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**Anti-Phospho (Ser) PKC substrate -
Cryptate**

Eu³⁺ Cryptate-conjugated rabbit polyclonal anti-
body anti-Phospho (Ser) PKC substrate

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

www.htrf.com

HTRF® package insert

Document reference : 61P03KAE/Z rev03 (September 2004)

Packaging details :

	384-well low volume plate (20 µL)
61P03KAE	500 tests
61P03KAZ	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 still remain to be discovered.

Protein kinase C (PKC) isozyme family constitutes a group of signaling molecules whose activation is one of the earliest events in the cascade leading to a wide range of cellular responses, such as secretion, gene expression and proliferation. PKC family members are subdivided into three groups, based on co-factor requirements. Conventional PKCs (PKC α , β 1, β 2 and γ) require calcium and diacylglycerol (DAG). Novel PKC (PKC δ , ϵ , η , θ and μ) isoforms only require DAG. Finally, atypical PKCs (PKC ζ , τ and λ) require neither calcium nor DAG.

Phospholipases which generate IP₃ and DAG from PIP₂ are located upstream from PKC, whereas downstream PKC signalling regulates the MAPKs cascade.

Upon activation, conventional PKCs phosphorylate substrates containing serine or threonine, surrounded by arginine or lysine in the -3, -2 and +2 positions, and hydrophobic amino-acids at position +1.

The Anti-Phospho (Ser) PKC substrate antibody is a polyclonal antibody produced at Cell Signaling Technology (reference 2261). It recognizes phosphorylated serine with arginine or lysine residues at the -2 and +2 positions and a hydrophobic residue at the +1 position. It does not cross-react with non-phosphorylated serine residue or phosphorylated serine in a different configuration, nor it recognizes phospho threonine.

The antibody-Cryptate conjugate has been developed 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

Nishikawa K. et al. (1997) J. Biol. Chem. 272, 952-960.

Barnard D. et al. (1998) Oncogene 17, 1539-1547.

Mathis G. (1995). Clin Chem. 41,1391-1397.

2. Reagent description

The Anti-Phospho (Ser) PKC substrate polyclonal antibody is labeled with Eu³⁺ Cryptate by Cisbio international. Specific activity of the conjugate ranges from 4 to 7 Cryptates per antibody. This conjugate is supplied in 50 mM hepes buffer pH 7.0, 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 information 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 and miniaturization.

A scale-up of the Cryptate conjugate can be manufactured upon request on the same experimental basis as the 500 test size (ref 61P03KAZ).

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 (refer to the product information provided with the reagent). Mix gently.

Stock solution can be divided into aliquots and frozen for additional use (according to storage conditions, see §5).

3.2. Recommended buffer

We recommend to use any non phosphate containing buffer (i.e. hepes) for the preparation of the working solution, providing that the pH is maintained between 5.5 and 8.5. They can be supplemented 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®. KF plays an essential role in europium cryptate protection by preventing the action of possible quenchers contained in the assay at the time of fluorescence measurement. 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

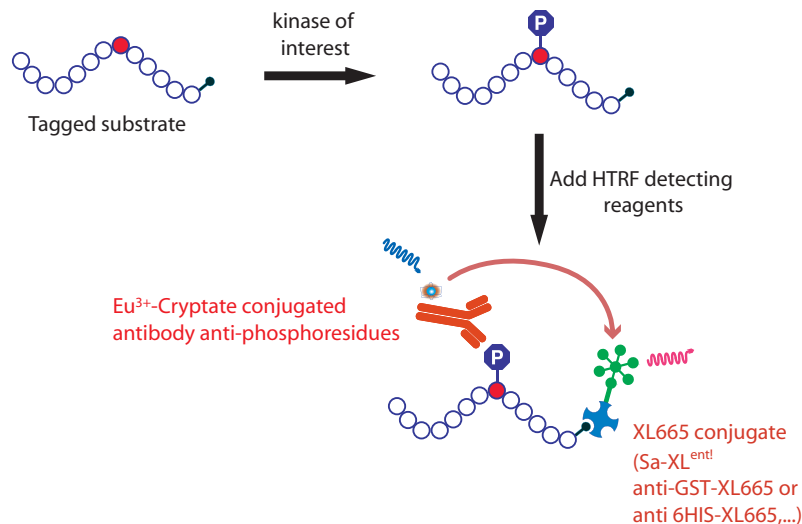
Plate references : 96 half-well plate (Costar # 3694 or equivalent), 384-well low volume plate (Greiner # 784076), 1536-well (Greiner # 782086).

5. Storage conditions and stability

-20°C	Long term storage
Freezing/Thawing	No more than one freeze / thaw 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 Eu³⁺ Cryptate with an XL665 conjugate such as streptavidin-XL^{entl} (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.