UbiQ
targeting the ubiquitin system

Proteomic activity profiling of Ub-conjugating enzymes
identification of enzymes reactive towards the biotin–Ub-Dha probe.

version	V1, July-2016
applicable for	UbiQ Triple E Probes, Activity-based probes for E1-E2-E3 enzymes
reference	Mulder, M. P. C. et al. a cascading activity-based probe sequentially targets E1–E2–E3 ubiquitin enzymes. *Nature chemical biology.* **12,** 523–530 (2016). doi:10.1038/nchembio.2084.

protocol

sample preparation

1. 15-cm dishes of subconfluent HeLa or MelJuSo cells were cultured under standard conditions
2. cells were washed with PBS
3. trypsinized
4. centrifuged at 1,500 r.p.m

5. the pellet was washed once with PBS,
6. resuspended in three pellet volumes: HR lysis buffer
   (50 mM Tris, pH 7.4, 5mM MgCl2, 250 mM sucrose, 1 mM DTT, and Protease Inhibitor Tablet (Roche))

7. lysed by sonication
8. cell extracts were clarified by centrifugation (20,000 r.p.m., 4 °C, 20 min)
9. total protein concentration was determined using Nanodrop

10. labeling was performed by adding
    - 10 µg biotin–Ub-Dha, 10 mM ATP, 10 mM MgCl2
    - to 10 mg cell lysates (total protein concentration)
    - in labeling buffer (50 mM HEPES, 100 mM NaCl, 1 mM DTT, pH 8.0)
    - in a volume of 400 µL for 1 h at 37 °C
    - while gently shaking.

    - 1 mM ATP and 1 mM MgCl2 were added to the reaction every 15 min. to ensure that the ATP supply is constant,
    - for the negative controls: ATP was depleted prior to the reaction using two units of apyrase (Sigma-Aldrich) for 15 min at 37 °C.
    - in case of the negative controls, the labeling reaction was performed as described above, but with the omission of ATP and MgCl2.

after reaction with the probe,

11. lysates were incubated with pre-equilbrated High Capacity Neutravidin Agarose (Thermo Fisher)
12. 3 h at 4 °C
13. in a total volume of 700 µL while rotating.

14. subsequently, the supernatant was removed and the resin was washed with 1 mL each of the following wash buffers:
    - buffer 1 (2% SDS in dH2O),
    - buffer 2 (50 mM HEPES, pH 7.5, 1 mM EDTA, 500 mM NaCl, 1% TritonX-100, 0,1% deoxycholate),
    - buffer 3 (10 mM Tris, pH 8.0, 1 mM EDTA, 0,5% NP-40, 250 mM LiCl) and buffer 4 (50 mM Tris, pH 7.4, 50 mM NaCl).

after washing

15. samples were eluted in
    - 40 µL 3× SDS PAGE Loading Buffer (Invitrogen)
    - with additional 2-mercaptoethanol
16. boiled at 95 °C for 5 min
17. before loading onto a 10% gel (NuPAGE, Invitrogen)

18. the proteins were run 1 cm into the gel
19. stained overnight with Coomassie Brilliant Blue
20. destaine in H2O
21. the band was cut into small pieces using a sterile scapel
22. processed further for LC-MS/MS analysis

LC-MS/MS analysis

In-gel digested peptides were

1. cleaned and desalted by 1 ml 0.1% formic acid twice on OASIS HLB cartridge (Waters)
2. eluted twice with 100 µL 80% acetonitrile in 0.1% formic acid

3. desalted peptides were vacuum centrifuged at room temperature until complete dryness
4. then dissolved in 0.1% formic acid prior to online nanoflow LC-MS/MS

5. the analysis of in-solution-digested peptides was performed using an EASYnLC system (Proxeon) connected to a QExactive (Thermo Scientific) using higher-collisional dissociation (HCD) fragmentation

6. separation of peptides was performed using 13-cm long analytical columns (ID 75 µm, Polymicro Avantes) packed inhouse with 1.8 µm C18 beads (Reprospher-DE, Pur, Dr. Maisch), using a 30-min gradient from 2% to 95% acetonitrile in 0.1% formic acid and a flow rate of 200 nL per minute

7. the mass spectrometer was operated in data-dependent acquisition mode using a top 10 method
8. full-scan MS spectra were acquired with a target value of 3E6 and a resolution of 70,000 with a scan range from 400 to 1,400 *m/z*.
9. HCD MS/MS spectra were acquired using a target value of 1E5, a resolution of 17,500, and a normalized collision energy of 25%
10. all charges lower than two and higher than six were rejected, and all unknown charges were rejected. The underfill ratio was set to 1.0%, and a dynamic exclusion of 60 s was used.

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data processing

1. MaxQuant version 1.5.1.2 was used to analyze all RAW data.
2. experiment was performed with biological triplicates and measured as three technical replicates
1. MS/MS spectra were filtered and deisotoped
2. the 18 most abundant fragments for each 100 m/z were retained

3. MS/MS spectra were filtered for a mass tolerance of 6 p.p.m. for precursor masses,
4. a mass tolerance of 20 p.p.m. was used for fragment ions

5. peptide and protein identification was performed through matching the identified MS/MS spectra versus a target-decoy version of the complete human UniProt database (as of 3 December 2015, 92,040 proteins),
6. in addition to a database of 245 commonly observed MS contaminants.

7. up to two missed tryptic cleavages were allowed
8. Cysteine carbamidomethylation was set as a fixed peptide modification

9. protein N-terminal acetylation and methionine oxidation were set as variable peptide modifications
10. peptides were accepted with a
    - minimum length of six amino acids,
    - maximum size of 6 kDa,
    - maximum charge of six.
11. the processed data were filtered by posterior error probability (PEP) to achieve a protein false discovery rate (FDR) <1%, a peptide-spectrum match FDR <1%.
12. modified peptides were additionally filtered to have an Andromeda score of at least 40

Label-free quantification (LFQ)

Label-free quantification was performed using MaxQuant LFQ.

1. quantification was performed over three biological replicates.
2. The fast LFQ algorithm was used with default settings (minimum ratio count of two, minimum neighbors of three, average neighbors of six). Protein
3. LFQ values were log2transformed for further processing

4. proteins identified by site only, reverse sequence only and potential contaminants were removed.
5. Proteins identified by at least fourMS/MS spectra, at least two razor + unique peptides and at least one unique peptide were retained.
6. proteins detected in at least two out of the three biological replicates in at least one group were retained. To ensure biological reproducibility.
7. subsequently, missing values were imputed using Perseus software version 1.5.1.6 with standard settings.
    - LFQ values were averaged within all treatment conditions.
8. proteins were considered to have a specific interaction with the Ub ligase probe when they were enriched at least twofold compared to the negative control with a t-test P value <0.05

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