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TECHNICAL NOTE

Phosphorylation Site Specific Aptamers for Cancer Biomarker Peptide Enrichment and Detection in Mass Spectrometry Based Proteomics

INTRODUCTION

The development of renewable capture reagents that could be produced more efficiently and cost-effectively than monoclonal antibodies is a key topic in diagnostics and therapeutics. The cancer research community has demonstrated a critical need for these reagents for biomarker detection. In collaboration with the National Cancer Institute's Office of Cancer Clinical Proteomics Research, **Base Pair Biotechnologies** has developed anti-peptide capture reagents that can be used to identify and quantify proteins containing a specific target peptide sequence and specific modification. Such reagents could be extremely useful in biomarker validations using powerful technologies, such as Stable Isotope Standard Capture with Anti-Peptide Antibodies (SISCAPA) in conjunction with Multi-Reaction Monitoring Mass Spectrometry (MRM-MS)[1-3]. The scientists at **Base Pair Biotechnologies** have developed high-affinity aptamers against specific phosphorylated and unmodified peptide target sequences (Table 1) of certain cancer biomarkers of interest by utilizing a ***patented, multiplexed*** "Systematic Evolution of Ligands by Exponential enrichment" (SELEX) approach.

TARGETS

Protein phosphorylation is one of the most common post-translation modifications and plays an essential role in nearly every cellular process[4,5]. Many diseased states, such as cancer, arise from the disruption of protein phosphorylation events[4,5]. As a result, one particular class of cancer biomarkers of interest are extracellular signal related kinases (ERKs), also known as mitogen activated protein kinases (MAPKs). Phosphorylation of these enzymes and subsequent proteins in the ERK/MAPK cascade leads to their activity which plays a role in the initiation and regulation of meiosis, mitosis, and post-mitotic functions in differentiated cells. The cascade may be initiated by a diverse range of external stimuli, including mitogens, growth factors, and cytokines. Cancers arise when the proteins in the pathway become unregulated via mutations, therefore it is important in the cancer diagnostic and therapeutic community to develop binding agents that may distinguish the phosphorylated versions of certain proteins[6-11].

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APTAMER SELECTION

Aptamers are single-stranded DNA or RNA oligonucleotides selected to have unique three dimensional folding structures for binding to a variety of targets such as proteins, peptides, and even small molecules with an affinity and specificity rivaling that of antibodies. They are typically selected by an *in vitro* process referred to as "SELEX" [12,13] as depicted in ***Figure 1***. Using a proprietary variant of this process, **Base Pair Biotechnologies** is developing aptamers to multiple targets simultaneously, decreasing development time and cost. Our novel, multiplexed SELEX platform also presents a unique advantage for developing binding agents against peptides. Due to the low immunogenicity of molecules such as peptides (<5000 Daltons), raising antibodies against these targets requires coupling the peptide to a carrier molecule, potentially affecting the affinity and specificity of the binding agent towards the desired target[14]. SELEX is well-suited for generating binders against peptides and other small molecules, and counter-selections can be employed to remove binders of structurally related molecules.

We were able to find specific, high affinity aptamers towards peptides phosphorylated at one particular residue from its non-phosphorylated version. We also were able to determine site specificity by challenging a phosphorylation specific aptamer to the same peptide with a proximate alternate phosphorylation site. We verified the binding affinity via Microscale Thermophoresis (MST)[15-17] which allows fast, immobilization-free and label-free quantification of biomolecule interactions with high sensitivity and accuracy as well as low sample consumption. A previous technical note entitled "Binding Analytics of an Aptamer to a Small Molecule Determined by Microscale Thermophoresis" details the MST analysis of Base Pair aptamers.

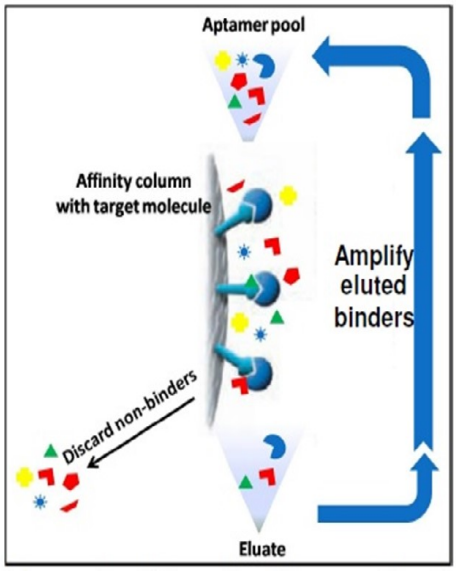

Figure 1: *Overview of SELEX for Production of DNA Aptamers. A randomized library, flanked by two constant regions for PCR priming is constructed. The library is allowed to bind with the target and partitioned from the non-binding population. Following repeated rounds of selection and enrichment, high affinity DNA ligands are cloned and sequenced.*

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METHODS

To select aptamers binding with a high affinity and specificity to these targets, the target peptide molecules were synthesized and multiplexed, and multiple rounds of selection were performed using a 32-nucleotide randomized DNA library. A proprietary approach to counter-selection was also performed to increase the likelihood of phosphorylation specificity. After all rounds of selection were completed, the targets and their corresponding bound DNA sequences were subjected to next generation sequencing. An *in silico* analysis of the secondary structure (using either Vienna or mfold[18,19]) and sub-sequence motifs as well as enrichment of particular sequences was performed in an effort to predict which candidate sequences are most likely to exhibit strong binding. Furthermore, sequence alignments and consensus sequences were determined to reduce the number of unique candidates per target to be tested. Selected clones were then characterized by MST binding studies in both PBS and 20% human serum.

RESULTS

We were able to find aptamers towards both phosphorylated and unmodified versions of the same peptide sequence (ERK1* peptide and ERK1 peptide, respectively), as well as aptamers against peptides that were phosphorylated at two different locations (ERK3* peptide and ERK4* peptide). We determined the binding affinity (K_d) of the aptamers to these peptides using MST binding studies in duplicate using a nonlinear fit of the data according to the law of mass action.

Table 1 *Comparison of binding affinities of aptamers in PBS versus 20% serum. * indicates phosphorylated protein. Lowercase, underlined letters in Target Peptide Sequence indicates a phosphorylated amino acid.*

Aptamer Name	Parent Protein	Selected Target	K_d PBS	K_d 20% Serum	Cross reactivity	K_d 20% Serum
D5A4Z	ERK1*	IADPEHDHTGFL**t**EYVATR	16.9 nM	12.9 nM	IADPEHDHTGFL**T**EYVATR	No binding
EBSGH	ERK1	IADPEHDHTGFL**T**EYVATR	13.2 nM	10.1 nM	IADPEHDHTGFL**t**EYVATR	No binding
D4MWF	ERK3*	VADPDHDHTGFL**t**EYVATR	8.0 nM	7.7 nM	VADPDHDHTGFLTE**y**VATR	No binding
D46VZ	ERK4*	VADPDHDHTGFLTE**y**VATR	160 nM	~uM	VADPDHDHTGFL**t**EYVATR	No binding

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RESULTS

Peptide aptamers selected in 20% serum were proven to bind similarly in both serum and PBS. ***Figure 2*** shows the binding of ERK1* peptide aptamer (D5A4Z) against the ERK1* peptide target sequence in Table 1 in both PBS (Figure 2a) and 20% serum (Figure 2b). We achieved a binding of 16.9 nM and 12.9 nM, respectively, with an R^2 fit of >0.97.

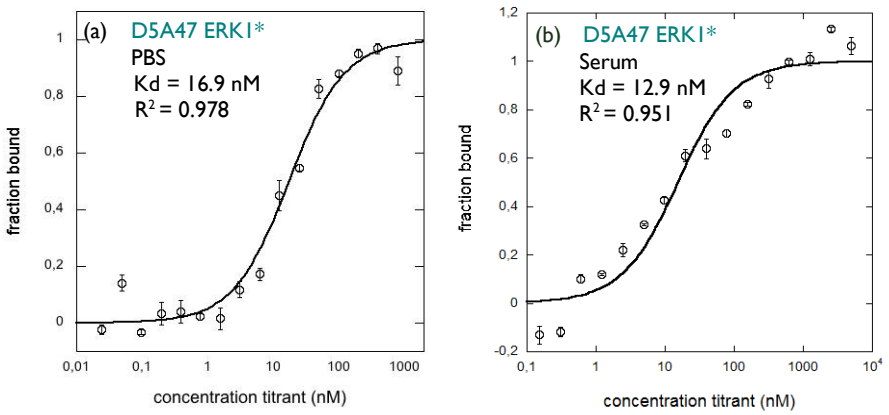
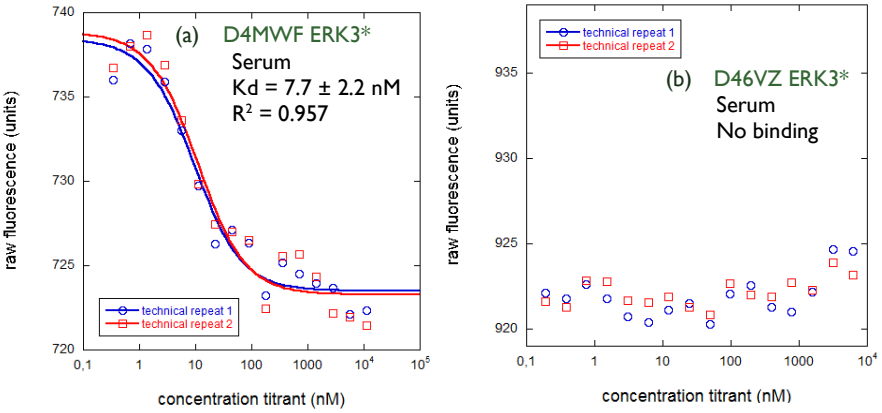


Figure 2: *Equilibrium binding constants (Kd's) of aptamers and target peptides as determined by microscale thermophoresis (MST). (a) ERK1* aptamer (D5A4Z) against ERK1* peptide sequence in PBS buffer, Kd = 16.9 ± 3.5nM, R^2 = 0.978. (b) ERK1* peptide aptamer (D5A4Z) against ERK1* peptide sequence in serum, Kd = 12.9 ± 4.3 nM, R^2 = 0.951 .*

Figure 3 shows the raw data binding events of the ERK3* peptide aptamer to the ERK3* peptide sequence in 20% serum (Figure 4a). However upon addition of the ERK4* peptide aptamer to the ERK3* peptide, (Figure 4b) no binding is indicated, showing lack of cross-reactivity of this aptamer against site-specific phosphorylation. Raw data is used due to inability to determine binding curves for cross reactive aptamers.

Figure 3*: Raw MST data of aptamers and target peptides in serum (a) ERK3* aptamer (D4MWF) against ERK3* peptide and (b) ERK4* aptamer (D46VZ) against ERK3* peptide (no binding indicated).*



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CONCLUSION

Base Pair Biotechnologies has characterized the binding affinity (dissociation constant, K_d) of several aptamer-peptide interactions as well as their off-target interactions (cross-reactivity). We have developed novel affinity reagents which tightly bind clinically relevant peptides and have showcased our abilities to provide highly specific and sensitive affinity agents in comparison to monoclonal antibodies in a short period of time and at low cost compared to conventional SELEX. The resulting reagents will be provided to researchers at the National Cancer Institute and will be made available to the larger research community. In the near term, they will be evaluated as surrogates for antibodies in a SISCAPA MRM-MS approach. Based on the representative binding performance above, we expect the reagents to be of great value to the cancer research community. More importantly, we will have demonstrated the ability to rapidly and inexpensively generate anti-peptide affinity reagents – something that has proved quite challenging for conventional antibody technology.

Talk to the aptamer selection specialists at Base Pair about our ability to address sophisticated affinity reagent goals with unique aptamer selection strategies.

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