistry on a 25 or 50 µmol scale. Starting with the pre-loaded Fmoc amino acid Wang resin (0.2 mmol/g, Applied Biosystems), each successive amino acid (Novabiochem) was coupled in 4 molar excess for 45 min with pyBOP and DiPEA. The thiolysine building block could also be used in 3 molar excess. Deprotection of the Fmoc-group was achieved with 20% piperidine in NMP (3×1.2 mL, 2×2 and 1×5 min). Peptides were cleaved with TFA/iPr_3SiH/H_2O (95/2.5/2.5 v/v/v), precipitated in cold n-hexane/diethyl ether and washed 3× with diethylether. The peptides were obtained in good purity and required no additional purification.

General protocol one-pot Ubl ligation of 5-thiolysine modified peptides

To 0.1 M sodium phosphate pH 8.0 were added: 5 mM $MgCl_2$ (from 0.2 M aq. stock), 150 mM NaCl (from 5 M stock), 50 mM MESNa (from 2 M stock), 5 mM ATP (from 0.5 M aq. stock), Ub/Ubl (50 µM Ub or 10 µM Ub-like protein), TAMRA-thiolysine modified peptide (5 eq, *i.e.* 250 µM in the case of Ub or 50 µM in the case of a Ub-like protein). After adjusting the pH to 7.5 – 8.0 using 1N NaOH, the corresponding E1 (150 nM) was added and the ligation mixture was incubated at 37°C for >6 hrs. Next, 20 mM VA-044 (from freshly prepared 0.1 M stock in buffer), 40 mM GSH and 0.25 M TCEP (0.5 M stock, pH 7.0) were added to the crude ligation mixture. The pH was corrected to 7.0 using 1N NaOH and the reaction mixture was incubated at 37°C. Analysis by LC-MS showed that complete desulfurization took place in 3 – 6 hrs. However, it is important to limit this incubation period to 1 day since prolonged reaction times (>2 days) can lead to loss of the methyl groups on the TAMRA moiety. This was not only observed by LC-MS, but also a color change as the solution turns from the characteristic pink/purple to a more orange color.

LC-MS

LC-MS measurements were performed on a system equipped with a Waters 2795 Separation Module (Alliance HT), Waters 2996 Photodiode Array Detector (190-750nm), Phenomenex Kinetex C18, (2.1×50 mm), 2.6µm and LCTTM Orthogonal Acceleration Time of Flight Mass Spectrometer. Samples were run at a flow rate of 0.8 mL/min using 2 mobile phases: A= 0.1% aq. formic acid and B= 0.1% formic acid in CH_3CN. Column T= 40°C. Gradient: 5%→95%B over 3.5 min. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1 (deconvulation with maxent1 function).

SDS-PAGE analysis

A sample of the reaction mixture was diluted in *NuPAGE® LDS sample buffer* (3×, Invitrogen), heated at 71°C for 5-10 minutes and loaded on *12% NuPAGE® Novex® Bis-Tris Mini Gels* (Invitrogen) using MES-SDS running buffer. As marker was used the *SeeBlue Plus2 Pre-stained Standard* (Invitrogen, cat# LC5925).

SDS-PAGE analysis of Ub/Ubl ligation reactions

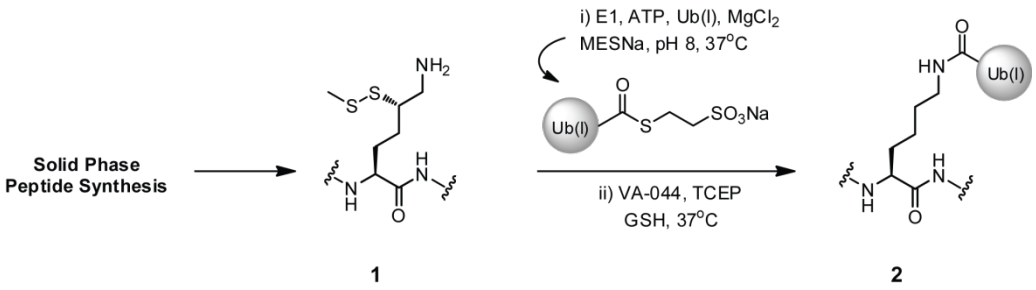


Figure S1. *One-pot Native Chemical Ub/Ubl ligation*.

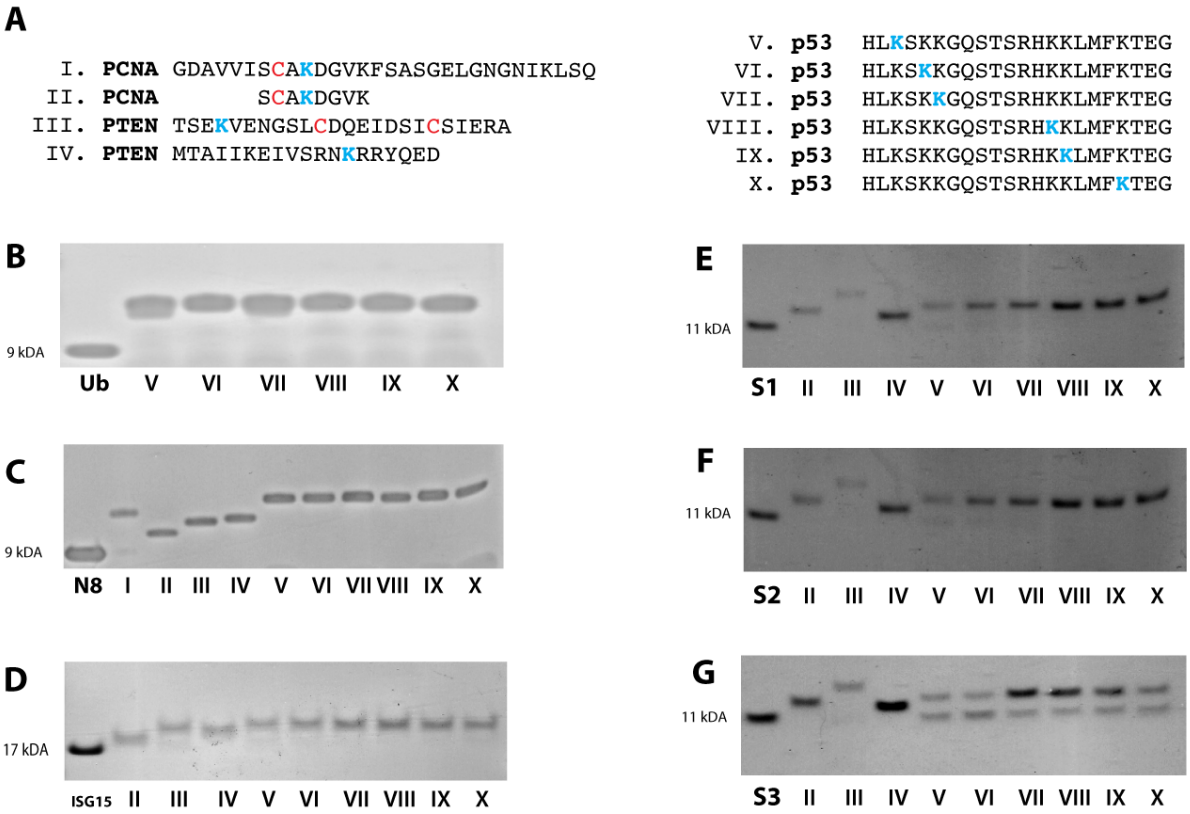


Figure S2. SDS-PAGE analysis of Ub/Ubl modifications of (**A**) 5-thiolysine modified peptides derived from monoubiquitinated proteins. The 5-thiolysine residue is indicated in cyan; in the case of peptides I – III, the thiol of the Cys residue was protected with a photolabile 2-nitrobenzyl group. (**B**) Ub ligation peptides V - X; (**C**) Nedd8 ligation peptides I - X; (**D**) ISG15 ligation peptides II - X; (**E**) SUMO-1 ligation peptides II - X; (**F**) SUMO-2 ligation peptides II - X; (**G**) SUMO-3 ligation peptides II – X.

Although the majority of ligation experiments proceeded to completion, in some cases (e.g. SUMO-3 ligation of peptides V – X) incomplete ligations were observed. In these cases completion can be achieved by treatment with fresh E1 and ATP.

Fluorescence Polarization Assay reagents

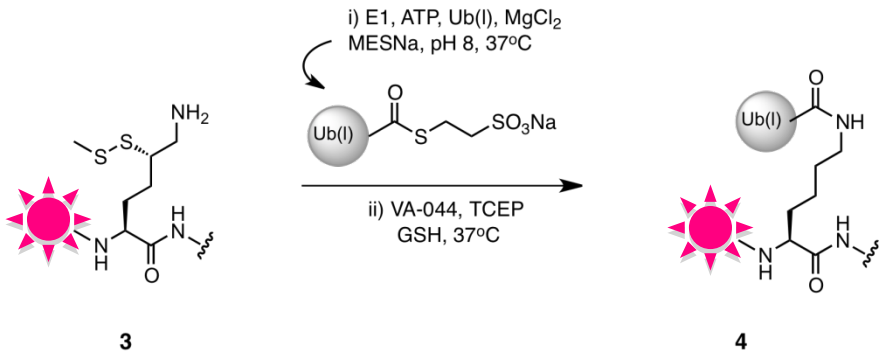


Figure S3. One-pot Native Chemical Ub/Ubl ligation and desulfurization - synthesis of FP reagents

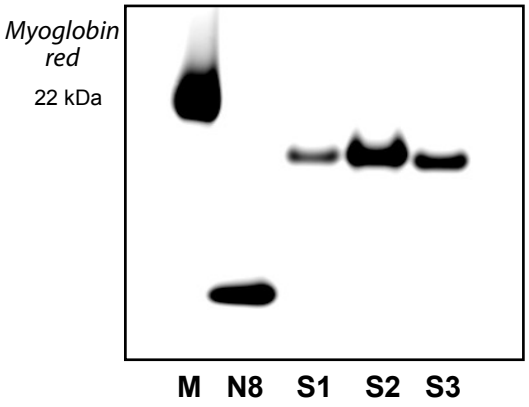


Figure S4. Fluorescence scan of SDS gel with Ubl FP reagents (exc 560 nm, em 590 nM).

Preparative HPLC TAMRA-Lys(Ub/Ubl)-Gly-OH FP reagents.
Shimadzu LC-20AD/T using a C8 Vydac column (Grace Davison Discovery Sciences™). Column Mobile phases: A= 0.05% aq. TFA and B= 0.05% TFA in CH_3CN. T= 40°C. Flow rate= 5 mL/min. Gradient: 30→75%B over 18 min.

5-TAMRA-Lys(Ub)-Gly-OH R_t= 13 min.
5-TAMRA-Lys(Nedd8)-Gly-OH R_t= 15.5 min.
5-TAMRA-Lys(SUMO-1)-Gly-OH R_t= 14 min.
5-TAMRA-Lys(SUMO-2)-Gly-OH R_t= 15 min.
5-TAMRA-Lys(SUMO-3)-Gly-OH R_t= 15 min.

Yield: 20 mg TAMRA-Lys(Ub)-Gly-OH FP reagent from 40 mg Ub (≈50%)
Yield: 0.4 mg TAMRA-Lys(Nedd8)-Gly-OH FP reagent from 1 mg Nedd8 (≈37%)
Yield: 0.3 mg TAMRA-Lys(SUMO-1)-Gly-OH FP reagent from 1 mg SUMO-1 (≈30%)
Yield: 0.3 mg TAMRA-Lys(SUMO-2)-Gly-OH FP reagent from 1 mg SUMO-2 (≈28%)
Yield: 0.11 mg TAMRA-Lys(SUMO-3)-Gly-OH FP reagent from 0.5 mg SUMO-3 (≈20%)

LC-MS analysis TAMRA-Lys(Ubl)-Gly-OH FP reagents.

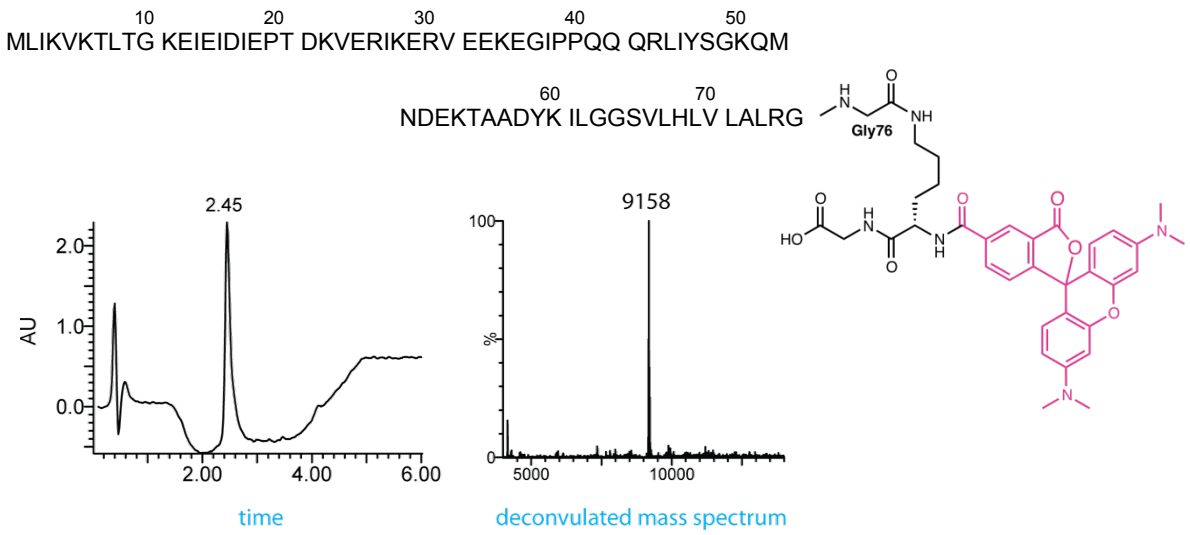


Figure S5. LC-MS analysis of 5-TAMRA-Lys(Nedd8)-Gly-OH. Found: 9158 Da, calc 9158 Da.

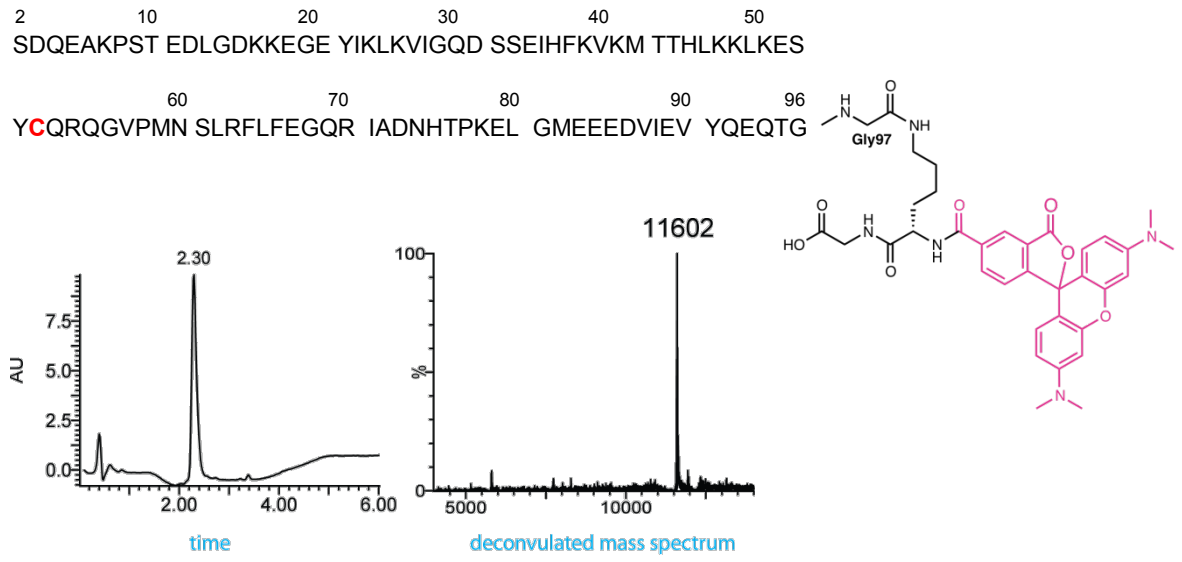


Figure S6. LC-MS analysis of 5-TAMRA-Lys(SUMO-1)-Gly-OH. Found: 11602 Da, calc 11600 Da. According to MS analysis, the N-terminal Methionine is missing and Cys52 is retained during the preparation of this conjugate. LC-MS analysis of the SUMO-1 protein showed deletion of the N-terminal methionine residue, indicating it had been removed during expression.

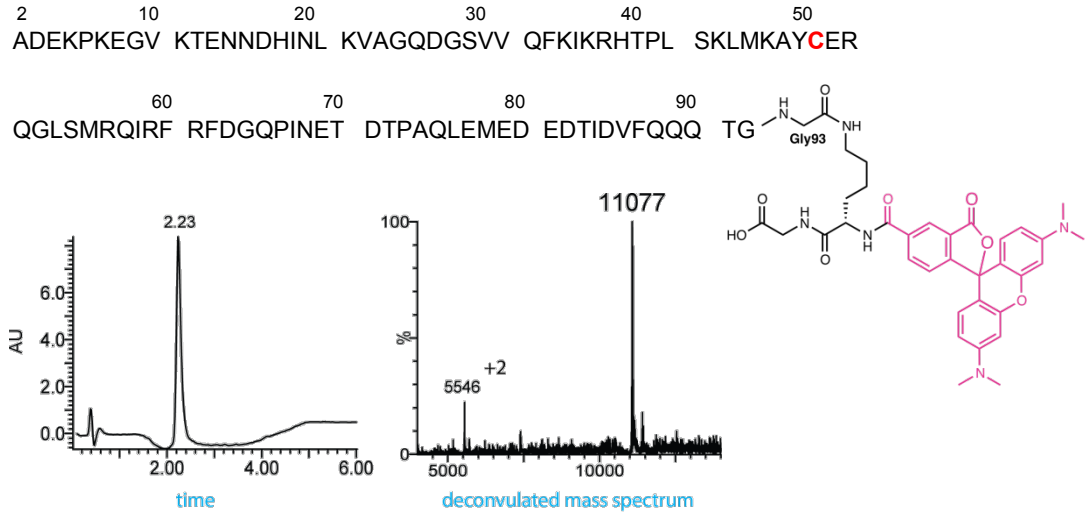


Figure S7. LC-MS analysis of 5-TAMRA-Lys(SUMO-2)-Gly-OH. Found: 11077 Da, calc 11076 Da. According to MS analysis, the N-terminal Methionine is missing and Cys52 is retained during the preparation of this conjugate. LC-MS analysis of the SUMO-2 protein showed deletion of the N-terminal methionine residue, indicating it had been removed during expression.

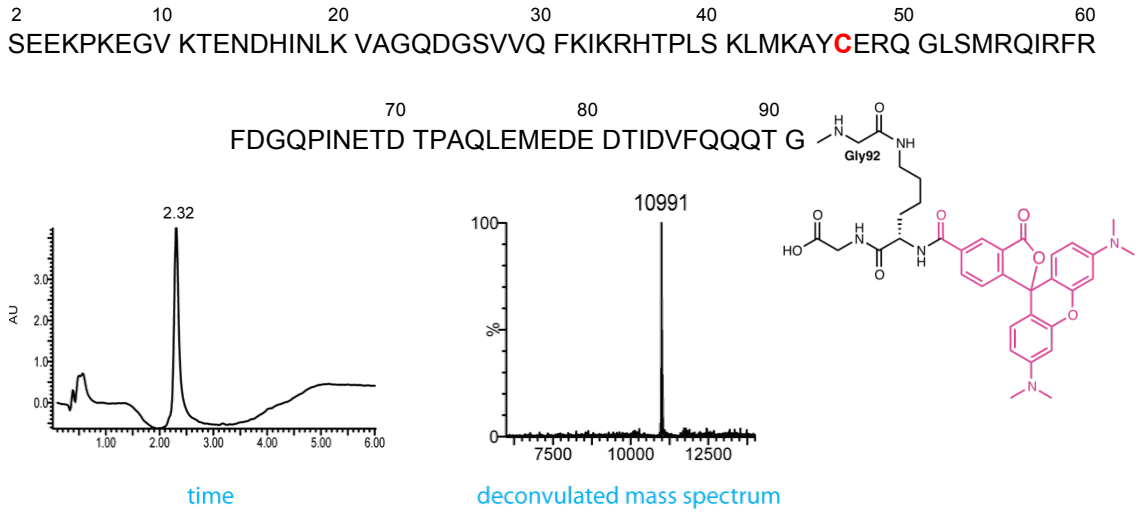


Figure S8. LC-MS analysis of 5-TAMRA-Lys(SUMO-3)-Gly-OH. Found: 10991 Da, calc 10992 Da. According to MS analysis, the N-terminal Methionine is missing and Cys52 is retained during the preparation of this conjugate. LC-MS analysis of the SUMO-3 protein showed deletion of the N-terminal methionine residue, indicating it had been removed during expression.

Preparative HPLC PTEN[281-297] and PTEN[5-21] FP reagents.
Shimadzu LC-20AD/T using a C18 Atlantis Prep T3 (5 µm, 10×150 mm). Column Mobile phases: A= 0.05% aq. TFA and B= 0.05% TFA in CH_3CN. T= 40°C. Flow rate= 7.5 mL/min. Gradient: 25→47%B over 30 min.

5-TAMRA-PTEN[281-297]-Ub R_t= 17.3 min.
5-TAMRA-PTEN[5-21]-Ub R_t= 17.5 min.

Yield: 1.0 mg TAMRA-PTEN[281-297]-Ub FP reagent from 2 mg Ub (≈40%)
Yield: 2.5 mg TAMRA-PTEN[5-21]-Ub FP reagent from 3 mg Ub (≈65%)

LC-MS analysis PTEN[5-21] and PTEN[281-297] FP reagents

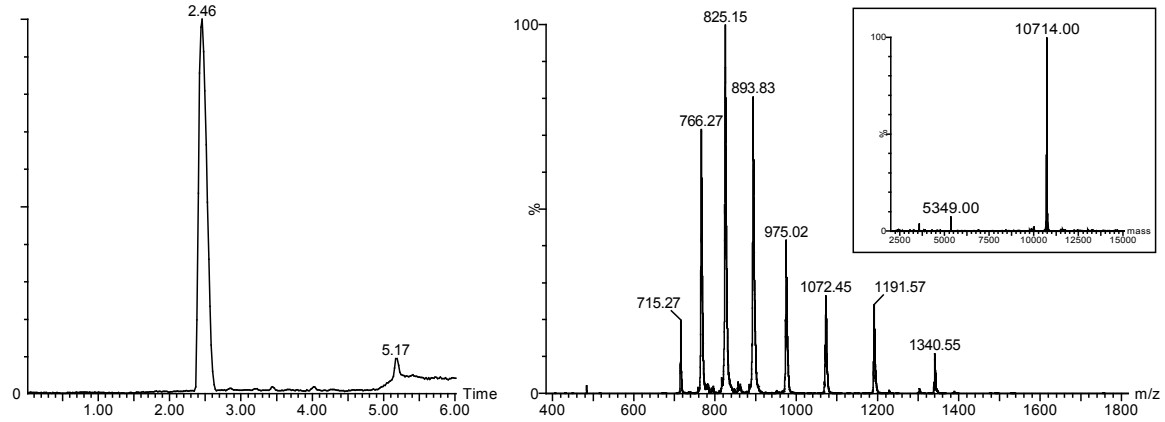


Figure S9. LC-MS analysis of the PTEN[281-297] FP reagent. Left: UV trace. Right: MS analysis. Insert: deconvoluted mass of product peak at 2.46 min.

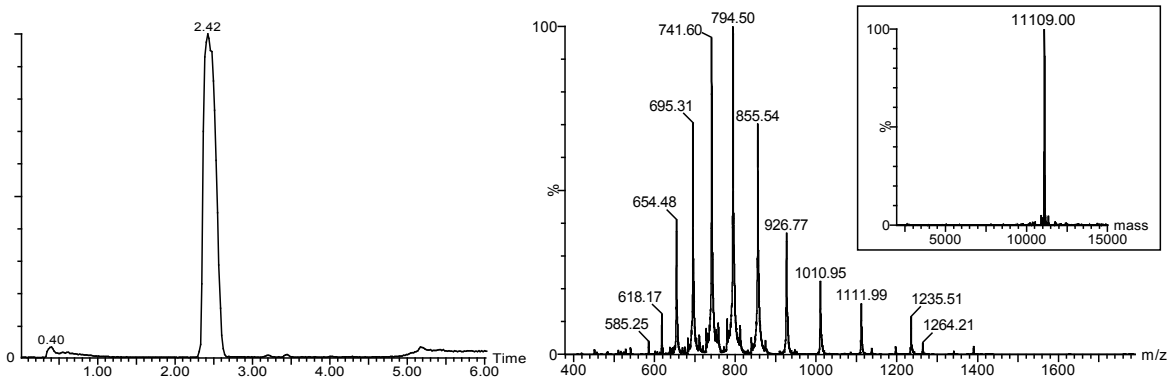


Figure S10. LC-MS analysis of the PTEN[5-21] FP reagent. Left: UV trace. Right: MS analysis. Insert: deconvoluted mass of product peak at 2.42 min. This mass corresponds to the product containing oxidized methionine (+16 Da).

Fluorescence polarization assay set-up

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-KG and the grating factor (G) was determined using a polarization value (L) for TAMRA-KG (25 nM) 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 TrisHCl, 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. The obtained data was fitted according to a ‘one phase exponential decay’ using Prism 5.01 (Graphpad Software, Inc.). From the obtained polarization values (P_t) the amount of processed substrate (S_t) was calculated with the following equation (Levine *et al. Anal. Biochem.* **1997**, *247*, 83):

$$S_t = S_0 - S_0 \times \frac{P_t - P_{min}}{P_{max} - P_{min}}$$

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 5.01 (GraphPad Software, Inc.).

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

Fluorescence polarization experiments

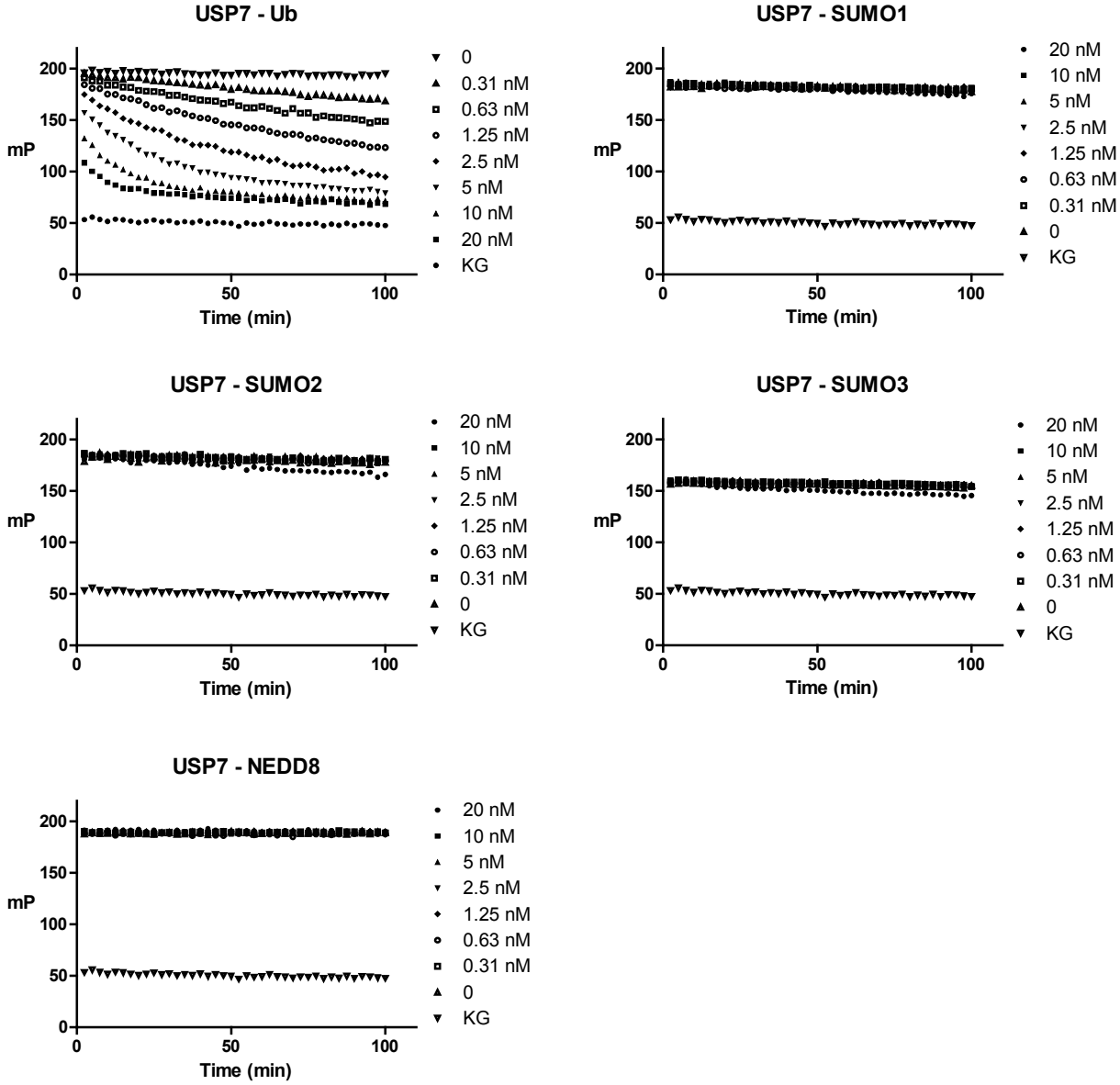


Figure S11. Fluorescence polarization assay results for USP7 (0.31-20 nM) with all Ub/Ubl FP reagents (fixed concentration of 100 nM).

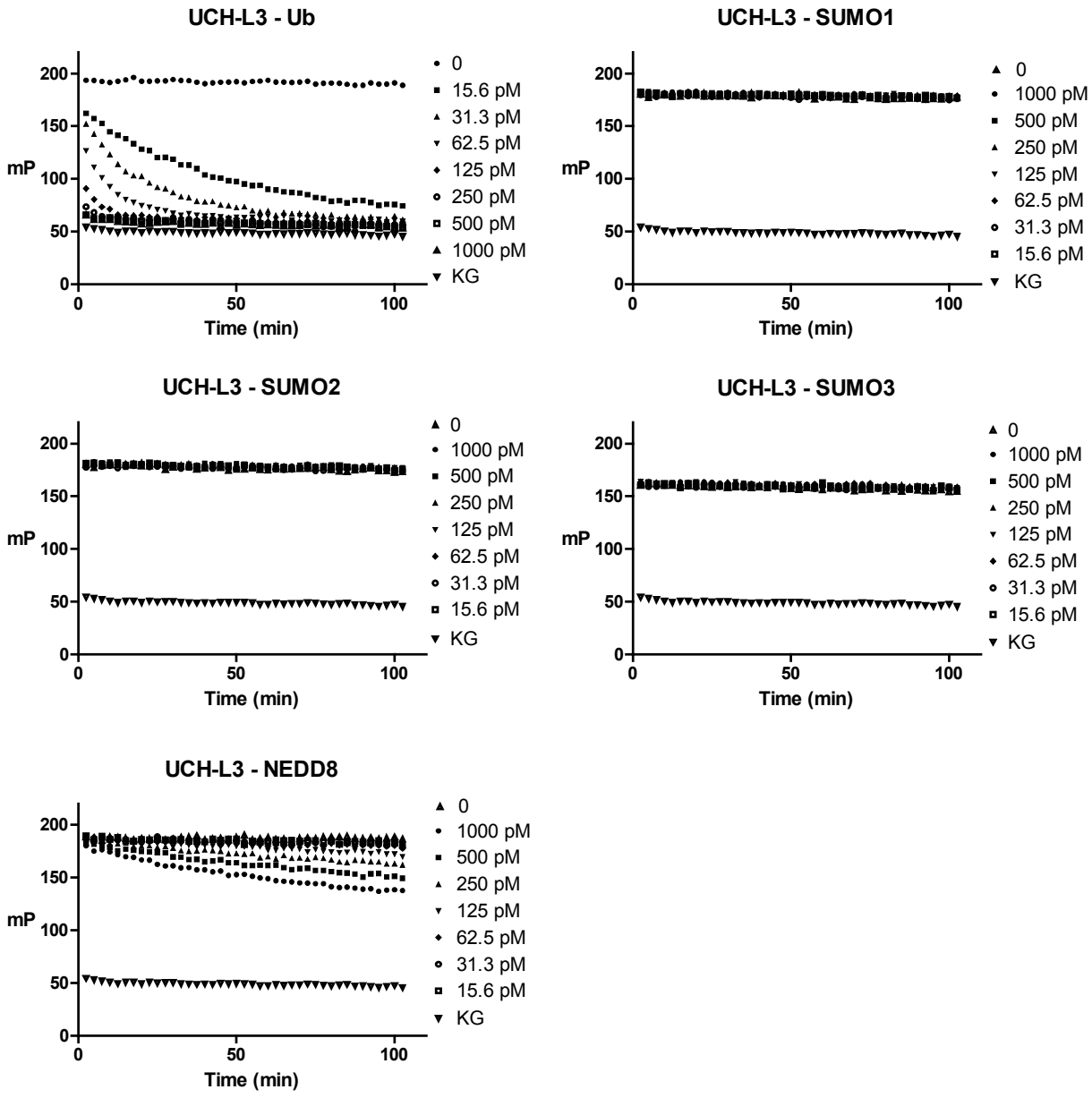


Figure S12. Fluorescence polarization assay results for UCH-L3 (15.6 pM – 1 nM) with all Ub/Ubl FP reagents (fixed concentration of 100 nM).

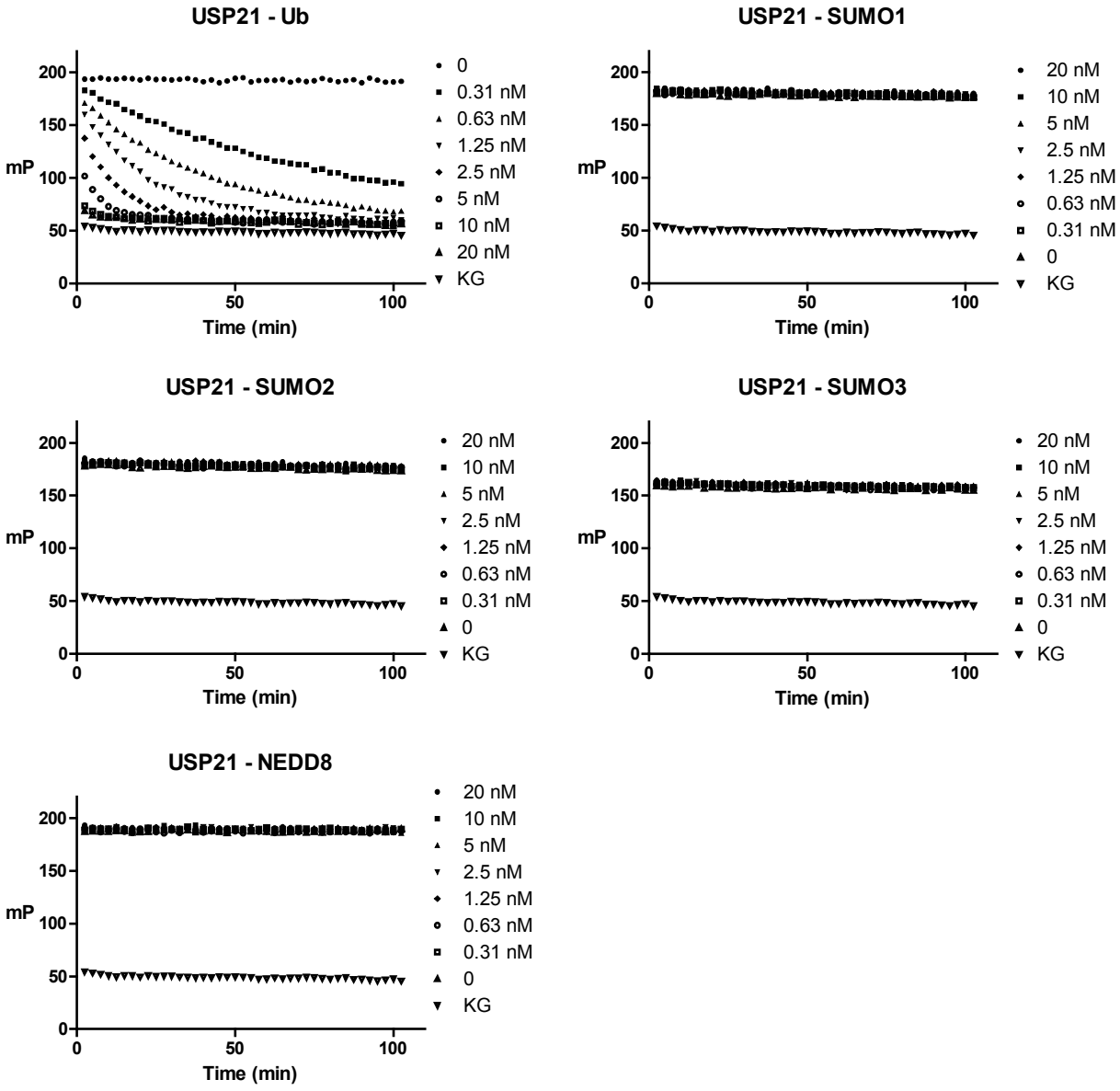


Figure S13. Fluorescence polarization assay results for USP21 (0.31-20 nM) with all Ub/Ubl FP reagents (fixed concentration of 100 nM).

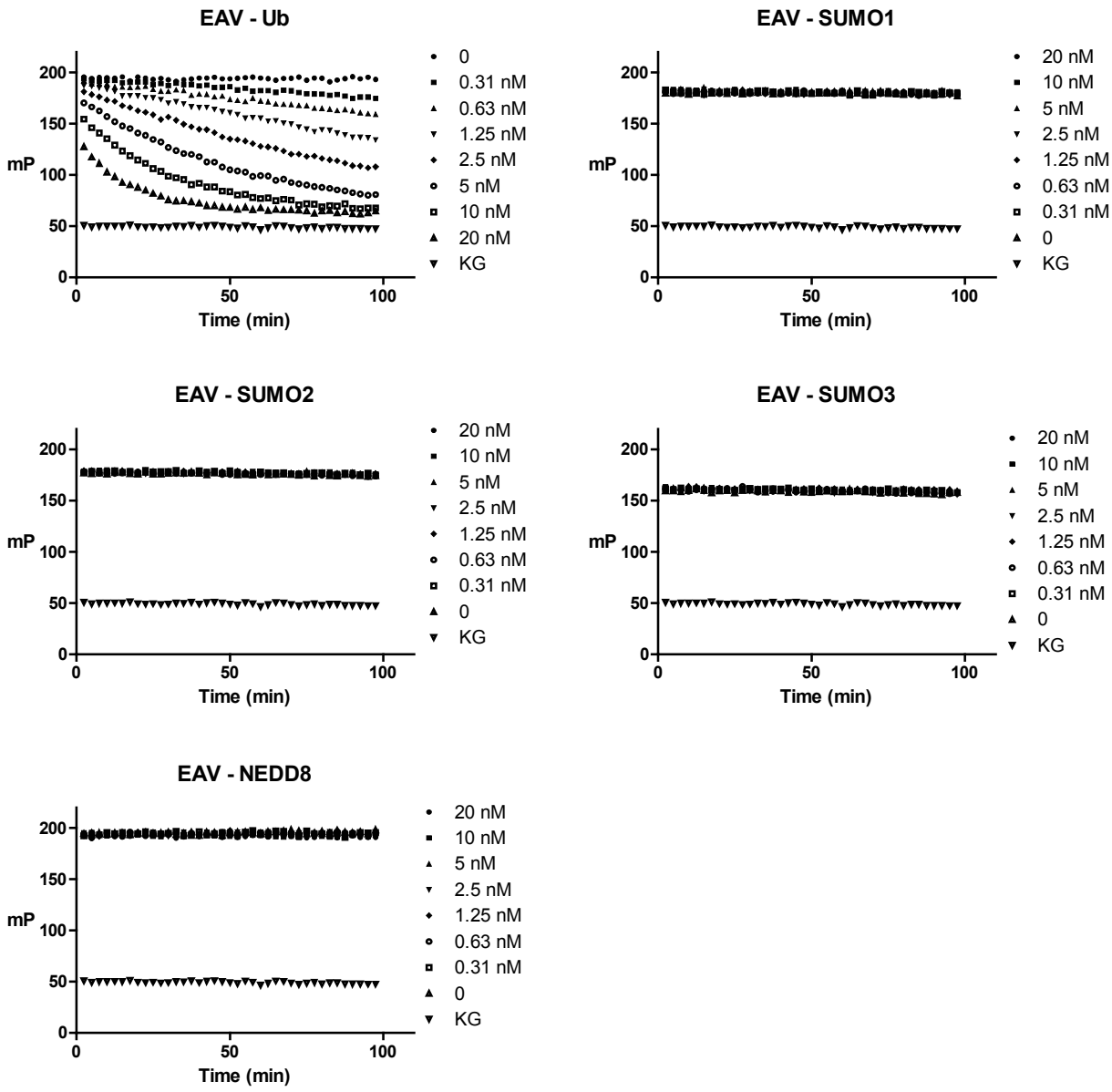


Figure S14. Fluorescence polarization assay results for the OTU derived from EAV (0.31-20 nM) with all Ub/Ubl FP reagents (fixed concentration of 100 nM).

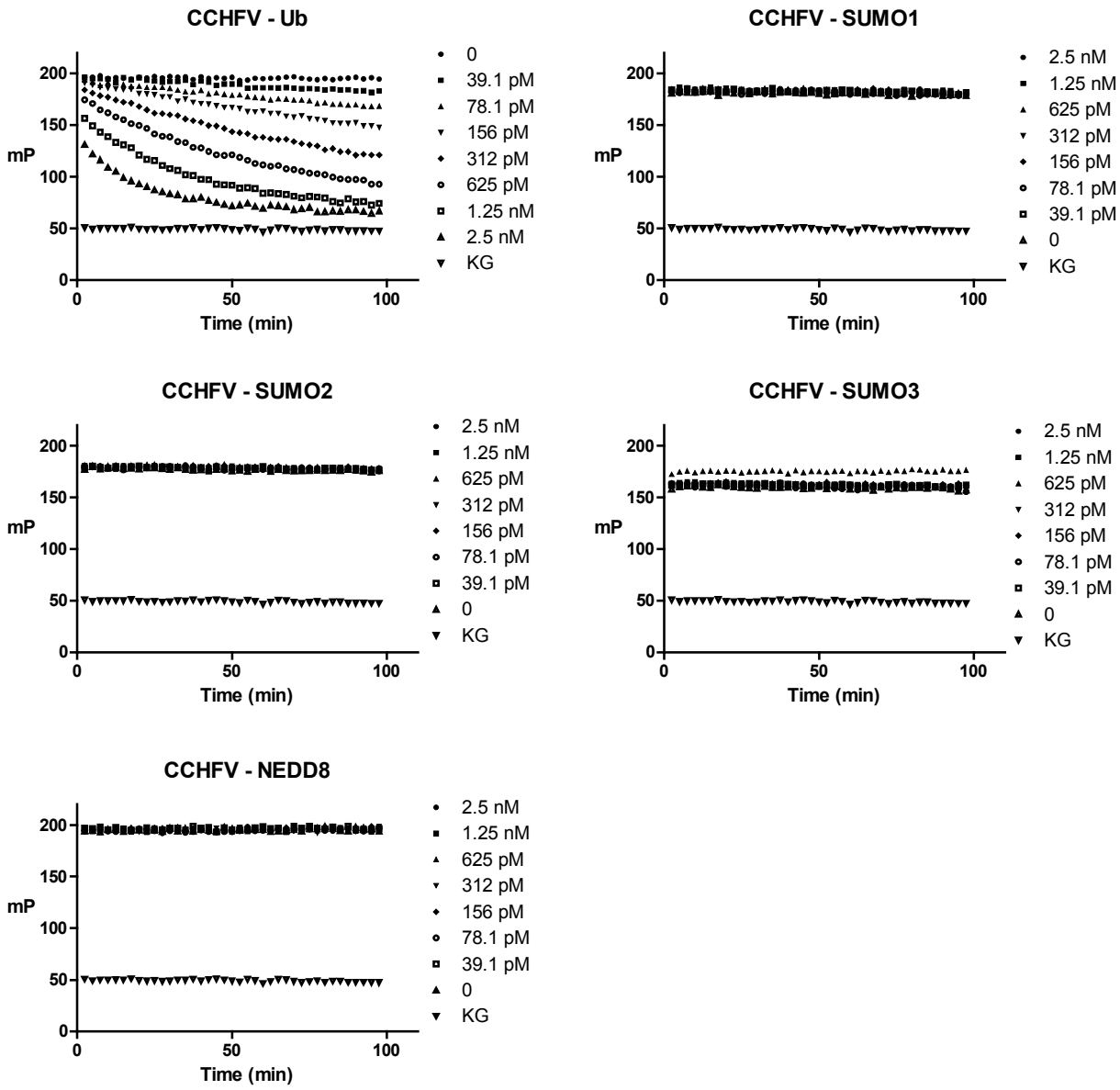


Figure S15. Fluorescence polarization assay results for the OTU derived from CCHFV (0.31-20 nM) with all Ub/Ubl FP reagents (fixed concentration of 100 nM).

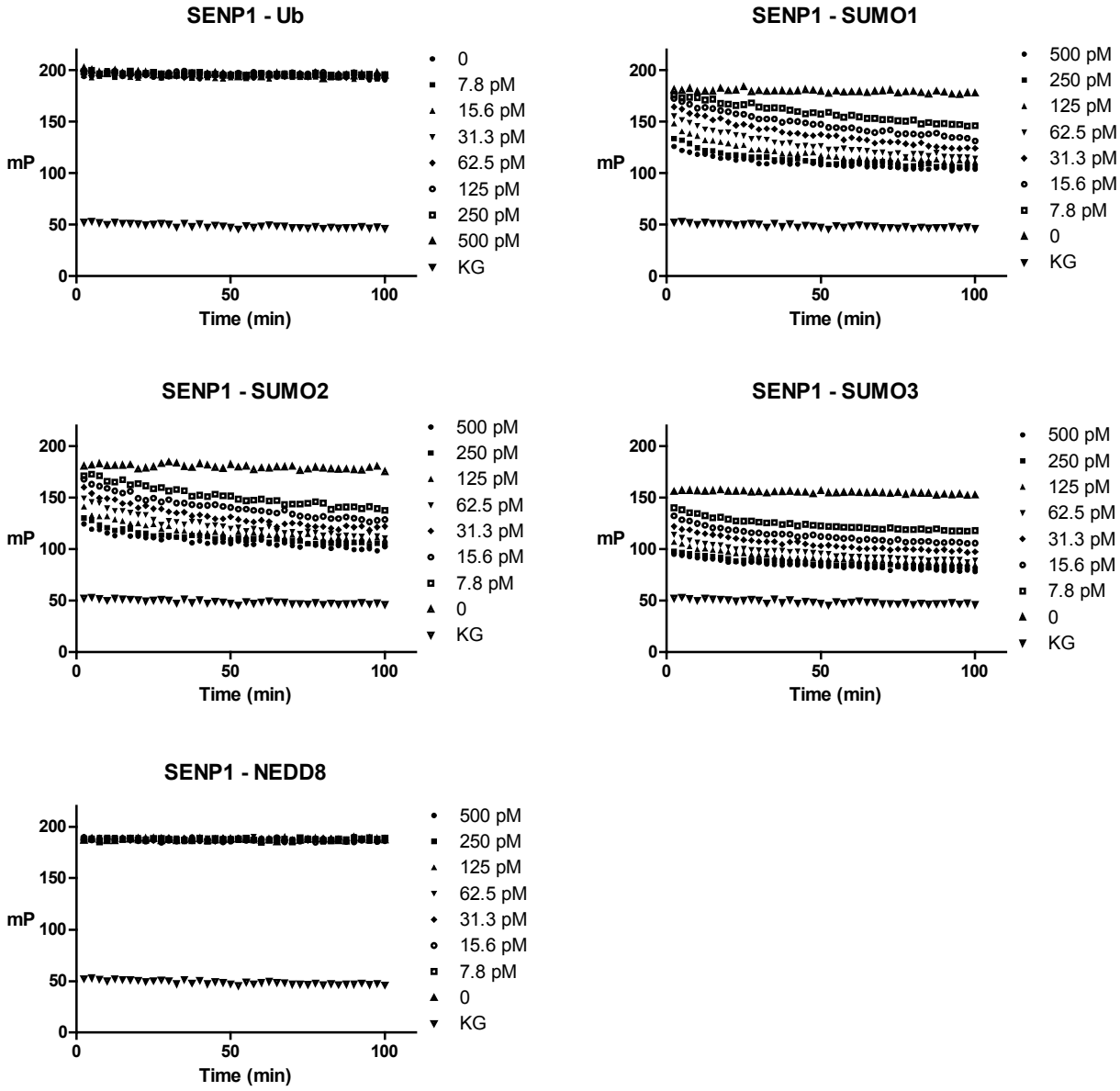


Figure S16. Fluorescence polarization assay results for SENP1 (7.8-500 pM) with all Ub/Ubl FP reagents (fixed concentration of 100 nM).

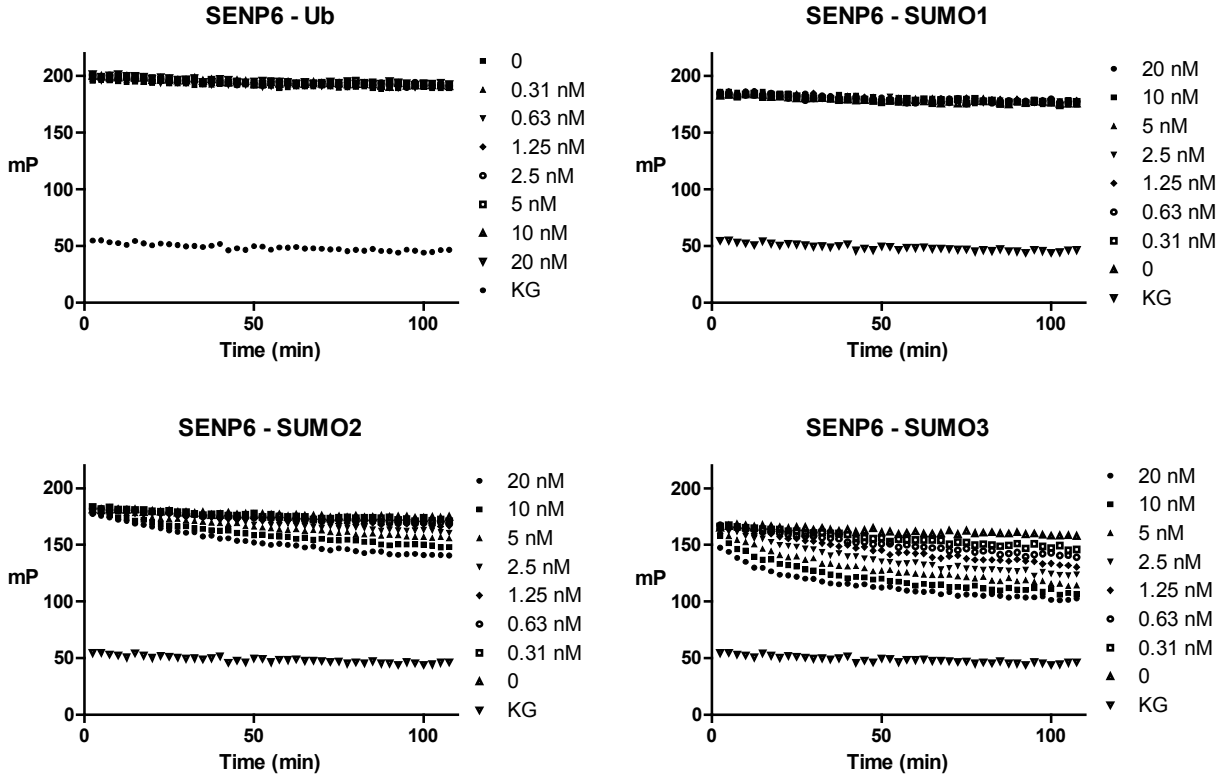


Figure S17. Fluorescence polarization assay results for SENP6 (0.31-20 nM) with the Ub and all SUMO FP reagents (fixed concentration of 100 nM).

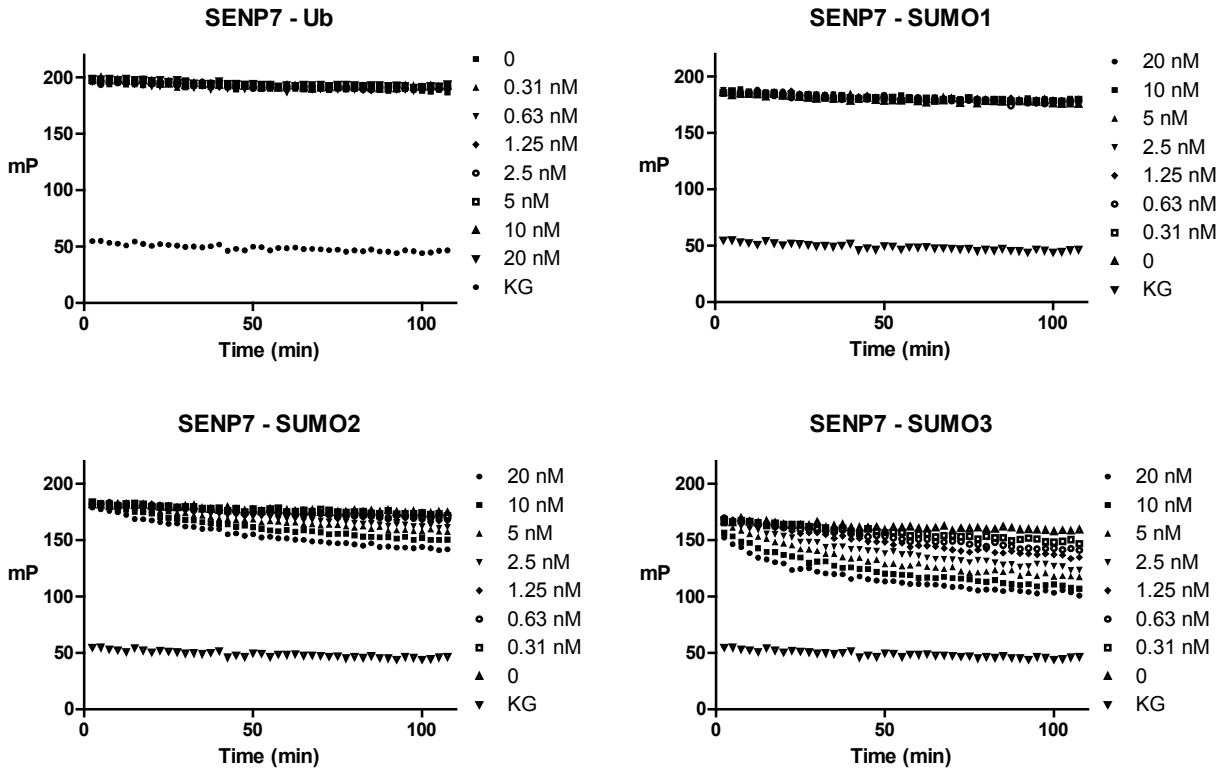


Figure S18. Fluorescence polarization assay results for SENP7 (0.31-20 nM) with the Ub and all SUMO FP reagents (fixed concentration of 100 nM).

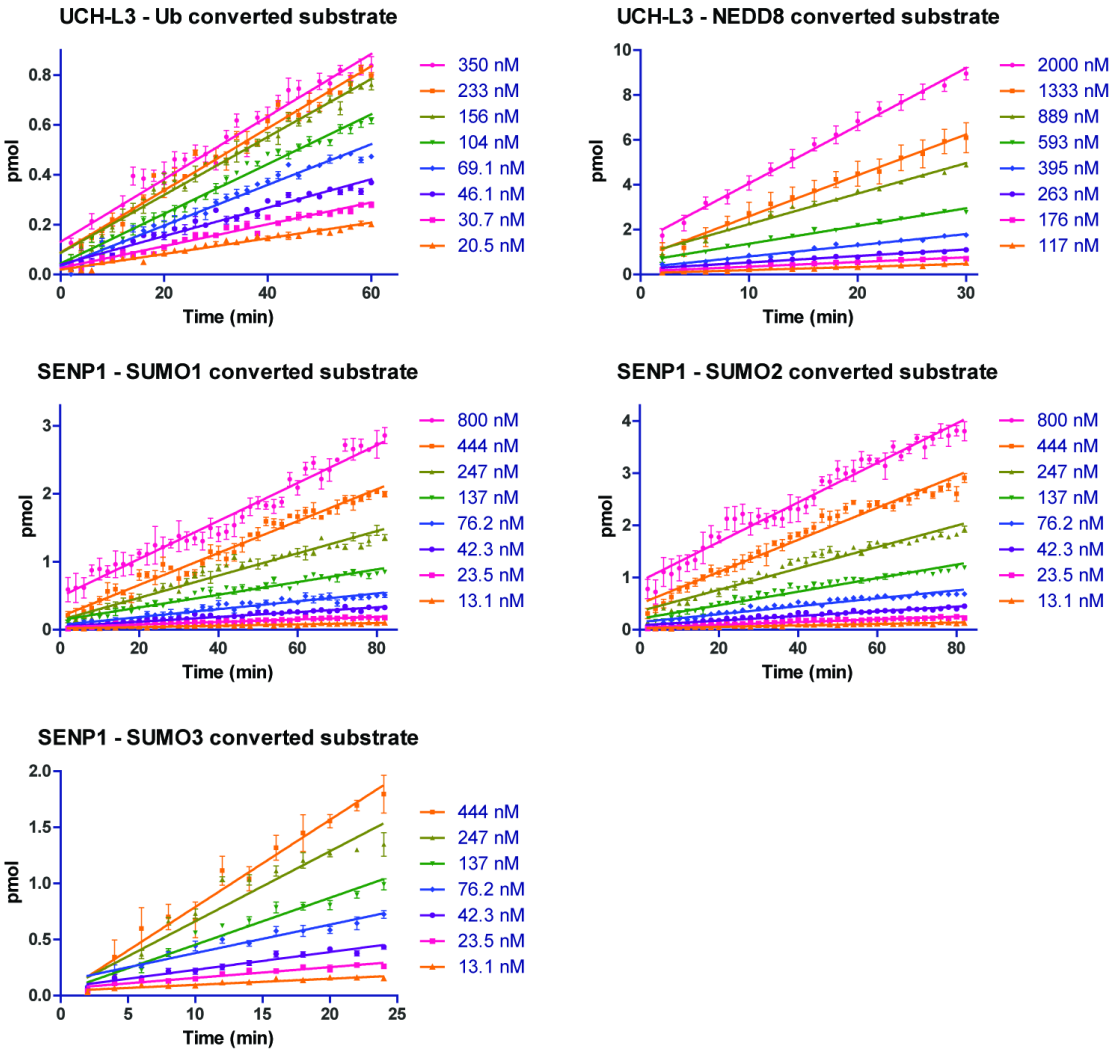


Figure S19. Substrate titration. Different concentrations of the Ub, Nedd8 and SUMO1,2,3 FP reagents were incubated with the depicted Ub(l) deconjugating enzymes (UCH-L3: 5 pM for Ub; UCH-L3: 700 pM for Nedd8; SENP1: 10 pM) and the change in fluorescence polarization was monitored. The resulting data were transformed to express the converted substrate as a function of time (shown here). From this the rates of initial velocity were calculated.

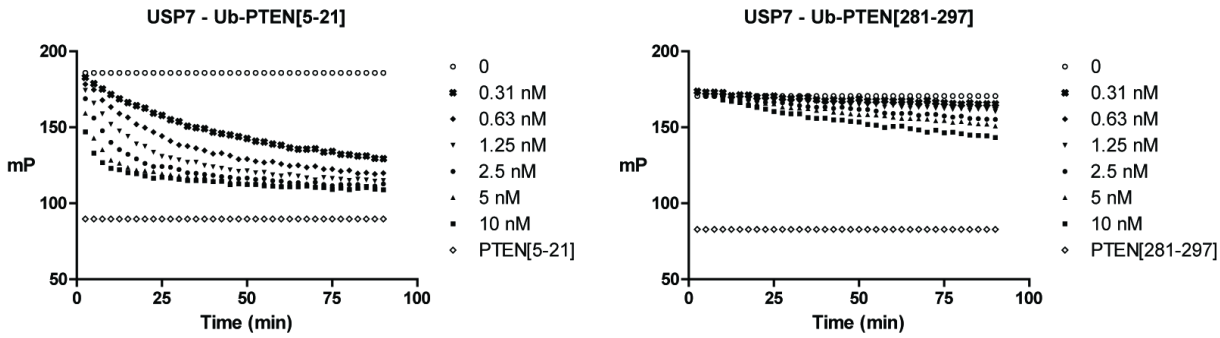


Figure S20. Fluorescence polarization assay results for USP7 (0.31-20 nM) with the PTEN-derived Ub FP reagents (fixed concentration of 100 nM).

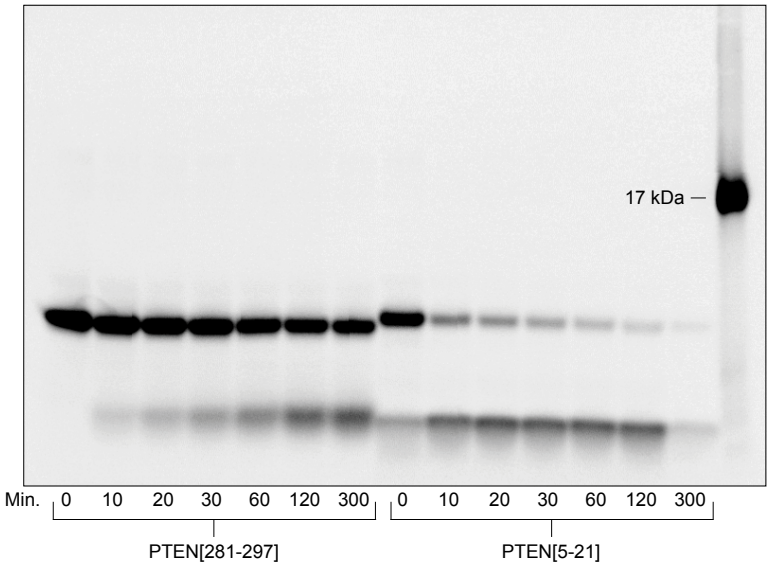


Figure S21. USP7 mediated hydrolysis of the PTEN-derived FP reagents, analyzed by gel electrophoresis USP7FL [1-1102] was incubated (10 nM final concentration) with the PTEN-derived FP reagents (10 µM final concentration) in a buffer containing 50 mM Tris.HCl (pH 8.0), 5 mM DTT and 150 mM NaCl. Samples were taken after 10, 20, 30, 60, 120 and 300 minutes and the reaction was stopped by addition of sample buffer containing β-mercaptoethanol, followed by heating the sample to 80 °C for 5 minutes. As a control (0) 10 µM of the PTEN-derived FP reagents were incubated in buffer in the absence of USP7 for 300 minutes. The samples were resolved by gel electrophoresis (12% bis-tris precast gel, MES buffer). Loading: 0.1 nmol/lane. Read-out by fluorescence scanning ($\lambda_{ex/em}$ 550/590 nm).

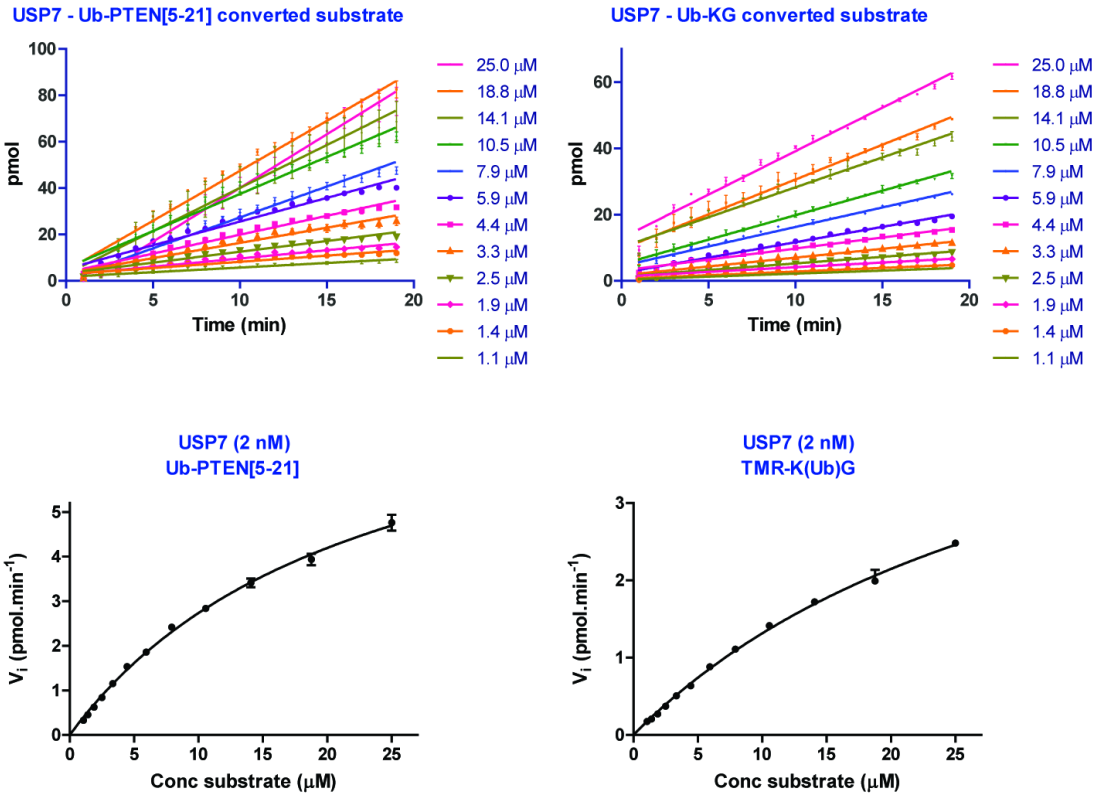


Figure S22. Substrate titration. Different concentrations of the Ub-PTEN[5-21] (left) and Ub-KG (right) FP reagents were incubated with 2 nM full lengthUSP7 and the change in fluorescence polarization was monitored. The resulting data were transformed to express the converted substrate as a function of time (top figure). From this the rates of initial velocity were calculated and these data were fitted to Michaelis-Menten kinetics (bottom figure).

Figure S23 shows that addition of excess ubiquitin does not inhibit the enzymatic activity of USP7, indicating that product inhibition does not take place during the FP assay.

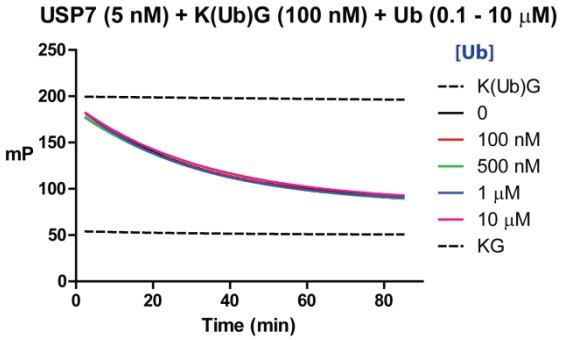


Figure S23. USP7 (5 nM) was incubated with different concentrations ubiquitin (0-10 μM). After addition of the substrate TAMRA-K(Ub)G (100 nM) the change in fluorescence polarization was monitored.