Time-resolved fluorometry with the DELFIA method user guide

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Introduction

Time-resolved fluorometry (TRF), as applied in the DELFIA® technology from Revvity, Inc., is well-established in High Throughput Screening (HTS) and research laboratories. Its popularity stems from the high sensitivity and wide dynamic range of measurement it affords in various assay designs. Ideal for secondary screening, DELFIA assays are separation assays, free from possible interference by sample matrices.

The technology is extremely flexible. It suits both 96-well and 384-well plate formats and can be applied in coated plates and in filter assays. Its general robustness and automation friendliness have also led to its widespread use in clinical screening and diagnostics. A notable benefit in many applications is its suitability for multi-analyte assays.

Labeling procedures for creating high-sensitivity time-resolved fluorometric assays are extremely easy. The labeled compounds have a high specific activity and a good stability with a minimal influence on biological activity.

This guide provides an introduction to the DELFIA method, and to the labeling procedures involved.

1. Principle of the TRF technology

1.1 Unique fluorescence properties of lanthanide chelates

The technology described here is based on the use of lanthanide chelate labels with unique fluorescence properties. The fluorescence lifetime of the specific signal is several orders of magnitude longer than the non-specific background. This enables the label to be measured at a time when the background has already decayed (temporal resolution). The large Stokes' shift, i.e. the difference between excitation and emission wavelengths, and the narrow emission peak contribute to increasing the signal-to-noise ratio. The sensitivity is, furthermore, increased because of the dissociation-enhancement principle: the lanthanide chelate is dissociated into a new highly fluorescent chelate inside a protective micelle.

Suitable lanthanide (Ln) metals for use as chelate labels are europium, samarium and terbium. The europium ion, Eu^{3+}, is the one that is mainly used. In particular it has frequently been used as the label for antibodies in solid-phase immunoassays. It has also been the choice when labeling DNA probes and it is used in cytotoxicity assays. Samarium, Sm^{3+}, has been introduced as a second choice, opening up the fascinating possibility of dual labeling. Terbium Tb^{3+} enables the unique concept of triple labeling.

In short, the special fluorescence properties of lanthanides are:

- Long decay time
- Large Stokes' shift
- Sharp emission peak
- High fluorescence intensity

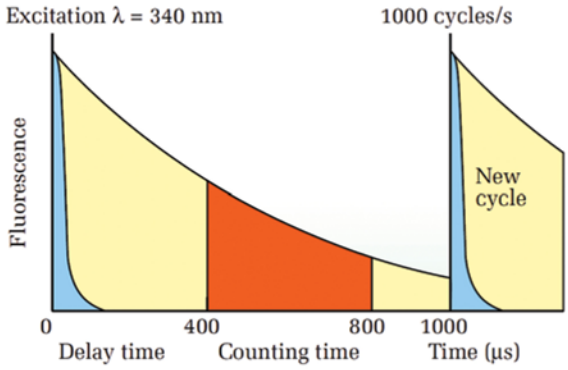
Decay times of lanthanide-chelates can exceed 1,000,000 ns

Non-specific background fluorescence has a decay time of only about 10 ns. It thus dies away before the sample fluorescence is measured. In a timeresolved fluorometer or multilabel plate reader, the sample is pulsed 1,000 times per second with excitation light of 340 nm. In the period between flashes the sample fluorescence is measured for 400 µs after a delay time of 400 µs. This explains the high sensitivity and gives statistically accurate results after a short and convenient measuring time. (See Figure 1(a)).

Lanthanide-chelates have large Stokes' shifts

The Stokes' shift for europium is almost 300 nm. This big difference between excitation and emission peaks means that the fluorescence measurement is made at a wavelength where the influence of background is minimal. In addition, the emission peak is very sharp which means that the detector can be set to very fine limits and that the emission signals from different lanthanide chelates can be easily distinguished from each other (See Figure 1(b)).

1(A)



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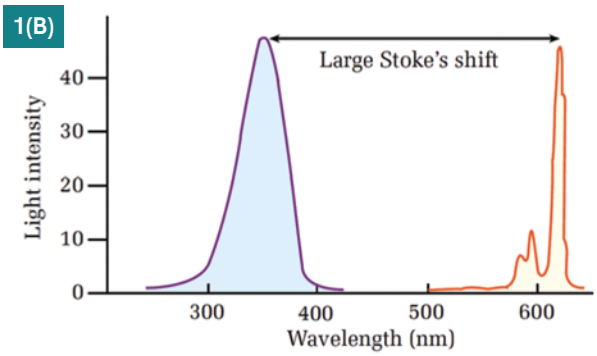


Figure 1: Unique fluorescence properties of lanthanides, (a) long fluorescence decay time, (b) large Stokes' shift.

1.2 Dissociation-Enhancement

In most DELFIA assays, the labeled biomolecule as such is practically non-fluorescent. However, after the binding reaction is complete, fluorescence is developed by the addition of either Enhancement Solution or DELFIA Inducer (Table 1). The low pH of these formulations efficiently dissociates the europium from the labeled compound within a few minutes. The free Eu^{3+} rapidly forms a new, highly fluorescent chelate inside a protective miscelle with components of the Enhancement Solution or DELFIA Inducer (Figure 2). The fluorescence of the lanthanide chelate is amplified 1-10 million times by the enhancement technique. The speed of this development process will depend on the original chelate used, the choice of Enhancement Solution or DELFIA Inducer, and whether or not shaking is used.

Table 1: Suitability of enhancement solution and DELFIA inducer for use with various europium chelate types. Times required to reach 98% of maximum signal.

Chelate	DELFIA enhancement solution		DELFIA Inducer	
	Shaking (slow)	Without shaking	Shaking (slow)	Without shaking
N1	5 min	45 min	5 min	30 min
DTPA	30 min	180 min	5 min	30 min
W1024	45 min	120 min	5 min	30 min
W2014	45 min	120 min	5 min	30 min

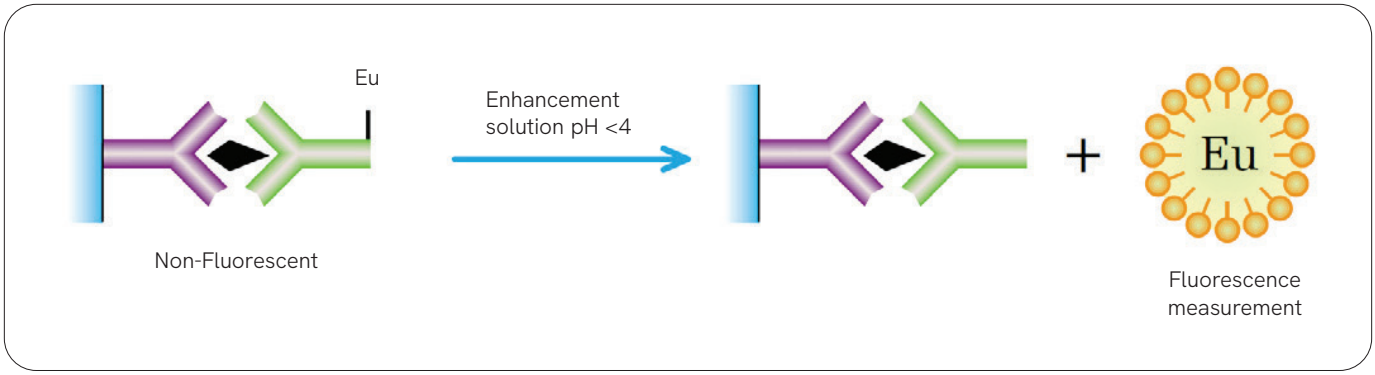


Figure 2: Dissociation enhancement.

Multianalyte assays may involve the use of terbium chelates as secondary or tertiary labels. A different enhancing substance, DELFIA Enhancer is then needed in addition to the formulation used (i.e. either Enhancement Solution or DELFIA Inducer) to release the europium primary label. DELFIA Enhancer is not needed in a dual label assay involving samarium with europium. Both Enhancement Solution and DELFIA Inducer will effectively release the samarium as well as the europium.

1.3 Suitability of the DELFIA method

The DELFIA method brings high sensitivity TRF separation assays to areas such as secondary screening and other pre-clinical phases in drug discovery, as well as routine clinical diagnostics, clinical screening, and clinical research.

In addition to DELFIA separation assays, homogeneous, non-dissociation TRF systems are also available. Revvity's LANCE™ and Alpha™ homogeneous assays are widely used for primary screening in drug discovery laboratories.

2. Applications

2.1 General

The DELFIA method is versatile in its application. Eu-, Sm- and Tb-labeling reagents are used for protein, peptide and oligonucleotide labeling and the resulting labeled compounds are suitable for various types of assay based on solid-phase separation. Reagents immobilized to microtitration plates allow an easy and efficient separation of the unbound fraction by using the DELFIA Platewash. The bound fraction of the labeled reagent is quantified with an instrument capable of detecting time-resolved fluorescence, such as Revvity's EnVision™ Multilabel Plate Reader (See Section 5 on Page 18 for a review of suitable detection instruments).

Examples of application areas include:

- Immunoassays
- Biomarker detection
- Immunogenicity
- Receptor-ligand binding
- Cell-mediated cytotoxicity
- ADCC assays
- Cell proliferation
- Kinase assays
- Biodistribution studies

2.2 Kinase assays

Protein kinases use ATP to phosphorylate certain amino acid residues (tyrosine, serine or threonine) on their substrates. In receptor-mediated signalling the phosphorylation forms an important secondary messenger system. Phosphorylation can be measured following radioactive ^{32}P incorporation. This, however, involves the safety procedures and disposal problems of working with radionuclides. Eu-labeled anti-phosphotyrosine, -threonine and threonineproline are available for the sensitive detection of phosphorylation levels on various substrates using time-resolved fluorometry and DELFIA as the detection technology.

In tyrosine kinase assays, for example (Figure 3), the enzymatic reaction is first carried out with biotinylated substrate. Then the reaction mixture is diluted and transferred to a streptavidin plate. The biotinylated substrate binds to the plate, and a europium-labeled antibody against phosphorylated tyrosine is added. This binds to the phosphorylated substrate. Detection is performed in the usual way after the enhancement process.

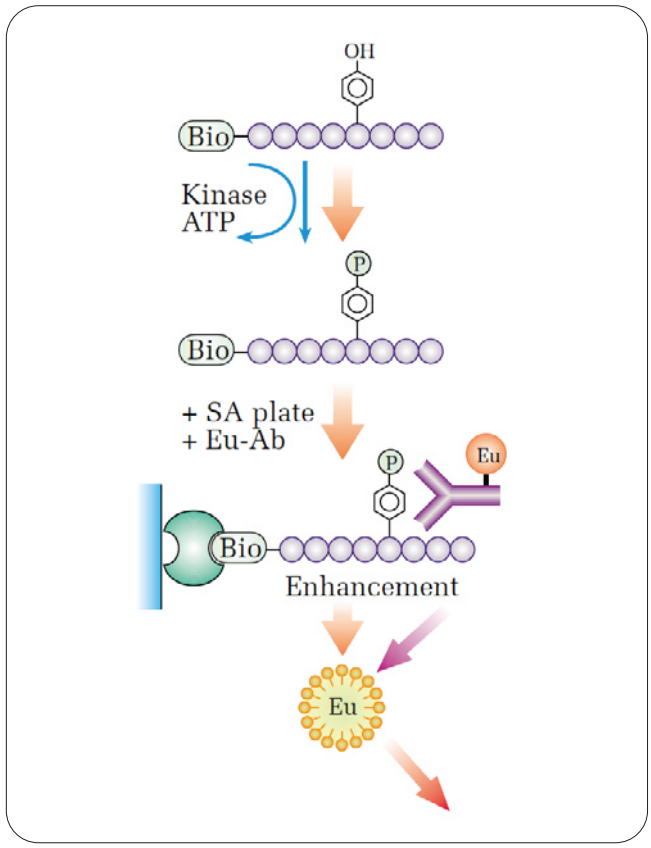


Figure 3: Principle of the DELFIA tyrosine kinase assay.

2.3 Ligand receptor assays

The DELFIA chemistry has been applied with success using various assay formats in the detection of receptor ligand interactions. In the galanin assay, for example, a non-filtration method involves the incubation on streptavidin coated plates of europium-labeled galanin, the galanin receptor, and biotinylated wheat germ agglutinin. Such an assay is easy to automate, works with 384-well plates, and is highly suitable for HTS laboratories.

Motilin assay may be performed in a similar fashion using streptavidin coated plates, with tissue-culture plates for cell-based assays, or with filter plates for cell membrane or suspension cell assays. These provide an excellent means of separating the receptor ligand complex from excess reagents.

2.4 Cell proliferation assay

Cell proliferation is an important parameter when studying live cell function, especially, when the effect of growth regulatory substances or cytotoxic agents is under study. Methods developed to measure the proliferation of cells have been based on microscopic detection, incorporation of radioactive precursors, uptake of chromogenic dyes or measurement of metabolic activity of proliferating cells. Since cellular proliferation requires the replication of cellular DNA, methods based on DNA synthesis measurement can be used as an accurate indicator of cell growth. Traditionally, tritium-labeled thymidine has been used to label DNA. The DELFIA cell proliferation assay follows a similar approach, but employs 5-bromo-2′-deoxyuridine (BrdU) as an alternative label.

As a pyrimidine analog, BrdU can be incorporated into newly synthesized DNA instead of thymidine, and detected immunochemically using europium-labeled monoclonal antibody. The DELFIA assay employs Fix Solution to fix the cells and denature the DNA, and DELFIA Inducer for the development of fluorescence. An assay procedure appropriate for most applications is illustrated in Figure 4. The actual protocol will be dependent on the cell line used, and exact incubation times have to be optimized for each experimental setup individually.

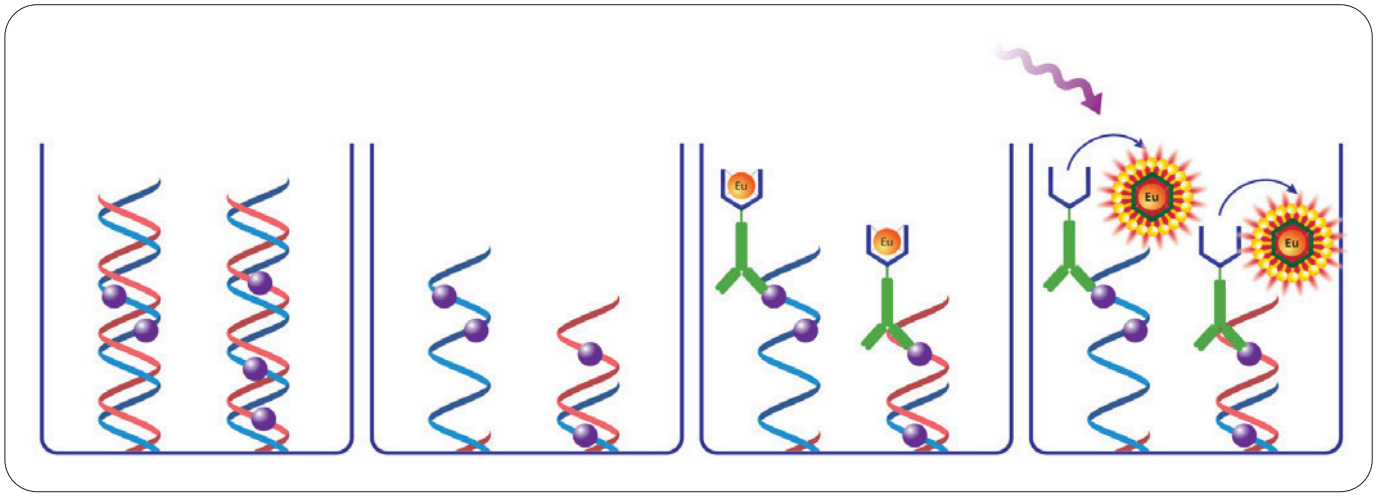


Figure 4: Cells are incubated with the non-radioactive pyrimidine analog BrdU to allow its incorporation into newly synthesized DNA in place of thymidine. Subsequently, Europium-labeled anti- BrdU antibodies are used to detect the level of BrdU incorporation via time-resolved fluorescence, a measurement used as an accurate indicator of cell proliferation.

2.5 Cytotoxicity assay

The DELFIA cytotoxicty assay is based on loading target cells with an acetoxymethyl ester of fluorescence enhancing ligand (BATDA). The ligand penetrates the cell membrane quickly. Within the cell the ester bonds are hydrolyzed to form a hydrophilic ligand (TDA) which no longer passes the membrane. After cytolysis the ligand is

released and allowed to react with an Eu-solution. The Eu and the ligand form a highly fluorescent and stable chelate (EuTDA). The measured signal correlates directly with the amount of lysed cells.

Benefits of BATDA as a cell marker

- Non-radioactive label
- Fast accumulation of label in cells
- Excellent recovery of labeled cells
- Fast release of label
- Fast measurement
- Stable fluorescent signal (at least 5 h)

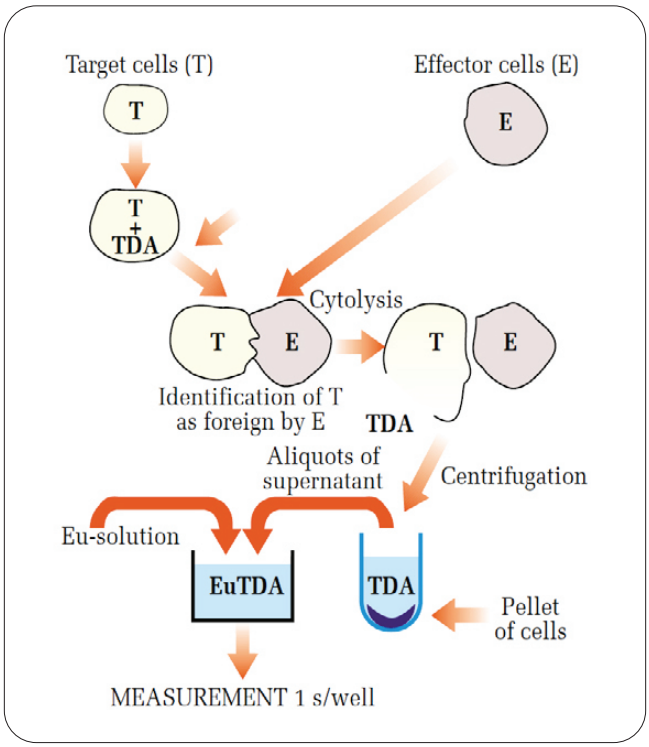


Figure 5: Principle of the DELFIA cytotoxicity assay.

2.6 Adherent cell assays

DELFIA provides an excellent method for assays in which the expression of receptors on cell surfaces is investigated. The europium label used does not interfere with the function of the cell.

Labeling cells with europium and detection with time-resolved fluorometry also provides a very simple and highly sensitive way to quantitate cell adhesions. Cell-to-cell and cell-to-matrix adhesion has an important role in cell homing, stimulated cell activation, cellular migration (leukocytes), etc. *In vitro* cell-to-matrix adhesion studies are performed using, e.g. fibronectin-coated microtitration plates.

2.7 Hybridization assays

Simple and user friendly methods for detection of gene mutation or viruses in routine laboratories can be designed using Eu-labeled oligonucleotides. Dual or even triple-label assays can be set up using DELFIA technology to include internal control for the sample or increase throughput of the assay. Streptavidin coated DELFIA plates as solid phase eliminate laborious sample preparation and electrophoresis procedures.

2.8 Immunoassays

Lanthanide labeled immunoreagents can be applied in non-competitive (Figure 6(a)) or competitive (Figure 6(b)) time-resolved fluorescence assays. The design of each type of assay depends on the analyte, the antibodies, the required sensitivity and the required dynamic range.

As a general rule, about 25-100 ng of labeled antibodies per well is enough for noncompetitive sandwich-type assays, but the actual optimal level depends on the purity and affinity of the antibodies and the desired signal levels. For competitive assays no general rules can be given, but the listed references may be valuable when optimizing an assay.

To achieve low fluorescence background, addition of 20-50 mmol/l DTPA in the assay buffer is recommended.

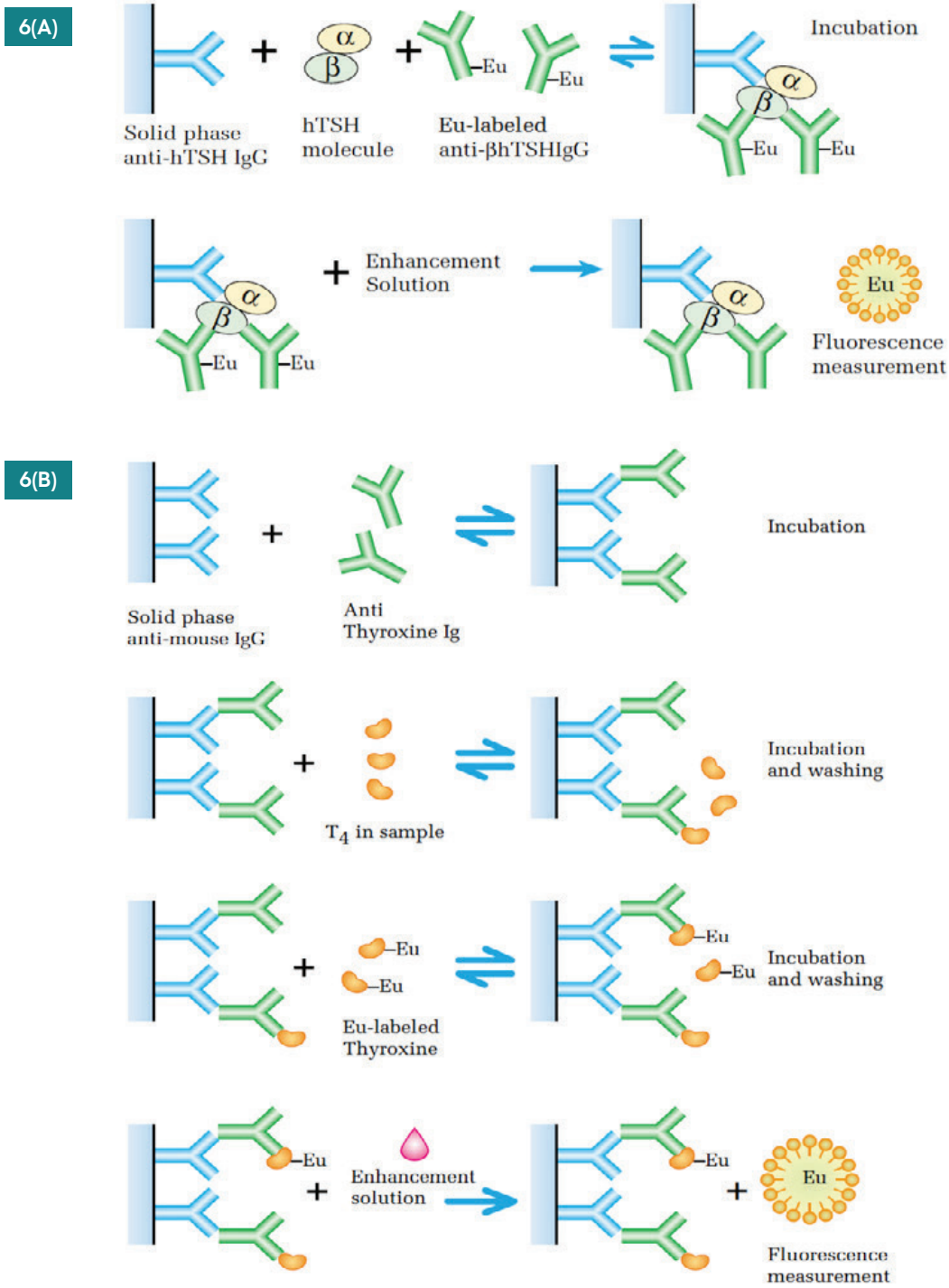


Figure 6: Examples of immunoassays types, (a) non-competitive "sandwich-type", (b) competitive.

2.9 Multianalyte assays

Simultaneous quantification of two or more analytes in one sample can save time and reduce the volume of samples required.

Lanthanides are suitable for multi-label assays because of their narrow emission peaks at different wavelengths (613 nm for Eu^{3+}, 643 nm for Sm^{3+}, 545 nm for Tb^{3+}) (See Figure 7) and their different fluorescence lifetimes (e.g. 730 ms for Eu^{3+} and 50 ms for Sm^{3+}). These features can be utilized for the optimization of the measurement conditions in order to get maximal sensitivity and to minimize the signal spillover.

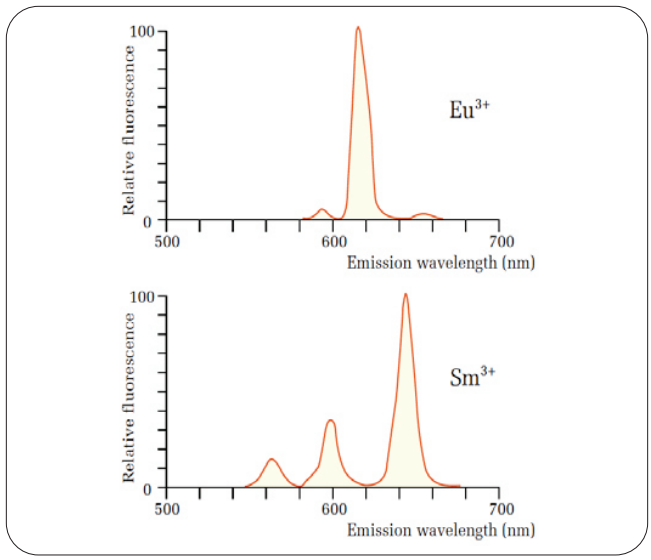


Figure 7: Emission wavelength (nm) spectra for europium and samarium.

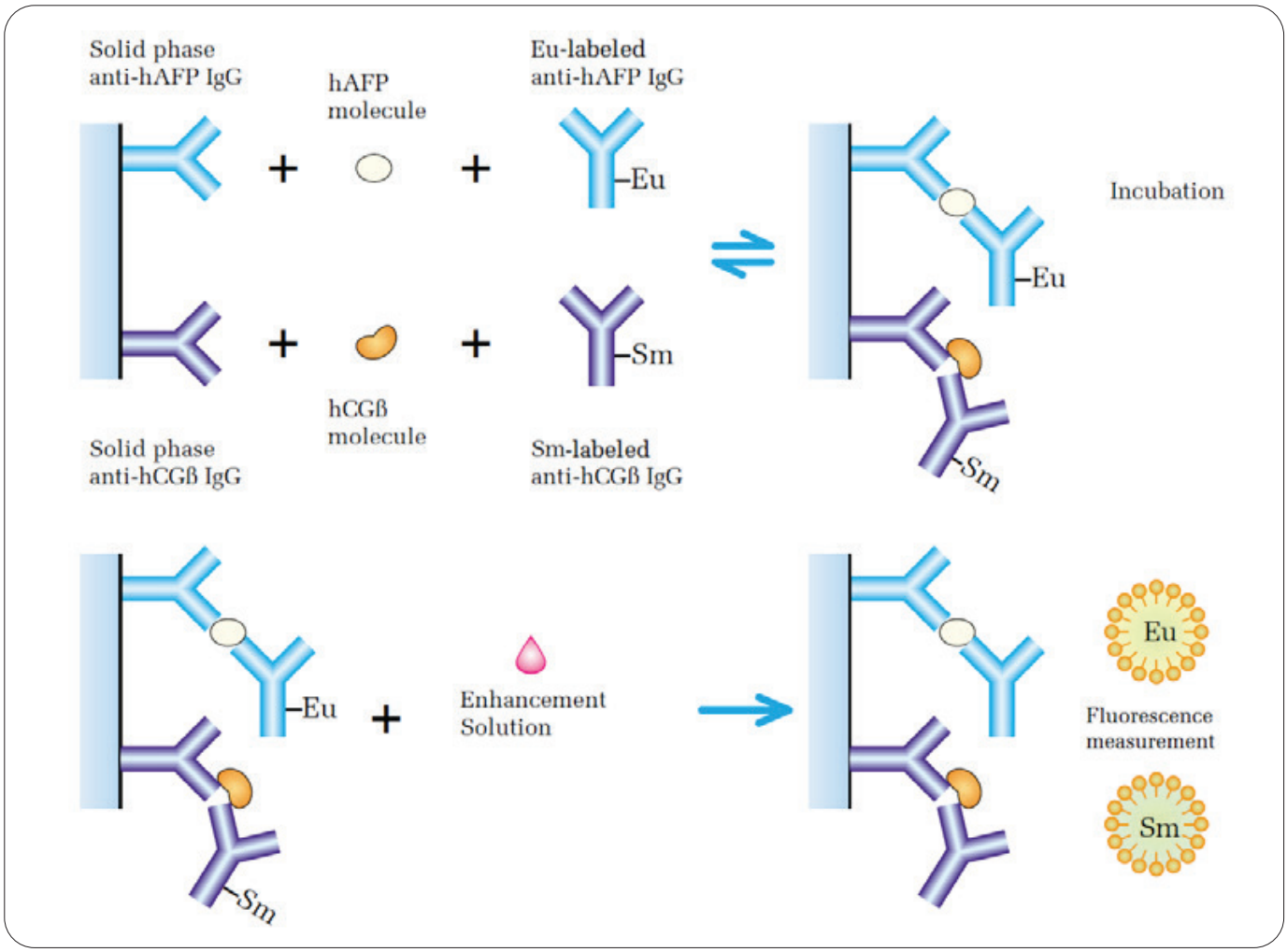


Figure 8: Example of a dual label "sandwich-type" immunoassay employing europium and samarium chelate labels.

Sm-labeled 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 (Figure 8). For detection of Tb^{3+} another highly fluorescent chelate needs to be formed by adding DELFIA Enhancer.

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

3. Labeling with lanthanide chelates

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

Labeling 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 labeling 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 labeled proteins. Thus, the labeling procedure is easy to perform and yields labeled 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 labeling kit (1244-302/303) is recommended. The labeling kits are intended for labeling of 0.2-1 mg of a "typical IgG". The labeling reagents (1244-301, AD0001, AD0005 and AD0009) are more suitable products for large scale labeling of proteins and peptides.

Advantages of the labeling kits and reagents are:

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

In addition to providing the appropriate reagents and other requisites, which allow customers to perform labeling, Revvity, Inc. also offers a customized labeling service. On a laboratory scale, we conjugate biological compounds with lanthanide chelates and perform custom coatings of microtitration plates. Through our labeling service you can have your compound, protein, peptide, or antibody labeled with Eu^{3+}, Sm^{3+}, or Tb^{3+}.

3.1 What compounds can be labeled?

Any stable compound with an amino group can, in principle, be labeled with Eu^{3+}, Sm^{3+} and Tb^{3+}. The method of separation of the labeled protein is determined by the molecular weight of the compound. In order to use a simple gel filtration for separation of a labeled 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 labeled, alternative purification systems need to be found.

3.1.1 Proteins

When labeling 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 labeling yield gives acceptable results. For polyclonal antibodies the suitable number of chelate molecules coupled is 3-5. Labeling 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 labeled with fewer chelates than, for example, monoclonal antibodies. Proteins with molecular weight 30-70,000 are preferably labeled 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 labeling. Europium labels can be introduced practically anywhere into the sequence using the standard "labeling in solution" method, as described in detail in the section on protein labeling. The label attaches specifically to an amino terminus, or to a lysine or cysteine side chain.

Our OnPoint custom service team has considerable experience in peptide synthesis, purification and labeling, and is happy to put this expertise at the disposal of customers. Our Labeling 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 labeling fairly insoluble peptides. Normal deliveries are from 50 µg to 1 mg of labeled purified peptide, and in some cases we can supply as much as 10 mg. All peptides are purified by HPLC, and the characterization includes analytical HPLC, lanthanide measurement and mass spectrometry. Delivery time is about 4 weeks from order.

In addition to DELFIA labelings, LANCE Quenching peptides, which are mainly used for protease assays, may be prepared either by your lab or through OnPoint custom services. 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-labeled 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 labeled with Eu^{3+} (or Sm^{3+} or Tb^{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-labeled Streptavidin (1244-360).

3.1.4 Other small molecules

Small bio-organic molecules (haptens MW < 1000) can be labeled using the same activated chelate derivatives as proteins and peptides. The molecule to be labeled (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 (e.g. immunoreaction). If the molecule has no such available group, a suitable derivative has to be synthesized. In immunoassays, for example, the same derivative that was used for antigen preparation is often suitable for labeling, too. The compound to be labeled normally has to be water soluble (though this is not always necessary) and stable under the labeling conditions (this depends on the activation of the chelate). Labeled compounds can usually be purified using HPLC and gel filtration-, RP- or ion exchange columns and have to be optimized on a case-by-case basis. Contact your local Revvity representative for more information on the labeling possibilities for your own molecule and application.

3.2 How labeling is done – an example for proteins

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

3.2.1 Labeling conditions and labeling yield

The labeling depends on the nature and concentration of the protein to be labeled, the temperature and pH of the reaction and the intended final labeling yield. The proteins to be labeled 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 labeling yield is affected by several factors:

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

The recommended reaction conditions for labeling of proteins are pH 9-9.3, +4 °C and overnight incubation. Under these conditions, the following calculations are valid for the labeling 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.

Table 2: 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.

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

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 labeling degree of about 8 Ln-N1 ITC chelates per protein.

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

Table 3 gives examples of expected labeling yields obtained with different proteins, when labeled with the DELFIA Eu/Sm Labeling Kits (1244-302/303) according to the kit instructions.

Table 3: Expected labeling yield with protein with different molecular weight and isoelectric point between 4-7 when labeling is done with the DELFIA Eu/Sm labeling Kits (1244-302/303) according to the kit instructions.

Molecular weight of protein	Expected labeling 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

Peptides (size up to about 40 amino acids) are labeled like proteins except that the molar excess of chelate over peptide is lower than in protein labeling. 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). Labeling is usually performed at +4 °C but, if the peptide to be labeled is very stable, it can be labeled 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 Labeling Kits (1244-302/303) include all of the needed reagents (Eu- or Sm-standard, Stabilizer, Enhancement Solution, Assay Buffer and Wash Concentrate). When using Labeling Reagents the needed reagents have to be obtained individually:

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

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

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

3.2.3 Labeling procedure

The labeling procedure consists of:

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

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

Pre-treatment of the protein

If the protein or peptide to be labeled 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 labeling. Suitable methods for removing interfering compounds include gel filtration (e.g. NAP and PD-10 columns by GE Life Sciences), 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 labeling, 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 labeling reaction (less than 1 mg/mL), a vacuum centrifuge can be used to concentrate the peptide solution.

Labeling

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

If labeling with the Eu-Labeling 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 labeling with Eu- or Sm-Labeling kits (1244-302/303), the reagent is dissolved directly with 0.5 mL of the pre-treated protein in the labeling 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 labeled proteins

Separation of the labeled 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. 1x10 cm) layered on Sepharose 6B (e.g. 1x30-40 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.

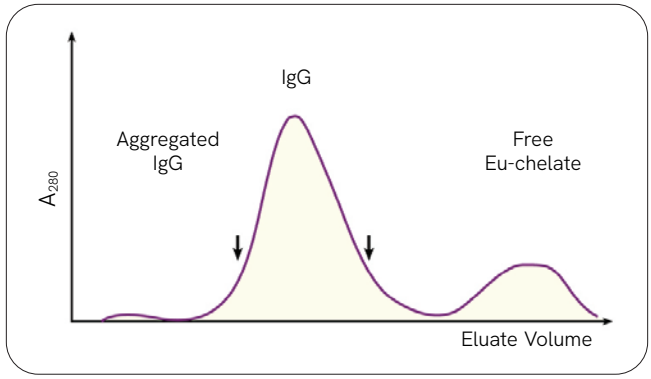


Figure 9: The elution profile of labeled IgG from a column of Sephadex G-50 and Sepharose 6B. It is recommended that the fractions between the arrows (monomeric IgG) be pooled.

The gel filtration eluate can be monitored by UV-absorbance at 280 nm. The first peak contains the labeled protein and the second peak unreacted chelate (Figure 9).

When labeling 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 unlabeled peptide by using reverse phase HPLC. The labeled 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 labeled 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.

Table 4: Recommended columns for purification of proteins and peptides after labeling with Eu-N1 ITC chelate.

Proteins MW above 100,000	Proteins MW 30-100,000	Proteins MW 15-30,000	Proteins and peptides MW 2,500-15,000	Peptides MW below 2,500 (< 25 aa)
Superdex 200	Superdex 75	Sephadex G-50	Sephadex G-25	RP-HPLC
Sephadex G-50/ Sepharose 6B	Sephadex G-50		RP-HPLC	

There should be dedicated columns for each lanthanide (Eu^{3+}, Sm^{3+} or Tb^{3+} and Dy^{3+}) used for labeling. 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 labeled proteins

The concentration of Eu3+, Sm3+ or Tb3+ 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 8,000 at 280 nm. To remove particles and possible aggregates the labeled compound should be filtered through a 0.22 mm low protein binding membrane.

3.2.5 Storage of labeled compounds

Labeled 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 labeled compound during storage. Storage should be at the optimal temperature for the protein or peptide. If the labeled 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 labeled proteins or peptides. If during storage the background level of the assay tends to increase due to aggregation formation, the labeled compound should be filtered through a 0.2 µM membrane.

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.

4.1.1 Assay buffer

The use of a Tris-based buffer is recommended. HEPES and phosphate buffers can be used with N1-chelates, but lower signals might be obtained compared to Tris-based buffers. For storage purposes, phosphate buffers must not be used due to their chelating nature.

To avoid non-specific binding the buffer should contain a blocking agent such as bovine serum albumin (BSA). There are many different grades of BSA and some of these contain a considerable amount of heavy metals that will eventually show as high levels of background in the assay. Using purified BSA is highly recommended, or alternatively high grade of casein or ovalbumin can be used to block non-specific binding.

A detergent such as Tween 20 or Tween 40 is also needed in the buffer to further prevent non-specific binding to the plate.

To keep the fluorescence background low as well as for maintaining good precision the assay buffer should contain low concentrations of chelator such as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA). It is, however, essential to remember that too much chelator present in the assay buffer will eventually start competing for the lanthanide and will destroy the assay. As a general rule, no more than 50 µmol/L of chelator may be used in the assay buffer when working with compounds labeled with N1-chelate.

An example of an assay buffer composition for a DELFIA assay could be 50 mM Tris-HCl, pH 7.5-8, containing 0.9% NaCl, 0.2-0.5% of purified BSA, 0.01-0.1% Tween (20 or 40) and 20 µM EDTA.

4.1.2 Hybridization buffers

Hybridization assays require additional sodium chloride in the buffer. To achieve successful hybridization a standard assay buffer (as described above) can be used, but should be supplemented with 0.5-1 M NaCl. On the other hand, with sticky oligonucleotides, additional reagent to prevent non-specific binding is needed. Reagents such as polyacrylic acid (up to 1 mg/mL) or polyvinylpyrrolidone, PVP (0.05-0.2%, MW about 360,000 g/mol) are recommended in this case.

4.1.3 Wash solutions for DELFIA assays

To enable the high sensitivity of the DELFIA assays, automated plate washers should be used, with typically 4-6 wash cycles prior to addition of Enhancement Solution. To avoid dissociation of the lanthanide during washes, neutral buffered solutions like Tris-HCl (pH 7.5-8) with detergents are recommended.

4.2 Types of plates

DELFIA assays are separation assays using either coated microplates or AcroWell™ filtration plates. The main critical factors in achieving high sensitivity and good precision are the background fluorescence of the plate, and in the case of coated plates, the coating properties of the well surface.

AcroWell™ filtration plates have a uniquely low fluorescence background and are therefore well suited for DELFIA assays, particularly for DELFIA receptor ligand binding assays.

The plate consists of two parts, a clear polystyrene lid and a chemically resistant and biologically inert polypropylene filter plate assembly.

In solid phase separation assays, low fluorescence background is achieved through careful selection of the plastic material (usually polystyrene), proper well surface treatment (usually high protein binding treatment) and high quality coating.

A number of coated and uncoated plates, optimized for DELFIA assays, are available as catalog items. Both 96- and 384-well format plates are supplied with different pigments to fulfill each assay need.

DELFIA yellow plates have a special facility to reduce fluorescence background in DELFIA assays. The UV-absorbing agent in the plastic material prevents the excitation of the plastic, giving lower background and increased sensitivity. The advantage of the yellow plate is especially strong in multi-analyte DELFIA assays where samarium or terbium is used as a second label alongside europium.

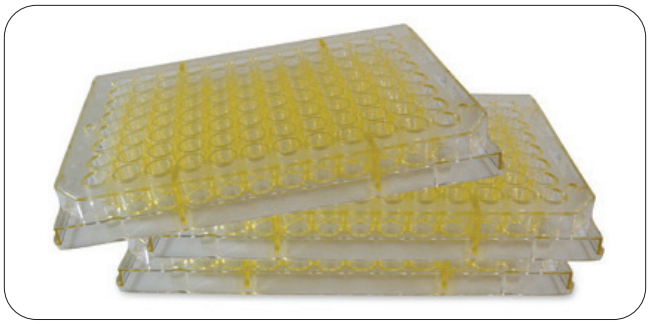


Figure 10: The DELFIA yellow plate is characterized by exceptionally low fluorescence background.

Revvity offers a variety of coated plates in different formats and plate pigments that have been tested and optimized for DELFIA assays.

Streptavidin coated plates

- Bind all biotinylated products

Anti-mouse, anti-rabbit and anti-sheep antibody-coated plates

- Anti-mouse-antibody is raised in rabbit and reacts with all mouse IgG subclasses, IgA and IgM. Reaction with human serum and fetal calf serum is less than 0.1%
- Anti-rabbit- and anti-sheep-antibodies are raised in goat.

5. Measurement of DELFIA assays

DELFIA assays may be measured by modern multilabel plate readers that have a TRF option, or with dedicated instruments.

5.1 Today's versatile instruments

Revvity's top-of-the-range EnVision™ and EnSpire® TRF multilabel plate readers meet all of today's needs for efficient processing of single or multilabel DELFIA assays. Various models are available, and it may be supplied with or without a stacker. As an alternative, the Victor™ multilabel plate reader is also available.

For ultra-fast processing of samples the ViewLux™ ultraHTS microplate imager allows detection of all samples on a microplate simultaneously.

For fully automatic performance of assays the AutoDELFIA™ automatic immunoassays system performs all of the other assay stages as well as detection.

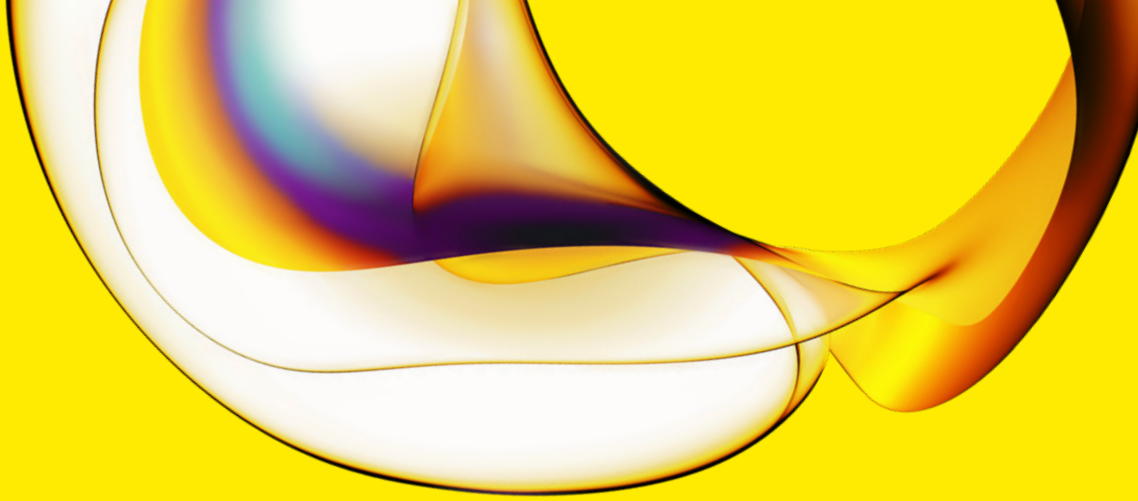
5.2 Legacy instruments

Instruments described in this section are no longer supplied by Revvity, but may be available in your laboratory. The 1234 DELFIA Fluorometer is an automatic bench top, time-resolved fluorometer allowing measurement of a dynamic range of up to five orders of magnitude with a high precision. Single or dual labeled samples, i.e. europium and samarium, can be measured with a high sensitivity (e.g. 10^{17} moles Eu^{3+} per well). With dual labels, automatic spillover correction is performed. In addition, a third lanthanide, terbium, can be measured as a single label.

5.3 Automation

For automation of the DELFIA assays various options are available. Both the EnVision multilabel counter and the ultra high throughput ViewLux microplate imager are detectors designed for easy integration into robotic systems such as the JANUS®, SciClone, and Zephyr® automated workstations.

Please visit our website to learn about the DELFIA method: www.revvity.com



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