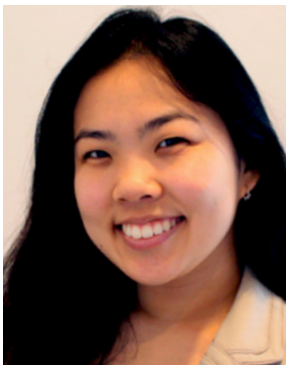
Development of Quantitative Förster Resonance Energy Transfer (qFRET) based High Throughput (HTS) screening for PD-1/PD-L1 immune-checkpoint Assay

Amanda Xaypraseuth, Vipul Madahar, & Jiayu Liao
Department of Bioengineering

ABSTRACT

Programmed cell death protein 1 (PD-1) and programmed cell death 1 - ligand 1 (PD-L1) are immune-checkpoint proteins that play an important part in cancer immunity. PD-1 is a protein on the surface of cells that down-regulates the immune system[1] while PD-L1 is a protein on some normal and cancer cells. The interaction of these proteins play a major role in tumor immune escape, inhibiting T lymphocyte proliferation and survival functions. To combat this issue, targeting these immune checkpoint proteins with monoclonal antibodies (mAbs) has become the turning point in cancer treatment. However, limitations were found using mAbs such as the cost of administration, its high molecular weight, and its lack of clinical efficacy. Recently, researchers are investigating small molecule inhibitors to target the PD-1/PD-L1 mechanism instead. With CA-170 as the only small-molecule modulator in clinical trials targeting PD-1, it is essential to research options that can contribute to cancer treatments. This study provides a novel, rapid assessment for PD-1/PD-L1 interaction with the use of FRET-based kinetic analysis. PD-1/PD-L1 binding will be quantified by fluorescence using donor and acceptor pairs, CyPet and Ypet, which were bound to PD-L1 and PD-1, respectively. From this study, we calculated a K_d value of 0.31±0.13 and developed an HTS assay with a Z' value > 0.7, values that validate the robustness and efficacy of this assay. With the development of this type of screening, it will be easy to contribute to small molecule inhibitor discovery and the growing field of cancer immunotherapy.

KEYWORDS: *PD-1; PD-L1; Immunotherapy; qFRET; HTS; Oncology*



Amanda Xaypraseuth

Department of Bioengineering

Amanda Xaypraseuth is a fourth year Bioengineering major. She has been involved in research for three years with experience developed at the UCR School of Medicine, Keck Graduate Institute, and the UCR Department of Bioengineering. Under the guidance of Dr. Jiayu Liao, Amanda's current research focuses on developing a high-throughput screening method for PD-1/PD-L1 interaction to assist in oncological and biopharmaceutical research. After graduating, she aspires to gain more experience in industry before pursuing higher education.

FACULTY MENTOR

Jiayu Liao, *Department of Bioengineering*

Professor Jiayu Liao obtained his Ph.D. degree from Dept. of Biological Chemistry, School of Medicine at UCLA, where he discovered the first SUMO E3 ligase family of genes, and then conducted his post-doctoral training of human genomics and chemical biology at the Scripps Research Institute. Shortly, he joined the Genomic Institute of Novartis Research Foundation (GNF) as the principle investigator and founding scientist of GPCR platform, where he was involved in the identification of target and side-effect the novel immunosuppressant drug, Gilenya from Novartis, and led a high-throughput screening for S1P1 receptor for the discovery of SEW2871, which led to the second generation of drugs, Siponimod from Novartis and Ozanimod from BMS. Dr. Liao moved to UCR as a founding faculty for the Department of Bioengineering in 2006. His work at UCR mainly focus on development of quantitative FRET technology platform for biochemical/pharmaceutical parameter determinations and high-throughput drug discoveries, focusing on SUMOylation and Ubiquitin-like pathways involved in anti-viruses and anti-cancers.

Development of Quantitative Förster Resonance Energy Transfer (qFRET) based High Throughput (HTS) screening for PD-1/PD-L1 immune-checkpoint assay

INTRODUCTION

The World Health Organization predicts that worldwide cancer rates are set to double by 2020[2]. With 10 million new cancers being diagnosed each year worldwide, it is essential to find an effective prevention campaign. A current method being used to address the cancer crisis is cancer immunotherapy, which has successfully brought exponential progress to the development of cancer treatments. Cancer immunotherapy is a method of eliminating cancer cells by enhancing or modulating the host immune system. One of the many types of cancer immunotherapies is using immune checkpoint molecules to regulate the immune balance, and the neutralization of immunosuppressive checkpoints[3]. Among these immune checkpoints, it was found that the blockade of programmed death protein 1 (PD-1) and programmed cell death 1 - ligand 1 (PD-L1) led to one of the most successful immunotherapies by enhancing T cell immune responses against tumor cells[3].

PD-1 is highly expressed by activated T cells, whereas PD-L1 is expressed on several types of tumor cells. The interaction of these two proteins results in the inhibition of T-cell activation and proliferation, providing an immune escape mechanism for tumor cells. Further studies then demonstrated that by blocking the interaction of PD-1 and PD-L1, it subjects tumor cells to attack by cytotoxic T cells **(see Figure 1)**. With the presence of PD-L1 on several types of tumor cells such as lung cancer, breast cancer, melanoma, and ovarian cancer, efforts are being made to develop PD-1/PD-L1 inhibitors for the treatment of these high-risk diseases.

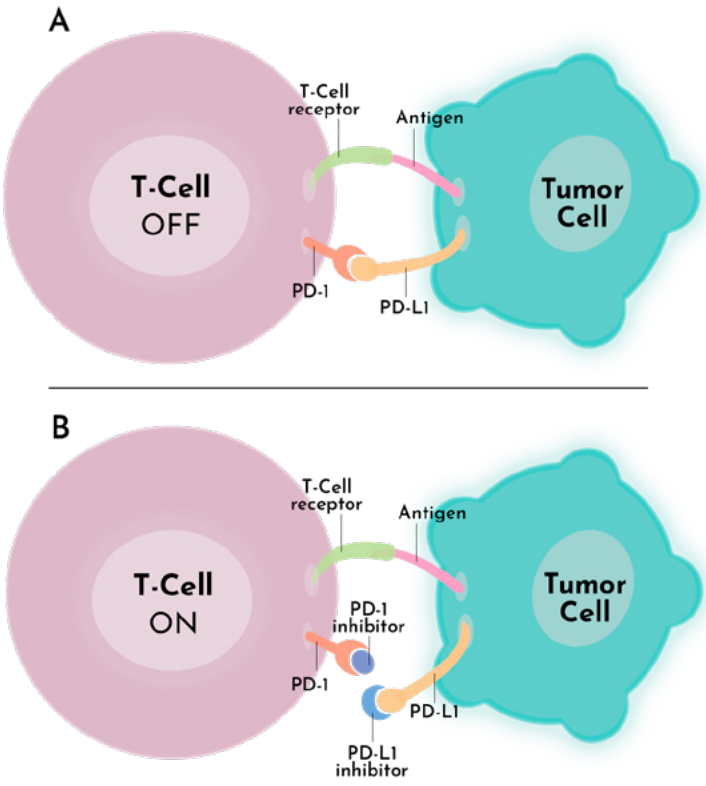


Figure 1. PD-1/PD-L1 Pathway Mechanism of Action (a) Tumor Uses PD-L1 as Defense Mechanism. (b) PD-1/PD-L1 Inhibitor Blocks Tumor's Defense Mechanism

Since the discovery of these proteins, monoclonal antibodies (mAbs), which are antibodies that enlist natural immune system functions to fight cancer, have been used for cancer immunotherapy. Ipilimumab was the first mAb monotherapy to be approved for advanced melanoma. By combining a combination of nivolumab (anti-PD-1-mab) and ipilimumab, Bristol-Myers Squibb (an American pharmaceutical company) was able to create the first approved immunotherapy combination with a high response rate. Despite this novel breakthrough, it was found that utilizing mAbs has its disadvantages, such as its production cost, instability, and immunogenicity[4]. Thus, small molecule immune checkpoint inhibitors of PD-1 and its ligand PD-L1 have become an active research field in drug discovery.

Currently, there are very few such inhibitors reported, one reason being the elusivity of the structural information of immune checkpoint proteins. Because of the increasing need to find a small molecule inhibitor to halt the interaction of PD-1 and PD-L1, this study is focused on developing a Förster Resonance Energy Transfer (FRET) based high throughput screening method to provide a rapid assessment for PD-1/PD-L1 interaction, in hopes of making the search for a small-molecule inhibitor more efficient and easier.

FRET has been widely used in biological and biomedical research and is a very powerful tool in elucidating protein interactions in many cellular processes[6]. FRET offers real-time monitoring and spatial information on molecular interactions in living cells[5] while being highly sensitive to nanoscale changes in donor/acceptor separation distance. This distance-dependent physical process measures the energy transfer emitted from the donor as it excites the acceptor. The FRET signal strength is generally determined by two major factors: the intrinsic FRET efficiency of the donor and acceptor and the amounts of the interactive donor and acceptor[5]. This technology can detect particle interactions within a range of 1-10 nm, an ability that allows this study to quantitatively describe the molecular interactive events between PD-1 and PD-L1.

Using this FRET technology by binding donor fluorophore CyPet (cyan fluorescent protein for energy transfer) and acceptor fluorophore Ypet (yellow fluorescent protein for energy transfer) to PD-L1 and PD-1, respectively, this study will provide insight to

how these two proteins interact and are used to develop a high throughput screening method for inhibitor screening. The development of this screening method will be subjected to a K_d measurement and a Z' factor measurement. K_d measurement is an important parameter to quantitatively assess the binding affinity of protein-protein interactions. Although there are traditional methods to calculate for K_d, such as surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC), FRET can serve as an additional tool that produces comparable values to these older, aforementioned approaches[5]. The Z' factor is another important value that determines whether the assay results are reproducible, i.e. that the variability of key endpoints of the assay is acceptably low[7]. This statistical method will be used in this study to quantify the potency and efficacy of the assay setup.

With the exploitation of FRET technology, it is obvious to see that there is potential to develop new methods to assist in biological research. This study will report the experimental developments of K_d determination of PD-1 and PD-L1 interaction and HTS assay development using the engineered FRET pair, CyPet and YPet. It is hoped that the use of this developed screening method will be applied in industrial applications, providing a rapid assessment for small molecule inhibitors that have the potential to become a cancer immunotherapy.

METHODOLOGY

Cloning and Expression of CyPet-PDL1 and YPet-PD1

The CyPet-PDL1 and YPet-PD1 plasmids used in this study were from the Hungxi Hospital located in China and were cloned into a pET28(b) vector (Novagen). Following cloning, BL21(DE3) *Escherichia coli (E.coli)* cells were transformed via electroporation with the pET28 vectors. The transformed *E.coli* BL21(DE3) were then plated on LB plates supplemented with 50 µg/mL kanamycin to ensure proper transformation.

Single isolated clones of CyPet-PDL1 and YPet-PD1 were then inoculated into loosely capped 10 mL LB tubes and incubated overnight at 37° with 250 rpm (revolutions per minute). Once the LB tubes have been incubated, the 10 mL starting cultures were then inoculated into a 1 L culture of prepared 2 × YT medium (there was 1L of 2 × YT medium made per 10 mL per each type of protein). Then, the 1 L cultures were grown in an incubator at 37° with 280 rpm for about 3 hours, which was the time it took for the culture to reach its exponential phase in its cell cycle. Afterward, the 1 L cultures were induced with 0.1 mM Isopropyl ß-D'1-thio-galactopyranoside (IPTG) and were continued to be incubated at 25° overnight, but no longer than 16 hours. The addition of IPTG enhances the expression of the protein. Aliquots were collected before and after the addition of IPTG for SDS-PAGE analysis.

Purification of CyPet-PDL1 and YPet-PD1

CyPet-PDL1 and YPet-PD1 cells were harvested by centrifugation for 3 minutes at 4° at 8,000 xg per each 300 g of culture. Post-centrifugation, the 1 L of pelleted cells were washed and resuspended with 40 mL of lysis buffer. Once resuspended, the cells underwent sonication for a 5 minute total process time with 5 seconds on and 5 seconds off intervals in an ice bath. The sonicated cells were then placed in a centrifuge and spun down at 35,000 xg for 20 minutes to separate cellular debris and inclusion bodies, leaving other proteins in solution. The supernatant and pellets were then collected to be examined using 10% SDS-PAGE to verify the successful expression of CyPet-PDL1 and YPet-PD1.

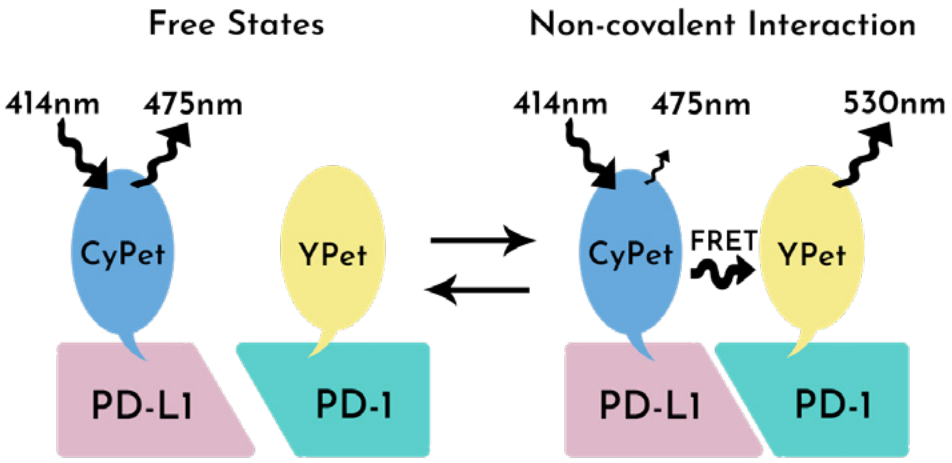


Figure 2. Design of FRET-based detection for PD1/PDL1 protein interactions

Development of Quantitative Förster Resonance Energy Transfer (qFRET) based High Throughput (HTS) screening for PD-1/PD-L1 immune-checkpoint assay

The supernatant was then subjected to be further purified by Ni-NTA column chromatography to capture histidine-tagged (His-Tag) proteins, PD-1 and PD-L1. To begin purification, 10 mL columns were filled with 300 µL of Ni-NTA agarose beads (QIAGEN). These beads were washed with 1 column volume (CV) of ddH20. The supernatant was then poured into the column and drained to the level of beads. Once finished, 1 CV of Wash Buffer 1 was poured into the column and drained to the level of beads. This step was then repeated with 1 CV of Wash Buffer 2 and 1 CV of Wash Buffer 3. After draining the wash buffers, a 1.5 mL Eppendorf tube was placed under the column to collect protein as it was being eluted with an Elution buffer. Once eluted, the protein solution was injected into a dialysis tube that was clamped at one end. The dialysis tube filled with protein solution was then placed in a large beaker filled with PBS for 16 hours, which is a step needed to remove any remaining imidazole concentration and wash buffer. These protein purification steps were performed according to the QIAGEN standard protocol[8].

FRET and K_d Measurement Assay

60 µL mixtures of recombinant CyPet-PDL1 and YPet-PD1 and phosphate-buffered saline (PBS) were created to begin FRET measurements. The final concentration of CyPet-PDL1 was fixed to 1 µM and the final concentration of YPet-PD1 was varied from 0 to 4 µM. The rest of the volume was supplemented with PBS, creating a total volume of 60 µL. These mixtures were then transferred to a 384-well plate (Grenier black) to measure the fluorescence emission spectrum of each sample. The two excitation wavelengths used to measure the fluorescence emission (emitted at 530 nm) were 414 nm to excite CyPet and 475 nm to excite YPet **(see Figure 2)**.

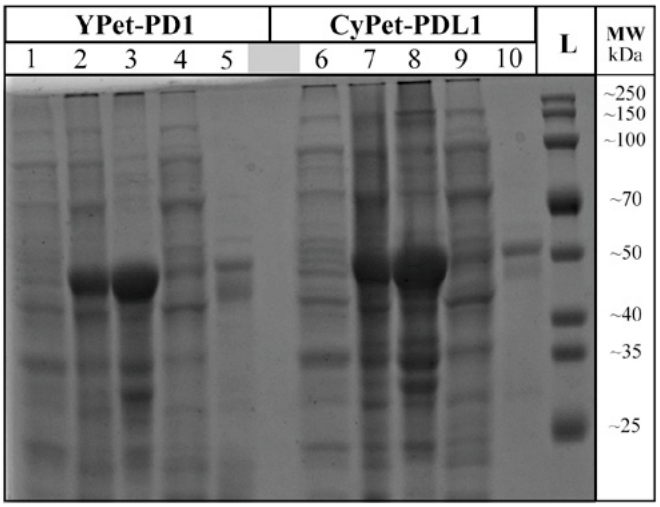


Figure 3. SDS-PAGE gel, Coomassie stain for determination of protein expression and validation of protein purification. **1**-YPet-PD1 Uninduced, **2**-YPet-PD1 Induced, **3**-YPet-PD1 Cell Pellet, **4**-YPet-PD1 Cell Supernatant, **5**-YPet-PD1 Purified and Dialyzed Protein, **6**-CyPet-PDL1 Uninduced, **7**-CyPet-PDL1 Induced, **8**-CyPet-PDL1 Cell Pellet, **9**-CyPet-PDL1 Cell Supernatant, **10**-CyPet-PDL1 Purified and Dialyzed Protein, L-Protein Ladder

The fluorescence emission was then calculated by solving for Em_{FRET}, which is proportional to the amount of YPet-PD1 bound to CyPet-PDL1. Em_{FRET} was solved by using Equation 1[9], where FLDD is the excited fluorescence signal of the donor, FLAA is the excited fluorescence signal of the acceptor, 'x' is the CyPet ratio factor, and 'y' is the YPet ratio factor. After Em_{FRET} has been calculated for each sample, the K_d value between CyPet-PDL1 and YPet-PD1 was calculated based on the algorithm that Song et. al developed[9]. This data was then processed and analyzed by using the GraphPad Prism software.

$$EM_{FRET}=(EM_{Total}) - (x * FL_{DD}) - (y * FL_{AA})$$

Equation 1

Z Prime Assay

The Z' factor for this study's assay was done once each day over three consecutive days with an assessment of three reaction conditions that produced a Max, Mid, and Min signal. The reaction was done in a 60 µL volume with 0.5 µM of CyPet-PDL1 and YPet-PD1 for the Max signal, 0.25 µM of CyPet-PDL1 and YPet-PD1 for the Mid signal, and 0.5 µM of CyPet-PDL1 and YPet-PD1 with the addition of Guanidine-HCl for the Min signal. The Max and Mid reactions were supplemented with PBS to achieve a volume of 60 µL whereas the Min reaction was supplemented with Guanidine-HCl. The addition of Guanidine-HCl mimics the presence of a small molecule inhibitor, which will produce no signal of energy transfer emission from CyPet to YPet. After the reactions were created, they were incubated for 30 minutes at 37°C. These reactions were then done in a 384 well plate format and set in a sequence outlined within NIH assay guidelines. After proper plate setup, the fluorescence emissions and the Em_{FRET} of all the reactions were measured and calculated. After calculating Em_{FRET}, Equation 2[7] was used to calculate the Z' Factor.

$$Z'=\frac{(AVG_{Max} - 3SD_{Max}/\sqrt{n}) - (AVG_{Min} - 3SD_{Min}/\sqrt{n})}{AVG_{Max} - AVG_{Min}}$$

Equation 2

RESULTS

Expression, Purification, and Dialysis of Recombinant Proteins

Induced, un-induced, cell pellet, cell supernatant, and purified samples for both YPet-PD1 and CyPet-PDL1 were examined by using 10% SDS-PAGE to detect the active expression products **(see Figure 3)**. The uninduced bands for YPet-PD1 and CyPet-PDL1 (Lane 1 and 6) show little to no presence of protein. However, once these samples were induced with IPTG (Lane 2 and 7), there was increased protein presence as indicated by the band around 48 kDa and 60 kDa for Lane 2 and 7, respectively, which are the molecular weights for YPet-PD1 and CyPet-PDL1, respectively. The cell pellet and cell supernatant were also collected for examination (Lanes 3,4,8, and 9). As expected, there is protein presence in each sample indicated by bands located around 48 kDa and 60 kDa. Most of the protein is in the cell pellet, therefore the cell pellet samples (Lane 3 and 8) are expected to have a thicker band than the cell supernatant samples (Lane 4 and 9).

The purified and dialyzed protein samples of YPet-PD1 and CyPet-PDL1 were also collected to be examined (Lanes 5 and 10). These lanes have the clearest protein bands compared to the other samples, which have lighter bands scattered down the lane in addition to the protein band. Despite being purified and dialyzed, Lanes 5 and 10 still have a light band beneath the 48 kDa and 60 kDa mark. Because His-Tag purification was used to capture our protein, these light bands can only be presumed to be degraded purified protein. Since the His-Tag is located at the N terminal of the protein, the C terminal can get degraded or cleaved during the process of purification. This phenomenon would yield purified proteins that aren't full-length. Thus, the bands present in Lanes 5 and 10 are YPet-PD1 and CyPet-PDL1 with the lighter bands being the same protein but cleaved at the C terminal.

K_d Measurements

The K_d Measurement experiment was done by fixing the CyPet-PDL1 concentration to 1 μM and increasing the concentration of YPet-PD1 from 0 to 4 μM in a total volume of 60 μL. After the FRET emission intensity under each condition was calculated, the data were fitted to a nonlinear regression curve fit (see **Figure 4**). The GraphPad Prism software was then used to calculate for our K_d and R2 value, which was 0.31 ± 0.13 μM and 0.9136, respectively. Typically, the smaller the K_d value, the greater the binding affinity of the ligand for its target. The larger the K_d value, the weaker the target molecule and ligand are attracted to.

Z Prime

The Z' factor for our assay was done once each day over three consecutive days with three reaction conditions that produced a Max, Mid, and Min signal. The Max signal contained 0.5 μM of CyPet-PDL1 and YPet-PD1. The Mid signal contained 0.25 μM of CyPet-PDL1 and YPet-PD1. The Min signal contained 0.5 μM of CyPet-PDL1 and YPet-PD1, including Guanidine-HCl to mimic the presence of a small molecule inhibitor within the reaction. The Z' assay was done 3 times with 3 plates to ensure a lack of variation with our samples **(see Figure 5)**. The Z' factor for each day and plate was then calculated according to the NIH guidelines, which was well within the criteria of Z' factor ≥ 0.4 and < 1 (see **Table 1**). All conditions of the study's Z' assay, which collectively had an average Z' value of 0.98, met the NIH criteria.

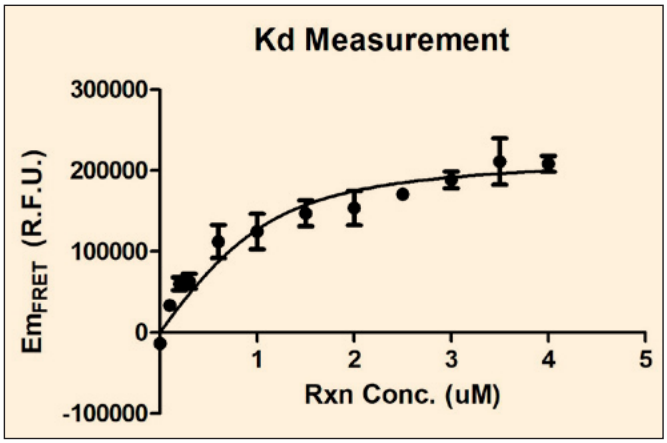


Figure 4. YPet-PD1 FRET emission (RFU-Relative Fluorescent Units) vs. YPet-PD1 conc. (μM)

DISCUSSION

With the growing number of cases of cancer every year, it is important to discover small molecule inhibitors for cancer immunotherapies. This study reports the development of a FRET-based HTS screening method for small molecule inhibitors to assist in PD-1/PD-L1 inhibitor discovery and oncological research. By measuring the K_d and the Z' values of our developed assay, we were able to quantify the robustness and validity of our HTS setup. The K_d value that this assay received was 0.31 ± 0.13 μM, which not only indicates that there is a high binding affinity between the expressed proteins, PD-1 and PD-L1, but also is comparable to previous experimental data[10,11]. This number also validates that the expression of our protein was successful as it is expected that these complementary proteins would bind readily to each other. Further validation was done by running an SDS-PAGE gel, which indicated a thick band around 48 kDa and 60 kDa, the molecular weight of YPet-PD1 and CyPet-PDL1, respectively. The SDS-PAGE gel also

Development of Quantitative Förster Resonance Energy Transfer (qFRET) based High Throughput (HTS) screening for PD-1/PD-L1 immune-checkpoint assay

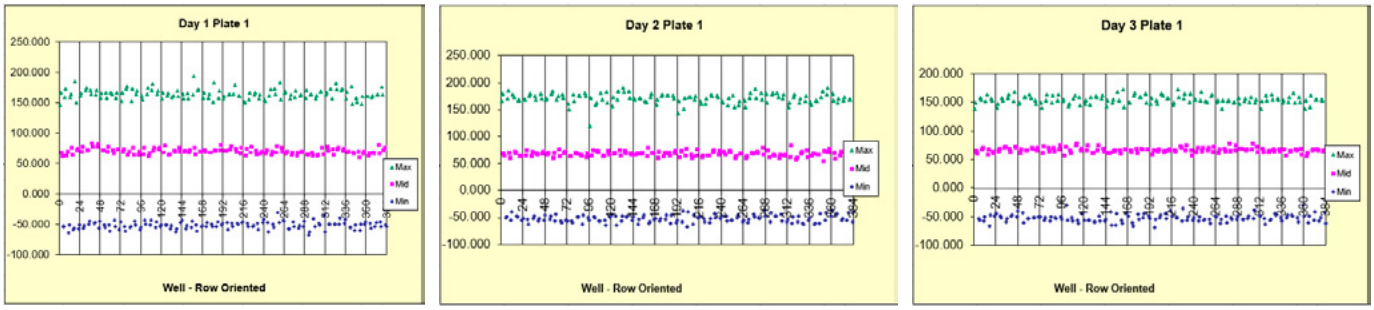


Figure 5. Z' for Plate 1 of 3 over days 1, 2, and 3. Each condition has an N of 128 for each plate, for a total of 384 reactions per plate. Plate replicates for each day resembles the figure shown.

showed that there was another light band present within Lanes 5 and 10 in addition to the thick protein band. This light band is usually indicative of protein degradation, but a western blot should be done to confirm what that band indicates. Although the presence of protein degradation may have affected our experimental data, it was confirmed that our purified samples sufficed to perform our experiments due to the comparable K_d value that was measured. The reported Z' assay utilized Em_{FRET} as the metric for hits. The Z' data demonstrated that the signal window between the Max and Min signal fell around 205 R.F.U., as seen by the gap between the Max and Min signal in each graph in **Figure 5**. This size range indicates confidence in each hit, making analysis of the screening data easier and less error-prone. **Figure 5** also demonstrates the lack of variability in our samples, indicated by the horizontal line trend that is present in each plate measurement. It is aspired to see this linearity to validate that the assay can produce accurate and consistent data. Further data analysis determined that the Z' over the 3 days had an average of 0.98 which confirms the assay's fidelity and robustness in the duration of a drug screening.

The robustness of this study's performance does not only hold oncological relevance to PD-1 and PD-L1. It also serves as a platform for other types of assays used in preclinical drug discovery including but not limited to antibody and other protein-protein interaction research. The utilization of FRET technology for HTS development provides a more cost-efficient and robust method for rapid assessment of discovering immunotherapeutic agents. With its wide Em_{FRET} range and Z' factor values that fall within NIH criteria, this FRET-based assay acknowledges and combats the limitations that some HTS methods have, such as the reduction in the quality and specificity of data as throughput increases[12]. For future studies, a western blot can be performed on our purified protein sample to further validate the presence of the correct protein. In addition, protein inhibitor, phenylmethylsulphonyl fluoride (PMSF), can be included in the binding buffer to potentially yield less protein degradation. Once that has been done, an actual screening with drug libraries can be performed to begin the search for a potential small-molecule inhibitor of PD-1 and PD-L1. With its high efficacy and tolerability, FRET techniques are sure to make a mark in biological research and discovery.

Table 1. Z' > 0.9 for each plate over three days, all Z' are within the set criteria

	Z' FACTOR MEASURED		
	Day 1	Day 2	Day 3
Plate 1	0.98	0.99	0.98
Plate 2	0.98	0.98	0.98
Plate 3	0.98	0.98	0.98

ACKNOWLEDGMENTS

I would like to sincerely thank Dr. Jiayu Liao and Vipul Madahar of Bioengineering for the continual guidance throughout this project. This work was supported by the Undergraduate Education Quarterly Minigrant from the University of California, Riverside.

REFERENCES

1. Syn, N. L., Teng, M. W. L., Mok, T. S. K., & Soo, R. A. (2017, December 1). De-novo and acquired resistance to immune checkpoint targeting. *The Lancet Oncology*. Lancet Publishing Group. https://doi.org/10.1016/S1470-2045(17)30607-1

2. Eaton L. (2003). World cancer rates set to double by 2020. BMJ (Clinical research ed.), 326(7392), 728. https://doi.org/10.1136/bmj.326.7392.728/a

3. Wu, Y., Chen, W., Xu, Z. P., & Gu, W. (2019). PD-L1 Distribution and Perspective for Cancer Immunotherapy-Blockade, Knockdown, or Inhibition. Frontiers in immunology, 10, 2022. https://doi.org/10.3389/fimmu.2019.02022

4. Chames, P., Van Regenmortel, M., Weiss, E., & Baty, D. (2009). Therapeutic antibodies: successes, limitations and hopes for the future. *British Journal Of Pharmacology*, 157(2), 220–233. https://doi.org/10.1111/j.1476-5381.2009.00190.x

5. Liao, J. Y., Song, Y., & Liu, Y. (2015, December 3). A new trend to determine biochemical parameters by quantitative FRET assays. Acta *Pharmacologica Sinica*. Nature Publishing Group. https://doi.org/10.1038/aps.2015.82

6. Zhang, J., et al. Creating new fluorescent probes for cell biology. Nat. Rev. Mol. Cell Biol. 3(12):906–918, 2002.

7. HTS Assay Validation - Assay Guidance Manual - NCBI Bookshelf. (n.d.). Retrieved March 22, 2020, from https://www.ncbi.nlm.nih.gov/books/NBK83783/

8. The QIAexpressionist - (EN) - QIAGEN. (n.d.). Retrieved April 18, 2020, from https://www.qiagen.com/us/resources/resourcedetail

9. Song, Y., Madahar, V., & Liao, J. (2011). Development of FRET assay into quantitative and high-throughput screening technology platforms for protein-protein interactions. *Annals of Biomedical Engineering*, 39(4), 1224–1234. https://doi.org/10.1007/s10439-010-0225-x

10. Chang, H.-N., Liu, B.-Y., Qi, Y.-K., Zhou, Y., Chen, Y.-P., Pan, K.-M., … Gao, Y.-F. (2015). Blocking of the PD-1/PD-L1 Interaction by a D-Peptide Antagonist for Cancer Immunotherapy. *Angewandte Chemie*, 127(40), 11926–11930. https://doi.org/10.1002/ange.201506225

11. Lin, D. Y. W., Tanaka, Y., Iwasaki, M., Gittis, A. G., Su, H. P., Mikami, B., … Garboczi, D. N. (2008). The PD-1/PD-L1 complex resembles the antigen-binding Fv domains of antibodies and T cell receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 105(8), 3011–3016. https://doi.org/10.1073/pnas.0712278105

12. Roberts, S. A. (2001). High-throughput screening approaches for investigating drug metabolism and pharmacokinetics. *Xenobiotica*, 31(8–9), 557–589. https://doi.org/10.1080/00498250110060978