

LanthaScreen™ Frequently Asked Questions and Technology Comparison

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I. Filter Settings, Instruments and Plates

What filters/dichroics do I need to read a LanthaScreen™ assay?

We recommend using a 340 nm excitation filter with a 30 nm bandwidth, but an excitation filter with similar specifications will perform well. The exact specifications of the emission filters are more important. In a LanthaScreen™ assay the donor (terbium) intensity is measured using a 495 nm filter (10 nm bandwidth), and the fluorescein (acceptor) intensity is measured using a 520 nm filter (25 nm bandwidth). Filters suitable for performing LanthaScreen™ assays can be obtained from Chroma as filter set PV001, or from other vendors. A filter module for the BMG PHERAstar can be obtained direct from BMG Instruments.

The dichroic mirror should ideally have a cutoff of less than 490 nm. A dichroic mirror meeting this specification is available from Chroma as part of filter set PV001, or from the instrument manufacturer. In some instruments (Tecan instruments, for example) the fluorescein dichroic performs well but must be manually selected. In other instruments a 50:50 mirror may be selected, but in general will not give as high performance as a dedicated dichroic. The LanthaScreen™ filter module from BMG contains an optimal dichroic mirror.

Can I use a standard fluorescein filter to measure LanthaScreen™ emission?

In general, no. The typical fluorescein emission filter has a bandwidth that is too large for optimal performance in a LanthaScreen™ assay. Such filters not only measure the intensity of the acceptor fluorophore, but also signals from terbium itself. Having this “contaminating” intensity present in the data will make the acceptor signal only a small portion of the raw intensity. This will have a negative impact on the data.

Can I use a monochromator based instrument to read LanthaScreen™ assays?

We have found two monochromator-based instruments that work well with LanthaScreen™ biochemical assays; the Tecan Safire²™ and the Molecular Devices SpectraMax M5. For the Safire²™, we recommend exciting the samples at 332 nm with a 20 nm bandwidth. The donor (terbium) signal is measured at 485 nm with a 20 nm bandwidth and the acceptor (fluorescein) signal is measured at 515 nm with a 20 nm bandwidth. With the SpectraMax M5, we recommend exciting at 332 nm and measuring intensity at 488 and 518 nm (the band width is built in at 15 nm and cannot be adjusted). When using a monochromator based instrument, white assay plates often show better performance. In filter-based instruments the color of the plate usually shows a negligible effect on data quality.

If you are using a LanthaScreen™ GFP Cellular Assay, we do not recommend the use of monochromator-based instruments, as the sensitivity of these instruments is not sufficient to adequately detect the endogenously expressed GFP fusion proteins.

Are there specific plates I should use for my LanthaScreen™ Assay?

The LanthaScreen™ biochemical assay format is suitable for nearly any brand or volume of assay plate. Invitrogen has developed many assays using Corning low volume black polystyrene 384 well plates (#3676). We have also successfully used Corning low volume white polystyrene 384 well plates (#3673), Thermo LabSystems Microfluor 1 Black 96 well plates (#7005), and Thermo Electron Cliniplate black polypropylene 384 well Plates (#95040020). When performing the assay on monochromator based readers such as the Tecan Safire² or the Molecular Devices M5, assay performance may be increased by using

white microtiter plates. With filter-based instruments, the differences seen between black and white plates are often negligible.

We recommend the use of white assay plates for LanthaScreen™ GFP Cellular Assays to achieve optimal signal detection. We have successfully used Corning low-volume white polystyrene plates (Corning Cat. no. 3674) and Corning tissue culture-treated white polystyrene plates (Corning Cat. no. 3704). The tissue culture-treated plate is recommended for assay formats that perform cell treatment, lysis, and detection in the same well/plate. The low-volume plate provides excellent results in assay formats where the cell lysate and terbium-labeled detection antibody are combined in a plate different from the cell cultivation plate. We expect LanthaScreen™ GFP Cellular Assays to be compatible with equivalent plates from all major manufacturers.

Certain LanthaScreen™ GFP Cell Lines, which exhibit poor adherence to standard tissue culture-treated plates, may require the use of poly L-lysine coated plates, to prevent loss of cells during medium removal.

II. Applications of LanthaScreen™ TR-FRET Technology

What applications are the LanthaScreen™ reagents appropriate for?

Terbium-labeled phosphospecific antibodies have been applied to assays for over 150 kinases. Antibodies specific to epitope tags such as 6-His or GST have been applied to nuclear receptor assays. Terbium labeled streptavidin is optimal for developing protease assays using biotinylated fluorescein labeled peptides. Ubiquitin assays for E3s have been developed using labeled ubiquitin proteins and anti-epitope tags, and deubiquitination assays can be performed using our DUB substrate.

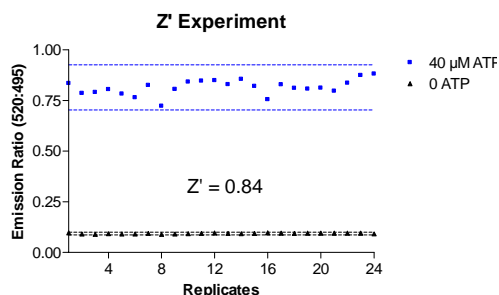
See the new brochure ("Simplify your TR-FRET assay development") and website (www.invitrogen.com/lanthascreen) for data and examples.

Can I use LanthaScreen™ reagents in cell lysate assays, and do you have data or protocols for this application?

We have not yet developed products specific to cell-lysate based applications. However, we have shown in several cases that the Tb-based LanthaScreen™ signal is indeed stable in the presence of cell lysates. So, for customers building their own assays using the LanthaScreen™ Toolbox, the presence of cell lysate components is not expected to interfere with the LanthaScreen™ signal.

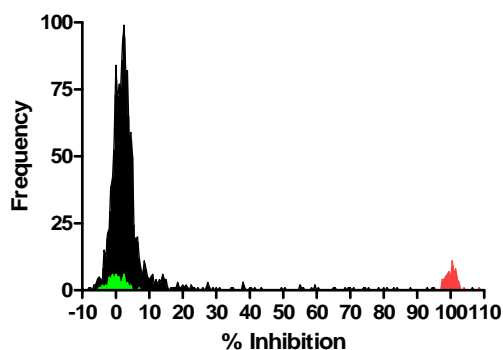
How does LanthaScreen™ perform in a screening environment?

Typical Z' values observed are 0.7 or greater.



The kinase reaction was performed using 30 ng/mL Src, 40 μM ATP, and 400 nM fluorescein-polyGAT substrate in 1X kinase buffer. The reaction was incubated for 90 minutes at room temperature. The reaction was stopped by adding 5 μL 40 mM EDTA in TR-FRET dilution buffer. This was immediately followed by the addition of 5 μL of 8 nM Tb-PY20 antibody in TR-FRET dilution buffer. The reaction was allowed to incubate at room temperature for 60 minutes before it was read on a Tecan Ultra

EGFR LOPAC Screen

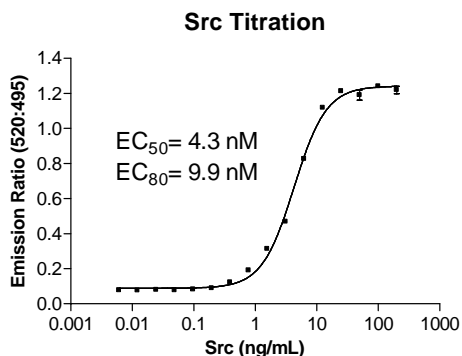


The sample screen was performed using 250 ng/mL of EGFR, 5 μ M ATP, and 400 nM fluorescein-polyGATsubstrate in 1X kinase buffer, 2 nM of Terbium labeled p-Y20 Ab was added and the reaction was stopped using 10 mM EDTA. The LOPAC¹²⁶⁰® (Library of Pharmacologically Active Compounds) obtained from Sigma Aldrich were tested at a concentration of 10 μ M. 30 hits above 50% inhibition were identified, including known kinase inhibitors found in the library. The screen resulted in a Z' of 0.87.

III. Assay Parameters

How do I determine how much kinase to use in a LanthaScreen™ Assay?

To determine the proper amount of kinase to use under a given set of substrate and/or ATP concentrations, you should perform the assay against a serial dilution of the kinase using a constant concentration of substrate and ATP. From the data generated, determine the EC₈₀ (or the amount of kinase that will generate 80% of the maximal change in the FRET ratio). Use this amount of kinase for any additional optimizations. You may want to begin your kinase titration at a concentration lower than is typical in other kinase assay formats, as the LanthaScreen™ assay is extremely sensitive.



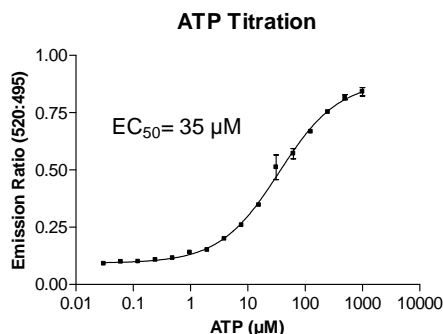
The 2-fold serial dilution of kinase was performed in the presence of 100 μ M ATP and 400 nM fluorescein-polyGAT substrate in 1X kinase buffer. The reaction was incubated for 90 minutes at room temperature. The reaction was stopped by adding 5 μ L 40 mM EDTA in TR-FRET dilution buffer. This was immediately followed by the addition of 5 μ L of 8 nM Tb-PY20 antibody in TR-FRET dilution buffer. The reaction was allowed to incubate at room temperature for 60 minutes before it was read on a Tecan Ultra

When performing a kinase titration, why does the FRET ratio sometimes decrease at high concentrations of kinase?

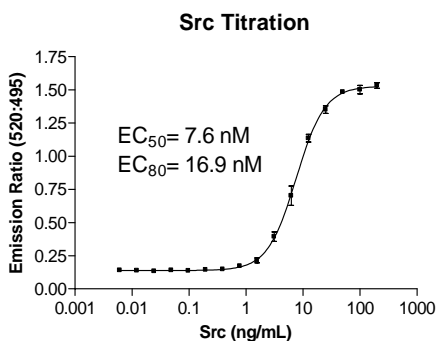
At high concentrations of kinase a decrease in signal ratio may be observed, especially with tyrosine kinases. Because the kinase itself is often phosphorylated (or can be autophosphorylated), at high concentrations it can be bound by the Tb-labeled antibody, thereby competing with fluorescein labeled phosphorylated substrate for binding to the antibody. If this phenomenon is observed, it indicates that a lower concentration of kinase should be used in the assay.

How do I determine how much ATP to use in a LanthaScreen™ Assay?

The amount of ATP to use depends on the goals of the specific assay. In an assay designed to identify ATP-competitive inhibitors, the assay is typically run at the ATP K_m apparent. To determine the ATP K_m apparent, perform the assay using a serial dilution of ATP and a constant amount of kinase and substrate. The EC_{50} value of this curve is the ATP K_m apparent. Now, using the determined concentration of ATP and the same amount of substrate, run the assay using a dilution series of kinase. From the kinase titration curve, determine the EC_{80} concentration of kinase and use this concentration of kinase in subsequent assays.



The kinase reaction was performed using 10 pg/µL Src with 400 nM fluorescein-polyGAT substrate in 1X kinase buffer with a 2-fold serial dilution of ATP. The reaction was incubated for 90 minutes at room temperature. The reaction was stopped by adding 5 µL 40 mM EDTA in TR-FRET dilution buffer. This was immediately followed by the addition of 5 µL of 8 nM Tb-PY20 antibody in TR-FRET dilution buffer. The reaction was allowed to incubate at room temperature for 60 minutes before it was read on a Tecan Ultra.



The 2-fold serial dilution of kinase was performed in the presence of 40 µM ATP and 400 nM fluorescein-polyGAT substrate in 1X kinase buffer. The reaction was incubated for 90 minutes at room temperature. The reaction was stopped by adding 5 µL 40 mM EDTA in TR-FRET dilution buffer. This was immediately followed by the addition of 5 µL of 8 nM Tb-PY20 antibody in TR-FRET dilution buffer. The reaction was allowed to incubate at room temperature for 60 minutes before it was read on a Tecan Ultra.

How much substrate should I use in a LanthaScreen™ assay?

In general, 200 nM to 1 µM peptide or physiological protein substrate is often used in a LanthaScreen™ kinase assay. We recommend starting with 200 or 400 nM in the assay reaction. The molar concentration of substrate can be calculated from the molecular weight of the substrate, by dividing the mg/ml concentration by the molecular weight. This will give a molar concentration of the substrate. Multiplying by 1,000,000 will give a µmolar concentration. The molecular weight of each substrate can be found on the certificate of analysis for each individual substrate or on our website.

$$[\text{Substrate}] \text{ in } \mu\text{M} = \frac{\text{mg/ml}}{\text{molecular weight}} \times 1,000,000$$

Which LanthaScreen™ antibody should I use?

For tyrosine kinase assays, we offer four different terbium labeled anti-phosphotyrosine antibodies. When using fluorescein labeled poly-GAT or poly-GT, performance using the Tb-labeled PY20 antibody is excellent. When using specific peptide substrates, performance may vary between the different varieties of anti phosphotyrosine antibodies, and as such we recommend evaluating the assay with a selection of antibodies in order to find the antibody that gives optimal performance. However, for tyrosine kinase assays, any of the anti-phosphotyrosine antibodies will likely perform well.

For serine/threonine kinases, we provide a reactivity chart at www.invitrogen.com/lanthascreen which shows the performance of various kinases using suitable substrate/antibody pairs.

I already have an antibody that I would like to use with in a LanthaScreen™ Assay. Is there a way I can determine if it will work without directly labeling it?

Yes, you may "indirectly" label your primary antibody by using a terbium labeled, species-specific anti-IgG antibody. As a starting point for further optimization, we recommend a final concentration of 2 nM primary and 5 nM terbium-labeled secondary antibody in the assay well. The reaction conditions can be further optimized by increasing and/or decreasing the amount of terbium-labeled secondary antibody to determine the optimal amount to use. Check with the supplier of your primary antibody to determine its stock concentration. While in a majority of cases the unlabeled primary and terbium labeled secondary antibody may be pre-mixed prior to addition to the assay well, in some cases separate additions (primary first, followed by secondary) are required. In general, assays using directly labeled antibodies may come to equilibrium faster or have a more stable signal (longer read window) than assays that use indirect labeling techniques. Also, although the response window (Z') is often acceptable when using an indirect labeling strategy, it may sometimes be improved by using a directly labeled primary antibody.

I would like to my antibody directly labeled with the LanthaScreen™ terbium chelate, how do I do that?

There are a couple of different ways to directly label your antibody. First, you can have our custom labeling service prepare the material for you. You send us 1 mg of material or more and we will perform the labeling reaction for you. As an alternative to that, you may buy our terbium chelate in an amine or thiol reactive form and perform the labeling reaction in your lab.

How much antibody should I use for my kinase assay?

In general, we recommend 2 nM as the final concentration of antibody in the detection reaction. The molecular weight of a Tb labeled antibody is 150,000 Da so a concentration of a 2 nM solution corresponds to 0.3 µg/mL. This is a 1:3,300 dilution of a 1 mg/mL stock solution.

How many assays can I run with a given amount of antibody?

Again, this depends on the volume of the assay and the concentration of antibody used. At 2 nM antibody in a final volume of 20 µL, 25 µg of antibody will run >4000 assays, and 1 mg of antibody will run >165,000 assays.

Which substrate should I use to assay my tyrosine kinase?

If you want a specific peptide substrate, we offer two synthetic peptides determined to be effective substrates for src-family kinases; peptide 1 (YIYGSFK) and peptide 2 (KVEKIGEGTYGVVYK). Peptide 1 was a random peptide identified to be a pp60^{c-src} substrate and peptide 2 is a sequence from p34^{cdc2}. Alternatively, fluorescein labeled peptide substrates can be readily synthesized by Invitrogen to suit your particular needs. In addition to peptide substrates, we offer fluorescein labeled Poly-GT and Poly-GAT. We

have had 100% success rate with these substrates to-date when assaying Tyrosine Kinases (TKs).

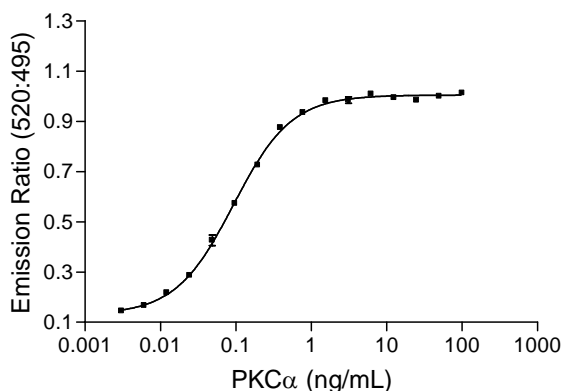
Should I use Poly-GT or Poly-GAT?

Try both or check our data to see if we have run your kinase of interest against these substrates (see reactivity chart at www.invitrogen.com/lanthascreen). In general, either substrate will work for a given TK. However, one of the substrates will probably show a lower EC₅₀ with the kinase (i.e. be a better substrate for the kinase), allowing less kinase to be used in the assay. Additionally, one substrate may show a larger assay window than the other (usually, but not always, Poly-GAT shows a larger window). However, either substrate will give an assay with an excellent Z' value.

I already have a biotinylated substrate that I know works with my kinase. Can I use this in a LanthaScreen™ assay?

Yes, a biotinylated substrate can be used in a LanthaScreen™ assay provided you have fluorescein-labeled streptavidin (available from Invitrogen part number SA100-02). A typical assay should contain 200-400 nM biotinylated substrate in the kinase reaction, and 1-2 nM Tb-labeled antibody and 50 nM fluorescein labeled streptavidin to detect the product. These conditions will need to be optimized for your particular system, but the recommended amounts are a good starting place. This format does, however, increase the cost associated with screening because of the additional component compared to the directly labeled substrate format. Also, it adds another layer of complexity with the additional component requiring optimization.

PKC α Trimolecular LanthaScreen™



The kinase reaction was performed using 10 μ M ATP and 200 nM substrate in 1X kinase buffer. The reaction was incubated for 90 minutes at room temperature. The reaction was stopped by adding 5 μ L 200 nM fluorescein-streptavidin with 40 mM EDTA in TR-FRET dilution buffer. This was immediately followed by the addition of 5 μ L of 4 nM Tb-pSer (PKC) antibody in TR-FRET dilution buffer. The reaction was allowed to incubate at room temperature for 60 minutes before it was read on a Tecan Ultra

How many assays can I run with a given amount of substrate?

It varies, depending on the concentration of substrate used in the assay. But in general, for the PEPTIDE substrates, 1 mg of peptide will run *approximately* 250,000 wells (10 μ L reaction, 200 nM peptide). For Poly GT or GAT, the 1 mL, 30 μ M size we sell is *approximately* 1 mg. With these substrates, 1 mL of 30 μ M will run *approximately* 16,700 wells (10 μ L reaction, 200 μ M substrate).

20nmol of our physiological protein substrates is sufficient for *approximately* 10,000 wells (10 μ L reaction, 200 μ M substrate).

IV. Donor/acceptor distance, interferences

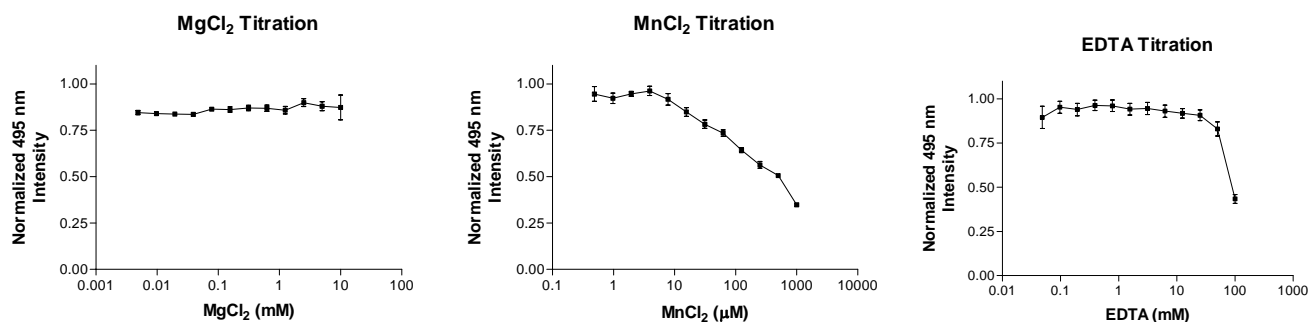
What is the optimal and/or maximum distance for a Tb-fluorescein pair?

The Förster radius-- the distance at which energy transfer efficiency is half-maximal-- is around 50-angstroms for the terbium→ fluorescein pair. However, the Förster radius does not give a complete indication of energy transfer efficiency when using long lifetime fluorophores such as terbium chelates. When using terbium chelates, energy transfer efficiency is determined by the distance of closest approach between the donor and acceptor *during the excited state lifetime of the donor*. In many assay systems, such as those designed using antibodies or peptides, there is a large degree of conformational freedom that allows the donor and acceptor to approach one another, effectively enhancing the FRET signal. Additionally, it is important to note that as the donor/acceptor pair approach one another and the efficiency of energy transfer increases, the fluorescent lifetime decreases to a comparable extent. From a practical standpoint, this means that when energy transfer is extremely efficient, FRET cannot be measured in time-resolved mode (because the energy transfer is complete before the measurement is made). This is another reason why TR-FRET assays based around terbium labeled antibodies or streptavidin perform so well, because there exist a range of donor/acceptor distances, several of which are optimal for measuring FRET.

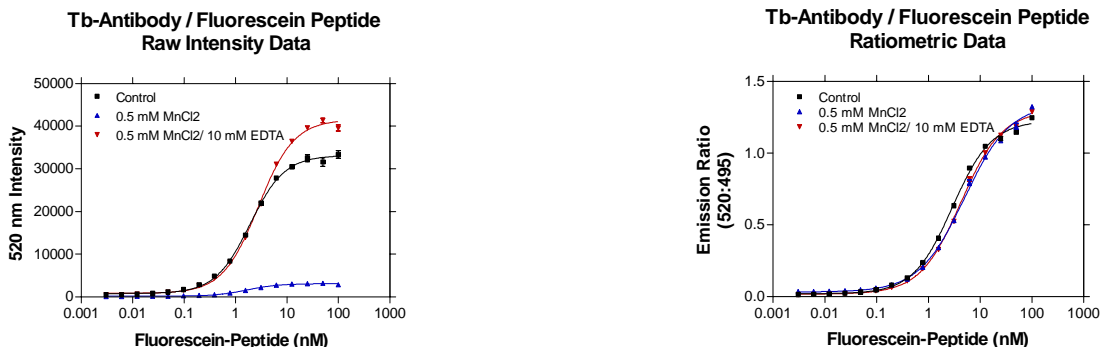
Are the LanthaScreen™ reagents stable to interference from Mg^{2+} , Mn^{2+} , and EDTA?

The chelate is completely stable to Mg^{2+} . The amount of Mn^{2+} or EDTA that the chelate can tolerate depends largely on how long they are mixed together and the combination of additives used in the reaction. See the figure below for an indication of the relative intensity change in the presence of these additives. If a reaction requires either Mg^{2+} or Mn^{2+} for activation, it is best to stop the reaction by adding an equimolar amount (or slight excess) of EDTA to chelate the metal ions present. This will then essentially eliminate any interference on the terbium chelate by EDTA or Mn^{2+} . Regardless, when LanthaScreen™ assays are performed using a ratiometric readout (division of the acceptor signal by the donor signal), any interference caused by Mn^{2+} or EDTA is largely cancelled out.

A: Mn^{2+} or EDTA can affect raw signal intensity at high concentrations



B: Interferences are compensated for by the ratiometric readout



For my kinase assay, can I pre-mix the Tb-Ab and EDTA so that I can stop the kinase assay and begin detection with a single reagent addition?

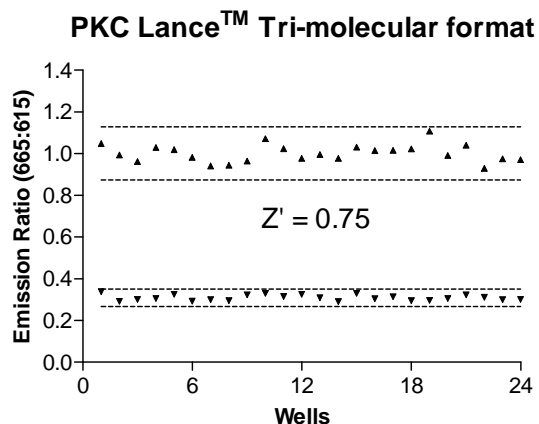
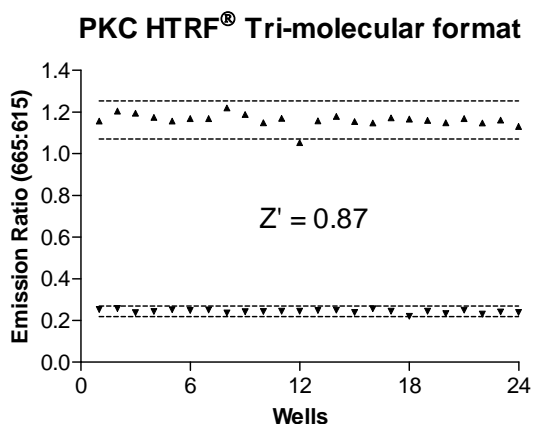
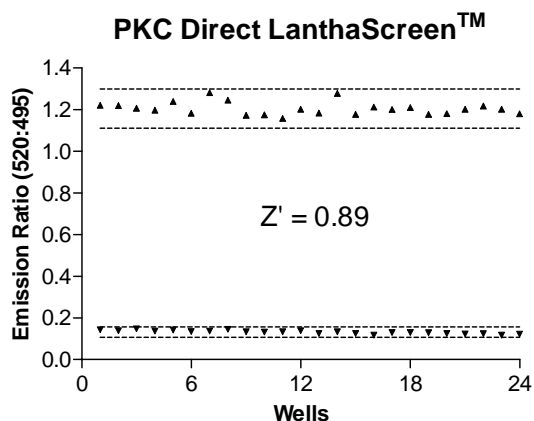
Yes, depending on the concentrations of reagents used and the time for which they are mixed. We recommend developing and optimizing the assay by using separate reagent additions, because this method will work under the widest range of conditions. Once the assay is optimized, the performance of the assay using pre-mixed antibody and EDTA can be evaluated. We have successfully developed robust assays in which the antibody and EDTA were pre-mixed and then stored overnight at 4° prior to use the following day. A loss of signal intensity was observed in this case, however, by using the ratiometric readout, this effect was minimal.

V. Assay comparison

How does the LanthaScreen™ technology compare to other TR-FRET assay formats?

We performed a comparison between the LanthaScreen™ Assay and other commercially available TR-FRET assays from 2 different suppliers for the PKC kinase target. Our data revealed that the assays performed comparably, but that the LanthaScreen assay was simpler to optimize and contained fewer components that required optimization. The LanthaScreen™ Assay is a two component system, whereas the other assay formats utilize a trimolecular mechanism which was more time consuming to optimize and has added costs. Assay conditions and data results follow.

The PKC alpha was diluted to 20 ng/mL in 2X kinase buffer (prepared from PV3189, 100 mM Hepes (pH7.5), 20 mM MgCl₂, 2 mM EGTA, 0.02% BRIJ-35) and 5 µl was dispensed per well. The no enzyme control was prepared using 2X kinase buffer. The kinase reaction was started by adding 5 µl of a 0.8 µM substrate, 20 µM ATP solution in water. The reaction was allowed to run 90 minutes at room temperature. The reactions were stopped by adding 5 µl of 40 mM EDTA. In the case of the trimolecular formats, the 40 mM EDTA solution also contained 200 nM acceptor-labeled streptavidin. Then 5 µl of a 4 nM lanthanide labeled antibody was added. Reactions were incubated for one hour at room temperature then read on a Tecan Ultra™ using a 340/30 filter to excite. The LanthaScreen™ samples were measured using 495/10 and 520/25 filters while the Lance™ and HTRF® samples were measured using 615/10 and 665/10 filters. The HTRF® samples also contained 200 mM KF final.



Analysis of data

Tecan Ultra™		LanthaScreen™ Direct		HTRF®		Lance™	
		10 ng/mL	0	10 ng/mL	0	10 ng/mL	0
Average		1.205	0.132	1.162	0.243	1.001	0.309
Stdev		0.031	0.008	0.030	0.008	0.043	0.014
Fold Increase		9.1		4.8		3.2	
S/N		26.91		23.64		12.20	
S/B		9.1		4.8		3.2	
Z'		0.89		0.87		0.75	

$$\text{Signal to Noise} = \frac{(\text{Mean Positive Control} - \text{Mean Negative Control})}{\sqrt{((\text{Positive Control STD})^2 + (\text{Negative Control STD})^2)}$$

$$\text{Signal to Background} = \frac{\text{Mean Positive Control}}{\text{Mean Negative Control}}$$

$$Z' = 1 - \frac{3(\text{Positive Control STD} + \text{Negative Control STD})}{\text{Mean Positive Control} - \text{Mean Negative Control}}$$

Cost Analysis - Components

Product Description	Catalog Number	Price	Size	Cost/Well
Lance™ EU1024 phospho-(Ser) PKC Substrate Ab	AD0188	\$2,538.00	10 µg	\$0.758
SureLight™ APC SA	CR130-100	\$320.00	1 mg	\$0.0176
Cryptate Anti-phospho-(Ser) PKC-substrate Pab [CST#2261]	61P03KAE 61P03KAZ	\$420.00	500 wells 10,000 wells	\$0.84
Streptavidin-XL ^{ent!}	611SAXLA	\$770.00	20,000 wells	\$0.0423
LanthaScreen™ Tb-pSer (PKC Substrate) Antibody	PV3560 PV3561	\$1,950.00 \$12,000.00	25 µg 1 mg	\$0.23 \$0.036
Fluorescein-PKC Substrate	PV3506	\$495.00	1 mg	\$0.0038
[Ser25]-PKC Biocytin (19-31) K(Biotin)-RFARKGSLRQKNV	Anaspec 23019	\$320.00	5 mg	\$0.00049

Cost Analysis – Complete Assay

Format	Reagents required	Small Ab Cost/well	Large Ab Cost/well
Direct LanthaScreen™	Tb-pSer (PKC Substrate) Antibody, Fluorescein-PKC Substrate	\$0.2338	\$0.0398
Lance™ Trimolecular Format	Lance EU1024 phospho-(Ser) PKC Substrate Ab, [Ser25]-PKC Biocytin (19-31) K(Biotin)-RFARKGSLRQKNV, SureLight APC SA	\$0.77609	Not listed
HTRF® Trimolecular Format	Cryptate-Anti-phospho-(Ser) PKC-substrate Pab [CST#2261], [Ser25]-PKC Biocytin (19-31) K(Biotin)-RFARKGSLRQKNV, Streptavidin-XLent!	\$0.88279	Not listed