Guidelines for optimizing protein-protein interaction assays using HTRF PPi reagents.

This technical note reviews the general principles and steps in developing a protein-protein interaction assay using HTRF PPi reagents from Revvity assays.

Abstract

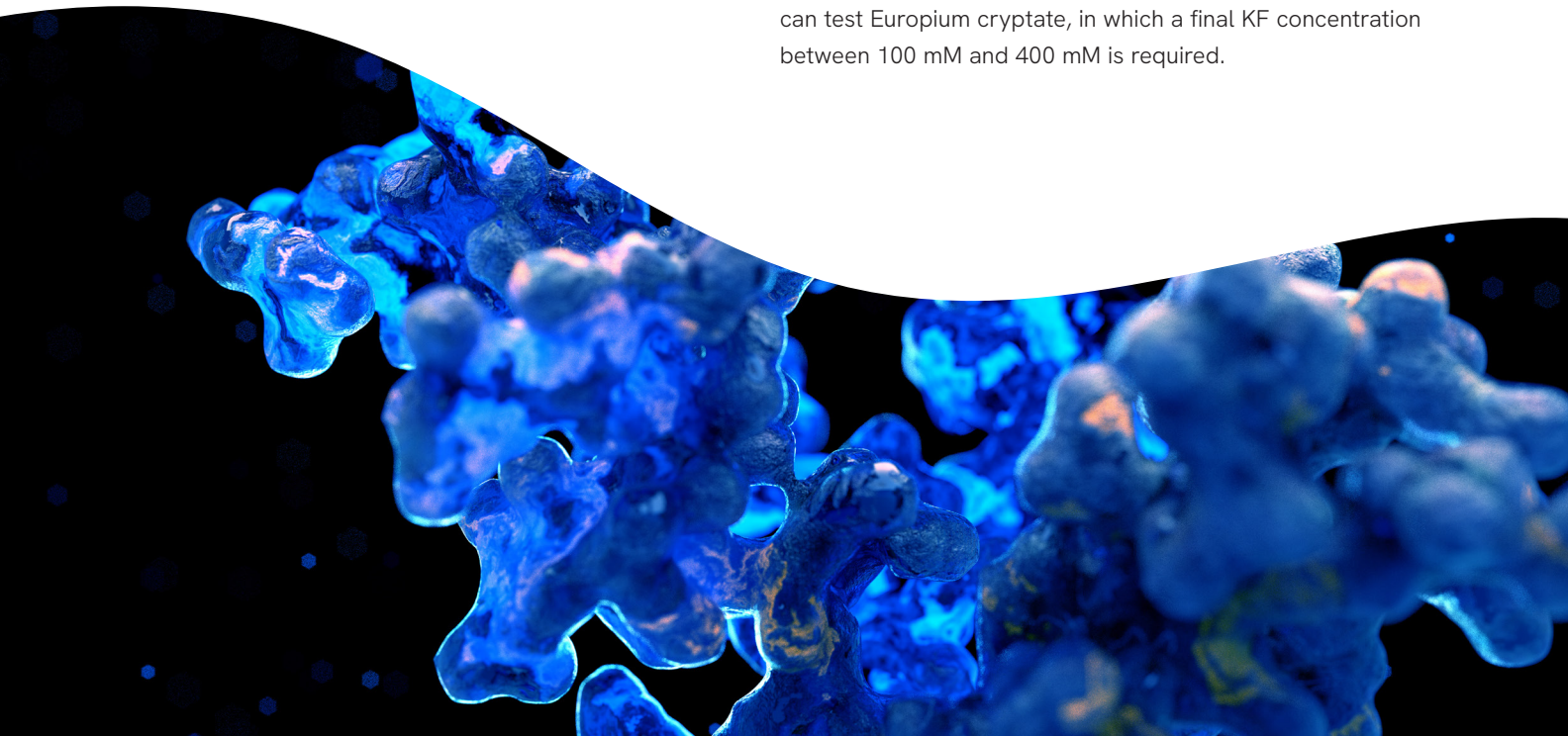
This technical note reviews the general principles and steps in developing a protein:protein interaction assay using HTRF® PPi reagents from Revvity. In an HTRF protein:protein interaction assay, one protein is labeled (directly or indirectly) with the donor, and the other protein is labeled (directly 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.

Before you begin

Revvity PPi buffers have been optimized for maximmum assay performance. Therefore, if you do not have specific buffer requirements, we recommend using the Revvity PPi - Terbium detection buffer Cat # 61DB10RDF and Revvity PPi - Europium detection buffer Cat # 61DB9RDF for Revvity PPi reagents preparation.

If your experimental conditions include constraints in terms of compounds and environment which complicate detection steps, remember that Revvity's HTRF cryptate reagents are resistant to most buffer conditions and additives (DMSO up to 5%, chelators, divalent cations, ionic strength, and pH).

Terbium cryptate is a recommended donor if the interaction you are studying is sensitive to ionic strength. Otherwise, you can test Europium cryptate, in which a final KF concentration between 100 mM and 400 mM is required.



For all assays, we recommend testing several reagent combinations, since FRET efficiency is dependent on the relative position of the donor and the acceptor and thus will vary on conformation with the detected complex.

You will find more information about dye characteristics and buffers in the full guide entitled Protein:Protein Interaction Assays with HTRF®.

Choosing the right assay format

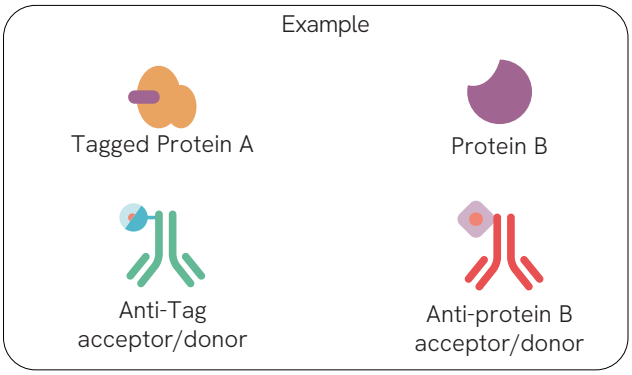
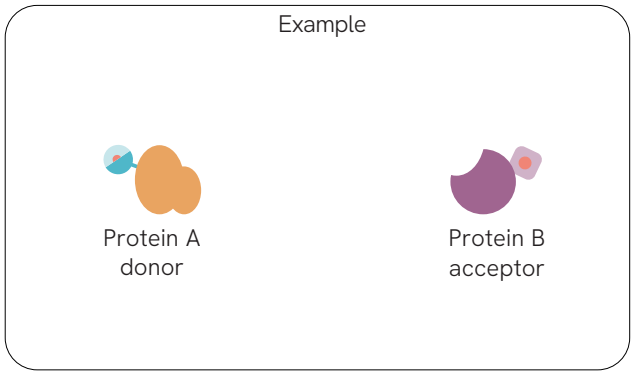
Indirect assays	Direct assays
Binding partners (protein A and protein B) are detected using intermediary dyed reagents	Binding partners (protein A and B) are directly labeled with HTRF dyes
	
▪ Separated control of detection reagent and protein concentrations ▪ Suitable for a wide range of PPI Kd ▪ Antibodies used have to be labeled	▪ Give access to true Kd values and binding kinetics parameters ▪ Unsuitable for very low affinity interactions with Kd in the µM range ▪ Proteins of interest have to be labeled

Fig 1: You can use pre-labeled antibodies and proteins. Label them on your own, or have them labeled for you by Revvity's custom service team (www.Revvity.com/biomolecule-labeling-service).

Detection assays can be in one of two formats: indirect and direct. Each features characteristics, advantages and disadvantages that should be taken into account when selecting the one for your assays

Indirect assay format

a. Experiment 1: specific signal generation

The first assay is a cross-test of all possible donor/acceptor combinations to determine the best option.

Then to ensure that the optimal FRET signal is detected, 6 concentrations of each protein are tested in a matrix assay: C0, C1, C2, C3, C4, and C5. Note that "C0" represents detection reagents + one protein only, and is used to assess the non-specific signal and background (noise) of the assay. Table 1 displays a plate plan for this step.

Donor-labeled reagents should be used at the recommended concentration indicated in the Revvity datasheet.

Acceptor-labeled reagents should be used at the recommended concentration indicated in the Revvity datasheet. Acceptor labeled concentration should be further optimized for matching that of the molecule to be detected for each condition (1:1 ratio). When using a biotin tag, maintain a constant 4:1 ratio between biotin and streptavidin.

Note that a background increase is observed when the acceptor-labeled reagent is used above 300-500 nM.

Test the following concentrations when the expected Kd is known: 0.1 Kd, 0.3 Kd, 1 Kd, 3 Kd, and 10 Kd.

When the expected Kd is not known, test a wide range of concentrations from low µM to low nM: e.g. 5 µM, 1 µM, 0.2 µM, 40 nM, and 8 nM.

Table 1

Anti-Tag-Donor/ Anti-Protein B-Acceptor		Tagged-Protein A or B			
		C0	C1	...	C5
Protein B or A	C0	X	X	...	X
	C1	X	X	...	X
	...	...	...	...	...
	C5	X	X	...	X

b. Result analyses

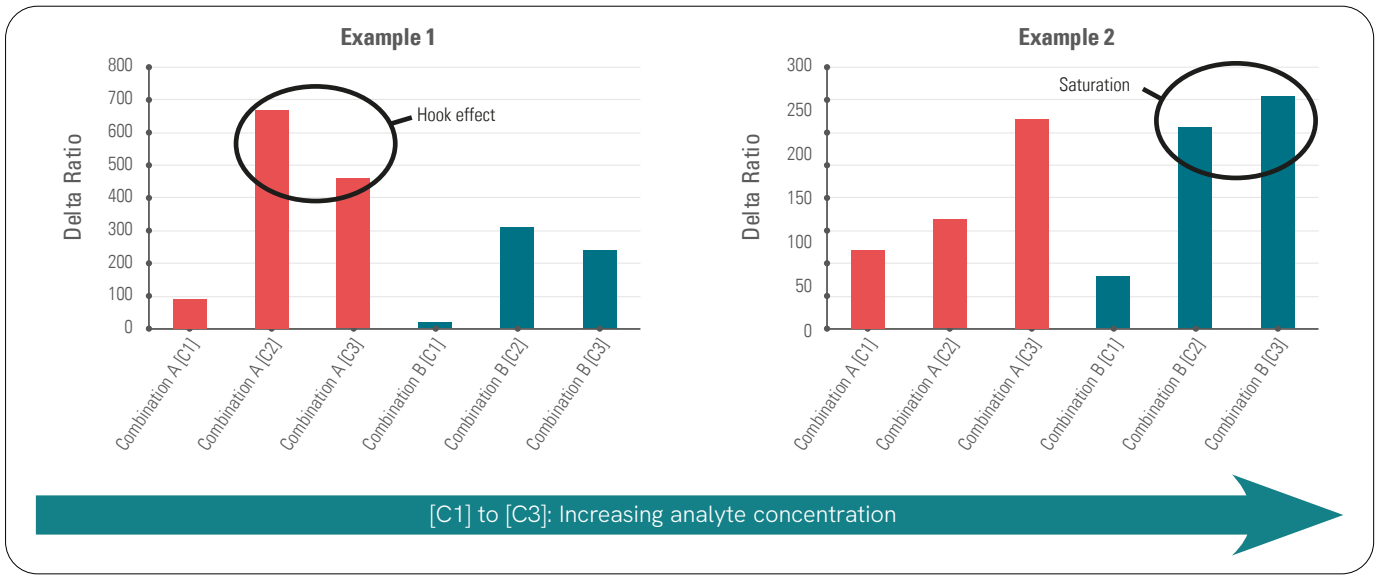


Fig 2: Combination A depicts the tagged-protein A / protein B assay format (Cf fig 1) while combination B refers to the tagged-protein B / Protein A pair.

Example 1: Analyte concentration falls partially within the detection range

Combination A gives the best HTRF results and is thus selected as the best pair.

[C3] does not give a higher HTRF signal than [C2], even though that was expected. This means that the Hook Effect has been reached and the assay is no longer in the linear range for [C3].

Example 2: Analyte concentration falls within the detection range

No combination reaches the Hook, meaning the analyte concentrations are well within the assay's linear range.

Combination B gives a higher HTRF signal. However, it reaches a plateau between [C2] and [C3], which prevents it from being selected as the best pair.

Even though pair A gives a slightly lower HTRF signal than pair B, it is selected as the best pair.

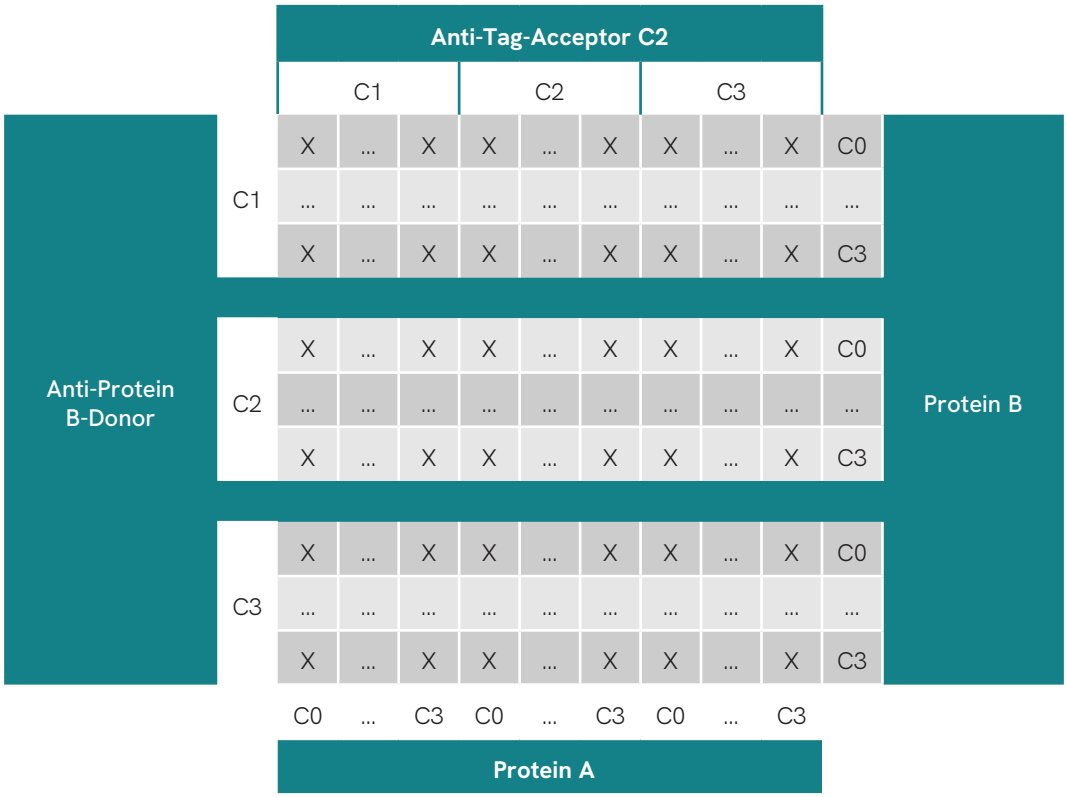
In all cases, we recommend further optimization of the chosen pair by testing different concentrations of proteins and acceptors/donors as described below.

C. Experiment 2: Assay optimization

Run the following experiments to refine the concentrations obtained from the first step:

- Optimization of the concentration of the protein detected by the labeled acceptor: test a range of 9 concentrations around the concentration obtained in step 1: 16X, 8X, 4X, 2X, 1X, 0.5X, 0.25X, 0.125X, and 0.0625X.
- Optimization of the concentration of the protein detected by the donor: test 3 concentrations around the optimal concentration obtained in step 1: 2X, 1X, and 0.5X.
- Optimization of the concentrations of both acceptor- and donor-labeled conjugates: this test is not mandatory, as the recommended doses are usually sufficient, but you may wish to modify the recommended value slightly to see if there is any possible improvement. To do this, prepare concentrations of the donors at 2X, 1X, and 0.5X the recommended concentrations, and the acceptors at 1X, 0.5X, and 0.25X the recommended concentrations.

Table 2: Below summarizes these tests in a single matrix.



Direct assay format

A) Experiment 1: Specific signal generation

As for an indirect assay, the first step is to test all different donor/acceptor combinations to find the best one.

In the same way, several labeled protein concentrations (C1 to 5) are tested in a matrix assay to ensure that the optimal FRET signal is detected. Table 3 displays an example plate organization for this step.

When Kd is known, test 10-fold dilutions for each labeled protein (0.01 Kd, 0.1 Kd, 1 Kd, 10 Kd, 100 Kd). When Kd is not known, refer to the indirect assay format to select the concentrations to be tested.

Table 3

Protein A-Donor/Protein B-Acceptor	
Protein B or A	C0
	C1
	...
	C5

Protein A or B			
C0	C1	...	C5
X	X	...	X
X	X	...	X
...	...	...	...
X	X	...	X

Note that donor-labeled proteins should not be tested above 20-40 nM. Also, black plates are highly recommended at donor-conjugate concentrations above 10 nM

B) Experiment 2: Assay optimization

To further determine the exact protein concentrations to use, repeat the experiment while testing tighter ranges of each binding partner concentration around the most promising concentrations which you determined previously.

Signal specificity

You can run a displacement assay to evaluate assay specificity: use an excess amount (100:1) of one unlabeled partner to disrupt the interaction and decrease the HTRF signal.

We invite you to check out the **Protein:protein Interaction Assays with HTRF** full guide for further information on reagents, more detailed optimization procedures, and extensive examples from Revvity's R&D studies.

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