LANCE™ Eu-W1024 ITC Chelate & Europium Standard
AD0013
Development grade

INTRODUCTION

Fluorescent isothiocyanato-activated (ITC-activated) Eu-W1024 chelate is optimized for labelling proteins and peptides containing at least one primary aliphatic amino group. The labelled compound can be used in homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) assays (LANCE™ assays).

PACKAGE CONTENTS

1 vial (1 mg, 1.4 µmol) of Eu-W1024 ITC Chelate
1 vial (0.5 mL) of 100 nmol/L Europium 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 14 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 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-W1024 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	40 %
2.5	20 %
1	8 %

Table 1. The effect of protein concentration on the percentage of Eu-W1024 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 15-fold molar excess of chelate over protein would give a labelling degree of about 6 Eu-W1024 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 15-fold molar excess of chelate during overnight reaction for the introduction of 6 chelates per protein, 45-fold molar excess of W1024 is needed to obtain the same label incorporation during a 4-hour reaction.

   Suitable number of W1024 chelates coupled to a protein depends on the molecular weight (MW). When the MW of a protein is higher than 100 000, 4 - 10 chelates per protein is a good labelling yield. For proteins with a MW in the range of 30 000 - 70 000, the preferred number of coupled chelates is 2 - 6. Proteins with a MW less

than 30 000 should be labelled with 1 - 2 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 - 4 (peptide concentration 5 - 20 mg/mL), 4 - 6 (peptide concentration 2.5 - 5 mg/mL) or 6 - 12 (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, 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[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.

3. Reconstitution of chelate

   After calculating the molar excess of chelate needed in the reaction, Eu-W1024 ITC chelate is dissolved either in distilled water (for immediate use only) or in 10 mmol/L sodium succinate pH 5.0 or 10 mmol/L sodium acetate pH 4.8 (in case some of the chelate will be stored at -20°C for future purposes). Suitable concentration for reconstituted Eu-W1024 ITC chelate is 5 - 25 mmol/L (1 mg of Eu-W1024 ITC is 1.4 µmol). For example, dissolving 1 mg of Eu-W1024 ITC chelate in 200 µL water gives a concentration of 7 mmol/L. After dissolving the chelate it is kept on ice for immediate use.

[1] 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 W1024.

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 the 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 DNA Grade (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 DNA Grade. Sephadex G-50 DNA Grade is suitable also for purification of proteins with a MW between 15 000 and 30 000.

Peptides having at least about 25 amino acid residues can be purified from the unreacted chelate on Sephadex G-25 DNA Grade 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.

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

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 DNA Grade	Sephadex G-25 DNA Grade	RP-HPLC
Sephadex G-50 /Sepharose 6B	Sephadex G-50 DNA Grade		RP-HPLC	

Table 2. Recommended columns for purification of proteins and peptides after labelling with Eu-W1024 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

Determination of the Eu^{3+} concentration in the Eu-W1024-labelled proteins (or peptides) can be performed as follows. First the labelled protein (peptide) is diluted in 0.1 mol/L HCl (e.g. 1:100) and incubated at room temperature for 10 minutes. The final dilution is performed in DELFIA Enhancement Solution (prod. no. 1244-105) and the dilution factor has to be at least 1:100 to dilute out the effect of HCl on the pH of Enhancement Solution. Eu fluorescence of the sample is measured against 100 nmol/L Eu standard (supplied with the chelate) diluted 1:100 in 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 in 1234 DELFIA Research Fluorometer or 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 contribution of the reacted Eu-W1024 ITC chelate to absorbance at 280 nm is 0.016 per 1 µmol/L chelate (molar absorptivity 16 000).

FILTRATION

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

VICTOR is a trademark of Wallac Oy.

STORAGE OF LABELLED COMPOUNDS

To ensure stability, the W1024-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.

LANCE SETTINGS FOR VARIOUS VICTOR MODELS

A typical LANCE measurement in TR-FRET includes measuring of both donor (Eu at 615 nm) and acceptor (APC at 665 nm) emissions using identical counting parameters exept the filters. Both values are needed if quench correction is required (for more detailed information please refer to Application note "Quench Correction for TR-FRET").

Counting parameters for LANCE labels are instrument dependent because each instrument is individually calibrated; the following table is for your reference.

When using europium as a donor and APC as an acceptor the following parameters should be used. First measurement is done with Eu filter (615) and second with 665 filter.

Parameter	**VICTOR**	**VICTOR LANCE Upgraged**	**VICTOR^2**	**VICTOR^2 HTS (LANCE model)**	**VICTOR^{2}V (LANCE protocol 615/665)**
Flash Energy area	copy Eu	copy Eu	copy Eu	copy Eu	copy Eu
Flash Energy level	copy Eu	copy Eu	copy Eu	copy Eu	copy Eu
Excitation filter	340	'390'	320	340	340
Integrator cap.	1	1	1	1	1
Integrator level	copy Eu	copy Eu	copy Eu	copy Eu	copy Eu
Emission filter	1) 615 2) 665	1) 615 2) 665	1) 615 2) 665	1) 615 2) 665	1) 615 2) 665
Delay time	70 µs	50 µs	50 µs	50 µs	50 µs
Window	200	100	100	100	100
Cycle	1000	1000	1000	1000	1000

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

Both the chemical structure and the LANCE type assays are covered by following patents:

US 4,925,804
US 5,637,509
PCT WO 87/07955
PCT WO 98/15830
US 4,761,481
US 4,920,195
US 5,032,677
US 5,202,423
US 5,324,825
US 5,457,186
US 5,571,897

Wallac Oy, P.O. Box 10, FIN-20101 Turku, Finland. Tel. +358-2-2678111 Fax +358-2-2678357