DELFIA® Eu-Labelling Reagent 1244-301

INTENDED USE

DELFIA® Eu-Labelling Reagent is optimized for the europium labelling of proteins and peptides (1) for use in dissociation-enhanced time-resolved fluorometric assays (2,3,4,5). The reagent is the Eu^{3+}-chelate of N^1-(p-isothiocyanatobenzyl)diethylenetriamine-N^1, N^2, N^3, N^3-tetraacetic acid (1). 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 (1 mg, ~ 1.5 µmol) of lyophilized Eu-Labelling Reagent (Eu-N1 ITC chelate)

STORAGE

The expiry date is stated on the vial label. The reagent should be stored at +8°C or lower before reconstitution.

REAGENT RECONSTITUTION

Dissolve the lyophilized Labelling Reagent in distilled water (e.g. 200 µL giving 7.5 mmol/L solution of the chelate) for immediate use. Keep at 0°C (icebath).

RECONSTITUTED STABILITY

The reconstituted reagent should be used the same day or it should be divided into aliquotes and stored frozen at -20°C for future use.

WARNINGS AND PRECAUTIONS

This labelling 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 labelling 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.

LABELLING 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 labelling reaction

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

2. Labelling

   The recommended reaction conditions for labelling of proteins are pH 9 - 9.3, +4°C and overnight incubation. Under these conditions, the following calculations are valid for labelling of 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 at pH 9 – 9.3, +4°C, overnight incubation.

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 labelling degree of about 8 Eu-N1 ITC chelates per protein.

If the protein to be labelled is not stable in the labelling conditions (+4°C, pH 9 - 9.3, overnight incubation), it is possible to run a 4-hour reaction (+4°C, pH 9 - 9.3) 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 labelling 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 the same label incorporation during a 4-hour reaction. Note that the separation of unreacted chelate from labelled protein is more difficult with four hour labelling because of the greater amount of chelate in the reaction.

If the protein to be labelled is not stable at pH 9 – 9.3 labelling can also be done at pH 8.4 – 8.5. Reactivity of Eu-N1 chelate at pH 8.4 – 8.5 is only about 25 % of the reactivity at pH 9 – 9.3. For example, if a protein (pI around 6, molecular weight 160 000) is reacted 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 labelling 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 labelling 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 labelled with 1 - 3 chelates. The given values may be higher for basic proteins (pI between 8 and 10).

LABELLING OF PEPTIDES

Peptides (size up to about 40 amino acids) are labelled like proteins except that the molar excess of chelate over peptide is lower than in protein labelling. 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). Labelling is usually performed at +4°C but, if the peptide to be labelled is very stable, it can be labelled at room temperature (+20 - +25°C). Suitable number of chelates coupled to a peptide is 1 - 2 depending on the peptide.

LABELLING PROCEDURE

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

1. Pretreatment

   If the buffer including the protein or peptide to be labelled 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 labelling. Suitable methods for removing interfering compounds include gel filtration (e.g. NAP[1] and PD-10 columns by Amersham Pharmacia Biotech), 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 labelling, 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 labelling 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 Labelling Reagent is dissolved in water. Suitable concentration for reconstituted Labelling Reagent is 5 – 30 mmol/L. For example, dissolving 1 mg of Eu-N1 ITC chelate in 200 µL gives a concentration of 7.5 mmol/L. After dissolving the chelate should be kept on ice for immediate use.

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

4. Labelling

If the protein or peptide is already in a labelling buffer (50 - 100 mmol/L sodium carbonate, pH 9 - 9.3) after the pretreatment or reconstitution, the calculated amount of chelate is added into the protein (peptide) solution on ice.

If the protein (peptide) is not in a labelling buffer, 1 mol/L sodium carbonate (pH 9 - 9.3) 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 9 – 9.3 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 labelled 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 (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 labelled protein and the second peak unreacted chelate (Note: when labelled 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 labelling 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.

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

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 unlabelled peptide by using reverse phase HPLC. The labelled peptide is eluted from the column in acetonitrile gradient in 0.02 - 0.1 mol/L triethylammonium acetate (pH 7.5). After collecting the labelled 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 labelling with Eu-N1 ITC chelate.

There should be dedicated columns for each lanthanide (europium, terbium, samarium, dysprosium) used for labelling. 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 LABELLED 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 LABELLED PROTEINS AND PEPTIDES

To determine the Eu^{3+} concentration in the labelled proteins (peptides), the labelled protein (peptide) is diluted in DELFIA Enhancement Solution, mixed gently and let stand for about 2 minutes. Eu fluorescence is then measured in a time-resolved fluorometer. 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).

VICTOR is a trademark of Wallac Oy.

FILTRATION

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

STORAGE OF LABELLED COMPOUNDS

To ensure stability, the labelled 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-labelling kit, prod. no. 1244-302, and Sm-labelling kit, prod. no. 1244-303; available also as a specialty product from Wallac Oy, 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 LABELLED PROTEINS

The amount of proteins, incubation time, temperature and the buffers used must be optimized for each particular analyte (2,3). As a general rule about 5 - 100 ng of the labelled 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.

WARRANTY

Purchase of this reagent gives the purchaser the right to use this material in his own research. Further distribution of this reagent is expressly prohibited. Purchase of this product implies agreement with these conditions of sale.

PATENTS

This reagent is covered by patents on both the chemical structure (4,5,6,7) and the dissociation enhancement principle.

REFERENCES

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