Supporting Information for

Probing affinity and ubiquitin linkage selectivity of ubiquitin-binding domains using mass spectrometry

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Determination of Ub•UBD binding affinity by the ESI-MS technique

The equilibrium expression for the dissociation of a Ub•UBD complex is given by eq. 1, where $[Ub]_{eq}$, $[UBD]_{eq}$, $[Ub•UBD]_{eq}$ are the equilibrium concentration of Ub, UBD and their complex, respectively.

$$K_d = \frac{[Ub]_{eq}[UBD]_{eq}}{[Ub \bullet UBD]_{eq}} \quad (1)$$

The free and the bound Ub ions as measured by mass spectrometry in positive ion mode are Ub^{n+} and $Ub•UBD^{n+}$, respectively. Providing that electrospray response factors for Ub^{n+} and $Ub•UBD^{n+}$ are similar, it can be assumed that the ratio (R) of the ion intensity (I) of the bound and unbound protein ions represent solution equilibria (eq. 2)

$$R = \frac{[Ub]_{eq}}{[Ub \bullet UBD]_{eq}} = \frac{\sum_n I(Ub^{n+})}{\sum_n I((Ub \bullet UBD)^{n+})} \quad (2)$$

Assuming that Ub molecules can be either free or UBD-bound the mass conservation is given by eq. 3, where $[Ub]_0$ is the initial concentration of Ub.

$$[\mathrm{Ub}]_0 = [\mathrm{Ub}]_{\mathrm{eq}} + [\mathrm{Ub} \bullet \mathrm{UBD}]_{\mathrm{eq}} \quad (3)$$

Combining eq. 2 and 3, the equilibrium concentration of Ub•UBD complex can be determined from the ratio R and the initial concentration of Ub (eq. 4).

$$[\mathrm{Ub} \bullet \mathrm{UBD}]_{\mathrm{eq}} = \frac{[\mathrm{Ub}]_0}{1 + \mathrm{R}} \quad (4)$$

Similarly, the mass conversion for UBD is given by eq.5, where $[\mathrm{UBD}]_0$ is the initial concentration of UBD.

$$[\mathrm{UBD}]_0 = [\mathrm{UBD}]_{\mathrm{eq}} + [\mathrm{Ub} \bullet \mathrm{UBD}]_{\mathrm{eq}} \quad (5)$$

Combining eq. 2, 4 and 5 and replacing to eq. 1, the K_{d} will be determined from the ratio (R) and the initial concentrations of Ub and UBD (eq.6).

$$K_{\mathrm{d}} = \mathrm{R}\left([\mathrm{UBD}]_0 - \frac{[\mathrm{Ub}]_0}{1 + \mathrm{R}}\right) \quad (6)$$

Table S1. Sequences of UBDs used in this study

UBD	Sequence of thrombin cleaved product
Rabex-5-MIU	GSQKQIQEDWELAERLQREEEEAFASSQS
UQ1 -UBA	GSVRFQQQLEQLSAMGFLNREANLQALIATGGDINAAIERLLGS
hHR23A -UBA2	GSQVTPQEKEAIERLKALGFPESLVIQAYFACEKNENLAANFLLSQNFDDE
IsoT-ZnF	GSKQEVQAWDGEVRQVSKHAFSLKQLDNPARIPPCGWKCSKCDMRENLWLNL TDGSILCGRRYFDGSGGNNHAVEHYRETGYPLAVKLGTITPDGADVYSYDED DMVLDPSLAEHLSHFGIDMLKMQKTDK

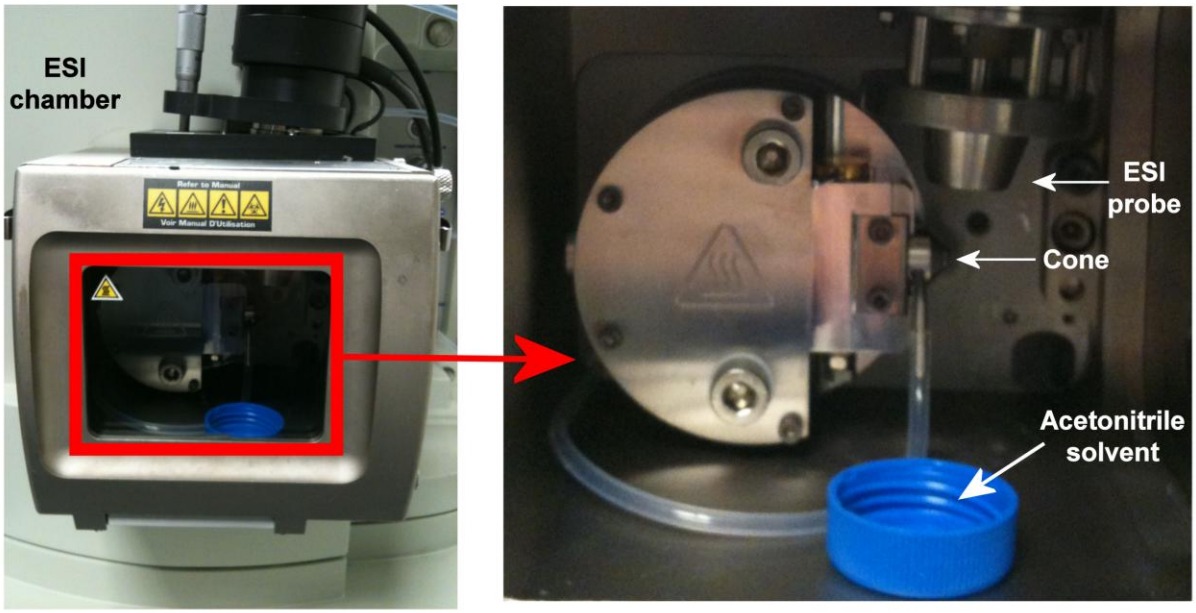


Figure S1. Photograph of the acetonitrile solvent container placed inside the doorway of the ESI source chamber.

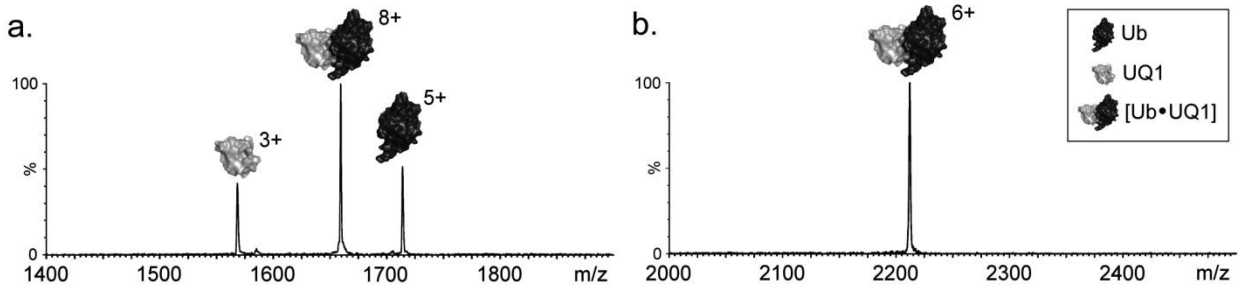


Figure S2. Spectra of quadrupole isolated Ub-UQ1-UBA complex charge states (a) 8+ and (b) 6+ of Ub (0.5 μM) and UQ1-UBA (4 μM) complex.

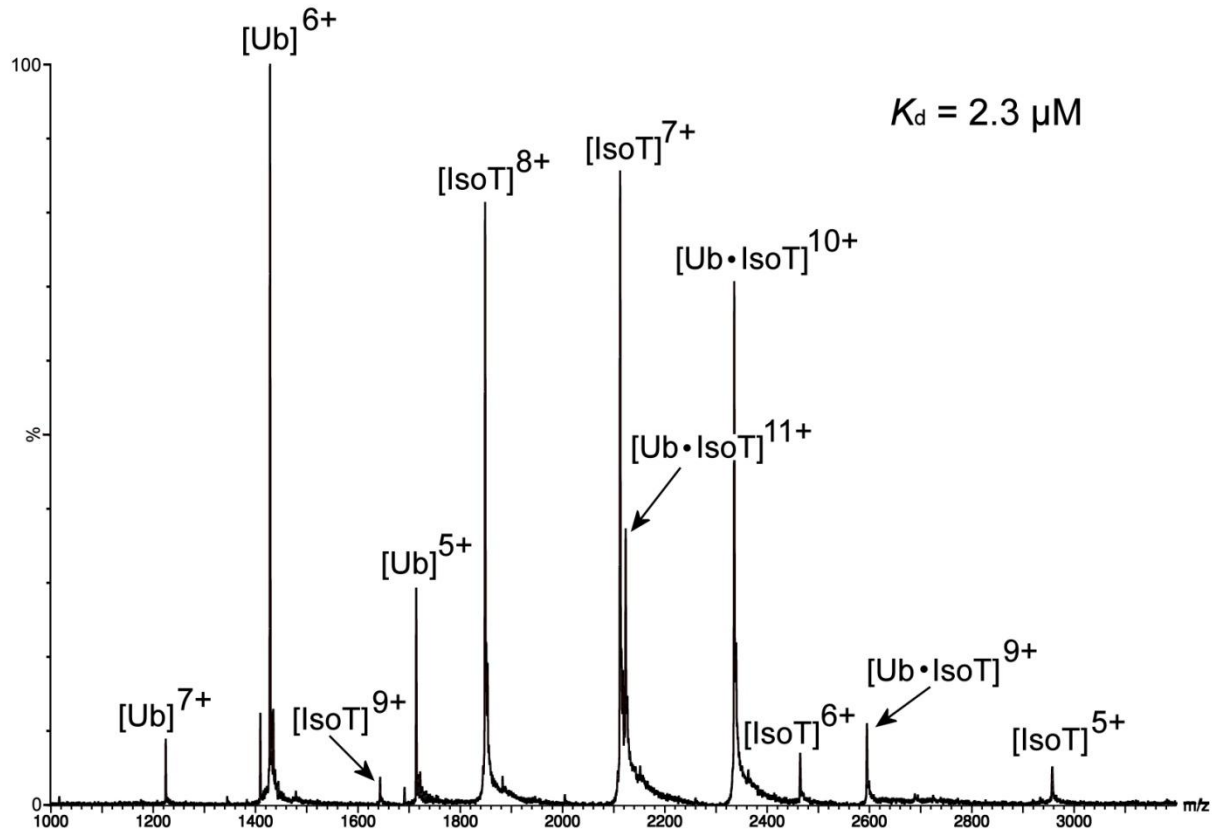


Figure S3. ESI mass spectrum of IsoT-ZnF (2 μM) and Ub (0.5 μM) sprayed from 25 mM ammonium acetate.

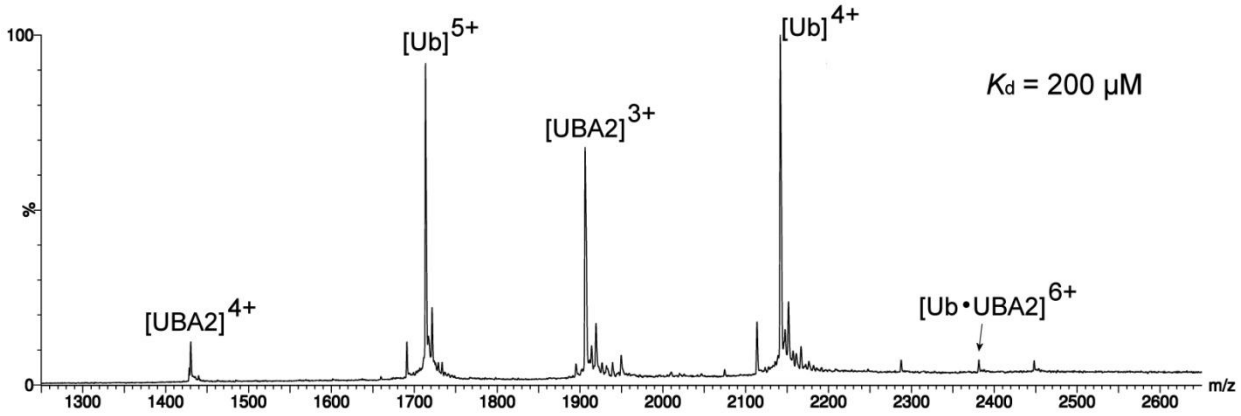


Figure S4. ESI mass spectrum of 0.5 μM Ub and 4 μM UBA2 complex, sprayed from 25mM ammonium acetate solution under acetonitrile vapor.

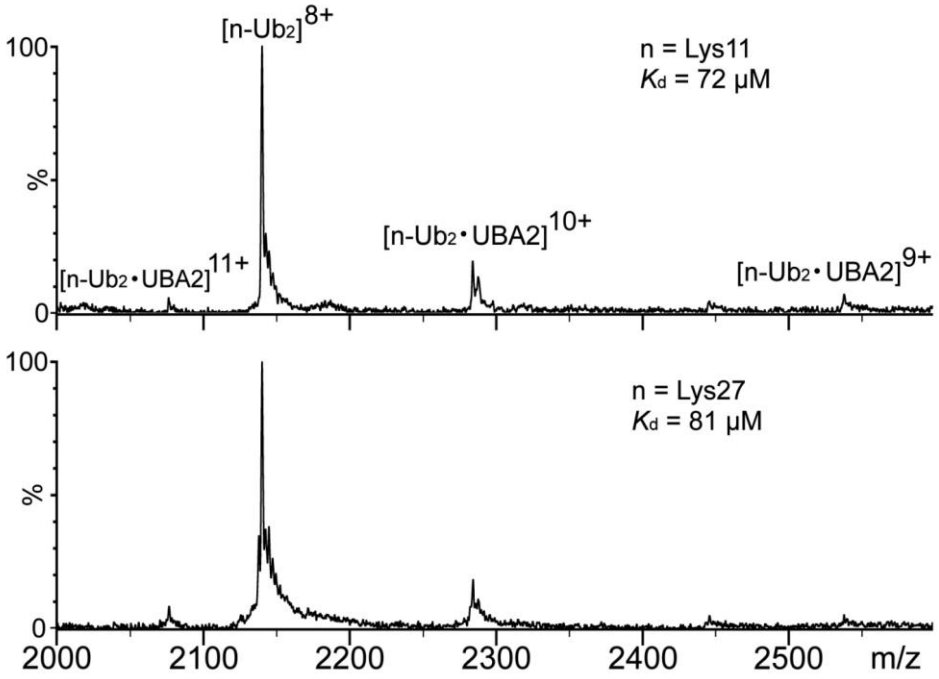
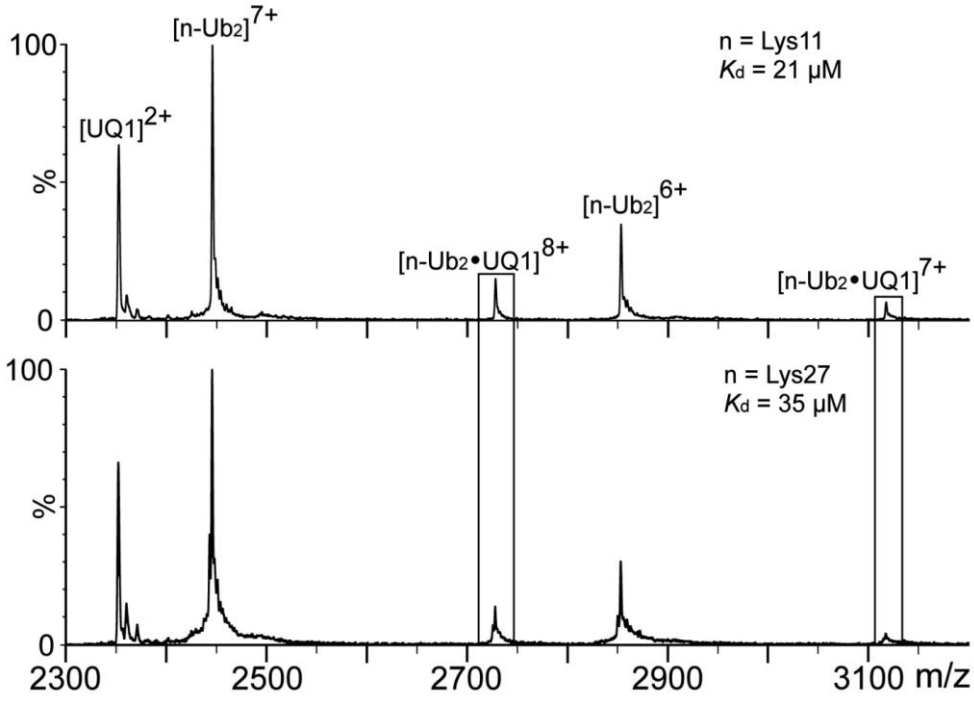


Figure S5. ESI mass spectra of UBA2 (4 μM) with n-Ub_2 (0.5 μM) (where n is the lysine linkage type) sprayed from 25 mM ammonium acetate.



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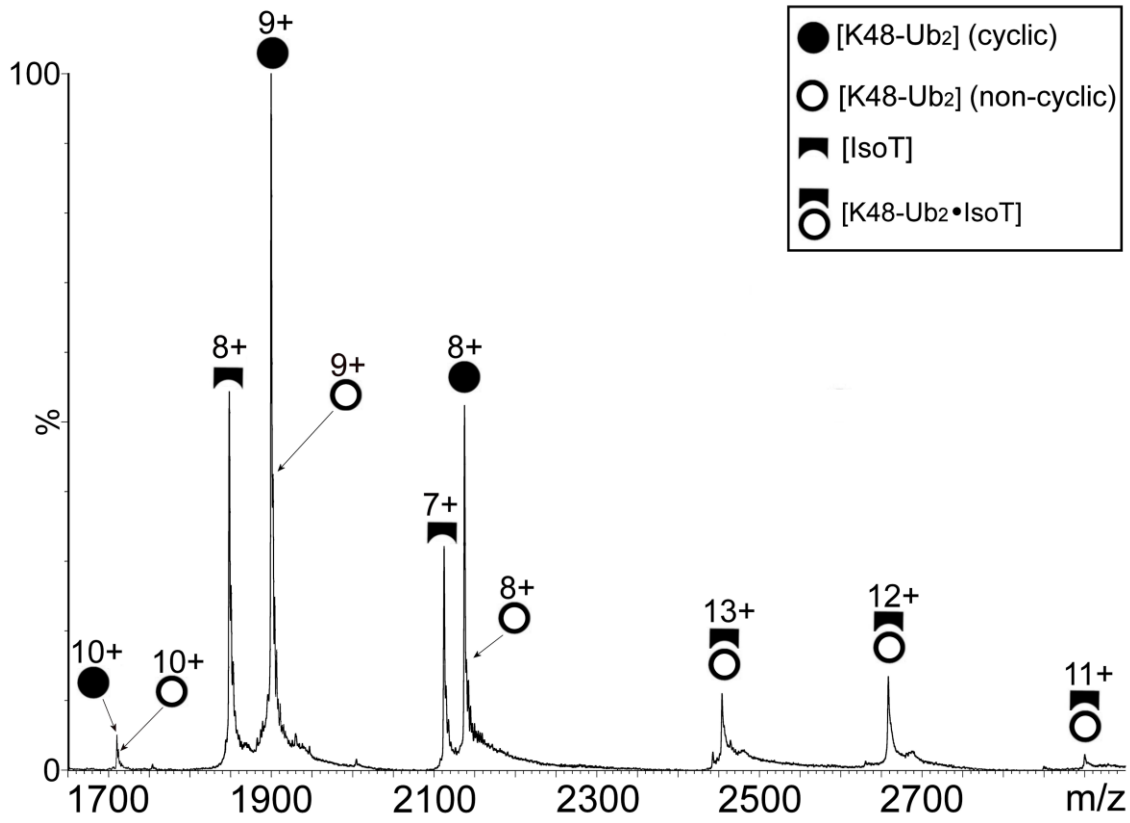

Figure S7. Native ESI-MS of commercial Lys48 di-Ub (0.5 μM) in the presence of IsoT-ZnF domain (1 μM) showing that only the acyclic population of Lys48 di-Ub is capable of forming a complex.

Table S2. Individual apparent K_d values for domains binding to cyclic and acyclic Lys48 di-Ub.

	K_d (μM)	
UBD	acyclic Lys48-Ub2	cyclic Lys48-Ub2
UQ1-UBA	15 ± 2	15 ± 2
UBA2	15 ± 2	21 ± 2
MIU	9 ± 1	14 ± 2
IsoT-ZnF	3.6 ± 0.8	Not observed

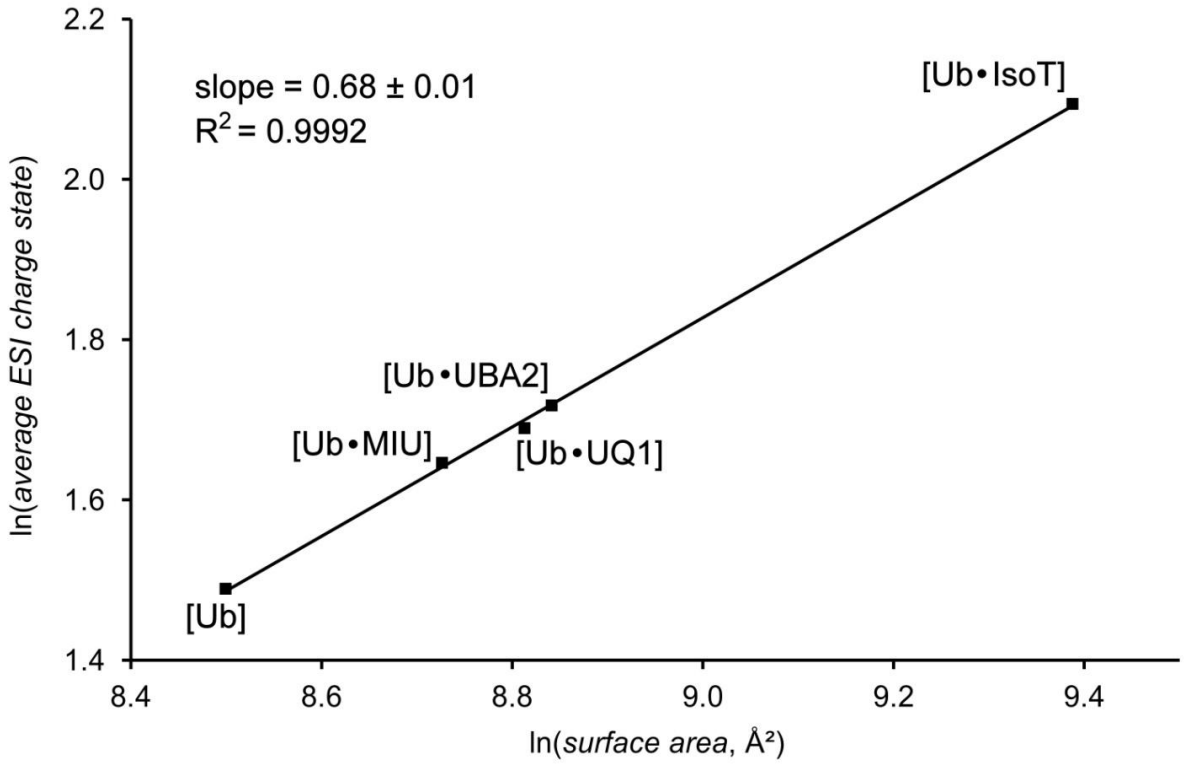

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Table S3. Comparison of biophysical methods used for studying ubiquitin • UBD noncovalent interactions.

	Method [a]				
	ESI - MS	**NMR $^{15}N/^{1}H$ CSPs**	**SPR**	**ITC**	**Fluorescence anisotropy**
K_d range [b]	10^{-3} – 10^{-7} M	10^{-3} – 10^{-7} M	10^{-5} – 10^{-12} M	10^{-5} – 10^{-8} M	10^{-6} -10^{-11} M
Sample consumption [c]	10 – 15 μg	500 – 5000 μg	50 – 400 μg	500 – 5000 μg	1 – 500 μg
Instrument setup time (optimization)	1 day [d]	1 day	2 – 3 h (immobilization of one partner)	minutes	~ 4 h
Analysis time [e]	~ 1 h	~ 1-2 days	1 – 2 h	2 – 3 h	1 – 6 h
Sample preparation / requirements [f]	Very short Sample requires desalting	Long One binding partner requires isotopic labelling	Long Ligand/protein ratio : 30 – 50	Short sample volume ≥ 2 mL protein ≥ 0.5 mL ligand	Long One binding partner requires labelling with a fluorophore
Data interpretation	Simple and rapid Individual signals seen for all species	Relatively complex	Curve fitting	Curve fitting Assumptions required	Curve fitting Assumptions required
Achievable information	K_d, identity of all species, rate constants, stoichiometry	K_d, mapping binding sites	K_d and rate constants	K_d, ΔH, ΔS, rate constants stoichiometry	K_d
Costs [g] **1. Capital**	~ 300,000 USD (for ESI-TOF)	~600,000 USD	~250,000 USD	~130,000 USD	~20,000 USD
2. Per sample	Low	Very High	High	Very High	Medium - High
Limitations / remarks	Low salt tolerance	MW limit : ~40,000 Da Requires isotope labeling	1. Nonspecific interactions with the matrix. 2. Some proteins may lose their activity after immobilization 3. Artifactual chelate effects	Weak (>1μM) interactions require sample concentration of at least 5 times the K_d.	1. Require labeling with a fluorophore. 2. Require control experiments to verify that labeling does not affect the interaction. 3. System dependent.

[a] According to recent reviews on ubiquitin binding domains [1,2], these biophysical techniques; NMR, SPR and ITC, have been used in studies of Ub•UBD interactions. Moreover, additionally to these methods, fluorescence anisotropy has been also used in some recently published studies.

[b] The K_d values for ESI-MS, SPR, ITC and fluorescence anisotropy methods, are taken from a recent review by Erba and Zenobi [3].

[c] The figures for sample consumption for ESI-MS, SPR and ITC adapted from reference 3. The sample consumption for the determination of a K_d value of a given Ub•UBD system as reported in previously published work is within these limits (all the related examples are referenced in the main manuscript of this work). For example, for the determination of the K_d of Ub•IsoT complex by ESI-MS, we have used ~ 10 μg of samples in total (~ 2 μg of Ub (250 μL at 1 μM) and ~ 8 μg of IsoT Znf domain (250 μL at 2 – 8 μM)). In contrast, > 2 mg were used for the determination of the K_d of the same Ub•UBD system by ITC [4] (~ 1.2 mg of Ub and ~ 1 mg of IsoT-Znf domain). The sample consumption for fluorescence anisotropy is highly dependent on the binding affinity of the Ub•UBD complex. The range given in this table is an estimate of the sample that is required to determine the binding affinity of Ub•UBD complex with a K_d value between 1 and 100 μM. For example, the K_d of Lys63-Ub_4•hHR23A-UBA1 complex (K_d = 30 μM) was determined by fluorescence anisotropy. In this experiment, the titration was performed by adding 0.1 μM of the fluorophore carrier domain into increasing concentrations of Lys63-Ub_4 varying between 0.1 μM and ~150 μM [5]. Assuming that the minimum sample volume that is required is ~ 20 μL (total volume of the two interacting partners) and at least 10 - 15 points are needed to plot the titration curve, the total sample consumption is > 150 μg. For weaker Ub•UBD interactions, more sample will be required. In contrast to fluorescence anisotropy, we have shown that sample consumption of the ESI-MS method, does not depend on the binding affinity of the tested system, and it uses very low sample quantities (10 – 15 μg) to determine the K_d of Ub•UBD complexes within the range of 2 – 200 μM.

[d] The instruments needs to be optimized only once using one Ub•UBD system. In this study the instrument conditions were optimized using the Ub•UQ1 complex.

[e] The estimated time that is required to determine the K_d of one system.

[f] This does not include the time that is required to express and purify the proteins, since this step is necessary for all the methods.

[g] The figures for the capital cost are from the National Center for the Research Resources. *http://ncrr.nih.gov/biomedical_technology/shared_instrumentation/arra_instrumentation_awards.asp*
The cost per sample relates to sample consumption.

Supporting Information References

(1) Hurley, J. H. Lee, S.; Prag, G. *Society* **2006**, *372*, 361-372.

(2) Dikic, I. Wakatsuki, S.; Walters, K. J. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 659-671.

(3) Erba, E. B.; Zenobi, R. *Annu. Rep. Prog. Chem., Sect. C* **2011**, *107*, 199-228.

(4) Reyes-Turcu, F. E. Horton, J. R. Mullally, J. E. Heroux, A. Cheng, X.; Wilkinson, K. D. *Cell* **2006**, 1197-1208.

(5) Sims, J. J. Haririnia, A. Dickinson, B. C. Fushman, D.; Cohen, R. E. *Nat. Struct. Mol. Biol.* **2009**, *16*, 883-9.