MANUAL

HTRF Human cIAP1 BIR2 Binding Kit

Part # 64BDIAPB2PEG & 64BDIAPB2PEH

Test Size#: 500 TESTS (64BDIAPB2PEG), 10,000 TESTS (64BDIAPB2PEH) - assay volume: 20 µL

Revision: #03 of September 2023 **Store at:** ≤-60°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 cIAP1 BIR2 binding assay is only intended for cIAP1 ligands binding measurement on the BIR2 protein domain using HTRF® technology.

cIAP1 ligands are detected in a competitive assay format using a specific GST antibody labelled with Terbium Cryptate (donor) which binds to Human cIAP1 BIR2 GST-tagged and LCL161-Biotin ligand associated to a Streptavidin labelled with d2 (acceptor). The detection principle is based on HTRF® technology. 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). Your compound competes with the LCL161-Biotin, and thereby prevents FRET from occurring. The specific signal is inversely proportional to the compound concentration (Fig. 1).

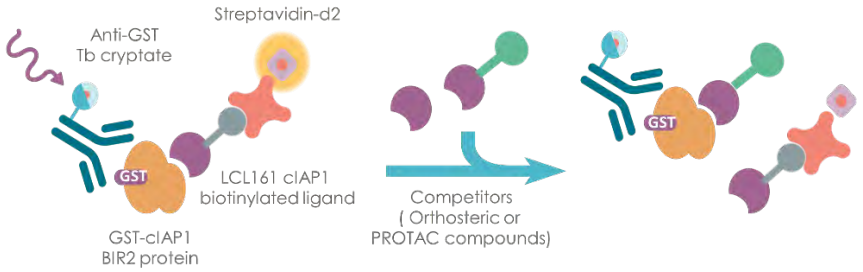
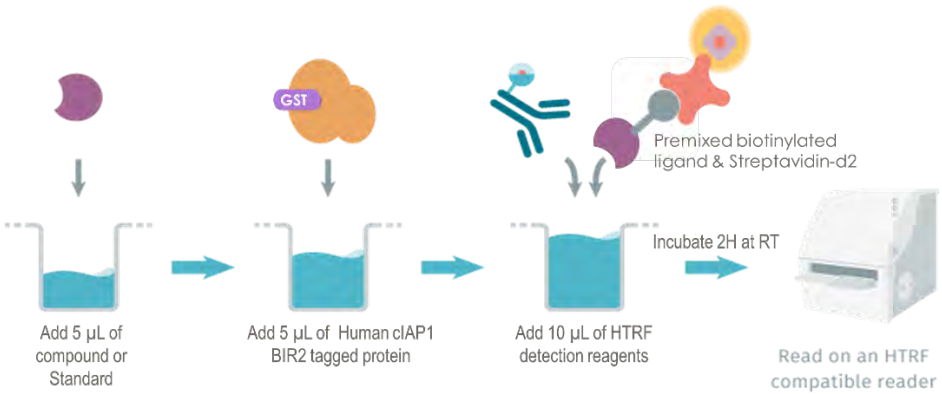


Figure 1: Principle of HTRF® cIAP1 BIR2 binding competitive assay.

MANUAL AT A GLANCE



Make sure you use the appropriate setup for Tb Cryptate. For more information about setup and HTRF® compatible readers, please visit our website at: www.revvity.com

MATERIALS PROVIDED:

KIT COMPONENTS	500 TESTS* CAT # 64BDIAPB2PEG	10,000 TESTS* CAT # 64BDIAPB2PEH
cIAP1 BIR2 binding standard Frozen - 10X	1 vial - 30 µL	2 vials - 30 µL
GST Tb Cryptate Antibody	1 vial - 50 µL Frozen - 50X	1 vial - 1 mL Frozen - 50X
LCL161-Biotin ligand	1 vial - 50 µL Frozen - 50X	1 vial - 1 mL Frozen - 50X
Streptavidin-d2	1 vial - 50 µL Frozen - 50X	1 vial - 1 mL Frozen - 50X
Human cIAP1-BIR2 GST-tagged	1 vial - 125 µL Frozen - 20X	4 vials - 625 µL Frozen - 20X
Diluent #9 - 5X	3 vials - 2 mL	1 vial - 100 mL
PROTAC Binding Buffer 2 – 1X ready-to-use	1 vial - 20 mL	1 vial - 220 mL

* When used as advised, the two available kit sizes will provide sufficient reagents for 500 and 10,000 tests respectively in 20 µL final.
Assay volumes can be adjusted proportionally to run the assay in 96 or 1536 well microplates.

PURCHASE SEPARATELY:

- Low volume white (only) microplate*
- HTRF®-Certified Reader **. Make sure the setup for Tb 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

Storage upon reception:
Store the kit at -60°C or below until the expiration date indicated on the package.

Storage and stability of thawed material:
When you are ready to use the kit, take the reagents out and prepare them following the manual provided in this document. Unused thawed reagents can be stored and conserved for future use. Refer to the table below for storage options and corresponding shelf life.

	Storage after Thawing/reconstitution
Lysis Buffer / Blocking Reagent / Detection buffer	2-8°C until the expiration date indicated on the package
Antibodies*	2-8°C for 48h or freeze at -16°C or below until the expiration date indicated on the package for long term storage
Protein/standard /Control Lysate*	freeze at -60°C or below until the expiration date indicated on the package for long term storage

*For Antibodies, Protein, Standard & control lysate, Stock solutions may be thawed and frozen only once. Freeze in aliquots to avoid multiple freeze/thaw cycles (once aliquoted, single use of the reagent). Volume of antibodies aliquots should not be under 10µL. Volume of Protein, Standard & control lysate aliquots should not be under 20µL.
Working solutions should be prepared the day of the experiment and not stored for longer period.

REAGENT PREPARATION

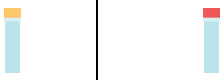
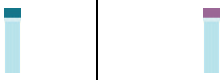
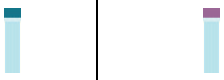
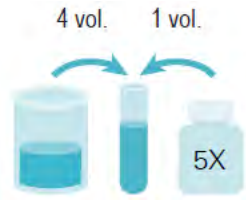
BEFORE YOU BEGIN:

- It is very important to prepare reagents in the specified buffers. The use of an incorrect diluent or Binding buffer may affect reagent stability and assay results.
- Thaw protein on ice, other reagents can be thawed at room temperature
- Before use, allow buffer to warm up at room temperature and homogenize it with a vortex.

cIAP1 BIR2 binding standard (for standard curve) must be prepared in Diluent 9. The cIAP1 BIR2 binding standard is the LCL161.

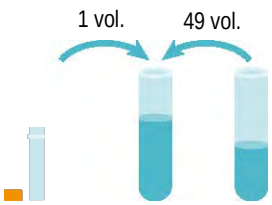
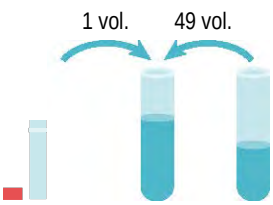
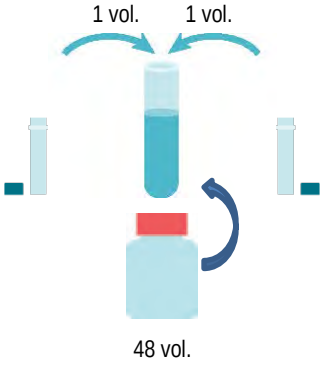
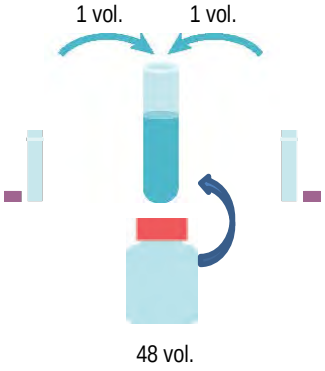
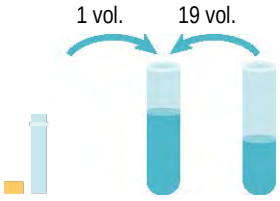
TAKE CARE TO PREPARE STOCK AND WORKING SOLUTIONS ACCORDING TO THE DIRECTIONS FOR THE KIT SIZE YOU HAVE PURCHASED.

TO PREPARE REAGENT STOCK SOLUTIONS:

500 TESTS KIT - 64BDIAPB2PEG		10,000 TESTS KIT - 64BDIAPB2PEH	
GST Tb Cryptate antibody			
Thaw the GST Tb Cryptate antibody. Centrifuge. This 50X stock solution can be frozen and stored at -16°C or below.			Thaw the GST Tb Cryptate antibody. Centrifuge. This 50X stock solution can be frozen and stored at -16°C or below.
Streptavidin-d2			
Thaw the Streptavidin-d2. Centrifuge. This 50X stock solution can be frozen and stored at -16°C or below			Thaw the Streptavidin-d2. Centrifuge. This 50X stock solution can be frozen and stored at -16°C or below
LCL161-Biotin ligand			
Thaw the LCL161-Biotin ligand. Centrifuge. This 50X stock solution can be frozen and stored at -16°C or below.			Thaw the LCL161-Biotin ligand. Centrifuge. This 50X stock solution can be frozen and stored at -16°C or below.
cIAP1 BIR2 binding standard			
Thaw the cIAP1 BIR2 binding standard. Centrifuge. This 10 X stock solution can be frozen and stored at -60°C or below.			Thaw the cIAP1 BIR2 binding standard. Centrifuge. This 10 X stock solution can be frozen and stored at -60°C or below.
Human cIAP1 BIR2 GST-tagged			
Thaw the Human cIAP1 BIR2 GST-tagged on ice. Centrifuge the vial. To avoid freeze/thaw cycles, it is recommended to aliquot the remainder of this 20X stock solution under 10 µL minimum in disposable plastic vials for storage at ≤-60°C.			Thaw the Human cIAP1 BIR2 GST-tagged on ice. Centrifuge the vial. To avoid freeze/thaw cycles, it is recommended to aliquot the remainder of this 20X stock solution under 10 µL minimum in disposable plastic vials for storage at ≤-60°C.
Diluent #9			
Dilute 5-fold the 5X diluent #9 with distilled water: Homogenize the 5X diluent #9 with a vortex and add 1 volume of stock solution in 4 volumes of distilled water, e.g. 1 mL of diluent + 4 mL of distilled water. Mix gently after dilution.			Dilute 5-fold the 5X diluent #9 with distilled water: Homogenize the 5X diluent #9 with a vortex and add 1 volume of stock solution in 4 volumes of distilled water, e.g. 10 mL of diluent + 40 mL of distilled water. Mix gently after dilution.
PROTAC Binding buffer 2			
Ready to use.			Ready to use.

TO PREPARE WORKING SOLUTIONS:

Each well requires 5 µL of each reagent. Prepare in separate vials.

<table>
<tr><th colspan="2">500 TESTS KIT - 64BDIAPB2PEG</th><th colspan="2">10,000 TESTS KIT - 64BDIAPB2PEH</th></tr>
<tr><td colspan="4">GTP Tb Cryptate antibody</td></tr>
<tr><td>Dilute 50-fold the 50X stock solution (thawed reagent) of GST Tb cryptate antibody with PROTAC Binding buffer 2 (1X), eg 10 µL of thawed Tb cryptate antibody stock solution + 490 µL of PROTAC Binding buffer 2 (1X).</td><td> 
</td><td> 
</td><td>Dilute 50-fold the 50X stock solution (thawed reagent) of GST Tb cryptate antibody with PROTAC Binding buffer 2 (1X), eg 10 µL of thawed Tb cryptate antibody stock solution + 490 µL of PROTAC Binding buffer 2 (1X).</td></tr>
<tr><td colspan="4">Streptavidin-d2 and LCL161-Biotin acceptor solution Premix</td></tr>
<tr><td>To prepare acceptor solution, dilute 50-fold the 50X stock solutions (thawed reagents) of Streptavidin-d2 and LCL161-Biotin ligand with PROTAC Binding Buffer 2 (1X), eg 10 µL of thawed Streptavidin-d2 stock solution + 10 µL of thawed LCL161-Biotin ligand + 480 µL of PROTAC Binding buffer 2 (1X).</td><td> 
</td><td> 
</td><td>To prepare acceptor solution, dilute 50-fold the 50X stock solutions (thawed reagents) of Streptavidin-d2 and LCL161-Biotin ligand with PROTAC Binding Buffer 2 (1X), eg 10 µL of thawed Streptavidin-d2 stock solution + 10 µL of thawed LCL161-Biotin ligand + 480 µL of PROTAC Binding buffer 2 (1X).</td></tr>
<tr><td colspan="4">Human cIAP1 BIR2 GST-tagged</td></tr>
<tr><td>Dilute 20-fold the 20X stock solution (thawed reagent on ice) of Human cIAP1 BIR2 GST-tagged protein with PROTAC Binding buffer 2 (1X), eg 10 µL of thawed protein stock solution + 190 µL of PROTAC Binding buffer 2 (1X).</td><td colspan="2"> 
</td><td>Dilute 20-fold the 20X stock solution (thawed reagent on ice) of Human cIAP1 BIR2 GST-tagged protein with PROTAC Binding buffer 2 (1X), eg 10 µL of thawed protein stock solution + 190 µL of PROTAC Binding buffer 2 (1X).</td></tr>
</table>

TO PREPARE WORKING STANDARD SOLUTIONS:

- Each well requires 5 µL of standard.
- Dilute the standard stock solution serially with Diluent#9 (1X) **.**
- In order to counteract any standard sticking, we recommend changing tips between each dilution.

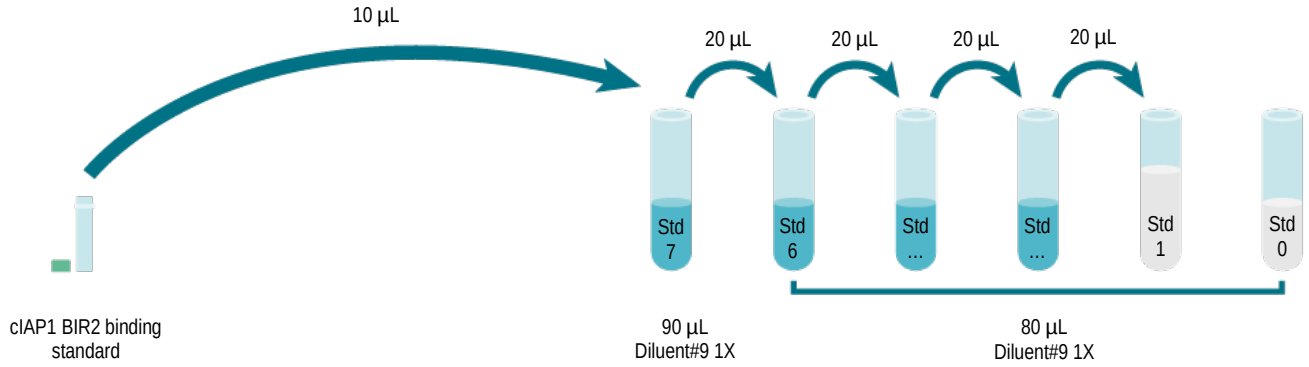
A recommended standard dilution procedure is listed and illustrated below:

Dilute the standard stock solution 10-fold with Diluent#9 (1X) to prepare high standard (Std 7): take 10 µL of standard stock solution and add it to 90 µL of Diluent#9 (1X) . Mix gently.

Use the high standard (Std 7) to prepare the standard curve using 1/5 serial dilutions as follows:

- Dispense 80 µL of Diluent#9 (1X) into each vial from Std 6 to Std 0.
- Add 20 µL of standard to 80 µL of Diluent#9 (1X) , mix gently and repeat the 1/5 serial dilution to make standard solutions: Std6, Std5, Std4, Std3, Std2, Std1.

This will create 7 standards for the analyte. Std 0 (Positive control) is Diluent#9 (1X) buffer alone.



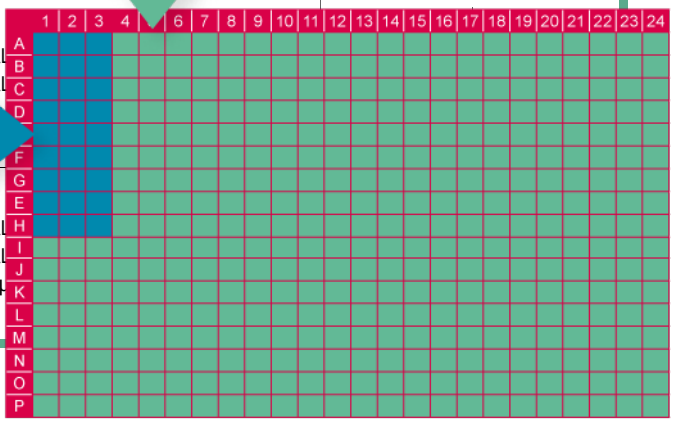
STANDARD	SERIAL DILUTIONS	"cIAP1 BIR2 BINDING STANDARD WORKING SOLUTION (µM)"	"cIAP1 BIR2 BINDING STANDARD FINAL CONCENTRATION (µM)"
Standard Stock solution	Thawed stock solution	20 000	-
Standard 7	10µl standard stock solution + 90 µL Diluent#9 (1X)	2 000	500
Standard 6	20 µL standard 7 + 80 µL Diluent#9 (1X)	400	100
Standard 5	20 µL standard 6 + 80 µL Diluent#9 (1X)	80	20
Standard 4	20 µL standard 5 + 80 µL Diluent#9 (1X)	16	4
Standard 3	20 µL standard 4 + 80 µL Diluent#9 (1X)	3.2	0.8
Standard 2	20 µL standard 3 + 80 µL Diluent#9 (1X)	0.64	0.16
Standard 1	20 µL standard 2 + 80 µL Diluent#9 (1X)	0.13	0.032
Standard 0	100 µL Diluent#9 (1X)	0	0

ASSAY MANUAL

	NEGATIVE CONTROL (OR CRYPTATE CONTROL)	STANDARD (STD 0 - STD 7)	COMPOUND
Step 1 	Dispense 5 µL of diluent into each negative control well.	Dispense 5 µL of each cIAP1 BIR2 binding standard (Std 0 - Std 7) into each standard well.	Dispense 5 µl of Compound diluted in Diluent#9 (1X)
Step 2 	Add 5 µL of PROTAC Binding buffer 2 to all wells	Add 5 µL of Human cIAP1 BIR2 GST-tagged protein to all wells	
Step 3 	Add 10 µL of a mix (vol:vol) of acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody to all wells		
Step 4 	Seal the plate and incubate 2 hours at RT		
Step 5 	Remove the plate sealer and read on an HTRF® compatible reader		

EXAMPLE OF PLATE MAP

	1	2	3	4	5	6
A	(Negative control) 5 µL of diluent #9 5 µL of PROTAC Binding buffer 2 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well A1	Repeat Well A1	5 µL Compound 1 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well A4	Repeat Well A4
B	5 µL Std 0 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well B1	Repeat Well B1	5 µL Compound 2 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well B4	Repeat Well B4
C	5 µL Std 1 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well C1	Repeat Well C1	5 µL Compound 3 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well C4	Repeat Well C4
D	5 µL Std 2 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well D1	Repeat Well D1	5 µL Compound ... 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well D4	Repeat Well D4
E	5 µL Std ... 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well E1	Repeat Well E1	5 µL Compound ... 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well E4	Repeat Well E4
F	5 µL Std ... 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well F1	Repeat Well F1	5 µL Compound ... 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb anti	Repeat Well F4	Repeat Well F4
G	5 µL Std ... 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well G1	Repeat Well G1	5 µL 5 µL		
H	5 µL Std ... 5 µL of cIAP1 BIR2 GST-tagged 10 µL of mix (vol:vol) acceptor solution (Streptavidin-d2 / LCL161-Biotin ligand) and GST Tb antibody	Repeat Well H1	Repeat Well H1	5 µL 5 µL 10 µ		



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.

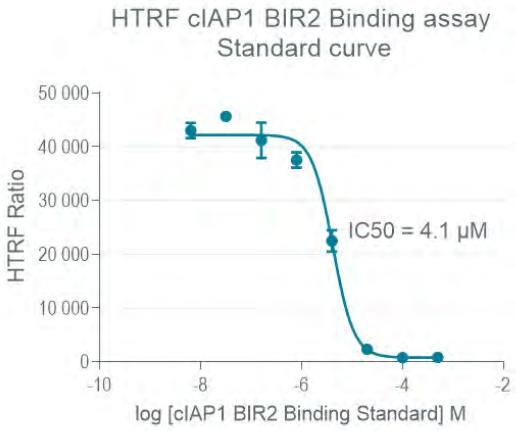
$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

For more information about data reduction, please visit 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.

	RATIO (1)	CV % (2)
Negative control	753	5
Std 0 – 0 µM	43001	3
Std 1 - 0.032 µM	45648	1
Std 2 – 0.16 µM	41181	8
Std 3 – 0.8 µM	37536	3
Std 4 – 4 µM	22489	9
Std 5 – 20 µM	2298	17
Std 6 - 100 µM	766	2
Std 7 - 500 µM	803	9



ANALYTICAL CHARACTERISTICS

HTRF cIAP1 BIR2 biotin Ligand (LCL161-biotin) Kd	10 nM
HTRF cIAP1 BIR2 biotin Ligand (LCL161-biotin) concentration	10 nM
HTRF cIAP1 BIR2 Binding Kit - Standard IC50 value	4100 nM
HTRF cIAP1 BIR2 Binding Kit - Standard Ki value	2050 nM

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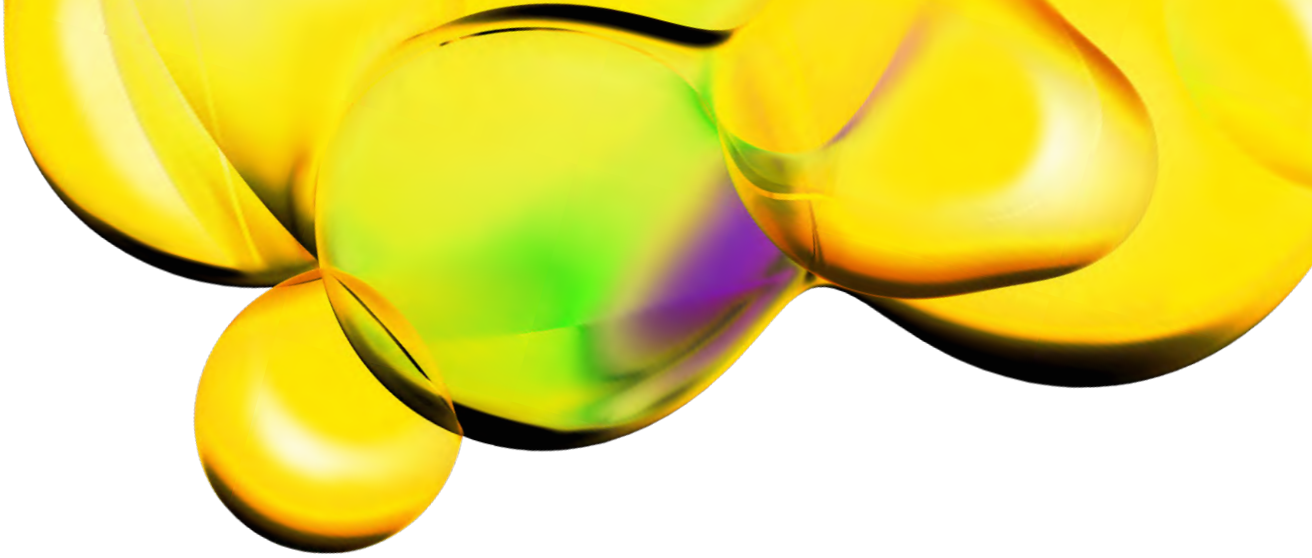

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www.revvity.com

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

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