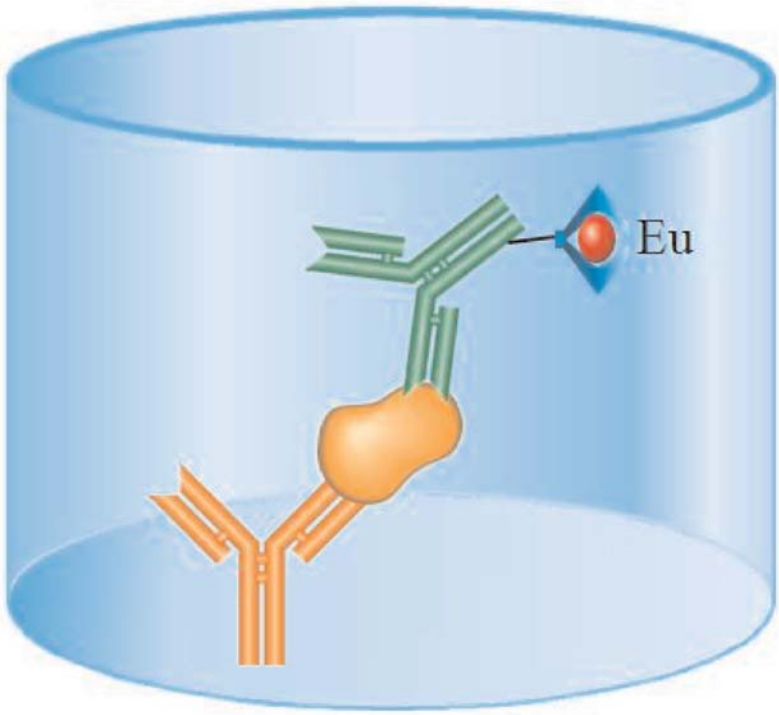
DELFIA immunoassays

Guide to Converting ELISA Assays to DELFIA



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Contents

1. Enhanced performance for your applications with DELFIA

The first part of this guide presents a simple conversion of an existing TNF-α (Tumor Necrosis Factor-α) ELISA (Enzyme-Linked Immunosorbent Assay) to DELFIA (Dissociation Enhanced Lanthanide Fluoroimmunoassay). The following sections describe how to set up and further optimize DELFIA assays.

DELFIA

Time-resolved fluorometry (TRF) is a well-established technique in drug discovery as well as in other research areas. Providing high sensitivity and wide dynamic range, the method is characterized by lack of sample interference during measurement and is easy to automate. The TRF-based DELFIA system from PerkinElmer is an optimal tool for bio-affinity assays.

DELFIA features and benefits:

- Well-proven technology – published data in many application areas.
- Very high sensitivity – minimizes reagent use and very sensitive to smaller analyte quantities.
- Wide dynamic range – no need for additional dilutions.
- Multi-label assay capability – enables cost-effect assays with high information value.
- High signal stability over 24 hours – improves reliability and ease of use.

The experience of PerkinElmer in clinical diagnostics provides a solid background for DELFIA research reagents and proves the versatility of this technology. There are over 1,000 published reference articles describing immunoassays alone. Some example articles in Table 1 from many different assay fields highlight the advantages of DELFIA over competing methods.

Table 1. Examples of scientific references highlighting the advantages of DELFIA over traditional techniques.

Analyte	Article title	Extract from Article abstract	Reference
Cytokines	A new highly sensitive immunoassay for cytokines by dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA).	Compared to ELISA, *the sensitivity and range of measurement were significantly increased* in DELFIA TNF alpha and IL-6.	Ogata A , J Immunol Methods, , 1992 Apr 8;148(1-2):15-22.
	A time-resolved fluorescence immunoassay (DELFIA) increases the sensitivity of antigen-driven cytokine detection.	The DELFIA *enhanced the sensitivity* of a mouse IL-2 assay 8- to 27-fold, and a human GM-CSF assay 10-fold, as compared to colorimetric ELISA.	Allicotti G, J Immunoassay Immunochem. 2003;24(4):345-58.
Hormone and protein detection	Validation of an ultrasensitive and specific immunofluorometric assay for mouse follicle-stimulating hormone.	The mFSH IFMA *lowered the detection limit 34-fold* (5 vs. 170 pg/sample) compared with standard mFSH RIA. The IFMA has a wide analytical range, with a *good precision profile*. The greatly *enhanced sensitivity, specificity*, and *technical convenience* will allow wider application of FSH measurements to very small blood samples in immature and mature mice as well as transgenic models	Jimenez M, Biol Reprod. 2005 Jan;72(1):78-85. Epub 2004 Sep 1.
	Development of a sensitive and specific new plasma 4-androstene-3.17- dione time-resolved fluoroimmunoassay (TR-FIA).	The main advantages of TR-FIA assay were its *greater sensitivity* compared to radioimmunoassay and its *higher precision*.	Fiet J, Steroids. 2001 Aug;66(8):609-14.
	A new specific and sensitive time resolved-fluoroimmunoassay of 11-deoxycortisol in serum.	*More sensitive than RIA*, thus well-suited to accurate measurement in endocrinological studies. Moreover, this non-isotopic assay is *cheaper than RIA*.	Fiet J, J Steroid Biochem Mol Biol. 2001 May;77 (2-3):143-50.
	A time-resolved fluorescence immunoassay for insulin in rodent plasma.	The TR-FIA method had higher sensitivity and *required only one-tenth as much sample as in ELISA*.	Daijo JE, J Pharm Biomed Anal. 1999 Mar;19(3-4):335-42.
	A sensitive time-resolved fluorescent immunoassay for metallothionein protein.	The method *allows measurement of* low MT levels that are *undetectable using current RIA and ELISA protocols*.	Butcher H, J Immunol Methods. 2003 Jan 15;272(1-2):247-56.
Biological warfare agents	Rapid and sensitive detection of biological warfare agents using time-resolved fluorescence assays.	DELFIA TRF assays are significantly *better in terms of sensitivity, linear range, and run time* than standard capture ELISAs and should facilitate early detection of potential biological warfare agents in clinical and environmental samples.	Peruski AH, J Immunol Methods. 2002 May 1;263(1-2):35-41.

2. Converting ELISA assays to DELFIA

As shown in Table 2, the steps in a DELFIA assay are very similar to those in an ELISA assay. But the DELFIA format offers excellent sensitivity over a wider dynamic range (1 – 10,000 pg/ml), eliminating the need to use multiple ELISA kits to span a wide concentration range. This section demonstrates a straightforward conversion of an ELISA TNF-α assay to the DELFIA format. Conversion of other assays can be adapted from this example by referring to Table 3, which provides a specific recipe for the TNF-α assay.

Table 2. Principles of ELISA and DELFIA TNF-α immunoassays

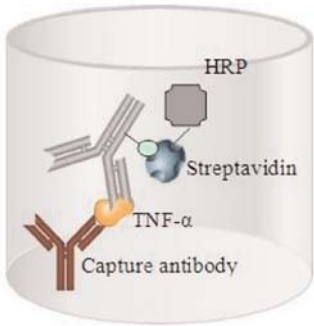
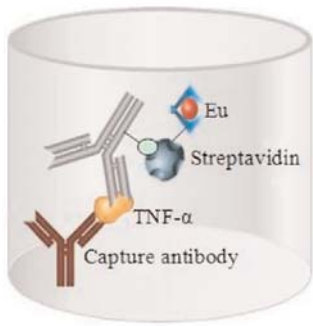
	ELISA	DELFIA
Assay schematic		
Assays	An hTNF-α ELISA assay was performed using a commercial kit (Biosource, part number KHC3012) as directed in the kit insert.	The hTNF-α DELFIA assay used most of the same components, though substituting Eu-SA and some optimized DELFIA buffers (see section 2.1) for SA-HRP.
Principles of assays	Both assays used a monoclonal antibody specific for human tumor necrosis factor alpha (hTNF-α) coated in microplate wells. Samples, including calibration standards of known concentration of hTNF-α, are added to these wells, followed by a biotinylated monoclonal antibody to bind to the hTNF-α antigen. A four-member sandwich forms upon the addition of the detection molecule.	
Detection molecule	Streptavidin-Peroxidase (SA-HRP)	Europium – Streptavidin (Eu-SA)
Signal development	During the substrate solution incubation, a yellow color forms in the presence of HRP. The enzymatic reaction must be stopped after 30 min.	In the Enhancement Solution, non-fluorescent DELFIA chelates are converted to stable fluorescent chelates, developing a signal in 5 minutes that is stable up to 8 hours.
Measurement	Absorbance was measured with a VICTOR™ at 450 nm using a factory-set absorbance protocol.	Time-resolved fluorescence was measured with a VICTOR using the factory-set DELFIA Europium protocol (excitation at 340 and emission at 615 nm).

Table 3. Comparison of ELISA and DELFIA assay steps.

	Step	ELISA	DELFIA
1	Add standards and samples onto coated microplate	50 µl incubation buffer and 100 µl calibration standards and samples	
2	Incubate	2 hours (slow shaking) at RT	
3	Wash	4 wash cycles	
4	Add a biotinylated component	100 µl of antigen specific biotinylated antibody	
5	Incubate	1 hour (slow shaking) at RT	
6	Wash	4 wash cycles	
7	Add a tracer	Add 100 µl Streptavidin-Horse Radish Peroxidase (SA-HRP)	Add 100 µl Europium-Streptavidin (Eu-SA) (100 ng/ml) in assay buffer
8	Incubate	30 minutes (slow shaking) at RT	
9	Wash	6 wash cycles	
10	Add a detection solution	100 µl of substrate	200 µl Enhancement Solution
11	Incubate	30 min at RT in the dark	5 min slow shake at RT
12	Stop the enzyme reaction	100 µl Stop solution	–
13	Measure	Absorbance at 450 nm (within two hours)	TR-fluorescence at 615 nm

2.1 Materials and methods

Materials

- ELISA TNF-α kit (Biosource, cat. no. KHC3012)
- DELFIA Eu (europium) - labeled streptavidin (Eu-streptavidin) (PerkinElmer cat. no. 1244-360)
- DELFIA Assay buffer (PerkinElmer cat. no. 1244-111)
- DELFIA Enhancement Solution (PerkinElmer cat. no. 1244-104)
- DELFIA Wash solution (PerkinElmer cat. no. 1244-114)
- TNF-α antigen (PeproTech EC, Cat. #300-01A)
- DELFIA-compatible microplate (See PerkinElmer's Microplate overview guide 007097_01)

Instruments

- Time-resolved fluorometer e.g. PerkinElmer 1420 VICTOR or 2102 EnVision™
- Automatic shaker e.g. PerkinElmer DELFIA Plateshake (1296-001/002 or 1296-003/004).
- Automatic washer e.g. PerkinElmer DELFIA Platewash (1296-026).

Methods

- The two assays were done in parallel as presented in Table 3.
- DELFIA Wash Solution and DELFIA Assay Buffer were used instead of equivalent ones in ELISA.
- All the ELISA component dilutions (except the TNF-α calibration standard) were done according to the kit instructions.
- The ELISA kit recommends a range of calibration standards from 15.6 pg/ml to 1000 pg/ml. However, to obtain a wider assay range, the first dilution (2000 pg/ml) of TNF-α was spiked with 191 µl of TNF-α antigen diluted to 0.1 µl/ml with 1% TSA-BSA, raising the concentration to 20,000 pg/ml. This was diluted 1:2 providing a final calibration point of 10,000 pg/ml. Other calibration standards diluted with the buffer provided with the ELISA kit were 4641, 1000, 500, 125, 62.5, 31.2, 15.6, and 0 pg/ml.
- Eu-SA was diluted to 100 ng/ml with DELFIA Assay buffer.
- DELFIA Wash Concentrate (25x) was diluted 25-fold by adding distilled water to give a buffered wash solution (pH 7.8).

2.2 Results and discussion

Using two replicates, background values from the blank calibration standard were subtracted from the average counts (DELFIA) and absorbance values (ELISA). These corrected values are plotted in Figure 1 against standard concentration on a log-log scale with a best line fit provided by GraphPad Prism (GraphPad Software, Inc.)

As shown, the TNF-α ELISA assay was successfully converted to an equivalent DELFIA assay; in fact, even without parameter optimization, the DELFIA assay displays substantially wider dynamic range. The DELFIA assay accommodated very high TNF-α concentrations without additional dilutions while the ELISA signal saturated after 1000 pg/ml. At the other end, the sensitivity of the TNF-α DELFIA assay (15 pg/ml) was at least equivalent to the sensitivity obtained with the commercial ELISA kit. With the optimization steps taught in the following sections, the sensitivity of the DELFIA assay was improved to 0.4 pg/mL and dynamic range extended to span 1 – 100,000 pg/mL. This enhanced sensitivity could be used to detect smaller concentrations of analyte or to minimize reaction volumes to reduce reagent costs.

The DELFIA signal develops quickly and is stable over long periods of time, providing flexibility in work flow planning. In contrast, in ELISA assays, the color-producing reaction requires the addition of stop solution followed by measurement within 30 min. Enzyme-based assays are also sensitive to sample contamination since both horse radish peroxidase (HRP) and alkaline phosphatase (AP) enzymes are commonly found in biological samples. Some improvements in sensitivity and dynamic range over chromogenic ELISA assays are provided by the chemiluminescent immunoassay (CLIA). However CLIA assays also suffer from poor signal stability arising from their enzymatic approach. In contrast to ELISA, DELFIA technology also enables multiplexing assays using four different chelates (see 3.6.1). Table 4 succinctly summarizes the three approaches.

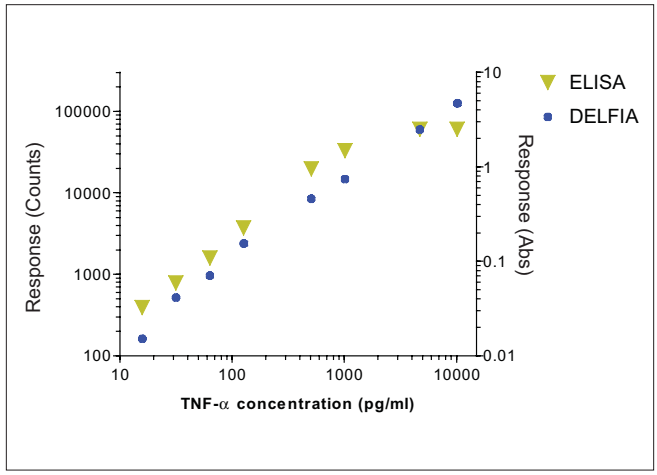


Figure 1. Comparison of TNF-α DELFIA (left y-axis) and ELISA (right y-axis) standard curves.

Table 4. A comparison of DELFIA to colorimetric and chemiluminescent ELISA assays.

Aspect	DELFIA	ELISA Colorimetric	ELISA Chemiluminescence
Dynamic range	Over 4 logs	< 3 logs	< 4 logs
Sensitivity	Very good	good	Very good
Signal stability	8 h	0.5 h	0.5 h
Temperature	Dependent	Very dependent	Dependent
Assay time	< 2.5 h	> 3.5 h	> 3 h
Sample consumption	Very small	High	Small
Multiplexing	Yes	No	No

3. Optimizing DELFIA assays

This chapter provides both additional theory and practical examples of DELFIA assays that demonstrate the better sensitivity, reproducibility and stability of the assay. The TNF-α immunoassay is used as a specific and typical example to show how different assay designs (see Figure 2) influence the outcome of the assay. As in all immunoassays, several parameters for each design need to be optimized.

3.1 Microplates

Minimizing the background fluorescence of the microplate containing the assay through careful selection of the plastic material is critical to achieving high sensitivity and good precision. PerkinElmer provides microplates designed and tested for DELFIA assays (See *PerkinElmer Microplates: A Complete Overview*, 007097_01) in both solid and strip formats. The DELFIA technology is extremely flexible and suited to both 96-well and 384-well coated and uncoated plate formats as well as filter assays. The DELFIA TNF-α assays described here were carried out in 96-well format, but the 384-well plate would work as well. When using antibodies with limited availability, DELFIA Streptavidin-coated 384-well plates (CC11-H10) may be the best format as they require less antibody than do directly coated plates. They are based on our proven low fluorescence background plates and all coating procedures are tested and optimized to give high sensitivity and good precision in DELFIA assays.

The converted DELFIA assay was performed with microplate strips provided with ELISA kit, causing a high assay background signal (1300-1700 counts) and compromising the sensitivity of the assay. The background of the plate can easily be measured with a plain plate containing no coating, buffer, or Enhancement Solution. This should give less than 500 and preferably less than 200 counts with VICTOR and EnVision (100 flashes).

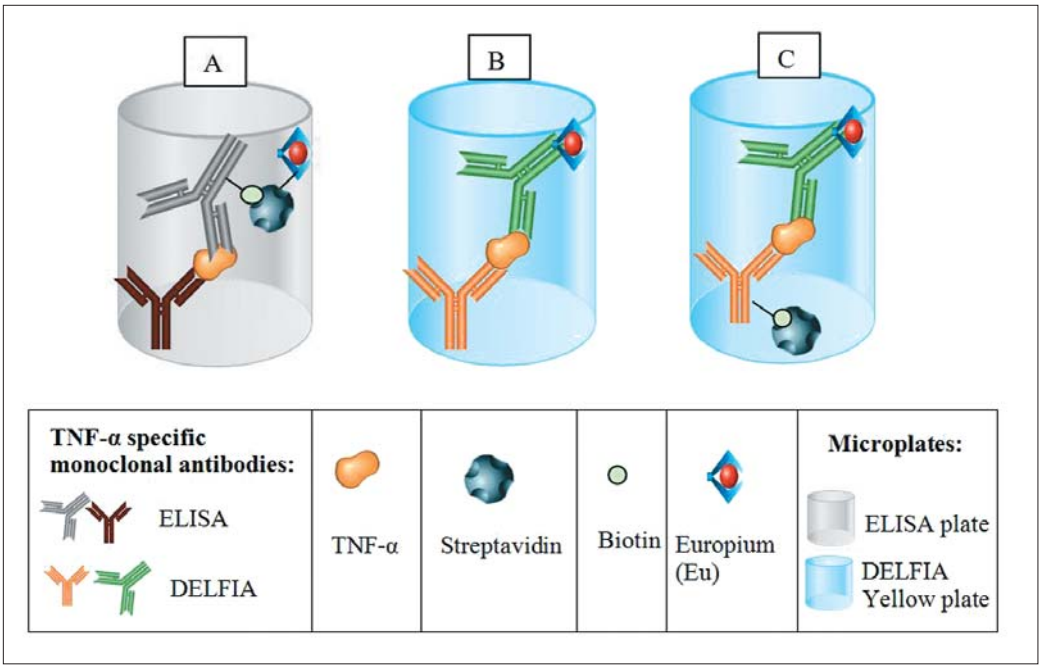


Figure 2. Different DELFIA TNF-α immunoassay designs. The first schematic on the left (A) shows the assay performed in chapter 2.

3.2 Capture antibody

3.2.1 Immobilizing the capture antibody

Capture antibodies can be immobilized to a solid surface directly or indirectly. In direct immobilization, the monoclonal antibody specific for an antigen is bound onto a solid surface through non-covalent bonds (see Figure 2, A and B). Indirect immobilization uses a secondary antibody coating or Streptavidin-coated plates with biotinylated biomolecules; e.g. peptides, proteins, antibodies and haptens (see Figure 2, C). Indirect coating reduces the cost of the immobilized antibody and thus many DELFIA assays are based on Streptavidin-coated plates. This approach was optimized below to enhance the sensitivity of the TNF-α assay.

Table 5. Protocols and components used to evaluate different capture and detection antibody pairs for optimization of the DELFIA TNF-α assay. All part numbers are PerkinElmer unless otherwise noted.

Plates	Indirect coatings: DELFIA streptavidin coated yellow strip plates (AAAND-0005) Direct antibody coatings: uncoated DELFIA yellow plate (AAAND-0001)
Biotinylation of capture antibodies	Required TNF-α antibodies (used with streptavidin coated plates) were labeled with Biotin-NHS. The labeling protocol: Label: Biotin-NHS Label excess: 15x pH: 9.3 Buffer: 0.1 mol/L sodium carbonate Incubation time: 2 h Incubation temperature: on ice MAb concentration during labeling: 1.5 mg/ml Purification: NAP-5 and NAP-10 gel filtration
Tracer	Antibodies specific for TNF-α were directly labeled with Eu-DTPA-ITC (AD0021). Labeling or label concentration in the assay was not optimized. The labeling was performed using the following labeling protocol aiming to a labeling degree of 7-8 Eu/IgG. Label: Eu-DTPA-ITC (AD0021) Chelate excess: 250x pH: 9.3 Buffer: 0.1 mol/L sodium carbonate Incubation time: overnight Incubation temperature: +4°C MAb concentration: 1.2 mg/mL Purification: Superdex 200 HR10/30 LPLC gel filtration
Calibration standards	The TNF-α calibration standard was diluted in human male serum and the reaction buffer (hTSH Ultra Assay Buffer).
Direct coating protocol	1. Treat the wells with 1 µg/100µl ml of antibody preparation in 0.2 M NaH2PO4 buffer 2. Incubate 20 h at room temperature 3. Incubate 5 min RT with saline solution (16 µl HCl/10 ml H2O), Wash three times. 4. Saturate wells* with saturation buffer and incubate overnight (50 mM NaH2PO4, 6% trehalose, 0.1% BSA-TSA, 0.1% Germal II). Aspirate the saturation solution and store strips humid at 4 °C until used. * *It is critical to avoid heavy metal contamination in the reagents, particularly, bovine serum albumin (BSA) or gelatine used for saturation of the plates. Use only purified BSA (e.g. CR84-100).*
The assay protocols	For streptavidin-coated plates: 1. add 50 µl of serum, plasma, cells or supernatant including TNF-α 2. add 50 µl biotinylated TNF-alpha antibody (200 ng/well) + Eu-labeled TNF-α antibody (100 ng/well) 3. incubate 2 h at RT with slow shaking 4. wash 4 x with DELFIA wash solution with DELFIA plate wash 5. add 100 µl DELFIA Inducer 6. incubate 15 min at RT slow shaking 7. measure with VICTOR For direct-coated plates: 1. add 50 µl of serum, plasma, cells or supernatant including TNF-α 2. add 50 µl Eu-labeled TNF-α antibody (100 ng/well) 3. incubate 2 h at RT slow shaking 4. wash 4 x with DELFIA wash solution 5. add 100 µl DELFIA Inducer 6. incubate 15 min at RT slow shaking 7. measure with VICTOR

3.2.2 Selecting the capture antibody

Because the choice of the capture and detection antibody pair greatly influences ultimate assay performance, the suitability of different monoclonal antibody pairs for DELFIA TNF-α were tested. All antibody pairs evaluated proved functional. Capture antibodies were immobilized in microplates both directly and indirectly. Though the composition of the coating solution affects antibody immobilization and should be optimized (pH, saturation protein, etc.), a general coating protocol for DELFIA, shown in Table 5 along with the full assay protocol, was used without optimization.

As shown in Table 6, the sensitivity of the TNF-α DELFIA is typically better than 1 pg/ml and can be further improved by careful optimization of conditions (label concentration, for example). Typically, the total signal and sensitivity is better using plates directly coated with antibody rather than with streptavidin. However, antibody consumption is significantly higher in direct coating (e.g. 1 µg of an antibody / well) than in indirect coating (e.g. 200 ng of biotinylated antibody / well) and coating plates with secondary antibody or streptavidin may often be the only viable option due to cost or limited availability of a specific antibody.

Table 6. Signal and sensitivity* of TNF-α assays with different antibody pairs and coating protocols.

Antibody pair		Counts (calibration standard 1000)		Sensitivity (pg/ml)	
Coated	Labeled	Direct coating	SA-coating	Direct coating*	SA-coating*
AHC3912 (Biosource)	610 (R & D)	61 844	40 173	0.5	0.4
610 (R & D)	AHC3912 (Biosource)	75 487	52 337	0.4	1.0
M303E (Pierce)	F6C5 (HyTest)	85 427	37 844	0.5	1.4
F6C5 (HyTest)	CH8810 (Anogen)	58 045	37 629	1.6	2.4

* The sensitivity was calculated using the following equation:
Detection limit cut-off (counts) = Assay background + 2*standard deviation (background)

Table 7. Measurement data obtained with the antibody pair AHC3912 + 610 (direct coating).

TNF-α pg/ml	Mean Counts	CV%	Specific signal*	S/B**
0	551	2.6		
3	732	1.5	181	1.3
10	1158	5.1	607	2.1
30	2375	0.0	1824	4.3
50	3604	1.7	3053	6.5
100	6788	0.7	6236	12.3
500	31079	6.1	30527	56.4
1000	61844	4.5	61293	112.2
10000	592997	1.8	592445	1075.9

* Specific signal is calculated by subtracting background (0) counts from results
** Signal to background (S/B) is obtained by dividing the counts by the 0-calibration standard counts

3.2.3 Optimizing the amount of capture antibody

After selecting the plate, coating method, and antibody pair, the concentrations for the reaction components need to be adjusted. In the example shown in Figure 3 and Table 8, the effect of different Bio-Mab (Ab5) concentrations was tested with the protocol described in Table 5 using 100 ng per well of Eu-labeled Mab (F6C5). The calibration standards were diluted in human male serum.

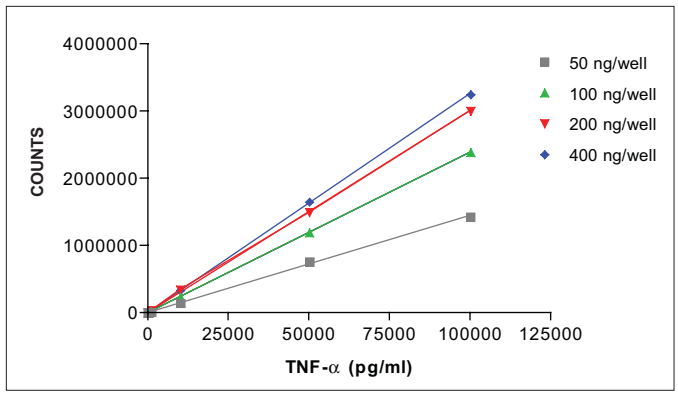


Figure 3. Calibration curves of the DELFIA TNF-α assay for different concentrations of biotinylated TNF-α antibody.

Table 8. Calibration curves of the DELFIA TNF-α assay for different concentrations of biotinylated TNF-α antibody.

TNF-α pg/ml	Concentration of Biotinylated antibody							
	50 ng/well		100 ng/well		200 ng/well		400 ng/well	
	COUNTS	S/B	COUNTS	S/B	COUNTS	S/B	COUNTS	S/B
0	262		318		268		298	
1	296	1.1	301	0.9	384	1.4	350	1.2
10	399	1.5	495	1.6	649	2.4	674	2.3
100	1551	6	2594	8	3723	14	3975	13
1 000	12149	46	23332	73	33809	126	35615	120
10 000	145969	557	238464	750	344871	1289	335370	1127
50 000	760047	2901	1194040	3758	1495854	5592	1654447	5561
100 000	1431434	5463	2390947	7525	3007722	11244	3252618	10933
Sensitivity(pg/ml)	2.5		9.2		0.5		0.6	

All of the Bio-Mab concentrations tested worked well and the calibration standard curves were linear up to 100,000 pg/ml. The dynamic range of the DELFIA TNF-α assay proved to be 100 times better than that of the commercial ELISA assay (up to 1000 pg/ml). The background of the assays (with no TNF-α) was approximately 300 counts, independent of the Bio-Mab concentration. The Bio-Mab concentration had a significant contribution to S/B, especially when measuring higher calibration standards in the range of 100 – 100,000 pg/ml. The S/B-values were much lower with 50 ng/well of Bio-Mab than with 200 or 400 ng/well and yet 50 ng/well was sufficient to provide assay linearity up to 100,000 pg/ml.

3.3 Tracer

In the simple TNF-α conversion from ELISA to DELFIA presented at the beginning of this Note, Eu-streptavidin (1244-360) was used as a detection molecule because it provides a fast and straightforward way to benefit from the advantages of DELFIA. Eu-streptavidin is intended for use as a generic reagent to detect biotinylated compounds, e.g. antibodies or antigens.

More often immunoassays are performed with directly labeled biomolecules. The direct labeling of the detection antibody with a lanthanide chelate can be performed exploiting PerkinElmer's labeling service or by labeling the molecules in-

house using PerkinElmer's DELFIA labeling kits (e.g. DELFIA Eu-labeling kit 1244-302). For more information on this topic, see brochure 1244-1126, *Applications of time-resolved fluorometry with DELFIA methods*. The labeling procedure is extremely simple providing high labeling yields and stable compounds. Labeling with lanthanides typically has minimal effect on biological activity.

In this work, ITC-activated Eu-DTPA chelate (AD0021) was used for labeling of TNF-α specific antibodies using the protocol presented in Table 5. The DTPA-chelate was chosen in order to provide an assay capable of analyzing EDTA-plasma samples. A labeling kit with N1-chelate is available and is recommended when there is no need to analyze EDTA-plasma samples. Labeling with Eu-N1-ITC chelate is performed exactly as described in Table 5 for Eu-DTPA-ITC chelate.

Ideally, the amount of labeled antibody used should be optimized when developing a DELFIA assay. As a general rule, 25-100 ng/well of labeled antibody is enough for a non-competitive sandwich assay, but the optimal level varies with the purity and affinity of the antibody and the signal level desired. In the TNF-α DELFIA assay presented here, the concentration of the labeled antibody was fixed at 100 ng/well, providing excellent dynamic range and sensitivity without further optimization.

3.4 Optimizing assay conditions

The effects on reaction rate of temperature, component concentration, dynamics, and quality of immunoreagents have been investigated in Application Note 1234-976 *How to optimize rapid and simple Immunoassays*. The optimization of other assay conditions are presented here.

3.4.1 Suitable assay buffer

Ready-made DELFIA buffers (1244-106, 1244-111 and 4002-0010) designed to minimize non-specific background are a good starting point for the development of a new DELFIA assay. For more information see DELFIA Buffers Guide (P10978).

When commercial DELFIA assay buffers are not suitable, Tris-HCl-based buffers at neutral or slightly alkaline pH are recommended. Buffers containing chelating agents, e.g. HEPES and phosphate can be used with N1-chelates if the incubation time is short. In cell based assays, the background fluorescence can be decreased by increasing the DTPA concentration to 50 µmol/l or by using 100 µmol/l EDTA in the assay buffer.

3.4.2 Washing

Because lanthanide detection is very sensitive, high background and significant sample-to-sample variation can occur if the plate is not washed adequately. Using a well-maintained automatic plate washer (e.g. DELFIA Platewash, 1296-026) and an optimized wash protocol is thus recommended for optimal results. Since the DELFIA chelates are pH sensitive, buffered solutions like Tris-HCl with detergents are suitable for washing, though DELFIA Wash concentrate (1244-114) is optimized specifically for DELFIA assays. Before the addition of Enhancement Solution, six washing cycles are recommended.

3.4.3 Shaking

Shaking improves assay kinetics significantly and is an important contributor to a successful enhancement step in DELFIA. The time needed for efficient enhancement of lanthanide fluorescence should be separately checked for each shaker model. As an example, the DELFIA Plateshake (1296-003/004) is a general-purpose microplate shaker specially optimized for use with DELFIA kits. Shaking speeds ranging from 100 to 1350 rpm can be manually selected and there are two preset shaking speeds optimized to give best results for DELFIA assays. Overly vigorous shaking should be avoided as it may cause air bubbles which interfere with the measurement. Enhancement times with the Plateshake for different chelates are presented in Table 9.

Table 9. Shaking times for the DELFIA Plateshake to reach 98% of maximum signal.

	DELFIA Enhancement Solution		DELFIA Inducer	
Chelate	Shaking (slow) (min)	Without shaking (min)	Shaking (slow) (min)	Without shaking (min)
N1	5	45	5	30
DTPA	30	180	5	30
W2014	45	120	5	30

3.5 Sample matrix

The suitability of the assay for measuring different sample matrices was evaluated testing the assay linearity (a cell culture sample) and recovery (serum, heparin and EDTA samples).

Linearity of the TNF-α assay was evaluated with a stimulated cell supernatant sample which was diluted with a cell culture medium RPMI (Figure 4). The assay was performed using biotinylated 610 on a Streptavidin-coated plate with Eu-labeled AHC3912. The calibration standards (1 – 100,000) were diluted in RPMI medium.

The DELFIA TNF-α assay linearity was very good and the sensitivity was excellent (0.2 pg/ml). Together with the extremely broad dynamic range (1 – 100,000) the DELFIA TNF-α assay is an excellent method for measuring cell culture samples without any additional dilutions.

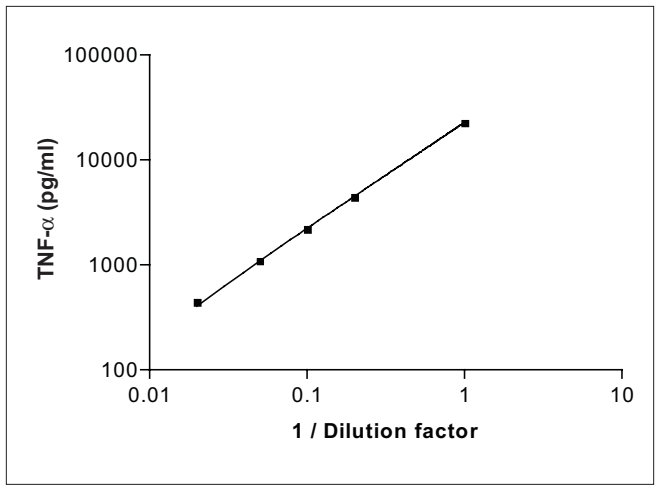


Figure 4. The DELFIA TNF-α assay linearity tested with a cell culture sample.

TNF-α pg/ml	Mean Counts	CV%	Specific signal	S/B
0	363	8.8		
1	634	1.2	270	1.7
10	1053	9.1	690	2.9
100	5811	1.7	5447	16.0
1,000	51253	4.9	50889	141.1
10,000	494540	2.9	494177	1361.1
25,000	1130044	1.0	1129680	3110.2
50,000	2062236	0.1	2061872	5675.9
75,000	2791579	1.8	2791216	7683.2
100,000	3311597	0.4	3311234	9114.5

Table 10. Values for the calibration curve of the linearity test.

The assay recovery was evaluated by adding known concentrations of analyte to the samples. Untreated serum, heparin and EDTA plasma samples were spiked with different concentrations of TNF-α (500, 100, 20 and 10 pg/ml). The assays were run using an 610 -coated plate and AHC3912-Eu. As shown in Table 11, recoveries were excellent in all of the assays.

Table 11. The DELFIA TNF-α assay recovery was tested with untreated serum, heparin and EDTA plasma samples spiked with TNF-α concentration 500, 100, 20 and 10 pg/ml.

Spiked samples	TNF-α (pg/ml)	Recovery %
serum 2 + 500 pg/ml	491.8	97.1
serum 2 + 100 pg/ml	106.9	100.5
serum 2 + 20 pg/ml	26.8	102.0
serum 2 + 10 pg/ml	16.4	100.0
serum 2	6.4	
Heparin plasma 3 + 500 pg/ml	509.3	100.4
Heparin plasma 3 + 100 pg/ml	107.7	100.3
heparin plasma 3 + 20 pg/ml	25.6	91.0
heparin plasma 3 + 10 pg/ml	18.6	112.0
heparin plasma 3	7.4	
EDTA-plasma 3 + 500 pg/ml	553.6	109.0
EDTA-plasma 3 + 100 pg/ml	107.1	98.5
EDTA-plasma 3 + 20 pg/ml	28.8	101.0
EDTA-plasma 3 + 10 pg/ml	18.0	94.0
EDTA-plasma 3	8.6	

3.6 Detection

3.6.1 Enhancement

While no optimization of the enhancement step is needed, close adherence to the recommended protocol (see the Dos and Don'ts section below) is critical to the assay's sensitivity.

In DELFIA assays, the native lanthanide chelate is essentially non-fluorescent. However, after the binding reaction is complete the lanthanide fluorescence is developed by the addition of either DELFIA Enhancement Solution or DELFIA Inducer. The lanthanide ions are released into solution at low pH where they rapidly form new, highly fluorescent chelates inside a protective micelle with components of the Enhancement Solution or DELFIA Inducer. The fluorescence of the lanthanide chelate is amplified 1-10 million times by this enhancement step. DELFIA Inducer is used for all DELFIA chelates, while Enhancement Solution is recommended only for N1 chelates (see Table 9).

Multianalyte assays may involve the use of samarium, dysprosium or terbium chelates as secondary, tertiary or quaternary labels. Like europium, samarium requires only DELFIA Enhancement Solution or Inducer to develop its lanthanide fluorescence. Dysprosium and terbium however require an additional secondary addition of DELFIA Enhancer (C 500-100) (See Figure 5).

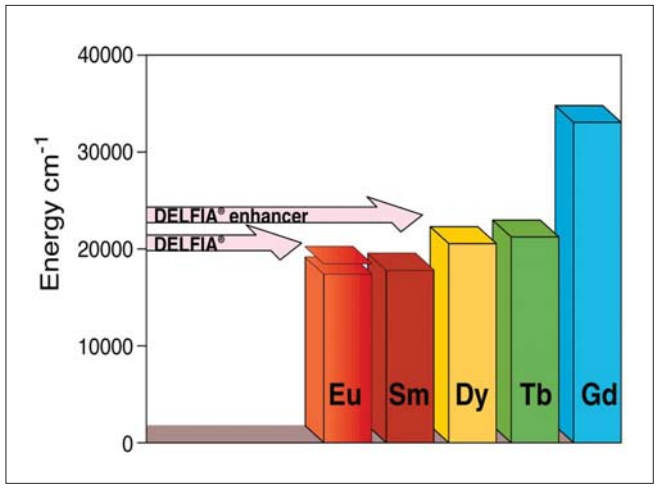


Figure 5. Europium and samarium can be measured by adding Enhancement Solution while dysprosium and terbium require the secondary addition of DELFIA Enhancer.

The speed of the dissociation enhancement process will depend on the original chelate used, the choice of DELFIA Enhancement Solution or DELFIA Inducer, and whether shaking is used (See Table 9). If the plate cannot be measured after finishing the assay it should be stored empty and Enhancement Solution added immediately prior to measurement.

3.6.2 Detection Instruments

Various models of EnVision and VICTOR plate readers are available from PerkinElmer to meet most speed and capacity requirements. When

measuring DELFIA Eu-fluorescence on a 96-well plate, both instruments allow a detection limit better than 10 amol Eu/well in a measurement time of approximately 2 minutes/plate. With a 384-well plate a detection limit better than 5 amol Eu/well is achieved with reading times of 3 and 5 minutes/plate for EnVision and VICTOR respectively. The EnVision enables measurement of more than 90,000 wells per day. For ultra-fast processing of 384 well plates the PerkinElmer ViewLux™ ultraHTS microplate imager measures all samples on a microplate simultaneously.

PerkinElmer instruments can be calibrated to give approximately the same signal count, which is useful when different instruments are used in assay development and HTS.

Aside from the obvious requirement of TRF capability, other plate readers may be used if they are of sufficient sensitivity. This can be easily tested by measuring 200 µl of a 1 nmol/L Europium standard solution (B119-100) in a clear 96-well plate in TRF mode. This should provide a signal to background ratio (S/B) > 10,000:1.

4. Dos and Don'ts

Assay step	Do	Don't
General	• Allow reagents to reach room temperature (+20 - +25 °C) before performing an assay. • Avoid europium contamination and resulting high fluorescent background through careful pipetting and washing techniques. • Use optimized DELFIA assay buffers to assure good results.	• Use microplates with high fluorescent background (see 3.1).
Tracer	• Store labeled proteins and peptides at a high concentration and in the absence of chelators or competing metals in the buffer. In most cases, 50 mmol/L Tris-HCl buffered saline solution (pH 7.5-8.0) containing 0.1-0.5% purified BSA will ensure the stability of the labeled compound during storage. • Avoid carry-over when pipeting a tracer solution by holding the pipette tip slightly above the top of the well and avoid touching the plastic strip or the surface of the liquid.	• Expose the tracer to chelating agents like EDTA during incubations. (Reagents labeled with N1 chelates tolerate chelating agents up to 50 µM and reagents labeled with DTPA chelates tolerate chelating agents up to 5 mM). • Store labeled proteins or peptides in DELFIA Assay Buffer (1244-106, 1244-111, 4002-0010) or phosphate buffers. • Store diluted reagents.
Enhancement	• Use 100-200 µl of Enhancement Solution for 96 and 50 µl for 384-well plates. • Use a dedicated Eppendorf Multipette or other pipeting instrument for the Enhancement solution and discard the first aliquot. • Dispense the Enhancement Solution slowly to avoid air bubbles. • Flush pipette or dispenser tips and tubing thoroughly with DELFIA Enhancement Solution before use. • Protect the plate from dust with a plate lid. • Add Enhancement Solution just prior to shaking and measuring the plate.	• Dispense Enhancement Solution from labware which might have been contaminated with Eu • Seal the plate with tape after the addition of the Enhancement Solution. The adhesive might quench the signal. • Use the same reservoir for Enhancement Solution and tracer. • Use glass reservoir for Enhancement Solution.
Washing and shaking	• Optimize the number of wash cycles. • Use an automatic plate washer for optimal results. • Ensure that each well is filled up completely to the top edge • Check that the wells are dry after washing. If not, invert the plate and tap it firmly against absorbent paper. • Check the time needed for efficient enhancement of lanthanide fluorescence for each shaker model.	• Shake too vigorously, which may cause air bubbles that interfere in the fluorescence measurement
Measurement	• Measure with a sensitive time-resolved fluorometer like the VICTOR or EnVision.	• Measure plates with their plate covers.

5. Troubleshooting

Problem	Possible cause/effect	Solution
High background signal	Background of the plain plate (no coating, no buffer, no Enhancement solution) > 500	• Change plates
	Inadequate washing prior to measurement	• Use 4-6 washing cycles in a DELFIA Plate Wash after incubation with Eu-labeled compound
	Inadequate blocking of the plates	• Saturate overnight at RT, or > 2 hours + 37°C • Use a saturation volume greater than that of the coating solution
	Binding of Eu-labeled polyclonal Ab to the plate	• Add 0.1 % Tween 20 or 40 to assay buffer
	Contamination of pipettes and tables with label	• Clean pipettes and tables carefully
Inadequate sensitivity	High background	• See above
	Low maximal signal	• Increase sample volume • Use two different tracer antibodies • Increase antibody concentration in a sandwich assay • Change antibody used
Poor reproducibility	Inadequate incubation with Enhancement Solution	• Use at least 5 minutes incubation on a shaker before measurement. Check time needed for maximum signal.
	Antibody aggregation	• Filter the antibody or the antibody diluted in Assay buffer through 0.22 µM filter
	Low affinity of antibody	• For immunometric assays, increase the amount of tracer per well, increase incubation time, check coating procedure, etc.
	Uneven coating	• Test with PerkinElmer microplates
	Plate sealed with tape during Enhancement and measurements	• Do not cover a plate containing Enhancement Solution with tape
	Trace amounts of Eu in sample	• Add 50 µmol/l DTPA or EDTA to assay buffer
	Labeled antibody stored at wrong temperature	• Optimize storage conditions
Decrease in assay counts after storage of labeled compound.	Heavy metal contamination of BSA	• Add purified stabilizing agents like glycerol, glucose or BSA (CR84-100)
	Unsuitable storage conditions	• Do not expose Eu-labeled antibody to chelating agents like EDTA or phosphate-based buffers during storage. Store Eu-Antibody undiluted in Tris-HCl buffer (50 mM Tris-HCl, 0.9% NaCl, 0.05% NaN3, pH 7.8)
	Instability of antibody or other reagents	• Optimize reagents

6. Supplemental material

6.1 Assay reagents

Cat.No.	Product	Package
Plates:		
AAAND-0001	DELFIA Yellow Plate, 96-well	60 plates
AAAND-0005	DELFIA Streptavidin-coated yellow plate, 96-well	10 plates
Labels:		
1244-360	DELFIA Eu-labeled streptavidin	250 µg
AD0021	DELFIA Eu-DTPA ITC chelate and Eu standard	1 mg
Buffer:		
1244-111	DELFIA Assay Buffer	250 mL
1244-114	DELFIA Wash Concentrate	250 mL
Enhancement:		
1244-104	DELFIA Enhancement Solution	50 mL
4013-0010	DELFIA Inducer	250 mL
Other:		
CR84-100	Stabilizer (DTPA-purified BSA), 7.5%	50 mL
#300-01A	PeproTech EC, TNF-α	
ELISA Kit:		
KHC3012	Biosource, Human tumor necrosis factor alpha (hTNF-α)	

6.2 Additional information

Material	Cat.No.	Description
Brochure	006837	*DELFIA Research Reagents*
Brochure	1244-1126	*Applications of time-resolved fluorometry with DELFIA methods* (Labeling booklet)
Brochure	007097	*PerkinElmer Microplates: A Complete Overview*
Brochure	P10978	*DELFIA Buffers Guide*
Application Note	1234-965	*Eu-labeled oligonucleotides are stable and sensitive as probes and primers*
Application Note	1234-966	*DELFIA assays bring convenience in monoclonal antibody development: Immunoassay designs and a model assay protocol.*
Application Note	1234-976	*How to optimize rapid and simple immunoassays (DELFIA)*
Application Note	1234-9847	*Multiplexing DELFIA assays using lanthanide-labeled probes*
Application Note	1420-1000	*The Acrowell Plate: Low fluorescence background using the DELFIA system*

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007650_01 Printed in USA