UNIVERSITY OF TURKU

THE PEPTIDE-BREAK TECHNOLOGY

A Novel Approach for Protein Post-translational Modification Assays in Drug Discovery

Natalia Tong-Ochoa

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In all affairs it is a healthy thing now and then to hang a question mark
on the things you have long taken for granted.
- *Bertrand Russell (1872-1970)*

TURUN YLIOPISTO
Luonnontieteiden ja tekniikan tiedekunta
Kemian laitos
Natalia Tong-Ochoa: Peptide-break teknologia: Uusi lääkeseulontaan soveltuva menetelmä proteiinien jälkitranslationaalisten modifikaatioiden havainnointiin
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Tiivistelmä

Tämän tutkimuksen tavoitteena oli kehittää universaali biokemiallinen ja ilman vasta-aineita toteutettu määritysalusta proteiinien jälkitranslaationaalisten modifikaatioiden (PTM) havainnointiin. PTM-reaktion havainnoinnissa käytettiin sammutus-resonanssi-energiansiirtoa (QRET), joka perustuu sammuttajamolekyylin kykyyn erotella kohteeseen sitoutunut ja sitoutumaton lantanidikelaatin sisältämä reportterimolekyyli ja luoda tähän perustuva luminesenssi-signaalin muutos.

Ensimmäisenä QRET teknologian soveltuvuus PTM havainnointiin osoitettiin käyttäen lyhyttä repotteripeptidiä ja leimaamatonta vasta-ainetta, joka tunnisti epidermaalisen kasvutekijäreseptorin (EGFR) luoman fosfotyrosiinin. Universaalin määritysalustan luomiseksi, PTM havainnointiin kehitettiin vasta-aineista riippumaton ”peptide-break” teknologia, joka perustuu luontaisiin kahden peptidin muodostamiin leusiinivetoketju-rakenteisiin. Perustilassaan sitoutuneet muokkaamattomat peptidit tuottavat korkean luminesenssi-signaalin, mutta aktiivisen entsyymin ollessa läsnä, muokkaamattoman reportteripeptidin ja entsyymin muokkaaman vasta-peptidin sitoutuminen estyy ja luminesenssi-signaali laskee. Menetelmän toiminnallisuus ja nanomolaarinen herkkyys havainnollistettiin lukuisien eri entsyymien ja entsyymiryhmien kanssa.

Tämän jälkeen peptide-break teknologian toiminnallisuus ja monipuolisuus osoitettiin käyttämällä varauksellisia peptidejä leusiinivetoketju-peptidien sijasta. Näin helpotettiin peptidien suunnittelua, tinkimättä menetelmän suorituskyvystä. Lisäksi peptide-break teknologiasta kehitettiin lämpötilaan perustuva muunnos, jossa nostamalla entsymaattisesti muokattavan peptidin määrää kyettiin havainnoimaan huonon toiminnallisuuden omaavia entsyymejä. Tämän tutkimuksen perusteella, peptide-break teknologia soveltuu lukuisten PTM-ryhmien havainnointiin ja luo uudentyyppisen lähestymistavan, joka on hyödynnettävissä niin akateemisesti kuin teollisuudessakin.

Avainsanat: sammutusenergiansiirto (QRET), tehoseulonta (HTS), peptide-break teknologia, jälkitranslationaaliset modifikaatiot (PTM)

UNIVERSITY OF TURKU
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Abstract

The work presented in this thesis describes the development of a universal platform for the biochemical detection of protein post-translational modifications (PTM) using the quenching resonance energy transfer (QRET) technique in an antibody-free system. The QRET technique employed is based on the difference in the luminescence signal produced by the target bound and unbound to a Ln^{3+}-ligand in the presence of soluble quenchers.

In the project, PTM monitoring was first demonstrated in a simple QRET tyrosine EGFR assay using antibodies to recognize the modified substrate peptide, a commonality in FRET assays, yet in a single-label approach. On the basis of this, a universal PTM detection method called "the peptide-break technology" was further developed as an antibody-free system. The approach relied on the peptide dimerization concept of leucine zipper (LZ) coiled-coils. The dimer was composed of an enzyme-substrate peptide and a detection Eu^{3+}-peptide. In the assay, the PTM addition to the substrate peptide disrupted the dimer, leading to a low luminescence signal, whereas without PTM addition the dimer formation proceeds and provides Eu^{3+}-chelate protection leading to a high luminescence. The peptide-break technology was successfully developed for a variety of enzymes for phosphorylation, dephosphorylation, deacetylation, and citrullination, using nanomolar concentrations without the need for antibodies or enzyme reporters.

Later, the peptide-break technology was simplified and optimized to increase the freedom for substrate peptide selection and design. The peptide dimerization was mediated by the interaction between oppositely charged peptides. Finally, the technology was further studied in a thermal shift assay, allowing the use of high substrate peptide concentrations that are needed in assays with low affinity or activity enzymes. The reported data suggest that this technology is potentially applicable to other challenging PTM types, possibly providing new assay solutions within academia as well as for the pharmaceutical industry.

Keywords: quenching resonance energy transfer (QRET), high throughput screening (HTS), the peptide-break technology, post-translational modification (PTM)

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Natalia Tong-Ochoa

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II Härmä, H., Tong-Ochoa, N., Van Adrichem, AJ., Jelesarov, I., Wennerberg, and Kopra, K. (2018). Toward universal protein post-translational modification detection in high throughput format. *Chem Commun* 54: 2910-2913

III Kopra, K., Tong-Ochoa, N., Laine, M., Eskonen, V., Koskinen, P., and Härmä, H. (2018). Homogeneous peptide-break assay for luminescent detection of enzymatic protein post-translational modification activity utilizing charged peptides. *Anal Chim Acta* doi:10.1016/j.aca.2018.12.041

IV Eskonen, V., Tong-Ochoa, N., Kopra, K., and Härmä, H. (2018). Thermal dissociation assay for time-resolved luminescence detection of peptide post-translational modifications (*Submitted manuscript*)

1 INTRODUCTION

Protein post-translational modifications (PTMs) are essential regulatory mechanisms involved in cellular signal transduction processes and are catalyzed by a vast number of enzymes. Alterations in protein PTMs are reflected in deregulation of cellular processes, e.g. cell proliferation, gene expression, protein-protein interaction and apoptosis, which can result in different pathological conditions. In this regard, the targeting of deficient enzymes to treat distinct diseases has long been a primary goal in the drug discovery field.

The discovery and development process of a drug can take approximately 15 years with an investment leastways of $ 1 billion (Hughes *et al.* 2011). To expedite this process, a considerable effort is focused on the improvement of technology to produce data of sufficient quantity and quality. The purpose of high throughput screening (HTS) assays during the hit discovery process is to diminish the need for repetitive manual tasks, and remove the potential errors, with a significant time thrift. Nowadays, more than 100 000 compounds can be tested per day using a conventional HTS assay (Schneider 2018).

A variety of sensitive HTS assays have been applied to measure enzyme activity using traditionally radiometric methods. Currently, the tendency is for the use of luminescence-based systems, as they provide a method that overcome the major problems with radiometric assays related to the shelf life of radioligands and radioactive waste. These luminescence systems include dual and single label assays, with a variety of distinct labels, including organic dyes, biological fluorophores and lanthanide probes. Most of the commercially available methods, either radiometric or luminometric systems, rely on the use of specific antibodies as a detection component. The need for specific antibodies is typically the limiting factor for these assays as there is an insufficient range available to target the whole PTM spectrum (Li *et al.* 2008). Antibody-free systems, based on metal ion coordination, have been extensively developed for phosphorylation (Loomans *et al.* 2003; Shults & Imperiali 2003). However, the nature of this approach has limited its expandability to other PTM types. New complex targets for drug discovery are constantly emerging, suggesting new versatile HTS technologies are also in demand. Sensitive, cost-effective and adaptable HTS methods would provide new assay solutions to the scientific community within academia as well as the pharmaceutical industry.

2 REVIEW OF LITERATURE

A detection technology which provides a method for assaying a wide variety of PTM types in a single platform is referred to here as "universal technology." A universal biochemical PTM enzymatic assay for inhibitor or activator screening would use a generic detection component for PTM enzyme activity monitoring. In a luminescence-based strategy, the universal variable of the assay, i.e., the detection component, has to carry the reporter label, as substrate peptides will differ from enzyme to enzyme due to their specificity. Several approaches have been proposed following this design, but their development for universal applicability has been challenging. In this review of the literature, universal and target-specific detection technologies that have been applied to PTM enzymatic HTS assays are presented together with a brief introduction of protein PTMs and the enzymes involved. Also, a brief introduction about enzymes as therapeutic targets for inhibition or activation in different disease treatments is presented.

2.1 Protein post-translational modifications

Our human gene set has been estimated at approximately 25,000 genes (International Human Genome Sequencing Consortium 2004), while the size of the human proteome comprises over 1 million proteoforms (Jensen 2004). This diversity is gained after mRNA alternative splicing and is enriched by the PTMs increasing also the range of protein function. Protein PTMs are covalent additions or proteolytic cleavage events which represent a significant regulatory mechanism of the proteome. The modification of single or multiple amino acids defines protein function, localization, interaction, turnover, and the tertiary and quaternary structures. The occurrence of these events rely on the spatial orientation and surrounding of the amino acid (AA), leading to the specificity and selectivity of the protein (in a time and signal dependent manner) (Hirano *et al.* 2016). There are more than 300 types of PTMs that yield the highly complex variety of protein forms (proteoforms) and diversify protein function (The UniProt Consortium 2015; Kim *et al.* 2016). Statistical analysis of PTM frequency from the Swiss-Prot

database indicates that phosphorylation, acetylation, and glycosylation are the three most abundant experimentally identified PTMs (Khoury *et al.* 2014).

A protein can be modified by more than one PTM type or by the same PTM at different residues. Since PTMs affect the molecular mass of proteins, technologies such as mass spectrometry (MS) can provide information regarding identification, location and quantity of PTMs (Kim *et al.* 2006; Aebersold & Mann 2016). The expression dynamics of this assortment of PTMs (**Figure 1**) is catalyzed by enzymes. These are called 'writers' when they add a certain PTM, such as kinases, or 'erasers' if they remove, such as phosphatases (Seet *et al.* 2006; Lim & Pawson 2010). Additionally, protein domains called 'readers' can modulate signaling through PTMs by recognizing them and trigger a cellular response. There is an active interplay between writers, erasers and readers that regulates a variety of cellular processes. In this sense, the understanding of this interplay is the basis for addressing the deregulated biological processes that underline different diseases. In the following section, the most common PTMs are briefly described including the modular recognition domains and catalytical enzymes.

Phosphorylation is a process that catalyzes the transfer of a phosphate group from adenosine triphosphate (ATP) to the acceptor residue, serine, threonine, tyrosine (mainly in eukaryotes) and histidine or aspartate (mainly in prokaryotes), generating adenosine diphosphate (ADP) (Deribe *et al.* 2010). Phosphorylation is the most studied PTM and was one of the first to be described (Hunter & Cooper 1985; Pawson 2002). This reversible PTM is regulated by kinases and phosphatases, and due to its rapid and transient nature, acts as the primary response to stimuli within a cellular process enhancing or depleting enzyme activity (Audagnotto & Dal Peraro 2017).

Every time a phosphoryl group is added to a specific AA residue, a -2 negative charge is added to the modified protein, altering its structure and electrostatic properties (Deribe *et al.* 2010). This characteristic of phosphorylation enables modulation of the molecular interactions, either facilitating protein-protein interactions (PPIs) (Langeberg & Scott 2015) or dissociation (Rubin *et al.* 2005). The phosphorylated residues mediating PPIs are recognized by certain modular domains such as the Src Homology 2 (SH2). This domain represents the largest class of domains and allows all the SH2-containing proteins to localize phosphorylated tyrosine (pTyr) sites and trigger different cellular responses (Flynn 2001; Beltrao *et al.* 2013). The specificity of these PPIs derives from the ability of the SH2 domains to recognize additional residues surrounding the pTyr of the binding site. Structural studies have reported that this specificity can be mediated by secondary binding sites even located outside the pTyr binding pocket (Deribe *et al.* 2010).

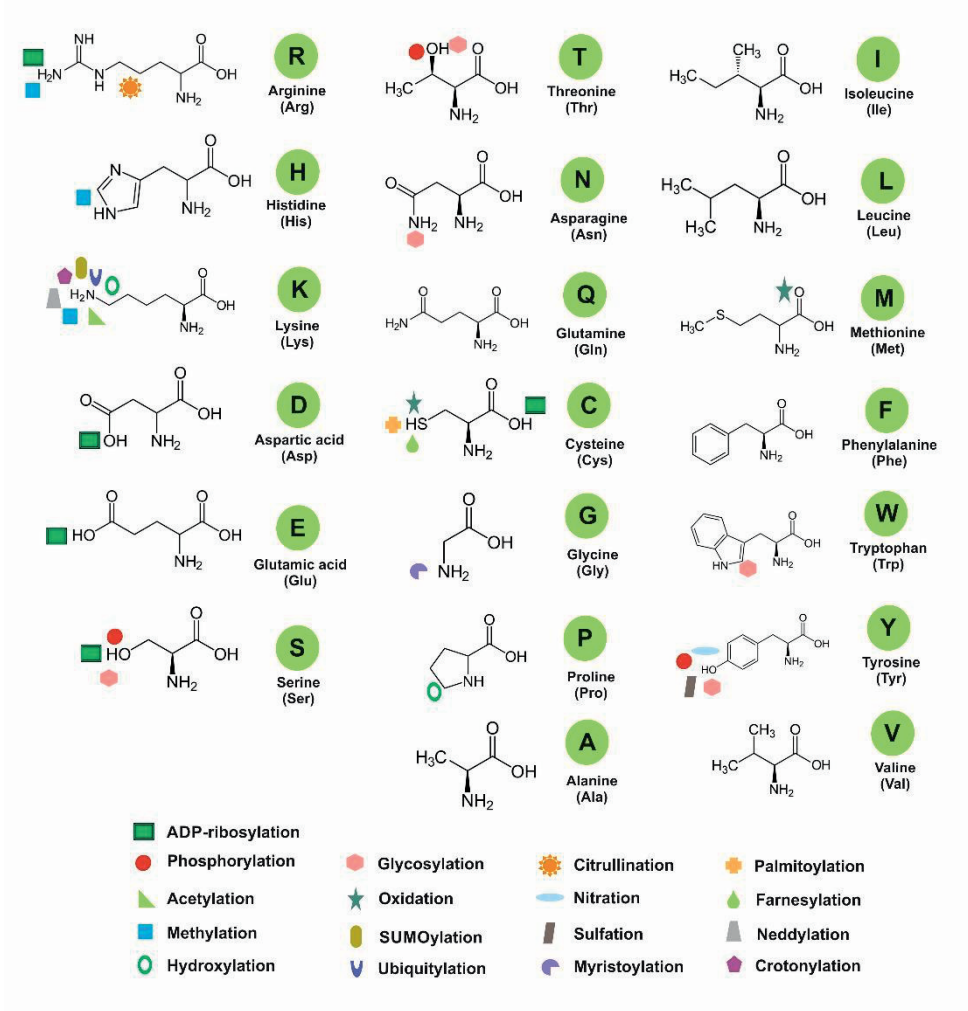

Figure 1. The most common enzymatic post-translational modifications (PTMs) in eukaryotes and their amino acid targets. The chemical structures of the 20 amino acids together with their chemical symbol, and the most important PTMs occurring at each residue are depicted.

Glycosylation is one of the more complex PTM processes, and involves the transfer of a glycosyl group from a substrate donor to a protein. Based on the type of linkage between the sugar and AA, it is classified in four categories: O-glycosylation (the sugar is attached with the hydroxyl group of a serine, threonine or tyrosine), N-glycosylation (the sugar is attached with the amino group of an asparagine), glycosylphosphatidylinositol (GPI) anchors (a GPI is attached to the C-terminal of a protein chain), and C-mannosylation (the sugar moiety is attached to a carbon on a tryptophan) (Moremen *et al.* 2012; Krasnova & Wong 2016;

Audagnotto & Dal Peraro 2017). The attached glycan (many sugars) can be highly complex with multiple different combinations of glycan branching, providing a substantial structural diversity (Moremen *et al.* 2012). The main purpose of this glycan diversity is to alter protein thermodynamic and kinetic properties in distinct manners to regulate protein stability, aggregation, cell surface interactions, enzyme activity and protein trafficking (Hanson *et al.* 2009; Sun *et al.* 2016; Audagnotto & Dal Peraro 2017).

Acetylation is dynamic PTM that transfers acetyl groups using acetyl coenzyme A (acetyl CoA) to the ε-amino group of lysines. Another type of acetylation corresponds to the N-terminal (Nt) modification, which occurs at the N-termini of the nascent polypeptide chains and proteins (Drazic *et al.* 2016). Lysine acetylation is considered as a reversible PTM, and is catalyzed by lysine acetyltransferases (KATs) and regulated by lysine deacetyltransferases (KDACs). Whereas Nt-acetylation is irreversible and catalyzed by Nt-acetyltransferases (NATs).

It has been widely reported that lysine acetylation plays an important role in chromatin structure regulation and its transcriptional activity (Xu *et al.* 2014). The effect of acetylation on chromatin structure modulation may be due to an electrostatic mechanism, since the addition of an acetyl group neutralizes the positive lysine charge (Martin & Zhang 2005). Besides the effects of writers and erasers modulating cellular functions, modular domains, such as the bromodomain reader, a part of certain transcription factors and KATs, can bind to acetylated lysine and build large transcriptional and chromatin-remodeling complexes (Seo & Lee 2004; Filippakopoulos & Knapp 2012).

Crosstalk between PTMs. Coordination between PTMs is essential for the regulation of different cellular pathways (Beltrao *et al.* 2013). PTM crosstalk can create a "code" on the protein surface that is recognized by specific domains leading to the activation or inhibition of downstream events (Lothrop *et al.* 2013). A certain PTM code delivers or retains the signal only if the surrounding input is present at the same time and place (Yang & Seto 2008; Venne *et al.* 2014; Yang & Qian 2017). PTM crosstalk has been classified into positive and negative events. In the positive form, the initial PTM actively triggers either the second PTM or serves as an interaction site for other proteins (Hunter 2007; Venne *et al.* 2014). In the negative form, there is direct competition between two PTMs for the same AA or an indirect effect when a specific PTM blocks the recognition of the second PTM, thus avoiding its addition or removal (Hunter 2007; Beltrao *et al.* 2013). The importance of the interplay between different PTMs relies on the efficient coordination between the PTMs, which allow signal translation into functional cellular processes. The most common enzymatic PTMs are summarized in **Table 1**.

Table 1. Most common enzymatic PTMs in eukaryotes and their characteristics and functions.

PTM type	Change in mass (Da)	Target amino acid (s)	Common motifs	Function
Phosphorylation	80	Y/S/T	> 320 possibilities	Signal transduction, enzyme activity regulation, PPIs
N-Glycosylation	>800	N	NxS/T[ε]	Protein folding, stability, cellular interaction
O-Glycosylation	203, >800[*]	S/T/Y	α	
Acetylation	42	K	GK, KP	Protein stability, PPIs
Methylation	14	K/R/H[±]	MK, RGG	Transcription, protein-nucleic acid interactions
Ubiquitylation	>1000	K	varies	Protein degradation
SUMOylation	12,000	K	ΨKxD/E[μ]	Subcellular transport
Neddylation	9072	K	varies	Protein stability
Myristoylation	210	G	MGxxxS/T[β]	Protein localization, PPIs, protein-membrane interactions
Farnesylation	204	C	CAAx[Θ]	
Palmitoylation	238	C	varies	
Sulfation	80	Y	N.D	Signalling, protein localization, PPIs
Citrullination	<1	R	GxRGΨ	Protein folding
Oxidation	16	C/M	C/S/TxxC	Protein oxidative regulation
Nitration	45	Y	N.D	Oxidative damage
Hydroxylation	16	K/P	N.D	Structural stability (collagens)
Crotonylation	68	K	N.D	Protein structure

References provided in the text except where noted. [*]A single sugar: 203 or a chain: >800. [ε]x cannot be P (Moremen *et al.* 2012). [α]It varies according to the enzyme, with P as important AA residue (Moremen *et al.* 2012). [±] (Clarke 2013) Ψ, a hydrophobic residue. [μ]x, any AA (Liddy *et al.* 2013). [Θ]CAAX: C, Cys; A, an aliphatic AA; x, any AA (Cho & Park 2016). [β]Glycine in the N-terminal MGXXXS/T: M, Met; G, Gly; S, Ser; T, Thr; x, any AA (Cho & Park 2016). N.D, not determined (Jensen 2006; Kim *et al.* 2016).

2.1.1 The role of protein PTMs

In this section, phosphorylation cascades and crosstalk between distinct PTMs are presented to recognize how their interplay affects such functions as protein activity, cellular location, and protein interaction. As an example, the downstream signaling of the epidermal growth factor receptor (EGFR) kinase is briefly described from its initiation till its termination (**Figure 2**).

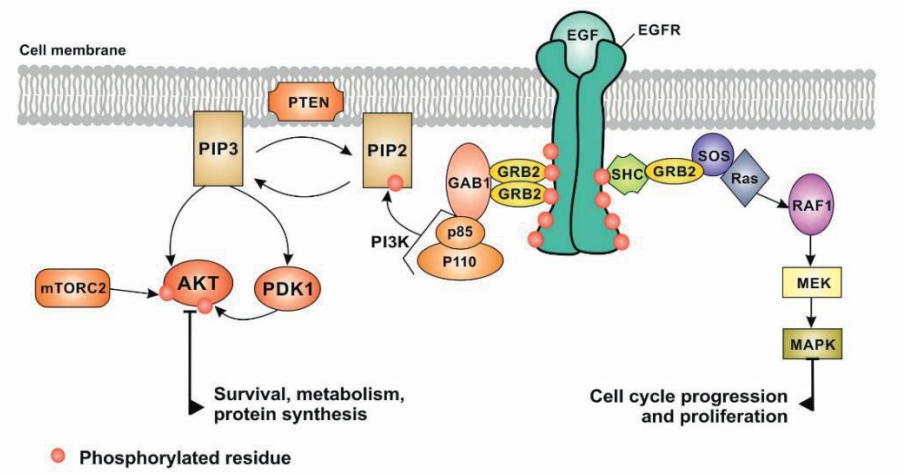

Figure 2. A simplified representation of the downstream signaling of the epidermal growth factor receptor (EGFR) kinase. After EGF binding to EGFR kinase, several phosphorylation events occur to mediate activation of several other protein kinases including AKT and MAPK. This activation drives cellular processes such as survival, protein synthesis, and proliferation.

Several signaling pathways between the membrane and nucleus are activated by external stimuli upon the receptor tyrosine kinases (RTKs). This signal transmission can be attributed to the RTKs activity and the scaffolding and docking function of the receptors (Li & Hristova 2006). EGFR kinase is a receptor activated by epidermal growth factor (EGF) ligand binding, which results in autophosphorylation of tyrosine residues on the cytoplasmic receptor portion of EGFR (Wells 1999; Seo & Lee 2004; Kolch & Pitt 2010). Modification of the catalytic domain activates and potentiates the receptor functionality, while the modified non-catalytic domain builds docking sites for downstream targets, which bind to specific phosphotyrosine residues on the receptor, e.g. SH2 (Ge *et al.* 2002; Ferguson 2004; Seo & Lee 2004). For instance, active EGFR can recruit SH2-containing proteins such as the growth factor receptor-bound protein 2 (Grb2) adaptor. This in turn recruits the guanidine nucleotide exchange factor (GEF) SOS, which stimulates a series of events that lead to the initiation of the downstream signaling pathway of mitogen-activated protein kinase (MAPK) for cell cycle progression and proliferation (**Figure 2**) (Deribe *et al.* 2010). Besides protein recruitment, active EGFR can also attract enzymes, such as the phosphatidylinositol 3-kinase (PI3K). This recruitment activates PI3K, which phosphorylates membrane phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP2) becoming later in phosphatidylinositol (3,4,5)-trisphosphate (PIP3). This phosphorylation generates docking sites on PIP3 for the enzymes phosphoinositide-dependent kinase-1 (PDK1) and protein kinase B (PKB), also

known as AKT (**Figure 2**) (Deribe *et al.* 2010; Rodon *et al.* 2013; Dienstmann *et al.* 2014). The result of this series of phosphorylation-mediated activation is the regulation of several cellular processes, including cell survival, metabolism, and protein synthesis (Rodon *et al.* 2013).

Active EGFR and in general other RTKs are regulated by the correct amount of signal they have to deliver upon a stimulus. Inhibition of the EGFR catalytic activity is regulated by serine and threonine phosphorylation of its juxtamembrane domain exerted by the protein kinase C (PKC) (Chen *et al.* 1996). Moreover, signal attenuation is controlled by protein tyrosine phosphatases (PTPs), which dephosphorylate activated RTKs and downstream effectors (Tonks 2003). The main regulator of EGFR is rapid endocytosis and degradation of the EGF-EGFR complex. This involves EGFR ubiquitylation, which triggers the receptor internalization to early endosomes and future degradation (Deribe *et al.* 2010; Savio *et al.* 2016). Additionally, α-tubulin acetylation can control the velocity of the early endosomes motility, while deacetylation of the Lys40 of α-tubulin slows the EGFR degradation (Deribe *et al.* 2010). The interplay between distinct PTMs along a protein lifespan is determinant in the regulation of different cellular processes and is the foundation of the biological mechanisms underlying different diseases.

2.1.2 PTMs impact in health and disease

As Steffen *et al*. explained carefully in their review (Steffen *et al.* 2016), despite that the protein lifespan is strictly controlled by enzymes catalyzing almost all conversions with a defined activity, a perturbation is always possible. This perturbation can alter the quantity or quality of an enzyme which is in most of the cases linked to a pathological condition (Steffen *et al.* 2016). In the next section, perturbations in different stages of the protein lifespan and its association with some diseases are briefly reviewed.

Protein synthesis, 10–50% of protein synthesis are estimated to result in defective ribosomal products due to incorrect coding or transfer, or both, within the ribosomal complex (Steffen *et al.* 2016). The quality of protein synthesis is controlled by different cellular systems located in the cytosol, endoplasmic reticulum (ER), mitochondria and nucleus. These systems are comprised of chaperones, which mediate the folding of the newly synthesized and refolding of the damaged proteins, and enzymes of the ubiquitin system, which identify and ubiquitylate misfolded proteins and prepare them for degradation (Steffen *et al.* 2016). Accumulation of misfolded proteins causes cytotoxic effects in the cell,

which has been linked to the starting point of neurodegenerative diseases such as Alzheimer's and Parkinson's (Thommen *et al.* 2017).

Proteolytic processing of a protein is considered as an irreversible PTM which involves the cleavage of peptide bonds in a protein by enzymes called proteases. This can occurs several times during a protein lifespan and affects the activity of the protein target. For instance, during the maturation process of the transmembrane protein β-amyloid precursor protein (APP), a protein expressed at high concentration in neurons, three proteases enable protein maturation by converting the APP to the amyloid β-peptide (Aβ) (Haass *et al.* 2012). An error in this dynamic proteolytic event can cause precipitation of Aβ leading to diseases such as Alzheimer's, which in this particular case is considered the major cause of the disease (Haass *et al.* 2012). To date, there is a lack of efficacy of the existing Aβ-targeted therapies for Alzheimer disease, so interest has been switched to Tau protein as a potential alternative target (Li & Götz 2017).

Addition or removal of PTMs. As phosphorylation is the primary response of most of the cellular pathways, its dysregulation is observable in most human diseases. As an example, more than 150 kinases have been targeted for cancer anti-cancer drug development (Fleuren *et al.* 2016; Oliveira *et al.* 2018). For instance, mutation of upstream receptors such as EGFR, and downstream mediators such as phosphatase and tensin homolog (PTEN) have been reported especially in lung cancer (Rodon *et al.* 2013). Targeting drugs for specific kinases has been challenging since in most pathways there is usually a feedback loop that recovers the inhibited function upon drug exposure. Dual inhibitors can block alternative responses by targeting a pathway at two levels providing an advantage over specific inhibitors. Lately, effort has been focused on targeting not only enzymes from the same family, but also from different ones. For example, the CUDC-101 inhibitor, a developing drug for solid tumors, which blocks histone deacetylase (HDAC), EGFR, and human epidermal growth factor receptor 2 (HER2) enzymes (Falkenberg & Johnstone 2014). The development of dual inhibitors has been challenging due to the complex cellular networks that each protein represents. However, such cases as the CUDC-101 inhibitor have been able to reach the clinical phase, giving promising results in the treatment of patients with squamous cell carcinoma, when used in combination with chemoradiation (Falkenberg & Johnstone 2014; Galloway *et al.* 2015).

The formation of protein complexes is mediated by a variety of PTMs that modulate the binding affinities by altering electrostatic or structural properties of the protein interaction sites (Seet *et al.* 2006). It has been reported that more than

60% of PTM sites participate in PPIs, modulating interaction and hence function (Duan & Walther 2015). Drug molecules targeting pathological PPIs are at the beginning of development. Despite the challenge of using small molecules for blocking the wide areas often associated with PPIs, the JQ1 inhibitor has been reported to disrupt the interaction between the bromodomain and acetylated lysine (Verdin & Ott 2015). JQ1 inhibitor has been initially tested in squamous cell carcinoma and has also been found to function as an immunosuppressant, suggesting other possibilities beyond cancer for this drug class (Verdin & Ott 2015; Zaware & Zhou 2017).

Protein degradation plays an essential role in the regulation of protein abundance, and as indicated earlier is a control system for misfolded proteins. Ubiquitylation is a key PTM signal that delivers receptors from the cell surface to the lysosomes for degradation and is regulated by deubiquitylases, which catalyze the removal of K48-linked chains inhibiting degradation (Yau & Rape 2016). This process has been vastly studied for EGFR, in mammalian cells (Savio *et al.* 2016; Harrigan *et al.* 2018). The ubiquitin carboxyl-terminal hydrolase 8 (USP8) enzyme deubiquitylates EGFR on early endosomes and avoids its degradation (D'Arcy *et al.* 2015). In tumors such as glioblastoma, lung and breast cancer, EGFR is amplified or mutated at the tyrosine domain affecting the signal propagation and hence resulting in tumor cell proliferation (Harrigan *et al.* 2018). Inhibitors against USP8 have been reported, e.g., HBX90659 and its derivatives which have been shown to be efficacious in mouse models of lung cancer (Harrigan *et al.* 2018).

One of the challenges in drug discovery and development is to identify the correct proteins that participate in a particular disease. In this regard, understanding protein roles, cellular networks of proteins or pathways in a cellular context can help to identify the best target in a disease state. Furthermore, with the development of improved technologies for proteomics, the identification of pathological proteins would be eased and could bring in the future new disease markers which can aid on the drug discovery and development process.

2.2 The drug discovery and development process

The drug discovery process (**Figure 3**) is a long journey which usually initiates with a scientific basis coming from academia. When developing a drug, sufficient research data is gathered to mainly prove that the modulation of a target (a protein) results in a therapeutic effect over a specific disease. Proper identification and validation of the targets and further hit-to-lead processes before the clinical development stage are needed. In this section, the generalities of early drug

discovery are discussed, covering the steps of the drug development process before the clinical development phase.

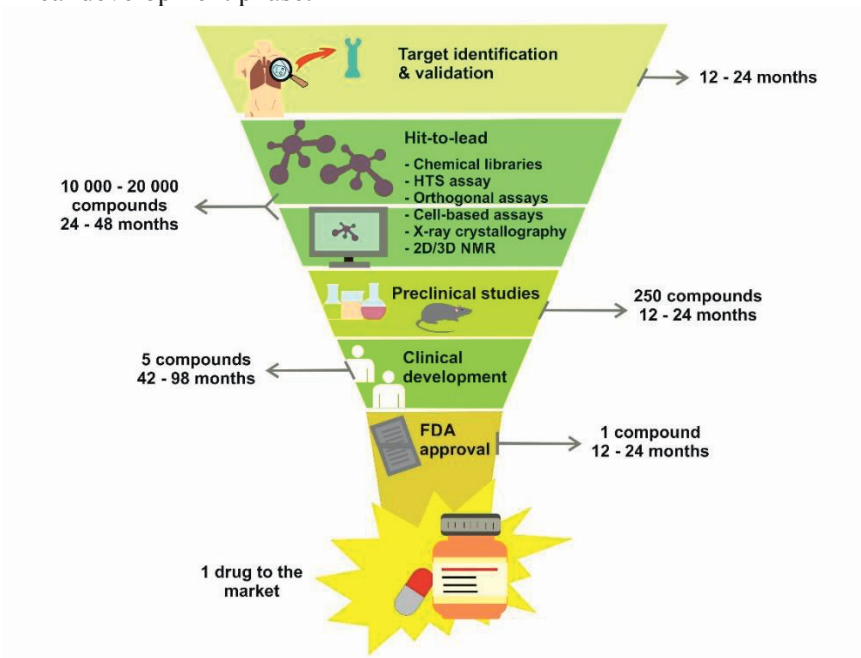

Figure 3: An overview of the drug discovery and development process. The first step is to identify the best target involved in a disease, which can take between 12 to 24 months. After that, chemical libraries are screened to find a hit that is then refined to improve its safety and effectiveness. Subsequent preclinical and clinical stages, including studies conducted in humans, take about 120 months, and it can take about 24 months for the FDA approval of the new medicine.

2.2.1 Target identification and validation

The selected target should meet clinical considerations, and most importantly, be druggable, i.e., accessible to the drug molecule to-be-developed, either small molecule or large biologicals. The challenge is to identify which target is relevant in a disease and in which form (Hughes *et al.* 2011). Enzymes, which are the most commonly targeted proteins, are naturally very dynamic targets as a result of substrate bindings, intermediates and products during the activity. A single target can represent a variety of different possibilities for drug intervention. In this context, the understanding of the mechanisms of activity of the target, covering structural characterization, partner interactions, environment characterization where the catalytic process occurs, and its related protein family, can render relevant information for compound design (Barnash *et al.* 2017; Holdgate *et al.* 2018). Presently, drugs with different activities have been designed to target many

of different drug target forms through different modes (Figure 4). After the target has been selected, validation is assessed with techniques involving *in vitro* assays and eventually using animal models (Hughes *et al.* 2011).

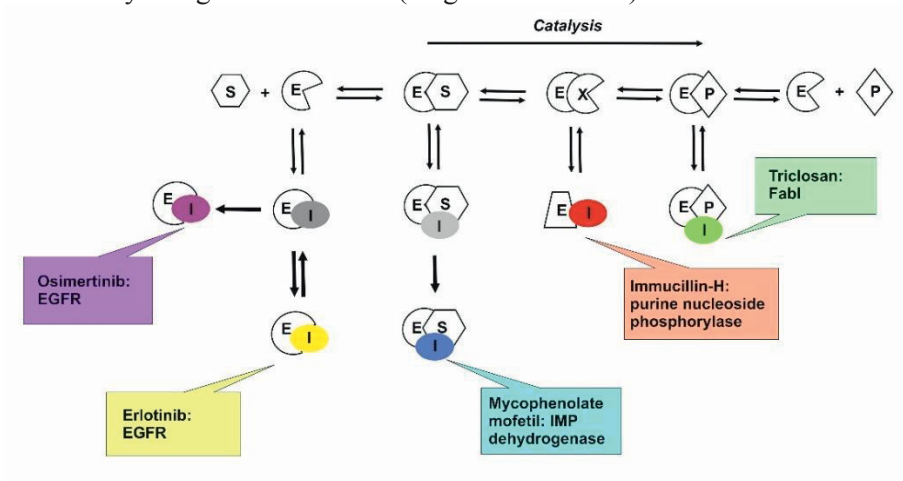

Figure 4. The kinetics of drug-target interactions. A single enzyme (E) may represent different targets for drug intervention. Enzymes bind substrates (S), intermediates (X) and products (P) during the catalytic cycle. Some examples of inhibitors (I) which bind to these different enzyme forms, either covalently (unidirectional arrow) or non-covalently, are shown. Figure modified from (Holdgate *et al.* 2018).

2.2.2 Hit-to-lead generation

In this phase, compound screening assays are used to find the 'hit' molecule. A hit is defined as the compound that has the desired activity in a screen and whose activity is confirmed upon retesting (Bleicher *et al.* 2003; Hughes *et al.* 2011). The primary screening begins with the library design or selection considering the structural similarity of the native binders and the binding site of each target. After that, HTS assays and computer-based software are run to find compounds or biologics that bind to the identified target. Although the HTS assay should give minimal false positive and negative hits, counter screen assays are performed to identify interfering compounds that could otherwise appear as hits. These screens are performed by removing the key component of the assay, such as the target. Additionally, orthogonal assays like isothermal titration calorimetry (ITC), MS, thermal shift assays, among others are performed to support and confirm the preliminary hits. Along this process, compound clusters are generated and evaluated to identify a structure-activity relationship (SAR) between the clusters and the target (Barnash *et al.* 2017). Additionally, cellular studies are performed to determine the selectivity and mechanism of action (MOA) of the hit series

providing information about the pharmacological effect of the drug under development (Barnash *et al.* 2017). The refined hit series gives more potent and selective compounds with pharmacological or biological activity, but requires more optimization in order to fit the target better. These are the lead compounds.

2.2.3 Lead optimization

In this phase, the aim is to maintain desired lead compound properties while reducing the deficiencies in the structure. The structure is modified to, e.g., improve the permeability of the compound through cellular epitheliums (Hughes *et al.* 2011). After the final characterization of the refined compound (preclinical candidate), *in vivo* model studies are performed before the clinical phase. The preclinical candidate should be tested for genotoxicity, high-dose pharmacology, pharmacokinetic and pharmacodynamic studies, dose linearity, drug-induced metabolism, and chemical stability (Hughes *et al.* 2011). All this information forms the basis of a regulatory submission to allow the human administration to begin in a phase called clinical development.

2.3 Considerations for HTS assay development

Several methods have been utilized for HTS assay development, including enzyme activity measurements from purified proteins, a reconstituted complex, a cellular extract or a phenotype in intact cells (Inglese *et al.* 2007). Regardless of the selected method, assays designed for HTS aim to obtain significant biological responses from the compound activities in a rapid manner. In this section, considerations for HTS assay development and post-screening analysis are reviewed.

2.3.1 The in vitro assay components

The target. A deep understanding of the physiological environment of the target is needed to mimic its natural condition and to be able to control its activity, substrate specificity and the compound structure activity relationship. Comprehensive characterization between the different target forms is also required in order to decide between the use of a truncated or full-length native enzyme, or multimeric protein complexes (Holdgate *et al.* 2018). Domain architecture, PTM status, activation state and artificial modification, such as affinity tags, can affect the target properties. Furthermore, the use of only the catalytic domain rather than the full-length protein can sometimes overlook allosteric sites that could provide a druggable site for inhibitor discovery. For example, it has been reported that allosteric inhibitors against the AKT serine/threonine protein kinases (AKT1-3)

can modulate the 'native' kinases with higher affinity than inhibitors developed with a truncated enzyme (Lindsley *et al.* 2005). Conversely, a catalytic domain of a protein can replace the use of the full-length version as in the case of the human β-amyloid precursor protein cleaving enzyme (BACE1) catalytic domain, which was reported to function independently of the other protein domains (Holdgate *et al.* 2018). Binding partners of the drug target can also influence its catalytic activity, as in the case of the binders of the histone-lysine *N*-methyltransferase enhancer of zeste homolog 2 (EZH2). The methyltransferase EZH2 catalyzes histone H3 methylation only in the presence of its binding partners forming a pentameric complex (Holdgate *et al.* 2018). In this context, a profound research has to be invested in knowing the drug target to ensure that the right form of enzyme is represented. The more the target diverges from its natural state the more likely it is to identify compounds that lack a physiologically relevant mechanism.

The target substrate refers to the modifiable peptide or protein recognizable by the drug target. When designing a substrate peptide for a target, some targets require a consensus sequence in the substrate in order to bind, while others are sequence insensitive. It is important to note that *in vitro* sequence selectivity may not reflect *in vivo* physiological relevance. For instance, the best known deacetylation substrate for SIRT6 is H3K9, which possess an arginine at the -1 position, a residue that is not preferred *in vitro*, according to the work of Steegborn *et al.* (Rauh *et al.* 2013). Based on this, considering additional residues beyond the immediate consensus sequence may perhaps play an important role in recognition by the target. Moreover, for some targets, a native protein substrate is necessary to identify inhibitors that target the desired activity as in the case of the histone-lysine N-methyltransferase NSD2. Full-length nucleosome (which is the basic unit of deoxyribonucleic acid (DNA) packaging in eukaryotes, consisting of a DNA and histone protein cores), was reported to be required as the NSD2 substrate for lysine methylation of the histone H3 to provide fidelity of the biological target in a panel of kinetic isotope effect studies (Holdgate *et al.* 2018). The use of peptides as substrates could facilitate easier *in vitro* assays, but if no relevant response is obtained for the studied target, protein substrates should be considered.

Cofactors are defined as any non-protein substance required for an enzyme to be catalytically active. Cofactors mediate the catalytic reaction or influence the enzyme structure. A cofactor may remain unchanged along the enzymatic reaction or may undergo through distinct states during the reaction cycle. It may strongly bind to the enzyme, or it can be transient, dissociating in equilibrium. Cofactors may be inorganic metal ion, e.g., Mg^{2+} as in the case of kinases (Adams 2001), or Zn^{2+} for deacetyltransferases (Wang *et al.* 2007), or organic compounds, e.g.

FAD/FADH2 involved in hydride transfer (Acker & Auld 2014). The enzyme-cofactor affinity may be overly high that enzyme is purified with the cofactor, e.g., a Zn^{2+} ion bound to the active site in the case of HDAC enzymes (Boskovic *et al.* 2016) or sometimes additional cofactor is needed in the HTS assay in order to maximize enzyme activity (Acker & Auld 2014). The concentration of the cofactor in the assay is also important as it influences the ability to identify a competitive cofactor compound in the screening.

2.3.2 The HTS assay parameters

Any HTS assay must fulfill the following parameters: a) sufficient sensitivity to allow detection of low potency compounds, b) reproducibility to support the validity of the obtained data, c) accuracy with regards to the positive and negative controls, and d) cost-effectiveness (Inglese *et al.* 2007). In order to determine if the developed assay is suitable for HTS, performance has to be assessed as part of the validation process. A series of statistical parameters evaluate assay performance including among the most common the Z'-value, signal-to-background (S/B) ratio, and the coefficient of variation (**Table 2**) (Zhang *et al.* 1999). In addition to these parameters, the developed assay should be tolerant to DMSO, as the chemical libraries are typically stored in that solvent. Usually, cell-based assays are intolerant to concentrations higher than 1% DMSO, while biochemical assays can tolerate up to 10% (Hughes *et al.* 2011).

Table 2 The most important equations for determining assay performance.

Parameter	Equation	Comment
Coefficient of variation	$\%CV = \frac{\sigma}{\mu} \times 100$	This measures the precision relative to the mean value, and is calculated for the maximum and minimum signals. An acceptable limit is <15%.
Signal to background	$S/B = \frac{\mu_{max}}{\mu_{min}}$	Usually calculated using control compounds, acceptable value >2-fold.
Z' factor	$Z' = 1 - \frac{(3\sigma_{max} + 3\sigma_{min})}{\lvert\mu_{max} - \mu_{min}\rvert}$	Represents the signal window using a score where a value >0.5 represents an acceptable assay.

σ, standard deviation of the assay signal; μ, mean of the assay signal; max, maximum signal; min, minimum signal.

2.3.3 Post-screening

After the HTS assay development, screening and hit identification, it is necessary to quantify the inhibitory effect of the hit compounds to determine which ones should progress for further testing and development. The quantification is determined by measuring the concentration at which the compound obtains 50% of its maximal inhibitory effect (IC_{50}). Dose-response measurements are performed in which the enzymatic inhibition degree is monitored at increasing concentrations of the compound. It should be noted that the IC_{50} may not be a reflection of inhibitor affinity, since the value may be significantly affected by experimental conditions (Inglese *et al.* 2007). Usually, in HTS screening, the compound is premixed with the enzyme for a specific period, such thatno time-dependent inhibition effect is detectable. In some cases, non-specific inhibition may occur due to impurities, aggregators, precipitators, or compounds that bind to unfolded proteins (Holdgate *et al.* 2018). In general, these compounds are called pan-assay interference compounds (PAINS) and can result in misleading IC_{50} values as the inhibitory activity due to other effects leads to denaturation, inactivation or removal of the active protein from its catalytic function (Holdgate *et al.* 2018).

Computational approaches, enzyme kinetics-based methods, and biophysical methods can help to identify such compounds. In general, the mechanism of inhibition can be either reversible or irreversible. Reversible inhibitors usually lead to full inhibition rapidly since non-covalent interaction is needed. Among this type there are competitive inhibitors, which usually have structural similarity with the substrate that accesses to the active site of the enzyme, uncompetitive inhibitors, which bind only to the enzyme-substrate complex, mixed type inhibitors, which bind both the free enzyme and the enzyme-substrate complex, and non-competitive inhibitors, which affect the enzyme activity without affecting the substrate binding. In the latter type, we find the allosteric inhibitors or activators, which bind and lead a change in the target conformation that leads to an inactive or active form of the target. In the case of irreversible compounds, they include non-specific protein denaturing agents that mostly interact with functional groups of the enzyme.

2.3.4 Compound interference in HTS assays

Every assay is prone to compound interference, which can be lead to false positive or false negative results. Commercially available assays have been developed with methods to rule out screening artifacts, for example, as with the ALPHAScreen TruHits kit from ALPHAScreen technology. This kit lists the possible types of interferences and strategies for assessing them including spectrophotometric analysis, biotin mimetics assays, and so forth. Compounds can interfere in a non-specific manner with one of the assay reagents or with the signal generated from

the assay detection type. Fluorescence and absorbance interferences are related to signal increase or reduction. For example, heterocyclic compounds fluoresce in the blue-green range, and can thereby interfere with coumarin-based assays which also excite or emit in that range (Inglese *et al.* 2007). Approaches including separation steps can minimize interferences as compounds are removed before the detection step. Nonetheless, these approaches are difficult to miniaturize and require high concentrations of reagents especially when using liquid handling robots. These assays are sometimes used as part of a hit confirmation process. Additionally, the use of lanthanide reporters, red-shifted fluorophores, and additional measurements along the screening can allow background subtraction in homogeneous assays. These fluorophores have a long lifetime luminescence that can be measured after the background fluorescence has decayed (Comley 2003; Inglese *et al.* 2007).

Compound aggregation may affect signal increment or reduction due to enzyme denaturation, ligand sequestration or light scattering. Aggregation usually occurs at concentrations >1 μM, and to solve this, detergents such as Triton X-100 (0.01-0.1%) can be added to disrupt the micellar structures of aggregates (Thorne *et al.* 2010; Acker & Auld 2014). Reporter enzyme-based assays such as the well-known luciferase systems can be affected if the tested compound inhibits the detection enzyme. For instance, it has been reported that resveratrol, an activator of sirtuin 1, can inhibit luciferase, which has been used as a reporter in several assays (Inglese *et al.* 2007).

2.4 HTS assays technologies for enzyme activity monitoring

In this section, the principle of the approaches, and the advantages and disadvantages of the different HTS technologies are presented. The methods are exemplified considering mainly phosphorylation as a PTM model. The section is divided into radiometric and luminometric assays, and examples of HTS methods that combine biochemical and biophysical approaches are also briefly described. A summary of the most common PTM enzymes and their advantages and limitations is shown in **Table 3** at the end of this section.

2.4.1 Radiometric assays

Radiometric assays traditionally applied to enzyme activity measurements, mainly in kinase activity monitoring. This technology relies on the conversion of a radioactively-labeled substrate (referred to here as the cofactor required for the enzyme activity) to a radioactive secondary product (the processed cofactor). The assay measures the radioactivity from the released product or the residual substrate after conversion. The advantage of this method is its specificity and sensitivity.

Radioisotopes allow the specific labeling of a single substrate without perturbing the biology of the reaction being studied, and the amount of radioactivity per labeled substrate can be measured making the system quantifiable. Some major requirements are needed in order to obtain a functional assay, including a simple method for quantitative separation of substrate and product, and availability of a labeled substrate with known specific radioactivity. These requirements can sometimes become limitations if they are not fully achieved. Additionally, the limited shelf-life of the radioactive labels leads to increased radioactive substrate production and therefore, relatively large amounts of radioactive waste, which have limited the use of the technique. A variety of radioactively labeled substrates for enzyme assays have been reported, including ^{32}P-ATP, ^{14}C-acetyl CoA, PAP^{35}S and [^{3}H]-acetylated oligopeptides for kinase (Ko *et al.* 2011), acetyltransferase (Rekowski & Giannis 2010), sulfotransferase (Paul *et al.* 2012), and histone deacetylase (Heltweg *et al.* 2005) assays, respectively. In the following section, the principle of two radiometric assay formats for enzyme activity monitoring are briefly described.

The scintillation proximity assay

The scintillation proximity assay (SPA) is a homogeneous method that uses microscopic beads containing scintillant that can be stimulated to emit light (Cook 1996). Stimulation occurs when the radiolabeled molecules bind to the SPA bead surfaces bringing them into close proximity. When the radioisotopes decay, there is a release of β-radiation (or Auger electrons for ^{125}I) that stimulates the scintillant in the SPA bead producing light which can be further detected using a scintillation counter or with a charge-coupled device (CCD) camera detector for imaging detection systems (**Figure 5A**) (Cook 1996; Glickman *et al.* 2008). As the free radioactive substrates are not in enough close proximity to the SPA bead to stimulate it, no washing steps are required. To improve the performance of the approach, SPA beads with red-shifted light emission have been implemented in order to reduce compound interferences related to their emission and absorption properties (Ma *et al.* 2008; Yu *et al.* 2014). Additionally, different versions of microtiter plates coated with polystyrene-based scintillant have been adapted for a direct assay, eliminating the need to add a scintillation cocktail for detection.

Filtration binding assay

Since the assay includes washing steps (heterogeneous assay type) it has been questioned whether this system can be considered as an HTS assay or not. However, a few commercially available platforms have been developed for large

kinase panels in HTS formats, for example, the Reaction Biology Corporation's HotSpot^{SM} (Anastassiadis *et al.* 2011) and Millipore's KinaseProfiler (Gao *et al.* 2013). In a standard assay, the enzymatic reaction is first performed in one assay plate and then filtered through a filter plate using a vacuum manifold. The radioactive product attaches to a special filter, while the residual unreacted material is washed away. The filters are dried, and a scintillation cocktail is added for detection. A variety of different filter materials are available for this technology, e.g., negatively charged phosphocellulose (PH) filters, which can retain phosphorylated peptides containing basic AA residues. The main advantage of this approach is its free-of-interference feature, however, the requirement for washing steps are a major limitation when applying this method on a large scale. In comparison with SPA assays, there is a greater assay variability and more radioactive waste, suggesting that the filtration binding assay format is better suited to a low-throughput format (Carpenter *et al.* 2002; Van Der Hee *et al.* 2005).

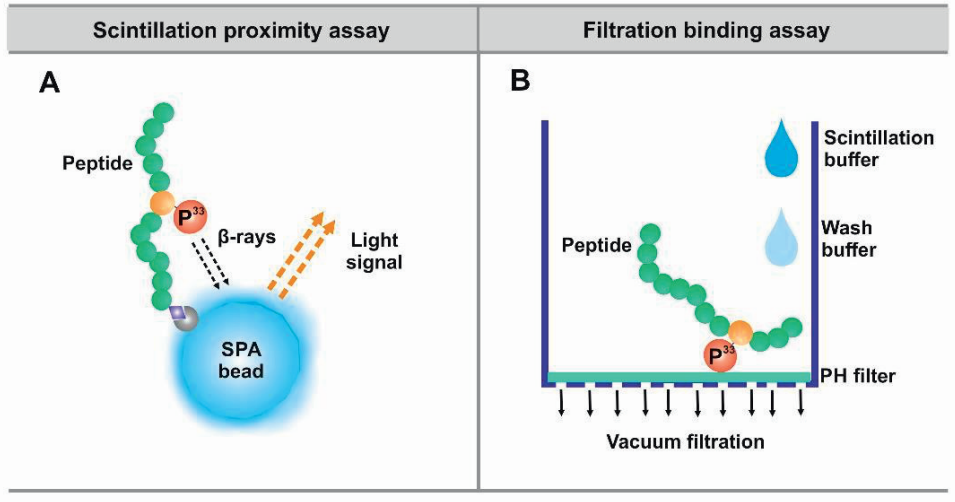

Figure 5. Radiometric assays for kinase activity monitoring. (**A**) In a scintillation proximity assay (SPA), the radioactively phosphorylated peptide binds to the SPA beads. After radioactive decay, β-particles from the ^{33}P interact with the scintillant embedded within the beads and light is emitted. (**B**) In a filtration binding assay, the radioactively phosphorylated substrate peptide is retained in the phosphocellulose (PH) filter, and the residual reagents are washed away using vacuum filtration. A scintillation cocktail is used to react the radioactive molecules and detect the phosphorylated peptide.

2.4.2 Luminometric assays

Most of the currently available HTS assays are based on fluorescence and luminescence systems, since these are simple, relatively cost-effective, easy to automate, and are performed in a homogeneous format. This technology overcomes some of the problems related to the use of radioactivity, as it employs nonradioactive labels and only requires low sample volumes. A broad variety of traditional fluorophores, which have relatively short fluorescence half-lives (< μsec) are available. These include organic dyes, e.g., fluorescein, rhodamine, coumarin, Texas red and cyanine5, and biological fluorophores, such as green fluorescence protein (GFP), among others (Lavis & Raines 2007; Choulier & Enander 2010; Chudakov *et al.* 2010). The benefit of organic dyes over biological fluorophores is their small size, which allows their conjugation to macromolecules such as antibodies, without interfering with the biological function of the labeled target. Alternatively, biological fluorophores have the advantage that they can either be expressed alone or fused with proteins within cells, bacteria, or entire organisms (Li *et al.* 2008).

Presently, longer half-life luminophores (μsec – msec), such as the lanthanide (Ln^{3+})-based probes, are widely used due to their favorable properties. Lanthanides provide a longer luminescence signal and an apparently wider Stokes shift when compared to the traditional fluorophores, which have mostly overlapping excitation and emission spectra (Hemmilä & Laitala 2005; Allen & Imperiali 2010; Charbonniere 2011). These properties provide a substantial reduction in background fluorescence from autofluorescence and help circumvent problems related to light scattering and self-quenching (Hagan & Zuchner 2011).

Time-resolved luminescence (often referred as fluorescence - TRF) is a technology that was developed based on the use of Ln^{3+}-based probes (**Figure 6**) (Hemmilä *et al.* 1984a). With this system, plate readers are set-up to include a delay time between the excitation and the reading time of the lanthanide emission signal, thus enabling autofluorescence subtraction. The difference with fluorescence intensity (FI) technology is the timing of the excitation and emission measurement processes being in the FI in a simultaneous manner which leads to a high background outcome (Hemmilä & Laitala 2005). Among the lanthanide series, europium and terbium probes have been widely applied in different assay formats (Hemmilä *et al.* 1997; Hewitt & Butler 2018).

A variety of luminometric methods are available based on single and dual-labels, antibodies, reporter enzymes, secondary products, the use of traditional fluorophores or TRF technology, and so forth. In the following section, a description of some of the techniques and their impact on HTS is presented.

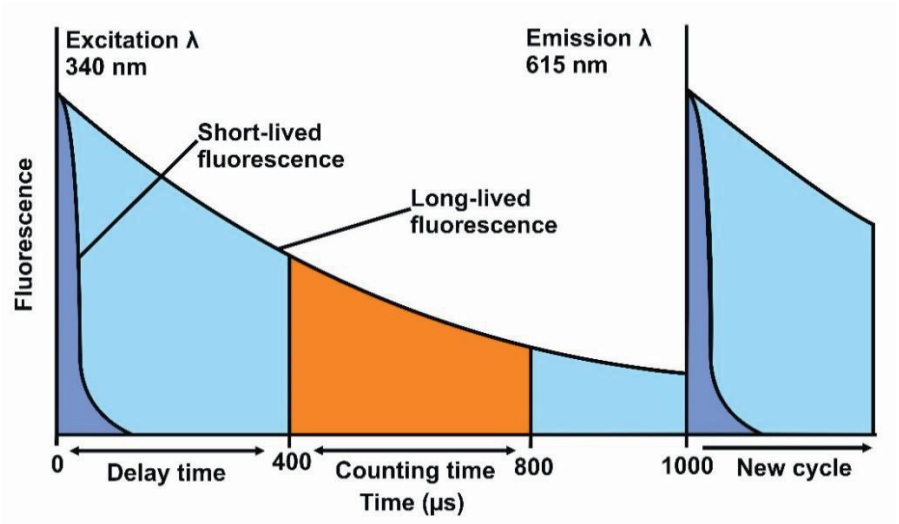

Figure 6. The time-resolved luminescence (TRF) technology. After excitation by a pulse of light, the fluorescence decay of a lanthanide is emitted in µsec – msec, which allows subtraction of background fluorescence interferences that often occur on a time sub µsec time scale.

Dissociation-enhanced lanthanide fluorescence immunoassays

The dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) is a TRF-based method that involves substrate peptide binding and separation steps prior to detection (**Figure 7**) (Hemmilä *et al.* 1984a). In this heterogeneous kinase assay, a biotinylated substrate peptide is attached to a streptavidin-coated plate before the enzymatic reaction. For detection, the plate is washed several times before the addition of the non-fluorescent europium-labeled antibody that binds to phosphorylated peptides. More washing steps are then performed to remove the unbound labeled reagent. In the next step, an enhancement solution is added to dissociate the non-fluorescent europium ions into solution to form a highly fluorescent chelate with the components from the enhancement solution, and final measurement of the signals is performed (Takalo *et al.* 1994).

This assay can decrease the interferences from compound fluorescence or fluorophore labeling since washing steps are included. The main disadvantage is that since it is a multistep process, which includes multiple washing cycles, it may not be a time- and cost-effective method (Ma *et al.* 2008).

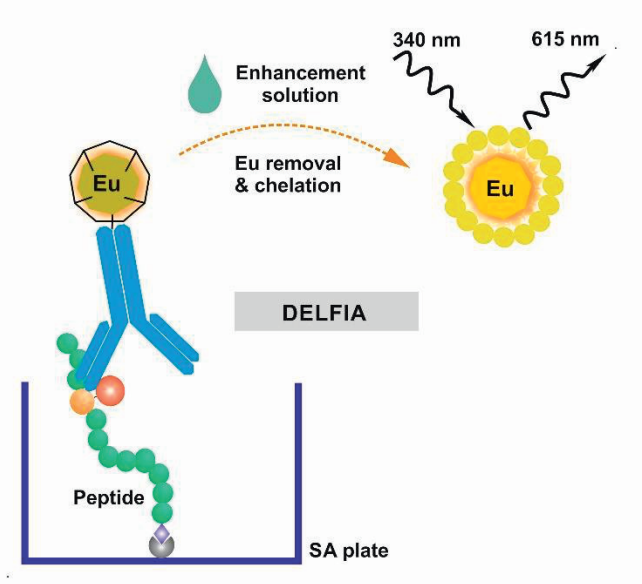

Figure 7. The principle of the dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA). After the enzymatic reaction and washing steps, the non-fluorescent europium (Eu)-labeled antibody is added to recognize the modified peptide. After the washing steps, the enhancement solution is added to dissociate the non-fluorescent europium ions and to form a highly fluorescent chelate that are detected after excitation.

Fluorescence intensity assays

The FI assay is based on the use of traditional fluorophores, and has been widely applied among assays that use protease-based detection reactions (Salisbury *et al.* 2002). Transferase activity monitoring, such as kinase assays, have been developed using enzyme cascades for detection (Ma *et al.* 2008; Janzen 2014). For instance, in the commercially available method called ADP Hunter™, kinase activity is detected by means of the production of ADP, using reactions in cascades involving pyruvate kinase, pyruvate oxidase, and horseradish peroxidase. After enzyme activity, the pyruvate kinase detection enzyme converts ADP to ATP and phosphoenolpyruvate to pyruvate. Later the pyruvate oxidase converts pyruvate to hydrogen peroxide (H_2O_2), which is detected by using the fluorescent Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) and horseradish peroxidase, resulting in the production of highly fluorescent resorufin (Charter *et al.* 2006; Helenius *et al.* 2012). In a different approach, the Promega's ProFluor® method uses a rhodamine-110 fluorophore which is conjugated to the substrate peptide. The rhodamine-110-peptide is in a non-fluorescent form, and after the phosphorylation reaction, a

protease solution is added to stop the reaction and to digest the non-phosphorylated peptide. The digestion releases the fluorescent rhodamine-110 from the non-phosphorylated peptide while the phosphorylated peptide is resistant to digestion and remains non-fluorescent. In this sense, the fluorescent intensity measured correlates to the kinase activity in the presence of the active protease (Ma *et al.* 2008). The main disadvantages of this approach are related to autofluorescence interferences and the multistep nature of the detection process, which includes several reporter enzymes that could be inhibited by the tested compound. Also, it can be challenging to develop peptides that are suitable for the studied target and the detection enzymes (Ma *et al.* 2008; Simeonov *et al.* 2008).

Fluorescence polarization assays

Fluorescence polarization (FP) is a single-label approach that depends on differences in the rotational properties of small vs. large molecules (Li *et al.* 2008). Fluorescent molecules are excited by a plane of polarized light and the emitted light is in the same polarization plane when the molecules do not substantially move or rotate during the excited state. Conversely, if the molecules rotate within the excited state lifetime, the polarized light is emitted in a different plane from the excitation polarization plane. In this sense, small molecules rotate rapidly thus becoming depolarized (low FP); and large molecules (or the association between small and large molecules) rotate insignificantly, and the emitting polarized light is in the same plane of the excitation polarization plane (high FP) (Checovich *et al.* 1995; Nasir & Jolley 1999). The polarization change is detected by monitoring the amount of fluorescence in the parallel vs. perpendicular plane after excitation in the parallel plane. This single-label approach encompasses direct and competitive assay formats, which have been developed for a variety of enzyme targets (Zhang *et al.* 2007; Mazitschek *et al.* 2008; Brandt *et al.* 2013; Lewallen *et al.* 2014).

In a direct measurement, the substrate peptide is fluorescently labeled, and after enzymatic activity, the modified peptide is recognized by a specific antibody leading to an increased polarization signal due to the decreased complex mobility (Kristjánsdóttir & Rudolph 2003). For example, in the competitive PolarScreen kinase assay, after the enzymatic reaction, the antibody and tracer are added to the reaction to initiate the competition for the antibody binding. The antibody recognizes the molecule in high concentration, either the fluorescently labeled tracer, which is a synthetically phosphorylated peptide (high FP value), or the phosphorylated substrate peptide produced by the kinase (lower FP value) (**Figure 8A**) (Seethala & Menzel 1997; Kristjánsdóttir & Rudolph 2003; Li *et al.* 2008).

In the case of the Transcreener kinase assay, the kinase activity is detected by measuring the ADP production. Antibodies bind either to the fluorescently labeled

ADP tracer (high FP value) or the ADP produced by the kinase activity (lower FP value) (**Figure 8B**) (Kumar & Lowery 2017). The Transcreener assay has been developed to detect a more extensive enzyme set, including non-nucleotide-dependent enzymes such as methyltransferases. The detection is performed by using a secondary enzyme cascade, which produces AMP that is detectable with an antibody and using fluorescently labeled AMP as a tracer (Kumar *et al.* 2015).

The advantage of the assays relies on the use of far-red tracers, which reduce interference from fluorescent compounds and light scattering due to their excitation and emission maxima above 600 nm (red wavelengths) (Vedvik *et al.* 2004). However, the use of enzyme cascades, and specifically the need of antibodies is typically the limiting factor. Anti-phosphotyrosine specific antibodies are relatively insensitive to the surrounding AA context. However, certain antibodies require a specific consensus sequence to bind to the modified target. For example, phosphoserine or phosphothreonine antibodies are highly dependent on the AA sequence surrounding the binding site, thus limiting the diversity of phosphorylated sites that can be recognized (Goddard & Reymond 2004; Li *et al.* 2008).

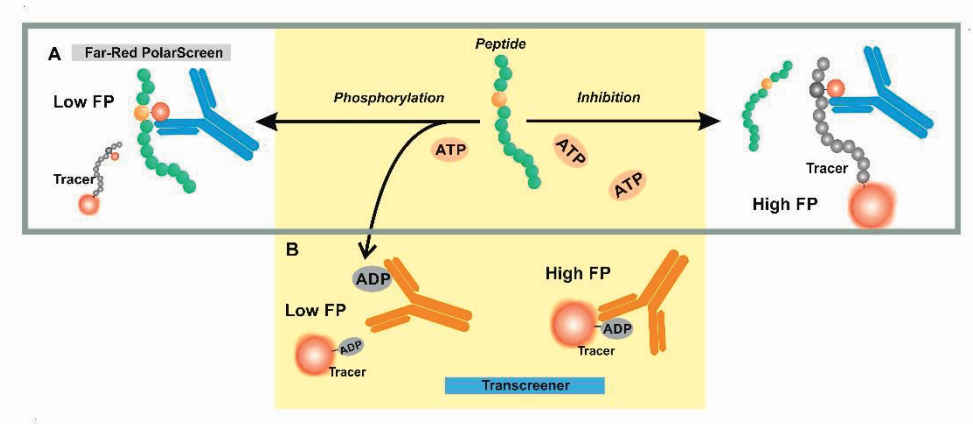

Figure 8. The principle of the competitive fluorescence polarization (FP) assay. After phosphorylation, the product competes out the tracer and binds the specific antibody (low FP), while in an inhibited enzymatic reaction, the tracer is in higher concentration leading to antibody binding (high FP). (**A**) The PolarScreen assay uses an antibody to recognize either the phosphorylated peptide modified by the kinase, or the tracer (fluorescently labeled phosphorylated peptide). (**B**) The Transcreener assay detects the ADP product formation. The antibody recognizes either the ADP produced by the enzymatic reaction, or a labeled ADP (the tracer).

Antibody-free systems using FP technology have been developed using metal ion systems, as for example with the IMAP method (Loomans *et al.* 2003; Klumpp *et al.* 2006). IMAP is based on a high-affinity covalent coordination of the trivalent metal-containing nanoparticles with phosphogroups (Loomans *et al.* 2003). The

phosphogroups can be free or linked to serine, threonine or tyrosine residues, or other molecules, which provides an advantage for measuring kinase, phosphatase or phosphodiesterase activity (**Figure 9**). Despite being an antibody-free system, the need for ATP concentration optimization and a specifically designed substrate peptide have limited the applicability of the IMAP method. Additionally, the number of carboxylic acid groups in the substrate peptide interfere with the detection component binding. Thus, long binding incubation is needed if more than five acid groups are present in the peptide sequence. Like IMAP, other FP assays have been developed using cationic polyamino acids (Coffin *et al.* 2000), or a mixture of ions and proteins that interact with the phosphorylated product (Scott & Carpenter 2003; Chopra *et al.* 2008). However, the nature of these approaches has limited their applicability to other enzyme types.

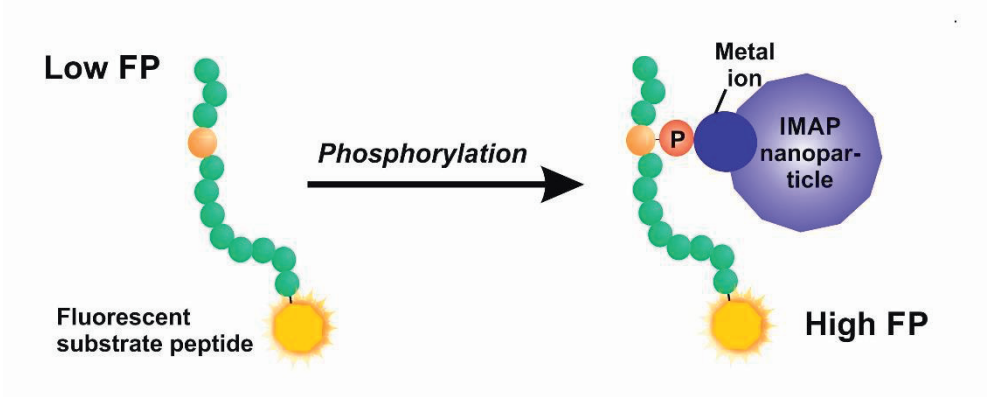

Figure 9. The principle of the IMAP fluorescence polarization (FP) assay. The IMAP detection approach is based on the high-affinity binding between the phosphate group and the metal ion immobilized on the nanoparticles. After phosphorylation, detection component interacts with phosphate groups on the phosphorylated peptides. This interaction causes a change in the complex motion avoiding significant rotation and leading to high FP. Conversely, the non-phosphorylated peptide can freely move, and low FP is monitored.

Förster resonance energy transfer assays

Förster resonance energy transfer (FRET) is a dual-label technique that relies on the energy transfer from an excited donor to an acceptor fluorophore (Li *et al.* 2008). Some elemental requirements are needed, such as spectral overlap between donor emission and acceptor excitation, the distance between the donor and acceptor should be between 1 – 10 nm, and the labels should be favorably orientated. In a FRET assay, the FRET signal is monitored by measuring changes in the sensitized emission intensity of the acceptor (Berney & Danuser 2003; Roda

et al. 2009). Moreover, signals can be monitored by measuring the intensity decrease of the donor fluorescence due to FRET. This measurement is usually employed to corroborate FRET events. TRF technology has been applied to FRET, thus leading to the TR-FRET method.

This method has proven its functionality and suitability for different enzymes, including transferases and hydrolases, generally utilizing the energy transfer from a lanthanide donor to an organic dye acceptor (Moshinsky *et al.* 2003; Carlson *et al.* 2009; Degorce *et al.* 2009; Horton & Vogel 2010; Gauthier *et al.* 2012; Zielinski *et al.* 2016). For instance, in the LANCE kinase assay, a Ln^{3+}-labeled phosphotyrosine antibody is used to monitor phosphorylation of an acceptor-labeled peptide. Close proximity between the donor and acceptor leads to energy transfer and a high time-resolved luminescence (TRL) signal (**Figure 10A**) (Hemmilä 1999; Karvinen *et al.* 2002; Degorce *et al.* 2009; Horton & Vogel 2010; Gauthier *et al.* 2012).

The main limitation of the FRET method is the need for specific antibodies. Furthermore, due to the close-proximity factor, incorrect orientation of the labels and free fluorophores can mask energy transfer by quenching the signal assay optimization can be challenging.

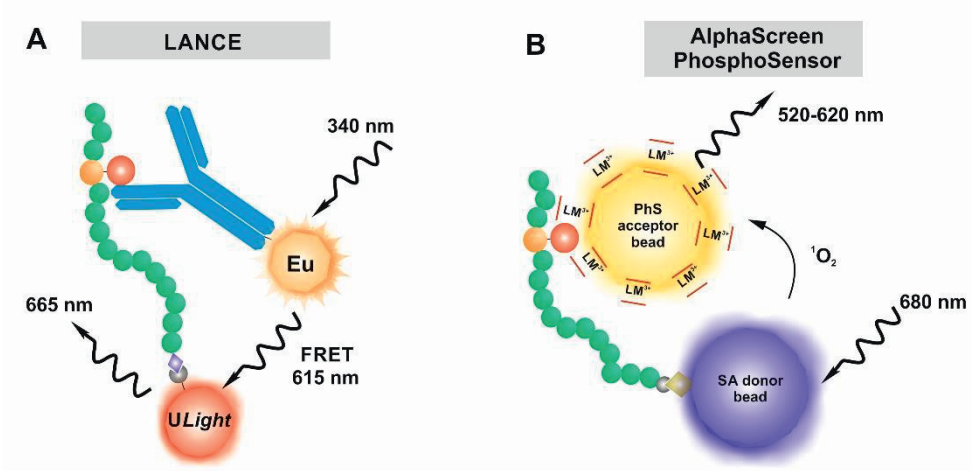

Figure 10. The principle of two homogeneous proximity- and energy transfer-based assays. (**A**) The LANCE kinase assay is a Förster resonance energy transfer (FRET) method that uses a lanthanide (Eu)-labeled antibody (donor) to recognize the phosphorylated substrate peptide (acceptor) and transfer energy from the excited donor to the acceptor molecule yielding to a high luminescence signal. (**B**) The AlphaScreen kinase assay relies on a chemical energy transfer between the donor and the acceptor beads. In the kinase assay, the phosphorylated peptide binds to the Lewis metal chelate (LMC^{3+}) on the phosphosensor (PhS) acceptor and the streptavidin (SA) coated on the donor bead. After excitation, a singlet oxygen reacts with the acceptor bead leading to a high the luminescence signal.

Amplified luminescent proximity homogeneous assay

The amplified luminescent proximity homogeneous assay (ALPHA) technology is a proximity-based method like FRET that uses donor and acceptor beads to mediate the chemical energy transfer (**Figure 10B**) (Glickman *et al.* 2002). There are two technologies that have been developed using this concept, the AlphaScreen, and the AlphaLISA assays. After the donor and acceptor beads are brought into close proximity, a laser excitation at 680 nm activates a photosensitizer in the donor bead, which converts ambient oxygen to a more excited singlet state. The singlet oxygen reacts with a thioxene derivative in the acceptor bead generating chemiluminescence at 370 nm, which activates luminophores contained in the same bead emitting light at 520-620 nm, in the case of AlphaScreen, or 615 nm, with AlphaLISA beads. Since the lifetime of the singlet oxygen is around 4 μs, it can diffuse approximately 200 nm, which gives freedom to measure larger molecules compared to FRET. Besides, the emission wavelength is lower than the excitation, which allows reducing the background interference significantly.

However, this technology may be sensitive to other interferences such as heavy metals, which act as singlet oxygen quenchers. These ions in solution can react with the singlet oxygen forming insoluble oxides and avoiding the chemical energy transfer (Eglen *et al.* 2008; Bielefeld-Sevigny 2009).

Chelation-enhanced fluorescence assays

The chelation-enhanced fluorescence (CHEF) assay, called Omnia, is a single-label antibody-free method that relies on the use of sulfonamide-oxine (Sox) as an artificial AA and physiological concentrations of Mg^{2+} (**Figure 11**) (Shults & Imperiali 2003). In this approach, the chelation-sensitive fluorophore Sox has heteroatoms that can coordinate with biologically or artificially available metal ions, such as Ca^{2+} and Mg^{2+} (González-Vera 2012). The fluorophore possesses a different electronic structure when coordinated than in the absence of the metal ion, and this is reflected in a change of its fluorescence properties (Krueger & Imperiali 2013). In this context, the chelating properties of the phosphate group have been harnessed for kinase sensing under this approach. 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 (Luković *et al.* 2008). The kinase assays are monitored with 360 nm excitation and 485 nm emission with an average Mg^{2+} concentration of 10 mM. The key point is that before phosphorylation, Mg^{2+} binding is weak (100 mM < K_D < 300 mM), while after phosphorylation, the affinity is increased (4 mM < K_D < 20 mM). A high

concentration of phosphorylated peptide exists in the bound and fluorescent state in the presence of 10 mM Mg^{2+}.

The challenge of the approach is to achieve an effective metal-binding after phosphorylation. For this, careful placement of the CHEF fluorophore is needed to achieve high-affinity and selective metal ion binding. Hence, the initial peptide sequence has to be modified several times (Shults *et al.* 2006). Other assays have been developed for kinase activity using different indicators such as Ca^{2+}, and Zn^{2+}. Although CHEF is an antibody-free system, the approach has been limited to kinase assays (González-Vera 2012).

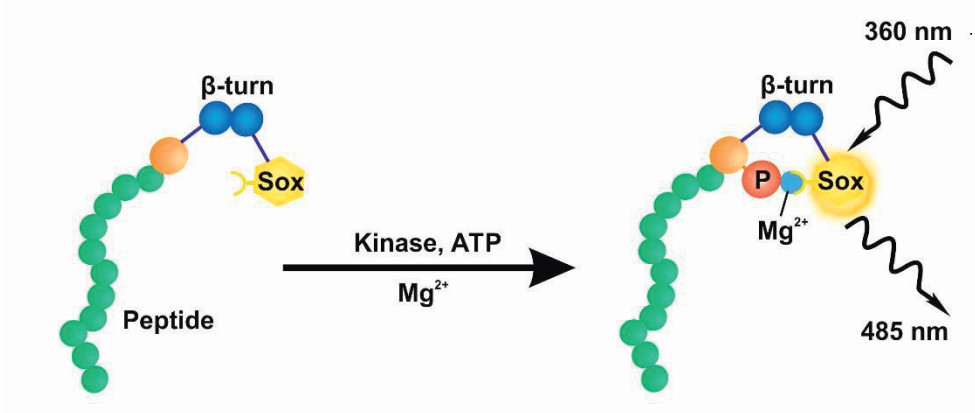

Figure 11. The principle of the chelation-enhanced fluorescence (CHEF) assay. The assay uses a substrate peptide which contains the artificial Sox amino acid for sensing. After phosphorylation, the Sox residue undergoes chelation-enhanced fluorescence upon Mg^{2+} binding. The Sox residue is pre-organized for Mg^{2+} binding by a β-turn sequence. The chemosensor can be appended to either the C-terminal, as in the figure, or N-terminal of the modification site (Shults & Imperiali 2003).

Chemiluminescent assays

The firefly luciferase has been used as a reporter enzyme in HTS kinase assays. This enzyme catalyzes the oxidation of luciferin into oxyluciferin using ATP, oxygen, and Mg^{2+}. The oxyluciferin, which is in an excited state, subsequently emits a yellow-green light with a spectral maximum of 560 nm (Lundin 2000). The commercially ADP-Glo™ assay measures kinase activity by monitoring the ADP production using a luciferase reporter (**Figure 12**) (Zegzouti *et al.* 2009). The phosphorylation detection is performed in two steps. First, a reagent is added to stop the kinase reaction and to deplete the remaining ATP, and secondly a reagent is added to simultaneously convert ADP to ATP and allow this newly synthesized ATP to be measured using a luciferase reaction (Larson *et al.* 2009; Zegzouti *et al.*

2009). Since the light emission is derived from a biochemical reaction, no excitation source is needed, allowing the use of a simple luminometer for signal recording. Chemiluminescent assays can be used for any combination of kinase and substrate, regardless of its nature such as peptide, protein, sugar, and lipid (Ma *et al.* 2008).

Although luciferase inhibitors may hamper the detection, there is already deep knowledge including an extended list of identified inhibitors that could help to address interference problems (Auld et al. 2009). A disadvantage of the system includes its low sensitivity and long assay times. The assay is not particularly time effective as the detection depends on two chemical reactions which consume an averaged time of approximately 90 min.

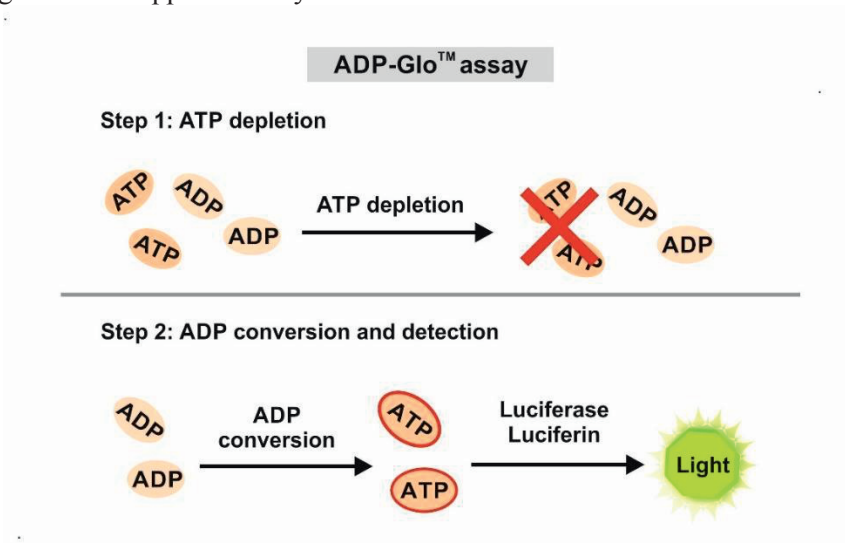

Figure 12. The principle of a chemiluminescent kinase assay. In the ADP-Glo™ kinase assay, after phosphorylation, the detection is performed in two steps. First, the remaining ATP has to be depleted, and second, the ADP produced by the kinase reaction is converted to ATP and utilized by the luciferase enzyme to convert luciferin in oxyluciferin. The luciferase reaction generates a yellow-green light which can be measured by a conventional luminometer.

2.4.3 Other HTS assays

Other assay types have been developed using combined biochemical and biophysical approaches. The mobility shift assay is a method that harnesses the charge difference between the modified status and the non-modified status of a fluorescently-labeled substrate peptide after the enzymatic reaction (Ma *et al.* 2008). When the peptides are separated by electrophoresis, different mobilities are observed due to the charge difference of the modified vs. non-modified peptides.

Caliper Life Sciences developed a microfluidic chip-based assay for kinases using this approach. The enzymatic reaction occurs in the chip and is subsequently detected by electrophoretic separation, this is called an on-chip assay. The detection is performed using a microfluidic capillary electrophoresis instrument that measures the change in the relative fluorescence intensities of the substrate and product peaks (representing enzyme activity) (Elkins *et al.* 2016). Another version of the method is available, off-chip assay, where the enzymatic reaction occurs in a conventional microtiter plate and is further sipped into a microfluidic chip for separation and detection (Ma *et al.* 2008). The off-chip assay allows the measurement of temporal enzyme kinetics as the reaction mixture can be sipped at the different time points. Contrarily, the on-chip type needs a high conversion rate (~20%) in a short reaction time, which impedes kinetic measurements.

Table 3. Summary of the most common PTM enzyme assays

Method	Commercially available	Peptide substrate	Detection component	Advantages	Limitations
SPA	PerkinElmer's SPA assay	Biotin-peptide	Scintillant-coated plate	No compound/reagent interferences. No need of scintillant cocktail addition.	Radioactive materials. Needs large volumes in plate.
Filtration binding	KinaseProfiler™	Peptide	PH filter, scintillant cocktail.	No compound/reagent interferences.	Radioactive materials. Multi-washing steps.
DELFIA	Perkin Elmer's DELFIA®	Biotin-peptide	Eu^{3+}-Ab. SA-plate, enhancement solution.	No compound/reagent interferences. Sensitive.	Multi-washing steps. Specific Ab.
FI	ADP Hunter™	Peptide	Enzyme cascades, resorufin fluor production	For all ADP production systems	Multistep assays. Many additional detection enzymes. Possible inhibitor for detection enzymes.
FP	PolarScreen™	Peptide	Ab, phosphorylated R-peptide (tracer)	Red-shift tracer to reduce compound interference	Specific Ab. Sensitive to buffer components. Specific Ab. Long antibody incubation for detection.
	Transcreener™	Peptide	Ab, R-ADP (tracer). Enzyme cascades in certain assays.	For all ADP production systems.	Specific Ab. Not suitable for high ATP concentration (mM). Many detection enzymes.
	IMAP™	F-peptide	Metal-based beads bind to phosphate groups.	Ab free	Fluorescence compound interference. Specifically designed substrate peptide. ATP concentration optimization.
TR-FRET	LANCE	Biotin-peptide	Ln^{3+}-Ab (donor). R-SA (acceptor)	Sensitive. Red-shift tracer to reduce compound interference	Specific Ab. Ln^{3+} quenched by some divalent ions and chelated by EDTA at high concentrations (mM).
	HTRF®	Biotin-peptide	Eu^{3+} -cryptated Ab (donor). R-SA (acceptor)	Sensitive. Red-shift tracer to reduce compound interference.	Specific Ab. High KF concentrations to reduce cryptate quenching by water.
ALPHA	AlphaScreen®	Biotin-peptide	LM^{3+}-Acceptor, SA-	Measure large molecules	Beads are light sensitive. Specific

			donor beads.	(labels close proximity <200 nm). Sensitive. Long λ_{ex} and shorter λ_{em}: low background interference.	reader. Specific Ab in some cases. Interferences from singlet oxygen quenchers (heavy metals).
CHEF	Omnia®	Sox-XX-peptide	Sox chelation through Mg^{+2} binding	Ab free	Fluorescence compound interference. Sensitive to buffer components. Developed only for kinases.
Chemilumi nescense	ADP-Glo™	Peptide	Two chemical reactions before luciferase assay	For all ADP production systems. No fluorescence interference	Two-step assay. Possible luciferase inhibition.
Mobility shift assay	Caliper chip-based platform	F-peptide	On-chip- or off-chip-based assay	Accurate detection. Automation friendly	Needs special substrate peptide development. Relatively expensive.

Abbreviations: SPA, scintillation proximity assay; PH, phosphocellulose; DELFIA, dissociation-enhanced lanthanide fluorescence immunoassay; Ab, antibody; SA, streptavidin; FI, fluorescence intensity; ADP, adenosine diphosphate; FP, fluorescence polarization; R, red shifted dye; ATP, adenosine triphosphate; F, organic fluorophore; TR-FRET, timed-resolved luminescence energy transfer; Ln^{3+}, lanthanide chelate; KF, potassium fluoride; ALPHA, amplified luminescent proximity homogeneous assay; LM^{3+}, Lewis metal chelate; λ_{ex}, excitation wavelength; λ_{em}, emission wavelength; CHEF, chelation-enhanced fluorescence; Sox, a chelation-enhanced fluorophore; XX, structural amino acid sequence.

2.5 Concluding remarks

Cell signaling networks are orchestrated by a variety of different proteins, each one interacting with several other proteins in various pathways and affecting the overall cellular function. The presence of protein isoforms with diversified roles in distinct cellular contexts, and of feedback loop mechanisms, which maintain a cellular event despite, for instance, the inhibition of a specific protein reveals the complexity of these cellular systems. It is clear that a profound understanding of this network helps in finding the most appropriate drug target for a disease, and the best drug design and screening approaches.

Nowadays, the available HTS technologies offer the freedom to the end-user to select methods that suit best to their drug discovery goals. In general, an ideal HTS assay should be robust, sensitive, easy to optimize, cost-effective, and should be able to provide a significant measurable biological response. A variety of different HTS technologies for PTM enzyme assays rely on the detection of the modified or the secondary product produced after enzyme activity. In the case of phosphorylation, radiometric and some luminescence approaches measure enzyme activity by detecting the phosphorylated product. Other HTS assays detect the secondary product, in this case, ADP. As ADP is a universal kinase secondary product, the ADP detection approach can be applied to other different kinases. These PTM assays have been robustly developed for kinases and phosphatases and some other small-size PTMs. Moreover, in the case of the ADP detection method, indirect detection systems have been developed to expand the assay applicability to other PTMs. For instance, in methylation, the secondary product (SAH) is converted to an intermediate product and then to ADP, which can be then detected by the same approach. In general, the mentioned PTM detection approaches are based on antibodies, enzyme cascades, or chemical reactions, which may hamper their applicability to other PTM types. The current HTS technologies are yet not adaptable to some of the most important and complex targets involving SUMOylation, NEDDylation or glycosylation. The challenge to expand an HTS method to other PTM types depends perhaps on the enzymes involved. The selection of the detection method is target specific as the enzyme requirements and the enzymatic reaction itself vary among protein families.

New complex targets for oncology, (Fleuren *et al.* 2016) including epigenetics (Filippakopoulos & Knapp 2014) or PPIs (Scott *et al.* 2016), neurodegenerative disorders (Li & Götz 2017), or rare diseases (Jen *et al.* 2016; Boycott & Ardigó 2018), are continually emerging, suggesting there is a demand for new strategies and more versatile technologies capable of fulfilling the unmet drug development needs for novel targets.

The quenching resonance energy transfer (QRET) technique is a homogeneous, simple and single-label assay concept based on a luminescence difference between target molecule bound to a Ln^{3+}-ligand and a free Ln^{3+}-ligand (Kopra & Härmä 2015). When the Ln^{3+}-ligand is free in solution, the chelate is exposed to the soluble quencher molecule which attenuates the luminescence signal. Contrarily, when the Ln^{3+}-ligand is in complex with the target molecule, the chelate is protected against quenching and a high luminescence signal is detected. The ligand can be a peptide, aptamer, nucleotide or any low molecular weight molecule incapable of protecting the Ln^{3+} luminescence from quenching (Kopra & Härmä 2015). This technique has been extensively reported for screening assays for different targets, such as GPCRs proteins, estrogen receptor-ligand binding, and small GTPases (Rozwandowicz-Jansen *et al.* 2010; Huttunen *et al.* 2011; Martikkala *et al.* 2011; Kopra *et al.* 2014b, 2017). The advantage of the technique relies on its simplicity and sensitivity. The QRET is simple as it is designed in a single-label approach, there is no need for long incubations, and the detection component can be added in a single step. It is sensitive due to the use of the TRL detection, and due to all these attributes, it can be optimized in a cost-effective manner. The challenge relies on the need for small ligands for Ln^{3+} conjugation. The labeled molecule should preferably be a low molecular weight ligand to avoid self-protection of the Ln^{3+}. Additionally, depending on the application, the use of a high concentration of total protein in the tested reaction can sometimes affect the system by quenching the overall luminescence signal, thus affecting the assay sensitivity. Sometimes a tested compound can act as a quencher by absorbing light at the emission wavelength of the Ln^{3+}. However, the influence of such quencher compounds are associated with all HTS technologies that rely on a luminescence-based detection. The implementation of the QRET technique approach presents a possibility to develop QRET-based assays for different PTM targets that may have found difficulties in the existing HTS technologies.

If the drug discovery and development community are willing to embrace new approaches, there would be more opportunities to obtain the desired outcome, perhaps in the earliest stages of the drug discovery and development, thus possibly resulting in a better clinical translation and subsequent better treatments.

3 AIMS OF THE STUDY

The main objective of the work presented from this doctoral thesis was to develop a universal biochemical HTS method for protein PTMs monitoring using the QRET technique in an antibody-free system. Ultimately, this would provide a simple, sensitive and versatile detection method that allows measurement of a wide range of different PTM types. The applicability of the QRET technique for PTM enzymatic activity assays was first demonstrated in an antibody-based system for tyrosine phosphorylation monitoring. On the basis of this, the universal detection method was further developed using the QRET technique in an antibody-free system. Under this framework, a novel tool is presented, which is referred to as "the peptide-break technology."

More specifically, the aims of the original publications were:

I To demonstrate the suitability of the QRET technique for PTM enzymatic activity monitoring in a homogeneous single-label tyrosine EGFR activity assay.

II To develop a universal homogeneous single-label detection technology for PTM monitoring using the QRET technique and the leucine zipper concept.

III To simplify the universal PTM detection technology using a peptide dimer approach exclusively based on electrostatic interactions to enable freedom for substrate peptide design.

IV To study the applicability of the universal peptide-break technology in a thermal shift assay to enable the use of high peptide concentrations.

4 MATERIALS AND METHODS

A brief summary of the materials and methods used in this project are presented here. For more detailed information refer to the original publications **(I-IV)**.

4.1 Labels and quencher molecules

All the assays were developed using the QRET technique as a detection system which consisted of a Eu^{3+}-labeled molecule and a soluble quencher.

4.1.1 Lanthanide chelate

The nonadentate Eu^{3+}-chelate-9d, {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). The excitation and emission maximum of the Eu^{3+}-chelates are approximately 340 nm and 615 nm, respectively.

Eu^{3+}-chelate conjugation to peptides ***(I-IV)*****.** In all the label conjugations, the isothiocyanate group of the Eu^{3+}-chelate reacted with the primary amino group of the peptides. The lysine amino groups present in the sequence were synthetically protected with Fmoc groups. Subsequent Fmoc removal was performed after label conjugation and before purification using 30% of piperidine. All the HPLC purifications were performed using reversed-phase adsorption chromatography (Dionex 3000 LC system), and an Ascentis RP-amide C18 column. An eluent system containing 50 mM triethylammonium acetate buffer (TEAA) pH 7.0 and an acetonitrile linear gradient was used. The Eu^{3+}-peptide concentration was determined based on the Eu^{3+}-ion concentration by comparing observed luminescence signals to a commercial Eu^{3+}-standard (Hemmilä *et al.* 1984b). In publication **I**, the Eu^{3+}-chelate was conjugated to a substrate peptide for a single-peptide EGFR assay **(I)**, and to a detection peptide for a dual-peptide assay **(II-IV)**.

4.1.2 Soluble quencher molecule

Quenchers were chosen according to the absorption spectra. All the quenchers were stored in DMF (QRET Technologies). The Quench III was used in publication **I**, MT2 was utilized in **II-III,** and MT10 in **IV.**

4.2 Instrumentation and instrument settings

4.2.1 Plate reader for TRL-signal measurements

The TRL-signal from the Eu^{3+}-chelate was monitored with a Victor 1420 multilabel counter (PerkinElmer) or a plate reader from Labrox Ltd. Measurements were performed at 615 nm, using 340 nm excitation wavelength with 400 µs **(I)** or 600 µs delay time, and 400 µs decay time **(I-IV)**. Black Optiplate 384F **(I-III)** or black Framestar 96-well microtiter **(IV)** plates were used for homogeneous assays, while an anti-mouse IgG 96-well plate for heterogeneous assays **(I)**.

4.2.2 Circular dichroism spectroscopy

Circular dichroism (CD) measurements of the non-labeled peptides **(II)** were carried out at room temperature (RT) in a 1 mm cuvette using a Chirascan CD spectrometer from Applied Photophysics Ltd (Leatherhead, UK). The CD signal was monitored from 190 to 250 nm in 1 nm steps with an averaging time of 5 seconds at each wavelength.

4.2.3 Thermal shift assay

The thermal shift assays were conducted using a PTC-100 thermal cycler for the heating process and a Victor multilabel counter for TRL-signal measurements. The signals were collected after a 3 min incubation at the chosen temperature.

4.3 Assay buffer selection and peptide design

All buffers were selected according to the enzyme requirements (**Table 4**). The substrate peptides were designed in-house by considering the enzyme's consensus sequence and the assay's detection approach **(I-IV)**. The detection peptides followed the Fos/Jun leucine zipper (LZ) sequences **(II, IV)** and the peptide net charge **(III)** (**Table 5**).

Table 4. Buffers used for the PTM enzyme assays.

Publication	PTM	Buffer
I	Phosphorylation	**AB1:** 25 mM Tris, pH 7.45, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.01% Triton X-100
II, III, IV	Phosphorylation, dephosphorylation	**AB2:** 10 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.01% Triton, 5 mM NaCl, and 1 mM $MgCl_2$
II, III, IV	Deacetylation	**AB3:** 10 mM HEPES (pH 8), 0.1 mM EDTA, 0.01% Triton, 25 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$
II, III	Citrullination	**AB4:** 50 mM HEPES (pH 7.5), 25 mM NaCl, and 0.01% Triton X-100

In publications II-IV, the detection component was added in AB2.

Table 5. Peptides used for PTM monitoring in this doctoral work. Modification sites of the substrate peptides are underlined.

Publica-tion	Name	Type	Peptide function	AA[¤] (n)	Peptide charge[*]	PTM modification site
I	PEP1[**]	-	Phosphorylation			Eu^{3+}-EEE**Y**EEEVEEEVEEE
I	PEP2[**]	-	Phosphorylation			Eu^{3+}-EE**Y**EEEEVEEEVEEE
I	PEP3[**]	-	Phosphorylation			Eu^{3+}-E**Y**EEEEEVEEEVEEE
II	Jun	LZ	Phosphorylation	36	-	-**Y**-
II	p-Jun	LZ	Phosphorylated	36	-	-p**Y**-
II	Fos	LZ	Detection	36	-	-
II	Tyro-LZ	LZ	Phosphorylation	35	-	-**Y**-
II	TyroP-LZ	LZ	Dephosphorylation	35	-	-**pY**-
II, III	Ser-LZ	LZ	Phosphorylation	35	-	-RRx**S**-
II, III	LysAc-LZ	LZ	Acetylated	35	-	-$^{(Ac)}$**K**GA-
II, III	ArgLZ	LZ	Citrullination	35	-	- SG**R**GK -
II, III, IV	Eu^{3+}-LZ	LZ	Detection	35	-	-
III	Eu^{3+}-CPR1	Charge	Detection	15	-12	-
III	Eu^{3+}-CPR2	Charge	Detection	27	-23	-
III	CP1	Charge	Phosphorylation	19	+10	GGRRRV**S**RRVRRRV**S**RRGG
III	CP2	Charge	Phosphorylation	21	+13	GGRRRV**S**RRVRRRV**S**RRVR RR
III	CP3	Charge	Phosphorylation	34	+23	GGRRRVRRRV**S**RRVRRRVR RRVRRRV**S**RRVRRRK
III	CP4	Charge	Citrullination	17	+10	- SG**R**GK -
III	CP5	Charge	Citrullinated	17	+8	-SG$^{(CIT)}$**R**GK-
III, IV	CP6	Charge	Acetylated	17	+8	GRAR$^{(Ac)}$**K**GARRAR$^{(Ac)}$**K**GAR RR
III, IV	CP7	Charge	Acetylation	17	+10	GRAR**K**GARRAR**K**GARRR
IV	CP8	Charge	Methylation	19	+10	GRART**K**QTRRART**K**QTRRR
IV	CP9	Charge	Methylated	19	+10	GRART$^{(met-1)}$**K**QTRRART$^{(met-1)}$**K**QTRRR
IV	LZ-Y	LZ	Phosphorylation	35	-	REELRKRRAELRRR**Y**AQLRQ RREQLRQR**Y**ANLRKE
IV	LZ-pY	LZ	Dephosphorylation	35	-	REELRKRRAELRRR**pY**AQLR QRREQLRQR**pY**ANLRKE

[*]Net charge at pH 7.5. [**]PEP4-6 represented the phosphorylated version of PEP1-3, respectively. [¤]AA= amino acid

4.4 Optimization of the homogeneous EGFR activity assay (I)

A series of assays were performed to understand and demonstrate the functionality of the approaches and to select the optimal assay conditions. Later, assays were performed with the optimized conditions for validation purposes.

Substrate peptide selection was first assessed in a *homogeneous QRET binding assay* using PEP4-6 (10 nM) and 4G10 antibody (187 ng/well). First, PEP4-6 and the antibody were incubated in 40 µl for 5 min. Next, 10 µl of Quench III (3.6 µM) was added, and the TRL-signal was measured during 60 min. Later, an *EGFR enzymatic assay* was performed using 10 nM PEP1-3, 50 µM ATP, 2 nM EGFR, and AG-1478 inhibitor (0 and 2 µM). All the enzymatic components were incubated in 10 µl for 30 min at RT. Next, the antibody and Quench III were added in 40 µl using the same concentrations and measurements as in the binding test.

Antibody concentration selection. An antibody titration (0-374 ng/well) was performed with phosphorylated PEP4 (10 nM), EGFR (0 and 2 nM), ATP (0 and 50 µM) and Quench III (3.6 µM) in a *homogeneous binding assay*. First, all reagents except the antibody were added in 40 µl, incubated 5 min and measured. Next, 10 µl antibody was added and measured as previously described. Also, an EGFR titration (0-50 nM) was performed with 40 ng/well of 4G10 antibody in a *heterogeneous binding assay*. First, 50 µl of antibody was added to the anti-mouse IgG plate, incubated for 60 min and washed (4x). Next, a 50 µl mixture of PEP4 (10 nM) and varying EGFR concentrations was added, incubated 15 min, washed and measured. The assay used Kaivogen buffer and Kaivogen wash solution.

EGFR concentration selection and assay validation. An EGFR titration (0.5, 1.0, 2.0, and 5.0 nM) with PEP1 was performed in an enzymatic assay to find the suitable EGFR concentration. The optimized assay condition consisted of 2 nM EGFR, 10 nM PEP1, 50 µM ATP, 187 ng/well antibody and 3.6 µM Quench III. Next, the assay was validated with a panel of EGFR inhibitors (AG-1478, compound 56, erlotinib, PD174265, and staurosporine). Inhibitor titration (0 to 5 µM) was performed with the optimized assay condition.

4.5 Optimization of the peptide-break technology (II-IV)

All the assays were performed in 50 µl per well unless otherwise stated. Standard protocols for binding and enzymatic assays were used along the optimizations. In the *homogeneous QRET binding assay,* peptides (substrate and detection peptides) were incubated for 5 min before TRL-signal measurement. Next, quencher was

added, and the TRL-signal was measured multiple times during 60 min. In the *homogeneous PTM enzymatic assays* (**Table 6**), the reaction was performed in 10 µl, and the detection component (Eu^{3+}-peptide and quencher) was added in 40 µl. First, the inhibitor or activator was added, followed by the enzyme, substrate peptide and cofactor and incubated at RT during 30-45 min. Next, the detection component was added and measured as in the binding assay. Citrullination assays were performed with 1 mM $CaCl_2$ added together with the enzyme, and in HDAC3 deacetylation assays, the inhibitor was pre-incubated with the enzyme for 10 min before the substrate peptide addition.

Table 6. Assay conditions for the PTM enzymatic assays (II-IV).

Publication	PTM	Enzyme (nM)	Substrate peptide (nM)	Cofactor (µM)	Detection peptide (nM)	Quencher (µM)
II	Phosphorylation	0.5, EGFR	10	50, ATP	10	3, MT2
II, III	Phosphorylation	0.5, PKA	10	50, ATP	10	3, MT2
II	Dephosphorylation	3.0, PTP1B	10	-	10	3, MT2
II	Deacetylation	0.5/0.05*, Sirt1	20	500, NAD+	5	3, MT2
II, III	Deacetylation	0.5, HDAC3	10	-	10	3, MT2
II, III	Citrullination	1.0, PAD4	10	-	10	3, MT2
III	Phosphorylation	2.0, PIM1-3	10	50, ATP	10	3, MT2
IV	Dephosphorylation	1.5, PTP1B	10000	-	1	500, MT10
IV	Deacetylation	1.5, HDAC3	10000	-	1	500, MT10

*A low enzyme concentration was used to obtain an improved assay window for the activator. Concentrations are given considering the final volume of 50 µl per well.

4.5.1 The peptide-break technology using the leucine zipper peptides (II)

Helicity of the leucine zipper (LZ) peptides was studied using circular dichroism (CD) spectroscopy. The peptide dimers, Jun/p-Jun with Fos and Tyro-LZ/TyroP-LZ with EuLZ, were measured at 30 µM using PBS (pH 7.4). Three replicates for each sample were prepared, and the final signal was the triplicate average corrected for the buffer signal.

Affinity studies of the peptide dimers was studied in a *homogeneous QRET binding assay*. Titration (0.1 nM-15000 nM) of Jun/p-Jun with 2 nM Eu^{3+}-Fos and Tyro-LZ/TyroP-LZ with 0.5 nM Eu^{3+}-LZ using MT2 quencher was performed in AB2 buffer.

Homogeneous PTM enzymatic assays in an end-point mode were performed to optimize the assay conditions and to validate the optimized assays. Inhibitor

titrations (0-100 µM) were performed against EGFR (AG-1478, compound 56, erlotinib, PD174265, and staurosporine), PKA (staurosporine, H-7, and H-89), PTP1B (Na_3VO_4, and TCS-401), HDAC3 (TSA), Sirt1 (EX527) and a Sirt1 activator (SRT1720). For PAD4 assay, $CaCl_2$ titration (0.5, 1.0, and 5.0 mM) was performed using GSK484 (5 µM) as a negative control. The assay conditions are shown in **Table 6**. Additionally, the peptide-break technology was demonstrated for kinetic measurements in PKA and EGFR assays using 20 µl final volume. In the PKA assay, 2 nM Ser-LZ peptide, 1 nM Eu^{3+}-LZ, and 1.5 µM MT2 quencher were incubated for 5 min. Next, a mixture of 1 nM PKA and 50 µM ATP was added to start the enzymatic reaction and the direct TRL-signal measurement ("0-time point"). The EGFR assay was performed using 8 nM Tyro-LZ, 1 nM EGFR, 50 µM ATP, 3 nM Eu^{3+}-LZ, and 1.5 µM MT2 in the same manner.

Inhibitor screening assay using the PKIS library was performed to validate the approach. Before the screen, EGFR and PKA assay miniaturization was performed from 50 to 10 µl final volume. DMSO tolerance was studied with varying DMSO concentrations (0-10%) in the 10 µl miniaturized assay. The assay conditions were 10 nM peptide, 50 µM ATP, and 0.5 nM PKA/1 nM EGFR. For detection, 10 nM/5 nM Eu^{3+}-LZ and 3 µM/2 µM MT2 quencher were used in PKA/EGFR assays. The screening assays used 356 compounds from the Published Kinase Inhibitor Set (PKIS) library in a single data point (1 µM) for each compound. Also, 96 points for 0 and 1 µM of staurosporine were used as controls and to measure the assay robustness (*Z*' factor) from the three assayed plates.

4.5.2 The peptide-break technology using the charge-based peptides (III)

Substrate peptide selection. First, a *homogeneous QRET binding assay* was performed to define the optimal charge and affinity for the charge-based (CP) peptides. In the assay, CP1-3 and Ser-LZ peptides (10 nM) were tested with Eu^{3+}-LZ detection peptide (10 nM) and MT2. Next, a *homogeneous QRET enzymatic assay* with PKA kinase, H-89 inhibitor (0 and 2 µM), and CP1-3 and Ser-LZ peptides was performed to obtain assay window between modified and non-modified peptide. The assay conditions are shown in **Table 6**. Later, binding tests with CP-peptides for citrullination (CP4/CP5) and deacetylation (CP6/CP7) and the same detection component were performed to optimize the assays.

Homogeneous PTM enzymatic assays. Inhibitor titrations (0-25 µM) were performed against PKA, PIM1-3, PAD4, and HDAC3 enzymes to validate the assays. H-89 and staurosporine inhibitors were used in the PKA assay, while staurosporine in the PIM1-3 assays, both assays with CP1 peptide. The PAD4

assays used CP4 and Arg-LZ peptides and GSK484 inhibitor. The HDAC3 assay used CP6 and LysAc-LZ peptides and TSA inhibitor. The assay conditions are shown in **Table 6**.

Detection peptide selection was performed using binding and enzymatic assays. Different charged detection peptides, Eu^{3+}-CPR1 and Eu^{3+}-CPR2, and a LZ-detection peptide Eu^{3+}-LZ were studied with CP1-3. The assay conditions are shown in **Table 6**.

4.5.3 The thermal dissociation assay using the peptide-break technology (IV)

Homogeneous thermal dissociation assay was performed to measure the thermal detachment profile of the peptide dimers. LZ-peptides for dephosphorylation (LZ-Y/LZ-pY), CP-peptides for deacetylation (CP6/CP7) and demethylation (CP8/CP9), and Eu^{3+}-LZ detection peptide were used in the experiments. In the assay, substrate peptides (10 μM) and a mixture of Eu^{3+}-LZ (1 nM) and MT10 (0.5 mM) were added to a 96-well plate and incubated 5 min. Next, the plate was heated from 25 °C (RT) to 90 °C using a thermal cycler, and the TRL-signal was recorded every 5 °C with a 3 min incubation at the chosen temperature. Later, a thermal binding assay using different ratios of phosphorylated (LZ-pY) and non-phosphorylated (LZ-Y) peptides and Eu^{3+}-LZ (1 nM) was performed to understand the assay sensitivity. In the assay, LZ-Y peptides were added to the LZ-pY solution in varying percentage (0, 0.5, 1, 5, 7, and 100%) keeping a 10 μM final concentration of LZ-Y and LZ-pY in the well.

PTM enzymatic monitoring using the thermal dissociation assay. Inhibitor titrations (0-1 μM) were performed against PTP1B and HDAC3 enzymes using Na_3VO_4 and TSA inhibitors, respectively. The enzymatic assay was performed as previously described. Assay conditions are shown in Table 6. Additionally, a *thermal dissociation assay using single-flash heating* was performed to increase the assay throughput. The optimal temperature was reached using a single flash heating, and the plate was kept 3 min at it before the measurement. The Z' factor was determined for PTP1B and HDAC3 assays using 18 positive and 18 negative controls (Na_3VO_4 and TSA at 5 μM).

5 RESULTS AND DISCUSSION

The studies presented from this doctoral thesis were focused concentrated on the development of a universal detection method for PTM enzymatic assays using the QRET technique in an antibody-free and HTS-compatible format. A summary of the results and discussion of publications **I-IV** is presented here.

The QRET technique is a detection method that has previously demonstrated its potential for HTS assays. It was first presented for receptor-ligand interaction screening assays in 2009 (Härmä *et al.* 2009) and successfully developed for other applications (Kopra & Härmä 2015). The simplicity of the method relies on its single-label approach and easy assay set-up, while its sensitivity is based on the use of the time-gated monitoring. This combination makes QRET a viable option for the development of PTM enzyme assays.

In this thesis, the main goal was to develop a universal detection platform for PTM enzyme activity monitoring using the QRET technique. The universal method, called the peptide-break technology, would be simple, sensitive, and versatile, allowing the monitoring of a wide range of different PTM types. Before proceeding with the peptide-break technology development, the QRET suitability for PTM monitoring was first evaluated in a homogeneous tyrosine EGFR assay **(I)**. The developed assay used antibodies to recognize the modified substrate peptide, which is a commonality in FRET, however in a single-label approach. On the basis of this, the peptide-break technology was further developed and optimized using QRET and a peptide-peptide binding approach. The approach was based on the leucine zipper **(II)** and electrostatic interaction **(III)** concepts, which allowed the use of concentrations in the nanomolar range. In the last stage of the project, the peptide-break technology was applied to a thermal dissociation assay allowing the use of substrate peptide concentrations in the micromolar range **(IV)**.

5.1 The QRET technique in a homogeneous EGFR kinase assay (I)

In publication **I**, a homogeneous QRET tyrosine EGFR kinase assay was introduced using a Eu^{3+}-chelate conjugated substrate peptide and a phosphotyrosine

specific antibody (**Figure 13**). The QRET technique distinguishes between the non-phosphorylated Eu^{3+}-peptide and the antibody bound to the phosphorylated Eu^{3+}-peptide in the presence of soluble quencher molecules.

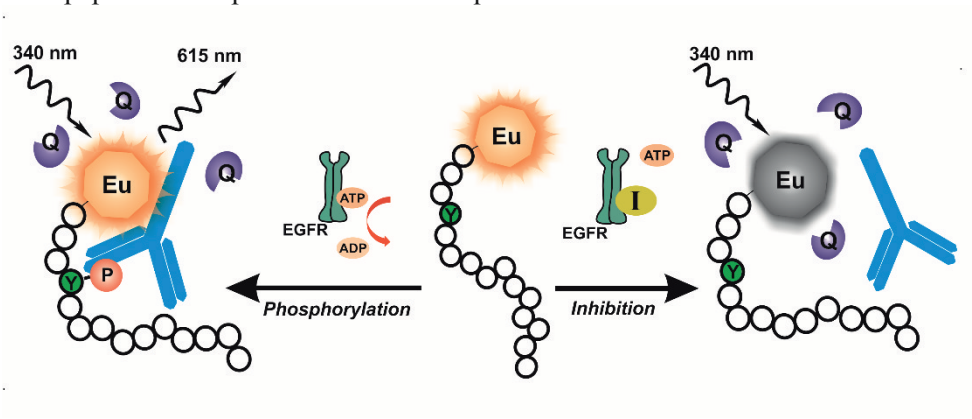

Figure 13. The principle of the homogeneous single-label epidermal growth factor receptor (EGFR) activity assay. Upon phosphorylation, a specific antibody binds to the phosphotyrosine Eu^{3+}-peptide and simultaneously protects the Eu^{3+}-chelate from quenching, resulting in a high TRL-signal. Conversely, in an inhibited reaction, the non-phosphorylated Eu^{3+}-peptide is preferentially quenched by the quencher molecules (Q) leading to low TRL-signal. Figure from the original publication **I**.

Three different peptides with phosphotyrosines at positions -2, -3 or -4 (PEP4-6) from the amino-terminal Eu^{3+}-chelate (**Table 5**) were designed to study the optimal Eu^{3+}-chelate protection from the antibody recognition. In the QRET binding assay, no significant differences between the peptides were found. All phosphotyrosine positions protected equally the Eu^{3+}-chelate from quenching after antibody recognition, yielding an averaged S/B of five. Despite this, the orientation or the proximity of the Eu^{3+}-chelate with regards to the phosphorylation site could interfere with the EGFR ability to phosphorylate. In this sense, PEP1-3 peptides were studied in a QRET enzymatic assay using the EGFR catalytic domain. The substrate peptide with the furthest tyrosine position (-4) (PEP 1) from the Eu^{3+}-chelate, showed the best S/B=4. This may be due to the increased distance between Eu^{3+}-chelate and tyrosine position providing less interference in the phosphorylation reaction and detection. PEP1 was selected to optimize the enzyme and antibody concentrations.

The utilized EGFR catalytic domain had multiple phosphotyrosine sites, which were found to increase the required antibody concentration in the assay. The relation between antibody and EGFR concentration was studied in titration assays. From these assays, a higher antibody concentration was required in the presence of

constant EGFR (2 nM) and phosphorylated peptide (10 nM) compared to assays without EGFR (**Figure 14A**). This was since the antibody recognizes equally all phosphotyrosines and not only the ones in the Eu^{3+}-peptide. Next, an EGFR titration assay was performed using 10 nM of phosphorylated peptide and 40 ng/well of antibody in a heterogeneous format to rule out the potential QRET effect. It was found that the antibody interaction with the phosphorylated Eu^{3+}-peptide was completely blocked at 25 nM EGFR concentration (**Figure 14B**). In this regard, about 5-fold higher antibody concentration (187 ng/well) was utilized for subsequent assays.

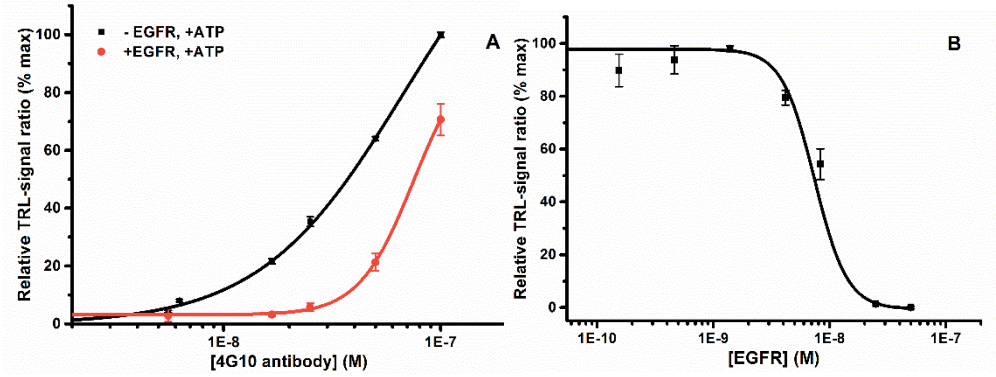

Figure 14. The relation between epidermal growth factor receptor (EGFR) and 4G10 antibody concentrations. (**A**) In a QRET assay with phosphorylated Eu^{3+}-peptide (PEP 4), the anti-phosphotyrosine antibody was titrated without EGFR (square) and with EGFR (circle) in the presence of ATP. (**B**) EGFR was titrated in the presence of PEP 4 and the antibody in a heterogeneous assay. Increasing the EGFR concentration increments also the number of available phosphotyrosine sites, thus diminishing the antibody binding to PEP 4. Data are the means ± SD (n=3). Figure modified from publication **I**.

In an antibody-based EGFR assay, high kinase concentrations would lead to high antibody concentrations, increasing the overall assay cost. In this regard, an EGFR titration was performed in a QRET enzymatic assay to assess the assay sensitivity. In the assay, the best S/B ratio was 3.9 achieved with 2 nM of EGFR, while 5 nM EGFR gave a S/B of 1.9, due to the increased antibody binding to the enzyme phosphotyrosines. In the assay using 2 nM EGFR, the degree of phosphorylation was approximately 70% of the total substrate peptide (calculated by comparing the TRL-signals of the synthetically phosphorylated peptide control under equal conditions, in the presence of EGFR and antibody). Validation of the developed assay was demonstrated using 2 nM of EGFR with a small panel of known ATP-competitive EGFR inhibitors with different potencies in the pico-to-nanomolar

range (Figure 15). The monitored IC_{50} values ranged between 0.08 and 155 nM with a S/B between 3.0 to 4.3.

In publication **I**, the suitability of QRET for enzyme activity monitoring in an antibody-based system was studied and proven. In the reported kinase assay, the substrate peptide was labeled with Eu^{3+}-chelate, and the phosphorylated peptide was detected by using a specific antibody. In all the antibody-based methods, the need for specific antibodies limits the applicability of the approach to other PTM types.

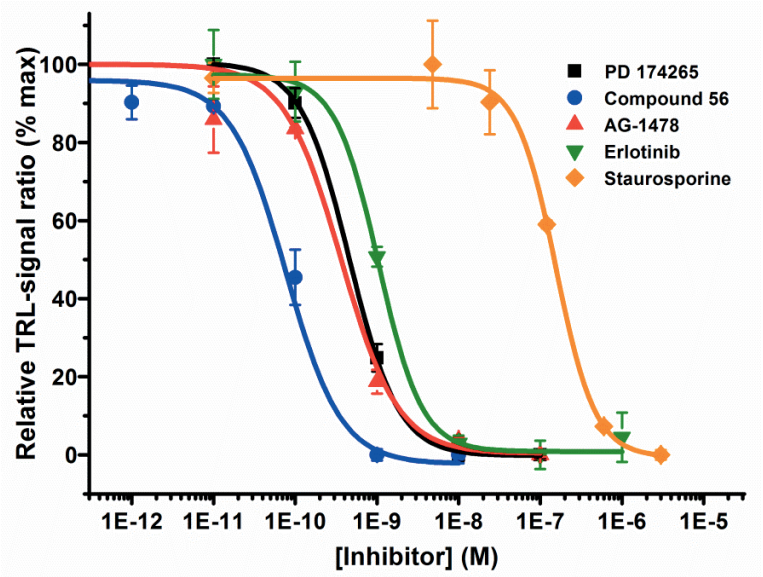

Figure 15. Dose-response curves for epidermal growth factor receptor (EGFR) inhibitors using the optimized QRET antibody-based kinase assay. The inhibitors were titrated from 0 to 5 µM concentration. Data are the means ± SD (n=3). Figure from publication **I**.

Antibody-based detection assays have been successfully developed for certain PTMs like tyrosine phosphorylation (Seethala & Menzel 1997; Varkondi *et al.* 2005; Degorce *et al.* 2009; Horton & Vogel 2010). Since the anti-phosphotyrosine antibodies are relatively insensitive to the surrounding AA context, they are broadly reactive. However, phosphoserine or phosphothreonine antibodies require a specific AA sequence surrounding the binding site, thus limiting the diversity of phosphorylated sites being recognized (Li *et al.* 2008). Also, developing pan-specific antibodies can be challenging for small size PTMs, as they are poorly antigenic (Zhao & Jensen 2009). In general, an antibody would have to be generated for each tested substrate peptide, which may also be time-consuming and expensive. The dependency on suitable antibodies limits the reported QRET kinase assay. Additionally, when extrapolating the system to other PTM types, the

conjugation of a Eu^{3+}-chelate for every tested peptide would have to be performed. Considering controls and additional peptides for assay optimization would mean a tremendous amount of work and an expensive approach. This work has proven the functionality of QRET in a PTM enzymatic assay. The assay is potentially applicable to other kinases with a substrate peptide containing a suitable sequence for the studied kinase and a specific antibody for the phosphopeptide detection. However, having an antibody-based detection system with a substrate peptide being labeled for every enzyme studied does not resemble the best approach for a universal PTM enzymatic assay.

5.2 The peptide-break technology as a universal detection method for a wide spectrum of PTMs (II, III, IV)

As challenging new targets are emerging, new screening strategies should also be raised to fill the unmet HTS technology needs. While antibody-based detection assays provide a sensitive system for enzyme activity monitoring, there are limiting factors related to the availability of suitable antibodies for the desired targets. Antibody-free methods have been developed such as IMAP (Loomans *et al.* 2003), or Omnia (Shults & Imperiali 2003), which have been actively developed for kinases, but the nature of its approach has limited expandability to other PTM types. Other technologies, such as Transcreener, LANCE, or AlphaScreen, have been able to provide tools for a more extended PTM set, e.g., kinases, methyltransferases, and deacetylases, although at the cost of having a detection system which depends on antibodies or enzyme cascades.

The peptide-break technology was developed using QRET and a peptide-peptide binding approach in an antibody-free detection system. The binding approach was based on the leucine zipper **(II)** and electrostatic interaction **(III)** concepts, which were optimized to be used in the nanomolar range concentrations. Later, the peptide-break technology was applied to a thermal shift assay, which allowed the use of substrate peptide concentrations this time in the micromolar range **(IV)**.

5.2.1 The peptide-break technology using the leucine zipper approach (II)

Leucine zippers (LZ) are coiled-coil dimerization domains of the basic-region leucine zipper (bZIP) transcription factor proteins. These bZIP proteins are regulators of cellular processes including cell proliferation, tissue differentiation, and the response to oxygen or AA deprivation (Kaplan *et al.* 2014). Their activities depend on the specific DNA recognition and the stability and specificity of protein dimer formation (O'Shea *et al.* 1991). The LZ motifs mediate protein dimerization,

which is required to promote DNA binding and subsequently influence the RNA synthesis rate (Daugherty & Gellman 1999). In vitro studies have shown that LZ sequences between only 30 to 40 residues are sufficient for coiled-coil heterodimeric formation (O'Shea *et al.* 1991), having K_D values ranging from pM to high µM (Daugherty & Gellman 1999; Kaplan *et al.* 2014). The LZ sequences are arranged by heptad repeats denoted as **(abcdefg)*n***, where ***a*** and ***d*** are usually hydrophobic residues, with leucines in ***d*** positions. The ***e*** and ***g*** positions are usually long, charged or polar amino acids that contribute to the electrostatic interactions. The remaining positions ***b***, ***c,*** and ***f*** are located at the surface, away from the coiled coil (Daugherty & Gellman 1999; Kaplan *et al.* 2014) (**Figure 16A**).

In the peptide-break technology, a LZ dimer is formed by a Eu^{3+}-labeled detection peptide and the substrate peptide designed for the studied enzyme. The LZ dimer formation leads to Eu^{3+}-chelate protection and a high TRL luminescence signal, while the addition of a PTM to the substrate peptide leads to dissociation of the peptide dimer enabling signal attenuation from the soluble quenchers. The peptide pair re-associates when the PTM is cleaved from the substrate peptide providing a method to investigate transferases and hydrolases (**Figure 16B**).

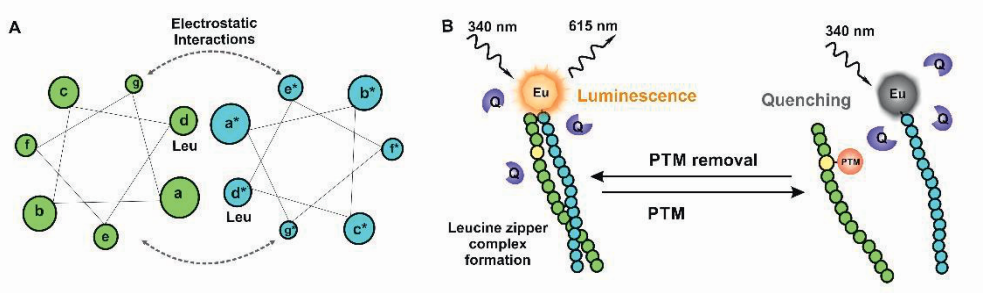

Figure 16. The principle of the universal peptide-break technology and leucine zipper interaction. (**A**) In the LZ dimer structure, ***d***-positions are occupied with leucines and typically ***a***-positions with hydrophobic amino acids. Positions ***e*** and ***g*** contain typically charged residues contributing to the binding. (**B**) In the peptide-break technology, a high TRL-signal is detected upon leucine zipper (LZ) dimer formation, and dimer detachment due to a PTM addition promotes luminescence quenching. Figure modified from publication **II**.

The highly characterized LZ from the Fos and Jun proteins, which form the heterodimeric complex AP-1 transcription factor, was used as a starting point for the assay development (Kaplan *et al.* 2014). Jun and Fos sequences of 36 AA length from the LZ domain were selected to serve as substrate peptide and detection peptide, respectively. Helicity studies of the peptides at 30 µM showed

that the Jun/Fos pair formed a typical helical structure, while the helical structure was lost for the p-Jun/Fos pair, giving a 222 nm/208 nm ratio of 0.89 and 0.56, respectively (**Figure 17A**). This corroborated that the PTM presence in the substrate peptide is able to destabilize the peptide dimer helicity. Homogeneous binding assays showed an EC_{50} value of 2.5 µM for the Jun/Fos, while no binding was detectable under the tested conditions for the p-Jun/Fos pair **(Figure 17A**, inset). On this basis, higher affinity peptides were designed to enable the measurement of PTMs with higher sensitivity by shifting the peptide break-down point to nanomolar concentrations. This was a critical point in the assay development, as the new peptides should have optimal peptide-pair binding with sufficient affinity and dissociation properties. Helicity studies of the redesigned peptides, tyrosine phosphorylated (TyroP-LZ), non-phosphorylated (Tyro-LZ) and detection peptide (EuLZ), indicated substantial helicity for both dimers Tyro-LZ/EuLZ and TyroP-LZ/EuLZ at 30 µM, giving a 222 nm/208 nm ratio of 0.75 and 0.77, respectively (**Figure 17B**). Higher affinity binding was indeed achieved as even the phosphorylated residue in the TyroP-LZ/EuLZ dimer was unable to destabilize the typical helical structure when using 30 µM of peptides.

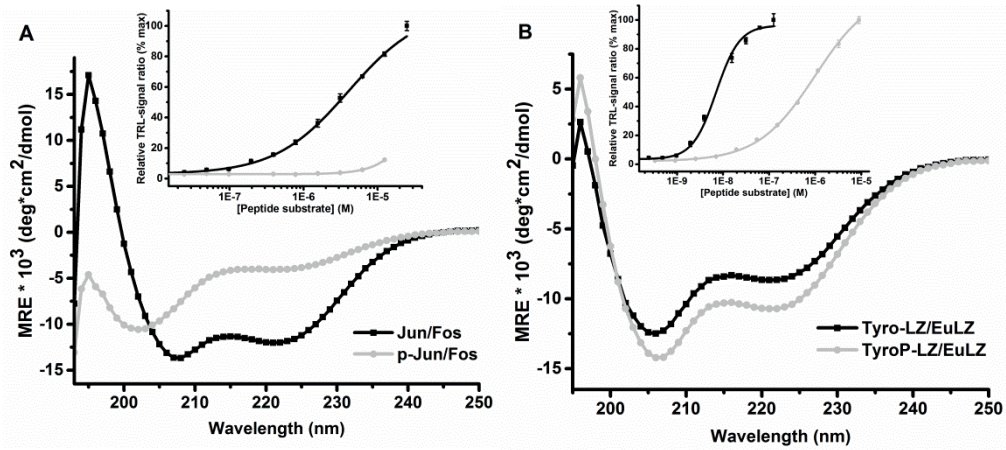

Figure 17. Structural and binding studies of phosphorylated and non-phosphorylated LZ peptides. (**A**) CD spectra of Jun/Fos and p-Jun/Fos dimers at 30 µM show no helicity for the phosphorylated p-Jun/Fos dimer, (**B**) while CD spectra of Tyro-LZ/EuLZ and TyroP-LZ/EuLZ dimers at 30 µM indicate substantial helicity for both dimers. Homogeneous QRET binding tests for all the peptide pairs are shown in the insets. Figure modified from publication **II**.

The homogeneous binding assays gave EC_{50} values of 0.3 µM and 0.006 µM for the TyroP-LZ/EuLZ and the Tyro-LZ/EuLZ pair, respectively (**Figure 17B**, inset). The EC_{50} value differences between the phosphorylated and non-phosphorylated

pair provide the basis to measure PTMs at nanomolar level with a suitable assay window.

The functionality of the peptide-break technology was first tested with a small panel of selective and non-selective inhibitors against EGFR and PKA kinases. Later, the universality of the technology was studied using PTP1B phosphatase, PAD4 amidinotransferase, Sirtuin 1 and HDAC3 deacetylases (**Figure 18**). Substrate peptides were designed for each studied enzyme considering the required consensus sequences and the LZ sequences to maintain the binding with the detection peptide. The obtained IC_{50} values were comparable to the literature values, and the S/B ratios ranged between 4–97. The generated data proved the versatility of the assay, indicating potential applicability to other PTMs using this single-label and antibody-free approach. Additionally, the peptide-break technology was further adapted to a real-time kinetic mode using the phosphorylation developed assays for EGFR and PKA kinases. The adapted method applies to a continuous signal reading as previously demonstrated in QRET-based assays (Kopra *et al.* 2014a, 2014b).

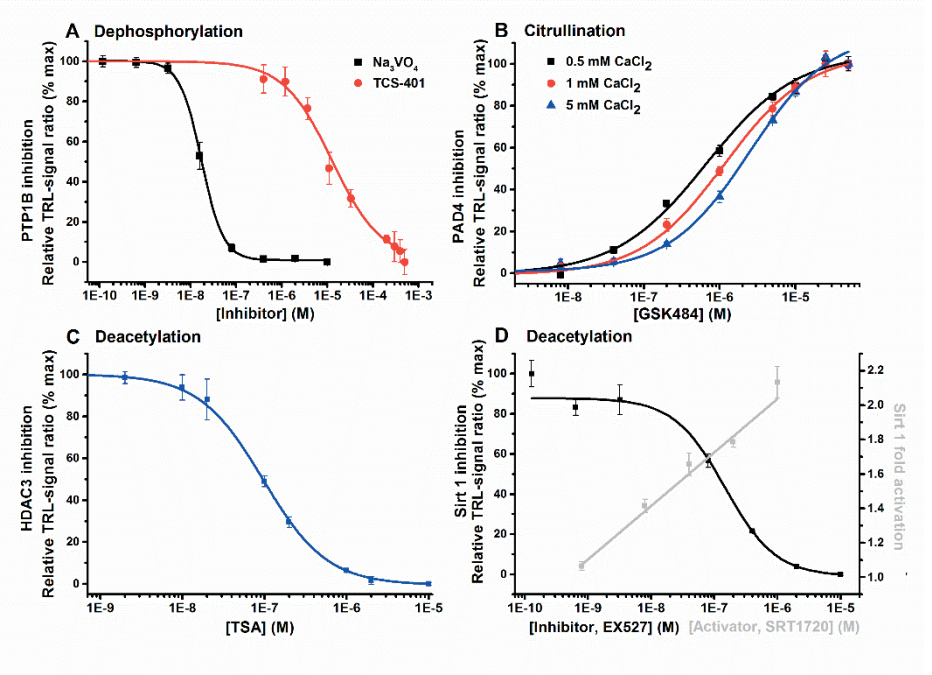

Figure 18. Dose-response curves for inhibitors and an activator against the studied enzymes. Compounds were run against (**A**) PTP1B phosphatase, (**B**) PAD4 amidinotransferase at varying $CaCl_2$ concentrations, (**C**) HDAC3 deacetylase, and (**D**) Sirtuin1 deacetylase. Typical sigmoidal curves are shown in the figures. Data represent the means ± SD (n=3). Figure modified from publication **II**.

Assay validation was performed by running a small-scale HTS screen with 356 compounds of the PKIS library against EGFR and PKA kinases. Before the screening, DMSO tolerance was evaluated in a 10 µl miniaturized assay, showing tolerance of 10% DMSO. From the screening, 10/13 PKA inhibitors (**Figure 19A**) and 42/48 EGFR inhibitors (**Figure 19B**) were found with IC_{50} values lower than 1 µM. Assay robustness was investigated during the PKA screen, and an average Z' value of 0.67 was obtained using 96 negative and positive controls.

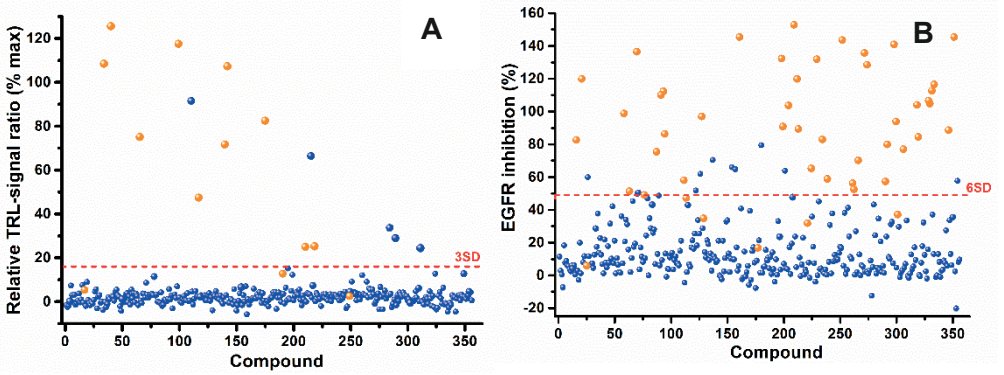

Figure 19. Validation of the universal peptide-break technology using the PKIS library in PKA and EGFR kinase assays. The screening was performed at 1µM compound concentration and a (**A**) 3SD threshold for hit identification was set for PKA and a (**B**) 6SD threshold for EGFR assays. The number of detected compounds are comparable with the Nanosyn Caliper screening information found in the literature (Elkins *et al.* 2016). The S/B ratio was 12 and 6 for the PKA and EGFR kinase assays, respectively. Figure modified from publication **II**.

In publication **II,** a simple, sensitive, and versatile luminescent assay was reported, which enabled the monitoring of small-size PTMs. The key benefit of the peptide-break technology relies on its versatility. The assay development is simple as the detection peptide sequence remains the same regardless the substrate peptide sequence of the studied enzyme. Using this approach, it could be assumed that any PTM could be measurable. However, inserting a consensus sequence in the LZ peptide can present a great challenge in the peptide design. The leucines located at ***d***-positions, which are spaced 7 residues apart along an α-helix and the coiled-coil interface of the LZ, have to be conserved to provide sufficient peptide-peptide binding affinity (Daugherty & Gellman 1999; Kaplan *et al.* 2014). Simultaneously, the modifiable AA should provide sufficient dissociative properties only upon PTM addition. These challenges lead to the following studies reported in the publication **III** and summarized in the following section.

5.2.2 The peptide-break technology using the charged peptides approach (III)

While some enzymes are insensitive to the peptide sequence context they modify, other enzymes may require specific amino acids flanking the modification site for substrate peptide recognition (Bheda *et al.* 2016). For example, enzymes such as O-GlcNAc glycotransferases (OGTs), which glycosylate serine or threonine, require specific amino acids such as proline, which is known to be the most sterically restricted of all the amino acids (Pathak *et al.* 2015). Proline gives conformational rigidity to the peptide sequence, which would disrupt the LZ dimer coiled-coil structure in the peptide-break system. This would result in loss of the dimer formation between the substrate peptide and the detection peptide, thus, limiting the use of the technology for this enzyme target.

In publication **III**, we introduced the peptide-break technology based on a peptide dimerization utilizing electrostatic interactions between oppositely charged peptides. In the system, the affinity of the peptide dimer required optimization to obtain dimer dissociation upon PTM addition. There was no need to maintain a leucine in a specific position, and the coiled-coil structure was not required for peptide-peptide binding. Since only the overall peptide charge gives the basis of peptide dimerization, the freedom for enzyme substrate peptide selection was increased. Charge-based (CP) peptides were studied using sequences without leucines, and the optimal peptide architecture was adjusted in PKA- and PIM-mediated serine phosphorylation assays. After that, the concept was tested in arginine citrullination and lysine deacetylation assays. Three substrate peptides (CP1-3) with two phosphorylation sites, varying length (19 to 34 residues), and different charges (+10 to +23) were designed for PKA activity monitoring (**Table 5**). The CP1-3 affinities were studied by their binding to the previously described Eu^{3+}-LZ detection peptide, which was negatively charged, and compared to the LZ substrate peptide (Ser-LZ) for PKA kinase **(II).** Binding assays using 10 nM of peptide showed that the affinity of the duplex formation followed the order of the positive charge of the substrate peptide (**Figure 20A**).

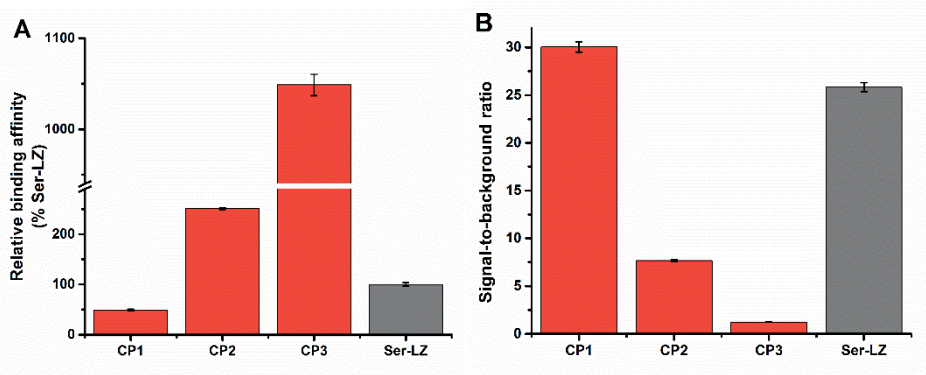

Figure 20. Binding and functionality properties of the charge-based (CP) and leucine zipper-based peptides. (**A**) The affinity of the CP-peptides was determined against Eu^{3+}-LZ, and compared to Ser-LZ for PKA kinase. The affinity of the peptides was in order CP3 >> CP2 > Ser-LZ > CP1. (**B**) Enzymatic PKA assay with CP and LZ peptides was performed to obtain assay window between modified and non-modified peptide. The lowest binding affinity (CP1) provides the highest separation after PTM addition. Data represent the means ± SD (n=3). Figure from publication **III**.

Based on the data, a net charge between +10 (CP1) and +13 (CP2) in the CP-peptide is needed to obtain the binding affinity comparable to the Ser-LZ control with Eu^{3+}-LZ. The longest peptide, CP3 (+23), showed ultra-high affinity in binding compared to Ser-LZ. Since the phosphorylated residues may not be able to disrupt this high-affinity complex formed with Eu^{3+}-LZ, CP3 was not expected to be functional in the enzymatic assays. In the PKA kinase assay, the highest S/B ratio was obtained with the 19 AA CP1 (S/B=30) (**Figure 20B**), which showed the lowest affinity in binding to Eu^{3+}-LZ reporter (**Figure 20A**). This pair gave an S/B ratio comparable to the control LZ-pair (S/B=26). The two phosphorylated sites on CP1 seemed to disrupt the peptide dimer efficiently, allowing sufficient TRL-signal quenching and thus a low background. CP2 and CP3 peptides also showed high binding levels after phosphorylation, leading to lowered peptide-pair dissociation efficiency with S/B ratios of 7.7 and 1.2 for CP2 and CP3, respectively (**Figure 20B**). This points out that the substrate peptide design should be optimized considering the peptide affinities and the characteristics of studied PTM, as small-size PTMs such as methylation (Hamamoto *et al.* 2015) may not disrupt a high-affinity dimer. Based on these results, CP1 was selected for further assay functionality studies in dose-response measurements using PKA kinase and three PIM family kinase assays. In the inhibitor titrations, both PKA and PIM assays showed typical sigmoidal inhibitor curves with IC_{50} values in line with the literature.

The concept was also tested in citrullination and deacetylation assays using CP and LZ peptides side-by-side. Based on CP1, CP-peptides were selected for PAD4 (citrullination) and HDAC3 (deacetylation) assays containing two modification sites and keeping the +10 charge and 19 AA-length of CP1 (**Table 5**). Similar S/B ratios were obtained between the binding and enzymatic assays for both PTMs using the negatively-charged Eu^{3+}-LZ detection peptide (**Figure 21A**), confirming the correct assay functionality. Later, the assay validation was performed in dose-response assays. The obtained IC_{50} values were comparable between the CP- and the LZ peptide assays and the S/B ratios range between 6 and 8 for all the assays (**Figure 21B**).

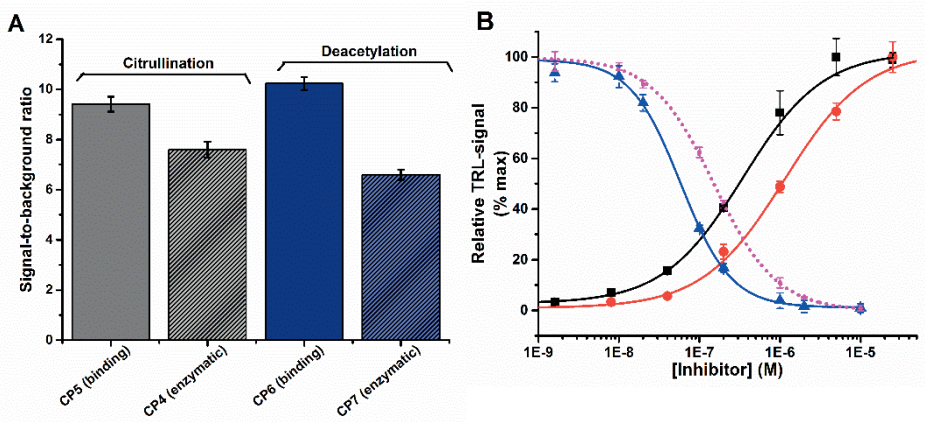

Figure 21. Binding and functionality properties of the charge-based citrullination and deacetylation peptides. (**A**) Similar S/B ratios were obtained between binding (plain bars) and enzymatic (stripped bars) assays for citrullination and deacetylation. (**B**) Inhibitor titrations against PAD4 and HDAC3 enzymes gave characteristic sigmoidal curves. GSK484 titration was performed for citrullination with the CP4 (squares) and Arg-LZ (circles) peptides, while TSA titration for deacetylation with CP6 (triangles) and LysAc-LZ (dotted line). Similar IC_{50} values were obtained between the CP- and LZ-peptides. Data represent the means ± SD (n=3).

Additionally to the substrate peptide studies, charge-based detection peptides were studied with CP1-3 substrate peptides. The 36 AA-length of the previously studied Eu^{3+}-LZ detection peptide was necessary for dimerization. However, as the CP-peptides bind through electrostatic interactions, the net charge is crucial and not the peptide length. Besides, the CP1-3 substrate peptides contained 19 AA, with which a 36 AA-length detection peptide was not needed. Two negatively-charged Eu^{3+}-peptides of 15 AA (Eu^{3+}-CPR1) and 27 AA (Eu^{3+}-CPR2) were studied using the CP1-3 peptides in binding and enzymatic PKA assays. No differences in the binding affinity were found within the CP1-3 peptide series and the Eu^{3+}-CPR2 due

to the apparent high affinity of the peptide (-23). However, the affinity increased with the increasing positive charge of the substrate peptide with the 15 AA Eu^{3+}-CPR1 (-12). In the PKA assays, the CP1/Eu^{3+}-CPR1 pair gave S/B=8.9, while the CP2/Eu^{3+}-CPR1 pair gave S/B=3.6. The higher affinity of CP2 compared to CP1 showed decreased separation between phosphorylated and non-phosphorylated peptides, while the ultra-high affinity of CP3 prevented any PTM dependent separation (S/B=1).

These data demonstrate that with CP-peptides the sequence length can be significantly reduced without losing the required affinity or assay window between modified and non-modified peptides, which is not possible when using LZ peptides. The CP peptides can be easily modified as they are not restricted to specific amino acids in certain positions or to a structural conformation, but the peptide net charge is crucial. In this sense, the peptide affinity can be adjusted by simply changing the number of charges in the peptide and their distribution. This versatility provides to the peptide-break technology the possibility of extrapolating the system from one PTM to another, as in the case of phosphorylation, deacetylation and citrullination assays **(III)**. Working with charged peptides could bring some disadvantages. Charged molecules present in the assay would potentially bind to the oppositely charged peptides. However, we have not observed those interferences in the reported assays. The labeled detection peptide binds to the substrate charged peptide with high affinity. Additionally, charged amino acids in the substrate peptide could negatively affect the substrate recognition by the enzyme, if the enzyme is highly dependent on a consensus sequence. But working with high-affinity substrate peptides and in high concentration would perhaps favor the peptide recognition. More studies on the charged-based system would be needed to know its limitations and disadvantages. However, this system shows so far the advantage of higher freedom to modify the substrate peptide and brings options for detection, as the Eu^{3+}-peptides can be selected freely.

5.2.3 A luminescence thermal dissociation assay using the peptide-break technology (IV)

The thermal shift assay (TSA) is a biophysical method applied to drug discovery as well as structural studies (Redhead *et al.* 2017). TSA for drug discovery relies on the stabilization of the complex formed by an interaction such as a ligand and a protein. By increasing the unfolding temperature, a fluorescence signal change is monitored in the presence of a fluorescent dye sensitive to protein denaturation (Scott *et al.* 2016). The ligand-binding affinity is estimated by the change of the unfolding temperature detected in the presence and absence of the ligand. A

fluorescence-based TSA has been proposed as an easy-to-develop method for hit identification compared to other biophysical methods (Lo *et al.* 2004). It can be performed using a standard thermal cycler instrument for PCR and a plate reader for fluorescence monitoring.

In publication **IV**, the peptide-break technology was applied to a luminescence TSA that harnessed the thermal stability difference of the post-translationally modified and non-modified peptide dimer (**Figure 22**). A high peptide concentration forces peptide dimerization independent of the PTM presence (**Figure 17B**), and the PTM addition reduces the affinity between dimer-forming peptides. In the luminescence TSA approach, the peptide dimer stability decreases at elevated temperature, being less stable the dimer with a PTM. As a result, higher TRL-signals are detected for the peptide dimer without a PTM.

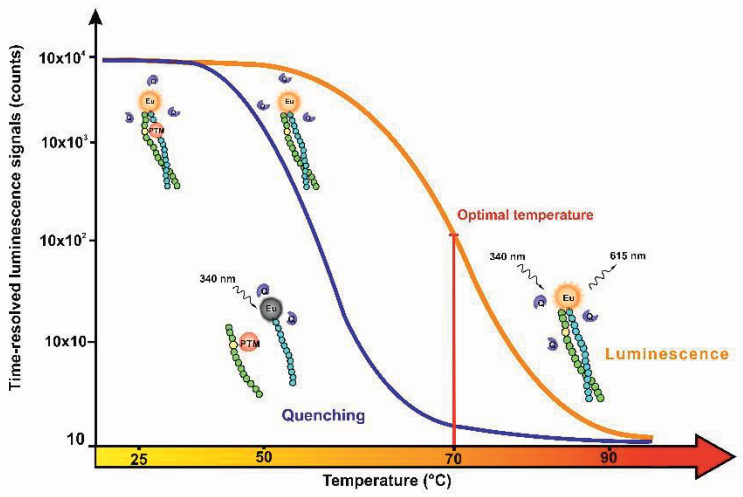

Figure 22. The principle of the thermal dissociation assay using the peptide-break technology. At room temperature, high peptide concentration drives the peptide dimerization irrespective of the PTM presence. As the PTM induces dimer disruption, the affinity between the dimer-forming peptides with a PTM (blue line) is lower than the dimer without a PTM (orange line). At the optimal temperature, the dimer with a PTM is disrupted allowing signal attenuation due to the quenchers in solution, while the non-modified dimer remains intact resulting in Eu^{3+}-chelate protection and high TRL-signal.

The peptide-break approach was evaluated for the TSA using LZ- and CP-peptides. The thermal profile of the non-modified LZ-Y and phosphorylated LZ-pY with Eu^{3+}-LZ showed an increase in the monitored S/B ratio being 2.5 at RT and 36 at 70 °C (**Figure 23A**). This demonstrated that there was PTM-independent binding of the peptides at RT when 1.5 nM Eu^{3+}-LZ and 10 µM substrate peptide were

used. However, at 70 °C, the LZ-pY/Eu^{3+}-LZ dimer stability was lower than the stability of the LZ-Y/Eu^{3+}-LZ. As a result, a difference in the recorded luminescence signals was observed due to the presence of the quenchers which lead to a high S/B ratio. The assay sensitivity was evaluated using LZ-pY and LZ-Y peptides in different ratios keeping the overall peptide concentration to 10 µM. At 70 °C, 1% (100 nM) of the non-phosphorylated dimer was detected from the phosphorylated population giving a S/B=7 (**Figure 23B**). The peptide-break technology was optimized for PTM monitoring in the nanomolar range to increase the assay sensitivity. Micromolar concentrations of the peptides in the assay forces peptide dimerization independent of the PTM presence. Using temperature-induced dimer disruption allowed the use of peptide concentrations in the micromolar range and detection of nanomolar concentrations of the modified substrate peptide.

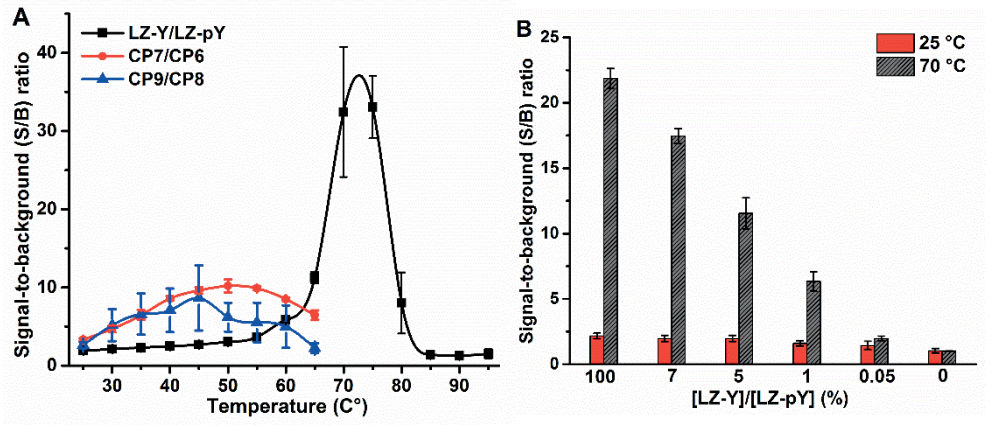

Figure 23. Thermal dissociation response of the leucine zipper (LZ)- and charge based (CP)-peptides. (**A**) The thermal profile of the LZ-Y/LZ-pY peptides for dephosphorylation showed an optimal temperature of 70 °C, while the CP-peptides profile showed 50 °C for deacetylation (CP7/CP6) and 45 °C for demethylation (CP9/CP8) using 10 µM of substrate peptides and 1.5 nM Eu^{3+}-LZ in the system. (**B**) The thermal assay sensitivity was evaluated using different ratios of LZ-pY and LZ-Y peptides considering 10 µM total concentration. At 70 °C, less than 1% (100 nM) of non-phosphorylated peptide LZ-Y was detected from the 10 µM LZ-pY pool with a S/B=7. Data are shown as means ± SD (n=3). Figure modified from publication **IV**.

The TSA was tested for deacetylation and demethylation using CP6/CP7 and CP8/CP9 peptides, respectively. The thermal profile showed that the optimal temperature for demethylation and deacetylation was 45 °C (S/B=5.1) and 50 °C (S/B=10.5), respectively (**Figure 23A).** Comparing the optimal temperatures

between the CP- and LZ-peptides, indicated that a higher temperature is needed to affect the LZ dimer (70 °C). This difference may be explained by the different binding mechanisms and differential affinity of the peptide pairs. The apparent stability of the LZ dimer against thermal-induced dimer disruption is greater than the CP dimer stability. The LZ dimer formation is based on a specific binding through leucines and electrostatic interactions (Kaplan *et al.* 2014), while the charged peptide dimerization is based on non-specific electrostatic interaction. The quenching effect is crucial to obtain the S/B ratio, since no differences were observed when the thermal profiles were measured without the quencher.

The TSA was tested for enzyme activity monitoring using PTP1B for dephosphorylation and HDAC3 for deacetylation. In the dephosphorylation assay, a maximal S/B ratio of 25 was monitored at 70 °C (Figure 24A), while in the deacetylation 3.8 was monitored at 55 °C (**Figure 24B**). In the case of CP-peptides, the thermal curve had a broader maximum, which was observed in both synthetic and enzyme-catalyzed reactions. This may be a thermal profile proper of the CP-peptides as this was not observed with the LZ-peptides.

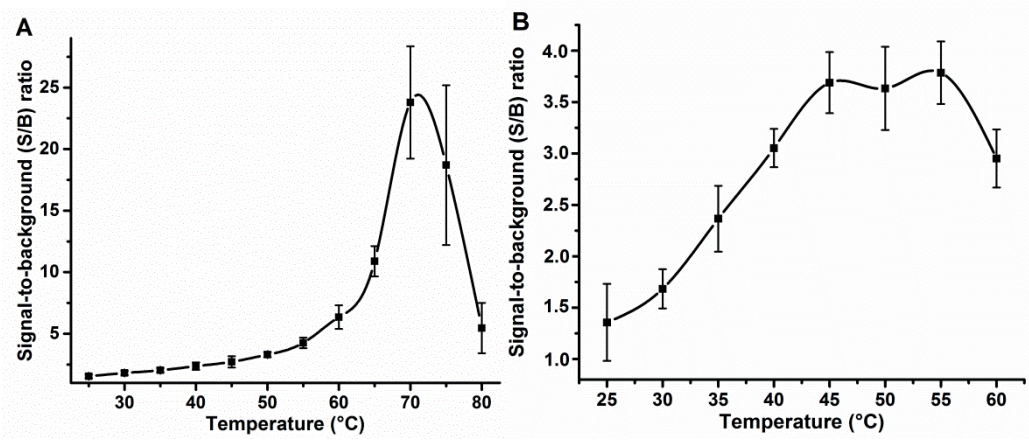

Figure 24. Enzymatic dephosphorylation and deacetylation assays using the thermal shift based peptide-break technology. (**A**) Dephosphorylation assay using LZ-pY and Eu^{3+}-LZ peptides gave a S/B=25 at 70 °C, while (**B**) deacetylation assay using CP6 and Eu^{3+}-LZ showed a broad peak in the temperature profile being optimal at 55 °C with a S/B ratio of 3.8. Data are shown as the means ± SD (n=3). Figure modified from publication **IV**.

The thermal dissociation assay was validated by performing dose-response measurements for inhibitors against the studied enzymes. The calculated IC_{50} values were 3.8 nM for Na_3VO_4 and 9.1 nM for trichostatin A, used to inhibit PTP1B and HDAC3, respectively. The monitored values were in line with the

published literature (Gordon 1991; Muthyala *et al.* 2015). Additionally, the proposed system was tested using single temperature flash heating. This protocol was expected to be better suited for HTS, as only 3 min at the selected temperature was needed to induce the separation between modified and non-modified peptides. Z' factor values > 0.6 were monitored with the flash-heating for both enzymatic assays.

The QRET-based thermal dissociation assay using the peptide-break technology was presented as a proof-of-principle for dephosphorylation and deacetylation enzymatic assays, but could potentially be applied to other PTM types. The assay enabled the use of high substrate peptide concentrations (µM range) to enable the monitoring of low activity or affinity enzymes. Enzymes with low activity are challenging to measure as low concentrations of the modified product are produced. In this sense, a high peptide concentration would enable the full enzyme activity. The majority of the TSA applications have been focused on ligand-binding affinity studies. In drug discovery, TSA can be used to identify compounds that bind to a protein and stabilize it against thermal denaturation (Redhead *et al.* 2017). In this project, TSA for enzyme activity monitoring was developed using the peptide-break technology. The method can use µM concentrations of substrate peptides and detect nM concentrations of the post-translationally modified peptide.

6 SUMMARY AND CONCLUSIONS

Nowadays, the available HTS technology offers several options for the drug discovery and development community to select methods that better suit their goals. However, the current technologies are not yet adaptable to the emerging complex targets. There is a demand for new screening strategies and versatile technologies capable of addressing the novel targets issues.

The doctoral study concentrated on the development of a universal detection platform for PTM monitoring using the QRET technique in an antibody-free system. All the assays were constructed using Eu^{3+}-chelates in a single-label approach and HTS compatible format. The suitability of QRET for PTM monitoring was first evaluated in a conventional homogeneous EGFR kinase assay **(I)** using antibodies to recognize the modified substrate peptide. On the basis of this the peptide-break technology was further developed using an antibody-free and peptide-peptide binding approach. This was assessed based on the leucine zipper **(II)** and electrostatic interaction **(III)** concepts, which allowed the use of nanomolar concentrations in the assays. In the publication **IV**, the peptide-break technology was applied to a thermal shift assay, allowing the use of peptide concentrations now in the micromolar range.

The main conclusions based on the original publications are:

I In this article, the suitability of QRET for PTM enzymatic monitoring was demonstrated in a phosphorylation assay. Although, as with FRET assays in general, an antibody (anti-phosphotyrosine) was used for detection, the QRET-based kinase assay was achieved in a single-label approach. Moreover, the data indicated that the assay could potentially be applied to other enzymes, with the correct substrate peptide and antibody for detection. However, this would need the expensive and time consuming production of a Eu^{3+}-labeled peptide for every enzyme. Also, as in all antibody-based

detection systems, the extension of the approach would be limited by the availability of suitable PTM targeting antibodies. In this sense, this approach does not represent the best system for a universal PTM enzymatic assay.

II The current HTS methods for PTM monitoring face detection limitations, including antibody dependency, dual-labeling, and so forth, which constrains their applicability to other complex PTMs. Based on publication I, the peptide-break technology was developed using QRET in an antibody-free system and a peptide-peptide binding approach following the LZ concept. Its key benefit relies on its simplicity and versatility, as a unique detection Eu^{3+}-peptide was used for assaying different PTMs. The main challenge with this was for the substrate peptide design, as the enzyme consensus sequence had to be adapted to the LZ concept for dimerization. Despite this limitation, the peptide-break technology was developed for a variety of enzymes for phosphorylation, dephosphorylation, deacetylation, and citrullination without the need for antibodies or enzyme reporters.

III In this article, the peptide-break technology was simplified and optimized to increase the freedom of substrate peptide selection. The peptide dimerization was based on electrostatic interactions between oppositely charged peptides. In the system, no specific residues in certain positions nor structural conformations were needed for dimerization, but the peptide net charge was crucial. This feature provided freedom to modify the peptide length and sequence without losing the required affinity or assay window. The peptide-break technology with charged peptides was optimized using PKA- and PIM-mediated phosphorylation assays, and further developed for lysine deacetylation, and arginine citrullination assays. The simplified and expanded peptide-break technology can potentially be applied to a variety of PTMs by simply introducing an enzyme-specific sequence to the charged substrate peptide.

IV In this article, the peptide-break technology was applied to a QRET-based thermal shift assay for dephosphorylation and deacetylation. In a standard peptide-break assay, micromolar peptide concentrations force peptide dimerization independent of the PTM presence. However, applying increasing temperature to that system induced dimer disruption yielding S/B ratios > 4 for deacetylation and > 20 for dephosphorylation assays. The peptide-break thermal shift assay allows the use of high peptide concentrations needed in assays for low affinity enzymes with and low activity enzymes. The method can be used with micromolar concentrations

of substrate peptides and detect nanomolar concentrations of the post-translationally modified peptide. The approach was demonstrated for two hydrolases but could potentially be applied to other targets.

As new targets are emerging, new HTS technologies for the primary screening phase are in demand. The QRET-based peptide-break technology developed in these studies aimed to provide a versatile biochemical assay adaptable to challenging PTMs. Even though the presented data was limited to small PTMs, the groundwork presented here suggests the technology could be potentially applicable to other PTM types, e.g., glycosylation, and perhaps to the simultaneous assaying of multiple enzyme targets. The path from developing a novel HTS technology to launching a robust commercial kit was beyond the scope of this doctoral work, but we truly expect that the method can be used by the scientific community within academia as well as to the pharmaceutical industry.

7 ABBREVIATIONS

AA	amino acid
Acetyl CoA	acetyl coenzyme A
ADP	adenosine diphosphate
ALPHA	amplified luminescent proximity homogeneous assay
APP	β-amyloid precursor protein
ATP	adenosine triphosphate
Aβ	amyloid β-peptide
BACE1	β-amyloid precursor protein cleaving enzyme
bZIP	basic-region leucine zipper
CCD	charge-coupled device
CD	circular dichroism
CHEF	chelation-enhanced fluorescence
DELFIA	dissociation-enhanced lanthanide fluorescence immunoassay
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
EZH2	enhancer of zeste homolog 2
FI	fluorescence intensity
FP	fluorescence polarization
FRET	fluorescence resonance energy transfer
GEF	guanidine nucleotide exchange factor
GFP	green fluorescence protein
GPI	glycosylphosphatidylinositol
Grb2	growth factor receptor-bound protein 2
HDAC	histone deacetylase
HER2	human epidermal growth factor receptor 2
HPLC	high performance liquid chromatography
HTS	high throughput screening
ITC	isothermal titration calorimetry
KATs	lysine acetyltransferases

KDACs	lysine deacetyltransferases
LMC^{3+}	lewis metal chelate
Ln^{3+}	lanthanide
LOD	limit-of-detection
LZ	leucine zipper
MAPK	mitogen-activated protein kinase
MOA	mechanism of action
MRE	mean residue ellipticity
MS	mass spectrometry
NATs	Nt-acetyltransferases
Nt	N-terminal
OGTs	O-GlcNAc glycotransferases
PAINS	pan-assay interference compounds
PDK1	phosphoinositide-dependent kinase-1
PH	phosphocellulose
PhS	phosphosensor
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKIS	published kinase inhibitor set
PPIs	protein-protein interactions
PTEN	phosphatase and tensin homolog
PTM	post-translational modification
PTPs	protein tyrosine phosphatases
pTyr	phosphorylated tyrosine
QRET	quenching resonance energy transfer
RT	room temperature
RTKs	receptor tyrosine kinases
SA	streptavidin
SAR	structure-activity relationship
SH2	Src homology 2
Sox	sulfonamide-oxine
SPA	scintillation proximity assay
S/B	signal-to-background
TEAA	triethylammonium acetate
TRF	time-resolved fluorescence
TRL	time-resolved luminescence
TSA	thermal-shift assay

8 REFERENCES

Acker, M.G. & Auld, D.S. (2014) Considerations for the design and reporting of enzyme assays in high-throughput screening applications. *Perspect Sci* **1**, 56–73.

Adams, J.A. (2001) Kinetic and Catalytic Mechanisms of Protein Kinases. *Chem Rev* **101**, 2271–2290.

Aebersold, R. & Mann, M. (2016) Mass-spectrometric exploration of proteome structure and function. *Nature* **537**, 347–355.

Allen, K.N. & Imperiali, B. (2010) Lanthanide-tagged proteins - an illuminating partnership. *Curr Opin Chem Biol* **14**, 247–254.

Anastassiadis, T., Deacon, S.W., Devarajan, K., Ma, H. & Peterson, J.R. (2011) Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. *Nat Biotechnol* **29**, 1039–45.

Audagnotto, M. & Dal Peraro, M. (2017) Protein post-translational modifications: In silico prediction tools and molecular modeling. *Comput Struct Biotechnol J* **15**, 307–319.

Auld, D.S., Zhang, Y.Q., Southall, N.T., Rai, G., Landsman, M., MacLure, J., Langevin, D., Thomas, C.J., Austin, C.P. & Inglese, J. (2009) A basis for reduced chemical library inhibition of firefly luciferase obtained from directed evolution. *J Med Chem* **52**, 1450–1458.

Barnash, K.D., James, L.I. & Frye, S. V. (2017) Target class drug discovery. *Nat Chem Biol* **13**, 1053–1056.

Beltrao, P., Bork, P., Krogan, N.J. & Van Noort, V. (2013) Evolution and functional cross-talk of protein post-translational modifications. *Mol Syst Biol* **9**, 1–13.

Berney, C. & Danuser, G. (2003) FRET or no FRET: a quantitative comparison. *Biophys J* **84**, 3992–4010.

Bheda, P., Jing, H., Wolberger, C. & Lin, H. (2016) The Substrate Specificity of Sirtuins. *Annu Rev Biochem* **85**, 405–29.

Bielefeld-Sevigny, M. (2009) AlphaLISA Immunoassay Platform— The "No-Wash" High-Throughput Alternative to ELISA. *Assay Drug Dev Technol* **7**, 90–92.

Bleicher, K.H., Böhm, H.J., Müller, K. & Alanine, A.I. (2003) Hit and lead generation: Beyond high-throughput screening. *Nat Rev Drug Discov* **2**, 369–378.

Boskovic, Z. V., Kemp, M.M., Freedy, A.M., Viswanathan, V.S., Pop, M.S., Fuller, J.H., Martinez, N.M., Figueroa Lazu, S.O., Hong, J.A., Lewis, T.A., Calarese, D., Love, J.D., Vetere, A., Almo, S.C., Schreiber, S.L. & Koehler, A.N. (2016) Inhibition of Zinc-Dependent Histone Deacetylases with a Chemically Triggered Electrophile. *ACS Chem Biol* **11**, 1844–1851.

Boycott, K.M. & Ardigó, D. (2018) Addressing challenges in the diagnosis and treatment of rare genetic diseases. *Nat Rev Drug Discov* **17**, 151–152.

Brandt, M., Szewczuk, L.M., Zhang, H., Hong, X., McCormick, P.M., Lewis, T.S., Graham, T.I., Hung, S.T., Harper-Jones, A.D., Kerrigan, J.J., Wang, D.-Y., Dul, E., Hou, W., Ho, T.F., Meek, T.D., Cheung, M.H., Johanson, K.O., Jones, C.S., Schwartz, B., Kumar, S., Oliff, A.I. & Kirkpatrick, R.B. (2013) Development of a high-throughput screen to detect inhibitors of TRPS1 sumoylation. *Assay Drug Dev Technol* **11**, 308–25.

Carlson, C.B., Horton, R. a & Vogel, K.W. (2009) A toolbox approach to high-throughput TR-FRET-based SUMOylation and DeSUMOylation assays. *Assay Drug Dev Technol* **7**, 348–55.

Carpenter, J.W., Laethem, C., Hubbard, F.R., Eckols, T.K., Baez, M., Mcclure, D., Nelson, D.L.G. & Johnston, P.A. (2002) Configuring Radioligand Receptor Binding Assays for HTS Using Scintillation Proximity Assay Technology. In Janzen, W.P. (ed), *High Throughput Screening: Methods and Protocols*, Humana Press, pp. 31–49.

Charbonniere, L.J. (2011) Luminescent Lanthanide Labels. *Curr Inorg Chem* **1**, 2–16.

Charter, N.W., Kauffman, L., Singh, R. & Eglen, R.M. (2006) A generic, homogenous method for measuring kinase and inhibitor activity via adenosine 5′-diphosphate accumulation. *J Biomol Screen* **11**, 390–399.

Checovich, W.J., Bolger, R.E. & Burke, T. (1995) Fluorescence polarization — a new tool for cell and molecular biology. *Nature* **375**, 254–256.

Chen, P., Xie, H. & Wells, A. (1996) Mitogenic signaling from the egf receptor is attenuated by a phospholipase C-gamma/protein kinase C feedback mechanism. *Mol Biol Cell* **7**, 871–81.

Cho, E. & Park, M. (2016) Palmitoylation in Alzheimer's disease and other neurodegenerative diseases. *Pharmacol Res* **111**, 133–151.

Chopra, P., Nanda, K., Chatterjee, M., Bajpai, M., Dastidar, S.G. & Ray, A. (2008) An improved zinc cocktail-mediated fluorescence polarization-based kinase assay for high-throughput screening of kinase inhibitors. *Anal Biochem* **380**, 143–5.

Choulier, L. & Enander, K. (2010) Environmentally sensitive fluorescent sensors based on synthetic peptides. *Sensors* **10**, 3126–3144.

Chudakov, D.M., Matz, M. V., Lukyanov, S. & Lukyanov, K.A. (2010) Fluorescent Proteins and Their Applications in Imaging Living Cells and Tissues. *Physiol Rev* **90**, 1103–1163.

Clarke, S.G. (2013) Protein methylation at the surface and buried deep: thinking outside the histone box. *Trends Biochem Sci* **38**, 243–252.

Coffin, J., Latev, M., Bi, X. & Nikiforov, T.T. (2000) Detection of phosphopeptides by fluorescence polarization in the presence of cationic polyamino acids: application to kinase assays. *Anal Biochem* **278**, 206–212.

Comley, J. (2003) Assay Interference a limiting factor in HTS. *Drug Discov World* 91–98.

Cook, N.D. (1996) Scintillation proximity assay: a versatile high-throughput screening technology. *Drug Discov Today* **1**, 287–294.

D'Arcy, P., Wang, X. & Linder, S. (2015) Deubiquitinase inhibition as a cancer therapeutic strategy. *Pharmacol Ther* **147**, 32–54.

Daugherty, D.L. & Gellman, S.H. (1999) A fluorescence assay for leucine zipper dimerization: Avoiding unintended consequences of fluorophore attachment. *J Am Chem Soc* **121**, 4325–4333.

Degorce, F., Card, A., Soh, S., Trinquet, E., Knapik, G.P. & Xie, B. (2009) HTRF : A Technology Tailored for Drug Discovery – A Review of Theoreti- cal Aspects and Recent Applications. *Curr Chem Genomics* **3**, 22–32.

Deribe, Y.L., Pawson, T. & Dikic, I. (2010) Post-translational modifications in signal integration. *Nat Struct Mol Biol* **17**, 666–672.

Dienstmann, R., Rodon, J., Serra, V. & Tabernero, J. (2014) Picking the Point of Inhibition: A Comparative Review of PI3K/AKT/mTOR Pathway Inhibitors. *Mol Cancer Ther* **13**, 1021–1031.

Drazic, A., Myklebust, L.M., Ree, R. & Arnesen, T. (2016) The world of protein acetylation. *Biochim Biophys Acta - Proteins Proteomics* **1864**, 1372–1401.

Duan, G. & Walther, D. (2015) The Roles of Post-translational Modifications in the Context of Protein Interaction Networks. *PLoS Comput Biol* **11**, 1–23.

Eglen, R.M., Reisine, T., Roby, P., Rouleau, N., Illy, C., Bossé, R. & Bielefeld, M. (2008) The Use of AlphaScreen Technology in HTS: Current Status. *Curr Chem Genomics* **1**, 2–10.

Elkins, J.M., Fedele, V., Szklarz, M., Abdul Azeez, K.R., Salah, E., Mikolajczyk, J., Romanov, S., Sepetov, N., Huang, X.P., Roth, B.L., Al Haj Zen, A., Fourches, D., Muratov, E., Tropsha, A., Morris, J., Teicher, B.A., Kunkel, M., Polley, E., Lackey, K.E., Atkinson, F.L., Overington, J.P., Bamborough, P., Muller, S., Price, D.J., Willson, T.M., Drewry, D.H., Knapp, S. & Zuercher,

W.J. (2016) Comprehensive characterization of the Published Kinase Inhibitor Set. *Nat Biotechnol* **34**, 95–103.

Falkenberg, K.J. & Johnstone, R.W. (2014) Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nat Rev Drug Discov* **13**, 673–91.

Ferguson, K.M. (2004) Active and inactive conformations of the epidermal growth factor receptor. *Biochem Soc Trans* **32**, 742–5.

Filippakopoulos, P. & Knapp, S. (2012) The bromodomain interaction module. *FEBS Lett* **586**, 2692–2704.

Filippakopoulos, P. & Knapp, S. (2014) Targeting bromodomains: epigenetic readers of lysine acetylation. *Nat Rev Drug Discov* **13**, 337–356.

Fleuren, E.D.G., Zhang, L., Wu, J. & Daly, R.J. (2016) The kinome "at large" in cancer. *Nat Rev Cancer* **16**, 83–98.

Flynn, D.C. (2001) Adaptor proteins. *Oncogene* **20**, 6270–6272.

Galloway, T.J., Wirth, L.J., Colevas, A.D., Gilbert, J., Bauman, J.E., Saba, N.F., Raben, D., Mehra, R., Ma, A.W., Atoyan, R., Wang, J., Burtness, B. & Jimeno, A. (2015) A phase I study of CUDC-101, a multitarget inhibitor of HDACs, EGFR, and HER2, in combination with chemoradiation in patients with head and neck squamous cell carcinoma. *Clin Cancer Res* **21**, 1566–1573.

Gao, Y., Davies, S.P., Augustin, M., Woodward, A., Patel, U.A., Kovelman, R. & Harvey, K.J. (2013) A broad activity screen in support of a chemogenomic map for kinase signalling research and drug discovery. *Biochem J* **451**, 313–328.

Gauthier, N., Caron, M., Pedro, L., Arcand, M., Blouin, J., Labonté, A., Normand, C., Paquet, V., Rodenbrock, A., Roy, M., Rouleau, N., Beaudet, L., Padrós, J. & Rodriguez-Suarez, R. (2012) Development of Homogeneous Nonradioactive Methyltransferase and Demethylase Assays Targeting Histone H3 Lysine 4. *J Biomol Screen* **17**, 49–58.

Ge, G., Wu, J., Wang, Y. & Lin, Q. (2002) Activation mechanism of solubilized epidermal growth factor receptor tyrosine kinase. *Biochem Biophys Res Commun* **290**, 914–20.

Glickman, J.F., Schmid, A. & Ferrand, S. (2008) Scintillation Proximity Assays in High-Throughput Screening. *Assay Drug Dev Technol* **6**, 433–455.

Glickman, J.F., Wu, X., Mercuri, R., Illy, C. & Bowen, B.R. (2002) A Comparison of ALPHAScreen , TR-FRET , and TRF as Assay Methods for FXR Nuclear Receptors. *J Biomol Screen* **7**, 3–10.

Goddard, J.-P. & Reymond, J.-L. (2004) Recent advances in enzyme assays. *Trends Biotechnol* **22**, 363–70.

González-Vera, J. a. (2012) Probing the kinome in real time with fluorescent peptides. *Chem Soc Rev* **41**, 1652–64.

Gordon, J.A. (1991) Use of Vanadate as Protein - Phosphotyrosine Phosphatase Inhibitor [41]. In *Methods in Enzymology*, pp. 477–482.

Haass, C., Kaether, C., Thinakaran, G. & Sisodia, S. (2012) Trafficking and Proteolytic Processing of APP 1–26.

Hagan, A.K. & Zuchner, T. (2011) Lanthanide-based time-resolved luminescence immunoassays. *Anal Bioanal Chem* **400**, 2847–2864.

Hamamoto, R., Saloura, V. & Nakamura, Y. (2015) Critical roles of non-histone protein lysine methylation in human tumorigenesis. *Nat Rev Cancer* **15**, 110–24.

Hanson, S.R., Culyba, E.K., Hsu, T.-L., Wong, C.-H., Kelly, J.W. & Powers, E.T. (2009) The core trisaccharide of an N-linked glycoprotein intrinsically accelerates folding and enhances stability. *Proc Natl Acad Sci* **106**, 3131–3136.

Härmä, H., Rozwandowicz-Jansen, A., Martikkala, E., Frang, H., Hemmilä, I., Sahlberg, N., Fey, V., Perälä, M. & Hänninen, P. (2009) A new simple cell-based homogeneous time-resolved fluorescence QRET technique for receptor-ligand interaction screening. *J Biomol Screen* **14**, 936–43.

Harrigan, J.A., Jacq, X., Martin, N.M. & Jackson, S.P. (2018) Deubiquitylating enzymes and drug discovery: Emerging opportunities. *Nat Rev Drug Discov* **17**, 57–77.

Van Der Hee, R.M., Deurholt, T., Gerhardt, C.C. & De Groene, E.M. (2005) Comparison of 3 AT1receptor binding assays: Filtration assay, ScreenReady™ target, and WGA Flashplate®. *J Biomol Screen* **10**, 118–126.

Helenius, M., Jalkanen, S. & Yegutkin, G.G. (2012) Enzyme-coupled assays for simultaneous detection of nanomolar ATP, ADP, AMP, adenosine, inosine and pyrophosphate concentrations in extracellular fluids. *Biochim Biophys Acta - Mol Cell Res* **1823**, 1967–1975.

Heltweg, B., Trapp, J. & Jung, M. (2005) In vitro assays for the determination of histone deacetylase activity. *Methods* **36**, 332–337.

Hemmilä, I. (1999) LANCETM: Homogeneous assay platform for HTS. *J Biomol Screen* **4**, 303–307.

Hemmilä, I., Dakubu, S., Mukkala, V., Siitari, H. & Lövgren, T. (1984a) Europium as a Label in Time-Resolved lmmunofluorometric Assays. *Anal Biochem* **137**, 335–343.

Hemmilä, I., Dakubu, S., Mukkala, V.M., Siitari, H. & Lövgren, T. (1984b) Europium as a label in time-resolved immunofluorometric assays. *Anal Biochem* **137**, 335–343.

Hemmilä, I. & Laitala, V. (2005) Progress in lanthanides as luminescent probes. *J Fluoresc* **15**, 529–542.

Hemmilä, I., Mukkala, V.M. & Takalo, H. (1997) Development of luminescent lanthanide chelate labels for diagnostic assays. *J Alloys Compd* **249**, 158–162.

Hewitt, S.H. & Butler, S.J. (2018) Application of lanthanide luminescence in probing enzyme activity. *Chem Commun* **54**, 6635–6647.

Hirano, A., Fu, Y.H. & Ptáek, L.J. (2016) The intricate dance of post-translational modifications in the rhythm of life. *Nat Struct Mol Biol* **23**, 1053–1060.

Holdgate, G.A., Meek, T.D. & Grimley, R.L. (2018) Mechanistic enzymology in drug discovery: A fresh perspective. *Nat Rev Drug Discov* **17**, 115–132.

Horton, R.A. & Vogel, K.W. (2010) Multiplexing terbium- and europium-based TR-FRET readouts to increase kinase assay capacity. *J Biomol Screen* **15**, 1008–15.

Hughes, J.P., Rees, S.S., Kalindjian, S.B. & Philpott, K.L. (2011) Principles of early drug discovery. *Br J Pharmacol* **162**, 1239–1249.

Hunter, T. (2007) The Age of Crosstalk: Phosphorylation, Ubiquitination, and Beyond. *Mol Cell* **28**, 730–738.

Hunter, T. & Cooper, J.A. (1985) Protein-tyrosine kinases. *Cancer Res* **54**, 897–930.

Huttunen, R., Shweta, Martikkala, E., Lahdenranta, M., Virta, P., Hänninen, P. & Härmä, H. (2011) Single-label time-resolved luminescence assay for estrogen receptor--ligand binding. *Anal Biochem* **415**, 27–31.

Inglese, J., Johnson, R.L., Simeonov, A., Xia, M., Zheng, W., Austin, C.P. & Auld, D.S. (2007) High-throughput screening assays for the identification of chemical probes. *Nat Chem Biol* **3**, 466–479.

International Human Genome Sequencing Consortium. (2004) Finishing the euchromatic sequence of the human genome. *Nature* **431**, 931–945.

Janzen, W.P. (2014) Screening technologies for small molecule discovery: The state of the art. *Chem Biol* **21**, 1162–1170.

Jen, J.C., Ashizawa, T., Griggs, R.C. & Waters, M.F. (2016) Rare neurological channelopathies-networks to study patients, pathogenesis and treatment. *Nat Rev Neurol* **12**, 195–203.

Jensen, O.N. (2004) Modification-specific proteomics: Characterization of post-translational modifications by mass spectrometry. *Curr Opin Chem Biol* **8**, 33–41.

Jensen, O.N. (2006) Interpreting the protein language using proteomics. *Nat Rev Mol Cell Biol* **7**, 391–403.

Kaplan, J.B., Reinke, A.W. & Keating, A.E. (2014) Increasing the affinity of selective bZIPbinding peptides through surface residue redesign. *Protein Sci* **23**, 940–953.

Karvinen, J., Hurskainen, P., Gopalakrishnan, S., Burns, D., Warrior, U. & Hemmilä, I. (2002) Homogeneous Time-Resolved Fluorescence Quenching Assay (LANCE) for Caspase-3. *J Biomol Screen* **7**, 223–231.

Khoury, G.A., Baliban, R.C. & Floudas, C.A. (2014) Proteome-wide post-translational modification statistics: Frequency analysis and curation of the swiss-prot database. *Sci Rep* **1**, 1–5.

Kim, M.S., Zhong, J. & Pandey, A. (2016) Common errors in mass spectrometry-based analysis of post-translational modifications. *Proteomics* **16**, 700–714.

Kim, S.C., Sprung, R., Chen, Y., Xu, Y., Ball, H., Pei, J., Cheng, T., Kho, Y., Xiao, H., Xiao, L., Grishin, N. V., White, M., Yang, X.J. & Zhao, Y. (2006) Substrate and Functional Diversity of Lysine Acetylation Revealed by a Proteomics Survey. *Mol Cell* **23**, 607–618.

Klumpp, M., Boettcher, A., Becker, D., Meder, G., Blank, J., Leder, L., Forstner, M., Ottl, J. & Mayr, L.M. (2006) Readout technologies for highly miniaturized kinase assays applicable to high-throughput screening in a 1536-well format. *J Biomol Screen* **11**, 617–633.

Ko, K.-C., Choi, M.H. & Park, S.H. (2011) A phosphorylation assay using [γ-32P]ATP: A highly sensitive detection of protein kinase C. *J Label Compd Radiopharm* **54**, 105–109.

Kolch, W. & Pitt, A. (2010) Functional proteomics to dissect tyrosine kinase signalling pathways in cancer. *Nat Rev Cancer* **10**, 618–629.

Kopra, K., Van Adrichem, A.J., Salo-Ahen, O.M.H., Peltonen, J., Wennerberg, K. & Härmä, H. (2017) High-Throughput Dual Screening Method for Ras Activities and Inhibitors. *Anal Chem* **89**, 4508–4516.

Kopra, K. & Härmä, H. (2015) Quenching resonance energy transfer (QRET): a single-label technique for inhibitor screening and interaction studies. *N Biotechnol* **00**.

Kopra, K., Ligabue, A., Wang, Q., Syrjänpää, M., Blaževitš, O., Veltel, S., van Adrichem, A.J., Hänninen, P., Abankwa, D. & Härmä, H. (2014a) A homogeneous quenching resonance energy transfer assay for the kinetic analysis of the GTPase nucleotide exchange reaction. *Anal Bioanal Chem* **406**, 4147–56.

Kopra, K., Syrjänpää, M., Hänninen, P. & Härmä, H. (2014b) Non-competitive aptamer-based quenching resonance energy transfer assay for homogeneous growth factor quantification. *Analyst* **139**, 2016–23.

Krasnova, L. & Wong, C.-H. (2016) Understanding the Chemistry and Biology of Glycosylation with Glycan Synthesis. *Annu Rev Biochem **85**, (599-630)*.

Kristjánsdóttir, K. & Rudolph, J. (2003) A fluorescence polarization assay for native protein substrates of kinases. *Anal Biochem* **316**, 41–49.

Krueger, A.T. & Imperiali, B. (2013) Fluorescent Amino Acids: Modular Building Blocks for the Assembly of New Tools for Chemical Biology. *ChemBioChem* **14**, 788–799.

Kumar, M. & Lowery, R.G. (2017) A High-Throughput Method for Measuring Drug Residence Time Using the Transcreener ADP Assay. *SLAS Discov* **22**, 915–922.

Kumar, M., Zielinski, T. & Lowery, R.G. (2015) Biochemical Assay Development for Histone Methyltransferases Using a Transcreener-Based Assay for S-Adenosylhomocysteine. *Assay Drug Dev Technol* **13**, 200–209.

Langeberg, L.K. & Scott, J.D. (2015) Signalling scaffolds and local organization of cellular behaviour. *Nat Rev Mol Cell Biol* **16**, 232–244.

Larson, B., Banks, P., Zegzouti, H. & Goueli, S.A. (2009) A Simple and Robust Automated Kinase Profiling Platform Using Luminescent ADP Accumulation Technology. *Assay Drug Dev Technol* **7**, 573–584.

Lavis, L.D. & Raines, R.T. (2007) Bright Ideas for Chemical Biology. *ACS Chem Biol* **3**.

Lewallen, D.M., Bicker, K.L., Madoux, F., Chase, P., Anguish, L., Coonrod, S., Hodder, P. & Thompson, P.R. (2014) A FluoPol-ABPP PAD2 high-throughput screen identifies the first calcium site inhibitor targeting the PADs. *ACS Chem Biol* **9**, 913–921.

Li, C. & Götz, J. (2017) Tau-based therapies in neurodegeneration: Opportunities and challenges. *Nat Rev Drug Discov* **16**, 863–883.

Li, E. & Hristova, K. (2006) Role of Receptor Tyrosine Kinase Transmembrane Domains in Cell Signaling and Human Pathologies. *Biochemistry* **45**, 6241–6251.

Li, Y., Xie, W. & Fang, G. (2008) Fluorescence detection techniques for protein kinase assay. *Anal Bioanal Chem* **390**, 2049–57.

Liddy, K.A., White, M.Y. & Cordwell, S.J. (2013) Functional decorations: Post-translational modifications and heart disease delineated by targeted proteomics. *Genome Med* **5**, 1–12.

Lim, W.A. & Pawson, T. (2010) Phosphotyrosine Signaling: Evolving a New Cellular Communication System. *Cell* **142**, 661–667.

Lindsley, C.W., Zhao, Z., Leister, W.H., Robinson, R.G., Barnett, S.F., Defeo-Jones, D., Jones, R.E., Hartman, G.D., Huff, J.R., Huber, H.E. & Duggan, M.E. (2005) Allosteric Akt (PKB) inhibitors: Discovery and SAR of isozyme selective inhibitors. *Bioorganic Med Chem Lett* **15**, 761–764.

Lo, M.C., Aulabaugh, A., Jin, G., Cowling, R., Bard, J., Malamas, M. & Ellestad, G. (2004) Evaluation of fluorescence-based thermal shift assays for hit identification in drug discovery. *Anal Biochem* **332**, 153–159.

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

Lothrop, A.P., Torres, M.P. & Fuchs, S.M. (2013) Deciphering post-translational modification codes. *FEBS Lett* **587**, 1247–1257.

Luković, E., González-Vera, J.A. & Imperiali, B. (2008) Recognition-Domain Focused (RDF) Chemosensors: Versatile and Efficient Reporters of Protein Kinase Activity. *J Am Chem Soc* **130**, 12821–12827.

Lundin, a. (2000) Use of firefly luciferase in ATP-related assays of biomass, enzymes, and metabolites. *Methods Enzymol* **305**, 346–70.

Ma, H., Deacon, S. & Horiuchi, K. (2008) The challenge of selecting protein kinase assays for lead discovery optimization. *Expert Opin Drug Discov* **3**, 607–621.

Martikkala, E., Rozwandowicz-Jansen, A., Hänninen, P., Petäjä-Repo, U. & Härmä, H. (2011) A homogeneous single-label time-resolved fluorescence cAMP assay. *J Biomol Screen* **16**, 356–62.

Martin, C. & Zhang, Y. (2005) The diverse functions of histone lysine methylation. *Nat Rev Mol Cell Biol* **6**, 838–849.

Mazitschek, R., Patel, V., Wirth, D.F. & Clardy, J. (2008) Development of a fluorescence polarization based assay for histone deacetylase ligand discovery. *Bioorganic Med Chem Lett* **18**, 2809–2812.

Moremen, K.W., Tiemeyer, M. & Nairn, A. V. (2012) Vertebrate protein glycosylation: Diversity, synthesis and function. *Nat Rev Mol Cell Biol* **13**, 448–462.

Moshinsky, D.J., Ruslim, L., Blake, R. a & Tang, F. (2003) A widely applicable, high-throughput TR-FRET assay for the measurement of kinase autophosphorylation: VEGFR-2 as a prototype. *J Biomol Screen* **8**, 447–52.

Muthyala, R., Shin, W.S., Xie, J. & Sham, Y.Y. (2015) Discovery of 1-hydroxypyridine-2-thiones as selective histone deacetylase inhibitors and their potential application for treating leukemia. *Bioorganic Med Chem Lett* **25**, 4320–4324.

Nasir, M.S. & Jolley, M.E. (1999) Fluorescence polarization: an analytical tool for immunoassay and drug discovery. *Comb Chem High Throughput Screen* **2**, 177–190.

O'Shea, E.K., Klemm, J.D., Kim, P.S. & Alber, T. (1991) X-ray Structure of the GCN4 Leucine Zipper, a Two-Stranded, Parallel Coiled Coil. *Science (80-)* **254**, 539–544.

Oliveira, F.M.S. de, Mereiter, S., Lönn, P., Siart, B., Shen, Q., Heldin, J., Raykova, D., Karlsson, N.G., Polom, K., Roviello, F., Reis, C.A. & Kamali-

Moghaddam, M. (2018) Detection of post-translational modifications using solid-phase proximity ligation assay. *N Biotechnol* **45**, 51–59.

Pathak, S., Alonso, J., Schimpl, M., Rafie, K., Blair, D.E., Borodkin, V.S., Schüttelkopf, A.W., Albarbarawi, O. & Van Aalten, D.M.F. (2015) The active site of O-GlcNAc transferase imposes constraints on substrate sequence. *Nat Struct Mol Biol* **22**, 744–749.

Paul, P., Suwan, J., Liu, J., Dordick, J.S. & Linhardt, R.J. (2012) Recent advances in sulfotransferase enzyme activity assays. *Anal Bioanal Chem* **403**, 1491–500.

Pawson, T. (2002) Regulation and targets of receptor tyrosine kinases. *Eur J Cancer* **38**, S3-10.

Rauh, D., Fischer, F., Gertz, M., Lakshminarasimhan, M., Bergbrede, T., Aladini, F., Kambach, C., Becker, C.F.W., Zerweck, J., Schutkowski, M. & Steegborn, C. (2013) An acetylome peptide microarray reveals specificities and deacetylation substrates for all human sirtuin isoforms. *Nat Commun* **4**, 1–10.

Redhead, M., Satchell, R., McCarthy, C., Pollack, S. & Unitt, J. (2017) Thermal Shift as an Entropy-Driven Effect. *Biochemistry* **56**, 6187–6199.

Rekowski, M.V.W. & Giannis, A. (2010) Histone acetylation modulation by small molecules: a chemical approach. *Biochim Biophys Acta* **1799**, 760–7.

Roda, A., Guardigli, M., Michelini, E. & Mirasoli, M. (2009) Nanobioanalytical luminescence: Förster-type energy transfer methods. *Anal Bioanal Chem* **393**, 109–23.

Rodon, J., Dienstmann, R., Serra, V. & Tabernero, J. (2013) Development of PI3K inhibitors: Lessons learned from early clinical trials. *Nat Rev Clin Oncol* **10**, 143–153.

Rozwandowicz-Jansen, A., Laurila, J., Martikkala, E., Frang, H., Hemmilä, I., Scheinin, M., Hänninen, P. & Härmä, H. (2010) Homogeneous GTP binding assay employing QRET technology. *J Biomol Screen* **15**, 261–7.

Rubin, S.M., Gall, A., Zheng, N. & Pavletich, N.P. (2005) Structure of the Rb C-Terminal Domain Bound to E2F1-DP1 : A Mechanism for Phosphorylation-Induced E2F Release 1093–1106.

Salisbury, C.M., Maly, D.J. & Ellman, J.A. (2002) Peptide Microarrays for the Determination of Protease Substrate Specificity. *J Am Chem Soc* **124**, 14868–14870.

Savio, M.G., Wollscheid, N., Cavallaro, E., Algisi, V., Di Fiore, P.P., Sigismund, S., Maspero, E. & Polo, S. (2016) USP9X Controls EGFR Fate by Deubiquitinating the Endocytic Adaptor Eps15. *Curr Biol* **26**, 173–183.

Schneider, G. (2018) Automating drug discovery. *Nat Rev Drug Discov* **17**, 97–113.

Scott, D.E., Bayly, A.R., Abell, C. & Skidmore, J. (2016) Small molecules, big targets: Drug discovery faces the protein-protein interaction challenge. *Nat Rev Drug Discov* **15**, 533–550.

Scott, J.E. & Carpenter, J.W. (2003) A homogeneous assay of kinase activity that detects phosphopeptide using fluorescence polarization and zinc. *Anal Biochem* **316**, 82–91.

Seet, B.T., Dikic, I., Zhou, M.M. & Pawson, T. (2006) Reading protein modifications with interaction domains. *Nat Rev Mol Cell Biol* **7**, 473–483.

Seethala, R. & Menzel, R. (1997) A homogeneous, fluorescence polarization assay for src-family tyrosine kinases. *Anal Biochem* **253**, 210–8.

Seo, J. & Lee, K.-J. (2004) Post-translational modifications and their biological functions: proteomic analysis and systematic approaches. *J Biochem Mol Biol* **37**, 35–44.

Shults, M.D., Carrico-Moniz, D. & Imperiali, B. (2006) Optimal Sox-based fluorescent chemosensor design for serine/threonine protein kinases. *Anal Biochem* **352**, 198–207.

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

Simeonov, A., Jadhav, A., Thomas, C.J., Wang, Y., Huang, R., Southall, N.T., Shinn, P., Smith, J., Austin, C.P., Auld, D.S. & Inglese, J. (2008) Fluorescence Spectroscopic Profiling of Compound Libraries. *J Med Chem* **51**, 2363–2371.

Steffen, P., Kwiatkowski, M., Robertson, W.D., Zarrine-Afsar, A., Deterra, D., Richter, V. & Schlüter, H. (2016) Protein species as diagnostic markers. *J Proteomics* **134**, 5–18.

Sun, S., Shah, P., Eshghi, S.T., Yang, W., Trikannad, N., Yang, S., Chen, L., Aiyetan, P., Höti, N., Zhang, Z., Chan, D.W. & Zhang, H. (2016) Comprehensive analysis of protein glycosylation by solid-phase extraction of N-linked glycans and glycosite-containing peptides. *Nat Biotechnol* **34**, 84–88.

Takalo, H., Mukkala, V.M., Mikola, H., Liitti, P. & Hemmilä, I. (1994) Synthesis of europium(III) chelates suitable for labeling of bioactive molecules. *Bioconjug Chem* **5**, 278–282.

The UniProt Consortium. (2015) UniProt: A hub for protein information. *Nucleic Acids Res* **43**, D204–D212.

Thommen, M., Holtkamp, W. & Rodnina, M. V. (2017) Co-translational protein folding: progress and methods. *Curr Opin Struct Biol* **42**, 83–89.

Thorne, N., Auld, D.S. & Inglese, J. (2010) Apparent activity in high-throughput screening: origins of compound-dependent assay interference. *Curr Opin Chem Biol* **14**, 315–324.

Tonks, N.K. (2003) PTP1B: From the sidelines to the front lines! *FEBS Lett* **546**, 140–148.

Varkondi, E., Schäfer, E., Bökönyi, G., Gyökeres, T., Orfi, L., Petak, I., Pap, A., Szokoloczi, O., Keri, G. & Schwab, R. (2005) Comparison of ELISA-based tyrosine kinase assays for screening EGFR inhibitors. *J Recept Signal Transduct Res* **25**, 45–56.

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

Venne, A.S., Kollipara, L. & Zahedi, R.P. (2014) The next level of complexity: Crosstalk of posttranslational modifications. *Proteomics* **14**, 513–524.

Verdin, E. & Ott, M. (2015) 50 years of protein acetylation: From gene regulation to epigenetics, metabolism and beyond. *Nat Rev Mol Cell Biol* **16**, 258–264.

Wang, D., Helquist, P. & Wiest, O. (2007) Zinc binding in HDAC inhibitors: a DFT study. *J Org Chem* **72**, 5446–5449.

Wells, A. (1999) EGF receptor. *Int J Biochem Cell Biol* **31**, 637–643.

Xu, Y.-M., Du, J.-Y. & Lau, A.T.Y. (2014) Posttranslational modifications of human histone H3: An update. *Proteomics* **14**, 2047–2060.

Yang, X. & Qian, K. (2017) Protein O-GlcNAcylation: Emerging mechanisms and functions. *Nat Rev Mol Cell Biol* **18**, 452–465.

Yang, X.J. & Seto, E. (2008) Lysine Acetylation: Codified Crosstalk with Other Posttranslational Modifications. *Mol Cell* **31**, 449–461.

Yau, R. & Rape, M. (2016) The increasing complexity of the ubiquitin code. *Nat Cell Biol* **18**, 579–586.

Yu, W., Eram, M.S., Hajian, T., Szykowska, A., Burgess-Brown, N., Vedadi, M. & Brown, P.J. (2014) A scintillation proximity assay for histone demethylases. *Anal Biochem* **463**, 54–60.

Zaware, N. & Zhou, M.M. (2017) Chemical modulators for epigenome reader domains as emerging epigenetic therapies for cancer and inflammation. *Curr Opin Chem Biol* **39**, 116–125.

Zegzouti, H., Zdanovskaia, M., Hsiao, K. & Goueli, S.A. (2009) ADP-Glo: A Bioluminescent and Homogeneous ADP Monitoring Assay for Kinases. *Assay Drug Dev Technol* **7**, 560–572.

Zhang, J.-H., Chung, T.D.Y. & Oldenburg, K.R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* **4**, 67–73.

Zhang, S., Chen, L., Kumar, S., Wu, L., Lawrence, D.S. & Zhang, Z.Y. (2007) An affinity-based fluorescence polarization assay for protein tyrosine phosphatases. *Methods* **42**, 261–267.

Zhao, Y. & Jensen, O.N. (2009) Modification-specific proteomics: Strategies for characterization of post-translational modifications using enrichment techniques. *Proteomics* **9**, 4632–4641.

Zielinski, T., Reichman, M., Donover, P.S. & Lowery, R.G. (2016) Development and Validation of a Universal High-Throughput UDP-Glycosyltransferase Assay with a Time-Resolved FRET Signal. *Assay Drug Dev Technol* **14**, 240–51.

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