APPLICATION NOTE

Simple conversion of ELISA to high sensitivity DELFIA technology.

Authors

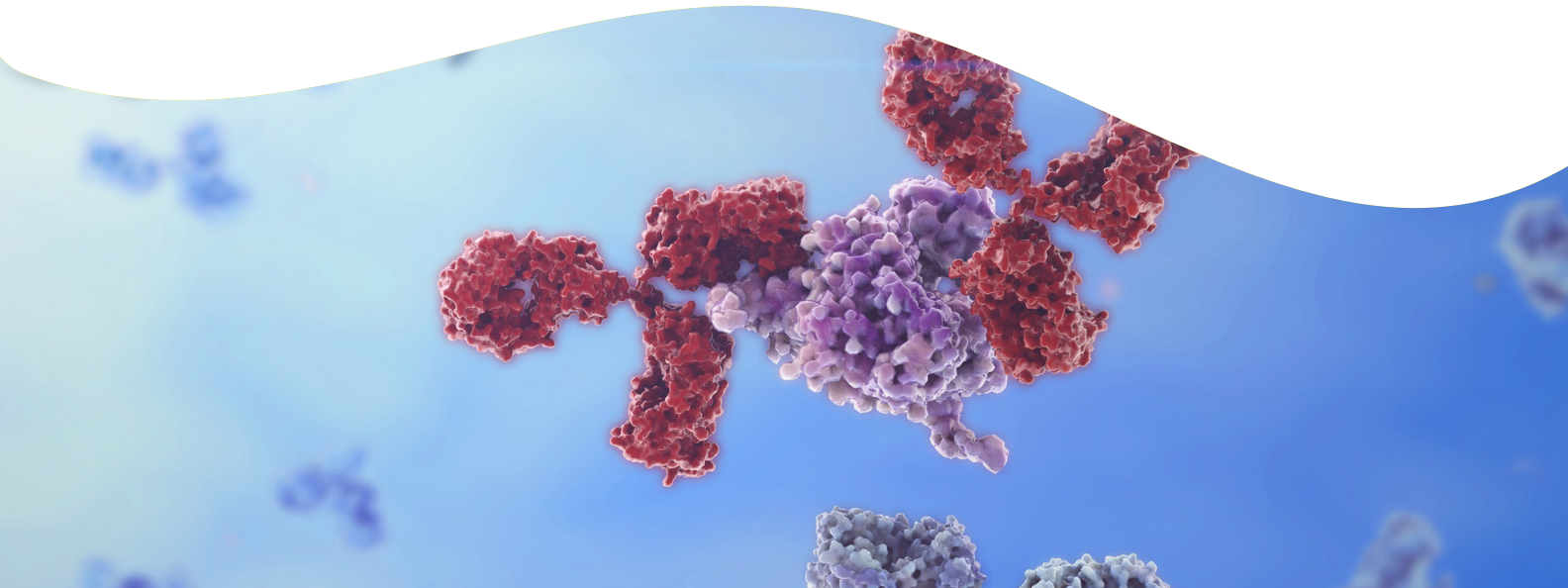
Matthew Marunde
Stephen Hurt

Revvity, Inc.

Introduction

Immunoassays are a highly utilized set of methods to quantify and detect specific biological molecules. These assays are particularly useful when detecting biomarkers present in complex biological matrices (i.e. human serum, cerebrospinal fluid, or blood) where an abundance of other proteins and molecules are present. One traditional technology used is enzyme linked immunosorbent assays (ELISA) which uses a microplate-bound capture antibody and an enzyme-labeled detection antibody. In the assay, both antibodies bind, sandwiching an antigen, and then a substrate is added to generate color, which is later measured by an absorbance reader generating a signal that is proportional to the amount of the target molecule present.

Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIA®) technology is very powerful, providing highly sensitive and robust immunodetection based on time resolved fluorescence (TRF). This detection method, similar to ELISA, is special due to its unique chemical properties of lanthanide metals that incur DELFIA with several distinct advantages over ELISA technology. DELFIA affords a combination of improved sensitivity, wider dynamic range, and easily developed signal that does not require a stop solution and is stable and long lasting, allowing samples to be read long after assay completion.



Here we demonstrate and provide clear instructions to simply and swiftly convert ELISA to DELFIA technology. There are thousands of R&D Systems DuoSet® ELISA antibody pairs that can be readily converted to DELFIA assays to provide a higher sensitivity assay (lower LDL) with broader dynamic range assay (3 - 5 log). We purchased Human IL-2 (DY202) and Mouse IL-5 (DY405) ELISA DuoSets® from R&D Systems. Each DuoSet® provides the necessary capture antibody, biotinylated detection antibody, and standard analyte for straightforward conversion to DELFIA. Capture antibodies were either directly adsorbed (direct coating) or indirectly captured to a 96-well microplate. Then followed by the addition of standard antigen and biotinylated detection antibody to the microplate. Subsequently, streptavidin- Europium was added to each well. A final dissociation step is then performed with Enhancement Solution to release and activate europium. Light emission is generated by excitation of europium at 340 nm to generate stable and robust 615 nm signal proportional to antigen presence (Figure 1).

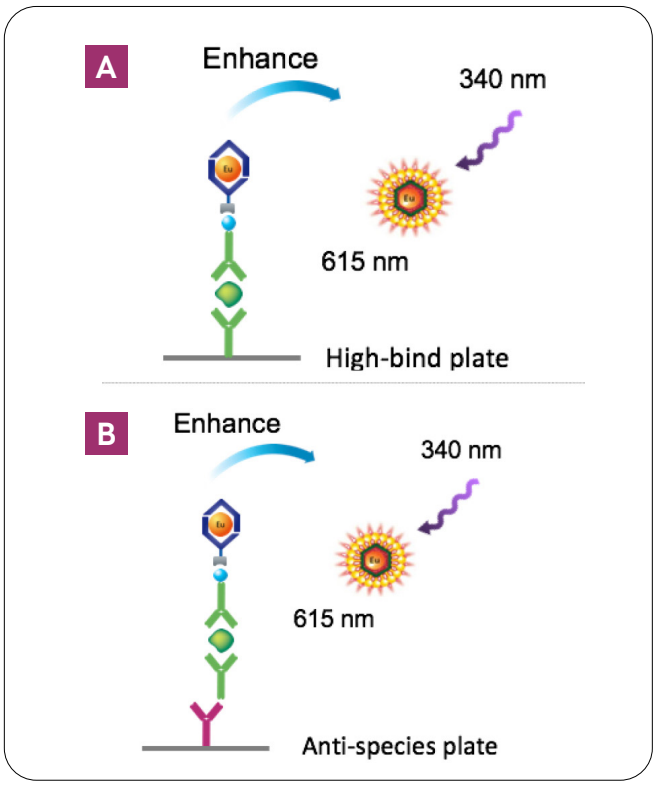


Figure 1: DELFIA TRF assay. A) Direct assay configuration using capture antibody directly-adsorbed to a high bind DELFIA plate. Biotinylated-detection antibody binds to DELFIA Europium-streptavidin. Addition of Enhancement Solution releases Europium to form a new fluorescent chelate, that can be excited at 340 nm to emit signal at 615 nm. B) Indirect assay configuration using a pre-coated anti-species DELFIA plate (anti-mouse IgG). The mouse IgG capture antibody binds to the anti-mouse IgG pre-coated plate.

Conversion and performance of human IL-2 and mouse IL-5 DELFIA assays

DELFIA immunoassays for Human IL-2 and Mouse IL-5 were performed with R&D Systems DuoSets® for Human IL-2 (R&D Systems #DY202) and Mouse IL-5 (R&D Systems #DY405) using the recommended protein concentrations and amounts suggested in the kit manuals. All proteins and analytes were reconstituted according to the ELISA kit manuals. All buffers were warmed to room temperature (RT) before use. For direct adsorption assays, capture antibody was diluted in 1X

Materials and methods

Table 1: List of materials and instrumentation necessary for ELISA conversion to DELFIA.

Item Name		Catalog #	
		5-plate assay	15-plate assay
R&D Systems DuoSet® Human IL-2		DY202-5	DY202
R&D Systems DuoSet® Mouse IL-5		DY405-5	DY405
Microplates	DELFIA yellow plates (direct adsorption)	AAAND-0001	
	DELFIA anti-mouse IgG plates	4007-0010	2 x 4007-0010
Plate seals and covers	TopSeal-A Plus	6050185	
	Plate covers	6000027	
DTPA purified BSA (for blocking plate after direct adsorption)		CR84-100	
DELFIA Wash Buffer, 25X (or use your own)		1244-114 (250 mL)	
DELFIA Assay Buffer (or use your own)		1244-106 (50 mL)	1244-111 (250 mL)
DELFIA Enhancement Solution		1244-105 (250 mL)	2 x 1244-105 (250 mL)
DELFIA Europium-Streptavidin		1244-360	
DELFIA plate shaker (optional) *Recommended for decreasing incubation times		1296-003 (240 volt for Europe use) 1296-004 (120 volt for US use)	
DELFIA plate washer (optional) *Recommended for consistency or multiple plates		1296-0010	
TRF-capable plate reader		We recommend using a Revvity Victor®, EnVision®, EnSpire®, or EnSight® multimode detector	

Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium (GIBCO #14190144) and 100 µL was added to each well of yellow DELFIA 96-well plate (Revvity #AAAND- 0001), incubated overnight at RT, and covered with Topseal-A PLUS (Revvity #6050185). For indirect capture assays, add 100 µL of capture antibody diluted in 1x DPBS and incubate two hours at RT on a plate shaker set to a slow speed (~300 rpm). Afterwards, wells were washed three times with 200 µL of 1x DELFIA wash buffer (Revvity #1244-111). For consistency, we recommend using a plate washer (Revvity #1296-0010). Next for the direct assay, each well was blocked with 200 µL of 1X DPBS supplemented with 1% BSA for at least one hour at RT on a plate shaker. Note, BSA quality is critical for performance and we recommend Revvity #CR84-100. The blocking step is not required for indirect assays. Repeat plate washing procedure. Add 100 µL of standard or sample prepared in DELFIA assay buffer (Revvity #1244-111) to each well and incubate for two hours at RT on a plate shaker. Repeat plate washing procedure. Add 100 µL of biotinylated detection antibody solution prepared in DELFIA assay buffer to each well and incubate for one hour at RT on a plate shaker. Repeat plate washing procedure. Add 100 µL of 100 ng/mL Europium-Streptavidin (Eu-SA, Revvity #1244-360) prepared in DELFIA assay buffer to each well and incubate 20 minutes at RT on a plate shaker. Important, once Eu-SA has been added it is recommended to not use TopSeal-A and use a plate cover instead from this point on. Repeat wash procedure two times through for a total of six well washes. Add 200 µL of Enhancement Solution to each well, cover with a plate lid (no top seal), and incubate at least five minutes at RT on a plate shaker prior to reading TRF signal using a 2104 EnVision equipped with a flash lamp and necessary optics (see Table 2). See the Appendix for checklist protocol. If the plate is to be stored, it is recommended to store after washing away unbound Eu-SA and prior to the addition of Enhancement Solution.

Instrumentation

DELFIA assays require a TRF-capable plate reader that can excite at 320 or 340 nm and measure emission at 615 nm. In this example, DELFIA assays were measured on an EnVision-2104 multimode plate reader. DELFIA Europium time resolved fluorescence signal was measured using the appropriate equipment and parameters highlighted in Table 2.

For TRF, the EnVision reader includes flash lamp based excitation as standard and a high energy laser option is available for higher speed and sensitivity. The laser delivers a faster decaying pulse that enhances long-lived dye signal, but decays completely by the time of measurement. In comparison, a Xenon flash lamp excitation pulse decays more slowly, with increased background during measurement. The TRF laser gives superior signal-to-noise ratios and exceptionally fast measurement times.

In addition to TRF, the EnVision system detects 12 assay technologies including Alpha technology, fluorescence intensity, fluorescence polarization, ultra-sensitive luminescence and absorbance. To validate the instrument, 1 nM Europium standard solution (#B119-100) was used to perform plate optimization.

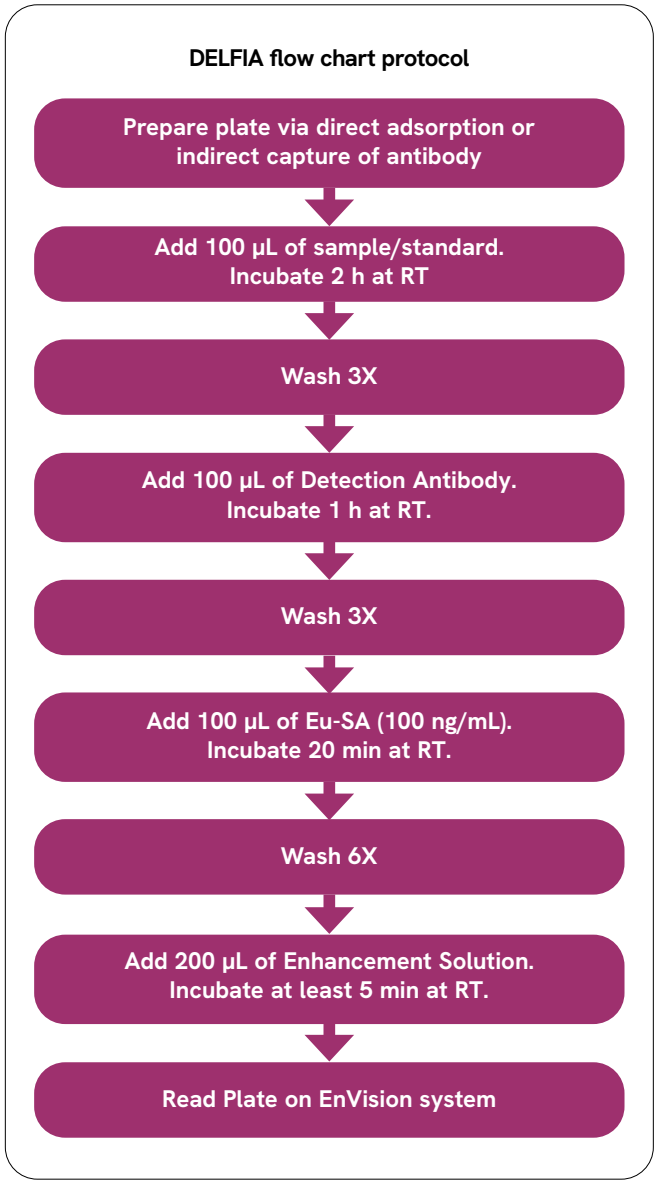


Figure 2: EnVision multilabel plate reader.

Table 2: EnVision optics and optimal measurement parameters for DELFIA.

Excitation source	Flash lamp	TRF laser unit (337 nm)
Top Mirror	#402 (D400)	#446 (D400/D630)
Excitation Filter	#101 (X340)	Not Applicable.
Emission Filter	#203 (M615)	#203 (M615)
Measurement Height (mm)	6.5	6.5
Excitation Light (%)	100	100
Delay (µs)	400	400
Window time (µs)	400	400
Time between flashes (µs)	2000	2000
Number of flashes	100	100

Standard curve and data analysis

Standard curves for each converted DELFIA immunoassay were performed using recombinant proteins provided in the DuoSet® kits or Revvity Human IL-2 (#AL221S) and Mouse IL-5 (#AL569S). Curves were plotted in GraphPad Prism Version 7.0 according to nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and the $1/Y^2$ weighting method. Lower limit of detection (LDL) was calculated using the following equation:

LDL = mean (blanks) + 2 * SD

Results

1. Human IL-2 and Mouse IL-5 ELISA DuoSets® can be easily converted to DELFIA

Both human IL-2 and mouse IL-5 ELISAs DuoSets® were acquired from R&D Systems. Antibody concentrations were used as suggested in the ELISA protocol and altered to incorporate the use of Eu-SA for conversion to DELFIA instead of the horseradish peroxidase enzyme for colorimetric ELISA. As shown in Figure 3A and B human, IL-2 was successfully converted to a direct orientation DELFIA where the mouse monoclonal capture antibody was directly adsorbed to a 96-well DELFIA plate. The human IL-2 DELFIA assay displayed an assay sensitivity of 1.2 pg/mL and R^2 value of 0.996. In Figure 3C and D, mouse IL-5 was also successfully converted to a direct adsorption DELFIA displaying an assay sensitivity of 10 pg/mL and R^2 value of 0.998.

2. Human IL-2 DELFIA delivers high performance in both direct and indirect orientations

Another way to orient the assay is to indirectly capture one of the antibodies to the microplate. Indirect capture assays provide the benefit of only requiring a short incubation to attach the capture antibody to the plate as opposed to an overnight incubation for direct assays. One approach to design an indirect assay is through the utilization of anti-species antibodies and is the easiest way to transition converted DELFIA assays into the indirect capture format. However, this indirect format is only possible when the capture and detection antibodies are differing species. In the case of human IL-2, the capture antibody is a mouse IgG and the detection antibody a goat IgG, thus an anti-mouse IgG plate can be used for an indirect binding assay. In the case of mouse IL-5, a rat polyclonal antibody is used as both the capture and detection antibody limiting the assay to a direct orientation. Figure 4 compares Human IL-2 performed directly and indirectly using the same antibody, analyte concentrations and volumes with a yellow 96-well or clear 96-well anti-mouse IgG microplate, respectively. When the capture antibody was directly adsorbed to the plate, the assay demonstrated a sensitivity of 1.2 pg/mL, R^2 value of 0.996, signal-to-background (S/B) of 61 at 3000 pg/mL and dynamic range between 3000 pg/mL – 1.4 pg/mL, while indirectly bound antibodies elicited sensitivity of 6.8 pg/mL, R^2 value 0.993, S/B of 24 at 3000 pg/mL and the same dynamic range as the direct assay.

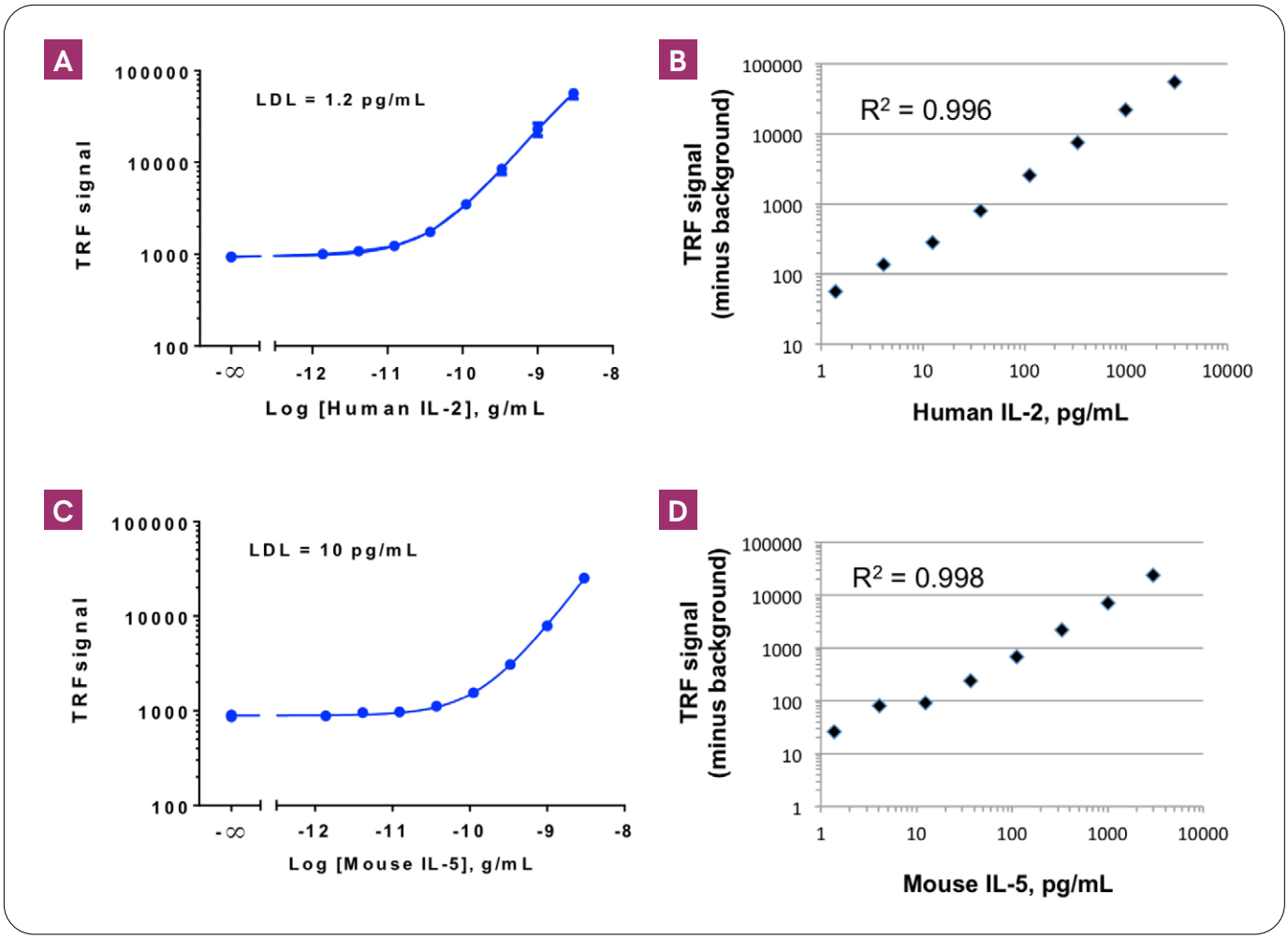


Figure 3: Conversion of R&D Systems ELISA DuoSet® for human IL-2 and mouse IL-5 to DELFIA technology. Both assays were performed in yellow 96-well DELFIA plates. A and B demonstrate standard curves for human IL-2 presented using non-linear regression and linear regression, respectively. C and D demonstrate standard curves for mouse IL-5 presented using non-linear regression and linear regression, respectively. DELFIA assay was performed with manual washing. Assays were run using the R&D DuoSet® standard analytes.

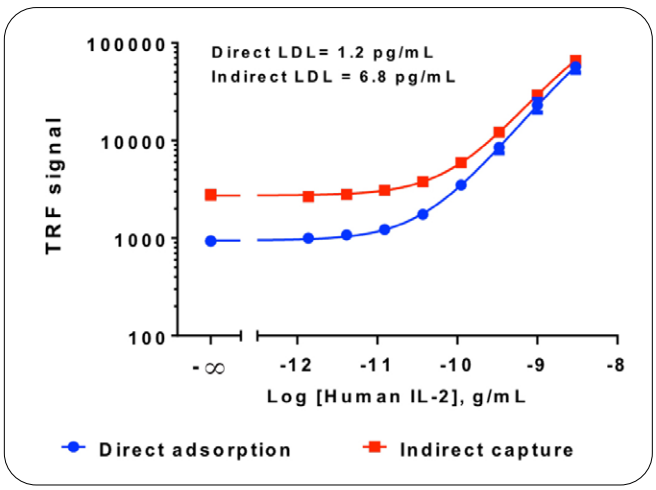


Figure 4: Comparison of human IL-2 performed using a direct adsorption assay (blue) in a yellow 96-well DELFIA plate, and indirect capture (red) in a clear 96-well anti-mouse IgG stripwell plate. Assays were run using the R&D Systems DuoSet® analytes.

3. DELFIA converted assays show as good or improved sensitivity compared to ELISA

DELFIA is a highly sensitive technology that can confer improved sensitivity. Assay sensitivity is the lowest detectable concentration of antigen in the assay, calculated by interpolating the mean of background + 2 x standard deviation. Comparing the sensitivity performance between converted DELFIA assays and the performance published by R&D Systems ELISAs, we observed ~ 13-fold improved sensitivity for human IL-2 and ~ 3-fold improved sensitivity for mouse IL-5 using the suggested protein amounts in the ELISA protocol (Table 3). Further, the human IL-2 performed using the indirect orientation also afforded low sensitivity.

Table 3: Comparison between sensitivity results for each orientation tested for the same ELISAs converted to DELFIA technology. All antibodies and analytes used for the comparison were from the R&D System DuoSet®. All sensitivities are reported as pg/mL.

Antigen	Orientation	DELFIA	ELISA
Human IL-2	Direct	1.2	15.6
Human IL-2	Indirect	6.8	N/A
Mouse IL-5	Direct	10.0	31.2

4. DELFIA technology incurs enhanced dynamic range for human IL-2 and mouse IL-5

One significant advantage of DELFIA is the wide dynamic range it can offer compared to ELISA technology. A broad dynamic range increases the likelihood that your sample concentration will fall within the curve, preventing the hassle of having to repeat assays due to missed detection windows. A broad dynamic range also reduces or eliminates the extensive sample preparation. For both human IL-2 and mouse IL-5, using Revvity analytes, the standard ranges were able to be extended from the ELISA suggested range of 2000 – 31.3 pg/mL to 100,000 – 1 pg/mL for human IL-2 and from 1000 – 15.6 pg/mL to 100,000 – 1 pg/mL for mouse IL-5 (Figure 5).

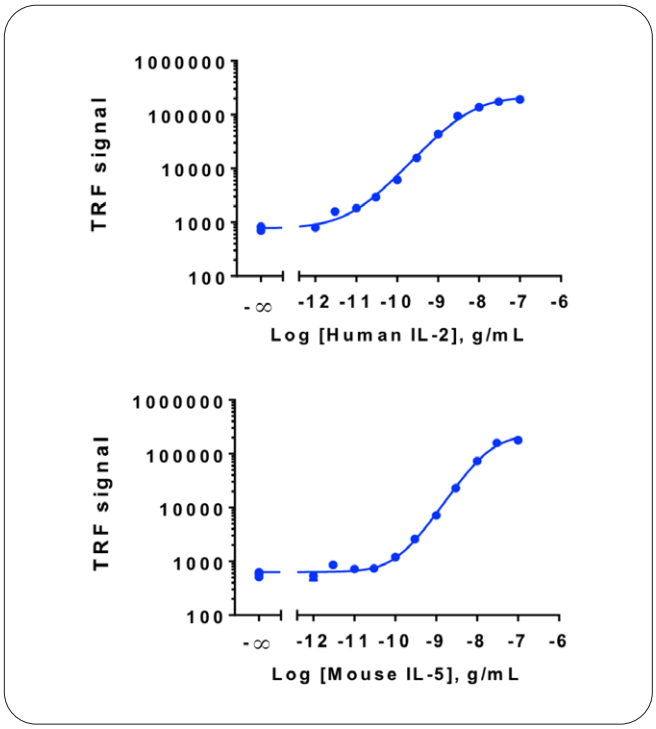


Figure 5: Both human IL-2 and mouse IL-5 demonstrated much broader dynamic range compared to the ELISA assay. On the left, human IL-2 DELFIA was performed using Revvity's human IL-2 (#AL221S) analyte. On the right, mouse IL-5 DELFIA was performed using Revvity's mouse IL-5 (#AL569S) analyte. Data depicted using direct adsorption assay format.

5. DELFIA demonstrates highly stable signal with no change after 48 hours

Another advantage of DELFIA is the stability of the assay, which requires no stop solution. The human IL-2 assay converted to DELFIA was used to perform standard curve in triplicate with indirect capture using anti-mouse IgG clear 96-well strip plates. Once the Enhancement Solution was added and incubated on a DELFIA plate shaker for 15 minutes, the plate was read. To prevent evaporation, the plate was covered with parafilm and a plate lid until the next read time; note that no cover or seal with adhesive should be used for storage. Adhesives from the seals can significantly quench fluorescence signal and will affect assay results. The assay demonstrates stable signal for at least 24 hours, with calculated sensitivities of 5.1, 2.0, 3.3, 4.1, 3.5, and 4.0 pg/mL at the immediate, 1, 2, 4, 24, and 48 hour time points and no change in signal to background (Figure 6).

Conclusion

In this application note, we were able to successfully and simply convert both human IL-2 and mouse IL-5 ELISA DuoSets® to DELFIA time-resolved fluorescence assays. The ELISA antibodies were used to create functional DELFIA assays by simply replacing the Streptavidin-HRP with the Europium-Streptavidin reagent. In compatible kits, we demonstrated the ease of switching from direct to indirect orientation using anti-species IgG plates. Further, we showed that switching from ELISA to DELFIA technology with no further optimization can afford the improved sensitivity, wider dynamic range and long lasting stable signal that DELFIA technology is known for.

There are thousands of different ELISA DuoSets® available f rom R&D Systems. These data demonstrate how easily ELISA DuoSets® can be converted using the protocols provided to incorporate DELFIA technology and instantly harness the many benefits of DELFIA to empower your research.

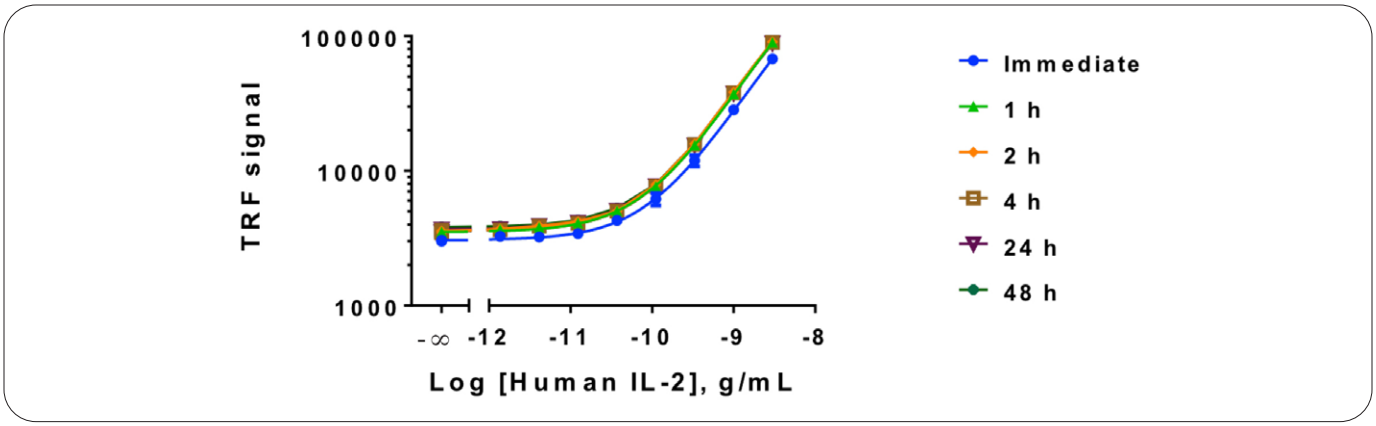


Figure 6: DELFIA signal is highly stably with no change after 48 hours. Human IL-2 was performed in an indirect orientation using clear 96-well anti-mouse IgG strip well plates.

Appendix

Converting R&D systems ELISA DuoSet® to DELFIA technology

Step 1. Preparing your microplates
Indirect coating of capture antibody:

- Add 100 µL of antibody to each well
  - Reconstitute and store antibody according to data sheet
  - Determine the amount of ng/well from the R&D system protocol or your optimized values
- Incubate 2 hours plate shaker to ensure plates bind the capture antibody
- Wash x three times with 1x DELFIA wash solution
  - We recommend using a plate washer for consistency. If being done by hand it is simplest to dispense 200 – 300 µL of wash solution per well.

Direct adsorption of capture antibody:

- Add 100 µL of antibody to each well in DPBS without the presence of a carrier protein.
  - Reconstitute and store antibody according to R&D Systems data sheet.
  - Determine the amount of ng/well from the R&D system protocol or your optimized values
- Incubate overnight at room temperature
- Wash x 3 times with 1x DELFIA wash solution
  - We recommend using a plate washer for consistency. If being done by hand, it is simplest to dispense 200 – 300 µL of wash solution per well.
- Block the plate with 200 µL of DPBS + 1% BSA in each well
  - We recommend using Revvity CR84-100
- Incubate with blocking solution for at least 1 hour at room temperature on a plate shaker
- Wash x 3 times with 1x DELFIA wash solution

Step 2. Performing the assay

- Add 100 µL of standard analyte or sample to each well.
  - Prepare standards and any sample dilutions in DELFIA assay buffer
  - Reconstitute and store standard analyte according to data sheet
- Incubate 2 hours at room temperature on a plate shaker set to a slow speed (300 rpm)
- Wash x 3 times with 1x DELFIA wash solution
- Add 100 µL of biotinylated detection antibody to each well
  - Prepare working detection antibody solution in DELFIA assay buffer
  - Determine the amount of ng/well from the R&D system protocol or your optimized values
  - Reconstitute and store detection antibody according to data sheet
- Incubate 1 hour at room temperature on a plate shaker set to a slow speed (300 rpm)
- Wash x 3 times with 1x DELFIA wash solution

- Add 100 µL of Europium-Streptavidin (100 ng/mL)
  - Eu-SA solution stock concentration is 100 µg/mL
  - Prepare in DELFIA assay buffer
- 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 from this point forward
- Wash x 6 times with 1x DELFIA wash solution
  - The extra wash steps are necessary for removing any unbound Eu-SA
- Add 200 µL of Enhancement Solution and cover with a plate lid
  - If the plate is to be stored prior to reading, it is recommended to cover the plate and add Enhancement Solution just prior needing to read the assay.
- Incubate at least 5 minutes at room temperature on a plate shaker set to a slow speed (300 rpm)
- Read plate using TRF settings (see Table 2 in Materials and Methods). We recommend the EnVision system.
  - The developed signal will be stable for at least 24 hours when stored properly by covering tightly with parafilm. Note that seals or tapes with adhesives should be avoided after Enhancement Solution has been added to the plates.

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
(800) 762-4000
www.revvity.com