Sm-labelled immunoreagents are suitable for use in dual-label assays together with Eu^{3+} as the second label because the same enhancement formulation (Enhancement Solution or DELFIA Inducer) is optimal for their measurement. Eu^{3+} gives higher fluorescence and, therefore, Sm^{3+} should be used as the second label in dual-label assays for measuring the analyte requiring less sensitivity (See Figure 8). For detection of Tb^{3+} and Dy^{3+} another highly fluorescent chelate needs to be formed by adding DELFIA Enhancer.

Together, these four lanthanides open up novel ways of rationalizing tedious, expensive and time-consuming assays.

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3. Labelling with lanthanide chelates

In this section we provide basic information on practical aspects of labelling with lanthanide chelates. As an example (section 3.2), we will consider the labelling of proteins in somewhat greater detail.

Labelling with the lanthanide chelates offers obvious advantages. The very mild coupling reaction, as well as the overall hydrophilic nature and negative net charge of the chelate allow labelling to a high specific activity without decrease in affinity or immunoreactivity, or increase in non-specific binding. The thermodynamic stability of the chelate allows long-term storage of the labelled proteins. Thus, the labelling procedure is easy to perform and yields labelled compounds, which are stable enough to allow their use in research for a long time.

For convenience and an easy start, the use of a labelling kit (1244- 302/303) is recommended. The labelling kits are intended for labelling of 0.2 - 1 mg of a “typical IgG”. The labelling reagents (1244-301, AD0001, AD0005 and AD0009) are more suitable products for large scale labelling of proteins and peptides.

Advantages of the labelling kits and reagents are:

- Ready to use
- Easy labelling procedure
- Hydrophilic chelate (no need for organic solvents)
- Efficient but mild reaction conditions
- Minimal influence on immunoreactivity or biological activity
- High labelling yield
- Stable label
- Long shelf-life of the labelled proteins
- Safe reagents and no radioactive waste

In addition to providing the appropriate reagents and other requisites, which allow customers to perform labelling, PerkinElmer Life Sciences also offers a customized labelling service. On a laboratory scale, we conjugate biological compounds with lanthanide chelates and perform custom coatings of microtitration plates. Through our labelling service you can have your compound (protein, peptide, DNA, RNA, hapten, cells) labelled with Eu^{3+}, Sm^{3+}, Tb^{3+} or Dy^{3+}.

3.1 WHAT COMPOUNDS CAN BE LABELLED?

Any stable compound with an amino group can, in principle, be labelled with Eu^{3+}, Sm^{3+}, Tb^{3+} and Dy^{3+}. The method of separation of the labelled protein is determined by the molecular weight of the compound. In order to use a simple gel filtration for separation of a labelled protein from free lanthanide chelates (as discussed in the following sections), the molecular weight of the protein or peptide has to be at least 2500. If smaller compounds are to be labelled, alternative purification systems need to be found.

3.1.1 Proteins

When labelling antibodies, generally about 6- 12 Ln^{3+} per monoclonal antibody IgG is an optimal yield giving high sensitivity with low background. For many assays even a lower labelling yield gives acceptable results. For polyclonal antibodies the suitable number of chelate molecules coupled is 3 – 5. Labelling of antibodies with over 20 Ln^{3+}/IgG may occasionally cause aggregation and an elevated background, especially after storage. Proteins with a lower molecular weight should be labelled with fewer chelates than, for example, monoclonal antibodies. Proteins with molecular weight 30-70,000 are preferably labelled with 2 – 6 chelate molecules and proteins and peptides with molecular weights less than 30,000 with 1 – 3 chelate molecules.

3.1.2 Peptides

Peptides of length from 4 to about 40 amino acids are suitable for DELFIA labelling. Europium labels can be introduced practically anywhere into the sequence using the standard “labelling in solution” method, as described in detail in the section on protein labelling. The label attaches specifically to an amino terminus, or to a lysine or cysteine side chain.

The Wallac Labelling Service has considerable experience in peptide synthesis, purification and labelling, and is happy to put this expertise at the disposal of customers. Our Labelling Service also has access to a DELFIA peptide building block, which makes it possible to introduce the label into the peptide during synthesis. This helps in particular when labelling fairly insoluble peptides. Normal deliveries are from 50 µg to 1 mg of labelled purified peptide, and in some cases we can supply as much as 10 mg. All peptides are purified by HPLC, and the characterisation includes analytical HPLC, lanthanide measurement and mass spectrometry. Delivery time is about 4 weeks from order.

In addition to DELFIA labellings LANCE Quenching peptides, which are mainly used for protease assays, may be prepared either by your lab or through the Wallac Labelling Service. Quenching peptides should be no more than 14 amino acids long. A LANCE Eu-label is attached to one end of the peptide, and a quenching molecule attached to the other. These peptides are non-fluorescent but when a protease cleaves the peptide, the signal may rise up to 1000-fold (signal-to-background level is over 1000).

3.1.3 Oligonucleotides and DNA

Lanthanide-labelled oligonucleotide probes are ideal for detection and quantification of amplification products. The robust, sensitive technology, and its multi-analyte capability is especially useful in screening assays on microtitration plates. Oligonucleotides are synthesized with appropriate amino groups, and then labelled with Eu^{3+} (or Sm^{3+}, Tb^{3+} or Dy^{3+}). Another common approach in DNA hybridization assays is to label the probes with biotin. The biotinylated probes can easily be detected by using Eu-labelled Streptavidin (1244-360).

3.1.4 Other small molecules

Small bio-organic molecules (haptens MW < 1000) can be labelled using the same activated chelate derivatives as proteins and peptides. The molecule to be labelled (eg. steroid, amino acid, drug compound, etc.) has to contain either an amino- or a carboxy-group that is not essential for the further bio-reaction (eg. immunoreaction). If the molecule has no such available group, a suitable derivative has to be synthesised. In immunoassays, for example, the same derivative that was used for antigen preparation is often suitable for labelling, too. The compound to be labelled normally has to be water soluble (though this is not always necessary) and stable under the labelling conditions (this depends on the activation of the chelate). Labelled compounds can usually be purified using HPLC and gel filtration-, RP- or ion exchange columns and have to be optimised on a case-by-case basis. Contact your local PerkinElmer representative for more information on the labelling possibilities for your own molecule and application.

3.2 HOW LABELLING IS DONE – AN EXAMPLE FOR PROTEINS

The following section is intended to provide an example of how labelling is carried out. These general instructions are for labelling of proteins and peptides with isothiocyanate (ITC) activated N1-chelates. More detailed instructions are supplied with the individual kit or reagent being used. Specific labelling instructions for labelling of proteins and peptides with iodoacetamido, amino and dichlorotriazine activated N1 and DTPA-chelates are also supplied with the respective reagent.

3.2.1 Labelling conditions and labelling yield

The labelling depends on the nature and concentration of the protein to be labelled, the temperature and pH of the reaction and the intended final labelling yield. The proteins to be labelled must be in a buffer that does not contain any amines or sodium azide. The protein or peptide must not be stabilized with a protein (e.g. BSA, casein or gelatine).

The labelling yield is affected by several factors:

- The number of amino groups, isoelectric point, and nature and concentration of the protein
- Composition of the labelling buffer (pH, molarity, etc.)
- Reagent composition
- Reaction time
- Temperature

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 the labelling of a protein with N1 ITC chelates (1244-301, 1244-302, 1244-303, AD0001, AD0005, AD0009), assuming an isoelectric point (pI) between 4 and 7 and a lysine residue density of 1 per each 3000 molecular weight units, e.g. 50 lysines for a molecular weight of 150-160,000.

Protein concentration (mg/mL)	Percentage of chelate reacted
5	20 %
2.5	10 %
1	4 %

Table 3. The effect of protein concentration on the percentage of Ln-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 Ln-N1 ITC chelates per protein.

The labelling yield needs to be optimized separately for each particular protein and the assay requirements. Especially monoclonal antibodies may behave individually.

Table 4 gives examples of expected labelling yields obtained with different proteins, when labelled with the DELFIA Eu/Sm Labelling Kits (1244-302/303) according to the kit instructions.

Molecular weight of protein	Expected labelling yield
160 000, monoclonal antibody	6 – 10
160 000, polyclonal antibody	2 – 6
100 000	4 – 7
50 000	1 – 4
30 000	1 – 3

Table 4. Expected labelling yield with protein with different molecular weight and isoelectric point between 4 – 7 when labelling is done with the DELFIA Eu/Sm Labelling Kits (1244-302/303) according to the kit instructions.

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.

3.2.2 Reagents and materials needed

The Eu/Sm Labelling Kits (1244-302/303) include all of the needed reagents (Eu- or Sm-standard, Stabilizer, Enhancement Solution, Assay Buffer and Wash Concentrate). When using Labelling Reagents the needed reagents have to be obtained individually:

- CR84-100 Stabilizer
- 1244-104 DELFIA Enhancement Solution
- C500-100 DELFIA Enhancer (if labelling with terbium chelates)
- Labelling buffer

In addition, for purification of the labelled proteins or peptides, you will need:

- Chromatographic system
- Elution buffer
- Column decontamination buffer
- Spectrophotometer for measurement of protein concentrations.

3.2.3 Labelling procedure

The labelling procedure consists of:

1. Pre-treatment of the protein
2. Labelling
3. Purification by removal of unbound chelates

The straightforward gel filtrations needed in steps 1 and 3 are part of any labelling procedure. The labelling itself is a simple pipetting step - the pretreated protein is combined with the appropriate labelling reagent and incubated overnight at +4 °C.

Pre-treatment of the protein

If the protein or peptide to be labelled is in a solution that contains primary amines (e.g. Tris, ammonium ions), sulfhydryl groups (e.g. mercaptoethanol) or sodium azide, a pretreatment step is necessary since these compounds interfere with labelling. Suitable methods for removing interfering compounds include gel filtration (e.g. NAP and PD-10 columns by Amersham Pharmacia Biotech), dialysis and reverse phase HPLC (RP-HPLC).

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.

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.

Labelling

The process is slightly different, depending on whether you are working with individual labelling reagents or a labelling kit.

If labelling with the Eu-Labelling reagent (1244-301), Eu-N1 chelate (AD0001), Sm-N1 chelate (AD0005) or Tb-N1 chelate (AD0009), the reagent is first dissolved in water. The amount of reagent to be added is calculated according to the amount of protein and its molecular weight.

If labelling with Eu- or Sm-Labelling kits (1244-302/303), the reagent is dissolved directly with 0.5 mL of the pre-treated protein in the labelling buffer (100 mmol/L Na_2CO_3, pH 9.3). The maximum amount of protein is approximately 1 mg.

After gently mixing the protein and reagent, the mixture is incubated overnight at +4 °C.

Note. Always carefully follow the specific instructions supplied with the reagent or kit.

Purification of labelled proteins

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 8 000 and 30 000.

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 (Figure 9).

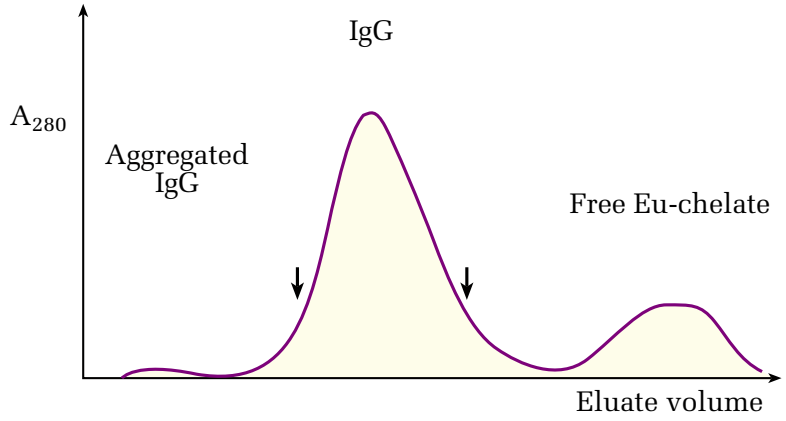

Figure 9. The elution profile of labelled IgG from a column of Sephadex G-50 and Sepharose 6B. It is recommended that the fractions between the arrows (monomeric IgG) be pooled.

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.

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 with an 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 of 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 5. Recommended columns for purification of proteins and peptides after labelling with Eu-N1 ITC chelate.

There should be dedicated columns for each lanthanide (Eu^{3+}, Sm^{3+} or Tb^{3+} and Dy^{3+}) 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). After adding the BSA the column should be equilibrated overnight. RP-HPLC columns can be washed using the phthalate buffer described above.

3.2.4 Characterization of labelled proteins
The concentration of Eu^{3+}, Sm^{3+} or Tb^{3+} is determined from an aliquot, which is diluted with Enhancement Solution (1:1 000 – 1:100 000) The fluorescence is measured in microtitration wells (200 mL/well, in duplicate). The signal is compared to the signal of stock standards diluted 1:100 in Enhancement Solution.

The resulting concentrations and the approximate signals will be as follows. The figures are for clear 96-well plates, 200 mL/well. For development of the Tb signal, C500-100 DELFIA Enhancer is also required.

For Eu^{3+}: 1 nmol/L Eu - signal about 1 000 000 cps
For Tb^{3+}: 1 nmol/L Tb - signal about 500 000 cps
For Sm^{3+}: 10 nmol/L Sm – signal about 100 000 cps

The protein concentration can be measured with any appropriate method, e.g. Lowry's method, or it can be calculated from the absorbance at 280 nm, which has to be corrected for the absorption of the chelate. The molar absorptivity of reacted N1-ITC chelate is 8000 at 280 nm. To remove particles and possible aggregates the labelled compound should be filtered through a 0.22 mm low protein binding membrane.

3.2.5 Storage of labelled compounds
Labelled proteins and peptides should be stored at a high concentration and in the absence of chelators or competing metals in the buffer. Do not store diluted reagents. In most cases, 50 mmol/L Tris-HCl buffered saline solution containing 0.1-0.5% purified BSA will ensure the stability of the labelled compound during storage. Storage should be at the optimal temperature for the protein or peptide. If the labelled protein requires storage at +4°C, it is advisable to add a bacteriostatic agent such as sodium azide (NaN_3) at concentration of 0.05-0.1%. Neither DELFIA Assay Buffer (Prod. No. 1244-106, 1244-111, 4002-0010) nor phosphate buffers (see section 4.) are suitable for storage of labelled proteins or peptides. If during storage the background level of the assay tends to increase due to aggregation formation, the labelled compound should be filtered through a 0.2 µm membrane.

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4. Setting up DELFIA assays

4.1 TYPES OF BUFFERS

To achieve the best results in a DELFIA assay, the optimal buffer composition should be chosen. A number of ready-to-use buffer products are available as catalog items, or users may prepare their own formulations based on the following guidelines.