

# MANUAL

Technology: HTRF®

Protein-Protein Interaction

# HTRF Human XIAP BIR3 Red Ligand

Part number	64XIAPFLRED
Test size	10 binding assays (Kd determination)

Storage:  $\leq -16^{\circ}\text{C}$

Assay volume: 20  $\mu\text{L}$

Version: 26

Date: February 2024

## ASSAY PRINCIPLE

Revvity' XIAP BIR3 Red Ligand (#64XIAPFLRED) is primarily intended to perform affinity binding curves using HTRF® technology and can be used in association with the HTRF XIAP BIR3 Binding kit (#64XIAPB3PEG/H).

The HTRF XIAP BIR3 Red Ligand (A410099.1 derivative) is particularly suited for cooperativity binding studies to assess the effect of a PROTAC protein substrate on the affinity of the XIAP BIR3 Red Ligand for XIAP BIR3 protein. HTRF XIAP BIR3 Red Ligand binding is detected in a direct binding assay format using an anti-GST antibody labelled with Terbium Cryptate which binds to Human XIAP BIR3 GST-tagged. 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  $K_d$  for HTRF XIAP BIR3 Red Ligand (Fig. 1)

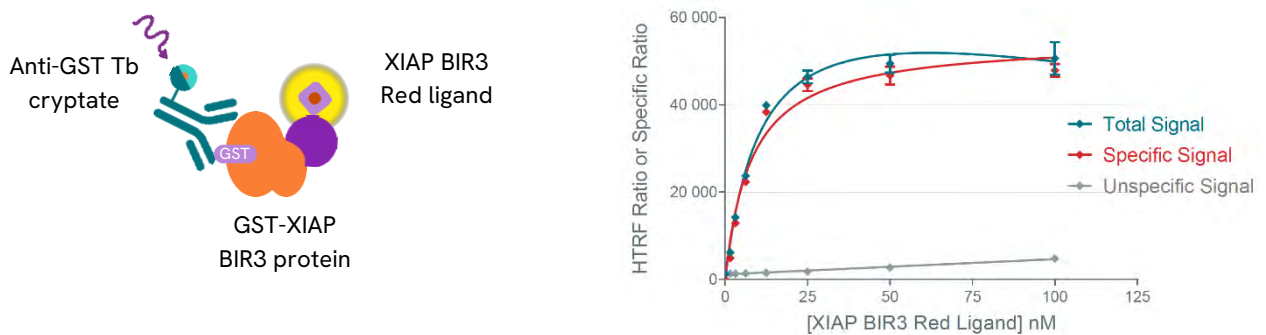
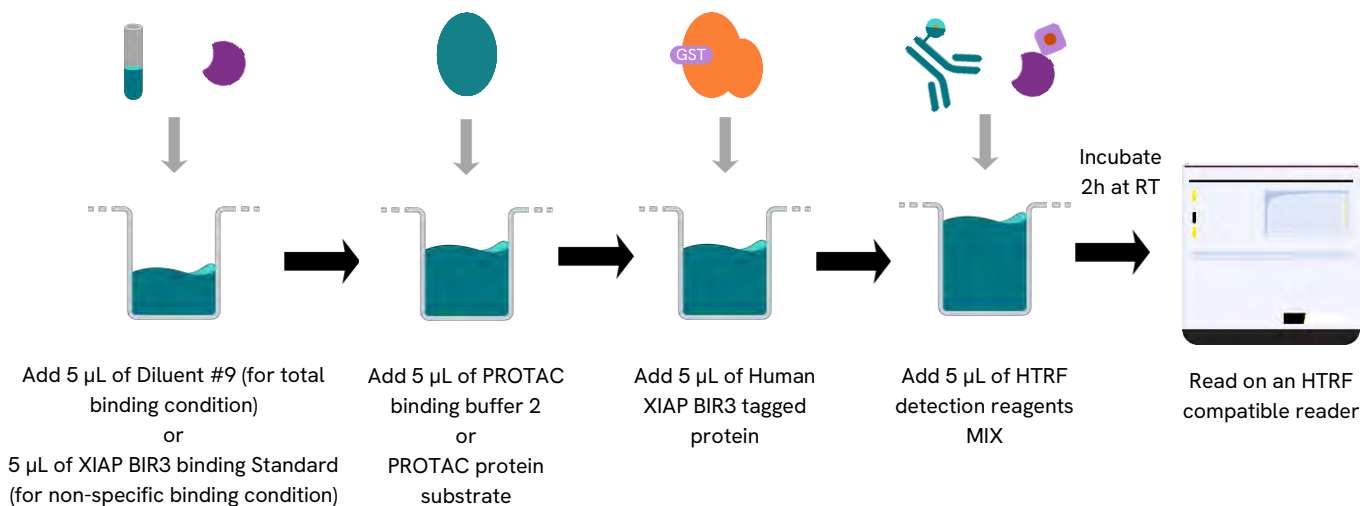


Figure 1: Principle of  $K_d$  determination with HTRF XIAP BIR3 Red Ligand.

## PROTOCOL 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 Terbium Cryptate. For more information about setup and HTRF® compatible readers, please visit our website.

## MATERIAL PROVIDED

### KIT COMPONENTS

HTRF XIAP BIR3 Red Ligand - 5X  
(A410099.1 derivative)

1 vial - 200  $\mu$ L  
Frozen

### Purchase separately

- XIAP BIR3 Standard (#Revvity 64XIAPB3CDA)
- HTRF PROTAC Binding Buffer 2 (#Revvity 64BDE32RDF)
- Diluent 9 (5X) (#Revvity 62DL9DDA/C)
- HTRF XIAP BIR3 Binding kit (#Revvity 64XIAPB3PEG/H)
- Low volume white (only) microplate\*
- HTRF®-Certified Reader \*\*. Make sure the setup for Terbium Cryptate is used.

\* For HTRF microplate recommendations, please visit our website.

\*\* For a list of HTRF-compatible readers and setup recommendations, please visit our website.

## STORAGE AND STABILITY

Store the HTRF XIAP BIR3 Red Ligand at  $-16^{\circ}\text{C}$  or below. Under appropriate storage conditions, HTRF XIAP BIR3 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  $-16^{\circ}\text{C}$  or below. Aliquot and store rapidly.

Volume of HTRF XIAP BIR3 Red Ligand aliquots should not be under 10  $\mu$ L.

Once thawed, aliquoted HTRF XIAP BIR3 Red Ligand can be frozen once.

Working solutions should be prepared the day of the experiment and not stored for longer period.

## REAGENT PREPARATION

### Before you begin

- It is recommended to prepare all reagents in the specified buffers. The use of incorrect buffers may affect reagent stability and assay results.
- XIAP BIR3 binding standard will be used to determine the non-specific binding signal.
- PROTAC Binding Buffer 2 is ready-to-use.
- XIAP BIR3 GST tagged protein must be prepared following instructions from the package insert of the HTRF XIAP BIR3 Binding Kit.
- Diluent 9 (5X) must be homogenized with a vortex then diluted 5-fold with distilled water (e.g. 200 $\mu$ L of Diluent 9 (5X) + 800  $\mu$ L of distilled water) and mixed gently to obtain the Diluent 9 (1X).

### XIAP BIR3 binding standard

#### To prepare the XIAP BIR3 binding standard stock solution:

Thaw the XIAP BIR3 binding Standard. Centrifuge.

The stock solution (200  $\mu$ M) can be frozen and stored at  $-16^{\circ}\text{C}$  or below.

#### To prepare the XIAP BIR3 binding standard working solution:

Homogenize the XIAP BIR3 binding Standard stock solution then dilute it 10-fold with Diluent 9 (1X) (e.g. 20 $\mu$ L of XIAP BIR3 binding Standard stock solution + 180 $\mu$ L Diluent 9 (1X)). Mix gently. XIAP BIR3 binding standard working concentration is 20  $\mu$ M.



## XIAP BIR3 red ligand

### To prepare the XIAP BIR3 red ligand stock solution:

Thaw the HTRF XIAP BIR3 Red Ligand. Centrifuge.

This 5X stock solution can be frozen and stored at -16°C or below.

### To prepare the XIAP BIR3 red ligand working solutions:

Procedure to prepare one binding curve of the HTRF XIAP BIR3 Red Ligand (for monitoring Total and Non-specific Binding):

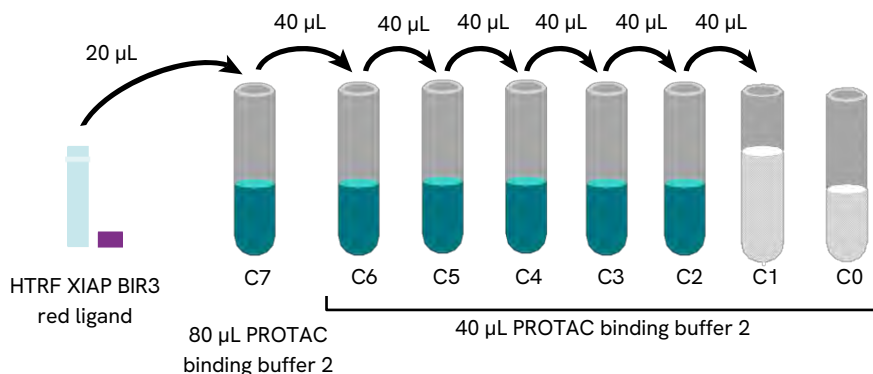
Dilute the XIAP BIR3 Red Ligand stock solution 5-fold with PROTAC Binding Buffer 2 to prepare high XIAP BIR3 Red Ligand (C7), (e.g. 20 µL of XIAP BIR3 Red Ligand stock solution + 80 µL of PROTAC Binding Buffer 2). Mix gently.

Use the high XIAP BIR3 Red Ligand (C7) to prepare XIAP BIR3 Red Ligand recommended range of concentrations using 1/2 serial dilutions as follows:

- Dispense 40 µL of PROTAC Binding Buffer 2 into each vial from C6 to C1.
- Add 40 µL of XIAP BIR3 Red Ligand to 40 µL of PROTAC Binding Buffer 2, mix gently and repeat the 1/2 serial dilution to make standard solutions: C6, C5, C4, C3, C2, C1.
- C0 is PROTAC Binding Buffer 2 buffer alone (Negative control).

This will create 7 concentrations for the XIAP BIR3 red ligand to be used for total and non-specific binding conditions.

*In order to counteract any sticking, we recommend changing tips between each dilution.*



## GST Tb cryptate antibody

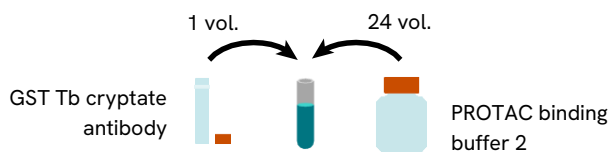
### To prepare the GST Tb cryptate stock solution:

Thaw the GST Tb Cryptate antibody included in the XIAP BIR3 Binding kit (#Revvity 64XIAPB3PEG/H). Centrifuge.

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

### To prepare the GST Tb cryptate working solution:

Homogenize the GST Tb Cryptate antibody stock solution then dilute it 25-fold with PROTAC Binding Buffer 2, (e.g. 80µL of GST Tb Cryptate antibody stock solution + 1920µL of PROTAC Binding Buffer 2). Mix gently.

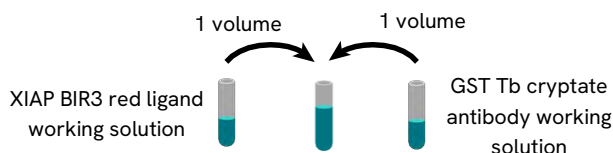


**WARNING:** Do not prepare the mAb anti-GST Tb Cryptate working solution following instructions from the package insert of the XIAP BIR3 Binding kit (#Revvity 64XIAPB3PEG/H).

## Detection reagent mix solutions of HTRF XIAP BIR3 red ligand + GST Tb cryptate antibody:

Just prior dispensing, mix each working solution of HTRF XIAP BIR3 Red Ligand and GST Tb Cryptate antibody by adding 1 volume of GST Tb Cryptate antibody working solution to 1 volume of each HTRF XIAP BIR3 Red Ligand working solutions (C7 to C0) (e.g. 50  $\mu$ L of HTRF XIAP BIR3 Red Ligand working solution + 50  $\mu$ L of GST Tb Cryptate antibody working solution).

This will create 7 Mix for the XIAP BIR3 Red Ligand and GST Tb Cryptate antibody.



Mix	Mix preparation	XIAP BIR3 red ligand concentration in the mix solution (nM)	XIAP BIR3 red ligand final concentration (nM)
Mix 7	50 $\mu$ L C7 XIAP BIR3 Red Ligand + 50 $\mu$ L of working solution of GST Tb Cryptate antibody	400	100
Mix 6	50 $\mu$ L C6 XIAP BIR3 Red Ligand + 50 $\mu$ L of working solution of GST Tb Cryptate antibody	200	50
Mix 5	50 $\mu$ L C5 XIAP BIR3 Red Ligand + 50 $\mu$ L of working solution of GST Tb Cryptate antibody	100	25
Mix 4	50 $\mu$ L C4 XIAP BIR3 Red Ligand + 50 $\mu$ L of working solution of GST Tb Cryptate antibody	50	12.5
Mix 3	50 $\mu$ L C3 XIAP BIR3 Red Ligand + 50 $\mu$ L of working solution of GST Tb Cryptate antibody	25	6.25
Mix 2	50 $\mu$ L C2 XIAP BIR3 Red Ligand + 50 $\mu$ L of working solution of GST Tb Cryptate antibody	12.5	3.12
Mix 1	50 $\mu$ L C1 XIAP BIR3 Red Ligand + 50 $\mu$ L of working solution of GST Tb Cryptate antibody	6.25	1.56
Mix 0	50 $\mu$ L PROTAC Binding Buffer 2 + 50 $\mu$ L of working solution of GST Tb Cryptate antibody	0	0

## ASSAY PROTOCOL

		NON-SPECIFIC BINDING SIGNAL	TOTAL BINDING SIGNAL
Step 1		Dispense 5 $\mu$ L of XIAP BIR3 Binding Standard into each well	Dispense 5 $\mu$ L of Diluent#9 (1X) into each well
Step 2		Add 5 $\mu$ L of PROTAC Binding Buffer 2 or 5 $\mu$ L of PROTAC substrate protein (diluted in the PROTAC Binding Buffer 2)	
Step 3		Add 5 $\mu$ L of Human XIAP BIR3 GST Tagged protein	
Step 4		Add 5 $\mu$ L of premixed HTRF XIAP BIR3 Red Ligand and GST Tb Cryptate antibody working solutions to all wells	
Step 5		Seal the plate and incubate 2 hours at RT	
Step 6		Remove the plate sealer and read on an HTRF® compatible reader	

## Example of map plate

	1	2	3	4	5	6
A	5 µL of XIAP BIR3 Binding Standard 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 0 (PROTAC Binding Buffer 2 and GST Tb Cryptate antibody)	Repeat Well A1	Repeat Well A1	5 µL of Diluent #9 (1X) 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 0 (PROTAC Binding Buffer 2 and GST Tb Cryptate antibody)	Repeat Well A4	Repeat Well A4
B	5 µL of XIAP BIR3 Binding Standard 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 1 (XIAP BIR3 Red Ligand C1 and GST Tb Cryptate antibody)	Repeat Well B1	Repeat Well B1	5 µL of Diluent #9 (1X) 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 1 (XIAP BIR3 Red Ligand C1 and GST Tb Cryptate antibody)	Repeat Well B4	Repeat Well B4
C	5 µL of XIAP BIR3 Binding Standard 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 2 (XIAP BIR3 Red Ligand C2 and GST Tb Cryptate antibody)	Repeat Well C1	Repeat Well C1	5 µL of Diluent #9 (1X) 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 2 (XIAP BIR3 Red Ligand C2 and GST Tb Cryptate antibody)	Repeat Well C4	Repeat Well C4
D	5 µL of XIAP BIR3 Binding Standard 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 3 (XIAP BIR3 Red Ligand C3 and GST Tb Cryptate antibody)	Repeat Well D1	Repeat Well D1	5 µL of Diluent #9 (1X) 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 3 (XIAP BIR3 Red Ligand C3 and GST Tb Cryptate antibody)	Repeat Well D4	Repeat Well D4
E	5 µL of XIAP BIR3 Binding Standard 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 4 (XIAP BIR3 Red Ligand C4 and GST Tb Cryptate antibody)	Repeat Well E1	Repeat Well E1	5 µL of Diluent #9 (1X) 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 4 (XIAP BIR3 Red Ligand C4 and GST Tb Cryptate antibody)	Repeat Well E4	Repeat Well E4
F	5 µL of XIAP BIR3 Binding Standard 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 5 (XIAP BIR3 Red Ligand C5 and GST Tb Cryptate antibody)	Repeat Well F1	Repeat Well F1	5 µL of Diluent #9 (1X) 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 5 (XIAP BIR3 Red Ligand C5 and GST Tb Cryptate antibody)	Repeat Well F4	Repeat Well F4
G	5 µL of XIAP BIR3 Binding Standard 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 6 (XIAP BIR3 Red Ligand C6 and GST Tb Cryptate antibody)	Repeat Well G1	Repeat Well G1	5 µL of Diluent #9 (1X) 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 6 (XIAP BIR3 Red Ligand C6 and GST Tb Cryptate antibody)	Repeat Well G4	Repeat Well G4
H	5 µL of XIAP BIR3 Binding Standard 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 7 (XIAP BIR3 Red Ligand C7 and GST Tb Cryptate antibody)	Repeat Well H1	Repeat Well H1	5 µL of Diluent #9 (1X) 5 µL of PROTAC Binding Buffer 2 or PROTAC protein substrate 5 µL Human XIAP BIR3 GST Tagged protein 5 µL MIX 7 (XIAP BIR3 Red Ligand C7 and GST Tb Cryptate antibody)	Repeat Well H4	Repeat Well H4

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
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## 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 XIAP BIR3 Red Ligand.

$$\text{Specific binding signal} = \text{total binding signal} - \text{non-specific binding signal}$$

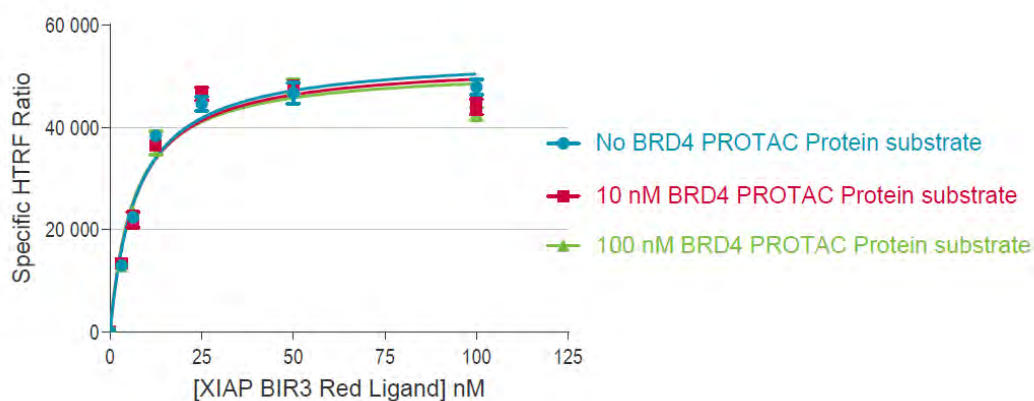
For more information about data reduction, please visit our website.

## 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 affinity binding (Kd) of the XIAP BIR3 Red Ligand to GST-XIAP BIR3 protein was assessed in the absence or presence of the targeted protein BRD4 (10nM or 100nM). This result indicates that the presence of BRD4 does not change the Kd of the Red Ligand.

Consequently, this example shows that a cooperativity experiment with a PROTAC molecule composed of a XIAP ligand and a BRD4 warhead can be set up. Finally, the alpha factor can be established by dividing the Ki of the PROTAC in the binary complex (XIAP BIR3 -PROTAC) by the Ki of the PROTAC in the ternary complex (XIAP BIR3 -PROTAC-BRD4).



HTRF XIAP BIR3 Red Ligand Kd  
(reference without PROTAC protein substrate)

4 nM ± 3

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