UBIQUIGENT™

Validation and Experimental Utility of the E2^{scan}™ Kit version 2

BACKGROUND

The E2^{scan} Kit version 2 is designed to facilitate the identification of E2 conjugating enzymes which support the ubiquitylation of substrate proteins catalysed by an E3 ligase and/or the auto-ubiquitylation of the E3 ligase itself. The kit contains an E2^{scan} plate (a panel of 34 E2 conjugating enzymes arrayed in duplicate across a 96 well plate) plus all the components (E1 enzyme, control E3 ligase, ubiquitin, ATP and assay buffer) required to perform a complete substrate ubiquitylation or auto-ubiquitylation assay using your E3 ligase and/or E3 ligase/substrate combination of choice. Through the addition of a substrate mix (E1, E3, substrate, and ubiquitin) and then ATP to the wells of an E2^{scan} plate you can screen the E2 conjugating enzyme panel in just one hour. Reaction products may be analysed as required, for example by SDS-PAGE or Western blotting using specific antibodies. One kit contains all the reagents and buffers necessary to perform two 'E2^{scan}s' of the panel of 34 E2 conjugating enzymes. You need only provide your E3 ligase to be evaluated and substrate of interest (if required). A control E3 ligase (C-terminus of Hsc70 Interacting Protein; CHIP) and various control wells are also provided in order to carry out control auto-ubiquitylation assays or any other controls that you may wish to perform.

Why use the E2^{scan} Kit version 2?

- E2 conjugating enzymes can be critical in determining the ubiquitin chain linkage type(s) generated in a ubiquitylation reaction (Ye and Rape 2009).
- The linkage type can determine the fate of the ubiquitylated substrate; for example to be degraded or to function in a signalling capacity (Ye and Rape 2009).

E2^{scan} experiments facilitate:

- The rapid identification of the E2s that couple with your E3 ligase (auto and/or substrate ubiquitylation modes).
- Investigation of ubiquitin 'priming' and 'chain extension' on substrate proteins.
- The identification and investigation of novel E2 activities such as substrate N-terminal ubiquitylation (Tatham *et al.* 2013).
- The development of additional assay formats for the E3 ligase.
- Exploration of how E2s may interact with and modify the activity of deubiquitylase enzymes (DUBs) and *vice versa* (DiBello *et al.*, 2013; Wiener *et al.*, 2013).
- The identification of E2 enzymes where the formation of E2 thioester intermediates is prevented by small molecule inhibition (Strickson *et al.* 2013).

Figure 1a

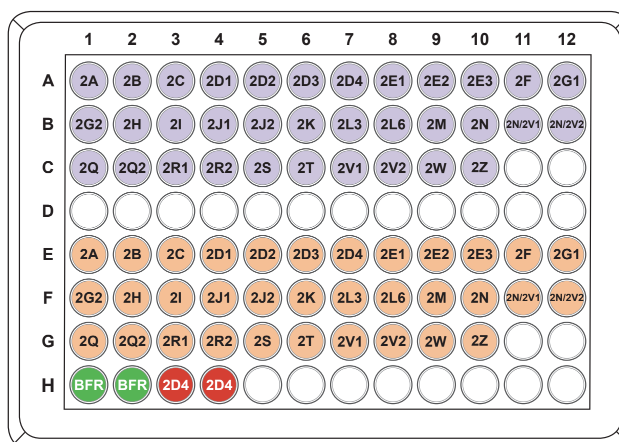

Figure 1b

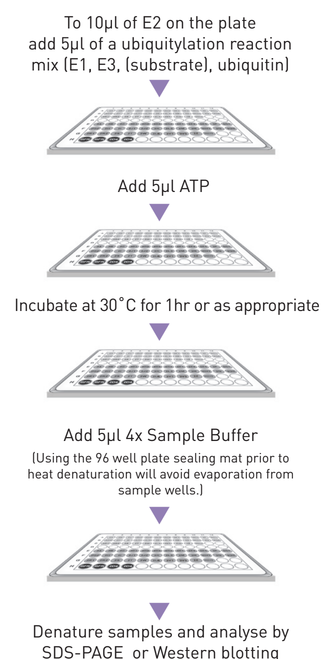

Figure 1a: E2^{scan} **Plate Configuration:** 2 replicates of the E2^{scan} panel (0.05nmoles E2/well in 10µl); buffer controls; positive controls; empty and available for testing user E2s; e.g. different species, mutants etc.

Figure 1b: E2^{scan} **Experimental Procedure:** Summary of the E2^{scan} protocol for analysing ubiquitylation reaction products 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:

Results from Typical E2^{scan} experiments

E2^{scan}: NEDD4.2 Catalyses hDCNL1 Ubiquitylation

CHIP Auto-Ubiquitylation: Ube2W Priming and Ube2N/Ube2V1 Extension

E2^{scan} Kit version 2 E2 Conjugating Enzyme Activity Validation

Ubiquitin thioester loading assay

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

Correspondence:
tech.support@ubiquigent.com

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Application Note - 002

E2^{scan} Kit version 2: NEDD4.2 Catalyses DCNL1 Ubiquitylation

Example E2^{scan} and priming/extension experiments conducted at Ubiquigent are presented in Figures 2 and 3 respectively. Data is shown using the E2^{scan} Kit version 2 run in the presence (Figs. 2a) and 3a)) and absence (Figs. 2b) and 3b)) of an E3 ligase NEDD4.2 (Neural precursor cell Expressed Developmentally Down-regulated protein 4.2) - a Homologous to the E6-AP Carboxyl Terminus (HECT) domain E3 ligase - in substrate ubiquitylation mode (substrate was hDCNL1; human Defective in Cullin Neddylation 1-Like 1). A 'no E3 ligase control' experiment (with hDCNL1 substrate present) was also run to serve as a control to enable one to determine which products in the E3-containing reactions were generated in an E3-dependent manner (compare Figs. 2a) to 2b) and Figs. 3a) to 3b)). The NEDD4.2 catalysed and no E3 ligase hDCNL1 ubiquitylation reactions were split into two equal aliquots for analysis using anti-hDCNL1 (Figs. 2a) and 2b)) and anti-ubiquitin conjugate antibodies (Figs. 3a) and 3b)).

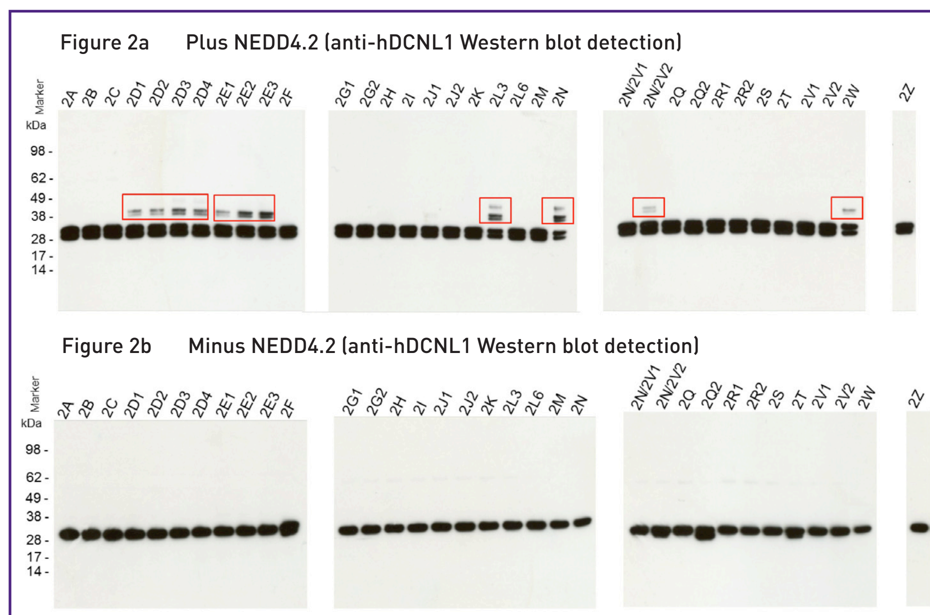


Figure 2a) and b) E2^{scan} Results. hDCNL1 substrate ubiquitylation reactions contained; ATP, ubiquitin, Ube1 (6His-tagged) (E1), E2 (on the E2^{scan} plate), NEDD4.2 (E3; where indicated) and hDCNL1 (substrate). Ubiquitylation reactions were run in the supplied E2^{scan} plate and analysed by Western blotting (after resolving the proteins by 4-20% SDS-PAGE under denaturing and reducing conditions) with an anti-hDCNL1 antibody. The E2 in the reaction is identified above each well (eg 2A=Ube2A). The antibody detects the hDCNL1 substrate in all reactions and ubiquitylated hDCNL1 only with certain E2s which couple with the E3 NEDD4.2 (see red boxes).

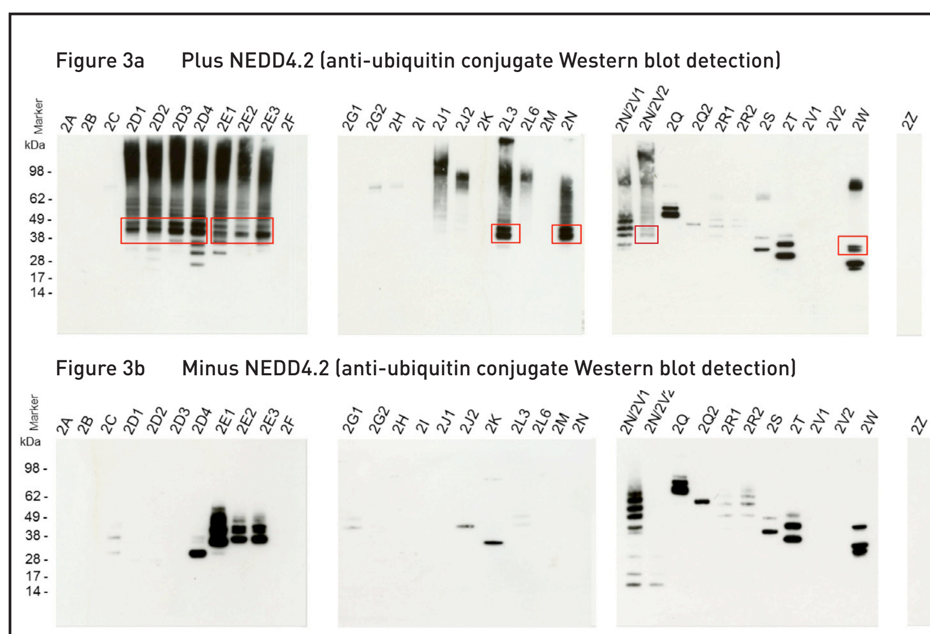


Figure 3a) and b): E2^{scan} Results. hDCNL1 substrate ubiquitylation reactions contained; ATP, ubiquitin, Ube1 (6His-tagged) (E1), E2 (on the E2^{scan} plate), NEDD4.2 (E3; where indicated) and hDCNL1 (substrate). Ubiquitylation reactions were run in the supplied E2^{scan} plate and analysed by Western blotting (after resolving the proteins by 4-20% SDS-PAGE under denaturing and reducing conditions) with an anti-ubiquitin conjugate antibody. The E2 in the reaction is identified above each well (eg 2A=Ube2A). The antibody detects mono- and poly-ubiquitin conjugates in reactions with only certain E2s which couple with the E3 NEDD4.2 (plus NEDD4.2), these should be distinguished from those E2s that become auto-ubiquitylated or generate free poly-ubiquitin chains in the absence of the E3 NEDD4.2 (minus NEDD4.2).

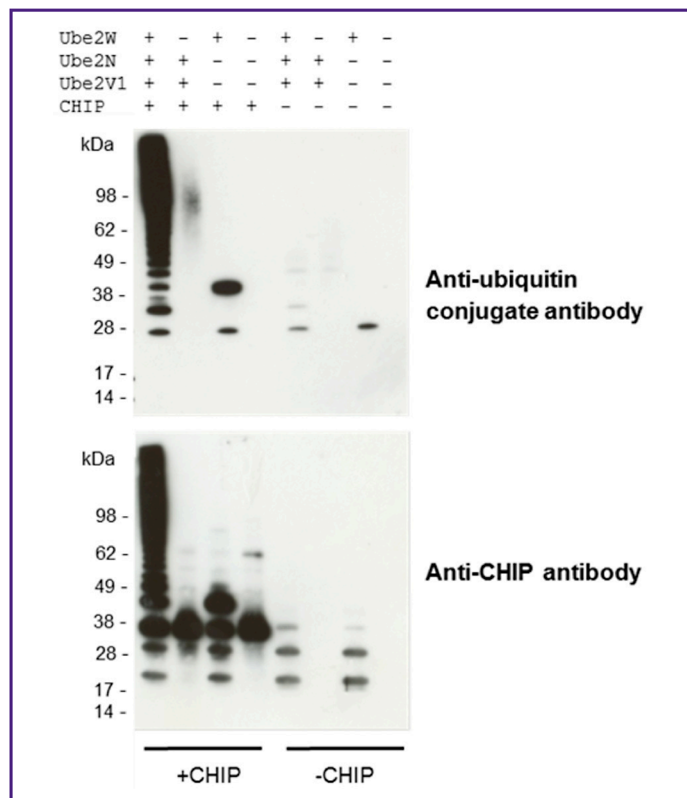
DISCUSSION

Of the 34 E2 conjugating enzymes tested in the NEDD4.2 catalysed hDCNL1 substrate ubiquitylation assay, Ube2D1, Ube2D2, Ube2D3, Ube2D4, Ube2E1, Ube2E2, Ube2E3, Ube2L3 and Ube2N were all identified as E2s that couple to NEDD4.2 to support the ubiquitylation of the hDCNL1 substrate. An hDCNL1 substrate specific antibody (Fig. 2a) and b)) and an anti-ubiquitin conjugate antibody (Fig. 3a) and b)) were used to detect the reaction products. From the data it seems likely that a NEDD4.2 catalysed mono-ubiquitylation event has occured on the hDCNL1 substrate (Fig. 2a). It is possible also that polyubiquitylation has occured but that the hDCNL1 antibody cannot detect this event as the epitope to which the antibody would bind is obscured by ubiquitin conjugates and/or the additional ubiquitylation reaction products are not sufficiently abundant to be detected. This data extends on the number of E2 conjugating enzymes identified in the literature that couple to NEDD4.2, as reported by Fotia *et al.* (2006). In this paper Ube2D1, Ube2D2 and Ube2L3 were shown to support extensive ubiquitylation of a substrate Nedd4-binding protein 1 (N4BP1) while Ube2E1 and Ube2E3 supported less extensive ubiquitylation, this was based on the intensity of the bands observed in Figure 5 of Fotia *et al.* (2006).

Data derived from the NEDD4.2 E2^{scan} experiment (Fig. 2 and 3) is consistent with that reported in the literature in which several E2 conjugating enzymes (Ube2D1, Ube2D2, Ube2E3 and Ube2L3) were found to support the NEDD4.2 catalysed *in vitro* ubiquitylation of a substrate N4BP1 (Fotia *et al.* 2006).

E2^{scan} Kit version 2: CHIP Auto-Ubiquitylation; Ube2W Priming and Ube2N/Ube2V1Extension

An E2 coupled to an E3 ligase may regulate the type of ubiquitylation event that occurs on a substrate; this can be a mono-ubiquitylation (which may 'prime' a site for poly-ubiquitylation) or poly-ubiquitylation - either directly on the substrate or by chain extension of a 'primed' site. Certain E2s may initiate priming of the substrate with ubiquitin and then a second group of E2s can promote ubiquitin chain extension (Christensen *et al.* 2007; Soss *et al.* 2011; Ye and Rape 2009; Tatham *et al.* 2013). Priming and extension reactions were run using the E3 ligase CHIP and E2s identified from a previous E2^{scan} experiment (data not shown). The E2 priming and extension reactions were conducted in the presence or absence of CHIP.



DISCUSSION

To demonstrate the E2 dependent priming and extension events, controls were also run in the presence or absence of the priming (Ube2W) and extension (Ube2N/Ube2V1) E2 conjugating enzymes. From the Western blot data (see Figure 4) it can be see that auto-polyubiquitylation of the E3 ligase CHIP by Ube2N/Ube2V1 requires the presence of Ube2W which mono-ubiquitylates – 'primes' – the CHIP substrate thus enabling chain extension.

Figure 4: **Ube2W priming and chain extension results.** Reactions contained; ATP, ubiquitin, Ube1 [6His-tagged] (E1), E2 (as defined), CHIP (E3; as defined). Ubiquitylation reactions were analysed by Western blotting probed with the antibodies indicated. Auto-polyubiquitylation of the E3 ligase CHIP by Ube2N/Ube2V1 requires the presence of Ube2W which mono-ubiquitylates – 'primes' – the CHIP substrate thus enabling chain extension with Ube2N/Ube2V1.

E2^{scan} Kit version 2 E2 Conjugating Enzyme Activity Validation

The activity of each ubiquitin E2 conjugating enzyme in the E2^{scan} kit version 2 was validated by loading Ube1 [6His-tagged] activated ubiquitin onto the active cysteine of the E2 conjugating enzymes via a transthiolation reaction. The sensitivity of this bond to the reducing agent DTT was determined. Example data is provided for four E2s (see Fig. 5 compare A and B).

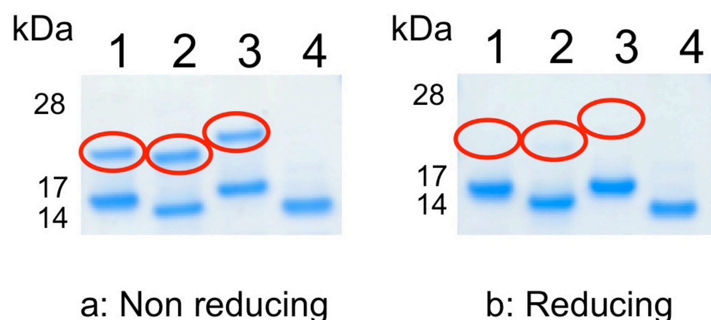


Figure 5: **E2 Ubiquitin thioester loading assay.** The Ube1 [6His-tagged] and the E2 conjugating enzyme were incubated in the presence of ubiquitin and ATP at 30˚C for 20 minutes and then the reactions were analysed by SDS-PAGE under reducing or non-reducing conditions. Lane 1 Ube2G1 (Cat#62-0028), Lane 2 Ube2G2 (Cat#62-0030), Lane 3 Ube2H (Cat#62-0032) and Lane 4 Ube2I (Cat#62-0034). a) Non-reducing SDS-PAGE: E2 conjugating enzymes 'loaded' with ubiquitin via a thioester bond are outlined with a red circle; b) Reducing SDS-PAGE: Sensitivity of the ubiquitin~E2 thioester bond to the reducing agent DTT was confirmed by analysing the same samples as in Fig. 5a) under reducing conditions. Note the absence of the ubiquitin loaded E2 enzyme (see red circles) in Fig. 5b). Note that as expected the SUMO loading E2 Ube2I (lane 4) shows no ubiquitin thioester loading.

ADDITIONAL RESOURCES

For complete information on the E2^{scan} Kit version 2, including kit contents, detailed user protocols, example data and data interpretation, call

+44-(0)1382-381147

and request a copy of the 32-page

E2^{scan} Kit version 2
User Protocol Manual.

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

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