Ubiquigent

WHITE PAPER

DUB*profiler*–Cell

Overview

The DUB*profiler*-Cell™ platform supports the development of novel deubiquitinase (DUB) enzyme inhibitors by revealing the 'active DUBome' in a given cell type, and by reporting the target engagement of test compounds to that 'active DUBome'.

The DUB*profiler*-Cell assay utilises activity-based probes (ABPs) to capture active DUBs (i.e., DUBs with reactive catalytic site cysteine residues) within a cell type of interest. ABPs are tools that can be used to monitor the activity of specific classes of enzymes. ABPs have three components: a reactive electrophile for covalent modification of the enzyme active site, a linker, or a specificity group for directing probes to specific enzymes, and a reporter for visualizing or enriching for enzymes bound to the probe.

For DUBs, many different probe architectures have been reported with different selectivity toward individual DUBs. Probes using a molecule of Ubiquitin (Ub) as the specificity motif have been the more popular ones[1,2,3,4], with a Cysteine-reactive electrophilic group such as a vinyl sulfone (VS), vinyl methyl ester (VME) or propargylamide (PA) replacing the C-terminal glycine residue. However, di-Ubiquitin ABPs mimicking the different poly-Ub linkages recognised by DUBs have also been generated and studied[5], and Ward et al[6] have reported on the synthesis of a cell-permeable ABP that is reactive with a number of DUBs. Since their introduction, DUB ABPs have become a valuable tool in drug discovery through the identification of new DUB families, characterisation of DUB inhibitor selectivity and investigating changes in DUB activity in response to exogenous stimuli.

By pre-incubating lysates or live cells with test compounds, the assay can be used to evaluate target engagement by compounds, including those with either covalent or non-covalent mechanisms of action. Employing both Western blotting and multiplexed tandem mass spectrometry (MS) the assay can be configured to evaluate compounds at either single or multiple concentrations (reporting relative EC_{50} values) and examining one, several or all active DUBs in the sample. The DUB*profiler*-Cell platform has been extensively optimised and validated for detecting target engagement to any active DUB. Proof of concept data is currently available in respect of compounds selective for two example DUBs; USP7 and USP30 (important

targets in oncology and neurodegeneration, respectively).

Main applications of DUB*profiler*–Cell

DUB target identification and validation

The DUB*profiler*-Cell platform can be employed as part of target identification / validation approaches to reveal which DUBs are expressed – and active – in any given cell type and under any given circumstance. Customers can supply diseased versus healthy cells, an isogenic cell line pair, or cells treated under different conditions. Using Western blotting and MS, the relative abundance of individual DUBs of interest or the entire active DUBome can be reported in samples to reveal novel disease-relevant DUB targets.

Determination of compound-DUB in-cell target engagement

With the small molecule chemical tractability of DUBs demonstrated by a number of groups through the development of high affinity and selective inhibitors (supported by the DUB*profiler*™ service) it is now becoming increasingly important to determine the in-cell DUB target engagement profile of these molecules as drug discovery programmes progress towards the clinic. DUB*profiler*-Cell provides for the "single point" (single concentration) screening of many compounds, e.g., to establish a rank order of their potency, or analysis of a full compound dilution series to establish relative EC_{50} values against selected DUBs or the entire MS-detectable active DUBome of any cell type.

Key benefits

- **Physiologically relevant** - use unmodified, disease-relevant cells; assess compound potency and specificity against endogenous DUBs.
- **Informative** - ability to address one or two DUBs or the entire active DUBome: unbiased analysis by multiplexed MS.
- **Flexible** - either combine multiple compounds in one analysis to rank according to potency or determine EC50 values for individual compounds against all endogenous DUBs to address potency and selectivity.
- **Efficient** - generate EC50 curves simultaneously across multiple DUBs with different orders of magnitude of abundance due to the large dynamic range of the MS platform employed.
- **Robust** - probe binding conditions optimised for each compound and validated prior to preparation of MS samples; aliquot of MS samples verified by Western blotting prior to submission to ensure validity of data.

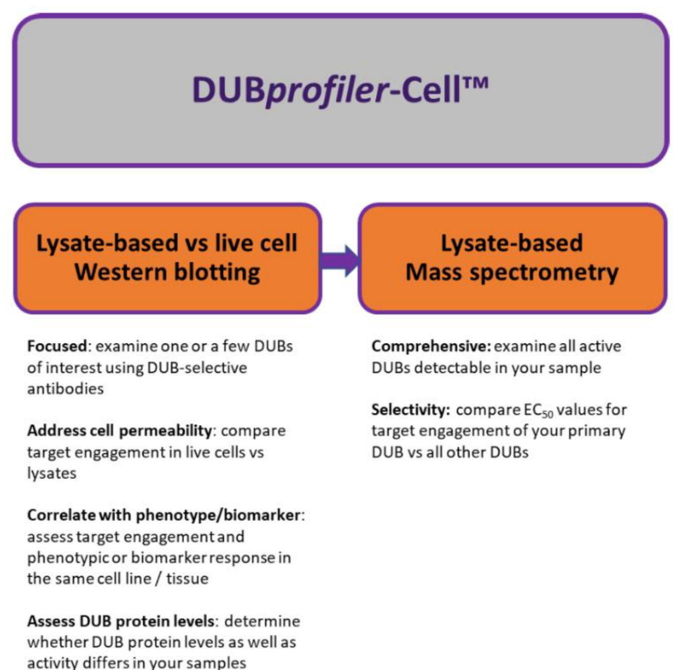

Figure 1: DUBprofiler-Cell project modules

DUB*profiler*–Cell options

DUB*profiler*-Cell is a highly flexible platform that is available in two main formats: lysate-based and live cell; target engagement (TE) can be analysed by Western blotting or mass spectrometry (MS). The parallel analysis of probe binding in lysates incubated in vitro with compounds, vs live cells treated with compounds, has many advantages (detailed below in Figure 1), including the investigation of cellular permeability of compounds, and measurement of associated biomarkers where TE has been demonstrated. Typically, conditions are finalised in the lysate-based assay and live cell assays using Western blotting focussing on one or a few DUBs of interest, before progressing to MS analysis for a more comprehensive view of the active DUBome.

A typical DUB*profiler*-Cell project is described. Following the selection of the appropriate cell line, the DUB target(s) and compounds of interest, the conditions for binding of the activity probe to the DUB target will be optimised in lysates for future experiments. Optimal conditions are determined by Western blotting using antibody(ies) to the DUB(s) of interest. If customers do not have a validated antibody to the DUB target of interest, Ubiquigent™ may undertake evaluation of antibodies for each DUB of interest on the customer's behalf.

After selecting the most appropriate probe binding conditions to ensure (i) sub-maximal binding of the DUB target of interest and (ii) successful prevention or inhibition of binding by one or more compounds, a small dilution series of each compound of interest is examined using the above conditions. Typically, this will be a three-point, 10-fold dilution series, but the customer may choose the top concentration and an alternative dilution series if required. From the analysis of the ratio of probe-bound DUB to "free" DUB, compound-target engagement EC_{50} values can be reported for any compound against any DUB that is examined. Importantly at this stage, the lysate-based and live cell analyses are conducted in parallel, which yields important information about the cell permeability and cellular efficacy of the compounds. In addition, DUB protein levels are analysed to establish whether the compound treatment(s) affect the abundance of the DUB as well as binding (and modulation of DUB activity).

Customers may then continue to a full analysis of all active DUBs in their cell line or tissue of choice by exploiting the benefits of MS. A more detailed description of the experimental workflow for the MS module is described in the next section.

As outlined, this is the typical DUBprofiler-Cell workflow; however, customers are encouraged to discuss their individual project objectives and the workflow can be adapted accordingly. For example, as mentioned previously, DUB*profiler*-Cell can be applied to examine the differences in the activity of DUBs in diseased versus healthy cells as part of a target identification or validation approach. Similarly, customers may be interested in comparing different cell lines or substituting compound treatments with different perturbations with the aim of examining their impact on DUB activity.

Mass Spectrometry: Enabling an unbiased readout of compound-target engagement

The ability to identify the spectrum of proteins interacting with a small molecule at an early stage in the drug discovery process can support decision-making as compounds progress. However, this type of data may also drive the repositioning of existing drugs by the identification of additional targets. For selectivity assessment the compound of interest is typically used as a competitor over a range of concentrations in a lysate of a disease-relevant cell line. The affinity of the compound to all members of the target class is determined by quantifying the (reduced) abundance of proteins

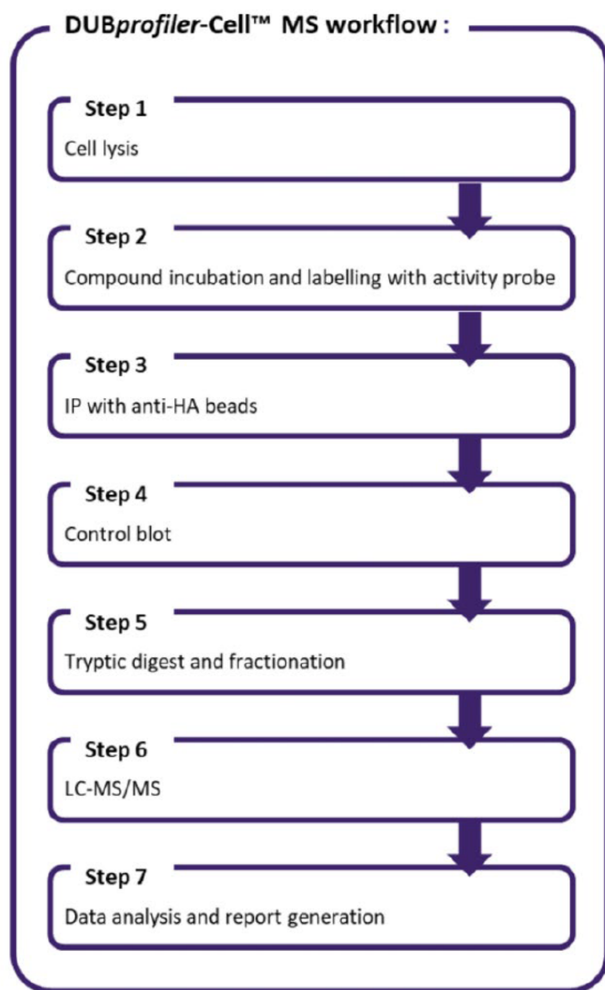

Figure 2: DUBprofiler-Cell mass spectrometry workflow

captured by the ABP. More specifically, inhibition curves of ABP binding are obtained, from which apparent compound-target engagement EC_{50} values can be calculated. This is a very powerful approach because proteins are assayed under physiological conditions (e.g., use of relevant cell line; proteins at endogenous expression levels and in their native structural form and modification status). In addition, the multiplexing capability of MS for protein identification and quantification provides ranked affinities of a compound engagement against all members of the MS detectable target class in one experiment.

- Test compounds are incubated with cell lysate to enable engagement with their DUB target(s), then the HA-tagged ABP is added to engage the active DUBome (sub-proteome) in a covalent manner (Step 2).
- The DUBome is then enriched via immunoprecipitation (IP) using an anti-HA antibody (Step 3).
- ABP-labelled samples are probed with antibodies by Western blotting to visualize the effect of the compounds on ABP target(s) engagement and confirm the quality of the samples, prior to processing for MS (Step 4).
- The enriched, immunoprecipitated samples (in the presence or absence of compound treatment) are enzymatically digested for MS analysis (Step 5).
- Offline fractionation of samples prior to LC-MS/MS analysis is performed to reduce sample complexity and improve the detection of lower abundance targets (Step 5, 6).
- Prevention of ABP engagement to a specific DUB(s) due to test compound prebinding to the DUB(s) is reported as a ratio relative to the no compound control samples for each protein identified by MS (Step 7).
- Where multiple concentrations of a single compound are used, a target engagement EC_{50} may be provided for each DUB that is detected, thus reporting compound selectivity.

How do we define the detectable DUBome?

As part of the validation of the MS workflow in DUB*profiler*-Cell, it was important to establish whether it was possible to measure prevention of probe binding to any DUB that was identified by MS, regardless of its abundance.

In order to do this, lysates were incubated with increasing concentrations of the untagged version of the activity probe, to mimic a pan DUB

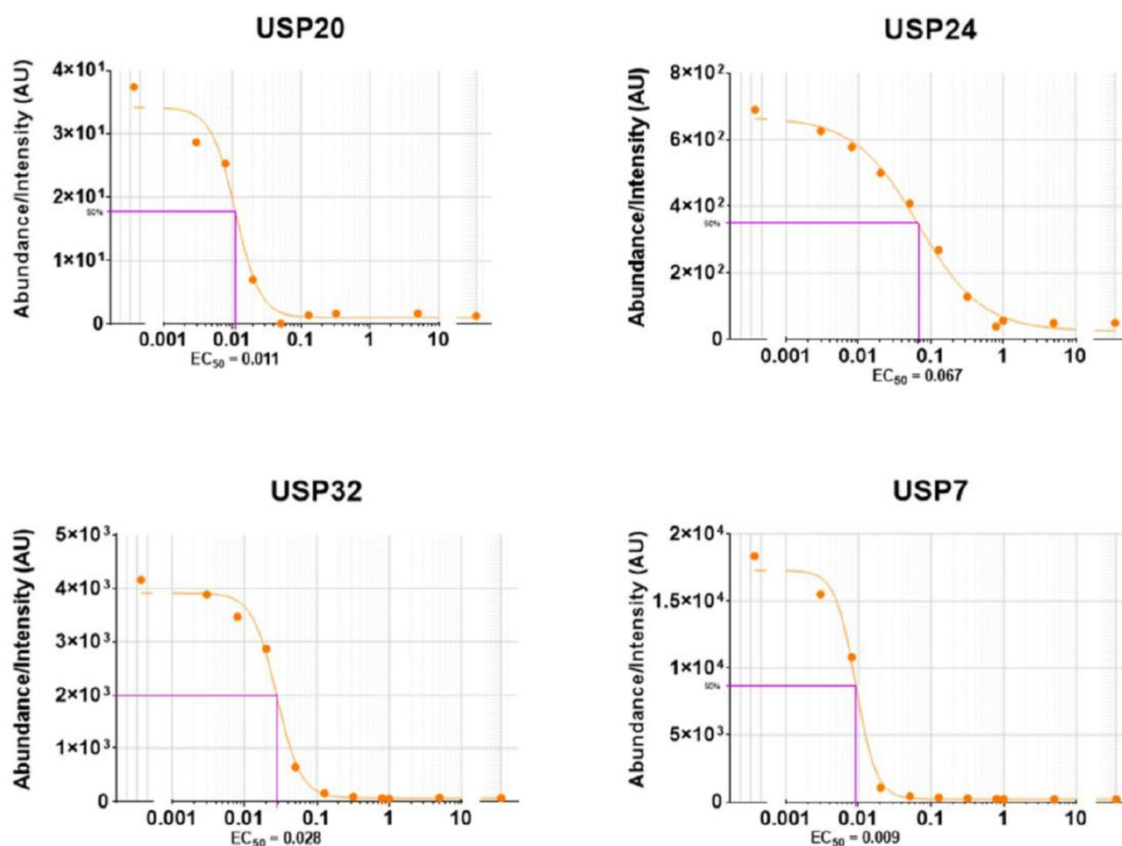

Figure 3: DUBprofiler-Cell large dynamic range.

Prevention of probe binding by compound can be monitored, irrespective of protein abundance. Lysates were pre-incubated with increasing concentrations of untagged ABP lacking the reporter tag (to simulate a pan-DUB inhibitor compound) and this prevented binding of tagged ABP in a dose-responsive manner. EC50 values can be calculated from proteins with significant differences in abundance (almost 3 orders of magnitude; intensities ranging from ≈ 40 to 20,000).

inhibitor. Lysates were analysed according to the workflow in Figure 2 and the data generated is presented in Figure 3.

The data indicates that it is possible to measure the impact of compounds on probe-binding to DUBs, irrespective of their abundance, so long as the DUB is detectable by MS via the workflow described in Figure 2. In the light of this validation, the EC50 values for compound-DUB engagement are reported for any DUB that is detected in at least two positive control samples (plus ABP) in the MS experiment.

References

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Contact us

We look forward to discussing your DUB*profiler*–Cell project with you. Please email: services@ubiquigent.com

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