High complexity protease and kinase profiling solution assays with readout on randomly assembled microarrays

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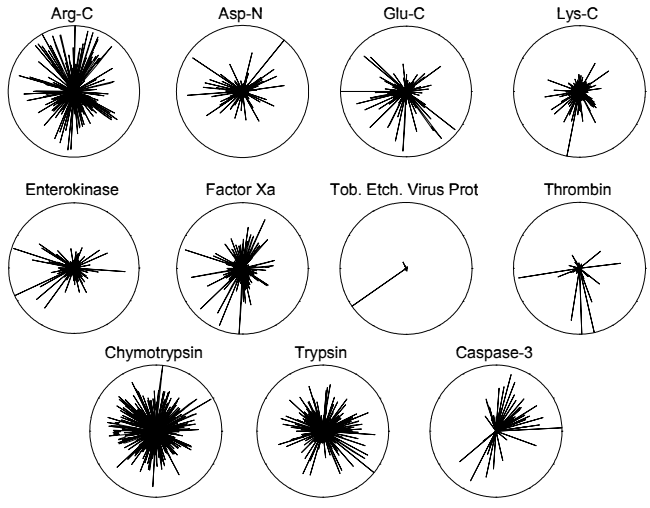
Introduction

We have developed miniaturized and multiplexed assays for the measurement of protease and kinase activities in complex samples. This technology will accelerate research in functional proteomics and enable biologist to carry out multiplexed protease and/or kinase inhibitor screens on a large scale. The assay readout is based on Illumina's universal Sentrix® BeadArrays [1,2].

Results and Discussion

In our protease assay, unlike in the earlier techniques based on hybridizable peptide nucleic acid (PNA) tags readable on DNA arrays [3,4], the peptide portion in our assay is C-terminally labeled with a biotin residue and contains a sequence of five histidine residues on the amino terminus. Upon protease cleavage, the portion of the peptide containing the biotin residue is detached from the oligonucleotide-peptide conjugate. Following the reaction, all biotin containing species are captured and removed by incubation with streptavidin beads. The cleaved conjugates that remain in solution are captured by hybridization of their oligonucleotide sequence to Sentrix BeadArrays and detected by a labeled antibody against the pentahistidine tag. We have generated multiple sets of oligonucleotide-tagged peptide substrates of different complexity (100 to 1000 substrates in a mixture) and have shown that the response of individual substrates is independent of the complexity of the mixture. Our initial results demonstrated the possibility to perform the protease assay in a multiplexed substrate environment with high sensitivity. Figure 1 shows the response of various model proteases.

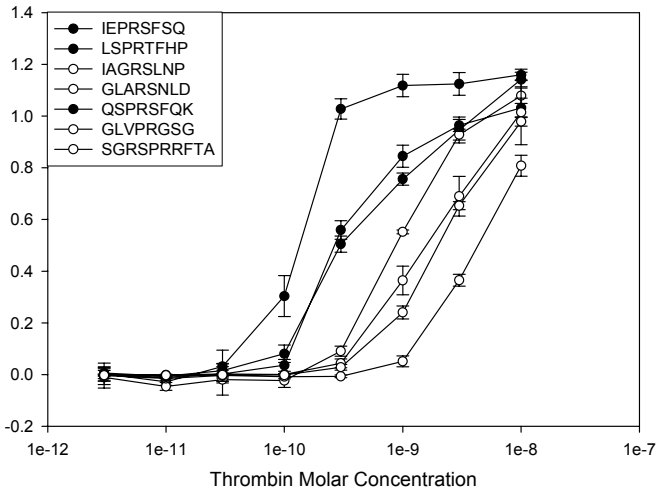
Figure 1. Radial plots of 1000-plex substrate pool with model proteases.



We have used thrombin as a model enzyme for characterization of our assay using the 1000-plex substrate pool (substrate concentration ~0.25nM each). The limit of detection (LOD) of thrombin activity using the best substrate in the pool (IEPRSFSQ) is 27 pM. An important characteristic of the enzymatic assay is the linear dynamic range of enzyme concentrations which could be quantified. As can be seen from the Fig. 2, even though

the linear dynamic range defined by an individual substrate is limited (roughly one order of magnitude), the use of various substrates combined can extend the dynamic range to over three orders of magnitude. Thus, the multiplexed assay provides a significant improvement over the assays utilizing only single substrate. The reproducibility of the assay allows the user to determine a measurable fold-change of <1.5 in thrombin concentration.

Figure 2. Titration curves of the best thrombin substrates found in 1000-plex assay. Error bars indicate the standard deviation of three hybridization replicates.



To detect kinase-dependent phosphorylation, we developed a specific chemical modification of the electrophilic phosphate group with an amino-functionalized dye, utilizing 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) as the coupling agent. We achieve selective phosphate labeling in a one-tube reaction, with a carboxylate blocking step (EDC, pH 6) prior to phosphate labeling as a key feature. Following labeling, the substrate mixture is deconvolved by hybridization to the complementary DNA sequences on a microarray. The approach is simple, mild, and labels all three phosphorylated amino acid residues in a variety of peptide substrates. Our substrate pool contains a diverse set of substrates targeted at over 50 kinases from all over the kinome. Similar to the protease assay, a broader range of enzyme concentrations can be quantified than possible with a single substrate. We characterized the phosphorylation signature for 26 different kinases acting on the mixture of 900 peptide substrates (~10 nM each), and showed that these signatures support detection of multiple kinase activities (at least 4) simultaneously. The ability to test kinase inhibition against multiple kinases at once provides economical screening of both novel and off-target inhibition by drug candidates. In addition, the analysis of kinase activities in a cell extract can be performed.

Acknowledgement
The project was partially supported by NIH SBIR grants R44 AI056869-02 and R43 GM071272-01A1.

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