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High-Throughput MALDI-TOF Mass-Spectrometry-based Deubiquitylating Enzyme Assay for Drug Discovery

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Running head: Quantification of deubiquitylating activity by MALDI-TOF Mass Spectrometry

Key Words: Ubiquitin; Deubiquitylating Enzyme; DUBs; MALDI-TOF Mass Spectrometry; Drug Discovery; High-Throughput Screening

ABSTRACT

Ubiquitylation (or ubiquitination) is the reversible conjugation of a 76-amino-acid polypeptide (ubiquitin) to a target protein to modulate various biological processes. Deubiquitylating enzymes (DUBs) are a class of enzymes that specifically remove ubiquitin from a substrate. In recent years DUBs have garnered significant attention as a new class of targets in multiple therapeutic areas. The recent development of high-throughput Matrix-Assisted Laser Desorption/Ionization-Time of Flight mass spectrometry (MALDI-TOF MS) has provided new tools to perform drug discovery screening. Here we present a facile and high-throughput step-by-step protocol of the MALDI-TOF MS-based DUB assay for screening the activity of DUBs in vitro. In a MALDI-TOF DUB assay, we quantitate the amount of mono-ubiquitin generated by the *in vitro* cleavage of ubiquitin chains. The presented protocol takes advantage of nanolitre dispensing robotics and automated MALDI-TOF MS analysis to screen large and diverse compound libraries.

1. Introduction

Ubiquitylation is a post-translational modification that impacts multiple cellular processes. Removal of ubiquitin from its substrate is the activity of specific ubiquitin proteases, called deubiquitylating enzymes (DUBs). The human genome encodes approximately 100 DUBs that are divided into seven families [1-3]: the USPs family, with 56 members, 17 ovarian tumour proteases (OTUs), 12 JAB1/MPN/MOV34 Zn-dependent metalloprotease (JAMMs), four Machado-Josephin domain proteases (MJD), four ubiquitin c-terminal hydrolases (UCHs), five motif interacting with ubiquitin-containing novel DUB family (MINDYs) and one zinc finger-containing ubiquitin peptidase 1 (ZUP1).

In 2014, Ritorto *et al.* [4] employed MALDI-TOF MS to characterize the *in vitro* activity and substrate specificity of a panel of 32 DUBs. This method, named the MALDI-TOF DUB assay, relies on detecting the free ubiquitin signal generated by the activity of a DUB toward ubiquitin dimers. Quantification of DUB activity is achieved by using heavy labelled ^{15}N ubiquitin as an

internal standard. The method was initially performed in standard 0.5 mL tubes and was therefore low-throughput in nature. Here, we report a step-by-step protocol of a high-throughput version of the original MALDI-TOF DUB assay, adapted to employ a nanolitre pipetting system, fast automatic detection and semi-automatic data analysis [5-9].

Initially, compounds are deposited on 384-well plates using acoustic delivery systems and incubated with the DUB of interest. The reaction is started by adding a ubiquitin dimer of suitable linkage and stopped by adding trifluoroacetic acid. Positive hits are then confirmed by dose-response analysis, allowing calculation of IC50 values. Cherry-picked positive 'hits' are then tested against a panel of different DUBs to assess their specificity within the same phylogenetic family and across six out of the seven DUB families. The 384-plate format is typically employed for extensive compound screening, and up to 3,072 data points can be acquired daily.

2. Materials

Prepare all solutions using milli-Q water and Mass Spectrometry grade solvents (ethanol, acetonitrile and isopropanol) for all stages. Low-protein-binding tubes are recommended for the handling and storage of ubiquitin substrates.

2.1. Solutions and reagents

1. Prepare MALDI-TOF matrix by weighing 7.6 mg of 2, 5-dihydroxyacetophenone (DHAP) in a 1.5 mL tube. Add 375 µl of 100% ethanol. In a separate tube, weight 25 mg diammonium hydrogen citrate and resuspend in 1 mL Milli-Q water. Add 125 µl of the aqueous diammonium hydrogen citrate to the ethanol/DHAP matrix solution. Shake the mixture for at least an hour. Store the matrix solution for up to 1 week at room temperature (20° - 25° C). Do not store at +4° or -20°C.
2. Enzyme buffer: 40 mM Tris pH 7.5, BSA 0.01%, 1 mM dithiothreitol (DTT) or tris(2-

carboxyethyl)phosphine (TCEP). To prepare 10 mL of enzyme buffer, mix 0.4 mL of 1 M Tris-HCl stock solution and 10 µl of 10% bovine serum albumin (BSA). Bring to volume with milliQ water. Keep on ice. Add 20 µl of 0.5 M TCEP or DTT before using this solution to dilute the enzyme of choice.

3. Substrate solution: 40 mM Tris pH 7.5, 0.01% BSA. Use enzyme buffer (without the DTT or TCEP) to dilute the substrate of choice to a final concentration of 0.1 mg/mL. To be kept on ice until use. Substrate solution can be stored at 4°C for several days in low binding tubes.
4. Stopping solution: 6% trifluoroacetic acid (TFA). To prepare 100 mL of stopping solution, add 6 mL of 100% TFA to 94 mL of MilliQ Water. Agitate gently and keep on ice until use. The stopping solution can be stored at 4 °C for up to two weeks. See **Note 1** regarding TFA and the care taken when handling this material.
5. ^{15}N ubiquitin solution: produce and purify the ^{15}N ubiquitin as previously described [8] and then dilute in milli-Q water to a final concentration of 4 µM.
6. MALDI target cleaning solution: to be prepared under chemical fumehood. For 1L: mix 300 mL of LC-MS grade Acetonitrile and 699 mL of Milli-Q water. Add 1 mL of 100% Trifluoroacetic Acid (30% acetonitrile, 0.1% TFA (v/v) solution). Refer to **Note 1** for TFA risks and handling.
7. High-purity nitrogen gas.
8. Recombinant deubiquitylase enzyme (*in vitro* active, see **Table 1**).
9. Ubiquitin dimer (or trimer/tetramer), produced and purified as previously described [10, 11].

2.2. Equipment, supplies and software

1. MTP AnchorChip 1536 BC (Part Number # 8280787).

2. 384-well microplate, PS, small volume, hibase, white (Greiner bio-one, Cat. number 784904).
3. 384-well LSVD plate, non-sterile polypropylene (TTP Labtech, Cat. number 4150-05828)
4. Microplate 384/V-PP, Protein LoBind (Eppendorf, Order no 0030 624.300).
5. Silverseal sealer aluminium foil.
6. Rapiflex MALDI-TOF mass spectrometer (Bruker – equipped with Compass software for FlexSeries 2.0).
7. FlexControl software (Bruker).
8. FlexAnalysis software (Bruker).
9. ZERO_Script file (see **Note 2** for code availability), to be imported into the Flex Analysis Methods folder.
10. UW Ultrawave QS18 ultrasonic cleaning bath.
11. 20 cm x 13 cm x 4 cm stainless steel box for sonicating MALDI target.
12. FluidX, XRD-384 reagent dispenser.
13. TTP Labtech Mosquito HTS equipped with five positions plate deck.
14. 16 Channel Electronic VIAFLO Pipette, 2 – 50 µl volume range and appropriate tips.

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1. Preliminary Preparations

1. Clean the MALDI target. To do so, place the MALDI target (384 or 1536 AnchorChip) in a stainless steel box of adequate size. Pour 100% isopropanol onto the target to completely cover it and sonicate for 3 minutes (using the UW Ultrawave QS18 Ultrasonic Cleaning Bath). Remove isopropanol and add 30% acetonitrile, 0.1% TFA (v/v) solution, sonicate for further 3 minutes, then dry the MALDI target plate using a stream of high-purity

nitrogen.

3.2. MALDI-TOF DUB Assay for HTS Screening

Compound libraries are usually delivered by non-contact acoustic transfer on 384 well plates of choice. The final concentration of the compounds varies depending on the type of library in use. 10-50 µM final is a commonly used range. Depending on the material starting concentration, 5-500 nL can be deposited on the assay plate. Higher amounts can also be spotted. However, the final DMSO concentration should not exceed 3% (compounds are usually resuspended in 100% DMSO).

1. Aliquot compounds into assay plate (384-well microplates) using non-contact acoustic delivery. Reserve two columns for both positive (DMSO only) and negative (no enzyme) controls (see **Figure 1**).
2. Using FluidX, XRD-384 reagent dispenser, aliquot 3 µl of enzyme solution into each assay plate column except for the negative control column (see **Figure 1**).
3. Using FluidX, XRD-384 reagent dispenser, aliquot 3 µl of enzyme buffer into the negative control column (see **Figure 1**).
4. Cover the plate with aluminium sealing foil to prevent evaporation and incubate at room temperature for 5-30 minutes.
5. Using FluidX, XRD-384 reagent dispenser, add 3 µl of the substrate to each column of the entire assay plate.
6. Cover the assay plate with aluminium sealing foil to prevent evaporation and incubate at room temperature for the time suggested by initial optimization experiments (See **Note 3**).
7. Stop the reaction by adding 3 µl TFA to a final 2% (v/v) concentration to each column (see **Figure 1**).

3.3 Spot the Assay Plates on the MTP AnchorChip 1536 MALDI Target

This step is performed using a TTP Labtech Mosquito HTS (equipped with five positions plate deck). The instrument aliquots 1,050 nL of sample from the assay plate into the mixing plate (384-well LSVD plate, non-sterile polypropylene). The sample will be extensively mixed with 1,200 nL of DHAP matrix (taken from reservoir plate) and spotted on the MTP AnchorChip 1536 MALDI target (see **Figure 2**).

1. Aliquot a sufficient amount of DHAP and ^{15}N ubiquitin into a reservoir plate (microplate 384/V-PP) and cover the plate with aluminium sealing foil to prevent evaporation.
2. On the TTP Labtech Mosquito HTS, position the reservoir plate on deck position 1, assay plate on deck position 2, mixing plate on deck position 4 and the MTP AnchorChip 1536 BC MALDI target on deck position 5.
3. Aliquot 1050 nL of assay plate column 1 into mixing plate column 1.
4. Add 500 nL of ^{15}N ubiquitin to mixing plate column 1.
5. Add 1.2 µL of DHAP matrix to mixing plate column 1.
6. Mix thoroughly using 2x 10 mixing cycles of 1000 nL.
7. Spot 260 nL on the MTP AnchorChip 1536 MALDI target.
8. Repeat Step 3 to 7 for all subsequent assay plate columns.
9. Allow the MALDI target to dry completely at room temperature.

3.4. Automatic MALDI-TOF MS Analysis

1. Open Flex Control and select a fingerprint (.par file) Reflectron Positive (RP) acquisition (acquitistion window 8-9 kDa).
2. Insert and dock the freshly spotted MALDI target into the instrument and select appropriate MALDI target geometry.
3. Wait for the instrument to reach an adequate vacuum level.
4. Calibrate the instrument using the ^{15}N ubiquitin signal (8669.47 *m/z*).

5. In the Automation run, select “New” and start a new run using the automatic run wizard.

3.5. Statistical Analysis for HT MALDI-TOF DUB Assay

1. Download and allocate scripts necessary for analysis as indicated in Note 2.
2. Open the Flex Analysis software and select one spectrum within the folder containing the raw data of interest.
3. Open the “Method” menu and select the Flex Analysis method (extension. FAMSMethod) “ZERO” script.
4. Open the “Method” menu and select “Edit Script”
5. Modify the destination folder (strOutputDir) and result file name (StrOutputFileName) as desired, save the modified script.
6. Open the flexAnalysis Batch Process tool and select spectra to process using the previously modified “ZERO” script. The zero script will export mass areas of each spot from the raw data into a .csv column format. For the negative controls, the zero script considers the area of the background noise detectable in the ubiquitin *m/z* window.
7. Copy and paste the zero script output columns (Spectrum, Compound, m/z, S/N, Resolution and Area) into the Input sheet of the Baseline data check file.
8. Convert Output zero deleted sheet (the last sheet of Baseline data check file) to .txt plain format.
9. Drag text file onto the Grid .exe file. The script will produce a new file with GRID included in the file name. The GRID file will select ubiquitin and ^{15}N ubiquitin area values and report them in the same position as spotted on the MALDI plate.
10. Calculate ubiquitin over ^{15}N ubiquitin area ratio.
11. Evaluate data quality by calculating Z’ scores

4. Notes

1. TFA should be handled in a chemical fumehood using protective eyewear and gloves. TFA is used as a component of the stopping solution and for cleaning the MALDI target.
2. The Zero Script in The in-house scripts are publicly available in GitHub at https://github.com/Vdecesare/GRID-script.git and https://github.com/Vdecesare/Zero-Script.git). Locate the FAMSMethod in the Data\Methods\flexAnalysisMethods.

3. Before starting an HTS campaign, it is fundamental to identify ideal enzyme and substrate concentrations, incubation times and temperatures. This optimization can be done by testing several enzyme concentrations over a time course experiment (reaction progress curve, see figure 3). Data points should be collected at the beginning of the reaction to identify active compounds when an excess of the substrate results in linear product formation. In figure 3, representative results of a reaction progress curve of USP7 tested at three different concentrations (34.4 nM, 13.76 nM and 6.9 nM). The percentage of a cleaved substrate (% of cleavage) is obtained by applying the equation in **Note 9**. At the final concentration of 34.4 nM, the reaction is linear for up to 15 minutes. The reaction incubation time can be extended to 20 minutes by reducing the final concentration of the enzyme down to 13.76 nM. Further dilution to 6.9 nM results in a non-linear product formation curve.
4. All liquid handling systems should be tested for accuracy before starting the main procedure. Quality control of the FluidX, XRD-384 reagent dispenser should report a Coefficient of Variation (CV%) ≤5. If the CV% is >5, a new liquid handling head might be required. Accurate calibration of the TTP Labtech Mosquito HTS is also fundamental to ensure optimal mixing and spotting on the MALDI target.
5. High-throughput screening campaigns should be performed using the same batch of both enzyme and substrate, to be partitioned prior to use into single-use aliquots. If this is not

possible, new protein batches should be tested side-by-side before the screening.

6. Extensive mixing cycles of sample and DHAP matrix are required to activate the formation of sample/DHAP matrix crystals and ensure optimal MALDI-TOF MS signal. The number of mixing cycles and the volume can be adjusted depending on sample concentration and complexity.
7. The DHAP matrix is prepared with 75% ethanol, the evaporation of mixed samples is time-sensitive. The mixture of a sample and matrix must be immediately spotted onto the MALDI target before the next steps can take place. Once spotted, the reaction mixture should be analyzed as soon as possible to avoid degradation of the sample.
8. DTT and TCEP are strong nucleophiles and may therefore compete for the compound with the active cysteine of the DUBs. Carefully evaluate DTT or TCEP presence in the reaction buffer depending on the DUB and compound library in use. Enzymatic activity should be evaluated in the presence or absence of a reducing agent in a side-by-side comparison.
9. Ubiquitin/^{15}N ubiquitin ratio can be translated into % of substrate consumed (% of cleavage) using the previously reported equation [4]:

Equation 1: $$x = \left(\frac{\text{AreaUbi}}{\text{Area } {}^{15}N \text{ Ub}} \times \left[{}^{15}N \text{ Ubi}\right])/2\right)/[DiUbi] \times 100$$

10. Both positive (DMSO only) and negative controls (no enzyme or presence of strong DUB inhibitor) are necessary to calculate the reliability and quality of data from each plate. A positive control indicates the maximum signal achieved without any interference, while the negative control determines the background signal. In absence of ubiquitin signal, as for in the negative control and in case of strong inhibition, the “Zero script” will integrate the signal background area to allow Z’ Prime calculations.
11. The aim of HTS statistical analysis is the robust identification of positive hits. The Z-Prime (or Z’, See Equation 2), extensively used in the HTS field, takes into account both the

signal window between positive and negative controls and their statistical variability [12] Z-Prime is defined as three times the sum of positive and negative control standard deviations (σ_p, σ_n) divided by the difference of the averages of positive and negative controls (μ_p, μ_n)

Equation 2: $\mathrm{Z-Prime} = 1 - \frac{3(\sigma_{p+\sigma_n})}{|\mu_{p-\mu_n}|}$

12. Z-Prime scores higher than 0.5 are considered acceptable.

Contributions

V.DC. and P.D. wrote the manuscript.

Data availability

The data that support the anticipated results are available from the corresponding author upon reasonable request. Other enquiries can also be addressed to https://dub-screen.mrc.ac.uk/

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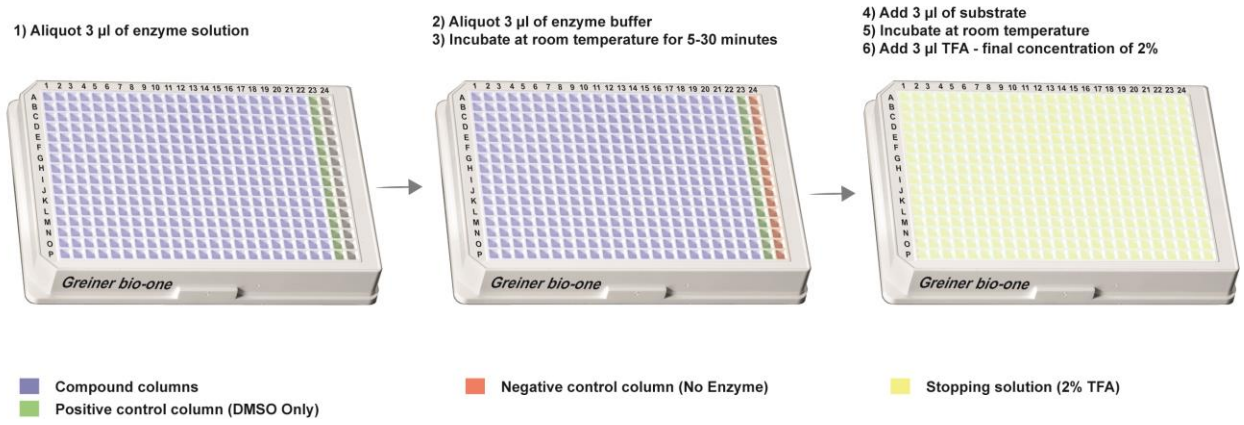
Figures

Figure 1



297

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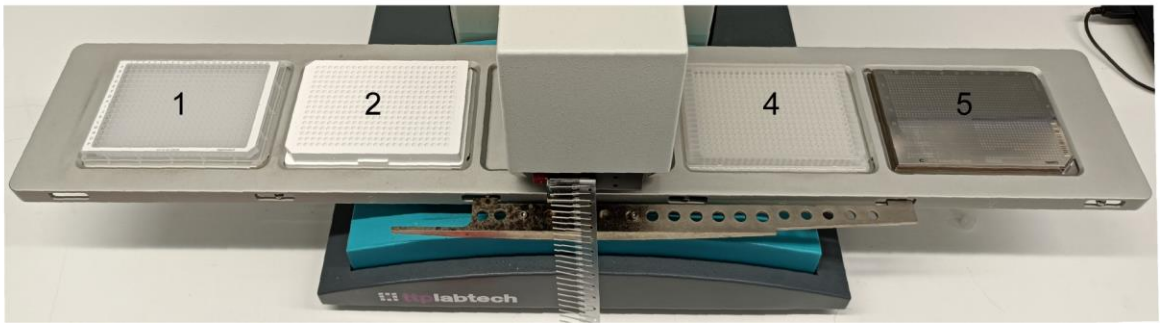
Figure 2



299

300

Figure 3

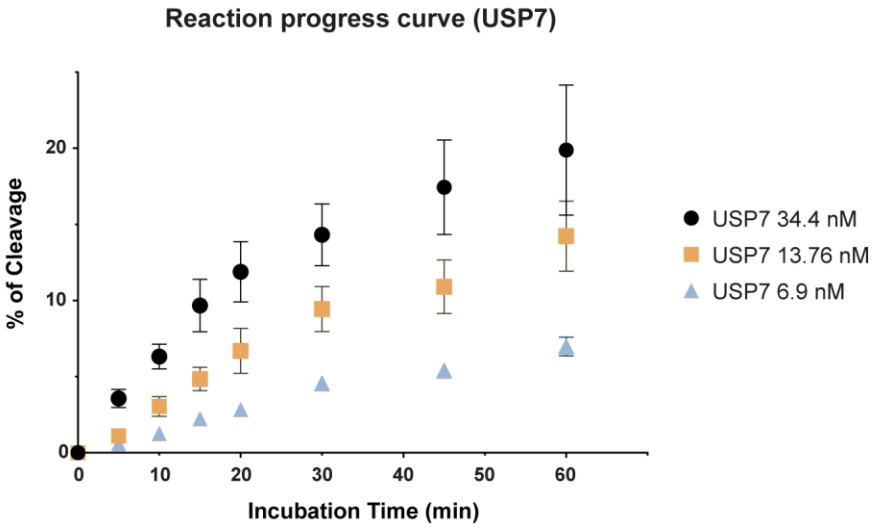


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Figure Legends

Figure 1. MALDI-TOF MS HTS Workflow.

Figure 2. TTP Labtech Mosquito HTS plates disposition. Reservoir plate with ^{15}N Ubiquitin and DHAP matrix (position 1). Assay plate (position 2). Mixing plate (position 4). MALDI target (position 5).

Figure 3. Reaction progress curve of USP7. Different enzymatic concentrations are tested over time. The linearity of the reaction is evaluated to identify the ideal enzyme concentration and incubation time.

Table 1| *In vitro* active deubiquitylases currently available for selectivity profiling in the MRC-PPU DUB Profiling service

ENZYME	LENGTH	ACCESSION	TAG	DU NUMBER	EXPRESSION SYSTEM	SUBSTRATE
USP1 G670A G671A / WDR48	1-785 / 1-732	O94782.1 / Q8TAF3-1	His6 / None	DU23056	Insect cells	K63
USP2	1-396	O75604-4	GST	DU13025	*E. coli*	K63
USP4	1-963	Q13107-1	His6	DU14350	*E. coli*	K48
USP5	1-858	P45974-1	His6	DU15641	*E. coli*	K63
USP6	529-1406	P35125-1	GST	DU37745	Insect cells	K63
USP7	1-1102	Q93009-1	His6	DU15644	*E. coli*	K63
USP8	1-1118	P40818-1	His6	DU15645	Insect cells	K48
USP9X	1553-1995	Q93008-3	GST	DU20628	*E. coli*	K11
USP9Y	1550-2000	O00507-1	GST	DU21846	*E. coli*	K11
USP10	1-798	Q14694-1	His6	DU15647	Insect cells	K63
USP11	1-963	P51784-1	GST	DU20016	*E. coli*	K48
USP12 / WDR48	1-370 / 1-732	O75317-1 / Q8TAF3-1	His6 / None	DU24373	Insect cells	K48
USP15	1-952	Q9Y4E8-2	GST	DU37753	Insect cells	K48
USP16	1-823	Q9Y5T5-1	His6	DU25349	*E. coli*	K63
USP19	1-1318	O94966-1	GST	DU37789	Insect cells	K63
USP20	1-914	Q9Y2K6-1	GST	DU15664	*E. coli*	K63
USP21	196-565	Q9UK80-1	GST	DU22385	*E. coli*	K48
USP25	1-1055	Q9UHP3-2	GST	DU21610	*E. coli*	K48
USP27X	1-438	A6NNY8-1	GST	DU21193	*E. coli*	K11
USP28	1-1077	Q96RU2-1	GST	DU20233	*E. coli*	K48
USP30	57-517	Q70CQ3-1	GST Clvd	DU36294	*E. coli*	K48
USP35	390-978	Q9P2H5-1	GST	DU67487	*E. coli*	K63
USP36	81-461	Q9P275-1	GST	DU4944	*E. coli*	K11
USP38	1-1042	Q8NB14-1	GST	DU35473	*E. coli*	K11
USP45	1-814	Q70EL2-1	GST	DU15681	*E. coli*	K48
USP46 / WDR48	1-366 / 1-732	P62068-1 / Q8TAF3-1	His6 / None	DU24347	Insect cells	K48
USP47	1-1355	Q96K76-4	His6	DU15682	Insect cells	K48
USP48	1-1053	Q86UV5-1	GST	DU27626	Insect cells	K11
CYLD	2-956	Q9NQC7-1	His6	DU1834	*E. coli*	K63
OTULIN	1-352	Q96BN8-1	GST Clvd	DU43487	*E. coli*	M1
OTU1	1-348	Q5VVQ6-1	GST	DU36559	*E. coli*	K11
OTUD1	270-481	Q5VV17-1	His6	DU25080	*E. coli*	K63
OTUD3	1-398	Q5T2D3-1	GST	DU21323	*E. coli*	K11
OTUD5 phospho Ser 177	1-571	Q96G74-1	GST Clvd	DU21450	*E. coli*	K63
OTUB1	1-271	Q96FW1-1	GST	DU19741	*E. coli*	K48
OTUB2	1-234	Q96DC9-1	GST	DU32795	*E. coli*	K63
VCPIP1	25-561	Q96JH7-1	GST	DU44386	*E. coli*	K48
vOTU	1 - 183	3ZNH_A*	GST	DU45351	*E. coli*	K63
TRABID	245-697	Q9UGI0-1	His	DU22468	*E. coli*	K63
A20	1-366	P21580-1	GST	DU32912	*E. coli*	K48
CEZANNE	1-843	Q6GQQ9-1	GST	DU20899	*E. coli*	K11
UCHL1	1-223	P09936-1	His	DU15693	*E. coli*	Ub-W
UCHL3	1-230	P15374-1	GST	DU21015	*E. coli*	Ub-W
UCHL5	1-329	Q9Y5K5-1	GST	DU12810	*E. coli*	Ub-W
BAP1	1-240	Q92560-1	GST	DU63658	*E. coli*	Ub-W
JOS2	1-188	Q8TAC2-1	His	DU20941	*E. coli*	K11
AMSH	256-424	O95630-1	GST	DU15719	*E. coli*	K63
AMSH-LP	1-436	Q96FJ0-1	GST	DU15780	*E. coli*	K63
MINDY1	1-469	Q8N5J2-1	MBP	DU59325	*E. coli*	K48 (4mer)
MINDY2	1-621	Q8NBR6-1	GST	DU63404	*E. coli*	K48
MINDY3	1-445	Q9H8M7-1	GST	DU47870	*E. coli*	K48

* vOTU – viral homologue of the ovarian tumour protease superfamily from the Crimean-Congo haemorrhagic fever virus. Substrate: ubiquitin dimer of the indicate linkage or alternative ubiquitin substrate. 4mer: tetramer.