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Eu<sup>3+</sup> Cryptate-conjugated Rabbit polyclonal antibody anti-Phosphothreonine

For in vitro research use only  
 Storage temperature : -20°C

[www.htrf.com](http://www.htrf.com)

## HTRF® package insert

Document reference : 61PTRKAE/Z rev07 (July 2008)

**Packaging details :**

	384-well low volume plate (20 µL)
61PTRKAE	500 tests
61PTRKAZ	10,000 tests

## 1. Background

Protein phosphorylation and dephosphorylation are basic mechanisms for the modification of protein function in eukaryotic cell. Studies on the role of phosphorylated proteins have been hampered by their low abundance and the problem of distinguishing between the various types of phosphorylated proteins. Many important substrates of Serine/Threonine kinase cascades remain to be discovered. Phospho-specific antibodies allow the measurement of the activity in intracellular signaling pathways by correlating phosphorylation at a specific site with protein and pathway activity.

The Anti-Phosphothreonine antibody is produced and characterized at Cell Signaling Technology Inc. using a new patented technology designed to produce antibodies that bind phosphorylated threonine in a highly context-independent fashion (reactivity is largely independent of the surrounding amino acid sequences). It reacts with a large number of phosphothreonine containing peptides or proteins.

The Anti-Phosphothreonine antibody is a polyclonal antibody which was produced by immunizing rabbits with a phosphothreonine peptide. It is highly specific for the presence of phosphothreonine in peptides or proteins and shows no cross-reactivity with the corresponding non-phosphorylated threonine residues. This Anti-Phosphothreonine antibody does not react with phosphotyrosine or phosphoserine.

This reagent has been manufactured for High Throughput Screening using the HTRF® technology. HTRF® is an homogeneous time-resolved fluorescence technique, based on the non-radiative energy transfer of the long-lived emission from the Europium Cryptate donor to the cross-linked allophycocyanin (XL665) acceptor.

**References**

- Hunter, T. A thousand and one protein kinases. *Cell*. 1987; 50(6):823-9.
- Edelman, A.M. et al. Protein serine/threonine kinases. *Ann Rev Biochem*. 1987; 56:567-613.
- Heffetz, D. et al. Generation and use of antibodies to phosphothreonine. *Methods Enzymol*. 1991; 201:44-53.
- Mathis G. Probing molecular interactions with homogeneous techniques based on rare earth cryptates and fluorescence energy transfer. *Clin Chem*. 1995;41:1391-7.

## 2. Reagent description

The IgG fraction of Anti-Phosphothreonine antibody has been isolated by immunoaffinity chromatography over a phospho-threonine affinity column. The purified polyclonal antibodies were labeled with Eu<sup>3+</sup> Cryptate by Cisbio international. Specific activity of the conjugate ranges from 4 to 7 Cryptates per antibody.

This conjugate is supplied in 100 mM phosphate pH 7.0, 0.1% Tween 20, 0.1% bovine serum albumin (BSA) protease free.

The concentration of this conjugate has been calibrated in order to obtain a 620 nm signal within the 30,000-50,000 count range. This calibration was run in a 384-well low volume plate format using a final volume of 20 µL, a buffer containing 0.1% BSA and 0.4M KF, and a PHERAstar Plus reader (BMG LABTECH). A recommended amount of antibody per well is specified in the product description sheet attached to the product. On this basis, each vial enables the assessment of 500 tests. Actual amount per well will be dependent on optimized assay conditions.

A scale-up of the cryptate conjugate can be manufactured upon request on the same experimental basis as the 500 test size (ref 61PTRKAZ)

## 3. Reagent handling

### 3.1. Preparation of the working solution

- Allow the stock solution to thaw at room temperature.
- Mix the solution gently by pipeting several times. Do not vortex.
- Dilute the stock solution to the working concentration. Mix gently.
- Stock solution can be divided into aliquots and frozen for additional use (according to storage conditions, see §5).

## 3.2. Recommended buffer

Most common buffers can be used for the preparation of the working solution, providing that the pH is maintained between 5.5 and 8.5. They can be complemented with BSA (0.1%) to prevent reagent coating, and detergents such as Tween 20, Triton X100, CHAPS (up to 0.5%)... may also be added. Avoid SDS, due to its denaturing effect on XL665. It is recommended to check the background signal by counting the buffer blank.

**Potassium fluoride (KF) at a final concentration of 100 to 400 mM must be used for HTRF®. Optimal concentration must be determined experimentally. KF plays an essential role in trisbipyridine europium cryptate protection by preventing the action of possible quenchers contained in the assay at the time of fluorescence measurement. KF can be added to the conjugate working solutions. However, **the presence of KF during all phases of the assay is NOT mandatory** since all potential quenching processes with europium cryptate are reversible. **KF is only required during fluorescence readout** and it may therefore be dispensed in a separate step, just before counting.**

## 4. Assay flexibility and miniaturization

When used as suggested, one small size vial (ref xxxxxKAE) will provide sufficient reagent for 500 tests using a 384-well low volume plate in 20 µL final assay volume (HTRF® packaged basis).

To change the plate formats (96 half-well or 1536-well) and final volumes (100 µL to less than 10 µL), the volume of each assay component is simply proportionally adjusted in order to maintain the reagent concentrations as for the 20 µL final assay volume. For instance, in the case of the 1536-well format in 10 µL final volume, half as much material per well is used, thereby allowing 1,000 tests to be run. The performances of the HTRF® assay remain the same whatever the level of miniaturization.

Assay components	Volume proportion	Assay format		
		1536-well (10 µL)	384-well low volume (20 µL)	96 half-well (100 µL)
Other assay components	2 volume	5 µL	10 µL	50 µL
XL665 conjugate	1 volume	2.5 µL	5 µL	25 µL
Cryptate conjugate	1 volume	2.5 µL	5 µL	25 µL
		1,000 tests	500 tests	100 tests

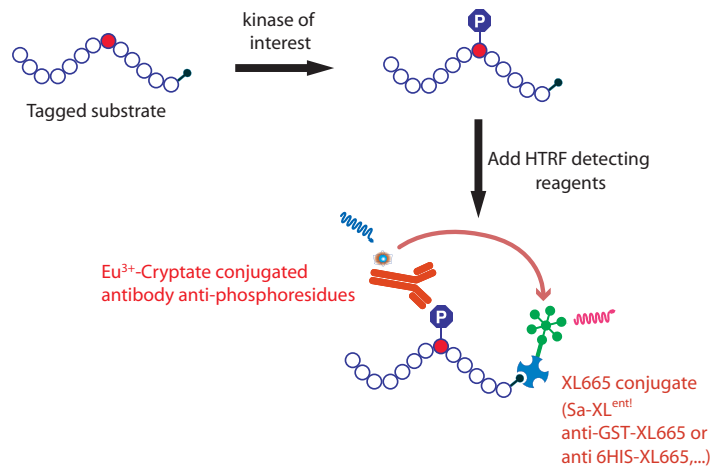
Plate references : 96 half-well plate (Costar # 3694 or equivalent), 384-well low volume plate (Greiner # 784076), 1536-well (Greiner # 782086).

## 5. Storage conditions and stability

- 20°C	long term storage
freezing ↙ thawing ↘	not recommended (loss of ~15% after one cycle)

**Caution ! It is possible to combine Cryptate and XL665 conjugates immediately before use. Do not store Cryptate and XL665 conjugates mixed together for extended periods of time.**

## 6. Companion reagents



As illustrated above, all kinase assays are based on the same template. The enzymatic reaction is usually carried out with a biotinylated substrate (protein, peptide and the enzyme itself in the case of autophosphorylation). The phosphorylated substrate is then detected using the specific anti phosphoresidue antibody coupled to Eu<sup>3+</sup> Cryptate with an XL665 conjugate such as streptavidin-XL<sup>entl</sup> (ref 611SAXLA) or Streptavidin-XL665 (ref 610SAXLA). Alternatively, other moieties such as GST-, 6HIS, c-myc or DNP may be used instead of biotin to label the kinase substrate.

The HTRF® application note # 11 describes how to develop an HTRF® kinase assay. This document is available online at [www.htrf.com](http://www.htrf.com).