WHITE PAPER

Targeting IDOL-catalysed ubiquitylation of LDLR

Overview

Ubiquigent has successfully developed and validated a fully automated, HT-compatible IDOL-catalysed LDLR ubiquitylation assay based on a TR-FRET readout. Customers can submit their compounds for screening at Ubiquigent, or we can provide all the reagents in kit format for you to conduct your HT campaign in-house. We have also validated suitable assays for hit confirmation and deconvolution, including autoubiquitylation of IDOL and IDOL-catalysed ubiquitylation of myosin regulatory light chain. (MRLC) which are also available to access or purchase. Inhibitors of LDLR ubiquitylation, leading to its stabilisation may be therapeutically beneficial in cardiovascular disease and Alzheimer's disease.

IDOL Assay principle: HTRF Substrate Ubiquitylation Assay
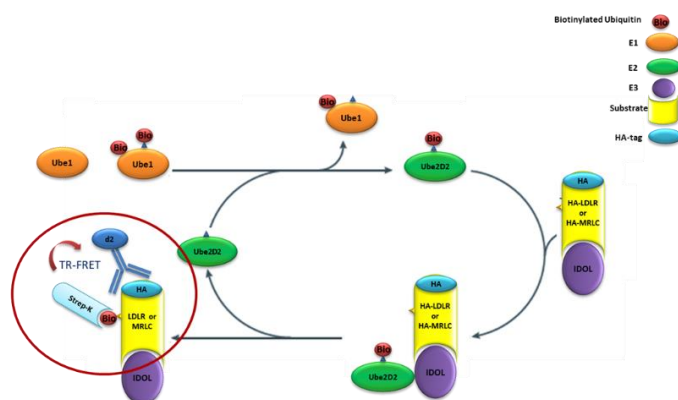
HTRF assay: Strep-K donor and anti-HA-d2 acceptor

The reaction begins with the binding of biotinylated Ubiquitin to the E1, Ube1. The biotinylated Ubiquitin is then transferred from Ube1 to the E2 conjugating enzyme, Ube2D2. The E3 ligase IDOL brings the E2- biotinylated complex into close proximity with the substrate HA-LDLR (or an alternative substrate HA-MRLC). TR-FRET transfer occurs when the donor – Streptavidin Eu Cryptate – which has a high affinity towards biotin, is in close proximity to the acceptor, Anti HA-d2, which binds to the HA-LDLR substrate. Thus, only biotinylated ubiquitin that has been attached to the substrate HA-LDLR (or HA-MRLC) will be measured. A FRET signal is therefore only achieved upon the specific ubiquitylation of HA-LDLR

Why target IDOL–catalysed LDLR Ubiquitylation?

In cardiovascular disease

- Inducible Degrader of LDLR (IDOL) is a RING finger domain ubiquitin E3 ligase; ubiquitylation and degradation of the low density lipoprotein (LDL) receptor (LDLR) modulates cholesterol homeostasis (Zelcer et al., 2009; Zhang et al., 2011)
- An inherited loss–of–function mutation in the LDLR gene in humans – or poor diet – can elevate plasma LDL levels, reduce LDL clearance and accelerate atherosclerosis and the risk of cardiovascular disease (Tolleshaug et al., 1983; Brown and Goldstein 1986).
- Reduction in IDOL–catalysed ubiquitylation of LDLR via knockdown increases cell surface [LDLR] and LDL uptake in cells (Zelcer et al., 2009) and confers protection against atherosclerosis in a hamster model of hypercholesterolemia (Liang et al., 2022).
- Serum IDOL levels decrease after statin therapy, and atorvastatin significantly decreased the levels of IDOL in a dose–dependent manner in vitro with a concomitant increase in LDLR expression (Chan et al., 2022).
- **These studies highlight the potential benefit in targeting the IDOL–LDLR axis for the treatment of cardiovascular disease.**

In Alzheimer's disease

- IDOL is also the principal regulator of LDLR in brain, controlling the clearance of both ApoE–containing high–density lipoprotein (HDL) particles and Aβ.
- Apolipoprotein E (ApoE) is the strongest genetic risk factor for Alzheimer's disease (AD) (Kim et al., 2009a).
- Transgenic overexpression of LDLR in mouse brain led to a ~50–90% decrease in ApoE levels, dramatically reduced Aβ aggregation and enhanced Aβ clearance from the brain extracellular space and attenuated the plaque–associated neuroinflammatory responses (Kim et al., 2009b).
- Moreover, IDOL deficiency in a transgenic mouse model of Aβ. amyloidosis increased brain LDLR, decreased ApoE, decreased soluble and insoluble Aβ, reduced amyloid plaque burden, and ameliorated neuroinflammation (Choi et al., 2015).
- **These findings identify IDOL as a major determinant of LDLR–dependent ApoE and Aβ clearance in the brain, and a potential key target for therapeutic intervention in AD.**

Service Offerings

Ubiquigent offers the IDOL-catalysed LDLR substrate ubiquitylation in 2 formats:

- **Assay kits** for between 1000 and up to 1 million data points to allow the customer to perform their own screening in-house at their own facility.

The assay kits are sent with all the proteins required to run the assay along with a detailed assay set up which includes buffer compositions and screening assay concentrations. To complement this kit, we also offer MRLC as an alternate substrate and an autoubiquitylation IDOL assay kit.

- **Screening at Ubiquigent** using the established optimised HTS robotic platform.

The assay has been fully optimised and automated for HTS screening, using a continuous assay set up. Continuous refers to the constant re-loading of the E2, Ube2D2. By adding the assay components altogether, it allows the assay to be easily transferred onto various robotic systems.
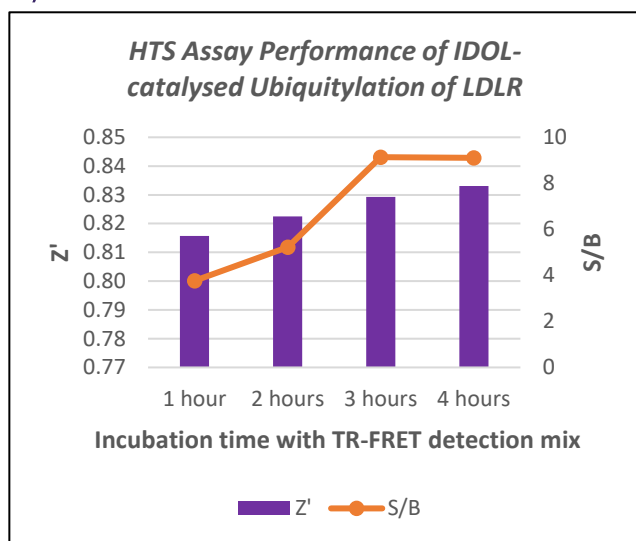
Details on the Ubiquigent HTS offering:
- Options to screen in singlicate (352 compounds/ plate) or duplicate (176 compounds/ plate).
- HT-IC_{50} follow-up is available for any hit compounds identified in the primary screen.
- A data report will be provided, with the compound's activity measured against the % of IDOL activity relative to the control.
- QC criteria of Z'>0.7, S/B > 5/1 applied.

The continuous IDOL-catalysed LDLR substrate ubiquitylation assay workflow:
- 60 nl of test compound spotted onto assay plates using a mosquito liquid handler.
- 100X of test compound incubated with Assay Mix (Ube1, Ube2D2, Ubiquitin, Biotinylated Ubiquitin, HA-LDLR and T7-IDOL) for 15 mins.
- Addition of ATP to start the reaction. Incubation for 1 hour.
- Addition of Detection Mix. Signal allowed to develop for 4 hours before reading the TR-FRET signal on a plate reader.

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HTS Assay Performance

Evaluation of the incubation time of the reaction with the detection mix for up to 4 hours revealed that both S/B and Z' are improved by longer incubation times (3-4h) and that the reagents are stable over this time. Note that the assay reaction is stopped by the addition of the detection mix.

HTS Assay Performance of IDOL-catalysed Ubiquitylation of LDLR

Z' vs Incubation time with TR-FRET detection mix (1 hour, 2 hours, 3 hours, 4 hours), with Z' axis ranging from 0.77 to 0.85 and S/B axis ranging from 0 to 10.

Z' — S/B

To validate the assay for determination of IC50 values, a serial dilution of a 'FRET-dead' LDLR (T7-LDLR), (no HA-tag) was run in the assay and the IC_{50} determined.

LDLR-T7 (FRET-dead) IC50= 1.8 µM

Mean % Activity vs Concentration (µM)
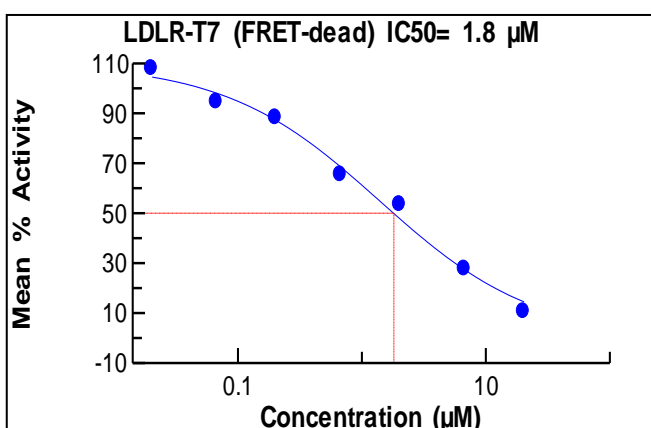
Ubiquigent also offers 3 other complementary assays to assist in deconvolution of any hit compounds from the initial screen:

- **MRLC (Myosin Regulatory Light Chain) substrate Ubiquitylation assay**. Uses an alternative substrate to LDLR, to establish if the hit compound is inhibiting IDOL-mediated LDLR ubiquitylation specifically.
- **Autoubiquitylation IDOL assay**. IDOL is known to auto-ubiquitinate, and this assay will assist in pinpointing whether the hit compound is a specific inhibitor of the E3 ligase, IDOL.
- **Pre-loaded assay**. This assay enables separation of the 1st step of the ubiquitylation reaction from the IDOL-mediated ubiquitylation of LDLR. The E2 is pre-loaded and the reaction is then stopped. IDOL and LDLR are then added to the reaction. A compound that is confirmed as a hit in this assay is likely inhibiting IDOL activity or the interaction of IDOL with LDLR, rather than being an 'off-target' inhibitor of E1 or E2.

References

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