DELFIA® Tb-N1 Iodoacetamido Chelate & Terbium Standard AD0010

For Research Use Only

INTRODUCTION

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

PACKAGE CONTENTS

1 vial (1 mg, 1.3 µmol) of Tb-N1 Iodoacetamido Chelate
1 vial (0.5 mL) of 100 nmol/L Terbium Standard

STORAGE

The manufacturing date of the chelate is stated on the vial label. Store the chelate with a desiccant 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 13 mmol/L solution of the chelate) for immediate use. Keep at 0°C (icebath).

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 (icebath).

RECONSTITUTED STABILITY

The chelate reconstituted in water should be used the same day.

For a longterm storage at -20°C, the chelate should be dissolved in succinate or acetate buffer and aliquoted.

WARNINGS AND PRECAUTIONS

This labelling reagent is intended for research use only.

The handling of concentrated Tb^{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

1. Parameters of labelling reaction

   Parameters of labelling reaction include protein concentration, pH, temperature, reaction time, molar excess of chelate over protein and the possible use of a reducing agent.

2. Labelling

   Usually only one or a few of the sulfhydryl groups that are naturally present on the protein or introduced using chemical methods or genetic engineering to the protein are modified.

   If a reducing agent is not used, the following protocol for labelling is recommended. Labelling with iodoacetamido-activated chelates is carried out in sodium hydrogen carbonate at pH 8 - 8.5 or HEPES at pH 8 - 8.5. EDTA (final concentration 1 - 5 mmol/L) can be added to the solution to prevent oxidation of sulfhydryl groups. Reaction time can be 18 - 48 hours. The long reaction time is needed because without treatment with a reducing agent most cysteine residues form S-S bridges. Only a very small fraction of cysteines are in those cases in sulfhydryl form. The suitable temperature is +4°C to maintain the stability of the protein to be labelled.

   It is advantageous to treat the protein with a reducing agent to expose sulfhydryl groups by using e.g. dithiothreitol (DTT), ß-mercaptoethanol or tris(2-carboxyethyl)phosphine hydrochloride (TCEP) prior to labelling. After incubating for about 30 minutes with a reducing agent, the protein is purified using a small gel filtration column. Immediately after gel filtration, reaction with IAA-activated chelate is set up as above. When sulfhydryl groups are exposed the reaction with iodoacetamido-activated chelates is faster than without the incubation with a reducing agent. Reaction times from 3 hours to overnight are sufficient.

   Dithiothreitol and ß-mercaptoethanol must not be present when iodoacetamido-activated chelate is used for labelling. If a reducing agent must be present during labelling, TCEP is the reagent of choice. TCEP does not interfere with labelling but efficiently reduces S-S bridges to sulfhydryl groups. It should be used at a concentration which is 2 - 10 times higher than the molar concentration of protein (e.g. 0.4 - 2 mmol/L). In the presence of TCEP reaction proceeds efficiently giving a good labelling yield already after 3 - 4 hours at +4°C. Same buffers as above can be used to adjust pH to 8 - 8.5. EDTA can be added as described above.

Protein concentration (mg/mL)	Molar excess of chelate over protein
5	20 - 30
2.5	30 - 40
1	40 - 60

Table 1. Guidelines for adjusting the molar excess of chelate over protein at different protein concentrations (labelling at pH 8 - 8.5 and at +4°C).

LABELLING OF PEPTIDES

Peptides (size up to about 40 amino acids), containing a cysteine residue, 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), 4 - 6 (peptide concentration 2.5 - 5 mg/mL) or 6 - 20 (peptide concentration 1 - 2.5 mg/mL). If a reducing agent TCEP is used during labelling, its molar concentration should be 2 - 10 times higher than the peptide concentration. 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).

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 protein or peptide solution contains primary amines (e.g. Tris, ammonium ions), sulfhydryl groups (e.g. mercaptoethanol) or sodium azide, they must be removed as they react with the IAA-activated chelate and interfere with labelling. Such interferences may be removed by using dialysis, gel filtration or reverse phase HPLC (RP-HPLC).

   If the protein or peptide is treated with a reducing agent (DTT or ß-mercaptoethanol), the reducing agent must also be removed prior to labelling using gel filtration or 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[1].

   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.

[1] Centricon and Centriprep are registered trademarks of Millipore Corp.

3. Reconstitution of chelate

After calculating the amount of chelate needed in the reaction, Tb-N1 IAA 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 Tb-N1 IAA chelate is 5 - 30 mmol/L (1 mg of Tb-N1 IAA is 1.3 µmol). For example, dissolving 1 mg of Tb-N1 IAA chelate in 200 µL gives a concentration of 6.5 mmol/L. After dissolving the chelate it is kept on ice for immediate use.

4. Labelling

For labelling a protein or a peptide already in 50 - 100 mmol/L sodium hydrogen carbonate or HEPES (pH 8 - 8.5) labelling buffer, the following reagents are added on ice:

- EDTA (pH 8) to a final concentration of 1 - 5 mmol/L from a stock solution of 10 - 100 mmol/L EDTA in water
- calculated amount of chelate (see Table 1, p. 3)
- if a reducing agent is used, TCEP is added to a final concentration of 0.4 - 10 mmol/L from a stock solution of 10 - 100 mmol/L TCEP in water

If the protein (peptide) is not in a labelling buffer, 1 mol/L sodium hydrogen carbonate (pH 8.4) or 1 mol/L HEPES (pH 8 - 8.5) is added to adjust the buffer concentration to 50 - 100 mmol/L. EDTA (possibly TCEP) and the required amount of chelate are then added into the protein (peptide) solution kept on ice.

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 carbonate or HEPES buffers 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 - 8.5 using either 0.5 - 1 mol/L NaOH or 0.5 - 1 mol/L HCl.

After adding all necessary components and checking pH, incubate at +4°C for 3 - 4 hours (labelling in the presence of TCEP), 3 - 24 hours after a separate reduction step (labelling in the absence of TCEP) or 18 - 48 hours without the use of a reducing agent.

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) [2]. 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.

[2] 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 Tb-N1 IAA 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 Tb^{3+} concentration in the labelled proteins (peptides), dilute them first (e.g. 1:8000) in DELFIA Enhancement Solution (prod. no. 1244-105). Wait for 5 minutes to allow Tb to dissociate and then add 1/5 of the final volume (e.g. 0.2 mL into 0.8 mL to give a final volume 1 mL; final dilution 1:10 000) DELFIA Enhancer (prod. no. CR09-100). After mixing and incubating for 2 minutes measure in a time-resolved fluorometer against 100 nmol/L Tb standard diluted 1:100 in the same way as the sample (1 nmol/L Tb in Enhancement Solution plus DELFIA Enhancer in a clear 96-well plate, 200 µL per well, gives about 500 000 cps in 1420 VICTOR™ Multilabel Counter).

Protein (peptide) concentration can be measured with a suitable method (e.g. Lowry) or calculated from absorbance at 280 nm. The molar absorptivity of reacted Tb-N1 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 protein should be filtered through a 0.22 µm low protein binding membrane.

STORAGE OF LABELLED COMPOUNDS

For good stability the lanthanide-labelled proteins should be stored at high concentration and in the absence of competing metals or chelators 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 LABELLED 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 labelled proteins per tube or well can be used. The DELFIA Assay Buffer (product 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 Tb ions from labelled protein into solution. For detection of Tb-labelled proteins first add 200 µL of Enhancement Solution per well and shake **slowly** for 5 minutes. Then add 50 µL of DELFIA Enhancer to each well and shake **slowly** for 5 minutes before measuring with the time-resolved fluorometer (1420 VICTOR or 1234 DELFIA Research Fluorometer).

WARRANTY

Purchase of the product gives the purchaser the right to use this material in his own research, development, and investigational work. The product is not to be injected into humans or used for diagnostic procedures. Wallac Oy reserves the right to discontinue or refuse orders to any customer who plans to use these products for any other purposes.

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All information supplied with the product and technical assistance given is believed to be accurate, but it remains the responsibility of the investigator to confirm all technical aspects of the application. We appreciate receiving any additions, corrections, or updates to information supplied to the customer.

PATENTS

The reagents are covered by the following patents on both the chemical structure and the dissociation enhancement principle:

Patent 1. Mikola, H., Mukkala, V-M. and Hemmilä, I. (1987): Eur. Patent No. 139,675.
Mikola, H., Mukkala, V-M. and Hemmilä, I. (1989): US Patent No. 4,808,541.

Patent 2. Hemmilä, I. and Dakubu S. (1982): Eur. Patent No. 64,484.
Hemmilä, I. and Dakubu S. (1982): US Patent No. 4,565,790.

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