revvity

Protein-protein interaction assays with HTRF

A guide to developing biochemical assays with HTRF PPi reagents

Introduction

HTRF® is the premier TR-FRET (Time-resolved Fluorescence Resonance Energy Transfer) technology on the market. It brings together the sensitivity of fluorescence with the homogenous nature of FRET (Fluorescence Resonance Energy Transfer) and the low background of time resolution. HTRF uses two fluorophores, termed a donor and an acceptor dye, that transfer energy when in close proximity to each other. The excitation of the donor source (e.g. flash lamp or laser) triggers an energy transfer towards the acceptor, which in turn emits specific fluorescence at a given wavelength. This creates a homogenous assay format in which bound and unbound partners do not need to be separated. Fluorescence emission from the acceptor is generated only upon proximity with the donor.

In an HTRF protein-protein interaction assay, one protein is labeled (directly or indirectly) with the donor, and the other protein is labeled (directly or indirectly) with the acceptor. When the two proteins interact, the donor molecule is brought within proximity of the acceptor molecule. Excitation of the donor will result in signal generation proportional to the binding of proteins.

HTRF assays offer many advantages over other technologies:

- Homogeneous add-and-read format
- No wash steps
- Low background
- Straightforward miniaturization from 96- or 384-well microplates to high density assay formats such as 384-well low volume and 1536-well plates
- Stable signal, providing flexibility in time of readout or size of assays

This User Guide reviews the general principles and steps in developing a protein-protein interaction assay using HTRF PPi reagents from Revvity. For more information on the theory and benefits of Revvity's HTRF technology, including protein-protein interaction assays, please visit our website at www.Revvity.com.

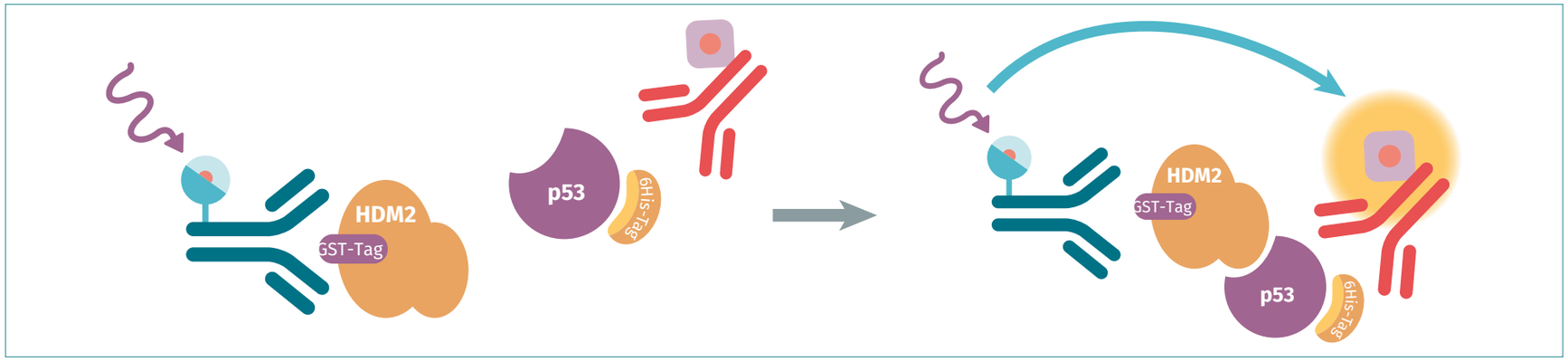

Figure 1: Illustration of an HTRF protein-protein interaction assay, using anti-GST terbium cryptate donor, anti-6His d2 acceptor, GST-tagged HDM2, and 6His-tagged p53.

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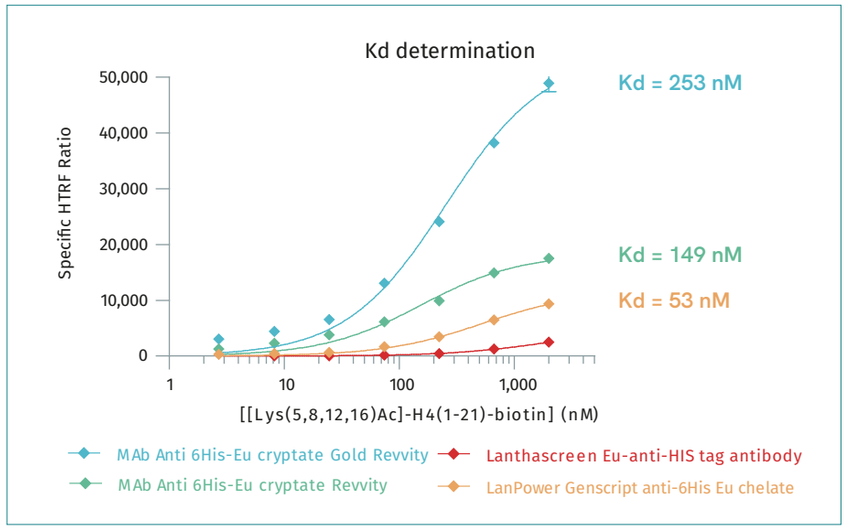
HTRF PPi Reagents

The HTRF PPi is a line of reagents labeled with HTRF fluorophores that are used to develop in-house assays when a ready-to-use assay kit is not available. PPi reagents include anti-tag antibodies, streptavidins, and anti-immunoglobulin antibodies for detecting a broad variety of tag motifs. These reagents are labeled with different HTRF tracers: Europium or Terbium cryptates as donors and XL665 or d2 as acceptors. Specific PPi reagents are chosen depending on the specific assay being developed. Frequently used tags consist of protein and peptide structures such as GST, 6His, c-myc, FLAG®, HA, maltose binding protein (MBP), and small organic motifs such as biotin and dinitrophenyl (DNP).



Data demonstrate an improved assay window of Mab Anti 6His-Eu cryptate Gold Revvity.

Experiments in this guide demonstrating assay development and optimization will utilize only terbium (Tb) cryptate donors. Europium (Eu) cryptate-based HTRF assays require the addition of potassium fluoride (KF) as a fluorescence booster just before readout.

For additional information and a complete list of HTRF PPi reagents, visit www.Revvity.com/protein-protein-interaction. Custom labeling and assay development services are also available to help meet your research needs.

	CRYPTATE DONORS		ACCEPTORS	
	EUROPIUM	TERBIUM	XL665	D2
Anti-GST (GSS11)	✓	✓	✓	✓
Anti-6His Gold	✓	✓	✓	✓
Anti-6His Gold	✓	✓	✓	✓
Anti-c-myc (9E10)	✓	✓	✓	✓
Anti-FLAG™ (M2)	✓	✓	✓	✓
Anti-HA (HAS01)	✓	✓	✓	✓
Anti-MBP	✓	✓	✓	✓
Anti-DNP	✓	✓	✓	✓
Anti-human IgG	✓	✓	✓	✓
Anti-mouse IgG	✓	✓	✓	✓
Anti-rabbit IgG	✓	✓	✓	✓
Protein A	✓	✓	✓	✓
Streptavidin	✓	✓	✓	✓
XLent!	✓	✓	✓	✓

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ASSAY DESIGN BASICS

HTRF Acceptors and Donors

Generally, the goal during assay development is to determine which acceptor-donor pair generates the best possible Signal/Background (S/B). PPi reagents allow you to test a large number of possible combinations to find the optimal conditions for your assay. The number of possible assay formats is quite large since each detecting reagent may be used as either the donor or acceptor.

The example below shows some different assay combinations for a protein-protein interaction assay between protein A and protein B, using all the possibilities offered by the PPi reagents. For the purpose of exemplification, protein A is GST-tagged, protein B is native but may be biotinylated.

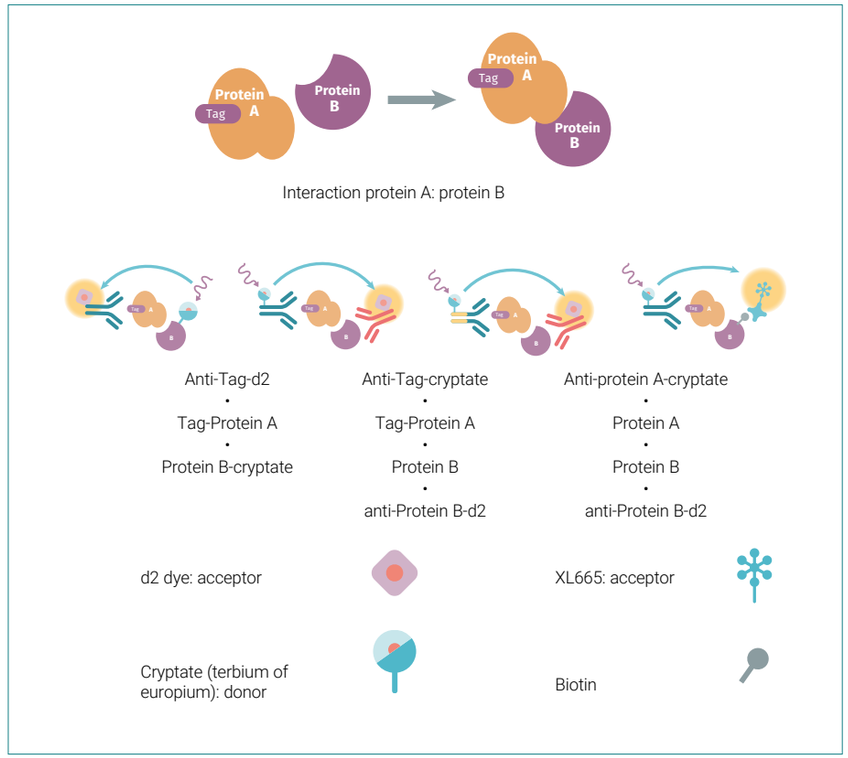
A. Use an anti-tag labeled with a dye (here a d2 as acceptor) and directly label protein B with a cryptate donor to create the FRET signal. You can build the same assay by inverting the two dyes.

B. Use an anti-tag labeled with a cryptate donor to reveal protein A, and an anti-protein B labeled with a d2 acceptor (with custom labeling services or labeling kits). You can build the same assay by inverting the two dyes.

C. Use the two antibodies directed against their respective proteins; an anti-protein A labeled with a cryptate donor and an anti-protein B labeled with a d2 or a XL665 acceptor. You can build the same assay by inverting the two dyes.

D. Because of the biotin on protein B, you can use a streptavidin labeled with XL665 to reveal the protein B. The anti-Tag cryptate donor is used for the anti-Tag-labeled protein. In this case as well, the labels can be inverted.

Biotin present in culture medium may interfere with the binding of biotinylated proteins to acceptor-labeled Streptavidin. We recommend using biotin-free culture medium if you use this detection format.



Click to Enlarge

Figure 3: Example of possible acceptor-donor combinations for a protein A;protein B interaction assay.

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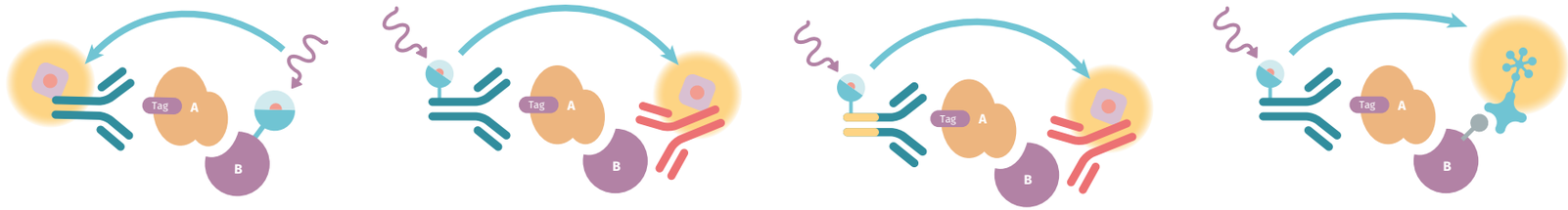
Interaction protein A: protein B



Anti-Tag-d2
•
Tag-Protein A
•
Protein B-cryptate

Anti-Tag-cryptate
•
Tag-Protein A
•
Protein B
•
anti-Protein B-d2

Anti-protein A-cryptate
•
Protein A
•
Protein B
•
anti-Protein B-d2

d2 dye: acceptor

Cryptate (terbium of europium): donor

XL665: acceptor

Biotin

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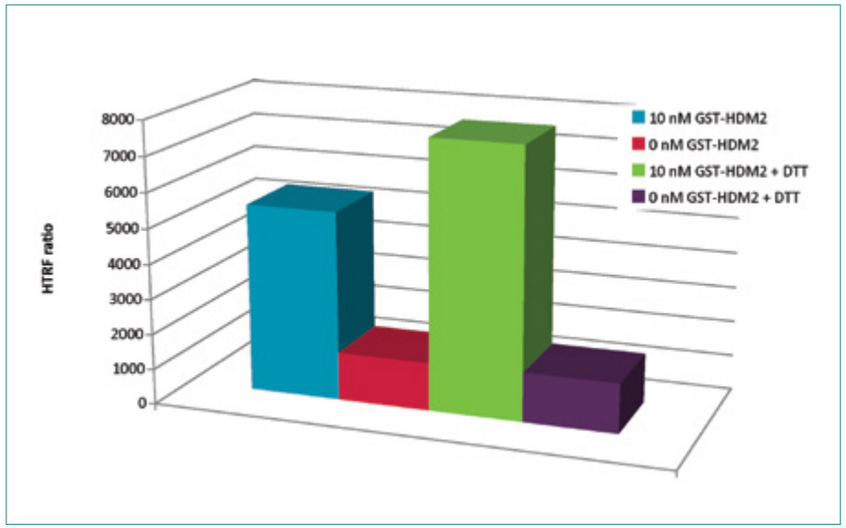
ASSAY DESIGN BASICS

Buffers

Revvity PPi detection buffers have been optimized for maximum performance. When using specific home made buffers, we recommend using a basic buffer of 1X PBS, pH 7.4, with 0.1-0.5% BSA. You may also want to consider running a small experiment initially with and without a small concentration of a reducing agent such as Dithiothreitol (DTT) to determine if there is any signal window boost with either formulation. Figure 4 shows an example of a p53-HDM2 protein-protein interaction assay, where the addition of DTT gave a small boost to the signal and the assay window.

Revvity's HTRF cryptate reagents are resistant to most buffer conditions and additives (e.g., DMSO, pH, chelators, divalent cations, and ionic strength). Your primary consideration should be selecting a buffer most suitable for the proteins you are studying, where interferences may be a consideration.



Instrumentation

Measuring HTRF assays requires an instrument capable of:

- Supplying energy with the spectral characteristics necessary for europium and terbium cryptate excitation.
- Detecting fluorescence in a time-resolved mode (the introduction of a delay between the excitation pulse and a time-gated measurement window).
- Quantifying fluorescence selectively and efficiently at 620 and 665 nm.

Revvity collaborates with a variety of instrument manufacturers to validate microplate readers for use with HTRF. A list of HTRF approved readers can be found at www.revvity.com.

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ASSAY DESIGN BASICS

Microplates

Most brands of available microplates are compatible with HTRF technology. However, background levels and signal-to-noise may differ among plate types. White plates from different suppliers remain the most appropriate standard. See Appendix A for recommended microplates and associated assay volumes. Assays developed using 96- or 384-well plates are easily miniaturized.

Stability and Storage

HTRF reagents and assays are not sensitive to light exposure and do not require lab light shading during manipulation or setting up assays. Revvity assays and reagents are also not affected by storage temperature or temperature changes, although protein components in your assay may be temperature sensitive. Of course, assay kinetics are usually influenced by incubation temperature. Additionally, HTRF assays do not require temperature equilibration before readout.

HTRF assays are remarkably robust. Extended signal stability provides flexibility in read times, ease of scale-up for HTS campaigns, and increased sample compatibility.

Data reduction

HTRF technology uses either Eu3+ or Tb3+ cryptate as the donor fluorophore, and either XL665 or d2 as the acceptor. For these fluorophores, we recommend measuring the fluorescence emission at 620 nm for the donor and at 665 nm for the acceptor.
The measurement of HTRF emissions at two different wavelengths (620 nm and 665 nm) allows the ratiometric reduction of data. This feature of HTRF is extremely advantageous, particularly for reducing well-to-well variations that may arise in homogeneous assay formats where a separation or wash step is not performed. Compounds and/or media additives left in the plate may change the photophysical properties in a given sample, and the degree to which this occurs can vary from sample to sample. By using the ratio of the donor and acceptor emission signals, it is possible to eliminate compounds that are simply interfering with detection.

See: www.revvity.com

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MICROPLATE READERS

Choosing the Right Plate Reader

Choosing the suitable microplate reader ensures you'll get an optimal readout. A multimodal reader gives your research the ideal flexibility and expands the field of possibilities by measuring a wide range of technologies.

Revvity offers multimode plate readers that are certified for use with HTRF technology. Certification involves rigorous testing of plate reader with HTRF technology to ensure optimal readout. HTRF assays are also compatible with multimode readers from other providers as well.

EnVision® Nexus™

Provides lightning speed and superior sensitivity across all established detection technologies with advanced options for ultimate performance. It is the next generation of high-throughput screening, ideal for your most demanding assays.

VICTOR® Nivo™

Packs all the latest major detection technologies into the industry's smallest benchtop footprint. The perfect microplate reader for everyday biochemical and cell-based assays.

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Assay Development

The following section contains a series of experiments to develop and optimize a biochemical HTRF protein-protein interaction assay. Included are sample dilution scheme, plate layouts, protocols, and some example data for two representative protein-protein interaction assays:

- p53 and HDM2 (with 6His and GST tags respectively)
- EGF and EGFR (with biotin and human IgG Fc tags respectively)

Assays can be easily developed using proteins with other species-specific antibodies or tags, such as c-myc, maltose binding protein (MBP), dinitrophenyl (DNP), etc. In these cases, follow experimental processes similar to those that follow, selecting the appropriate HTRF streptavidin, anti-tag, or anti-species reagents.

Additionally, for the purpose of simplicity, we will limit acceptors included in these experiments to those conjugated with d2 or XL665 and donors studied to those conjugated with terbium cryptate. If you are developing a protein-protein assay with the europium cryptate-conjugated reagents, follow the same steps, while also including potassium fluoride (KF) in the detection buffer.

We recommend performing assays during assay development in duplicate (two wells per condition) or triplicate (three wells per condition) whenever possible. This will account for any pipetting errors that could create false or misleading results.

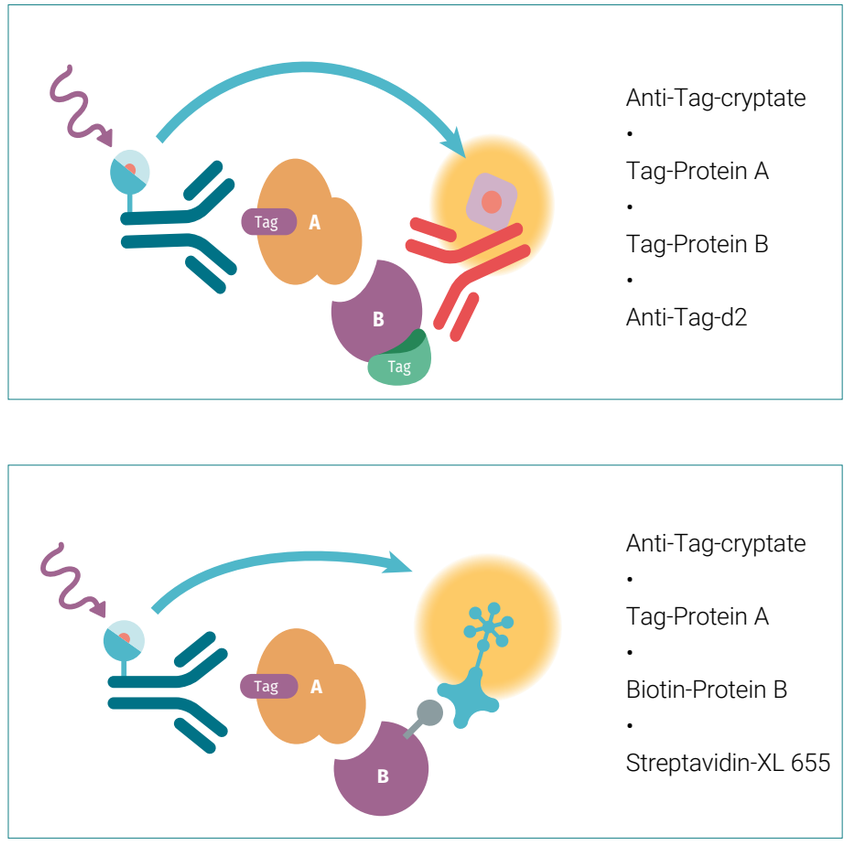

Figure 5: Illustration of assay interaction combinations demonstrated in this guide.

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ASSAY DEVELOPMENT

Experiment One: Select the best Acceptor-Donor pairs

This experiment explores a small protein-protein cross-titration versus multiple combinations of the donor and acceptor reagents. It is designed to identify the best acceptor-donor pairs for the protein-protein interaction. We recommend that you try all combinations of acceptor and donor pairs, as you might otherwise miss results with the best signal window.

When developing a protein-protein interaction assay for GST-HDM2 and 6His-p53 tagged proteins, we examined the following acceptor-donor reagent combinations:

A:D PAIRING COMBINATIONS	ACCEPTOR	DONOR
1	Anti-GST d2	Anti-6His Tb Cryptate Gold
2	Anti-GST XL665	Anti-6His Tb Cryptate Gold
3	Anti-6His d2	Anti-GST Tb
4	Anti-6His XL665	Anti-GST Tb

When developing a protein-protein interaction assay for biotin-EGF and EGFR-Fc tagged proteins, we examined the following acceptor-donor reagent combinations:

A:D PAIRING COMBINATIONS	ACCEPTOR	DONOR
1	Streptavidin-d2	Anti-Human IgG-Tb
2	Streptavidin- XL665	Anti-Human IgG-Tb
3	Anti-Human IgG-d2	Streptavidin-Tb
4	Anti-Human IgG-XL665	Streptavidin-Tb

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A. Plate Layout

Prepare one 384-well plate as laid out below. Run samples in duplicate or triplicate (triplicates shown here).

		1	2	3	4	5	6	7	8	9	10	11	12
		300 nM Protein X	300 nM Protein X	300 nM Protein X	30 nM Protein X	30 nM Protein X	30 nM Protein X	3 nM Protein X	3 nM Protein X	3 nM Protein X	0 nM Protein X	0 nM Protein X	0 nM Protein X
A	300 nM Protein Y												
B	30 nM Protein Y					A:D Pairing #1 Anti-tag-d2 bind to Protein X-tag Anti-tag-Tb bind to Protein Y-tag							
C	3 nM Protein Y												
D	0 nM Protein Y												
E	300 nM Protein Y												
F	30 nM Protein Y					A:D Pairing #2 Anti-tag-Tb bind to Protein X-tag Anti-tag-d2 bind to Protein Y-tag							
G	3 nM Protein Y												
H	0 nM Protein Y												
I	300 nM Protein Y												
J	30 nM Protein Y					A:D Pairing #3 Anti-tag-XL665 bind to Protein X-tag Anti-tag-Tb bind to Protein Y-tag							
K	3 nM Protein Y												
L	0 nM Protein Y												
M	300 nM Protein Y												
N	30 nM Protein Y					A:D Pairing #4 Anti-tag-Tb bind to Protein X-tag Anti-tag-XL665 bind to Protein Y-tag							
O	3 nM Protein Y												
P	0 nM Protein Y												

Rest of plate left empty

Figure 4.2.1A: 384-well plate map for acceptor-donor selection experiment.

Tips:

- If you know the Kd of your protein-protein interaction, you may want to test an alternate range of concentrations. For example, if the Kd is known to be in a low nM, or even a pM range, you may want the highest dose <50 nM. Likewise, if the protein interaction is known to exhibit a high Kd, you may need to test higher doses. If you do not know the Kd of your protein interaction we suggest the 3–300 nM range as a good starting point.
- Be sure to include 0 nM wells, as these serve as initial controls for interaction specificity. For example, if a signal is seen in the absence of either protein, this may suggest some non-specific contaminant allowing energy transfer.

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B. Reagent Preparation

Per well, add the following for 20 µL final volume:

- 5 µL Protein X, prepare at 4X*
- 5 µL Protein Y, prepare at 4X
- 5 µL Donor conjugate
- 5 µL Acceptor conjugate

* Assay volumes can be adjusted as long as the reagent concentrations are kept constant.

1. Prepare a 4X initial concentration of Proteins X and Y in assay buffer.

2. Perform 10-fold serial dilutions from the highest concentration as follows (include a buffer only sample):

PROTEIN X		
Tube #	Final Concentration	4X Concentration
1	300 nM	1200 nM
2	30 nM	120 nM
3	3 nM	12 nM
4	0 nM	0 nM

PROTEIN Y		
Tube #	Final Concentration	4X Concentration
1	300 nM	1200 nM
2	30 nM	120 nM
3	3 nM	12 nM
4	0 nM	0 nM

3. During the first incubation (see protocol following) prepare working solutions of donor and acceptor reagents. Refer to specific lot information (available at www.Revvity.com) for recommended quantity per well (ng/well) for use in a 20 µL assay (based on a 384-well low volume plate). Calculate the concentration of the reagent to prepare based on this information and the volume per well used. This recommendation is based on the active moiety concentration and is tested by Revvity for each lot of HTRF reagents.

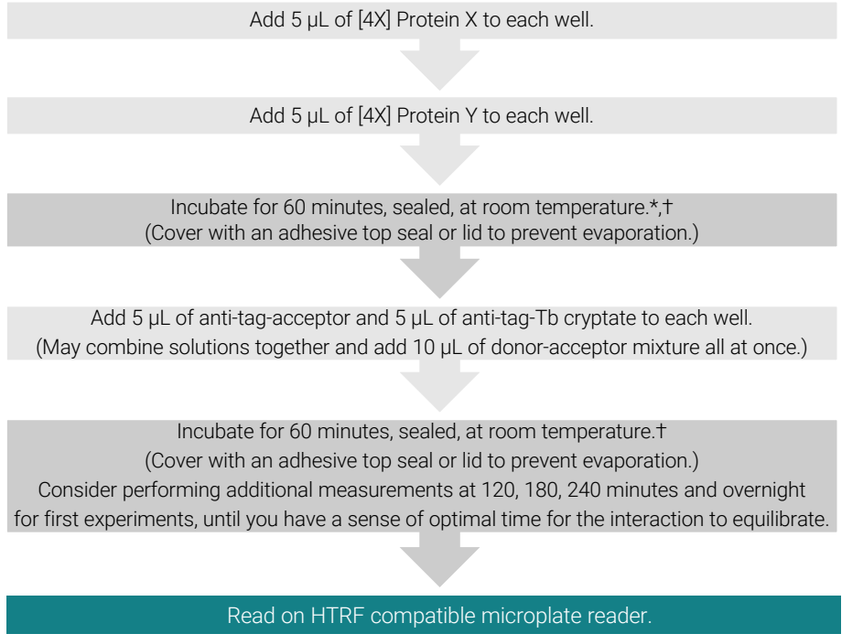
Note:

When using a protein with a biotin tag, it is important to maintain a 4:1 ratio of biotin to streptavidin (SA). Therefore, if you are titrating the biotinylated protein you should also titrate the SA-HTRF reagent. More information on this can be found in Experiment #2.

C. Assay Protocol for a 384-well Low Volume Plate

Add reagents to microplate, using an electronic multi-channel repeat pipettor to dispense reagents. Refer to plate map in Figure 4.2.1A.

D. Assay Example



* You may need to optimize incubation times, but, in general, 60 minutes is adequate for the first incubation. Some assays need less time. Once the HTRF reagents are added, the reaction will continue. This does not interfere with the protein-protein interaction. Pre-incubation of proteins is not always required and this step can be omitted.

† Experiments in this guide were performed at room temperature. You can incubate at higher or lower temperatures if you have prior knowledge of specific temperature requirements for your protein-protein interaction. Additionally in some cases, if no or low signal is seen, increasing or decreasing the incubation temperature will boost signal without increasing the background.

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D. Assay Example

In this example (Figures 4.2.1B and 4.2.1C), an assay was performed to measure the interaction of 6His-p53 and GST-HDM2. Proteins were combined first and incubated. After 60 minutes, anti-GST (acceptor) and anti-6His terbium cryptate (donor) reagents were added, followed by incubation for 60, 120, 240 minutes and overnight. Note that for Revvity HTRF assays there is no need to create additional sample assay plates to measure the assay at multiple time points. The assay signal is stable and not light sensitive.

In Figure 4.2.1B, a 3-D bar graph demonstrates the HTRF ratio signal when comparing the four acceptor:donor pairings and varying concentrations of proteins. In Figure 4.2.1C, comparing the four acceptor:donor pairings and varying concentrations of proteins for this specific assay, the signal to background shows that the use of anti-GST-d2 as the acceptor and anti-6His Tb cryptate Gold as the donor generates the largest signal window, followed by use of the same donor with an anti-GST-XL665 donor. This data also shows that a very low concentration of protein produces the largest signal windows. For higher concentrations of proteins, we observed the hook effect, which is why the signal decreases. However, the assay is not yet optimized for protein concentrations; thus we chose two of these combinations for the next experiment. The background for each combination was obtained in the absence of protein p53-6His or HDM2-GST.

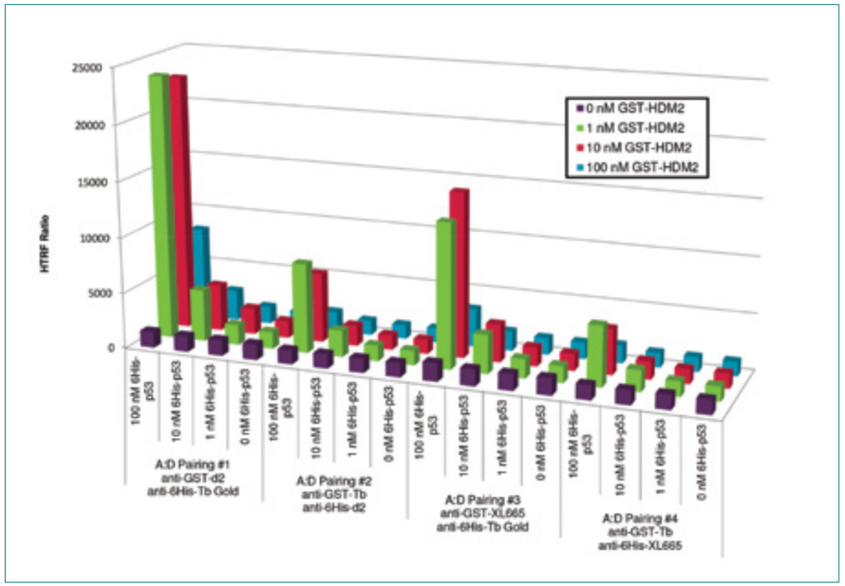

Figure 4.2.1B: Comparison of four combinations of acceptor and donor pairs, for an assay measuring the p53 and HDM2 protein-protein interaction, where HDM2 is GST-tagged and p53 is 6His-tagged. We adjusted the concentration based on the Kd of the proteins used. The largest signal ratio is seen when using the anti-6His terbium cryptate Gold as the donor and either an anti-GST-d2 (A:D pairing #1) or an anti-GST- XL665 (A:D pairing #3) acceptor.

Signal : Background	[GST-HDM2] nM			
[6His-p53] nM	100	10	1	
100	5.7	15.8	16.3	A:D Pairing #1 anti-GST-d2 anti-6His-Tb Gold
10	1.9	2.9	3.3	
1	1.1	1.7	1.3	
100	1.4	4.9	6.3	A:D Pairing #2 anti-GST-Tb anti-6His-d2
10	1.0	1.4	1.9	
1	1.0	1.0	1.1	
100	2.1	8.9	7.8	A:D Pairing #3 anti-GST-XL665 anti-6His-Tb Gold
10	1.2	2.3	2.3	
1	1.0	1.1	1.1	
100	1.2	3.1	4.1	A:D Pairing #4 anti-GST-Tb anti-6His-XL665
10	1.0	1.3	1.5	
1	1.0	1.0	1.0	

Figure 4.2.1C: Comparison of signal to background for data generated with the four combinations of acceptor-donor pairs, for an assay measuring the p53 and HDM2 protein: protein interaction, where HDM2 is GST-tagged and p53 is 6His-tagged. A significantly larger signal to background is seen when using the anti-6His Tb cryptate Gold as the donor rather than the anti-GST Tb cryptate (pairings #1, #3). The largest signal windows were found when also using the anti-GST-d2 (A:D pairing #1) acceptor, though the anti-GST-XL665 also generates a good signal to background for a first assay.

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ASSAY DEVELOPMENT

Experiment Two: Confirm Signal Specificity

This experiment was performed to confirm that the signal seen is specific to the interaction of the two proteins, and not due to a non-specific effect (allowing energy transfer in absence of protein-protein interaction).

We also recommend that you perform a displacement assay, using a high concentration of one of the two proteins untagged, versus several concentrations of the other protein tagged.

The dose of the untagged protein should be 100-fold greater than the highest concentration of the tagged protein in the assay. If the interaction is specific, you can expect to see a significant signal decrease to background signal level.

If you are unable to inhibit the signal significantly even at low concentrations of tagged protein, you should explore if proteins or other reagents, such as buffer components are causing non-specific results. For example, if a protein has a residual GST tag in addition to the expected 6His tag, you might see an HTRF signal when incubating the protein on its own (no second protein) with the anti-GST and anti-6His tagged acceptor and donor reagents. If you suspect possible tag contamination, Revvity has tag check kits available for detecting and measuring all GST, 6His and FLAG tagged proteins. If you cannot determine the cause of the non-specificity with the proteins you are using, you may want to source proteins from another vendor, or go back and check another acceptor-donor pairing for any non-specific response. While the acceptor-donor pair is typically chosen based on the largest signal, there is a possibility it may not always be best and you may need to try another pairing.

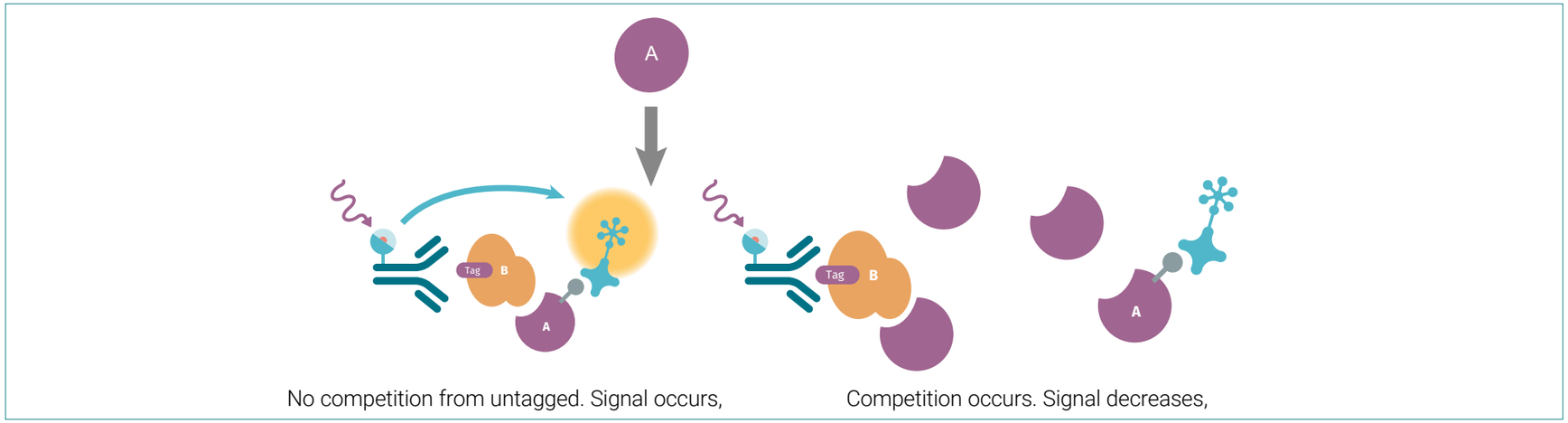

Figure 4.2.2A: Untagged Protein A (purple) competes with the biotin-tagged Protein A (purple) for binding to Protein B (orange). The excess of untagged protein competes with the complex of tagged Proteins A and B. The greater the displacement of tagged-Proteins, the lower the HTRF signal.

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A. Plate Layout

Prepare one plate as shown in example below, on a 384-well plate. Run samples in duplicate or triplicate (duplicates shown here).

		1	2	3	4	5	6	7	8	
		100 nM Protein X	100 nM Protein X	10 nM Protein X	10 nM Protein X	1 nM Protein X	1 nM Protein X	0 nM Protein X	0 nM Protein X	
Plus untagged Protein X	A	100 nM Protein Y								Rest of plate left empty
	B	10 nM Protein Y			A:D Pairing #1					
	C	1 nM Protein Y								
	D	0 nM Protein Y								
	E	100 nM Protein Y								
	F	10 nM Protein Y			A:D Pairing #2					
	G	1 nM Protein Y								
	H	0 nM Protein Y								
Plus Buffer	I	100 nM Protein Y								
	J	10 nM Protein Y			A:D Pairing #3					
	K	1 nM Protein Y								
	L	0 nM Protein Y								
	M	100 nM Protein Y								
	N	10 nM Protein Y			A:D Pairing #4					
	O	1 nM Protein Y								
	P	0 nM Protein Y								

Figure 4.2.2B: 384-well plate map for specificity experiment.

B. Reagent Preparation

Per well, add the following for 20 µL final volume:

- 2 µL buffer or untagged Protein X, prepared at 10X
- 4 µL Protein X, prepare at 5X
- 4 µL Protein Y, prepare at 5X
- 5 µL Donor conjugate, prepare at 4X
- 5 µL Acceptor conjugate, prepare at 4X

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1. Prepare a 5X initial concentration of Proteins X and Y in assay buffer.

2. Perform 10-fold serial dilutions from the highest concentration as follows (include a buffer only sample):

PROTEIN X AND PROTEIN Y		
Tube #	Final Concentration	5X Concentration
1	100 nM	500 nM
2	10 nM	50 nM
3	1 nM	5 nM
4	0 nM	0 nM

UNTAGGED PROTEIN X AND PROTEIN Y		
Tube #	Final Concentration	10X Concentration
1	10 µM	100 µM

3. In Eppendorf tubes, prepare a 10X concentration of untagged Protein X in assay buffer. The final concentration should be 100 times the highest concentration of the tagged protein concentration used in the assay. In this assay, we prepared a stock solution at 100 µM to have a final concentration of 10 µM (100-times 100 nM).

4. During the first incubation (see protocol following) prepare working solutions of acceptor and donor reagents. Refer to specific lot information (available at www.Revvity.com) for recommended quantity per well (ng/well) for use in a 20 µL assay (based on a 384-well low volume plate). Calculate the concentration of the reagent to prepare based on this information and the volume per well used. This recommendation is based on the active moiety concentration and is tested by Revvity for each lot of HTRF reagents.

   When using a biotin-conjugated protein and a streptavidin-conjugated HTRF reagent, maintain a 4:1 ratio of biotin to streptavidin for initial assay development. You can modify the ratio in a later experiment when the assay conditions are further established. When titrating the biotinylated protein, also titrate the SA-HTRF reagent. In this case, convert the streptavidin reagent concentration to molar concentration, and add the appropriate amount based on the biotin protein reagent as opposed to using the recommended quantity found in the lot-specific product sheet. An example of a titration of streptavidin reagent, along with decreasing concentrations of biotin can be found in the table below.

BIOTIN REAGENT CONCENTRATION, nM (FINAL)	STREPTAVIDIN REAGENT CONCENTRATION, nM (FINAL)	STREPTAVIDIN REAGENT CONCENTRATION, nM (4X PREP)
300	75	300
100	25	100
33	8.3	33
11	2.8	11
3.7	0.93	3.7
1.2	0.30	1.2
0.4	0.10	0.4
0	*	*

* When the biotin reagent concentration is 0 nM, use the concentration of streptavidin reagent for one of the highest biotin concentrations to serve as a control (do not use 0 nM streptavidin reagent). For example, here we used the 75 nM final concentration of the streptavidin reagent for both the 300 nM and 0 nM samples.

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C. Assay Protocol for a 384-well Low Volume Plate

Reagents added to microplate, using an electronic multi-channel repeat pipettor to dispense reagents. Refer to Figure 4.2.2B for the plate map.

Add 2 µL of buffer or of [10X] untagged Protein X to wells.

↓

Add 4 µL of [5X] Protein X at 4X to each well.

↓

Add 4 µL of [5X] Protein Y at 4X to each well.

↓

Incubate 60 minutes, sealed, at desired temperature.*†
(Cover with an adhesive top seal or lid to prevent evaporation.)

↓

Add 5 µL of anti-tag-acceptor and 5 µL of anti-tag-Tb cryptate to each well.
(May combine solutions together and add 10 µL of acceptor-donor mixture all at once.)

↓

Incubate for 60 minutes, sealed, at desired temperature.†
(Cover with an adhesive top seal or lid to prevent evaporation.)
You may want to perform additional measurements at 120, 180, 240 minutes and overnight for the first experiments, until you have a sense of the optimal time the interaction needs to equilibrate.

↓

Read on HTRF compatible microplate reader.

* 60 minutes is usually adequate for first incubation. However, use the optimal time identified in Experiment One. Pre-incubation of proteins is not always required and this step can be omitted.

† Experiments in this guide were performed at room temperature. Incubate at higher/lower temperatures if you have prior experimental knowledge of specific temperature requirement for your protein-protein interaction.

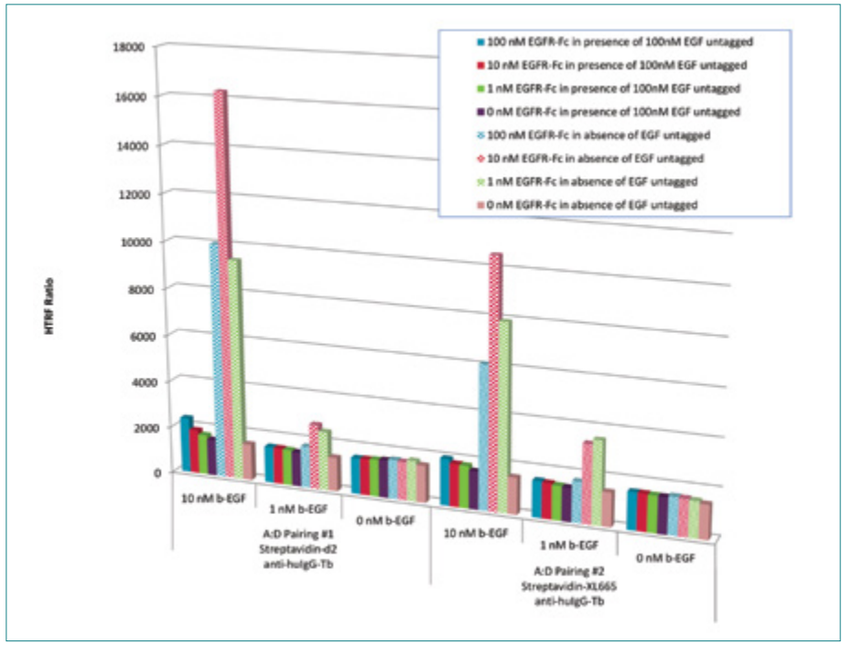

Figure 4.2.2C: Comparison of signal derived from the biotin-EGF:EGFR-Fc protein-protein interaction assay in the presence or absence of 100 nM untagged EGF protein. Competition with the untagged protein significantly decreased the HTRF ratio value in all experiments, essentially to background levels, demonstrating the signal is specific to the protein-protein interaction.

Signal : Background	[EGFR-FC] nM			
[b-EGF] nM	100	10	1	
10	6.5	10.5	6.1	A:D Pairing #1 Streptavidin-d2 anti-huIgG-Tb
1	1.2	1.9	1.7	
0	1.1	1.0	1.1	
10	3.9	6.8	5.1	A:D Pairing #2 anti-GST-Tb anti-6His-d2
1	1.2	2.3	2.5	
0	1.1	1.1	1.1	

Figure 4.2.2C: Comparison of signal derived from the biotin-EGF:EGFR-Fc protein-protein interaction assay in the presence or absence of 100 nM untagged EGF protein. Competition with the untagged protein significantly decreased the HTRF ratio value in all experiments, essentially to background levels, demonstrating the signal is specific to the protein-protein interaction.

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Experiment Three: Cross-titration to optimize concentrations of proteins

The third experiment is a more extensive cross-titration of the proteins, in order to determine the best concentration of proteins to use in the assay. In this assay you will use the one or two best acceptor-donor pairs determined from Experiment One.

A. Plate Layout

Each condition is run in triplicate.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
		300 nM Protein X	300 nM Protein X	100 nM Protein X	100 nM Protein X	33 nM Protein X	33 nM Protein X	11 nM Protein X	11 nM Protein X	3.7 nM Protein X	3.7 nM Protein X	1.2 nM Protein X	1.2 nM Protein X	0.4 nM Protein X	0.4 nM Protein X	0 nM Protein X	0 nM Protein X	
A	300 nM Protein Y																	Rest of plate left empty
B	100 nM Protein Y																	
C	33 nM Protein Y																	
D	11 nM Protein Y					Acceptor-Donor Pair #1												
E	3.7 nM Protein Y																	
F	1.2 nM Protein Y																	
G	0.4 nM Protein Y																	
H	0 nM Protein Y																	
A	300 nM Protein Y																	
B	100 nM Protein Y																	
C	33 nM Protein Y																	
D	11 nM Protein Y					Acceptor-Donor Pair #2												
E	3.7 nM Protein Y																	
F	1.2 nM Protein Y																	
G	0.4 nM Protein Y																	
H	0 nM Protein Y																	

Figure 3.2.3A: 384-well plate map for protein cross-titration experiment

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Tips:

- Based on the results from the two prior experiments, or if you know the Kd of your protein-protein interaction, you may want to test an alternate range of concentrations.
- Be sure to include 0 nM wells, as these serve as controls for interaction specificity. For example, if a signal is seen in the absence of either protein, this may suggest some non-specific contaminant resulting in energy transfer.

B. Reagent Preparation

Per well, add the following for 20 µL final volume:

- 5 µL Protein X, prepare at 4X
- 5 µL Protein Y, prepare at 4X
- 5 µL Donor conjugate
- 5 µL Acceptor conjugate

1. Prepare a 4X initial concentration of Proteins X and Y in assay buffer.
2. Perform 3-fold serial dilutions from the highest concentration as follows (include a buffer only sample):

PROTEIN X AND PROTEIN Y		
Tube #	Final Concentration	5X Concentration
1	100 nM	500 nM
2	10 nM	50 nM
3	1 nM	5 nM
4	0 nM	0 nM
5	1 nM	5 nM
6	0 nM	0 nM
7	1 nM	5 nM
8	0 nM	0 nM

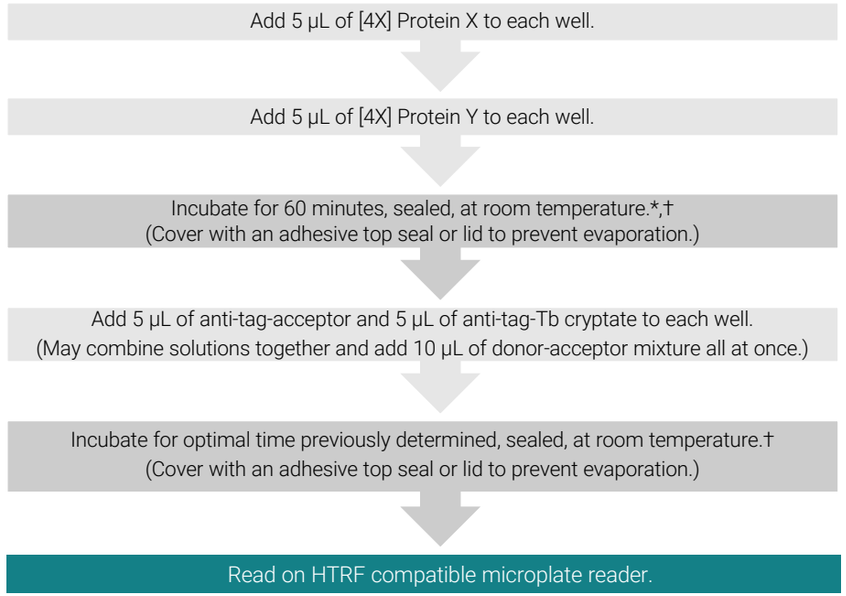
3. During the first incubation prepare working solutions of acceptor and donor reagents, again using each at the recommended concentration per well per the product lot information. (See Experiment One, Step 3 for more information on this).

Note:

When using a protein with a biotin tag, it is important to maintain a 4:1 ratio of biotin to streptavidin. Therefore, if you are titrating the biotinylated protein you should also titrate the SA-HTRF reagent. More information on this can be found in Experiment Two, Step 4.

C. Assay Protocol for a 384-well Low Volume Plate

Add reagents to microplate, using an electronic multi-channel repeat pipettor to dispense reagents, refer to plate map in Figure 4.2.3A.



* You may need to optimize incubation times, but, in general, 60 minutes is adequate for the first incubation. Some assays need less time. Once the HTRF reagents are added, the reaction will continue. This does not interfere with the protein-protein interaction. Pre-incubation of proteins is not always required and this step can be omitted.

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† Experiments in this guide were performed at room temperature. You can incubate at higher or lower temperatures if you have prior knowledge of specific temperature requirements for your protein-protein interaction. Additionally in some cases, if no or low signal is seen, increasing or decreasing the incubation temperature will boost signal without increasing the background.

D. Assay Example

Assays were performed to measure the specificity of the interaction of the X:Y assays. An 8-point dose response curve, including 0 nM, was generated for each protein and then they were combined per the 384-well plate map in a cross-titration. After 60 minutes, acceptor and donor reagents were added, followed by incubation for 60 minutes (the optimal incubation time previously determined). We ran the assay with the two best combinations of acceptor and donor pairs, as determined in Experiment One. Where the protein was biotin-conjugated and acceptors are streptavidin conjugates, the biotin:streptavidin ratio was kept constant at 4:1. See Figures 4.2.3B, 4.2.3C and 4.2.3D.

For the p53-HDM2 interaction, the signal increases proportionally to the binding until we reach the hook effect, and the signal starts to decrease.

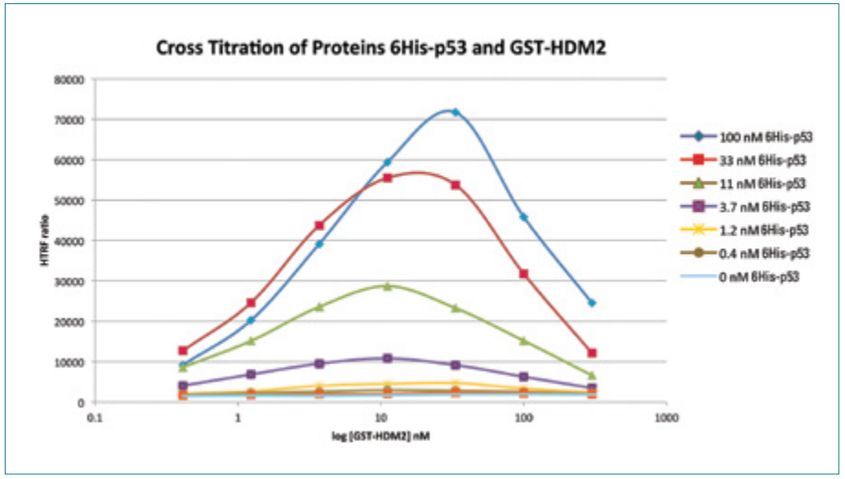

Figure 4.2.3B Cross-titration results for binding of 6His-p53 and GST-HDM2, using anti-6His Tb Gold donor reagent and anti-GST-d2 acceptor reagent. The expected Kd for this interaction is 300 nM. (Zhang Q, Zeng S, Lu H. Targeting p53-MDM2-MDMX Loop for Cancer Therapy. Subcell Biochem. 2014 ; 85: 281–319. doi:10.1007/978-94-017-9211-0_16.)

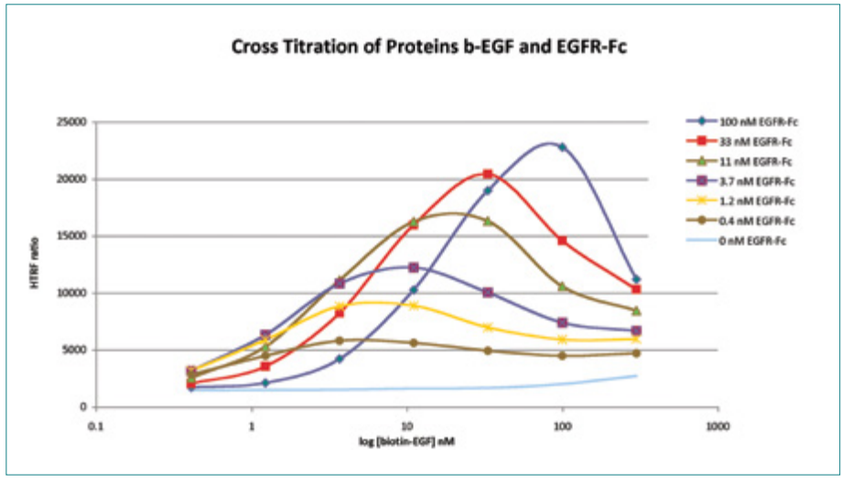

Figure 4.2.3C Cross-titration results for binding of biotin-EGF to EGFR-Fc, using streptavidin-d2 and anti-hulgG-Tb reagents. The Kd for this interaction is approximately 2.8 nM. (Lax I, et al. Chicken epidermal growth factor (EGF) receptor: cDNA cloning, expression in mouse cells, and differential binding of EGF and transforming growth factor alpha. Mol Cell Biol. 1988. May;8(5):1970-8.)

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Signal : Background		EGFR-FC						
		100 nM EGFR-Fc	33 nM EGFR-Fc	11 nM EGFR-Fc	3.7 nM EGFR-Fc	1.2 nM EGFR-Fc	0.4 nM EGFR-Fc	0 nM EGFR-Fc
A:D Pairing #1	300 nM biotin-EGF	4.1	3.8	3.1	2.5	2.2	1.7	1.0
	100 nM biotin-EGF	11.4	7.3	5.3	3.7	2.9	2.2	1.0
	33 nM biotin-EGF	11.4	12.3	9.8	6.1	4.2	3.0	1.0
	11 nM biotin-EGF	6.5	10.0	10.2	7.7	5.6	3.5	1.0
	3.7 nM biotin-EGF	2.8	5.5	7.4	7.2	5.8	3.8	1.0
	1.2 nM biotin-EGF	1.4	2.4	3.6	4.3	4.0	3.0	1.0
	0.4 nM biotin-EGF	1.2	1.4	1.7	2.2	2.2	1.9	1.0
	0 nM biotin-EGF	0.9	1.0	1.0	1.0	1.2	1.0	1.0
A:D Pairing #12	300 nM biotin-EGF	3.2	3.5	3.0	2.6	1.9	1.5	1.0
	100 nM biotin-EGF	6.4	4.3	3.4	2.8	2.3	1.8	1.0
	33 nM biotin-EGF	5.6	6.1	5.0	5.6	3.0	2.2	1.0
	11 nM biotin-EGF	2.7	5.1	5.3	4.5	3.8	2.9	1.0
	3.7 nM biotin-EGF	1.4	2.7	3.6	4.2	3.9	3.2	1.0
	1.2 nM biotin-EGF	1.2	2.7	2.2	2.9	3.0	2.9	1.0
	0.4 nM biotin-EGF	1.1	1.2	1.5	1.7	1.9	1.8	1.0
	0 nM biotin-EGF	1.0	1.0	1.0	1.0	1.0	1.0	1.0

The final choice of protein concentrations is based on several parameters:

- Select as high an S:B as possible.
- Protein concentrations should be on the linear part of the curve (before reaching the hook effect).
- Avoid a large excess of proteins compared to expected Kd.
- Reduce the quantity of proteins used.

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Experiment Four: Optimization of Acceptor:Donor Reagent Concentrations

The fourth experiment is optional if the S/B is satisfactory with reagent concentrations previously used. The experiment typically run is an optimization of the concentrations of the acceptor and donor reagents. Though the recommended values provided in the lot specific data sheets are generally sufficient, you may want to look at slight modifications above or below the recommended concentrations to see if there can be additional improvement in the signal. Of course, it is important that the signal to background is maintained or improved; your aim is to increase the signal while not increasing the background.

To run these assays, choose the best condition for one of the two proteins from Experiment Three, and incubate this with a titration of the second protein. Next, prepare concentrations of the donors at 2X, 1X, and 0.5X the recommended concentrations (according to the specific lot information for the reagent), and the acceptors at 1X, 0.5X and 0.25X the recommended concentrations and add these to the protein-protein interaction as described in the plate layout on the next page.

A. Plate Layout

Prepare one plate as shown in example below, on a 384-well plate. Run samples in duplicate or triplicate (triplicates shown here).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
		100 nM	33 nM	11 nM	3.7 nM	1.2 nM	0.4 nM	0 nM	100 nM	33 nM	11 nM	3.7 nM	1.2 nM	0.4 nM	0 nM	100 nM	33 nM	11 nM	3.7 nM	1.2 nM	0.4 nM	0 nM
A	2X Donor																					
B																						
C																						
D	1X Donor																					
E																						
F																						
G	0.5X Donor																					
H																						
I																						
		1X Acceptor							0.5X Acceptor							0.25X Acceptor						

Figure 4.2.4A 384-well plate map for Experiment Four to optimize acceptor-donor reagent concentrations..

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B. Reagent Preparation

Per well, add the following for 20 µL final volume:

- 5 µL Protein X, prepare at 4X
- 5 µL Protein Y, prepare at 4X
- 5 µL Donor conjugate
- 5 µL Acceptor conjugate

1. Prepare a 4X initial concentration of Protein X in assay buffer.
2. Prepare a 4X initial concentration of Proteins Y in assay buffer.
3. Perform 1:3 serial dilutions of Protein Y, from the initial dilution, as follows (include a buffer only sample):

PROTEIN X AND PROTEIN Y		
Tube #	Final Concentration	4X Concentration
1	100 nM	400 nM
2	33 nM	132 nM
3	11 nM	44 nM
4	3.7 nM	14.8 nM
5	1.2 nM	4.8 nM
6	0.4 nM	1.6 nM
7	0 nM	0 nM

4. During the first incubation (see protocol following) prepare working solutions of acceptor and donor reagents. Refer to specific lot information (available at www.Revvity.com) for recommended quantity per well (ng/well) for use in a 20 µL assay (based on a 384-well low volume plate). Calculate the concentration of the reagent to prepare based on this information and the volume per well used. This recommendation is based on the active moiety concentration and is tested by Revvity for each lot of HTRF reagents.

Note:

For assays using a biotin-conjugated reagent, you may want to look at optimizing the biotin:streptavidin ratio rather than preparing the reagent at varied quantity per well. As an example, when using an 11 nM concentration of biotinylated protein, you would use the following amounts of streptavidin reagent to generate 2:1, 4:1 and 8:1 ratios of the number of biotin molecules per each streptavidin molecule.

RATIO OF MOLECULES BIOTIN TO MOLECULES STREPTAVIDIN	BIOTIN REAGENT CONCENTRATION, NM (FINAL)	STREPTAVIDIN REAGENT CONCENTRATION, NM (FINAL)	STREPTAVIDIN REAGENT CONCENTRATION, NM (4X PREP)
2:1	11	5.5	22
4:1	11	2.8	11
8:1	11	1.4	5.6

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C. Assay Protocol for a 384-well Low Volume Plate

Add reagents to microplate, using an electronic multi-channel repeat pipettor to dispense reagents. Refer to plate map (Figure 4.2.4A) for the plate map.

Add 5 µL of [4X] Protein X to each well.

Add 5 µL of [4X] Protein Y to each well.

Incubate for 60 minutes, sealed, at room temperature.*,†
(Cover with an adhesive top seal or lid to prevent evaporation.)

Add 5 µL of anti-tag-acceptor and 5 µL of anti-tag-Tb cryptate to each well.
(May combine solutions together and add 10 µL of donor-acceptor mixture all at once.)

Incubate for 60 minutes, sealed, at room temperature.†
(Cover with an adhesive top seal or lid to prevent evaporation.)

Read on HTRF compatible microplate reader.

* You may need to optimize incubation times, but, in general, 60 minutes is adequate for the first incubation. Some assays need less time. Once the HTRF reagents are added, the reaction will continue. This does not interfere with the protein-protein interaction. Pre-incubation of proteins is not always required and this step can be omitted.

† Experiments in this guide were performed at room temperature. You can incubate at higher or lower temperatures if you have prior knowledge of specific temperature requirements for your protein-protein interaction. Additionally in some cases, if no or low signal is seen, increasing or decreasing the incubation temperature will boost signal without increasing the background.

D. Assay Example

An assay was performed to explore the optimal concentrations of anti-GST-d2 acceptor and anti-6His Tb Gold donor in the GST-HDM2, 6His-p53 protein-protein interaction assay. A single 11 nM final concentration of GST-HDM2 was added to the assay, while the 6His-p53 was titrated from 3–0.4 nM (final concentration). Proteins were combined first and incubated for 60 minutes. The acceptor and donor reagents were added as described in the plate map, and the assay was further incubated and measured on an HTRF microplate reader. See Figure 4.2.4B.

Signal : Background		[6HIS-P53] nM					
		33	11	3.7	1.2	0.4	
Donor	2X	2.5	1.5	1.1	1.0	1.0	1X Acceptor
Donor	1X	5.3	2.4	1.4	1.1	1.1	1X Acceptor
Donor	0.5X	9.2	3.6	2.0	1.3	1.1	1X Acceptor
Donor	2X	2.4	1.6	1.2	1.1	1.0	0.5X Acceptor
Donor	1X	5.2	2.3	1.5	1.2	1.1	0.5X Acceptor
Donor	0.5X	9.6	3.9	2.1	1.4	1.1	0.5X Acceptor
Donor	2X	2.3	1.4	1.2	1.0	1.1	0.25X Acceptor
Donor	1X	4.6	1.7	1.5	1.2	1.1	0.25X Acceptor
Donor	0.5X	8.5	3.1	2.0	1.3	1.1	0.25X Acceptor

Figure 4.2.4B. Comparison of the signal: background changes when titrating the acceptor and donor.

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When determining which conditions of the acceptor and donor to proceed with, one important parameter is the signal to background. In the acceptor data comparison seen in Figure 4.2.4B, there is little difference in the signal to background between the results with 1X and 0.5X acceptor conditions, whereas results from the 0.25X acceptor show a decreased S:B compared to both the 1X and 0.5X. Without further testing of additional lower concentrations around 0.25X, the assay might be on the edge of good results and so we chose to move ahead with the 0.5X concentration of acceptor.

With regard to choosing the optimal donor condition, it may initially appear that the 0.5X concentration is best, as it gives the larger signal:background window. However when optimizing the donor signal, you should also take into account the signal from the donor (represented by the 620 channel readout in raw data), the microplate reader and light source in use, and the color of the microplate material. In this case, we were using the BMG Pherastar® FS microplate reader, with the lamp as the light source. We were also using white microplates. With those parameters in mind, we recommend that when developing an assay, you have at least 40 times more counts at 620 nm than the buffer blank (which represents the background of the plate and buffer). This will ensure you subsequently read at the most robust assay conditions. Figure 4.2.4C, represents the average signal of the donor across 21 wells, at each of the donor conditions. In this case, when reviewing both the signal:background with the signal from the donor, we recommend you use the 1X donor as it has both a reasonable S:B and greater than the recommended level of donor signal.

AVERAGE SIGNAL RAW DATA 620 nM	
2X Donor	127854
1X Donor	45747
0.5X Donor	17267
Blank	535

Figure 4.2.4C. Comparison of the raw 620 nm channel signal data; this represents the counts derived from the donor reagent.

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ASSAY DEVELOPMENT

Experiment Five: Competition Assay

The fifth experiment is to further validate the assay by competing against the protein interaction with expected inhibitors. This is done by running a full dose response curve of one of the untagged proteins or small compounds, antibodies or other known inhibitors that might be used. Increasing concentrations of the untagged protein or compound should demonstrate decreased signal, as they disrupt the interaction of the proteins and thereby the energy transfer leading to the HTRF signal.

To run these assays, choose the best condition for the two proteins from prior experiments, and incubate. Next prepare acceptor and donor at concentrations determined in Experiment Four.

A. Plate Layout

The following is an example of how you might set up a plate with inhibitors and controls. Actual plate layout will vary depending on number and potency of inhibitors. Test a minimum 8-pt dose response curve of one or more expected inhibitors of the protein-protein interaction. Run samples in duplicate or triplicate.

Run assay controls on the plate as follows, with minimally three wells of each.

a. Both proteins, no inhibitor

b. Protein X only, no inhibitor (no Protein Y)

c. Protein Y only, no inhibitor (no Protein X)

d. No proteins, no inhibitor

B. Reagent Preparation

	1	2	3	4	5	6	7	8	9	10	11	12
	CONTROLS			[Un-tagged Protein] nM			[Compound Inhibitor 1] nM			[Antibody Inhibitor 1] nM		
A	Both Proteins			500			75			100		
B	No Protein Y			200			30			40		
C	No Protein X			80			12			16		
D	Neither Protein			32			4.8			6.4		
E				12.8			1.9			2.6		
F				5.1			0.768			1.0		
G				2.0			0.307			0.410		
H				0.819			0.123			0.164		
I				0.328			0.049			0.066		
J				0.131			0.020			0.026		
K				0.052			0.008			0.010		
L				0.021			0.003			0.004		
M				0			0			0		

Figure 4.2.5A: 384-well plate map for competition experiment.

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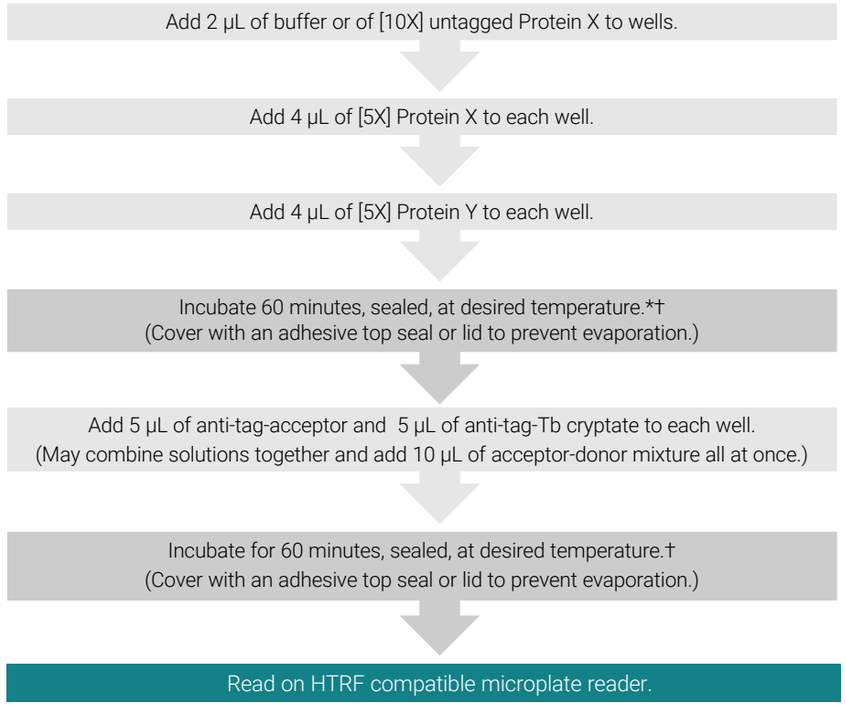
Per well, add the following for 20 µL final volume:

- 2 µL buffer, untagged protein, compound or antibody prepared at 10X
- 4 µL Protein X, prepare at 5X
- 4 µL Protein Y, prepare at 5X
- 5 µL Donor conjugate, prepared at optimized concentration (from Experiment Four)
- 5 µL Acceptor conjugate, prepared at optimized concentration (from Experiment Four)

1. Prepare 5X stock concentration of Proteins X and Y in assay buffer.
2. Prepare 10X concentrations of dose response competitors in assay buffer.
3. During first incubation prepare working solutions of acceptor and donor reagents, based on results from Experiment Four. It is also possible to combine these two reagents together and add 10 µL of the combined solution rather than 5 µL of each separately.

C. Assay Protocol for a 384-well Low Volume Plate

Add reagents to microplate, using an electronic multi-channel repeat pipettor to dispense reagents, as in Experiment Two.



* 60 minutes is usually adequate for first incubation. However, use the optimal time identified in Experiment One. Pre-incubation of proteins is not always required and this step can be omitted.

† Experiments in this guide were performed at room temperature. Incubate at higher/lower temperatures if you have prior experimental knowledge of specific temperature requirement for your protein-protein interaction.

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D. Assay Example

An assay was performed measuring the disruption of the biotin-EGF and EGFR-FC protein interaction by untagged EGF and two known antibody inhibitors, Cetuximab and anti-EGFR Antibody-10 (clone 111.6). See Figure 4.2.5B and Table 4.2.5-1. The results correlated with literature values for a cell-based EGFR HTRF assay as seen in table 4.2.5-1.

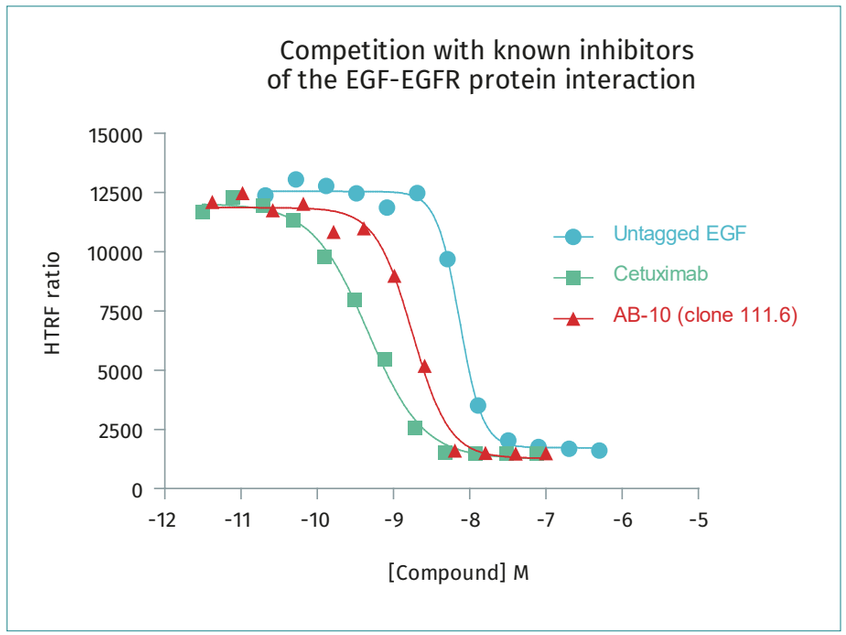

Figure 4.2.5B. Competition of the biotin-EGF and EGFR-FC interaction with known inhibitors; untagged EGF was titrated from 21 pM to 500 nM, Cetuximab was titrated from 3 pM to 75 nM, and EGFR antibody was titrated from 4 pm to 100 nM. Increasing concentrations of the protein and the antibodies resulted in decreasing signal as a result of decrease in protein interaction.

Table 4.2.5-1. Comparison of IC50 results to that derived in a cell-based EGFR HTRF assay. IC50s and rank potency correlate well with a previous study. (F. Charrier-Savournin, J. Vallaghé, S. Douzon, S. Martinez, E. Trinquet, Revvity provides a full Cell-Based Platform for the investigation of therapeutic antibodies and small molecule action mechanisms: an EGFR case study. Poster presented at: MipTec, September 2011.)

	IC50 (nM)		
	Untagged EGF	Cetuximab	AB-10 (111.6)
Experiment #5 results	7.18	0.47	1.73
Cell Based HTRF EGFR assay	2.4	0.19	0.5

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ASSAY DEVELOPMENT

Other Considerations for Assay Development

Depending on assay requirements, you may need to consider other factors to further optimize the protein-protein interaction assay. Descriptions of how to assess additional assay factors are not reviewed in detail here; however, typical best practices for assay optimization should be followed. Factors to evaluate might include any of the following:

- Order of addition: With HTRF protein interaction assays, the order of addition of the HTRF reagents is generally not critical. There is no need to add the acceptor before the donor or vice versa. In some instances, acceptor and donor conjugates can be combined and then added as one solution mix to the protein reagents. Typically the proteins are combined first, with any inhibitors, allowed to incubate a short time, and then the HTRF reagents are added.
- Incubation temperature: HTRF protein-protein assays are typically incubated at room temperature. HTRF reagent stability is not a limitation.
- Adjusting volumes: Whatever the plate type, assay volume should match well volume as closely as possible so that focalization of the reader excitation beam gives maximum energy delivery. For instance, when assay volumes are equal to or lower than 20 µL, it is better to use 384 low volume or 1536-well plates rather than regular 384-well plates.
- DMSO and additive tolerance: HTRF assays are resistant to most standard buffer conditions and additives such as DMSO, pH, chelators and ionic strength. Evaluate as you would for any assay.
- Z'-factor: Evaluate as you would when optimizing any assay.

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APPENDICES

Appendix A: Recommended Microplates and Volumes for HTRF Assays

Most available microplate brands are compatible with HTRF technology and products. Nevertheless, background levels may differ substantially among various plate types. FIA white plates from many suppliers remain the most appropriate standard. Tissue-culture treated, or surface-treated plates may also be used. Glass bottom plates produce suboptimal results and should not be used. The following plates produced excellent results during our validation process for a broad range of HTRF assays:

PROVIDER	FORMAT	RECOMMENDED VOLUME	TYPE	CAT#
Revvity	96-well low volume	15-20 µL	White	66PL96001
Greiner	384-well	80-100 µL	Black	781076
Greiner	384-well	80-100 µL	White	781075
Greiner	384-well low volume	15-20 µL	Black	784076
Greiner	384-well low volume	15-20 µL	White	784075
Greiner	96-well	160-200 µL	White, TC-sterile	655083
Greiner	96-well	160-200 µL	Black	655209
Greiner	96-well	160-200 µL	Black, TC-sterile	655086
Greiner	96-well	160-200 µL	Black, TC-sterile	655079
Greiner	96-well	160-200 µL	White, TC-sterile	655073
Greiner	384-well	80-100 µL	Black, TC-sterile	781086
Greiner	384-well	80-100 µL	Black, TC-sterile	781079
Greiner	384-well	80-100 µL	White, TC-sterile	781073
Greiner	384-well	80-100 µL	Black	781209
Greiner	384-well	80-100 µL	White, TC-sterile	781080
Greiner	384-well low volume	15-20 µL	White, TC-sterile	784080
Greiner	1536-well	5-10 µL	White	783075
Greiner	1536-well	5-10 µL	Black	783076
Greiner	1536-well	5-10 µL	White , TC-sterile	782080
Greiner	1536-well	5-10 µL	Black , TC-sterile	782086
Greiner	1536-well	5-10 µL	White , TC-sterile	782078
Greiner	1536-well	5-10 µL	Black, sterile	782092
Greiner	1536-well	5-10 µL	White, sterile	782073
Greiner	1536-well	5-10 µL	White, TC-sterile	782093
Corning	96-well	160-200 µL	Black, NT	3915
Corning	96-well	160-200 µL	White, NT	3912

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PROVIDER	FORMAT	RECOMMENDED VOLUME	TYPE	CAT#
Corning	96-well half-area	80-100 µL	Black, NT	3694
Corning	96-well half-area	80-100 µL	White, NT	3693
Corning	384-well	80-100 µL	Black, NT	3573
Molecular Devices	96-well half area and half volume	30-40 µL	Black, NT	42-000-0117
NUNC	384-well low volume	15-20 µL	Black, NT	264705
NUNC	384-well low-volume	15-20 µL	White, NT	264706

The signal-to-noise ratios obtained using white microplates do not differ greatly from those of black plates. Absolute counts, however, are significantly higher in white plates, thereby improving count statistics and subsequent assay reproducibility and sensitivity. We therefore recommend the systematic use of white plates.

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APPENDICES

Appendix B: Recommended Conjugates

The following is a list of Revvity materials used to produce the data shown in this guide. Catalog numbers listed correspond to the smallest package size. Additional larger sizes may be available. Please be sure you are ordering the appropriate catalog number (size and quantity) for your assay needs.

ITEM	DESCRIPTION	CATALOG NUMBER
Streptavidin-d2	d2-conjugated streptavidin	610SADLA
Streptavidin-XL665	XL665-conjugated streptavidin	610SAXLA
Streptavidin-Tb	Tb cryptate-conjugated streptavidin	610SATLA
Anti-Human IgG-d2 (PAb)	d2-conjugated polyclonal goat anti-human IgG	61HFCDAA
Anti-Human IgG-XL665 (PAb)	XL665-conjugated polyclonal goat anti-human IgG	61HFCXLA
Anti-Human IgG-Tb (PAb)	Tb cryptate-conjugated polyclonal goat anti-human IgG	61HFCTAA
Anti-GST-Tb	Tb cryptate-conjugated mouse monoclonal antibody anti-glutathione S-transferase	61GSTTLA
Anti-6His-Tb cryptate Gold	Tb cryptate-conjugated monoclonal antibody anti-polyhistidine	61HI2TLA
Anti-GST-XL665	XL665-conjugated mouse monoclonal antibody anti-glutathione S-transferase	61GSTXLA
Anti-GST-d2	d2-conjugated mouse monoclonal antibody anti-glutathione S-transferase	61GSTDLA
Anti-6His-d2	d2-conjugated mouse monoclonal antibody anti-6 histidine	61HISDLA
Anti-6His-XL665	XL665-conjugated mouse monoclonal antibody anti-6 histidine	61HISXL

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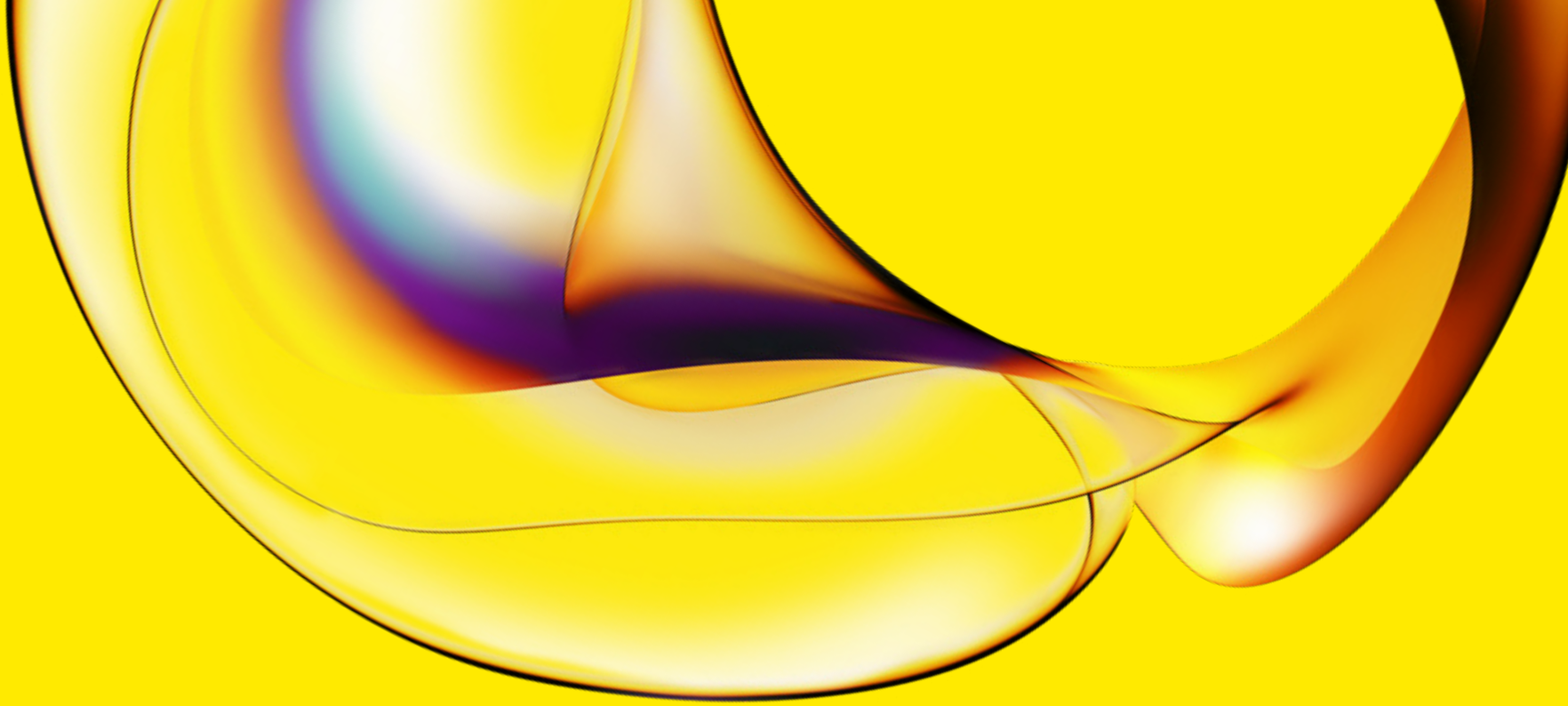

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