BRD4/VHL PROTAC BINDING ASSAY KITS

PROTOCOL

Part # 63ADK000CB32PEG & 63ADK000CB32PEH

Test size: 500 tests (63ADK000CB32PEG), 10,000 tests (63ADK000CB32PEH) - assay volume: 20 µL

Revision: 01 (May 2021)

Store at: -60°C or below

For research use only. Not for use in diagnostic procedures.

ASSAY PRINCIPLE

The HTRF BRD4/VHL PROTAC Binding Assay is designed to measure the ternary complex formation between BRD4, PROTAC degrader and VHL proteins. The BRD4 contains both bromodomain 1 and 2. Utilizing HTRF (Homogeneous Time-resolved Fluorescence) technology, the assay enables simple and rapid characterization of PROTAC degraders in a high throughput format.

As shown in Figure 1, the interaction between Tag1-BRD4 and Tag2-VHL is detected by using anti-Tag1-Europium (HTRF donor) and anti-Tag2-XL665 (HTRF acceptor). When the donor and acceptor antibodies are brought into close proximity due to the BRD4, PROTAC degrader and VHL binding, excitation of the donor antibody triggers fluorescent resonance energy transfer (FRET) towards the acceptor antibody, which in turn emits specifically at 665 nm. This specific signal is directly proportional to the extent of PROTAC degrader bind to both BRD4 and VHL protein. Thus, PROTAC degrader forming a ternary complex will cause an increase in HTRF signal.

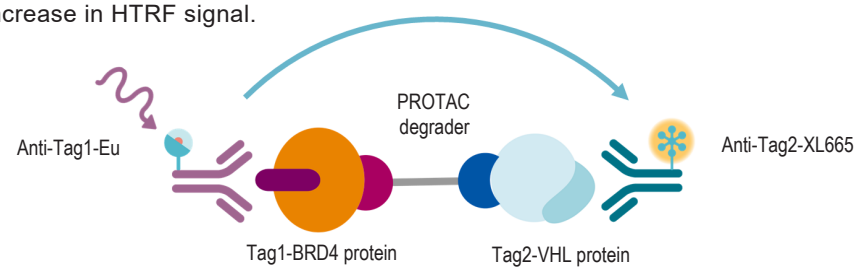
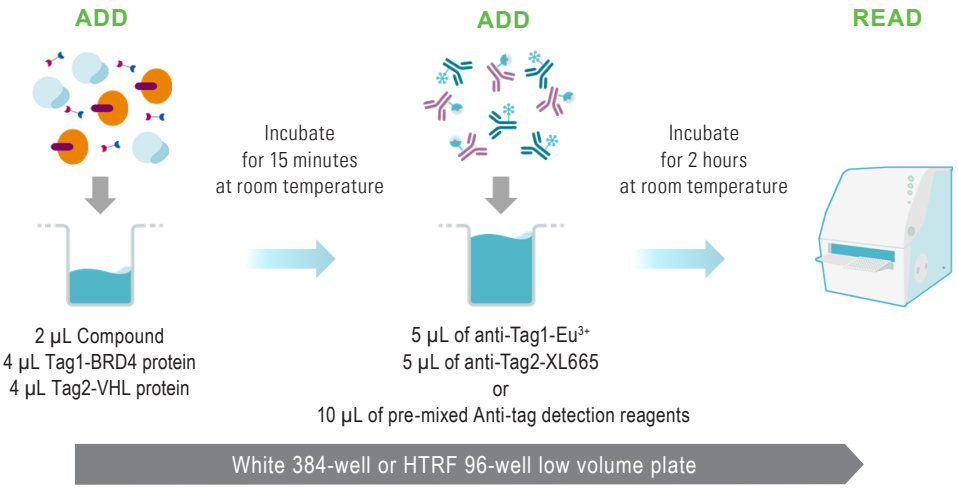


Figure 1: Principle of the HTRF BRD4/VHL PROTAC binding assay.

PROTOCOL AT A GLANCE



Make sure to use the setup for Eu^{3+} Cryptate. For more information about set-up and compatible HTRF® readers, please visit our website at: http://www.cisbio.com/readers

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MATERIALS:

KIT COMPONENTS	500 TESTS CAT # 63ADK000CB32PEG	10,000 TESTS CAT # 63ADK000CB32PEH
Tag1-BRD4* MW: 73.2 kDa	1 vial 25 µL 500X Frozen	1 vial 420 µL 500X Frozen
Tag2-VHL (CUL2/ELOB/ELOC/RBX1) Complex* MW: 153.0 kDa	1 vial 25 µL 500X Frozen	1 vial 420 µL 500X Frozen
PROTAC Standard MW: 1002.6 Da	1 vial 10 µL 3 mM DMSO Solution Frozen	2 vials 10 µL 3 mM DMSO Solution Frozen
Anti-Tag1-Eu^{3+}	1 vial 25 µL 100X Frozen	1 vial 0.5 mL 100X Frozen
Anti-Tag2-XL665	1 vial 25 µL 100X Frozen	1 vial 0.5 mL 100X Frozen
Diluent	1 vial 20 mL Cat# 62DLBDDD (200 mL) ready-to-use	1 vial 200 mL Cat# 62DLBDDD (200 mL) ready-to-use
Detection Buffer	1 vial 10 mL Cat# 62DB1FDG (130 mL) ready-to-use	1 vial 130 mL Cat# 62DB1FDG (130 mL) ready-to-use

* The amounts of Tag1-BRD4 and Tag2-VHL provided are sufficient for the validated amounts of tagged proteins suitable for PROTAC ternary complex formation study: optimized concentrations of BRD4 and VHL in 20 µL final assay volume.

For reading, an HTRF®-Certified Reader is needed.
For HTRF microplate recommendations, please visit http://www.cisbio.com/microplate-recommendations
For a list of HTRF-compatible readers and setup recommendations, please visit http://www.cisbio.com/readers

STORAGE AND STABILITY

Store the kit at -60°C or below. Under appropriate storage conditions, reagents are stable until the expiry date indicated on the label.

Reagents

Once thawed, tagged BRD4 & VHL stock solution may be frozen, and can be thawed only once.
Once thawed (or reconstituted), anti-Tag solutions can be frozen once.
To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -60°C or below.

Thawed diluent and detection buffer can be stored at 2-8°C on your premises.

REAGENT PREPARATION

BEFORE YOU BEGIN:

- It is very important to prepare reagents in the specified buffers. The use of an incorrect diluent may affect reagent stability and assay results.
- Thaw the frozen reagents at room temperature.
- Before use, allow all reagents to warm up to room temperature then homogeneize buffer and diluent. It is recommended to filter buffers before use.
- The tagged protein solutions must be prepared in individual vials - DO NOT premix tagged solutions prior to dispensing.
- The anti-Tag solutions must be prepared in individual vials and can be premix prior to dispensing.
- Compounds may be prepared in diluent. We recommend keeping DMSO below 1% during the assay (20 µL final volume).

TO PREPARE WORKING SOLUTIONS:

Take care to prepare stock and working solutions according to the directions for the kit size you have purchased.

500 TESTS	10,000 TESTS
Tag1-BRD4 protein 500X stock solution of optimized final concentration	
Thaw the Tag1-BRD4 protein* solution. Dilute 100-fold the 500X Tag1-BRD4 protein stock solution with diluent buffer to prepare a 5X working solution. e.g. 5 µL of thawed Tag1-BRD4 protein stock solution + 495 µL of diluent buffer. (20 µL final volume).	
Tag2-VHL protein 500X stock solution of optimized final concentration	
Thaw the Tag2-VHL protein* solution. Dilute 100-fold the 500X Tag2-VHL protein stock solution with diluent buffer to prepare a 5X working solution. e.g. 5 µL of thawed Tag2-VHL protein stock solution + 495 µL of diluent buffer. (20 µL final volume).	
Anti-Tag1-Eu^{3+}	
Thaw the anti-Tag1-Eu^{3+} solution. This 100X stock solution can be frozen and stored at -60°C or below. Dilute 100-fold the 100X anti-Tag1-Eu^{3+} stock solution with detection buffer.	
e.g. 25 µL of thawed anti-Tag1-Eu^{3+} stock solution + 2475 µL of detection buffer.	e.g. 0.5 mL of thawed anti-Tag1-Eu^{3+} stock solution + 49.5 mL of detection buffer.
Anti-Tag2-XL665	
Thaw the anti-Tag2-XL665 solution. This 100X stock solution can be frozen and stored at -60°C or below. Dilute 100-fold the 100X anti-Tag2-XL665 stock solution with detection buffer. e.g. 25 µL of thawed anti-Tag2-XL665 stock solution + 2475 µL of detection buffer.	Thaw the anti-Tag2-XL665 solution. This 100X stock solution can be frozen and stored at -60°C or below. Dilute 100-fold the 100X anti-Tag2-XL665 stock solution with detection buffer. e.g. 0.5 mL of reconstituted anti-Tag2-XL665 stock solution + 49.5 mL of detection buffer.

*Titration of Tag1-BRD4 or Tag2-VHL can be performed if necessary.

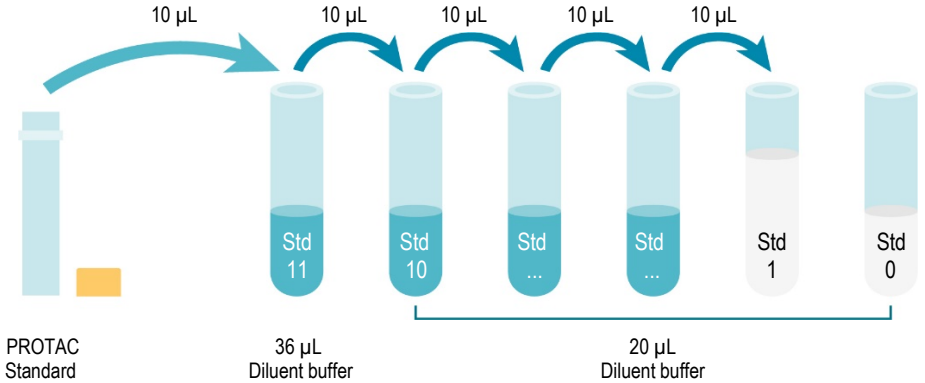
TO PREPARE WORKING PROTAC STANDARD SOLUTIONS:

- Each well requires 2 µL of standard.
- In order to counteract any standard sticking, we recommend changing tips between each dilution.

A recommended standard dilution procedure is listed and illustrated below:

- Dilute the PROTAC standard stock solution 10-fold with diluent buffer to prepare high standard (Std 11):
  Take 4 µL of standard stock solutionand add it to 36 µL of diluent buffer. Mix gently.
- Use the high standard (Std 11) to prepare the standard curve using 3-fold serial dilutions, as follows:
  - Dispense 20 µL of diluent buffer into each vial from Std 11 to Std 0.
  - Add 10 µL of standard to 20 µL of diluent buffer, mix gently, and repeat the serial dilution to make the other standard solutions: Std10, Std9, Std8, Std7, Std6, Std5, Std4, Std3, Std2, Std1.

This will create 11 standards for the analyte. Std 0 (Negative control) is diluent buffer.



STANDARD	SERIAL DILUTIONS	WORKING SOLUTIONS	FINAL CONCENTRATIONS
Standard stock solution	Thawed stock solution	3 000 µM	
Standard 11	4 µL standard stock solution + 36 µL Diluent buffer	300 µM	30 µM
Standard 10	10 µL Standard 11 + 20 µL Diluent buffer	100 µM	10 µM
Standard 9	10 µL Standard 10 + 20 µL Diluent buffer	33 µM	3.3 µM
Standard 8	10 µL Standard 9 + 20 µL Diluent buffer	11 µM	1.1 µM
Standard 7	10 µL Standard 8 + 20 µL Diluent buffer	3.7 µM	0.37 µM
Standard 6	10 µL Standard 7 + 20 µL Diluent buffer	1.2 µM	0.12 µM
Standard 5	10 µL Standard 6 + 20 µL Diluent buffer	0.41 µM	0.041 µM
Standard 4	10 µL Standard 5 + 20 µL Diluent buffer	0.14 µM	0.014 µM
Standard 3	10 µL Standard 4 + 20 µL Diluent buffer	0.046 µM	0.0046 µM
Standard 2	10 µL Standard 3 + 20 µL Diluent buffer	0.015 µM	0.0015 µM
Standard 1	10 µL Standard 2 + 20 µL Diluent buffer	0.005 µM	0.0005 µM
Standard 0	20 µL Diluent buffer	0 µM	0 µM

ASSAY PROTOCOL

	Cryptate control	Standard (Std 0 - Std 11)	Compound
Step 1	Dispense 10 µL of diluent buffer into each cryptate control well.	Dispense 2 µL of each PROTAC standard (Std 0 - Std 11) into each standard well.	Dispense 2 µL of compound into each compound well.
Step 2		Add 4 µL of Tag1-BRD4 protein and 4 µL of Tag2-VHL protein to all wells	
Step 3	Incubate for 15 minutes at room temperature.		
Step 4	Dispense 5 µL of anti-Tag1-Eu3+ and 5 µL of detection buffer.	Dispense 10 µL of pre-mixed anti-Tag1-Eu3+ and anti-Tag2-XL665.	
Step 5	Seal the plate and incubate for 2 hours at room temperature.		
Step 6	Remove the plate sealer and read on an HTRF® compatible reader.		

EXAMPLE OF PLATE MAP

	1	2	3	4	5	6
A	**Buffer control**: 10 µL diluent 10 µL detection buffer	Repeat Well A1	Repeat Well A1	**Compound...:** 2 µL compound... 4 µL Tag1-BRD4 4 µL Tag2-VHL 10 µL pre-mix anti-Tag reagents	Repeat Well A4	Repeat Well A4
B	**Cryptate control:** 10 µL diluent 5 µL detection buffer 5 µL anti-Tag1-Eu	Repeat Well B1	Repeat Well B1	**Compound...:** 2 µL compound... 4 µL Tag1-BRD4 4 µL Tag2-VHL 10 µL pre-mix anti-Tag reagents	Repeat Well B4	Repeat Well B4
C	**Negative control:** 2 µL diluent 4 µL Tag1-BRD4 4 µL Tag2-VHL 10 µL pre-mix anti-Tag reagents	Repeat Well C1	Repeat Well C1	**Compound...:** 2 µL compound... 4 µL Tag1-BRD4 4 µL Tag2-VHL 10 µL pre-mix anti-Tag reagents	Repeat Well C4	Repeat Well C4
D	**Positive control:** 2 µL PROTAC standard 4 µL Tag1-BRD4 4 µL Tag2-VHL 10 µL pre-mix anti-Tag reagents	Repeat Well D1	Repeat Well D1	**Compound...:** 2 µL compound... 4 µL Tag1-BRD4 4 µL Tag2-VHL 10 µL pre-mix anti-Tag reagents	Repeat Well D4	Repeat Well D4
E	**Compound 1:** 2 µL compound 1 4 µL Tag1-BRD4 4 µL Tag2-VHL 10 µL pre-mix anti-Tag reagents	Repeat Well E1	Repeat Well E1	**Compound...:**	Repeat Well E4	Repeat Well E4
F	**Compound 2:** 2 µL compound 2 4 µL Tag1-BRD4 4 µL Tag2-VHL 10 µL pre-mix anti-Tag reagents	Repeat Well F1	Repeat Well F1	**Compound...:**	Repeat Well F4	Repeat Well F4
G	**Compound...:** 2 µL compound... 4 µL Tag1-BRD4 4 µL Tag2-VHL 10 µL pre-mix anti-Tag reagents	Repeat Well G1	Repeat Well G1	**Compound...:**	Repeat Well G4	Repeat Well G4
H	**Compound...:** 2 µL compound... 4 µL Tag1-BRD4 4 µL Tag2-VHL 10 µL pre-mix anti-Tag reagents	Repeat Well H1	Repeat Well H1			

DATA REDUCTION & INTERPRETATION

1. Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

2. Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

For more information about data reduction, please visit http://www.cisbio.com/data-reduction

The data should be fitted with Bell-shaped equation in GraphPad Prism software.

RESULTS

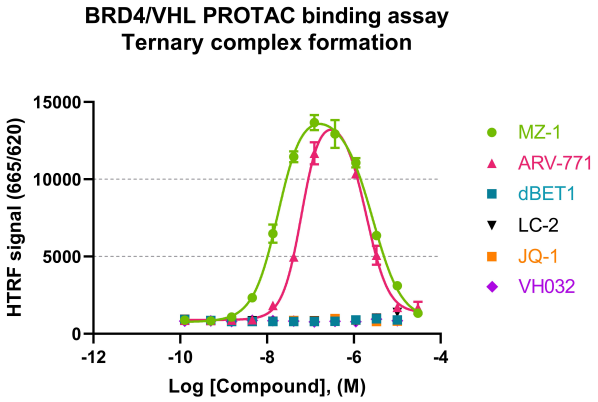
The data shown below must not be substituted for the data obtained in the laboratory, and should be considered only as an example.

The formation of ternary complex were tested at optimized concentrations of VHL and BRD4.

Readouts on **PerkinElmer® EnVision** with a flash lamp.

Note that results may vary from one HTRF® compatible reader to another.

Compound	Vender	Cat#	Description
MZ-1	MCE	HY-107425	A long-lasting and selective removal degrader based PROTAC between BRD4 and VHL.
ARV-771	MCE	HY-100972	A potent BET degrader in cellular models of CRPC by recruiting VHL E3 ubiquitin ligase.
dBET1	MCE	HY-101838	A hybrid of the highly selective degrader based PROTAC between BRD4 and CRBN.
LC-2	MCE	HY-137516	A potent degrader based PROTAC between KRAS G12C and VHL.
JQ-1	MCE	HY-13030	A potent, specific and reversible BET bromodomain inhibitor which has potential anti-cancer activity.
VH032	MCE	HY-120217	A ligand used in the recruitment of the VHL protein. It is used as a precursor to synthesize MZ-1.



The concentration of MZ-1 at peak, 123.6 nM

The concentration of ARV-771 at peak, 370.7 nM

FOR MORE INFORMATION
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