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Development of an Eg5 assay using the HTRF® Transcreener® ADP kit

Based on Cisbio Bioassays' patented HTRF® technology, HTRF Transcreener® ADP assay is a universal method for identifying and characterizing the phosphotransferase activity of ATPases and kinases. ADP is directly detected by a specific monoclonal antibody labeled with europium cryptate and correlates closely with the amount of phosphorylated substrate in a kinase assay. Here we describe a typical four-step assay development with the Eg5 ATPase using the HTRF Transcreener ADP assay.

ADP is the universal product of kinase and ATPase activity. ADP measurement with HTRF technology avoids the limitations imposed by traditional methods of measuring the accumulation of ADP from kinases and ADP-producing enzymes. Examples of these include the use of radioactivity (for example, ^{33}P) or the reliance upon a secondary enzyme (such as luciferase, peroxidase or pyruvate kinase) for detection. With radioactive methods, particular attention must be paid to safety and the proper disposal of materials, which can be costly. Additionally, assay sensitivity can be compromised as a result of high background. Data obtained by enzyme-coupled detection methods can have high false-positive rates, and sensitivity limitations can arise if high turnover by the target enzyme is required for the product's detection by the secondary enzyme.

HTRF Transcreener ADP assay is a homogeneous, nonradioactive screening alternative to these various methods. Enzyme-free detection enables a low false-positive rate, and for kinases, this kit is a highly flexible solution as it is compatible with any substrate.

HTRF Transcreener ADP assay is a competitive immunoassay performed in two steps, the enzymatic step followed by the detection step (**Fig. 1a**). Standard curves are generated to mimic ADP generation during an enzymatic reaction: total adenosine concentration (ATP plus ADP) is kept constant for each sample while the percentage of ADP in each sample is changed (**Fig. 1b**).

The four-step development of the human Eg5 assay

Human Eg5, a member of the kinesin super family, has a key role in mitosis and is responsible for the formation and maintenance of the bipolar spindle. Eg5 has garnered substantial interest as a potential chemotherapeutic target in cancer treatment.

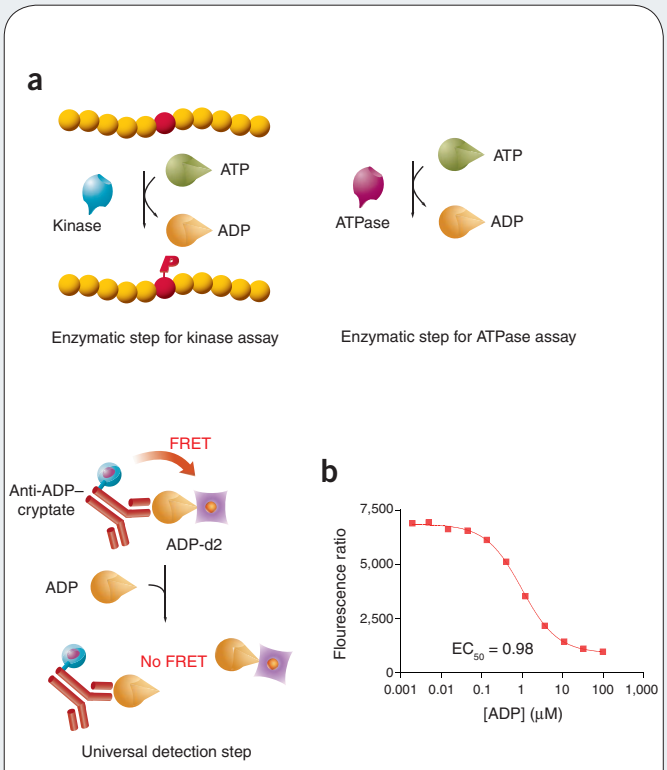

Figure 1 | HTRF Transcreener ADP assay principle and standard curve. (**a**) During the enzymatic step, a kinase catalyzes the transfer of a phosphate group from ATP to a substrate, whereas ATPases convert ATP into ADP and inorganic phosphate. Then in the detection step, native ADP and d2-labeled ADP compete for antibody to ADP (anti-ADP) labeled with Eu^{3+} cryptate. The fluorescence resonance energy transfer (FRET) signal is inversely proportional to the concentration of ADP in the sample. (**b**) The standard curve determined by assaying FRET in mixtures of various concentrations of ATP and ADP. ATP solutions were serially diluted in ADP solutions to mimic the conversion of ATP into ADP (from 0 to 100%). EC_{50}, half-maximal effector concentration.

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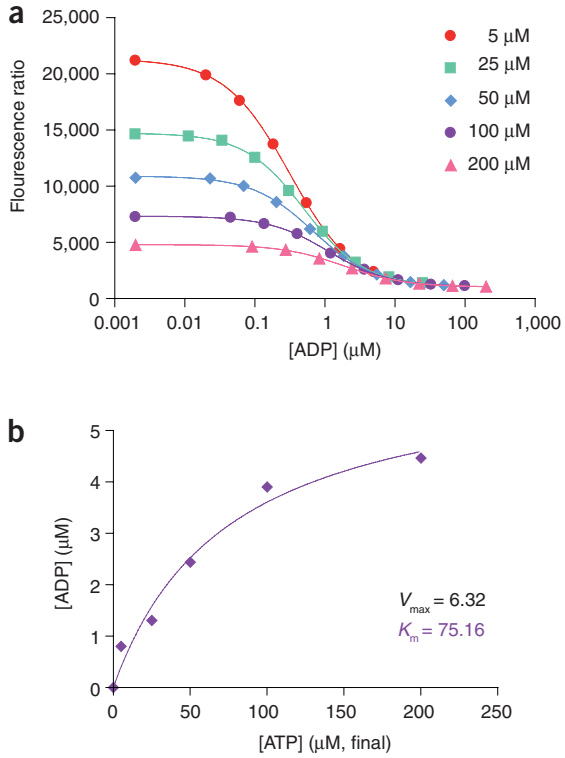

Figure 2 | Standard curves and K_m calculation. (**a**) The concentration of ADP produced for each concentration of ATP was calculated based on the corresponding standard curve, that is, the ratio obtained for Eg5 with 5 μM final ATP concentration was read on the standard curve obtained with 5 μM final ATP plus ADP to obtain a concentration of ADP. (**b**) K_m was calculated using the Michaelis-Menten equation from the resulting plot of the ADP concentration produced versus ATP concentration.

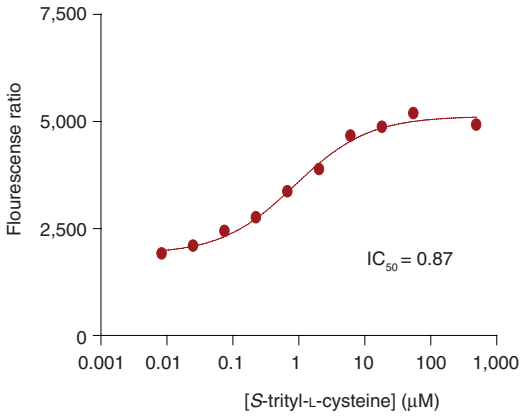

Figure 3 | *S*-trityl-L-cysteine inhibition. For the inhibition experiment, we first preincubated the Eg5 enzyme (60 nM) at 37 °C for 90 minutes in presence of various concentrations of *S*-trityl-L-cysteine from 500 μM to 30 nM, with a threefold dilution between each concentration. Then we added ATP (100 μM) and HTRF detection reagents and incubated the plate at room temperature for 30 minutes.

In this assay all reagents were kit components except Eg5 (purchased from Cytoskeleton), Eg5 inhibitor *S*-trityl-L-cysteine (Calbiochem-Merck), and ATP and $MgCl_2$ (Sigma). We prepared Eg5, ATP and compounds in the enzymatic buffer provided with the kit, supplemented with 10 mM $MgCl_2$. We prepared HTRF detection reagents in the detection buffer, which contains 60 mM EDTA and 400 mM KF. For each step, we performed an ATP-ADP titration with the amount of ATP used in the assay.

Because the EDTA contained in the detection buffer does not stop Eg5 activity, we performed all experiments in one step: we mixed 2 μl of the Eg5 enzyme, 4 μl of ATP and 4 μl of enzymatic buffer with 5 μl of ADP labeled with the fluorophore d2 and 5 μl of Eu^{3+} cryptate–labeled antibody to ADP.

The first step of the assay development was an enzyme titration performed to obtain the optimal enzyme concentration. We ran a twofold dilution series of Eg5 (250 nM to 7.8 nM final reaction concentrations in 20 μl), which we incubated with a nonlimiting concentration of ATP (100 μM final concentration in 20 μl). We added the HTRF detection reagents at the same time and incubated the mixtures at room temperature (20–24 °C) for 30 minutes. The optimal Eg5 concentration thus determined was 60 nM.

In the second step, to determine the enzyme kinetics, we used three different concentrations of Eg5 close to the optimum (30, 60 and 90 nM) and other reagents as above. We read the plate at different time points: 3, 6, 10, 15, 20, 25, 35 and 45 minutes and 1, 2, 3, 4, 5 and 22 hours. The optimal incubation period for the three concentrations of Eg5 to achieve maximum signal and a linear time course was 25 minutes (R^2 = 0.955 for 60 nM Eg5).

The third step was the ATP titration. We generated a standard curve for each ATP concentration used in the assay. We ran the assays with a fixed concentration of enzyme (60 nM) and different concentrations of ATP from 5 μM to 200 μM final for 25 minutes (**Fig. 2a**). We plotted the amount of ADP produced against ATP concentrations using the Michaelis-Menten equation. The apparent Michaelis constant (K_m) was 75 μM (**Fig. 2b**).

Using the inhibition curve (**Fig. 3**), we calculated the half-maximal inhibitory concentration (IC_{50}) to be 0.87 μM.

A recent study[1] identified *S*-trityl-L-cysteine as a reference inhibitor of Eg5 activity, with an IC_{50} of 1.0 μM. Using HTRF Transcreener ADP assay, the IC_{50} value (0.87 μM) was in the same range as that published.

Conclusion

Here we described guidelines for development of an ATPase assay with the HTRF Transcreener ADP kit. A universal and nonradioactive alternative, this solution is well suited to a broad range of targets such as lipid kinases, ATPases and heat shock proteins. The homogeneous format and enzyme-free detection enable high-throughput applications.

HTRF Transcreener ADP assay completes the existing HTRF solution portfolio for kinases and oncology, with the HTRF KinEASE™ platform, dedicated to serine/threonine and tyrosine kinases, and the HTRF toolbox reagents, including a line of antibodies to phosphorylated residues.

1. DeBonis, S. *et al. In vitro* screening for inhibitors of the human mitotic kinesin Eg5 with antimitotic and antitumor activities. *Mol. Cancer Ther.* **3**, 1079–1090 (2004).