DELFIA®

Eu-N1 DTA Chelate & Europium Standard

Product number: AD0004 Lot number: 3327768

Material provided: 1 vial (2 mg, 2.6 µmol) of lyophilized Eu-N1 DTA Chelate

1 vial (0.5 mL) of 100 nmol/L Europium Standard

Introduction:
DELFIA® Eu-N1 DTA Chelate is optimized for the europium labelling of proteins and peptides for use in dissociation-enhanced time-resolved fluorometric assays. The reagent is the Eu^{3+}-chelate of N1-[p-(3,5 dichlorotriazinyl)benzyl]diethylenetriamine-N^1, N^2, N^3, N^3-tetraacetic acid. The dichlorotriazinyl group reacts with free amino groups on the proteins and peptides, forming a stable, covalent bond.

Storage:
The manufacturing date of the chelate is stated on the vial label. Store the chelate at -20°C before reconstitution. Store the standard at +2 - +8°C.

Reagent Reconstitution:
Dissolve the chelate in distilled water (e.g. in 100 µL giving 26 mmol/L solution of the chelate) for immediate use. Keep at 0°C (ice bath). If all chelate is not used during the same day, it can be dissolved either in 10 mmol/L sodium succinate (pH 5.0) or in 10 mmol/L sodium acetate (pH 4.8) (pH of the reconstituted chelate should be below 7 for storage purposes). Keep at 0°C (ice bath).

Reconstituted Stability:
The chelate reconstituted in water should be used the same day. For long term storage at -20°C, the chelate should be dissolved in succinate or acetate buffer and aliquoted.

Precautions:
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.

Labeling of Proteins:
Eu-N1 DTA chelate has a dichlorotriazinyl group as a reactive arm. Dichlorotriazinyl 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	50 %
2.5	25 %
1	10 %

Table 1. The effect of protein concentration on the percentage of Eu-N1 DTA 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 labelling degree of about 8 Eu-N1 DTA 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 16-fold molar excess of chelate during overnight reaction for the introduction of 8 chelates per protein, 48-fold molar excess of Eu-N1 DTA chelate is needed to obtain the same label incorporation during a 4-hour reaction.

Suitable number of Eu-N1 DTA 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).

Labeling 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 - 4 (peptide concentration 5 - 20 mg/mL), 3 - 5 (peptide concentration 2.5 - 5 mg/mL) or 5 - 15 (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.

Labeling 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, dialysis and reverse phase HPLC (RP-HPLC).

2. Concentrating protein and peptide

If a protein is too dilute (less than 1 mg/mL) or 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. 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 a peptide solution.

3. Reconstitution of chelate

After calculating the amount of chelate needed in the reaction, Eu-N1 DTA chelate is dissolved either in water (for immediate use only) or in 10 mmol/L sodium succinate pH 5.0 or in 10 mmol/L sodium acetate pH 4.8 (in case some

of the chelate will be stored for future purposes). Suitable concentration for reconstituted Eu-N1 DTA is 5 - 30 mmol/L (2 mg of Eu-N1 DTA is 2.6 µmol). For example, dissolving 2 mg of Eu-N1 DTA chelate in 100 µL gives a concentration of 26 mmol/L. After dissolving the chelate is kept on ice for immediate use.

4. Labeling

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 labeling 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). 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 10 000 and 30 000.

Peptides having at least about 25 amino acid residues can be purified from the unreacted chelate 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 10-30 000	Proteins and peptides MW 2500-10 000	Peptides MW below 2500 (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 DTA 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 Labeled Proteins and Peptides:

To determine the Eu^{3+} concentration in the labelled proteins (peptides), the labelled 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.

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 labelled protein should be filtered through a 0.22 µm low protein binding membrane.

Storage of Laebeld 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 PerkinElmer Life Sciences, 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 Labeld Compounds:
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 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.

The labelled protein as such is practically non-fluorescent. After binding assay DELFIA Enhancement Solution dissociates Eu ions from labelled 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).
Temperature during storage is determined by the stability of the protein. Suitable temperatures are e.g. +4°C, -20°C and -70°C.

AD0004-R Rev01

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com