Development of Protein-Catalyzed Capture (PCC) Agents with Application to the Specific Targeting of the E17K Point Mutation of Akt1

Thesis By:

Kaycie Marie Deyle

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, CA

2014

(Defended: May 19, 2014)

"There is no passion to be found playing small - in settling for a life that is less than the one you are capable of living." – Nelson Mandela

"To give anything less than your best is to sacrifice the gift." – Steve Prefontaine

Abstract

This thesis describes the expansion and improvement of the iterative *in situ* click chemistry OBOC peptide library screening technology. Previous work provided a proof-of-concept demonstration that this technique was advantageous for the production of protein-catalyzed capture (PCC) agents that could be used as drop-in replacements for antibodies in a variety of applications. Chapter 2 describes the technology development that was undertaken to optimize this screening process and make it readily available for a wide variety of targets. This optimization is what has allowed for the explosive growth of the PCC agent project over the past few years.

These technology improvements were applied to the discovery of PCC agents specific for single amino acid point mutations in proteins, which have many applications in cancer detection and treatment. Chapter 3 describes the use of a general all-chemical epitope-targeting strategy that can focus PCC agent development directly to a site of interest on a protein surface. This technique utilizes a chemically-synthesized chunk of the protein, called an epitope, substituted with a click handle in combination with the OBOC *in situ* click chemistry libraries in order to focus ligand development at a site of interest. Specifically, Chapter 3 discusses the use of this technique in developing a PCC agent specific for the E17K mutation of Akt1. Chapter 4 details the expansion of this ligand into a mutation-specific inhibitor, with applications in therapeutics.

Acknowledgements

I must start by thanking my family, without whom I would not be where I am today. My parents, Ed and Diane, have supported me in every way possible since the day I was born. You both have raised me and the world's best brothers (both pretty incredible people), Matt and Shawn, to be like yourselves – strong, confident, intelligent, and hard-working. With the tools with which you have equipped me for this life, anything is possible. The closeness of my family astounds me, and I have the honor of saying that some of my best friends were destined to be a part of my life from birth. To my immediately family, but also my extended family, including my grandparents (Clarence and Mary Ann Badar), and my vast network of aunts, uncles, and cousins, thank you for always being there and for helping to mold me into the person I am today.

To my husband, Casey, who has been there for every day of this journey, cheering me on through the best days while reminding me that there is more to life during the worst. Major thanks for being a constant source of support and laughter, and for ensuring that we were always well-stocked with ice cream and wine.

Next, I must thank my advisor, Jim Heath, for his well-timed guidance throughout the course of my six years in his lab. I greatly appreciate the ability to explore and learn on my own and at my own pace, being roped in only when necessary. Thank you for showing me that nothing in science is impossible if you can think about it the right way, and for ensuring that we always had more than enough funding to do the best possible work.

To my committee, Dennis Doughtery, Doug Rees, and John Bercaw, I greatly appreciate the guidance and direction that you have provided throughout the years. Your insightful questions and candid advice have expanded my range as a scientist. To my previous mentors, Carl LeBlond and LeRoy Whinnery, thank you for taking a raw student under your wings and

turning her into a scientist, and to Dr. LeBlond, thank you for instilling in me a love for organic chemistry and science in general. To LeRoy, your constant support throughout all of the years in which I have known you has meant more to me than you will ever know.

I must next thank my colleagues in and around the Heath group. I have had the sincere pleasure these past six years to work with the hands-down smartest, most capable and genuinely nicest lab mates I have ever seen assembled. You have all enriched my Caltech experience beyond measure and have encouraged me every single day to be better than I ever thought possible. A special thanks to Blake Farrow, Jessica Pfeilsticker, Aiko Umeda, Steve Millward, Heather Agnew and Bert Lai for being such capable and absolutely amazing people to work with, not to mention some of my best friends. I would get my hands dirty again in a lab with any of you at any time. To the rest of the capture agents group: Rosemary Rohde, JingXin Liang, Arundhati Nag, Samir Das, Ryan Henning, Joey Varghese, and Mary Beth Yu, thank you for being incredible lab mates and really helping to push our technology to the limits. And to all of the Heath group members past and present, for our interactions both personal and professional, thank you for being there to both enhance my Caltech experience and keep me sane: Alex Sutherland, Min Xue, Jing Yu, Jing Zhou, Wei Wei, Jun Wang, Kevin Kan, Habib Ahmed, Udi Vermesh, Ophir Vermesh, Slobadon Mitrovik, Ann Cheung, Kiwook Huang, John Nagarah, and Jen-Kan Yu. I have met some of the best people I hope to ever have the pleasure of calling friends in this lab.

Life at Caltech ran so smoothly for me, mostly due to the amazing support system in place around me. I cannot thank the people that make up that system, and have allowed me to focus more on research and less on paperwork: Elyse Garlock, Joe Drew, Carlos Rodriquez, Cora Carriedo, Ron Koen, and Steve Gould, to name a few. I also must thank Jost Vielmetter and his team at the Protein Expression Center (PEC) for being so amazingly helpful all of the time. Felicia Rusnak and Jie Zhou at the Protein and Peptide Mass Analysis Lab (PPMAL) for all of their help

and experimental input over the years. And Mona Shahgholi for always be willing to chat, and her advice and assistance with MS techniques and experiments.

There is sometimes a life outside of lab, and mine was far richer for the people that were in it. To Tim Mui, thank you for being the world's greatest roommate, running buddy and sounding board. To Kristina Daeffler, Chris Daeffler, Renee Thomas, Catrina Pheeney, and Matthew Van Wingerden, for always being up for a good game – be it board or football – or a good bottle of wine. Both of these are better with friends. To the Robinsons – Diane, Brent, Katherine and Brenna – for opening up your lives and your home (and grill!) to Casey and me, whenever we wanted or needed the company. Your support has been indescribable these past few years, and we consider you our extended family. To my twin, Joe Zewe, for being never more than a phone call away, and for always somehow knowing exactly what to say. I raise my phone-shot of Jack to you, sir! To my soul mate and maid-of-honor, Jessica Nichol, for…everything. Your support, whether by phone, email, gchat or random cards and care packages has certainly seen me through my fair share of tough times. Thank you for being one of the most caring and considerate people I have ever met. And to Marc, whom I still miss as a lab partner to this day, Whoomp! There it is!

Last but not least, to AJ. You have shown me the very definition of bravery and strength, and I strive to face much lesser adversity with your grace and courage. I miss you every day, and you remain, as you will forever, the never-ending source of my inspiration. Love you always.

Table of Contents

List of Figures and Tables

Chapter 2:

Chapter 3:

Chapter 4

Chapter 5

Appendix A:

Chapter 1

Introduction

1.1 Protein-Catalyzed Click (PCC) Peptide Capture Agents for Biomarker Detection and Therapeutics

Detecting cancer-associated biomarkers is a necessary step on the road to personalized medicine, as emerging therapeutics require the identification of specific patient populations that will respond to targeted therapies[1]. Methods for protein biomarker detection are highly desirable for rapidly screening changes in protein mutation status, monitoring patient treatment[2], and simple point-of-care diagnostics[3]. Techniques that rely on detecting or monitoring protein levels mainly use antibodies for the capture and measurement of these proteins[4]. Antibodies, however, are biological reagents that are inherently unstable, vary from batch to batch, can exhibit high levels of cross-reactivity with other antibodies, and are expensive to produce[5]. Diagnostic assays are frequently prohibitively limited in both cost and stability due to the restrictions of the gold-standard antibody detection agents.

Peptides can be the missing link for both inexpensive biomarker detection and targeting traditionally undruggable proteins. Peptide - protein interactions cover a large surface area, producing antibody-like affinities with unsurpassed specificities[6]. To date, most peptide discovery techniques use genetically-encoded libraries, which allow for ease of library generation and rapid and simple sequencing. These techniques permit screening of enormous numbers of compounds against a target of interest without any complicated syntheses or detailed knowledge of the target[7]. These libraries, however, are limited by the biological system from which they are derived, both in terms of screening elements and library size. Most of these systems, such as phage display, bacterial display and yeast display, are confined to the natural amino acids because they use the cell machinery to make and express their libraries. These systems limit the suitability of the resulting peptide capture agents due to the instability of biological peptides, which are comprised of naturally-occurring L -amino acid monomers that can be degraded in biological systems and fluids.

The Heath group has sought to alleviate the issue of peptide capture agent instability by relying exclusively on the use of unnatural amino acids. Because biological libraries are not conducive to this type of work, we have instead adopted a peptide screening method utilizing One-Bead, One-Compound (OBOC)[8] chemically synthesized libraries on 90μm polystyrene beads. This technique trivializes the inclusion of any unnatural amino acid or structure that can be chemically synthesized, allowing for the use of biologically stable D - amino acids and azide-alkyne click chemistry handles in the library[9].

The Sharpless group showed that the typical azide - alkyne click catalyst, Cu(I)[10], speeds up the reaction but is only barely necessary for it to occur, and demonstrated the ability to replace this catalyst with the surface of a protein. They took advantage of this to assemble small molecule inhibitors for proteins by breaking up known inhibitors into two components and assembling two libraries – each one comprised of pieces similar to its original half of the inhibitor. One of these libraries of molecules was appended with a click handle, the other library with the opposite click handle. When two click reactants bound tightly to the protein surface and in close enough proximity to each other, the long dwell time of these reagents allowed for the click to occur without the use of Cu(I)[11]. In this way, they were able to bring the two libraries, which consisted of variations on the original inhibitor, together and use the surface of the protein to assemble the best possible small molecule inhibitor.

We have adapted this technology to assemble 5-mer peptide sequences displayed on OBOC libraries using the surface of the target protein itself to catalyze a click reaction between peptides that bind tightly to this surface. Hence, we have termed these capture agents “protein catalyzed capture” (PCC) agents. This strategy requires that the two compounds are high-affinity, selective binders for the target that is acting as a catalyst because the click reaction does not occur without a long dwell time between the two agents. PCC agents have been developed against a

number of protein targets, and have been shown to exhibit a selectivity and affinity similar to those of monoclonal antibodies. They also can be readily integrated into all standard protein assay formats.

Chapter 2 of this thesis describes the technology development process that was undertaken to optimize the screening stages for the production of high-affinity ligands to targets of interest. Optimizing the in-depth screening procedure has allowed for the rapid expansion of this project in the past few years. This detailed *in situ* azide-alkyne click screening technology is now regularly used to develop peptide affinity agents that mimic the performance of antibodies[9-15]. These affinity agents that maintain the stability of small molecules can be made to replace biological reagents[9,12,15], lowering the cost and increasing the robustness of detection assays[13,14].

1.2 Epitope Targeting Strategies

The detection of single amino acid point mutations in proteins is critical in the identification of specific patient populations that will respond to targeted therapies in the new era of personalized medicine[1]. The current techniques for mutation detection rely on either capture and measurement of these proteins through antibodies,[4] or on DNA sequencing. DNA sequencing is currently an expensive and time-consuming route to take for mutation screening, especially as most patients need to be screened for mutations before the proper course of their treatment is even decided[16]. Antibodies can provide a faster route for mutation detection and treatment monitoring, as there are methods currently in place for their use as rapid point-of-care diagnostics[3]. These diagnostic tests also provide information about the levels of protein expression in a body, something that cannot be tested through sequencing, which can be used to monitor the response level of a patient to a certain treatment, potentially detecting ineffective

medications immediately after they are given. In a diagnostic setting, such binders can be used to assay for the mutant protein within diseased tissues, and thus potentially provide clinical guidance for treatment decisions[3].

A more ambitious application is the development of drugs that can selectively inhibit mutant proteins, and thus avoid those toxic side-effects that stem from the inhibition of the wild-type (WT) variants[17] that reside in non-diseased tissues. Patients on therapies targeted very specifically to the mutations characteristic of their disease could show significant improvements without the toxic side-effects that stem from of the inhibition of the healthy, wild-type versions of these proteins[17]. A relevant example is compound CO-1686, which is an a epidermal growth factor receptor (EGFR) inhibitor specific for the T790M point mutation associated with certain non-small cell lung carcinomas. That drug, which is currently in clinical trials, is designed to minimize the toxicities (such as skin rash) that can appear when WT EGFR is targeted, since WT EGFR is expressed throughout the healthy tissues in the body[18].

A challenge of drug targeting a single point mutation is that the mutation may not be directly associated with a binding pocket. The presence of a binding pocket is traditionally required for small molecule inhibitor development as is serves as a thermodynamic sink that can attract binders. This requirement does not hold for antibodies and, in fact, several examples of monoclonal antibodies directed against epitopes containing single amino acid mutations do exist[19,2,20]. However, antibodies do not readily enter the living cells that can harbor the mutated proteins[21,22], and so, mutation-selective antibodies are typically only used as diagnostic reagents for staining fixed cells or tissues.

Thus, there is a need for an approach that can identify small molecules that can be generally targeted against epitopes containing single amino acid point mutations to allow for the rapid detection and assessment of tumor status, and can also potentially be developed into cell-

penetrant inhibitors[5]. Our approach is inspired by the technique for developing an epitope-targeted monoclonal antibody (mAb). Such mAbs are made by injecting a small portion of the protein of interest containing the mutation (the epitope) into an animal and screening for an immune response that has the desired selectivity[2,20,19]. This approach can yield an antibody that exhibits focused binding to the specific designated area of the protein surface.

An all-chemical strategy for targeting PCC agent development against epitopes near phosphorylates sites was developed recently[15]. For that approach, an approximately 30-amino fragment representing the phosphorylated epitope of interest was synthesized, and a metalloorganic Zn-chelator was utilized to bind to the phosphate group and present an azide near that site. That epitope was then screened against a large (1 million element) one-bead-one-compound (OBOC) library of 5-mer alkyne-presenting peptides. Hits were defined as those compounds that bound to the synthesized epitope, and that were coupled to that epitope through a triazole linkage. PCC Agents with high selectivity for the epitope and the full protein, and with affinities as low as 19nM, were developed.

The bulk of my thesis work focuses on the generalization of the epitope targeting strategy by directly substituting an alkyne click handle into the chemically synthesized peptide epitope (around the E17K residue of Akt1) of interest. Chapter 3 describes how this technique was used to develop a 5-mer PCC agent selective for the E17K mutant Akt1 protein. This PCC agent was able to be used as a drop in antibody replacement for the detection of this single amino acid mutation in various assays. It was also possible to render this agent cell-membrane permeable, and this allowed it to be used as a focused imaging agent in live cell experiments. Chapter 4 describes the expansion of this PCC agent into a biligand and then a triligand through the use of iterative *in situ* click chemistry in order to make a bulkier PCC agent. The final triligand PCC agent is capable of blocking the binding of the mutant protein to its substrate at the cell membrane,

rendering it inactive and demonstrating the ability of these PCC agents to serve as targeted therapeutics.

1.3 *In Situ* Click Screening Using Azide-Containing Phage Display Libraries

Peptide screening technology has expanded incredibly in the past ten years since the inception of the PCC agent project. Using the protein-catalyzed click screens described above, PCC agents have been developed against only small chunks, or "epitopes" of proteins[15], and various PCC agents that have shown to be unique inhibitors and activators of Akt kinase[23,15], molecular imaging agents[24], detection agents for anthrax[14], suitable as third world detection agents for HIV[13], as well as the single amino acid point mutation specific E17K agents.

The OBOC libraries have their drawbacks, however. The physical size of the library limits the number of total sequences that can be screened. A full library usually contains up to 10^6 members – only a portion of which are screened. The library screening and hit picking methods are exceptionally time-consuming and labor-intensive, hindering rapid peptide discovery. The sequencing of OBOC libraries is also done by either Edman degradation or MALDI TOF/TOF, rendering the sequencing process expensive, time-consuming, and reliant on expert knowledge. Many of these drawbacks are also a huge barrier to entry in this field, limiting the labs that would be able to assist in the advancement of the science. PCC agents could be produced significantly faster and cheaper with library display technology that would combine the advantages of the OBOC product screening techniques and library design with the rapid screening and sequencing of genetically displayed libraries.

Recent advances in biology have made it possible to incorporate unnatural amino acids into the genetic code[25]. Schultz has shown that through the use of amber suppression, azide-containing amino acids can be incorporated in specific locations into the pIII coat protein on an

M13 phage[26]. The *Methanococcus jannaschii* amber suppressor tRNATyr (MjtRNA) and the mutant *M.jannaschii* tyrosyl-tRNA synthetase (MjTyrRS) DNA can be contained in one plasmid that can be used to express these amber suppression tools in *E.coli*. In this system, the mutant synthetase is used to attach the unnatural amino acid azidophenylalanine to the tRNA in vivo, allowing for its incorporation into proteins. This tRNA recognizes the amber stop codon and should insert the amino acid in only that location, creating a new amino acid/tRNA combination that can be encoded into proteins.

Chapter 5 discusses the ongoing development of a screening technology that combines the *in situ* click screen advantages of the OBOC process with the rapid screening of large libraries characteristic of biological display systems. For this project, a phage display library containing azidophenylalanine for use in *in situ* click chemistry screening has been made and is being used to develop a PCC agent. These phage libraries can be screened in place of the OBOC peptide libraries described in previous chapters for the more rapid development of PCC agents.

1.4 References

1. Hanash, S. M.; Baik, C. S.; Kallioniemi, O., Emerging molecular biomarkers--blood-based strategies to detect and monitor cancer. *Nature reviews. Clinical oncology* **2011,** *8* (3), 142-50.
2. Yu, J.; Kane, S.; Wu, J.; Benedettini, E.; Li, D.; Reeves, C.; Innocenti, G.; Wetzel, R.; Crosby, K.; Becker, A.; Ferrante, M.; Cheung, W. C.; Hong, X.; Chirieac, L. R.; Sholl, L. M.; Haack, H.; Smith, B. L.; Polakiewicz, R. D.; Tan, Y.; Gu, T.-L.; Loda, M.; Zhou, X.; Comb, M. J., Mutation-Specific Antibodies for the Detection of EGFR Mutations in Non–Small-Cell Lung Cancer. *Clinical Cancer Research* **2009,** *15* (9), 3023-3028.
3. Rusling, J. F.; Kumar, C. V.; Gutkind, J. S.; Patel, V., Measurement of biomarker proteins for point-of-care early detection and monitoring of cancer. *Analyst* **2010,** *135* (10), 2496-2511.
4. Borrebaeck, C. A., Antibodies in diagnostics - from immunoassays to protein chips. *Immunology today* **2000,** *21* (8), 379-82.
5. Kodadek, T.; Reddy, M. M.; Olivos, H. J.; Bachhawat-Sikder, K.; Alluri, P. G., Synthetic molecules as antibody replacements. *Accounts of chemical research* **2004,** *37* (9), 711-8.
6. Edwards, P. J.; LaPlante, S. R., Peptides as Leads for Drug Discovery. In *Peptide Drug Discovery and Development*, Wiley-VCH Verlag GmbH & Co. KGaA: 2011; pp 1-55.
7. Sun, N.; Funke, S. A.; Willbold, D., Mirror image phage display – Generating stable therapeutically and diagnostically active peptides with biotechnological means. *Journal of Biotechnology* **2012,** *161* (2), 121-125.

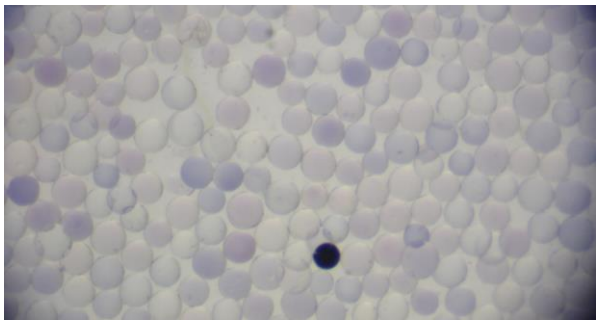
8. Lam, K. S.; Lebl, M.; Krchňák, V., The "One-Bead-One-Compound" Combinatorial Library Method. *Chemical Reviews* **1997,** *97* (2), 411-448.
9. Agnew, H. D.; Rohde, R. D.; Millward, S. W.; Nag, A.; Yeo, W.-S.; Hein, J. E.; Pitram, S. M.; Tariq, A. A.; Burns, V. M.; Krom, R. J.; Fokin, V. V.; Sharpless, K. B.; Heath, J. R., Iterative In Situ Click Chemistry Creates Antibody-like Protein-Capture Agents. *Angewandte Chemie International Edition* **2009,** *48* (27), 4944-4948.
10. Kolb, H. C.; Finn, M. G.; Sharpless, K. B., Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angewandte Chemie International Edition* **2001,** *40* (11), 2004-2021.
11. Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radić, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B., Click Chemistry In Situ: Acetylcholinesterase as a Reaction Vessel for the Selective Assembly of a Femtomolar Inhibitor from an Array of Building Blocks. *Angewandte Chemie International Edition* **2002,** *41* (6), 1053-1057.
12. Millward, S. W.; Henning, R. K.; Kwong, G. A.; Pitram, S.; Agnew, H. D.; Deyle, K. M.; Nag, A.; Hein, J.; Lee, S. S.; Lim, J.; Pfeilsticker, J. A.; Sharpless, K. B.; Heath, J. R., Iterative in situ click chemistry assembles a branched capture agent and allosteric inhibitor for Akt1. *Journal of the American Chemical Society* **2011,** *133* (45), 18280-8.
13. Pfeilsticker, J. A.; Umeda, A.; Farrow, B.; Hsueh, C. L.; Deyle, K. M.; Kim, J. T.; Lai, B. T.; Heath, J. R., A Cocktail of Thermally Stable, Chemically Synthesized Capture Agents for the Efficient Detection of Anti-Gp41 Antibodies from Human Sera. *PLoS ONE* **2013,** *8* (10), e76224.
14. Farrow, B.; Hong, S. A.; Romero, E. C.; Lai, B.; Coppock, M. B.; Deyle, K. M.; Finch, A. S.; Stratis-Cullum, D. N.; Agnew, H. D.; Yang, S.; Heath, J. R., A Chemically Synthesized Capture Agent Enables the Selective, Sensitive, and Robust Electrochemical Detection of Anthrax Protective Antigen. *ACS Nano* **2013,** *7* (10), 9452-9460.
15. Nag, A.; Das, S.; Yu, M. B.; Deyle, K. M.; Millward, S. W.; Heath, J. R., A Chemical Epitope-Targeting Strategy for Protein Capture Agents: The Serine 474 Epitope of the Kinase Akt2. *Angewandte Chemie International Edition* **2013,** *52* (52), 13975-13979.
16. Mardis, E. R., A decade's perspective on DNA sequencing technology. *Nature* **2011,** *470* (7333), 198-203.
17. Chong, C. R.; Janne, P. A., The quest to overcome resistance to EGFR-targeted therapies in cancer. *Nat Med* **2013,** *19* (11), 1389-1400.
18. Tjin Tham Sjin, R.; Lee, K.; Walter, A. O.; Dubrovskiy, A.; Sheets, M.; St Martin, T.; Labenski, M. T.; Zhu, Z.; Tester, R.; Karp, R.; Medikonda, A.; Chaturvedi, P.; Ren, Y.; Haringsma, H.; Etter, J.; Raponi, M.; Simmons, A. D.; Harding, T. C.; Niu, D.; Nacht, M.; Westlin, W. F.; Petter, R. C.; Allen, A.; Singh, J., In vitro and In vivo Characterization of Irreversible Mutant-Selective EGFR Inhibitors that are Wild-type Sparing. *Molecular Cancer Therapeutics* **2014**.
19. Capper, D.; Zentgraf, H.; Balss, J.; Hartmann, C.; von Deimling, A., Monoclonal antibody specific for IDH1 R132H mutation. *Acta neuropathologica* **2009,** *118* (5), 599-601.
20. Capper, D.; Preusser, M.; Habel, A.; Sahm, F.; Ackermann, U.; Schindler, G.; Pusch, S.; Mechtersheimer, G.; Zentgraf, H.; von Deimling, A., Assessment of BRAF V600E mutation status by immunohistochemistry with a mutation-specific monoclonal antibody. *Acta neuropathologica* **2011,** *122* (1), 11-9.
21. Marschall, A. L. J.; Frenzel, A.; Schirrmann, T.; Schüngel, M.; Dubel, S., Targeting antibodies to the cytoplasm. *mAbs* **2011,** *3* (1), 3-16.
22. Rondon, I. J.; Marasco; A., W., Intracellular AntibodiesS (Intrabodies) For Gene Therapy Of Infectious Diseases. *Annual Review of Microbiology* **1997,** *51* (1), 257-283.
23. Millward, S. W.; Henning, R. K.; Kwong, G. A.; Pitram, S.; Agnew, H. D.; Deyle, K. M.; Nag, A.; Hein, J.; Lee, S. S.; Lim, J.; Pfeilsticker, J. A.; Sharpless, K. B.; Heath, J. R., Iterative in Situ Click

Chemistry Assembles a Branched Capture Agent and Allosteric Inhibitor for Akt1. *Journal of the American Chemical Society* **2011,** *133* (45), 18280-18288.
24. Millward, S. W.; Agnew, H. D.; Pitram, S.; Lai, B. T.; Rohde, R. D.; Hardman, N., Protein catalysed capture agents for molecular imaging. *Internation Hospital Equipment and Solutions* **2013**.
25. Wang, L.; Xie, J.; Schultz, P. G., Expanding the Genetic Code. *Annual Review of Biophysics and Biomolecular Structure* **2006,** *35* (1), 225-249.
26. Tian, F.; Tsao, M. L.; Schultz, P. G., A phage display system with unnatural amino acids. *J Am Chem Soc* **2004,** *126* (49), 15962-3.

Chapter 2

Evolution of the OBOC Peptide Library

Screening Protocol



2.1 Introduction

2.1.1 Iterative In Situ Click Chemistry for Protein-Catalyzed Capture (PCC) Agent Development

Previous work in the Heath lab demonstrated that the flexibility of chemically synthesized One-Bead, One-Compound (OBOC) peptide libraries could be combined with the selective power of the *in situ* click process to develop multi-peptide ligand capture agents that can serve as drop-in antibody replacements in assays[1]. These peptide ligands can be made in large quantities entirely by robots, making the scale-up cheap and robust. They are also highly stable agents that can be used in a variety of assays, removing the need for the gold-standard antibodies in a variety of protein detection techniques[2,3].

The iterative *in situ* click screen to develop a capture agent starts with the discovery of a peptide ligand that binds to a protein target through the use of OBOC library screening. Once a peptide has been discovered, labeled the "anchor peptide," it is appended with a click handle and screened again against the protein in the presence of a new OBOC library that contains the opposing click handle, as seen in **Figure 2-1**. When a library member binds to the surface of the protein in close proximity to the anchor ligand and is held in place through a high-affinity for the protein target, a click reaction between the anchor and library-bound ligand can occur without the use of the Cu(I) catalyst. The addition of this new ligand, the secondary ligand, forms a "biligand" in complex with the original anchor. This selection technique allows the protein target itself to catalyze the formation of the peptide ligands that bind to it with the highest affinity and selectivity. This iterative process can be performed as many times as necessary to produce a ligand with the desired affinity and specificity for the target, and serves as the basis for the iterative *in situ* click chemistry technique for protein-catalyzed capture (PCC) agent production. After a PCC agent has been discovered using this technique, the Cu(I) catalyst can be brought back in order to scale-up the final click triazole-containing product in high quantities.

The technology as presented by Agnew, *et al*[1] provided a solid foundation for the construction of these PCC agents, but the methods, discussed in section 2.3.1, were time-consuming and labor-intensive, making rapid ligand discovery very difficult. After this BCAii proof-of-concept PCC agent was completed, the next stage of technology development required an optimization of the techniques involved in order to increase the robustness and output of the overall process. This chapter describes the transformation of the OBOC iterative *in situ* click technology into an efficient and robust technique.

2.1.2 Prostate Specific Antigen (PSA)

Prostate Specific Antigen (PSA) is a serum protease produced by the prostate. The accurate detection of PSA levels in the blood can be a strong indicator of the presence of prostate cancer, but this result is confounded by the elevated PSA levels also seen in Benign Prostate Hyperplasia (BPH), a non-cancerous condition[4]. In serum, PSA is partially in complex with α_1-antichymotrypsin (ACT), with 60-95% generally found as a PSA-ACT complex while the rest of the PSA remains free. It has also been discovered that the PSA-ACT fraction is larger in prostate cancer, whereas BPH has more PSA free in serum[4]. It was hypothesized, therefore, that a better PSA detection test could be designed to measure this through the use of PCC agents, and much of the screening strategies developed in this chapter were focused on the design of this agent.

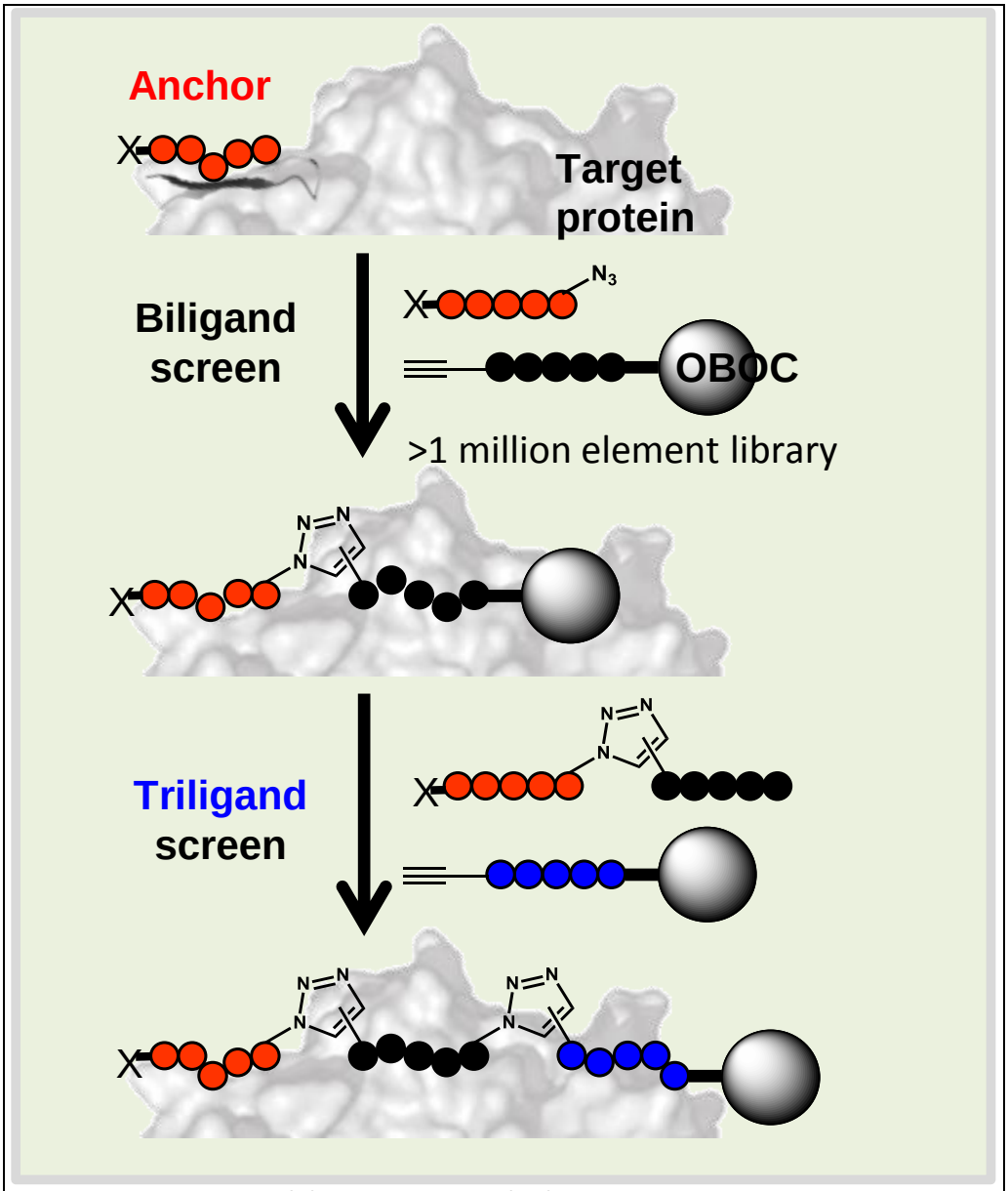


Figure 2-1: Iterative In Situ Click Screening Core Technology.
An anchor ligand that binds to the protein target can be appended with a click handle. In the presence of the protein and a OBOC library appended with the opposite click handle, the anchor can click onto the library to form a biligand. The click only occurs when the anchor and library bead are held long enough on the protein surface, so the protein selects ligands with high affinities and selectivities. This process can be repeated as many times as necessary.

2.2 Materials and Methods

2.2.1 *Standard Materials*

All amino acids were purchased from Aapptec as the FMOC carboxylic acid with the standard TFA side-chain protecting groups. HATU (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and PEG_5 (Fmoc-NH-PEG_5-CH_2CH_2COOH, Fmoc-18-amino-4,7,10,13,16-pentaoxaoctadecanoic acid) were purchased from ChemPep. DIEA (diisoproylethylamine), TES (triethylsilane), and TFA (trifluoroacetic acid) were purchased from Sigma. TentaGel beads were purchased as 90µm S-NH2 beads, 0.29mmol/g, $2.86x10^6$ beads/g from Rapp Polymere (Germany), and Rink Amide resin was purchased from Anaspec.

2.2.2 *Peptide Library Construction*

Peptides and peptide libraries were synthesized by hand until the summer of 2009, when they were then synthesized on a Titan 357 split-and-mix automated peptide synthesizer (Aapptec) via standard FMOC SPPS coupling chemistry[5] using 90µm TentaGel S-NH_2 beads. Libraries contain 18 D-stereoisomers of the natural amino acids, minus cysteine and methionine (unless otherwise stated), at each of five randomized positions and an azide or alkyne *in situ* click handle. At least a five-fold excess of beads is used when synthesizing libraries to ensure efficient oversampling of each sequence. Amino acid side-chains are protected by TFA labile protecting groups that are removed all at once following library synthesis.

Figure 2-2: OBOC Peptide Library *constructed on TentaGel Resin. Where X is comprised of all of the naturally occurring D – amino acids except Cys and Met.*

2.2.3 *Bulk Peptide Synthesis*

Bulk synthesis of peptide sequences was performed using standard FMOC SPPS peptide chemistry on either the Titan 357 automated peptide synthesizer (AAPPTEC) or a Liberty 1 microwave peptide synthesizer (CEM Corporation). The typical scale was 300mg on Rink Amide Resin, unless otherwise noted. Peptides were cleaved from the beads with side-chains deprotected using a 95:5:5 ratio of TFA: H_2O: TES. The peptides were purified on a prep-scale Dionex U3000 HPLC with a reverse-phase C18 column (Phenomenex).

2.2.4 *Typical Screening Protocol for Fluorescent Dye-labeled Protein Target Detection*

Hit beads in the initial OBOC screens were detected via a fluorescent probe attached to the protein target of interest. The target protein was labeled using an Alexa-Fluor 647 Microscale Protein Labeling Kit, following all manufacturer's instructions. The activity of the target enzymes was then tested before screening to ensure that the dye label did not disturb function or folding.

Screens were conducted using a OBOC library of 5-amino-acid-long peptides composed of the D - isomers of 19 naturally occurring amino acids (no Cys, for stability reasons). 100mg of dried library was weighed for screening (~280,000 unique sequences, ~42% sampling of sequence space) and swelled in 1xTBS buffer (25mM Tris, 150mM NaCl, 10mM $MgCl_2$, pH = 7.5) containing 0.05% NaN_3, 0.1% BSA, and 0.1% Tween-20 (TBSTBNaN_3). The library was then blocked for one hour in this buffer, then 50nM protein in 1.5mL TBSTBNaN_3 was added, the screen wrapped in foil to protect the light-sensitive dye label, and incubated overnight on a 180° shaking arm. In the morning, the buffer containing the protein was drained from the beads, which were then washed three times with TBSTBNaN_3, three times with TBS + 0.1% Tween-20 (TBST), then three times with 1xTBS. The beads were then dried on a vacuum and spread to a monolayer on approximately 10 clean microscope slides for about 10mgs of beads per slide. The slides were imaged on a GenePix

Pro 5.1 microarray scanner at 635nm to view beads containing bound fluorescent protein target. The dye saturated the color signal of the GenePix, and the hit "beads" that were considered appeared white in a sea of red, due to the background auto fluorescence of the TentaGel library (**Figure 2-4**). These hit beads were then removed from the microscope slides using a needle, stripped of protein with 7.5M pH = 2.0 Guanadine-HCl buffer, rinsed in water, and sequenced via Edman degradation on an Applied Biosystems Procise CLC 494 system.

2.2.5 *Typical Screening Protocol for Antibody Signal Amplification Target Only Screens*

100mg of library beads were prepared, washed and blocked for one hour as for the fluorescent detection screen. The library was then incubated with about 50nM, which differed slightly based on the exact screen, of protein overnight at room temperature. In the morning, the library was washed five times with 1xTBS + 0.1% BSA + 0.1% Tween-20 (1xTBSTB). The primary anti-protein target antibody was incubated with the library for 1 hour, washed five times with the 1xTBSTB buffer, then incubated with the secondary anti-mouse alkaline-phosphatase antibody for one hour. The library was then washed five times with the TBSTB buffer, three times five minutes each in high salt buffer (1xTBS + 600mM NaCl), and five times in 1xTBS. The screen was developed with a two part BCIP/NBT system: 10mL TBS + 26 µL BCIP + 13 µL NBT. This detection cocktail was mixed with the library beads, which were poured into a large polystyrene dish for visualization of the color change under an optical microscope. Hit library members appear as dark purple among the normally clear beads (**Figure 2-6**), and are removed using a pipet. They are washed, stripped, and sequenced as above.

2.2.6 *Typical Screening Protocol for an Anti-Screen*

The library beads (typically 250-500mg) swelled in 1xTBS were blocked 2 hours to overnight in 5% milk in 1xTBS, washed three times with 1x TBS, then incubated with an off-target protein in 0.5% milk in 1xTBS for one hour on the shaking arm at room temperature. The beads were washed three times with 1x TBS, then incubated with the anti-off-target protein - alkaline phosphatase conjugated antibody in 0.5% milk for one hour at room temperature. The antibody used here must be the same antibody used in the target screen in order to ensure that the library members that bind to this antibody are removed and not mistaken for hits. The library resin was then washed three times with high salt buffer and let shake for one hour in high salt at room temperature before being washed three times with BCIP buffer (100mM Tris-Cl, 150mM NaCl, 1mM $MgCl_2$, pH = 9.0) and developed by adding 15mL BCIP buffer plus 13µL BCIP and 26µL NBT. The beads that turned purple bound to both mutant and wildtype protein or to the detection antibodies, and were discarded. The beads that remained clear after this step were picked and washed with guanidine-HCl to remove any bound proteins.

The off-target protein can be a different version of the target, such as a wildtype protein when detecting for a mutation, or a protein lacking a certain domain or post-translational modification of interest, such as a phosphorylation site or glycosylation. Anti-screens can also be designed to clear against any number of interferents, such as whole human serum, to remove any generally sticky peptide sequences. For these anti-screens, the antibody used for detection is an anti-whole human serum antibody followed by a secondary alkaline-phosphatase conjugated antibody.

2.2.7 *Typical Target Screening Procedure During a Multi-Step Screen (**Figure 2-3**)*

The library beads were blocked in 5% milk in 1x TBS for two hours to overnight. They were then washed three times with 1x TBS. The target protein and anchor peptide or small molecule targeting agent[6] were pre-incubated in 3-5mL of 0.5% milk in an approximately a 10:1 ratio, ensuring the same concentration of anchor peptide used in the preclear. This solution was added to the blocked library beads and incubated for either 5 hours or overnight to allow an *in situ* click reaction to occur. In the morning, the beads were washed three times with 1x TBS, then incubated with the same dilution of an anti-target alkaline phosphatase conjugated antibody that was used in the anti-screen in 0.5% milk for one hour. The beads were then washed three times with a high salt TBS, then incubated on the shaking arm for one hour with the high salt buffer. They were then washed three times with BCIP buffer and developed as previously. Hit beads turned purple and were removed and washed in NMP for four hours to decolorize, then guanidine-HCl to denature and remove and remaining protein.

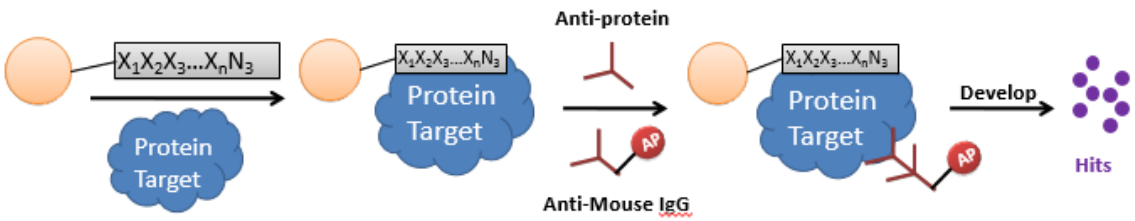


Figure 2-3: Typical Antibody-Detected Target Screen. *The library is incubated with the protein target, which is detected via antibodies conjugated to alkaline phosphatase. The screen is developed with BCIP/NBT, and hit beads turn purple.*

2.2.8 *Typical Screening Protocol for a Preclear*

Swelled library beads (250-500mg) were blocked overnight in 5% w/v dried non-fat milk in 1x TBS, then washed with 1x TBS three times. The beads were incubated with a µM solution of any anchor peptide or small molecule for one hour, then washed 3x with 1xTBS. Five milliliters of either a 1:10,000 dilution of streptavidin-alkaline phosphatase conjugate in 0.5% milk in TBS or an anti-biotin antibody were added to the beads and incubated with shaking at room temperature for one hour. If the anti-biotin antibody was used, a secondary antibody conjugated to alkaline

phosphatase was then incubated with the library for 1 hour after it was washed three times in 1xTBS. The beads were washed with a high-salt TBS buffer three times, then were left to shake in high salt buffer for one hour. The beads were then washed three times with BCIP and developed as for the anti-screen. After one hour, the purple beads were removed by pipette and discarded. The remaining beads were incubated in NMP 4 hours to remove trace purple precipitate from the BCIP/NBT reaction, then were washed five times with methanol, five times with water, five times with TBS and blocked overnight in 5% milk.

2.2.9 *Typical Screening Protocol for a Click Product Screen*

The beads that pass through the target and anti-screen were washed three times with 1x TBS. They were then incubated with a 1:10,000 dilution of either streptavidin – alkaline phosphatase conjugate or anti-biotin antibody (whichever was used in the preclear) in 0.5% milk for one hour. The beads were washed three times with high salt TBS then let shake for one hour with high salt buffer before being washed three times with BCIP buffer and developed as previously. The beads that turned purple contained the anchor peptide covalently bound to the bead and had formed a protein-catalyzed *in situ* click reaction. These beads were collected and stripped with guanidine-HCl for one hour, washed ten times with water, and sequenced via Edman degradation.

2.2.10 *Peptide Sequencing Strategies*

The OBOC peptide library sequencing method most commonly used by Caltech is Edman degradation. This process involves treating a peptide with a free amine terminus with phenylisothiocyanate, which reacts stoichiometricly with the N-terminus of the peptide to form a phenylthiocarbamyl (PTC)-peptide derivative. This PTC derivative is then treated with TFA to cleave it off from the rest of the peptide, leaving behind a new N-terminus to react during the

next cycle. Meanwhile, the PTC amino acid is then analyzed via HPLC, and the peak is compared to standards of all of the PTC-amino acids in order to determine the residue. One cycle per amino acid residue is performed and analyzed, providing the sequence of the peptide on the hit library bead[7]. This method is slow, but highly accurate and has been automated by Applied Biosystems into the Procise CLC 494 Automated Edman Degradation machine used by Caltech.

Hit peptide sequences can also be determined through MALDI-TOF/TOF MS. For this method, the library must be specially made. The peptide must be attached to the library through a methionine amino acid, and no other methionine can be present in the library. The isobaric amino acids, isoleucine and leucine, lysine and glutamine, are doped by anther amino acid in order to properly call the sequence by mass. Glutamine is doped with a 6% molar equivalent of glycine, and isoleucine is doped with a 7% molar equivalent of alanine. While reading the mass of these amino acids on the MALDI, any residue that has one of these amino acids can be distinguished by the presence or absence of the small satellite parent mass corresponding to the same sequence plus glycine or alanine[8].

In order to sequence the library hit by MALDI-TOF/TOF, the bead is first treated with cyanogen bromide in order to cleave the peptide from the bead at the methionine amino acid. It can then be dissolved in MALDI matrix and spotted onto the plate. The peptide parent peak is first discovered using MALDI-TOF, then is fragmented again in order to break it up into smaller amino acid ions. These ions can be analyzed using standard peptide MS techniques to determine the sequence[8].

2.3 Results and Discussion

2.3.1 *Screening via Fluorescent Dye-labeled Protein Target Detection*

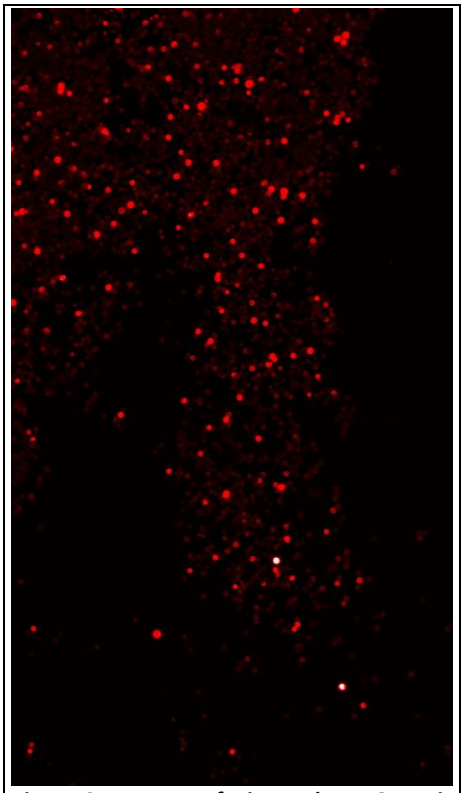


Figure 2-4: Image of Hit Beads on GenePix Microarray Scanner*. The bright white beads are saturating the fluorescence and are considered "hits" above the background TentaGel auto fluorescence.*

The initial OBOC peptide screening strategies developed by Heather Agnew[1] relied on a fluorescent dye-labeled protein in order to detect hit binding. The target protein of interest was labeled with a dye, and any library beads that bound to the target were detected on a GenePix microarray reader. As seen in **Figure 2-4**, the TentaGel library beads also auto-fluoresce, meaning that all screens conducted in this fashion were highly subjective, and the hit quantity depended entirely on the gain settings of the microarray. AlexaFluor-647 was also the only dye that was used, as the beads auto fluoresce the least in the range of this dye. These hits were mostly picked using a light microscope, meaning that the images from the microarray had to be used as a "map" to guide the bead picker to the correct clear bead on a slide of thousands. This process was highly inefficient, requiring up to an hour to pick each individual hit bead. These picked hits were always imaged again on the GenePix to ensure that each bead that had been selected was a highly fluorescent bead, indicating that the correct one had been chosen based on the map. It was possible to use a COPAS automatic bead sorter to separate out the hit beads, though one was not available at Caltech.

The sequences from a typical fluorescent target screen are shown in **Table 2-1**. The hits were generally dominated by the positively charged residues, arginine and lysine. This overwhelming charged signal is most likely due to the overall (-3) charge on the AlexaFluor 647

dye,[9] which is attracting the positively charged amino acid sequences and creating a significant level of noise in the final hits. Most screens had to be run many times in order to find enough quality hit sequences, meaning ones that did not contain almost exclusively arginine and lysine residues, because of this high background. Generally, a hit that contained 3 or more positively charged amino acids was considered background and removed from the pool. One screen rarely yielded more than a handful of hits that appeared to be binding to the surface of the protein and not just to the dye.

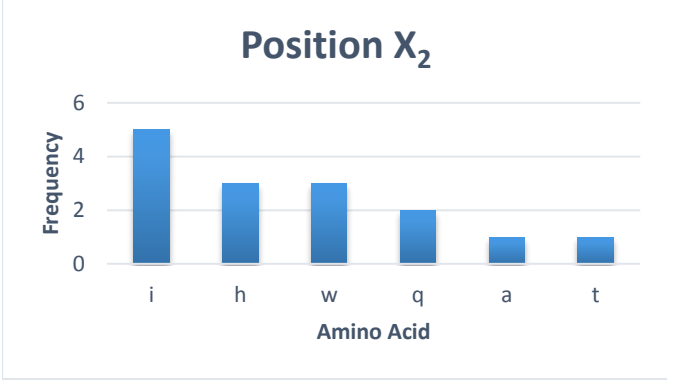


***Figure 2-5: Histogram of Position X_2 in PSA Screens**. The hits from multiple PSA screens were pooled and analyzed. This sample chart shows the frequency of an amino acid at position X2 in the library, and is used to synthesize the focused library.*

Focused screens were also used in order to hone in on target-binding peptide sequences. The focused libraries used in these screens were designed based on histograms of the amino acids that were seen at each library position, meaning X_1 -> X_5 as seen in **Figure 2-2**, after the removal of the dye label background sequences. As can be seen in **Figure 2-5**, in this particular PSA screen, there were only six amino acids that were seen at position 2, so only these six amino acids were built into the focused library at position 2. This reduction in total amino acids present in each position allowed for the synthesis of a much smaller library that could be oversampled in each screen to permit a more thorough sampling of the sequence space. Only about 100mg of beads were usually screened, but 100mg could frequently oversample the sequence space of a focused library, compared to that of naïve libraries where less than half of the space was sampled. Due to this increase in sequence space sampling, focused libraries were generally extended by one or two amino acid positions in the hopes that a slightly longer peptide would have a higher affinity

and selectivity for the protein target. The screening was then repeated with the focused libraries, and the same process for analyzing hits was repeated until the peptide sequences converged in sequence homology and produced a peptide ligand that showed near μM affinity for the protein target.

This convergence frequently required the use of two to three separate focused libraries with accompanying screening and sequencing. The overall time required to determine one peptide ligand that bound to the target protein of interest could easily take more than six months. These ligands also regularly bound in the range of low μM affinities, which are generally considered to be fairly weak binders.

Table 2-1: Screens from Sample Target Screen Using Fluorescent Protein Detection. *This screen was performed against PSA protein labeled with AlexaFluor 647 dye. Note the high prevalence of "r" and "k" positively charged amino acids. See Figure 2-2 for a visualization of the X amino acid positions on bead.*

X_1	X_2	X_3	X_4	X_5
y	r	r	r	r
r	i	f	r	r
r	f	l	r	a
r	r	k	r	f
m	r	r	w	r
r	r	r	w	p
r	r	w	i	r
r	r	r	f	l
r	l	r	w	r
r	f	r	i	r
l	s	r	r	r
r	r	r	y	t
r	r	m	r	w
r	r	k	p	r
f	y	r	r	r
r	k	w	l	w
k	r	r	m	r

2.3.2 *Screening via Antibody Signal Amplification Target Only Screens*

Detecting hit peptides via fluorescence was a very time-consuming process in which the high noise from the overwhelming presence of positively charged amino acids meant that very little meaningful output was obtained. For this reason, a new method of screening was developed using a tag-less protein to switch the screening focus from the charged dye label back to the target. This technique relied on anti-target antibodies conjugated to alkaline phosphatase, which is an enzyme that can form a dark purple precipitate in the presence of its BCIP/NBT substrate. This meant that any "hit" now showed up as a very dark purple bead in a sea of clear. The label-less detection technique, therefore, provided the additional benefit of a colorimetric readout of a hit, allowing for the much easier separation of these beads from the rest of the library.

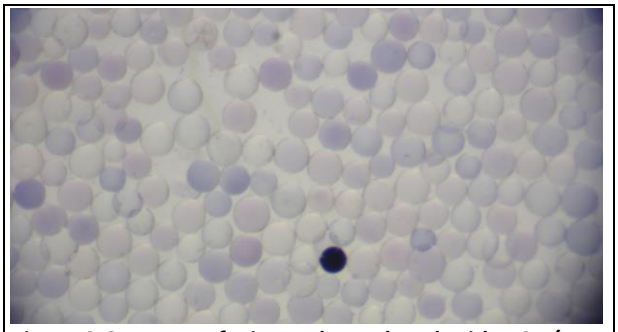


Figure 2-6: Image of Hit Bead Developed with BCIP/NBT. *High background lighter purple surrounding beads could be removed through later preclear and antiscreen steps.*

As can be seen in sample screen results in **Table 2-2**, the high prevalence of positively charged amino acids is gone. In fact, the comparison between **Table 2-1** and **Table 2-2** is startling, considering that the only difference between these two screens is the target detection method. This demonstrates that the dye label was having a dramatic effect on the quality of hit sequences and was responsible for much of the large time investment that was devoted to screening. This huge reduction in noise now meant an instant reduction in the number of screens that needed to be run and sequenced in order to see homology. The colorimetric hit visualization also permitted larger numbers of beads to be screened much faster, so the overall number of library sequences that were sampled went up even though fewer screens were run. One BCIP/NBT-developed screen could sample the same number of beads as up to five different fluorescent screens in less

time, as all of the hits could be picked in the time it used to take to pick one. With this increase in both sampled sequence space and in the overall signal to noise seen in the sequences, hit quality and screening speed improved dramatically in a much shorter overall time.

Table 2-2: Screens from Sample Antibody Amplification Screen Using BCIP/NBT Protein Detection*. Screen was performed against unlabeled (PSA), detected with PS2 mouse mAb anti-PSA antibody and anti-mouse-AP secondary antibody with BCIP/NBT readout.*

X_1	X_2	X_3	X_4	X_5
n	g	m	e	d
e	t	q	m	d
w	t	d	e	m
s	e	d	d	t
a	n	d	e	e
n	y	d	p	e
G	n	m	d	d
e	d	v	l	i
f	e	n	d	a
e	i	n	e	l
v	e	f	G	e
e	h	d	a	y
d	e	t	a	t
i	w	n	m	e
y	d	d	s	l
d	d	e	a	G
e	n	t	i	d

2.3.3 Introduction of an Anti-screen

The antibody development technique dramatically improved the quality of hit peptides by visual inspection (**Table 2-1** versus **Table 2-2**), but also introduced a hidden source of noise into the screens. The presence of several different antibodies and a new detection agent in the screen itself provided more "off-target" sources of library binding. This was conclusively demonstrated by Steve Millward while screening for an Akt capture agent. He developed a biligand using the standard *in situ* click chemistry technique with antibody development, and proceeded to test the affinity of this ligand via SPR. The SPR was set up to immobilize an anti-FLAG antibody (the same used in screening) to the flow cell in order to capture the much less stable Akt protein that might

not survive the required EDC/NHS coupling step. A blank flow cell of only Anti-FLAG antibody without Akt was used as a chip blank. The data from these SPRs is seen in **Figure 2-7**. The sensorgram on the right shows binding to the Akt, as to be expected, but the sensorgram from the blank flow cell on the left shows an identical signal. In conjunction with data (not shown) from the anchor ligand that has almost no binding to the anti-FLAG flow cell, we can conclude that the biligand is actually binding to the anti-FLAG antibody, present in both of those flow cells, and not to the desired Akt target protein. It is only logical that we would see "hits" of peptide sequences that bind to these antibodies, because the presence of the detection antibody bound to a library bead would show BCIP precipitation exactly like the presence of the target protein. A new screening step was needed that would remove the signal seen from the binding of these other proteins used in the screening process.

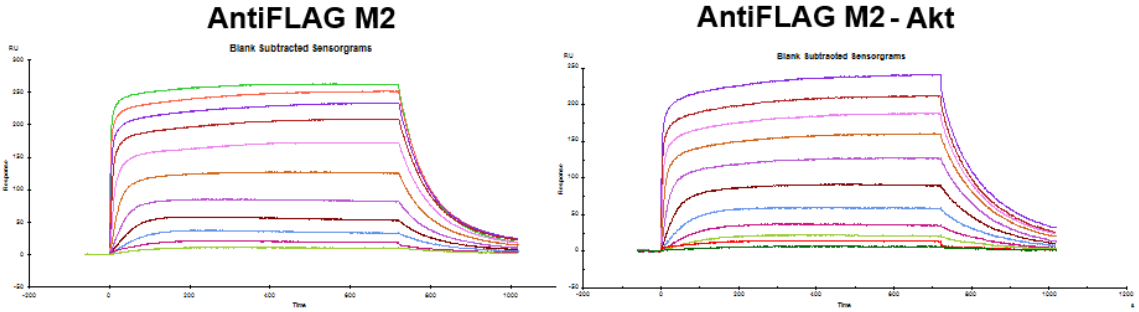


Figure 2-7: SPR Data from Akt Biligand. Data from Steve Millward's Akt Biligand Capture Agent. *An anti-FLAG antibody was immobilized onto an SPR chip via standard EDC/NHS coupling techniques. It was used to capture a FLAG-tagged Akt protein for testing. As seen from the figure on the left, the capture agent bound equally as well to the flow cell immobilized with only Anti-FLAG antibody, supposed to be the chip blank, as to the one on the right that also contained immobilized Akt indicating that the biligand's affinity actually stems from the Anti-FLAG antibody and not the Akt protein target.*

Around this time, there was interest in developing capture agents for proteins containing post-translational modifications, such as phosphorylations or glycosylations. It was hypothesized that hits specific for a post-translational modification could be discovered by screening against the protein target containing the modification, then *anti-screening* against the protein target with

the post-translational modification removed, since everything else in the screen would be identical (**Figure 2-8**). These screens entailed first "target screening," as per usual antibody detection screens, to find all of the hit beads that have an affinity for the target. These beads were then be stripped of their purple color and bound proteins and incubated with the off-target protein that had the post-translational modification removed. Any purple hits from the anti-screen were thrown out as not specific for the modification, since they demonstrated binding in a screen that did not contain the site of interest. This new screening step has the added benefit of removing all of the hits that also have an affinity for the antibodies or developing solution that was used in the screen. An anti-screen like this would have prevented the development of a biligand with an affinity for the anti-FLAG antibody, as these hits would have been detected in both the target and the anti-target screen, and would have been discarded.

The anti-screen is an important step that is now incorporated into each screen that is run in the lab, and is responsible for a significant reduction in background hits. For example, an anti-screen that was run for the PSA protein eliminated 91% of the hit beads from the target screen, indicating that approximately 91% of what was previously considered to be a target hit was just background. For visualization purposes (**Table 2-3**), this means that a screen run with 250mg of beads went from 167 hits down to 15 after this step. This cut down on not only sequencing and hit analysis/testing time, but also eliminated the time that was usually spent trying to tease out signal from noise. Focused screens were also no longer necessary, as that step was designed to help enrich for signal, eliminating a significant chunk of time necessary for developing a capture agent.

Current screening protocols have evolved significantly to include stringent anti-serum anti-screens in order to make capture agents that can function in the most complex mediums, such as out of blood and in cells. For these anti-screens, the decolorized target hit beads are

incubated with anywhere from 1% - 25% human serum to remove even the marginally sticky peptides from the pool of potential candidates.

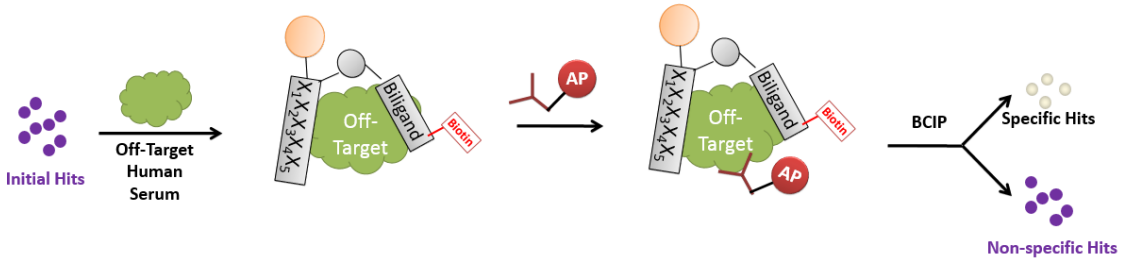


Figure 2-8: Sample Anti-Screen Step. *The target hits are stripped of target, decolorized, and incubated with an off-target or general interferent such as human serum. The off-target is detected with an off-target antibody (the target antibody should also be included if it is different), and these purple beads are non-specific binders, which are removed. The clear beads are specific for the target.*

2.3.4 Introduction of a Click Product Screen

The *in situ* screening process has an inherent screening advantage that had not yet been exploited. A covalently-linked product is formed on the surface of the bead during the screen that can be detected separately from target binding. This means that in addition to probing the library for beads that bind to the target, the library can be searched additionally for the presence of the *in situ* click product – a completely complementary screen.

Once an anchor ligand has been discovered, the next step in the *in situ* screening process (**Figure 2-1**) involves the clicking of a new peptide ligand onto this anchor ligand. In order to accomplish this, the anchor peptide is appended with a click handle and pre-incubated with the target protein, and then both are incubated with the OBOC library. This step searches for a library peptide that binds in close proximity to the anchor peptide on the surface of the protein target, and will "click" onto the anchor if held in position long enough. This click reaction covalently attaches the anchor peptide onto the library bead. By first appending the anchor peptide with a biotin tag, the presence of the anchor peptide on bead, or the ability of this library candidate to "click" to the anchor, can be probed independently of the presence of the target on bead. These screens involve harsh, denaturing wash steps that ensure that everything not covalently attached

to the library will be removed and not detected by either the streptavidin conjugated to alkaline phosphatase or an anti-biotin antibody. These detection agents will bind to the biotin label on the anchor that will only be present after a covalent reaction has occurred, and can therefore detect which library members have formed a click product (**Figure 2-9**).

Table 2-3: PSA Screening Statistics. *These hit bead statistics are taken from a screen against PSA. The percent column indicates the percent of beads that passed from one stage of the screen to the next.*

	Beads	**Percent**
Start	375,500	
Target Screen	167	0.04%
Anti-screen	15	9%
Product Screen	7	47%

Continuing the comparison with the PSA screens from above, only 7 of the 15 remaining beads after the anti-screen showed the presence of a click product. The other 8 beads could very easily have been hits that would be a different anchor ligand – a peptide ligand that is binding specifically to the target protein, but is not close enough to the original anchor for a click to form. The sequences from these hits, shown in **Table 2-4**, are very nearly identical peptides, and contrast sharply with the previously identified hits from the anti-screen in **Table 2-2**. This indicates that the sequences are more than likely all binding very strongly to the exact same location and in close proximity to the anchor ligand, allowing for the formation of the click product.

Table 2-4: PSA Hit Bead Sequences from Product Screen. *The product hits shown in Table 2-3 were sequenced. There is an enormous sequence homology, meaning that the same part of the target is being targeted. The end of the last sequence and the 7th hit were lost due to machine error.*

X_1	X_2	X_3	X_4	X_5
Y	G	w	r	e
Y	d	w	r	q
L	G	w	r	e
e	G	w	r	e
a	d	w	r	q
a	G	-	-	-

The product screen is an elegant step in the screening process that allows for the very specific narrowing of the sequence space. It has become such a huge part of the success of the OBOC capture agent development process that naïve anchor screens, which inherently cannot

include product screens, have been completely eliminated. This switch to all *in situ* click screens has greatly increased both the specificity and affinity of the original anchor ligands, dramatically improving the quality of the final PCC agent. Details of the rationale and results from these more targeted screens can be seen in Chapter 3.

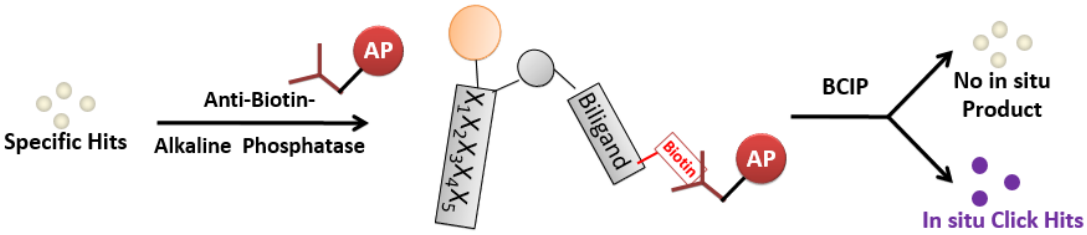


Figure 2-9: Sample Product Screening Step. *The specific hits that survive the anti-screen are stripped of all non-covalent binders and incubated with anti-biotin alkaline phosphatase (or streptavidin). The purple hits from this screen indicate those in which a click reaction has covalently attached the anchor ligand onto the library bead.*

2.3.5 *Introduction of a Preclear*

Three of the candidates from **Table 2-4** were scaled up. In order to do this, the secondary arm is clicked to the original anchor using Cu(I) to form a "biligand," and is tested for binding to the PSA protein. Unfortunately, none of the biligand candidates shown in **Table 2-4** demonstrated binding to the PSA protein in either ELISA assays or SPR, even though the anchor ligand by itself was still able to bind (indicating that all of the parts of the assays were working). The secondary ligands themselves also did not show any binding to the PSA protein, independent of the anchor ligand. These ligand sequences from the click screen, however, were very homologous, indicating that they were all binding in the same place, which was somewhere they could click onto the anchor peptide. It would be impossible to see that level of similarity in the hit sequences, otherwise. Unfortunately, during the screening process, the anchor ligand itself is present in ten times higher quantity than the protein target, and can also bind to the library beads. It was hypothesized, therefore, that the anchor ligand itself bound to those library sequences tightly enough to catalyze the click product that was detected in the final screen. This scenario would

explain why the biligands showed no binding to the protein – the anchor could no longer even bind to the target with another ligand, potentially blocking those binding sites. It also explains why the secondary ligands showed no affinity for PSA. They were not ligands that bound to the target, and wouldn't have an affinity for it.

To counter this effect, a new screening step was added at the beginning of the process to remove all of the library peptides that bound to the anchor ligand before the anchor ligand even saw the target protein (**Figure 2-10**). These screens still detect the biotin label on the anchor ligand, and the detection with streptavidin or anti-biotin in this "preclear" step eliminates the need to use these detection agents in the anti-screen. The preclear screens generally remove 1-10% of the library beads, depending on the library, and also reduce the percentage of beads that need to be removed in the anti-screen.

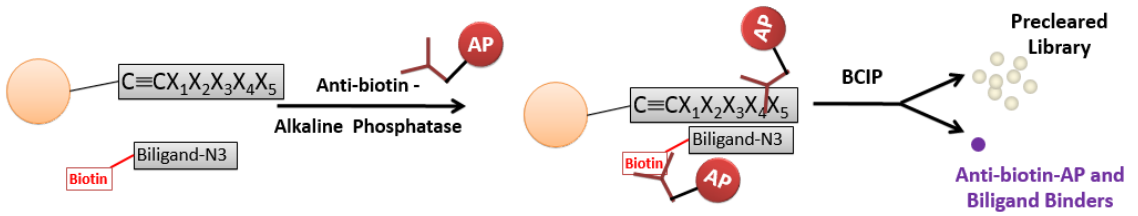


***Figure 2-10: Sample Preclear Screening Step**. The library is incubated with an anchor or biligand, which is then detected with an anti-biotin alkaline phosphatase conjugated antibody (or streptavidin), and developed with BCIP. The purple beads from this screen bind to either the initial ligand or the detection antibody.*

2.3.6 Use of Alkyne Versus Azide Libraries

Throughout the course of technology development, certain seemingly trivial details become important. For the OBOC screens, different libraries and slightly different conditions produced vastly different results. The first issue with the propargylglycine alkyne-containing amino acids surfaced initially after the addition of multiple stages to the screening process. After undergoing more than three rounds of screening, washing and denaturing, the libraries containing the alkyne were no longer able to be successfully sequenced via any method - Edman degradation or MALDI TOF/TOF. The Edman spectra were entirely blank, indicating that the amino acid

residues were probably not cleaving from the beads, and the MALDI TOF/TOF was unable to identify a parent peak that contained the fixed alkyne amino acid. The alkyne-containing amino acid was the N-terminal residue, the first residue that needed to cleave via Edman, and anything modifying this amino acid would affect the cleavage. It was hypothesized that the BCIP/NBT developing solution was modifying these amino acids, which was confirmed by the use of C-terminal alkyne libraries. Even after undergoing four screening steps, the libraries still sequenced correctly using Edman degradation up to the alkyne amino acid. These same library hits, though, were not able to be sequenced using MALDI-TOF/TOF. Because the TOF/TOF would be greatly affected by an unknown change to an amino acid, it was assumed that the alkyne was somehow being modified during these screening steps. For this reason, azide-containing libraries are now always used when undergoing more than three screening steps, unless a C-terminal alkyne library with Edman degradation sequencing is appropriate.

It was also noticed that the libraries that contained a propargylglycine seemed to have more difficult preclears, meaning more purple hits to remove, than the libraries that contained the Lys(N_3) azide amino acid. To test this, two libraries, identical except for their N-terminal azide or alkyne click handle, were blocked in 5% milk in TBS. The libraries were washed three times in TBS, then developed with the BCIP/NBT solution used in the methods section. After 45 minutes, about 5% of the beads in the alkyne library turned bright yellow, indicating binding of the NBT substrate. The azide library did not show this background substrate turnover/binding, and it was assumed that this was related to the sequencing issues with the alkyne libraries. If the NBT substrate is somehow changing or appending to the propargylglycine amino acid, it could explain why the sequences no longer appear as they should during screening.

2.3.7 Typical Flow of Screening

With a multi-stage screening process now in place, the some of the steps need to be conducted in a certain order to achieve the correct results. The first step is the preclear. This occurs before the anchor ligand sees the protein target, and has a chance to form legitimate clicked-hit peptides on bead. These screens look for anything that binds to streptavidin, alkaline-phosphatase, BCIP/NBT, and the anchor peptides. Usually, a screen begins with 300-500mg of library beads, and 1-10% are removed. Typically, any bead that has turned even the lightest shade of purple is removed in order to reduce the overall background as much as possible. This means that any bead that passes through this stage of the screening process has remained clear.

The next step is the target and click-catalyzed screen. The beads that remained clear in the preclear are incubated with the target of interest and the anchor ligand overnight for a click reaction to occur. These beads are then probed for the presence of target. Any bead bound to target will turn purple, and passes through to the next stage of screening. Even though the on-bead click has occurred during this screen, probing for the click product occurs at a later stage.

The hits from the target screen are then decolorized and incubated with an off-target protein or proteins. Any library bead that binds and turns purple in this screen demonstrates off-target interactions with other proteins, and is removed from the pool. At the end of this screen, only beads that remain entirely clear are kept. Even slight purple can indicate undesirable interactions and background binding, and are removed from the pool of hits.

The final screening stage probes for the presence of the clicked product on bead. After harsh denaturing and washing conditions, the beads are probed for the presence of biotin. These beads will turn purple only if biotin is linked to the bead, which is only possible if the *in situ* click reaction was successful. These purple hits have proven to have no affinity to the screening agents in the preclear, an affinity for the target but not off-target interactions in the target and anti-screens, and then have also shown involvement in the covalent click reaction. The clear-purple-

clear-purple pattern of hit detection also ensures that the beads are behaving properly at each stage in the process.

Screens following this pattern now have several produced high-affinity ligands that are very selective to their target of interest. This methodology has an incredibly high success rate that is only getting better as the process continues to grow and develop.

2.4 Conclusions

Over the past ten years in which the project has been in existence, protein-catalyzed capture (PCC) agents have proven to be highly effective detection agents that are incredibly stable and easy to synthesize[1,2,3,6,10]. These agents can be made almost entirely with robotics for ease of scale-up, and the capture agents are highly modular, so the addition of labeling tags is trivial. The exact chemical structures of each of these capture agents are known, eliminating the batch to batch variability that is common with antibodies and can cause multiplexed assays to be expensive and difficult to produce. A spin-off company, InDi Molecular, is in place for commercialization of these agents. PCC agents are also completely stable, demonstrating no degradation upon incubation with mouse liver enzymes, and full functionality after being stored at 65°C as a powder for weeks[2], demonstrating their excellence for use in anything from clinical work to detection of diseases in third world countries[3]. The technology development discussed in this chapter has revolutionized how screening for PCC agents occurs, and the robustness of these techniques has provided a solid foundation for the rapid discovery of a multitude of additional agents for a wide range of purposes [1,2,3,6,10].

2.5 Acknowledgements

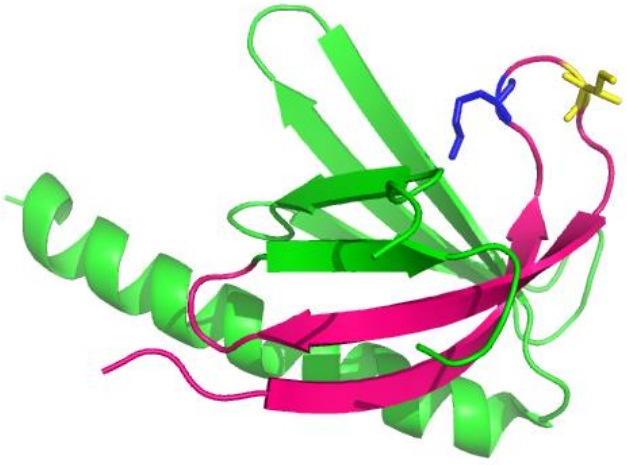
The work described in this chapter was done in conjunction with Heather Agnew and Steve Millward. The initial OBOC screening techniques used were developed by Heather, and the colorimetric screening development was done by Steve. The rest of the work described herein was performed in conjunction with both Heather and Steve, as well as Arundhati Nag and Rosemary Rohde.

2.6 References

1. Agnew, H. D.; Rohde, R. D.; Millward, S. W.; Nag, A.; Yeo, W.-S.; Hein, J. E.; Pitram, S. M.; Tariq, A. A.; Burns, V. M.; Krom, R. J.; Fokin, V. V.; Sharpless, K. B.; Heath, J. R., Iterative In Situ Click Chemistry Creates Antibody-like Protein-Capture Agents. *Angewandte Chemie International Edition* **2009,** *48* (27), 4944-4948.
2. Farrow, B.; Hong, S. A.; Romero, E. C.; Lai, B.; Coppock, M. B.; Deyle, K. M.; Finch, A. S.; Stratis-Cullum, D. N.; Agnew, H. D.; Yang, S.; Heath, J. R., A Chemically Synthesized Capture Agent Enables the Selective, Sensitive, and Robust Electrochemical Detection of Anthrax Protective Antigen. *ACS Nano* **2013,** *7* (10), 9452-9460.
3. Pfeilsticker, J. A.; Umeda, A.; Farrow, B.; Hsueh, C. L.; Deyle, K. M.; Kim, J. T.; Lai, B. T.; Heath, J. R., A Cocktail of Thermally Stable, Chemically Synthesized Capture Agents for the Efficient Detection of Anti-Gp41 Antibodies from Human Sera. *PLoS ONE* **2013,** *8* (10), e76224.
4. Stenman, U.-H.; Leinonen, J.; Zhang, W.-M.; Finne, P., Prostate-specific antigen. *Seminars in Cancer Biology* **1999,** *9* (2), 83-93.
5. Coin, I.; Beyermann, M.; Bienert, M., Solid-phase peptide synthesis: from standard procedures to the synthesis of difficult sequences. *Nature protocols* **2007,** *2* (12), 3247-56.
6. Nag, A.; Das, S.; Yu, M. B.; Deyle, K. M.; Millward, S. W.; Heath, J. R., A Chemical Epitope-Targeting Strategy for Protein Capture Agents: The Serine 474 Epitope of the Kinase Akt2. *Angewandte Chemie International Edition* **2013,** *52* (52), 13975-13979.
7. Procise Protein Sequencing System. http://tools.lifetechnologies.com/content/sfs/manuals/cms_041125.pdf (accessed May 1).
8. Lee, S. S.; Lim, J.; Tan, S.; Cha, J.; Yeo, S. Y.; Agnew, H. D.; Heath, J. R., Accurate MALDI-TOF/TOF sequencing of one-bead-one-compound peptide libraries with application to the identification of multiligand protein affinity agents using in situ click chemistry screening. *Anal Chem* **2010,** *82* (2), 672-9.
9. Sobek, D. J.; Aquino, C.; Schlapbach, D. R., Analyzing the Properties of Fluorescent Dyes Used for Labeling DNA in Microarray Experiments. In *BioFiles*, Simga-Aldrich: Online, 2011; Vol. 6.3.
10. Millward, S. W.; Henning, R. K.; Kwong, G. A.; Pitram, S.; Agnew, H. D.; Deyle, K. M.; Nag, A.; Hein, J.; Lee, S. S.; Lim, J.; Pfeilsticker, J. A.; Sharpless, K. B.; Heath, J. R., Iterative in situ click chemistry assembles a branched capture agent and allosteric inhibitor for Akt1. *Journal of the American Chemical Society* **2011,** *133* (45), 18280-8.

Chapter 3

Development of a PCC Agent Selective for the E17K Mutant Akt1 Protein



3.1 Introduction

3.1.1 The E17K Mutation in the Pleckstrin Homology Domain of Akt1

Akt1 kinase plays a critical role in the PI3K signaling pathway,[1] the activation of which is closely linked to tumor development and cancer cell survival[2]. The phosphorylation of regulatory amino acids (Ser474 and Thr308) on Akt occur through the localization of Akt to the cell membrane through its membrane-binding Pleckstrin Homology Domain (PH Domain). These phosphorylations activate the Akt protein, which can then activate many other downstream signaling pathways[2]. The recently discovered E17K mutation in the PH Domain of Akt1 results in an increased affinity for the phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3, or PIP3) substrate at the cell membrane (**Figure 3-1**)[3]. This switch from a negatively charged glutamic acid to a positively charged lysine amino acid in the PIP3 binding pocket causes this mutant protein to have a four times higher affinity for the PIP3 substrate. This increased affinity causes the Akt1 to be bound to the cell membrane, and hence activated four times longer than in healthy, wildtype cells. Consequently, this deregulated recruitment of Akt1 to the cell membrane causes constitutive activation of the PI3K pathway, which has been shown to be sufficient to induce leukemia in mice[3]. The oncogenic properties of the driving E17K single point mutation make it a target for specific detection and inhibition.

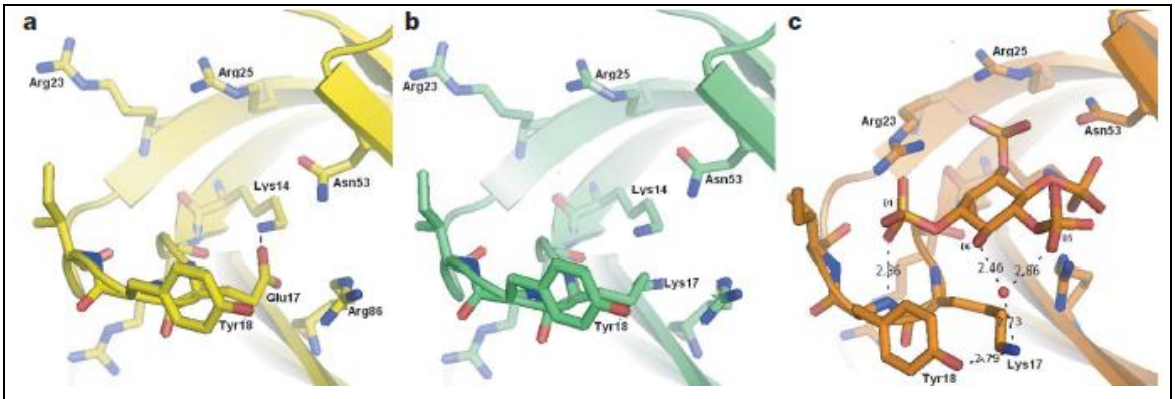


Figure 3-1: PH Domain Binding Pocket Changes upon E17K Mutation: *a.) Interaction between Lys14 and Glu17 in binding pocket of wildtype PHD, b.) repellent interaction between Lys14 and Lys17 in the binding pocket of the E17K mutant, and c.) new hydrogen bonds to water with the E17K mutation in complex with the PIP3 substrate.3*

3.1.2 A General Strategy for Targeting Single Amino Acid Point Mutations in Proteins

Targeting single amino acid point mutations in proteins is becoming a necessary step in the era of personalized medicine, and methods for the detection of these mutant protein biomarkers are highly desirable for guiding treatment decisions[4]. Thus, there is a need for an approach to identify small molecules that can be generally targeted against epitopes containing single amino acid point mutations, and can also potentially be developed into cell-penetrant inhibitors. Previously, a strategy was developed for targeting the phospho-epitopes by chemically synthesizing the surrounding chunk of protein and focusing the site of the *in situ* click screen by attaching an azide click handle to a phosphate chelating group.[5] This method has been generalized by directly substituting an alkyne click handle into the chemically synthesized peptide epitope. For this work, the peptide represents the epitope of Akt1 containing the E17K mutation, an attractive target due to the oncogenic nature of this mutation[3]. That target is subjected to an *in situ* click screen against an OBOC peptide library of 5-mers (comprehensive in 18 amino acids), each terminated in an azide presenting amino acid.

This generalized technique allows us to focus our PCC agent development to a location on the PH Domain that is adjacent to the E17K oncogenic mutation. The approach yielded a 5-mer peptide that exhibited a 10:1 selectivity for E17K Akt1 relative to wild-type (WT). We exploited the chemical flexibility and modularity of the PCC agent to append a dye and a cell penetrating peptide. The resultant ligand could selectivity image the E17K Akt1 protein in live cells, again with high selectivity relative to WT. The technique for epitope targeting described herein provides a general approach for the synthesis of small molecule peptides that are capable of selectively distinguishing between WT and mutant proteins in cancer. These small molecule peptides would be useful tools for disease detection assays, as well as provide a path towards the inhibition of their target proteins.

3.2 Materials and Methods

3.2.1 Akt1 PH Domain Expressions

Akt1 Pleckstrin Homology Domain DNA was purchased from DNA2.0, and the codons were optimized for expression in *E.coli*. The first 124 N-terminal amino acids from full-length Akt1 were used as the PH Domain DNA, and a 6-his tag separated by a thrombin cleavage site was added at the C-terminus of the protein for purification. In order to make the E17K mutant of the PH Domain, the glutamic acid in position 17 was mutated to a lysine via QuikChange (Stratagene), following all of the manufacturer's protocols. The DNA was synthesized in a pJexpress 414 vector containing an ampicillin resistant gene to be expressed in *E.coli* cells. Protein expression was performed by the Protein Expression Center at Caltech using their standard bacterial expression protocol, and purified via Ni-NTA column. The proteins expressed in this manner were used for the pull-down assays confirming the anchor binding via immunoprecipitation assays, and for the biligand screens. These PH Domain proteins were unsuitable for long-term storage under a large variety of tested conditions, so a GST tag was added to hopefully improve the long term stability.

For that reason, the DNA from DNA 2.0 was amplified out of the pJExpress vector using polymerase chain reaction (PCR) to insert the restriction enzyme sites EcoRI and NotI for insertion into a pGEX-4T-1 vector containing a GST tag. The primers used were:

5' - AGAGAATCCATGTCCGACGTCGCGATCGTAAAGGAAGGG – 3'

5' - TCTGCGGCCGCTTAGTGGTGATGATG – 3'

Both the wildtype and E17K mutant DNA were amplified out of the pJExpress vector, restriction enzyme digested, and ligated overnight into a pGEX-4T-1 vector that attached an N-terminal GST tag to the PH Domain protein. BL21-DE3-pLys cells were transformed with the DNA, confirmed correct via sequencing. An overnight starter colony from each protein was grown in 5mL LB + 100 µg/mL Amp overnight. 4mL of this starter culture was used to inoculate 500mL of LB+Amp, and grown to mid-log phase. The cultures were inoculated with 1mM IPTG and grown

5 hours at 28°C. The cells were spun down for 10 minutes at 8,000 RPM and lysed with lysis buffer (1x TBS, 1mM DTT, 1mg/mL Lysozyme, 1% Triton-X), and left for 30 minutes on ice before flash freezing in liquid nitrogen. Upon thawing on ice, the lysate was sonicated for 5 minutes, then centrifuged for 30 minutes at 10,000 RPM to remove cellular debris. The supernatant was then purified on a HisPur Co column (Pierce) using the recommended protocol. These GST-tagged proteins were used to confirm the biligand binding via immunoprecipitation assays, and for the triligand screens. They were also used to obtain the full ELISA curves of all three ligands. These proteins, however, were also not suitable for long term storage and needed to be re-expressed for all assays.

The imaging experiments required that the PH Domain protein be expressed in mammalian cells and have a GFP tag for visualization. Because of this, Akt1 DNA with codons optimized for use in mammalian cells was obtained from InvivoGen as a pUNO-hAKT1 plasmid. The DNA was mutated via QuikChange as before so that both a wildtype and E17K version were on hand. The primers used to clone the DNA from this vector into a TOPO C-terminal GFP mammalian vector (Life Technologies) were:

5’ – AAGATGGGGATGAGCGACGTGGCT – 3’

5’ – TCCCCGACCGGAAGTCCATCTCCTC – 3’

Cloning into the TOPO vector was performed by following all of the manufacturer’s recommended instructions. Because the GST-PH Domain proteins expressed in *E.coli* were still not stable for long term storage, this DNA was used to express the PH Domain in mammalian cells to test the storage suitability of this recombinant fusion protein. The expressions were performed by transfecting a suspension culture of HEK-293-6E cells with XtremeGene HD by the Protein Expression Center at Caltech following their standard protocols. These proteins were not purified,

and were used as-is out of cell lysates. This protein was used in triligand pull-down and inhibition assays, and was still not stable for long term storage.

3.2.2 Design and Synthesis of Epitope-Targeting Anchor/Target Peptide

Epitope targeting for the point mutation of the PH Domain of Akt1 was accomplished by screening against a 33-mer peptide fragment derived from the N-terminus of the PH Domain, highlighted in **Figure 3-6,** that contained the E17K point mutation as well as a propargylglycine (Pra) alkyne click-handle substitution (I19[Pra]) for directing the *in situ* click reaction near the mutated site. The peptide fragment epitope sequence used in these studies was:

MSDVAIVKEGWLKKRGKY[Pra]KTWRPRYFLLKNDG

This 33-mer fragment was capped with an N-terminal biotin label for detection in the screen, and was purified on a prep-scale Dionex U3000 HPLC with a reverse-phase C4 column (Phenomenex). MALTI-TOF MS showed a peak for m/z = 4215.93 for the pure product, expected m/z = 4219.9.

3.2.3 CD Spectroscopy of 33-mer Target Peptide Epitope

Lyophilized powder of the 33-mer biotin-tagged target fragment that was used for screening was dissolved in 500µL of 1x PBS to a concentration of 0.5mg/mL. Concentrations were estimated by weight, and confirmed by A280 measurement on a NanoDrop. Experiments were performed using an Aviv 62 CD Spectrometer. The machine was purged for 20 minutes with N_2; then, the 1xPBS blank in a 500µL 1cm cuvette was added, and the machine was purged with N_2 for another five minutes. The spectra was acquired by taking three measurements/minute from wavelengths 199-250nM. The 33mer fragment sample was then added, purged for 5 minutes, and was measured exactly as the blank. The 33mer cuvette was then removed, and 500µL of

7.0M Guanadine-HCl (pH = 2.0) was added to denature the sample. This spectra was acquired as above.

To work up the data, the signal in **ΔA** from the sample was subtracted from the blank at each wavelength. Then the mean residue molar circular dichroism $\mathbf{\Delta\varepsilon_{MR}}$ was calculated from this readout using the number of residues in the fragment (33) and the concentration in mg/mL (0.5 for the folded sample, or 0.25 for the denatured sample, since it was diluted with Guanadine-HCl) using the equation: $\mathbf{\Delta\varepsilon_{MR} = \Delta A/((residue\ \#\ x\ concentration\ mg/mL)\ x\ l)}$[6]**.** The spectra were graphed by plotting this number against the wavelength.

3.2.4 Screen for Initial Anchor Ligand Peptide

Screens were performed using a library containing 100% Met coupled at the C-terminus for potential MALDI TOF/TOF sequencing[7]. The peptide library was a comprehensive 5-mer containing 18 unnatural D-amino acids, excluding Met and Cys due to stability reasons. The N-terminus consisted of an azide click handle with varying carbon chain lengths – 2 carbon, 4 carbon and 8 carbon – for in vivo click with the Pra on the target 33-mer epitope fragment. Screens were completed using with 300mg of dried library beads swelled at least six hours in 1x TBS (25mM Tris-Cl, 150mM NaCl, 10mM $MgCl_2$, pH = 7.5) buffer.

Preclear (**Figure 3-2a)**:

Swelled library beads were blocked overnight in 5% w/v dried non-fat milk in 1x TBS, then washed with 1x TBS three times. Five milliliters of a 1:10,000 dilution of streptavidin-alkaline phosphatase conjugate in 0.5% milk in TBS was added to the beads, and incubated shaking at room temperature for one hour. The beads were washed with a high-salt TBS buffer (1x TBS with 750mM NaCl) three times, then let shake in high salt buffer for one hour. The beads were then

washed three times with BCIP buffer (100mM Tris-Cl, 150mM NaCl, 1mM $MgCl_2$, pH = 9.0) and developed by adding 15mL BCIP buffer plus 13µL BCIP and 26µL NBT (Two part system, Promega). After one hour, the purple beads were removed by pipette and discarded. The remaining library beads were incubated in NMP for four hours to remove trace purple precipitate from the BCIP/NBT reaction, then were washed five times with methanol, five times with water, five times with TBS and blocked overnight in 5% milk.

Product Screen (**Figure 3-2b):**

Beads remaining from the preclear were washed three times with 1x TBS, then incubated with 5 mL of a 100 nM dilution of the 33-mer epitope target in 0.5% milk for either 5 hours or 12 hours to allow for an *in situ* click reaction to occur. The beads were then washed three times with 1x TBS, and incubated for one hour with a 7M Guanadine-HCl buffer (pH = 2.0) to remove all of the 33-mer epitope target that was not attached covalently to the beads. These beads were then washed ten times with 1x TBS, blocked for two hours in 5% milk, then incubated for one hour with a 1:10,000 dilution of streptavidin- alkaline phosphatase conjugate in 0.5% milk in TBS to detect for the presence of the 33-mer epitope target clicked to a bead. The beads were washed three times with a high-salt TBS buffer, then let shake in high salt buffer for one hour. Afterwards, the beads were again washed three times in BCIP buffer, and developed as per the preclear. Purple beads are removed from the screen via pipette, and are considered hit beads. These hits were incubated in the guanidine-HCl buffer to remove attached streptavidin, washed ten times with water, and sequenced via Edman degradation on a Procise CLC 494 system from Applied Biosystems. See **Table 3-2** for the 5-hour sequences and **Table 3-3** for the 16 hour, overnight sequences.

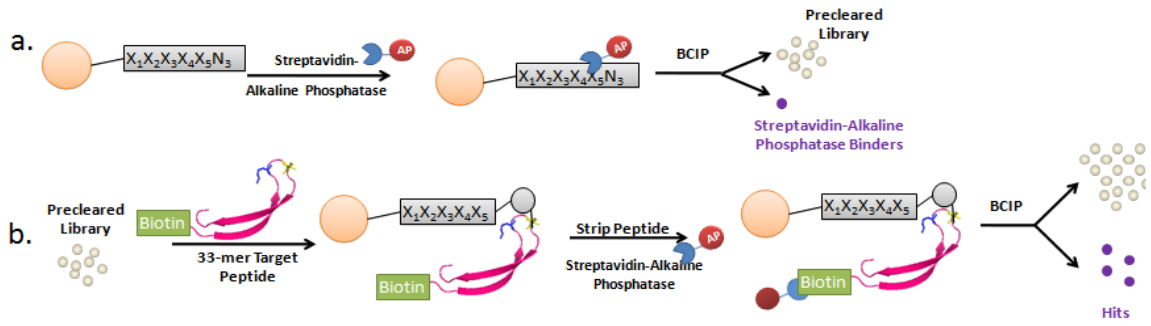


Figure 3-2: Screening Strategy for Anchor Ligand Determination *(a) Preclear: Library beads are incubated with streptavidin - alkaline phosphatase conjugate to remove any library beads that bind to this or the BCIP reagents. (b) Product Screen: Precleared library beads are incubated with the 33-mer target peptide containing an azide in situ click handle. The fragment catalyzes triazole formation between the alkyne on the 33-mer target and the azide on beads that contain peptide sequences that bind specifically to the 33-mer in a close enough proximity to the alkyne substitution for a click reaction to occur without copper. The unclicked peptide is then stripped from the beads, and the remaining covalently attached 33-mer is detected by streptavidin – alkaline phosphatase with BCIP development.*

3.2.5 Hit Library Bead Sequence Analysis

Hit sequences were segregated based on their hydrophobicity and sequence homology using principal component analysis. The algorithm analyzes a series of peptides via hydrophobicity and sequence homology, and graphs them on a 2D sequence map (**Figure 3-7**). Clusters of hits were circled, and one peptide from each cluster was scaled-up and tested for binding to both wildtype and E17K mutant PH domain. The ligands chosen for scale-up were: dqntr, ypwve, eefef, yleaf, and elnhy. Any ligand candidates that were difficult to call on the sequencing were not chosen for scale-up and testing.

3.2.6 Streptavidin-Agarose Immunoprecipitation (Pull-down) Assays for Binding Affinity

Pull-down assays were done on streptavidin agarose resin (Invitrogen). The resin was incubated with N-terminal biotinylated anchor peptide candidates identified via the principle component analysis seen in **Figure 3-7**. The anchor candidate-coated beads were then incubated with both wildtype and E17K mutant protein to compare the selectivity of the ligands as well as the binding ability.

Assays were performed using 50µL of streptavidin-agarose slurry (25µL resin) in Spin-X tubes (Sigma) to allow for the easy removal of the solutions. Resin was aliquotted into 14 tubes – six ligands plus a blank tested against two different proteins – then washed three times with 1x TBST (1x TBS + 0.1% Tween-20). Each set of tubes was incubated with a 10x excess of the appropriate biotinylated ligand to streptavidin binding sites in 200 µL 1x TBS or plain buffer for the blank. Ligand binding was done for one hour at room temperature, and then resin was washed three times with 1x TBS. Resin was blocked with 1x TBS with 5% BSA for two hours. The anchor-coated resin was then incubated with either wildtype or mutant expressed PH domain protein overnight (~16 hours) in cold room (4°C). Protein was spun out of tubes, and the resin was washed three times with high salt TBS, then incubated for five minutes in the high salt buffer. The resin was then washed three times with the 1x TBS buffer, and spun out to dry completely. Fifty µL of denaturing SDS gel loading buffer with 10% B-mercaptoethanol was added to the sample,s and they were incubated at 95° C for ten minutes to denature from the resin. The gel loading buffer was spun out of the Spin-X tubes, and the samples were run on an Any KD BioRad Premade Gel under denaturing conditions. Gel was transferred to nitrocellulose membrane, blocked for one hour in 5% milk (4°C), and western blotted[8]. Proteins were detected using rabbit polyclonal anti-Akt1 antibody (ab64148, Abcam) and an anti-rabbit HRP conjugated secondary anti-body (Cell Signaling), then developed with West Pico Chemilluminescent substrate (Pierce). Relative protein band sizes were analyzed to compare binding between the anchor candidates, and were used to determine selectivity for either wildtype or mutant PH Domain.

3.2.7 Point ELISAs with Anchor Ligand and 33-mer Epitope (Epitope Targeting Verification)

The 33-mer epitope used in screening was resynthesized without the alkyne click handle and with a 6-His tag as an orthogonal tag to the biotin on the anchor ligand. This tag was added after a PEG_5 on the N-terminus of the peptide, and was made and purified as was previously described. The mutant fragment had an expected m/z of 5160.72, observed MALDI-TOF MS m/z for [M+H] of 5161.61. The WT fragment has an expected m/z of 5161.72, and an observed MALDI-TOF MS for [M+H] of 5162.78.

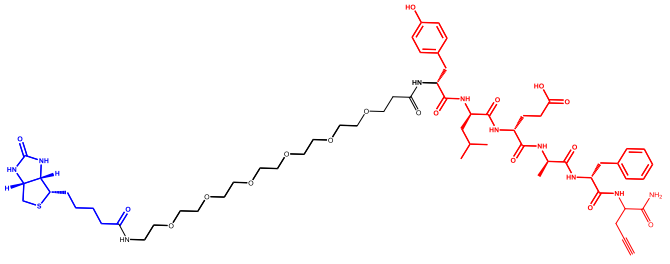


Figure 3-3: Biotin – PEG_5 – yleaf – Pra Anchor ligand: *As analyzed by MALDI-TOF MS, expected m/z = 1298.62, observed [M+Na] = 1319.89.*

For these assays, 100nM Biotin-PEG_5-yleaf-Pra (**Figure 3-3**) was immobilized for one hour on a Neutravidin-coated ELISA plate (Pierce). The plate was blocked in 5% BSA in 1xTBS for one hour, then again overnight at room temperature. The immobilized anchor was then incubated with either 1µM or 100nM wildtype 33-mer epitope or 1µM or 100nM E17K mutant 33-mer epitope for one hour. The plate was washed three times with 1xTBS + 0.1% Tween-20, and tapped dry. The epitope was then detected by a 1:1,000 dilution of an anti-his mouse mAb (ab18184, Abcam) for one hour, washed as above, and then detected with 1:10,000 dilution of an anti-mouse HRP-conjugated goat pAb (Abcam) for one hour. The plate was once again washed and developed with a 1:1 TMB substrate (KPL) for 15 minutes. To graph the data, the blank (epitope and antibodies binding to plate with no anchor ligand present) was subtracted from the triplicate sample values. The fraction bound was found by setting the highest value to 100% and normalizing the rest accordingly. The triplicate values were then graphed with their error bars, and the p-values were calculated by GraphPad using a two-way ANOVA test.

3.2.8 HPLC-Detected Immunoprecipitation (Pull-down) Assays (Epitope Targeting Verification)

Pull-down assays with the biotinylated anchor and his-tagged 33-mer epitope were performed to verify epitope targeting. As with the full-protein assays, the biotinylated anchor ligand was incubated for one hour with 50µL of streptavidin agarose slurry that had been washed three times with 1xTBS. The anchor ligand was washed out, and the resin was blocked for an hour in 5% BSA in 1xTBS. Two hundred µL of a 50µM solution of his-tagged 33-mer epitope in 1xTBS was added to the blocked resin, and this was incubated overnight (~16 hours) at 4°C. Because small peptide fragments like the 33-mer epitope are difficult to transfer to and detect on the nitrocellulose membrane as for a traditional Western blot, the amount of binding in these assays was detected via HPLC. In order to do this, the bound 33-mer peptide fragments were washed three times with 1xTBS + 0.5% BSA, and one time with 1xTBS. The resin was then incubated with 200µL of the 7M guanadine-HCl (pH = 2.0) buffer used to strip beads in the screen. The guanidine buffer was spun out of the beads in Spin-X tubes and injected onto a Beckman Coulter semi-prep HPLC with a reverse phase C18 analytical column. The peak seen on the HPLC illustrated how much of the 33-mer epitope bound to either the yleaf anchor or to blank beads.

3.2.9 Ligand-Directed Tosyl Labeling Experiments

For these assays, the yleaf anchor was appended with an N-terminal FMOC-piperidine-4-carboxylic acid (pip) as a linker on 300mg of rink amide resin in NMP using standard FMOC amino acid coupling techniques. The resin was equilibrated in anhydrous DCM, and 250µL of 3-(chlorosulfonyl)benzylchloride (tosyl) was added with 450µL of DIEA and shaken for 30 minutes at room temperature. Then, 250µL of 2-(2-(2-aminoethoxy)ethoxy)ethanol (EG), 450µL of DIEA and 19mg DMAP in anhydrous DMC were added and shaken overnight. The resin was washed and equilibrated in NMP, and 2eq Cy5 carboxylic acid (Lumiprobe) was coupled at 37°C overnight

using standard FMOC coupling techniques. The resin was washed, TFA cleaved and HPLC purified as usual; see **Figure 3-4** for image.

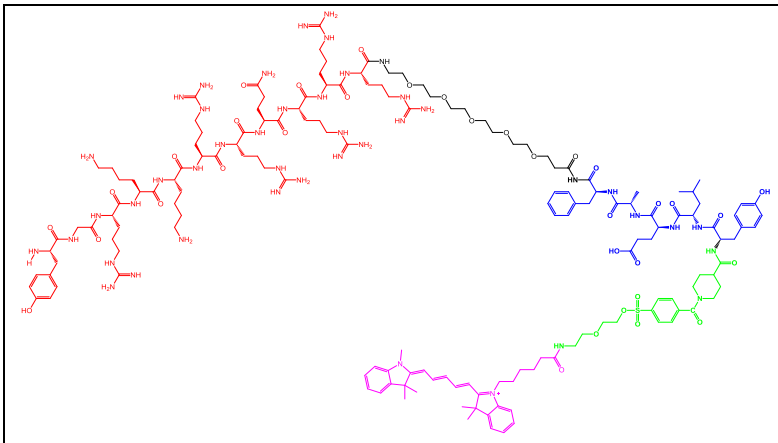


Figure 3-4: TAT – PEG$_5$ – yleaf – pip- tosyl – EG – Cy5: Labeling experiments were performed using a yleaf anchor ligand (blue) that was built onto a TAT peptide (red) with a PEG spacer (black). The labeling arm consisted of a pip spacer, tosyl labeling group, and EG spacer (green) with a Cy5 dye (pink) payload. MALDI-TOF MS, expected m/z = 3316, observed m/z [M+H} = 3317.50.

In order to label the protein, 50µL of full-length GST-E17K PH Domain from SignalChem was treated with 10x molar excess of the anchor ligand with the tosylate dye label and incubated for two days at room temperature. The mixture was lyophilized after two days, and then denatured by boiling in SDS-PAGE loading buffer. The labeled protein was run alongside an unlabeled control on an Any-KD gel (Biorad), then imaged on an Odyssey fluorescent gel reader (**Figure 3-13**). After confirming that labeling had occurred, the gel was stained with BioSafe Coomassie blue stain (BioRad), and the blue protein bands were cut out. The gel pieces were trypsin digested using the Pierce In-gel Digest Kit, following all of the manufacturer's instructions. The tryptic fragments from both the unlabeled and labeled protein digests were lyophilized to concentrate them, taken up in 2µL of 50% H_2O/50% Acetonitrile, and were analyzed by MALDI TOF MS.

Initially, analyses were performed by considering any peak that was present in the labeled protein sample that was not present in the unlabeled sample. The weight of the dye labeling arm – 552.3 g/mol – was subtracted from these peaks, and the corresponding tryptic fragment was located. This provided four potential fragment candidates that were all located near the 33-mer

epitope in the PH domain of the protein. Next, every MALDI peak in the labeled sample was analyzed by subtracting the weight of the dye label and comparing it to a potential tryptic fragment. One other fragment was identified using this method, and corresponded to the doubly labeled peak of one of the previously identified labeled fragments. These results confirmed multiple previous experiments done using LC/MS techniques that proved not strong enough to fragment the tryptic peptides into individual amino acids.

These tryptic peptide samples were then analyzed by MALDI TOF/TOF MS to identify the exact amino acid that contained the dye label. Only YFLLK was able to be successfully fragmented, and the TOF/TOF confirmed that the tyrosine was the label-containing amino acid. This confirms the results, seen in the original publication,[9] that only Y, H, and E nucleophilic amino acids are labeled using this technique. The remaining tryptic fragments all contain at least one of these amino acids, with the doubly-labeled fragment containing two.

The labeling sites were then plotted onto a Pymol image that combined the Akt1 protein (PDB ID: 3096) and the E17K PH Domain (PDB ID: 2UZR) with the N-terminal GST tag (PDB ID: 1UA5) that was present on the full-length protein from SignalChem that was used in these labeling assays. This Pymol-made fusion protein was used to approximate what the commercial protein looked like in solution and give an idea of the extent of the selectivity of this assay. The concentration of labeling sites only surrounding the epitope demonstrate the exclusive binding of this ligand in solution.

3.2.10 Details of the MALDI-TOF Analysis of Tryptic Peptide Fragments

All of the peaks from the MALDI-TOF spectra of the labeled tryptic digests were analyzed for their potential to contain a dye label. The MALDI spectra were manually calibrated to ensure the least possible error. Each peak was then analyzed by zooming in on the spectra on the

computer and obtaining the exact mass for the monoisotopic peak, which is recorded as “MALDI peak” in **Table 3-1** below. The mass of the dye, 552.37g/mol, was subtracted from this peak, and it was compared to the closest possible theoretical tryptic digest fragment (“Digest”). The “expected” mass of the digest plus the dye was calculated and subtracted from the observed mass, “MALDI peak,” and the absolute value of this difference was recorded in “P/M 1.” The peak area was obtained from the MALDI data and added to the spreadsheet as “Peak Area” to allow for a cutoff (4500) of any peaks that looked to be within the noise. Any peak below this value is shown in red italics, and was not considered for this study. Any peak that was within 0.1% of the mass of the expected digest mass was considered to be within error of the instrument, was considered a hit dye-labeled fragment, and colored blue in the table. There were no more new peaks seen using this method than were discovered by looking for peaks that grew in from the unlabeled MALDI to the labeled MALDI. The labeled sites seen in this MALDI-TOF experiment were all seen previously in at least 2 LC/ESI-MS experiments attempting to identify the labeled region.

Table 3-1: Excel Table of Tryptic Fragment Analysis

MALDI Peak	Peak - dye	Expected	Digest	Peak Area	P/M 1	Corresponding Fragment
1053.15	*500.78*	*1051.6349*	*499.265*	*4296.69*	*1.5151*	
1090.15	537.78	*1114.6007*	562.2307	5813.86	24.4507	
1118.11	565.74	*1114.6007*	562.2307	12649.91	3.5093	
1142.16	*589.79*	*1132.6993*	*580.329*	*4217.63*	*9.4607*	
1179.14	*626.77*	*1173.6565*	*621.287*	*4393.2*	*5.4835*	
1194.14	641.77	*1201.732*	649.362	5139.51	7.592	
1202.16	*649.79*	*1201.732*	*649.362*	*4103.69*	*0.428*	
1234.66	682.29	*1234.7826*	682.4126	8193.47	0.1226	YFLLK
1300.08	747.71	*1303.7273*	751.3573	6445.8	3.6473	
1302.09	*749.72*	*1303.7273*	*751.357*	*4496.81*	*1.6373*	
1308.09	755.72	*1303.7525*	751.3825	5926.62	4.3375	
1320.57	768.2	*1320.7691*	768.3991	7886.31	0.1991	EGWLHK
1440.11	887.74	*1447.8246*	895.4546	6406.74	7.7146	
1475.16	922.79	*1477.9158*	925.5458	10131.17	2.7558	
1493.13	940.76	*1477.9158*	925.5458	9276.21	15.2142	
1499.13	*946.76*	*1507.814*	*955.444*	*4112.05*	*8.684*	
1515.1	962.73	*1507.814*	955.444	4687.71	7.286	
1567.65	1015.28	*1565.8591*	1013.489	7907.73	1.7909	
1639.2	1086.83	*1645.9403*	1093.57	21961.13	6.7403	
1707.53	1155.16	*1701.0101*	1148.64	12923.9	6.5199	
1791.09	1238.72	*1795.9606*	1243.591	5200.25	4.8706	
1802.79	1250.42	*1800.0105*	1247.641	8149.76	2.7795	
1851.79	*1299.42*	*1841.9813*	*1289.61*	*4331.77*	*9.8087*	
1995.47	*1443.1*	*1957.1459*	*1404.78*	*4368.22*	*38.3241*	
2212.04	1659.67	*2213.208*	1660.838	95735.94	1.168	EEWTTAIQTVADGLK
2225.51	1673.14	*2213.208*	1660.838	17712.89	12.302	
2233.95	1681.58	*2213.208*	1660.838	12256.12	20.742	
2284.12	1731.75	*2344.242*	1791.872	5711.7	60.122	
2306.92	1754.55	*2344.242*	1791.872	6553.24	37.322	
2344.23	1791.86	*2344.242*	1791.872	4506.1	0.012	EAPLNNFSVAQCQLMK
2383.46	1831.09	*2362.2571*	1809.887	8608.79	21.2029	
2406.7	*1854.33*	*2362.2571*	*1809.89*	*4338.6*	*44.4429*	

The peak at ~2212 was not seen on the unlabeled mass spec, but is seen on the labeled fragment, and was considered a hit. The peak at 2211 is also, however, a common mass seen for trypsin. We do see this particular unlabeled fragment fly in the MALDI-TOF MS (1659), and know that this is a site that can be labeled, based on the ESI-MS experiments that were conducted with a biotin and not Cy5 labeling arm (which therefore have a different labeled mass) that this is a site that can be labeled. In attempting to zoom in for the monoisotopic mass, we see a broad peak with no clearly-identifiable mass peak – unlike all of the other peaks in the spectrum, which showed the distribution of masses very clearly. This led us to believe that we are, in fact, seeing this peak labeled in the MALDI, especially since this site was seen as labeled by the ESI, and that the MALDI spectra is showing an overlap of the trypsin peak with the labeled fragment. The ESI labeling experiments were done using the biotin labeling arm, so this mass did not overlap with trypsin in these experiments, which confirms this. We just cannot exactly call this mass in the MALDI due to the similarity of this peak to that of trypsin.

3.2.11 Images of Anchor Ligand in HEK-293T Cells Expressing PH Domains

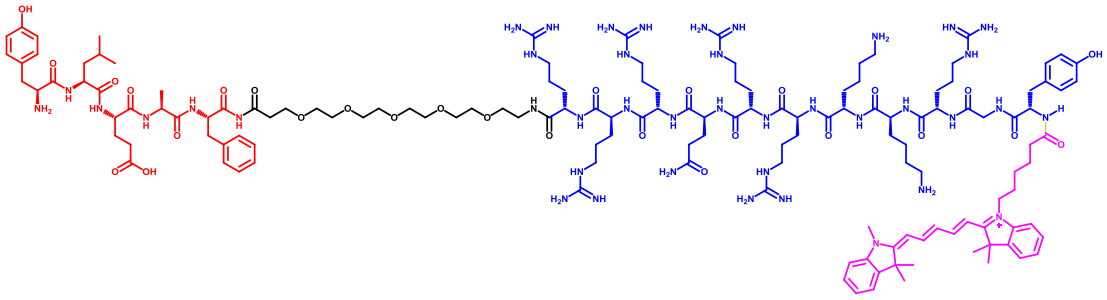


Figure 3-5: yleaf – PEG$_5$ – TAT – Cy5: *The yleaf anchor ligand (red) was appended with a TAT cell penetrating peptide (blue) and a Cy5 dye (pink) separated by a PEG5 linker (black). MALDI-TOF MS, expected m/z: 2937.72, observed m/z = 2937.83.*

These experiments were designed to visualize the dye-labeled anchor ligand in cells overlapping with the GFP-labeled PH Domain proteins. For this reason, the yleaf anchor ligand was synthesized with an N-terminal PEG$_5$, TAT (YGRKKRRQRR), and Cy5 dye (**Figure 3-5**). GFP-

tagged protein DNA was also cloned as described above. HEK-293T cells were grown in DMEM media supplemented with 10% FBS (both Invitrogen), 100x non-essential amino acid solution (Sigma), and PenStrep antibiotic (Invitrogen). Once the cells reached ~80% confluency, they were treated with trypsin to remove from the plate and split into a 12-well flat bottom cell culture plate with a D-poly-lysine (BD) coverslip at approximately a 50% confluency in 1mL total volume. The cells were allowed to attach to the coverslips for approximately 24 hours, then were transfected to express either wildtype GFP-PH domain or E17K mutant GFP-PH domain proteins using XtremeGene HD transfection agent at a ratio of 3:1 transfection agent to DNA. Several wells were left untreated as no protein blanks. The cells were given 24 hours to express protein. They were then serum starved for one hour in DMEM media prepared as above, but without the FBS. After one hour, the Cy5-labeled anchor was added to the wells to a final concentration of 50nM. As the HEK-293T cells express endogenous Akt1 protein, this level was adjusted to give the lowest background signal possible. The protein blank cells were also incubated with 50nM of the yleaf anchor to ensure that binding was due to the presence of the E17K mutant protein. A blank of PEG_5-TAT-Cy5 was also added to wells expressing either wildtype or E17K mutant to ensure that ligand binding was due to the presence of the yleaf anchor. After a one-hour incubation with the peptide, the cells were washed once in serum starved media, then incubated thirty minutes in serum starved media to wash out any excess peptide. During this time, the cells were also treated with 10µg of Hoescht 33342 dye to stain the nuclei. After the thirty-minute period, one well of each wildtype or mutant protein with peptide was activated with PDGF for 10 minutes. The cells were then washed twice with cold PBS buffer, fixed with 10% Neutral Buffered Formalin Solution (Sigma) and glued onto microscope slides. Images were taken on a Zeiss LSM 510 Meta NLO with Coherent Chameleon confocal microscope. A 40x Plan-apochromat lens was used. The laser

intensity and gain were fixed for all pairs of images between wildtype and mutant samples to ensure that the differences seen were not artificially created.

3.3 Results and Discussion

3.3.1 In situ Click Epitope-Targeted Screening Strategy for E17K PH Domain-Specific Ligand

Using FMOC SPPS peptide synthesis techniques,[10] a peptide epitope representing residues 1-32 of the E17K PH Domain of Akt1 was synthesized. From the crystal structure (PDB ID: 2UZR), these residues form a β-sheet around the E17K mutation (blue). The epitope fragment was appended with an N-terminal PEG_5-biotin to serve as a detection handle when screening. This manual synthesis of the epitope allowed for an I19Pra substitution (Pra – propargylglycine) to provide an alkyne click handle on the most proximal side-chain residue to the E17K mutation. Following chromatographic purification, and characterization via mass spectrometry, HPLC and circular dichroism, the modified epitope was ready for screening.

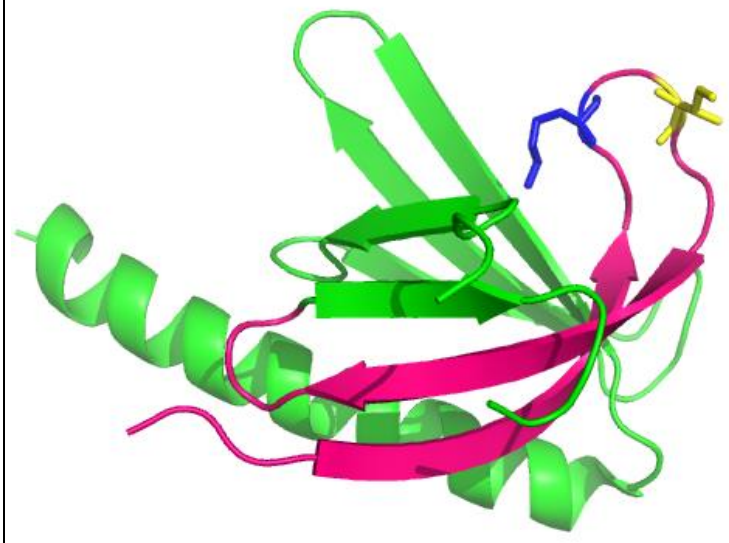


Figure 3-6: Design of Screening Target Epitope: *PH Domain (green) with highlighted screening target epitope (pink). The epitope was designed to surround the E17K mutation shown in blue. The amino acid in position 19 was substituted with a propargylglycine (Pra) alkyne-containing amino acid (yellow) for focusing the site of the in situ click screen.*

A single generation in situ click screen can yield ligands with a high selectivity for the target. Hits from such a screen are those library elements that are covalently coupled to the synthetic epitope through a triazole linkage. The *in situ* click reaction itself is low yielding[12], but the biotin handle on the synthetic epitope permits enzymatic

amplification of those hit beads using a colorimetric streptavidin-linked alkaline phosphatase assay. The basic screening strategy is shown in **Figure 3-2**. Out of the 1.5 million library members that were screened against the alkyne-containing 33-mer E17K PH Domain fragment, only 21 beads (0.0014%) showed the presence of the covalently coupled epitope. These beads were sequenced using Edman degradation (

Table *3-2*, Table 3-3). The hits were segregated based on their hydrophobicity and sequence homology using principal component analysis (**Figure 3-7**). Based upon this analysis, five ligands that represented the diversity of hits (**circled in Figure 3-7**) were scaled-up with a biotin tag and tested for binding to both E17K and WT full-length PH Domain. These hits were dqntr, ypwve, eefef, yleaf, and elnhy. Here, the lowercase sequence letters indicate that the amino acids that comprise the peptide are non-natural D-stereoisomers.

Table 3-2: Hit Sequences from Anchor Screen against 33-mer peptide epitope (5hr click screen):

Az2	G	v	e	k	f
Az8	y	h	e	w	f
Az4	i	s	e	y	e
Az2	p	h	w	l/k	f
Az8	d	l	l	t	f
Az4	a	r	s	d	f
Az8	f	k/l		G	t
Az8	f	e	i	q	
Az8	e	e	p	d/n	f

Table 3-3: Hit sequences from Anchor screen against 33-mer peptide fragment (16hr screen):

Az4	e	e	f	e	f
Az8	f	e	e	a	i
Az2	e	l	n	h	y
Az2	h	a	r	h	q
Az2	h	e	w	v	t
Az4	n	w	y	a	w
Az4	n	l	v	p	n
Az2		r	r	r	f
Az4	a	l	n	s	k
Az8	p		a	y	h
Az2	n	r	y	v	r
Az8	y	l	e	a	f

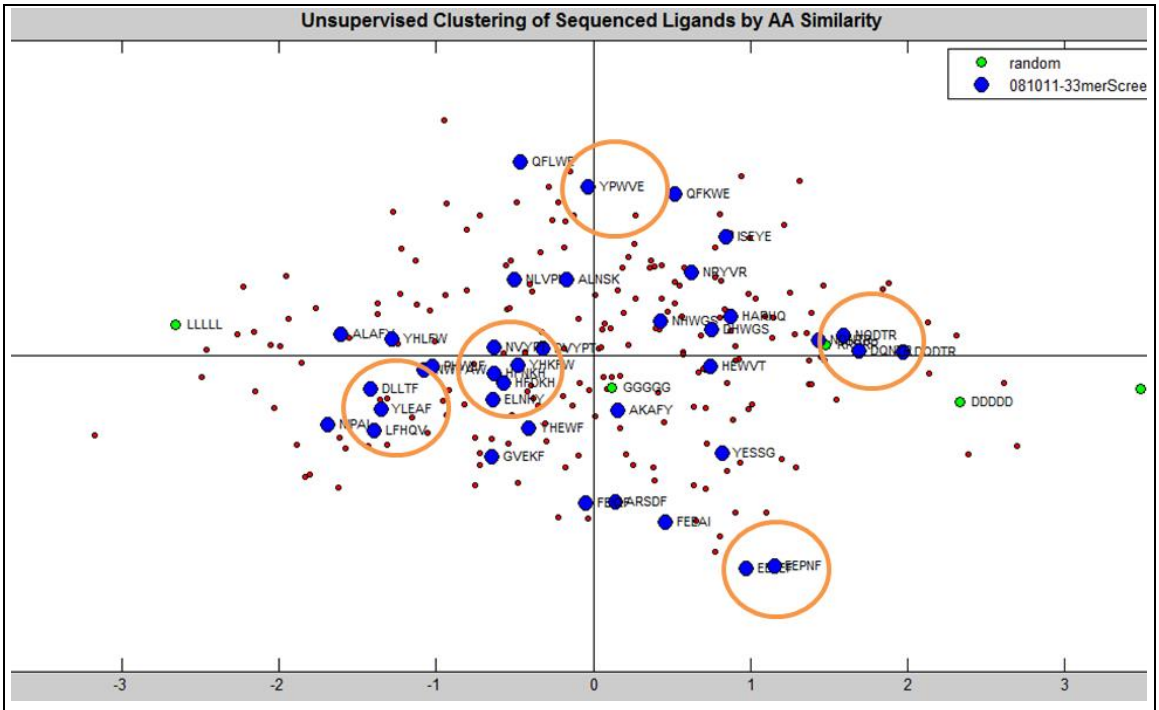


Figure 3-7: Clustering of Anchor Sequence Ligands by AA Similarity: *Hit sequences from the anchor screen were segregated based on their hydrophobicity and sequence homology using principal component analysis. Circled clusters indicate regions where a peptide was selected and scaled-up as a possible anchor sequence. The potential anchor sequences that were tested are: dqntr, ypwve, eefef, yleaf and elnhy.*

Streptavidin – agarose immunoprecipitation assays (**Figure 3-8**) were used to probe for the ability of the anchor candidates to recognize and bind to the proteins in buffer. One ligand

candidate showed a distinctively stronger binding to the E17K protein relative to the WT, seen in Lane 5. This peptide sequence, "yleaf," (**Figure 3-3**) was carried forward for additional investigations. Two out of the four other candidates, though, also showed a preference for the E17K mutant protein. One candidate showed a preference for the WT protein, and one candidate showed no strong binding to either fragment. This result was to be expected, because it is possible for a ligand to bind to the fragment in a way that is not directly accessible on the surface of the full protein. It can also be hypothesized that the three fragments that showed a preference for the E17K fragment bound at or around the site of the mutation, and the peptide that shows binding to the WT protein must bind away from the mutation.

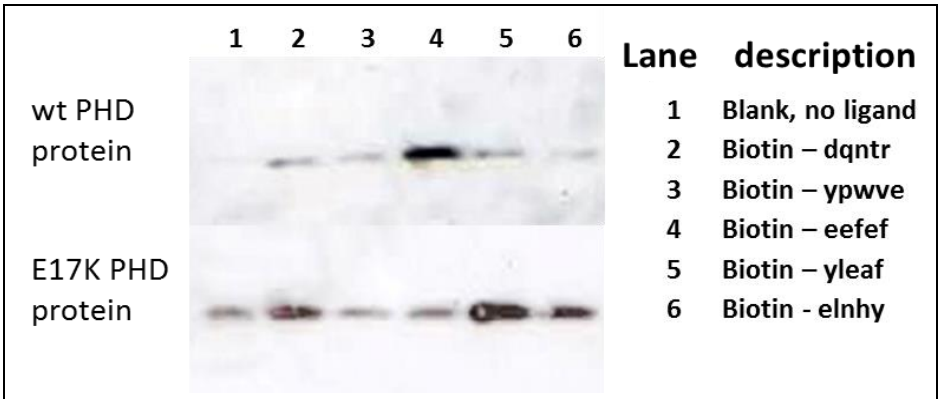


Figure 3-8: Streptavidin-Agarose Pull-down Assays for Anchor Ligand Binding Affinity: *Streptavidin-agarose was incubated with a panel of potential anchor sequences that were synthesized with biotin tags. These resins were then incubated with either wildtype or E17K Mutant PH Domain to measure the amount of protein that is pulled down by each potential anchor ligand.*

3.3.2 CD Spectroscopy of 33-mer Target Peptide Epitope

In order to determine whether the epitope used in the screen would retain the secondary structure of the full protein, CD spectroscopy was performed (**Figure 3-9**). The biotin-tagged, alkyne-containing fragment that was used as a screening target was tested, and the resultant spectra do show the presence of secondary structure. This result is confirmed by the

disappearance of this structural signature upon the addition of a denaturing guanidine-HCl buffer. More importantly, the characteristic dip at 217nM of the blue, fully-natured fragment spectra is the signature of a β-sheet, which is the expected structure of this part of the full protein. We can assume, then, that this fragment is maintaining a structure similar to that of the natured protein, even with the incorporation of the click handle. Therefore, peptide binding to the E17K fragment should see a surface similar to that of the full-length PH Domain protein. The messiness of the spectra from 200 – 210 nm could be due to the biotin label that has been attached to the fragment, or be due to the absence of the rest of the protein, causing random coiling or unfolding.

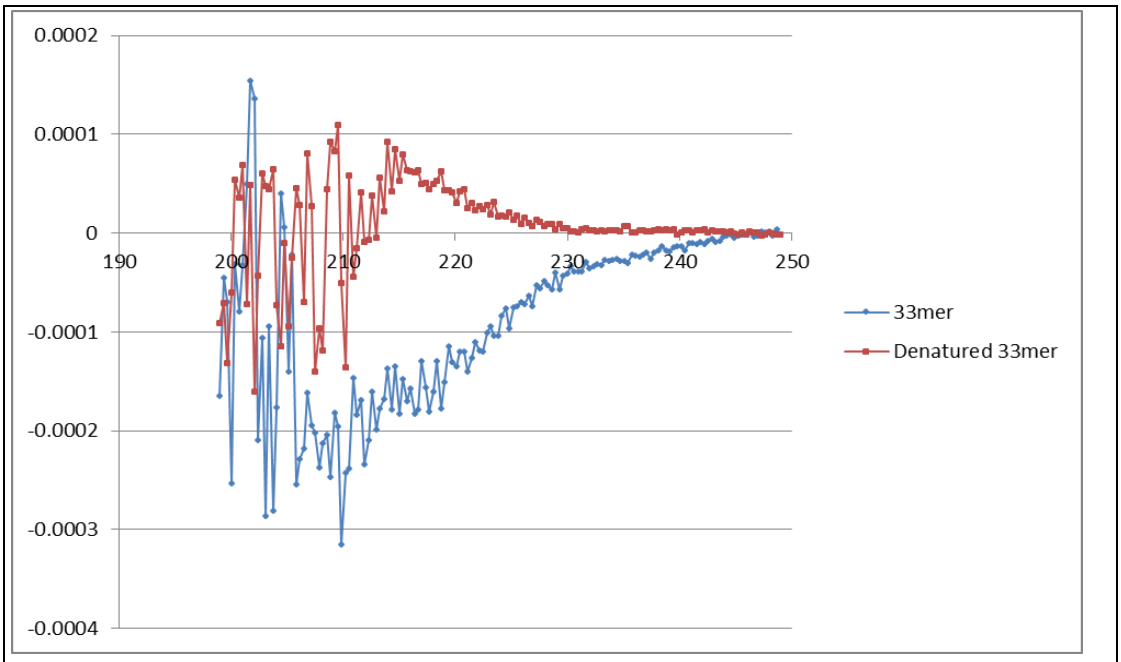


Figure 3-9: CD Spectra of 33mer Epitope Fragment used in Screening: *The blue spectra indicates the 33mer target fragment that was used in screening; the red indicates the denatured epitope.*

3.3.3 Verification of the Epitope Targeting Strategy

The biotin-modified yleaf peptide (**Figure 3-3**) was subjected to a variety of binding assays against the synthesized WT and E17K 33-mer PH Domain fragments prepared without the biotin label and alkyne click handle. These assays were instead labeled with an N-terminal 6-His tag in

order to have an alternative detection handle to the anchor ligand. First, the yleaf peptide was used in immunoprecipitation assays to pull-down either the WT or E17K mutant 6His-tagged 33-mer peptide fragments, as opposed to the full-length proteins that were used to initially validate the candidates. Typical immunoprecipitation assays involve western blotting to estimate the amount of protein binding, but peptide fragments are too small to be consistently captured or quantified on a blot. Because of this, the amount of peptide epitope precipitated in these assays was quantified via injection onto an analytical HPLC. These unique immunoprecipitation assays further confirmed preferential yleaf ligand binding to the E17K mutation relative to the WT epitope (**Figure 3-10**). As an assay control, another candidate ligand that, in initial testing, did not exhibit preferential E17K binding to the full protein (lane 4, **Figure 3-8**), was tested, and yielded consistent results to the full-protein pull-down assays (**Figure 3-8**). The first immunoprecipitation assays demonstrated that the anchor bound selectively to the full-protein, and these HPLC-detected pull-downs confirm that the binding and the selectivity are due to the interaction between the anchor and 33-mer epitope.

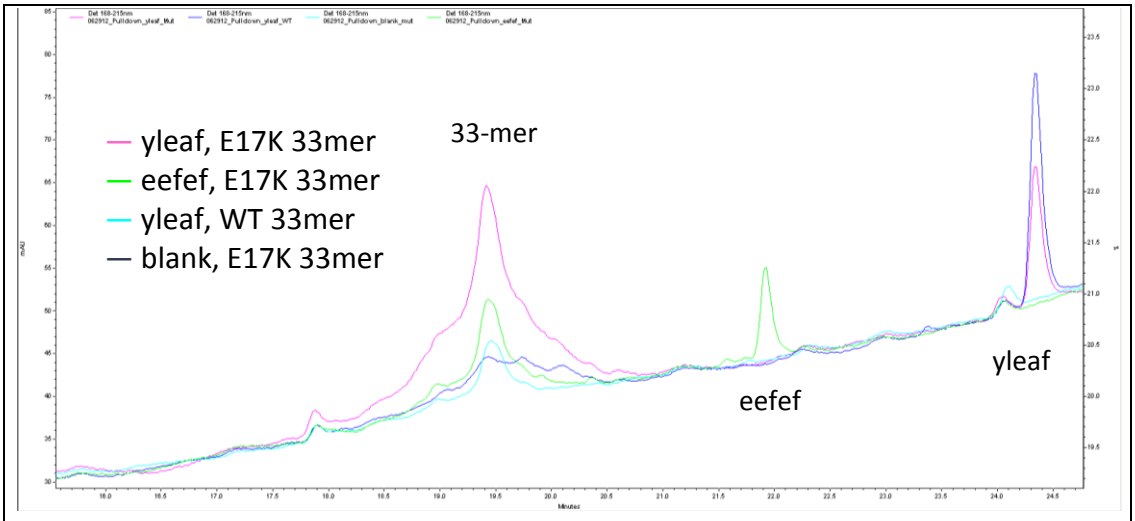


Figure 3-10: HPLC-detected Immunoprecipitation Assay for Epitope Targeting Verification. *The HPLC traces show the quantity of the 33-mer fragment that was pulled-down in the assay. The anchor ligand, yleaf, pulls down the most E17K 33-me,r and the least WT 33-mer. The amount of pull-down by the eefef discarded anchor candidate demonstrates the validity of the assay. This corroborates what was seen in the full-protein pull-downs in Figure 3-8.*

The selectivity of the yleaf peptide for the E17K 33-mer epitope was also tested in an ELISA assay format. For these assays, the WT or E17K 33-mer peptide fragments were captured using the PEG-biotin-modified yleaf ligand immobilized on a Neutravidin-coated plate. The yleaf ligand exhibited significant selectivity for the E17K fragment over the WT across a 100 nM – 1 µM concentration range (**Figure 3-11**), further demonstrating the binding of this ligand to the specific epitope of interest and providing additional validation of the epitope targeting strategy.

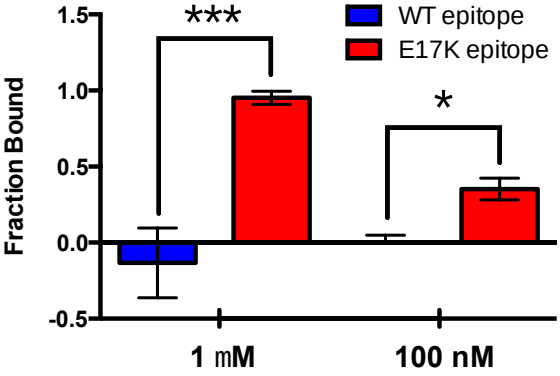


Figure 3-11: ELISA Assay Verification of Epitope Targeting. *The WT and E17K 33-mer fragments were incubated with the yleaf anchor immobilized on an ELISA plate. Strong preference for the E17K fragment is demonstrated.*

3.3.4 Ligand-Directed Labeling Experiment to Confirm Epitope Targeting and Ligand Selectivity

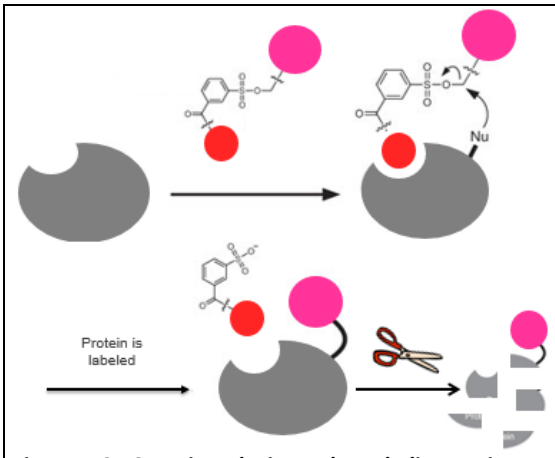


Figure 3-12: Ligand-Directed Labeling Diagram, *modified from Tsukiji et al9. The anchor (red) with the Cy5 dye (pink) attached through an electrophilic tosyl group. The reaction with nucleophilic side-chains on the protein can indicate where the ligand is binding. These sites can be confirmed through trypsin digestion and MS.*

The selectivity of the yleaf anchor ligand was further verified using the directed labeling technique reported by Tsukiji *et al*[9]. The approach yields information relative to the binding location of the ligand on the protein target. For this method, a payload is attached to the N-terminus of the targeting yleaf ligand through an electrophilic tosylate linker. Upon ligand binding to the protein target, the payload is transferred onto the protein through a nucleophilic S_N2 reaction with proximal nucleophilic amino acid side chains (**Figure 3-12**). The protein can then be trypsin-digested, and the identity of the fragments containing the payload can be mapped on the protein surface using mass spectrometry (MS). Thus, the site of ligand binding can be estimated. The assay also serves as an independent validation of the immunoprecipitation and ELISA binding assays discussed above.

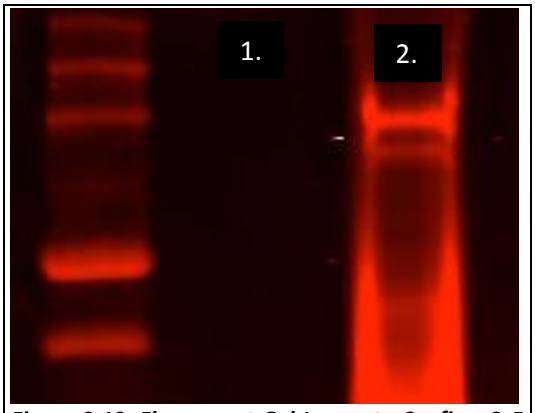


Figure 3-13: Fluorescent Gel Image to Confirm Cy5 Labeling. *Lane 1 shows the control protein with no label, and Lane 2 shows the labeled protein. The large band corresponds to the labeled GST-E17K-Akt1.*

For the assay, yleaf was modified at the N-terminus to contain a tosylate linker attached to a Cy5 dye molecule to enable easy identification of the labeled and digested protein fragments (**Figure 3-4**). A Glutathione S-Transferase (GST)-Akt1(E17K) protein (SignalChem) was incubated with the Cy5-appended yleaf peptide. The labeling of the protein target was initially confirmed by visualization on a fluorescent gel reader

(**Figure 3-13**). The labeled protein and an unlabeled control were then trypsin-digested from the gel, and were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS (**Figure 3-14**). Five peaks appeared in the MS of the labeled protein that were not present in the unlabeled protein digests, which all corresponded to an expected trypsin fragment plus the weight of the linker and dye.

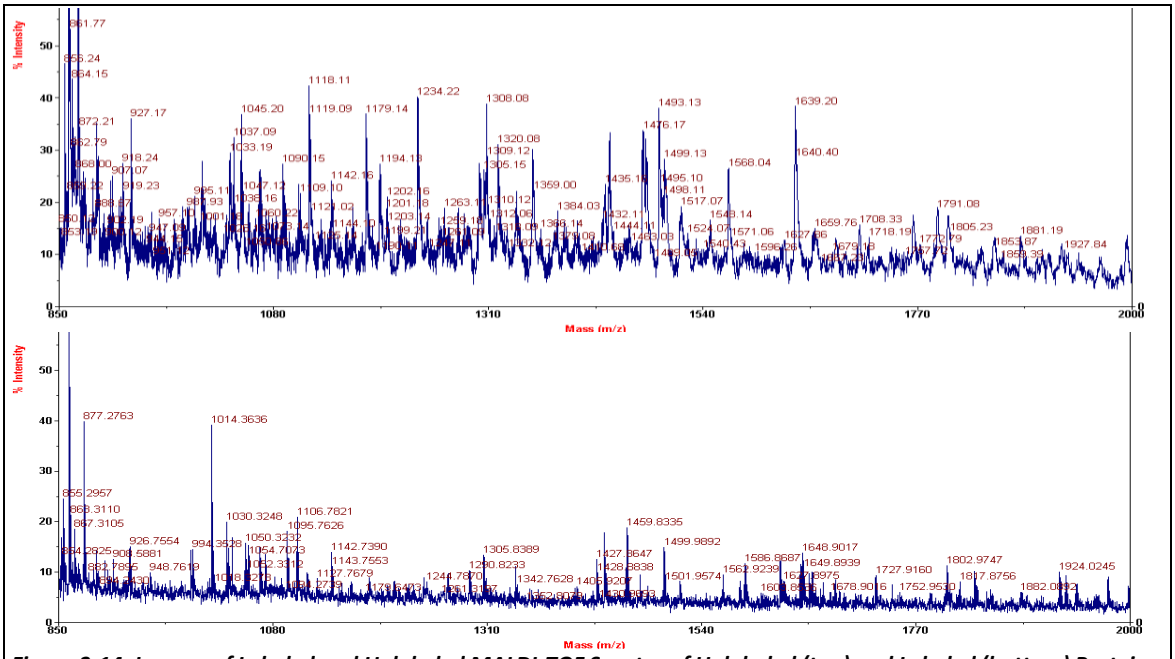


Figure 3-14: Images of Labeled and Unlabeled MALDI-TOF Spectra of Unlabeled (top) and Labeled (bottom) Proteins.

These peaks were then analyzed by MALDI TOF/TOF MS to extract sequence information for the labeled regions of the protein. All but one of the dye-labeled peptides were difficult to fragment, as is characteristic of cationic peptide labels[11]. The labeled digest YFLLK could be fragmented, and indicated the presence of the dye on the Y amino acid (**Figure 3-15**). This is consistent with the original literature on the labeling technique,[9] which showed that Y, E and H amino acids are the nucleophiles that can be labeled. The other labeled Akt1 fragments that were identified contain at least one of these amino acids. One fragment contains two such amino acids and, in fact, there were MALDI peaks corresponding to the masses of both the singly-and doubly-

labeled fragments. **Figure 3-16** shows the location of the labeled fragments in the PH Domain sequence, as well as the amino acids that should contain the label.

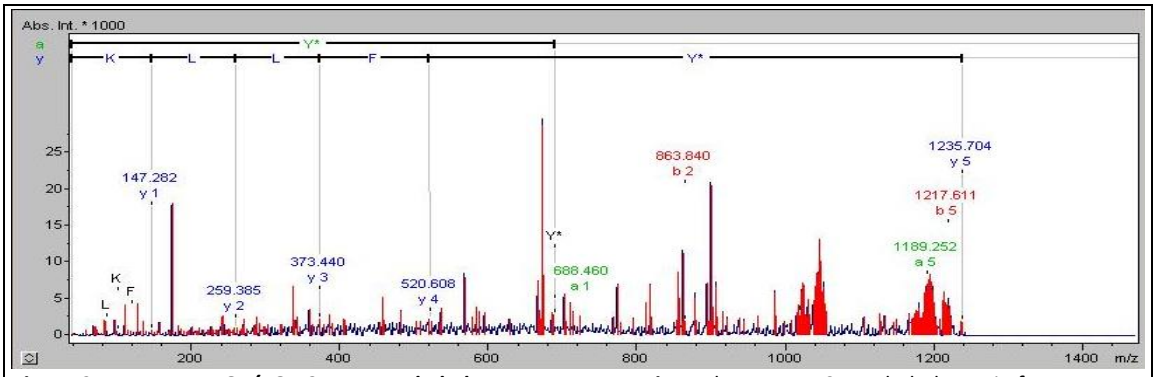


Figure 3-15: MALDI-TOF/TOF Cy5 Dye-Labeled YFLLK Fragmentation: The YFLLK – Cy5 Labeled trypsin fragment analyzed by MALDI-TOF/TOF MS. The fragments shown above demonstrate that the Cy5-dye is on the Y amino acid, which corresponds to the results found by authors of the original technique[9].

The labeling sites were then mapped on a composite crystal structure of GST (PDB ID: 1UA5) and Akt(E17K) (Akt PDB ID: 3096, E17K PDB ID: 2UZR) (**Figure 3-17**). All labeled sites surround the anticipated binding site of the yleaf ligand. A thorough search of the entire MALDI spectra was conducted to identify any other labeled fragments anywhere on the large protein, but none were found. Thus, this labeling experiment demonstrates that only sites around the expected N-terminal binding site of the yleaf ligand are labeled, confirming the very specific binding of the peptide ligand at the site directed by the epitope-targeted in situ click screening process.

MSDVAIVK|EGWLHK|R|GK|YIK|TWR|PR|YFLLK|
NDGTFIGYK|ER|PQDVDQR|EAPLNNFSVAQCQLMK|TER|
PR|PNTFIIR|CLQWTTVIER|TFHVETPEER|
EEWTTAIQTVADGLK|K|QEEEEMDFR|SGLVPR|GSAG

Figure 3-16: Trypsin-Digested Sequence of PH Domain Protein. *The red dashed lines show where the trypsin cuts are located. The fragments highlighted in red are the ones that showed labels, and the amino acids colored in cyan are the ones containing the labels.*

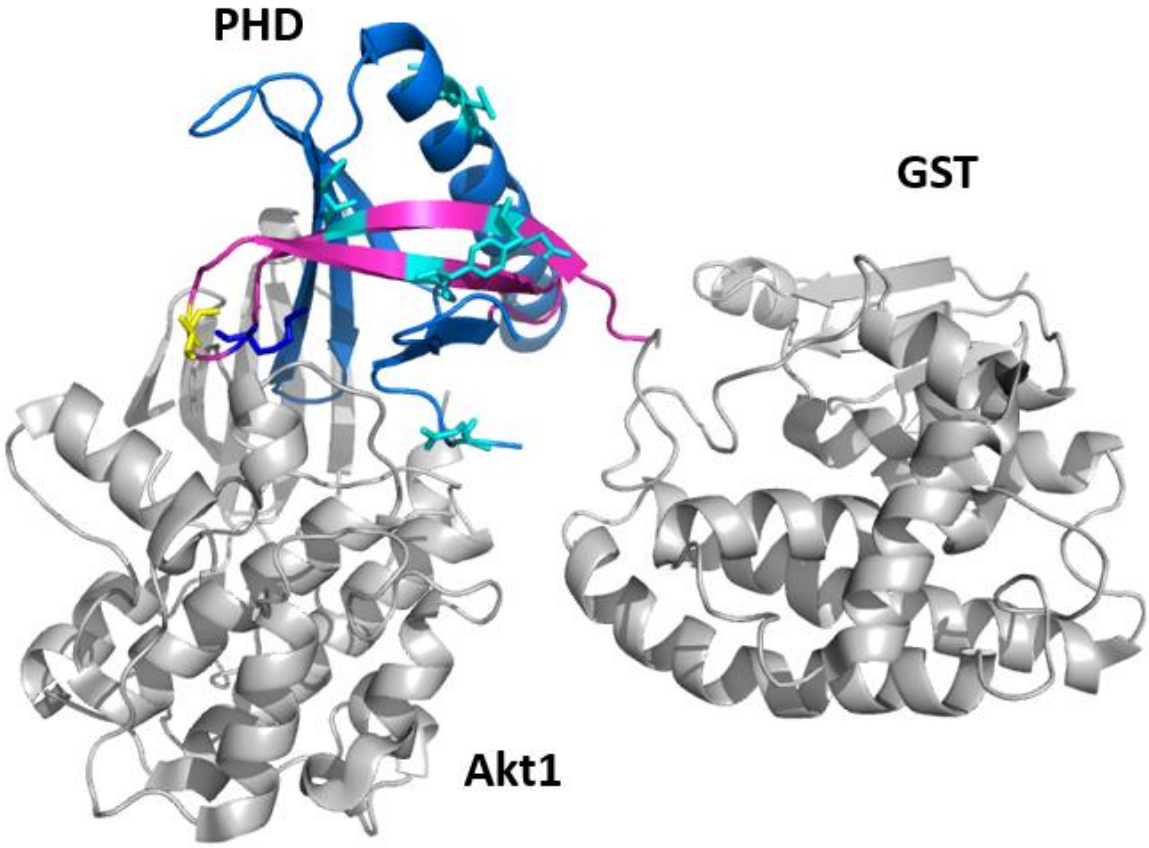


Figure 3-17: Compiled Crystal Structure of Fully Labeled Protein*. The cyan amino acids labeled in cyan are all clustered immediately around the pink 33-mer fragment of the blue PH Domain. No labeled sites were found in any other part of the large fusion protein.*

3.3.5 In Cell Imaging

Live cell-based assays can provide a demanding environment for demonstrating the selectivity of the yleaf PCC agent to the E17K Akt1. In addition, they also can demonstrate the value of a small, epitope targeted ligand relative to a similarly targeted antibody, since antibodies cannot enter live cells. To demonstrate target binding in live cells, HEK-293T cells were transfected to express GFP-tagged E17K or GFP-tagged WT PH Domain proteins. The yleaf ligand was then labeled with both a Tat cell-penetrating peptide and a Cy5 dye (**Figure 3-5**). The combination of the GFP label on the protein, and the Cy5 label on the dye, permitted the use of multi-color fluorescence microscopy for interrogating any spatial registry between the two

fluorescent labels. Live HEK-293T cells expressing these GFP-tagged proteins were exposed to varying concentrations of the modified yleaf ligand for one hour. The cells were then thoroughly washed with PBS to equilibrate the concentration, and fixed for fluorescence microscopy measurements.

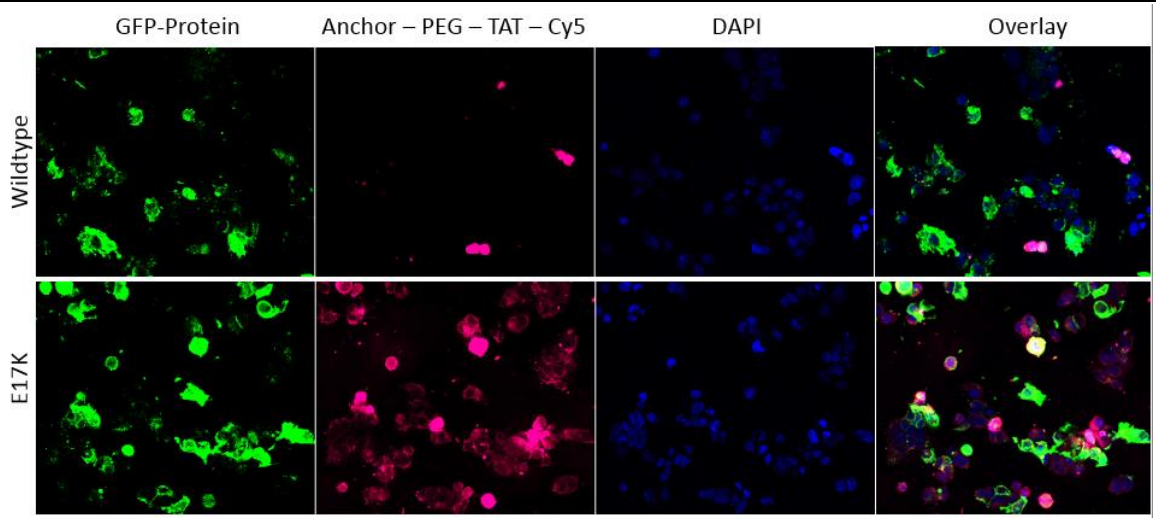


Figure 3-18: Images of Anchor Ligand in GFP-tagged WT and E17K PH Domain. *The green fluorescence shows the equivalent expression of the WT and Mut proteins in cells. The pink anchor ligand is distributed throughout almost all of the E17K – expressing proteins, but shows only background binding to the WT – expressing cells.*

Confocal microscopy images of the two differentially-expressing Akt1 PH Domain cells showed a consistent level of expression between the GFP-WT PH Domain and GFP-E17K PH Domain. However, the level of the PCC agent retained by the cells was substantially different (**Figure 3-18**). Nearly all of the cells expressing the mutant protein show some level of capture agent retention and demonstrate co-localization of capture agent and GFP-PH Domain protein (**Figure 3-19**). The GFP-WT cells, however, show very low levels of capture agent retention, and do not

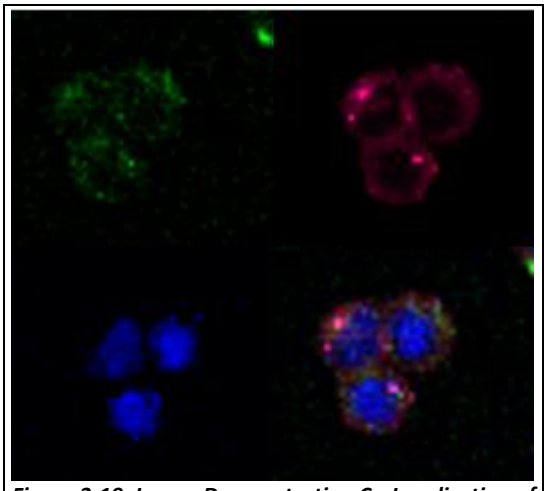


Figure 3-19: Image Demonstrating Co-Localization of Cy5 - *PCC Agent with GFP - tagged PH Domain proteins in cells.*

seem to have any co-localization of the two. These measurements demonstrate the selectivity of the E17K capture agent for its target within the demanding environment of live cells.

3.4 Conclusions

The *in situ* click-focused epitope screen for capture agent development presents a rapid strategy for discovering peptide ligands that bind to any site of interest on a protein surface. This method is not limited to conserved binding pockets, post-translational modifications, or structured regions of proteins. By only accepting hits wherein the target epitope catalyzed the formation of a covalent bond, it was ensured not only that the candidate ligands bound to the site of interest, but that they also bound tightly and in an exact orientation so that this triazole could form. In this way, the *in situ* click screen became not only a screen for ligand affinity, but also for ligand specificity. The peptide hits developed using this method are very specific for the exact location on the protein where the click reaction was centered. The PCC Anchor, yleaf, developed using this technique has demonstrated the ability to detect the E17K mutant PH Domain in conditions ranging from simple assays in buffer to complex imaging experiments in cells. Assays validating the peptide-peptide binding of the epitope target and the yleaf anchor ligand also highlight the exquisite selectivity of this ligand for the E17K mutation. This technology provides an ideal solution for the discovery of selective ligands to an area of interest on a protein surface, and demonstrates the ability to produce agents capable of distinguishing even the slightest change in protein structure – a single point mutation.

3.5 Acknowledgements

The labeling and imaging experiments shown were done with Blake Farrow. Steve Millward and Aiko Umeda assisted with the protein expression and cell culture. Ying Qiao Hee and Jeremy Work made many of the peptides used in these experiments. Bert Lai performed the

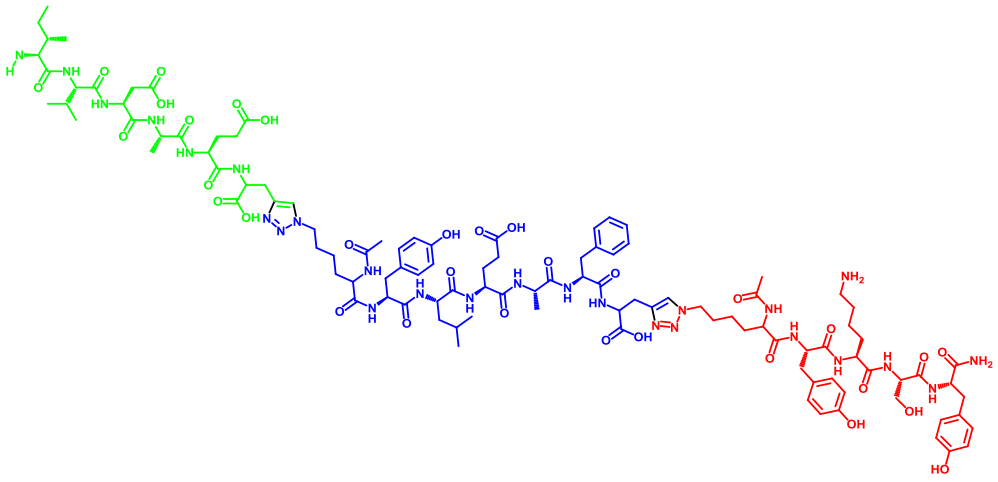
MALDI-TOF/TOF sequencing of the tryptic fragments. Jost Vielmetter, Angela Ho, and Sravya Keremane of the Protein Expression Center were indispensable in the expression of these proteins. Felicia Rusnak and Jie Zhou performed the trypsin digests and LC/MS for the protein labeling experiments. The advice from Mona Shahgholi on MS techniques and experimental set-up was essential to the success of the labeling experiments.

3.6 References

1. Testa, J. R.; Tsichlis, P. N., AKT signaling in normal and malignant cells. *Oncogene* **2005,** *24* (50), 7391-3.
2. Vivanco, I.; Sawyers, C. L., The phosphatidylinositol 3-Kinase-AKT pathway in human cancer. *Nat Rev Cancer* **2002,** *2* (7), 489-501.
3. Carpten, J. D.; Faber, A. L.; Horn, C.; Donoho, G. P.; Briggs, S. L.; Robbins, C. M.; Hostetter, G.; Boguslawski, S.; Moses, T. Y.; Savage, S.; Uhlik, M.; Lin, A.; Du, J.; Qian, Y.-W.; Zeckner, D. J.; Tucker-Kellogg, G.; Touchman, J.; Patel, K.; Mousses, S.; Bittner, M.; Schevitz, R.; Lai, M.-H. T.; Blanchard, K. L.; Thomas, J. E., A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature* **2007,** *448* (7152), 439-444.
4. Rusling, J. F.; Kumar, C. V.; Gutkind, J. S.; Patel, V., Measurement of biomarker proteins for point-of-care early detection and monitoring of cancer. *Analyst* **2010,** *135* (10), 2496-2511.
5. Nag, A.; Das, S.; Yu, M. B.; Deyle, K. M.; Millward, S. W.; Heath, J. R., A Chemical Epitope-Targeting Strategy for Protein Capture Agents: The Serine 474 Epitope of the Kinase Akt2. *Angewandte Chemie International Edition* **2013,** *52* (52), 13975-13979.
6. Corrêa, D. H.; Ramos, C. H., The use of circular dichroism spectroscopy to study protein folding, form and function. *African J Biochem Res* **2009,** *3* (5), 164-173.
7. Lee, S. S.; Lim, J.; Tan, S.; Cha, J.; Yeo, S. Y.; Agnew, H. D.; Heath, J. R., Accurate MALDI-TOF/TOF sequencing of one-bead-one-compound peptide libraries with application to the identification of multiligand protein affinity agents using in situ click chemistry screening. *Anal Chem* **2010,** *82* (2), 672-9.
8. Towbin, H.; Staehelin, T.; Gordon, J., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences* **1979,** *76* (9), 4350-4354.
9. Tsukiji, S.; Miyagawa, M.; Takaoka, Y.; Tamura, T.; Hamachi, I., Ligand-directed tosyl chemistry for protein labeling in vivo. *Nat Chem Biol* **2009,** *5* (5), 341-343.
10. Coin, I.; Beyermann, M.; Bienert, M., Solid-phase peptide synthesis: from standard procedures to the synthesis of difficult sequences. *Nature protocols* **2007,** *2* (12), 3247-56.
11. Pashkova, A.; Moskovets, E.; Karger, B. L., Coumarin Tags for Improved Analysis of Peptides by MALDI-TOF MS and MS/MS. 1. Enhancement in MALDI MS Signal Intensities. *Analytical Chemistry* **2004,** *76* (15), 4550-4557.

Chapter 4

Expansion of E17K Selective Anchor Ligand into an Inhibitor



4.1 Introduction

As described in previous chapters, there is increased interest in compounds that can selectively inhibit a disease-associated mutant protein target while sparing the wildtype (WT) variant[1]. PHD inhibiting compounds[2,3] with selectivity for the E17K variant of Akt have not been reported. The specificity of the yleaf anchor ligand described in Chapter 3 for the E17K Akt1 in live cells, coupled with the proximity of the E17K mutation to the PIP3 binding site, prompted the consideration of further developing this PCC Agent into a compound capable of blocking the E17K PH Domain interaction with its PIP3 substrate. The yleaf anchor peptide itself did not exhibit evidence of inhibition (**Figure 4-11**). It was reasoned that a similarly targeted, but bulkier PCC Agent might serve as a steric blocker of the PH Domain - PIP3 interaction. To this end, two cycles of iterative *in situ* click chemistry screens, as described in Chapter 2, were executed in order to develop the yleaf ligand into a biligand and then a triligand, which was capable of successfully blocking this binding interaction. This showed that these larger PCC Agents could serve as highly selective inhibitors of E17K Akt1 by blocking binding of the Pleckstrin Homology Domain of Akt1 to the PIP3 substrate.

4.2 Materials and Methods

4.2.1 Screen for Biligand Peptide

The anchor determined above – yleaf – was scaled up with a biotin on the N-terminus for detection, a PEG_5 linker between the biotin and the peptide, and a d-propargylglycine (Pra) on the C-terminus as the *in situ* click handle (Biotin-PEG_5-yleaf-Pra). Screens were performed using a library with 100% Met coupled at the C-terminus for potential MALDI TOF/TOF sequencing. The library consisted of a comprehensive 5-mer containing 18 unnatural D-amino acids, excluding Met and Cys due to stability reasons. The N-terminus of the library was appended with an azide click handle with a 4 carbon chain (Lys(N_3))– for in vivo click with the Pra on the anchor peptide.

Screens were performed with 300mg of dried library beads swelled at least six hours in 1x TBS (25mM Tris-Cl, 150mM NaCl, 10mM $MgCl_2$, pH = 7.5) buffer.

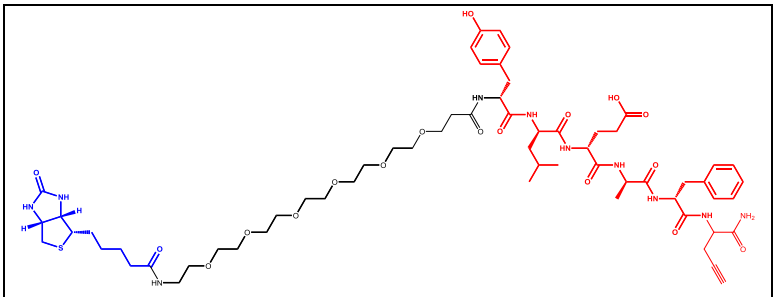


Figure 4-1: Biotin – PEG5 – yleaf – Pra: *Biligand screens were performed using the yleaf anchor (red) with a PEG5 spacer (black) and a biotin tag (blue). MALDI-TOF MS, expected [M+Na] 1319.62, observed 1319.89.*

Preclear (**Figure 5a**):

Swelled library beads were blocked overnight in 5% w/v dried non-fat milk in 1x TBS, then washed with 1x TBS three times. The beads were incubated with a 7.15µM solution of the anchor peptide - Biot-PEG_5-yleaf-Pra for one hour, then washed three times with 1xTBS. Five milliliters of a 1:10,000 dilution of streptavidin-alkaline phosphatase conjugate in 0.5% milk in TBS was added to the beads, and incubated with shaking at room temperature for one hour. The beads were washed with a high-salt TBS buffer (1x TBS plus 600mM NaCl) three times, then let shake in high salt buffer for one hour. The beads were then washed three times with BCIP buffer (100mM Tris-Cl, 150mM NaCl, 1mM $MgCl_2$, pH = 9.0) and developed by adding 15mL BCIP buffer plus 13µL BCIP and 26µL NBT to the beads in a 150mm polystyrene tray. After one hour, the purple beads were removed by pipette and discarded. The remaining beads were incubated in NMP for four hours to remove trace purple precipitate from the BCIP/NBT reaction, then were washed five times with methanol, five times with water, five times with TBS and blocked overnight in 5% milk.

Target Screen (**Figure 5b**):

The clear beads remaining from the preclear were blocked in 5% milk in 1x TBS for two hours. They were then washed three times with 1x TBS. A pre-incubated solution of E17K mutant

protein (715nM) and anchor ligand (7.15µM) in 3mL of 0.5% milk was added to the blocked library beads and incubated for either five hours or overnight to allow an *in situ* click reaction to occur. In the morning, the beads were washed three times with 1x TBS, then incubated with a 1:4,000 dilution of an anti-His Alkaline Phosphatase conjugated antibody (Abcam) in 0.5% milk for one hour. The beads were then washed three times with a high salt TBS, then incubated on the shaking arm for one hour with the high salt buffer. They were then washed three times with BCIP buffer, and developed as previously. Hit beads turned purple and were removed and washed in NMP for four hours to decolorize, then guanidine-HCl to denature and remove and remaining protein. The beads were then washed ten times with water and blocked in 5% milk overnight.

Off-Target Anti-Screen (**Figure 5c**):

The beads from the target screen were washed three times with 1x TBS, then incubated with the off-target, wildtype PHD protein in 0.5% milk for one hour on the shaking arm at room temperature. The beads were washed three times with 1x TBS, then incubated with a 1:4,000 dilution of Anti-His Alkaline Phosphatase conjugated antibody in 0.5% milk for one hour at room temperature. They were then washed three times with high salt buffer and let shake for one hour in high salt at room temperature before being washed three times with BCIP buffer and developed as previously. The beads that turned purple bind to both mutant and wildtype protein or to the anti-his antibody, and were set aside. The beads that remained clear were picked and washed with guanidine-HCl to remove any bound proteins, and blocked in 5% milk overnight.

Product Screen (**Figure 5d**):

The beads specific for the mutant PH domain were washed three times with 1x TBS. They were then incubated with a 1:10,000 dilution of Streptavidin – Alkaline Phosphatase conjugate in

0.5% milk for one hour. The beads were washed three times with high salt TBS, then let shake for one hour with high salt buffer before being washed three times with BCIP buffer and developed as previously. The beads that turned purple contained the anchor peptide covalently bound to the bead, and had formed a protein-catalyzed in situ click reaction. These beads were collected and stripped with guanidine-HCl for one hour, washed ten times with water, and sequenced via Edman degradation as per the anchor candidate hits. There were 22 total hit beads.

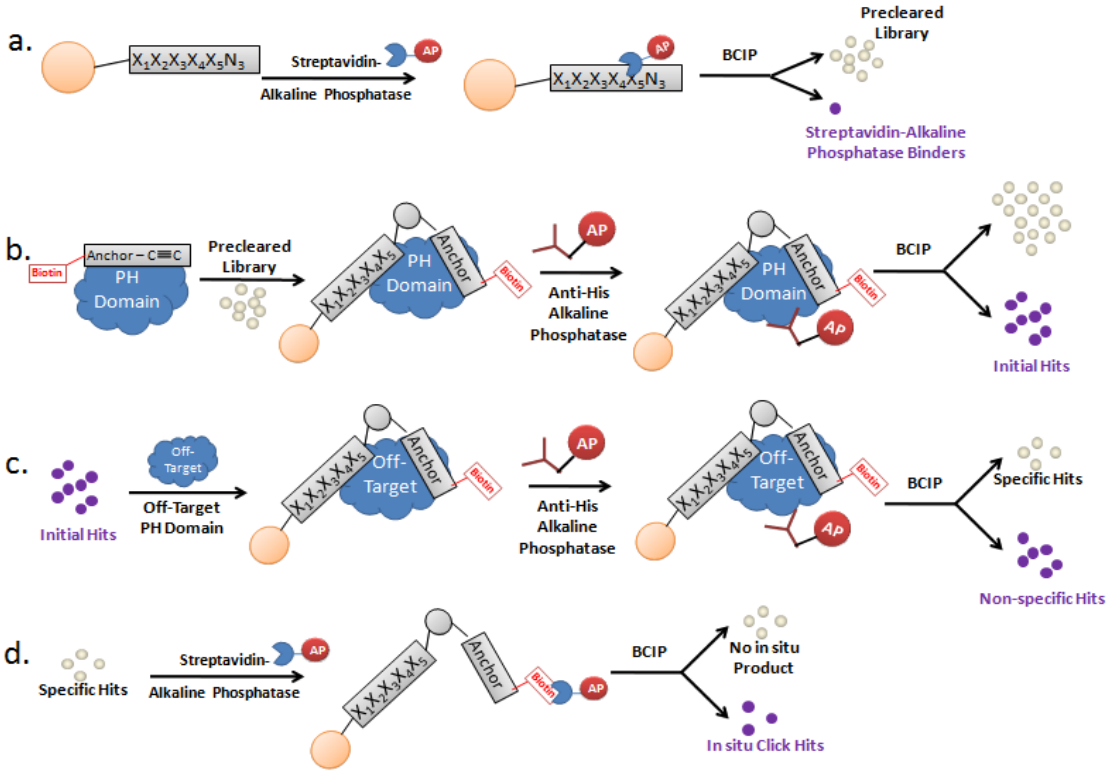


Figure 4-2: Screening Strategy for Biligand Determination: (a) *Preclear: Any library beads that bind to the screening reagents are removed. (b) Target Screen: Precleared beads are incubated with the target and anchor ligand and allowed to "click." The presence of the his-tagged PH Domain is detected via an anti-His antibody. (c) Anti-Screen: Hit beads from the target screen are incubated with the off-target PH Domain and anti-his antibody. These hit beads bind to both the target and off-target (WT and E17K mutant). (d) Product Screen: The beads are probed with streptavidin-alkaline phosphatase to determine which contain the click product and, thereby, have shown biligand formation.*

4.2.2 Streptavidin-Agarose Immunoprecipitation (Pull-down) Assays to Test Biligand Candidates (Figure 4-6)

Four biligand candidates were segregated based on their hydrophobicity and sequence homology using principal component analysis as for the anchor ligands screened in Chapter 3. Biligands were synthesized by coupling the 2° ligand onto Rink Amide Resin on the Titan peptide synthesizer. The amide group on the end of the Lys(N_3) was capped by shaking the resin with 2mL acetic anhydride, 2mL NMP and 0.5mL DIEA for three times for 10 minutes each time, then washed with NMP. FMOC-Propargylglycine-Otbu (Pra) was clicked onto the Lys(N_3) on the 2° ligand by incubating 2 equivalents of the Pra amino acid with 2 equivalents of CuI and 2 equivalents of ascorbic acid with 1 equivalent of azide on the resin in 20% piperidine/NMP for 3

hours. The resin was washed five times with 4 mL of a chelating solution consisting of 1g sodium diethyldithiocarbamate in 20mL NMP and 1mL DIEA. The anchor was then built onto the 2° ligand on bead, and an N-terminal PEG_5-biotin tag were added.

Assays were performed exactly as for the anchor ligands, except for two key differences. The biligand assays were performed using 6ug of GST-tagged PHD protein, instead of the untagged PHD that was used in the anchor pull downs. The pull-downs were also conducted out of 1% serum in 1x TBS, as opposed to just 1x TBS. This is a much more demanding assay that tests not only the affinity, but also the selectivity of the ligands because they need to be able to bind to their target protein in a very complex medium. The performance of the biligand candidates in this challenging assay indicates that they have little off-target interactions with a multitude of other proteins, and would perform well in the condition in which they will need to be detecting these ligands – in cells and out of cell lysates.

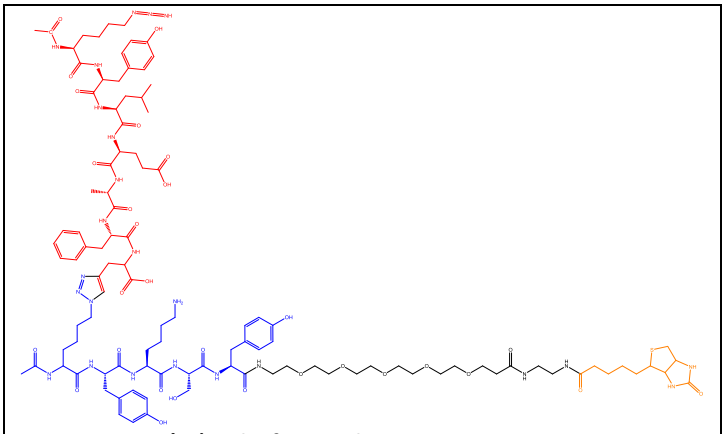


Figure 4-3: Lys(N_3) - yleaf - Tz - yksy - PEG_5 – Biotin: *MALDI-TOF MS expected 2248.1, observed [M+H] 2249.1.*

4.2.3 Screen for Triligand Peptide

The best biligand candidate as determined in immunoprecipitation assays – yleaf–(triazole, Tz)-yksy - was scaled up (**Figure 4-3**) with a C-terminal PEG_5-biotin for detection during the assay by coupling PEG_5 onto NovaTag Biotin resin (EMD). Lys(N_3)-yksy was coupled onto the resin on the Titan peptide synthesizer, and FMOC-Pra-Otbu was clicked on as previously. The remaining "Lys(N_3)-yleaf" portion was then synthesized on the Titan, the Lys(N_3) serving as the click handle for the triligand screen. The screens (**Figure 4-4**) were completed using a random 5 D-amino acid library with a C-terminal D-propargylglycine alkyne click handle, and were otherwise performed exactly as for the biligand, including all concentrations. Three hit beads were discovered in this screen, and the first hit had a nonsensical sequence, so it could not be used. Both of the usable hits were scaled up and tested for binding using ELISA assays.

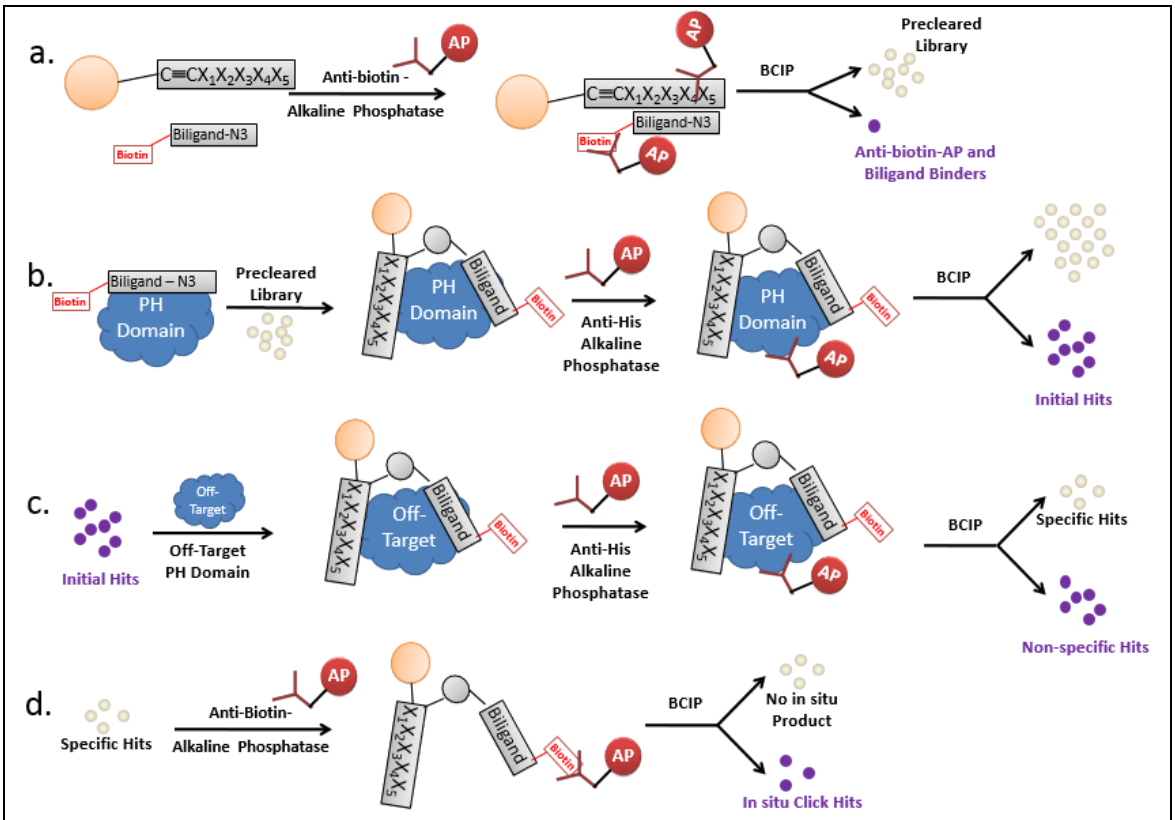


Figure 4-4: Screening Strategy for Triligand Determination: *(a) Preclear: Any library beads that bind to the screening reagents are removed. (b) Target Screen: Precleared beads are incubated with the target and biligand and allowed to "click." The presence of the his-tagged PH Domain is detected via an anti-His antibody. (c) Anti-Screen: Hit beads from the target screen are incubated with the off-target PH Domain and anti-his antibody. These hit beads bind to both the target and off-target (WT and E17K mutant). (d) Product Screen: The remaining beads are probed with streptavidin to determine which contain the click product and, thereby, have shown triligand formation.*

4.2.4 Full ELISA Curves for Ligands

The full curve ELISAs were obtained using streptavidin coated ELISA plates (Pierce). The ligands – anchor, biligand, two triligand candidates (ivdae and iryrn) and "eflya" scrambled anchor peptide blank - were laid down on the plate at a concentration of 1µM for one hour. Two lanes of each ligand were used on the plate for both proteins – WT and E17K GST-PHD. The plates were blocked with 5% BSA for two hours. Dilutions of both WT and E17K GST-PHD proteins were made in 0.5% BSA in 1xTBS ranging from 1µM down to 0.5nM by serially diluting 1:2 down a series of 8 samples. For each ligand, a no protein blank was also used. The proteins were incubated with

the blocked plate for one hour, washed three times with 1xTBST + 0.5% BSA and tapped dry, then detected with a 1:10,000 dilution of an HRP conjugated anti-GST ab. The plate was again washed three times with 1xTBST and tapped dry. It was developed with a 1:1 solution of TMB substrate, and development was stopped with 1M H_2SO_4 and read on a plate reader. The curves were plotted by normalizing the signal by the blank wells, and were fitted by a Hill function in GraphPad using a common saturation and slope (Bmax = 1.466 +/- 0.03, h = 0.7383 +/- 0.025).

4.2.5 Point ELISA Assays for Triligand Binding to Akt1 and Akt2 Wildtype and E17K Mutant Proteins

These assays were conducted to test the binding of the triligand to the off-target Akt2 wildtype and mutant proteins. For this assay, all samples were taken in triplicate for statistical purposes. Triligand peptide was first immobilized onto Neutravidin ELISA plates (Pierce) for one hour. A scrambled anchor peptide, eflya, was used as the no-ligand blank, as the GST proteins have significant background binding to a blank Neutravidin plate. The plates were then blocked with 5% BSA overnight. Protein was laid down on the plate at a concentration of 100nM for samples wells and the blank, scrambled peptide wells. GST protein alone (Abcam) was also incubated with the triligand and scrambled peptide as a control. The proteins were incubated for one hour, then washed three times with 1xTBST. The protein was then detected with 1:10,000 anti-GST mouse mAb (Fisher, #MA4-004) for one hour, washed three times with 1xTBST, and developed with a 1:1 mixture of TMB substrate for ten minutes. The samples were plotted by subtracting the blanks and averaging the sample wells. The highest signal was considered 100% binding, and the other samples were normalized accordingly.

4.2.6 PIP3 Agarose Inhibition Assays

PIP_3 Agarose beads (Echelon) were used to detect for the inhibition of PH Domain binding to its substrate, PIP_3, upon incubation with the anchor candidate peptide ligands. To test the inhibition of each of the ligands, anchor biligand and triligand, 20µL of resin slurry was added to each of four tubes, and washed three times with 1x TBS. Protein, 2µg (234nM) of E17K mutant, was pre-incubated for one hour at room temperature with either DMSO (no peptide ligand blank), anchor, biligand or triligand at 2.38µM (10x in relation to protein) in 200µL of 1x TBS. For the control, mutant PH Domain was incubated with 1x TBS and 1µL DMSO to mimic the ligand conditions. These protein samples were then added to PIP_3 agarose in a Spin-X tube and incubated at room temperature for two hours. The resin was washed three times with 1x TBS with 0.25% IGEPAL CA-630, spun out to dry completely, then denatured with 50µL 3x SDS gel loading buffer for 10 min at 95°C. The gel loading buffer was spun out of the resin and detected via western blot as per the streptavidin – agarose pull downs. Inhibition was indicated by a decrease in the amount of PH Domain that was pulled down by the resin.

Expanded inhibition blots with either wildtype of E17K mutant protein were performed in a similar fashion. Twelve tubes of 20µL of PIP3 agarose were washed three times with 1x TBS. 2µg of either wildtype or mutant PHD-GFP protein (234nM) in 200uL 1xTBS were pre-incubated for 30 minutes with differing concentrations of triligand: 0.1eq (23.4nM), 1eq (234nM), 10eq (2.34µM), 100eq (23.4µM), and 1000eq (234µM). The protein and triligand solutions were then incubated with the PIP3 resin for 2 hours at room temperature. The resins were washed, eluted, and blotted as per all PH Domain western blots.

4.3 Results and Discussion

4.3.1 Biligand Development

It was hypothesized that a similarly targeted, but bulkier PCC Agent might serve as a steric blocker of the PHD-PIP3 interaction. To this end, two cycles of iterative *in situ* click chemistry screens were designed to develop the yleaf ligand into a biligand and then a triligand. To identify the biligand (the first iterative cycle, **Figure 4-2**), the yleaf ligand was modified to present an alkyne at the C-terminus, and a PEG_5-biotin group at the N-terminus (**Figure 4-1**). This modified ligand (called an anchor ligand) was then co-incubated with an alkyne-presenting OBOC library and the (unmodified) E17K PHD. Successful hits are those in which the E17K PHD promotes the click coupling of the anchor ligand onto a library peptide, and those hits are detected by screening for the formation of this clicked product (**Figure 4-2**). Those hits are candidate 2^o ligands (**Table 4-1**). As with the discovery of the anchor ligands, the biligand hits are clustered according to their hydrophobicity and sequence homology using principle component analysis. These hits cluster into groups, as seen in **Figure 4-5**, and unique clusters are circled. Hits from these different clusters, thus representing the sequence diversity of the screen, were chosen to be scaled up and tested for both affinity and selectivity to the E17K mutant PH Domain protein. For testing, the 2^o ligand candidates are appended to the yleaf anchor ligand via a Cu(I) catalyzed 1,4 triazole, to mimic the triazole formed by the protein target during the screen to form a biligand. The biligand candidates are then subjected to immunoprecipitation assays (pull down) to identify a candidate biligand in a manner that is similar to what was done to identify the original yleaf ligand (**Figure 4-6**). The biligand in lane 6 in **Figure 4-6**, yleaf – yksy, shows the highest affinity for the E17K mutant protein while still maintaining the selectivity over the wildtype protein. This sequence was chosen as the biligand.

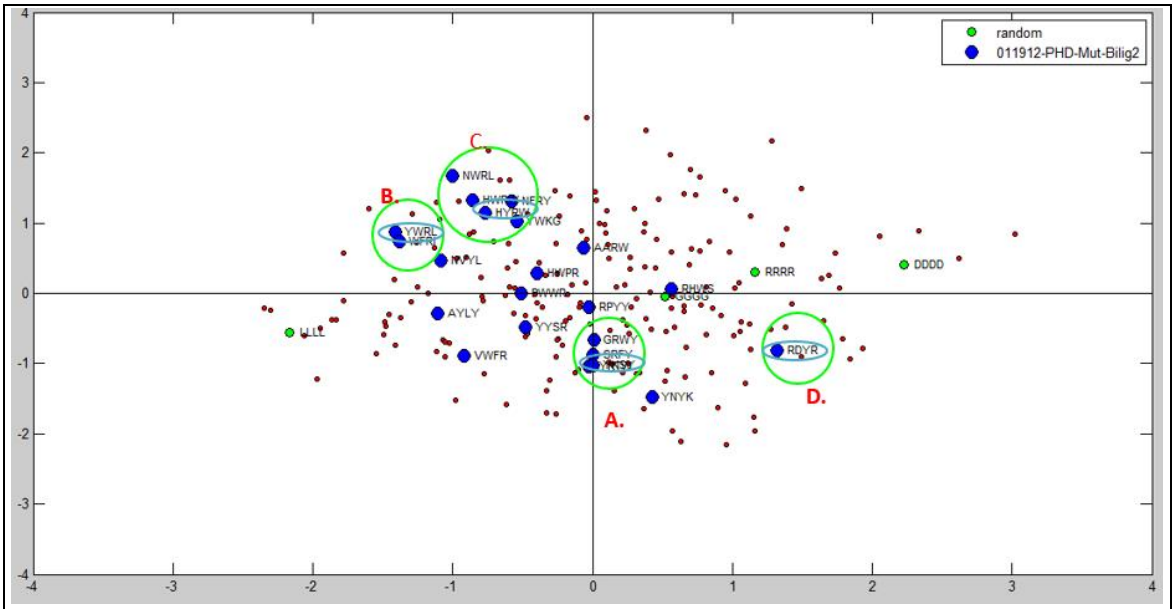


Figure 4-5: Clustering of Biligand Sequence Ligands by AA Similarity: *Hit sequences from the biligand screen were analyzed by their hydrophobicity and sequence homology using principal component analysis. Clusters circled in green indicate clustered regions, and the cyan circles indicate the peptide that was selected and scaled-up as a possible biligand sequence. The potential biligand sequences that were tested are: yleaf-ywrl, yleaf-yksy, yleaf-rdyr, and yleaf-hyrw, where "yleaf" is the anchor ligand and the "-" indicates the location of the triazole linkage.*

Table 4-1: Hit Sequences from Biligand Screen

Az4	h	w	p	r
Az4	n	v	y	l
Az4	h	y	r	w
Az4	r	d	y	r
Az4	y	n	y	k
Az4	y	k	t	w
Az4	s	r	f	y
Az4	y	k	s	y
Az4	y	y	s	r
Az4	r	h	w	s
Az4	p	w	w	r
Az4	n	f	r	y
Az4	y	w	r	l
Az4	y	w	k	G
Az4	a	y	l	y
Az4	h	w	r	w
Az4	n	w	r	l
Az4	a	a	r	w
Az4	G	r	w	y
Az4	w	f	r	i
Az4	r	p	y	y
Az4	v	w	f	r

Figure 4-6: Pull-down Assay Results for Biligand Candidates: *Note that all of the biligand candidates improve upon the binding of the anchor ligand, but yleaf-yksy shows the greatest signal in binding the E17K protein and the lowest in binding the WT protein. This biligand was chosen as the candidate biligand and carried on to triligand screening.*

4.3.2 Triligand Development

Once a candidate biligand has been identified, it is then similarly modified to form a new anchor ligand (**Figure 4-3**), which is then similarly screened (**Figure 4-4**) to identify a triligand. There were only 3 hit sequences from this triligand screen, and one sequence was not able to be called due to low signal and an irregular sequence, seen in **Table 4-2**. Because there were only two valid hits from this screen, both were scaled up and tested in a full-curve ELISA assay (**Figure 4-7**). The iryrn triligand showed significantly improved affinity for the E17K PH Domain, but this benefit was offset by the drastic increase in affinity for the wildtype PH Domain. The ivdae triligand maintains the selectivity seen in the anchor ligand and was chosen as the triligand, whose structure is shown in **Figure 4-8**.

Table 4-2: Hit Sequences from Triligand Screen

G	l	-	-	m	-
i	r	y	r	n	Pra
i	v	d	a	e	Pra

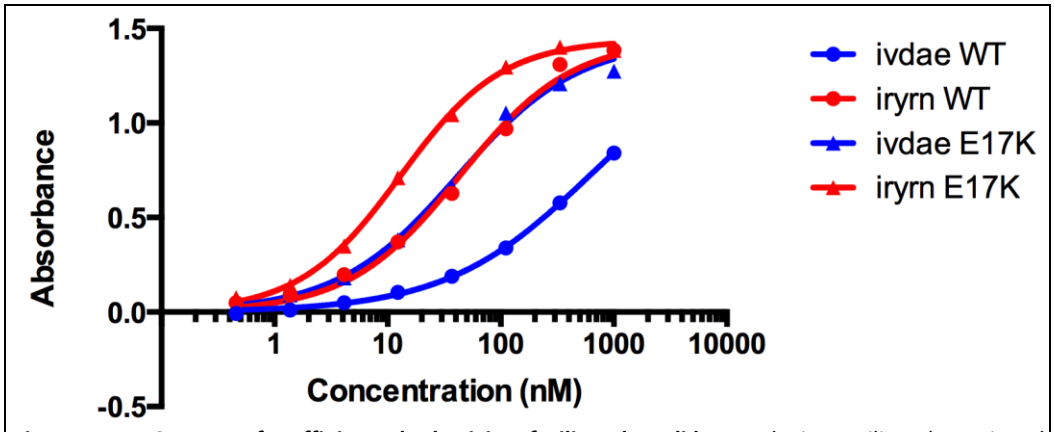


Figure 4-7: ELISA assays for affinity and selectivity of triligand candidates. *The iryrn triligand seen in red shows a significant affinity increase, but loses much of the selectivity for the E17K mutant PH Domain (triangles). The ivdae maintains the selectivity and affinity of the anchor ligand, and was carried forward as the triligand.*

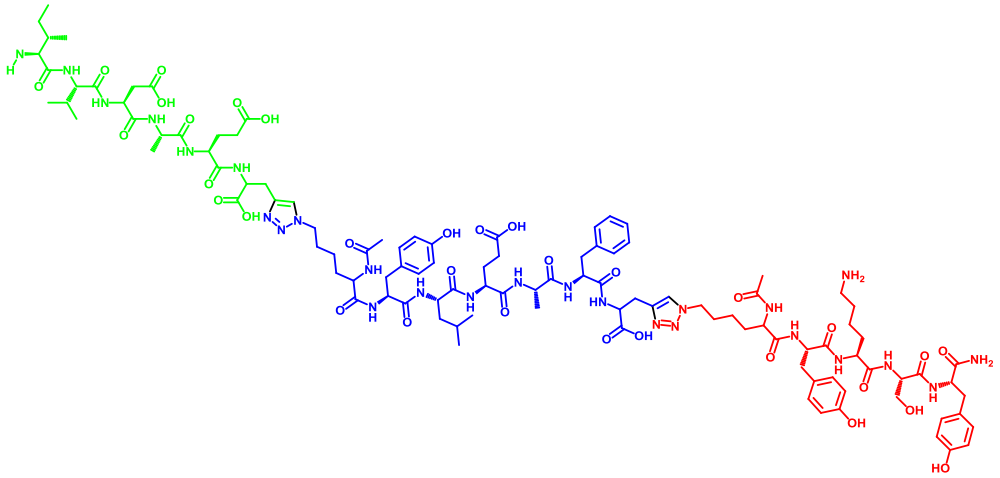


Figure 4-8: Structure of final triligand: ivdae – Pra – Lys (N_3) – yleaf – Pra – Lys (N_3) - yksy

Binding curves that compare the yleaf ligand with the biligand and triligand PCC agents are shown in **Figure 4-9**. Likely because the expanded binding site for these larger PCC agents grows away from the location of the E17K point mutation, increasing the affinity while maintaining the selectivity of the final PCC agent upon the addition of these secondary and tertiary arms proved challenging. For example, the biligand exhibited an increase in affinity for the E17K mutant protein, but this is offset by an even larger increase in affinity for the WT protein. However, at the triligand stage, the selectivity for E17K Akt1 relative to WT Akt1 is largely recovered.

Additionally, there is a slight preference for E17K Akt1 relative to E17K Akt2 (**Figure 4-10**). The homology of the PHD between these isoforms is 79%, as calculated by a pairwise sequence analysis using Blast2Seq between the Akt1 E17K structure (PDB ID: 2UZR) and the Akt2 PH Domain structure (PDB ID: 1P6S). The binding curves of 4B yield EC_{50} values for the E17K Akt1 of 61nM, 19nM, and 45nM for the yleaf ligand, the biligand and the triligand, respectively.

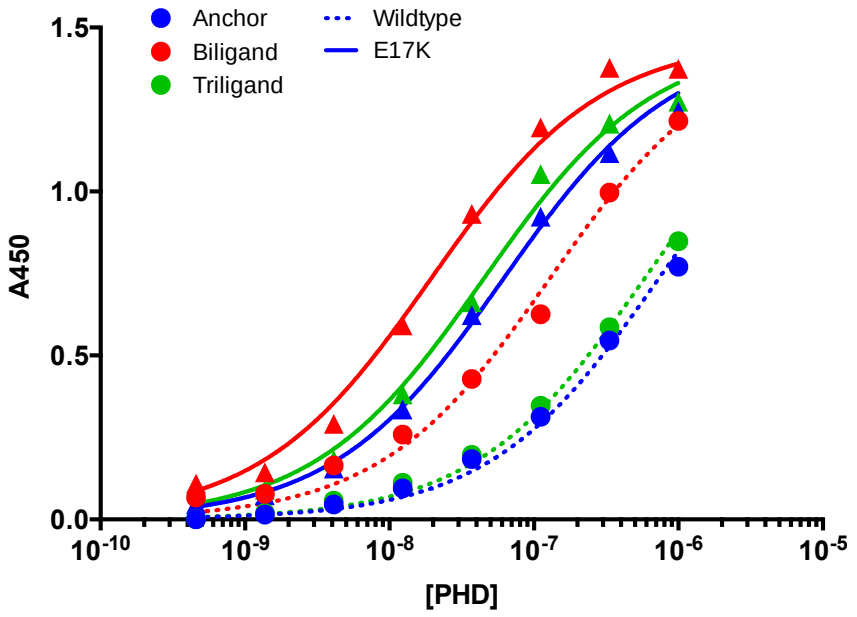


Figure 4-9: Full ELISA curves of Anchor, Biligand, and Triligand. *The ELISAs show that the biligand (red) has an increased affinity for the E17K PH Domain from the anchor ligand (blue), but also for the wildtype protein. The triligand (green) restores the affinity and selectivity of the anchor ligand.*

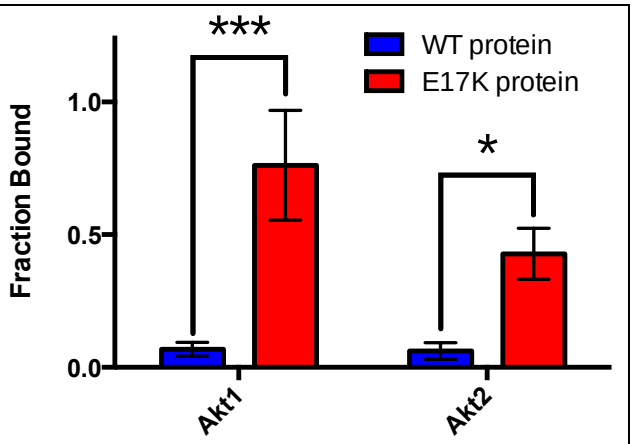


Figure 4-10: Point ELISA of Triligand binding to Akt1 and Akt2. *The binding to both the wildtype and E17K mutant PH Domains was tested for both Akt1 and Akt2. The triligand maintains the selectivity for the E17K in both proteins, and shows only a slight preference for the Akt1 isoform.*

4.3.3 Inhibition Assays

The yleaf ligand, the biligand, and the triligand were all tested for their ability to block the E17K PHD binding with PIP3 (**Figure 4-11**). For this test, PIP3-coated resin (Echelon Biosciences) was used to mimic the PHD interaction with the cell membrane, and could be used to bind the protein as in an immunoprecipitation assay[3]. The presence of an effective blocking compound would reduce the ability of the resin to capture the protein, and would thus appear as a diminished signal in the corresponding western blot assay. A control lane containing no capture agent was used to show baseline binding of the protein to the PIP3 resin. As mentioned above, the yleaf ligand produced no change in E17K binding ability, but both the biligand and triligand exhibited the ability to block the PHD-PIP3 interaction, with the triligand being the most effective (Fig 4D). In an expanded study, we compared the amount of E17K and WT PHD binding relative to the amount of added triligand. This assay shows significant selective inhibition (by around 10^3) of the E17K mutant relative to the WT.

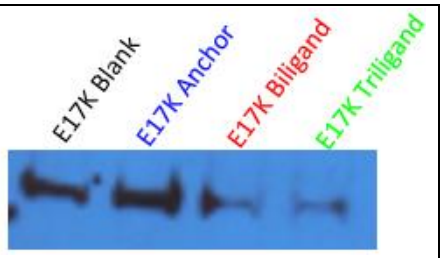


Figure 4-11: PH Domain membrane binding in the presence of each ligand. *The first lane, blank, shows the amount of protein binding to PIP3 with no ligand. Anchor ligand in respect to protein shows no decrease in binding, but binding is starting to be blocked with the biligand and triligands.*

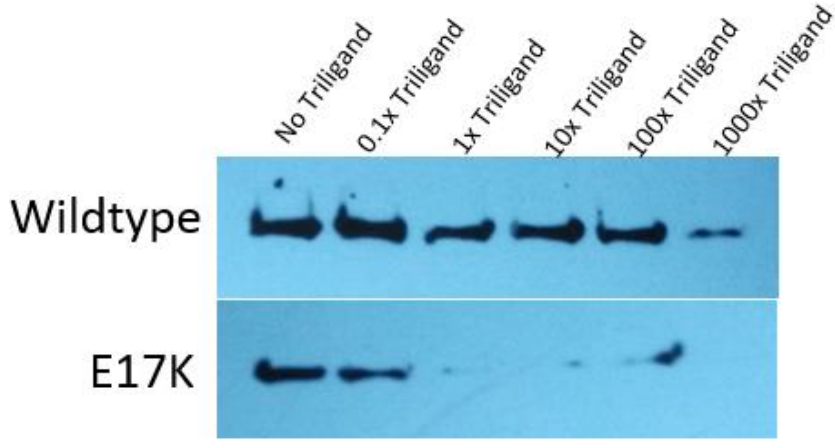


Figure 4-12: Expanded Inhibition Assay. *The wildtype or E17K mutant PH Domains were incubated with varying amounts of triligand. The wildtype protein shows very little reduction in binding until 1000x triligand to protein. The E17K PH Domain, however, shows a significant drop in binding almost immediately.*

4.4 Conclusions

The epitope-targeting strategy allowed for such selective targeting of the E17K mutant PH Domain that selectively blocking the oncogenic activation of this protein was the logical next step. Bulking up the original anchor into a triligand covered more of the PH Domain PIP3 binding pocket, therefore blocking PIP3 binding. By ensuring that the triligand maintained its selectivity for the E17K mutant PH Domain, the ligand demonstrates significantly reduced interference with the wildtype PH Domain protein. The blocking of the E17K protein binding to the cell membrane demonstrates the ability of this click-focused epitope screening technology to produce not only selective binding agents, but also potential therapeutics that could show significantly decreased toxic side-effects due to the reduction in off-target and healthy-cell interactions.

4.5 Acknowledgements

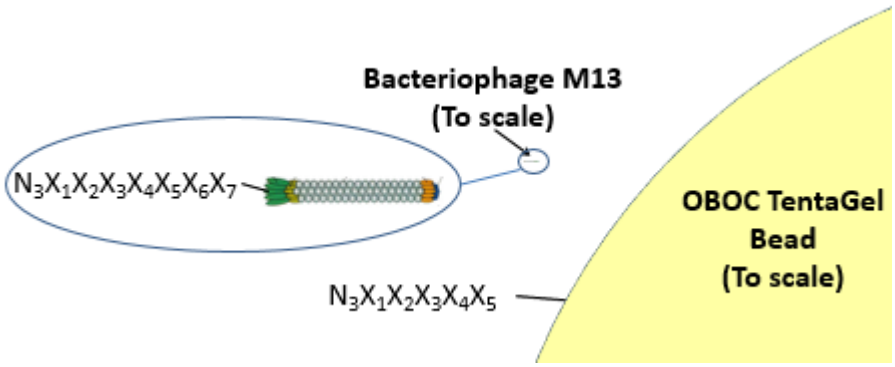
Ying Qiao Hee and Jeremy Work made many of the peptides used in these experiments. Blake Farrow graphed several of the images in GraphPad and fitted the curves.

4.6 References

1. Chong, C. R.; Janne, P. A., The quest to overcome resistance to EGFR-targeted therapies in cancer. *Nat Med* **2013,** *19* (11), 1389-1400.
2. Mahadevan, D.; Powis, G.; Mash, E. A.; George, B.; Gokhale, V. M.; Zhang, S.; Shakalya, K.; Du-Cuny, L.; Berggren, M.; Ali, M. A.; Jana, U.; Ihle, N.; Moses, S.; Franklin, C.; Narayan, S.; Shirahatti, N.; Meuillet, E. J., Discovery of a novel class of AKT pleckstrin homology domain inhibitors. *Molecular Cancer Therapeutics* **2008,** *7* (9), 2621-2632.
3. Hiromura, M.; Okada, F.; Obata, T.; Auguin, D.; Shibata, T.; Roumestand, C.; Noguchi, M., Inhibition of Akt kinase activity by a peptide spanning the betaA strand of the proto-oncogene TCL1. *The Journal of biological chemistry* **2004,** *279* (51), 53407-18.

Chapter 5

Extending OBOC *in situ* Click Chemistry into a Phage Display System



5.1 Introduction

5.1.1 Azide-Containing Phage Display Libraries

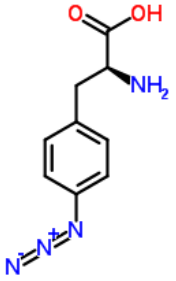


Figure 5-1: Azido - phenyl alanine *amino acid to be incorporated in phage library.*

Recent advances in chemical biology have made it possible to incorporate unnatural amino acids into recombinant proteins[1]. Schultz *et al.* has shown that through the use of amber suppression, azide-containing amino acids can be incorporated at specific locations into the pIII coat protein on an M13 phage[2]. The goal of this project is to synthesize a randomized 7-amino acid phage display peptide library containing an azide amino acid at a fixed position coded by an amber stop codon (TAG) to translate the click product screening technology into a biological library screening, and to demonstrate the use of resulting azide-phage library in *in situ* click screening.

The 7-randomized amino acid library will dramatically increase the number of peptide sequences that can be panned in each screen. For comparison, a complete OBOC library of 5 amino acids is sampled in 660 mg of library beads. To sample each sequence one time in a library the size of the 7-mer, it would require 447g of beads –-a practically impossible task using our current methods! In the phage display screen, however, it is trivial to completely oversample this 7-mer library 100 times in each screen–a huge advantage of the biological display technique. The strategies for discovering PCC agents with these libraries should be nearly identical to those previously developed for the OBOC click libraries, except that a much greater sequence space can be sampled with each screen due to the physical size of the library, and screening and sequencing steps can be performed significantly faster.

5.1.2 Mirror-Image Phage Display

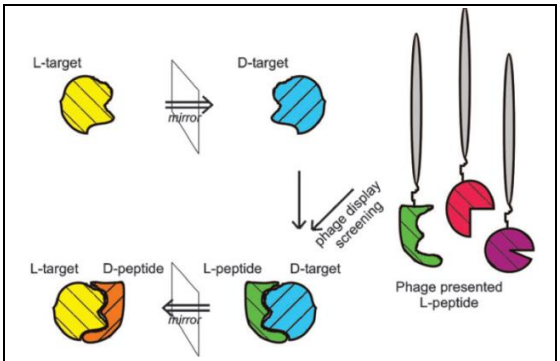


Figure 5-2: Mirror-Image Phage Display Technique[3].

One of the disadvantages common to phage display is that the library will contain exclusively L-amino acids, and will therefore produce a peptide sequence that is susceptible to protease cleavage. In order to avoid this potential problem, a technique called "mirror-image phage display" (**Figure 5-2**) has been developed. In this technique, an L-amino acid phage library is screened against a target synthesized from D-amino acids in order to find a D-peptide that binds to the L-target[4], where the D-target forms an exact mirror image of the L-target. Therefore, screening against a mirror image of the target and reversing the stereochemistry of the hit peptide binder produces a D-ligand that binds to the original L-target. With the epitope-targeting strategy currently used for PCC agent discovery in the lab, where a chemically-synthesized portion of the protein is used as a target for screening, it is trivial to prepare a D-amino acid epitope for use in a mirror image phage display screen. Using this technique, an azide-containing phage library can be screened in order to develop D-amino acid PCC agents, effectively converting the click screening so crucial to our success in PCC ligand discovery from an expensive and time-consuming OBOC library method to a simpler, quicker biological screening process.

5.1.3 G6PD Capture for Malaria Eradication

The Bill and Melinda Gates foundation is calling for the eradication of malaria, but this is a serious challenge that can only be addressed through the elimination of asymptomatic and chronic infections[5]. There exists currently a family of drugs, the 8–aminoquinolines such as primaquine, which can completely clear a person of infection (termed a "radical cure" regimen) and can thereby reduce the transmission of the disease[6]. Unfortunately, people with a glucose–

6–phosphate dehydrogenase (G6PD) deficiency, the most common enzymatic deficiency in the world, risk severe and life-threatening reactions to the standard treatment with this medication, which in turn requires a significantly different dosing method to effectively cure patients with a G6PD deficiency[7]. Therefore, in order to successfully employ the primaquine "radical cure" strategy for malaria eradication, effective methods to rapidly determine a patient's G6PD activity need to be developed immediately so that it is possible to administer the appropriate dose of the medication as quickly as possible[6].

The G6PD is found in red blood cells, and assists in the formation of NADPH from $NADP^+$, conferring protection from oxidative stress. Deficiencies normally arise from mutations in the G6PD gene that produce proteins with less than optimal function, reducing the overall enzymatic activity[6]. There are about 140 known mutations of this protein, most of which are single base changes, which can adversely affect the G6PD activity[7]. Current gold standard assays measure a deficiency by performing enzymatic tests on blood samples, measuring the amount of converted NADPH per unit of blood. Unfortunately, patients who have recently undergone a hemolytic event, causing a mass death of old red blood cells, show a false normal test. This is due to the higher than normal prevalence of young red blood cells, which express a higher G6PD copy number than mature red blood cells, compensating for the reduced activity of the enzyme caused by the mutation[6].

The goal of this project is to develop a capture agent that universally detects G6PD in all of the possible mutant forms in order to capture the protein to a chip where the concentration and the activity of the G6PD in the blood can be measured simultaneously. Such a diagnostic test can normalize the G6PD activity to protein copy number as opposed to activity per unit of blood, resulting in much fewer false positives and faster treatment of malaria. This combination test

should help reduce the number of false normal blood test results, allowing for significant progress toward the eradication of malaria.

5.2 Materials and Methods

5.2.1 Preparation of Plasmid for Incorporation of Azidophenylalanine and Amp Resistant Gene

The plasmid pAC-DHPheRS-6TRN (**Figure 5-3**) containing the coding sequences for *Methanococcus jannaschii* amber suppressor tRNATyr (MjtRNA) and the mutant *M.jannaschii* tyrosyl-tRNA synthetase (MjTyrRS) was a gift from Dr. Zhiwen Zhang at Santa Clara University. This plasmid was originally designed for the site-specific incorporation of 3,4-dihydroxy-L-phenylalanine (DOPA); its mutant MjtRNA recognizes TAG as a codon, and the mutant MjTryRS recognizes DOPA as a substrate. It also harbors a tetracycline-resistance selection marker. Since the host *E. coli* strains used for the phage production and in subsequent studies carry a Tet^R marker in their F' episomes, the Tet^R gene in this plasmid was first replaced with a beta-lactamase (Amp^R) gene for ampicillin selection, to be compatible with the antibiotic resistance of the host. Nine point mutations based on the description by Tian *et al.*[2] were then introduced into the MjTyrRS synthetase so that the resultant synthetase recognizes the azidophenylalanine amino acid instead of the DOPA.

To perform the Tet^R -> Amp^R switch, the Amp^R gene was amplified from a pET-3a plasmid by polymerase chain reaction (PCR) using the primers shown in **Table 5-1.** The PCR product and the original Tet^R-containing suppression plasmid were digested with HindIII and EagI, and ligated to produce slow-growing colonies on an LB-agar plate supplemented with 100 µg/mL ampicillin. Final clones were confirmed by DNA sequencing (Laragen).

Table 5-1: Primers for AmpR Switch.

Primer	Sequence
Hind_bla_5	GCG AAG CTT TAA TGC GGT AGT TTA TCA CAG TTA AAT TGC TAA CGC AGT CAG GCA CCG TGT ATG AGT ATT CAA CAT TTC CGT GTC GCC C
Eag_bla_3	ATA CGG CCG TTA CCA ATG CTT AAT CAG TGA GGC ACC TAT CTC AGC G

Based on the previous work by Tian *et al*.[2], nine point mutations were introduced into the mutant MjTyrRS gene to change its substrate specificity from DOPA to azidophenylalanine: E25K, L32T, S67A, N70H, E107N, D158P, I159L, L162Q, and Q167A. All mutagenesis reactions were carried out using QuikChange Kit (Stratagene) according to the manufacturer's instructions. **Table 5-2** lists the primers used in the order of performed reaction. Each QuikChange reaction was independently verified for the correct sequence, and the final plasmid was completely sequenced to ensure correctness. The resulting final plasmid was named pAC-AzPherS-6TRN.

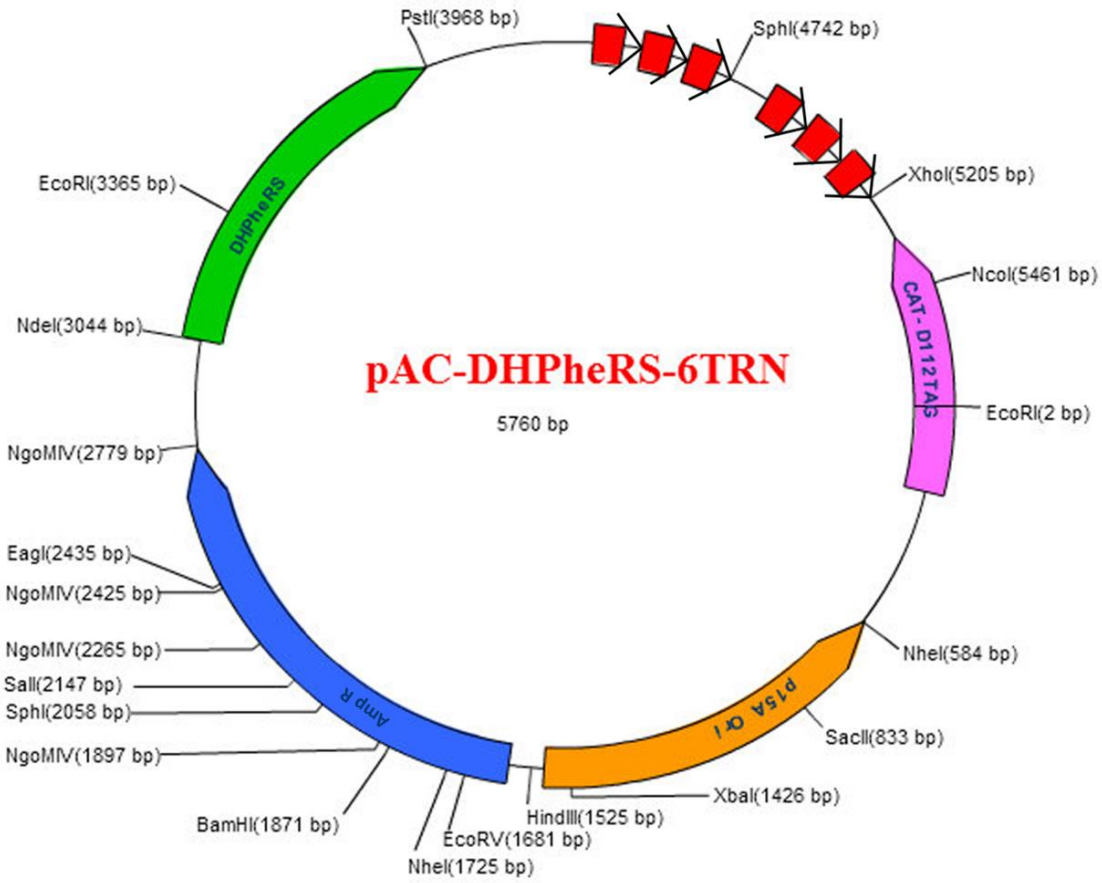


Figure 5-3: pAC-DHPheRS-6TRN Plasmid. *Plasmid for the incorporation of Azidophenylalanine. Red boxes are the six copies of the M. jannaschii tRNATyrCUA. Green indicates the synthetase. Blue is the Amp resistant gene, and the pink is the Chloramphenicol resistant marker containing an amber codon used to indicate successful incorporation.*

Table 5-2: Primers used for the DOPA -> Azidophenylalanine Synthetase QuikChange Reactions. *The number in the primer name indicates the order in which the QuikChanges were performed, and the mutation is included in the name of the primer.*

Primer Name/Mutation	**Sequence**
1_G73A_Upper	AGG AAG AGT TAA GAG AGG TTT TAA AAA AAG ATG AAA AGT CTG CTC T
1_G73A_Lower	AGA GCA GAC TTT TCA TCT TTT TTT AAA ACC TCT CTT AAC TCT TCC T
2_G319A_A321C_U	TTA AAG GCA AAA TAT GTT TAT GGA AGT AAC TTC CAG CTT GAT AAG GAT TAT ACA CTG
2_G319A_A321C_L	CAG TGT ATA ATC CTT ATC AAG CTG GAA GTT ACT TCC ATA AAC ATA TTT TGC CTT TAA
3_T199G_A208C_U	TGC TGG ATT TGA TAT AAT TAT ATT GTT GGC TGA TTT ACA TGC CTA TTT AAA CCA GAA AGG AG
3_T199G_A208C_L	CTC CTT TCT GGT TTA AAT AGG CAT GTA AAT CAG CCA ACA ATA TAA TTA TAT CAA ATC CAG CA
4_C94A_T95C_T96C_U	GAG GTT TTA GAA AAA GAT GAA AAG TCT GCT ACC ATA GGT TTT GAA CCA AGT GGT AAA ATA CAT
4_C94A_T95C_T96C_L	ATG TAT TTT ACC ACT TGG TTC AAA ACC TAT GGT AGC AGA CTT TTC ATC TTT TTC TAA AAC CTC
5_G472C_A473C_T474G_A475C_U	GGT TGC TGA AGT TAT CTA TCC AAT AAT GCA GGT TAA TCC GCT TCA TTA TTT AGG CGT CGA TGT
5_G472C_A473C_T474G_A475C_L	ACA TCG ACG CCT AAA TAA TGA AGC GGA TTA ACC TGC ATT ATT GGA TAG ATA ACT TCA GCA ACC
6_T484C_T485A_U	TAT CCA ATA ATG CAG GTT AAT GAT ATT CAT TAT CAA GGC GTC GAT GTT CAG G
6_T484C_T485A_L	CCT GAA CAT CGA CGC CTT GAT AAT GAA TAT CAT TAA CCT GCA TTA TTG GAT A
7_C499G_A500C_U	CAT TAT TTA GGC GTC GAT GTT GCG GTT GGA GGG ATG GAG C
7_C499G_A500C_L	GCT CCA TCC CTC CAA CCG CAA CAT CGA CGC CTA AAT AAT G

5.2.2 Test of Azidophenylalanine Incorporation into a Protein in E.coli

The ampicillin-resistant vector pAC-AzPheRS-6TRN developed above also carries a chloramphenicol acetyltransferase (CAT or Cm^R) gene where residue D112 is mutated to an amber stop codon. *E. coli* TOP10-F' cells were transformed with pAC-AzPheRS-6TRN plasmid and plated on an LB agar plate supplemented with ampicillin (100 μg/mL and tetracycline (12.5 μg/mL). Colonies were slow-growing, and were therefore incubated for 24 hours at 37C°. An LB broth culture of 5 mL supplemented with ampicillin (100 μg/mL) and tetracycline (12.5 μg/mL) was inoculated with a single colony and grown overnight at 37°C. The overnight culture (1 mL each) was diluted into two 7 mL cultures with appropriate antibiotics. An azidophenylalanine stock solution was prepared freshly by dissolving the amino acid in 50% DMSO and 50% acidic water (pH = 2.0 with HCl). A blank stock solution containing only the DMSO/acid without amino acid was also prepared. The amino acid (or blank) stock solution was added to the diluted culture to the final concentration of 2 mM (or 0 mM) azidophenylalanine. After incubating at 37°C for two hours, 34 μg/mL of chloramphenicol was added to each culture. Each culture was allowed to grow further at 37°C, and the level of growth was assessed by OD_{600} at 6 and 18 hours of incubation.

5.2.3 Test of Azidophenylalanine Incorporation into M13KE Phage

Four phage clones with variable display sequences of [Amber]-AHEATH, [Amber]-SHEATH, [Amber]-RHEATH, [Amber]–THEATH in M13KE phagemid were purchased from Antibody Design Labs. M13KE phagemid also contains the lacZα gene for blue/white plaque screening. A naturally-amber-suppressing strain of *E. coli* XL1 Blue was transformed with each individual clone, and plated on LA agar plate supplemented with tetracycline (12.5 μg/mL), IPTG (50 μg/mL) and X-gal (40 μg/mL) in top agar following the procedure described in Ph.D. Phage Display Libraries Manual (New England Biolabs, ref). An overnight starter culture of *E. coli* Top10-F' transformed with pAC-AzPheRS-6TRN was diluted 10-fold into 10 mL of LB supplemented with ampicillin (100 μg/mL)

and tetracycline (12.5 µg/mL) and grown at 37°C in the presence of 2mM azidophenylalanine for two hours. A blue plaque from each individual clone was added to the culture from the fresh plates, and cultures were further incubated for five hours. The cultures were centrifuged at 4500 rpm to remove the cells, and the phages were precipitated overnight at 4°C by collecting the top 8 mL of the supernatant and mixing it with 1.6 mL of 20% (w/v) PEG-8000 in 2.5M NaCl (PEG/NaCl). An alkyne-labeled TAMRA dye was clicked onto the azide moiety on the azidophenylalanine-containing phages using the Click-It Kit (Invitrogen) according to the manufacturer's instructions, and the resulting phages were resolved by SDS-PAGE. The gel was then imaged on a Typhoon imager using the preset settings for TAMRA dyes.

5.2.4 Synthesis of M13KE Azidophenylalanine-Terminated 7-mer Random Library

The peptide library was designed to have the following sequence when expressed on the surface of the pIII coat protein of the M13KE phage: A-[Amber]–$X_1X_2X_3X_4X_5X_6X_7$–G-L-V-P-R-G-S-pIII protein. The N-terminus of the display sequence has a fixed alanine amino acid to provide a non-charged spacer at the signaling peptide cleavage site to ensure proper expression of the azide-incorporated final pIII protein. The amber codon, the site of the azidophenylalanine incorporation, follows the N-terminal alanine, and precedes then the randomized 7-mer amino acid region. This library sequence is separated from the rest of the pIII protein by a thrombin cleavage site (LVPRGS) to allow the enzymatic cleavage of a "clicked" phage hit from a matrix-immobilized target.

The phage library was built by Antibody Design Labs using standard NNK codon, where N encodes an equimolar amount of cytosine, guanine, thymine or adenine bases, and K encodes only guanine or thymine. By designing the library in this fashion, some of the redundancy of the third codon as well as two of the three possible stop codons, TAA and TGA, are eliminated.

The final DNA library contained about 7.65 x 10^8 total transformants, and about 90% of that library contained inserts for a total sequence diversity of 6.9 x 10^8. It should be noted that there is a significant prevalence for guanine at the first position of a codon, as can be seen in **Figure 5-4**. This leads to a greater prevalence of the Glycine (G) amino acid in the overall distribution (**Figure 5-5**). Stop codons and Cysteine (C) amino acids are repressed since the production of functional phages is prohibited with these sequences, which is to our benefit. The statistical distribution of the library sequences is otherwise unremarkable.

Position	A	T	G	C	Total
First	84	60	128	57	329
Second	82	88	92	67	329
Third		176	153		329

Figure 5-4: Distribution of Random Nucleotides. *There is a significant prevalence for G in the first position, for unknown reasons. Recall that the third position is limited to T and G due to the NNK format of the library.*

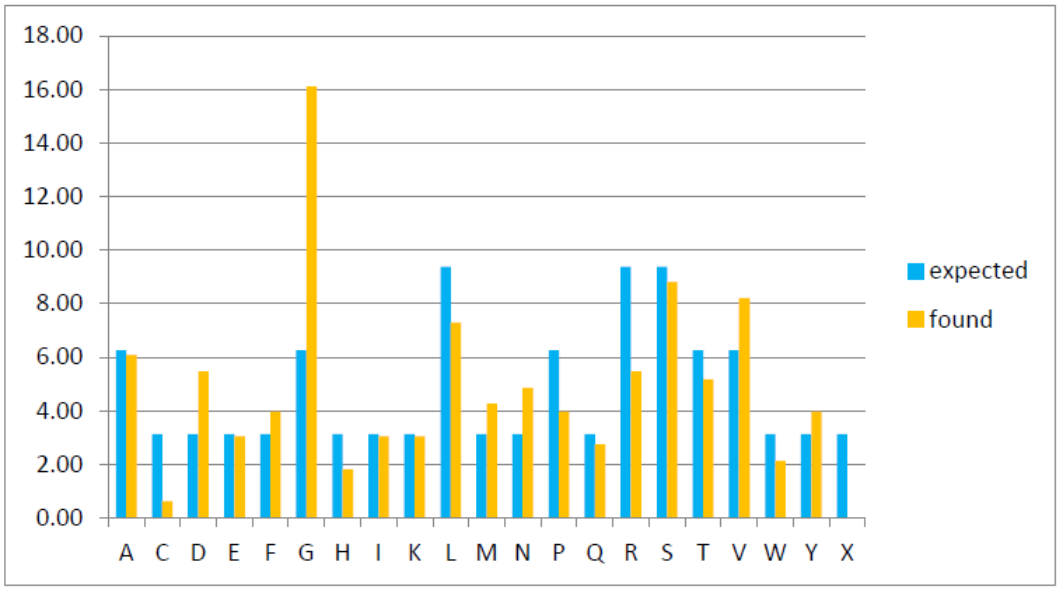


Figure 5-5: Distribution of Amino Acids. *The high prevalence of the G base pair in the first position lead to a much higher prevalence of the G amino acid than was expected. Otherwise, the distribution is as to be expected.*

5.2.5 Design and Synthesis of G6PD Target and Scrambled Target for Screening

The capture agent that will effectively detect G6PD in clinical settings must be able to bind to a site on the protein surface that has the least rate of mutation in order to ensure the most efficient capture of the protein with diverse sequence variance. As seen in **Figure 5-6**, exons 2 and 3 have the least number of total mutations identified in the polypeptide sequence. There are also no Class I mutations that correlate to the most severe deficiencies in enzymatic activity. Therefore, these two exons are the ideal location to target in order to capture the most diverse pool of mutant G6PD proteins. The region that is targeted by the capture agent should

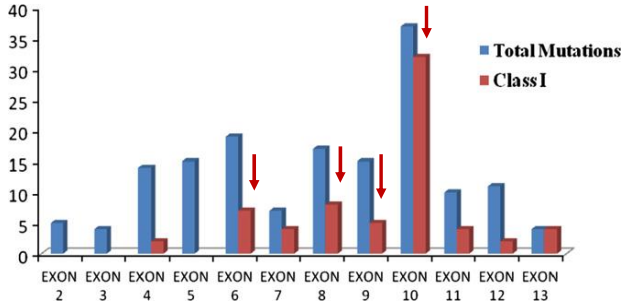


Figure 5-6: Location of G6PD Mutations in protein. *Exons 2 and 3 show the least number of mutations and no Class I mutations, so provide an ideal location to target.*

also be distant from the $NADP^+$ binding site in order not to interfere with the enzyme activity after capture. In the crystal structure (PDB ID: 1QKI) in **Figure 5-8**, the region encoded by exons 2 and 3 is unstructured in red in the bottom right of the protein. This region is far from the active site seen in complex with the $NADP^+$ at the top left of the protein, and the enzyme is expected to retain its original level of activity after being captured at this position.

The region including these exons, seen in **Figure 5-7**, has a few sites commonly mutated, which are shown in red. Therefore, the capture agent must be targeted to the amino acids shown in black in order to ensure it is not binding to a highly mutated section of the protein. The epitope that was selected for screening consists of amino acids 20 – 31 corresponding to the sequence LFQGDAFGQSDT, and was synthesized on TentaGel resin using D – amino acids in order to use the mirror image phage display technique to produce a D - amino acid peptide to bind to the L - G6PD protein. The synthetic target epitope was appended with propargylglycine (Pra) residue at the N-terminus, providing an alkyne click handle. A scrambled epitope also made of D – amino acids, [Pra]-GDAHSFQDTLQF, was synthesized on TentaGel as well, to be used in a preclear/antiscreen step for the library. The target and scrambled target on TentaGel resin will allow the phage library to click onto the permanently-immobilized target so that very harsh washing conditions can be used to remove all but the covalently bound phages. These peptide sequences were tested for their correctness and purity via Edman degradation sequencing.

Figure 5-7: Amino Acid Sequence of Exons 1 and 2 of G6PD. *The amino acids highlighted in red indicate frequently mutated positions, and the residues in black are generally conserved.*

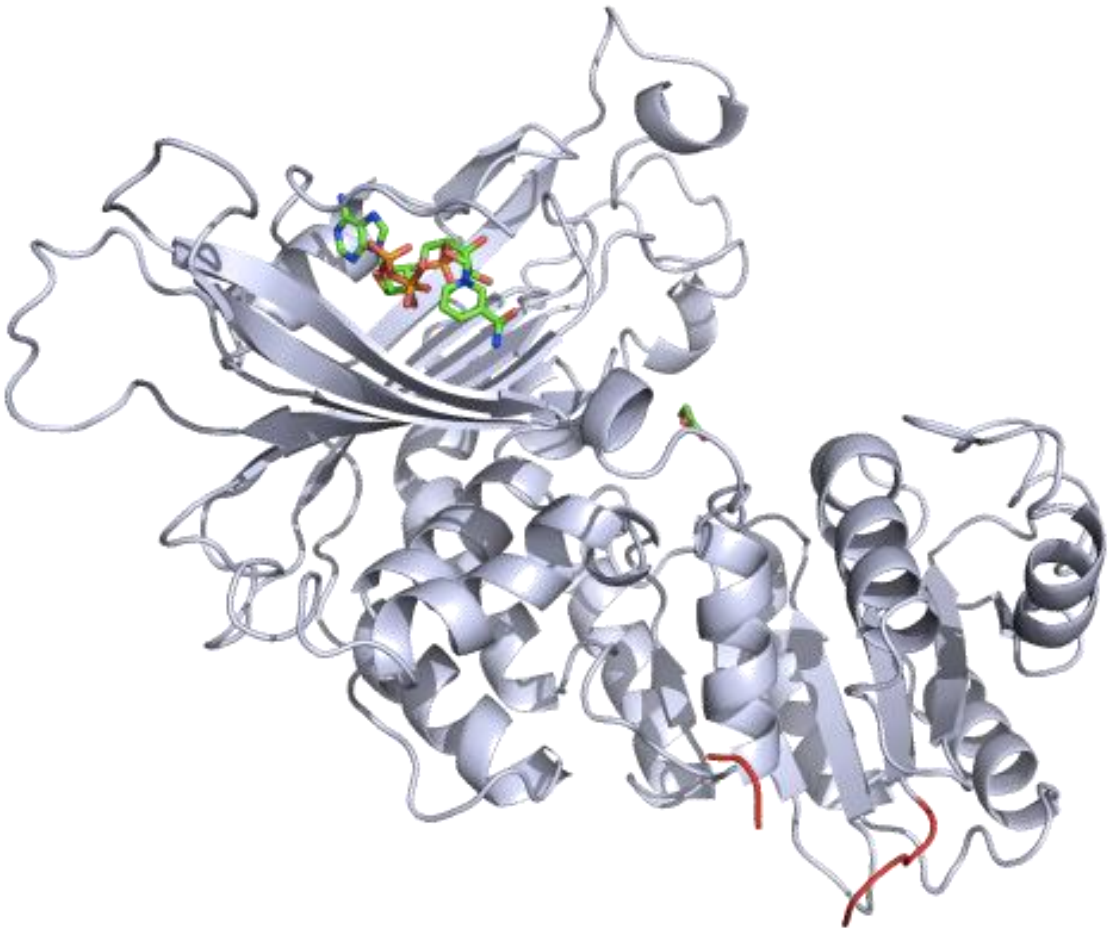


Figure 5-8: Crystal Structure of G6PD (1QKI). *This mutant G6PD in complex with NADP+ indicates that the unstructured regions encoded by exons 2 and 3 (bottom right in red, not entirely present in structure) are far from the active site of the protein. Capturing the protein at this location should not affect enzyme activity.*

5.2.6 Optimized Phage Library Target Screening Conditions

General protocols for phage library screening procedures including phage titering, scaling up, and sequencing as well as preparation of materials such as buffers, PEG/NaCl solution and top agar adopted from the NEB Ph.D. Phage Display Library System manual for M13KE, unless otherwise noted.

The library from Antibody Design Labs was produced in a naturally-suppressing strain of *E. coli* TG1 that inserts Glu, or E, at the site of amber codon. This library cannot be used for click screening, as there is no azide amino acid, but can be used for "target" screening to find peptide

sequences that bind to the G6PD epitope. The target screening must be completed first, also because approximately 35% of this phage pool contains no insert, or "naked phages." Naked phages have a significant selection advantage over those that would require the amber suppression. The target screen is designed, therefore, to enrich for phage containing the library insert, and remove the naked phage from the phage pool before incorporation of azidophenylalanine is performed.

The TentaGel beads containing the target and scrambled epitopes are stored in the form of 50% (v/v) slurry in 1:1 ethanol:water solution. One hundred µL each slurry of the target and scrambled epitopes were washed with 1 mL Tris-buffered saline (TBS, 25mM Tris-HCl, 150mM NaCl, 10mM $MgCl_2$) with 0.1 % Tween-20 (TBST), then blocked with 1mL of 5% (w/v) bovine serum albumin (BSA) in TBS for one hour at room temperature. An antiscreen was performed by incubating the scrambled peptide resin with a phage screening solution containing 2 µL sterile filtered human serum, 2 µL of the original library (~5×10^{11} total phages), and 196 µL TBS for 30 minutes at room temperature. The beads are spun down and the supernatant, containing the phage that did not bind to the scrambled peptide, is added to the target epitope resin and incubated for one hour at room temperature. Extensive washes are performed to ensure that the least number of non-specific and naked phages remain: five times with TBST, 30 minutes shaking in TBST, five times with TBST, five times with high salt TBS (2.5 M NaCl), 30 minutes shaking in high salt TBS, five times with high salt TBS, five times with TBST, five times with TBS, 10 minute shaking with TBS, five times with TBS, and two times with $CaCl_2$ thrombin buffer (1 mM $CaCl_2$ in TBS). The last wash step is retained and titered to ensure no phage are eluting. The target beads are then incubated with 4 units of thrombin in 200 µL $CaCl_2$ buffer for 24 hours at room temperature to elute hit phages. This step should elute only the phages that have a thrombin cleavage tag. After incubation, the 200 µL of thrombin cleavage cocktail was removed from the

beads, and the beads were washed five times with 200 µL TBS to remove all of the cleaved phages. The washes were combined with the thrombin eluent. The target beads are then acid-bumped by adding 1mL of 0.2 M glycine-HCl + 1mg/mL BSA buffer (pH = 2.0) for 10 minutes in order to ensure that all thrombin cleaved peptides were removed previously, and that only naked phage remain. The 1mL of acid eluent was removed from the beads and neutralized with 150 µL of 1 M Tris (pH = 9.1). Both the thrombin cleavage and acid bump eluents are titered to determine the number of phages eluted in each step.

5.2.7 Testing Phage Plaques for Library Inserts

The titered phage from a library or final eluent of a screen can be rapidly assessed for the presence of library inserts by plaque PCR before sequencing each individual clone from titered plaques. The first two primers listed in **Table 5-3** were designed by Antibody Design Labs, to recognize the DNA sequence on the M13KE phagmid on the either side of the cloning site, allowing the screening for the presence of insert based on the increased size of the PCR product compared to the one from a naked phage. The last PCR primer is an alternate 3' primer designed to recognize part of the insert DNA sequence, and therefore, a positive PCR reaction only occurs when the template phage contains the insert, and not with a naked phage. At each stage of the screening process, plaques were picked, and plaque PCR was performed to determine the percent of naked phage.

Table 5-3: Primers for Colony PCR of Inserts. *The first two primers show a slightly heavier PCR band if the insert is present. The last primer is an alternate 3' primer designed to sit inside the insert and, therefore, the PCR will show nothing if the insert is not present.*

Primer Name	Sequence
M13gV_5	GTC AGG GCA AGC CTT ATT CAC TG
Psi_3	GCG TAA CGA TCT AAA GTT TTG TCG
Thrombin_3	CGA ACC ACG CGG AAC CAG AC

5.2.8 Incorporation of Azidophenylalanine into Phage Libraries

The TOP10-F' cells transformed with the suppressor plasmid pAC-AzES-6TRN were streaked from a glycerol stock on an LB agar plate supplemented with ampicillin (100 µg/mL) and tetracycline (12.5 µg/mL). A single colony was inoculated into LB broth with appropriate antibiotics, and grown overnight at 37°C. The starter culture was diluted 100-fold into 20mL of LB + 12.5 µg/mL Tet + 100 µg/mL Amp, and allowed to grow for two hours at 37°C to early log phase (OD_{600} = ~0.1). A stock solution of azidophenylalanine was freshly prepared by dissolving 8 mg of amino acid in 250 µL of DMSO and 250 µL of acidic water (pH = 2.0) The entire volume of the stock azidophenylalanine solution was added to the culture to achieve the final concentration of 2 mM. For a control culture with no amino acid, only the 250 µL DMSO and 250 µL pH = 2.0 water are added. The cultures were then allowed to grow for additional one hour before the phage stock was added. For the target screen hits, the entire 1.2 mL phage elution was added to the growing culture. For the previously amplified library, phage amounts of 100 times the library diversity were added. The phage culture was then allowed to grow for 5 hours, centrifuged at 4500 rpm to remove the cells, and the top 80% of the supernatant was collected and precipitated with 1/6th volume of PEG/NaCl overnight at 4°C. The mixture containing the precipitated phage was centrifuged at 14,000 rpm for 15 minutes. The pellet was dissolved in 200 µL TBS and spun down again at 14,000 rpm to remove cellular debris. The total number of recovered phages was estimated before titering by measuring the absorbance at 269 nm and 320 nm with a NanoDrop spectrophotometer and using the formula in **Figure 5-9** from the Antibody Design Laboratories website[8], where the vector size was set to 7200bp for M13KE.

$$\frac{(A_{269}-A_{320}) \cdot 6\times10^{16}}{\text{number of bases/virion}} = \text{virions/ml}$$

Figure 5-9: Formula for Estimation of Phage Concentration.

5.2.9 *Optimized Phage Library Click Screening Conditions*

The click phage screens were performed on the library containing the azidophenylalanine after the incorporation step. These screens were performed exactly as the target screen, except that the phage library was incubated with the target on resin overnight at room temperature to allow sufficient time for the click reaction to occur. A diagram of this screen is seen in **Figure 5-10**.

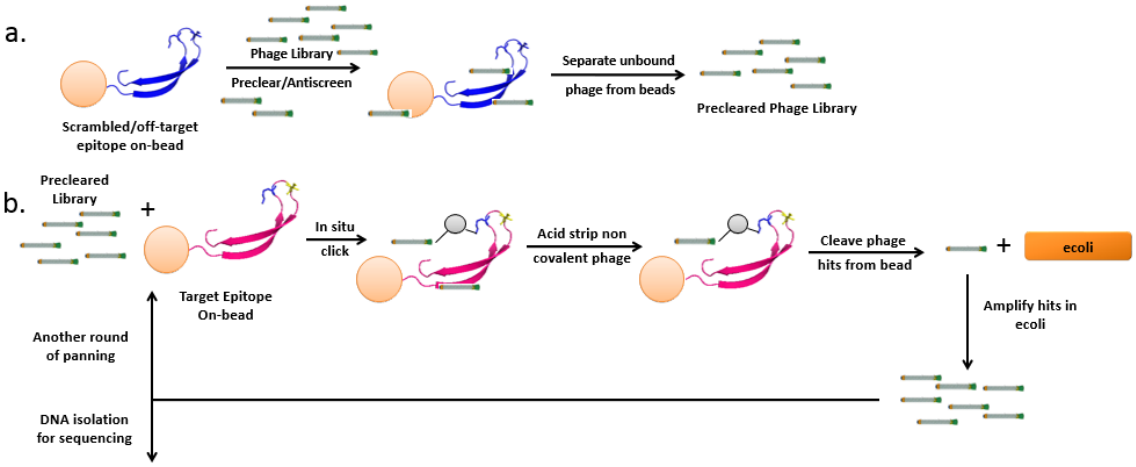


Figure 5-10: Phage Click Screening Strategy. *a.) The phage library is precleared for TentaGel and sticky binders by preincubating the library with a scrambled epitope on resin. b.) The phage are then incubated with the target overnight for a click to form, washed extensively, and eluted from the resin by thrombin cleavage for amplification in e.coli or titering and sequencing.*

5.3 Results and Discussion

5.3.1 *Test of Azidophenylalanine Incorporation into a Protein in E.coli*

The first test was to determine whether, and if so, in which condition the suppressor plasmid pAC-AzPheRS-6TRN created by the series of mutagenesis described in 5.2.1 is effective in incorporating azidophenylalanine into a recombinant protein in *E. coli* in response to an amber codon. Because the plasmid contains a chloramphenicol resistant marker (Cm^R) with an amber codon in its coding sequence (CAT-D112TAG), this antibiotic resistance is only conferred upon successful suppression of amber codon to synthesize the full-length protein. When grown in the presence of chloramphenicol in a liquid medium, only the culture containing the

azidophenylalanine amino acid was able to survive, as seen in **Figure 5-11**, with measured ODs of 0.639 with added azidophenylalanine as opposed to 0.108 with control. This result indicates that the mutated MjTyrRS for azidophenylalanine is functional and incorporating azidophenylalanine amino acid into the amber stop codon of the protein. It also indicates that the conditions used for this incorporation are sufficient to keep the background suppression without the unnatural amino acid to a negligible level.

Figure 5-11: Test of azide incorporation in e.coli.

5.3.2 Test of Azidophenylalanine Incorporation into M13KE Phage

After the successful incorporation of the azidophenylalanine amino acid into a protein in *E.coli*, the incorporation was tested using a small number of M13KE phage clones that contain representative display peptide sequences similar to the library that was designed for the subsequent *in situ* click screening. The azidophenylalanine incorporation was performed using four phage clones individually, and the presence of azide moiety in the collected phages was tested by clicking on an alkyne-containing TAMRA dye using a Click-It Kit (Invitrogen). This allowed for the fluorescence visualization of the dye-labeled pIII protein resolved by SDS-PAGE. As seen in **Figure 5-12**, there is one band across three of the four lanes corresponding to ~65kDa, or the running weight of the pIII phage coat protein, indicating that three of the four phage clones showed incorporation of the azidophenylalanine. The fourth phage could have been incorporating improperly or could have been lost in the multiple steps of the experiment, since the quantities of phage used were very small. Also, there are no other bands present in the gel which would indicate nonspecific azide incorporation in other proteins in the phage. This suggests that the incorporation of

azidophenylalanine is specific to the pIII protein where amber codon is introduced, and that peptides containing azidophenylalanine at a precise location can be displayed on the phage surface.

5.3.3 Phage Library Screening Conditions and Results

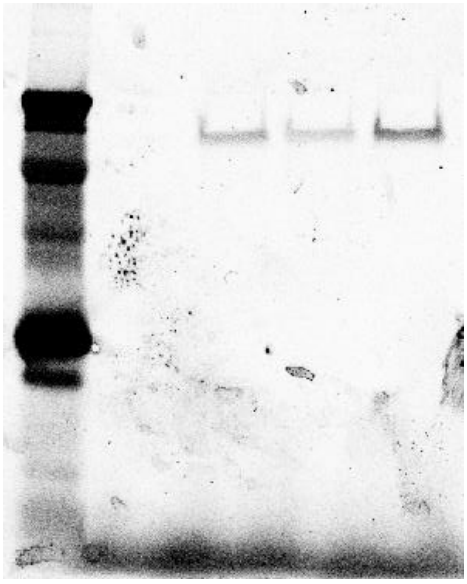


***Figure 5-12: Click-It Kit Visualization of Azide in pIII Coat Protein**. The band seen above corresponds to ~65kDa, the known running mass of the pIII protein.*

The original phage library produced by Antibody Design Labs contained about 35% naked phage determined by titering followed by plaque PCR, and cannot be directly carried onto the azidophenylalanine incorporation. The high prevalence of naked phage in the library complicates the subsequent azidophenylalanine incorporation, as the rapidly infecting naked phages that do not require suppression of the amber codon have a significant growth advantage over the properly-inserted phages, and can overwhelm the relatively slow synthesis of azidophenylalanine-containing phages by amber suppression. For this reason, the traditional phage display screening methods–"target" screens searching only for binding of the library component to the target of interest–were used to minimize the number of naked phages present in the incorporation step. The target screens eluted with thrombin cleavage were able to reduce the total amount of naked phage seen on titer plates to about 18%. Unfortunately, when this pool of eluted target hits was amplified using a naturally-suppressing *E. coli* strain XL1-Blue, this number rose to 85%-too high to use for any incorporation. An enrichment strategy that can reduce the number of naked phage to a manageable level has yet to be achieved.

5.3.4 Focused Library Screening

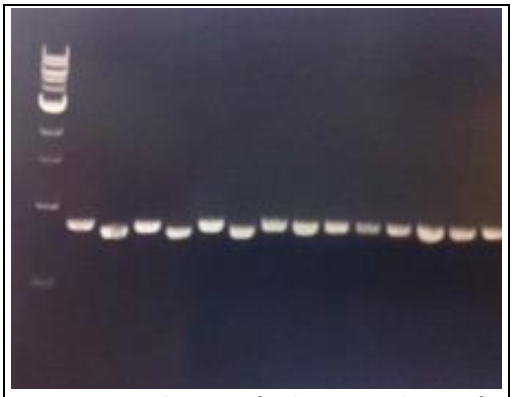


Figure 5-13: Gel Image of Colony PCR*. This test for library insert shows only 3 naked phage, lanes 2, 4, and 6. Out of 16 plaques total (not all shown on this gel), 13 contained an insert, and were used for the "focused" library.*

Because the amount of naked phages cannot be reduced to a level that would allow for successful azidophenylalanine incorporation, a "focused" library was designed from the insert-containing hit phages isolated from a target screen. **Figure 5-13** shows a result from the plaque PCR that was completed after a target screen to search for phages containing inserts. In this gel, each lane represents a single hit clone, and lanes 2, 4, and 6 show naked phages, as indicated by the PCR product of lower molecular weight. The phage plaques that were tested in this PCR were also individually amplified in 1mL cultures in order to create a small pool of each sequence. The 13 insert-containing plaques were then sequenced to ensure that each one contained an insert. As can be seen in **Table 5-4**, only 11 of the 13 hits contained one clean sequence. The rest appeared to have multiple sequences and may have had some naked phage contamination, and were thus eliminated from our pool of potential hits. Therefore, out of this target screen, 11 of the amplified stocks that contained inserts (**Table 5-4**) were pooled to create a "focused" library of phage that are known to bind to the target. This focused library has the added advantage of containing no naked phage, so it is the ideal library to be used to test the conditions for the azidophenylalanine incorporation. It will also be used for the subsequent focused library click screen with the target to test the *in situ* click on a phage library.

Table 5-4: Hits from 13 Insert-Containing Phages, Figure 5-13

#1	Amber	D	A	L	L	P	T	V
#3	Amber	N	S	T	Y	A	N	S
#4	Amber	I	S	A	Y	L	I	Q
#5	Uncallable, multiple sequence							
#6	Amber	A	F	S	A	L	D	L
#8	Amber	M	L	V	P	L	K	P
#10	Amber	M	D	T	W	L	M	T
#11	Amber	T	L	M	G	Q	W	W
#12	Amber	S	Y	T	T	M	E	V
#13	Amber	G	V	G	G	P	G	P
#14	Uncallable, multiple sequence							
#15	Amber	E	W	W	P	G	V	W
#16	Amber	V	L	H	G	G	R	A

5.4 Conclusions

The difficulties associated with the original library itself have delayed the testing of the described technology, though a roundabout solution to that problem has been discovered. The focused library is currently undergoing testing as the proof-of-concept for the click phage screening.

Future libraries for azidophenylalanine incorporation should be built using a phage vector that is out of frame, so that the insert is required in order to correctly synthesize the pIII coat protein. This will entirely eliminate the problem with the naked phage, and the complicated screening strategies will no longer be as important.

5.5 Acknowledgements

This ongoing work is being done in conjunction with Dr. Aiko Umeda. JingXin Liang performed the background research on the G6PD deficiency in malaria, and assisted in the selection of the screening target.

5.6 References

1. Wang, L.; Xie, J.; Schultz, P. G., EXPANDING THE GENETIC CODE. *Annual Review of Biophysics and Biomolecular Structure* **2006,** *35* (1), 225-249.
2. Tian, F.; Tsao, M. L.; Schultz, P. G., A phage display system with unnatural amino acids. *J Am Chem Soc* **2004,** *126* (49), 15962-3.
3. Funke, S. A.; Willbold, D., Mirror image phage display-a method to generate d-peptide ligands for use in diagnostic or therapeutical applications. *Molecular BioSystems* **2009,** *5* (8), 783-786.
4. Schumacher, T. N. M.; Mayr, L. M.; Minor, D. L.; Milhollen, M. A.; Burgess, M. W.; Kim, P. S., Identification of d-Peptide Ligands Through Mirror-Image Phage Display. *Science* **1996,** *271* (5257), 1854-1857.
5. Liu, J.; Modrek, S.; Gosling, R. D.; Feachem, R. G. A., Malaria eradication: is it possible? Is it worth it? Should we do it? *The Lancet Global Health* **2013,** *1* (1), e2-e3.
6. Domingo, G. J.; Satyagraha, A. W.; Anvikar, A.; Baird, K.; Bancone, G.; Bansil, P.; Carter, N.; Cheng, Q.; Culpepper, J.; Eziefula, C.; Fukuda, M.; Green, J.; Hwang, J.; Lacerda, M.; McGray, S.; Menard, D.; Nosten, F.; Nuchprayoon, I.; Oo, N. N.; Bualombai, P.; Pumpradit, W.; Qian, K.; Recht, J.; Roca, A.; Satimai, W.; Sovannaroth, S.; Vestergaard, L. S.; Von Seidlein, L., G6PD testing in support of treatment and elimination of malaria: recommendations for evaluation of G6PD tests. *Malaria journal* **2013,** *12*, 391.
7. Cappellini, M. D.; Fiorelli, G., Glucose-6-phosphate dehydrogenase deficiency. *Lancet* **2008,** *371* (9606), 64-74.
8. Phage Concentration Calculator. http://www.abdesignlabs.com/technical-resources/phage-calculator/ (accessed May 2).

Appendix A

Use, Care and Maintenance of Procise 494 CLC

Edman Degradation Protein Sequencer

A.1 Regular Use

A.1.1 Loading a Bead

There are 4 cartridges on the Edman, and each can be loaded with a bead. The cartridge is unscrewed from the machine, and the lid with the hose coming out of the top should ALWAYS be rested on the cartridge holder, and not left to dangle. This stretches out that hose, and it will need to be replaced. The cartridge top unscrews. Inside, there are two quartz sample holders, and the bead fits in between them. With gloves on, use a finger on each side of the cartridge to hold the second quartz sample holder in place while you turn the cartridge upside-down and let the top part slide out. These quartz sample holders are VERY expensive, and chip easily. Do not drop them, and be very careful when handling them. Once the top sample holder is out, there should be a used filter and cartridge seal that can be removed and thrown out. A new cartridge seal is slid into the cartridge on top of the bottom sample holder that was left in place. A new filter gets tapped into the groove in the top sample holder that was removed. It can be gently tapped in place with tweezers. The bead is placed onto this filter, and the whole cartridge is then turned upside down, with the bottom sample holder and cartridge seal held in place by a finger on each side of the cartridge and slid down on top of the top sample holder that has the bead on top. Then, the cartridge can be turned right-side-up, the lid can be screwed back on, and it can be placed back into its slot on the Edman.

The run is set up on the Procise software on the computer. Each run should be set to use the “PulsedLiquid cLC” method, unless a special one is required. The cycles should be set to equal the number of desired amino acids plus 3 – one warm-up cycle, one blank cycle and one standard cycle – that run automatically. All of the solvent bottles should be checked to ensure that the levels are appropriate for running the desired amount of cycles. You can check the amount of

each solvent required by clicking the button on the bottom of the screen. It will provide an amount in mL required to run the set cartridges and cycles.

A.1.2 Solvents

Most of the solvents used are purchased from somewhere other than Applied Biosystems, and mixed in house. See

Table *A-1* for details. When refilling solvents, the bottle should be screwed on until you hear three clicks. Over-tightening can damage the assembly, such as the R1 bottle. To loosen or tighten this bottle, you must first push up hard on the rachet assembly to hold it in place while you screw or unscrew the bottle.

Table A-1: Solvent Compositions

Letter	**Name**	**Composition**
HPLC Solvents		
B2	Acetonitrile in Isopropanol	9:1 Acetonitrile:Isopropanol
A3	THF in water	3.5% THF in water, 100uL stock Na2PO4, 1mL 1% Acetone, 10mL Buffer premix, 20uL TFA
Machine Solvents		
R1	PITC in Heptane	2.5mL PITC in 50mL heptane
R2B	N-methylpiperidine/MeOH/H2O	
R3	TFA, neat	*purchased from Sigma*
R4A	25% TFA in Water, DTT	12.5mL TFA in 37.5mL water with 0.01% DTT (32uL 1M stock)
R5	Acetonitrile with 0.0001% DTT	
S1	N-heptane	*purchased from Sigma*
S2B	Ethyl Acetate	450mL Ethyl Acetate with 225uL 1M DTT
S3	N-Butyl Chloride	*purchased from Sigma*
S4B	20% Acetonitrile in water	20% Acetonitrile in water
Stocks		
	Na2PO4 stock	2g in 15mL 3.5% THF in water
	1M DTT stock	0.231g in 1.5mL Ethyl acetate or water

A.1.3 Ordering

Table A-2: Parts and Chemicals Commonly Ordered from Applied Biosystems

Part/Chemical	Part Number
Premix Buffer Concentrate, 100mL	401446
R5B, Acetonitrile/n-Acetylcysteine, 40 mL	4340966
Seal, Cylinder Head	200240
Seal, 5mm Micro Pump	201399
Procise Cartridge Seals (50/pk)	401611
PTH-C18 Column, 0.8mm x 250mm	401882
PTH Amino Acid Standards	4340968
R2C, N-Methylpiperidine in isopropanol, butanol, water	4310689
9mm TFA Filters	401111

Table A-3: Parts and Chemicals Commonly Ordered from Sigma Aldrich

Part/Chemical	Part Number
Ethyl Acetate CHROMASOLV® Plus, 99.9%	650528-4L
Phenyl Isothiocyanate	78780
Heptane, CHROMASOLV Plus, 99.9%	650536-4L
2L Chromasolve N-Butyl Chloride for HPLC	34958-2L
Trifluoroacetic Acid, 99% reagent plus	T6508-1L

A.1.4 Contacting AB

The number to call to place a service call with Applied Biosystems is (800) – 831 – 6844, press #4, then #1. Our serial number is 4CL000092. Ask for service on the instrument and get a reference number for the call. Type this onto the purchase order, and have the purchasing department fax it to (650) – 554 – 2193. Include a description of the problem on the purchase order.

A.1.5 Idle machine

The machine should never be left completely idle, or the column can dry out and will need to be replaced. If the machine is not running, press the "Manual" button on the front of the pumps. It should turn the pumps on to 5uL/min at 50%B, and the bottom of that window will change to say "free running." This low flow of solvent keeps the solvents moving, and the column wet.

A.1.6 Settings

These are our common settings for the machine in the off chance that something is lost or rest and needs to be reprogrammed. These are also the settings used for all of the amino acid sequencing in this thesis.

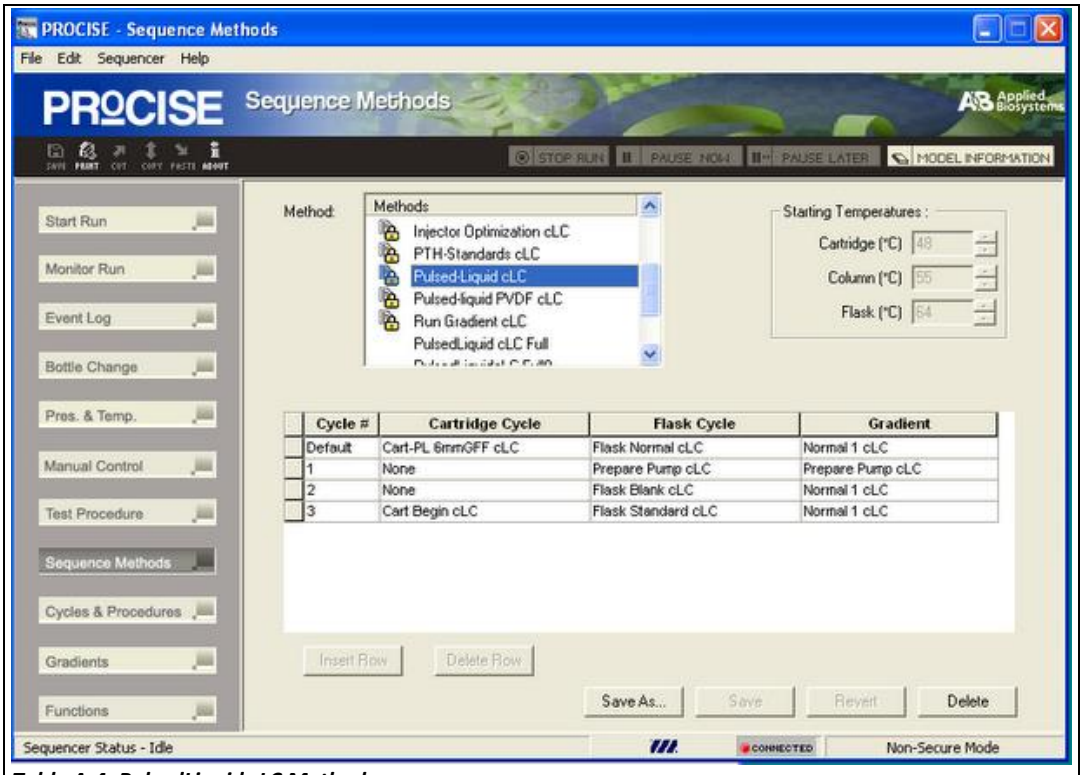


Table A-4: PulsedLiquid cLC Method

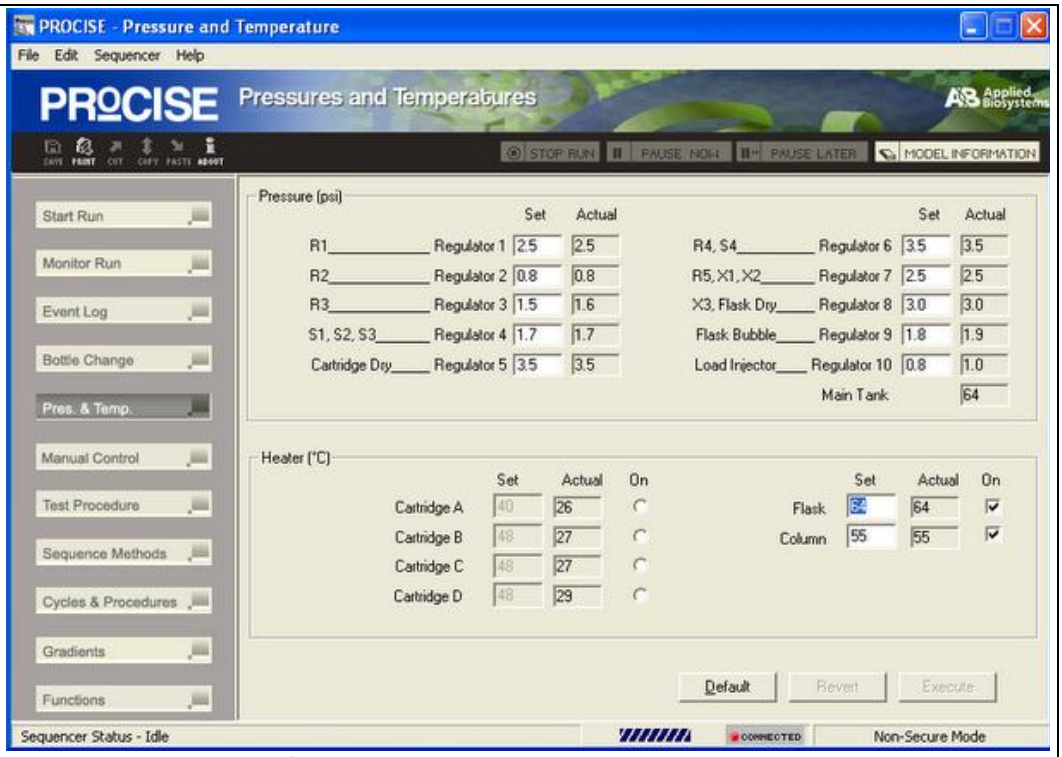


Figure A-1: Temperature and Pressure Settings

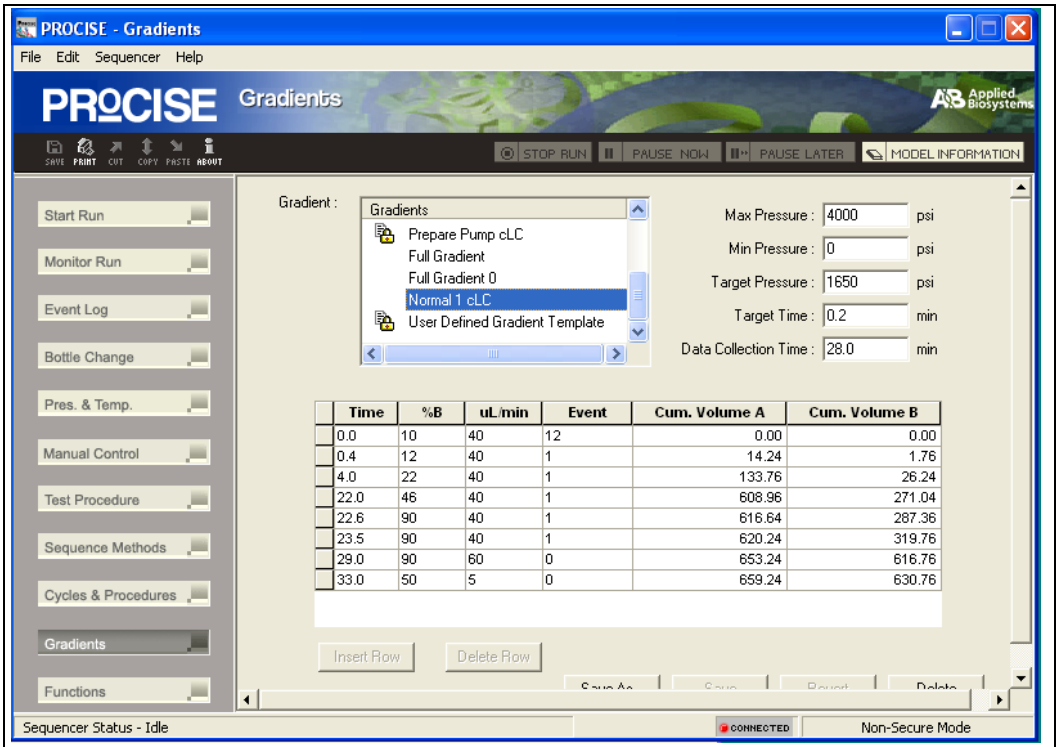


Figure A-2: Normal1 cLC Gradient

A.2 Troubleshooting

A.2.1 Computer Errors, Freezing, or Not Saving Spectra

The computer needs to be restarted frequently, more so when people use the computer for other uses. Try to restart the computer once a week to prevent the loss of spectra, and keep people from using the computer for any other purpose, especially while the machine is running. If a computer error occurs, or the computer or sequencer freeze, shut down the computer and both the Procise machine and the pumps. Turn them both back on, and they should sync up properly and continue running.

A.2.2 Machine Bottle Runs Dry

When a bottle on the machine runs dry, air bubbles can get into the lines, and it will not be able to detect the solvent even after the bottle is refilled. In order to fix this, the line will need to be backflushed. After the solvent bottle has been refilled, go to the tab in the Procise software called "Manual Control" and find the solvent bottle that needs to be backflushed in either the "Flask Functions" tab or "Cartridge Functions" tab. For example, in **Figure A-3**, the Flask Function "Backflush S3" is highlighted. Once it is highlighted, click "execute" on the bottom of the screen, and watch the bottom of the hose in the solvent bottle until bubbles can be seen coming out. Then press "All Off" to shut off the backflush, and the solvent should now be detected. The run can now be restarted.

A.2.3 HPLC Bottle Runs Dry

Sometimes the HPLC solvent bottles run dry, and this is not as easily fixed as the machine bottles. First, the HPLC bottles need to be refilled. Then the pump must be purged manually using the buttons on the front of the pumps. Push the function key for purge, then select the pump to be purged. When the purge is complete, push "Manual" on the front of the pump to turn the pumps on to free running. The pumps should be allowed to run freely until bubbles are no longer coming from the end of the column (as seen in the waste line). If the machine is needed immediately, the flow rate can be turned up to move this process along faster.

Everything should be done to minimize the times that these bottles are allowed to run dry, so that the pumps are not pumping dry and the column does not dry out.

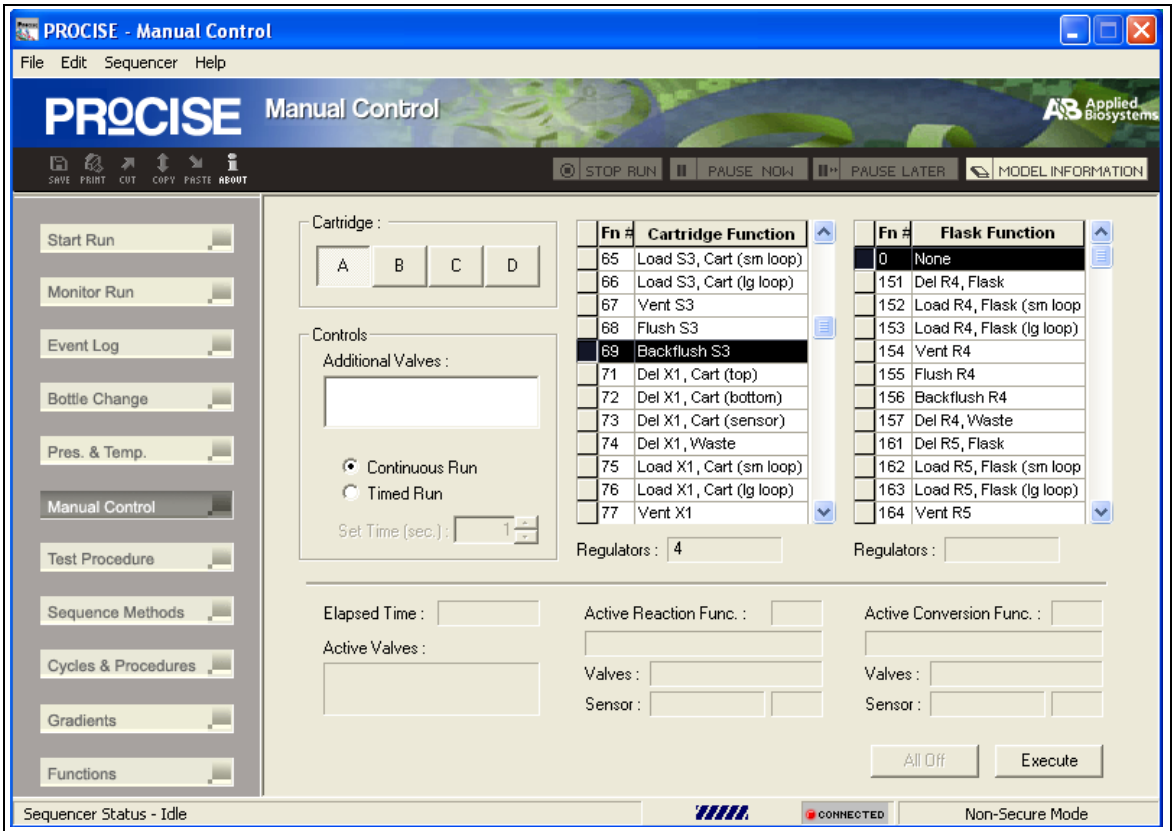


Figure A-3: Procise Screen for Backflushing a Line

A.2.4 Baseline Errors – Dip at front of Spectra

A dip at the front of the spectra, as seen in **Figure A-4**, causing difficulty in reading the amino acid sequence, is a fairly common problem that indicates that the pump seals need to be changed. In order to do this, you must unscrew the grey metal screw going into the pump cylinder, then unscrew the black ring around the top of the cylinder. Remove the cylinder by using the purge function of the pumps to purge the pump you are working on, and PUSH the cylinder out. NEVER PULL the cylinder out – it could damage the pumps, which are nearly impossible to replace on this machine. Once the cylinder has been pushed out fully and starts getting pulled back in, push stop to keep it out. Gently pull the cylinder off of the post. One of the pump seals that needs to be changed is the one on the end of the post. It is frequently hard to remove, and

can be sliced off or yanked with pliers. The second pump seal is the ring inside of the metal part at the top of the cylinder – pull this off to expose the seal.

To put the cylinder back onto the post, do NOT just push. If it is not aligned correctly, this can damage the inside of the cylinder, which cannot be replaced. Use the metal alignment tool in the spare parts drawer. This part has two pieces that come together to form a tunnel that fits onto the end of the cylinder, through which you can slide the cylinder post. Before doing this, take a piece of Parafilm to cover the hole into the pump cylinder. If one of the metal alignment pieces falls in, it will be VERY difficult to remove. Then, while holding the metal pieces to the end of the cylinder, align this with the post, and apply steady but hard pressure to push the cylinder onto the post. You will feel a click when it is on properly, and it will not pull back off if you tug on it. Then, by tilting the cylinder back and forth, pull off the metal alignment pieces, then the Parafilm. Use the purge function to pull this cylinder back down into the pump. One that is in, it will need twisted so that it aligns properly and slides into place. There is a notch it must fit into. Then, the black ring can be screwed back on, followed by the metal screw.

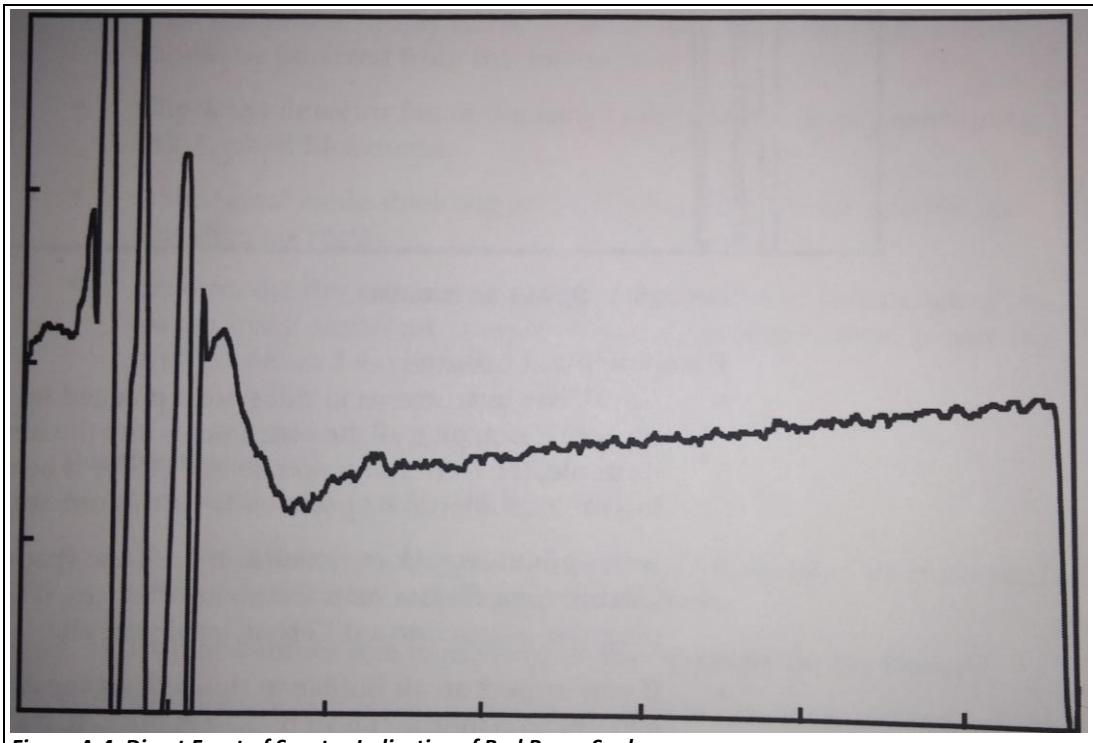


Figure A-4: Dip at Front of Spectra Indicative of Bad Pump Seal

A.2.5 Baseline Errors – Stretched Out Amino Acid Standards

If the amino acid standards look very stretched out and are not completing by the end of the HPLC run, or if the DPTU peak is shifting from cycle to cycle, there is most likely a leak in the pumps. This is a fairly easy fix, which requires looking at all of the screws around the pumps. One of them will more than likely have a ring of salt around it. Wash the salt off, and tighten the screw ¼ turn. It should fix the spectra problems immediately.

A.2.6 Pressure Errors – Change Bottle Seals

Occasionally, when the machine has difficulty adding a solvent, it is due to a faulty rachet cap assembly, or the part that the bottle screws into on the machine. In order to test this, go to the "Test Procedure" tab on the Procise software, and select "Leak" test. Scroll down to the appropriate bottle, and try a leak test. If it fails, but the bottle is screwed in tightly, this assembly should be replaced. See page 9-19 in the User Manual for details on this procedure. Once this

assembly has been replaced, the seal should no longer leak, and the solvent delivery should function as normal.

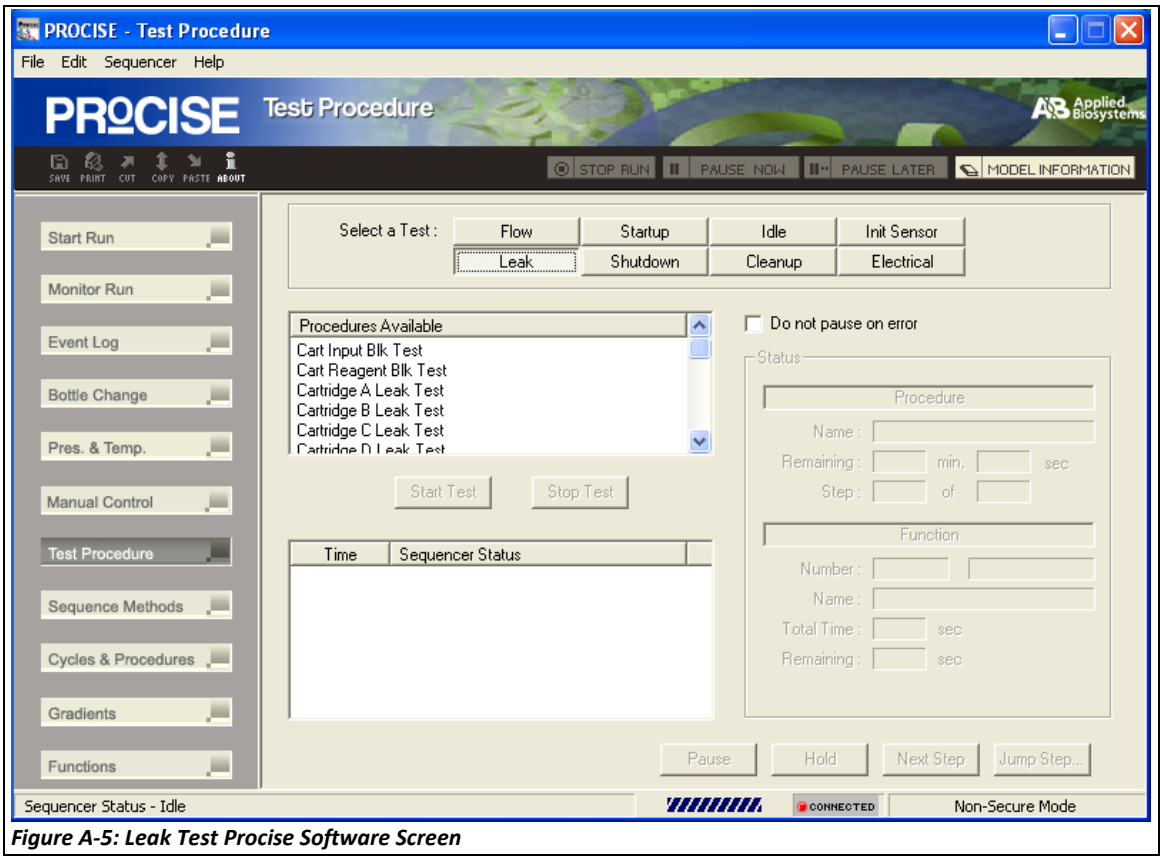


Figure A-5: Leak Test Procise Software Screen

A.2.7 Pressure Errors – Clogged Lines

Overpressure errors on the Edman can sometimes be related to a clogged line. Use the error message to try to find the line that might be clogged. The line can either be replaced by new line from stocks in the spare-parts drawers, or flushed clear with methanol.