Phosphatase Assays Using the LANCE *Ultra* Technology

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1 Abstract

Protein phosphorylation and dephosphorylation events are involved in the regulation of many aspects of the cell cycle, including proliferation, differentiation, secretion and apoptosis. Aberrant protein phosphorylation is a cause or consequence of many human diseases. As a result, kinases and phosphatases have become critical targets for drug discovery and this has prompted the development of many assay technologies suitable for high-throughput screening (HTS). LANCE® *Ultra* time-resolved fluorescence energy transfer (TR-FRET) reagents were developed primarily for detecting Ser/Thr and Tyr phosphorylation events. An extensive series of europium chelate (Eu)-labeled anti-phospho antibodies were developed with their specific unphosphorylated U*Light*™-labeled peptide counterparts. In the classical LANCE *Ultra* homogeneous assay set-up, peptide phosphorylation events cause an increase of the TR-FRET signal while kinase inhibitors lead to signal decrease.

We present here proof of concept data showing that the LANCE *Ultra* technology can be adapted for detecting the activity of Ser/Thr and Tyr phosphatases. This is done by using the phosphorylated version of U*Light*-labeled peptides as enzyme substrates. LANCE *Ultra* phosphatase reactions are thus signal decrease assays in which peptide dephosphorylation is detected. In these reactions, phosphatase inhibitors will restore partly or totally the original TR-FRET signal.

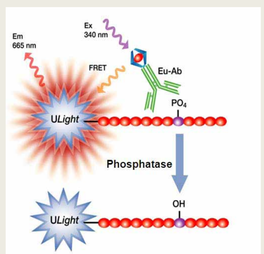
The activity of two Ser/Thr phosphatases, PP1A and PP2A, was monitored using as substrates three phosphorylated U*Light*-labeled peptides: TAU (pThr231), TOPOIIa (pThr1342) and 4E-BP1 (pThr46). In these assays, TR-FRET signal decreases gradually as the two phosphatases dephosphorylate the U*Light*-phospho-peptide substrates. Our data indicate that LANCE *Ultra* Ser/Thr phosphatase assays are extremely sensitive, requiring about 10-fold less enzyme than assays using P^{32} labeled peptide substrates. IC_{50} values obtained for the inhibition of PP2A by okadaic acid were consistent with literature data (0.5 nM for TOPOIIa (pThr1342) and 0.2 nM for 4E-BP1 (pThr46)). Robustness of the PP2A phosphatase assays was demonstrated with the TOPOIIa (pThr1342) and 4E-BP1 (pThr46) substrates, with Z'-factor values above 0.7. A LANCE *Ultra* Tyr phosphatase assay was also performed with TCPTP and a phospho-U*Light*-JAK1 (pTyr1023) peptide as substrate; efficient dephosphorylation of the peptide was demonstrated with sub-picogram amounts of enzyme.

Overall, our results demonstrate the LANCE *Ultra* technology is well suited for the development of robust and sensitive signal decrease phosphatase assays and for characterizing the effect of inhibitors.

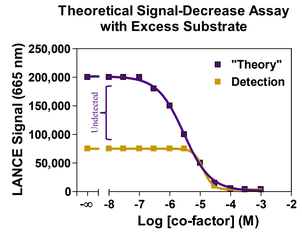
2 Assay Principle

In LANCE *Ultra* assays, Eu-labeled anti-phospho-target antibodies bind to phosphorylated U*Light*-labeled substrates, which brings Eu donor and U*Light* acceptor dye molecules into close proximity. Following excitation of the assay reaction at 320 or 340 nm, the energy from the Eu donor is transferred to the U*Light* acceptor dye which, in turn, emits light at 665 nm. The intensity of the emission output is proportional to the level of U*Light*-substrate phosphorylation.

In LANCE *Ultra* phosphatase assays, peptide phosphorylation decreases over time, resulting in a reduced interaction between the Eu-labeled antibodies and U*Light*-labeled peptides. Phosphatase activity thus leads to TR-FRET signal decrease, while phosphatase inhibitors prevent this decrease in a concentration-dependent manner.

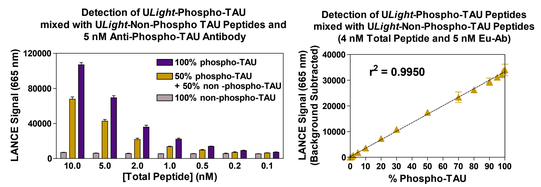


3 Signal-Decrease Phosphatase Assay



In phosphatase signal-decrease assays, initial enzymatic conditions are critical for the development of a sensitive assay. If the amount of labeled substrate is too high relative to the Eu-antibody concentration, small changes in phosphorylation will remain undetected, as in the theoretical example depicted above (in brackets). In phosphatase signal-decrease assays, U*Light*-phospho-peptide and Eu-anti-phospho-substrate antibody concentrations must thus be carefully titrated in order to allow detection of low levels of dephosphorylation.

Optimization of Antibody/Phospho-Peptide Ratio

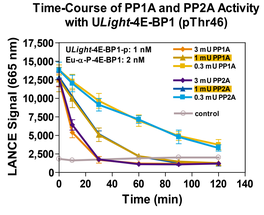


Optimal concentration of TAU (pThr231) phospho-peptide was determined above using 5 nM Eu-anti-phospho-TAU antibody. We first evaluated different total amounts of TAU peptides including either 100% or 50% of phosphopeptide, and measured the extent of signal decrease in samples containing 50% phospho-peptide (left panel). We wanted to determine which peptide concentrations would generate ~50% net signal reduction with 50% of phospho-peptide while yielding a high TR-FRET signal. Total concentrations of peptides ranging from 2 to 5 nM appeared suitable. On the right, a standard curve of TAU phospho-peptide was made by mixing 4 nM total of phospho and non-phospho TAU in different proportions with 5 nM of Eu-anti-phospho-TAU antibody. Excellent linearity was observed (r^2 value of 0.9950), indicating that these peptide and antibody concentrations are ideal for monitoring peptide dephosphorylation in a signal decrease assay.

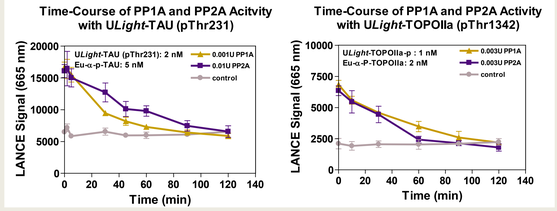
4 Serine/Threonine Phosphatase Assays

PP1A and PP2A Ser/Thr Phosphatases

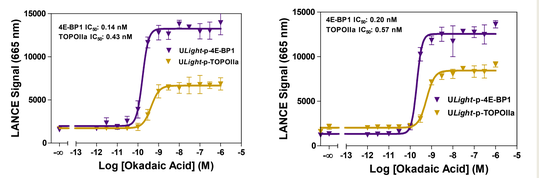
PP1A and PP2A assays were performed with U*Light*-TAU (pThr231), TOPOIIa (pThr1342) and 4E-BP1 (pThr46) peptides. Various enzyme concentrations were tested. Selected PP1A and PP2A concentrations are highlighted in the figure legend. The pattern of signal decrease was similar to the ones observed in assays using ^{32}P radio-labeled peptides, but the LANCE *Ultra* assays used much less enzyme (not shown). S/B ratios for the PP1A and PP2A phosphatase assays were of ~3 for U*Light*-TAU and TOPOIIa and ~8 for U*Light*-4E-BP1.



PP1A and PP2A Ser/Thr Phosphatases (cont'd)

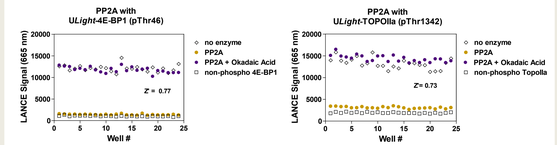


PP2A : Okadaic Acid Dose-Response Curves



Dose-response curves were performed with PP2A using U*Light*-TOPOIIa (pThr1342) and 4E-BP1 (pThr46) as substrates. For TOPOIIa, 0.004U of PP2A was used for 90 min. For 4E-BP1, 0.001U of PP2A was used for 60 min. Assays gave reproducible IC_{50} values (left vs. right panels). Ab and peptide concentrations were slightly modified for the TOPOIIa assay: left) 2 nM Ab and 1 nM peptide; right) 3 nM Ab and 2 nM peptide. These concentration changes yielded higher signal but similar S/B ratios and IC_{50} values.

Z' Study of the PP2A Assay

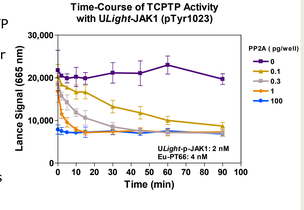


PP2A assay robustness was evaluated in a Z'-factor analysis with the U*Light*-4E-BP1 and U*Light*-TOPOIIa phosphorylated peptides. Okadaic acid was used at a concentration inhibiting 100% of the enzyme activity (100 nM). Z'-factor was calculated using the PP2A (gold circles) and PP2A + Okadaic Acid (purple circles) data. Controls were the phospho- or non-phospho-peptides alone (open symbols). The two assays gave Z' factors above 0.7.

5 Tyrosine Phosphatase Assay

TCPTP Tyrosine Phosphatase

A similar assay design was applied to measure TCPTP tyrosine phosphatase activity. JAK1 is reported in the literature to be a natural substrate for TCPTP. We show here that TCPTP can indeed dephosphorylate the U*Light*-JAK1 (pTyr1023) peptide. For this assay, we used the generic Eu-labeled-PT66 generic anti-phospho-tyrosine antibody. Although signal decrease is observed, assay background is relatively high, indicating that further assay optimization might be needed. The amount of enzyme sufficient for this experiment is below one picogram per well.



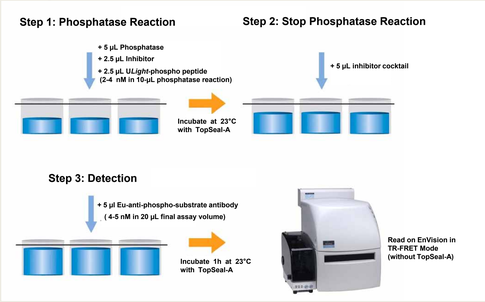
6 Materials and Methods

Materials

- U*Light*-phospho-peptides (custom products) PerkinElmer
- Eu-anti-P-Tau (pThr231) (custom product) PerkinElmer
- Eu-anti-P-TopoIIa (pThr1342) PerkinElmer #TRF0218
- Eu-anti-P-4E-BP1 (pThr46) PerkinElmer #TRF0216
- Eu-anti-PT66 (anti-P-Tyrosine) PerkinElmer #AD0068
- LANCE® Detection Buffer, 10X PerkinElmer #CR97-100
- OptiPlate™-384, white opaque PerkinElmer #6007299
- TopSeal™-A PerkinElmer #6005185
- Envision® Multi-label Reader PerkinElmer #2104-0010

- All phosphatases (human recombinant) were from Millipore.
- Concentrations of peptides are calculated for 10 µL reactions, while antibody concentrations are for 20 µL total assay volumes.
- Assay Buffer: 50 mM HEPES pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 3 mM $MnCl_2$ 2 mM DTT, and 0.01% Tween-20.

Phosphatase Protocol



7 Summary and Conclusions

- We developed three Ser/Thr and one Tyr Phosphatase assays using as peptide substrates the phosphorylated versions of four LANCE *Ultra* U*Light*-peptides derived from TAU (residues surrounding Thr231), 4E-BP1 (residues surrounding Thr46), TOPOIIa (residues surrounding Thr1342) and JAK1 (residues surrounding Tyr1023).
- The optimized PP2A/U*Light*-TOPOIIa (pThr1342) and PP2A/U*Light* 4E-BP1 (pThr46) assays were reproducible and robust, as confirmed by Z'-factors above 0.7 in 384-well format.
- The IC_{50} values obtained for the inhibition of PP2A by okadaic acid were of the same order as that reported in the literature (IC_{50} value = 0.1nM). Average values obtained for P-4E-BP1 and P-TOPOIIa were of ~0.2 and 0.5 nM, respectively. These data suggest that the use of a variety of phosphorylated substrates might allow for pathway selective inhibitor screening.
- Preliminary work with the TCPTP/U*Light*-JAK1 (pTyr1023) assay shows that tyrosine phosphatase LANCE *Ultra* assays can be performed using a generic Eu-labeled anti-phospho-Tyr antibody, such as PT66.
- Overall, our results demonstrate the LANCE *Ultra* technology is well suited for the development of robust and sensitive signal decrease phosphatase assays and for characterizing the effect of their inhibitors.

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