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A Practical Experimental Protocol for Identification and Validation of UFMylation Substrate in Human Cells

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Abstract

UFMylation is an evolutionarily conserved ubiquitin-like modification that covalently conjugates UFM1 to lysine residues of substrates via a sequential E1-E2-E3 enzymatic cascade. UFMylation plays a pivotal role in maintaining cellular homeostasis, and its dysregulation is closely linked to multiple major diseases, including malignant tumors, hematopoietic defects, neurodegenerative disorders, and congenital developmental defects, highlighting its important biological significance. However, few substrates of UFMylation have been reported to date, limiting our deep understanding of the mechanistic functions of this modification. This major bottleneck stems from two major technical limitations: the overwhelming abundance of ribosomal protein L26 (RPL26)-UFM1 conjugates masks signals from low-abundance substrates, and conventional methods rely on cumbersome cotransfection of multiple pathway components with poor efficiency and specificity in UFMylated peptides enrichment. To address these challenges, we have developed an effective and specific experimental protocol for UFMylation detection and large-scale substrate identification. This protocol employs CRISPR-Cas9-mediated gene editing to generate *UFSP1/UFSP2* double-knockout (*UFSP1*^{KO}/*UFSP2*^{KO}, DKO) HEK293T cells, which completely abrogate de-UFMylation and thus significantly elevate global protein UFMylation levels upon exogenous introduction of mature UFM1-ΔC2. In addition, exogenous co-expression of the E3 ligase core components UFL1 and DDRGK1 can further improve the sensitivity of substrate detection. This protocol enables large-scale identification of UFMylation substrates with modification sites via high-efficiency enrichment with the K-ε-VG antibody and LC-MS/MS analysis.

Key features

- Employs *UFSP1/UFSP2* DKO HEK293T cells with exogenous mature UFM1-ΔC2 to enhance UFMylation.
- Simplified transfection via single-factor assays using UFM1-ΔC2, UFL1, and DDRGK1.
- Combines K-ε-VG antibody enrichment with LC-MS/MS for large-scale substrate identification.
- Verifies UFMylation sites via site-directed mutagenesis and UFSP2-mediated de-UFMylation.

Keywords: UFMylation, Substrate, UFSP1/2, UFL1, DDRGK1

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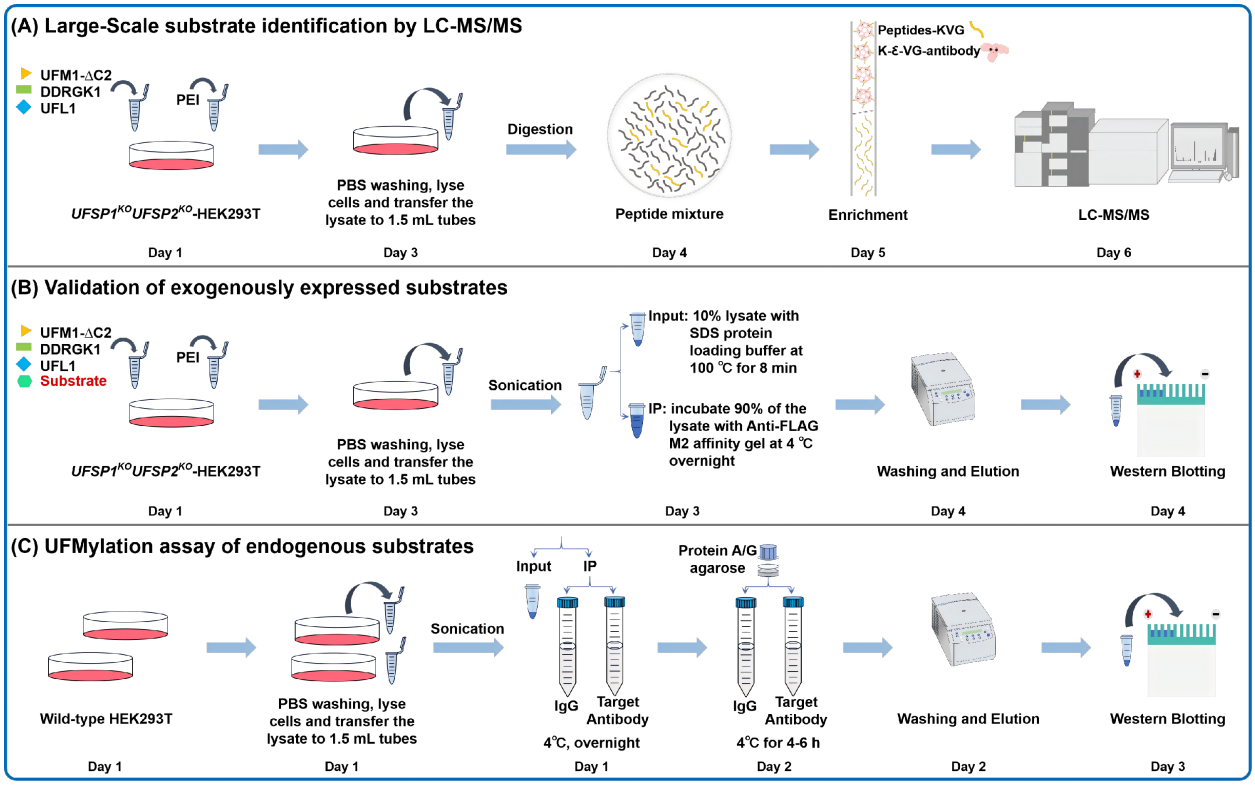
Graphical overview



Graphical overview of the workflow for identifying and validating UFMylation substrates in human cells. This graphical overview summarizes the key steps of the optimized protocol. (A) Large-scale substrate identification by LC-MS/MS: Performed in *UFSP1/UFSP2* double-knockout (DKO) HEK293T cells, followed by proteomic identification of UFMylated substrates via LC-MS/MS. (B) Validation of exogenously expressed substrates: Performed in DKO HEK293T cells, using immunoprecipitation (IP) and western blotting to confirm substrate UFMylation. (C) Endogenous substrate UFMylation assay: Conducted in wild-type (WT) HEK293T cells, to detect endogenous UFMylation via immunoprecipitation (IP) and western blotting.

Background

UFMylation is an evolutionarily conserved ubiquitin-like post-translational modification, in which ubiquitin-fold modifier 1 (UFM1) is covalently attached to lysine residues of target substrates through the sequential catalytic cascade involving three core components: the E1 activating enzyme UBA5, the E2 conjugating enzyme UFC1, and the multi-subunit E3 ligase complex comprising UFL1, UFBP1, and CDK5RAP3 [1–3]. Aberrant UFMylation is closely associated with cancers, neurodevelopmental disorders, and skeletal dysplasia [1,4,5].

In human cells, UFM1 is initially synthesized as an 85-amino-acid precursor (pro-UFM1). Its C-terminal Ser-Cys dipeptide is proteolytically trimmed by UFM1-specific proteases (UFSPs) to generate the mature 83-residue UFM1 variant termed UFM1-ΔC2, a truncated form competent for subsequent substrate conjugation [6]. Reversible UFMylation homeostasis is tightly regulated by two UFSP isoforms: UFSP1 mainly mediates pro-UFM1 maturation, whereas endoplasmic reticulum (ER)-resident UFSP2 predominantly catalyzes de-UFMylation to cleave conjugated UFM1 from modified substrates [7,8]. Conventional UFMylation detection methods require cumbersome cotransfection of multiple core components and suffer from low efficiency; meanwhile, the dominant high-abundance RPL26-UFM1 conjugates mask signals of low-abundance substrates, resulting in fewer than 30 verified UFMylation substrates to date, greatly hindering mechanistic studies [9,10]. Additionally, the shortage of modification-specific antibodies capable of capturing UFMylated peptides has long impeded high-throughput proteome-wide identification of UFMylation sites. The K-ε-VG antibody is a modification-specific affinity antibody raised against the remnant K-ε-VG epitope retained on substrates after proteolytic digestion; this reagent enables specific enrichment of UFM1-modified peptides and serves as the core tool for large-scale UFMylation site mapping via

liquid chromatography–tandem mass spectrometry (LC-MS/MS) [11].
This optimized protocol employs *UFSP1/UFSP2* double-knockout HEK293T cells combined with exogenous mature UFM1-ΔC2 to boost UFMylation and simplifies transfection by only using UFM1-ΔC2, UFL1, and DDRGK1 through single-factor optimization. It integrates K-ε-VG antibody enrichment and LC-MS/MS for high-throughput substrate identification, and validates modification sites via site-directed mutagenesis and UFSP2-mediated de-UFMylation assays, enabling the identification of over 600 UFMylation substrates with high specificity and efficiency [11].
This protocol has three minor limitations relevant to experimental execution: (1) It is primarily validated in HEK293T cells, (2) it has limited sensitivity for extremely low-abundance substrates, and (3) overexpression of modification components may introduce minor stoichiometric artifacts. A detailed mechanistic discussion is available in the *Limitations of the study* section of our accompanying JBC article [11]. Beyond substrate identification, this protocol can be widely applied to verify UFMylation sites, explore regulatory mechanisms of the UFMylation system, and screen disease-related UFMylation substrates in diverse mammalian cell models.

Materials and reagents

Biological materials

1. HEK293T cell line (ATCC, catalog number: CRL-3216)
2. *UFSP1*^{KO}/*UFSP2*^{KO}-HEK293T cell line was generated as a single-cell clone from parental HEK293T cells (CRL-3216) via CRISPR-Cas9-mediated genome editing and validated by Sanger sequencing. This cell line is available upon reasonable request from the corresponding author. Alternatively, users may generate this cell line independently following the detailed CRISPR-Cas9 editing protocol described in our previous publication [7].

Reagents

1. Polyethylenimine (PEI) (Sigma-Aldrich, catalog number: 765090-1G)
2. $Na_2HPO_4 \cdot 12H_2O$ (Sigma-Aldrich, CAS number: 10039-32-4)
3. KH_2PO_4 (Sigma-Aldrich, CAS number: 7778-77-0)
4. Tris-HCl (1 M, pH 6.8) (Beyotime Biotechnology, catalog number: ST768)
5. Tris-HCl (1 M, pH 7.4) (Beyotime Biotechnology, catalog number: ST774)
6. Tris-HCl (1 M, pH 8.0) (Beyotime Biotechnology, catalog number: ST780)
7. Sodium dodecyl sulfate (SDS) (BioFroxx, catalog number: 3250GR500)
8. Glycerol (Sangon Biotech, CAS number: 56-81-5)
9. Dithiothreitol (DTT) (Macklin, catalog number: D806827)
10. Bromophenol blue (Diamond, CAS number: 115-39-9)
11. NaCl (Sinopharm, CAS number: 7647-14-5)
12. Nonidet P-40 (NP-40) (Biosharp, catalog number: BS205)
13. EDTA (0.5 M, pH 8.0) (Beyotime Biotechnology, catalog number: C0196)
14. Tris base (BioFroxx, catalog number: 1115KG001)
15. Glycine (Sinopharm, CAS number: 56-40-6)
16. Methanol (Sinopharm, CAS number: 67-56-1)
17. KCl (Sinopharm, CAS number: 7447-40-7)
18. Iodoacetamide (BBI Life Sciences, CAS number: 144-48-9)
19. Acetone (Sinopharm, CAS number: 67-64-1)
20. Triethylammonium bicarbonate (Sigma-Aldrich, catalog number: T7408)
21. $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich, CAS number: 10035-04-8)
22. Trypsin Gold, mass spectrometry grade (Promega, catalog number: V5280)
23. Trifluoroacetic acid (TFA) (Sigma-Aldrich, CAS number: 76-05-1)
24. Tween-20 (BioFroxx, catalog number: 1247LT001)
25. Nonfat powdered milk (EpiZyme Biotechnology, catalog number: PS112L)
26. ProClean 950 (Beyotime Biotechnology, catalog number: ST855)
27. Bovine serum albumin (BSA) (BioFroxx, catalog number: 4240GR500)
28. DMEM with high glucose (Viva Cell, catalog number: C3113-0500)

29. Fetal bovine serum (FBS) (ExCell, catalog number: FSP500)
30. Penicillin-streptomycin solution (100×) (Biological Industries, catalog number: 03-031-1B)
31. Trypsin-EDTA solution (Biological Industries, catalog number: 03-050-1BCS)
32. Opti-MEM (Gibco, catalog number: 31985-070)
33. MG132 (Selleck Chemicals, catalog number: S2619)
34. Anti-FLAG M2 affinity gel (Sigma-Aldrich, catalog number: A2220)
Note: Storage: -20 °C. Shelf life: ≥6 months unopened. Handling: Centrifuge briefly at 500× g for 30 s to collect beads before use; do not freeze-thaw the resin repeatedly.
35. Protein A/G agarose (Proteintech, catalog number: PR40025)
Note: Storage: 4 °C. Shelf life: 24 months from the date of manufacture. Handling: Prewash the beads three times with ice-cold PBS by brief low-speed centrifugation (500× g for 30 s) before use.
36. Rabbit IgG control antibody (Proteintech, catalog number: 30000-0-AP)
37. UFMylation Remnant Motif (K-ε-VG) kit (Micrometer Biotechnology, catalog number: WM302)
Note: Storage: Antibody-conjugated agarose beads at -20 °C; all other kit components at 4 °C. Shelf life: 12 months from the date of manufacture. Handling: Avoid repeated freeze-thaw cycles of the agarose beads; gently mix by inverting before use, do not vortex.
38. Color PAGE Gel Rapid Preparation kit (EpiZyme Biotechnology, catalog number: PG112)
39. Prestained protein ladder (EpiZyme Biotechnology, catalog number: WJ103)
40. Protease inhibitor cocktail (Roche, catalog number: 04693132001)
41. Anti-Myc affinity gel (Beyotime Biotechnology, catalog number: P2285)
42. Anti-DDRGK1 (Sigma-Aldrich, catalog number: HPA013373)
43. Anti-FLAG (Sigma-Aldrich, catalog number: F7425)
44. Anti-UFM1 (Abcam, catalog number: ab109305)
45. Anti-UFSP2 (Abcam, catalog number: ab185965)
46. Anti-Myc (Proteintech, catalog number: 60003-2-Ig)
47. Anti-HA (Cell Signaling Technology, catalog number: 3724)
48. Anti-GAPDH (ABclonal, catalog number: A19056)
49. Mouse IgG-agarose (Beyotime Biotechnology, catalog number: P2265)
50. Immobilon ECL Ultra Western HRP substrate (Millipore, catalog number: WBKLS0500)
51. Triethylammonium bicarbonate buffer (1 M) (Sigma-Aldrich, CAS number: 15715-58-9)
52. $CaCl_2$ (Sigma-Aldrich, CAS number: 10043-52-4)
53. Trichostatin A (10 mM) (Selleck Chemicals, catalog number: S1045)
54. Nicotinamide (Sigma-Aldrich, CAS number: 98-92-0)
55. Urea (Sigma-Aldrich, CAS number: 57-13-6)
56. Sodium deoxycholate (Sigma-Aldrich, CAS number: 302-95-4)
57. 2-D Quant kit (Cytiva, catalog number: 80-6483-56)

Solutions

1. PBS (10×) (see Recipes)
2. PEI transfection reagent (see Recipes)
3. Lysis buffer (see Recipes)
4. SDS protein loading buffer (5×) (see Recipes)
5. Buffer A (see Recipes)
6. Denaturing lysis buffer (see Recipes)
7. NETN buffer (see Recipes)
8. SDS-PAGE running buffer (10×) (see Recipes)
9. Tris-glycine transfer buffer (10×) (see Recipes)
10. TBS (20×) (see Recipes)
11. 20% Tween-20 (see Recipes)
12. TBST (1×) (see Recipes)
13. Blocking buffer (see Recipes)
14. Antibody dilution buffer (see Recipes)
15. DMEM complete medium (see Recipes)

Recipes

1. PBS (10×)

Reagent	Final concentration	Quantity or volume
$Na_2HPO_4·12H_2O$	100 mM	36.28 g
NaCl	1370 mM	80 g
KCl	27 mM	2 g
KH_2PO_4	20 mM	2.72 g
ddH_2O	n/a	To 1,000 mL

After the powder is completely dissolved, adjust the pH to 7.4, then vacuum-filter the solution for later use. Dilute the 10× PBS stock solution to a 1× working solution with ddH_2O.

2. PEI transfection reagent

Reagent	Final concentration	Quantity or volume
PEI	1 mg/mL	100 mg
ddH_2O	n/a	To 100 mL

Dissolve 100 mg of PEI powder in approximately 80 mL of ultrapure water with continuous stirring. Slowly add concentrated HCl to the PEI solution until pH < 2 and keep stirring until the powder is fully dissolved. Next, adjust the pH to 7.0 with NaOH solution, then top up with ultrapure water to a final volume of 100 mL. Mix well and filter the solution through a 0.22 μm sterile membrane filter in a biosafety cabinet. Prepare 1 mL aliquots and store at -20 °C. Avoid repeated freeze-thaw cycles.

3. Lysis buffer

Reagent	Final concentration	Quantity or volume
Urea	8 M	48.05 g
Tris-HCl (1 M, pH 8.0)	50 mM	5 mL
NP-40	1% (v/v)	1 mL
Sodium deoxycholate	1% (m/v)	1 g
DTT (1 M)	5 mM	500 μL
EDTA (0.5 M, pH 8.0)	2 mM	400 μL
Nicotinamide	30 mM	0.366 g
Trichostatin A (10 mM)	3 μM	30 μL
ddH_2O	n/a	To 100 mL

This lysis buffer is used for cell lysis in large-scale substrate identification by LC-MS/MS. Add 1× protease inhibitor cocktail before use.

4. SDS protein loading buffer (5×)

Reagent	Final concentration	Quantity or volume
Tris-HCl (1 M, pH 6.8)	250 mM	25 mL
SDS	10% (m/v)	10 g
Glycerol	50% (v/v)	50 mL
DTT	500 mM	7.72 g
Bromophenol blue	0.2% (m/v)	0.2 g
ddH_2O	n/a	To 100 mL

After complete dissolution and thorough mixing, aliquot the solution at 1 mL per tube and store at -20 °C.

5. Buffer A

Reagent	Final concentration	Quantity or volume
Tris-HCl (1 M, pH 7.4)	50 mM	5 mL
NaCl (5 M)	120 mM	2.4 mL
NP-40	0.5% (v/v)	0.5 mL
ddH_2O	n/a	To 100 mL

Store at 2–8 °C for up to 1 month.

6. Denaturing lysis buffer

Reagent	Final concentration	Quantity or volume
Tris-HCl (1 M, pH 7.4)	50 mM	5 mL
EDTA (0.5 M, pH 8.0)	0.5 mM	100 µL
DTT (1 M)	1 mM	100 µL
20% SDS	2% (v/v)	10 mL
ddH_2O	n/a	To 100 mL

Store at 2–8 °C for up to 1 month.

7. NETN buffer

Reagent	Final concentration	Quantity or volume
Tris-HCl (1 M, pH 8.0)	20 mM	10 mL
EDTA (0.5 M, pH 8.0)	1 mM	1 mL
NaCl (5 M)	100 mM	10 mL
NP-40	0.5% (v/v)	2.5 mL
ddH_2O	n/a	To 500 mL

Store at 2–8 °C for up to 1 month. The final pH of the prepared NETN buffer is 8.0 (adjust if necessary).

8. SDS-PAGE running buffer (10×)

Reagent	Final concentration	Quantity or volume
Tris base	250 mM	30.3 g
Glycine	1920 mM	144.1 g
SDS	10 g/L	10 g
ddH_2O	n/a	To 1,000 mL

Dilute the 10× running buffer to 1× running buffer with ddH_2O.

9. Tris-glycine transfer buffer (10×)

Reagent	Final concentration	Quantity or volume
Tris base	250 mM	30.3 g
Glycine	1920 mM	144.1 g
ddH_2O	n/a	To 1,000 mL

Dilute the 10× transfer buffer to 1× transfer buffer with methanol and water to make a solution containing 25 mM Tris, 192 mM glycine, and 20% methanol.

10. TBS (20×)

Reagent	Final concentration	Quantity or volume
NaCl	3000 mM	175 g
Tris base	500 mM	60.6 g
KCl	60 mM	4.5 g
ddH_2O	n/a	To 1,000 mL

After complete dissolution, adjust the pH to 7.4 with dropwise diluted HCl under constant stirring, then bring the solution to a final volume of 1 L and store at room temperature.

11. 20% Tween-20

Reagent	Final concentration	Quantity or volume
Tween-20	20%	10 mL
ddH_2O	n/a	40 mL
Total	n/a	50 mL

Store at 2–8 °C for up to 1 month.

12. TBST (1×)

Reagent	Final concentration	Quantity or volume
20% Tween-20	0.1%	5 mL
TBS (20×)	n/a	50 mL
ddH_2O	n/a	940 mL

13. Blocking buffer

Reagent	Final concentration	Quantity or volume
Nonfat dry milk	50 g/L	5 g
1× TBST	n/a	To 100 mL
ProClean 950	0.1% (v/v)	100 µL
Total	n/a	100 mL

Store at 2–8 °C for up to 1 month.

14. Antibody dilution buffer

Reagent	Final concentration	Quantity or volume
BSA	50 g/L	5 g
TBST (1×)	n/a	To 100 mL
ProClean 950	0.1% (v/v)	100 µL
Total	n/a	100 mL

Store at 2–8 °C for up to 1 month.

15. DMEM complete medium

Reagent	Final concentration	Quantity or volume
DMEM with high glucose	89% (v/v)	445 mL
FBS	10% (v/v)	50 mL
Penicillin-streptomycin solution	1% (v/v)	5 mL
Total	n/a	500 mL

Store at 2–8 °C for up to 1 month.

Laboratory supplies

1. Immobilon®-PSQ PVDF membrane (Millipore, catalog number: ISEQ00010)
2. 100 mm cell culture dishes (Nunc, catalog number: 150466)
3. 1.5 mL microtubes (Axygen, catalog number: MCT-150-C)
4. 15 mL conical tube (KIRGEN, catalog number: KG2611)
5. 10 mL pipettes (Nunc, catalog number: 170356N)
6. 100–1,000 µL tips (KIRGEN, catalog number: KG1313)
7. 1–200 µL tips (KIRGEN, catalog number: KG1212)
8. 0.1–10 µL tips (KIRGEN, catalog number: KG1011)
9. Cell scraper (Corning, catalog number: 3010)
10. C18 SPE column (Phenomenex, catalog number: 8B-S100-AAK)
11. Vacuum concentrator (Thermo Fisher Scientific, catalog number: SPD111V-230)
12. C18 ZipTips (Merck Millipore, catalog number: ZTC18S096)

Equipment

1. Magnetic stirrer (IKA, model: C-MAG HS 7)
2. pH meter (Sartorius, model: PB-11)
3. Analytical balance (Mettler Toledo, model: MS105DU)
4. Biological safety cabinet (Thermo Scientific, model: 1386)
5. Pipette fillers (Thermo Scientific, catalog number: 9501)
6. Heating block (AOSHENG, model: K30)

7. 2–8 °C refrigerator (Thermo Scientific, model: PLR-386)
8. Biomedical freezer (Haier, model: DW-40L262)
9. Ultra-low temperature freezers (Thermo Scientific, model: 994)
10. Water-jacketed CO_2 incubator (Thermo Scientific, model: 3111)
11. Vortex (Scientific Industries, model: G-560E)
12. Refrigerated centrifuge (Thermo Scientific, model: Sorvall ST 16R)
13. Centrifuge (Eppendorf, model: 5424)
14. Refrigerated centrifuge (Eppendorf, model: 5424R)
15. Electric water bath (Yuexin, model: HH-6)
16. Shaker (QILINBEIER, model: TS-8)
17. Ultrasonic cell disruptor (Scientz Biotechnology, model: SCIENTZ08 - IIIC)
18. Mixer (QILINBEIER, model: WH-986)
19. PowerPac™ Basic Power Supply (Bio-Rad Laboratories, catalog number: 1645050)
20. ChemiDoc MP Imaging System (Bio-Rad Laboratories, catalog number: 12003154)

Software and datasets

1. Image Lab (Bio-Rad Laboratories, Version 6.0, free to use)
2. MS proteomics data have been deposited to PRIDE (https://www.ebi.ac.uk/pride/archive/projects/PXD070408, PXD070408, 3/29/2026).

Procedure

A. UFMylation assay for global and exogenously expressed substrates

This protocol takes the *UFSP1* and *UFSP2* double-knockout HEK293T cell line (***UFSP1^{KO}/UFSP2^{KO}*-HEK293T**) cultured in 100-mm cell culture dishes as an example for operational description. When using cell culture vessels of other specifications (e.g., 60 mm cell culture dishes), adjust the dosage of all reagents proportionally.

A1. Cell preparation

1. Cell seeding

a. Seed cells into culture dishes 24 h prior to transfection. Prepare the required cells and harvest them as follows:

i. Prewarm DMEM complete medium and 1× PBS in a 37 °C water bath.

ii. Aspirate the spent medium, slowly add 5 mL of 1× PBS along the dish wall, swirl the dish, and aspirate the PBS completely.

iii. Add 2 mL of trypsin-EDTA solution, swirl the dish to distribute trypsin evenly over the bottom, and incubate for 2 min at 37 °C. Add complete medium to terminate trypsin digestion, collect the cell mixture into a 15 mL centrifuge tube, and centrifuge at 150× *g* at room temperature for 5 min.

b. When *UFSP1^{KO}/UFSP2^{KO}*-HEK293T cells reach 90% confluency, seed 2.5 × 10^6 cells with 10 mL of medium per dish (equivalent to a 1:3 split ratio). Swirl the dishes gently immediately after seeding to ensure uniform cell distribution.

Note: UFSP1^{KO}/UFSP2^{KO}*-HEK293T cells have a ~25% slower proliferation rate than wild-type HEK293T cells. For wild-type HEK293T cells, use the density of 2 × 10^6 cells per 100 mm dish, which will achieve 80% confluency post-seeding prior to transfection.*

2. Cell transfection

a. Aspirate the spent medium, then add 9 mL of fresh DMEM complete medium to maintain cell viability and support healthy growth after transfection.

b. Prepare Transfection Premix A and Premix B.

i. Premix A: Combine Opti-MEM medium with PEI to a final volume of 500 µL.

ii. Premix B: Combine Opti-MEM medium with the indicated plasmids (**Table 1**) to a final volume of 500 µL.

Note: The volume-to-mass ratio of PEI to plasmid is 2:1 (μL/μg). Adjust the amount of PEI according to the plasmid dosage. HEK293T cells used in this assay tolerate a maximum PEI concentration of 5 μg per mL medium.

Table 1. Plasmid composition and dosage for transfection groups in the exogenous UFMylation assay. This table lists the plasmids and corresponding dosages used for each transfection group in the exogenous UFMylation assay. All dosages refer to the amount per 100 mm cell culture dish. Control group 1 (UFMylation machinery only) contains UFMylation factors plus Flag-Vector, serving as a control for background signals from the modification machinery alone. Control group 2 (substrate only) contains HA-Vector (to balance total DNA) plus Flag-substrate, assessing basal UFMylation levels without overexpressed modification machinery. The experimental group 1 (complete UFMylation system) combines UFMylation factors with Flag-substrate to promote substrate UFMylation. The experimental group 2 (complete UFMylation system and UFSP2) permits substrate UFMylation; however, overexpressed UFSP2 drives a de-UFMylation process that results in the complete loss or marked reduction of substrate UFMylation. Notably, UFSP2 can be substituted with highly active UFSP1 to attain an even stronger de-UFMylation effect. All groups receive equal total amounts of plasmid DNA. All dosages refer to the amount of plasmid per 100 mm cell culture dish. – indicates plasmid omitted.

Plasmid[†]	Control group 1	Control group 2	Experimental group 1	Experimental group 2[‡]
HA-UFL1	2 μg	–	2 μg	2 μg
DDRGK1-HA	2 μg	–	2 μg	2 μg
HA-UFM1-ΔC2	6 μg	–	6 μg	6 μg
HA-UFSP2	–	–	–	2 μg
HA-Vector	2 μg	12 μg	2 μg	–
Flag-Vector	5–8 μg	–	–	–
Flag-substrate	–	5–8 μg	5–8 μg	5–8 μg

[†] The plasmids used in this protocol were constructed in our laboratory.
[‡] For de-UFMylation validation and confirmation of substrate UFMylation.

c. Add each Premix A to the matched Premix B, vortex briefly for thorough mixing, and incubate at room temperature for 15 min. Dispense the plasmid/PEI/Opti-MEM mixture dropwise into the dishes, swirl gently to achieve even distribution, and then place the dishes back in the incubator.

3. Medium replacement: Aspirate the spent medium containing transfection complexes at 12 h post-transfection, then add fresh complete medium.
Note: If cell confluency is excessively low at the time of transfection, cells tend to grow poorly, displaying a rounded morphology with partial cell detachment and floating.

A2. Sample preparation

Exogenously transfect substrate expression vectors together with key UFMylation factors (UFL/DDRGK1/UFM1-ΔC2) into *UFSP1*^{KO}/*UFSP2*^{KO}-HEK293T cells to enhance the UFMylation of substrate proteins.
Note: If substrate protein stability is regulated by UFMylation, add MG132 to a final working concentration of 10 μM 6–8 h prior to cell harvesting. MG132 treatment could be omitted if UFMylation does not regulate substrate protein stability.

1. Harvest cells at 48–60 h post-transfection; PEI-mediated transfection routinely yields ~80% efficiency in HEK293T cells. Aspirate culture supernatant, slowly add 5 mL of pre-chilled 1× PBS along the dish wall to wash off residual medium, and repeat the washing step twice.
Note: Remove residual PBS completely to ensure consistent solution volume across all samples. Tilt the cell culture dish briefly and aspirate the remaining liquid with a pipette.

2. Large-scale substrate identification by LC-MS/MS
a. Protein extraction
i. Digest cells with 2 mL of trypsin-EDTA solution, add complete medium to terminate trypsin digestion, and collect the cell suspension into a 15 mL centrifuge tube. Wash the cell pellet twice with ice-cold 1× PBS, then centrifuge at 150× *g* for 5 min at 4 °C.
Pause point: Snap-freeze the sample immediately in liquid nitrogen, then store at -80 °C for subsequent use.
ii. Resuspend the cell pellet in lysis buffer (100 μL per dish, supplemented with 1× protease inhibitor cocktail), then mix thoroughly by vortexing.

iii. Perform sonication on ice under the following parameters: 25% power, 3 s sonication followed by a 5 s interval, with a total treatment duration of 3 min. Perform two sonication cycles until the solution becomes clear and particle-free. Keep the sample on ice for 30 min, followed by centrifugation at 20,000× *g* for 10 min at 4 °C to remove unbroken cells and debris; collect the supernatant (cell lysate).
iv. Quantify protein concentration using the 2-D Quant kit and collect a small aliquot of protein sample for western blot analysis. Under our experimental setup, the concentration of the obtained protein samples typically ranges from 5 to 10 μg/μL. A 5 mg aliquot of the total protein is then used for subsequent mass spectrometry analysis.
v. Perform protein reduction, alkylation, and acetone precipitation on the remaining cell lysate. Reduce the cell lysate with 5 mM DTT at 37 °C for 45 min, followed by alkylation with 30 mM iodoacetamide at room temperature in the dark for 45 min. For protein precipitation, add 5 volumes of pre-chilled acetone and incubate overnight at -20 °C.
vi. For acetone washing: centrifuge at 20,000× *g* for 10 min at 4 °C, then discard the supernatant. Resuspend the pellet thoroughly in 1 mL of pre-chilled 80% acetone (-20 °C), incubate the mixture at -20 °C for 60 min, and centrifuge again at 20,000× *g* for 10 min at 4 °C. Discard the supernatant and repeat the acetone washing procedure twice more.
vii. After the final centrifugation, carefully aspirate all supernatant acetone completely and allow the protein pellet to air-dry at room temperature for 2 min to eliminate residual acetone.

b. Protein digestion, peptide desalting, and drying
i. Resuspend the washed protein pellets in 1 mL of 0.1 M triethylammonium bicarbonate solution. Add 2 μL of 1.5 M $CaCl_2$ solution (final concentration 3 mM), then sonicate the suspension at 20% power with a pulse pattern of 3 s on/5 s off for a total of 2 min. Fully disperse the protein pellet until it forms a homogeneous milky suspension.
ii. Digest proteins with Trypsin Gold at a substrate-to-enzyme ratio of 25:1 (w/w) at 37 °C for 12 h.
iii. Terminate enzymatic digestion by adding 20% (v/v) TFA solution dropwise to a final pH of 2.5–3.0.
iv. Desalt the resultant peptides using a C18 SPE column according to the manufacturer's instructions.
v. Dry the desalted peptides in a vacuum concentrator at low temperature (heat off) for 2–3 h.

c. Affinity enrichment
i. Dissolve tryptic peptides in 300 μL of NETN buffer, vortex, centrifuge, then adjust the sample pH to 7.0–7.5.
Note: Desalted peptides obtained after acidification are poorly soluble upon initial addition into the buffer. Adjust pH to approximately 7.0 using Tris solution, followed by 10 min of non-contact sonication to fully dissolve peptides.
ii. Fully resuspend agarose beads from the UFMylation Remnant Motif (K-ε-VG) kit. Transfer 40 μL of bead suspension (for 2.5 mg input peptides) into a 1.5 mL tube. Wash beads once with 500 μL of PBS, centrifuge at 2,000× *g* for 30 s at 4 °C, and repeat one wash with 500 μL of NETN buffer under the same centrifugation conditions.
iii. Combine the peptide solution from step A2.2c.i with prewashed beads and incubate for 4 h at 4 °C with gentle shaking.
iv. After incubation, wash agarose beads four times with 500 μL of NETN buffer and twice with 500 μL of ddH_2O and centrifuge at 2,000× *g* for 30 s at 4 °C every time after each wash.

d. LC-MS/MS analysis: Prior to LC-MS/MS analysis, desalt the prepared peptides using C18 ZipTips according to the manufacturer's protocol. LC-MS/MS experiments were performed by Shanghai Applied Protein Technology Co., Ltd.

3. Validation of exogenously expressed substrates by western blotting
a. Cell lysis and sample denaturation
i. Add 400 μL of denaturation lysis buffer to each cell culture dish and swirl the dish rapidly to fully cover all adherent cells.
Note: Adjust the volume of denaturation lysis buffer within a range of 350–450 μL according to cell confluency per dish; increase the volume for high cell confluency and decrease it for low cell confluency.
ii. Scrape the lysed cells with a cell scraper and transfer the cell lysate into a 1.5 mL centrifuge tube.
iii. After sample collection, perform a brief pulse spin, then heat the samples in a 100 °C heating block for 10 min. Gently flick the centrifuge tubes halfway through heating to keep the solution well mixed.

b. Lysate sonication: Sonicate the samples with a non-contact ultrasonic processor under the following parameters: 80% power, 10 s sonication followed by a 10 s interval, with a total treatment duration of 10 min. Stop sonication when the samples turn clear and transparent; prolong the ultrasonic treatment if sample turbidity remains.
Note: Pre-cool the ultrasonic processor to 4 °C before use to avoid protein degradation caused by temperature rise during sonication.
c. Centrifuge samples at 13,000× *g* for 10 min at room temperature.

d. Input sample preparation
i. Aliquot 40 µL of whole cell lysate from each sample into a new 1.5 mL centrifuge tube. Add 20 µL of 5× SDS protein loading buffer together with 40 µL of denaturation lysis buffer and mix thoroughly.
ii. Perform a brief pulse spin and heat the mixture at 100 °C in a heating block for 8 min. Allow the samples to cool to room temperature for immediate electrophoresis or store at -80 °C until further use.

e. Immunoprecipitation (IP) sample preparation
i. Pre-clearing: Transfer the remaining 360 µL supernatant (from step A2.3c) to a new 1.5 mL centrifuge tube and add 900 µL of Buffer A (supplemented with 1× protease inhibitor cocktail), followed by 20 µL of mouse IgG-agarose. Incubate the mixture for 30 min at 4 °C with gentle rotation to remove nonspecific binding proteins.
ii. Centrifuge the mixture at 2,000× *g* for 30 s at 4 °C. Transfer the supernatant to a new 1.5 mL tube and add 20 µL of pre-equilibrated Anti-FLAG M2 affinity gel.
iii. Incubate the mixture overnight at 4 °C with gentle rotation.

f. Washing of affinity beads
i. On the following day, centrifuge the mixture at 2,000× *g* for 30 s at 4 °C and discard the supernatant.
ii. Add 1 mL of NETN buffer to resuspend and wash the beads. Centrifuge the mixture at 2,000× *g* for 30 s at 4 °C; repeat this washing procedure three times.

g. Elution and denaturation of IP products
i. Carefully remove residual liquid with a 1 mL syringe.
ii. Add 100 µL of 1× SDS protein loading buffer (prepared by diluting 5× SDS protein loading buffer with NETN buffer) to the beads, mix thoroughly, and perform a brief pulse spin.
Note: Adjust the volume of 1× SDS protein loading buffer between 60 and 100 µL according to the UFMylation level of the target substrates; use a larger volume for high UFMylation levels and a smaller volume for low UFMylation levels.
iii. Heat the mixture in a 100 °C heating block for 8 min to denature the IP products.
iv. Cool the samples to room temperature before electrophoresis, or store at -80 °C if not used immediately.

B. UFMylation assay of endogenous substrates

Except for ribosomal protein L26 (RPL26, the major endogenous UFMylation substrate), the UFMylation level of most other endogenous substrates is pretty low. Therefore, a large number of cells is required for this assay. This protocol uses wild-type HEK293T cells cultured in twenty 100 mm cell culture dishes as an example for operation.

1. Cell harvest and treatment: Harvest cells when the cell confluency reaches approximately 90%. Follow steps A2.3a–d for cell treatment (including cell collection, lysis, denaturation, centrifugation, and input sample preparation).

2. IP sample grouping and pre-clearing
a. Divide the cell lysates into two groups for endogenous substrate enrichment:
i. Group 1: IgG control group, serving as the negative control.
ii. Group 2: Substrate antibody group, for specific enrichment of target substrate proteins.

b. Pre-clearing: For each group, pool the lysates derived from ten 100-mm culture dishes into a single 15 mL centrifuge tube. Add 5 mL of Buffer A (supplemented with 1× protease inhibitor cocktail), followed by 20 µL of protein A/G agarose. Incubate the mixture for 30 min at 4 °C with gentle rotation to remove nonspecific binding proteins.
c. Centrifuge the mixture at 500× *g* for 5 min at 4 °C using a swing-bucket rotor. Discard the agarose bead pellet and keep the supernatant.

3. Antibody incubation: Add 4 µg of IgG antibody (for the control group) or target substrate protein antibody (for the experimental group) to the corresponding pre-cleared supernatants. Incubate the mixtures overnight at 4 °C with gentle rotation.
4. Protein A/G agarose incubation: On the following day, add 50 µL of protein A/G agarose per tube. Incubate the mixtures for 4–6 h at 4 °C with gentle rotation.
5. Bead transferring and washing: Centrifuge the samples at 500× *g* for 5 min at 4 °C using a swing-bucket rotor. Discard most of the supernatant, then resuspend the agarose beads in 1 mL of NETN buffer and transfer the suspension from the 15

mL tube to a new 1.5 mL centrifuge tube. Wash the beads three times with 1 mL of NETN buffer per wash. After each wash, centrifuge at 2,000× *g* for 30 s at 4 °C and aspirate the supernatant carefully to avoid disturbing the bead pellet.
6. Product collection, denaturation, and detection: Follow the procedures described in step A2.3g for subsequent sample elution, denaturation, and detection.

C. Western blotting

1. Prepare SDS-PAGE gel: Prepare SDS-PAGE gels using the Color PAGE Gel Rapid Preparation kit following the manufacturer's instructions. Select an appropriate separating gel concentration according to the molecular weight of the target proteins.
2. Electrophoresis
a. Assemble the prepared SDS-PAGE gel into the electrophoresis tank, add 500 mL of 1× running buffer, and check for leakage. Remove the comb when no obvious drop in liquid level is observed.
b. Load 10–20 µL of prepared protein sample into each well as needed. For frozen samples, heat at 100 °C for 2 min, cool to room temperature, mix gently, and perform a brief pulse spin before loading. Conduct electrophoresis at 80 V for the stacking gel and 120 V for the separating gel. Stop electrophoresis when the bromophenol blue front migrates to the bottom edge of the separating gel.

3. Membrane transfer
a. Prepare and pre-cool 1× transfer buffer in advance.
b. Disassemble the gel cassette after electrophoresis and rinse off residual electrophoresis running buffer with deionized water.
c. Activate the PVDF membrane with methanol for 60 s, then rinse it quickly with ultrapure water to remove residual methanol. Subsequently, equilibrate the activated PVDF membrane in pre-cooled 1× transfer buffer for 5–10 min.
d. Assemble the standard transfer sandwich in pre-cooled 1× transfer buffer in the following order: negative electrode > filter paper > gel > PVDF membrane > filter paper > positive electrode. Carefully remove all trapped air bubbles. Place the transfer cassette into the transfer tank, then submerge the entire tank in an external ice water bath and place pre-chilled ice packs inside the tank adjacent to the cassettes. Perform wet transfer at 100 V for 90–150 min; adjust the transfer time according to the molecular weight of target proteins.

4. Blocking: Take out the PVDF membrane after transfer, rinse off residual transfer buffer with 1× TBS, and incubate the membrane in blocking buffer at room temperature for 1 h to block nonspecific binding.
5. Primary antibody incubation: After blocking, rinse off residual blocking buffer with 1× TBS. Dilute the primary antibody in antibody dilution buffer at the manufacturer-recommended dilution ratio and incubate the membrane in the primary antibody solution overnight at 4 °C or for 2 h at room temperature. The antibody dilution ratios applied in validation experiments are as follows: anti-DDRGK1 (1:1,000), anti-FLAG (1:1,000), anti-UFM1 (1:1,000), anti-UFSP2 (1:1,000), anti-Myc (1:1,000), anti-HA (1:1,000), and anti-GAPDH (1:5,000).
6. Secondary antibody incubation: Recover the primary antibody solution. Wash the membrane with TBST for 5 min and repeat this washing step three times. Discard the used TBST, then incubate the membrane in secondary antibody diluted with blocking buffer at room temperature for 1 h.
7. Chemiluminescence detection: Discard the secondary antibody solution. Wash the membrane with TBST for 5 min and repeat this washing step four times. Prepare fresh ECL chemiluminescence working solution and detect the signals of target protein bands using the ChemiDoc MP Imaging System.

Data analysis

The LC-MS/MS data analysis workflow for large-scale UFMylation substrate identification is fully detailed in the *Experimental procedures* section (*Database search* and *Bioinformatics analysis* subsections) of [11]. Supporting analytical results and visualizations are presented in Figure 4, Supplemental Figures S4 and S5, and Datasets S1–S4 of the same publication. MS proteomics data were deposited to ProteomeXchange/PRIDE with identifier PXD070408.

Validation of protocol

1. UFMylation assay of exogenously expressed substrates

We identified 617 substrates using our optimized protocol as reported in [11]. This substrate list includes multiple previously reported substrates, such as RPL26/RPL26L1, RPL10, cytochrome b5 reductase 3 (CYB5R3), valosin-containing protein (VCP/p97), fatty acid synthase (FASN), poly (ADP-ribose) polymerase-1 (PARP1), activating signal cointegrator 1 (ASC1), Ribophorin1 (RPN1), 14-3-3ε, and histone H4. We selected five representative proteins from this list for further validation: heat shock protein 90-alpha (HSP90α), YEATS domain-containing protein 2 (YEATS2), annexin A5 (ANXA5), annexin A6 (ANXA6), and protein arginine N-methyltransferase 5 (PRMT5). All candidate proteins were successfully conjugated by UFM1, and their UFMylation modification could be deconjugated by UFSP2. Supporting analytical results and visualizations are presented in Figure 4D–H of [11].

Figure 1 shows the unpublished replicate experimental results.

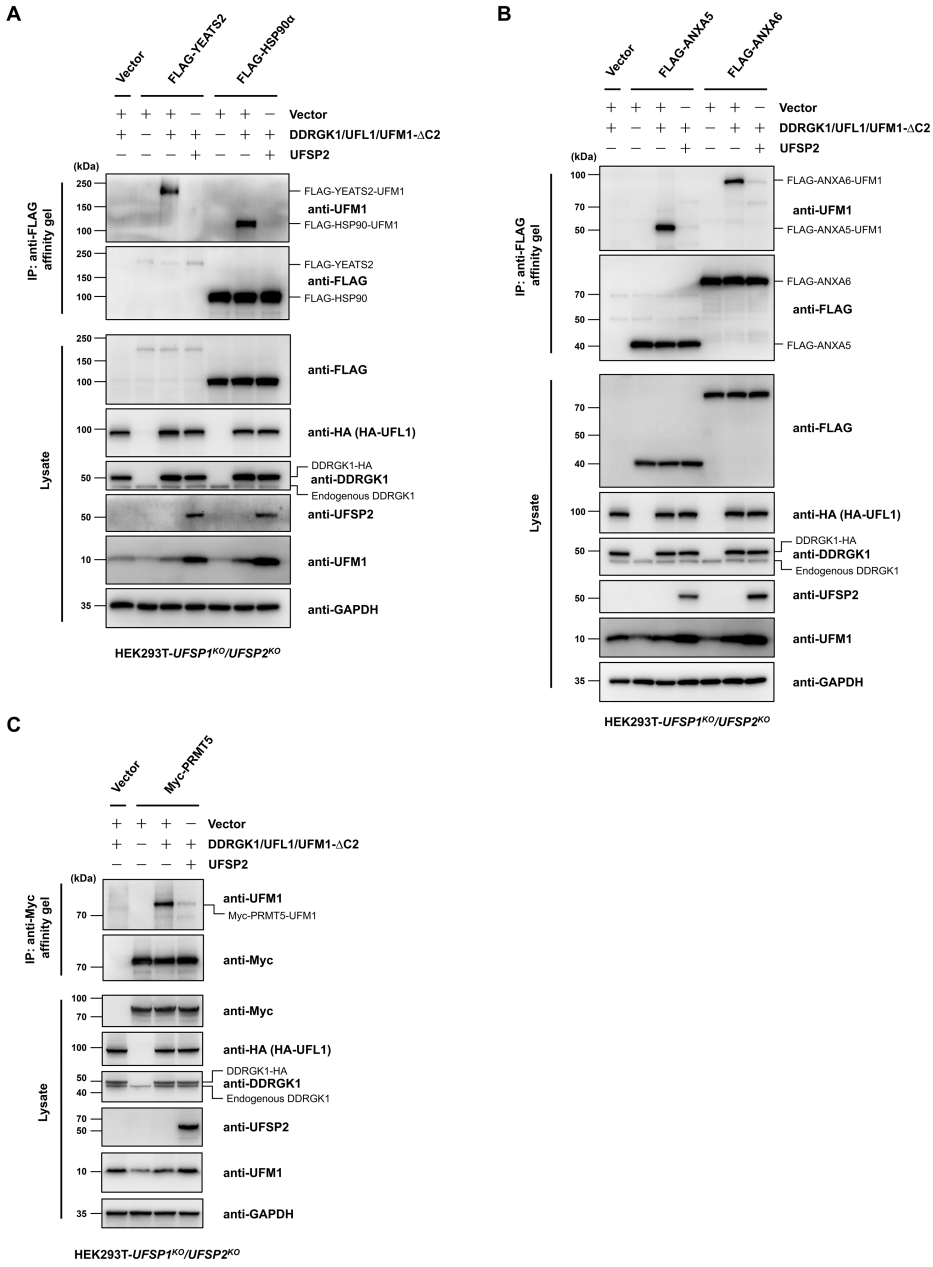


Figure 1. UFMylation assay of exogenously expressed substrates. To validate the reliability of the UFMylation of five selected substrate proteins [HSP90α and YEATS2 (A), ANXA5 and ANXA6 (B), and PRMT5 (C)], the UFM1-specific

protease UFSP2 construct was cotransfected during protein modification assay in *UFSP1*^{KO}/*UFSP2*^{KO}-HEK293T cells. After transfection and cultivation, cells were lysed, and the lysates were subjected to immunoprecipitation using anti-FLAG M2 (A, B) or anti-Myc (C) affinity gels. The precipitated proteins were analyzed via western blotting with the indicated antibodies.

2. Large-scale substrate identification by LC-MS/MS
Supporting analytical results and visualizations are presented in Figure 4A–C and Supplemental Figures S5 of [11].

3. UFMylation assay of endogenous substrates
Supporting analytical results and visualizations are presented in Figure S4A–E of [11].

4. This protocol has been used and validated in the following research articles:
• Fang et al. [11]. Optimization of protein UFMylation modification method and its application in substrate identification in human cells. *Journal of Biological Chemistry*, 2026, 302(4). (Figure 4; Supplemental Figures S4–S5).
• Liang et al. [12]. UFMylation deficiency in hepatocytes activates the Keap1-Nrf2 pathway and contributes to hepatocarcinogenesis. *Redox Biology*, 2026: 104046. (Figure 5D, E).

General notes and troubleshooting

General notes

1. This protocol was established and validated exclusively in HEK293T cells, and is designed to be generally applicable for UFMylation substrate identification in mammalian cell models; cross-application to primary cells, animal tissues, or other cell lines requires optimization of transfection, lysis, and incubation parameters according to cellular characteristics.
2. The method shows limited sensitivity to extremely low-abundance UFMylation substrates, which may lead to omission of some low-expression, weakly modified functional substrates; expanding cell culture scale (e.g., 20–30 × 100 mm dishes per sample) can significantly improve enrichment efficiency and detection sensitivity for low-abundance substrates. This strategy has been successfully used in our laboratory to identify several low-abundance transcription factors and signaling molecules as novel UFMylation substrates.
3. Exogenous overexpression of UFMylation components (UFL1, DDRGK1, UFM1-ΔC2) may slightly alter the stoichiometry of the intracellular modification system and generate a small amount of non-physiological background signals; empty vector controls must be strictly included in all experiments to exclude nonspecific effects caused by overexpression of the modification machinery. All candidate substrates identified in this overexpression system should be further validated by endogenous UFMylation assays in wild-type cells without any exogenous overexpression.
4. *UFSP1/UFSP2* double-knockout (*UFSP1*^{KO}/*UFSP2*^{KO}) HEK293T cells are the key experimental material for the UFMylation assay of exogenously expressed substrates.
5. This protocol is mainly used for identification and modification site validation of UFMylation substrates; further endogenous knockdown and rescue experiments are required to explore the physiological functions of UFMylation.

Troubleshooting

Problem 1: Massive cell death and floating after transfection.
Possible causes: 1) Excessively high PEI concentration. 2) Low cell seeding density. 3) No timely medium replacement after transfection.
Solutions: 1) Control PEI final concentration to no more than 5 μg/mL. 2) Ensure cell confluency reaches 80% before transfection. 3) Replace with fresh complete medium 12 h post-transfection.

Problem 2: Weak or no UFMylation band signal.
Possible causes: 1) UFMylation promotes proteasomal degradation of substrate proteins. 2) Incorrect plasmid ratio/dosage for transfection. 3) Protein degradation caused by improper lysis or IP operation.
Solutions: 1) Treat cells with MG132 before harvesting. 2) Strictly follow the plasmid ratio in Table 1 and maintain PEI:plasmid ratio at 2:1 (μL/μg). 3) Add fresh protease inhibitor to the lysis buffer, operate on ice throughout, and shorten the processing time.

Problem 3: Excessive nonspecific bands and high background interference.
Possible causes: 1) Insufficient blocking. 2) Insufficient IP washing times. 3) Impure cell lysate with debris.
Solutions: 1) Extend blocking time to 1 h and use blocking buffer with ProClean 950. 2) Increase IP washing time to 8 min to remove nonspecific binding proteins. 3) Sonicate until the lysate is clear and centrifuge to remove cell debris.

Problem 4: Undetectable UFMylation signal of endogenous substrates.
Possible causes: 1) Extremely low abundance of endogenous substrates. 2) Poor specificity of substrate antibody. 3) Low IP enrichment efficiency.
Solutions: 1) For most endogenous substrates, expand cell culture to 10 × 100 mm dishes; for extremely low-abundance substrates, further expand to 20–30 × 100 mm dishes per sample. 2) Use commercially validated or in-house-verified substrate-specific antibodies. 3) Extend the incubation time of primary antibody with cell lysate to 16–18 h at 4 °C and increase the amount of Protein A/G agarose to 60–80 μL per sample.

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Competing interests

The authors declare no conflicts of interest.

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References

1. Millrine, D., Peter, J. J. and Kulathu, Y. (2023). A guide to UFMylation, an emerging posttranslational modification. *FEBS J.* 290(21): 5040–5056. https://doi.org/10.1111/febs.16730
2. Wei, Y. and Xu, X. (2016). UFMylation: A Unique & Fashionable Modification for Life. *Genom Proteom Bioinf.* 14(3): 140–146. https://doi.org/10.1016/j.gpb.2016.04.001
3. Zhou, X., Mahdizadeh, S. J., Le Gallo, M., Eriksson, L. A., Chevet, E. and Lafont, E. (2024). UFMylation: a ubiquitin-like modification. *Trends Biochem Sci.* 49(1): 52–67. https://doi.org/10.1016/j.tibs.2023.10.004
4. Komatsu, M., Noda, N. N. and Inada, T. (2026). The mechanistic basis and cellular functions of UFMylation. *Nat Rev Mol Cell Biol.* 27(6): 419–432. https://doi.org/10.1038/s41580-025-00944-y
5. Gerakis, Y., Quintero, M., Li, H. and Hetz, C. (2019). The UFMylation System in Proteostasis and Beyond. *Trends Cell Biol.* 29(12): 974–986. https://doi.org/10.1016/j.tcb.2019.09.005
6. Komatsu, M., Chiba, T., Tatsumi, K., Iemura, S., Tanida, I., Okazaki, N., Ueno, T., Kominami, E., Natsume, T., Tanaka, K., et al. (2004). A novel protein-conjugating system for Ufm1, a ubiquitin-fold modifier. *EMBO J.* 23(9): 1977–1986. https://doi.org/10.1038/sj.emboj.7600205
7. Liang, Q., Jin, Y., Xu, S., Zhou, J., Mao, J., Ma, X., Wang, M. and Cong, Y. S. (2022). Human UFSP1 translated from an upstream near-cognate initiation codon functions as an active UFM1-specific protease. *J Biol Chem.* 298(6): 102016. https://doi.org/10.1016/j.jbc.2022.102016
8. Millrine, D., Cummings, T., Matthews, S. P., Peter, J. J., Magnussen, H. M., Lange, S. M., Macartney, T., Lamoliatte, F., Knebel, A., Kulathu, Y., et al. (2022). Human UFSP1 is an active protease that regulates UFM1 maturation and UFMylation. *Cell Rep.* 40(5): 111168. https://doi.org/10.1016/j.celrep.2022.111168

9. Zhou, J., Liang, Q., Dong, M., Ma, X., Jin, Y., Guan, D., Liu, J., Wang, M. and Cong, Y. S. (2022). Optimized protocol to detect protein UFMylation in cells and in vitro via immunoblotting. *STAR Protoc.* 3(1): 101074. https://doi.org/10.1016/j.xpro.2021.101074
10. Banerjee, S., Kumar, M. and Wiener, R. (2020). Decrypting UFMylation: How Proteins Are Modified with UFM1. *Biomolecules.* 10(10): 1442. https://doi.org/10.3390/biom10101442
11. Fang, Y., Wang, X., Dong, J., Yi, X., Lv, Z., Bu, C., Ma, X., Cong, Y. S. and Liang, Q. (2026). Optimization of protein UFMylation modification method and its application in substrate identification in human cells. *J Biol Chem.* 302(4): 111320. https://doi.org/10.1016/j.jbc.2026.111320
12. Liang, Q., Xu, S., Fang, Y., Wang, X., Xiao, Y., Wang, Y., Li, S., Guo, Q., Cao, Y., Cao, Y., et al. (2026). UFMylation deficiency in hepatocytes activates the KEAP1-NRF2 pathway and contributes to hepatocarcinogenesis. *Redox Biol.* 90: 104046. https://doi.org/10.1016/j.redox.2026.104046