LANCE TR-FRET immunoassay conversion guide.

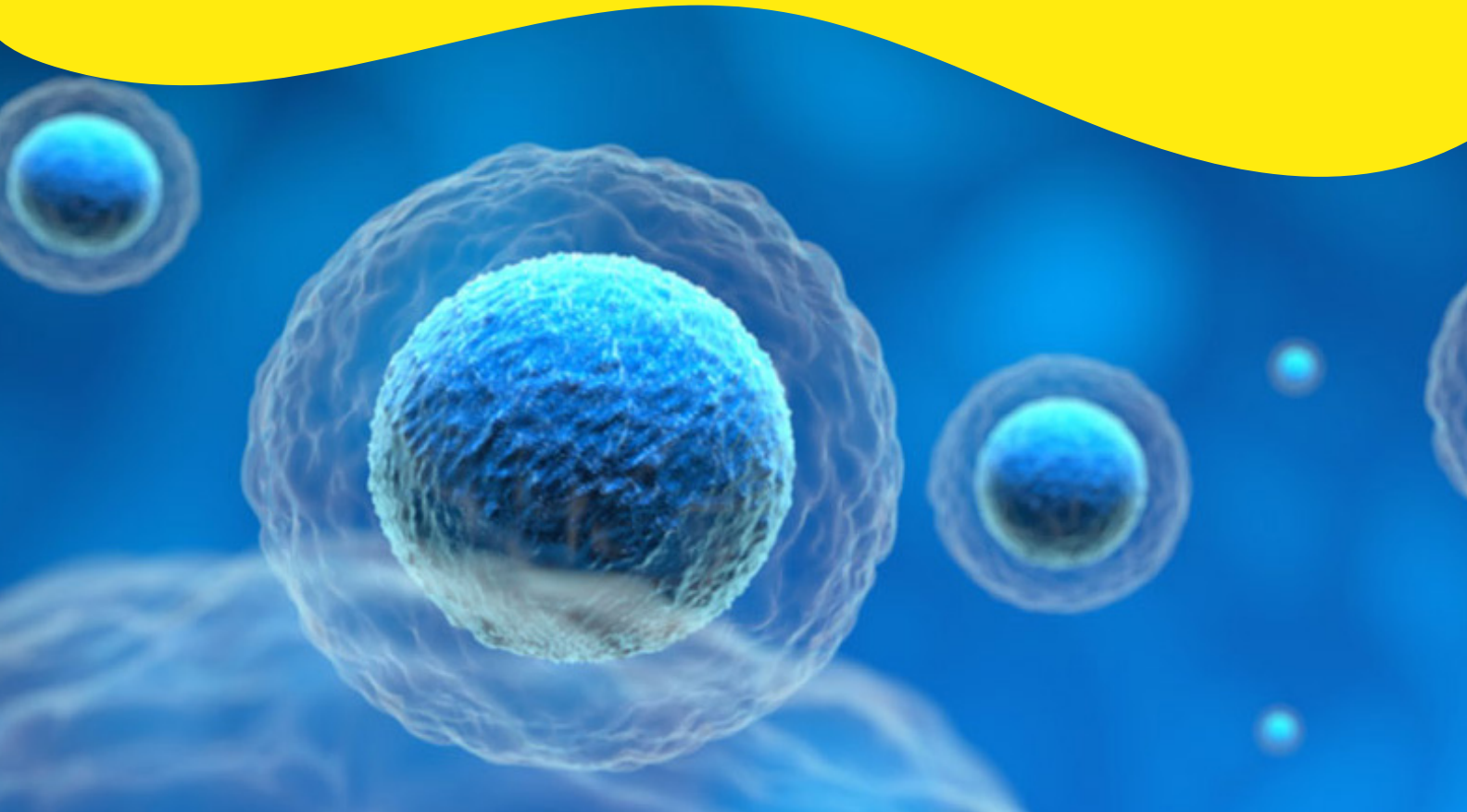


Table of contents

Immunoassay conversion

LANCE TR-FRET is a fast and sensitive alternative to your existing immunoassay. This guide outlines the simple conversion of an ELISA or other immunoassay to a LANCE TR-FRET assay with a few optimization steps. LANCE *Ultra* uses a LANCE Europium chelate donor fluorophore and a U*Light* dye acceptor fluorophore. LANCE uses a LANCE Europium chelate donor fluorophore and a SureLight™ allophycocyanin (APC) acceptor fluorophore. For LANCE TR-FRET immunoassays, a target specific antibody is conjugated to (direct method) or is captured by an antibody conjugated to (indirect method) a Europium chelate, and then a second target specific antibody is conjugated to (direct method, Figure 1A) or is captured by an antibody conjugated to (indirect method, Figure 1B) the U*Light* or APC acceptor dye.

These two target specific antibodies are used to capture the target analyte. When the analyte is present in the sample, the Europium chelate donor and U*Light* or APC acceptor dye are brought into proximity. Upon excitation at 320 nM or 340 nM, the Europium chelate is excited and energy is transferred to the U*Light* or APC dye, which both emit light at 665 nM. The emission of the Europium chelate can also be detected at 615 nM. Unknown concentrations of an analyte can be easily quantified by running the LANCE TR-FRET assay with a standard curve using a known amount of analyte as a reference. This guide will focus on developing LANCE *Ultra* TR-FRET immunoassays using the U*Light* acceptor dye; however the principles can be applied to developing assays using the APC acceptor dye as well.

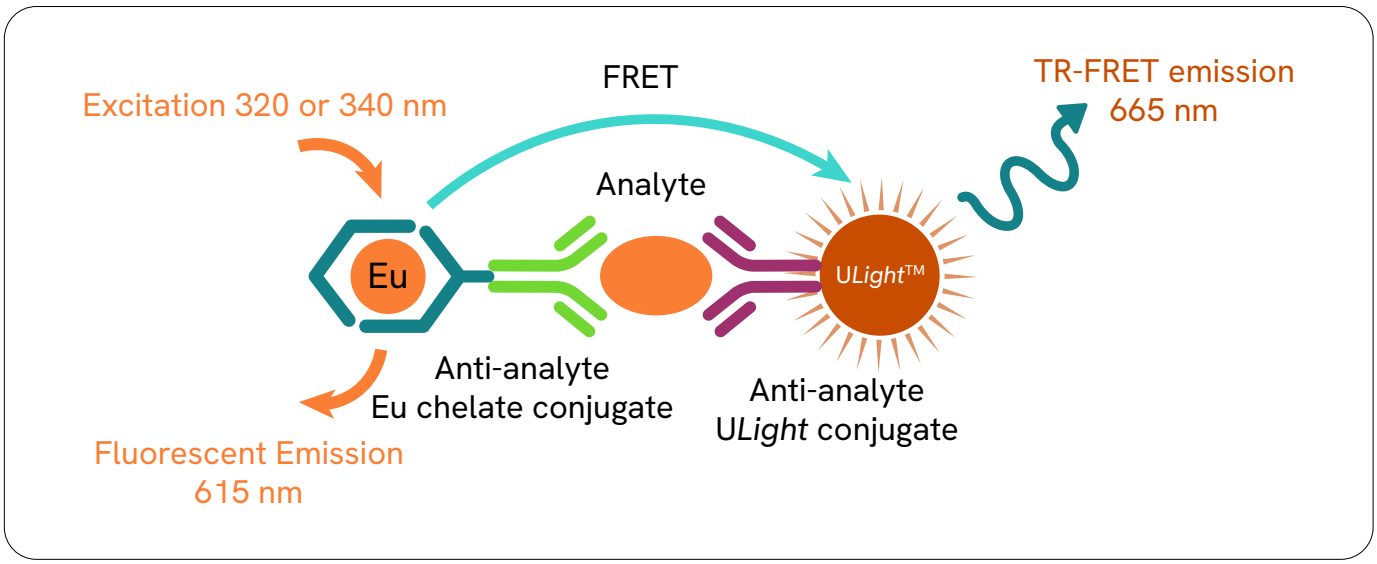


Figure 1A: Direct TR-FRET method for capturing the analyte.

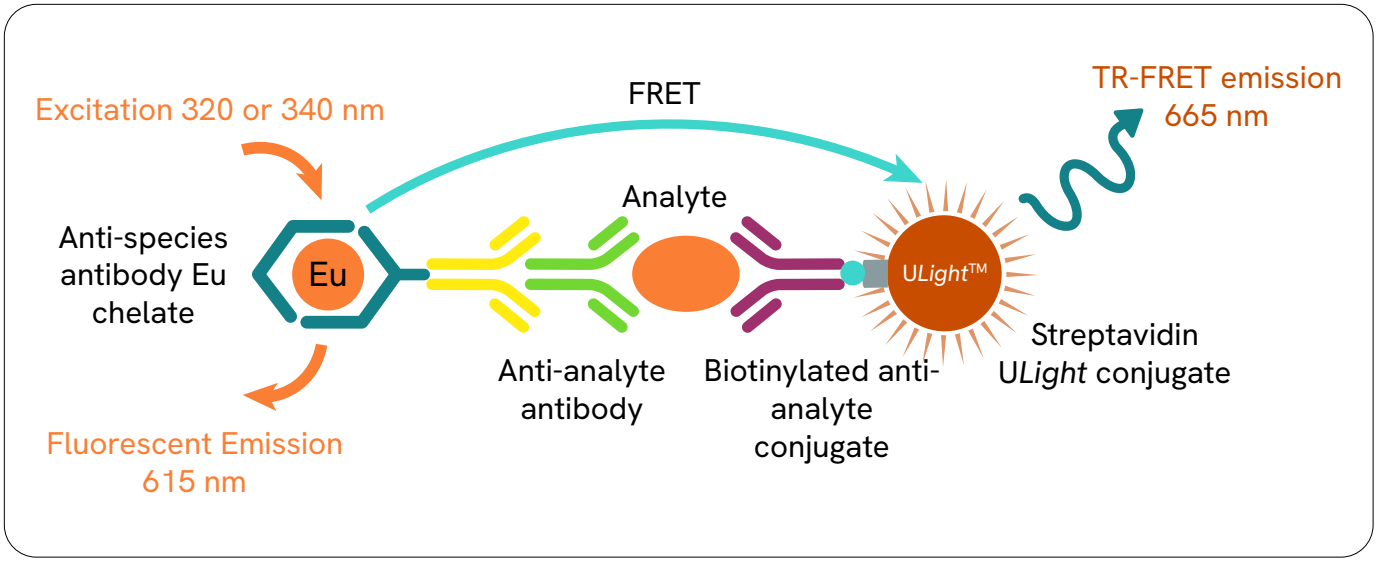


Figure 1B: Example of an Indirect TR-FRET method for capturing an analyte.

Advantages of LANCE TR-FRET assays are as follows:

- Eu chelates have a high quantum yield, large Stokes' shift, a narrow-banded emission, and a long lifetime making them ideal energy donors in TR-FRET assays. Long fluorescence decay after excitation (at least a millisecond) allows time-delayed signal detection that virtually eliminates background originating from microplates and buffer components. The large Stokes shift minimizes crosstalk, resulting in a high signal-to-background ratio.
- *ULight*™ is a small, light- resistant acceptor dye that has red shifted emission and is therefore less sensitive to quenching by colored compounds.
- Most importantly, LANCE is a homogeneous assay which means that the reduced number of assay steps compared with standard immunoassays offers maximum precision and improved CVs while speeding up the process and simplifying the workflow (Figure 2).

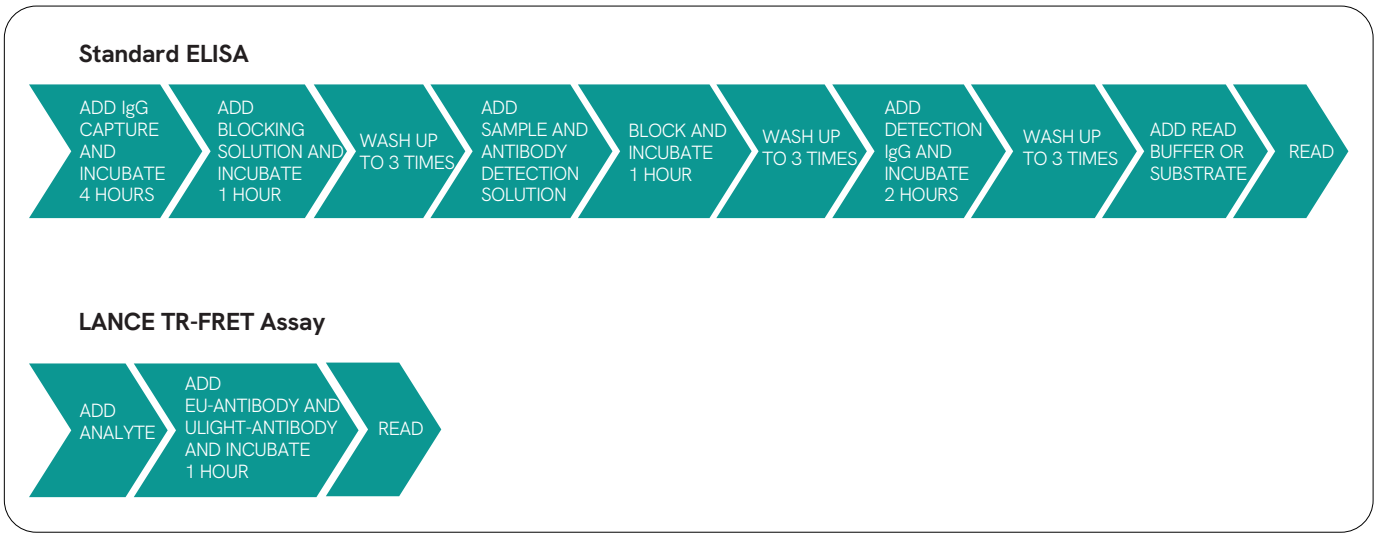


Figure 2: A workflow comparison between LANCE TR-FRET and standard immunoassays. LANCE TR-FRET assays can be performed in three simple steps.

Assay design

The most straightforward LANCE *Ultra* TR-FRET immunoassay set-up is based on directly labeling the capture antibodies with a Europium chelate and a U*Light* conjugate (Figure 1A). Revvity offers custom labeling services to directly label antibodies with a Europium chelate or U*Light*. Customers also have the option of labeling their own antibodies with Europium chelates (see Appendix C). There are also indirect capture methods that can be used directly with antibodies already validated in another immunoassay technology. For example, a biotinylated antibody can be captured by a Streptavidin Europium chelate or a Streptavidin U*Light* conjugate. An anti-species Europium chelate or an anti-species U*Light* conjugate are also options for capturing an unlabeled antibody which is specific for the target. Figure 1B shows an indirect schematic utilizing an anti-species Eu chelate and a Streptavidin-U*Light* conjugate. Table 1 shows the various toolbox reagents that revvity offers for setting up an indirect TR-FRET assay.

LANCE toolbox table

Table 1: LANCE TR-FRET Toolbox Reagents for Designing Assays.

Coating	Eu-conjugated	U*Light*-conjugated	APC-conjugated
Streptavidin	AD0062, AD0063	TRF0102	AD201, AD202
Anti-FITC	AD0270, AD0271, AD0272	TRF503	
Anti-Mouse IgG	AD0076, AD0077	TRF501	AD0059M
Anti-V5	AD0277,AD0278, AD0276	TRF505	
Protein A		TRF0103	
Anti-GST	AD0252, AD0253, AD0254	TRF0104	AD0059G
Anti-Rabbit IgG	AD0082, AD0083	TRF502	AD0059R
Anti-FLAG	AD0273, AD0274, AD0275	TRF0059	AD0059F
Protein G	AD0070, AD0071, AD0211		
Anti-c-myc	AD0114, AD0115, AD0116	TRF504	
Anti-C-tag	AD0279, AD0280, AD0281	TRF506	
Anti-6XHis	AD0110, AD0111, AD0205	TRF0105	AD0059H
Anti-human IgG	AD0074, AD0075, AD0212	TRF500	
Anti-HA	AD0084, AD0085		

Go to www.revvity.com for a full list of LANCE products.

There are a few simple steps that can be done to optimize a new TR-FRET assay. When converting from another immunoassay, the same antibody pairs can be used. The first step would be to determine the best antibody concentration and configuration for the assay. Next, the preliminary lower detection limit (LDL) is determined. Then other parameters, such as incubation time, buffer conditions, and protocol variations can be tested to improve sensitivity if the initial assay needs further optimization.

Things to consider

1. Due to the distance requirement for efficient TR-FRET (~10 nM), the directly labeled capture method is the most likely to be successful as it minimizes the distance between the Eu donor and U*Light*/APC acceptor. Contact your local revvity representative for more information about LANCE custom labeling.
2. If using the indirect capture method with anti-species Eu chelates or U*Light*/APC conjugates, make sure that the two antibodies in the assay are of different species so as not to create a competition between donor and acceptor for the antibodies.
3. When using toolbox Eu chelates or U*Light*/APC conjugates, make sure that they cannot interact with each other in the absence of analyte. For example, if using an anti-species Eu and a different anti-species U*Light*/APC conjugate, make sure that the anti-species antibody conjugated to Eu or U*Light*/APC cannot be recognized by the other anti-species antibody.

Before you begin

- An instrument capable of reading time-resolved fluorescence is required (i.e. Revvity's VICTOR™ ViewLux® (for high throughput), EnVision® or EnSight® Multilabel Plate Reader). If you are using an instrument manufactured by another company, we recommend that you contact their technical support team to see if they have optimized settings, filter recommendations, etc. for a LANCE TR-FRET assay. For revvity recommended instrument settings, refer to Appendix B.
- Use an excitation wavelength of 320 or 340 nM to excite the LANCE Europium chelate. We recommend you read this assay in dual emission mode, detecting both the emission from the Europium donor fluorophore at 615 nM, and the acceptor fluorophore (at 665 nM for APC, ULight dye, and Alexa Fluor 647). The raw FRET signal at 665 nM can be used to process your data or you can analyze the data using the ratio of 665 nM to 615 nM (see Appendix A). The ability to analyze the signal at 615 nM can also be helpful when troubleshooting.
- Use a microplate suitable for fluorescence (we recommend white OptiPlates™ or white ProxiPlates™ for TR-FRET).
- TopSeal-A PLUS (or another adhesive plate seal) can be used during incubation steps to prevent evaporation, however, it must be removed prior to reading the plate in the instrument.

Optimization step 1: Eu-Ab and U*Light*-Ab configuration and concentration titration

I. Direct method

For the direct assay format, a cross-titration should be performed using three concentrations of Eu-antibody and three to four concentrations of U*Light*-antibody with and without analyte. For this step, a mid-range concentration of analyte can be used to get an idea of the best antibody concentrations. Then full titration curves of the analyte can be done to assess the maximum signal, the dynamic range and the sensitivity of the assay.

It is recommended to test both possible assay configurations as the sensitivity and the level of counts may differ dramatically depending on the set-up. In this case, the two configurations are as follows:

1. Eu antibody A + U*Light* antibody B
2. Eu antibody B + U*Light* antibody A

We recommend starting with 0.3 nM, 1 nM, and 3 nM (final) of the Eu-antibody and 1 nM, 3 nM, 5 nM, and 10 nM (final) of the U*Light*-antibody. Figure 3 shows the basic workflow for setting up the assay. Figure 4 shows a plate map for testing 10 ng/mL and 0 ng/mL of analyte for each combination.

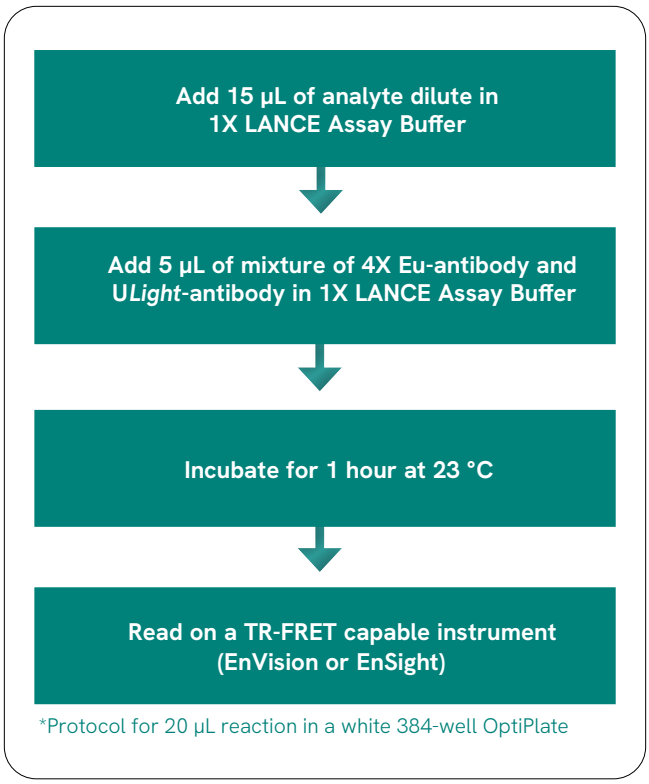


Figure 3: Basic LANCE TR-FRET protocol.

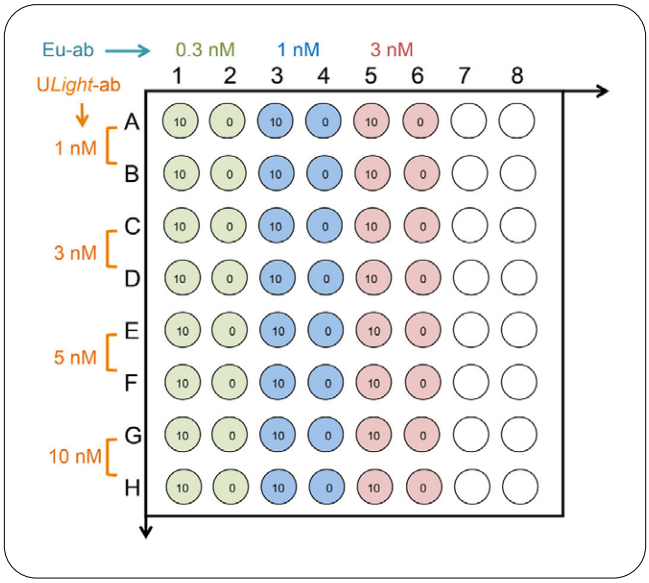


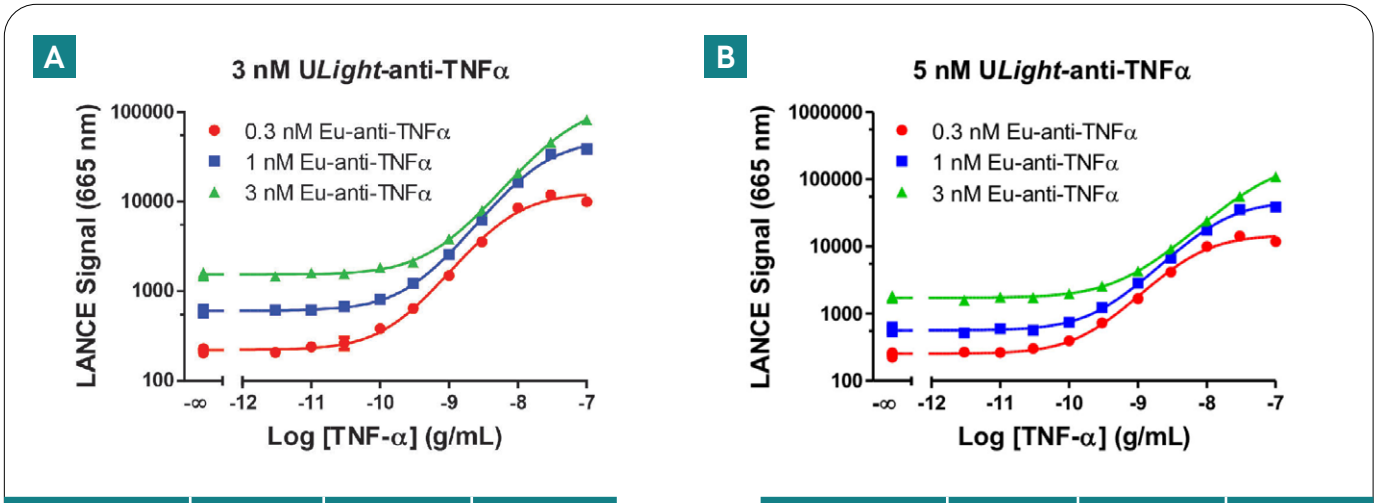
Figure 4: Plate map for setting up cross-titration of Eu-antibody (Eu-ab) and U*Light*-antibody (U*Light*-ab) in a 384-well plate in duplicate. The concentrations of analyte shown are 10 ng/mL (10) and 0 ng/mL (0).

Table 2 shows an example of a cross-titration for detecting TNFα using one configuration of antibodies. The signal to background for each concentration was calculated by taking the ratio of signal at 10 ng/mL of the TNFα analyte to 0 ng/mL of analyte. A few concentrations of analyte may be tested if needed. In addition, it may be useful to test 0 nM of each component in the presence of the other (i.e. 0 nM Eu-antibody in the presence of 1, 3, and 5 nM U*Light*-antibody) for troubleshooting.

Table 2: Cross-titration of Eu-anti-TNFα antibody and a U*Light*-anti-TNFα antibody. The signal to background is listed for each combination using 10 ng/mL of TNFα analyte. The best signal to background from the initial cross titration is highlighted in yellow.

	Eu-ab (0.3 nM)	Eu-ab (1 nM)	Eu-ab (3 nM)
U*Light*-ab (1 nM)	25.76	19.81	10.16
U*Light*-ab (3 nM)	39.21	27.4	13.60
U*Light*-ab (5 nM)	40.62	30.22	13.54
U*Light*-ab (10 nM)	37.06	28.4	11.96

Figure 5 shows full titration curves for two of the U*Light*-ab concentrations (3 nM and 5 nM) and all three Eu-ab concentrations. The signal to background, EC_{50}, and sensitivity for each curve was calculated and used to assess which antibody concentrations would be optimal for the assay. Although the higher concentrations of Eu-anti-TNFα show a higher signal to background, the sensitivity (LDL) was best with the lower (0.3 nM) Eu-antibody concentration. For LDL calculations, see Appendix A. This was due to the higher background seen using the higher concentrations of Eu-anti-TNFα. From the curves above, the best antibody concentrations are 0.3 nM Eu-anti-TNFα and 5 nM U*Light*-anti-TNFα. Here, we only show data from one configuration. As stated before, it is best to test the alternate configuration (swapping the antibodies conjugated to Eu and U*Light*) in order to fully assess which components will give the best assay.



3 nM ULight-ab	0.3 nM Eu-ab	1 nM Eu-ab	3 nM Eu-ab
EC_{50} (ng/mL)	12.3	19.4	54.5
LDL (pg/mL)	43.7	43	97.3
S:B (@Max)	46	65	54

5 nM ULight-ab	0.3 nM Eu- ab	1 nM Eu-ab	3 nM Eu-ab
EC_{50} (ng/mL)	11.8	16.1	78.9
LDL (pg/mL)	34	67.8	106
S:B (@Max)	48	67	62

Figure 5: Full titration curves for three different concentrations of Eu-antibody and two concentrations of ULight-anti-TNFα antibody. In yellow is the best assay condition.

II. Indirect method

The fastest and most convenient way to convert the immunoassay from a working ELISA to LANCE *Ultra* TR-FRET assay is to use antibodies from the ELISA with LANCE toolbox reagents. We refer to this as the "indirect method" for TR-FRET. However, this assay set-up has a few additional challenges compared with the direct method. First, TR-FRET is highly sensitive to the distance between the donor and acceptor. When using the indirect method, the distance between donor and acceptor increases because you are adding in additional capture molecules on the detection reagents (i.e. streptavidin or anti-species antibody). Therefore, the overall signal is expected to be lower than with the direct method. However, in many cases, the indirect method can still provide an excellent assay.

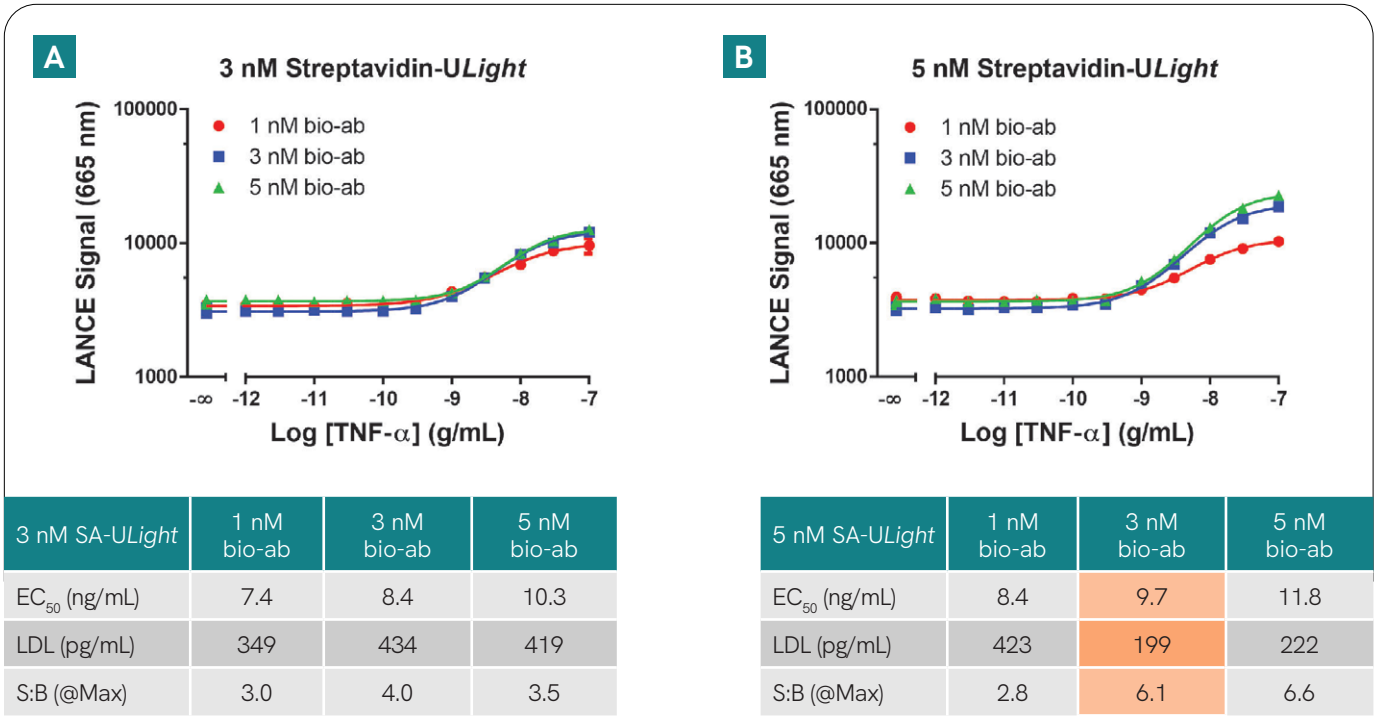
Another challenge with using the indirect method is that there are additional components whose concentrations must be optimized. For example, when setting up an indirect method for detecting TNFα, we had to optimize not only the concentrations of the detection reagents (Eu and ULight toolbox), but also both antibodies that bind to the TNFα. We recommend starting with 0.3, 1, and 3 nM for the Eu and 1, 3, and 5 nM for the ULight conjugates. In addition, we recommend starting with the same concentrations of antibody that bind to each of these components. For example, when using Streptavidin-ULight, use 1, 3, and 5 nM biotin-antibody. Table 3 shows the signal to background ratio of a cross-titration using a Eu-anti-mouse antibody, a mouse anti-TNFα antibody, a Streptavidin (SA)-ULight conjugate, a biotinylated anti-TNFα antibody, and 10 ng/mL TNFα or buffer to determine signal to background.

Table 3: Cross-titration of TR-FRET components for the indirect method. The best signal to background from the initial cross titration is highlighted in yellow.

	Bio-ab (5 nM)	Bio-ab (3 nM)	Bio-ab (1 nM)		
Mouse ab (3 nM)	1.23	1.81	1.56	Eu-anti-mouse (3 nM)	SA U*Light* (5 nM)
Mouse ab (3 nM)	1.06	1.67	1.63	Eu-anti-mouse (3 nM)	SA U*Light* (3 nM)
Mouse ab (3 nM)	1.22	1.18	1.59	Eu-anti-mouse (3 nM)	SA U*Light* (1 nM)
Mouse ab (3 nM)	1.69	2.08	1.79	Eu-anti-mouse (1 nM)	SA U*Light* (5 nM)
Mouse ab (3 nM)	0.67	1.68	1.60	Eu-anti-mouse (1 nM)	SA U*Light* (3 nM)
Mouse ab (3 nM)	2.70	1.47	1.76	Eu-anti-mouse (1 nM)	SA U*Light* (1 nM)
Mouse ab (3 nM)	1.97	2.33	1.87	Eu-anti-mouse (0.3 nM)	SA U*Light* (5 nM)
Mouse ab (3 nM)	1.23	2.01	1.91	Eu-anti-mouse (0.3 nM)	SA U*Light* (3 nM)
Mouse ab (3 nM)	1.04	1.29	1.58	Eu-anti-mouse (0.3 nM)	SA U*Light* (1 nM)
Mouse ab (1 nM)	0.98	2.30	2.08	Eu-anti-mouse (3 nM)	SA U*Light* (5 nM)
Mouse ab (1 nM)	1.97	1.80	2.17	Eu-anti-mouse (3 nM)	SA U*Light* (3 nM)
Mouse ab (1 nM)	1.50	1.43	2.01	Eu-anti-mouse (3 nM)	SA ULight (1 nM)
Mouse ab (1 nM)	2.67	2.61	2.36	Eu-anti-mouse (1 nM)	SA U*Light* (5 nM)
Mouse ab (1 nM)	1.87	2.42	2.73	Eu-anti-mouse (1 nM)	SA U*Light* (3 nM)
Mouse ab (1 nM)	1.19	1.61	2.44	Eu-anti-mouse (1 nM)	SA U*Light* (1 nM)
Mouse ab (1 nM)	2.59	3.01	2.86	Eu-anti-mouse (0.3 nM)	SA U*Light* (5 nM)
Mouse ab (1 nM)	1.98	2.59	2.97	Eu-anti-mouse (0.3 nM)	SA U*Light* (3 nM)
Mouse ab (1 nM)	1.31	1.73	2.60	Eu-anti-mouse (0.3 nM)	SA U*Light* (1 nM)
Mouse ab (0.3 nM)	1.23	1.39	1.81	Eu-anti-mouse (3 nM)	SA U*Light* (5 nM)
Mouse ab (0.3 nM)	1.04	1.46	1.62	Eu-anti-mouse (3 nM)	SA U*Light* (3 nM)
Mouse ab (0.3 nM)	0.98	1.30	1.56	Eu-anti-mouse (3 nM)	SA U*Light* (1 nM)
Mouse ab (0.3 nM)	1.97	2.95	3.03	Eu-anti-mouse (1 nM)	SA U*Light* (5 nM)
Mouse ab (0.3 nM)	1.50	2.48	3.10	Eu-anti-mouse (1 nM)	SA U*Light* (3 nM)
Mouse ab (0.3 nM)	1.16	1.62	2.84	Eu-anti-mouse (1 nM)	SA U*Light* (1 nM)
Mouse ab (0.3 nM)	3.48	3.80	4.09	Eu-anti-mouse (0.3 nM)	SA U*Light* (5 nM)
Mouse ab (0.3 nM)	2.34	3.36	4.00	Eu-anti-mouse (0.3 nM)	SA U*Light* (3 nM)
Mouse ab (0.3 nM)	1.48	1.75	3.23	Eu-anti-mouse (0.3 nM)	SA U*Light* (1 nM)

Although the signal to background is lower than for the direct method, we ended up seeing the best signal to background with similar concentrations of U*Light* (5 nM) and Europium (0.3 nM) conjugates. In this case, the 1 nM biotinylated anti-TNFα and 0.3 nM mouse anti-TNFα antibodies gave the best signal to background.

As with the direct method, this cross-titration should be repeated with the alternate configuration (Streptavidin-Eu and anti-Mouse-U*Light*) to determine the best configuration for the assay. Next, full titration curves should be performed with a few of the best conditions from the initial cross-titration to fully assess the signal to background, the dynamic range, and the sensitivity of the assay as shown in Figure 6.



3 nM SA-*ULight*	1 nM bio-ab	3 nM bio-ab	5 nM bio-ab
EC_{50} (ng/mL)	7.4	8.4	10.3
LDL (pg/mL)	349	434	419
S:B (@Max)	3.0	4.0	3.5

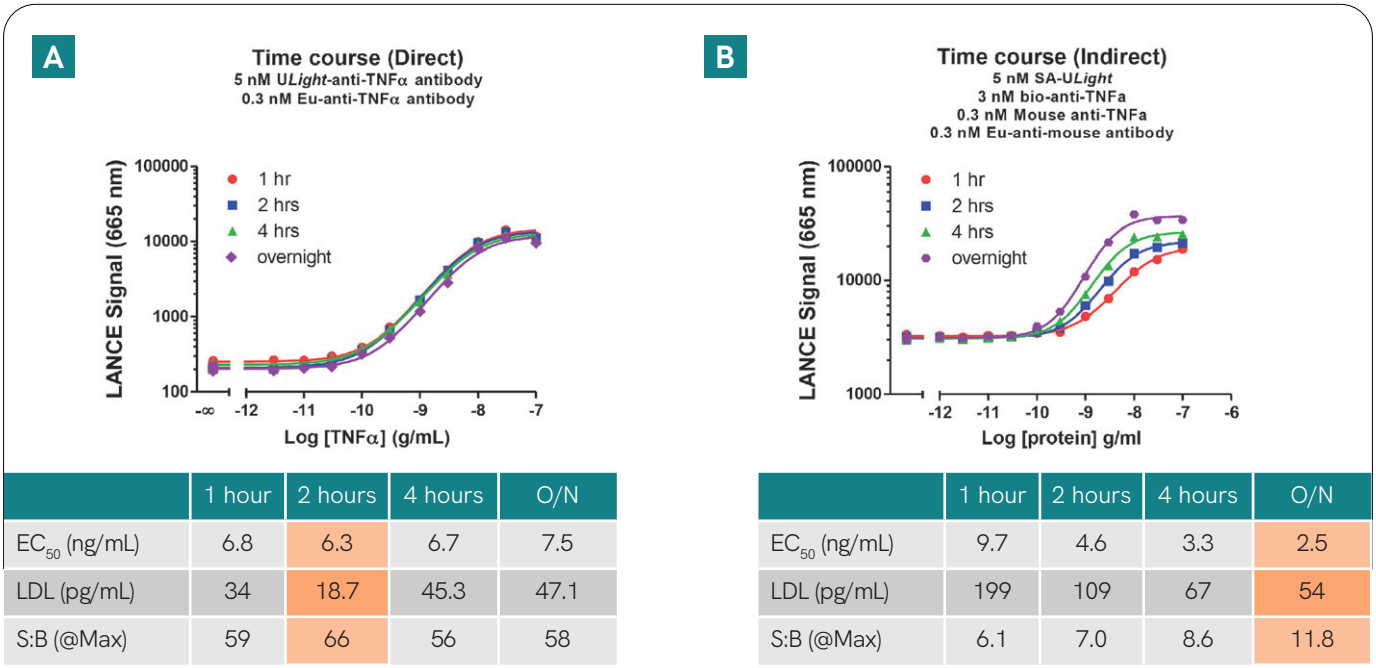
5 nM SA-*ULight*	1 nM bio-ab	3 nM bio-ab	5 nM bio-ab
EC_{50} (ng/mL)	8.4	9.7	11.8
LDL (pg/mL)	423	199	222
S:B (@Max)	2.8	6.1	6.6

Figure 6: Full titration curves for three different concentrations of Biotin-antibody and two concentrations of Streptavidin-U*Light*. In yellow is the best assay condition.

Optimization step 2: Incubation time

Another parameter that is useful to optimize is the incubation time for the reaction. In many cases, one hour is sufficient for the reaction to come to equilibrium. However, in order to find the optimal assay conditions, it may be necessary to incubate for longer times, including testing an overnight incubation. One of the advantages of LANCE *Ultra* TR-FRET is that the signal is very stable over time and not susceptible to quenching after reading. Therefore, the same plate can be read multiple times over a period of time to find the optimal incubation time. Testing the signal stability over time will also give information on the stability of the overall reaction (i.e. proteins and antibodies). This information is useful if a plate is to be sitting for a period of time waiting to be read, such as in the case of a screen using multiple plates.

Figure 7 shows a comparison between data collected with the direct method and the indirect method over time. The plate was re-read three additional times and the data plotted as a function of TNFα concentration. Figure 7A shows that the directly labeled reaction is already close to equilibrium after 1 hour incubation. However, as the data in the table below it shows, there is a slight advantage to the two hour incubation. Interestingly, the indirect method (Figure 7B) shows a much greater difference between the various time-points. This may be because there are five components that must all bind to create the signal as opposed to only three in the direct method. The overnight incubation significantly increases the signal to background and lowers the LDL. This indicates that the incubation time can greatly affect the assay.



	1 hour	2 hours	4 hours	O/N
EC_{50} (ng/mL)	6.8	6.3	6.7	7.5
LDL (pg/mL)	34	18.7	45.3	47.1
S:B (@Max)	59	66	56	58

	1 hour	2 hours	4 hours	O/N
EC_{50} (ng/mL)	9.7	4.6	3.3	2.5
LDL (pg/mL)	199	109	67	54
S:B (@Max)	6.1	7.0	8.6	11.8

Figure 7: Time course for direct and indirect methods. A) Direct method. The same plate was re-read after 1, 2, 4, and overnight (~18 hours). B) Indirect method. The same plate was re-read after 1, 2, 4 and overnight (~22 hours).

Optimization step 3: Assay buffers

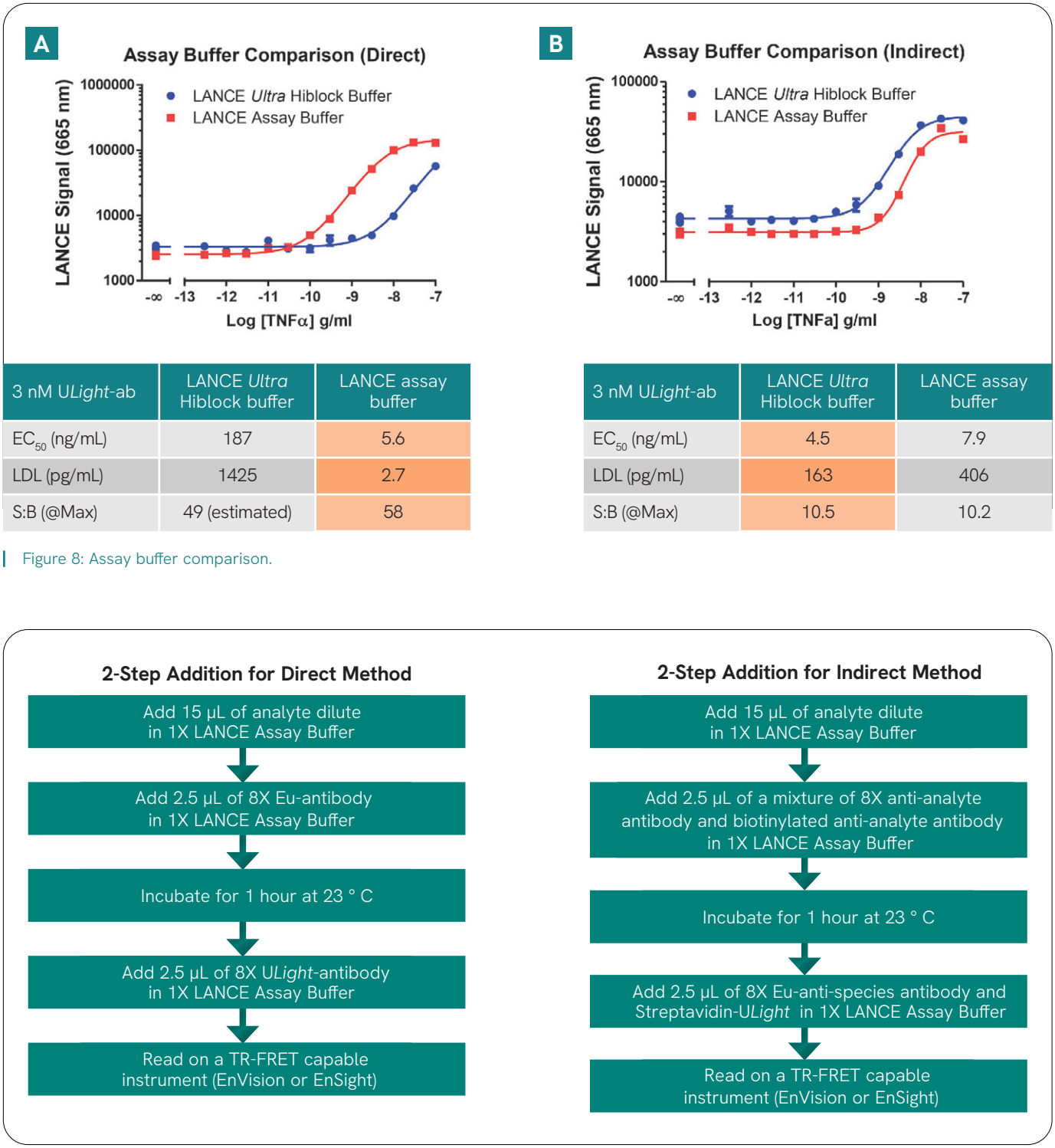
The buffer used to dilute the components of the reaction can have a dramatic effect on the assay performance. For LANCE *Ultra* TR-FRET assays, it is critical that the buffer used be highly pure and free from any chelates or contaminating metal ions that may interfere with the Europium chelate. Revvity offers highly pure buffer options that can be used when developing an assay. Two of these buffers are the LANCE Assay buffer (cat #TRFLAB-002, TRFLAB-100) and the LANCE *Ultra* Hiblock buffer (cat #TRF1011). Even though both of these are highly pure, the difference in the components of the buffer can also affect the assay and should be compared.

As shown in Figure 8A and 8B, the LANCE Assay buffer works best for the direct assay set-up using our TNFα (human) LANCE *Ultra* TR-FRET Detection kit (cat #TRF1208C). However, when using the indirect method for detecting TNFα that we optimized, the buffers gave similar results, with LANCE *Ultra* Hiblock buffer having a slight advantage in sensitivity

Optimization step 4: Protocol variations

The most time efficient protocol for doing a LANCE *Ultra* TR-FRET assay is to add a mixture of the Europium labeled component and the U*Light* labeled components directly to the sample and incubating for a period of time prior to reading. However, sometimes the sensitivity of an assay, dynamic range, and even the assay signal to background can be increased by adding in additional incubation steps with individual components of the assay.

For the direct method, the "2-step method" involves adding the Europium antibody or the U*Light* antibody and incubated for 1 hour prior to adding the other reagent. For the indirect method, a mixture of the target antibodies can be added to the sample and incubated prior to adding the detection reagents. In addition, for the indirect method, the reaction could be split up into a 3-step additions where the Europium and U*Light* are added in separate steps with an incubation period between the additions. The optimal order of addition should be determined for each particular assay.



3 nM U*Light*-ab	LANCE *Ultra* Hiblock buffer	LANCE assay buffer
EC_{50} (ng/mL)	187	5.6
LDL (pg/mL)	1425	2.7
S:B (@Max)	49 (estimated)	58

3 nM U*Light*-ab	LANCE *Ultra* Hiblock buffer	LANCE assay buffer
EC_{50} (ng/mL)	4.5	7.9
LDL (pg/mL)	163	406
S:B (@Max)	10.5	10.2

Figure 8: Assay buffer comparison.

Figure 9: Examples of alternate order-of-addition protocols for LANCE TR-FRET assays.

Possible further optimization

Table 4: Other Parameters that can be optimized.

TR-FRET Optimization parameter	Recommendations and Comments
Sample Volume	If sample is limiting, 5 µL or 10 µL can be used and detection reagents added at a higher volume. However, we have seen that 15 µL sample volume usually gives the best sensitivity.
Assay Volume	Due to the brightness of the Europium chelate, LANCE TR-FRET assays can be miniaturized to 10 or 5 µL final volumes and still give highly accurate data. In addition, increasing assay volume may allow for addition of a larger volume of sample.
Ratio of Europium or U*Light* to Antibody	For the direct method, the ratios used for Europium and U*Light* conjugations may be optimized. This is usually done through the custom services, but can also be done by the customer if labeling your own antibody.

Tips and troubleshooting

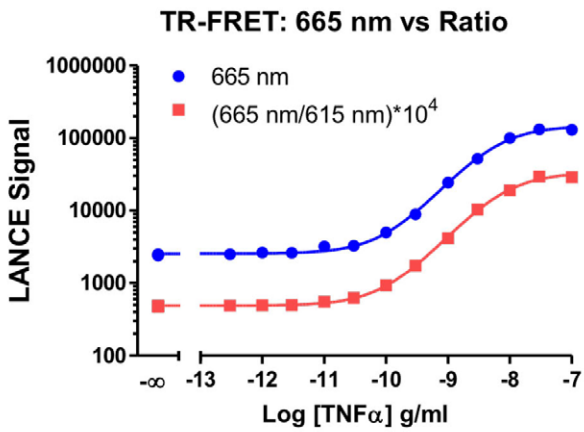
Table 5: Tips and Troubleshooting.

Problem	Cause	Solution
High background	Concentration of Eu conjugate or U*Light* conjugate is too high	Test lower concentrations of Europium and/or U*Light*
	Antibodies interacting in the absence of analyte	If using indirect capture method, test alternate detection reagents
	Did not remove TopSeal prior to reading	Reading plate with TopSeal can give high background
Low Signal	Assay conditions not optimal	Test longer incubation times or alternative buffers
	Buffer made with low quality water or low quality BSA	Contaminating heavy metal cations at high concentrations interact with the Europium chelate and quench fluorescence. Only use ultrapure laboratory grade water (and BSA) for reagent preparation
	Using black microplates	White opaque microplates are recommended for obtaining the highest signal.
	Distance between Europium and U*Light* is too large	Use direct capture method instead of indirect capture or test alternate antibodies/configurations
	Instrument settings not optimal	Check instrument settings
Signal decreases with increasing analyte concentration	Starting analyte concentration is too high	If analyte concentration is too high, you may be already over the Hook point. Test lower analyte concentrations
No specific signal	Direct Europium conjugation is interfering with binding to analyte	Test lower ratios of Europium to antibody conjugation or test indirect capture method
	Distance between Europium donor and U*Light* acceptor is too large	Use direct capture method instead of indirect capture or test alternate antibodies/configuration

Appendix A: Data analysis

I. Plotting standard curves

Typically, LANCE *Ultra* TR-FRET data is fit to a dose-response curve using a sigmoidal or four-parameter with variable slope fit. This method of fitting takes advantage of the full dynamic range of the assay. These types of curves can be fit using standard statistical software, such as GraphPad Prism® or Microsoft® Excel® with Solver plug-in. TR-FRET data can be analyzed using the emission of U*Light* at 665 nM or alternatively, it can be analyzed using the ratio of the emission from the Europium donor (at 615 nM) to the emission of the U*Light* at 665 nM. Since the emission of Europium at 615 nM is typically much higher signal than that of the acceptor dye at 665 nM, it is often convenient to multiple the ratio by a factor, such as 10^4, so as not to have to plot a fraction on the y-axis. Using the ratio can minimize error in replicates from well to well. In addition, if there are components in the sample that slightly quench the fluorescence, using the ratio can normalize the data to account for differences in quenching from sample to sample. Figure 10 shows an example of data analyzed using the two different methods. In this case, the differences are minimal.



3 nM U*Light*-ab	665 nM	(665 nM/ 615 nM)*104
EC_{50} (ng/mL)	5.6	7.3
LDL (pg/mL)	2.7	3.8
S:B (@Max)	58	68.5

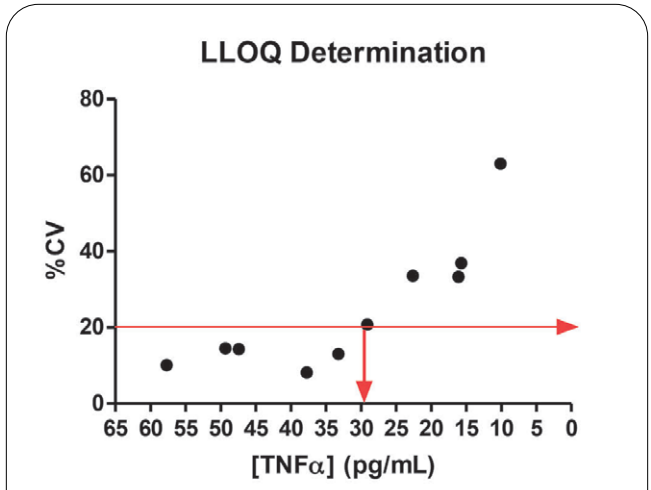
Figure 10: Comparison of data analyzed with the 665 nM or the Ratio of 665 to 615 nM signals using the standard curve from TNFα (human) LANCE *Ultra* TR-FRET detection kit (cat #TRF1208C).

If your standard curve begins to decrease at high concentrations of analyte tested you are probably seeing a "hook effect". The hook effect occurs when you have saturated your detection reagents with analyte. When excess analyte is present, it may bind one or the other antibody, but not both at the same time, disrupting the association between the Europium donor and the U*Light* acceptor. Any concentration points beyond the hook should be removed prior to analysis. After fitting the standard curve, you can interpolate the lower detection limit, the lower limit of quantification, and the concentrations of analyte for your unknowns.

II. Determining the LDL and LLOQ

Assessing the true sensitivity of the assay requires determining not only the lower detection limit (LDL or LLD) but also the lower limit of quantification (LLOQ). The LDL is equivalent to the concentration interpolated from a signal corresponding to the mean of your "zero analyte" signals + two standard deviations. A basic method for determining LLOQ is the concentration interpolated from a signal corresponding to the mean of your "zero analyte" signals + 10 standard deviations. However, below we describe a more accurate way to determine the LLOQ.

In order to more accurately determine the LLOQ for the assay, at least ten data points spanning from below the LDL to at least 4X the LDL were tested in the assay using 10 replicates of each concentration. A standard curve was run with the samples and each data point was interpolated from the standard curve to give the most accurate TNFα concentration. The two lowest data points could not be accurately quantified using the standard curve and therefore were not used in the determination of LLOQ. The average, standard deviation, and % CV were calculated for the 10 replicates of each concentration using the equation: %CV = (standard deviation/mean)*100%. Finally, a scatterplot was made of the %CV as a function of the mean concentration of the 10 replicates. The LLOQ is the point which the %CV becomes tighter than 20%. In this case, concentrations larger than 30 pg/mL show %CVs less than 20%.



[TNFα] (pg/mL) (10 replicates)	% CV
57.7	10.1
49.3	14.5
47.4	14.3
37.7	8.2
33.2	13.0
29.1	20.8
22.6	33.6
15.7	36.9
16.1	33.3
10.1	63

Figure 11: Scatterplot and table of % CV as a function of TNFα concentration for accurate LLOQ determination. The data was collected using the from TNFα (human) LANCE *Ultra* TR-FRET detection kit (cat #TRF1208C).

Appendix B: Instrument requirement

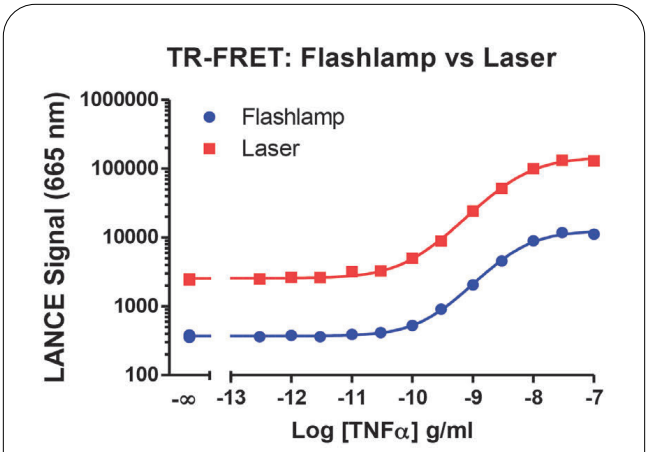
LANCE TR-FRET assays require a microplate reader that is capable of time-resolved fluorescence. Below is a table for recommended instrument setting for running a LANCE TR-FRET Immunoassay.

Revvity EnVision can be equipped with either a laser or a flashlamp. Below are setting for EnVisions equipped with a laser. Figure 12 shows data collected using either the laser or the flashlamp on the EnVision. The data is fairly comparable, with the laser giving better sensitivity and a slightly higher signal to background. Figure 13 shows data collected on the EnVision using a flashlamp and comparing it to data generated on the EnSight (which also uses a flashlamp). Again, the data are fairly comparable.

Table 6: Instrument Settings.

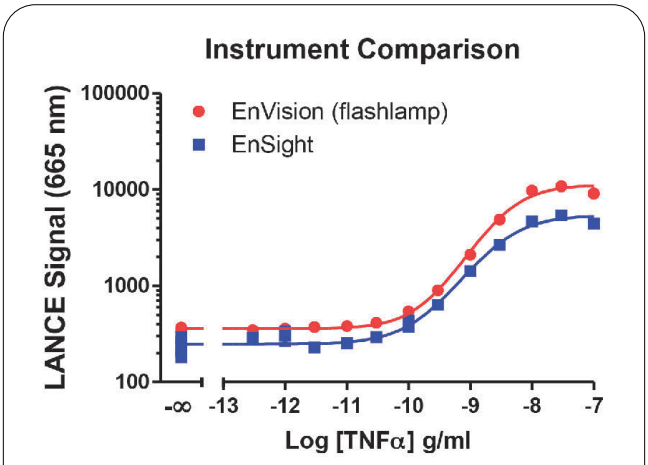
Parameter	EnVision	EnSight	VICTOR	ViewLux
Flash Energy Area	n/a	n/a	High	n/a
Flash Energy Level	100%	n/a	150	*800,000/600,000
Excitation Filter	UV2 320	UV (TRF) 320 (111)	320/340	DUG11 (UMB, AMC)
Integrator Cap	n/a	n/a	2 (3 if signal is low)	n/a
Integrator Level	n/a	n/a	2X the setting in LANCE High Count 615 label	n/a
Emission Filter	203- Eu 615	Monochromator 615 nM	615 nM	618/8 (Eu)
Emission Filter	205- APC 665	Monochromator 665 nM	665 nM	671/8 (LANCE)
Delay Time	60 µs	70 µs	50 µs	50 µs
Readout speed, gain and binning	n/a	n/a	n/a	Medium, high, and 2X
Measurement Time/ Number of Flashes	100 (200 if signal too low)	100	n/a	20 us exposure time
Window	100 µs (200-300 µs if signal too low)	100 µs	100 µs (200-300 µs if signal too low)	354 µs
Mirror module	462 (D400/D630) or 412 (D400)	n/a	n/a	*Mirror 2 (UV dichroic) or n/a
Cycle	2000 µs	n/a	1000/2000 µs	n/a

* ViewLux with flat field correction, bias correction, cosmic ray detection, excitation energy compensation



	Flashlamp	Laser
EC_{50} (ng/mL)	5.1	5.6
LDL (pg/mL)	30	2.7
S:B (@Max)	34	58

Figure 12: Comparison of data read using a revvity EnVision with either a laser or a flashlamp. The standard curve is from the TNFα (human) LANCE *Ultra* TR-FRET detection kit (cat #TRF1208C).



	EnVision (Flashlamp)	Ensight
EC_{50} (ng/mL)	3.8	3.1
LDL (pg/mL)	36	80
S:B (@Max)	31	22

Figure 13: Comparison of data from the same plate read using either a revvity EnVision (flashlamp) or a revvity EnSight. The standard curve is from the TNFα (human) LANCE *Ultra* TR-FRET detection kit (cat #TRF1208C).

Laser settings for LANCE assays on an EnVision

Delay: 50 µs (you could measure delay 10 µs to get slightly improved S/B)

Window 100 µs (it is important not to use too

Time: long window time)

Time Between Flashes: 16600 µs

Focus Height: 6.5 mM

Appendix C: Europium labeling antibodies

Customers can develop their own TR-FRET immunoassay by directly labeling their antibody with a Europium lanthanide chelate. For labeling antibodies for an immunoassay, we recommend the Isothiocyanate (ITC)-activated chelates. These react primarily with free amine groups, including the N-terminus of a protein or peptide as well as lysine residues on antibodies, proteins, and peptides.

Below is a guide on how to directly label an antibody with a Eu-chelate. Protein concentration, pH, temperature, reaction time, and molar excess of chelate are critical parameters that must be met in order to have an efficient labeling.

Calculations for labeling antibodies

The recommended reaction conditions for labelling of antibodies are pH 9-9.3, 4 °C, and overnight incubation. Under these conditions, the following calculations are valid for labeling a protein with an isoelectric point (pI) between 4 and 7.

For example, if an antibody (pI around six, molecular weight 160kDa) is reacted at a concentration of five mg/mL under the conditions described above, a 24-fold molar excess of chelate over protein would give a labelling degree of about six Eu-W1024 chelates

per protein. If the antibody to be labelled is not stable under the labeling conditions (+4 °C, pH 9-9.3, overnight incubation), it is possible to run a four-hour reaction (+4 °C, pH 9-9.3) by increasing the molar excess of chelate over protein. A suitable amount of chelate is three times higher for the four-hour reaction than for the overnight reaction. For example, if a protein (5 mg/mL in the labelling reaction) requires 24-fold molar excess of chelate during overnight reaction for the introduction of six chelates per protein, 72-fold molar excess of W1024 is needed to obtain the same label incorporation during a four-hour reaction. Suitable number of W1024 chelates coupled to a protein depends on the molecular weight (MW). When the MW of an antibody is higher than 100kDa, 4-10 chelates per protein is a good labeling yield.

Table 7: The effect of protein concentration on the percentage of lyophilized Eu-W1024 ITC chelate reacting with the antibody.

Antibody concentration	Percentage of Chelate Reacted
5 mg/mL	25%
2.5 mg/mL	12%
1 mg/mL	5%

Labeling procedure

The antibody to be labelled must not be stabilized with a protein (i.e. bovine serum albumin (BSA), casein, or gelatin).

1. Pre-treatment

If the buffer including the antibody to be labelled contains primary amines (i.e. Tris, ammonium ions), sulfhydryl groups (i.e. mercaptoethanol), or sodium azide, a pre-treatment step is necessary. The above mentioned compounds interfere with the labelling. Suitable methods for removing interfering compounds include gel filtration, dialysis, or reverse phase HPLC.

2. Concentrating the antibody

If the antibody is too dilute (less than 1 mg/mL) or it is preferable to use less chelate to facilitate purification after labeling, a concentration step is necessary.

3. Reconstitution of Chelate

After calculating the molar excess of chelate needed in the reaction, the Eu-W1024 ITC is dissolved in distilled water. Suitable concentration for reconstituted Eu-W1024 ITC chelate is 1-5 mM (0.1 mg of Eu-W1024 ITC is 0.14 µmol). For example, dissolving 0.1 mg of Eu-W1024 ITC chelate in 100 µL water gives a concentration of 1.4 mM. After dissolving the chelate it is kept on ice for immediate use.

4. Labeling

If the antibody is already in labelling buffer (50-1100 mM sodium carbonate, pH 9-9.3) after pretreatment or reconstitution, the calculated amount of chelate is added into the antibody solution on ice. If it is not in labelling buffer, 1 M sodium carbonate, pH 9-9.3, is added to adjust the buffer concentration to 50-100 mM followed by the calculated amount of reconstituted chelate.

5. Purification

Separation of the labeled antibody from the unreacted chelate is performed by gel filtration. Elution buffer should be Tris-HCL based, i.e. 50 mM Tris-HCL (pH 7.8) containing 0.9% NaCl and 0.05% sodium azide (TSA buffer). Most antibodies can be purified using Superdex 200 column or a combination os Sephadex G-50 DNA Grade layered on Sepharose 6B.

Characterization of labeled antibody

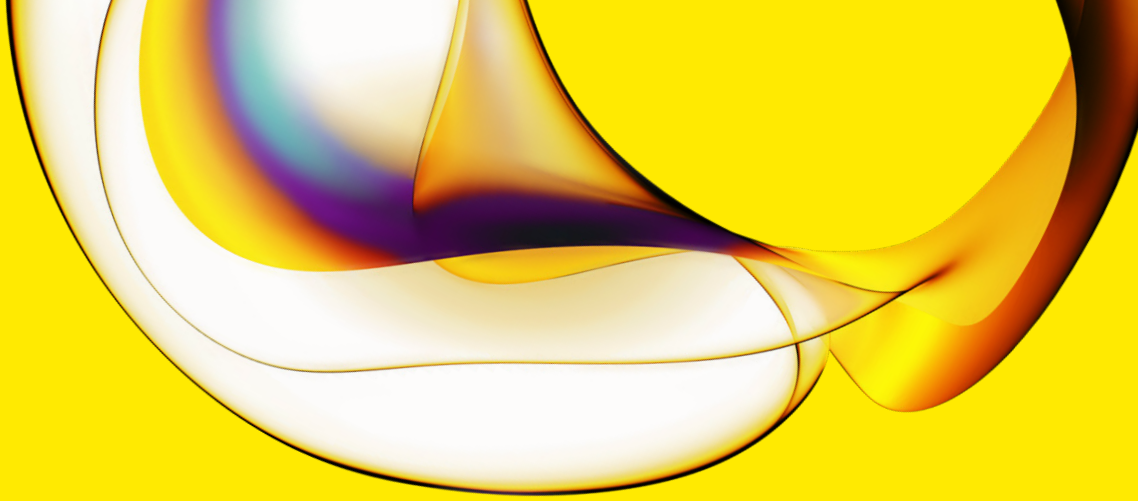
Determination of Eu^{3+} concentration in the labeled antibody can be performed as follows. First, the labeled antibody is diluted in 0.1 M HCL (1:100) and incubated at room temperature for 10 minutes. The final dilution is performed in DELFIA Enhancement Solution (cat #1244-105) and the dilution factor has to be at least 1:100 to dilute out the effect of HCl on the pH of Enhancement Solution. Eu fluorescence of the sample is measured against 100 nMol/L Eu standard (supplied with the chelate) diluted 1:100 in Enhancement Solution. Antibody concentration can

be calculated from absorbance at 280 nM.
The contribution of Eu-W1024 ITC chelate to absorbance at 280 nM is 0.016 per 1 µmol/L chelate.

Storage of labeled antibody

To remove particles and possible aggregates, the labelled antibody should be filtered through a 0.22 um low protein binding membrane. To ensure stability, the labeled antibody should be stored at a high concentration and in the absence of chelators or competing metals in the buffer. A concentrated solution (0.1 mg/mL or higher) can be stored without any stabilizer. Temperature during storage is determined by the stability of the antibody. Suitable temperatures are 4 °C, -20 °C, and -70 °C.

For the most updated LANCE TR-FRET products and catalog numbers, see www.revvity.com



www.revvity.com

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

For a complete listing of our global offices, visit www.revvity.com

013401_01