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

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HTRF® package insert

Document reference : 62EUSPEA rev08 (Oct.2010)

Packaging details :

62EUSPEA	5 nmoles Cryptate / kit
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1. Kit description and intended use

The macrocycle structure of Europium trisbipyridine Cryptate (Eu-TBP-K) allows them to be derivatized by a variety of reactive functions that can target the best represented groups on proteins or oligonucleotides. Initially, both the Cryptate and the target molecule had to be activated for a covalent conjugation to occur (e.g. using heterobifunctional coupling agents). Recently, a mono-derivatized NHS ester version of Eu-TBP-K was synthesized. The spacer grafted onto one of the pyridine rings includes a highly reactive NHS group that targets the conjugation to primary amine groups under mild conditions.

Note: The kit was designed as a tool for assessing the biological activity of Cryptate-labeled entities, and should not be considered as a test size labeling. For any enquiry about custom labeling services, please directly contact Cisbio international.

2. Equipment and materials required but not included

- Precision micropipettes with disposable tips, capable of dispensing 10-1000 µL.
- Column stand and clamp.
- Vortex.
- Test tubes.
- HTRF® compatible reader (more information about compatible reader at htrf-assays.com/readers).
- Calibrator and Conjugate buffer complemented with BSA and KF (see § 4.4).

3. Supplied reagents and stability

3.1 Supplied reagents

Eu Cryptate labeling reagent (5 nmoles – desiccated)	1 microtube + desiccant
Purification column	1 column
Elution buffer (50 mM phosphate buffer pH7, NaN ₃ 2 mM)	1 vial, 20 mL
620nm Calibrator *	1 vial, lyophilized



- See indications on label for reconstitution volume
- Reconstitute with 50 mM phosphate buffer pH7, 0.4 M KF, 0.1% BSA (not supplied)
- Mix gently after reconstitution

* the 620nm Calibrator is designed for the calibration of the labeled protein (see § 4.4). Allow the reagent to come to room temperature and reconstitute the vial as indicated above, 30 minutes before use.

3.2 Reagent stability

This kit should be stored at 2-8°C before use. Under these conditions, the kit is stable until the expiry date indicated on the box label.

4. Protocol

4.1. Protein preparation and labeling conditions

The protein to be labeled should be conditioned in 50 mM phosphate buffer pH 8.0, by dialysis or other buffer exchange procedure. The labeling process is pH and concentration sensitive. Make sure that the pH of the buffer is 8.0 and that the concentration of the protein is at 6.67 µM (e.g. 1 mg/mL for an antibody).

Depending on the molecular weight of the target molecule, each kit enables the labeling of 50 µg (antibody) to 5 µg (15 kDa MW protein) with an initial molar ratio of 15 Cryptates per molecule.

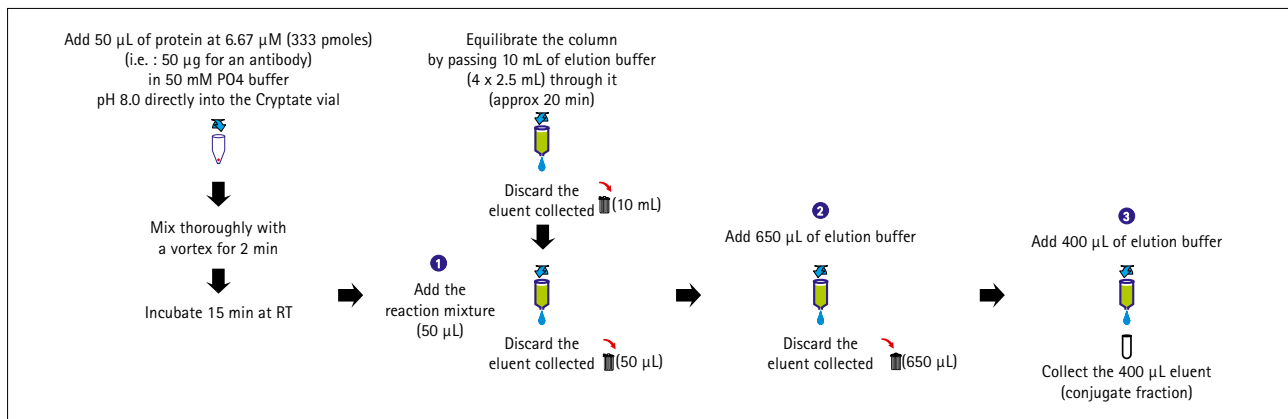
Concentration of the molecule to be labeled should be determined at its maximum absorption using the corresponding molar extinction coefficient, i.e. for a protein :

4.2. Labeling procedure

$$[\text{protein}] \text{ mole/L} = \frac{\text{OD}_{280\text{nm}}}{\epsilon_{\text{protein}} \cdot \text{path length}}$$

The complete procedure is described alongside. The equilibration of the column takes approximately 20 min.

The column supplied with this kit cannot dry out. The volume of eluent recovered at each step corresponds exactly to the volume loaded. Wait until the elution of each step is completed before starting the next one.



4.3. Preparation of the conjugate stock solution

Prepare the conjugate stock solution by adding 0.1% Tween 20 and when possible 0.1% BSA to the conjugate fraction recovered, e.g. to the 400 μL conjugate fraction add 4 μL of a 10% Tween 20 solution and 4 μL of a 100 mg/mL BSA solution. Proceed to the determination of the conjugate working dilution before dividing into aliquots and freezing at -20°C .

4.4. Determination of the conjugate assay dilution

In general, the labeling procedure allows 70% of initial material to be recovered on average as Cryptate conjugate. However, because of the small quantities involved (e.g. 50 μg of antibody in one run), the measurement by optical density is far too inaccurate, and the best way to achieve this quantification consists in calculating the conjugate assay dilution from the Cryptate specific fluorescence at 620 nm.

The calibrator enclosed with the kit facilitates the reader normalization and enables this determination to be done using any HTRF[®] compatible instrument. From serial dilutions of the stock solution, a comparison of their respective 620 nm fluorescence will be made with that of the calibrator, and the assay dilution will be deduced by linear interpolation. For instance, this calibrator gives a 620 nm fluorescence within the 30,000-40,000 cps range using PHERAstar Plus, the HTRF[®] dedicated reader (BMG LABTECH). The conjugate assay dilution should be the dilution which yields a 620 nm fluorescence equivalent to that of the calibrator.

This determination should be carried out as follows :

- Prepare the conjugate buffer : 50 mM phosphate pH 7.0, 0.1% BSA, 0.4 M KF (potassium fluoride).
- Reconstitute the calibrator with conjugate buffer. Leave the solution at RT for at least 30 mins. **These 2 steps may be carried out before labeling.**
- Prepare 3 successive dilutions of the conjugate stock solution ranging from 1/500 to 1/2000 with the conjugate buffer.
- Dispense 20 μL of each dilution and of the calibrator in triplicate in a 384-well low volume plate.
- Read on a HTRF[®] compatible reader.
- Note the signal obtained at 620 nm for the calibrator, and deduce the conjugate assay dilution by linear interpolation.

If the dilution series fails to delimit the 620 nm signal of the calibrator (i.e. all dilutions yield a 620 nm fluorescence inferior to the calibrator's), extrapolate the assay dilution from the results obtained, and verify that its 620 nm fluorescence matches the calibrator's.

4.5. Preparation of the working solution

The assay dilution deduced above corresponds to the final conjugate dilution in the assay. In order to prepare the working solution to be dispensed, the dilution factor of the Cryptate conjugate in the assay should be taken into account, i.e. the volume of Cryptate conjugate / the total assay volume. For instance, if the assay dilution is 1/1000 and the volume of conjugate is 5 μL for a total volume of 20 μL , then the conjugate should be four times more concentrated when dispensed, i.e. the working dilution should be 1/250.

4.6. Conjugate storage conditions and handling

Divide the stock solution into suitably sized aliquots and store at -20°C . Avoid repeated thaw/freeze cycles.

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 or CHAPS (up to 0.5%) may also be added. Avoid SDS, due to its denaturing effect on proteins, and particularly on XL665.

4.7. Recommendations

- Always store the Cryptate under a desiccated atmosphere. Cryptate is stable for 3 months at 2-8 $^{\circ}\text{C}$. However, it should be kept frozen at -20°C for longer storage.
- Strictly follow the instructions. Always start working with a 15-fold amount of Cryptate per molecule to be labeled.

- Do not elute more than 400 µL of conjugate fraction (step 3)
- Do not re-use the column.

5. Case study : Labeling of MAb anti GST

5.1. Reconstitution of the 620 nm calibrator

The calibrator was brought to room temperature for 30 minutes.

The vial was then reconstituted with conjugate buffer (50 mM Phosphate buffer pH7, 0.4 M KF, 0.1% BSA) as indicated on the label.

The calibrator was finally left for 30 minutes at room temperature before dispensing started.

5.2. Labeling

The MAb anti GST was labeled following the indications given in paragraph 4.2.

	Before labeling	After labeling *
Quantity of protein	50 µg	35 µg
Volume	50 µL	400 µL
Concentration	1 mg/mL	87.5 µg/mL

* A 70% labeling yield was considered as the basis for this calculation

5.3. Determination of the conjugate assay dilution

5.3.1. Dilution of the conjugate

Three dilutions of the conjugate in the conjugate buffer (50mM Phosphate buffer pH7, 0.4M KF, 0.1% BSA) were made:

Dilution 1	1/500	175 ng/mL	5 µL of conjugate + 2495 µL of conjugate buffer
Dilution 2	1/1000	87.5 ng/mL	1 mL of dilution 1 + 1 mL of conjugate buffer
Dilution 3	1/2000	43.7 ng/mL	1 mL of dilution 2 + 1 mL of conjugate buffer

5.3.2. Distribution and readout

20 µL of each dilution and of the 620 nm calibrator were dispensed in triplicate in a 384-well low volume plate.

The plate was then read on an HTRF® compatible instrument.

	Volume / well	Quantity / well	Mean Counts at 620 nm*	
Dilution 1	20 µL	3.5 ng	86102	
Dilution 2		1.75 ng	43051	⇐ <i>Bounding dilution</i>
Dilution 3		0.875 ng	21525	⇐ <i>Bounding dilution</i>
620 nm calibrator		-	36876	

* These results were obtained on PHERAstar Plus

5.3.3. Calibration of the conjugate vs the 620 nm calibrator by linear interpolation

Working dilutions were deduced by linear interpolation of bounding dilutions (i.e.: dilutions 2 and 3).

	Counts at 620 nm	Quantity /well	Dilution
620 nm calibrator	36876	-	-
Deduced assay dilution	36876	1.50 ng	1/1166

5.3.4. Preparation of the conjugate for the assay

The following assay format was used:

5 µL proteinX-GST

10 µL anti proteinX-XL665

5 µL anti-GST-Cryptate ⇒ **vol Cryptate / total volume = 1/4**

The Cryptate conjugate was diluted to 1/4 in the well. The working solution was therefore four times more concentrated (4 x 1/1166).

Thus the working dilution for the Cryptate conjugate was 1/291, in order to match the 620 nm level of the calibrator.