

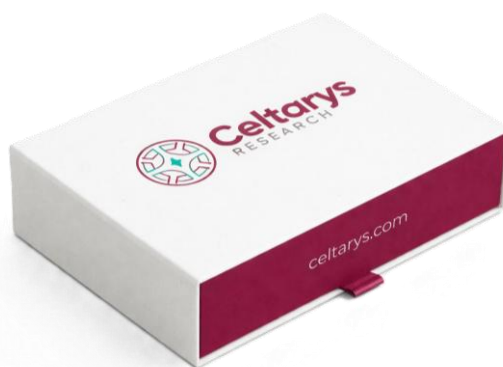
## VHL COOPERATIVITY ASSAY KIT

Celtarys' **VHL Cooperativity Assay Kit** is a homogeneous fluorescence polarization (FP)-based assay kit designed to evaluate how a Protein of Interest (POI) influences the apparent binding affinity of VHL-recruiting PROTACs for the VHL E3 ligase complex.

The assay uses **CELT-504** as a fluorescent tracer, together with the ElonginB/ElonginC-VHL (VBC) complex, to compare PROTAC binding in the absence (binary condition) and presence (ternary condition) of the POI.

By comparing concentration-response displacement curves under both conditions, the kit enables the determination of apparent binding affinities (IC<sub>50</sub> and K<sub>i</sub>) and the estimation of a **cooperativity factor ( $\alpha$ )**, providing valuable insights into PROTAC-induced ternary complex formation.

The kit provides researchers with the essential VHL-specific reagents and a validated experimental protocol to assess PROTAC cooperativity directly in their own laboratories, supporting the characterization and optimization of targeted protein degraders.



### Why this assay?

This assay kit provides the following advantages:

- **Quantitative cooperativity assessment:** Enables the comparison of binary and POI-containing binding conditions to determine apparent binding affinities and cooperativity factors ( $\alpha$ ).
- **Ternary complex characterization:** Supports the identification of positive, neutral or negative cooperativity associated with PROTAC-mediated interactions between VHL and the POI.

- **Homogeneous fluorescence polarization readout** with no washing or separation steps.
- **High-affinity fluorescent tracer:** CELT-504 has a low nanomolar affinity for the VBC complex, providing a sensitive readout of VHL binding interactions.
- **Easy to implement** in laboratories equipped with a fluorescence polarization-compatible microplate reader.
- **Ready-to-use assay format:** The kit provides the essential VHL-specific components and a detailed protocol to facilitate experimental setup and implementation.
- **Flexible experimental design:** Compatible with different VHL-recruiting PROTACs and user-supplied recombinant proteins of interest.
- **384-well format** suitable for comparative profiling of multiple PROTAC candidates.
- The **VHL cooperativity assay kit** has been validated using the well-characterized **PROTAC MZ1 and BRD4-BD2** as the protein of interest (POI), with **VH298** included as a monovalent VHL-binding reference compound. As shown in Table 1, **MZ1** exhibited strong positive cooperativity in the presence of BRD4-BD2 ( $\alpha \approx 23.6$ ), in close agreement with the reported literature value<sup>1</sup> ( $\alpha \approx 22$ ). In contrast, **VH298** showed no positive cooperativity ( $\alpha \approx 0.71$ ), supporting the assay's ability to distinguish cooperative ternary-complex formation from conventional binary binding.

|               |                   | <b>Ki binary<br/>(nM)</b> | <b>Ki + BRD4<br/>(nM)</b> | <b><math>\alpha</math><br/>(assay)</b> | <b><math>\alpha</math><br/>(literature)</b> |
|---------------|-------------------|---------------------------|---------------------------|----------------------------------------|---------------------------------------------|
| <b>MZ1</b>    | PROTAC            | 148.5                     | 6.30                      | 23.6                                   | 18 <sup>1</sup>                             |
| <b>VH 298</b> | Small<br>Molecule | 63.2                      | 89.1                      | 0.71                                   |                                             |

**Table 1. Experimental validation of the VHL cooperativity assay kit.** Ki values calculated from fluorescence polarization competition assays in the absence and presence of 2  $\mu$ M BRD4-BD2. Cooperativity ( $\alpha$ ) was calculated as  $Ki(\text{binary})/Ki(+\text{BRD4})$ .

## What does the kit include?

- **Assay Buffer (10×)** for the preparation of the assay buffer.
- **ElonginB/ElonginC-VHL (VBC) protein complex.**

- **VHL fluorescent tracer (CELT-504).**
- **VH298 reference competitor**, included as a control for VHL binding and cooperativity assessment.
- **384-well black, low-volume, round-bottom plate.**
- **Detailed assay protocol** including reagent preparation, experimental setup, recommended controls, data analysis and storage instructions.

*Note: The Protein of Interest (POI) and test PROTACs are not included in the kit and must be supplied separately. The kit is designed to accommodate different recombinant POIs, allowing researchers to select the target protein most relevant to their studies.*

## What equipment is required?

To perform the assay, the laboratory should have access to a microplate reader capable of fluorescence polarization (FP), equipped with the appropriate filters or monochromator modules for parallel and perpendicular fluorescence detection. Recommended optical characteristics:

- **Excitation:** approximately 560 nm.
- **Emission:** approximately 570–590 nm.
- **Detection mode:** Fluorescence polarization (FP).

*Note: The optimal excitation and emission settings should be verified according to the specifications of the microplate reader and the assay window obtained with the provided controls. Standard laboratory equipment, including micropipettes, reagent tubes and materials for preparing serial dilutions, is also required.*

## How does FP (fluorescence polarization) work?

The principle of the assay is based on the **change** in **rotational mobility** that occurs when a **fluorescent ligand binds to a larger target protein**. Free CELT-504 rotates rapidly in solution and, when excited by plane-polarised light, produces a low fluorescence polarisation signal. When CELT-504 binds to the target protein VHL, its effective molecular volume increases, rotational relaxation slows down and the **emitted light** retains a **higher degree of polarisation**, resulting in a higher FP signal (Figure 1). VHL-binding compounds compete with CELT-504 for the same binding site, displacing the tracer in a concentration-dependent manner and reducing the FP signal.

To evaluate cooperativity, the **same PROTAC is tested under two experimental conditions:**

- **Binary condition:** VHL + PROTAC + CELT-504.
- **Ternary condition:** VHL + PROTAC + POI + CELT-504.

The displacement curves obtained under both conditions are compared to determine whether the presence of the POI modifies the apparent affinity of the PROTAC for VHL. The **cooperativity factor ( $\alpha$ )** is calculated as the ratio between the binary and ternary apparent binding affinities:

- **$\alpha > 1$ : Positive cooperativity.** The presence of the POI enhances the apparent affinity of the PROTAC for VHL.
- **$\alpha \approx 1$ : No detectable cooperativity.**
- **$\alpha < 1$ : Negative cooperativity.** The presence of the POI reduces the apparent affinity of the PROTAC for VHL.

Positive cooperativity may favor ternary complex formation, whereas negative cooperativity indicates less favorable interactions under the tested conditions.

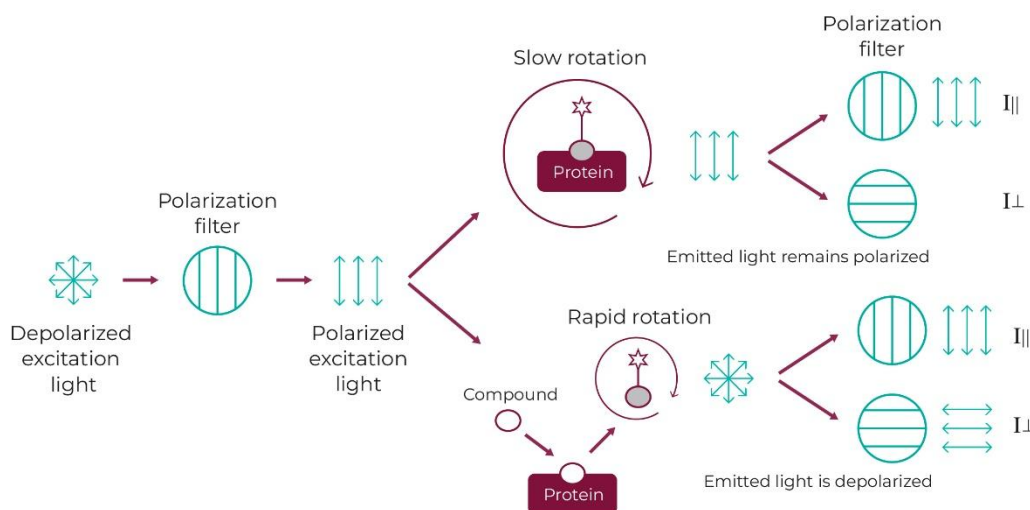


**Figure 1. Depiction of the basis of FP assays.**

### Scientific References

1. Gadd, M. S., Testa, A., Lucas, X., Chan, K. H., Chen, W., Lamont, D. J., Zengerle, M., & Ciulli, A. (2017). Structural basis of PROTAC cooperative recognition for selective protein degradation. *Nature chemical biology*, 13(5), 514–521. <https://doi.org/10.1038/nchembio.2329>