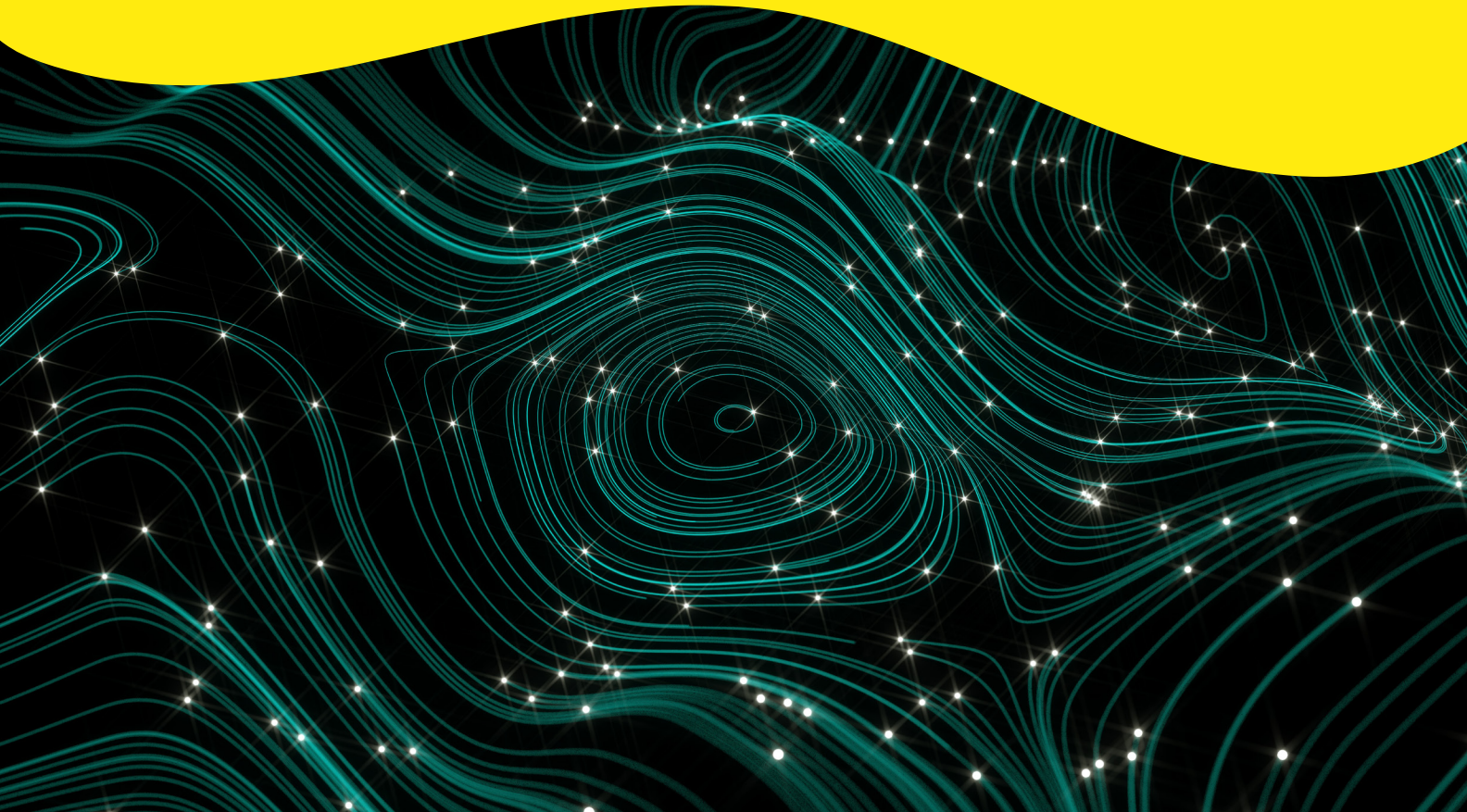
User's guide to DELFIA™ immunoassays



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DELFIA assay principle

Traditional enzyme-linked immunosorbent assays (ELISAs) are often used to detect and quantify the levels of an analyte from a range of complex sample matrices (e.g. human serum, urine, or blood) where an abundance of other proteins and molecules are present. While compatible with complex matrices, a colorimetric ELISA is susceptible to the constraint of a narrow linear range for the optical density (OD), which is common to absorbance-based measurements. The smaller dynamic range (~ 2 logs) of the assay decreases the likelihood that the unknown sample concentration will fall within the standard curve and introduces the challenge of testing multiple dilutions from the same, potentially limited, sample. Repeat dilution testing of the same sample also requires additional assay wells and often leads to purchasing another kit or coating additional assay plates to accurately quantify all samples. Chemiluminescent ELISAs can be significantly more sensitive, but require vast amounts of antibody, increasing the cost of the assay, and lack signal stability due to the nature of the rapid decline in luminescence over time.

Time-resolved fluorometry (TRF) is a well-established technique in drug discovery and basic research, first becoming widely used in the 1980s for immunoassays. Providing high sensitivity and wide dynamic range, this method is characterized by decreased background autofluorescence during measurement. TRF-based DELFIA™ (Dissociation-enhanced lanthanide fluorescence immunoassay) technology provides a highly-sensitive wash-based immunoassay technology that offers significant advantages over traditional ELISA:

High sensitivity

Ideal for complex sample matrices; accurately detect femtogram quantities of analyte (see Figure 1).

Wide dynamic range

No need for additional dilutions (see Figure 1).

Superior stability

Read plates months later after proper storage, with a stable time-resolved fluorescent signal that does not photobleach with repeat measurements.

Proven technology

Developed by Turku University of Finland in collaboration with Wallac Oy, Turku, Finland in the early 1980s, DELFIA technology has a track record of thousands of peer-reviewed publications, representing disease diagnostics, neonatal screening, and drug discovery (see "References" section in this guide).

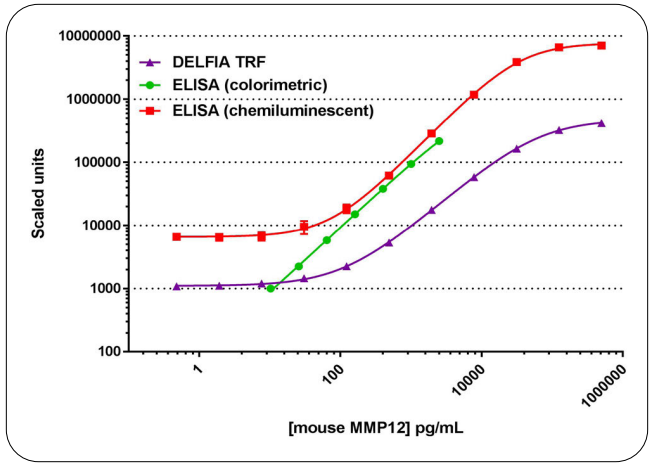


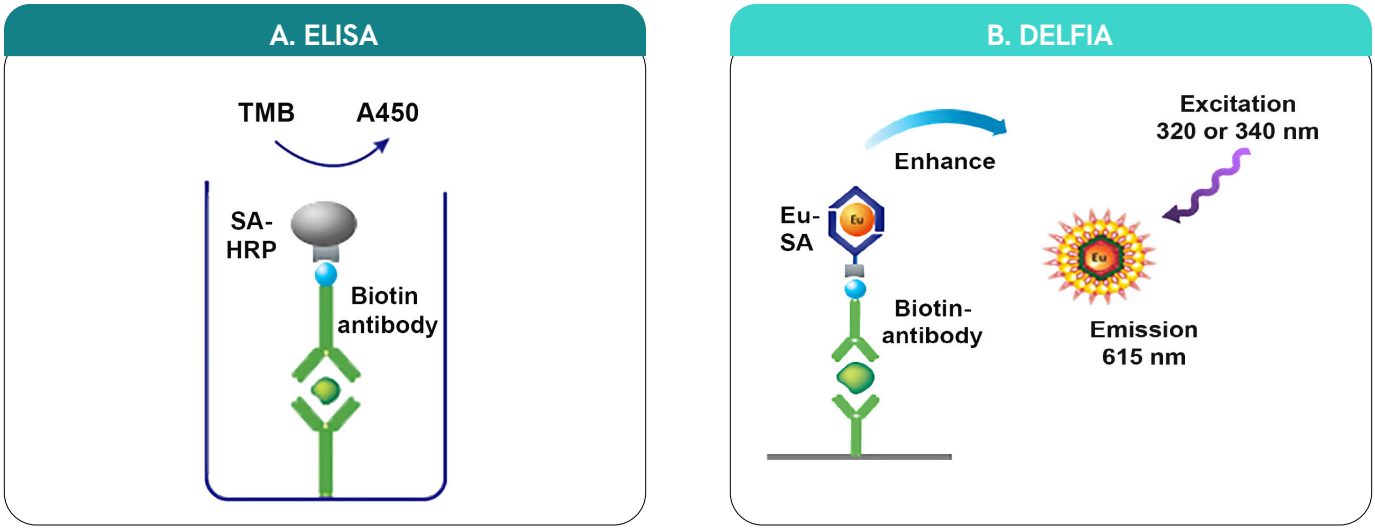
Figure 1: DELFIA assay performance compared to colorimetric and chemiluminescent ELISA. Assays were performed using 25 µL sample for DELFIA and 75 µL sample for ELISAs with the same polyclonal antibody as both the capture and detection antibody, in sandwich assay format using direct coated plates and a biotinylated version of the anti-mouse MMP12 antibody with DELFIA Europium-streptavidin (Eu-SA). The biotinylated detection antibody was detected with streptavidin-HRP for the ELISA assays.

The DELFIA assay principle provides a time-resolved fluorescence readout with a protocol that is virtually identical to that of a standard sandwich ELISA (Figure 2). Antibody is first captured on a coated microplate, followed by addition of sample and then detection antibody representing a typical sandwich assay. Intervening wash steps ensure thorough isolation of target analyte from the sample matrix for optimal sensitivity in complex sample types. Unlike ELISA (Figure 2A), DELFIA (Figure 2B) utilizes a Europium lanthanide chelate-labeled detection reagent conjugate (e.g., Eu-streptavidin or Eu-antibody) in place of horseradish peroxidase (HRP)-conjugate. The Europium chelate provides stable chemistry that allows the assay to be read overnight or days later given proper storage with no change in signal, as opposed to HRP-catalyzed readouts that require measurement within 30 minutes. An enhancement step unique to DELFIA releases the lanthanide from the antibody complex upon addition of DELFIA Enhancement Solution, producing a new, highly fluorescent lanthanide chelate contained within a protective micelle. The amplified fluorescent signal is detected using time-resolved fluorometry. This detection method eliminates the prompt, non-specific autofluorescent background signal, hence enables DELFIA to be the superior technology with superior sensitivity.

With standard fluorescent detection, reagent and microplate interference can contribute to high background and reduced sensitivity. DELFIA uses the principle of time-resolved fluorometry to eliminate background interference. Lanthanide chelates possess both long fluorescence decay times (Figure 3A) and large Stokes shifts (Figure 3B), properties that allow delayed signal measurement at a wavelength with little background interference. In addition, lanthanides emit a stable fluorescent signal that exhibits a sharp emission peak and high fluorescence intensity.

Table 1: Advantages of DELFIA in comparison to ELISA.

	DELFIA	Chemiluminescent ELISA	Colorimetric ELISA
Sensitivity	<0.5 pg/mL	<0.5 pg/mL	>5 pg/mL
Dynamic range	4-5 logs	4-5 logs	2-3 logs
Signal-to-Noise	>100	>100	~2.5
Signal stability	**Excellent** ▪ No stop reaction required ▪ Signal can be measured months later with proper microplate storage	**Poor** ▪ Luminescent signal declines rapidly, hence measurements must be made within a five minute window after substrate addition	**Poor** ▪ Reaction termination required ▪ Prompt measurement of colorimetric signal required
Reagent stability	**Excellent** ▪ Lanthanide-based assay: performance characteristics intrinsic to TRF technology	**Poor** ▪ Enzymatic assay: performance characteristics dependent on enzyme conjugate quality and activity	**Poor** ▪ Enzymatic assay: performance characteristics dependent on enzyme conjugate quality and activity ▪ Incomplete stop reaction causes continuous generation of color, affecting data quality



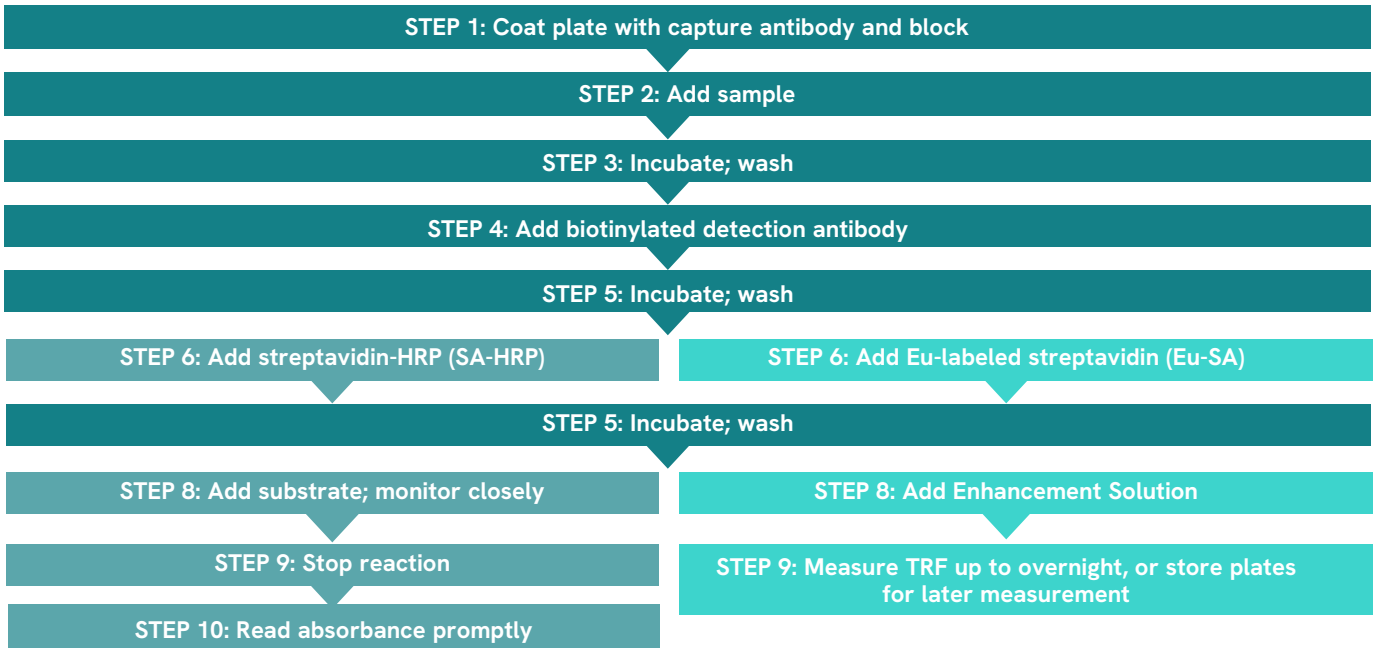


Figure 2: DELFIA vs. ELISA assay protocol comparison.

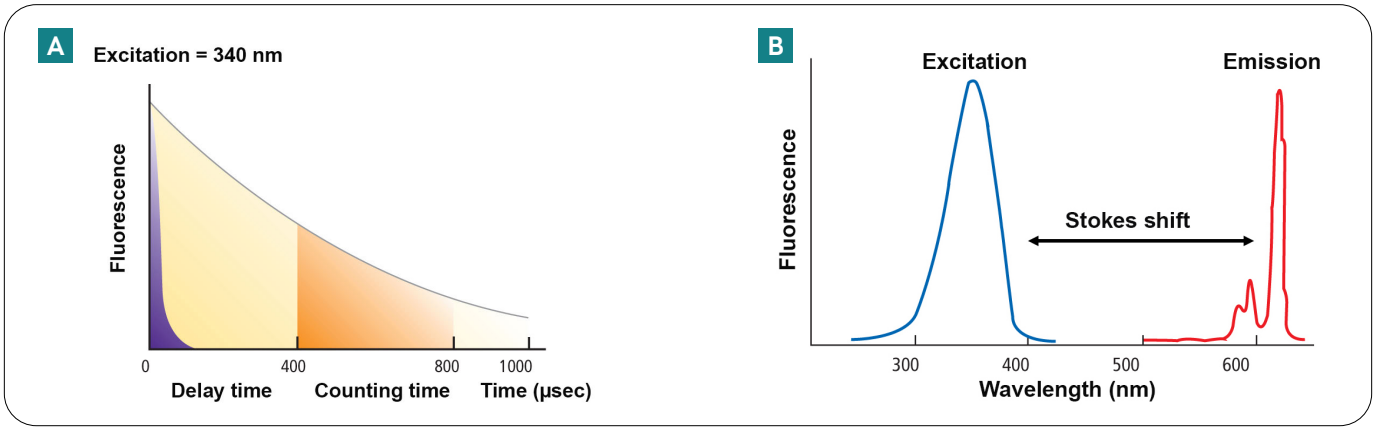


Figure 3: A) Fluorescence from lanthanide chelates may last up to 1000 µsec, significantly longer than conventional fluorophores. B) Lanthanide chelates exhibit a large Stokes shift, the difference between the maximum absorption and emission spectra of a fluorophore. The sharp emission peak and high fluorescence intensity give DELFIA an advantage over alternative technologies, as background autofluorescence decays before measuring specific signal from the Europium lanthanide chelate (see purple shaded region vs. orange shaded region in A). Additionally, the large Stokes shift enables plate reader optics to be chosen that cover the full peak for maximal energy from both the excitation and emission wavelengths, in contrast to classical fluorophores.

DELFIA immunoassays are a superior alternative to traditional ELISAs. The use of Revvity's validated DELFIA in diagnostics applications can be extended to research assay applications representing it as a versatile platform, with over 1,000 publications on immunoassays alone (see "References" section of this guide for a selection). DELFIA delivers lower detection limits in the femtogram range, representing sensitivities that are either better or comparable to superior chemiluminescent detection systems. DELFIA also offers a broad dynamic range, minimizing the need for time-consuming sample preparation and sample dilution. Because DELFIA is not an enzyme-based technology, assay performance is not susceptible to degradation of enzyme conjugate activity or substrate signal. Lanthanide fluorescent signals are strong and stable, providing consistent signal measurement up to 24 hours at room temperature, or even months after an assay has been completed if plates are stored properly (dry, protected from dust in dark at 4 °C).

Assay formats

DELFIA immunoassays can be performed in 96- and 384-well formats. Antibodies or antigens can be coated directly or indirectly to DELFIA plates. Detection can occur with Europium-labeled anti-analyte detection antibody, or via indirect detection (e.g., biotinylated antibody + Europium-streptavidin). Sandwich antibody assays, bridging/double-antigen assays, and fixed-cell assays have all been performed. Common DELFIA assay formats are discussed below; note that the DELFIA Toolbox Kits assume use of sandwich assay format.

Direct assay format

A common DELFIA assay configuration involves direct coating of the sample containing antigen to the microplate. This format requires the use of only one antibody: the detection antibody. In this assay configuration, antigen is directly coated to the wells. A Europium-labeled antibody against the target antigen is used as a tracer. Increasing amounts of

antigen in the sample lead to increased signal. This assay format enables a simplified protocol (one-step protocols requiring only one round of wash steps), however, has disadvantages when working with complex samples as the entire sample is coated to the well before blocking (i.e., target antigen in the sample competes with other proteins in the sample for binding to plate). (See Figure 4A).

Indirect assay format

The indirect DELFIA format again involves direct coating of antigen to the microplate, but instead of using a Europium-labeled antibody against target antigen, a biotinylated or unlabeled antibody against the target antigen is used in combination with Europium-streptavidin or a Europium-anti-species antibody as appropriate (the "tracer"). This format is often more convenient, as there is no need to Europium-label the detection antibody. Additionally, signal amplification can occur as a result of use of a binary detection antibody/Eu-anti-species antibody pair (See Figure 4B).

Sandwich assay format

In a DELFIA sandwich immunoassay, the analyte of interest is bound between two antibodies. The capture antibody is directly or indirectly coated to the microplate. A biotinylated or unlabeled detection antibody can be used in combination with Eu-streptavidin or Eu-anti-species antibody as a tracer. Alternative DELFIA formats exist where the detection antibody is directly labeled with Europium. The sandwich format offers high specificity, since two antibodies are required to bind target antigen. It is also suitable for complex sample types. (See Figures 4C and 4D).

Competition assay format

In a competition assay, a known amount of Europium-labeled analyte competes with possible analytes from the sample. The more (unknown) analyte present in the sample, the lower the signal will be. The less analyte in the sample, the higher the signal will be, as the Europium-labeled analyte will have less competition binding to capture antibody. After the Eu-analyte and sample analyte are left to compete for the capture site on the antibody, the unbound analyte is washed away. Only analyte bound to the capture antibody remains in the well. Addition of Enhancement Solution will then release Europium so that it can form a bright enhancing chelate, which can be measured via TRF. (See Figure 4E).

Direct vs. Indirect coating of capture antibody

Similar to ELISA, capture antibody can be directly coated to a high-bind plate or indirectly associated with an appropriate streptavidin-coated or anti-species antibody-coated microplate (see Figures 4C and 4D). For most assays, the total signal and sensitivity is better using plates directly coated with capture antibody rather than with streptavidin or anti-species antibody for indirect capture (see Table 2). However, antibody consumption is significantly higher in direct coating (e.g., 1 µg of antibody/well) than in indirect coating (e.g., 200 ng of biotinylated antibody/well). Therefore, coating plates with secondary antibody or streptavidin may often be the only viable option due to cost or limited availability of a specific antibody.

For the DELFIA Toolbox Kits, uncoated high-bind plates are provided for direct coating of the capture antibody of interest to the plate. For indirect coating, DELFIA plates are available as streptavidin-coated plates and anti-rabbit, anti-sheep, or anti-mouse coated plates (see "Microplates" section of this guide).

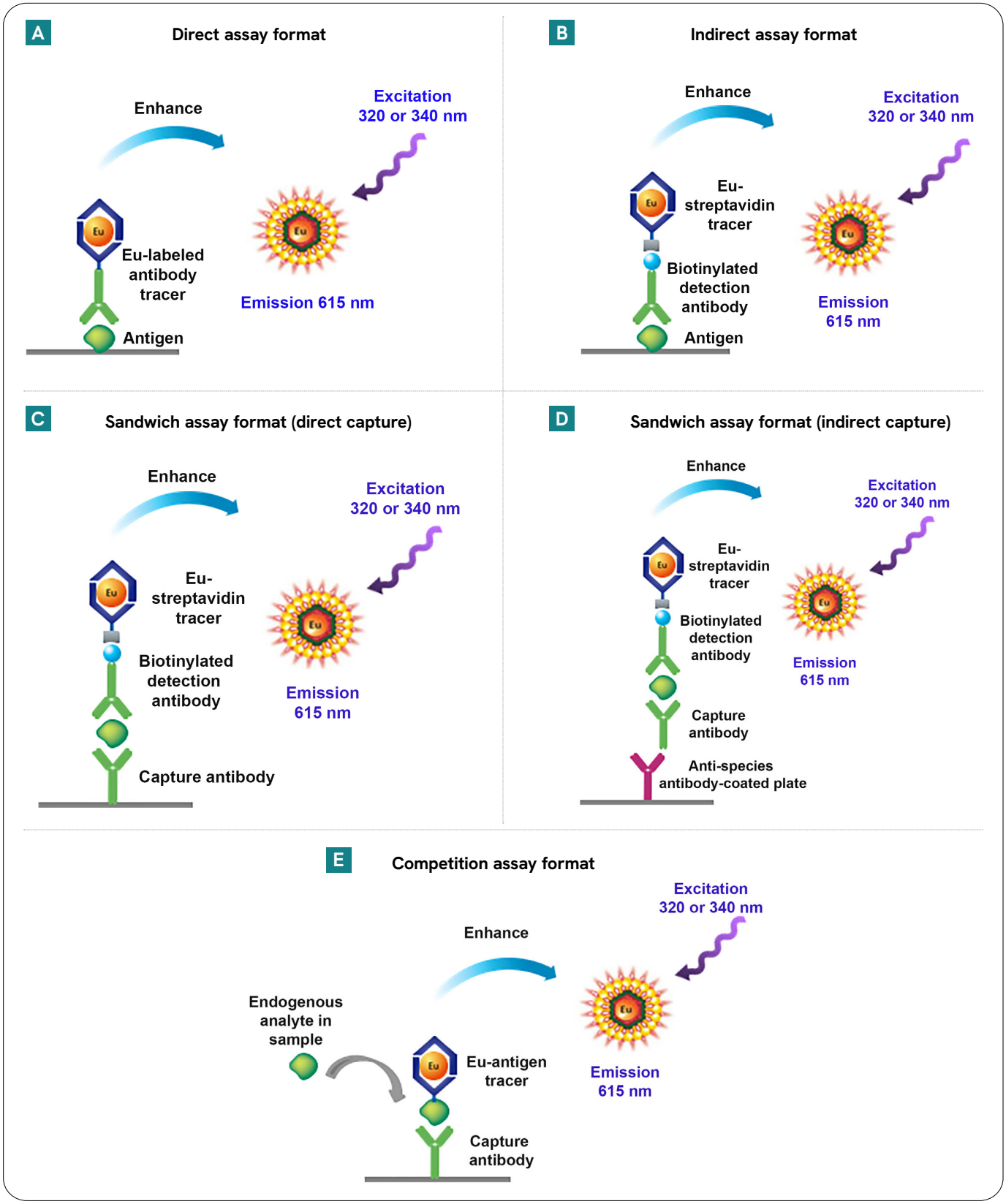


Figure 4: DELFIA immunoassay formats.

Table 2: Sensitivity of DELFIA TNFα assays with different antibody pairs and coating protocols.

Antibody Pair		Sensitivity (pg/mL)	
Capture	Detection	Direct coating	Indirect coating (Streptavidin-coated Plate)
Antibody 1	Antibody 2	0.5	0.4
Antibody 2	Antibody 1	0.4	1
Antibody 3	Antibody 4	0.5	1.4
Antibody 4	Antibody 5	1.6	2.4

Antibody considerations

If converting from an existing ELISA assay, the same antibodies can be used for the DELFIA assay. If developing a new immunoassay, antibody selection should carefully be considered.

Antibody pairs

Several antibody companies sell antibody pairs designed for sandwich ELISA assays. Antibody pairs such as **Abcam's ELISA matched antibody pairs** or **R&D Systems DuoSet® ELISA Systems** can easily be adapted to DELFIA using recommended ELISA concentrations. The advantage of such pairs is that the antibodies are already selected to bind simultaneously to target analyte for sandwich assay formats. ELISA plates coated with capture antibodies can be used if the plastic of the plate does not show high TRF background. This can be assessed by measuring an empty plate using DELFIA TRF instrument settings (see Instrument Settings section in this guide). Background with empty DELFIA clear stripwell or DELFIA yellow microplates plates is ~100-200 counts using our EnVision® or VICTOR multimode plate readers. Normal background in biochemical DELFIA assays in the same plates is 300-1500 counts using the same plate readers.

If selecting a new antibody pair, it may be necessary to screen several antibodies in both orientations (capture vs. detection antibody) as potential pairs to ensure simultaneous and optimal binding to target analyte.

Specificity

The specificity of the antibody(ies) used in the immunoassay will help discriminate the target analyte from other proteins within the sample, hence enabling the accuracy of results. Antibody specificity is partly dependent on the type of the immunogen used to generate the antibody. **Synthetic peptide immunogens** are often used to target a specific epitope, though they can fail to adequately represent the intact 3D protein structure or post-translational modifications that occur on the native protein. Many antibodies generated for use in Western blotting may only recognize the **denatured form of the protein**, which can pose a problem when working with serum and plasma samples or cellular secretions that don't involve a denaturing step during sample preparation. Note that antibodies that have been validated in ELISA, immunohistochemistry (IHC), and immunoprecipitation (IP) can be considered for use in DELFIA. Specificity is also dependent on the method used for antibody purification. **Affinity purification** yields a more homogeneous product compared to purification by either **Protein A or Protein G resins**. One can also expect that the antibody provided may vary between production lots, particularly if the antibody is polyclonal. **Polyclonal antibodies** represent a pool of antibodies against the immunogen and typically show a higher probability for detection in a range of different conditions, whereas a **monoclonal antibody** is more likely to work in only one set of conditions.

Sensitivity

The sensitivity of the antibody itself will impact overall assay sensitivity. Antibody sensitivity is determined by the **affinity** of the antibody for its target antigen, and is often assessed by quantitating the amount of antigen required for antibody recognition/detection in a given format. Sensitivity becomes critical when the protein is expressed at low **endogenous levels**.

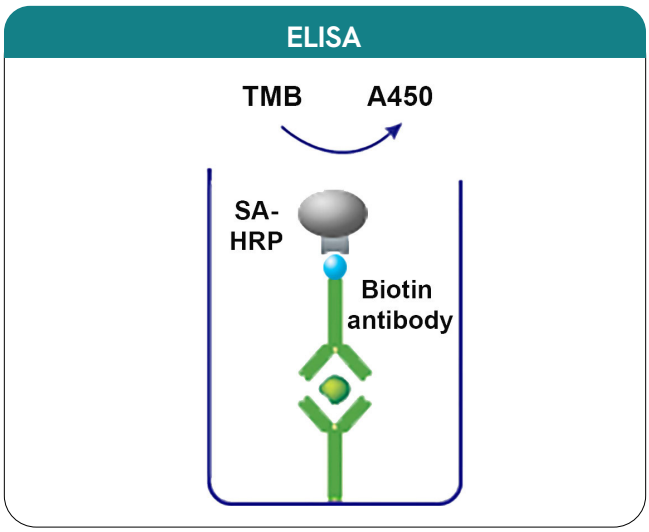
Reproducibility

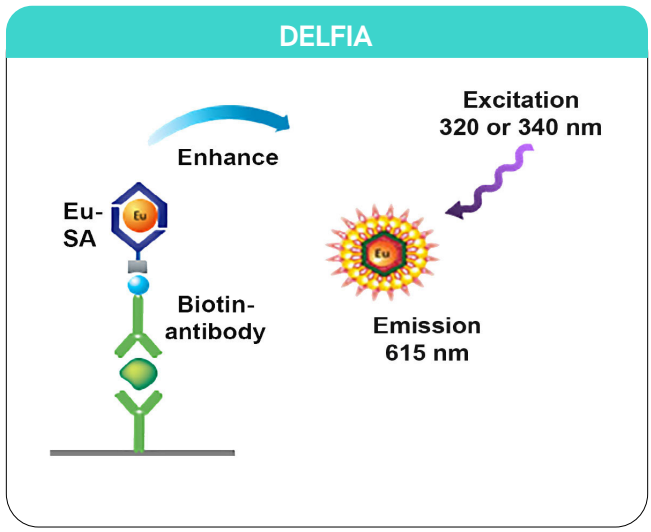
Another important criterion for antibody selection is antibody reproducibility. Reproducibility refers to binding kinetics and detection by the same antibody over time with different lots on different days.

Poor correlation between antibody lots can be problematic. **Monoclonal antibodies** are considered highly specific reagents that generate the most reproducible results. **Polyclonal antibodies** have a higher batch-to-batch variation and therefore may require that every new lot of material be evaluated prior to use.

Assay conversion: ELISA to DELFIA

Converting an existing ELISA to a DELFIA protocol is straightforward. As shown in Figure 5, the steps in a DELFIA assay are very similar to those in an ELISA assay while offering excellent sensitivity over a wider dynamic range.





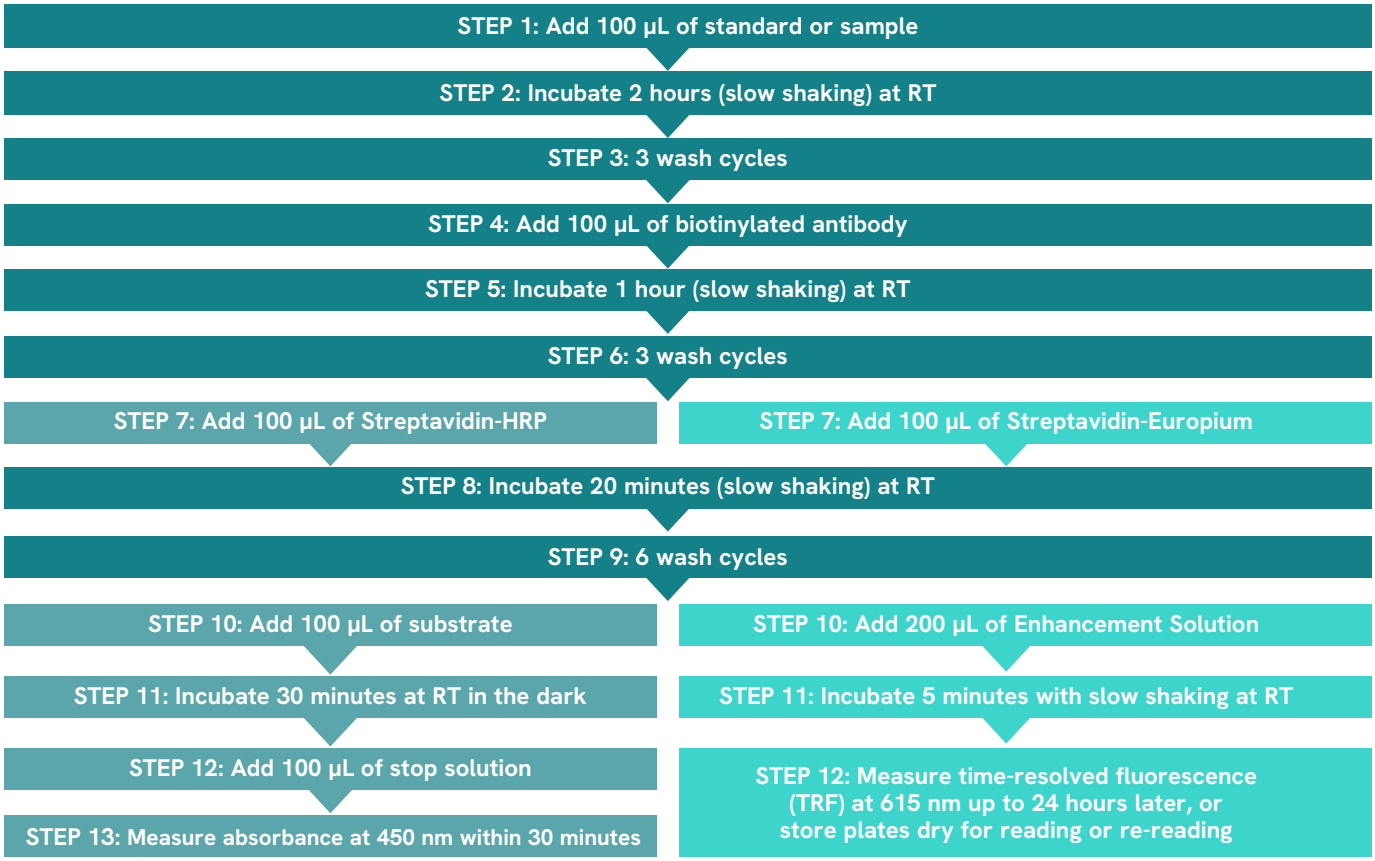


Figure 5: ELISA vs. DELFIA sandwich immunoassay protocol (96-well format) using biotinylated detection antibody with streptavidin-HRP (ELISA) or Europium-streptavidin (DELFIA).

Materials and methods

DELFIA toolbox kit components

- Eu-labeled streptavidin or Eu-labeled anti-species antibody
- DELFIA Wash Solution
- DELFIA Assay Buffer
- DELFIA Enhancement Solution
- DELFIA uncoated (high-bind) 96-well stripwell plate or ½ areaplate (high-bind)
- DTPA-purified BSA, 7.5% (for plate blocking)

Materials to be supplied by user

- Sandwich antibodies against analyte of interest (may be capture and detection antibodies previously optimized for ELISA assay)
- PBS (we recommend Gibco #10010-023) or other buffer
- Plate lid (Revvity #6000027)
- TopSeal™-A Plus Adhesive Sealing Film (Revvity #6050185; remove prior to reading plate)
- Plate Reader with TRF Option (Revvity EnVision®, VICTOR Nivo™, EnSight™ multimode plate readers; see section on "Instrument Settings")

Sample preparation

- This section provides general guidelines as a resource for preparing various sample types for use in DELFIA. Optimal sample preparation procedures will vary depending on the target and assay.

Cell culture supernatant

- If needed, pipet cell culture media into a centrifuge tube and centrifuge to spin debris at 1,500 rpm for 10 minutes at 4 °C.
- Immediately aliquot and store samples at -80 °C. Avoid freeze/ thaw cycles.

Cell lysate

- Place tissue culture plates on ice.
- Aspirate media and wash cells with ice-cold PBS.
- Aspirate PBS and add lysis buffer.
- Scrape cells or shake plate and pipet into a pre-chilled tube.
- Vortex briefly and centrifuge at 13,000 rpm for 10 minutes at 4 °C.
- Aliquot supernatants on ice and store at -80 °C. Avoid freeze/thaw cycles.

Plasma

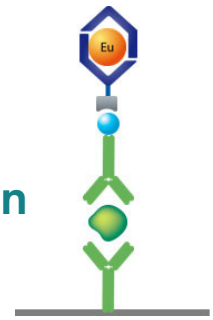
- Collect whole blood by venipuncture and treat with anticoagulant. Hemolytic, lipemic, and icteric serum samples should not interfere with the assay.
- Centrifuge at 1,000 – 2,000 x g for 10 minutes at 4 °C to remove cells.
- Collect supernatant (plasma) and aliquot. Store at -80 °C. Avoid freeze/thaw cycles.

Serum

- Collect whole blood (treated or untreated) and allow to clot.
- Incubate at room temperature without shaking for 20 minutes.
- Centrifuge at 1,000 – 2,000 x g for 10 minutes at 4 °C.
- Immediately aliquot supernatant (serum) and store samples at -80 °C. Avoid freeze/thaw cycles.

Tissue

- Place tissue in microfuge tubes and immerse in liquid nitrogen to snap freeze. Store samples at -80 °C for later use or keep on ice.
- Add extraction buffer (~300 µL per 5 mg of tissue; final concentration of protein extract should be > 1 mg/mL) and homogenize with electric homogenizer.
- Agitate for 2 hours at 4 °C on an orbital shaker.
- Centrifuge for 20 minutes at 13,000 rpm at 4 °C. Place on ice, aliquot supernatant to a pre-chilled tube, and store samples at -80 °C. Avoid freeze/ thaw cycles.



Protocol 1: DELFIA sandwich immunoassay in 96-well plate using europium-streptavidin

Reagent preparation

Plate coating, for one 96-well plate:

Day 1: Prepare 12 mL of 10 µg/mL capture antibody in buffer (e.g., 0.2 M sodium phosphate buffer pH 6.8, 1X PBS, 50 mM Tris-HCl buffer 8.0, etc.).

- *Note:* The calculated volume of required capture antibody is 9.6 mL, according to this protocol.

Day 2: Prepare 100 mL of 1X DELFIA wash solution for the wash steps involved in plate coating.

- Dilute 4 mL of 25X DELFIA wash concentrate in 96 mL of Milli-Q water.
- *Note:* The calculated volume of required 1X DELFIA wash solution is 86.4 mL, according to this protocol.

Prepare 35 mL of 1% BSA blocking solution.

- Dilute 4.6 mL of 7.5% BSA in 30.4 mL of buffer (e.g., 1X PBS or other neutral pH buffer).
- *Note:* The calculated volume of required blocking buffer is 28.8 mL, according to this protocol.

DELFIA assay, for one 96-well plate:

1. Prepare 400 mL of 1X DELFIA wash solution for assay.
   - Dilute 16 mL of 25X DELFIA wash concentrate in 384 mL of Milli-Q water.
   - *Note:* The calculated volume of required 1X DELFIA wash solution is 345.6 mL, according to this protocol.
2. Dispense 12 mL DELFIA assay buffer to clean, 50 mL conical tube if using as diluent for standard curve.
   - *Note:* The calculated volume of required DELFIA assay buffer to prepare the standard curve is 9.83 mL according to this protocol – see Table 3.
3. Prepare 12 mL of 100 ng/mL (or ELISA concentration) biotinylated detection antibody in DELFIA assay buffer.
   - *Note:* The calculated volume of required biotinylated detection antibody is 9.6 mL, according to this protocol.
4. Prepare 12 mL of 100 ng/mL Eu-streptavidin in DELFIA assay buffer.
   - Dilute 12 µL of 100 µg/mL Eu-streptavidin in 12 mL DELFIA assay buffer.
   - *Note:* The calculated volume of required Eu-streptavidin is 9.6 mL, according to this protocol.
5. Dispense 25 mL of Enhancement Solution into a clean, 50 mL conical tube for dispensing.
   - *Note:* The calculated volume of required Enhancement Solution is 19.2 mL, according to this protocol.

Step 1: Coating microplates

1. Add 100 µL of capture antibody (10 µg/mL, or ELISA coating concentration) to each well.
   - Reconstitute and store antibody according to the data sheet. Antibodies can be prepared in 0.2 M sodium phosphate buffer pH 6.8, 1X PBS, 50 mM Tris-HCl buffer 8.0, or other buffers for coating.

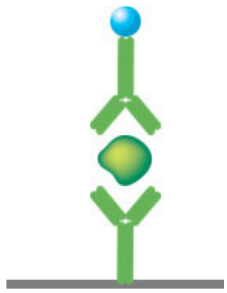
   - We recommend coating with 1 µg antibody per well in 96-well format.
   - To optimize an unknown ELISA capture antibody concentration, we recommend testing undiluted, 1-in-2 dilution, 1-in-5-dilution, and 1-in-10 dilution of capture antibody.

2. Cover with a lid or TopSeal-A adhesive seal and incubate overnight at 23 °C to ensure the capture antibody binds to the plate.
3. Wash each well 3 times with 300 µL of 1X DELFIA wash solution or other wash solution.
   - If being done by hand, it is simplest to dispense 300 µL of wash solution per well.
4. Block plates by adding 300 µL of 1X PBS +1% BSA or other blocking solution to each well. We strongly recommend Revvity's DTPA-purified BSA for this step – this BSA is included in the DELFIA Toolbox Kits. Incubate at room temperature on a plate shaker set to a slow speed (300 rpm) for a minimum of 1 hour.
5. Remove blocking solution by inverting the plate and blotting it against clean paper towels. Plates can be sealed with adhesive and used the next day, or dried in a laminar flow hood and stored dry at 4 °C in a foil bag with a desiccant bag.

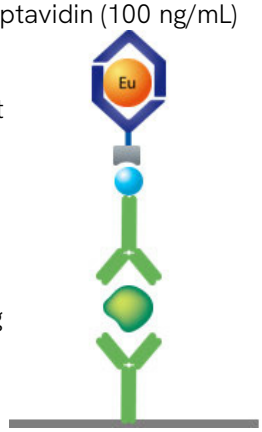
Step 2: Performing the assay

1. Remove adhesive film from the microplate, if the plate has been covered.
2. Add 100 µL of standard analyte or sample to each well. The dynamic range of the assay will be antibody-dependent, but broader than a standard colorimetric ELISA assay. Suggested initial concentrations range from 100 fg/mL to 100 µg/mL. See an example for preparing serial dilutions of analyte in Table 3.
   - Prepare standards and any sample dilutions in the appropriate diluent for the sample type, as identified by linearity and recovery experiments (see "Sample validation" section in this guide).
   - Reconstitute and store standard analyte according to the manufacturer's data sheet

3. Cover and incubate plate for 2 hours at room temperature on a plate shaker set to a slow speed (300 rpm).
4. Wash each well 3 times with 300 µL of 1X DELFIA wash solution.
5. Add 100 µL of biotinylated detection antibody to each well (100 ng/mL for biotinylated antibody, or ELISA concentration).
   - Prepare working detection antibody solution in DELFIA assay buffer.
   - For optimizing biotinylated antibody concentration, we recommend testing 1-100 ng per well in 96-well format.



   - Reconstitute and store detection antibody according to the manufacturer's data sheet.
6. Cover and incubate 1 hour at room temperature on a plate shaker set to a slow speed (300 rpm).
7. Wash each well 3 times with 300 µL of 1X DELFIA wash solution.
8. Add 100 µL of Europium-Streptavidin (100 ng/mL) to each well.
   - Prepare Eu-labeled reagent in DELFIA assay buffer.
   - For optimizing Eu-streptavidin concentration, we recommend testing 1-20 ng per well in 96-well format.



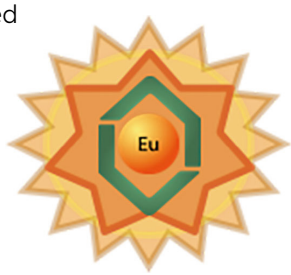
9. Incubate 20 minutes at room temperature on a plate shaker set to a slow speed (300 rpm).
   - Cover the plate with a plate lid.
   - Do not cover the plate with TopSeal-A from this point forward, as the adhesive can quench the signal.

10. Wash each well 6 times with 300 µL of 1X DELFIA wash solution.

- The extra wash steps are necessary for removing any unbound Europium-labeled reagent.

11. Add 200 µL of Enhancement Solution to each well and cover the plate with a plate lid.

- Remember to not add TopSeal-A or adhesive seal, as this can quench the signal.
- If the plate is to be stored prior to reading, it is recommended to cover the plate with a lid and add Enhancement Solution just prior needing to read the assay.



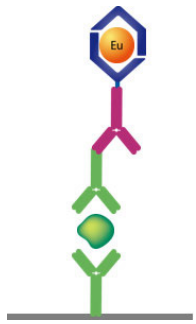
12. Incubate at least 5 minutes at room temperature on a plate shaker set to a slow speed (300 rpm).

13. Read plate using TRF settings (refer to "Instrument settings" section).

- The developed signal will be stable for at least 24 hours at 23-25 °C when stored properly by covering tightly with parafilm.
- Important Note: seals or tapes with adhesives should be avoided after Enhancement Solution has been added to the plates, as adhesive chemicals can quench the signal.

Table 3: Example of analyte serial dilution for running a standard curve for DELFIA assay.

Tube	Vol. of analyte (µL)	Vol. of diluent (µL)	[Analyte] in standard curve	
			(g/mL)	(pg/mL)
A	70 µL of 10 µg/mL stock analyte solution	630	1.00E-06	1,000,000
B	300 µL of Tube A	700	3.00E-07	300,000
C	300 µL of Tube B	600	1.00E-07	100,000
D	300 µL of Tube C	700	3.00E-08	30,000
E	300 µL of Tube D	600	1.00E-08	10,000
F	300 µL of Tube E	700	3.00E-09	3,000
G	300 µL of Tube F	600	1.00E-09	1,000
H	300 µL of Tube G	700	3.00E-10	300
I	300 µL of Tube H	600	1.00E-10	100
J	300 µL of Tube I	700	3.00E-11	30
K	300 µL of Tube J	600	1.00E-11	10
L	300 µL of Tube K	700	3.00E-12	3
M (Blank)	0	500	0	0
N (Blank)	0	500	0	0
O (Blank)	0	500	0	0
P (Blank)	0	500	0	0



Protocol 2:

DELFIA sandwich immunoassay in 96-well plate using europium-anti-species antibody

Reagent preparation

Plate coating, for one 96-well plate:

Day 1: Prepare 12 mL of 10 µg/mL capture antibody in buffer (e.g., 0.2 M sodium phosphate buffer pH 6.8, 1X PBS, 50 mM Tris-HCl buffer 8.0, etc.).

- *Note:* The calculated volume of required capture antibody is 9.6 mL, according to this protocol.

Day 2: Prepare 100 mL of 1X DELFIA wash solution for the wash steps involved in plate coating.

- Dilute 4 mL of 25X DELFIA wash concentrate in 96 mL of Milli-Q water.
- *Note:* The calculated volume of required 1X DELFIA wash solution is 86.4 mL, according to this protocol.

Prepare 35 mL of 1% BSA blocking solution.

- Dilute 4.6 mL of 7.5% BSA in 30.4 mL of buffer (e.g., 1X PBS or other neutral pH buffer).
- *Note:* The calculated volume of required blocking buffer is 28.8 mL, according to this protocol.

DELFIA assay, for one 96-well plate:

1. Prepare 400 mL of 1X DELFIA wash solution for assay.
   - Dilute 16 mL of 25X DELFIA wash concentrate in 384 mL of Milli-Q water.
   - *Note:* The calculated volume of required 1X DELFIA wash solution is 345.6 mL, according to this protocol.
2. Dispense 12 mL DELFIA assay buffer to clean, 50 mL conical tube if using as diluent for standard curve.
   - *Note:* The calculated volume of required DELFIA assay buffer to prepare the standard curve is 9.83 mL according to this protocol – see Table 4.
3. Prepare 12 mL of 2 µg/mL (or ELISA concentration) primary detection antibody in DELFIA assay buffer.
   - *Note:* The calculated volume of required detection antibody is 9.6 mL, according to this protocol.
4. Prepare 12 mL of 200 ng/mL Eu-anti-species antibody in DELFIA assay buffer.
   - Dilute 48 µL of 50 µg/mL Eu-anti-species antibody in 12 mL DELFIA assay buffer.
   - *Note:* The calculated volume of required Eu-anti-species antibody is 9.6 mL, according to this protocol.
5. Dispense 22 mL of Enhancement Solution into a clean, 50 mL conical tube for dispensing.
   - *Note:* The calculated volume of required Enhancement Solution is 19.2 mL, according to this protocol.

Step 1: Coating microplates

1. Add 100 µL of capture antibody (10 µg/mL, or ELISA coating concentration) to each well.
   - Reconstitute and store antibody according to the data sheet. Antibodies can be prepared in 0.2 M sodium phosphate buffer pH 6.8, 1X PBS, 50 mM Tris-HCl buffer 8.0, or other buffers for coating.

- We recommend coating with 1 µg antibody per well in 96-well format.

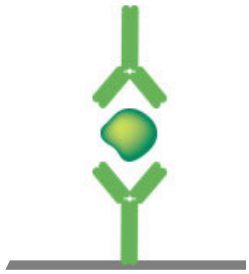
- To optimize an unknown ELISA capture antibody concentration, we recommend testing undiluted, 1-in-2 dilution, 1-in-5-dilution, and 1-in-10 dilution of capture antibody.

2. Cover with a lid or TopSeal-A adhesive seal and incubate overnight at 23 °C to ensure the capture antibody binds to the plate.
3. Wash each well 3 times with 300 µL of 1X DELFIA wash solution or other wash solution.
   - If being done by hand, it is simplest to dispense 300 µL of wash solution per well.
4. Block plates by adding 300 µL of 1X PBS +1% BSA or other blocking solution to each well. We strongly recommend Revvity's DTPA-purified BSA for this step – this BSA is included in the DELFIA Toolbox Kits. Incubate at room temperature on a plate shaker set to a slow speed (300 rpm) for a minimum of 1 hour.
5. Remove blocking solution by inverting the plate and blotting it against clean paper towels. Plates can be sealed with adhesive and used the next day, or dried in a laminar flow hood and stored dry at 4 °C in a foil bag with a desiccant bag.

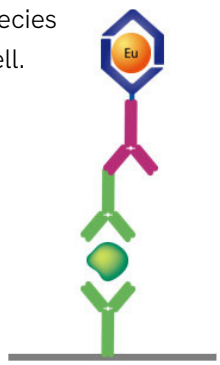
Step 2: Performing the assay

1. Remove adhesive film from the microplate, if the plate has been covered.
2. Add 100 µL of standard analyte or sample to each well. The dynamic range of the assay will be antibody-dependent, but broader than a standard colorimetric ELISA assay. Suggested initial concentrations range from 100 fg/mL to 100 µg/mL. See an example for preparing serial dilutions of analyte in Table 4.
   - Prepare standards and any sample dilutions in the appropriate diluent for the sample type, as identified by linearity and recovery experiments (see "Sample validation" section `in this guide).

   - Reconstitute and store standard analyte according to the manufacturer's data sheet.
3. Cover and incubate plate for 2 hours at room temperature on a plate shaker set to a slow speed (300 rpm).
4. Wash each well 3 times with 300 µL of 1X DELFIA wash solution.
5. Add 100 µL of primary detection antibody to each well (2 µg/mL of primary detection antibody, or ELISA concentration).
   - Prepare working detection antibody solution in DELFIA assay buffer.



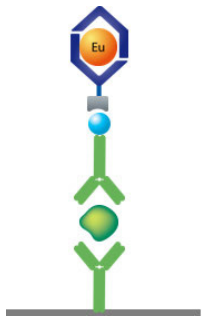
   - For optimizing primary detection antibody concentration, we recommend testing 10-200 ng per well in 96-well format.
   - Reconstitute and store detection antibody according to the manufacturer's data sheet.
6. Cover and incubate 1 hour at room temperature on a plate shaker set to a slow speed (300 rpm).
7. Wash each well 3 times with 300 µL of 1X DELFIA wash solution.
8. Add 100 µL of Europium-anti-species antibody (200 ng/mL) to each well.
   - Prepare Eu-labeled reagent in DELFIA assay buffer.
   - For optimizing Eu-anti-species antibody concentration, we recommend testing 10-100 ng per well in 96-well format.



9. Incubate 60 minutes at room temperature on a plate shaker set to a slow speed (300 rpm).
   - Cover the plate with a plate lid.
   - Do not cover the plate with TopSeal-A from this point forward, as the adhesive can quench the signal.
10. Wash each well 6 times with 300 µL of 1X DELFIA wash solution.
    - The extra wash steps are necessary for removing any unbound Europium-labeled reagent.
11. Add 200 µL of Enhancement Solution to each well and cover the plate with a plate lid.
    - Remember to not add TopSeal-A or adhesive seal, as this can quench the signal.
    - If the plate is to be stored prior to reading, it is recommended to cover the plate with a lid and add Enhancement Solution just prior needing to read the assay.
12. Incubate at least 5 minutes at room temperature on a plate shaker set to a slow speed (300 rpm).
13. Read plate using TRF settings (refer to "Instrument settings" section).
    - The developed signal will be stable for at least 24 hours at 23-25 °C when stored properly by covering tightly with parafilm.
    - Important Note: seals or tapes with adhesives should be avoided after Enhancement Solution has been added to the plates, as adhesive chemicals can quench the signal.

Table 4: Example of analyte serial dilution for running a standard curve for DELFIA assay.

Tube	Vol. of Analyte (µL)	Vol. of Diluent (µL)	[Analyte] in Standard Curve	
			(g/mL)	(pg/mL)
A	70 µL of 10 µg/mL stock analyte solution	630	1.00E-06	1,000,000
B	300 µL of Tube A	700	3.00E-07	300,000
C	300 µL of Tube B	600	1.00E-07	100,000
D	300 µL of Tube C	700	3.00E-08	30,000
E	300 µL of Tube D	600	1.00E-08	10,000
F	300 µL of Tube E	700	3.00E-09	3,000
G	300 µL of Tube F	600	1.00E-09	1,000
H	300 µL of Tube G	700	3.00E-10	300
I	300 µL of Tube H	600	1.00E-10	100
J	300 µL of Tube I	700	3.00E-11	30
K	300 µL of Tube J	600	1.00E-11	10
L	300 µL of Tube K	700	3.00E-12	3
M (Blank)	0	500	0	0
N (Blank)	0	500	0	0
O (Blank)	0	500	0	0
P (Blank)	0	500	0	0



Protocol 3: DELFIA sandwich immunoassay in ½ areaplate using europium-streptavidin

Reagent preparation

Plate coating, for one ½ areaplate:

Day 1: Prepare 6 mL of 10 µg/mL capture antibody in buffer (e.g., 0.2 M sodium phosphate buffer pH 6.8, 1X PBS, 50 mM Tris-HCl buffer 8.0, etc.).

- *Note:* The calculated volume of required capture antibody is 4.8 mL, according to this protocol.

Day 2: Prepare 50 mL of 1X DELFIA wash solution for the wash steps involved in plate coating.

- Dilute 2 mL of 25X DELFIA wash concentrate in 48 mL of Milli-Q water.
- *Note:* The calculated volume of required 1X DELFIA wash solution is 43.2 mL, according to this protocol.

Prepare 17 mL of 1% BSA blocking solution.

- Dilute 2.3 mL of 7.5% BSA in 14.7 mL of buffer (e.g., 1X PBS or other neutral pH buffer)
- *Note:* The calculated volume of required blocking buffer is 14.4 mL, according to this protocol.

DELFIA assay, for one ½ areaplate:

1. Prepare 200 mL of 1X DELFIA wash solution for assay.
   - Dilute 8 mL of 25X DELFIA wash concentrate in 192 mL of Milli-Q water.
   - *Note:* The calculated volume of required 1X DELFIA wash solution is 172.8 mL, according to this protocol.
2. Dispense 6.5 mL DELFIA assay buffer to clean, 50 mL conical tube if using as diluent for standard curve.
   - *Note:* The calculated volume of required DELFIA assay buffer to prepare the standard curve is 4.96 mL according to this protocol – see Table 5.
3. Prepare 6 mL of 100 ng/mL (or ELISA concentration) biotinylated detection antibody in DELFIA assay buffer.
   - *Note:* The calculated volume of required biotinylated detection antibody is 4.8 mL, according to this protocol.
4. Prepare 6 mL of 100 ng/mL Eu-streptavidin in DELFIA assay buffer.
   - Dilute 6 µL of 100 µg/mL Eu-streptavidin in 6 mL DELFIA assay buffer.
   - *Note:* The calculated volume of required Eu-streptavidin is 4.8 mL, according to this protocol.
5. Dispense 12.5 mL of Enhancement Solution into a clean, 50 mL conical tube for dispensing.
   - *Note:* The calculated volume of required Enhancement Solution is 9.6 mL, according to this protocol.

Step 1: Coating microplates

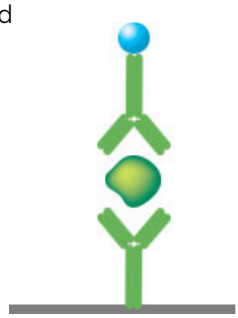
1. Add 50 µL of capture antibody (10 µg/mL, or ELISA coating concentration) to each well.
   - Reconstitute and store antibody according to the data sheet. Antibodies can be prepared in 0.2 M sodium phosphate buffer pH 6.8, 1X PBS, 50 mM Tris-HCl buffer 8.0, or other buffers for coating.

   - We recommend coating with 0.5 µg antibody per well in ½ areaplate format.
   - To optimize an unknown ELISA capture antibody concentration, we recommend testing undiluted, 1-in-2 dilution, 1-in-5-dilution, and 1-in-10 dilution of capture antibody.
2. Cover with a lid or TopSeal-A adhesive seal and incubate overnight at 23 °C to ensure the capture antibody binds to the plate.
3. Wash each well 3 times with 150 µL of 1X DELFIA wash solution or other wash solution.
   - If being done by hand, it is simplest to dispense 150 µL of wash solution per well.
4. Block plates by adding 150 µL of 1X PBS +1% BSA or other blocking solution to each well. We strongly recommend Revvity's DTPA-purified BSA for this step – this BSA is included in the DELFIA Toolbox Kits. Incubate at room temperature on a plate shaker set to a slow speed (300 rpm) for a minimum of 1 hour.
5. Remove blocking solution by inverting the plate and blotting it against clean paper towels. Plates can be sealed with adhesive and used the next day, or dried in a laminar flow hood and stored dry at 4 °C in a foil bag with a desiccant bag.

Step 2: Performing the assay

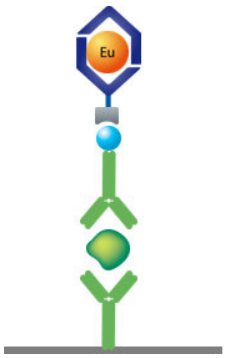
1. Remove adhesive film from the microplate, if the plate has been covered.
2. Add 50 µL of standard analyte or sample to each well. The dynamic range of the assay will be antibody-dependent, but broader than a standard colorimetric ELISA assay. Suggested initial concentrations range from 100 fg/mL to 100 µg/mL. See an example for preparing serial dilutions of analyte in Table 5.
   - Prepare standards and any sample dilutions in the appropriate diluent for the sample type, as identified by linearity and recovery experiments (see "Sample validation" section in this guide).

   - Reconstitute and store standard analyte according to the manufacturer's data sheet.
3. Cover and incubate plate for 2 hours at room temperature on a plate shaker set to a slow speed (300 rpm).
4. Wash each well 3 times with 150 µL of 1X DELFIA wash solution.
5. Add 50 µL of biotinylated detection antibody to each well (100 ng/mL for biotinylated antibody, or ELISA concentration).
   - Prepare working detection antibody solution in DELFIA assay buffer.
   - For optimizing biotinylated antibody concentration, we recommend testing 0.5-50 ng per well in ½ areaplate format.
   - Reconstitute and store detection antibody according to the manufacturer's data sheet.



6. Cover and incubate 1 hour at room temperature on a plate shaker set to a slow speed (300 rpm).

7. Wash each well 3 times with 150 µL of 1X DELFIA wash solution.



8. Add 50 µL of Europium-Streptavidin (100 ng/mL) to each well.
   - Prepare Eu-labeled reagent in DELFIA assay buffer.
   - For optimizing Eu-streptavidin concentration, we recommend testing 0.5-10 ng per well in ½ areaplate format.

9. Incubate 20 minutes at room temperature on a plate shaker set to a slow speed (300 rpm).
   - Cover the plate with a plate lid.
   - Do not cover the plate with TopSeal-A from this point forward, as the adhesive can quench the signal.

10. Wash each well 6 times with 150 µL of 1X DELFIA wash solution.
    - The extra wash steps are necessary for removing any unbound Europium-labeled reagent.

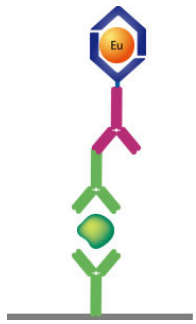
11. Add 100 µL of Enhancement Solution to each well and cover the plate with a plate lid.
    - Remember to not add TopSeal-A or adhesive seal, as this can quench the signal.
    - If the plate is to be stored prior to reading, it is recommended to cover the plate with a lid and add Enhancement Solution just prior needing to read the assay.

12. Incubate at least 5 minutes at room temperature on a plate shaker set to a slow speed (300 rpm).

13. Read plate using TRF settings (refer to "Instrument settings" section).
    - The developed signal will be stable for at least 24 hours at 23-25 °C when stored properly by covering tightly with parafilm.
    - Important Note: seals or tapes with adhesives should be avoided after Enhancement Solution has been added to the plates, as adhesive chemicals can quench the signal.

Table 5: Example of analyte serial dilution for running a standard curve for DELFIA assay.

Tube	Vol. of Analyte (µL)	Vol. of Diluent (µL)	[Analyte] in Standard Curve	
			(g/mL)	(pg/mL)
A	40 µL of 10 µg/mL stock analyte solution	360	1.00E-06	1,000,000
B	150 µL of Tube A	350	3.00E-07	300,000
C	150 µL of Tube B	300	1.00E-07	100,000
D	150 µL of Tube C	350	3.00E-08	30,000
E	150 µL of Tube D	300	1.00E-08	10,000
F	150 µL of Tube E	350	3.00E-09	3,000
G	150 µL of Tube F	300	1.00E-09	1,000
H	150 µL of Tube G	350	3.00E-10	300
I	150 µL of Tube H	300	1.00E-10	100
J	150 µL of Tube I	350	3.00E-11	30
K	150 µL of Tube J	300	1.00E-11	10
L	150 µL of Tube K	350	3.00E-12	3
M (Blank)	0	250	0	0
N (Blank)	0	250	0	0
O (Blank)	0	250	0	0
P (Blank)	0	250	0	0



Protocol 4: DELFIA sandwich immunoassay in ½ areaplate using europium-anti-species antibody

Reagent preparation

Plate coating, for one ½ areaplate:

Day 1: Prepare 6 mL of 10 µg/mL capture antibody in buffer (e.g., 0.2 M sodium phosphate buffer pH 6.8, 1X PBS, 50 mM Tris-HCl buffer 8.0, etc.).

- *Note:* The calculated volume of required capture antibody is 4.8 mL, according to this protocol.

Day 2: Prepare 50 mL of 1X DELFIA wash solution for the wash steps involved in plate coating.

- Dilute 2 mL of 25X DELFIA wash concentrate in 48 mL of Milli-Q water.
- *Note:* The calculated volume of required 1X DELFIA wash solution is 43.2 mL, according to this protocol.

Prepare 17 mL of 1% BSA blocking solution.

- Dilute 2.3 mL of 7.5% BSA in 14.7 mL of buffer (e.g., 1X PBS or other neutral pH buffer).
- *Note:* The calculated volume of required blocking buffer is 14.4 mL, according to this protocol.

DELFIA assay, for one ½ areaplate:

1. Prepare 200 mL of 1X DELFIA wash solution for assay.
   - Dilute 8 mL of 25X DELFIA wash concentrate in 192 mL of Milli-Q water.
   - *Note:* The calculated volume of required 1X DELFIA wash solution is 172.8 mL, according to this protocol.
2. Dispense 6.5 mL DELFIA assay buffer to clean, 50 mL conical tube if using as diluent for standard curve.
   - *Note:* The calculated volume of required DELFIA assay buffer to prepare the standard curve is 4.96 mL according to this protocol – see Table 6.
3. Prepare 6 mL of 2 µg/mL (or ELISA concentration) primary detection antibody in DELFIA assay buffer.
   - *Note:* The calculated volume of required detection antibody is 4.8 mL, according to this protocol.
4. Prepare 6 mL of 200 ng/mL Eu-anti-species antibody in DELFIA assay buffer.
   - Dilute 24 µL of 50 µg/mL Eu-anti-species antibody in 6 mL DELFIA assay buffer.
   - *Note:* The calculated volume of required Eu-anti-species antibody is 4.8 mL, according to this protocol.
5. Dispense 11 mL of Enhancement Solution into a clean, 50 mL conical tube for dispensing.
   - *Note:* The calculated volume of required Enhancement Solution is 9.6 mL, according to this protocol.

Step 1: Coating microplates

1. Add 50 µL of capture antibody (10 µg/mL, or ELISA coating concentration) to each well.
   - Reconstitute and store antibody according to the data sheet. Antibodies can be prepared in 0.2 M sodium phosphate buffer pH 6.8, 1X PBS, 50 mM Tris-HCl buffer 8.0, or other buffers for coating.

- We recommend coating with 0.5 µg antibody per well in ½ AreaPlate format.
- To optimize an unknown ELISA capture antibody concentration, we recommend testing undiluted, 1-in-2 dilution, 1-in-5-dilution, and 1-in-10 dilution of capture antibody.

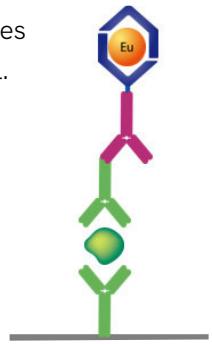
2. Cover with a lid or TopSeal-A adhesive seal and incubate overnight at 23 °C to ensure the capture antibody binds to the plate.
3. Wash each well 3 times with 150 µL of 1X DELFIA wash solution or other wash solution.
   - If being done by hand, it is simplest to dispense 150 µL of wash solution per well.
4. Block plates by adding 150 µL of 1X PBS +1% BSA or other blocking solution to each well. We strongly recommend Revvity's DTPA-purified BSA for this step – this BSA is included in the DELFIA Toolbox Kits. Incubate at room temperature on a plate shaker set to a slow speed (300 rpm) for a minimum of 1 hour.
5. Remove blocking solution by inverting the plate and blotting it against clean paper towels. Plates can be sealed with adhesive and used the next day, or dried in a laminar flow hood and stored dry at 4 °C in a foil bag with a desiccant bag.

Step 2: Performing the assay

1. Remove adhesive film from the microplate, if the plate has been covered.
2. Add 50 µL of standard analyte or sample to each well. The dynamic range of the assay will be antibody-dependent, but broader than a standard colorimetric ELISA assay. Suggested initial concentrations range from 100 fg/mL to 100 µg/mL. See an example for preparing serial dilutions of analyte in Table 6.
   - Prepare standards and any sample dilutions in the appropriate diluent for the sample type, as identified by linearity and recovery experiments (see "Sample validation" section in this guide).
   - Reconstitute and store standard analyte according to the manufacturer's data sheet.

3. Cover and incubate plate for 2 hours at room temperature on a plate shaker set to a slow speed (300 rpm).
4. Wash each well 3 times with 150 µL of 1X DELFIA wash solution.
5. Add 50 µL of primary detection antibody to each well (2 µg/mL of primary detection antibody, or ELISA concentration).
   - Prepare working detection antibody solution in DELFIA assay buffer.
   - For optimizing primary detection antibody concentration, we recommend testing 5-100 ng per well in ½ AreaPlate format.
   - Reconstitute and store detection antibody according to the manufacturer's data sheet.

6. Cover and incubate 1 hour at room temperature on a plate shaker set to a slow speed (300 rpm).
7. Wash each well 3 times with 150 µL of 1X DELFIA wash solution.
8. Add 50 µL of Europium-anti-species antibody (200 ng/mL) to each well.
   - Prepare Eu-labeled reagent in DELFIA assay buffer.
   - For optimizing Eu-anti-species antibody concentration, we recommend testing 5-50 ng ng per well in ½ areaplate format.



9. Incubate 60 minutes at room temperature on a plate shaker set to a slow speed (300 rpm).
   - Cover the plate with a plate lid.
   - Do not cover the plate with TopSeal-A from this point forward, as the adhesive can quench the signal.

10. Wash each well 6 times with 150 µL of 1X DELFIA wash solution.

- The extra wash steps are necessary for removing any unbound Europium-labeled reagent.

11. Add 100 µL of Enhancement Solution to each well and cover the plate with a plate lid.

- Remember to not add TopSeal-A or adhesive seal, as this can quench the signal.
- If the plate is to be stored prior to reading, it is recommended to cover the plate with a lid and add Enhancement Solution just prior needing to read the assay.

12. Incubate at least 5 minutes at room temperature on a plate shaker set to a slow speed (300 rpm).

13. Read plate using TRF settings (refer to "Instrument settings" section).

- The developed signal will be stable for at least 24 hours at 23-25 °C when stored properly by covering tightly with parafilm.
- Important Note: seals or tapes with adhesives should be avoided after Enhancement Solution has been added to the plates, as adhesive chemicals can quench the signal.

Table 6: Example of analyte serial dilution for running a standard curve for DELFIA assay.

Tube	Vol. of Analyte (µL)	Vol. of Diluent (µL)	[Analyte] in Standard Curve	
			(g/mL)	(pg/mL)
A	40 µL of 10 µg/mL stock analyte solution	360	1.00E-06	1,000,000
B	150 µL of Tube A	350	3.00E-07	300,000
C	150 µL of Tube B	300	1.00E-07	100,000
D	150 µL of Tube C	350	3.00E-08	30,000
E	150 µL of Tube D	300	1.00E-08	10,000
F	150 µL of Tube E	350	3.00E-09	3,000
G	150 µL of Tube F	300	1.00E-09	1,000
H	150 µL of Tube G	350	3.00E-10	300
I	150 µL of Tube H	300	1.00E-10	100
J	150 µL of Tube I	350	3.00E-11	30
K	150 µL of Tube J	300	1.00E-11	10
L	150 µL of Tube K	350	3.00E-12	3
M (Blank)	0	250	0	0
N (Blank)	0	250	0	0
O (Blank)	0	250	0	0
P (Blank)	0	250	0	0

Assay optimization (DELFIA sandwich immunoassay)

An existing ELISA assay can easily be converted to DELFIA without optimization, using the same concentrations as in the original ELISA. If desired, the DELFIA assay can be further optimized to improve assay performance or reduce antibody consumption to save costs. All optimizations can be tested using a standard curve run in triplicate.

Microplates

Minimizing the background fluorescence from microplates used to perform the assay through careful selection of the microplate material is critical to achieving high sensitivity and good precision. Microplates provided in the DELFIA Toolbox Kits are high-bind, uncoated plates designed and validated for DELFIA assays. The use of ELISA microplates (e.g., microplates provided in an ELISA kit) can result in high DELFIA assay background signal, and is generally not recommended. In some cases, it may be possible to use an ELISA plate pre-coated with the capture antibody. In this case, the plate background with TRF measurement needs to be evaluated. Background with empty DELFIA clear stripwell or DELFIA yellow microplates plates is 100-200 counts using our EnVision or VICTOR plate readers. Normal background in biochemical DELFIA assays in the same plates is 300-1500 counts using the same plate readers.

Capture antibody

Amount of capture antibody

For direct coating, we recommend utilizing 1 µg of capture antibody per well in 96-well format (0.5 µg of capture antibody per well in ½ areaplate format). This is similar to or lower than the recommended amount of antibody required in ELISA (1-10 µg per well). The enhancement step of the DELFIA assay enables use of less antibody with similar or better sensitivity. If converting from an ELISA where the concentration of the capture antibody directly coated is unknown, we recommend testing undiluted, and diluted (1-in-2, 1-in-5, and 1-in-10) of the capture antibody for direct coating in the DELFIA assay.

Note: For indirect coating to streptavidin- or anti-species antibody-coated DELFIA plates, we recommend testing concentrations of 50 ng, 100 ng, 200 ng, and 400 ng per well of capture antibody (for 96-well plates) and running a titration curve of analyte at each amount of capture antibody.

Immobilizing the capture antibody

The plate coating protocol provided in the "Protocol" sections is an example protocol for plate coating for DELFIA assays. Other plate coating protocols could be used as well. Increasing the amount of antibody coated to the plate may improve assay sensitivity. Note: We strongly recommend Revvity's DTPA-purified BSA (#CR84-100) if blocking with BSA.

When plates are coated with 1 µg of antibody per well, then 150-250 ng of biologically active antibody is typically coated per well. Some of the coated antibody may not be capable of binding. For instance, in direct coating format some antibodies either are difficult to coat or others could lose most of their biological activity during the coating process. In these cases, anti-species coated plates or streptavidin–coated microplates are recommended.

[*Note: some grades of BSA may contain a considerable amount of heavy metals that will eventually show high levels of background in the assay. Hence, use of purified BSA is highly recommended; alternatively, a high grade of casein or gelatin can be used for saturation of the plates. Stabilizer, 7.5% purified BSA (#CR84-100) is available from Revvity.]

Detection antibody

When using biotinylated or unlabeled antibody as the detection antibody we recommend testing 1-200 ng detection antibody per well in 96-well format (0.5-100 ng detection antibody per well in ½ areaplate format). If converting from an ELISA where the concentration of the capture antibody directly coated is unknown, we recommend using undiluted and a 10-fold dilution of the detection antibody in the DELFIA assay.

Note: If Europium-labeling the detection antibody, please see recommendations in the "Eu-labeled tracer" section below for recommended concentrations. We offer a DELFIA Europium labeling kit, #1244-302, for labeling your own reagents.

Eu-labeled tracer

For easy conversion from ELISA to DELFIA, Eu-streptavidin or Eu-anti-species antibody is used as a tracer for detection as each provides a straightforward way to immediately benefit from the advantages of DELFIA. We recommend testing 10-100 ng Eu-antibody or 1-20 ng Eu-Streptavidin per well in 96-well format (5-50 ng Eu-antibody or 0.5-10 ng Eu-Streptavidin per well in ½ areaplate format) using a sandwich or non-competitive assay format.

Assay conditions

Buffers

Ready-made DELFIA buffers designed to minimize non-specific background are recommended for the development of a new DELFIA assay. DELFIA assay buffer and DELFIA wash solution are provided in our DELFIA Toolbox Kits.

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, Revvity #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 (Revvity #1244-114) is optimized specifically for DELFIA assays. Before the addition of Enhancement Solution, six washing cycles are recommended.

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 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 DELFIA Plateshake are presented in Table 7. If a different plate shaker is used, the incubation time after addition of Enhancement Solution should be optimized by reading the plate every five minutes until the signal is stable.

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 DELFIA Enhancement Solution. 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. The fluorescence of the lanthanide chelate is amplified 1-10 million times by this enhancement step.

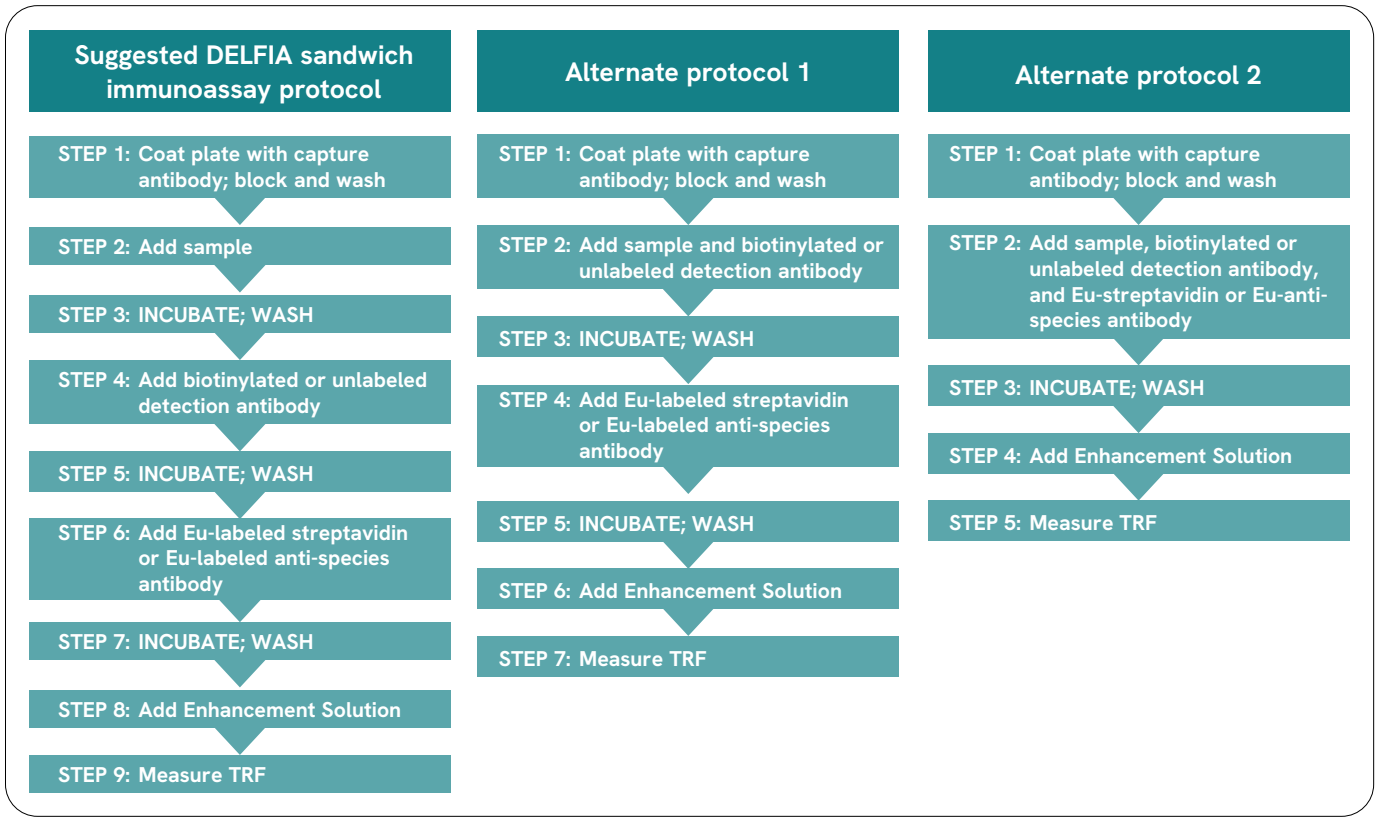
Table 7: Shaking times with DELFIA plateshake to reach 98% maximal signal with wnhancement solution.

DELFIA chelate	Shaking (Slow) (Minutes)	Without shaking (Minutes)
DELFIA Eu-N1 chelate (Eu label in DELFIA ELISA Toolbox)	5	45

Protocol variations

For some assays, the number of wash steps can be reduced by altering the order-of-addition. The effect of alternate protocols on assay sensitivity and reproducibility should be tested beforehand. Additionally, complex sample types may benefit from the additional wash steps provided in the suggested protocol. Examples of DELFIA sandwich immunoassay protocol variations are shown in Table 8.

Table 8: DELFIA sandwich immunoassay protocols.



Evaluating assay performance

The performance of a DELFIA immunoassay is determined by parameters such as sensitivity, dynamic range, reproducibility, linearity, and recovery.

Sensitivity: Lowest amount/concentration of analyte that can be detected in the assay (refer to "Data analysis" section in this guide for more information).

Dynamic range: Range of amounts/concentrations of analyte that can be detected by the assay, defined by the upper limit of detection and lower limit of detection.

Reproducibility, as represented by %CV (coefficient of variation): A statistical method used to evaluate intra- or inter-assay precision, and is used to assess how consistent results will be with the assay (refer to "Data analysis" section in this guide for more information).

Linearity (Linearity of dilution): Linearity refers to the extent to which the dose-response curve for a spike or sample (in a particular diluent) is linear, and helps determine sample compatibility (refer to "Sample validation" section in this guide for more information).

% Recovery: Recovery is used to assess the compatibility of the assay with a particular type of sample (e.g., cell lysates, serum, blood), and also provides a measure of how accurate quantitated results will be with that sample type (refer to "Sample validation" section in this guide for more information).

Data analysis

Standard curve fitting

DELFIA non-competitive (sandwich) immunoassay data can be fit to either a linear curve (using just the linear portion of the data) or a dose-response curve. A dose response curve (or sigmoidal or 4PL curve) is typically used to process DELFIA immunoassay data in order to take advantage of the full dynamic range of the assay (Figure 6). These types of curves can be fit using standard statistical software, such as MyAssays®, GraphPad Prism® or Microsoft® Excel® with Solver plug-in. For dose-response curves, $1/Y^2$ weighting should be used for curve fitting. After fitting a standard curve, the concentration of analyte for the unknowns can be interpolated. Please note that it is recommended to run a new standard curve for each assay.

$1/Y^2$ data weighting is required because errors are not the same across the curve. If the error emerged mainly from the instrument (e.g. ± 1,000 counts everywhere), the weighting wouldn't be required. At the bottom of the curve, values would be 1,000 ± 1,000 and at the top of the curve, values would be 1,000,000 ± 1,000. However, if the error is more a percentage of the signal and emerges from sample handling and experimental variation (e.g. X ± 5%, i.e. 1,000 ± 50 and 1,000,000 ± 50,000), data weighting is recommended.

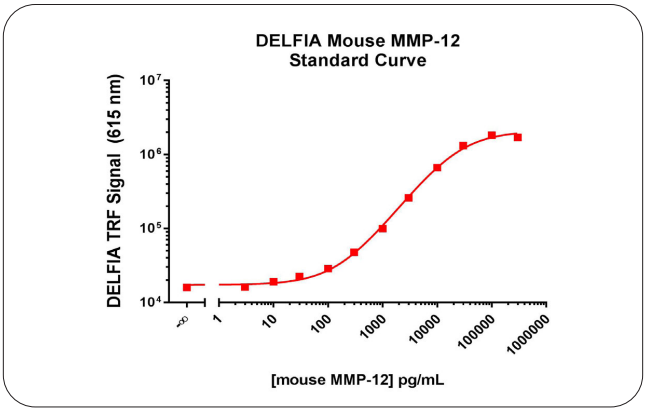


Figure 6: Standard curve in DELFIA assay buffer for sandwich DELFIA assay for mouse MMP-12. The assay was developed using polyclonal anti-mouse MMP12 antibody from R&D Systems (#AF3467) as both the capture and detection antibody. The antibody was directly coated to a 96-well clear stripwell DELFIA plate for capture. The antibody was also biotinylated for use as a detection antibody in conjunction with DELFIA Eu-streptavidin. The plate was measured on an EnVision multimode plate reader using laser settings.

Calculating sensitivity

Using the standard curve, sensitivity (LDL: Lower Detection Limit) for the assay can be calculated using the following equation:

LDL = mean (blanks) + 3 SD

The lower detection limit (LDL or LLD) is equivalent to the concentration interpolated from a signal corresponding to the mean of the "blank" signals + 3 standard deviations (Table 9).

Table 9. Calculation of mean (blanks) + 3 SD. The signal from samples corresponding to 0 ng/mL analyte is recorded. The mean (as shown, 280.5) and the standard deviation (as shown, 33.15332) are calculated. The value corresponding to the mean plus three standard deviations is calculated (as shown, 379.96), which is used as an "unknown". The concentration of analyte corresponding to this signal is then interpolated from the standard curve (not shown).

	Signal	
0 ng/mL Analyte	324	300
	260	308
	304	272
	240	236
Mean (Blank)	280.5	
Standard Deviation	33.15332	
Mean + 3 SD	379.96	

The LLOQ for the assay can be calculated using the following equation:

LLOQ = mean (blank) + 10 SD

The lower limit of quantification (LLOQ) is equivalent to the concentration interpolated from a signal corresponding to the mean of "blank" signals + 10 standard deviations.

Calculating coefficient of variation (%CV)

To determine the variability of the assay, we recommend testing three concentrations of standard analyte (low, medium, and high concentrations) by running 20 replicates of each concentration.
The coefficient of variation (expressed as %CV) is calculated using the following equation:

% CV = (Standard Deviation / Mean) * 100

Control samples

The use of appropriate assay controls enables validation of the assay and eliminates false positive results. Positive and negative controls are also helpful in troubleshooting the DELFIA assay.

Positive control: Use a sample type known to contain endogenous levels of the analyte being detected. This control helps verify the specificity and sensitivity of the antibody(ies) used in the DELFIA assay for analyte.

Negative control: Use a sample that is known to not contain detectable levels of analyte

Standards: Standards with known concentrations of analyte are used as calibrators to enable accurate quantitation of analyte from unknowns (samples). Typically, recombinantly-expressed proteins or synthetic standards are used.

Spike: A "spike" is a control that contains a known amount of standard (analyte) added ("spiked") as a small volume to a larger volume of diluent or sample. Spikes are used in recovery experiments (see "Sample validation" section in this guide).

Sample validation

For quantitation of an analyte in samples, it is necessary to create a standard curve of the analyte in a matrix that matches the samples. For example, when working with RPMI medium-based cell culture supernatants, a standard curve of the analyte should be generated using RPMI. The suitability of the DELFIA assay developed for measuring different sample matrices should be evaluated by testing the assay linearity and recovery. A suitable diluent will exhibit good recovery (values between 70% and 130%) and linearity (no change upon dilution) (method below).

Linearity experiment

1. Spike one of the experimental samples with a high concentration (e.g., 3 ng/mL) of the standard analyte.
2. Perform a two-fold serial dilution of this spike-in using the proposed diluent. At least five dilutions are recommended.
3. In a separate set of tubes, create a standard curve of the standard analyte in the proposed diluent.
4. Run the DELFIA protocol with the standards, spike-in, and spike-in dilutions (in triplicate). Plot the standard data and interpolate the concentration of the undiluted spike-in and respective dilutions within the curve.
5. To determine linearity, plot the interpolated concentrations of the undiluted spike-in and respective dilutions versus the dilution factor (e.g., 1, 0.5, 0.25, 0.125, 0.0625, 0.03125). Perform a linear regression and determine linearity by correlation coefficient (Figure 7).

If good linearity (R2>0.995) is not achieved with the proposed diluents, then two-fold dilutions of the experimental samples would need to be performed to minimize interference from the sample matrix. Subsequently, adjust the calculated final sample concentrations by multiplying by a dilution factor of 2. The standard curve would be tested in the same diluent used for sample dilution.

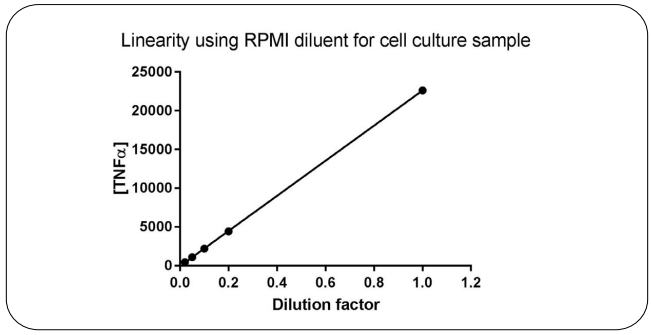


Figure 7: Linearity for a DELFIA TNFα assay using cell culture supernatant samples.

Spike-and-recovery experiment

1. In one set of tubes, spike one of the experimental samples with a low, a medium, and a high concentration of the analyte (include one tube that contains sample only, without spike-in). The low, medium, and high concentrations of analyte should be selected based on the dynamic range of the assay.
2. In a second set of tubes, in the proposed diluent (from linearity experiment), spike with the same concentrations of analyte (include one tube that contains diluent only, without spiked analyte).
3. In a third set of tubes, create a standard curve of the standard analyte in the proposed diluent (refer to step 3 in Linearity Experiment, above).
4. Run the DELFIA protocol with the standards and spike-ins. Plot the standard data and interpolate the concentration of the spike-ins from the curve.
5. Compare the interpolated concentrations of the sample spike-ins and diluent spike-ins (Table 10). Correct the interpolated concentrations of the spiked sample by subtracting the amount of analyte measured in the "No spike" sample from the low, medium, and high spike-in samples. Percent recovery is calculated using the following equation:

$$\text{Percent Recovery} = \frac{\text{Calculated concentration of analyte in spiked sample}}{\text{Calculated concentration of spiked diluent}} \times 100$$

A good diluent will result in a quantitative recovery range (between 70% and 130%).

Table 10: Spike-and-recovery for DELFIA TNFα assay. Untreated serum, heparin, and EDTA plasma samples were spiked with different concentrations of TNFα (500, 100, 20 and 10 pg/mL).

Samples spiked with TNFα	Interpolated concentration	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
Serum 2 + 10 pg/mL	16.4	100
Serum 2 (Unspiked)	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
Heparin Plasma 3 + 10 pg/mL	18.6	112
Heparin Plasma 3 (Unspiked)	7.4	
EDTA-Plasma 3 + 500 pg/mL	553.6	109
EDTA-Plasma 3 + 100 pg/mL	107.1	98.5
EDTA-Plasma 3 + 20 pg/mL	28.8	101
EDTA-Plasma 3 + 10 pg/mL	18	94
EDTA-Plasma 3 (Unspiked)	8.6	

Tips and troubleshooting

General tips for DELFIA assays

Assay Step	Do	Don't
General	Allow reagents to reach room temperature (20 to 25 °C) before performing an assay.	Do not use microplates with high fluorescent background. Background with DELFIA clear stripwell or DELFIA yellow microplates plates is 100-200 counts using our EnVision or VICTOR plate readers. Normal background in biochemical DELFIA assays in the same plates is 300-1500 counts using the same plate readers.
	Avoid Europium contamination and resulting high fluorescent background through careful pipetting and washing techniques. Use optimized DELFIA assay buffers to ensure good results.	
Tracer	Store labeled proteins and peptides at high concentration and in the absence of chelators or competing metals in the buffer. In most cases, 50 mM Tris-HCl buffer saline (pH 7.5-8.0) containing 0.1-0.5% purified BSA will ensure stability of the labeled compound during storage.	Do not expose the Eu-labeled reagent (tracer) to chelating agents like EDTA.
	Avoid carry-over when pipetting a Eu-labeled reagent (tracer) solution by holding the pipet tip slightly above the top of the well and avoiding touching the plastic strip or the surface of the liquid.	Do not store labeled proteins or peptides in DELFIA assay buffer or phosphate buffers.
Enhancement	Use 200 µL of Enhancement Solution for 96-well and 50 µL for 384-well plates.	Do not dispense Enhancement Solution from labware which might have been contaminated with Europium.
	Pour the required amount of Enhancement Solution into a 15-mL or 50-mL conical tube. The conical tube and tip for dispensing should be dedicated for this purpose, and should be stored properly in a sealable plastic bag to prevent contamination.	Do not store Enhancement Solution in glassware.
	Use a dedicated Eppendorf Multipipette or another pipetting instrument for the Enhancement Solution and discard the first aliquot.	Do not seal the plate with tape after the addition of the Enhancement Solution. The adhesive might quench the signal.
	Dispense the Enhancement Solution slowly to avoid air bubbles.	Do not use the same reservoir for Enhancement Solution and Eu-labeled reagent (tracer).
	Flush pipet or dispenser tips and tubing thoroughly with DELFIA Enhancement Solution prior to use.	
	Protect the plate from dust with a plate lid.	
	Add the Enhancement Solution just prior to shaking and measuring the plate.	
Washing and shaking	Optimize the number of wash cycles.	Do not shake too vigorously, which may cause air bubbles that interfere in the fluorescence measurement.
	Use an automatic plate wash 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.	
Measurement	Measure with a sensitive time-resolved plate reader such as a VICTOR Nivo, VICTOR X, EnSight, or EnVision.	Do not measure plates with their plate covers.

Troubleshooting DELFIA assays

Problem	Cause	Solution
High background signal	Background of the plain plate is >500 (no coating, no buffer, no Enhancement Solution)	Change the plates. We recommend using DELFIA-branded plates for DELFIA assays. Regular ELISA plates may exhibit high background when measuring TRF signal.
	Inadequate washing prior to measurement	Use 4-6 washing cycles in a DELFIA Platewash or other plate washer after incubation with Eu-labeled compound.
	Inadequate blocking of the plates	Saturate overnight at room temperature, or >2 hours at 37 °C.
		Use a saturation volume greater than that of the coating solution.
	Binding of the Eu-labeled polyclonal antibody to the plate	Use recommended DELFIA buffers.
	Contamination of pipettes and bench spaces with label	Clean pipettes and bench spaces carefully.
	Cross-reactivity of antibodies	Consider alternative assay formats.
Inadequate sensitivity	High background	See above.
	Low maximal signal	Increase the sample volume.
		Use two different tracer antibodies.
		Increase the antibody concentration in a sandwich assay.
		Change the antibody used.
Poor reproducibility	Inadequate incubation with Enhancement Solution	Incubate for at least 5 minutes on a shaker before measurement. Check the 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 or the incubation time, check the coating procedure, etc.
	Uneven coating	Test with Revvity DELFIA microplates.
	Plate sealed with tape during the enhancement and measurement steps	Do not cover the plate containing Enhancement Solution with tape.
	Trace amounts of Eu in sample	Add 20 µM DTPA or EDTA to the assay buffer, or use pre-optimized DELFIA buffers.
	Labeled antibody stored at wrong temperature	Optimize the 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 (catalog number CR84-100).
	Unsuitable storage conditions	Do not expose the Eu-labeled antibody to chelating agents like EDTA or phosphate-based buffers during storage. Store a 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 the reagents.

Instrument settings

DELFIA assays require a plate reader that is capable of TRF (time-resolved fluorescence) detection. This is a time-resolved fluorescence intensity assay. We recommend using a Revvity VICTOR Nivo™, VICTOR X, EnSight™ or EnVision™ multimode plate reader with factory installed and tested measurement protocols (view our Instrument product pages). If using a plate reader from another manufacturer, Revvity's Europium standard solution (catalog #B119-100) can be used. A serial dilution in DELFIA Enhancement solution should be performed to determine the sensitivity of the instrument. We recommend checking with the manufacturer of the plate reader for additional suggested instrument settings if a plate reader manufactured by another company is used.

Fluorometers (Filter Fluorometer) vs. Spectrofluorometers/Monochromators

We recommend a time-resolved filter-based plate reader for DELFIA assays. Very sensitive time-resolved spectrofluorometers may be satisfactory for certain assays. Most plate readers are capable of TRF-type assays; a flash lamp is needed for TRF analysis, which is an option on most readers. One could also use laser excitation on an appropriate EnVision instrument.

- A TRF filter fluorometer plate reader utilizes bandwidth filters in front of the fiber optic for excitation and in front of the detector for emission wavelength choice. The grating-type spectrofluorometers are generally less sensitive than filter fluorometers, thus can compromise sensitivity with highly sensitive assays such as TRF.
- A TRF-capable spectrofluorometer utilizes a monochromator (usually an excitation and emission grating) to discriminate wavelength positions of study for fluorescence excitation and emission. Spectrofluorometers can be up to 1000-fold less sensitive than filter fluorometers, though spectrofluorometers may work in some DELFIA applications.
- The new double-monochromator instruments claim to have almost similar sensitivity as the 'original' filter readers. These may be suitable for most DELFIA assays.
- For cell-based assays, top illumination is recommended, as some clear-bottom plates may absorb excitation at 340 nm. Top illumination is required with white and black plates.

DELFIA instrument settings when using DELFIA Europium

Generic default instrument settings are recommended for all DELFIA Europium assays. If using instrument from another manufacturer, it is recommended to review additional requirements for DELFIA assays. Table 11 indicates recommended settings for Revvity instruments.

Table 11: Revvity multimode plate reader settings for DELFIA measurements.

	EnVision	VICTOR Nivo	EnVision Nexus
			
Excitation	320 or 340 nm	320 or 340 nm	330 nm DELFIA module for Flash lamp : HH36794001 DELFIA module for Eu Laser : HH36794006
Emission	615 nm	615 nm	615 nm
Delay	400 µsec	400 µsec	400 µsec
Window	400 µsec	400 µsec	400 µsec
Cycle Time (lamp)	2000	N/A	
Flash Energy	N/A	10 µJ	
Measurement Time			400 msec
Laser Excitation Cycle Time	16600		
Number of Windows	1		1

Microplates

DELFIA Toolbox Kits contain DELFIA uncoated stripwell plates or ½ areaplates that are high protein binding. Other plates are also available for DELFIA assays (Table 12).

Clear, white, black, or DELFIA yellow plate colors can all be used for DELFIA TRF assays. Because the time-resolved measurement decreases background autofluorescence, clear, black, or white plates are equally suitable. DELFIA yellow plates were specifically designed for best performance with DELFIA assays. If working in 384-well format, we recommend using white plates to help maximize signal.

Typically, the total signal and sensitivity is better using plates directly coated with antibody rather than with streptavidin or anti-species antibody. However, antibody consumption is significantly higher in direct coating (e.g. 1 µg of antibody/well in 96-well format) than in indirect coating (e.g. 200 ng of biotinylated antibody/well in 96-well format), and using coated plates with secondary antibody or streptavidin may often be the only viable option due to cost or limited availability of a specific antibody.

Table 12: Standard coated and uncoated plates for DELFIA assays

Product	Well format	Sample volume (µL)	Wash volume (µL)	Enhancement solution volume (µL)	Catalog number	Number of plates
½ areaplate, high bind (uncoated)	96-well ½ Area	50	150	100	6002520	50
DELFIA yellow plate, uncoated*	96-well	100	300	200	AAAND-0001	60
DELFIA streptavidin-coated yellow plate	96-well	100	300	200	AAAND-0005	10

Table 12: Standard coated and uncoated plates for DELFIA assays (continued)

Product	Well format	Sample volume (µL)	Wash volume (µL)	Enhancement solution volume (µL)	Catalog number	Number of plates
DELFIA streptavidin-coated clear plate	96-well stripwell	100	300	200	4009-0010	10
DELFIA streptavidin-coated white plate	384-well	25	90	50	CC11-H10	10
DELFIA anti-mouse coated clear plate	96-well stripwell	100	300	200	4007-0010	10
DELFIA anti-rabbit coated yellow plate	96-well	100	300	200	AAAND-0004	10
DELFIA anti-rabbit coated clear plate	96-well stripwell	100	300	200	4008-0010	10
DELFIA anti-sheep coated yellow plate	96-well	100	300	200	CC33-1210	10
OptiPlate, high bind (uncoated)	96-well (Black)	100	300	200	6005320	50
					6005329	200
	96-well (White)	100	300	200	6005500	50
					6005509	200
OptiPlate, high bind (uncoated)	384-well (White)	25	90	50	6005620	50
					6005629	200

**Uncoated DELFIA plates are high-bind plates that can be used for direct plate-coating procedures (coating with proteins, antibodies, etc.).*

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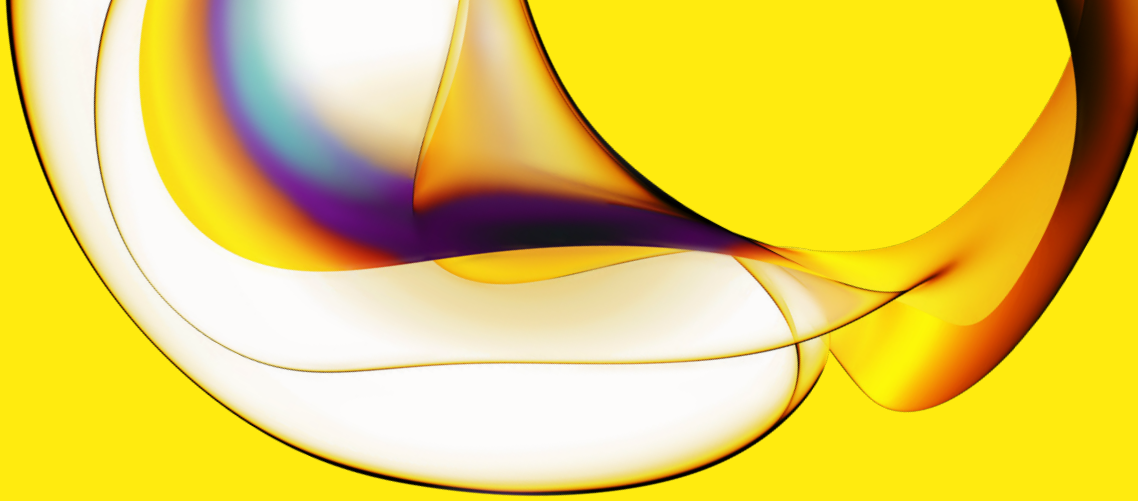

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