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Perspectives on the Development of the PhosphoSens™ Kinase Assay Technology

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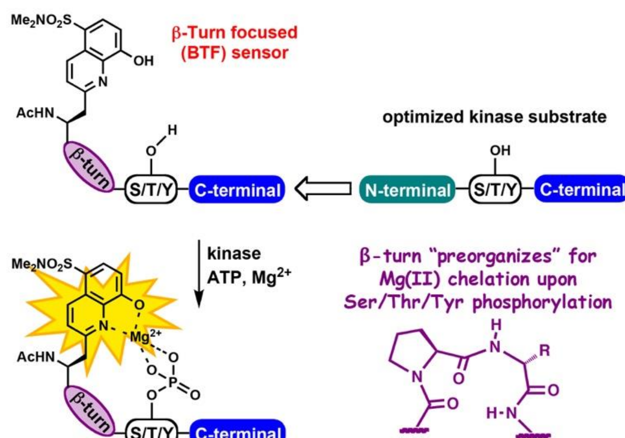
INTRODUCTION – Protein kinase sensing via ChEF

The Chelation-Enhanced Fluorescence (ChEF) method for protein kinase sensing, filed under the PhosphoSens trademark, was first introduced by Imperiali and coworkers in 2003 (1,2). The sensing mechanism exploits a synthetic amino acid with a side chain bearing an 8-hydroxyquinoline derivative that, together with divalent magnesium, relays information on the phosphorylation status of proximal serine, threonine or tyrosine residues in peptide-based substrates. In early studies the chelating quinoline group was diversified by chemical synthesis; the studies ultimately afforded 5-sulfonamido-substituted quinolines, which showed advantageous fluorescence properties (ε_{max} and quantum yield - Φ) (3). The ChEF method provides continuous fluorescence-based monitoring of target protein kinases and the quinoline-based sensors represent tools that provide valuable information-rich data and enable rigorous kinetic analysis.

FIRST GENERATION ChEF KINASE SENSORS - β-turn focused design with the Sox amino acid

The first generation ChEF kinase sensors (Figure 1) were designed to include constrained two-residue β-turn motifs to preorganize the bidentate 8-hydroxyl-quinoline from the synthetic sulfonamide-oxine (Sox) amino acid and the transferred phosphoryl group and maximize the stability of the fluorescent chelate thereby enhancing the fluorescence signal. This design is designated as the β-turn focused (BTF) design. Sensor designs start with optimized kinase substrates identified from the literature or developed in house. Then, based on knowledge of the kinase binding preferences, either the N- or C-terminal binding determinants are preserved and the sensing motif, including the Sox amino acid and the β-turn, is appended in place of the other terminal sequence. This design is illustrated in Figure 1 for a sensor with C-terminal binding determinants. This design formed the basis for the OMNIA kinase assay product line until the end of 2015.

Figure 1 – ChEF kinase sensors - BTF Design



Sensors made using the BTF design share the following attributes:

a) Assays are continuous and data are acquired in real time, rapidly affording complete kinetic parameters for enzymes, substrates and inhibitors.
b) Assays use physiological ATP (0.1-1 mM) and Mg(II) concentrations and are compatible with essential trace transition metal ions including Mn(II).
c) Consumable materials are non-radioactive and do not involve custom antibodies.

d) The design rules are simple and can be broadly applied to serine, threonine, and tyrosine kinases.
e) The assays can be run in high throughput (96-, 384- and 1536-well low-volume plates) and afford high confidence data (Z' >0.85) due to low background and fluorescence enhancements in the 3-10-fold range.

Sensors based on the BTF design have been used extensively for several years (see Sox-User Literature pdf). The assays perform very well with purified and recombinant enzymes (1,4) and, in select cases, are also amenable to the analysis of protein kinases in unfractionated cell and tissue lysates (2).

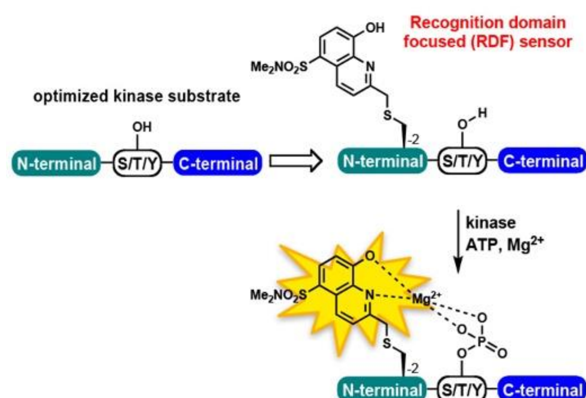
A critical analysis of the technology in 2008 highlighted the following opportunities for improving the ChEF-based kinase sensor reagents:

a) The first generation sensors relied on the availability of a specialized amino acid building block, designated as Fmoc-L-Sox (L-N-fluorenylmethoxycarbonyl-5-sulfonamido-oxine-alanine), that necessitated preparation via a stereoselective approach. A simpler synthetic approach would be advantageous.
b) The perceived requirement for the preorganizing β-turn motif placed limits on the selectivity of the kinases sensors in complex samples – due to the loss of either C- or N-terminal selectivity determinants, therefore a sensor design that maximally preserves specificity determinants would likely improve selectivity and sensor activity.
c) The photophysical properties of the sulfonamido-oxine (λ_{ex} 380 nm and λ_{em} 485 nm) could potentially be improved as it would be useful to have higher ε_{max} and longer λ_{ex} and λ_{em} wavelengths to further minimize interference from background signals.

SECOND GENERATION - ChEF KINASE SENSORS – Recognition-domain focused design

Major advances, addressing key aspects of the ChEF technology, have been integrated in the second generation recognition-domain focused (RDF) design. Based on this design, sensors can be assembled is a more straightforward fashion using post solid-phase peptide synthesis (SPPS) cysteine alkylation to introduce the sulfonamide oxide fluorophore, thereby exploiting the chiral pool building block and circumventing the stereoselective synthesis of Fmoc-L-Sox. Most importantly, with the Cys alkylation strategy, sensors are made by simply incorporating a cysteine residue either in the (-)2 or (+)2 position, relative to the phosphorylation site and protein kinase sensors with advantageous properties are easily made by thiol alkylation before or after resin deprotection (5). With these two technical developments in place, the RDF design kinase sensors, integrating the CSx (Sulfonamido-oxine-Cys) residue, then emerged as the preferred sensor design. With the second generation design significant improvements in sensor specificity were achieved due to the incorporation of both N- and C-terminal kinase specificity determinants. The new sensors are frequently

Figure 2 – ChEF kinase sensors - RDF design



characterized by single-digit or sub-μM K_M values and robust k_{cat} values, which has broadened the scope for sensor application in unfractionated cell and tissue lysates (6,7). An additional advantage of the design is that the "post-synthesis" Cys alkylation of bead-based libraries is greatly simplified presenting a versatile method for identifying optimized target kinase substrates and sensors (8).

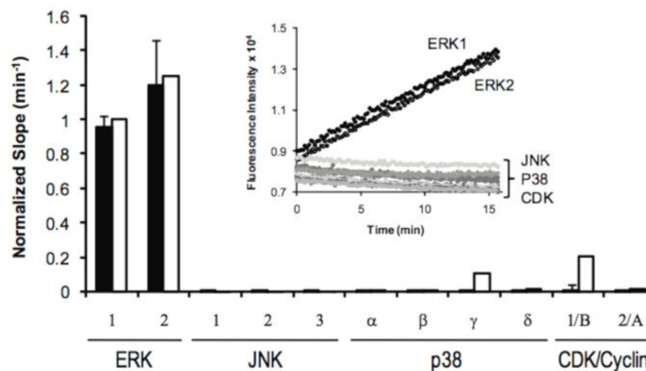
Progress is also being made with the development of 8-hydroxyquinoline derivatives with advantageous photophysical properties including, in particular, longer λ_{emMax}. A wide variety of bicyclic and tricyclic quinoline derivatives have been synthesized and screened. Currently, the most promising results are with synthetic quinolines prepared via Cu-catalyzed "click" chemistry between a 5-azido-8-hydroxy quinoline and terminal aryl alkynes (9). The novel quinolines have also been integrated into kinases sensors and the best member of the click-chemistry library of fluorophores, thus far, displays a 40 nm red shift in emission wavelength (485 vs 525 nm) (Figure 3). Future research will emphasize the development of red-shifted RDF design kinase sensors.

Figure 3 – Extended wavelength ChEF sensors via "click" chemistry

ADVANCING ChEF SENSOR SELECTIVITY THROUGH DOCKING DOMAIN STRATEGIES

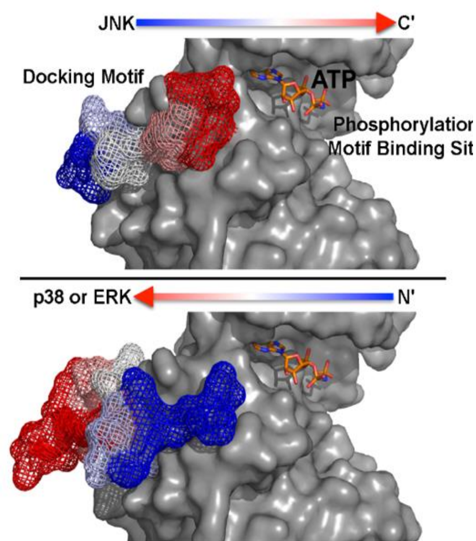
Exciting recent advances focus on chimeric kinase sensors based on protein and peptide "docking domains" conjugated to ChEF-based sensing modules. Of the human kinases that are associated with disease, many of the critical players invoke extended binding contacts, beyond simple peptidic determinants, with their physiological substrates and therefore docking domain strategies are particularly relevant. The initial proof-of-concept illustrating the advantage of exploiting a docking-domain-based was demonstrated with a ChEF-based sensor for selected epidermal growth factor-regulated kinase isoforms (ERK1/2) (10). The ERK1/2 sensor included a modular protein domain known as the PNT domain conjugated, via native chemical ligation (NCL) at the N-terminus, to a sensing module that included the serine phosphorylation site proximal to CSx in a short peptide. As illustrated in Figure 4, the chimeric ERK1/2 sensor shows excellent selectivity for the target kinase relative to the various isoforms of the JNK and p38 MAP kinases and selected cyclin-dependent kinases.

Figure 4 – Specificity of the semisynthetic ERK sensor (5 μM) comprising a PNT domain conjugated via NCL to a peptidic CSx module



Although the selectivity of the PNT-based chimeric ERK1/2 sensor was excellent, semisynthesis of this sensor, involving both recombinant and synthetic components, was cumbersome, suggesting that sensor assembly might not be readily amenable to scale up and broader commercial distribution. Therefore, we sought to identify simpler alternatives. In particular, reports in the literature between 2009 and 2012 presented the concept of small peptidic docking domains acting as surrogates for the larger (100-120 residue) protein domains in binding to MAP kinase family members (11). Based on these developments we established a series of "bipeptide" MAP kinase activity sensors exploiting the CSx kinase sensing modality together with directionally programmable docking domain (D domain) motifs (12). A critical feature of the design included recognition of the orientation of the D domain peptide binding, relative to the kinase active site and the serine-containing phosphorylation substrate. This feature necessitated careful consideration in the construction of the MAP kinase sensors. For JNK, bipeptide sensors can be assembled via standard SPPS approaches (from C-terminus to N-terminus). In contrast, for ERK and p38 sensor assembly an N-terminus to N-terminus ligation strategy was required for optimal positioning of the D domain peptide.

Figure 5 – Design of bipeptide MAP kinase probes requires recognition of D domain peptide orientation



The new MAP kinase probes show excellent activity with their target kinases and the low (single-digit μM to sub-μM) K_M values (Figure 6A), allow the probes to be used at concentrations that ensure excellent selectivity even in unfractionated cell lysates. For example, the family of probes has been exploited for studying the changes in ERK, JNK and p38 in the MCF10A, MCF10-AT, MCF10AT.KC12 and MCF10CA1 cell lines, which are a series of breast cancer cell lines with increasingly malignant phenotypes (Figure 6B).

Figure 6 – A. Sequences and Km values for bipeptide MAPK sensors; **B**. Kinetic analysis of unfractionated cell lysates from MCF10 breast cancer cell lines.

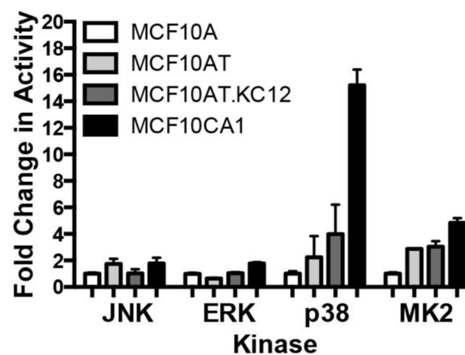
A

JNK1/2/3: ERPSRDHLYLPLEP-PEG$_2$-SANLLSP(CSx)PA
K_M = 3.3 μM (JNK1), 0.95 μM (JNK2), 2.8 μM (JNK3)

ERK1/2: GLKRVRRQALISSEIPKLQP-PEG$_2$-Triazole-PEG$_2$-VP(CSx)LTPGGRR
K_M = 1.1 μM (ERK1/2)

p38αβ: C'-KKRRKLLLPNSADEIKKIKI-PEG$_2$-Triazole-PEG$_2$QP(CSx)ASPVV-C'
K_M = 0.31 μM (p38a), 0.14 μM (p38β)

Blue = D Domain Red = CSx sensing module S/T = phosphorylation site



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