MANUAL

HTRF VHL - Red Ligand

Part # 64BDVHLRED

Test Size#: 10 binding assays (Kd determination) - assay volume: 20 µL

Revision: 02 of September 2023 **Store at:** ≤-16°C

This product is intended for research purposes only. It is not intended to be used for therapeutic or diagnostic purposes.

ASSAY PRINCIPLE

Revvity' HTRF VHL-Red Ligand (#Revvity 64BDVHLRED) is primarily intended to perform affinity binding curves using HTRF® technology and can be used in association with the HTRF Human VHL binding kit (#Revvity 64BDVHLPEG/H).

The HTRF VHL-Red Ligand is particularly suited for cooperativity binding studies to assess the effect of a PROTAC protein substrate on the affinity of the VHL-Red Ligand for VHL protein. HTRF VHL-Red Ligand binding is detected in a direct binding assay format using an anti 6His antibody gold labeled with Europium Cryptate which binds to Human VHL 6his-tagged protein complex. When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). The specific binding signal is calculated by subtracting the non-specific binding signal from the total signal, enabling determination of Kd for HTRF VHL-Red Ligand (Fig. 1).

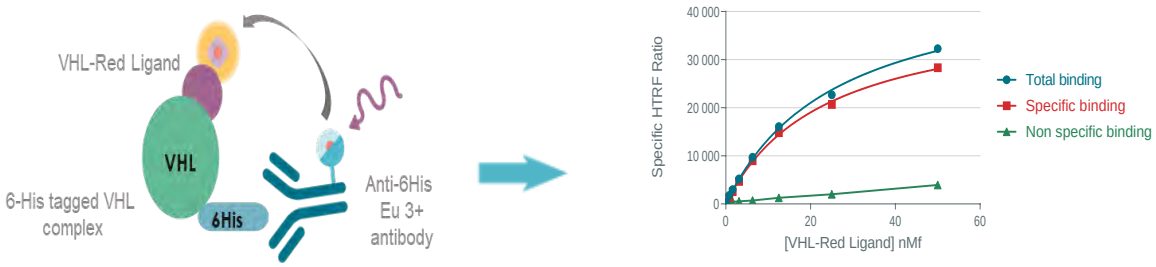
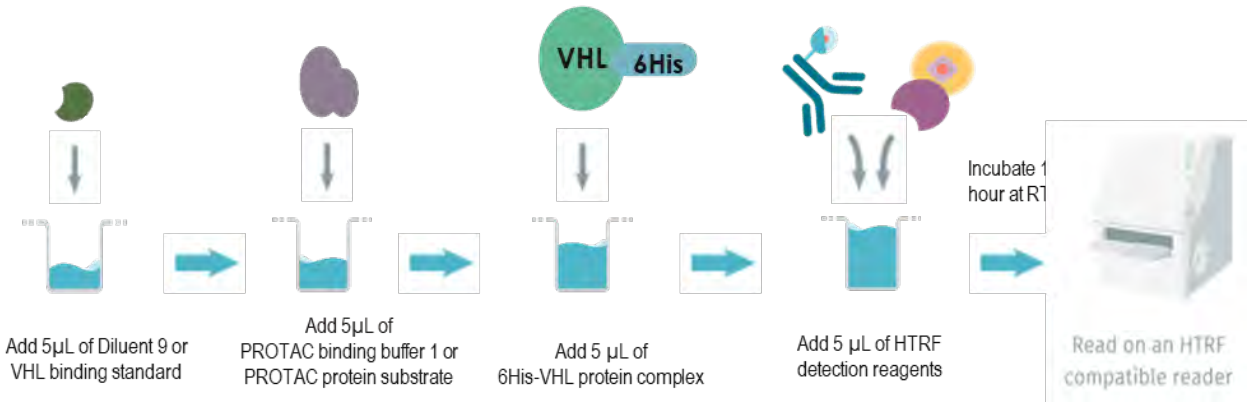


Figure 1: Principle of Kd determination with HTRF VHL-Red Ligand.

MANUAL AT A GLANCE



Assay volumes can be adjusted proportionally to run the assay in 96 or 1536 well microplates.
Make sure you use the appropriate setup for Eu 3+ Cryptate. For more information about setup and HTRF® compatible readers, please visit our website at: www.revvity.com

MATERIAL PROVIDED:

HTRF VHL - Red ligand - 25X	1 vial - 50 µL Frozen	

PURCHASE SEPARATELY:

- VHL Standard (#Revvity 64BDVHLCDA)
- MAb Anti-6HIS-Eu cryptate Gold (#Revvity 61HI2KLA/B)
- HTRF PROTAC binding buffer 1 (#Revvity 64BDE31RDF)
- Diluent 9 (5X) (#Revvity 62DL9DDA/C)
- HTRF Human VHL binding kit (#Revvity 64BDVHLPEG/H)
- Low volume white (only) microplate*
- HTRF®-Certified Reader **. Make sure the setup for Eu3+ Cryptate is used.

* For HTRF microplate recommendations, please visit www.revvity.com
** For a list of HTRF-compatible readers and setup recommendations, please visit www.revvity.com

STORAGE AND STABILITY

Store the HTRF VHL - Red Ligand at -16°C or below. Under appropriate storage conditions, HTRF VHL - Red Ligand is stable until the expiry date indicated on the label.

To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.

Volume of HTRF VHL - Red Ligand aliquots should not be under 10 µL.

Once thawed, aliquoted HTRF VHL - Red Ligand can be frozen once.

REAGENT PREPARATION

BEFORE YOU BEGIN:

- It is very important to prepare all reagents in the specified buffers. The use of incorrect buffers may affect reagent stability and assay results.
- VHL binding standard will be used to determine the non-specific binding signal.
- PROTAC binding buffer 1 is ready-to-use.
- 6His-VHL protein complex must be prepared following instructions from the package insert of the HTRF Human VHL binding kit.
- Diluent 9 (5X) must be homogenized with a vortex then diluted 5-fold with distilled water (e.g. 200µL of Diluent 9 (5X) + 800µL of distilled water) and mixed gently to obtain the Diluent 9 (1X).

► VHL BINDING STANDARD

TO PREPARE THE VHL BINDING STANDARD STOCK SOLUTION:

Thaw the VHL binding Standard. Centrifuge.

This 1mM stock solution can be frozen and stored at -16°C or below.

TO PREPARE THE VHL BINDING STANDARD WORKING SOLUTION:

Homogenize the VHL binding Standard stock solution then dilute it 25-fold with Diluent 9 (1X) (e.g. 10µL of VHL binding Standard stock solution + 240µL Diluent 9 (1X)). Mix gently.

► HTRF VHL-RED LIGAND

TO PREPARE THE HTRF VHL-RED LIGAND STOCK SOLUTION:

Thaw the HTRF VHL-Red Ligand. Centrifuge.

This 25X stock solution can be frozen and stored at -16°C or below.

TO PREPARE THE HTRF VHL-RED LIGAND WORKING SOLUTION:

The HTRF VHL-Red Ligand working solution (C7) is obtained by diluting 25-fold the 25X stock solution of HTRF VHL-Red Ligand with PROTAC binding buffer 1 e.g. 5µL of thawed HTRF VHL-Red Ligand stock solution + 120µL of PROTAC binding buffer 1.

Use the HTRF VHL-Red Ligand working solution to prepare the recommended range of concentrations by using serial dilutions (in order to counteract any sticking, we recommend changing tips between each dilution).

Procedure for preparing 1 range of HTRF VHL-Red Ligand (for monitoring total and non-specific binding signals):

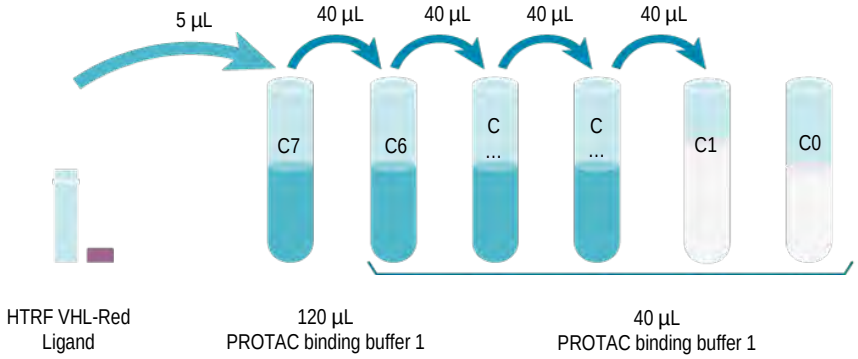
Prepare 100µL of HTRF VHL-Red Ligand working solution (C7).

Use the high concentration (C7) to prepare the range of concentrations using ½ serial dilutions as follows:

- Dispense 40µL of PROTAC binding buffer 1 into each vial from C6 to C0.

Add 40µL of C7 of HTRF VHL-Red Ligand to 40µL of PROTAC binding buffer 1 to obtain the C6, mix gently and repeat the ½ serial dilution to make the concentrations: C5, C4, C3, C2 and C1. C0 (Negative control) is PROTAC binding buffer 1 alone.

This will create 7 concentrations of HTRF VHL-Red Ligand to be used for Total and non-specific binding conditions.



HTRF VHL-RED	SERIAL DILUTIONS	"HTRF VHL-RED WORKING SOLUTION (nM)"	"HTRF VHL-RED FINAL CONCENTRATION (nM)"
Standard Stock solution	Thawed stock solution	10 000	-
C7	5µL stock solution + 120 µL PROTAC binding buffer 1	400	50
C6	40 µL C7 + 40 µL PROTAC binding buffer 1	200	25
C5	40 µL C6 + 40 µL PROTAC binding buffer 1	100	12.5
C4	40 µL C5 + 40 µL PROTAC binding buffer 1	50	6.25
C3	40 µL C4 + 40 µL PROTAC binding buffer 1	25	3.125
C2	40 µL C3 + 40 µL PROTAC binding buffer 1	12.5	1.562
C1	40 µL C2 + 40 µL PROTAC binding buffer 1	6.25	0.781
C0	80 µL PROTAC binding buffer 1	0	0

► MAB ANTI-6HIS-EU CRYPTATE GOLD:

TO PREPARE THE MAB ANTI-6HIS-EU CRYPTATE GOLD STOCK SOLUTION:

Allow each vial of lyophilized conjugate to warm up at room temperature.
The mAb Anti-6HIS-Eu cryptate Gold stock solution is obtained by reconstituting the lyophilizate of ref 61HI2KLA with 0.25mL of distilled water or the lyophilizate of ref 61HI2KLB with 1mL of distilled water. After reconstitution mix gently.

This stock solution can be frozen and stored at -16°C or below.

TO PREPARE THE MAB ANTI-6HIS-EU CRYPTATE GOLD WORKING SOLUTION:

Homogenize the mAb Anti-6HIS-Eu cryptate Gold stock solution then dilute it 50-fold with PROTAC binding buffer 1 (e.g. 40µL of mAb Anti-6HIS-Eu cryptate Gold stock solution + 1960µL of PROTAC binding buffer 1). After dilution mix gently.

WARNING: Do not prepare the mAb Anti-6HIS-Eu cryptate Gold working solution following instructions from the package insert of the mAb Anti-6HIS-Eu cryptate Gold (#Revvity 61HI2KLA/B).

► PRE-MIXED SOLUTION OF HTRF VHL-RED LIGAND + MAB ANTI-6HIS-EU CRYPTATE GOLD:

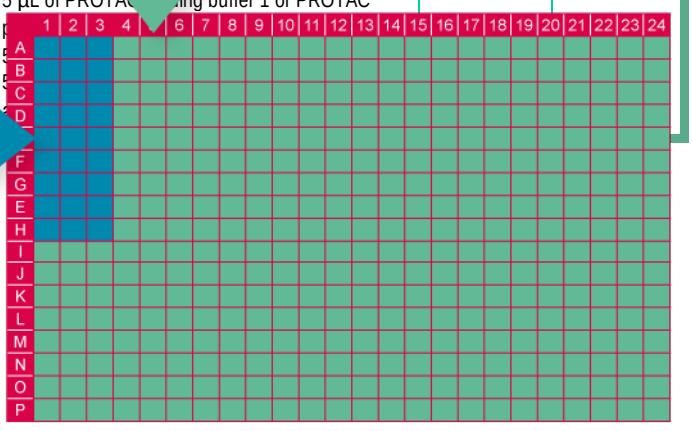
Just prior dispensing, pre-mix the working solutions of HTRF VHL-Red Ligand and mAb anti-6HIS-Eu cryptate gold by adding 1 volume of mAb anti-6HIS-Eu cryptate gold working solution to 1 volume of each HTRF VHL-Red Ligand working solutions (C7 to C0) (e.g. 25µL of HTRF VHL-Red Ligand working solution + 25µL of mAb anti-6HIS-Eu cryptate gold working solution).

ASSAY MANUAL

	NON-SPECIFIC BINDING SIGNAL	TOTAL BINDING SIGNAL
Step 1 	Dispense 5 µL of VHL binding Standard into each well	Dispense 5 µL of PROTAC binding buffer 1 into each well
Step 2 	Add 5 µL of PROTAC binding buffer 1 or 5µL of PROTAC substrate protein (diluted in the PROTAC binding buffer 1)	
Step 3 	Add 5µL of 6His-VHL protein complex	
Step 4 	Add 5 µL of premixed HTRF VHL-Red Ligand and mAb anti-6HIS-Eu cryptate gold working solutions to all wells	
Step 5 	Seal the plate and incubate 1 hour at RT	
Step 6 	Remove the plate sealer and read on an HTRF® compatible reader	

EXAMPLE OF MAP PLATE

	NON-SPECIFIC BINDING			TOTAL BINDING		
	1	2	3	4	5	6
A	5 µL of VHL Binding Standard 5 µL of PROTAC binding buffer 1 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C0 and mAb anti-6His Eu cryptate gold premixed	Repeat Well A1	Repeat Well A1	5 µL of diluent #9 5 µL of PROTAC binding buffer 1 or PROTAC protein substrate 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C0 and mAb anti-6His Eu cryptate gold premixed	Repeat Well A4	Repeat Well A4
B	5 µL of VHL Binding Standard 5 µL of PROTAC binding buffer 1 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C1 and mAb anti-6His Eu cryptate gold premixed	Repeat Well B1	Repeat Well B1	5 µL of diluent #9 5 µL of PROTAC binding buffer 1 or PROTAC protein substrate 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C1 and mAb anti-6His Eu cryptate gold premixed	Repeat Well B4	Repeat Well B4
C	5 µL of VHL Binding Standard 5 µL of PROTAC binding buffer 1 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C2 and mAb anti-6His Eu cryptate gold premixed	Repeat Well C1	Repeat Well C1	5 µL of diluent #9 5 µL of PROTAC binding buffer 1 or PROTAC protein substrate 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C2 and mAb anti-6His Eu cryptate gold premixed	Repeat Well C4	Repeat Well C4
D	5 µL of VHL Binding Standard 5 µL of PROTAC binding buffer 1 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C3 and mAb anti-6His Eu cryptate gold premixed	Repeat Well D1	Repeat Well D1	5 µL of diluent #9 5 µL of PROTAC binding buffer 1 or PROTAC protein substrate 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C3 and mAb anti-6His Eu cryptate gold premixed	Repeat Well D4	Repeat Well D4
E	5 µL of VHL Binding Standard 5 µL of PROTAC binding buffer 1 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C4 and mAb anti-6His Eu cryptate gold premixed	Repeat Well E1	Repeat Well E1	5 µL of diluent #9 5 µL of PROTAC binding buffer 1 or PROTAC protein substrate 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C4 and mAb anti-6His Eu cryptate gold premixed	Repeat Well E4	Repeat Well E4
F	5 µL of VHL Binding Standard 5 µL of PROTAC binding buffer 1 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C5 and mAb anti-6His Eu cryptate gold premixed	Repeat Well F1	Repeat Well F1	5 µL of diluent #9 5 µL of PROTAC binding buffer 1 or PROTAC protein substrate 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C5 and mAb anti-6His Eu cryptate gold premixed	Repeat Well F4	Repeat Well F4
G	5 µL of VHL Binding Standard 5 µL of PROTAC binding buffer 1 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C6 and mAb anti-6His Eu cryptate gold premixed	Repeat Well G1	Repeat Well G1	5 µL of diluent #9 5 µL of PROTAC binding buffer 1 or PROTAC protein substrate 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C6 and mAb anti-6His Eu cryptate gold premixed	Repeat Well G4	Repeat Well G4
H	5 µL of VHL Binding Standard 5 µL of PROTAC binding buffer 1 5µl 6His-VHL protein complex 5 µL of HTRF VHL-Red Ligand C7 and mAb anti-6His Eu cryptate gold premixed	Repeat Well H1	Repeat Well H1	5 µL of diluen 5 µL of PROTA ing buffer 1 or PROTAC		



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.

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

3. Calculate the specific binding signal for each concentration used of HTRF VHL-Red Ligand.

Specific binding signal = Total binding signal – Non-specific binding signal

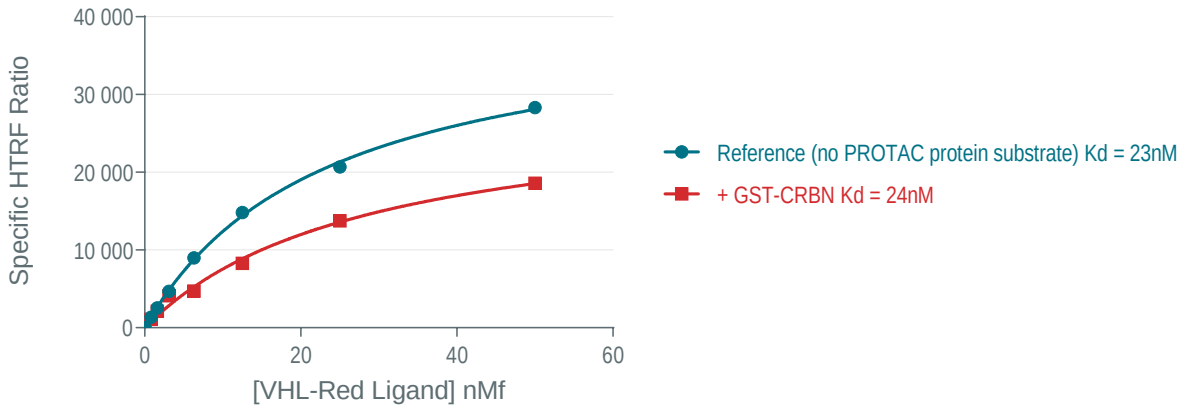
For more information about data reduction, please visit www.revvity.com

RESULTS

This data must not be substituted for the data obtained in the laboratory and should be considered only as an example (readouts on an HTRF compatible reader). Results may vary from one HTRF® compatible reader to another.

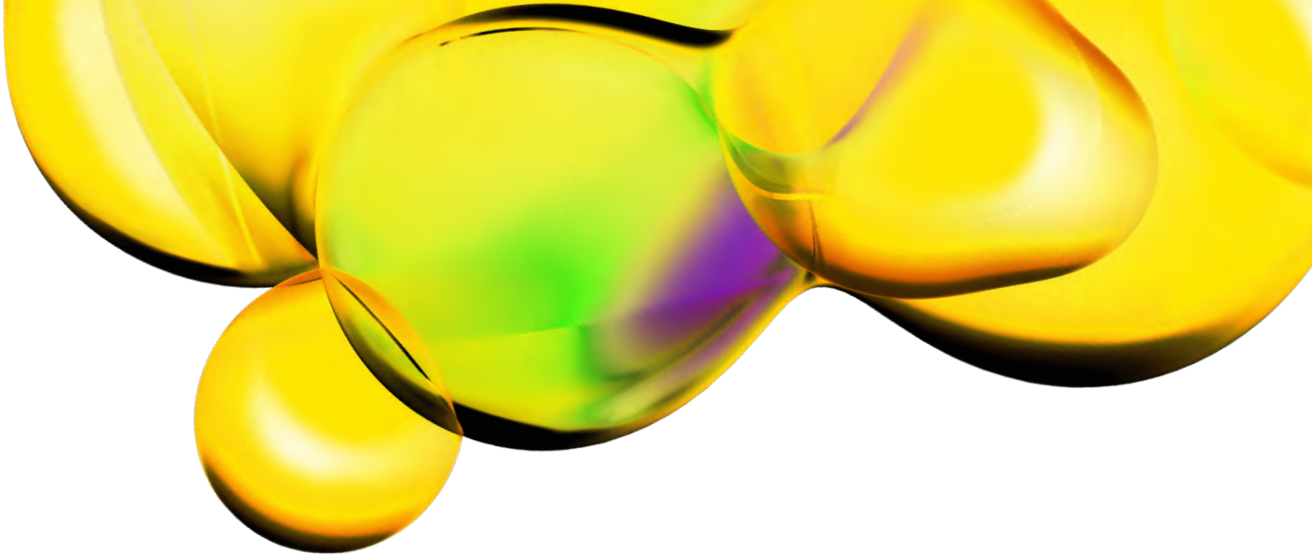
In this example, the binding affinity (Kd) of the VHL-Red Ligand to 6His-VHL protein complex was assessed in the absence or presence of the target protein CRBN as example of preliminary cooperativity studies of CRBN/VHL PROTAC compounds. These results indicate that the presence of 65nM of CRBN protein does not change the Kd of the Red Ligand.

This example shows that cooperativity studies can be set up with the HTRF VHL binding kit and the alpha factor can be calculated by dividing the Ki of the studied CRBN-6-5-5-VHL PROTAC compound in the binary complex (VHL-PROTAC) by the Ki of the PROTAC compound in the ternary complex (VHL -PROTAC-CRBN). As shown here, the signal max may vary in presence of the target protein.



ANALYTICAL CHARACTERISTICS

HTRF VHL-Red Ligand Kd (reference without PROTAC protein substrate)	24.8nM ±9.7 (2SD)



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