

WHITE PAPER

REDOXprofiler

DUBs and wider cysteine protease screening hit triage assays for identifying false positive REDOX cycling compounds

Summary

The REDOXprofiler™ service comprises two complementary assays for identifying REDOX Cycling Compounds (RCCs). It is critically important to identify RCCs as they may generate false positive 'hits' in screens against enzymes that utilise a catalytic cysteine such as deubiquitinases (DUBs) and other cysteine proteases.

Key features of Ubiquigent's REDOXprofiler service

- Critical triage assays – it is important to understand whether the activity of your compound against a target enzyme may be due (at least in part) to RCC properties.
- Cross confirmatory methods – two complementary RCC detection assays; REDOXprofiler-Resazurin and REDOXprofiler-Cat/SOD.
- Context specific – ability to determine RCC effects in the context of specific DUBs.
- Enzyme target agnostic – the REDOXprofiler-Resazurin assay is useful for the detection of RCCs independent of target class (DUB, cathepsin, caspase, other Cys protease etc). REDOXprofiler-Cat/SOD is designed for the analysis of compound activity towards DUBs but may be adapted to address non-DUB targets.
- Full curve analysis – REDOXprofiler-Resazurin: one EC50 curve reported, REDOXprofiler-Cat/SOD: two DUB IC50 curves reported (plus and minus Cat/SOD).
- Flexible – screen any combination of compound(s) in either REDOX assay vs. any DUBs (in the context of REDOXprofiler-Cat/SOD).
- Scalable – any scale and complexity of screening available.
- Efficient – simple service procedure using one electronic submission form (similar to that used in our DUBprofiler™ service and supplied on request) to order either or both assays.
- Fast – rapid screening cycles.

The validation of the mechanism of action of hits from screens and knowing which to progress and which to discard is critical to the prosecution of a

successful enzyme targeted drug discovery programme. Many factors can result in test compounds generating 'false positive' data including target aggregation, interference with the assay readout and nonspecific target modifications resulting from compound catalysed reactions in the assay. In respect of the latter category, of critical importance in DUB – and other cysteine protease – drug discovery is the identification of RCCs which may generate Reactive Oxygen Species (ROS) such as hydrogen peroxide (H_2O_2) or superoxide (O_2^-), particularly in the presence of reducing agents including dithiothreitol (DTT). In screening campaigns, such compounds can cause the false positive inhibition of enzymes that utilise a catalytic cysteine. Such enzymes include five of the six sub-families of DUBs as well as other cysteine proteases including cathepsins (Mirkovic et al. 2011) and caspases and also protein tyrosine phosphatases (Johnston 2011) and are generally – although not always – unwanted and discarded. This RCC- catalysed enzyme inhibition occurs through the oxidation of the catalytic cysteine to a reversible sulphenic acid (SOH) or an irreversible sulphinic (SO_2H) or sulphonic (SO_3H) acid. Thus, it is important to determine whether those compounds identified as hits (such as via Ubiquigent™'s DUBprofiler service) have an RCC or a non-RCC mechanism of action prior to further progression of that compound or series of compounds in hit-to-lead programmes.

The REDOXprofiler service was developed and validated by Ubiquigent following a collaboration with Medivir AB, a leading pharmaceutical company with an expertise in protease inhibitor research.

REDOXprofiler–Resazurin

Assay summary

This is a surrogate assay for measuring the ability of compounds to oxidise susceptible targets

such as enzymes including DUBs, leading to a reduction in their catalytic activity. In the presence of a reducing agent and potentiated by RCCs, resazurin is reduced and converted to resorufin and thus its fluorescence is dequenched (Lor et al. 2007). This is the basis of the REDOXprofiler-Resazurin service. Due to the primary purpose of the platform being to triage hit compounds identified in Ubiquigent's DUBprofiler screening service the assay conditions for REDOXprofiler-Resazurin have been selected to represent those that the test compound is exposed to in DUBprofiler.

Applications

- Determine whether hit compounds from your in-house and/or DUBprofiler screens may have RCC properties.
- Screen for RCC properties in compounds designed to target enzymes other than DUBs.

Compound submission

See the last page of this brochure for details on how to submit compounds for profiling in the REDOXprofiler-Resazurin service.

The service and data reporting

Customer-submitted compounds are tested in the assay at eight concentrations (two technical replicates at each concentration), the top concentration being 1:100 of the submitted compound concentration and the remaining seven concentrations comprising half-log dilutions from the selected top concentration. Compounds may be submitted for resazurin turnover and/or autofluorescence analysis. The former service reports on the compound's ability to modulate the conversion of resazurin to resorufin in the presence of a reducing agent. Compound test data is reported as a percentage relative to positive (100%) and negative (0%) controls where the positive control data is the assay signal generated in the presence of 3µM of

Example data: Compound titration in a REDOX_{profiler}-Resazurin assay

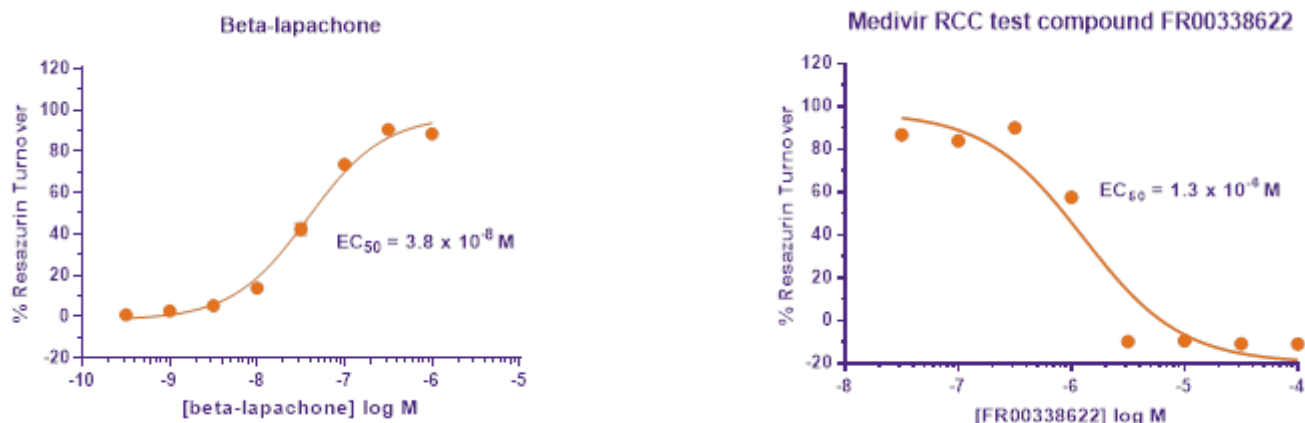


Figure 1: REDOX_{profiler}-Resazurin dose response curves for beta-lapachone and one test compound provided by Medivir AB.

The tested compounds are both RCCs. Note that curves may appear as an 'up' or a 'downslope'. In the latter case resazurin turnover decreases as the compound's concentration is increased, a phenomenon thought to be due to trapping of some of the ROS radicals by the compound competitively with the resazurin (Lor et al. 2007; Figure 10b). In the data shown above compound FR00338622 is active in this surrogate REDOX assay even at the lowest concentration tested (30nM). FR00338622 was also evaluated in a wider titration from 100µM to 1nM and this data showed that the REDOX activity of the compound began to decrease below the 30nM concentration presented here but was still showing an effect of ~50% Resazurin Turnover even at the lowest concentration tested (1nM) (data not shown). Together these observations underline the importance of profiling potential RCCs in this assay format starting at perhaps more than one top concentration in order to determine the 'upslope' EC₅₀ (in the REDOX_{profiler}-Resazurin assay, compounds are tested at 8 half log concentrations).

beta-lapachone (an RCC) and the negative control data is the assay signal generated in the absence of beta-lapachone. The autofluorescence analysis assay reports the fluorescence readout in the assay in the presence of the test compound and the absence of the substrate (resazurin). The customer's compound(s) will be analysed alongside the RCC control compound beta-lapachone. The beta-lapachone data is provided free of charge.

REDOX_{profiler}-Cat/SOD

Assay summary

In this – a modified DUB_{profiler} assay – the IC₅₀ values of compounds at selected DUBs are reported both in the presence and absence of catalase (Cat) and superoxide dismutase (SOD). Catalase catalyses the disproportionation of

H₂O₂ to O₂ and H₂O while SOD disproportionates O₂⁻ to O₂ and H₂O₂. Thus, if the compound has an RCC mechanism of action then in the presence of catalase and SOD an elimination or a reduction in the target inhibitory activity of the compound may be seen if there is an elimination or a sufficient fall in ROS respectively, whereas if the compound does not have RCC activity then the IC₅₀ will remain the same in the presence or absence of catalase and SOD. Mixed mechanisms of action may also be detected. Due to the primary purpose of the platform being to triage hit compounds identified in Ubiquigent's DUB_{profiler} screening service, the assay conditions for REDOX_{profiler}-Cat/SOD have been selected to represent those that the test compound is exposed to in DUB_{profiler}.

Applications

Example data: Beta-lapachone titration in a REDOX_{profiler}-Cat/SOD USP7 assay

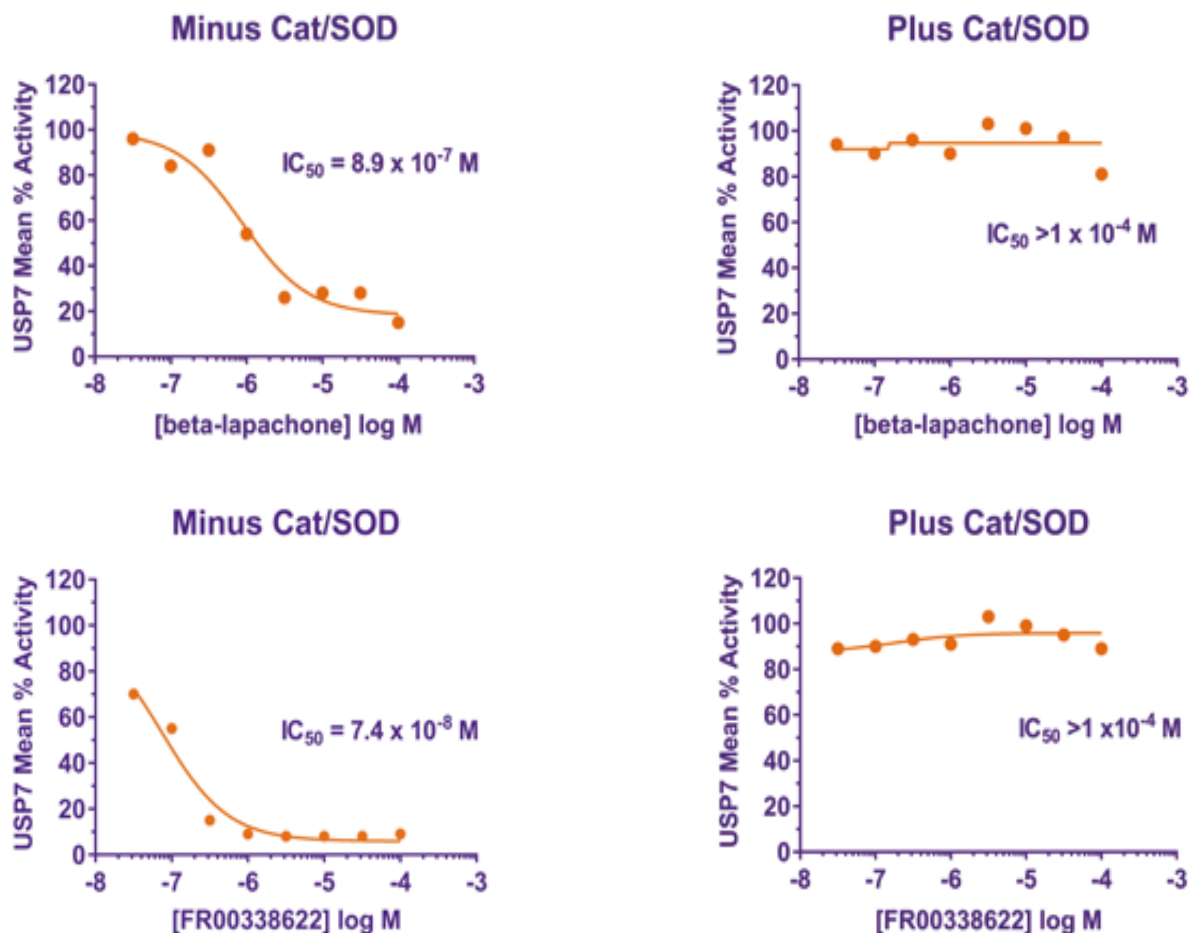


Figure 2: REDOX_{profiler}-Cat/SOD USP7 IC₅₀ curves for beta-lapachone and one RCC test compound provided by Medivir AB.

Both of the compounds tested are RCCs. Note that the catalase/SOD in the assay fully reverses the inhibition seen with both compounds. Thus the USP7 inhibitory activity observed in the absence of catalase/SOD is due to compound RCC ROS generating activity and not direct DUB engagement and inhibition of the enzyme by the compound. If a compound had a mixture of RCC and true DUB inhibitory activity then one would expect still to see some DUB inhibitory activity even in the presence of catalase/SOD, although such data could in theory also be generated if the catalase/SOD present was insufficient to disproportionate all of the H₂O₂ or O₂ - generated by the potential RCC (compounds generating such data could be further investigated by Ubiquigent to clarify these possible interpretations).

- Determine whether hit compounds from your in-house and/or DUB_{profiler} screens may be inhibiting a target DUB(s) through an RCC ROS generating property that is reversible - or at least partially reversible - in the presence of catalase and SOD.
- Screen for the effect of your test compound in a DUB-specific context: this is important

since different DUBs may have differing sensitivities to ROS.

Compound submission

See the last page of this brochure for details on how to submit compounds for profiling in the REDOX_{profiler}-Cat/SOD service.

The service and data reporting

Customer submitted compounds are tested in IC₅₀ assays versus selected DUB(s) in the presence and absence of catalase and SOD. The compounds are tested in both assays at eight half-log dilutions with the top concentration being 1:100 of the submitted compound concentration. Compound test data is reported as a percentage relative to positive (100%) and negative (0%) controls where the positive control data is the assay signal generated in the presence of the DUB and the absence of the test compound and the negative control data is the assay signal generated in the absence of the DUB. The customer's compound(s) will be analysed alongside the RCC control compound beta-lapachone. The beta-lapachone data is provided free of charge.

Accessing REDOXprofiler

- A Master Services Agreement (MSA) will be put in place between your company and Ubiquigent.
- The following will be provided to you: dedicated test compound submission tubes, an Excel-based service submission form which provides you with the flexibility to generate quotes for and then to order any combination of REDOXprofiler-Resazurin and/or REDOXprofiler-Cat/SOD assays providing live quotation and volume-based discounting as you complete the form.
- Then simply email your completed submission form to us, send the compounds and we will undertake your study and email you your data report.
- To access our quick guide that takes you through the process step-by-step, contact us using the details below.

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Contact us

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