Caution: For Laboratory Use. A research chemical for research purposes only.

LANCE® Eu-W1284 Iodoacetamido Chelate & Europium Standard
AD0107
Development grade

INTRODUCTION

Iodoacetamido-activated (IAA-activated) Eu-W1284 chelate is optimized for labelling proteins and peptides containing at least one sulfhydryl group. The labelled compound can be used in homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) assays (LANCE™ assays).

PACKAGE CONTENTS

1 vial (0.2 mg, 0.24 µmol) of lyophilised Eu-W1284 Iodoacetamido 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. 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 2.4 mmol/L solution of the chelate). Keep at 0°C (ice bath).

RECONSTITUTED STABILITY

The chelate reconstituted in water should be stored at -20°C for future purposes.

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.

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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 from 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 the iodoacetamido-activated chelate is used for labelling. If a reducing agent is required 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 (e.g. 0.4 - 2 mmol/L) which is 2 - 10 times higher than the molar concentration of protein (peptide). 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.

3. Reconstitution of chelate

   After calculating the amount of chelate needed in the reaction, Eu-W1284 IAA chelate is dissolved in water. Suitable concentration for reconstituted Eu-W1284 IAA chelate is 0.5 - 3 mmol/L. For example, dissolving 0.2 mg of Eu-W1284 IAA chelate in 100 µL gives a concentration of 2.4 mmol/L. After dissolving the chelate it should be kept on ice for immediate use.

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

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 < 0.1 % 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-W1284 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

Determination of the Eu^{3+} concentration in the Eu-W1284-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 PerkinElmer

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.

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 Upgraded**	**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