MANUAL

β-arr2 recruitment KIT

Part # 62BDBAR2PEB & 62BDBAR2PEC

Test Size#: 1,000 TESTS (62BDBAR2PEB), 20,000 TESTS (62BDBAR2PEC)

Revision: #03 of September 2023 **Store at:** ≤-60°C

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

ASSAY PRINCIPLE

The HTRF β-arr2 recruitment assay is a sandwich immunoassay intended to measure the recruitment of β-arrestin 2 mediated by GPCR activation in cells. It enables the direct pharmacological characterization of compounds acting on β-arrestin 2- coupled receptors in various cellular models.

The principle is based on HTRF® technology. The interaction of native β-arrestin 2 and AP2 (as partner) expressed by cells is detected with Europium cryptate-labeled AP2 antibody (Europium donor) and d2-labeled B-arrestin2 antibody (acceptor). 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 signal modulates positively in proportion to the recruitment of β-arrestin 2 by interacting with AP2 (Fig. 1).

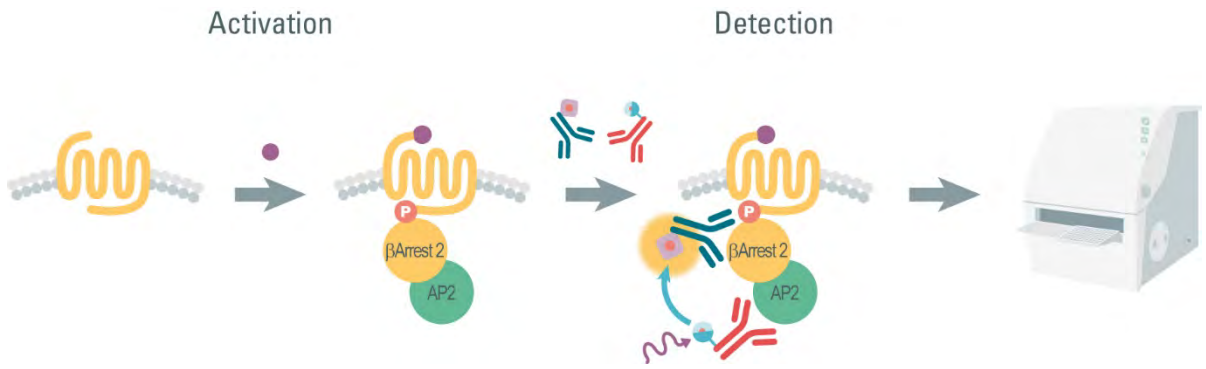
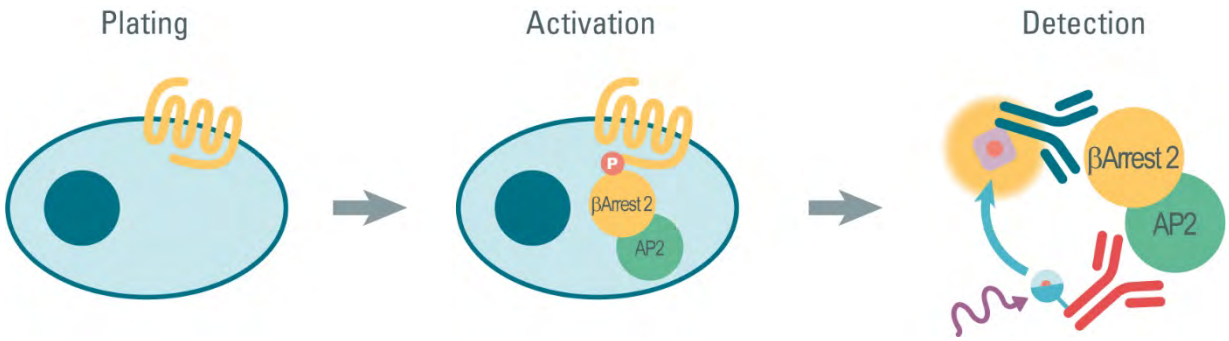


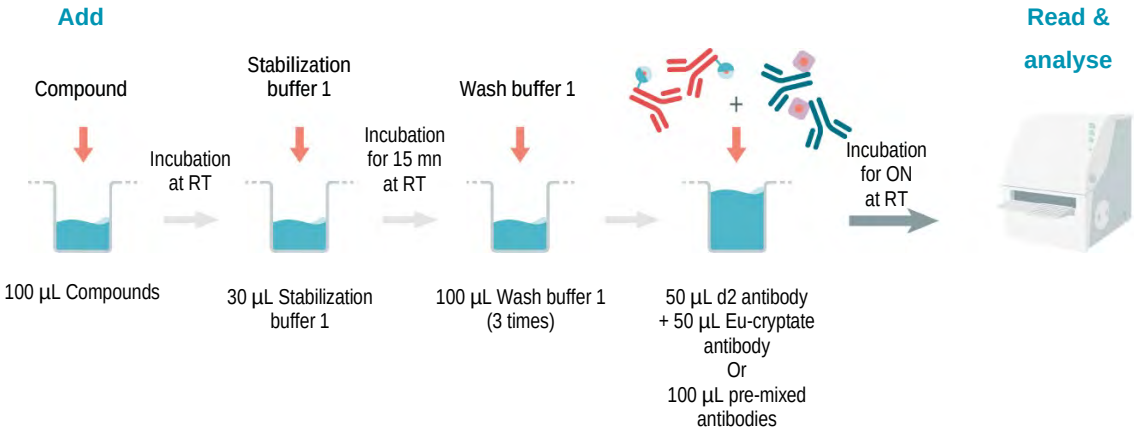
Figure 1: Principle of HTRF β-arr2 recruitment assays

Our technical support team can help you to set up this manual, or another. Please contact us at www.revvity.com

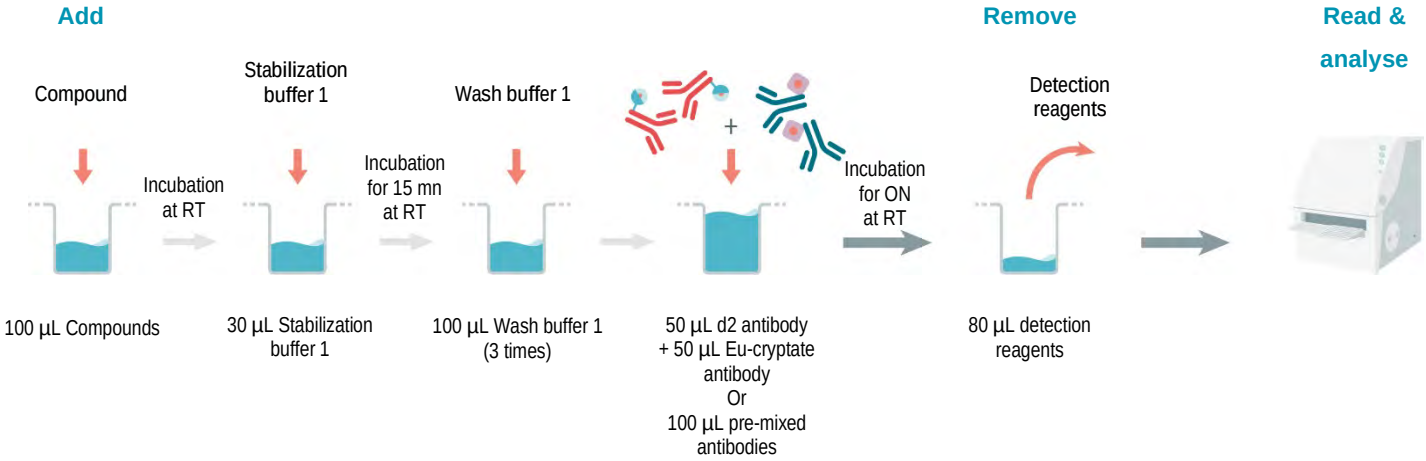
MANUAL AT A GLANCE



► ASSAY MANUAL FOR ADHERENT CELLS:



► ***Assay manual with optional step:*** *Optional step may improve detection.*



MATERIALS

KIT COMPONENTS	STORAGE	1,000 TESTS CAT # 62BDBAR2PEB	20,000 TESTS CAT # 62BDBAR2PEC
B-arr2 recruitment Eu Cryptate-antibody	≤-16°C	1 vial – 1mL 50X	1 vial - 20 mL 50X
B-arr2 recruitment d2-antibody	≤-16°C	1 vial – 1mL 50X	1 vial - 20 mL 50X
Stimulation buffer 4	≤-16°C	1 vial - 20 mL 5X	2 vials – 200mL 5X
Wash buffer 1	2-8°C	1 vial - 30 mL 10X	3 vials - 200 mL 10X
Stabilization buffer 1	2-8°C	1 vial - 30 mL Ready-to-use	3 vials - 200 mL Ready-to-use
Detection buffer 14	2-8°C	1 vial - 10 mL 10X	1 vial - 200 mL 10X

For reading, an HTRF®-Certified Reader is required. Make sure to use the setup for Eu Cryptate. For a list of HTRF-compatible readers and setup recommendations, please visit our website at: www.revvity.com

For HTRF microplate recommendations, please visit www.revvity.com

STORAGE AND STABILITY

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

Anti-Tag stock solutions can be frozen and thawed only twice.

To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage and ≤-16°C (anti-tags).

Thawed HTRF buffers can be stored at 2-8°C on your premises. Stimulation buffer 4 must be stored at ≤-16°C.

REAGENT PREPARATION

BEFORE YOU BEGIN:

- It is very important to prepare detection reagents in the Detection Buffer 14. The use of an incorrect buffer may affect assay results.
- Thaw the frozen reagents at room temperature.
- Before use, allow all reagents to warm up to room temperature, then homogenize buffer.
- The anti-Tag solutions must be prepared in individual vials and can be premixed prior to dispensing.
- Compounds may be prepared in Stimulation buffer 4. We recommend keeping DMSO below 1% in the assay (100 µL final volume).

TO PREPARE STOCK SOLUTIONS:

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

1,000 TESTS		20,000 TESTS	
d2 antibody			
Thaw the d2 antibody. Mix gently. This 50X d2 stock solution can be frozen and stored at ≤ -16°C.			Thaw the d2 antibody. Mix gently. This 50X d2 stock solution can be frozen and stored at ≤ -16°C.
Eu Cryptate-antibody			
Thaw the Eu Cryptate-antibody. Mix gently. This 50X Eu cryptate stock solution can be frozen and stored at ≤-16°C.			Thaw the Eu Cryptate-antibody. Mix gently. This 50X Eu cryptate stock solution can be frozen and stored at ≤-16°C.
Stimulation buffer 4			
Thaw the Stimulation buffer 4. This 5X thawed buffer can be stored at ≤-16°C on your premises.			Thaw the Stimulation buffer 4. This 5X thawed buffer can be stored at ≤-16°C on your premises.
Stabilization buffer 1			
Thaw the Stabilization buffer 1. This thawed buffer can be stored at 2-8°C on your premises.			Thaw the Stabilization buffer 1. This thawed buffer can be stored at 2-8°C on your premises.
Wash buffer 1			
Thaw the Wash buffer 1. This 10X thawed buffer can be stored at 2-8°C on your premises.			Thaw the Wash buffer 1. This 10X thawed buffer can be stored at 2-8°C on your premises.
Detection buffer 14			
Thaw the Detection buffer 14. This 10X thawed buffer can be stored at 2-8°C on your premises.			Thaw the Detection buffer 14. This 10X thawed buffer can be stored at 2-8°C on your premises.

TO PREPARE WORKING SOLUTIONS:

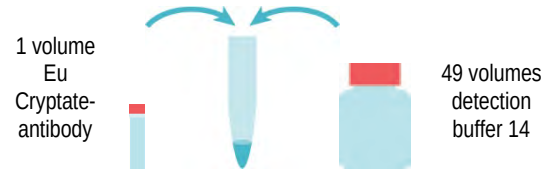
► TO PREPARE WORKING STABILIZATION BUFFER SOLUTION:

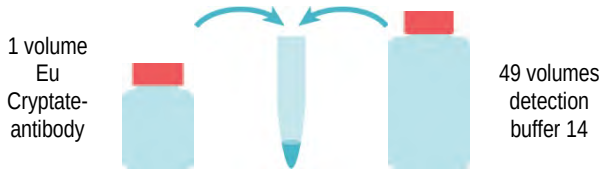
Thaw the Stabilization buffer 1 solution. Mix gently, and it is ready to use.

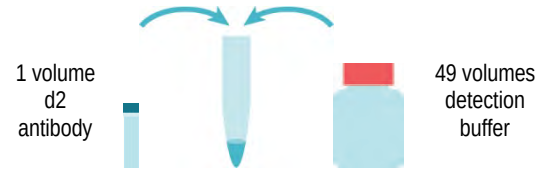
► TO PREPARE WORKING ANTIBODY SOLUTIONS:

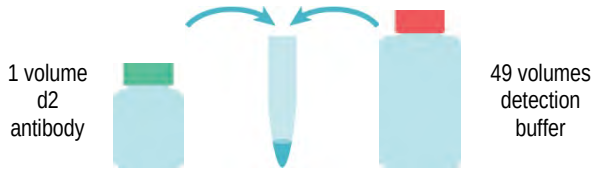
HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the d2 and Europium Cryptate-antibodies will impair the assay's quality. Be careful, as working solution preparation for the antibodies may differ between the 1,000 and 20,000 tests data point kits.

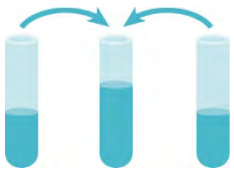
Antibody working solutions are stable for 2 days at 2-8°C. Dilute the antibodies with detection buffer 14. In practice:

<table>
<tr><th>1,000 TESTS</th><th>20,000 TESTS</th></tr>
<tr><td colspan="2">B-arr2 recruitment kit Eu Cryptate-antibody</td></tr>
<tr><td> 

Dilute 50-fold the frozen stock solution with detection buffer: e.g add 49 mL of detection buffer to the 1 mL of Eu cryptate-antibody stock solution.</td><td> 

Dilute 50-fold the frozen stock solution with detection buffer: e.g add 490 mL of detection buffer to the 10 mL of Eu cryptate-antibody stock solution.</td></tr>
<tr><td colspan="2">B-arr2 recruitment kit d2 antibody</td></tr>
<tr><td> 

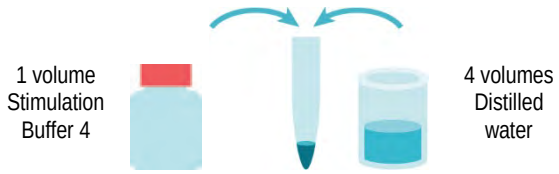
Dilute 50-fold the frozen stock solution with detection buffer: e.g add 49 mL of detection buffer to the 1 mL of d2-antibody stock solution.</td><td> 

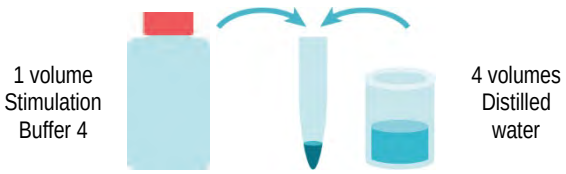
Dilute 50-fold the frozen stock solution with detection buffer: e.g add 490 mL of detection buffer to the 10 mL of d2-antibody stock solution.</td></tr>
<tr><td colspan="2">Antibody mix</td></tr>
<tr><td>It is recommended to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents, by adding 1 volume of d2-antibody solution to 1 volume of Eu Cryptate-antibody solution.</td><td>  </td><td>It is recommended to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents, by adding 1 volume of d2-antibody solution to 1 volume of Eu Cryptate-antibody solution.</td></tr>
</table>

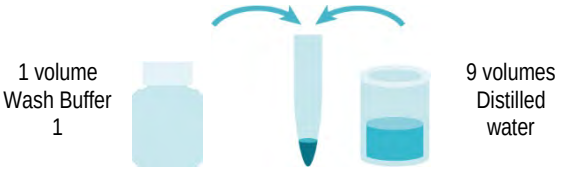
TO PREPARE WORKING BUFFER SOLUTIONS:

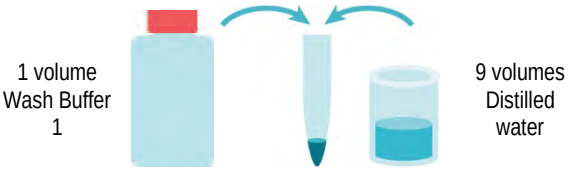
Buffers differ between HTRF kits. Make sure to use the appropriate buffers according to the manual in the section “Manual at a Glance”.

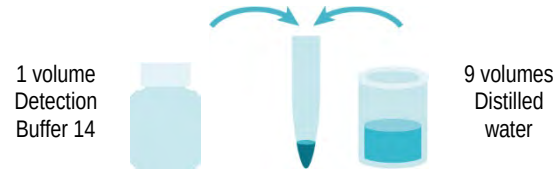
Prepare the required amount of each buffer before running the assay. Working solutions are stable at 2-8°C. It is recommended to filter each buffer before use.

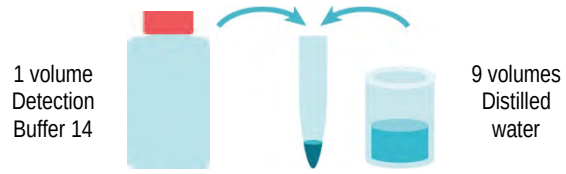
<table>
<tr><th>1,000 TESTS</th><th>20,000 TESTS</th></tr>
<tr><td colspan="2">Stimulation Buffer 4</td></tr>
<tr><td> 

Dilute 5-fold the Stimulation buffer 4 5X with distilled water: e.g add 1 mL of Stimulation buffer 4 5X to 4 mL of distilled water.</td><td> 

Dilute 5-fold the Stimulation buffer 4 5X with distilled water: e.g add 10 mL of Stimulation buffer 4 5X to 40 mL of distilled water.</td></tr>
<tr><td colspan="2">Wash buffer 1</td></tr>
<tr><td> 

Dilute 10-fold the Wash buffer 1 10X with distilled water: e.g add 1 mL of Wash buffer 1 10X to 9 mL of distilled water.</td><td> 

Dilute 10-fold the Wash buffer 1 10X with distilled water: e.g add 10 mL of Wash buffer 1 10X to 90 mL of distilled water.</td></tr>
<tr><td colspan="2">Detection buffer 14</td></tr>
<tr><td> 

Dilute 10-fold the Detection buffer 14 10X with distilled water: e.g add 1 mL of Detection buffer 14 10X to 9 mL of distilled water.</td><td> 

Dilute 10-fold the Detection buffer 14 10X with distilled water: e.g add 10 mL of Detection buffer 14 10X to 90 mL of distilled water.</td></tr>
</table>

ASSAY MANUAL

- Various cell parameters must be optimized depending on the type of compound to be screened (agonist or antagonist) and on the GPCR. These parameters include cell density, agonist concentration (for antagonist mode assay), and stimulation time.
- White opaque 96-well tissue-culture treated microplates are required for these assays. We recommend you use CulturPlate-96, White Opaque 96-well Microplate, Sterile and Tissue Culture Treated from Revvity - ref#6005680; 6005688; 6005689.

	NEGATIVE CONTROL*	NON-STIMULATED CELLS**	STIMULATED CELLS
Step 1 	Dispense 100 µL of cells into all wells.	Dispense 100 µL of cells into all wells.	Dispense 100 µL of cells into all wells.
Step 2 	Remove cell culture medium and add 100 µL Stimulation Buffer 4.	Remove cell culture medium and add 100 µL Stimulation Buffer 4.	Remove cell culture medium and add 100 µL compound.
Step 3 	Seal the plate and incubate the appropriate time at room temperature.	Seal the plate and incubate the appropriate time at room temperature.	Seal the plate and incubate the appropriate time at room temperature.
Step 4 	Remove Stimulation buffer 4 and add 30µL Stabilization buffer 1.	Remove Stimulation buffer 4 and add 30µL Stabilization buffer 1.	Remove compound and add 30µL Stabilization buffer 1.
Step 5 	Seal the plate and incubate for 15mn at room temperature.	Seal the plate and incubate for 15mn at room temperature.	Seal the plate and incubate for 15mn at room temperature.
Step 6 	Remove Stabilization buffer 1 and wash 3 times with 100µL Wash buffer 1.	Remove Stabilization buffer 1 and wash 3 times with 100µL Wash buffer 1.	Remove Stabilization buffer 1 and wash 3 times with 100µL Wash buffer 1.
Step 7 	Remove Wash buffer 1 and add 50µL Eu cryptate antibodies + 50µL detection buffer 14.	Remove Wash buffer 1 and add 100µL pre-mixed d2 and Eu cryptate antibodies.	Remove Wash buffer 1 and add 100µL pre-mixed d2 and Eu cryptate antibodies.
Step 8 	Seal the plate and incubate ON at room temperature.	Seal the plate and incubate ON at room temperature.	Seal the plate and incubate ON at room temperature.
Optional Step 9	Remove 80µL	Remove 80µL	Remove 80µL
Step 10 	Read the plate with an HTRF compatible reader.	Read the plate with an HTRF compatible reader.	Read the plate with an HTRF compatible reader.

*The Negative Control wells represent the non-specific fluorescence stemming from the Cryptate.
**Data with non-stimulated cells indicate the basal cellular level of B-arr2 recruitment before compound treatment.

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.

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

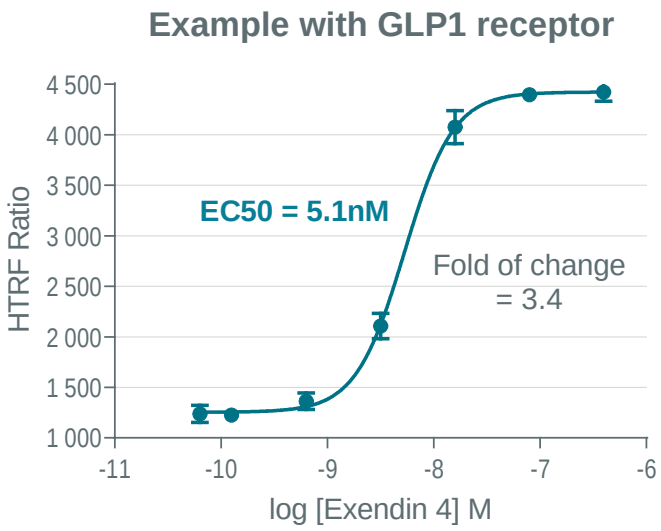
For more information about data reduction, please visit www.revvity.com

RESULTS

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

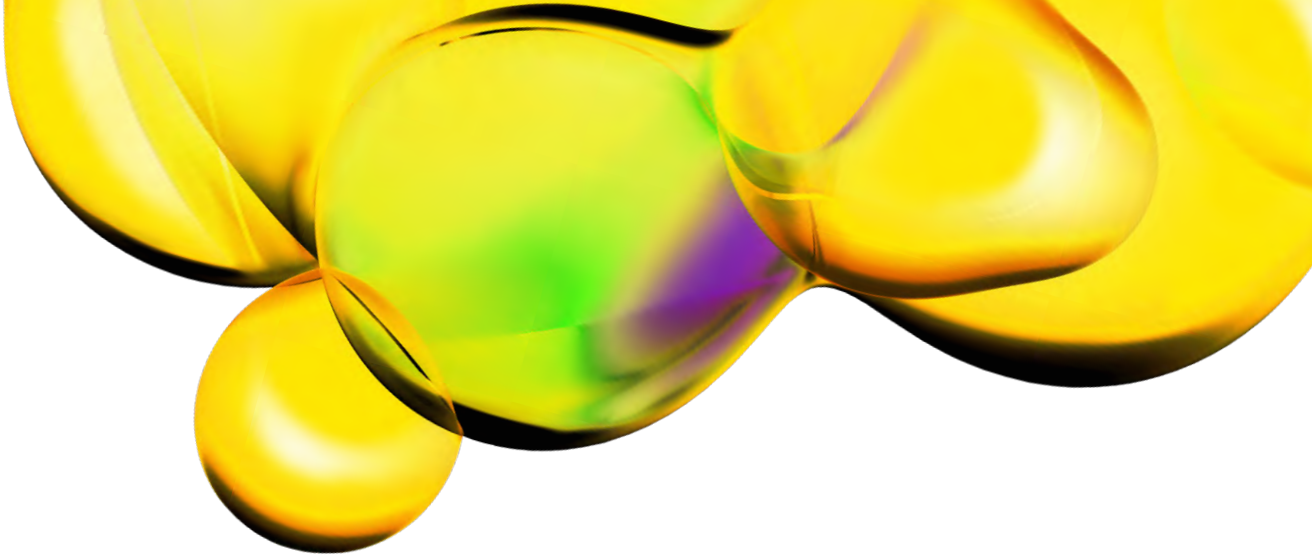
Readouts obtained on an HTRF compatible reader with a flash lamp.

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



GENERAL LAB WORK PRIOR TO USING B-ARR2 RECRUITMENT KIT: CELL PREPARATION - FREQUENTLY ASKED QUESTIONS / TROUBLESHOOTING PARAMETERS

Using adherent cells, allow time for your cells to recover after plating.	Allow cells to regain full signaling capacity by plating them at least 20 hours before starting the pharmacological treatment.
Depending on GPCRS and cell types, a serum starving step may be essential to reduce the basal level activity. This step should be optimized case-by-case.	Before treating the cells with compounds, remove culture media from the plate and replace it with serum-free media before incubating from 2 hours up to overnight at 37°C.
Adherent/suspension cells	The β-arr2 recruitment kit is suitable for adherent cells, but not compatible with suspension cells.
Using an improper cellular model can induce a low signal.	Check that the β-arrestin 2 and AP2 are well expressed in the cellular model used for the B-arr2 recruitment assays. HTRF β-arrestin 2 and HTRF AP2 total kits are suitable for these investigations. The assay can be used for many adherent cell types, including transfected cell lines if endogenous level of β-arr2 and/or AP2 is very low. Transfection in cells highly expressing β-arr2 and/or AP2 may induce a low signal.
Using an improper cell density can induce poor sensitivity and a low signal.	Check that the cell density is correct. Too high or low cell numbers can affect assay performances.
Stimulation time should be optimized for each sample used.	Depending on the type of treatment and the temperature, the stimulation time can vary widely. Because of this, we recommend a time course study to determine the optimal compound incubation time.
Fluorescence reading	Using an inappropriate setup may seriously impair the results. For information about HTRF® compatible readers and for setup recommendations, please visit our website at: www.revvity.com



Manufactured by Cisbio Bioassays - Parc Marcel Boiteux - 30200 Codolet – FRANCE

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