# Optimization and miniaturization of DELFIA TRF assays converted from ELISA.

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## 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 (e.g. human serum, cerebrospinal fluid, or blood) where an abundance of other proteins and molecules are present. Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIA®) is a very powerful immunoassay technology, providing highly sensitive and robust immunodetection based on time resolved fluorescence (TRF). This detection method is similar to a traditional ELISA, however DELFIA's utilization of lanthanide metals endows DELFIA with several distinct advantages over ELISA technology. DELFIA affords a combination of improved sensitivity, wide dynamic range, easily developed signal that does not require a stop solution, and long lasting durable signal that allows samples to be read long after assay completion.

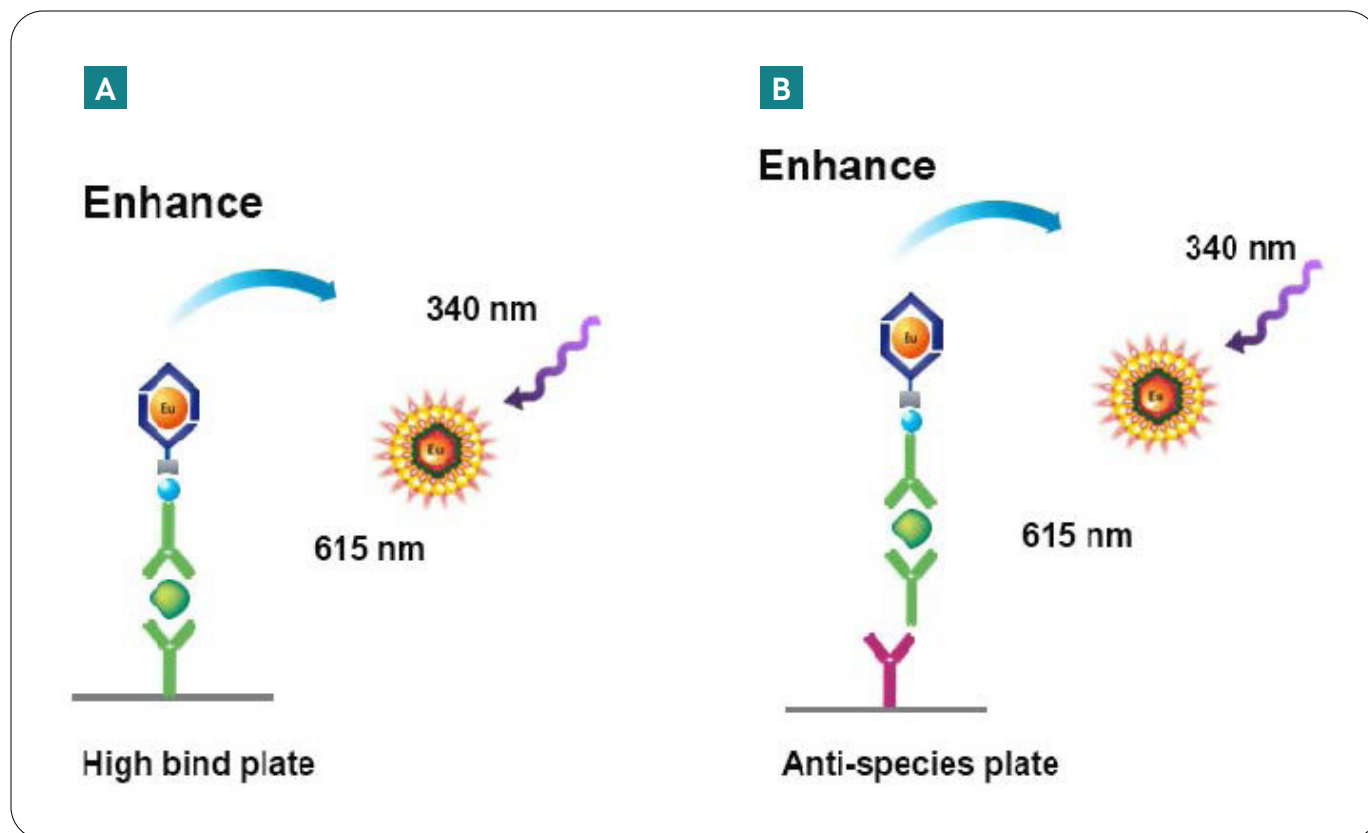For research purposes only. Not for use in diagnostic procedures.
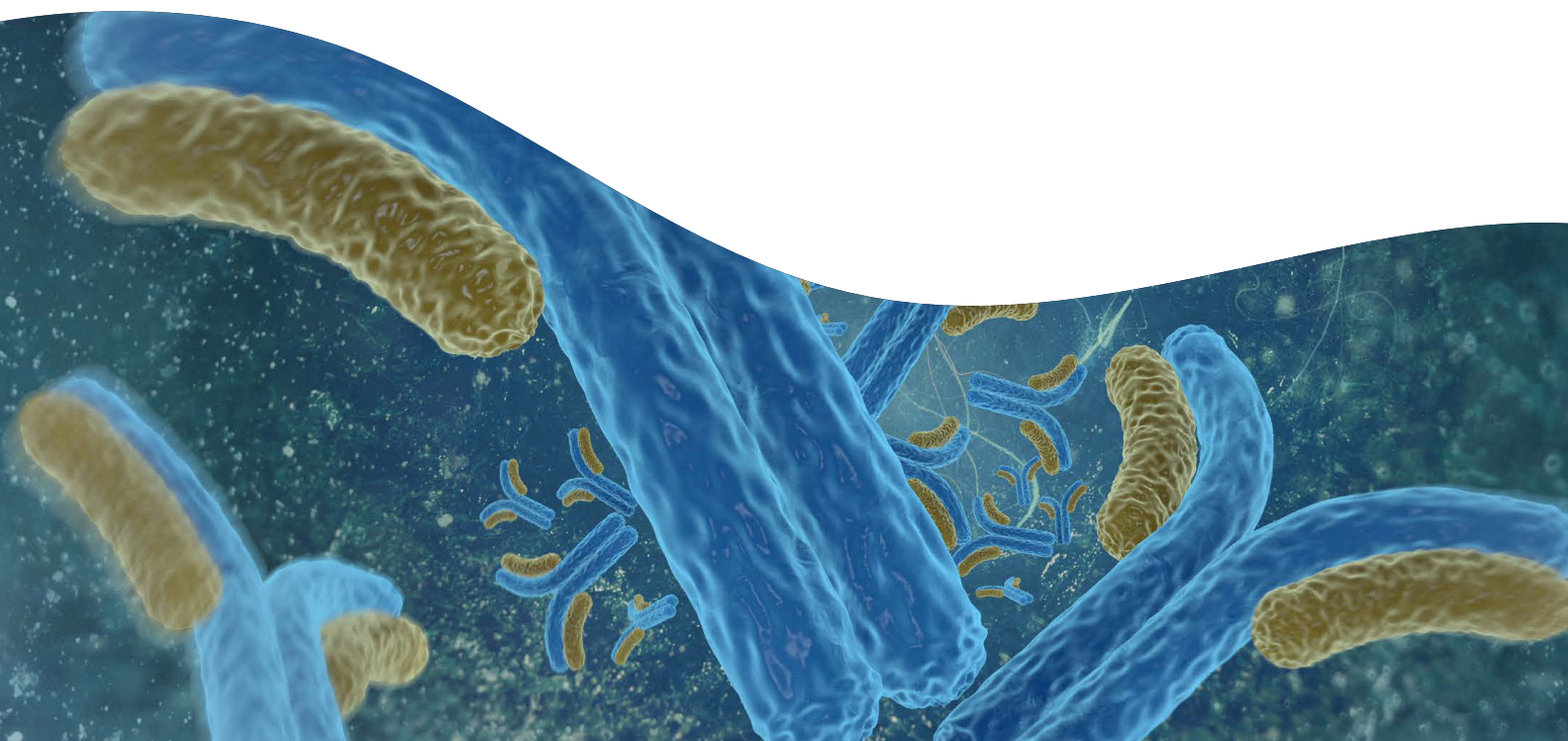
Figure 1: DELFIA TRF assay. A) Direct assay configuration using capture antibody directly adsorbed to a high bind DELFIA plate. Biotinylated-detection antibody binds to DELFIA Europium-streptavidin. Addition of Enhancement Solution releases Europium to form a new fluorescent chelate that when excited at 340 nM emits signal at 615 nM. B) Indirect assay configuration using a pre-coated anti-species DELFIA plate (anti-mouse IgG). The mouse IgG capture antibody is bound by the anti-mouse IgG pre-coated plate.

Conveniently, ELISA assays can be easily converted to and optimized on DELFIA. We used Human IL-2 (R&D Systems #DY202) and Mouse IL-5 (R&D Systems #DY405) ELISA DuoSets® for conversion to DELFIA. Each DuoSet® provided the necessary capture antibody, biotinylated detection antibody, and standard analyte for straightforward conversion to DELFIA. Capture antibodies were either directly adsorbed or indirectly captured onto 96-well microplates. Standard analyte and biotinylated detection antibody were then added to the microplate. Subsequently, Europium-streptavidin was added to each well. A final dissociation step was then performed with Enhancement Solution to release europium, forming a highly fluorescent Europium signal. Light is generated by excitation of europium at 340 nM to generate stable and robust 615 nM signal proportional to antigen presence (Figure 1).

We compared the results generated when using either flash lamp or TRF laser module as an excitation source on the EnVision™ multilabel plate reader (Figure 2). To further demonstrate DELFIA's capabilities, we also performed titration experiments that stretched antibody usage to achieve the same sensitivity, and miniaturized the assay on 96-well ½ AreaPlates® to highlight cost benefits against ELISAs.



Figure 2: EnVision Multilabel Plate Reader.

## Materials and methods

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

| Item name | | Catalog # | |
|---|---|---|---|
| | | **5-plate assay** | **15-plate assay** |
| R&D Systems DuoSet® human IL-2 | | DY202-05 | DY202 |
| R&D Systems DuoSet® mouse IL-5 | | DY405-05 | DY405 |
| Microplates | DELFIA yellow plates (direct adsorption) | AAAND-0001 | |
| | DELFIA anti-mouse IgG plates | 4007-0010 | 2 x 4007-0010 |
| | ½ AreaPlates (high bind) | 6057890 | |
| Plate seals and covers | TopSeal-A Plus | 6050185 | |
| | Plate covers | 6000027 | |
| DTPA purified BSA | | CR84-100 | |
| DELFIA wash buffer | | 1244-114 | |
| DELFIA assay buffer | | 1244-106 (50 mL) | 1244-111 (250 mL) |
| DELFIA enhancement solution | | 1244-104 (50 mL) | 1244-105 (250 mL) |
| 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. | |

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

DELFIA immunoassays were performed using R&D Systems DuoSet® kits (Human IL-2: R&D Systems #DY202 and Mouse IL-5: R&D Systems #DY405) at the recommended antibody concentrations and amounts suggested in the kit manuals. All antibodies and analytes were reconstituted according to the ELISA kit manuals. All buffers were warmed to room temperature before use. For direct binding (adsorption) assays, capture antibody was diluted in 1X Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium (GIBCO #14190144). 100 µL of capture antibody was added to each well of a yellow DELFIA 96-well high bind plate (Revvity #AAAND-0001) and incubated overnight at room temperature covered with TopSeal-A® PLUS (Revvity #6050185). For indirect assays, 100 µL of capture antibody diluted in 1X DPBS was added to the anti-species plate and incubated two hours at room temperature on a plate shaker set to a slow speed (~300 rpm). 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 room temperature 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. Plate washing was repeated with 1X DELFIA wash buffer. Then, 100 µL of standard or sample prepared in DELFIA assay buffer (Revvity #1244-111) was added to each well and incubated for two hours at room temperature on a plate shaker. Plates were washed again with 1X DELFIA wash buffer. 100 µL of biotinylated detection antibody solution prepared in DELFIA assay buffer was added to each well and incubated for one hour at room temperature on a plate shaker. Plates were washed with 1x DELFIA wash buffer. 100 µL of 100 ng/mL Europium-Streptavidin (Eu-SA, Revvity #1244-360) prepared in DELFIA assay buffer was added to each well and incubated 20 minutes at room temperature on a plate. Once Eu-SA has been added it is recommended to not use TopSeal and use a plate cover instead from this

point on to prevent signal quenching. Plates were washed six times with 1X DELFIA wash buffer. 200 µL of Enhancement Solution was added to each well.

The plate was covered with a plate lid (no TopSeal) and incubated 5 minutes at room temperature on a plate shaker. Plates were read using a 2104 EnVision equipped with a flash lamp and TRF laser unit and necessary optics (see Table 2). See the Appendix for a flow chart and 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 and a final incubation period on a plate shaker.

## DELFIA protocol

## Titration experiments to reduce amount of antibody required in the assay

DELFIA immunoassays for Human IL-2 and Mouse IL-5 were performed using R&D Systems DuoSets® for Human IL-2 (R&D Systems #DY202) and Mouse IL-5 (R&D Systems #DY405). The recommended protocol antibody concentrations were used as a starting point (1X) for both the capture and detection antibodies and diluted 1.25X, 1.5X, 1.75X, 2X, 2.5X, 3X, and 4X the suggested ELISA amounts. The assays were performed following the DELFIA protocol described above.
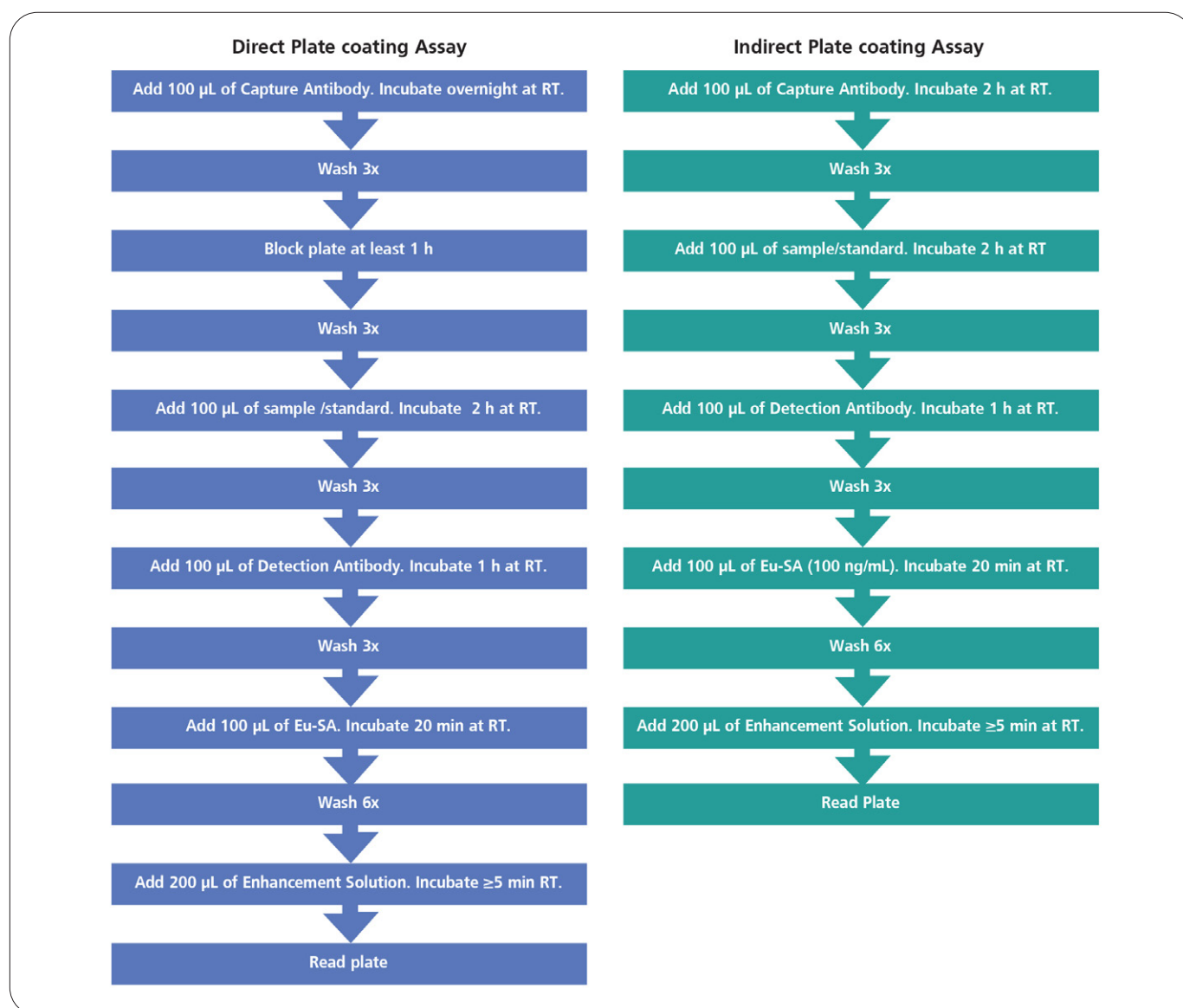


Figure 3: DELFIA protocol overview.

| 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 |

## Instrumentation

DELFIA assays require a TRF-capable plate reader than 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. To validate the instrument, 1 nM Europium standard solution (B119-100) was used to perform plate optimization.

## 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) analytes. Curves were plotted in GraphPad Prism Version 7.0 and analyzed with nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) with $1/Y^2$ weighting method. Lower limit of detection (LDL) and percent coefficient of variance (CV%) was calculated using the following equations:

**LDL = mean (blanks) + 2 * SD**
**CV% = (standard deviation / mean) * 100**

## Results

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

Human IL-2 and mouse IL-5 ELISA DuoSets® were acquired from R&D Systems. Antibody concentrations were used as suggested in the ELISA protocol and altered to incorporate the use of DELFIA Europium-streptavidin in place of the horseradish peroxidase enzyme. As shown in Figures 4A and 4B, human IL-2 was successfully converted to a direct orientation DELFIA (where the mouse monoclonal capture antibody was directly adsorbed to a 96-well high bind 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 Figures 4C and 4D, mouse IL-5 was also successfully converted to a direct orientation DELFIA displaying an assay sensitivity of 10 pg/mL and $R^2$ value of 0.998.

### 2. TRF laser module improves signal-to-background and assay CV% compared to flash lamp

The Revvity Envision (#2104-0010A) comes standard with a flash lamp installed. However, a TRF laser module can be equipped and used as an alternative excitation source. The TRF laser emits at a 337 nM wavelength and does not require a specialized excitation filter (see Table 2). Both the human IL-2 and mouse IL-5 DELFIA assays were tested to
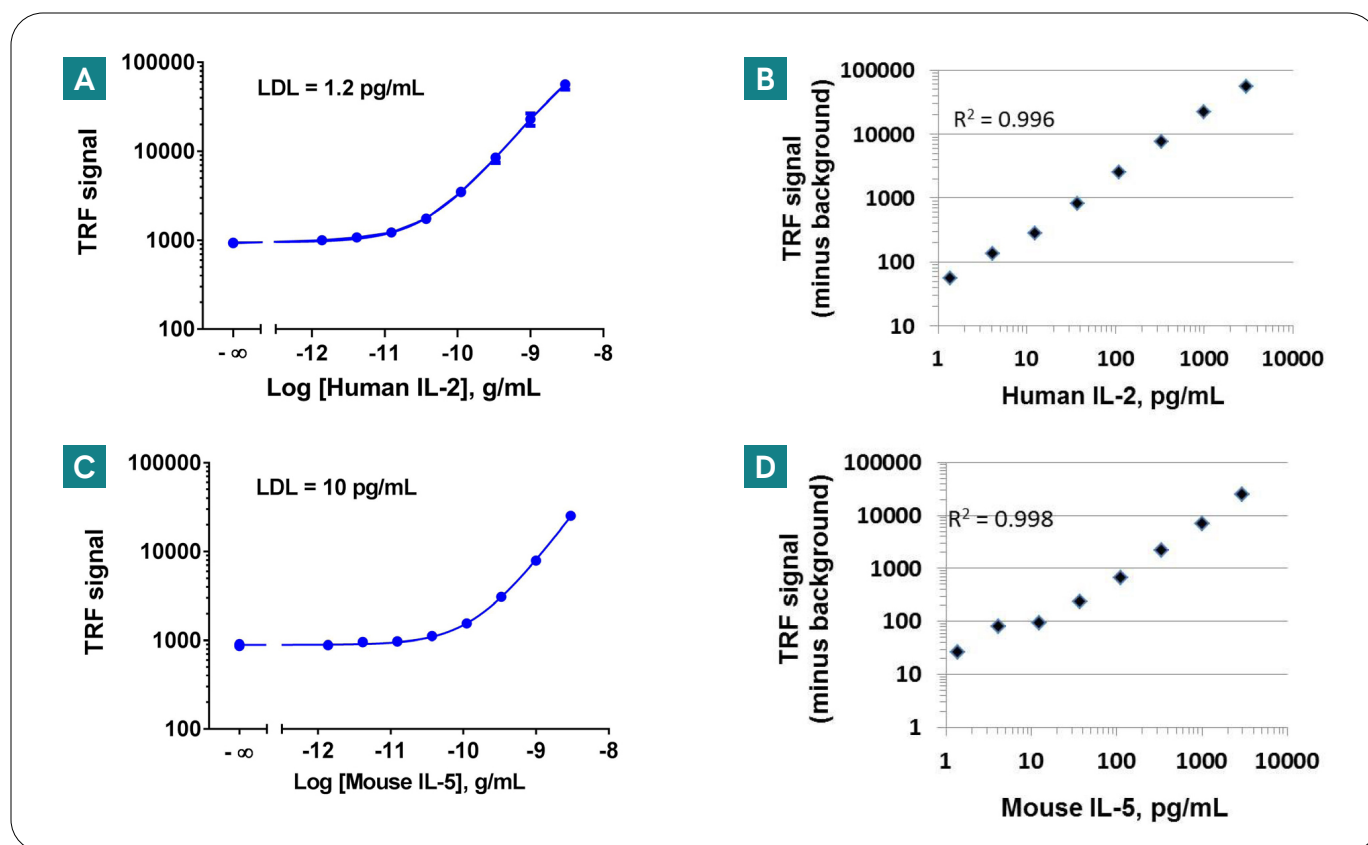
Figure 4: Conversion of R&D Systems DuoSet® ELISA to DELFIA TRF for human IL-2 and mouse IL-5. Assays were performed in yellow 96-well DELFIA high bind plates. A and B show standard curves for human IL-2 analyzed using non-linear regression and linear regression, respectively. C and D show standard curves for mouse IL-5 analyzed using non-linear regression and linear regression, respectively. DELFIA assays were performed with manual washing. Assays were run using the R&D DuoSet® standard analytes.

compare the flash lamp and laser modules. The assays were performed using the antibody concentrations recommended in R&D Systems ELISA protocol. In Figure 5, human IL-2 (left) and mouse IL-5 (right) TRF signal was measured using both the flash lamp and laser module as the excitation source. For both assays, the flash lamp and laser module generated similar sensitivities (1.2 and 1.6 pg/mL with lamp and laser, respectively, for human IL-2 and 10.0 and 8.8 pg/mL with lamp and laser, respectively, for mouse IL-5). Assay improvements were observed in both signal-to-background and average CV% when using the installed laser module. As indicated in the tables beneath the graphs, there was approximately a 40% increase in signal-to- background when using the laser compared to the flash lamp for both kits (data point at 3 ng/mL analyte used for calculation). The average CV% across six assays was calculated. The flash lamp revealed an average CV% of 5.5%, while the laser gave an improved 3.1% CV%.

**3. Antibody consumption can be minimized while maintaining assay performance**

Although DELFIA assays can be easily converted from an ELISA using pre-defined ELISA assay antibody concentrations, these concentrations may not be optimal for efficient consumption of materials and assay performance. To demonstrate an approach to determine more optimal antibody concentrations for a DELFIA assay, capture and biotinylated detection antibodies were tested at 1, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, and 4.0-fold dilutions in comparison to the suggested concentrations in the R&D Systems ELISA protocol.

Figure 6 shows the standard curves (left) generated from these titration assays, while the associated tables (right) show the signal- to-background (S/B) at a high, middle, and low concentrations of analyte along the curve. Assay sensitivity is also indicated. A balance between assay sensitivity and efficient consumption of materials can aid in choosing optimal concentrations of capture and detection antibodies.
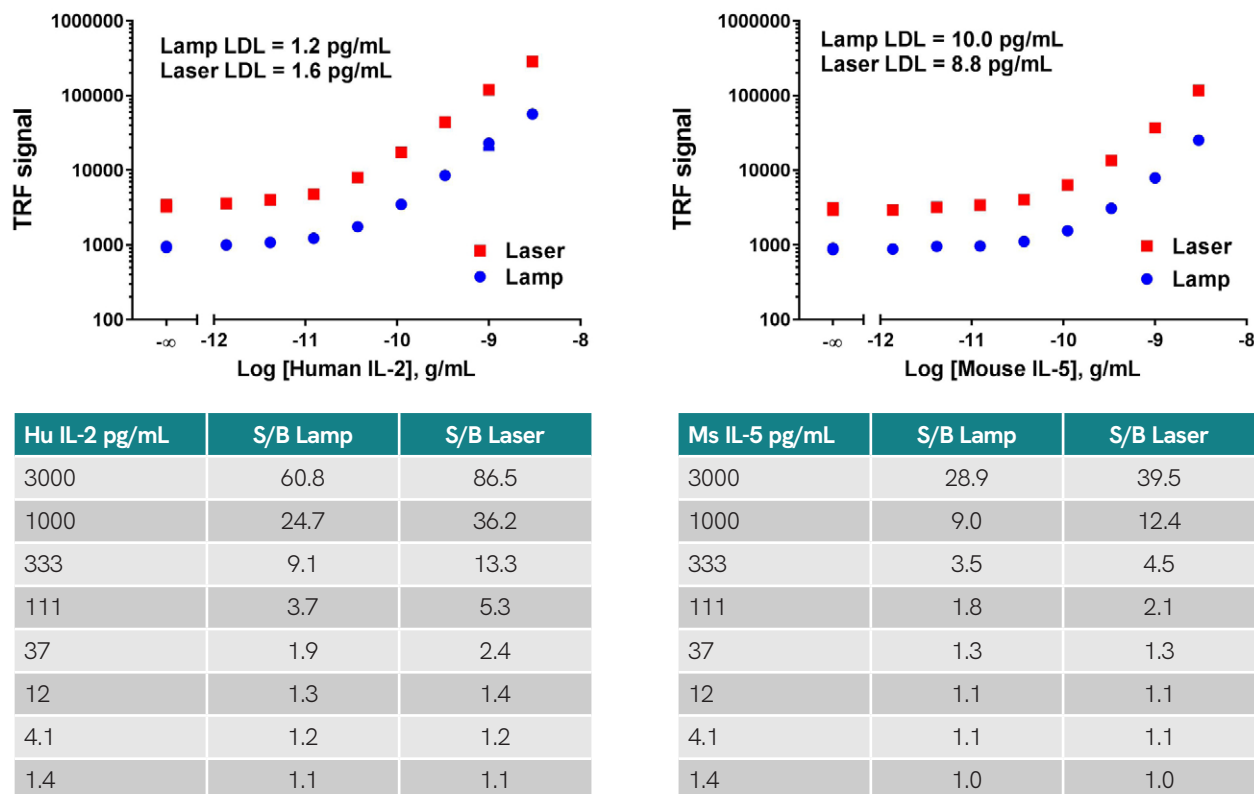
Figure 5: Human IL-2 and mouse IL-5 DELFIA assays measured using the flash lamp and TRF laser module. The top graphs show standard curves of human IL-2 (left) and mouse IL-5 (right) read back-to-back with flash lamp and the laser module. The standard curves were generated with non-linear regression using the 4-parameter logistic equation. The tables below the figures display signal-to-background signals for human IL-2 (left) and mouse IL-5 (right) at each concentration of the standard curve tested.

## 4. Miniaturization of human IL-2 DELFIA assay using 96-well ½ AreaPlate

Another approach to reduce cost is to decrease the volume of the assay through assay miniaturization. Miniaturization of the DELFIA assay can be used to improve assay throughput and decrease consumption of precious samples. Assays can be miniaturized by using a smaller, white 96-well ½ AreaPlate (Revvity #6057890). These plates have wells that are spaced to mimic the spacing of standard 96-well plates, but the actual area of each well is reduced to permit use of lower assay volumes compared to what would be used in a typical 96-well plate. The human IL-2 DELFIA assay was performed using the recommended concentrations in R&D Systems ELISA protocol; however, the volume of each step was reduced 2-fold, thereby reducing the consumption of antibody, analyte, and buffers in the assay.
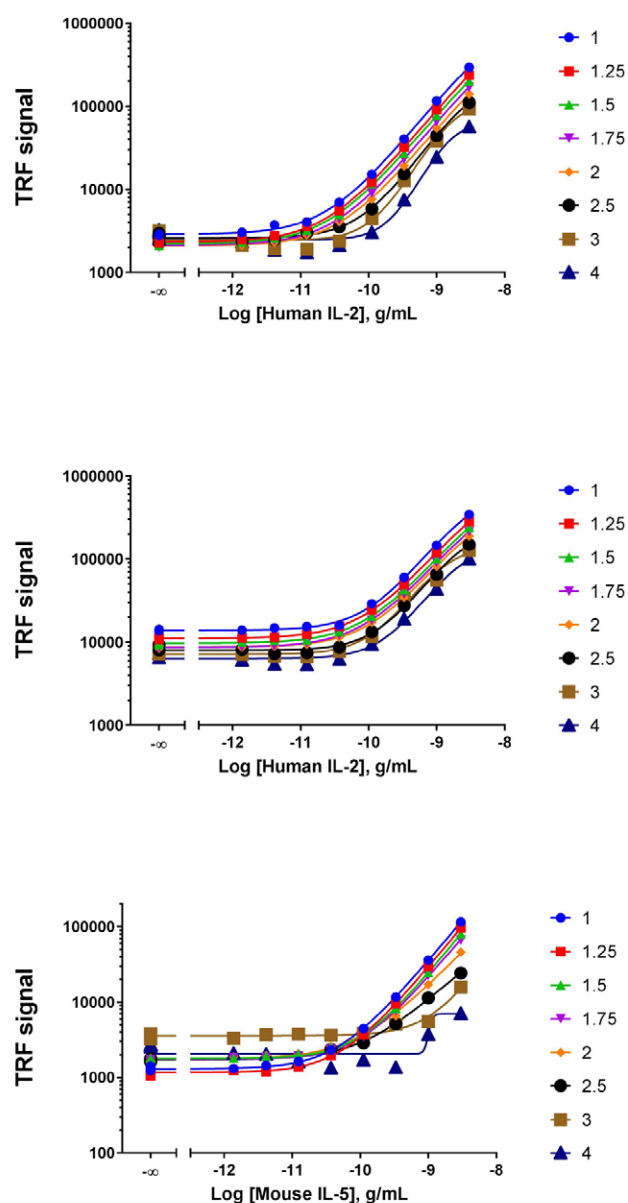
| Direct adsorption - human IL-2 | | | | |
|---|---|---|---|---|
| Antibody dilution (fold) | Signal-to-background | | | Sensitivity pg/mL |
| | 3000 pg/mL | 111 pg/mL | 12 pg/mL | |
| 1X | 105 | 5.4 | 1.4 | 0.2 |
| 1.25X | 104 | 5.4 | 1.6 | 0.2 |
| 1.50X | 91 | 5.3 | 1.5 | 1.8 |
| 1.75X | 81 | 4.3 | 1.4 | 1.6 |
| 2.0X | 67 | 3.6 | 1.4 | 1.4 |
| 2.5X | 42 | 2.2 | 1.1 | 28.0 |
| 3.0X | 31 | 1.5 | 0.6 | 71.0 |
| 4.0X | 19 | 1.0 | 0.6 | 148.1 |

| Indirect capture - human IL-2 | | | | |
|---|---|---|---|---|
| Antibody dilution (fold) | Signal-to-background | | | Sensitivity pg/mL |
| | 3000 pg/mL | 111 pg/mL | 12 pg/mL | |
| 1X | 25 | 2.1 | 1.1 | 5.9 |
| 1.25X | 25 | 2.2 | 1.1 | 4.4 |
| 1.50X | 25 | 2.1 | 1.1 | 3.7 |
| 1.75X | 25 | 2.1 | 1.1 | 4.9 |
| 2.0X | 21 | 1.9 | 1.1 | 13 |
| 2.5X | 18 | 1.6 | 0.9 | 52 |
| 3.0X | 16 | 1.5 | 0.9 | 50 |
| 4.0X | 14 | 1.3 | 0.8 | 72 |

| Direct adsorption - mouse IL-5 | | | | |
|---|---|---|---|---|
| Antibody dilution (fold) | Signal-to-background | | | Sensitivity pg/mL |
| | 3000 pg/mL | 111 pg/mL | 12 pg/mL | |
| 1X | 89 | 3.4 | 1.3 | 7.1 |
| 1.25X | 83 | 3.2 | 1.2 | 9.6 |
| 1.50X | 48 | 2.3 | 1.2 | 7.3 |
| 1.75X | 40 | 2.1 | 1.2 | 10.6 |
| 2.0X | 27 | 2.3 | 1.2 | 4.5 |
| 2.5X | 14 | 1.7 | 1.3 | 7.6 |
| 3.0X | 5 | 1.1 | 1.1 | 241 |
| 4.0X | 5 | 1 | 0.6 | 933 |

Figure 6: "Stretching" of capture and detection antibody in human IL-2 and mouse IL-5 DELFIA assays. DELFIA assays were measured using the TRF laser module on the EnVision. Left: standard curves generated for human IL-2 (direct adsorption; top), human IL-2 (indirect capture; middle), and mouse IL-5 (direct adsorption; bottom) at a various antibody dilutions (1X representing the suggested capture and detection antibody amounts in R&D Systems ELISA DuoSet® protocol). Right: tables that are associated with each of the standard curve titrations performed for human IL-2 (direct adsorption; top), human IL-2 (indirect capture; middle), and mouse IL-5 (direct adsorption; bottom). S/B at a high, middle, and low concentrations of analyte (3000, 111, and 12 pg/mL, respectively) is indicated. These tables also show the assay sensitivity calculated using the average background + 2x standard deviation.

Using the laser module on the EnVision and direct adsorption of capture antibodies on the ½ AreaPlate, the human IL-2 DELFIA assay exhibited sensitivity of 0.9 pg/mL (Figure 7). Due to increased background observed in the white ½ AreaPlates, assay S/B was reduced by about 35% compared to the assay run in DELFIA yellow 96-well plates (data point using 3000 pg/mL analyte used for calculation). However, the results generated in the ½ Area Plates were similar to those obtained with 2-fold dilution of antibodies in regular 96-well plates, except half the volume of buffer was used, thereby reducing assay cost.



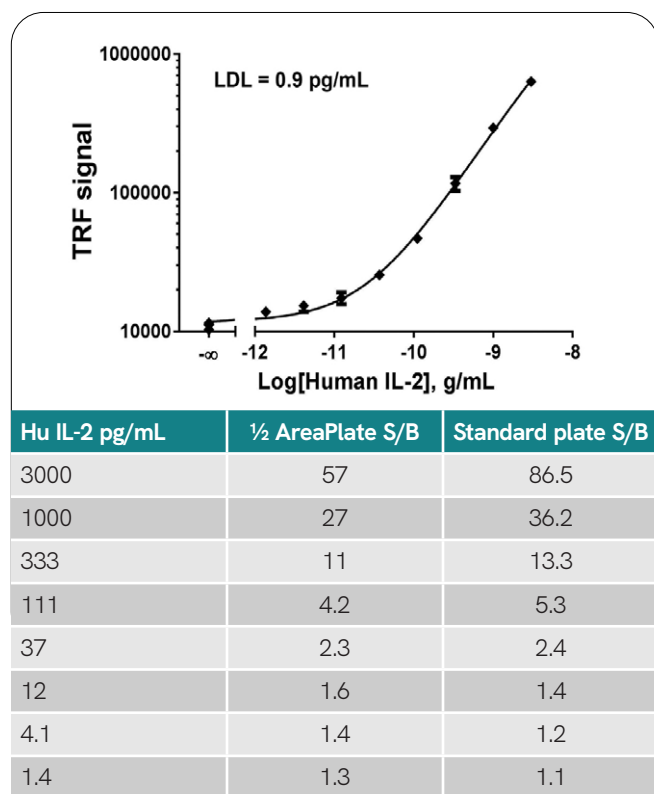| Hu IL-2 pg/mL | ½ AreaPlate S/B | Standard plate S/B |
|---|---|---|
| 3000 | 57 | 86.5 |
| 1000 | 27 | 36.2 |
| 333 | 11 | 13.3 |
| 111 | 4.2 | 5.3 |
| 37 | 2.3 | 2.4 |
| 12 | 1.6 | 1.4 |
| 4.1 | 1.4 | 1.2 |
| 1.4 | 1.3 | 1.1 |

Figure 7: Human IL-2 DELFIA assay standard curve was performed in white 96-well ½ AreaPlates and measured using the TRF laser module on the EnVision. Standard curves presented used non-linear regression with the 4-parameter logistic calculation. The table on the right represents the human IL-2 DELFIA signal-to-background ratios at each point of the standard curve when using the ELISA recommended concentrations in a white 96-well ½ AreaPlate or standard 96-well yellow DELFIA plate.

## 5. Cost analysis comparison of optimized DELFIA assays

Assays were performed to reduce the amount of capture and detection antibody required ("stretching") and to miniaturize the assay volume by altering microplate type. Cost savings per-well can be calculated by factoring the cost of required assay reagents and consumables. Costs were calculated for 500 assay points (~ 5 plates; 500 wells).

The following assay materials were included in the calculation: R&D Systems ELISA DuoSet® (Human IL-2), microplates and seals, DELFIA assay buffer, DELFIA wash buffer, DTPA purified BSA, and Eu-streptavidin. These are the core materials required for converting an ELISA assay to a DELFIA assay. Neither instrument nor labor costs were included in these calculations. Table 3 shows percent savings per-well when performing a DELFIA assay using direct adsorption in a DELFIA yellow 96-well plate or indirect capture in an anti-mouse IgG 96-well clear strip well plate compared to using the recommended ELISA concentrations. Although the cost of indirect capture assays appears slightly more expensive, these costs do not take into account the overall time saved (e.g., no overnight incubation is required for indirect assays).

Costs were also calculated for assays using 96-well ½ AreaPlates at half the normal 96-well volumes (miniaturization; no antibody "stretching"). Cost-per-well was calculated to be reduced by 22% per-well (500 wells) when compared to the cost of performing the human IL-2 DuoSet® ELISA. This reduced cost was attributed to reduced amounts of buffer, antibodies and analyte required for these assays.

## Conclusion

In this application note we were able to successfully convert human IL-2 and mouse IL-5 ELISA DuoSets® to DELFIA time-resolved fluorescence (TRF) technology. Results indicated that the EnVision plate reader TRF laser module improved both assay signal-to-background and average coefficient of variance (CV%). Due to the highly sensitive nature of DELFIA technology, reduced amounts of capture and detection antibody can be tolerated. It was shown that the human IL-2 DELFIA assay could be extended ("stretched") to use 2-fold less antibody while still maintaining exceptional performance. Similar results were obtained for mouse IL-5, where antibody could be stretched 1.5 – 1.75-fold while maintaining S/B and good assay sensitivity. Furthermore, we showed that the DELFIA assay can be miniaturized using 96-well ½ AreaPlates. Assay miniaturization with DELFIA demonstrated high quality assay performance while only using half the required reagents, compared to a typical 96-well assay. Finally, we performed a cost analysis of DELFIA assays and showed how altering the consumption of materials from the DuoSet® kit can lead to significant cost savings on a per-well basis.

These data demonstrate that an ELISA DuoSet® from R&D Systems can be converted to DELFIA technology. The assay can be converted using the recommended concentrations in the ELISA protocol and a standard EnVision flash lamp. The assay can also be optimized by using a laser module and reducing antibody, protein, and buffer consumption, reducing cost while maintaining extraordinary assay performance.

Table 3: Cost analysis of Direct Adsorption and Indirect Capture Human IL-2 DELFIA assay

| Antibody dilution (fold) | # of assay points | % savings per well (Direct) | % savings per well (Indirect) |
|---|---|---|---|
| 1 | 500 | - | - |
| 1.25 | 625 | 7.7 | 6.1 |
| 1.5 | 750 | 12.9 | 10.2 |
| 1.75 | 875 | 16.6 | 13.1 |
| 2.0 | 1000 | 19.3 | 15.3 |
| 2.5 | 1250 | 23.2 | 18.4 |
| 3.0 | 1500 | 25.8 | 20.4 |
| 4.0 | 2000 | 29.0 | 23.0 |

## 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 on plate shaker to ensure plates bind the capture antibody

Wash 3x 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 coating 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 3x 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 3x 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 3x 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 3x 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 6x with 1X DELFIA wash solution

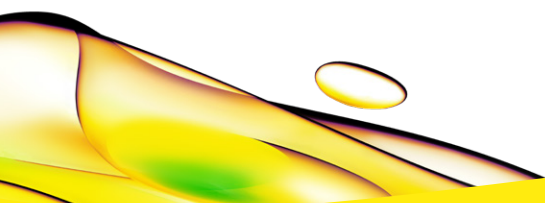- The extra wash steps are necessary for removing any unbound Eu-SA

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)

- 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