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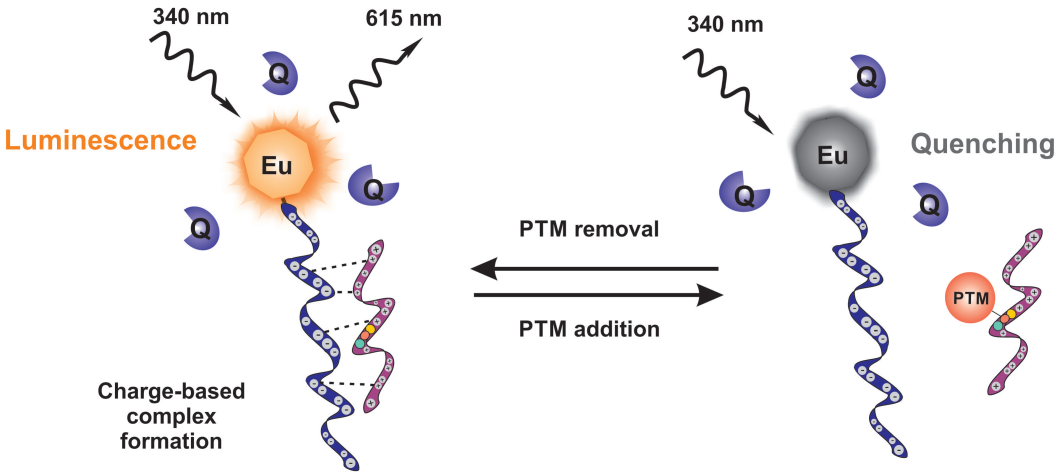

# Homogeneous peptide-break assay for luminescent detection of enzymatic protein post-translational modification activity utilizing charged peptides

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*KEYWORDS : protein post-translational modification, phosphorylation, acetylation, citrullination, peptide-break technology, time-resolved luminescence*

**Abstract**

We have developed a rapid and sensitive universal peptide-based time-resolved luminescence assay for detection of enzymatic post-translational modifications (PTMs). PTMs play essential roles in intracellular signaling and cell regulation, thus providing functional protein diversity in cell. Due this, impaired PTM patterns have been linked to multiple disease states. Clear link between PTMs and pathological conditions have also driven assay development further, but still today most of the methodologies are based on single-specificity or group-specific PTM-recognition. We have previously introduced leuzine-zipper based peptide-break technology as a viable option for universal PTM detection. Here, we introduce peptide-break technology utilizing single-label homogeneous quenching resonance energy transfer (QRET) and charge-based peptide-peptide interaction. We demonstrate the functionality of the new assay concept in phosphorylation, deacetylation, and citrullination. In a comparable study between previously introduced leucine-zipper and the novel charge-based approach, we found equal PTM detection performance and sensitivity, but the peptide design for new targets is simplified with the charged peptides. The new concept allows the use of short < 20 amino acid peptides without limitations rising from the leucine-zipper coiled-coil structure. Introduced methodology enables wash-free PTM detection in a 384-well plate format, using low nanomolar enzyme concentrations. Potentially, the peptide-break technique using charged peptides may be applicable for natural peptide sequences directly obtained from the target protein.

## 1. Introduction

Post-translational modifications (PTMs) of protein side- and main-chain peptide-bonds are covalent and usually enzyme-catalyzed events. PTMs play pivotal roles in generating protein diversity for cellular function and regulation [1,2]. PTMs are involved in reader-writer-eraser systems, in which the modifying enzymes recognize short linear motifs in their target protein. There are around 200 different PTMs introduced, which greatly increase the complexity of the proteome. The main function of these PTMs is to regulate activity, localization, and interactions with other cellular molecules [1,2]. PTMs can occur throughout the protein life cycle and act in a reversible or irreversible manner. The most important and frequent PTMs are phosphorylation, glycosylation, and acetylation, from which the phosphorylation, principally on serine, threonine, and tyrosine, is the most intensively studied PTM [3].

The impaired PTM function is linked to several human diseases like heart disease, cancer, neurodegenerative diseases, and diabetes [4-6]. PTMs also possess a role in aging and age-related diseases [7]. Thus, there is a constant need for new and improved assay methodologies, potentially applicable for the detection of multiple different PTMs. Single assay platform would enable cost-effective assays with fixed reagents, providing more efficient screen for novel drug candidates from a large molecular library. There are numerous methods available to measure PTMs. Methods based on chromatographic separation (e.g. HPLC), western blotting, ELISA, and mass spectrometry have been used widely for years [8-10]. Lately, homogeneous methods based on luminescence have been developed. However, most of the currently used methods are based on antibodies, making them group-specific for a given PTM or even specific PTM in certain sequential context [11-13]. This need for antibodies complicates the assay development for new targets and PTMs. More recently, antibody-independent assays have been developed based on the detection of PTM reaction products, most often nucleotide diphosphates. These methods are applicable e.g. to phosphorylation, ribosylation, and glycosylation monitoring, thus these reactions produce nucleotide diphosphates as a side product [14-16]. However, due to indirect nature of the assay, the readout is sensitive for target independent false positives related to e.g. kinase autophosphorylation. Previously, we have developed leucine-zipper based technology called peptide-break, utilizing homogeneous quenching resonance energy transfer (QRET) as readout [17-20]. Using this antibody-free single-label technology, we were able to monitor a number of different PTMs in a simple, sensitive, and real-time fashion [17].

Here we introduce the peptide-break approach for PTM detection based on non-leucine-zipper concept. In our previous work, the used peptide pairs were constructed on leucine driven α-helix, which partially limited the peptide design by restricting addition of longer consensus sequence for the studied enzyme [7,21,22]. To increase the freedom of enzyme substrate selection, charged amino acids were introduced to the peptide sequence. Utilizing opposite charge of the two peptides, we were able to form structurally simple non-

leucine-zipper based peptide-peptide pair. With the charge-based peptides (CP), we studied Protein kinase A (PKA) and Serine/threonine-protein kinase pim (PIM) -mediated serine phosphorylation to adjust optimal peptide architecture. Thereafter, concept was further tested using enzymes for arginine citrullination and lysine deacetylation to highlight the simple peptide design for new targets. The assay allowed nanomolar enzyme and peptide concentrations in a high throughput (HTS) compatible 384-well plate format.

## 2. Materials and methods

### *2.1 Materials and instrumentation*

All peptides were purchased from Pepmic Co., Ltd (Suzhou, China) or QRET Technologies (Turku, Finland). Used enzymes; catalytic domain of PKA (part no. P6000L), recombinant human histone deacetylase 3/nuclear receptor corepressor 2 (HDAC3/NcoR2) (part no. H85-38G) hereafter HDAC3, and recombinant human protein-arginine deiminase 4 (PAD4, part no. 10500), were from New England Biolabs (Ipswich, MA), BPS Bioscience (San Diego, CA), and Cayman Chemical (Ann Arbor, MI). Wild-type human PIM1-3 proteins were produced in bacteria as a GST-fusion protein and purified using glutathione sepharose beads as described previously [23]. Enzyme inhibitors; TSA, GSK484, and H-89 dihydrochloride were from Cayman Chemical and Santa Cruz Biotechnology (Dallas, TX). Nonadentate europium-chelate, {2,2',2'',2'''-{[2-(4-isothiocyanatophenyl)ethylimino]-bis(methylene)bis{4-{[4-(agalactopyranoxy)phenyl]ethynyl}-pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato}europium(III) was purchased from BN Products & Services (Turku, Finland), and conjugated as described elsewhere [17]. The soluble modulator MT2 was from QRET Technologies and it was used according to the manufacturer instructions. Black Optiplate 384F microtiter plates and $Eu^{3+}$-standard solution were from PerkinElmer Life and Analytical Sciences (Groningen, Netherlands) and PerkinElmer Wallac (Turku, Finland). All other reagents, including analytical-grade solvents, ATP, and staurosporine were acquired from Sigma-Aldrich (St. Louis, MO).

All $Eu^{3+}$-peptide purifications were carried out using reversed-phase adsorption chromatography, Dionex ultimate 3000 LC system from Thermo Fischer Scientific, Dionex, and Ascentis RP-amide C18 column from Sigma-Aldrich, Supelco Analytical. Time-resolved luminescence (TRL) emission signals were measured at 615 nm, using excitation at 340 nm (600 µs delay and 400 µs decay). All measurements were performed using standard microtiter plate reader developed by Labrox Ltd. (Turku, Finland) or with Victor 1420 multilabel counter from PerkinElmer Wallac.

### *2.2 Charge-based peptide substrate selection*

All assays were performed in black 384-well plate and in 50 µL final volume using triplicates. CP-peptide binding tests were performed with PKA substrate peptides (CP1-3), by utilizing previously introduced $Eu^{3+}$-LZ reporter peptide in readout [17]. All used peptides are listed in Table 1. PKA assays were performed in

assay buffer containing 10 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.01% Triton, 5 mM NaCl, and 1 mM $MgCl_2$. In the CP-peptide binding test, 10 µL CP-peptide (10 nM or 100 nM) were incubated with 10 nM $Eu^{3+}$-LZ (20 µL) for 5 min. After signal monitoring, the MT2 modulator was added finalizing the 50 µL total volume. TRL-signals were monitored multiple times during 60 min incubation. After binding affinity comparison, peptides were used in enzymatic PKA assay, to define optimal charge and affinity for the peptides. First, single concentration inhibitor assay using H-89 (2 µM) was performed with PKA. Inhibitor was added in 2 µL volume, and thereafter 0.5 nM PKA (4 µL), and 10 nM CP-peptide together with 50 µM ATP (4 µL) were added. 10 µL enzyme reaction was incubated for 30 min, before addition of 20 µL $Eu^{3+}$-LZ (10 nM) and 20 µL MT2. TRL-signals were monitored multiple times during 60 min incubation.

*2.3 CP-peptide selection for different enzymes*

We used the assays performed with PKA-peptides as a basis in selection for PAD4 (citrullination) and HDAC3 (deacetylation) substrate peptides and conditions (Table 1). All citrullination related assays were carried out in a buffer containing 50 mM HEPES (pH 7.5), 25 mM NaCl, and 0.01% Triton X-100, and HDAC3 assays in buffer with 10 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.01% Triton, 25 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$. Optimization was carried out with commercially modified and non-modified peptides (Table 1), using similar protocol as with PKA peptides (CP1-3).

*2.4 Enzymatic PTM monitoring using CP-peptides*

The dose-response measurements for PKA, PIM1-3, PAD4, and HDAC3 were performed to validate the enzymatic assays. PKA assay was performed as previously described using H-89 (0-10 µM) and staurosporine (0-5 µM). PIM, PAD4, and HDAC3 assays were performed with minor modifications made for PKA protocol, and in their selected assay buffer. PIMs (2 nM) were assayed with the same buffer and CP1-peptide (10 nM) used also for PKA, using staurosporine (0-5 µM) as inhibitor. PAD4 (1 nM) assays were performed in the presence of 1 mM $CaCl_2$ added together with the enzyme. Enzyme reactions (10 µL) using CP4-peptide (10 nM) and GSK484 inhibitor (0-25 µM) were incubated for 45 min before addition of detection components. In the HDAC3 (0.5 nM) assays, inhibitor (TSA) was preincubated for 10 min with the enzyme, before substrate peptide (CP6) addition and further 30 min enzyme reaction.

*2.5 Charge based $Eu^{3+}$-peptide selection*

$Eu^{3+}$-peptide length and complexity needs were tested in PKA phosphorylation assay. Two shorter negatively charged non-leucine-zipper based $Eu^{3+}$-peptides, 15 aa $Eu^{3+}$-CPR1 and 27 aa $Eu^{3+}$-CPR2, were compared side-by-side with the 36 aa $Eu^{3+}$-LZ used in all other assays. Binding tests were performed with CP-substrates having different length and change (CP1-3) and using single fixed H-89 PKA inhibitor concentration (2 µM) in an enzymatic PKA assay.

*2.6 Data analysis*

In all assays, the signal-to-background ratio (S/B) was calculated as $\mu_{max}/\mu_{min}$ and coefficient of variation (CV%) as ($\sigma/\mu$) x 100. In all formulas $\mu$ is the mean value, and $\sigma$ is the standard deviation. Data were analyzed using Origin 8 software and the $IC_{50}$ (half maximal inhibitory concentration) values were obtained using standard sigmoidal fitting functions.

## 3. Results and discussion

*3.1 Charge-based assay platform enables the use of short nonstructural substrate peptides*

We have previously introduced the antibody-free peptide-break technology utilizing leucine-zipper (LZ) peptides [17]. The binding between LZ-peptides is based on coiled-coil interface created by leucine at *d*-position in the seven amino acid repeats. The strict demand of *d*-position leucine and conservation in coiled-coil structure, however, creates uncertainty to changes in binding upon amino acid sequence modification [21,22]. Thus the need to provide sufficient peptide-peptide binding and sufficient dissociative properties upon PTM addition might be demanding [17]. Here we utilize the homogeneous single-label peptide-break principle to HTS-compatible PTM detection using charge-based peptides (CP). Charge-based interactions are independent of leucine and the coiled-coil structure, and thus it was expected to be simpler to control (Fig. 1). Peptide-break technology utilizes single-label QRET signaling principle, in which the directly luminescent $Eu^{3+}$-chelate is attached to the detection peptide. In the assay, the differentiation between free peptides and peptide-duplex comes solely from the quencher/$Eu^{3+}$-chelate distance. Thus the technology is not restricted to certain PTM class, but only modification induced break in the peptide-duplex [24].

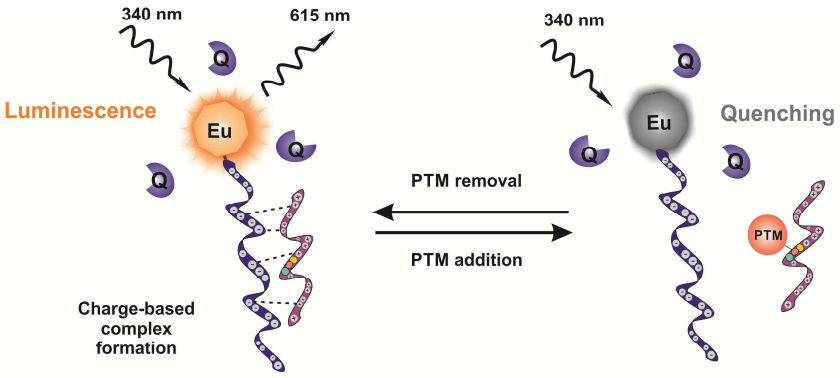


**Figure 1.** Principle of the charge-based peptide-break technology for PTMs. In the homogeneous dual-peptide platform, the $Eu^{3+}$-conjugated reporter interacts with high affinity to the substrate peptide carrying an enzymatic modification site. In the assay, high time-resolved luminescence is detected upon peptide-duplex formation and the dimer detachment upon modification promotes luminescence quenching in the presence of a soluble quencher molecule.

The advantage of the CP-peptides compared to LZ-peptides is their high freedom to tolerate modifications made in their sequence. This was expected to enable simple addition of new PTM sites and changes in peptide affinity without a fear of losing the duplex-structure. First, we demonstrate the functionality of the CP-peptide approach using three substrate peptides in varying length (19 to 34 aa) and charge (+10 to +23). All substrate peptides (CP1-3) contained the RRXS-sequence sequence applicable to PKA catalyzed serine phosphorylation (Table 1) [17,25]. The CP-peptide series was first characterized by their binding to the previously described negatively charged $Eu^{3+}$-LZ reporter peptide [17]. In the peptide-peptide binding test, the affinity of the duplex formation followed the order of the substrate peptide positive charge (Fig. 2a). Based on the duplex formation results, a net charge between +10 (CP1) and +13 (CP2) produced comparable affinity pair with $Eu^{3+}$-LZ, as Ser-LZ (35 aa) control peptide. Ser-LZ binding affinity has been previously adjusted to enable PKA activity monitoring, and thus we expected that the optimal affinity also with the CP-peptides is in the same range (Fig. 2a) [17]. The longest and most positive CP3-peptide showed ultra-high affinity in binding test compared to Ser-LZ. Based on this, CP3 is expected to be non-functional in the enzymatic assays where peptide separation after phosphorylation plays a key role.

**Table 1.** Peptides used to monitor PTMs. Modification sites of the substrate peptides highlighted as red.

| *Name* | *Type* | *Peptide function* | *Number of amino acid* | *Peptide charge*[1] | *PTM modification site* |
|---|---|---|---|---|---|
| **$Eu^{3+}$-LZ** | **LZ** | Detection | 36 | - | - |
| **$Eu^{3+}$-CPR1** | Charge | Detection | 15 | -12 | - |
| **$Eu^{3+}$-CPR2** | Charge | Detection | 27 | -23 | - |
| CP1 | Charge | Phosphorylation | 19 | +10 | GGRRRV**S**RRVRRRV**S**RRGG |
| CP2 | Charge | Phosphorylation | 21 | +13 | GGRRRV**S**RRVRRRV**S**RRVRRR |
| CP3 | Charge | Phosphorylation | 34 | +23 | GGRRRVRRRV**S**RRVRRR-VRRRVRRRV**S**RRVRRRK |
| CP4 | Charge | Citrullination | 17 | +10 | - SG**R**GK - |
| CP5 | Charge | Citrullinated | 17 | +8 | -SG$^{(CIT)}$**R**GK- |
| CP6 | Charge | Acetylated | 17 | +8 | -$^{(Ac)}$**K**GA- |
| CP7 | Charge | Acetylation | 17 | +10 | -**K**GA- |

[1]Net charge at pH 7.5

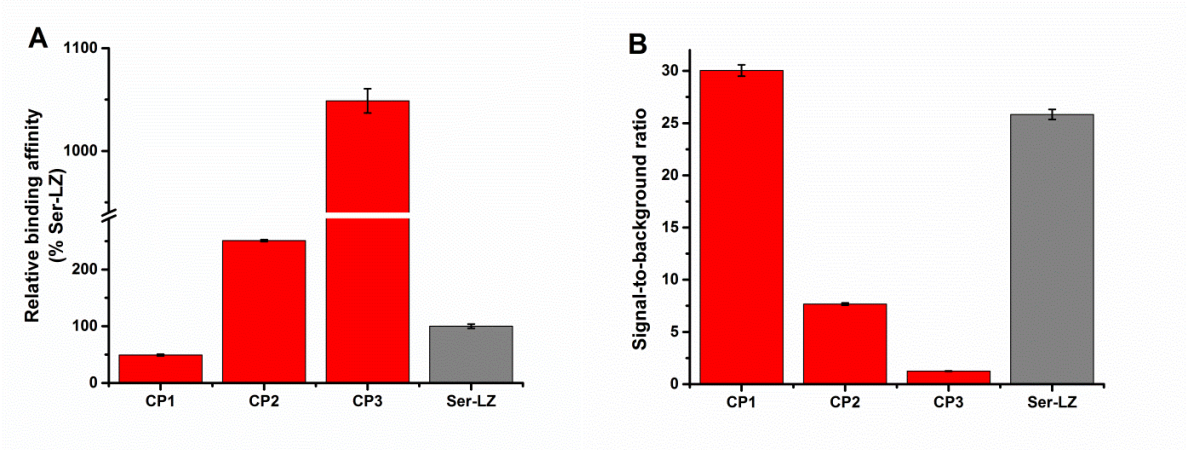


**Figure 2.** Binding and functionality properties of the CP-peptides compared to LZ-peptide control. (a) CP-peptide (10 nM) affinities were determined against $Eu^{3+}$-LZ (10 nM) and it compared to the previously described Ser-LZ (10 nM). CP-peptide affinities monitored were in order CP3 >> CP2 > Ser-LZ > CP1. (b) Single concentration H-89 inhibition assay (2 µM) with PKA (0.5 nM) and charge-based peptides (10 nM) was performed to obtain assay window between modified and non-modified peptide. The lowest binding affinity (CP1) provides highest separation after PTM addition. CP1 functionality was comparable to Ser-LZ control. Data represent mean ± SD (n=3).

Based on these binding tests and first PKA assays, we selected CP1 as a substrate for all forthcoming phosphorylation assays. To further validate the assay, inhibitor titrations were performed with H-89, and staurosporine. The $IC_{50}$ values for staurosporine and H-89 were 56 ± 4 nM and 230 ± 34 nM, respectively (Fig. 3a). These $IC_{50}$ values were in line with the ones reported earlier [17,26,27]. To address the assay functionality for other basophilic kinases than PKA, we performed staurosporine titrations also with the three PIM family kinases (PIM1-3). PIM inhibitors are attractive new targets and with high potential for cancer therapy, and similarly as PKA, PIMs target arginine-rich sequences in their substrates [28]. Therefore, CP1 was directly used for PIMs to demonstrate the suitability of a single substrate for a variety of enzymes, even the maximal enzyme activity may not be achieved [29,30]. In the PIM assays, staurosporine showed concentration dependent inhibition of PIM kinase activity (Fig. 3b). All PIMs showed nearly equal sensitivity for staurosporine with the $IC_{50}$ values between 183 nM and 295 nM, which are somewhat higher than the previously reported values [31]. This can be at least partly explained by the use of higher staurosporine competitive ATP concentration in the phosphorylation assay [31]. The calculated S/B ratios for PIM1-3 were between 8.4 and 10.8. This indicates that CP1-peptide can be applied to monitor *in vitro* enzyme activity without a need to acquire substrate sequences for all the enzymes separately.

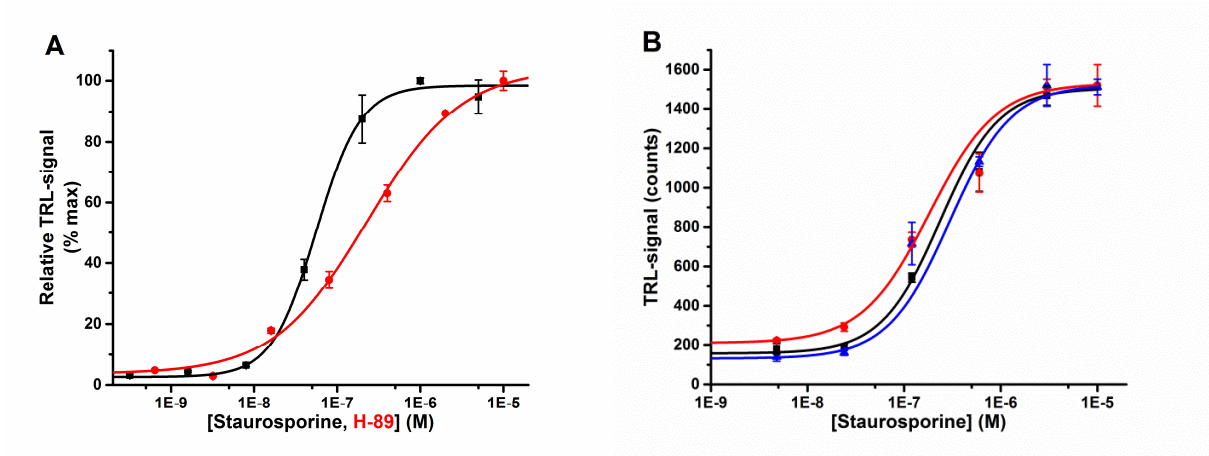


**Figure 3.** Inhibitor titrations with serine kinases, PKA and PIM1-3. (a) Staurosporine (black) and H-89 (red) titrations were performed with CP1-peptide (10 nM) and PKA (0.5 nM). Both inhibitors showed characteristic sigmoidal inhibition curves with nanomolar $IC_{50}$ values. (b) PIM1-3 (2 nM) were titrated with ATP competitive inhibitor (staurosporine), using PKA substrate peptide CP1. PIM 1 (black), PIM2 (red), and PIM3 (blue) showed similar staurosporine sensitivity. Data represent mean ± SD (n=3).

*3.2 Enzymatic citrullination and deacetylation can be monitored with CP-peptides*

Based on the PKA substrate optimization, we selected 17 aa CP-peptides for PAD4 (citrullination) and HDAC3 (deacetylation) assays (Table 1). Selection was made by introducing two modification sites for PAD4 and HDAC3 in the CP1-based sequence, keeping the charge and the length similar as in CP1. The substrate peptides were synthesized with and without the given modification, to perform binding studies before enzymatic reaction in a selected buffer condition suitable for the given enzyme. As shown in Figure 2a, S/B ratios monitored with $Eu^{3+}$-LZ and selected substrate were 9.4 (citrullination, CP4/CP5) and 10.2 (deacetylation, CP7/CP6), indicating applicability for enzymatic PTM monitoring (Fig. 4a).

Next, we studied the substrate peptides CP4 and CP6 in the corresponding enzymatic assays, citrullination and deacetylation. These assays were first performed with and without relevant inhibitor (GSK484 or TSA) at saturating concentration. The calculated S/B ratios monitored using PAD4 (1 nM) and HDAC3 (0.5 nM) were 7.8 and 6.6, respectively (Fig. 4a). These values are similar to the values obtained using synthetized peptides, confirming the assay functionality. Thereafter, enzyme activity inhibition with the CP4- and CP6-peptides in comparison with Arg-LZ and LysAc-LZ were studied. In the GSK484 inhibitor titration with PAD4 enzyme, CP4 and Arg-LZ peptides showed similar behavior with the monitored $IC_{50}$ values of 0.35 ± 0.02 µM and 1.14 ± 0.16 µM, respectively (Fig. 4b) [17,32]. Also the monitored S/B ratios with both peptides were highly similar (6.5-7.0). In the TSA inhibitor titration with HDAC, CP6 and LysAc-LZ showed equally similar behavior with the observed S/B ratios

6.5 and 8.0, respectively. Also the $IC_{50}$ values for CP6 and LysAc-LZ were comparable, 59 ± 2 nM and 152 ± 5 nM, respectively (Fig. 4b). Observed $IC_{50}$ values from both PAD4 and HDAC3 assays were similar to those reported earlier [17,32,33].

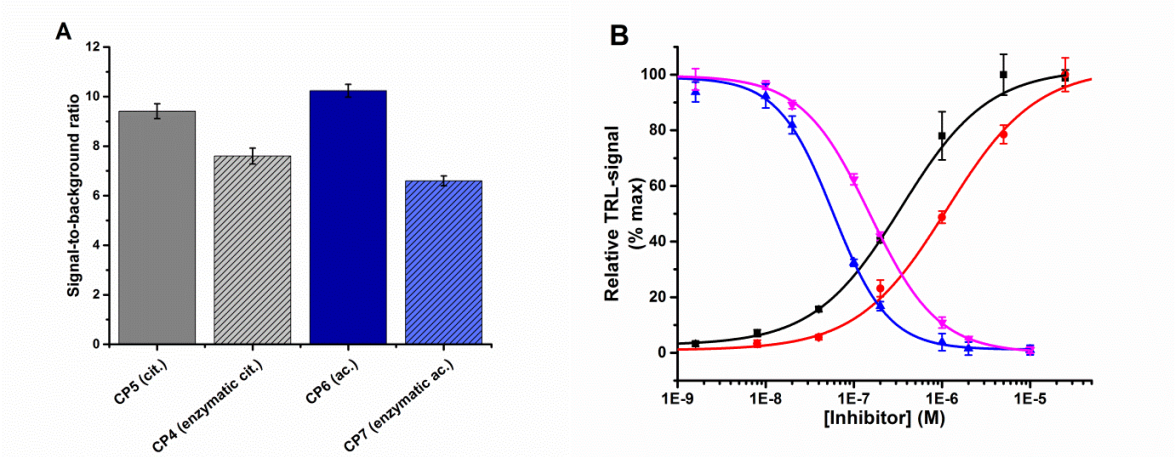


**Figure 4.** Binding and functionality properties of the citrullination and deacetylation peptides. (a) Citrullinated CP5- and acetylated CP6-peptides showed lowered binding to $Eu^{3+}$-LZ (10 nM) compared to their non-modified counter peptides CP4 and CP7, respectively. Enzymatically citrullinated CP4 (1 nM PAD4) and deacetylated CP6 (0.5 nM HDAC3) showed similar PTM dependency as their synthetically modified counter peptides. (b) GSK484 titration (0-25 µM) with PAD4 (1 nM) and TSA titration (0-10 µM) with HDAC3 (0.5 nM) were performed with CP- and LZ-peptides. Citrullination with the CP4 (black) and Arg-LZ (red) and deacetylation with CP6 (blue) and LysAc-LZ (magenta) gave characteristic sigmoidal inhibitor curves with similar $IC_{50}$ values, respectively. Data represent mean ± SD (n=3).

*3.3 $Eu^{3+}$-conjugated reporter peptide sequence can be shortened and selected only based on its charge*

All previous assays with PKA, PIMs, PAD4, and HDAC3 were performed with 36 aa $Eu^{3+}$-LZ and by modifying only the substrate peptides charge and length [17]. As the now used substrate peptides are short (17-19 aa), long $Eu^{3+}$-LZ peptide which was needed for LZ formation is not anymore obligatory. Sometimes, it may also be more convenient to modify the $Eu^{3+}$-peptide. This was demonstrated with two shorter negatively-charged $Eu^{3+}$-peptides, 15 aa ($Eu^{3+}$-CPR1) and 27 aa ($Eu^{3+}$-CPR2). These reporters were first tested with PKA substrates CP1-3 to confirm the peptide-peptide interaction. With the long $Eu^{3+}$-CPR2, we did not observe any change in peptide-peptide binding affinity within the CP1 to CP3 series (data not shown). With all tested CP-peptides the observed $Eu^{3+}$-CPR2 binding affinity was very high, which indicates that $Eu^{3+}$-CPR2 is not suitable for PTM monitoring. This was already expected based on the previous observations with $Eu^{3+}$-LZ and CP3, showing high affinity and low PTM induced separation (Fig. 2). With 15 aa $Eu^{3+}$-CPR1, trend in binding to CP1-3 was similar to what was previously monitored with 36 aa $Eu^{3+}$-LZ (Fig. 2a and Fig. 5a). Both $Eu^{3+}$-CPR1 and $Eu^{3+}$-LZ showed increasing

affinity from CP1 to CP3 responding to increasing positive charge of the substrate peptide. However, the response with $Eu^{3+}$-LZ was much steeper as with $Eu^{3+}$-CPR1 the change was modest. This difference relates directly to the $Eu^{3+}$-peptide length. The short $Eu^{3+}$-CPR1 cannot benefit from the increased charge of the CP-peptide with longer sequence than 15 aa, as $Eu^{3+}$-LZ has no such length related restrictions. These observation were further confirmed with synthetically acetylated and non-acetylated peptides, which showed PTM-dependent separation with $Eu^{3+}$-CPR1 but not with $Eu^{3+}$-CPR2 (data not shown).

Based on these observations, we selected the short $Eu^{3+}$-CPR1 for the enzymatic PKA assays with CP1-3. In the PKA assay using DMSO or 2 µM PKA inhibitor (H-89), CP1 gave the best S/B ratio of 8.9. This was already expected based on the binding data where $Eu^{3+}$-CPR1 and $Eu^{3+}$-LZ showed nearly equal affinity to CP1 (Fig. 5b). CP2 showed decreased but measurable separation between phosphorylated and non-phosphorylated peptides (S/B 3.6), as the CP3 showed no PTM dependent separation (Fig. 5). The data demonstrate that with the charged peptides, length of the used peptides can be significantly reduced without losing the affinity or assay window between modified and non-modified peptides. However, one must keep in mind the distribution of the charged amino acids, especially if reporter and substrate peptides length differs significantly. The data clearly highlights that the charge-based peptides can be easily modified for different enzymatic targets without considering structural but only charge related features of the used peptides.

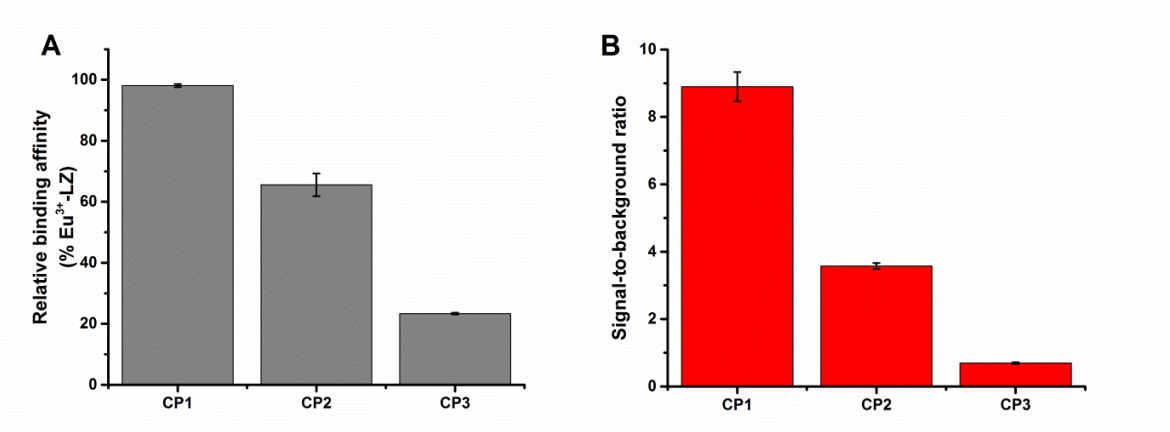


**Figure 5.** Binding and functionality of $Eu^{3+}$-CPR1 with different CP-peptides. (a) The affinity of different CP-substrates to the $Eu^{3+}$-CPR1 (10 nM) was determined and compared against $Eu^{3+}$-LZ (10 nM). CP1 showed equal binding to both $Eu^{3+}$-peptides, while the longer CP2- and CP3-peptides preferred the longer $Eu^{3+}$-LZ over $Eu^{3+}$-CPR1. (b) Assay window with $Eu^{3+}$-CPR1 was monitored with or without H-89 (2 µM) in an enzymatic PKA (0.5 nM) assay. Results indicate preference for the CP1 substrate over longer more positively charged CP2 and CP3. Data represent mean ± SD (n=3).

Lack of simple and robust HTS-compatible assay systems for the monitoring of multiple different PTMs has slowed advancements in the PTM targeted drug discovery. Previously, we introduced a new homogeneous peptide-break technology for the monitoring of different PTMs [17]. Now we introduce a continuum for the peptide-break technology using short charged peptides. The new peptide architecture enables more freedom in peptide design compared to structure dependent LZ-peptides. Charged peptides also allow simple assays extrapolation from one PTM to another. We highlighted this by using phosphorylation peptides as a basis of the study, and further modifying the substrate to allow citrullination and deacetylation monitoring. This change was made by simply introducing a new consensus sequence for the enzyme, but without any other change in substrate sequence or $Eu^{3+}$-reporter. We also showed that the peptide affinity adjustment with the CP-peptides is simple, and can be done by changing the number of charged residues and their distribution. Compared to the LZ-peptides, now the charged amino acids directly change the affinity of the CP-peptide-duplex, without the possible unwanted effect to the duplex structure. LZ-peptides are also less robust regarding the PTM position, thus not only leucine in *d* position but also amino acids in positions *a* and *g* affect to the leucine-zipper interaction core [22,23]. Homodimerization, which might occur with LZ-peptides, is also non-existing with CP-peptides due to repulsion, providing a simple signal interpretation and peptide modification [22,34-36].

## 4. Conclusions

In this work, we have highlighted the functionality of the homogeneous HTS-compatible peptide-break PTM-detection platform using charged peptides. Our aim was to simplify and expand the functionality of the peptide-breaktechnology, and provide a higher freedom for end-users to select the wanted substrate peptide. Compared to the previously presented LZ-peptide approach, change from one PTM class to another, can now be simply done by introducing the new PTM site. The detection platform is potentially applicable to large variety of PTMs, monitoring solely PTM on the desired peptide without interferences from e.g. autophosphorylation. Moreover, a high sensitivity is obtained using the luminescent lanthanide labels and time-gated measurement. This enables the use of sub or low sub nanomolar enzyme concentration in a HTS compatible format. We have now proved the concept functionality with purified enzymes and artificial substrate peptides, and it remains to be seen whether natural, charged peptides can be directly used as a substrates utilizing this technique.

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**Competing financial interests**

The authors declare the following competing financial interest(s): Kari Kopra and Harri Härmä have commercial interest through QRET Technologies Ltd.

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**Author Contributions**

⊥These authors contributed equally to this work.

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**Highlights:**

- Antibody-free strategy for the detection of protein post-translational modifications (PTMs)
- Charge-based peptides enable simple assay design and re-editing for different PTMs
- High throughput compatible method with nanomolar sensitivity for variety of PTMs
- Time-gated single-label luminescence monitoring enables low background and high S/B ratio