Thermal Dissociation Assay for Time-Resolved Fluorescence Detection of Protein Post-Translational Modifications

Ville Eskonen,* Natalia Tong-Ochoa, Salla Valtonen, Kari Kopra, and Harri Härmä

Materials Chemistry and Chemical Analysis, Department of Chemistry, University of Turku, Vatselankatu 2, FI-20014 Turku, Finland

ABSTRACT: Post-translational modifications (PTMs) of proteins provide an important mechanism for cell signal transduction control. Impaired PTM control is a key feature in multiple different disease states, and thus the enzyme-controlling PTMs have drawn attention as highly promising drug targets. Due to the importance of PTMs, various methods to monitor PTM enzyme activity have been developed, but universal high-throughput screening (HTS), a compatible method for different PTMs, remains elusive. Here, we present a homogeneous single-label thermal dissociation assay for the detection of enzymatic PTM removal. The developed method allows the use of micromolar concentration of substrate peptide, which is expected to be beneficial when monitoring enzymes with low activity and peptide binding affinity. We prove the thermal dissociation concept functionality using peptides for dephosphorylation, deacetylation, and demethylation and demonstrate the HTS-compatible flash isothermal method for PTM enzyme activity monitoring. Using specific inhibitors, we detected literature-comparable IC_{50} values and Z’ factors from 0.61 to 0.72, proving the HTS compatibility of the thermal peptide-break technology.

INTRODUCTION

Protein post-translational modifications (PTMs) are covalent additions or proteolytic cleavage events which represent an essential regulatory mechanism involved in cellular signal transduction processes. Protein PTM dynamics are regulated by a vast number of enzymes called “writers” and “erasers” depending on the nature and function of the PTM. 1,2 Targeting of deficient enzymes to treat different diseases, e.g., cancer, diabetes, heart, and neurodegenerative diseases, has been one of the main goals in modern drug discovery. 3-5 Due to the apparent medical need for enzyme activity modulators, e.g., kinase inhibitors, 6 there is a constant necessity for improved methodology. A variety of sensitive high-throughput screening (HTS) assays have been developed to measure PTM enzyme activity. Nowadays, the tendency is for the use of fluorescence-based methods since they are simple, easy to automate, and relatively cost-effective. A broad range of reporter labels including traditional fluorophores with short fluorescence half-lives (<μs), e.g., fluorescein or rhodamine, 7,8 and longer half-life luminesophores (μs to ms), such as the lanthanide (Ln^{3+})-based probes, have been widely used in PTM enzyme assays. The interest for the use of Ln^{3+}-probes has increased as they can improve the assay sensitivity due to their properties. Lanthanides provide longer luminescence lifetime and a wider Stokes shift compared to traditional fluorophores, which have mostly overlapping excitation and emission spectra. 9-11 Time-resolved luminescence (TRL) is a technology developed based on Ln^{3+} probes. 12,13 In this system, luminescence signal is read after a selected delay time set between the excitation pulse and the lanthanide emission signal monitoring window, thus enabling short-lived autoluminescence subtraction. 14 The time-resolved Förster resonance energy transfer (TR-FRET) is a dual-label based method that utilizes energy transfer from a lanthanide donor to an organic dye acceptor. 15-19 Despite its high sensitivity, challenges appear during optimization in relation to label conjugation and close proximity between the used labels. Fluorescence polarization (FP) offers an easier-to-optimize option as it is a single-label method. FP is based on the rotational property differences of small vs large molecules and has been mostly applied for kinases as good antibodies are available on the market. 20-22 Whether single- or dual-label-based technique, most of the PTM assays use antibodies for detection, creating the need to produce a particular antibody for each studied PTM. For instance, phosphoserine or phosphothreonine antibodies require a specific amino acid sequence near the binding site. In this sense, the phosphosite diversity being recognized is limited. 23 Moreover, developing pan-specific antibodies can be difficult due to the small size of some PTMs. 23 Since the antibody availability is limited, the PTM monitoring has been indirectly assayed using antibodies against secondary products, e.g., UDP antibody in the case of target glycosylation. 24 This affects the assay optimization and

Received: July 11, 2019
Accepted: September 10, 2019
Published: September 24, 2019

DOI: 10.1021/acsomega.9b02134
ACS Omega 2019, 4, 16501−16507

Cite This: ACS Omega 2019, 4, 16501−16507

© 2019 American Chemical Society

16501
sensitivity as enzyme cascades or chemical reactions are part of the detection. FP and TR-FRET have been reported for ligases, kinases, methyltransferases, fucosyltransferases, and glycosyltransferases. Antibody-free fluorescent methods have also been reported. The immobilized metal-ion affinity particle (IMAP) technology is an FP-based method that relies on the high-affinity and covalent coordination of trivalent metal-containing nanoparticles with phosphogroups. Omnia, on the other hand, is a chelation-enhanced fluorescence method developed for phosphorylation. In a kinase activity assay, the phosphate group located in the vicinity of the coordinating fluorophore promotes the metal-ion binding, producing an increase in the fluorescence signal. Although the methods are antibody-free, the nature of the approaches has limited their applicability to phosphorylation.

Previously, we have developed an antibody-free PTM monitoring method called the peptide-break technology which allows the detection of a variety of PTMs in a single platform. The peptide-break technology is based on the peptide dimerization monitored using the single-label quenching resonance energy transfer (QRET) technology demonstrated previously for the detection of various PTM targets. Peptide dimer is formed between the Eu³⁺-labeled detection peptide and the selected substrate peptide designed for the studied PTM enzyme. In the absence of PTM, peptides form a complex leading to high TRL signal due to Eu³⁺-chelate protection from the soluble quenchers, while the PTM addition to the substrate peptide dissociates the peptide complex and the TRL signal is quenched. We have demonstrated that the peptide complex can be formed using either specific leucine zippers or merely charge-based interactions. This concept provides versatility to the system and allows assay optimizations of a variety of PTM types using nanomolar concentration of reagents.

Herein, we introduce a fluorescent single-label thermal dissociation assay for the monitoring of enzymatic PTM removal using the peptide-break technology. The fluorescence thermal dissociation assay harnesses the stability difference between the post-translationally modified and the nonmodified peptide dimer forced together at high substrate peptide concentration. The increase of the temperature decreases the peptide complex stability, being more stable without a PTM. As a result, a higher TRL signal is detected with a complex without a PTM at higher temperatures. The proposed antibody-free HTS method enables the use of high peptide concentrations, which is essential for some low-affinity or -activity enzymes. The fluorescent thermal dissociation assay is now demonstrated with methylation, dephosphorylation, and deacetylation assays.

RESULTS AND DISCUSSION

We have previously developed the universal single-label peptide-break technology for the detection of enzymatic PTM reactions using leucine zippers or charge-based peptides. These assays were optimized for detection at room temperature using low nanomolar peptide concentrations. However, not all enzymes possess high peptide binding and enzymatic activity allowing low substrate concentrations. This might reduce the applicability of the peptide-break technology with some enzymes. To address this potential shortcoming, we have now developed a thermal dissociation-based peptide-break approach. The new assay protocol enables the use of high substrate peptide concentration of the selected peptide substrate for the studied PTM enzyme, which promotes the peptide–enzyme interaction. The formed duplex of the detection peptide with the modified and unmodified substrate can then be distinguished using the difference in dissociation rates of the duplexes at higher temperatures. Such a detection scheme is potentially suitable for low-activity or -affinity enzyme monitoring. The principle of this method is presented in Figure 1.

Figure 1. Principle of the thermal peptide-break technology. At room temperature, high peptide concentration drives the peptide duplex formation irrespective of the PTM. At increasing temperatures, PTM-reduced duplex affinity (purple) is disrupted at lower temperature compared to the dimer without a PTM (orange line). At the optimal temperature, the duplex with a PTM is dissociated, allowing signal attenuation due to the quenchers in solution, while the nonmodified dimer remains intact, resulting in Eu³⁺ chelate protection and high TRL signal.

Thermal Dissociation-Based Peptide-Break Technique Enables the Detection of Small Nonmodified Peptide Population from the Large Modified Peptide Pool. The peptide-break technology relies on the affinity difference between modified and unmodified substrate peptide and the Eu³⁺-labeled reporter peptide upon enzymatic PTM addition or removal. The method enables detection of different PTMs by simply changing the PTM-modified peptide, while the Eu³⁺-labeled reporter peptide remains the same. Affinity difference of the modified vs nonmodified peptide provides the basis to distinguishing bound vs nonbound peptide fractions using a homogeneous single-label QRET detection. We have previously shown that high peptide concentration efficiently promotes the modification-independent peptide pair formation, leading to peptide paring at all time. It is well known that temperature affects the equilibrium of reactants, and at high temperatures, proteins and peptides are denatured and biomolecular interactions are weakened. We decided to use this temperature-related effect to discriminate the binding of modified and nonmodified peptides to the Eu³⁺-labeled reporter (Figure 1). This was developed to improve the applicability of the peptide-break technology for PTM enzymes possessing low activity or peptide binding affinity, as it enabled the use of micromolar substrate peptide.

To demonstrate the concept functionality, we performed a binding test with a number of peptides in high concentration without a PTM and peptides containing two phosphoryls, acetyl, or methyl sites. First, we studied the effect of phosphoryl groups using Eu–LZ detection peptide in complex
with LZ-Y and LZ-pY substrates. TRL signal was monitored using 5 °C measurement interval from RT until 95 °C (Figure 2a,b). A clear difference between the signals of LZ-Y and LZ-pY was observed between 60 and 80 °C. Thereafter, similar thermal ramping was performed with the two charge-based peptide pairs for deacetylation and demethylation, CP7/CP6 and CP9/CP8 (Figure 2b). These peptides did not contain a leucine zipper structure similar to LZ-Y/LZ-pY, and the optimal temperature for these peptide pairs was significantly lower (Figure 2b). The observed S/B ratios for LZ-Y/LZ-pY, CP7/CP6, and CP9/CP8 in RT at 10 μM concentration were 1.8, 3.3, and 2.8, respectively. With increasing temperature, heat-induced separation between Eu−LZ and the substrate peptides gave the maximal S/B ratio of 36, which was monitored with the leucine zipper LZ-Y/LZ-pY peptides at the optimal temperature of 70 °C. In case of both charge-based peptide pairs, the peak shape was broader and the monitored S/B ratios were lower compared to LZ peptides. The optimal temperatures for the charged-based CP7/CP6 peptide pair were 50 and 45 °C for CP9/CP8. The observed S/B ratios for CP7/CP6 and CP9/CP8 were 10.5 and 5.1, respectively. The affinity difference between peptide pairs partly explains the difference in the optimal temperatures and also the higher S/B ratio observed at room temperature for CP peptides compared to LZ peptides. Also the nature of CP and LZ peptides is different, explaining the temperature differences and the sharp temperature profile of LZ peptides compared to the shallow profile of the CP peptides (Figure 2b). The difference in the S/B ratios and temperature curves confirmed the previous observation that LZ complex possesses higher affinity than CP complexes to Eu−LZ peptide. Data also clearly showed the functionality of the nonenzymatic thermal-break concept using modified and nonmodified peptides.

Next, the sensitivity of the thermal peptide-break assay was evaluated using LZ-Y and LZ-pY peptides at RT and at the optimal 70 °C temperature using the slow step-by-step heating. The data are collected at 70 °C. In the assay, LZ-Y and LZ-pY were added in different ratios keeping the overall peptide concentration at 10 μM to mimic the enzymatic reaction. The resulting TRL signals were then compared to the TRL signals measured for 100% LZ-pY. As previously, the difference between LZ-Y and LZ-pY was barely detectable at 25 °C, and LZ-Y could not be separated from LZ-pY at any concentration (Figure 3). However, at 70 °C, a separation between peptides was detected with the maximal S/B ratio over 20, when only LZ-Y was present. Already less than 1% of LZ-Y was clearly detected at 70 °C with an S/B ratio of ~7. This 1% corresponds to 100 nM concentration of LZ-Y in the presence of 9.9 μM LZ-pY, providing the basis to detect low degree of enzymatic conversion. Based on these data, the linear range of detection is from 1 μM to 10 nM. More than 1 μM of LZ-Y gives S/B ratio similar to the 100% LZ-Y and less than 10 nM LZ-Y results in S/B ratio close to 1 (Figure 3).

**Figure 2.** Temperature dependence of the thermal peptide-break approach using the QRET detection. (A) TRL signals of 10 μM LZ-Y (black) and LZ-pY (red) in complex with Eu−LZ showed a clear temperature-dependent decrease and a PTM-dependent separation as a function of temperature. In the presence of soluble quencher MT10, efficient dissociation of LZ-pY from the Eu−LZ complex is observed at 10−15 °C lower temperature as for LZ-Y. The maximal S/B ratio with LZ peptides was achieved at 70 °C. Data represent mean ± SD (n = 3). (B) In the presence of 10 μM LZ-Y or LZ-pY, CP7 or CP6, and CP9 or CP8, the binding behavior was monitored with Eu−LZ (1 nM). At RT, high TRL signals and low S/B ratios were observed for all peptide complexes in the presence of soluble quencher. TRL signals of LZ-Y, CP7, and CP9 complexes were compared to the TRL signals of LZ-Y, CP6, and CP8, respectively. At elevated temperature, the PTM-modified lower-affinity peptides, LZ-pY, CP6, and CP9, were dissociated from the Eu−LZ reporter inducing a drastic TRL signal decrease compared to their counterparts still forming a relatively stable complex with Eu−LZ.

**Figure 3.** Temperature dependence of the thermal peptide-break approach with high peptide concentration. The detection sensitivity of the thermal peptide-break approach was monitored by mimicking the enzymatic activity by varying LZ-Y and LZ-pY ratio at fixed 10 μM total peptide concentration. At 25 °C, no separation between PTM-modified LZ-pY and unmodified LZ-Y peptide was monitored. At 70 °C, high degree of separation was monitored and already less than 1% of LZ-Y was efficiently detected from the large pool of LZ-pY. Data represent mean ± SD (n = 3).
functionality with the nonenzymatic assays, we decided to prove the concept functionality with two enzymatic reactions, dephosphorylation (PTP1B) and deacetylation (HDAC3). First, the assays were performed in the presence and absence of saturating concentration of specific inhibitors Na₃VO₄ and trichostatin A for PTP1B and HDAC3, respectively. Using a temperature ramping protocol from 25 to 80 °C (LZ-pY) and from 25 to 60 °C (CP6), a highly similar temperature profile in the enzymatic assay was monitored as previously with the nonenzymatic reactions (Figures 2b and 4). In the assay with 10 μM LZ-pY and 1 nM PTP1B, the highest S/B ratio with 1 μM Na₃VO₄ inhibited vs noninhibited reaction was 25 when the measurement was performed after heating at 70 °C (Figure 4a). This is in line with those obtained without enzyme reaction, indicating highly efficient enzyme reaction under these conditions. In the HDAC3 assay with CP6, the peak shape was again broader compared to the LZ-pY profile, and the S/B ratio was 3.8 as monitored between 45 and 55 °C using 1 and 0 μM trichostatin A (Figure 4b). Also in this case, the thermal peak shapes and S/B ratios were highly similar to nonenzymatic assays.

To analyze the assay concept functionality further, we performed inhibitor titrations with PTP1B and HDAC3, using Na₃VO₄ and trichostatin A, respectively. The same temperature cycle with 5 °C incremental steps was carried out as previously, and the maximal responses were detected at the optimal temperatures, 70 °C for LZ-pY and 50 °C for CP6.

Single-Temperature Flash Heating Assay Showed Good HTS Compatibility. In all previous assays, thermal ramping was applied to monitor PTM-dependent thermal separation of the used peptides. As the optimal assay conditions were peptide-pair-dependent and equal-temperature profiles were measured for nonenzymatic and enzymatic reactions, it is expected that single temperature can be used to obtain PTM-dependent separation. The thermal ramping protocol is time-consuming, and thus a protocol with a single heating step is favored, as it is readily adaptable to HTS. We tested the assay at a single flash temperature, using the peptidesspecific optimal temperatures determined earlier, 70 °C for the

Figure 4. Temperature profiles of the enzymatic PTM removal assays using PTP1B and HDAC3. TRL signals of enzymatic reactions without an inhibitor were compared to the TRL signals of the inhibited enzymatic reactions. (A) High S/B ratio was observed at 70 °C for LZ-pY in the PTP1B enzyme reaction without or with 1 μM Na₃VO₄. (B) S/B ratio of nearly 4 was observed at 45–55 °C when CP6 was assayed with HDAC3 and 0 or 1 μM trichostatin A. Highly similar peak shape inherent to each peptide pair was found for enzymatic and nonenzymatic reactions. Data represent mean ± SD (n = 3).

Figure 5. Inhibitor titration curves for Na₃VO₄ at 70 °C and trichostatin A at 50 °C. (A) The enzymatic reactions with LZ-pY were heated in 5 °C intervals, and the peak maximum temperature was used to show the dose response for the used inhibitor. Based on the dose–response curves, the IC₅₀ value for PTP1B inhibitor Na₃VO₄ was 3.8 nM. (B) From the deacetylation reaction with CP6, dose–response curve at 50 °C showed IC₅₀ of 1.1 nM for HDAC3 inhibitor trichostatin A. Data represent mean ± SD (n = 3).
Figure 6. Thermal peptide-break assay using flash heating. (A) Plate cooling lowered the S/B ratio of the peptide assays approximately 20%, but due to the lower variation, the Z′ factor was significantly improved. Data represent mean ± SD (n = 24). (B) Z′ factor measurement for the enzymatic dephosphorylation and deacetylation assays with 18 replicates. Dephosphorylation assay with (red) and without (black) 5 μM Na3VO4 and deacetylation assays with (green) and without (blue) trichostatin A gave Z′ values of 0.72 and 14.6 and S/B ratios of 0.61 and 3.0, respectively.

LZ-pY and 50 °C for the CP6. With the flash heating mode, the complex was kept at selected temperature for 3 min before the immediate measurement. Thereafter, the plate was allowed to cool down to RT and measurements were repeated after 20 and 60 min at RT. These measurements with the commercial peptides at RT were beneficial for the reproducibility compared to the high-temperature measurement directly after 70 °C (Figure 6a).

We also observed that EuLZ complexes are not capable of reassociating after heating at RT, as cooling did not significantly change the S/B ratio. When the plate is measured hot, the S/B ratio is higher than if the plate is allowed to cool down (Figure 6a). The TRL signals are low and the deviation is high when measured directly after 70 °C. When the plate is allowed to cool down, the signal levels rise, but the S/B ratio remains. Also the deviation decreases. This seems to be due to the time the plate reader takes to measure a large assay. Because of the increase in the TRL signal levels during the cooling down, the first measured wells give a lower signal value than the last measured wells.

Next we converted the flash heating experiment with the peptides to enzymatic reactions and determined Z′ factors for both dephosphorylation and deacetylation, by running 18 reactions under fully inhibited (5 μM Na3VO4 and trichostatin A) and noninhibited conditions for PTP1B and HDAC3 (Figure 6b). We allowed the plate cooling to RT before measuring due to the previous result shown in Figure 6a. In the enzymatic dephosphorylation assay, the S/B ratio and Z′ factor monitored after dissociation at 70 °C were 15 and 0.72, respectively. As a control, the nonenzymatic assay with LZ-Y and 50 °C was 4.6 with or without 5 μM trichostatin A and the Z′ factor was 0.61 (Figure 6b). The results clearly imply that the flash heating is amenable to monitor enzymatic reactions with increased simplicity and good HTS compatibility.

With the CP peptides, the substrate sequence is simplified compared to LZ, where leucines need to be positioned in the seven repeats potentially limiting the use of long consensus sequences extending to the crucial leucine positions. However, we observed that the LZ strategy is beneficial in the detection of thermal dissociation as the affinity is higher and peak shape is sharper compared to shallow peak behavior of the CP peptides. Some differences in the measured S/B ratios and Z′-factor values were recorded between the measurements for both the CP peptides and the LZ peptide. This is possibly due to the low background signal levels. The detected background signal levels were close to the signal levels from an empty well so even a small change in this signal level will affect the S/B ratio greatly. In this study, the method was merely applied to detect the removal of a PTM, but the concept is also expected to function with enzymes adding a PTM to substrate peptides.

■ CONCLUSIONS

In this work, we have developed a thermal peptide-break platform for single-label detection of enzymatic PTM removal utilizing TRL signal readout. The assay utilizes the previously introduced concept, peptide-break, with nanomolar sensitivity but now by applying temperature-controlled approach, which enables the use of micromolar peptide concentrations. The method is based on heat-induced dissociation distinguishing a proportion of peptide where PTM is enzymatically removed from a large pool of peptide substrate carrying PTM. The thermal peptide-break technique was proved to be functional in monitoring of PTP1B and HDAC3 enzymatic activities, and the results obtained from nonenzymatic demethylation indicates broad suitability for a variety of PTMs. Method functionality was first demonstrated with heat-ramping scheme to optimize the heat-induced detection, and thereafter the assay was converted to flash mode to enable HTS. In conclusion, we expect that the developed thermal peptide-break assay platform is useful addition for advanced approaches to study PTMs and enzyme inhibitors and activators in a single peptide-based platform.

■ EXPERIMENTAL SECTION

Materials. Peptides used in this research were purchased from Pepmic Co., Ltd (Suzhou, China) (Table 1). Eu–LZ and the soluble modulator, MT10, were from QRET Technologies and were used according to the manufacturer’s instructions.
Table 1. Peptide Sequences Used<sup>26,29</sup>

| name  | sequence                                      |
|-------|-----------------------------------------------|
| LZ-pY | REELKRRKAELRRPYAQLRQRREQLQRQRpYANLRLKE       |
| LZ-Y  | REELKRRKAELRRYAAQRQRREQLQRQRYANLRLKE         |
| CP7   | GRARKGARRAKGARRR                              |
| CP6   | GRARK(Ac)GARRAK(Ac)GARRR                      |
| CP9   | GRARTK(Met-1)QTRRTARK(Met-1)QTRRR            |
| CP8   | GRARTQRTARRTQKTRRR                           |

(EurLZ peptide is labeled with a nonadentate (9d) europium chelate: \(2,2',2''\)-terpyridine-6,6''-diyl]bis(methylene-nitrito))tetraakis(acetato)europium(III). Recombinant human PTP1B and recombinant human HDAC3/NCOR2 were purchased from Thermo Fisher Scientific (Waltham, CA). Inhibitors Na<sub>3</sub>VO<sub>4</sub> and trichostatin A were from MP Biomedicals, LLC (Illkirch, France) and Santa Cruz Biotechnology (Dallas, USA). Black Framestar 96-well microtiter plates, used in all assays, were from 4ttitude (Surrey, U.K.). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO).

**Instrumentation.** TRL signals were measured using Victor 1420 multilabel counter from PerkinElmer Life and Analytical Sciences, Wallac (Turku, Finland), using 340 nm excitation and 615 nm emission wavelengths and 400 μs delay and integration times. Temperature cycling was carried out with a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA).

**Assay Concept Functionality with Synthetic Peptides.** The peptide binding tests were performed for LZ-Y and LZ-pY peptides in assay buffer 1 (10 mM HEPES (pH 7.5), 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 5 mM NaCl, and 0.01% Triton X-100) and for CP7 and CP6 peptides and CP9 and CP8 peptides in assay buffer 2 (20 mM HEPES (pH 8.0), 1 mM MgCl<sub>2</sub>, 25 mM NaCl, 2.7 mM KCl, and 0.01% Triton X-100). All assays were performed as triplicate. Buffers were selected based on the PTP1B (buffer 1) and HDAC3 (buffer 2) preferences<sup>27</sup>. In the assays, 20 μL of varying peptides (10 μM) and 30 μL of detection solution, containing Eu–LZ (1 nM) and soluble modulator MT10, were added to the wells. The microtiter plate was heated and measured in 5 °C intervals from +25 °C until the TRL signal levels reached the background signal level.

**Enzymatic PTM Monitoring.** Enzyme reactions were carried out in the enzyme-specific selected assay buffers described above. All assays were performed as triplicate in the final volume of 50 μL. We performed inhibitor titration (0–1 μM) with Na<sub>3</sub>VO<sub>4</sub> for PTP1B and trichostatin A for HDAC3. Inhibitors and enzyme solution were added, both 5 μL. Thereafter, 10 μL of LZ-pY (PTP1B) or CP6 (HDAC3) was added and reactions were incubated for 30 min (PTP1B) or 45 min (HDAC3) at RT. Detection solution (1 nM Eu–LZ and MT10) was added in 30 μL, and after 5 min of incubation, heating cycle with 5 °C interval was started and TRL signals were monitored in every step until the signal reached the background level. Similar assays were also performed by using single saturating (1 μM) inhibitor concentration to achieve full inhibition and maximal signal-to-background (S/B) ratio.

The flash mode protocol was performed in a single selected temperature using 18 individual reactions. The enzyme reaction with PTP1B and HDAC3 were performed with a protocol described previously by comparing the reaction with 5 μM or no inhibitor. In the flash heating mode, 50 μL reactions with detection component were heated for 3 min at the selected temperature, 70 °C for LZ-pY (PTP1B) and 45 °C for CP6 (HDAC3). For LZ-pY in PTP1B and for CP6 in HDAC3 reactions, the selected temperatures were 70 and 45 °C, respectively, after the plate had time to cool down. The TRL signals were monitored directly after flash heating and after the plate was cooled down to RT (20 and 60 min).

**Data Analysis.** S/B ratio was calculated by \( \mu_{\text{max}}/\mu_{\text{min}} \) and coefficient of variation (CV%) as \( (\sigma/\mu) \times 100 \). \( \mu_{\text{max}} \) and \( \mu_{\text{min}} \) are the mean values of three triplicates of luminescence intensity signals. The \( Z' \) factor was calculated as \( 1 - \frac{3(\sigma + \sigma_c)}{\sigma - \sigma_c} \).

In all calculations, \( \sigma \) refers as standard deviations (SD) and \( \mu \) as mean values. The half-maximal inhibitor concentration (IC<sub>50</sub>) values and all other curves were fitted using standard sigmoidal fitting functions with Origin 2016 (OriginLab, Northampton, MA).

**AUTHOR INFORMATION**

*E-mail: vijues@utu.fi.*

**ORCID**

Ville Eskonen: 0000-0001-5214-5904

Kari Kopra: 0000-0001-7585-6020

**Notes**

The authors declare the following competing financial interest(s): K.K. and H.H. have commercial interest through QRET Technologies Ltd.

**ACKNOWLEDGMENTS**

This work was supported by the Academy of Finland (270010, 296093, and 296225), Drug Research Doctoral Programme (University of Turku Graduate School), and Finnish Academy of Science and Letters (foundation of Vilho, Yrjö, and Kalle Väisälä).

**REFERENCES**

1. Mann, M.; Jensen, O. N. Proteomic Analysis of Post-Translational Modifications. *Nat. Biotechnol.* 2003, 21, 255–261.
2. Venne, A. S.; Kollipara, L.; Zahedi, R. P. The next Level of Complexity: Crosstalk of Posttranslational Modifications. *Proteomics* 2014, 14, S13–S24.
3. Huq, M.; Wei, L.-N. Protein Posttranslational Modification: A Potential Target in Pharmaceutical Development. *Pharm. Biotechnol.* 2010, 1–26.
4. Steffen, P.; Kwiatkowski, M.; Robertson, W. D.; Zarrine-Afsar, A.; Deterra, D.; Richter, V.; Schlüter, H. Protein Species as Diagnostic Markers. *J. Proteomics* 2016, 134, 5–18.
5. Fleuren, E. D. G.; Zhang, L.; Wu, J.; Daly, R. J. The Kinome “at Large” in Cancer. *Nat. Rev. Cancer* 2016, 16, 83–98.
6. Rodon, J.; Dienstmann, R.; Serra, V.; Tabernero, J. Development of PI3K Inhibitors: Lessons Learned from Early Clinical Trials. *Nat. Rev. Clin. Oncol.* 2013, 10, 143–153.
7. Lavis, L. D.; Raines, R. T. Bright Ideas for Chemical Biology. *ACS Chem. Biol.* 2007, 3, 142–153.
8. Chouliler, L.; Enander, K. Environmentally Sensitive Fluorescent Sensors Based on Synthetic Peptides. *Sensors* 2010, 10, 3126–3144.
9. Hemmilä, I.; Laiala, V. Progress in Lanthanides as Luminescent Probes. *J. Fluoresc.* 2005, 15, S29–S42.
10. Allen, K. N.; Imperiali, B. Lanthanide-Tagged Proteins - an Illuminating Partnership. *Curr. Opin. Chem. Biol.* 2010, 14, 247–254.
11. Charbonniere, L. J. Luminescent Lanthanide Labels. *Curr. Inorg. Chem.* 2011, 1, 2–16.
(12) Sitari, H.; Hemmilä, I.; Soini, E.; Lövgren, T.; Koistinen, V. Detection of Hepatitis B Surface Antigen Using Time-Resolved Fluorimunosay. Nature 1983, 301, 258−260.

(13) Hemmilä, I.; Dakubu, S.; Mukkala, V.; Sitari, H.; Lövgren, T. Europium as a Label in Time-Resolved Immunofluorometric Assays. Anal. Biochem. 1984, 137, 355−343.

(14) Hewitt, S. H.; Butler, S. J. Application of Lanthanide Luminescence in Probing Enzyme Activity. Chem. Commun. 2018, 54, 6635−6647.

(15) Moshinsky, D. J.; Rulisim, L.; Blake, R.; Tang, F. A Widely Applicable, High-Throughput TR-FRET Assay for the Measurement of Kinase Autophosphorylation: VEGFR-2 as a Prototype. J. Biomol. Screening 2003, 8, 447−452.

(16) Carlson, C. B.; Horton, R. A.; Vogel, K. W. A Toolbox Approach to High-Throughput TR-FRET-Based SUMOylation and DeSUMOylation Assays. Assay Drug Dev. Technol. 2009, 7, 348−355.

(17) Degorce, F.; Card, A.; Soh, S.; Triquenet, E.; Knapik, G. P.; Xie, B. HTRF: A Technology Tailored for Drug Discovery — A Review of Theoretical Aspects and Recent Applications. Curr. Chem. Genomics 2009, 3, 22−32.

(18) Horton, R. A.; Vogel, K. W. Multiplexing Terbium- and Europium-Based TR-FRET Readouts to Increase Kinase Assay Capacity. J. Biomol. Screening 2010, 15, 1008−1015.

(19) Gauthier, N.; Caron, M.; Pedro, L.; Arcand, M.; Blouin, J.-S.; Labonté, A.; Normand, C.; Paquet, V.; Rodenbrock, A.; Roy, M.; et al. Development of Homogeneous Nonradioactive Methyltransferase and Demethylase Assays Targeting Histone H3 Lysine 4. J. Biomol. Screening 2012, 17, 49−58.

(20) Li, Y.; Xie, W.; Fang, G. Fluorescence Detection Techniques for Protein Kinase Assay. Anal. Bioanal. Chem. 2008, 390, 2049−2057.

(21) Vedvik, K. L.; Elason, H. C.; Hoffman, R. L.; Gibson, J. R.; Kupcho, K. R.; Somberg, R. L.; Vogel, K. W. Overcoming Compound Interference in Fluorescence Polarization-Based Kinase Assays Using Far-Red Tracers. Assay Drug Dev. Technol. 2004, 2, 193−203.

(22) Kristjánsson, K.; Rudolph, J. A Fluorescence Polarization Assay for Native Protein Substrates of Kinases. Anal. Biochem. 2003, 316, 41−49.

(23) Zhao, Y.; Jensen, O. N. Modification-Specific Proteomics: Strategies for Characterization of Post-Translational Modifications Using Enrichment Techniques. Proteomics 2009, 9, 4632−4641.

(24) Zielinski, T.; Reichman, M.; Donover, P. S.; Lowery, R. G. Development and Validation of a Universal High-Throughput UDP-Glycosyltransferase Assay with a Time-Resolved FRET Signal. Assay Drug Dev. Technol. 2016, 14, 240−251.

(25) Loomans, E. E. M. G.; van Doornmalen, A. M.; Wat, J. W. Y.; Zaman, G. J. R. High-Throughput Screening with Immobilized Metal Ion Affinity-Based Fluorescence Polarization Detection, A Homogeneous Assay for Protein Kinases. Assay Drug Dev. Technol. 2003, 1, 445−453.

(26) Shults, M. D.; Imperiali, B. Versatile Fluorescence Probes of Protein Kinase Activity. J. Am. Chem. Soc. 2003, 125, 14248−14249.

(27) Härmä, H.; Tong-Ochoa, N.; Van Adrichem, A. J.; Jelesarov, I.; Wennberg, K.; Kopra, K. Toward Universal Protein Post-Translational Modification Detection in High Throughput Format. Chem. Commun. 2018, 54, 2910−2913.

(28) Kopra, K.; Tong-Ochoa, N.; Laine, M.; Eskonen, V.; Koskinen, P. J.; Härmä, H. Homogeneous Peptide-Break Assay for Luminescent Detection of Enzymatic Protein Post-Translational Modification Activity Utilizing Charged Peptides. Anal. Chim. Acta 2019, 1055, 126−132.

(29) Kopra, K.; Eskonen, V.; Seppälä, T.; Jakovleva, J.; Huttunen, R.; Härmä, H. Peptidic Fluorescent “Signal-On” and “Signal-Off” Sensors Utilized for the Detection Protein Post-Translational Modifications. ACS Omega 2019, 4, 4269−4275.

(30) Härmä, H.; Rozwandowicz-Jansen, A.; Martikka, E.; Frang, H.; Hemmilä, I.; Sahling, N.; Fey, V.; Perälä, M.; Hänninen, P. A New Simple Cell-Based Homogeneous Time-Resolved Fluorescence QRET Technique for Receptor-Ligand Interaction Screening. J. Biomol. Screening 2009, 14, 936−943.