Technology: HTRF™

Manual | Protein-Protein Interaction

Terbium Cryptate labeling kit

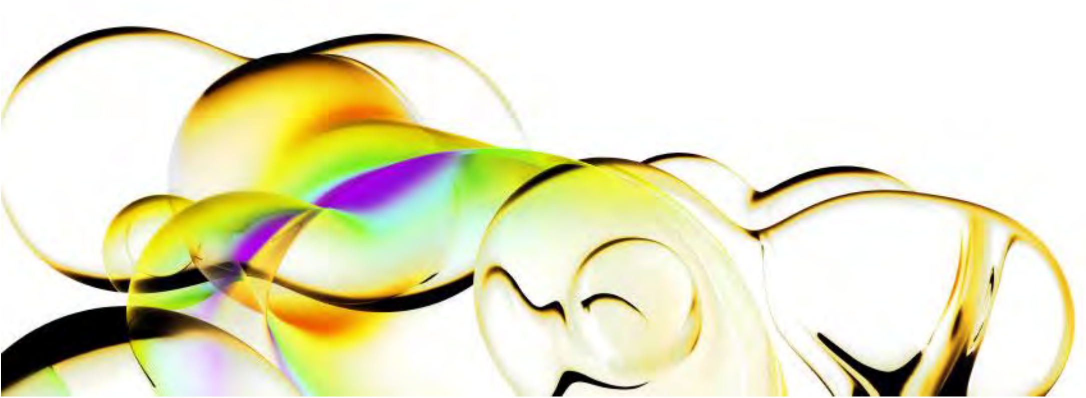
Part number: 62TBSPEA

Packaging, shipping & storage details		Storage upon receipt
Set sent in dry ice	5 nmoles Cryptate and calibrator	-60°C or below
Set sent at room temperature (RT)	Buffers and column	2-8°C

Version: 06

Revision date: July 2024

For research only. Not for use in diagnostic procedures.



KIT DESCRIPTION AND INTENDED USE

The structure of Lumi4®-Terbium Cryptate (Lumi4®-Tb-K) allows it to be derivatized by a variety of reactive functions that can target the best represented groups on proteins. The mono-derivatized NHS ester version of Lumi4®-Tb-K allows the conjugation to primary amine groups under mild conditions.

Note: This kit is an assay development tool enabling the selection of appropriate assay components, e.g. a specific antibody. Based on this selection, Revvity can then carry out a labeling scale-up to provide larger conjugate batches. Labeling conditions for this scale-up may differ from those used with this kit.

This kit is for research use and for antibody conjugation only. The conjugate generated through this labeling may not be used for diagnostic purposes.

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 on our website).
- BSA and tween20 to complement the conjugate buffer (see § 4.3 et 4.4).

SUPPLIED REAGENTS AND STABILITY

Supplied reagents

Reagent	Quantity	Storage
Lumi4-Tb cryptate labelling reagent	1 microtube + desiccant	Store at -60°C or below until use
Purification column	1 column	Store at 2-8°C
Elution buffer (100 mM PO4 buffer pH7, 2 mM NaN_3)	1 vial, 20 mL	Store at 2-8°C until use
620nm Calibrator	1 microtube + desiccant	Store at -60°C or below until use
Conjugate buffer (50mM Hepes buffer, 2 mM NaN_3)	1 vial, 10 mL	Store at 2-8°C until use

*the 620nm Calibrator is designed for the calibration of the labeled protein (see § 4.4 for reconstitution and use).

Reagent stability

The Lumi4®-Tb-K and the 620nm calibrator should be stored at - 60°C or below, the other components must be stored at 2-8°C before use. Under these conditions, the kit is stable until the expiry date indicated on the box label.

PROTOCOL

Protein preparation and labeling conditions

The protein to be labeled should be conditioned in 50 mM PO4 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).

Each kit enables the labeling of 75 µg of antibody with an initial molar ratio of 10 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 an antibody:

$$[\text{antibody}]\ \text{mole/L} = \frac{OD_{280nm}}{210{,}000}$$

Where 210,000 is the molar extinction coefficient (ε) in cm-1M-1 of a typical IgG at 280nm.

Labeling procedure

The complete procedure is described alongside and takes approximately 1h.

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.

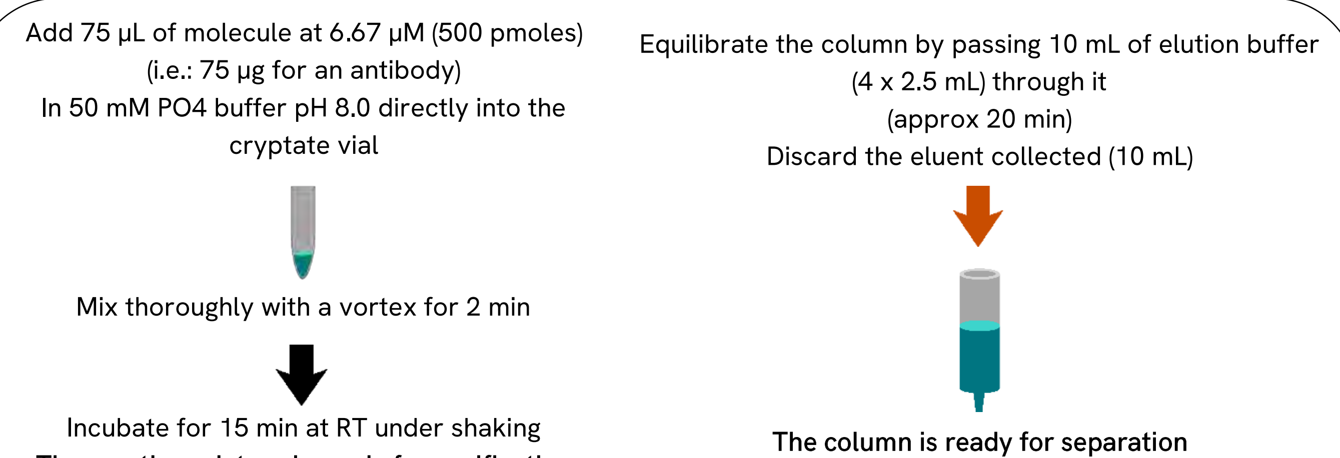
Add 75 µL of molecule at 6.67 µM (500 pmoles)
(i.e.: 75 µg for an antibody)
In 50 mM PO4 buffer pH 8.0 directly into the cryptate vial

Mix thoroughly with a vortex for 2 min

Incubate for 15 min at RT under shaking
The reaction mixture is ready for purification

Equilibrate the column by passing 10 mL of elution buffer
(4 x 2.5 mL) through it
(approx 20 min)
Discard the eluent collected (10 mL)

The column is ready for separation

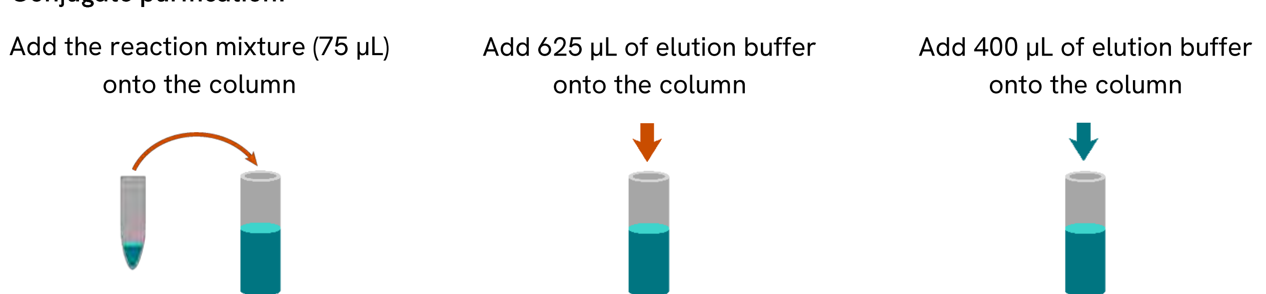


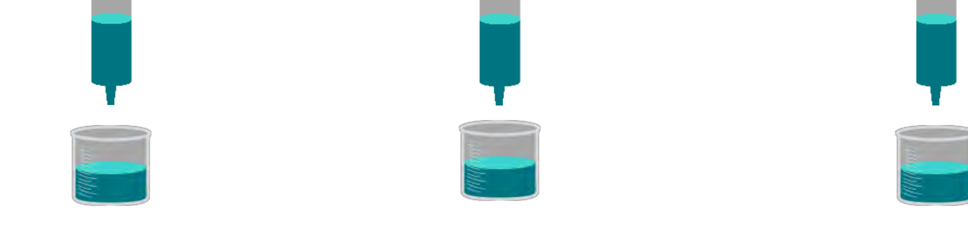
Conjugate purification:

Add the reaction mixture (75 µL) onto the column

Add 625 µL of elution buffer onto the column

Add 400 µL of elution buffer onto the column





Discard the eluent collected (75 µL)

Discard the eluent collected (625 µL)

Collect the 400 µL eluent (conjugate fraction)

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.

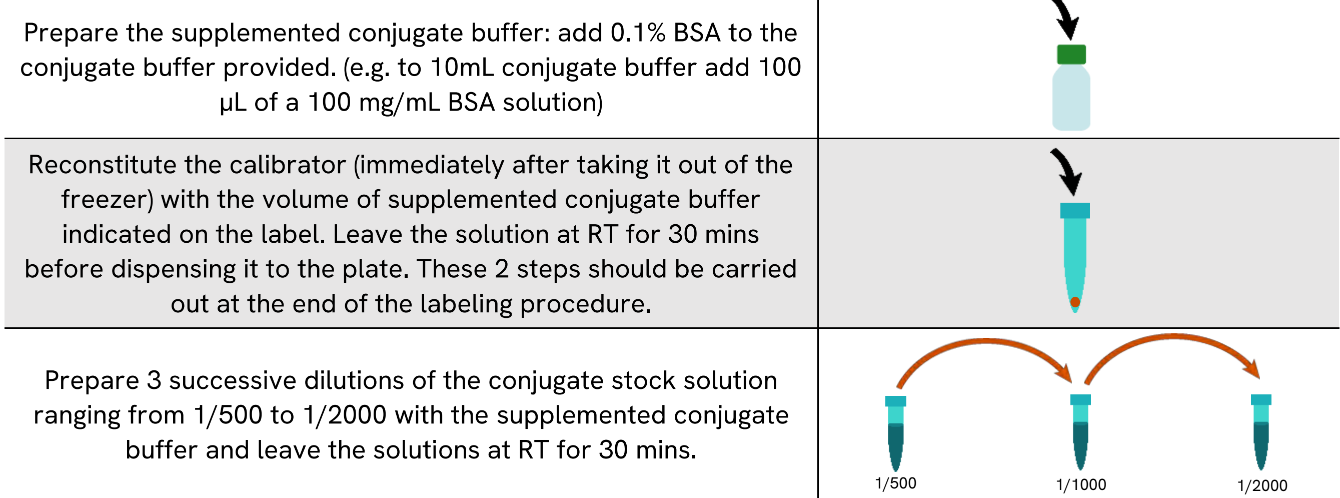
Determination of the conjugate assay dilution

In general, the labeling procedure allows 60% of initial material to be recovered on average as cryptate conjugate. However, because of the small quantities involved (e.g. 75 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 PHERAstarPlus (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:



Step	
Prepare the supplemented conjugate buffer: add 0.1% BSA to the conjugate buffer provided. (e.g. to 10mL conjugate buffer add 100 µL of a 100 mg/mL BSA solution)	
Reconstitute the calibrator (immediately after taking it out of the freezer) with the volume of supplemented conjugate buffer indicated on the label. Leave the solution at RT for 30 mins before dispensing it to the plate. These 2 steps should be carried out at the end of the labeling procedure.	
Prepare 3 successive dilutions of the conjugate stock solution ranging from 1/500 to 1/2000 with the supplemented conjugate buffer and leave the solutions at RT for 30 mins.	
Dispense 20 µL of each dilution and of the calibrator in triplicate in a 384-well low volume plate.	
Leave the plate for 30mins at RT on the bench	
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.

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 determined 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.

Conjugate storage conditions and handling

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

For preparation of working solutions it is recommended to use Hepes buffer (with pH buffer around 7.0) complemented with BSA (0.1%) to prevent reagent coating. 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.

Recommendations

- Always store the cryptate and calibrator under a desiccated atmosphere.
- Strictly follow the instructions. Always start working with a 10-fold amount of cryptate per molecule to be labeled to ensure an efficient coupling (labeling efficacy is concentration dependent).
- The molecule to be labeled must be in a buffer free of ammonium ions or primary amines, as they will compete with the amine groups of the protein for the reactive dye.
- If the molecule is in Tris or glycine buffer or purified with ammonium sulphate, the buffer needs to be replaced with phosphate-buffered saline (PBS).
- Impure molecules or material stabilized with bovine serum albumin (BSA) cannot be labeled reliably, unless pre-purified.
- Do not elute more than 400 µL of conjugate fraction (step 4)
- Do not re-use the column.

CASE STUDY: LABELING OF MAB ANTI-6HIS

Reconstitution of the 620 nm calibrator

The vial was reconstituted with supplemented conjugate buffer (50 mM Hepes pH7, 0.1% BSA) as indicated on the label (see § 4.4).

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

Labeling

The MAb anti-6HIS was labeled following the indications given in paragraph 4.2.

	Before labeling	After labeling*
Quantity of protein	75 µg	45 µg
Volume	75 µL	400 µL
Concentration	1 mg/mL	110 µg/mL

*a 60% labeling yield was considered as the basis for this calculation

Determination of the conjugate assay dilution

Dilution of the conjugate

Three dilutions of the conjugate in the supplemented conjugate buffer (50mM Hepes pH7, 0.1% BSA) were made:

Dilution 1	1/500	220 ng/mL	5 µL of conjugate + 2495 µL of conjugate buffer
Dilution 2	1/1000	110 ng/mL	1 mL of dilution 1 + 1 mL of conjugate buffer
Dilution 3	1/2000	55 ng/mL	1 mL of dilution 2 + 1 mL of conjugate buffer

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 kept at RT for 30mins and then read on an HTRF™ compatible instrument (please note that for laser-based instruments, white plates should be avoided).

	Volume/well	Quantity/well	Mean counts at 620nm*
Dilution 1	20 µL	4.4 ng	71486
Dilution 2		2.2 ng	33530
Dilution 3		1.1 ng	17454
620nm calibrator			30000

*Results obtained on PHERAstar^{plus} (BMGlabtech) in black plates

Calibration of the conjugate versus 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 620nm*	Quantity/well	Dilution
620nm calibrator	30000	-	-
Deduced assay diluton	30000	1.91 ng	1/1141

Preparation of the conjugate for the assay

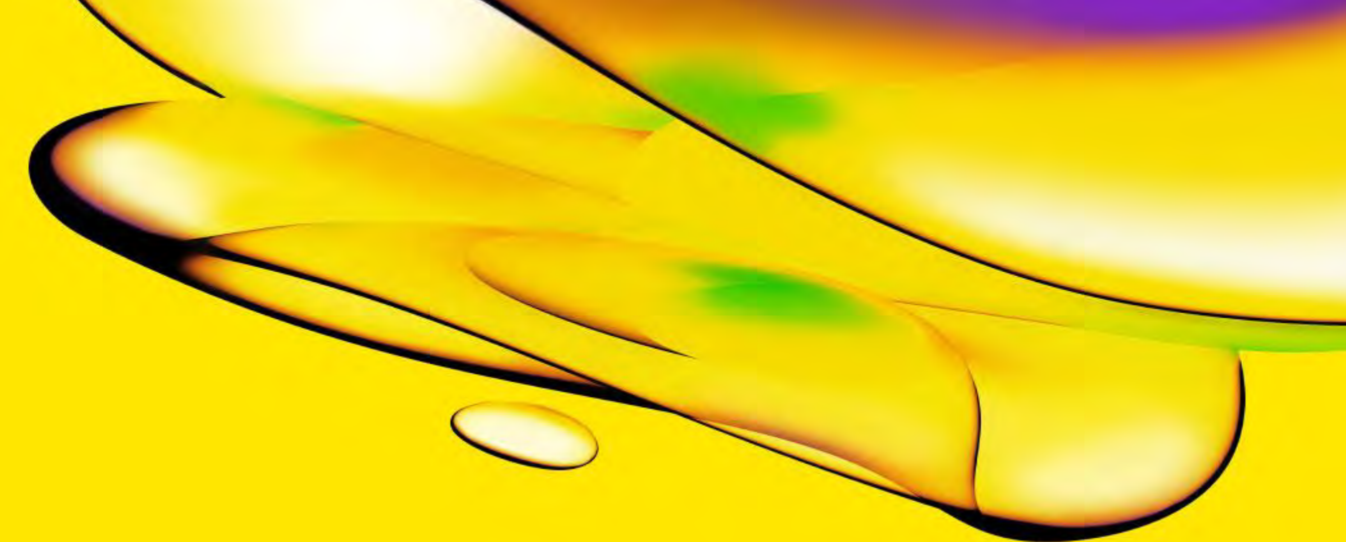
The following assay format was used:

- 5 µL proteinX-6HIS
- 10 µL anti proteinX-XL665
- 5 µL anti-6HIS-Cryptate

The Cryptate conjugate was diluted to 1/4 in the well. The working solution was therefore four times more concentrated. Thus the working dilution for the Cryptate conjugate was 1/285, in order to match the 620 nm level of the calibrator.

REACH European regulations and compliance
This product and/or some of its components include a Triton concentration of 0.1% or more and as such, it is concerned by the REACH European regulations. We recommend researchers using this product to act in compliance with REACH and in particular: to only use the product for in vitro research in appropriate and controlled premises by qualified researchers, ii) to ensure the collection and the treatment of subsequent waste, and iii) to make sure that the total amount of Triton handled does not exceed 1 ton per year.

This product contains material of biologic origin. Use for research purposes only. Do not use in humans or for diagnostic purposes. The purchaser assumes all risk and responsibility concerning reception, handling and storage.



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