APPLICATION NOTE

Improve PROTAC targeting kinase discovery with HTRF kinase binding kits.

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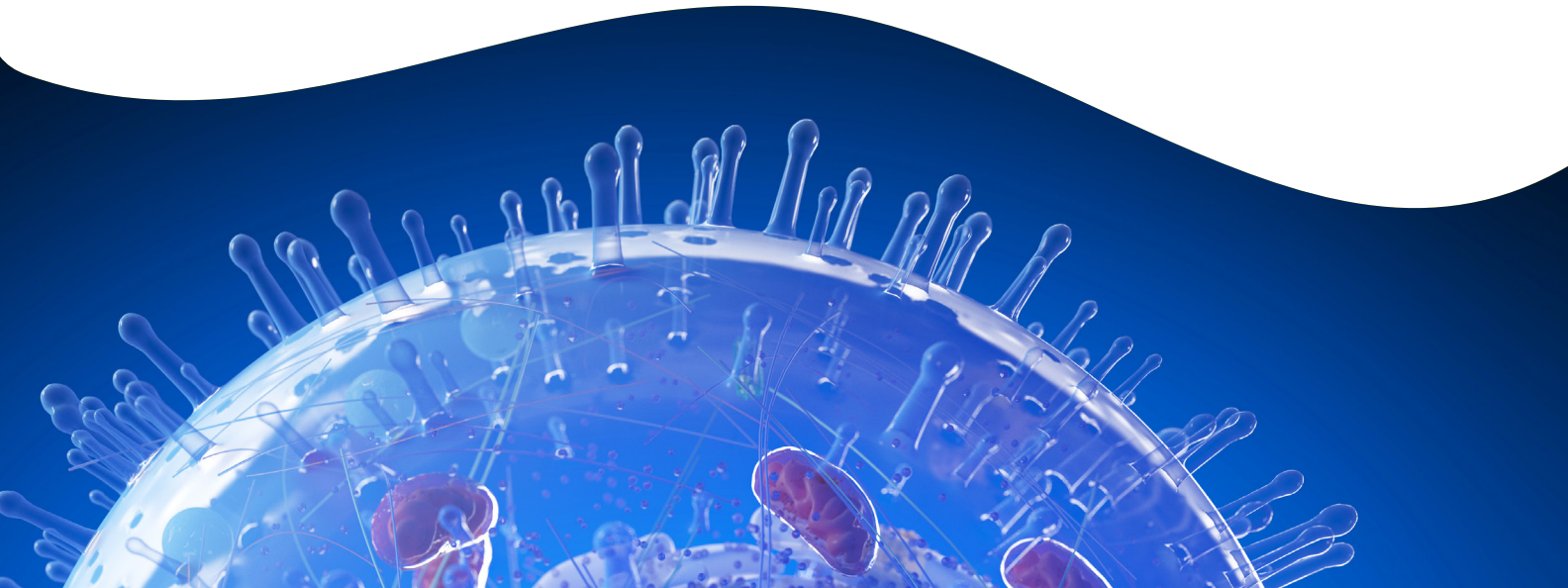
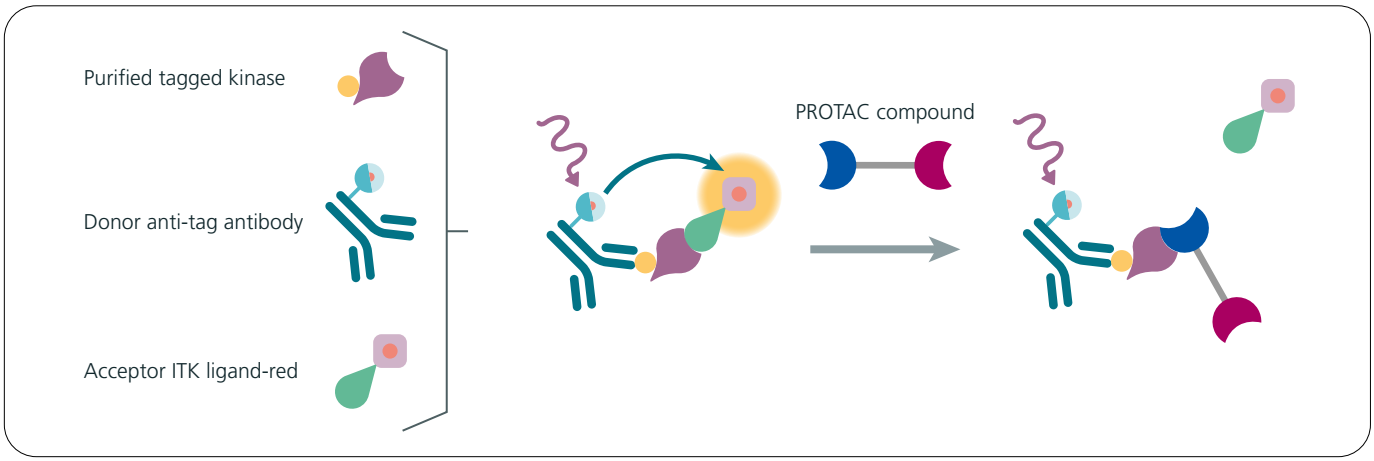
Kinases are essential proteins involved in signal transduction, regulating numerous biological events such as cell growth and proliferation, cell differentiation, and migration. Consequently, kinase dysfunctions due to gene amplification or mutations are associated with numerous diseases, such as cancer, immune and metabolic disorders, and cardiovascular and neurodegenerative diseases. From a therapeutic perspective, kinase inhibitors competing with ATP binding, as well as monoclonal antibodies, have been developed extensively and mainly approved for cancer treatment. However, these drugs suffer from several limitations, such as drug resistance, off-target effects, and other side effects. More recently, promising new approaches relying on induced protein degradation, such as PROTAC, have gained traction because they are expected to overcome most of the limitations inherent to small molecule inhibitors and even target the "undruggable" proteome. Proteolysis-targeting chimera (PROTAC) compounds are hetero-bifunctional molecules that bind both to the targeted protein and to E3 ubiquitin ligase, forming a ternary complex that ultimately leads to POI ubiquitination and its subsequent proteasome degradation. To date, PROTAC technology has been used to induce the degradation of various proteins, including kinases.

Here, we demonstrate how HTRF® kinase binding assays are used to efficiently and accurately identify, characterize, and optimize PROTAC targeting kinases on two relevant models: BTK and CDK4.



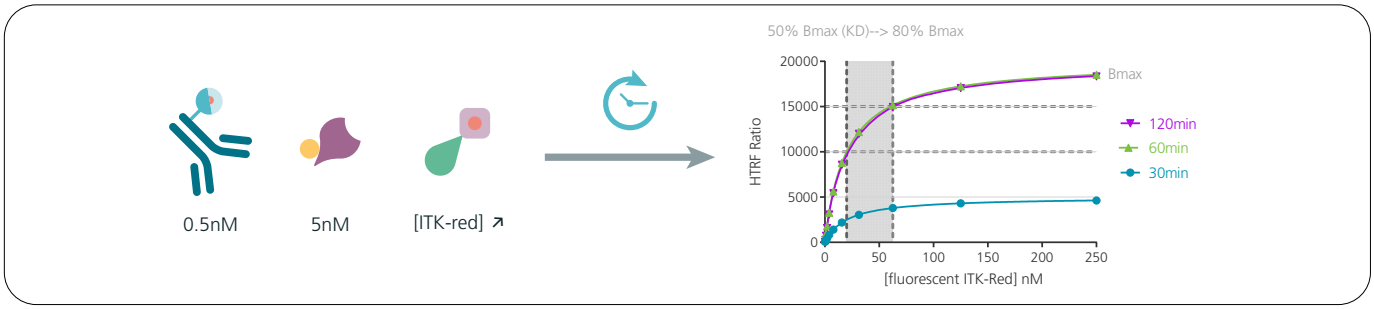
Assay principle and workflow

The assay is based on an HTRF sandwich format relying on HTRF donor anti-tag antibody or streptavidin, which recognize the corresponding tagged kinase and an HTRF acceptor fluorescent derivative of kinase inhibitor. In the presence of competitive inhibitors such as ITK-derived PROTAC compounds, the binding of fluorescent ITK ligand-red is abrogated, leading to HTRF signal decrease.

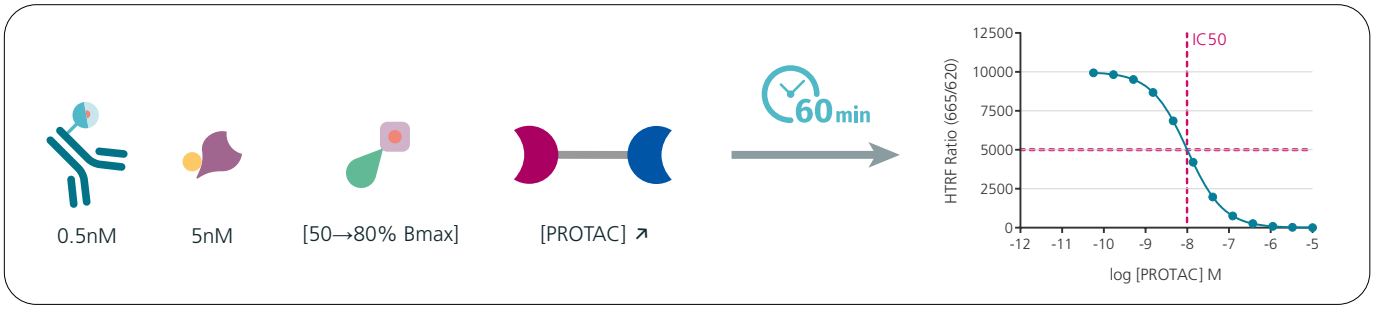


2-step experiment workflow:

1. HTRF saturation binding curve is evaluated to determine the KD of acceptor fluorescent ligand towards the kinase of interest at equilibrium.



2. HTRF competitive binding assay is performed by adding a fixed concentration of fluorescent ITK-red from 50% (KD) to 80% of the Bmax to a dilution series of the compound of interest.



The measured IC_{50} values are then used to calculate apparent Ki potency using the Cheng-Prussoff equation:

$$K_i = \frac{IC_{50}}{\left(1+\left(\frac{\text{Dasatinib-Red}}{K_d}\right)\right)}$$

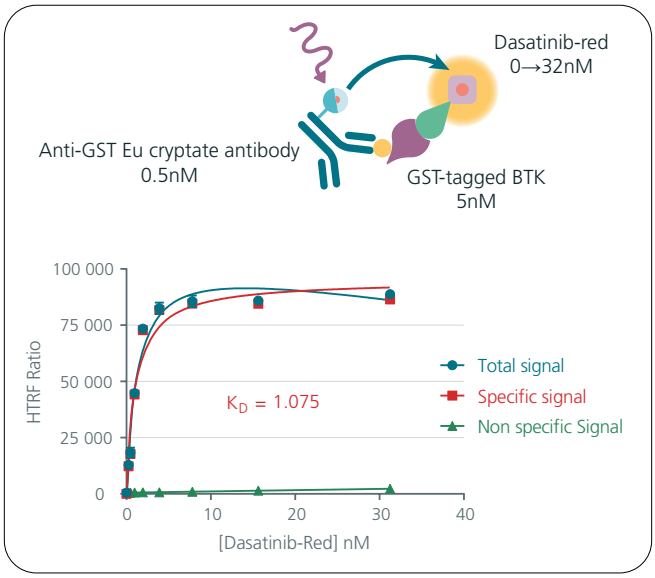
PROTAC compound screening through HTRF BTK kinase binding assay

GST-tagged BTK kinase binding assay

1. Saturation binding experiment: Determination of dasatinib-red Kd

The first step in developing the kinase inhibitor assay is to determine the optimal fluorescent ITK concentration for the kinase of interest, in this case the GST-BTK. The assay's dynamic range depends on the kinase concentration.

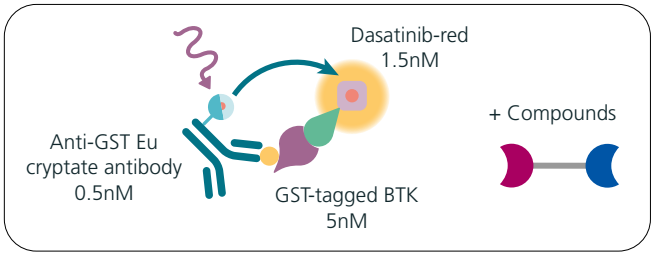
Here, 5 nM of GST-BTK, 0.5nM of anti-GST Eu cryptate antibody, and a concentration ranging from 0-32 nM of dasatinib-red were used.

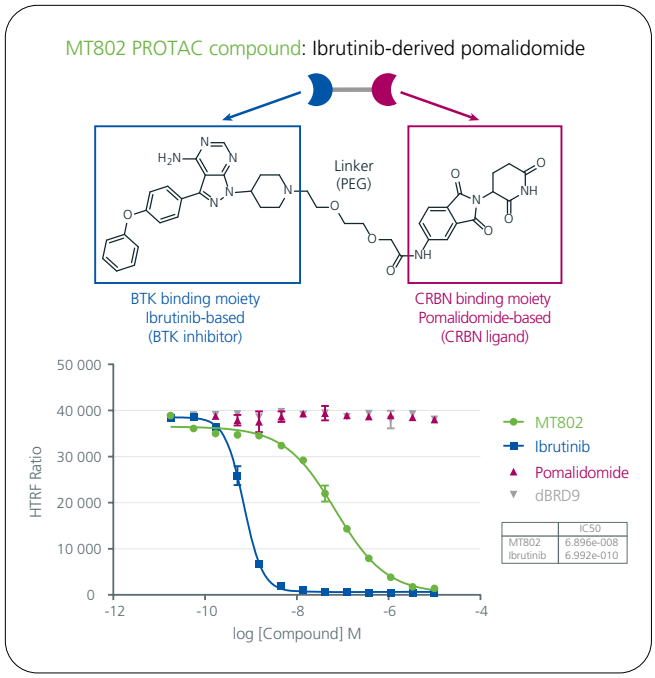


The Kd of dasanitib-red for GST-BTK is 1.075nM

2. Assessment of MT802 PROTAC compound binding through HTRF competitive inhibition experiment

From the above results, dasatinib-red was used at 1.5 nM in a competitive assay where ibrutinib and its PROTAC derivative MT802 were tested. Two irrelevant compounds were also included in the experiment: Pomalidomide, corresponding to the cereblon ligand and comprised in MT802; and dBRD9, another PROTAC compound targeting BRD9 protein.





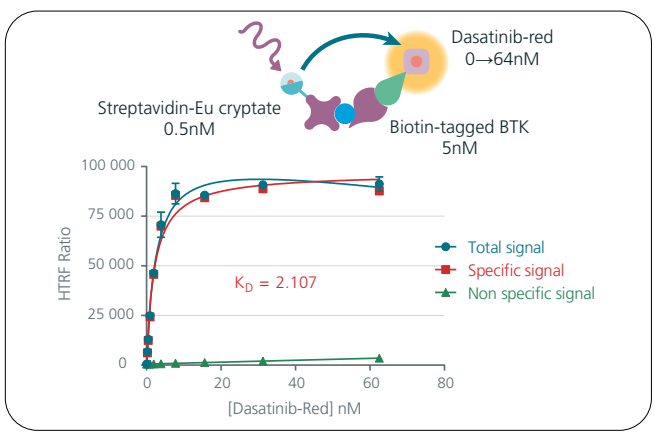
As expected, unlike the irrelevant dBrd9 and pomalidomide compounds, both ibrutinib and MT-802 compounds efficiently compete with the dasatinib-red ligand.
This experiment revealed that the potency of the MT802 compound is approximately 100 times lower than ibrutinib, likely explained by the greater size and sterical hindrance from the PROTAC compound.

Biotin-tagged BTK kinase binding assay

1. Saturation binding experiment: Determination of dasatinib-red Kd

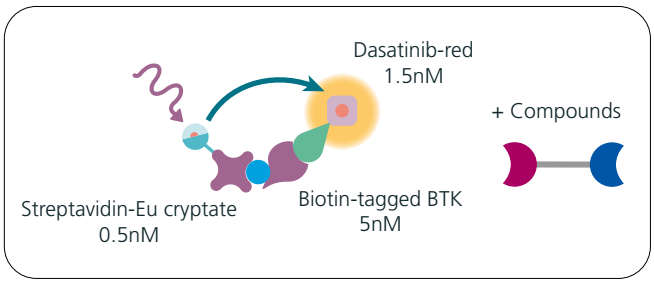
The same principle as that described above was applied to a recombinant biotinylated-BTK.

Here, 5 nM of biotin-BTK, 0.5nM of streptavidin-Eu cryptate, and a concentration ranging from 0-64 nM of dasatinib-red were used.

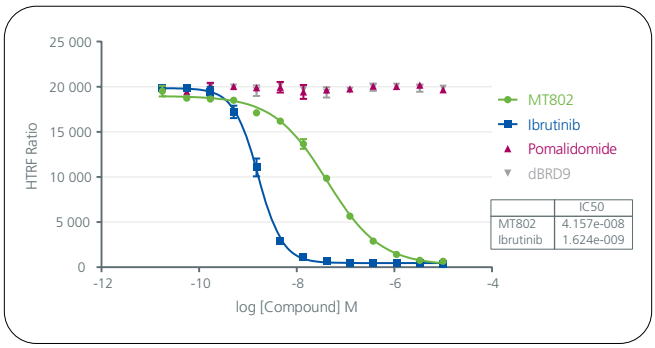


Here, the Kd of dasanitib-red for biotin-BTK is 2.107nM.

2. Assessment of MT802 PROTAC compound binding through HTRF competitive inhibition experiment.



From the above results, dasatinib-red was used at 1.5 nM in a competitive assay where ibrutinib and its PROTAC derivative MT802 were tested. Two irrelevant compounds were also included in the experiment: Pomalidomide, corresponding to the cereblon ligand and comprised in MT802; and dBRD9, another PROTAC compound targeting BRD9 protein.



Comparable results were obtained with the biotinylated-BTK enzyme. In this experiment, MT802 compound potency is approximately 25 times lower than that of the Ibrutinib.

Materials and methods:

- GST-tagged BTK, Carna Biosciences (ref. 08-180)
- MAb Anti GST-Eu kinase binding cryptate, Revvity (ref. 62KBGSTKAF)
- Biotinylated-tagged BTK, Carna Biosciences (ref. 08-480-23N)
- Dasatinib-red, Revvity (ref. 62KB02REDC)
- Streptavidin-Eu cryptate Kinase Binding, Revvity (ref. 62KBSAKAF)
- Kinase Binding Buffer, Revvity (ref. 62KBBRDD)
- MT802, Revvity (not commercially available)
- Ibrutinib Selleckchem (ref. S2680)
- Pomalidomide, Tocris (ref. 6302)
- dBRD9, Tocris (ref. 6606)
- ProxiPlate-384 Plus, PKI (ref. 6008280)

HTRF saturation binding experiment:

- + 5 µL kinase binding buffer (or 5µL of unlabeled dasatinib 3µMf for non-specific signal)
- + 5 µL recombinant GST or biotin-tagged BTK (5 nMf)
- + 5 µL HTRF anti-GST or streptavidin-Eu cryptate
- + 5 µL HTRF dasatinib-red serial dilutions
- 1 hr incubation at RT before reading HTRF signal

HTRF competitive experiment:

- + 5 µL of serial dilution compounds
- + 5 µL recombinant GST or biotin-tagged BTK (5 nMf)
- + 5 µL HTRF anti-GST or streptavidin-Eu cryptate
- + 5 µL of HTRF dasatinib-red (1.5nMf)
- 1 hr incubation at RT before reading HTRF signal

Table 1 shows the results obtained using either a GST or biotin detection system. Both assay formats provide correlated results and enable c

Table 1	GST-BTK	Biotin-BTK	Reference values in literature
Dasatinib-red Kd (nM)	1.075	2nM	nd*
MT802 Ki / IC50 / SB	29nM / 69nM / 120	23nM / 41nM / 88	IC50=48.88nM (1)
Ibrutinib Ki / IC50 / SB	0.29nM / 0.69nM / 60	0.9nM / 1.6nM / 43	IC50=0.5nM (2)

*nd: not determined

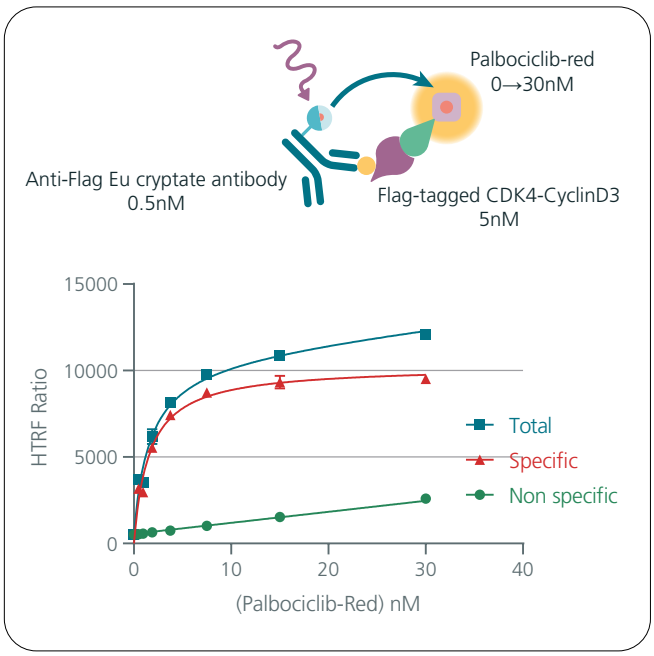
1. Buhimschi et al. Targeting the C481S Ibrutinib-Resistance Mutation in Bruton's Tyrosine Kinase Using PROTAC-Mediated Degradation. Biochemistry 2018, 57, 26, 3564-3575
2. Akinleye et al. Ibrutinib and novel BTK inhibitors in clinical development. Journal of Hematology & Oncology 2013, 6:59

PROTAC compound screening through HTRF CDK4 kinase binding assay

Flag-CDK4 kinase binding assay

1. Saturation binding experiment: Determination of palbociclib-red Kd

This experiment was performed with 5 nM of Flag-CDK4, 0.5nM of anti-Flag Eu cryptate antibody, and a concentration ranging from 0-30 nM of palbociclib-red.

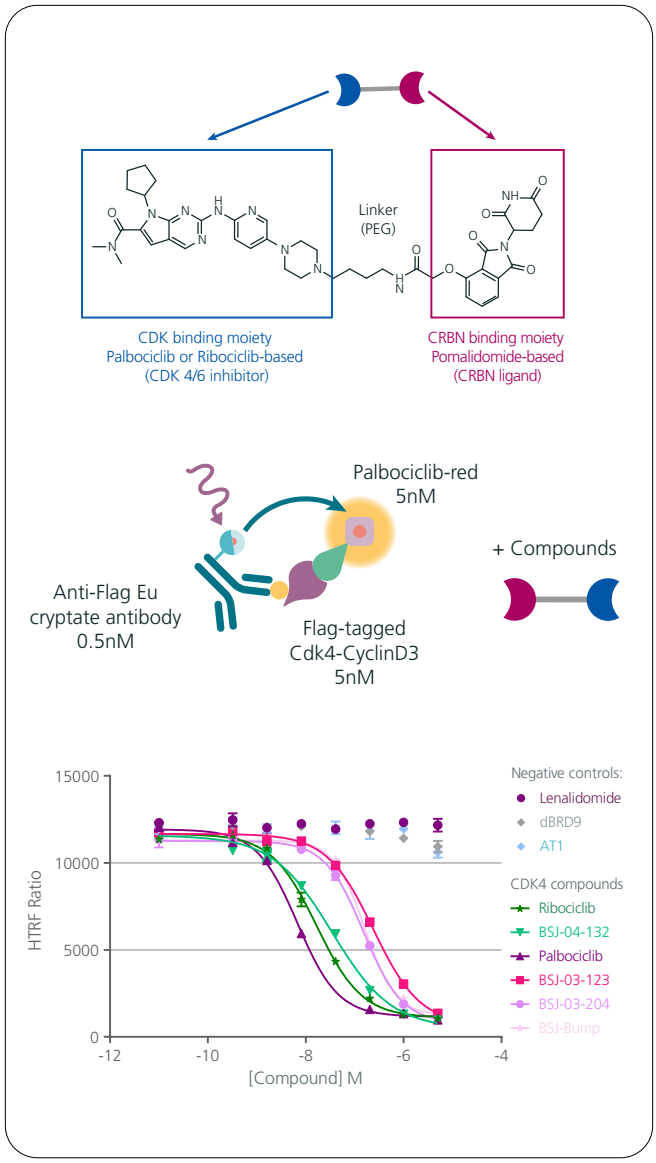


The Kd of palbociclib-red for Flag-CDK4 is 1.57nM; the 80% Bmax is 5nM.

2. Assessment of CDK4 degrader binding through HTRF competitive inhibition experiment.

From the above results, a competitive experiment was carried out using palbociclib-red at 5 nM. The following compounds were tested:

- Palbociclib and ribociclib, which are orthosteric inhibitors of CDK4 and CDK4/6 kinases, respectively.
- BSJ-03-123, BSJ-03-204, and BSJ-Bump, which are palbociclib derived PROTAC compounds including a thalidomide moiety.
- BSJ-04-132, which is a ribociclib-derived thalidomide-based PROTAC compound.
- Lenalidomide, dBrd9, and AT1, which are irrelevant compounds.

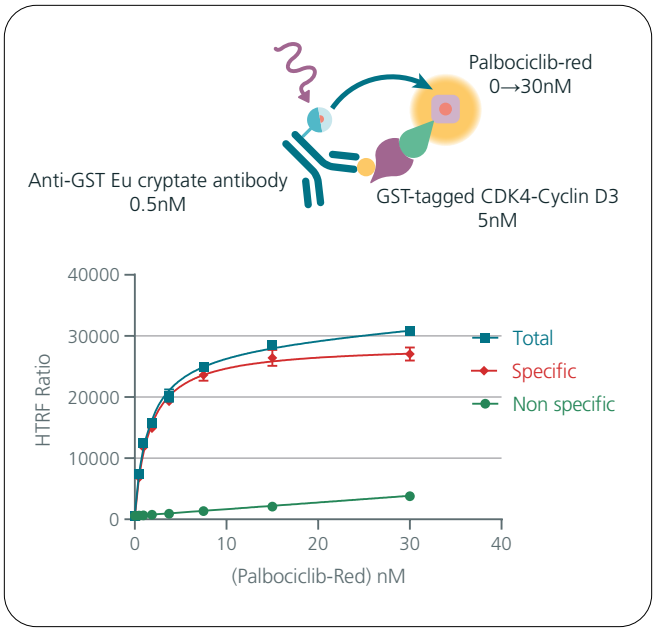


As expected, unlike the irrelevant dBrd9, lenalidomide or AT1 compounds, palbociclib and ribociclib as well as their PROTAC derivatives efficiently compete with Palbociclib-Red ligand binding. Interestingly, derivatization of palbociclib with thalidomide (BSJ-03-123, BSJ-03-204, BSJ-Bump) significantly impacts its affinity (from 25 to 36-fold), whereas derivatization of ribociclib (BSJ-04-132) shows less impact (2-fold). Pharmacological parameters are reported in Table 2.

GST-CDK4 kinase binding assay

1. Saturation binding experiment: Determination of the Palbociclib-red Kd

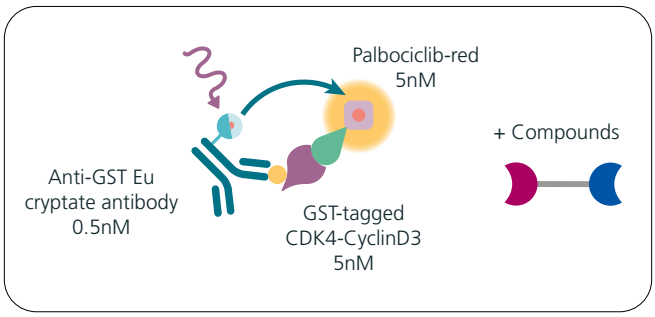
This experiment was performed with 5 nM of GST-CDK4, 0.5nM of anti-GST Eu cryptate antibody, and a concentration ranging from 0-30 nM of palbociclib-red.

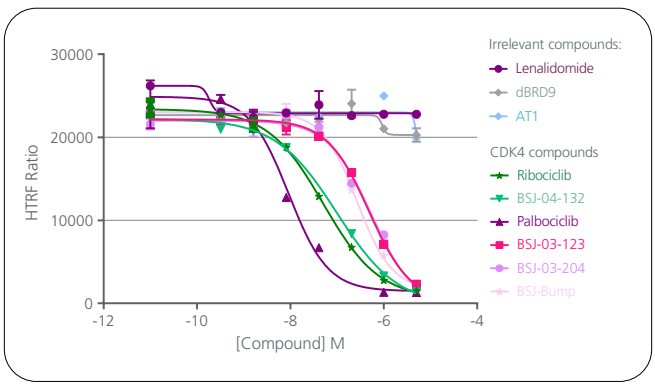


The Kd of palbociclib-red for Flag-CDK4 is 1.57nM; the 80% Bmax is 5nM

2. Assessment of CDK4 degrader binding through HTRF competitive inhibition experiment.

From the above results, palbociclib-red was used at 5 nM in a competitive assay using the same panel of compounds as previously described.





Comparable results were obtained using the GST tagged CDK4 enzyme. Palbociclib and ribociclib, as well as their PROTAC derivatives, exhibit efficient competition with palbociclib-red ligand binding. Again, the derivatization of palbociclib with thalidomide (BSJ-03-123, BSJ-03-204, BSJ-Bump) compounds leads to decreasing their potencies, whereas thalidomide derivatization of ribociclib (BSJ-04-132) does not impair its potency. Pharmacological parameters are reported in Table 2

Materials and methods:

- Flag-tagged CDK4, Carna Biosciences (ref #04-405-20N)
- MAb Anti FLAG M2-Eu cryptate, Revvity (ref. 61FG2KLA)
- GST-tagged CDK4, Carna Biosciences (ref #04-105)
- MAb Anti GST-Eu cryptate, Revvity (ref. 61GSTKLA)
- Palbociclib-red, Revvity (not commercially available)
- Kinase Binding Buffer, Revvity (ref. 62KBBRDD)
- ProxiPlate-384 Plus, PKI (ref. 6008280)

HTRF saturation binding experiment:

- + 5 µL kinase binding buffer
- (or 5µL of unlabeled palbociclib 3µMf for non-specific signal)
- + 5 µL recombinant GST-tagged CDK4 (5 nMf)
- + 5 µL HTRF Mab anti GST-Eu cryptate (0.5nMf)
- + 5 µL of HTRF palbociclib-red serial dilutions
- 2 hrs incubation at RT before reading HTRF signal

HTRF competitive experiment:

- + 5 µL of serial dilution compounds
- + 5 µL recombinant GST-tagged CDK4 (5 nMf)
- + 5 µL HTRF anti GST-Eu cryptate
- + 5 µL of HTRF palbociclib-red (5nMf)
- 2 hrs incubation at RT before reading HTRF signal

Table 2 shows the results obtained using either a Flag or GST detection system. Despite the imperfect match between experimental and reported pharmacological values, both assay formats provide results consistent with literature, with palbociclib being twice as potent as ribociclib. They both enable convenient and robust screening of PROTAC targeting CDK4 compounds, as well as their pharmacological characterization.

Table 2	Flag-CDK4	GST-CDK4	Reference values in literature
Palbociclib-red Kd (nM)	1.55	1.57	nd*
Palbociclib Ki / IC50 / SB	1.56nM / 6.76nM / 10	2.1nM / 9.23nM / 17	IC50 5-20 nM (1, 2) Ki 0.26nM (3)
BSJ-04-123 Ki / IC50 / SB	55.7nM / 241 nM /17	109.7nM / 475 nM /119	IC50 31.2nM (1)
BSJ-04-204 Ki / IC50 / SB	35.1nM /152nM / 14	125.9nM / 1.6nM / 13	nd*
BSJ-Bump Ki / IC50 / SB	37nM / 160nM / 9	61.4nM / 266nM / 14	IC50 41.1nM (1)
Ribociclib Ki / IC50 / SB	3.8 nM / 16.7nM / 11	11.5nM / 50nM / 42	IC50 30nM (2) Ki 0.53nM (3)
BSJ-04-132 Ki / IC50 / SB	7.9nM / 34.3 / 24	22.6nM / 98.1nM/ 16	

*nd: not determined

1. Brand M. et al., Homolog-Selective Degradation as a Strategy to Probe the Function of CDK6 in AML. Cell Chemical Biology; 26, 300-306
2. O'Leary B. et al., Treating cancer with selective CDK4/6 inhibitors. Nature Reviews Clinical Oncology; 13,417–430
3. Chen et al., Spectrum and Degree of CDK Drug Interactions Predicts Clinical Performance. Molecular Cancer Therapeutics; 15(10); 2273–81

Conclusions

This application note clearly demonstrates the applicability of HTRF kinase binding kits in a PROTAC context. By combining different anti-tag antibodies and fluorescent ligands, as well as different sources of recombinant protein kinases, it is possible to easily screen and characterize new PROTAC compound targeting kinases. The highly versatile kinase binding kits offer an accurate and straightforward method for exploring and expanding the possibilities of targeted protein degradation strategies.

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