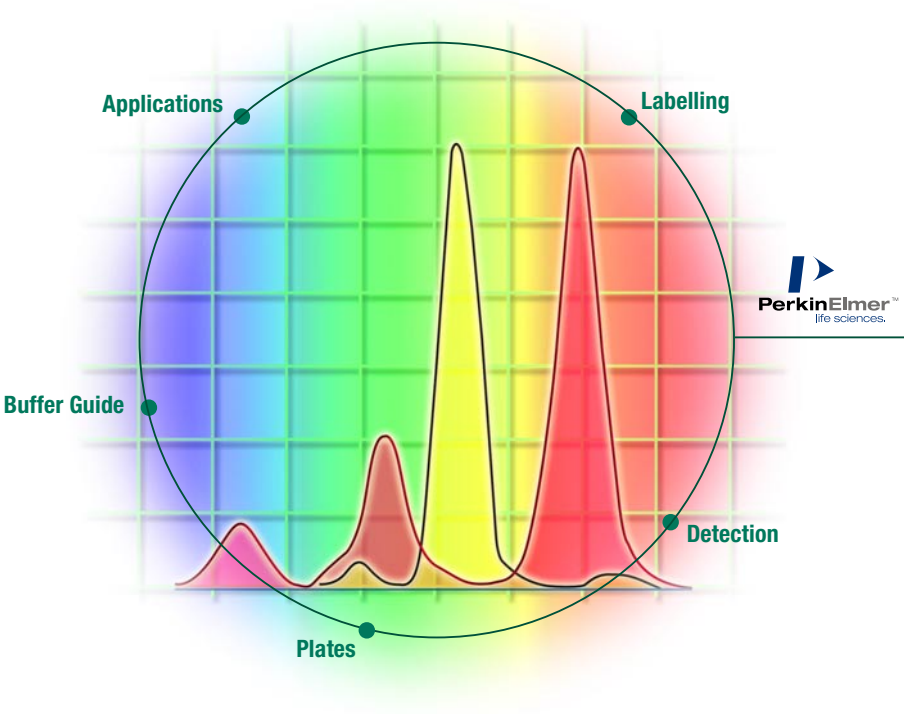
Life Sciences

Drug Discovery | Research | Clinical Screening

Applications of time-resolved fluorometry with the DELFIA® method



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Foreword

Time-resolved fluorometry (TRF), as applied in the DELFIA® system from PerkinElmer Life Sciences, is well-established in high throughput screening (HTS) 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 plate 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.

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

This booklet provides an introduction to the DELFIA method, and to the labelling procedures involved.

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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, terbium, and dysprosium. 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 labelling 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 labelling. Terbium, Tb^{3+} and dysprosium, Dy^{3+} enable the unique concept of triple and quadruple labelling.

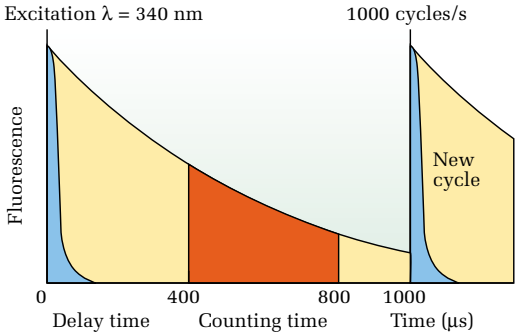
In short, the special fluorescence properties of lanthanides are:

- 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 time-resolved fluorometer or multilabel reader, the sample is pulsed 1000 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)



1 (b)

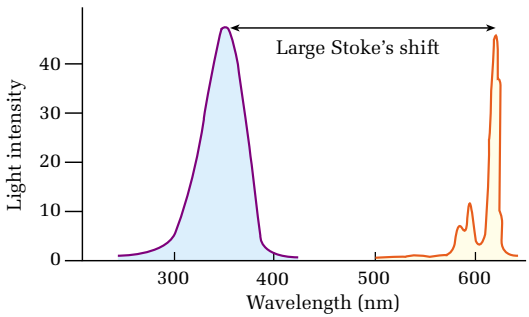


Figure 1. Unique fluorescence properties of lanthanides, (a) long fluorescence decay times, (b) large Stokes' shift.

1.2 DISSOCIATION-ENHANCEMENT

In most DELFIA assays, the labelled 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 (see Table 1). The low pH of these formulations efficiently dissociates the europium from the labelled 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 (See 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.

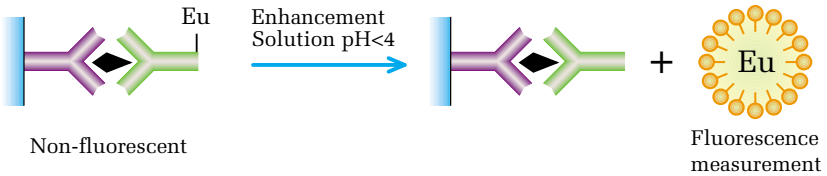


Figure 2. Dissociation enhancement.

Table 1. Suitability of Enhancement Solution and DELFIA Inducer for use with various europium chelate types. Times required to reach 98% of maximum signal.

	DELFIA Enhancement Solution		**DELFIA Inducer**	
Chelate	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

Multianalyte assays may involve the use of dysprosium or terbium chelates as secondary, tertiary or quaternary 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. PerkinElmer Life Sciences' LANCE™ homogeneous assays are widely used for primary screening in drug discovery laboratories.

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2. Applications

2.1 GENERAL

The DELFIA method is versatile in its application. Eu-, Sm- and Tb-labelling reagents are used for protein, peptide and oligonucleotide labelling and the resulting labelled 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 labelled reagent is quantified with a instrument capable of detecting time-resolved fluorescence, such as PerkinElmer's EnVision™ multilabel reader (see section 5 on page 22 for a review of suitable detection instruments).

Examples of application areas are shown in Table 2. Copies of the application notes mentioned are available from your PerkinElmer representative or may be downloaded from the PerkinElmer website. (http://lifesciences.perkinelmer.com/library/literature.asp)

Table 2. Some application areas for DELFIA assays.

Application	For more information
General	➤ Advice for setting up robust DELFIA binding assays (1234-979) ➤ Multiplexing DELFIA assays using lanthani de-labelled probes (1234-9847) ➤ DELFIA cell adhesion assays (1234-969) ➤ Brochure DELFIA and LANCE reagents (1234-990) ➤ DELFIA buffers guide (P10978)
Kinase assays	➤ DELFIA protein kinase assays (1234-968) ➤ Sensitive DELFIA Abl tyrosine kinase assay using poly(Glu, Ala, Tyr) substrate (1234-9844)
Ligand receptor assays	➤ The AcroWell plate: low fluorescence background using the DELFIA system (1420-1000)
Cytotoxicity assays	➤ A new simplified, gentle cell-labelling method for non-radioactive cytotoxicity assays (1234-967)
Hybridization assays	➤ Eu-labelled oligonucleotides are stable and sensitive as probes and primers (1234-965)
Immunoassays	➤ How to optimize rapid and simple immunoassays (1234-976) ➤ DELFIA assays bring convenience in monoclonal antibody development (1234-966)
Functional assays	➤ A simple, more HTS-friendly DELFIA time-resolved fluorescence assay for cAMP determination (1234-9859) ➤ Time-resolved fluorescence-based GTP binding assay for G-Protein coupled receptors (1234-9858)

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-labelled anti-phosphotyrosine, -threonine and threonine-proline 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 assay, for example (see 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-labelled antibody against phosphorylated tyrosine is added. This binds to the phosphorylated substrate. Detection is performed in the usual way after the enhancement process.

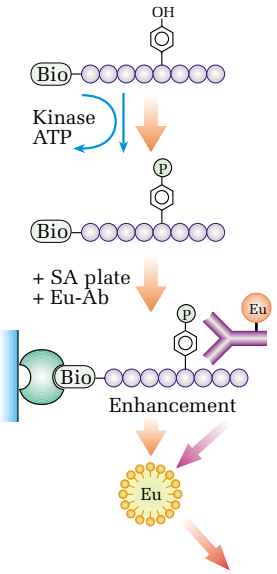


Figure 3. Principle of the DELFIA tyrosine kinase assay.

2.3 LIGAND RECEPTOR ASSAYS

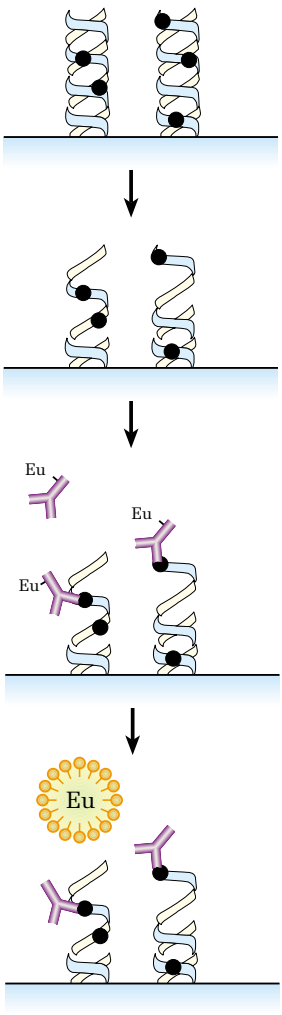
The DELFIA chemistry has been applied with success using various assay formats in the detection of receptor ligand interactions. In galanin assay, for example, a non-filtration method involves the incubation on streptavidin coated plates of europium-labelled galanin, the galanin receptor, and biotinylated 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, or it may involve filter plates. This provides 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-labelled 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-labelled 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.



Cell culture (1-5- days, +37 °C)

BrdU labelling (2-24 h, +37 °C)

Remove labelling medium (suspension cells have to be centrifuged)

Cell fixation and denaturation (30 min. RT)

Remove Fix Solution

Anti-BrdU-Eu incubation (30 - 120 min. RT)

Wash 4 times
Add DELFIA inducer and incubate 15 min.

Measure TF-fluorescence

Figure 4. DELFIA cell proliferation assay.

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 hydrolysed 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 labelled cells
- Fast release of label
- Fast measurement
- Stable fluorescent signal (at least 5 h)

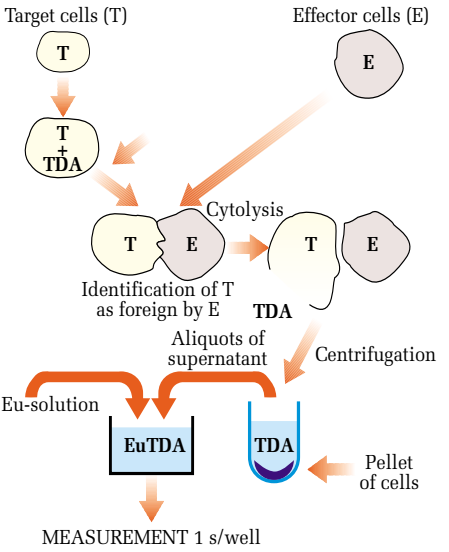


Figure 5. Principle of the DELFIA cytotoxicity assay

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.

Labelling 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-labelled 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 labelled 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 labelled antibodies per well is enough for non-competitive 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.

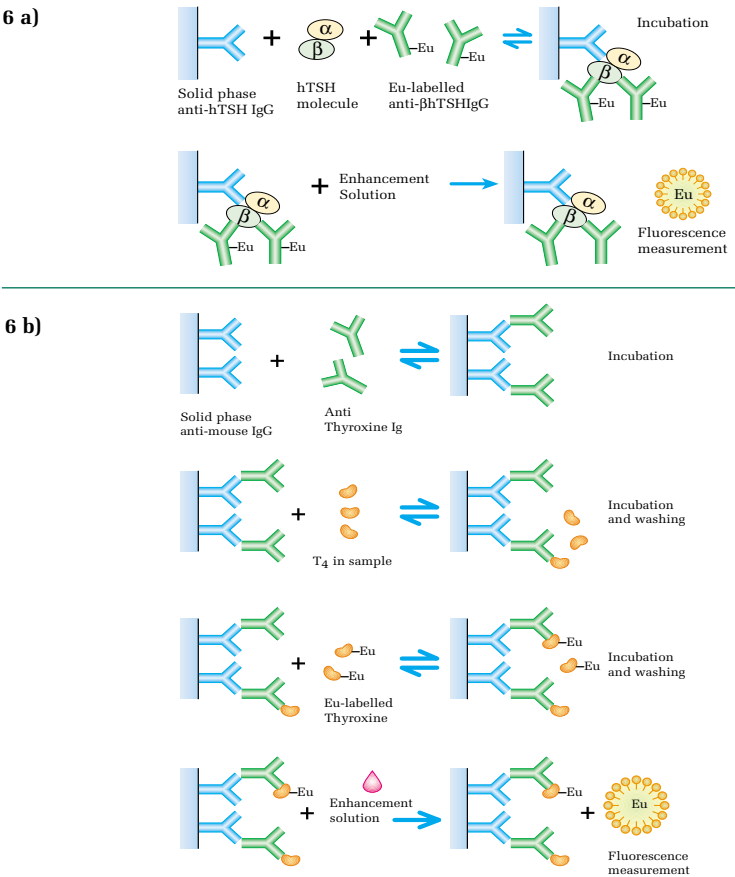


Figure 6. Examples of immunoassays types, (a) non-competitive "sandwich-type", (b) competitive.

2.9 MULTIANALYTE ASSAYS

Simultaneous quantification of two or more analytes in one sample can save time and reduce the volume of samples required. Although radioactive isotopes may be used for dual label assays, no other strong alternative exists apart from TRF. Conventional fluorescent labels have high quantum yields but they are not suitable for multianalyte assays because of the background problem and the difficulty in distinguishing between their emission bands.

Lanthanides, however, are suitable because of their narrow emission peaks at different wavelengths (613 nm for Eu^{3+}, 643 nm for Sm^{3+}, 545 nm for Tb^{3+} and 572 nm for Dy^{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.

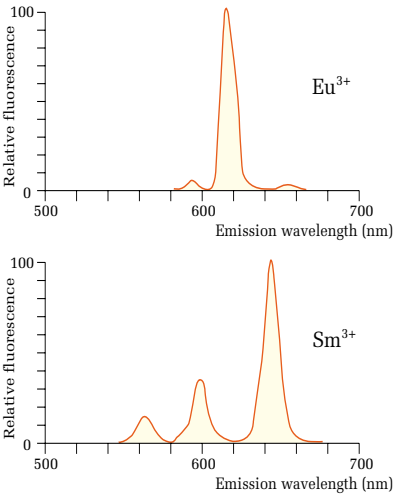


Figure 7. Emission wavelength (nm) spectra for europium and samarium.

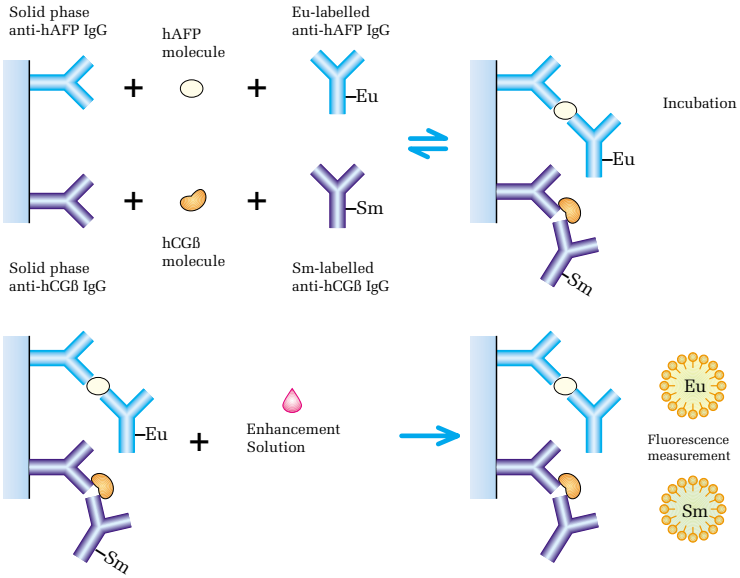


Figure 8. Example of a dual label "sandwich-type" immunoassay employing europium and samarium chelate labels.

Sm-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).

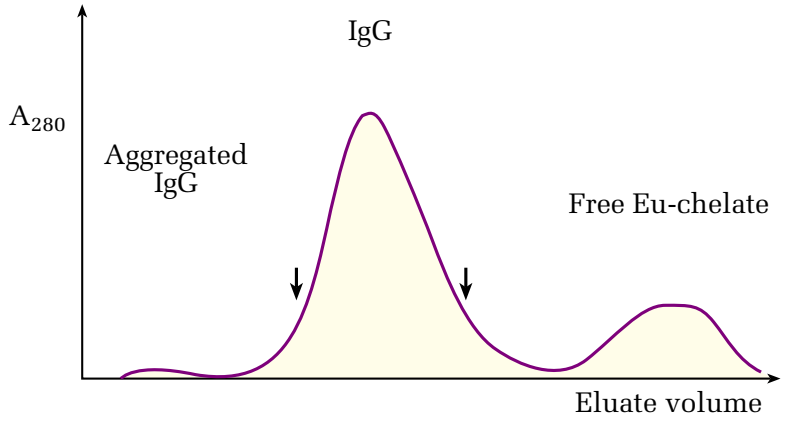

Figure 9. The elution profile of labelled IgG from a column of Sephadex G-50 and Sepharose 6B. It is recommended that the fractions between the arrows (monomeric IgG) be pooled.

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.

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 diethylenetriamine-pentaacetic 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 labelled with N1-chelate.

An example of an assay buffer composition for a DELFIA assay could be 50mM 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 20µM 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, and

DELFIA GTP 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.

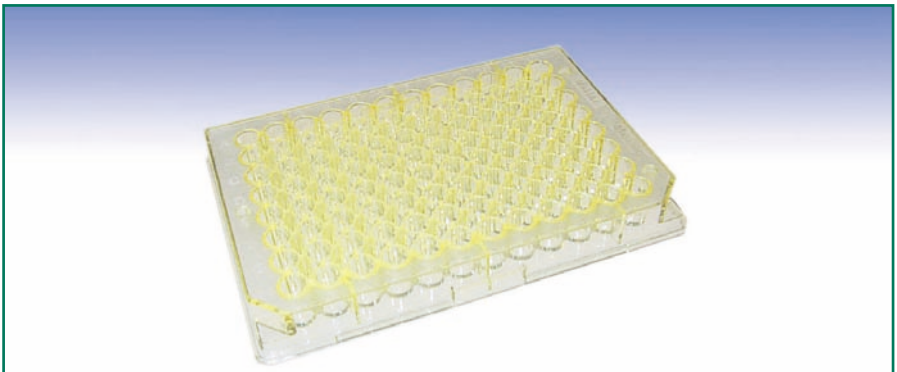

Figure 10. The DELFIA yellow plate is characterized by exceptionally low fluorescence background.

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.

PerkinElmer 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-GST antibody coated plates

– Used to bind fusion proteins containing glutathione-S-transferase from *Schistosoma japonicum* expressed from the pGEX vector in *E. coli*. The antibody is raised in goat.

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 readers that have a TRF option, or with dedicated instruments.

5.1 TODAY'S VERSATILE INSTRUMENTS

PerkinElmer's top-of-the-range EnVision™ multilabel counter meets all of today's needs for efficient processing of single or multilabel DELFIA assays. Fast and application-oriented, the instrument can also measure any other non-radioactive label in vessels ranging from Petri dishes to 384 well plates. Various models of the instrument are available, and it may be supplied with or without a stacker. As alternatives the Fusion and VICTOR^2 instruments, also available in various versions, are highly suitable.

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 PerkinElmer, 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 labelled 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. The VICTOR multilabel counter is able to measure all four lanthanides in standard clear and opaque microtitration plates.

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 MiniTrak and PlateTrak platforms.

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