Application Note

How to Optimize a Tyrosine Kinase Assay Using Time Resolved Fluorescence-Based LANCE™ Detection

Protein kinases regulate several important functions within cells including metabolism, cell cycle progression, angiogenesis, cell adhesion, etc. Due to the fact that kinases are involved in such a wide variety of cellular functions, their role in disease states has drawn considerable interest as drug discovery targets. Traditional methods of detecting kinase activity rely on the transfer of radioactive phosphate from ATP to an acceptor molecule, either a peptide substrate or protein. As the drug discovery industry continues the effort to move to non-radioactive technology platforms, the need exists for sensitive, reliable, and cost efficient assays to measure kinase activity.

Time-resolved fluorometry (TRF) is a well-established technology that exploits the unique fluorescence properties of lanthanide chelates to provide a powerful alternative to radioisotopic assays in many HTS applications. TRF assays exhibit low background and high signal-to-background ratios, two attributes critical for robust HTS assays. Long fluorescence decay after excitation allows time-delayed signal detection (microseconds) to virtually eliminate all natural fluorescent background caused by cells and cell debris, screening compounds, plates, and other reagents (half-life of nanoseconds). A large Stokes shift (e.g., excitation and emission wavelengths for the Eu-chelate are ~340 nm and ~615 nm, respectively) minimizes crosstalk, resulting in a high signal-to-background ratio. Therefore, because of their excellent temporal and spectral resolution, lanthanide chelate labels can provide high sensitivity assays.

LANCE™ refers to homogeneous time-resolved fluorometry applications using techniques such as TR-FRET (time-resolved fluorescence resonance energy transfer assay) and TR-FQA (time-resolved fluorescence quench assay). Homogeneous assays particularly benefit from time-resolved fluorometry because the sample constituents present during detection cause very high background fluorescence when conventional fluorochromes are used. TR-FRET is based on the proximity of a Eu chelate and the fluorophore that have been brought together by a specific binding reaction. The excited energy of the Eu-chelate is transferred by a resonance mechanism to an acceptor within a short distance (~ 15 nm). Fluorescent lanthanide chelates with long excited-state lifetimes are used to avoid interference caused by emission from acceptor molecules excited directly rather than by energy transfer. TR-FRET is widely used for kinase and protein-protein interaction assays.

This note will describe some of the steps necessary in order to optimize a kinase assay, specifically tyrosine kinase assay, using the LANCE technology platform. Included will be steps describing the optimizing of peptide and enzyme concentrations, kinase assay time, as well as the Eu-labeled antibody and SureLight™ streptavidin-allophycocyanin (SA-APC) concentrations. As an example, the optimization of the tyrosine kinase, c-Src, will be described.

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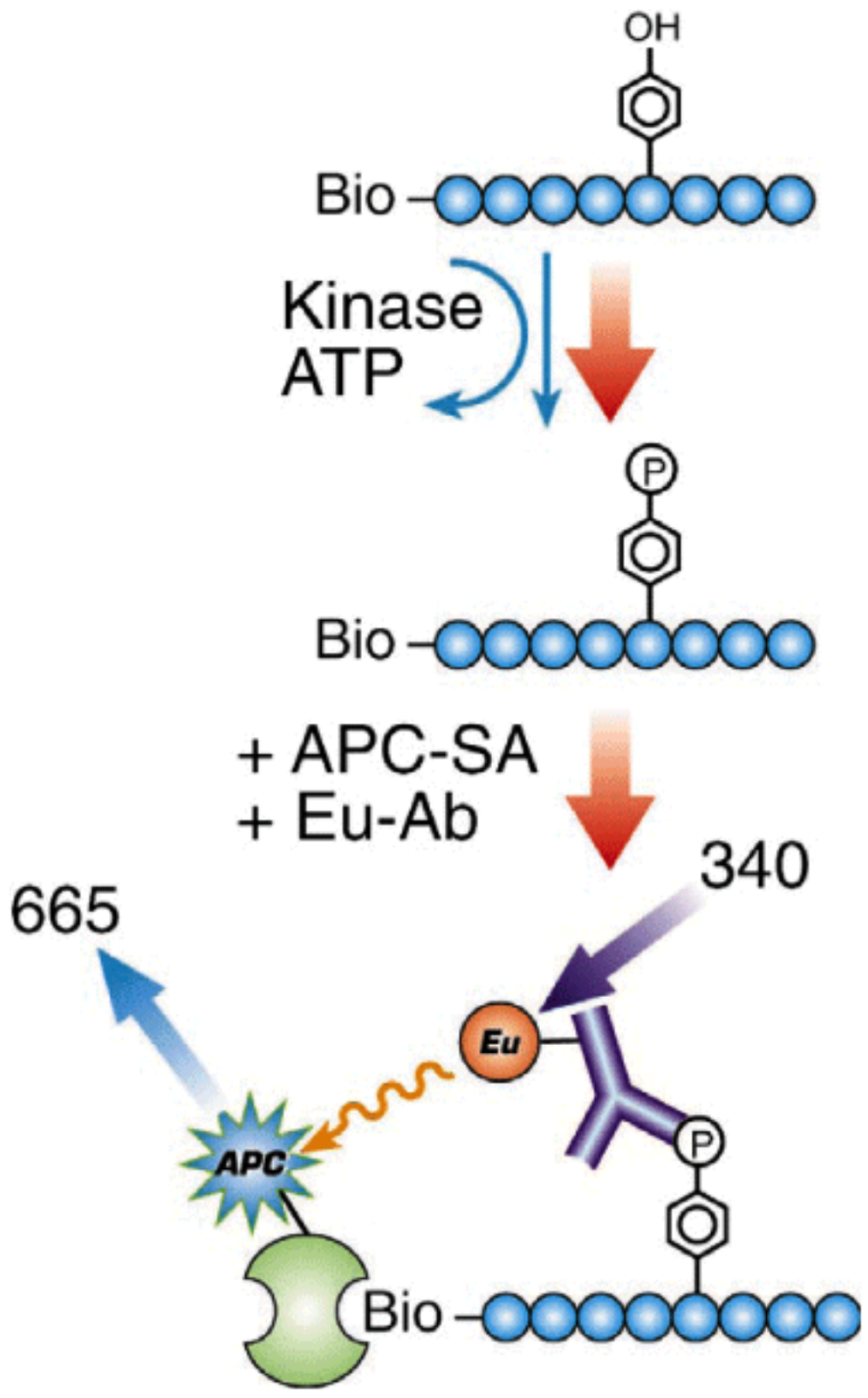
ASSAY PRINCIPLE



Add to microtiter plate:

buffer components peptide, ATP, kinase, and compounds

Add EDTA, SA-APC and Eu-labeled Ab

Measure at 665 and 615 nm using time-resolved fluorescence

ASSAY PROTOCOL

As indicated in the assay principle for the kinase assay, the LANCE technology is a platform for detection of phosphorylated substrates using energy transfer from a phosphospecific Eu-labeled antibody to an acceptor, in this case SA-APC. This is a non-radioactive detection technology so [^{33}P]ATP is NOT used, but other than that the kinase reaction is mainly unchanged from other technology platforms.

Like any other enzyme assay, the buffer constituents and conditions are critical for obtaining desired results. Important things to consider when optimizing your enzyme of interest prior to detection by LANCE are the buffer components including ATP, Mg^{2+}, NaCl, Mn^{2+}, phosphatase inhibitors such as Na_3VO_4, EGTA, glycerol, and DTT. When using purified enzyme, the need for phosphatase inhibitors, such as Na_3VO_4, is most likely unnecessary.

For LANCE, it is highly important to determine whether Mn^{2+} is essential for your specific kinase as it interferes with the fluorescence of Eu. Free Mn^{2+} binds to the Eu-containing chelate, and causes chemical quenching. If the kinase requires Mn^{2+} for enzyme activity, then chelate the Mn^{2+} with EDTA, or the like, prior to addition of the Eu-labeled Ab and SA-APC to prevent chemical quenching of the Eu. However, it is important to remember to use only an amount necessary to chelate the Mn^{2+}, as free EDTA will compete for the Eu, reduce the excitation energy, and thus diminish the energy transfer to the APC acceptor. See application note "Stability of the WALLAC LANCE™ Eu-Chelates" (1234-9861) for more information.

LANCE kinase assays can be performed in 96-, 384-, and 1536-well formats using either white or black plates. General observations are that higher energy transfer counts are obtained when using white plates, but that black plates yield higher S:B ratios. Choose a plate format and color in accordance with the needs of your assay.

Optimization of Substrate and Kinase amount

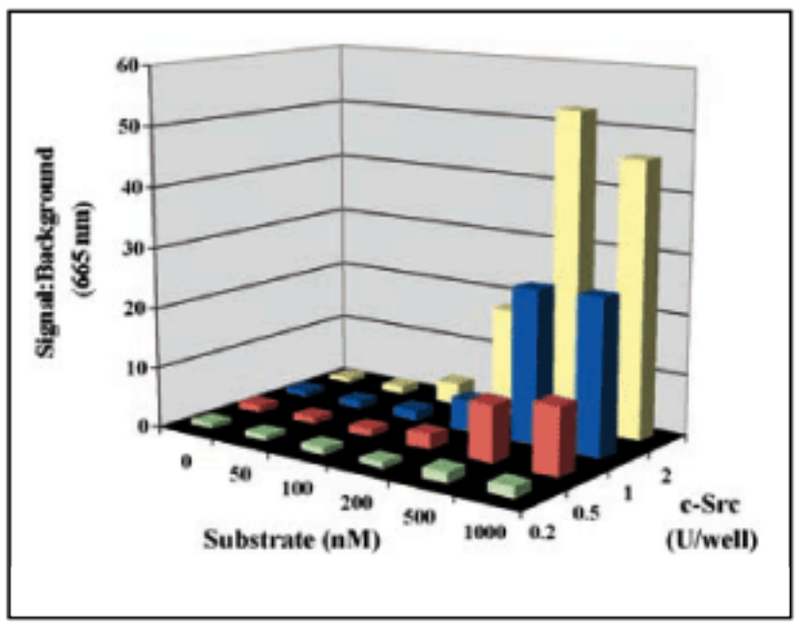


Figure 1. Optimization of biotinylated peptide substrate and c-Src enzyme amount. c-Src enzyme ranging between 0.2 and 2 U/well was incubated with biotinylated-peptide substrate ranging from 0 to 1000 nM in assay buffer consisting of: 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 500 µM ATP, 1 mM EGTA, 2 mM DTT, and 0.01 % Brij 35. Reaction was terminated after 60 min by the addition of 15 mM EDTA and phosphorylation detected by the addition of 2 nM W1024-PY100 Ab and 200 nM SA-APC and measured 30 min after addition at 665 and 615 nm on VICTOR²V using the LANCE 615/665 counting protocol.

As expected, higher amounts of enzyme yielded more phosphorylated product during the 60 min incubation. However, as you can see the level of substrate phosphorylation did not change when the substrate concentration was increased above 500 nm regardless of the amount of enzyme used. Excellent signal to background values of greater than 10:1 were obtained using at least 0.5 U c-Src/well and 500 nM biotinylated substrate. In all further studies described here, we used 500 nM peptide and 1 U/well c-Src enzyme.

Time Course of peptide phosphorylation

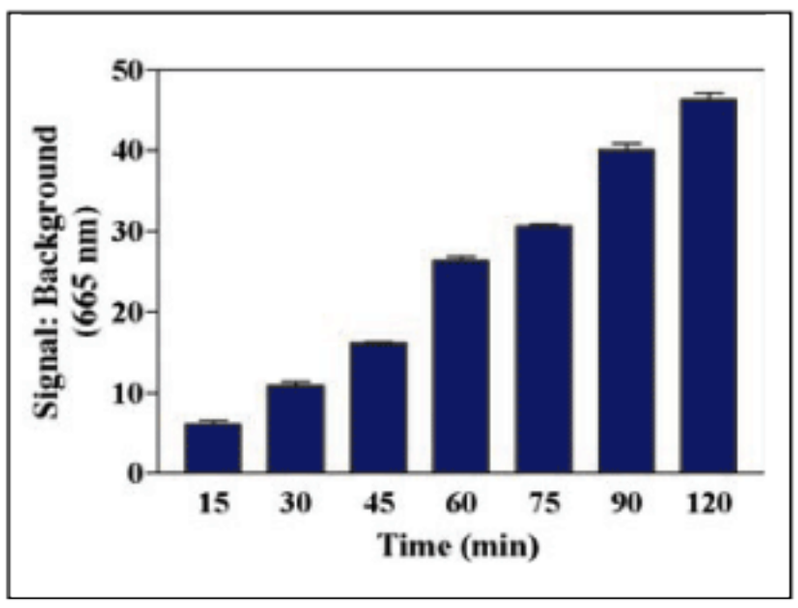


Figure 2. Time course of c-Src phosphorylation of biotinlyated peptide substrate. c-Src enzyme (1 U/well) was incubated with 500 nM biotinylated-peptide substrate in assay buffer consisting of: 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 500 µM ATP, 1 mM EGTA, 2 mM DTT, and 0.01 % Brij 35. Reactions were terminated at the specified time points by the addition of 15 mM EDTA and phosphorylation detected by the addition of 2 nM W1024-PY100 Ab and 75 nM SA-APC. It was measured after 60 min at 665 and 615 nm on a VICTOR²V multilabel plate reader using LANCE 615/665 counting protocol.

Using the above conditions at room temperature (21°C), kinase assay time points as short as 30 min yielded signal to background values of 10:1 or greater. Again the use of higher enzyme amounts will allow you to obtain more phosphorylated product in a shorter time period. The time constraints of your assay will dictate the ability to use less enzyme or necessitate the need for more than 1 U/well enzyme. The

optimal assay time requirements will need to be determined for each kinase and will be very dependent upon the amount of enzyme and substrate utilized.

Optimization of Eu-labeled Antibody and SA-APC amounts

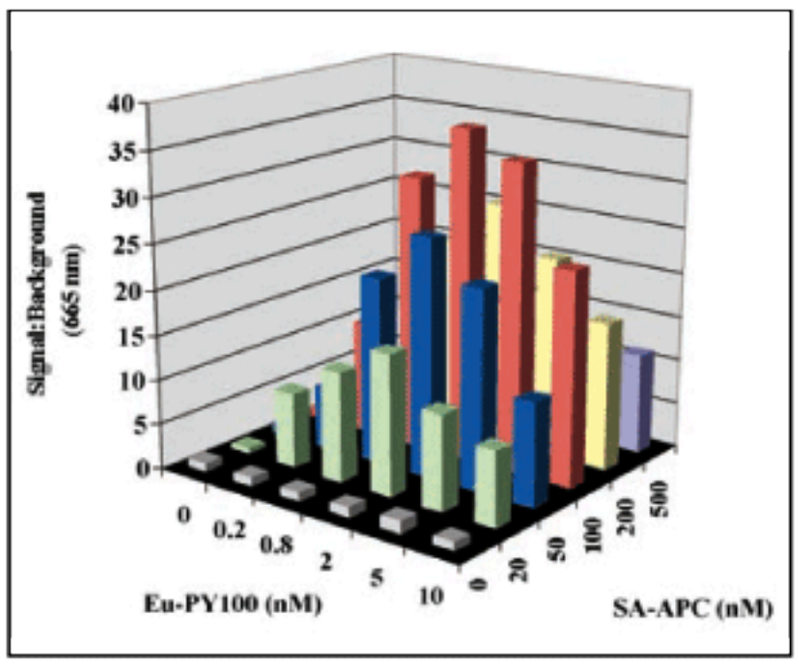


Figure 3. Cross-titration of Eu-PY100 antibody and SA-APC. c-Src enzyme (1 U/well) was incubated with 500 nM biotinylated-peptide substrate in assay buffer consisting of: 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 500 µM ATP, 1 mM EGTA, 2 mM DTT, and 0.01 % Brij 35. Reactions were terminated after 60 min by the addition of 15 mM EDTA and phosphorylation detected by the additions between 0 and 10 nM of W1024-PY100 Ab and 0-500 nM of SA-APC and measured 30 min after addition at 665 and 615 nm on a VICTOR²V multilabel plate reader using LANCE 615/665 counting protocol.

Figure 3 represents a cross titration of antibody and acceptor to determine the optimal amount of both constituents for detection of the phosphorylated tyrosine on the biotinylated substrate. From this experiment, the optimal amount of Eu-labeled phosphotyrosine specific antibody, W1024-PY100, is 2 nM whereas the optimal amount of SA-APC is 100 nM, as measured by SA concentration. Under these conditions, a signal to background of greater than 30:1 was obtained. Using as low as 0.2 nM W1024-PY100 antibody and 100 nM SA-APC, a signal:background of almost 13 was achieved.

Optimization of Antibody Incubation Time

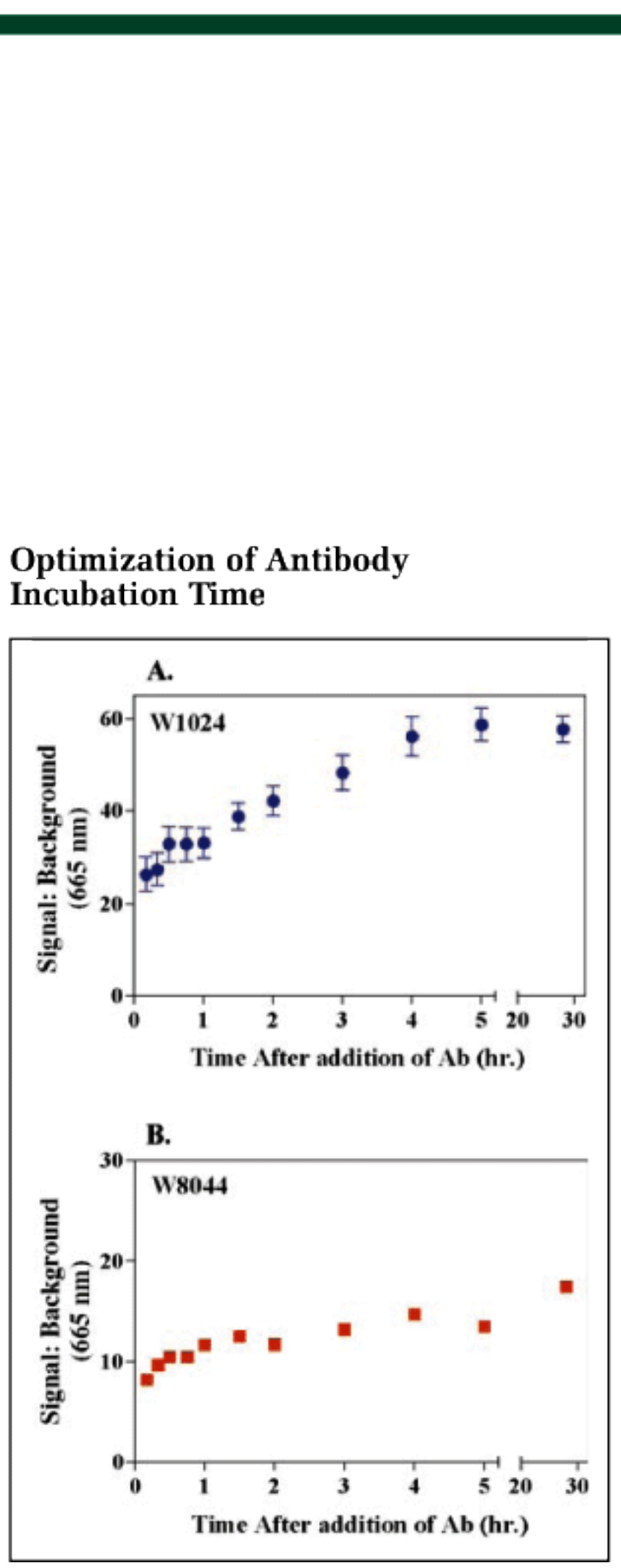


Figure 4. Examination of Signal:Background dependence on incubation time following antibody addition. c-Src enzyme (1 U/well) was incubated with 500 nM biotinylated-peptide substrate in assay buffer consisting of: 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 500 µM ATP, 1 mM EGTA, 2 mM DTT, and 0.01 % Brij 35. Reactions were terminated after 60 min by the addition of 15 mM EDTA and phosphorylation detected by the addition of either A) 2 nM W1024-PY100 Ab or B) 2 nM W8044-PY100 Ab and 75 nM SA-APC and measured at the indicated times after antibody addition at 665 and 615 nm on a VICTOR²V multilabel plate reader using LANCE 615/665 counting protocol.

At times points as early as 10 minutes after the addition of the detection mixture, signal:background values of greater than 20:1 can be obtained with the W1024 chelate and nearly 10:1 with the W8044 chelate. However, we recommend at least 30 minutes to allow for further product detection. Optimal detection times will need to be determined for each enzyme of interest and will be dependent on the assay conditions used.

Effect of EDTA Concentration and Time of Incubation with W1024-PY100 and W8044-PY100

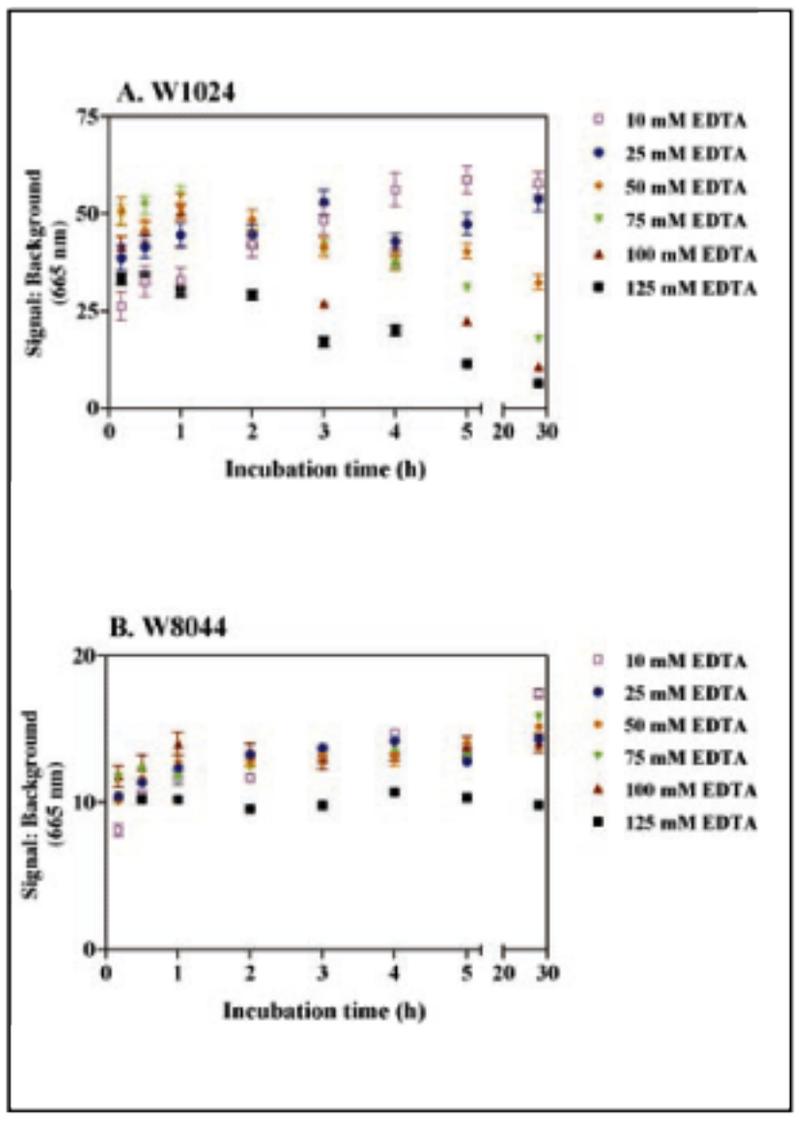


Figure 5. Effect of EDTA and incubation time on W1024-PY100 and W8044-PY100 energy transfer. c-Src enzyme (1 U/well) was incubated with 500 nM biotinylated-peptide substrate in assay buffer consisting of: 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 500 µM ATP, 1 mM EGTA, 2 mM DTT, and 0.01 % Brij 35. Reactions were terminated after 60 min by the addition of EDTA ranging between 10 and 125 mM and phosphorylation detected by the addition of either A) 2 nM W1024-PY100 or B) 2 nM W8044-PY100 Ab and 75 nM SA-APC and measured at the indicated times after antibody addition at 665 and 615 nm on a VICTOR²V multilabel plate reader using LANCE 615/665 counting protocol.

A time and dose-dependent decrease in energy transfer as indicated by APC signal :background measured at 665 nm was observed with excess EDTA with the Eu-W1024 chelate-labeled Ab (Figure 5A). This effect was not observed with the more stable W8044 chelate-labeled PY100 Ab (Figure 5B). It is important to avoid the use of excess EDTA to terminate the kinase reaction unless a W8044 chelate labeled Ab is used or the assay plates are read within 2 h of termination.

Counting Time Delay (µsec)	Signal : Background
	Avg. ± S.D.
0	1.1 + 0.0
40	42.9 + 3.4
60	59.2 + 6.1
80	51.6 + 3.9
100	55.3 + 6.2
150	36.6 + 2.6
200	35.2 + 2.3

Table 1. Optimization of LANCE counting delay time on the VICTOR²V. Kinase assay was performed using c-Src enzyme (1 U/well) along with 500 nM biotinylated-peptide substrate using the following assay buffer: 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 500 µM ATP, 1 mM EGTA, 2 mM DTT, and 0.01 % Brij 35. The reaction was terminated after 60 min by the addition of 15 mM EDTA and phosphorylation detected by the addition of 2 nM Eu-PY100 Ab and 75 nM SA-APC prior to measurement at 665 and 615 nm on a VICTOR²V multilabel plate reader using counting delay times ranging from 0-200 µsec.

Depending on the plate reader you are using, the time delay can significantly affect the signal to background values obtained in TR-FRET. For the VICTOR²V, the optimal time turns out to be around 60 µsec using a counting window of 100 µsec. The ideal time delay for each brand of plate reader will need to be determined.

Parameter	VICTOR²V
Excitation Filter	340 nm (BW 50 nm)
Emission Filter 1	615 nm (BW 8.5nm)
Emission Filter 2	665 nm (BW 10 nm)
Delay Time (µsec)	60
Window (µsec)	100
Cycle (µsec)	1000

Table 2. Counting parameters for VICTOR²V multilabel plate reader

EXAMPLE OF A LANCE ASSAY FOR c-SRC KINASE

Materials:

10X NEBuffer for Abl PTK	New England Biolabs (Cat. # B6050S)
Tyrosine kinase biotinylated peptide substrate 1	Pierce (Cat # 29912)
c-Src ($p60^{c\text{-}src}$)	Upstate Biotechnology (Cat.# 14-117)
100 mM ATP	
LANCE Eu-W1024 anti-pTyr antibody (PY100)	PerkinElmer (Cat. #AD0161)
SureLight Allophycocyanin-Streptavidin, kinase assay	PerkinElmer (Cat. #CR130-100)
10X LANCE detection buffer	PerkinElmer (Cat. #CR97-100)
0.5 M EDTA solution	
OptiPlate-384 F (384-well black plate)	PerkinElmer (Cat. #6007270)

Reagents:

Prepare 2X Assay Buffer

Dilute 1 ml of 10X buffer to 5 ml final volume with dH_2O

Prepare 4X biotinylated peptide substrate + ATP

Dilute stock peptide substrate to concentration of 2 µM in dH_2O
Dilute ATP 50-fold to a concentration of 2 mM dH_2O

Prepare 4X Src Enzyme

Dilute stock enzyme 15-fold dH_2O to yield a concentration of 0.2 U/µl

Prepare 4X stop mixture

Prepare 1X LANCE detection buffer from 10X stock solution
Supplement 1X LANCE detection buffer with 30 mM EDTA

Prepare 4X detection mixture

Supplement 1X LANCE detection buffer with 400 nM SA-APC
(concentration based on SA molarity) and 8 nM (1.3 µg/ml) W1024-labeled PY100 Ab

Protocol

Pipet 10 µl of 2X assay buffer into all wells
Add 5 µl of 4X Src peptide + ATP to all wells
Add 5 µl of dH_2O to all blank wells
Add 5 µl of 4X Src Enzyme to all sample wells
Cover plate with TopSeal and incubate 60 min at room temp.
Add 10 µl of 4X stop mixture to all wells and incubate 10 min at room temp
Add 10 µl of 4X detection mixture to all wells
Mix and incubate 60 min prior to reading on VICTOR^{2}V, EnVision, ViewLux, Fusion or other time-resolved fluormeter

CONCLUSIONS:

- Important to optimize substrate and kinase amounts
- Determine streptavidin-APC concentration to maximize signal
- Establish kinase reaction time needed
- Minimize the EDTA used to stop kinase reaction when using W1024 chelate
  OR perform detection step using W8044 chelate

REFERENCES

Tyrosine kinases

A. LANCE Scientific publications:

Biazzo-Ashnault, D.E., Park, Y.-W., Cummings, R.T., Ding, V., Moller, D.E., Zhang, B.B., and Qureshi, S.A. (2001) Detection of insulin receptor tyrosine kinase activity using time-resolved fluorescence energy transfer technology. Anal. Biochem. 291, 155-158.

B. LANCE Scientific posters:

An Automated Simultaneous Screen for the Identification of Inhibitors of the Catalytic Kinase Domains of two Unrelated Receptor Tyrosine Kinases.
Paper Presented at the 7th Annual Conference and Exhibition of the Society for Biomolecular Screening. Baltimore, Sept (2001).
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Effect of assay technology and ATP concentration on IC_{50} values of protein kinase inhibitors.
Paper Presented at the 7th Annual Conference and Exhibition of the Society for Biomolecular Screening. Baltimore, Sept (2001).
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Performance of Corning's 2 µl 1536 Microplate in Miniturized TR-FRET Tyrosine Kinase and Fluorescence Polarization Assays.
Paper Presented at the 7th Annual Conference and Exhibition of the Society for Biomolecular Screening. Baltimore, Sept (2001).
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Corning White 10 µl 1536 Assay Plate Performance in a Miniaturized TR-FRET Tyrosine Kinase Assay.
Paper Presented at the 7th Annual Conference and Exhibition of the Society for Biomolecular Screening. Baltimore, Sept (2001).
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Miniaturization of a LANCE™ assay.
Paper Presented at the 4th Annual Conference and Exhibition of The Society for Biomolecular Screening, Baltimore MD, Sept (1998).
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Homogeneous Time-Resolved Fluorometric Energy Transfer Assay (LANCE™) for Protein Tyrosine Kinase.
Abstract of Papers Presented at the 3 rd Annual Conference of the Society for Biomolecular Screening, California, Sept (1997).
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Serine/threonine kinases

A. LANCE Scientific publications:

Bader, B., Butt, E., Palmetshoer, A., Walter, U., Jarchau, T., and Drueckes, P. (2001) A cGMP-dependent protein kinase assay for high throughput screening based on time-resolved fluorescence energy transfer. J. Biomol. Screening 6, 255-264.

B. LANCE Scientific posters:

Screening for Specific Antibodies for Ser/Thr Kinase Assays Based on Time-Resolved Fluorescence.
Paper Presented at the 7th Annual Conference and Exhibition of the Society for Biomolecular Screening. Baltimore, Sept (2001).
To download pdf file: http://lifesciences.perkinelmer.com/library/literature.asp

APPLICATION NOTES

Quench correction for LANCE Time Resolved Fluorescence Resonance Energy Transfer (1234-9860)
Stability of Wallac LANCE Eu-chelates (1234-9861)

PRODUCTS AVAILABLE

Product description		Catalog number
LANCE Tyrosine Kinase Start Up Reagents		AD0121
Includes:	LANCE Eu-W1024 anti-pTyr antibody (PY100) SureLight Allophycocyanin-Streptavidin, kinase assay Biotinylated poly(Glu, Tyr) kinase substrate Biotinylated phosphotyrosine peptide Tris-HCl buffer OptiPlate-384-well white plate (2 plates)	
LANCE Eu-W1024 anti-pTyr antibody (PY20)		AD0066 (50 µg) AD0067 (1 mg)
LANCE Eu-W1024 anti-pTyr antibody (PT66)		AD0068 (50 µg) AD0069 (1 mg)
LANCE Eu-W1024 anti-pTyr antibody (PY100)		AD0161 (50 µg) AD0162 (1 mg)
SureLight Allophycocyanin-Streptavidin, kinase assay		CR130-100 (1 mg) CR130-150 (50 mg)
Biotinylated poly(Glu, Tyr) kinase substrate		6760624R
LANCE detection buffer		CR97-100
OptiPlate-96 F (96-well black plate)		6005270
OptiPlate-96 (96-well white plate)		6005290
OptiPlate-384 F (384-well black plate)		6007270
OptiPlate-384 (384-well white plate)		6007290
OptiPlate-1536 F (1536-well black plate)		6005235
OptiPlate-1536 (1536-well white plate)		6005228
TopSeal-A Microplate sealing film		6005185
ViewLux Plate Imager		1440
EnVision Multilabel Plate Reader		2100
VICTOR²V Multilabel Counter		1420
Fusion Multilabel Reader		

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