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Eu³⁺ Cryptate-conjugated mouse monoclonal antibody (PT66) anti-Phosphotyrosine

For in vitro research use only
 Storage temperature : 2-8°C

www.htrf.com

HTRF® package insert

Document reference : 61T66KLA/B rev07 (July 2008)

Packaging details :

	384-well low volume plate (20 µL)
61T66KLA	5,000 tests
61T66KLB	20,000 tests

1. Background

The largest known protein family is made up of protein kinases identified largely from eukaryotic sources. These enzymes use the gamma phosphate of ATP (or GTP) to generate phosphate monoesters utilizing protein phenolic groups (on Tyrosine) as phosphate group acceptors.

The «conventional protein-tyrosine» kinase PTK group includes a large number of enzymes with quite closely related kinase domain that specifically phosphorylate tyrosine residues. These enzymes have been found only in metazoan cells where they are widely recognized for their roles in transducing growth and differentiation signals.

Phosphorylated tyrosine on a substrate can be easily detected by phosphotyrosine specific PT66 antibodies. PT66 is a mouse monoclonal IgG1 which was produced using phosphotyrosine conjugated to BSA as immunogen. This monoclonal was shown to react specifically with phosphorylated tyrosine, both as free amino acid or when conjugated to carriers. No cross-reactivity is observed with non-phosphorylated tyrosine, phosphothreonine, phosphoserine, AMP or ATP. It is suitable for use in a wide variety of tyrosine kinase activity tests involving receptor or non-receptor enzymes and, specific or ubiquitous substrates.

This toolbox reagent 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

Hardie G and Hanks S. The Protein Kinase. Protein-tyrosine kinases. FactsBook Series. London: Academic Press Ltd; 1995.

Mathis G. Probing molecular interactions with homogeneous techniques based on rare earth cryptates and fluorescence energy transfer. Clin Chem. 1995;41:1391-7

Sarmay G Pecht I and Gergely J. Protein-tyrosine kinase activity tightly associated with human type II F_{cγ} receptors. Proc Natl Acad Sci USA. 1994;91:4140-4

2. Reagent description

PT66 monoclonal antibody anti-Phosphotyrosine is labeled with Eu³⁺ Cryptate at Cisbio international. Specific activity of the conjugate is around 6 Cryptates/antibody.

This conjugate is lyophilized in 20 mM Veronal pH 7.4, 0.1% protease free bovine serum albumin (BSA) and stabilizers.

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 from the two available sizes enables the assessment of 5,000 and 20,000 tests respectively. Actual amount per well will be dependent on optimized assay conditions.

3. Reagent handling

3.1. Preparation of the working solution

- Allow the lyophilized reagent to warm up at room temperature for at least 30 minutes.
- For the 5,000 test vial (61T66KLA) : reconstitute the product with 250 µL of distilled water .
- For the 20,000 test vial (61T66KLB) : reconstitute the product with 1 mL of distilled water .
- Vortex the solution gently.
- After reconstitution, the stock solution can be divided into aliquots and frozen for additional use (according to storage conditions, see §5).
- Dilute the stock solution to the working concentration. Mix gently.

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 vial from the two available sizes will provide sufficient reagent for 5,000 and 20,000 tests respectively using a 384-well low volume plate in 20 µL final assay volume (HTRF® packaged basis).

To move to other 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, 2 times less material per well is used, thereby allowing 10,000 and 40,000 tests respectively 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
	Small size	10,000 tests	5,000 tests	1,000 tests
	Bulk size	40,000 tests	20,000 tests	4,000 tests

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

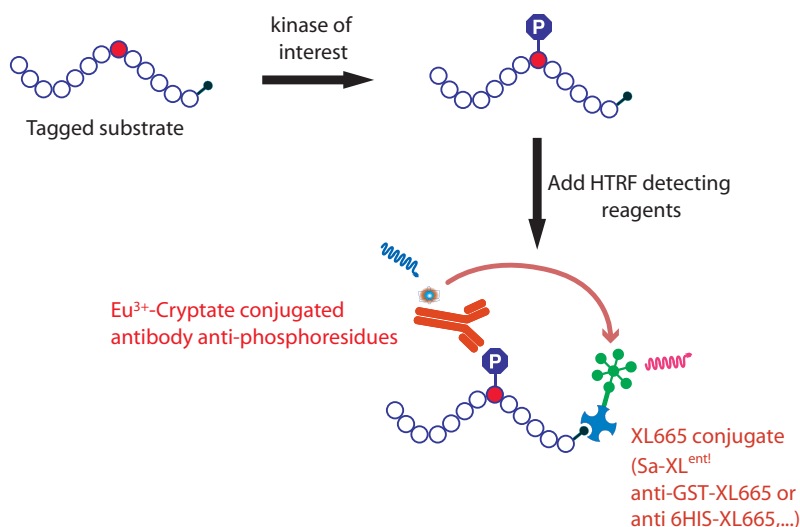
5. Storage conditions and stability

Lyophilized PT66-Cryptate conjugate should be stored at 2-8°C until reconstituted. Under proper storing conditions, this reagent is stable until the expiry date indicated on the label.

Once reconstituted, stock solutions are stable 1 week at 2-8°C. They can be refrozen (at -20°C) and thawed at least two more times.

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^{ent} (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.