TECHNICAL NOTE

LANCE TR-FRET

Development of LANCE TR-FRET Kits for Cellular Phosphoprotein Detection

Author:

Cat Lautenschlager

PerkinElmer, Inc.
Hopkinton, MA

Introduction

Protein phosphorylation is a critical process involved in cell signal transduction. It is a key regulatory mechanism that triggers essential cellular signaling events through signaling cascades. Many intracellular processes are regulated by protein phosphorylation, including cellular growth, proliferation, and division. Studies of protein phosphorylation in cells enable the elucidation of complex signaling pathways involved in the progression of many diseases such as cancer, inflammatory diseases, immune diseases, and metabolic diseases. This makes protein phosphorylation an attractive target for therapeutic intervention. The ability to mediate the selective regulation of specific kinases offers promising therapeutic potential.

Cell-based assays provide a physiologically-relevant means of studying target proteins under the influence of a variety of regulatory factors that naturally exist in a complex cellular environment. Studying and screening for phosphorylation activity in a cell-based format offers significant advantages over traditional biochemical approaches, as cell-based assays give information on a compound's activity in an appropriate biological context. Performing assays in a cell-based format can also reduce the rate of false positives as compared to biochemical approaches.

LANCE® *Ultra* TR-FRET cellular phosphoprotein detection kits (Figure 1) provide physiologically-relevant, homogeneous mix-and-read assays that can be measured without the need for specially-engineered cell lines. These kits were developed with highest stringency. All kits utilize carefully-researched, highly specific and selective antibodies. Selected antibodies were then screened in multiple configurations for optimal kit performance. Assays were then rigorously validated in cellular models using well-established pathway-specific stimulators and inhibitors. Finally, all kits were assessed for assay reproducibility and screening suitability by assessing Z'.

Development of each kit was performed using an EnVision® multilabel plate reader using default values for TR-FRET detection for laser excitation. In addition to fast, sensitive LANCE TR-FRET technology detection, the EnVision multilabel microplate reader provides fluorescence intensity, luminescence, absorbance, fluorescence polarization, and AlphaLISA® and AlphaPlex™ assay technologies. The system is based on hybrid technology, combining filters and a monochromator for enhanced flexibility. It incorporates proprietary Direct Double Optics™ technology for high speed and sensitivity in simultaneous filter-based readouts, such as FRET assays, and for TRF, lamp-based excitation is standard, with the option of a high energy laser for higher speed and sensitivity.

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Antibody Selection

LANCE *Ultra* TR-FRET cellular phosphoprotein detection kits rely on high quality antibodies for accurate results. For a given phosphoprotein target, numerous phosphorylation sites and isoforms may exist. It is therefore critical that phosphoprotein assays utilize highly specific and selective antibodies. All antibody pairs used in LANCE *Ultra* cellular phosphoprotein assays were chosen for specificity, selectivity, and detectability in multiple cell lines.

Antibody Screening

In order to validate an antibody, it must be shown to be specific, selective, and reproducible in the context where it is used. LANCE *Ultra* assays are dependent on not just one, but a pair of antibodies labeled with fluorophores for TR-FRET measurement. After carefully selecting antibodies, each possible antibody pair was screened in both orientations (e.g, Europium-labeled antibody #1 and U*Light*-labeled antibody #2, vs. Europium-labeled antibody #2 and U*Light*-labeled antibody #1) at different fluorophore:antibody labeling ratios. The best antibody pairs were chosen based on assay performance.

Validation in Cellular Models

All LANCE *Ultra* TR-FRET cellular phosphoprotein detection kits were then validated in cellular models using pathway-specific stimulators and inhibitors. Two examples are described below.

The LANCE *Ultra* pAKT (Ser473) kit was initially validated on NIH3T3 cells by stimulation with PDGF-AA (Figure 2A). Active PDGF-AA signaling has been shown to result in phosphorylation of AKT at both the Ser473 and Thr308 phosphorylation sites[1]. Data in Figure 2A generated with the LANCE *Ultra* kit show a dose-dependent effect of PDGF-AA on AKT (Ser473) phosphorylation. A signal-to-background (S/B) of nine was achieved for this assay. The LANCE *Ultra* pAKT (Ser473) kit was further validated by inhibition with wortmannin, a known PI3K inhibitor[1]. Data in Figure 2B show a dose-dependent inhibitory effect on AKT (Ser473) phosphorylation with increasing amounts of wortmannin. An S/B of 11.5 was achieved for this assay.

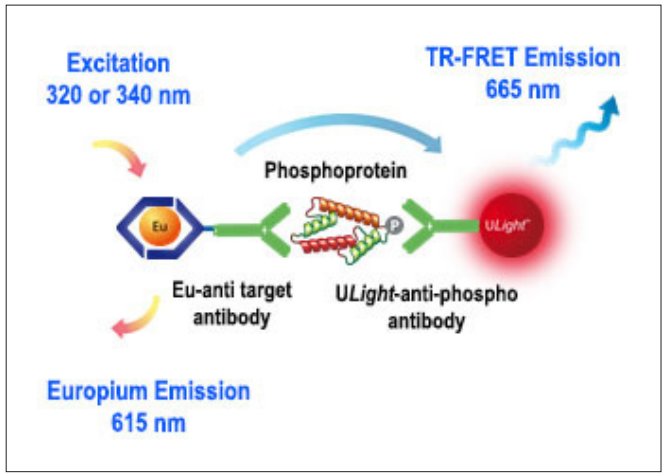

Figure 1. LANCE *Ultra* TR-FRET assay schematic. In the presence of phosphoprotein, antibodies sandwich the target protein and bring the Europium and U*Light*™ fluorophores within close proximity. Upon excitation at 320 or 340 nm, energy is transferred via FRET from Europium to the U*Light* fluorophore, generating light at 665 nm that is proportional to the amount of phosphoprotein in the cell lysate.

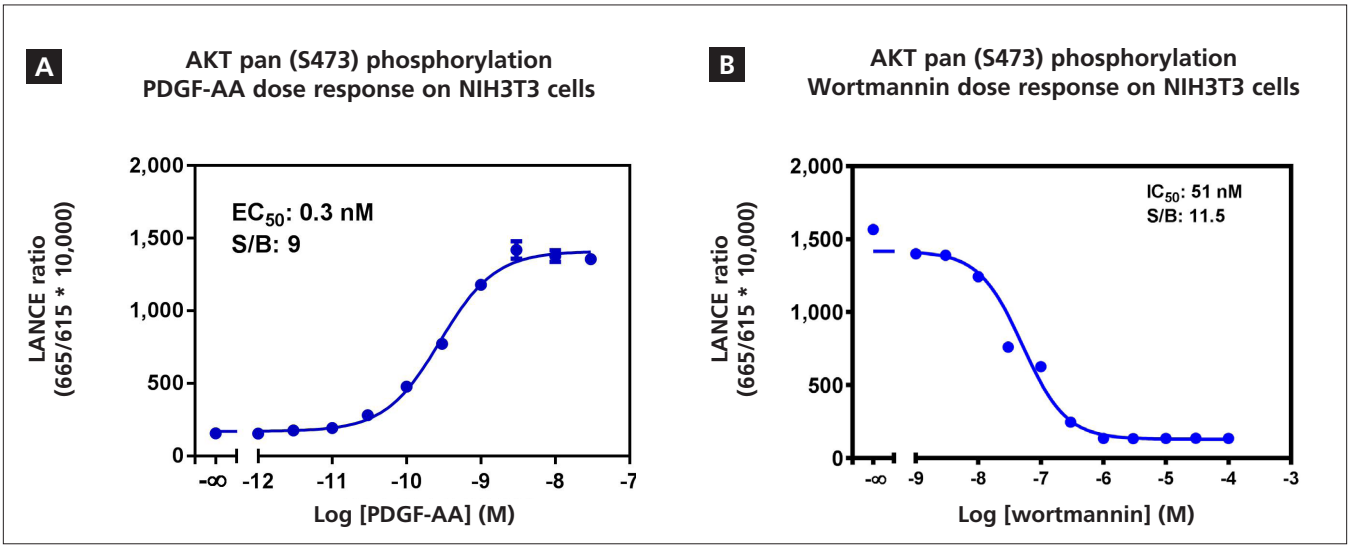

Figure 2. (A) Validation of LANCE *Ultra* pAkt (Ser473) assay using PDGF-AA, a known stimulator for pAKT (Ser473). NIH3T3 cells (60K/well) were treated with increasing concentrations PDGF-AA for 30 min. Cells were then lysed with 50 µL of LANCE *Ultra* Lysis Buffer 2 containing 1 mM NaF and 2 mM Na_3VO_4 for 30 min at room temperature. Incubation time: 4 h. (B) Validation of LANCE *Ultra* pAkt (Ser473) assay wortmannin, a PI3K inhibitor, in NIH3T3 cells. NIH3T3 cells (60K/well) were treated with increasing concentrations wortmannin for 30 min. The cells were then stimulated with 1 nM of PDGF-AA for 30 min before lysis with 50 µL of LANCE *Ultra* Lysis Buffer 2 containing 1 mM NaF and 2 mM Na_3VO_4 for 30 min at room temperature. Incubation time: 4 h. All data were measured using the EnVision multilabel plate reader with laser excitation.

Next, we describe the validation of the LANCE *Ultra* pERK (Thr202/Tyr204) kit. This assay was initially validated with HEK293 cells by stimulation with epidermal growth factor (EGF, Figure 3A). EGF binds and activates epidermal growth factor receptor (EGFR), inducing the MAPK/ERK pathway[2]. Data in Figure 3A generated with the LANCE *Ultra* kit show a dose-dependent effect of EGF on ERK phosphorylation. A signal-to-background (S/B) of 7.2 was achieved for this assay. The LANCE *Ultra* pERK kit was further validated by inhibition with erlotinib, an EGFR inhibitor[3]. Data in Figure 3B show a clear dose-dependent inhibitory effect on ERK phosphorylation with increasing amounts of erlotinib. An S/B of 12.8 was achieved for this assay.

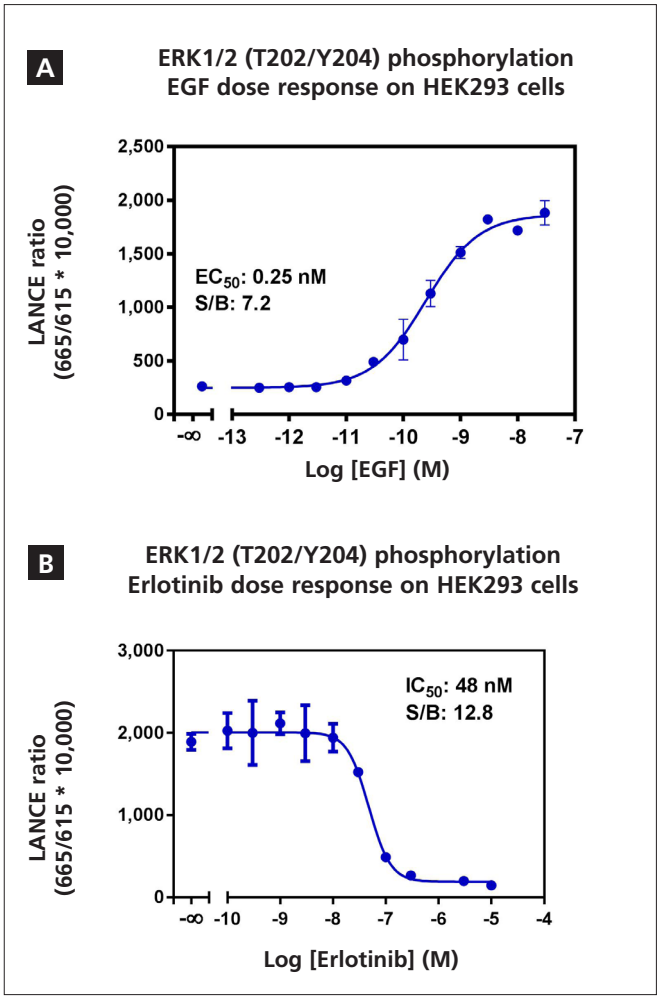

Figure 3. (A) Validation of LANCE *Ultra* pERK 1/2 (Thr202/Tyr204) assay using EGF, a known stimulator of the MAPK/ERK signaling pathway. HEK293 cells (50K/well) were treated with increasing concentrations EGF for 10 min. Cells were then lysed with 50 µL of LANCE *Ultra* Lysis Buffer 1 for 15 min at room temperature. Incubation time: 4 h. (B) Validation of LANCE *Ultra* pERK 1/2 (Thr202/Tyr204) assay using Erlotinib, an EGFR inhibitor, in HEK293 cells. HEK293 cells (50K/well) were treated with increasing concentrations of Erlotinib for 15 min. The cells were then stimulated with 1 nM of EGF for 10 min before lysis with 50 µL of LANCE *Ultra* Lysis Buffer 1 for 15 min at room temperature. Incubation time: 4 h. All data were measured using the EnVision multilabel plate reader with laser excitation.

Assay Reproducibility

To ensure all LANCE *Ultra* cellular phosphoprotein assays yield highly-reproducible data suitable for screening, each assay was tested in a Z' experiment (Figures 4 and 5). Stimulated vs. unstimulated cells were used for these measurements. All assays produced a robust Z' value (Z' > 0.70) suitable for screening assays.

Conclusion

We describe here the development and validation of LANCE *Ultra* TR-FRET cellular phosphoprotein detection kits for kinase and cell signaling assays. These kits provide a physiologically-relevant cellular assay in a homogeneous format with no wash steps and no separation steps. All kits were rigorously developed using highly specific and selective antibodies screened for optimal performance and validated in cellular models using known pathway stimulators and inhibitors. LANCE *Ultra* TR-FRET assays are suitable for both basic research and screening, showing high reproducibility.

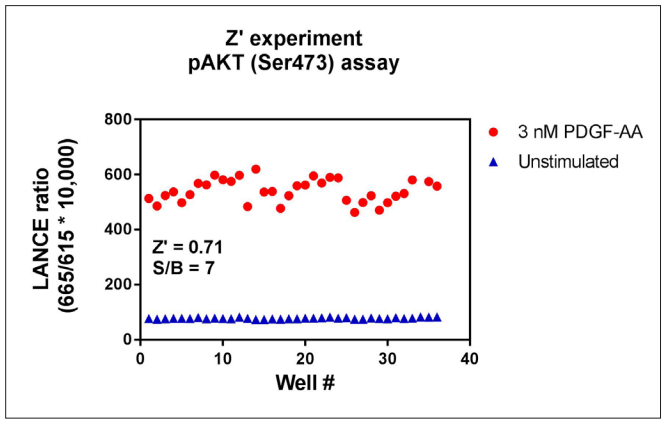

Figure 4. Z' assay for pAkt (Ser473) using NIH3T3 cells (60,000 per well) treated with 3 nM PDGF-AA for 30 min prior to lysis.

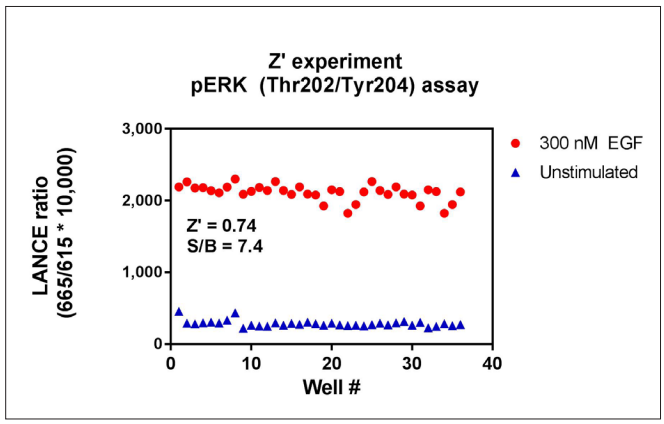

Figure 5. Z' assay for pERK 1/2 (Thr202/Tyr204) using HEK293 cells (50,000 per well) treated with 300 nM EGF for 10 min prior to lysis.

References

1. Franke, T. F. et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 81, 727–736 (1995).
2. Williams, R. et al. Identification of a human epidermal growth factor receptor-associated protein kinase as a new member of the mitogen-activated protein kinase/extracellular signal-regulated protein kinase family. J. Biol. Chem. 268, 18213–18217 (1993).
3. Hidalgo, M. Erlotinib: preclinical investigations. Oncology (Williston Park, N.Y.) 17, 11–16 (2003).

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

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