# Enhancement of PD-1 Binding Affinity Through Affinity Maturation and Mutagenesis

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## ABSTRACT AND INTRODUCTION

In order to further understand affinity maturation, this project attempted to improve binding between PD-1 and PDL-1. PD-1 and PDL-1 are important in the regulation of cell death, especially in the case of tumor prevention, and an improved interaction between ligand and receptor could improve cancer treatment. Using randomized mutation of the original binding interfaces, the aim of the project was to use affinity maturation to produce a new protein with improved affinity to PDL-1.

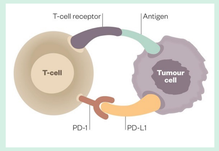


**Figure 1. Interaction between PD-1 and PDL-1**
PDL-1 acts as a suppressor to T-cells, preventing T-cell detection—and destruction—of tumor cells.
(http://www.pharmaceutical-journal.com/news-and-analysis/feature/immune-checkpoint-inhibitors-bring-new-hope-to-cancer-patients/20067127.article)

The human immune system has means of detecting foreign bodies and defective cells, but some dysfunctional cells, like tumor cells, can avoid an immune response altogether. These cancer cells avoid an immune response by evading T cells (Schreiber, 2011), through the activation of the PD-1 receptor on T cells. PD-1 negatively regulates T cell activity, and this receptor is stimulated by PDL-1 and PDL-2 ligands. PD-1 is a cell-surface protein which modulates the cell cycle; PD stands for Programmed Death. PDL-1 and -2 ligands limit the activity of T cells during inflammation, as to avoid additional damage by overexpression of T cell activity (Freeman, 2000; Francisco, 2010). Cancer cells, however, exploit this negative feedback system to evade detection--and destruction--by T cells by expressing PDL-1 and PDL-2. A new approach to cancer therapy, through immune checkpoints, can help the immune system regain an advantage over tumor cells (McDermott, 2013). This experiment aims toward designing a protein--a PD-1 domain--to bind to ligands released by tumor cells. In order to produce such a therapy, however, the binding affinity of the protein must be improved. The protein's affinity will be improved with affinity maturation, with techniques such as using mutagenesis libraries and ELISA assays.

## MATERIALS & METHODS

In order to affinity mature an inhibiting reagent that can bind to PDL-1, the project first focused on mutating all the peptide positions in the binding interfaces. Before the production of the library, however, the original affinity between PDL-1 and PD-1 were tested using ELISA assays. Assay plates were coated with PDL-1, and the binding of PD-1 was observed. The two interfaces were the focus of where mutagenesis was targeted. There are 56 amino acid positions which can be varied, thus a library of differing amino acid combinations is produced. The mutagenesis was performed through PCR with mutated primers. Each amino acid in the interface will get a mutated primer, designed to mutate the specific position. Three DNA nucleotides code for one amino acid, so the primers replaced the target trio of nucleotides with the sequence NNK.

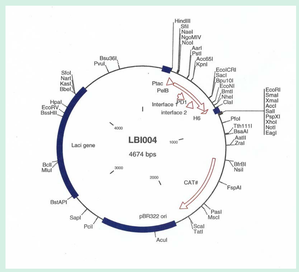


**Figure 2. Plasmid Map of the Protein Library**
After ligation, this plasmid has a variety of mutated PD-1 interfaces. The plasmid will be transformed into *E. coli*.
(Diagram courtesy of Dr. Bo Yu)

The N and K code denotes not one nucleotide from the four options, ATGC, but rather ambiguous nucleotides options. N denotes any of the four nucleotides, and K denotes either G or T. In order to perform bridge PCR, two primers, one forward and one backward, are designed and ordered. The production of a library starts with PCR of the original PD-1 coding region with forward and backward primers separately. This will produce complementary fragments, which are PCRed one final time to complete the desired region. Since DNA is prone to binding to non-target regions, the project also extracted the final PCR product from a gel, to make sure our product was free of extraneous fragments. So far, the project has been completed to this point. Once the fragments cut with restriction enzymes and ligated into a plasmid, this library will be transformed into *E. coli*. With a small focal area, this experiment can afford to screen each cell individually.

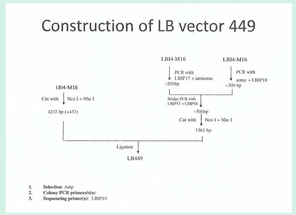


**Figure 3. Schematic of Vector Creation**
The diagram features specific names of primers (LBIP17 and LBIP18) and corresponding templates (LB14-M16). This process is completely for all 56 amino acid positions, then added together for ligation.
(Diagram courtesy of Dr. Bo Yu)

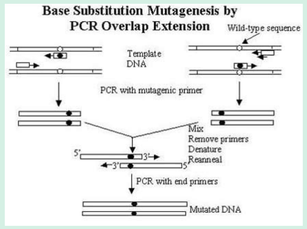


**Figure 4. Process of Bridge PCR**
Forward and backward primers are used to produce two halves of the target sequence, then bridge PCR combines them.
(http://www.scrigroup.com/limba/engleza/100/Sitedirected-mutagenesis63218.php)

The *E. coli* will express the proteins in its periplasm, making it easier to screen the proteins. Unlike cell-surface proteins--which take extra steps to release into the environment--this expression in the periplasm means most proteins will be detectable without extra effort. Plasmids are transformed into *E. coli*, streaked onto plates, and individual colonies are sorted into 96 well plates. The proteins produced by the *E. coli*, which are regulated by a Laci gene, will be used in an ELISA assay with a PDL-1-coated plate. By comparing the fluorescence of individual samples, we can determine which mutated positions have improved affinity. Once those specific sequences are established, we may have them sequenced and optimized.

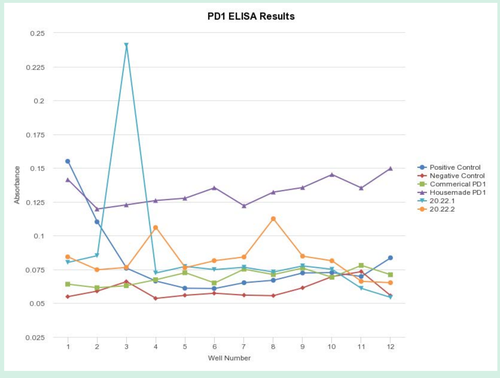


**Figure 5. Graph of ELISA Assay Results**
Each line denotes a different sample, including positive and negative control, commercial and housemade PD1, and overnight IPTG induced PD1 (20.22.1 and 20.22.2). As shown, all PD1 binding has very low absorbance, meaning the binding affinity is low as well.

## RESULTS

Although this project has not been completed, our initial ELISA assays, and the Bridge PCR, have been successful. In line with the established knowledge, the ELISA assays found the binding of the original PD-1 to be very weak, with very low affinity for PDL-1. In addition, all but one amino acid position was successfully bridge PCRed.
Electrophoresis gels after each PCR also showed a high amount and concentration of replicated DNA to work with, which can help later on in ligation. This also means the designated primers had successful binding with their target areas. Despite the high success of the primers, there also seems to be minor off-target binding, resulting in slightly smeared gels. As a counterbalance to potential extraneous DNA fragments, however, our specific sized fragment was extracted with a gel extraction protocol.

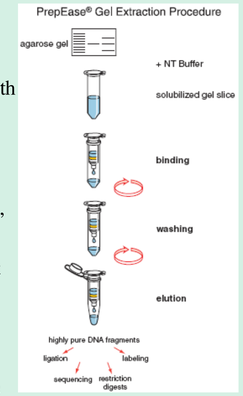


**Figure 6. Gel Extraction Method**
Using spin columns, one can extract DNA from a gel. Gel extraction ensures collection of a specific fragment size, the target fragment rather than extraneous fragments.
(http://www.affymetrix.com/catalog/131351/USB/PrepEase+Gel+Extraction+Kit#1_1)

## SUMMARY / DISCUSSION

Despite the unfinished nature of this project, the potential in affinity mutagenesis is still waiting to be released. Immunotherapy for cancer is an ongoing field of research, searching for a non-destructive approach to recovery. PD-1 and PDL-1 seemed to be a promising candidate for further studies into inhibition of tumor growth. The low affinity, however, hindered the potential for a successful treatment. As shown in previous papers, Dr. Bo Yu's research, and in our preliminary data, PD-1 and PDL-1 are not suitable for use as a therapy--with high doses and low effectiveness. Even beyond this project, affinity mutagenesis may be useful in improving efficiency of any number of binding molecules. This exploration, specifically, also exhibits the simplicity and efficacy of designing libraries for mutagenesis. From the preliminary results, the library looks promising, with few obstacles. This research is ongoing--exploration can further scientific knowledge of cancer and open paths to deeper understanding.

## ACKNOWLEDGEMENTS / REFERENCES

Dolan DE, Gupta S. 2014. PD-1 pathway inhibitors: changing the landscape of cancer immunotherapy. Cancer Control. 21. 231-237.
(http://www.ncbi.nlm.nih.gov/pubmed/24955707)

Francisco LM, Sage PT, Sharpe AH. 2010. The PD-1 pathway in tolerance and autoimmunity. Immunol Rev 236: 219-42.
(http://www.ncbi.nlm.nih.gov/pubmed/20636820)

Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Honjo T. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192: 1027-1034.
(http://www.ncbi.nlm.nih.gov/pubmed/11015443)

McDermott DF, Atkins MB. 2013. PD-1 as a potential target in cancer therapy. Cancer Med. 2(5). 662–673.
(http://www.ncbi.nlm.nih.gov/pubmed/24403232)

Schreiber RD, Old LJ, Smyth MJ. 2011. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. Mar 25;331(6024):1565-70
(http://www.ncbi.nlm.nih.gov/pubmed/21436444)

Swaika A, Hammond WA, Joseph RW. 2015. Current state of anti-PD-L1 and anti-PD-1 agents in cancer therapy. Mol Immunol. Oct;67(2 Pt A):4-17.
(http://www.ncbi.nlm.nih.gov/pubmed/25749122)