TECHNICAL DATA SHEET

DELFIA® Research Reagents

Research Use Only. Not for use in diagnostic procedures

Eu-N1 ITC Chelate & Europium Standard

Product Number: 1244-301

INTRODUCTION:

DELFIA® Eu-N1 ITC Chelate is optimized for the europium labeling of proteins and peptides for use in dissociation-enhanced time-resolved fluorometric assays. The reagent is the Eu^{3+}-chelate of N^1-(p-isothiocyanatobenzyl) diethylenetriamine-N^1, N^2, N^3, N^3-tetraacetic acid. The DTTA group (diethylenetriaminetetraacetic acid) forms a stable complex with Eu^{3+} and the isothiocyanato group reacts with free amino groups on the proteins and peptides, forming a stable, covalent thiourea bond.

PACKAGE CONTENTS:

1 vial (2 mg, ~ 3 µmol) of lyophilized Eu-N1 ITC Chelate
1 vial (0.5 mL) of 100 nmol/L Europium Standard

STORAGE:

The expiry date of this kit is stated on the outer kit label. The chelate should be stored at -20 ºC and the standard at 2-8 ºC.

REAGENT RECONSTITUTION:

Dissolve the lyophilized material in distilled water (e.g. 200 µL giving 15 mmol/L solution of the Chelate since the vial was slightly overfilled) for immediate use. Keep at 0 °C (icebath). To determine the exact concentration, the solution is diluted 500 times with distilled water, i.e., 10.0 µL to 5.00 mL, and measured by UV-Vis. A volume of at least 10 µL for dilution is recommended for accurate measurement. Record the absorbance at λ_{max} at ~270 nm. The concentration is calculated by the equation below:

$$[\text{Chelate}] = [(A_{270nm} \times 500) / 18] \text{ mM (mmol/L)}$$

For example, if A270 nm = 0.250

$$[\text{Chelate}] = (0.250 \times 500)/18 = 6.9 \text{ mmol/L (or nmol/µL)}$$

RECONSTITUTED STABILITY:

The reconstituted reagent should be used within 24 hours if stored at 4 °C or divided into aliquots and stored frozen at -20 °C for future use up to 3 days.

WARNINGS AND PRECAUTIONS:

This labeling reagent is intended for research use only.

The handling of concentrated Eu^{3+}-solutions constitutes a contamination risk, which may cause elevated backgrounds in an assay based on time-resolved fluorometry. Keep the labeling reagents and required accessories separated from the place and accessories where the actual assay is performed.

Disposal of all waste should be in accordance with local regulations.

LABELING OF PROTEINS:

Eu-N1 ITC chelate has an aromatic isothiocyanato group as a reactive arm. Isothiocyanato group reacts with primary aliphatic amino groups at alkaline pH.

1. Parameters of labeling reaction
   Parameters of labeling reaction include protein concentration, pH, temperature, reaction time and molar excess of chelate over protein.

2. Labeling
   The recommended general reaction conditions for labeling of proteins are pH 8.9 - 9.1, 4 °C and overnight incubation. Under these conditions, the following calculations are valid for labeling a protein with an isoelectric point (pI) between 4 and 7.

Protein concentration (mg/mL)	Percentage of chelate reacted
5	20 %
2.5	10 %
1	4 %

Table 1. The effect of protein concentration on the percentage of Eu-N1 ITC chelate reacting with the protein.

For example, if a protein (pI around 6, molecular weight 160 000) is reacted at a concentration of 5 mg/mL under the conditions described above, a 40-fold molar excess of chelate over protein would give a labeling degree of about 8 Eu-N1 ITC chelates per protein.
The labeling efficiency is very pH dependant and the resulted Eu/protein ratio varies even within a small pH range. At higher pH, *i.e.,* pH ~10 if the protein is stable, the percentage of chelate reacted could increase to 50-60%, at protein concentration of 5 mg/mL. At pH ~8.5

the labeling efficiency decreases significantly to ~ 25% of that at pH 9, requiring 4 times the amount of chelate to achieve similar labeling ratio.

If the protein to be labeled is not stable under the recommended labeling conditions (4 °C, pH ~9 overnight incubation), it is possible to shorten the reaction time to 4 hours (4 °C, pH 9) by increasing the molar excess of chelate over protein. A suitable amount of chelate is three times higher for the 4-hour reaction than for the overnight reaction. For example, if a protein (5 mg/mL in the labeling reaction) requires 40-fold molar excess of chelate during overnight reaction for the introduction of 8 chelates per protein, 120-fold molar excess of Eu-N1 ITC chelate is needed to obtain similar label incorporation during a 4-hour reaction. Note that the separation of unreacted chelate from labeled protein is more difficult with four hour labeling because of the greater amount of chelate in the reaction.

If the protein to be labeled is not stable at pH 9, labeling can also be done at pH 8.4 - 8.5. However, labelling efficiency of Eu-N1 chelate at pH 8.4 - 8.5 is only about 25 % compared with that at pH 8.9- 9.1. For example, if a protein (pI around 6, molecular weight 160 000) is allowed to react at a concentration of 5 mg/mL, pH 8.4 - 8.5, 4 °C and overnight reaction, a 160-fold molar excess of chelate over protein would give a labeling degree of about 7 - 9 Eu-N1 ITC chelates per protein.

Suitable number of Eu-N1 ITC chelates coupled to a protein depends on the molecular weight (MW). When the MW of a protein is higher than 100 000, 4 - 15 chelates per protein is a good labeling yield. For proteins with a MW in the range of 30 000 - 100 000 the preferred number of coupled chelates is 2 - 10. Proteins with a MW less than 30 000 should be labeled with 1 - 3 chelates. The given values may be higher for basic proteins (pI between 8 and 10).

LABELING OF PEPTIDES:

Peptides (size up to about 40 amino acids) are labeled like proteins except that the molar excess of chelate over peptide is lower than in protein labeling. Recommended molar excess of chelate over peptide is 3 - 6 (peptide concentration 5 - 20 mg/mL), 5 - 10 (peptide concentration 2.5 - 5 mg/mL) or 8 - 30 (peptide concentration 1 - 2.5 mg/mL). Labeling is usually performed at 4 °C but, if the peptide to be labeled is very stable, it can be labeled at room temperature (20 - 25 °C). Suitable number of chelates coupled to a peptide is 1 - 2 depending on the peptide.

LABELING PROCEDURE:

The protein or peptide to be labeled must not be stabilized with a protein (e.g. bovine serum albumin (BSA), casein or gelatin).

1. Pre-treatment
   If the buffer including the protein or peptide to be labeled contains primary amines (e.g. Tris, ammonium ions), sulfhydryl groups (e.g. mercaptoethanol) or sodium azide, a pretreatment step is necessary. The above mentioned compounds interfere with labeling. Suitable

methods for removing interfering compounds include gel filtration (e.g. NAP[1] and PD-10 columns), dialysis and reverse phase HPLC (RP-HPLC).

2. Concentrating protein and peptide
   If a protein is too dilute (less than 1 mg/mL) or if it is preferable to use less chelate to facilitate purification after labeling, a concentration step is necessary. Suitable concentrators are e.g. Centricon and Centriprep concentrators[2].

   If the concentration of a peptide is too low for an efficient labeling reaction (less than 1 mg/mL), a vacuum centrifuge can be used to concentrate the peptide solution.

3. Reconstitution of chelate
   After calculating the amount of chelate needed in the reaction, the chelate is dissolved in water. Suitable concentration for reconstituted chelate is 5 - 30 mmol/L. For example, dissolving 1 mg of chelate in 200 µL gives a concentration of 7.5 mmol/L. The exact concentration is determined by UV-Vis measurement and calculated by the equation given in the Reagent Reconstitution section. After dissolving the chelate should be kept on ice for immediate use.

4. Labeling
   If the protein or peptide is already in a labeling buffer (50 - 100 mmol/L sodium carbonate-sodium bicarbonate buffer, pH 9) after the pre-treatment or reconstitution, the calculated amount of chelate is added into the protein (peptide) solution on ice.

   If the protein (peptide) is not in a labeling buffer, 1 mol/L sodium carbonate/sodium bicarbonate buffer (pH 9.2, Na_2CO_3/$NaHCO_3$ = 10/90) is added to adjust buffer concentration to 50 - 100 mmol/L followed by the calculated amount of reconstituted chelate.

   In both cases, pH is checked after adding the chelate by applying a 0.5 µL sample on a pH-paper or pH-stick. It is advisable to check the performance of the pH-paper or pH-stick with 50 - 100 mmol/L sodium carbonate buffer of known pH. A suitable pH-paper is Spezial Indikatorpapier pH 8.2 - 10.0 (Merck Art. No. 9558). If necessary, pH of the reaction mixture is adjusted to 8.9 - 9.1 using either 0.5 - 1 mol/L HCl or 0.5 - 1 mol/L NaOH.

   After adding all necessary components and checking pH, incubate at 4 °C overnight (or for 4 hours).

5. Purification
   Separation of the labeled protein from unreacted chelate is performed by gel filtration. Elution buffer should be Tris-HCl based, e.g. 50 mmol/L Tris-HCl (pH 7.8) containing 0.9 % NaCl and 0.05 % sodium azide (TSA buffer). Proteins with a molecular weight over 100 000 can be purified using Superdex 200 column or a combination of Sephadex G-50

[1] NAP is a trademark of Amersham Pharmacia Biotech.
[2] Centricon and Centriprep are registered trademarks of Millipore Corp.

(e.g. 1 x 10 cm) layered on Sepharose 6B (e.g. 1 x 30 - 40 cm) [3]. Proteins with a MW in the range of 30 000 - 100 000 are best purified using Superdex 75 or Sephadex G-50. Sephadex G-50 is suitable also for purification of proteins with a MW between 15 000 and 30 000.

In gel filtration the collected fraction size should be about 1 mL. The gel filtration eluate can be monitored by UV-absorbance at 280 nm. The first peak contains the labeled protein and the second peak unreacted chelate(Note: when labeled IgG is purified using either Superdex 200 or a combination of Sephadex G-50 and Sepharose 6B, aggregated IgG is first eluted in the void volume followed by monomeric IgG which is pooled and unreacted chelate). The Eu concentration of the fractions can be measured by making a 1:1000 - 1:10000 dilution in DELFIA Enhancement Solution (prod. no. 1244-105). The dilutions should be mixed gently and let stand for about 2 minutes before measuring in a time-resolved fluorometer. The fractions from the first peak with the highest Eu counts are pooled and characterized. Collecting of fractions should be stopped at least two fractions before the signal of unreacted chelate starts to rise.

When labeling only a small amount of antibody (< 0.5 mg) the purification can be done with a PD-10 column by applying the reaction mixture in the equilibrated column and collecting 0.5 mL fractions. The fractions from the first peak with the highest Eu counts should be pooled and characterized.

Peptides having at least about 25 amino acid residues can be purified from the unreacted chelate on Sephadex G-25 or alternatively using reverse phase HPLC. Small peptides (less than 25 amino acid residues) can be purified from the unreacted chelate and at least in some cases also from the unlabeled peptide by using reverse phase HPLC. The labeled peptide is eluted from the column in acetonitrile gradient in 0.02 - 0.1 mol/L triethylammonium acetate (pH 7.5). After collecting the labeled peptide acetonitrile is evaporated. It is advisable to add 50 µL/mL 1 mol/L Tris-HCl (pH 8.5) before evaporation of acetonitrile to make sure that pH stays neutral.

Proteins MW above 100 000	Proteins MW 30-100 000	Proteins MW 15-30 000	Proteins and peptides MW 2500-15 000	Peptides MW below 2500 (< 25 aa)
Superdex 200	Superdex 75	Sephadex G-50	Sephadex G-25	RP-HPLC
Sephadex G-50 /Sepharose 6B	Sephadex G-50		RP-HPLC	

Table 2. Recommended columns for purification of proteins and peptides after labeling with Eu-N1 ITC chelate.

[3] Superdex, Sephadex and Sepharose are trademarks of Amersham Pharmacia Biotech.

There should be dedicated columns for each lanthanide (europium, terbium, samarium, dysprosium) used for labeling. After purification, the gel filtration column should be decontaminated by washing with 10 mmol/L phthalate buffer (pH 4.1) containing 0.01 % DTPA. Before each run, it is advisable to saturate and further purify the gel filtration column on the previous day by applying concentrated BSA solution of high purity (e.g. 0.5 mL 7.5 % BSA; for suitable BSA see section STORAGE OF LABELED COMPOUNDS). After adding the BSA the column should be equilibrated overnight. RP-HPLC columns can be washed using the phthalate buffer described above.

CHARACTERIZATION OF LABELED PROTEINS AND PEPTIDES:

To determine the Eu^{3+} concentration in the labeled proteins (peptides), the labeled protein (peptide) is diluted in DELFIA Enhancement Solution (prod. no. 1244-105), mixed gently and let stand for about 2 minutes. Eu fluorescence is then measured in a time-resolved fluorometer against 100 nmol/L Eu standard (supplied with the chelate) diluted 1:100 in DELFIA Enhancement Solution (1 nmol/L Eu in Enhancement Solution in a clear 96-well plate, 200 µL per well, gives about 1 000 000 cps when measured in 1234 DELFIA Research Fluorometer or 1420 VICTOR™ Multilabel Counter).

The protein (peptide) concentration can be measured with a suitable method (e.g. Lowry) or calculated from the absorbance at 280 nm. The molar absorptivity of reacted Eu-N1 ITC chelate is 8000 at 280 nm (1 µmol/L reacted chelate gives an absorbance of 0.008 at 280 nm).

FILTRATION:

To remove particles and possible aggregates the labeled compound should be filtered through a 0.22 µm low protein binding membrane.

STORAGE OF LABELED COMPOUNDS:

To ensure stability, the labeled proteins and peptides should be stored at a high concentration and in the absence of chelators or competing metals in the buffer. A concentrated solution (0.1 mg/mL or higher) can be stored without any stabilizer. With lower concentrations, the stability can be increased by adding purified BSA (a component in the DELFIA Eu-labeling kit, prod. no. 1244-302, and Sm-labeling kit, prod. no. 1244-303; available also as a specialty product from PerkinElmer, prod. no. CR84-100 Stabilizer) to a final concentration of 0.1 %. Temperature during storage is determined by the stability of the protein. Suitable temperatures are e.g. 4 °C, -20 °C and -70 °C.

USE OF LABELED PROTEINS:

The amount of proteins, incubation time, temperature and the buffers used must be optimized for each particular analyte. As a general rule about 5 - 100 ng of the labeled proteins per tube or well can be used. The DELFIA Assay Buffer (prod. no. 1244-106) is optimal for most assays. It contains NaCl, Tris-HCl, bovine serum albumin (BSA), bovine gamma globulins, Tween 40, diethylenetriaminepentaacetic acid (DTPA), < 0.1 % NaN_3, and an inert red dye. If this assay buffer cannot be used, it is recommended to use a Tris-HCl buffer containing 20 µmol/L EDTA or DTPA to keep the fluorescence background low.

The labeled protein as such is practically non-fluorescent. After binding assay DELFIA Enhancement Solution dissociates Eu ions from labeled protein into solution, where they form highly fluorescent chelates with components of the Enhancement Solution. The strips should be shaken **slowly** for 5 minutes before measuring with the time-resolved fluorometer (1420 VICTOR or 1234 DELFIA Research Fluorometer).

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