Application Note - 003

Validation and Experimental Utility of the DUB^{scan}™ Kit (Cat. No. 67-0006-001)

BACKGROUND

The DUB^{scan} Kit has been flexibly designed for many potential applications. One application that might be of greatest interest is the identification of DUB enzymes which support the deubiquitylation of substrate proteins. The kit contains a DUB^{scan} plate (a panel of DUBs arrayed across 47 wells of a 96 well plate) plus a control ubiquitylation reaction mix for performing both positive and negative control deubiquitylation reactions. Through the addition of your own DUB substrate of interest to the wells of a DUB^{scan} plate you can screen the DUB enzyme panel in just one hour. Reaction products may be analysed as required, for example by SDS-PAGE and Western blotting using specific antibodies. One kit contains all the reagents and buffers necessary to perform a DUB^{scan} of the panel of 47 DUB assay wells.

Utility of the DUB^{scan} Kit

- Examine the cleavage of *in vitro* ubiquitylated substrates generated using different E2/E3 combinations (refer to the Ubiquigent E2^{scan} kit version 2 for identifying E2s that 'couple' with your E3 of interest).
- Examine the cleavage of *in vivo* ubiquitylated substrates after capture from a cell lysate (refer to Ubiquigent ubiquitin chain binding proteins for capturing ubiquitylated proteins).
- Investigate DUB ubiquitin-ubiquitin linkage cleavage specificity (refer to Ubiquigent di-ubiquitin and ubiquitin chains for use as substrates).
- Examine the relative position of cleavage within ubiquitin chains (distal *versus* proximal).
- Explore mono and poly-deubiquitylation DUB specificity (refer to specific Ubiquigent E2s – such as Ube2W – for mono-ubiquitylating activity).
- Explore potential DUB inhibitors and/or activators.
- Identify novel DUB binding proteins.
- Explore how DUBs may interact with and modify the activity of other ubiquitin system proteins such as E2s and *vice versa* (Wiener *et al.*, 2012).

Figure 1a

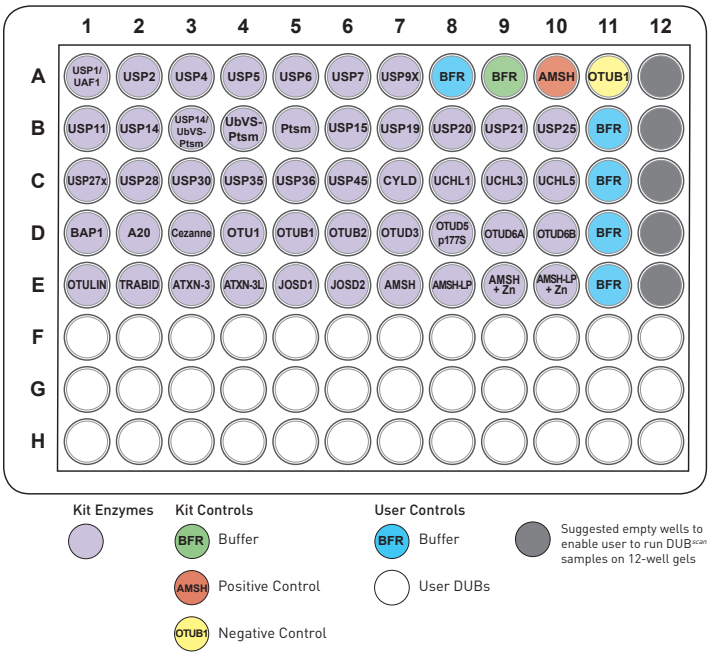


Figure 1a: DUB^{scan} Plate Configuration

Figure 1b

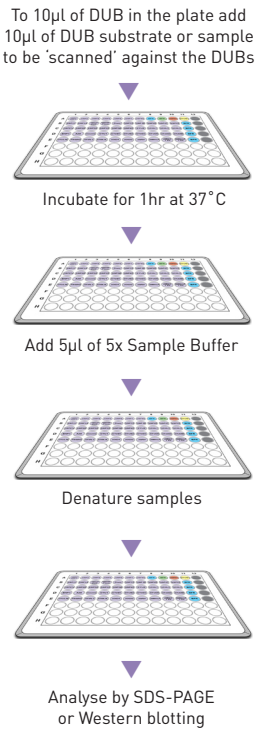


Figure 1b: DUB^{scan} Experimental Procedure: Summary of the DUB^{scan} protocol for analysing the DUB substrate (such as a ubiquitylated protein) or the sample to be 'DUB scanned' by SDS-PAGE/Western blotting. If you wish to analyse your reaction products by another method then simply proceed to the incubation step then analyse using your method of choice.

INSIDE:

Authors: Sian Armour and Jason Brown.
Ubiquigent Ltd.
Sir James Black Centre
Dow Street, Dundee DD1 5EH
Scotland, UK

Correspondence:
tech.support@ubiquigent.com
Version 2.1 6 May 2014
www.ubiquigent.com
©2014 Ubiquigent, Ltd.

DUB^{scan} Kit: Cleavage of K63 ubiquitin chain-linked *in vitro* auto-ubiquitylated CHIP

An example DUB^{scan} experiment conducted at Ubiquigent is presented in Figure 2. This dataset examines the cleavage of an auto-ubiquitylated substrate (E3 ligase; CHIP) generated using a specific E2/E3 combination (Ube2W/Ube2N/Ube2V1) known in the literature to generate both free and substrate-linked K63 chains. The DUB^{scan} reaction samples were analysed by SDS-PAGE and Western blotting and were probed with either an FK2 anti-mono and poly-ubiquitylated conjugates antibody (Figs. 2a) or an anti-CHIP antibody (Figs. 2b).

Figure 2a

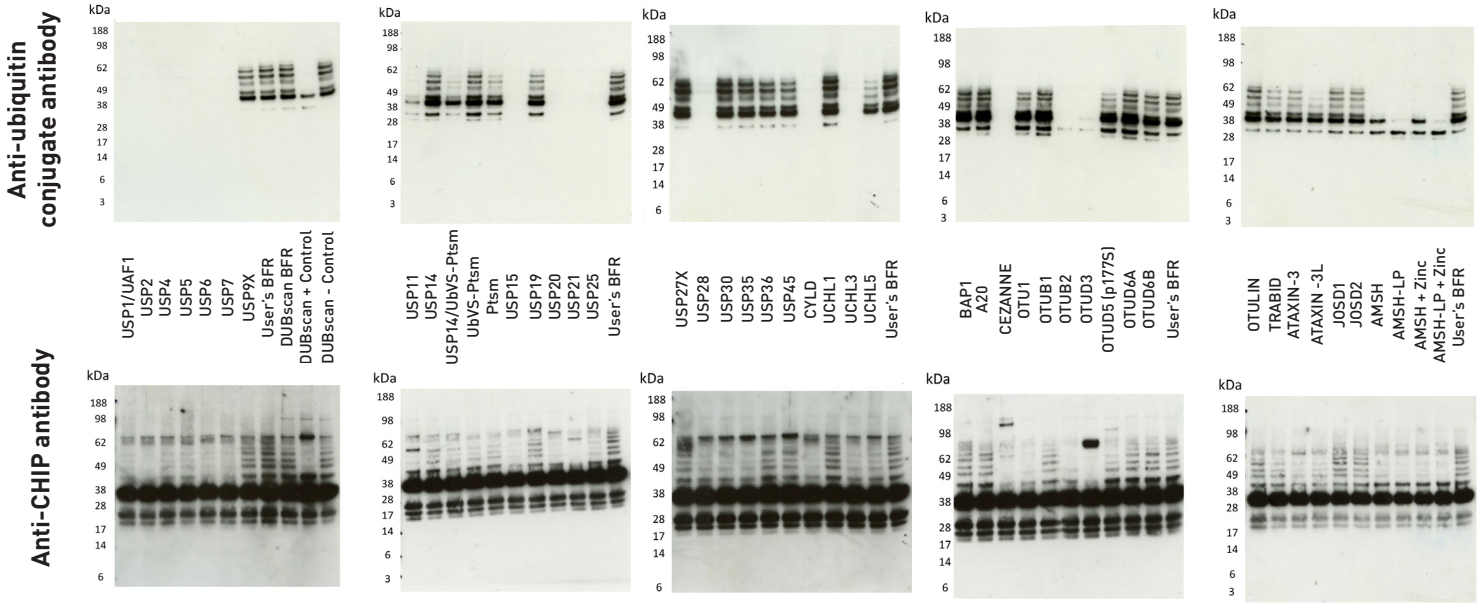


Figure 2b

Figures 2a) and 2b): DUB^{scan} **Results:** CHIP auto-ubiquitylation reactions contained; E1 activating enzyme Ube1, three E2 conjugating enzymes (Ube2N, Ube2V1, Ube2W), E3 ligase CHIP, ubiquitin and ATP and were incubated at 37˚C for 1 hour. This stopped reaction was then added across the DUB^{scan} plate, incubated for 1 hour at 37˚C and then analysed by Western blotting (after resolving the proteins by 4-20% SDS-PAGE under denaturing and reducing conditions). The DUB in the reaction is identified below and above each well. This data determines which DUBs do and don't cleave both free and substrate-linked K63 ubiquitin chains (Figs. 2a) and CHIP E3 ligase-linked K63 chains (Figs. 2b).

DISCUSSION

K63-linked auto-ubiquitylated CHIP DUB^{scan} control data

The data derived from the DUB^{scan} control wells is consistent with that reported in the literature in which the deubiquitylase AMSH (DUB^{scan} positive control) cleaves K63-linked ubiquitin chains (Sato *et al.*, 2008) and OTUB1 (DUB^{scan} negative control) is reported not to cleave K63 chains (Messick *et al.*, 2008).

K63-linked auto-ubiquitylated CHIP DUB^{scan} data

The K63-linked CHIP auto-ubiquitylation Control Reaction (Cat# 67-0010-030) which was 'scanned' across the whole DUB^{scan} plate contains a mixture of both free K63-ubiquitin chains and chains linked to the E3 ligase CHIP. Example data is shown probing with both the anti-mono and poly-ubiquitylated conjugates antibody (FK2) and an anti-CHIP antibody. The FK2 antibody data-set enabling one to determine which DUBs do and don't cleave K63 chains (assuming that the free and CHIP linked chains are exclusively K63 in nature as a result of using the appropriate E2 enzymes to generate K63-linked chains as reported in the literature (Windheim *et al.*, 2008)) through the loss of multiple – ubiquitin chain – bands visualised above the molecular weight for ubiquitin compared to the user's buffer controls (no DUB controls). The anti-CHIP antibody data enables one to determine which DUBs specifically cleave CHIP E3 ligase-linked K63 chains through the loss of multiple bands above the molecular weight of CHIP compared with the user's buffer controls (no DUB controls).

The example DUB^{scan} experimental data show that the majority of DUBs that cleave K63-linked ubiquitin chains can cleave both free and E3 ligase (substrate) linked chains.

DUB^{scan} Kit: Cleavage of cell-derived ubiquitylated IRAK1

An example DUB^{scan} experiment conducted at Ubiquigent is presented in Figure 3. This dataset examines the cleavage of a ubiquitylated substrate (Interleukin-1 receptor-associated kinase 1; IRAK1) captured from a cell lysate. The DUB^{scan} reaction samples were analysed by SDS-PAGE and Western blotting and were probed with the FK2 anti-mono and poly-ubiquitylated conjugates antibody provided with the kit.

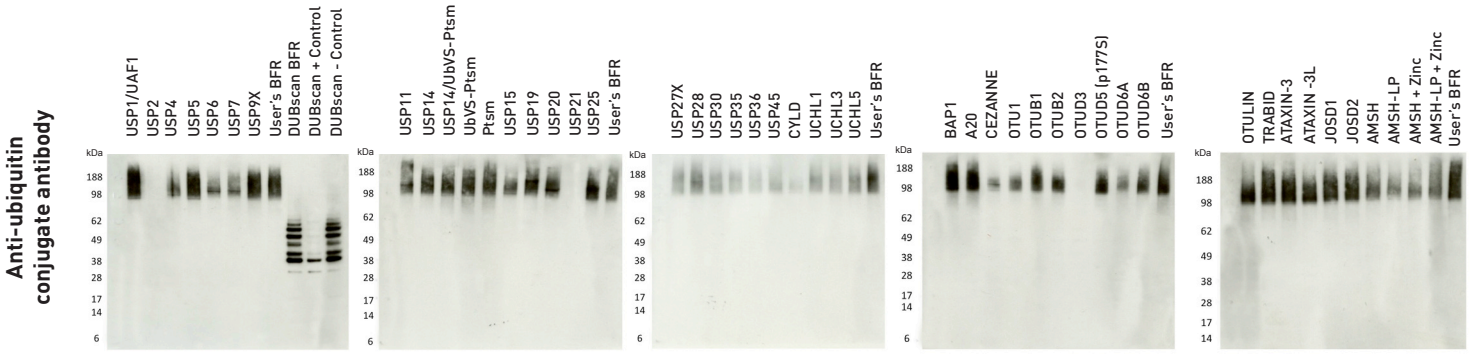


Figure 3: **DUB^{scan} Results:** Cell lysate derived from HEK293 Interleukin-1 (IL-1) stimulated cells was incubated for 45 min at 4˚C with a substrate specific antibody (anti-IRAK1). Protein G-Dynabeads were added and the sample incubated for a further 2 hr at 4˚C with continuous stirring. Dynabead complex was then washed thoroughly and added to the DUB^{scan} plate which was incubated on a plate shaker for 1 hr at 37˚C. Samples were then analysed by Western blotting (after resolving the proteins by 4-20% SDS-PAGE under denaturing and reducing conditions). The DUB in the reaction is identified above each well. This data determines which DUBs do and don't cleave IRAK1-substrate-linked ubiquitin chains.

DISCUSSION

Cellular ubiquitylated IRAK1 DUB^{scan} data

The ubiquitylated IRAK1 sample captured from a cell lysate was 'scanned' across the DUB^{scan} plate. Example data is shown probing with the anti-mono and poly-ubiquitylated conjugates antibody (FK2). This data-set enables one to determine which DUBs do and don't cleave ubiquitin chains on a cell-derived ubiquitylated substrate through the loss of multiple – ubiquitin chain – bands visualised above the molecular weight for the IRAK1 substrate compared to the user's buffer controls (no DUB controls; 'User's BFR').

The example DUB^{scan} experimental data shows that a number of DUBs can remove some percentage of the total IRAK1 -linked ubiquitin chains. Emmerich *et al.*, (2013) recently reported that the ubiquitin chains formed in response to IL-1 stimulation are linear ubiquitin chains covalently attached to K63 ubiquitin chains either directly as K63/linear hybrids or indirectly by attachment to IRAK1, perhaps indicating a reason why most DUBs cannot fully cleave the chains due to their chain cleavage specificity.

DUB^{scan} Kit Deconjugating Enzyme Activity Validation

The activity of each deconjugating enzyme in the DUB^{scan} kit was validated through their ability to 'turn over' either the fluorogenic substrate ubiquitin-rhodamine110-glycine or a di-ubiquitin substrate.

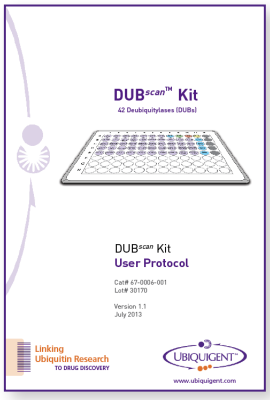
DUB	Ubiquitin-Rhodamine110-glycine turnover initial rate (RFU/min)	Di-ubiquitin
UCHL3	24191	-
USP6 CD(529-1406)	23244	-
USP7	20488	-
UCHL5	20395	-
UCHL1	20262	-
BAP1	20096	-
USP20	19891	-
USP4	19045	-
OTUD6A	18805	-
CEZANNE	18253	-
USP15	17430	-
USP2	17385	-
26S Proteasome	16359	-
USP21 CD(196-565)	14898	-
USP28	12048	-
OTUD3	11492	-
USP1/UAF1	6349	-
CYLD	5598	-
USP5	4940	-
USP25	4203	-
USP11	3363	-
ATAXIN-3L	2103	-
AMSH-LP CD(264-436) +Zinc	1918	-
OTUB2	1787	-
USP30	1573	-
JOSD2	1420	-
USP35	1098	-
OTUD5 (p177S)	976	-
OTUD6B	934	-
USP14/26S Proteasome [Ub-VS]	908	-
ATAXIN-3	818	-
USP36 CD(81-461)	788	-
OTU1	743	-
USP45	691	-
JOSD1	690	-
USP27X	536	-
USP9X CD(1554-1995)	487	-
USP19 -TM(1-1290)	454	-
AMSH-LP CD(264-436)	358	-
USP14	-	-
26S Proteasome [Ub-VS]	-	-
OTULIN	-	Di-ubiquitin (linear)[1]
OTUB1	-	Di-ubiquitin (K48-linked)[1]
A20	-	Di-ubiquitin (K48-linked)[1]
TRABID	-	Di-ubiquitin (K63-linked)[1]
AMSH CD(252-424)	-	Di-ubiquitin (K63-linked)[1]
AMSH CD(252-424) + Zinc	-	Di-ubiquitin (K63-linked)[1]

[1] Where a di-ubiquitin is referenced no detectable turnover of Ubiquitin-Rhodamine110-glycine was determined however the DUB enzyme did cleave the di-ubiquitin noted in the table

Table 1: DUB^{scan} Kit Enzyme Quality Control data

REFERENCES

Emmerich CH *et al.* (2013) *PNAS* **110**, 15247-15252.
Messick TE *et al.* (2008) *J Biol Chem* **283**, 11038-11049.
Sato Y *et al.* (2008) *Nature* **455**, 358-362.
Wiener R *et al.* (2012) *Nature* **483**, 618-22.
Windheim M *et al.* (2008) *Biochem J* **409**, 723-729.



ADDITIONAL RESOURCES

For complete information on the DUB^{scan} Kit including kit contents, detailed user protocols, example data and data interpretation, call **+44-(0)1382-381147** and request a copy of the 24-page

DUB^{scan} Kit User Protocol Manual.

Technical Support: +44-(0)1382-381147